Immunological Assessment of Viral Vectors Expressing Multiple Cytokine Transgenes in Nonclinical Studies

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Outline

- Central question: Are current nonclinical immunological assessments adequate?
- Example: Multi-armed oncolytic Adenoviral vectors
- Assessment strategy
- Future directions

Adenovirus: Viral Vector Ally in Immunotherapy

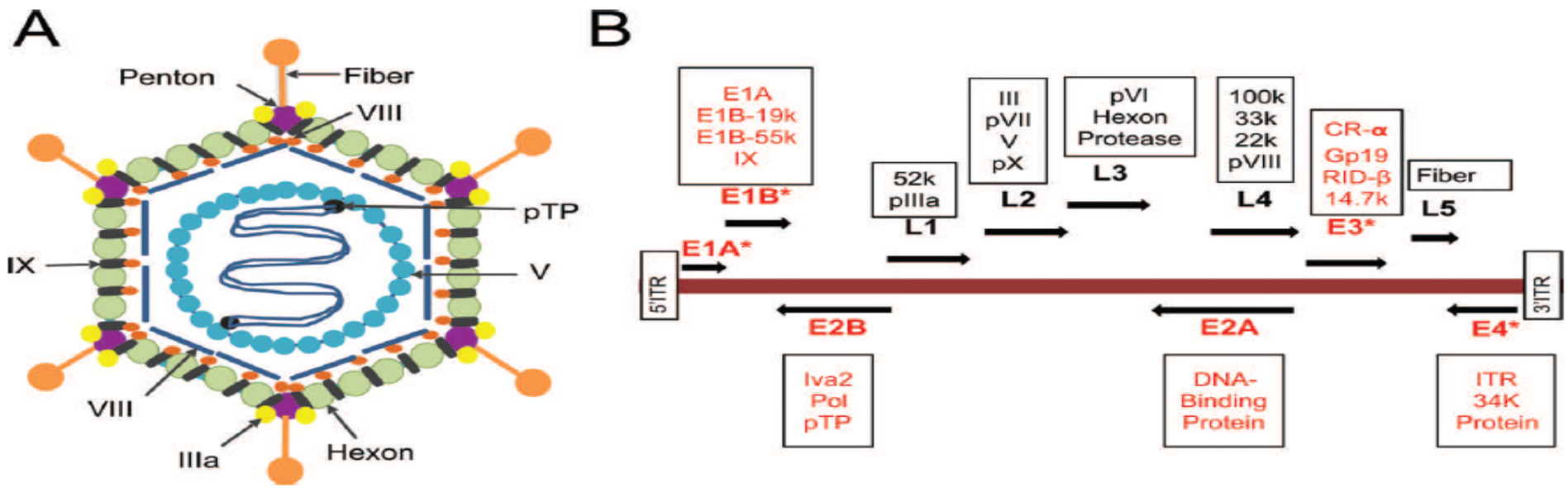


Figure 1. Adenovirus structure and genome organization. (A) Graphical representation of adenovirus structure and various proteins. (B) Adenovirus genome organization showing various early (E) and late (L) transcripts and proteins encoded by each transcript. Regions indicated by red with (*) are deleted in various adenoviral vectors. E1 and E3 regions were deleted in first generation and E1, E2, E3, and/or E4 were deleted in second-generation adenoviral vectors. Most recent adenoviral vectors called helper-dependent adenoviral vectors only contain ITRs and packaging signals. Figure is adapted from Ref. [209].

Adapted from: "Adenoviral Vector-Based Vaccines and Gene Therapies: Current Status and Future Prospects" (2018) http://dx.doi.org/10.5772/intechopen.79697

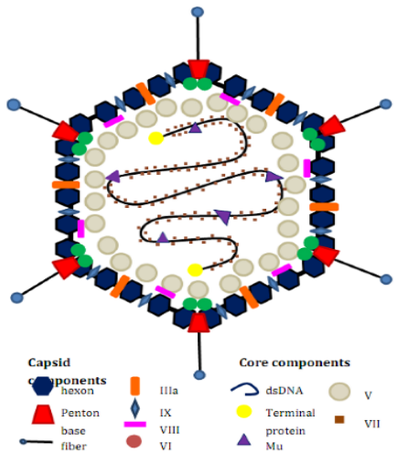
Adenovirus Immunology: Overview

Table 1 Summary of immune responses in viral gene transfer

Vector	*Innate immune response*	*Adaptive immune response*
AAV	Low and highly transient inflammation Complement activation TLR-9 dependent DNA sensing DC activation	Pre-existing NAB Memory CD8+ T-cell responses to capsid NAB formation after vector administration Antibody and T-cell responses against transgene product depending on route of vector administration and other factors Treg and immune tolerance induction to the transgene product for hepatic gene transfer
Adenovirus	Inflammation, immunotoxicity in target organ Thrombopenia, platelet activation Hemodynamic changes Inflammasome-dependent cell death Induction of inflammatory cytokines and IFN-α,β Activation of TLR-9-dependent and TLR-9-independent pathways of DNA sensing Activation of TLR-2 NK cell activation Endothelial cell activation Complement activation DC activation	Pre-existing NAB NAB formation after vector administration Transduction of APCs CTL responses against viral gene products (unless gutted vectors are used) and transgene product Antibody and T-cell responses against transgene products, especially if nonspecific promoters are used

Adapted from: Nayak S. and Herzog R.W. *Gene Therapy* (2010) 17. 295-304

Adenovirus Immunology: Digging a Little Deeper



I.V.

