Time Course of Signaling Profiles of Blood Leukocytes in Acute Pancreatitis and Sepsis

Antti Turunen[a] (antti.turunen@hus.fi), Antti Kuuliala[b] (antti.kuuliala@helsinki.fi), Anne Penttilä[a] (anne.penttila@hus.fi), Kirsi-Maija Kaukonen[c] (maija.kaukonen@hus.fi), Harri Mustonen[a] (harri.mustonen@helsinki.fi), Ville Pettilä[c] (ville.pettila@hus.fi), Pauli Puolakkainen[a] (pauli.puolakkainen@hus.fi), Leena Kylänpää[a] (leena.kylanpaa@hus.fi) and Krista Kuuliala[b] (krista.kuuliala@helsinki.fi)

[a]Abdominal Center, Department of Abdominal Surgery, Helsinki University Hospital and University of Helsinki, Helsinki, Finland; [b]Bacteriology and Immunology, Helsinki University Hospital and University of Helsinki, Helsinki, Finland; [c]Department of Anaesthesiology, Intensive Care and Pain Medicine, University of Helsinki and Helsinki University Hospital, Helsinki, Finland

Corresponding author: Antti Turunen (antti.turunen@hus.fi)

Word Count: 3736

Abstract

Activation of intracellular signaling pathways in circulating leukocytes represents an early step in systemic immune-inflammatory response occurring e.g. in acute pancreatitis (AP) and sepsis. Previously, we found aberrations in the phosphorylation of leukocyte signaling proteins in patients with sepsis or AP (measured <48 hours from hospital admission) resembling each other and associating with AP severity. Of these patients, those with sepsis or severe AP complicated by persistent organ dysfunction (OD+, n=17) and patients with moderately severe AP (OD-, n=6) were followed up in this study by measuring the phosphorylations at two additional time points (2-4 and 5-8 days after the initial sample) using phosphospecific whole blood flow cytometry. 28 healthy subjects served as controls (HC). Constitutive STAT3 phosphorylation (pSTAT3) declined in monocytes and neutrophils of OD-/OD+ and in lymphocytes of OD+ and remained higher in OD- than HC. Monocytes of OD-/OD+ showed low pSTAT3 and pSTAT1 levels in response to IL-6 through follow-up. Monocyte pNF-κB levels in response to TNF, LPS and *E. coli* in OD+, to *E. coli* in OD-, and lymphocyte pNF-κB levels in response to TNF in OD- increased during follow-up but remained lower than in HC, and neutrophil pNF-κB levels in response to TNF declined in OD-. Phorbol myristate acetate+Ca^{2+} ionophore-stimulated pERK1/2 decreased in neutrophils of OD-/OD+. To conclude, in patients with moderately severe or severe AP or sepsis, improvement and molecular events contributing to OD can be assessed at the level of blood leukocyte signaling.

Keywords: Pancreatitis, Sepsis, Leukocytes, Flow cytometry, Signal transduction

Introduction

Acute pancreatitis (AP) is a relatively common disease resulting from alcohol abuse, or biliary or idiopathic reasons. AP is usually mild and self-limiting, but it can also be locally or systemically complicated and develop into a severe systemic inflammation with dysfunction of vital organs. Systemic inflammation is considered to involve an early proinflammatory stage which is in turn counteracted by the release of anti-inflammatory cytokines that can lead to excessive immunosuppression and potentially, secondary infections [1]. Organ dysfunction (OD) enduring less than 48 hours is determined as transient, whereas persistent OD endures over 48 hours and is associated with an up to 50% mortality rate [2,3].

In order to optimize the treatment of patients who develop OD, the immune status and appropriate immunomodulation strategy should be determined as early as possible [4,5]. The current clinical scoring systems such as acute physiology and chronic health evaluation (APACHE) II or measuring acute phase reactants have been shown to have only limited use in predicting clinical outcome [5-7]. We have previously shown that circulating levels of IL-8 and hepatocyte growth factor (HGF) predict the risk of developing OD during hospital stay in AP patients [8]. However, the generation of cytokines requires protein synthesis and is therefore time-consuming. This process is preceded by activation of intracellular signaling pathways in leukocytes [9]. Indeed, the molecular mechanisms of the development of systemic inflammation and immunosuppression comprise various intracellular signaling pathways, suggesting that these can provide biomarkers for evaluating the course of the systemic inflammatory process. We have previously shown aberrant phosphorylation of nuclear factor-κB (NF-κB), signal transducer and activator of transcription (STAT) 1 and STAT3 and extracellular signal -regulated kinase (ERK) 1/2 in leukocytes of patients with severe AP [10-12]. Recently, we also showed that signaling aberrations in sepsis with OD

resemble those of severe AP with OD, associate with the severity of AP and may provide novel markers in predicting which AP patients will develop OD [13].

This far, studies are lacking in which the signaling markers are determined during the hospitalization period in patients with AP or sepsis. Hence, it is not known whether these markers could help in assessing the predominance of proinflammatory or immunosuppressive mechanisms during the disease course, if they are associated with persistent organ dysfunction and if they reach the levels of healthy controls rapidly or remain aberrant for a longer time. Knowing these aspects can ultimately affect successful treatment decisions. In the current study we followed the time course of the leukocyte phosphorylation markers in the patients from [13] who had moderately severe AP without persistent OD or sepsis or severe AP complicated with persistent OD.

Materials and methods

Patients

The original study [13] included 18 patients with AP and 14 with sepsis. Of the original AP patients, only those who had moderately severe (n=6) or severe (n=3) AP were included in the current study because the patients with mild AP (n=9) recovered rapidly and were not hospitalized at the time of the subsequent blood sampling points. The sepsis patients were enrolled at the intensive care unit (ICU) and the AP patients at the Emergency Department at Helsinki University Hospital. Twenty-eight healthy volunteers served as a control group (age 24-68, eight men and 19 women). All patients and control subjects were recruited between February 2010 and May 2012. The study was approved by the Surgical Ethical Review Board of the Joint Authority for the Hospital District of Helsinki and Uusimaa. Written informed consent was obtained from each patient or his/her next of kin.

AP was diagnosed as described in the revised Atlanta Classification: epigastric pain typical of AP, plasma amylase level more than three times the upper reference limit and/or typical findings in computed tomography (CT). The severity of AP was retrospectively classified according to the revised Atlanta Classification as either mild with no local complications, moderately severe (transient OD lasting less than 48 h and/or local complications and/or worsening of chronic illness) or severe with persistent OD (over 48 h). OD was assessed to be present if the patients Modified Marshall Score (MMS) was ≥2 [14]. Exclusion criteria were more than two previous AP attacks and the onset of symptoms more than 72 h before admission.

Sepsis patients were recruited within 48 h of admission to hospital. Exclusion criteria were known malignancy, immunological deficiency or hospital acquired septic infection. Three patients were assessed to have OD with an acute increase in Sequential Organ Failure Assessment (SOFA) score of two or more points and 11 patients had septic shock with the need of vasopressor to maintain mean arterial pressure ≥ 65 mmHg and serum lactate level <2 mmol/l [15]. Thus, all sepsis patients had persistent OD.

Blood sampling

A 4-ml venous blood sample was obtained within 48 h after admission to hospital as described previously [13]. A second sample was taken 2-4 days and third 5-8 days after the first sample if the patient was still in hospital. The samples were collected into Falcon polypropylene tubes (Becton Dickinson, Lincoln Park, NJ, USA) and supplemented with 1:10 pyrogen-free citrate phosphate dextrose (Baxter Health Care Ltd, Norfolk, UK). They were kept at room temperature until stimulations within three hours of sampling. Each sample was compared to a sample obtained from a healthy control within two weeks.

Biological agents and leukocyte agonists

We used fluorescein isothiocyanate (FITC)-conjugated anti-CD14 (clone MφP9 IgG_{2b}), phycoerythrin (PE)-conjugated anti-HLA-DR (clone L243, IgG_{2a}) and its isotype control (mouse IgG_{2a}) for surface marker staining. AlexaFluor647-conjugated anti-pNF-κB p65 (pS529) (clone K10-895.12.50, IgG_{2b}), anti STAT1 (pY701) (clone 4a, IgG_{2a}), anti-ERK1/2 (pT202/pY204) (clone 20A, IgG_1) and PE-conjugated anti-STAT3 (pY705) (clone 4/P-STAT3, IgG_{2a}) were used for intracellular phosphospecific labelling. The antibodies were from Becton Dickinson Biosciences (San Jose, CA).

