AALBORG UNIVERSITY

DENMARK

Regulation of pro-resolving biological systems in rheumatoid arthritis

investigations on the melanocortin system and resistin

Andersen, Marlene

Link to publication from Aalborg University

REGULATION OF PRO-RESOLVING BIOLOGICAL SYSTEMS IN RHEUMATOID ARTHRITIS

– INVESTIGATIONS ON THE MELANOCORTIN SYSTEM AND RESISTIN –

BY
MARLENE ANDERSEN

DISSERTATION SUBMITTED 2017

AALBORG UNIVERSITY
DENMARK

Regulation of pro-resolving biological systems in rheumatoid arthritis
-investigations on the melanocortin system and resistin-

by

Marlene Andersen

AALBORG UNIVERSITY
DENMARK

2017

.

Explore.
DREAM.
Discover.

Mark Twain

ENGLISH SUMMARY

Regulation of pro-resolving biological systems in rheumatoid arthritis -investigations on the melanocortin system and resistin-

Rheumatoid arthritis (RA) is a debilitating, autoimmune disease affecting about 1% of the population in western countries. In RA, auto-reactive T cells expand in the synovium, where they induce inflammation, leading to cartilage damage and bone resorption. There is no cure for RA. Despite biologic disease modifying agents, have improved disease outcome, severe rheumatoid arthritis can still cause physical disabilities and 40% of RA patients do not respond satisfactorily to treatment. Due to these shortcomings and the hazards of life-long immunosuppressive treatment, we attempted to explore the basis for curative induction of immune tolerance in RA.

The melanocortin system consists in five melanocortin receptors (MC1-5R) and their ligands, α-, β- and γ-melanocyte stimulating hormones (α-, β- and γ-MSH), and corticotropin (ACTH), all derived from a common precursor protein: pro-opio-melanocortin (POMC) peptide. The MCRs are present in many tissues including the central nervous system and control varied bodily functions, such as pigmentation, libido and hunger. MCR and POMC genes and proteins are also expressed in cells of the immune system, exerting anti-inflammatory, tolerance inducing and pro-resolving actions. MCR expression is stimulated by the melanocortins, the synthesis of which is upregulated by inflammatory cytokines and inhibited by TGF-β. Interestingly, in animal experiments stimulation of MCRs in effector CD4$^+$T helper (Th) cells and CD8$^+$ T cytotoxic (Tc) cells turned these into T cells with regulatory function.

In this study, we wanted to explore the possibilities of inducing immune tolerance to auto-antigens based on the melanocortin system in RA. To this end, we searched for signs of melanocortin signaling in leukocyte subtypes in RA by measuring changes in MC1-5R gene expressions as a result of adalimumab induced normalization of the cytokine environment.

Our findings unanimously pointed at regulated melanocortin signaling in leukocyte subsets in RA. Thus, in RA patients responding to TNFα inhibition, MCR gene expressions reacted with a differentiated fall, while increasing in a non-responding patient. Significant fall in MC2,3,4 and 5 R gene expressions was found in cytotoxic T cells and in B-cells. Strong associations between changes in disease driving cytokine and MCR gene expressions in cytotoxic T cells and helper T cells, indicated a central role for the melanocortin system in the disease process.

Resistin is a recently detected, cytokine with pleiotropic effects. Originally, resistin was found to be produced by murine adipocytes and induce resistance to insulin. In man, resistin is primarily synthesized by monocytes and exerts pro-inflammatory, fibrogenic and regulatory functions. In our RA patients, TNFαI resulted in a significant fall in resistin gene expression in monocytes and helper T cells. Change in gene expressions of resistin and TGF-β in monocytes correlated, supporting the perception of fibrogenic and regulatory roles.

In summary, our results point at a future role for both the melanocortin system and resistin in immune tolerance induction in RA.

DANSK RESUMÉ

Regulering af naturligt forekommende anti-inflammatoriske processer ved leddegigt, -undersøgelser af melanocortin systemet og resistin-.

Leddegigt (Reumatoid artrit (RA)) er en invaliderende autoimmun sygdom, der rammer omkring 1% af befolkningen i de vestlige lande. Ved RA findes auto-reaktive T-celler, der migrerer til leddenes synovium, hvor de inducerer inflammation der medfører bruskskader og knoglenedbrydning. Der findes ingen helbredende behandling for RA. I de seneste år er der kommet nye behandlinger med biologiske lægemidler, der reducerer forekomsten af led forandringer ved RA. Fyrre procent af RA patienter responderer ikke tilfredsstillende på de kendte behandlinger og der er stadig RA patienter, der får svære fysiske handicaps. Med dette in mente og de risici, der er forbundet med livslang immunsupprimerende behandling, har vi udforsket melanocortin systemet og resistin med henblik på at inducere immuntolerance.

Det humane melanocortin system består af 5 receptorer: melanocortin receptor 1-5 (MC1-5R) og disses ligander α-, β- og γ-melanocytstimulerende hormoner (α-, β- og γ-MSH), og corticotropin (ACTH); som stammer fra precursorn: pro-opio-melanocortin (POMC) peptid. MC1-5R findes i mange forskellige celler og væv, inklusiv centralnervesystemet. Melanocortin systemet har betydning for så forskellige funktioner som pigmentering, libido og sult. MC1-5R og POMC gener og proteiner er også udtrykt i immunsystemets celler, hvor systemet udøver sine anti-inflammatoriske, pro-resolverende og tolerance inducerende virkninger. MCR'erne opreguleres af melanocortinerne, hvis syntese stimuleres af pro-

inflammatoriske cytokiner og inhiberes af TGF-β. Interessant er det, at stimulering af MCR på CD4$^+$ T helper celler og CD8$^+$ T cytotoksiske celler, forvandler cellerne til T celler med regulatorisk funktion, in vivo.

I dette studie ønskede vi at udforske grundlaget for at inducere immun tolerance mod auto-antigener med hjælp af melanocortin systemet ved RA. Med dette mål for øje søgte vi efter beviser for melanocortin signalering i forskellige leukocyt subtyper ved RA. Dette ved at måle forandringer i MC1-5R genudtryk til følge af adalimumab induceret normalisering af cytokinmiljøet.

Vores resultater pegede på, at melanocortin systemet reguleres i forskellige leukocyt subtyper ved RA. Hos RA patienter, der responderer på TNFα inhibering faldt MCR genudtrykket, medens MCR genudtrykket steg hos non-responder. Vi fandt desuden at MC1-5R genudtrykkene reguleres nuanceret i forskellige typer af leukocytter. MC2-5R genudtrykket faldt signifikant i T cytotoksiske celler og B celler. Yderligere var der stærke korrelationer mellem forandringer i genudtrykket af sygdomsdrivende cytokiner og MC1-5R i T cytotoksiske celler og T helper celler, hvilket tyder på at reguleringen af melanocortin systemet er nært knyttet til sygdomsprocessen ved RA.

Resistin er et nyligt opdaget cytokin med pleiotrope virkninger. Oprindeligt blev resistin påvist i fedtceller fra mus, hvor resistin øgede insulinresistensen. Hos mennesket derimod, dannes resistin frem for alt i monocytter og udøver pro-inflammatoriske, fibrosedannende og immunregulatoriske virkninger. I vores studie resulterede TNFα inhibering i et signifikant fald i resistin

genudtrykket i monocytter og T hjælper celler. Vi fandt desuden en korrelation mellem forandringer i resistin og TGF-β genudtrykket i monocytter. Dette fund støtter hypotesen om, at resistin har fibrotiserende og immunregulerende egenskaber.

Sammenfattet peger vores resultater på en mulig rolle for både melanocortin systemet og resistin ved fremtidige forsøg til induktion af immun tolerance ved RA.

ACKNOWLEDGEMENTS

CONTENTS

ABBREVIATIONS

Abbreviations	Extended version
ACTH	Adrenocorticotropin
ACPA	Anti-citrullinated peptide antibody
bDMARD	Biologic disease modifying agent
cDMARD	Conventional disease modifying agent
cDNA	Complementary deoxyribonucleic acid
CD	Cluster of differentiation
CD4$^+$ Th ly	CD4+ T helper lymphocytes
CD8$^+$ T ly	CD8+ T cytotoxic
CD14$^+$	CD14+ monocytes
CD19$^+$ B ly	CD19+ B lymphocytes
CRP	C reactive protein
Ct	Cycle threshold
DAS28	Disease activity score 28
DC	Dendritic cell
DMARD	Disease modifying antirheumatic drug
DNA	Deoxyribonucleic acid
ESR	Erythrocyte sedimentation rate
Foxp3	Forkhead box p3
Ig	Immunoglobulin
IL	Interleukin
INF	Interferon
MCR	Melanocortin receptor
mRNA	Messenger ribonucleic acid
MSH	Melanocortin stimulating hormone
NFκB	transcription factor nuclear factor kappa B
NK	Natural killer
PBMC	Peripheral blood mononuclear cell
PCR	Polymerase chain reaction
POMC	Proopiomelanocortin
qRT-PCR	
RA	Rheumatoid arthritis
RF	Rheumatoid factor
RNA	Ribonucleic acid
RT-PCR	Reverse transcriptase-polymerase chain reaction
TCR	T cell receptor
TGF	Transforming growth factor
Th	T helper

TNF	Tumour necrosis factor
TNFR	Tumour necrosis factor receptor
Treg	CD4+CD25+ regulatory T lymphocytes

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which are referred to in the text by roman numerals (I-III), and three posters (IV-VII).

I. **Melanocortin 2, 3 and 4 receptor gene expressions are downregulated in CD8^{+} T cytotoxic lymphocytes and CD19^{+}B lymphocytes in rheumatoid arthritis responding to TNFα inhibition**. Nagaev I, Andersen M, Olesen MK, Nagaeva O, Wikberg J, Mincheva-Nilsson L and Andersen GN. Scand J Immunol 2017;86:31-9.

II. **Resistin gene expression is downregulated in CD4^{+} T helper lymphocytes and CD14^{+} monocytes in rheumatoid arthritis responding to TNFα inhibition.** Nagaev I, Andersen M, Olesen MK, Nagaeva O, Wikberg J, Mincheva-Nilsson L and Andersen GN. Scand J Immunol 2016;84:229-36.

III. **Melanocortin receptor gene expressions in CD56^{+} NK cells from two rheumatoid arthritis patients respond to TNFα inhibition by adalimumab..** Andersen M, Meyer MK, Nagaev I, Nagaeva O, Wikberg J, Mincheva-Nilsson L and Andersen GN (in manus).

Poster abstracts.

IV. **Resistin Gene Transcription Is Regulated in Adaptive and Innate Immunity in Rheumatoid Arthritis**. Andersen M, Kruse M, Nagaev I, Nagaeva O, Wikberg J, Mincheva-Nilsson L, Andersen GN. Rheum Dis 2016;75(Suppl2):904

V. **The Melanocortin System Is Responsive in Disease Driving Immune Cells in Rheumatoid Arthritis and May Offer A Pathway To Curative Treatment.** Andersen M, Kruse M, Nagaev I, Nagaeva O, Wikberg J, Mincheva-Nilsson L, Andersen GN. Ann Rheum Dis 2016;75(Suppl2):903

VI. **TNF-alpha Inhibition Normalizes Melanocortin Receptor Subtype 2, 3 and 4 Expression in CD8^{+}, CD14^{+} and CD19^{+} Leukocyte Subsets in Rheumatoid Arthritis**. Marlene Andersen, Michael Kruse Meyer, Ivan Nagaev, Olga Nagaeva, Jarl E.S. Wikberg, Lucia Mincheva-Nilsson and Grethe N. Andersen. Arthritis Rheumatol 2014; Suppl.:675-676.

VII. **Adalimumab (Humira®) normalizes melanocortin receptor subtype 2, 3 and 4 expression in CD8^{+}, CD14^{+} and CD19^{+} leukocyte subsets in rheumatoid arthritis.** Andersen M, Olesen MK, Nagaev I, Nagaeva O, Wikberg J, Mincheva-Nilsson L, Andersen GN. Scand J Rheumatol 2014;43:Suppl 127 p. 25-26.

Additional Scientific work not included in the PhD Thesis

1. **Effect of IL-6R Inhibition with Tocilizumab on the Proteome of Peripheral Blood Mononuclear Cells from a Rheumatoid Arthritis Patient.** Meyer MK., **Andersen M**, Bennike TB, Birkelund S, Andersen GN, Stensballe A. Proteomics & Bioinformatics 2015;8(12), 274–82.

2. **Effectiveness and drug adherence of biologic monotherapy in routine care of patients with rheumatoid arthritis: a cohort study of patients registered in the Danish biologics registry.** Jorgensen T, Kristensen L, Christensen R, Bliddal H, Lorenzen T, Hansen MS, Østergaard M, Jensen J, Zanjani L, Laursen T, Butt S, Dam MY, Lindegaard HM, Espesen J, Hendricks O, Kumar P, Kincses A, Larsen LH, **Andersen M**, Næser EK, Jensen DV, Grydehøj J, Unger B, Dufour N, Sørensen V, Vildhøj S, Hansen IMJ, Raun J, Krogh NS, Hetland M. Rheumatology 2015;54:2156-65.

3. **Effectiveness and Drug Adherence of Biologic Monotherapy in Danish Rheumatoid Arthritis Patients: A Cohort Study of Clinical Practice in the Danbio Registry**. Jorgensen T, Kristensen L, Christensen R, Bliddal H, Lorenzen T, Hansen MS, Østergaard M, Jensen J, Zanjani L, Laursen T, Butt S, Dam MY, Lindegaard HM, Espesen J, Hendricks O, Kumar P, Kincses A, Larsen LH, **Andersen M**, Næser EK, Jensen DV, Grydehøj J, Unger B, Dufour N, Sørensen V, Vildhøj S, Hansen IMJ, Raun J, Krogh NS, Hetland M. Ann Rheum Dis 2014;73(Suppl 2):613–4.

4. **Cellular Responses of IL6 Inhibition (Tocilizumab) in Rheumatoid Arthritis Using High-Accuracy Tandem Mass Spectrometry.** Meyer MK, **Andersen M,** Andersen GN, Stensballe A Arthritis Rheumatol 2014; Suppl:33-4.

5. **The Prevalence of Biological Monotherapy among Rheumatoid Arthritis Patients in Denmark: Results from the Danish Nationwide DANBIO Registry.** Joergensen TS, Kristensen LE, Lorenzen T, Jensen J, Zanjani L, Laursen T, Butt S, Dam MY, Lindegaard MH, Espesen J, Hendricks O, Kumar P, Kincses A, Larsen LH, **Andersen M,** Næser EK, Jensen DV, Grydehøj J, Unger B, Dufour N, Sørensen V, Vildhøj S, Hansen IM, Raun J, Hetland ML. Abstract, ACR/ARHP Annual Scientific Meeting 2013.

6. **Andersen M**. På ACR for første gang. Best Practice. Maj 2013

7. **Dermal melanocortin rebound in diffuse systemic sclerosis after anti-TGF beta1 antibody therapy.** Andersen GN, **Andersen M**, Nagaeva O, Wikberg JE, Mincheva-Nilsson L. Scand J Immunol 2012;478-82.

8. **The melanocortin system: a new and important actor on the scene of systemic sclerosis**. Andersen GN, **Andersen M**, Nagaeva O, Wikberg JE, Mincheva-Nilsson L. Arthritis Rheum 2011;63: S908-908.

1. REVIEW OF THE LITTERATURE

1.1. Rheumatoid Arthritis

1.1.1. Introduction

Rheumatoid arthritis (RA) is an autoimmune disorder characterized by chronic inflammation and loss of self-tolerance. In RA, the auto-reactive immune system mounts a cellular Th1 response, but with important participation of the humoral Th2 response, as RA is also characterized by the presence of auto-antibodies.

