Role of Serosal Cavity Resident Leukocytes in the Orchestration of Leukocyte Recruitment Following the Induction of Experimental Inflammation.

Jean-Francois Henry Cailhier

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Abstract

This study evaluated the role of resident peritoneal and pleural macrophages (Mϕ) in neutrophil (PMN) recruitment in acute peritoneal and pleural inflammation. I also investigated the role of lymphocytes (Lρ) in peritoneal inflammation by studying experimental peritonitis in mice deficient in various Lρ populations.

The conditional Mϕ ablation mice used in these studies are transgenic for the human diphtheria toxin receptor (DTR) under the CD11b promoter (CD11b-DTR mice) and exhibit >97% depletion of resident serosal Mϕ following intraperitoneal (IP) administration of diphtheria toxin (DT). I determined leukocyte numbers by flow cytometry in peritoneal or pleural lavage exudates at various time points after the initiation of inflammation with various agents following Mϕ depletion (peritoneum: Brewer's thioglycollate [BTG], zymosan; pleural cavity: carrageenan and fixed staphylococci). I also induced BTG peritonitis in RAG-1 knockout (KO) mice (mature B and T Lρ deficient), NUDE mice (T Lρ deficient), μMT mice (B Lρ deficient) and their respective controls.

Mϕ ablation markedly inhibited peritoneal and pleural PMN recruitment at early time points compared to wild type (WT) controls. Administration of Mϕ-rich resident cells, unlike Mϕ-depleted resident cells, significantly restored PMN infiltration. Analysis of PMN C-X-C chemokines in lavage exudate showed that Mϕ-depleted mice had significantly reduced levels of peritoneal and pleural MIP-2 and KC at the 1hr time point compared to control mice with more marked MIP-2 reduction compared to KC (>90% reduction vs 25-40%). Reduced levels of monocyte C-C chemokine and various cytokines were evident in the Mϕ-depleted

mice at early time points. *In vitro* studies demonstrated that the production of these chemokines and cytokines from peritoneal and pleural cells was Mϕ-dependent. RAG-1 KO mice exhibited increased early PMN infiltration and blunted Mϕ infiltration. NUDE exhibited increased early PMN infiltration and increased Mϕ infiltration whilst μMT KO mice exhibited decreased PMN influx and a reduced Mϕ influx. Although chemokine analysis of peritoneal exudates in RAG-1 KO mice and NUDE mice demonstrated some differences in MCP-1 levels, there were no clear differences evident in μMT KO mice.

These data suggest that resident Mϕ play a pivotal role in the orchestration of PMN infiltration with Mϕ-dependent production of MIP-2 being important. The data suggests that Lρ can modulate leukocyte recruitment in experimental peritonitis with T cells possibly acting as suppressor cells and B cells facilitating Mϕ recruitment. However, the exact mechanisms of Lρ action remain elusive.

Aux femmes de ma vie,
Sabine et Florence,
de même que Celle à venir.

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I want to acknowledge the work of Richard Lang's group that generated the CD11b-DTR mice and the experiments around its characterisation included in the first publication but not in this thesis. I want to acknowledge Debbie Sawatzky's collaboration in the pleurisy project. My work has resulted in two papers published in peer-reviewed journals and the URLs referring to the papers are included at the end of this thesis.

I declare that I have composed this thesis and that the work I have included is mine and was done by myself with the technical help of people I have acknowledged in the appropriate section.

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Abbreviations

AGE : Advanced Glycation End-products
AA : Arachidonic Acid
ADP : Adenosine Diphosphate
APC : Allophycocyanin
ATP : Adenosine Triphosphate
BTG : Brewer's Thioglycollate
CAPD : Chronic Ambulatory Peritoneal Dialysis
CBA : Cytometric Bead Array
CINC-1 : Cytokine-Induced Neutrophil Chemoattractant-1
CSF-1 : Colony Stimulating Factor-1
DARC : Duffy antigen receptor for chemokines
DC : Dendritic Cells
DT : Diphtheria Toxin
DTR : Diphtheria Toxin Receptor
FITC : Fluorescein Isothiocyanate
GM-CSF : Granulocyte-Marophage Colony Stimulating Factor-1
GRO-α : Growth-Related Oncogene-α
hbEGF : Heparin-Binding Epidermal Growth Factor
ICAM-1 : Intercellular Adhesion Molecule-1
IFN-γ : Interferon-γ
IL : Interleukin
IL-1Ra : IL-1 receptor antagonist
IL-1RI Interleukin-1 receptor type I
IL-1RII Interleukin-1 receptor type II
IP : Intraperitoneally
Ipl : Intrapleurally
KC : Keratinocyte-derived Chemoattractant
KO : Knock Out
LPS : Lipopolysaccharide
Lρ : Lymphocyte
LSP-1 : Leukocyte-Specific Protein-1
LT : Leukotriene
MC : Mast Cells
MCP-1 : Macrophage Chemoattractant Protein-1
MDMϕ : Monocyte-Derived Macrophage
MeC : Mesothelial Cells
Mϕ : Macrophages
MIP-1α : Macrophage Inflammatory Protein-1α

MIP-2 : Macrophage Inflammatory Protein-2
NF-κB : Nuclear Factor κB
PBS : Phosphate-Buffered Saline
PE : Phycoerythrin
PECAM-1 : platelet endothelial cell adhesion molecule-1
PG : Prostaglandins
PMA : phorbol 12-myristate 13-acetate
PMN : Polymorphonuclear Cells, Neutrophils
PSGL-1 : P-Selectin Glycoprotein Ligand-1
RAGE : Receptor for the Advanced Glycation End-products
RANTES : Regulated upon Activation, Normal Tcell Expressed and Secreted
SDF-1 : stromal-derived factor-1
SEM : standard error from the mean
sIL-6R : soluble IL-6 receptor
SOCS : Suppressors of Cytokine Signalling
TcR : T Cell Receptor
TGF-β : Transforming Growth Fractor-β
TK : Thymidine-Kinase
TLR : Toll-like Receptor
TNF-α : Tumour Necrosis Factor-α
Tx : Thromboxane
VCAM-1 : Vascular Cell Adhesion Molecule-1
WT : Wild-Type

Chapter 1: Introduction

1.1 Historical background

Celsus (1st century AD) described a situation characterised by *rubor, calor, tumor* and *dolor*. This was the first description of the cardinal signs of a process now known as inflammation, from the Latin *inflammare* (to set on fire). Galen (3rd century AD) proposed that inflammation was an essential part of the response to injury but emphasised the humoral aspect of it. More than 1400 years later, Virchow (1799-1878) saw inflammation as a consequence of an initial 'irritant' (Virchow, 1971). The aggression of the body from the 'irritant' would result in a change or injury in the tissue known as the 'irritament' or the inflammatory stimulus as a passive consequence of the irritant. This will subsequently affect other nearby parts of the body not directly in contact with the 'irritant'. These distant consequences would be an active condition relying on the intrinsic physiology of the local milieu and represented the 'irritation' (Virchow, 1971). Virchow regarded 'irritation' as the starting point of inflammation leading to pathological consequences that he described as the fifth cardinal sign of inflammation: *functio laesae*, loss of function (Scott et al., 2004; Virchow, 1971).

Cohnheim, Virchow's former student, proposed the 'vascular' theory as he believed that the exudate cells were derived from the blood such that the most important aspect of inflammation was the vessel wall lesion secondary to the injurious agent. He proposed that the vessel walls became more permeable and were

unable to retain leukocytes within the vessel. Any other phenomena evident were of secondary importance to this passive leukocyte extravasation (Besredka, 1921).

Metchnikoff was one the first scientists to actually see chemotactic movement: either positive (attraction) or negative (repulsion). He also noted that phagocytic cells (the phagocytes) were the first cells to arrive at an inflamed site and believed that their role was primarily 'digestive' i.e. the phagocytes were attracted specifically to clear inflammatory material and protect the attacked organism. This inflammatory reaction was also found in vessel-free organisms such as the larva of the starfish, thereby indicating that a vascular lesion was not a necessary first step of inflammation and suggesting that vessels acted to accelerate the arrival of these phagocytes through diapedesis. The chemotactic behaviour of phagocytes was considered to be the *primum movens* of the inflammatory reaction and inflammation was considered to be a salutary reaction to protect the organism against the morbid agent by mobilising phagocytes (Besredka, 1921). Metchnikoff described different types of phagocytes. The "big eaters" or macrophagocytes became known as macrophages (Mϕ) whilst the 'smaller eaters' or microphagocytes became known as neutrophils (PMN)(Segal, 2005). With this "phagocyte *armamentum*, Nature had its *Natura Medicatrix*!" (Besredka, 1921). The concept of 'disharmony' was at the core of Metchnikoff's view of inflammation such that "the sentinel phagocyte was directed to regaining disrupted harmony" (Tauber, 2003). The resident phagocyte or Mϕ thus represented a gatekeeper that functioned to maintain harmony under normal conditions and acted to rectify the 'dysregulated disharmony' that occurs in the context of inflammation after the injurious agent has been eliminated (Tauber, 2003).

Inflammation is therefore a body response to injury and is usually followed by tissue repair until tissue function is regained. This inflammatory response is composed of altered blood flow, the recruitment and activation of leukocytes, followed by eradication of the inflammatory stimulus and removal of tissue debris leading to tissue repair (Henson, 2005). Acute inflammation is characterized by an initial influx of PMN followed by inflammatory monocytes that will differentiate into Mϕ (Serhan and Savill, 2005). Beside resident cells, PMN rapidly arrive at an inflamed site and have a crucial role in acute inflammation. PMN are intimately involved in host defense against microbial invasion and also play an important role in other inflammatory disease states (Segal, 2005). When recruited PMN are activated and produce a myriad of cytokines, reactive oxygen species and antimicrobial enzymes and are potent phagocytes. Activated and cytokine-stimulated PMN can secrete chemokines and other chemoattractant proteins that are important in the recruitment of other leukocytes (Yamashiro et al., 2001). PMN are also responsible for a large degree of host tissue destruction (Cochrane, 1968; Weiss, 1989) that may be evident at inflammatory sites. In view of the importance of PMN accumulation in various pathophysiological states, it is crucial to understand the mechanism of their recruitment and identify the cellular network involved in this process. This is particularly important because an increased knowledge of the initiation of inflammation may provide insights into preventing injury and promoting resolution thereby reducing loss of organ function.

1.2 Description of the serosal cavities and their resident cells:

The peritoneal, pleural and pericardial cavities are serosal or serous cavities. They constitute a protective barrier against damage and reduce friction between the organs and tissues within them (Mutsaers, 2004). They are constituted of mesothelial cells (MeC), resident leukocytes and fibroblasts. All of these cells are important as they are metabolically active and act as a barrier to invading pathogens by generating an innate and adaptive immunological response (reviewed in (Faull, 2000)). The serosal cavities are lined by a monolayer of MeC, which constitute the most abundant cell type. They were originally described as the "epithelial lining of mammalian mesodermic cavities", hence the term mesothelium (Mutsaers, 2004). The serosal cavities also contain fibroblasts situated between the MeC lining and the capillaries in the interstitium(Faull, 2000). These cells are also capable of cytokine and chemokine production (Loghmani et al., 2002; Witowski et al., 2001).

The peritoneal membrane is composed of three layers: mesothelium, interstitium and blood microvessels The peritoneal membrane exhibits a variable thickness according to the examined area. The interstitium contains various leukocytes and fibroblasts as well as lymphatic vessels. The peritoneal microcirculation is derived from the systemic circulation and the vascular endothelium is mostly continuous. The lymphatic drainage of the peritoneal cavity is accomplished by specialized lymphatic pores called 'stomata' in the subdiaphragmatic peritoneum as well as by milky spots predominantly located in the

omentum. The lymphatics present in the peritneal interstitium do not contribute significantly to drainage of fluid from the peritoneal cavity (Cameron et al., 1992).

The vasculature and lymphatics of the pleural space are rather unusual. The capillaries of the visceral pleura in large mammals are supplied by the systemic bronchial circulation whereas in smaller animals the blood supply comes from the pulmonary circulation (Albertine et al., 1984). In humans, the visceral pleural microcirculation is derived from both the systemic and pulmonary circulation (Miserocchi, 1997). In humans, the parietal pleura is thinner than the visceral pleura and its capillaries originate from the systemic microcirculation (Miserocchi, 1997) and are closer to the MeC layer than the visceral capillaries (Albertine et al., 1984). Moreover, the visceral pleura contains lymphatics that directly open into the pleural space through specialized openings called stomata similar to those found in the subdiaphragmatic peritoneum of the peritoneal space (Albertine et al., 1984 ; Miserocchi, 1997).

The resident leukocytes are comprised mainly of Mϕ and these cells are also present in specialised cellular aggregates called milky-spots in the peritoneal space (Daems and de Bakker, 1982; Takahashi, 1994). The equivalent structures in the pleural cavity are called 'black-spots' (Boutin et al., 1996). There is, however, a significant population of free Mϕ within the serosal cavities that are not associated with either milky spots or black-spots. In the pleural space of CD1 mice, 82% of leukocytes were Mϕ-like cells, 15% were lymphocytes, 1-2% were PMN whilst there were also few mast cells (Peao et al., 1992). However, different mouse strains

exhibited significant variation in the number of Mϕ and lymphocytes (Festing et al., 1990). In rats, Mϕ represent around 72% of the recoverable cells from the pleural and peritoneal cavities (5% and 13.2% lymphocytes; 19% and 12.8% PMN respectively for the peritoneal and pleural space) (Gjomarkaj et al., 1999). In humans, the peritoneal resident leukocyte population is composed of 45-90% Mϕ, 10-47% lymphocytes [45% T (mostly memory T cells) and 2% B lymphocytes], 8% NK cells, 2-6% dendritic cells and <5% PMN (Broche and Tellado, 2001; Kubicka et al., 1996; Lewis and Holmes, 1991). Thus, resident leukocytes constitute the majority of cells recoverable by lavage. A more detailed analysis of the different constituents of the serosal membranes and their role in the initiation of inflammation will be further discussed below.

1.2.1 Description and role of resident Mϕ:

Mϕ are dispersed throughout the tissues and have an important role in innate immunity (Gordon, 2002), apoptotic cell clearance (Savill et al., 2002), development (Gouon-Evans et al., 2000) and morphogenesis (Diez-Roux et al., 1999; Lang and Bishop, 1993). Mϕ are heterogeneous cells and can be found in tissues where they are known as resident Mϕ. The local microenvironment will provide tissue-specific signals such as apocrine secretion products from neighbouring resident cells and extracellular matrix and this is likely to play a prominent role in the development of the different phenotypes found in the various resident Mϕ populations (Gordon, 2003). These various resident Mϕ include serosal (peritoneal and pleural) Mϕ, Kupffer cells in the liver, alveolar Mϕ in the lung, microglia in the brain and

osteoclasts within bone (Wiktor-Jedrzejczak and Gordon, 1996). Resident Mϕ differ from the other monocyte-derived Mϕ (MDMϕ) as they are long-lived in tissue and possess a proliferative capacity such that they may be sustained by self-renewal (Daems and de Bakker, 1982; Lawson et al., 1992; Takahashi et al., 1996). Although resident Mϕ number was shown to be independent of the bone marrow and the level of circulating monocytes based on studies involving strontium^{89} ablation (Volkman et al., 1983), recent evidence suggests that the situation is more complex. The renewal of resident Mϕ could be derived from colony forming unit-granulocyte/Mϕ or earlier precursor cells (Naito et al., 1996; Takahashi, 1994) in peripheral tissues or by extravasation and differentiation of circulating monocytes (Bruno et al., 2001; Geissmann et al., 2003; Lawson et al., 1992).

Recent work has suggested that there are two distinct monocyte populations that give rise to either resident Mϕ or inflammatory Mϕ and that these monocytes may be distinguished by their surface markers. Resident Mϕ are derived from CX_3CR1^{Hi}, $Gr1^-$ and $CCR2^-$ monocytes whilst inflammatory Mϕ are derived from CX_3CR1^{Lo}, $Gr1^+$, CD62L and $CCR2^+$ monocytes (Geissmann et al., 2003). Intra-peritoneal migration of resident Mϕ-precursor monocytes is not prominent in the absence of inflammation. During inflammatory peritonitis, however, there is some recruitment of the long-lived CX_3CR1^{Hi}, $Gr1^-$ and $CCR2^-$ monocytes (Geissmann et al., 2003) and this may be to replace the resident Mϕ that emigrate to the parathymic lymph nodes during and following inflammation (Bellingan et al., 2002). The nature of the originating cells for both monocyte subsets is not entirely clear and it is not known if the CX_3CR1^{Hi}, $Gr1^-$ and $CCR2^-$ MDMϕ would be able to replenish normal

tissue resident Mϕ (Taylor and Gordon, 2003). However, a bone marrow Mϕ-DC progenitor has been recently described that may give rise to splenic and brain resident Mϕ, splenic resident DC and inflammatory Mϕ and DC (Fogg et al., 2006).

The osteopetrotic mouse exhibits a deficiency of the primary Mϕ growth factor colony stimulating factor (CSF)-1 (Wiktor-Jedrzejczak et al., 1990). Study of this mouse has allowed the identification of CSF-1-dependent and CSF-1-independent Mϕ populations further highlighting the complexity of the Mϕ system (Cecchini et al., 1994; Wiktor-Jedrzejczak and Gordon, 1996; Witmer-Pack et al., 1993). Interestingly, Ly-1 positive resident peritoneal Mϕ are derived from the same precursor cells as Ly-1 positive B cells present in the peritoneum (Takahashi, 1994). In addition, careful analysis of PU.1 mutant mice showed that the transcription factor PU.1 was essential for normal differentiation of both Mϕ and B cells further linking the origins B cells and Mϕ (McKercher et al., 1996).

Resident Mϕ have an important role in the immune protection of their respective organ of residence and resident tissue Mϕ are regarded as sentinels of the innate immune system. They are implicated in the clearance of pathogens and the repair of injured tissue (Sean Eardley and Cockwell, 2005). Although resident Mϕ are heterogeneous, the comparison of the phagocytic activity and prostaglandin release of resident peritoneal and pleural Mϕ exhibit considerable similarity. In contrast, both peritoneal and pleural Mϕ are functionally and phenotypically different from resident alveolar Mϕ (Dorger et al., 2001; Gjomarkaj et al., 1999; Sestini et al., 1984). In addition, adhesion and phagocytosis of apoptotic cells also differs between

resident peritoneal Mϕ and alveolar Mϕ (Hu et al., 2004; Hu et al., 2000). Monocytes modify their expression of adhesion molecules as they differentiate into monocyte-derived Mϕ (MDMϕ). They downregulate various integrins [$\alpha_4\beta_1$, $\alpha_6\beta_1$, $\alpha_L\beta_2$, platelet endothelial cell adhesion molecule-1 (PECAM-1)and $\alpha_{IIb}\beta_3$ integrins] and upregulate $\alpha_v\beta_5$ integrin reflecting their different adhesive requirements (Faull et al., 1996). Resident Mϕ and MDMϕ, or elicited Mϕ, differ in their production of hyaluronan (Hodge-Dufour et al., 1997) and oxygen radicals (Rugtveit et al., 1995), caveolae formation and endocytosis (Kiss et al., 2002) and expression of laminin (Wicha and Huard, 1983). However, these variations might reflect differences in the activation state of the cell rather than differences between resident and elicited Mϕ.

Resident peritoneal Mϕ are an important source of cytokines and chemokines involved in the immune defense of the peritoneal cavity. Key players include the cytokines interleukin (IL)-1, IL-6 and tumour necrosis factor (TNF)-α (Lewis and Holmes, 1991; Topley et al., 1993c) and the chemoattractants IL-8, monocyte chemotactic protein (MCP)-1 (Bauermeister et al., 1998; Topley et al., 1993c; Topley et al., 1994), prostaglandins (Topley et al., 1993c) and leukotrienes (Kolaczkowska et al., 2002; Mackenzie et al., 1990; Mackenzie et al., 1991). It is believed that this "cytokine network" facilitates communication between peritoneal Mϕ and MeC that act in concert to protect the peritoneum (Topley et al., 1996).

In addition, TNF-α and IL-1β are key cytokines in the development of pleural inflammation as they act to enhance IL-8 and MCP-1 production from pleural MeC (Antony et al., 1995; Frode et al., 2001; Goodman et al., 1992; Mohammed et al.,

1998a; Park et al., 2003). Studies using function-blocking antibodies suggest that activated resident pleural Mϕ could be responsible for this TNF-α and IL-1β secretion (Frode et al., 2001; Park et al., 2003). To date, there has been limited study of the role of the resident pleural Mϕ in the initiation of inflammation and orchestration of PMN recruitment. Previous work has demonstrated that the pleural eosinophil influx induced by the administration of lipopolysaccharide (LPS) was reduced in mice previously treated with diphosphonate-containing liposomes in order to deplete resident pleural Mϕ (Bozza et al., 1994) thereby suggesting an important role for the resident pleural Mϕ. In contrast, the role of resident peritoneal Mϕ in the initiation of inflammation and orchestration of PMN recruitment is more controversial. Previous studies have produced conflicting results suggesting that the resident peritoneal Mϕ play a key role (Ajuebor et al., 1999; Knudsen et al., 2002; Souza et al., 1988), an inhibitory role (Ajuebor et al., 1999) or no role at all (Ajuebor et al., 1999) depending on the model used. Although these studies do suggest that resident Mϕ may play a key role in the initiation of inflammatory responses, there are no definitive data available for PMN infiltration and pro-inflammatory cytokine production in serosal inflammation.

1.2.2 Description and role of resident dendritic cells (DC):

Dendritic cells (DC) represent 1% of the resting resident peritoneal cells in rats (van Vugt et al., 1991). DC are also described as the sentinel cells of the immune system, at least with regard to their key interactions with the adaptive arm of the immune response i.e. T and B lymphocytes (Banchereau and Steinman, 1998).

DC can be located in non-lymphoid tissues and constantly sample their environment to detect infectious or non-self stimuli. These immature resident DC have a limited plasticity and life span (Foti et al., 2004). Similar to Mϕ, splenic resident DC exhibit different phenotypes and share numerous Mϕ markers and functions that highlight the close relationship between Mϕ and DC (Leenen et al., 1998). The sensing function of DC is mediated through various innate immune receptors such as the Toll-like receptors (TLR). These receptors can react to different classes of infectious agents including bacteria and viruses. Upon receptor ligation, DC can up-regulate genes leading to expression of various cytokines and chemokines involved in the inflammatory and immune response such as IL-1β, IL-1 receptor antagonist (IL-1RA), TNF-α, IL-2, IL-6, IL-12 p40, MIP-1α, MIP-1β, MIP-1γ, MIP-2α, MCP-5, macrophage migration inhibitory factor (MIF), inducible protein (IP)-10 and growth-related oncogene (GRO)-1 (Dubois et al., 1995; Granucci et al., 2001).

1.2.3 Description and role of lymphocytes:

Although lymphocytes are present in serosal cavities, their function during inflammation has not been explored in detail (Faull, 2000). A short description of the importance of the lymphocytes in the serous cavities will follow in the next two sections and I will then discuss the different murine models that lack various lymphocyte populations.

1.2.3.1 Description and role of B lymphocytes:

B cells represent 2.3% of total cells in the human peritoneum (Hartman et al., 1995). Peritoneal B lymphocytes are divided into 4 different subpopulations according to their expression of CD5 (Ly-1) and MAC-1 as follows:

(i) B-1a cells ($CD5^+$, $MAC\text{-}1^+$ and $C45RA^{Low}$) (Youinou et al., 1999),

(ii) B-1b cells ($CD5^-$, $MAC\text{-}1^+$ and $C45RA^{Low}$, these cells comprise 5-10 % of the total B-1 cell population) (Youinou et al., 1999),

(iii) B-1c cells ($CD5^+$, $MAC\text{-}1^-$) (Hastings et al., 2006)

(iv) B-2 cells ($CD5^-$, $MAC\text{-}1^-$; comparable to normal circulating B cells) (Youinou et al., 1999).

The B-1 cells represent approximately 50% of the B cell population in the murine peritoneum (10-20% of total peritoneal cells are B-1 cells) (Hayakawa et al., 1985). A key function of the B1 cells is the synthesis of immunoglobulins (Ig) and natural antibodies (IgM) (Herzenberg, 2000). Peritoneal B cells are derived from different progenitors (Herzenberg, 2000) and the B-1a cells exhibit the capacity for self-renewal (Youinou et al., 1999). The maintenance of B-1 cells in the peritoneum depends on interactions between MeC-derived stromal cell-derived factor (SDF)-1 (Foussat et al., 2001) and constitutive IL-10 production (Balabanian et al., 2002). In addition, CXCL13 production by peritoneal Mϕ has an important role in the peritoneal homing of B-1 cells (Ansel et al., 2002). B-1 cells play a role in the initiation of the early phase of skin delayed-type hypersensitivity and are essential

for subsequent T cell recruitment (Szczepanik et al., 2003); a finding that can also be found in filarial models of inflammation (Ramalingam et al., 2003). B-1 cell-dependent IL-10 production can affect peritoneal Mϕ phagocytosis and reactive oxygen species production (Popi et al., 2004). B-1 cells play an important role in diseases mediated by filarial parasites (Paciorkowski et al., 2000) as well as in autoimmunity (Boes et al., 2000), autoimmune haemolytic anemia (Watanabe et al., 2002), atherosclerosis (Shaw et al., 2003; Shaw et al., 2000), and granuloma formation (Bogsan et al., 2005).

1.2.3.2 Description and role of T lymphocytes:

The majority of peritoneal T cells express the αβ T cell receptor (TcR) with around 17% expressing the γδ TcR. The majority of T cells exhibit markers of thymus-dependent origin and surface antigens compatible with an activation and memory phenotype. This peritoneal T cell phenotype indicates that the peritoneum is capable of thymus-independent differentiation of T cells (Hartman et al., 1995). The CD4/CD8 ratio also differs from that evident in the blood where most of the T cells are CD8+. Although the majority of peritoneal CD8+ cells secrete a T_h1 pattern of cytokines such IL-2 as well as the cytotoxic mediators IFN-γ and TNF-α, some peritoneal CD8+ cells secrete the cytokines IL-4 and IL-5 that are characteristic of T_h2 cells (Birkhofer et al., 1996). These T_h2-type cells are able to support B cell differentiation and secretion of IgG and IgA (Birkhofer et al., 1996), whilst γδ T cells would play an important role in monocyte/Mϕ differentiation (Skeen et al., 2004). These γδT cells would have an important role in regulation of the early

inflammatory response and it is of interest that γδ T cell knock-out (KO) mice exhibit greater pulmonary inflammation with increased numbers of PMN and Mϕ after aerosolised *Bordetella pertussis*(Zachariadis et al., 2006). Similarly, increased numbers of PMN and elevated cytokine levels were found in γδ T cell KO after IP *Listeria* inoculation (Skeen et al., 2001). This evidence suggests that γδ T cells are important in regulating the extent of the inflammatory response in order to prevent excessive damage. It is currently unclear why the lymphocyte population of the peritoneal cavity is so different from the blood. However, the cytokine IL-15 may be involved in the generation of such differences because it affects the maturation and proliferation of NK and CD8 T cells and can be produced by Mϕ and MeC (Kobayashi et al., 2005; Rapoport et al., 1999; Tagaya et al., 1996). Pleural T cells play an important role in eosinophil recruitment induced by LPS as selective ablation of T cells inhibited pleural eosinophil influx (Bozza et al., 1994).

1.2.3.3 Description of mice deficient in various lymphocyte populations:

Different mice models to study lymphocyte functions are available. A naturally occurring mutation in mice resulted in mice with absent hair, a congenital absence of the thymus due to a defect of the thymic epithelium and absent T cell development such that they are used as a model of T cell deficiency (Pantelouris, 1968; Wortis et al., 1971). There is some extrathymic T cell production present but this is very marginal. The natural killer (NK) cells and Mϕ of NUDE mice exhibit increased cytotoxic activity (Budzynski and Radzikowski, 1994). The μMT KO

mice were generated by disruption of one of the exons coding for the μ-chain and resulted in B cell development stopping at the stage of pre-B-cell maturation, resulting in an absence of mature B cells (Kitamura et al., 1991). RAG-1 KO mice were generated by mutating the RAG-1 gene involved in the V(D)J recombination reaction of immunoglobulin and T cell receptors. RAG-1 KO lack mature T and B cells but other immune mediators are increased such as complement activity and NK cell activity (Mombaerts et al., 1992; Shultz et al., 2000).

1.2.4 Description and role of resident mast cells:

There are two classes of mast cells in the mouse: connective tissue MC and mucosal MC. As is the case for resident Mϕ, MC complete their maturation in the tissue of residence under the influence of the local microenvironment. MC exist only as "resident" cells and can produce inflammatory mediators such as histamine, platelet-activating factor, prostaglandins, thromboxane, leukotriene, chymase, cytokines (GM-CSF, TNF-α, IL-1,-3, -4 and -6) and chemokines (MCP-1 and MIP-1α) (He and Walls, 1998; Metcalfe et al., 1997). Previous work has examined the role of MC in the initiation and amplification of "non-immunologic" inflammation (Wershil et al., 1988) (Metcalfe et al., 1997). In a model of phorbol 12-myristate 13-acetate (PMA)-induced cutaneous inflammation in MC-deficient W/W^v mice, MC were involved in tissue swelling and leukocyte recruitment as adoptive transfer of MC resulted in increased swelling and PMN influx (Wershil et al., 1988). The induction of BTG peritonitis in MC-deficient W/W^v mice resulted in a reduced PMN recruitment compared to controls and this defect could be corrected with adoptive

transfer (Qureshi and Jakschik, 1988). However, an inflammatory response was still present in both models with PMN recruitment being reduced by 55% and 70% at 18h in the PMA and the BTG model respectively and this suggests that MC are not the only cell involved in the initiation of inflammation but are capable of modulating inflammatory responses (Metcalfe et al., 1997; Qureshi and Jakschik, 1988). The production of leukotrienes by MC is an important facet of the early PMN recruitment in a model of infectious peritonitis (Malaviya and Abraham, 2000; Malaviya et al., 2001). Another study examined the role of MC in PMN recruitment in three experimental models of peritonitis after MC 'depletion' was induced by the activating MC compound 48/80 (which induces excessive degranulation). MC depletion had no effect upon BTG induced peritonitis, reduced PMN infiltration in LPS induced peritonitis (though no chemokine differences were evident) and inhibited PMN influx and chemokine secretion in zymosan induced peritonitis (Ajuebor et al., 1999). In addition to playing a role in zymosan peritonitis (Ribeiro et al., 2000), MC were essential for PMN recruitment in a cutaneous model of T cell-mediated delayed hypersensitivity reaction (Biedermann et al., 2000) and in an experimental model of bullous pemphigoid (Chen et al., 2002). In the study by Chen et al, MC activation was crucial for the development of Mϕ-dependent PMN influx (Chen et al., 2002). The IL-8-induced PMN recruitment into the air-pouch cavity is MC-dependent and mediated by MC release of cytokine-induced neutrophil chemoattractant (CINC)-1 (Ramos et al., 2003). Furthermore, PMN recruitment has been found to be MC-dependent in zymosan-induced pleurisy (Takeshita et al., 2003). In this context it is of interest that PMN recruitment is MC-independent in carrageenan-induced pleurisy (Horakova et al., 1980; Takeshita et al., 2003). MC

degranulation did not affect eosinophil recruitment in LPS-induced pleurisy (Bozza et al., 1994). In summary, MC play a role in the initiation of inflammation but many studies indicate that the prime action of MC is the modulation of the inflammatory reaction.

1.2.5 Description and role of resident natural killer cells:

The NK cell population is enriched in the human peritoneal cavity of healthy CAPD patients; 20% of the peritoneal lymphocytes would be NK cells whilst 4.2% would be CD8+ NK cells (Lewis et al., 1993). In C57BL/6 mice, NK cells represent 3.2±0.8% of total unstimulated peritoneal cells whereas NK 1.1^{+} T cells represent 3.5±1.1% (Kawamura et al., 1999). Activated NK cells produce a spectrum of cytokines such as TNF-α, IFN-γ, GM-CSF, lymphotoxin and IL-8 (Das and Khar, 2002), whereas NK T cells can produce T_h1-type cytokines (including IFN- γ and TNF- α), as well as T_h2-type cytokines (including IL-4 and IL-13) (Smyth and Godfrey, 2000; Wilson and Delovitch, 2003). The production of IFN-γ by NK T cells is induced by Mϕ-dependent IL-12 production (Kawamura et al., 1999; Kobayashi et al., 1989). NK T cells can mediate cytotoxicity through Fas–Fas Ligand and perforin/granzyme pathways (Wilson and Delovitch, 2003). NK T cells are involved in many important biological processes including the suppression of tissue destruction (allograft tolerance, GVHD), autoimmune responses, antitumour responses and host defence against viral, bacterial and parasitic pathogens (Godfrey and Kronenberg, 2004). NK T cells might be also implicated in allergy and contact hypersensitivity (via IL-4 secretion in the latter). NK T cells may modulate B-1 B

and mast cell activation and have also been implicated in atherosclerosis (Godfrey and Kronenberg, 2004). It remains to be determined if NK T cells are regulators or effectors of immune-mediated damage (reviewed in (Godfrey and Kronenberg, 2004)). Peritoneal resident NK cell activation would facilitate Mϕ clearance of bacteria (Scott et al., 2003) via IL-12 regulation (Godshall et al., 2003).

