Regulated Melanocortin Receptor 1-5 Gene Expressions in CD56+ NK Cells from Two Rheumatoid Arthritis Patients Treated with Adalimumab

Marlene Andersen1, Michael Kruse Meyer1, Ivan Nagaev2, Olga Nagaeva2, Jarl Erik Sylvester Wikberg3, Lucia Mincheva-Nilsson4 and Grethe Neumann Andersen5
1Department of Rheumatology, North Denmark Regional Hospital/Department of Health Science and Technology, Aalborg University, Denmark
2Department of Clinical Microbiology, Division of Clinical Immunology, Norrland’s University Hospital, Umea, Sweden
3Department of Pharmaceutical Biosciences, Uppsala University, Uppsala, Sweden
4Department of Rheumatology, North Denmark Regional Hospital/Department of Clinical Medicine, Aalborg University, Denmark

Abstract

The role of the endogenous, proresolving melanocortin system is still essentially unexplored in human auto-immune diseases. Melanocortin receptor (MCR) 1-5 genes and proteins are expressed in several leukocyte subsets and MCR1-3 and MCR5 mRNAs have been found in CD56+ natural killer (NK) cells from healthy humans. CD56+ NK cells may be decisive in the induction of auto-immunity and their cytokine profile assists in the direction of naïve CD4+ T cells into specific effector or regulatory T cell subsets. We explored the possibilities of affecting CD56+ NK cell function through MCR1-5. Thus, we determined the response of MCR1-5 gene expression in active rheumatoid arthritis (RA) to TNFα inhibition (I) with adalimumab. CD56+ NK cells from RA patients treated with adalimumab were separated by immune-magnetic beads before and after three months of adalimumab treatment. Total RNA was extracted and mRNAs for MCR1-5 and a panel of cytokines were measured by qRT-PCR. All MCR genes, including MCR4, not previously described in NK cells, were expressed in CD56+ NK cells in active RA and reacted to adalimumab by down-regulation. In addition, the gene expression of cytokines important for CD56+ NK cell activity, i.e. especially TNFα and IFNγ were down-regulated. NK cell activity in RA may thus be modulated by MCR signalling. The response of MCR1-5 gene expressions in NK cells to adalimumab reinforces this perception. We propose that MCR signalling in NK cells may represent a new endogenous pathway to counteract autoimmune inflammation in RA.

Introduction

This is the first description of the regulation of the anti-inflammatory, immune tolerance inducing and tissue preserving melanocortin system in CD56+ natural killer (NK) cells in rheumatoid arthritis (RA).

Rheumatoid arthritis is widespread and affects about 1% of the population in western countries. RA is T cell mediated, implying activation and expansion of autoreactive T cells, their migration into the synovium and induction of local inflammation with monocyte activation and osteoclastogenesis, leading to cartilage damage and bone resorption. The immune response in RA is predominantly of the T helper (h) 1 mediated type [1]. At present RA patients face life-long treatment with immunosuppressive agents often in the form of biologic disease modifying agents (bDMARDS), such as for example monoclonal antibodies against pro-inflammatory cytokines: tumor necrosis factor alpha (TNFα), IL-6, IL-1 or membrane protein CD20 on B-cells [2].

The melanocortin system comprises five G protein-coupled seven-helix transmembrane receptors, MCR1-5, discovered in the early 1990ties [3] and are products of their own ligands, the melanocortins [4]. We have previously demonstrated MCR1-3 and MCR5 gene expressions in various leukocyte subsets including CD56+ NK cells from healthy persons [5,6]. Beyond exerting anti-inflammatory and immune tolerance inducing properties in immune cells [7], MCR1-5 have diverse other physiological roles [8]. Thus, MCR1 was primarily found on melanocytes controlling pigmentation, while MCR2 is the adrenocorticotropic (ACTH) receptor of the adrenal cortex per se. MCR3 and MCR4 are expressed in the central nervous system, especially in the hypothalamus,
where they are involved in energy homeostasis and control of libido. Of peripheral human cells, the expression of MCR4 has been demonstrated in melanocytes, keratinocytes [9] and dendritic cells, in which MCR4 partake in the control of cell adhesion and antigen presentation [10]. MCR5 was primarily found in exocrine glands, controlling secretion.

