Novel Experimental Therapeutics and PET Imaging of Activated Macrophages in Rheumatoid Arthritis

Durga MSH Chandrupatla
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Durga Chandrupatla

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Novel Experimental Therapeutic and PET Imaging of Activated Macrophages in Rheumatoid Arthritis

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door

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Chapter 1

Aim and thesis outline
The aim of this thesis is to study the feasibility of positron emission tomography (PET) of folate receptor β (FRβ) on activated macrophages for (early) diagnosis and assessment of disease activity in rheumatoid arthritis (RA) as well as monitoring of (new) therapeutics. These studies were conducted in an antigen-induced arthritis model in rats. To target FRβ with PET imaging, we applied folate-based PET tracer ([18F]fluoro-PEG-folate).

The thesis has been divided in three subsections: I - PET therapeutic evaluation in an arthritic rat model, II - Evaluation of novel therapeutics in an arthritic rat model, and III - Immunophenotyping monocytes/macrophages in RA patients. In each section we focused on the role of macrophages and FRβ in relation to either imaging or targeting in RA.

Section I

Chapter 2 gives an overview on the position of FRβ as imaging and therapeutic target on (activated) macrophages, discussing its functional properties, macrophage expression and polarization.

Chapter 3 describes the establishment and validation of an improved rat model of RA for PET-guided therapy evaluation with sustained articular macrophage infiltration thereby providing a prolonged window for therapy response monitoring with macrophage PET tracers.

Chapter 4 describes the first response monitoring study for a currently used therapeutic agent in RA (MTX) with the folate-based PET tracer ([18F]fluoro-PEG-folate targeting FRβ in the improved rat model of RA and moreover the systemic inflammation seen in this model.

Chapter 5 describes results in this model with [18F]fluoro-PEG-folate and PET targeting articular inflammation. Specifically, the impact of the folate antagonist methotrexate (MTX), the anchor drug in RA treatment, was investigated by PET imaging, ex vivo tissue distribution of the tracer, and reduction of synovial macrophage infiltration examined by ED1, ED2, immunohistochemistry and FRβ immunofluorescence.

Section II

In Chapter 6, we report on alkaline phosphatase (AP) as a prophylactic or therapeutic modality both as single agent and in combination with MTX, using [18F]fluoro-PEG-folate PET.

In Chapter 7, we studied the targeted delivery of radiolabeled F8-IL10, a conjugate of an antibody fragment F8 (binding to the extra-domain-A of fibronectin) and the anti-inflammatory cytokine IL10, at inflammatory sites in the RA rat model as well as in RA patients.
Section III

Chapter 8, the expression of FRβ was studied on monocyte/macrophage subpopulations in peripheral blood of RA patients compared with healthy controls. Moreover, we examined FRβ expression on macrophages in RA synovial tissue sections in conjunction with other macrophage marker expression related to macrophage polarization.

Chapter 9 gives a summary of the work described in this thesis with discussions and future perspectives followed by key points highlighting the most important findings of this thesis.
Chapter 2

Folate receptor $\beta$ as a macrophage mediated imaging and therapeutic target in rheumatoid arthritis

Submitted

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Abstract

Macrophages play a key role in the pathophysiology of rheumatoid arthritis (RA). Notably, positive correlations have been reported between synovial macrophage infiltration and disease activity as well as therapy outcome in RA patients. Hence, macrophages can serve as an important target for both imaging disease activity and drug delivery in RA. Folate receptor β (FRβ) is a glycosylphosphatidyl (GPI)-anchored plasma membrane protein being expressed on myeloid cells and activated macrophages. FRβ harbours a nanomolar binding affinity for folic acid allowing this receptor to be exploited for RA disease imaging (e.g. folate-conjugated PET tracers) and therapeutic targeting (e.g. folate antagonists and folate-conjugated drugs). This review provides an overview of these emerging applications in RA by summarizing and discussing properties of FRβ, expression of FRβ in relation to macrophage polarization, FRβ-targeted in vivo imaging modalities, and FRβ-directed drug targeting.

Keywords: Rheumatoid Arthritis, Macrophages, Folate Receptor β, Positron Emission Tomography (PET), Synovial Tissue

Abbreviations
CD - Cluster of Differentiation, CTLA4 - Cytotoxic T-Lymphocyte Antigen 4, DMARDs - Disease Modifying anti-Rheumatic Drugs, FRβ - Folate Receptor β, GPI - Glycosylphosphatidylinositol, GM-CSF - Granulocyte Macrophage - Colony Stimulating Factor, HLA-DRB1 - Human Leucocyte Antigen DRB1, IL - Interleukin, “M1-type” macrophage - pro-inflammatory macrophages, “M2-type” macrophages - anti-inflammatory macrophages, M-CSF - Macrophage - Colony Stimulating Factor, MTX - Methotrexate, MRI - Magnetic Resonance Imaging, NSAIDs - Nonsteroidal Anti-Inflammatory Drugs, PADI4 - Peptidyl Arginine Deaminase type 4, PET - Positron Emission Tomography, PTPN22 - Protein Tyrosine Phosphatase, Non-Receptor type 22, RA - Rheumatoid Arthritis, SE - Shared epitope, STAT4 - Signal Transducer and Activator of Transcription 4, TNFα - Tumor Necrosis Factor α, TNFAIP3 - TNF Alpha Induced Protein 3.
Chapter 2 Folate receptor β in RA

Figure 2.1 – Onset of rheumatoid arthritis and positioning of macrophage imaging for early disease monitoring

1. Rheumatoid arthritis

Rheumatoid arthritis (RA) is an autoimmune disease, which affects approximately 0.5-1.0% of the world population [1]. Although the exact aetiology of RA is unknown, the currently accepted hypothesis consists of two stages [2]. In genetic susceptible individuals, the first stage of development of RA consists of accelerated citrullination of proteins in extra-articular sites, e.g. due to smoking or infection, including formation of rheumatoid factor (RF), anti-citrullinated protein antibodies (ACPA) and anti-carbamylated proteins (α-CarP) [3–6]. Only 40% of ACPA positive arthralgia individuals will eventually develop RA [7]. A second trigger seems to be needed for development of clinical disease. Up to 15 years later, the second trigger could be an unrelated episode of otherwise self-limiting synovial inflammation and associated locally induced citrullination. In the presence of pre-existing anti-citrullinated protein/peptide antibodies this event may induce chronic synovitis evolving into clinical RA through binding of the antibodies to autoantigens in the joints [8–10] (Figure 2.1).

To detect development of (subclinical) synovitis, advanced imaging techniques may have diagnostic value on top of detection of ACPA. Application of ultrasonography and MRI techniques in preclinical RA have been discussed in recent reports [11,12], whilst
application of Positron Emission Tomography (PET) will be discussed in detail below. RA’s main characteristics include (chronic) inflamed synovium and joint destruction, which, when left untreated, can lead to permanent joint deformities and comorbidities, such as cardiovascular disease and osteoporosis [10]. Early identification and treatment of RA is currently recommended to prevent further joint damage and disability [13]. To this end, the European League Against Rheumatism (EULAR) guidelines indicate treatment with classical Disease Modifying Anti-Rheumatic Drugs (DMARDs) (e.g. methotrexate (MTX)), biologicals DMARDs (e.g. Infliximab, Rituximab, Tocilizumab and Secukinumab) and targeted synthetic DMARDs (e.g. Janus kinase inhibitors), either as monotherapy or in combination therapy [14]. Despite this wide spectrum of potential therapeutic agents that are currently available, response to treatment usually varies between 50 and 70%. This is probably related to factors such as the heterogeneous character of RA, the stage of the disease and the presence of anti-drug antibodies. To increase treatment efficacy and to reduce costs, monitoring tools, e.g. imaging, are needed in order to select responders and non-responders in an early phase of treatment.

2. Immune cells & RA

In RA, the inflamed synovium harbours several immune cell types, especially B and T lymphocytes, neutrophils and macrophages [8]. T lymphocytes orchestrate production of pro-inflammatory cytokines such as IL17, triggering activation of synovial fibroblasts and production of tumour necrosis factor α (TNFα), IL15 and IL18 [15]. B lymphocytes primarily release autoantibodies such as rheumatoid factor and promote T cell activation [16]. Synovial macrophages are dominant producers of TNF-α [17–23] and mediate the crosstalk with B and T lymphocytes via production of pro-inflammatory cytokines such as IL23 and immune complexes, respectively [22]. Moreover, macrophage production of IL1β and TNFα mediates synovial fibroblast proliferation and activation. These promote osteoclast formation and activation, which drives bone and cartilage destruction [22]. Given the prominent role of macrophages in RA pathophysiology, their non-invasive visualization can hold promise for early RA disease monitoring (Figure 2.1).

3. Macrophage PET imaging in RA

In RA, synovial macrophage infiltration is a hallmark of the disease, reflecting disease activity in early and established stages, being a sensitive biomarker for assessment of response to therapy [24–26]. Therefore, macrophage imaging could serve as an important clinical and diagnostic tool as well as a tool for guiding therapy in RA. PET is a non-invasive, in vivo imaging modality, with high sensitivity to detect active arthritis both at early or advanced stages of RA. It also has the ability to quantify tracer uptake, which is essential for intervention studies, i.e. for monitoring disease activity and therapy response in the whole body [27–30]. While ultrasound and MRI cover mostly detection of anatomical changes in synovial tissue [31], PET imaging allows for quantitative detection and monitoring of molecular targets. Various PET tracers have been developed to image RA. Initial macrophage-directed PET studies used [18F]FDG (measuring glucose metabolism in inflammatory sites) to visualize inflamed RA joints with results corresponding to clinical findings, thus providing evidence for the usefulness of
PET in detecting synovitis [32–34]. This tracer showed high sensitivity, but low specificity for arthritis imaging [32]. Subsequently, PET studies were extended by using more macrophage-specific tracers (Table 2.1).

The first class of potential macrophage tracers was targeted towards the 18-kDa translocator protein (TSPO, formerly known as peripheral benzodiazepine receptor), an outer mitochondrial membrane protein that is upregulated in activated macrophages [45,46]. \(^{(R)}\)\([-^{11}C]\)PK11195 is the prototypical TSPO tracer that was employed in preclinical RA models [36,47–51]. In a clinical setting, significantly higher \((R)\)\([-^{11}C]\)PK11195 uptake was observed in severely inflamed joints of RA patients than in moderately or mildly inflamed joints, which correlated with the extent of macrophage infiltration in excised synovial tissue [37]. In addition, subclinical disease activity could be shown when contralateral uninflamed knee joints of RA were compared with non-inflamed joints of healthy controls [37]. However, \((R)\)\([-^{11}C]\)PK11195 showed limitations in detecting subclinical synovitis in RA. In particular, considerable background uptake was seen in peri-articular tissue both in a rat model of arthritis [42] and in RA patients [29]. To overcome these limitations, a second generation of TSPO tracers was developed, with \([^{11}C]\)DPA713 and \([^{18}F]\)DPA714 [50,51] having been evaluated in preclinical RA models [36][52]. Herein, both \([^{11}C]\)DPA713 and \([^{18}F]\)DPA714 were superior to \((R)\)\([-^{11}C]\)PK11195, but this still needs to be confirmed in a clinical setting.

In search for novel macrophage PET tracers in RA, macrophage markers identified on activated microglia can be helpful, e.g. CB2R and A2AR (G-protein-coupled receptors), P2X7R (purinergic ion channel receptor) or matrix metalloproteinases [53]. The focus of the present review is on another emerging (activated) macrophage marker, i.e. the folate receptor, which potentially could also be exploited for imaging and therapeutic targeting purposes in RA [54,55].

4. Folate receptors (general properties)

Folate receptors (FR) belong to a family of two other proteins, i.e. reduced folate carrier (RFC) and proton-coupled folate transporter (PCFT). RFC and PCFT have an established function in membrane transport/internalization of folates required for a variety of biosynthetic reactions and DNA synthesis [56–59] (Table 2.2).

FR, RFC and PCFT differ in membrane orientation, folate substrate affinity, pH optimum and tissue distribution [56,59–61] (Table 2.2). While RFC and PCFT are transmembrane carrier proteins, FR is anchored to the plasma membrane via a glycosylphosphatidylinositol (GPI) anchor [62]. At least 3 isoforms of FR exist; FR\(\alpha\), FR\(\beta\) and FR\(\gamma\), of which the latter is a soluble secreted form because it lacks a GPI-anchoring signal [63]. FR\(\alpha\) and FR\(\beta\) display high binding affinity for folic acid (Kd: 0.1-1.0 nM), but low binding affinity for the folate antagonist methotrexate (MTX) [56,61,64,65]. FRs internalize their substrates via a process of receptor-mediated endocytosis [66,67] or potocytosis [68]. FR\(\alpha\) has a relatively broad tissue distribution profile in normal cells (e.g.
Table 2.1 – PET tracers for macrophage imaging in rheumatoid arthritis

<table>
<thead>
<tr>
<th>Name</th>
<th>PET isotope</th>
<th>Half-time (min)</th>
<th>Binding target</th>
<th>Use</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>FDG</td>
<td>18F</td>
<td>110</td>
<td>Glucose transporter</td>
<td>Glucose metabolism</td>
<td>[33-35]</td>
</tr>
<tr>
<td>(R)-PK11195</td>
<td>11C</td>
<td>20</td>
<td>TSPO</td>
<td>Neuro-inflammation</td>
<td>[36-38]</td>
</tr>
<tr>
<td>DPA713</td>
<td>11C</td>
<td>20</td>
<td>TSPO</td>
<td>Neuro-inflammation</td>
<td>[36–39]</td>
</tr>
<tr>
<td>DPA714</td>
<td>18F</td>
<td>110</td>
<td>TSPO</td>
<td>Neuro-inflammation</td>
<td>[36,40,41]</td>
</tr>
<tr>
<td>PEG-folate</td>
<td>18F</td>
<td>110</td>
<td>Folate</td>
<td>RA, artherosclerosis</td>
<td>[42–44]</td>
</tr>
</tbody>
</table>

Table 2.2: Overview and expression profiling and transport kinetic features of folate transporters

<table>
<thead>
<tr>
<th>PCFT (Proton-Coupled Folate Transporter)</th>
<th>RFC (Reduced Folate Carrier)</th>
<th>FR (Folate Receptor α, β, γ isoform)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Membrane orientation</td>
<td>Localization</td>
<td>pH optimum</td>
</tr>
<tr>
<td>Transmembrane</td>
<td>Enterocytes</td>
<td>7.2 - 8.0</td>
</tr>
<tr>
<td></td>
<td>Immune cells, Tumor cells</td>
<td>7.4 - 8.0</td>
</tr>
<tr>
<td></td>
<td>Kidney (FRα)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Tumor cells (FRα)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Myeloid cells / Activated</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Macrophages (FRβ)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Hematopoietic cells (FRγ, soluble, secreted form)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>KM: 200-400 μM</td>
<td>KM: 1-5 μM</td>
</tr>
<tr>
<td></td>
<td>KD: 0.1-1 nM</td>
<td>KD: 5-10 nM</td>
</tr>
<tr>
<td></td>
<td>KM: 2-10 μM</td>
<td>KM: 50-100 nM</td>
</tr>
</tbody>
</table>
kidney) and cancer cells (e.g. ovarian carcinoma cells) [69], whereas FRβ expression is restricted to hematopoietic cells of the myeloid lineage [70,71]. In fact, FRβ is expressed on monocytes [72], activated macrophages of RA patients [73,74], tumour-associated macrophages [75] and acute myeloid leukaemia (AML) cells [76]. A number of substances have been reported to upregulate FRβ expression, e.g. retinoic acid [77] and curcumin [78], whereas a pluripotent growth factor like activin-A down-regulates FRβ expression [79].

Given the fact that RFC is constitutively expressed on immune cells [80,81], including macrophages [79], and exhibits a much greater folate transport capacity than FRβ [61,74] it is still an unresolved issue whether the primary function of FRβ in macrophages is folate transport rather than other homeostatic or immune-regulatory functions. In addition, considering that macrophages are non-proliferating cells, a role for FRβ in folate uptake for DNA synthesis does not seem of primary importance. In this regard, alternative functions for FRβ have been suggested, although they still lack experimental evidence: (a) delivery of folates for biopterin metabolism, which facilitates reactive oxygen species (ROS) production in macrophages [82] (b) FRβ-mediated scavenging of folates from sites of inflammation to deprive pathogens from nutrients [73], or (c) involvement in signalling processes consistent with the notion that FR, as GPI-anchored protein, is localized in specialized cholesterol-rich membrane invaginations called caveolae, which harbour multiple proteins involved in signalling processes [56,59]. With respect to the latter, a recent study reported that FRβ on macrophages had a functional interaction with CD11/CD18 to regulate cellular adhesion to collagen [83].

Beyond RA synovium, FRβ-expression has been identified on macrophages in inflamed atherosclerotic lesions [84–87] and tumour-associated macrophages [75,88–90], underscoring the fact that FRβ plays a role on macrophages regulating inflammatory processes. Lastly, in mice FRβ expression has been noted on LyC6 myeloid derived suppressor cells (MDCS), a myeloid subset capable of suppressing T-cell activity. So far, expression of FRβ on human MDSCs counterparts has not been examined.

5. Role of folate receptor β in RA

Consistent with FRβ being expressed in hematopoietic cells of the myeloid lineage [70,71], peripheral blood monocytes (PBM’s) from healthy donors and RA patients express FRβ. Based on their CD14/CD16 expression, 3 subclasses of PBM’s were identified; classical (CD14+/CD16-), non-classical (CD14-/CD16+) and intermediate (CD14+/CD16+) monocytes, of which the pro-inflammatory classical monocytes expressed FRβ and were capable of binding folate-linked molecules [72].

This finding provides a rationale for targeting pro-inflammatory FRβ+ monocytes to suppress their infiltration into sites of inflammation, e.g. RA synovium [72]. FRβ positive macrophages were originally identified in RA synovial fluid and assigned a functional role in methotrexate transport [91]. A study by van der Heijden et al [74] showed that FRβ mRNA expression in synovial fluid macrophages and synovial tissue from RA patients was an order of magnitude higher than that of T cells from the same patient.
Immunohistochemical evaluation of synovial biopsies from RA patients confirmed strong FRβ staining of CD68 positive macrophages both in synovial lining and sublining [74]. Importantly, a study by Xia et al [73] revealed that especially activated macrophages rather than quiescent macrophages, in RA synovial fluid had high FRβ expression and concomitant folate-conjugate binding activity. Macrophage FRβ expression is not only restricted to RA, but has also been reported in other arthritis related diseases. In temporal artery biopsies of giant cell arteritis patients, severe inflammation coincided with FRβ-positive macrophages in the adventitia [92]. In two murine models of systemic lupus erythematosis, the number of FRβ-positive macrophages correlated with disease activity [93].

Also in two experimental models of autoimmune uveitis and autoimmune encephalomyelitis in rats, FRβ-positive macrophages were detected at local and systemic sites (e.g. peritoneal cavity) of inflammation [94]. Lastly, several studies reported the presence of FRβ on macrophages in knee sections of osteoarthritis patients [95,96].

6. Folate receptor β and macrophage polarization

Macrophage heterogeneity is a common feature in RA inflamed synovial tissue [20–23]. Micro-environmental factors may affect both activation status and skewing of macrophages into various subsets with distinct immunophenotypes and specialized immune-regulatory and homeostatic functions. Polarization of macrophages covers the broad spectrum from pro-inflammatory to anti-inflammatory macrophages, which have been designated “M1-type” (classical activation, pro-inflammatory) macrophages and “M2-type” (alternatively activated, anti-inflammatory) macrophages, respectively [97]. Whereas M1- and M2-type macrophages represent the extremes of polarization, macrophages harbour plasticity of skewing in either direction. There are many markers that may help to differentiate M1/M2 macrophages. M1 macrophages are involved in tumour inhibition and are resistant to pathogens, whereas M2 macrophages promote tumour growth and have immunoregulatory properties [98]. Classical activation stimuli for M1-type macrophages include IFNγ, LPS and GM-CSF, those for M2-type macrophages include M-CSF, IL-4, IL-10, IL13, glucocorticoids and immune complexes [99,100]. Immunophenotypically, M1-stimulated macrophages display increased cell surface expression of CD80 (provides a costimulatory signal necessary for T cell activation and survival) and CD64 (Fc-gamma receptor 1, FcγRI), whilst M2-stimulated macrophages have increased expression of CD163 (haemoglobin scavenger receptor), CD206 (mannose receptor), CD200R (orexin receptor 2) and CD32 (FcγRIIa) [101]. CD68 is acknowledged as one of the most common markers for identifying human macrophages [101], although its expression can also be detected on fibroblasts [102]. CD169 (Siglec-1) is a macrophage marker that is implicated in immune tolerance and antigen presentation [103]. Although CD169 has been found on activated macrophages in inflammatory diseases [104,105], its function in RA is still unknown.

During the past decade, several studies have explored FRβ expression in the context of macrophage polarization. Initially, studies from Puig-Kroger et al [106] showed that FRβ was preferentially expressed on M2-type macrophages following in vitro skewing.
of monocytes with M-CSF compared with M1-type macrophages with GM-CSF. Moreover, RA synovial fluid macrophages showed an activin A-dependent skewing to pro-inflammatory M1 macrophages and reduced expression of FRβ [107]. In synovial tissue of osteoarthritis patients, however, FRβ expression was not exclusively observed on either M1- or M2-type macrophages [108]. Some recent studies add complexity to this issue by reporting that M-CSF polarized FRβ expressing M2 macrophages demonstrated a high pro-inflammatory response to TLR-ligands and complex IgG and/or autoantibodies to citrullinated protein immune complexes (ACPA-IC) as commonly present in RA [109], [110]. Together, these data suggest that FRβ is differentially expressed on in vitro M-CSF skewed M2-type monocyte-derived macrophages, with is in line with FRβ expression on tumour associated macrophages [75,89,90]. However, in RA (and OA) synovial inflammatory conditions alter macrophage phenotypes along with FRβ expression (Figure 2.2).

7. Imaging folate receptor β in RA

The high binding affinity of folate receptors for folic acid has been exploited for the design of multiple imaging agents [111] to either detect FRα expression in tumours [112,113]
8. Therapeutic targeting of folate receptor β in RA

FRs have not only been exploited for imaging, but also for therapeutic targeting in cancer and inflammation [58]. Targeting of FRα expressing tumours has included folate-conjugated: (a) radionuclides (α-emitters) for cancer treatment, (b) anticancer drugs,
(c) nanoparticles containing either anticancer drugs, siRNAs, miRNAs or genes, or (d) folate antagonists for which FRα has a high affinity [58,61,129] For FRβ, similar targeting approaches are applicable [130]. Table 2.3 provides a selection of approaches that have been reported for targeting FRβ-expressing macrophages in RA and RA-related diseases as well as for FRβ-expressing tumour associated macrophages and FRβ-expressing acute myeloid leukaemia cells. Conceivably, applications in the cancer setting may be translatable to the RA setting. Table 2.3 describes several modalities for FRβ targeting, including folate antagonists, folate-conjugated immunotoxins, folate-conjugated drugs, folate-conjugated nanoparticles containing drugs or genetic material, and via chimeric antigen receptor (CAR) T-cells. With respect to antifolates, several drugs inhibiting key enzymes in folate metabolisms, e.g. dihydrofolate reductase (DHFR), thymidylate synthase (TS) and glycinamide ribonucleotide formyltransferase (GARTFase) [80] were evaluated for FR targeting and anti-arthritic activity in vitro or in arthritic animals. In general, FR has a low affinity for DHFR inhibitors, including MTX, as compared with TS and GARTFase inhibitors [61,74]. Antifolates with selectivity for FRα and FRβ rather than other folate transporters (RFC or PCFT) include BGC-945 and selected GARTFase inhibitors. As illustrated in Table 2.3, folic acid conjugation to a variety of (anti-inflammatory) drugs, drug-containing liposomes, proteins, siRNAs and miRNAs provided a bona fide vehicle for targeted delivery to FR-positive tumour cells and activated macrophages in different autoimmune inflammatory animal models. CAR-T cell therapies with T cells transduced with a high affinity FRβ-specific single chain antibody represents a novel approach for selective targeting and lysis of FRβ-positive AML cells [131,132]. Experimental therapeutics with anti-FRβ CAR T-cells has as yet not been explored in relation to FRβ-positive macrophages targeting in auto-immune inflammatory diseases.
Table 2.3: FRβ therapeutic targeting in rheumatoid arthritis

<table>
<thead>
<tr>
<th>Category</th>
<th>Remarks</th>
<th>Reference</th>
</tr>
</thead>
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<tr>
<td><strong>Antifolates</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MTX</td>
<td>DHFR inhibitor, low FR affinity, High RFC/PCFT affinity</td>
<td>[91]</td>
</tr>
<tr>
<td>CH-1504</td>
<td>DHFR inhibitor, low FR affinity, High RFC affinity</td>
<td>[133]</td>
</tr>
<tr>
<td>EC0746</td>
<td>Aminopterin-folate conjugate DHFR inhibitor, activity in RA mouse model</td>
<td>[134]</td>
</tr>
<tr>
<td>EC0746</td>
<td>Aminopterin-folate conjugate DHFR inhibitor, activity in animal uveitis and encephalomyelitis model</td>
<td>[94]</td>
</tr>
<tr>
<td>Alimta/pemetrexed</td>
<td>TS inhibitor, moderate FR affinity, High RFC/PCFT affinity</td>
<td>[136]</td>
</tr>
<tr>
<td>BGC945</td>
<td>TS inhibitor, FR$\beta$/β specific</td>
<td>[74,135]</td>
</tr>
<tr>
<td>LY399887</td>
<td>GARTFase inhibitor, high FR and RFC affinity, activity in mouse RA model</td>
<td>[137]</td>
</tr>
<tr>
<td>LY329201 &amp; LY399886</td>
<td>GARTFase inhibitors, in vitro activity and activity in rat RA model</td>
<td>[138]</td>
</tr>
<tr>
<td><strong>Immunotoxins</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anti-FRβ-PE38</td>
<td>Recombinant immunotoxin dsFv anti-FRβ-Pseudomonas endotoxin A (PE38). Reduction RA synovial macrophages and fibroblasts</td>
<td>[140-142]</td>
</tr>
<tr>
<td>Anti-FRβ-PE38</td>
<td>Targeting FRβ-positive tumor associated macrophages in mouse glioma</td>
<td>[143]</td>
</tr>
<tr>
<td>Anti-FRβ-PE38</td>
<td>Targeting FRβ-positive macrophages mouse atherosclerotic lesions</td>
<td>[144]</td>
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<td><strong>Folate-conjugated nanoparticles</strong></td>
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<td>G5 dendrimer MTX</td>
<td>Targeting mouse primary FRβ-macrophages</td>
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<td>Liposomes + MTX</td>
<td>Activity to FRβ-positive macrophages in mouse collagen-induced arthritis</td>
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<td>Dextran-MTX</td>
<td>Activity to FRβ-positive macrophages in mouse collagen-induced arthritis</td>
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<td>Liposomes + anti-inflammatory drugs</td>
<td>Targeting activated macrophages in inflammatory diseases</td>
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<td>NFkB decoy</td>
<td>Delivery to murine macrophages</td>
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<td>G5 dendrimers MTX</td>
<td>Targeting FRβ-positive tumor-associated macrophages</td>
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<td>Liposomes + zoledronate</td>
<td>Targeting FRβ-positive tumor-associated macrophages</td>
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<td>Targeting FRβ-positive AML cells</td>
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<td><strong>Folate drug conjugates</strong></td>
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<td>FDG-FA</td>
<td>Targeting FRα-positive tumors and FRβ-positive macrophages</td>
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<td>FA-liposomes</td>
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<td>FA-conjugated microRNAs for delivery to FR-positive cells</td>
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<td><strong>CAR-T cells</strong></td>
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<td>High affinity FRβ- specific</td>
<td>For eradication FRβ-positive AML cells</td>
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<td>CAR-T cells</td>
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9. Conclusion

There is growing evidence that FRβ expression on activated macrophages represent an important biomarker in various autoimmune inflammatory diseases, including RA. FRβ expression in relation to macrophage polarization warrants further investigations under conditions mimicking inflamed RA synovium. FRβ holds promise as a target for imaging with various modalities including PET and optical imaging with rationally designed tracers. This will allow disease monitoring studies and, ideally, early identification of arthritis and PET-guided therapy response monitoring. With respect to therapy, FRβ serves as an excellent target for delivery of therapeutics to macrophages; these may include folate antagonist and folate-conjugated drugs. In conclusion, FRβ expression on activated macrophages may be exploited to guide future diagnostics, targeted therapies and therapy response monitoring in RA.
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Section I

PET-guided therapeutic evaluation in an arthritic rat model
Chapter 3

Sustained macrophage infiltration upon multiple intra-articular injections: an improved rat model of rheumatoid arthritis for PET guided therapy evaluation

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Abstract

To widen the therapeutic window for PET guided evaluation of novel anti-RA agents, modifications were made in a rat model of rheumatoid arthritis (RA). Arthritis was induced in the right knee of Wistar rats with repeated boosting to prolong articular inflammation. The contralateral knee served as control. After immunization with methylated bovine serum albumin (mBSA) in complete Freund’s adjuvant and custom Bordetella pertussis antigen, one or more intra-articular (i.a.) mBSA injections were given over time in the right knee. Serum anti-mBSA antibodies, DTH response, knee thickness, motion, and synovial macrophages were analyzed and $[^{18}\text{F}]$FDG (general inflammation) and ($R$-$[^{11}\text{C}]$PK11195 (macrophages) PET was performed followed by ex vivo tissue distribution. Significant anti-mBSA levels, DTH, swelling of arthritic knee, and sustained and prolonged macrophage infiltration in synovial tissue were found, especially using multiple i.a. injections. Increased $[^{18}\text{F}]$FDG and ($R$-$[^{11}\text{C}]$PK11195 accumulation was demonstrated in arthritic knees as compared to contralateral knees, which was confirmed in ex vivo tissue distribution studies. Boosting proved advantageous for achieving a chronic model without remission. The model will offer excellent opportunities for repeated PET studies to monitor progression of disease and efficacy of novel therapeutic agents for RA in the same animal.
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1. INTRODUCTION

Rheumatoid arthritis (RA) is an autoimmune disease that results in chronic and systemic inflammation of the joints, affecting approximately 0.5–1% of the adult population [1]. It is characterized by inflammation of the joints resulting in synovial hyperplasia by infiltration of immune cells further leading to cartilage and bone destruction [2]. Timely recognition of RA will allow for earlier start of therapy preventing more severe expansion of the disease. Moreover, several studies have shown that tight control as a treatment strategy in individual RA patients seems promising in achieving predefined level of low disease activity or preferably remission within a reasonable period of time [3, 4]. To this end, noninvasive imaging modalities may serve as sensitive and accurate tools for assessment and monitoring of disease activity during therapy to evaluate therapeutic efficacy.

