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Predicting outcome of acute kidney transplant rejection using

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Citation

Rekers, N. V. (2014, December 3). *Predicting outcome of acute kidney transplant rejection using*. Retrieved from <https://hdl.handle.net/1887/29900>

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Title: Predicting outcome of acute kidney transplant rejection using molecular markers

Issue Date: 2014-12-03

Increased metallothionein expression reflects steroid resistance in renal allograft recipients

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Abstract

Steroid-refractory acute rejection is a risk factor for inferior renal allograft outcome. We aimed to gain insight into the mechanisms underlying steroid resistance by identifying novel molecular markers of steroid-refractory acute rejection. Eighty-three kidney transplant recipients (1995-2005), who were treated with methylprednisolone during a first acute rejection episode, were included in this study. Gene expression patterns were investigated in a discovery cohort of 36 acute rejection biopsies, and verified in a validation cohort of 47 acute rejection biopsies. In the discovery set, expression of metallothioneins (MT) was significantly ($P < 0.000001$) associated with decreased response to steroid treatment. Multivariate analysis resulted in a predictive model containing MT-1 as an independent covariate (AUC=0.88, $P < 0.0000001$). In the validation set, MT-1 expression was also significantly associated with steroid resistance ($P = 0.029$). Metallothionein expression was detected in macrophages and tubular epithelial cells. Parallel to the findings in patients, *in vitro* experiments of peripheral blood mononuclear cells from 11 donors showed that non-response to methylprednisolone treatment is related to highly elevated MT levels. High expression of metallothioneins in renal allografts is associated with resistance to steroid treatment. Metallothioneins regulate intracellular concentrations of zinc, through which they may diminish the zinc-requiring anti-inflammatory effect of the glucocorticoid receptor.

Introduction

The occurrence of steroid-refractory acute rejection is a risk factor for adverse outcome in renal allograft recipients ¹⁻⁵. First rejection episodes are most of the time treated by high-dose steroids, leading to reversal of the acute rejection. However, approximately 30% of patients have no or an inadequate response to corticosteroid therapy alone and require therapy with anti-thymocyte globulin (ATG) ^{2,5-7}. Such steroid resistant patients may show progression of chronic damage to the graft, which has a detrimental effect on graft outcome ^{4,5,8}.

Cellular and molecular markers in the graft tissue may complement clinical parameters and histomorphology when assessing steroid resistance of the acute rejection episode. Indeed, expression of markers, particularly those of inflammatory cell types, was found to be associated with therapy response (reviewed in ⁸). Recently, we showed that the combination of T-cell activation markers CD25:CD3 ratio and LAG-3 offers a superior prognostic value for assessing steroid response, compared with conventional parameters ⁹. We observed considerable molecular heterogeneity among the biopsy samples with acute rejection, underlining the complexity of the mechanisms involved in response to steroid therapy.

In the current study we aimed to gain further insight into the mechanisms underlying steroid resistance by identifying novel molecular markers of steroid-refractory acute rejection using genome wide expression profiling. Our results reveal that relatively high intra-graft expression of metallothioneins (MT), a group of small cysteine-rich molecules that regulate intracellular zinc concentrations, during acute rejection is associated with resistance to steroid treatment. These proteins were further studied in biopsies and steroid-treated leukocyte cultures.

Materials and methods

Patient information

We reviewed all 873 patients who received a renal allograft in our center between 1995 and 2005. We selected patients suffering from a histologically proven, first rejection episode, from whom a renal biopsy had been taken prior to the start of anti-rejection treatment. Only patients receiving pulse therapy with methylprednisolone

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(3 days with 1 g bolus) as anti-rejection treatment were included. The primary clinical endpoint was response to anti-rejection treatment with methylprednisolone, as described previously ⁹. Steroid resistant acute rejection was defined as lack of clinical response to methylprednisolone, and a requirement for anti-thymocyte globulin treatment within 14 days after the start of the steroid therapy. The response to steroid therapy was monitored by creatinine concentration measurements. Maintenance immunosuppression consisted of prednisone and a calcineurin inhibitor (Cyclosporine A or Tacrolimus), with or without Mycophenolate Mofetil.

In total, 83 patients were included in the study. For genome-wide expression profiling, a discovery set (n=36) was studied: 18 patients with steroid responsive acute rejection and 18 patients with steroid resistant acute rejection. As microarray analysis requires high-quality RNA ¹⁰⁻¹³, biopsy samples that had generated the highest quality of RNA were included in the discovery set. Four biopsies, taken during acute decrease of graft function but with no histomorphologic indication of rejection, were included as controls. Results from the discovery set were verified in a validation set (n=47): 31 patients with steroid responsive acute rejection and 16 patients with steroid resistant acute rejection.

Biopsy samples

Rejection severity of all biopsy specimens was assessed on paraffin-embedded biopsy tissue according to Banff 2011 criteria ¹⁴. A second biopsy core was snap frozen in liquid nitrogen and stored at -80°C. In addition to the biopsies taken on clinical indication, paired pretransplant biopsy samples (stored at -80°C) were available from 14 steroid resistant and nine steroid responsive patients.

RNA extraction and quality assessment

Eight to ten 10-µm sections were cut with a cryomicrotome from each snap frozen biopsy core. Total RNA was extracted using the RNeasy Mini Kit (Qiagen, Chatsworth, CA). RNA quality was determined on Nano LabChips with the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). The mean RNA concentration was 2.76 ± 1.11 µg, mean 28S:18S ratio was 2.1 ± 0.04 , and mean RNA integrity number (RIN) was 8.11 ± 0.92 .

Microarray Analysis

Gene expression profiling was performed with Illumina HumanRef-8 v3.0 BeadChips (Illumina, San Diego, CA), as previously described¹⁵. Only samples with a RNA quality index > 8 were included in the microarray analysis. Illumina GenomeStudio software was used to verify the adequacy of signal-background ratio and hybridization controls. All data have been deposited in the Gene Expression Omnibus public database, accession number: GSE47097.

Real-time quantitative PCR analysis

Synthesis of cDNA, primer design, and quantitative PCR (qPCR) analysis were performed according to previously described protocols^{9,16}. An average of 0.92 ± 0.15 μ g of RNA from each frozen biopsy core was transcribed into cDNA. Primer sequences can be provided on request. PCR efficiencies were between 90% - 110%. Differences in mRNA expression levels were normalized to the geometric mean signal of the reference genes glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and β -actin.

Immunohistochemistry

Immunohistochemical analyses were performed on 32 paraffin-embedded biopsy samples: 17 from steroid responsive patients and 15 from steroid resistant patients. Protein staining for metallothionein, CD68, and CD163 was performed on 4- μ m thick sections. Primary monoclonal mouse anti-human antibodies against metallothionein (Abcam, Cambridge, UK; clone UC1MT), CD163 (Abcam; clone 10D6, prediluted), and CD68 (Dako, Glostrup, Denmark; clone KP1) were used for immunohistochemistry. Standardized protocols¹⁷ were applied. Details concerning antigen retrieval and staining procedure are provided in Table 1. Photos were taken with a Leitz DMRD microscope (Leica Microsystems, Rijswijk, the Netherlands).

Table 1. Characteristics of antibodies.

Protein	Antigen retrieval	Type	Dilution	Blocking	Pre-incubation	Incubation time	Secondary antibody	Detection
MT	Citrate (pH 6.0)	Monoclonal	1:300	0.1% H ₂ O ₂	5% NGS, 1 h ¹	1 h	Mouse-EnVision+	DAB
CD68	Tris/EDTA (pH 9.0)	Monoclonal	1:1000	0.03% H ₂ O ₂		1 h	Mouse-EnVision+	DAB
CD163	Citrate (pH 6.0)	Monoclonal	1:10	0.03% H ₂ O ₂		1 h	Mouse-EnVision+	DAB

¹ In the MT staining the slides were pre-incubated for 1 h with 5% normal goat serum (NGS), diluted in PBS.

***In situ* hybridization for Y chromosome**

The presence of male, Y-chromosome-expressing cells was determined by *in situ* hybridization, as described previously ¹⁸. Briefly, 4- μ m sections were hybridized overnight with digoxigenin (DIG) labeled, Y-chromosome-specific DNA probe, followed by consecutive incubations with mouse anti-DIG monoclonal antibody (Sigma-Aldrich, St. Louis, MO) and rabbit anti-mouse Ig-horseradish peroxidase (anti-mouse Envision-HRP, Dako). Sections were developed with Nova Red and counter-stained with hematoxylin. Photos were taken with a Leitz DMRD microscope (Leica Microsystems).