In western countries, RA affects about 1% of the population with a female:male ratio of approximately 4:1 (Silman 2002). RA is characterized by synovial inflammation and hyperplasia, cartilage and bone destruction (Koch 2007), typically located to the peripheral joints symmetrically, especially to the small joints of the hands and feet. Extra-articular organ involvement such as pleurisy, pericarditis, cutaneous rheumatoid nodules, interstitial lung disease, vasculitis and secondary Sjögrens syndrome as well as systemic manifestations such as cardio-vascular disease and fatigue are especially frequent in patients with auto-antibodies. Other features are morning stiffness and elevated inflammatory markers such as C-reactive protein (CRP) and erythrocyte sedimentation rate (ESR) as well as inflammation induced anemia and auto-antibodies, especially rheumatoid factor (RF) and anti-citrullinated protein antibodies (ACPAs).

The diagnosis of RA is based on the criteria settled by the American College of Rheumatology (ACR) in 1988 (Table 3.1) (Arnett 1988) and by ACR/European League Against Rheumatism (EULAR) in 2010 (Aletaha 2010). As 1988's ACR criteria included late disease manifestations *i.e*. joint erosions and

rheumatoid nodules (table 3.1), they were found insufficient in the diagnosis of early

RA. To meet the demands of modern early treatment, new diagnostic criteria were

established (Table 1.1).

Table 1.1. 1988 American College of Rheumatology classification criteria for rheumatoid arthritis.

Criterion	Definition
1. Morning stiffness	Morning stiffness in and around the joints, lasting at least one hour before maximal improvement
2. Arthritis of 3 or more joint areas	At least three joint areas simultaneously have had soft tissue swelling or fluid observed by a physician. (PIP, MCP, wrist, elbow, knee, ankle, and MTP joints)
3. Arthritis of hand joints	At least one area swollen in a wrist, MCP, or PIP joint
4. Symmetric arthritis	Simultaneous involvement of the same joint areas on both sides of the body
5. Rheumatoid nodules	Subcutaneous nodules, over bony prominences, or extensor surfaces, or in juxta-articular regions, observed by a physician
6. Serum rheumatoid factor	Demonstration of abnormal amounts of serum rheumatoid factor
7. Radiographic changes	Postero-anterior hand and wrist radiographs showing juxta-articular bony decalcification or erosions

Table 1.1: For classification purposes, a patient shall be said to have rheumatoid arthritis if he/she has satisfied at least 4 of these 7 criteria. Criteria 1 through 4 must have been present for at least 6 weeks.

Table 1.2. 2010 ACR/EULAR classifications criteria for RA

Classifications criteria for RA.	
A. Joint involvement (0-5)	
• **1 large joint**	0
• **2-10 large joints**	1
• **1-3 small joints**	2
• **4-10 small joints**	3
• **> 10 joints (at least one small joint)**	5
B. Serology (0-3)	
• **Negative RF and negative ACPA**	0
• **Low-positive RF or low-positive ACPA**	2
• **High-positive RF or high-positive ACPA**	3
D. Duration of symptoms	
• **< 6 weeks**	0
• **≥ 6 weeks**	1
D. Acute-phase reactants (0-1)	
• **Normal CRP and normal ESR**	0
• **Abnormal CRP or abnormal ESR**	1

A score ≥ 6 points is required for classification as definite RA. RF = rheumatoid factor. ACPA = anti-citrullinated protein antibodies. ESR = erythrocyte sedimentation rate. CRP = C-reactive protein

1.1.2. Etiology and Pathogenesis

RA results from a complex interaction between genes and environment, leading to a breakdown of immune-tolerance. 50 % of risk of developing RA is attributable to genetics. Especially factors influencing antigen presentation, T-cell and B-cell activation are involved. The antigen binding major histocompatibility complex (MHC)II, expressed on antigen presenting cells (APCs) consists in an alpha- (DRA) and a beta chain (DRB). Out of hundreds of different HLADRB1 alleles available,

the DRB1 chains that increase the risk of RA, share a sequence of amino acids in the antigen binding groove, the so-called shared epitope. Although about 100 single nucleotide polymorphisms (SNPs) in the genome associate with RA, only SNPs in the protein tyrosine phosphatase, non-receptor type 22 (PTPN22) gene is related to disease activity (Balsa 2010). PTPN22 affects the responsiveness of T-and B-cell receptors (Bottini 2014).

Even environmental factors, especially smoking, influence the risk. Smoking may promote citrullination of lung proteins, leading to the synthesis of antibodies against citrullinated proteins (ACPA)s, that cross-react with similar proteins in the joints (Ytterberg 2015). In line with this, MHCIIs with shared epitope have been suggested to favor the presentation of citrullinated proteins, leading to auto-immunity (Hill 2003, Auger 2005).

The hallmark of RA joint involvement is synovial cell hyperplasia and proliferation forming an invasive pannus at the cartilage-bone border, resulting in bone destruction. Prior to this, however, APCs *i.e.* dendritic cells, macrophages and B-cells by presenting self-antigens have activated T and B cells. Consequently, CD4^{+} and CD8^{+} T cells are thought to invade the synovium and through the synthesis of interferon gamma (IFNγ) and IL-17 attract and activate monocytes/macrophages, promoting synovial cell transformation into invasive fibroblast like synoviocytes as well as osteoclastogenesis (Choy 2012). Synovial germinal centers may be formed, where further activation of T- and B-cells can take place (Takemura 2001).

Activated macrophages produce large quantities of inflammatory cytokines TNF-α, IL-1 and IL-6 that maintain inflammation as well as osteoclastogenesis. Fibroblast-like synoviocytes beyond producing inflammatory cytokines, release prostaglandins and matrix metalloproteinases (MMPs), promoting bone degradation and cellular invasion. Attracted granulocytes release their granule content of tissue damaging enzymes, especially myeloperoxidase (MPO) and MMP9. Furthermore, NETS produced by neutrophils may represent a source of epigenetically modified auto-antigens stmulating the production of ACPAs (Corsiero 2016). Invading B-cells contribute to the inflammatory process by the synthesis of RF and ACPA forming large immunocomplexes,that further stimulate cytokine production (Choy 2012).

1.1.3. Measurement of Disease Activity and Severity

1.1.3.1 Health Assessment Questionnaire for Rheumatoid Arthritis

The Health Assessment Questionnaire (HAQ), introduced in 1984 (Meenan 1984) ascertains disease activity based on the patients´ own experience. The HAQ assesses 20 difficulties in daily living on a 4-point Likert scale and groups these into 8 functional categories. Each category gives a single score equal to the maximum value of their component activities (0, 1, 2, or 3). The higher the score, the more compromised function. The HAQ has been found a reliable predictor of long-term outcomes, i.e mortality, work disability and economic loss (Leigh 1991, Young 2000, Maska 2011).

1.1.3.2 Disease Activity Score 28

The Disease Activity Score 28 (DAS28), developed by EULAR, measures disease activity in RA. DAS28 ranges from 2.0 to 10.0, higher scores meaning more active disease (Prevoo 1995). A value below 2.6 is consistent with remission and above 5.1 with high disease activity. Twenty-eight is the number of joints evaluated as to tenderness (TEN28) and swelling (SW28). The patient estimates global health on a visual analogue scale (VAS global) between 0 and 100, where 100 is the worst imaginable.

DAS28 is calculated based on CRP as follows:

DAS28-CRP = 0.56 X $\sqrt{}$(TEN28) + 0.28 X $\sqrt{}$(SW28) + 0.36 X ln(CRP+1) + 0.014 X VAS Global + 0.96

The formula above is used in our studies and in the Danish database "Danbio" including patients receiving treatment with biologic disease modifying drugs (bDMARDs). DAS28 is neither an exact nor exhaustive measure of disease processes as synovitis in the feet and ankles is not included and chronic pain as well as joint tenderness is weighted twice as high as synovitis. Yet, DAS28(CRP) is a strong predictor of disease progression.

1.1.4. ACPA

In 1998 for the first time, antibodies against proteins containing posttranslationally modified arginine, namely citrulline, were defined in RA. The antibodies related to RA with a good sensitivity and a very high specificity (Schellekens 1998). Because

of indications that the well-known anti-keratine (AKA) and anti-perinuclear factor antibodies (AFPA) were targeting citrullinated fillagrin in keratinocytes, antibodies were searched for by means of a library of synthetic citrullinated fillagrin derivatives. It was demonstrated that the presence of anti-cyclic citrullinated protein (anti-CCP) antibodies and AKA/AFPA coincided in RA patients (Schellekens 1998). The former tests for AKA/AFPA were very inconvenient and therefore not in common use. Because AKA/AFPA had been demonstrated in preclinical RA (Kurki 1992), a pathogenetic role for anti-CCP antibodies in RA was suspected. A later examination of a Swedish cohort of RA patients, who had had blood samples taken several years prior to disease debut as part of a regional health program, supported the findings by Kurki (Rantapaa-Dahlqvist 2003).

In the following years antibodies to a long array of citrullinated proteins such as vimentin, enolase, fibrinogen derivatives etc. were discovered and the name changed to anti citrullinated protein antibodies (ACPAs). In recent time a pathogenetic role for ACPAs has been searched for. Evidence has accumulated that several factors harmful to bodily tissues, such as smoking (Mahdi 2009), silica- (Stolt 2010) and dust inhalation (Too 2016) as well as high physical workload (Zeng 2017) promote citrullination of proteins in the lungs and other organs, functioning as antigenic epitopes for the development of ACPAs. These findings have led to the theory that immune cells recognizing citrullinated proteins in the lungs migrate to the joints, where they are reactivated by local citrullinated epitopes, resulting in a fierce immune reaction, leading to the well-known bone destructive synovitis found in RA (Snir 2010).

1.1.5. Pharmacological Treatment of Rheumatoid Arthritis

There is no cure for RA. The goal of treatment is to achieve remission, minimizing joint damage, and enhancing physical function and quality of life. The strategy includes early diagnosis, the use of DMARDs and the aim "treat to target", *i.e* the goal of the treatment is no arthritic joint.

Four classes of drugs are used: non-steroidal anti-inflammatory drugs (NSAIDs), corticosteroids, conventional (c)DMARDs and bDMARDs. NSAIDs and corticosteroids act immediately, while the clinical effects of DMARDs appear after several weeks. Glucocorticoids are used systemically and/or intra-articularly for flares and as a "bridging" therapy.

Methotrexate (Mtx) is the cornerstone cDMARD and is recommended as first line therapy in moderate to high disease activity. Improvement is expected in 6-12 weeks. Methotrexate prevents pyrimidine and purine syntheses and consequently inhibits proliferation of immune cells. Other cDMARDS used are: sulfasalazine, leflunomide and antimalarials.

Biological DMARDS are increasingly used in the treatment of RA resistant to cDMARDs. They target specific steps in the inflammatory process and rapidly suppress disease activity. Of bDMARDs, tumor necrosis factor alpha (TNFα) inhibitors (I) were invented first. Five different TNFαI are available: etanercept, infliximab, adalimumab, certolizumab and golimumab. Etanercept is a fusion protein, joining the TNF receptor (R) to the Fc part of IgG1. The other TNFαIs are monoclonal antibodies (mAbs), -the newest of them humanized- that

neutralize soluble and membrane bound TNF, preventing interaction with the TNFRs. Other bDMARDs act on other targets. Tocilizumab binds IL-6R, rituximab causes B-cell depletion by binding to CD20, abatacept, a fusion protein between CTLA-4 and the Fc region of IgG1, prevents co-stimulation of T-cells. Lately, small molecule, janus kinase (JAK) inhibitors, such as tofacitinib, have been found effective in RA (Tanaka 2012).

1.1.5.1 Adalimumab.

Adalimumab is the TNFαI used in our study. Adalimumab (Humira®) is a recombinant, fully humanized IgG1 mAb specific for human TNFα. It is approved by the European Medicines Agency and U.S. Food and Drug Administration, for treatment of moderately to severely active RA.

Adalimumab blocks the interaction of TNF-α with its cell surface receptors TNFR1,2 by binding to soluble and membrane-bound TNFα. Adalimumab blocks TNFα signaling, which via a series of events involving several adaptor proteins activates the phosphorylation of inhibitory nuclear factor kappa B (IκB), leading to its degradation and the release of transcription factor NFκB, which then translocates to the nucleus and initiates the inflammatory cascade by transcribing the genes of inflammatory cytokines, chemokines, adhesion molecules, inducible nitric oxide synthase, metalloproteinases a.o. (Gravestein 1998). The aim of adalimumab treatment is to achieve physiological levels of TNFα in blood and inflammation sites, as abnormally low levels favor infections and tumor cell growth.

Forty mg adalimumab given as a subcutaneous injection every other week should induce a rapid reduction in synovitis. Absorption and distribution of

adalimumab are slow, resulting in a smooth concentration-time profile (Nestorov 2005). The effects of adalimumab and two other TNF-α inhibitors *i.e.* etanercept and infliximab in combination with methotrexate or leflunomide, have been found to be equal (De Stefano 2010).

1.1.6. Immune Cells in RA

1.1.6.1 T helper Cells, -CD4$^+$ Th1, 2, 17, 22 and CD4$^+$CD25$^+$Regulatory T cells-

T helper (Th) cells express the surface protein CD4 in close proximity to the T cell receptor (TCR) and are referred to as CD4$^+$ Th cells. The CD4 glycoprotein is necessary for binding of the TCR to the MHCII antigen complex on the surface of APCs and for the amplification of the signal created thereby. CD4$^+$T cells develop in the thymus and migrate to peripheral tissues. Encounter with their specific antigen activates CD4$^+$ T cells to become cytokine secreting effector Th or T regulatory (Treg) cells. Helper T cells are named so, because one of their main roles is to activate the immune cells, most appropriate, to kill the infectious intruder in question, by means of cytokine secretion. The importance of functioning CD4$^+$ Th cells has been made obvious by HIV infections, in which CD4$^+$ Th cells are depleted. Subtypes of CD4$^+$Th cells may be characterized by their cytokine profile and their expression of specific transcription factors and homing receptors.

In 1986 the Th1/Th2 paradigm was proposed based on the findings in mice and is used to explain how the host elicits an adequate adaptive immune response (Mosmann 1986). Thus, Mosmann et al found that CD4$^+$ Th cells can be divided into two distinct lineages based on their cytokine profile: Th1 cells, which

by producing interferon gamma (IFNγ) activate the cellular immune response, including monocytes and cytotoxic CD8^{+} T cells, which kill intracellular pathogens and the Th2 cells, which by the production of IL-4 stimulate B-cell proliferation, maturation, immunoglobulin production and Ig class switch, in order to clear extracellular pathogens.

In recent years, Th cell types with other functions have been identified: Th17, regulatory T cell (Treg), Th22, Th9, and T follicular helper (Tfh) cells. Th17 cells support cellular immunity by secreting IL-17, Tregs support immune tolerance by the secretion of IL-10 and by the secretion and expression of membrane bound TGFβ.

Th17 cells were first characterized in 2005-2006 (Harrington 2005, Betteli 2006) as IL-17 producing CD4^{+} Th cells with tissue injuring abilities in autoimmune diseases. About the same time point regulatory T cells (Tregs) were defined as CD4^{+}CD25^{+}Foxp3^{+} Th cells with tissue preserving and immune tolerance inducing properties (Sakaguchi 1995, 2003). Both cell types demanded the presence of TGFβ for their differentiation. However, while both TGFβ and IL-6 were needed for the development of Th17 cells, Treg differentiation was inhibited by the presence of IL-6. In this way a dichotomy between the generation of autoimmunity and tissue harming Th17 cells and immune tolerance inducing Tregs cells was demonstrated. As the only CD4^{+} T cell, Tregs express the transcription factor Foxp3. Experiments have shown that induction of Foxp3 expression and IL-17 production in T cells are mutually exclusive (Betelli 2006).

