Systemic inflammatory response syndrome (SIRS) after major abdominal surgery is predicted by early upregulation of TLR4 and TLR5

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Mini Abstract

Patients undergoing major surgery are at risk of life-threatening complications including systemic inflammatory response syndrome (SIRS) and sepsis. Early post-operative expression of TLR4 and TLR5 and their downstream signalling pathways in monocytes leads to over-expression of IL-6 and can predict SIRS in patients undergoing hepatopancreaticobiliary surgery.

Running Title – Monocyte dysfunction in post-operative SIRS

Abstract

Objective

To study innate immune pathways in hepatopancreaticobiliary (HPB) surgical patients to understand mechanisms leading to enhanced inflammatory responses and identifying biomarkers of adverse clinical consequences.

Summary Background Data

Patients undergoing major abdominal surgery are at risk of life-threatening systemic inflammatory response syndrome (SIRS) and sepsis. Early identification of at-risk patients would allow tailored post-operative care and improve survival.

Methods

Two separate cohorts of patients undergoing major HPB surgery were studied (combined n=69). Bloods were taken pre-operatively, on day 1 and day 2 post-operatively. Peripheral blood mononuclear cells and serum were separated and immune phenotype and function assessed *ex vivo*.

Results

Early innate immune dysfunction was evident in 12 patients who subsequently developed SIRS (post-operative day 6) compared to 27 who did not, when no clinical evidence of SIRS was apparent (pre-operatively or days 1 and 2). Serum interleukin (IL)-6 concentration and monocyte TLR/NF-κB/IL-6 functional pathways were significantly upregulated and

overactive in patients who developed SIRS (p<0.0001). Interferon alpha-mediated STAT1 phosphorylation was higher pre-operatively in patients who developed SIRS. Increased *TLR4* and *TLR5* gene expression in whole blood was demonstrated in a separate validation cohort of 30 patients undergoing similar surgery. Expression of TLR4/5 on monocytes, particularly intermediate CD14^{++}CD16^{+} monocytes, on day 1 or 2 predicted SIRS with accuracy 0.89-1.0 (areas under receiver operator curves).

Conclusions

These data demonstrate the mechanism for IL-6 overproduction in patients who develop post- operative SIRS and identify markers that predict patients at risk of SIRS 5 days before onset of 30 patients undergoing similar surgery. Expression of TLR4/5 on monocytes, particularly intermediate CD14^{++}CD16^{+} monocytes, on day 1 or 2 predicted SIRS with accuracy 0.89-1.0 (areas under receiver operator curves).

Introduction

The systemic inflammatory response syndrome (SIRS) is associated with significant patient morbidity and mortality.[1] SIRS is a clinically-defined state that represents activation of inflammatory, innate immune, coagulation and repair pathways and is frequently observed in hospitalised patients. Overall, there is a 7-fold increase in 28 day mortality in hospitalised patients with SIRS compared to those without.[2] The incidence of post-operative sepsis (SIRS plus presumed or confirmed infection) is high in major operations; 16.7% following distal pancreatic resections,[3] or 32-46% in digestive and gynaecological tumour resections.[4] Identification of patients at risk of SIRS would allow pre-emptive therapy that may improve outcomes.

A key step in the initiation of SIRS is activation of pattern recognition receptors (PRRs) by pathogen associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs).[5] A wide range of PRRs have been described including membrane bound Toll-like receptors (TLRs) and C-type lectin receptors (CLRs), or cytoplasmic receptors such as NOD-like receptors (NLRs) and RIG-I-like receptors (RLRs). The paradigm for PRR activation is TLR4 interacting with lipopolysaccharide (LPS), a component of Gram-negative bacteria. There are at least 11 other members of the TLR family in humans and each is associated with activation by particular PAMPs.[6] TLR5 is activated by flagellin, which is a component of motile bacteria.[7] Following receptor ligation, adapter proteins are recruited and intracellular signalling pathways eventually lead to the activation (phosphorylation) of key transcription factors such as NF-κB and ERK1/2 amongst others.[8, 9] These in turn drive the production of important pro- and anti-inflammatory cytokines such as IL-6, TNF-α and IL-10.[10]

Following surgery, dysregulated release of pro-inflammatory cytokines has been associated with post-operative complications. Many groups have attempted to identify biomarkers that predict poor outcomes following surgery, but the majority of these tests are inconvenient to run in a clinical setting or involve unwieldy or expensive techniques.[11, 12] Despite this complexity, several reports have suggested that an elevated IL-6 concentration in the early post-operative period is a good predictor of post-operative SIRS.[4, 13]

Complex abdominal surgery can lead to the release of a broad array of PRR agonists including DAMPs released from the trauma itself, PAMPs released from commensal (e.g. due to gut translocation) or environmental contamination. In specialist surgical centres with technical expertise and dedicated peri-operative care, it is likely that the release of DAMPs and PAMPs related to the surgical procedure is relatively constant across patients, suggesting that this is not the only determinant that distinguishes patients who develop SIRS from those who do not. We hypothesise that the development of SIRS is a function of the responsiveness of host innate immune systems – principally circulating monocytes – to a given DAMP or PAMP load. In this study, we have focused on TLR4 and TLR5 and their bacterial ligands as examples of PRR sensing systems and also because Gram negative (LPS) and motile (flagellin) bacteria are representative of the majority of commensal bacteria likely to be liberated during surgery, for example *Escherichia coli*. Our aim was to study the TLR pathways involved in IL-6 production in patients at high risk of SIRS with the goal of identifying early biomarkers of an enhanced inflammatory response with adverse clinical consequences.

Materials and Methods

Patient selection
We included adult patients who were undergoing major liver or pancreatic
(non-emergency) resection at Barts Health HPB Centre between August 2011 and November
2013 and who gave written informed consent. Exclusion criteria were active concurrent
inflammatory disease (e.g. inflammatory bowel disease, rheumatoid arthritis), pre-operative
sepsis and treatment with chemotherapeutic or regular anti-inflammatory medications.

Data collection
Demographic, clinical, haematological and histological data were collected prospectively
from patient notes and the Electronic Patient Record system and included information on co-
morbidities, indication for surgery, cancer diagnosis, duration of the procedure, length of
hospital stay and in-hospital mortality. Patients were examined and observations charts
reviewed daily by the clinical team for the presence of SIRS or infection, starting on the
morning after surgery (Day 1). SIRS was defined according to international convention when
two or more of the following criteria were present: 1. body temperature <36°C or >38°C; 2.
heart rate >90 beats per minute; 3. tachypnoea with breathing rate >20 breaths per minute and
4. peripheral white cell count <4000 cells/mm^3 or >12,000 cells/mm.[3,14] Definitions of
infection were agreed *a priori* by the investigators and were based on the Center for Disease
Control and Prevention definitions.[15]

Venous blood sampling and separation
Bloods were taken at clinically indicated times pre-operatively, and on day 1 and day 2 post-
operatively. Serum was collected and stored at -80°C. Peripheral blood mononuclear cells
(PBMC) were separated by centrifugation over Ficoll Paque (GE Healthcare, Little Chalfont,
UK) to provide a source of monocytes. The resulting interface was aspirated, washed in PBS
and stored in 10% dimethyl sulfoxide (DMSO) freezing solution at -80°C.

TLR stimulation and cytokine production
A total of 2 x 10^6 PBMCs were re-suspended in 1ml complete medium (RPMI 1640
containing 10% fetal calf serum (FCS) 10U/ml penicillin G sodium, 10μg/ml streptomycin
sulfate), then rested for two hours and equally divided into a 12 well plate. Each well
contained 1ml complete medium per well. The cells were stimulated with 2ng/ml *S. typhi*
lipopolysaccharide (LPS, Sigma, Gillingham, UK) and 10μg/ml *S. typhi* flagellin (Invivogen,
Toulouse, France) for 24h. Supernatants were stored at -80°C. IL-6, TNF-α and IL-10
concentrations were measured in thawed supernatants and patient serum samples using
commercially available enzyme linked immunosorbent assay (ELISA) kits (R&D Systems,
Minneapolis, USA).

Measurement of TLR4 and TLR5 cell surface expression on monocytes
Thawed PBMCs were incubated at room temperature in the dark for 45min with a
combination of antibodies specific for the following: CD14 (Pacific Blue, clone M5E2),
CD16 (AlexaFluor 647®, clone 3G8), TLR4 (Phycoerythrin (PE), clone HTA125)
(Biolegend, Cambridge, UK) and TLR5 (Fluorscein isothiocyanate (FITC), clone 85B152.5)
(Abcam, Cambridge, UK). Specific antibody staining was determined with reference to an
isotype-matched control obtained from the same manufacturers. Cells were fixed in 1%
paraformaldehyde (PFA) and analysed immediately using a Beckton Dickinson Canto II flow
cytometer. TLR4 and TLR5 expression was determined and expressed as median
fluorescence intensity (MFI) of each marker on CD14$^+$, using Beckton Dickinson FACS Diva
software.

*Quantification of NF-ĸB, ERK1/2 and STAT1 phosphorylation in CD14$^+$ cells (Multiplex
PhosFlow)*

PBMCs were prepared as for TLR stimulation above and the contents of each well of a 6 well
plate were re-suspended in 200μl of complete medium. Following incubation, the cells were
aspirated from each well and transferred into FACS tubes. Each well was washed with 1ml
complete medium to ensure complete removal of any cells that had adhered to the base of the
well. The samples were then centrifuged at 300*g* for 5min and the cells re-suspended in
complete medium complete medium to ensure complete removal of any cells that had
adhered to the base of the well. The samples were then centrifuged at 300*g* for 5min and
the cells re-suspended in complete medium.

The following were added to the PBMCs in FACS tubes and incubated for 15min in a water
bath at 37°C; 10μg/ml flagellin, 2ng/ml LPS, 10,000u/ml IFN2α, or no agonist. Cells were
fixed in 1% PFA, permeabilized with Perm buffer III (BD Biosciences, San Jose, USA) on ice
in the dark for 30min. PBMCs were then washed in FACS buffer (500ml PBS, 10ml FCS,
0.1g sodium azide, 0.18g ethylenediaminetetraacetic acid (EDTA)) and the antibody cocktail

added: anti-phosphorylated STAT1 (Fluorscein isothiocyanate (FITC)), anti-phosphorylated NF-kB (Phycoerythrin (PE)), anti-phosphorylated ERK1/2 (Alexa Fluor 647 (AF647)) (BD Biosciences, Oxford, UK) and anti-CD14 (Pacific Blue) (Biolegend, Cambridge, UK). The PBMCs were incubated at room temperature for 45min in the dark, washed with FACS buffer and re-suspended in 300µl FACS buffer and immediately analysed on a Canto II flow cytometer. Colour compensation and gating were performed to select CD14$^+$ cells for analysis of NF-$\mathcal{D}$B, STAT1 and ERK1/2 phosphorylation. In each experiment specific antibody staining was determined with reference to an isotype-matched control. The data were analysed using WinList™ 3D version 3.1. (Verity Software House) and expressed as the percentage of CD14$^+$ cells that were positive for each phospho-antigen above isotype control.

