Cytokine 90 (2017) 73–79

Contents lists available at ScienceDirect

Cytokine

journal homepage: www.journals.elsevier.com/cytokine

Cytokine correlation analysis based on drug perturbation

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ARTICLE INFO

Article history:
Received 13 June 2016
Received in revised form 20 October 2016
Accepted 25 October 2016

Keywords:
Clustering
PCA
Profile
Compound
T cell

ABSTRACT

Cytokines and chemokines play a crucial role in regulating the immune system. Understanding how these molecules are co-regulated is important to understand general immunology, and particularly their role in clinical applications such as development and evaluation of novel drug therapies. Cytokines are today widely used as therapeutic targets and as biomarkers to monitor effects of drug therapies and for prognosis and diagnosis of diseases. Therapies that target a specific cytokine are also likely to affect the production of other cytokines due to their cross-regulatory functions and because the cytokines are produced by common cell types. In this study, we have perturbated the production of 17 different cytokines in a preclinical rat model of autoimmune arthritis, using 55 commercially available immunomodulatory drugs and clinical candidates. The majority of the studied drugs was selected for their anti-inflammatory role and was confirmed to inhibit the production of IL-2 and IFN-γ in this model but was also found to increase the production of other cytokines compared to the untreated control. Correlation analysis identified 58 significant pairwise correlations between the cytokines. The strongest correlations found in this study were between IL-2 and IFN-γ ($r = 0.87$) and between IL-18 and EPO ($r = 0.84$). Cluster analysis identified two robust clusters: (1) IL-7, IL-18 and EPO, and (2) IL-2, IL-17 and IFN-γ. The results show that cytokines are highly co-regulated, which provide valuable information for how a therapeutic drug might affect clusters of cytokines. In addition, a cytokine that is used as a therapeutic biomarker could be combined with its related cytokines into a biomarker panel to improve diagnostic accuracy.

1. Introduction

Cytokines and chemokines are secreted molecules that are involved in a range of different functions that regulate the immune system. Today, more than 100 different cytokines have been identified [1]. Cytokines have generally been classified into specific groups based on their functions or because they are produced by the same cell type. The pro-inflammatory cytokines include interleukine-1 (IL-1), IL-6 and tumor necrosis factor alpha (TNF-α). These cytokines are mainly produced by activated macrophages in response to infection, which induce inflammation, fever and the release of acute phase proteins [2,3]. The pro-inflammatory cytokines are also involved in chronic inflammatory diseases such as rheumatoid arthritis and inflammatory bowels disease. Inhibiting the activity of the pro-inflammatory cytokines by agents such as neutralizing antibodies and receptor antagonists have been shown to be successful in patients with inflammatory diseases [3,4]. Another large group of cytokines is mainly involved in the adaptive immunity, and especially in T-cell development. Activation of T helper (Th) cells initially results in IL-2 production, which stimulates T-cell proliferation [2]. After a few rounds of divisions, Th cells develop into either Th1 or Th2 cells [5,6]. The differentiation of the Th cells is highly controlled by cytokines where IL-12 and interferon gamma (IFN)-γ promote Th1 development whereas IL-4 promote Th2 development [6–8]. The Th cells are classified according to the cytokines they secrete. Th1 cells produce mainly IFN-γ and IL-2, whereas Th2 cells produce cytokines such as IL-4, IL-5 and IL-13 [9–11]. In addition, Th17 cells are defined by their production of IL-17, which is a cytokine that induces inflammation [9]. A range of different drugs have been developed to inhibit lymphocyte activation. For example, calcineurin inhibitors such as cyclosporine A and tacrolimus efficiently inhibit the production of cytokines involved in the adaptive immunity [12]. Such immunosuppressive drugs are mainly used to prevent rejection after organ transplantation, and have a primarily inhibitory effect on IL-2 secretion [13]. Drugs that block IL-4 and IL-5 signaling have shown promising effects in allergy treatment [14]. Although most cytokines are involved in stimulating the immune system, there are critical cytokines that have an opposing effect. IL-10 is a strong