Positron Emission Tomography (PET) is a promising noninvasive imaging modality that can be used to visualize active arthritis at a molecular level in RA [5] via targeting macrophages [6,7]. Most human studies targeting macrophages by PET have been performed with the macrophage tracer \((R)-[^{11}C]PK11195\) in various inflammatory diseases [8]. \((R)-[^{11}C]PK11195\) targets the 18-k translocator protein (formerly known as peripheral benzodiazepine receptor) a mitochondrial membrane protein that is upregulated in activated macrophages [8]. Histological studies have shown that macrophages are an important biomarker for prediction and monitoring of therapeutic effects of a wide range of disease modifying antirheumatic drugs and biologics [9, 10]. Jahangier et al. demonstrated a clear positive clinical effect in RA patients after intra-articular treatment with Yttrium-90 and glucocorticoids correlating effect with a decrease in total numbers of macrophages [11].

Animal models can be applied for in vivo evaluation of efficacy of new therapeutic agents for RA [12]. As it takes some time for most antirheumatic drugs to read out their mode of action on arthritis activity with macrophage infiltration as a biomarker, a chronic RA animal model is required with sustained arthritis activity characterized by macrophage infiltration in synovial tissue. As currently no suitable rat model is available that would allow noninvasive macrophage PET guided evaluation of the therapeutic agents, we have optimized an antigen induced model with persistent arthritis in rats offering sufficiently sized inflamed joints to enable quantitative measurements of PET tracer uptake in inflamed joints as well as the opportunity for comparison to contralateral noninflamed control joints within the same animals.

2. MATERIALS AND METHODS

2.1. Animals.

Wistar rats (male, 150–200 grams, Charles River International Inc, Sulzfeld, Germany) were provided with standard food (16% protein rodent diet, Harlan Laboratories Inc., Madison, WI, USA) and water ad libitum. Rats were housed in groups of three or four in conventional cages and kept in a room with a 12-hour light/dark cycle and constant room temperature (21°C) and humidity level (50%). All animal experiments were carried out in accordance with the Dutch law.
on animal experimentation and were approved by the VU Medical Center Institutional Committee on Animal Experimentation.

2.2. Antigen Induced Rat Model.

As reference in this paper, the methylated bovine serum albumin (mBSA) induced rat model as described by van de Putte et al. [13] and Dijkstra et al. [14] was applied (Table 1), indicated hereafter as “original model.” In short, according to the descriptions of the original model, rats were immunized subcutaneously (s.c.) twice at days 0 and 7 with an emulsion containing mBSA (Sigma-Aldrich Chemie BV, Zwijndrecht, The Netherlands) dissolved with complete Freund’s adjuvant (CFA) (Sigma Aldrich, Steinheim, Germany) and custom Bordetella pertussis (CBP) antigen (Becton Dickinson, Breda, The Netherlands) [14]. Rats were immunized with two administrations of 200 uL solution containing 50 mg mBSA in 1 mL 0.9% NaCl emulsified with an equal volume of complete Freund’s adjuvant antigen (CFA) and custom Bordetella pertussis (CBP) antigen (1x10^{11} cells/mL). Both the first and the second immunization were performed in the tail base. At day 21, local arthritis was induced by injecting 20 uL mBSA solution containing 10 mg mBSA [15] in 1 mL 0.9% NaCl intra-articular (i.a.) in the right knee (RA knee); the contralateral left knee served as an internal control (Con-RA). The i.a. injection was situated between femur and tibia and behind the patella tendon.

2.3. Modifications of Original Rat Model.

Three modifications were performed as compared to the original model (Table 3.1). At first, the second immunization (initially 200 uL in the original model) step was adapted. To minimize animal discomfort by multiple immunizations at a single location (as was performed in the original model), second immunization was divided into two injections with one in the neck and one in the upper flank (away from the knees) with each injection consisting a volume of 100 uL. Secondly, the i.a. injection volume of mBSA was increased to 60 uL while in the original it was 20 uL. Both modifications were applied in groups A, B, C, and D. The last modification comprised repeated i.a. injections (resp., 3x (group C) and 5x (group D)) while in group A and B no boosts were applied. The difference between groups A and B was the sacrificing day: 6 and 28 days after i.a. injection for groups A and B, respectively (Figure 3.1). Control rats received an i.a. injection in the right knee with sterile physiological saline instead of mBSA. Subgroups of control rats were sacrificed at 6 (group E) and 28 (group F) days, respectively.

2.4. Validation Experiments.

(i) Examination of Immunization Status Serum Levels of Anti-mBSA

Blood samples before and after the immunization procedure were obtained from the tail vein with a Microvette (cb300, Sarstedt BV, Etten-Leur, The Netherlands). After centrifugation at room temperature (5 min/2500 xg), serum samples were stored at −80°C until use. Anti-mBSA levels were determined by ELISA [16]. Briefly, 96-well microplates (Greiner bio-one, Alphen a/d Rijn, The Netherlands) were precoated overnight with 100 uL/well of 5 g mBSA/uL phosphate buffered saline (PBS) at 37°C. After washing with PBS (100 uL/well, 5 times) wells were blocked with 0.1% gelatin (Baker Chemical Co,
Figure 3.1 – Time line of RA rat model. 1st (■) and 2nd (■) immu-nization, DTH (◇), i.a. injection (□), boost i.a. injections (▲), PET and/or, ex vivo tissue distribution, and (immuno) histopathology of groups A (6 d), B (28 d), C (19 d), D (28 d), E (saline, 6 d), and F (saline, 28 d) (▼).
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Austria, Texas, USA) in PBS for 30 minutes at 37°C. Subsequently, 1:100, 1:200, and 1:400 diluted serum samples were added and incubated for 1 hour at room temperature. After washing with PBS, horseradish peroxidase (HRP) labeled to rabbit-anti-rat (RaR) IgG1 antibody 1:1000 (Invitrogen, NY, USA) was added to the wells and incubated for 2 hrs at room temperature. Enzyme reaction was visualized with 0.8 mg aminosalicylic acid (Sigma-Aldrich Chemie BV, Zwijndrecht, The Netherlands) and 0.5 μL of 30% hydrogen peroxide (H₂O₂) dissolved in 1 mL distilled water. Absorption at 450 nm was measured using an ELISA reader (Tecan, Spectra Fluor, MTX Labsystems, Inc, Vienna, USA).

**Delayed Type Hypersensitivity Test (DTH)**

At day 19, immunization status was examined by DTH [17, 18] response. CBP antigen (25 μL, 2.7 × 10^10 cells/mL 0.9% NaCl) was injected s.c. in the right ear of the rat. The left ear served as an internal control. A control group of rats was injected with sterile physiological saline in the left ear. Subsequently, ear thickness was measured at 0, 6, 24, and 48 hours after injection using a digital micrometer.

(ii) **Macroscopic Evaluation of Arthritis Activity**

Macroscopic evaluation of the severity of arthritis was assessed by knee measurements prior to (at the same day) every i.a. injection. In between, knees were measured 3 times a week until rats were sacrificed. Knees were measured by caliper measurement of knee thickness in mediolateral direction.

**2.5. Histopathology and Immunohistochemistry**

Both knees were dissected in toto and fixed for 7 days at 4°C in 10% freshly made paraformaldehyde in PBS with 2% sucrose (pH = 7.3) prior to decalcification in 123 mM sodium ethylenediaminetetraacetic acid (Na₂-EDTA 2H₂O) (Merck, Darmstadt, Germany) and 113 mM sodium hydroxide (NaOH) (Sigma-Aldrich Chemie BV, Zwijndrecht, The Netherlands) (pH = 7.2) for ~5.5 weeks at 4°C. Decalcified knees were rinsed for 24 hours in 2% sucrose (Sigma-Aldrich Chemie BV, Zwijndrecht, The Netherlands) in PBS (pH = 7.2) and 24 hours in 2% sucrose in PBS and 50 mM NH₄Cl (Sigma-Aldrich Chemie BV, Zwijndrecht, The Netherlands) (pH = 7.1). Thereafter, knees were embedded in paraffin. Sections of 5 μm were cut through the center of the joint in longitudinal direction and stained with haematoxylin and eosin to assess the degree of inflammation in synovial tissue.

Immunohistochemical localization of rat macrophages was determined by a mouse anti-rat monoclonal antibody ED1 (HM3029, Hycult, PA, USA) a lysosomal membrane related antigen on rat macrophages and by a mouse anti-rat monoclonal antibody ED2 (MCBI, VU University Medical Center, Amsterdam) cell surface glycoprotein related antigen on rat macrophages [19]. An IgG1 isotype antibody (HI1016, Hycult, PA, USA) was used as negative control antibody. Briefly, after antigen retrieval with a solution of 0.1% pepsin (Sigma-Aldrich Chemie BV, Zwijndrecht, The Netherlands) with 0.1% of HCl 37% in PBS per slide for 30 min at 37°C, sections were incubated for 1 h with 1:100 diluted ED1, ED2, or isotype control antibody in 0.1% BSA/PBS for a period of 1 h. The detection EnVision kit (K4063, dual-link-HRP, rabbit/mouse, DAKO, Glostrup, UK) was used according to instructions of the manufacturer for a period of 30 min. After
washing with PBS, slides were stained for peroxidase activity with 3,3'-diaminobenzidine tetrahydrochloride (DAB) containing 0.01% H$_2$O$_2$. Subsequently, sections were counterstained with haematoxylin, dehydrated, and mounted. Negative controls were included by replacement of the primary antibody with an isotype specific control antibody. Images were captured using a Leica 4000B microscope and Leica digital camera DC500 (Microsystems B.V. Rijswijk, The Netherlands).

2.6. PET and Ex Vivo Tissue Distribution Studies.

$[^{18}F]$FDG with a radiochemical purity of $>97\%$ was purchased from BV Cyclotron VU (Amsterdam, The Netherlands). $(R)$-$[^{11}C]$PK11195 was synthesized as described previously [20], with radiochemical purity of $>98\%$ and a mean specific activity of 95.7 ± 28.4 GBq/μmol. Rats were anesthetized using inhalation anesthetics (isoflurane 2–2.5% and oxygen 0.45 volume %). The jugular vein was cannulated with a polyurethane 3 French cannula. During all procedures vital body signs like, body temperature, heart-beat, respiratory rate, and blood oxygen saturation were monitored continuously using a rectal temperature probe and pulse oxygen meter with SpO$_2$ sensor. Anesthetized rats were positioned in a double-layer LSO high resolution research tomograph (HRRT) (Siemens/CTI, Knoxville, TN, USA), a small animal, and human brain 3D scanner with high spatial resolution (2.3–3.4 mm full width at half maximum) and high sensitivity [21]. First, a 6-minute transmission scan was acquired using a 740 MBq 137Cs rotating point source. Next, $[^{18}F]$FDG (21.1 ± 5.1 MBq) or $(R)$-$[^{11}C]$PK11195 (10.5 ± 2.9 MBq) was administered i.v. through the cannula and a dynamic emission scan of 1 hour was acquired. PET data were normalized and corrected for scatter, random, attenuation, decay, and dead time. Data were acquired in 64-bit list mode and converted into 16 sinograms with frame durations increasing from 15 up to 300 seconds.

Images were reconstructed using an iterative 3D ordinary Poisson ordered-subsets expectation maximization (OSEM) algorithm with 8 iterations and 16 subsets and a matrix size of 256 × 256 × 207, resulting in a cubic voxel size of 1.21 × 1.21 × 1.21 mm$^3$. PET images were made of rats from the original model and of rats in group A. Sixty minutes after PET scanning, rats were sacrificed and knees, blood, and various tissues were excised and weighed and the amount of radioactivity was determined using an LKB 1282 CompuGamma CS gamma counter (LKB, Wallac, Turku, Finland). Rats without PET scanning (groups B–E) were sacrificed 60 minutes after tracer injection followed by ex vivo tissue biodistribution. Results were expressed as percentage of the injected dose per gram tissue (%ID/g).

PET images were analyzed using AMIDE software (Amide’s Medical Image Data Examiner, version 0.9.2) [22]. Fixed size ellipsoidal shaped regions of interest (ROI) (dimensions: 6.0 × 17.7 × 7.4 mm$^3$) were manually drawn over the area of the left and right knees in the last frame of the image. ROIs were projected onto the dynamic image sequence, and time-activity curve (TAC) data were extracted. TACs were expressed as standardized uptake values (SUV): mean ROI radioactivity concentration normalized for injected dose and body weight [7].
2.7. Statistical Analysis.

Statistical tests were performed using IBM SPSS version 20. A one-sample Kolmogorov-Smirnov test was to test for normal distribution taking (P value) criteria for t-test. A Wilcoxon signed rank (exact) test was used to determine differences between paired observations (e.g., tracer uptake in right versus Con-RA knee, thickness of the antigen-induced versus control ear, and mediolateral thickness of RA knee versus Con-RA knee). A Mann-Whitney (exact) test was used to determine differences in absorbance before and after immunization. A value <0.05 was considered statistically significant. A Bonferroni correction was applied when necessary.

RESULTS

During the entire study, no major change in body weight was observed and knee functionality was never dramatically impaired during the course of the induction of arthritis in the RA knee of the rats.

3.1. Immunization Status.

All rats showed a significant increase (P < 0.001) in the level of mBSA antibody titers as compared with mBSA levels before immunization (Figure 3.2(A)). In addition, a DTH test was executed and all rats showed a good DTH response with a significant (P = 0.001) increase in ear thickness of the right ear at 6, 24, and 48 hours after injection compared with the control left ear (Figure 3.2(B)) and compared to control rat ear's injected with saline (data not shown).

3.2. Arthritis Evaluation of No-Boost Model.

As negative control, healthy rat knee sections, stained with the ED1 and ED2 rat macrophage specific antibodies, showed no signs of inflammation in the synovial tissue (Figure 3.3, left panels). Some macrophages were found in the single layered synovial lining. In contrast, the RA knees of the rats in the firstly adapted no-boost group (group A, 0x boost panels) showed a moderate influx of inflammatory cells in the synovium with a hyperplasia of synovial tissue consisting of 3-4 layers. PET tracer [18F]FDG uptake in the RA knees of rats from group A was also low and showed no obvious difference as compared to the Con-RA knees, as shown in Figure 3.5(A).

3.3. Arthritis Evaluation of the Modifications in the Rat Model

3.3.1. Macroscopic Evaluation of Arthritis Activity. In the no-boost group A, a significant difference was observed in knee thickness between the RA knees compared to the Con-RA knees (P = 0.01), which persisted until day 6 after i.a. injection (P = 0.01) (Figure 3.2(C)). In the no-boost group followed for 28 days (group B, Figure 3.2(C)), differences between the RA versus Con-RA knee were significant (P = 0.001) at day 4 but gradually decreased (P = 0.1) in 28 days. Continued observation without boosting demonstrated that the RA knee thickness decreased significantly (P < 0.0001) between 6 and 28 days (group A versus group B). Although significantly decreased in size, the knee thickness did not normalize completely at 28 days when compared to the Con-RA knees of the same rats. When the rats were dosed with 3 boost injections

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(group C) significant differences were found in knee thickness of the RA versus Con-RA knee ($P = 0.002$ and $P = 0.003$ at day 4 as well as at the sacrificing day, resp.). In the group of rats dosed with 5 boost injections (group D), also significant differences were observed between the RA and Con-RA knees ($P = 0.0001$ and $P < 0.0001$ at days 4 and 28, resp.). Comparing the boost groups C and group D (3x and 5x boosts) on the day those rats were sacrificed, a significant difference in RA knee thickness was observed, with group C < group D ($P = 0.024$). Comparing group A (no boost) with both boost groups (C and D) with respect to RA knee measurements, significant differences were observed (group A versus group C and group A versus group D: $P = 0.003$ and $P < 0.0001$, resp.).

![Figure 3.2](image)

**Figure 3.2** – (A) Measurement of anti-mBSA in serum in rats before immunization (left) and after immunization (right) ($P < 0.001$). (B) Caliper measurement of right ear swelling of (■) A (6 d); (♦) B (28 d); (○) C (19 d); (▲) D (28 d), compared to the control ear of (□) A (6 d); (◇) B (28 d); (○) C (19 d); (▲) D (28 d), as a response to s.c. injection of antigen ($< 0.001$). (C). Knee thickness of arthritic knee of (□) A (6 d); (◇) B (28 d); (○) C (19 d); (▲) D (28 d), compared to control Con-RA knee of (■) A (6 d); (♦) B (28 d); (○) C (19 d); (▲) D (28 d). All results depicted represent mean ± SD.
3.3.2. Immunohistochemistry. As shown in Figure 3.3, synovial tissue of healthy rat knees showed no signs of inflammation (Figure 3.3, left panel) and RA knees in group A (no boost) showed a moderate influx of inflammatory cells in the synovium with a hyperplasia of synovial tissue consisting of 3-4 layers. In contrast, the representative images of the knees from rats in group C and group D demonstrated a clear increase in ED1 and ED2 positively stained macrophages and multilayered synovial tissue (>5; see also next paragraph). The infiltration of macrophages was quantified in the different groups of rats (between 2 and 4 rats per group). In Figure 3.4(A), the results for ED1 positive macrophages in group A and group B are shown. In comparison to group A, group B showed significantly lower numbers of ED1+ macrophages ($P = 0.028$) (but still significantly higher than the control group F ($P < 0.0001$)). In Figure 3.4(B) (ED1) and Figure 3.4(C) (ED2), the ED1 and ED2 positive macrophages in knees of rats in all groups are represented, including rats injected with saline (showing very low numbers of macrophages). On comparing those numbers in group C and group B, RA knees from rats in group C clearly displayed more ED1 and ED2 positive macrophages. For those in group D an even higher amount of ED2+ macrophages was found as compared to the no-boost groups (Figure 3.4(C)). In Figure 3.3, representative images of ED1 and ED2 staining sections of different groups are shown.
Figure 3.4 – Macrophage counting in histological knee sections. (A) Total number (±SD) of ED1 positive macrophages in the lining and sublining of the knee synovial tissue from the no boost rats (A (6 d) and B (28 d)); RA knee, light grey bars, Con-RA knee, black bars. (B) Total number (±SD) of ED1 positive macrophages in the lining and sublining of the knee synovial tissue from the groups B (28 d), C (19 d), and D (28 d) are added next to saline control rats group F (28 d). (C) Total number (±SD) of ED2 positive macrophages in the lining and sublining of the knee synovial tissue from the groups B (28 d), C (19 d), and D (28 d) are added next to saline control rats group F (28 d).

PET and Ex Vivo Tissue Distribution Studies.

The feasibility of PET evaluation of arthritis activity in the rats of group A (0x boost) as compared to the original model was assessed. Figures 3.5(A) and 3.5(B) show representative $^{18}$F$\text{FDG}$ and $(R)$-$^{11}$C$\text{PK11195}$ images. The uptake of $^{18}$F$\text{FDG}$ in the RA knee of the group A was clearly higher than that in the original model (Figure 3.5(C), left; original model).
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Figure 3.5 – Representative coronal PET images of (a) $^{18}$F-FDG and (b) $(R)$-$^{11}$C-PK11195 in arthritic rats group A (6 d). Uptake of both tracers is clearly shown in the right arthritic knee (arrow) compared with the contralateral knee (arrowhead). (c) PET images of $^{18}$F-FDG uptake in arthritic rats, original model (left) and group A (6 d) (right).

Time-activity curves (SUV versus time after tracer injection) of the tracers up to 6 days are depicted in Figure 3.6. The SUV in the RA knee as compared to the Con-RA knee for $^{18}$F-FDG in group A rats was significantly increased (2.78±0.366 versus 1.38±0.189; $P = 0.001$; Figure 3.6(A)) and the ratio of uptake in the RA knee was 2 times higher than the Con-RA knee (Figure 3.6(C)). The SUV for $(R)$-$^{11}$C-PK11195 (Figure 3.6(B)) also increased in the RA knee (2.00±0.55 versus 1.11±0.45) and the ratio in the RA knee uptake is 1.8 times more than the Con-RA knee in group A (Figure 3.6(C)).

After PET, ex vivo tissue distribution of group A rats was performed and both $^{18}$F-FDG and $(R)$-$^{11}$C-PK11195 (Figure 3.7) showed increased accumulation in the RA knee (1.6 and 1.4 times higher, resp.) compared to uptake in Con-RA knee $^{18}$F-FDG and $(R)$-$^{11}$C-PK11195 (0.52±0.06 and 0.79 ± 0.05 in RA knees; 0.33 ± 0.04 and 0.57 ± 0.05, resp.). Furthermore, both tracers showed increased uptake in macrophage rich tissues such as spleen, liver, and bone (marrow). Moreover, increased uptake of $(R)$-$^{11}$C-PK11195 in the heart could be related to TSPO expression on myocardial cells. Both tracers showed accumulation in the intestinal system and renal system (Figure 3.7) due to excretion of the tracers via hepatobiliary and renal route. Physiological uptake of $^{18}$F-FDG was noted in heart and brain tissue.

Results from the ex vivo tissue distribution one hour after injection of $(R)$-$^{11}$C-PK11195 in the no-boost groups (A and B) and the boost groups C and D are depicted in Figure 3.8. For all groups, extra-articular tracer uptake was again observed in macrophage rich tissue and physiological uptake in the intestines. No-boost groups (A and B) (Figure 3.8(a)): a 1.45 times higher uptake of $(R)$-$^{11}$C-PK11195 was measured in group A between the RA and Con-RA knee. In group B, a 1.2 times higher uptake in the RA knee (0.46±0.05) was found compared to Con-RA knee (0.39 ± 0.03) ($P = 0.05$). Comparing group A with B, it was observed that the tracer uptake in the RA knee from rats in group B was a little bit less (although not significantly different from that of group A).
Figure 3.6 – Time-activity-curves of tracer uptake expressed as SUV (± SD) in RA knee (●) and Con-RA knee (○) of (A) $[^{18}F]$FDG and (B) (R)-$[^{11}C]$PK11195 in rats of group A (6 d). (C) SUV ratios for RA/Con-RA are depicted.
Figure 3.7 – Ex vivo tissue distribution of $^{18}$F]FDG ($n=6$), light grey bars, and (R)-$^{[11}$C]PK11195 ($n=5$, black bars) at 1 h after injection in rats of group A (6 d). Results are expressed as percentage of the injected dose per gram (%ID/g ± SD).

Multiple boost group C versus group D (Figure 3.8(b)): the uptake of (R)-$^{[11}$C]PK11195 in RA knees was higher in rats from group C and group D as compared to that in group B, although a level of significance was not reached between the no-boost B and group C. However, comparing no-boost (group B) with the boost group (group D) a significant difference in tracer uptake was found ($P = 0.01$). Zooming in on the rats sacrificed at day 28 ((groups B, D, E, and F) and for group C at day 19) after the last i.a. injection a linear correlation was found between uptake and total number of ED1 positive macrophages. In contrast, no correlation could be demonstrated with respect to ED2 positive macrophages.

Zooming in on the rats sacrificed at day 28 ((groups B, D, E, and F) and for group C at day 19) after the last i.a. injection a linear correlation was found between uptake and total number of ED1 positive macrophages. In contrast, no correlation could be
demonstrated with respect to ED2 positive macrophages.

Figure 3.8 – Ex vivo tissue distribution of (R)-[\(^{11}\text{C}\)]PK11195 at 1 h after injection in rats of (a) no boost groups A (6 d) and B (28 d); (b) no boost group B (28 d), boost groups C (19 d), and D (28 d). Results are expressed as percentage of the injected dose per gram (\%ID/g ±SD).
DISCUSSION

In this study an mBSA-induced RA rat model was described with sustained and prolonged RA condition. Adaptations with respect to the original model described by van de Putte et al. [13] and Dijkstra et al. [14] resulted in a model that allows evaluation of arthritis activity and therapeutic efficacy of novel antirheumatic drugs with PET. The modifications resulted in significantly higher influx of macrophages in synovial tissue, corresponding to improved visualization of arthritis with PET.

So far, no animal model was available for PET guided imaging and monitoring of therapeutic efficacy with a sustained and prolonged arthritic condition, long enough to test new drugs as well as showing some level of systemic disease. With our focus on detection of (sub)clinical arthritis versus noninflamed joints, animal models with predominant bone destruction and those with polyarticular distribution are not favored. Various rodent models of RA (acute and chronic) have been described where the very acute models are not very useful for monitoring therapeutic efficacy [23]. Chronic models such as collagen induced arthritis (CIA), bacterial (streptococcal) cell wall contents induced arthritis (SCW) and adjuvant induced arthritis (AIA) have their specific characteristics [24]. In AIA, rats develop polyarthritis with prominent bone destruction. In CIA [24] and SCW [25], swelling of paws and limbs appears with periods of remission dampening the readouts for therapeutic intervention, whereas SCW shows low significant systemic effect.

Several advantages of our present RA model are presented: it is monoartritic; hence the contralateral knee could be used as an internal control, its robustness, and, after the immunization period, the relative rapid development of arthritis within a week characterized by clear macrophage infiltration in the synovium of one joint, resembling human RA, and with the other joints as internal control. Using the boosting procedure, the therapeutic window was largely enhanced. Macrophages play an important role in early RA and may therefore be exploited as potential targets for the development of new treatment and imaging agents [26, 27]. This model might also be particularly useful for PET purposes, since arthritis is induced in a relatively large knee joint, which is an advantage as detection of arthritis in smaller joints could be hampered by limited spatial resolution of PET scanners. Also, injection of larger volumes associated with low specific activity of some PET tracers is more problematic in mice than in rats. For monitoring response with PET imaging, quantification is essential. To this end, assessment of tracer levels in blood will be needed at any time after injection of the PET tracer which poses a problem with respect to frequent blood sampling in mice.

Limitations of the mBSA induced RA rat model are possibly the longer total time period (immunizations, i.a., and boost injections, frequent knee measurements). Also, skilled biotechnicians are needed to perform the precise intra-articular injection in the knee joint. Although the model consists of only one macroscopically inflamed knee joint, the contralateral knee also showed a low level of microscopic abnormalities related to the infiltration of macrophages in the synovium, being indicative for some systemic inflammatory effects, as also observed in a study by Meyer et al. [28]. Nevertheless, the clear difference of macrophage infiltration in the arthritic versus the noninflamed knee allowed internal comparison on PET.
PET studies showed increased uptake of both $^{18}$F-FDG and $(R)$-$^{11}$C-PK11195 in RA compared to that in Con-RA knees. Both tracers, however, have limitations for clinical imaging of arthritis activity. $^{18}$F-FDG has high sensitivity but low specificity for imaging of arthritis. In a previous clinical study, absolute uptake of RA joints and osteoarthritic joints was comparable [29]. Although $(R)$-$^{11}$C-PK11195 is a more specific tracer targeting mainly macrophages a limitation of this tracer is the background uptake in periarticular tissues [30]. The background binding of the PET tracer to both the noninflamed knee joint and periarticular bone (marrow) (in both knees and other physiological locations) could lead to underestimation of targeting properties of the applied PET tracer since PET imaging of a target relies on the contrast between the target and its background. Obtaining high, arthritic to background ratios is of particular clinical relevance to detect very early (sub)clinical synovitis as it is often only subtly present at this stage of the disease. Therefore, further research of new specific targets on activated macrophages in the early phase of RA remains warranted. An alternative candidate target in this respect is the folate receptor being selectively expressed on activated macrophages [31, 32]. Based on nanomolar binding affinities for folate and folate-conjugated ligands, this receptor might be an attractive target for both imaging and therapeutic applications, with folate linked therapeutic agents showing little or no collateral toxicity to normal tissue [33] and specifically target the activated macrophages rather than the non activated macrophages [34].

Conclusions
In conclusion, in this study, a rat arthritis model suitable for PET guided evaluation of antirheumatic drugs was optimized and validated. The boost regimen proved advantageous for achieving a chronic model with sustained arthritis activity. This model is excellent for in vivo testing of novel PET tracers for their suitability for imaging of arthritis (activity) as well as for PET monitoring of efficacy of novel therapeutic agents for RA.

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Chapter 3  An improved rat model of arthritis for PET

References


Chapter 4

Imaging and methotrexate response monitoring of systemic inflammation in arthritic rats employing the macrophage PET tracer $[^{18}\text{F}]\text{fluoro-PEG-folate}$

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Abstract

Background
In rheumatoid arthritis, articular inflammation is a hallmark of disease, while the involvement of extra-articular tissues is less well defined. Here, we examined the feasibility of PET imaging with the macrophage tracer $[^{18}\text{F}]$fluoro-PEG-folate, targeting folate receptor (FR $\beta$), to monitor systemic inflammatory disease in liver and spleen of arthritic rats before and after methotrexate (MTX) treatment.

Methods
$[^{18}\text{F}]$fluoro-PEG-folate PET scans (60 min) were acquired in saline- and MTX-treated (1 mg/kg, 4x) arthritic rats, followed by tissue resection and radiotracer distribution analysis. Liver and spleen tissues were stained for ED1/ED2-macrophage markers and FR $\beta$ expression.

Results
$[^{18}\text{F}]$fluoro-PEG-folate PET and ex vivo tissue distribution studies revealed a significant ($P < 0.01$) 2-fold lower tracer uptake in both liver and spleen of MTX-treated arthritic rats. Consistently, ED1- and ED2-positive macrophages were significantly ($P < 0.01$) decreased in liver (4-fold) and spleen (3-fold) of MTX-treated compared with saline-treated rats. Additionally, FR $\beta$-positive macrophages were also significantly reduced in liver (5-fold, $P < 0.005$) and spleen (3-fold, $P < 0.01$) of MTX- versus saline-treated rats.

Conclusion
MTX treatment reduced activated macrophages in liver and spleen, as markers for systemic inflammation in these organs. Macrophage PET imaging with $[^{18}\text{F}]$fluoro-PEG-folate holds promise for detection of systemic inflammation in RA as well as therapy (MTX) response monitoring.

Abbreviations
RA - Rheumatoid arthritis, mBSA - Methylated bovine serum albumin, MTX - Methotrexate, HRRT - High resolution research tomography, PEG - Polyethylene glycol, SD - Standard deviation, %ID/g - Percentage of the injected dose per gram, i.a. - Intra-articular, FR - Folate Receptor, ROI - Region of interest, EC20 - A folate-linked chelator of $^{99}\text{Tc}$. 
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INTRODUCTION

Rheumatoid arthritis (RA) is a chronic inflammatory disease involving mainly the synovium of the joints, although other tissue/organ involvement has been recognized [1, 2]. Extra-articular manifestations occur in active and severe RA, including skin, eye, heart, lung, renal, nervous, and gastrointestinal systems [3, 4]. Therefore, early detection and treatment of systemically affected organs in RA could benefit in achieving predefined low disease activity and remission [5, 6]. To this end, in a preclinical setting, animal models of arthritis may serve a valuable tool for imaging (extra) articular and nonarticular inflammation and for monitoring the response to therapeutic interventions.

