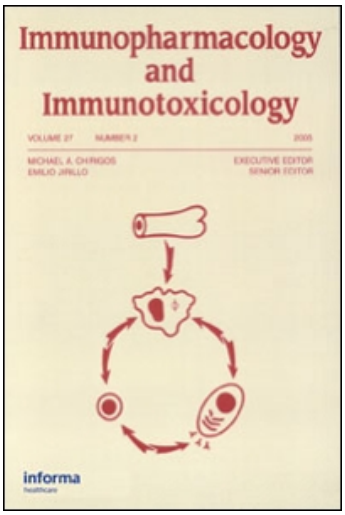

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Combination of immunosuppressive drugs leaves specific "fingerprint" on gene expression in vitro

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RESEARCH ARTICLE

Combination of immunosuppressive drugs leaves specific "fingerprint" on gene expression *in vitro*

Brigitta Rumberger[1], Clemens Kreutz[2], Christian Nickel[1], Marinella Klein[1], Severine Lagoutte[1], Sven Teschner[1], Jens Timmer[2], Peter Gerke[1], Gerd Walz[1], and Johannes Donauer[1]

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Abstract

Following organ transplantation many patients suffer from drug-related side effects, or receive more immunosuppression than necessary to prevent rejection. Hence, parameters are needed to tailor the immunosuppressive therapy to the individual needs of an organ recipient. The aim of this study was to determine whether drug combinations provoke specific gene expression patterns in a simple assay system *in vitro*. Stimulated peripheral blood lymphocytes were cultured in the presence of cyclosporine A, tacrolimus, mycophenolic acid, everolimus and sirolimus, or combinations thereof. Using a cDNA microarray, we found that all samples clustered in drug-specific groups. Gene expression profiles were almost identical in PBL treated with either cyclosporine A or tacrolimus, and with either sirolimus or everolimus. More than 50 genes were synergistically affected by combinations of calcineurin-inhibitors and TOR-inhibitors and drug-specific regulated genes could be identified for both substance groups. Our data suggest that *in vitro* gene profiling can be used to describe synergistic effects of immunosuppressive drugs. Furthermore, our approach may help to identify marker genes urgently needed to optimize and individualize immunosuppressive drug regimens after organ transplantation.

Introduction

Methods to analyze the response to drug combinations in a given individual are urgently needed in order to eventually develop protocols of individually "tailored" immunosuppression.[1] Recently it has been shown that in many cases synergistic effect of drugs *in vitro* correlates well with *in vivo* data. Furthermore, mRNA expression correlates well with protein expression levels and functional tests.[2,3] Since effects of drugs are complex, the array technology is well suited to study impact of drugs without a priori knowledge of genes of interest.

It is well documented that different states of lymphocyte activation can be identified as "functional fingerprints" using microarray technology.[4] There is evidence that gene expression patterns from PBL (peripheral blood lymphocytes) can predict presence of tolerogenic cells.[5] Microarray-based gene expression profiles of organs have been shown to classify states of rejection in several models.[6–8] Lamb et al. [9] showed that shared mechanisms of action can be deduced from gene expression data of *in vitro* experiments.

The aims of this study were to show that specific gene expression patterns of immunosuppressive drugs can be generated in a short *in vitro* experiment, and furthermore to identify specific or synergistic effects of the calcineurin-inhibitors cyclosporine A (CsA) and tacrolimus (FK), the TOR-inhibitors (target-of-rapamycin-inhibitors) everolimus (EVL) and sirolimus (SRL) and mycophenolic acid (MMF) or clinically used combinations thereof. For that purpose global gene expression of *in vitro* cultured PHA(phytohaemagglutinin) stimulated PBLs was monitored with 7.5k cDNA-microarrays.

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Materials and methods

Cell culture

PBL from three different healthy adults were isolated from whole blood samples using Ficoll density gradient centrifugation and cultured in RPMI medium (supplemented with fetal calf serum 10% and penicillin/streptomycin 1%) in 12-well plates at 2×10^6 cells/well. After 24 hours PHA and immunosuppressive drugs were added to yield the following final concentration of drugs: PHA 1 µg/ml, CsA 50 ng/ml, FK 1 ng/ml, MMF 50 ng/ml, EVL 1 ng/ml, SRL 1 ng/ml and combinations of CsA or FK respectively with MMF or EVL or SRL. Drug dilutions were done in medium from stocks immediately before being added to culture. Parallel cultures were grown containing PHA only or PHA plus tween-ethanol or methanol at the same concentrations used for the drug dilutions, respectively.

Cells were harvested 24 h later, homogenized in 4 M guanidinium isothiocyanate with and 0.72% ß-mercaptoethanol. RNA was isolated in a cesiumchloride gradient instantaneously. After ethanol precipitation RNA was resuspended at a concentration of 0.4–4 µg/µl and stored at – 80°C. We investigated 5 single drugs and 6 drug combinations, each repeated in a paired design by the same three biological replications. Three controls were done, one for each solvent condition. Additionally, we performed technical duplicates for one donor for incubations with two single drugs and their combination (complete with all controls) to compensate for lost pellets from another experiment. Finally, our study involved a total of 48 cDNA microarrays.