Serum conditioning of healthy control PBMCs
PBMCs from healthy controls were prepared in the same way as patient samples. In serum conditioning experiments, 500µl patient serum was added to each well of a 12-well plate prior to stimulation with the same agonists as above. Supernatants were stored at -20°C until analysis by ELISA. PBMCs were then used for flow cytometry as described above.

Gene Expression
Blood was collected at each time point in a PAXGene™ blood RNA tube (PreAnalytix, Hilden, Germany). Total RNA was extracted using PAXGene™ blood RNA kits (PreAnalytix). Samples were analysed for RNA integrity and reverse transcribed to complementary DNA (cDNA). Gene expression was quantified by quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) using TLR4 and TLR5 Taqman assays (Applied Biosystems, Foster City, CA), which spanned the final two exons of the most common isoform of each gene, and were carried out on a 7900HT apparatus, Life Tech (Applied Biosystems, Foster City, CA) as previously described.[16] Each sample was assayed in triplicate. Reference genes (β2 microglobulin (*B2M*) and Ubiquitin C (*UBC*)) were selected empirically from a panel of six.[17] Relative quantification was calculated using the standard delta-delta methodology. Results were expressed as a normalized ratio of candidate gene / reference gene.

Statistical analysis
All data presented in the results section were tested for normality using the D'Agostino Pearson test. Continuous data were analysed using appropriate statistical tests (unpaired t test, Mann Whitney test) based on normality of distribution. Differences in categorical variables were calculated using a chi-squared or Fisher's exact test as appropriate. All statistical analysis was performed using GraphPad Prism and $p < 0.05$ was considered significant.

Results

Serum IL-6 concentrations predict post-surgical SIRS
We examined immune parameters associated with the development of SIRS in a cohort of 39 patients undergoing 'high risk' surgical procedures (Table 1). Mean patient age was 63.2 years (range 22-85), 52% (n=20) were male, and 95% (n=37) were Caucasian (Table 1).

A total of 24 patients underwent pancreaticoduodenectomy, 9 major liver resection, 4 distal pancreatectomy, 1 palliative bypass procedure and 1 excision of choledochal cyst. Of these 39 patients, 12 (30.7%) subsequently developed SIRS between post-operative days 2 and 12 (median day 6). The cause of each complication and the outcome are shown in Table 2. There were no differences in pre-operative haematology (haemoglobin/haematocrit, white cell count, platelets) or biochemistry (urea, creatinine, bilirubin) between patients who developed SIRS and those who have an uneventful recovery. The median length of hospital stay for patients who developed post-operative SIRS was significantly longer than for those who had an uneventful recovery (22 (range 12-52) vs 12 (range 6-24) days, p<0.001). All patients were managed with the same anaesthetic regime, and there were no differences in SIRS rates among the three surgeons who performed the procedures. There were no significant differences in intraoperative transfusion of blood products or key clinical parameters (haemoglobin, white cell count, creatinine, bilirubin or C-reactive protein (CRP)) between patients who developed SIRS and those who did not (Table 1). There were also no significant differences in operative time and blood loss volume, except in 6 patients who underwent right hemi-hepatectomy, which is a complex procedure with longer duration (295min vs 198min, p<0.05) and greater blood loss (2695ml vs 1344ml, p<0.01).

We examined post-operative serum concentrations of selected inflammatory cytokines (Fig. 1). Prior to the overt development of SIRS we found that serum concentrations of IL-6 were higher in patients who developed SIRS after surgery compared with those who did not (Fig. 1A, 122.4pg/ml vs 92.1pg/ml on day 2, p<0.05). Of note these changes in IL-6 cytokine levels pre-dated changes in any of the clinical parameters assessed. Post-operative serum concentrations of the anti-inflammatory cytokine IL-10 were reduced in both groups of patients compared to preoperative levels (Fig. 1B. 211.5 pg/ml pre-operatively vs 151.1 pg/ml on day 2, p<0.02), but there were no significant differences between groups.

Monocyte TLR expression is associated with post-operative SIRS
Ligation of microbial products by TLRs, including TLR4 and TLR5, leads to rapid production of pro-inflammatory cytokines such as IL-6. We tested the hypothesis that monocytes (a major producer of pro-inflammatory cytokines) expressed different levels of TLRs in patients who went on to develop SIRS. Expression levels (median fluorescence intensity, MFI) of TLR4 and TLR5 on CD14^{+} monocytes sampled on post-operative day 1 were 2.1-fold and 2.4-fold greater respectively in patients who developed post-operative SIRS than in those with an uneventful recovery (Fig. 2, p<0.0001). Similar results were seen on post-operative day 2, with 2.1-fold and 2.7-fold higher TLR4 and TLR5 expression (MFI) in patients who developed SIRS. There were no significant pre-operative differences in TLR4 or in TLR5 expression between the two groups of patients. This level of TLR expression in patients did not differ significantly from that seen in monocytes from 14 healthy control volunteers (7 males and 7 females, mean age 37.4 years (range 21-62)). In patients who did not develop post-operative SIRS, there was no significant change in monocyte TLR4 and TLR5 expression in the peri-operative period. However, in the SIRS group, there was a significant increase in expression of TLR4 (p<0.0002) and TLR5 (p<0.0001) early after surgery (individual patient data from post-operative days 1 and 2 are shown in Supplemental Digital Content 1).

Consistent with these data, on post-operative day 2 (but not pre-operatively, Fig. 3A) monocytes from patients who developed SIRS produced significantly more IL-6 following *ex vivo* stimulation with the TLR4 agonist LPS (1.11 ng/ml vs 300 pg/ml, p<0.0001) or TLR5 agonist flagellin (892 pg/ml vs 148 pg/ml, p<0.0001) when compared with monocytes from patients who did not develop SIRS (Fig. 3C). Stimulation with flagellin on post-operative day 1 also induced more IL-6 in patients who went on to develop SIRS compared to those who did not (Fig. 3B, 252 pg/ml vs 132 pg/ml, p=0.02), as was the case with LPS-induced IL-10 production on day 1 (Fig. 3E, 275pg/ml vs 182 pg/ml, p=0.03). No differences were seen in TLR-mediated TNF-α (Fig. 3G-H) although the tonic production of TNF-α by unstimulated monocytes was significantly reduced in the immediate post-operative period compared with pre-operatively (pre-operative mean 200pg/ml, 109pg/ml on day 1, 91pg/ml on day 2, p<0.0001 ANOVA). This change was noted in both patient groups. In contrast, IL-6 or IL-10 production by unstimulated monocytes did not differ between pre-operative and early post-operative samples.

These findings were unlikely to be due to the effects of stable circulating soluble mediators

(including IL-6 itself), since changes in TLR4 or TLR5 expression and ligand-stimulated cytokine production could not be replicated by culturing blood monocytes from healthy controls in patient serum, regardless of whether or not the serum was derived from a patient who subsequently developed SIRS (Supplemental Digital Content 2). Following LPS and flagellin stimulation, there was no significant difference in IL-6, TNF-α or IL-10 production between PBMCs treated with sera from patients who develop SIRS or patients who did not (Supplemental Digital Content 3).

We next sought to determine whether the observed intrinsic enhancement of monocyte TLR activation and cytokine production was associated with activation of canonical signalling pathways. We developed a multiplex assay based on the BD Bioscience PhosFlow system to study the phosphorylation of the key intracellular signalling molecules NF-κB, ERK1/2 and STAT1 (Supplemental Digital Content 4 and Fig. 4). In-line with our *ex vivo* cytokine production data, monocyte stimulation with LPS or flagellin resulted in greater NF-κB phosphorylation in patients who developed SIRS compared with patients who did not on post-operative day 1 and day 2 (Fig. 4B-C). LPS stimulation resulted in a 3.7-fold increase in the percentage of phosphorylated NF-κB-positive CD14+ cells on day 1 compared with non-SIRS patients ($p<0.01$) and 11.2-fold increase on day 2 ($p<0.005$). Flagellin stimulation resulted in a 3.8 fold increase in NF-κB phosphorylation on day 1 compared with non-SIRS patients ($p<0.05$) and an 8.9 fold increase on day 2 ($p<0.005$).

ERK1/2 phosphorylation in blood monocytes was consistently lower in patients who developed SIRS than in patients who did not when assessed in unstimulated ($p<0.04$), LPS-stimulated ($p<0.03$) or flagellin-stimulated cells ($p<0.03$) as assessed on post-operative day 2 (Fig. 4I). A similar trend was observed in monocytes from day 1, but these comparisons did not reach statistical significance. There were no pre-operative differences in unstimulated, LPS- or flagellin-induced NF-κB or ERK 1/2 phosphorylation. However, pre-operative induction of STAT1 phosphorylation by IFNα cytokine (but not LPS or flagellin) was 1.9 fold greater in monocytes from patients who developed SIRS compared with those who did not (Fig. 4D, $p<0.05$ by univariate analysis).

Taken together, these data suggest that patients who proceed to develop SIRS have increased TLR4 and TLR5 expression on blood monocytes in the immediate post-operative period. The functional consequence of this enhanced post surgical response is increased activation of the NF-κB-IL-6 signalling pathway. The strong positive correlations between TLR expression and agonist-induced IL-6 production suggest that these phenomena are linked *in vivo* (Supplemental Digital Content 5).

Elevated TLR expression on intermediate monocytes from patients who develop SIRS
To determine which monocyte subset was involved in the altered expression of TLR4 or TLR5 we studied CD16 expression to distinguish classical (CD14++,CD16-) intermediate (CD14++,CD16+) and non-classical (CD14+,CD16++) monocytes (Supplemental Digital Content 6). Pre-operatively, the proportions of intermediate monocytes were higher ($p<0.01$, Fig. 5) and those of classical monocytes lower ($p<0.05$) in patients who developed SIRS compared to those who did not, but there were no significant post-operative differences in these proportions. In all patients analysed here, monocyte expression of TLR4 ($p<0.0001$) and TLR5 ($p<0.0001$) were highest in the intermediate subset, and the increases in TLR4 and TLR5 levels seen in patients who proceeded to develop SIRS were restricted to this subset (Supplemental Digital Content 7). In keeping with the data from total monocytes, there were no pre-operative differences in TLR4 or TLR5 expression in any subset. However, on both post-operative days 1 and 2, TLR4 ($p<0.0004$) and TLR5 ($p<0.0001$) expression on intermediate monocytes was significantly higher in patients who developed SIRS compared with patients who had an uneventful recovery (Fig. 5). On day 2, TLR4 and TLR5 expression levels were 1.5-fold higher in patients who developed SIRS compared with those who did not. Thus, TLR4 and TLR5 expression on intermediate monocytes progressively increased over time in patients who subsequently developed SIRS, whereas they remained constant in patients who had a non-eventful recovery.

