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Folate-targeted nanoparticles for rheumatoid arthritis therapy

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Abstract

Rheumatoid arthritis (RA) is the most common inflammatory rheumatic disease, affecting almost 1% of the world population. Although the cause of RA remains unknown, the complex interaction between immune mediators (cytokines and effector cells) is responsible for the joint damage that begins at the synovial membrane. Activated macrophages are critical in the pathogenesis of RA and showed specifically express a receptor for the vitamin folic acid (FA), folate receptor β (FRβ). This particular receptor allows internalization of FA-coupled cargo. In this review we will address the potential of nanoparticles as an effective drug delivery system for therapies that will directly target activated macrophages. Special attention will be given to stealth degree of the nanoparticles as a strategy to avoid clearance by macrophages of the mononuclear phagocytic system (MPS). This review summarizes the application of FA-target nanoparticles as drug delivery systems for RA and proposes prospective future directions.

Key words: Drug delivery; Folate; Nanoparticles; Rheumatoid arthritis; Stealth

Arthritis may be defined as inflammation of the joints causing pain, swelling and stiffness. The broad category of arthritis includes diseases that can be classified as inflammatory, metabolic, degenerative or infectious. These conditions affect joints and the surrounding tissues, as well as the connective tissue of the skin, bones, and muscles.[1] RA is the most common form of chronic inflammatory arthritis, characterized by inflammation of the joints, resulting in synovial hyperplasia by infiltration of activated immune cells further leading to cartilage and bone destruction.[1]

The history of RA, as many chronic diseases, started around 1500 BC when Ebers Papyruralies describe a condition similar to RA. Several reports along time suggest that mummies were found to bear deformities similar to arthritis, however this condition was identified as RA by Garrod only in 1800, replacing the old terms arthritis deformans and rheumatic gout.[2]

RA occurs worldwide, although the estimated prevalence ranges from nearly 1% of the adult population in northern Europe and USA to 0.5% in other geographic areas. The highest prevalence is observed in certain Northern-American Indian populations. The annual incidence of RA varies from 8 to 50 cases per 100,000 inhabitants, with considerable differences according to the diagnostic criteria used and the geographic area. RA is clearly more common in women than in men with a ratio of approximately of 3:1.[3] RA can develop in persons of any age, with a typical age at onset of about 55 years.[4]

Arthritis in general, and RA in particular, is a common cause of disability. Mortality rates in RA patients are higher than in the general population (mortality ratios ranges from 1.28 to 2.98).[5] Life expectancy is shortened by up to 3 to 5 years, especially in patients that develop treatment-related adverse effects including

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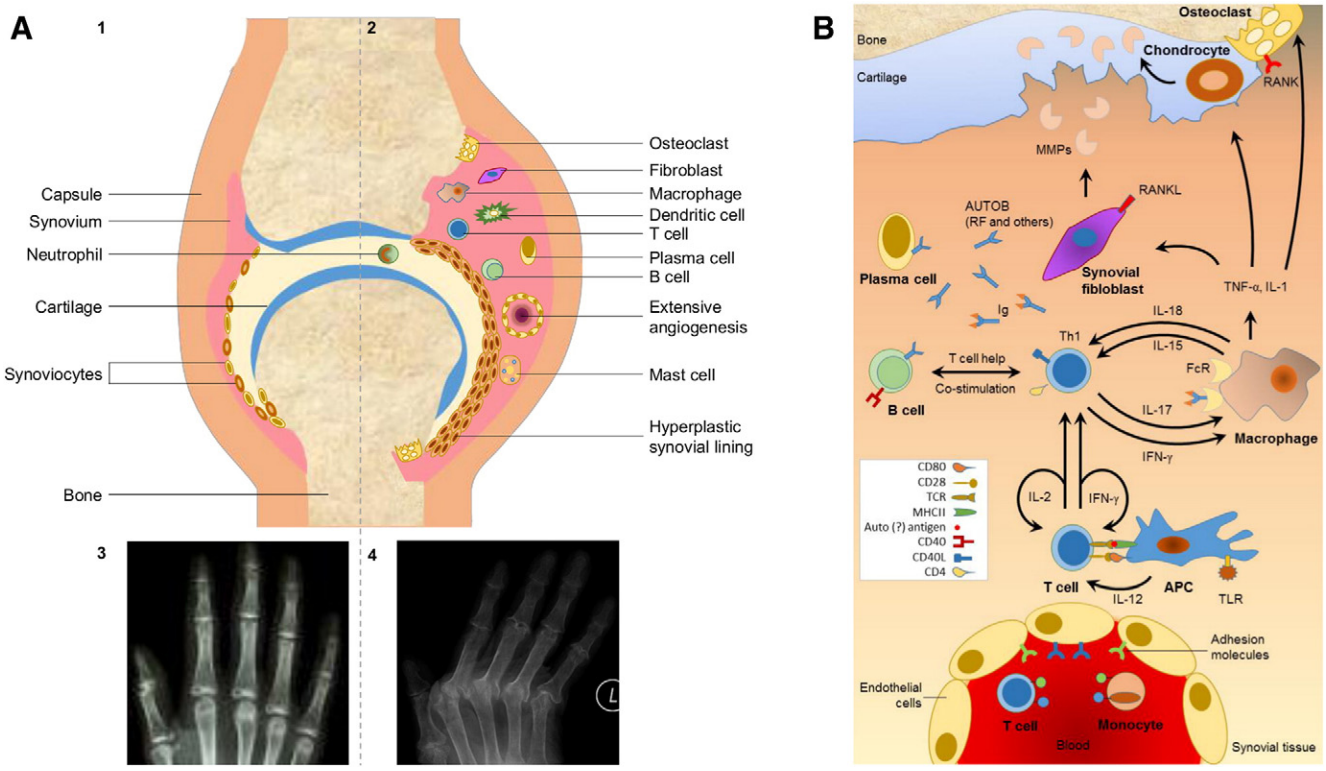


Figure 1. **(A)** Schematic view of (1) a normal joint and (2) its changes in RA. The "radiographic joint space" of metacarpophalangeal joints in (3) a normal hand and (4) from a patient with established RA. **(B)** Schematic representation of events occurring in RA.

infections, tumors and gastrointestinal toxicity from drugs used in RA therapy.[4,6] Furthermore, it was known that patients with RA are at higher risk of suffering from acute cardiovascular events, such as myocardial infarction, compared with the general population.[7] Therefore, the most common cause of death in RA are concomitant cardiovascular diseases, accounting for more than 50% of the mortality.[1]

Immunopathogenesis

Like many other rheumatic diseases, RA is an autoimmune disease. In these disorders, the affected organism has a defective ability to distinguish self from foreign molecules. Humoral and cellular immune responses to autoantigens, such as production of rheumatoid factors (RFs), occur in RA.[1] RFs are the first autoantibodies described in RA, which was posteriorly found to be directed to the Fc region of immunoglobulin (Ig) G.[8] The immune and inflammatory systems are intimately linked to the destruction of cartilage and bone. Although the cause of RA is unknown, many pathways involved in the generation of the disease have been recognized and identified as important by therapeutic proof-of-concept studies.[9] RA is a combination of genetic and environmental factors that when present increase the susceptibility to develop clinical manifestations.[2] The complex interaction of immune modulators (cytokines and effector cells) is responsible for the joint damage that begins at the synovial membrane and affects other structures (Figure 1). Synovitis is caused by the influx and/or local activation of mononuclear cells (including T cells, B cells, plasma cells, dendritic cells, macrophages and mast cells) and by the formation of new blood vessels[10].

