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Invited Perspective

Correlation and cluster analysis of immunomodulatory drugs based on cytokine profiles

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ABSTRACT

Drug discovery is a constant struggle to overcome hurdles posed by the complexity of biological systems. One of these hurdles is to find and understand the molecular target and the biological mechanism of action. Although the molecular target has been determined, the true biological effect may be unforeseen also for well-established drugs. Hence, there is a need for novel ways to increase the knowledge of the biological effects of drugs in the developmental process. In this study, we have determined cytokine profiles for 26 non-biological immunomodulatory drugs or drug candidates and used these profiles to cluster the compounds according to their effect in a preclinical *ex vivo* culture model of arthritis. This allows for prediction of functions and drug target of a novel drug candidate based on profiles obtained in this study. Results from the study showed that the JAK inhibitors tofacitinib and ruxolitinib formed a robust cluster and were found to have a distinct cytokine profile compared to the other drugs. Another robust cluster included the calcineurin inhibitors cyclosporine A and tacrolimus and the protein kinase inhibitors fostamatinib disodium and sotrastaurin acetate, which caused a strong overall inhibition of the cytokine production. The results of this methodology indicate that cytokine profiles can be used to provide a fingerprint-like identification of a drug as a tool to benchmark novel drugs and to improve descriptions of mode of action.

1. Introduction

One of the most important steps in drug discovery is to identify and validate the target of a new compound. Identifying the target as well as description of possible alternative targets provide valuable information since the therapeutic effect and possible side effects of the drug candidate can be predicted in the early phase [1]. Even in the present days of big data and rational drug design, there is a constant need of deeper knowledge of the biological effects of drugs and drug candidates. In addition, there is a need for tools to deal with the complex drug mechanism as well as identification of valuable biomarkers and clinical endpoint phenotypes for evaluation of drug effects. This is especially true for compounds developed through phenotypical assays, but also for compounds where the molecular target is known. The complexity of biological systems with interconnected pathways, feedback loops, off-target effects, and individual variations often convolutes the active mechanisms. Also, the importance of polypharmacology is increasingly acknowledged adding another level of complexity [2].

One way to increase the knowledge gained from data is to extract patterns and similarities from a multitude of inputs and correlate different compounds and disease mechanisms such as exemplified by the "connectivity map" created by Lamb et al. [3]. The connectivity map is based on gene expression profiles from cultured human cells treated by bioactive compounds to enable the functional connections between drugs, genes and diseases. By using a wide variety of small-molecule perturbagens, they created a system capable of connecting biological processes and therapeutics. This system could then be used to find compounds with common mechanism of action in order to find target pathways and to identify potential therapeutic opportunities. Recently, the connectivity map was vastly extended to include over a million profiles, which could be used to predict mechanism of action of small molecules [4]. With these promising results, we set out to create a more focused system, based on immunologically active compounds and cytokine profiles as a measure of immunomodulation. Our approach was to

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use cytokine pattern profiling in primary cells extracted from preclinical rat models of autoimmune inflammation [5]. Using *ex vivo* cultures as a model system of inflammation provides the advantage of mimicking the *in vivo* system while excluding complex factors such as drug formulation and bioavailability [6].

Characterization of the immune regulation can be accomplished by measuring molecular biomarkers such as cytokines, chemokines, large scale proteomics [7], gene expression profiling [8] or more recently by micro RNA profiling [8,9]. As a medium scale system to depicture the immune regulating complexity and network we have chosen profiling of cytokine response in our preclinical model. Cytokines are central molecules that regulate the immune system and are usually classified into groups based on their function or because they are produced by the same immune cell. The pro-inflammatory cytokines, which induce inflammation, include interleukine-1 (IL-1), IL-6 and tumor necrosis factor alpha (TNF-α) [10,11]. Cytokines are also highly involved in the development and activation of the adaptive immunity, and especially in T-cell development. IL-2 stimulates the activation of T-cells [10], which develop into either Th1 or Th2 cells. Th1 cells are characterized by their production of IL-2 and IFN-γ whereas Th2 cells produce the cytokines IL-4, IL-5 and IL-13 [12,13]. In addition, Th17 cells are defined by their production of IL-17, which is a cytokine that induces inflammation [14]. Many chemokines, such as RANTES, MIP-3α, GRO/KC are examples of molecules that are involved in inflammation by recruiting immune cells to the infected site [11]. Also, cytokines such as IL-7, erythropoietin (EPO) and vascular endothelial growth factor (VEGF) are important growth factors for the immune system [15–17].