In summary, although the role of NK cells in peritonitis or pleurisy remains ill defined, it is likely that the modulation of the inflammatory response via IFN-γ secretion and the subsequent effects upon cytokine and chemokine production might be of some importance.

1.2.6 Description and role of the mesothelial and stromal cells:

The mesothelial cell (MeC) lining constitutes a protective barrier to physical and infectious injury and has a complex role in the immunity of the serosal cavity (Mutsaers, 2004). Although peritoneal and pleural MeC may be similar in many regards, there have been no studies that have directly compared them and therefore their functions will be presented separately. MeC play an important role in peritoneal inflammation (Topley, 1995b; Topley et al., 1993c; Topley and Williams, 1994) and tissue repair (Mutsaers et al., 1997; Rennard et al., 1984). *In vivo* and *in vitro* studies have demonstrated that peritoneal MeC produce pro-inflammatory cytokines such as IL-1β (Lanfrancone et al., 1992) and IL-6 (Topley et al., 1993b), anti-inflammatory molecules such as IL-10 and the decoy molecule IL-1 receptor type II (IL-1RII) (Yao et al., 2004a; Yao et al., 2004b) and prostaglandins (Topley et

al., 1994)((Jayne et al., 2000) (reviewed in (Broche and Tellado, 2001)). Stimulated MeC increase the expression of adhesion molecules and produce chemokines [IL-8, MCP-1 and Regulated upon Activation, Normal T Expressed and Secreted (RANTES)] (Jonjic et al., 1992; Li et al., 1998; Topley et al., 1993a; Zeillemaker et al., 1995) which represent essential steps in order to mount an inflammatory response. CD40 is expressed at the MeC surface and is induced by IFN-γ and CD40L (CD154). CD40 activation increases the expression of intercellular adhesion molecule-1 (ICAM-1) by peritoneal mesothelial cells (Yang et al., 2004), which facilitates ICAM-1-dependent PMN migration across the mesothelial membrane (Li et al., 1998).

During pleural inflammation, it has been reported that MeC are predominantly responsible for the secretion of C-X-C chemokines such as IL-8 and C-C chemokines such as MIP-1α and MCP-1 that act to recruit PMNs and mononuclear cells (Antony et al., 1995; Mohammed et al., 1998a; Mohammed et al., 1999; Mohammed et al., 1998b).

Fibroblasts are present in the interstitial stroma beneath the MeC layer and are also implicated in serosal inflammation (Faull, 2000). Recent studies demonstrated that activated peritoneal and pleural fibroblasts may be a source of C-X-C and C-C chemokine production (Loghmani et al., 2002; Witowski et al., 2001). In addition, resident cells, specifically fibroblasts, can play an important role in leukocyte recruitment in the air-pouch model of inflammation (Garcia-Ramallo et al., 2002).

MeC involvement in the initiation of inflammation has been widely discussed whilst the underlying population of fibroblasts may also be involved. However, most studies involve the study of chemokine and cytokine production after the activation of the MeC and this raises the possibility that other cells may well be involved in the initial production of these activating cytokines. A "cytokine network" between the resident cells is therefore essential for this initiation of inflammation (Topley, 1995a). Some studies have suggested that resident Mϕ-derived pro-inflammatory cytokines such as TNF-α would be essential for the secretion of C-X-C and C-C chemokines from mesothelial cells and fibroblasts (Antony et al., 1995; Cuzzocrea et al., 1999b; Frode et al., 2001; Goodman et al., 1992; Mohammed et al., 1998b; Pace et al., 1999; Park et al., 2003; Topley et al., 1994; Witowski et al., 2001). However, there have been no definitive experiments performed to address this issue. T_h1 (IL-2, IFN-γ and lymphotoxin) and T_h2 (IL-4, IL-5, IL-6 and IL-10) cytokines also appear to have a role in the regulation of chemokine production, suggesting that leukocytes other than Mϕ might be involved (Kunkel, 1996; Mohammed et al., 1999). This provides an exciting environment to investigate the role of the resident Mϕ and lymphocytes in the orchestration of leukocyte recruitment in serosal inflammation.

1.3 Description of the Mϕ ablation systems:

The simplest method to reduce Mϕ number in the peritoneal cavity is peritoneal lavage in order to wash out Mϕ from the peritoneal cavity. This has been shown to reduce the number of recoverable Mϕ number by about 80% and block

PMN recruitment induced by various inflammatory agents (Barja-Fidalgo et al., 1992; Souza et al., 1988). These studies therefore suggested that Mϕ might be involved in the control of PMN recruitment. In addition to these methods, various depletion strategies have been developed to dissect Mϕ function *in vivo*. Mϕ depletion induced by the administration of liposomal clodronate is probably the most common strategy used (Van Rooijen, 1989). Following the phagocytosis of liposome encapsulated clodronate, the phospholipid bilayers are disrupted by the action of lysosomal phospholipases and the clodronate is then released in the intra-cytoplasmic compartment (Van Rooijen and Sanders, 1994). Clodronate accumulation induces cell death by inhibiting mitochondrial adenosine triphosphate (ATP)/adenosine diphosphate (ADP) translocase, thereby causing loss of the mitochondrial membrane potential and direct induction of apoptosis (Green, 2003). Clodronate released in the circulation from dead Mϕ or by leakage from liposomes is ineffective and does not kill bystander cells (Schmidt-Weber et al., 1996; Van Rooijen and Sanders, 1994). Mϕ and activated monocytes are the only cells to die after phagocytosis of clodronate-containing liposomes as resting and activated PMN did not undergo apoptosis despite phagocytosis of liposomes (Schmidt-Weber et al., 1996). Resident Mϕ ablation rates are high using this reagent but can be somewhat variable. Examples of the efficacy of macrophage ablation in different organs include the following; 78-88% ablation in the lung (Cheung et al., 2000), >95% ablation in the liver and spleen, around 55% ablation of circulating monocytes (Zito et al., 2001), 70% ablation in the eye (Pouvreau et al., 1998) and 85% ablation in the peritoneum (Ajuebor et al., 1999). Recently, a modified version of this protocol has been described using clodronate-loaded erythrocytes with a Mϕ ablation rate of

around 70% (Rossi et al., 2005). Using clodronate-based Mϕ ablation, Mϕ were shown to be important in various situations including glomerulonephritis (D'Souza et al., 1999), lung inflammation (Hashimoto et al., 1996), arthritis (Lawlor et al., 2005), allograft rejection (Wyburn et al., 2005), diabetes (Jun et al., 1999), neointimal formation (Danenberg et al., 2003), liver injury (Schumann et al., 2000), experimental allergic encephalomyelitis (Huitinga et al., 1990), ischaemia-reperfusion injury in the gut (Chen et al., 2004), uveitis (Baatz et al., 2001) and pancreatitis (Shifrin et al., 2005). It is of interest that previous work using clodronate to study the role of resident peritoneal Mϕ in the initiation of experimental peritonitis has produced conflicting results (Ajuebor et al., 1999; Knudsen et al., 2002). Ajuebor *et al* showed that Mϕ ablation had no effect upon PMN recruitment in the BTG and zymosan model of peritonitis. In contrats, Knudsen *et al* demonstrated that Mϕ ablation blunted PMN influx in their casein model of peritonitis. The latter work is in accord with a study using peritoneal lavage as a Mϕ depleting strategy as this demonstrated an important role of resident Mϕ (Souza et al., 1988). Table 1 summarises the details and conclusions of various studies that have examined the role of resident Mϕ.

Table 1-Summary of relevant studies on Mϕ ablation and peritoneal inflammation

Studies	Mϕ Ablation Method	% Mϕ Ablation	Animals	Inflammatory Model	Impact on PMN Recruitment
Ajuebor *et al* *(Ajuebor et al., 1999)*	Clodronate Liposomes	89%	Mice	BTG	No Effect
			Mice	LPS	Reduced
			Mice	Zymosan	Enhanced
Knudsen *et al* *(Knudsen et al., 2004)*	Clodronate Liposomes	85%	Rat	Casein Digest Bacto-Tryptone	Reduced
Souza *et al* *(Souza et al., 1988)*	Saline Lavage	80%	Rat	Carrageenan	Reduced
			Rat	Zymosan	Reduced
			Rat	E. Coli endotoxin	Reduced
			Rat	Mϕ-derived neutrophil chemotactic factor	No Effect

A conditional Mϕ ablation strategy has advantages over the available naturally occurring and induced Mϕ-deficient mutant mice as the timing of Mϕ elimination can be chosen. Despite their limitations, non-conditional Mϕ-deficient mice have proven valuable for analysis. For example, the *Csfm*^{op}/*Csfm*^{op} (osteopetrosis) mouse is a naturally occurring mutant of the colony stimulating factor-1 gene and exhibits a Mϕ deficiency at a level that permits viability in homozygotes (Cecchini et al., 1994). These mice have provided significant insight into Mϕ function during development (Gouon-Evans et al., 2000) as well as inflammation (Lenda et al., 2003). In addition, mice targeted at the *PU.1* locus exhibit multiple defects in development of hematopoietic lineages including a complete absence of tissue Mϕ and B cells (McKercher et al., 1996; Scott et al., 1994). Although the *PU.1* mutation results in perinatal lethality, these mice have been used to demonstrate that mesenchymal cells are able to clear apoptotic cells in the absence of tissue Mϕ during embryonic regression of inter-digital tissues (Wood et al., 2000).

Previous strategies used to eliminate specific cell types in a living organism have included the generation of transgenic lines that express diphtheria toxin (DT) A-chain (Breitman et al., 1987; Pappenheimer, 1977) or the ricin polypeptide (Landel et al., 1988). However, even low levels of unanticipated transgene expression can give unpredictable consequences in such mice (Breitman et al., 1987). The alternative ablation strategy of killing thymidine-kinase (TK) expressing cells with gancyclovir (Heyman et al., 1989; Minasi et al., 1993) is useful but is limited by

the fact that it only permits the elimination of proliferating cells. More recently, conditional Mϕ ablation has been achieved using transgenic expression of Fas under the control of the c-*fms* promoter coupled with drug inducible Fas dimerization to induce cell death (Burnett et al., 2004). Identification of the human receptor for DT (also known as heparin-binding epidermal growth factor (hbEGF) (Naglich et al., 1992) or referred to as DTR for diphtheria toxin receptor) created an opportunity for a unique ablation strategy. The murine form of hbEGF binds DT poorly, but murine cells can be rendered sensitive through transgenic expression of human hbEGF or DTR. Richard Lang's group generated transgenic mice expressing human hbEGF lineage specifically under the CD11b promoter (CD11b-DTR) and demonstrated that cell ablation results from toxin injection (Cailhier et al., 2005). In addition, since DT is a protein synthesis inhibitor this strategy results in the induction of death in both mitotic and terminally differentiated cells. The death mechanism is apoptosis as 65% of the F4/80 positive peritoneal Mϕ are Annexin-V positive 6 hours after DT administration (Cailhier et al., 2005). The generation of CD11b-DTR transgenic mice used in this study have also been used to demonstrate the importance of Mϕ in progressive renal inflammation (Duffield et al., 2005b) and the progression of liver injury and subsequent resolution of fibrosis (Duffield et al., 2005a). This strategy has also been used to generate transgenic mice in which hepatocytes (Saito et al., 2001) or dendritic cells (Jung et al., 2002) may be conditionally ablated.

1.4 Overview of leukocyte recruitment:

Leukocytes are essential during acute inflammation and must migrate from the blood to the site of inflammation. Circulating leukocytes migrate toward inflammatory stimuli in response to a gradient of released chemoattractants or chemokines. Resident cells such as Mϕ, MeC and fibroblasts constitute a reservoir of such chemokines and upon cytokine activation will release them (Loghmani et al., 2002; Topley et al., 1996; Witowski et al., 2001) and establish the necessary gradient to attract leukocytes passing through the serosal vasculature (Figure 1.1 adapted from (Luster, 1998)). The combined effects of inflammatory cytokines and chemoattractants induce the following sequence of events:

1) Leukocyte rolling along endothelial cells,
2) Activation of leukocyte integrins,
3) Firm adhesion to the vascular endothelium (adherence),
4) Transmigration out of the vessel by passing between the endothelial cells,
5) Activation of the recruited leukocyte.

The rolling phase is mediated by the main leukocyte selectin ligand P-selectin glycoprotein ligand-1 (PSGL-1) engaging with endothelial adhesion molecules known as selectins (E-, L-, and P-selectin). Adherence involves the adhesive interactions between integrins on the leukocytes (e.g. $\alpha_L\beta_2$, $\alpha_M\beta_2$, $\alpha_4\beta_1$) and

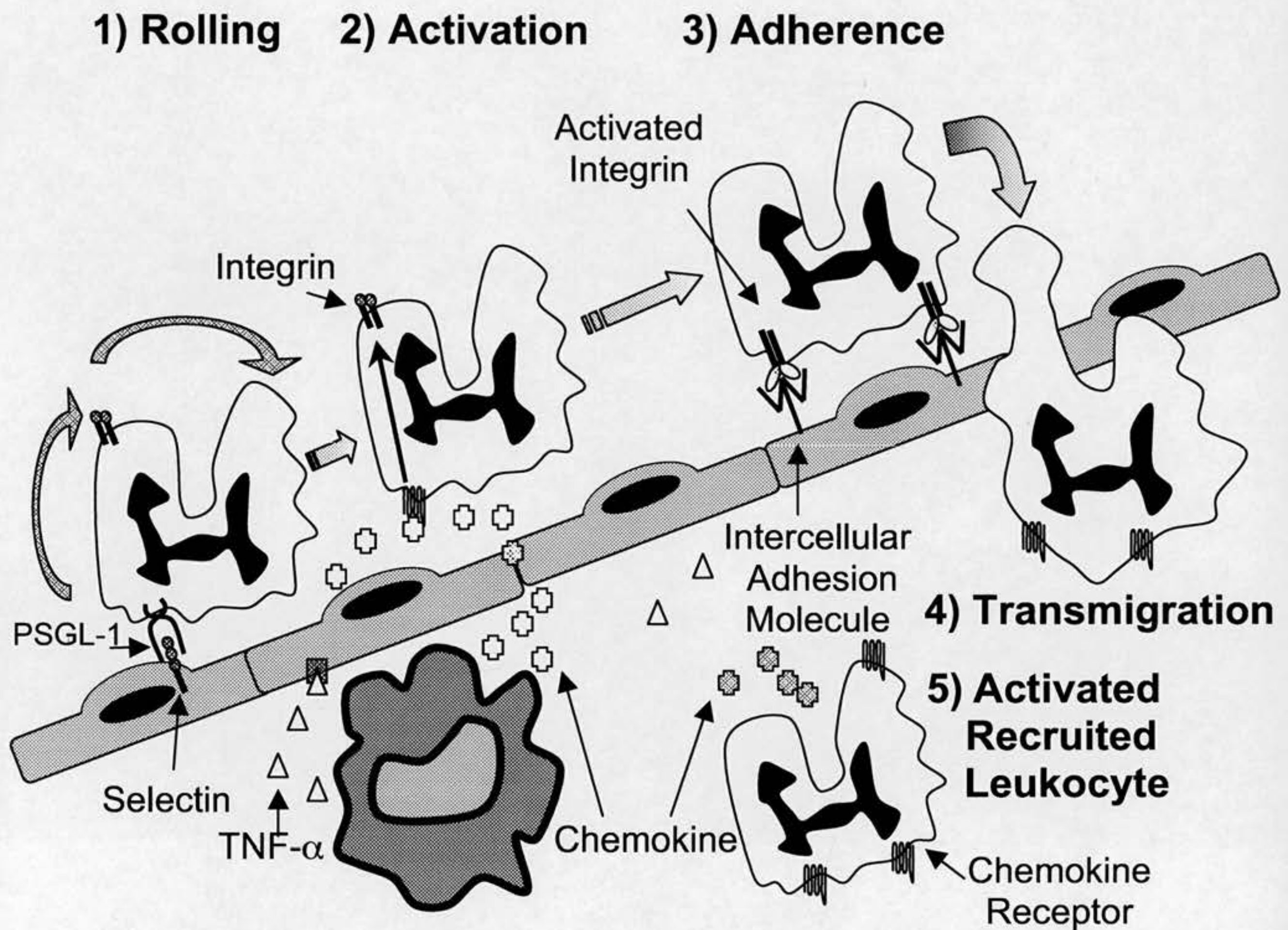


Figure 1.1 Overview of leukocyte recruitment

The rolling phase is mediated by the main leukocyte selectin ligand P-selectin glycoprotein ligand-1 (PSGL-1) engaging with endothelial adhesion molecules known as selectins (E-, L-, and P-selectin). Adherence involves adhesive interactions between integrins on the leukocytes (e.g. $\alpha_L\beta_2$, $\alpha_M\beta_2$, $\alpha_4\beta_1$) and members of the immunoglobulin superfamily on the endothelial cells [e.g. intercellular adhesion molecule (ICAM)-1 and -2, vascular cell adhesion molecule (VCAM)-1]. Both ICAM-1 and VCAM-1 are induced on endothelial cells by inflammatory cytokines (such as TNF-α) whilst integrins act as tethering molecules on the leukocyte surface. The leukocyte transmigration is mediated by interactions between platelet endothelial cell adhesion molecule-1 (PECAM-1) and members of the junctional adhesion molecule family with their leukocyte counter-ligands. The final step of diapedesis results in the leukocytes entering the extravascular connective tissue having traversed the basement membrane with the aid of secreted matrix-degrading metalloproteinases.

members of the immunoglobulin superfamily on the endothelial cells [e.g. intercellular adhesion molecule (ICAM)-1 and -2, vascular cell adhesion molecule (VCAM)-1] (Carlos and Harlan, 1994; McIntyre et al., 2003). Both ICAM-1 and VCAM-1 are induced on endothelial cells by inflammatory cytokines (such as TNF-α) whilst integrins act as tethering molecules on the leukocyte surface. The leukocyte transmigration is mediated by interactions between PECAM-1 and members of the junctional adhesion molecule family with their leukocyte counter-ligands. The final step of diapedesis results in the leukocytes entering the extravascular connective tissue having traversed the basement membrane with the aid of secreted matrix-degrading metalloproteinases (Nourshargh and Marelli-Berg, 2005).

1.5 Description of relevant cytokines:

Cytokines are a group of secreted proteins that carry a message from cell to cell ("cyto") and induce a targeted cell to accomplish something ("kine" from the same root as kinetics). They were called lymphokines or monokines depending of the cell of origin. Many of these molecules are now known as interleukins (proteins talking to leukocytes) (Sigal, 2004). Cytokines are multifunctional proteins and are involved in cell growth and activation, inflammation, immunity and cell differentiation (Feldmann et al., 1996). Many cytokines are secreted simultaneously and their action can compete against each other. They can enhance or inhibit production of cytokines or other proteins such as chemokines and can either increase or decrease the inflammatory response (Gouwy et al., 2005). As it was discussed

previously, cytokines are essential stimulators of resident cells in order to initiate chemokine production. There are numerous cytokines that are grouped in different families but I will describe only the main cytokines that have been involved in models of peritonitis and pleurisy.

1.5.1 IL-1β and TNF-α:

IL-1β and TNF-α are potent pro-inflammatory cytokines produced by Mϕ, epithelial, NK and T cells that are systemically involved in the acute-phase response. They increase body temperature, hepatic production of acute-phase proteins, which play a crucial role in opsonisation, mobilise energy to allow increase in temperature, allow PMN mobilisation and promote signal to involve adaptive immunity. They also have an important local effect. They contribute to endothelium and leukocyte activation (Janeway et al., 2001; O'Shea et al., 2002). TNF-α can induce production of other pro-inflammatory cytokines such as IL-1β and IL-6 (O'Shea et al., 2002). Furthermore, IL-1β and TNF-α play a crucial role in the initiation and modulation of the inflammatory response in serosal cavities as it was demonstrated earlier through induction of chemokine secretion by resident cells (Antony et al., 1995; Frode et al., 2001; Goodman et al., 1992; Lewis and Holmes, 1991; Mohammed et al., 1998a; Park et al., 2003; Tessier et al., 1997; Topley et al., 1993c).

1.5.2 IFN-γ:

IFN-γ is produced mainly by T and NK cells, its production by Mϕ, DC and PMN remains ill-defined. It is a major pro-inflammatory cytokine and it has an important role in antiviral activity but also in regulating function of innate and adaptive immunity (Renauld, 2003; Trinchieri et al., 2003). IFN-γ has been implicated as a potent regulator of C-X-C and C-C chemokines in MeC and acts to downregulate IL-8 and upregulate MCP-1 and RANTES (Robson et al., 2001). Cytokine production can also be induced (Trinchieri et al., 2003). IFN-γ can control PMN recruitment and sIL-6R signalling *in vivo* (McLoughlin et al., 2003). Therefore, it might be important in regulating leukocyte recruitment in experimental peritonitis and pleurisy.

1.5.3 IL-6:

IL-6 has a more complex role and can have pro-inflammatory (Cuzzocrea et al., 1999b; Romano et al., 1997) and anti-inflammatory proprieties (Tilg et al., 1997; Tilg et al., 1994; Xing et al., 1998) depending on the model used. IL-6 is produced by a variety of cells (Mϕ, MeC, endothelial cells and fibroblasts) in response to inflammatory stimuli or other cytokines such as IL-1β and TNF-α. Previous work in experimental models of pleurisy suggested that the initiation of inflammation is dependent upon endogenous IL-6 secretion that subsequently stimulates the additional production of TNF-α and IL-1β from resident pleural cells (Cuzzocrea et

al., 1999b). In contrast, increased IL-1β levels have been reported to precede elevated IL-6 levels (Utsunomiya et al., 1991) and this suggests that IL-1β might induce IL-6 production, so the exact temporal production of cytokines is not entirely clear. IL-6 has also been described as an important inducer of chemokine production. Moreover, the administration of IL-6-sIL-6R complex to cells unable to respond to IL-6 was able to generate a pro-inflammatory response (Romano et al., 1997). However, these complexes have been involved in down-regulation of C-X-C chemokines and up-regulation of C-C chemokines production by MeC thereby stopping PMN recruitment and facilitating mononuclear cell recruitment (Hurst et al., 2001). Furthermore, IL-6 production has been linked to production of anti-inflammatory molecules by Mϕ such as circulating IL-1 receptor antagonist (IL-1ra) and soluble TNF receptor p55, which neutralize their respective ligand (Tilg et al., 1994).

1.5.4 IL-10:

IL-10 is produced by Mϕ, lymphocytes and keratinocytes. It has an immunomodulatory role and can inhibit T_h1 responses, promote T_h2 responses, down-regulate expression of activating and co-stimulatory molecules, suppress Mϕ pro-inflammatory cytokine production (such as TNF-α and IL-1β)and inhibit chemokine production. IL-10 can downregulate IL-8 production and increase soluble TNF-α receptor and IL-1Ra and MCP-1 production by monocytes (Seitz et al., 1995). It is considered as an anti-inflammatory cytokine, thereby representing

the counter-balance for TNF-α and IFN-γ pro-inflammatory activities (Pestka et al., 2004; Renauld, 2003).

1.5.5 IL-12:

IL-12 is mainly produced by Mϕ, B and dendritic cells. It is an important immunoregulatory and pro-inflammatory cytokine, that links innate to adaptive immunity. It is essential for cell-mediated immunity (T_h1 differentiation), resistance to infections and tumours. Many of its pro-inflammatory functions are mediated by IL-12-induced IFN-γ production (Trinchieri et al., 2003).

1.6 Description of relevant chemokines:

The presence of inflammatory stimuli will induce secretion of cytokines that will lead to release of chemotactic cytokines that recruit leukocytes. These chemotactic cytokines or chemoattractants are small proteins that lead to leukocyte accumulation and are called chemokines. They are divided into four families (Gouwy et al., 2005; Luster, 1998; Mackay, 2001; Rossi and Zlotnik, 2000; Zlotnik and Yoshie, 2000):

1) C-X-C chemokines
2) C-C chemokines
3) C-X_3-C chemokine
4) C chemokine

1.6.1 C-X-C chemokines:

C-X-C chemokines are named as such because of the presence of a single amino acid between the two cysteine residues nearest to the N-terminal position. They are divided into ELR+ and ELR- because of the presence of a glutamate-leucine-arginine motif before the first two cysteines. ELR+ C-X-C chemokines attract PMN and are represented by IL-8 (CXCL8) and GRO-αin humans and byMIP-2 and KC in mice. IL-8 can be produced by monocytes, T cells, PMN, fibroblasts, endothelial cells and MeC. IL-8 is a potent PMN attractant but can also attract T cells. GRO-α is a specific and potent PMN chemoattractant. IL-8 and GRO-α acts through the receptors CXCR1 and CXCR2. There is no equivalent of IL-8 in mice. However, KC and MIP-2 are two homologues of human GRO-α. In murine cells, KC and the MIP-2 receptor, CXCR2, plays a major role in PMN recruitment (McColl and Clark-Lewis, 1999) and this receptor also mediates human IL-8 chemoattractant signal.

ELR- C-X-C chemokines attract mostly lymphocytes and are represented by IFN-γ-inducible protein (CXCL10), monokine induced by IFN- γ (CXCL9), IFN-inducible T cell α-chemoattractant (CXCL11) and stromal-derived factor (SDF)-1.

1.6.2 C-C chemokines:

In members of the C-C chemokine family, the two final cysteines are adjacent and these chemokines attract a broader range of cells. MCP-1 was the first C-C chemokine to be identified. It is a potent monocyte chemoattractant but can also attract PMN and T cells. MCP-1 (CCL2) interacts with CCR2 and this receptor is essential for the recruitment of monocytes to the peritoneum after BTG administration (Kuziel et al., 1997). MIP-1α is included in this family and was called macrophage inflammatory protein as it was identified from LPS-stimulated monocytes. MIP-1α attracts monocytes, but is less potent in this regard than MCP-1. MIP-1α also attracts PMN and T cells. Another important member of this family is RANTES which can attract monocytes, T cells and NK cells. The actions of both MIP-1α and RANTES are mediated by CCR1.

1.6.3 C-X_3-C chemokine:

The C-X_3-C chemokine family is characterised by the presence of three amino acids between the two cysteines and has only one member called fractalkine. Fractalkine is an integral membrane protein that can be cleaved in order to exert its chemoattractant effect on monocytes and T lymphocytes.

1.6.4 C chemokine:

The C chemokine family is named as such because of the presence of only cysteine in the N-terminal domain. So far, the chemokine lymphotactin is the only member of this family and this chemokine appears to be an exclusive T cell attractant.

The most important function of chemokines is chemoattraction. Chemokine secretion will create a chemokine gradient that will give recruitment directions to leukocytes. They also promote the integrin activation that is an essential requirement for firm leukocyte-endothelial interactions. Furthermore, chemokines are involved in degranulation and mediator release from attracted leukocytes. They also have a role in lymphoid organ development, metastasis, angiogenesis and angiostasis (Mackay, 2001; Rossi and Zlotnik, 2000). Chemokines are therefore crucial to the development of inflammation and their differential regulation will affect the nature of the inflammatory cells present at sites of inflammation. It is therefore important to determine and understanding the temporal sequence of chemokine secretion in order to fully appreciate the complexity of this cellular ballet.

1.7 Other inflammatory mediators:

Leukocytes can metabolise arachidonic acid (AA) into prostaglandins (PG), thromboxanes (Tx), leukotrienes (LT) and prostacyclins and all of these members of the eicosanoid family are key regulators of inflammation (Harizi and Gualde, 2005;

Stenson and Parker, 1980). The name is derived from the characteristic twenty carbon fatty acid derivatives, *eicosa* or twenty in Greek (Funk, 2001). PGs mediate several of the cardinal signs of inflammation including vasodilation, oedema and pain (Morley, 1974). Mϕ can produce all the AA metabolites upon different stimuli (Stenson and Parker, 1980) and represent a major source of PGs (Ferraris and DeRubertis, 1974). Vascular permeability has usually been associated with mast cells, but recent data suggest that Mϕ-derived LT may represent important mediators of vasopermeability (Kolaczkowska, 2002; Kolaczkowska et al., 2002). LTB4 has been shown to be present in pleural effusions and has important chemotactic properties for PMN. Pleural Mϕ appear to be an important source of LTB4 (Pace et al., 2004). LTB4 also appears to play a role in peritonitis but exerts more marked effects upon eosinophils compared to other leukocytes (Tager et al., 2000).

Although the complement system was initially considered as an additional weapon or 'complement' to humoral immunity, it now clear that it is also associated with cellular immunity. Complement can enhance innate immunity by promoting phagocytosis of apoptotic cells (Mevorach et al., 1998) and bacteria (Brown et al., 1983; Brown et al., 1982) as well as bridging innate to adaptive immunity and regulating the adaptive arm by enhancing B and T cells responses (reviewed in (Carroll, 2004)). Complement production is mostly hepatic and is upregulated by IL-6 as part of the acute phase response. Some of the early components, such as C1q, are also produced locally by Mϕ (Loos et al., 1980; Rabs et al., 1986). Peritoneal resident and BTG-stimulated Mϕ have been showed to increase their production of C1q following IL-6 stimulation whereas IL-1 and IFN-γ inhibited C1q production

(Faust and Loos, 2002). Production of the initial members (C1, C2, C3 and C4) of the complement system by cells of the innate immune system is crucial to the formation of the membrane attack complex. An additional role includes the opsonisation of both bacteria and apoptotic cells as well as enhancement of adhesion molecule expression on the endothelial cell surface (Kishore et al., 2004; Kishore and Reid, 2000). Furthermore, complement also plays a crucial role in inflammation *per se*. For example, the tissue damage and inflammation induced by the reperfusion phase following ischemia is complement-dependent (Barrington et al., 2001). The activation of C3 and C5, which are key members of the complement pathway, liberates the fragments C3a (Bokisch et al., 1969) and C5a (Kay et al., 1973) that are also called anaphylotoxins as a result of their important pro-inflammatory functions. Mϕ are able to generate these two fragments (Huber-Lang et al., 2002; Taylor-Robinson et al., 1978). The production of anaphylotoxins is common to all three activation pathways of complement and underscores their importance in the inflammatory reaction. They play an important role in the initial PMN (Snyderman et al., 1971) and monocyte recruitment (Zwirner et al., 1998), modulate cytokine/chemokine release from Mϕ (Goodman, 1984; Guo and Ward, 2005) and exert immunomodulatory effects (Chenoweth et al., 1982) by ligation to their respective surface receptors, C3aR and C5aR (Ames et al., 1996; Gerard and Gerard, 1991; Kildsgaard et al., 2000). An increased expression of the anaphylotoxin receptor C5aR was found at the surface of peritoneal resident Mϕ and PMN compared to the surface of BTG-recruited Mϕ (Chenoweth et al., 1982). This could demonstrate the importance of 'C5a awareness' in the gate-keeping leukocytes such

as resident Mϕ in the initiation of inflammation secondary to anaphylotoxin production.

Thus these two classes of inflammatory mediators, eicosanoids and complement proteins, can play an adjuvant role to cytokines and chemokines in order to promote an adequate inflammatory response to any type of tissue insult.

1.8 Inflammation Models:

The models described in these studies were used as tools to assess the 'gate-keeper' functions of serosal resident leukocytes. Although the models of thioglycollate and zymosan peritonitis have been extensively used to study inflammatory processes, such models may not accurately reflect the clinical situation in patients with infection or inflammation of the peritoneal space. For example, in the clinical context, patients undergoing chronic ambulatory peritoneal dialysis develop peritonitis following the exogenous introduction of bacteria or yeast. It is therefore apparent that these experimental models do have significant limitations such that the findings must be interpreted with some caution in extrapolating to the clinical situation. Despite this, it is undoubtedly the case that these experimental models have provided great insights into the biology of peritoneal inflammation.

A similar criticism of the model of carrageenan pleurisy may be made as clinical pleural inflammation is generally seen in association with pulmonary infection. However, clinical situations do arise where intra-thoracic or intra-mediastinal tubes

are inserted e.g. following a pneumothorax or cardiac surgery and in such circumstances pleural inflammation can originate from a defect in the thoracic wall without a primary lung lesion. Therefore, the administration of exogenous *Staphylococcus aureus* into the pleural space does recapitulate some aspects of such a situation.