Investigation of metallothionein expression in various cell types

Metallothionein expression was investigated in RNA from a panel of various cell types ¹⁹. This panel included peripheral blood mononuclear cell (PBMC) fractions (n=9), cultured dendritic cells (n=6), monocytes (n=3), differentiated macrophages (n=7), stimulated macrophages (n=6), T cells (n=5), stimulated T cells (n=5), B cells (n=6), proximal tubular epithelial cells (n=2), human aorta endothelial cells (n=5), and human umbilical vascular endothelial cells (n=5). RNA from pretransplant biopsy samples (n=65) was included as a reference.

***In vitro* methylprednisolone response experiments**

Blood was obtained from healthy blood bank donors (n=11; Sanquin, Leiden, the Netherlands) after informed consent. PBMC were isolated by Ficoll Hypaque density gradient centrifugation, cultured in 96-well plates (10^5 /well) in RPMI 1640 medium with 10% fetal calf serum (Gibco), and stimulated for 50 h with PHA (2 μ g/ml). This was done either in the absence or presence of 10^{-4} M methylprednisolone (Solu-Medrol® from Pfizer, Capelle a/d IJssel, the Netherlands; powder for intravenous infusion). After the culture, the cells were harvested and stored in RNAlater (Qiagen) until RNA extraction and qPCR analysis.

Statistical analysis

Comparison of categorical data between groups was evaluated using the Fisher's Exact test. Microarray data were normalized using the Quantile Normalization method. Only genes showing expression above background ($P < 0.05$) in at least 20 of the 40 samples were included. Hierarchical clustering was performed with the

gplots R package (version 2.10.1). The Mann-Whitney U-test was performed to select differentially expressed genes between the steroid response groups. Multiple testing correction was performed with the Benjamini-Hochberg method ²⁰.

Lasso regression analysis, performed with the *penalized* R package (version 0.9-33) ²¹, was used to analyze the discriminative value between groups of genes analyzed in the microarrays. Leave-one-out cross-validated receiver operating characteristics (ROC) analysis, using the R package *ROCR* (version 1.0-2) ²², was applied to evaluate the robustness of the multivariate model from the lasso regression analysis. The enrichment of specific functional groups of genes was analyzed on the basis of hypergeometric tests using the online gene set analysis toolkit WebGestalt (<http://bioinfo.vanderbilt.edu/webgestalt/>) ^{23,24}.

PCR data are expressed as mean $\pm$ standard error of the mean. Differences in mean mRNA and protein expression between groups were tested with independent samples t-tests and Mann-Whitney U-tests. Logistic regression analysis and ROC analysis were performed for multivariate analysis of qPCR data. All statistical tests were two-sided, and P values less than 0.05 were considered as significant. Statistical analyses were performed using the IBM SPSS statistical package (version 20.0.0, Amsterdam, the Netherlands) and the R statistical package (version 2.12.0, <http://www.r-project.org/>).

Results

Demographics and clinical data

No significant differences in the demographic and clinical characteristics were found between steroid responsive and steroid resistant patient groups in the discovery cohort (n=36) and validation cohort (n=47) (Table 2 and Table S1). There was no significant difference in the RNA concentration, purity, and quality of the samples from the patient groups (data not shown).

Table 2. Demographic and clinical data of patients with steroid responsive and steroid resistant acute rejection in the discovery set and the validation set.

Variable	Discovery set			Validation set		
	Steroid responsive (n=18), n (%)	Steroid resistant (n=18), n (%)	P value	Steroid responsive (n=31), n (%)	Steroid resistant (n=16), n (%)	P value
Patient age ≥ 50 years	8 (44.4%)	8 (44.4%)	1.00	14 (45.2%)	7 (43.8%)	1.00
Patient gender Female	4 (22.2%)	6 (33.3%) 0.15	0.71	13 (41.9%)	5 (31.2%)	0.54
Donor age ≥ 50 years	3 (17.6%)	8 (44.4%)		13 (41.9%)	7 (43.8%)	1.00
Donor gender Female	11 (64.7%)	10 (55.6%)	0.73	20 (64.5%)	13 (81.2%)	0.32
Year of transplantation 1995 through 1999	12 (66.7%)	14 (77.8%)	0.71	23 (74.2%)	13 (81.2%)	0.73
2000 through 2005	6 (33.3%)	4 (22.2%)		8 (25.8%)	3 (18.8%)	
Donor type Living	5 (27.8%)	4 (22.2%)	1.00	4 (12.9%)	6 (37.5%)	0.07
Post mortal	13 (72.2%)	14 (77.8%)		27 (87.1%)	10 (62.5%)	
HLA-A matching ≥ 1 mismatch	10 (58.8%)	14 (77.8%)	0.29	18 (58.1%)	9 (56.2%)	1.00
HLA-B matching ≥ 1 mismatch	13 (76.5%)	14 (77.8%)	1.00	22 (71.0%)	12 (75.0%)	1.00
HLA-DR matching ≥ 1 mismatch	9 (52.9%)	13 (72.2%)	0.31	19 (61.3%)	11 (68.8%)	0.75
Virtual PRA	4 (23.5%)	5 (27.8%)	1.00	7 (22.6%)	6 (37.5%)	0.32
Immunized (6-100%)	8 (57.1%)	11 (68.8%)	0.71	20 (76.9%)	10 (76.9%)	1.00
Cold ischemia time > 18 hours	4 (22.2%)	4 (22.2%)	1.00	8 (25.8%)	3 (18.8%)	0.73
Induction therapy Daclizumab	14 (77.8%)	14 (77.8%)		23 (74.2%)	13 (81.2%)	
None	8 (44.4%)	8 (44.4%)	1.00	14 (45.2%)	10 (62.5%)	0.36
Maintenance therapy Double therapy	10 (55.6%)	10 (55.6%)		17 (54.8%)	6 (37.5%)	
Triple therapy	3 (16.7%)	2 (11.1%)	1.00	10 (32.3%)	2 (18.8%)	0.49
Delayed graft function Yes	18 (100.0%)	17 (94.4%)	1.00	30 (96.8%)	16 (100.0%)	1.00
Rejection time <3 months post Tx	0 (0.0%)	1 (5.6%)		1 (3.2%)	0 (0.0%)	
3-6 months post Tx	3 (17.6%)	3 (16.7%)	1.00	2 (7.1%)	4 (25.0%)	0.17
C4d staining Positive	7 (38.9%)	8 (44.4%)	1.00	7 (22.6%)	4 (25.0%)	1.00
Vascular rejection Yes						

PRA, panel reactive antibodies; Tx, transplantation



Microarray analysis

Transcriptional profiles of 40 renal allograft biopsy samples were analyzed using whole genome microarray slides. Expression profiles were investigated in the discovery cohort of 18 steroid resistant rejections and 18 steroid responsive rejections, and in 4 control biopsy samples. Six thousand eight hundred and thirty two genes with significant expression ($P < 0.001$) above background were included for further analysis.

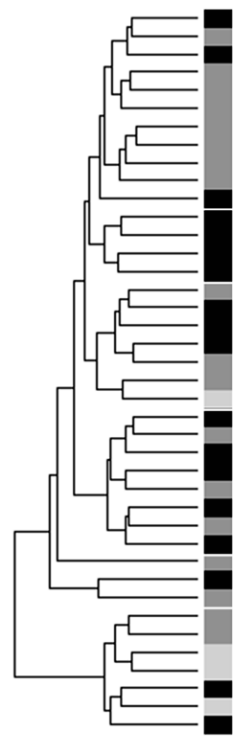
To test for associations between the gene expression profile in the biopsy samples and clinical parameters, we first clustered the patients based on similarities in the expression pattern of the corresponding kidney allograft biopsies (Figure 1A). This unsupervised hierarchical clustering analysis did not result in a clear distinction between steroid responsive and steroid resistant acute rejection biopsies. The control biopsies largely clustered together. Comparison of patients in different clusters did not reveal differences of the clinical parameters (Figure 1B).

Relation between molecular variables and steroid response

The expression of 249 genes differed significantly ($P < 0.05$ with the unadjusted Mann-Whitney U-test) between steroid responsive and steroid resistant rejections: 158 transcripts were upregulated and 91 transcripts were downregulated in the steroid resistant rejections.