The immune system with its specific cytokine pathways is obviously a very important target for disease interventions. Immunomodulation includes both activating and suppressing immunotherapies and is achieved using both cells, biological agents and small molecules. Activating immunotherapies are increasingly used, especially with the novel cell therapies for treating cancer. Also antibodies and cytokines as well as small molecules are used to activate the immune response to restore it in patients with compromised immunity. Immunosuppressive drugs are used to suppress the immune response, to treat e.g. allergic, inflammatory and autoimmune diseases or to prevent rejection after organ transplantation [18–20]. Immunosuppression involves reduction of the activation and efficiency of the immune systems by suppressing mainly signaling pathways of the immune cells. A range of different drugs have been developed to inhibit the immune system. Four interesting groups can be devised based on their mode of action, namely immunophilin targeting drugs, glucocorticoids, protein kinase inhibitors and antibodies [18,19]. Immunophilin targeting drugs include the calcineurin inhibitors tacrolimus and cyclosporine A as well as the mammalian targets of rapamycin (mTOR) inhibitors such as sirolimus and everolimus [18]. The glucocorticoids are steroids that bind to the glucocorticoid receptor and thereby up-regulate the expression of several anti-inflammatory proteins, leading to both immunosuppressive and anti-inflammatory effects [18]. Examples of glucocorticoids include the drugs prednisolone, dexamethasone and fluticasone propionate. The protein kinase inhibitors block the activity of the protein kinase enzymes, which are central enzymes for many signaling pathways in immune cells. The protein kinase inhibitors include, for example, the janus kinase (JAK) inhibitors tofacitinib and ruxolitinib as well as the tyrosine kinase inhibitors nilotinib and fostamatinib disodium [19,20]. Antibodies used in immunosuppression are often targeting specific molecules of the signaling system, such as the IL-2 receptor, CD3 and TNF-α, which thereby downregulating the immune response [18].

Given the fact that immunosuppressive drugs have distinct targets it is likely that different groups of drugs will modulate the cytokine production differently. At the same time, its complexity, feedback mechanisms and redundancy makes it difficult to assess a distinct mechanism of action also for well-characterized drugs without engaging and analyzing the whole immune system reaction. However, by assessing the cytokine profile of a specific condition such as a drug treatment or a disease state, a lot can be learned about which immune pathways that are affected. This allows knowledge to be made about the disease mechanism and drug targets, similarly to the gene expression profiles in the connectivity map. The aim of this study was to analyze how different drugs affect the cytokine production and to compare the drugs based on their cytokine profiles. Data obtained from this study could then be used to predict molecular targets and mode of action of immunomodulatory drug candidates by comparing their cytokine profiles to the profiles generated by the well-characterized immunomodulatory drugs included in this study.

2. Materials and methods

2.1. Animals

Rats, Dark Agouti (DA) (Janvier, Europe), were kept in animal facilities in a climate-controlled environment with 12 h light/dark cycles, housed in polystyrene cages containing wood shavings and fed standard rodent chow and water *ad libitum* in the animal house of Medicon Village, Lund, Sweden. The rats were found to be free from common pathogens. The experiments were approved by the local ethical committee (Malmö/Lund, Sweden, M167-12).

2.2. Cell preparation and activation

Female DA rats, 8–10 weeks of age, were injected with 500 μl of the adjuvant pristane (Acros Organics, Geel, Belgium) s.c. at the base of the tail at day 0 in order to pre-stimulate an immune response [21]. At day 14, at the onset of arthritis, rats were sacrificed and spleens were collected from 9 rats. Single cell suspensions were prepared by passing the cells through a 40 μm cell strainer (BD Falcon, San Jose, CA, USA) with a piston from a 5 ml syringe. Red blood cells were lysed in BD pharmlyse buffer and the remaining cells were washed with HBSS [21]. Cells were diluted to 4.5×10^6 cells per ml of RPMI medium containing 3 μg/ml of ConA (Sigma) and selected drug (see below). Cells were incubated in a 96 well plate in a standard incubator (37 °C and 5% CO_2) for 44 h. Supernatants were harvested from cell culture plates and frozen at −20 °C and stored at −80 °C until assayed (supernatant was analyzed within five months from preparation). Drugs were analyzed on nine separate biological replicates.

2.3. Drugs

The tested drugs were diluted to 10 mM stock solutions in DMSO (stored in dark at room temperature for long time use) or in mqH_2O (prepared fresh prior to assay). The final concentrations of the drugs were determined as the maximal concentration that did not induce cell apoptosis but still caused decrease of IFN-γ or IL-2 levels according to dose response curves measured by ELISA during prior experimental calibration studies (data not shown). The final concentration of DMSO in analyzed samples was 0.5%. The following compounds and their concentrations were used in the cell cultures: apremilast (Selleck Chemicals; 0.125 μM), apilimod mesylate (Axon MedChem; 125 nM), astaxanthin (SantaCruz BT; 50 μM), bardoxolone methyl (Toronto Research Chemicals; 6.25 nM), bortezomib (Selleck Chemicals; 6.25 nM), cyclosporine A (Sigma; 125 nM), dexamethasone (Sigma; 125 nM), dimethyl fumarate (Sigma; 125 nM), everolimus (Selleck Chemicals; 2.5 μM),

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fostamatinib disodium (Selleck Chemicals; 2.5 μM), fluticasone propionate (mcule; 50 μM), glatiramer acetate (Toronto Research Chemicals; 50 μM), levalbuterol (mcule; 50 μM), losmapimod (Selleck Chemicals; 50 μM), morniflumate (AK Scientific; 50 μM), mycophenolic Acid (Sigma; 50 μM), nilotinib (Selleck Chemicals; 2.5 μM), pilocarpine hydrochloride (Sigma; 125 nM), prednisolone (Sigma; 2.5 μM), rosiglitazone (Cayman; 50 μM), ruxolitinib (Selleck Chemicals; 125 nM), sirolimus (Cayman; 2.5 μM), sotrastaurin acetate (Axon; 2.5 μM), tacrolimus (Toronto Research Chemicals; 125 nM), tofacitinib (Axon; 125 nM), triptolide (Toronto Research Chemicals; 6.25 nM).

