

Supplementary Appendix

This appendix has been provided by the authors to give readers additional information about their work.

Supplement to: Suntharalingam G, Perry MR, Ward S, et al. Cytokine storm in a phase 1 trial of the anti-CD28 monoclonal antibody TGN1412. *N Engl J Med* 2006;355. DOI: 10.1056/NEJMoa063842.

Suntharalingam 06-3842

Appendix 1

Methods

The cytokines were evaluated on serum samples by cytometric bead array (CBA, BD Biosciences, San Jose, CA, USA; Human Th₁/Th₂ cytokine kit and Human Inflammation Kit) using a FACSCalibur flow cytometer (Becton Dickinson [BD]) equipped with a 488 nm Argon-ion laser. Blood samples from all 8 volunteers were taken according to the study protocol (available at http://www.mhra.gov.uk/home/idcplg?IdcService=GET_FILE&dID=20366&noSaveAs=0&Rendition=WEB) before treatment at Day 0, and then 1 and 4 hours post-infusion. Following the serious adverse events, cytokine sampling was subsequently done on the 6 patients at approximately 26 hours, 40 hours and then every 6 hours until Day 4 following infusion. Daily samples for cytokine levels were then obtained until Day 10 following the infusion. The normal values and detection limits of cytokines using this technique are given below. The assays were done at an independent certified laboratory arranged through PAREXEL.

Cell subset analysis: The immunophenotyping results for T cell subsets were from 3 different sources. The Day -7 and -10 pre-dose and Day 0 cell numbers were measured on a FACSCalibur flow cytometer (BD) by an outside laboratory on behalf of TeGenero AG per study protocol. A whole blood assay was used and subsets were based on double-staining (i.e., CD3⁺CD4⁺=CD4⁺ and CD3⁺CD8⁺=CD8⁺ T cells); absolute numbers were obtained by comparison with a fixed number and volume of TrueCount Beads (BD). The Day 1 cell subsets were done at an accredited reference laboratory

following Good Laboratory Practice (GLP) in London, arranged through PAREXEL. All subsequent time points for cell subsets (daily from Day 2 post-dosing until Day 6, then Days 8, 10, 11, 12, 15, 18, 22, 25 and 31 post-infusion) were evaluated at the clinical pathology accredited hematology laboratory at North West London Hospitals NHS Trust, Northwick Park & St. Mark's Hospital site. The data were acquired on a Coulter Epics XL flow cytometer (Beckman Coulter, Fullerton, CA, USA) using GLP and standard operating procedures (SOPs) for acquisition and analysis using a whole blood assay. For cell subset analysis, double positive events (i.e. CD3⁺/CD4⁺ and CD3⁺/CD8⁺) gated on the CD45⁺/side scatter (SSC)^{low} region were counted as CD4⁺ and CD8⁺ T cells, respectively.

Normal ranges for cytokines in cytometric bead array.

Cytokine	Normal level (pg/mL)	Limits of detection (pg/mL)
IFN- γ	<10.9	7.1-5000
TNF- α	<3.7	2.8-5000
IL-10	<3.7	2.8-5000
IL-8	<40	3.6-5000
IL-6	<5.6	3.0-5000
IL-4	<4.1	2.6-5000
IL-2	<4.9	2.6-5000
IL-1 β	<48	7.2-5000
IL-12p70	<9.4	1.9-5000

Results (supplemental, patients 5 and 6)

Patient 5 was intubated at 15 hours post-infusion due to acute delirium and non-compliance with supportive treatment. He subsequently became hypotensive with a blood pressure of 70/35 and acidemic with a peak lactate of 11.3 mmol/L, and required inotropic and vasopressor support until day 4. He developed ARDS with bilateral pulmonary infiltrates and a minimum P/F ratio of 180 mmHg on Day 2, requiring

mechanical ventilation until Day 9 when he was extubated. Steroid therapy was completed by Day 24.

Patient 6, the index case, was intubated 12 hours after infusion of TGN1412 and ventilated for a total of 16 days. He had a labile blood pressure for the first 48 hours of his ICU stay (80/50 – 180/100) and required high fluctuating doses of vasopressors and inotropes, with lactate 3.9 – 5.4 mmol/L on CVVHDF. He had severe respiratory impairment with extensive bilateral pulmonary infiltrates (Figure 2A) and a minimum P/F ratio of 50 mmHg. He developed recurrent and persistent culture-negative pyrexia and was treated empirically with escalating antibiotic, antifungal and antiviral agents with eventual resolution of the fever. Computed tomography of the chest on day 11 demonstrated mild alveolar edema and a left lower lobe pneumonia, with minimal evidence of posterior consolidation despite the clinical presentation of ARDS. He was extubated on day 16 and the steroid taper was completed on Day 33.

From the laboratory results: a close comparison of Th₁ and Th₂ cytokines for the 6 patients revealed that the elevated blood TNF- α , usually short-lived, was sustained and still high at 24 hours post-infusion in patients 5 and 6 (supplemental Figure 10). In patient 6, who was the most ill, IL-10 levels were disproportionately low when compared with the pro-inflammatory cytokine elevation in the same individual and with the high IL-10 levels observed in the other 5 patients.

Figure Legends

Supplemental Figure 4. Patient 1: timecourse of level of CRP, Creatinine, ALT, platelets, T cell subsets, monocytes, neutrophils and cytokines from the time of administration of TGN1412 (0 days) until 30 days post-dose. (A) A rapid rise in creatinine with concomitant decline in platelet count was seen within 8 hours of exposure to the drug. CRP increased during day 1. A close analysis of the first 5 days shows that the peak creatinine was reached within the first day, and the CRP peak was delayed to 2 days, occurring just prior to the platelet trough at day 2-3 which was maintained until day 5. For the use of ventilatory assistance with continuous positive-airway pressure (CPAP) and renal support with continuous venovenous hemodialysis (CVVHD), “+” and “-” denote “on” and “off” respectively. **(B)** An overview of the 30 days post-dose reveals a delayed rise in ALT followed by an increase of platelets after the initial recovery phase. **(C, D) T cell, monocyte and neutrophil numbers relative to cytokine response after administration of TGN1412. (C)** Pre- and post-dose levels of CD3⁺, CD4⁺, CD8⁺ T cell subsets and monocytes over the first 5 days indicated a drop in all subsets such that they were undetectable by the first 24 hours after administration of TGN1412 and coincided with a rapid (within 4 hours) rise of all cytokines measured, mostly for IFN- γ , TNF- α and IL-10 and least for IL-4. TNF- α was the first cytokine to rise, within an hour of infusion of TGN1412, and also rapidly fell to normal levels. At 2 days post-infusion, lymphocyte subsets and monocytes began to recover with normalization of cytokine levels. Timing of treatment with steroids and daclizumab is shown. A steroid taper thereafter lasted until day 21. The start of recovery of lymphocytes and monocytes does not appear to correlate with the discontinuation of high-dose steroids. **(D)** After initial recovery of lymphocytes and monocytes, the CD4⁺:CD8⁺ T cells recovered in a

1:1 ratio, and exceeded the expected “normal” starting range of the patient as indicated at Day -10. The neutrophils increased during the acute and reactive phases over the first 5 days and in response to steroid treatment, as expected. All cell subsets shown returned to the normal baseline level by Day 25. HC = hydrocortisone 100 mg; MP = methylprednisolone 1000 mg iv; D = daclizumab iv, 70 mg for all 3 doses.

Supplemental Figure 5. Patient 2: timecourse of level of CRP, Creatinine, ALT, platelets, T cell subsets, monocytes, neutrophils and cytokines from the time of administration of TGN1412 (0 days) until 30 days post-dose. (A-D) Reaction following infusion was similar to that of Patient 1 (see legend for supplemental figure 4) except that the platelet trough occurred at Day 3 and was maintained until Day 6. Also, the peak ALT occurred later, at Day 12. The steroid taper in this patient lasted until day 21. HC = hydrocortisone 100 mg; MP = methylprednisolone 1000 mg iv; D = daclizumab iv, 84 mg for the first dose, then 85 mg for doses 2 and 3.

Supplemental Figure 6. Patient 3: timecourse of level of CRP, Creatinine, ALT, platelets, T cell subsets, monocytes, neutrophils and cytokines from the time of administration of TGN1412 (0 days) until 30 days post-dose. (A-D) Reaction following infusion was similar to that of Patient 1 (see legend for supplemental figure 4) except that the platelet trough occurred at Day 3-4 and started to recover at Day 5. The peak ALT occurred later, similar to that of patient 2, at Day 9. A steroid taper in this patient lasted until day 21. HC = hydrocortisone 100 mg; MP = methylprednisolone 1000 mg iv; D = daclizumab iv, 85 mg for the first dose, then 75 mg for doses 2 and 3.