For more than 25 years ago, several researchers concluded that Th1 cells outnumbered Th2 cells especially in the synovium, but also in the blood of patients with RA. By cloning synovial membrane and synovial fluid CD4^{+}T cells and examining their cytokine profile, almost all were found to produce IFNγ, not IL-4 (Miltenburg 1992, Quayle 1993). These early findings still hold as in a recent work upon the production of granulocyte-macrophage stimulating growth factor (GM-CSF) by synovial and peripheral blood T cells in RA, the number of IFNγ producing Th1 cells dominated convincingly over Th2, Th17 and Th22 cells (Yamada 2017).

For the last decade the Th1/Th2 paradigm in RA has been challenged by the Th17/Treg dichotomy. There seems to be no doubt that Th17 cells play a pathogenetic role in RA. Thus, an increased number of Th17 cells in peripheral blood and especially in synovial fluid correlating to DAS28 and CRP was demonstrated in RA, with an increased ratio of Th17/Th1 cells in peripheral blood and in synovial fluid of 0.10 and 0.40, respectively (Leipe 2010). Furthermore, Th17 cells have been found to activate synovial fibroblasts to produce matrix metalloproteinases (van Hamburg 2011). IL-17 treated fibroblast like synoviocytes may furthermore upregulate RANKL (also called osteoclast differentiation factor) synthesis, thereby promoting osteoclastogenesis from monocytes and subsequent bone destruction (Kim 2015).

The human immune system is potentially very harmful, and needs to be controlled by suppressor- cells and/or biologic regulatory systems. Absence or

malfunction of Tregs resulting in the deleterious immunodysregulation, polyendocrinopathy, enteropathy X-linked (IPEX) syndrome, consisting in a myriad of auto-immune disorders, clearly demonstrates their necessity. In RA, several researchers have found Tregs enriched in the synovial membrane (Cao 2003, Moradi 2014,), while it is uncertain if the number Tregs is within normal limits in peripheral blood. Different mechanisms of malfunction of Tregs in RA have been proposed, like epigenetic changes of the CTLA-4 molecule, rendering suppression of effector T cells defective (Cribbs 2014).

Lately, increased plasticity of Th17cells, meaning that Th17 cells transform into non-classical, IFNγ producing, Th1 cells was detected in RA synovium. Furthermore, it was proposed that these ex-Th17 cells were resistant to suppression by Tregs (Kotake 2017, Basdeo 2017).

1.1.6.2 T Cytotoxic Cells

T cytotoxic(c) lymphocytes express the surface protein CD8 and are referred to as CD8^{+} Tc cells. CD8^{+} Tc cells have primarily been perceived as effector cells in cell-mediated immunity, with the ability to kill infected cells by means of granzyme B and perforin. However, Tc cells also seem to be involved in the pathogenesis of autoimmune diseases.

CD8^{+} Tc cells, upon recognition of their specific antigen presented by the MHCI of infected cells, undergo expansion and mediate pathogen clearance by killing infected cells and secreting effector cytokines, which activate monocytes/macrophages. After this, the majority of CD8^{+} Tc cells dies by apoptosis,

leaving 5-10% behind as memory cells. IFN-γ is the key effector cytokine produced by CD8^{+} T cells. IFN-γ increases the sensitivity of infected cells to apoptosis.

CD8^{+} Tc cells may play a greater role in RA pathogenesis and maintenance of RA than has been recognized. Although the principal purpose of CD8^{+} T cells is to protect the host from non-self and altered self, growing evidence implicates CD8^{+} Tc cells in the pathogenesis of autoimmune disorders, including RA, type 1 diabetes, systemic lupus erythematosus, multiple sclerosis, and inflammatory bowel disease (Kang 1999, Santamaria 2003, Babbe 2000, Brimmes 2005).

In the synovium, CD8^{+} Tc cells have been found prior to overt arthritis in persons producing RF and/or ACPA and have therefore been linked to the pathogenesis of RA. The presence of CD8^{+} Tc cells in the synovium correlated to the presence of ACPA in the blood and was more common in persons with more than one type of ACPA (de Hair 2014). Others found that IFN-γ production by circulating CD8^{+} Tc cells correlated to disease activity measured by DAS28 (Carvalheiro 2015). These findings indicate that not only MHCII restricted CD4^{+} Th cells play a role in the pathogenesis and maintenance of RA.

Interestingly, the promptness of IFN-γ synthesis by CD8^{+} Tc cells in virus infections decides which CD8^{+} Tc cells will dominate the later memory cell cohort (Liu 2004). Applying this finding to RA, the CD8^{+} Tc cells most suited for IFN-γ production upon auto-antigen recognition might dominate the memory Tc cell cohort and assure the maintenance of auto-immunity.

1.1.6.3 B Cells

B cells are derived from multipotent stem cells in the bone marrow. Before the B cells enter the circulation, they undergo several maturation stages and express the B cell receptor (BCR). Although B cells reacting to self-antigens are cleared by clonal deletion in the bone marrow, auto-reactive, however often anergic, B-cells, may enter the periphery, where they will die. When an antigen is presented on the BCR to CD4^{+} T cells, dependent on antigen type and cytokine milieu, a Th2 response might be elicited, resulting in IL-4, IL-6 and IL-5 release, which instigates naïve B cells to proliferate and differentiate into antibody secreting plasma cells, affinity maturation and later on memory cell status.

However, human B-cells may play several roles. B-cells are central to adaptive immunity, conducting several tasks, such as antigen presentation, T-cell activation, antibody production, cytokine synthesis, immune tolerance induction and ectopic germinal center establishment. B cells express several membrane molecules, among these CD19, which enhances the susceptibility to antigen stimulation by assembling with the BCR.

There are several indications of a pathogenetic role for B-cells in RA. Thus, the removal of self-reactive B cells seems inappropriate in RA (Samuels 2005). Moreover, levels of circulating B-cell activating factor (BAFF), which stimulates the survival of auto-reactive B cells and their production of RF and ACPA, are elevated (Moura 2011). Furthermore, B cells activate T-cells in the germinal centers of RA synovium (Vidard 1996)

Considering cytokine synthesis, mRNA for several cytokines, such as IFN-γ, TNF-α, IL-6, IL-10, IL-2, BAFF, RANKL and others have been demonstrated in human B-cells (Yeo 2011).

As to tolerance induction, IL-10 and/or TGFβ producing regulatory B cell populations have been demonstrated in mice and humans (Iwata 2011). Cui et al found that the number of potentially IL-10 producing CD19$^+$CD5$^+$CD1dhi B cells is low in RA and correlates inversely with DAS28 (Cui 2014).

1.1.6.4 Natural Killer Cells

In 1975, NK cells were identified in mice as cells capable of killing leukemia cells without prior sensitization to antigens (Kiessling 1975). In man, natural killer cells are large, granular lymphocytes of innate immunity, that develop in the bone marrow and react to stressed, infected or cancer transformed cells. They are identified by the expression of the surface protein: neural cell adhesion molecule (CD56) and lack of the T cell co-receptor CD3. NK cells are equipped with both activating and inhibitory receptors, which interact in an intricate manner, including calibration to constitutional MHCI expression in different individuals and so-called licensing (Kim 2005). Activating receptors recognize signs of cell damage and/or infection, such as Fas ligand (L), viral ligands, tumor cell ligands and decrease in MHCI expression. NK cells express several inducers of apoptosis such as perforin and FasL. Moreover, Fc receptor expression allows NK cells to participate in antibody-dependent cell-mediated cytotoxicity (ADCC). NK cells are found in the lymphoid system, lung, liver, bone marrow, epithelial linings and other tissues. They

account for 10-15 % of lymphocytes in the blood. Their most important functions are cytotoxicity and cytokine production. Constitutionally high NK cell activity, due to efficient NK cell receptor interplay, may predispose to autoimmune disease, for example RA, by increased release of auto-antigens from killed cells (Yen 2001).

NK cells may, through their ability to produce cytokines, direct the immune response. However, the role of NK cells in RA is controversial, and it is not established whether NK cells contribute to disease development or act to protect from tissue damage. Thus, Gulan et al reported an increased frequency of perforin expression as well as number of CD56$^+$ NK cells in the blood, synovial fluid and synovium in RA. These cells had high perforin content per cell, except for NK cells in the synovial tissue (Gulan 2003). However, others have counted a low number of CD56$^+$ NK cells in the blood of patients with active RA, increasing upon treatment with the bDMARD rituximab and correlating inversely with measures of disease activity (Lurati 2012). These findings are supported by a recent report, describing a lower number of circulating 56$^+$ NK cells in RF and/or ACPA positive RA patients compared to seronegative RA. Moreover, NK cell apoptosis could be elicited by triggering of the FcγRIII, pointing at autoantibodies as the cause of low NK cell count (Chalan 2016).

2. BIOLOGIC SYSTEMS WITH IMMUNE TOLERANCE INDUCING POTENTIAL

2.1. The melanocortin system

2.1.1. The melanocortin 1-5 receptors

The first melanocortin receptor (MC1R) was discovered in 1992 (Chhajlani 1992, Montjoy 1992), in the following years four more MCRs were sequenced (Chhajlani 1993, Schiøth 1996, Gantz 1993). MCRs are seven-helix, small transmembrane G protein-coupled receptors, which activate the adenylate cyclase pathway, resulting in increased levels of intracellular cAMP. Moreover, MCR signaling results in inhibition of the degradation of IκBα, leading to less NFκB translocation to the nucleus and downregulation of the transcription of genes in the inflammatory cascade (Mandrika 2001). Each receptor is the product of a small, intronless gene.

The MCRs are widespread in human tissues and cells, including the brain stem and influence so different physiological functions as skin pigmentation, corticosteroid synthesis, inflammation, hunger, sexual arousal and exocrine glandular secretion (Wikberg 2000, 2008 (reviews)). The melanocortin receptor 2 (MC2R) only binds ACTH and is the adrenal gland ACTH receptor, but is also found in many immune cell types (Neumann-Andersen 2001, Andersen 2005).

2.1.2. Melanocortins

The ligands of the MCRs are the melanocortins, all derived from pro-opio-melanocortin (POMC), which is split into α-, β-, and γ- melanocyte stimulating hormone (MSH) and ACTH by protein convertases 1 and 2. (Figure 2.1).
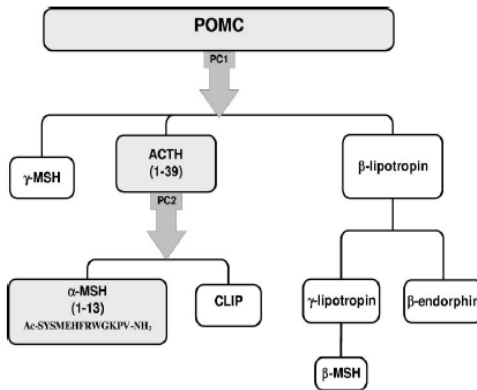
Figure 2.1. Melanocortin peptides, ACTH and α-, β-, and γ-MSH, derive from post-translational processing of POMC.

While MC2R binds ACTH only, the remaining MCRs bind ACTH and MSHs with differential affinity (table 2.1). The differences in affinity have been used to pinpoint, which MCR is responsible for cell functions, elicited by melanocortin stimulation.

Table 2.1. Affinity of the melanocortins to melanocortin receptors.

Receptors	Bindings affinity for melanocortins
MC1R	A-MSH = ACTH > β-MSH > γ-MSH
MC2R	ACTH
MC3R	γ-MSH = ACTH ≥ α-MSH = β-MSH
MC4R	A-MSH = ACTH > β-MSH > γ-MSH
MC5R	A-MSH > ACTH > β-MSH

2.1.2.1 Anti-inflammatory Effects of MCR stimulation

Melanocortin receptor expression has been reported in a wide range of human immunologically active cells, that is in neutrophils (Catania 1996), macrophages (Bhardwaj 1997), dendritic cells (Renalls 2010), CD19^{+} B-lymphocytes, CD8^{+}

cytotoxic T-cell and CD56^{+} NK cells (Neumann-Andersen 2001, Andersen 2005, Loser 2010), as well as in endothelial cells (Kalden 1999) and fibroblasts (Böhm 1999).

The actions of the melanocortin system in immune cells are predominantly anti-inflammatory, pro-resolving and immune tolerance inducing. It has been under debate, which MCR is the most important mediator of these effects. Thus anti-inflammatory effects mediated by MC1R have been reported in several human immune cell types. For example, in a MC1R expressing macrophage cell line, stimulation with α-MSH decreased lipopolysaccharide (LPS) induced TNFα synthesis (Taherzadeh 1999). Under similar conditions, NFκB nuclear translocation and nitrogen oxide (NO) release was reduced (Mandrika 2001). Furthermore, in a LPS stimulated endothelial cell line, α-MSH reduced the expression of adhesion molecules significantly (Kalden 1999). Several steps in the inflammatory process, *i.e.* inflammatory cytokine production, vasodilation and leukocyte migration were thus inhibited by MC1R signaling.

Lately, focus has been on the anti-ischemic effects of melanocortin signaling in models of ischemic reperfusion injury (IR) (Giuliani 2012). In an IR model in MC3R null mice, treatment with a selective MC1R agonist diminished tissue injury by decreasing leukocyte adhesion and migration (Leoni 2010). The aim of these studies was to develop selective MSH analogues to treat/prevent tissue injury in cardio- and cerebrovascular catastrophes.

Signaling through the MC3R has also been reported to elicit anti-inflammatory effects. In an animal model of urate induced arthritis, neutrophil influx, increase in joint size, synovial fluid IL-1β and IL-6 synthesis were inhibited by injection of ACTH and γ-MSH, which is semi-selective for MC3R (Getting 2002). In a model of peritonitis in a mouse strain with naturally defective MC1R similar results were achieved (Getting 2003). The anti-inflammatory, tissue-preserving effects of MC1R and MC3R stimulation were found equal in a model of IR injury, as the response to treatment with a synthetic, MC3R selective γ-MSH analogue resembled the results achieved by the stimulation of the MC1R (Leoni 2008).

2.1.3. Immune Tolerance Induction by Melanocortin Signaling

2.1.3.1 T-cells

Evidence is accumulating, that melanocortin system signaling is able to induce immune tolerance. In early experiments, it was shown that MC1R signaling decreases CD86 expression in human monocytes (Bhardwaj 1997). As CD86 co-stimulation is necessary as the second signal in T cell activation, a reduction in CD86 number might impede T cell auto-reactivity.

Most interestingly however, it was found that MC5R signaling could induce immune tolerance in a murine model of auto-immune uveoretinitis. In this model, treatment of IFNγ producing effector CD4^{+} Th cells reactive to retinoid binding peptide with α-MSH in the presence of auto-antigen presenting APCs, transformed the effector CD4^{+}Th cells into TGFβ producing CD4^{+}CD25^{+} Tregs

(Taylor 2001). This transformation was shown to proceed through MC5R signaling. Equally promising as to the induction of tolerance to auto-antigens are the findings in a study on murine experimental autoimmune encephalomyelitis (EAE), a model of multiple sclerosis. In this study myelin protein-specific T cells by α-MSH gene bearing virus transfection were rendered transgenic for α-MSH, thereby manipulated to produce α-MSH. This maneuver caused transformation of the myelin protein-specific effector Th1 into Treg cells, producing significantly less IFNγ and IL-2 and significantly more TGFβ. Also, injection of the α-MSH transgenic myelin protein-specific T cells resulted in far less EAE than the injection of their non-transgenic counterpart (Han 2007).