Many experimental animal models have been exploited to unravel the pathophysiology of inflammatory arthritis [7–12]. However, in most of these studies the primary research focus was on disease pathways and immune cells of the synovium rather than extra-articular manifestations. Also, depending on the modality and time frame of arthritis induction, extra-articular manifestations were not monitored or underreported. Macrophages are known to play central role in RA disease progression [13]. Several studies have shown a direct correlation between disease remission and lower numbers of macrophage infiltration incidents into the synovium [14–16]. In patients, tissue resident macrophages in macrophage-rich organs such as liver and spleen may also be involved in extra-articular inflammation in RA [17]. Recent studies indicated that up to 50% of RA patients were reported with abnormal liver symptoms, including elevated alkaline phosphatase and small foci of necrosis and fatty liver [18]. Moreover, liver resident macrophages in an animal model were implicated in regulating chronic inflammation of arthritis through interacting with synovial phagocytes [19]. Not limiting to liver, spleen has also been reported in systemic inflammation in RA. Studies have shown manifestations of spleen enlargement and histological changes in either early or longstanding RA [20, 21].

Macrophage Positron Emission Tomography (PET) has been proposed as a noninvasive modality to monitor disease activity and therapy response in the whole body [22]. Beyond the prototypical macrophage tracer (R)[11C]-PK11195, targeting the translocator protein (TSPO) on activated macrophages, second-generation TSPO tracers showed improved properties over (R)[11C]-PK11195 to visualize arthritis [23]. Other interesting macrophage PET tracers to visualize arthritis are 4-[18F]-fluorophenylfolate, [68Ga]-DOTA-folate [24], and [18F]fluoro-PEG-folate [25]. These folate-based tracers bind with high affinity to folate receptor (FRβ) expressed on activated macrophages [26–28]. FR is also of interest from a therapeutic perspective as it can bind and internalize antifolates and folate-conjugated antiarthritic therapeutics [26–31].

Recently we reported that the macrophage tracer [18F]fluoro-PEG-folate allowed visualizing arthritis in the inflamed knee joints of arthritic rats and also was able to monitor the response to the anchor drug in RA therapy, methotrexate (MTX) [32]. In the present study we extend on these observations by exploiting [18F]fluoro-PEG-folate PET to monitor potential systemic inflammation in liver and spleen of arthritic rats before and after MTX therapy, hypothesizing that MTX therapy also impacts systemic inflammatory effects in the organs. These studies were complemented with histological
and immunofluorescence assessment of macrophage infiltration in liver and spleen.

METHODS

2.1. Animals.

The European community council directives 2010/63/EU for laboratory animal care and the Dutch law on animal experimentation criteria were fulfilled for performing the animal experiments. Wistar rats (male, 150–200 grams, Charles River International Inc., Sulzfeld, Germany) were provided with standard food, water (ad libitum), and conditions as described previously [32]. The local committee on animal experimentation of the VU University Medical Center (DEC PET13-07) validated and approved experimental protocols.

2.2. Arthritic induction and therapeutic interventions.

Wistar rats were immunized [33] and arthritis was induced via 4x intra-articular (i.a.) methylated bovine serum albumin (mBSA) injections, 4 or 5 days apart in the arthritic (right) knee with the contralateral (left, nonarthritic) knee serving as control knee essentially as described before [33]. Rats were anesthetized during immunization and arthritic induction using inhalation anesthetics (isoflurane: 2–2.5% and oxygen: 1 L/min). After the last i.a. injection the rats (n= 4/group) were treated 4x (d0, d7, d14, and d21) either with saline (500 μL, intraperitoneal (i.p.) injection) or with MTX (VU University Medical Centers’ Pharmacy) (i.p.) at 1.0 mg/kg. Healthy rats (nonarthritic) (n=3) did not receive either arthritic induction or therapeutic interventions [32].

Six days after the last saline or MTX treatment, $^{18}$F-fluoro-PEG-folate PET scans were performed, immediately after which rats were sacrificed and tissues were excised for further processing and various analyses described hereafter.

2.3. $^{18}$F-fluoro-PEG-folate and PET.

$^{18}$F-fluoro-PEG-folate was synthesized as previously described [25], with a radio-chemical purity of >97% and mean specific activity of 49.7 ± 2.1GBq/μmol. Saline- and MTX-treated arthritic rats were anesthetized using inhalation anaesthetics (isoflurane: 2–2.5% and oxygen: 1 L/min). The jugular vein was cannulated with a polyurethane 3-French cannula (0.7 mm × 19 mm, BD Angiocath, Breda, Netherlands). During all procedures body temperature, heartbeat, respiratory rate, and blood oxygen saturation were monitored continuously using a rectal temperature probe and a pulse oxygen meter with SpO$_2$ sensor. Anesthetized rats (n=2, from saline- and MTX-treated groups) were positioned in a high resolution research tomograph (HRRT) (Siemens/CTI, Knoxville, TN, USA) and $^{18}$F-fluoro-PEG-folate (20.5 ± 3.4MBq) was administered i.v. through the cannula and a dynamic PET scan was acquired for 60 min. Next, PET scans were normalized (for scatter, random, attenuation, decay, and dead time) and reconstructed as described before [25]. AMIDE software (version 0.9.2) [34] was used to analyse the images and data were expressed as standardized uptake values (SUV). The last frame was used to manually draw fixed size ellipsoidal shaped ROI over the area of liver and
spleen (dimensions: $4 \times 4 \times 4$ mm$^3$) and arthritic and contralateral knees (dimensions: $7 \times 4 \times 7$ mm$^3$). The ROI for knees was drawn on top of the knee area [25] whereas, for liver and spleen, first a dotted line was drawn to represent the organ and then ROI was drawn approximately at the same spot in the saline- and MTX-treated rats. Through projecting ROIs onto the dynamic image sequence the time activity curve (TAC) was generated. TACs were expressed as standardized uptake values (SUV), that is, mean ROI radioactivity concentration normalized to injected dose and body weight.

2.4. Ex vivo tissue distribution studies.

Rats (saline (n=4), MTX (n=4)) were sacrificed sixty minutes after $[^{18}F]$fluoro-PEG-folate tracer administration [33]. Upon sacrificing, the knees, liver, and spleen were excised, rinsed, dipped dry, weighed, and the amount of radioactivity determined using an LKB 1282 Compgamma CS gamma counter (LKB, Wallac, Turku, Finland). Tissue radioactivity was expressed as percentage of the injected dose per gram tissue (%ID/g).

2.5. Histopathology and immunohistochemistry.

The liver and spleen sections from all rats (n=3 for healthy rats and n=4 for saline- and MTX-treated rats) were fixed in 4% neutral buffered paraformaldehyde for 24 h before embedding in paraffin wax. Sections of 5 μm were cut and stained initially with haematoxylin and eosin and then with an ED1 (homologous to human CD68), ED2 (homologous to human CD163), or isotype control antibody [32]. ED2/CD163 serves as marker for M2-type (anti-inflammatory) macrophages. Images were captured using a Leica 4000B microscope and Leica digital camera DC500 (Microsystems B.V. Rijswijk, Netherlands).

2.6. FR immunofluorescence and microscopy (frozen rat tissue).

At the end of the study, liver and spleen tissues were collected from healthy rats (n=3) and saline- and MTX-treated rats (n=4) and snap frozen in liquid nitrogen and stored at $-80^\circ$C. Tissues were embedded in appropriate media (OCT; SKU4583, Tissue-Tek, Netherlands) and were cut using cryotome ($-20^\circ$C) (Leica, Netherlands) and placed on Superfrost (4951PLUS4, ThermoFisher, Netherlands) glass slides for immunofluorescence (IF) staining. Sections of 8 μm were cut and stained with haematoxylin and eosin, and staining for FRβ-positive macrophages was performed with a mouse anti-rat FRβ antibody [29] or isotype control antibody. For immunostaining, liver and spleen tissue sections were first brought to room temperature (RT) for 30 min, fixed in acetone (439126, Sigma-Aldrich, Netherlands) for 10 min at $-20^\circ$C, and air-dried for 10 min at RT. A DAKO pen was used to mark the sections (S2002, DAKO, Santa Carla, CA, USA) which were subsequently washed 3x with PBS on a shaker. Next, sections were incubated with 100% fetal bovine serum (FBS) for 30 min at RT to avoid nonspecific binding and washed again in PBS (3 x 5 min). Thereafter, sections were incubated with mouse anti-rat FRβ IgM (final concentration 1 μg/ml) or isotype control IgM (ab35768, Abcam, Cambridge, UK; final concentration 1 μg/ml) in 10% FBS/PBS for 24 hours at 4°C or with 10% FBS/PBS. After washing (3 x 5 min in PBS on a shaker), sections were incubated with goat-anti-mouse Alexa 488 ((final concentration 1 μg/ml) (A21042)
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ThermoFisher Scientific, Netherlands) in 10% FBS/PBS, washed (3 × 5 min in PBS on a shaker), air-dried, and mounted with 2 μL of MOWIOL mounting medium (81381, Merck, Zwijndrecht, The Netherlands). The 2D IF slides were imaged with a Zeiss Axiovert 200 M MarianasTM inverted microscope (40x oil-immersion lens). The microscope, camera, and data processing were controlled by SlideBook software (SlideBook version 6 (Intelligent Imaging Innovations, Denver, CO)) as described previously [35].

2.7. Quantification of macrophages.

The identity of all stained slides was hidden from and counted by two independent observers for FRβ and ED1- and ED2-positive macrophages. For quantification, representative areas of liver and spleen sections were divided into 4 regions and counted at 400x magnification for FRβ and ED1- and ED2-positive macrophages in the saline- and MTX-treated rats. The average numbers of macrophages per area from all four regions were combined and depicted as total numbers of FRβ, ED1, or ED2 macrophages.

2.8. Statistical analysis.

Statistical analysis was performed using SPSS (version 15) for Windows (SPSS Inc., Chicago, IL, USA). Mann–Whitney (exact) tests were performed to analyse differences in tracer uptake (tissue distribution) macrophage infiltration in saline- versus MTX-treated groups. A $P$ value <0.05 was considered as statistically significant. All results are presented as mean ± standard deviation (SD).

RESULTS AND DISCUSSION

3.1. Arthritis induction and MTX therapeutic interventions.

Upon arthritic induction all rats showed macroscopic thickening of the arthritic knee compared with the contralateral control knee (data not shown). As shown earlier, arthritis induction was well tolerated and allowed a window for therapeutic intervention with MTX, which was also well tolerated and not associated with any adverse effects [32].

3.2. [$^{18}$F]Fluoro-PEG-Folate PET.

In a recent study we showed that imaging with the macrophage tracer [$^{18}$F]fluoro-PEG-folate could visualize decreased accumulation of the tracer in the knee joints of arthritic rats treated with MTX. In the present study, we particularly focussed on macrophage-rich organs such as liver and spleen for their potential involvement in systemic inflammation and the impact of MTX therapy upon this. The coronal PET images visualized higher tracer uptake in liver and spleen of the saline-treated arthritic rats (Figure 4.1(A)) compared to the MTX-treated rats (Figure 4.1(B)). Standard uptake values (SUV) of [$^{18}$F]fluoro-PEG-folate were quantified for liver and spleen with ROIs (colored ellipsoid) demonstrating decreased liver (1.5-fold) (Figure 4.1(C)) and spleen (2-fold) (Figure 4.1(D)) tracer uptake in MTX-treated compared to saline-treated rats. The relatively high uptake in the intestinal area, kidney, and bladder was due to the known clearance of the folate tracer [25, 32].
Figure 4.1 - Representative coronal PET images of $[^{18}F]$fluoro-PEG-folate in (A) saline-treated (n=2) and (B) MTX-treated (n=2) rats. Orange ellipsoid: ROI drawn around the synovium of the knee joint, liver (white arrow), spleen (red arrow), and arthritic (right (R); yellow arrow) and contralateral knees (left (L)) depicted on each image. Spleen and liver areas are indicated by dashed lines. Standardized uptake value (SUV) scale bar from minimum 0 to maximum 1 represents the uptake of the tracer. Clearance organs intestine (I) and bladder (B) are also depicted. $[^{18}F]$Fluoro-PEG-folate uptake is expressed as SUV (±SD) in (C) liver and (D) spleen of the saline- and MTX-treated group.
The MTX treatment results showed that, beyond knee joints, folate tracer binding is also inhibited by methotrexate in the extra-articular tissues, liver, and spleen, which suggests local anti-inflammatory effects on macrophage activity as part of systemic inflammation in these organs. These results are consistent with data from another arthritic rat model wherein $^{99m}$Tc]-EC20 folate scans also showed increased tracer uptake in liver and spleen [36] as compared to healthy rats. The increased tracer uptake in liver and spleen in arthritic rats coincided with increased tissue FR levels as measured by $[^3H]$folic acid binding studies. Notably, a clinical study with $[^{18}F]$-FDG, an indicator of active metabolism, in patients with collagen vascular disease-associated arthritis also showed significantly increased tracer uptake in the spleen, pointing to its inflammatory involvement [37]. In a clinical study in RA patients with $^{99m}$Tc]-EC20 folate, articular inflammation as well as liver and spleen involvement were demonstrated [38], further corroborating systemic inflammatory effects in arthrits.

3.3. Ex Vivo Tissue Distribution Studies.

To further establish the usefulness of therapeutic monitoring of systemic inflammation via $[^{18}F]$fluoro-PEG-folate PET and regular MTX treatment (the anchor drug in RA), ex vivo tissue distribution studies were performed on selected tissues 60 minutes after tracer injection. In excised liver and spleen sections of MTX-treated rats, tracer uptake was significantly 3- and 16-fold lower ($P < 0.01$ and $P < 0.001$), respectively, compared to the saline-treated rats (Figure 4.2). For comparison, previously reported tracer uptake in liver and spleen of healthy rats was ~1.4-fold lower [32] than in arthritic rats also pointing at presence of systemic inflammation/macrophage activity in liver and spleen. The markedly lower tracer uptake (5-fold, $P < 0.01$) in the MTX-treated arthritic rat knees [32] is depicted as a reference (Figure 4.2). Plasma levels of $[^{18}F]$fluoro-PEG-folate were low and comparable between both groups. Uptake of $[^{18}F]$fluoro-PEG-folate in kidney (2.92±0.33 versus 3.34±0.63 %ID/g) and intestine (1.06±0.49 versus 0.84±0.56 %ID/g) is not significantly altered after MTX therapy. This is consistent with the notion that kidney constitutively expresses another FR isoform (i.e., FRα, implicated in renal retention of folates) [32].

It is of importance to note that tissue distributions data were obtained 6 days after the last MTX administration; thus it is unlikely that lowered tracer uptake is due to FR blocking by MTX as residual plasma levels of MTX will be very low (<10 nM) at that stage [32]. Moreover, $[^{18}F]$fluoro-PEG-folate binding affinity towards FRβ outweighs MTX by at least 2-3 orders of magnitude [25, 27]. Together, PET and tissue distribution data illustrate that MTX treatment has a marked effect on macrophage tracer uptake in liver and spleen of arthritic rats.

3.4. Effect of MTX on Systemic Macrophage Infiltration.

To extend on the PET and ex vivo tissue distribution data with $[^{18}F]$fluoro-PEG-folate, the level of macrophage infiltration was examined in saline-treated and MTX-treated rats. Macrophage numbers were quantified in liver and spleen sections of saline-treated versus MTX-treated rats by immunohistochemical assessment of the abundance of total ED1-positive macrophages and ED2-positive macrophages, the latter, as CD163 homologue, serving as a proposed marker for anti-inflammatory macrophages. Figures 4.3
Figure 4.2 – Ex vivo tissue distribution of $[^{18}\text{F}]$fluoro-PEG-folate in liver, spleen, plasma, and arthritic knee of saline-treated (n=4) and MTX-treated (n=4) rats at 60 min after tracer injection. Results expressed as mean percentage injected dose per gram (\%ID/g). Error bars indicate SD. **$P < 0.01$ and ***$P < 0.001$. 

- **Saline**
- **MTX**
and 4.4 show representative images of ED1- and ED2-positive macrophages in liver and spleen sections. In liver and spleen of arthritic rats the numbers of ED1- and ED2-positive macrophages were ∼5-fold higher ($P < 0.01$) than those of healthy rats.

For both ED1- and ED2-positive macrophages in liver and spleen, a marked decrease in macrophage infiltration is noted for MTX treatment compared to saline-treated rats. This was confirmed by a significantly (4-fold, $P < 0.01$) lower numbers of ED1- and ED2-positive macrophages in the liver of MTX-treated rats (Figures 4.3(i) and 4.3(j)), compared to saline-treated rats. Similarly, spleen sections of MTX-treated rats revealed significantly (3-fold, $P < 0.01$) lower quantifications of ED1- and ED2-positive macrophages, compared to saline-treated rats (Figures 4.4(i) and 4.4(j)). Antibody control stained liver and spleen sections were clearly negative for both ED1- and ED2-positive macrophages (Figures 4.3(c), 4.3(d), 4.3(g), 4.3(h), 4.4(c), 4.4(d), 4.4(g), and 4.4(h)). It is of interest to note that MTX impacted the infiltration of both ED1 and ED2 macrophage in liver and spleen of arthritic rats. For ED2 macrophages this may be counterintuitive given their assigned anti-inflammatory phenotype [13]. However, in the context of RA, recent evidence suggests that M2 macrophages can be skewed to produce proinflammatory cytokines [39], which can shift the balance of M2 to a more M1 phenotype. An alternative explanation could be that the MTX impacts circulating proinflammatory subsets of FR expressing circulating monocytes [40] to suppress overall infiltration and polarization of macrophages in arthritic knees, liver, and spleen. Unravelling the exact mechanism of action of how MTX impairs macrophage infiltration awaits further research.

### 3.5. Effect of MTX on FRβ-Positive Macrophages.

FRβ-positive synovial macrophages were shown to be highly infiltrated in the synovium of RA patients [27]. Given that $[^{18}\text{F}]$fluoro-PEG-folate binds to FRβ [25], we examined the and MTX-treated arthritic rats to verify the data of the PET and tissue distribution studies. In liver and spleen of arthritic rats the number of FRβ-positive macrophages was significantly ($P < 0.01$) higher than those of healthy rats. Representative immunofluorescence images of FR β expression in cryosections of liver (Figures 4.5(a)–4.5(d)) and spleen (Figures 4.6(a)–4.6(d)) after saline and MTX therapeutic interventions revealed a markedly lower FRβ expression in both liver and spleen of MTX-treated versus saline-treated rats. This was confirmed by quantitative assessments showing significant 5-fold ($P < 0.005$) and 3-fold ($P < 0.01$) lower numbers of FRβ-positive macrophages in the liver (Figure 4.5(e)) and spleen (Figure 4.6(e)) of MTX-treated rats. The FRβ levels in MTX-treated rats approximated FRβ-positive macrophages in liver and spleen of healthy rats. Antibody control stained liver and spleen sections were negative (Figures 4.5(c), 4.5(d), 4.6(c), and 4.6(d)). Results for FRβ staining were consistent with ED1 and ED2 stainings (Figures 4.3 and 4.4). Together, these results underscore that macrophage infiltration in liver and spleen is implicated in inflammation and response to therapy, similar to that shown for RA synovium in patients [14–16].

In addition to MTX, antiarthritic effects elicited through FR targeting have been reported for folate-conjugated immunotoxins [29] and various folate-conjugated drugs [30, 41, 42]. Since FRβ is primarily expressed on activated macrophages [27, 28], microenvironmental conditions in liver and spleen will be of importance for FRβ expression and macrophage polarization. FRβ expression has been reported on both M1- and M2-
Figure 4.3 – Representative immunohistochemical (HE) images of ED1+ and ED2+ macrophages in liver sections of healthy (n=3) (ED1 and ED2), saline-treated (n=4), and MTX-treated (n=4) rats. ((a), (b)) Images represent ED1+ macrophages in the liver of saline-treated and MTX-treated rats, respectively. ((c), (d)) Isotype control stained liver sections of saline-treated and MTX-treated rats, respectively. ((e), (f)) Images of ED2+ macrophages in the liver of saline-treated and MTX-treated rats, respectively. ((g), (h)) Images of isotype control stained liver sections of saline-treated and MTX-treated rats, respectively. ((i), (j)) Bar graph representations of quantifications of ED1+ and ED2+ macrophages in liver of healthy, saline-treated, and MTX-treated rats. Values depict mean numbers of macrophages counted in predefined areas of the liver. Error bars indicate SD. **P < 0.01.
Figure 4.4 – Representative immunohistochemical (HE) images of ED1+ and ED2+ macrophages in spleen sections of healthy (n=3) (ED1 and ED2), saline-treated (n=4), and MTX-treated (n=4) rats. ((a), (b)) Images represent ED1+ macrophages in the spleen of saline-treated and MTX-treated rats, respectively. ((c), (d)) Isotype control stained spleen sections of saline-treated and MTX-treated rats, respectively. ((e), (f)) Images of ED2+ macrophages in the spleen of saline-treated and MTX-treated rats, respectively. ((g), (h)) Images of isotype control stained spleen sections of saline-treated and MTX-treated rats, respectively. ((i), (j)) Bar graph representations of quantifications of ED1+ and ED2+ macrophages in spleen of healthy, saline-treated, and MTX-treated rats. Values depict mean numbers of macrophages counted in predefined areas of the spleen. Error bars indicate SD. $P < 0.01.$
Figure 4.5 – Representative immunofluorescence images of FRβ+ macrophages in liver sections of healthy (n=3) and saline- (n=4) and MTX-treated (n=4) rats. ((a), (b)) Images represent FR β+ macrophages in the liver of saline-treated and MTX-treated rats, respectively. ((c), (d)) Isotype control stained liver sections of saline-treated and MTX-treated rats, respectively. (e) Bar graph representation of quantifications of FRβ+ macrophages in liver of saline-treated and MTX-treated rats. Values depict mean numbers of macrophages counted in predefined areas of the liver. Error bars indicate SD (blue color: DAPI (nucleus staining); green color: FRβ staining). $P < 0.01$. 
Figure 4.6 - Representative immunofluorescence images of FRβ+ macrophages in spleen sections of healthy (n=3) and saline- (n=4) and MTX-treated (n=4) rats. ((a), (b)) Images represent FRβ+ macrophages in the spleen of saline-treated and MTX-treated rats, respectively. ((c), (d)) Isotype control stained spleen sections of saline-treated and MTX-treated rats, respectively. (e) Bar graph representation of quantifications of FRβ+ macrophages in spleen of saline-treated and MTX-treated rats. Values depict mean numbers of macrophages counted in predefined areas of the spleen. Error bars indicate SD. (blue color: DAPI (nuclear staining); green color: FRβ staining). $P < 0.01$. 
type macrophages [43], and in rat RA synovium FRβ expression has been also observed on a mixed M1- and M2-type [44]. As indicated above, in the RA microenvironment with circulating complex IgG autoantibodies and/or ACPA antibodies, FRβ expressing activated macrophages can release proinflammatory cytokines [39, 45] and thus be a bonafide target. More detailed investigations on the specific polarization and phenotypic properties of FRβ expressing tissue macrophages in liver and spleen may assist optimal targeting of this receptor for imaging and therapeutic exploitations. These premises do not only hold for arthritis but also for cancer [46].

4. Conclusion

MTX treatment reduced activated macrophages in liver and spleen, as markers for systemic inflammation in these organs. Macrophage PET imaging with $^{[18}F]$fluoro-PEG-folate holds promise for detection of systemic inflammation in RA as well as therapy (MTX) response monitoring.

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References


Chapter 5

In vivo monitoring of anti-folate therapy in arthritic rats using $^{18}\text{F}]$fluoro-PEG-folate and positron emission tomography

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Abstract

Background
Folate receptor β (FRβ) is involved in facilitating cellular uptake of folates and anti-folates (such as methotrexate (MTX)). In rheumatoid arthritis, FRβ is expressed on synovial macrophages and recently has been explored as a biomarker for imaging in arthritic rats using the folate-based positron emission tomography (PET) tracer $[^{18}\text{F}]$fluoro-PEG-folate. The purpose of this study was to examine whether this folate tracer can also be used to monitor therapeutic efficacy of MTX in arthritic rats.

Methods
Arthritic rats received either no treatment or MTX therapy (1 mg/kg, either 2× or 4×). Healthy rats did not receive any arthritic induction or therapy. $[^{18}\text{F}]$fluoro-PEG-folate PET-CT scans (60 min) were performed before and after MTX therapy. Following PET, the ex vivo tissue distribution of radioactivity was determined in excised knees and multiple tissues. Synovial macrophage infiltration in knee sections was quantified by immunohistochemistry using ED1 and ED2 antibodies.

Results
PET scans clearly visualized increased uptake of $[^{18}\text{F}]$fluoro-PEG-folate in arthritic knees compared with contralateral knees. Significantly lower standard uptake values (1.5-fold, \(p < 0.01\)) were observed in arthritic knees of both MTX-treated groups after therapy, approximating the levels seen in healthy rats. Consistently, ex vivo tissue distribution demonstrated a 2–4-fold lower tracer uptake in the arthritic knee of 2× and 4× MTX-treated rats, respectively, compared with control rats. These results were corroborated with significantly reduced (2-4 fold, \(p < 0.01\)) ED1-positive and ED2-positive synovial macrophages in arthritic knees of the MTX-treated rats compared with those of the control rats.

Conclusion
This study in arthritic rats underscores the potential and usefulness of $[^{18}\text{F}]$fluoro-PEG-folate PET as a therapeutic monitoring tool of MTX therapy and potentially other anti-folate treatment of arthritis.

Keywords: $[^{18}\text{F}]$fluoro-PEG-folate, Folate receptor β, Methotrexate, Rheumatoid arthritis, Macrophages

Abbreviations
FDG - FluoroDeoxyGlucose; FRβ - Folate receptor beta; i.a. - Intra-articular; ID/g - Injected dose/gram; M-CSF - Macrophage-colony stimulating factor; MTX - Methotrexate; PEG - Polyethylene glycol; PET-CT - Positron emission tomography–computer tomography; RA - Rheumatoid arthritis; ROI - Region of interest; SD - Standard deviation; SUV - Standardized uptake value.
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BACKGROUND

Methotrexate (MTX) is the anchor drug in rheumatoid arthritis (RA) therapy, either as a single agent or in combination with disease-modifying anti-rheumatic drugs and biological agents [1–4]. Membrane transport via carrier-mediated and receptor-mediated routes is the first regulatory step in the mechanism of action of MTX in immunological target cells [5–7]. Notably, in RA (synovial) macrophages, the folate receptor \( \beta \) (FR\( \beta \)) has been recognized as a major transport route for MTX, next to the reduced folate carrier [8, 9]. FR\( \beta \) expression is confined to cells of the myeloid lineage [10, 11] as opposed to the \( \alpha \)-isoform of FR (FR\( \alpha \)), which is selectively expressed in specific types of cancer (ovary, breast) [12–14]. Given the high binding affinity (low nanomolar Kd) of FR for folic acid, this receptor has been exploited for therapeutic targeting with folate-conjugated drugs [15,16] as well as imaging of FR\( \alpha \)-positive tumours and activated FR\( \beta \)-positive macrophages in RA [17–19]. FRs harbour several interesting properties for targeting with folate-based positron emission tomography (PET) tracers; for example, easy accessibility as an extracellular GPI-anchored membrane protein, high binding affinities for folates, and specific expression on activated macrophages in inflammatory diseases, allowing receptor targeting for imaging with folate-based PET tracers [9, 10].

In humans, macrophages have been identified as a sensitive biomarker for therapy monitoring, regardless of the choice of treatment [20]. Moreover, RA remission has been positively correlated with lower numbers of synovial macrophages [21]. These findings, however, were obtained by invasive histological studies. Clearly, non-invasive imaging of macrophages may be an attractive alternative approach to detect and monitor synovial activity in body tissues [22]. Animal models of arthritis can serve as a pre-clinical step to explore macrophage expression through novel imaging modalities. Beyond successful application of single-photon imaging agents for example, EC20, a \(^{99m}\)Tc-labelled folate [23–25] to image arthritis, recently a folate-based PET tracer, \([^{18}F]\)fluoro-PEG-folate, has been synthesized [26]. Such a tracer could potentially employ the higher sensitivity of PET and its ability to quantify uptake, which is essential for intervention studies. The potential of \([^{18}F]\)fluoro-PEG-folate for imaging macrophages has been demonstrated in an arthritic animal model [26]. However, the potential of \([^{18}F]\)fluoro-PEG-folate to monitor the efficacy of therapeutic interventions, in particular using anti-folates, such as MTX, has not been explored.

In the present study, the potential of \([^{18}F]\)fluoro-PEG-folate as a macrophage-targeted PET agent for monitoring MTX therapy efficacy in arthritic rats was examined.

METHODS

Animals

Wistar rats (male, 150–200 g; Charles River International Inc, Sulzfeld, Germany) were provided with standard food (16% protein rodent diet; Harlan Laboratories Inc., Madison, WI, USA) and water ad libitum. Rats were housed in groups of three to six in conventional cages and kept in a room with a 12-hour light/dark cycle, and constant room temperature (20°C) and humidity level (50%). Animal experiments were performed
in accordance with the European Community Council Directive 2010/63/EU for laboratory animal care and the Dutch Law on animal experimentation. The experimental protocol was validated and approved by the local committee on animal experimentation of the VU University Medical Center (DEC PET13-07).

Arthritic induction and therapeutic interventions

Wistar rats received 4 intra-articular (i.a.) mBSA injections, 4 or 5 days apart, in the arthritic knee as described previously [27]. This model had resemblance to human arthritis because after immunization and an i.a. mBSA injection in one knee, arthritis develops within a week as manifested by an increased knee thickness and synovial macrophage infiltration in the arthritic vs contralateral knee. This adds another major advantage to this model because the contralateral knee serves as an internal control over the arthritic knee. Additionally, by applying successive intra-articular mBSA injections after the first i.a. mBSA injection, a prolonged chronic phase of arthritis is maintained allowing assessments of therapeutic interventions. MTX (VU University Medical Center’s pharmacy, the Netherlands) was administered (1 mg/kg) intraperitoneally (i.p.) once at days 22, 24, 29 and 33 (after the first i.a. injection) for the 4-MTX group (n = 4 rats) or once 3 days before (day 31) and once 3 days after (day 37) the fourth i.a. injections (2-MTX group, n = 4). Control rats (untreated control group, n = 4 rats) received phosphate-buffered saline (PBS) i.p. once at days 22, 24, 29 and 33 (after the first i.a injection). Healthy rats did not undergo arthritis induction or receive any treatment. At the end of therapy (day 40), all rats were sacrificed and tissues were excised for further processing. Figure 5.1 summarizes the schedule of arthritis induction, therapeutic interventions and various analyses.

