Peripheral lymphocyte multidrug resistance activity as a predictive tool of biological therapeutic response in rheumatoid arthritis

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Running head: MDR activity in RA

ABSTRACT

Objective: Multidrug resistance (MDR) transporters may be used as biomarkers to monitor disease progression in RA and as a predictive tool to establish responsiveness to biological therapy. In this multicenter clinical trial, we aimed to assess the predictive value of MDR1, MRP1 and BCRP activity measurement for biological therapeutic response in RA before as well as 4 to 6 and 12 weeks after the initiation of biological therapy.

Methods: Peripheral blood samples were collected from 27 bDMARD Responders and 12 Non-responders at the indicated time points as well as from 35 healthy controls. MDR activity (MAF) of MDR1, MRP1 and BCRP was measured in CD3+ and CD19+ cells using the Solvo MDQ Kit™ and cell surface staining by flow cytometry following PBMC isolation.

Results: At the start of therapy, MAFC (composite MAF of MRP1 and MDR1) and MAFMDR values and at 4 to 6 weeks of treatment, MAFC, MAFMRP and MAFMDR values of CD3 cells were higher in Non-responders compared to Responders. ROC analysis revealed that RA patients with MAFC values above 21.3 in CD3 cells at the start of bDMARD therapy are likely to be Non-responders. At 4 to 6 weeks of treatment, MAFC values above 20.3, MAFMRP values above 6.0 and MAFMDR values above 13.9 in CD3 cells also predict unfavorable response.

Conclusions: Our results indicate that the determination of MAFC values in CD3 cells of RA patients may be of predictive value prior to the initiation of biological therapy to establish whether the patient will demonstrate sufficient therapeutic response.

KEYWORDS

BCRP, bDMARD, MDR1, MRP1, multidrug resistance, T cells, therapeutic response

INTRODUCTION

Rheumatoid arthritis (RA) affects approximately 0.5-1% of the population and causes chronic synovial inflammation eventually leading to joint destruction and disability [1]. Early diagnosis and immediate, effective therapy are crucial in order to prevent joint deterioration, functional disability and unfavorable disease outcome. The optimal management of RA is needed within 3-6 months after the onset of disease, therefore a very narrow „window of opportunity" is present to achieve remission or at least low disease activity (LDA) [2,3]. Therefore, it is very important to predict the efficacy of expensive biologicals at early stages of treatment. Although a new generation of drugs is available, there are no validated circulating biomarkers of prognostic use or to predict response to specific therapies [4].

Multidrug resistance (MDR-ABC) transporters (MDR1/P-gp/ABCB1; MRP1/ABCC1; BCRP/ABCG2) are important components in the development of drug resistance in malignancies [5] and in autoimmune conditions, such as RA [6]. Although studies of the crystal structure and function of MDR-ABC transporters suggest that they are not directly involved in the release of cytokines and chemokines, they may extrude other intracellular small molecules influencing the inflammatory balance. Thus they may play an important role in the pathogenesis of RA via influencing cell migration, proliferation and inflammation in an indirect manner. Therefore, MDR-ABC transporters may also be important biomarkers of disease progression in RA. The assessment of MDR protein activity may help physicians to evaluate how patients will respond to biological treatment and may support the decision whether there is a necessity to modify the treatment.

The most important csDMARDs (including methotrexate, sulfasalazine, leflunomide and hydroxychloroquine) are substrates of MDR proteins. For this reason, MDR activity of RA patients on csDMARD therapy has been extensively studied and the expression, polymorphisms and activity of MDR proteins has been linked to therapeutic success of csDMARDs, especially that of methotrexate. However, little is known about the relation of MDR proteins to therapeutic success of biologicals, such as anti-TNF agents. Although these molecules do not enter the cell, and are therefore not substrates of MDR proteins, the endobiotics, such as the cytokines they target are known to interact with these transporters [7-9]. Through influencing the distribution of cytokines and other soluble factors within the cell and in its most proximal environment, the function of T- and B-cells may be affected by the activity of the transporters, balancing the effect of such factors on lymphocyte activation, proliferation, production of other cytokines and antibodies, etc. Therefore, MDR activity may be used as a biomarker of therapeutic success in RA and other autoimmune disorders.