TLR5 expression predicts SIRS
The markers of immune dysfunction identified in this cohort of patients correctly identified all patients who subsequently developed clinical SIRS. We calculated the area under the receiver-operator curve (AUROC, Fig. 6) to estimate the accuracy of those tests that could be performed conveniently in a clinical pathology setting as predictors of SIRS. The AUROC, sensitivity, and specificity for IL-6, TLR4 and TLR5 expression on day 1 and day 2 were substantially greater than that for WCC on day 1, indicating greater accuracy for these tests compared to routine clinical parameters (Table 3). To confirm that increased monocyte expression in the post-operative period was associated with SIRS we took advantage of the availability of a second, independently collected cohort of 30 different patients from the same hospital undergoing similar surgery in whom whole blood gene expression profiling by

Taqman PCR had been performed along with stored serum to assess IL-6 levels. The baseline demographic and clinical characteristics of patients in the confirmatory cohort were similar to those of the index group with no significant differences in sex, age, ethnicity, intra-operative time, receipt of intra-operative blood products, pre-operative biochemistry and haematology (Table 1 and Table 4). In the confirmatory cohort, 8 patients (26.7%) developed SIRS in the post-operative period, all associated with subsequent development of infection. As in the original cohort, patients who developed SIRS had a significantly longer in-patient stay (median 22 vs 9 days, $p<0.001$). Patients in the confirmatory cohort who developed SIRS had significantly greater IL-6 concentration in the serum (233.9 pg/ml vs 71 pg/ml, $p=0.002$) on day 2 but this did not reach statistical significance on day 1 (Supplemental Digital Content 8). *TLR5* gene expression increased significantly post-operatively compared to pre-operative samples in this cohort. In keeping with the findings from our earlier protein-based studies in selected monocytes, the expression of *TLR5* mRNA in whole blood was 35% greater in patients who went on to develop SIRS compared to those who did not (Fig. 7, $p<0.01$).

Discussion

SIRS is a common complication of major surgery and identifying patients who will develop SIRS early in the post-operative period may allow changes in management that may lead to improved outcomes. Here we report that serum concentration of IL-6 cytokine on post-operative days 1 and 2 can be used to distinguish patients who will develop SIRS from those who will not - a median of 5 days before the onset of clinical signs in our series. We have studied the mechanisms that drive IL-6 production and show that in patients who develop post-operative SIRS, both LPS and flagellin can drive monocyte IL-6 production to a greater extent than in patients who do not, and this is likely to be mediated by upregulated TLR gene expression, as indicated both by studies of protein expression in an experimental cohort and confirmed by mRNA Taqman PCR in whole blood from patients in a confirmatory cohort. As such, we have identified TLR expression on monocytes, especially TLR5 on intermediate monocytes, as early and accurate biomarkers for post-operative SIRS.

IL-6 is a pleotropic inflammatory cytokine that forms an important part of the early response to injury or infection. It activates vascular endothelial cells increasing permeability and vascular leakage, stimulates the hepatic production of acute phase proteins (including C-reactive protein), promotes bone marrow production of inflammatory and immune cells, and can activate B and T lymphocytes.[18] We and others have observed that IL-6 is elevated in patients who develop SIRS, and although IL-6 concentrations were lower in our series than those reported by studies of similar patient groups,[4] it is a consistent finding that IL-6 levels on day 1 can distinguish patients who will develop SIRS from those who will not.[13] We further investigated the mechanisms underlying these data, and detected that the TLR/NF-κB/IL-6 axis is over-active in patients who develop SIRS. While responses to both TLR4 and TLR5 agonists were increased, the abnormality is specific to the NF-κB but not ERK1/2 signalling, and only IL-6 production was elevated (not TNF-α or IL-10), suggesting that additional intracellular factors determine selectivity of response. Activation of NF-κB in CD14$^+$ monocytes has recently been shown to be associated with poor recovery following orthopaedic surgery (recovery from fatigue, functional impairment of the hip, and pain after surgery), although this study did not explore the mechanisms or pathways that result in transcription factor activation.[19]

We have focussed on two key TLRs that mediate host responses to bacterial products, LPS and flagellin that together represent a large proportion of bacteria found in the gut and elsewhere, although the exact source of these products is as yet unclear. The role of additional DAMPs or PAMPs and any of the over 40 PRRs (e.g. lipoteichoic acid ligation of TLR2 or uric acid and NALP3) is currently unknown. We postulate that pro-inflammatory signals (PAMPs or DAMPs), perhaps from dead cells or from host commensal bacteria are liberated during surgery and may act as potential triggers of inflammation.

TLR-mediated production of type 1 IFN (IFNα/β) is a very early step in the initiation of inflammation. Autocrine and paracrine type 1 IFN binds to the cognate receptor and activates signalling mediators including STAT1 and STAT2, which in-turn drive
IRF-mediated gene expression and amplify the response.[20] It is therefore of particular interest to note pre-operative differences in IFNα-induced STAT1 phosphorylation between patients who develop SIRS and those who do not. One attractive hypothesis is that individuals who develop SIRS are pre-disposed to excessive inflammation with a 'primed' STAT1 response. For a given degree of tissue damage, endogenous bacterial translocation (or an alternative stimulus), this 'pre-primed' state facilitates a more robust inflammatory response, which may include expression of TLRs (known IFN stimulated genes).[21] In keeping with this, we have shown an increased abundance of TLR5 mRNA in patients who develop SIRS. However our data do not allow us to identify the mediator of the change, although inhibitors of TLR and IFN signalling – such as suppressor of cytokine signalling (SOCS) and protein inhibitor of activate STATs (PIAS) proteins are potential candidates.

Patients who develop SIRS have a greater proportion of CD14^{++}/CD16$^+$ (intermediate) monocytes pre-operatively. Intermediate monocytes are considered pro-inflammatory with greater production of TNF-α and IL-1β following stimulation with LPS, increased interaction with endothelial cells and greater chemokine production compared to other subsets.[22] We show that intermediate monocytes express higher levels of TLR4[23] and TLR5 (previously not known) than CD14$^+$/CD16$^-$ classical or CD14$^+$/CD16^{++} non-classical monocytes at all time points. The increased expression of TLR4 and TLR5 detected on monocytes from patients who develop SIRS was restricted to the intermediate subset, and accordingly, the accuracy of predicting SIRS is increased by measuring TLR4 and TLR5 on intermediate monocytes only (compared with total monocytes).

The incidence of post-operative SIRS in our main cohort was 31.7%, and SIRS was associated with a significantly increased length of hospital stay (22 days vs 13 days) and a higher mortality rate (15.3%). Despite the heterogeneity of the operations included in this study, these outcomes are in keeping with published data[24, 25] suggesting that our conclusions are applicable more widely. The diagnostic accuracy of the tests proposed in this study will now require validation in a larger, independent cohort that includes patients undergoing a broader range of surgery, and it may be possible to refine our protocols yet further to enable these tests to be conducted in a routine clinical setting.

The patients who developed SIRS in this study had worse outcomes than those who did not develop SIRS, in terms of length of hospital stay, use of intravenous antibiotics, return to HDU or further radiological or surgical procedures. Organ dysfunction was not a common feature and only seen in the patients who died. This highlights the potential clinical utility of the markers we identify as sensitive indicators of events that can be avoided or whose impact can be reduced (e.g. by the early introduction of antibiotics), rather than indicators of extreme complications that are likely to be irredeemable.

Currently-used 'early warning' scoring systems are based on routinely collected clinical data, but these can be difficult to interpret in post-operative patients in whom clinical observations may already be deranged. These scores are therefore unable to identify patients at risk until SIRS is established, or nearly established. Our current experience suggests that this is too late to implement preventative measures such as low-dose glucocorticoids, antibiotics and/or prolonged stay on the high-dependency unit. In contrast, the novel approach that we propose may permit the use of interventions that are targeted against key inflammatory mediators of SIRS pathogenesis early after surgery, particularly given that none of the patients in the current study had met SIRS criteria at the time of blood sampling. This suggests that at this time, general or specific interventions can be implemented to avert SIRS. Although targeted therapies such as specific TLR4 inhibitors did not alter outcomes in patients with severe sepsis,[26, 27] this may be due to the fact that patients were already critically unwell and at advanced stages of SIRS when given a TLR4 antagonist. We hypothesise that treating patients with TLR4 inhibitors or agents that impair signalling through TLR5 or NF-kB may provide therapeutic benefit before the clinical manifestations of SIRS or sepsis become apparent.

In conclusion, we have identified a novel mechanism by which innate immune dysfunction occurs early in the post-operative period in patients who develop SIRS – long in advance of clinical signs. Abnormally elevated IL-6 levels in patients who will develop SIRS are related to upregulated TLR4 and TLR5 expression and preferential activation of NF-DB. We hypothesise that prior to surgery, patients who develop SIRS exhibit an increased proportion of intermediate monocytes that are primed to react in this way. Clearly more work is needed to develop the clinical application of the markers we have identified here. However, we believe that the strategy we have employed here to study the mechanisms of disease have identified novel biomarkers as well as potential targets for therapeutic intervention.

Figure & Table Legends:

Figure 1. Serum IL-6 concentration is greater in patients who develop post-operative SIRS.

Figure 2. Increased expression of TLR4 and TLR5 on CD14$^+$ monocytes sampled in the early post-operative period predicts development of post-operative SIRS.

Figure 3. Effects of TLR stimulation on peripheral blood mononuclear cell cytokine production following major HPB surgery.

Figure 4. Increased TLR-mediated NF-kB phosphorylation in CD14+ monocytes sampled in the early post-operative period predicts development of post-operative SIRS.

Figure 5. Expression of TLR4 and TLR5 on intermediate monocytes sampled in the early post-operative period predicts development of post-operative SIRS.

Figure 6. Accuracy of markers of innate immune dysfunction in predicting SIRS.

Figure 7. Taqman TLR gene expression in whole blood sampled in the pre-operative period predicts the development of SIRS in a confirmatory cohort of patients.

Table 1. Demographic and clinical parameters - experimental cohort.

Table 2. Causes of SIRS and outcome in patients who developed post-operative SIRS in the experimental cohort.

Table 3. Area under the receiver-operator curve for prediction of SIRS in patients undergoing complex HPB surgery.

Table 4. Demographic and clinical parameters - confirmation cohort.