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http://dx.doi.org/10.1016/j.cyto.2016.10.015
1043-4666/

anti-inflammatory cytokine that suppresses the production of a range of cytokines [15]. IL-10 derived from CD4(+) regulatory T cells provides an important mechanism for controlling the immune response [16,17]. In addition to cytokines, chemokines and growth factors play an important role in the immune system. Chemokines are particularly involved in selective migration of immune cells through chemotaxis and upregulation of adhesion molecules. Chemokine ligands (CCL) such as CCL5 (RANTES), CCL20 (MIP-3α) and CXCL1 (GRO/KC) are examples of chemokines that are involved in inflammation by recruiting immune cells to the infected site [2]. In addition, cytokines such as IL-7 and erythropoietin (EPO) have an important role in the hematopoiesis by inducing lymphocyte and erythrocyte development, respectively [18,19]. Understanding how different cytokines and chemokines are connected and co-regulated is of great medical importance. Cytokines have shown to be useful biomarkers for preliminary diagnosis of a range of different diseases and to monitor the effect during drug therapies [20–22]. Drugs that target a specific cytokine are likely to affect the production of other cytokines due their overlapping pathways and cross-regulatory mechanisms. In this study, we have investigated how 55 different commercially available immunomodulatory drugs affect the cytokine production of *ex vivo* stimulated autoreactive splenocytes in order to analyze the relationship between 17 different cytokines and chemokines. Cytokines that correlate under such treatments are likely to be co-regulated and similarly affected during drug therapies.

2. Materials and methods

2.1. Animals

Rats, Dark Agouti (DA) (Charles River 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 (Malmö/Lund, Sweden, M167-12) ethical committee.

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 [23]. At day 14, at the onset of arthritis, rats were sacrificed and spleens were collected from 4 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 remaining cells were washed with HBSS [23]. 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 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 by two 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% if not otherwise stated. The following compounds and their concentrations were used in the cell cultures: Actarit (AK Scientific; 50 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), Bucillamine (Toronto Research Chemicals; 50 μM), Chloroquine Phosphate (Sigma; 2.5 μM; no DMSO added), Cinchophen (Sigma; 50 μM), Clodronate (SantaCruz BT; 50 μM; no DMSO added), Cyclosporine A (Sigma; 125 nM), D-Penicillamine (Sigma; 50 μM; no DMSO added), Dexamethasone (Sigma; 125 nM), Diacerein (AK Scientific; 2.5 μM), Dimethyl Fumarate (Sigma; 125 nM), Doramapimod (Selleck Chemicals; 2.5 μM), Emorfazone (Sigma; 50 μM), Enbrel (Wyeth, Pfizer; 5.14 μM; used at concentration and buffer received), Escin (Sigma; 2.5 μM), Esonarimod (Civentichem; 50 μM), Ethyl Pyruvate (Sigma; 50 μM), Fingolimod Hydrochloride (Selleck Chemicals; 2.5 μM), Fostamatinib Disodium (Selleck Chemicals; 2.5 μM), Givinostat (Selleck Chemicals; 6.25 nM), Glatiramer Acetate (Toronto Research Chemicals; 50 μM), Imatinib Mesylate (SantaCruz BT; 2.5 μM), Laquinimod (CiVentiChem; 50 μM), Leflunomide (SantaCruz BT; 50 μM), Lisofylline (Cayman; 50 μM), Maraviroc (Selleck Chemicals; 50 μM), Masitinib (Selleck Chemicals; 2.5 μM), Methotrexate (Sigma; 50 μM), Mizoribine (Toronto Research Chemicals; 50 μM), Morniflumate (AK Scientific; 50 μM), Mycophenolic Acid (Sigma; 50 μM), Nilotinib (Selleck Chemicals; 2.5 μM), Phenylbutazone (Cayman; 50 μM), Pilocarpine Hydrochloride (Sigma; 125 nM), Plerixafor (Toronto Research Chemicals; 50 μM), Pomalidomide (Selleck Chemicals; 50 μM), Prednisolone (Sigma; 2.5 μM), Risedronate Sodium (Toronto Research Chemicals; 125 nM; no DMSO added), Romazarit (Peakdale; 50 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), Talmapimod (Axon; 2.5 μM), Talniflumate (AK Scientific; 2.5 μM), Tarenflurbil (Sigma; 125 nM), Temsirolimus (Sigma; 2.5 μM), Thalidomide (Santa Cruz BT; 50 μM), Tofacitinib (Axon; 125 nM), Triptolide (Toronto Research Chemicals; 6.25 nM), Zoledronic Acid (AK Scientific; 2.5 μM; no DMSO added).