Furthermore, even allergen sensitized CD8^{+} Tc cells seem amenable to immune tolerance induction by α-MSH treatment as shown in murine models of contact hypersensitivity dermatitis (Grabbe 1996, Loser 2010). In one experiment, CD8^{+} Tc cells sensitized to 2,4-dinitro-fluorobenzene (DNFB) were treated with α-MSH in the presence of DNFB pulsed dendritic cells and transferred to mice with DNFB specific contact dermatitis. Upon cutaneous application of DNFB, these mice developed less ear swelling than controls (Loser 2010). Furthermore, unlike CD4^{+} T cells, CD8^{+}Tc cells increased the expression of MC1R upon α-MSH stimulation (Loser 2010).

2.1.3.2 B Cells

The reported effects of melanocortins on B cells seem contradictory, thus ACTH stimulated the functions of human tonsillar B-cells in the presence of IL-2 (Alvrez-Mon 1985). In a murine pro-B-lymphocyte cell line, NDP-MSH, a potent synthetic α-MSH analogue, stimulated JAK/STAT signaling and cell growth, probably via MC5R (Buggy 1998).

Recently however, in a murine SLE model, NDP-MSH treatment reduced IgG1, IgG2a levels and ANA. In the glomeruli, IgG deposits, cellularity and expression of inducible nitric oxide synthase diminished, pointing at a suppressive effect of melanocortins on auto-reactive B cell functions (Botte 2014).

2.2. Resistin

Resistin is a peptide discovered in 2001 with hormone and cytokine properties. In mice, resistin is secreted by adipocytes and was named for its ability to induce insulin resistance in these (Steppan 2001). The origin and effects of resistin are however controversial in humans. Thus, resistin gene expression was found absent in human adipocytes, but could be demonstrated in monocytes (Nagaev 2001). In human peripheral blood monocytes (PBMCs), expression of resistin mRNA was markedly increased by the pro-inflammatory cytokines IL-1, IL-6, TNFα and by LPS (Kaser 2003). In turn, resistin, was found to exert pro-inflammatory effects such as upregulation of the gene expression and release of TNFα, IL-1β and IL-6 in human PBMCs, via NFκB activation (Silswal 2005, Bokarewa 2005). Furthermore, resistin was found able to induce arthritis in mice (Bokarewa 2005) and levels were found elevated in RA synovial fluid, correlating with joint destruction (Bokarewa 2005, Senholt 2007). TNFα inhibition has been demonstrated to decrease serum resistin levels in RA patients (Gonzales-Gay 2008).

The presence of resistin mRNA and protein has scarcely been examined in other cell types than monocytes or PBMCs. However, in a study on differences in grade of inflammatory reaction between lean and obese asthma patients, resistin release by CD4^{+} T cells was demonstrated (Rojas-Dotor 2013). In a study on frequencies of CD4^{+} Th1, 2, 17, 22 and Treg subtypes in the healthy human intestinal tract, resistin gene expression was found to be enriched together with CD4^{+} Th17 cells in the cecum (Wolff 2012). In a co-culture of CD4^{+} T cells and DCs stimulated with resistin, gene expression for markers of Tregs, *i.e.* TGFβ,

CTLA-4 and FoxP3 increased as did the number of CD4$^+$CD25$^+$ Tregs (Son 2010).

These findings point at a role for resistin in immune response direction and immune

tolerance induction.

3. AIM OF THE INVESTIGATIONS

The overall aim of the study was to explore whether immune tolerance inducing, pro-resolving, anti-inflammatory biological systems are activated in rheumatoid arthritis and amenable to changes in the cytokine environment.

The more specific aims were to

1. examine the impact of TNFαI induced normalization of the cytokine milieu in RA on MC1-5R gene expression in order to explore the possibilities of influencing melanocortin system activities in RA.

2. examine the gene expression of resistin in leukocyte subsets not formerly examined, to achieve an improved understanding of resistin potentials.

3. search for regulated MC1-5R gene expression in NK cells, as the functions of NK cells in RA are uncertain and MC1-5R may constitute a new pathway to treatment.

4. METHODOLOGY

4.1. Patients and Methods

The present investigations were performed on lymphocyte subsets donated by patients with active RA. All patients gave their informed consent, orally and in writing. The study was approved by the Ethics Committee of Northern Jutland, Denmark, (N20100060).

At the time of inclusion, the diagnosis was secured by clinical estimation of number of swollen and tender joints, standard blood samples including RF, ACPA and ANA. X-rays of the wrists, hands, feet and chest were performed.

Patients with active RA, starting adalimumab treatment, were consecutively included according to the advice of the Danish 'Council for Expensive Hospital Medicine', which recommends bDMARD to RA patients with DAS28 (CRP) ≥ 3.2 for more than 3 months, not responding to two cDMARDs or DAS28 (CRP) >5.1 at two consecutive consultations. Seven patients, six females and one male, with definite RA according to the 2010 ACR/EULAR criteria (Alehtaha 2010) were included. Their mean age was 45.4 ± 7.5 (mean $\pm$ SD) years and mean disease duration 4.0 years (1–14 years). Six patients produced RF and ACPA. Median DAS28 (CRP) was 5.1 (4.3–6.7) [interquartile range (IQR)]. Patients with cancer, congestive heart disease functional class NYHA III-IV, and other severe diseases were excluded.

Peripheral blood samples were collected at two time-points: just before start and after three months of adalimumab therapy.

Inflammatory activity was estimated by conventional laboratory tests such as CRP, hemoglobin, leucocytes with differential and platelets. Levels of rheumatoid factor and ACPA were measured by ELISA technique.

4.1.1. Electronic Patient-Self-Reported Outcome (DANBIO)

DANBIO is a nation-wide Danish database, which has been established with the aim to follow the use and results of bDMARD treatment in Danish patients with rheumatic diseases. Moreover, DANBIO allows patients with rheumatic disease to report their status before the consultation, thereby improving the communication with the rheumatologist and documenting rheumatic disease activity from the patient`s perspective.

In our patients, demographic data such as age, sex, disease duration, co-morbidity and previous medication were registered in DANBIO at diagnosis.

Just before the start of adalimumab treatment and at three months of therapy, the patients reported pain-, fatigue- and patient´s global on a 100 - mm visual analogue scale (VAS), as well as estimated their functional abilities in a health assessment questionnaire (HAQ), electronically, to DANBIO. At the same time-points, the examining doctor noted the number of swollen and tender joints, doctor´s global assessment as well as current medication in DANBIO.
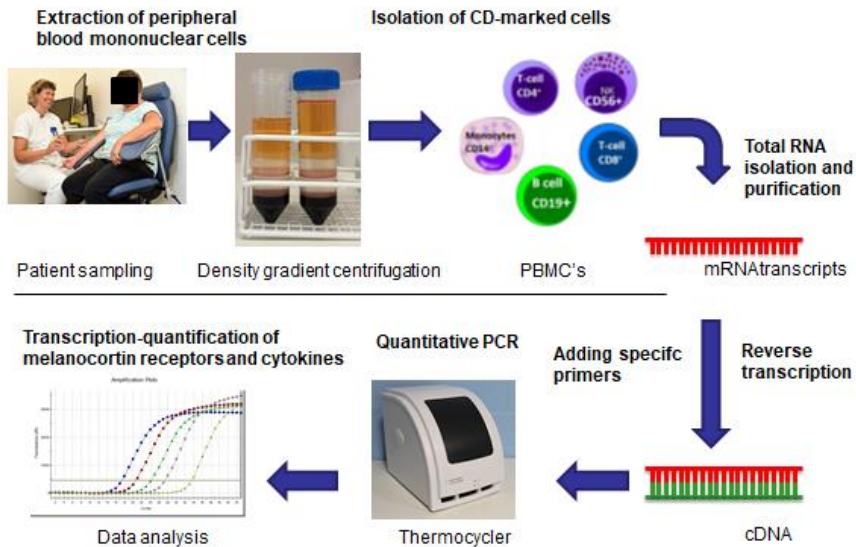
Figure 4.1. The workflow of the laboratory experiments used herein.

4.1.2. Isolation of Peripheral Mononuclear Blood Cells (PBMC)

For cell isolation, 10 mL of whole blood was diluted 1:1 with PBS and subjected to Ficoll-isopaque (Lymphoprep, Nycomed, Oslo, Norway) gradient centrifugation. The interface containing PBMC was collected, diluted 1:5 with PBS and washed from Lymphoprep by centrifugation. Next, cells were resuspended in 1.5 mL Roswell Park Memorial Institute (RPMI) medium and left at 4 °C overnight. Cells were counted and the concentration was adjusted to 10^6/mL using RPMI. Subpopulations of PBMC's were separated by positive selection with specific monoclonal antibody-coated Dynabeads (Dynal, Oslo, Norway) according to the manual. The workflow of the laboratory experiments used herein can be seen in figure 4.1.

4.1.3. Study I and II: Monoclonal Antibodies

All of the monoclonal antibodies used herein were purchased from DAKO A/S (Glostrup, Denmark). The specificities of the monoclonal antibodies were as follows: CD4, CD8 and CD19 and their negative isotype control were all IgG1 isotype and derived from Mab/clone MT310, DK25, HD37 and DAK-G01, respectively. CD14 and its negative isotype control were of IgG2a isotype and derived from Mab/clone TUK4 and DAK-G05, respectively.

4.1.4. Total RNA Extraction

Lysates from $CD4^+$, $CD8^+$, $CD14^+$ and $CD19^+$ cell subsets (> 95 % purity) were used to extract total RNA by the acid guanidinum thiocyanate– phenol–chloroform method.

4.1.5. Study III. Monoclonal Antibodies

The monoclonal antibody against the IgG1 MY 31 isoform of neural cell adhesion molecule (NCAM, CD56), which defines the NK cell subset was purchased from Becton-Dickinson, Mountain View, CA, USA, while its negative isotype control IgG1 used herein was derived from Mab/clone DAK-G0, DAKO A/S Glostrup, Denmark.

4.1.6. Total RNA Extraction.

Lysates from $CD56^+$ (> 95 % purity) were used to extract total RNA by the acid guanidinum thiocyanate-phenol-chloroform method.

4.1.7. **Quantitative Reverse Transcription-Polymerase Chain Reaction**

Real-time qPCR analysis was used to examine changes in receptor and cytokine mRNA levels. Real-time qPCR is a simple method, which gives reliable results. Messenger RNA levels provide information about the transcription of genes and changes in the regulation of transcription.

We performed our custom gene expression analysis according to the MIQE guidelines (Bustin 2009). Lymphocyte subpopulations separated by positive Dynabead selection were used for total RNA extraction. Cell-bead pellet was resuspended in 10 µl of phosphate buffered solution (PBS), lysed in 350 µl of RLT lysis buffer (RNeasy Mini kit, QIAGEN, Germany) and kept at -80 ℃ until further use. To make our tests as uniform as possible, we did RNA preparation, reverse transcription and overall quality assessment for all samples at the same time in the same experiment. The monoclonal antibody covered magnetic beads were removed prior to RNA extraction. RNA yield was on average 2005 ng in a total volume of 30 µl, and purity on average A260/A280 was 1.7 assessed by NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific Inc., USA). For each sample, 400 ng of total RNA in reaction volume 20 µl was transcribed into cDNA by using random hexamer primers, MultiScribe™ MuLV reverse transcriptase, reaction buffer and dNTP mix (High-Capacity cDNA Reverse Transcription Kit, Catalog # 4368813, Life Technologies) according to the manufacturer´s manual. RNA integrity, inhibition testing, efficiency of reverse transcription and cDNA stability were evaluated by the constancy of threshold cycle (Ct) values for 18S rRNA in every

single RT-qPCR test. This is a classical reference gene as well as the rational quality indicator for the entire study. Therefore, as previously optimized and tested by the standard curve assessment, we used Eukaryotic 18S rRNA Endogenous Control (VIC®/MGB probe, primer limited, Catalog # 4319413E, Life Technologies) according to the manufacturer's recommendations. Details for each TaqMan® Assay regarding sequence accession numbers and the targeted splice variants, location and length of amplicon as well as location and identity of any modifications can be retrieved on the producer's web site www.lifetechnologies.com using assay ID (or official gene symbol) as next: Hs00267168_s1 (MC1R), Hs00300820_s1 (MC2R), Hs00252036_s1 (MC3R), Hs00271877_s1 (MC4R), Hs00271882_s1 (MC5R), Hs01555410_m1 (IL1B), Hs00961622_m1 (IL10), Hs00989291_m1 (IFNG), Hs00174128_m1 (TNF), Hs04188773_g1 (LTA) and Hs99999918_m1 (TGFB1), Hs00174103_m1 (IL8), Hs01003716_m1 (IL15) and Hs524809_ (RETN). Eukaryotic 18S rRNA (Catalog # 4319413E) (Thermo Fisher, Scientific, Inc.). However, *in silico* specificity screen by BLAST is impossible because the producer does not disclose the primer/probe sequences. We performed uniformly multiplexed tests with cDNA input equal to 5 ng of total RNA in 96-well plates and reaction volume 20 µl/well using 2x Universal Master Mix on a 7900HT instrument (all from Life Technologies) with factory default settings. Each plate included both the no template control (NTC) and the PMA/ionomycin-stimulated PBMC (sPBMC) as an internal positive control. The data were analyzed with a ddCt method resulting in relative quantities (RQ) comparative to a reference expression level in stimulated

PBMC that is equal to 1. Fold-change value is a result of division RQ after therapy by RQ before therapy.

4.2. Statistics

Because of a rather small number of patients, Wilcoxon´s matched-pairs signed rank test for continuous data was used to evaluate differences in receptor and cytokine gene expression levels as well as disease activity variables before and after three months of adalimumab treatment. Thus, the patients served as their own controls. This set-up is advantageous as it eliminates confounding factors due to badly matched controls.

Correlation analyses were performed using the non-parametric Spearman rank correlation test. All P-values were two-sided; $P < 0.05$ was considered significant. All statistical analyses were performed using the SPSS computerizing program (Amonk, NY, USA version 22).

5. RESULTS

5.1. Results in Paper I-III

In this result section, at first the effects of adalimumab treatment on disease activity in RA patients will be reported.

Next the effects of adalimumab treatment on gene expressions of the biomarkers of interest, that is MC1-5R and resistin will be presented, then the effects on gene expression of context cytokines will be stated. Finally, correlations between fold changes in the gene expressions of the biomarkers of interest and the context cytokines will be explained.

In these three original papers, TNFαI induced changes in gene expressions of the biomarkers of interest were related to changes in gene expressions of cytokines, representing important disease mechanisms in RA. The cytokines examined were: Th1 response cytokines: IFNγ and TNFβ; inflammatory cytokines: IL-1β and TNFα as well as regulatory cytokines: IL-10 and TGFβ.

Fold change in gene expressions were examined in important disease driving immune cell subsets of adaptive immunity that is CD4^{+} Th cells, CD8^{+} Tc cells and CD19^{+} B cells as well as in CD14^{+} monocytes, representing innate immunity.

In paper III, MC1-5R gene expression in another cell subset of innate immunity, namely the CD56^{+} NK cell subset was examined.

Effects of Adalimumab Treatment on Measures of Disease Activity in RA Patients

All patients injected themselves subcutaneously with adalimumab in the form of Humira 40 mg every other week during all 3 months without interruption.