In this multicenter clinical trial, we aimed to assess the predictive value of flow-cytometry based multidrug resistance activity measurement for biological therapeutic response in rheumatoid arthritis. We aimed to assess the activity of three clinically relevant MDR proteins (MDR1, MRP1, BCRP) in CD3+ and CD19+ lymphocytes of RA patients before as well as 4 to 6 and 12 weeks after the initiation of biological therapy.

METHODS

Patient Recruitment

39 RA patients were recruited at the outpatient clinics of the Department of Rheumatology, University of Debrecen, Hungary and the Department of Rheumatology and Clinical Immunology, Charité, Berlin, Germany. Patients were sampled before the start of biological treatment as well as between 4 and 6 weeks and at 12 weeks of treatment. DAS28 and CRP values were also recorded at this time. Patients were regarded as non-responders (n = 12) if DAS28 values showed a decrease of less than 25% between the start of biologicals and at 12 weeks of treatment (arbitrary cut-off). Patient characteristics as well as details of the therapy received are included in Table 1.

Healthy controls (n = 35) were sampled at the Department of Rheumatology, University of Debrecen, Hungary on a single occasion. They had a negative history of autoimmune disorders including RA and a negative status upon physical examination as well as no infectious symptoms within three weeks before sampling.

Exclusion criteria for all participants included chronic infectious diseases requiring systemic treatment, autoimmune diseases other than RA, immunodeficiencies, allergic diseases and hematological malignancies or solid tumors.

Written informed consent was obtained from all participants and the study adhered to the tenets of the most recent revision of the Declaration of Helsinki. Ethical approval for the study was granted by local ethics committees of the University of Debrecen (TUKEB 21018/2014/EKU) and Charité (EA1/193/10), respectively.

Peripheral Blood Mononuclear Cell (PBMC) isolation

6 mls of EDTA anticoagulated peripheral blood sample was collected. PBMCs were separated by density gradient centrifugation using Ficoll Histopaque-1077 (Cat. No: H8889, Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer's instructions.

Flow Cytometry

Measurements were conducted on a BD FACSCalibur flow cytometer (BD Biosciences, San Diego, CA, USA) equipped with 488 nm and 635 nm lasers or on a Miltenyi MACSQuant flow cytometer, equipped with 405nm, 488 nm and 638 nm lasers, respectively.

The Solvo MDQ Kit™ was used strictly following the manufacturer's instructions. In this assay, fluorescent reporter substrates are trapped in the cytoplasm and pumped out by MDR proteins depending on the presence or absence of highly selective inhibitors, allowing for quantitative, standardized assessment. PBMCs were loaded with fluorescent MDR activity reporter substrates (1.25 uM working solution of Calcein-AM for MDR1 and MRP1, em: 515 nm, incubated for exactly 10 minutes followed by rapid centrifugation and 1 mM working solution of mitoxantrone for BCRP, em: 684 nm, respectively, incubated for exactly 30 minutes followed by rapid centrifugation) and treated with highly selective MDR protein inhibitors (12.7 mM working solution of verapamil for MDR1 and MRP1, 2 mM working solution of indomethacin for MRP1 and 0.1 mM working solution of KO134 for BCRP,

respectively) to obtain multidrug activity factor (MAF) values (Figure 1). Note that the above working concentrations are further diluted upon addition to the cell suspension to avoid cell toxicity as described in the manufacturer's instructions.

Cell surface staining was applied to select CD3+ and CD19+ cells using anti-human CD3-PerCP and CD19-PE monoclonal antibodies (Cat. No: 345766 and 345789, respectively, both BD Biosciences) in case of Calcein-AM stained cells and anti-human CD3-FITC and CD19-PE monoclonal antibodies (Cat. No: 345764 and 345789, respectively, both BD Biosciences) in case of mitoxantrone stained cells according to the manufacturer's instructions. Samples were run on a flow cytometer immediately following cell surface staining.

Activities of multidrug transporters are reflected by the difference between the amount of Calcein/mitoxantrone accumulated in the presence or absence of the selective inhibitor(s). When calculating the MAF values, this accumulation difference is normalized to the dye uptake measured in the presence of the inhibitor. Thus, the result of the test becomes independent from factors influencing the cellular accumulation of Calcein/mitoxantrone other than the activity of the multidrug transporters. The inter-assay variability of the test is CV <10%.