The ligands of the MCRs are the melanocortins, a family of hormone peptides, derived from a common precursor protein: pro-opio-melanocortin (POMC). POMC is cleaved into α-, β- and γ- melanocyte stimulating hormone (MSH) and ACTH and several other peptides including β-endorphin. POMC was originally found in the pituitary, from where melanocortins are released into the blood stream. Recently, POMC mRNA has been detected in several leukocyte subsets, e.g. helper and cytotoxic T cells, B cells, monocytes and NK cells [6]. The synthesis of the melanocortins in various peripheral tissues and cells is stimulated by pro-inflammatory TNFα and inhibited by TGFβ [11]. Hypothetically therefore, in leukocytes, the melanocortins may act in an autocrine and paracrine manner to reduce inflammatory cytokine production and leukocyte trafficking [12]. Alpha-MSH may even direct the immune reaction by inducing regulatory T-lymphocyte differentiation [7]. Furthermore, α-MSH decreases antibody production by plasma cells [13]. In monocytes, α-MSH predominantly inhibits the nuclear translocation of NFκB, which controls the transcription of multiple inflammatory mediators such as TNFα [14].

NK cells are large granular lymphocytes with cytotoxic and cytokine producing potentials, which constitute 5-25 % of peripheral blood lymphocytes [15]. They express multitudes of receptors enabling the identification and lysis of stressed, infected, or tumour cells and are part of the innate immune system. Interestingly, accumulating evidence points at NK cells as important in autoimmunity due to their capacity to direct the adaptive immune response by cytokine release [16]. The low frequency of NK cells in the circulation has made their isolation challenging and limited the number of reports on their function.

The role of NK cells in RA is still controversial and it is not established whether NK cells contribute to disease development or act to protect from tissue damage. In active RA, the circulating number of CD56+ NK cells is reduced, but increase in patients responding to bDMARDs such as etanercept [17], tocilizumab [18] and rituximab [19]. A very low number of NK cells is characteristic of active disease in rheumatoid factor (RF) and anti-cyclcic citrullinated protein antibody (ACPA) positive patients [20] and predicts a poorer response to bDMARD [18]. Not only the number, but also phenotypic characteristics of NK cells such as IFNγ expression [21], perforin A and granzyme contents are restored by bDMARDs [18].

Still, there are many question marks concerning the function of NK cells in RA. Although some NK functions have been elucidated, the question whether NK cells are friends or foes in RA remains [21]. Considering this, we examined the gene expression of MCR1-5, the activation of which has anti-inflammatory, tolerance inducing and pro-resolving properties in other leukocyte subsets. To evaluate the regulation of MCR1-5 gene expressions in NK cells in RA, we examined the reaction of MCR1-5 gene expressions to TNFα inhibition with adalimumab in the context of changes in Th1, inflammatory and regulatory cytokine gene expressions.

Patients and Methods

The study was approved by the ethics committee of Northern Jutland, Denmark (N-20100060). All patients gave their informed consent both orally and in writing.

Patients

Two patients starting adalimumab were included according to the advice of the “Council for Expensive Hospital Medicine” (RADS). RADS recommends bDMARD to RA patients with disease activity score on the examination of 28 joints and using C-reactive protein (CRP) levels as a measure of inflammatory activity (DAS28) (CRP > 3.2 for more than three months not responding to two conventional DMARDS or DAS28(CRP) > 5.1 at two consecutive consultations. Both patients, included in the study, were females with definite RA according to the American College of Rheumatology (ACR)/ European League against Rheumatism (EULAR) criteria from 2010 [22]. The mean age was 40.5 ± 10.5 (mean ± SD) years. Mean disease duration was 21.5 ± 0.5 months. Both patients were RF positive and antinuclear antibody (ANA) negative, one produced anti-citrullinated protein antibody (ACPA). Disease activity score in 28 joints (CRP) in the patients at start of adalimumab was 4.7 ± 0.2 (mean ± SD). At the time of inclusion in the study, both patients medicated with methotrexate, mean dose 21.25 mg/week (17.5 and 25 mg/week, respectively) and in addition one of the patients medicated with 5 mg prednisolone/day. One of the patients was a smoker with 10 pack years. Both patients had normal BMI and no co-morbidity.

Treatment with adalimumab

The patients were instructed in the use of adalimumab (Humira®) 40 mg injection pens. The first injection of 40 mg adalimumab was taken subcutaneously into the thigh or abdomen at the clinic under supervision of a specialized nurse. Subsequently, 40 mg adalimumab was administered every other week by the patients. Humira® injection pens were handed for free to the patients monthly at the clinic. The patients accounted for their use. Follow-up blood samples were gathered 12 days after the sixth injection of adalimumab.