$[^{18}\text{F}]$fluoro-PEG-folate synthesis and PET-CT

$[^{18}\text{F}]$fluoro-PEG-folate was synthesized as described previously [26], with a radiochemical purity $> 97\%$ and mean specific activity of $31.4 \pm 5.5 \text{ GBq/\mu mol}$. Rats were anaesthetized using inhalation anaesthetics (isoflurane 2–2.5\% and oxygen 0.45 volume \%). The tail vein was cannulated with a poly-urethane 3-French cannula. During PET-CT (Mediso nanoPET-CT, Budapest, Hungary) rats were placed in an integrated heating bed while monitoring respiratory function. A computed tomography (CT) scan was performed for -5 min, followed by tracer administration ($10.5 \pm 1.1 \text{ MBq}$) at the start of a dynamic PET scan of 60 min. PET data were normalized, and corrected for scatter, randomization, attenuation, decay and dead time. List-mode PET data were rebinned in 19 successive frames ($4 \times 5, 4 \times 10, 2 \times 30, 3 \times 60, 2 \times 300, 3 \times 600$ and $1 \times 900 \text{ s}$), which were reconstructed using an iterative 3D Poisson ordered-subsets expectation-maximization algorithm with four iterations and six subsets. Resulting images had a matrix size of $170 \times 170 \times 157$ voxels, each with a dimension of $0.6 \times 0.6 \times 0.6 \text{ mm}^3$.

PET data analysis

Images were analysed using AMIDE software (A Medical Image Data Examiner, version 0.9.2) [28]. CT and PET images were superimposed for drawing regions of interest (ROI). Fixed-size ellipsoidal-shaped ROIs (dimensions: $7 \times 4 \times 7 \text{ mm}^3$) were drawn
Figure 5.1 – Timeline depicting the induction of arthritis in rats and therupon methotrexate (MTX) interventions. On day 7 and day 14 the firstand second immunization (Im1 and Im2) was administered, on day 19 the delayed type hypersensitivity (DTH) test and thereupon four intra-articular (i.a.) injections were administered. Upon arthritic induction the rats were administered saline (black arrows) or 2× MTX (dark grey arrows) or 4× MTX (light grey arrows). PET-CT (#) was performed before (day 22) and after (day 40) MTX therapy. At the end of the study (day 40), ex-vivo tissue distribution (TD) was performed. Healthy rats did not receive any arthritic induction or MTX treatment and were sacrificed on day 40.

manually over the area of both arthritic and contralateral knees in the last frame. Next, ROIs were projected onto the dynamic image sequence and time–activity curves (TACs) were generated. TACs were expressed as standardized uptake values (SUV); that is, mean ROI radioactivity concentration normalized to injected dose and body weight.

Ex vivo tissue distribution studies

All rats were sacrificed (60 min after tracer administration) and knees, blood and various internal organs were excised, rinsed, dipped dry, weighed and the amount of radioactivity determined using an LKB 1282 Compu-gamma CS gamma counter (LKB, Wallac, Turku, Finland). Results were expressed as percentage of the injected dose per gram of tissue (%ID/g) [27].

Histopathology and immunohistochemistry

Both knees were dissected in toto and fixed for 7 days at 4°C in 10% freshly made paraformaldehyde in PBS with 2% sucrose (pH 7.3) prior to decalcification in osteosoft (101728; Merck, Darmstadt, Germany) for ~2.5 weeks at room temperature. Thereafter, knees were embedded in paraffin. Sections of 5 μm were cut through the centre of the joint in a longitudinal direction and stained with haematoxylin and eosin to assess the degree of inflammation in synovial tissue. Staining for macrophages was performed as described previously [27]. Briefly, after antigen retrieval, sections were incubated with the specific mouse anti-rat monoclonal antibodies ED1, homologous to human CD68, and ED2, homologous to human CD163, or isotype control antibody for 1 hour at RT.
All antibodies were obtained from Hycult (Plymouth Meeting, PA, USA). The detection EnVision™ kit (K4063 dual-link-HRP rabbit/mouse; DAKO, Glostrup, UK) was used according to the instructions of the manufacturer with 3,3’-diaminobenzidine tetrahydrochloride (DAB; DAKO) containing 0.01% H\textsubscript{2}O\textsubscript{2}. Subsequently, sections were counterstained with haematoxylin, dehydrated and mounted. Images were captured using a Leica 4000B microscope and Leica digital camera DC500 (Microsystems B.V. Rijswijk, the Netherlands).

All stained slides were blinded and counted by two independent observers, guided by an experienced pathologist, for ED1-positive and ED2-positive synovial macrophages. For this purpose, knee sections were divided into four quadrants (Q1–Q4), each representing the joint capsule with synovial tissue lining on either side of the proximal and distal side of the bone. Under the microscope (Leica, Amsterdam, the Netherlands) at 400× magnification, in each quadrant two to three areas were evaluated for macrophages in the lining and sub-lining (1–10 layers) of the synovium. The average number of macrophages per area from all four quadrants were combined and presented as total number of ED1 or ED2 macrophages (± standard deviation (SD)).

Statistical analysis
Statistical analysis was performed using SPSS (version 15) for Windows (SPSS Inc., Chicago, IL, USA). The Wilcoxon signed-rank (exact) test was used to determine differences in paired observations, such as uptake of $[^{18}\text{F}]$fluoro-PEG-folate in arthritic versus contralateral knees. Mann–Whitney (exact) tests were performed to analyse differences in $[^{18}\text{F}]$fluoro-PEG-folate uptake in groups (i.e. arthritic versus normal and control knees). $p < 0.05$ was considered statistically significant. All results are represented as mean ± SD.

RESULTS
Arthritis induction and therapeutic interventions
Arthritis induction in rats (see Fig. 5.1 for timeline) was associated with macroscopic thickening of the arthritic knee compared with the contralateral knee (data not shown). Therapeutic interventions with MTX at the time points depicted in Fig. 5.1 were not associated with any adverse effects or visible effects on knees and no significant changes in body weight were observed.

$[^{18}\text{F}]$fluoro-PEG-folate PET studies in untreated and MTX-treated arthritic rats
At baseline, $[^{18}\text{F}]$fluoro-PEG-folate PET scans (Fig. 5.2a–c) clearly visualized high uptake in the arthritic knee of control rats, which decreased in both the 2 × -MTX and 4 × -MTX groups. Before treatment, $[^{18}\text{F}]$fluoro-PEG-folate SUV in the arthritic knee (1.01 ± 0.07) was significantly ($p < 0.01$) higher than in the contralateral knee (0.67 ± 0.04) (Fig. 5.2a, d). After MTX treatment, both the 2 × -MTX (0.67 ± 0.11) (Fig. 5.2b) and 4 × -MTX (0.70 ± 0.10) groups (Fig. 5.2c) showed a significantly ($p < 0.01$)
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Figure 5.2 – Representative coronal PET-CT scans of $[^{18}\text{F}]$fluoro-PEG-folate in control and arthritic rats. (a) Control (before therapy, day 22); (b) 2× MTX and (c) 4× MTX (after therapy, day 40). Orange ellipsoid: ROI drawn around the synovium of the knee joint. Arthritic (right) and contralateral knees (left) depicted on each image. Standardized uptake value (SUV) scale bar from minimum 0 to maximum 1, represent the uptake of the tracer. TACs of $[^{18}\text{F}]$fluoro-PEG-folate uptake are expressed as SUV (±SD) in arthritic and contralateral knees of the control group (d), 2× MTX group (e) and 4× MTX group (f).

1.5-fold lower uptake of $[^{18}\text{F}]$fluoro-PEG-folate compared with untreated arthritic rats. In fact, SUV values in the arthritic knee of both 2× -MTX and 4× -MTX rats (Fig. 5.2e, f) were comparable with the level of uptake in knees of healthy rats (0.67 ± 0.07) (data not shown).

Ex vivo tissue distribution studies

Before treatment, control rats showed a significantly 1.5-fold ($p < 0.05$) higher uptake (expressed as %ID/g) of $[^{18}\text{F}]$fluoro-PEG-folate in arthritic knees (0.22 ± 0.04) compared with contralateral knees (0.14 ± 0.04). After MTX treatment, $[^{18}\text{F}]$fluoro-PEG-folate uptake in arthritic knees was significantly 2-fold ($p < 0.05$) lower in the 2× -MTX group (0.11 ± 0.01) and 4-fold ($p < 0.01$) lower in the 4× -MTX group (0.06 ± 0.03) compared with untreated arthritic rats (Fig. 5.3).

Uptake of $[^{18}\text{F}]$fluoro-PEG-folate in plasma did not differ between control vs 2× -MTX and 4× -MTX rats (0.010 ± 0.006 vs 0.009 ± 0.001 and 0.009 ± 0.001, respectively) (Table 5.1). MTX treatment, however, did reduce $[^{18}\text{F}]$fluoro-PEG-folate uptake in high macrophage resident organs, as illustrated in Table 5.1 for the control
Figure 5.3 – Ex-vivo tissue distribution of $^{18}$F-fluoro-PEG-folate in arthritic and contralateral knees of healthy (white bars), control rats (black bars) and $2\times$ -MTX (dark grey) and $4\times$ -MTX (light grey) rats at 60 min post tracer injection. Results expressed as mean percentage injected dose per gram (%ID/g). Error bars indicate SD. MTX methotrexate

group vs both $2\times$ -MTX and $4\times$ -MTX groups in the liver, heart, spleen, lung and bone. In the kidney and intestine, MTX treatment had no major impact on tracer uptake in control, $2\times$ -MTX and $4\times$ -MTX rats (Table 5.1).

Immunohistochemistry of synovial macrophages

To examine whether the lower $^{18}$F-fluoro-PEG-folate uptake in arthritic knees after MTX treatment was due to reduced infiltration of synovial macrophages, ED1-positive and ED2-positive macrophages in synovial tissue were quantified. Microscopically, synovial tissue of arthritic rats showed cellular influx of ED1+ and ED2+ macrophages (Fig. 5.4a–l), the latter of which were significantly ($p < 0.01$) more abundant: 3-fold ($42 \pm 9$ vs $15 \pm 4$) and 2-fold ($36 \pm 8$ vs $13 \pm 3$), respectively, in the arthritic and contralateral knees (Figs. 5.4a vs d, g vs j and 5.5). Both the $2\times$ -MTX and $4\times$ -MTX treatment groups of arthritic rats showed a marked and significant reduction of ED1+ and ED2+ synovial macrophages in the arthritic knees compared with the untreated counterparts (Fig. 5.4).

Quantification showed a 4-fold and 3-fold ($p < 0.01$) reduction in ED1+ synovial macrophages after $2\times$ -MTX and $4\times$ -MTX treatments (Fig. 5.5a), respectively. For ED2+ the reduction was 3-fold ($p < 0.01$) for both groups (Fig. 5.5b). Notably, MTX treatment reduced ED1+ and ED2+ synovial macrophages in arthritic knees to levels observed in contralateral knees (Fig. 5.5).
Figure 5.4 – Representative images of ED1+ and ED2+ synovial macrophages in knee sections of control and MTX-treated rats. a, b, c Images represent ED1+ synovial macrophages in the arthritic knee of control, 2 × -MTX and 4 × -MTX rats, respectively. d, e, f ED1+ synovial macrophages in the contralateral knee of control, 2 × -MTX and 4 × -MTX rats, respectively. g, h, i images of ED2+ synovial macrophages in the arthritic knee of control and 2 × -MTX and 4 × -MTX rats, respectively. j, k, l ED2+ synovial macrophages in the contralateral knee of control, 2 × -MTX and 4 × -MTX rats, respectively. All images captured at 200× magnification.
Table 5.1 – Ex vivo tissue distribution of $[^{18}\text{F}]$fluoro-PEG-folate in various tissues of healthy control, $2\times$ -MTX and $4\times$ -MTX rats at 60 min post tracer injection. Results expressed as mean percentage injected dose per gram (%ID/g) ± standard deviation. MTX methotrexate

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<th>4x -MTX group</th>
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<tr>
<td>Heart</td>
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<tr>
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DISCUSSION

The present study, using $[^{18}\text{F}]$fluoro-PEG-folate, investigated the feasibility of non-invasively monitoring efficacy of antifolate therapeutic interventions in RA. Lower accumulation of $[^{18}\text{F}]$fluoro-PEG-folate in arthritic knees corroborated with decreased numbers of active macrophages in MTX-treated rats compared with the untreated rats. This was illustrated for MTX, because this is the golden standard in clinically active RA treatment [1, 3, 4]. Folate receptor expression on activated macrophages has been exploited for imaging and therapeutic monitoring of arthritis with various folate PET tracers including $4[^{18}\text{F}]$fluorophenylfolate and $[^{68}\text{Ga}]$-DOTA-folate [29]. These PET tracers showed a significantly improved specificity over a general inflammation tracer $[^{18}\text{F}]$FDG, which relates to increased glucose metabolism in, for example, activated macrophages. In the present study, we made use of a pegylated folate tracer, $[^{18}\text{F}]$fluoro-PEG-folate, which harbours improved plasma pharmacokinetic properties over other folate tracers. In a side-by-side comparison in a rat model for RA [27], $[^{18}\text{F}]$fluoro-PEG-folate demonstrated a 1.5× improved target to background ratio compared with the mitochondrial translocator protein targeted macrophage tracer $(R)-[^{11}\text{C}]$PK11195 [26]. Moreover, $[^{18}\text{F}]$fluoro-PEG-folate also displayed promising PET imaging potential [26], which was taken a step further in the present study for monitoring therapeutic interventions, such as MTX therapy.

$[^{18}\text{F}]$fluoro-PEG-folate PET combined with a CT has advantage over the previous reported $[^{18}\text{F}]$fluoro-PEG-folate PET study [26], because the region of interest (ROI) around the synovium can be depicted more precisely. $[^{18}\text{F}]$fluoro-PEG-folate showed a marked reduction in tracer uptake in arthritic knees of the rats following two different MTX treatment regimens. It is unlikely that reduced tracer uptake in the MTX-treated rats is due to direct competition of the radiolabelled tracer with MTX for FRβ for various reasons: PET scans were acquired in the last week after the last MTX dose and, based on MTX pharmacokinetics [30] at that time, residual plasma levels will be <10
Figure 5.5 – Quantification of ED1+ and ED2+ synovial macrophages in knee sections of control and treated rats. a ED1+ synovial macrophages, 6 days after the third boost, of control rats (black bars) and 2 × -MTX (dark grey) and 4 × -MTX (light grey) rats. b ED2+ synovial macrophages, 6 days after the third boost, of control rats (black bars) and 2 × -MTX (dark grey) and 4 × -MTX (light grey) rats. Values represent mean number of macrophages counted in predefined areas of the synovium. Error bars indicate SD. MTX methotrexate, SD standard deviation

nM; the binding affinity of FR3 for [18F]fluoro-PEG-folate outweighs the binding affinity for MTX by at least 100-fold; and also the binding affinity of the natural circulating plasma folate (i.e. 5-methyltetrahydrofolate) is 3-fold higher than the tracer [9, 26], and thus competitive effects are not anticipated. In addition, immunohistochemical analysis of the arthritic joints showed a significant reduction of macrophages in synovial tissue which was in line with reduced joint uptake of the folate tracer. Consistent with our PET results, Kelderhouse et al. [31] also demonstrated a markedly lower accumulation of the SPECT folate targeted imaging agent [99mTc]-EC20 in a collagen-induced arthritis (CIA) model upon administration of anti-rheumatic drugs. In the same CIA model, OTL0038, a novel folate-conjugated near-infrared dye, also showed low accumulation following anti-rheumatic therapies [32]. Together, whereas SPECT and optical imaging each has proven value with folate-based imaging agents, PET folate harbours advantages over SPECT (low-resolution and low-sensitivity images) [26] and optical imaging (no deep tissue imaging) [32]. Although costs of PET are relatively high at this moment, it is anticipated that with the widespread application of PET technology worldwide, costs will come down in the near future as also happened in the past decennia for the other imaging techniques such as CT and MRI.

Previously, apart from prominent arthritis induction in the arthritic knee, signs of systemic inflammation were also observed [27], reflected by tracer uptake in macrophage-rich organs, especially the liver and spleen. Ex vivo tissue distribution data indicated that MTX therapy also had systemic effects by reducing [18F]fluoro-PEG-folate uptake in these organs as well as in the contralateral knee. Independent of MTX therapy, the
increased accumulation of $[^{18}\text{F}]$fluoro-PEG-folate in kidneys and intestinal tissue could be attributed to tracer clearance and/or high expression of FRα on kidney proximal tubule cells [15, 17] and intestinal tissue [33] to which receptor the folate tracer also binds.

Immunohistochemical analyses indicated that markedly reduced numbers of macrophages in the synovium of MTX-treated arthritic rats accounted for reduced $[^{18}\text{F}]$fluoro-PEG-folate tracer uptake. Interestingly, reduction in macrophages upon MTX treatment involved both ED1-positive and ED2-positive macrophages although CD68 (and possibly its rat homologue ED1 used in the present study) is not an absolute marker for macrophages because its expression has also been demonstrated on fibroblasts. To extend on this point, an experienced pathologist examined the (arthritic) knee sections for morphological assessment of cellularity and immunohistochemical staining of ED1+ cells. ED1+ synovial fibroblasts were identified but morphologically discerned from macrophages and were not taken into account for macrophage scoring assessments. Moreover, from the perspective of macrophage polarization, we also examined numbers of ED2+ macrophages (the rat homologue of human CD163, so-called ‘M2’, ‘anti-inflammatory macrophages’) in synovial tissue before and after therapy. Notably, studies by Puig-Kroger et al. [34] showed that FRβ is differentially expressed on M2 macrophages upon ex-vivo skewing of monocytes with macrophage-colony stimulating factor. However, studies by Tsuneyoshi et al. [35] in human RA synovial tissue demonstrated mixed patterns of FRβ expression on ‘M1’ (‘pro-inflammatory’) and M2 macrophages. In this context it is also important to note that M2 macrophages in an RA environment with complex IgG autoantibodies and/or ACPA antibodies are triggered to produce pro-inflammatory cytokines [36, 37]. Thus, MTX targeting with respect to polarization of macrophages and the role of FRβ therein warrant further investigations. Given the fact that beyond MTX several other second-generation anti-folates have been developed which have demonstrated potential pre-clinical anti-arthritic activity [7, 9, 38–40], $[^{18}\text{F}]$fluoro-PEG-folate PET imaging may be useful for monitoring their efficacy.

CONCLUSION

The present study demonstrates the feasibility of in vivo monitoring of MTX therapy in arthritic rats using $[^{18}\text{F}]$fluoro-PEG-folate PET, paving the way for its future use in human clinical RA.

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References


Section II

Evaluation of novel therapeutics in an arthritic rat model
Chapter 6

Prophylactic and therapeutic activity of alkaline phosphatase in arthritic rats: single agent effect of alkaline phosphatase and synergistic effects in combination with methotrexate

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Abstract

Alkaline Phosphatase (AP) is a gate-keeper of innate immune system responses by detoxifying inflammation triggering moieties released from endogenous and external sources. We examined whether AP’s broad mechanism of action constitutes a safe therapeutic, either as single agent or combined with methotrexate (MTX), for chronic inflammatory disorders, e.g. rheumatoid arthritis (RA).

A rat model for RA was used with repeated intra-articular methylated Bovine Serum Albumin (mBSA) injections in one knee (“arthritic” knee), the contralateral knee serving as internal control. Recombinant human placental AP (200 µg, subcut) was administered before mBSA injections (prophylactic setting) or after arthritis induction (therapeutic setting) or combined with MTX (0.3 mg/kg or 1 mg/kg; i.p.). As endpoint of treatment outcome, macrophage infiltration in knees, liver and spleen was assessed by immunohistochemistry (ED1 and ED2 expression), immunofluorescence (macrophage marker Folate Receptor-β, FRβ), and [18F]fluoro-PEG-folate PET (macrophage imaging) and ex-vivo tissue distribution.

Single agent AP treatment and combinations with MTX were well tolerated. Both prophylactic and therapeutic AP markedly reduced synovial macrophage infiltration in arthritic knees (ED1; 3.5-4 fold, ED2; 3.5-6 fold), comparable with MTX treatment. AP/MTX combinations slightly improved on single agent effects. PET monitoring and ex-vivo tissue distribution studies corroborated the impact of AP, MTX and AP/MTX on reducing synovial macrophage infiltration. Beyond localized articular effects, AP also revealed systemic anti-inflammatory effects by a 2-fold reduction of ED1, ED2 and FRβ-positive macrophages in liver and spleen of arthritic rats. Collectively, single agent AP and combined with MTX elicited local and systemic anti-arthritic activity in arthritic rats.

Keywords: Rheumatoid arthritis, Alkaline phosphatase, Methotrexate, [18F]fluoro-PEG-folate, Folate Receptor β, PET-CT, macrophages, systemic inflammation.

Abbreviations

AP - Alkaline Phosphatase; DMARD - Disease Modifying Anti-Rheumatic Drug; EULAR - European league against rheumatism; FR - Folate Receptor; hRESCAP - human rescue alkaline phosphatase; i.a. - intra articular; ID/g - injected dose/ gram; i.p. - intraperitoneally; MTX - Methotrexate; mBSA - methylated Bovine Serum Albumin; PEG - Polyethylene glycol; PET-CT - Positron Emission Tomography – Computer tomography; RA -Rheumatoid Arthritis; s.c. - subcutaneously.
INTRODUCTION

Rheumatoid arthritis (RA) is an auto-immune disease with hallmarks of synovial and systemic inflammation which, when left untreated, leads to progressive bone and joint destruction\(^1\)\(^-\)\(^3\). Current treatment options for RA include chemical disease modifying anti-rheumatic drugs (cDMARDs) with methotrexate (MTX) and glucocorticoids as predominant initial treatments\(^4\),\(^5\). MTX has an established place in RA treatment based on its low costs, safety profile, efficacy and longstanding clinical experience\(^6\),\(^7\). Still, a considerable fraction (30-40%) of RA patients are faced with MTX intolerance or inefficacy early on in the treatment or loss of efficacy during chronic treatment\(^8\)\(^-\)\(^1\)\(^1\). At this stage, treatment with biological DMARDs (bDMARDs) is indicated\(^1\), but these come with higher costs and are also prone to development of resistance\(^1\)\(^0\),\(^1\)\(^2\). Therefore, identification of novel modalities that could reinforce longer-lasting efficacy of MTX, or have alternative mechanisms of action, deserve continued interest.

Alkaline Phosphatase (AP), a glycosylphosphatidylinositol (GPI) anchored protein, is a member of the family of ecto-phosphatase proteins\(^1\)\(^3\)\(^-\)\(^1\)\(^7\). At least 4 different enzyme isoforms of AP are differentially expressed in various tissues (e.g. liver, intestine, placenta, bone) and immune cells (e.g. macrophages) from where AP may be released in soluble form by GPI-specific phospholipase D\(^1\)\(^6\),\(^1\)\(^8\). Since ectophosphatases act extracellularly by dephosphorylating inflammation triggering moieties (ITMs), AP is thought to have a gate-keeper function in the innate immune system by detoxifying well-known ITMs from external and internal sources such as lipopolysaccharides (LPS), CpG oligodeoxynucleotides (CpG-DNA) and nucleotide phosphates (adenosine tri-, di- and mono-phosphates (ATP, ADP and AMP, respectively)\(^1\)\(^9\)\(^-\)\(^2\)\(^5\). Whereas increased concentrations of extracellular nucleotide phosphates exert pro-inflammatory signals\(^2\)\(^6\),\(^2\)\(^7\), their ectophosphatase-mediated conversion to adenosine conveys a well-recognised anti-inflammatory effect via interactions with adenosine receptors\(^2\)\(^8\). This mechanism of action is reminiscent of the dominant anti-arthritic mechanism of action of MTX\(^8\)\(^-\)\(^1\)\(^0\),\(^2\)\(^9\), which involves the non-lytic extracellular release of adenosine and extrusion of adenine nucleotides (AMP, ADP, ATP) that are converted to adenosine by the action of ectophosphatases CD39 and CD73 on immune-competent cells\(^3\)\(^0\). Recent evidence indicates that down-regulation of CD39 on regulatory T cells during MTX treatment inhibits local generation of adenosine, thereby conferring MTX resistance in RA\(^3\)\(^1\). Under conditions of attenuated CD39 and CD73 function, AP may compensate for their function. AP therapeutic interventions have been studied in preclinical and clinical models of local and systemic inflammatory diseases. In fact, in animal models, exogenous ectophosphatase interventions by AP resulted in near complete inhibition of systemic TNF\(\alpha\), IL6 and IL8 response after a systemic inflammatory insult with LPS\(^2\)\(^3\),\(^3\)\(^2\). In humans AP administration prevented the induction of these pro-inflammatory cytokines/chemokines peri- and postsurgical in patients undergoing open heart surgery\(^3\)\(^3\),\(^3\)\(^4\). In the RA setting, AP intervention is relatively unexplored. As a preliminary account, a safety assessment (phase 1/2a) study of multiple injections with bovine intestinal AP (twice daily, 2000U, sc, for 3 days) in 6 RA patients (treatment-resistant, severe active RA, including anti-TNF failures) with 3 months follow up showed no safety events, and 1 sustained and 1 transient clinical response with temporary improvement of DAS response (A Hammond, data on file). Bovine AP, however, is rather short-lived (\(T_{1/2}\)max: 2hr) which hampers
proper repeated treatments.

In this study, the potential anti-arthritic activity of recombinant human placental AP (hRESCAP) was examined in an established rat model of RA \(^{35}\). This model is characterized by extensive synovial macrophage infiltration upon arthritis induction, which is also a hallmark of RA disease in humans \(^{36}\). Moreover, upon therapeutic intervention, reduction of synovial macrophage infiltration indicates efficacy of therapy in human RA \(^{37,38}\). The purpose of this study was to assess the impact of longer-lived recombinant human placenta AP as standalone treatment and in combination with MTX on synovial macrophage infiltration in arthritic rats. Additionally, to assess potential systemic anti-inflammatory effects, the impact of AP on macrophage infiltration in liver and spleen in arthritic rats was investigated. Macrophages were imaged primarily by immunohistochemistry (ED1 and ED2 markers) and immunofluorescence (Folate Receptor \(\beta\) (FR\(\beta\)) expression). For selected experiments complementary PET-CT and ex vivo tissue biodistribution studies were performed with the macrophage tracer \(^{[18}F\)fluoro-PEG-folate which is targeted to FR\(\beta\) on activated macrophages \(^{39-41}\).

**MATERIALS AND METHODS**

**Animals**

Groups of 3-6 Wistar rats (male, 150-200 grams, Charles River International Inc, Sulzfeld, Germany) were provided with standard food and water (ad libitum) under conditions described previously \(^{38}\). All animal experiments were performed according to the criteria and guidelines of European Community Council Directive 2010/63/EU for laboratory animal care and the Dutch Law on animal experimentation. The experimental protocol was approved by the local committee on animal experimentation of the VU University (DEC PET13-07).

**Arthritic induction and therapeutic interventions**

All (except healthy control) rats were immunized and arthritis was induced via 1x or 4x intra-articular (i.a) methylated Bovine Serum Albumin (mBSA) injections, 4 or 5 days apart in the arthritic (right) knee, with the contralateral (left, non-arthritic) knee serving as control as described before \(^{35}\). For therapeutic interventions, AP (human recombinant placenta AP, TNO, Zeist, The Netherlands) was administered subcutaneously (s.c.) at a dose of 700 U/kg (\(\approx 200 \mu g\)), MTX (Pharmacy VU University Medical Center) was administered intra-peritoneal (i.p.) at two dosages: 0.3 mg/kg (low dose) and 1.0 mg/kg (high dose).

Rats were divided in 8 groups, depicted in Figure 6.1, based on different treatments and treatment schedules. In a prophylactic setting, two rats received AP twice prior to intra-articular (i.a.) arthritis induction and four rats received AP twice prior to intra-articular injections and 4x PBS between i.a. injections. In the treatment groups, arthritic rats were administered AP twice or 4 times after arthritis induction, either as standalone therapy or in combination with low or high dose MTX. Control rats received
500µL of PBS (i.p.). Healthy rats didn’t receive arthritic induction or any treatment. At the end of each study, all rats were sacrificed and tissues excised for further analysis.

**Alkaline phosphatase activity**

An enzymatic assay was used to determine plasma concentrations of alkaline phosphatase prior to and 0-4 hours after administration of a dose of 700U/kg AP to healthy rats and arthritic rats. At time points 0, 15, 60, 120 and 240 min after AP administration, a blood sample was drawn from the tail vein of the rats and transferred to a 1 mL Eppendorf microtube containing heparin (454081, Greiner bio-one, Charlotte, USA) as anti-coagulant. As a control, blood was drawn at the same time points from arthritic rats that were injected a t=0 with 500 µl PBS. Eppendorf tubes were centrifuged at 3,000 x g for 5 min at 4°C, after which the plasma was collected and stored at -80°C until use.

The enzymatic assay for AP was based on the conversion of the substrate paranitrophenolphosphate (PNP; 104105, Sigma-Aldrich, Zwijndrecht, the Netherlands) to paranitrophenol, which is measured spectrophotometrically at 405 nm at 25°C. To a 3 mL reaction cuvette was added 2.9 mL substrate solution (containing final concentrations of 25 mM glycine, 10 mM MgCl₂, 3 mM PNP, adjusted to pH 9.6 with NaOH). The reaction was started by adding 30 µL of plasma sample, 1:1 diluted in enzyme diluent buffer (25 mM glycine, 1 mM MgCl₂, 0.1 mM ZnCl₂, 10% (v/v) glycerol, adjusted to pH 9.6 with NaOH). In parallel, a reference cuvette was assayed without substrate. The reaction was followed on line for 5 min at 25°C with continuous monitoring increase of absorbance at 405 nm using a (10037-434, VWR, Radnor, PA, USA) spectrophotometer. From the linear phase of A405 increments, AP activity in plasma samples (in U/L) was calculated from a standard curve with serial dilutions of a human recombinant placenta AP stock solution. One Unit of activity was defined as the amount of enzyme decomposing 1 µmol of PNP/min at 25°C and pH 9.6.

**Histopathology and immunohistochemistry**

The arthritic and contralateral knees from all rats were dissected in toto and fixed for 7 days at 4°C in 10% freshly made paraformaldehyde in PBS with 2% sucrose (pH=7.3) prior to decalcification in osteosoft (101728, Merck, Germany) for ~2.5 weeks at room temperature. Thereafter, knees were embedded in paraffin. Sections of 5 µm were cut through the centre of the joint in longitudinal direction and stained with haematoxylin and eosin (HE) to assess the degree of inflammation in synovial tissue.