C3 + C4

Factor X

Opsonized Virus

Spleen

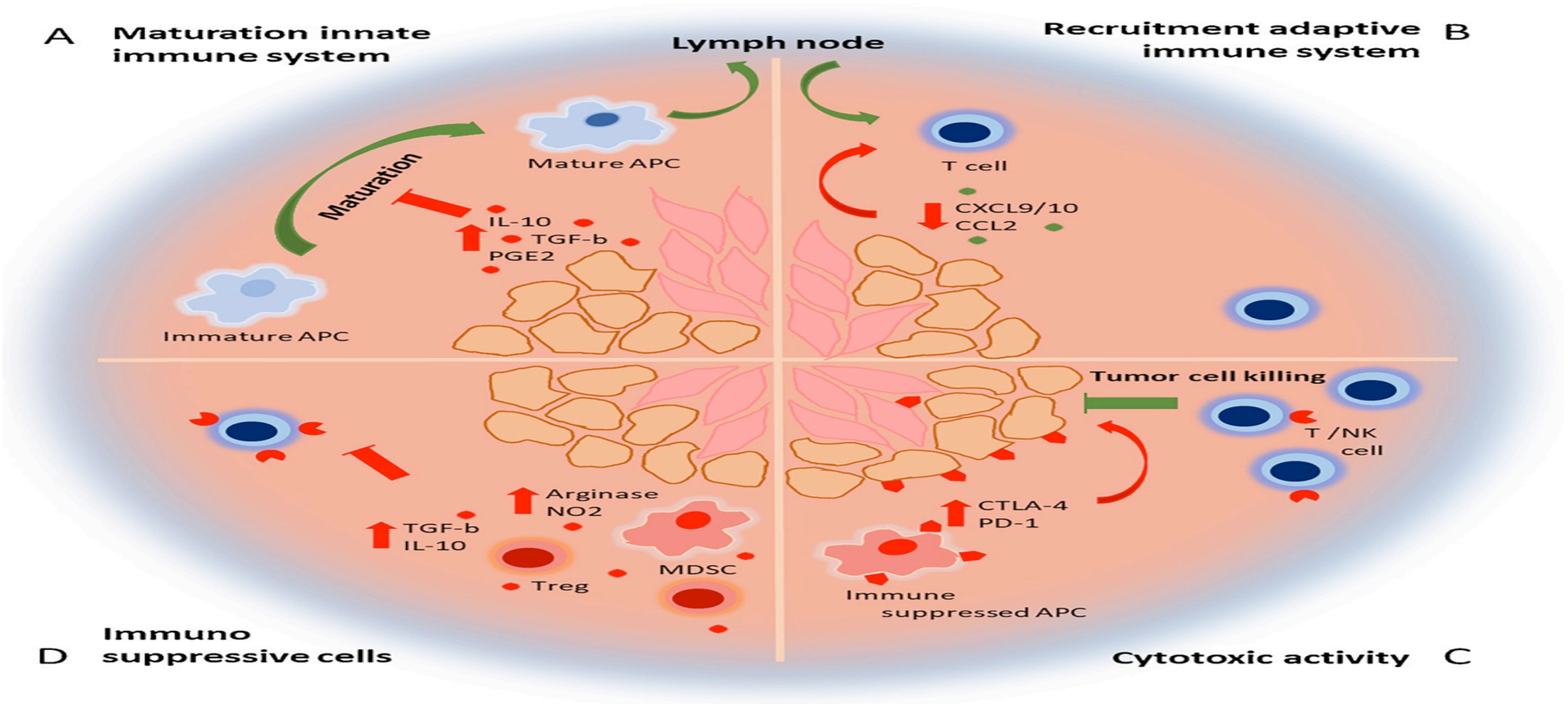
Liver

Target Cell

Ref #1: Atasheva S. and Shayakhmetov D. "Adenovirus Sensing by the Immune System." *Curr Opin Virol*. 2016 December; 21: 109–113. doi:10.1016/j.coviro.2016.08.017

Ref #2: "Adenoviral Vector-Based Vaccines and Gene Therapies: Current Status and Future Prospects" (2018) http://dx.doi.org/10.5772/intechopen.79697

Solid Tumor Immunosuppressive Microenvironment



Adapted from: J.F. de Graaf et al. *Cytokine & Growth Factor Reviews*. 41 (2018) 28-39

Adenovirus: Multi-armed Vectors

Table 1 (*continued*)

Transgene	Virus	Tumor	Additive immunologic effects	Toxicity
Combinations				
GM-CSF + IL-12	AdV [104,105]	Melanoma	Secreted cytokine profile shifted from Th2 to Th2 response [105] Infiltration of T helper, CTL, NK and DC [104], [105] Immunity against rechallenge with tumor cells [105]	Not reported
IL-12 + IL-18	AdV [106]	Melanoma	Infiltration of T helper, CTL, NK	Not reported
IL-12 + CCL2	HSV [67]	Neuroblastoma	Reduced tumor growth	Not reported
B7.1 + IL-12	AdV [41]	Melanoma	Infiltration of T helper, CTL and DC	Not reported
B7.1 + IL-18	HSV [132]	Neuroblastoma, Prostate	Reduced tumor growth No significant difference in survival	Not reported
B7.1 + GM-CSF	AdV [85]	Melanoma	Infiltration of T helper, CTL and DC Immunity against rechallenge with tumor cell	Not reported
4-1BBL + IL-12	AdV [42]	Melanoma	Infiltration DC, T helper and CTL	No signs

Adapted from: J.F. de Graaf et al. *Cytokine & Growth Factor Reviews*. 41 (2018) 28-39

Typical Approach

Toxicology Study of Dual Armed Ad5 Vector in Immunocompetent Tumor-Bearing BALB/c Mice

