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Choice of immunoassay to evaluate porcine cytokine levels

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Abstract

*Background***:** In order to adequately monitor cytokines in experimental models, currently available methods and commercially available kits should be compared.

Aim: To compare the plasma and tissue concentrations of IL-1β, IL-6, IL-8, IL-10, and TNF as a measure of systemic inflammation in septic pigs.

Methods: Cytokines were quantified from blood and tissue samples obtained at 0, 60, 120, 180, and 240 min, and in postmortem biopsies of the liver, kidney, lung, heart, and spleen from 26 anesthetized landrace pigs. (24 with experimental sepsis, two sham controls). Porcine-specific ELISAs (R&D) and multiplex (9-plex from Thermo Fischer, 13-plex from Millipore) immunoassays were compared.

Results: The assays differed for the different cytokines and between blood and tissue. In blood, the highest concentration of TNF and IL-6 was in ELISA, IL-1β equal in ELISA and 13-plex, IL-8 in 13-plex and IL-10 in 9-plex. In tissue, the highest concentration of TNF and IL-1β was in ELISA, IL-6 and IL-8 in 13-plex and IL-10 in 9-plex.

Conclusion: The choice of analysis impacts the quantified cytokine responses in porcine models. ELISA and multiplex techniques supplement each other and our data suggest which assays to use for the quantification of the different cytokines.

Keywords: Cytokines, ELISA, multiplex, porcine, sepsis

Introduction

Inflammation is the process initiated by the immune system to restore homeostasis when the host encounters threats such as invading microorganisms and tissue damage (Medzhitov, 2008). However, disseminated infection can trigger systemic inflammation and cause sepsis, and it is presumed that systemic infection triggers an initial “cytokine storm” (Huber-Lang et al., 2014). In spite of much research, specific therapies in sepsis are still missing.

Large-animal models in general and porcine models in particular serve as life-like systems for sepsis because of highly comparable anatomical and physiological properties (Swindle and Smith, 1998). To what extent the currently available methods and commercially available kits reflect systemic inflammation in the pig remains to be determined. Relatively few commercial kits are available for analysis of porcine samples, compared to the many available for murine and human samples.

The aim of this study was to compare the return concentrations for plasma and tissue cytokines in septic pigs as quantified by enzyme-linked immunosorbent assays (ELISA) and two different multiplex kits for the five central cytokines interleukin- (IL-) 1β, IL-6, IL-8, IL-10 and tumor necrosis factor (TNF), which are known to increase in sepsis.

Materials and methods

Blood and tissue samples

EDTA-plasma and serum samples were obtained from a total of 26 anesthetized landrace pigs of which 24 were subjected to experimental continuous intravenous

meningococcemia and 2 served as sham controls, as described in detail by Hellerud et al. (Hellerud et al., 2017). In brief, samples were obtained at 15 minutes prior to inoculation, at start of the bacterial infusion and then at 60, 120, 180, and 240 minutes. Postmortem biopsies of the kidney, liver, left lung, ventricles of the heart and spleen were collected and homogenized for cytokine quantification. The samples analyzed by Hellerud et al. were reanalyzed in thus study to compare the various immunoassays. Importantly, the experiments and the animal welfare were deemed satisfactory by the Norwegian Animal Research Authority of the Norwegian Food Safety Authority.

Immunoassays

Porcine ELISA kits from R&D (Minneapolis, MN), and two luminex-based assays: porcine 13-plex for Millipore (Billerica, MA), and porcine 9-plex from Invitrogen (Thermo Fisher Scientific Inc., Waltham, MA) were used. All analyses were carried out according to the manufacturer's kit instructions. Samples out of reading range were set to the upper detection limit, UDL. Blood samples were processed to either EDTA-plasma or serum. The IL-1β and IL-8 ELISA were carried out using serum, all other with EDTA-plasma, as recommended.

Statistics

Data was analyzed in GraphPad Prism 8 (GraphPad Software, San Diego, CA). Cytokine levels in blood samples were compared by two-way ANOVA or a mixed effects model if values were missing and then the Tukey's post-hoc test. $P < 0.05$ was considered significant. Blood sample data are represented as means with 95% confidence intervals. Cytokine levels in tissue samples were compared by one-way analysis of variance (ANOVA) or a mixed effects model if values were missing and

then the Tukey's post-hoc test. Non-normally distributed data were compared with the Friedman non-parametric test and Dunn's multiple comparisons test. Tissue sample data are represented as box and whisker-plots with median, quartiles, min to max, and all points shown.