Electronic patient self-reported outcome (DANBIO)

Patients reported pain-fatigue and patient’s global on a 100 mm visual analogue scale (VAS), as well as answered the health assessment questionnaire (HAQ) electronically before and three months after start of adalimumab treatment. Recordings were saved in DANBIO, a national electronic data register initiated to survey RA patients treated with bDMARDS.

Methods

Isolation of peripheral blood mononuclear leukocytes (PBMC)

Peripheral blood samples were donated by two patients with definite, active RA, before and after three months of adalimumab. For lymphocyte isolation, whole blood was diluted 1:1 with Tris-buffered Hank’s salt solution, pH 7.2, and subjected to Ficoll-isopaque (Lymphoprep, Nycomed, Oslo, Norway) gradient centrifugation. The interface containing PBMC was collected. The CD56+ NK subpopulation of PBMC was isolated by Mab coupled to Dynabeads (Dynal, Oslo, Norway). In brief, Dynabeads coated with Dynabeads®
with anti-CD56 mAb, were incubated with the isolated PBMC for 2 h with end-to-end rotation at 4°C. The cell fraction bound to the beads was washed five times with PBS and used for RNA extraction.

Monoclonal antibodies

The monoclonal antibody against the IgG1 MY 31 isofrom of neural cell adhesion molecule (NCAM, CD56), NK cells 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.

Total RNA extraction

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

Quantitative reverse transcription-polymerase chain reaction (RT-qPCR)

Custom gene expression analysis according to the MIQE guidelines (23) was performed. Both RNA yield (on average 2005 ng in total volume 30 µl) and purity (average A260/280 was 1.7) were assessed by NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, 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 Single RT-qPCR Test). Therefore, as previously optimized and tested constancy of threshold cycle (Ct) values for 18S rRNA in every transcriptase, reaction buffer and dNTP mix (High-Capacity cDNA reverse transcriptase, reagent buffer and dNTP mix (High-Capacity cDNA Reverse Transcription Kit, Catalog # 4368813, Thermo Fisher Scientific Inc.). RNA integrity, inhibition testing efficiency of reverse transcription and cDNA stability were evaluated at once by the constancy of threshold cycle (Ct) values for 18S rRNA in every single RT-qPCR test. 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, Thermo Fisher Scientific Inc.).

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 accession numbers and the targeted splice variants, location and identity of any accessions and the targeted splice variants, location and identity of any

Statistics

Our data, obtained by examining gene expressions in the CD56+ NK cell subset in two patients with active RA, are presented as the mean, and standard deviation (SD). As can be seen from the figures, the measurements were very similar in the two patients. The patients functioned as their own controls. The use of paired observations is an advantage as this method rules out bias due to physiological differences and external factors, which are inevitable when using controls.

Results

Patients

Both patients responded to adalimumab (Table 1). Methotrexate was continued at the same dose as at baseline to avoid the production of antibody against adalimumab. The patient treated with prednisolone stopped this treatment three weeks after start of adalimumab. Both patients took a total of six injections of adalimumab. The two patients both subjectively and clinically had a good response to adalimumab and all the measured parameters improved, DAS28 from 4.7 ± 0.2 (mean ± SD) to 2.9 ± 0, HAQ from 1.37 ± 0.375 to 0.812 ± 0.437, number of tender joints decreased from 9.0 ± 1 to 2.5 ± 0.5 and number of swollen joints from 5.5 ± 1.5 to 1.5 ± 0.5. Also biochemical parameters showed improvement, thus leukocyte number was reduced from 9.75 ± 3.55 (mean ± SD) to 9 ± 3.3, platelets from 406.5 ± 10.5 to 370.0 ± 18.0 and CRP from 11.0 ± 8.0 to 2.2 ± 1.6.

Fold change in MCR1-5 gene expressions in CD56+ NK cells due to adalimumab treatment in RA

All five MCR genes were expressed in CD56+ NK cells.