Hybridization

All hybridizations were performed in the presence of an equal amount of reference RNA (Stratagene, LaJolla, CA, USA) as recently described by Boldrick et al.[10] In the presence of Cy3- labeled dUTP, 12 µg of PBL RNA were transcribed into cDNA using Stratascript reverse transcriptase (Stratagene, La Jolla, CA, USA). Likewise, 12 µg of reference RNA (Stratagene) were Cy5-labeled. All other steps, including hybridization, were performed following the protocol published by P.Brown et al. (see http://cmgm.stanford.edu/pbrown for details) except that a PCR-purification kit (Qiagen, Hilden, Germany) was used for cDNA purification. (For a detailed protocol of hybridization see www.genomics.uni-freiburg.de)

BrdU-test

BrdU-(5-Brom-2-desoxyuridin)-tests were purchased from Roche (Grenzach-Wyhlen, Germany) and done according to the manufacturer's specification simultaneously using the same cells, same drug dilutions and identical culture conditions.

Preparation and storage of drugs

CsA and EVL were kindly provided by Novartis Pharma (Basel, Switzerland), FK by Astellas Pharmaceuticals (Munich, Germany) and SRL by Wyeth (Münster, Germany), mycophenolic acid and p-PHA were purchased from Sigma (Munich, Germany).

Drugs were kept lyophilized at – 20°C until preparation of stocks at a concentration of 1 mg/ml for SRL and 10 mg/ml for all others. Stocks were prepared in ethanol containing 20% of tween 20 for CsA, FK, SRL, and EVL and in methanol for MMF and also kept at – 20°C until dilution in medium. The first step was done in pure medium, all following steps in medium containing fetal calf serum.

Drug concentrations were chosen from *in vitro* data published previously.[11–13]

Microarrays

cDNA microarrays were produced and processed essentially according to the Stanford protocol described by Eisen and Brown 1999.[14] Approximately 7,700 annotated genes from the RZPD (Resource Center and Primary Database, Berlin, Germany) including 1152 clones from the so-called lymphochip (https://www.rzpd.de) were obtained as bacterial stocks. Plasmids were purified using the Qiagen 96-well Turbo Kit (Qiagen, Hilden, Germany), and inserts were purified by PCR using vector primers flanking the individual inserts (5′-CTG CAA GGC GAT TAA GTT GGG TAA C-3′ and 5′-GTG AGC GGA TAA CAA TTT CAC ACA GGA AAC AGC-3′).

PCR products were purified by ethanol precipitation and resuspended in ddH2O. Aliquots were transferred into 384-well plates, dried and resuspended in 3 × SSC/1.5 M betain to a final concentration of approximately 40 ng/µl. Printing was performed on aminosilane coated slides (CMT-GAP II Slides, Corning, NY, USA), using an arrayer that was assembled according to specifications by the Stanford group using software provided by Joe de Risi (http://cmgm.stanford.edu/pbrown).

Semiquantative RT-PCR

Total RNA (1 µg) from all samples of one donor was purified with the RNase free DNase set (Qiagen, Hilden, Germany) and reversely transcribed into cDNAs using oligo (dT) 12-18 primer and Superscript II reverse transcriptase (Invitrogen,Carlsbad, CA, USA). Each single-stranded cDNA was diluted for subsequent PCR amplification. PCR was carried out with GAPDH as an internal control in each reaction vial.

The following primers were used (5′-3′): GAPDH: TGG AAA TCC CAT CAC CAT CT and GTC TTC TGG GTG GCA GTG AT; E4BP4 (X64318): GAA CCC TCG ATG GTG TCA AG and TCT TGG CTC CCT TGT GTA GC; Fructose Bisphosphatase (U21931): GCC GTG TTA GAC GTC ATT CC and TAA GGT GCA CAG CAG GTC AG; MCP 2 (Y16645): AGC CAC TTT CAG CCC TCA G and AAT CCC TGA CCC ATC TCT CC; IL-2: GCA ACT CCT GTC TTG CAT TG and CAG TTC TGT GGC CTT CTT GG.

To determine the optimal number of PCR cycles for all primer pairs a set of preliminary tests was performed using RNA of one donor. An initial denaturation at 94°C for 5 min followed by 28 cycles of denaturation for 30 sec, annealing for 1 min at 55°C and elongation for 2 min at 72°C was found to give best results in all reactions. Products were run on 3% agarose gels and bands revealed using ethidium bromide staining. Bands were evaluated in relation to the corresponding GAPDH-band using the Scion Imaging software (Scion, Frederick, MD, USA).

Data analysis

Signal intensities were measured by an Axon 4000A scanner using GenePix 3.0 software (Axon Instruments Inc., Union City, CA, USA). Artifacts, if not detected by the software, were excluded manually. Image and data files, array layout, as well as all relevant information according to the MIAME guidelines (Minimum Information About a Microarray Experiment[15]) were transferred into the GeneTrafficDuo database (Microarray Data Management and Analysis Software, Iobion Informatics, LLC, La Jolla, CA, USA).