Liver and spleen sections from all rats were dissected and fixed in 4% paraformaldehyde for 24 h before embedding in paraffin. Sections of 5 µm were cut and stained initially with HE and then for macrophages. Staining for macrophages with ED1 (homologous to human CD68) and ED2 (homologous to human CD163, a marker for M2 anti-inflammatory macrophages), or isotype control antibody was performed as described in detail previously. Images were captured using a Leica 4000B microscope and Leica digital camera DC500 (Microsystems B.V. Rijswijk, The Netherlands).
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**Figure 6.1** – Timeline of arthritis induction in rats and interventions with alkaline phosphatase (AP), MTX and AP/MTX combinations. On d7 and d14, the 1st and 2nd immunization (Im1 and Im2) with mBSA was performed; on d19 the Delayed Type Hypersensitivity (DTH) test, followed by four (unless otherwise indicated) intra-articular (i.a) injections as depicted. Interventions were made with AP (700 U/kg), MTX (0.3 mg/kg or 1.0 mg/kg), or AP/MTX combinations, as depicted. To one group of rats prophylactic administration of AP was given, i.e. twice before arthritis induction. All other groups received therapeutic administration of AP and/or MTX after arthritis induction. As a control, untreated arthritic rats received injections with PBS. PET-CT (#) was performed before (d22) and after (d40) AP therapy. At the end of the study (d40), rats were sacrificed after ex-vivo tissue distribution (TD) was performed. Healthy rats didn’t receive any arthritic induction and treatment and were sacrificed on day 26. (Black arrows = i.a. injections; Light grey arrows = AP and dark grey arrows = MTX).
FRβ immunofluorescence and microscopy

Liver and spleen tissues collected at the end of the study were snap frozen in liquid nitrogen and stored at -80°C. Tissues were embedded in appropriate media (OCT; SKU4583, Tissue-Tek, Netherlands), cut using a cryotome cryostat (-20°C) (Leica, The Netherlands) and placed on Superfrost (4951PLUS4, ThermoFisher, The Netherlands) glass slides for immunofluorescence (IF) staining. Sections of 8 µm were cut and stained with haematoxylin and eosin.

Immunostaining of FRβ was performed with a mouse anti-rat FRβ antibody or isotype control antibody. Specifically, liver and spleen tissue sections were first brought to room temperature (RT) for 30 min, fixed in acetone (439126, Sigma-Aldrich, Netherlands) for 10 min (-20°C) and air dried for 10 min (RT). A DAKO pen was used to mark sections (S2002, DAKO, Santa Carla, CA, USA), which subsequently were washed 3x with PBS on a shaker. Hereafter, sections were incubated with 100% fetal bovine serum (FBS) for 30 min (RT) to block non-specific binding and washed again in PBS (3x 5min). Next, sections were incubated with anti-rat FRβ (1:50) in 10% FBS/PBS or with 10% FBS/PBS for 24 h at 4°C. After washing (3x 5 min in PBS on a shaker), sections were incubated for 1 hour at RT with secondary antibody goat-anti-mouse Alexa 488 (1:500) (R37120; ThermoFisher Scientific, Netherlands) in 10% FBS/PBS, air dried and mounted (2µL of MOWIOL mounting medium (81381, Merck, Zwijndrecht, The Netherlands).

The 2D IF slides were imaged with a Zeiss Axiovert 200M Marianas™ inverted microscope, (40x oil-immersion lens). The microscope, camera and data processing were controlled by SlideBook™ software (SlideBook™ version 6 (Intelligent Imaging Innovations, Denver, CO)) as described previously.

Quantification of macrophages in knee sections, liver and spleen

All stained slides were blinded and counted by two independent observers for ED1- and ED2-positive synovial macrophages. To this end, the knee section was divided into four quadrants (Q1 to Q4), each representing the joint capsule with synovial tissue lining on either side of the proximal and distal side of the bone. Under the microscope (Leica) at 400x magnification, 2-3 areas in each quadrant were counted for macrophages in lining and sub-lining (1-10 layers) of the synovium. The average number of macrophages per area from all four quadrants were combined and used as total number of ED1 or ED2 macrophages (±SD).

Stained slides of liver and spleen sections of arthritic rats and AP-treated arthritic rats were blinded and counted by two independent observers for FRβ, ED1- and ED2-positive synovial macrophages. For quantification, representative areas of liver and spleen sections were divided into 4 regions, each representing a central pulp and vein, respectively. The FRβ, ED1- and ED2-positive macrophages were counted at 400x magnification as described above. The average number of macrophages per area from all four regions were combined and used as total number of FRβ, ED1 or ED2 macrophages. As a control liver and spleen sections of healthy rats were analyzed as control.
[\textsuperscript{18}F]fluoro-PEG-folate and PET-CT

The macrophage PET tracer [\textsuperscript{18}F]fluoro-PEG-folate was synthesized as previously described\textsuperscript{41}, with a radiochemical purity of >96.5% and a mean specific activity of 27.6 ± 3.5 GBq/µmol. Untreated and 4x AP-treated arthritic rats were anesthetized using inhalation anaesthetics (isoflurane 2-2.5% and oxygen 0.45 volume %). The tail vein was cannulated with a poly-urethane 3 French cannula (0.7mm x 19mm, BD Angiocath, Breda, The Netherlands). During PET-CT (Mediso nanoPET-CT, Budapest, Hungary) rats were place in an integrated heating bed (\textdegree C) while monitoring respiratory function. A CT scan was performed for 5 min, followed by tracer administration (10.7±1.8 MBq) at the start of a dynamic PET scan of 60 min. PET data were normalized, and corrected for scatter, randoms, attenuation, decay and dead time. List mode PET data were rebinned in 19 consecutive frames (4x5, 4x10, 2x30, 3x60, 2x300, 3x600 and 1x900 s), which were reconstructed using an iterative 3D Poisson ordered-subsets expectation-maximization algorithm with 4 iterations and 6 subsets. Resulting images had a matrix size of 225 × 225 × 236 voxels, each with a dimension of 0.40 × 0.40 × 0.46 mm\textsuperscript{3}.

Images were analysed using AMIDE software (A Medical Image Data Examiner, version 0.9.2)\textsuperscript{44} and were expressed as standardized uptake values (SUV), i.e. mean ROI radioactivity concentration normalized to injected dose and body weight. CT and PET images were superimposed for drawing the regions of interest (ROI). Using the last frame fixed size ellipsoidal shaped ROI (dimensions: 7 × 4 × 8 mm\textsuperscript{3}) were manually drawn over the area of both arthritic and contralateral knees. Time activity curves (TAC) were extracted by projecting ROI onto the dynamic image sequence.

Ex vivo tissue distribution studies

At the end of the treatment period, both arthritic rats receiving 4x AP, 4x AP/low dose MTX, 4x AP/high dose MTX treatment and untreated rats were administered with [\textsuperscript{18}F]fluoro-PEG-folate tracer (10.7±1.8 MBq). Sixty minutes after tracer administration, rats were sacrificed. Low and high dose MTX treated arthritic rats were sacrificed without tracer administration. Next, knees, blood and various internal organs were excised, rinsed, dipped dry, weighed and the amount of radioactivity determined using an LKB 1282 Compugamma CS gamma counter (LKB, Wallac, Turku, Finland). Tracer uptake in the various tissues was expressed as percentage of injected dose per gram tissue (%ID/g)\textsuperscript{38}.

Statistical analysis

Statistical analyses were performed using SPSS (version 15) for Windows (SPSS INc, Chicago, IL, USA). The Wilcoxon signed rank (exact) test was used to determine differences in paired observations, such as macrophage infiltration in arthritic versus contralateral knees. Mann-Whitney (exact) tests were performed to analyse differences in macrophage infiltration in groups, e.g. arthritic versus PBS treated knees. A p-value <0.05 was considered as statistically significant. All results are presented as mean ± standard deviation (SD).
RESULTS

Arthritis induction and AP/MTX therapeutic interventions

Arthritis induction in rats was associated with measurable macroscopic thickening (swelling) of the arthritic knee compared with the contralateral control knee (data not shown). Therapeutic interventions with AP, MTX or their combination, as depicted in Fig 6.1, were well tolerated and not associated with any adverse effects. No significant changes in the body weight were observed. Accurate assessments of macroscopic decrease of swelling in the arthritic knee joints during treatment were not feasible due to interference of repeated i.a. injections. For this reason, microscopic assessments of knee joints were performed (see below).

Alkaline phosphatase pharmacokinetics

Plasma AP pharmacokinetics were assessed in healthy rats and arthritic rats following i.p. injection of 700 U/kg human recombinant placenta AP, the same amount as used in therapeutic interventions depicted in Figure 6.1. As a control, plasma AP levels were determined in arthritic rats injected with PBS. Baseline plasma levels of AP were slightly but significantly (p<0.05) higher in healthy rats (0.27 ± 0.01 U/mL) than in arthritic rats (0.21 ± 0.02 U/mL, Figure 6.2). After AP administration, plasma AP levels increased over 1 hour to reach a maximum that was 1.5- and 1.7-times higher than baseline levels in healthy and arthritic rats, respectively. It should be noted that increased plasma AP levels in healthy rats were retained for at least 4 hours, whilst in arthritic rats AP plasma levels steadily returned to baseline levels within this time frame. AP levels in PBS injected arthritic rats were unchanged over the 4 hour sampling period (Fig 6.2).

Effects of AP, MTX and AP/MTX combination therapy on synovial macrophage infiltration

The ability of AP, MTX and AP/MTX to suppress synovial macrophage infiltration in knee joints of arthritic rats was used as a primary endpoint for therapy efficacy assessment. To this end, macrophage numbers were quantified in arthritic knee section versus the contralateral knee section by immunohistochemical assessment of the abundance of both total ED1- and ED2-positive macrophages, the latter being a representative marker for anti-inflammatory macrophages\textsuperscript{45}. Representative images and quantification of ED1- and ED2-positive macrophages in arthritic and contralateral knees, before and after therapeutic interventions are shown in Fig 6.3 and 6.4. First, two AP administrations prior to intra-articular mBSA injection elicited potential prophylactic activity by suppressing arthritis induction as indicated by markedly reduced infiltration of both ED1+ (Fig 6.3A) and ED2+ (Fig 6.3B) macrophages in the arthritic knees. This was confirmed by 4-fold (p<0.01) and 8-fold (p<0.01) lower levels of ED1+ (Fig 6.3C) and ED2+ (Fig 6.3D) macrophages in arthritic knees of AP-pretreated arthritic rats as compared with untreated rats. These reduced levels were comparable with macrophage counts in contralateral knees.

Two AP administrations (followed by 4x PBS) resulted in a significant 2-fold (p<0.01) and 3-fold (p<0.01) reduction in synovial ED1+ (Fig 6.4A) and ED2+ (Fig 6.4B)
macrophage infiltration in arthritic knees. In a therapeutic setting, 4x AP administration further reduced these macrophage counts in arthritic knees by another 8-fold (p<0.001) (Fig 6.4). Next, effects of AP treatment in combination with MTX on synovial macrophage infiltration were examined using either a dose of 1 mg/kg MTX that was previously shown to be effective or a lower dose of 0.3 mg/kg MTX. AP/MTX combinations were well tolerated and resulted in numerically increased reductions of ED1-positive synovial macrophages when compared with standalone MTX or AP treatment (Fig 6.4A). The differences for did not reach statistical significance for individual groups, but when MTX 0.3 and 1.0 mg/kg MTX groups were combined, ED1-positive macrophages were significantly reduced (p<0.01) in AP/MTX combinations over MTX standalone treatment (Fig 6.4A). Also for ED2-positive macrophages MTX/AP combinations numerical reductions were observed, but these did not reach statistical significance over standalone MTX or AP treatment as these treatments were already very effective (Fig 6.4B).

[18F]fluoro-PEG-folate macrophage PET

Possible reduction of synovial macrophage infiltration in arthritic rats by AP treatment was also monitored by [18F]fluoro-PEG-folate PET in one of the 4x AP-treated rats and in an untreated (only PBS) arthritic rat (Fig 6.5). The coronal PET-CT image showed higher tracer uptake in the untreated arthritic rats than in the 4x AP-treated rats (Fig 6.5A). [18F]fluoro-PEG-folate SUV in the synovial region of interest (white ellipsoid) was 1.5-fold higher in the arthritic knee of the untreated rat (Fig 6.5B) as compared with to the arthritic knee of 4x AP-treated rat (Fig 6.5C).
Figure 6.3 – Representative images and quantification of ED1- and ED2-positive macrophages in knee sections of rats receiving AP (2x) prophylactic treatment. (A) ED1 images of arthritic and contralateral knees of untreated and 2x AP treated rats. (B) Quantification of ED1+ macrophages of untreated and 2x AP treated rats. (C) ED2 images of arthritic and contralateral knees of untreated and 2x AP treated rats. (D) Quantification of ED1+ macrophages of arthritic and contralateral knees of untreated and 2x AP treated rats. Error bars indicate SD. **: p<0.01

Ex vivo tissue distribution studies

Following PET studies, tissues were collected from untreated (only PBS), AP-treated, AP + 0.3 mg/kg MTX-treated and AP + 1.0 mg/kg MTX-treated arthritic rats, and tracer uptake quantified as %ID/g. In excised knee sections of untreated (only PBS) arthritic rats, tracer uptake was 1.5-fold higher (p<0.05) in the arthritic knee than in the contralateral knee (0.408 ± 0.01 vs 0.283 ± 0.01) (Fig 6.5D). After AP treatment, $[^{18}F]$fluoro-PEG-folate uptake in the arthritic knee was 20% lower (0.342 ± 0.04 %ID/g). AP combined with either 0.3 or 1.0 mg/kg MTX further reduced tracer uptake by 1.6-1.8-fold (p<0.05) in arthritic knees compared with PBS controls (Fig 5D). These levels were comparable to those in contralateral knees of untreated rats (results not shown). The impact of AP treatment, either standalone or combined with MTX, on $[^{18}F]$fluoro-PEG-folate tracer uptake in other tissues is depicted in Table 6.1. In all treatment groups, $[^{18}F]$fluoro-PEG-folate was rapidly cleared from plasma (Table 6.1). Notably, AP and AP/MTX treatments also showed reduced $[^{18}F]$fluoro-PEG-folate uptake in high
Figure 6.4 – Quantification of ED1- and ED2-positive macrophages in knee sections of arthritic rats receiving AP, MTX, and AP/MTX combination therapy. Quantification of (A) ED1+ macrophages and (B) ED2+ macrophages of healthy, arthritic and contralateral knees of PBS, 2x AP + 4x PBS, 4x AP, MTX (0.3 mg/kg), MTX (1.0 mg/kg), AP + MTX (0.3 mg/kg), AP + MTX (1 mg/kg) treated rats. Error bars indicate SD. * Indicates significance of AP or MTX treatment compared to PBS controls (*: p<0.05, **: p<0.01, ***: p<0.001). # Indicates significance of AP/MTX(combined 0.3 + 1.0) treatment compared with standalone MTX treatment (combined 0.3 + 1.0), ##: p<0.01; *: p<0.05; **: p<0.01; ***: p<0.001.
Figure 6.5 – $[^{18}\text{F}]$fluoro-PEG-folate PET-CT scans and ex vivo tissue biodistribution of knees and selected tissues of arthritic rats. (A) Representative coronal $[^{18}\text{F}]$fluoro-PEG-folate PET-CT scans untreated (left) and 4x AP treated (right) arthritic rats. The white ellipsoid depicts the ROI which was drawn around the synovium of the arthritic knee joint to construct the time activity curves of $[^{18}\text{F}]$fluoro-PEG-folate uptake, expressed as SUV, for (B) untreated (only PBS) and (C) 4x AP treated arthritic rats with the contralateral knee as reference. The SUV scale bar from min 0 to max 1 represents the uptake of the tracer. (D) $[^{18}\text{F}]$fluoro-PEG-folate ex vivo tissue distribution was performed at 60 min post tracer injection for arthritic rats that received 4 times: PBS (control groups), 4x AP, AP + MTX (0.3 mg/kg) and AP + MTX (1.0 mg/kg). Results are expressed as mean percentage injected dose per gram (%ID/g) for 4 rats. Error bars indicate SD. *: p<0.05.
Table 6.1: Ex vivo tissue distribution of $^{18}$F]fluoro-PEG-folate in various tissues of arthritic rats. Tissue distribution was performed at 60 min post tracer injection for arthritic rats that had received 4 times treatment with PBS (control groups), AP, AP + MTX (0.3 mg/kg) and AP + MTX (1.0 mg/kg). Results are expressed as mean percentage injected dose per gram (%ID/g) for all rats. Error bars indicate SD of 4 rats/group.

macrophage resident organs, i.e. lung, heart, liver and spleen (Table 6.1). Consistent with high expression of folate receptor α in kidney and intestine$^{46}$ tracer uptake was high in these organs, but not affected by AP and AP/MTX treatments (Table 1).

Systemic anti-inflammatory effects of AP in arthritic rats

To examine whether the reduced tracer uptake in liver and spleen of AP-treated arthritic rats was associated with reduced macrophage infiltration in those organs, ED1 and ED2 immunohistochemistry was performed on liver (Fig 6.6) and spleen (Fig 6.7) section of saline-treated vs AP-treated arthritic rats with liver and spleen tissue of healthy rats as a reference. Representative images of ED1- and ED2-positive macrophages in liver and spleen sections are shown in Fig 6.6A-H and Fig 6.7A-H, respectively. Quantifications of ED1- and ED2-positive macrophages in liver (Fig 6.6 I/J) and spleen (Fig 6.7 I/J) showed significantly higher (4-5 fold, p<0.001) levels in the organs of arthritic rats compared with those of healthy rats, pointing to a systemic inflammatory component. Following AP treatment a marked and significant decrease (50%, p<0.01) of both ED1- and ED2- positive infiltrating macrophages was observed in liver of arthritic rats (Fig 6.6I/J). Similarly, in spleen of arthritic rats, AP treatment also resulted in a significant reduction (30%, p< 0.01) of both ED1- and ED2- positive infiltrating macrophages (Fig 6.7I/J. Antibody control stained liver and spleen sections were clearly negative for both ED1- and ED2- positive macrophages (Fig 6.6 & 6.7 A,B,E,F).
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Figure 6.6 – Representative immunohistochemical (HE) images of ED1+ and ED2+ macrophages in liver sections of healthy rats, saline-treated (control) arthritic rats and AP-treated arthritic rats. A,B: Isotype control stained liver sections of saline-treated and AP-treated rats, respectively. C,D: ED1+ macrophages in liver of saline-treated and AP-treated rats, respectively. E,F: Isotype control stained liver sections of saline-treated and AP-treated rats, respectively. G,H: ED2+ macrophages in liver of saline-treated and AP-treated rats, respectively. I,J: Quantifications of ED1+ and ED2+ macrophages in liver of healthy, saline-treated and AP-treated rats. Values depict mean number of macrophages counted in predefined areas of the liver. Error bars indicate SD. **: p<0.01, ***: p<0.001.
Effect of AP on FRβ-positive macrophages in liver and spleen of arthritic rats

To verify whether reduced [$^{18}$F]fluoro-PEG-folate tracer uptake in liver and spleen of AP-treated arthritic rats (Table 6.1) was associated with reduced numbers of FRβ-positive macrophages, cryosections of liver and spleen tissues were examined for FRβ expression by immunofluorescence. Representative images and quantitative results are shown in Fig 6.8 A-E (for liver) and Fig 6.8 E-J (for spleen). In liver and spleen of arthritic rats, the number of FRβ positive macrophages was significantly higher (4-6 fold, p<0.001) than in corresponding organs of healthy rats. After AP therapeutic interventions, the number of FRβ-positive was markedly reduced in both liver (60% reduction, p<0.001) and spleen (50% reduction, p<0.001) of arthritic rats. Antibody control stained liver and spleen sections were negative (Fig 6.8 A,B/F,G).
DISCUSSION

The main finding of this study is that interventions with alkaline phosphatase (AP) elicit prophylactic anti-arthritic activity in rats by suppressing arthritis induction after intra-articular antigen injection. Moreover, in a therapeutic setting, i.e. after arthritis induction, AP intervention also conveyed local anti-arthritic effects represented by a marked reduction of synovial macrophage infiltration as well as systemic anti-arthritic effects as represented by lowered macrophage infiltration in liver and spleen of arthritic rats. Lastly, AP activity is preserved in treatment combinations with MTX.

Multiple administrations of human recombinant placenta AP (hRESCAP) spaced over
4 days were well tolerated by arthritic rats. Based on our previously reported data, a once every 4 days schedule was used taking into account the biological half-life of hRESCAP in rats of ≈ 3 days. Monitoring AP plasma pharmacokinetics after a single i.p. dose of 700 U/kg AP in healthy and arthritic rats showed peak plasma levels after 1 hour, which were about 50-70% above baseline (Fig 6.2). In healthy rats this rise increased hRESCAP level in plasma remained stable for 4 hours, whereas in arthritic rats hRESCAP plasma levels dropped to basal levels within 4 hours. These results confirm previous data on consumption of available AP during condition of oxidative stress due to endotoxin release such as in RA, and organ injury as with cardiovascular events and cardiothoracic surgery. During its action AP is consumed, proposedly by conjugating to its ITM substrates, and eliminated by Kupffer cells. AP after reacting to ITMs and removal from circulation by Kupffer cells does not return to plasma. Studies by Tuin et al showed that under these circumstances de novo synthesis of AP was initiated. Therapy with an AP harboring a long plasma residence time will then only be consumed when needed, specifically during exacerbations when other ectophos- phatases cannot cope with the ITM insult. Alternatively, AP can be administrated as an injectable during exacerbations to assist combating the surge in ITM, rather than using/consuming AP reserves in the body. Consistently, this mode of action may also be involved in the prophylactic activity of AP in dampening antigen-induced arthritis in rats. Acting as an anti-inflammatory protein, the net effect of AP will be to prevent pro-inflammatory cytokines like TNFα and IL6 to be produced by activated immune cells, thereby preventing downstream effects in the inflammatory cascade. The same mode of action of AP may contribute to the therapeutic activity of AP in rats with established arthritis and reflected by reduced macrophage infiltration in the synovium. Removal of ITMs will suppress production of pro-inflammatory cytokines and reduce chemotaxis to attract monocyte/macrophage cells. In addition, systemic and local inflammation are associated with increased vascular permeability and leucocyte extravasation. In this regard, AP has also been thought to improve barrier dysfunction by restoring tight junctions between polarized cells, thereby attenuating cell migration. Studies addressing the impact of bDMARD treatment in human RA did not influence monocyte migration into RA synovium but rather impacted macrophage efflux from the synovium. Given these observations, the effect of AP on the dynamics of monocyte influx and macrophage retention in RA synovium warrants further investigations.

Inflamed RA synovium is characterized by the presence of differentiated/polarized macrophages covering a spectrum of pro-inflammatory (M1) and anti-inflammatory (M2) macrophages. Both AP and MTX interventions reduced both ED1- and ED2-positive macrophage infiltrations in synovial tissue. ED2 represents the rat homologue of human CD163, which has been assigned a marker for M2-type macrophages. This classification may not be that rigid since M2-macrophages in an arthritic synovial microenvironment with ACPA antibodies and complex IgG autoantibodies were found to produce pro-inflammatory cytokines. AP, just like MTX may thus impact synovial infiltration of polarized inflammatory macrophages.

As many cDMARD and bDMARD treatments in RA are combined with MTX, in the present study the efficacy of AP and MTX combinations in arthritic rats were studied specifically. AP/MTX combinations were well tolerated and slightly more effective.
in terms of reducing synovial macrophage infiltration. Immunohistochemistry data (Fig. 6.4) provided some indication of a synergistic effect of AP + MTX although both treatments already showed clear efficacy as single agents. These results indicate that it may be possible to reduce MTX dosages even further in optimisation of the AP/MTX combination. Mechanistically, it is conceivable that AP can complement the anti-arthritic effects of MTX by supporting the extracellular conversion of pro-inflammatory AMP, ADP and ATP into anti-inflammatory adenosine by the action of the ectophosphatases CD39 and CD73 on immune-competent cells\textsuperscript{30,31}. Under normal conditions, CD39 and CD73, acting locally as high affinity but low capacity ectophosphatases, have sufficient governing power over low-pathogenicity increases of extracellularly exposed nucleotides. However, under pathological conditions, e.g. during exacerbations in chronic inflammatory disorders, they become stoichiometrically occupied. Systemically, the responding immune system has a backup system in place with the broad-acting ectophosphatase AP. In contrast to its reduced efficacy, AP has a high capacity, providing a safety net in pathological conditions or post-operative inflammatory effects\textsuperscript{33,48,49} where high affinity CD39 and CD73 cannot sufficiently cope with surges of nucleotides.

\textsuperscript{18}Ffluoro-PEG-folate PET\textsuperscript{38,41} and ex vivo tissue distribution studies proved to be of added value to monitor the effects of AP and MTX treatment in arthritic rats (Fig 6.5). It should be taken into account that imaging and ex vivo tissue distribution studies include the whole knee joint instead of only synovial tissue as in immunohistochemical studies. This may explain why differences in untreated and treated arthritis are less pronounced when comparing tracer uptake in knee sections and macrophage counts in synovial tissue. \textsuperscript{18}Ffluoro-PEG-folate ex vivo tissue distribution studies indicate that AP and AP/MTX combinations had systemic effects beyond reducing synovial macrophage infiltration. Systemic inflammation, indicated by increased macrophage infiltration in liver and spleen, has been reported in rats with adjuvant-induced arthritis\textsuperscript{64}. In the present study, liver and spleen of arthritic rats also showed increased infiltration of macrophages, which were markedly decreased upon AP treatment (Fig 6.6-6.8). This reduction of macrophages included FR\textsubscript{β}-positive macrophages which constitute a marker for activated macrophages\textsuperscript{39,40}. The lowered number of FR\textsubscript{β}-positive macrophages after AP treatment may account for reduced tracer uptake in liver and spleen in the ex vivo tissue distribution studies (Table 1). These results point to systemic anti-inflammatory effects elicited by AP, which underscores systemic activity of AP observed in other animal models of inflammatory diseases\textsuperscript{32}.

Altogether, conceptually, ectophosphatase intervention by AP may fulfill a novel, unique and unmet niche in RA treatment by combining different, yet synergistic mode of actions with MTX and other cDMARDs and bDMARDs. AP as anti-inflammatory protein could be positioned as a promising new RA treatment either as standalone therapy or in combination with MTX. Being an endogenous protein, AP lacks resistance formation or tolerization effects. AP’s potential is further supported by a wide “safety window of use” and proven safety of recombinant human AP in human volunteer safety studies when AP was administered once every 24 hr for 3 consecutive days\textsuperscript{64}. Moreover, also in pregnant women plasma AP levels increase up to 30-fold over baseline levels without any signs of toxicity\textsuperscript{66}. Notwithstanding these facts, prior to application in a RA setting, safety of repeated AP dosing will have to be confirmed. Anticipating therapeutic
applications of AP in RA treatment, macrophage PET imaging may be an attractive non-invasive modality to monitor therapy response\textsuperscript{38,67}

In conclusion, AP, both as prophylactic and as therapeutic intervention, demonstrated favourable articular and systemic anti-arthritic efficacy in a rat model of arthritis. These studies warrant further preclinical and clinical evaluation of AP as a putative novel therapeutic entity for arthritis.

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Chapter 7

F8-IL10: A new potential anti-rheumatic drug evaluated by a PET-guided translational approach

Submitted

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Abstract

Background
Antibody fragment F8-mediated IL10 delivery is a novel treatment for rheumatoid arthritis (RA). F8 binds to the extra-domain-A of fibronectin (ED-A), overexpressed in inflammation, allowing for local delivery of IL10. In this study, the in-vivo biodistribution and arthritis targeting of radiolabeled F8-IL10 were investigated in RA patients, followed by further mechanistic studies in animals.

Methods
Three RA patients with active disease (DAS28 >3.2) received a microdose of 0.4 mg, 30-74 MBq $^{124}$I-F8-IL10, followed by PET-CT and blood sampling. Regions of Interest (ROIs) were drawn on visually identified PET-positive joints and target-to-background (T/B ratios) were calculated. Healthy mice, rats, and arthritic rats were injected with iodinated (labeled according to Iodogen or Chloramine-T) F8-IL10 or KSF-IL10 control antibody. Various organs were excised, weighed and counted for radioactivity. Tissue sections were stained for fibronectin ED-A.

Results
In RA patients, $^{124}$I-F8-IL10 cleared rapidly from the circulation. Within 5 min <1% was present in blood. PET-CT showed targeting in a total of 38 joints (range 11-15 per patient) and high uptake in liver and spleen. Out of 38 $^{124}$I-F8-IL10 PET-positive joints, 19 (50%) joints were also clinically active. In 17/19 (89%) of PET-negative joints, PET outcome corresponded with absence of clinical symptoms of arthritis. Mean T/B ratios of PET-positive joints were 2.5±1.2, which were 50% higher for clinically active than clinically silent joints. Biodistribution of radioiodinated F8-IL10 in healthy mice showed no effect of radioiodination method on in vivo biodistribution. $^{124}$I-F8-IL10 joint uptake was also demonstrated in arthritic rats, -14 fold higher than control antibody $^{124}$I-KSF-IL10 (p<0.001). Interestingly, liver and spleen uptake was twice as high in arthritic than in healthy rats and was related to increased fibronectin ED-A expression (~7x) in these tissues of arthritic rats.

Conclusion
Promising uptake of $^{124}$I-F8-IL10 was found in arthritic joints in RA patients. Unexpected high liver and spleen uptake and fast blood clearance were observed. Animal experiments revealed increased expression of fibronectin ED-A in liver and spleen in arthritic rats most likely due to systemic inflammation. This translational study demonstrated the value of in-vivo biodistribution imaging of new and potential anti-rheumatic drugs by PET-CT.

Keywords: Fibronectin (ED-A), IL10, rheumatoid arthritis, pharmacokinetics, positron emission tomography, computed tomography

Abbreviations
RA - Rheumatoid arthritis, MTX - Methotrexate, DMARD - disease-modifying anti-inflammatory drugs, EULAR - European League Against Rheumatism, ARC - American
College of Rheumatology, F8- F8 antibody fibronectin , IL10 - Interleukin 10, PET-CT -
Positron Emission Tomography - Computed tomography, SD - standard deviation, ID/g
- injected dose/ gram, i.a.- intra-articular, ED-A - extra-domain-A of fibronectin, p.i. -
post injection.
Rheumatoid arthritis (RA) is a chronic inflammatory and destructive joint disease [1] for which treatment options currently include chemical and biological disease modifying anti-rheumatic drugs (DMARDs) [2,3]. Nevertheless, 30-40% of patients are refractory to these treatments leaving an unmet need for alternative therapies [4]. Targeted delivery of cytokines at the site of disease is an emerging novel approach to treat chronic inflammation, especially RA [5] and has also been successfully investigated in the cancer setting over the last few years [6–9]. Immunocytokines (cytokine-antibody fusion proteins) represent a class of therapeutic agents that have previously shown their usefulness for targeting antigens at the site of inflammation followed by local activity of the cytokine [5]. Along these lines, target specificity and dose mediated therapy response can be achieved, especially at synovium in RA.