References

1. Ferraris VA, Ballert EQ, Mahan A. The relationship between intraoperative blood transfusion and postoperative systemic inflammatory response syndrome. *Am J Surg* 2013; 205:457-65.
2. Comstedt P, Storgaard M, Lassen AT. The Systemic Inflammatory Response Syndrome (SIRS) in acutely hospitalised medical patients: a cohort study. *Scand J Trauma Resusc Emerg Med* 2009; 17:67.
3. Kelly KJ, Greenblatt DY, Wan Y, et al. Risk stratification for distal pancreatectomy utilizing ACS-NSQIP: preoperative factors predict morbidity and mortality. *J Gastrointest Surg* 2011; 15:250-9, discussion 259-61.
4. Mokart D, Merlin M, Sannini A, et al. Procalcitonin, interleukin 6 and systemic inflammatory response syndrome (SIRS): early markers of postoperative sepsis after major surgery. *Br J Anaesth* 2005; 94:767-73.
5. Akira S, Takeda K. Toll-like receptor signalling. *Nat Rev Immunol* 2004; 4:499-511.
6. Fallon PG, Allen RL, Rich T. Primitive Toll signalling: bugs, flies, worms and man. *Trends Immunol* 2001; 22:63-6.
7. Hayashi F, Smith KD, Ozinsky A, et al. The innate immune response to bacterial flagellin is mediated by Toll-like receptor 5. *Nature* 2001; 410:1099-103.
8. Akira S, Uematsu S, Takeuchi O. Pathogen recognition and innate immunity. *Cell* 2006; 124:783-801.
9. Poltorak A, He X, Smirnova I, et al. Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. *Science* 1998; 282:2085-8.
10. Chang L, Karin M. Mammalian MAP kinase signalling cascades. *Nature* 2001; 410:37-40.
11. Durila M, Bronsky J, Harustiak T, et al. Early diagnostic markers of sepsis after oesophagectomy (including thromboelastography). *BMC Anesthesiol* 2012; 12:12.
12. Hinrichs C, Kotsch K, Buchwald S, et al. Perioperative gene expression analysis for prediction of postoperative sepsis. *Clin Chem* 2010; 56:613-22.
13. Mokart D, Capo C, Blache JL, et al. Early postoperative compensatory anti-inflammatory response syndrome is associated with septic complications after major surgical trauma in patients with cancer. *Br J Surg* 2002; 89:1450-6.
14. American. American College of Chest Physicians/Society of Critical Care Medicine Consensus Conference: definitions for sepsis and organ failure and guidelines for the use of innovative therapies in sepsis. *Crit Care Med* 1992; 20:864- 74.
15. Horan TC, Andrus M, Dudeck MA. CDC/NHSN surveillance definition of health care-associated infection and criteria for specific types of infections in the acute care setting. *Am J Infect Control* 2008; 36:309-32.
16. Torrance HD, Brohi K, Pearse RM, et al. Association Between Gene Expression Biomarkers of Immunosuppression and Blood Transfusion in Severely Injured Polytrauma Patients. *Ann Surg* 2014.
17. Vandesompele J, De Preter K, Pattyn F, et al. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol* 2002; 3:RESEARCH0034.
18. Dienz O, Rincon M. The effects of IL-6 on CD4 T cell responses. *Clin Immunol* 2009; 130:27-33.
19. Gaudilliere B, Fragiadakis GK, Bruggner RV, et al. Clinical recovery from surgery correlates with single-cell immune signatures. *Sci Transl Med* 2014; 6:255ra131.
20. Yarilina A, Ivashkiv LB. Type I interferon: a new player in TNF signaling. *Curr Dir Autoimmun* 2010; 11:94-104.
21. Khoo JJ, Forster S, Mansell A. Toll-like receptors as interferon-regulated genes and their role in disease. *J Interferon Cytokine Res* 2011; 31:13-25.
22. Ziegler-Heitbrock L. The CD14+ CD16+ blood monocytes: their role in infection and inflammation. *J Leukoc Biol* 2007; 81:584-92.
23. Pedraza-Sanchez S, Hise AG, Ramachandra L, et al. Reduced frequency of a CD14+ CD16+ monocyte subset with high Toll-like receptor 4 expression in cord blood compared to adult blood contributes to lipopolysaccharide hyporesponsiveness in newborns. *Clin Vaccine Immunol* 2013; 20:962-71.
24. Cameron JL, Riall TS, Coleman J, et al. One thousand consecutive pancreaticoduodenectomies. *Ann Surg* 2006; 244:10-5.
25. Mukherjee S, Kocher HM, Hutchins RR, et al. Impact of hospital volume on outcomes for pancreaticoduodenectomy: a single UK HPB centre experience. *Eur J Surg Oncol* 2009; 35:734-8.
26. Opal SM, Laterre PF, Francois B, et al. Effect of eritoran, an antagonist of MD2-TLR4, on mortality in patients with severe sepsis: the ACCESS randomized trial. *JAMA* 2013; 309:1154-62.
27. Shirey KA, Lai W, Scott AJ, et al. The TLR4 antagonist Eritoran protects mice from lethal influenza infection. *Nature* 2013; 497:498-502.

28.	Mokart D, Merlin M, Sannini A, et al. Procalcitonin, interleukin 6 and systemic inflammatory response syndrome (SIRS): early markers of postoperative sepsis after major surgery. *Br J Anaesth* 2005; 94:767-73.
29.	Akira S, Takeda K. Toll-like receptor signalling. *Nat Rev Immunol* 2004; 4:499-511.
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31.	Hayashi F, Smith KD, Ozinsky A, et al. The innate immune response to bacterial flagellin is mediated by Toll-like receptor 5. *Nature* 2001; 410:1099-103.
32.	Akira S, Uematsu S, Takeuchi O. Pathogen recognition and innate immunity. *Cell* 2006; 124:783-801.
33.	Poltorak A, He X, Smirnova I, et al. Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. *Science* 1998; 282:2085-8.
34.	Chang L, Karin M. Mammalian MAP kinase signalling cascades. *Nature* 2001; 410:37-40.
35.	Durila M, Bronsky J, Harustiak T, et al. Early diagnostic markers of sepsis after oesophagectomy (including thromboelastography). *BMC Anesthesiol* 2012; 12:12.
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40.	Torrance HD, Brohi K, Pearse RM, et al. Association Between Gene Expression Biomarkers of Immunosuppression and Blood Transfusion in Severely Injured Polytrauma Patients. *Ann Surg* 2014.
41.	Vandesompele J, De Preter K, Pattyn F, et al. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol* 2002; 3:RESEARCH0034.
42.	Dienz O, Rincon M. The effects of IL-6 on CD4 T cell responses. *Clin Immunol* 2009; 130:27-33.
43.	Gaudilliere B, Fragiadakis GK, Bruggner RV, et al. Clinical recovery from surgery correlates with single-cell immune signatures. *Sci Transl Med* 2014; 6:255ra131.
44.	Yarilina A, Ivashkiv LB. Type I interferon: a new player in TNF signaling. *Curr Dir Autoimmun* 2010; 11:94-104.
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47.	Pedraza-Sanchez S, Hise AG, Ramachandra L, et al. Reduced frequency of a CD14+ CD16+ monocyte subset with high Toll-like receptor 4 expression in cord blood compared to adult blood contributes to lipopolysaccharide hyporesponsiveness in newborns. *Clin Vaccine Immunol* 2013; 20:962-71.
48.	Cameron JL, Riall TS, Coleman J, et al. One thousand consecutive pancreaticoduodenectomies. *Ann Surg* 2006; 244:10-5.
49.	Mukherjee S, Kocher HM, Hutchins RR, et al. Impact of hospital volume on outcomes for pancreaticoduodenectomy: a single UK HPB centre experience. *Eur J Surg Oncol* 2009; 35:734-8.
50.	Opal SM, Laterre PF, Francois B, et al. Effect of eritoran, an antagonist of MD2-TLR4, on mortality in patients with severe sepsis: the ACCESS randomized trial. *JAMA* 2013; 309:1154-62.
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Supplemental Digital Content:

Supplemental Digital Content 1. Daily change in TLR4 and TLR5 expression in patients undergoing hepatobiliary surgery.

Supplemental Digital Content 2. TLR4 and TLR5 expression on CD14$^+$ monocytes from healthy volunteers with and without incubation with patient sera.

Supplemental Digital Content 3. Cytokine production in supernatants from peripheral blood mononuclear cells from healthy volunteers with and without incubation with patient sera.

Supplemental Digital Content 4. Representative FACS plot showing increase in proportion of cells with expression of phosphorylated NF-κB following stimulation with lipopolysaccharide (LPS) and phosphorylated STAT1 following stimulation with interferon(IFN)-γ.

Supplemental Digital Content 5. TLR expression and TLR-mediated cytokine production are correlated.

Supplemental Digital Content 6. Representative FACS plot showing classical (CD14^{++}CD16$^-$), intermediate (CD14^{++}CD16$^+$) and non-classical (CD14$^+$CD16^{++}) monocytes.

Supplemental Digital Content 7. Expression of TLR4 and TLR5 is higher on intermediate monocytes compared to classical monocytes at all time points. Supplemental Digital Content 8. Serum IL-6 concentration is greater in patients who develop post-operative SIRS in the confirmatory cohort

	No SIRS (n=27)		SIRS (n=12)		P value
Sex	Male	Female	Male	Female	p=ns
	14	13	6	6	
Ethnicity	Caucasian	Other	Caucasian	Other	p=ns
	26	1	11	1	
Operative Factors					
Operative time (mins)	268 (135-460)		327 (170-585)		p=ns
Blood loss (mls)	773 (190-2010)		1088 (150-3200)		p=ns
Intra-operative transfusion (n(%))	7 (26)		6 (50)		p=ns
Pre-operative values					
Haemoglobin (g/dl)	12.8 (11-14.5)		13.1 (12-14.5)		p=ns
WCC (x10³/ml)	7.9 (5.2-10)		8.1 (5.2-10.4)		p=ns
Platelets (x10³/ml)	265 (129-402)		274 (138-411)		p=ns
Urea (mmol/l)	4.8 (3.8-8.6)		5.1 (3.5-8.4)		p=ns
Creatinine (Dmol/l)	81 (48-147)		79 (59-102)		p=ns
Post-operative day one					
Haemoglobin (g/dl)	10.2 (9.0-12.5)		10.2 (8.6-12.5)		p=ns
WCC (x10³/ml)	14.1 (8.0-17.9)		13 (9.2-15.1)		p=ns
Platelets (x10³/ml)	210 (98-429)		221 (104-442)		p=ns
Urea (mmol/l)	6.9 (3.9-10.5)		6.6 (4.4-10.2)		
Creatinine (Dmol/l)	81 (41-143)		75 (49-100)		p=ns
CRP (mg/l)	53 (5-108)		49 (21-76)		p=ns
Post-operative day two					
Haemoglobin (g/dl)	9.4 (7.8-12.8)		9.6 (7.9-13.0)		p=ns
WCC (x10³/ml)	14.5 (7.8-24.1)		14.1 (7.5-20.3)		p=ns
Platelets (x10³/ml)	198 (83-389)		202 (99-400)		p=ns
Urea (mmol/l)	6.2 (3.3-8.5)		6.3 (3.4-8.8)		p=ns
Creatinine (Dmol/l)	78 (41-150)		81 (48-167)		p=ns
CRP (mg/l)	119 (48-257)		113 (23-203)		p=ns

Table 1. Demographic and clinical parameters - experimental cohort. Data presented as mean (range) unless indicated otherwise. WCC=white cell count, CRP = C-reactive protein.