Table 1: Characteristics of the two rheumatoid arthritis patients.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Before treatment</th>
<th>After 3 months of adalimumab</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Age, years</td>
<td>51</td>
<td>30</td>
</tr>
<tr>
<td>Disease duration, months</td>
<td>22</td>
<td>21</td>
</tr>
<tr>
<td>Satisfied 2010 ACR/EULAR criteria for RA</td>
<td>yes</td>
<td>yes</td>
</tr>
<tr>
<td>CRP mg/L (&lt;3 mg/L)</td>
<td>19</td>
<td>3</td>
</tr>
<tr>
<td>Hemoglobin mmol/L (7.3 – 9.5 mmol/L)</td>
<td>5.83</td>
<td>8.1</td>
</tr>
<tr>
<td>Leukocytes x10⁹/L (3.5 – 10.0 x 10⁹/L)</td>
<td>13.3</td>
<td>6.2</td>
</tr>
<tr>
<td>Neutrophils x10⁹/L (2.0 – 7.0 x 10⁹/L)</td>
<td>6.87</td>
<td>3.32</td>
</tr>
<tr>
<td>Lymphocytes x10⁹/L (1.3 – 3.5 x 10⁹/L)</td>
<td>5.22</td>
<td>2.25</td>
</tr>
<tr>
<td>Platelets, x10¹²/L (165 – 400 x 10¹²/L)</td>
<td>396</td>
<td>417</td>
</tr>
<tr>
<td>IgM-RF (&lt; 3.5 kIU/L)</td>
<td>64</td>
<td>267</td>
</tr>
<tr>
<td>ACPA (&lt;14 AE/L)</td>
<td>0</td>
<td>74</td>
</tr>
<tr>
<td>ANA (0)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Methylxol, mg/week</td>
<td>17.5</td>
<td>25</td>
</tr>
<tr>
<td>Prednisolone, mg/day</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>DAS28(CRP)</td>
<td>4.9</td>
<td>2.90</td>
</tr>
<tr>
<td>HAQ score (0)</td>
<td>1.750</td>
<td>1.000</td>
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<tr>
<td>Swollen joint count out of 40</td>
<td>7</td>
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</tr>
<tr>
<td>Tender joint count out of 40</td>
<td>8</td>
<td>10</td>
</tr>
<tr>
<td>Smoker</td>
<td>yes</td>
<td>never</td>
</tr>
</tbody>
</table>

IgM-RF = IgM-rheumatoid factor; ACPA = anti-citrullinated protein antibodies; ANA = antinuclear antibodies; ACR = American College of Rheumatology; DAS28 = Disease Activity Score in 28 joints. HAQ = Health Assessment Questionnaire.
(figures 1 and 2). The MCR4 gene was, to the best of our knowledge, demonstrated for the first time in NK cells. Fold changes in the two patients with active RA after three months of adalimumab were MCR1 0.82 ± 0.40 (mean ± SD); MCR2 0.55 ± 0.04; MCR3 0.33 ± 0.12; MCR4 0.39 ± 0.16 and MCR5 0.50 ± 0.19, where 0.5 denotes a twofold decrease in mRNA and 0.25 a fourfold decrease, etc.

The down-regulation of MCR gene expression due to adalimumab was most pronounced in MCR3 and MCR4. MCR1 gene expression was down-regulated in one patient and slightly up-regulated in the other.

Fold change in Th1-, inflammatory- and regulatory cytokine gene expressions, IL-8, IL-15 and resistin gene expressions in CD56+ NK cells due to adalimumab in RA

Fold changes after three months of adalimumab were: IFNγ 0.23 ± 0.04 (mean ± SD); TNFβ 0.80 ± 0.06; IL-1β 0.06 ± 0.04; TNFα 0.18 ± 0.14; IL-10 0.04 ± 0.01; TGFβ 0.49 ± 0.01; IL-8 0.06 ± 0.06; IL-15 0.89 ± 0.77 and for resistin 0.12 ± 0.01, where 0.5 denotes a twofold decrease in mRNA and 0.25 a fourfold decrease, etc. (Figures 1 and 2).

Thus IFNγ, TNFα, IL-1β, IL-10, TGFβ, IL-8 and resistin gene expressions were all downregulated in the CD56+ NK cell subset of both patients with active RA after three months of adalimumab.

TNFβ and IL-15 gene expressions were downregulated in one patient and slightly upregulated in the other.

Discussion

We here present the results of our systematic evaluation of the gene expressions of MCR1-5 in CD56+ NK cells and the reaction of MCR1-5 gene expressions to adalimumab in two patients with active RA responding to treatment. Our examination showed that the MCR1-5 genes were expressed and reacted with down-regulation to adalimumab. Also the cytokines of the Th1 response, inflammatory and regulatory cytokines were down-regulated during adalimumab in CD56+ NK cells, accompanied by a decrease in clinical signs and biochemical measures of disease...
activity. In summary, our findings indicated that adalimumab resulted in the downregulation of MCR1-5 as well as of Th1, inflammatory and regulatory cytokine gene expressions.