Whole *Escherichia coli* (*E. coli*) bacteria were from the National Institute of Health and Welfare (Helsinki, Finland). They were grown in brain-heart infusion medium, pelleted and washed twice. A diluted culture was made to quantify viable bacteria. They were pelleted, resuspended in glycerol-tryptone soya broth medium and stored at -70°C. The stimuli were diluted in PBS before use. Tumor necrosis factor (TNF) and IL-6 were from R&D (Minneapolis, MN, USA). Phorbol-12-myristate-13-acetate (PMA), CA^{2+}-ionophore A_{23187} and *E. coli* O111:B4 lipopolysaccharide were from Sigma (St. Louis, MO, USA).

Ex vivo stimulation and immunolabeling

The whole blood samples were stimulated as described previously [13]. The blood samples were divided into 100 µl aliquots in Falcon polystyrene tubes (Becton Dickinson). The tubes were supplemented with TNF (final concentration 10 ng/ml) or IL-6 (100 ng/ml) for 5 minutes, or with LPS (100 ng/ml) or *E. coli* (50 cells/leukocyte) for 10 minutes at +37°C. Reference tubes were left without stimulus.

Next leukocyte fixation, red cell lysis and leukocyte permeabilization were performed according to BD Phosflow Protocol III for Human Whole Blood (http://www.bdbiosciences.com/pharmingen/products/display_product.php?keyID=94#3). Then the samples were washed with BD Pharmingen Stain Buffer and resuspended in 100 µl

of the buffer. Aliquots of AlexaFluor647- and PE-labelled phosphospecific monoclonal antibodies were added. The samples were then incubated for 20 min in the dark at room temperature and then washed and resuspended in 300 µl of the buffer. They were stored in the dark on ice. Flow cytometry was done within three hours.

The proportion of HLA-DR-positive monocytes was determined as described previously [16].

Flow cytometry

Flow cytometry was done as described previously [13]. Data acquisition and analysis were done by FACSCanto II flow cytometer with FACSDIVA software (BS Sciences, San Jose, CA, USA). The anti-CD14-FITC label was used for identifying monocytes. CD14-FITC positive monocytes were excluded using electronic gates and neutrophils and lymphocytes were delineated according to their light-scattering properties.

Flow cytometric data on the intracellular targets were determined as the mean of relative fluorescence units (RFU) of all monocytes or lymphocytes and the proportion (%) of positively fluorescing cells [10]. To determine the latter, an electronic gate covering the brightest fluorescing cells was set to include less than but as close as possible to 5% of the cells in the non-stimulated sample. The same gate was used to determine the proportion of positively fluorescing cells in the respective stimulus-treated sample.

The proportion of constitutively STAT3-phosphorylated cells (pSTAT3+%) and their mean RFU were determined in the following way: a marker was set on each cell population histogram of the healthy subject´s sample so that it encompassed less than but as close as possible to 5 % of the brightest events. The markers were then copied to the respective patient´s sample histograms [11].

Statistical analysis

Demographic and clinical data are shown as medians with ranges or number of patients (proportion). The fluorescence intensities or percentages of positive cells are shown as means with 95% confidence intervals obtained by bias-corrected bootstrapping (5000 replications). Mann-Whitney U-test was used to compare the healthy subjects and patients. The changes in fluorescence intensity were evaluated using Page's trend test. Results were considered statistically significant if p value was ≤ 0.05 and two-tailed tests were used. No correction was made for multiple testing. SPSS 22 (IBM, Chicago, IL, USA) and Stata 15.1 (StataCorp, College Station, TX, USA) software packages were used for statistical analyses.

Results

Basic clinical features of the groups are shown in Table 1. The etiology in AP was alcohol (n=2 in moderately severe and n=1 in severe group) or biliary (n=4 moderately severe, n=2 severe). Three AP patients underwent endoscopic retrograde cholangiopancreatography (ERCP) and one had a laparotomy. In the sepsis group, seven patients had pneumonia, one cervical abscess and five intra-abdominal infection. Five patients had a positive bacterial culture in blood and four in intra-abdominal fluid taken during laparotomy. Five sepsis patients had a laparotomy and one cervical drainage was done. One patient with severe AP and three patients with sepsis died within the follow-up period. A flow chart covering the follow-up and composition of OD- and OD+ groups is shown in Figure 1.

[Table 1 near here]

[Figure 1 near here]

Time course of signaling profiles in patients with AP (OD- and OD+) and with sepsis (OD+)

NF-κB

Monocyte NF-κB phosphorylation was generally lower in patients than healthy controls (Figure 2A-C), with an increasing trend in OD+ group following TNF-, LPS-, and *E. coli* stimulation, and in OD- group following *E. coli* stimulation. However, at the end of follow-up the levels remained decreased compared to controls in both OD- and OD+ groups following stimulation by TNF (both p levels <0.001), LPS (p=0.002 and p=0.013, respectively), and *E. coli* (both p<0.001).

Neutrophil NF-κB phosphorylation was generally similar in patients at entry and controls (Figure 2D), but a decreasing trend was seen in OD- group, and at the end of follow-up the levels in both OD- and OD+ groups were lower than controls (p=0.033 and p=0.047, respectively).

Lymphocyte NF-κB phosphorylation was generally lower in patients than healthy controls at entry (Figure 2E), but an increasing trend was seen in OD- group and at the end of follow-up the level in OD+ group remained lower than in the healthy controls (p=0.014).

The corresponding results shown in terms of proportion of pNF-κB positive cells are shown in Supplementary Figure 1 and are generally comparable to the fluorescence intensity results above.

[Figure 2 near here]

STAT1

Monocyte IL-6-stimulated STAT1 phosphorylation remained lower in patients than healthy controls throughout follow-up (Figure 3A; both p<0.001). In the patients' lymphocytes, the levels did not differ from those of the healthy controls and showed no trend during follow-up (Figure 3B). The corresponding results shown in terms of proportion of pSTAT1 positive

cells are shown in Supplementary Figure 2 and are generally comparable to the fluorescence intensity results above.

[Figure 3 near here]

STAT3

Non-stimulated STAT3 phosphorylation of monocytes and neutrophils was increased in patients compared to healthy controls at entry (Figure 4A,C). During follow-up, the levels decreased in both OD- and OD+ groups, and at the end of follow-up only monocyte non-stimulated STAT3 phosphorylation remained elevated compared to healthy controls ($p=0.007$). IL-6-stimulated STAT3 phosphorylation of monocytes, which was decreased in patients compared to healthy controls at entry, increased during follow-up in both OD- and OD+ group, but still did not reach the level of the healthy controls in either OD- or OD+ group (Figure 4B, $p=0.033$ and $p=0.014$, respectively).

In neutrophils there were virtually no increase in pSTAT3 levels in response to IL-6 in the samples of either patients or healthy controls (Figure 4D), which is in good accordance with the novel finding that mature neutrophils do not express the transmembrane signaling complex gp130 required for IL-6 signaling [17].

In lymphocytes, non-stimulated pSTAT3 levels, which were increased in the patients compared to the controls at entry, decreased in OD+ group, but did not decline to the level of the healthy controls (Figure 4E, $p=0.001$ for OD- and $p<0.001$ for OD+). In IL-6-stimulated lymphocytes, pSTAT3 levels of the patients did not differ from those of the healthy controls and showed no trend during follow-up (Figure 4F).

The corresponding results shown in terms of proportion of pSTAT3 positive cells are shown in Supplementary Figure 3 and are generally comparable to the fluorescence intensity results above.

[Figure 4 near here]

ERK1/2

Monocyte ERK1/2 phosphorylation following stimulation by PMA and Ca^{2+} ionophore A23187 was lower in patients compared to controls at entry with no change during follow-up (Figure 5A). Neutrophil pERK1/2 levels were comparable in patients and controls, with a decreasing trend in OD- group (Figure 5B). In lymphocytes, the levels were comparable in patients and controls with no change during follow-up. The corresponding results shown in terms of proportion of pERK1/2 positive cells are shown in Supplementary Figure 4 and are generally comparable to the fluorescence intensity results above.

[Figure 5 near here]

Time course of monocyte HLA-DR expression

Monocyte HLA-DR expression was lower in patients than in healthy controls (Figure 6). In OD+ group there was an increasing trend, but the levels remained low during follow-up. The two patients who died shortly after the second blood sampling showed a steep decline in monocyte HLA-DR expression between the first and second sampling.

Discussion

The results of the current study show how the phosphorylation of the crucial intracellular signal transduction molecules, STAT3, STAT1, NF-κB and ERK1/2 changes in whole blood leukocytes during hospitalization period in patients with moderately severe acute pancreatitis (AP) without persistent organ dysfunction (OD- group), and patients with sepsis or severe AP complicated by persistent OD (OD+ group). Recently, we reported the patients' phosphorylation results on admission to hospital with special reference to the severity of AP

and similarities in AP and sepsis [13]. Now we extended the focus to observe the time course of the phosphorylations, considering their applicability in the follow-up and associations with the complex immunological changes (from hyperinflammation to immunosuppression) occurring during the disease course of AP and sepsis.

