Trafficking of monocytes to the peritoneum after abdominal surgery and their role in the development of septic complications.

Dr Nick Bunker

MD(Res)

Imperial College London, Department of Surgery & Cancer

Declaration of Originality

This work is my own all others are referenced appropriately.

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Acknowledgements

I would like to thank the staff and students of Imperial College London, Chelsea campus for the help and assistance both when undertaking the experimental work and the preparation of this manuscript. In particular Kieran O'Dea and Kate Tatham have been readily available with helpful advice throughout. Professor Masao Takata was available to guide the direction of the work.

I would not have started this MD without the encouragement and support of Jonathan Handy, his mentorship and advice continues even after the completion of my MD.

Abstract

Background

Peritoneal infection that follows surgery is associated with SIRS and the development of multi-organ dysfunction. It has a high mortality. The dynamics of leucocyte trafficking into the peritoneal cavity and the expression of cytokines in response to either endotoxin or abdominal surgery will effect the development of both local and systemic inflammation. Firstly the leucocyte trafficking in response to intra-peritoneal LPS (lipopolysaccharide) is compared to an abdominal incision. Then, using the expression of TNFα on the cell membrane as a marker of pro-inflammatory status, this expression is described in animals undergoing surgery followed by an 'second hit' of ip LPS. Finally the ability of these trafficked cells to trigger SIRS leading to pathophysiological effects in the lungs was assessed.

Methods

C57/B6 mice were exposed to either an intra-peritoneal dose of 20ng LPS or abdominal surgery and the leucocytes identified and counted in both blood and peritoneal lavage fluid by flow cytometry. A two-hit model was devised, abdominal surgery the primary hit and 24 hours later ip LPS the second hit. The expression of TNFα was measured with flow cytometry. Using two intravenous dyes a lung permeability index was created comparing mice primed by surgery and those just receiving LPS. The data was analysed using student t-test and one-way ANOVA as appropriate.

Results

The time course of migration was different for the various leucocyte sub-populations in response to both LPS and surgery. There was a significant increase in the expression of membrane bound TNFα on monocytes and macrophages in the two-hit model than with surgery or LPS alone. There was a trend to greater lung permeability in the two-hit group.

Conclusions

Leucocytes migrate to the peritoneal cavity after surgery and are primed to respond vigorously to a subsequent dose of LPS. This may have effects on lung permeability.

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Chapter 1

Introduction

Introduction

Sepsis continues to be one of the commonest causes of mortality worldwide and is represented by a number of physiological changes that can take place in response to any infection. At a molecular level the physiological changes are a result of identification of non-self (ie microbial cells) or damaged cells followed by activation of various cells of the immune system leading to an increase in production of cytokines, facilitating cell to cell communication. This communication is a complex balance between pro- and anti-inflammatory cytokines, which in health regulate the appropriate response to infection but when this response becomes disproportionate the syndrome of sepsis occurs. Surprisingly in many cases it is not the effects of the original infective insult but a dysregulated immunological response that leads to the majority of the cellular damage. The picture is further complicated by the fact that it is not only sepsis that activates these cells and the cytokine cascade. Sepsis is just one trigger for the systemic inflammatory response, it can also occur after tissue injury in surgery or trauma and non-infective inflammatory conditions such as pancreatitis. Infection and the sepsis that follows one of these inflammatory events can be devastating and has a high mortality.

The innate immune system is the first part of the immune response and the mononuclear phagocyte system comprising monocytes and tissue resident macrophages play a central role within it as orchestrators of the overall response. Recently using new identification techniques several phenotypically and functionally distinct subsets of monocytes have been found in both humans and mice. The cell surface expression of receptors aids identification of these cells and also gives some clues as to their role during inflammation.

This work tests the hypothesis that an abdominal surgical incision provides a stimulus for inflammatory cell accumulation within the peritoneal cavity and that these cells are primed to respond to any subsequent infection, producing effects that are distant from the origin of either surgery or infection, leading to multi-organ dysfunction.

The current definitions and hypotheses of sepsis and systemic inflammation, the basics of innate immunity and the role of monocyte subsets in humans and mice are first reviewed.

Sepsis, SIRS and surgery

The definitions of the systemic inflammatory response syndrome (SIRS) and sepsis were developed at a consensus conference in North America in 1992 (1). In fact it was at this conference that the term SIRS was first coined. It had become obvious that unchecked inflammation could cause widespread organ dysfunction and that there were many diverse causative factors including trauma, cardiopulmonary bypass, surgery, pancreatitis and infection.

In the years preceding this conference some researchers included all of these under the banner of sepsis whilst others had many variations within their inclusion criteria, not only was this confusing for clinical research it also made estimating the size of the epidemiological problem very difficult.

From this conference the consensus was that a single common inflammatory pathway existed and should be termed SIRS. Sepsis was re-defined as SIRS with a documented infective cause.

SIRS was defined as more than one of the following clinical criteria present at any one time

(i) Heart rate >90 bpm

(ii) Respiratory rate > 20 or a $paCO_2$ < 32mmHg

(iii) Temperature < 36°C or >38°C

(iv) WCC < 4 or > 12 $x10^9$/L or more than 10% immature neutrophils

There were also attempts to stratify the degree of insult with definitions for severe sepsis (sepsis with organ dysfunction), septic shock (hypotension that does not resolve with the administration of an appropriate amount of intravenous fluid) and multi-organ dysfunction syndrome.

These landmark definitions have been used for the last 20 years to improve the epidemiological assessments of sepsis and SIRS, to stratify patients into various sub-groups allowing some individual risk assessments to be made and improve the recruitment of patients into trials aimed at the management of severe sepsis without including patients at lower risk of benefit but equal risk of harm.

Although the ability to stratify patients has had some success and sepsis trials include more homogenous populations there are still many problems. Infection and sepsis appear to be an increasing problem within intensive care. Mortality from sepsis is decreasing although the incidence continues to rise at a much more rapid rate, so the total number of deaths continues to rise. From 1979 to 2003 the mortality rate from sepsis decreased from 27.8% to 17.9% in the USA. Over the same time period the incidence increased from 82.7 per 100,000 patients to 240.4 per 100,000(2). If you just examine patients with severe sepsis there were over 750,000 cases with an associated mortality of 28.6%(3). The incidence is increasing for many reasons; some related to the patients and some related to the infecting organisms. People are living

longer, in part due to our ability to control chronic disease states, but they undergo more high risk debilitating surgery and many survive in relative states of immunosuppression. Infectious organisms are also evolving rapidly, some would argue at a much fast pace than technology; multi-drug resistant bacteria are now commonplace both in hospital and the community and there are few new antibiotics in development. Over-use of antibiotics not just within medicine is a considerable problem leading to more and more resistance(2)(4). In the worldwide point prevalence intensive care study, EPIC 2, infection was present in 51% of patients with 75% of patients receiving antibiotics on the day of study. Mortality was significantly higher in the infected patients compared with those not infected (25.3% vs 18.2% $p<0.001$). Multivariate logistical regression identified emergency surgery, age, cancer, heart failure, cirrhosis and immunosuppression as independent risk factors for death in the infected cohort(5).

Surgery itself is a risk factor for SIRS but may also be the cornerstone of its management. The injury induced by surgery shares many of the pathways activated in septic patients(6). The production of inflammatory cytokines such as IL-1β, IL-8, IL-10 and TNFα increases, with some evidence that the levels of these cytokines correlate with outcome in both sepsis(7) and wound healing(8). Pattern recognition receptors (eg Toll-like receptors) identify both bacterial products(9) and intracellular proteins released from necrotic host cells(10) triggering the same intracellular cascade irrespective of the source. Finally the response of the cells of the innate immune system including neutrophils and monocytes are similar regardless of whether the source of activation is sepsis or tissue damage(11).

In a recent European study on surgical outcomes the overall crude mortality after surgery was 4.0% and a multivariate logistical regression identified emergency

surgery, age, cancer, heart failure and cirrhosis as independent risk factors(12). It is perhaps not surprising given the common pathway of inflammation shared by surgical trauma and infection that the risk factors for death are almost identical in these two large observational studies (EPIC 2 and EUSOS). The type of surgery also influences the risk, highest risks borne by vascular, upper gastrointestinal, lower gastrointestinal and hepato-biliary surgery(12). The size of incision, tissue trauma involved and risk of infection from intra-luminal contents make abdominal surgery particularly risky.

Within the peritoneal cavity, surgery is often the mainstay of treatment for infection leading to peritonitis but infection may also follow and complicate routine or emergency abdominal surgery. There is likely to be complex interplay between the inflammatory effects of the surgery itself and the septic process. This may be why patients who develop peritonitis have a significant degree of mortality and morbidity. In EPIC 2, 20% of the documented infections were intra-abdominal(5) and Marshall previously found that the mortality of intra-abdominal infection was 30%(13).If the infection follows surgery and is persistent then mortality can rise to between 30 and 64%(14)(15)**.** This is tertiary peritonitis.

Primary peritonitis is a spontaneously occurring event in patients with ascites and may have its origin in the translocation of bacteria across the gut wall. Secondary peritonitis is associated with perforation of a hollow viscus, as occurs with diverticulitis or a duodenal ulcer; it is a common diagnosis managed by acute surgeons. Those patients who succumb at an early stage from secondary peritonitis do so because of overwhelming breach of the gastrointestinal tract and massive release of bacterial products and toxins. Those who survive the early stages, usually with prompt surgical intervention and early use of antibiotics, either recover or develop a chronic inflammatory condition. The latter is associated with SIRS, multi-

organ failure and a higher mortality and is known as tertiary peritonitis(14). There are patients who respond much better to treatment despite a similar insult and this heterogeneity is likely to be a function of both genetic polymorphisms(16), the so called "survivor genes" and the number and severity of co-morbid conditions.

Surgical management of intra-abdominal sepsis

The traditional mainstay of treatment for many of the causes of peritonitis is source control, commonly involving surgery. However recently there has been an acceptance that there are significant risks associated with surgery and the benefits that initially appeared intuitive may be less so. A good example of this is the management of appendicitis. Ten years ago this was managed in the vast majority of patients with open surgery**.** More recently laparoscopic surgery has become the standard of care in many centres due to lower morbidity, lower complication rate and lower hospital length of stay using this approach(17). In fact since the 1970's there has been a growing amount of evidence that not all patients with suspected appendicitis need surgery at all and many can be managed with a course of antibiotics(18)(19)(20). The key to this is patient selection, and the ability to make the right choice for individual patients has significantly improved over the last 30 years using imaging modalities such as ultrasound and computer tomography. This more conservative approach to the management of not just appendicitis but other causes of intra-abdominal sepsis has come about as we understand more about the morbidity associated with all types of surgery.

Supportive management of intra-abdominal sepsis

In those patients who require abdominal surgery the role of Intensive Care has grown over the last decade. In the UK there have been attempts to stratify patients in whom higher nursing ratio's, intensive monitoring, close attention to fluid balance and antibiotic husbandry would reduce significant morbidity and mortality. There is a growing amount of evidence that higher levels of care can reduce complications following high risk surgery(21)(22).

The improvements in mortality that have been made over the last 30 years have occurred not because of a specific targeted therapy but by improvements in the quality of supportive care offered within Intensive Care units. In fact one could argue that we are just causing less harm in our efforts to cure. This includes interventions such as low tidal volume ventilation(23), high PEEP(24), prone positioning(25), goal directed therapy(26), resuscitation fluid selection(27)(28) and volumes(29) as well as many more. Along the way we have stopped or adapted interventions that have been found to be ineffective or harmful such as tight glycaemic control(30)(31), albumin for fluid resuscitation(32) and high frequency oscillation(33)(34).

The innate immune system and potential therapeutic targets in sepsis

The innate immune system was thought of as a blunt tool in the immunological response that responded in a very non-specific way to a variety of insults. However in the last 20 years there have been numerous advances in our understanding of this system including the discovery of a superfamily of receptors identifying both microbial products and damged host cells (35), the links between the innate and the adaptive systems (36) and the biology of cell surface markers allowing the identification of subtle subsets of the cells involved.

There are several different facets to the innate immune system. It must be able to recognise invading organisms and the toxins they produce as well as discriminate between damaged and healthy cells. On recognition, the cellular components of the system must translate this message into the nucleus, increasing DNA transcription to produce substances that will eradicate the identified problem. These sensing and effector functions must also have the ability to turn off when the problem is controlled. This system is very sensitive and effective, persistent infections are rare but there are many instances where the response to infection not only eradicates the invading organism but also causes significant damage to the host (37).

Ligands - PAMP's and DAMP's

The recognition of pathogens is primarily cell-based. Receptors on the cell surface are able to recognise pathogen associated molecular patterns (PAMPs) and/or damage associated molecular patterns (DAMPs)(38). PAMP's can take many different forms including dsDNA from viral organisms, peptidoglycan and lipoteichoic acid from Gram positive bacteria and endotoxin from Gram-negative bacteria. In the 1800's Pfeiffer named the latter with the discovery that a constituent of bacteria was able to kill experimental animals even if the bacteria itself had been rendered non-viable; he named this endotoxin (39). This has been found to be the most potent of the several bacterial components that can trigger inflammation and this has pushed researchers to try and block its effects prior to the interaction with cells of the innate immune system. An anti-endotoxin monoclonal antibody called centoxin did show effectiveness in animal models and a phase 2 trial. However the first large randomized trial did not show benefit, except in the sub-group with confirmed gram negative sepsis (40). Further larger trials with *a-priori* defined subgroups failed to

find any mortality advantage and finally a large French study not only demonstrated no benefit but a signal towards harm, the product was rapidly withdrawn (41).

Damage associated molecular patterns (DAMP's) are endogenous ligands that can be recognised by pattern recognition receptors on cells of the innate immune system. They are released following cell damage or necrosis.

There are many of these that have been identified including High mobility group box protein (HMGB), heat shock proteins, S100 proteins, RAGE, formyl peptides, ATP and nuclear factors (42).

HMGB has probably been the most studied, it is a component of the nucleus of cells and is released by cells undergoing necrosis. It is also released by macrophages and monocytes, without cell death, as a signal to recruit more inflammatory cells to areas of damage (10). It is identified by TLR-4 in a very similar way to the identification of LPS and the downstream activation effects in monocytes may be similar(43).

Pattern recognition receptors are able to identify both PAMPS and DAMPs and although there is some small differences in the downstream effects, most are conserved regardless of the stimulus. The initial identification is usually made by either sentinel macrophages or patrolling monocytes(44)(45) and these respond by sending out a chemokine signal to attract neutrophils. Neutrophils contain numerous granules, the first released aid their migration to areas of inflammation, subsequently released chemicals aid the clearance of microbes/damaged cells. Neutrophils also alter the chemokine gradients and signal to endothelial cells to driving the attraction of monocytes (46)(47). In an animal model of sepsis where neutrophils were depleted the numbers of monocytes recruited to areas of infection were significantly reduced (48). Finally to limit the degree of inflammation, macrophages prevent further

neutrophil sequestration, send signals to actiavte neutrophil apopotosis and then scavenge the remaining cell debris (49).

The immune system was traditionally viewed as differentiating between self and non-self but perhaps should be considered as a response to triggers that may cause harm be they self or non-self.

Receptors

Following the identification of the ligands that activate the innate immune system the obvious next step was to search for the receptors. It had been noticed that a strain of mice (C3H/HEJ) was resistant to the effect of injected endotoxin, suggesting that a single receptor existed (50). It was only later that the mutation in these animals was found to code for part of the cytoplasmic domain of a transmembrane receptor called Toll-like receptor 4 (51). The Toll-like receptors were first discovered in the fruit fly, *Drosophilia Melangastor* (35) and they are remarkably conserved across many species. Their role as receptors was established in the 1990's (52) but their full function is only just beginning to be understood. In humans there are several Toll-like receptors that are configured either to identify single ligands or in some cases multiple consitituents of the invader. TLR-1,-2 and -6 recognise several bacterial lipoproteins, TLR-4 recognises LPS, TLR-5 responds to flagellin - an intracellular component of bacteria and TLR-3, -7, -8, -9 all recognise demethylated nucleic acids and are thus able to differentiate between DNA/RNA from bacterial or viral organisms and mammals (53).

TLR-4 typifies the toll-like receptors. It is a transmembrane protein with a extracellular portion that forms a hydrophobic pocket to bind LPS. When released from bacterial cell walls, LPS rapidly forms micelles in solution and so, to aid its

presentaton to TLR-4, it first binds to Lipopolysaccharide binding protein (LBP) (54)(54) and a co-receptor CD14 (55) found either on monocytes or in a free soluble form. These facilitate the delivery of single molecules of LPS to TLR-4 and promote binding. Once LPS is bound the intracellular portion of the Toll-like receptor is activated. This involves a Toll/IL-1 receptor (TIR) domain interacting with several bridging adaptors such as MyD88 and activating downstream proteins such as the Interleukin receptor associated kinase (IRAK) family (53). One of the common targets of this intracellular signalling process is to activate Nf-κB, promoting its translocation into the nucleus and the transcription of pro-inflammatory cytokines such as Tumour Necrosis Factor (TNFα) and IL-6.

In humans TLR-4 receptor polymorphisms are known but often make the individual more susceptible to sepsis rather than less. This may be in part due to the presence of multiple pathways for the detection of microbial DNA, debris and endotoxin that include but are not limited to the TLR system such as fmLP and the intracellular sensors NOD1 and NOD2.

Downstream effects

TNFα is part of a genetic superfamily that has evolved with a wide range of diverse functions including organogenesis, apopotosis, inflammation and host defense (56). There are two receptors found on cell membranes for TNFα, the 55kD TNFR-1 and the 75kD TNFR-2. Evidence is beginning to emerge that these two receptors may have opposite downstream effects which may explain why non-specific TNFα anatagonists have proven ineffective to manage sepsis (57). The control of TNFα is complex and involves a membrane bound enzyme TNFα converting enzyme (TACE) also known as CD156b. This is an ADAM-17 metalloprotease and is involved in the

cleavage of more than 40 proteins from the cell surface including both TNFα and TNFR. TNFα is synthesised and transported to the cell surface as a 26kD membrane bound precursor, this is subsequenty cleaved to form the 17kD soluble form by TACE (58). By cleaving both TNFα and its receptor TNFR from the cell membrane, it can regulate both the pro- and anti-inflammatory properties of TNFα (59). One example of this is the ability to dampen down the response to infection by binding soluble TNFα to free TNFR, therefore neutralising it. The effects of TNFα binding to membrane bound TNFR are many and diverse. In the context of sepsis they promote programmed cell death in some target cells yet, delay apoptosis in others (56)(60). They also have a chemokine function and co-ordinate the recruitment and activation of a variety of cells to the areas of infection maximising the response (56). Animals infused with a recombinant form of TNFα exhibit many of the signs of sepsis including hypotension, metabolic acidosis, endothelial leak and hyperglcaemia. On post mortem examination of the lungs there are the characteristic changes of pulmonary oedema and inflammatory cell infiltration. Taken together this suggests a central role for TNFα in the pathophysiology of sepsis(61). In animal models the levels of TNFα correlate well with the degree of insult and mortality(62) and there is some evidence that this is also true in children with meningococcal disease(63). However there is little correlation in many of the other causes of sepsis, in fact in many studies on septic patients researchers failed to detect TNFα at all(64).

Given it's central role a substantial amount of time and money has been invested in trying to find an anti-TNFα drug that is effective in sepsis. A product was developed and studied in a multi-centered, randomized, double blind, placebo controlled trial. Unfortunately although there was a mortality benefit at 3 days this was lost by 28 days, the *a priori* primary outcome measure(65). In 2001 another multi-centre

randomized controlled trial was published, this time the intervention group receiving the p55 soluble TNFα receptor, with the aim of binding free TNFα in plasma preventing its effects(66). There was strong animal data for a significant mortality benefit and a phase II study that suggested if used early then mortality was reduced at 28 days(67). Unfortunately once fully randomized and properly powered to examine mortality as an end point there was no statistical difference between the p55 TNFα receptor and placebo(66).

The downstream response does not just involve TNFα; there are many cytokines that have effects in inflammation. The system is highly complex and each cytokine may have a role in both the pro- and anti-inflammatory response, one explanation of this is the competition between cytokine cell surface receptors and circulating forms. This is well illustrated by IL-6. It is probably the most studied pro-inflammatory cytokine and many researchers have demonstrated its increase in sepsis, during surgery and following trauma (68). There is an asoociation between high levels of IL-6 and mortality in sepsis (69). However it also has some anti-inflammatory properties and given the right conditions it downregulates the production of IL-1 and TNFα without effecting the production of other anti-inflammatory cytokines such as IL-10 (70).

IL-10 is commonly studied as an anti-inflammatory cytokine and is able to reduce the production of inflammatory cytokines such as TNFα and IL-6 in all myeloid cells both at a translational and post-translational level (71). In patients with an infective exacerbation of COPD there is a clear timeline of cytokine release, TNFα appears early and is followed by IL-6 and then IL-10 much later on (72).