Group	Virus	IV Dose (Total PFU)[a] D: 1, 4, 8, 11, 15	Number of Animals		
			D2	D28	D42
1	VC	NA	5 M / 5 F	10 M / 10 F	10 M / 10 F
2	V	1×10^6	5 M / 5 F	10 M / 10 F	10 M / 10 F
3	V	1×10^7	5 M / 5 F	10 M / 10 F	10 M / 10 F
4	V	1×10^8	5 M / 5 F	10 M / 10 F	10 M / 10 F

a. VC = Vehicle Control
b. V = Virus encoding 3 transgenes

Immunology Assessments:
1. Transgene Expression: Serum + Tissue Homogenates D28 + 42
2. ADA/NAB: D28 + D42
3. Mutliplex Cytokine Analysis: INF-γ, IL-2, IL12p70, IL-1β, IL-6, IL-10, and TNF-α on D2

End Result of the Typical Approach

- Tabular data
- Standard stats: Shapiro-Wilk test and homogeneity of variance using the Levene test. Based on the results of these tests, a parametric or a nonparametric one-way ANOVA will be performed, followed by a *post hoc* pairwise test (e.g., Dunnett's, Wilcoxon's) as appropriate. The level of significance is $p < 0.05$ ($p < 0.01$ for normality and variance tests).
- These types of analyses may not be optimal for immunological analyses in safety studies.

Future Directions: A Different Approach to Data

- How do we answer questions of the impact of interacting system components to address immunology data?
- Example client question: The cytokine data indicate significant increases in proinflammatory cytokines in mid- and high-dose animals at 6 hrs post-dose. Is this a normal antiviral cytokine response or cytokine storm?

Yiu HH, Graham AL, Stengel RF (2012) “Dynamics of a Cytokine Storm.” *PLOS ONE* 7(10): e45027. doi:10.1371/journal.pone.0045027

Dynamics of a Cytokine Storm

Yiu HH, Graham AL, Stengel RF (2012) "Dynamics of a Cytokine Storm." *PLOS ONE* 7(10): e45027. doi:10.1371/journal.pone.0045027

- Study: Can linear modeling using differential equations be applied to cytokine storm data to determine temporal/concentration cause and effect relationships between cytokines and how each cytokine inhibits or induces the other?
- Serum cytokine data collected over five days post-dose was evaluated from six male subjects that received TGN1412 (0.1 mg/kg IV infusion 2mg/min).
- Cytokines: INF-γ, IL-1, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12, TNF-α measured by Luminex.
- 4 hr PD, 1, 4, 26, and 40 hrs PD, then every 6 hrs through D4, daily until D10.

Approach

- Selected Linear Time Invariant Model with parameter estimates from the median time course data over the five-day time course.
- 2nd Order equations were used to model concentration (1st Order) and rate of change (2nd Order) for each cytokine. Best fit time course constants were found by numerical search.
- All nine cytokines then analyzed concurrently in an 18th Order system.
- A unified coupled model was applied to calculate cytokine class interactions, induction/inhibition, and the impact of variability.

Can the Model Predict Individual Cytokine Response?