Of the seven patients, six responded to treatment (Table 5.1). To avoid adverse effects, prednisolone was stopped after two weeks and cDMARDs were tapered when possible in accordance with disease activity. Methotrexate however, was continued to avoid anti-adalimumab antibody synthesis. If methotrexate was not tolerated, another DMARD was chosen. In two patients treated with sulfasalazine combined with leflunomide and methotrexate, respectively, sulfasalazine medication was stopped. The four patients, treated with a mean dose of 18.75 mg/week methotrexate at inclusion, continued this medication. Also the three patients taking leflunomide continued their medication.

DAS28 (CRP), HAQ, number of tender and swollen joints, pain and global VAS decreased significantly in patients with RA responding to adalimumab. Median C-reactive protein and ACPA level, neutrophil and platelet number decreased insignificantly.

The non-responding patient was a 47-year-old male with RA since half a year with DAS28(CRP) 4.4, highly positive RF145 kIU/l(<10 kIU/ l) and highly positive ACPA >600 AE/l (<14 AE/l) at inclusion. This patient did not respond to adalimumab, despite combination with 25 mg methotrexate s.c. a week. At 3-months follow-up of adalimumab treatment, the patient had developed

rheumatoid nodules and had six tender and swollen joints. The patient was not

smoking.

Table 5.1. Characteristics of six patients with active rheumatoid arthritis, responding to TNF-α inhibition

Variable	Median (25–75 percentile) before TNF-α inhibition $N = 6$	Median (25–75 percentile) during TNF-α inhibition $N = 6$	P
Pain VAS	68.0 (42.5–89.5)	12.5 (8.8–37.5)	0.03[a]
Fatigue VAS	77.0 (52.3–82.0)	44.5 (13.0–67.5)	0.06
Global VAS	85.00 (47.8–92.3)	26.5 (13.5–57.0)	0.03[a]
HAQ	1.50 (0.78–2.03)	0.44 (0.19–1.25)	0.04[a]
CRP (<3 mg/l)	4.0 (2.75–23.75)	3.0 (2.00–4.68)	0.38
Swollen joint number	8.0 (6.3–21.5)	2.0 (0.0–2.8)	0.04[a]
Tender joint number	9.0 (4.5–21.5)	0.5 (0.0–2.8)	0.04[a]
DAS28 (CRP) (<2.6 in remission)	5.10 (4.38–6.73)	2.20 (1.61–3.90)	0.03[a]
Hgb (7.3–9.5 mmol/l)	8.55 (8.13–8.83)	8.90 (8.18–9.03)	0.16
Platelets (165–400 ×10^9/l)	336 (232–461)	319 (196–362)	0.16
Leukocytes (3.5–10.0 × 10^9/ml)	8.7 (5.58–12.3)	7.6 (6.18–9.10)	0.31
Neutrophils (2.0–7.0 × 10^9/ml)	5.2 (3.2–8.1)	4.4 (3.0–6.8)	0.06
ACPA ($N = 5$) (<14 AE/l)	52.0 (9.8–206.0)	47.0 (9.8–151.3)	0.25

VAS, visual analogue scale; Hgb, haemoglobin; DAS28CRP, 28 joints disease activity score, based on C-reactive protein; ACPA, anti-citrullinated protein antibody; CRP, C-reactive protein; HAQ, health assessment questionnaire.
Results are shown as median values (interquartile range) before and 3 months after start of TNF-α inhibition.
Statistic: Wilcoxon matched-pairs signed rank test for continuous data.

PAPER I

In paper I, entitled: "**Melanocortin 2, 3 and 4 receptor gene expressions are downregulated in CD8^{+} T cytotoxic lymphocytes and CD19^{+} B lymphocytes in rheumatoid arthritis responding to TNFα inhibition",** we explored the differential reactions of MC1-5R gene expressions in CD4^{+} Th cells, CD8^{+} Tc cells, CD19^{+} B cells and CD14^{+} monocytes to adalimumab treatment in the context of changes in Th1 response, inflammatory- and regulatory cytokine gene expressions in active RA.

Changes in MC1-5R Gene Expressions (figure 5.1)

In cell subsets of adaptive immunity, our analyses showed downregulation of MC1-5R gene expressions in CD8^{+} Tc cells and CD19^{+} B-cells. There was a significant downregulation of MC2R, MC3R and MC4R gene expressions in CD8^{+} Tc cells in RA patients responding to TNFαI. The expressions of these MCR genes as well as the MC5R gene were also significantly downregulated in CD19^{+} B-cells from RA patients responding to TNFαI.

In CD4^{+} Th cells, the transcription of all MCR genes, except for MC1R was downregulated in RA patients responding to therapy, however the changes did not reach significance.

In the patient, not responding to TNFαI, MC1-5R gene expression increased impressively in all cell subsets of adaptive immunity.

In innate immunity, i.e. in CD14^{+} monocytes, MC1-5R gene transcription decreased in the RA patients responding to adalimumab treatment, however the results were not significant.

Also in the RA patient not responding to TNFαI, MC1-5R gene transcription in CD14^{+} monocytes decreased.
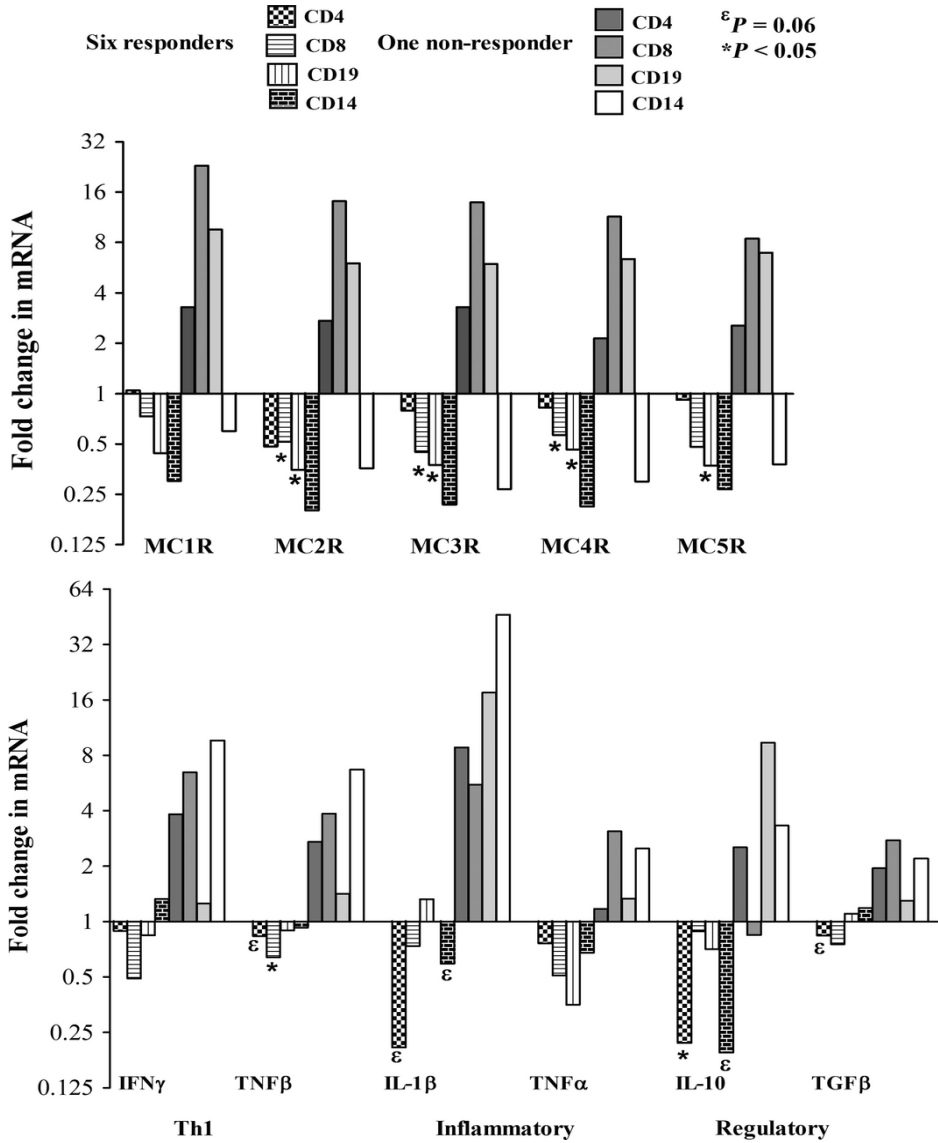
Figur 5.1. RT-qPCR analyses of MC1-5R, IFNγ, TNF-β, IL-1β, TNF-α, IL-10 and TGF-β mRNAs in CD4$^+$ Th ly, CD8$^+$ Tc ly, CD19$^+$ B ly and CD14$^+$ monocytes, showing downregulation of mRNA expression upon treatment with adalimumab in responders and upregulation in the non-responder, preferentially. The data are presented as fold change in a logarithmic scale, where 1 denotes the expression before treatment, 0.5 denotes twofold decrease and 0.25 a fourfold decrease etcetera. 2 denotes a twofold increase and 4 a fourfold increase etcetera. * = $P < 0.05$; $\varepsilon = P < 0.06$. *Statistic*: Wilcoxon's matched-pairs signed rank sum test.

PAPER II

In paper II, entitled: "**Resistin gene expression is downregulated in CD4^{+} T helper lymphocytes and CD14^{+} monocytes in rheumatoid arthritis responding to TNFα inhibition"**, we examined the effects of adalimumab treatment on resistin (RETN) gene expression in cell subsets of innate and adaptive immunity in the context of Th1-, inflammatory- and regulatory cytokine gene expressions as outlined above.

Changes in resistin gene expression (figure 5.2 and 5.3)

We found that CD14^{+} monocytes contained the highest number of RETN gene transcripts of the examined leucocyte subtypes both before and during TNFαI. In CD14^{+} monocytes RETN gene expression was significantly down-regulated in the six RA patients responding to TNFαI and up-regulated in the non-responding RA patient.

In CD4^{+} Th cells RETN gene expression decreased significantly in RA patients responding to TNFαI, while in the non-responding patient there was a slight decrease. The RETN gene expression increased, however insignificantly, in CD8^{+}Tc lymphocytes and CD19^{+} B lymphocytes from RA patients responding to therapy, and decreased in both cell subsets from the non-responding patient.
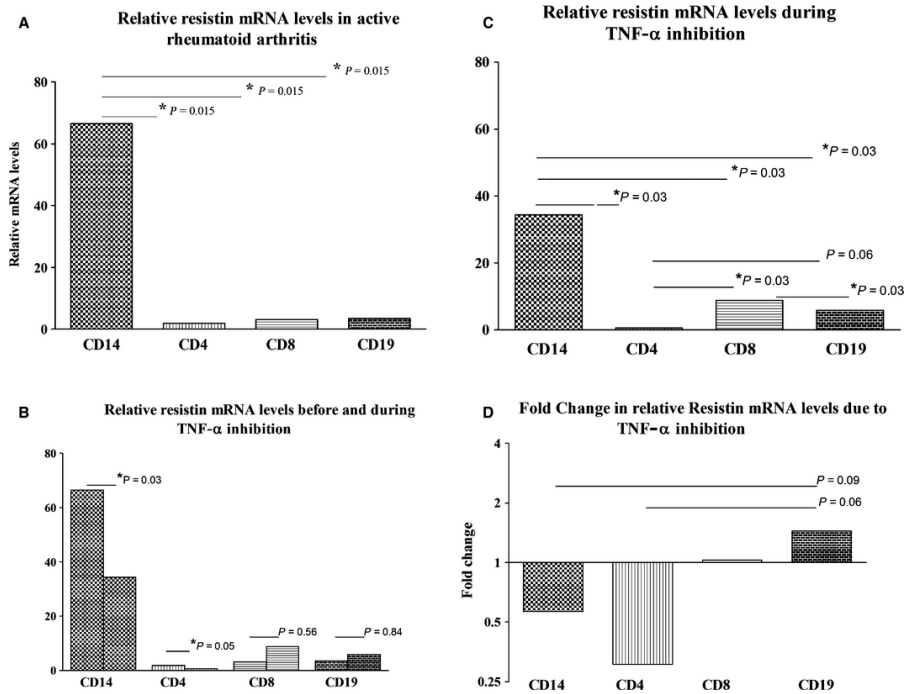
Figure 5.2. (A) Relative resistin mRNA levels in CD14+ monocytes, CD4+ Th lymphocytes, CD8+ Tc lymphocytes and CD19+ B-lymphocytes in active rheumatoid arthritis. (B) Relative resistin mRNA levels in CD14+ monocytes, CD4+ Th lymphocytes, CD8+ Tc lymphocytes and CD19+ B-lymphocytes in rheumatoid arthritis before and during TNF-α inhibition. (C) Relative resistin mRNA levels in CD14+ monocytes, CD4+ Th lymphocytes, CD8+ Tc lymphocytes and CD19+ B-lymphocytes in rheumatoid arthritis patients responding to TNF-α inhibition. (D) Fold change in relative resistin mRNA levels in CD14+ monocytes, CD4+ Th lymphocytes, CD8+ Tc lymphocytes and CD19+ B-lymphocytes in rheumatoid arthritis due to TNF-α inhibition. Statistic: Wilcoxon′s matched-pairs signed rank-sum test.
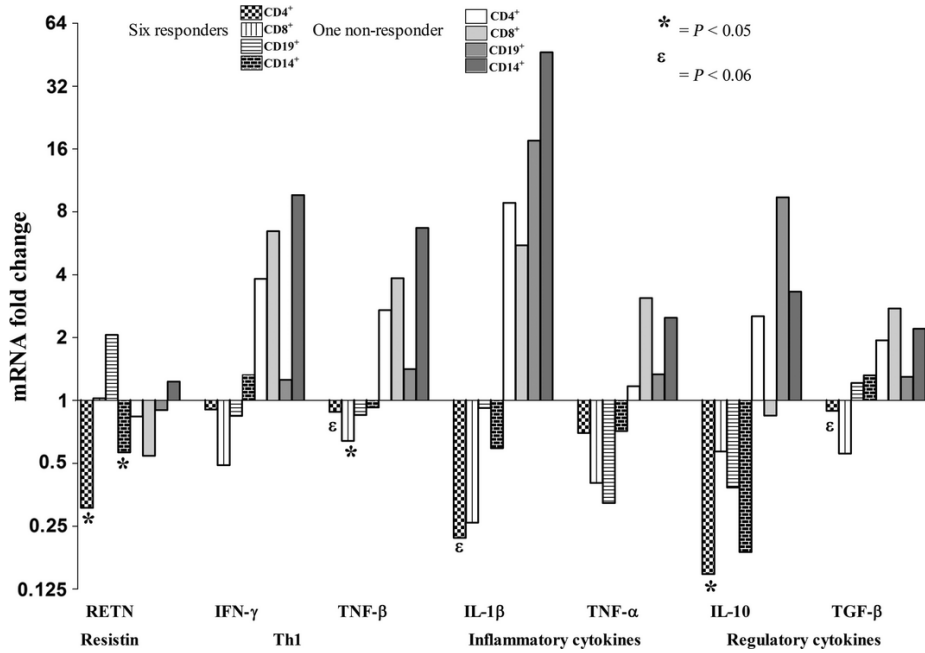
Figure 5.3. Fold change due to TNF-α inhibition in RETN, IFN-γ, TNF-β, IL-1β, TNF-α, IL-10 and TGF-β gene expressions in six patients with active rheumatoid arthritis, responding to treatment and one patient with active rheumatoid arthritis, who did not respond. Cytokine mRNA levels were measured before start of TNF-α inhibition and after 3 months of treatment. IFN-γ, interferon gamma; IL, interleukin; RETN, resistin; TNF-β, tumor necrosis factor beta; TGF-β, transforming growth factor beta. *P < 0.05, εP < 0.06.