2.4. Multiplex immunoassays

Measurement of the cytokines and the growth factors in supernatant was performed using the Bio-Plex Pro™ rat cytokine assay, 24-plex assay, (Bio-Rad Laboratories, Cat#171-K1001 M) on the instrument Bio-Plex 200 system (Bio-Rad Laboratories, Hercules, CA, USA). This system measures the following cytokines and growth factors: EPO, G-CSF, GM-CSF, GRO/KC, IFN-γ, IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-10, IL-12p70, IL-13, IL-17A, IL-18, MCP-1, M-CSF, MIP-1α, MIP-3α, RANTES, TNF-α and VEGF. The drugs were divided on three separate 96-well plates, where each plate included three biological replicates of the 26 different drugs and controls as well as standards. Standard curves were generated and the cytokine concentrations were estimated with the Bio-Plex Manager software V.4.0 (Bio-Rad Laboratories, Hercules, CA, USA) using the five-parameter logistic model.

2.5. Statistical analysis

The R version 3.3.2, language and environment for statistical computing [22] and Python version 3.4.5 [23] with the packages Pandas [24], seaborn [25], matplotlib [26], rpy2 [27] and scipy [28] were used for statistical analyses and preparation of the figures. Due to that many drugs dramatically affected the cytokine concentrations, leading to cytokine levels that were both above and below the detection range based on the standard curve, the fold change (FC) values were based directly on the fluorescence intensity values. Pairwise Pearson's correlation between the drugs was based on the log2 FC values from 9 biological replicates of the 24 cytokines. For all analyses involving calculations of the Pearson's correlation coefficient, which is very sensitive to outliers, the data set was first filtered by removing outliers as detected by boxplots (data points outside 1.5 interquartile range above the upper quartile or below the lower quartile). To account for multiple pairwise correlations, the p-values were adjusted based on Bonferroni correction. An adjusted p-value less than 0.01 was considered statistically significant.

2.6. Cluster analysis

Hierarchical agglomerative clustering was performed on the log2 FC values obtained from the cytokine expression profiles induced by the different drugs. The cytokines were clustered based on one minus the pairwise Pearson's correlation coefficients. Thus, drugs with a strong positive correlation to each other will fall in the same cluster. The distance between two clusters was defined by the average linkage method, the average distance between members of the clusters. To assess the robustness of the generated dendrograms, bootstrapping (n = 10.000) was performed using the Pvclust package in R [29]. Other linkage methods such as complete, single and Ward function were evaluated. However, the average linkage method was found to be a robust method with the highest cophenetic correlation coefficient.

2.7. Factor analysis

Factor analysis using PCA to extract components was used to reduce the correlated data of the drugs. The final number of components was determined by extraction of components with an eigenvalue greater or equal to one. To increase the interpretation of the factors, the Varimax rotation procedure with Kaiser Normalization was used [30], using the psych package in R [31]. The coefficients generated from this procedure, linking the cytokines to the factors, are the correlation coefficients (Pearson's correlation under Varimax rotation) between the drugs and the factors.

3. Results

3.1. Drug cytokine profiles

Splenocytes were collected from pristane-induced arthritis (PIA) DA rats. The PIA rat model is a T cell-dependent model of rheumatoid arthritis (RA) that fulfills the American College of Rheumatology classification criteria for RA [5]. The induced disease state can be transferred by T cells through *ex vivo* stimulation with ConA [6], which provides a good model for studying drug effects on cytokine production *in vitro*. The splenocytes were *ex vivo* stimulated by ConA for 44 h, in the absence (positive control) or presence of a drug. The levels of 24 different cytokines where measured by the Bio-Plex rat assay. The effect on the cytokine production for each drug was analyzed by the fold change (FC) where the cytokine levels from cultures in the presence of drug were divided by the corresponding positive control. Biological drugs were excluded from the study to allow a more homogenous treatment and to avoid a possible immune response against protein addition. Also, biologicals are usually specific for the human system. In total, cytokine profiles from 26 different non-biological drugs were generated. These drugs were selected as the ones that showed a strong effect on the cytokine production based on our previous study that included 55 immunomodulatory drugs [32] and a preliminary follow up experiment. The fewer number of drugs allowed us to fit three biological replicates on each of the three Bio-Plex assays. Hence, each drug cytokine profile is based on nine biological replicates. In Fig. 1, four different examples of cytokine profiles are shown. The two calcineurin inhibitors tacrolimus and cyclosporine A show similar cytokine profiles, where both drugs inhibit most of the cytokines, especially the Th1 cytokines IL-2 and IFN-γ as well as the Th17 cytokine IL-17. In contrast, the JAK inhibitors tofacitinib and ruxolitinib increase the expression of IL-2 and MIP-3α but cause a strong inhibition of IFN-γ. This indicates that drugs with similar targets generate similar cytokine profiles. Cytokine profiles from all 26 drugs can be found in the supplemental material (supplementary Fig. 1). Generally, the drugs suppress the overall production of cytokines, in particular the inflammatory cytokines (IL-1, TNF-α, IL-6), the Th1 cytokines (IL-2 and IFN-γ) and the Th17 cytokine (IL-17).