Operation	Day SIRS/Sepsis criteria met	Cause	Outcome	Discharge
PPPD	POD 6	Lower respiratory tract infection	IV antibiotics and transferred to HDU for positive pressure ventilation	POD 27
PPPD	POD 8	Infected intra-abdominal collection	IV antibiotics. Did not require drainage	POD 16
PPPD	POD 6	Lower respiratory tract infection	Oral antibiotics	POD 14
PPPD	POD 5	Infected post-operative collections	IV antibiotics and radiological drainage. Returned to HDU for supportive care	POD 52
DP	POD 4	Lower respiratory tract infection	IV antibiotics. Prolonged HDU stay	POD 28
Excision of choledochal cyst	POD 2	Tachycardia and hypotensive with a raised WCC	Supportive measures. No evidence of sepsis	POD 12
Right hemi-hepatectomy	POD 6	Liver abscess	IV antibiotics. Radiological drain inserted.	POD 18
DP	POD 6	Lower respiratory tract infection	IV antibiotics.	POD 15
PPPD	POD 4	Infected post-operative collection	IV antibiotics. Radiological drain inserted.	POD 20
PPPD	POD 12	Bibasal lower respiratory tract infection	IV antibiotics.	POD 22
Right hemi-hepatectomy	POD 10	Lower respiratory tract infection	IV antibiotics.	POD 19

		Mortality		
Operation	**Day SIRS/Sepsis criteria met**	**Cause**	**Outcome**	**Death**
PPPD	POD 2	Adult respiratory distress syndrome (ARDS) secondary to pancreatic leak	IV antibiotics and ventilator and dialysis support. Re-look laparotomy on POD 3.	POD 4
PPPD	POD 1	Grade 3 pancreatic leak. SMA thrombus leading to small bowel ischaemia	Re-look laparotomy. Degree of damage to small bowel unsalvageable.	POD 3

Table 2. Causes of SIRS and outcome in patients who developed post-operative SIRS in the experimental cohort. PPPD – pylorus-preserving pancreaticoduodenectomy, DP – distal pancreatectomy, WCC – white cell count, SMA – superior mesenteric artery, IV – intravenous.

Parameter	AUROC	95% CI	P value	Cut off*	Sensitivity (%)	Specificity (%)
Day 1						
WCC	0.71	0.55 – 0.88	0.03	13.35×10^3/ ml	61.5	82.1
IL6	0.88	0.72 – 1.03	0.006	97.7 pg/ml	75	83
Total TLR4	0.87	0.75 – 0.99	0.0004	2000	75.0	68.2
Intermediate TLR4	0.95	0.86 – 1.04	0.001	8793	88.9	88.9
TLR5	0.92	0.83 – 1.00	<0.0001	2399	75.0	81.2
Intermediate TLR5	0.89	0.74 – 1.00	0.005	5437	88.9	77.8
Day 2						
IL-6	0.98	0.93-1.03	0.0004	97.7 pg/ml	100	83.3
TLR4	0.93	0.83 – 1.02	<0.0001	2997	83.3	71.4
Intermediate TLR4	0.98	0.92 – 1.04	0.0007	9515	100	88.9
TLR5	0.97	0.92 – 1.03	<0.0001	3739	91.7	100
Intermediate TLR5	1.00	1.00 – 1.00	0.0003	7426	100	100

Table 3. Area under the receiver-operator curve for prediction of SIRS in patients undergoing complex HPB surgery. * mean fluorescence intensity units unless otherwise indicated. WCC=white cell count

	No SIRS (n=22)		SIRS (n=8)		P value
Sex (n)	Male 13	Female 9	Male 7	Female 1	p=ns
Age (years)	66 (46-84)		64 (47-81)		p=ns
Ethnicity (n)	Caucasian 20	Other 2	Caucasian 6	Other 2	p=ns
Operative Factors					
Operative time (mins)	306 (120-620)		300 (150-470)		p=ns
Intra-operative transfusion (n(%))	4 (18.2)		2 (25)		p=ns
Pre-operative values					
Haemoglobin (g/dl)	12.9 (9.2-15.5)		13.8 (7.3-15.0)		p=ns
WCC (x10^3/ml)	8.4 (4.4-13.3)		9.4 (5.7-15.9)		p=ns
Platelets (x10^3/ml)	319 (180-474)		254 (132-397)		p=ns
Urea (mmol/l)	5.1 (2.8-9.2)		5.9 (4.7-6.7)		p=ns
Creatinine (Dmol/l)	75 (39-141)		80 (51-101)		p=ns
Post-operative day one					
Haemoglobin (g/dl)	11.3 (9.9-11.7)		11.4 (9.7-12.3)		p=ns
WCC (x10^3/ml)	14.1 (8.1-23.9)		16.0 (9.2-22.3)		p=ns
Platelets (x10^3/ml)	248 (141-471)		240 (149-441)		p=ns
Urea (mmol/l)	5.5 (3.3-9.4)		6.7 (5.2-8.7)		p=ns
Creatinine (Dmol/l)	70 (39-146)		78 (57-101)		p=ns
CRP (mg/l)	72 (40-95)		46 (20-85)		p=ns
Post-operative day two					
Haemoglobin (g/dl)	10.5 (8.9-11.1)		10.5 (10.1-11.7)		p=ns
WCC (x10^3/ml)	12.8 (9.2-18.2)		16.0 (10.7-22.0)		p=0.02
Platelets (x10^3/ml)	239 (149-441)		210 (117-283)		p=ns
Urea (mmol/l)	5.1 (2.3-8.7)		8.2 (5.4-12.1)		P<0.001
Creatinine (Dmol/l)	66 (35-159)		80 (55-106)		p=ns
CRP (mg/l)	146 (127-243)		198 (110-244)		p=ns

Table 4. Demographic and clinical parameters - confirmation cohort. Data presented as mean(range) unless indicated otherwise. WCC=white cell count, CRP = C-reactive protein.
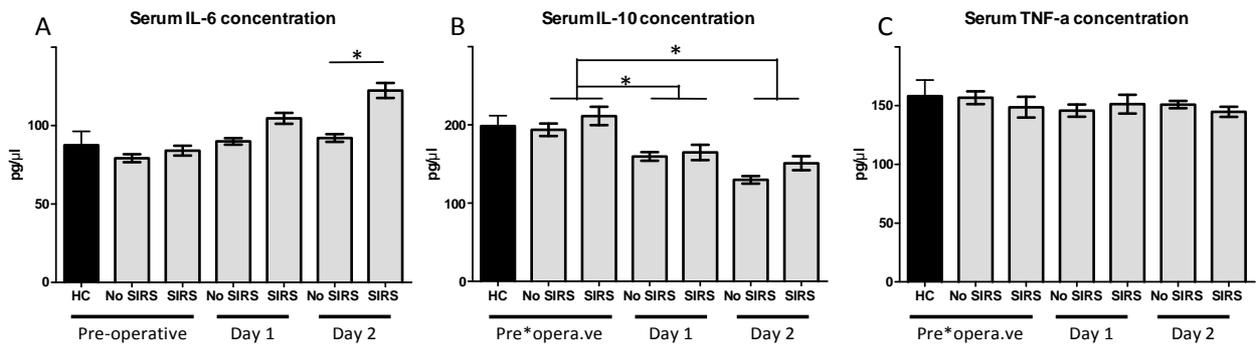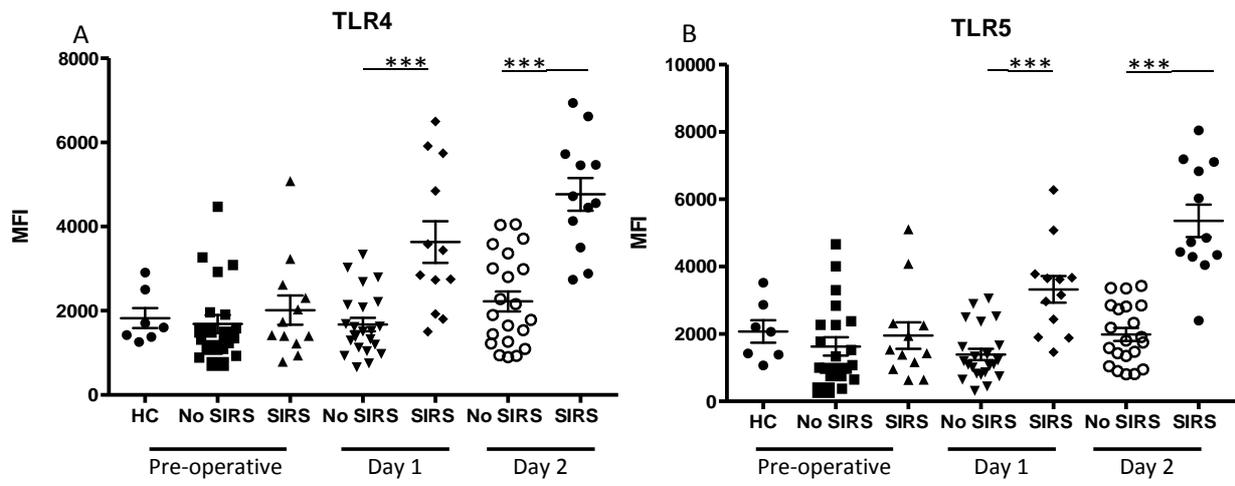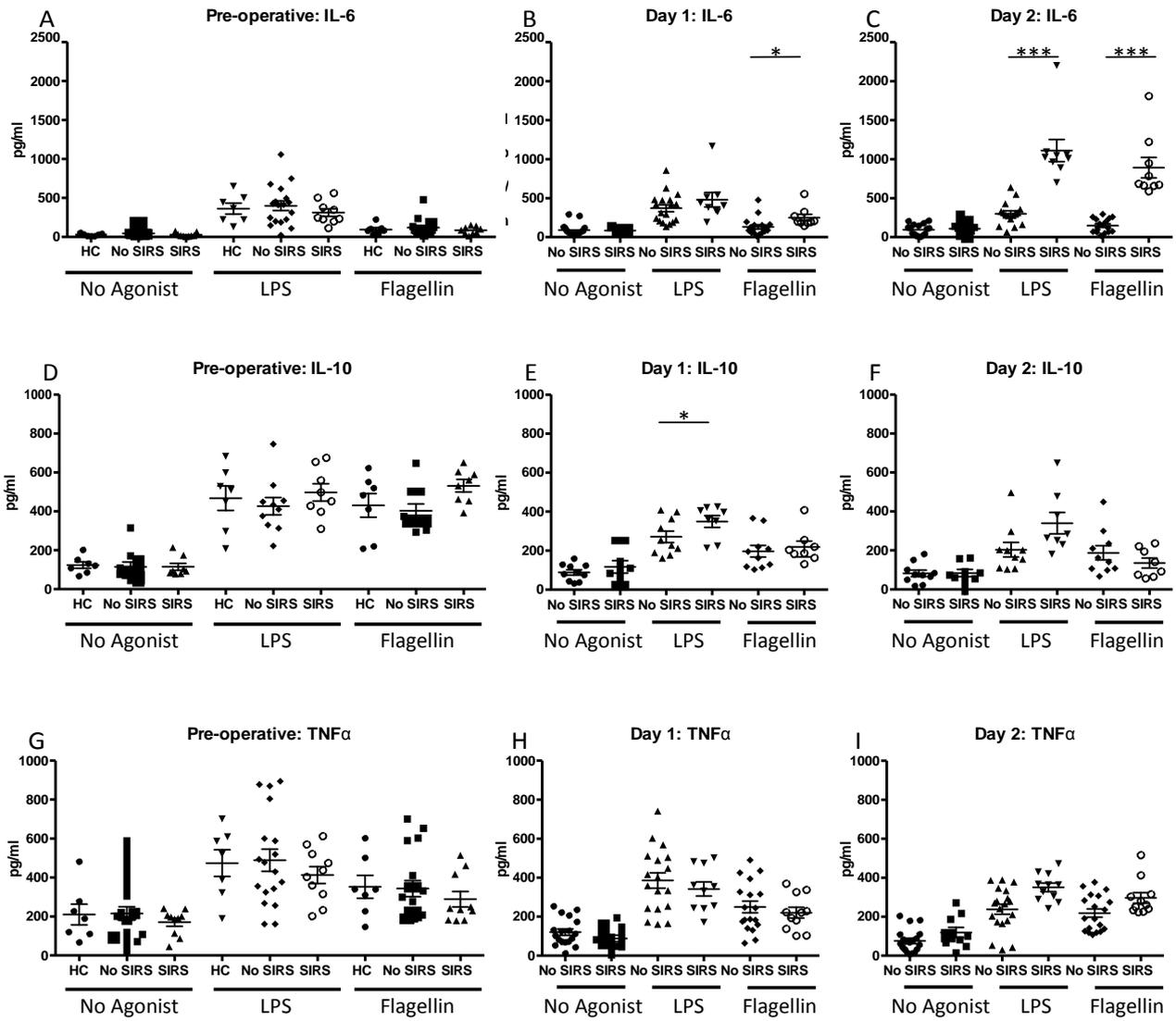
Figure 1