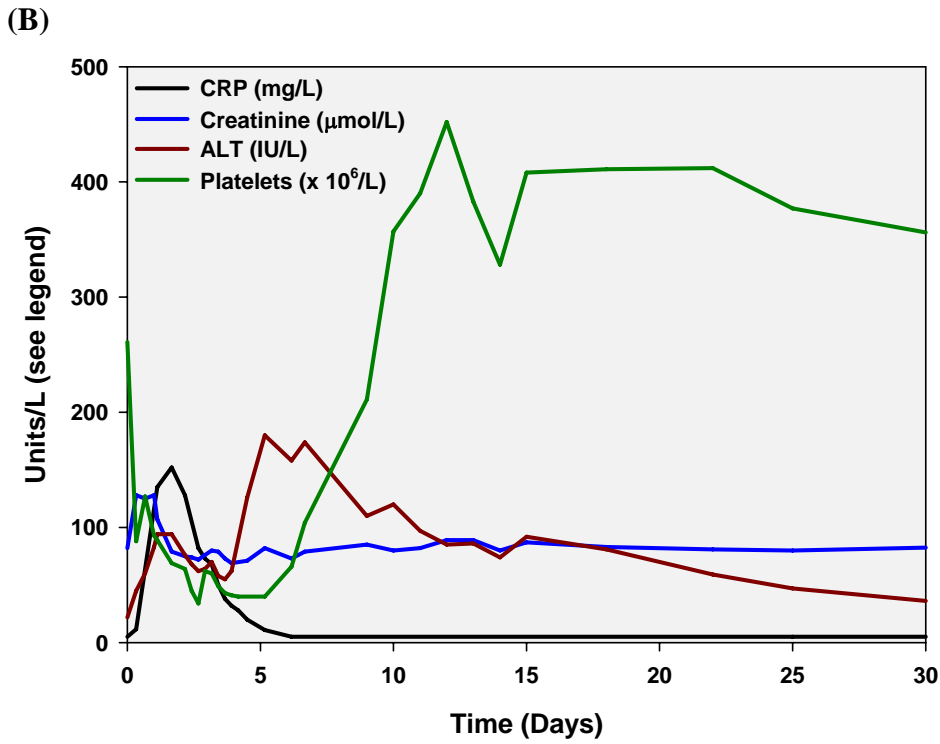
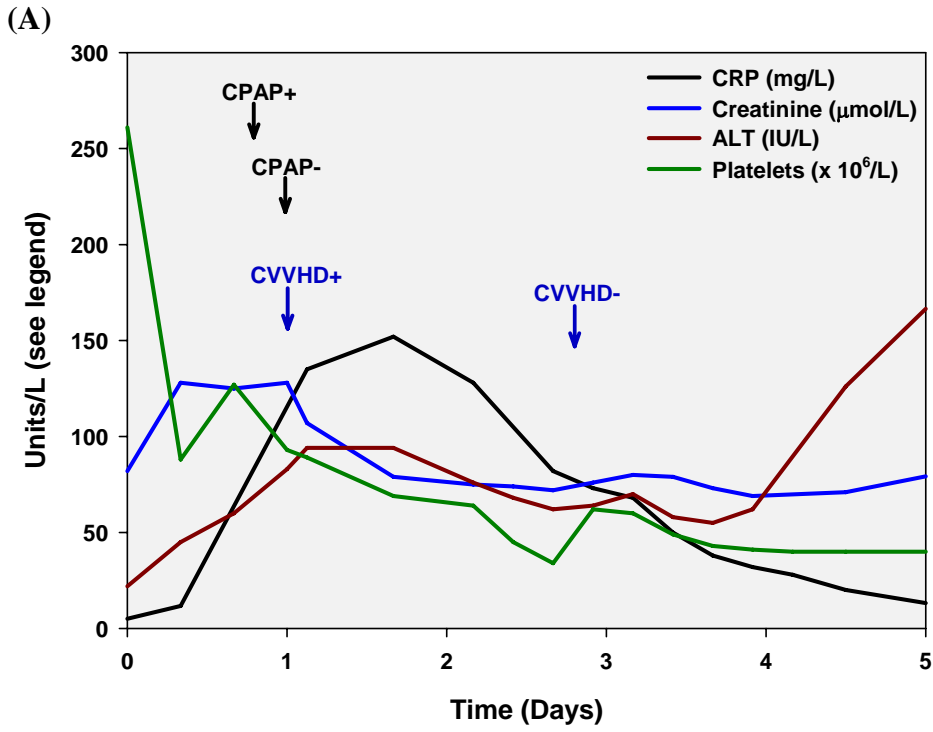
Supplemental Figure 7. Patient 4: timecourse of level of CRP, Creatinine, ALT, platelets, T cell subsets, monocytes, neutrophils and cytokines from the time of administration of TGN1412 (0 days) until 30 days post-dose. (A-D) Reaction following infusion was similar to that of Patient 1 (see legend for supplemental figure 4) except that the platelet trough occurred at day 3 and was maintained until day 5. A steroid taper in this patient was continued until day 21. HC = hydrocortisone 100 mg; MP = methylprednisolone 1000 mg iv; D = daclizumab iv, 75 mg for all 3 doses.

Supplemental Figure 8. Patient 5: timecourse of level of CRP, Creatinine, ALT, platelets, T cell subsets, monocytes, neutrophils and cytokines from the time of administration of TGN1412 (0 days) until 30 days post-dose. (A-D) Reaction following infusion was similar to that of Patient 1 (see legend for supplemental figure 4) except that the platelet trough at day 3-4 and was maintained until day 8. This patient was the only one of the 6 with an early ALT peak (within the first 8 hours) in addition to a later rise at Day 15. He also differed in the recovery of CD4⁺:CD8⁺ T cell subsets which occurred in a ratio of 2:1. The cytokine response was unusual in this individual compared with patients 1-4 but similar to that of patient 6 in that the level of IL-4 was high and the IL-6 and TNF- α elevations prolonged. For the use of ventilatory assistance with IPPV and renal support with CVVHD, “+” and “-” denote “on” and “off” respectively. The steroid taper was completed in this patient on day 24. HC = hydrocortisone 100 mg; MP = methylprednisolone 1000 mg iv; D = daclizumab iv, 75 mg for the first dose, then 85 mg for doses 2 and 3.

Supplemental Figure 9. Patient 6: timecourse of level of CRP, Creatinine, ALT, platelets, T cell subsets, monocytes, neutrophils and cytokines from the time of administration of TGN1412 (0 days) until 30 days post-dose. (A-D) Reaction following infusion was similar to that of Patient 1 (see legend for supplemental figure 4) except that the timing of peak levels occurred over a prolonged period of time. The peak creatinine was reached within the first day, and the CRP peak was delayed to 4-5 days, occurring after the platelet trough at Day 3-4, which was maintained until Day 6-7. Early apparent peaks in the monocyte counts at Day 4, 7, and 10 were due to dysplastic neutrophils being counted as monocytes by the automated counter. Recovery of lymphocytes and monocytes was slow and may have been partially due to the extra use of steroids in this patient (in order to control the inflammatory reaction) compared with the doses used for the others. Lymphocyte subsets and monocyte numbers reached baseline levels by Day 30. The cytokine response was pronounced and prolonged in this individual, in particular for the cytokines IL-6, TNF- α and IL-4, despite the extra dose of high-dose methylprednisolone given. For the use of ventilatory assistance with IPPV and renal support with CVVHD, "+" and "-" denote "on" and "off" respectively. This patient received an extra dose of high-dose methylprednisolone and completed the subsequent steroid taper on day 33. HC = hydrocortisone 100 mg; MP = methylprednisolone 1000 mg iv; D = daclizumab iv, 75 mg for all doses.

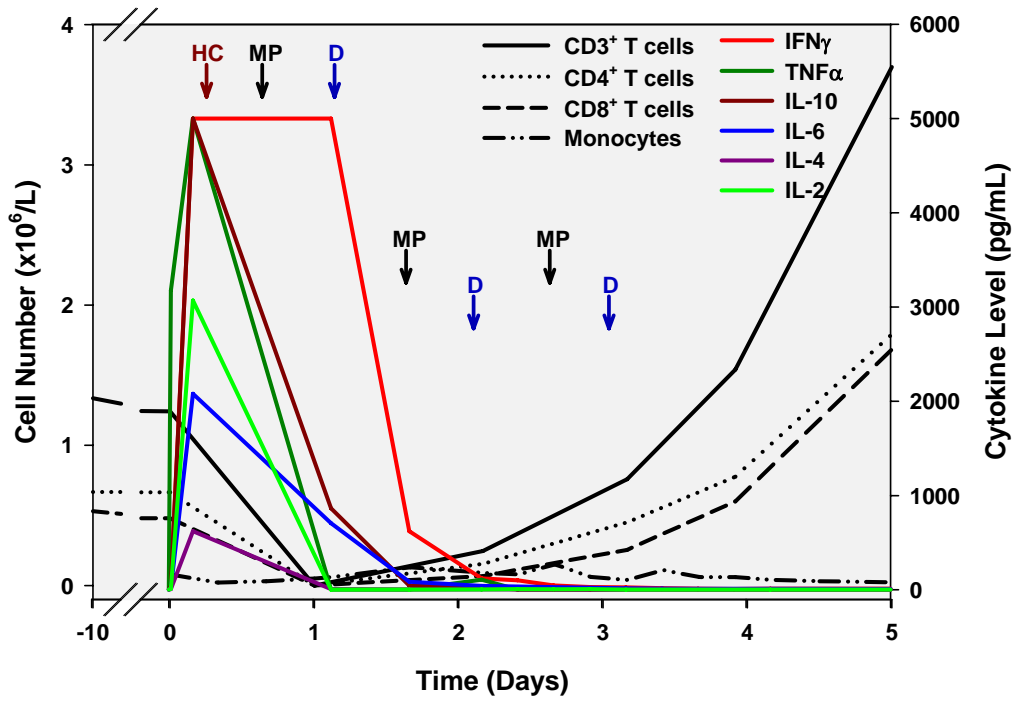
Supplemental Figure 10. Comparison of Th₁ and Th₂ cytokine levels for the first 5 days following infusion of TGN1412. Cytokines for Patient 1 (A, B), Patient 2 (C, D),

Patient 3 (E, F), Patient 4 (G, H), Patient 5 (I, J) and Patient 6 (K, L) are shown. Th₁ cytokines IFN- γ , TNF- α , IL-8, IL-6, IL-2 and IL-12p70 are shown in figures A, C, E, G, I, and K whereas Th₂ cytokines IL-10, IL-4 (shown together with the Th₁ cytokine IL-1 β for comparison) are depicted in figures B, D, F, H, J and L. High proinflammatory, Th₁ cytokines were observed in all patients within the first 4 hours following infusion, with a more prolonged course in patients 5 and 6 (I, K). Prolonged and high levels of IL-6, IL-4 and TNF- α were particularly noted in the latter. The level of IL-10 appears proportionate in all patients except in patient 6 (L) where the response was less pronounced. Of note, the 4 hour time-points for the following cytokines could not be analysed due to sample limitations: Patient 2 IL-12p70 (C) and IL-1 β (D), Patient 3 IL-8 (E) and IL-1 β (F), Patient 4 IL-1 β (H), and Patient 5 IL-8, IL-12p70 (I) and IL-1 β (J). The Day 1 post-infusion sample for IL-12p70 (I) in Patient 5 was also not evaluable due to sample limitation.