3.2. Correlation analysis

In order to identify drugs with similar cytokine profiles, the Pearson's correlation coefficient was calculated for all possible pairwise combinations of the drugs. Each correlation coefficient was based on the log2 FC of 24 different cytokines from 9 biological replicates (216 data points). Since Pearson's correlation coefficient is very sensitive to extreme values, outliers identified in each cytokine profile were removed before the correlation coefficients were computed. On average, 7.5% of the data points were identified as outliers (see boxplots, supplementary Fig. 1). All scatter plots indicated a linear relationship between the drugs (data

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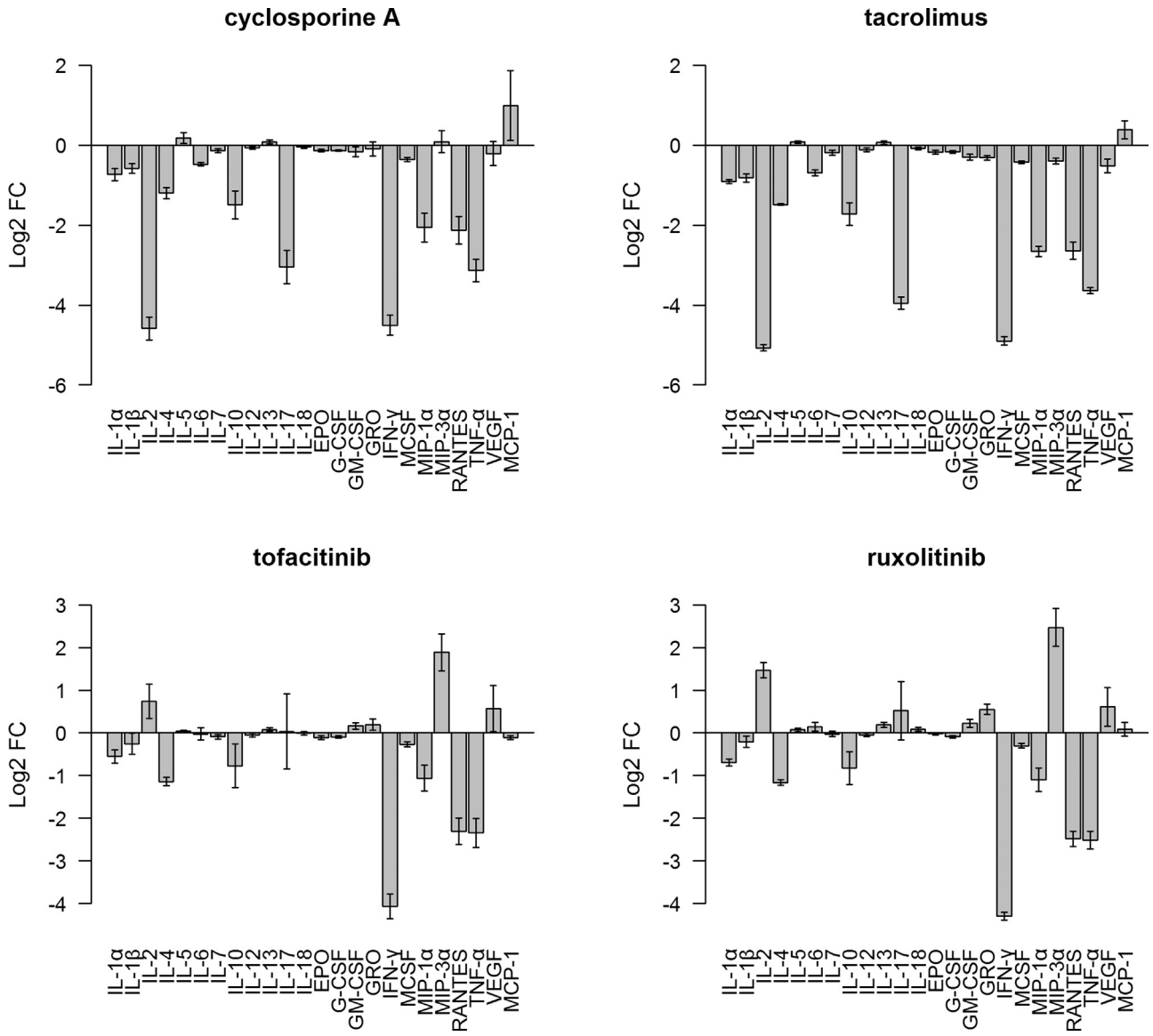

Fig. 1. Cytokine profiles shown as log2 fold change (FC) for four selected drugs. Cytokine levels from ConA stimulated splenocytes from PIA rats were measured for each of the drugs. The FC was calculated by dividing the cytokine level of cultures with drugs by the positive control (ConA in the absence of drug). A log2 FC less than zero indicates that the drug suppresses the cytokine whereas a value greater than zero indicates that the drug increases the production of the cytokine. Bars represent mean log2 FC ± 1 SE from 9 biological replicates (9 individual rats). Two very extreme values were omitted before generating the bar chart: one IL-2 measurement for tofacitinib (log2 FC = −5.1) and one IL-2 measurement for ruxolitinib (log2 FC = −4.9).