2.4. Multiplex immunoassays

Measurement of the cytokines and the growth factors in supernatant was performed using the Bio-Plex Pro™ rat cytokine assay, 23-plex, (Bio-Rad Laboratories, Cat#171-K1001M) on the instrument Bio-Plex 200 system (Bio-Rad Laboratories, Hercules, CA, USA). This system measures the following cytokines and growth factors: IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-10, IL-12p70, IL-13, IL-17A, IL-18, IFN-γ, TNF-α, EPO, G-CSF, GRO/KC, GM-CSF, M-CSF, MIP-3α, VEGF and RANTES. The drugs were divided on two separate 96-well plates, including 33 and 22 drugs in the first and second plate, respectively. Each plate included two biological replicates for each drug 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 3.2.3, language and environment for statistical computing (https://www.R-project.org/), was used for statistical analyses and preparation of figures. After inspecting the raw data, the following cytokines were removed from subsequent analyses due to too low concentration that were below or close to the lower

detection limit: IL-4, IL-12p70, GM-CSF, M-CSF and G-CSF. Due to that some drugs dramatically affected the cytokine concentration, 1.3% of the cytokine measurement values were above or below the detection limit. The majority of these values included measurements of IL-2 (14 out of 110 measurements) and IL-13 (9 out of 110 measurements). Measurements that were above or below the detection limit were set to the maximum or minimum detection limit, respectively, in order to use as much information as possible. Since most cytokines were found to have a skewed distribution, statistical analyses involving Pearson's correlation, cluster analysis and principal component analysis (PCA) were based on log10-transformed data. Pairwise Pearson's correlation was used to generate the correlation matrix of the cytokines. To account for multiple testing, 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 log-transformed cytokine expression profiles induced by the different drugs. The cytokines were clustered based on one minus the pairwise Pearson's correlation coefficients. Thus, cytokine pairs with a strong positive correlation will fall in the same cluster. The distance between two clusters was defined by the average linkage, 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 [24]. Other linkage methods such as complete, single and median method were evaluated. However, the average linkage method was found to be the most robust method according to the mean percentage of times the original cluster was identified on re-sampled data.

2.7. Factor analysis

Factor analysis using PCA to extract the components was used to reduce the correlated data of the cytokines. The final number of components was determined by extraction of components with an eigenvalue greater than one. To increase the interpretation of the factors, the Varimax rotation procedure with Kaiser Normalization was used [25], using the psych package [26]. The coefficients generated from this procedure, linking the cytokines to the factors, are the correlation coefficients (Pearson's correlation under Varimax rotation) between the cytokines and the factors.

3. Results

3.1. Cytokine concentration and effect of drugs

Cytokine levels were examined from ConA-stimulated autoreactive splenocytes in the presence or absence of the drugs. The splenocytes were taken from DA rats with pristane-induced arthritis (PIA) in order to create a more disease relevant environment for the drugs and to elicit a stronger cytokine response. PIA is a reproducible, T cell-dependent, rat model of rheumatoid arthritis that fulfills the criteria for RA as defined by the American College of Rheumatology [27–29]. It has been shown that the induced disease state of the T cells can be carried through *ex vivo* stimulation with ConA [23], indicating the relevance of the cytokine levels measured. In total, 55 different commercially available drugs were added into separate culture wells to study their effect on the cytokine production. After 44 h of *ex vivo* stimulation, the supernatant was harvested and the cytokine concentrations were determined by the Bio-Plex rat assay. After removal of cytokines expressed below reliable levels (see Section 2), totally 17 different cytokines, chemokines and growth factors were analyzed. Table 1 shows the descriptive statistics of the cytokine concentration from the cultures with drug treatment. The mean concentrations are generally greater than the median concentrations, which indicate in a skewed distribution of the cytokines. The skewed distribution was also confirmed by histograms for each cytokine (data not shown). The coefficient of variation (CV), which describes the variability relative to the mean, indicates how much each cytokine is affected by the drugs. The cytokines IL-2, IL-13, GRO and TNF-α are highly affected by the drugs since their standard deviations are equal or greater than their means.