T cells

CD4+ helper T (Th) cells make a crucial contribution to the development of inflammatory arthritis, where two T-cell subsets have been well characterized. T cells undergo polarization into either Th1 or Th2 cells, which is mutually exclusive. Th1 cells mainly secrete interferon (IFN) γ and tumor-necrosis factor (TNF)-α; Th2 cells produce interleukin (IL)-4, IL-5, IL-13 and IL-10. The polarity of Th cells is pivotal for the type of B-cell activation. Th1 cells have pro-inflammatory potential and promote certain humoral responses, whereas Th2 cells besides exerting anti-inflammatory events also stimulate other types of humoral responses, such as immunoglobulin (Ig) E production.[9] RA is clearly characterized by a shift toward the proinflammatory Th1 phenotype, with overproduction of IFNγ and inadequate production of Th2 cytokines such as IL-4 and IL-13. However, a Th1 phenotype does not explain all the mechanisms involved in RA, such as the contradictory role for IFNγ in experimental arthritis.[11,12] The model attributing a key role to a Th1/Th2 imbalance in RA was clarified by the identification of Th17 and T-regulatory (Treg) lymphocyte subsets.[13]

The differentiation of lymphocytes to Th17 cells is dependent on the nuclear transcription factor RORγt. Th17 cells produce the proinflammatory cytokine IL-17, which acts on several cell types found in rheumatoid joints: monocytes, macrophages, fibroblasts, osteoclasts and chondrocytes. Furthermore, this cytokine also induces a wide range of effector molecules implicated in joint damage, such as proinflammatory cytokines (e.g., IL-1β, IL-6, and TNF-α), multiple chemokines, matrix metalloproteinases (MMPs), cyclooxygenase-2 and prostaglandin E2. In this way, the cellular targets and biological effects of IL-17 are in agreement with the theory that Th17 cells have a vital role in mediating synovitis and articular damage.[14] The importance of IL-17 in the pathogenesis of arthritis in animal models has been demonstrated quite consistently. IL-17 knockout mice develop significantly less severe arthritis than wild-type mice, and neutralizing anti–IL-17 antibody reduces the severity of arthritis. Furthermore, in the streptococcal cell wall model of arthritis, IL-17 receptor signaling is required in radiation-resistant cells in the joint for full progression of chronic synovitis and bone erosion.[15]

The immune response needs to be controlled to avoid a chronic inflammation. For this purpose, Treg cells, known to have suppressor activity, are pivotal in the maintenance of self-tolerance.[16] Treg cells exhibit a CD4+CD25 high phenotype and express the transcription factor FOXP3. Although Treg cells can regulate any Th subset, special attention has been put on the Th17/Treg balance. It is therefore clear that Th17 and Treg cells have a functional antagonism, in which Tregs act as immunosuppressive cells and Th17 cells are involved in inducing autoimmunity.[17] Treg cells can suppress inflammation and immune responses through several mechanisms including cell-contact-dependent and -independent ones.[16]

B cells

B cells play several key roles in the pathogenesis of RA. Their primary function is the production of autoantibodies, RF and anti-citrullinated peptide/protein antibodies (ACPA), that contribute to form larger immune complexes that can further stimulate the production of pro-inflammatory cytokines, including TNF-α, through complement and Fc-receptor activation.[1,10] Furthermore, B cells with specificity for self-immunoglobulin can bind and internalize immunoglobulin–antigen complexes and enhance antigen-presenting function by generating a wider range of peptides.[18] In this way, besides production of pathologic autoantibodies and proinflammatory cytokines, B cells can also present antigens to T cells and supply costimulatory signals which are essential for T cell activation, clonal expansion and effector functions.[19] These findings on the role of B cells and their immunoglobulin products in self-sustaining chronic inflammatory processes have effectively contributed to the development of therapies. Targeted B cell therapies attenuate the function of secreted and membrane associated factors that contribute to B cell accumulation and survival at sites of the disease.[20] The clinical efficacy of an anti-CD20 monoclonal antibody, designated as rituximab, has confirmed the essential role of B cells in RA pathogenesis, as demonstrated in experimental models.[11]

Synovial fibroblasts

There is growing evidence that activated synovial fibroblasts (SFs), largely present in rheumatoid synovium, are one of the main players in the destructive process of RA.[21] In healthy tissue, the physiological function of SFs is to provide nutritive plasma proteins and lubricating molecules such as hyaluronic acid to the joint cavity and the adjacent cartilage. Furthermore, SFs are involved in continuous matrix remodeling by the production of matrix components such as collagen, hyaluronan and a variety of matrix-degrading enzymes.[22] Studies indicate the involvement of Toll-like receptors (TLRs), key recognition structures of the innate immune system, at an initial stage of synovial activation. In theory, microbial fragments or RNA released from necrotic cells within the synovial fluid acts as endogenous TLR ligands in the stimulation of pro-inflammatory gene expression in SF of synovial membrane.[23] Once activated, SFs produce increased amounts of cytokines, chemokines and matrix-degrading enzymes that mediate the interaction with neighboring inflammatory and endothelial cells and are responsible for the progressive destruction of articular cartilage and bone.[23] In this way, the production of cytokines and chemokines helps to recruit macrophages, neutrophils and T cells to the rheumatoid synovium, which attracts more inflammatory cells and, which, in turn, enhances the activated state of SFs and osteoclasts.[1] Furthermore, SFs also stimulate synovial vascularization through the release of proangiogenic factors. In this way, angiogenesis supports the influx of immune cells into affected joints, perpetuating the inflammatory processes and facilitating the access of SFs to the bloodstream, thus increasing dissemination of RA.[24] SF hyperplasia also contributes to the pathogenesis of RA, however the molecular mechanisms that sustain it are incompletely understood.[25]

Osteoclasts

Osteoclasts are multinucleated cells of hematopoietic origin and are the primary bone resorbing cells, essential for the remodeling of bone throughout life. Osteoclasts have two pivotal molecular machineries that allow them to resorb bone.[26] Osteoclasts utilize a proton pump to acidify the environment deep to the ruffled border and solubilize mineral from bone. In addition, proteolytic enzymes including matrix metalloproteinases (MMPs) and cathepsin K are secreted that degrade the organic bone matrix.[27] Macrophage--driven osteoclastogenesis requires the presence of macrophage colony-stimulating factor (M-CSF) and results from the interaction of the receptor activator of nuclear factor-κB (RANK) and the RANK ligand (RANKL).[10] RANKL expression is regulated by inflammatory cytokines, such as TNF-α, IL-1β, IL-6 and IL-17, but is also influenced by non-cytokine inflammatory mediators such as prostaglandin E2. The interaction of RANKL with its receptor RANK is modulated by osteoprotegerin (OPG), a soluble decoy receptor, which is expressed by mesenchymal cells in the rheumatoid arthritis synovium. In RA, an imbalance between OPG and RANKL expression promotes RANKL-induced bone loss.[28]

Chondrocytes

Adult human articular cartilage, which covers the articulating surfaces of long bones, is populated exclusively by chondrocytes

that are somewhat unique to this tissue. Under physiological conditions, the chondrocytes maintain a stable equilibrium between the synthesis and the degradation of matrix components.[29] Under the influence of synovial cytokines (particularly IL-1 and IL-17A) and reactive nitrogen intermediates, cartilage is progressively deprived of chondrocytes, which undergo apoptosis.[25] Chondrocytes switch from an anabolic matrix-synthesizing state to a catabolic state that is characterized by the formation of ADAMTS (a disintegrin and metalloproteinase with thrombospondin motifs) and MMPs that cleave the cartilage components proteoglycan and collagen fibers, respectively.[28] These processes ultimately lead to the destruction of the surface cartilage and the radiographic appearance of joint-space narrowing.