PAPER III

In paper III, entitled: "**Melanocortin receptor gene expressions in CD56$^+$ NK cells from two rheumatoid arthritis patients respond to TNFα inhibition by adalimumab",** we examined the expression and differential reactions of MC1-5R gene expressions to adalimumab treatment in CD56$^+$ NK cells from two patients with active RA.

Changes in MC1-5R Gene Expressions (figure 5.4)

MC1-5R gene expression was demonstrated in CD56$^+$NK cells from two patients with active RA. MC4R gene expression for the first time was found to be present in human CD56$^+$NK cells. Adalimumab treatment resulted in the downregulation of MC2-5R in both patients, while MC1R only was downregulated in one patient.
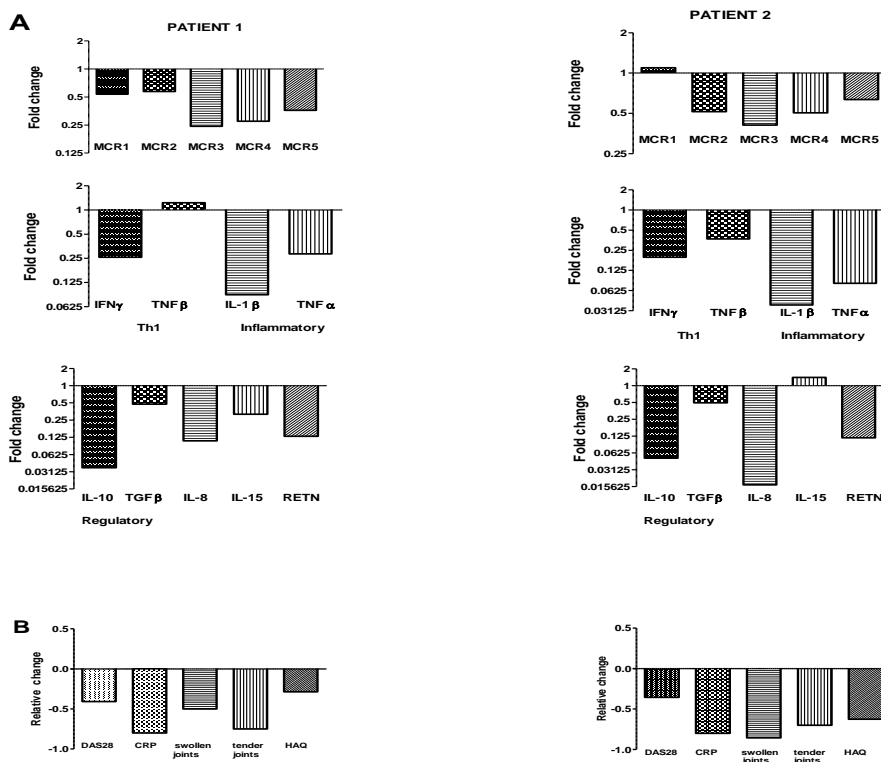
Figure 5.4. Fold changes of melanocortin 1-5 receptor and cytokine gene expressions in CD56⁺NK cells in 2 RA patients, responding to adalimumab treatment. A. Real time RT-qPCR analyses of MCR1-5, IFNγ, TNFβ, IL-1β, TNFα, IL-10, TGFβ, IL-8, IL-15 and resistin (RETN) mRNAs in CD56⁺ natural killer cells, showing down-regulation of mRNA expression upon treatment with adalimumab. The data are presented as fold change in a logarithmic scale, where 1 denotes the expression before treatment, 0.5 denotes two-fold decrease and 0.25 a four-fold decrease etcetera. **B**. Relative change in clinical sign and symptoms of rheumatoid arthritis activity presented as a ratio between the value after three months of treatment and the value before treatment.

PAPERS I, II and III

Changes in Th1-response, Inflammatory and Regulatory Cytokine Gene Expressions (figure 5.1)

Th1 Response Cytokines IFN-γ and TNF-β

Th1 response cytokines IFN-γ and TNF-β gene expressions were downregulated in lymphocyte subsets of adaptive immunity, *i.e* CD4^{+} Th ly, CD8^{+} Tc ly and CD19^{+} B ly, in patients with RA responding to adalimumab. TNF-β gene expression decreased significantly in CD8^{+} Tc ly and tended to decrease significantly in CD4^{+} Th ly (P = 0.06).

In the patient with RA not responding to adalimumab, IFN-γ and TNF-β gene expressions were upregulated in all examined cell subsets of adaptive immunity.

Inflammatory Cytokines IL-1β and TNF-α

Inflammatory cytokine IL-1β and TNF-α gene expressions were downregulated, however insignificantly, (except for a slight increase in IL-1β gene expression in CD19^{+} B cells), in lymphocyte subsets of adaptive immunity, in patients with RA responding to adalimumab. In the RA patient not responding to adalimumab, IL-1β and TNF-α gene expressions increased in all cell subsets of adaptive immunity.

In cells of innate immunity, *i.e*. CD14^{+} monocytes, the gene expressions of inflammatory cytokines IL-1β and TNF-α decreased in patients with RA responding to adalimumab. Fall in IL-1b gene expression almost reached significance (P = 0.06).

In the non-responding patient, the gene expressions of IL-1β and TNF-α increased in monocytes.

Regulatory Cytokines IL-10 and TGF-β

In all examined cell subsets of adaptive immunity, regulatory cytokine IL-10 gene expression was downregulated, in CD4$^+$ Th cells significantly, in patients with RA responding to adalimumab. In the non-responding patient, IL-10 gene expression increased in all cell subsets of adaptive immunity, except CD8$^+$ Tc cells.

In monocytes IL-10 gene expression was downregulated in patients responding to therapy and upregulated in the non-responding patient.

TGF-β gene expression was downregulated in CD4$^+$ Th and CD8$^+$ Tc cell subsets and slightly upregulated in CD19$^+$ B-cells due to TNFαI in RA responding to therapy. In the non-responding patient TGF-β gene expression increased in all examined cell subsets of adaptive immunity.

In CD14$^+$ monocytes, TGF-β gene expression increased in both the responding and non-responding patients, however upregulation was more pronounced in the non-responding patient.

Correlations between Fold Change in MC1-5R, Resistin-, and Context Cytokine Gene Expressions

Correlations between Fold Change in MC1-5R and Cytokine Gene Expressions (figure 5.5)

The TNFαI induced fold change in MCR gene expressions correlated to fold change in IFNγ gene expression in the CD8^{+} Tc cell subset and to fold change in IL-1β gene expression in CD4^{+} Th cells. The correlations, we found between TNFαI induced fold change in gene expressions between MC1-5R and cytokines, are in accordance with previously described mechanisms, regulating MC1-3R and MC5R gene transcription. Thus, MCR gene expression in various cells and tissues is stimulated by their ligands, i.e the melanocortins. The synthesis of melanocortins in turn is stimulated by inflammatory cytokines and inhibited by TGF-β.

A CD8+ T cytotoxic lymphocytes

- MC1R R$_s$ 0.93; *P* = 0.007
- MC2R R$_s$ 0.86; *P* = 0.02
- MC3R R$_s$ 0.82; *P* = 0.03
- MC4R R$_s$ 0.93; *P* = 0.007
- MC5R R$_s$ 0.82; *P* = 0.03

Fold change MC1-5R mRNA

Fold change IFN γ mRNA

B CD4+ T helper lymphocytes

- MC1R R$_s$ 0.89; *P* = 0.01
- MC2R R$_s$ 0.71; *P* = 0.09
- MC3R R$_s$ 0.93; *P* = 0.007
- MC4R R$_s$ 0.57; *P* = 0.20
- MC5R R$_s$ 0.89; *P* = 0.01

Fold change MC1-5R mRNA

Fold change IL-1 β mRNA
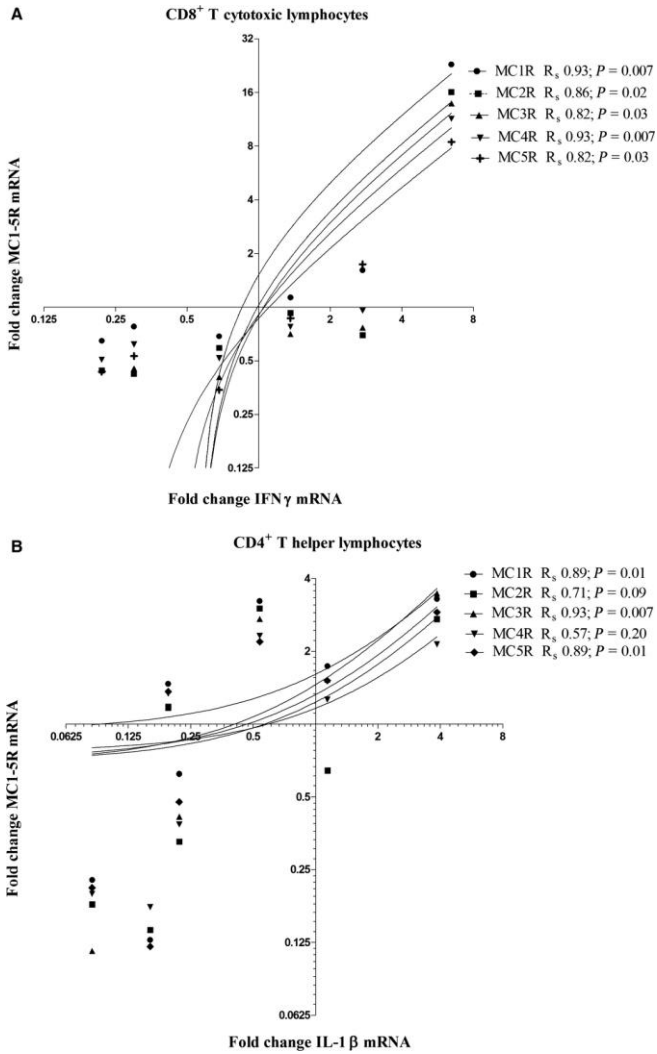
Figure 5.5. A. Correlations between fold change in MC1-5R and IFN-γ mRNA levels in CD8+ T cytotoxic cells in RA at 3-months follow-up of adalimumab treatment. **B.** Correlations between fold change in MC1-5R and IL-1β mRNA levels in CD4+ T helper cells in RA at 3-months follow-up of adalimumab treatment. *Statistic:* Spearman rank correlation test. *P* values <0.05 are considered significant. Although this graph has a log axis, the regression lines are fitted to the actual data.

Correlations between Fold Change in Resistin and TGF-β Gene Expressions (figure 5.6)

There was a significant positive correlation between fold change in RETN and TGF-β gene expressions in CD14$^+$ monocytes, pointing at a relation between resistin gene expression and immune tolerance.
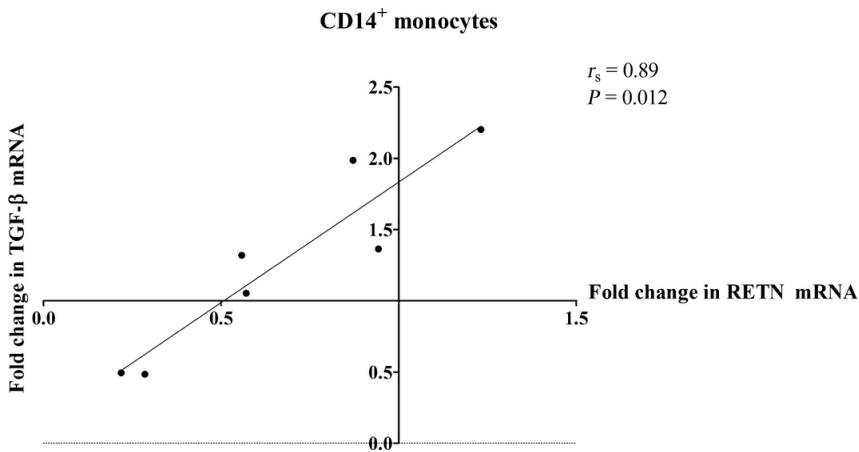
Figure 5.6. Correlation between fold change in gene expression of resistin and transforming growth factor beta (TGF-β) in patients with active rheumatoid arthritis treated with TNF-α inhibition. Messenger RNAs were measured before and 3 months after start of treatment. Statistic: Spearman′s rank correlation test.

7. DISCUSSION

The studies in this thesis were aimed to find signs of activation of anti-inflammatory, immune tolerance inducing, pro-resolving biologic systems in human autoimmune, inflammatory disease. As the melanocortin system has only been very scarcely examined in human disease, we examined this system in active RA as it is known to possess tissue preserving, immune tolerance inducing qualities. We chose to measure adalimumab induced fold change in MC1-5R gene expressions in cell subsets of the adaptive and innate immune system. Moreover, in the same experiments we explored RETN gene expression as RETN has been suggested to play a role in the induction of Tregs.

The effects of adalimumab on the immune system in active RA is to dampen and normalize a pathogenetic immune process. Thus, in RA patients responding to three months of adalimumab therapy, immune markers are expected to have approached normality. The favorable effects are evidenced by the fact that adalimumab treatment prevents joint destruction in responding patients (REF).

Evidence of regulation of the melanocortin system and resistin in immune cells in RA

Concerning the melanocortin system, we found that adalimumab treatment induced a decrease in MC1-5R gene expressions in all examined immune cell subsets, except for MC1R in CD4$^+$ Th cells. The production of melanocortins, that is of α-, β-, γ-MSH and ACTH is upregulated by inflammatory cytokines, especially TNFα and inhibited by TGFβ. Melanocortins in turn upregulate MCR

expression. Our findings indicate upregulation of the melanocortin system in active RA, approaching the resting state upon adalimumab treatment.

In CD8^{+} Tc cells and CD19^{+} B-cells, adalimumab caused a significant decrease in MC2,3,4R gene expressions, pointing at a special role for MC2,3,4Rs in the function of these cell subsets. Even MC5R gene expression decreased significantly in CD19^{+} B-cells. The anti-inflammatory actions of MC3R activity in monocytes are well-known from animal experimental studies of urate crystal induced peritonitis and arthritis (Getting 2002, 2003). In experimental murine uveoretinitis, immune tolerance was induced by stimulating MC5R on CD4^{+} Th cells with α-MSH, while the specific antigen was presented to these on DCs. This maneuver turned effector CD4^{+} Th cells cells into TGFβ secreting CD4^{+}CD25^{+} Tregs (Taylor 2001). Our finding of significant reactions of MC2-5R gene expressions in B cells to TNFαI, points at a path to the transformation of pathogenetic B cell functions in RA. Promisingly, melanocortins have been shown to decrease kidney damage in lupus mice, by influencing B-cell activities such as ANA- and overall immunoglobulin production (Botte 2014).

Resistin is a recently discovered cytokine, the effects of which are still only sporadically described. Surprisingly, RETN seems able to elicit seemingly opposing effects, that is exert pro-inflammatory, pro-fibrotic as well as immune tolerance and pro-resolving actions. The suspicion of pro-fibrotic qualities was based on a correlation between myocardial RETN and TGFβ gene expressions in a model of fibrosis and the ability of RETN to induce connective tissue growth factor (CTGF)

synthesis (Chemaly 2013). However most interesting, Son et al showed that treatment of a co-culture of CD4$^+$ T cells and DC with RETN resulted in enhanced differentiation of CD4$^+$ T cells into CD4$^+$CD25$^+$ Tregs (Son 2010).