Coagulation and sepsis

More recently the connection between coagulation and sepsis has been identified as a possible area for therapeutic intervention. During sepsis there is an imbalance between pro- and anti- coagulant factors, with a decrease in the synthesis of protein C, protein S and anti-thrombin III as well as thrombomodulin, the latter a molecule required for protein C activation. Activated protein C has an important role in turning off the coagulation cascade by decreasing plasminogen activator inhibitor 1 (PAI-1) and inhibiting factor Va and VIIIa; it also has a role in decreasing apoptosis. By inhibiting both the synthesis and activation of protein C, endotoxin both increases fibrin whilst inhibiting fibrinolysis. This creates microvascular thrombi, areas of ischaemia and further cell damage (73). This was the premise for the 2001 PROWESS trial that compared recombinant activated protein C with placebo (74). This trial demonstrated a very impressive 6% absolute risk reduction in the primary outcome of 28-day mortality; however later studies failed to find benefit and there was a suggestion of excess mortality from bleeding complications. This led to an unprecedented second randomized controlled trial in 2011, PROWESS-shock. The drug was trialed in units with little prior use to ensure equipoise and after recruiting 1697 patients, all with septic shock they found there was no statistical mortality benefit of activated protein C at 28 days (75). The pharmaceutical company Eli Lilly promptly withdrew the drug from the worldwide market.

SIRS vs CARS

In 1972 Lewis Thomas proposed that bacteria were simply "bystanders" in sepsis and the real enemy was an overactive immune response (76). The obvious extension to this is that by preventing over-activity, mortality should reduce. Many different targets and potential therapies have been tried in the last 30 years with very little if any success, and this has led to questions about whether this original hypothesis was true.

Bone introduced the term compensatory anti-inflammatory response syndrome (CARS) to explain why many sepsis patients do not succumb to the original infection but at day 3 or 4 often from a secondary insult (77). Hotchkiss and Karl in their 2003 review suggest that there is a fine balance between pro- and anti- inflammatory responses and the same cell types or cytokines in different situations may produce both types of effect (16). One hypothesis is that there is over-stimulation of the anti-inflammatory compensatory response leading to a state of relative immunoparalysis. This could help explain why many of the intervention trials for sepsis have failed to show any mortality benefit. What remains controversial is the timeline of events; does either SIRS or CARS dominant, do these processes occur sequentially or is CARS simply the normal process of turning off of inflammation. These questions have clear therapeutic implications; improvements in organ support have decreased early death from sepsis but the development of immunomodulatory drugs continues to be directed towards reducing activation of the pro-inflammatory arm. Many authors believe that future developments need to be targeted to an individual's immune status using genetic profiling.

The compensatory response is complex and includes both cellular and soluble

mediators. Monocytes from septic patients exposed to LPS, produce less TNFα, IL-1α, IL-1β, IL-6 and IL-12 but significantly more IL-10 (78). This also seems to be the case following trauma (79). It has also been known for several decades that serum taken from either burns (80) or septic patients (81) is able to blunt the pro-inflammatory response of leucocytes from healthy volunteers. These circulating factors include soluble CD14 and LPS binding protein, that act by "mopping up" circulating LPS. Soluble TNFα receptors perform a similar role with TNFα (78).

To demonstrate these effects in patients Hotchkiss compared lung and splenic tissue from patients who died of sepsis with that from alive trauma patients including those who were brainstem dead (82). Splenic cells from patients who died from sepsis produced less cytokines than control patients. This was true for both the pro- and anti-inflammatory cytokines including TNFα, IFNγ, IL-6 and IL-10. At a molecular level antigen presenting cells express less HLA-DR and more of the programmed cell death family in the septic patients. This provides some support for the theory of a general decrease in immunity related to a change in innate immune cells surface expression and an increase in apoptosis. Clinically an alteration in the ratio of pro- and anti-inflammatory cytokines can have an effect on outcome. In meningococcal disease, mortality is higher in patients and their 1st degree relatives who have either a raised IL-10:TNFα ratio (83) or a raised TNFα to soluble TNFα receptor ratio (84).

In summary the effects of CARS may manifest in several ways. It may simply be over-production; persistently raised IL-10 levels in sepsis confer a worse outcome(85). It may be that a cytokine signal triggers apoptosis and increased cell death; with a knock on effect on immune cells leading to anergy, or a state of unresponsiveness(86). It may be that the cells of organs with significant dysfunction

simply hibernate and could be available to function normally given the right metabolic conditions. Autopsy evidence suggests that there is actually very little cell death in sepsis, a finding that seems at odds with the degree of macro organ dysfunction. It is likely to be a combination of several diverse mechanisms.

Innate Immune system

The complexity and redunancy within the system with several different pathways leading to the same end-point is both a strength and a weakness. It gives the immune system the flexibility to react to many different pathogens but when imbalance between the pro- and anti inflammatory responses occurs, blocking one facet of the pathway is unlikely to fix the problem and may conversely lead to harm. The "magic bullet" probably does not exist for the management of SIRS or sepsis due to significant interpatient heterogenity but for some disease states modicfication of this innate immune response has been effective. These include Rheumatoid arthritis (87) and hepatitis B (88). It is interesting that although beneficial at reducing the impact of the underlying disease these modifications are often accompanied by a predisposition to sepsis (89).

Neutrophils

The leuckocyte subpopulations involved in the innate immune system arise from common progenitor cells within bone marrow. They employ a range of killing strategies to remove invaders from the host. The polymorphic neutrophils (PMN's) have a short half live, in resting conditions most will undergo apopotosis in around 6 hours. Interestingly apoptosis in neutrophils can be delayed by high circulating levels of TNFα to facilitate bacterial clearance (60). In response to infection there is a rapid mobilisation of PMN's from bone marrow and following cytokine and chemokine

gradients they migrate to the infected areas. Phagocytosis occurs either via receptors on the cell surface or complement enhanced opsonization, followed by the release from lysozomes of several intracellular compounds including reactive oxygen species, hydrogen peroxide and peroxynitrite (37). Once phagocytosis has occurred there is an immediate signal to begin apoptosis allowing control of this powerful immune response. More recently the concept of neutrophil extracellular traps (NETs) has been developed as another putative mechanism by which neutrophils eliminate bacteria and cell debris (90). NETs are formed by the ejection of nuclear material including histones from the cell a process that may or may not lead to neutrophil cell death. This occurs in response to either whole bacteria or component parts from a wide range of organisms. They trap bacteria in the peripheral circulation, limiting spread as well as being bacteriocidal. Abnormal NET release has been implicated in the aetiology of chronic infective conditions and some autoimmune diseases including vasculitis (91) and SLE (92).

Macrophages

Macrophages are mostly tissue based and have a much longer half life than other cells of the innate immune system (37). They are replenished by circulating monocytes that are not just delivery cells but biologically active with diverse roles depending on the subset. The killing mechanisms are very similar to neutrophils, although the target and time course often differ. Tissue macrophages act as sentinel cells that are the first part of the innate immune system to detect either PAMP's or DAMP's, triggering an increase in production of inflammatory mediators such as TNFα and IL-6 (93). Chemokine gradients then develop to attract neutrophils and monocytes to these areas of inflammation. The same cells also help in the repair of damaged tisue as

inflammation subsides. The act of phagocytosis of apoptic cells increases the production of anti-inflammatory cytokines such as TGFβ and a variety of growth factors whilst decreasing the production of pro-inflammatory cytokines (94). Specialised macrophages in the liver (Kupffer cells) and spleen are involved in clearance of systemic pathogens by phagocytosis. They are also implicated in some chronic disease states such as alcohol induced cirrhosis (95).

Monocytes

Initially, monocytes were thought of as cells that only became activated when they had marginalised out of the circulation and differentiated into macrophages (96)(45). However recently it has become apparent that there are subsets of monocytes with very different roles in the sepsis response and these can be clearly documented in many different species (97). Rather than a passive, reactive role they may actually orchestrate some of this intertwined complex process. These subsets have been demonstrated in both mice and humans and although some similarity exists the functional characteristics are not identical between species.

Human Subsets

Several groups had identified different subsets of human monocytes on the basis of countercurrent centrifugal elutration (98) (99) but it wasn't until Passlick et al described a novel combination of surface antibodies by flow cytometry that the current method of a characterization of the subsets could be achieved (100). Using two colour flow cytometry and cell sorting they identified two subsets of monocytes in volunteers blood. The larger, more granular cells make up 90-95% of the monocyte numbers and express mostly CD14; they were termed classical monocytes.

The smaller cells representing 5-10% of the total monocyte count express both CD14 and CD16. The functional characteristics of both of these subsets remain controversial. As already discussed, CD14 is a cell surface receptor acting as a co-receptor with soluble LBP to present LPS to TLR-4. CD16 or FcγIIIR is a cell surface receptor for the Fc portion of IgG. The initial characterization suggested that the $CD14^{+}/CD16^{+}$ cells were more efficient at antigen presentation but produced less TNF, IL-6 and IL-10 than the $CD14^{++}/CD16^{-}$ cells (101). In later experiments the same group failed to reproduce their findings; by measuring mRNA instead of soluble cytokines an equal amount of TNF, IL-1 and IL-6 was produced by the two subsets but the $CD14^{++/}CD16^{-}$ cells produced significantly more IL-10 (102). By using whole blood and flow cytometric analysis of intracellular TNF, they found a significantly greater production of TNF by the $CD14^{+}/CD16^{+}$ cells; this was especially marked following stimulation by the products of Gram Positive bacteria (103). These two studies led to the $CD14^{+}/CD16^{+}$ cells being labeled as pro-inflammatory. As flow cytometric technology improved, the surface expression of proteins on the subsets has been studied in much greater detail and become more complex. Grage-Griebenow et al defined monocytes using CD14, CD64 (a high affinity receptor for IgG) and CD16, identifying three populations. $CD64^{+}/CD14^{+}/CD16^{-}$ analogous to $CD14^{++}/CD16^{-}$, $CD64^{-}/CD14^{dim}/CD16^{+}$ analogous to $CD14^{+}/CD16^{+}$ and a third, novel group $CD64^{+}/CD14^{+}/CD16^{+}$ (104) (105). As well as differences in cytokine/chemokine production the subsets also differ in the mechanisms they employ to migrate into areas of inflammation. The $CD14^{++}/CD16^{-}$ subset express similar amounts of TLR-4 but significantly more TLR-2 (103) (106). They migrate into tissue via CCR2 and MCP-1 dependent mechanisms and are able to either produce myeloperoxidase and reactive oxygen species (ROS) or differentiate into macrophages (107). The

$CD64^+/CD14^+/CD16^+$ subset have previously been included with all the $CD16^+$ cells, they express both TLR-2 and TLR-4 and have the typical morphological appearance of a monocyte. However they have a high expression of HLA-DR and CD11c, greater T cell accessory capacity and a high production of IL-12, all traits of dendritic cells (DC's) and they may represent an intermediate form of either dendritic cell or $CD14^{dim}/CD16^+$ monocytes (104). The $CD14^{dim}/CD16^+$ subset expresses both TLR-4 and TLR-2 and are probably the most mature cell line with in vitro evidence of maturation from the $CD14^{++}$ cells via the intermediate $CD64^+/CD14^+/CD16^+$ form (106) (108). Both subsets expressing CD16 also express CX_3CR1 the receptor for fractalkine and CCR5 the receptor for macrophage inflammatory protein-1 (MIP-1) providing an alternative mechanism for adherence and migration.

Subsequently, there have been numerous observational studies examining the trafficking and in-vitro activity of the different subsets in both healthy volunteers and patients (109). It appears that the minority subset, $CD14^+/CD16^+$ increases dramatically in several clinical situations including active rheumatoid arthritis (110) neonatal sepsis (111), gram negative sepsis, erysipelas (112), HIV (113), lupus (114) and even with strenuous exercise (115). More recently a biphasic kinetic for the migration of monocytes into myocardium following myocardial infarction has been described (116). The experimental work in humans does need to be interpreted with caution, many of the methods involved in the earlier separations of subsets may have activated the cells and subsequent increases in cytokines may just represent in-vitro phenomena. This is a common criticism of all in-vitro work and has pushed investigators to find monocyte subsets in suitable laboratory animal models that will allow the study of in-vivo responses (117).

Murine subsets - heterogeneity

In 2001 during a study investigating the effect of the chemokine, monocyte chemoattractant protein 1(MCP-1/CCL2) Palframan et al discovered that there appeared to be at least two monocyte subsets in mice and they could be separated by their expression of CCR2, CX_3CR1 or L-selectin (118). There was also a suggestion that these different subsets also had different functions with only the $CCR2^+$ cells migrating to inflamed peripheral lymph nodes (119). Geissman et al expanded this significantly in 2003 using Rag deficient mice to increase the yield of monocytes and, like Palframan, used animals where a single allele of the gene coding for CX_3CR1 was replaced by green fluorescent protein (GFP) (120). They identified 2 subsets; CX_3CR1^{Lo} expressing high levels of CCR2, the Ly6C/G complex Gr-1 and CD62L (L-selectin); the other subset was CX_3CR1^{Hi}, low expression of the Gr-1 complex and minimal CCR2 or CD62L. Several groups have confirmed these results and murine monocytes are now clearly divided into 2 distinct subsets known as Gr-1^{Hi} and Gr-1^{Lo} (45).

In the same paper, Geissman examined the effect of inflammation using adoptive transfer of GFP labeled monocytes into the circulation of donor mice, which had previously had sterile peritonitis, induced with thioglycollate. In normal wild type recipient mice the Gr-1^{Lo} monocytes circulated for several days migrating into normal tissue such as liver, lung and spleen, whereas the Gr-1^{Hi} cells rapidly became undetectable in all tissues. In peritonitic mice the Gr-1^{Hi} monocytes migrated within 18 hours to the site of inflammation whereas the Gr-1^{Lo} cells mostly remained in the circulation and extravasated in much lower numbers. Gr-1^{Hi} monocytes were also the first cells to migrate in response to sterile peritoneal inflammation with an attractant signal from tissue expressed MCP-1 (119).

They hypothesised that the Gr-1^{Hi} cells migrated in response to inflammation whereas the Gr-1^{Lo} cells had a more patrolling role, maintaining normal tissue homeostasis. This was reinforced by the work of Auffray, who, using confocal intravital microscopy, demonstrated Gr-1^{Lo} monocytes crawling along the luminal border of endothelium (121). The process of rolling along endothelium in areas of inflammation is well known and allows the interaction of endothelial chemokine's with their cell surface receptors as well as encouraging extravasation at tight junctions (122). The pattern of movement of the Gr-1^{Lo} monocytes was different; it was present predominantly in healthy tissue and occurred both with and against the flow of blood and for a much further distance than the rolling of other leucocytes. The appearance was that of a 'patrolling' cell. Using antibodies against the integrin LFA-1 this attachment to the endothelial luminal border was disrupted, and CX3CR1 deficient mice had a significant reduction in both the number of patrolling cells and the distance they moved (121). This suggests a role for both of these cell surface proteins in this patrolling behavior. Experimental proof of the function of these patrolling Gr-1^{Lo} monocytes is lacking although the hypothesis that they phagocytose dead cells and circulating oxidized lipids during health is credible. The natural ligand for CX3CR1 is fractalkine and this interaction is likely to mediate the patrolling role, the union of these proteins also appears to send a survival signal to the monocyte significantly prolonging its lifespan although the mechanism for this is currently unknown. (121)(45,123)**.** Interestingly CX_3CR1 although minimally expressed also has some role on the Gr-1^{Hi} cells. In CX_3CR1 deficient mice that have been infected with Listeria, bacterial cell numbers are less well controlled by Gr-1^{Hi} cells and they migrate to the spleen in much fewer number (124)**.**

Intra-peritoneal injections of Listeria demonstrated that these patrolling cells

extravasated to areas of infection within 2 hours and rapidly became the prime producers of TNF and IL-1. The more traditional 'inflammatory' monocytes, Gr-1^{Hi} did not migrate to the peritoneum for up to 8 hours at which point they took over the role of inflammatory cytokine producer (121). By this stage the Gr-1^{Lo} monocytes had already changed to a more reparative role, switching to the production of mRNA coding for proteins involved in tissue remodeling (125). The end result is that the two monocyte subsets differentiate differently following the same stimulus, Gr-1^{Lo} cells mature into cells that resemble alternatively activated M2 macrophages and Gr-1^{Hi} cells have some of the features of pro-inflammatory circulating dendritic cells (121). There is evidence from other experimental models that this is indeed the case with the same stimulus causing the different subsets to mature into distinct cell types with distinct functions. Hypercholesterolaemia in mice triggers several responses including an increase in the numbers of Gr-1^{Hi} monocytes. These migrate into atheromatous areas and drive the inflammatory response within these plaques; they may also eventually differentiate into foam cells. Gr-1^{Lo} cells have a less defined role in atherosclerosis but they may have a stabilizing effect on plaques by differentiating into M2 macrophages and forming granulation tissue (126). Nahrendorf et al summarized the work done on monocyte subsets following a myocardial infarction as a bi-phasic response; an initial influx of pro-inflammatory Gr-1^{Hi} monocytes migrate using a CCR2 dependent pathway and are involved in the clearance of free lipids and dead myocytes. This is followed by migration of Gr-1^{Lo} cells, utilizing CX_3CR1, and leading to granulation, fibrosis and repair (127)(128). In muscle injury again it is the Gr-1^{Hi} monocytes that are recruited in the first 48hrs of necrosis producing significant amounts of IL-1β and TNFα. By day 4 the predominant cell type is Gr-1^{Lo} expressing more TGFβ and IL-10, and by day 10 when nucleated cells are beginning to reappear

few monocytes remain (129). In this model the Gr-1^{Lo} cells appear to be derived from the Gr-1^{Hi} cells, the signal triggering this switch may be the phagocytosis of dead cells. In mice depleted of monocytes around the time of injury very little phagocytosis or regeneration occurred.

Finally in acute lung injury there is clear evidence for a differential role of monocyte subsets. O'Dea et al demonstrated that there was a significant increase in the pro-inflammatory cytokine production, specifically TNFα, of monocytes that had marginated into the pulmonary vasculature and out of the central circulation, in response to an intra-venous injection of LPS (130). The lung has a unique response to systemic challenges as most leucocytes have to deform to pass through the narrow capillary beds (a process hampered by the action of LPS) and almost the entire cardiac output must pass through the lung vasculature (131). O'Dea et al demonstrated that Gr-1^{Hi} monocytes are mobilised from bone marrow and marginate within the pulmonary vasculature in response to a sub-clinical dose of IV LPS (132). These cells produce little membrane bound TNFα but have clearly formed a degree of cell-cell interaction because following a secondary stimulus of clinical significant LPS there was a 5 fold increase in the amount of membrane TNF expressed compared to non-primed monocytes. Using targeted inhibition of the monocytes response with clodronate-liposomes it was apparent that these effects were in the main mediated by the Gr-1^{Hi} subset rather than Gr-1^{Lo} monocyte or other leucocytes. Wilson demonstrated a similar effect to O'Dea using high stretch ventilation instead of a second dose of LPS (133). Mice pre-treated with a sub-clinical dose of LPS were liable to greater lung injury when subsequently exposed to high stretch ventilation. This injury could be attenuated using clodronate to deplete the circulating monocytes. The predominant subset recruited was Gr-1^{Hi}. This heterogeneity between the subsets

is determined by the phosphorolyation of p38 and activation of its substrate MAPkinase (Mitogen activated Protein) specifically MK2. TNFα production is consistently higher in the Gr-1^{Hi} monocytes and associated with higher levels of Phospho p38 and activated MK2. This production of TNFα can be reduced to baseline levels with the addition of MAPkinase inhibitors suggesting a significant role in the production of cytokines (134). The experiments of Wilson also combines tissue damage and infection to examine the effect of the two hit insult.

Murine subsets - trafficking

Monocytes arise from bone marrow, specifically from progenitor cells called macrophage/dendritic precursors (MDP). These are the parent cells for both subsets of monocytes, conventional dendritic cells, plasmacytoid dendritic cells and macrophages. There appears to be a role for both CX_3CR1 and the M-CSF receptor (CD115) during development as the former is first expressed on the MDP cells and the latter on the more pluripotent granulocyte/macrophage progenitors.

The expression of Gr-1^{Hi} on monocytes appears to peak just before release from bone marrow. These cells then appear to preferentially migrate into areas of either sterile inflammation (135) or infection (120). By selectively removing monocytes from the central circulation using liposomal clodronate and then sequentially examining their re-population it is possible to examine the maturation of the monocyte response. Following an intravenous injection of clodronate most monocytes have been removed from the central circulation by 18hours. At 48 hours re-population has begun almost universally with bone marrow derived Gr-1^{Hi} cells which, as they mature, express Gr-1 less and less resulting in the Gr-1^{Lo} subset. Labeling the initial Gr-1^{Hi} cells with Dil and following their maturation confirms the development of Gr-1^{Lo} cells from the Gr-

1^{Hi}. In times of inflammation the expression of Gr-1 actually increases representing a left ward shift to a more immature cell released from bone marrow, similar to the leftward shift of neutrophils (135).

This builds a picture of Gr-1^{Hi} cells released from bone marrow under the influence of CCR2, in times of no inflammatory stimulation there is a no activation of CX_3CR1 and these cells either undergo apoptosis or differentiate into the more mature Gr-1^{Lo} cells. In times of inflammation these Gr-1^{Hi} cells rapidly migrate to areas producing significant amounts of MCP-1 such as resident macrophages or the mesothelial cells lining the peritoneum (119). The Gr-1^{Lo} cells, which routinely patrol the endothelium, are immediately available to rapidly migrate into areas of inflammation and assist in this recruitment.