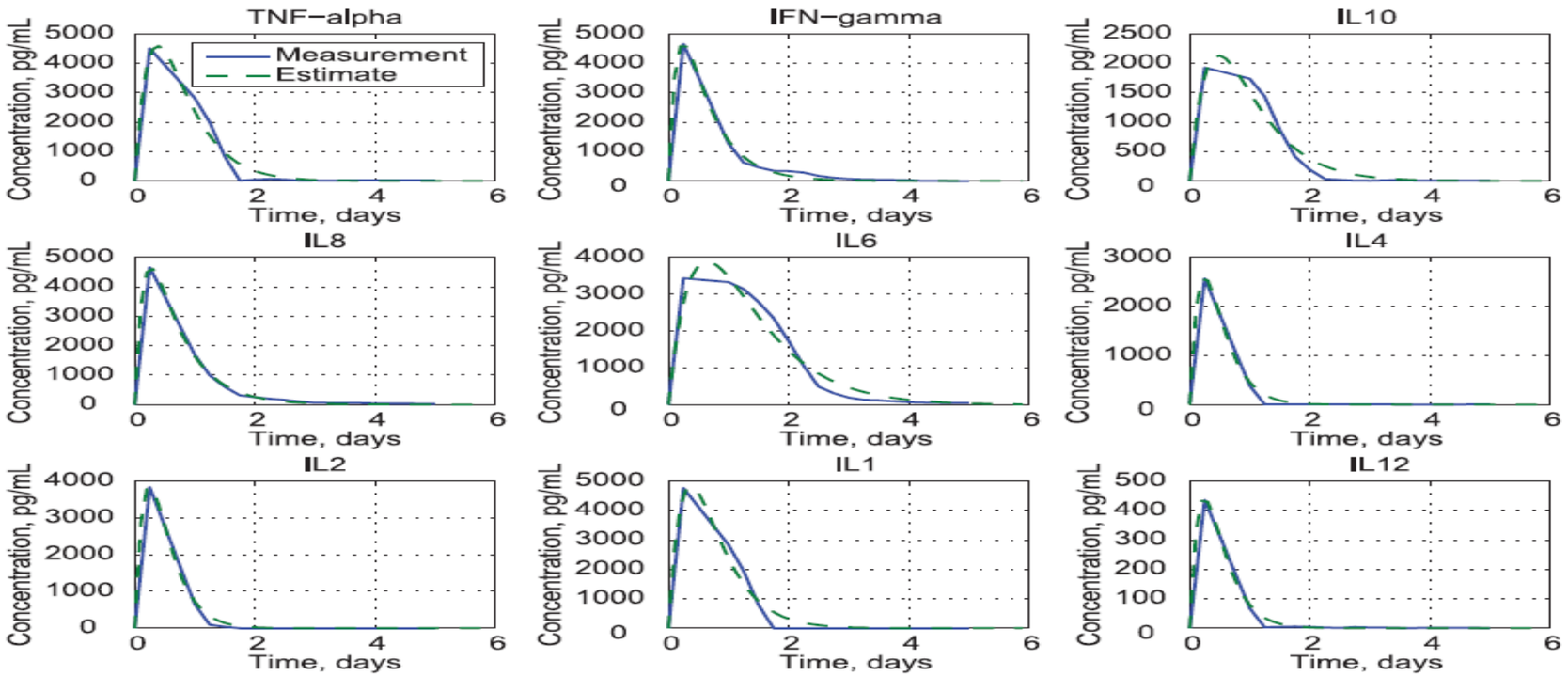


Figure 1. Comparison of clinical trial data [11] and estimates from uncoupled second-order models of cytokine response.
doi:10.1371/journal.pone.0045027.g001

$$J = \sum_{k=0}^{20} \varepsilon(t_k)^2 = \sum_{k=0}^{20} [z(t_k) - x_1(t_k)]^2$$

Takeaway: Yes.

Modeling Individual Cytokine Response

Table 1. Eigenvalues, Time Constants, Periods, Damping Ratios, and Initial Rates of Change for Uncoupled, Second-Order Cytokine Models.

Component	λ_1, d^{-1}	λ_2, d^{-1}	τ_1, d	τ_2, d	P, d	ζ, -	$x_2(0)$, pg/mL-d
TNF-α	−2.63	−2.63	0.38	0.38	2.39	1	32821
IFN-γ	−7.21	−2.05	0.14	0.49	1.63	1.2	55328
IL10	−2.08	−2.08	0.48	0.48	3.02	1	12047
IL8	−6.71	−1.84	0.15	0.54	1.79	1.22	50804
IL6	−1.55	−1.55	0.65	0.65	4.05	1	16437
IL4	−4.17	−4.17	0.24	0.24	1.51	1	29489
IL2	−4.08	−4.08	0.25	0.25	1.54	1	42780
IL1	−2.71	−2.71	0.37	0.37	2.32	1	35535
IL12	−4.13	−4.13	0.24	0.24	1.52	1	4947

doi:10.1371/journal.pone.0045027.t001

Takeaway: Proinflammatory INF-γ, IL-1, IL-2, IL-8, and TNF-α are produced faster than IL-6, IL-10, IL-4, and IL-12. The model will allow predictions based on change in concentration and the rate of change in cytokine concentration.

Modeling Individual Cytokine Response:

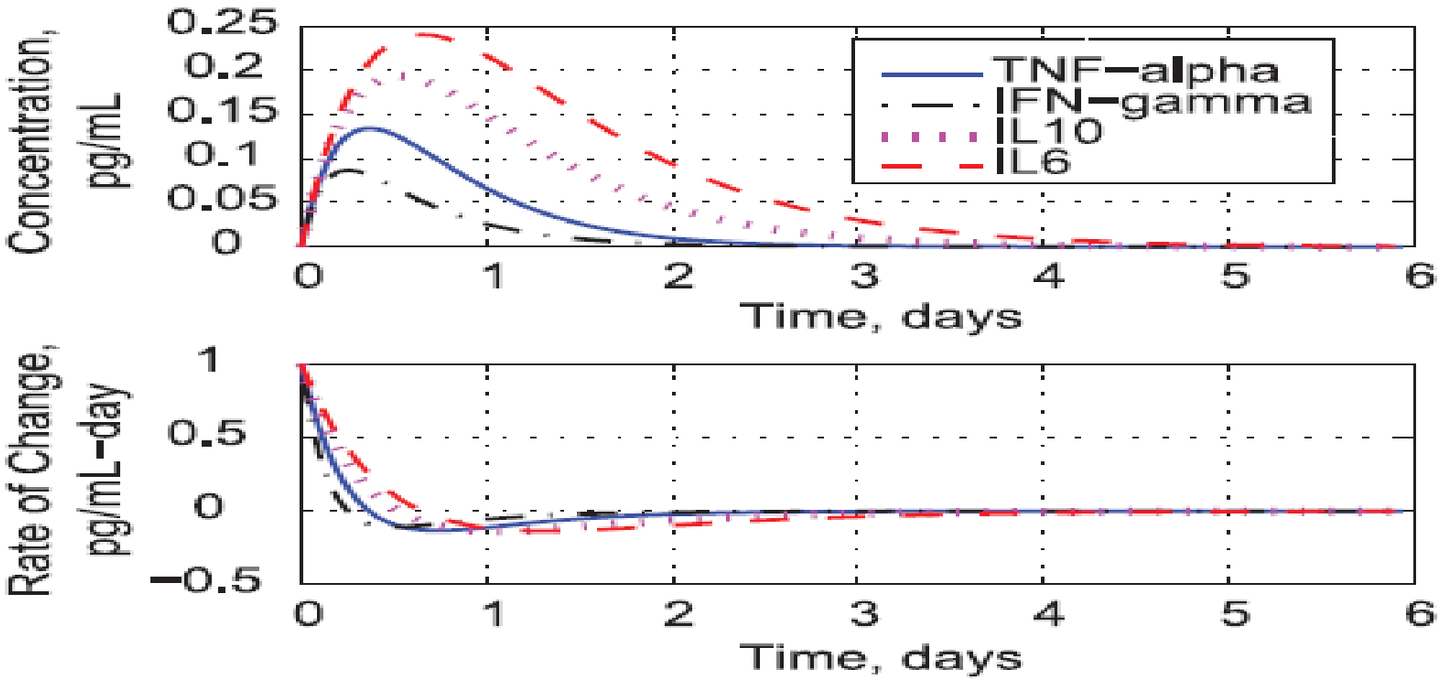


Figure 2. Response to unit initial rates of change for TNF-α, IFN-γ, IL10, and IL6.
doi:10.1371/journal.pone.0045027.g002

Takeaway: Increase in concentration and rate of change showed the following: INF-γ peaks first, then TNF-α, followed by IL-10, then IL-6. IL-6 is the last to peak, but reaches the highest concentration, and recedes by D4.