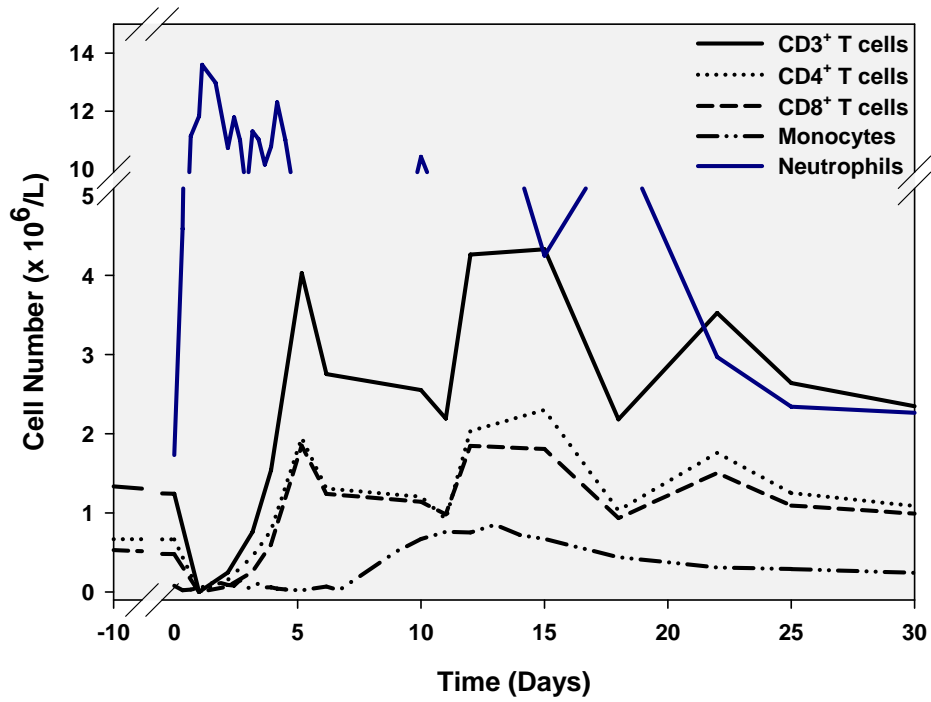


Supplemental Figure 4

(C)

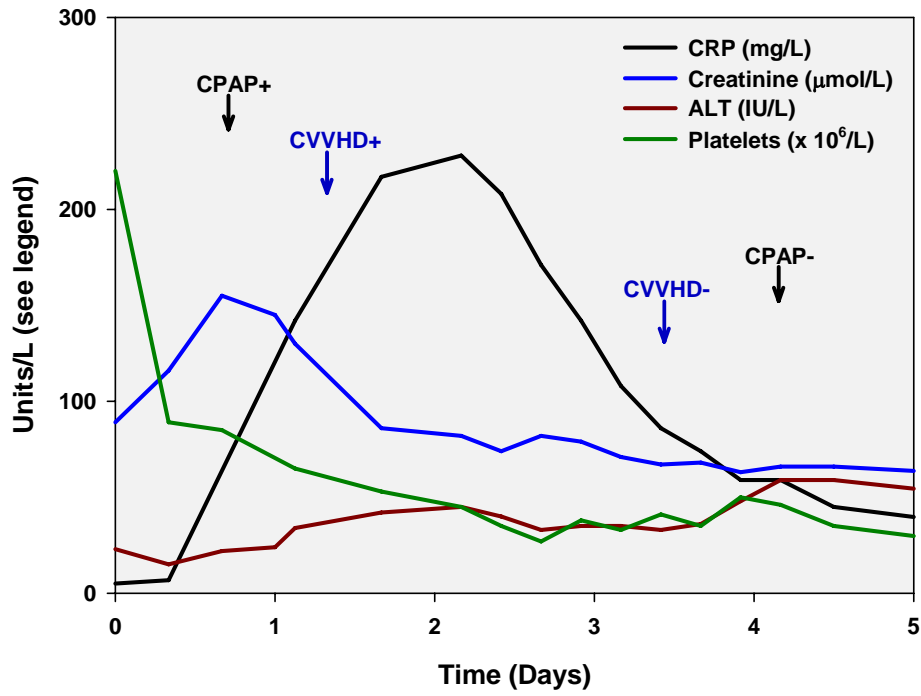


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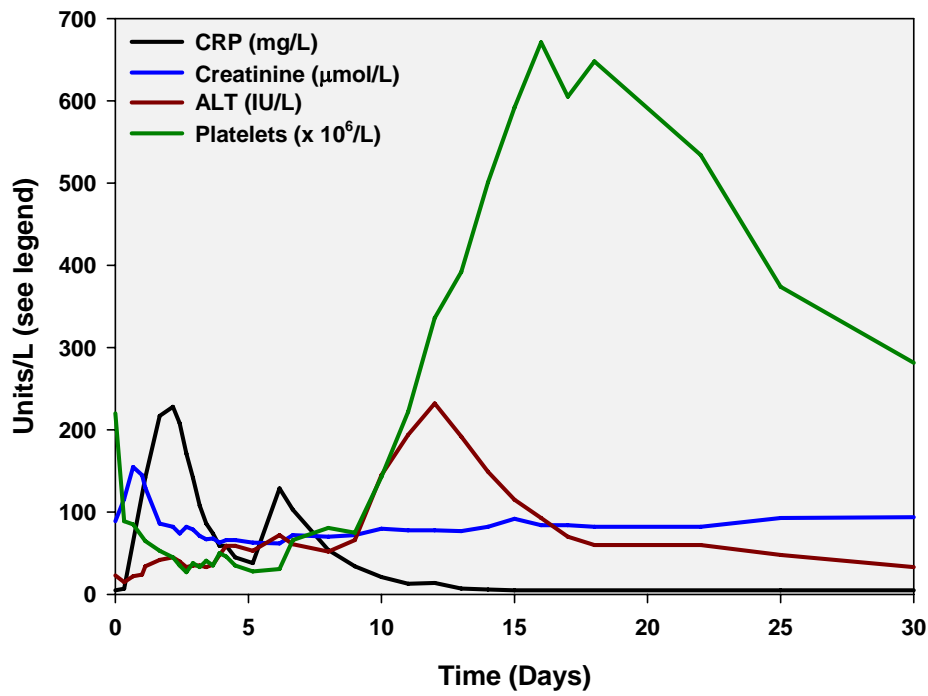