Cytokines

Cytokines are directly implicated in many of the immune processes that are associated with the pathogenesis of RA.[28] A large number of cytokines are found elevated in rheumatic joints. Indeed, it is now evident that these cytokines play an important role in the processes that cause inflammation, articular destruction and the comorbidities associated with RA.[30] Cytokines are small proteins with key roles in cell signaling, being secreted by several cells acting either in themselves (autocrine) or on surrounding cells (paracrine signaling).[31] Cytokines can be categorized into several classes, families or superfamilies. This has been done using either their numerical order of discovery or their functional activity.[31] In RA, the primary site of inflammation is the synovial tissue, from which cytokines may be released into the systemic circulation.[1] Cytokines however do not have one single effect and the phases of the inflammatory process depend on several cytokines. Therefore, the cytokine network is both pleiotropic and redundant. In RA inflammation, the effects of proinflammatory cytokines predominate over those of anti-inflammatory cytokines[32] (Table 1).

Plasma levels of cytokines in RA tissue revealed that many proinflammatory cytokines such as TNF-α, IL-1, IL-6, granulocyte-macrophage colony-stimulating factor (GM-CSF) and chemokines such as IL-8 (CXCL8) are abundant in all patients regardless of therapy. Through tangled signal pathways, these cytokines activate genes associated with inflammatory responses, including other cytokines and MMPs involved in tissue degradation.[10] On the other hand, this can be compensated through the increased production of anti-inflammatory cytokines, such as IL-10 and transforming growth factor beta (TGFβ), and cytokine inhibitors such as IL-1 receptor antagonist (IL-1Ra) and soluble TNF receptor.[1]

Macrophages

Macrophages are of central importance in the pathogenesis of RA, due to their higher presence in the inflamed synovial membrane and at the cartilage pannus junction, their activation status and their successful response to anti-rheumatic therapy.[1,33] Recent data demonstrate that resident tissue macrophages are established during embryonic development and persist into adulthood independently of blood monocyte input in the steady state. In the context of inflammation, classical monocytes readily differentiate to macrophages, and both recruited and resident macrophages share the capacity for proliferation in tissues.[34,35] Macrophages subsequently become "activated macrophages" displaying different phenotypes depending on the nature of the recruiting stimulus and the location.[36] Activated macrophages may release cytokines (IL-1, IL-6, TNF-α), chemokines (eg, monocyte chemotactic protein-1, MCP-1/CCL2), digestive enzymes (eg, collagenases), prostaglandins, and reactive oxygen species (ROS), which can aggravate or accelerate damage to the normal tissues.[37] Further, activated macrophages are known to participate in antigen presentation, and thereby they are thought to contribute to the activation and proliferation of antigen specific T-cells and their consequent destructive effects.[38] An increase in the levels of macrophage-derived proteases, such as leucocyte elastase, and MMPs, including MMP-1, MMP-3 and MMP-9, has also been described at the site of inflammation.[39] However, they possess broad proinflammatory, destructive and remodeling abilities, and contribute considerably to inflammation and joint destruction in both acute and chronic phases of RA.[1] Apart from the vital role of macrophages in RA inflammation, they are at the origin of pathological bone erosion due to their excessive differentiation into osteoclasts, unique cells specialized in bone resorption.[40]

Table 1
Cytokine roles categorized according to their contribution to inflammation in RA (Adaptaded from Chizzolini C. *et al*., 2009[1]).

Proinflammatory	Ambivalent	Anti-inflammatory
TNF-α	IFNγ	IL-1Ra
IL-1		IL-4*
IL-6		IL-13*
IL-12		IL-10
IL-15		TGFβ
IL-17		
IL-18		
IL-23		
CXCL8		
CCL3		
CCL2		

* IL-4 is anti-inflammatory in the context of RA synovial inflammation. By impacting on IgE production, however, IL-4 is a key cytokine in IgE-mediated inflammation. Similar considerations apply to IL-13.
[1] Chizzolini C, Dayer JM, Miossec P. Cytokines in chronic rheumatic diseases: is everything lack of homeostatic balance? Arthritis Res Ther 2009;11(5):14.

Folate receptor

Prolonged inflammatory states may last for weeks, months or even years. Macrophages can display different markers of activation and maturation depending on the type of activation, the immune cells involved, state of differentiation, type of aggression and the tissue where this all takes place. FRβ, whose expression is selectively elevated in RA synovial macrophages,[41] has been used as a target for immunotherapy in a number of clinical situations, such as autoimmunity and chronic inflammation.[42]

FRs include at least four isoforms, α, β, γ/γ′ and δ, exhibiting distinct patterns of tissue expression. FRα is

expressed at the luminal surface of polarized epithelial cells of normal adult tissues including proximal kidney tubules, uterus, fallopian tube, choroid plexus, epididymis, submandibular salivary, acinar cells of the breast, bronchial gland, type I and type II pneumocytes in the lung, and trophoblasts of the placenta. Furthermore, cancer types such as endometrial, cervix, ovary, testicular colorectal, choriocarcinoma, lung, pediatric ependymomas, mesotheliomas and renal cell carcinomas also show FRα expression.[43] In other malignant types of cancer such as breast, colon and renal, FRα expression is less frequent.[44] In turn, FRβ is a differentiation marker in the myelomonocytic lineage during neutrophil maturation and is amplified in activated monocytes and macrophages. However, FRβ in neutrophils is unable to bind the vitamin FA (or folate) due to dysfunctional posttranslational modifications.[45] In contrast, a functional FRβ with nanomolar affinity for folate has been identified on activated macrophages, key effect cells in chronic inflammatory diseases such as RA,[46] atherosclerosis,[47] systemic lupus erythematosus,[48] Crohn's disease[49] and osteoarthritis.[50] Furthermore, FRβ is expressed in a functional form in chronic myelogenous leukemia (CML) and in 70% of acute myelogenous leukemias (AML).[45] A functional FRβ has also been reported on macrophages induced by repeated treatment of human monocytes with macrophage colony-stimulating factor (M-CSF).[51] FRγ has been detected in normal and malignant hematopoietic cells present in the spleen, bone marrow and thymus, as well as ovarian, cervical and uterine carcinoma.[44] A polymorphism in the FRγ gene is caused by a mutation that results in a carboxyl-terminal truncation of the protein; the mutated protein is referred to as FRγ′.[52] FRδ has been found to be expressed on regulatory T cells in mice and has recently been proposed as a potential therapeutic target.[53] Recently, FRδ was renamed to Izumo1 egg receptor or Juno (Roman goddess of fertility and marriage), due to the expression of this protein on the egg surface, essential for female fertility.[54]

FRs are N-glycosylated proteins, of relative molecular mass (Mr) in the range of 38,000-45,000, with high binding affinity to folate. The FR isoforms are 220-237 amino acid polypeptides that share 68-79% amino acid sequence identity and contain eight conserved putative disulfide bonds.[44] The α, β, and δ isoforms are glycosyl phosphatidylinositol (GPI)-anchored membrane proteins, while FRγ/γ′ is constitutively secreted due lack of the signal for GPI-anchor attachment.