We found RETN gene expression in all examined immune cell types in active RA, with far the highest levels in CD14$^+$ monocytes. TNFαI resulted in a significant fall in RETN gene expression in CD14$^+$ monocytes and a rise in gene expression in the non-responding patient. In CD4$^+$ Th cells there was a significant fall in RETN gene expression in RA responding to therapy, however, RETN gene expression also decreased in the non-responding patient. Thus, we found clear signs of regulation of RETN gene expression by TNFαI in CD14$^+$ monocytes in RA. Also in CD4$^+$ Th cells, our results pointed at the regulation of RETN gene expression by the cytokine milieu, however the regulation may be more complex.

Also, CD56$^+$ NK cells from two patients with active RA were found to express MC1-5R genes. The role of the NK cell in RA is controversial. However, the number of CD56^{dim} NK cells, with high cytotoxic capacity seems decreased in the blood, while the number of CD56^{high} NK cells with high cytokine, especially IFNγ, secreting ability is increased in the inflamed synovium and synovial fluid (Flodström-Tullberg 2009, review). Thus, it seems that CD56$^+$ NK cells may play an active role in RA. The signs, we found of an activated melanocortin system in NK cells in RA, indicates that the melanocortin system might become a valuable new target for the regulation of NK cell activity in RA.

Hopefully, as the MC3R preferentially binds γ-MSH and MC4R has a higher affinity for β-MSH than other MCRs (Wikberg 2004) these MCRs can be targeted by synthetic, superselective γ- and β -MSH analogues, respectively, in the future (Getting 2006).

Signs of disease counteracting, pro-resolving roles for resistin and the melanocortin system in RA.

Considering our findings, there seems to exist yet unexplored, inherent mechanisms of immune cell functioning, acting to counteract the pathogenetic mechanisms in RA.

Interestingly, we found significant correlations between adalimumab induced fold change in MC1-5R- and Th1 response signature cytokine: IFNγ gene expressions in CD8^{+} Tc cells. Recently, the role of CD8^{+} Tc cells in RA has been highlighted by the finding that synovial invasion of just these cells in preclinical ACPA and/or RF positive individuals heralds overt RA (De Hair 2014). The fact that CD8^{+} Tc cells during their maturation increase IFNγ secretion to considerable amounts point at a role for Tc cells in immune response direction (Liu 2004). The correlations, we found, are suggestive of common regulatory mechanisms of melanocortin system activation and IFNγ synthesis in CD8^{+} Tc cells in RA.

Also in CD4^{+} Th cells, we found evidence that the regulation of melanocortin system activity might be closely related to important immune mechanisms in RA. Thus, in CD4^{+} Th cells, adalimumab induced fold changes in MC1,2,3,5R and IL-1β

gene expressions correlated significantly. As the CD4$^+$ Th cell is the classical helper cell, which directs the immune response exploiting differentiated cytokine synthesis, melanocortin system activity may be linked to disease driving mechanisms. The recent finding that IL-1β may exert Th1 cytokine functions, supports this notion (Bruchard 2015).

In CD14$^+$ monocytes, we found a significant correlation between adalimumab induced fold change in RETN and TGFβ gene expressions. In the light of the finding of a connection between RETN stimulated monocyte derived DCs and the differentiation of Tregs (Son 2010), our finding is interesting, yet, the meaning still unexamined and unclear.

In summary, we found signs of activation and regulation of the melanocortin and RETN pathways in leukocyte subsets of both innate and adaptive immunity in RA. Both these pathways have immune tolerance inducing, pro-resolving and anti-inflammatory potential, yet in the natural course of RA, their efforts were not powerful enough to resolve the disease. Our results, however, show that both pathways are amenable to therapeutic intervention in RA.

8. CONCLUSIONS

➢ The melanocortin system is activated in RA, and responds to changes in cytokine environment.

➢ The regulation of the melanocortin system is closely related to important pathogenetic mechanisms in RA.

➢ Resistin gene expression is regulated in monocytes and T helper cells in RA and responds to changes in cytokine milieu.

➢ The regulation of resistin is related to TGF-β gene expression in monocytes in RA, pointing at a regulatory function.

➢ NK cells might be targeted through melanocortin receptors.

REFERENCES

Alvarez-Mon M, Kehrl JH, Fauci AS. A potential role for adrenocorticotropin in regulating human B lymphocyte functions. J Immunol 1985;135:3823-6.

Aletaha D, Neogi T, Silman AJ, Funovits J, Felson DT, Bingham CO, et al. 2010 rheumatoid arthritis classification criteria: an American College of Rheumatology/European League Against Rheumatism collaborative initiative. Ann Rheum Dis 2010;69:1580–8.

Andersen GN, Hägglund M, Nagaeva O, Frängsmyr L, Petrovska R, Mincheva-Nilsson L, Wikberg JE. Quantitative measurement of the levels of melanocortin receptor subtype 1, 2, 3 and 5 and pro-opio-melanocortin peptide gene expression in subsets of human peripheral blood leucocytes. Scand J Immunol. 2005;61:279-84.

Arnett FC, Edworthy SM, Bloch D a, McShane DJ, Fries JF, Cooper NS, et al. The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. Arthritis Rheum 1988;31:315–24.

Auger I, Sebbag M, Vincent C, Balandraud N, Guis S, Nogueira L et al. Influence of HLA-DR genes on the production of rheumatoid arthritis-specific autoantibodies to citrullinated fibrinogen. Arthritis Rheum. 2005;52:3424-32.

Babbe H, Roers A, Waisman A, Lassmann H, Goebels N, Hohlfeld R, et al. Clonal expansions of CD8(+) T cells dominate the T cell infiltrate in active multiple sclerosis lesions as shown by micromanipulation and single cell polymerase chain reaction. J Exp Med. 2000;192:393–404.

Balsa A, Del Amo J, Blanco F, Caliz R, Silva L, Sanmarti R et al. Prediction of functional impairment and remision in rheumatoid arthritis patients by biochemical variables and genetic polymorphisms. Rheumatology 2010;49:458-66.

Basdeo SA, Cluxton D, Sulaimani J, Moran B, Canavan M, Orr C et al. Ex-Th17 (Nonclassical Th1) Cells Are Functionally Distinct from Classical Th1 and Th17 Cells and Are Not Constrained by Regulatory T Cells. J Immunol. 2017;198:2249-59.

Bettelli E, Carrier Y, Gao W, Korn T, Strom TB, Oukka M, et al. Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells. Nature. 2006;441:235-8.

Bhardwaj R, Becher E, Mahnke K, Hartmeyer M, Schwarz T, Scholzen T, et al. Evidence for the differential expression of the functional alpha-melanocyte-stimulating hormone receptor MC-1 on human monocytes. J Immunol 1997;158:3378–84.

Bokarewa M, Nagaev I, Dahlberg L, Smith U, Tarkowski A. Resistin, an adipokine with potent proinflammatory properties. J Immunol 2005;174:5789-95.

Botte DA, Noronha IL, Malheiros DM, Peixoto TV, de Mello SB. Alpha-melanocyte stimulating hormone ameliorates disease activity in an induced murine lupus-like model. Clin Exp Immunol 2014;33:1707-14.

Bottini N, Peterson EJ. Tyrosine phosphatase PTPN22: multifunctional regulator of immune signaling,development and disease. Annu Rev Immunol 2014; 32:83-119.

Brimnes J, Allez M, Dotan I, Shao L, Nakazawa A, Mayer L. Defects in CD8+ regulatory T cells in the lamina propria of patients with inflammatory bowel disease. J Immunol 2005;174:5814–22.

Bruce B, Fries JF. The Health Assessment Questionnaire (HAQ). Clin Exp Rheumatol. 2005;23(5 Suppl 39):S14-18.

Bruchard M, Rébe C, Derangère V. The receptor NLRP3 is a transcriptional regulator of Th2 differentiation. Nat Immunol 2015;16:859–70.

Buggy JJ. Binding of alpha-melanocyte-stimulating hormone to its G-protein-coupled receptor on B-lymphocytes activates the Jak/STAT pathway. Biochem J 1998;331:211–6.

Bustin SA, Benes V, Garson JA et al. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin Chem 2009;55:611–22.

Böhm M, Metze D, Schulte U, Becher E, Luger TA, Brzoska T. Detection of melanocortin-1 receptor antigenicity on human skin cells in culture and in situ. Exp Dermatol.1999;8:453-61.

Böhm M, Raghunath M, Sunderkötter C, Schiller M, Ständer S, Brzoska T, et al. Collagen metabolism is a novel target of the neuropeptide alpha-melanocyte- stimulating hormone. J Biol Chem 2004;279:6959–66.

Cao D, Malmström V, Baecher-Allan C, Hafler D, Klareskog L, Trollmo C. Isolation and functional characterization of regulatory CD25brightCD4+ T cells from the target organ of patients with rheumatoid arthritis. Eur J Immunol 2003;33:215–23.

Carvalheiro H, Duarte C, Silvia-Cardoso S, da Silva JA, Souto-Carneiro MM. CD8+ T cell profiles in patients with rheumatoid arthritis and their relationship to disease activity. Arthritis Rheumatol 2015;67:363-71.

Catania A, Rajora N, Capsoni F, Minonzio F, Star R a, Lipton JM. The neuropeptide alpha-MSH has specific receptors on neutrophils and reduces chemotaxis in vitro. Peptides 1996;17:675–9.

Catania A, Gatti S, Colombo G, Lipton JM. Targeting melanocortin receptors as a novel strategy to control inflammation. Pharmacol Rev 2004;56:1–29.

Chalan P, Bijzet J, Kroesen BJ, Boots AM, Brouwer E. Altered natural killer cell subsets in seropositive arthralgia and early rheumatoid arthritis are associated with autoantibody status. J Rheumatol. 2016;43:1008-16

Chemaly ER, Kang S, Zhang S, McCollum L, Chen J, Bénard L et al. Differential patterns of replacement and reactive fibrosis in pressure and volume overload are related to the propensity for ischaemia and involve resistin. J Physiol. 2013;591:5337-55.

Chhajlani V, Wikberg J. Molecular cloning and expression of the human melanocyte stimulating hormone receptor cDNA. FEBS Lett 1992;14:417-20.

Chhajlani V, Muceniece R, Wikberg JE. Molecular cloning of a novel human melanocortin receptor. Biochem Biophys Res Commun 1993;195:866-73.

Choy E. Understanding the dynamics: pathways involved in the pathogenesis of rheumatoid arthritis Rheumatology. 2012;51(Suppl 5):3-11.

Corsiero E, Pratesi F, Prediletto E, Bombardieri M, Migliorini P. NETosis as a source of antigens in rheumatoid arthritis. Front Immunol 2016;7:article 485.

Cribbs AP, Kennedy A, Penn H, Read JE, Amjadi P, Green P et al. Treg cell function in rheumatoid arthritis is compromised by ctla-4 promoter methylation resulting in a failure to activate the indoleamine 2,3-dioxygenase pathway. Arthritis Rheumatol.2014;66:2344-54.

Cui D, Zhang L, Chen J, Zhu M, Hou L, Chen B et al. Changes in regulatory B cells and their relationship with rheumatoid arthritis disease activity. J Exp Med 2015;15:282-95.

De Hair MJ, van de Sande MG, Ramwadhdoebe TH. Features of the synovium of individuals at risk of developing rheumatoid arthritis: implications for understanding preclinical rheumatoid arthritis. Arthritis Rheumatol 2014;66:513–22.

De Stefano R, Frati E, Nargi F, Baldi C, Menza L, Hammoud M, et al. Comparison of combination therapies in the treatment of rheumatoid arthritis: Leflunomide-anti-TNF-alpha versus methotrexate-anti-TNF-alpha. Clin Rheumatol. 2010;29:517–24.

Emery P, Smolen JS, Ganguli A, Meerwin S, Bao Y, Kupper H et al. Effects of adalimumab on the work-related outcomes scores in patients with rheumatoid arthritis receiving methotrexate. Rheumatology 2016;55:1458-65.

Flodström-Tullberg M, Bryceson YT, Shi FD, Höglund P, Ljunggren HG. Natural killer cells in human autoimmunity. Curr Opin Immunol. 2009; 21:634-40.

Gantz I, Miwa H, Konda Y, Shimoto Y, Tashiro T, Watson SJ, DelValle J, Yamada T. Molecular cloning, expression, and gene localization of a fourth melanocortin receptor. J Biol Chem. 1993;268:15174-9.

Getting SJ, Christian HC, Flower RJ, Perretti M. Activation of melanocortin type 3 receptor as a molecular mechanism for adrenocorticotropic hormone efficacy in gouty arthritis. Arthritis Rheum 2002;46:2765-75.

Getting SJ, Christian HC, Lam CW, Gavins FN, Flower RJ, Schiöth HB, Perretti M. Redundancy of a functional melanocortin 1 receptor in the anti-inflammatory actions of melanocortin peptides: studies in the recessive yellow (e/e) mouse suggest an important role for melanocortin 3 receptor. J Immunol. 2003;170:3323-30.

Getting SJ, Lam CW, Chen AS, Grieco P, Peretti M. Melanocortin 3 receptors control crystal-induced inflammation. FASEB J 2006;20:2234–41.

Grabbe S, Bhardwaj RS, Mahnke K, Simon MM, Schwarz T, et al. α-Melanocyte-stimulating hormone induces hapten-specific tolerance in mice. J Immunol 1996;156:473–8.

Gravestein LA, Borst J.Tumor necrosis factor receptor family members in the immune system. Semin Immunol. 1998;10:423-34.

Giuliani D, Minutoli L, Ottani A, Spaccapelo L, Bitto A, Galantucci M, Altavilla D, Squadrito F, Guarini S. Melanocortins as potential therapeutic agents in severe hypoxic conditions. Front Neuroendocrinol. 2012;33:179-93.

Gonzalez-Gay MA, Garcia-Unzueta MT, Gonzalez-Juanatey C, Miranda-Filloy JA, Vazquez-Rodriguez TR, De Matias JM et al. Anti-TNF-alpha therapy modulates resistin in patients with rheumatoid arthritis. Clin Exp Rheumatol 2008;26:311-6.

Gulan G, Ravlic-Gulan J, Strbo N, Sotosek V, Nemec B, Matovinovic D et al. Systemic and local expression of perforin in lymphocyte subsets in acute and chronic rheumatoid arthritis. J Rheumatol 2003;30:660-70.

Han D, Tian Y, Zhang M, Zhou Z, Lu J. Prevention and treatment of experimental autoimmune encephalomyelitis with recombinant adeno-associated virus-mediated a-melanocyte-stimulating hormone-transduced PLP139-151specific T cells. Gene Ther 2007;14:383–95.

Harrington LE, Hatton RD, Mangan PR, Turner H, Murphy TL, Murphy KM et al. Interleukin 17-producing CD4+ effector T cells develop via a lineage distinct from the T helper type 1 and 2 lineages. Nat Immunol. 2005;6:1123-32.

Hill JA, Southwood S, Sette A, Jevnikar AM, Bell DA, Cairns E. Cutting edge: the conversion of arginine to citrulline allows for a high-affinity peptide interaction with the rheumatoid arthritis-associated HLA-DRB1*0401 MHC class II molecule. J Immunol 2003;171:538–41

Iwata Y, Matsushita T, Horikawa M, et al. Characterization of a rare IL-10-competent B-cell subset in humans that parallels mouse regulatory B10 cells. Blood 2011; 117:530–41.

Kalden DH, Scholzen T, Brzoska T, Luger TA. Mechanisms of the antiinflammatory effects of alpha-MSH. Role of transcription factor NF-κB and adhesion molecule expression. Ann NY Acad Sci 1999; 885: 254–61.

Kang YM, Zhang X, Wagner UG, Yang H, Beckenbaugh RD, Kurtin PJ, et al. CD8 T cells are required for the formation of ectopic germinal centers in rheumatoid synovitis. J Exp Med 2002;195:1325–36.