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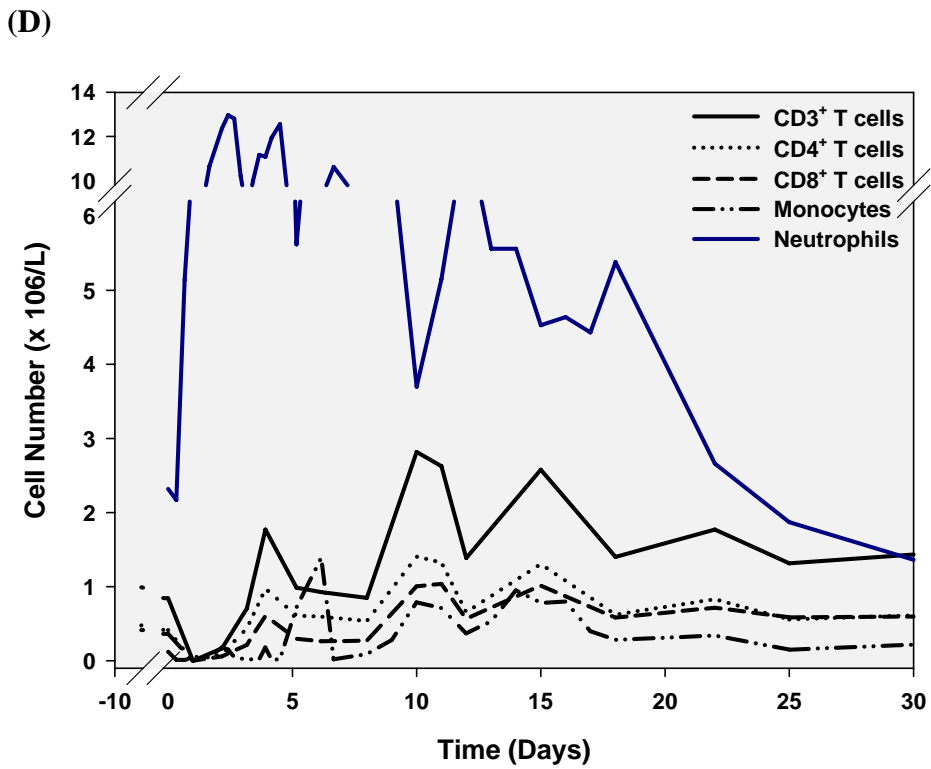
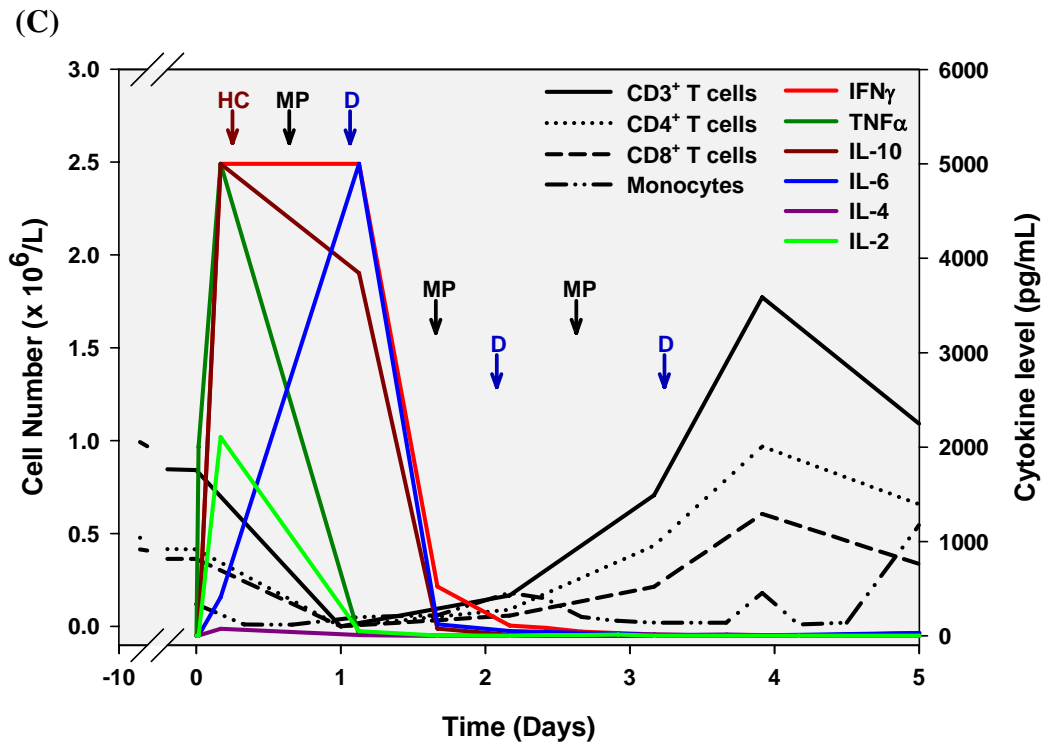
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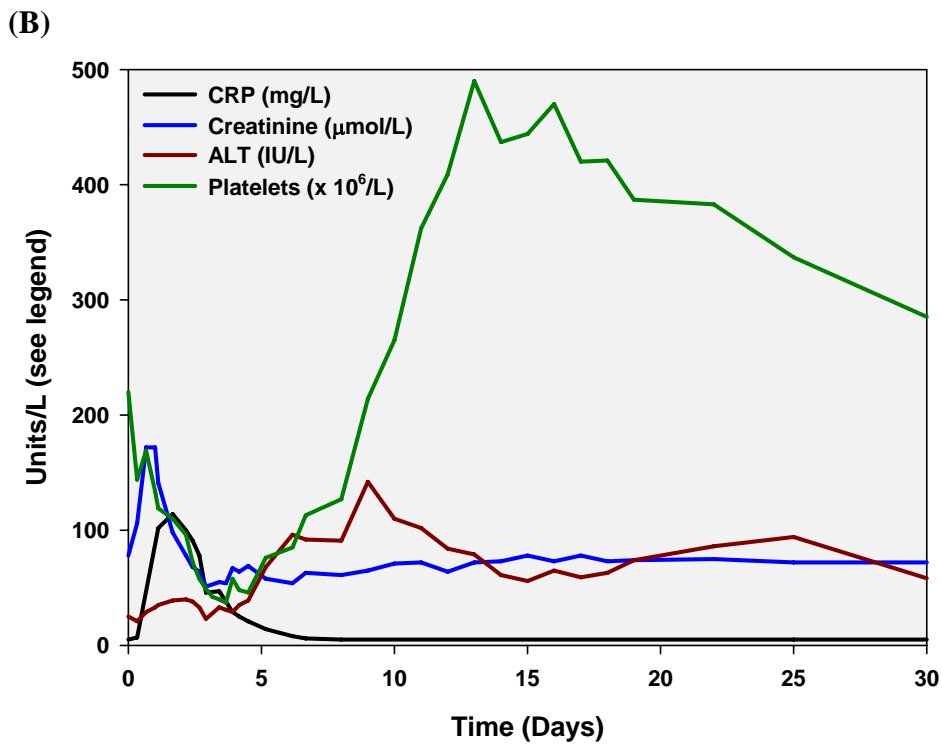
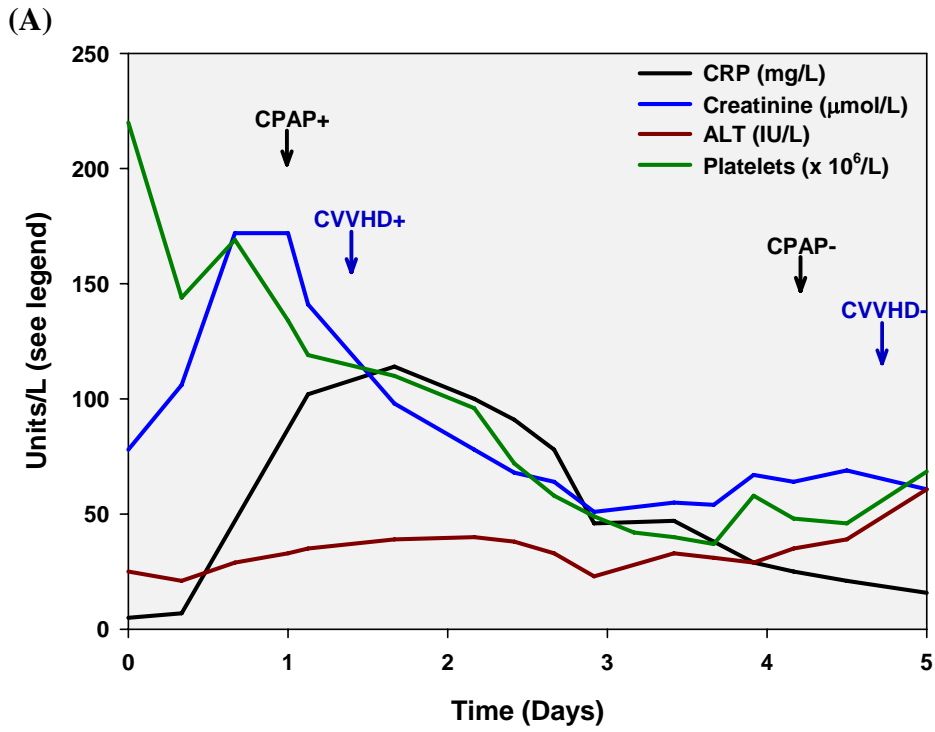
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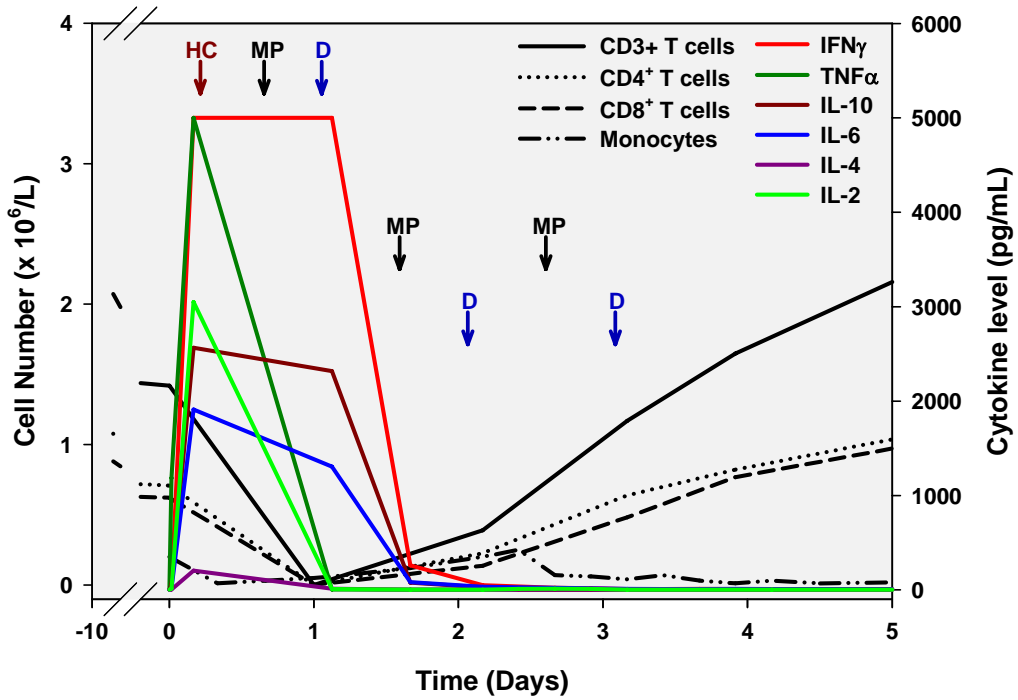