Modeling Individual Cytokine Response

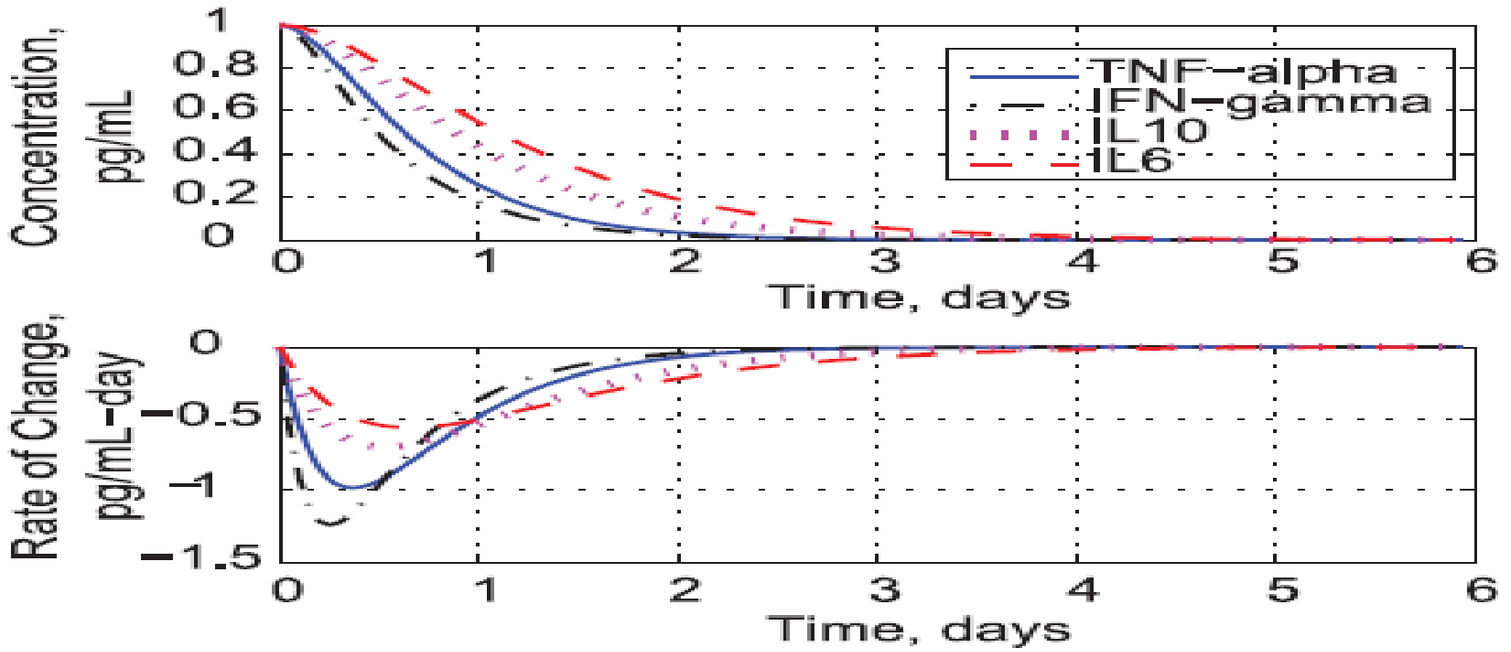


Figure 3. Response to unit initial concentrations for TNF-α, IFN-γ, IL10, and IL6.
doi:10.1371/journal.pone.0045027.g003

Takeaway: Change in cytokine concentration and rate of change match figure 2, peak on Day 1, and decay.

What Is the Inductive/Coupled Cytokine Response?

Table 2. Concentration Coefficients of the Fully Coupled Cytokine Model, C_C.

	TNF	IFN	IL10	IL8	IL6	IL4	IL2	IL1	IL12
TNF''	**−6.413**	0.345	**−0.383**	−0.186	**−0.632**	**−0.680**	−0.206	0.672	**−0.818**
IFN''	−0.554	**−18.641**	0.078	**1.576**	**1.542**	0.128	0.184	**0.696**	**−0.903**
IL10''	−0.487	**0.846**	**−3.320**	0.145	**−0.727**	−0.111	−0.030	−0.017	0.617
IL8''	**0.992**	−0.207	**1.566**	**−13.571**	0.058	**−0.823**	−0.316	0.046	**−3.356**
IL6''	0.412	**−1.688**	−0.303	0.042	**−2.784**	**0.640**	**0.769**	**0.955**	0.065
IL4''	**−1.129**	**−1.072**	−0.278	0.271	0.101	**−16.305**	**0.776**	**0.778**	−0.237
IL2''	−0.503	−0.775	**0.422**	**0.506**	−0.242	−0.022	**−15.226**	−0.181	−0.957
IL1''	0.053	−0.090	−0.376	**0.891**	−0.575	0.227	0.289	**−7.571**	0.604
IL12''	**−0.877**	−0.075	0.275	−0.228	0.320	0.343	**1.554**	−0.271	**−19.448**

Positive off-diagonal elements represent inductive acceleration of one cytokine by another; negative coefficients represent inhibitive acceleration. Input cytokines are listed in the first row. (.)'' represents $d^2(.)/dt^2$ in the first column of the table.

doi:10.1371/journal.pone.0045027.t002

Takeaway: Table shows the interactive stimulatory or suppressive effect of each cytokine relative to itself and other cytokines. Classical auto and cross regulatory cytokine network function confirmed.