A   Serum IL-6 concentration

pg/µl

HC   No SIRS   SIRS   No SIRS   SIRS   No SIRS   SIRS

Pre-operative   Day 1   Day 2

B   Serum IL-10 concentration

pg/µl

HC   No SIRS   SIRS   No SIRS   SIRS   No SIRS   SIRS

Pre*opera.ve   Day 1   Day 2

C   Serum TNF-a concentration

pg/µl

HC   No SIRS   SIRS   No SIRS   SIRS   No SIRS   SIRS

Pre*opera.ve   Day 1   Day 2

Figure 2

Figure 3

Figure 4

A — Pre-operative: NFkB

B — Day 1: NFkB

C — Day 2: NFkB

D — Pre-operative: STAT1

E — Day 1: STAT1

F — Day 2: STAT1

G — Pre-operative: ERK 1/2

H — Day 1: ERK 1/2

I — Day 2: ERK 1/2

Figure 5

A   **Pre-operative: Monocytes**

B   **Day 1: Monocytes**

C   **Day 2: Monocytes**

D   **TLR4: Intermediate Monocytes**

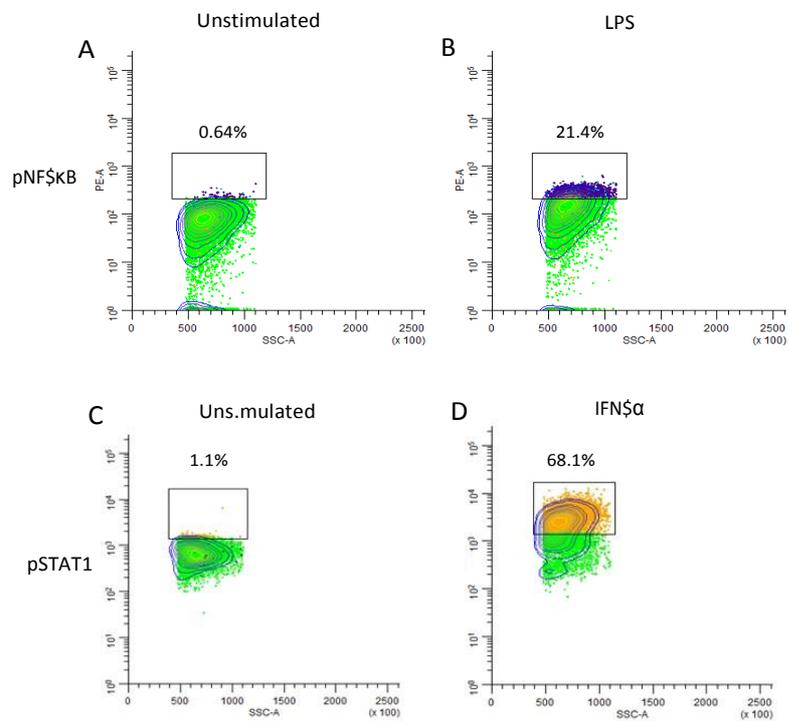E   **TLR5: Intermediate Monocytes**
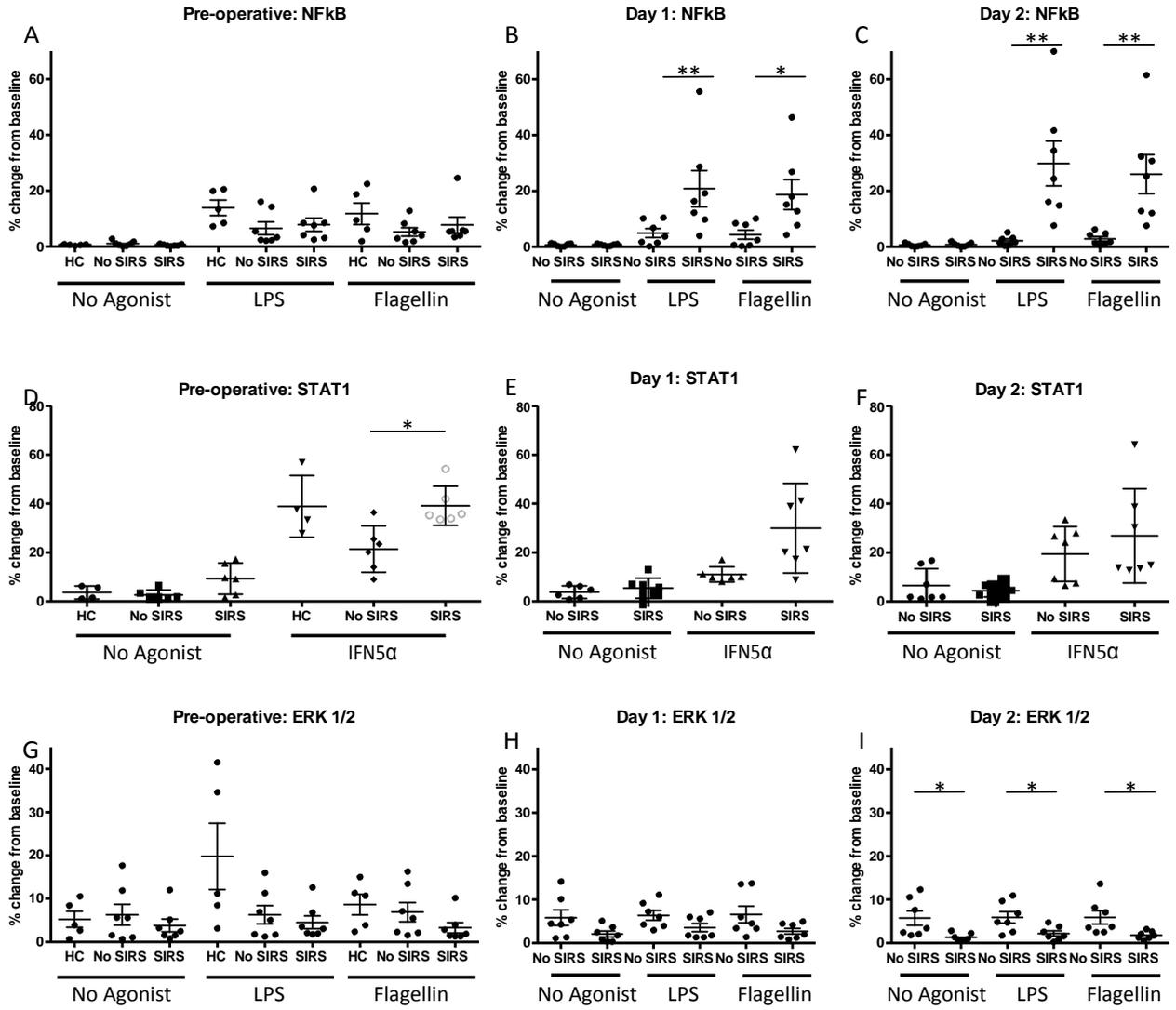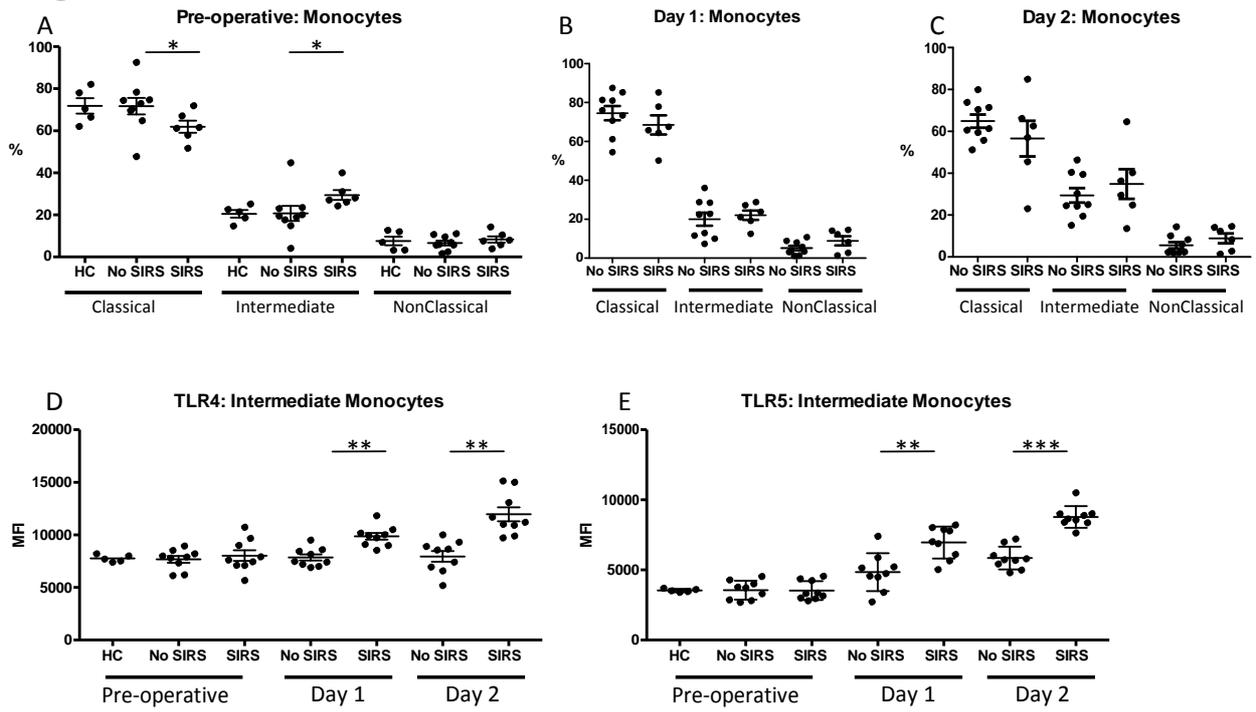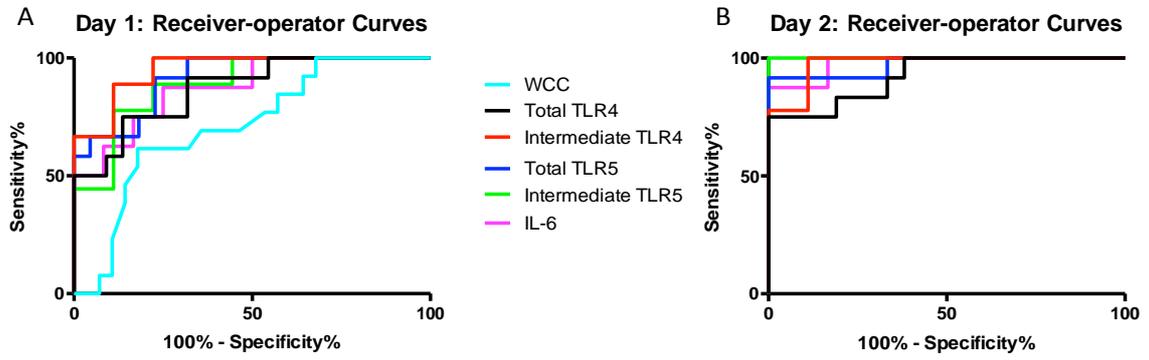
Figure 6