not shown). In total, 325 pairwise correlations were calculated (see correlation matrix, supplementary Table 1). After adjusting for multiple correlation analyses, 250 significant positive correlations were identified. No significant negative correlation was identified. Out of the significant correlations, 34 pairwise correlations were found to have a correlation coefficient greater than 0.8. Table 1 shows the top 20 correlations identified in this study. The calcineurin inhibitors tacrolimus and cyclosporine A show a strong correlation to each other (r = 0.93) as well as to the protein kinase inhibitors fostamatinib disodium, sotrastaurin acetate and nilotinib. In addition, the NSAID morniflumate also shows strong correlations to the calcineurin inhibitors and to the protein kinase inhibitors. Other expected strong correlations were found between the mTOR inhibitors sirolimus and everolimus (r = 0.87) as well as between the JAK inhibitors tofacitinib and ruxolitinib (r = 0.87). Several drugs showed relatively weak correlations to the other drugs, which indicates that some of the drugs generated distinct profiles. For example, the two strongest correlations identified by astaxanthin were 0.61 to apilimod mesylate and 0.49 to dexamethasone.

3.3. Principal component analysis

To identify groups of drugs that correlate, a PCA analysis was computed based on the cytokines profiles, using the Varimax rotation method. This analysis will reduce the dimensionality of the data in a way that provides simplified and interpretable factors, capturing the main patterns in the data set. The PCA resulted in four factors with an eigenvalue greater than or equal to one. In total, these four factors explained 86% of the variance. The Varimax factors and their loading scores are shown in Table 2. The loading scores in the table correspond to how well each drug correlates with the four factors. To interpret the factors, a loading score greater than 0.75 was set as a threshold value in order to determine if a drug was associated to the factor or not. The calcineurin inhibitors cyclosporine A and tacrolimus loaded on factor 2 together with

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Table 1
Top 20 significant Pearson's correlation coefficients.

Drug 1	Drug 2	r
tacrolimus	fostamatinib disodium	0.98
tacrolimus	sotrastaurin acetate	0.98
sotrastaurin acetate	fostamatinib disodium	0.98
sotrastaurin acetate	morniflumate	0.96
morniflumate	fostamatinib disodium	0.96
tacrolimus	morniflumate	0.95
fostamatinib disodium	cyclosporine A	0.93
tacrolimus	cyclosporine A	0.93
sotrastaurin acetate	cyclosporine A	0.92
morniflumate	cyclosporine A	0.92
fluticasone propionate	everolimus	0.91
nilotinib	fostamatinib disodium	0.90
sotrastaurin acetate	nilotinib	0.89
mycophenolic acid	Glatiramer acetate	0.88
nilotinib	morniflumate	0.88
nilotinib	losmapimod	0.87
sirolimus	everolimus	0.87
tofacitinib	ruxolitinib	0.87
nilotinib	mycophenolic acid	0.87
tacrolimus	nilotinib	0.86

Table 2
Varimax rotated factor loading matrix.

	Factor			
Drug	1	2	3	4
apilimod mesylate	0.51	0.54	0.41	0.09
apremilast	**0.80**[a]	0.12	0.27	0.37
astaxanthin	0.70	0.19	0.33	0.08
bardoxolone methyl	**0.79**[a]	0.14	0.26	0.08
bortezomib	0.59	0.42	0.32	0.21
cyclosporine A	0.29	**0.82**[a]	0.16	0.30
dexamethasone	**0.84**[a]	0.31	0.22	0.15
dimethyl fumarate	**0.90**[a]	0.04	0.14	0.16
everolimus	0.58	0.59	0.48	0.02
fluticasone propionate	0.62	0.53	0.42	0.05
fostamatinib disodium	0.14	**0.95**[a]	0.19	0.13
glatiramer acetate	0.30	0.64	0.62	0.07
levalbuterol	**0.79**[a]	0.30	0.35	0.15
losmapimod	0.49	0.48	0.60	−0.02
morniflumate	0.24	**0.88**[a]	0.24	0.18
mycophenolic acid	0.23	0.66	0.62	0.12
nilotinib	0.45	0.61	0.48	−0.34
pilocarpine hydrochloride	0.41	0.20	0.01	**0.87**[a]
prednisolone	**0.85**[a]	0.27	0.24	0.14
rosiglitazone	0.29	0.43	0.24	**0.77**[a]
ruxolitinib	0.28	0.22	**0.85**[a]	0.19
sirolimus	0.58	0.55	0.42	0.05
sotrastaurin acetate	0.19	**0.93**[a]	0.18	0.15
tacrolimus	0.17	**0.93**[a]	0.18	0.13
tofacitinib	0.39	0.26	**0.83**[a]	0.06
triptolide	**0.86**[a]	0.29	0.02	0.16

[a] Loading scores greater than 0.75.

the protein kinase inhibitors fostamatinib disodium, sotrastaurin acetate, and the NSAID morniflumate. The JAK inhibitors tofacitinib and ruxolitinib loaded on factor 3. The glucocorticosteroids prednisolone and dexamethasone loaded on factor 1 together with NF-kβ inhibitors triptolide, dimethyl fumarate and bardoxolone methyl as well as the drugs apremilast and levalbuterol. The forth factor included the drugs pilocarpine hydrochloride and rosiglitazone. The factor analysis indicates that the drugs can be divided into four groups, which show similar cytokine patterns.