1.8.1 Brewer's thioglycollate peritonitis:

Brewer's thioglycollate (BTG) peritonitis is frequently used as a model of leukocyte recruitment. It has been used for over 40 years as a source of recruited Mϕ (Gallily and Feldman, 1967; Gallily et al., 1964) and for over 20 years as a source of recruited neutrophils (PMN) (Baron and Proctor, 1982). It is a resolving model and is characterised by an initial influx of PMN peaking at 6-12h, which resolves by 24-36h (Baron and Proctor, 1982). Subsequently, monocytes are recruited by 24h and the inflammation has subsided by day 7-10 (Melnicoff et al., 1989). The exact nature of the innate inflammatory stimulus in this model is unknown. It has been described as 'non-specific' (Li et al., 1997) and to involve complement-independent pathways (White et al., 2002). The fact that thermically aged BTG induces an increased leukocyte recruitment compared to fresh BTG led Li *et al* to suggest that the accumulation of advanced glycation end-products (AGEs) induced by autoclaving might mediate the inflammatory stimulus (Li et al., 1997). Therefore, the inflammation may be mediated through the AGE receptor, RAGE (Chavakis et al., 2003). In this study, however, Chavakis *et al* looked at RAGE-Mac-1 interactions and not at the induction of inflammation. It is therefore difficult to be sure whether

RAGE is involved in peritonitis induction by BTG. It is generally accepted as an 'LPS-like' model of inflammation without exhibiting full Mϕ activation or the full bactericidal potential of the Mϕ (Eichner and Smeaton, 1983; Leijh et al., 1984).

1.8.2 Zymosan peritonitis:

Zymosan is a derivative of the fungal cell wall from *Saccharomyces cerevisiae*. As with BTG, this model is a resolving model and is characterised by an initial PMN influx that is followed by monocyte recruitment. The pro-inflammatory signals can be mediated directly to the Mϕ through binding of zymosan to TLR-2 (Sato et al., 2003) or indirectly by secretion of various pro-inflammatory mediators (recently reviewed in (Volman et al., 2005)). The phagocytic receptor dectin-1 has been shown to interact with TLR-2 in response to zymosan (Gantner et al., 2003). Resident Mϕ, along with MC, have been reported to play an important role in inducing PMN recruitment in a zymosan peritonitis model through TNF-α, IL-1β and IL-8 secretion (Ribeiro et al., 2000). However, as for the BTG model, there are still unresolved issues (Ajuebor et al., 1999).

1.8.3 Carrageenan pleurisy:

Carrageenan is an Irish sea-moss and induces complement dependent inflammation. Carrageenan-induced pleurisy is a well-established model of acute inflammation (Murai et al., 2003) and is characterized by a rapid influx of neutrophils (PMN) followed by mononuclear cell infiltration (Ackerman et al., 1980; Harada et al.,

1996). This model is often used to assess the anti-inflammatory effects of pharmaceutical agents that will eventually be used clinically (Cuzzocrea et al., 1999a; Cuzzocrea et al., 2000b; Cuzzocrea et al., 2004; Frode-Saleh and Calixto, 2000; Salvemini et al., 2001) and to assess the *in vivo* importance of established inflammatory mediators (Cuzzocrea et al., 2000a; Gilroy et al., 1999; Gilroy et al., 2004). Although the neutrophil influx evident in this model is generally used as an experimental readout of acute inflammation, there are data indicating that neutrophils are involved in the release of injurious enzymes and modulation of vascular permeability in carrageenan-mediated pleural inflammation (Dalmarco et al., 2002; Saito et al., 2002). Carrageenan has been referred to as a 'Mϕ-depleting agent' but, in the only specific paper on the subject, the authors were using ι-carrageenan (Ishizaka et al., 1989). However, despite one paper showing reduced splenic Mϕ after i.p. λ-carrageenan treatment (Goldmann et al., 2004), the evidence only implies functional modifications and no alteration in macrophage viability following λ-carrageenan administration (Vijayakumar and Muthukkaruppan, 1990; Vijayakumar et al., 1989).

1.8.4 Formalin Fixed *Staphylococcus aureus* pleurisy:

Staphylococcus aureus (S. aureus) is a Gram positive coccus that may cause severe infections (Caksen et al., 2000). The inflammatory reaction is mediated by peptidoglycan and lipoteichoic acid from the bacterial cell wall. The immune response against *S. aureus* leading to cytokine production is TLR-2- and MyD88-dependent (Takeuchi et al., 2000; Takeuchi et al., 1999; Texereau et al., 2005). *S.*

aureus stimulates TLR-2, which induces Rac1 GTPase activation that leads to nuclear factor (NF)-κB transactivation (Arbibe et al., 2000). Recent data indicate that CD36 mediates phagocytosis of *S. aureus* and facilitates direct interactions with TLR-2 and -6 in order to induce NF-κB activation and this is similar to the interaction of CD14-TLR-4 with LPS (Stuart et al., 2005). On the other hand, *S. aureus* is able to secrete a chemotaxis inhibitory protein that is able to blunt the leukocyte response to pro-inflammatory compounds secreted by *S.aureus* (de Haas et al., 2004).

1.9 The Aims of this Study

This thesis will evaluate the role of the resident Mϕ in both peritoneal and pleural cavities in orchestrating PMN recruitment and look into the cytokine/chemokine network involved in this process. These studies will be accomplished using a recently described conditional Mϕ ablation murine model. The contribution of lymphocytes in early leukocyte recruitment will be also looked at with the use of mice exhibiting various deficiencies in lymphocyte populations. This work was designed to address the following hypotheses:

1) The resident peritoneal and pleural Mϕ are key sentinel cells and orchestrate the initiation of inflammation by directing the generation of chemokines.

2) Resident peritoneal lymphocytes are capable of modulating innate immune responses.

In particular the questions addressed by the work include:

1) Are resident Mϕ responsible for the initiation of inflammation in peritonitis and pleurisy?

2) What are the key cytokines and chemokines involved in this process?

3) Can the in vivo cellular network between leukocytes initiated by Mϕ be studied *in vitro*?

4) How do the resident lymphocytes modulate leukocyte recruitment in peritonitis? In this regard I hypothesise that the lymphocytes are modulating innate immune processes.

5) What are the chemokines involved in lymphocyte-mediated modulation of leukocyte recruitment?

6) Can the modulating function of peritoneal resident lymphocytes be evaluated *in vitro*?

Chapter 2: Methods

2.1 Experimental animals:

The following experimental animals were used in these studies:

1) Homozygous CD11b-DTR mice on the FvB/N background were bred at the University of Edinburgh. In experiments involving homozygous CD11b-DTR mice, either FvB/N wild-type (WT) (Harland, UK) treated with DT or CD11b-DTR mice treated with vehicle served as controls.

2) RAG-1 KO mice were obtained from Charles River UK and exhibit a deficiency of mature B and T cells. The RAG-1 KO mice were on the C57BL/10 background and C57BL/10 mice (Charles River UK) were used as controls.

3) NUDE mice were obtained from Charles River UK and exhibit a deficiency in T cells. NUDE mice were on the CD1 background and CD1 mice (Charles River, UK) were used as controls.

4) μMT mice were a kind gift of Prof D. Gray (University of Edinburgh) and exhibit a deficiency in B cells. The μMT mice were on the C57BL/6 background and C57BL/6 mice (bred in-house at the University of Edinburgh) were used as controls.

2.2 Conditional Macrophage ablation:

Resident peritoneal and pleural Mϕ were ablated in homozygous CD11b-DTR mice by intraperitoneal (IP) injection of DT (25ng/g body weight) 24 h prior to

the administration of pro-inflammatory agents (see below). In the majority of experiments DT-treated FvB/N WT mice served as controls.

2.3 Induction of experimental peritonitis:

The well-established models of Brewer's thioglycollate or zymosan peritonitis were used to induce peritoneal inflammation. Mice were injected IP with 1 ml of 3% Brewer's thioglycollate (BTG) (DIFCO, Detroit, MI) or 0.2mg of zymosan (Sigma, St. Louis, MO) and underwent peritoneal lavage at various time points following the initiation of inflammation. Peritonea were lavaged at different time points with 5ml of cold PBS (Sigma, Dorset, UK). The undertaking of a peritoneal lavage is an invasive procedure and so called 'bloody lavages' were occasionally encountered. Peritoneal lavages were excluded from subsequent analysis if they exhibited gross contamination with blood that was evident on macroscopic inspection as circulating blood cells could affect the assessment of the recruited leukocyte number. Furthermore, serum or plasma may also interfere with the analysis of peritoneal cytokines or chemokines. Three peritoneal lavages were excluded in experiments involving BTG peritonitis after macrophage ablation. In addition, one lavage derived from a RAG-1 KO mouse, two lavages from CD1 mice and one lavage derived from a NUDE mouse were excluded.

2.4 Induction of experimental pleurisy:

The agents used to induce pleural inflammation included 1% carrageenan (a kind gift from Marine Colloids Inc, Philadelphia, USA) and formalin-fixed fluorescently labelled *Staphylococcus aureus* (Sigma, Dorset, UK). Mice were anaesthetised with halothane and a small incision was made between the 6th and 8th intercostal muscles. The pro-inflammatory agent was then injected intra-pleurally (IPl) with a blunted 21G needle and the wound closed with a Michel clip. Animals were subsequently killed by a rising concentration of CO_2 at various time points after pleurisy induction in order to prevent bleeding in the pleural cavity.

Mice underwent pleural lavage at various time points following inflammatory agent injection. Pleural lavage was performed lavaging the pleural cavity with 1 ml of 3.15% (weight/volume) sodium citrate (Sigma, Dorset, UK) in saline. Macroscopically 'bloody' pleural lavages were excluded for reasons similar to those mentioned in the previous section. Three pleural lavages were excluded in the carrageenan induced pleurisy time course for this reason.

2.5 Cell processing and flow cytometry analysis:

For flow cytometric analysis, peritoneal or pleural cells were incubated for 30 minutes in PBS containing 10% mouse serum. Conjugated antibodies at concentrations suggested by the supplier were then added and the mixture incubated

at $4^{o}C$ for 30 minutes. The cells were then washed before undergoing flow cytometry analysis. Circulating numbers of leukocytes were also determined from samples of whole blood. 25µl of whole blood was obtained by tail vein bleed into 3.9% sodium citrate. The blood was aliquoted into flow cytometry tubes, blocked with PBS containing 10% mouse serum and stained as for cells retrieved by pleural or peritoneal lavage. 2 ml of FACSLysis buffer (BD, UK) was then added to lyse erythrocytes and samples were spun and processed by flow cytometry. The following antibodies were used: anti-CD11b fluorescein isothiocyanate (FITC), anti-GR1 phycoerythrin (PE) and anti-c-kit PE (all from eBiosciences, UK), anti-B220 (mouse CD45R) PE and FITC and mouse anti-CD3 PE (both from Pharmingen, San Diego, CA) and F4/80 allophycocyanin (APC) and F4/80 PE (both from Caltag, UK). A known amount of fluorescent Flow-check fluorospheres (Beckman and Coulter, USA) was added to each sample prior to analysis and the ratio of cells to beads used to calculate the absolute number of any cell type in experimental fluid. Analyses were performed using a FACScan or FACScalibur instrument and analyzed using the FlowJo software (Treestar, Oregon, USA). Comparisons were made between different mice at each time point.

Flow cytometric assessment of leukocyte numbers was performed as it is an objective and rapid technique for counting cells (Backteman et al., 2002; Bono et al., 1999; Burgess and Davison, 1999; Deneys et al., 1994; Derer et al., 1983) compared to the analysis of more traditional cytospin smears where only a relatively small number of cells can be counted. It has been previously used in the literature to assess leukocyte number in inflammation (Henderson et al., 2003). Also, the use of flow

cytometry has been compared to analysis of cytospin smears to assess cell numbers and similar results were reported (Schweppe et al., 1992; van Zaanen et al., 1995) thereby suggesting that both techniques are valid. However, flow cytometry was better than cytospin smears if leukocyte numbers were low (van der Meer et al., 2001). Flow cytometry also has its limitations as antibody specificities and reactivities can be variable and the assessment of cell number is dependent upon the positioning of the gates. For this reason, I used the same gates for each time points in order to minimise variation due to this potentially confounding issue. Cytospin smears are also subject to technical difficulties such as distortion of cell morphology secondary to centrifugation that can prevent adequate cell identification. Flow cytometry plots of typical time course peritonitis patterns are depicted in Figure 2.1.

2.6 Adoptive transfer of resident peritoneal and pleural cells:

Cells derived from the lavage of serosal cavities of groups of naïve FvB/N control mice were pooled, centrifuged and either underwent positive or negative selection for macrophages. Peritoneal lavage samples from groups of FvB/N control mice were pooled, spun and either plated on tissue culture plastic for 90 minutes in order to deplete Mϕ by adhesion (Mϕ-depleted peritoneal cells) or resuspended in sterile PBS (Mϕ-rich peritoneal cells). Mϕ depletion by adhesion resulted in removal of 97±2.8% of Mϕ from the cell suspension. The Mϕ-depleted peritoneal cell population or the Mϕ-rich peritoneal cell population were subsequently adoptively transferred to the peritonea of mice 4 hr prior to the administration of BTG.

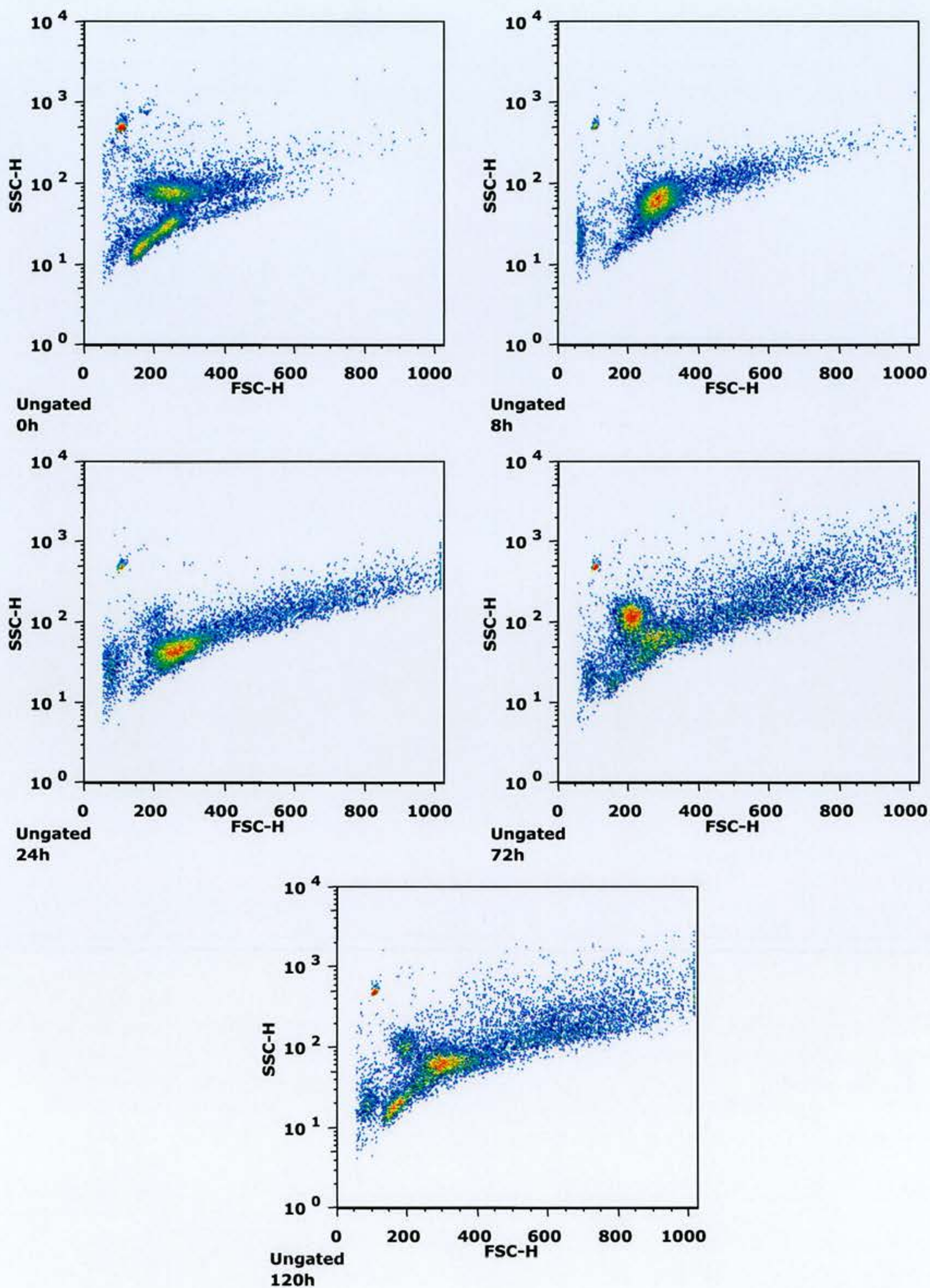


Figure 2.1- Representative flow cytometry plots of typical time course in peritonitis.

1 ml of 3% BTG was administered in C57BL/6 mice and peritoneal lavages performed at different time points.

Pleural lavages from groups of naive FvB/N control mice were resuspended in PBS with 0.5% mouse serum and 2mM ethylenediaminetetraacetic acid. Cells were then incubated for 10 minutes with PE-conjugated anti-F4/80 antibody to label Mϕ. Cells were then washed and spun at 350G for 10 minutes. The PE-conjugated anti-F4/80 labeled Mϕ were then incubated for 15 minutes with anti-PE conjugated MACS magnetic beads (Miltenyi Biotech Ltd, UK). Cells were then washed, spun for 10 minutes and Mϕ subsequently removed by passing the cells over a magnetic MACS column. As a control, pleural cells were incubated with an isotype control antibody and then processed exactly as for F4/80 labeled cells. This method of Mϕ depletion removed 98.2±0.7% Mϕ. This Mϕ-depleting strategy was also used once with peritoneal cells and yielded similar results to the Mϕ depletion by adhesion method. In addition, resident pleural Mϕ were purified by negative selection following incubation of pleural cells with anti-B220, anti-c-kit and anti-CD3 antibodies (all antibodies were PE conjugated) for 15 minutes in order to label B cell, mast cells and T cells. This was followed by incubation with anti-PE conjugated MACS magnetic beads and passage through the magnetic MACS column. The Mϕ purified by negative selection were >90% pure as assessed by F4/80 labelling. In experiments studying the effects of the reconstitution of Mϕ ablated mice by the adoptive transfer of non-transgenic FvB/N Mϕ, the purified Mϕ (purified by negative selection) as well as the Mϕ-depleted and Mϕ rich pleural cell populations were resuspended in 1% carrageenan and administered into the pleural cavity of each mouse.

Approximately 20% of the initial pleural resident Mϕ are lost following the passage of resident pleural cell populations over the MACS column irrespective of whether the cells had been stained with antibodies and this presumably reflects non-specific cell loss. The expression of the Mϕ marker F4/80 was compared between Mϕ that had been through the column to Mϕ that have been bound to the column and later eluted. F4/80 expression was comparable between these two groups (Figure 2.2). I did not undertake more extensive phenotyping.

2.7 Adoptive transfer of T cells into NUDE mice:

Resident peritoneal T cells were purified by negative selection. Peritoneal lavage cells from CD1 mice were pooled and the cells were incubated for 15 minutes with anti-B220, anti-c-kit and anti-F4/80 antibodies (all antibodies were PE conjugated) in order to label B cell, mast cells and Mϕ. This was followed by incubation with anti-PE conjugated MACS magnetic beads and passage through the magnetic MACS column. Approximately 50,000 T cells (a purity of 22% was obtained) were then transferred into NUDE mice at the same time as the administration of BTG and peritoneal lavages were performed 8h afterwards. Approximately 150,000 contaminating Mϕ were also transferred at the same time as T cells.

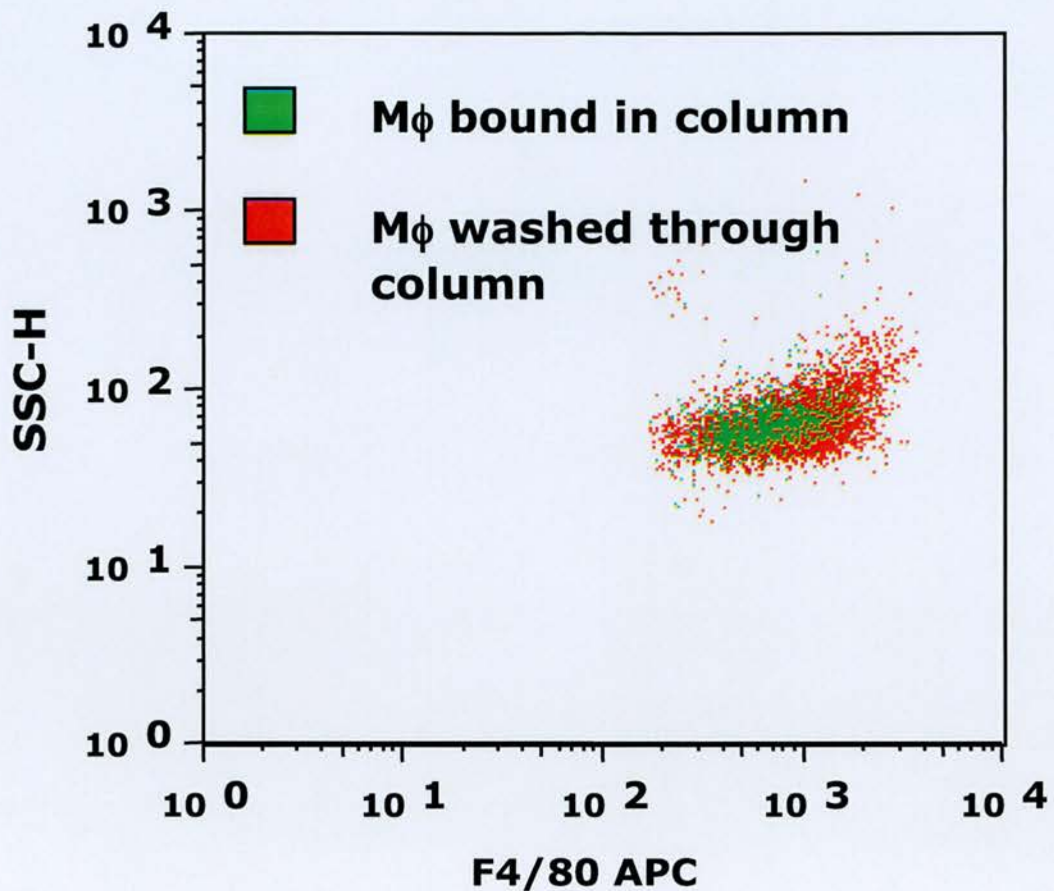


Figure 2.2- F4/80 expression of pleural resident Mϕ after passage through the magnetic column.
Pleural resident cells were incubated with a PE-conjugated isotype control antibody followed by incubation with anti-PE conjugated magnetic beads. Cells were then passed over a magnetic column with cells that bound to the column being subsequently eluted. Macrophages that either passed through or were bound in the column were then stained with F4/80 APC in order to analyse F4/80 expression.

2.8 Supernatant transfer studies:

NUDE and CD1 mice were injected with BTG and peritoneal lavage was performed after 3h with 3ml of PBS in order to reduce the dilution of chemoattractants. The peritoneal lavage fluid was centrifuged (1000G for 15 minutes) to remove cells and debris. The peritoneal supernatant fluid was then injected IP into NUDE and CD1 mice in a cross-over fashion in order to evaluate the capacity of the various mediators within the supernatant fluid to induce PMN recruitment. Peritoneal lavage and tail vein blood sample was performed 8h following the injection of peritoneal supernatant fluid and cells were stained for the PMN marker GR1 and counted by flow cytometry as previously described.

2.9 *In Vitro and In Vivo* Chemokine and Cytokine Studies:

Mice underwent peritoneal or pleural lavage at various time points following the administration of BTG or carrageenan in order to determine the levels of chemokines and cytokines. Lavage fluid supernatant was centrifuged and stored at -80°C until analysed by specific ELISA for MIP-2, KC, MIP-1α, MCP-1 and TNF-α, TGF-β (R&D, UK). The concentrations of IL–6, IL-10, IL12p70, IFN-γ and MCP-1 in lavage fluid or cell culture supernatant were determined using a Cytometric Bead Array (CBA) (BD Biosciences, UK). Cytokine-specific-antibody-coated beads were incubated with a fixed volume of experimental lavage fluid and followed by addition of the PE detection reagent. Samples were then washed and analysed by flow

cytometry to determine the cytokine concentrations according to the shift in fluorescence generated by the beads.

In order to dissect the role of various cells present in the peritoneum or pleural space in the generation of pro-inflammatory mediators, peritoneal cell populations that had been depleted of either Mϕ, MC, B cells or T cells was stimulated *in vitro*. Peritoneal cells were incubated for 15 minutes with either PE-conjugated anti-F4/80, PE-conjugated c-kit (CD117)(Pharmingen, UK) or an antibody to B-220 or CD3 in order to label Mϕ, MC, B or T cells respectively. Peritoneal cells were then incubated with anti-PE conjugated MACS magnetic beads and the Mϕ, MC, B or T cells removed by passing the cells over a magnetic MACS column (Miltenyi Biotech Ltd, UK). As a control, total peritoneal cells were incubated with an isotype control antibody followed by magnetic beads and subsequently passed over the magnetic MACS column. This method resulted in >97% depletion of the cell type of interest. Figure 2.3 illustrates flow cytometry analysis of the peritoneal cell populations obtained after magnetic immunodepletion. Pleural cells were only incubated with an anti-F4/80 antibody in order to deplete Mϕ and this depleted over 98% of Mϕ. Chemokine and cytokine production by intact control peritoneal/pleural cell populations or peritoneal/pleural cell populations depleted of Mϕ, MC or lymphocytes and subsequently stimulated with BTG or carrageenan *in vitro* was determined. In control experiments, cells were exposed to medium alone. Control peritoneal cells and peritoneal cells depleted of particular

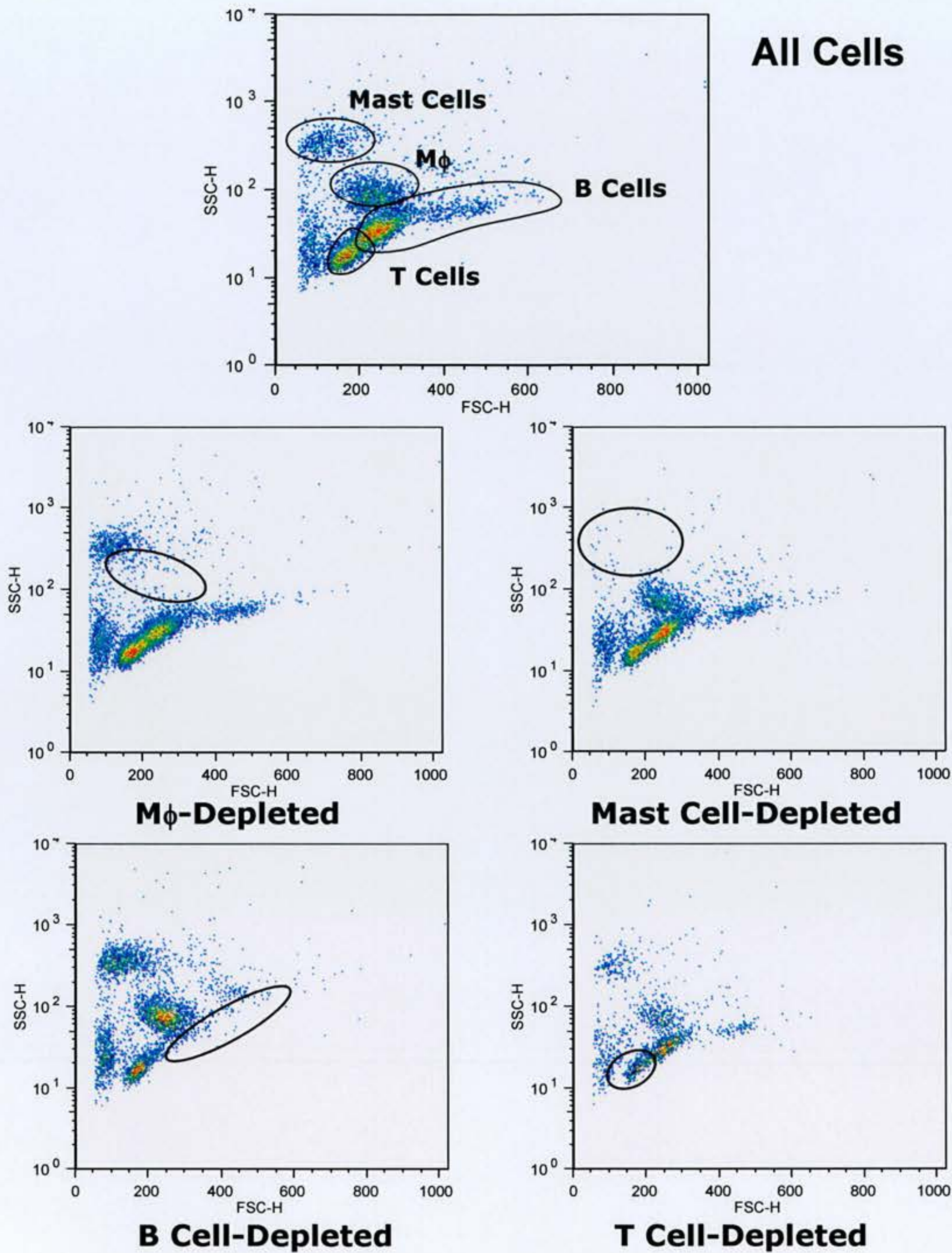


Figure 2.3- Peritoneal cells were selectively immunodepleted.
Peritoneal cells were depleted of either Mϕ, MC, B or T cells by incubation with PE-conjugated anti-F4/80, anti-c-kit, anti-B220 and anti-CD3 respectively followed by incubation with anti-PE conjugated magnetic beads and passage over a magnetic column (>97% depletion of cells of interest).

cell types were then plated in 48 well plates ($5x10^5$ cells per well) and exposed to 1% BTG or LPS (10ng/ml)(Sigma, Dorset, UK) for 3h and 6h. Control pleural cells and Mϕ-depleted pleural cells were plated in 48 well plates ($6x10^5$ F4/80 negative cells per well) and exposed to 0.25% carrageenan for 6h. Cell culture supernatant was centrifuged and stored at -80°C until analysed by specific ELISA for MIP-2, KC, MIP-1α, MCP-1 and TNF-α, TGB-β (R&D, UK). The concentrations of IL–6, IL-10, IL12p70, IFN-γ and MCP-1 in cell culture supernatant were determined using a Cytometric Bead Array (CBA) (BD Biosciences, UK). Cytokine-specific-antibody-coated beads were incubated with a fixed volume of experimental lavage fluid or cell culture supernatant and followed by addition of the PE detection reagent. Samples were then washed and analysed by flow cytometry to determine the cytokine concentrations according to the shift in fluorescence generated by the beads.

Intracellular TGF-β staining was performed on peritoneal cells in selected experiments. Peritoneal cells were fixed and permeabilised in paraformaldehyde 3%/sucrose 5%, washed and then preincubated with 5% bovine serum albumin in PBS for 1 hr. After washing, the cells were stained with either an anti-TGF-β antibody (R&D, UK) or a chicken Ig isotype control (R&D, UK) overnight. Cells were then washed and stained with an Alexa-633-conjugated anti-chicken antibody (Molecular Probes, Netherlands) and F4/80 PE for 1 hr. After washing, cells were processed by flow cytometry.

2.10 Statistical Analysis

Experimental data were analysed using a one-way ANOVA with Bonferroni multiple comparison *post hoc* test with a 95% confidence interval or a Student's T-test as appropriate. Statistical analysis including correlation analysis was performed using graph pad prism software. The significance level was set at $p < 0.05$. Data are presented as mean ± standard error from the mean (SEM). Values that were outside 2 standard deviations were suspected as 'outliers'. Such outliers may results from infrequent experimental problems such as unsuccessful peritoneal injections where the needle inadvertently hits the bowel such that the injected substance does not enter the peritoneal space. In order to confirm the true nature of 'outliers' the extreme studentized deviate (ESD) test or Grubb's test was performed on the data set in question. If the 'outliers' was identified as an outlier by a p value of less than 0.05, it was then excluded from subsequent analysis (Barnet and Lewis, 1998).