Figure 7
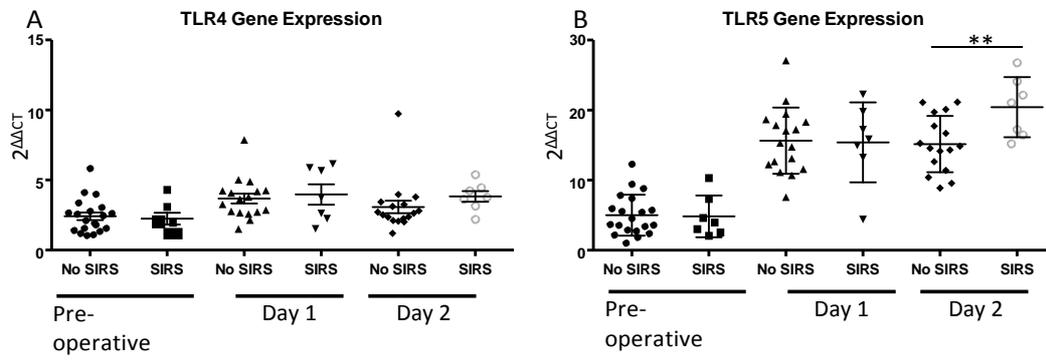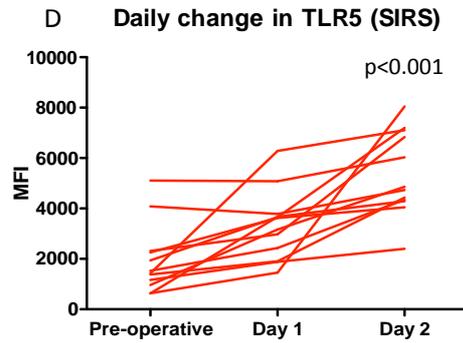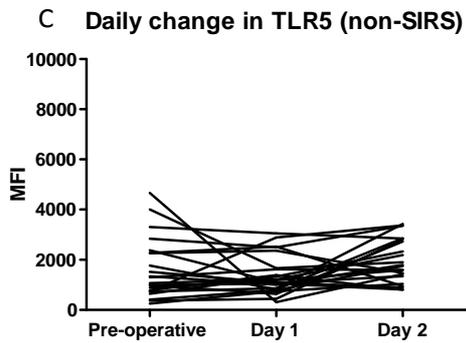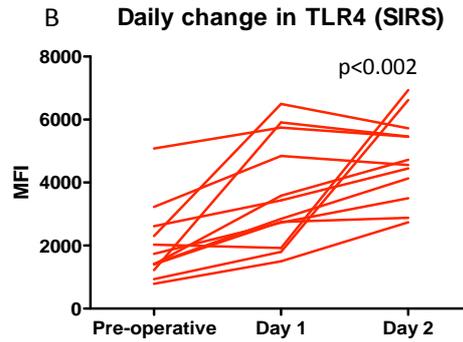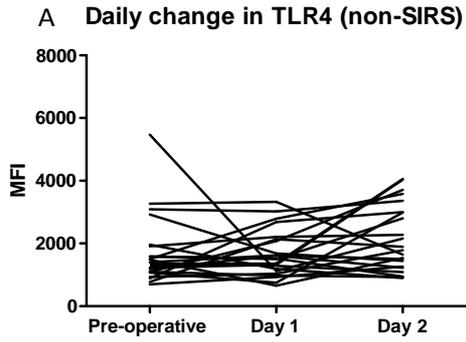
Supplemental Digital Content 1. Daily change in TLR4 (A, B) and TLR5 (C, D) expression in patients undergoing hepatobiliary surgery. Median fluorescence intensity of TLR4 and TLR5 in patients who have an uneventful recovery (No SIRS) and patients who develop post-operative SIRS (SIRS).
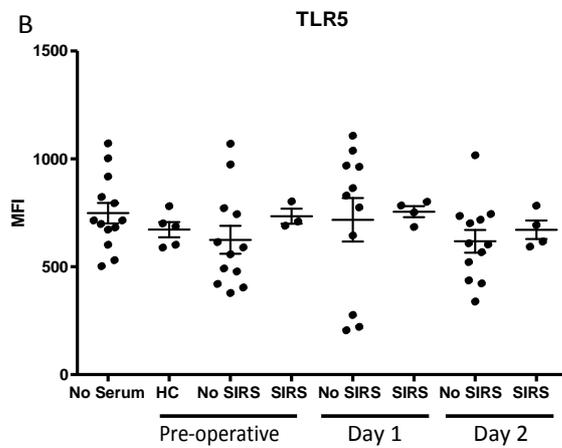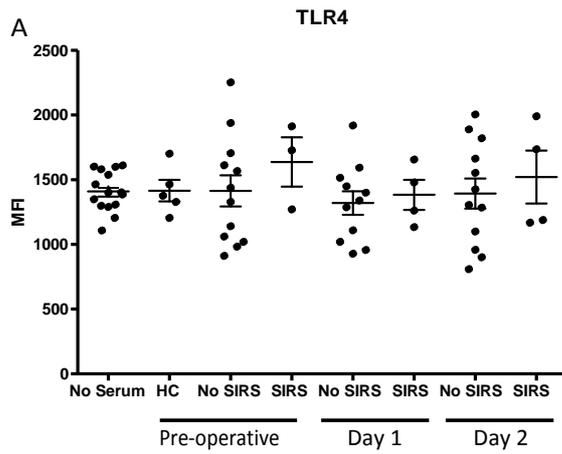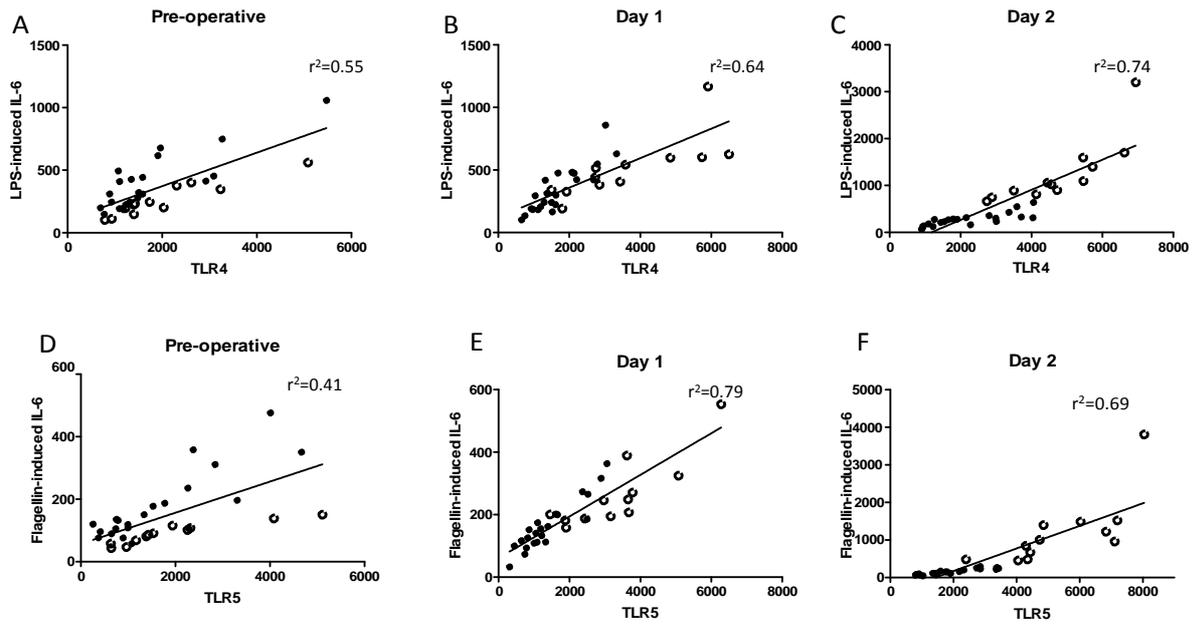
Supplemental Digital Content 2. TLR4 and TLR5 expression on CD14⁺ monocytes from healthy volunteers with and without incubaƟon with paƟent sera. TLR4 (A) and TLR5 (B) expression on CD14⁺ monocytes from healthy volunteers with and without incubation with patient sera. TLR expression was evaluated on healthy PBMCs without added serum (No Serum), maintained in media conditioned with serum from healthy controls (HC), patients who had an unevenful recovery (No SIRS) or patients who developed SIRS (SIRS).
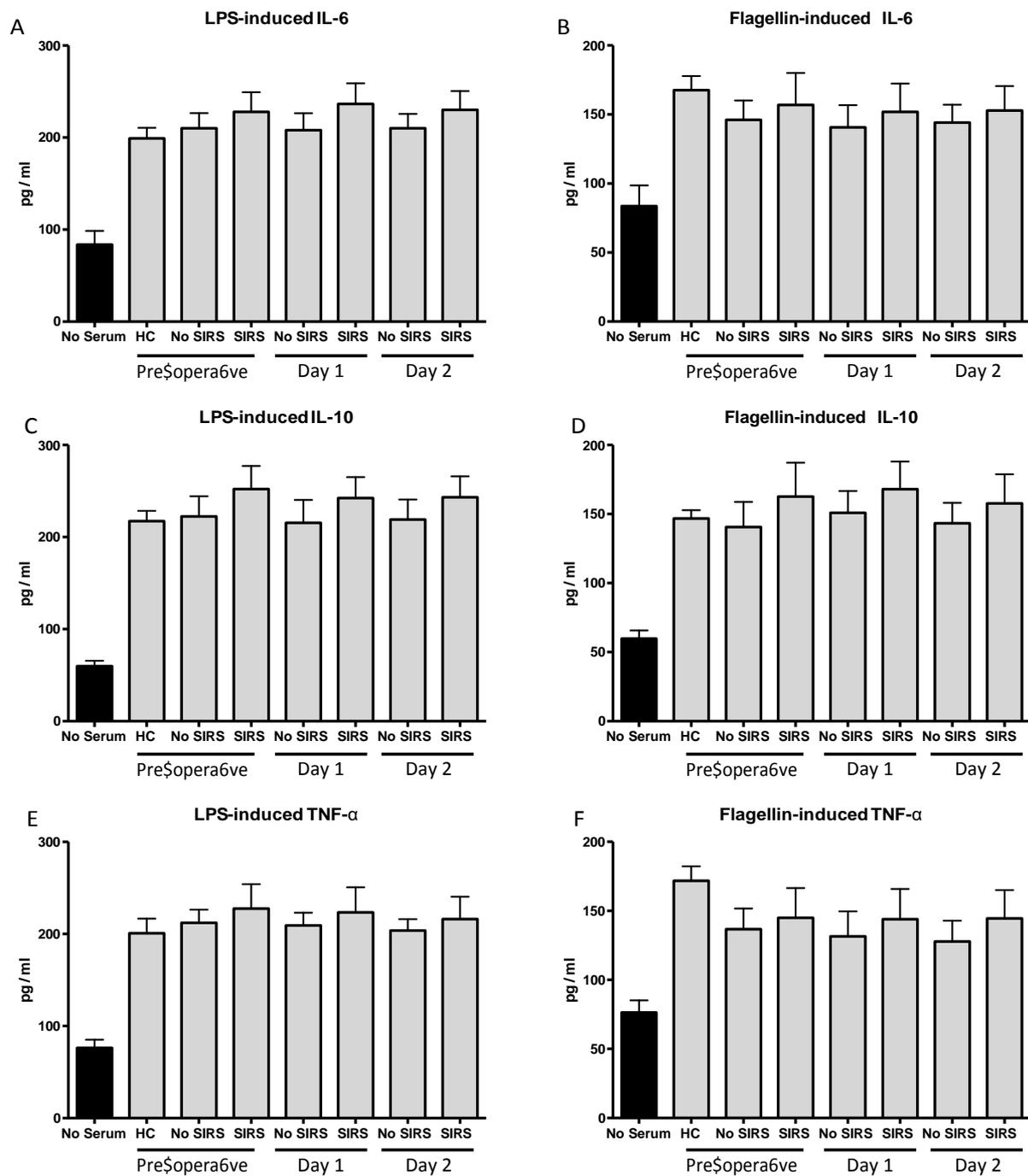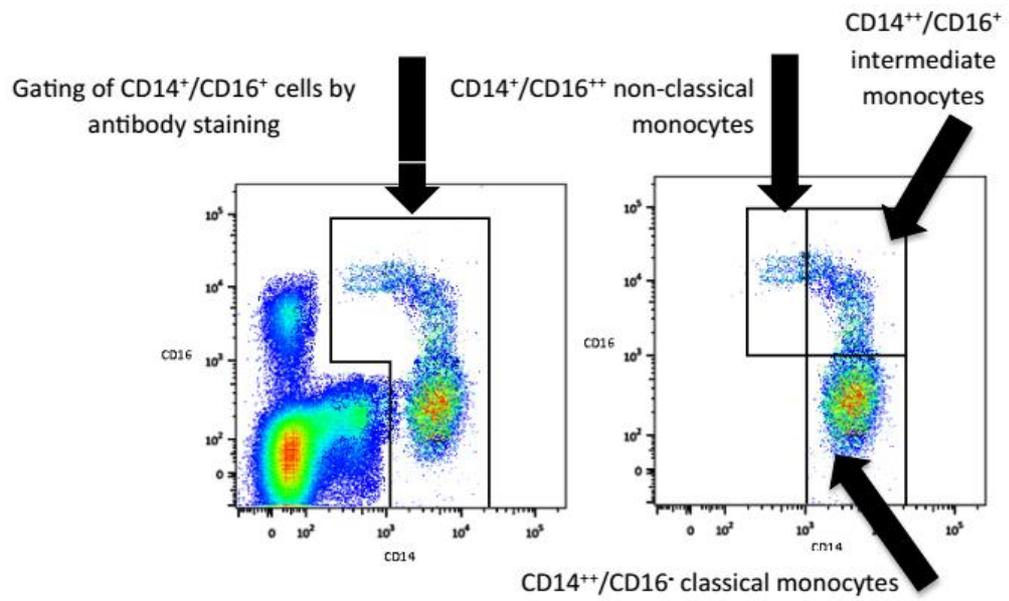
Supplemental Digital Content 3. Cytokine production in supernatants from peripheral blood mononuclear cells from healthy volunteers with and without incubation with patent sera.
IL-6 (**A, B**), IL-10 (**C, D**) and TNF-α (**E, F**) production expression was evaluated following stimulation with LPS (**A, C, E**) or flagellin (**B, D, F**) of PBMCs without added serum (No Serum), maintained in media conditioned with serum from healthy controls (HC), patients who had an uneventful recovery (No SIRS) or patients who developed SIRS (SIRS).
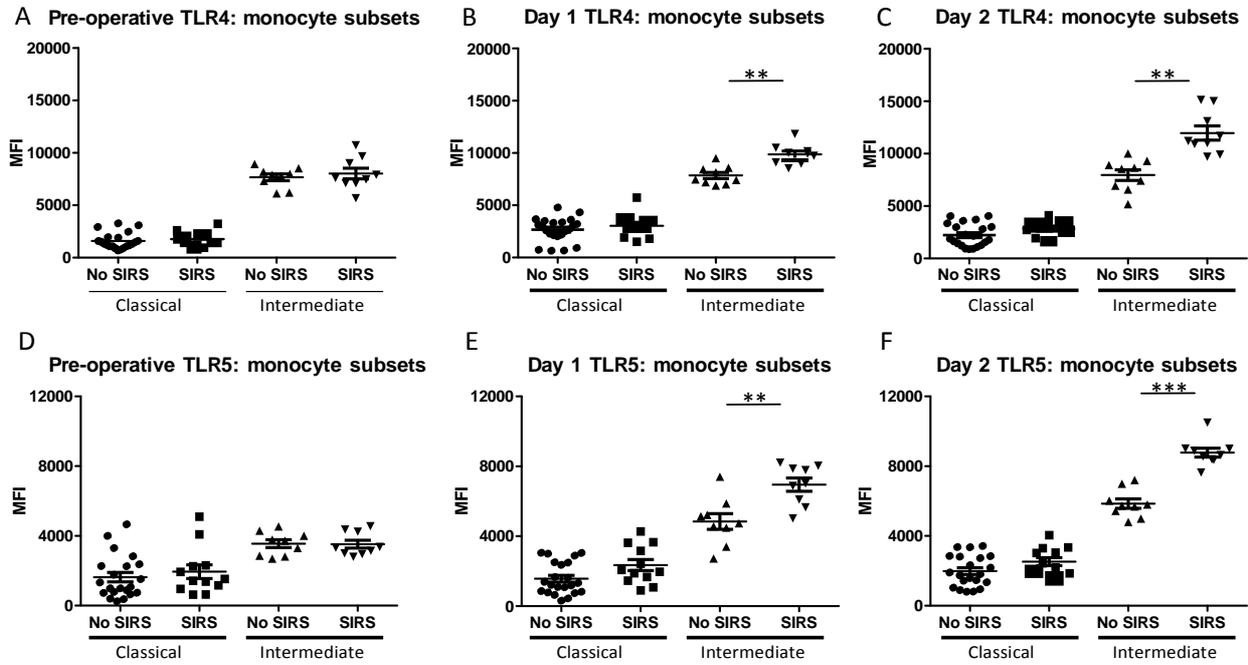
Supplemental Digital Content 4. Representative FACS plot showing PhosFlow. Increase in proportion of cells with expression of phosphorylated NFκB following stimulation with lipopolysaccharide (LPS, **B**) and phosphorylated STAT1 following stimulation with interferon(IFN)α (**D**) compared to unstimulated controls (**A, C**).