The 50 genes with the most significant differences between the two groups were included in two-way hierarchical clustering analysis (Figure 2). The dendrogram above the heatmap shows four clusters of patients: two clusters with mainly samples from steroid responsive patients (clusters 1 and 2), one cluster with mainly samples from steroid resistant patients (cluster 3), and one cluster containing only samples from steroid resistant patients (cluster 4). Comparison of the Banff scores from the steroid resistant patients in cluster 4 with the Banff scores of the steroid resistant patients in the other three clusters revealed no significant differences (data not shown). The dendrogram on the vertical axis revealed two distinct clusters of genes. A large number of genes from the metallothionein 1 (MT-1) family cluster together and show a similar expression pattern.

A



B

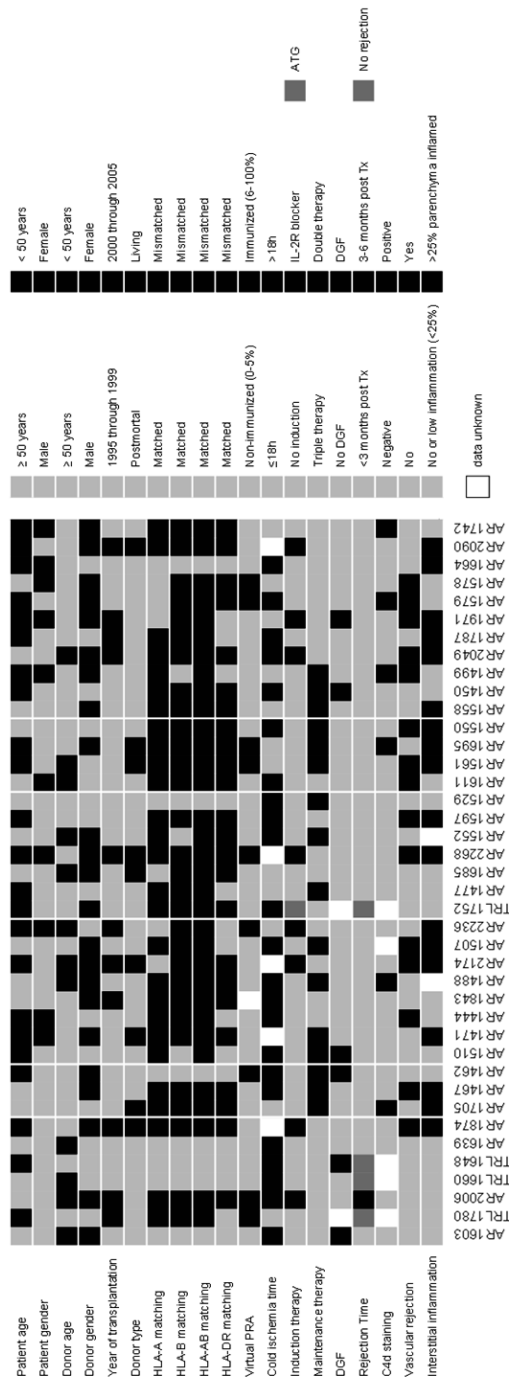


Figure 1. Analysis of associations between the gene expression profile in steroid resistant and steroid responsive kidney biopsy samples and clinical parameters. A) Unsupervised hierarchical clustering analysis of the kidney allograft biopsies in the discovery cohort and 4 control biopsy samples, based on similarities in the expression pattern of the 6,832 genes. B) Comparison of the clinical parameters of the patients in the identified clusters from the hierarchical clustering analysis.

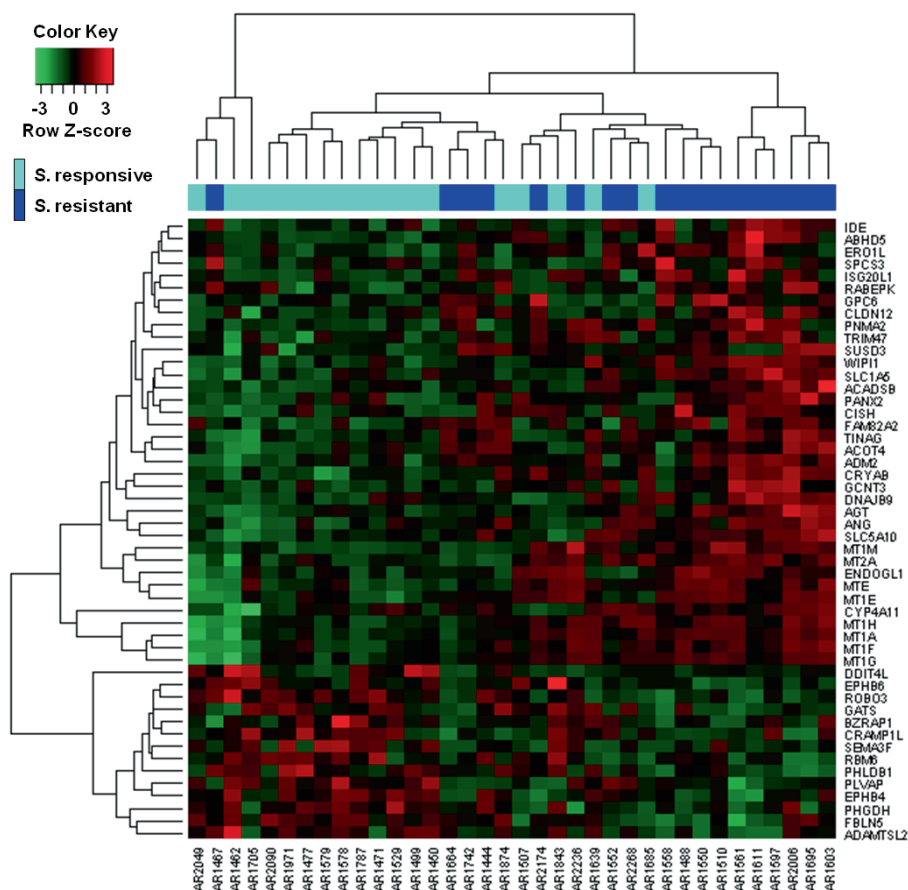


Figure 2. Hierarchical clustering and heatmap display of the 50 genes with the highest significant difference in expression between steroid responsive and steroid resistant acute rejection. Genes (rows) and samples (columns) were clustered in an unsupervised manner on the basis of the overall similarity in expression pattern between subjects in the discovery cohort. The dendrograms represent how close the relationship is between biopsy samples (x-axis) or different genes (y-axis). The color (scale indicated top left) in each cell reflects the level of expression of the corresponding gene in the corresponding sample. Color scale runs from bright green (relatively low mRNA expression) to bright red (relatively high mRNA expression). Patients with steroid responsive acute rejection are shown in light blue; patients with steroid resistant acute rejection are shown in dark blue.

Gene ontology

To gain insight into the function of differentially expressed genes, we included all 249 genes for pathway analysis by hypergeometric test in WebGestalt. One molecular function showed high enrichment in steroid resistant patients: cadmium ion binding ($P=0.000000004$). When adjusted for multiple testing, this finding remained highly

significant (ratio of enrichment of 20.1, $P < 0.000001$; Table S2). Seven of the nine genes involved in this molecular function were significantly higher expressed in steroid resistant rejection, and they were all members of the MT-1 gene family (MT-1A, MT-1E, MT-1F, MT-1G, MT-1H, MT-1M, MT-1X). Other pathways concerned amongst others the extracellular region ($P = 0.0003$), extracellular matrix part ($P = 0.0038$), and copper ion binding ($P = 0.0082$). The complete list of significant pathways is shown in Table S2.

Multivariate regression analysis

Multivariate analysis with lasso regression resulted in a model discriminating patient groups, containing as independent covariates MT-1G in combination with CYP4A11, TIMP1, FTHL7, and F2R. The predictive value of the multivariate model was significant ($AUC = 0.92$, $P = 0.00002$) when tested with receiver operating characteristics (ROC) curve analysis (Figure 3A). The predictive value of the multivariate model remained significant when validated with a leave-one-out cross-validated analysis ($AUC = 0.72$, $P = 0.048$; Figure 3B).

Quantitative PCR validation

Using real-time quantitative PCR (qPCR) we validated the microarray results for six mRNA transcripts, including the covariates from the multivariate lasso regression, in the acute rejection biopsy samples of the discovery cohort: MT-1, CYP4A11, TIMP1, F2R, AGT, and PHLDB1 (Figure 4). The qPCR results corresponded with the findings by microarray. The expression levels of MT-1 ($P = 0.018$, Figure 4A) and TIMP1 ($P = 0.002$, Figure 4C) were significantly increased in steroid resistant acute rejection, whereas those of PHLDB1 were significantly decreased ($P = 0.016$, Figure 4F) compared with the steroid responsive group. The steroid resistant samples showed higher expression of AGT ($P = 0.058$, Figure 4E) and of CYP4A11 ($P = 0.107$, Figure 4B), and lower expression of F2R ($P = 0.195$, Figure 4D).