We found that the constitutive STAT3 phosphorylation that regularly occurred in the patients' monocytes and neutrophils on admission to hospital [13], decreased during hospitalization, which was also seen in lymphocytes of OD+ group. These changes may result from changes in the levels of numerous circulating cytokines that are able to activate STAT3, e.g. IL-6 and IL-10 [18]. It has been reported that neutrophils of patients with sepsis, but not those of healthy individuals, express IL-10 receptor chain that makes responsiveness to IL-10 possible and leads to prompt STAT3 activation upon IL-10 exposure [19]. In the simplified model, based on several patient and animal studies, IL-6 typically peaks in the earlier, hyperinflammatory stage of systemic inflammation, while IL-10 levels increase thereafter and are associated with immunosuppression [1,20-23]. Although the constitutive STAT3 phosphorylation levels in monocytes decreased, in OD- group they were still higher than those of healthy controls whereas the levels in OD+ group reached the levels of healthy controls by the end of follow-up. In this context it may be noteworthy that in an induced septic peritonitis model, mice with targeted disruption of *Stat3* in monocytes and neutrophils had excessive local and systemic inflammation and hepatic and renal injury as compared to mice with normal *Stat3*, suggesting an advantageous role for STAT3 [24]. Altogether, future studies are required to reveal if some specific cytokines are involved in maintaining higher STAT3 phosphorylation in monocytes and associate with a less severe disease course in AP.

Instead, STAT3 phosphorylation upon IL-6 stimulation in monocytes was originally low and even though it increased in both OD- and OD+ groups, it did not reach the levels of healthy controls during follow-up. The patients' monocytes also showed low STAT1 phosphorylation

in response to IL-6 during the follow-up suggesting, altogether, a state of monocyte hyporesponsiveness to exogenous IL-6. This may result from membrane-bound IL-6 receptor shedding giving rise to soluble IL-6 receptors (sIL-6Rs). IL-6 can form a complex with sIL-6R, and the complex can activate, via the ubiquitously expressed signal-transducing transmembrane protein gp130, many cell types that do not express IL-6R. This is called IL-6 trans-signaling, and it is involved in many processes that are important in systemic inflammation, including activation of endothelial cells, recruitment of mononuclear cells and expression of chemokines. It has been shown in a mouse model that selective inhibition of IL-6 trans-signaling rather than complete blockade of IL-6 signaling significantly improves survival in sepsis. [25] Another mechanism that could contribute to IL-6 hyporesponsiveness is IL-6R down-regulation, which has been observed in monocytes of healthy subjects after interaction with bacterial antigens [26]. Furthermore, IL-6 hyporesponsiveness due to attenuated gp130 phosphorylation has been described in murine sepsis [27]. Regarding that the patients' IL-6 -stimulated pSTAT1 levels, as compared to the corresponding pSTAT3 levels, remained remarkably low throughout follow-up in the current study, one may conclude that there are additional disease-associated mechanisms affecting STAT1 phosphorylation. It has been reported, for example, in an *in vitro* sepsis model that miR-30a targets STAT1 and subsequently, attenuates TLR4 responses in human monocytes [28]. According to our results, monocyte IL-6 hyporesponsiveness persists in patients with moderately severe or severe AP or sepsis at least throughout the first hospitalization week. Thus, unraveling the exact underlying mechanisms could facilitate finding treatments that lead to more rapid amelioration. Finally, we cannot fully exclude the possibility that the hyporesponsiveness is endogenous and may even favor the development of systemic inflammation, which warrants further studies.

Low responsiveness was also observed in terms of NF-κB phosphorylation in monocytes, in which the phosphorylation levels in response to either TNF, LPS or whole *E. coli* showed some improvement but did not reach the levels of healthy controls during follow-up in either OD- or OD+ group. However, the improvement was significant in response to each of these three stimuli in monocytes in OD+ group, and in *E. coli* -stimulated monocytes and TNF-stimulated lymphocytes in OD- group. The mechanism dampening NF-κB phosphorylation may be, for example, induction of miR-146a, which has been reported to occur quickly after LPS stimulation in monocytes [29]. Also, the decreased NF-κB phosphorylation capacity may be connected to immunosuppression. This is supported by the finding that monocytes of sepsis patients exhibit a Wnt5a-induced phenotype in which the classical Toll-like receptor (TLR) 4-NF-κB signaling is inhibited [30]. HLA-DR expression on monocytes is a conventional marker for immunosuppression [31], and in the present study the low HLA-DR levels at entry improved significantly in OD+ but not in OD- group during follow-up, but the levels still remained lower than those of healthy controls. It remains to be elucidated if an improving NF-κB phosphorylation capacity, or signaling events closely related to it, in monocytes and lymphocytes represents an additional and even quicker or more sensitive marker for recovery from an immunosuppressive stage than HLA-DR is. In neutrophils, TNF-induced NF-κB phosphorylation levels of OD- group decreased significantly during follow-up. It may be noteworthy that delayed apoptosis belongs to the NF-κB -mediated responses to TNF and LPS in neutrophils [32], takes place during acute inflammation, and leads to accumulation of activated neutrophils contributing to the inflammatory response and host tissue damage. Resolution, on the other hand, is highly dependent on the induction of apoptosis in neutrophils [33]. Hence, the decrease in NF-κB phosphorylation stimulated by TNF in neutrophils may reflect the progression of recovery, which is more prominent in patients without persistent OD.

To the best of our knowledge, there are virtually no studies on the time course of activation abilities of signaling proteins in AP or sepsis, and NF-κB is among the rare ones studied. Böhrer *et al*. reported that nuclear extracts of peripheral blood mononuclear cells of nonsurviving sepsis patients have higher NF-κB binding activity than those of surviving patients do, as studied by electrophoretic mobility shift assay [34]. Seemingly, this contradicts our current results in which the NF-κB phosphorylation capability in monocytes and lymphocytes of the patients, also of those who died, is low. However, Böhrer *et al*. compared intrapersonal signal intensity in relation to day-one levels in a certain mass of nuclear extract. There was also considerable day-to-day variation. Moreover, patients with major trauma were included in their study while patients with AP and healthy controls were not, and vice versa in our study, which further complicates the comparison of the results.

Our results on ERK1/2 phosphorylation mostly concerned neutrophils, in which there was a significant decrease in the phosphorylation levels in the samples of patients with or without OD in response to phorbol myristate acetate and Ca^{2+} ionophore. It has been reported that priming of respiratory burst in neutrophils is inhibited by IL-10 in an ERK1/2-mediated way [35]. Thus, the decreasing capability of ERK1/2 phosphorylation could be contributed by the increasing IL-10 production along the course of systemic inflammation.

The most obvious limitation of this study is the low number of subjects. Hence, the results can be regarded only as preliminary. Larger studies including patients with mild AP hospitalized for more than a few days would enable the time-course comparison of signaling markers between mild AP, moderately severe AP and severe AP/sepsis. Also, other considerations such as signaling markers in prediction of mortality or risk of secondary infections, for example, would be possible in larger cohorts.

In conclusion, the results of this preliminary study suggest that the level of constitutive STAT3 phosphorylation in monocytes, responsiveness to IL-6 as determined by phosphorylation capacity of STAT1 and STAT3 in monocytes, NF-κB phosphorylation capacity in response to inflammatory stimuli in monocytes and lymphocytes, and ERK1/2 phosphorylation capacity in neutrophils are related to the disease course of moderately severe or severe AP and sepsis and occurrence of persistent OD. An important aspect is to find out if following the changes of the phosphorylation markers can, during the hospitalization time, help in guiding treatment decisions in AP and sepsis.

Acknowledgments

Ms Terhi Salonen is warmly acknowledged for excellent technical assistance.

Disclosure statement

The authors declare that they have no conflict of interest.

Funding

The study was supported, in part, by Helsinki University Hospital Research Funds, Helsinki, Finland.

Table 1

Demographic and clinical characteristics of the patients.

Characteristics	Moderately severe AP, n=6	Severe AP, n=3	Sepsis, n=14
Age, years	72 (50-84)	46 (26-82)	62 (31-75)
Gender, men:women	4:2	2:1	8:6
Onset of symptoms before hospital admission, hours	27 (8-72)	8 (3-48)	NA
CT on hospital admission	6 (109)	3 (100)	NA
On hospital (AP) or ICU (sepsis) admission			
APACHE II	8 (5-11)	7 (5-13)	15 (6-30)
SOFA	3 (2-5)	1 (0-8)	8 (3-16)
MMS	1 (0-1)	0 (0-4)	NA
CRP, mg/L	24 (3-274)	3 (3-598)	220 (0-412)
Creatinine, μmol/L	90 (77-153)	107 (69-667)	132 (36-324)
Highest CRP, mg/L	328 (255-481)	324 (322-598)	329 (100-670)
Highest SOFA	7 (3-12)	9(8-10)	9 (3-20)
ICU treatment	5 (83)	3 (100)	14 (100)
MIV	1	2	12
CRRT	0	1	4
Vasopressor agent	3	2	13
ICU entry after admission to hospital, hours	32 (14-122)	14 (5-38)	7 (1-32)
ICU length of stay, days	9 (4-14)	10 (4-20)	5 (2-22)
Hospital length of stay, days	20 (10-31)	23 (7-32)	12 (3-30)
30-day mortality, n (%)	0	1 (33)	3 (21)

Data are shown as median with range or number of patients (n) with percentage.
AP: acute pancreatitis, CT: computed tomography; ICU: intensive care unit; APACHE: Acute Physiologic and Chronic Health Evaluation; SOFA: Sequential Organ Failure Assessment; MMS: Modified Marshall Score; CRP: C-reactive protein; MIV: mechanical invasive ventilation; CRRT: continuous renal replacement therapy.