Supplemental Digital Content 5. TLR expression and TLR9mediated cytokine production are closely correlated. Correlation between the pre/operative, day 1 and day 2 expression of TLR4 and LPS/stimulated IL/6 production (**A9C**) or TLR5 and flagellin stimulated IL/6 production (**D9F**) in patients who have an uneventful recovery (closed circles) and those who develop SIRS (open circles). The r^2 coefficient is presented with each graph. $p < 0.0001$ for all correlations

Gating of CD14$^+$/CD16$^+$ cells by antibody staining

CD14$^+$/CD16^{++} non-classical monocytes

CD14^{++}/CD16$^+$ intermediate monocytes

CD14^{++}/CD16$^-$ classical monocytes

CD16

CD14

Supplemental Digital Content 6. Representative FACS plot showing gating strategy to identify classical (CD14^{++}CD16$^-$), intermediate (CD14^{++}CD16$^+$) and non-classical (CD14$^+$CD16^{++})monocytes.

Supplemental Digital Content 7. Expression of TLR4 and TLR5 is higher on intermediate monocytes compared to classical monocytes at all time points. Pre-operative (**A, D**), day 1 (**B, F**) and day 2 (**C, F**) TLR4 (**AEC**) and TLR5 (**DEF**) expression on classical and intermediate monocytes.