There are several similarities between the murine and human monocyte subsets. The Gr-1^{Hi} and $CD14^{+}/CD16^{-}$ cells are larger than Gr-1^{Lo} and $CD14^{dim}/CD16^{+}$ cells and express a similar variety of surface molecules. Gr-1^{Hi} and $CD14^{+}/CD16^{-}$ monocytes both express CCR2 whereas Gr-1^{Lo} and $CD14^{dim}/CD16^{+}$ cells express CX_3CR1. There are, however, some differences in subset function between species that appear contrary to this homology. In mice the main producer of TNFα is the Gr-1^{Hi} monocyte, whilst the Gr-1^{Lo} subset patrol blood vessels (45) and may have a reparative role in tissue injury (127). In humans however the $CD14^{+}/CD16^{-}$ cells have a more anti-inflammatory role with increased production of IL-10, whilst the $CD14^{dim}/CD16^{+}$ cells produce the majority of TNFα(136). Recent microarray genomic sequencing has discovered that the $CD14^{+}$ cells including $CD14^{+}/CD16^{-}$ and $CD14^{+}/CD16^{+}$ map a similar cluster of genes as the Gr-1^{Hi} murine cells (137). The expression of TNFα is actually highest on the intermediate $CD14^{+}/CD16^{+}$ cells when compared to either of the other 2 subsets. The $CD14^{dim}/CD16^{+}$ group do express

more anti-inflammatory cell surface markers such as IL-10 and match to their human counterpart $CD14^{dim}/CD16^{+}$.

Immune response to peritonism

It is surprising given the potential that the effect of surgery or tissue damage on the immune response has not been studied more. There are several different animal models that have demonstrated recruitment of cells to the peritoneal cavity but there has been little published regarding the effects this has in a more global sense. There are over 800 publications in pubmed that have used the ceacal ligation and puncture model and this is probably the most widely used (138). The problem with CLP is that there are several facets to it, incision, ligation and puncture all of which can be performed in a slightly different way dependent on local protocol. It remains a good model for examining the pathophysiology of sepsis in general but diificult to tease out which facet is responsible for recruitment or activation of leucocytes.

Other animal models have also looked at the trafficking of leucocytes into the periotneal cavity and the gut. To examine the effect this migration has on gut motility Kalff et al performed a laparotomy, handled the gut, closed and then removed the gut at various timepoints, measuring the amount of leucocyte activation in the muscularis mucosa (139). In a later study they found greater numbers of monocytes and macrophages in peritoneal lavage fluid after surgery upregulating COX-2 expression and suggested as a putative mechanism for post-op ileus (140). Many of the studies utilising CLP have a control group undergoing a sham laparotomy, these often demonstrate recruitment of monocytes and neutrophils when compared to wild type animals (141). These effects are a combination of the trafficked immune cells and the resident cells. There is some evidence that activation of immune cells in the

peritonal cavity can have deleterious effects at distant organ sites such as the heart (142) and lung (143). By removing at least some of the resident cells prior to introduction of LPS, Zhao et al found that mice could be partially protected (144).

Either LPS or surgery are able to recruit cells to the peritoneal cavity. Further stimulation provides a mechanism by which multi-organ failure could be triggered.

The kinetics and trafficking of leucocytes to the peritoneal cavity is difficult to study in humans not just because of patient heterogenaity but also the variation in surgery and technique. There are studies that have examined blood monocytes and the expression of HLA-DR following abdominal surgery (145) and more recently the influence of leucocytes on the development of multi-organ failure in the trauma patient (146). However the majority report the effect of surgery or its complications on hard outcomes for patients, which are clearly important, rather than the process of recruitment and activation within the peritoneal cavity and translation of this into a significant global SIRS response.

Hypotheses

1. In response to mild surgical trauma there is differential migration of the monocyte subsets into the peritoneal cavity.
2. Monocytes are primed by migration to produce more TNF when exposed to a post-operative infection. This production of pro-inflammatory cytokines, given the right circumstances, provides a mechanism for the development of multi-organ failure.

Monocyte subsets have been delineated for some time; there has been an appreciation more recently that the speed of migration into areas of inflammation is more rapid than originally thought. In combination with a priming effect from endothelial rolling/binding prior to extravasation this provides a discreet but potentially critical upstream event in subsequent peritonitis and sepsis. Several animal models have observed this infiltration of monocytes but not defined it.

Clinically, the description of cell migration and activity following minor surgery will increase our understanding of the differential response to minimally invasive and open surgery.

Chapter 2

Materials and Methods

Materials

General reagents

Invitrogen (Paisley, UK) supplied Dulbecco's phosphate buffered Saline (PBS), Foetal Calf serum (FCS) and Hanks Balanced salt solution (HBSS). Ethylenediaminetetraacetic Acid (EDTA), sodium azide and Trypsin-EDTA were purchased from Sigma chemical company (Dorset, UK). Lyse-fix solution was supplied by BDbioscience (Oxford, UK). The metalloproteinase inhibitor BB94 was obtained from British Biotech (Oxford, UK). FACS Wash buffer (FWB) was the main solution used to harvest, wash and suspend cells. It contains 2% foetal calf serum, 5 mM EDTA, 0.1%Azide diluted in PBS. LPS ultrapure (E. Coli 0111:B4) was purchased from Autogen Bioclear (Wiltshire, UK))

Animals

All protocols performed were in accordance with the Animals (Scientific Procedures) Act 1986, United Kingdom and had previously been reviewed and approved by the Home Office. Experiments were performed using male C57BL/6 mice (Charles River, Margate) between 8 to 12 weeks of age.

Antibodies

All antibodies were flurophore-conjugated for use in flow cytometry and directed against murine antigens. For determining expression of cell surface proteins (eg TNFα) appropriate isotype-matched controls were used utilizing flurophores not involved in cell identification. By subtracting the amount of flurophore detected with the isotype controls from the actual samples the signal from non-specific binding can be removed. The antibodies used are listed in **Table 1**.

Antibody	Flurophore	Clone	Manufacturer	Description	Isotype
F4/80	FITC	Cl:A3-1	Serotec	Rat, anti-mouse	IgG2b
Gr-1	PerCP	RB6-8C5	Biolegend	Rat, anti-mouse	IgG2b
XT22 (anti-TNF)	PE	MP6-XT22	eBioscience	Rat, anti-mouse	IgG1
CD11b	APC-Cy7	M1/70	Biolegend	Rat, anti-mouse	IgG2b
CD115	PE/APC	AFS98	Biolegend	Rat, anti-mouse	IgG2a
Isotype Control	PE	eBRG-1	eBioscience	Rat, anti-mouse	IgG1

Table 1. *Murine antibodies used with their associated flurophore and the manufacturer. FITC – Fluorescein isothiocyanate; PerCP – Peridinin Chlorophyll; PE – Phycoerythrin; APC – Allophycocyanin.*

Peritoneal LPS

LPS was diluted to the required concentration in sterile PBS. 200µL of solution was prepared in an insulin syringe to minimise damage to the peritoneal wall during injection. The solution was injected into the left upper quadrant of the animal's abdomen. They were allowed to recover in a warm cage with unlimited supply of water and food.

Surgery

Animals that underwent surgery were anaesthetized using a gas chamber filled with isoflurane until there was no response to pedal pressure, they were then transferred to a heated matt within a sterile area. Anaesthesia was maintained using isoflurane in a 50:50 O_2, and air mix via a sterile glove modified to act as a mask. Spontaneous breathing was maintained and therefore no muscle relaxant was required. Expired gas was analysed to keep the minimum alveolar concentration (MAC) between 1.6 and 2.0. Prior to surgery a lack of response to pedal pressure was again used to indicate sufficient depth of anaesthesia. Further monitoring included tail O_2 saturation and an intramuscular temperature probe.

Once anaesthetised and monitored the abdomen was shaved and the skin prepared using aqueous betadine (Ecolab, Leeds, UK). The area around the mouse remained sterile using a paper drape and the operator used sterile gloves to maintain an aseptic field. A longitudinal skin incision was performed in the upper left quadrant with a size 11-scalpel blade and extended to approximately 2cm. For peritoneal incision, blunt dissection to expose the bowel and intra-abdominal organs was performed and a further 2cm incision completed. The peritoneum was opened for a maximum of 3 minutes following the initial incision. The layers were then closed individually using 4.0 silk, interrupted sutures for the peritoneum and 2.0 silk continuous sutures for skin. A 5% eutectic mixture of local anaesthetic (Astra Zeneca, London, UK) was applied topically to the wound as analgesia. Anaesthesia was discontinued and the animal allowed to recover in a warm environment until fully awake.

Peritoneal Lavage Protocol

Mice were sacrificed by isoflurane overdose and exsanguinated by cardiac puncture including 10IU of heparin. An initial 5ml of FWB was injected using a 23G needle into the peritoneal cavity and the mouse was agitated for 1 minute to distribute cells. This initial lavage was aspirated, and the procedure repeated with a further 5ml of FWB. The recovered lavage and blood sample were stored on ice.

Flow cytometry

The lavage and blood samples were first centrifuged for 10mins at 2000rpm and re-suspend in 200uL of FWB. The samples were combined with 50µL of antibody or isotype control and incubated at 4°C in the dark for 30 minutes. The cells were washed and re-suspended in 50µL of FWB. Prior to re-suspension, red blood cells were lysed using BD lyse/fix and the samples washed again.

Flow cytometry was performed on a Cyan flow cytometer (Beckman Coulter, High Wycombe, Uk) and the data analysed using Flowjo software (Treestar, Oregon, US).

The gating strategies used were subjective (147), accepting that the gates may be altered to include cells forming distinct populations. They were based on the distribution of cells in the blood of normal untreated mice. Previous established gating strategies (120,132,148) were modified to identify the recruited subpopulations in the peritoneal lavage fluid. The results are present as the number of cells per ml.

Detection of membrane bound TNFα.

To try and reduce the confounding of LPS and TACE activity on the amount of membrane bound TNFα expressed the metalloproteinase inhibitor BB94 was included with the lavage fluid at a concentration of 10uM. Cell counts were not affected by its

inclusion in the lavage fluid. The process of preparation and flow cytometry then continues as above.

Expression levels are given as the geometric mean of fluorescence intensity (MFI) emitted by cells, with the isotype control value subtracted. Results are reported as mean of repeated experiments ± SEM.

Ex-Vivo stimulation of peritoneal lavage cells

The variation in the methodolgy with the ex-vivo experiments were as follows. In these animals, 24 hours after surgery lavage fluid was obtained using RPMI rather then Facs Wash Buffer (FWB). This allowed the cells to be maintained whilst stimulated by LPS ex-vivo. The cell counts were obtained using a haemocytometer and then incubated at 37°C either with the TACE inhibitor BB94 or 0.9% saline in equal volume and either 20ng LPS or an equal volume of 0.9% saline for one hour. The samples were centifuged at 1200rpm for 5 mins and the supernatant separated from the cells. The cells were re-suspended in FWB and then stained for flowcytometry in an identical way to that described above. In those samples not incubated with BB94 the supernatant was removed and stored at -20°C for later analysis of soluble TNFα by ELISA as descibed below.

ELISA of soluble TNFα

This was performed using our in-house assay with reagents purchased from R&D systems.

ELISA plates (Nunc, Roskilde, Denmark) were coated with capture antibody (R&D) at 1μg/ml and left at 4°C for 16 hours. Wells were then washed x 4 with PBS supplemented with 0.05% Tween-20 (Sigma), this washing protocol was

automated as standardised throughout the protocol. Wells were then blocked for an hour using a PBS supplemented with 1% BSA (Sigma) and 0.05% Tween-20 (Sigma). The washing step was repeated before standards consisting of TNF (R&D) were added. Samples were added and diluted in block buffer where appropriate and incubated for 2 hours at room temperature. Plates were then washed and biotinylated antibody (R&D) added at 200ng/ml and then left for a further two hours. Plates were washed once again and streptavidin-HRP (R&D) was added at a 1/200 dilution and left for 20 minutes. Substrate solution - Tetramethylbenzidine(TMB) (Sigma, UK) was added and plates incubated in the dark for a further 20 minutes before 2M Sulphuric acid (VWR, Leicestershire UK) was added as a stop solution.

Plates were read using a Dynex MRX II plate reader (Worthing UK) in conjunction with RevelationTM software (version 4.22, Dynex).

Wet/Dry Ratios

Animals were sacrificed by a recognised schedule one method and the lungs dissected out, ensuring the hilar structures were avoided. The lungs were then weighed to calculate the wet weight and then dried for 4 hours and weighed again to calculate the dry weight. The difference between these two weights represents the amount of fluid in the lungs. This technique proved difficult and the results often hard to interpret as there was wide variance in repeat experiments. Because of this wet/dry ratios were abandoned in favour of the alternative method of calculating lung water described below.

Lung Permeability Index

An alternative method of calculating the amount of peri-capillary leak is to utilise 2 separate dyes attached to flurophoes of different wavelengths (149).

The first dye, Alexa Fluro 594 (Invitrogen, Paisley, UK) is mixed with Bovine serum albumin (BSA) and injected into the tail vein, it circulates for 2 hours and a proportion crosses the pulmonary endothelium. To exclude the influence of intravascular BSA in the residual blood left in the lung samples, mice were injected i.v. with a second dye, Alexa Fluor 488-conjugated BSA (0.2 mg/mouse; Invitrogen) along with heparin (100 U/ml) 5 min before sacrifice.

Lungs were excised, rinsed briefly in saline, and swabbed gently with tissue to remove surplus blood. Lungs were then homogenized and incubated with collagenase type IV (Sigma-Aldrich) for 30 min at 37°C to ensure release of tissue-sequestered BSA. After further homogenization, lung suspensions were passed through a 50um filter and centrifuged, supernatants were collected. Fluorescence levels were measured in lung homogenate supernatants and diluted whole blood samples (1/100) using a fluorescence plate reader (Flx-800; Bio-Tek Instruments). An index of vascular permeability was calculated by subtracting the lung:blood ratio of BSA-Alexa Fluor 488 nm (intravascular content) from the lung:blood ratio of BSA-Alexa Fluor 594 nm (total lung content).

Statistics

The data is presented as the mean of the repeated experiments ± SEM. Each collection of data was analysed for normality using the Kolmogorov-Smirnov test. Parametric data was analysed using an unpaired t-test for 2 variables and one-way ANOVA with Bonferroni correction for multiple comparisons if there were 3 or more

variables. Non-parametric data were analysed with a Mann-Whitney U test for 2 variables and Kruskal-Wallis test with Dunn's test for multiple comparisons for more than 2 variables. The analysis was performed using Prism software (Graphpad, USA). p values less than 0.05 were considered to be significant.

In order to reduce the number of animals required for these experiments the control groups often only used 3 mice whereas intervention was performed on higher numbers.

Chapter 3

Dynamics of monocyte recruitment to the peritoneal cavity

Background and aims.

In-vivo studies on the recruitment of monocytes to areas of inflammation have been performed mostly in mice using both whole bacteria and specific PAMPs such as LPS. Van Furth described the use of thioglycollate broth as an intra-peritoneal injection, designed to increase the number of peritoneal macrophages for in-vitro co-cultures (150). Thioglycollate increases the number of peritoneal leucocytes within the first 48 hours but these cells are not activated (ie minimal opsonisation of bacteria or production of pro-inflammatory cytokines). More recently, Geissman's group used sterile intra-peritoneal thioglycollate to create an inflammatory response to observe the kinetics of the monocyte subsets. In a similar way Sunderkotter used ip sterile foetal calf serum (120,121,135). Although this sets up a sterile inflammatory response it does not represent the commonest cause of intra-peritoneal inflammation which is infection. The processes of mononuclear cell recruitment, migration and differentiation related to an infected stimulus are likely to be different to that from a sterile stimulus. In the abdomen most infections occur due to peritoneal seeding of gram negative commensal gut flora either via translocation or direct spill over from a perforated viscus. As previously mentioned the model of caecal ligation and puncture combines the effects of surgery and peritoneal infection making inferences about the effects of each component difficult to make. Feacal pellets are available but it is hard to standardise the numbers of microbes within them. Although less representative of the gut flora antigens, local administration of purified bacterial PAMPs such as LPS offers a more precise way of defining the peritoneal and systemic response to an infected peritoneum.

Van Furth also used sterile foetal calf serum to evoke peritoneal inflammation and demonstrated the flow of murine mononuclear cells from bone marrow through blood

monocytes to tissue macrophages (151)(96). This process invovles CCR2 (Chemokine receptor 2) and its ligand MCP-1 (Monocyte chemoattractant protein-1) (152). One trigger for release of monocytes appears to be from small amounts of microbial components such as LPS that enter the circulation and increase expression of MCP-1 on mesenchymal cells within the bone marrow (153). LPS injected into the peritoneum is able to rapidly pass into the circulation (154); Shi et al performed a dose response experiment and the intra-peritoneal dose mobilising the greatest number of bone marrow monocytes was 20ng (153). Monocytes are released at doses less than this but at higher doses monocytes are sequested in bone marrow and lung vasculature (155). The monocytes mobilised by intra-peritoneal LPS appear to have effects at distal sites such as the lung (156). This may be a function of monocytes released into the circulation by any inflammatory process or it may be unique to intra-peritoneal infection. Intra-peritoneal LPS has been shown to trigger monocyte release from bone marrow but there is also a suggestion that monocytes may also be released from a splenic store (126) in response to inflammation. The degree of LPS spill over from the peritoneal cavity may influence the response to the source infection as well as the level of systemic response. As TLR-4 is expressed on circulating immune cells and endothelial cells the pulmonary endothelium acts as sentinel cells for systemic infection leading to margination of leucocytes in the pulmonary vasculature. Barrier site infection such as within alveoli or peritoneal cavity relies on bone marrow derived immune cells such as monocytes and macrophages to detect microbes and recruit cells to the areas of inflammation. Ironically the global actiavation and sequestration of leucocytes with systemic disease may actually delay the clearance of the original infection (157).

Aim 1. Examine the dose-response relationship of intra-peritoneal LPS leading to monocyte recruitment and describe the time course for each of the monocyte subset's migration from blood into the peritoneal cavity

Monocytes migrate into the peritoneum in reponse not only to infection or sterile inflammation but also tissue damage, such as that caused by surgery or trauma (42,43,158). In response to microbial invasion the innate immune system is activated by pathogen associated molecular patterns (PAMP's) such as LPS. The cell damage as a result of surgery or trauma is sterile, but the innate immune system can still be activated via the recognition of damage associated molecular patterns (DAMP's) such as high mobility group box 1 protein (HMGB), ATP and nucleic acids. These constituent parts of cells are able to trigger pattern recognition receptors and activate the innate immune system. There is some cross-over in the recontion of DAMP's and PAMP's, for example TLR-4 recognioses both LPS and HMGB (43). Levels of HMGB-1 correlate with injury severity score and base deficit following trauma (159,160), and are raised after haemorrhagic shock (161). Although many of the other DAMPs do not correlate well with injury severity this provides some evidence for the triggering of the immune response by the insult of tissue damage (146). There are several other compounds that seem to act synergistically as DAMPs when triggered by tissue damage. These include ATP (162) which on release from necrotic cells recruits neutrophils to adhere to endothelium surrounding the areas of necrosis and formyl peptides that form a chemokine gradient promoting neutrophil migration to the areas of necrosis (163).

The response to PAMPs is subtly different to the response to DAMPs but there is also a degree of mutual activation, monocytes adherent to endothelium in repsosne to LPS

will upregulated a receptor for HMGB-1 (164). The differences may be related to the levels of potency. LPS is a potent stimulus that when present in the circulation will lead to a widespread response. DAMPs however are less potent and tend to limit the reaction of the innate immune cells to local areas of necrosis.

Aim 2. Compare and contrast the recruitment of monocytes to the abdominal cavity when stimulated by surgery to recruitment triggered by LPS.

Protocols

Intra-peritoneal LPS stimulation

The protocols for the injection of LPS and the harvest of cells from blood and peritoneal lavage were described in detail in chapter 2. C57/B6 mice received peritoneal LPS at 2 different doses. These doses were based on the work showing maximal monocyte margination to the lungs at a sub-clinical dose of 20ng LPS intra-venously and clear clinical symptoms produced with 20μg intra-venous LPS (132) and is consistent with Shi et al who subsequently found maximal release of monocytes from bone marrow with an intra-peritoneal dose of 20ng LPS (153).

By sacrificing the animals at a variety of time points up to 48 hours the time course of migration for each monocyte subset can be calculated.

Surgery

The surgical protocol developed has been described in detail in chapter 2. The animals undergoing peritoneal incision were compared to two control groups; one receiving a skin incision alone and the other receiving anaesthesia for a period

approximate to the duration of the anaesthesia for the surgical group. The animals were sacrificed at time points up to 48 hours.

Results

Identification of monocytes in blood and peritoneal lavage samples using flow-cytometry following ip LPS.