MAF values were calculated from the difference between the geometric mean fluorescent intensity (MFI) of cells with and without the highly selective inhibitors, respectively.

MAFC (composite MAF of MRP1 and MDR1) = 100 x (Fmax – Fo) / Fmax

MAF of MRP1 = 100 x (FMRP1 – Fo) / Fmax

MAF of MDR1 = MAFC – MAF of MRP1

MAF of BCRP = 100 x (FMX – F0) / FMX

Fmax/FMX: Calcein/mitoxantrone fluorescence with verapamil or KO134, respectively

Fo: fluorescence without inhibitor

FMRP1: Calcein fluorescence with indomethacin

Statistics

Comparisons were made using the Kruskall-Wallis test or the Mann-Whitney test as the distribution of data appeared to be non-normal according to the Shapiro-Wilk test. p values < 0.05 were considered significant. Statistics were calculated using the GraphPad Prism 5 software (La Jolla, CA, USA).

RESULTS

DAS28 values decreased upon treatment in Responders in contrast to Non-responders. Of note, initial DAS28 values were higher in the Responder group compared to Non-responders (Table 2, Figure 2). Neither differences were observed in CRP values between the two group, nor changes in CRP were demonstrated upon treatment.

MAF of MRP1 in CD3 cells was higher at 12wk in Non-responders compared to Controls. No other statistically significant difference was noted in MAF values between Controls and RA patients.

Control values were within the reference range established in our earlier study [10].

At the start of therapy, MAFC and MAFMDR values of CD3 cells were higher in Non-responders compared to Responders. At 6wk, MAFC, MAFMRP and MAFMDR values of CD3 cells as well as MAFMRP values of CD19 cells were higher in Non-responders compared to Responders (Table 2).

No significant changes were demonstrated in MAF values in the respective RA patient groups with the progress of treatment (Figure 3).

No difference was demonstrated in MAFBCRP values in CD3 or CD19 cells between Responders and Non-responders.

ROC analysis was performed to evaluate the predictive value of MAF for response to treatment in RA patients at the start of biological therapy and at 6wk. Cut-off thresholds were calculated for MAF values with ROCs of adequate p and AUC values (Figure 4). Patients with MAF values above the respective cut-off thresholds are likely to be Non-responders to treatment (MAFC of CD3 cells at 0wk: $p = 0.043$, AUC = 0.68; MAFC of CD3 cells at 6wk: $p = 0.033$, AUC = 0.72; MAFMDR on CD3 cells at 6wk: $p = 0.048$, AUC = 0.70; MAFMRP on CD3 cells at 6wk: $p = 0.049$, AUC = 0.69).

DISCUSSION

Our results indicate that at the start of therapy, MAFC and MAFMDR values, and later at 4 to 6 weeks of treatment, MAFC, MAFMRP and MAFMDR values of CD3 cells were higher in Non-responders to bDMARD compared to Responders among RA patients. ROC analysis revealed that RA patients with MAFC values above 21.3 in CD3 cells at the start of bDMARD therapy are likely to be Non-responders.

While MDR1 expression on healthy CD4+ and CD19+ lymphocytes is only marginal, significant upregulation was demonstrated in RA patients [11,12] as a result of the presence of danger-associated molecular patterns (DAMPs). Furthermore, the expression level of MDR1 was significantly elevated in methotrexate (MTX) non-responder patients compared to responders. The YB-1 transcriptional factor may have an essential role in the regulation of MDR1 in lymphocytes of RA patients by translocation from the cytoplasm into the nucleus. Inflammation-derived TNF-a appears to play a crucial role in this phenomenon [13].

In an earlier study, Rhodamine 123 was used as a substrate of MDR1 and verapamil as transporter inhibitor [14]. Flow cytometry based analysis did not find any correlations between MTX responders and non-responders at baseline, however in non-responders the functional activity was upregulated 4 months following therapy.