Chapter 3: The role of resident peritoneal macrophages in experimental inflammation

3.1 Introduction

MeC are important cells in peritoneal inflammation. However, many studies have used cytokines typically derived from Mϕ in order to stimulate MeC chemokine production (Robson et al., 2001; Topley et al., 1993a; Topley et al., 1993b). Therefore, it is likely that the resident peritoneal Mϕ may play an important role in the initiation of inflammation. Although, previous studies have examined the role of peritoneal Mϕ in the initiation of peritoneal inflammation, the generated data differed substantially between studies such that there was no consensus regarding the exact involvement of the peritoneal Mϕ. For example, previous work suggested that peritoneal Mϕ could either play a key role (Ajuebor et al., 1999; Knudsen et al., 2002; Souza et al., 1988), an inhibitory role (Ajuebor et al., 1999) or no role at all (Ajuebor et al., 1999) depending on the experimental model used. The CD11b-DTR mice developed by Richard Lang's group (Cailhier et al., 2005) provided an excellent resource to dissect the role of the resident peritoneal Mϕ in peritoneal inflammation and resolve these issues. Mϕ ablation is a useful technique in itself but also allows Mϕ repletion studies that can further demonstrate Mϕ function in inflammation. A complex 'cytokine network' (Topley et al., 1993c) is present in the peritoneal cavity and it is still incompletely understood. In this chapter I describe the results of a combination of *in vivo* and *in vitro* analysis of cytokine and chemokine production that facilitates the identification of the cells involved in

cytokine/chemokine production as well as the kinetics of secretion of cytokines/chemokines during peritoneal inflammation.

3.2 Results

3.2.1 The administration of DT to CD11b-DTR mice ablates transgenic Mϕ *in vivo.*

Previous work from Richard Lang's laboratory indicated that the administration of DT at a dose of 25 ng/g mouse body weight effectively ablated Mϕ systemically (Richard Lang, personal communication). Thus, flow cytometric analysis of peritoneal cells was performed 24 h following the injection of DT (25 ng/g mouse body weight). CD11b-DTR transgenic mice exhibited almost complete ablation of F4/80 positive peritoneal Mϕ (98.4%±0.9 Mϕ ablation, $p<0.00001$) after a single dose of DT with Mϕ numbers being compared to those evident in the peritoneal cavity of non-manipulated control CD11b-DTR mice (Figure 3.1). A dose titration was then performed with the administration of DT doses of 6.25 and 12.5 ng/g mouse weight and this resulted in Mϕ ablation of approximately 72% and 82% respectively whilst lower doses resulted in <50% ablation. 25ng/g body weight was therefore used for the *in vivo* studies as it induced marked Mϕ ablation.

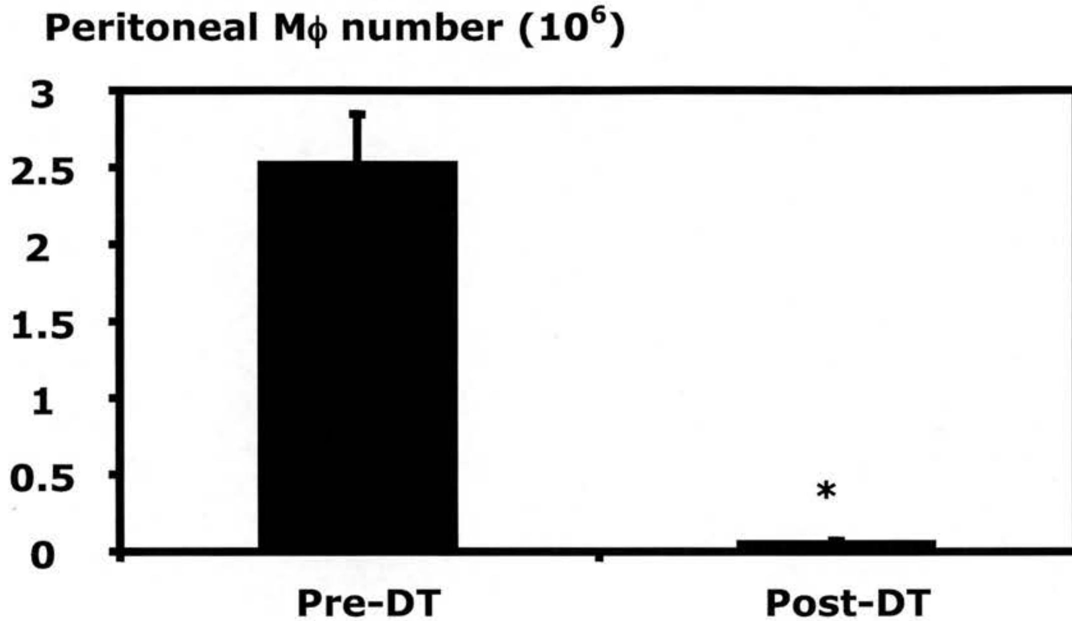


Figure 3.1 - The administration of DT ablates resident peritoneal Mϕ.
DTR mice underwent peritoneal lavage in order to establish the number of Mϕ present under normal non-manipulated conditions. Histogram of peritoneal Mϕ number in CD11b-DTR 24h after DT administration (25ng/g body weight) compared to the Mϕ number in the normal peritoneum (*=$p<0.00001$ vs pre-DT; n=7 per group).

3.2.2 Specificity of Mϕ elimination *in vivo*.

The CD11b-DTR model was generated to allow the selective ablation of CD11b+ cells especially monocytes and Mϕ. In order to investigate the specificity of cell ablation, CD11b-DTR mice were treated with either DT or PBS. Peritoneal lavage was performed and blood was obtained 24h after treatment with DT (25ng/g body weight) for cell count analysis. This demonstrated that, in addition to potent Mϕ ablation, DT administration significantly reduced the number of B cells and mast cells within the peritoneal cavity (Figure 3.2A). However, the numbers of peritoneal T cells were unaffected. DT administration did result in an increased number of PMN in the peritoneal cavity. This may be a consequence of the inevitable secondary necrosis of Mϕ that will occur under conditions promoting the widespread apoptosis of the professional resident phagocyte although some dying Mϕ may be cleared by 'amateur' phagocytes (Kurosaka et al., 2001; Misawa et al., 2001) as the resultant release of intracellular contents would be predicted to be pro-inflammatory and recruit PMN. Interestingly, review of the kinetics of pleural Mϕ ablation indicate that Mϕ depletion is almost complete 6h after DT administration at which time no significant difference in the number of B lymphocytes or MC was evident (for B cells: $2.8x10^5 \pm 0.7x10^5$ vs $4.6X10^5 \pm 0.2x10^5$; for MC: $1.10x10^4 \pm 0.09x10^4$ vs $1.4x10^4 \pm 0.25x10^4$; DT treatment vs PBS; $p>0.05$, n=4 per group). Although a significant subset of B lymphocytes and MC may express CD11b (Chevallier et al., 1998; Howell et al., 2002; Rosenkranz et al., 1998) this suggests that the loss of B cells and mast cells is not entirely secondary to transgene expression by these cell

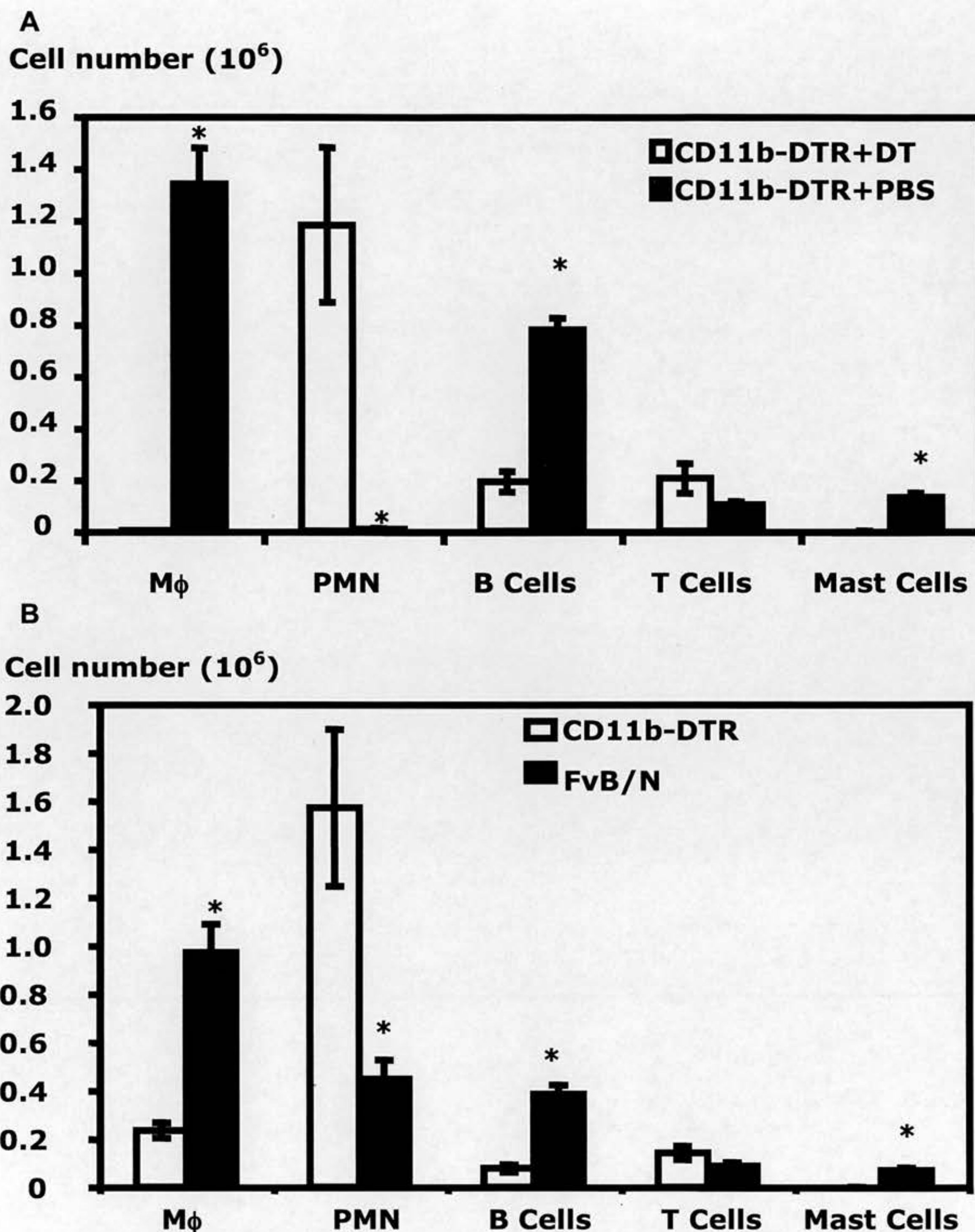


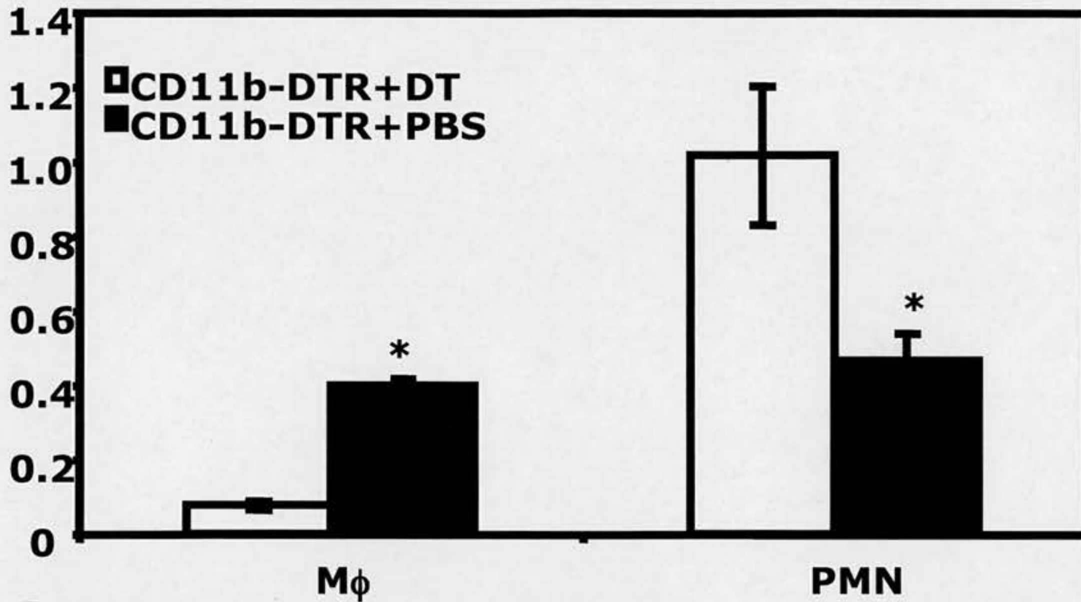
Figure 3.2 - Specificity of DT induced cell death: DT induces Mϕ ablation but also reduces B and mast cell numbers in the peritoneum.
A) DT or PBS was administered IP to CD11b-DTR mice and peritoneal lavage was performed 24h later. The number of Mϕ, PMN, B, T and mast cells was determined by flow cytometry (*=$p<0.01$ vs CD11b-DTR+DT; n=6). B) DT was administered IP to CD11b-DTR or FvB/N mice and peritoneal lavage was performed 24h later (*=$p<0.01$ vs CD11b-DTR; n=6 per group).

types but is mainly a consequence of the secondary necrosis of apoptotic Mϕ that occurs in the absence of a population of viable phagocytic Mϕ to ingest the dying cells.

To further address the specificity of the conditional ablation system, the differential effect of DT in CD11b-DTR and non-transgenic control FvB/N mice was examined. There was no difference in the baseline number of peritoneal Mϕ between CD11b-DTR and FvB/N control mice although CD11b-DTR mice exhibited an increased number of B and T cells. The administration of DT to CD11b-DTR mice resulted in a marked reduction in peritoneal Mϕ together with reduced numbers of B cells and mast cells (Figure 3.2B) compared to FvB/N mice. In addition, a mild influx of PMN was evident but there was no difference in T cell numbers (Figure 3.2B).

3.2.3 Circulating and recruited PMN are unaffected by DT administration.

Since CD11b is also expressed on granulocytes, I performed experiments to evaluate whether PMN were sensitive to DT. Flow cytometric analysis of whole blood performed 24 hours following DT administration indicated a significant 80% reduction in circulating monocyte numbers (Figure 3.3A). However, no reduction in the number of circulating PMNs was evident 24hr following DT administration (Figure 3.3A). In fact, an increased number of circulating PMN were evident at the 24 hr time point post DT. In addition, no reduction in circulating PMN number was



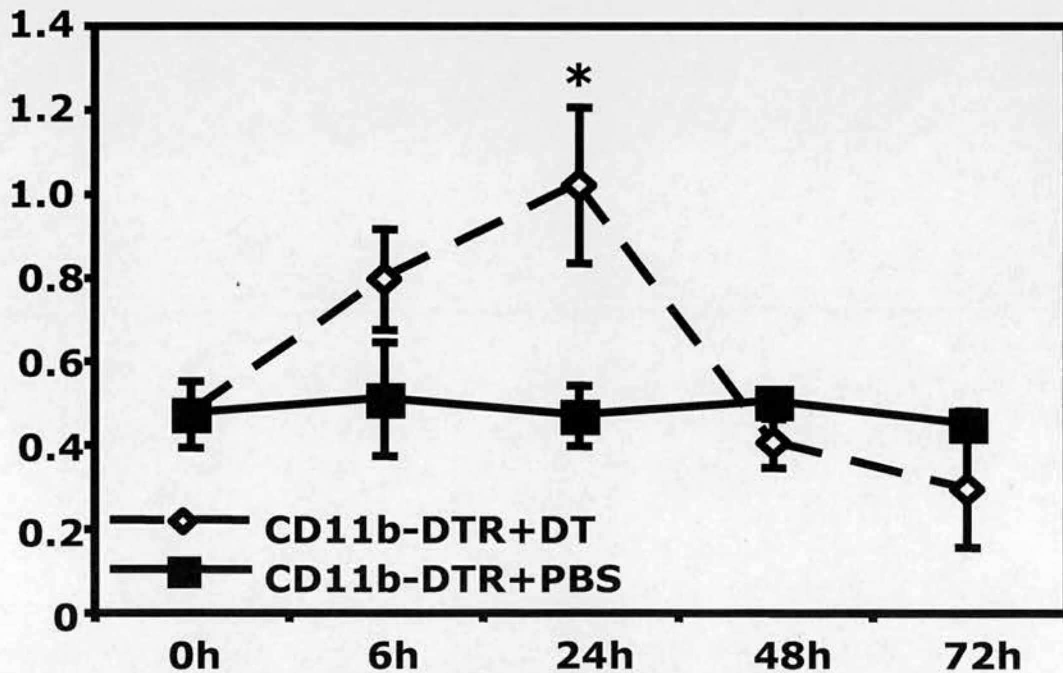


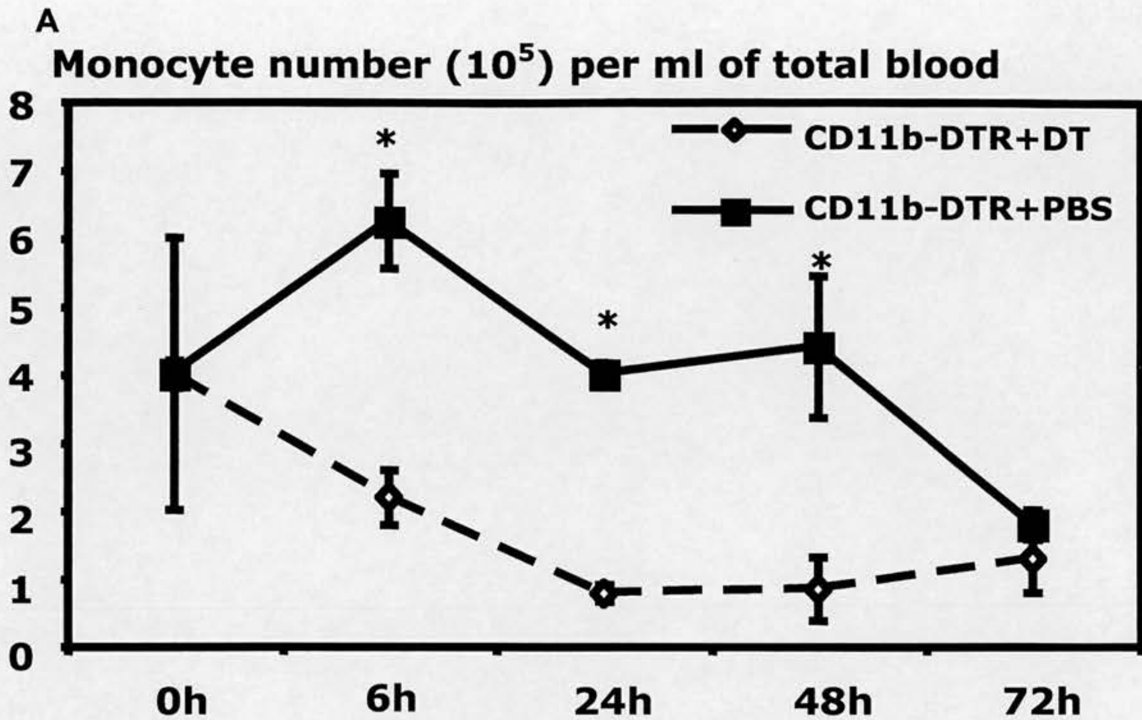
Figure 3.3 - The administration of DT reduces circulating blood monocytes but circulating PMN numbers are not reduced.
CD11b-DTR mice received either DT or PBS. Blood was obtained at 6, 24, 48 and 72h later and analysed by flow cytometry. A) Circulating monocytes and PMN numbers are compared at 24h. B) PMN kinetic following administration of DT or PBS (*=$p<0.05$ vs CD11b-DTR+DT, n=3-6 per group at each time point).

evident at 6, 48 or 72 hr following the administration of DT indicating an absence of any initial neutropenia or delayed effects of DT treatment (Figure 3.3B)

To determine whether recruited PMN were sensitive to DT, CD11b-DTR mice were injected with BTG in order to recruit a significant influx of PMN. DT (25 ng/g BW) or PBS was then injected 8 h after the initiation of peritonitis with peritoneal lavage being performed 12h later. Despite 90% ablation of recruited monocytes and recruited/resident Mϕ ($0.5x10^6 \pm 1.5x10^5$ vs $4.8x10^6 \pm 2.3x10^5$; DT treatment vs PBS; $p<0.005$) there was no difference in PMN number between experimental groups ($2.3x10^6 \pm 2.2x10^5$ vs $1.6x10^6 \pm 3.9x10^5$; DT treatment vs PBS; $p>0.05$).

3.2.4 Repopulation of monocytes and Mϕ in blood and the peritoneal cavity following DT administration.

Circulating monocyte numbers remained markedly reduced for 48 hr following the administration of DT compared to administration of PBS with almost a complete recovery of circulating monocyte numbers evident at 72 hrs (Figure 3.4A). In contrast, peritoneal Mϕ depletion persisted over this 72h period and this is likely to reflect the route of administration as the local intraperitoneal levels of DT may be higher than those in the systemic circulation (Figure 3.4B).



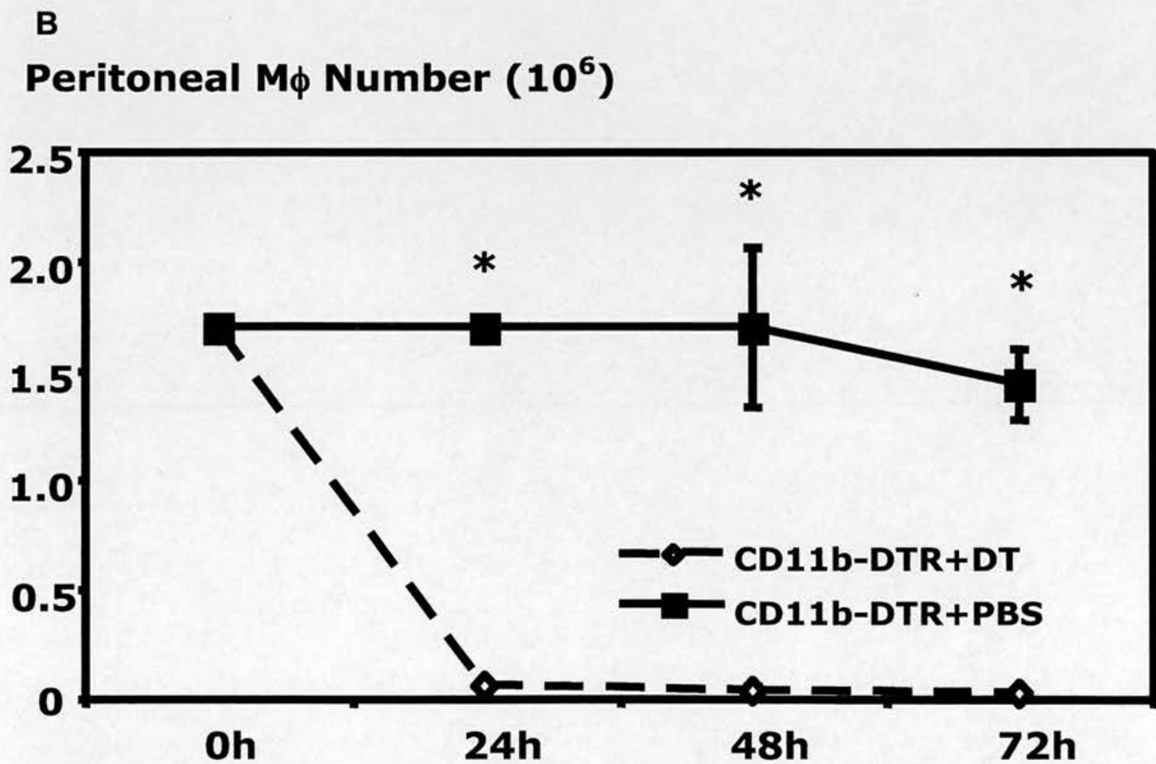


Figure 3.4 - Repopulation of monocytes/ Mϕ following DT administration
CD11b-DTR mice received either DT or PBS. Blood sampling and peritoneal lavage was performed at various time points to determine the repopulation kinetics of circulating monocytes (A) and peritoneal Mϕ (B). Cell numbers were determined by flow cytometry (*=$p<0.05$; n=3-5 per group at each time point).

3.2.5 Resident Mϕ ablation reduces PMN influx during experimental peritonitis induced by BTG.

The conditional ablation strategy was used to investigate the role of resident peritoneal Mϕ in sensing perturbation of the microenvironment and subsequent initiation of acute peritoneal inflammation and PMN recruitment in experimental peritonitis. DT treated FvB/N control mice exhibited a marked PMN influx that peaked 8h following the administration of 3% BTG (Figure 3.5). The ablation of resident peritoneal Mϕ markedly attenuated PMN infiltration following the administration of 3% BTG with significant differences being evident at both the 8h and 24h time points (Figure 3.5).

3.2.6 Adoptive transfer of non-transgenic resident peritoneal Mϕ to DT treated CD11b-DTR mice restores PMN recruitment in BTG peritonitis.

In light of the fact that the administration of DT did exert effects upon other cells within the peritoneum including B cells and MC, I went on to perform Mϕ repletion studies. In these experiments, I adoptively transferred either Mϕ-rich or Mϕ-depleted peritoneal cells derived from DT insensitive non-transgenic control FvB/N control mice. FvB/N control and 3 groups of CD11b-DTR mice were injected with DT (25 ng/g body weight) 24 hr prior to BTG injection in order to

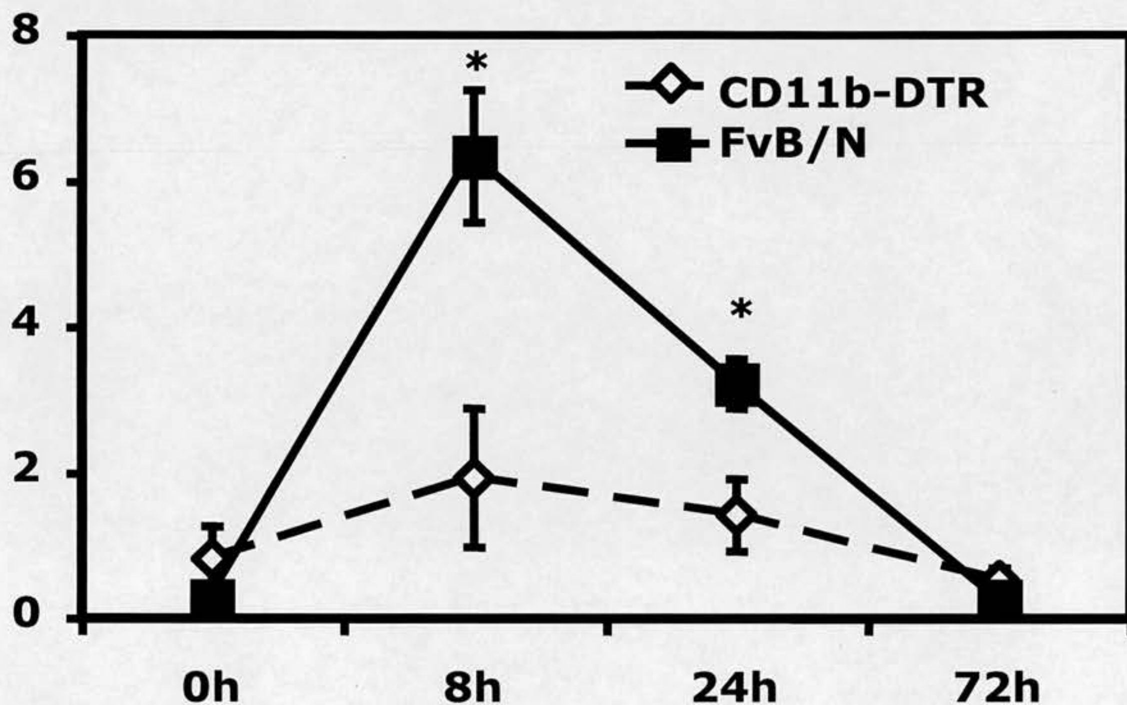


Figure 3.5 - Resident Mϕ ablation attenuates peritoneal PNN influx.
FvB/N and CD11b-DTR mice were injected IP with DT (25 ng/g body weight) in order to deplete resident peritoneal Mϕ. 1 ml of 3% BTG was injected IP 24h later with mice undergoing peritoneal lavage at various time points. Peritoneal cells were stained for the PMN marker GR1. Resident peritoneal Mϕ ablation induced a marked blunting of PMN infiltration of the peritoneal cavity (*=$p<0.001$ vs CD11b-DTR; n=7 per group at each time point).

ablate peritoneal Mϕ. Two groups of Mϕ-depleted CD11b-DTR mice were then reconstituted with either:

(i) Mϕ-rich peritoneal cells (MϕR) i.e. all peritoneal cells retrieved from the peritoneum ($7.9x10^5 \pm 2.7x10^5$ Mϕ were transferred per mouse in these experiments),

(ii) Mϕ-depleted peritoneal cells (MϕD) i.e. peritoneal cells that had been depleted of Mϕ by adhesion to tissue culture plastic prior to instillation into the peritoneal cavity of recipient mice.

These various cell populations were adoptively transferred 4h prior to the administration of BTG whilst the other groups (FvB/N and control Mϕ-ablated mice) received an identical volume of PBS IP in order to control for the IP injection. Mice were then injected with 3% BTG and underwent peritoneal lavage 8 hours later. Reconstitution of Mϕ-ablated mice with Mϕ-rich peritoneal cells fully restored PMN infiltration (Figure 3.6). In this experiment, the transfer of a Mϕ-rich peritoneal cell population alone was an important control as the transfer of Mϕ that have been manipulated *ex vivo* may have been inherently pro-inflammatory. However, the adoptive transfer of Mϕ-rich peritoneal cells to Mϕ ablated CD11b-DTR mice did not result in a significant PMN infiltrate (Figure 3.6) thereby highlighting the non-inflammatory nature of the adoptive transfer of cells and indicating that the cells were unlikely to have been significantly activated by the *ex vivo* manipulation.

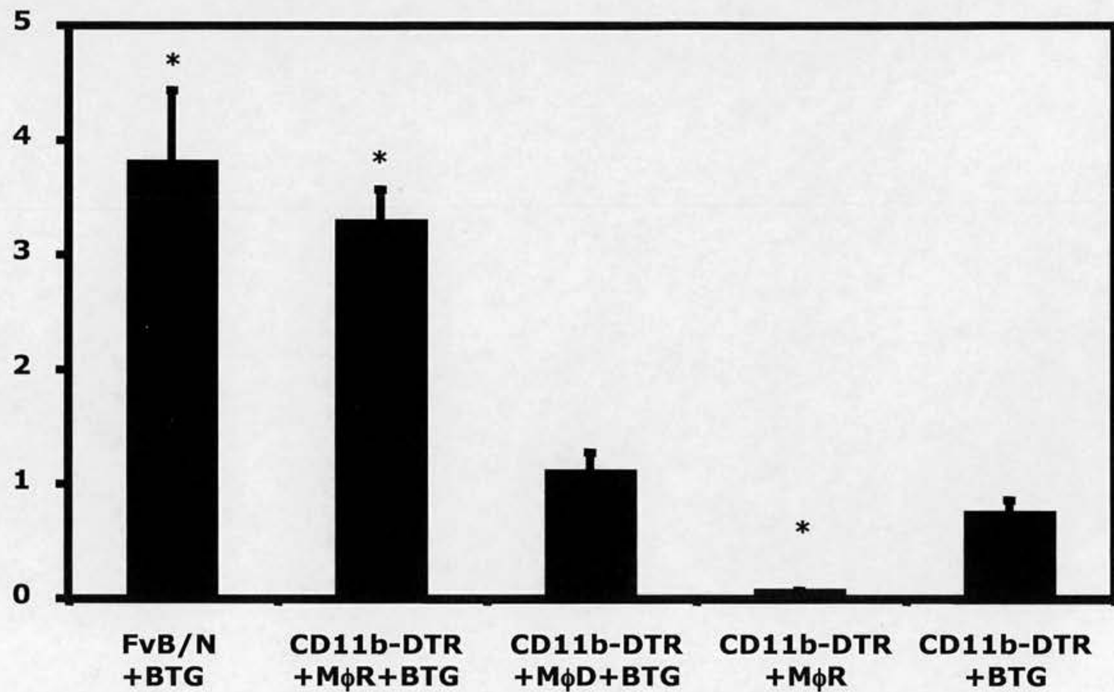


Figure 3.6 - Adoptive transfer of peritoneal Mϕ restores PMN influx in Mϕ ablated mice.

FvB/N control and 3 groups of CD11b-DTR mice were injected with DT (25 ng/g body weight) 24 hr prior to BTG injection. 2 groups of Mϕ-depleted CD11b-DTR mice were reconstituted with either (i) Mϕ-rich peritoneal cells (MϕR), (ii) Mϕ-depleted peritoneal cells (MϕD) 4h prior to BTG injection whilst the other groups received PBS. Mice underwent peritoneal lavage 8 hours after the induction of inflammation. Reconstitution of Mϕ-ablated mice with a Mϕ-rich peritoneal cell population fully restored PMN infiltration. The adoptive transfer of Mϕ-rich peritoneal cells alone in the 4th group of DT treated CD11b-DTR mice did not induce significant PMN infiltration highlighting the non-inflammatory nature of the adoptive transfer (*=$p<0.05$ vs CD11b-DTR+BTG; n=4-14 per group).