The affinities of FA for the FRs are: FRα, $K_d \sim 0.1$ nM; FRβ, $K_d \sim 1$ nM; and FRγ, $K_d \sim 0.4$ nM.[45] Importantly unlike the reduced folate carrier (RFC), which mediates transmembrane folate transport, has a K_d in the μM range and is ubiquitously expressed, FR is not normally required for cellular survival and for this reason their expression is highly restricted among tissues. Furthermore, the $>10^3$-fold higher affinity of FRs for folate enables *in vivo* targeting of the FRs via folate conjugation, not being affected by the presence of RFC in non-target tissues.[45]

Folate mediated targeting

FA is a high affinity ligand for the FR, and even after derivatization via one of its carboxyl groups, folate retains a high affinity. Although the K_d of folate conjugates for FRs is higher than FA (~10×), indicating a slight reduction in the binding affinity, this is still within the nM range. FRs mediate cellular internalization of folate conjugates via receptor-mediated endocytosis. In this way, folate conjugation constitutes a valid method for targeted delivery of therapeutic agents to FR expressing cells.[45] Receptor-mediated endocytosis of folate conjugates occurs through a succession of distinct steps, beginning with conjugate binding to a cell surface FR (Figure 2).[55] As a GPI anchored protein, FR internalization is thought to use clathrin independent carriers (CLIC) and GPI-anchored protein-enriched early endosomal compartment (GEEC) pathway.[56] After membrane invagination and internalization to form an endocytic vesicle, the pH of the vesicle lumen decreases through the action of proton pumps localized in the endosome membrane. This acidification mediates a conformational change in the FR protein allowing the release of bound ligand and its delivery in the cytosol.[57] Finally, the FR recycles back to the cell surface, allowing the delivery of additional folate conjugates into the cell.[55]

Besides their high affinity for its receptor, other properties make FA an attractive ligand for use in drug targeting. These features include its convenient availability, low molecular weight, easy conjugation chemistry, lack of immunogenicity, water solubility, stability in diverse solvents, pH and temperature. Furthermore, the small size of the folate ligand also allows good tissue penetration and rapid clearance from receptor negative tissues.[55] Therefore, these desirable properties render folate as one of the most studied ligands in targeted drug delivery. A wide range of molecules and drug carriers have been conjugated to folate and tested in FR targeting.[45]

Folate therapies

RA therapies, while directed at reducing joint inflammation and joint damage, have undesirable systemic effects that increase the risk of adverse events. Therefore, there is a need for improved measures of disease control, as well as methods to better target therapies just for involved tissues.[58] The selective neutralization of synovial macrophage activation is an appealing approach for diminishing local and systemic inflammation as well as for preventing irreversible joint damage.[59] Although macrophages are crucial to inflammation process, none of the available biological therapies specifically target synovial macrophages in RA. Their plasticity makes them an ideal target for the treatment of inflammation, especially arthritis.[40] Some therapies are designed to eliminate the entire population of macrophages. However, since they are involved in several processes, ranging from wound repair to defense against pathogens, this can cause severe adverse effects. Therefore, while depletion of cells from monocytic lineage might not cause toxicity over a short period of time, continuous elimination of these cells for long time would lead to severe consequences.[46] Specific elimination of the sub-population of chronic activated macrophages constitutes an alternative to the depletion of the entire macrophage population. Delivery of therapeutic agents specifically to pro-inflammatory cells would avoid toxicity and side damage to healthy cells.[60]

As described above, previous studies described that inflamed joints of RA patients present a subpopulation of activated macrophages expressing a receptor for the vitamin FA. Once just

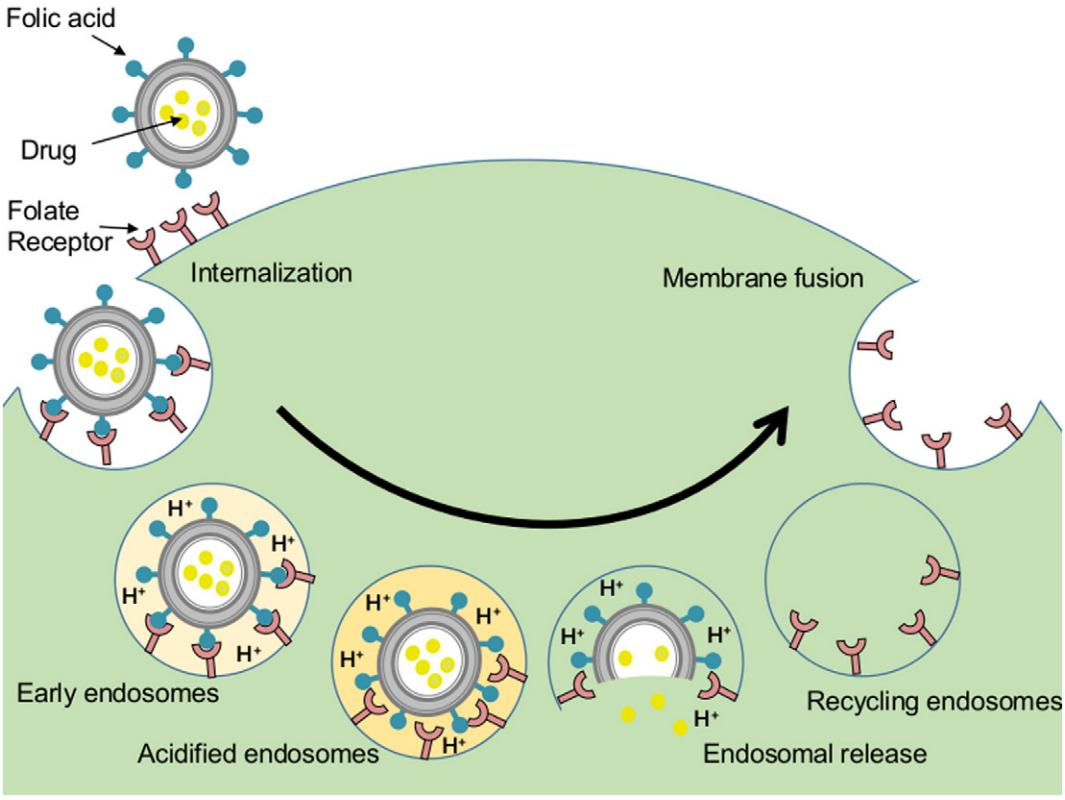


Figure 2. Schematic representation of folate mediated endocytosis.

few cell types also expressed the FRβ, accumulation of FR macrophages in arthritic joints allowed the selective targeting of folate linked imaging and therapeutic agents to these sites of inflammation.[37] In this way, folate-targeted therapies, selectively attack the pathologic cell type, leaving the healthy macrophages unharmed. Furthermore, since no other population of white cells appears to express a functional FRβ, the level of toxicity associated with folate-targeted therapy appears to be very low.[46] Recently, Low and colleagues developed a functional recombinant antibody with high specificity to human FRβ and demonstrated that this antibody selectively binding to inflammatory monocytes and activated macrophages from the synovial fluid of patients with rheumatoid arthritis.[61] Furthermore, other studies describe the development of dsFv anti-FRβ-targeted Pseudomonas exotoxin A (recombinant immunotoxin conjugated to a fragment of an anti-FRβ antibody),[62] folate hapten-mediated immunotherapies[63,64] and anti-folates designed to bind FR.[65] These data suggest that FRβ therapies can be applied as a research method for effective targeting of activated macrophages during inflammatory disease progression. Furthermore, arthritic joints are readily visualized with folate-targeted radiopharmaceuticals in patients with RA (^{18}F-polyethylene, PET tracer [66] and glycol-folate ^{99m}Tc-EC20, FolateScan[58]), constituting a good indication for a successful response to folate-targeted immunotherapy in humans. Although each of the abovementioned approaches holds promise for yielding new therapeutic options for RA patients, there have been few reports on the use of FA for targeting nanoparticles, as delivery systems of therapeutic agents to sites of inflammation.