In a more recent study, the activity of MDR1 and MRP1 was investigated on different leukocyte subsets, namely granulocytes, monocytes, lymphocytes, CD4+, CD8+ and CD19+ cells from RA patients and controls (traumatic injury patients and healthy volunteers, respectively). Based on DAS28 scores, RA patients fell into MTX responder and non-responder groups. Since side effects easily develop during MTX treatment, an additional MTX intolerant group was generated with intolerable side effects. In case of granulocytes, the functional activity of MRP1 was significantly higher in MTX responders vs. MTX non-responders. Furthermore, even higher functional activity was demonstrated in MTX intolerant individuals in comparison with MTX responders. Therefore, the authors concluded that determining MAF values might be useful in predicting MTX intolerance in order to avoid harmful side effects of MTX therapy [15].

Although the role of MDR transporter activity in the prediction of response to MTX has been characterized in RA, little is known about the relation of MDR proteins to therapeutic success of biologicals. In contrast to MTX and other csDMARDs, these molecules do not enter the cell, and are therefore not substrates of MDR proteins. However, the cytokines they target may indirectly interact with these transporters [7-9]. For instance, a recent study described that stimulation with TNF-a induced MDR1 and MRP1 expression via NF-kB signaling in astrocytes [16]. Therefore, we hypothesized that MDR activity may be used as a biomarker to predict therapeutic success in RA. A similar crosstalk in lymphocytes could provide the molecular basis of the findings of our clinical study, yet to be confirmed in future investigations.

Our ROC analysis revealed that the assessment of multidrug activity of peripheral blood lymphocytes carries predictive value for response to bDMARD treatment in RA patients at the start of therapy. Patients with MAF values above the cut-off thresholds are likely to be

Non-responders to treatment. Of note, these cut-off values are all below the respective reference ranges in healthy individuals established in our earlier study.

Although baseline MAF values before the start of bDMARD therapy did not differ between healthy controls and the RA patient groups, such differences were already present between Responder and Non-responder RA patients in case of T cells. Future studies may be able to explore whether differences in MAF values are present in therapy naïve RA patients compared to healthy controls as well as the changes in the activity of the studied transporters over the course of csDMARD treatment until reaching the need for bDMARD therapy. This information could provide more insight into the pathophysiological role of these transporters and may enable even earlier prediction of RA treatment response.

Interestingly, no significant changes of MAF values within the respective RA patient groups were demonstrated with the progress of treatment (Figure 3). However, considerable intra- and inter-patient variations were observed within both patient groups in these values. This observation may be related to limitations of our study, namely the relatively small number of patients included in each group and in particular the heterogeneity of the bDMARD treatment received. A larger number of patients in future studies will allow to create and compare homogenous patient groups in terms of the therapy applied.

In conclusion, our results indicate that the determination of MAFC values in CD3 cells of RA patients may be of predictive value prior to the initiation of biological therapy to establish whether the patient will demonstrate sufficient therapeutic response. Measuring MAFC, MAFMRP and MAFMDR values in CD3 cells at 4 to 6 weeks after the start of treatment further improves the accuracy of prediction as to whether adequate therapeutic response may be expected.

LIST OF ABBREVIATIONS

ABC	ATP-binding cassette
bDMARD	biological disease modifying antirheumatic drug
BCRP	breast cancer resistance protein
CRP	C-reactive protein
DAS	disease activity score
csDMARD	conventional synthetic disease modifying antirheumatic drug
MAF	MDR activity factor
MDR	multidrug resistance
MRP	multidrug resistance protein

MTX	methotrexate
PBMC	peripheral blood mononuclear cell
RA	rheumatoid arthritis

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FIGURE LEGENDS

Figure 1. The effects of highly selective inhibitors of MRP1 (indomethacin) and MRP1 + MDR1 (verapamil) on calcein substrate fluorescence intensity at high (A) and low (B) transporter activity, and the effects of the highly selective inhibitor of BCRP (KO134) on mitoxantrone substrate fluorescence intensity at high (C) and low (D) transporter activity in CD3 cells – representative samples of RA patients

Figure 2. DAS28 values at different sampling time points in Responder and Non-responder RA patients. *$p < 0.05$ vs. 0wk

Figure 3. Individual changes of MAF values on CD3 cells over time in Responder (n = 27) and Non-responder (n=12) RA patients. No significant changes were demonstrated in MAF values in the respective RA patient groups with the progress of treatment.