To exclude artifacts near background range, all spots were eliminated when sample intensity or reference intensity was less than 50 above the local background. Local background was subtracted from spot intensities. Normalization was performed with the Lowess (Locally weighted scatter plot smoother) sub-grid normalization method. Sub-grid normalization calculates the normalization factor for each of the 16 sub-grids independently and therefore is, compared to global normalization, relatively insensitive to local variations on the array.[16]

For statistical analysis of differentially expressed genes we fit a linear model to the gene expression data X to estimate the influence α_p of the patients and the effect β_d of the drugs using the following formula:

$$X_{pdi}=\alpha_p+\beta_d+\varepsilon_{pdi}$$
$$p=1,\ldots,npatient, d=1,\ldots,ndrugs, i=1,\ldots nreplicates.$$

p enumerates the patients treated with drug d and ε_{pdi} is Gaussian noise. The index i count the number of replicates.

Using this model we could eliminate patient specific effects on gene expression data. In the case of n=1 this corresponds exactly to a paired t-Test.

For each gene, the fit of the linear model provides a probability that the applied drug has a vanishing effect. To adjust these obtained p-values, the method by Benjamini[17] was applied to control for multiple testing (FDR = false discovery rate). For each drug 50 genes with the most significant effect (smallest pFDR = p-value adjusted for multiple testing) were selected and agglomerative hierarchical clustering introduced by Kaufman[18] was performed using the R statistical software package (www.r-project.org).

In order to screen on synergistic, possibly supra-additive effects, genes were selected which fulfilled the following three criteria: First, genes regulated either up or down less than 1.5-fold (+/- log 0.585) in the drug combination were eliminated. Second, pFDR for the change of expression level in the drug combination versus controls had to be <0.05. Third, regulation of combination versus each single substance and of combination versus expected fold-regulation (calculated as sum of effects of single substances) had to be at least 1.5-fold.

Results

One goal of this study was to measure the effects of immunosuppressants in various clinically relevant combinations on the gene expression profile of human PBL. We used the accepted model[11] of culturing PHA treated PBL in the presence of immunosuppressants.

Effects of immunosuppressants on cell proliferation and IL-2 production

We first tested the effect of each single substance on lymphocytic proliferation. Only MMF and none of the other single drugs in the chosen concentrations had an effect on lymphocyte proliferation. In combination of calcineurin-inhibitors with TOR-inhibitors there was a tendency to decrease proliferation in all cases (Figure 1a).

Semiquantitative RT-PCR was used to measure IL-2 expression. As expected and reported before[11] IL-2-production of PHA stimulated lymphocytes was reduced in presence of calcineurin-inhibitors and EVL and SRL. Combination of those drugs seemed to further reduce IL-2 production in all cases (Figure 1b). Mycophenolic acid did not affect the expression level of IL-2 in our system.

Effects of solvents and immunosuppressants on gene expression

The three different solvents constitute three different types of negative controls. Initially, we tried to ensure that

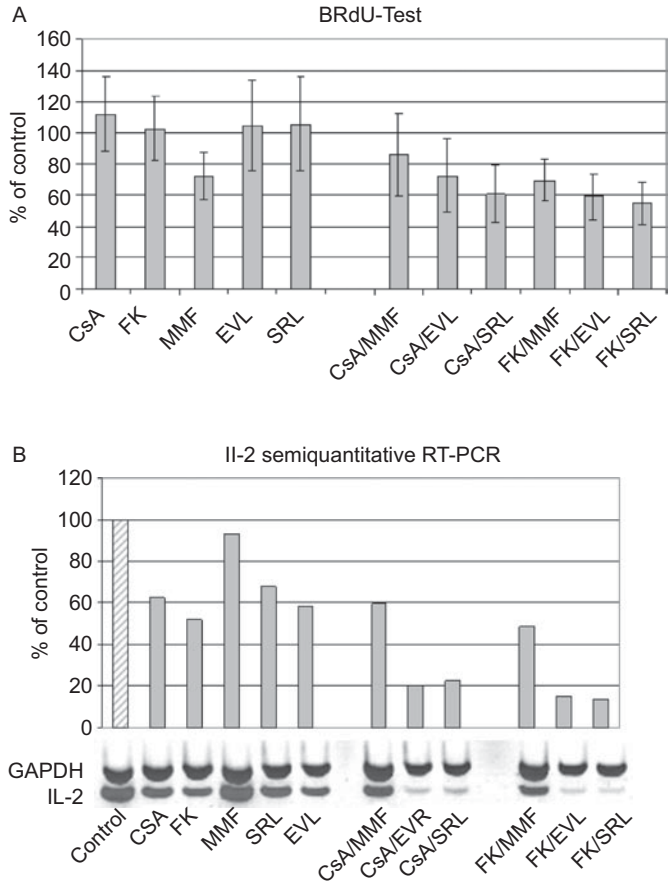

Figure 1. a: BrdU-Test was performed with each series of cell cultures. Pooled results of BrdU incorporation shown as percentage of control ± SD are given in figure 1a. b: Semi-quantitative RT-PCR using primers for IL-2 and GAPDH were performed on RNA from PHA stimulated PBL after incubation in presence of different immunosuppressive drugs. Expression level of GAPDH was used for internal normalisation. Expression levels of IL-2 were plotted as percentage of control (PHA only, no drugs).

the solvents did not lead to significant changes in gene expression measurements. No significant difference in gene expression was found by a t-test between the solvents after correction for multiple testing. Actually, all pFDR values were found to be close to one. The variability of the measurements within the negative control subset of the arrays (median SD=0.227) undercut the variability in the immunosuppressant samples (median SD=0.325) by a factor of 1.43. Figure 2 shows this difference in the measured variability. Hence, drug related changes in expression were estimated as fold induction in comparison to all three solvents as a common negative reference group.