Results and discussion

Blood samples (fig. 1)

Generally, inoculated pigs had increasing concentrations of all five cytokines during the observation period. Particularly, IL-1β, IL-6 and IL-8 continued to increase throughout the experiment. ELISA and the 13-plex yielded variable increases for the different cytokines, whereas the 9-plex differed by reporting lower concentrations, except for IL-10, where it was the only assay that detected an increase.

The IL-1β concentration peaked at 14.4 ng/mL in the ELISA and at 17.3 ng/mL in the 13-plex. The 9-plex detected virtually no increase (0.002 to 1.2 ng/mL). The IL-6 concentration peaked at 35.9 ng/mL in the ELISA, at 7.8 ng/mL in the 13-plex and at 0.97 ng/mL in the 9-plex. The TNF concentration peaked at 82.0 ng/mL in the ELISA, at 21.1 ng/mL in the 9-plex and at 14.7 ng/mL in the 13-plex. The IL-8 concentration peaked at 42.7 ng/mL in the 13-plex assay, at 29.3 ng/mL in the ELISA and 2.0 ng/mL in the 9-plex. IL-8 concentrations were above the upper detection limit for all samples at 180 and 240 minutes when analyzed with the 13-plex. The IL-10 concentrations did not change over time when quantified by ELISA and 13-plex, but the baseline values were significantly different, 0.08 ng/mL in the ELISA compared to 1.1 ng/mL in the 13-plex. Notably, samples analyzed with the 9-plex revealed increased IL-10 concentrations, from a baseline average value of 0.03 ng/mL where 17 samples were below the reading range of 0.001 ng/mL, to peaking at 1.4 ng/mL.

Tissue samples (fig. 2)

Postmortem biopsies of the kidney, liver, left lung, ventricles of the heart and spleen were collected and homogenized for quantification of the same cytokines measured in

blood. Interestingly, each assay's return values clearly differed between blood and tissue. However, for each cytokine one of the three assays consistently returned higher values than the others, regardless of the organ studied.

The all-organ, average IL-1β concentration was generally higher in ELISA (19.8 ng/mL) and 13-plex (10.2 ng/mL) compared to the 9-plex (1.8 ng/mL). ELISA quantified the highest IL-1β levels in kidney, liver, lung, and spleen whereas the 13-plex quantified marginally higher levels in the heart. The all-organ, average IL-6 concentration was higher when quantified by 13-plex (5.1 ng/mL) compared to 2.2 ng/mL for the ELISA and 1.5 ng/mL for the 9-plex. Per organ, 13-plex consequently yielded values 2-3 times higher than results from ELISA and 9-plex. The all-organ, average TNF concentration was higher in ELISA (5.5 ng/mL) compared to 13-plex (0.7 ng/mL) and 9-plex (0.9 ng/mL). ELISA showed consistently higher levels in all organs, although the differences in the kidney samples were non-significant. The all-organ, average IL-8 concentration was also significantly higher when quantified by 13-plex (41,504 pg/mL) compared to the 9-plex (2054 pg/mL) and the ELISA (5602 pg/mL). All organs, except for the spleen showed the highest levels of IL-8 in the 13-plex. The all-organ, average IL-10 concentration was considerably higher in samples analyzed with 9-plex (0.2 ng/mL) compared to ELISA (0.02 ng/mL) and 13-plex (0.1 ng/mL).

Discussion

Our study identifies large method-dependent discrepancies in the levels of cytokines detected after experimental bacteremia. This is both related to the particular cytokine quantified and to the sample source. In blood, ELISA and 13-plex showed comparable and consistent increases in all cytokines except IL-10 where only 9-plex showed a time-dependent increase, as would be expected in the clinical setting. In tissue samples, 13-plex reported higher levels of IL-8 and IL-6 whereas 9-plex reported higher levels of IL-10 and ELISA higher levels of TNF and IL-1β. Yet the pattern of increased concentrations between methods were very consistent both for blood and tissue samples.