Figure 4. ROC analysis was performed to evaluate the predictive value of MAF for response to treatment in RA patients at the start of biological therapy and at 6wk. Patients with MAF values above the respective cut-off thresholds are likely to be Non-responders to treatment. MAFC of CD3 cells at 0wk: $p = 0.043$, AUC = 0.68; MAFC of CD3 cells at 6wk: $p = 0.033$, AUC = 0.72; MAFMDR on CD3 cells at 6wk: $p = 0.048$, AUC = 0.70; MAFMRP on CD3 cells at 6wk: $p = 0.049$, AUC = 0.69

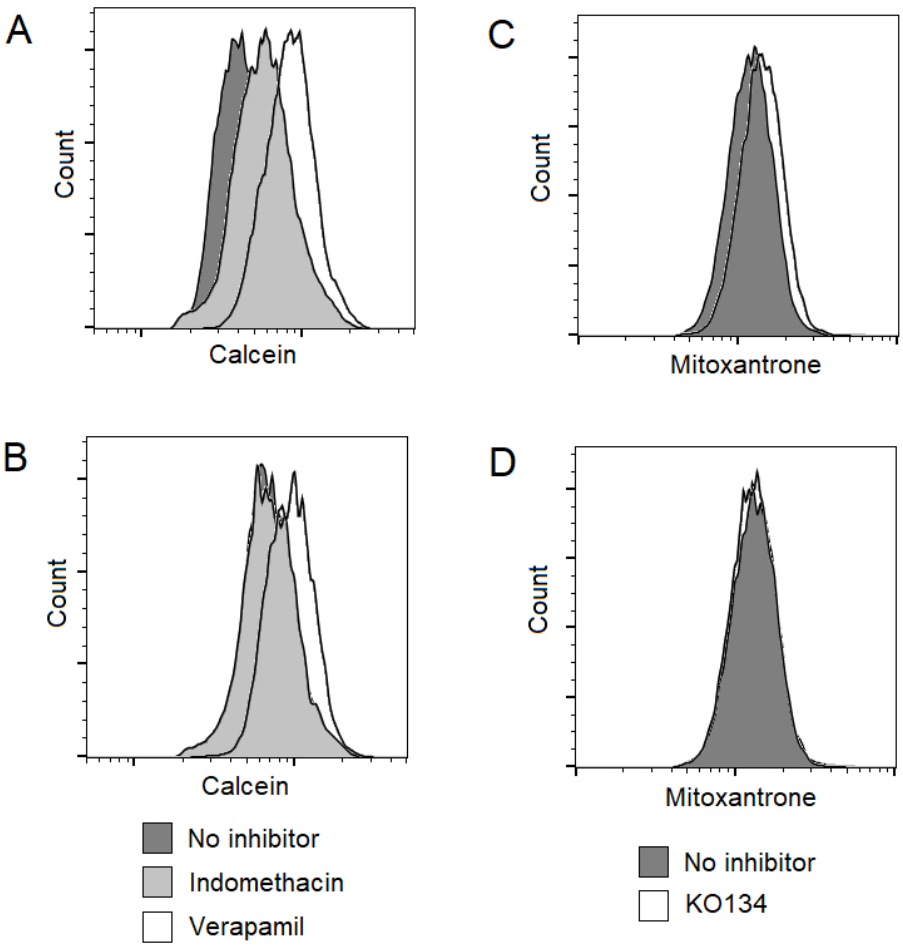

Figure 1

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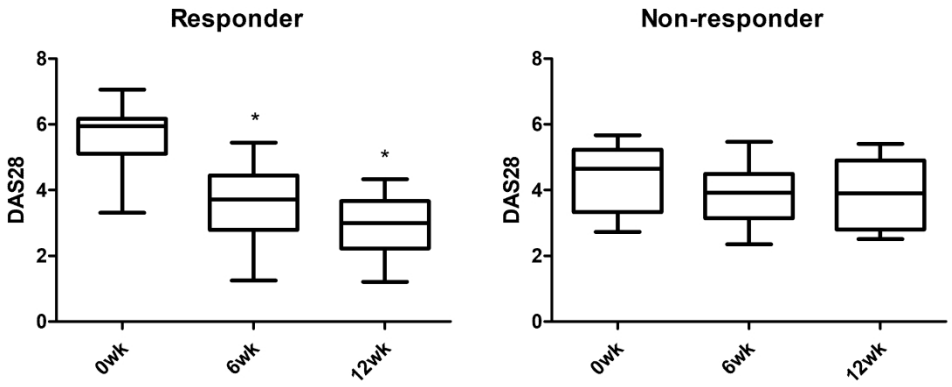