As previously reported there is an established method for identifying monocytes in murine blood samples with flow cytometry based on side scatter (cell granularity), the surface expression of CD11b and the monocyte/macrophage marker F4/80 (132,135,165). The $CD11b^{+}$ cells are neutrophils, monocytes, eosinophils or natural killer cells and these can be differentiated by the use of F4/80 and side scatter (granularity) (166). A clear reproducible population of monocytes is easily differentiated from lymphocytes ($CD11b^{-}$), neutrophils ($CD11b^{+}$, F4/80, Gr-1^{Hi++}), eosinophils ($CD11b^{+}$, $F4/80^{+}$, high side scatter) and natural killer (NK) cells ($CD11b^{+}$, $F4/80^{-}$, Gr-1^{Lo-Med}) (**Figure 1**). The monocyte population ($CD11b^{+}$, $F4/80^{+}$) can be divided into two subpopulations, Gr-1^{Lo} and Gr-1^{Hi}. In response to low dose (20ng) ip LPS, there wasn't a significant change in monocyte subset numbers (n=3-5), but the pattern of Gr-1 expression appeared to modified suggesting a small systemic effect.

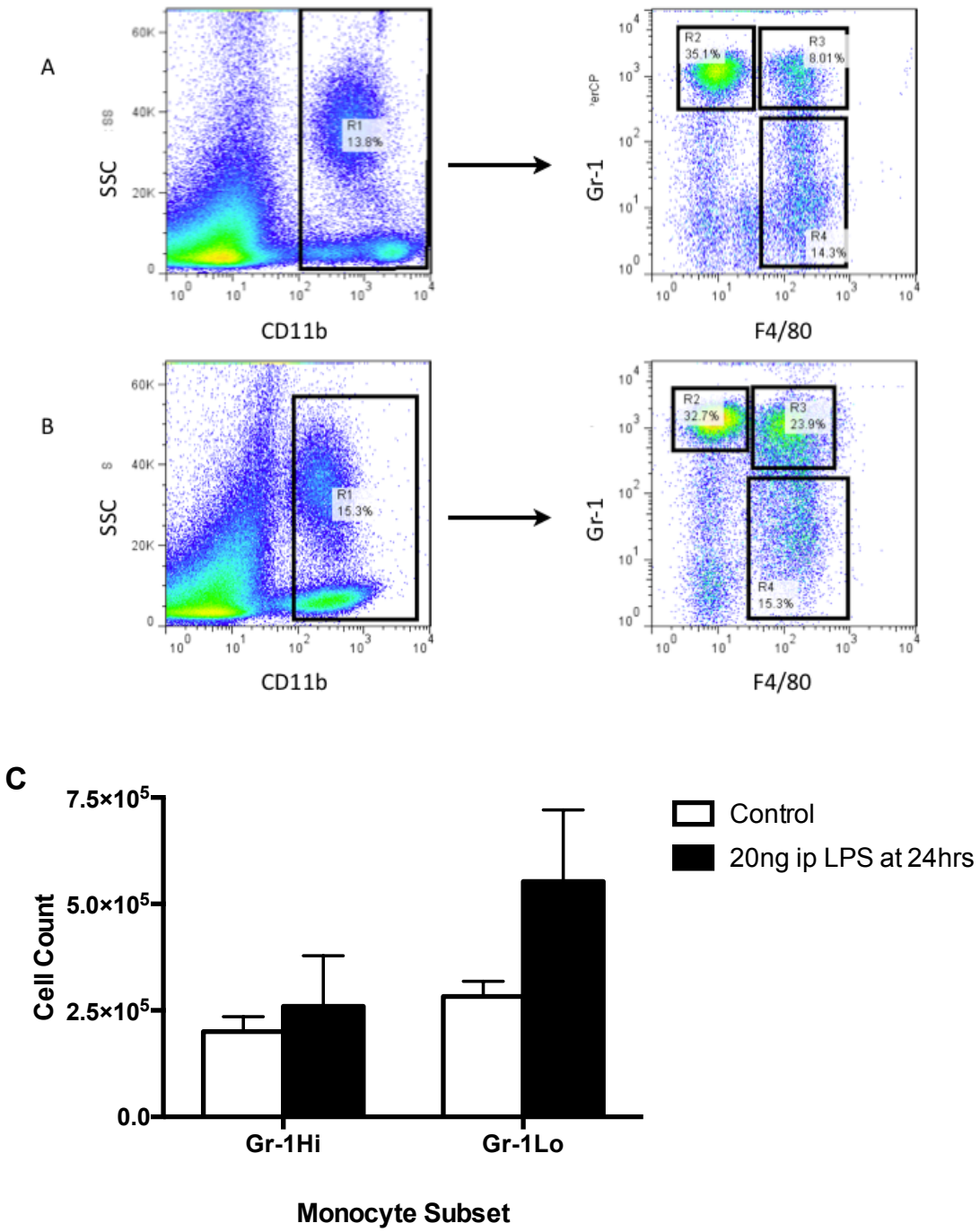
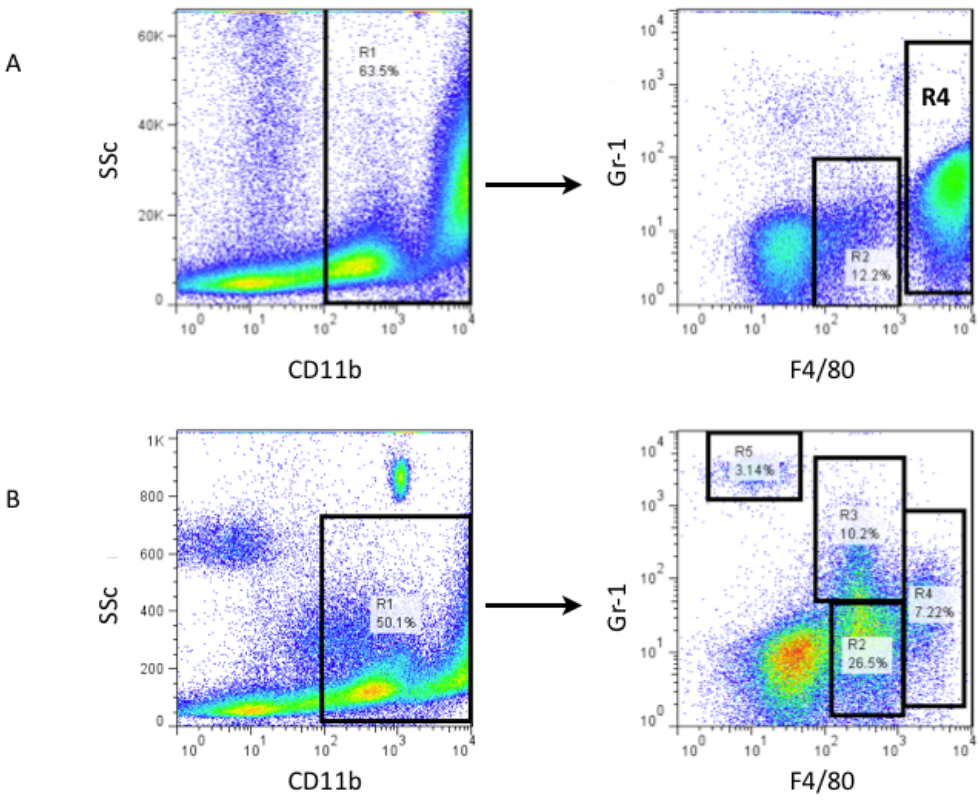


Figure 1. Identification and quantification of circulating monocytes. *Blood was obtained from controls (A) and from animals injected with 20ng ip LPS 24 hours previously (B). Monocytes were identified as CD11b^{+} and F4/80^{+}. R1 CD11b^{+}; R2 Neutrophils; R3 Gr-1^{Hi} monocytes; R4 Gr-1^{Lo} monocytes. There is no significant difference in blood monocyte cell number between controls and 20ng ip LPS (C). Un-paired t-test Gr-1^{Hi} p=0.6;Gr-1^{Lo} p=0.1; n=3$_{control}$ and 5$_{intervention}$.*

In peritoneal lavage from normal mice there was a resident population of cells that are strongly positive for CD11b and also express F4/80, these cells have previously been described as resident macrophages (135). There are also a resident population of Gr-1^{Lo} cells that resemble Gr-1^{Lo} monocytes in blood and are distinct from macrophages; there are few or no Gr-1^{Hi} monocytes. At 24 hours after a 20ng dose of ip LPS there was a rise in peritoneal lavage cell numbers with additional phenotypes clearly apparent. The $CD11b^{+}$ cells can be further separated by their expression of F4/80 and Gr-1. Neutrophils were evident with a similar phenotype as in blood of F4/80- and Gr-1Hi, while the monocytes classified by medium expression of F4/80 were mainly of the Gr-1Lo phenotype (**Figure 2**). Despite the prominence of Gr-1^{Lo} subset phenotype, relative increases in the Gr-1^{Hi} cells were much higher due to their virtual absence in the normal mouse peritoneal lavage.



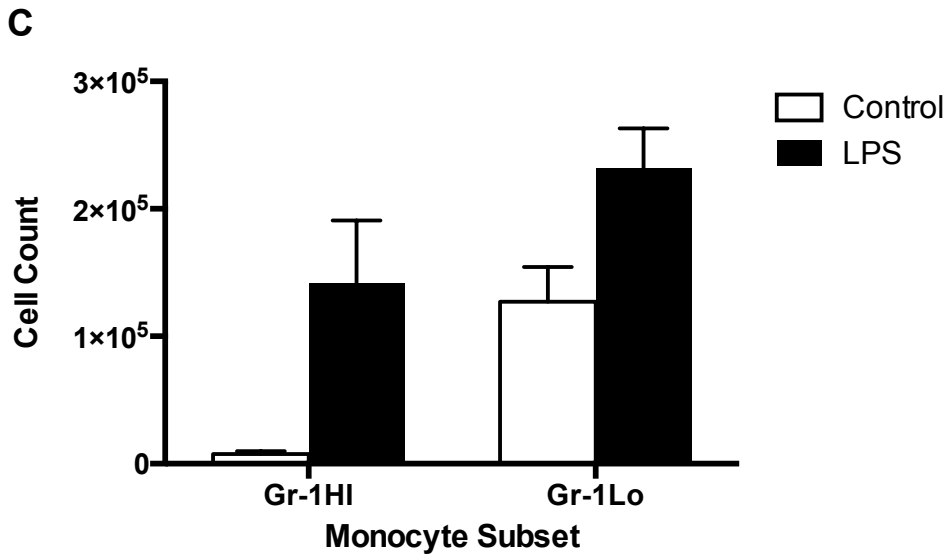


Figure 2. Identification and quantification of peritoneal lavage monocytes. *Lavage was obtained from the peritoneum of both controls (A) and mice that received a 20ng ip injection of LPS 24hrs previously (B). The cell populations were described in a similar way to the populations found in blood. R1 CD11b^{+}; R2 Gr-1^{Lo} monocytes; R3 Gr-1^{Hi} monocytes; R4 Peritoneal macrophages; R5 Neutrophils There are more monocytes of both subsets present in lavage following LPS exposure than are found in normal control mice(C). Un-paired t-test Gr-1^{Hi} p=0.009;Gr-1^{Lo} p=0.02; n=5$_{control}$ and 8$_{intervention}$.*

To further evaluate and understand the changes in peritoneal populations, peritoneal lavage fluid following injection of ip LPS was examined at various time points up to 48 hours (**Figure 3 and 4**). Blood monocyte counts appeared to rise at 16-24 hours, but no clear pattern emerged over the 48 hour observation period.

Mirroring this apparent increase in circulating cells, in peritoneal lavage fluid there is an initial rise in Gr-1^{Hi} monocytes peaking around 16 hours and subsequently declining by 48 hours. The peritoneal lavage fluid Gr-1^{Lo} monocytes initially decline and then peak later at around 24 hours; they remain elevated at 48 hours. These

results suggest that the Gr-1^{Hi} subset is preferentially recruited initially following an infective stimulus but this process is transient. The Gr-1^{Lo} monocytes numbers also increase somewhat later; this may represent either in-situ maturation or a second wave of migration.

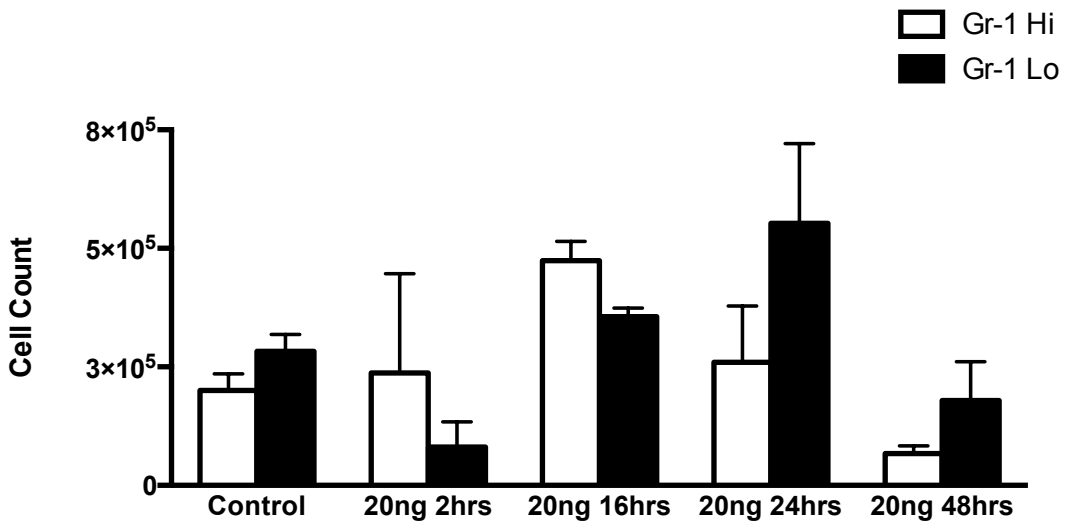


Figure 3. ***Monocyte subset kinetics in blood after ip LPS.*** *The change in monocyte subset numbers were followed in blood over 48hours after an ip injection of 20ng LPS and compared to control animals not exposed to LPS. One-way ANOVA Gr-1^{Hi} p=0.20; Gr-1^{Lo} p=0.07. n=$3_{control}$ and $5_{intervention}$*

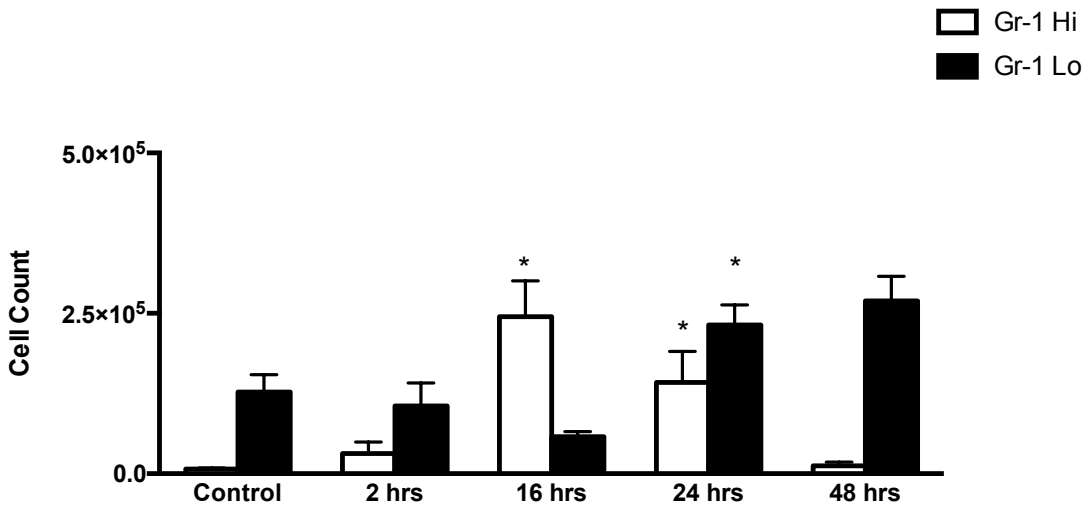


***Figure 4*. Recruitment kinetics of monocytes to the peritoneal cavity after ip LPS.** *The change in monocyte numbers was tracked in peritoneal lavage fluid at various time points over 48 hours. Kruskal-Wallis Gr-1^{Hi} p=0.0005; One-way ANOVA Gr-1^{Lo} p=0.01. *p<0.05. n=3$_{control}$ and 5$_{intervention}$.*

The dose of 20ng LPS recruits monocytes to the peritoneum following intra-peritoneal injection without any significant change in the number of circulating cells. In contrast, LPS triggered an early increase in the number of neutrophils in both blood and peritoneal lavage at 2 hours indicating that this dose was sufficient to elicit a systemic response (**Figure 5 and 6**). The increase in blood was completely reversed by 16 hours but persisted in the peritoneal lavage fluid. These results are consistent overall with the typical neutrophil then monocyte sequence of migration (49), without clear evidence of early monocyte emigration as described by Auffray (121). There were significantly more macrophages within the peritoneal lavage both in the control animals and after LPS when compared to monocytes or neutrophils. Following LPS there was a decrease in the number of peritoneal macrophages reaching a nadir

between 2 and 16 hours. This is likely to represent the previously described macrophage disappearance reaction (167).

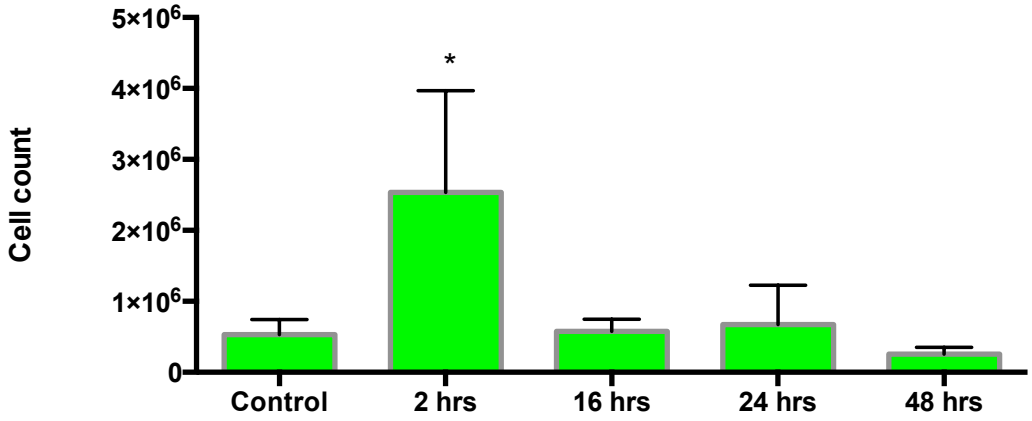


Figure 5. Neutrophil kinetics in blood after ip LPS. *The change in neutrophil numbers in blood over time following a 20ng intra-peritoneal LPS challenge compared to control animals. One-way ANOVA p=0.03; *p<0.05 compared to control. n=3$_{control}$ and 5$_{intervention}$*

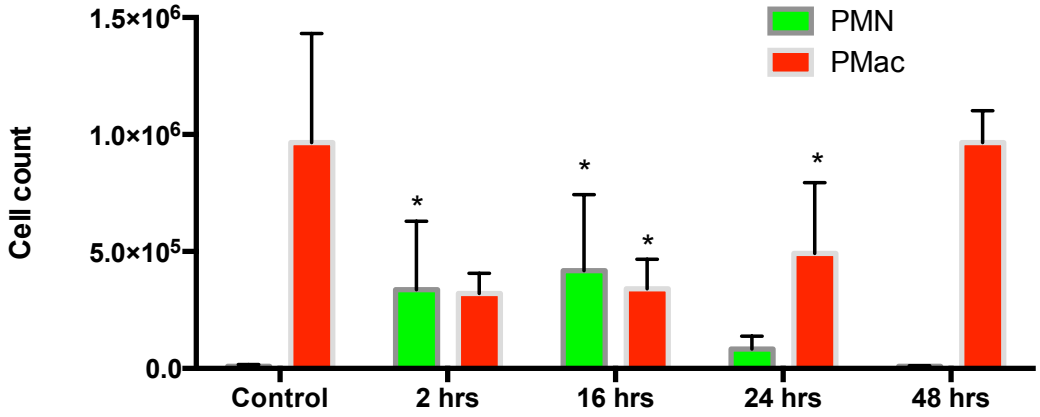
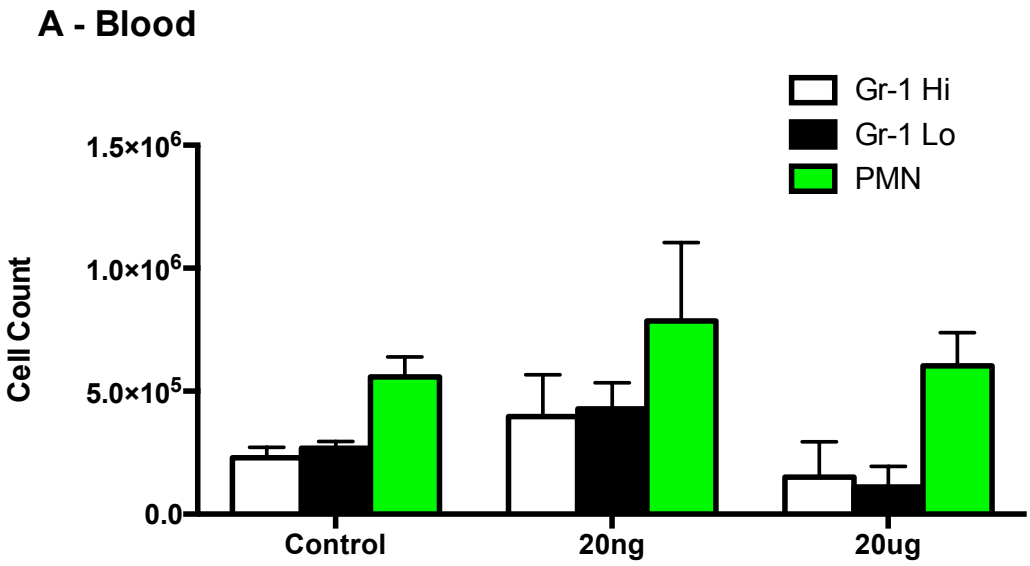


Figure 6. Neutrophil and resident macrophage kinetics in the peritoneal cavity after ip LPS. *A comparison of the absolute numbers of neutrophils and macrophages found in peritoneal lavage following a 20ng intra-peritoneal LPS challenge compared to control animals. One-way ANOVA PMac p=0.01; PMN p=0.002. *p<0.05 compared to control. n=3$_{control}$ and 5$_{intervention}$*

Leucocyte peritoneal migration with high dose ip LPS

The numbers of cells recruited in response to intra-peritoneal saline, a sub-clinical 20ng dose of LPS or a clinical 20µg dose of LPS were compared **(Figure 7).** Clinically there was a difference between the mice exposed to the 20ng dose and the mice exposed to the 20µg dose. The latter demonstrated piloerection and reduced movement whereas the former were indistinguishable from control animals.