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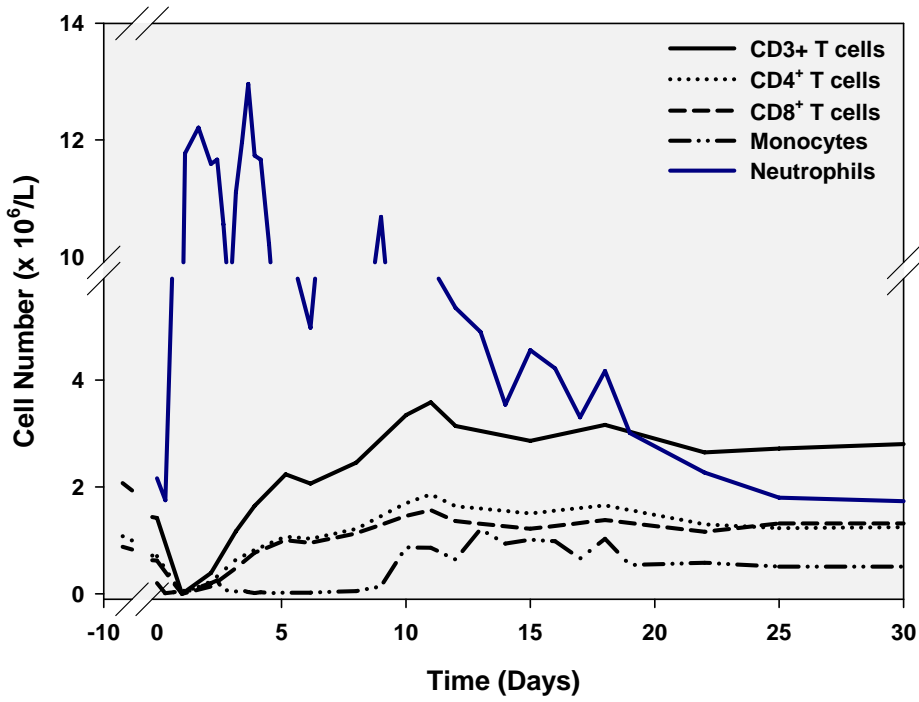


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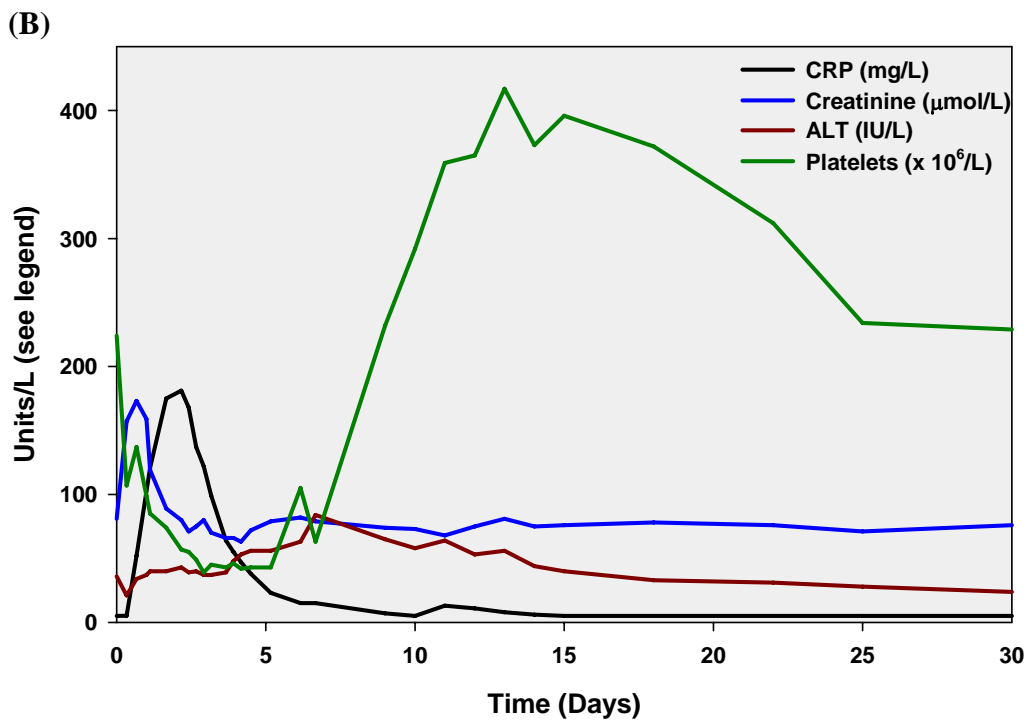
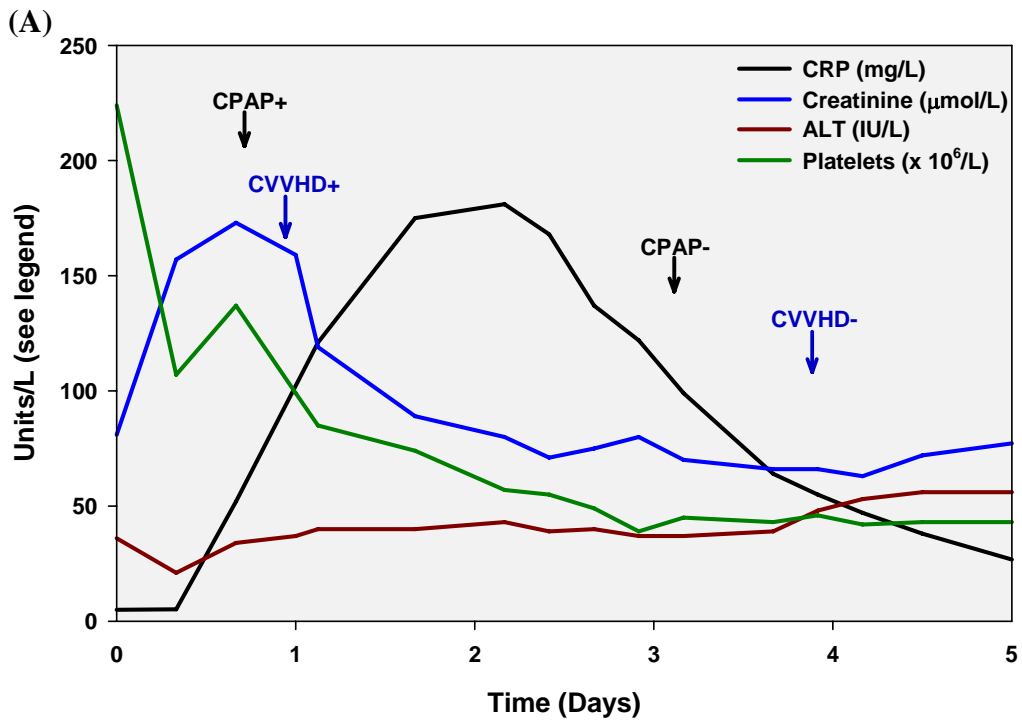
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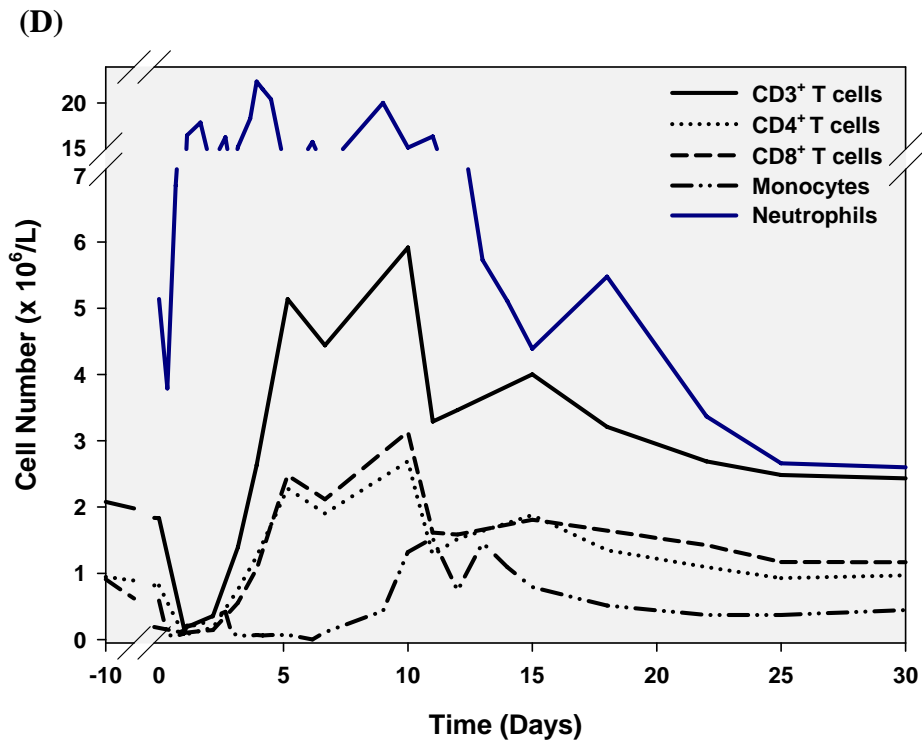
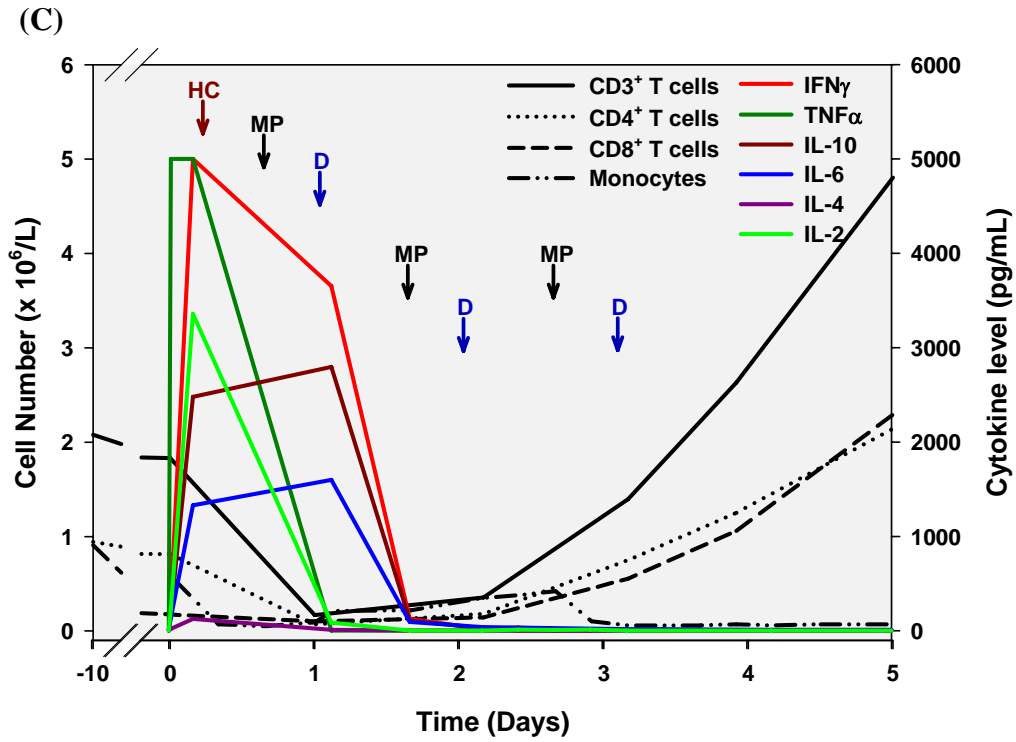
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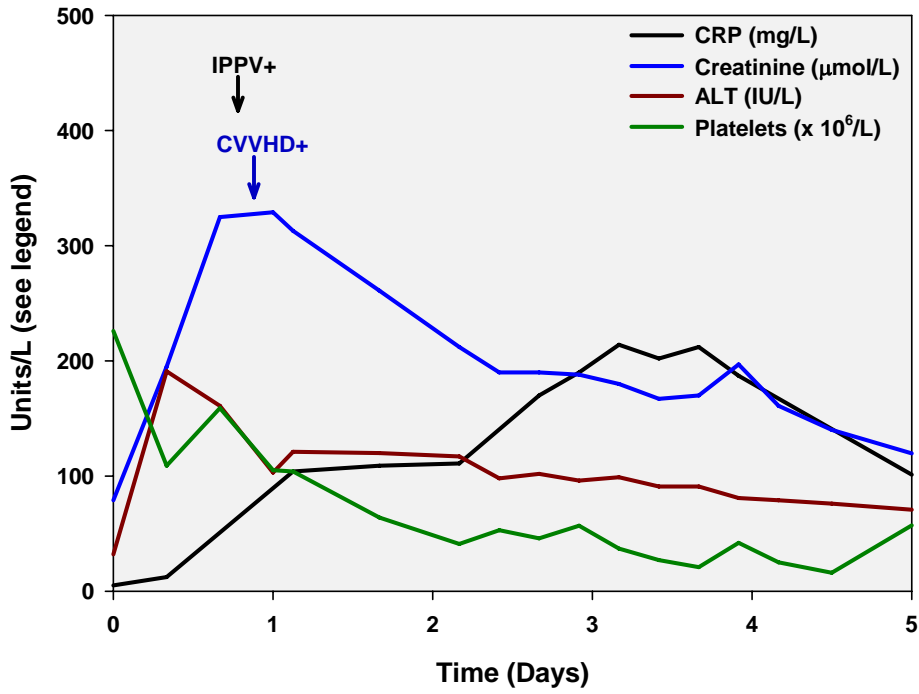