It was beyond the scope of this study to test the validity of the different kits, although it could be questioned with, at times, large differences. However, all kits are approved by the National Institute of Biological Standards and Control and analyses were carried out according to the kit instructions. The 13-plex and ELISA kits document a near 100% spiked recovery rate, whereas the manufacturers for the 9-plex would not share their spiked recovery data. The absolute quantities reported may be uncertain, especially in the tissue samples, and the protein bioactivity is unmeasured, but different absolute values is a well-known phenomenon in the laboratory, and has been documented before (Khan et al., 2004). To name a few things that influence immunoassay performance, manufacturers use different antibodies, absolute concentrations given by the assay standard-curves dictates assay accuracy, and many antibodies are made for reactivity with one species and then used in assays for other species due to documented cross-reactivity. The degree of cross-reactivity varies, and calculations

based on cross-reactivity may be difficult in lack of reliable controls from the original species.

For studies on human material, some relatively outdated studies presume ELISA and multiplex kits equal or multiplex even superior (Camilla et al., 2001; Chen et al., 1999). However, newer studies, as reviewed in (Tighe et al., 2015), question the use of multiplex before thorough validation as no standardized regulatory guidelines for the validation of multiplex biomarker assays currently exists. Approval typically rests on the capacity of the manufacturer to provide data, inferring adequacy, as is the case for the multiplexes here. Even so, this is the first study to compare ELISA and multiplex on porcine material. Although our findings are limited to the kits tested, we have summarized which assay generally identifies some, and preferably, the most cytokine in response to an inflammatory stimulus, and thus increases the resolution of the model system, in Table 1.

Conclusion

ELISA, 13-plex and 9-plex returned different quantities of IL-1β, IL-6, IL-8, IL-10 and TNF when measured in blood and biopsy replicates from experimentally septic pigs. Depending on the sample type and the cytokine in question, we recommend ELISA for the quantification of IL-6, TNF, and IL-1β in plasma, and for TNF and IL-1β in tissue homogenates. For IL-8 and IL-1β in blood, and IL-6 and IL-8 in tissue homogenates, we recommend reagents from the 13-plex, and lastly, for IL-10 in both blood and tissue homogenates, we recommend reagents from the 9-plex.

Statement on competing interests

The authors deny any competing interests.

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Recommended method for quantification of porcine cytokines in plasma and tissue

Cytokine	Plasma[1]			Tissue		
	ELISA	9-plex	13-plex	ELISA	9-plex	13-plex
TNF	X			X		
IL-1β	X[2]		X[2]	X		
IL-6	X					X
IL-8			X			X
IL-10		X			X	

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225 [1] For the ELISA, IL-1β and IL-8 were quantified in serum, according to kit instructions

226 [2] ELISA and 13-plex were comparable for IL-1β in blood samples

Figure legends

Figure 1. Plasma concentrations of IL-1β[1], IL-6 and TNF. The hourly concentrations of IL-1β, IL-6 and TNF from 0 to 4 hrs were quantified from n = 24 experimentally septic pigs and n = 2 sham animals using ELISA (open triangle), 9-plex (open square) and 13-plex (open circle). SHAM animals are shown as dotted lines. Results are shown as means with 95% confidence intervals. Samples were compared by two-way ANOVA or a mixed effects model if values were missing and then the Tukey's post-hoc test. $P < 0.05$ was considered significant. # = ELISA vs. 9-plex, † = ELISA vs. 13-plex, * = 9-plex vs. 13-plex. [1]The IL-1β ELISA was done in serum, as instructed.

Figure 2. Plasma concentrations of IL-8[2] and IL-10. The hourly concentrations of IL-8 and IL-10 from 0 to 4 hrs were quantified from n = 24 experimentally septic pigs and n = 2 sham animals using ELISA (open triangle), 9-plex (open square) and 13-plex (open circle). SHAM animals are shown as dotted lines. Results are shown as means with 95% confidence intervals. Samples were compared by two-way ANOVA or a mixed effects model if values were missing and then the Tukey's post-hoc test. $P < 0.05$ was considered significant. # = ELISA vs. 9-plex, † = ELISA vs. 13-plex, * = 9-plex vs. 13-plex. [2]The IL-8 ELISA was done on serum, as instructed.