Fig. 1 displays a box plot of the log2 fold change of the cytokine concentration from each drug relative to their corresponding positive control (ConA in the absence of drug). The overall effect of the drugs on the cytokines showed a reduced production of particularly IL-2, IL-17 and IFN-γ. In contrast, cytokines and growth factors such as IL-7, IL-13, IL-18, GRO, EPO and MIP-3α showed an increased expression. These results indicate that the drugs, at their present concentrations, modulate the cytokine production rather

Table 1
Descriptive statistics of the cytokine concentrations (pg/ml).

	Median	IQR	Mean	SD	CV
IL1α	227.9	94.2	243.6	184.8	0.8
IL1β	925.3	381	981.5	581.7	0.6
IL2	8729.7	7154	11201.5	11362.6	1
IL5	78.9	31.3	73.1	20.7	0.3
IL6	109.8	68	118.6	81.2	0.7
IL7	42.6	13.3	44.8	11.3	0.3
IL10	4818.9	2318.7	5063.8	2409.6	0.5
IL13	12.7	17.7	21	30.4	1.4
IL17	781.9	919.7	801.9	592.8	0.7
IL18	54.2	35.9	57.7	20.6	0.4
EPO	37.4	10.7	38.5	8.4	0.2
GRO	37.9	42.3	60.2	106.6	1.8
IFN-γ	3158.2	4506.9	3339.3	2795.8	0.8
MIP-3α	58.9	25.6	79.4	62.3	0.8
RANT	2271.5	1854.7	2505.1	1226.5	0.5
TNF-α	281.1	137.5	316.4	330	1
VEGF	112.4	92.3	118.5	76.3	0.6

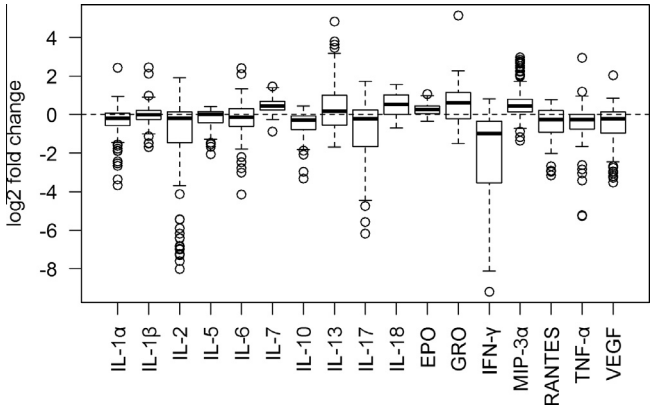

Fig. 1. The log2 fold change (FC) of the cytokine concentration. Cytokine concentration from autoreactive rat splenocytes was measured after 44 h of ConA-stimulation. In total, 55 different drugs were added to separate culture wells with two biological replicates. Hence, each box in the figure illustrates the fold change of the drug (110 measurements) relative the cytokine concentration in the absence of drug. Drugs diluted with or without DMSO were normalized to positive controls with or without DMSO, respectively. A log2 FC less than zero indicates that the drug inhibits the cytokine production whereas a log2 FC greater than zero indicates that the drug increases the production of the given cytokine. A reference line at zero is shown by the dashed line.

than causing a general global inhibition. Overall, the drugs reduced the mean and median cytokine concentration by 15% and 9%, respectively, compared to ConA-stimulated splenocytes in the absence of drugs.

The effect of each drug on each cytokine can be found in the supplemental material (Supplementary Table 1). As expected, the calcineurin inhibitors tacrolimus and Cyclosporine A induced dramatic reduction of the T cell cytokines IL-2 and IFN-γ, as did the glucocorticoids dexamethasone and prednisolone. The p38 inhibitors such as talmapimod and doramapimod caused a reduction in mainly IL-6 and TNF-α production, while the FDPS inhibitors risedronate and zoledronate increased the levels of IL-2, IL-13 and GRO. The majority of the drugs induced a fold change greater than 4 of at least one cytokine.