The reconstitution of DT-treated Mϕ ablated CD11b-DTR mice with Mϕ-rich peritoneal cells 4 hours prior to BTG treatment resulted in complete restoration of peak PMN infiltration at the 8 h time point; the time point exhibiting maximal PMN infiltration in this model and mouse strain. In contrast, the administration of Mϕ-depleted peritoneal cells was ineffective and resulted in levels of PMN recruitment directly comparable to that evident in the control Mϕ ablated CD11b-DTR mice. These data suggested that B cells and mast cells were not critically involved in orchestrating the PMN influx (Figure 3.6).

3.2.7 Resident Mϕ ablation reduces PMN influx during experimental peritonitis induced by zymosan.

Previous work suggested that the nature of the inflammatory stimulus may be a key factor in determining the involvement of Mϕ in experimental peritonitis (Ajuebor et al., 1999) and I therefore performed Mϕ depletion studies in the model of zymosan peritonitis. The depletion of resident peritoneal Mϕ resulted in a significant reduction in PMN infiltration 8 hours following the induction of zymosan peritonitis (Figure 3.7). Although I did not perform extensive time course studies or repeat the adoptive transfer experiments in the model of zymosan peritonitis, these data do suggest that resident Mϕ play an important role in the initiation of PMN infiltration in the peritoneum following the administration of pro-inflammatory stimuli.

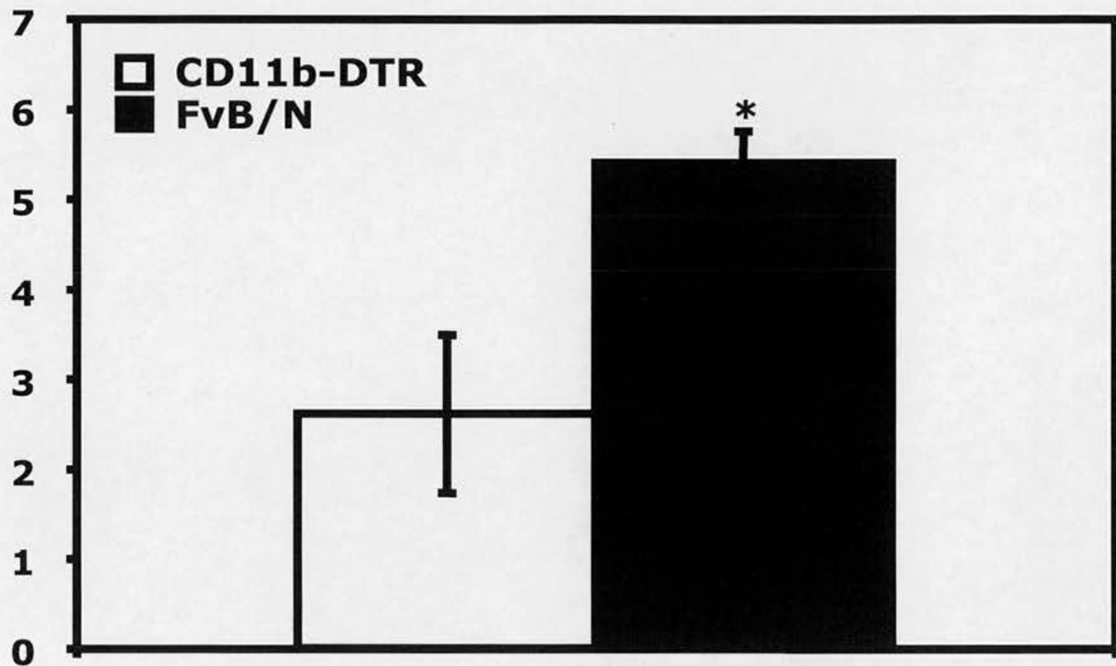


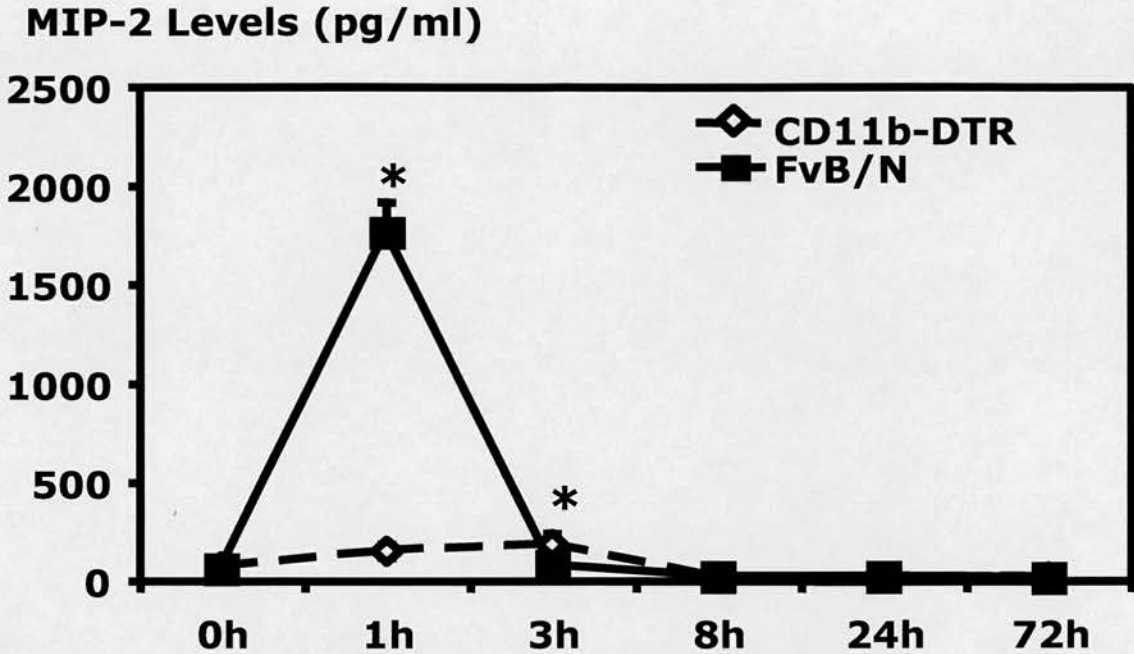
Figure 3.7 - Resident Mϕ ablation attenuates PMN influx in zymosan peritonitis.
FvB/N control and CD11b-DTR mice were injected with DT (25 ng/g body weight) 24 hours prior to the IP administration of zymosan. Peritoneal lavage was performed 8 hr following the administration of zymosan and PMN number assessment by GR1 staining flow cytometry. Mϕ ablation reduced the PMN influx (* $p<0.05$ vs CD11b-DTR; n=6 per group).

3.2.8 Peritoneal resident Mϕ ablation reduces secretion of key chemokines/cytokines *in vivo.*

In this model, peak levels of the PMN C-X-C chemokines MIP-2 and KC were found at the 1 h time point with levels being low at later time points. Resident peritoneal Mϕ ablation prior to the initiation of BTG peritonitis markedly reduced the elevation in MIP-2 levels (Figure 3.8A). There was a slight, albeit statistically significant, difference between DT-treated CD11b-DTR mice and DT-treated FvB/N control mice in the much lower levels of MIP-2 evident at 3 hours (Figure 3.8A). There were no differences in the MIP-2 levels at later time points between both groups as levels were low. This result strongly suggests that the production of MIP-2 *in vivo* is predominantly Mϕ dependent. In addition, a 50% reduction in the level of KC was evident in Mϕ-depleted mice at the 1 h time point. Interestingly, the levels of KC at 3h are higher in Mϕ-depleted mice compared to control mice thereby suggesting a source of KC other than resident Mϕ (Figure 3.8B). The mildly elevated levels of KC at the initiation of inflammation (24 hrs post DT treatment) may reflect the inflammatory reaction secondary to Mϕ ablation, which does result in a mild PMN influx (Figure 3.2B).

The C-C chemokines peaked at 8h in this model of peritoneal inflammation. Mϕ ablation completely inhibited the increased level of MIP-1α evident at 8h and reduced the level of MCP-1 at 8h by 55% (Figure 3.9A and 3.9B). However, there were no differences in the MIP-1α levels at any other time point (Figure 3.9A).

A



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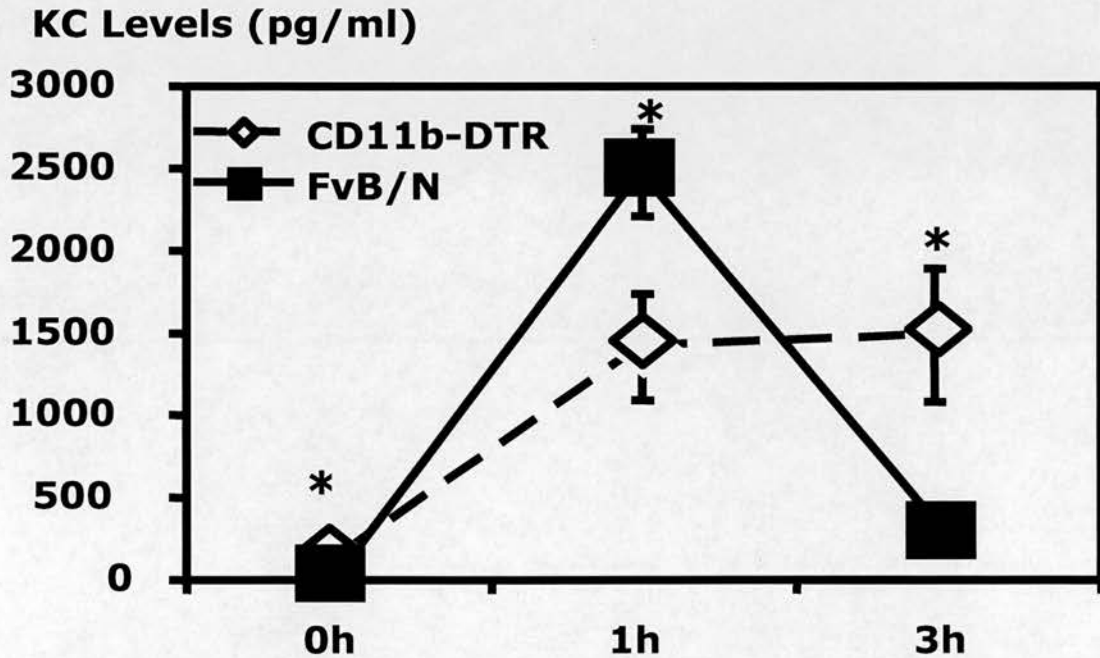

Figure 3.8 – Modulation of MIP-2 and KC production following Mϕ ablation in BTG peritonitis.
CD11b-DTR and FvB/N control mice were injected with DT (25 ng/g body weight) 24 hours prior to administration of BTG. Peritoneal lavage was performed at various time points after the induction of peritonitis as indicated. The levels of MIP-2 (A) and KC (B) were determined in the peritoneal lavage supernatant by specific ELISA. (*= $p<0.05$ vs CD11b-DTR group, n= 4-6 mice per group at each time point).

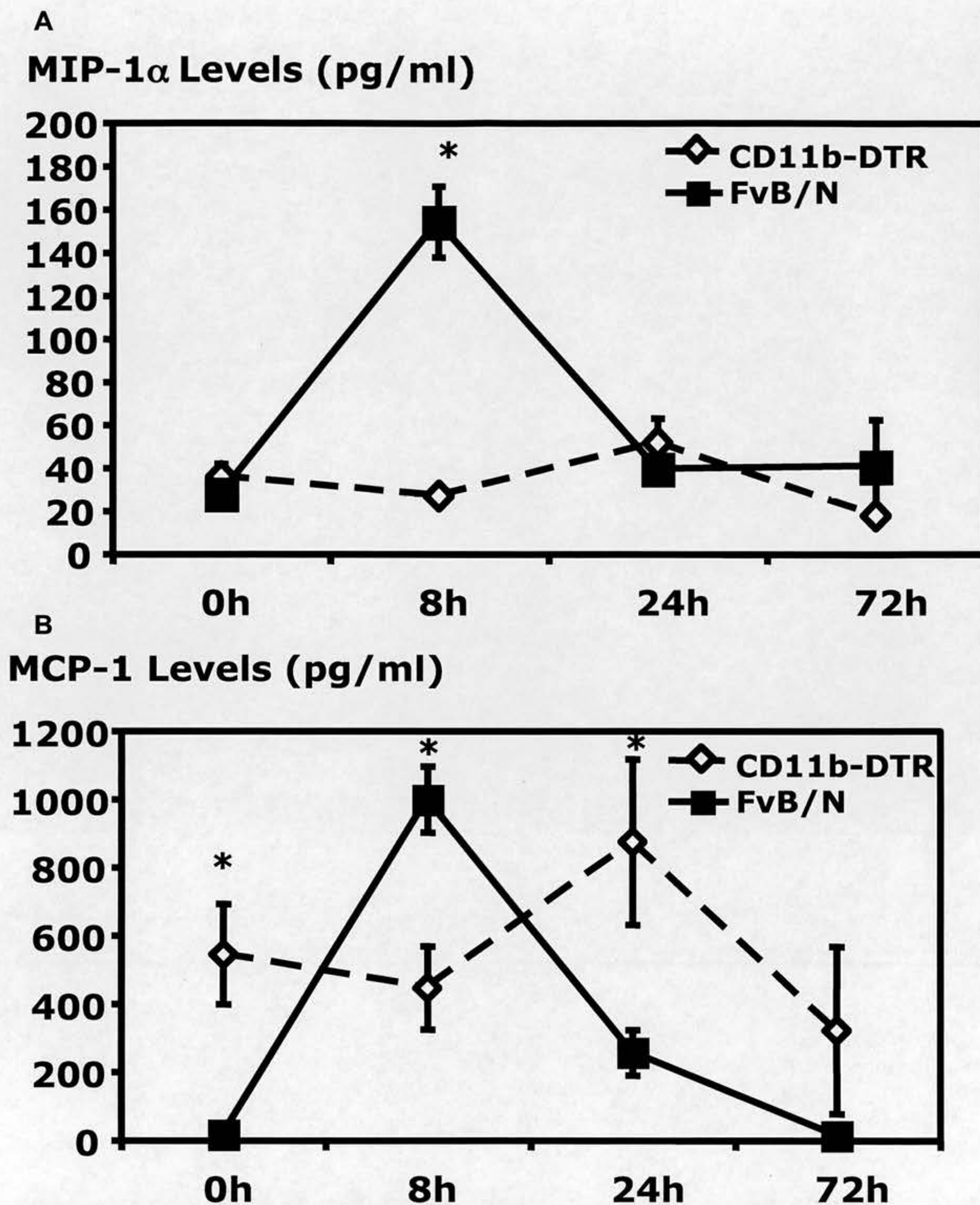

Figure 3.9 - Modulation of MIP-1α and MCP-1 production following Mϕ ablation in BTG peritonitis.

CD11b-DTR and FvB/N control mice were injected with DT (25 ng/g body weight) 24 hours prior to administration of BTG. Peritoneal lavage was performed at various time points after the induction of peritonitis. The levels of MIP-1α (A) and MCP-1 (B) were determined in the peritoneal lavage supernatant by specific ELISA. (*= $p<0.05$ vs CD11b-DTR group, n= 4-6 mice per group at each time point).

Interestingly, MCP-1 production was higher in Mϕ-ablated CD11b-DTR mice 24h after BTG administration (Figure 3.9B). Similar to the data for KC production, these data suggest that late MCP-1 production is probably derived from a source other than resident Mϕ. The MCP-1 difference at 0h is presumably a reflection of the pro-inflammatory milieu generated by the Mϕ ablation and subsequent secondary necrosis of the apoptotic Mϕ.

The levels of various cytokines were measured in the peritoneal fluid by ELISA or CBA. Mϕ ablation completely inhibited the increased level of TNF-α evident at 3h after BTG administration (Figure 3.10) with TNF-α levels being undetectable at late time points. However, there were no differences between CD11b-DTR mice and FvB/N controls for any of the other cytokines analysed (IL–6, IL-10, IL12p70 and IFN-γ) at the 8, 24 and 72h time points and the cytokine levels were relatively low compared to chemokine levels. The shorter time points were not looked at.

The level of the anti-inflammatory and reparative cytokine TGF-β was also analysed at various time points. A 70% reduction in TGF-β levels at 24h was observed in Mϕ-ablated CD11b-DTR mice compared to FvB/N control mice (113.3±21.8 pg/ml vs 424.9±48.3 pg/ml; DT-treated CD11b-DTR mice vs DT-treated FvB/N mice; $p<0.0001$). Intracellular staining indicated that TGF-β production was likely to be from differentiated MDMϕ as positive cells expressed a relatively high level of the Mϕ surface marker F4/80 whereas newly recruited F4/80

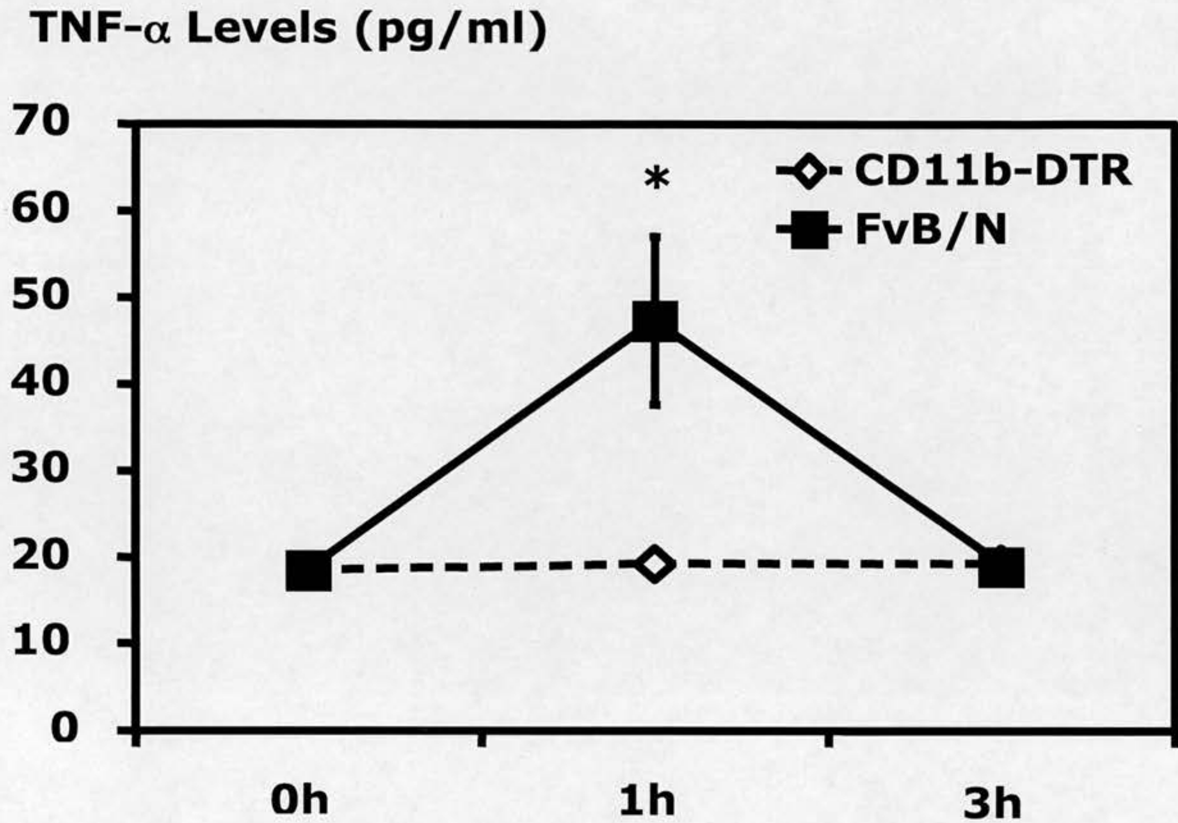

Figure 3.10- Modulation of TNF-α levels following Mϕ ablation in BTG peritonitis.
CD11b-DTR and FvB/N control mice were injected with DT (25 ng/g body weight) 24 hours prior to administration of BTG. Peritoneal lavage was performed at various time points after the induction of peritonitis. The levels of TNF-α were determined in the peritoneal lavage supernatant by specific ELISA. (*= $p<0.05$ vs CD11b-DTR group, n= 4-6 mice per group at each time point).

low monocytes were TGF-β negative (Figure 3.11A and 3.11B).

3.2.9 C-X-C chemokine responses are Mϕ dependent *in vitro.*

Previous studies of peritoneal and dermal inflammation have implicated the MC as playing an important role in the initiation of PMN infiltration (Ajuebor et al., 1999; Chen et al., 2002). I therefore performed additional *in vitro* studies to determine the production of C-X-C chemokines by BTG-stimulated peritoneal cell populations that had been depleted of either Mϕ or MC. Control non-depleted peritoneal cells produced significant levels of MIP-2 and KC following 3h stimulation with BTG (Figure 3.12). Chemokine production was completely unaffected by the specific immunomagnetic depletion of MC. In contrast, chemokine levels were dramatically reduced following the depletion of Mϕ thereby indicating that chemokine production was completely Mϕ dependent *in vitro* with no discernible involvement of MC (Figure 3.12).

I also investigated the production of MCP-1 *in vitro*. Although, MCP-1 levels obtained after *in vitro* BTG stimulation were much lower than those found *in vivo*, there was a complete absence of MCP-1 generation following Mϕ depletion whereas MC depletion had no effect (10.38±1.1 pg/ml vs 0±0 pg/ml vs 12.1±1.2 pg/ml; All cells vs Mϕ-depleted vs MC-depleted; $p<0.05$). These data suggest that MC do not significantly modulate chemokine production. It should also be stated that the low level of MCP-1 evident in these assays suggests that other cells such as MeC are likely to be involved in the production of MCP-1 *in vivo* during peritoneal

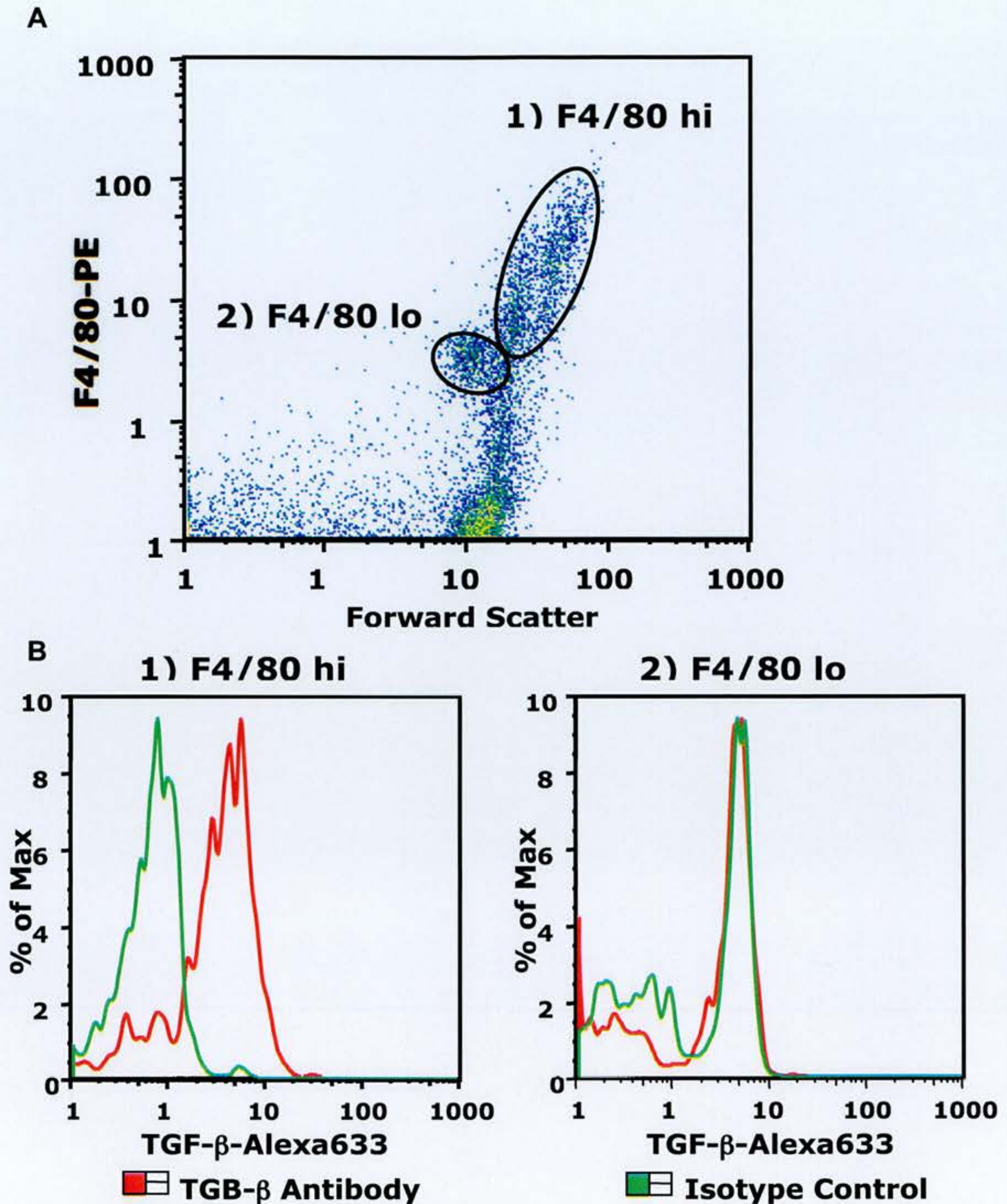

Figure 3.11- Differentiated monocyte-derived Mϕ express intracellular TGF-β 24h after administration of BTG.

Intracellular TGF-β staining was performed on peritoneal cells obtained from lavage 24h after the induction of BTG peritonitis. Peritoneal cells were fixed, permeabilised and stained with either an anti-TGF-β antibody or an isotype control. Cells were stained with an Alexa-633-conjugated secondary antibody and F4/80 PE. F4/80 analysis by flow cytometry revealed an F4/80 Hi and F4/80 Lo Mϕ populations (A). Subsequent TGF-β analysis demonstrated that F4/80 Hi monocyte-derived Mϕ strongly expressed TGB-β whereas F4/80 Lo monocytes did not (B).

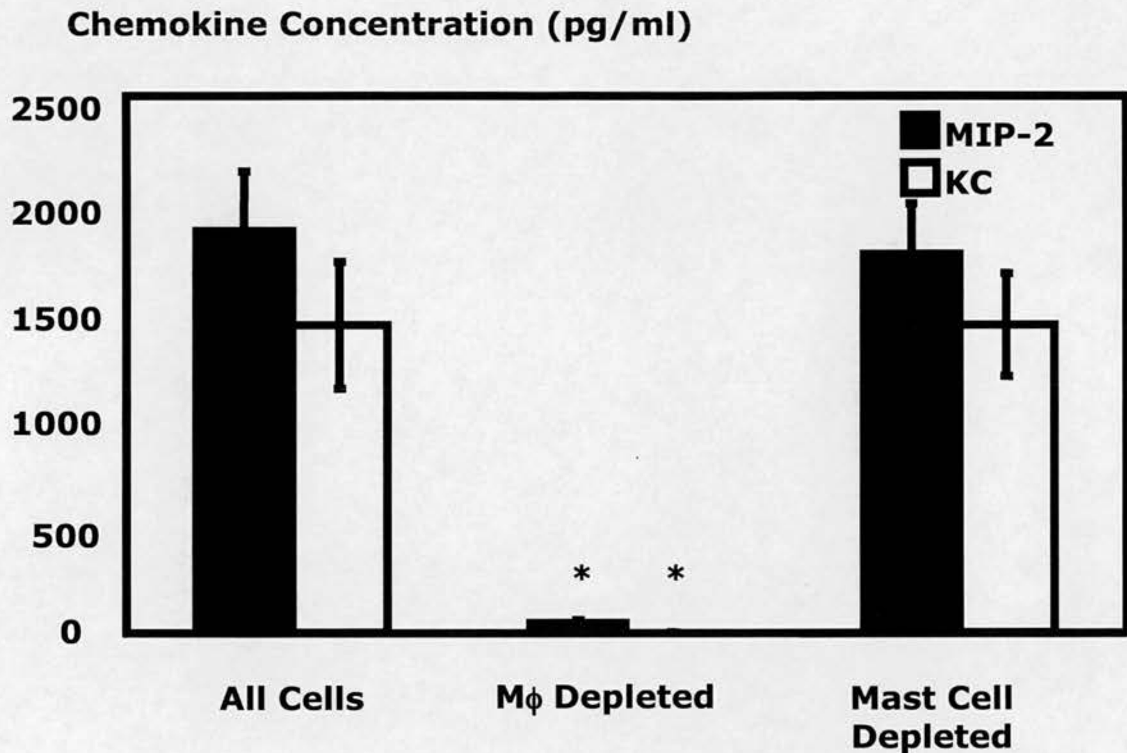

Figure 3.12 - C-X-C chemokine production in response to BTG stimulation is Mϕ dependent and MC independent *in vitro.*
Peritoneal cells were depleted of either Mϕ or MC by incubation with PE-conjugated anti-F4/80 or anti-c-kit (CD117) antibodies followed by incubation with anti-PE conjugated magnetic beads and passage over a magnetic column (>97% depletion of Mϕ or MCs achieved). Incubation of total peritoneal cells with an isotype control antibody followed by magnetic beads and passage over the magnetic column served as control. Cells were then plated in 48 well plates (5×10^5 cells per well) and exposed to 1% BTG for 3h. Peritoneal cell conditioned supernatants were harvested, spun and analyzed by specific ELISA for MIP-2 and KC. (*=$p<0.005$ vs All Cells; n=9 wells per conditions).

inflammation.

3.3 Summary

The CD11b-DTR mice are a potent experimental conditional Mϕ ablation system with DT inducing >98% depletion of resident peritoneal Mϕ 24h after administration. However, I noted that B and MC numbers are also reduced following DT treatment. In contrast, neither circulating nor recruited PMN exhibit significant sensitivity to DT. Using these transgenic mice, I was able to demonstrate that Mϕ ablation significantly blunted the recruitment of PMN that follows BTG-induced and zymosan-induced peritonitis. Importantly, Mϕ repletion studies demonstrated that the adoptive transfer of Mϕ-rich peritoneal cells fully restored PMN influx whereas Mϕ-depleted cells exhibited no significant effect upon PMN recruitment. This experiment reinforced the role of resident Mϕ in the initiation of inflammation. In addition, since both B cells and MC were present in the ineffective Mϕ-depleted peritoneal cell population, the adoptive transfer studies excluded a major contributing role of these cells despite their reduced numbers following DT administration.

The analysis of cytokine levels in peritoneal exudates revealed that TNF-α production *in vivo* was Mϕ dependent and was reduced following Mϕ ablation. Similar analysis of chemokine levels revealed that Mϕ-dependent *in vivo* production of MIP-2 plays a crucial role in PMN recruitment. In contrast, KC production was less affected by Mϕ depletion thereby suggesting a non-Mϕ origin *in vivo*.

Additional *in vitro* studies demonstrated Mϕ-dependent production of both C-X-C chemokines with no discernible role for MC evident.

In conclusion, these data suggest that the resident peritoneal Mϕ plays a crucial role in the initiation of inflammation and PMN recruitment in peritoneal inflammation.

Chapter 4: The role of resident pleural macrophages in experimental inflammation

4.1 Introduction

In the last chapter, it was demonstrated that resident peritoneal Mϕ are important for the initiation of inflammation. Despite the fact that both peritoneal and pleural Mϕ are similar functionally and phenotypically (Dorger et al., 2001; Gjomarkaj et al., 1999; Sestini et al., 1984), it cannot be assumed that resident pleural Mϕ will have the same role in the initiation of inflammation. Previous work demonstrated that the pleural eosinophil influx induced by the administration of LPS was reduced in mice previously treated with diphosphonate-containing liposomes in order to deplete resident pleural Mϕ (Bozza et al., 1994) thereby suggesting an important role for the resident pleural Mϕ. However, no studies have directly examined their contribution in the initiation of pleural PMN infiltration. Pleural MeC are also important in pleural inflammation and may generate important chemokines such as IL-8 and MCP-1. TNF-α and IL-1β are key cytokines involved in pleural MeC stimulation to initiate this chemokine production (Antony et al., 1995; Frode et al., 2001; Goodman et al., 1992; Mohammed et al., 1998a; Park et al., 2003). Studies using function-blocking antibodies suggest that activated resident pleural Mϕ may be responsible for this TNF-α and IL-1β secretion (Frode et al., 2001; Park et al., 2003). Therefore, it is likely that pleural resident Mϕ do have an important role in promoting PMN influx and the CD11b-DTR conditional Mϕ

ablation model provided an excellent biological tool to dissect the role of the resident pleural Mϕ in initiation of inflammation.