3.4. Cluster analysis

To further identify groups of drugs that have a similar cytokine profile and to gain an understanding of why they group together, hierarchical cluster analysis was computed, using one minus the Pearson's correlation coefficient as a distance measure. Hence, drugs that group into a cluster have a relatively strong positive correlation to each other. Out of the evaluated linkage functions the average linkage function was found to have the strongest cophenetic correlation coefficient (0.77; compared to single: 0.72, complete: 0.73 and Ward: 0.62). In addition, the average linkage method generated more distinct clusters that could be interpreted more easily compared to clusters from the other linkage methods (supplementary Fig. 2). Fig. 2 shows a heatmap of the log2 FC values where rows (drugs) and columns (cytokines) have been ordered based on correlation hierarchical clustering, using the average linkage method. The heatmap shows that the majority of the drugs cause an inhibitory effect on the cytokine production. The patterns give a detailed understanding of the resulting factors from the PCA analysis. For example, the JAK inhibitors tofacitinib and ruxolitinib (factor 3) have a unique pattern where a stimulatory effect is seen on the cytokines MIP-3α and IL-2 whereas a strong inhibitory effect is observed on RANTES, TNF-α and IFN-γ. The calcineurin inhibitors and the protein kinase inhibitors that loaded on the second factor are drugs that cause a general overall inhibition, especially on the cytokines IL-2, IL-17, TNF-α and IFN-γ. Drugs that loaded on the first factor have a general weak impact on the cytokine production (top seven rows in the heatmap). The heatmap also shows that the cytokines GM-CSF, IL-18, IL-13, IL-12, IL-7, EPO and IL-5 are barely affected by the drugs. The clustering of the cytokines shows that the pro-inflammatory cytokines IL-1, IL-6 and TNF-α can be found in the same cluster, whereas the IL-2 and IL-17 cluster together.

To access the uncertainty of the hierarchal cluster analysis, bootstrapping (n = 10.000) of the data was performed. Fig. 3 shows a dendrogram of correlation hierarchal cluster analysis, using the average linkage function. The bootstrap probability (bp) values in the dendrogram indicate the fraction of times the original clusters were identified from dendrograms generated by re-sampled data. As expected, drugs in a cluster with relatively strong correlation were found to be robust when the data was re-sampled. The most robust cluster (bp = 99) included the JAK inhibitors tofacitinib and ruxolitinib. Another robust cluster (bp = 98) was the cluster including the calcineurin inhibitors cyclosporine A and tacrolimus, the protein kinase inhibitors fostamatinib disodium and sotrastaurin acetate and the NSAID morniflumate. Two other interesting clusters with relatively high bp-value included the glucocorticoids prednisolone and dexamethasone together with levalbuterol whereas the mTOR inhibitors sirolimus and everolimus clustered together with the glucocorticosteroid fluticasone propionate.

4. Discussion

Cytokines play a crucial role during the immune response and are today used as direct or indirect drug targets. Drugs that inhibit the pro-inflammatory cytokines, such as the TNF-α inhibitor, play an important role in treating inflammatory diseases [18]. Another class of compounds that effectively block the adaptive immunity through cytokine modulation are the calcineurin inhibitors, which previously have been described as having a primary effect on the IL-2 production and thus reduce the T cell activation [33,34]. Such drugs play a key role to prevent rejection after organ transplantation. However, drugs that inhibit a certain cytokine will also affect other cytokines due to their overlapping signaling pathways. We have previously shown that the expression of cytokines are highly correlated during drug perturbation and that these correlations can be used to cluster cytokines [32]. The present study shows that drugs that inhibit the IL-2 production usually also reduce the production of especially IL-17 and IFN-γ. This is not surprising since activated Th1 cells secrete mainly IL-2 and IFN-γ, and that Th17 cells, which produce IL-17, are induced in parallel to Th1 cells [35].