3.2. Correlation analysis

Cytokine levels from ConA-stimulated splenocytes were analyzed in the presence of 55 different drugs from two separate biological replicates. Hence, in total 110 data points were collected for each cytokine. To identify cytokines that correlated during the different exposures of drugs, the Pearson's correlation coefficient on log-transformed data was computed for all pairwise cytokines. Table 2 shows the correlation coefficients between each pair of cytokines. The pro-inflammatory cytokines IL-1 (IL-1α and IL-1β), IL-6, IL-17 (IL-17A) and TNF-α were significantly correlated with each other. The strongest correlations identified were between the two Th1 cytokines IL-2 and IFN-γ (r = 0.87), and between IL-18 and EPO (r = 0.84). Other relatively strong correlations were found between IL-2 and IL-17 (r = 0.79) and between IL-7 and EPO (r = 0.77). The only cytokine that showed significant negative correlation to other cytokines (IL-2, IL-13, IL-18 and IFN-γ) was MIP-3α. Generally, all cytokines correlated with at least three other cytokines. Out of the 136 pairwise comparisons, 43% significant correlations were identified, which show that cytokines are highly co-regulated under drug perturbation.

3.3. Cluster analysis

To identify groups of cytokines that are similarly affected by the drugs, correlation hierarchical cluster analysis was performed by using the average linkage method. The method generates a dendrogram that relates cytokines based on their positive correlation (see Fig. 2). Using a cutoff value of 0.5 in the dendrogram, it was possible to identify five distinct clusters. As expected from the previous correlation analysis, the pro-inflammatory cytokines IL-1, IL-6 and TNF-α fall in the same cluster. The Th1 cytokines IL-2 and IFN-γ clustered together with IL-17 and IL-10. IL-5 formed cluster with the growth factor VEGF and the chemokine GRO. EPO and IL-18 clustered with IL-7 whereas IL-13 and MIP-3α formed a distinct cluster. The chemokine RANTES did not cluster with any group, given the cutoff value of 0.5. To evaluate the uncertainty of the hierarchical cluster analysis, bootstrapping (n = 10.000) was performed. The bootstrap probability (bp) value show the percentage of times that the original clusters were identified from the same type of cluster analysis based on re-sampled data. As expected, clusters of cytokines with strong correlations were found to be robust during re-sampling. The most robust cluster identified was the cluster of IL-7, IL-18 and EPO. The second most robust cluster included the cytokines IL-2, IL-17 and IFN-γ. On average, the bootstrap value was 65%. Using other linkage methods generated very similar clusters but with lower average robustness; complete (bp = 62%), single (bp = 55%) and median linkage method (bp = 56%). The two most robust clusters according the bp values; (1) IL-2, IL-17 and IFN-γ and, (2) IL-7, IL-18 and EPO were consistent for all tested linkages methods, indicating that these clusters are also robust to different types of linkage functions.

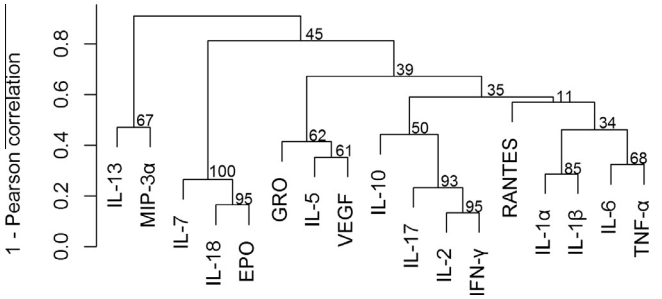

Fig. 2. Cluster analysis of the cytokine profiles. Hierarchical clustering was performed, using one minus Pearson's correlation coefficient as a distance measure, using the average linkage method. Thus, clusters in the lower part of the dendrogram represent groups of cytokines with strong positive correlation. Bootstrapping (n = 10.000) was performed to assess the robustness of the clusters and is shown as percentage at the tree nodes. A high percentage indicates a robust cluster since the same cluster is present in a large fraction of dendrograms generated by the re-sampled data.

3.4. Principal component analysis

To identify groups of cytokines that correlate, as a complement to the cluster analysis, a PCA analysis was performed on the

Table 2
Correlation matrix showing the Pearson's correlation coefficient between each cytokine pair.