4.2 Results

4.2.1 DT administration ablates pleural Mϕ .

In order to investigate the efficacy of Mϕ ablation in the pleural cavity, CD11b-DTR mice were treated with either DT (25ng/g body weight) or PBS IP. Pleural lavage was performed 24h after treatment with DT for cell count analysis by flow cytometry. CD11b-DTR transgenic mice exhibited almost complete ablation of F4/80 positive pleural Mϕ (96.1% ± 0.8 Mϕ ablation, $p<0.0001$) after a single dose of DT with Mϕ numbers being compared to those evident in the pleural cavity of non-manipulated control CD11b-DTR mice (Figure 4.1). Representative dot plots of pleural lavages performed 24h after DT administration in FvB/N and CD11b-DTR mice illustrate the potency of the F4/80 positive resident Mϕ ablation and the resistance of non-transgenic FvB/N resident Mϕ to DT treatment (Figure 4.2). 50% of the recoverable cells from a pleural lavage are resident Mϕ and this proportion was unchanged in FvB/N mice following DT administration. The majority of pleural cells retrievable by pleural lavage are F4/80 positive and studies of pleural cells following the intrapleural injection of dyes taken up by phagocytic cells indicate that the Mϕ marker F4/80 labels almost 100% of resident pleural Mϕ (Kris Houlberg, personal communication).

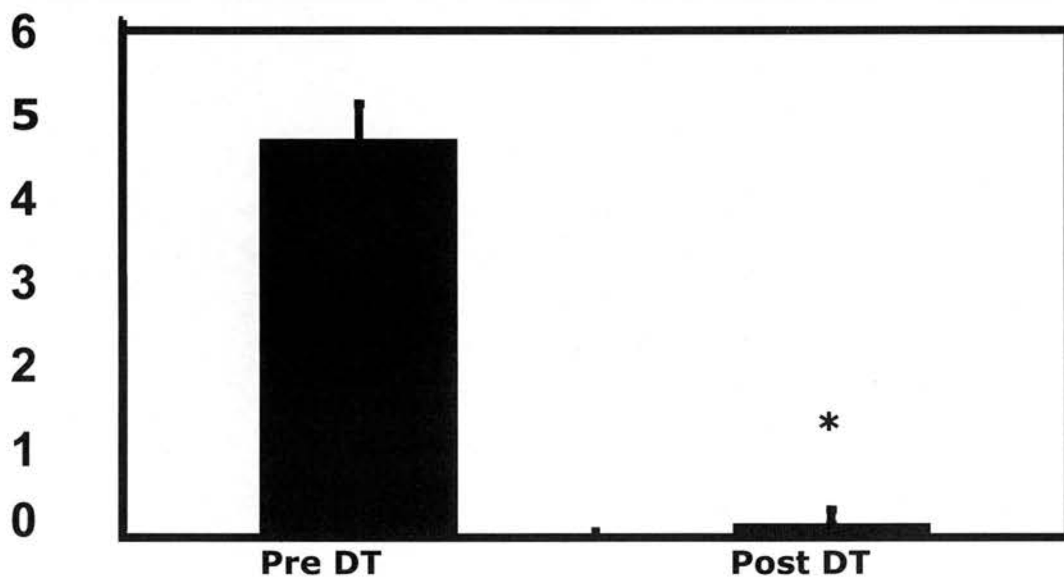

Figure 4.1- The administration of DT ablates resident pleural Mϕ.
DTR mice underwent pleural lavage in order to establish the number of Mϕ present under normal non-manipulated conditions. Histogram of pleural Mϕ number in CD11b-DTR 24h after DT administration (25ng/g body weight) compared to the Mϕ number in the normal non-manipulated pleural cavity (*=$p<0.00001$ vs pre-DT; n=3-4 mice per group).

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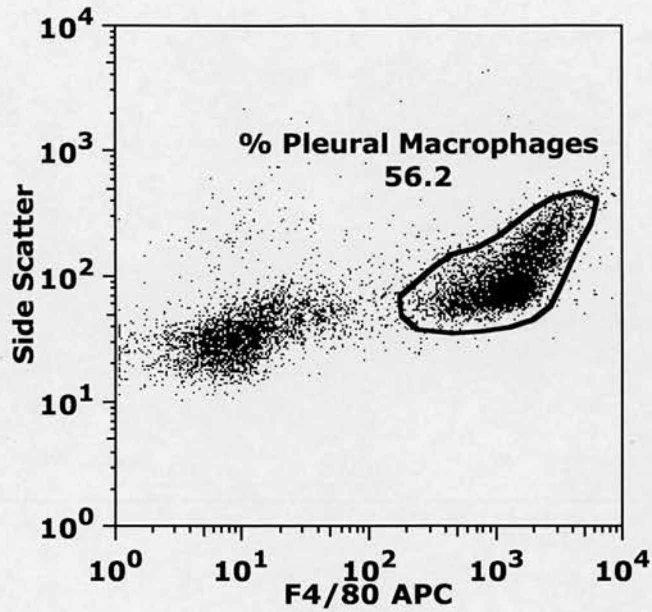

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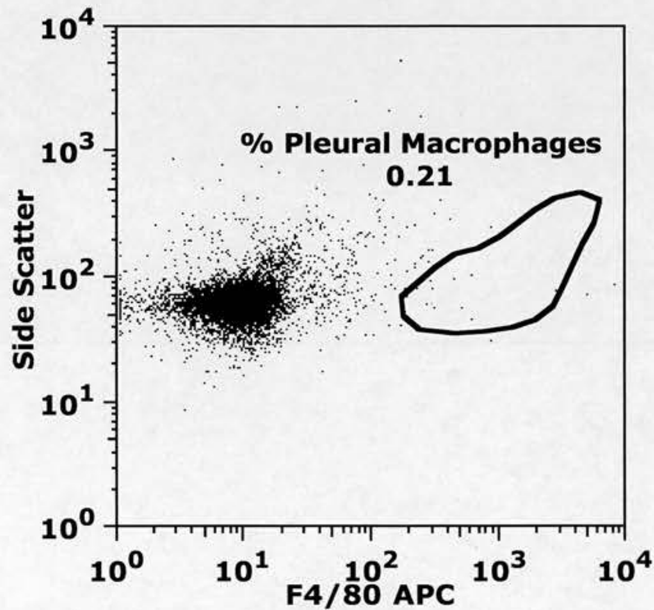

Figure 4.2 - DT administration to CD11b-DTR mice ablates F4/80 positive pleural Mϕ .

DT was injected IP to CD11b-DTR and FvB/N control mice (25 ng/g body weight) and pleural lavage performed 24h later. Cells were stained for F4/80 and analysed by flow cytometry. (A) Representative flow cytometry dot plot indicating that over 50% of pleural cells retrievable by pleural lavage from DT-treated FvB/N mice are F4/80 positive. (B) Administration of DT results in marked ablation of resident F4/80 positive pleural Mϕ in CD11b-DTR mice.

4.2.2 Specificity of Mϕ elimination *in vivo.*

To further address the specificity of the conditional ablation model in the pleural cavity, the numbers of other pleural cells was determined following DT administration. In these experiments I compared DT treated CD11b-DTR mice to CD11b-DTR mice treated with PBS and FvB/N mice treated with DT (Figure 4.3). There were no differences evident in the numbers of pleural Mϕ, B cells, T cells or MC between CD11b-DTR and FvB/N control mice. Although, the administration of DT to CD11b-DTR mice resulted in a profound reduction in pleural Mϕ, it also induced a reduction in the numbers of B cells and MC compared to both CD11b-DTR mice treated with PBS and FvB/N mice treated with DT (Figure 4.3). In addition, a mild influx of PMN was evident but there was no difference in T cell numbers (Figure 4.3). Potential reasons for such effects were discussed in chapter 3.

4.2.3 Repopulation of pleural Mϕ following DT administration.

Pleural Mϕ numbers remained markedly reduced for 48 hr following the administration of DT compared to administration of PBS with almost complete recovery of pleural Mϕ numbers evident at 72 hrs (Figure 4.4). This contrasts somewhat with the kinetics of repopulation of peritoneal Mϕ, which persisted at the 72h time point. As indicated previously, this difference in repopulation kinetics may well reflect the fact that the DT was administered directly into the peritoneum.

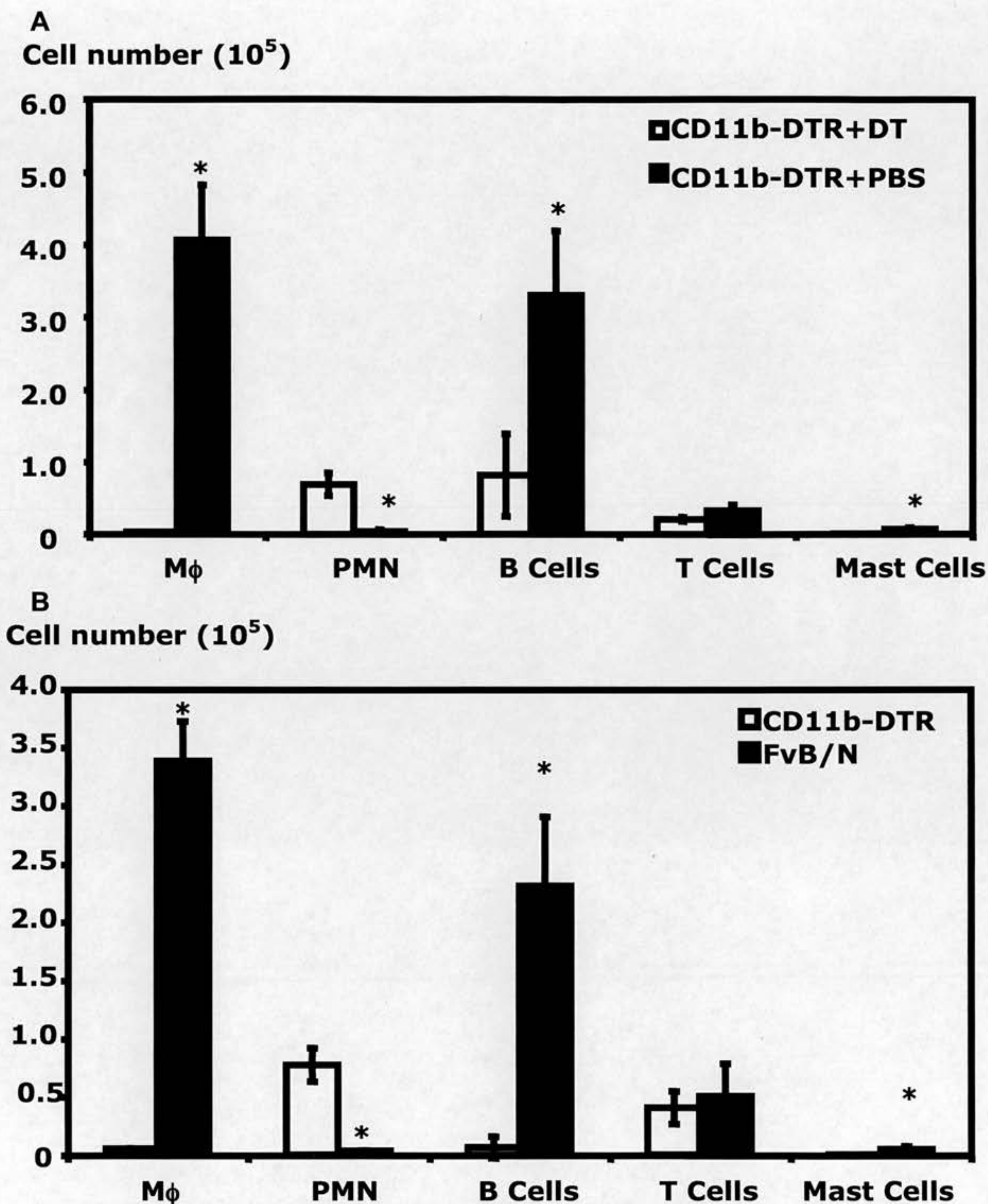

Figure 4.3 - Specificity of DT induced cell death: DT induces Mϕ ablation but also reduces also B and mast cell numbers in the pleural space.
DT or PBS was administered IP to CD11b-DTR mice and pleural lavage was performed 24h later. The numbers of Mϕ, PMN, B, T and mast cell was determined by flow cytometry (*=$p<0.05$ vs CD11b-DTR+DT; n=5-7 mice per group). B) DT was administered IP to CD11b-DTR or FvB/N mice and pleural lavage was performed 24h later (*=$p<0.05$ vs CD11b-DTR; n=5-7 mice per group).

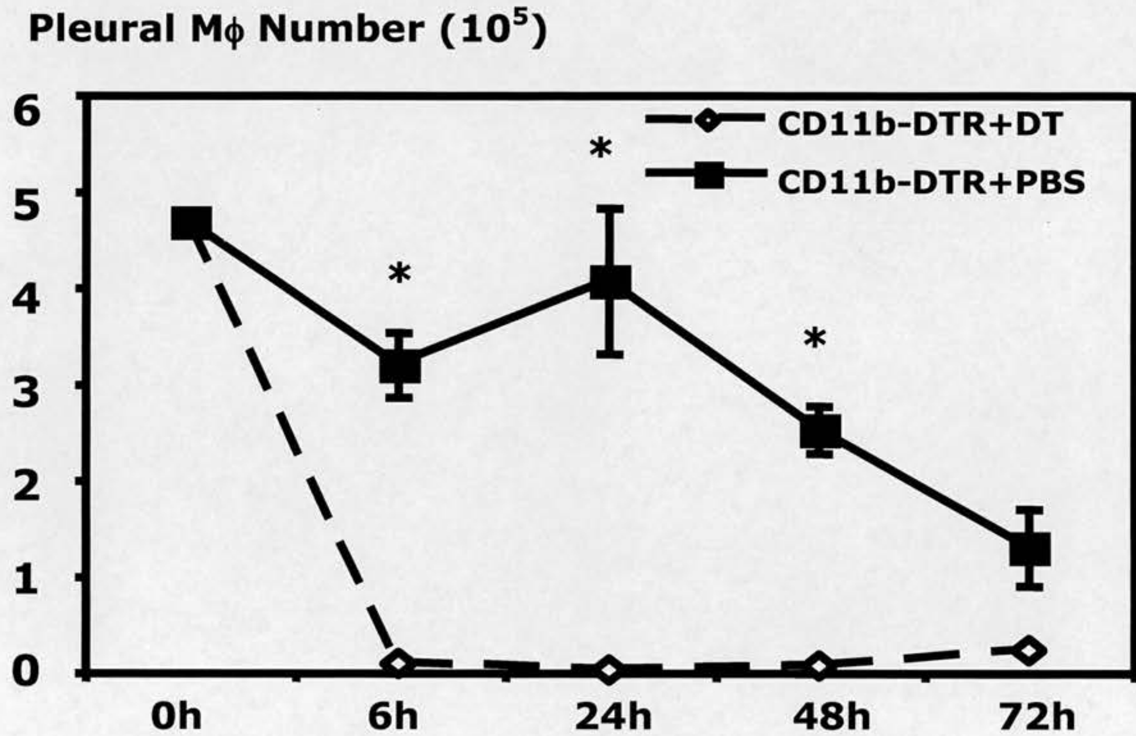

Figure 4.4 - Repopulation of monocytes/ Mϕ following DT administration CD11b-DTR mice received either DT or PBS. Pleural lavage was performed at various time points to determine the repopulation kinetics of pleural Mϕ. Cell numbers were determined by flow cytometry (*=$p<0.05$; n=3-5 mice per group per time point).

4.2.4 Resident pleural Mϕ ablation reduces PMN influx in carrageenan induced pleurisy.

The conditional Mϕ ablation strategy was also used to investigate the role of resident pleural Mϕ in initiating PMN recruitment following the administration of carrageenan. PMN infiltration following the administration of 1% carrageenan was markedly attenuated at all experimental time points following resident Mϕ ablation (Figure 4.5). It is particularly noteworthy that the early time points of 6 and 24 hours demonstrated a dramatic difference between groups. Although PMN infiltration in DT treated CD11b-DTR mice did reach approximately 50% of control levels at the later time point of 72 hours, this was still significantly less than DT-treated non-transgenic FvB/N control mice.

4.2.5 The adoptive transfer of non-transgenic purified Mϕ or Mϕ-rich pleural cell populations partially restores PMN influx in Mϕ-ablated CD11b-DTR mice following carrageenan administration.

In order to further analyse the role of resident pleural Mϕ in the initiation of acute pleural inflammation, we also performed Mϕ repletion studies. These experiments were also required as DT administration affected the numbers of B cells and MC within the pleural cavity. I adoptively transferred either Mϕ-rich or Mϕ-depleted pleural cell populations derived from DT insensitive non-transgenic FvB/N

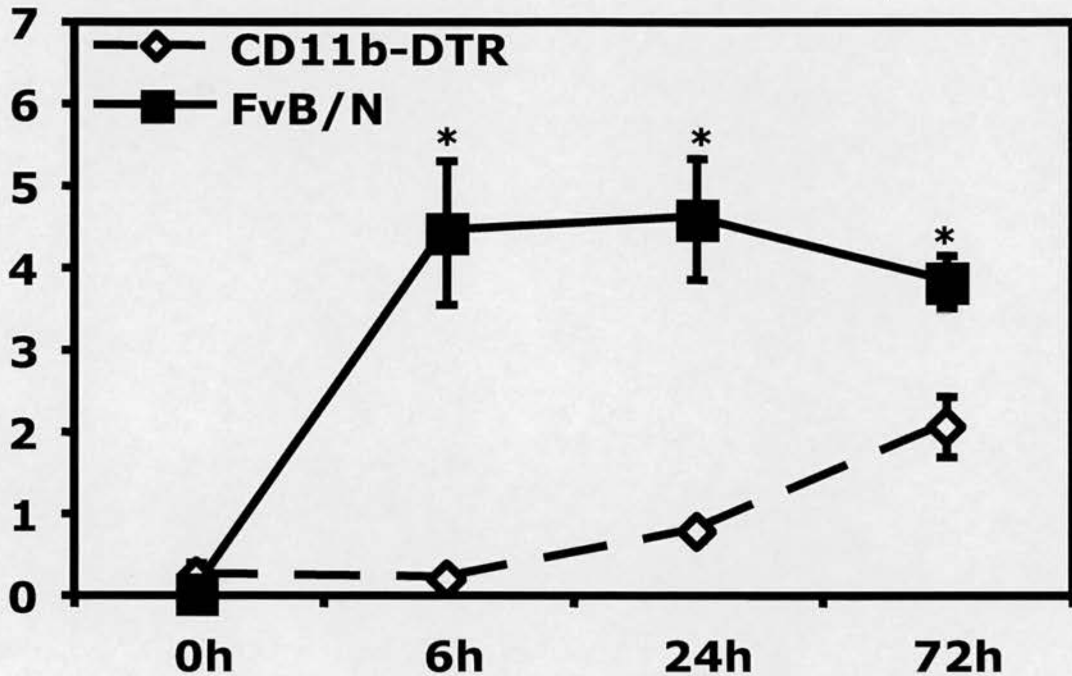

Figure 4.5 - Resident Mϕ ablation 24 hours prior to administration of carrageenan blunts PMN recruitment.
0.1ml of 1% carrageenan was administered to CD11b-DTR and FvB/N control mice 24h after DT treatment. Pleural lavage was performed at 0, 6, 24 and 72h following carrageenan. Lavaged cells were stained for GR1 and counted by flow cytometry (* = $p<0.05$ vs CD11b-DTR group, n=4-5 mice per group per time point).

control mice. In these experiments, two to three FvB/N mice were used to provide the pleural cells to adoptively transfer to one CD11b-DTR recipient mouse. The adoptive transfer of Mϕ-rich pleural cell populations resulted in the transfer of $1.5x10^5 \pm 0.3x10^5$ Mϕ and this restored Mϕ number to approximately 50% of the Mϕ number normally present in pleural lavage fluid. A full Mϕ transfer could not be accomplished as the numbers of FvB/N mice required were prohibitive. In addition, significant Mϕ loss occurred during the immunomagnetic depletion procedure. In view of the difficulty in performing multiple pleural injections, the Mϕ were injected at the same time as the carrageenan.

Despite the fact that Mϕ reconstitution of DT-treated CD11b-DTR mice was incomplete, the administration of Mϕ-rich pleural cells concurrently with carrageenan significantly increased the level of PMN infiltration at 6 hours compared to that evident in control Mϕ-ablated CD11b-DTR mice treated with carrageenan (Figure 4.6). The partial restoration of peak PMN infiltration was approximately 35% of levels present in control DT-treated FvB/N control mice at the same time point. In contrast, administration of Mϕ-depleted pleural cells concurrently with carrageenan made no significant impact upon PMN infiltration compared to Mϕ-depleted CD11b-DTR mice (Figure 4.6). Interestingly, the Mϕ number present (resident Mϕ plus any transferred Mϕ if performed) in the pleural space at the initiation of inflammation correlated with the PMN influx present at 6h (R^2 = 0.9979). This highlights the simple importance of the number of Mϕ present in the adoptive transfer. DT treated CD11b-DTR mice were also reconstituted with Mϕ

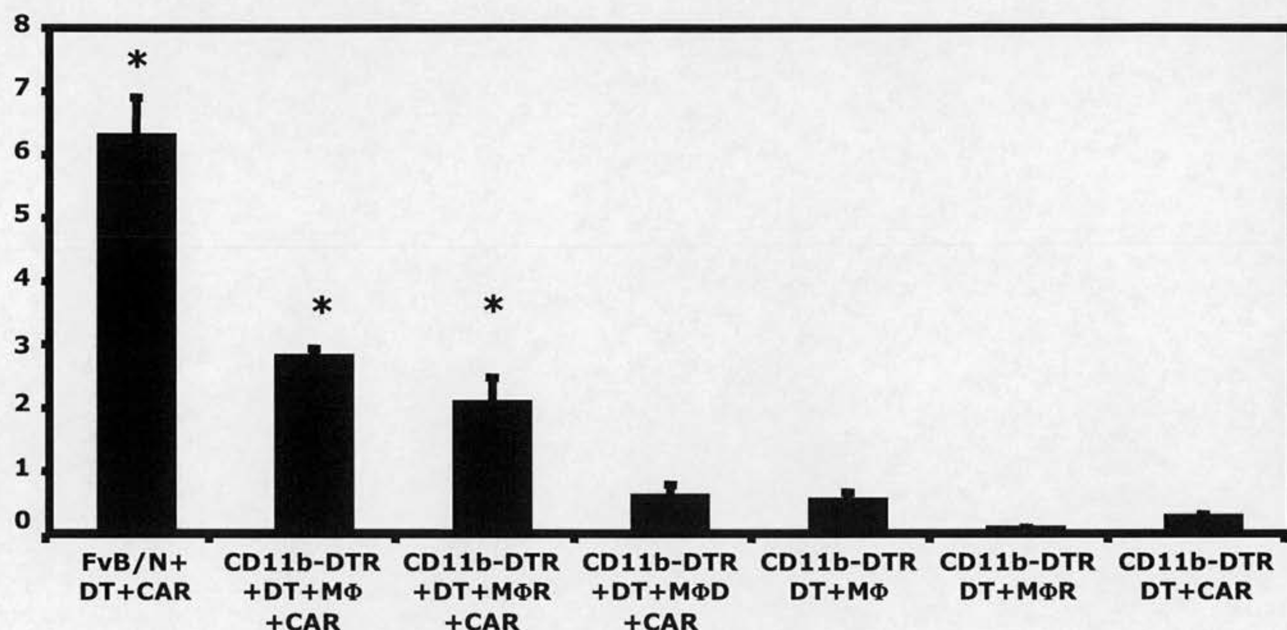

Figure 4.6 - Adoptive transfer of Mϕ-rich pleural cells and purified pleural macrophages partially restores PMN infiltration in carrageenan-induced pleurisy.
FvB/N control and 4 groups of CD11b-DTR mice were injected with DT (25 ng/g body weight) 24 hours prior to carrageenan injection. 3 groups of Mϕ-depleted CD11b-DTR mice were reconstituted with either (i) Mϕ-rich pleural cells (MϕR), (ii) Mϕ-depleted pleural cells (MϕD) or (iii) 90% pure Mϕ isolated by negative selection at the same time as the administration of carrageenan (CAR). Mice underwent pleural lavage 6 hours after the induction of inflammation (n=8-10 mice per group *$p<0.05$ vs CD11b-DTR+DT+CAR group). DT-treated CD11b-DTR mice exhibited a marked reduction in PMN infiltration whilst reconstitution of Mϕ-ablated mice with either purified Mϕ or a Mϕ-rich pleural cell population partially restored PMN infiltration. The adoptive transfer of either purified Mϕ or a Mϕ-rich pleural cell population without inflammatory agents did not induce significant PMN infiltration compared to DT-treated CD11b-DTR mice.

purified by negative selection (90% pure) concurrently with the administration of carrageenan and this resulted in a comparable PMN influx to that evident following reconstitution with Mϕ-rich pleural cells. It should be noted that, although DT-induced Mϕ ablation is associated with a reduction of B cell and MC number, the administration of Mϕ-depleted pleural cells comprising B cells, MC and T cells had no significant impact upon PMN infiltration. Lastly, the adoptive transfer of a control population of Mϕ-rich pleural cells or purified Mϕ was non-inflammatory (Figure 4.6).

4.2.6 Mϕ ablation modulates chemokine responses during carrageenan-induced pleurisy.

In this model, peak levels of the C-X-C chemokines MIP-2 and KC were found at the 1h and 3h time points respectively. Ablation of resident pleural Mϕ prior to administration of carrageenan markedly reduced MIP-2 levels at both 1h and 3h (Figure 4.7A) suggesting that the early production of MIP-2 *in vivo* is predominantly Mϕ dependent. In addition, Mϕ-ablated mice exhibited a delayed and significantly blunted peak in the level of MIP-2. There are very few Mϕ (<30,000) present within the pleural cavity of DT-treated CD11b-DTR mice at the 6 hr time point and this suggests that the delayed MIP-2 response may result from MIP-2 production by local cells such as mesothelial cells etc. MIP-2 levels are very low at the 24 hr time point and beyond in both experimental groups.

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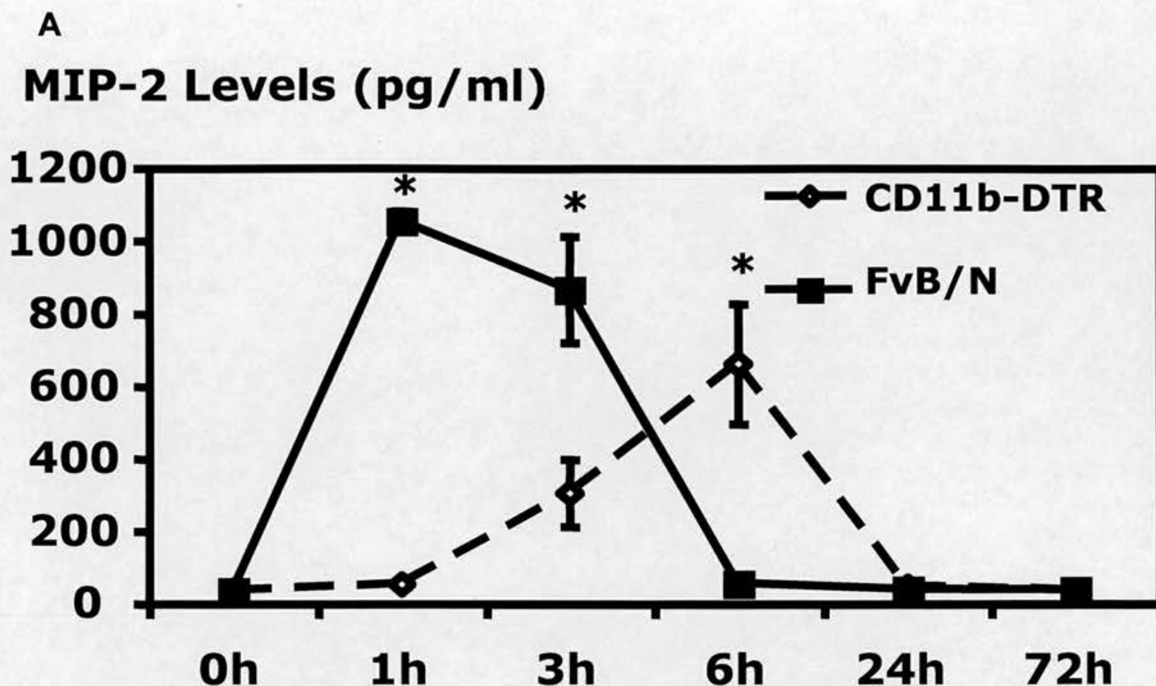

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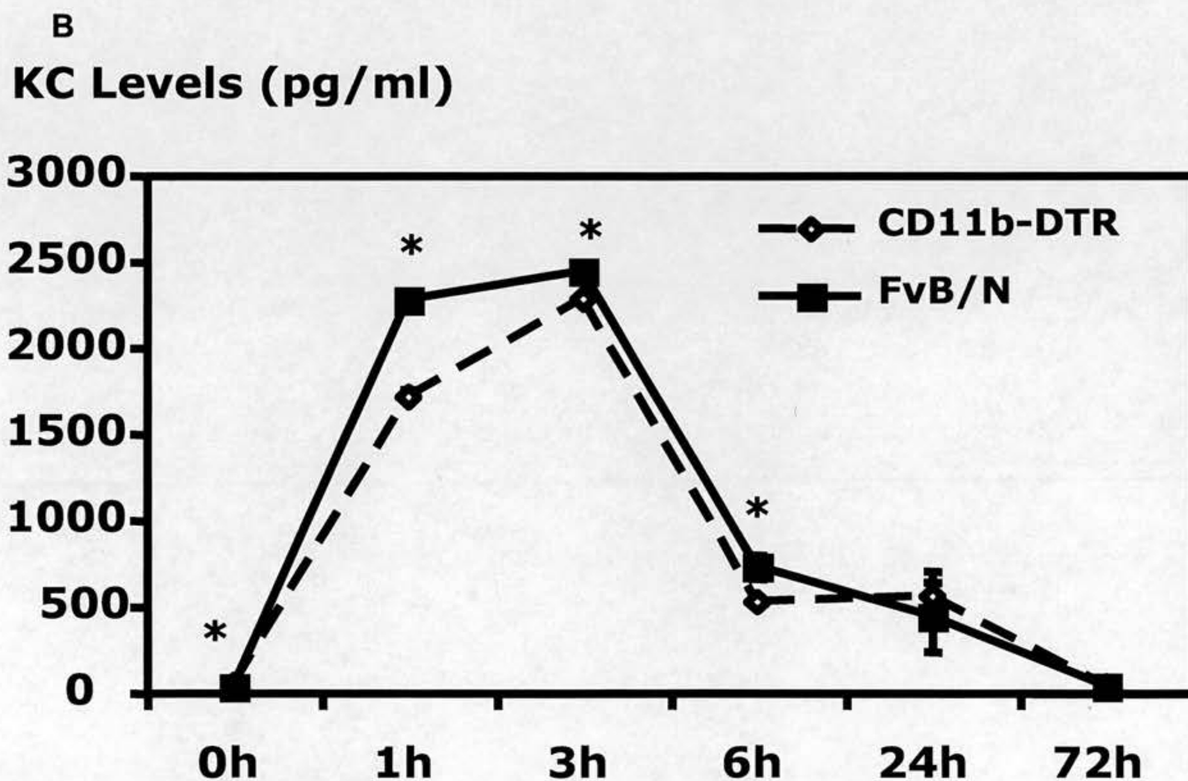

Figure 4.7 - Resident Mϕ ablation attenuates chemokine production in carrageenan-induced pleurisy.
CD11b-DTR and FvB/N control mice were injected with DT (25 ng/g body weight) 24 hours prior to administration of carrageenan. Pleural lavage was performed 1, 3, 6, 24 and 72 hr after the induction of pleurisy. The levels of MIP-2 (A) and KC (B) were determined in the pleural lavage supernatant by specific ELISA. (*= $p<0.05$ vs CD11b-DTR group, n= 5 mice per group per time point).

In contrast to the MIP-2 data, there was only a very modest, albeit statistically significant, reduction in KC levels evident in Mϕ-depleted mice at the 1, 3 and 6h time points (Figure 4.7B). No differences were evident at 24 or 72h. These data indicate that cells other than Mϕ are likely to be responsible for the production of KC. The kinetics of chemokine production in Mϕ-depleted and control mice needs to be reconciled with the data for PMN infiltration. The dramatic blunting of early PMN infiltration that follows resident pleural Mϕ ablation prior to the administration of carrageenan suggests that this early PMN influx is very much dependent upon resident Mϕ production of MIP-2.