Figure captions

Figure 1

Follow-up of the patients and composition of no persistent organ dysfunction (OD-) and persistent organ dysfunction (OD+) groups. Patient blood samples were taken at time points 0 (within 48h of admission to hospital, "Study entry"), 1 and 2 (2-4 and 5-8 days after time point 0, respectively). The patients with moderately severe AP comprise the OD- group, and patients with severe AP and sepsis comprise the OD+ group. The 2 sepsis patients referred to another hospital ("Referred") after Sample 1 are included in the OD+ group, but those patients who died during follow-up are analysed separately.

Figure 2

Time course of phosphorylated NF-κB (pNF-κB) fluorescence intensity (in relative fluorescence units, RFU) of monocytes stimulated with TNF (A), LPS (B), or *E. coli* (C), and neutrophils (D) and lymphocytes (E) stimulated with TNF, in patients without/with persistent organ dysfunction (OD-/OD+), patients who died, and healthy control subjects (HC). Patient blood samples were taken at time points 0 (within 48h of admission to hospital), 1 and 2 (2-4 and 5-8 days after time point 0, respectively). Patients with acute pancreatitis are shown as circles, sepsis patients as triangles, and healthy controls as squares. Group means are shown as horizontal bars with whiskers denoting 95% confidence intervals. The p-values are calculated by Page's trend test.

Figure 3

Time course of phosphorylated STAT1 (pSTAT1) fluorescence intensity (in relative fluorescence units, RFU) of monocytes (A) and lymphocytes (B) stimulated with IL-6 in patients without/with persistent organ dysfunction (OD-/OD+), patients who died, and healthy control subjects (HC). Patient blood samples were taken at time points 0 (within 48h of

admission to hospital), 1 and 2 (2-4 and 5-8 days after time point 0, respectively). Patients with acute pancreatitis are shown as circles, sepsis patients as triangles, and healthy controls as squares. Group means are shown as horizontal bars with whiskers denoting 95% confidence intervals. The p-values are calculated by Page's trend test.

Figure 4

Time course of phosphorylated STAT3 (pSTAT3) fluorescence intensity (in relative fluorescence units, RFU) of non-stimulated and IL-6-stimulated monocytes (A and B), neutrophils (C and D) and lymphocytes (E and F) in patients without/with persistent organ dysfunction (OD-/OD+), patients who died, and healthy control subjects (HC). Patient blood samples were taken at time points 0 (within 48h of admission to hospital), 1 and 2 (2-4 and 5-8 days after time point 0, respectively). Patients with acute pancreatitis are shown as circles, sepsis patients as triangles, and healthy controls as squares. Group means are shown as horizontal bars with whiskers denoting 95% confidence intervals. The p-values are calculated by Page's trend test.

Figure 5

Time course of phosphorylated ERK1/2 (pERK1/2) fluorescence intensity (in relative fluorescence units, RFU) of monocytes (A), neutrophils (B) and lymphocytes (C) stimulated with PMA with Ca-ionophore in patients without/with persistent organ dysfunction (OD-/OD+), patients who died, and healthy control subjects (HC). Patient blood samples were taken at time points 0 (within 48h of admission to hospital), 1 and 2 (2-4 and 5-8 days after time point 0, respectively). Patients with acute pancreatitis are shown as circles, sepsis patients as triangles, and healthy controls as squares. Group means are shown as horizontal bars with whiskers denoting 95% confidence intervals. The p-values are calculated by Page's trend test.

Figure 6

Time course of monocyte HLA-DR expression in patients without/with organ dysfunction (OD-/OD+), patients who died, and healthy control subjects (HC). Patient blood samples were taken at time points 0 (within 48h of admission to hospital), 1 and 2 (2-4 and 5-8 days after time point 0, respectively). Patients with acute pancreatitis are shown as circles, sepsis patients as triangles, and healthy controls as squares. Group means are shown as horizontal bars with whiskers denoting 95% confidence intervals. The p-values are calculated by Page's trend test.

References

1. Kylänpää M, Repo H, Puolakkainen PA. Inflammation and immunosuppression in severe acute pancreatitis. World J Gastroenterol 2010;16:2867-72.

2. Buter A, Imrie CW, Carter CR, et al. Dynamic nature of early organ dysfunction determines outcome in acute pancreatitis. Br J Surg 2002;89:298-302.

3. Johnson CD, Abu-Hilal M. Persistent organ failure during the first week as a marker of fatal outcome in acute pancreatitis. Gut 2004;53:1340-4.

4. Caldwell CC, Hotchkiss RS. The first step in utilizing immune-modulating therapies: immune status determination. Crit Care 2011;15:108.

5. Chakraborty S, Kaur S, Muddana V, et al. Elevated serum neutrophil gelatinase-associated lipocalin is an early predictor of severity and outcome in acute pancreatitis. Am J Gastroenterol 2010;105:2050-9.

6. Vincent J, Opal SM, Marshall JC. Ten reasons why we should NOT use severity scores as entry criteria for clinical trials or in our treatment decisions. Crit Care Med 2010;38:283-7.

7. Puolakkainen P, Valtonen V, Paananen A, et al. C-reactive protein (CRP) and serum phospholipase A2 in the assessment of the severity of acute pancreatitis. Gut 1987;28:764-71.

8. Nieminen A, Maksimow M, Mentula P, et al. Circulating cytokines in predicting development of severe acute pancreatitis. Crit Care 2014;18:R104.

9. Rakonczay Z, Hegyi P, Takács T, et al. The role of NF-κB activation in the pathogenesis of acute pancreatitis. Gut 2008;57:259-67.

10. Oiva J, Mustonen H, Kylanpaa ML, et al. Acute pancreatitis with organ dysfunction associates with abnormal blood lymphocyte signaling: controlled laboratory study. Crit Care 2010;14:R207.

11. Oiva J, Mustonen H, Kylänpää M, et al. Patients with acute pancreatitis complicated by organ failure show highly aberrant monocyte signaling profiles assessed by phospho-specific flow cytometry. Crit Care Med 2010;38:1702-8.

12. Oiva J, Mustonen H, Kylänpää M, et al. Patients with acute pancreatitis complicated by organ dysfunction show abnormal peripheral blood polymorphonuclear leukocyte signaling. Pancreatology 2013;13:118-24.

13. Kuuliala K, Penttilä AK, Kaukonen K-M, et al. Signalling Profiles of Blood Leukocytes in Sepsis and in Acute Pancreatitis in Relation to Disease Severity. Scand J Immunol 2018;87:88-98.

14. Banks PA, Bollen TL, Dervenis C, et al. Classification of acute pancreatitis--2012: revision of the Atlanta classification and definitions by international consensus. Gut 2013;62:102-11.

15. Singer M, Deutschman CS, Seymour CW, et al. The Third International Consensus Definitions for Sepsis and Septic Shock (Sepsis-3). JAMA 2016;315:801-10.

16. Kylänpää-Bäck ML, Takala A, Kemppainen E, et al. Cellular Markers of Systemic Inflammation and Immune Suppression in Patients with Organ Failure Due to Severe Acute Pancreatitis. Scand J Gastroenterol 2001;36:1100-7.

17. Wilkinson AN, Gartlan KH, Kelly G, et al. Granulocytes are unresponsive to IL-6 due to an absence of gp130. J Immunol 2018;200:3547-55.

18. Aggarwal BB, Kunnumakkara AB, Harikumar KB, et al. Signal transducer and activator of transcription-3, inflammation, and cancer. How intimate is the relationship? Ann N Y Acad Sci 2009;1171:59-76.

19. Tamassia N, Calzetti F, Menestrina N, et al. Circulating neutrophils of septic patients constitutively express IL-10R1 and are promptly responsive to IL-10. Int Immunol 2008;20:535-41.

20. Tao J, Gong D, Ji D, et al. Improvement of monocyte secretion function in a porcine pancreatitis model by continuous dose dependent veno-venous hemofiltration. Int J Artif Organs 2008;31:716-21.