The expression of the six transcripts was measured in pretransplant biopsies available from 23 of the patients studied during acute rejection (14 steroid resistant and nine steroid responsive). No significant differences were found in intragraft expression levels before transplantation, except for CYP4A11. The steroid resistant patients showed significantly higher CYP4A11 expression levels ($P = 0.0019$, Figure 4B).

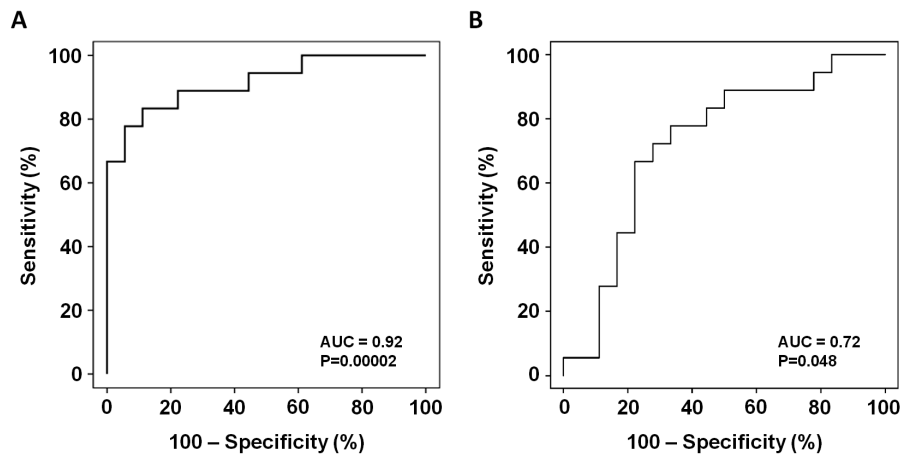


Figure 3. Predictive value of the lasso regression model for steroid resistant acute rejection. Multivariate regression analysis of the microarray data from the discovery set resulted in a predictive model with MT-1, CYP4A11, TIMP1, FTHL7, and F2R as independent covariates. A) The receiver operating characteristics (ROC) curve shows the true positive rate (sensitivity) and false positive rate (100 – specificity) for various cut-off levels of the multivariate model. The area under the curve (AUC) was 0.92 ($P=0.00002$). B) The predictive value of the multivariate model remained significant when validated with a leave-one-out cross-validated analysis (AU=0.72, $P=0.048$).

Validation cohort

To verify the findings that high MT-1 expression associates with resistance to steroid treatment, we used qPCR to analyze the expression of MT-1 in a validation cohort ($n=47$). The MT-1 expression level was significantly increased in steroid resistant acute rejection compared to steroid responsive acute rejection ($P=0.029$, Figure 5).

To validate the multivariate model obtained with microarray analysis, we performed multivariate logistic regression analyses on the qPCR measurements in the discovery and validation cohorts. The predictive value of the multivariate model, containing MT-1, CYP4A11, TIMP1, and F2R, was significant in both the discovery cohort (AUC=0.89, $P=0.00008$; Figure 6A) and the validation cohort (AUC=0.80, $P=0.0009$; Figure 6B).

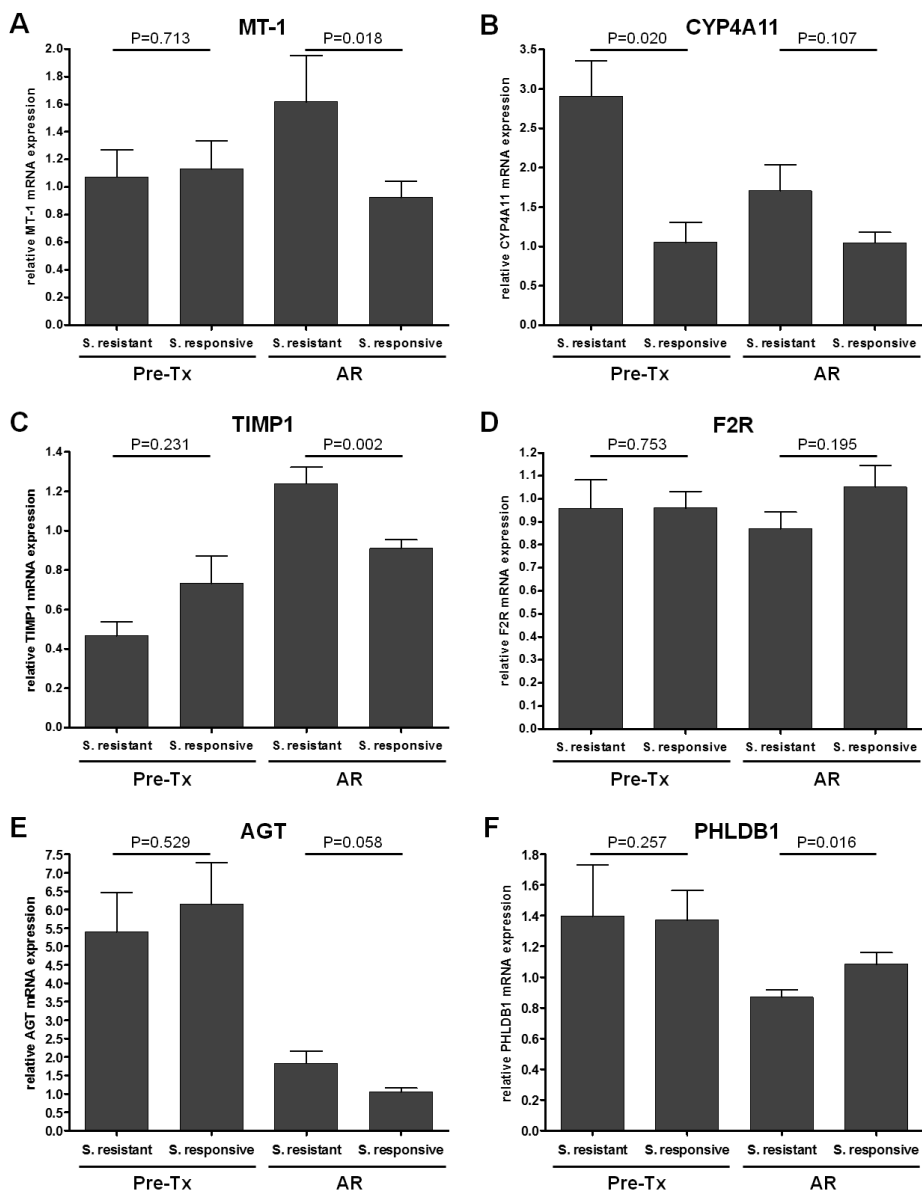


Figure 4. Quantitative real-time PCR validation of microarray results in the discovery patient cohort. RNA expression levels of MT-1 (A), CYP4A11 (B), TIMP1 (C), F2R (D), AGT (E), and PHLDB1 (F) measured in pretransplant biopsy samples (Pre-Tx) and acute rejection biopsy samples (AR) from the steroid resistant and steroid responsive patient groups in the discovery cohort. Data are expressed as mean $\pm$ SEM.

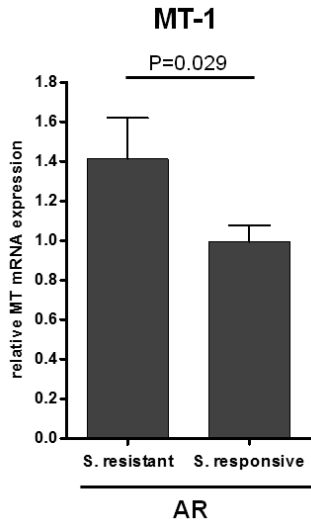


Figure 5. Metallothionein expression in the validation patient cohort. MT-1 mRNA levels were measured with qPCR in acute rejection (AR) biopsy samples from the steroid resistant and steroid responsive patients in the validation cohort. Data are expressed as mean $\pm$ SEM.