Reproducibility of array data

On our array there were several pairs of genes spotted twice with different cDNA-fragments. The results obtained from those duplicates were highly consistent (Figure 3) and emphasize the high reproducibility of the array data.

As only one concentration of each drug was tested the experiment was not laid out to detect differences between drugs belonging to the same class. In fact, comparison between the calcineurin-inhibitors (CsA versus FK) and the TOR-inhibitors (EVL versus SRL) yielded very little significant (pFDR<0.05) differences in gene expression. Comparing CsA and FK 63 significantly regulated genes were found. However, only for one gene the difference of expression exceeded the 1.5-fold (AB020644). The TOR-inhibitors differed significantly in 47 genes, again a difference>1.5-fold was found for 2 genes only.

Agglomerative clustering

Agglomerative hierarchical clustering using all 7700 genes separated the samples mostly in a donor specific way (Figure 4a). Focusing on changes of gene expression of treated PBL versus untreated controls from the respective donor, however, an expression pattern emerged by which the samples could be grouped according to treatment. The regulation of the genes involved in these drug specific patterns seems to be specific for the treatment condition and suggests the

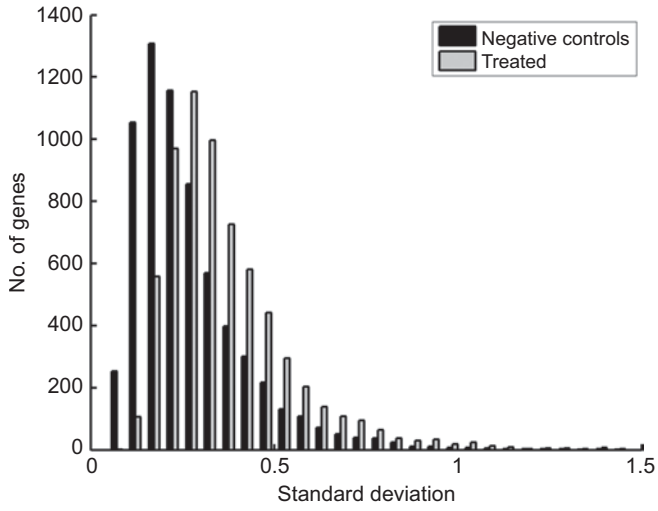

Figure 2. Frequency distribution of measurement variability. A clear decrease in standard deviation is observed within the negative controls (black) in comparison to the standard deviation within the treated subset (grey) of microarrays.

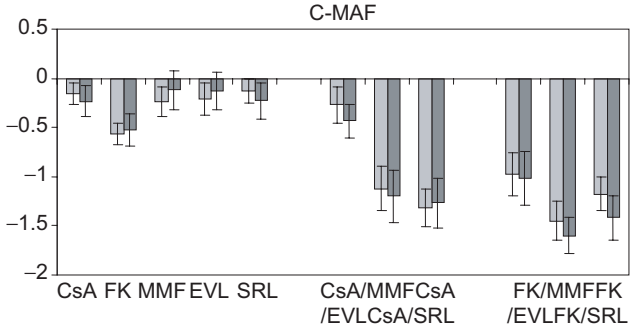

Figure 3. Histogram of expression levels determined by microarray with two different c-DNA fragments from the c-MAF gene from three donors including SD. The histogram shows the corresponding means of expression levels from microarray data of three donors (after subtraction of control) on a log 2 scale including SD.

presence of a transcriptional fingerprint (Figure 4b). We conclude that inter-individual variability can be overcome by deducting gene- and donor-specific control values for each gene. All further analyses to identify and describe "transcriptional fingerprints" or single "genes of interest" were done on "fold-changes" in gene expression compared to control (PHA stimulated PBLs) and their respective pFDR-values.

Group-specific effects of the immunosuppressants

Looking for group-specific effects of immunosuppressants genes were selected which were significantly regulated in the presence of one class of drugs, but not by other drugs. Calcineurin-inhibitors and TOR-inhibitors seemed largely to affect regulation of the same genes. However, still some genes could be identified which were regulated in a drug-specific way, for instance several monocyte chemoattractants (e.g., MCP-2 shown in Figure 5) were markedly down-regulated by TOR-inhibitors, but not affected in their regulation by calcineurin-inhibitors.

Additive effects of drug combinations

From the known biological properties of the drugs we expected that certain genes would be regulated in a synergistic ("supra-additive") fashion. To find such genes we selected for 1. significant regulation of the combination versus control and 2. changes of geneexpression of the combination exceeding the 1.5-fold (log 0.585) compared to control, either single substance or the sum of effects of the single substances. 309 genes fulfilled these criteria in at least one drug combination, calcineurin-inhibitors and MMF for 34 genes, calcineurin-inhibitor and TOR-inhibitor for 284 genes (including 9 genes which were selected in both kinds of combinations). We focused our further evaluation of supra-additive effects on the latter combinations, as the genes selected by our criteria were much more frequent.