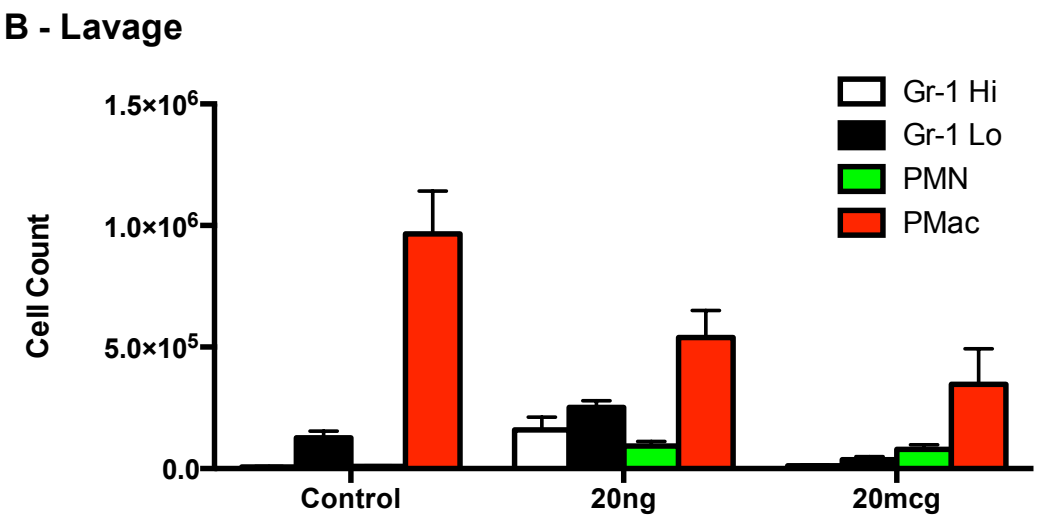


Figure 7. Leucocyte recruitment following high and low dose ip LPS challenge. *The recruitment effect of either control 0.9% saline, 20ng LPS or 20µg LPS on myeloid leucocyte subpopulations in (A) blood and (B) peritoneal lavage fluid 24 hours following injection. Blood – (One-way ANOVA) Gr-1^{Hi} 0.1; Gr-1^{Lo} 0.07; PMN 0.1. Lavage – Gr-1^{Hi} (unpaired t-test) p=0.04; Gr-1^{Lo} (One-way ANOVA) p=0.001; PMN (unpaired t-test) p=0.63; PMac (One-way ANOVA) p=0.03.*

With the high dose of 20µg ip LPS the circulating monocytes decrease whilst the neutrophil counts remain the same. In the peritoneal lavage fluid there was an un-expectant reduction in monocyte numbers with the high dose LPS. This may be related to more overspill of LPS into the systemic circulation leading to greater sequestration in the pulmonary vasculature or an effect similar to the macrophage disappearance reaction acting on the trafficked monocytes.

There was little difference in the numbers of neutrophils at either dose and there were less peritoneal macrophages at both doses than the control animals in keeping with the macrophage disappearance reaction.

Leucocyte recruitment to the peritoneal cavity following abdominal surgery and their identification by flow cytometry.

Following surgery the analysis of the flow cytometric data was more complex than the analysis following an LPS challenge. This complexity appeared to derive from the higher numbers of $CD11b^{+}$ cells that migrated into the peritoneal cavity and the more diverse phenotypes as based on the CD11b and side scatter (granularity) plots (**Figure 8 and 9**). The flow cytometry plots for the mice receiving a skin incision alone were similar to control animals **(see Figure 1).**

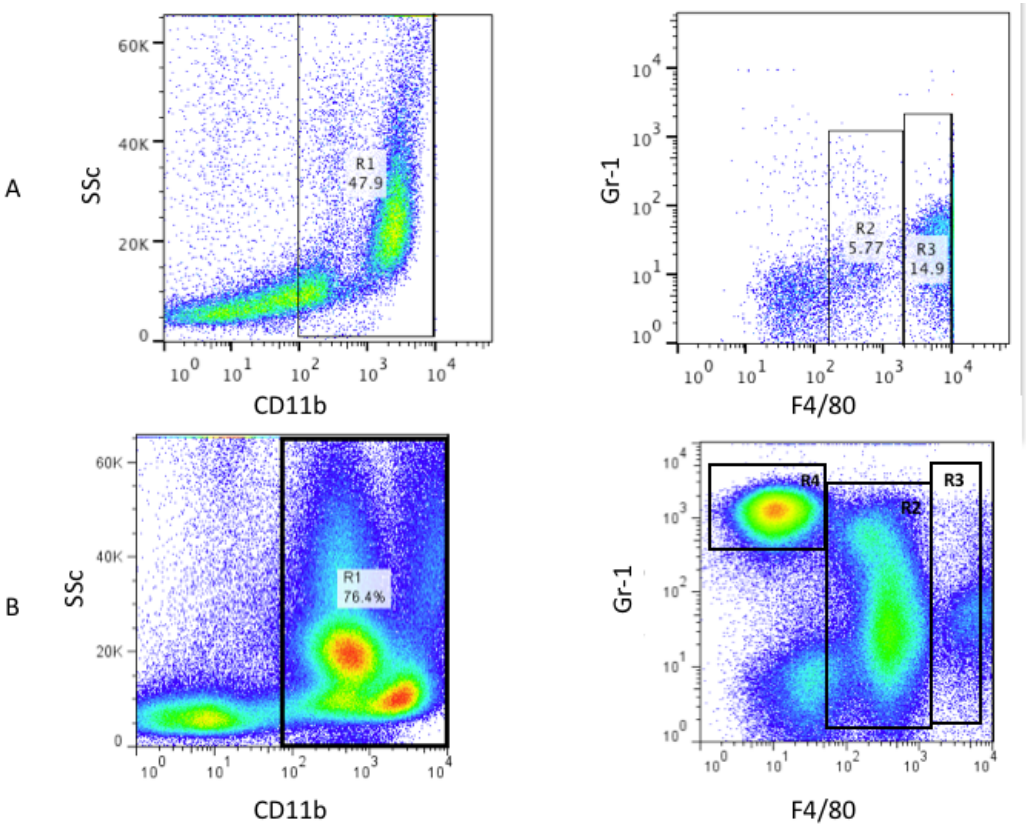


Figure 8*. *Identification of Cd11b^{+} cells in peritoneal lavage fluid. *Mice either received a skin incision alone (A) or a full thickness incision through peritoneum (B). At 24 hours peritoneal lavage samples were obtained. These populations are more difficult to differentiate due to significant overlap in expression of surface markers. R1 CD11b^{+}cells; R2 Monocytes and eosinophils; R3 Peritoneal macrophages; R4 neutrophils*

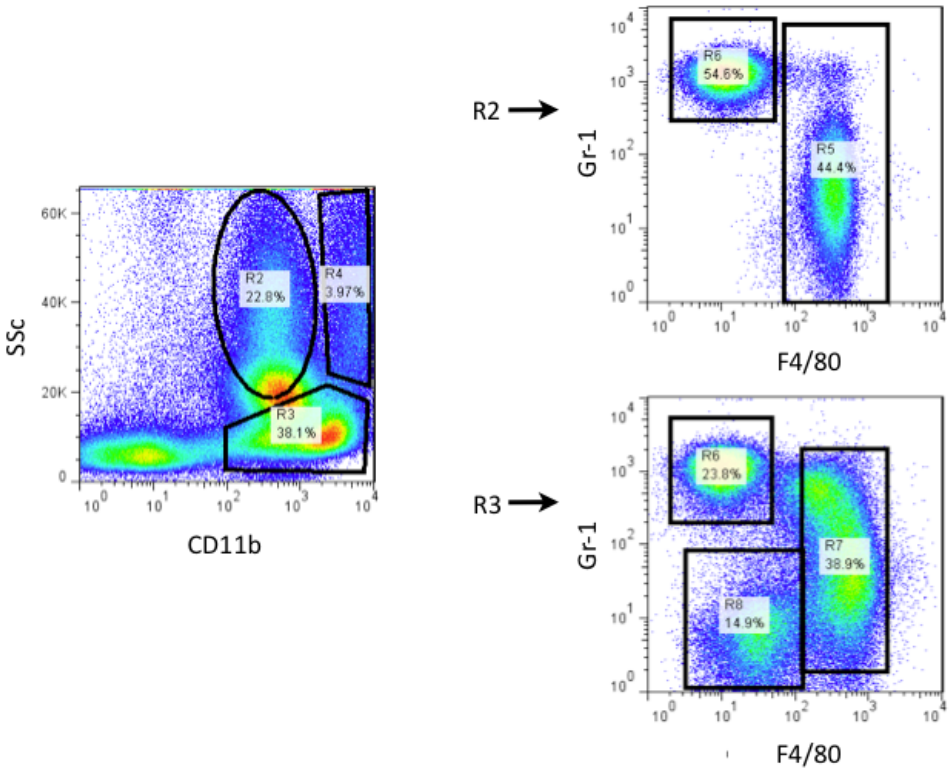


Figure 9. Flow cytometric analysis of peritoneal CD11b^{+} populations following abdominal surgery. *24 Hours after peritoneal incision mice were sacrificed and peritoneal lavage obtained. Several overlapping CD11b^{+} populations can be identified by flow cytometry. High side scatter cells would be consistent with neutrophils (Gr-1^{Hi}, F4/80^{-}) and eosinonphils (F4/80^{+}, Gr-1^{Med-Lo})* (148). *The cells with less side scatter would be consistent with monocytes (F4/80^{+}, variable Gr-1*

expression), and natural killer cells (F4/80$^-$, Gr-1$^-$). The population with the highest expression of CD11b and variable side scatter would be consistent with peritoneal macrophages. R4 Peritoneal macrophages; R5 Eosinophils; R6 Neutrophils; R7 Monocytes; R8 Natural killer cells.

A particular uncertainty was created by F4/80 monocyte/macrophage marker because of its known cross-reactivity with eosinophils (168). To improve delineation of mononuclear phagocytic population from other cell types in resting and inflamed peritoneum, staining for CD115, the monocyte colony stimulating factor (M-CSF) receptor present on cells of the monocyte/macrophage lineage was included. The populations were gated into CD11b$^+$, CD115$^+$ (monocytes, resident macrophages) or CD11b$^+$, CD115$^-$ (non monocyte/macrophage) (**Figure 10**). This strategy allows easy identification of both subsets of monocytes as well as peritoneal macrophages in lavage samples but it does not distinguish between resident and recruited macrophage populations.

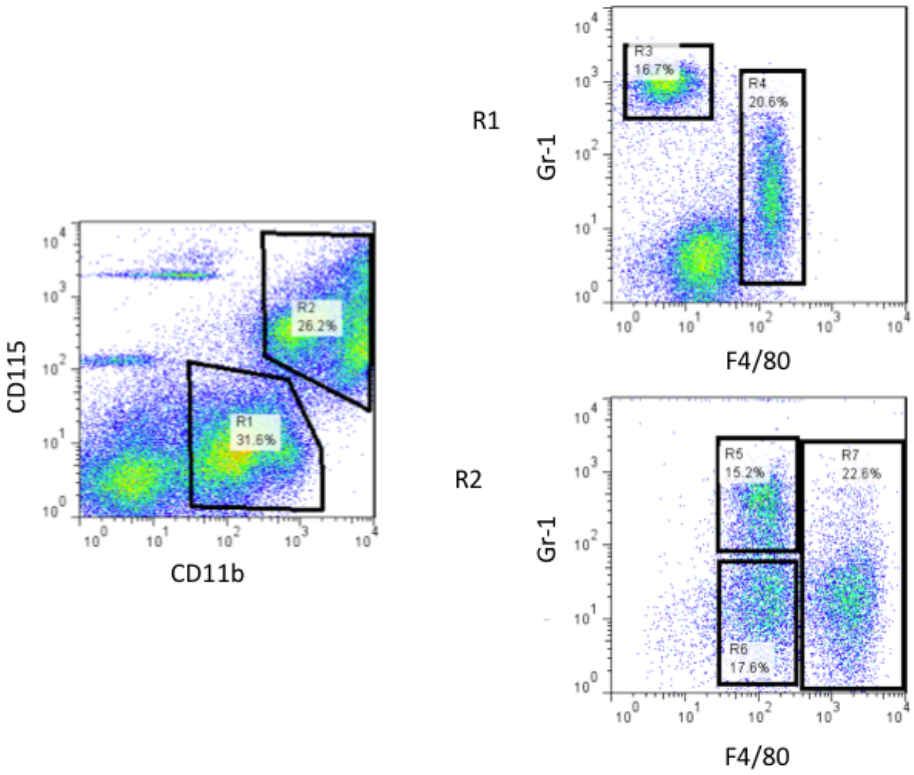


Figure 10. Flow cytometric analysis of peritoneal CD11b+ populations using CD115 expression to identify mononuclear phagocytic cells. *Animals were sacrificed 24 hours after peritoneal incision. $CD11b^+$ peritoneal lavage cells were stained and gated as either $CD115^+$ (monocytes and macrophages (MΦ)) or $CD115^-$ (neutrophils and eosinophils). These cells were then gated as previously with F4/80 and Gr-1. R1 $CD115^-$; R2 $CD115^+$; R3 Neutrophils; R4 Eosinophils; R5 Gr-1^{Hi} monocytes; R6 Gr-1^{Lo} Monocytes; R7 Peritoneal macrophages*

This new strategy allowed a more accurate estimation of monocyte migration following surgery. The essential stimulus for migration in the surgical model was the peritoneal incision; animals receiving skin incision alone or anaesthesia alone did not demonstrate any significant cell recruitment (**Figure 11**).

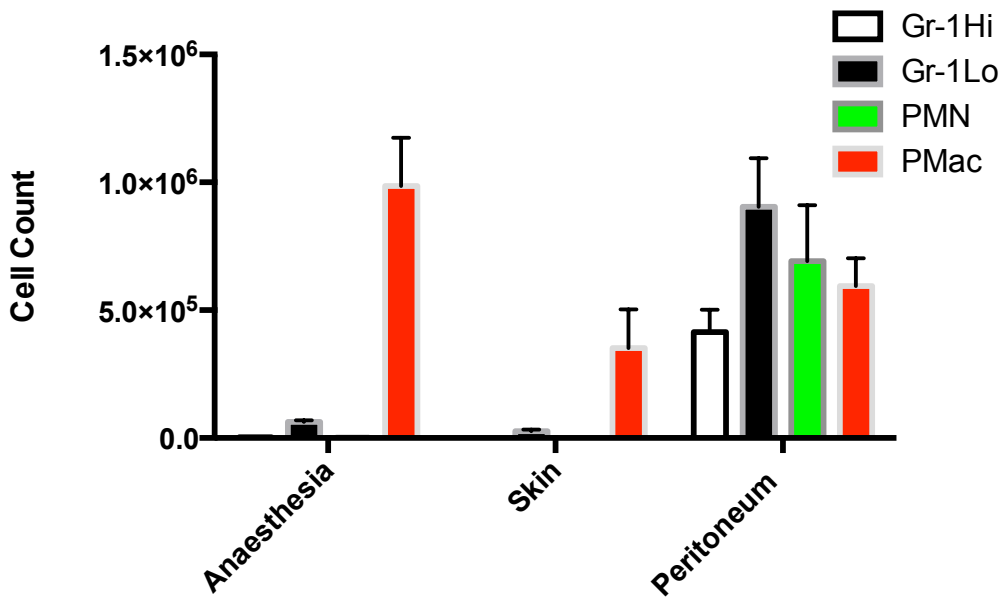


Figure 11. Leucocyte recruitment to the peritoneal cavity with variation in surgical stimulus. *Animals were separated into 3 groups. Anaesthesia alone, Anaesthesia + skin incision, Anaesthesia + skin incision + Peritoneal incision. At 24 hours peritoneal lavage was obtained. Kruskal-Wallis Gr-1^{Hi} p=0.003; Gr-1^{Lo} p=0.003; PMN p=0.003; PMac p=0.29. n=3$_{control}$ and 18$_{intervention}$*

Kinetics of monocyte trafficking to the peritoneal cavity following abdominal surgery.

Following laparotomy, monocyte subset dynamics in the blood and recruitment to the peritoneum was assessed at a variety of time points over 48hours.

There was little variation in Gr-1^{Hi} monocytes in blood although the Gr-1^{Lo} monocytes were significantly reduced in the first 4hrs following surgery **(Figure 12).**

There was a similar pattern in terms of recruitment of monocytes to the peritoneal cavity following surgery **(Figure 13)** as there was following intra-peritoneal LPS **(Figure 4)**, with an initial increase in Gr-1^{Hi} followed by Gr-1^{Lo} monocytes. It was

not possible to perform a time point that was comparable to that used in the LPS animals, as the mice needed time to recover from surgery. The onset of recruitment is slower when the stimulus is surgery than when the stimulus is LPS.

There were significantly more Gr-1^{Hi} monocytes recruited to the peritoneum after surgery than after ip LPS (20ng) challenge (LPS $2.45\pm0.55\times10^5$ vs. surgery $7.27\pm1.88\times10^5$ p=0.03) at 16h post-treatment. By 24 hours this difference between the two challenges was apparent with the Gr-1^{Lo} subset numbers (LPS, $1.90\pm0.34\times10^5$ vs. surgery $6.41\pm1.66\times10^5$ p=0.22).