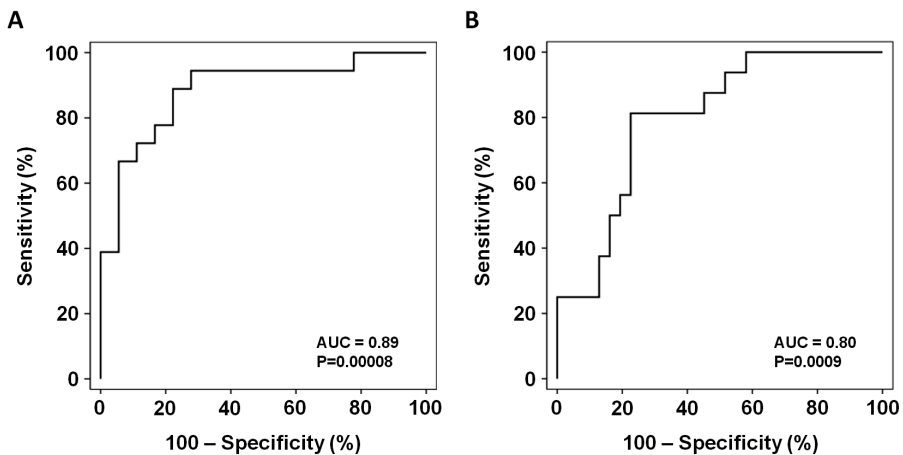


Figure 6. Predictive values of the multivariate regression model in the discovery set and validation set. Receiver operating characteristics (ROC) curve analysis of the multivariate regression model, containing MT-1, CYP4A11, TIMP1, and F2R, measured with qPCR. The area under the ROC curve was 0.89 ($P=0.00008$) in the discovery set (A) and 0.80 ($P=0.0009$) in the validation set (B). The ROC curves show the true positive rate (sensitivity) and false positive rate (100 – specificity) for various cut-off levels of the multivariate model.

Comparison of multivariate models

To investigate how the multivariate MT-1 model (MT-1, CYP4A11, TIMP1, and F2R) relates to the multivariate T-cell mediated model from our previous study (CD25:CD3e

ratio and LAG-3)⁹, we combined the discovery and validation cohorts. The predictive value of the MT-1 model was slightly higher (AUC=0.81, P=0.000002; Figure 7) than the predictive value of the T-cell activation model (AUC of 0.79, P=0.000007; Figure 7). Combination of the two models resulted in a predictive model with MT-1, TIMP1, F2R, CD25:CD3e ratio and LAG-3 as independent covariates (AUC=0.88, P<0.0000001; Figure 7).

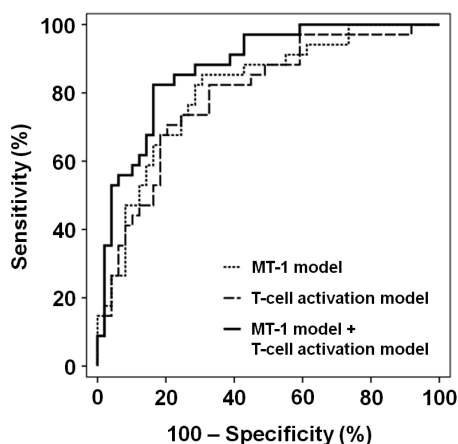


Figure 7. Comparison of the predictive values of the metallothionein and T-cell activation models for steroid resistant acute rejection. Receiver operating characteristics (ROC) curve analysis was used to compare the predictive value of the multivariate metallothionein-1 (MT-1) model (containing MT-1, CYP4A11, TIMP1, and F2R) and of the previously described multivariate T-cell activation model (containing CD25:CD3e ratio and LAG-3)⁹. The area under the ROC curve was 0.81 (P=0.000002, dotted line) for the MT-1 model and 0.79 (P=0.000007, striped line) for the T-cell activation model. Combination of the two models resulted in a multivariate model with MT-1, TIMP1, F2R, CD25:CD3e ratio and LAG-3 as independent covariates (AUC=0.88, P<0.0000001, solid line).

Localization of metallothionein expression in cell types and transplant biopsies

In order to determine which cell types express MT-1, we quantified MT-1 expression in a panel of various cell types (Figure 8). High MT-1 expression was seen in activated macrophages and proximal tubular epithelial cells (PTECs). The expression in PTECs was comparable to that in pretransplant biopsies (first bar). Endothelial cells from human aortas and umbilical veins showed intermediate MT-1 expression.

To investigate which cells express MT proteins within the grafts, we performed immunohistochemical stainings for MT on paraffin-embedded kidney allograft biopsy specimens. In accordance with the findings in the cell panel (Figure 8),

protein expression of MT was detected in tubular epithelial cells and inflammatory cells (Figure 9A-D). Co-localization of MT staining (Figure 9C and D) with expression of pan-macrophage marker CD68 (Figure 9E and F) and macrophage activation marker CD163 (Figure 9G and H) supported the observation that MT is expressed by activated macrophages. Stainings for the human Y-chromosome in a female-to-male transplantation revealed that MT is expressed by macrophages that are derived from the recipient (Figure 10).

No differences were found in the mRNA expression levels of macrophage marker CD68 in pretransplant ($P=0.75$) and acute rejection ($P=0.95$) biopsy samples, and in the CD68 protein expression ($P=0.58$) between the steroid response groups (data not shown).

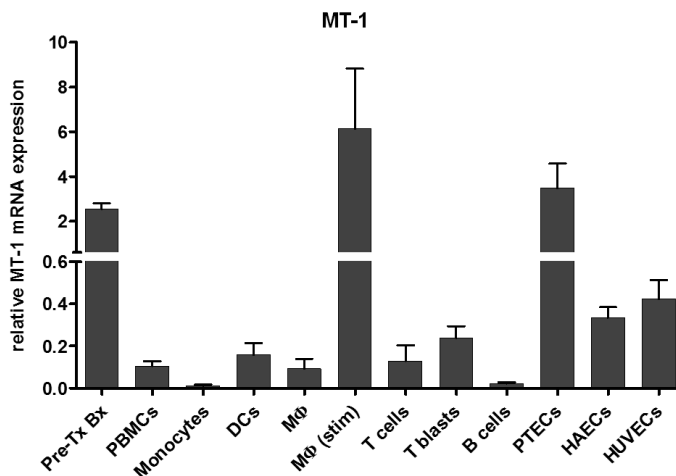


Figure 8. Localization of metallothionein (MT) expression in cell types. MT-1 expression was quantified by qPCR in mRNA from a panel of organs and cell types. High MT-1 expression was seen in activated macrophages (Mφ stim) and proximal tubular epithelial cells (PTECs). Data are expressed as mean ± SEM. Pre-Tx, pretransplant biopsy samples; PBMCs, peripheral blood mononuclear cells; DCs, dendritic cells; Mφ, differentiated macrophages; HAECs, human aorta endothelial cells; HUVECs, human umbilical vascular endothelial cells.