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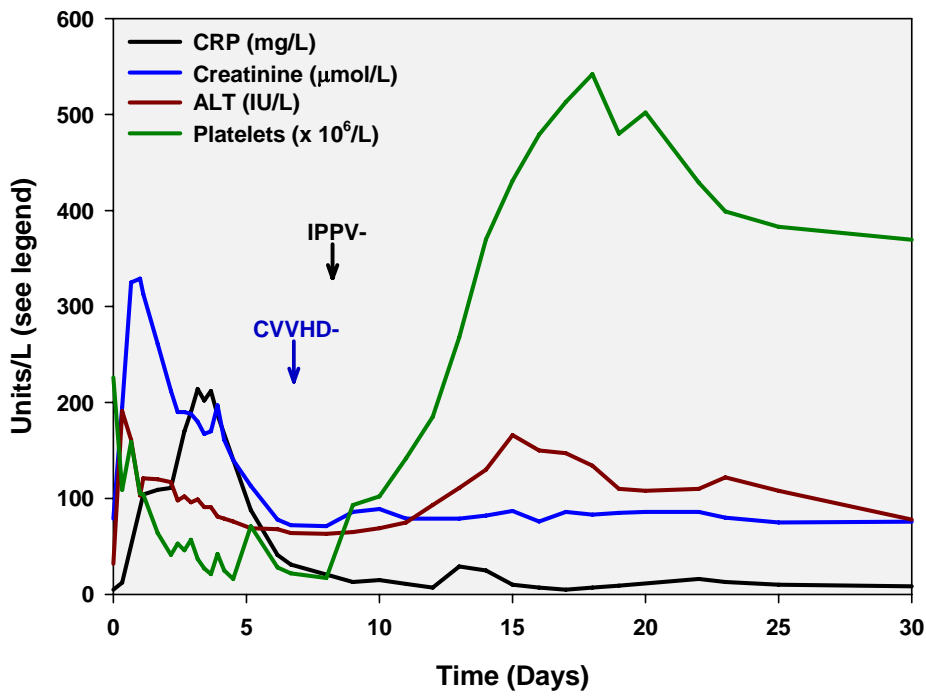


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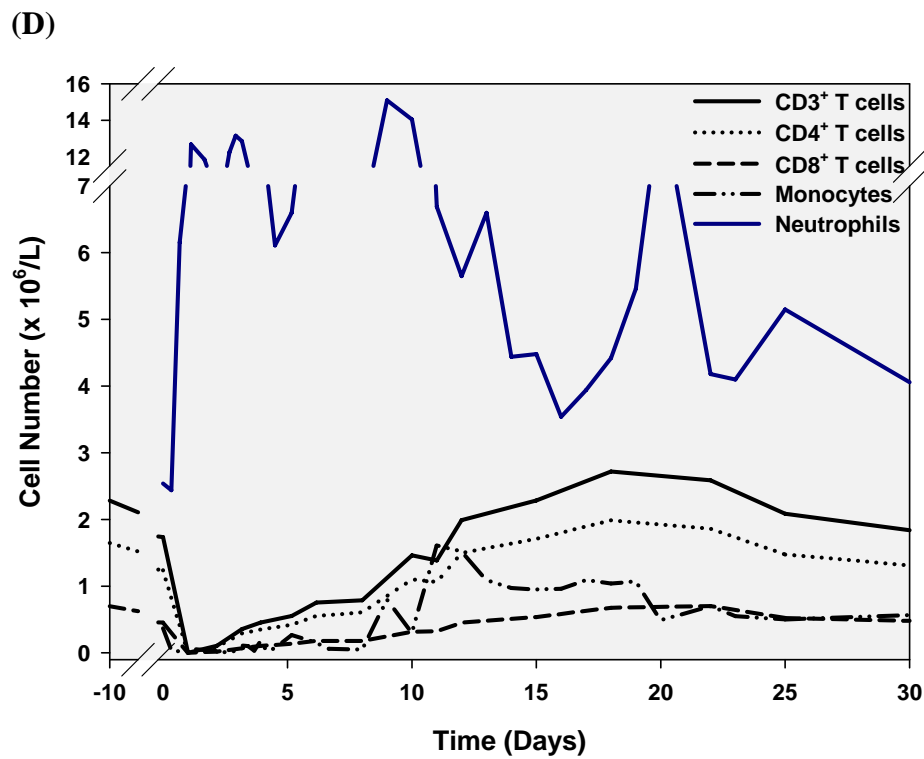
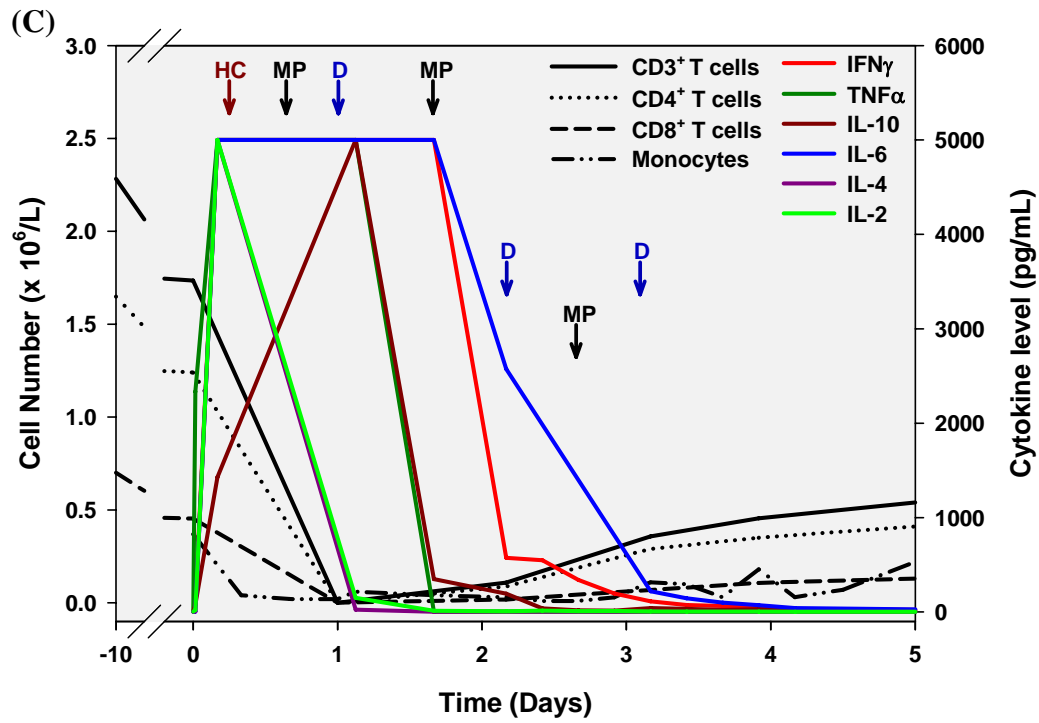
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(B)