21. Mole DJ, McFerran NV, Diamond T. Differential preservation of lipopolysaccharide-induced chemokine/cytokine expression during experimental pancreatitis-associated organ failure in rats shows a regulatory expressed phenotype. Pancreatology 2008;8:478-87.

22. Kieslichova E, Rocen M, Merta D, et al. The effect of immunosuppression on manifestations of sepsis in an animal model of cecal ligation and puncture. Transplant Proc 2013;45:770-7.

23. Boomer JS, Green JM, Hotchkiss RS. The changing immune system in sepsis: is individualized immuno-modulatory therapy the answer? Virulence 2014;5:45-56.

24. Matsukawa A, Takeda K, Kudo S, et al. Aberrant inflammation and lethality to septic peritonitis in mice lacking STAT3 in macrophages and neutrophils. J Immunol 2003;171:6198-205.

25. Barkhausen T, Tschernig T, Rosenstiel P, et al. Selective blockade of interleukin-6 trans-signaling improves survival in a murine polymicrobial sepsis model. Crit Care Med 2011;39:1407-13.

26. Dekkers PE, Juffermans NP, ten Hove T, et al. Endotoxin down-regulates monocyte and granulocyte interleukin-6 receptors without influencing gp130 expression in humans. J Infect Dis 2000;18:1055-61.

27. Abjeco AS, Andrejko KM, Raj NR, et al. Failed interleukin-6 signal transduction in murine sepsis: attenuation of hepatic glycoprotein (gp) 130 phosphorylation. Crit Care Med 2009;37:1729-34.

28. Wang Y, Li T, Wu B, et al. STAT1 regulates MD-2 expression in monocytes of sepsis via miR-30a. Inflammation 2014;37:1903-11.

29. Loubaki L, Chabot D, Paré I, et al. miR-146a potentially promotes IVIg-mediated inhibition of TLR signaling in LPS-activated human monocytes. Immunol Lett 2017;185:64-73.

30. Bergenfelz C, Medrek C, Ekström E, et al. Wnt5a induces a tolerogenic phenotype of macrophages in sepsis and breast cancer patients. J Immunol. 2012;188:5448-58.

31. Pfortmueller CA, Meisel C, Fux M, Schefold JC. Assessment of immune organ dysfunction in critical illness: utility of innate immune response markers. Intensive Care Med Exp 2017;5:49.

32. Nolan B, Kim R, Duffy A, et al. Inhibited neutrophil apoptosis: proteasome dependent NF-kappaB translocation is required for TRAF-1 synthesis. Shock 2000;14:290-4.

33. Paunel-Görgülü A, Kirichevska T, Lögters T, et al. Molecular mechanisms underlying delayed apoptosis in neutrophils from multiple trauma patients with and without sepsis. Mol Med 2012;18:325-35.

34. Böhrer HD, Ziegler R, Bierhaus A, et al. Role of NF-κB in the mortality of sepsis. J Clin Invest 1997;100:972-85.

35. Dang PM, Elbim C, Marie JC, et al. Anti-inflammatory effect of interleukin-10 on human neutrophil respiratory burst involves inhibition of GM-CSF-induced p47PHOX phosphorylation through a decrease in ERK1/2 activity. FASEB J 2006;20:1504-6.

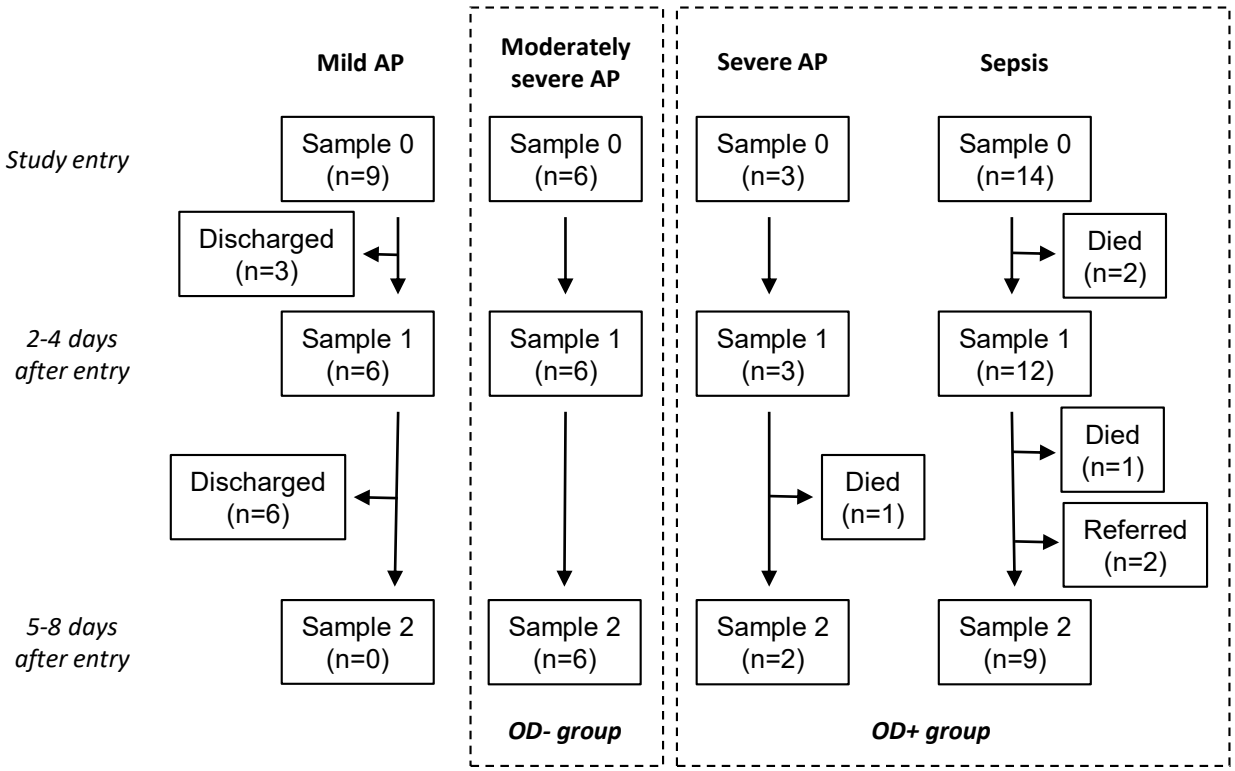

Mild AP
Moderately severe AP
Severe AP
Sepsis
Study entry
Sample 0 (n=9)
Sample 0 (n=6)
Sample 0 (n=3)
Sample 0 (n=14)
Discharged (n=3)
Died (n=2)
2-4 days after entry
Sample 1 (n=6)
Sample 1 (n=6)
Sample 1 (n=3)
Sample 1 (n=12)
Discharged (n=6)
Died (n=1)
Died (n=1)
Referred (n=2)
5-8 days after entry
Sample 2 (n=0)
Sample 2 (n=6)
Sample 2 (n=2)
Sample 2 (n=9)
OD- group
OD+ group