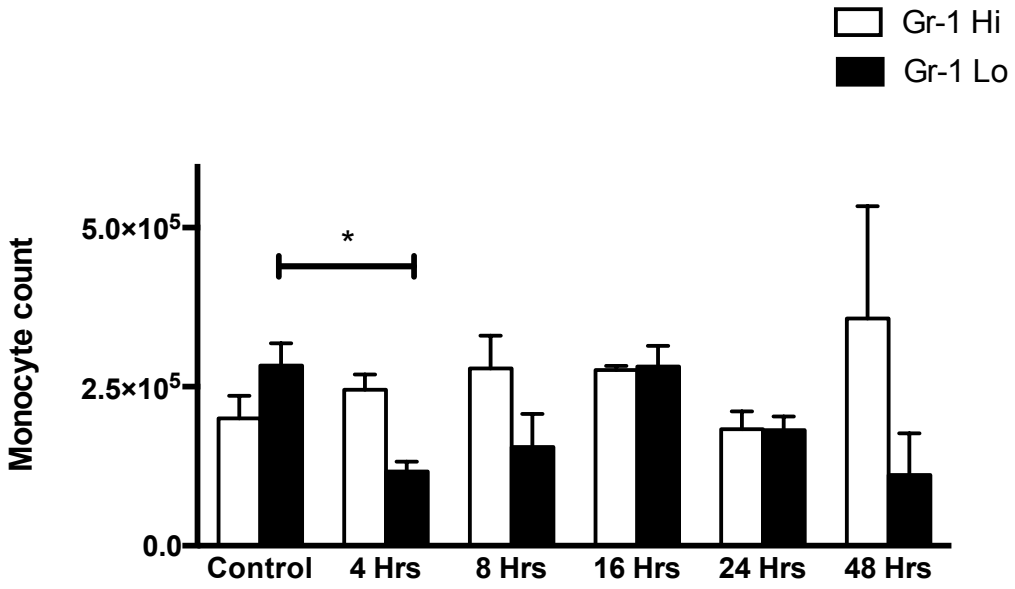


Figure 12. Monocyte subset kinetics in blood after abdominal surgery. *The change in monocyte numbers were followed in blood over 48 hours after an ip injection of 20ng LPS and compared to control animals not exposed to LPS. One-way ANOVA Gr-1^{Hi} p=0.22; Gr-1^{Lo} p=0.006. *p<0.05 compared to control animals. n=$5_{control}$ and $6_{intervention.}$*

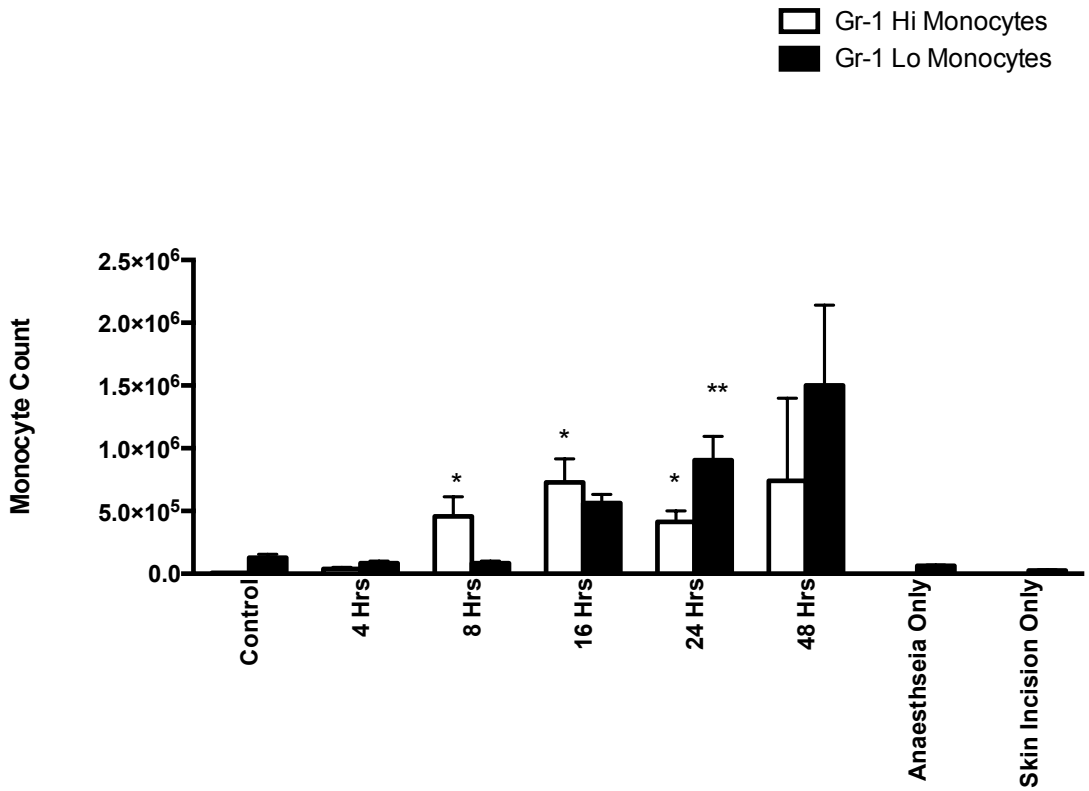


Figure 13. Recruitment kinetics of monocytes to the peritoneal cavity after abdominal surgery. *The change in monocyte numbers was tracked in peritoneal lavage fluid at various time points over 48 hours. They were compared to control animals that did not undergo surgery. Kruskal-Wallis Gr-1^{Hi} p=0.0001. *p<0.05 compared to control animals; Gr-1^{Lo} p=0.0001. **p<0.05 compared to control animals. n=5$_{control}$ and 6$_{intervention}$.*

In contrast to blood monocytes, neutrophil numbers present in blood increased significantly at 4 hours and appeared to remain elevated for the duration of the time course **(Figure 14)**. In peritoneal lavage fluid, neutrophils increased significantly following surgery but with much larger variation between mice than found with monocytes. There is a considerable reduction in the numbers of peritoneal

macrophages reaching a nadir between 8 and 16 hours suggesting surgery is also a trigger for the macrophage disappearance reaction. (**Figure 15**).

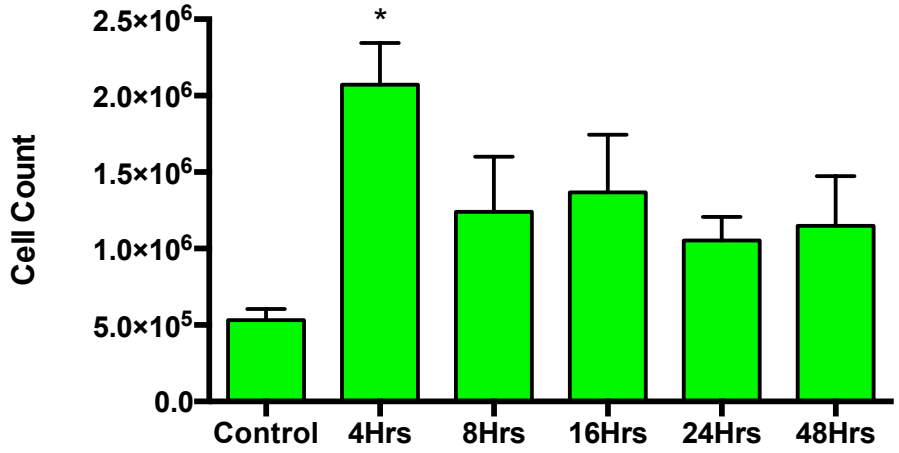


Figure 14. Neutrophil kinetics in blood after abdominal surgery. *The change in neutrophil numbers in blood over time following abdominal surgery compared to control mice. One-way ANOVA p=0.001. * p<0.05. n=3$_{control}$ and 5$_{intervention.}$*

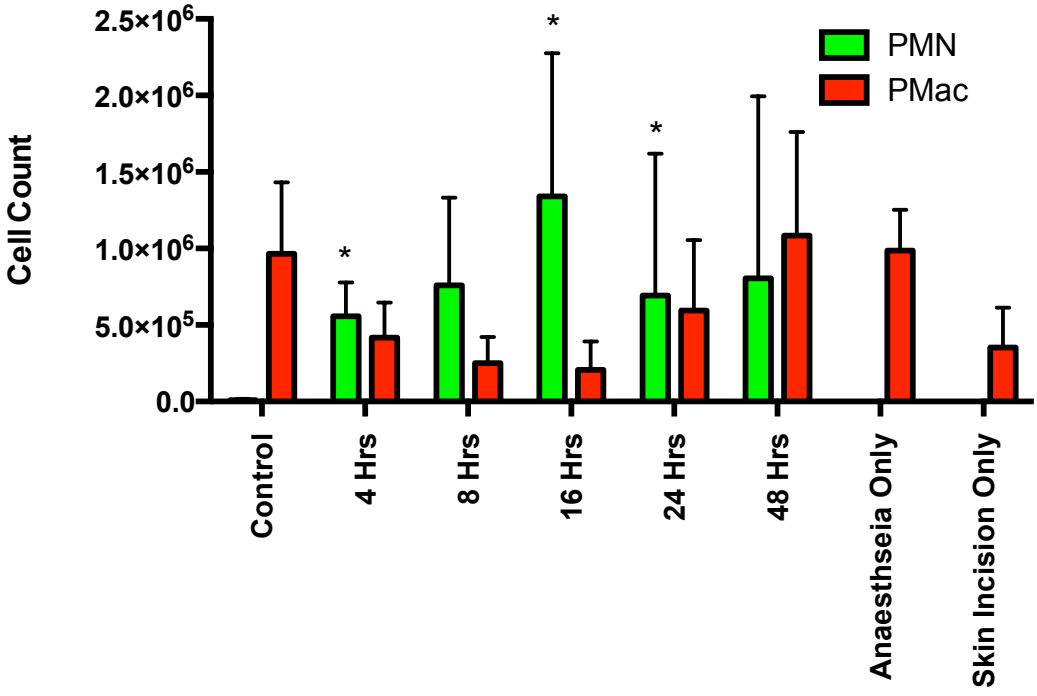


Figure 15. Neutrophil and resident macrophage kinetics in the peritoneal cavity after abdominal surgery. *A comparison of the absolute numbers of neutrophils and macrophages found in peritoneal lavage following abdominal surgery compared to control animals. PMN Kruskal-Wallis p=0.01; PMac One-way ANOVA p=0.03.*

** $p<0.05$ $n=3_{control}$ and $5_{intervention.}$*

Discussion

Murine monocyte subsets have been described in blood and have different functional phenotypes (120,123,126,148)**.** This heterogeneity can affect the inflammatory milieu at local sites of inflammation including the ovary (169) kidney (170), lung (132) and the peritoneal cavity (121)**.** The Gr-1^{Hi} subset are released from both bone marrow and spleen in response to inflammation and become the more pro-inflammatory phenotype when migrating into tissues. They are the predominant producers of TNFα (121,171)**,** they differentiate into macrophages of the classic M1 phenotype (172) and may also suppress tumor growth (173). The Gr-1^{Lo} phenotype may be more likely to differentiate in to cells with reparative capacity, in the peritoneum they differentiate into macrophages of the alternative pathway, M2 (172) and are late migrators into myocardium after ischemia promoting fibrosis (127)**.** The dynamics of monocyte subset recruitment to the peritoneum following surgery are likely to be highly significant in the development of subsequent peritonitis.

The differences in response of the monocyte subsets were demonstrated by comparing a conventional PAMP in the form of LPS to a sterile surgical procedure. The qualitative and quantitative differences observed were surprising as irrespective of the

dose of LPS, surgery elicited greater monocyte recruitment. Previous work has suggested that LPS from areas of inflammation triggers monocyte release from bone marrow even at ip doses as low as 2ng (153)**,** given the presence of DAMPS such as HMGB in plasma with a half-life of 17 minutes (174), a similar effect could also occur with surgery. These cells originating from the bone marrow increase the circulating pool; although our data confirm this for neutrophils at 2 hours there is no significant change in blood monocytes of either subset. This may be because the change is small and very transient; the released cells rapidly extravasate into the areas of infection along a chemokine gradient (49).

The pattern of recruitment of monocytes to the peritoneal lavage fluid mirrors that of blood although the numbers and degree of change is greater in lavage fluid, this may be because these cells are trapped in the cavity and, unlike the circulating cells cannot marginate to other capillary beds (132,157)**.** A similar pattern of recruitment is seen whether the stimulus is LPS or surgery. However there are many more leucocytes that migrate to the peritoneum after surgery with a greater proportion expressing CD11b, requiring an alternative strategy to identify the subpopulations.

Of note both the resident peritoneal macrophages and Gr-1^{Lo} monocytes (that may well be resident) are reduced from baseline at early time points following either LPS or surgery. Barth describes a peritoneal 'macrophage disappearance reaction' (MDR) that can occur with either inflammatory macrophages or as in this case with the resident population (167). LPS is known to trigger this reaction (175).There is some evidence that the mechanism of the "MDR" involves the coagulation system, it can be reversed by heparin and warfarin (176), fibrin is present on macrophages attached to the peritoneum (177) and there is evidence of fibrinolysis as the reaction resolves (178). It is hypothesized that the increased adhesion of macrophages to the peritoneal

lining makes accurate recovery difficult. Previous authors have found the reaction reverses by 48 hours (179) and our work would be consistent with this.

The process of recovery of cells from the peritoneal lavage is critical to this work. The risks of using systemic or local anti-coagulation in the immediate post-operative period outweighed the potential benefits in terms of cell recovery. The potential to improve the number of cells recovered enzymatically using trypsin was attempted but had no effect. The danger of manipulating the macrophage 'stickiness' is that the process may also affect the identification of cells or their inflammatory phenotype.

In humans there is clear myeloid migration to peritoneal fluid after gastrointestinal surgery although many are strongly adherent to peritoneum and difficult to harvest (180). These cells are the main producers responsible for the increase in soluble TNFα and IL-6, the increase in class II macrophages and the increased expression of HLA-DR on trafficked monocytes.

There are several candidates that may act as sentinel cells including macrophages, mast cells (181) and endothelium; via the expression of TLR-4. The toll-like receptors detect LPS but are also able to detect DAMPs such as HMGB and heat shock proteins (182,183). Monocytes are known to express pattern recognition receptors including TLR-4, their fate is dependent on the chemokine environment as well as their subset phenotype (184). For example monocytes exposed to LPS, IFNδ and M-CSF differentiate into M1 macrophages whereas exposure to IL-4 promotes differentiation into M2 (185). The differential effects of the monocyte subsets are mediated by expression of cell surface proteins, CCR2 on Gr-1^{Hi} and CX3CR1 on the Gr-1^{Lo} and the interaction with their ligands MCP-1 and fractalkine (97,186,187). There is no evidence in either LPS or surgically stimulated animals that the sentinel

event is early migration of patrolling Gr-1^{Lo} cells as described by Auffray but the cell counts in their experiments were at least one order of magnitude lower (121). This may be because the insult of surgery or LPS is different to the sterile injection of Thioglycollate used by Auffray. If this occurs at all then it is likely to be dwarfed by the later migration of Gr-1^{Hi} cells. Although there is evidence of in-situ maturation from the Gr-1^{Hi} monocytes to the Gr-1^{Lo} subset in other models of inflammation (127,129,135) it is not possible to clarify this with our model. It is possible that there is some maturation that occurs but also a separate kinetic of Gr-1^{Lo} monocytes from the circulating pool, the factors promoting each process are currently unknown.(121).

Surgery induces a greater recruitment of inflammatory cells than the doses of LPS studied. It may therefore prime the peritoneal cavity to respond more aggressively both locally and systemically to a subsequent breach of the gastrointestinal tract and release of bacteria. This in turn may create the ideal environment for the development of SIRS and multi-organ dysfunction.

Chapter 4

The local and systemic response of monocytes to LPS after abdominal surgery.

Background and aims

We found that a surgical incision which opens the peritoneal cavity produces a substantial infiltration of inflammatory cells including monocytes and neutrophils. The pattern of monocyte subset recruitment shares some similarities with the recruitment generated by the prescence of LPS, however, there are also important differences. There appeared to be a greater number of cells that traffic when stimulated by surgery than by LPS and there is a suggestion of a response from more diverse populations of leucocytes such as eosinophils.

Therefore although the surgical stimulus is relatively mild, a simple incision; and the chance of LPS contamination low there are still high numbers of leucocytes in the peritoneum. The clinical question is whether these recruited cells can create the inflammatory conditions within the peritoneum that lead to peritonitis and is this of sufficient magnitude to cause multi-organ failure.

Priming is the process by which cells of the immune system produce a heightened response following earlier low dose exposure to a stimulus (188). This can also occur as a spatial effect such as the augmented response of inflammatory cells following adherence to endothelium (189). The priming of neutrophils by this adherence may trigger de-granulation and release of superoxides and/or lipid mediators and delay apoptosis (190). The increase in the production of TNFα after consecutive doses of LPS is related to the site of injection with the intravenous route requiring a much lower dose than injections into tissue (191). It has been found previously by our group that monocytes recruited to the lung capillaries by a sub-clinical dose of LPS did not produce a significant amount of TNFα, but were primed to respond vigorously to a subsequent dose of LPS even if that was also below the threshold for clinical

signs to develop (132). Pre-treatment with LPS increased the numbers of Gr-1^{Hi} monocytes and neutrophils marginating in the lung vasculature, and that when ventilated with high stretch the signs of lung injury were significantly increased (133). Both of these studies suggest that monocytes mobilise from both the circulating pool and bone marrow in response to an initial insult and are then primed within the pulmonary vasculature to become more inflammtory or cause more cell damage if challenged again.

In tissues, macrophages are also available to be primed. Alveolar macrophages exposed to LPS in-vivo had a greater resposne when subsequently challenged ex-vivo, than macrophages only exposed to saline initially (192). Finally, in response to a spinal cord injury circulating $CD14^{dim}CD16^{+}$ monocytes are primed as they migrate into the cerebral spinal fluid to produce anti-inflammatory cytokines when activated by adhesion to cells of the chorid plexus (193).

The priming of leucocytes and their response to repeated inflammatory challenges has been examined in several different scenarios. The combination of insults used is varied and extensive but includes CLP and intra-tracheal LPS to study acute lung injury (194); sub-clinical iv LPS and iv zymosan (132); pancreatitis and iv LPS (195); intra-tracheal LPS followed by intravenous LPS to examine lung permeability (196); subclinical intravenous doses of LPS and Platelet activating factor to examine lung injury (197). The majority of these 2-hit models use insults that alone have a limited clinical or biochemical effect, together most result in profound changes.

Aim 1: Describe the effect of a secondary local challenge with LPS after abdominal surgery on systemic (plasma) and local (peritoneal lavage) soluble TNF levels.

Aim 2: Investigate the inflammatory response of a secondary exposure to LPS in subpopulations of recruited and resident cells following abdominal surgery.

It is well known in clinical practice that inflammatory insults that may appear innocuous can be have significant effects at organ sites distant from the origin of inflammation. There are many examples including pancreatitis, trauma, cardiopulmonary bypass, burns and sepsis that can require multi-organ support. The most common distant site to be affected is the lung. There are several reasons for this; the entire cardiac output has to pass through the pulmonary vasculature so any circulating factors produced by the SIRS response will come into contact with the endothelium; leucocytes are known to marginate to the lung (132,133); the lung capillaries are very small, 2-2.5μm in diameter causing leucocytes to deform (they are normally 8μm in diameter) to pass through, providing significant endothelial contact and potential hold up (198).

Peritonitis often leads to the development of an acute lung injury. This association has been known for a long time (199), it is especially common following surgery for abdominal emergencies such as a perforated viscus (200). Peritonitis has been exploited to investigate the soluble factors that trigger acute lung injury using many different animal models including mice (201), rats (202) and pigs (203).

If the recruited monocytes are primed to produce a greater response when challenged again by LPS even at low dose this may reach the threshold at which systemic effects begin to appear. The lung will be one of the first distant organ sites to be effected and is therefore reasonable to examine pulmonary tissue for the early changes of acute lung injury such as capillary leak and development of extravascular lung water.

Aim 3: Examine the effects on the lung of the recruitment of monocytes to the peritoneal cavity by surgery and subsequent 'second hit' by ip LPS.

Protocols

"Two hit" protocol

The surgical procedure has already been described in chapter 2. C57BL/6 mice were operated on and allowed to recover. They were kept in separate cages for the next 24 hours and then received no injection or an intra-peritoneal injection of 20ng LPS or saline also as previously described. One hour later they were sacrificed and peritoneal lavage fluid obtained.

Peak recruitment of cells for intra-peritoneal LPS and surgery occurred at slightly different time points for the two-monocyte subsets but was significant at 24 hours for both. As both subsets were present in lavage samples at 24 hours this was chosen as the time point to introduce the second hit. The rationale for using 20ng LPS as a sub-clinical none saturating dose of LPS was described in chapter 3 and therefore this was the dose used as the second hit.

Assessment of inflammatory status

To examine the effects of the 2-hit model on peritoneal inflammation, LPS challenge was performed either in vivo by ip injection of 20ng or ex-vivo following peritoneal lavage with LPS at a concentration of 20ng/ml.

To determine the amount of soluble TNFα present in the peritoneal lavage fluid, cells were not incubated with the metalloprotease inhibitor BB94 (TACE inhibitor)

allowing shedding of TNFα from the cell surface. They were also incubated with LPS for longer (4 hours), the supernatant collected and soluble TNFα measured by ELISA. To measure the amount of membrane bound TNFα, BB94 was added to the 20ng dose of LPS or the control dose of saline to prevent TACE-mediated release of TNFα from the cell surface and thereby determine the total memTNFα production by individual cells in the 1 hour period.

As described in chapter 2 using flow cytometry and antibodies directed against membrane bound TNFα the expression of this pro-inflammatory cytokine can be assessed on both monocyte subsets.

Assessment of lung permeability

Mice were divided into 2 groups, one underwent abdominal surgery as described in chapter 2. The other group, the controls, had no surgical procedure. 24 hours later both groups received LPS and were allowed to recover for 2 hours.

The mice from each group were sacrificed and underwent dye dilution experiments as described in chapter 2. Briefly two albumin-conjugated dyes of linked to different wavelength flurophores were injected intravenously at separate time points. The lungs were subsequently excised, homogenized and the supernatant measured for fluorescence. By measuring the lung:blood ratio for each flurophore, an index of lung permeability can be calculated (134).

Results

The effect of abdominal surgery on peritoneal TNFα levels following a secondary LPS challenge.

The in-vivo animals exposed to a 'primary hit' surgery and a 'secondary hit' LPS were compared to those exposed to LPS alone or surgery alone. A trend towards an increase in the level of soluble TNFα detectable in the peritoneal lavage fluid was apparent at 4 hours although this did not reach statistical significance (**Figure 1**).