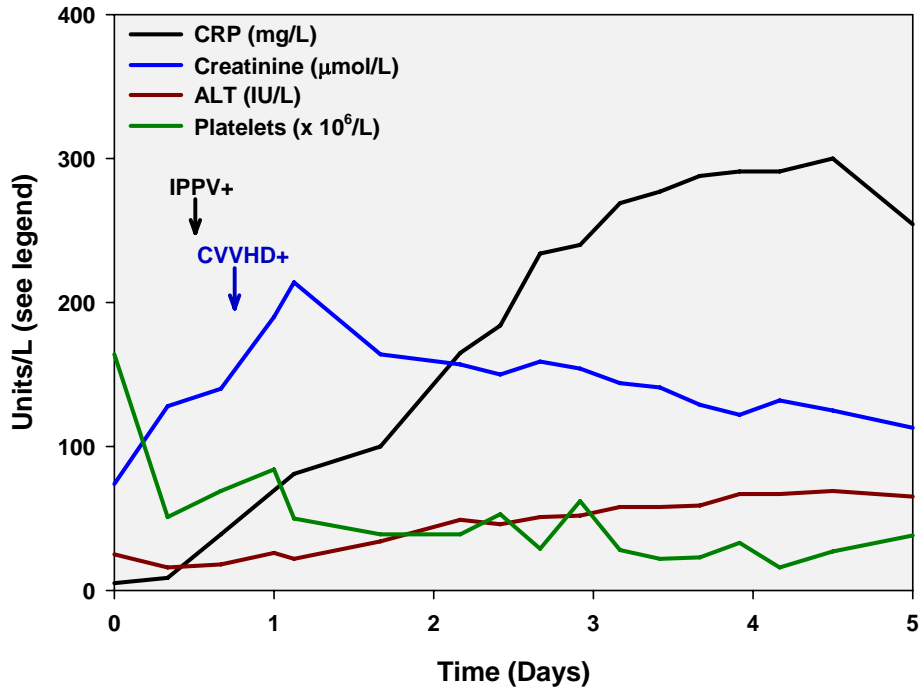


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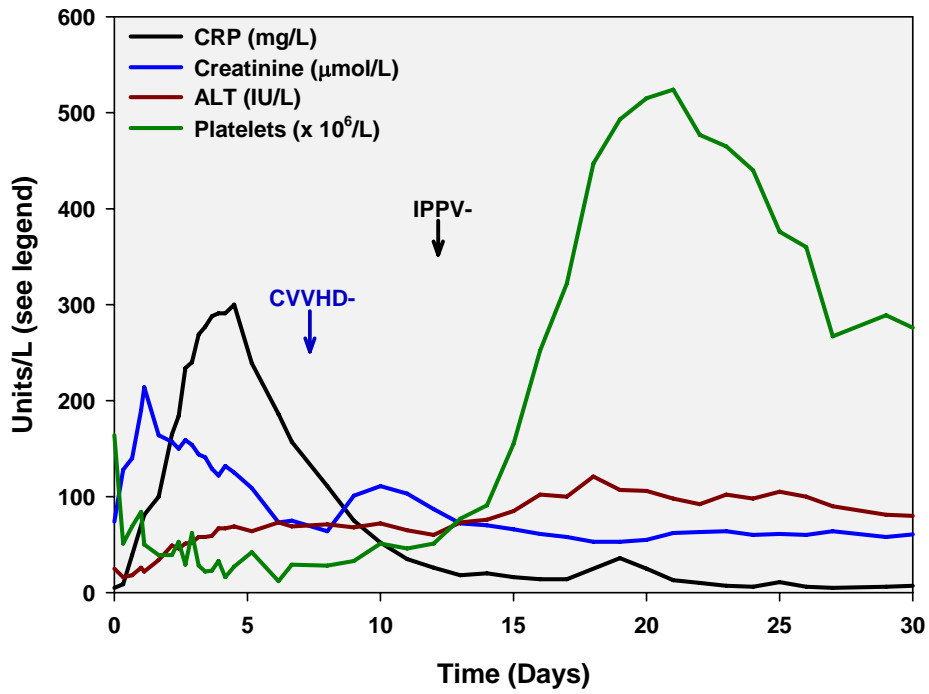


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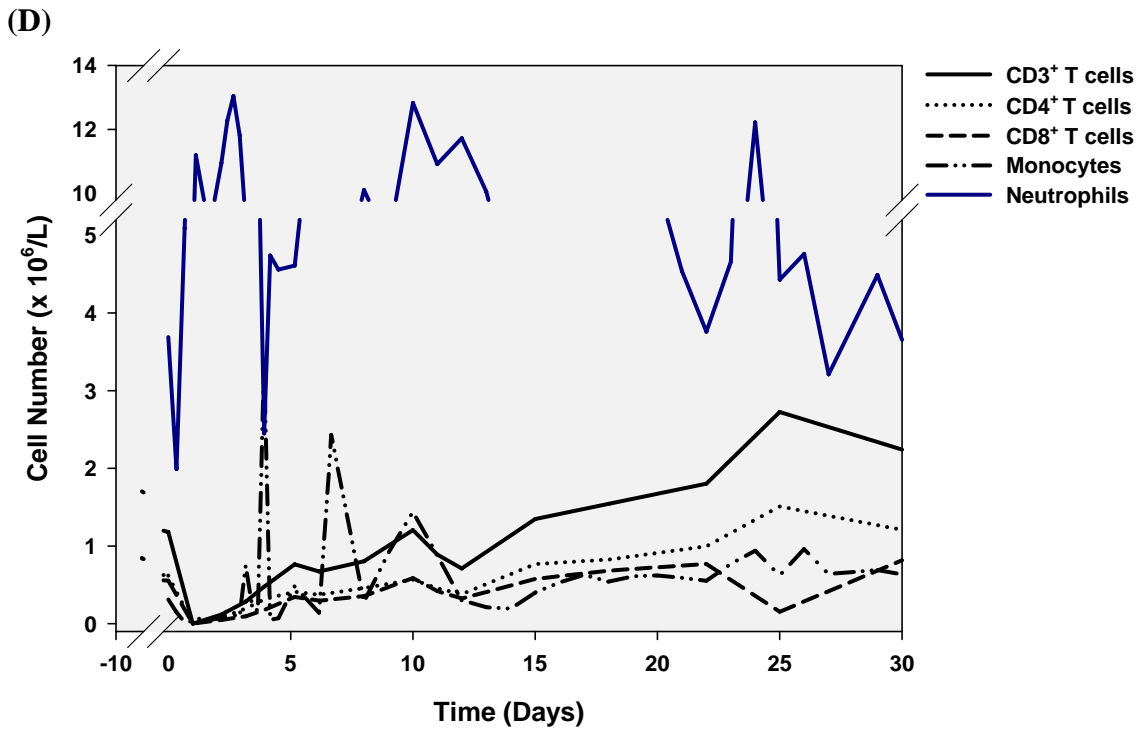
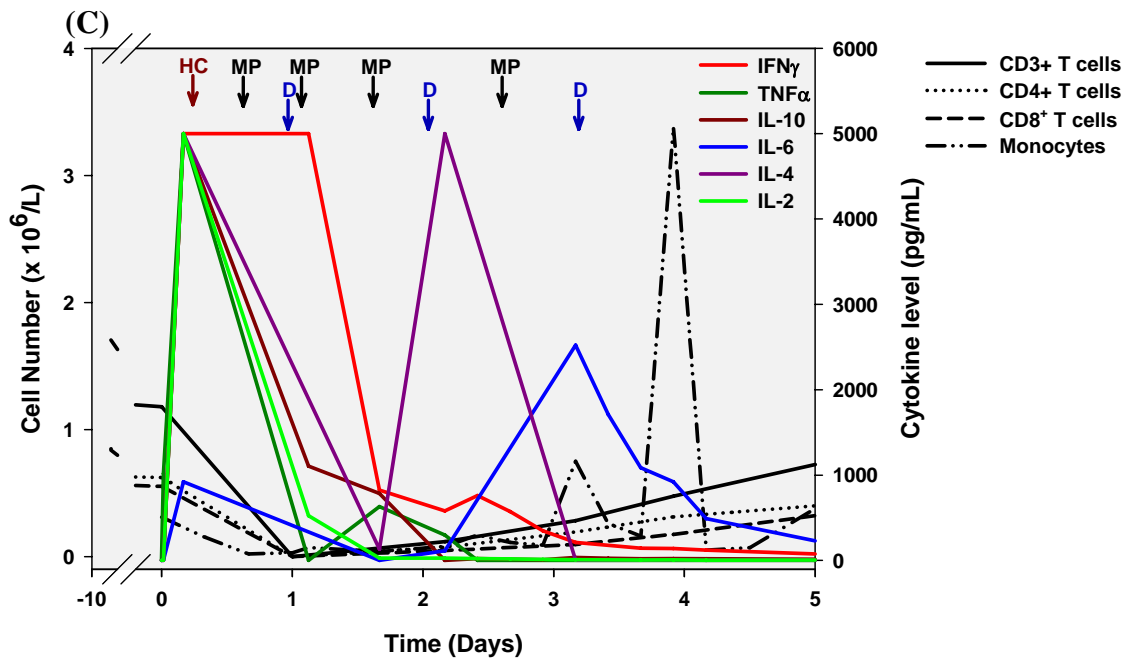
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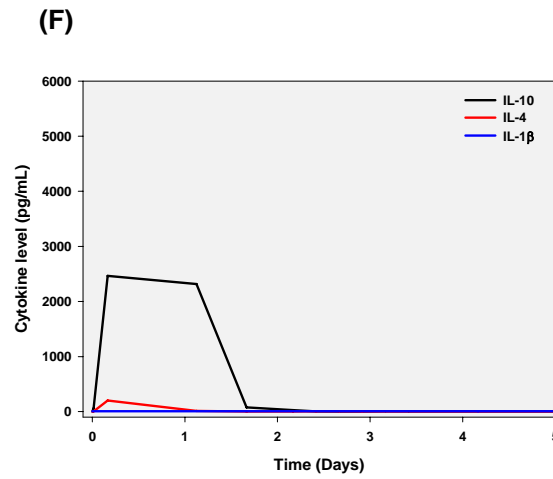
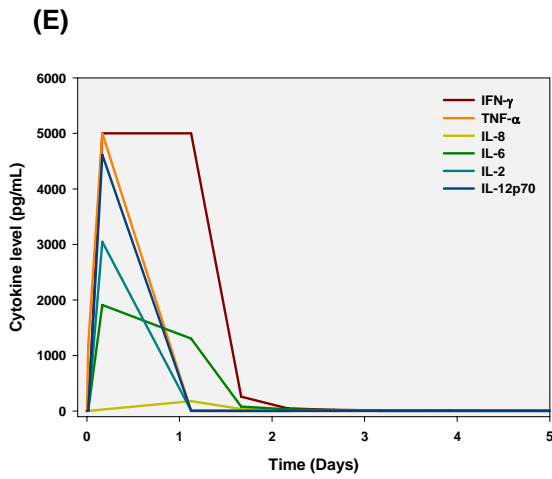
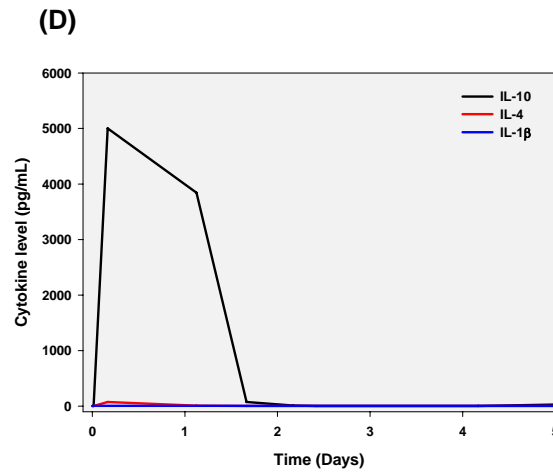
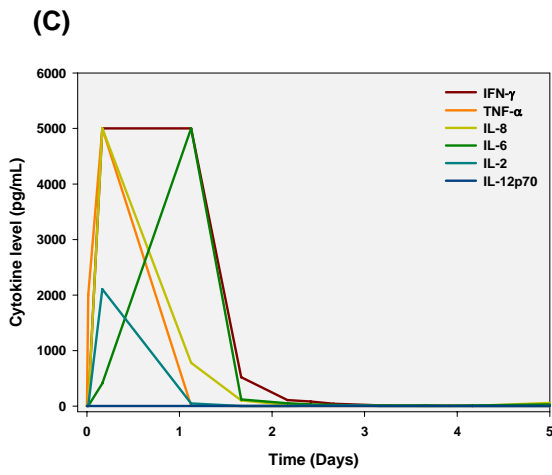
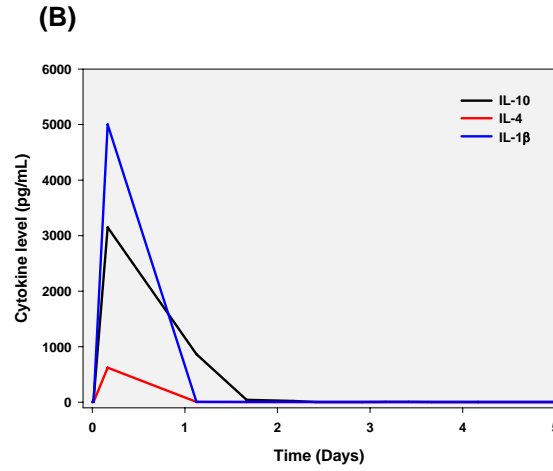
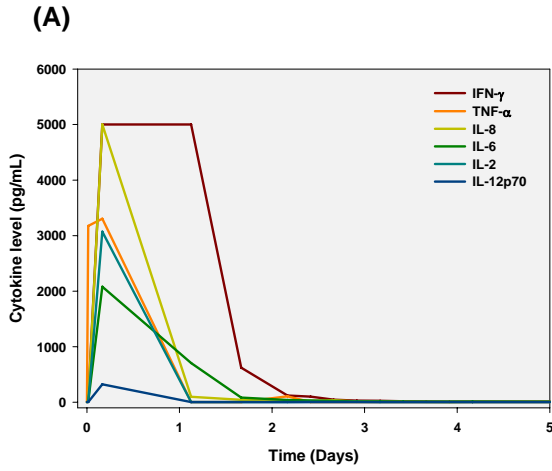
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Supplemental Figure 9