Figure 1

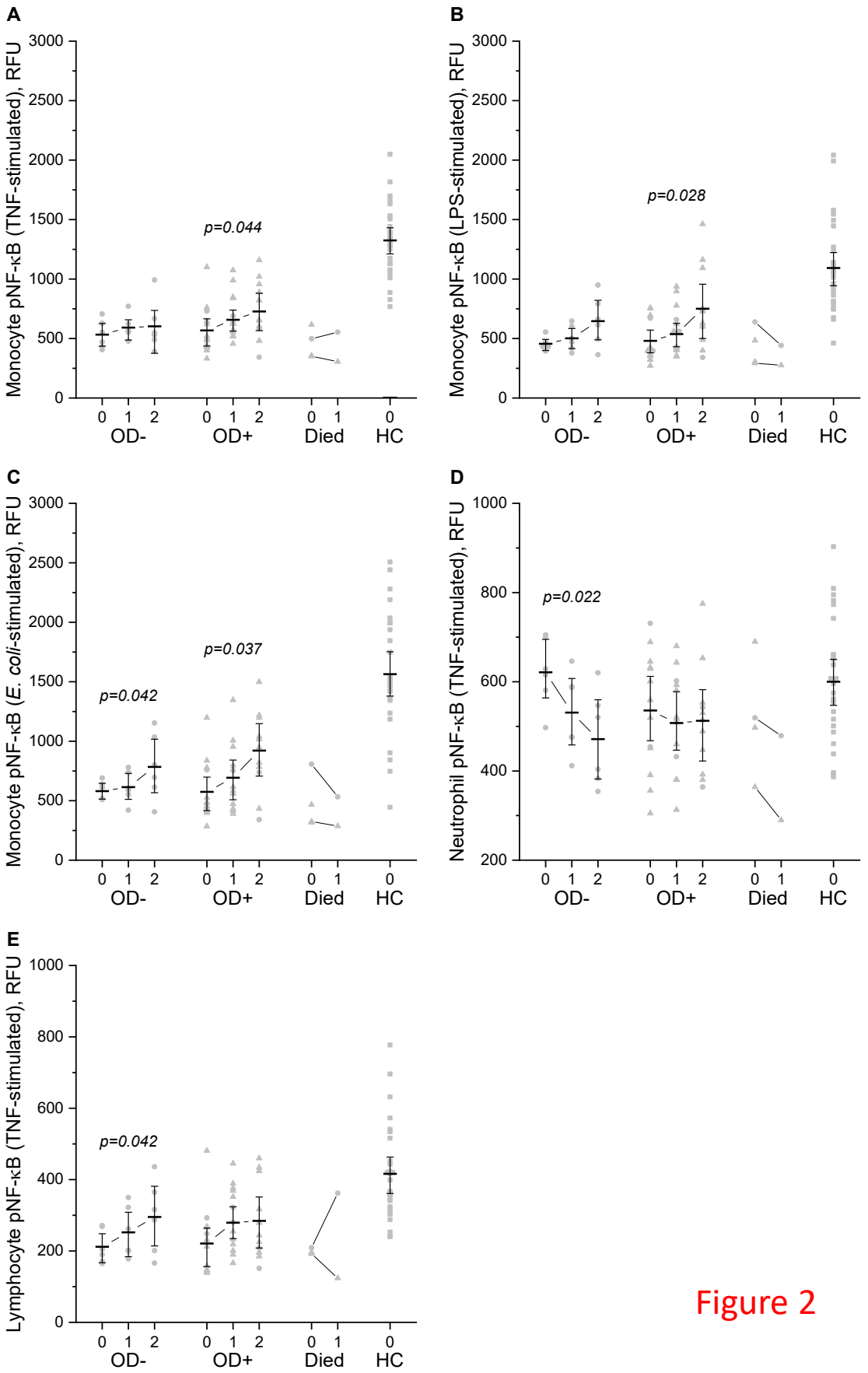
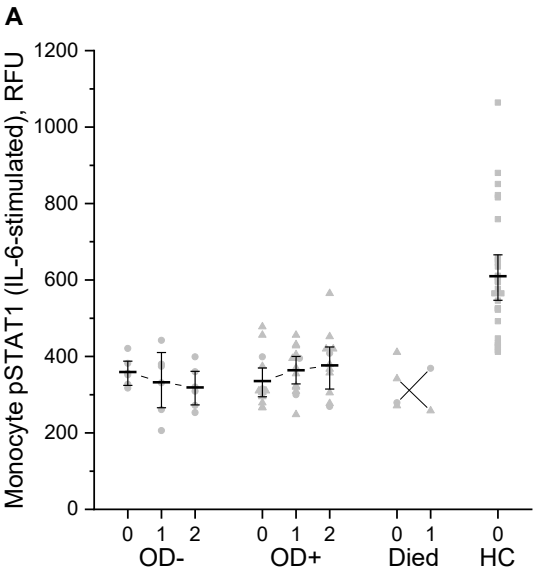

A
Monocyte pNF-κB (TNF-stimulated), RFU
3000
2500
2000
1500
1000
500
0
p=0.044
0 1 2 0 1 2 0 1 0
OD- OD+ Died HC
B
Monocyte pNF-κB (LPS-stimulated), RFU
p=0.028
C
Monocyte pNF-κB (E. coli-stimulated), RFU
p=0.042
p=0.037
D
Neutrophil pNF-κB (TNF-stimulated), RFU
1000
800
600
400
200
p=0.022
E
Lymphocyte pNF-κB (TNF-stimulated), RFU
p=0.042

Figure 2


A
Monocyte pSTAT1 (IL-6-stimulated), RFU
1200
1000
800
600
400
200
0
0 1 2
0 1 2
0 1
0
OD-
OD+
Died
HC

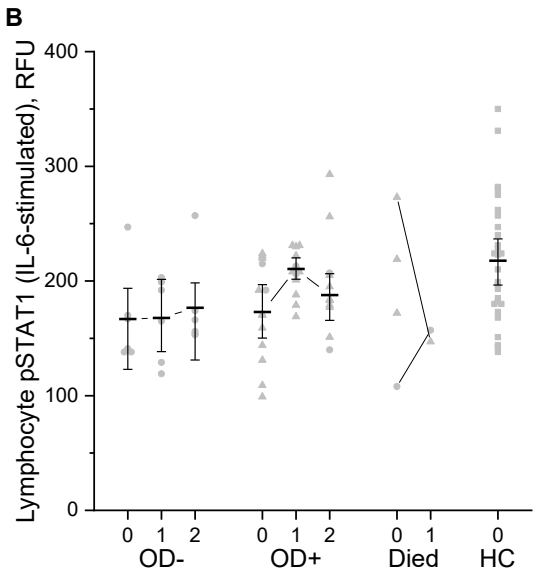
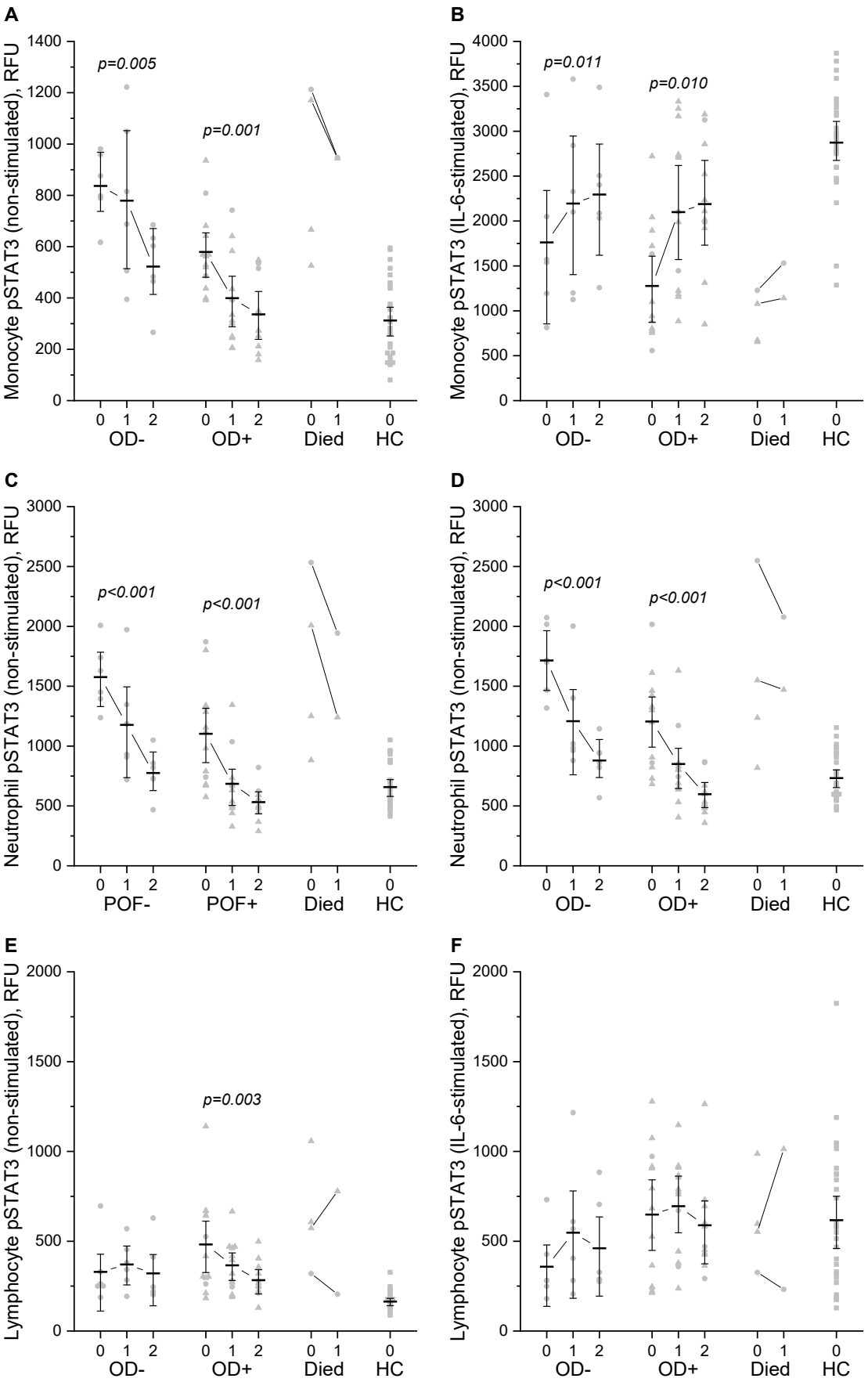