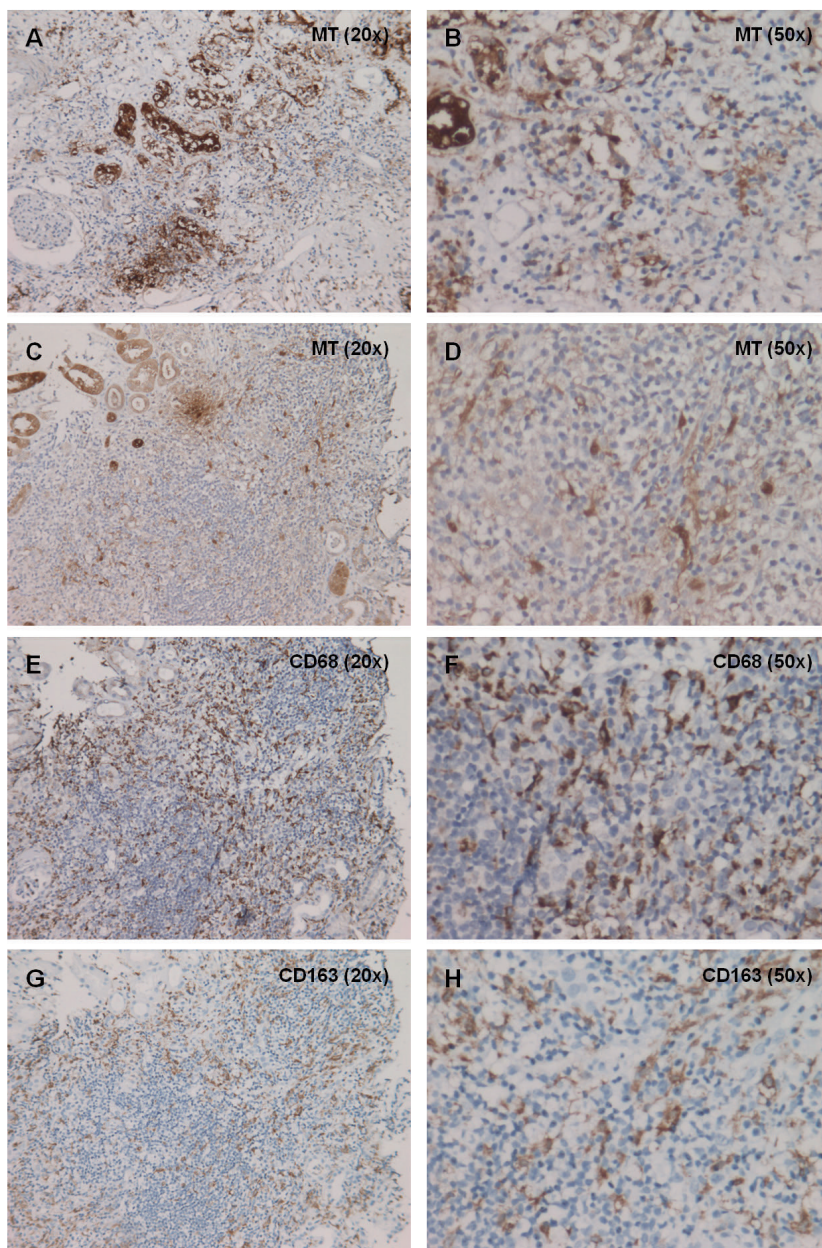


Figure 9. Immunohistochemical staining pattern of metallothionein (MT), CD68 and CD163 in sections from renal transplant biopsies with acute rejection. MT protein was detected in tubular epithelial cells and infiltrating lymphocytes (A-D). Sequential sections stained for MT protein (C and D), the pan-macrophage marker CD68 (E and F), and the macrophage activation marker CD163 (G and H) confirm that MT is expressed by activated macrophages. A, C, E, and G: overview recordings at 20x magnification; B, D, F, and H: enlarged recordings at 50x magnification.

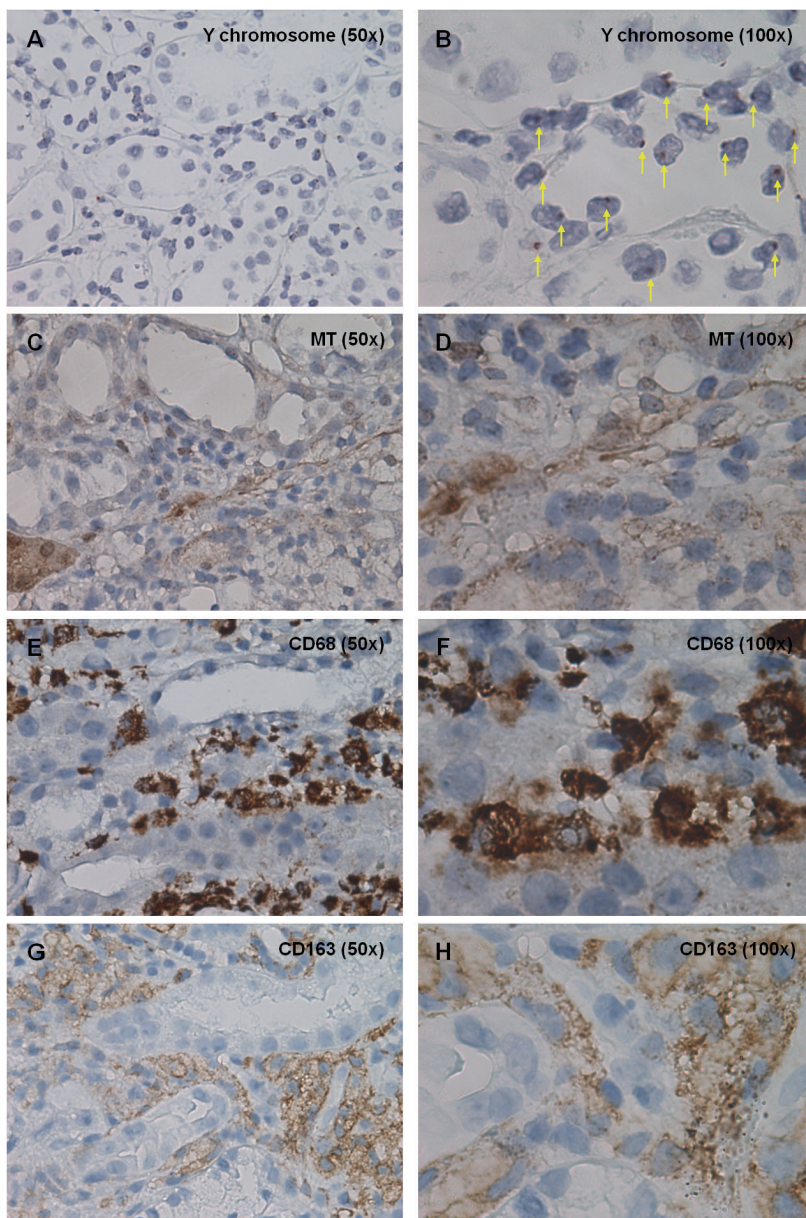


Figure 10. Analysis of the origin of MT positive macrophages. To detect the origin (donor or recipient) of the MT positive macrophages within renal transplant biopsies with acute rejection, we used in situ hybridization for the detection of the Y chromosome in transplant conditions where a male patient received a renal allograft from a female donor. Red-brown dots indicate the presence of Y chromosome-positive cells (yellow arrows) within the allograft (A-B). Sequential sections stained for MT protein (C-D), the pan-macrophage marker CD68 (E-F), and the macrophage activation marker CD163 (G-H) revealed that MT is expressed by macrophages that are derived from the patient. A, C, E, and G: overview recordings at 50x magnification; B, D, F, and H: enlarged recordings at 100x magnification.

***In vitro* methylprednisolone response experiments**

To further investigate the relationship between MT and response to steroid treatment of immune cells, peripheral blood mononuclear cells (PBMC) from healthy blood donors (n=11) were activated with PHA and cultured in the absence or presence of methylprednisolone. Using the *in vitro* cytokine response under the influence of methylprednisolone treatment, a distinction could be made between non-responders (n=3) and responders (n=8). Upon PHA stimulation, IFN γ and TNF α mRNA expression were increased compared to medium controls (Figure 11A and B). Responders to treatment (n=8) showed normalization of both IFN γ and TNF α expression levels when 10^{-4} M methylprednisolone was added during the PHA stimulation. Non-responders (n=3) still showed elevated IFN γ and TNF α mRNA expression even after methylprednisolone treatment (Figure 11A and B). Non-response to methylprednisolone treatment was associated with a significantly stronger increase in MT expression (48.8 fold) compared with what was seen in the responders (5.8 fold) (Figure 11C).

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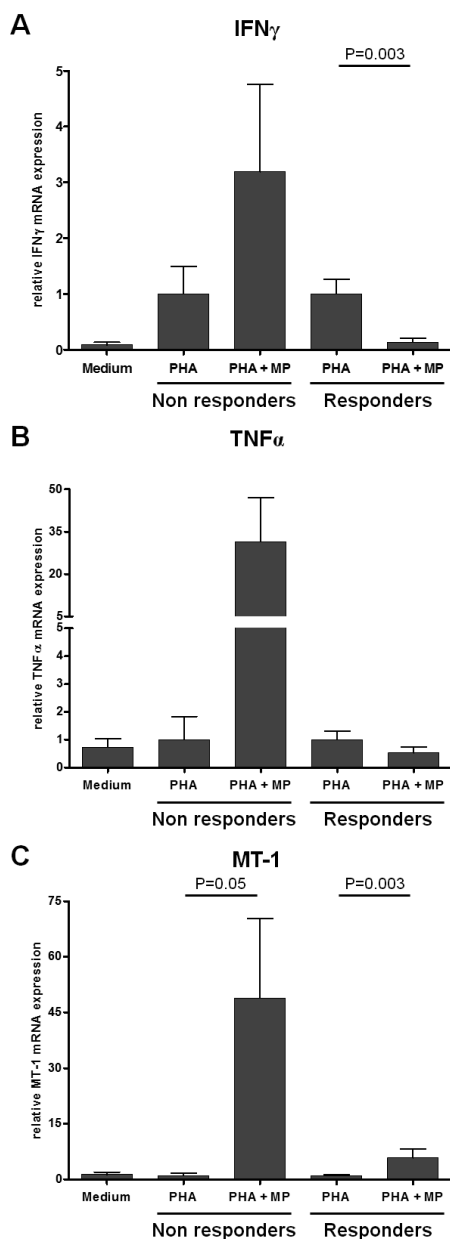


Figure 11. *In vitro* methylprednisolone (MP) response experiments. PBMC from healthy blood donors (n=11) were stimulated (50 h) with PHA or a combination of PHA and 10^{-4} methylprednisolone (PHA + MP). PBMC cultured in medium without a stimulus (50 h) were included as a control. After the culture period, RNA expression levels of IFN γ (A), TNF α (B), and MT-1 (C) were quantified by qPCR. Using the *in vitro* IFN γ and TNF α responses under the influence of methylprednisolone treatment, a distinction was made between non-responders (n=3) and responders (n=8). Data are expressed as mean $\pm$ SEM. Mean signals of PHA-stimulated PBMC were set to 1.