IL10 is an anti-inflammatory cytokine, produced by activated monocytes and T cells, and is involved in the regulation of inflammatory responses and immune reactions [10,11]. In the clinic, combination therapy of IL10 and MTX in a multicenter, placebo-controlled study in RA patients revealed clinical ACR 20 responses in 50-60% of patients compared with 10% for placebo [12]. Despite the anti-inflammatory properties of IL10, systemic administration in RA patients generated insufficient responses to pursue further development. However, when IL10 is fused with the single-chain antibody variable domain (Fv) fragment of antibody F8, targeted delivery of IL10 can be achieved locally especially at RA synovium. Antibody fragment F8 binds to the extra-domain A (ED-A) of fibronectin ED-A, selectively expressed at sites of inflammation in RA [13] and in tumors [14] in humans as well as in animals [15]. Previously, high levels of fibronectin ED-A have been demonstrated in the target tissues of arthritic as well as tumor-bearing animal models [15,16]. Besides, F8-IL10 displayed clear localization at sites of arthritis in a collagen-induced arthritis mouse model [16]. Recently, encouraging early signals of the therapeutic benefit of F8-IL10 was demonstrated in RA patients in a phase I study, underscoring the therapeutic potential of this new approach [17]. Positron emission tomography (PET) can provide unique whole-body molecular information about targeting and in vivo biodistribution of new therapeutic agents [18,19]. For imaging of antibodies, isotopes with long half-lives as $^{124}$I and $^{89}$Zr allow imaging of targeting and in vivo biodistribution over several days [20–22].

The purpose of the present translational study was to investigate the in vivo distribution and arthritis targeting of $[^{124}\text{I}]$F8-IL10 in RA patients, followed by mechanistic studies in animal models.

**METHODS**

**Patients**

Three RA patients (aged ≥ 18 years) who fulfilled the ACR 1987 classification criteria for rheumatoid arthritis [23] were included during 2013 and 2014 (Table 7.1). Patients had a high disease activity score across 28 joints (DAS28) of >3.2 and ≥2 clinically inflamed joints in hands. Exclusion criteria consisted of: any haematologic, liver or renal
Table 7.1 – Baseline patient demographics, clinical and functional characteristics.

<table>
<thead>
<tr>
<th></th>
<th>Patient 1</th>
<th>Patient 2</th>
<th>Patient 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex M/F</td>
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<td>F</td>
<td>M</td>
</tr>
<tr>
<td>Age (yrs)</td>
<td>53</td>
<td>50</td>
<td>18</td>
</tr>
<tr>
<td>Weight (kg)</td>
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<td>74</td>
<td>65</td>
</tr>
<tr>
<td>IgM RF positivity</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
</tr>
<tr>
<td>Anti-CCP positivity</td>
<td>yes</td>
<td>yes</td>
<td>no</td>
</tr>
<tr>
<td>Disease duration (yrs)</td>
<td>8</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>DAS28</td>
<td>4.8</td>
<td>4.1</td>
<td>5.3</td>
</tr>
<tr>
<td>Swollen joint count (DAS28)</td>
<td>7</td>
<td>3</td>
<td>12</td>
</tr>
<tr>
<td>Tender joint count (DAS28)</td>
<td>8</td>
<td>9</td>
<td>7</td>
</tr>
<tr>
<td>VAS (0-100)</td>
<td>70</td>
<td>70</td>
<td>80</td>
</tr>
<tr>
<td>CRP (mg/L)</td>
<td>28</td>
<td>&lt;2.5</td>
<td>25</td>
</tr>
<tr>
<td>ESR (mm/h)</td>
<td>8</td>
<td>4</td>
<td>11</td>
</tr>
<tr>
<td>DMARDs</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
</tr>
<tr>
<td>NSAIDs</td>
<td>no</td>
<td>no</td>
<td>no</td>
</tr>
</tbody>
</table>

function test abnormalities, concurrent active infections/autoimmune disease or severe (malignant) diseases (other than RA), immunodeficiency or HIV positivity, (history of) heart problems or insufficiency, uncontrolled hypertension, pregnancy or breastfeeding, major surgery within the previous 4 weeks, or any other treatment with investigational drugs within the previous three months. Stable doses of DMARDs and/or non-steroidal anti-inflammatory drugs (NSAIDs) were continued if used at inclusion. All clinical data were obtained by an experienced research nurse blinded to the imaging data.

Animals

Wistar rats, male, 150-200 grams, Charles River International Inc, Sulzfeld, Germany) and healthy mice, 6-8 weeks female, Envigo, Horst, NL) were provided with standard food (16% protein rodent diet, Harlan Laboratories Inc., Madison, WI, USA) and water ad libitum. Rats were housed in conventional cages and mice in filter top cages (in groups of 3-6 per cage) and kept in a room with a 12-hour light/dark cycle and constant room temperature (21°C) and humidity level (55±10%).

For arthritis induction, Wistar rats received 4x intra-articular (i.a) mBSA injections, 4 or 5 days apart in the arthritic (right) knee, with the contralateral (left) knee serve as a control, as described previously [24].

Tracer synthesis for administration to RA patients

F8-IL10 obtained from Philogen (Zurich, Switzerland) was first rebuffered to phosphate buffer saline (PBS) using PD-10 size exclusion chromatography. Hereafter 20 µL NaI in 20 mM NaOH was mixed with 280 µL 20 mM NaOH, containing the required amount
of $^{124}$I and 10 µL of the 1.41 mg/mL ascorbic acid solution. After 5 min 120 µL 0.5 M phosphate buffer pH 7.1 and 1.6 mL F8-IL10 in PBS (~1 mg) were added, followed by 30 µL of a freshly prepared 0.66 mg/mL Iodogen solution (20 µg). After 3 min the reaction was stopped by the addition of excess of ascorbic acid (100 µL of 25 mg/mL). After 10 min purification was performed using PD10 size-exclusion chromatography (eluent: 5 mg/mL ascorbic acid in 0.9% NaCl (pH 5.0)). The radiolabeling yield was 65.2±17.2 %. Finally $[^{124}\text{I}]$I-F8-IL10 was formulated with native F8-IL10 and 5 mg/mL ascorbic acid in 0.9% NaCl for human administration. The radiochemical purity was >95% according to iTLC and HPLC. The immune reactive fraction was determined by measuring binding to a serial dilution of ED-A coated beads and was 91.0±4.9 % at the highest resin concentration.

### Tracer synthesis for administration to animals

Radiolabeling of F8-IL10 and control antibody KSF-IL10 with $^{124}$I was applied in all experiments with rats essentially according to Tijink et al. [25]. In contrast, radiolabeling of F8-IL10 with $^{131}$I (for comparison with $[^{124}\text{I}]$I-F8-IL10 labeling according to chloramine-T, see below) was applied for experiments with healthy mice. For both species the Iodogen method was applied. Briefly, F8-IL10 obtained from Philogen (Zurich, Switzerland) was first rebuffered to phosphate buffer saline (PBS) using PD-10 size exclusion chromatography. Hereafter, 3 µL $^{131}$I in 20 mM NaOH (20 MBq) were mixed with 12 µL water and 285 µL 20 mM NaOH. After 5 min 120 µL 0.5 M phosphate buffer pH 7.1 and 1.6 mL F8-IL10 in PBS (~1 mg) were added, followed by 30 µL of a freshly prepared 0.66 mg/mL Iodogen solution (20 µg). After 3 min the reaction was stopped by the addition of an excess of ascorbic acid (100 µL of 25 mg/mL). After 10 min purification was performed using PD10 size-exclusion chromatography (eluent: 5 mg/mL ascorbic acid in 0.9% NaCl (pH 5.0)). The radiolabeling yield was 56.4±3.2% in case of $[^{131}\text{I}]$I-F8-IL10, while radiolabeling yields of $[^{124}\text{I}]$I-F8-IL10 and $[^{124}\text{I}]$I-KSF-IL10 were 61.7±9.3% and 69.8±5.4%, respectively. Finally $[^{131}\text{I}]$I-F8-IL10 was formulated with native F8-IL10 and 5 mg/mL ascorbic acid in 0.9% NaCl for in vivo administration. The radiochemical purity was >95% according to iTLC and HPLC. The immune reactive fraction was determined by measuring binding to a serial dilution of ED-A coated beads and was 85.2 % at the highest resin concentration. The radiochemical purity of all conjugates was >95% and immunoreactivity of 91.4±5.9% for $[^{131}\text{I}]$I-F8-IL10, and 91.2±1.5% and 92.2±0.1% for $[^{124}\text{I}]$I-F8-IL10 and $[^{124}\text{I}]$I-KSF-IL10, respectively.

For the study in healthy mice, apart from labeling of F8-IL10 with $^{131}$I according to Iodogen method (see above), F8-IL10 was also labeled with $^{124}$I according to the Chloramine-T method used earlier by Philogen [26]. In this method, 67 µL $^{124}$I in 10 M NaOH was mixed with 1.523 mL buffer A. Hereafter 0.25 mL (4 mg/mL, 1 mg) F8-IL10 and 0.25 mL (0.5 mg/mL, 0.125 mg) Chloramine-T in buffer A were added. After 45 sec the reaction was stopped by the addition of 0.25 mL (40 mg/mL) ascorbic acid in buffer A, pH 6.0 and immediately purified using PD10 size-exclusion chromatography (eluent: buffer A), followed by the addition of 40 mg/mL to arrive at a concentration of 4 mg/mL ascorbic acid in buffer A. The radiolabeling yield was 52.48±3.2%. Finally $[^{124}\text{I}]$I-F8-IL10 was formulated with native F8-IL10 and 40 mg/mL ascorbic acid in buffer A for in vivo administration (final concentration of ascorbic acid - 4 mg/mL). The radiochemical
Purity was >95% according to iTLC and HPLC. The immune reactive fraction was determined by measuring binding to a serial dilution of coated beads ED-A coated beads and was 98.4±3.7% at the highest resin concentration (formulation for in vivo experiment: 75 µg F8-IL10 ~30 kBq 150 µL/mouse; buffer A: 25 mM NaH₂PO₄·H₂O (3.900 g), 10mM NaOH (0.400 g), 30 mM NaCl (1.753 g), 2 mM KCl (0.149 g), 50 mM Mannitol (9.109 g), 5 mM EDTA (1.461 g), 1%, 10 mL glycerol (D=1.2613 g/mL 12.613 g) dissolved in 1 L water; for irrigation filter the solution through a 0.22 µm filter measure pH: 6.44)

[¹²⁴I]I-F8-IL10 PET-CT of RA patients

Before PET-CT scanning, patients received prophylaxis to block the thyroid by administration of 400 mg potassium perchlorate 48, 24 and 1 hour(s) before infusion of [¹²⁴I]I-F8-IL10 and 1 hour before PET-CT scanning at 24 hours and 72 hours. Next, a total of 3 whole body PET-CT scans per patient and detailed images of wrists/hands were obtained, and blood samples were withdrawn at 5 min, 30 min, 90 min, 3 hours, 24 hours and 72-hours post-injection (p.i.).

PET-CT scans were performed using both Gemini-64 and Ingenuity-128 PET-CT scanners (Philips Healthcare, Cleveland, USA). After intravenous (i.v.) injection of a single dose of [¹²⁴I]I-F8-IL10 (0.4 mg (~6 µg/kg), 59±25 MBq), patients were scanned at 1, 24 and 72 hours p.i. The administration system was flushed with 20 mL of 0.9% NaCl and, after administration of the tracer, residual activity in the injection device was measured to determine the net injected dose. Subsequently, whole body scans from skull to symphysis pubis were acquired with 5 minutes/field of view (FOV). In addition, detailed images of wrists/hands were obtained (1 FOV, 6 frames of 5 minutes each). PET scans were preceded by a 30 mAs low-dose CT for attenuation correction and anatomical localization of the PET signal. PET data were normalized and corrected for attenuation, decay and scatter. All scans were reconstructed according to international guidelines [27].

Image Analysis

An experienced nuclear medicine physician (OSH) qualitatively interpreted PET-CT data for whole body biodistribution and joints with visually enhanced tracer uptake (PET-positive joints). For quantitative comparison of tracer uptake, VOIs were drawn using software developed in-house [28] with the corresponding low dose CT as an anatomical reference. For biodistribution and dosimetry, VOIs were drawn over lungs, liver, spleen, kidneys, blood pool and vertebrae. For analysis of visually defined PET-positive joints, VOIs were drawn on top of joints with clear focal tracer enhancement using thresholds exceeding local background uptake (e.g., PET negative joints). In addition, for PET-negative hand joints, standardized spherical VOIs were drawn on wrists (~60 mL), metacarpophalangeal joints 1-5 (8 mL) and (proximal) interphalangeal joints 1-5 (2.0 mL) centered in the middle of the joints (n=22 joints per patient).

Standardized uptake values (SUVs) were calculated by dividing the PET tracer tissue concentration by the activity injected (MBq) per body weight (kg). Tracer uptake
I-F8-IL10 guided translational study in RA

The SUV peak (SUV) is defined as the highest average uptake within a sphere of 1.2 mL within the VOI [29,30]. In addition, spherical VOIs of 0.5 mL in the second metacarpal bone were used as background to calculate target to background (T/B) ratios.

Mechanistic studies in animals

**In vivo biodistribution of [124I/131I]I-F8-IL10 in healthy mice comparing radioiodination methods**

Healthy mice (n=14) were injected with either [124I]I-F8-IL10 (Chloramine-T method) or [131I]I-F8-IL10 (Iodogen method) at 10 minutes and 24 hours. In both cases, the F8-IL10 protein dose was 3 mg/kg. Each mouse received ~3.5 MBq [124I]I-F8-IL10 or [131I]I-F8-IL10. At the end of the study, all mice were sacrificed, and blood and various tissues were excised, rinsed, dipped dry and weighed. The amount of tissue radioactivity was determined using an LKB 1282 Compugamma CS gamma counter (LKB, Wallac, Turku, Finland). Results were expressed as a percentage of the injected dose per gram tissue (%ID/g) [24].

**In vivo biodistribution of [124I]I-F8-IL10 in healthy and arthritic rats**

Six days after the last i.a. injection of mBSA, all rats received -3.5 MBq tracer injection, with: group A (healthy, non-arthritic rats), tracer [124I]I-F8-IL10, 0.34 mg/kg injected F8-IL10 dose (healthy F8-IL10low) (n=4); group B (arthritic rats), tracer [124I]I-KSF-IL10 (control antibody), 0.34 mg/kg injected KSF dose (KSF-IL10) (n=6); group C (arthritic rats), tracer [124I]I-F8-IL10, 0.36 mg/kg injected F8-IL10 dose (F8-IL10low) (n=6); group D (arthritic rats), tracer [124I]I-F8-IL10, injected F8-IL10 dose 12.9 mg/kg (F8-IL10high) (n=6). Twenty-four hours post tracer injection, all rats were sacrificed, and knee joints, as well as various tissues, were removed and analyzed as described above in the mice experiment.

**Immunofluorescence of liver and spleen of arthritic versus healthy rats**

Tissues (liver and spleen) of arthritic rats (n=4) and healthy rats (n=4) were snap frozen in liquid nitrogen and stored at -80°C. Sections of 8 µm were cut and stained with hematoxylin and eosin (HE), whereas staining of splice isoforms of fibronectin ED-A and of tenascin-C was performed by F8-small immunoprotein (SIP) (Philogen, Siena, Italy) or isotype control antibody. Tissues were embedded in a suitable medium (OCT; SKU4583, Tissue-Tek, Netherlands), cut using a cryotome cryostat (-20°C) (Leica, Netherlands) and placed on Superfrost (4951PLUS4, ThermoFisher, Netherlands) glass slides for immunofluorescence staining.

For immunostaining, frozen tissue sections were first brought to room temperature (RT) for 30 min. The sections were fixed in acetone (439126, Sigma-Aldrich, Netherlands) for 10 min at -20°C and air dried for 10 min at RT. Sections were marked with DAKO pen (S2002, DAKO, Santa Clara, CA, USA). After 3x washing with PBS on a shaker, sections were treated with 100% fetal bovine serum (FBS) for 30 min at RT to avoid non-specific binding and then washed again with PBS (3x5 min). Thereafter, sections
were incubated with F8-small immunoprotein (SIP) (2 µg/ml) in 10% FBS/PBS for 24 hours at 4°C or with 10% FBS/PBS. After a washing step (3x5 min in PBS on a shaker), sections were incubated with rabbit anti-human IgE 1:1000 in 10% FBS/PBS (DAKO, A0094, Santa Clara, United States) for 60 min at RT. After a final washing step, sections were incubated with goat-anti-rabbit Alexa 488 (A-11008, ThermoFisher Scientific, Netherlands) in 10% FBS/PBS, air dried and mounted (2 µL of MOWIOL mounting medium (81381, Merck, Zwijndrecht, The Netherlands).

Microscopy and image analysis of immunofluorescent tissue sections

The slides were imaged with a Zeiss Axiovert 200M Marianas™ inverted microscope, (40X oil-immersion lens) equipped with a motorized stage (stepper-motor z-axis increments: 0.1 µm), and a turret of four epifluorescence cubes (FITC, Cy-5, Cy-3, AMCA as well as a DIC bright field cube). A cooled CCD camera (Cooke Sensicam SVGA [Cooke Co., Tonawanda, NY, USA], 1,280 × 1,024 pixels) recorded images with true 16-bit capability. The camera was linear over its full dynamic range (up to intensities of over 4,000) with dark/background currents (estimated by the intensity outside the cells) of typically <100. Exposure, objective, montage, and pixel binning were automatically recorded and stored with each image (Dell Dimension workstation: Quad-core processor, 16GB RAM). The microscope, camera, and data processing were controlled by SlideBook™ software (SlideBook™ version 6, Intelligent Imaging Innovations, Denver, CO, USA) [31].

All images obtained in 2D were first deconvolved (no neighbour), in order to improve the signal to noise ratio. The arthritic and healthy liver/spleen images were segment masked (using background from negative liver/spleen sections). Thereupon, mask statistics were performed on the entire mask, and the sum intensities were recorded. Sum intensities of all multiple representative images from arthritic and healthy rat liver/spleen were averaged (±SD).

Histology images were captured using a Leica 4000B microscope and a Leica digital camera DC500 (Microsystems B.V. Rijswijk, The Netherlands).

Statistical Analysis

PET data of RA patients were reported in a descriptive manner because of the small sample size. Quantitative human and RA-rat model data are presented as mean ± standard deviation (SD) or as median and interquartile range [IQR] in case of skewed distribution. Mann-Whitney U (exact) tests were performed to analyze differences in tissue distribution and fibronectin ED-A staining between different rat groups (A-D). A p-value <0.05 was considered as statistically significant. Statistical analyses were performed using SPSS version 22.0 for Windows (SPSS, Chicago, IL, USA).
RESULTS

Clinical data of RA patients

Clinical characteristics of the three patients included are summarized in Table 7.1. During the study there were no adverse events linked to the injected compound. All patients had high disease activity despite stable doses of DMARDs, with a DAS28 >4.0. Out of 66 evaluated hand/wrist joints of RA patients, 25 were clinically tender and/or swollen (38%), i.e., 5, 15 and 5 in patients 1, 2 and 3, respectively. Clinical symptoms of arthritis (tenderness and/or swelling) were present in wrists (5/66; 8%), metacarpophalangeal (MCP) joints (15/66; 22%) and proximal phalangeal (PIP) joints (5/66; 8%). $^{[124]}$I-F8-IL10 was injected without any side effects occurring during the observation period.

Joint targeting of $^{[124]}$I-F8-IL10 in RA patients

Detailed images of wrists/hands showed evident targeting in joints. The highest uptake and T/B ratios were determined at 24 hours p.i. Within the one FOV of the hands, a total of 57 hand joints across the 3 patients could be imaged using $^{[124]}$I-F8-IL10, and a cumulative number of 38 PET-positive joints were observed (11 to 15 joints per patient (Figure 7.1)). Out of 38 $^{[124]}$I-F8-IL10 PET-positive joints, 19 were also clinically swollen and/or tender (50%). In addition, there was enhanced uptake in 19 hand joints that lacked clinical signs of arthritis. The remaining 19/57 joints were PET-negative of which 17 (89%) joints were also clinically negative. Mean SUV of PET-positive joints was 0.25$\pm$0.15 and mean T/B ratio 2.5$\pm$1.2. PET-positive joints that were also clinically active had 1.5 fold higher T/B ratios than clinically silent PET-positive joints (i.e., subclinical joints) (T/B ratios 2.6$\pm$1.2 vs. 1.6$\pm$0.8, respectively).

Whole body distribution of $^{[124]}$I-F8-IL10 in RA patients

Blood sample analysis in RA patients indicated very fast clearance of $^{[124]}$I-F8-IL10 from the circulation after i.v. injection of the drug. In fact, after 5 minutes less than 1% of the injected dose was detected in blood (Figure 7.2). Another remarkable finding was high uptake of $^{[124]}$I-F8-IL10 in liver (48.0$\pm$3.5% of the injected dose, %ID) and, to a lower extent, spleen (5.3$\pm$3.5 %ID), as seen in the whole body PET-CT scans performed at hour (Figure 7.3). Thereafter, $^{[124]}$I-F8-IL10 liver uptake decreased from 48.0 (%ID) to 13.4 (%ID) at 72 hours p.i. (Table 7.2). To further understand the high uptake in liver and spleen, animal experiments were performed.

The effect of radioiodination on the in vivo biodistribution of F8-IL10 in healthy mice

To investigate whether radioiodination affected F8-IL10, resulting in increased uptake in liver and spleen, healthy mice were analyzed for biodistribution after i.v. injection of F8-IL10 labeled according to the Chloramine-T ($^{[124]}$I-F8-IL10) and Iodogen method ($^{[124]}$I-F8-IL10), respectively (Figure 7.4). At 10 min p.i., both tracers had comparable blood levels of 16-25 %ID/g, decreasing to low values <0.3 %ID/g at 24 hours p.i. Biodistribution in various organ tissues was also similar over time, with no accumulation
Figure 7.1 – Example of a $^{124}$I-F8-IL10 PET-CT scan of the hands of a clinically active RA patient.

Figure 7.2 – Pharmacokinetic analysis of $^{124}$I-F8-IL10 in (A) whole blood and (B) plasma of three RA patients.
Figure 7.3 – $^{[124]}$I-F8-IL10 PET scan of a RA patient showing clear tracer uptake in liver and spleen. %ID = percentage of the injected dose.

Table 7.2: Organ uptake of $^{[124]}$I-F8-IL10 expressed as a percentage of injected dose respectively 1 hour and 72 hours post-injection.

<table>
<thead>
<tr>
<th>Organ</th>
<th>%ID at 1 hour p.i.</th>
<th>%ID at 24 hour p.i.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean±SD</td>
<td>Mean±SD</td>
</tr>
<tr>
<td>Blood pool (heart)</td>
<td>0.005±0.002</td>
<td>0.001±0.0007</td>
</tr>
<tr>
<td>Kidney</td>
<td>1.5±0.4</td>
<td>0.5±0.3</td>
</tr>
<tr>
<td>Liver</td>
<td>48.0±3.5</td>
<td>13.4±2.7</td>
</tr>
<tr>
<td>Spleen</td>
<td>5.3±3.5</td>
<td>1.4±1.5</td>
</tr>
<tr>
<td>Bone marrow</td>
<td>0.2±0.1</td>
<td>0.004±0.001</td>
</tr>
</tbody>
</table>
in liver or spleen at 24 hours. All tissues showed clearance of both tracers within the time span of 24 hours. Urine excretion was similar.

**In vivo biodistribution of $^{124}$I-I-F8-IL10 in arthritic rats**

After arthritis induction, all rats showed macroscopic thickening of the right, arthritic knee compared with the contralateral knee (data not shown). None of the rats showed any adverse effects, and no major changes in body weight were observed. Similar to observations in RA patients, uptake in arthritic knee joints was found in the F8-IL10_{low} group. In this group, uptake in the arthritic knee joint of $^{124}$I-I-F8-IL10 was 2x higher than (p<0.01) in the (right) knee joint of a healthy (non-arthritic) rat (Figure 7.5). Specificity of uptake was demonstrated by a \approx 14 fold increased uptake of F8-IL10 in the arthritic knee joints as compared with uptake of the control antibody KSF-IL10 (p<0.001). Interestingly, up to two times higher uptake of F8-IL10 was also observed in liver and spleen of arthritic rats as compared with that in healthy rats, while uptake in other organs was comparable between arthritic and healthy rats. (Figure 7.5). When the dose of F8-IL10 was increased significantly (F8-IL10_{high} group), $^{124}$I-I-F8-IL10 uptake decreased in several tissues with a pronounced decrease in arthritic knee joints, liver and spleen (p<0.01) while blood levels were comparable at 24 hours p.i.
Figure 7.5 – Ex vivo tissue distribution (%ID/g) at 24 hr p.i. in healthy rats: $[^{124}]$I-F8-IL10$^{\text{low}}$ (white bars), and in arthritic rats: $[^{124}]$I-KSF-IL10-control (black bars), $[^{124}]$I-F8IL10$^{\text{low}}$ (light grey bars) and $[^{124}]$I-F8-IL10$^{\text{high}}$ (dark grey bars). The results are expressed as percentage injected dose per gram (%ID/g ± SD).

Investigation of the effect of the disease RA on liver and spleen expression of fibronectin ED-A

Increased expression of fibronectin ED-A in liver and spleen was observed in arthritic rats compared with healthy rats (Figure 7.6). In Figure 7.6 the white pulp (histology, Figure 7.6 A) with fibronectin ED-A expression in the spleen of healthy (Figure 7.6 B) and arthritic (Figure 7.6 C) rats is shown. Fibronectin ED-A expression in spleen was ~8x ($p<0.001$) increased in arthritic rats compared with healthy rats (Figure 7.6 D). Further, fibronectin ED-A expression in liver (hepatocytes) (histology, Figure 7.6 E) of healthy rats (Figure 7.6 F) and arthritic rats (Figure 7.6 G) was observed. Fibronectin ED-A expression in liver was ~7x ($p<0.001$) increased in arthritic rats compared with healthy rats (Figure 7.6 H).
Figure 7.6 – Histopathology and immunofluorescence images of fibronectin ED-A stainings on arthritic and healthy rats, spleen and liver sections. A, HE staining of spleen (10 x magnification). B, C fibronectin ED-A staining (40 x magnification) of healthy spleen (B) and arthritic spleen (C). D, The total fluorescence intensity of healthy spleen vs the arthritic spleen (Total Fluorescence intensity E+06 ± SD); E, HE staining of liver (10x magnification); F,G fibronectin ED-A staining (blue channel: nucleus, green channel: fibronectin ED-A) of healthy (F) and arthritic liver (G); H, The total fluorescence intensity of healthy liver vs the arthritic liver (Total Fluorescence intensity E+06 ± SD).
DISCUSSION

In this study, both targeting performance and in vivo biodistribution of the potential F8-IL10 anti-rheumatic drug were investigated using a translational approach. First of all, a feasibility PET-CT study in RA patients demonstrated that F8-IL10 targets clinically inflamed joints and also subclinically affected joints. Similarly, targeting was also found in arthritic joints of rats. The RA patient PET study revealed unexpected in vivo biodistribution. Very rapid blood clearance (less than 1% detectable < 5 minutes) and high liver and spleen uptake on PET-CT (within 1-hour p.i. of $^{[124]}$I-F8-IL10), were observed. Subsequent animal experiments showed that increased tracer uptake in liver and spleen is associated with increased, arthritis related, expression of fibronectin ED-A in in liver and spleen. The latter may be the main cause of local accumulation of the tracer in RA patients. In addition, a lower F8-IL10 administration dose (microdose of F8-IL10 administered in RA patients) may also result in relatively high uptake in liver and spleen, although our experiments did not allow definite conclusions for this mechanism.

Although our targeting data of F8-IL10 in arthritic joints were found in a small study of only 3 RA patients, these observations were supported by our arthritic rat data and are in line with targeting results previously found in a collagen-induced mouse model [16]. This provides further evidence of the feasibility of targeted delivery of IL10 by binding to fibronectin ED-A in arthritic joints, also reflected by the promising initial phase Ib therapeutic efficacy data of F8-IL10 in RA patients [17,32].

The observed high uptake in liver (and to some extent in spleen), as well as the fast blood clearance of $^{[124]}$I-F8-IL10 in RA patients, was unexpected. In previous experiments in tumor-bearing mice, no increased F8-IL10 uptake in liver and spleen was observed, and blood clearance was clearly slower shortly after i.v. injection [15]. There are several potential explanations for the observed differences: 1) the use of different radioiodination methods (i.e., Chloramine-T method in tumor-bearing mice and Iodogen in RA patients), 2) effect of the RA disease on fibronectin ED-A expression in liver and spleen, 3) different injected doses of F8-L10 and 4) species differences.

Firstly, the potential effect of the radioiodination method on the in vivo biodistribution was addressed in healthy mice. F8-IL10 can potentially be modified by oxidation during the radioiodination procedure, which could result in rapid blood clearance and uptake in the MPS system (liver/spleen) [33]. However, no differences in liver and spleen uptake of F8-IL10 were found between both radioiodination methods. In fact, in healthy mice, liver and spleen uptake at 24 hours p.i. was low, regardless of the radioiodination method. In addition, initial blood clearance was much slower (in line with previous observations in tumor-bearing mice) with 15-25 %ID/g present at 10 minutes p.i. in healthy mice as compared to < 1% ID/L in the blood compartment of RA patients at 5 minutes p.i.

Secondly, hepatic and splenic F8-IL10 uptake of arthritic rats was more than twice as high as that in healthy rats. A high dose of F8-IL10 significantly decreased liver and spleen uptake of $^{[124]}$I-F8-IL10 in rats with experimental arthritis, indicating specific
binding in these tissues. To explore whether this observation could be explained by increased fibronectin ED-A expression and hence increased F8-IL10 binding in arthritic rats, further immunohistochemical analyses were performed. Indeed, in arthritic rats significantly higher fibronectin ED-A expression was found in liver and spleen as compared with healthy rats.