Theoretically, such genes should be regulated in a similar way in every combination of a calcineurin-inhibitor with a TOR-inhibitor (CsA-EVL, CsA-SRL, FK-EVL, FK-SRL). As expected, 110 of those genes fulfilled our selection criteria in more than one tested combination and 55 genes in least at 3 of 4 (A list of those 55 genes is shown in Table 1, note that it contains three of the duplicate pairs mentioned above and shown in Figure 3). Re-examination of this selection based on means and p-values calculated for the entire substance group confirmed that gene expression levels between single substance (TOR-inhibitor and calcineurin-inhibitor respectively) and combination differed significantly in all cases.

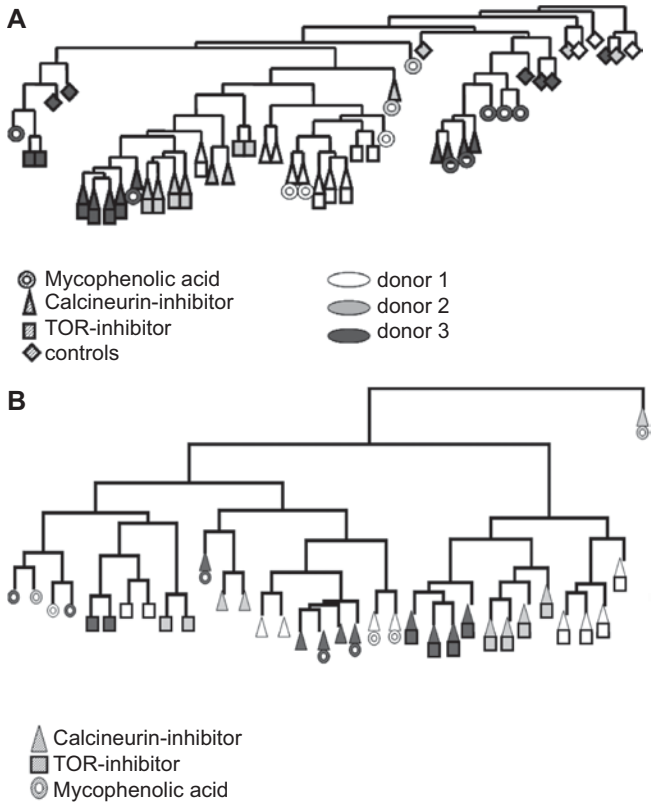

Figure 4. a: Hierarchical clustering based on Euclidean distance was done using all genes and all samples. The branches are characterised by origin from a common donor (color), modality of treatment (shapes) has a minor influence on the clustering. b: Hierarchical clustering based on Euclidean distance was done using the 50 genes with smallest pFDR-values calculated for drug or drug combination effects by fitting a linear model as described in Methods. Using this approach the modality of treatment (shapes) becomes the major determinant for the sorting of samples. The dendrogram's two major branches divide samples incubated with combination of calcineurin-inhibitor and TOR-inhibitor, from samples treated with single substances. The latter is subdivided in a branch with MMF samples, one with TOR-inhibitor samples and a third with calcineurin-inhibitor samples. The combinations of MMF with calcineurininhibitors are scattered among samples with single substance calcineurin-inhibitor treatment, one sample stands apart. Note that one set of duplicates for FK-MMF and two sets for MMF for donor three were combined in Figure 4b.

Approximately two thirds of these genes were up-regulated, one third was down-regulated. Functionally many of the up-regulated genes are involved in inflammation and innate immune response (e.g., fibroblast collagenase inhibitor, type IV collagenase, alpha-1 -antitrypsin-related protein), whereas many of the down-regulated genes are directly involved in the cell cycle, CTL (cytotoxic T-lymphocyte) function (such as granzyme B), apoptosis (bcl-xL), signaling (E4BP4) or transcription (c-MAF).

As shown in Figure 5 for 3 genes, microarray data matched the results of semiquantitative RT-PCR well.

Discussion

Long term immunosuppression after transplantation relies on the beneficial effects of immunosuppressive combination therapy. However, biological mechanisms of drug synergy are incompletely understood and an analytic basis for development of individually "tailored" immunosuppression is desirable.

With a systematic approach of collection of gene expression profiles Lamb et al.[9] created a resource to discover functional connections of actions of drugs from gene expression data of *in vitro* experiments. Hence, patterns of transcriptional response to immunosuppression most likely also indicate drug effects or possible adverse effects more precisely than single markers.

With our microarray experiments we could show that immunosuppressive drugs generate a distinctive gene expression pattern in an *in vitro* setting in only 24 h. By use of multiple biological controls we could reduce interindividual variance. Cluster analysis revealed a unique regulation in samples treated with combination immunosuppressive therapy. Genes with a strikingly supra-additive regulation under combination of calcineurin-inhibitors and TOR-inhibitors were extracted by a mathematical model. In many cases genes involved