Figure 2

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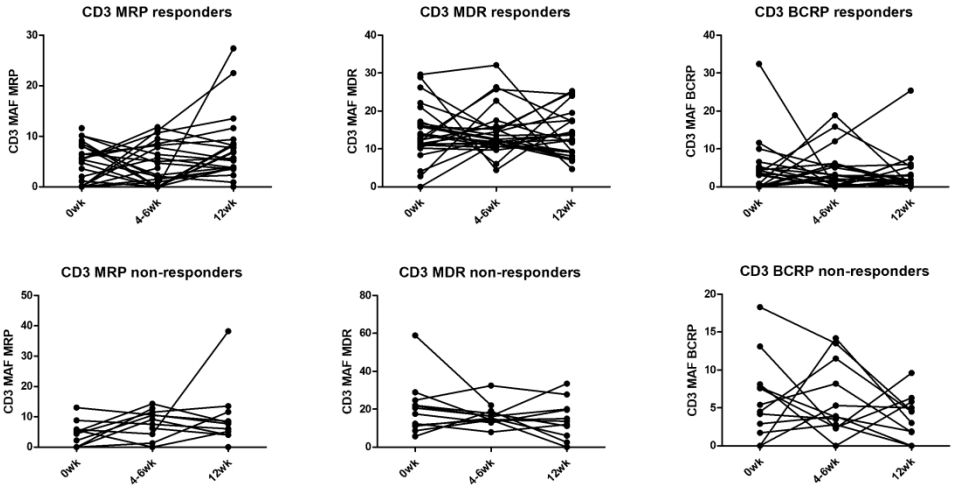

Figure 3.

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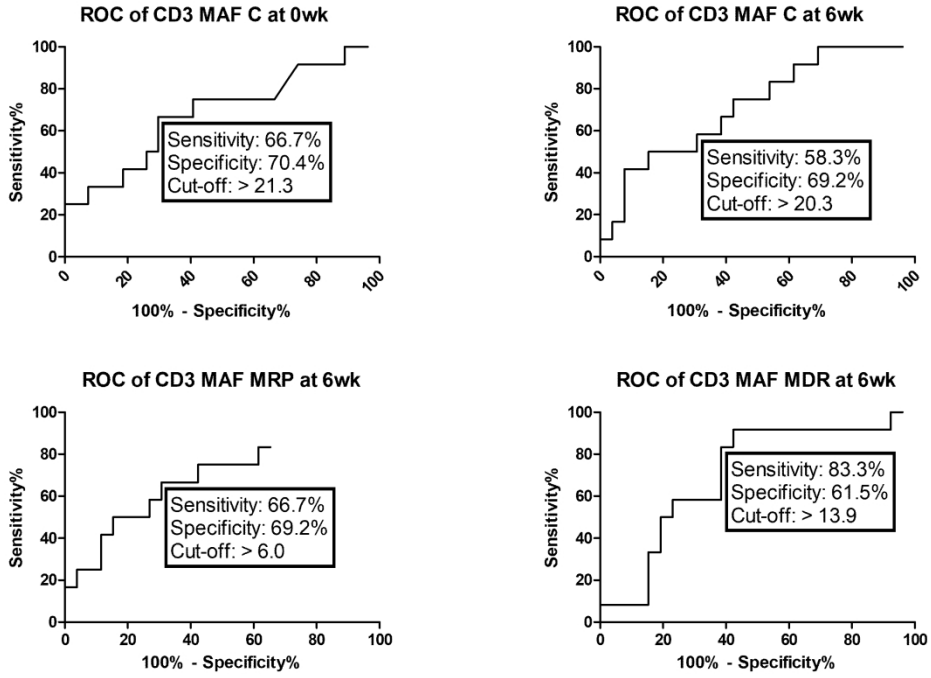

Figure 4.