The discontinuation of prednisolone in one of the patients, all else being equal, would diminish the fall in MCR1-5 gene expressions as prednisolone like adalimumab has anti-inflammatory effects. Thus discontinuation of prednisolone would counteract the fall in MCR1-5 gene expressions, reported in this study.

The fall in MCR1-5 gene expressions during adalimumab is not surprising as the MCR1-5 genes are upregulated by their own ligands, especially α-MSH, the synthesis of which is stimulated by pro-inflammatory TNFα [11], which drives inflammation in RA. The decrease in MCR1-5 as well as IL-10 and TGFβ gene expressions during adalimumab indicate that even regulatory, pro-resolving pathways are activated in CD56+ NK cells from two patients with active RA treated with adalimumab for three months. 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 four-fold decrease etcetera.

To the best of our knowledge, the finding of regulated MCR1-5 gene expression in CD56+ NK cells has not previously been reported. As the effects of the melanocortin system are immune tolerance inducing, anti-inflammatory, pro-resolving and tissue preserving, an upregulation of MCR1-5 genes points at an effort by the CD56+ NK cells to counteract/balance the pathogenetic immune reaction in RA. Our findings reveal a new immune regulating system in the NK cell subset. As a low number of NK cells heralds a poor response to bDMARD in RA [18], NK cells seem to influence disease activity, possibly through the melanocortin system.

The discovery of a new pathway, by which NK cell activity might be modified, may turn out to be attractive, as NK cells influence osteoclastogenesis in RA. Thus, osteoclastogenesis and bone erosion seem to be favoured by NK cell activity in RA [24,25].

In the CD56+ NK cells from both our patients, there was a greater fall in MCR3 gene expression during adalimumab than in any other MCR gene expression. Although a small number of patients, the finding points at a more pronounced responsiveness of MCR3 gene expression to changes in the inflammatory cytokine environment. Our finding is in accordance with the reported predominance of MCR3 signaling in monocytes in animal experimental inflammatory states [26,27]. Our findings support the notion of melanocortin system activity in cells of innate immunity, to which both NK cells and monocytes belong.

To the best of our knowledge, MCR4 gene expression in NK cells has not previously been reported in man. In human immune cells, the expression of the MCR4 gene has only been reported in dendritic cells, which express MCR4 mRNA as well as MCR1, 2, 3 and 5 mRNAs. When dendritic cells were stimulated with NDP-MSH, a potent synthetic derivative of α-MSH, several markers of dendritic cell central activities were down regulated. That is the expression of the antigen presenting non-classical MHC CD1a molecule was reduced together with the expression of intercellular adhesion molecule 1 and co-stimulatory molecule CD86 [10].

In active, inflammatory diseases such as RA, the production of melanocortins including α-, β-, γ-MSH and ACTH by immune cells will typically be increased [28]. The higher affinity for β-MSH compared to other naturally occurring melanocortins distinguishes MCR4 [29]. Differential affinity exhibited by MCRs for specific subtypes of MSH allows directed signalling. Thus signalling through MCR3, which has high affinity for γ-MSH, has been found to dominate the anti-inflammatory reaction in uurate-crystal induced arthritis and peritonitis in mice [30], while signalling through the MCR5 of Th cells dominates, when inducing regulatory T cells in mice with experimental autoimmune uveoretinitis [7]. Reports on specific effects of MCR4 signalling in immune cells are for good reasons still missing.

As NK cells, due to their ability to produce large quantities of cytokines, may take part in the early direction of the immune response in inflammatory, autoimmune diseases, it is important to map the pathways by which NK cell activity can be controlled. We here report of the presence of the proresolving melanocortin system, adjustable to changes in cytokine environment in human NK cells. Our findings may result in a new perception of the NK cell subset and highlight NK cells as a probable target for early therapeutic efforts in RA [31]. The findings in our two patients were in good accordance and, despite the small number, might inspire to further investigations.

Further studies are however needed, comprising a larger number of patients. Our quantitative measurements of MCR1-5
gene expressions could favorably be supplemented with flow cytometric measurements to estimate the relation between gene expressions and cell membrane expressions of MCRI-5.

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References