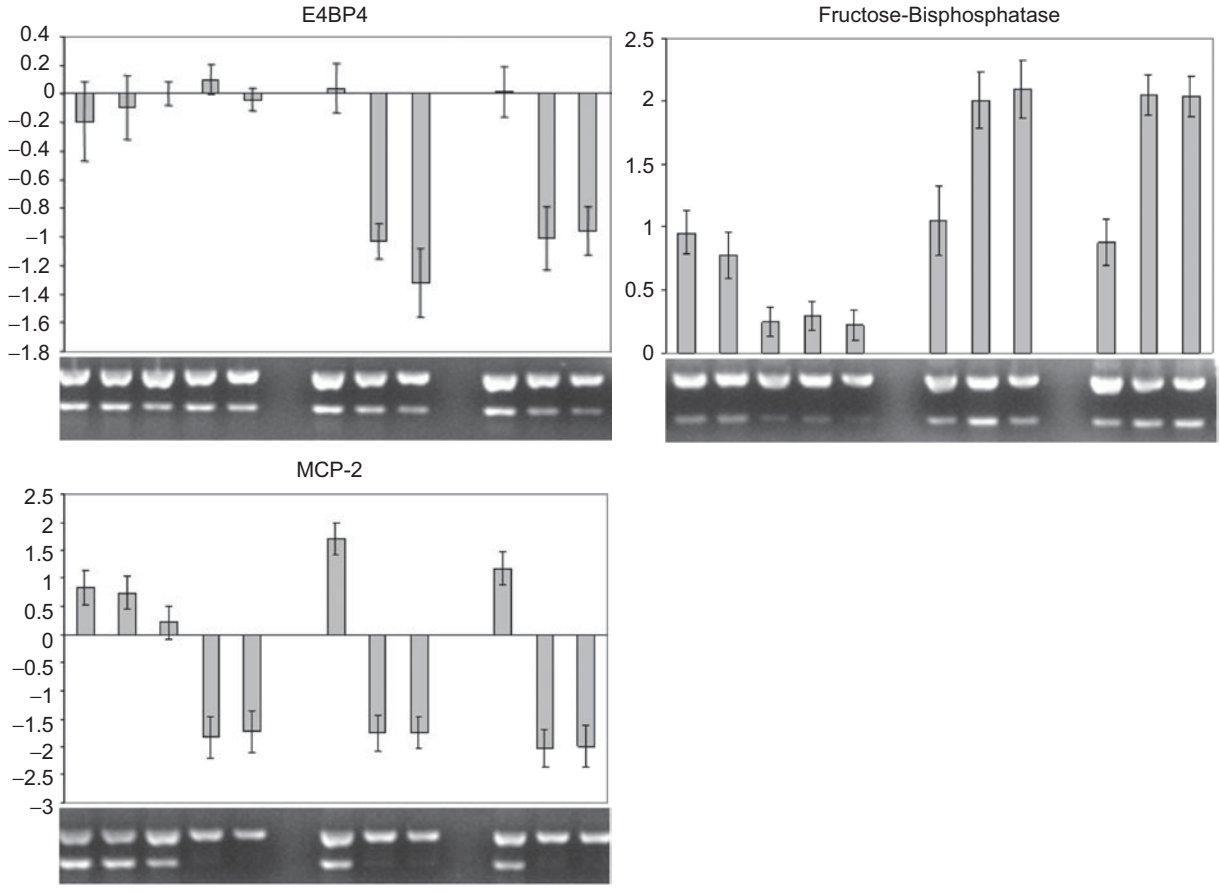

Figure 5. Verification of expression of candidate genes by semiquantitative reverse transcriptase polymerase chain reaction (RT-PCR). GAPDH (upper band) and candidate gene (lower band) were amplified simultaneously from cDNA from one donor. Above histogram shows the corresponding means of expression levels from microarray data of three donors (after subtraction of control) on a log 2 scale including SD. The samples are in the following order: CsA, FK, MMF, SRL, EVL, blank, CsA+MMF, CsA+SRL, CsA+EVL, blank, FK+MMF, FK+SRL, FK+EVL. 254×x190 mm.

in inflammation and innate immune response were up-regulated, whereas chemokines and genes involved in cell cycle or specific CTL function where down-regulated.

Distinctive patterns of gene expression induced by genetic alteration or external influences have been called "transcriptional fingerprints." They can be defined through cDNA microarrays by simultaneously screening thousands of genes in a non-hypothesis driven way. Microarray studies have already been used successfully *in vivo* and *in vitro* to identify such "fingerprints" of transplant rejection and lymphocyte activation.[7,19]

Immunosuppression works by influencing mechanisms of activation of lymphocytes and monocytes. The evaluation and prediction of drug effects *in vivo* is a difficult task. Large inter-individual differences in gene expression levels, different medical conditions or variable co-medications would render variance unacceptably high. An individually useable *in vitro* test system which could eventually be correlated with clinical outcome data appears to be a valuable tool.

We chose an easily reproducible *in vitro* cell culture assay of lymphocyte activation with standardized conditions to test whether "transcriptional fingerprints" are generated by treatment with immunosuppressants. We are aware of the limitations of *in vitro* assays in predicting biological processes in patients. Yet, Dudley et al. could successfully use an *in vitro* model of cyclosporine response in the prediction of individual response to immunosuppression.[20]

Another possible drawback to gene expression analysis is that a pattern described on a post-transcriptional level may be substantially distorted post-translationally. However, in a study of *in vitro* activated T-cells treated with and without calcineurin-inhibitors, microarray analysis proved to be a valid tool to identify novel transcriptionally regulated genes.[21] Results were confirmed on protein level and new therapeutic targets could be identified.