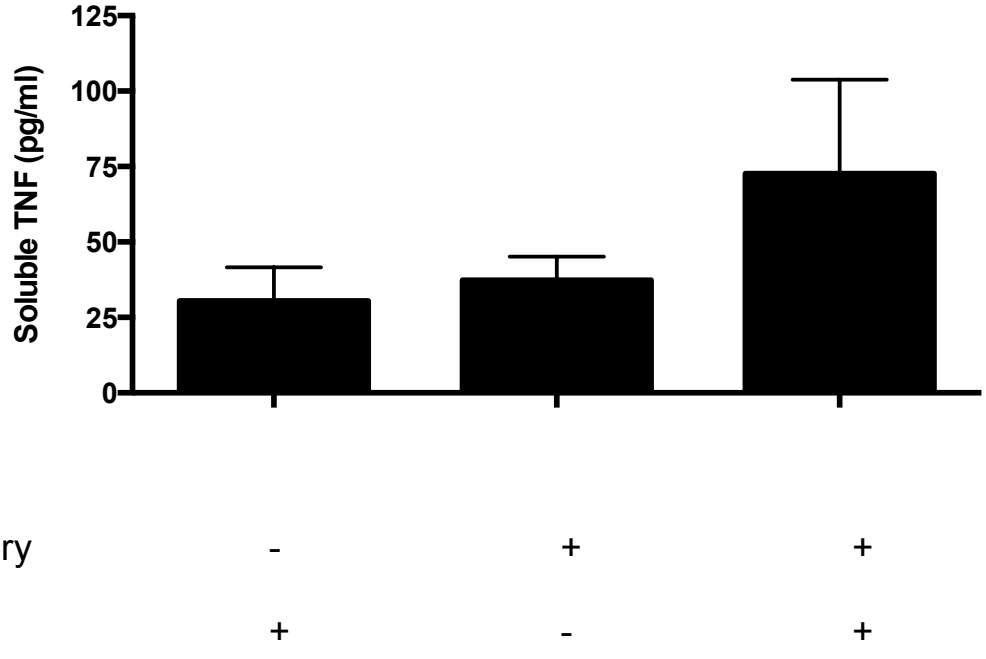


Figure 1. Soluble TNFα levels in peritoneal fluid an in-vivo 2-hit model*. Mice were divided into three groups, one had surgery and was allowed to recover, one received LPS and was sacrificed 4 hours later and the final group had surgery and 24 hours were exposed to 4 hours of ip LPS at 20ng. One-way ANOVA p=0.49. n=3 in all groups.*

Despite being able to detect TNFα in the peritoneal cavity at 4 hours levels in the plasma were below the detection limits of the ELISA. This result may not be surprising considering the low dose of LPS used and the reported short half life of TNF in blood of 18 minutes (204).

Is the increase in soluble TNFα in the peritoneal cavity of animals exposed to surgery and LPS related to a second wave of migration of inflammatory cells?

To discover whether there is any further recrutiment of inflammatory cells by the second LPS stimulation that might add to the response of the already recruited cells, three groups of mice were compared. 2 subjected to surgery, one of which received a secondary challenge with intra-peritoneal LPS and a third group receiving LPS only. LPS was injected 24 hours post-surgery and cells harvested by lavage 1 hour later.

There was no additional recruitment of monocytes to the peritoneal cavity in either the control animals that underwent just surgery and subsequently received ip saline or the 2-hit animals (Gr-1^{Hi}: Control $4.5\pm1.0x10^5$; 2-hit $5.6\pm1.2x10^5$ t-test p=0.48)(Gr-1^{Lo}: Control $11\pm2.7x10^5$; 2-hit $8.4\pm2.4x10^5$ t-test p=0.46). The only cell line to appreciably increase after surgery and secondary LPS were the neutrophils (Surgery alone $7.8\pm1.3x10^5$; LPS alone $3.2\pm1.0x10^5$ 2-hit $22\pm4.9x10^5$ p=0.0009). This additional recruitment suggests that a priming of the immune response to secondary 'infectious' challenge as a result of aseptic abdominal surgery (**Figure 2**).

As the neutrophil contribution to TNFα production is likely to be small (see below) the increase in soluble TNFα within peritoneal fluid following surgery and LPS can be attributed to the already recruited and resident monocytes and macrophages.

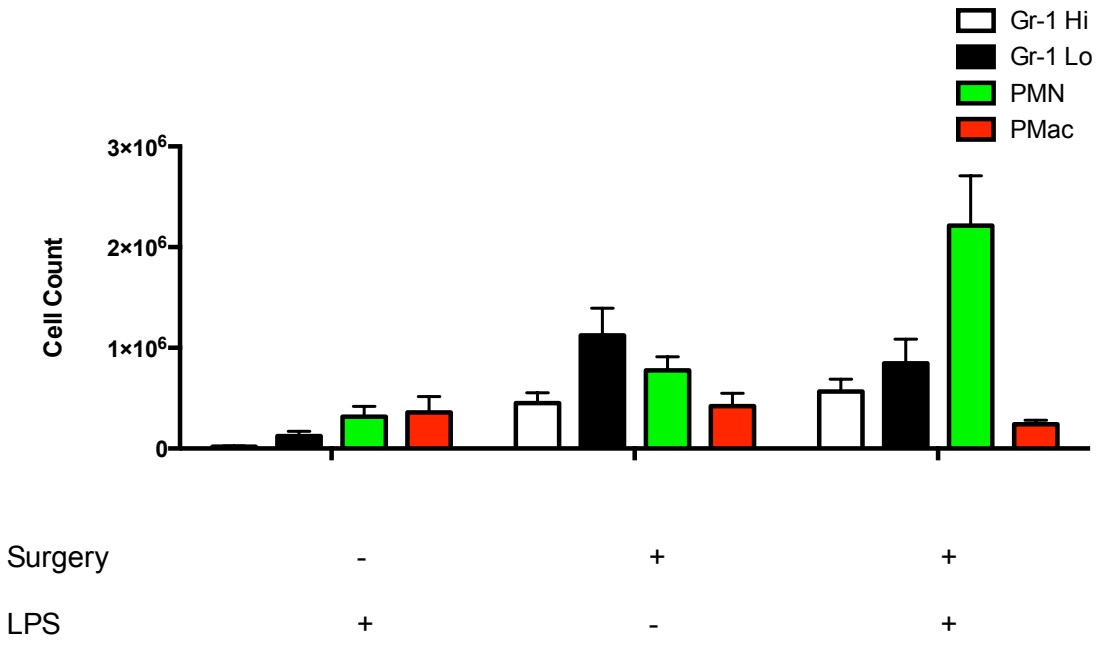


Figure 2. The effect of abdominal surgery and a second hit of LPS on leucocyte migration into the peritoneal cavity. *The effect of ip LPS on peritoneal leucocyte numbers was determined at 24hours post abdominal surgery. Lavage was performed 1 hour after 20ng LPS was injected and total leucocyte subpopulation numbers determined by flow cytometry. n=7$_{control}$ and 10$_{intervention.}$*

Expression of membrane bound TNFα on macrophages and monocytes recruited by surgery and subsequently exposed to LPS with BB94 in-vivo.

The expression of memTNFα in the 2-hit animals was compared to animals exposed to LPS without surgery and animals that underwent surgery but were not exposed to LPS. The secondary LPS challenge or control saline was performed in vivo with BB94 prior to cell harvest by peritoneal lavage after 1 hour. Shown here in (**Figure 3)** at 1 hour post LPS there are minimal Gr-1^{Hi} monocytes present in the peritoneal cavity and therefore it was not considered possible to determine their response in non surgical mice.

The Gr-1^{Hi} monocytes challenged in-vivo by LPS following surgery expressed high levels of membrane bound TNFα with those undergoing surgery only exhibiting negligible amounts in comparison (Surgery alone 4.6±1.1; Surgery + LPS 156.6±56.02). However it was not possible to assess a priming effect of surgery on the response to LPS because no Gr-1^{Hi} monocyte were present in LPS only challenged mice at 1 hour **(Figure 4).**

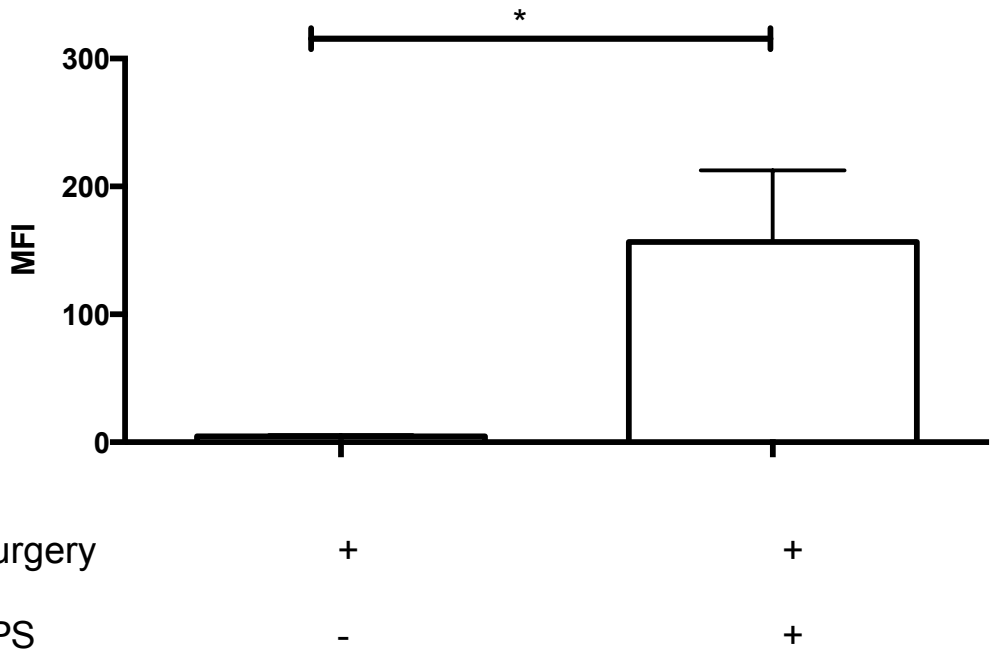


Figure 3. TNFα expression on Gr-1^{Hi} monocytes in-vivo. *Mice underwent abdominal surgery and recovered. At 24 hours they received either 20ng of LPS or 0.9% saline and were sacrificed one hour later. The MFI of membrane bound TNFα on Gr-1^{Hi} monocytes was measured. Unpaired t-test p=0.0074. n=9$_{control}$ and 11$_{intervention.}$*

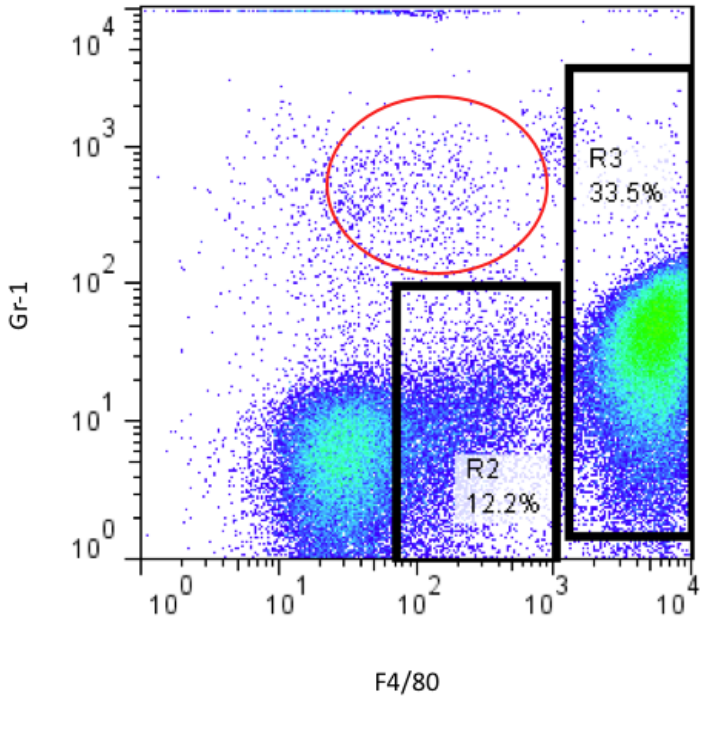


Figure 4. *In control mice challenged with LPS and sacrificed after 1 hour there are no resident Gr-1^{Hi} monocytes (see highlighted area)*

The Gr-1^{Lo} monocytes in the 2-hit animals demonstrated a similar, albeit slightly lower, TNFα response compared to the Gr-1^{Hi} subset (**Figure 5**). Unlike the case for the Gr-1^{Hi} monocytes, the presence of a resident Gr-1^{Lo} like phenotype in mice receiving LPS alone allowed some estimation of the effect of the prior surgery on LPS responsiveness. Based on this premise, the effect of surgery on responsiveness seemed to be substantial (LPS alone 16.5±9.1; Surgery + LPS 93.3±21.2 $p<0.001$).

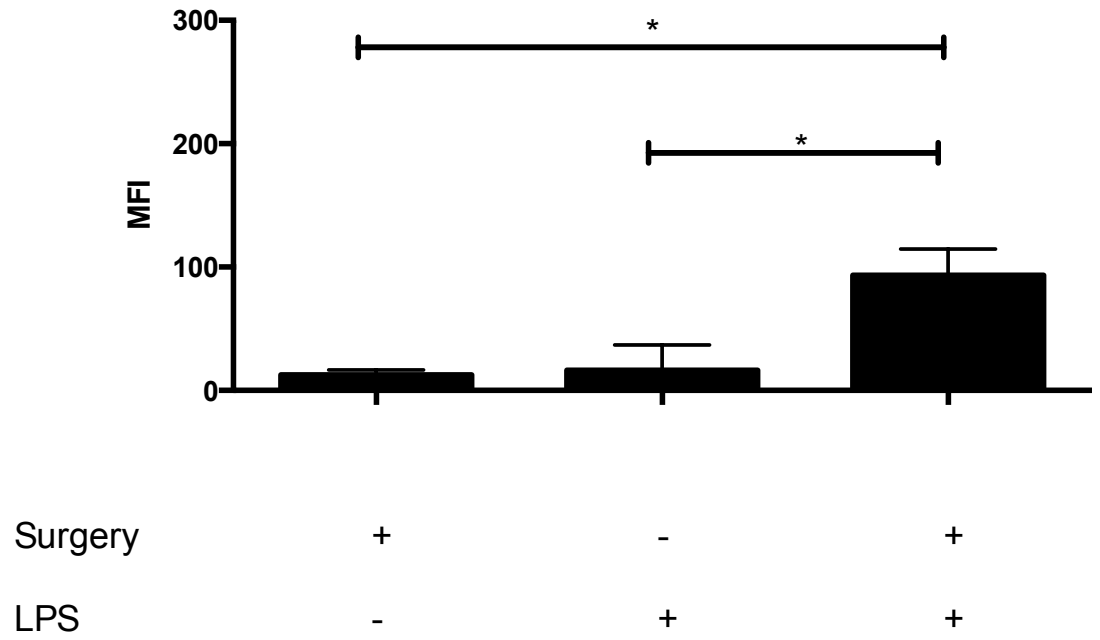


Figure 5. TNF expression on Gr-1Lo monocytes in-vivo. *Mice underwent abdominal surgery and recovered. At 24 hours they received either 20ng of LPS or 0.9% saline and were sacrificed one hour later. A further group did not undergo surgery but received LPS (20ng) 1 hour prior to sacrifice. The MFI of membrane bound TNFα on Gr-1^{Lo} monocytes was measured. One-way ANOVA p=0.0004. *p<0.05 n=9$_{control}$ and 11$_{intervention}$.*

Finally the expression of TNFα on the resident macrophages was assessed (**Figure 6**), again in the three groups of animals. These cells responded to the surgical insult by expressing the most TNFα of all the leucocyte sub-populations studied although, the intensity of response in the recruited monocyte subsets was of a comparably high magnitude. This direct comparison of resident cells provided the most clear cut evidence of priming of the inflammatory response within the peritoneal cavity due to aseptic surgery (Surgery alone 52.3±19.4; LPS alone 16.4±3.8; Surgery + LPS 249.5±79.8).

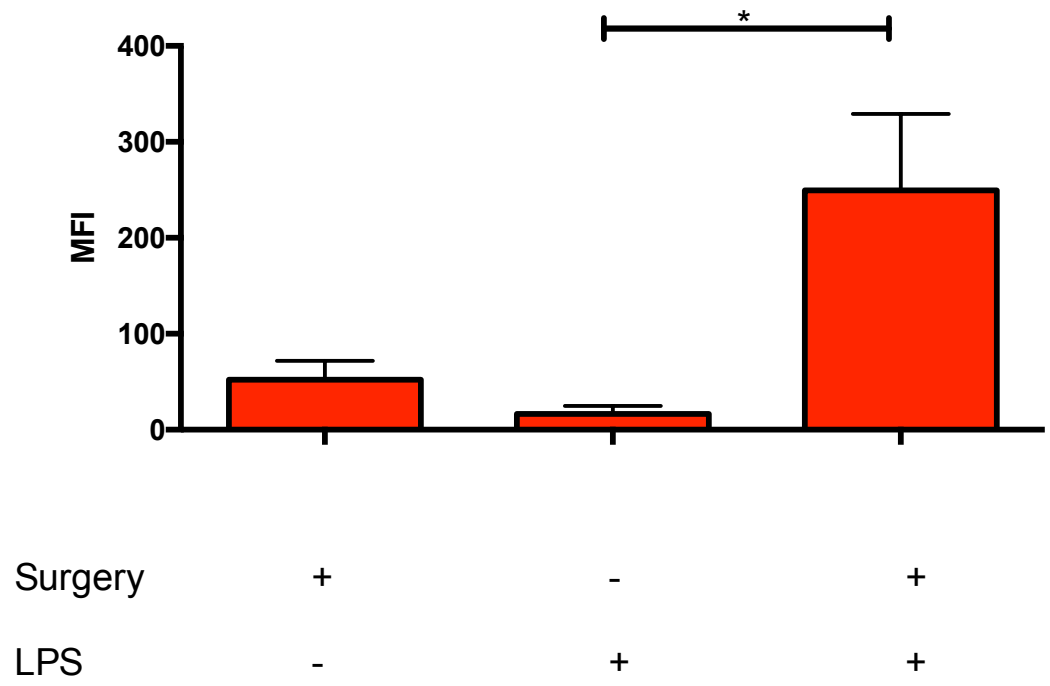


Figure 6. TNFα expression on resident macrophages. *Mice underwent abdominal surgery and recovered. At 24 hours they received either 20ng of LPS or 0.9% saline and were sacrificed one hour later. A further group did not undergo surgery but received LPS (20ng) 1 hour prior to sacrifice. The MFI of membrane bound TNFα on resident macrophages was measured. One-way ANOVA p=0.02. *<0.05. n=5$_{control}$ and 9$_{intervention}$.*

The expression of membrane bound TNFα on macrophages and monocytes recruited by surgery, harvested and then exposed ex-vivo to LPS.

In order to rule out the possibility that the results observed in vitro were influenced by the selective recovery of cells due to the 'macrophage disappearance reaction' and to consider the effect of the in vivo environment on priming, stimulation experiments were performed ex-vivo on cells recovered by lavage.