Figure 3. Average cytokine concentrations in tissue homogenates. The postmortem concentrations of *IL-1β, IL-6, TNF, IL-8, and IL-10* were quantified in kidney, liver, lung, heart and spleen biopsies from n = 24 experimentally septic pigs and n = 2 sham animals using ELISA, 9- and 13-plex. Results are represented as all-organ, pooled values in box and whisker-plots with corresponding SHAM values (grey circles) adjacent showing median, quartiles, min to max, and all points shown. Samples were

compared by two-way ANOVA or a mixed effects model if values were missing and then the Tukey's post-hoc test. $P < 0.05$ was considered significant. # = ELISA vs. 9-plex, † = ELISA vs. 13-plex, * = 9-plex vs. 13- plex.

Fig. 1

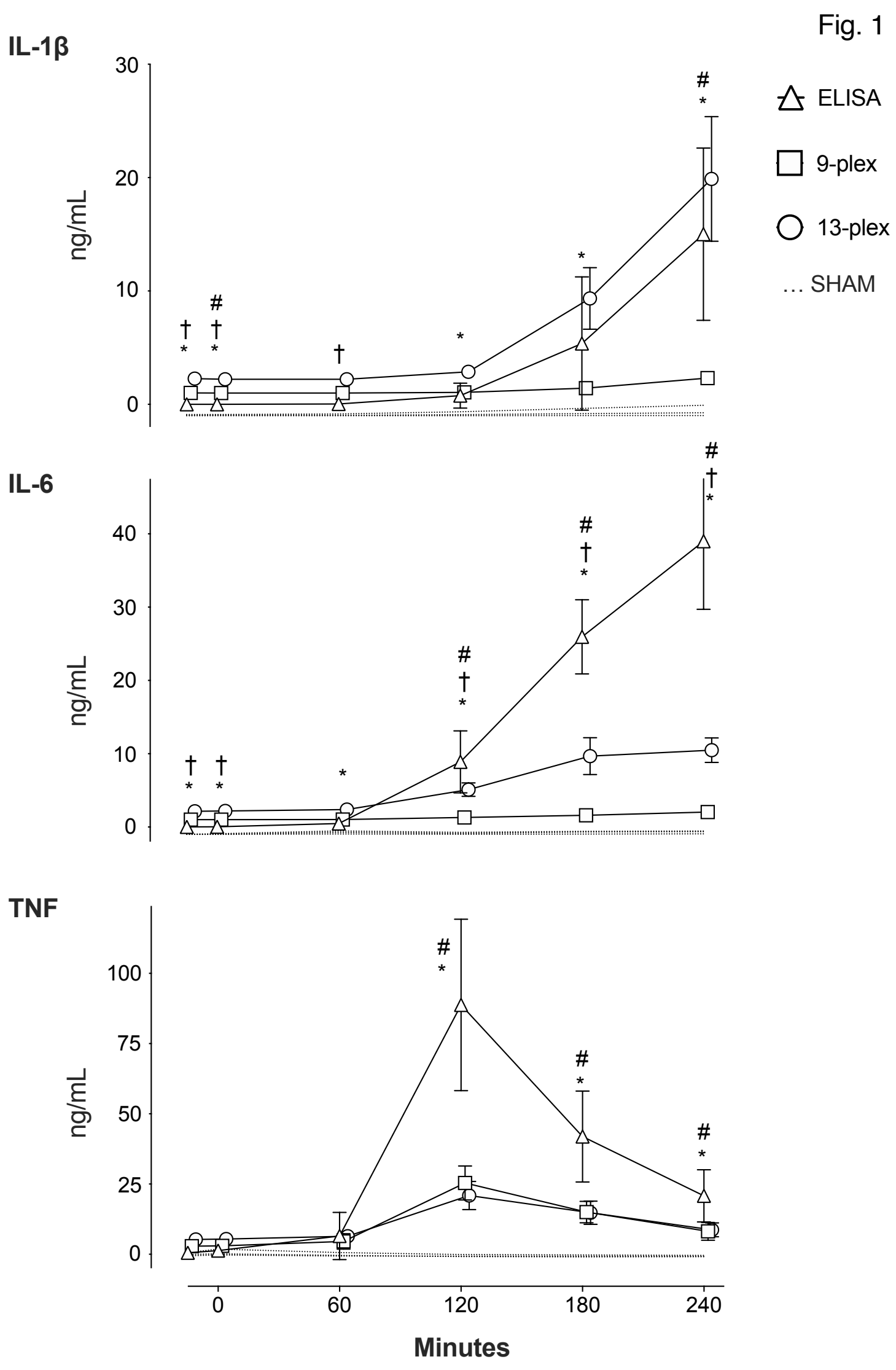
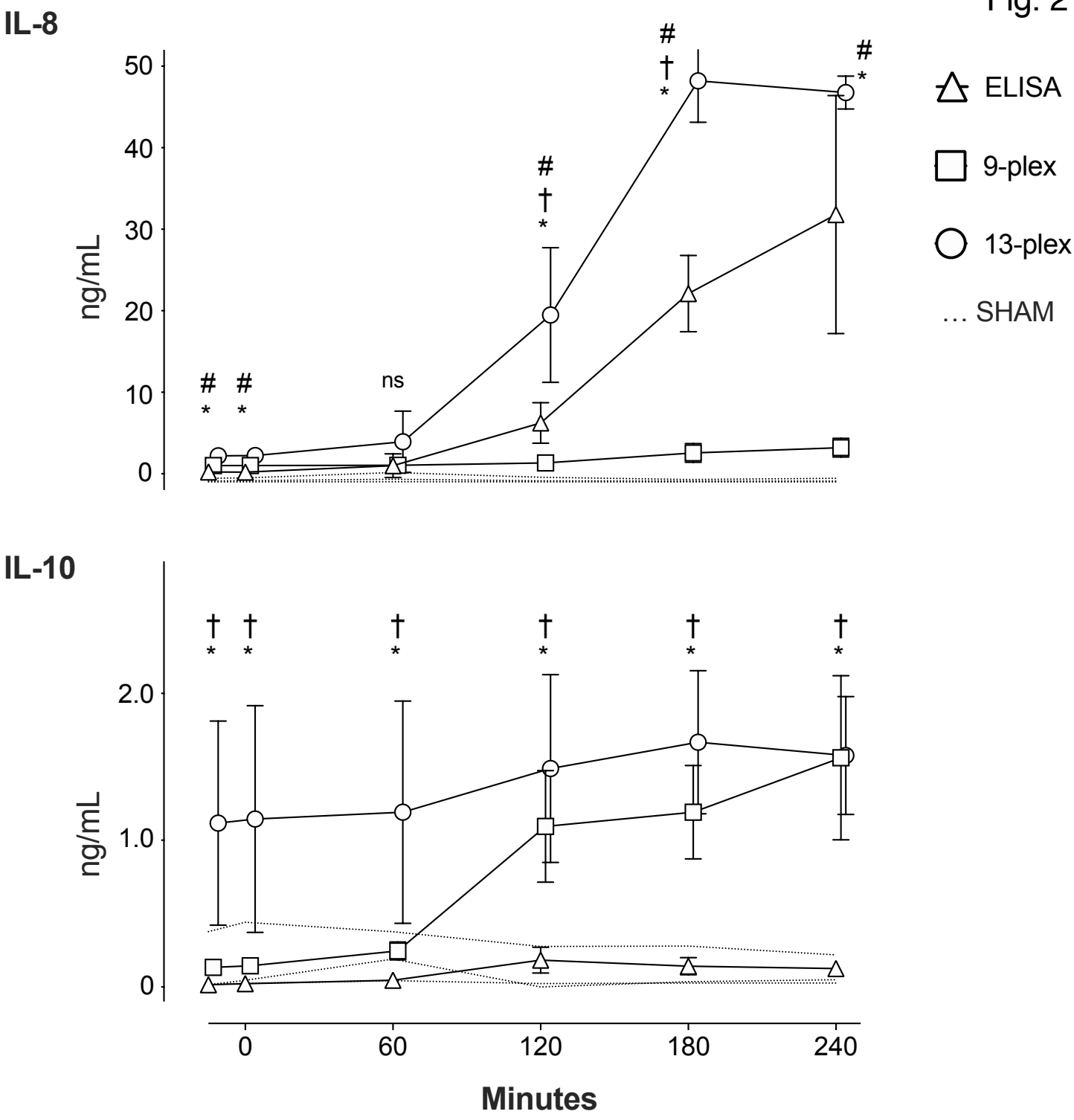