Using cut-off-values for minimal, presumably "relevant" regulation and p-value criteria to eliminate data of unclear significance we identified clear cut expression patterns for many genes. We realize that

Table 1. List of genes with supra-additive regulation by combination of calcineurin-inhibitors and TOR-inhibitors.

ID	Name	Category	CI	TI	CI+TI
D83920	Ficolin-1	Complex functions incl. cell cycle	1.55	0.38	3.23
Z36531	Fibrinogen-like protein (pT49)	Unknown function in immune response	1.13	0.84	3.21
N25218	Fibroblast collagenase inhibitor	Immune response/Inflammation	1.01	0.79	2.58
U64998	Ribonuclease k6 precursor	Transcription	−0.11	1.20	2.48
X62466	CAMPATH-1 (CDw52)	Unknown function in immune response	0.89	0.76	2.41
J05070	Type IV collagenase	Immune response/Inflammation	1.58	−0.31	2.33
X01683	alpha 1-antitrypsin	Immune response/Inflammation	0.71	0.53	2.25
L10333	Neuroendocrine-specific protein A	Vesicular trafficking	0.63	0.50	2.19
X62573	Fc receptor TC9	Fc-receptor	0.93	0.41	2.16
U21931	Fructose-1_6-biphosphatase	Carbohydrate metabolism	0.85	0.25	2.09
M32011	Neutrophil oxidase factor (p67-phox)	Redox regulation	0.87	0.06	2.05
U62858	Interleukin-13 receptor	cytokine receptor	0.35	0.76	2.02
M19684	Alpha-1-antitrypsin-related protein	Immune response/ Inflammation	0.53	0.60	1.94
X03663	c-fms proto-oncogene	cytokine receptor	0.75	0.02	1.87
T83429	similar to CD9	Unknown function in immune response	0.49	0.45	1.77
U03688	Dioxin-inducible cytochrome P450	Complex functions	0.49	0.45	1.77
T51594	Neuropilin 1	angiogenesis. regulatory T cells	0.75	0.00	1.73
U07802	Tis11d	RNA binding protein	0.49	0.53	1.71
M98398	CD36	Thrombospondin receptor	0.44	0.02	1.50
M28827	Thymocyte antigen CD1c	Antigen presentation	0.27	0.08	1.46
M32249	Unusual 5'- fetal A-gamma-globin	Cell cycle	0.36	0.50	1.45
U71213	mGST-1	Redox regulation	0.41	0.22	1.43
M29696	Interleukin-7 receptor (IL-7)	Cytokine receptor	0.31	0.03	1.40
AB011116	Mahogunin	Th2 differentiation	0.24	0.20	1.39
J03909	g-interferon-inducible protein (IP-30)	Immune response/ Inflammation	0.72	−0.09	1.39
N71535	FcRII mRNA for Ig G receptor	Fc-receptor	0.45	0.19	1.37
AI623784	Thymocyte antigen CD1c	Antigen presentation	0.40	0.16	1.33
J05593	TIMP-2	Immune response/ Inflammation	0.12	0.39	1.27
M14221	Cathepsin B proteinase	Antigen presentation	0.34	0.14	1.27
U90548	Butyrophilin (BTF3)	Unknown function in immune response	−0.01	0.26	1.18
X75593	rab 13	Vesicular trafficking	0.21	0.00	1.17
IMAGp958C21134	IMAGp958C21134	No annotation yet	0.01	−0.05	1.10
J03600	Lipoxygenase	Complex functions	0.26	−0.04	1.08
M16591	HCK	Cell cycle	0.13	−0.19	0.96
X57129	Histone H1	Histone	0.06	0.22	0.92
Y00433	Glutathione peroxidase	Redox regulation	0.05	0.23	0.91
U85625	Ribonuclease 6 precursor	Transcription	0.00	0.16	0.90
Z23115	bcl-xL	Apoptosis	−0.20	0.06	−0.89
N28416	GRS protein	Unknown function in immune response	−0.23	−0.04	−0.94
D14497	Proto-oncogene protein COT	Translation	−0.21	0.13	−0.94
X16396	MTHFD2	CH3 transfer	−0.16	−0.31	−1.08
D78335	5 -terminal region of UMK	DNA metabolism	0.07	−0.22	−1.10
X64318	E4BP4	Signaling	−0.13	0.04	−1.12
Z34289	Nucleolar phosphoprotein	Transcription	−0.42	−0.27	−1.27
AA496914	Short form C-MAF	Transcription	−0.36	−0.18	−1.32
AF055376	Transcription factor C-MAF	Transcription	−0.38	−0.19	−1.42
D84557	HsMcm6	Complex functions incl. cell cycle	−0.60	−0.19	−1.45
AI768839	Granzyme B	CTL-function	−0.21	−0.14	−1.73
W80984	B lymphocyte chemoattractant BLC	Chemokine	−0.65	0.02	−1.75
X66365	PLSTIRE	Cell cycle	−0.38	−0.66	−1.78
U31278	Madp2 homolog	Cell cycle	−0.72	−0.59	−1.97
D90145	Human LD78 beta gene	Chemokine	−0.17	−0.61	−1.98

Table 1. Continued on next page

Table 1. Continued.