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Table 1. Clinical characteristics of Responder and Non-responder RA patients as well as healthy controls. Data are expressed as median (IQR) for continuous variables and as number (percentage) for categorical variables. MTX – methotrexate

	Healthy controls (n = 35)	Responder (n = 27)	Non-responder (n = 12)
Age (years)	54 (42-62)	56 (49-61)	51 (39-61)
Gender (male/female)	4/31	2/25	1/11
RA duration (years)	-	10 (5-14)	8.5 (5-15)
No. of patients receiving MTX	-	15 (56%)	6 (50%)
No. of patients receiving prednisolone	-	9 (33%)	5 (42%)
No. of patients receiving adalimumab	-	2 (7%)	1 (8%)
No. of patients receiving certolizumab pegol	-	5 (19%)	3 (25%)
No. of patients receiving etanercept	-	7 (26%)	3 (25%)
No. of patients receiving abatacept	-	13 (48%)	5 (42%)

Table 2. Activity of various MDR transporters on CD3 and CD19 cells in RA patients and healthy controls. Data are expressed as median (IQR), $p < 0.05$ [a] vs Control, [b] vs Responder, [c] vs 0 wk value. MAFC – composite multidrug activity factor (of MRP1 and MDR1 activity), MAFMRP– multidrug activity factor of MRP1, MAFMDR – multidrug activity factor of MDR1, MAFBCRP – multidrug activity factor of BCRP

		0 wk		6 wk		12 wk	
	Control	**Responder**	**Non-responder**	**Responder**	**Non-responder**	**Responder**	**Non-responder**
DAS28	-	5.94 (5.11-6.17)	4.65[b] (3.33-5.23)	3.71[c] (2.79-4.45)	3.93 (3.14-4.50)	3.00[c] (2.23-3.67)	3.90[b] (2.81-4.90)
CRP	-	11.1 (2.6-16.6)	8.4 (1.4-15.1)	4.4 (1.3-7.9)	4.4 (1.5-10.4)	3.7 (2.1-5.6)	7.5 (2.7-11.6)
CD3 MAFC	18.3 (14.7-22.9)	18.9 (14.0-25.2)	23.5[b] (17.1-33.7)	17.1 (12.3-22.6)	22.7[b] (16.7-29.2)	18.3 (15.7-24.2)	25.2 (15.9-30.7)
CD3 MAFMRP	3.1 (1.2-5.7)	4.8 (0.0-8.0)	5.7 (2.2-8.0)	2.2 (0.0-7.9)	8.4[b] (2.1-11.3)	5.7 (3.7-8.5)	7.7[a] (4.0-11.6)
CD3 MAFMDR	14.6 (12.5-18.1)	12.9 (11.0-16.7)	19.1[b] (11.2-24.0)	12.4 (11.2-15.4)	15.8[b] (14.3-18.7)	12.5 (9.2-17.5)	13.6 (6.0-20.0)
CD3 MAFBCRP	2.5 (0.8-5.7)	3.1 (0.0-4.4)	5.0 (2.0-8.0)	2.0 (0.0-5.5)	3.9 (2.5-10.7)	1.4 (0.0-4.3)	4.5 (1.8-5.8)
CD19 MAFC	12.8 (8.9-17.9)	15.1 (8.1-22.1)	20.6 (13.5-31.0)	13.2 (9.3-20.4)	17.6 (11.4-27.2)	17.4 (13.1-22.3)	17.6 (9.2-25.9)
CD19 MAFMRP	2.2 (0.0-6.3)	0.9 (0.0-7.7)	4.4 (0.0-5.8)	0.6 (0.0-5.1)	6.8[b] (0.5-9.6)	3.2 (0.3-6.8)	5.1 (1.9-10.9)
CD19 MAFMDR	9.9 (8.0-14.0)	11.1 (6.0-16.3)	15.7 (8.4-25.4)	11.4 (5.3-14.8)	13.6 (8.6-17.7)	14.0 (7.1-17.7)	8.8 (1.9-15.7)
CD19 MAFBCRP	3.8 (1.0-6.3)	3.1 (0.7-7.0)	4.5 (0.0-11.0)	2.7 (0.0-5.2)	5.0 (3.1-8.4)	2.9 (1.3-5.1)	3.0 (1.8-3.7)