The levels of MCP-1 were also examined. The ablation of resident pleural Mϕ did not exert marked affects upon the production of MCP-1 with the MCP-1 levels being reduced by approximately 36% at the 3h time point only (Figure 4.8). This is similar to the results for KC and suggests that MCP-1 is primarily generated by cells other than Mϕ.

4.2.7 Mϕ ablation modulates cytokine responses during carrageenan-induced pleurisy.

Analysis of the levels of cytokines in pleural lavage samples indicated a key role for resident Mϕ in the early production of the cytokines TNF-α, IL-6 and IL-10 (Figures 4.9 and 4.10). Mϕ ablation resulted in greater than 90% reduction in TNF-α and IL-6 levels with low cytokine levels evident in Mϕ ablated mice (Figure 4.9A

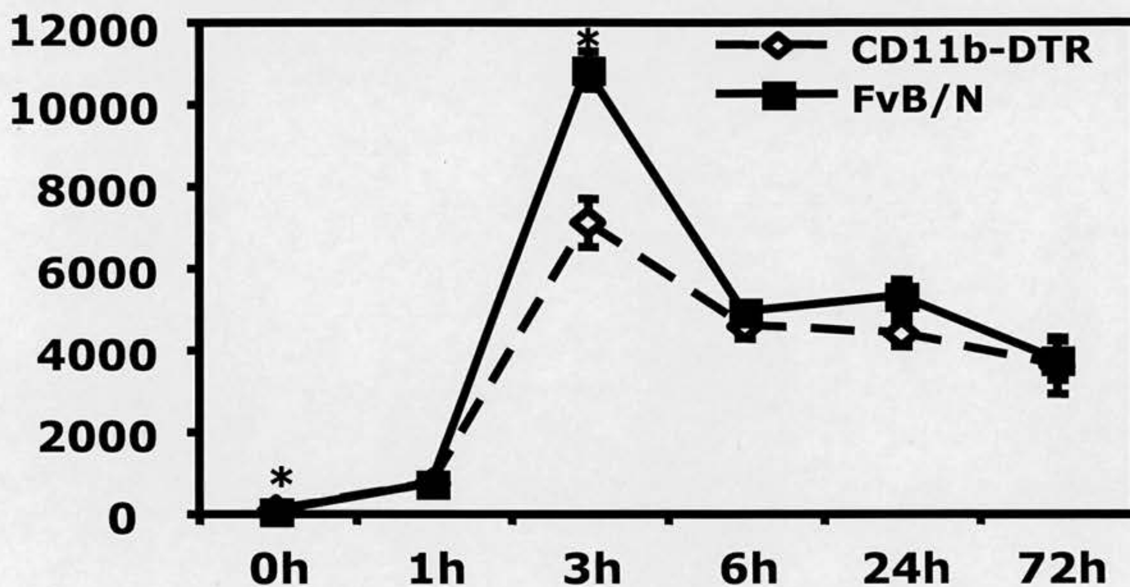

Figure 4.8 - Resident Mφ ablation attenuates MCP-1 production in carrageenan-induced pleurisy.
CD11b-DTR and FvB/N control mice were injected with DT (25 ng/g body weight) 24 hours prior to administration of carrageenan. Pleural lavage was performed 1, 3, 6, 24 and 72 hr after the induction of pleurisy. The level of MCP-1 in the pleural lavage supernatant was determined by CBA analysis (*= $p<0.05$ vs CD11b-DTR group, n= 5 mice per group per time point).

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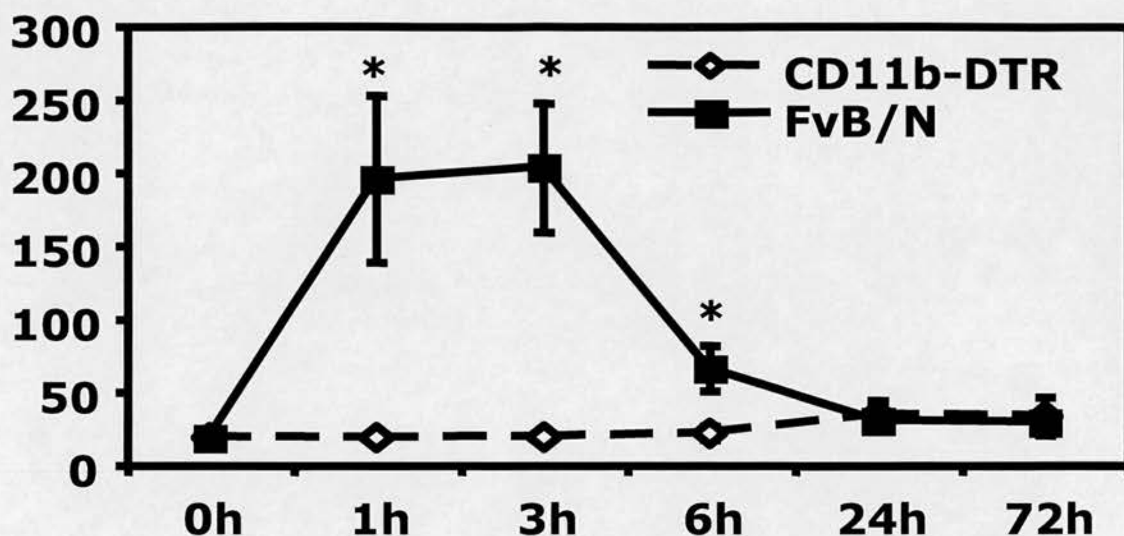

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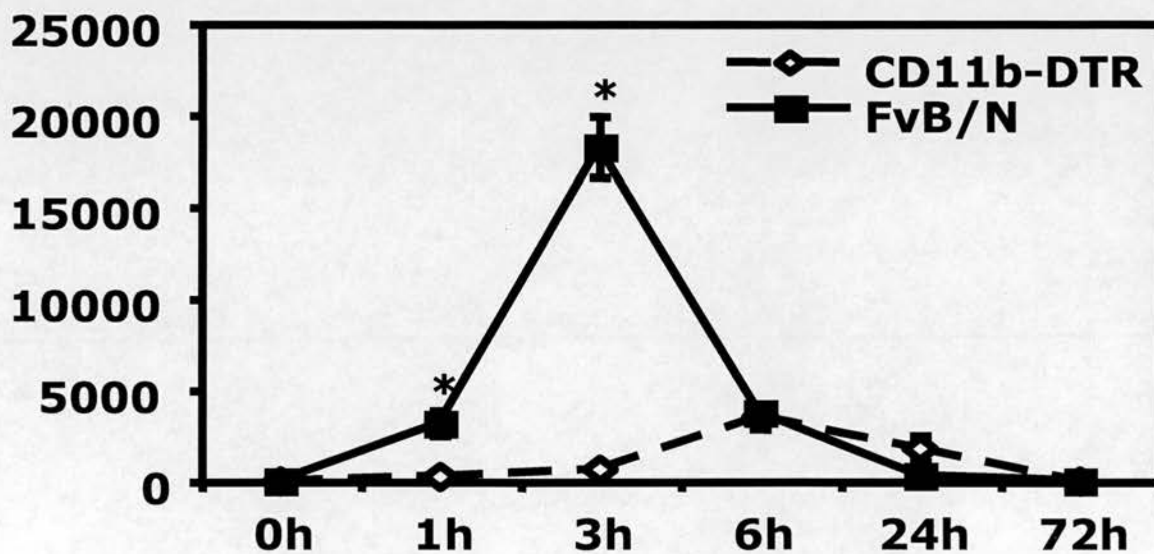

Figure 4.9 - Resident Mφ ablation attenuates TNF-α and IL-6 production in carrageenan-induced pleurisy.

CD11b-DTR and FvB/N control mice were injected with DT (25 ng/g body weight) 24 hours prior to carrageenan injection. Pleural lavage was performed 1, 3, 6, 24 and 72 hr after the induction of pleurisy. The level of TNF-α (A) in the pleural lavage supernatant was determined by specific ELISA whilst the level of IL-6 (B) was determined by CBA analysis (*= $p<0.05$ vs CD11b-DTR group, n= 5 mice per group per time point).

and 4.9B). The effect of Mϕ ablation upon IL-10 levels was less dramatic but a significant inhibitory effect upon IL-10 levels at the time points of 1, 3 and 6h was seen (Figure 4.10A). IL-12 levels were also reduced with Mϕ ablation at 24h (Figure 4.10B). In contrast, IFN-γ levels were low and comparable between DT treated CD11b-DTR mice and FvB/N control mice at each time point suggesting that resident Mϕ are not a significant source of IFN-γ in this model.

4.2.8 Chemokine and cytokine responses of pleural cell populations *in vitro* are Mϕ dependent.

Since pleural mesothelial cells may be an important source of chemokines, I performed additional *in vitro* studies to determine the production of chemokines and cytokines by carrageenan stimulated pleural cell populations that had been depleted of Mϕ. Immunomagnetic Mϕ depletion using antibodies for the Mϕ specific marker F4/80 resulted in 98% depletion of Mϕ from pleural cell populations whilst B cell and MC numbers were comparable between groups. Stimulation of control Mϕ-rich pleural cell populations for 6 hours with 0.25% carrageenan resulted in significant production of MIP-2 and KC (Figure 4.11). In contrast, no significant chemokine production was evident following stimulation of pleural cell populations depleted of resident Mϕ but containing B cells, T cells and MC thereby indicating that production of these C-X-C chemokines *in vitro* was completely Mϕ dependent. Indeed, since an equivalent number of cells were stimulated with carrageenan the Mϕ-depleted pleural cell population would have had an increased proportion of both

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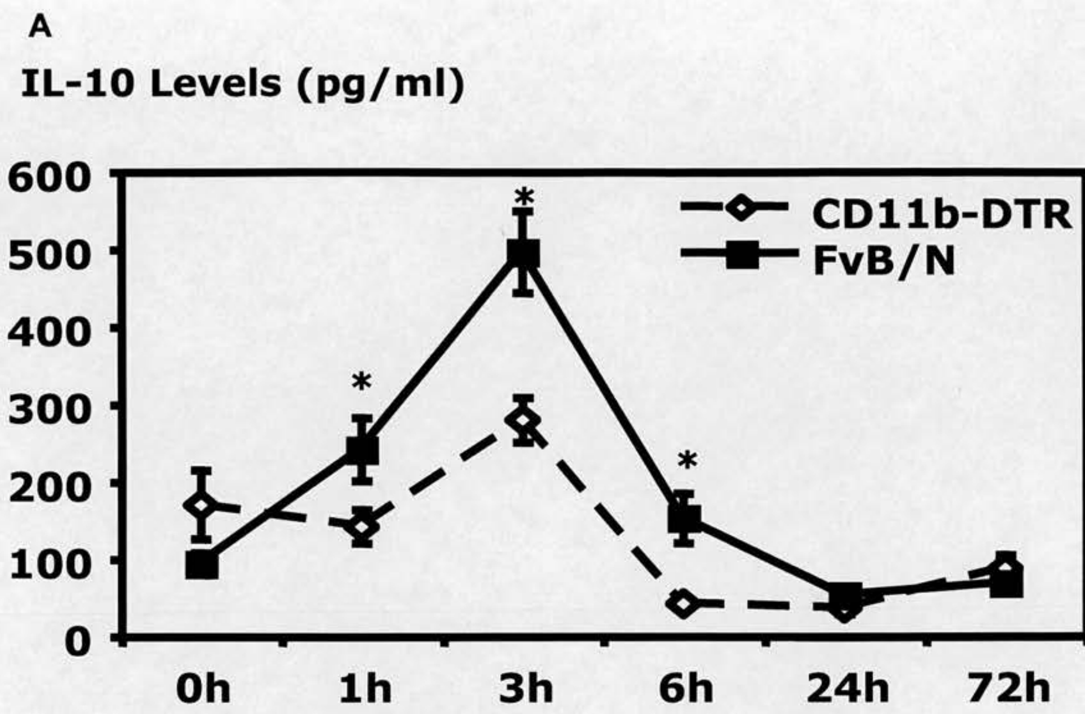

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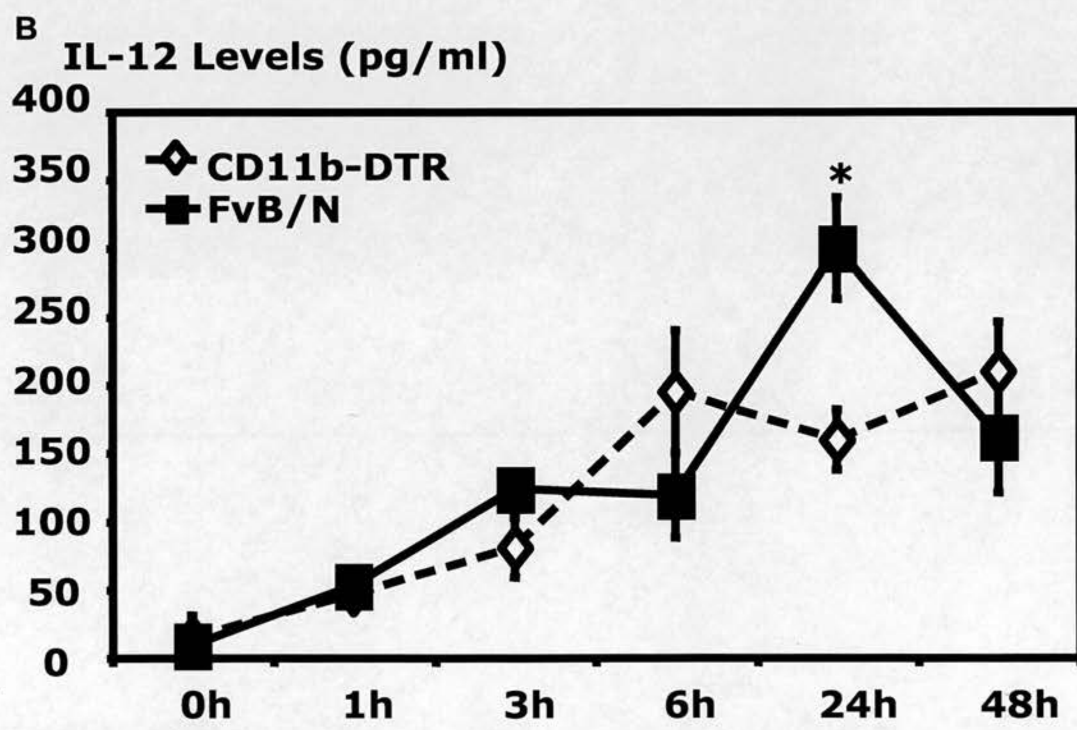


Figure 4.10 - Resident Mϕ ablation attenuates IL-10 and IL-12 production in carrageenan-induced pleurisy.

CD11b-DTR and FvB/N control mice were injected with DT (25 ng/g body weight) 24 hours prior to carrageenan injection. Pleural lavage was performed 1, 3, 6, 24 and 72 hr after the induction of pleurisy. The levels of IL-10 (A) and IL-12 (B) were determined by CBA analysis (*= $p<0.05$ vs CD11b-DTR group, n= 5 mice per group per time point).

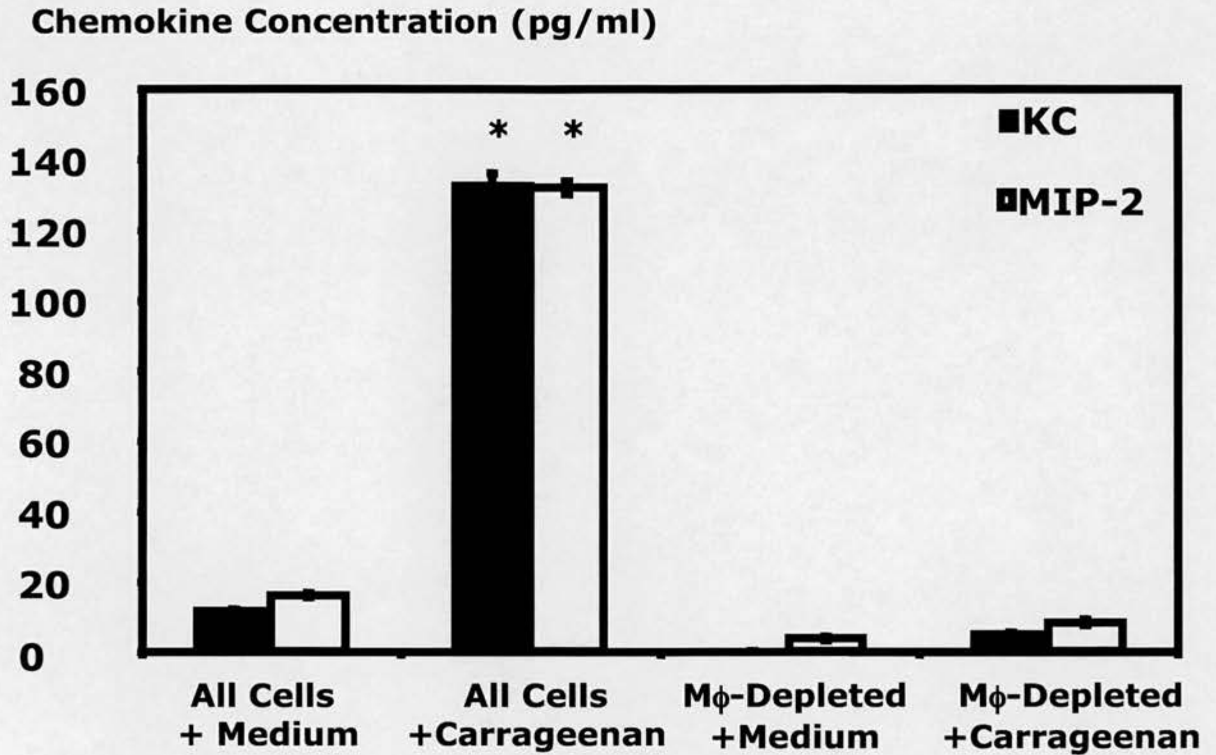


Figure 4.11 - *In vitro* production of MIP-2 and KC following carrageenan stimulation is Mϕ dependent.

Resident pleural cells were harvested and immunodepleted of resident pleural Mϕ by incubation with PE-conjugated anti-F4/80 followed by incubation with anti-PE conjugated magnetic beads and passage over a magnetic column. Equivalent numbers of cells were plated (60,000 cells per well) and stimulated with 0.25% carrageenan or normal medium for 6h. Supernatants were harvested and analyzed by specific ELISA for MIP-2 and KC (*=$p<0.05$ vs all cells with medium, n=4 wells per condition).

B cells and MC compared to the control non-depleted pleural cell population. Thus, the absence of significant chemokine production strongly suggests that B cells or MC *per se* are not key players in chemokine generation in response to carrageenan. Limited production of MCP-1 was evident *in vitro* but this was also significantly reduced by depletion of resident Mϕ (25.3±5.3 vs 7.1±4.7 pg/ml; Mϕ-rich pleural cells vs Mϕ-depleted pleural cells; $p<0.05$). Analysis of *in vitro* cytokine production demonstrated that resident Mϕ were key cytokine producers as Mϕ depletion prior to carrageenan stimulation resulted in a reduction of 63%, 67% and 92% in the production of TNF-α, IL-10 and IL-6 respectively (Figure 4.12).

4.2.9 Pleural resident Mϕ ablation reduces PMN influx in response to *Staphylococcus aureus*.

Although the carrageenan model of pleurisy is a useful model of inflammation and has been used by many investigators to dissect inflammatory pathways, it was important to assess if resident Mϕ were involved in models of inflammation that were more closely related to clinical disease. Formalin fixed, fluorescently labelled *Staphylococcus aureus* were therefore instilled into the pleural cavity and this induced a marked PMN infiltrate at the 4 hr time point (> 1.5×10^6 PMN). The ablation of resident Mϕ significantly reduced PMN infiltration following the administration of *Staphylococcus aureus* (Figure 4.13). Comparable PMN infiltration was also found in DT treated FvB/N control mice and PBS treated CD11b-DTR mice reinforcing the fact that the insertion of the transgene had no

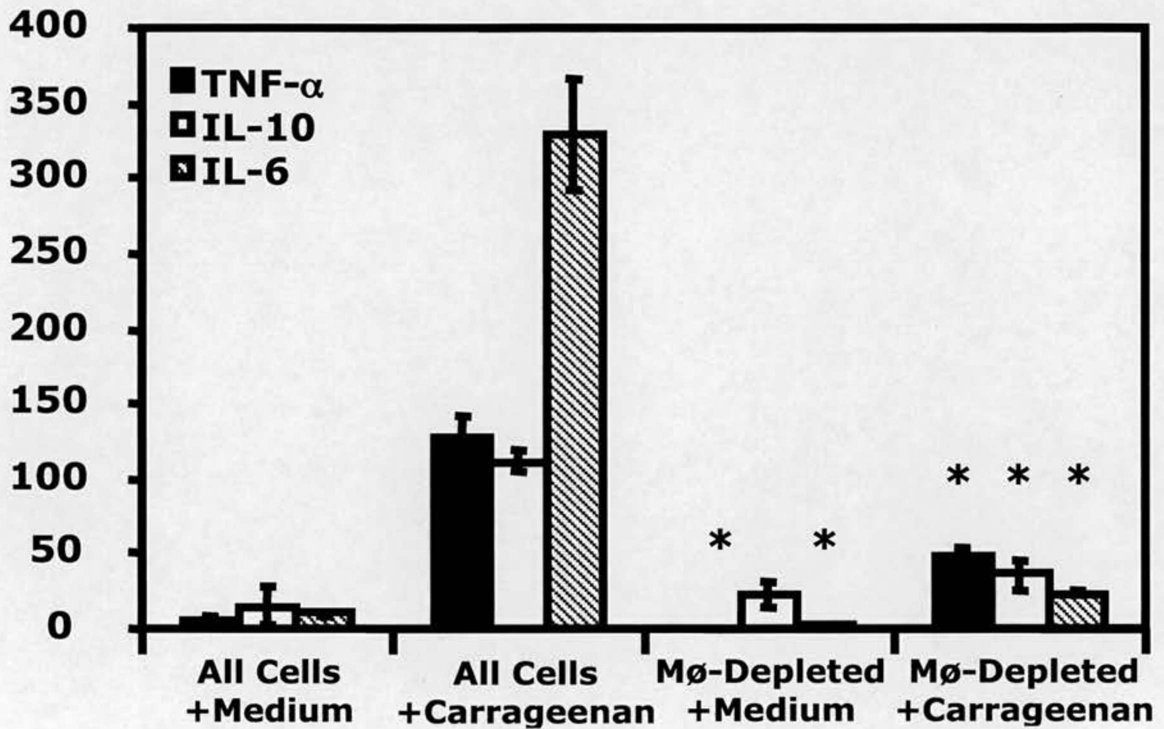


Figure 4.12 - *In vitro* production of the cytokines TNF-α, IL-10 and IL-6 following carrageenan stimulation is Mϕ dependent.

Resident pleural cells were harvested and immunodepleted of resident pleural Mϕ by incubation with PE-conjugated anti-F4/80 followed by incubation with anti-PE conjugated magnetic beads and passage over a magnetic column. Equivalent numbers of cells were plated (60,000 cells per well) and stimulated with 0.25% carrageenan or normal medium for 6h. Supernatants were harvested and analyzed by specific ELISA for TNF-α and by CBA for IL-10 and IL-6 (*=$p<0.05$ vs the corresponding all cells group, n=4 wells per condition).

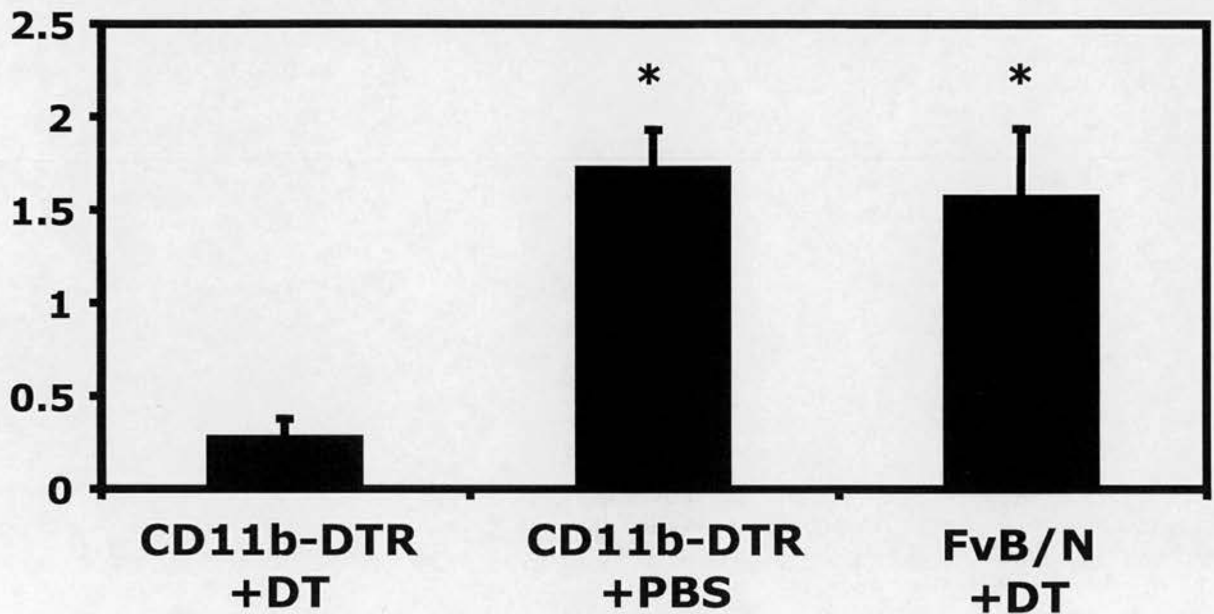


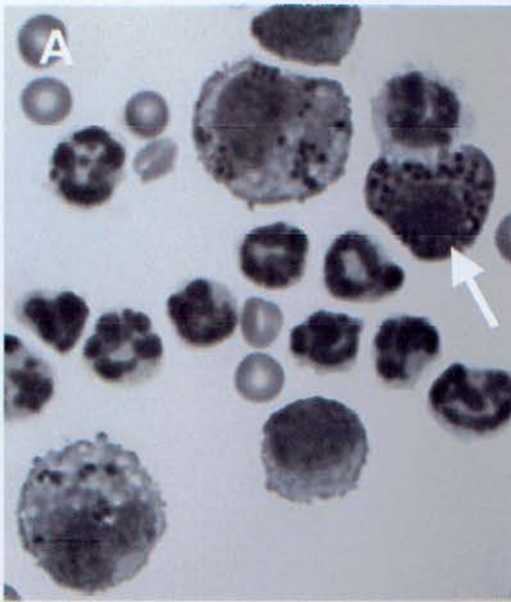
Figure 4.13 - Resident Mϕ ablation 24 hours prior to the administration of formalin fixed *Staphylococcus aureus* significantly blunts PMN recruitment.
$3x10^6$ formalin fixed fluorescently labelled *Staphylococcus aureus* bacteria were instilled into the pleural cavity of CD11b-DTR and FvB/N control mice 24h after DT treatment with PBS-treated CD11b-DTR serving as an additional control. Pleural lavage was performed at 4h following the administration of *Staphylococcus aureus*. Lavaged cells were stained for GR1 and counted by flow cytometry (* = $p<0.05$ vs DT-treated CD11b-DTR group, n=4 mice per group).

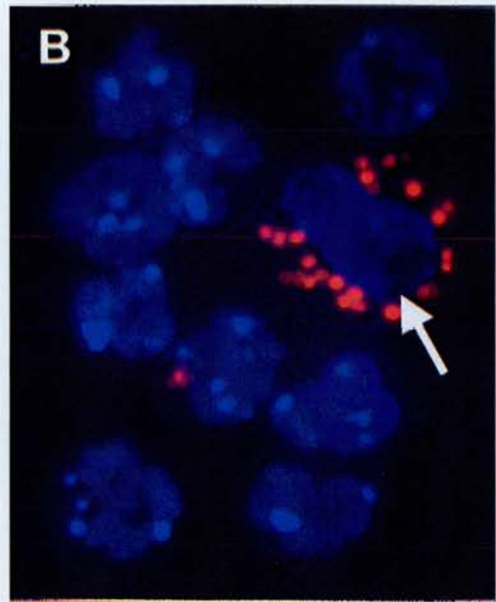
significant effect upon the generation of acute inflammatory responses (Figure 4.13) with comparable findings evident following the administration of carrageenan. An extended time course study was not undertaken in this *Staphylococcus aureus* mediated model but the significant reduction in PMN influx that followed prior ablation of resident pleural Mϕ provided 'proof of concept' that resident pleural Mϕ are likely to represent key orchestrators of PMN influx in pathogen induced inflammation.

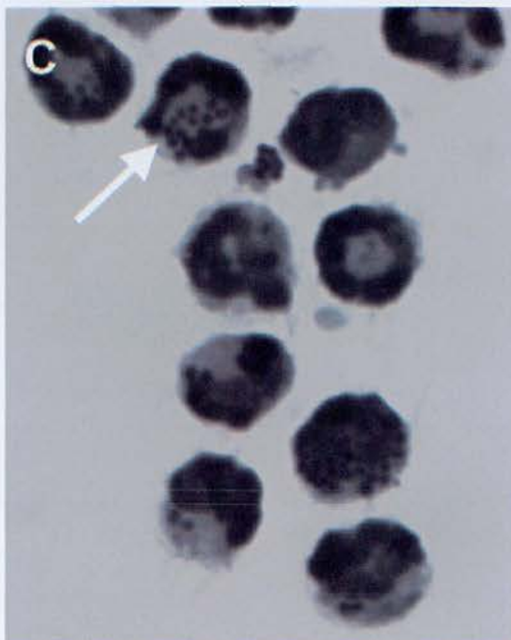
Cytospin preparations of pleural lavage cells from the 4h time point indicated prominent ingestion of *Staphylococcus aureus* particles by Mϕ in DT treated FvB/N control mice (Figure 4.14A and B) with very limited uptake by PMN. In contrast, in the absence of Mϕ, DT treated CD11b-DTR mice exhibited marked ingestion of *Staphylococcus aureus* particles by PMN (Figure 4.14C and D).

4.3 Summary

The CD11b-DTR mice are a potent experimental conditional Mϕ ablation system. DT administration depletes >96% of pleural resident Mϕ at 24h. In these transgenic mice, pleural Mϕ ablation was shown to blunt PMN recruitment following the intrapleural administration of carrageenan and formalin fixed *Staphylococcus aureus* bacteria. Mϕ repletion studies demonstrated that the adoptive transfer of Mϕ-rich pleural cells or purified pleural Mϕ alone at the same time as carrageenan administration resulted in a significantly greater PMN influx compared to that seen in







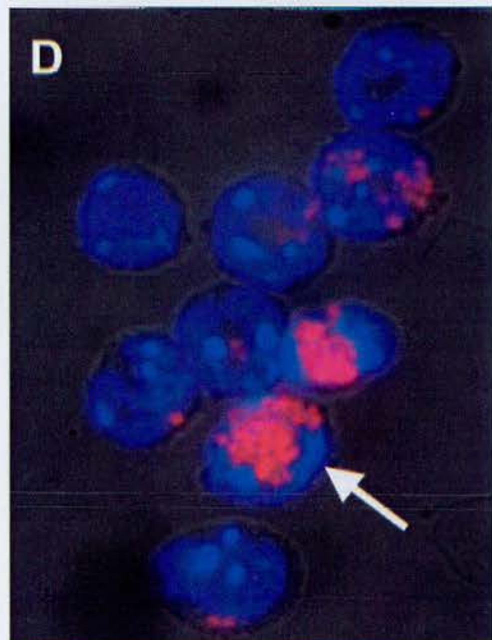


Figure 4.14 - Resident Mϕ exhibit prominent phagocytosis of formalin fixed *Staphylococcus aureus*.

Photomicrographs of Diffquick stained (A and C) or Hoechst stained (B and D) cytospin preparations of pleural lavage cells from either DT-treated FvB/N mice (A and B) or DT-treated CD11b-DTR mice (C and D) 4 hr after the administration of $3x10^6$ formalin fixed fluorescently labelled *Staphylococcus aureus* bacteria. Prominent ingestion of *Staphylococcus aureus* particles by Mϕ is evident in DT treated FvB/N control mice (examples arrowed in A and B) with very limited uptake by PMN. In contrast, DT-treated CD11b-DTR mice exhibit marked PMN ingestion of *Staphylococcus aureus* particles (examples arrowed in C and D)(100X magnification).