B
Lymphocyte pSTAT1 (IL-6-stimulated), RFU
400
300
200
100
0
0 1 2
0 1 2
0 1
0
OD-
OD+
Died
HC

Figure 3

Figure 4


A
Monocyte pSTAT3 (non-stimulated), RFU
p=0.005
p=0.001
OD-
OD+
Died
HC
B
Monocyte pSTAT3 (IL-6-stimulated), RFU
p=0.011
p=0.010
C
Neutrophil pSTAT3 (non-stimulated), RFU
p<0.001
p<0.001
POF-
POF+
D
Neutrophil pSTAT3 (non-stimulated), RFU
p<0.001
p<0.001
E
Lymphocyte pSTAT3 (non-stimulated), RFU
p=0.003
F
Lymphocyte pSTAT3 (IL-6-stimulated), RFU

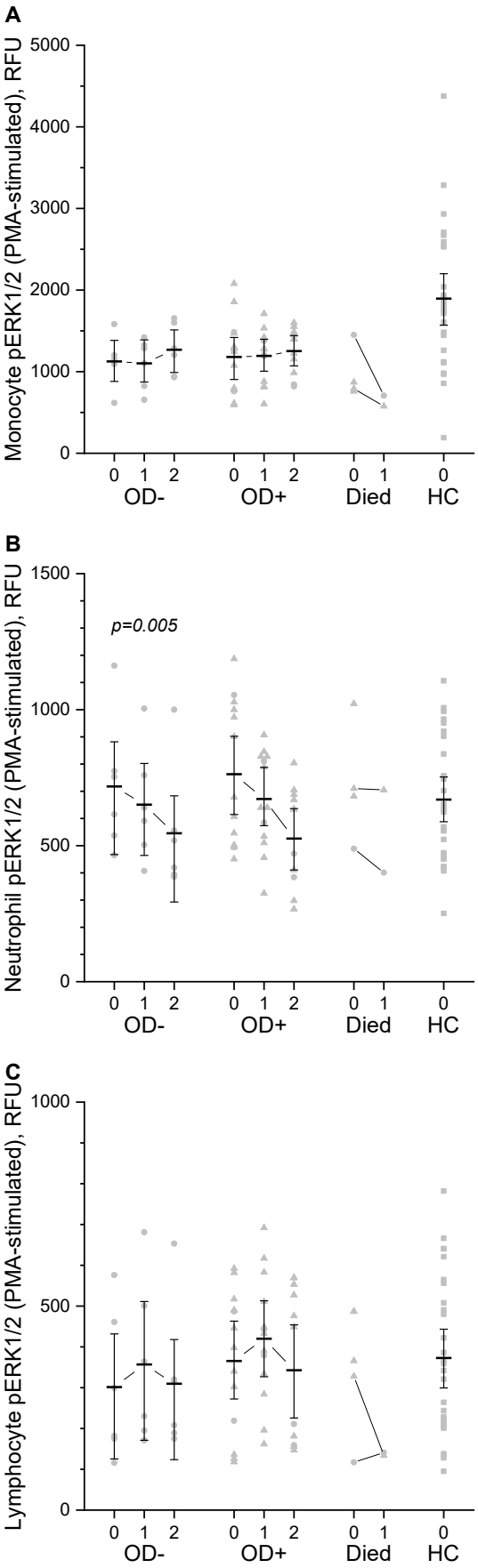

A
Monocyte pERK1/2 (PMA-stimulated), RFU
5000
4000
3000
2000
1000
0
0 1 2 0 1 2 0 1 0
OD- OD+ Died HC
B
Neutrophil pERK1/2 (PMA-stimulated), RFU
p=0.005
1500
1000
500
0
0 1 2 0 1 2 0 1 0
OD- OD+ Died HC
C
Lymphocyte pERK1/2 (PMA-stimulated), RFU
1000
500
0
0 1 2 0 1 2 0 1 0
OD- OD+ Died HC

Figure 5

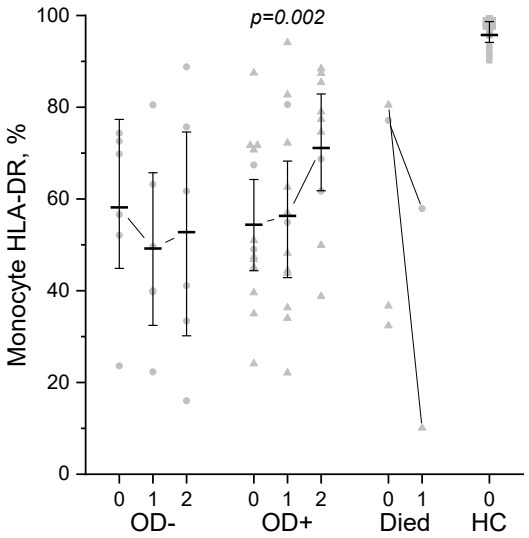


Figure 6

Time Course of Signaling Profiles of Blood Leukocytes in Acute Pancreatitis and Sepsis

Antti Turunen et al. Scandinavian Journal of Clinical and Laboratory Investigation. 2019.

Supplementary Figure Captions

Supplementary Figure 1

Time course of phosphorylated NF-κB (pNF-κB) fluorescence intensity (in proportion of positive cells) of monocytes stimulated with TNF (A), LPS (B), or E. coli (C), and neutrophils (D) and lymphocytes (E) stimulated with TNF, in patients without/with organ dysfunction (OD-/OD+), patients who died, and healthy control subjects (HC). Patient blood samples were taken at time points 0 (within 48h of admission to hospital), 1 and 2 (2-4 and 5-8 days after time point 0, respectively). Patients with acute pancreatitis are shown as circles, sepsis patients as triangles, and healthy controls as squares. Group means are shown as horizontal bars with whiskers denoting 95% confidence intervals. The p-values are calculated by Page's trend test.

Supplementary Figure 2

Time course of phosphorylated STAT1 (pSTAT1) fluorescence intensity (in proportion of positive cells) of monocytes (A) and lymphocytes (B) stimulated with IL-6 in patients without/with organ dysfunction (OD-/OD+), patients who died, and healthy control subjects (HC). Patient blood samples were taken at time points 0 (within 48h of admission to hospital), 1 and 2 (2-4 and 5-8 days after time point 0, respectively). Patients with acute pancreatitis are shown as circles, sepsis patients as triangles, and healthy controls as squares. Group means are shown as horizontal bars with whiskers denoting 95% confidence intervals. The p-values are calculated by Page's trend test.