Discussion

We aimed to identify novel molecular markers associated with steroid-refractory acute rejection, and to gain insight into the mechanisms underlying steroid resistance. For this, we investigated intragraft gene expression profiles of renal allograft recipients with a first rejection episode. Our study reveals that the expression of metallothioneins (MT) within the renal allograft may distinguish patients with steroid resistant acute rejection from patients with steroid responsive acute rejection. The findings, which were generated in a discovery cohort of 36 patients, were validated in a validation cohort of 47 patients.

Until now, research in the field of steroid-refractory acute rejection in renal allografts has been focused on markers of inflammatory cells (reviewed in ⁸). Recently, we found that the combination of T-cell activation markers CD25:CD3 ratio and LAG-3 offers a superior prognostic value for assessing steroid response compared with conventional parameters ⁹. In the current study, we found that relatively high intragraft MT expression during acute rejection is associated with steroid resistance, and that MT-1 isoforms are expressed in activated macrophages and in tubular epithelial cells. These findings are in line with earlier findings in lung allograft recipients with steroid-refractory acute rejection. Yousem and colleagues found increased percentages of MT-positive macrophages in transbronchial biopsy samples of lung allograft recipients who experienced steroid-refractory acute rejection ²⁵. Interestingly, several studies in the oncological research field have also demonstrated that elevated expression of MT is related to treatment resistance ²⁶⁻²⁸.

In multivariate analysis, the combination of MT-1 with CYP4A11, TIMP1 and F2R represented the best predictive model. This multivariate MT-1 model has a slightly higher predictive value than the T-cell activation model found in our previous study (see Figure 7) ⁹. Combination of the two models resulted in a predictive model with MT-1, TIMP1, F2R, CD25:CD3e ratio and LAG-3 as independent covariates, showing that our current findings strengthen the risk assessment of steroid resistance in patients having acute rejection. Patients with steroid-refractory acute rejection may benefit from immediate ATG treatment after the diagnosis of acute rejection.

Metallothioneins are a family of eleven proteins involved in the homeostasis of biologically essential metals ²⁹⁻³³. Under physiological conditions MT are mainly expressed in the kidney and liver ³⁰. MT can bind zinc ions, and by functioning as a

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zinc-donor or zinc-acceptor they can control cellular zinc distribution ^{29;30}. A variety of DNA-binding proteins rely on zinc finger motifs to bind to their target sequences ^{29;34}. Metallothioneins can influence the DNA-binding capacity of zinc-proteins by controlling the amount of zinc that is available for zinc finger domains ^{35;36}.

The glucocorticoid receptor (GR) is also a zinc-dependent protein ³⁷. Glucocorticoid effects depend on GR-mediated transcriptional regulation of genes encoding for pro-inflammatory proteins. Binding of GR to glucocorticoid response elements (GRE) in the promoter region of target genes relies on two zinc finger motifs ^{29;34;37}. Increased expression of metallothioneins may lead to removal of zinc ions that are normally complexed in the zinc finger domains of GR, preventing its binding to GRE and inhibiting the immunomodulatory effects of the steroid-based anti-rejection treatment.

Another mechanism of action of the GR that may be affected by increased MT expression is its recruitment of histone deacetylase (HDAC)-2 ³⁸. Upon ligand binding the GR becomes a target for HDAC-2, which in turn allows the GR to associate with and suppress the pro-inflammatory transcription factor NF- κ B ^{38;39}. In addition, the recruited HDAC-2 represses transcription from promoters through histone deacetylation, resulting in suppression of activated inflammatory genes within the nucleus ^{38;40}. As the recruitment of HDAC-2 to the GR relies on zinc, anti-inflammatory effects of this process may also be inhibited by MT.

In vitro tests with PBMC have been used to correlate gene expression profiles with clinical disorders, including steroid responsiveness ⁴¹⁻⁴³. Following this strategy, we compared the RNA level of pro-inflammatory cytokine responses (TNF α , IFN γ) between stimulated PBMC and stimulated PBMC treated with methylprednisolone. This allowed distinction between responders and non-responders to the steroid treatment. The concentration of 10^{-4} M methylprednisolone approximates the i.v. dosage of 1 g/day given in clinical practice. In all PBMC donors tested, treatment with methylprednisolone led to an increase in MT expression, which corresponds with earlier observations of MT as a steroid responsive gene ^{44;45}. However, non-responders displayed a significantly higher fold of MT upregulation upon methylprednisolone treatment than responders. These results are in line with the observation that patients who are steroid-resistant upon anti-rejection treatment have elevated MT expression. Further studies are needed to show that *in vitro* cultures of patient PBMC represent a useful indicator of the patient's response to steroid treatment *in vivo*.

Some comments need to be made with regard to the current findings. First, MT expression can be induced by the glucocorticoids ⁴⁴. To prevent an influence of steroid-based anti-rejection treatment on the intragraft mRNA and protein expression levels, the biopsy samples investigated in our study were collected before the patients received high-dose methylprednisolone. All patients did receive low-dose steroids as part of the maintenance therapy. Second, due to high homology in the gene sequence of the MT-1 subtypes, it has proven difficult to distinguish the MT-1 subtypes from each other. As our microarray assays rely on 50-bp long probes to measure specific mRNA transcripts, we were able to measure the mRNA expression of the individual MT-1 isotypes. However, no distinction could be made between the MT-1 isoforms with qPCR or immunohistochemistry. The finding by microarray, that most MT-1 isoforms were upregulated in steroid resistant acute rejection, shows that the difficulty to distinguish between MT-1 isoforms likely will not have affected our findings.

In conclusion, resistance to steroid treatment is associated with a relatively high intragraft expression of zinc-regulating MT during acute rejection. MT are mainly expressed by activated macrophages and tubular epithelial cells within the kidney. The relationship between MT and steroid resistance was confirmed *in vitro* by treatment of activated PBMC with corticosteroids. An increased expression of MT may lead to regulation of intracellular zinc concentrations, and to inactivation of the DNA binding capacity of the glucocorticoid receptor. The current findings point to MT as potentially novel therapeutic targets.

Acknowledgements

Part of this study was presented at the American Transplant Congress 2012 in Boston, MA, USA. This study was supported by a grant from the Dutch Kidney Foundation (C07.2238).

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Supplemental data

Table S1: Demographic and clinical data of the discovery set and validation set.

Variable	Discovery set (n=36), n (%)	Validation set (n=47), n (%)	P value
Patient age			1.00
≥ 50 years	16 (44.4%)	21 (44.7%)	
Patient gender			0.36
Female	10 (27.8%)	18 (38.3%)	
Donor age			0.36
≥ 50 years	11 (30.6%)	20 (42.6%)	
Donor gender			0.48
Female	22 (61.1%)	33 (70.2%)	
Year of transplantation			0.80
1995 through 1999	26 (72.2%)	36 (76.6%)	
2000 through 2005	10 (27.8%)	11 (23.4%)	
Donor type			0.79
Living	9 (25.0%)	10 (21.3%)	
Post mortal	27 (75.0%)	37 (78.7%)	
HLA-A matching			0.36
≥ 1 mismatch	25 (69.4%)	27 (57.4%)	
HLA-B matching			0.62
≥ 1 mismatch	28 (77.8%)	34 (72.3%)	
HLA-DR matching			1.00
≥ 1 mismatch	23 (63.9%)	30 (63.8%)	
Virtual PRA			1.00
Immunized (6-100%)	9 (25.7%)	13 (27.7%)	
Cold ischemia time			0.30
> 18 hours	20 (64.5%)	30 (76.9%)	
Induction therapy			1.00
Daclizumab	8 (22.2%)	11 (23.4%)	
None	28 (77.8%)	36 (76.6%)	
Maintenance therapy			0.66
Double therapy	16 (44.4%)	24 (51.1%)	
Triple therapy	20 (55.6%)	23 (48.9%)	
Delayed graft function			0.18
Yes	5 (13.9%)	13 (27.7%)	
Rejection time			1.00
<3 months post Tx	35 (97.2%)	46 (97.9%)	
3-6 months post Tx	1 (2.8%)	1 (2.1%)	
C4d staining			0.10
Positive	15 (41.7%)	11 (23.4%)	
Vascular rejection			1.00
Yes	7 (38.9%)	8 (44.4%)	

PRA, panel reactive antibodies; DGF, delayed graft function; Tx, transplantation

Table S2: Gene ontology molecular pathways overrepresented in the intra-graft expressed genes that significantly discriminate patient groups ^a.