Current RA therapy

The treatment of RA in the last years is characterized by a firm evolution of new agents and new approaches.[67] Progress in knowledge about cellular and molecular mechanisms of RA and the development of new therapies have changed the overview of scientific community about RA. Discoveries concerning its pathogenesis have led to the development of new agents with specific molecular targets, which have transformed the prognosis for numerous RA patients. Treatment paradigms in RA have shifted dramatically from controlling symptoms (using nonsteroidal anti-inflammatory drugs, NSAIDs, and corticosteroids) for controlling the disease process with the suppression of inflammation (disease-modifying antirheumatic drugs, DMARDs, and biologics),[68] in order to prevent joint damage. These changes in RA management result from growing evidences suggesting that early RA identification and treatment with DMARDs leads to improved prognosis and outcomes. Therefore the aims in RA management besides disease remission also include an improved functional status. The new criteria for the classification/diagnosis of RA proposed in 2010[69] reflect a probabilistic method to RA diagnosis and are specifically useful before the erosions that are typical of RA become detectable on X-rays. They include four scored areas: symptom duration (< or >6 weeks), number and type of joints involved, biomarkers of inflammation (acute-phase reactants), and biomarkers of specific autoimmunity (RF and ACPA).

Methotrexate (MTX) is the first line therapy indicated for the treatment of RA, but other options include leflunomide,

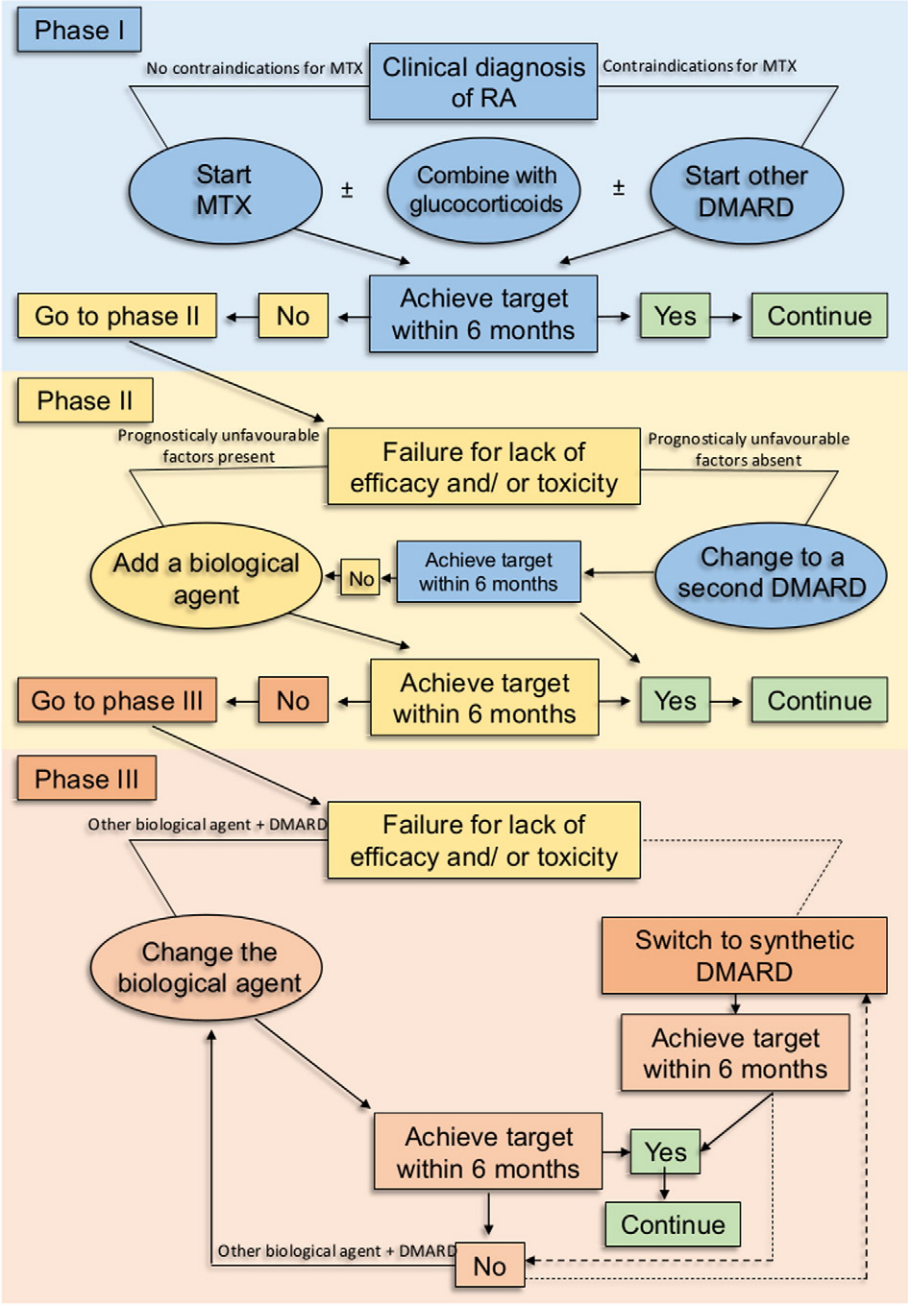


Figure 3. Algorithm based on the 2013 EULAR recommendations on RA management.

sulfasalazine, and hydroxychloroquine.[70] MTX is an analogue of folate and, hence, has structural and physiochemical properties considerably similar to those of folate; it has two carboxyl groups in its molecule and both of them are most completely dissociated in the physiological conditions.[71] The mechanisms proposed to explain the effects of MTX include (i) inhibition of proliferation of the inflammatory synovial cells due to inhibition of purine and pyrimidine synthesis; (ii) inhibition of the synthesis of polyamines; (iii) changes in cellular redox state and reduction in intracellular glutathione levels, leading to decreased macrophage and lymphocyte recruitment function and increased apoptosis sensitivity; and (iv) inhibition of the enzyme aminoimidazole carboxamide ribonucleotide (AICAR) transformylase, consequent elevation of AICAR cellular levels, resulting inhibition of AMP deaminase and ultimately leading to an increase in extracellular adenosine levels.[68]

Patients receiving MTX therapy should be reexamined after 3 months of therapy for symptomatic improvement (Figure 3). However, the toxicity associated with MTX administration can be minimized if it is dosed correctly and monitored appropriately. Major toxic effects, such as hepatic, pulmonary, renal and bone marrow abnormalities,[72] require careful monitoring. Minor