D13639	KIAK0002	Complex functions incl. cell cycle	−0.74	−0.53	−2.13
J04130	CCL4	Chemokine	−0.85	−0.75	−2.54
M38193	Granzyme B	CTL-function	0.08	−0.45	−2.92

Genes for which our quantitative selection criteria (compare Methods) were fulfilled in at least 3 out of 4 combinations of a calcineurin-inhibitor and a TOR-inhibitor are shown. Functional categories are taken from OMIM (Online Mendelian Inheritance in Man) database. A linear statistical model was used to estimate average fold-expression values for entire substance group (CsA and FK for calcineurin-inhibitors, SRL and EVL for TOR-inhibitors, and all four combinations thereof from three donors each) versus control. Results are shown on a log 2 basis. "CI" signifies fold-expression of calcineurin inhibitors, "TI" of TOR-inhibitors and "CI+TI" of the all combinations, respectively.

a consistent but lower difference in gene regulation may have biological significance. However, such small changes in gene expression level are not analyzable with the methods used.

Additive effects in an *in vitro* model may be the result of regulation of transcription or selection of different cell subpopulation in presence of immunosuppressive drugs, especially since the impact of immunosuppressive drugs on T-cell subsets is well documented.[22] Given the short observation period, the latter option seems rather unlikely.

Interestingly, cluster analysis of our data reveals that expression patterns of combined treatment of calcineurin-inhibitors and TOR-inhibitors clearly differ from the patterns of the single drugs. This distinction is caused by supra-additive rather than merely additive effects. The most conspicuous of these "synergisms" describing the transcriptional fingerprint of the drug combination are shown in Table 1.

Among the up-regulated genes many have a function in innate immune response or inflammation. Genes involved in cell cycle, CTL function, chemokines and several transcription factors with a well known role in survival and differentiation of lymphocytes such as E4BP4 (a transcription factor inhibiting apoptosis in pro-B lymphocytes) and c-MAF (a transcription factor involved in TH2-type differentiation) emerged among those genes with synergistic negative gene regulation.

In the above-mentioned study, Cristillo et al. were hoping to find genes regulated specifically by FK or CsA given the differences in structure and binding protein for FK and CsA.[21] Yet, their quest for substance specific regulation failed possibly because such genes fall under the detection threshold of the c-DNA microarray system. Likewise, we could not detect any substantial differences in regulation between FK and CsA in our system, neither or between SRL and EVL.

Our findings correspond well to published data. For instance, among the single substances only calcineurin-inhibitors enhance expression of the chaperones CD36 and CD68. In combination of calcineurin-inhibitors with TOR-inhibitors the effect was far more pronounced fulfilling our criteria for synergistic effects on CD36. A similar tendency was seen for CD68 (data not shown). These two scavenger receptors are involved in the uptake of oxidized LDL-cholesterol and possibly participate in the enhanced atherosclerotic risk in transplant recipients. Jin et al.[23] demonstrate an up-regulation of CD36 by CsA which could be verified on protein level for some assay conditions. However, they report different regulation by FK.[24] In our system we observed a similar regulation for both genes.

Similarly, in our system monocyte chemoattractant proteins were markedly down regulated by TOR-inhibitors. Downregulation of monocyte chemoattractant proteins by rapamycin has been extensively studied. The anti-artherogenic effect of rapamycin therapeutically exploited in drug-coated stents is partially attributed to this regulation.[25] Diverging effects of combined treatment of calcineurin-inhibitors and TOR-inhibitors versus single drug therapy on scavenger molecules have been reported before.[26]

To date, there are conflicting data on the effects of the combination of calcineurin-inhibitors and TOR-inhibitors on lipid metabolism.[21] It remains to be shown which of the observed effects (down-regulation of monocyte chemoattractant proteins or up-regulation of CD36 and CD68) may prevail *in vivo*.

By cluster analysis we could show that PBL, cultured with immunosuppressants for only 24 hours, clearly vary in their specific fingerprint-like gene expression pattern. In order to define such a "transcriptional fingerprint" we analyzed the "synergistic" effect of the combination of calcineurin-inhibitors and TOR-inhibitors focusing on very strongly supra-additive regulation. We were able to identify a number of genes relevant for either effects or side effects that are specifically regulated by combination of the drugs. It is feasible to assume that such patterns may be used to anticipate the immunosuppressive potential of a certain combination of drugs in a given patient once they have been correlated to clinical data.

Even though the feasibility of deciphering common mechanisms of action of small molecules by gene profiling has been demonstrated,[18] we are aware of the shortcomings of our *in vitro* model. Presumably, some of these effects may be even more pronounced on a post-translational level, at different time points or in purified lymphocyte subsets, while other effects may be overestimated. (INDENT)However, a non hypothesis

driven approach could lead to a better understanding of pharmacological mechanisms involved and identify potential patterns of biomarkers for effective therapies. The relevance of these patterns will have to be confirmed in correlation to clinical data. If "transcriptional fingerprints" described sufficient immunosuppression or absence of side effects, they could be used to choose the adequate combination of drugs in an individual. Ultimately, knowledge about expression patterns generated by immunosuppressive drugs may help in designing individually "tailored" therapies to render maximum effect and minimize side effects.

Acknowledgments

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