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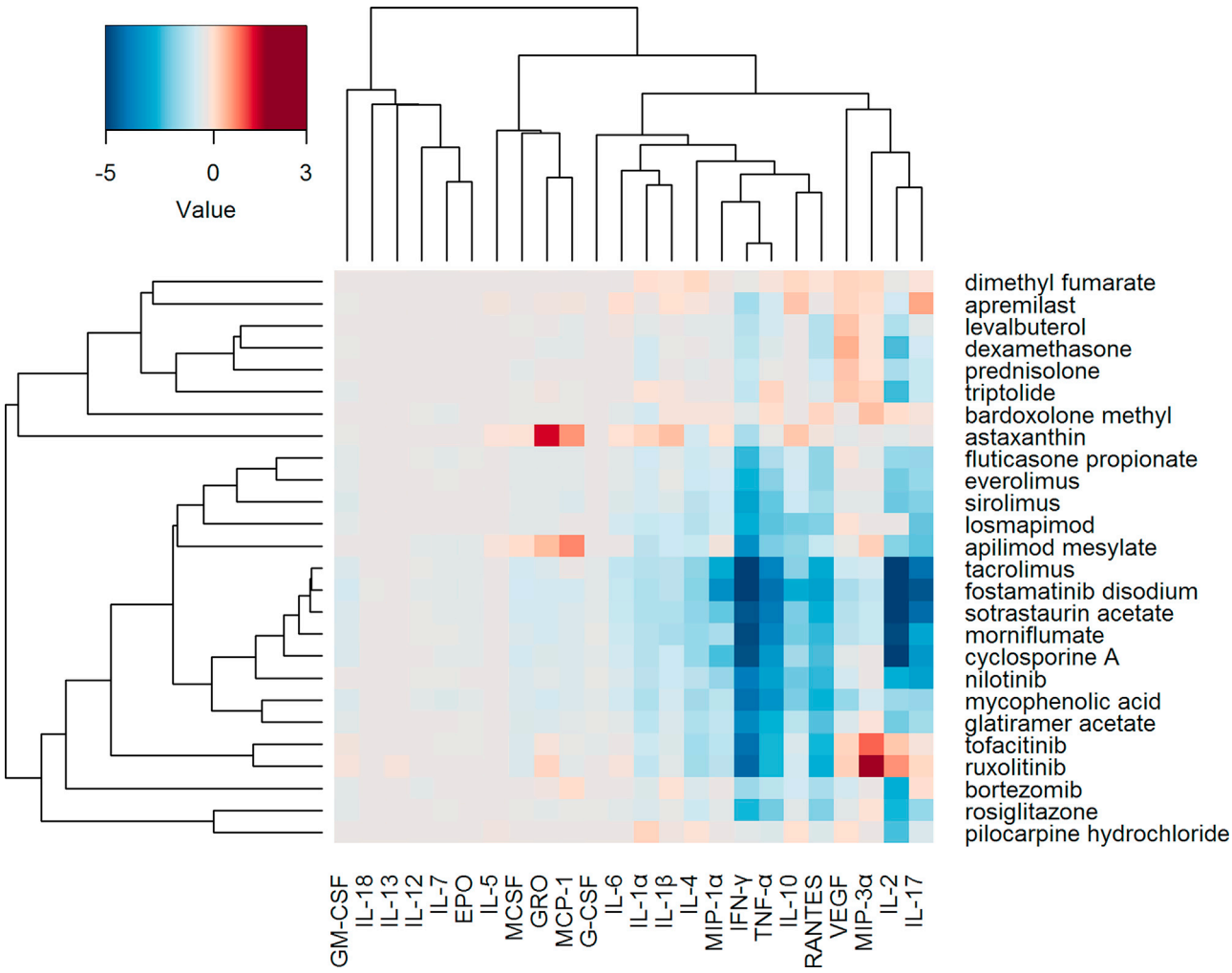

Fig. 2. Heatmap of the log2 FC values. The FC was calculated by dividing the cytokine level from ConA stimulated splenocytes in the presence of drugs by the positive control (ConA in the absence of drug). Red color indicates an increased cytokine production compared to the control, whereas a blue color indicates that the drugs suppress the production of the cytokine. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Both Th1 and Th17 cells are highly involved in autoimmune diseases and effective drugs for treating such diseases need to block the activity of these cells.

The correlation analysis showed that profiles from compounds with the same molecular target generally correlate very well. For example, the calcineurin inhibitors cyclosporine A and tacrolimus have a strong correlation ($r = 0.93$) as have the JAK inhibitors tofacitinib and ruxolitinib ($r = 0.87$) and the glucocorticoids dexamethasone and prednisolone ($r = 0.84$). In addition, there are compounds that correlate without sharing a common target, which indicates that drugs with different targets may generate similar cytokine profiles.

Grouping the profiles with PCA and hierarchical clustering increased the interpretability and we could for example see a large group of compounds with strong inhibition of especially IFN-γ, IL-2 and IL-17. This group included the drugs tacrolimus and cyclosporine A, together with fostamatinib (Syk inhibitor), sotrastaurin (PKC inhibitor) and morniflumate (COX inhibitor). The latter three drugs are all connected since PKC regulates the expression of COX [36] and Syk is able to activate PKC [37]. However, the calcineurin and the PKC pathway are complementary, which indicates that drugs with complete different targets may cause similar effect on the cytokine production.

Also the JAK inhibitors group very well together both in the clustering analyses and the PCA. These drugs show an interesting pattern in the heatmap where MIP-3α and IL-2 are increased while IFN-γ is strongly inhibited giving a very distinct profile for this group. The JAK family comprises of four tyrosine kinases: JAK1, JAK2, JAK3 and TYK2. These kinases are associated with different cytokine receptors that lack intrinsic kinase activity. JAK3 associates with JAK1 and the γ-chain receptor subunit, which associates with other subunits to form, for example, the IL-2 receptor. In contrast, the IFN-γ receptor is associated with JAK1 and JAK2 [38,39]. Although ruxolitinib has a strong selectivity towards JAK1 and JAK2 [39] and tofacitinib is mainly described as a JAK3 inhibitor [39], both drugs still induce similar cytokine profiles. Hence, the different JAK selectivity between tofacitinib and ruxolitinib does not seem to effect the cytokine production. Piscianz et al. [40] have found the same pattern when tofacitinib was added to PHA stimulated human PBMCs. Tofacitinib was shown to stimulate the IL-2 secretion but at the same time induced a dramatic inhibition of the IFN-γ production.