IL-1β
IL-6
TNF
ng/mL
Minutes
0
60
120
180
240
ELISA
9-plex
13-plex
… SHAM

Fig. 2


IL-8
50
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30
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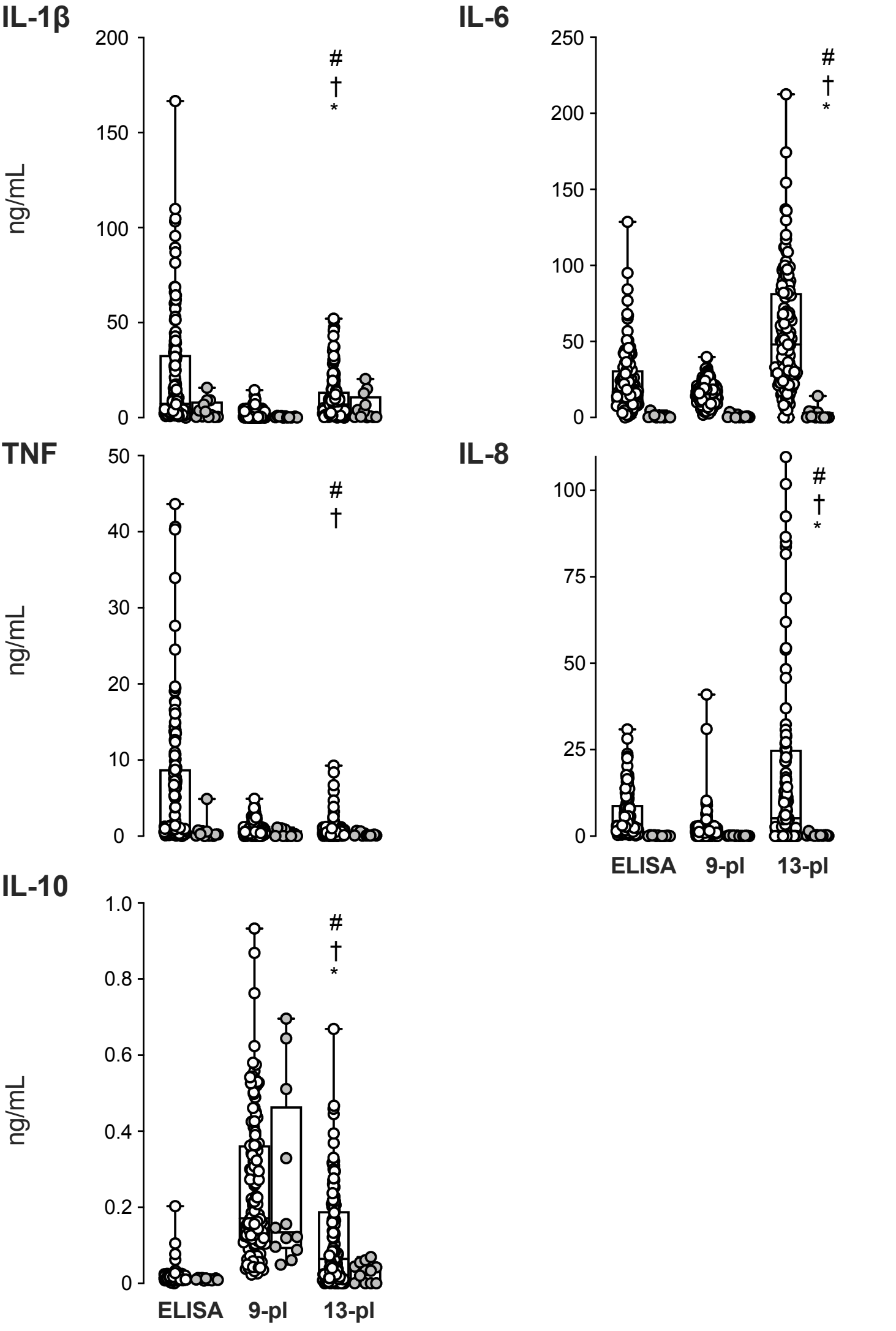
IL-1β
IL-6
TNF
IL-8
IL-10
ng/mL
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150
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ELISA
9-pl
13-pl