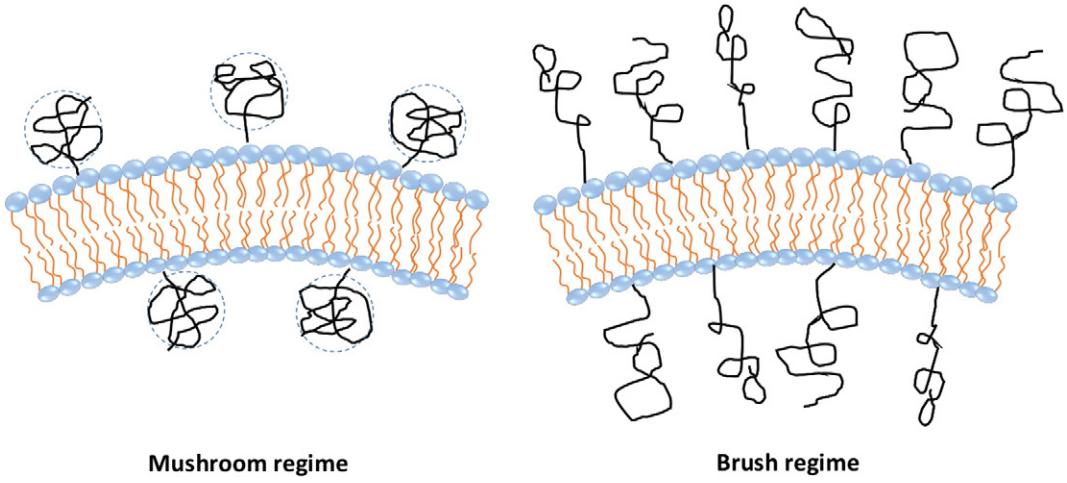


Figure 4. A schematic diagram of a PEG-grafted bilayer at low grafting concentration (mushroom regime) and a PEG-grafted bilayer at high grafting concentration (brush regime).

toxic effects, such as stomatitis, malaise, nausea, diarrhea, headaches and mild alopecia, are common but respond to folate supplementation.[68,73] Other effects include gastrointestinal or bone marrow toxicity, pneumonitis, hepatotoxicity and cirrhosis.

If patients are MTX intolerant or have moderate or high disease activity after 3 to 6 months of therapy another DMARD should be used or added, or alternatively biologic agents.[74] In RA therapy biological agents used are anti-TNF-α molecules, responsible for the neutralization of TNF-α, the master regulator of RA immunopathogenesis. Anti-TNF-α agents fall into three structural categories: anti-TNF-α IgG antibodies (the monoclonal antibodies (mAbs) infliximab, adalimumab, and golimumab), PEGylated Fab′ fragments (certolizumab), and modified TNF-R2 receptors (etanercept).[68,75] Furthermore, biological agents include an inhibitor of T-cell costimulation (fusion protein composed of the Fc region of the immunoglobulin IgG1 fused to the extracellular domain of CTLA-4, abatacept), an agent leading to B-cell depletion (chimeric monoclonal antibody against the protein CD20, rituximab) and the IL-6 receptor (IL-6R)-blocking monoclonal antibody (tocilizumab), as well as the IL-1 inhibitor (anakinra).[67] The implementation of these effective biological agents has been accompanied by ongoing health economic discussions regarding the implementation of these highly effective, but accordingly, highly priced drugs in the standard treatment guidelines of rheumatic diseases.[76] Despite their high clinical effectiveness, the cost efficacy of biologicals is questionable bearing in mind that this therapy costs are 20-200 fold compared to traditional DMARDs.[77]

Stealth nanoparticles

The application of nanotechnology in healthcare is an emerging area and the process of replacing traditional therapies has already begun.[78] Efficient drug delivery is one of the most prominent problems confronted by the biotechnological and pharmaceutical industries. Therefore, nanotechnology can repurpose the utilization of the myriad existing drugs produced by these industries.[78] Nanotechnology focuses on formulating therapeutic agents in biocompatible nanocarriers. Nanoparticles applied as drug delivery systems are submicron-sized particles, devices, or systems that can be made using a variety of materials. These materials include polymers (polymeric nanoparticles, micelles, or dendrimers), lipids (liposomes), viruses (viral nanoparticles), organometallic compound (nanotubes) and even inorganic compounds (gold nanoparticles, quantum dots).

Nanoparticle drug delivery systems constitute one of the most widely researched methods for improving circulation time, bioavailability and targeting of several therapeutic agents. Therefore, nanoparticles offer many advantages over free drug administration. Remarking, nanoparticles are capable of: (i) encapsulate and protect drugs from degradation or deactivation before to reaching target site *in vivo*, (ii) improve targeting over free drugs via presentation of tissue-specific targeting ligands, (iii) offer controlled drug release by altering nanoparticle composition, and (iv) be produced in large, reproducible, batches.[79] For all these reasons, nanoparticles hold the potential to be the ideal drug delivery carrier. However, the rapid clearance of nanoparticles from blood and limited targeting to specific tissues has prevented the widespread application of nanoparticles in the clinic.[80]

Application of unmodified nanoparticles is limited by their rapid recognition by macrophages of the MPS[81,82] within few hours of administration.[83] The main sites of nanoparticle clearance are liver and spleen, where macrophages are in direct contact with the bloodstream.[84] Numerous interesting approaches for design and engineering of long circulating nanoparticles have been described. However, the surface stabilization of nanoparticles with a range of nonionic surfactants or polymeric macromolecules has proved to be one of the most successful approaches for keeping the particles in the blood for long periods of time.[80,85] PEG has unique physical properties, being commonly used to improve the stability and biological performance of colloidal drug carriers. The grafting of PEG to the surface of a colloidal carrier is clearly shown to extend the circulation lifetime of the vehicle.[83] The ability of PEG to fulfil this role has been attributed mostly to its physical properties such

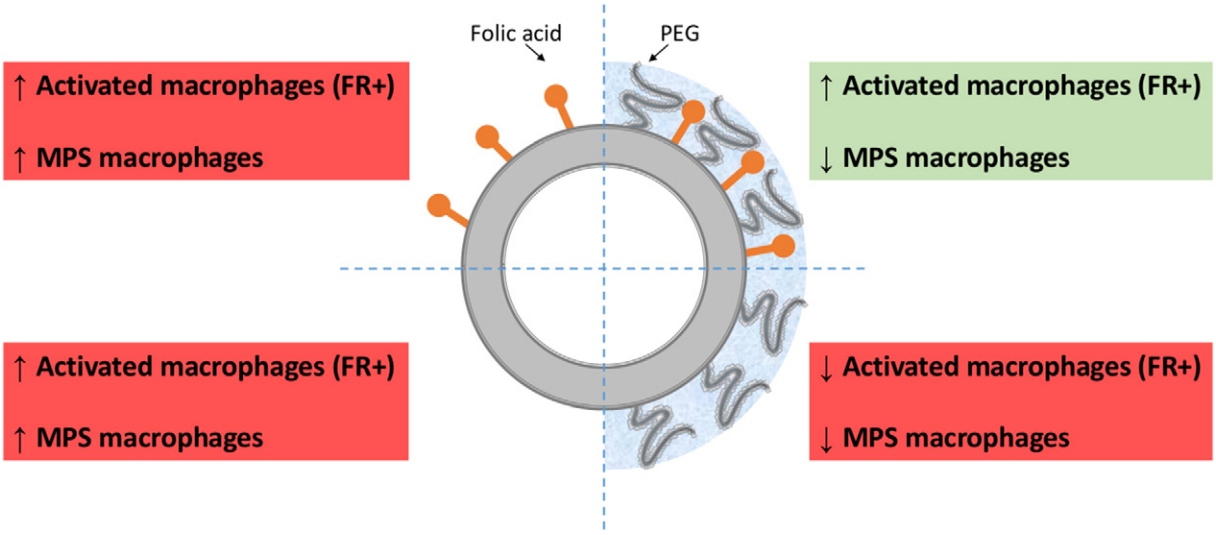


Figure 5. Influence of stealth degree in specificity of folate target nanoparticles to FRβ activated macrophages.