Kaser S, Kaser A, Sandhofer A, Ebenbichler CF, Tilg H, Patsch JR. Resistin messenger-RNA expression is increased by proinflammatory cytokines in vitro. Biochem Biophys Res Commun. 2003;309:286-90.

Kiessling R, Klein E, Pross H, Wigzell H. "Natural" killer cells in the mouse. II. Cytotoxic cells with specificity for mouse Moloney leukemia cells. Characteristics of the killer cell. Eur J Immunol 1975;5:117-21.

Kim S, Poursine-Laurent J, Truscott SM, Lybarger L, Song YJ, Yang L et al: Licensing of natural killer cells by host major histocompatibility complex class I molecules. Nature 2005;436:709-13.

Kim KW, Kim HR, Kim BM, Cho ML, Lee SH . Th17 cytokines regulate osteoclastogenesis in rheumatoid arthritis. Am J Pathol. 2015;185:3011-24.

Koch AE. The pathogenesis of rheumatoid arthritis. N Engl J Med. 2007;36:5–8.

Kotake S, Yago T, Kobashigawa T, Nanke Y. The Plasticity of Th17 Cells in the Pathogenesis of Rheumatoid Arthritis. J Clin Med. 2017;6. pii: E67. doi: 10.3390/jcm6070067.

Kurki P, Aho K, Palosno T, Heliovaara M. Immunpathology of rheumatoid arthritis: antikeratin antibodies precede the clinical disease. Arthritis Rheum 1992;35:914-7.

Lehrke M, Reilly MP, Millington SC, Iqbal N, Rader DJ, Lazar MA. An inflammatory cascade leading to hyperresistinemia in humans. PloS Med 2004;1(2):e45.

Leigh JP, Fries JF. Mortality predictors among 263 patients with rheumatoid arthritis. J Rheumatol. 1991;18:1307-12.

Leipe J, Grunke M, Dechant C, Reindl C, Kerzendorf U, Schulze-Koops H. Role of Th17 cells in human autoimmune arthritis. Arthritis Rheum. 2010 62:2876-85.

Leoni G, Patel HB, Sampaio AL, Gavins FN, Murray JF, Grieco P, Getting SJ, Perretti M. Inflamed phenotype of the mesenteric microcirculation of melanocortin type 3 receptor-null mice after ischemia-reperfusion. FASEB J. 2008;22:4228-38

Leoni G, Voisin MB, Carlson K, Getting S, Nourshargh S, Perretti M. The melanocortin MC(1) receptor agonist BMS-470539 inhibits leucocyte trafficking in the inflamed vasculature. Br J Pharmacol. 2010;160:171-80.

Liu F, Whitton JL, Slifka MK. The rapidity with which virus-specific CD8^{+} T cells initiate IFN-gamma synthesis increases markedly over the course of infection and correlates with immunodominance. J Immunol 2004;173:456–62.

Loser K, Brzoska T, Oji V, Auriemma M, Voskort M, Kupas V et al. The neuropeptide alpha-melanocyte-stimulating hormone is critically involved in the development of cytotoxic CD8+ T cells in mice and humans. PLoS One. 2010;5:e8958.

Lurati A, Bertani L, Marrazza M, Re KA, Bompane D, Scarpellini M. NK cell count as predictor of clinical response in patients with rheumatoid arthritis treated with rituximab. Biologics. 2012;6:83-7.

Mahdi H, Fisher BA, Källberg H, Plant D, Malmström V, Rönnelid J et al. Specific interactions between genotype, smoking and autoimmunity to citrullinated alpha-enolase in the etiology of rheumatoid arthritis. Nat Genet 2009;41:1319-24.

Mandrika I, Muceniece R, Wikberg JE. Effects of melanocortin peptides on lipopolysaccharide/interferon-gamma-induced NF-kappaB DNA binding and nitric oxide production in macrophage-like RAW 264.7 cells: evidence for dual mechanisms of action. Biochem Pharmacol. 2001;61:613-21.

Maska L, Anderson J, Michaud K. Measures of functional status and quality of life in rheumatoid arthritis: Health Assessment Questionnaire Disability Index (HAQ), Modified Health Assessment Questionnaire (MHAQ), Multidimensional Health Assessment Questionnaire (MDHAQ), Health Assessment Questionnaire II (HAQ-II), Improved Health Assessment Questionnaire (Improved HAQ), and Rheumatoid Arthritis Quality of Life (RAQoL). Arthritis Care Res (Hoboken). 2011;63 Suppl 11:S4-13.

Meenan RF, Anderson JJ, Kazis LE, Egger MJ, Altz-Smith M, Samuelson CO Jr. et al. Outcome assessment in clinical trials. Evidence for the sensitivity of a health status measure. Arthritis Rheum. 1984;27:1344-52.

Miltenburg AM, van Laar JM, de Kuiper R, Daha MR, Breedveld FC. T cells cloned from human rheumatoid synovial membrane functionally represent the Th1 subset. Scand J Immunol. 1992;35:603-10.

Moradi B, Schnatzer P, Hagmann S, Rosshirt N, Gotterbarm T, Kretzer JP et al. CD4^{+}CD25^{+}/highCD127low/[−] regulatory T cells are enriched in rheumatoid arthritis and osteoarthritis joints--analysis of frequency and phenotype in synovial membrane, synovial fluid and peripheral blood. Arthritis Res Ther. 2014;16:R97. doi: 10.1186/ar4545.

Mosmann TR, Cherwinski H, Bond MW, Giedlin MA, Coffman RL. Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. J Immunol 1986;136:2348-57.

Mountjoy KG, Robbins LS, Mortrud MT, Cone RD. The cloning of a family of genes that encode the melanocortin receptors. Science. 1992;257:1248-51

Moura RA, Cascao R, Perpetuo I, et al. Cytokine pattern in very early rheumatoid arthritis favours B-cell activation and survival. Rheumatology 2011; 50:278–82.

Nagaev I, Smith U. Insulin resistance and type 2 diabetes are not related to resistin expression in fat cells or skeletal muscle. Biochem Biophys Res Commun 2001;285:561-4.

Nestorov I. Clinical pharmacokinetics of TNF antagonists: How do they differ? Semin Arthritis Rheum 2005;34(5 Suppl 1)12-8.

Neumann Andersen G, Nagaeva O, Mandrika I, Petrovska R, Muceniece R, Mincheva-Nilsson L, et al. MC(1) receptors are constitutively expressed on leucocyte subpopulations with antigen presenting and cytotoxic functions. Clin Exp Immunol 2001;126:441–6.

Prevoo MLL, Van 'T Hof MA, Kuper HH, Van Leeuwen MA, Van De Putte LBA, Van Riel PLCM. Modified disease activity scores that include twenty-eight-joint counts: Development and validation in a prospective longitudinal study of patients with rheumatoid arthritis. Arthritis Rheum. 1995;38:44–8.

Quayle AJ, Chomarat P, Miossec P, Kjeldsen-Kragh J, Førre O, Natvig JB. Rheumatoid inflammatory T-cell clones express mostly Th1 but also Th2 and mixed (Th0-like) cytokine patterns. Scand J Immunol. 1993;38:75-82.

Rantapaa-Dahlqvist S, de Jong BA, Berglin E, Hallmans G, Sundin U, van Venrooij WJ. Antibodies against cyclic citrullinated peptide and IgA rheumatoid factor predict the development of rheumatoid arthritis. Arthritis Rheum 2003;48:2741-9.

Rennalls LP, Seidl T, Larkin JM, Wellbrock C, Gore ME, Eisen T, Bruno L.The melanocortin receptor agonist NDP-MSH impairs the allostimulatory function of dendritic cells. Immunology. 2010;129:610-9.

Rojas-Dotor S, Segura-Méndez NH, Miyagui-Namikawa K, Mondragón-González R. Expression of resistin, CXCR3, IP-10, CCR5 and MIP-1α in obese patients with different severity of asthma. Biol Res. 2013;46:13-20.

Sakaguchi S, Sakaguchi N, Asano M, Itoh M, Toda M. Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor-chains (CD25): breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. J Immunol 1995;155:1151–1164,

Sakaguchi S. Control of immune responses by naturally arising CD4+ regulatory T cells that express toll-like receptors. J Exp Med. 2003;197:397-401.

Samuels J, Ng YS, Coupillaud C, et al. Impaired early B cell tolerance in patients with rheumatoid arthritis. J Exp Med 2005; 201:1659–67.

Santamaria P. Effector lymphocytes in islet cell autoimmunity. Rev Endocr Metab Disord 2003;4:271–80.

Schellekens GA, de Jong BA, van den Hoogen FH, vand de Putte LB, van Venrooij WJ. Citrulline is an essential constituent of antigenic determinants recognized by rheumatoid arthritis-specific autoantibodies. J Clin Invest 1998;101:273-81.

Schiøth H, Chhajlani V, Muceniece R, Klusa V, Wikberg JE. Major pharmacological distinction of the ACTH receptor from other melanocortin receptors. Life Sci 1996;59:797-801.

Senolt L, Housa D, Vernerová Z, Jirásek T, Svobodová R, Veigl D, et al. Resistin in rheumatoid arthritis synovial tissue, synovial fluid and serum. Ann Rheum Dis. 2007;66:458–63.

Silman AJ, Pearson JE. Epidemiology and genetics of rheumatoid arthritis. Arthritis Res 2002;4:Suppl 3:S265-72.

Silswal N, Singh AK, Aruna B, Mukhopadhyay S, Ghosh S, Ehtesham NZ. Human resistin stimulates the pro-inflammatory cytokines TNF-alpha and IL-12 in macrophages by NF-kappaB-dependent pathway. Biochem Biophys Res Commun. 2005;334:1092–101.

Snir O, Widhe M, Hermansson M, von Spee C, Lindberg J, Hensen S et al. Antibodies to several citrullinated antigens are enriched in the joints of rheumatoid arthritis patients. Arthritis Rheum 2010;62:44-52.

Son YM, Ahn SM, Kim GR et al. Resistin enhances the expansion of regulatory T cells through modulation of dendritic cells. BMC Immunol 2010;11:33.

Steppan CM, Bailey ST, Bhat S, Brown EJ, Banerjee RR, Wright CM et al. The hormone resistin links obesity to diabetes. Nature. 2001;409:307-12.

Stolt P, Yahya A, Bengtsson C, Källberg H, Rönnelid J, Lundberg I et al. Silica exposure among male current smokers is associated with a high risk of developing ACPA-positive rheumatoid arthritis. Ann Rheum Dis 2010;69:1072-6.

Taherzadeh S, Sharma S, Chhajlani V, Gantz I, Rajora N, Demitri MT et al. Alpha-MSH and its receptors in regulation of TNFα production by human monocytes/macrophages. Am J Physiol 1999;276:R1289-94

Takemura S, Klimiuk PA, Braun A, Goronzy JJ, Weyand CM. T cell activation in rheumatoid synovium is B cell dependent. J Immunol. 2001;167:4710-8.

Tanaka Y, Maeshima K, Yamaoka K. In vitro analysis of a JAK inhibitor in rheumatoid arthritis. Ann Rheum Dis 2012;71 Suppl 2:i70-4.

Taylor A, Namba K. In vitro induction of CD25+ CD4+ regulatory T cells by the neuropeptide alpha-melanocyte stimulating hormone (alpha-MSH). Immunol Cell Biol. 2001;79:358-67.

Too CL, Muhamad NA, Ilar A, Padyukov L, Alfredsson L, Klareskog L et al. Occupational exposure to textile dust increses the risk of rheumatoid arthritis: results from a Malaysian population-based case-control study. RMD Open 2016;75:997-1002.

Van Hamburg JP, Asmawidjaja PS, Davelaar N, Mus AM, Colin EM et al. Th17 cells, but not Th1 cells, from patients with early rheumatoid arthritis are potent inducers of matrix metalloproteinases and proinflammatory cytokines upon synovial fibroblast interaction, including autocrine interleukin-17A production.

Vidard L, Kovacsovics-Bankowski M, Kraeft SK, et al. Analysis of MHC class II presentation of particulate antigens of B lymphocytes. J Immunol 1996; 156:2809–18.

Wikberg JE, Muceniece R, Mandrika I, Prusis P, Lindblom J, Post C, Skottner A. New aspects on the melanocortins and their receptors. Pharmacol Res. 2000;42:393-420.

Wikberg JE, Mutulis F, Mutule I, Veiksina S, Lapinsh M, Petrovska R et al. Melanocortin receptors: ligands and proteochemometrics modeling. Ann NY Acad Sci 2003;994:21-6.

Wikberg JE, Mutulis F. Targeting melanocortin receptors: an approach to treat weight disorders and sexual dysfunction. Nat Rev Drug Discov. 2008;7:307-23.

Wolff MJ, Leung JM, Davenport M, Poles MA, Cho I, Loke P. TH17, TH22 and Treg cells are enriched in the healthy human cecum. PLoS One 2012;7:e41373

Yamada H, Haraguchi A, Sakuraba K, Okazaki K, Fukushi JI, Mizu-Uchi H et al. Th1 is the predominant helper T cell subset that produces GM-CSF in the joint of rheumatoid arthritis. RMD Open. 2017;3(1):e000487. doi: 10.1136/rmdopen-2017-000487.

Yen JH, Moore BE, Nakajima T, Scholl D, Schaid DJ et al. Major histocompatibility complex class I-recognizing receptors are disease risk genes in rheumatoid arthritis. J Exp Med. 2001 May 21;193(10):1159-67.

Yeo L, Toellner KM, Salmon M, et al. Cytokine mRNA profiling identifies B cells as a major source of RANKL in rheumatoid arthritis. Ann Rheum Dis 2011;70:2022–8.

Young A, Dixey J, Cox N, Davies P, Devlin J, Emery P et al. How does functional disability in early rheumatoid arthritis (RA) affect patients and their lives? Results of 5 years of follow-up in 732 patients from the Early RA Study (ERAS). Rheumatology 2000;39:603–611.

Ytterberg AJ, Joshua V, Reynisdottir G, Tarasova NK, Rutishauser D, Ossipova E et al. Shared immunological targets in the lungs and joints of patients with rheumatoid arthritis: identification and validation. Ann Rheum Dis. 2015;74:1772-7.

Zeng P, Klareskog L, Alfredsson L, Bengtsson C. Physical workload is associated with risk of rheumatoid arthritis: results from a Swedish population-based case-control study. RMD open 2017;3:e00324. doi: 10.1136/rmdopen-2016-00324.

APPENDICES

Appendix A.:

Melanocortin 2, 3 and 4 Receptor Gene Expressions are Downregulated in CD8$^+$T Cytotoxic Lymphocytes and CD19$^+$ B Lymphocytes in Rheumatoid Arthritis responding to TNF-α Inhibition. Nagaev I, Andersen M, Olesen MK, Nagaeva O, Wikberg J, Mincheva-Nilsson L and Andersen GN

Appendix B.:

Resistin Gene Expression is Downregulated in CD4$^+$ T helper Lymphocytes and CD14+ Monocytes in Rheumatoid Arthritis Responding to TNF-α Inhibition. Nagaev I, Andersen M, Olesen MK, Nagaeva O, Wikberg J, Mincheva-Nilsson L and Andersen GN. Scandinavian Journal of Immunology, Vol. 84, Nr. 4, 2016, s. 229-236.

Appendix C.:

Regulated melanocortin receptor 1-5 gene expressions found in CD56$^+$ NK cell in rheumatoid arthritis Andersen M, Meyer MK, Nagaev I, Nagaeva O, Wikberg J, Mincheva-Nilsson L and Andersen GN.