	IL-1α	IL-1β	IL-2	IL-5	IL-6	IL-7	IL-10	IL-13	IL-17	IL-18	EPO	GRO	IFN-γ	MIP-3α	RANTES	TNF-α	VEGF
IL-1α	1																
IL-1β	0.71*	1															
IL-2	0.38*	0.11	1														
IL-5	0.57*	0.28	0.37*	1													
IL-6	0.51*	0.47*	0.51*	0.56*	1												
IL-7	0.25	0.47*	0.00	0.05	0.21	1											
IL-10	0.42*	0.16	0.55*	0.35	0.45*	−0.01	1										
IL-13	0.26	0.29	0.02	0.26	0.34	0.27	0.44*	1									
IL-17	0.51*	0.14	0.79*	0.66*	0.56*	0.12	0.53*	0.07	1								
IL-18	0.13	0.26	0.27	0.29	0.30	0.70*	−0.01	0.01	0.40*	1							
EPO	0.20	0.45*	0.12	0.21	0.31	0.77*	−0.12	0.08	0.25	0.84*	1						
GRO	0.28	0.20	0.24	0.61*	0.54*	−0.02	0.49*	0.53*	0.44*	0.10	−0.01	1					
IFN-γ	0.46*	0.14	0.87*	0.38*	0.53*	0.07	0.59*	−0.03	0.74*	0.28	0.14	0.17	1				
MIP-3α	0.16	0.28	−0.37*	0.08	0.19	−0.08	0.15	0.53*	−0.34	−0.38*	−0.18	0.17	−0.40*	1			
RANTES	0.49*	0.38*	0.46*	0.27	0.42*	0.14	0.31	−0.03	0.29	0.16	0.23	−0.18	0.56*	0.06	1		
TNF-α	0.64*	0.53*	0.47*	0.38*	0.68*	0.31	0.58*	0.30	0.53*	0.23	0.25	0.34	0.63*	0.05	0.44*	1	
VEGF	0.27	0.07	0.34	0.65*	0.43*	0.20	0.41*	0.51*	0.58*	0.34	0.23	0.56*	0.21	0.03	−0.05	0.26	1

* Indicates significant correlation (p < 0.01) after Bonferroni correction.

Table 3
Varimax rotated factor loading matrix.

	Factor			
	1	2	3	4
IL-1α	0.78[a]	0.27	0.14	−0.08
IL-1β	0.67[a]	0.07	0.43	−0.38
IL-2	0.52	0.32	−0.04	0.69[a]
IL-5	0.32	0.70[a]	0.13	0.14
IL-6	0.61[a]	0.52	0.18	0.05
IL-7	0.16	0.04	0.88[a]	−0.12
IL-10	0.52	0.55	−0.26	0.14
IL-13	0.15	0.64[a]	0.08	−0.52
IL-17	0.40	0.57	0.12	0.62[a]
IL-18	0.04	0.18	0.86[a]	0.34
EPO	0.15	0.04	0.93[a]	0.08
GRO	0.07	0.87[a]	−0.06	−0.08
IFN-γ	0.65[a]	0.20	−0.02	0.67[a]
MIP-3α	0.20	0.18	−0.22	−0.84[a]
RANTES	0.80[a]	−0.19	0.07	0.16
TNF-α	0.76[a]	0.32	0.15	0.07
VEGF	−0.02	0.85[a]	0.21	0.12
% var expl.	0.37	0.15	0.14	0.11

[a] Loading scores greater than 0.6.

cytokine data. PCA reduces the dimension of the data set into independent components to which the cytokines correlate with. The PCA resulted in four factors when the eigenvalue 1 criterion was applied. In total, these four factors explained 78% of the variance. The Varimax rotated factors and their loadings are shown in Table 3. The loading scores represent how well each cytokine correlates to the corresponding factors. To interpret the factors, a loading score greater than 0.6 was chosen as a threshold to determine if a cytokine qualified for loading a component. The pro-inflammatory cytokines IL-1α, IL-1β, IL-6 and TNF-α loaded on factor 1 together with RANTES and IFN-γ. Factor 2 included IL-5, IL-13, GRO and VEGF. The cytokines IL-7, IL-18 and EPO that formed a robust cluster in the cluster analysis loaded on factor 3. Finally, IL-2, IL-17, IFN-γ and MIP-3α loaded on factor 4. The PCA analysis showed a high similarity to the cluster analysis, where groups of cytokines in the dendrogram are also involved in the same factors in the PCA. MIP-3α was the only cytokine that showed relatively large negative loading scores, which is in agreement with the correlation analysis. Also, IL-10 was the only cytokine that did not load on any of the factors according to the given threshold.