Third, a microdose of i.v. administered agent may directly accumulate in high antigen expressing and well accessible organs upon the first passage through these organs (liver/spleen). This so-called antigen sink theory has also been observed by others in B-cell lymphoma patients injected with radiolabeled Rituximab [34]. The RA patients were injected with a 500-fold lower dose of F8-IL10 than healthy mice (0.006 mg/kg versus 3 mg/kg F8-IL10), where no accumulation of radioactivity in liver and spleen was observed. Thus, in the patient study liver and spleen may have acted as antigen sinks for the low dosage of i.v. administered F8-IL10, which could explain the fast blood clearance and relatively low uptake in arthritic joints. Upon increase of the F8-IL10 dose, liver and spleen may become saturated, resulting in higher plasma concentration, thereby leaving relatively more F8-IL10 available to bind to inflamed joints. To investigate this antigen sink theory, a high dose of F8IL10 (~2000-fold higher than that in humans) in arthritic rats was compared with the lowest feasible F8-IL10 dose for administration in rats (~50-fold fold higher than in humans). Indeed, a significantly lower uptake of radiolabeled F8-IL10 in the liver/spleen of the F8-IL10\textsuperscript{high} group was seen than in the F8-IL10\textsuperscript{low} group. However, uptake of radioactive F8-IL10 in several other tissues was decreased as well, which presumably was, at least in part, related to blockade of local F8-IL10 binding sites. Therefore, these results could not definitely prove or rule out a possible role of liver and spleen as antigen sink in case of F8IL10\textsuperscript{low} dose.

Finally, species differences between animals and humans cannot be ruled out. Findings in animals can be highly suggestive, but extrapolation to humans should be made with care. In the present study, direct dose comparisons were not possible since lowering the F8-IL10 dose (< 0.3 mg/kg) in rats to allow for a direct comparison with the dose in RA patients (0.006 mg/kg) was technically not possible. Apart from tissue observations, differences in pharmacokinetics, stability of the tracer and targeting between animals and patients may also play a role.

In conclusion, the imaging studies demonstrate that PET-guided drug development may provide significant information on the targeting potential of a new anti-rheumatic drug, although observations were made in a small group of 3 RA patients. [\textsuperscript{124}I]-F8-IL10 PET revealed promising targeting in arthritic joints in RA patients. In addition, PET whole body biodistribution data may point at tracer accumulation at other non-target sites, for F8-IL10 likely related to disease induced antigen expression at these sites and possibly related to antibody dosing. These insights can add to optimal dosing schedules for targeting sites of interest as well as inform on potential sites of concern for side effects. So far, the human phase 1b data have not revealed major side effects. F8-IL10 was generally well tolerated up to a dose of 600 μg/kg [32].

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**Ethics approval and consent to participate**
The study protocol was approved by the VU University Medical Center Medical Ethics Review Committee. All patients gave written informed consent prior to participation in the study. All animal experiments were performed in accordance with the European Community Council Directive 2010/63/EU for laboratory animal care and the Dutch Law on animal experimentation. The experimental protocol was approved by the local committee on animal experimentation of the VU University Medical Center.

**Trial registration**
EudraCT n°: 2010-023114-32
References


Section III

Immunophenotyping of monocytes/macrophages in rheumatoid arthritis
Chapter 8

Folate receptor beta (FRβ) expression on monocyte and macrophage populations in blood and synovial tissue of rheumatoid arthritis patients

Manuscript in preparation

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Abstract

Introduction
Folate receptor \( \beta \) (FR\( \beta \)) is expressed on monocyctic/macrophage cells in synovial fluid and synovial tissue of rheumatoid arthritis (RA) patients, which can potentially be exploited for imaging and therapeutic targeting. It is not fully understood how FR\( \beta \) expression is balanced between polarized macrophages of “M1” (pro-inflammatory) and “M2” (anti-inflammatory) types. Therefore, immunophenotyping was performed (FR\( \beta \), CD163, CD169, CD200r and CD206) in monocyte subpopulations from peripheral blood, synovial fluid (SF) and synovial tissue (ST) of RA and non-RA (arthritis of other origin) patients.

Methods
Peripheral blood mononuclear cells (PBMCs) (n=30), SF cells (n=10) and ST biopsy sections (n=5) were collected from early, DMARD (therapy-naïve) and/or treated established RA patients, with PBMC’s from healthy volunteers (n=8) and SF (n=9) and ST (n=2) of non-RA (Spondyloarthritis; SpA) as controls. Isolated PBMCs and SF cells were analyzed by flow cytometry to identify three subpopulations of monocytes: classical monocytes (CD14\textsuperscript{high}CD16\textsuperscript{low}), intermediate monocytes (CD14\textsuperscript{high} CD16\textsuperscript{+}) and non-classical monocytes (CD14\textsuperscript{low} CD16\textsuperscript{high}). Subsequently, these fractions were examined for FR\( \beta \) expression and M1 (CD169) or M2 (CD163, CD200r, CD206) macrophage markers. ST biopsy sections were stained by immunofluorescence for FR\( \beta \) and a combination of macrophage markers (CD163, CD206, and CD169), imaged by STED8 microscopy and analyzed quantitatively for percentages of cellular co-expression by image analysis software.

Results
FR\( \beta \) expression was markedly increased in non-classical monocytes (6-fold, p<0.001) and intermediate monocytes (2.5-fold, p<0.05) in monocyte subpopulations of early, DMARD (therapy-naïve), RA patients with clinically active disease, when compared with healthy FR\( \beta \) expression in classical monocytes. After long term treatment of established RA patients with suppressed disease activity FR\( \beta \) levels in all monocyte subpopulations were markedly lower and in the range of healthy controls. Expression of CD163, CD200r, CD206 and CD169 largely shared expression profiles of FR\( \beta \) expression, except that CD169 expression was significantly increased (4-fold, p<0.01) in non-classical monocytes of established RA patients as compared with their counterparts in early, DMARD (therapy-naïve) RA and healthy controls. In SF samples of established RA patients, macrophages FR\( \beta \) expression was 4-fold (p<0.05) higher compared with non-RA patient (SpA) samples. Similar trends were observed for CD200r (4.5 fold increased, p<0.01) and CD163, CD169 and CD206 expression (2-2.5-fold increased, p<0.05). In ST biopsies of 2 RA patients with clinically active systemic and local disease, FR\( \beta \) was strongly expressed and co-expressed with CD163, CD169 and CD206. In synovial tissue biopsies of 3 RA patients with mild disease activity, FR\( \beta \) expression and other marker expression was comparable with levels found in non-RA synovium.
Conclusion
FRβ constitutes a marker in RA for monocytes of early, DMARD (therapy-naïve), treatment-naive patients. Macrophage FRβ expression in RA ST is associated with active disease, but not exclusively coexpressed with designated M1 or M2 macrophage markers. These results underscore the importance of FRβ for imaging-guided disease activity monitoring and therapeutic targeting in RA.

Keywords: Folate receptor β, Rheumatoid arthritis, Macrophages, Monocytes, Synovial tissue.

Abbreviations
INTRODUCTION

Rheumatoid arthritis (RA) is an autoimmune disease, which affects approximately 0.5-1.0% of the world population [1]. Synovial inflammation and joint destruction, combined with infiltration of immune cells (lymphocytes and macrophages) and release of inflammatory cytokines (e.g. TNFα, IL6), are some of the key characteristics of RA [2]. Based on the emergence of auto-antibodies to post-translationally modified proteins in the blood of patients prone to RA, it is now recognized that the disease can be initiated 10-15 years before clinical symptoms are presented [3]. However, only 40% of the patients with positive serology will actually develop the disease [4]. In order to treat RA as early as possible, there is a need to identify subclinical synovitis at an early stage [5]. For this purpose, non-invasive imaging techniques such as Positron Emission Tomography (PET) are particularly suitable in RA [6]. Synovial macrophage infiltration has been validated as a good marker of RA disease activity, and reduction of synovial macrophage infiltration can be used as a measure of treatment response [7–9]. Over the past decade considerable efforts have been undertaken to visualize macrophage infiltration at sites of inflammation as a marker of disease severity [10].

Although macrophage PET tracers have merits in monitoring clinical disease activity in RA, their background uptake in peri-articular tissue hampers detection of subclinical synovitis [11–13]. In search of novel macrophage PET tracers in RA, folate receptor β (FRβ) has been identified as an attractive candidate [14,15]. FRβ is a glycosylphosphatidylinositol (GPI) anchored plasma membrane protein and one of 3 FR-isoforms (α, β, and γ-isoform) [16]. FRβ is differentially specifically expressed on monocytc/macrophage cells [17,18] and also identified on macrophages in human RA synovium [19,20]. Recently, it was demonstrated that an FRβ-targeted PET tracer, [18F]fluoro-PEG-folate, enabled visualization of arthritis in knee joints of arthritic rats [21]. In addition, following MTX treatment of these arthritic rats lower tracer uptake and concomitant reduction of synovial macrophage infiltration was observed, thus indicating that [18F]fluoro-PEG-folate PET may be used for therapy response monitoring [22,23]. Currently, [18F]fluoro-PEG-folate is evaluated in a first-in-man clinical application to visualize inflamed joints in RA patients [24].

An unresolved issue is FRβ expression in relation to macrophage polarization. This issue was first addressed by Puig-Kroger et al [25] in ex vivo studies for healthy volunteers. In this study, monocytes skewed by M-CSF to “M2”-type (anti-inflammatory) macrophages expressed markedly higher FRβ levels than monocytes skewed by GM-CSF to “M1”-type (pro-inflammatory) macrophages. This seems counterintuitive as FRβ targeted therapies with MTX [19,22] and FRβ-conjugated immunotoxins [26–28] elicited clear anti-arthritic effects. In addition, immunohistochemical studies of RA and osteoarthritis synovial tissue did not reveal exclusive FRβ expression on M2-type macrophages [29]. In addition, in peripheral blood monocytes of healthy volunteers, FRβ was expressed on CD14high CD16low classical monocytes rather than on intermediate (CD14high CD16+) and non-classical (CD14low CD16high) monocyte subpopulations [30].

The purpose of this study was to assess FRβ expression in subpopulations of monocytes from peripheral blood of early, DMARD (therapy-naïve) and established RA patients,
and synovial fluid (SF) monocytes from established RA patients using flow cytometry. Moreover, immunofluorescence staining for FRβ and macrophage subset markers (M1/M2) was performed in synovial tissue (ST) biopsies of RA patients with active disease, mildly active disease, and non-RA patients.

METHODS

Patients

Healthy volunteers (“Healthy”, n=8, age - 48±4 y, male:female 1:1); non-RA (n=9) (arthritis of other origin, i.e. Spondyloarthritis (SpA) (n=3), psoriatic arthritis (n=2), osteoarthritis (n=1), SLE-like disease (n=1), gout (n=1), reactive arthritis (n=1), early, DMARD (therapy-naïve) RA (treatment naïve), and established RA were included in the study. Table 1 provides an overview of demographics and clinical characteristics of the early, DMARD (therapy-naïve), established and non-RA groups (excluding patients of whom synovial tissue was collected, described separately below). Experiments were also performed in the group of early, DMARD (therapy-naïve) RA patients who had treatment for two weeks with a dose of 10 mg/week methotrexate (MTX) and 20 mg prednisone daily. Paired blood and synovial fluid samples (n=5) were obtained from the group of established RA patients. The protocol was approved by READE (Amsterdam) medical ethics committee. All patients gave written informed consent prior to participation in the study. All clinical data were collected by an experienced research nurse who was blinded for the experimental analyses. Five RA synovial tissue samples of RA patients with disease duration of 1-8 years, treated with DMARDs and/or NSAIDs were included in the study; 2 pts had very active systemic and local disease in the joint (either knee or ankle) that was biopsied, three patients had very mild disease activity either systemic and or local in the joints that were biopsied. As a reference two synovial tissue samples from non-RA patients (SpA) were included.

Isolation and staining of monocytes/macrophages

Freshly drawn peripheral blood from healthy volunteers and RA patients, or freshly drawn synovial fluid from RA patients was diluted in 0.1%BSA/PBS and layered on lymphoprep (STEMCELL, Vancouver, Canada) in equal proportions and centrifuged at 1000g for 15 min at room temperature. Isolated peripheral blood mononuclear cells (PBMC)/synovial fluid cell fractions were re-suspended in 50 mL 0.1% BSA/PBS and centrifuged at 600g for 10 min. Thereafter, PBMCs/synovial fluid cells were washed 2 times with 0.1% BSA/PBS at 300g for 8 min to remove platelets from the sample. Isolated PBMCs/synovial fluid cells were suspended at a density of 1x10⁶/mL in 0.1%BSA/PBS placed on ice. PBMCs/synovial fluid cells were stained for 30 min at 4°C with directly conjugated antibodies CD3-BV510 (UCHT1; 1:100), CD11b-PE-Cy7 (ICRF44; 1:50), CD14-PerCp-Cy5.5 (MFP9; 1:25), CD16- BV650 (3G8; 1:50), CD20-BV510 (2H7; 1:100), CD56- BV510 (NCAM16.2; 1:100) (all from BD eBioscience, San Jose, CA, USA). Fixable viability dye (FVD; efluor 506 1:1000) and streptavidin APC (1:100) were purchased from eBiosciences. Biotinylated mouse-anti human FR-β (m909; 1:25) primary antibody [30,31] was generously provided by Dr. P.S. Low (Purdue University, West Lafayette, IN, USA). After a washing step with 2 mL FACS buffer (PBS/0.1%
BSA/0.02% NaN₃), cells were centrifuged at 530g for 5 min at 4°C. For anti-FR-β incubated cells, streptavidin APC was added and incubated for 30 min at 4°C. Finally, cells were washed with 2 mL FACS buffer and centrifuged at 1000g for 5 min at 4°C and subsequently kept on ice.

Flowcytometry and gating procedures monocytes/macrophages

The BD LSRFortessa™ X-20 cell analyzer (BD Biosciences, San Jose, USA) was used for acquisition of the flow data. Kaluza software (Beckman coulter, Brea, USA) was used to analyze the flowcytometric data calculating specific (geometric) mean fluorescence intensity (MFI) (MFI; geometric mean fluorescence of marker – geometric mean fluorescence of FMO (fluorescence minus one)) control. The gating strategy (Figure 1) for selection of monocyte/macrophages population was as follows: forward vs side scatter (FSC vs SSC) was visualized and thereupon the lymphocyte and monocyte clouds were selected (Figure 8.1A). From this, the lineage (CD3, CD20, CD56 and FVD) negative population was selected (Figure 8.1B) and further gated based on CD11b vs CD14 expression. For further analysis, the CD14+ population was selected (Figure 8.1C) and gated for CD14 vs CD16, resulting in dissection of 3 monocyte subpopulations [32], classical monocytes (CD14^{high} CD16^{low}), intermediate monocytes (CD14^{high} CD16^{+}) and non-classical classical monocytes (CD14^{low}CD16^{high}) (Figure 8.1D). Synovial fluid cells were identified with FSC vs SSC, lineage negative population and thereupon with a similar CD14 vs CD16 gating strategy. In synovial fluid samples, the CD14^{+} monocytes/macrophages (Figure 1C: CD11b vs CD14) are gated further on CD14 vs CD16, wherein no subpopulations were visualized (Figure 8.1E). FRβ and macrophage markers CD163, CD169, CD200r and CD206 MFIs were assessed as stated above for the three monocyte/macrophage subpopulations. Representative histogram plots showing geometric mean fluorescence intensity (MFI) of FRβ, CD163, CD169, CD200r and CD206 in monocyte/macrophage subpopulations are shown in Supplementary Figure 8.1.

Immunofluorescence staining of synovial tissue macrophages

All study subjects underwent arthroscopic synovial tissue biopsy sampling as previously described [33–35]. In RA patients ST biopsy sampling was performed in either inflamed ankle or knee joints. The following demographics were collected: Disease Activity Score in 28 joints (DAS(28)); IgM-RF levels using IgM-RF ELISA (Sanquin, Amsterdam, the Netherlands (upper limit of normal (ULN) 12.5 IU/ml)) until December 2009 and thereafter using IgM-RF ELISA (Hycore Biomedical, Indianapolis, IN (ULN 49 IU/ml)); ACPA using anti-citrullinated cyclic peptide (CCP)2 ELISA CCPlus (Eurodiagnostica, Nijmegen, the Netherlands (ULN 25 kAU/l)); erythrocyte sedimentation rate (ESR); and serum levels of C-reactive protein (CRP). The study was approved by the Medical Ethics Committee of the AMC and performed according to the Declaration of Helsinki. All patients gave written informed consent. Frozen RA and non-RA ST sections were first defrosted at room temperature (RT) for 30 min and fixed for 10 min in acetone (Sigma-Aldrich, Missouri, USA) at -20°C. Upon washing 3x in 0.1% BSA/PBS for 5 min the tissue sections were blocked with 10% mouse serum (1:10 PBS) (DAKO, Santa Clara, USA) for 30 min at RT. Thereupon, sections were washed in 0.1% BSA/PBS and incubated with 1 mL of Biotin blocking system (X0907, DAKO, Santa Clara, USA) for
Figure 8.1 – Flow cytometry gating strategy for isolating monocytes/macrophages subpopulations from human peripheral blood. A - representative Forward vs Side scatter (FSC vs SSC) plot, B - representative lineage (lin CD19, CD4, CD56 & Fixable dead cells stains) vs FSC plot for exclusion of lymphocyte population and gating of monocyte population, C - CD14 vs CD11b gating of monocyte population providing the CD14$^+$ population, D - Classical monocytes (CD14$^{\text{high}}$ CD16$^{\text{low}}$), intermediate monocytes (CD14$^{\text{high}}$ CD16$^{+}$) and non-classical classical monocytes (CD14$^{\text{low}}$CD16$^{\text{high}}$). E - From C (CD14$^+$) synovial monocytes/macrophages population are gated against CD14 vs CD16.

Inhibiting non-specific binding of endogenous biotin. After washing, the sections were incubated overnight at 4°C with either 0.1% BSA/PBS as control or primary antibody cocktail (100 µL) (FRβ+CD163+CD169 or FRβ+CD163+CD206), prepared with Biotin FRβ (m909, 1 µg/mL), anti-human CD163 (2 µg/mL) (Ab182422, IgG1, Abcam, Cambridge, UK), anti-human CD206 (1 µg/mL) (555953, IgG1 BD, San Jose, USA), and anti-human CD169 (17-239, IgG1 MCBI, VUmc, NL) and added to separate sections and incubated overnight at 4°C. The sections were washed 3x in 0.1% BSA/PBS for 5 min at RT and incubated with the cocktail of secondary antibodies (100 µL); STREP 647 (1µg/ml) (S32357, Invitrogen, Strep 647, Carlsbad, USA) for FRβ, anti-rabbit STED 488 (1 µg/mL) for CD163, anti-mouse STED 580 (1 µg/mL) for CD169, anti-mouse STED 580 (1 µg/mL) for CD206, respectively, for the primary antibodies. After washing for 3x in 0.1% BSA/PBS for 5 min at RT, sections were incubated with 300 nM 4’,6-diamidino-2-phenylindole (1:1000, DAPI, 62248, ThermoFischer, Waltham, USA) for 2 min at RT, washed once with 0.1% BSA/PBS for 5 min, air dried, mounted with 2 µL medium (Mowiol, 81381, Sigma, Missouri, USA) and covered with coverslips. Images of ST were obtained with the Leica SP8 confocal microscope (Leica Microsystems, Wetzlar, Germany) [36]. A 40x oil objective with NA 1.4 was used to image the sample. Detection of the fluorescent signal (with control staining intensity used to determine background) was performed with gated Hybrid Detectors. Finally, images were deconvoluted using Huygens Professional software (Scientific Volume Imaging) and acquired.
using LasX imaging software (Leica Microsystems, Wetzlar, Germany) [36].

Quantification of synovial tissue macrophages

All RA and non-RA synovial tissue images were analyzed using Definiens studio image analysis software (Definiens, Carlsbad, USA) to identify and quantify FRβ, CD163, CD169 and CD206 positive cells in the tissues. First, the region of interest (ROI) was selected manually to exclude areas of inferior quality or fixation artifacts. Next, images were analyzed using Tissue Studio software (Definiens, Carlsbad, USA) for cellular analysis. A machine-learning approach [37] was defined to identify the synovial tissue region. Nuclei were identified through the Tissue Studio Nuclear algorithm and the membrane algorithm was used to draw the cytoplasm around the nuclei identified. Subsequently, the baseline expression (background being the baseline) of each marker was determined and then the RA and non-RA synovial tissues were processed with a novel rule set developed in Developer (Definiens) software. Finally, the percentage of cellular co-expression for FRβ and other markers was determined in various double or triple combinations for all the tissue samples [38].

Statistical analysis

An (unpaired) t-test was used to identify significant differences between RA and non-RA synovial fluid samples. Mann-Whitney (exact) tests were performed to analyse differences in classical, intermediate and non-classical monocyte subpopulations of healthy volunteers and RA patients groups, respectively. A p-value <0.05 was considered as statistically significant. Statistical analyses were performed using SPSS version 22.0 for Windows (SPSS, Chicago, IL, USA).

RESULTS

FRβ expression on PBMCs/monocyte subpopulations in blood of early, DMARD (therapy-naïve), treatment naïve RA patients

From PBMCs of early, DMARD (therapy-naïve) RA patients, CD14 vs CD16 gating as depicted in Figure 8.1, distinguished three subpopulations of monocytes - classical monocytes (CD14highCD16low), intermediate monocytes (CD14highCD16+) and non-classical monocytes (CD14low CD16). The classical monocyte population was most abundant, 84.5±3.1%, whilst the non-classical (7.1±1.9%) and intermediate fractions 7.5±2.1% constituted minor monocyte subpopulations. No significant changes in this distribution pattern were noted for healthy volunteers or established RA patients (data not shown). Examining FRβ expression on monocyte subpopulations revealed markedly high expression levels on non-classical monocytes > intermediate monocytes > classical monocytes of early, DMARD (therapy-naïve) RA patients (Figure 8.2). FRβ expression levels on non-classical monocytes in early, DMARD (therapy-naïve) were 5-12 fold higher (p<0.001) than those in healthy volunteers and established RA patients (Figure 8.2). Two weeks of treatment of early RA patients with a dose of 10 mg/week MTX and 20 mg prednisone daily did not show significant alternations in FRβ, CD163, CD169, CD200r and CD206 expression on monocyte subpopulations (results not shown). FRβ,
Figure 8.2 – Expression of FRβ in healthy volunteers (n=8), early, DMARD (therapy-naïve) RA patients (n=10), and established RA (n=10) for the 3 monocyte subpopulations Classical monocytes (CD14^{high} CD16^{low}), intermediate monocytes (CD14^{high} CD16^{+}) and non-classical classical monocytes (CD14^{low} CD16^{high}). Values depict mean fluorescence intensities (MFI). Error bars indicate SD. * p<0.05, *** p<0.001.

CD163, CD169, CD200r and CD206 expression levels in monocyte subpopulations did not correlate with clinical DAS scores or CRP levels in early, DMARD (therapy-naïve) or established RA (results not shown).

Expression profiling of CD163, CD169, CD200r and CD206 on monocyte subpopulations showed variable patterns (Figure 8.3 and Supplementary Figure 8.2). In early, DMARD (therapy-naïve) RA patient samples, both CD163 and CD169 expression were significantly higher in intermediate monocytes (4-8 fold, p<0.001 and 3-fold, p<0.05, respectively) and classical monocytes (4-fold, p<0.001 and 3-fold, p<0.05, respectively) than those in healthy volunteers and established RA patients. Remarkably, CD169 expression was 3-fold higher (p<0.05) on classical monocytes of established RA patients compared with other groups. CD200r expression was increased (4 fold, p<0.05) in early, DMARD (therapy-naïve) RA classical and intermediate monocytes, whereas CD200r levels in early, DMARD (therapy-naïve) non classical monocytes were comparable with those in healthy volunteers and established RA patients. CD206 expression levels were increased (2-5 fold, p<0.01) in all monocyte subpopulations of early, DMARD (therapy-naïve) RA patients compared with other groups (Supplementary Figure 8.2).
Chapter 8  FRβ-related immunophenotyping of monocytes/macrophages in RA

Figure 8.3 – Expression of FRβ and macrophage markers CD163, CD169, CD200r and CD206 on monocyte subpopulations of treatment naïve early, DMARD (therapy-naïve) RA patients (n=10). Monocyte subpopulations - Classical monocytes (CD14 high CD16 low), intermediate monocytes (CD14 high CD16 high) and non-classical classical monocytes (CD14 low CD16 high). Values depict mean fluorescence intensities (MFI). Error bars indicate SD. **p < 0.01, ***p < 0.001.

FRβ expression on RA synovial fluid monocytes

In addition to blood monocytes, FRβ expression was also examined by flowcytometry on CD14 + CD16 + monocytes/macrophages in synovial fluid from established RA patients (n=10). Herein, FRβ expression was 4-fold (p<0.05) higher than in a control group (n=9) of non-RA synovial fluid samples (Figure 8.4A). Similarly, expression of other markers was increased on RA synovial monocytes compared with non-RA controls; CD163 (2-fold) (Figure 8.4B), CD169 (2-fold) (Figure 8.4C), CD200r (4.5-fold, p<0.01) (Figure 8.4D), and CD206 (2.5-fold, p<0.05) (Figure 8.4E).

FRβ expression on monocytes of paired blood and synovial fluid samples of RA patients

FRβ expression was examined in paired blood (monocyte subpopulations) and synovial fluid samples from 5 established RA patients. For all 5 individual patients, FRβ expression levels were more then 4-fold higher on CD14 + macrophages/monocytes in synovial fluid samples than on paired 3 PBMC monocyte subsets (Figure 8.5). Similar rank orders of expression were observed for CD163, CD169, CD200r and CD206 markers; classical monocytes ≤ intermediate < non-classical monocytes < synovial fluid, except
Figure 8.4 – Monocytes/macrophage marker expression of (A) FRβ, (B) CD163, (C) CD169, (D) CD200r and (E) CD206 on synovial fluid monocytes/macrophages from established RA patients (n=10) and synovial fluid from non-RA controls (n=6). Values depict mean fluorescence intensities (MFI). Error bars indicate SD. *p<0.05, **p<0.01.

for CD169 expression, which was 1.5 fold higher in blood non-classical monocytes than in synovial fluid monocytes (Supplementary Figure 8.3).

Expression of FRβ on macrophage subpopulations in RA synovial tissue

ST biopsies of 5 RA and 2 non-RA patients (SpA) were stained by immunofluorescence for FRβ expression, other macrophage markers (CD163, CD169 and CD206), and their combinations. Two of the RA patients (pt 1 and pt 2) had very active systemic and local disease in the joints that was biopsied (either knee or ankle), while patients 3-5 had very mild disease activity either systemic and or local in the joints that were biopsied. Representative staining’s for RA patient 1 vs non-RA patient 1 are shown in Figure 8.6, depicting nuclear DAPI staining (Figure 8.6A and 8.6I, respectively), single staining for FRβ, CD163, CD169 and CD206 (Figure. 8.6B-E and 8.6J-M, respectively). These images reveal stronger staining of FRβ and the other markers in synovial tissue of the RA pt 1 as compared with the non-RA patient. In addition, 4-color staining’s for DAPI,
Figure 8.5 – Comparisons of FRβ expression in paired peripheral blood monocyte and synovial fluid samples from established RA patients (n=5). The individual FRβ expression for each patient is shown for Classical monocytes (CD14$^{\text{high}}$ CD16$^{\text{low}}$), intermediate monocytes (CD14$^{\text{high}}$ CD16$^{+}$) and non-classical classical monocytes (CD14$^{\text{low}}$CD16$^{\text{high}}$) and synovial fluid monocytes/macrophages. Values depict mean fluorescence intensities (MFI).

FRβ, CD163 and CD169 are shown (Figure. 8.6F and 8.6N, respectively). Co-expression images of DAPI, FRβ, CD163 and CD169, and for DAPI, FRβ, CD163 and CD206 are shown in Figure. 8.6G/H for RA pt 1 and Figs. 8.6O/N for non-RA pt 1.

Computer-assisted quantification of FRβ expression confirmed overall lower staining’s across all markers in ST of RA pts with mild disease and non-RA pts than in tissue sections of RA with active local and systemic disease (Figure 8.7). In the ST sections of RA pts 1 and 2 (with active disease) comparable percentages of co-expression were observed for both FRβ and CD163 (4-6%) and for FRβ and CD169 (3-7%), with slightly higher percentages of FRβ and CD206 co-expression (4-15%). Percentages of triple co-expression of FRβ, CD163 and CD169 (7-24%) and FRβ, CD163 and CD206 (39-49%) were markedly higher in the synovial tissue of active disease compared to mild RA (2-8% and 1-3%, respectively) and non-RA (1-2% and 0.5-1.5%, respectively).
Figure 8.6 – Representative images of active RA (top) and non-RA (bottom) synovial tissue for FRβ and macrophage markers. Top rows; DAPI nuclear staining and single staining’s for FRβ and macrophage markers CD163, CD169 and CD206. Fluorescence four-color staining’s (second row left) and co-expression quantification images (second row middle and right) are for FRβ, CD163, CD169 and CD206. **White cells** – FRβ⁺CD163⁺ CD206/CD169; **Purple** – FRβ⁺ CD206/CD169 and **Yellow** – FRβ⁺CD163
Figure 8.7 – Percentages of cellular co-expression of FRβ with single or multiple macrophage markers (CD163, CD169, CD206) in synovial tissue of RA patients (n=5) and non-RA patients (n=2).
DISCUSSION

The main finding in this study is that non-classical and intermediate subpopulations of blood monocytes of early, DMARD (therapy-naïve) RA patients express elevated levels of FRβ. This contrasted with established (and treated) RA, wherein non-classical and intermediate blood monocytes expressed low levels of FRβ. Furthermore, monocytes/macrophages in SF of active RA patients also expressed significantly higher FRβ than monocytes/macrophages in non-RA SF. In ST of RA patients with active and systemic disease, FRβ was highly expressed and co-expressed with macrophage markers covering the broad spectrum of macrophages from M1 to M2 types, but mostly with CD206 in active disease.

Blood monocyte heterogeneity is reflected by the presence of 3 subpopulations distinguished on the basis of CD14 and CD16 expression [32]. Classical (CD14\textsuperscript{high}CD16\textsuperscript{low}) monocytes harbor migratory capacities to sites of inflammation although in response to LPS stimuli they produce IL10 rather than TNFα [39]. Non-classical (CD14\textsuperscript{low}CD16\textsuperscript{high}) monocytes display pro-inflammatory features [40] and produce pro-inflammatory cytokines in SLE [41]. Intermediate (CD14\textsuperscript{high} CD16\textsuperscript{+}) monocytes are also considered to be pro-inflammatory [40], associated with active RA disease [42] and thought to play a role in RA disease pathogenesis by facilitating expansion of TH17 cells [43]. FRβ expression has been monitored on blood monocyte subpopulations of healthy human volunteers, revealing expression mainly on classical monocytes [30]. In the present study, low levels of FRβ expression were also observed in intermediate and non-classical blood monocytes of healthy volunteers. Remarkably, intermediate and non-classical blood monocytes in early, DMARD (therapy-naïve) clinically active RA patients had markedly increased FRβ levels, whereas in established (treated) RA with suppressed clinical disease activity, FRβ levels were low and even below healthy control levels, possibly due to extended treatment with anti-inflammatory drugs. This suggests that FRβ may constitute a marker for blood monocyte subpopulations with pro-inflammatory properties and may be used in immune-monitoring studies during RA treatment. It is important to mention that in this study only FRβ protein expression was assessed and not FRβ functional activity, e.g. folate binding capacity. This should be the subject of future studies as it has been reported that acquisition of FRβ functional activity is correlated with the activation status of monocyte/macrophages [44]. In this respect, in early (DMARD-naïve) RA blood monocyte subpopulations, we observed that HLA-DR expression levels were marginally higher than established RA patients (data not shown). In case of functional FRβ binding activity on clinically relevant blood monocyte subpopulations in early RA, this would enable their imaging with folate tracers using PET-CT or therapeutic targeting with folate antagonists or folate-conjugated drugs [14,15], albeit FRβ-targeted antibody approaches are also possible without FRβ functional binding activity [27,45].