Only about half of the 24 cytokines were affected by the drugs in this specific setup, diminishing the indicators available to separate the drugs efficiently. The pristane induced arthritis model in rats is known to induce Th1-mediated autoimmune disease [41], which explains why the drug cytokine profiles are dominated by the pro-inflammatory cytokines and the cytokines mainly produced by Th1 and Th17 cells. In addition, *ex vivo* activation of the splenocytes by ConA results in crosslinking of T cell receptors and induction of T cell

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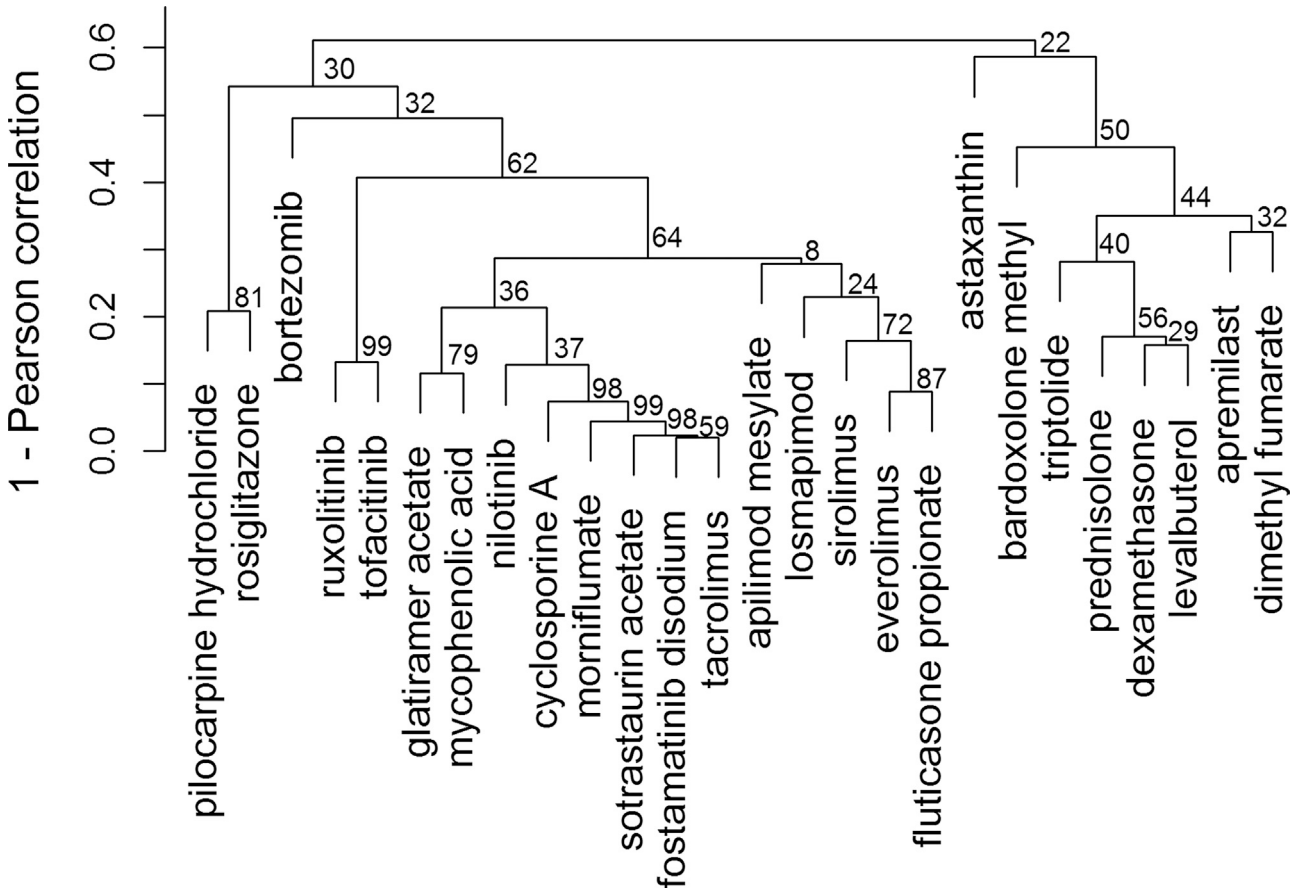

Fig. 3. Cluster analysis of the drug cytokine profiles. Agglomerative hierarchical clustering was performed based on one minus the Pearson's correlation coefficient, using the average linkage method. Clusters in the lower part of the dendrogram include drugs with a strong positive correlation. The bootstrap probability values are shown at the tree nodes and indicate how often the original clusters were generated from re-sampled data (n = 10.000).

mediated cytokine responses. Consequently, the established model is selected and validated for comparative studies of drugs with T cell regulatory effects. A further constraint is that the analyzed cytokine profiles are limited to those of the rat immune system. Future studies would benefit from a more diverse immune response where more cytokines are involved as well as including more variables in the measurements that may help to separate the profiles of drugs even further. Despite these limitations, our study shows that cytokine profiles from immunomodulatory drugs may provide valuable information where drugs with similar targets can be found in the same clusters. Computation of the correlation between a cytokine profile from a drug candidate and the cytokine profiles in this study may provide important information in the preclinical drug discovery process. If the cytokine profile from a new drug candidate match an existing drug, then the drug target and its clinical effect can be predicted based on the known information about the well-characterized drugs included in this study. The developed platform provides an efficient tool for predicting target and mode of action to facilitate earlier selection of drug candidates as well as identifying alternative effective drugs in preclinical models.

Conflict of interest

We declare that we have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.phrs.2017.10.012.

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