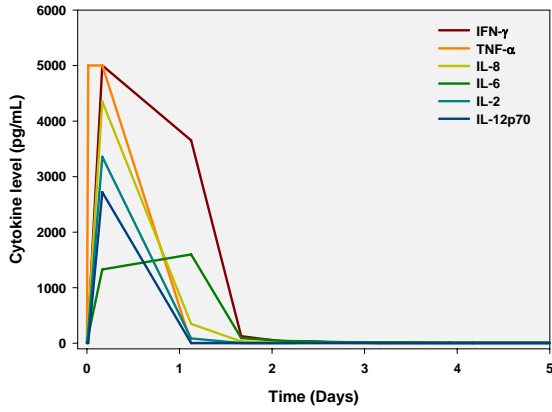


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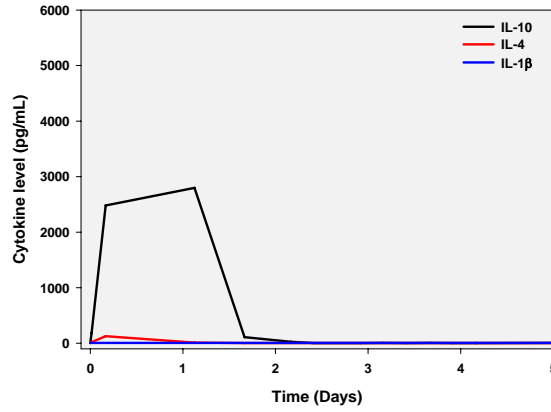


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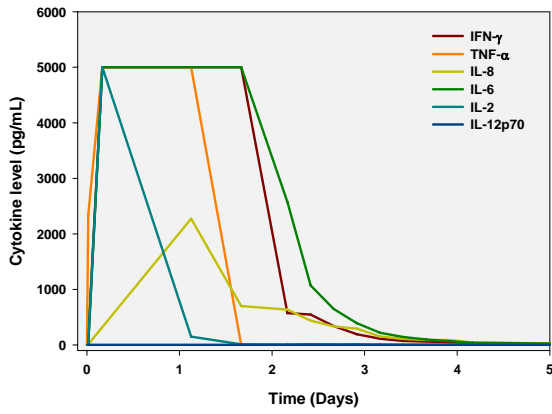
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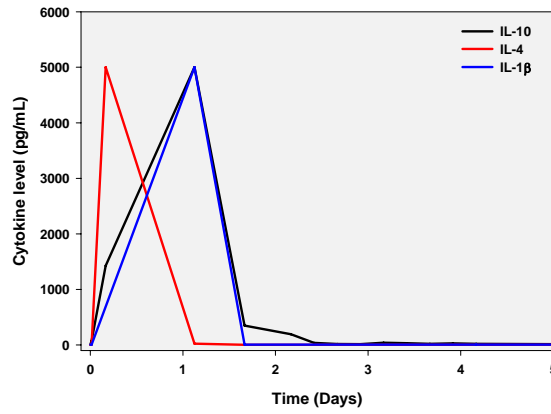
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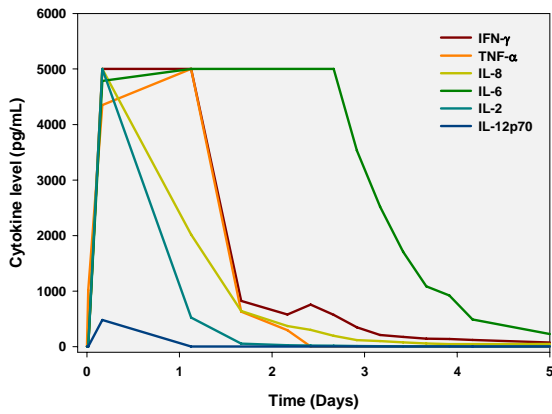
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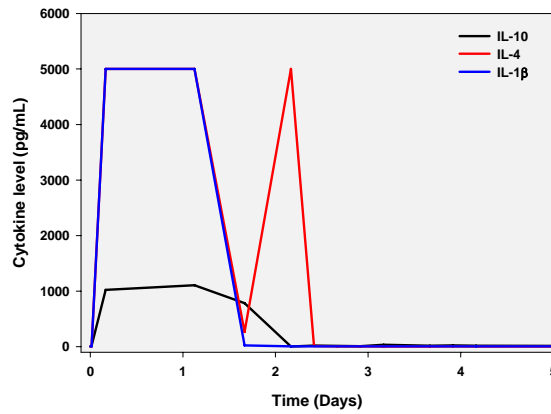
(J)



(K)



(L)



Supplemental Figure 10