Which Cytokines Interact and Is the Interaction Inductive or Inhibitive?

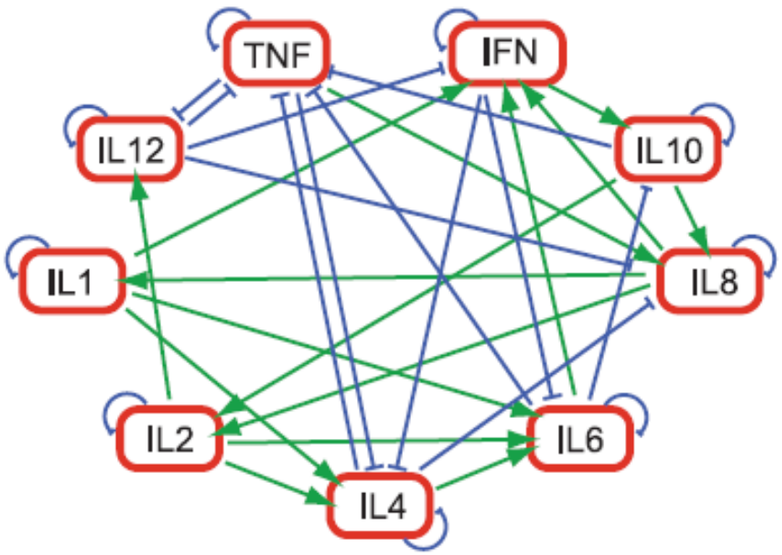


Figure 4. Most significant inductive and inhibitive accelerations in the cytokine coupling matrix. Arrowhead denotes induction; "T" represents inhibition.
doi:10.1371/journal.pone.0045027.g004

Takeaway:

a. TNF-α mutually inhibits IL-4 + IL-12
b. IL-6 is both enhanced and inhibited by INF-γ
c. TNF-α, INF-γ, and IL-4 have six cross regulatory
d. IL-6 and IL-8 participate in seven interactions
e. IL-1, IL-2, and IL-8 are strong inducers

Which Cytokines Have the Greatest Effect on Coupling?

Table 3. Eigenvalues, Periods, Damping Ratios, and Three Highest Eigenvector Magnitudes of A_C.

Mode	λ, d^{-1}	P, d	ζ, -	EV #1	EV #2	EV #3
1	−0.84	–	–	IL10	IL6	IL8
2	−1.4± j0.75	3.93	0.89	IL6	TNF	IL10
3	−1.88	–	–	IL8	TNF	IL1
4	−2.27± j0.61	2.66	0.97	IL1	IL8	IFN
5	−3.28± j0.60	1.89	0.98	IL1	IL10	IFN/IL4
6	−3.22± j0.98	1.86	0.96	IL1	IL4	TNF
7	−3.75	–	–	IL10	IL12	TNF
8	−4.02± j0.20	1.56	0.99	IL4	IL12	IL2
9	−4.41± j0.71	1.40	0.99	IL4	IL12	IFN/IL8
10	−5.29± j0.82	1.17	0.99	IL8	IFN	IL12
11	−5.82	–	–	IL8	IFN	IL12

doi:10.1371/journal.pone.0045027.t003

Takeaway: IL-8 and INF-γ and IL-4 are the cytokines most involved in coupling and have greatest cross regulatory impact.

Coupled Decay/Stimulation Effect?