GO identification	Ontology	P value	Pathway/function	Genes
GO:0046870	MF	9.98E-07	Cadmium ion binding	MT-1X, MT-1G, MT-1A, MT-1F, MT-1E, MT-1M, MT-1H
GO:0005576	CC	0.0003	Extracellular region	SVEP1, FRAS1, GPC6, LAMC3, LYGI, MMP11, ANG, TSKU, MFAP1, COL9A2, F12, REN, PROC, FXD6, PLA2G7, IDE, TCN2, GC, CHRD, PLA2G15, AGT, ADAMTSL2, TINAG, PLG, FBLN5, TNFRSF1A, AZGP1, SEMA3F, CPN2, PRAP1, ADIM2, SERPINA3, NUCB2, STC2, PLA1A, PHGDH, EPHB6, LAMA2, COL7A1, SPP1
GO:0044421	CC	0.0011	Extracellular region part	AGT, FRAS1, ADAMTSL2, TINAG, PLG, FBLN5, GPC6, LAMC3, SEMA3F, MMP11, ANG, MFAP1, COL9A2, F12, REN, PROC, NUCB2, FXD6, PLA2G7, LAMA2, IDE, COL7A1, TCN2, GC, CHRD, SPP1
GO:0044420	CC	0.0038	Extracellular matrix part	MFAP1, COL9A2, FRAS1, TINAG, LAMC3, LAMA2, COL7A1, ANG
GO:0005578	CC	0.0038	Proteinaceous extracellular matrix	MFAP1, COL9A2, FRAS1, ADAMTSL2, TINAG, FBLN5, LAMC3, GPC6, LAMA2, COL7A1, MMP11, ANG
GO:0005507	MF	0.0082	Copper ion binding	MT-1X, MT-1G, MT-1A, MT-1F, MT-1E, MT-1M, MT-1H, ANG
GO:0004091	MF	0.0082	Carboxylesterase activity	PLA2G15, IPO4, NLGN1, PLA2G4F, ACOT4, PLA2G7, PLA1A, MGLL
GO:0031012	CC	0.0122	extracellular matrix	MFAP1, COL9A2, FRAS1, ADAMTSL2, TINAG, FBLN5, LAMC3, GPC6, LAMA2, COL7A1, MMP11, ANG
GO:0005604	CC	0.0153	Basement membrane	LAMC3, LAMA2, FRAS1, COL7A1, TINAG, ANG
GO:0042598	CC	0.0175	Vesicular fraction	CYP4A11, HSP90B1, CYP8B1, ERO1L, SEC11C, UGT1A9, UGT1A6, PLG, TALDO1, KCNU8, SPCS3, RAB38
GO:0032787	BP	0.0223	Monocarboxylic acid metabolic process	PLA2G15, AGT, ACADSB, ACADL, AMACR, ALDH1L1, ACOT4, TNFRSF1A, ALDOB, CYP4A11, PCCB, RBP1, ABHD5, ECHS1, AGXT, AKR1C3, ACADS, MCCC2
GO:0006544	BP	0.0223	Glycine metabolic process	PHGDH, GCSH, SHMT2, AGXT
GO:0006082	BP	0.0223	Organic acid metabolic process	PLA2G15, AGT, ACADSB, GCSH, ACO1, ACADL, SHMT2, AMACR, ALDH1L1, ACOT4, TNFRSF1A, TMEM91, ALDOB, HPD, CYP4A11, ABHD5, DOCK6, PCCB, RBP1, ECHS1, CPVL, AGXT, PHGDH, AKR1C3, PEPD, MCCC2, ACADS
GO:0019752	BP	0.0282	Carboxylic acid metabolic process	PLA2G15, AGT, ACADSB, GCSH, ACO1, ACADL, SHMT2, AMACR, ALDH1L1, ACOT4, TNFRSF1A, TMEM91, ALDOB, HPD, CYP4A11, ABHD5, PCCB, RBP1, ECHS1, CPVL, AGXT, PHGDH, AKR1C3, PEPD, MCCC2, ACADS
GO:0042180	BP	0.0282	Cellular ketone metabolic process	PLA2G15, AGT, ACADSB, GCSH, ACO1, ACADL, SHMT2, AMACR, ALDH1L1, ACOT4, TNFRSF1A, TMEM91, ALDOB, HPD, CYP4A11, ABHD5, PCCB, RBP1, ECHS1, CPVL, AGXT, PHGDH, AKR1C3, PEPD, MCCC2, ACADS

GO:0043436	BP	0.0282	Oxoacid metabolic process	PLA2G15, AGT, ACADSB, GCSH, ACO1, ACADL, SHMT2, AMACR, ALDH1L1, ACOT4, TNFRSF1A, TMEM91, ALDOB, HPD, CYP4A11, ABHD5, PCCB, RBP1, ECHS1, CPVL, AGXT, PHGDH, AKR1C3, PEPD, MCCC2, ACADS PHGDH, GCSH, SHMT2, AGXT
GO:0009069	BP	0.0318	Serine family amino acid metabolic process	
GO:0006629	BP	0.0318	Lipid metabolic process	PLA2G15, AGT, ACADSB, IPO4, CYP27B1, ACADL, AMACR, ACOT4, TNFRSF1A, MGLL, AZGP1, ANG, CYP4A11, ABHD5, PCCB, RBP1, SERPINA3, ECHS1, PLA2G4F, PLA1A, PLA2G7, GDPD5, ETK2, AKR1C3, GC, SPTLC3, HSD17B8, MCCC2, ACADS
GO:0000267	CC	0.0325	Cell fraction	PLA2G15, STK39, CRYAB, AGT, SLC1A5, ERO1L, GSTM3, SEC11C, CHST10, SPTAN1, PLG, FBLN5, KCN8, ACY3, SLC16A2, GCHFR, RAB38, CYP4A11, CYP8B1, HSP90B1, GCNT3, ZFP106, UGT1A9, TALDO1, UGT1A6, SPCS3, EFN81, IDE, HSD17B8, CDH5
GO:0005792	CC	0.0357	Microsome	CYP4A11, HSP90B1, CYP8B1, ERO1L, SEC11C, UGT1A9, UGT1A6, PLG, TALDO1, KCN8, SPCS3
GO:0006631	BP	0.0376	Fatty acid metabolic process	CYP4A11, PLA2G15, ABHD5, PCCB, AGT, ACADSB, ECHS1, ACADL, ACOT4, TNFRSF1A, AKR1C3, MCCC2, ACADS
GO:0007155	BP	0.0385	Cell adhesion	SVEP1, SETBP1, AGT, FRAS1, AKAP8L, CHST10, CLDN8, CLDN12, TINAG, FBLN5, LAMC3, AZGP1, MUPCDH, BYSL, ACVRL1, CDH1, NLGN1, ADAM15, LAMA2, EFN81, COL7A1, SPP1, CHRDL, CDH5
GO:0050327	MF	0.0463	Testosterone 17-beta-dehydrogenase activity	AKR1C3, HSD17B8
GO:0008453	MF	0.0463	Alanine-glyoxylate transaminase activity	AGXT, AGXT2
GO:0005243	MF	0.0463	Gap junction channel activity	PANX2, GJB2
GO:0005212	MF	0.0463	Structural constituent of eye lens	CRYAA, CRYAB
GO:0022829	MF	0.0463	Wide pore channel activity	PANX2, GJB2
GO:0005615	CC	0.0490	Extracellular space	AGT, PLG, GPC6, SEMA3F, ANG, F12, REN, PROC, NUCB2, FXD6, PLA2G7, IDE, GC, TCN2, CHRDL, SPP1

^a All 249 genes significantly discriminating steroid responsive and steroid resistant patients were included for pathway analysis by hypergeometric test in WebGestalt. All P values are adjusted for multiple testing, using the Benjamini-Hochberg method. GO, gene ontology; BP, biological process; MF, molecular function; CC, cellular component.