as unlimited water solubility, large excluded volume and high degree of conformational entropy.[86] Although some describe the reduction or prevention of protein adsorption,[87] there is little evidence that the presence of PEG at the surface of a vehicle actually reduces total serum protein binding.[88] Others have shown that the steric barrier that PEG provides prevents aggregation of colloidal carriers and thus enhances their stability [89] More recently, some studies have suggested a "dysopsonization" phenomenon where PEG actually promotes binding of certain proteins that then act to mask the vehicle.[90] In a recent study we demonstrate a decrease internalization of PEGylated nanoparticles by activated MPS macrophages, which could be used for the development of drug delivery systems with improved qualities for systemic administration like intravenous application.[91] To remark that, the degree of macrophage uptake depends on the concentration of PEG in liposomes: a concentration of 10% PEG decreased uptake by macrophages to 13%, unlike 90% is observed for liposomes with 5% PEG. We showed that increasing PEG concentration clearly improved the stealth degree of nanoparticles, as the internalization of nanoparticles by macrophages is greatly reduced. This is in good agreement with the current scaling models for polymers at interfaces, which predict a mushroom-brush transition in PEG conformation at 5% of PEG-lipid, when PEG coils start to repel each other and extend out from the surface on which they are grafted.[88] The polymer density determines the regime: if the polymer density is low it is said to be in the mushroom regime, when the graft density is high the polymers are said to be in the brush regime (Figure 4).[92]

Nanoparticle populations bearing a predominant surface of PEG molecules as high brush configuration are most resistant to phagocytosis and poorly activated the human complement system. In opposition, those populations with a predominant surface PEG in a mushroom regime are potent activators of the complement system and are prone to phagocytosis.[93] Therefore, surface heterogeneity explains why liposomes with 5% PEG are rapidly internalized by macrophages, while the presence of 10% PEG reduces significantly their internalization. When we tested PEG concentration to improve the delivery specificity of folate based nanoparticles to activated FRβ-expressing macrophages, we verify that PEG at 10% greatly improved the stealth degree of the liposomal nanoparticles, thereby reducing the non-specific uptake, and promoted the specificity of FA-mediated targeting.[94] We also measured the uptake of liposomal nanoparticles with the improved PEG formulation in the monocytic cell line THP-1 with and without the overexpression of human FRβ. Compared to control, liposomal nanoparticles with folate were highly internalized in THP-1 cells retrovirally transformed with FRβ in comparison with the wild-type THP-1 cells that weakly express FRβ showing minimal uptake similar to the Jurkat T cells used as a negative control (Figure 5).[94]

Furthermore, our results (unpublished data) demonstrate that to contrast to MTX, the liposomes are selectively retained in plasma and are not subject to immediate absorption and filtering by the main organs (Figure 6). This means that the liposomes can circulate to their peripheral target tissue and be bound there instead of being non-selectively absorbed by the intestine, liver, kidney and brain. In an ideal pharmacological system the compound would be present at its target in low but stable amounts sufficient to exert an effect. Excess amounts would then be no longer available for the main metabolic organs, which are anyway not involved in the pathological response.

After systemic administration, the nanoparticle drug delivery system has to deliver the drug to the site of action. To achieve this, the so-called "passive targeting" phenomenon can be employed.[95] The most common passive targeting strategy is the Enhanced Permeability and Retention (EPR) effect exploited in oncology field, which take advantage of the leaky vasculature of tumor areas to enhance nanocarrier accumulation within the interstitial space of tumors.[96,97] The EPR phenomenon also occurs in other diseases where inflammatory processes have disrupted the permeability barrier of the vascular endothelium,

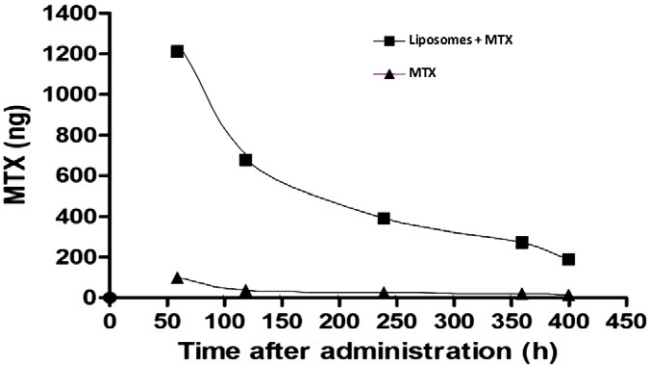


Figure 6. Pharmacokinetics of MTX when provided as free drug dissolved in serum, and encapsulated in liposomes. All materials were injected *i.v.* at a dose of 0.6 mg/kg MTX.

such as in RA.[98] Nanoparticles are retained in the extravascular space with a large portion being taken up by macrophages in the synovial layer.[95]

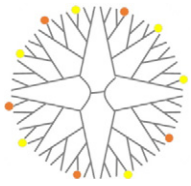
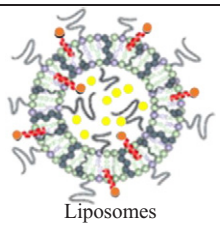
Folate-targeted nanoparticles

In the last years, two key studies describe the use of folate-nanoparticles to specific targeting of FRβ macrophages and improve MTX clinical benefit in arthritic mice (Table 2). Thomas T. and colleagues describe in 2011 the development of a folate-conjugated dendrimers to target macrophages in inflammatory disease of arthritis.[99] The poly(amidoamine) (PAMAM) dendrimer (generation 5 [G5]) nanoparticle covalently conjugated to polyvalent FA shown to be bound and internalized in a receptor-specific manner into both FRβ-expressing macrophage cell lines and primary mouse macrophages. Furthermore, the conjugate G5-FA-MTX acted as a potent anti-inflammatory agent and reduced arthritis-induced parameters of inflammation such as ankle swelling, paw volume, cartilage damage, bone resorption, and body weight decrease. Although dendrimers have been studied as drug delivery systems, some concerns remain regarding their safety for therapeutic use. In particular, the conjugation of ligands and therapeutic agents at the dendrimer surface do not protect them from degradation or deactivation prior to reaching target site *in vivo*. Furthermore, although the dendrimer from higher generations have some primary surface amino groups to conjugation, they limit broadly the molecules of ligands and therapeutic agents. Additionally, it was known that the size and charge of PAMAM dendrimers influence their cytotoxicity. The higher-generation (G4-G8) PAMAM dendrimers exhibit toxicity due to their high cationic charge density.[100] Finally, as described above, unmodified nanoparticles do not survive long in circulation, but instead are removed by macrophages of the RES. The dendrimers developed in this study are not stealth. This critical point could, at least in part, justify the incomplete inhibition of free FA to prevent target-dendrimers uptake by FRβ-expressing macrophages.

More recently, we report the encapsulation of MTX in a new liposomal formulation using a hydrophobic fragment of surfactant protein conjugated to a linker and folate to enhance their tolerance and efficacy.[94] Liposomes have gained extensive attention as carriers for a wide range of drugs due to being both nontoxic and biodegradable as they are composed of substances naturally occurring in biological membranes.[101] Biologically active materials encapsulated within liposomes are protected to a varying extent from immediate dilution or degradation, which makes them good drug delivery systems for the transport of bioactive compounds to pathologically affected organs.[102,103] The unique ability of liposomes to entrap drugs both in an aqueous and a lipid phase makes such delivery systems attractive for hydrophilic and hydrophobic drugs.[104] Our delivery system proved to be more efficient than classic systems where the FA is linked to liposomes by PEG.[105] The combination of all complementary characteristics of these tailored liposomes, including their small size, lack of cytotoxicity and their specific targeting of FRα-expressing cells[105], led us to evaluate the efficiency of this system to treat RA, by targeting FRβ present at the surface of activated macrophages. The presence of 10% PEG greatly improved the stealth degree of the liposomes, thereby reducing the non-specific uptake, and promoted the specificity of FA-mediated targeting. To test the specificity of these new liposomes in a pathological context, arthritis was induced in mice (CIA model), and the results shown that liposomes strongly accumulated in their joints (Figure 7, *A*).[94] Furthermore, the analysis of cell populations from inflamed joints of arthritic mice revealed that macrophages expressing high levels of FRβ are more prone to uptake FR-targeted than the non-targeted liposomes.