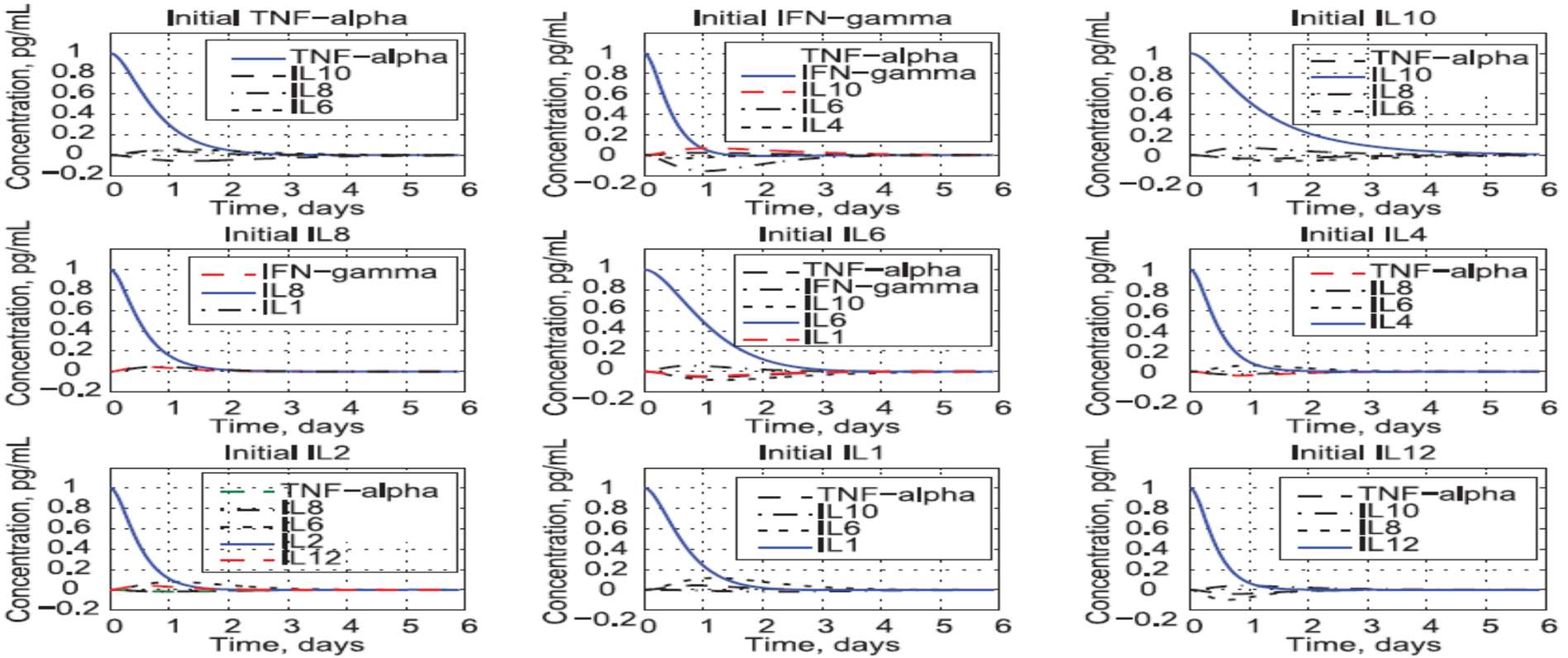


Figure 5. Unit initial-concentration response for nine cytokines based on the coupled.
doi:10.1371/journal.pone.0045027.g005

Takeaway: IL-1, IL-8, and TNF-α induce each other while downregulating IL-4 and IL-10. Changes in IL-4 and IL-10 inhibit TNF-α.

Inductive and Inhibitory Cytokine Effects

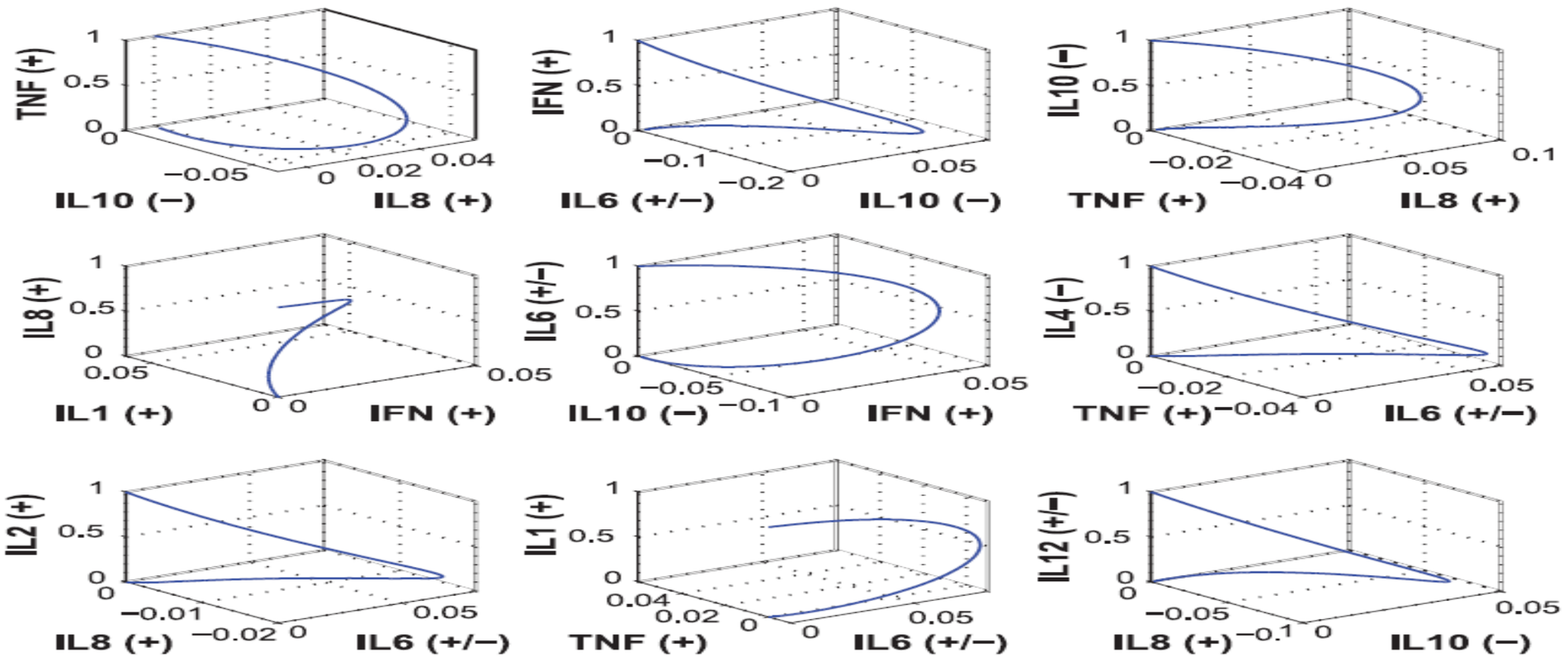


Figure 6. Motifs of response to unit initial cytokine concentrations.
doi:10.1371/journal.pone.0045027.g006

Takeaway: Space plot representation of graphic data in Figure 5.