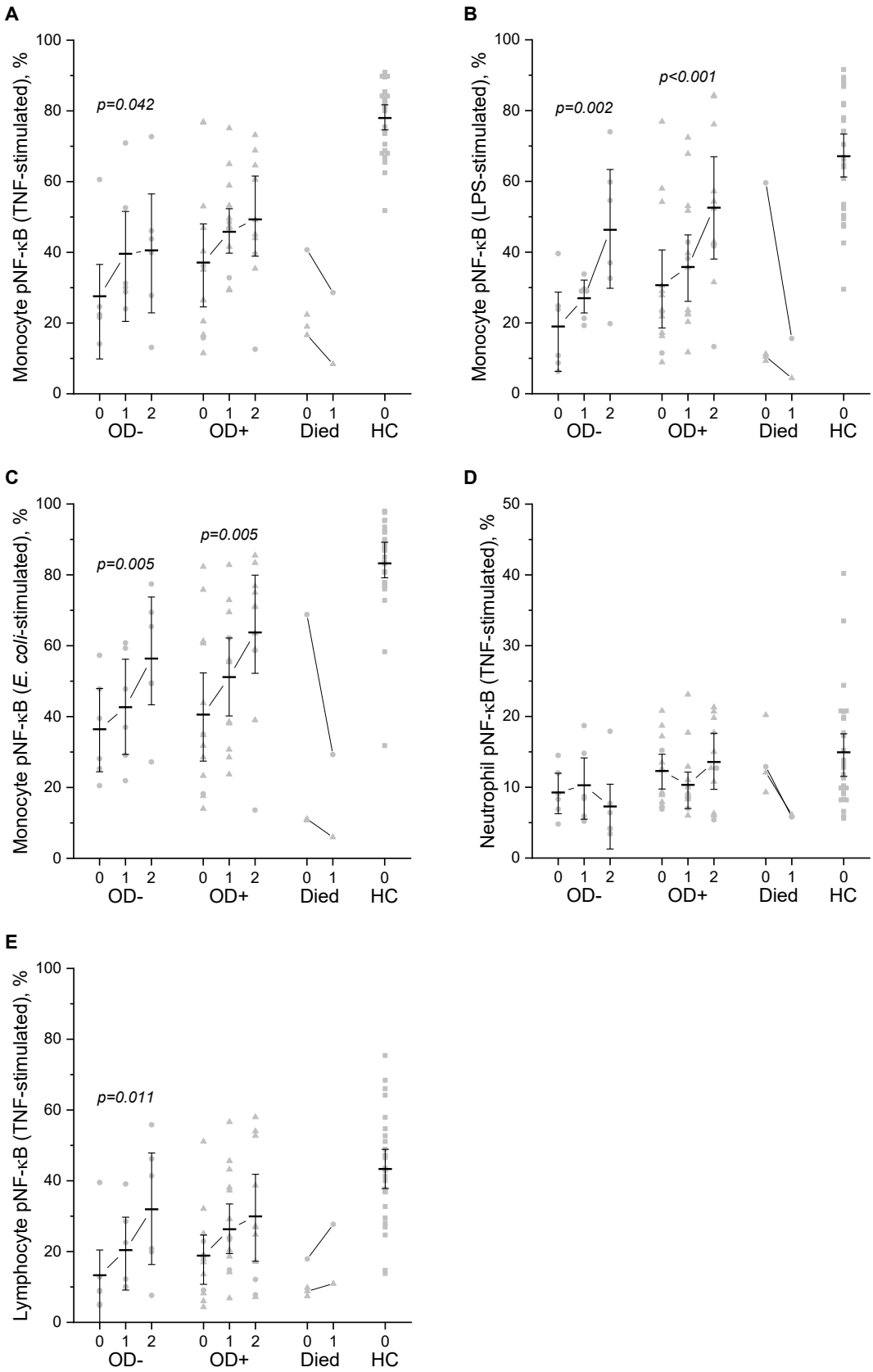
Supplementary Figure 3

Time course of phosphorylated STAT3 (pSTAT3) fluorescence intensity (proportion of positive cells) of non-stimulated and IL-6-stimulated monocytes (A and B), neutrophils (C and D) and lymphocytes (E and F) in patients without/with organ dysfunction (OD-/OD+), patients who died, and healthy control subjects (HC). Patient blood samples were taken at time points 0 (within 48h of admission to hospital), 1 and 2 (2-4 and 5-8 days after time point 0, respectively). Patients with acute pancreatitis are shown as circles, sepsis patients as triangles, and healthy controls as squares. Group means are shown as horizontal bars with whiskers denoting 95% confidence intervals. The p-values are calculated by Page's trend test.

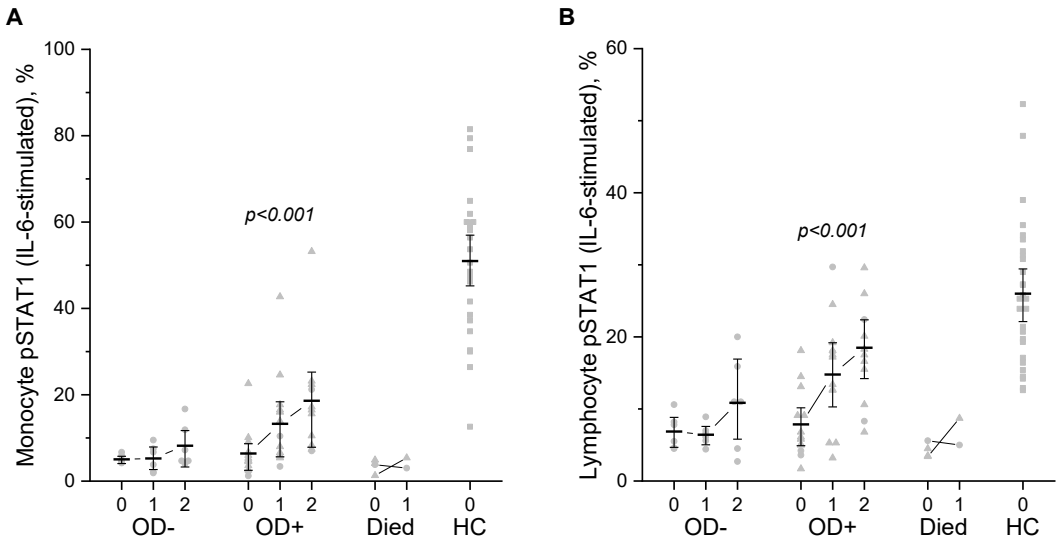
Supplementary Figure 4

Time course of phosphorylated ERK1/2 (pERK1/2) fluorescence intensity (proportion of positive cells) of monocytes (A), neutrophils (B) and lymphocytes (C) stimulated with PMA with Ca-ionophore in patients without/with organ dysfunction (OD-/OD+), patients who died, and healthy control subjects (HC). Patient blood samples were taken at time points 0 (within 48h of admission to hospital), 1 and 2 (2-4 and 5-8 days after time point 0, respectively). Patients with acute pancreatitis are shown as circles, sepsis patients as triangles, and healthy controls as squares. Group means are shown as horizontal bars with whiskers denoting 95% confidence intervals. The p-values are calculated by Page's trend test.

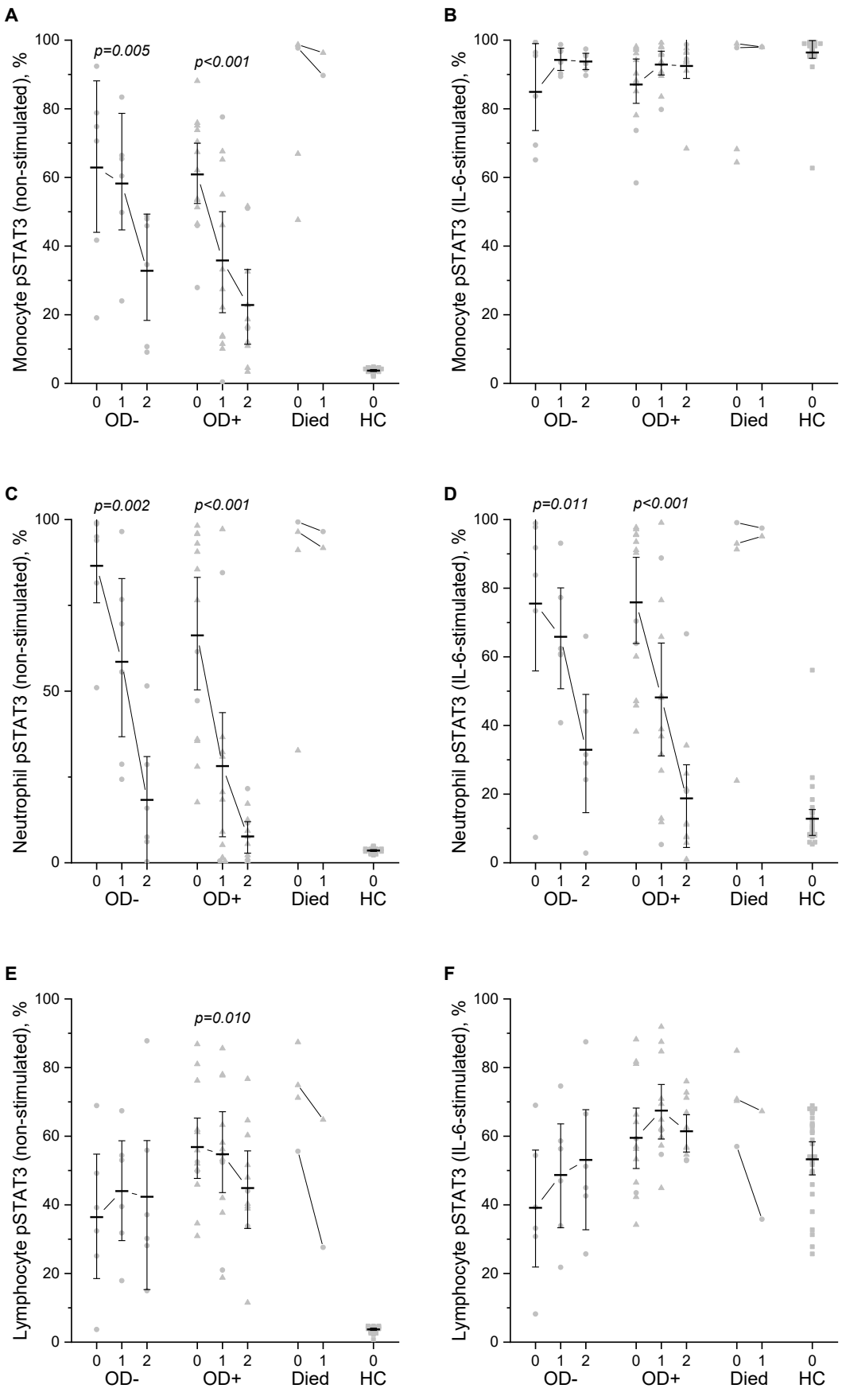
Supplementary Figure 1



Supplementary Figure 2



Supplementary Figure 3



Supplementary Figure 4