To prove the ability of our liposomal formulation as drug delivery system, liposomes encapsulating MTX were administered in arthritic mice, before disease onset. Complete prophylactic efficacy was observed in mice treated with FA-target liposomes encapsulating MTX, where mice did not shown any clinical signs of arthritis (Figure 7, *B*). Comparatively, when the drug was injected in a soluble form it only had a marginal effect and did not prevent the development of arthritis (Figure 7, *B*). This fact leads us to believe that encapsulation of MTX in our proposed formulation offers a cost effective way to treat arthritis

Table 2
Comparison of two folate-nanoparticles to specific targeting of FRβ macrophages studied to therapy of arthritic mice.

Nanoparticle	 PAMAM dendrimers (Thomas T. *et al*, 2011)	 Liposomes (Nogueira E. *et al*, 2015)
Drug protection	No *At surface*	Yes *Encapsulation*
Ligand/drug concentration	Limited *Conjugation to amino groups*	High *Integration/encapsulation*
Toxicity	High *High cationic charge density*	Low
Stealth	No	Yes *PEGylation*

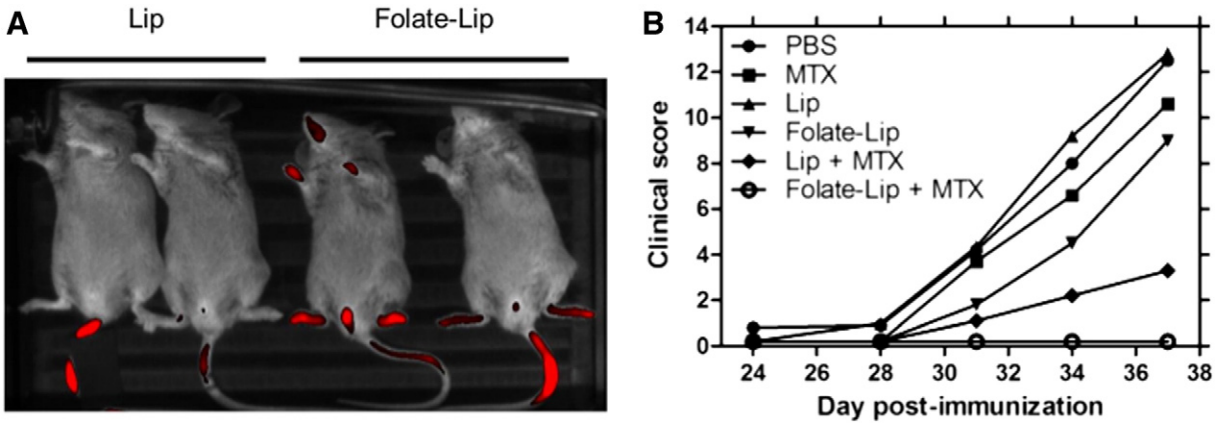


Figure 7. *In vivo* specific targeting and prophylactic efficiency of FR-targeted liposomes in arthritic mice. **(A)** *In vivo* uptake specificity of fluorescently labeled liposomes (30 min). **(B)** Clinical effects of liposomes encapsulating MTX on arthritis. Treatment started 14 days after immunization. The mean clinical score in each group over time is shown. Adapted with permission from Nogueira E. *et al.*[94]

and delay or reduce MTX intolerance. The results presented here might have an important implication in clinical practice, where available biological therapies could be delayed or completely replaced by the proposed MTX liposomal formulations tested in this work. It brings new hope to a large number of patients who become intolerant to MTX and require much more expensive treatments with biological agents.

Perspectives and future directions

As summarized above, activated but not resting macrophages express a FRβ, which can be exploited to deliver folate-targeted nanoparticles, as specific drug delivery systems to RA therapy. However, specific targeting of activated macrophages constitutes a big challenge, because they are phagocytic cells that internalize any strange particle. Thus it is imperative a total stealth degree, to avoid the clearance of nanoparticles by macrophages of the RES, thereby reducing the non-specific uptake. We verify that, to contrast to 5%, just 10% PEG ensures a proper stealth degree of the liposomal nanoparticles and promoted the specificity of FA-mediated targeting to FRβ activated macrophages. Furthermore, in contrast to free forms, encapsulated drugs are selectively retained in plasma and are not subject to immediate absorption and filtering by the main organs, suggesting that therapeutic agents would be present at its target in low but stable amounts sufficient to exert an effect.

This fact is particularly important in RA, where the first line therapy, MTX, presents several side effects, as potential toxicity and possible depression of the bone marrow or leading to hepatitis and liver function. Moreover, if patients are MTX intolerant another DMARD should be used or added, or alternatively biologic agents. However, the development of biological substances for the treatment of rheumatic conditions has been accompanied by ongoing health economic discussions regarding the implementation of these highly effective, but accordingly, highly priced drugs in the standard treatment guidelines of rheumatic diseases. In this way, the recent strategies of folate-targeted nanoparticles with MTX were effective to improve inflammatory disease treatments while decreasing the MTX side effects with an improved cost–benefit ratio. Furthermore, these nanoparticles exhibit outstanding pharmacokinetics relative to MTX in its current forms, which may prevent side effects due to specific FA-mediated targeting. The promising prophylactic results, obtained with liposomes encapsulating MTX, encourage to do further studies to analyze their therapeutic effect, after the disease onsets. MTX repurposing, an improvement by formulation, may have a number of research and development advantages such as reduced time to market, reduced development risk and cost (clinical safety and efficacy data are established), and the improved probability of success. Furthermore, folate-targeted nanoparticles open hope to repurposing of myriads of drugs used in RA therapy give up due their side effects. Besides of MTX, hydrophilic drug, FA-target liposomes demonstrate to be efficient in the encapsulation of hydrophobic drugs, like as celecoxib and carbon monoxide-r-eleasing molecules (CORM)-2, and specific delivery them in Caco-2 cancer cells.[105] In addition, our unpublished results demonstrate the success use of FA-targeted liposomes for specific delivery of small interfering RNA to activated macrophages. The effect of myeloid cell leukemia-1, Mcl-1, small interfering RNA (essential for synovial macrophage survival), either free or incorporated in liposomal formulation, was tested in primary human macrophages and successful inhibition of Mcl-1 expression was obtained.

Because the activated macrophages may contribute prominently to many other autoimmune and inflammatory diseases, these technologies may also be very useful for neglected patient classes in a range of orphan auto-immune diseases, like myasthenia gravis, primary biliary cirrhosis, Sjögren's syndrome, Behcet's disease, systemic lupus erythematosus, and Graves' disease. Some of these diseases also affect arthritis patients who would be especially benefited. If elimination or suppression of the activated macrophages can improve the symptoms of these autoimmune diseases also, we believe that folate-targeted nanoparticles, encapsulating other therapeutic agents might someday be available for the management of multiple unwanted inflammatory processes. Furthermore,

activated macrophages are pivotal cells in tumor-associated inflammation, a well-recognized hallmark of cancer progression.[106,107] Since FR is also overexpressed in many cancer cells, it constitutes one of the more attractive cancer molecular targets. In this way, folate-targeted nanoparticles also open new clinical avenues for diagnosis and treatment of cancer.

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