Variance Identification in Cytokine Response

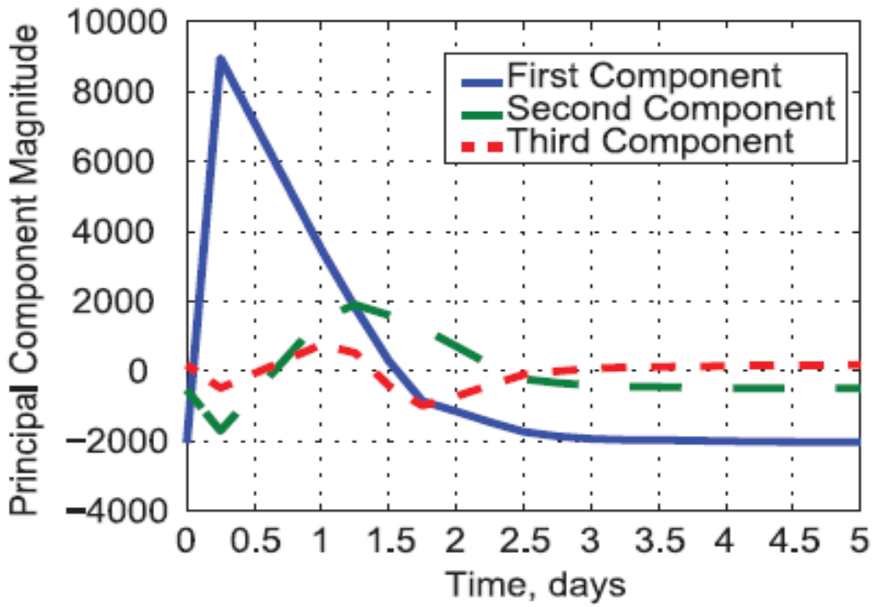


Figure 7. Shapes of the first three cytokine principal components, $y_1(t_k)$, $y_2(t_k)$, and $y_3(t_k)$.
doi:10.1371/journal.pone.0045027.g007

Takeaway: 92% of variance due to inter-patient variability and rate of change, 7% due to drug concentration, 1% remaining background variance.

Can the Model Show Group Pattern Cytokine Effects with Variance?

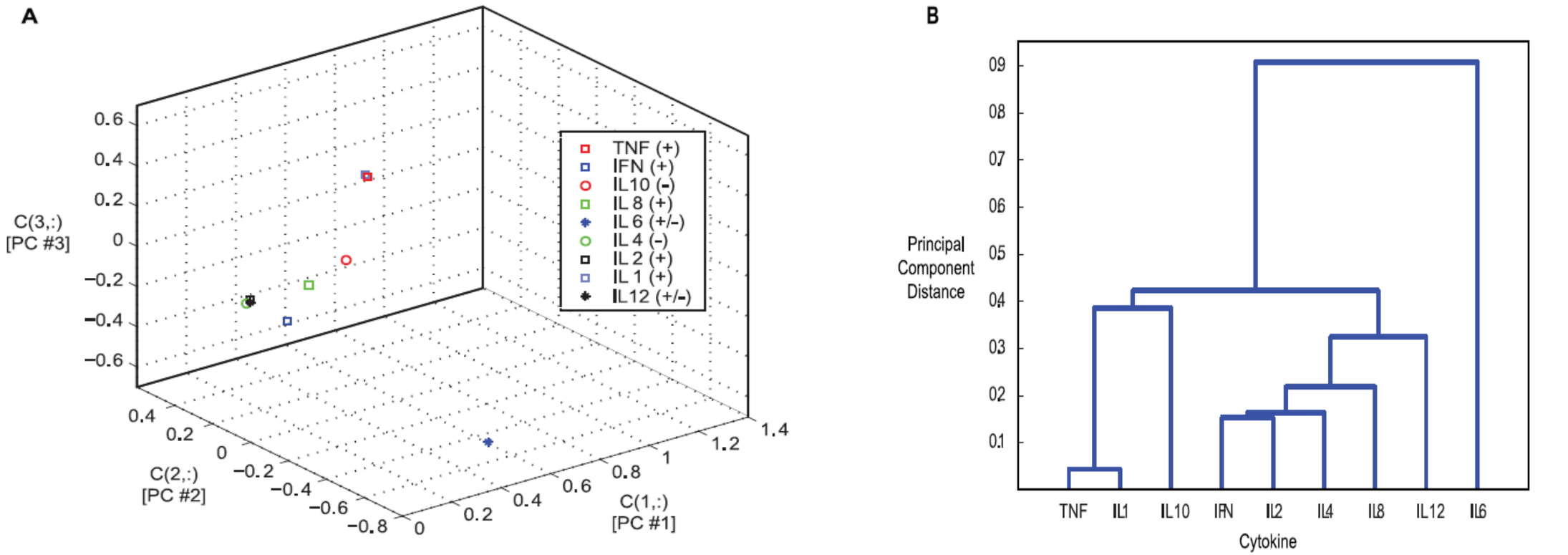


Figure 8. Similarity of cytokine response shapes as described by the first three principal component coefficients. A) Coefficients of the first three principal components. B) Dendrogram relating closeness of cytokine covariances.
doi:10.1371/journal.pone.0045027.g008

Takeaway: Yes, as shown in A and B.

Conclusions

- Taken together the data showed a 2nd Order Time Invariant Model applied to cytokine data can:
  - Accurately predict and measure cytokine concentrations and rates of change
  - Calculate the inductive or inhibitory effect of individual cytokines and determine functional cytokine network grouping
  - Describe cytokine network behavior and define and calculate the variances effecting cytokine network behavior
- Provides greater ability to rigorously answer immunological questions that arise during safety studies