The Gr-1^{Hi} monocytes expressed significantly more membrane bound TNFα following surgery and a 'second hit' of LPS than those cells exposed to surgery and 0.9% saline (Surgery only group 24.4±15.5; Surgery + LPS 198.5±85.7)**(Figure 7)**

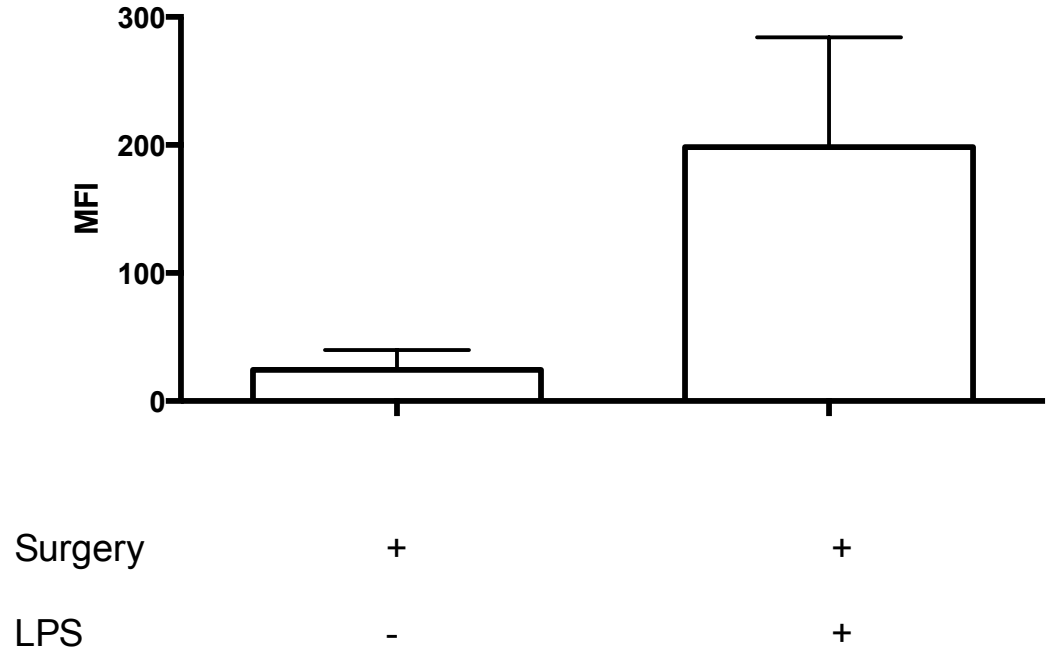


Figure 7. Expression of TNFα on Gr-1Hi monocytes after ex-vivo stimulation*. Mice underwent abdominal surgery, at 24 hours they were sacrificed and peritoneal lavage fluid obtained. The harvested cells were then exposed ex-vivo to either LPS or 0.9% saline and the MFI of TNFα expressed on Gr-1^{Hi} monocytes calculated by 1 hour later. Mann Whitney U p=0.03. n=$3_{control}$ and $8_{intervention}$*

Although the Gr-1^{Lo} cells demonstrated a similar trend towards higher expression of TNFα after LPS this did not reach significance (Surgery only group 38.6±21.4; LPS only group 29.7±2.5; Surgery + LPS 172.5±79.6). (**Figure 8**)

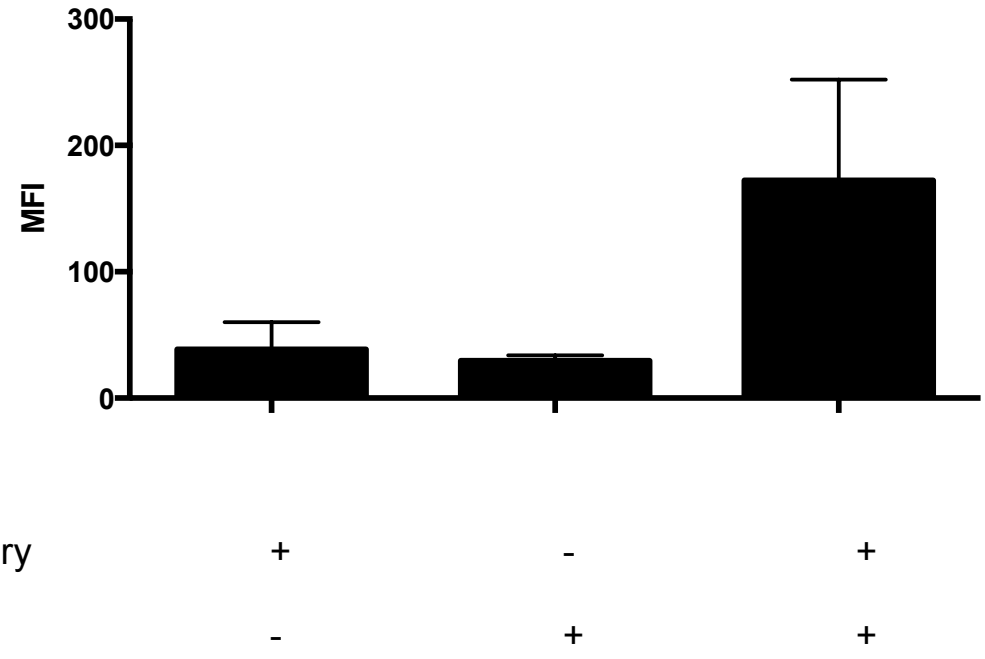


Figure 8. Expression of TNFα on Gr-1Lo monocytes after ex-vivo stimulation. *Mice underwent abdominal surgery, at 24 hours they were sacrificed and peritoneal lavage fluid obtained. The harvested cells were then exposed ex-vivo to either LPS or 0.9% saline. A further group did not undergo surgery but lavage was performed and the cells exposed to 20ng LPS for 1 hour. The MFI of TNFα on Gr-1^{Lo} monocytes was measured. One-way ANOVA p=0.13; n=3$_{control}$ and 8$_{intervention.}$*

As in the in-vivo groups the resident macrophages had the highest expression of membrane bound TNFα (Surgery only group 35.7±9.5; LPS only group 10.4±7.1; Surgery + LPS 238±49.5)(**Figure 9**). In conclusion, these ex-vivo experiments demonstrated a similar pattern to the challenges performed in vivo supporting their interpretation and suggesting that the intensity of responses was not dependent on cues from the in-vivo environment. The consistency of the effect between the in-vivo and the ex-vivo experiments suggests that this was not related to selective cell harvesting (the most responsive cells are likely to be the hardest to harvest).

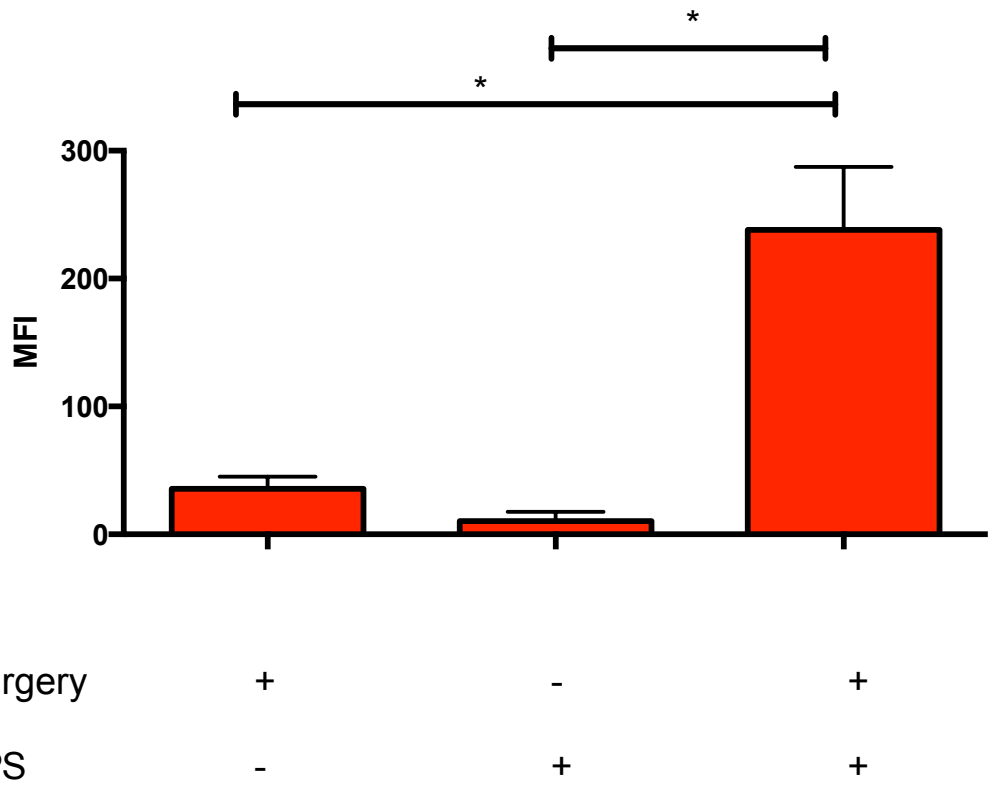


Figure 9. Expression of TNFα in macrophages after ex-vivo LPS stimulation. *Mice underwent abdominal surgery, at 24 hours they were sacrificed and peritoneal lavage fluid obtained. The harvested cells were then exposed ex-vivo to either LPS or 0.9% saline. A further group did not undergo surgery but lavage was performed and the cells exposed to 20ng LPS for 1 hour. The MFI of TNFα on the resident macrophages was measured. One-way ANOVA p=0.0001; *p<0.05; n=3$_{control}$ and 11$_{intervention}$.*

Neutrophils did not express any TNFα in either the in-vivo or ex-vivo models.

Is the insult of surgery and exposure to LPS sufficient to trigger SIRS and cause effects at distant organ sites.

The in-vivo and ex-vivo data strongly suggest that macrophages and the cells that replenish them and augment their inflammatory function, monocytes, are primed by a

simple surgical incision to increase their pro-inflammatory response to a subsequent challenge with LPS.

Peritonitis is known to lead to multi-organ failure especially lung injury. To investigate whether the migration and increased responsiveness of the monocytes in the peritoneal cavity was sufficient the 2-hit model was modified to include a test of lung permeability.

Using the low-dose LPS (20ng) used in the previous trafficking and priming experiments the mice were completely asymptomatic. Changes in lung permeability in mice who are well has little clinical significance. From the experiments performed in chapter 2 a 20μg intra-peritoneal dose of LPS is known to make mice symptomatic with piloerection and reduced movement, therefore this dose was used as the secondary challenge **(Figure 10).**

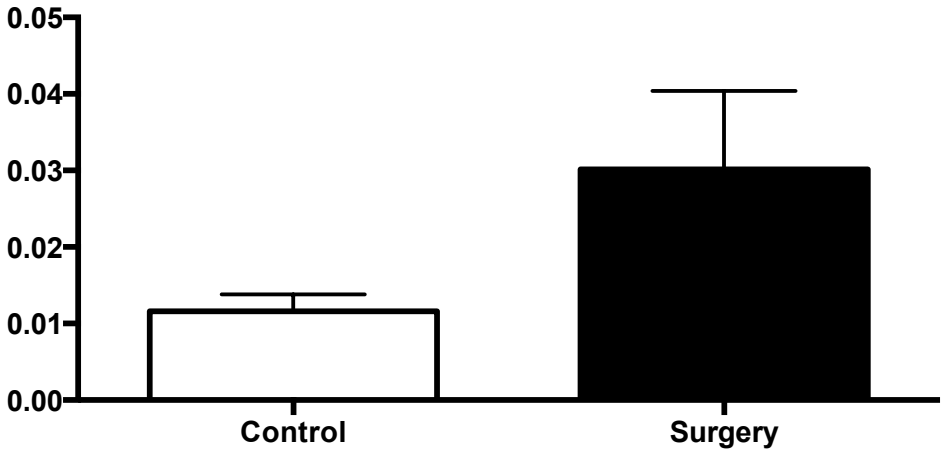


Figure 10. Dye dilution calculation of lung permeability. *Mice were divided into 2 groups, the first underwent surgery, recovered and then received a 20μg dose of ip LPS. The other received LPS alone. A permeability index was constructed using a 2-dye dilution technique. Unpaired t-test p=0.13 n=$8_{control}$ and $10_{intervention}$.*

There was a trend towards an increase in lung permeability in the group primed by surgery prior to LPS exposure although this did not reach significance.

This trend is important, as it is suggestive that monocytes and macrophages are primed by minor surgery to vigorously respond to LPS leading to systemic as well as local effects.

Discussion

Macrophages, as sentinel cells, are present in tissue to activate and respond to invaders. The fact that these cells have the highest expression of TNFα may represent this early response. Gr-1^{Lo} monocytes, when exposed to LPS demonstrate a much higher expression of TNFα than cells recruited by LPS or surgery alone. This provides strong evidence of a priming effect on the monocytes recruited, either via an interaction with the endothelial/peritoneal lining or via an internal priming mechanism within the monocyte. It is difficult to prove a priming effect by minor surgery for the Gr-1^{Hi} monocyte subset, as there are almost no cells of this phenotype in the peritoneal cavity one hour after LPS. Priming remains likely given the lack of an effect on the resident macrophages or Gr-1^{Lo} monocytes, an alternative comparison would be with the circulating blood Gr-1^{Hi} monocytes. This has important clinical connotations, the threshold for development of the systemic inflammatory response syndrome is lowered by minor surgery to the extent that at a local level within the peritoneal cavity doses of LPS (20ng) that would otherwise not trigger an inflammatory response, do so. There is also a systemic effect with pathophysiological changes in distant organs that would otherwise be unaffected by an equivalent dose of LPS (20μg).

The mechanism by which local inflammation within the peritoneal cavity leads to a systemic effect is related to not only the spill over of PAMPs and DAMPs but also the migration of soluble mediators produced by inflammatory cells into the circulation (183). Soluble TNFα levels in the peritoneal lavage fluid at 4 hours are higher but not statistical so in the 2-hit group, this is of interest as the levels of memTNFα are much higher in the 2-hit model. There are several reasons for this, the soluble levels are a snapshot at 4 hours, it is likely that more TNFα is synthesized and moved to the cell membrane. The shedding of TNF from the cell membrane occurs under the influence of TACE and this post-translational effect appears to be the rate-limiting step in soluble TNFα release. If TNFα is not cleaved it is rapidly recycled into the cell (205). Binding to soluble TNF receptors in the peritoneal lavage fluid may also neutralize the soluble TNFα; these receptors are also cleaved by the action of TACE (59) The presence of LPS may directly effect the amount and activity of TACE. Several authors have found that TACE mRNA is upregulated by LPS stimulation (206-208) although others who have found a decline in both TACE fluorescence and activity after LPS exposure dispute this (209). Finally, soluble TNFα is rapidly cleared from plasma with a half-life in LPS challenged mice of 6-7 mins (210). It initially re-distributes to areas of high blood flow including the liver, kidney and GI tract. Once bound rapid degradation occurs, so that the elimination half-life is 8-10 minutes. These mechanisms of TNF redistribution may have reduced the soluble levels especially in the 2-hit groups with the more activated cells.

The numbers of monocytes do not increase with further exposure to a second sub-clinical dose of LPS although there is a second recruitment of neutrophils. Wilson et

al demonstrated that in mice pre-treated with LPS and then ventilated with high stretch there is a second recruitment of monocytes to the pulmonary vasculature after the second stimulus (133). This inconsistency with our data may be related to the initial stimulus trapping monocytes in the pulmonary circulation that are then available to migrate following localized damage from high stretch ventilation.

Interestingly there are approximately 4 times as many Gr-1^{Lo} monocytes found in the lavage fluid as in the lung, this may be due to the longer time period of our experiments with more in-situ maturation from Gr-1^{Hi} cells.

Neutrophils were recruited early in response to surgery peaking at around 2 hours, un-expectantly a second wave responded to the secondary stimulus of LPS. These were significantly raised even at one hour post-injection suggestive that surgery has another form of priming affect, in terms of neutrophil numbers. Primed neutrophils have been shown to over expresses adhesion molecules on the promoting attachment to endothelium and therefore migrate to areas of inflammation much quicker (211)

The effect of surgery on the immune system has been extensively studied both in animals and humans. Caecal ligation and puncture (CLP) has been used extensively to model sepsis in animals including mice. It has been argued that CLP or a similar procedure colon ascendens stent procedure (CASP) most accurately models the formation of peritonitis and sepsis in humans (138). The ligation introduces an element of necrotic tissue, the reason 90% of animals die (212) and the size and number of punctures allows control of the speed of the septic insult. However there are variables related to the size of incision, the size of the needle, the extent of necrosis and the skill of the operator. Our two-hit model demonstrates that the surgery itself, in fact simply the incision, has a priming effect on monocytes that

increases the expression of TNFα and may effect the scale of subsequent pro-inflammatory responses.

As well as CLP there have been other surgical models used in the investigation of trauma (213)**,** cancer (214)and inflammation (194). Many of these examine the effects either in tissue, such as the lung or systemically in blood. There are a few studies that looked specifically at the effect of the trauma of surgery on cell recruitment and its effects. Van den Toll et al performed an incision and gut manipulation in rats and collected peritoneal lavage fluid. On inoculation of this fluid into rats, the cellular component was responsible for an increase in tumor load (214). This is the basis of the argument to reduce surgical trauma by utilising more laparoscopic techniques (215,216). This has been supported by trials in rats comparing laparoscopic surgery to open surgery; significantly more TNFα is produced by peritoneal lavage cells after open surgery than after laparoscopic surgery (217). These examples support our findings that the amount of memTNFα expressed in response to low-dose LPS is massively increased if preceded by surgery, even if the surgical insult is minor.

There have been many two-hit models that have investigated the mechanisms behind the development of acute lung injury some of which have used peritoneal inflammation as the primary insult (194,218,219). Lung permeability is increased when mice are primed with intra-tracheal LPS 18 hours before a second intra-tracheal dose (218)**.** A reduction in cardiac output of 40% by haemorrhage primes rabbits to increase their lung permeability to a similar extent as seen in our experiment, with further exposure to intravenous LPS (219). Of note, it is the development of sepsis after priming that triggers the increase in lung permeability this was demonstrated by

Czermak who compared CLP animals with sham laparotomy animals as the initial insult and showed evidence of increased permeability with subsequent LPS only in the CLP primed group (194). The mechanism of this systemic activation is likely to be related to spill over of not just LPS (154) but also DAMPs released by surgical trauma (146) and pro-inflammatory cytokines produced by the recruited cells (210).

In conclusion, there is evidence that a minor surgical incision is able to recruit leucocytes and following a 'second hit' of LPS activate both those sub-populations and the resident populations to significantly increase the expression of pro-inflammatory cytokines and their release as soluble mediators. Priming of leucocytes and the development of lung injury has been previously shown to occur but not with the relatively minor insult of a surgical incision and not with the 'second hit' confined to the peritoneal cavity.

Chapter 5

Final Discussion

The comparison of intra-peritoneal LPS with a surgical incision show that sub-populations of circulating leucocytes are recruited to the peritoneum in a broadly similar pattern. The comparison of cell counts between the groups is tricky as choosing a dose of LPS equivalent to the surgical incision is not possible, however the size of the recruitment with surgery remains surprising. Previously it was felt that PAMPs were the predominant recruiter of leucocytes when compared to DAMPs with faster mobilisation of neutrophils in greater number from bone marrow (220,221). This may be related to the location of the stimulus within the peritoneal cavity, capturing both DAMPs and migrated cells leading to greater cell recruitment. Zhang et al (38) have suggested a mechanism for lymphocyte recruitment following tissue damage. They used both rat and mouse models of injury detecting significant amounts of mitochondrial DNA following injury when compared to sham controls. They studied the activation of neutrophils at 2 hours via TLR-9, with a downstream effect of activating the p38 MAP-K system. This activation of the p38 MAP-K pathway is similar to that described by O'Dea et al (134) in Gr-1^{Hi} monocytes following intravenous LPS. This is likely to represent just one of many DAMPs and downstream pathways activated by tissue injury. This pathway is likely to increase the adhesion of Gr-1^{Hi} monocytes to the mesenteric endothelium leading to more margination/migration and trigger intra-cellular components of the p38 MAP kinase system that are already known to lead to greater expression of pro-inflammatory mediators such as iNOS and TNFα (222,223).

In a rat model of traumatic brain injury there is an increase at 6 hours in the levels of CCL2 mRNA within the CSF and this leads to a widening of tight junctions and migration of monocytes into the CSF by 24hours (224). CSF monocytosis is known to

trigger an inflammatory cascade, worsen oedema and prevent neuronal recovery (225). The inflammation following spinal cord injury appears to be mediated by the early infiltration of both neutrophils and monocytes and, in a murine model, blocking adhesion and subsequent migration improves neurological recovery (226). Seitz (227), using a rat model of chest trauma, showed that significant numbers of pro-inflammatory monocytes (analogous to Gr-1^{Hi} cells in mice) were recruited to the lung in areas releasing significant amounts of CCL-2. These three studies demonstrate that levels of CCL2 are increased in areas of tissue injury and this has an attractant effect on circulating monocytes. They suggest that the recruitment of leucocytes, the upgrading of pro-inflammatory cytokines and the change in clinical symptoms occur in many tissues. However the peritoneal cavity is unique, it provides an environment for co-culture of DAMP's or PAMP's with leucocytes, promoting their activation whilst reducing the opportunity for containment. Translocation or spillover from areas of injury of commensal bacteria makes infection a real possibility and the data suggests that SIRS in this situation would be highly likely. To further investigate these effects an analysis of the recruitment of pro-inflammatory cells to the liver and gut mesentery would be useful. The liver especially has a very good blood supply from the bowel via the portal venous system. In the context of liver surgery or injury, priming may occur in the resident macrophages (Kuppffer cells) that make a systemic response much more likely following a secondary infective challenge (228). Whether this is of the same magnitude as that seen in the peritoneal cavity would be interesting. Further animal work could also be directed towards exploring how the margination/migration kinetics of monocytes could be modified and what knock on effects this would have in terms of survival from the initial insult.

The differential functions of the monocyte subsets and the effect of priming have been seen in both animal models and real patients. In a mouse model of Crohns disease the dominant monocytes in the lamina propria are the Gr-1^{Hi} monocytes, they upregulate TLR-2 and having migrated into the gut wall become activated by bacterial components and differentiate into efficient antigen presenting cells priming T cells (229). In humans with Crohn's disease, monocytes are felt to be the most important leucocyte involved in the pathogenesis of gut wall inflammation. There is some evidence that monocytes from patients with Crohn's are primed, by an as yet unknown factor, to produce significantly higher levels of superoxide when further challenged by LPS (230). More recently monocytes from Crohn's patients and assumed to be primed by chronic inflammation produced much more IL-1β, CCL-2 and CCL-5 than those from healthy controls (231).

The difficulty of repeating this study in humans is the lack of control of the heterogenous population undergoing surgery. It is not possible to examine the leucocyte response to select parts of the surgical technique but intra-abdominal drains could be utilised to sample peritoneal fluid over time and describe the kinetics. This access to the peritoneal cavity is also available in longer-term liver disease patients requiring ascitic drainage or those receiving peritoneal dialysis. It may be wise to first describe the in-situ responses of mice to whole bacteria rather than a single PAMP such as LPS and compare and contrast both the recruitment and activation of the cells involved. There is some interdependence between the immune system and the neurohumoral system and this model allows further exploration of this especially the effect of regional anaesthesia on the propagation of systemic inflammation.

Conclusion

Should a similar effect be evident in human monocytes it suggests that the tissue damage caused by surgery or trauma can prime the innate immune system to vigorously respond to subsequent challenges? If the threshold of SIRS is significantly lowered by prior surgery then it provides further argument that by reducing tissue damage with minimally invasive techniques the impact of subsequent complications could be lessened.

However if open surgery is required are there any useful medical interventions that could reverse this process of recruitment and activation. Certainly the history of immunomodulatory therapies in the context of sepsis and intensive care is one of failure and it seems counter-intuitive that patients with healing wounds after surgery would benefit from immunosuppression. There are arrays of different molecules that can trigger several pattern recognition receptors acting in many distinct downstream pathways. The redundancy in the system is so great that an intervention at one specific point can be simply bypassed, with inflammation continuing unchecked. Perhaps future immune interventions will be designed based on individuals genetic make-up enabling real time targeted treatment, this may be some way off.

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