4. Discussion

Cytokines play a key role in regulating the immune system. Understanding how these cytokines are co-regulated is of particular importance for their use as biomarkers in certain diseases and to understand the effect of drug therapies. Cytokines that show a strong correlation under perturbation are likely to be co-regulated and thus induced via common pathways and/or secreted by the same cell type. Most of the previous studies involving analysis of cytokine profiles have been dedicated to different kinds of inflammatory diseases, where cytokines have been measured from blood samples. In this study, we have analyzed the perturbation of 17 different cytokines by using 55 different drugs in a preclinical rat model of autoimmune arthritis. The overall effect of the drugs on the cytokine production showed reduced secretion of IL-2 and IFN-γ whereas the growth factor IL-7 and the chemokines GRO and MIP-3α were found to be produced at a higher level compared to the control. The expression profiles indicate that the drugs, at the given concentrations, modulate the immune response rather than causing a general inhibition of the cytokines. In addition, 43% of the pairwise comparisons showed a significant correlation, indicating that production of the cytokines are highly connected or regulated by the same mechanisms. Hence, drugs that inhibit a certain cytokine are likely to also affect its related cytokines. The highest correlations found between pairs of cytokines were the ones between the Th1 cytokines IL-2 and IFN-γ, and between IL-18 and EPO. The relationships between these cytokines were also reflected in the clustering and the PCA analysis. The cytokines IL-7, IL-18 and EPO formed a robust cluster in the cluster analysis and were loaded on the same factor in the PCA analysis. IL-7 and IL-18 have recently been found to synergize to promote proliferation of naïve CD8 T cells [30]. In addition, a strong positive correlation between IL-18 and EPO has previously been observed in a rat model of type 1 diabetes [31]. Consistent with our study, the pro-inflammatory cytokines IL-1, IL-6 and TNF-α have previously been found to correlate and cluster together in studies of cytokine measurements from human blood samples [32–35]. The Th1 cytokines IL-2 and IFN-γ clustered together and showed a strong positive correlation. In addition, IL-17 clustered to these two cytokines. Previous studies have found a strong correlation between IL-2, IL-17 and IFN-γ [36–38]. IFN-γ production has been shown to be induced by IL-2 stimulation in both NK-cells and macrophages [39–41], and has a major role in favoring Th1 development [7,8,42]. Th17 cells have also been found to promote Th1 cell development through IL-17 induction [43], supporting the significant correlation between IL-17 and the Th1 cytokines IL-2 and IFN-γ. In addition, the drugs were found to cause the most inhibitory effect on the cytokines IL-2, IL-17 and IFN-γ, which support the robust cluster of these cytokines. Although the Th2 cytokines IL-5 and IL-13 were produced in response to ConA-stimulation, Th2 cell development seems to be suppressed in our system due to the very low amount of IL-4. The low level of IL-4 is likely due to that the pristane-treated DA rats are prone to Th1-mediated autoimmune disease [44]. Thus, our model system mainly captures the effect of drugs on the cytokine production involved in Th1 responses. Other limitations of our results are that the study was only based on *ex vivo* experiments, using drug concentrations based on their inhibitory effects *in vitro* rather than concentrations used in therapies. Secondly, our system does not capture the variation in cytokine production between different individuals. Due to the relatively large genetic variation within the cytokine genes [45,46], a considerable variation in cytokine concentration has been observed among human individuals [32,34,47–49]. Thirdly, the relationships of cytokines are also likely to be affected by the disease state. Hence, the effect of drug therapies on the cytokine network might be confounded by the underlying disease. In conclusion, our results predict the co-regulation of cytokines and suggest how drug therapies might affect the cytokine production within the specific disease model. Using the given correlations of the cytokines, one can predict a certain group of cytokines that is affected during a specific drug treatment. In addition, a cytokine used as a therapeutic biomarker could be combined with its related cytokines into a biomarker panel in order to improve diagnostic accuracy.

5. Funding

This work has been supported by the Swedish Knowledge Foundation, the Swedish Agency for Economic and Regional Growth through the European Regional Development Fund (SIM2020) and by Carl Tryggers Foundation (CTS 15/232). The research performed at Redoxis leading to these results has received funding from the European Community's Framework programs under grant agreements Neurinox (Health-F2-2011-278611), Innovative

Medicines Initiative (IMI) project BTCure (IMI Research Project 115142) as well as Swedish Governmental Agency for Innovation Systems.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.cyto.2016.10.015.

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