Short term (2 weeks) treatment of early, DMARD (therapy-naïve) RA patients with MTX and prednisone had no impact on FRβ expression on monocyte subpopulations. This could imply that MTX does not target FRβ-positive monocytes because of inefficient receptor binding, or because FRβ expression on monocyte subpopulations in early, DMARD (therapy-naïve) RA patients decreases only slowly during prolonged treatment,
as in established (treated) RA patients FRβ expression were decreased to healthy vol-
unteer levels. Of note, in established RA patients paired samples of blood and SF showed increased FRβ expression on SF macrophages over blood monocytes. As future
direction, particularly in view of imaging of subclinical RA, it would be of interest to
explore paired blood and synovial fluid monocytes of early, DMARD (therapy-naïve) RA
patients to examine how FRβ expression compares and also monitor whether changes
in FRβ expression levels during therapy are associated with diminished disease activ-
ity. Beyond FRβ expression, immunomonitoring studies after 2 weeks treatment also
included other markers (CD163, CD206, CD200r and CD169) for which differential ex-
pression in monocyte subpopulations of early (DMARD therapy-naïve) RA patients was
shown compared with healthy control and established RA patients. CD163, CD206 and
CD200r are recognized M2-type macrophage markers [46,47]. We did not follow up on
expression analysis of CD80 as proposed M1-type marker due to marginal expression
on monocytes subpopulations (data not shown). CD169 (Siglec-1, sialoadhesin) is rela-
tively unexplored in RA, but is expressed on circulating monocytes of systemic sclerosis
patients [48]. Moreover, CD169 elicits immunomodulatory properties [49] and is induced
by type I interferon-regulated genes [48], which can serve as biomarkers for RA [50].

Synovial macrophage infiltration is a hallmark of human RA and adopted as indica-
tor for disease activity or therapy response [7–9]. In the present study, the presence of
FRβ-positive macrophages in RA synovium [20,29,51] was confirmed, particularly in pa-
tients with active systemic and local disease. To further address the issue of FRβ being
exclusively expressed on M2-type macrophage [25] or also on M1-type macrophages [29],
the present study showed that FRβ is co-expressed with recognized M2-type markers
(CD163 and CD206). However, FRβ is also expressed on CD169-positive macrophages
with more M1-like properties [48] and even more abundantly on macrophage being posi-
tive for both CD163 and CD169. Together, these data suggest that FRβ may constitute
a broad marker for synovial macrophages within the M1 to M2 spectrum, which is in
line with the fact that M1 and M2 macrophages constitute a continuum with marked
plasticity [52,53]. Moreover, recent studies demonstrated that microenvironmental con-
ditions in RA synovium, i.e. presence of ACPAs and IgG immunocomplexes, can trigger
FRβ-expressing M2 macrophages to produce pro-inflammatory cytokines [54–56]. Thus,
the distinction of M1 and M2 types solely on the basis of standard marker expression for
defining pro- and anti-inflammatory macrophages should be made with caution. This
part of the study has limitations related to small sample size of synovial biopsy tissues
sections. Future studies should aim to include RA synovial samples with a broad range
of disease activities and various RA treatments for assessment of macrophage FRβ ex-
pression.

Collectively, this study underscores an important role of FRβ in blood monocytes and
synovial tissue of RA patients, which encourages ongoing studies of exploiting FRβ for
macrophage PET guided imaging studies in RA patients or as a therapeutic target [24].
Moreover, FRβ offers many opportunities for targeting of activated macrophages with
folate-conjugated drugs [14] or folate antagonists.

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Supplementary Figure 1: Representative histogram plots showing geometric mean fluorescence intensity (MFI) of FRβ, CD163, CD169, CD200r and CD206 in monocyte/macrophage subpopulations of healthy controls (HV), early DMARD (therapy-naive) RA (N-RA), established RA pts (E-RA) and synovial fluid macrophages pts are represented. The monocyte subpopulations refer to = Classical monocytes (CD14\textsuperscript{high} CD16\textsuperscript{low}), intermediate monocytes (CD14\textsuperscript{high} CD16\textsuperscript{+}) and non-classical classical monocytes (CD14\textsuperscript{low} CD16\textsuperscript{high}).
Supplementary Figure 2: Expression levels of (A) CD163, (B) CD169, (C) CD200r and (D) CD206 in monocyte subpopulations of healthy controls (n=8), early, DMARD (therapy-naïve) RA (t=0d) (n=10), (n=10) and established RA pts (n=10) are represented. The monocyte subpopulations refer to Classical monocytes (CD14\textsuperscript{high} CD16\textsuperscript{low}), intermediate monocytes (CD14\textsuperscript{high} CD16\textsuperscript{+}) and non-classical classical monocytes (CD14\textsuperscript{low} CD16\textsuperscript{high}). Values depict mean fluorescence intensities (MFI). Error bars indicate SD. *p<0.05 **p<0.01, ***p<0.001.
Supplementary Figure 3: Individual monocytes/macrophage marker expression of (A) CD163, (B) CD169, (C) CD200r and (D) CD206 in paired peripheral blood monocyte subpopulations and synovial fluid samples from (n=5) established RA patients. Classical monocytes (CD14$^{\text{high}}$ CD16$^{\text{low}}$), intermediate monocytes (CD14$^{\text{high}}$ CD16$^{+}$) and non-classical classical monocytes (CD14$^{\text{low}}$ CD16$^{\text{high}}$). Values depict mean fluorescence intensities (MFI).
Chapter 9

Summary, general discussion, future perspectives and key points of the thesis
9.1. Summary

After specifying the outline of the thesis in Chapter 1, Chapter 2 provides an overview of the chronic disease rheumatoid arthritis (RA), with a focus on the role of activated macrophages and the potential of the folate receptor beta (FRβ) as a macrophage mediated imaging and therapeutic target.

Chapter 3 describes the modification of an experimental model of RA in rats with the aim to create sustained articular macrophage infiltration, so that it could be used for both PET imaging and monitoring response to (new) therapeutic agents for RA. Adjustments consisted of a slightly adapted immunization procedure, increased volume of the first locally induced arthritis with methylated bovine serum albumin (mBSA) in the right knee of rats and, finally, repeat injections of mBSA to sustain arthritis for a longer period of time to be able to demonstrate efficacy of (multiple) administrations. The contralateral knee served as an internal control for each individual rat. These adjustments led to a relatively mild animal model of RA (no major body weight loss or other impairments). Significant anti-mBSA serum levels, DTH and knee swelling were observed with sustained and prolonged macrophage infiltration especially after repeated mBSA injections. To demonstrate the feasibility of PET evaluation of arthritis, two PET tracers were used $[^{18}\text{F}]$FDG (as a non-specific PET tracer for inflammation) and $(R)-[^{11}\text{C}]$PK11195 (binding to the macrophage marker translocator protein, TSPO, up-regulated in activates macrophages). Both tracers showed increased accumulation in the arthritic knee as compared with the contralateral control knee, which reflected microscopic macrophage infiltration as demonstrated by immunohistochemistry. Interestingly, the contralateral knee showed some macrophage infiltration as well, although much less than the arthritic knee, being indicative of a systemic component of RA in this model.

In Chapter 4, this rat model of RA was used to investigate the feasibility of visualising articular inflammation with the macrophage PET tracer $[^{18}\text{F}]$fluoro-PEG-folate, which targets FRβ. Moreover, in this study a systemic component of the inflammation was demonstrated in the liver and spleen of these rats. In addition, a first PET therapy response monitoring was performed during treatment of arthritic rats with MTX, the anchor drug in RA. After MTX treatment, $[^{18}\text{F}]$fluoro-PEG-folate PET images revealed attenuation of arthritis which was corroborated by markedly reduced macrophage infiltration in the arthritic knee of the rat. These studies were extended in Chapter 5 with ex vivo tissue distribution studies of $[^{18}\text{F}]$fluoro-PEG-folate: sections of arthritic knees and multiple (macrophage residing) organs were analysed histologically and by immunohistochemistry using specific antibodies to rat macrophages, as well as FRβ immunofluorescence measurements. It was demonstrated that MTX also reduced systemic inflammation in arthritic rats as confirmed by significant reductions in macrophage numbers in both spleen and liver of arthritic rats.

In Chapter 6, the arthritic rat model was employed to explore the potential anti-arthritic effects of a new agent, alkaline phosphatase (AP). AP functions as a gatekeeper of innate immune system responses by detoxifying (dephosphorylating) inflammation triggering moieties (e.g. ATP, ADP, LPS), released from endogenous and external sources. Administration of human recombinant AP was tested in both prophylactic
(before arthritis induction) and therapeutic (after arthritis induction) settings, the latter as single agent and in combination with MTX. Prophylactic and therapeutic schedules of single agent AP treatment, and combinations with MTX, were well tolerated. Both prophylactic and therapeutic AP administrations resulted in markedly reduced synovial macrophage infiltration in arthritic knees, comparable with MTX treatment effects. AP and MTX combinations slightly improved on single agent effects. \(^{18}F\)fluoro-PEG-folate PET scans and ex vivo tissue distribution studies confirmed the effects of AP and AP+MTX in reducing synovial macrophage infiltration. In addition to localized articular effects, AP also conveyed systemic anti-inflammatory effects by significant reductions in FR\(\beta\)-positive macrophages in liver and spleen of arthritic rats. Given these broad effects, single agent AP, or combined with MTX, deserves further evaluation as a new therapeutic modality in RA.

In Chapter 7, PET imaging was used to investigate targeting features and in vivo pharmacokinetics and pharmacodynamics of a new promising therapeutic agent, antibody fragment F8-mediated IL10 delivery (F8IL10), for RA. F8 binds to the extra-domain-A (ED-A) of fibronectin at sites of inflammation, allowing for local deposition of IL10. In a translational setting, radiolabelled F8-IL10 was injected in RA patients and in animals (with and without arthritis). Results in RA patients demonstrated clear targeting of radiolabelled F8-IL10 in the (sub)clinically inflamed joints. Remarkably, rapid clearance of F8-IL10 from the blood together with accumulation in liver, and to a lesser extent in spleen, were also noted. In addition, animal experiments were performed to elucidate the rapid uptake in liver (and spleen). These studies showed specific binding of F8-IL10 in liver and spleen of arthritic rats, which appeared to be due to increased fibronectin ED-A expression in these tissues as compared with similar tissues in healthy animals. Injection of a microdose of the PET tracer might result in increased (relative) uptake in liver and spleen upon first passage through these organs, but this remains to be proven in future studies. This translational study demonstrated the value of in vivo PET-CT biodistribution studies of new and potential anti-rheumatic drugs.

Chapter 8 reports on flow cytometric expression profiling of FR\(\beta\) on monocyte subpopulations (classical, intermediate, non-classical monocytes) in blood of early (treatment naive) and established RA patients, with healthy volunteers serving as controls. Moreover, using immunofluorescence studies, macrophage FR\(\beta\) expression was characterised in synovial tissue biopsies from RA patients with variable degrees of disease activity. These studies were accompanied with stainings for other macrophage markers (CD163, CD169 and CD206) to define FR\(\beta\) expression in relation to ‘M1’-type (pro-inflammatory) and ‘M2’-type (anti-inflammatory) macrophage polarizations. In early RA patients (i.e. early stage of disease), markedly elevated FR\(\beta\) expression levels were noted in intermediate and (pro-inflammatory) non-classical monocytes, which comprise 10-15% of the monocyte population in peripheral blood. Since FR\(\beta\) expression levels were not elevated in established RA patients and healthy volunteers, FR\(\beta\) may represent a marker for pro-inflammatory monocytes in early RA patients. In paired samples (blood and synovial fluid) from established RA patients, FR\(\beta\) expression was more prominent in monocytes/macrophages in synovial fluid as compared with blood. FR\(\beta\) expression was clearly increased in synovial tissue of RA patients with active systemic and local disease as compared with RA patients with milder disease activity and non-RA synovial tissue.
Co-expression studies of FRβ with other macrophage markers revealed co-expression with known M2-type macrophages (i.e. CD163 and CD206). However, FRβ co-expression was not exclusively with M2-type markers, as also co-expression with M1-like CD169 macrophages and triple staining of FRβ+CD169+CD163/CD206 were observed.
9.2 General Discussion and Future Perspectives

Animal models
For most anti-rheumatic drugs it takes time to evaluate their action on arthritis activity when using macrophage infiltration as a biomarker. Therefore, especially in case of (sub)clinical arthritis, most existing mice models of RA are not suitable (either short-term acute models or models with severe bone-destruction and/or poly-articular distribution) (1,2). An antigen induced arthritis model in the rat was chosen for several reasons. For monitoring of therapy efficacy using positron emission tomography (PET), a more chronic model was important and given the spatial resolution of most PET scanners, the larger rat knee is an advantage. Another advantage is the size of a rat compared with that of a mouse, providing the possibility to inject more radioactivity before running into problems with (cold) dose effects. In addition, full quantification of PET data is possible in rats, as repeated arterial blood sampling during a PET scan can be performed, a procedure that is not feasible in mice. The antigen-induced rat arthritis model adopted in the present studies is also reminiscent of human RA, which features synovial macrophage infiltration and moderate systemic inflammation (3-5). Taken together, this improved RA rat model is ideal to evaluate novel PET tracers, to image the degree of arthritis and to use PET as a tool for monitoring the efficacy of novel therapeutics agents for RA.

FRβ: folate PET tracer and macrophages
PET imaging has gained increasing interest as a non-invasive modality to visualize inflammation/arthritis (6). The work in this thesis focused on the application of $[^{18}\text{F}]$fluoro-PEG-folate as a novel macrophage PET tracer exploiting the fact that its target FRβ is expressed on activated macrophages. Tracer binding to a GPI-anchored external receptor protein like FRβ differs from commonly used (macrophage) PET tracers. $[^{18}\text{F}]$FDG, for example, monitors inflammation induced increased glucose metabolism, and $(R)-[^{11}\text{C}]$PK11195, targeting TSPO in the inner mitochondrial membrane of cells, makes use of the fact that TSPO expression is differentially high in monocyte/macrophage cells compared with lymphocytes. With respect to chemical properties, $(R)-[^{11}\text{C}]$PK11195 or second generation TSPO tracers (e.g. $[^{11}\text{C}]$DPA-713 and $[^{18}\text{F}]$DPA-714) differ from folate-based tracers. The first category are lipophilic compounds allowing easy cell penetration and diffusion, but are also prone to cellular extrusion by specific drug efflux transporters such as P-glycoprotein. In contrast, $[^{18}\text{F}]$fluoro-PEG-folate is an anionic compound that is not readily taken up by cells other than via a receptor or carrier mediated process. Linkage of the $[^{18}\text{F}]$PEG moiety to the γ-carboxyl group of folic acid abolishes substrate affinity for the reduced folate carrier (RFC) or proton-coupled folate transporter (PCFT) and retains binding affinity for folate receptor isoforms α or β. $[^{18}\text{F}]$FDG and $(R)-[^{11}\text{C}]$PK11195 are established tracers for monitoring clinical disease activity in RA, but background uptake in peri-articular tissue hampers detection of sub-clinical synovitis. A comparison with TSPO PET tracers in arthritic rats, showed that best contrast was obtained with $[^{18}\text{F}]$fluoro-PEG-folate (7), thereby encouraging further characterization and exploitation of this tracer. The studies in this thesis highlight its potential for monitoring systemic inflammation by increased macrophage infiltration in liver and spleen (chapter 4) and for therapy response monitoring (chapters 5 and 6). $[^{18}\text{F}]$fluoro-PEG-folate has also been investigated successfully in a first-in-man clinical application to visualize inflamed joints in RA patients (8). This paves the way for
future applications in other (FRβ-expressing) macrophage-driven inflammatory diseases such as systemic lupus erythematosus, autoimmune uveitis, autoimmune encephalomyelitis, giant cell arteritis, osteoarthritis and atherosclerotic lesions. Future improvements in folate-based PET tracers may also involve a more rapid synthesis time. Whereas [18F]fluoro-PEG-folate synthesis is a time-consuming, two-step process, recently Chen et al (9) reported a one-step synthesis of [18F]folate-PEG-NOTA-AI within < 1 hr. This new folate PET tracer warrants further (pre)clinical evaluation in RA.

Folate linker chemistry for FR targeting has made considerable progress over the past decade (10) enabling linkage of fluorochromes for near-infrared/optical imaging of FRα and FRβ expressing tissues, of which OTL-38 is a prototypical example (11-12). Conceivably, future studies may involve combined hybrid imaging modalities of PET, CT, SPECT and optical imaging for arthritic flare monitoring. Notwithstanding the major progress made in the development and application of FR-targeted imaging agents, the search for novel macrophage targeting agents will continue, e.g. specifically those targeting the M1-type of pro-inflammatory macrophages. In this respect, recent progress in the development ligands for the purinergic ion channel receptor P2X7R hold promise (13).

**FRβ:** monocytes, macrophage polarization and function

For optimal exploitation of FRβ as a target for imaging of and therapeutic interventions in RA, it is pivotal to understand the dynamics of its expression on macrophages and other myeloid/monocytic cells in both peripheral blood and synovium of RA patients. Macrophage heterogeneity is a common feature in RA inflamed synovial tissue represented by the presence of polarized macrophage subsets, distinct immunophenotypes and activation stages. The polarization spectrum of macrophages includes ‘M1-type’ (designated pro-inflammatory macrophages) and ‘M2-type’ (designated anti-inflammatory macrophages) as extremes, but this definition is not fixed as both types of macrophages can be skewed in either direction by a diversity of cytokines and other stimuli. Original studies reporting FRβ expression on macrophages in RA synovium (14,15) did not address whether FRβ was expressed on M1-type or M2-type macrophages, or both. Puig-Kroger et al (16) studied expression of FRβ ex vivo in relation to macrophage polarization in healthy individuals and found FRβ differentially expressed on the M2 type. In addition, in RA synovial tissue, FRβ colocalization was observed with CD163, a designated marker for M2-type macrophages. Finally, in various cancer tissues, FRβ expression was also identified on tumor-associated macrophages (TAMs), known to have an M2-type signature. However, detailed immunohistochemical studies by Tsuneyoshi et al (17) demonstrated that in synovial tissue of both RA and osteoarthritis patients, FRβ expression was not restricted to either M1- or M2-type macrophages. Given these discordant data, in this thesis the original studies from van der Heijden et al (15) were extended by re-examining human RA synovial tissue for FRβ expression along with co-expression analysis for other macrophage markers (chapter 8). In an exploratory series of 5 RA and 2 non-RA synovial tissues, FRβ expression was negible in non-RA synovium, but heterogeneous and quantitively diverse in RA synovium involving both lining and sublining. FRβ was more strongly expressed in synovial tissue of RA patients with active systemic and local disease than that of patients with mild disease. In RA synovium, FRβ colocalization with known ‘M2-markers’ CD163 and CD206 was
observed. However, co-expression of FRβ was also observed for CD169+ macrophages, featuring M1-like properties. Given these results, future studies should include additional synovial tissues of precisely defined RA patient groups with respect to disease stage, duration and treatment regimens, where possible with paired blood samples and synovial fluid collection for immunophenotyping. For the latter, multidimensional flow cytometric analysis may be an attractive tool (18).

The FRβ expression profiles in monocyte subpopulations do not provide clear leads into proposed putative functions of FRβ: (a) delivery of folates for biopterin metabolism, which facilitates reactive oxygen species production in macrophages, (b) FRβ-mediated scavenging of folates from sites of inflammation to deprive pathogens from nutrients, or (c) involvement in signalling processes consistent with the notion that a GPI-anchored protein like FRβ is co-localized with other GPI-anchored signalling proteins in plasma membrane lipid rafts. Future strategies to gain further insight into the functional role of FRβ should focus on those aspects.

FRβ and therapy

Synovial macrophage infiltration is a hallmark of RA disease, just as reduction of macrophage infiltration is a recognized indication of therapy response in RA patients. In the present arthritic rat model this notion was confirmed both by macrophage immunohistochemical analysis and folate-PET prior to and after therapeutic interventions. Considering FRβ-targeting for RA treatment, the question has been raised whether it would be beneficial if FRβ would be expressed on M2-type (anti-inflammatory) macrophages. In the context of cancer treatment, there would be a rationale to target M2-type tumour associated macrophages to boost the suppressed immune response to eradicate tumours. Importantly, recent studies indicated that in an RA (synovial) microenvironment of ACPAs (19) or complex IgGs (20-21) M2-type macrophages produced pro-inflammatory macrophages, which would argue in favour of targeting them. In fact, empirically, many therapeutic interventions with folate antagonists including MTX and FRβ-targeted drug conjugates elicited potent anti-arthritic effects (see also chapter 2). In addition, MTX-treatment in the arthritic rat studies (Chapters 4 and 5) conveyed marked reductions of synovial ED1 and ED2 macrophages and FRβ-positive macrophages in liver and spleen. For small molecule, folate antagonists like MTX it may be argued that folate transporters other than FRβ could internalize MTX into M1-type pro-inflammatory macrophages in order to contribute to MTX’s anti-arthritic effect. Some experimental evidence for this notion was presented by Municio et al (22), who showed that in ex vivo monocyte-derived M1-type macrophages from healthy donors, RFC was the dominant entry route for MTX. M1-type macrophages accumulated higher MTX-polyglutamate levels than ex vivo monocyte-derived M2-type macrophages expressing FRβ. It would be of interest to explore whether these observations could also be corroborated when tested under RA synovial microenvironmental conditions.

Whereas MTX can utilize multiple folate transport routes, including RFC, PCFT and FR (reviewed in Chapter 2), a new generation of folate antagonists has been synthesized that specifically utilize FRα and/or FRβ. Very recently, the results of a clinical phase I study of ONX-801/BGC945, a FRα/β targeted folate-based thymidylate synthase inhibitor, showed remarkable clinical responses in FRα expressing ovarian carcinoma pa-
tients (23). These results would encourage preclinical evaluation of ONX-801/BGC945 in arthritic animal models as a specific FRβ targeted drug for activated macrophages (24). Another interesting path could be microRNAs conjugated to folic acid (Folamirs), internalized by FRα expressing tumour cells (25). Conceptually, Folamirs could also be internalized by FRβ expressing macrophages, opening opportunities to manipulate their function.

**Experimental therapies & imaging**

The arthritic rat model and macrophage folate PET imaging proved useful in guiding efficacy assessments of novel experimental anti-arthritic therapeutics. In this thesis it was investigated whether alkaline phosphatase (AP) could serve as a safe therapeutic for RA. As an ectophosphatase, AP is thought to have a gate-keeper function in the innate immune system by detoxifying (i.e. dephosphorylating) well-known inflammatory triggering moieties (ITMs) from external and internal sources such as lipopolysaccharides (LPS), CpG oligodeoxynucleotides (CpG-DNA) and nucleotide phosphates (ATP, ADP and AMP) (26). AP-mediated conversion of nucleotide phosphates into adenosine then conveys an anti-inflammatory effect. This mechanism of action has overlap with the mechanism of action of MTX, which involves the non-lytic extracellular release of adenosine and extrusion of adenine nucleotides (AMP, ADP, ATP) that are converted to adenosine by the action of ectophosphatases CD39 and CD73 on immune-competent cells (see Figure 9.1).

Recent evidence indicates that down-regulation of CD39 during MTX treatment inhibits local generation of adenosine, thereby conferring MTX resistance in RA (27). As a high capacity/low affinity system, therapeutic interventions of AP may provide a safety net in pathological conditions (e.g. exacerbations of RA) when low capacity/high affinity CD39/CD73 cannot cope with nucleotide phosphate substrates. One other important feature of AP is that it is also involved in maintaining physiological barriers, which hold relevance for controlling extravasation of pro-inflammatory immune cells. These broad mechanisms of action of AP as an anti-inflammatory protein could have contributed to the marked anti-arthritic effects in arthritic rats following administration of human recombinant placental AP (hRESCAP). Mechanistically, as indicated in Figure 1, AP could complement the mechanism of action of MTX and indeed AP+MTX combinations had additive effects and allowed MTX dose reductions to still elicit optimal anti-arthritic effects (Chapter 6). Future studies should define optimal schedules for AP and minimal dosages of MTX for best efficacy. In arthritic rats, repeated administration of AP as single agent and in combination with MTX was well tolerated and it is also anticipated that in humans, AP (as an endogenous protein) will have a broad safety window. Indeed, safety of repeated dosing of hRESCAP has been proven in human volunteer studies, but will need to be confirmed in RA patients prior to clinical evaluation and application in RA. At present, AP treatment is used in cardiothoracic surgery to suppress post-surgery inflammation by neutralizing harmful endotoxins and pro-inflammatory extracellular adenine nucleotides (28-30). Altogether, AP’s mode of action and feasibility of combining with MTX, deserves further investigations as a novel promising therapeutic modality for RA.

The study on F8-IL10, a new promising therapeutic agent for RA, demonstrated the
Figure 9.1: Mechanism of action of Alkaline phosphatase (Aphos) and Methotrexate (MTX). MTX can enter cells via one of 3 transport routes; the reduced folate carrier (RFC), proton-coupled folate transporter (PCFT) or folate receptor (FR). After cell entry, MTX is metabolized to polyglutamate forms (MTX-PG) by the enzyme folylpolyglutamate synthetase (FPGS). MTX-PGs have a prolonged cellular retention and inhibit folate-dependent enzymes in the ‘de novo’ purine synthesis pathway, which provokes a rise in intracellular adenosine concentrations. Adenosine exerts anti-inflammatory signalling effects. In addition, MTX treatment causes cell and oxidative stress which results in the non-lytic extracellular release of ATP and ADP. These phosphorylated adenine nucleotides, along with other inflammatory triggering moieties elicit a pro-inflammatory response. This is neutralized by the action of ectophosphatases CD39 and CD73 on the cell membrane. Under conditions of CD39 and CD73 down-regulation, or saturation of these systems, Aphos can serve as back-up system to dephosphorylate ATP and ADP in adenosine. Beyond its dephosphorylating capacity, Aphos also functions in maintaining membrane integrity to preserve physiological barriers.
value of PET imaging to gain insight in both targeting and in vivo biodistribution of F8-IL10 (Chapter 7). Targeting of radiolabelled F8-IL10 was noticed in (sub)clinically inflamed joints, confirming delivery to and localization of F8-IL10 in the target of interest. Unexpectedly, PET data also revealed high uptake in liver and to a lesser extent in spleen, which stresses the importance of using whole body PET in development of new therapeutic agents. Traditionally, in drug development, only pharmacokinetics (blood compartment) and histology studies in target tissues are included. However, information on uptake in other body compartments is often lacking and may be crucial for future applications in patients. Aspects as appropriate therapeutic dose and development of side-effects may be related to such findings. Therefore, including PET in future drug development strategies is strongly recommended. For clinical application of a drug, up-front identification of those patients who will benefit from the treatment will contribute to increase in treatment efficacy, avoiding side-effects in non-responders and reducing treatment costs by not treating non-responders. At any time after injection, the tracer concentration depends on delivery (i.e input function) together with regional tissue characteristics (flow, extraction and delivery). Ideally, a kinetic model should account for all physiological factors contributing to the total measured radioactivity signal in tissue. However, such a model would most likely be too complex and impractical to use, as it could result in statistically unreliable parameter estimates. In general, based on known information of the underlying physiology, a model is composed in such a way that it contains the minimum number of compartments (and parameters) to fully describe the kinetics of the tracer. In fact, in tracer kinetic analysis, the main challenge is to identify which kinetic model is most appropriate for a given research question. In addition, sensitivity of the model to (deviations from) underlying assumptions needs to be assessed. Once the tracer behaviour in tissue can be described by a robust kinetic model, scan, tracer administration and blood sampling protocols can be optimised and simplified for clinical use.
9.3 Key points of this thesis

- The macrophage PET tracer $^{18}$F-fluoro-PEG-folate, which targets Folate Receptor $\beta$ (FR$\beta$) on activated macrophages, constitutes an excellent diagnostic tool for non-invasive monitoring of arthritis and therapy response monitoring.

- FR$\beta$ constitutes a marker for pro-inflammatory monocytes in early, treatment-naïve, RA patients.

- FR$\beta$-positive macrophages are markedly increased in synovial tissue of RA patients with active systemic and local disease in the joints as compared with RA patients with milder disease activity and non-RA synovial tissues. Co-expression of FR$\beta$ with known M2-type macrophage markers (i.e. CD163 and CD206), but also with M1-like CD169 macrophages.

- The anti-inflammatory protein Alkaline Phosphatase (AP) appears a promising new therapeutic compound against arthritic conditions. AP, as single agent and in combination with methotrexate, demonstrated marked local and systemic anti-arthritis activity in arthritic rats.

- Imaging studies with radiolabeled F8-IL10, an antibody-guided delivery system for the anti-inflammatory cytokine IL10, demonstrates clear targeting in the (sub)clinically inflamed joints of RA patients.
References


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Curriculum vitae

Durga M.S.H. Chandrupatla was born on November 1st 1988 in Kakinada, India. After completing his primary and secondary education in Hyderabad, India he started studying Pharmacy in 2006 at the Osmania University, Hyderabad, India. His bachelor internship was performed at the Lalita College of Pharmacy under the supervision of prof.dr. Prakash Diwan on corticosteroid drug therapy combined with natural ingredients for inflammatory skin disease. During his bachelor studies, he participated actively in several national pharmaceutical conferences and won best poster and oral presentation awards. Thereafter in 2010, he started his master program on drug discovery and safety at the VU University Amsterdam, The Netherlands. He specialised in pharmaceutical pharmacology and toxicology, with a minor in science business. For his major project he investigated the molecular basis of resistance to an aminopeptidase inhibitor prodrug in myeloid leukemia under the guidance of prof.dr. Frits Peters and dr. Gerrit Jansen. He graduated in 2012. In 2013, he started his PhD in the group of dr. Gerrit Jansen at the Department of Rheumatology, VU University medical center Amsterdam.

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List of publications