Mϕ ablated CD11b-DTR control mice after carrageenan administration. In contrast, the adoptive transfer of Mϕ-depleted pleural cells at the same time as carrageenan did not restore PMN infiltration. In these experiments, it was not possible to completely restore the numbers of resident pleural Mϕ at the initiation of inflammation and this may well account for the difference in PMN recruitment seen between FvB/N control mice and CD11b-DTR mice that had received either Mϕ-rich cells or Mϕ alone. Again, similar to the peritoneum, B and MC were present in the Mϕ-depleted transferred cell population but this cell transfer did not significantly recruit PMN thereby suggesting that B cells and MC did not make a major contribution to PMN recruitment despite the reduction in their numbers that follows DT administration. Chemokine analysis revealed that Mϕ-dependent *in vivo* production of MIP-2 plays a crucial role in PMN recruitment, whereas KC production was less affected by Mϕ depletion suggesting a non-Mϕ origin of KC. A similar conclusion could be reached for MCP-1 production. *In vivo* cytokine analysis revealed that TNF-α production was Mϕ dependent confirming the important role of Mϕ in the secretion of this initiator cytokine. IL-6 production was also reduced by Mϕ ablation and the major difference between the two groups was seen at the 3h time point, suggesting that TNF-α production probably precedes IL-6 production. The important cytokines, IL-10 and IL-12, were also reduced following Mϕ ablation, suggesting that Mϕ play a role in their production *in vivo*. *In vitro* studies demonstrated Mϕ-dependent production of both C-X-C chemokines and three cytokines (TNF-α, IL-6 and IL-10).

Mϕ ablation also reduced PMN recruitment after the instillation of formalin fixed *Staphylococcus aureus* bacteria into the pleural cavity. These experiments also highlighted the importance of the resident Mϕ as a phagocyte. Mϕ phagocytosis of bacteria was prominent in FvB/N control mice with scanty bacteria evident in PMN. In contrast, the bacteria were ingested by PMN in Mϕ ablated CD11b-DTR mice in the absence of the 'professional phagocyte' i.e. the Mϕ. In conclusion, the experiments highlighted in this chapter indicate that the pleural resident Mϕ plays a crucial role in the initiation of inflammation and PMN recruitment in pleural inflammation.

Chapter 5: The role of lymphocytes in experimental peritoneal inflammation

5.1. Introduction

The experiments outlined in chapter 3 examined the role of resident peritoneal Mϕ and MC in experimental peritonitis induced by administration of BTG. Resident peritoneal Mϕ were found to be critically important in the orchestration of PMN recruitment with adoptive cell transfer experiments and *in vitro* assays of chemokine production suggesting that PMN recruitment was MC independent. In addition to resident peritoneal Mϕ and MC the peritoneal space contains many lymphocytes which constitute nearly 50% of resident peritoneal leukocytes in humans (Broche and Tellado, 2001; Kubicka et al., 1996; Lewis and Holmes, 1991) and almost 14% of resident peritoneal leukocytes in mice although there are variations between strains (Gjomarkaj et al., 1999). The function of lymphocytes during peritoneal inflammation has not been explored in detail (Faull, 2000). Although the work outlined in chapter 3 indirectly examined the role of lymphocytes in experiments involving the adoptive transfer of Mϕ depleted peritoneal cells to Mϕ-depleted CD11b-DTR mice, I became interested in whether the presence of various lymphocyte populations may modulate the function of resident Mϕ including their subsequent chemokine and cytokine responses and resultant PMN recruitment. In addition, the previous studies involving conditional Mϕ ablation in experimental peritonitis did not explore the recruitment of monocytes and Mϕ to the inflamed peritoneum as the administration of DT resulted in a

dramatic reduction in the number of circulating monocytes. I was therefore interested in addressing the hypothesis that peritoneal lymphocytes may modulate the recruitment of monocytes/Mϕ to the inflamed peritoneum.

B cells represent an important component of the resident peritoneal lymphocyte population in mice (Hayakawa et al., 1985). The peritoneum contains B cells which are different from circulating B cells (Herzenberg, 2000). B-1 cells are involved in the synthesis of immunoglobulins (Ig) and natural antibodies (IgM) (Herzenberg, 2000) and have been shown to play an important role in different models of inflammation such as filarial (Paciorkowski et al., 2000) and bacterial infection (Boes et al., 2000) and delayed-type hypersensitivity (Szczepanik et al., 2003).

The T cell populations within the peritoneum are also quite different from circulating T cells with around 17% of peritoneal T cells expressing the γδ TcR (Hartman et al., 1995). The majority of peritoneal T cells exhibit markers of thymus-dependent origin and surface antigens compatible with an activation and memory phenotype. However there are peritoneal T cells with a phenotype indicating that the peritoneum is capable of thymus-independent differentiation of T cells (Hartman et al., 1995). The γδ T cells could have an important role in the regulation of the early inflammatory response and it is therefore of interest that γδ T cell KO mice exhibit increased pulmonary inflammation with increased numbers of recruited PMN and Mϕ (Zachariadis et al., 2006). It was suggested that γδ Tcells are important in regulating the extent of the inflammatory response in order to prevent excessive

tissue damage. However, to my knowledge there are no studies that have directly examined the role of T cells in experimental peritonitis.

There are a number of mice available that are deficient in various lymphocyte populations and such mice may be used to determine whether lymphocytes may modulate leukocyte recruitment. I therefore examined the inflammatory phenotype RAG-1 KO mice (mature B and T cell deficient), NUDE mice (T cell deficient), μMT mice (B cell deficient) and their respective controls in the experimental model of BTG peritonitis. I also wished to compare the experimental data between the three different lymphocyte deficient mice as previous studies have noted different effects on leukocyte recruitment depending on the inflammatory model used (Appelberg, 1992; Burne et al., 2001; Burne-Taney et al., 2003; Chen et al., 2002; Hancock et al., 2002; Morrison et al., 2006; Zwacka et al., 1997).

5.2. Results

5.2.1. RAG-1 knock-out mice exhibit increased early PMN infiltration and reduced monocyte recruitment at later time points.

The RAG-1 KO mice are on the C57BL/10 background and these mice were used as controls. C57BL/10 mice exhibited a peak in PMN numbers at 24h and a peak in monocyte/Mϕ numbers at 72h following the administration of BTG (Figure 5.1). In contrast, RAG-1 KO mice exhibited markedly increased PMN numbers at 8h

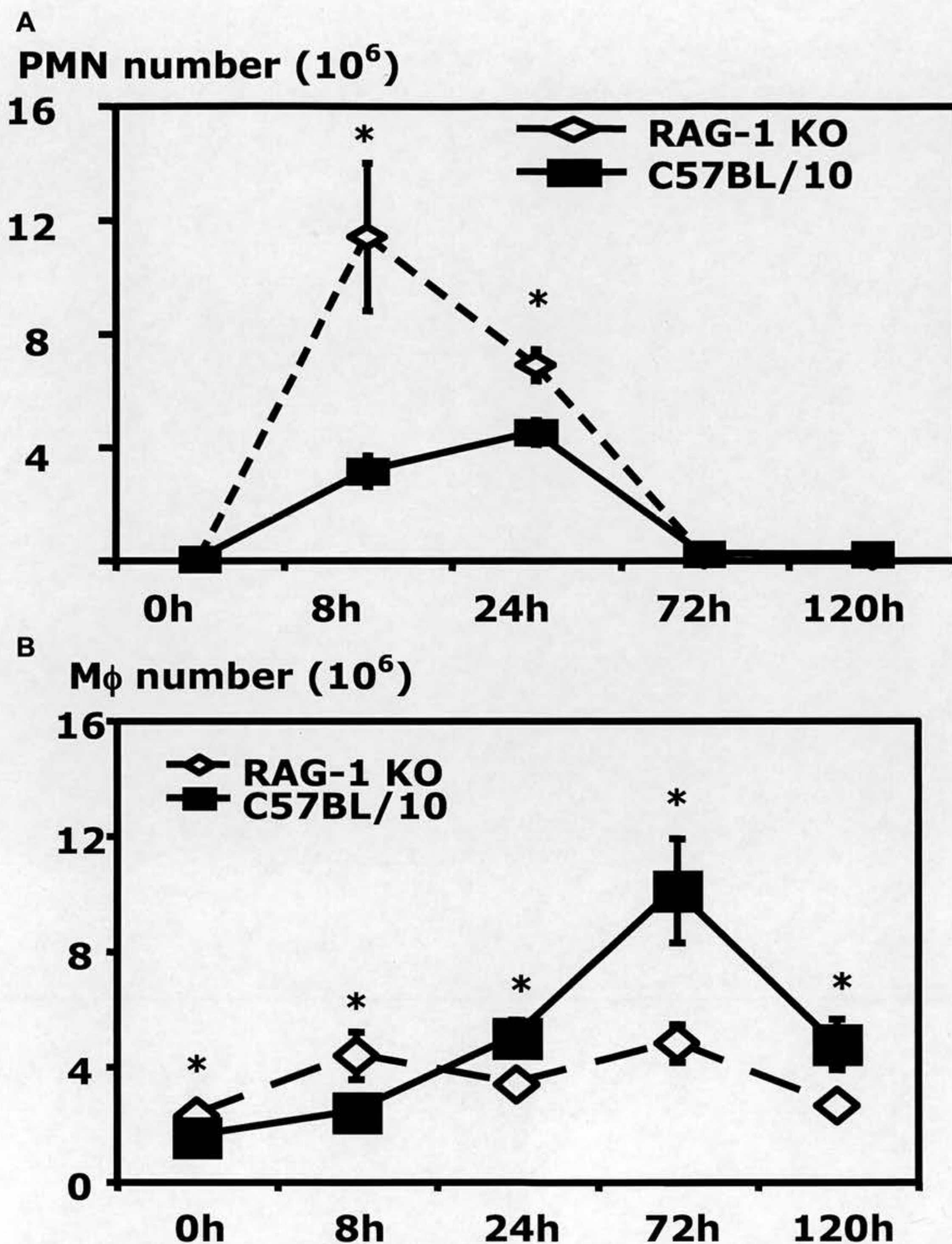


Figure 5.1 - RAG-1 KO mice exhibit increased PMN influx and reduced monocyte/Mϕ recruitment at later time points in BTG peritonitis.
1ml of 3% BTG was administered to RAG-1 KO and C57BL/10 WT mice and peritoneal lavage was performed at 0, 8, 24, 72 and 120h. Lavaged cells were stained for GR1 and F4/80 and counted by flow cytometry. The numbers of PMN (A) and monocyte/Mϕ (B) are depicted (* = $p<0.05$, RAG-1 KO mice vs C57BL/10 mice, n=6 mice per group per time point).

compared to C57BL/10 control mice with a significant difference still apparent at 24h (Figure 5.1A). The PMN infiltration had resolved by 72 h in both experimental groups. Examination of the numbers of monocyte/Mϕ indicated that RAG-1 KO mice exhibited an initial increase in monocyte/Mϕ number at 8h compared to C57BL/10 mice. Despite this initial increase, however, RAG-1 KO exhibited a profoundly reduced monocyte/Mϕ influx at all other time points examined with a particularly striking difference evident at 72 h (Figure 5.1B).

I also performed a preliminary experiment using the model of zymosan peritonitis in order to establish if this recruitment pattern could be reproduced using a different pro-inflammatory agent. PMN and monocyte/Mϕ numbers were determined 24h after the administration of zymosan. Although this preliminary experiment only comprised of 3 mice per group, there was a slight trend towards increased PMN numbers and reduced monocyte/Mϕ numbers in RAG-1 KO mice (Figure 5.2).

In view of the differences evident between RAG-1 KO mice and C57BL/10 mice in BTG peritonitis, I analysed the numbers of peritoneal Mϕ, PMN, MC, B cells and T cells in non-manipulated mice. This revealed that RAG-1 KO mice predictably had reduced peritoneal lymphocytes but also had significantly less MC and significantly more Mϕ than C57BL/10 mice (Figure 5.3).

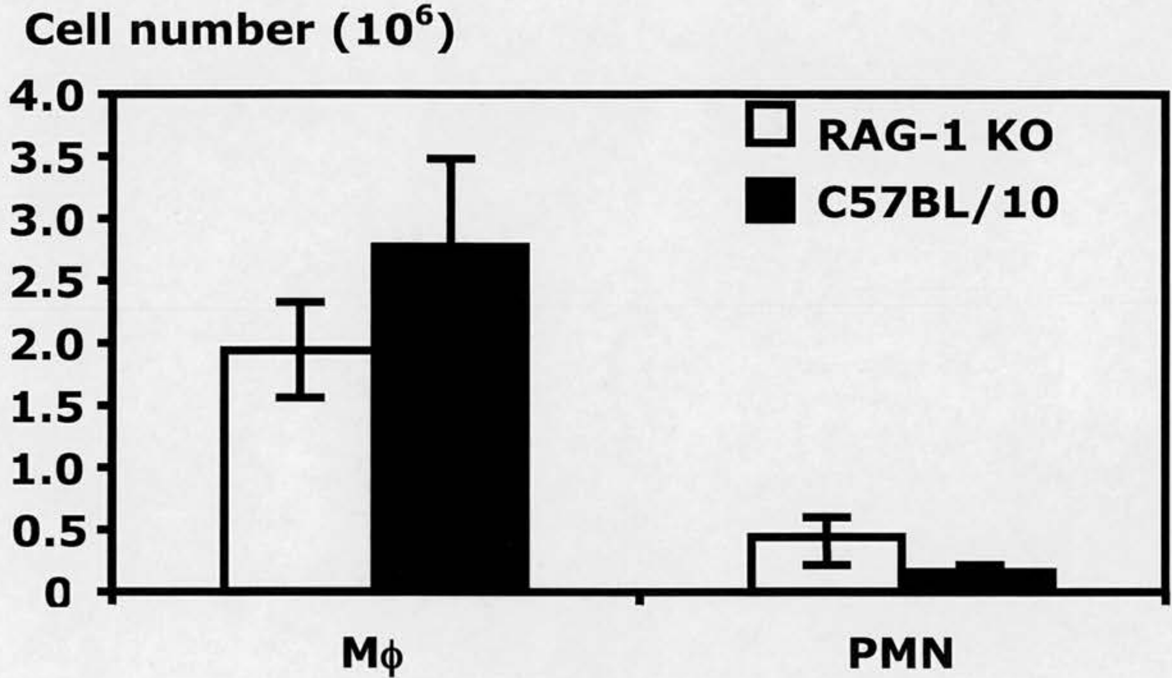


Figure 5.2 - RAG-1 KO mice exhibit a trend to an increased PMN influx and reduced Mϕ recruitment at the 24 hr time point in zymosan peritonitis.
0.2mg of zymosan was administered IP to RAG-1 KO and C57BL/10 WT mice. Peritoneal lavage was performed 24h following zymosan administration. Lavaged cells were stained for GR1 and F4/80 and counted by flow cytometry (n=3 mice per group).

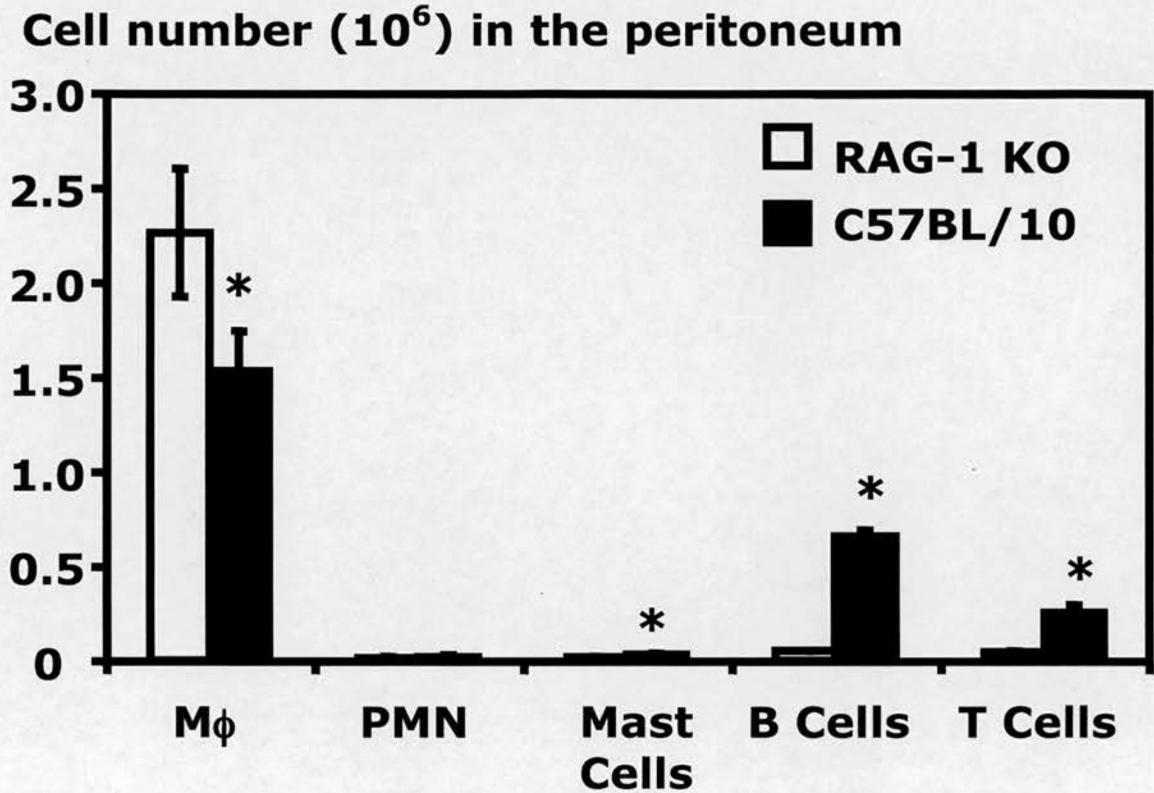


Figure 5.3 - RAG-1 KO mice exhibit an increased number of resident Mϕ compared to C57BL/10 mice but significantly less mast cells, B cells and T cells in the peritoneum.
Peritoneal lavage was performed in non-manipulated RAG-1 KO and C57BL/10 mice. The number of Mϕ, PMN, mast, B and T cells was determined by flow cytometry following staining for F4/80, GR1, c-kit, B220 and CD3 (*=$p<0.005$, RAG-1 KO mice vs C57BL/10 mice; n=6-12 mice per group).

I also examined the numbers of circulating leukocytes in non-manipulated RAG-1 KO and C57BL/10 mice. RAG-1 KO mice exhibited a profound B cell and T cell deficiency (Figure 5.4) but had comparable number of circulating PMN to C57BL/10 mice (Figure 5.4). Significant differences were found, however, in the numbers of circulating monocytes with RAG-1 KO mice exhibiting reduced monocyte numbers compared to C57BL/10 mice (Figure 5.4). These differences in the circulating and peritoneal leukocyte numbers may explain, at least in part, the phenotype of the RAG-1 KO mice during BTG peritonitis. Since the work outlined in chapter 3 indicated a key role for the resident peritoneal Mϕ in PMN recruitment, it is possible that the increased number of resident Mϕ present in RAG-1 KO mice underlies the significant increase in PMN recruitment seen at the 8h and 24h time point in RAG-1 KO mice. The differing numbers of circulating monocytes between RAG-1 KO and C57BL/10 mice also represents a confounding variable and may explain the reduced Mϕ numbers evident at late time points between RAG-1 KO and C57BL/10 mice. The increased monocyte/Mϕ numbers evident at 8h is not explained by the circulating monocyte counts but may reflect the differing numbers of resident peritoneal Mϕ evident in the non-manipulated peritonea of RAG-1 KO and C57BL/10 mice. I therefore went on to examine the levels of chemokines present during peritoneal inflammation in RAG-1 KO and C57BL/10 mice.

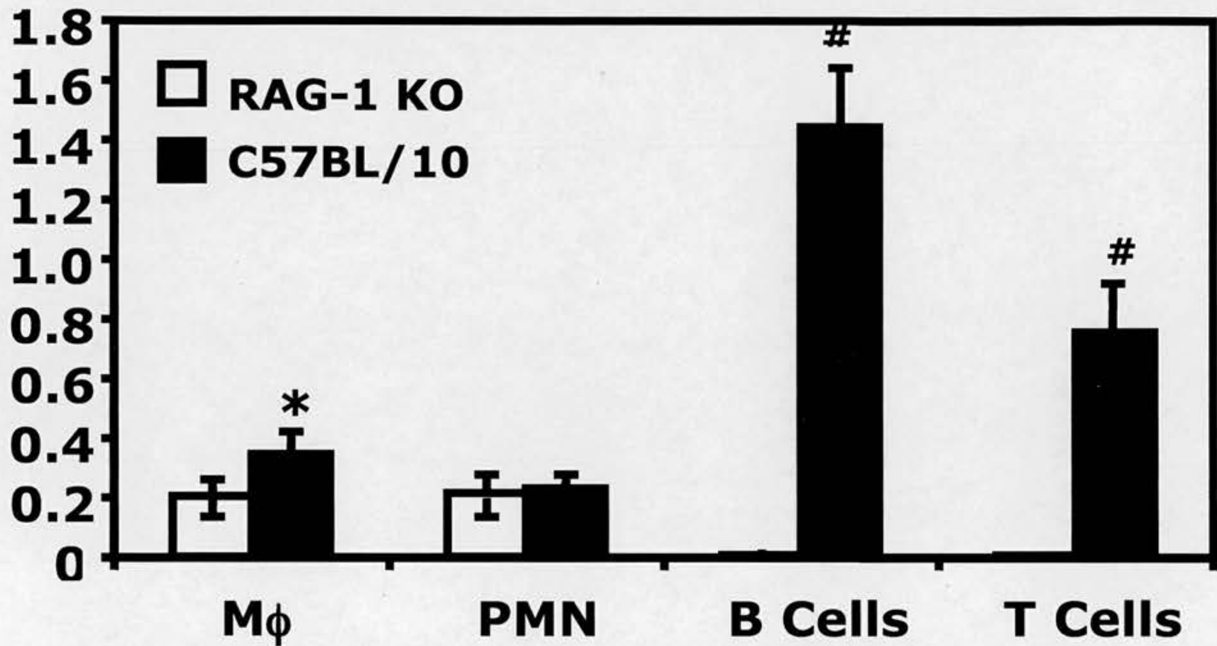


Figure 5.4 - RAG-1 KO have comparable circulating PMN numbers to C57BL/10 mice but significantly less circulating monocytes and lymphocytes.

Circulating blood leukocyte numbers were compared in non-manipulated RAG-1 KO and C57BL/10 mice. The number of Mϕ, PMN, B and T cells was determined by flow cytometry following staining for F4/80, GR1, B220 and CD3 (*=p<0.01 and #=p<0.0001, RAG-1 KO mice vs C57BL/10 mice; n=6 mice per group).

5.2.2. RAG-1 KO exhibit reduced MCP-1 levels at 24h.

RAG-1 KO exhibited a 50% reduction in MCP-1 levels 24h following BTG administration (20.4±2.3 pg/ml vs 42.8±7.2; RAG-1 KO vs C57BL/10; $p<0.01$; $n=4$ mice per group) and this may partly underlie the impaired monocyte/Mϕ recruitment evident at later time points. However, no statistically significant differences between experimental groups were found for any of the other cytokines and chemokines analysed. These included the cytokines TNF-α, IFN-γ, IL-10, IL-6 and IL-12 and the C-X-C chemokines MIP-2 and KC. Unfortunately, I was unable to perform complete cytokine analysis at the very early time points, however no differences were found in the levels of the C-X-C chemokines MIP-2 and KC at 1h and 3h after BTG administration. Since RAG-1 KO mice have an increased numbers of resident peritoneal Mϕ, I would have expected an increased MIP-2 and KC production based on the work outlined in chapter 3. Thus, other C-X-C chemokines might be involved.

5.2.3. NUDE mice recruited more PMN at 8h and 24h and more monocytes at 24h.

The NUDE mice are on the CD1 background and these mice were therefore used as controls. CD1 mice exhibited a peak in PMN numbers at 8h and 24h but monocyte/Mϕ numbers were not striking elevated with similar numbers evident at the 8h, 24 and 72h time points following the administration of BTG (Figure 5.5).

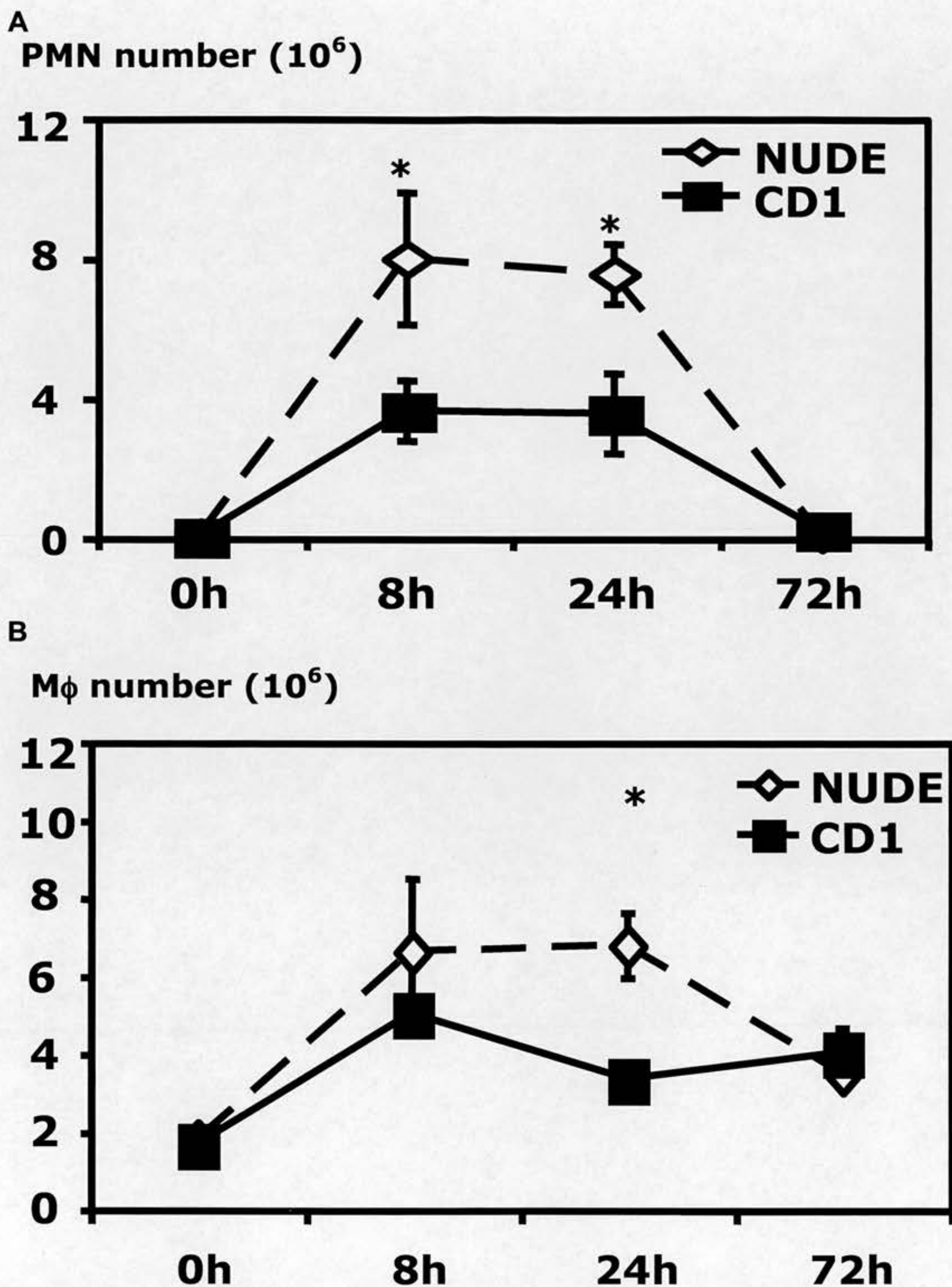


Figure 5.5 - NUDE mice have an increased PMN influx at 8 and 24h and increased monocyte/Mϕ recruitment at 24h following BTG peritonitis. 1ml of 3% BTG was administered to NUDE and CD1 mice. Peritoneal lavage was performed at 0, 8, 24 and 72 h following BTG. Lavaged cells were stained for GR1 and F4/80 and counted by flow cytometry. PMN (A) and monocyte/Mϕ (B) number progression are depicted (* = $p<0.05$, NUDE mice vs CD1 mice; n=6 mice per group per time point). There was one outlier at 8h and one at 24h in the CD1 group.

NUDE mice exhibited markedly increased PMN numbers at both 8h and 24h compared to CD1 mice (Figure 5.5A). The PMN infiltration had resolved by 72 h in both experimental groups. Examination of the numbers of monocyte/Mϕ indicated that NUDE mice exhibited increased numbers of monocyte/Mϕ at 24h compared to CD1 mice (Figure 5.5B).

Analysis of the numbers of peritoneal and circulating leukocytes in NUDE and CD1 mice was informative as they may be similar to RAG-1 KO mice and differ in the numbers of resident peritoneal Mϕ and circulating monocytes. Interestingly, however, NUDE mice have comparable numbers of resident peritoneal Mϕ to CD1 mice (Figure 5.6) but do exhibit significantly reduced numbers of peritoneal MC and B cells at baseline (figure 5.6). Thus the increased PMN recruitment in NUDE mice cannot be explained by a difference in resident peritoneal Mϕ number. Analysis of circulating leukocyte numbers indicated that NUDE and CD1 mice had comparable numbers of circulating blood monocytes but NUDE mice did exhibit a trend to increased circulating PMN numbers ($p=0.09$ between NUDE and CD1 mice (Figure 5.7). It may therefore be the case that the increased peritoneal PMN numbers at 8h and 24h may be explained by this trend towards more circulating PMN in the NUDE compared to CD1 mice. However, the increased numbers of peritoneal monocyte/Mϕ numbers in NUDE mice at the 24h time point during peritoneal inflammation is not secondary to differing numbers of circulating monocytes.

It is interesting that the pattern of increased PMN recruitment in NUDE mice is comparable to that observed in RAG-1 KO mice suggesting that T cells may be

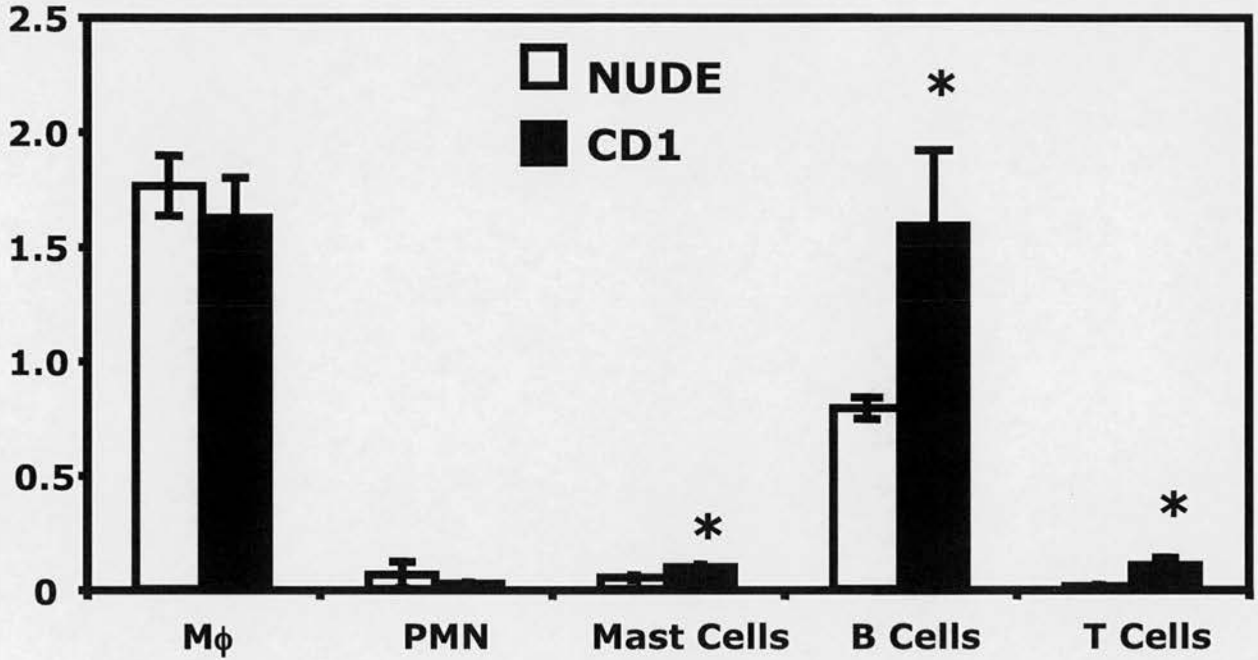


Figure 5.6 – NUDE mice have less peritoneal mast cells and B cells at baseline compared to C57BL/10 mice.
Peritoneal lavage was performed in non-manipulated NUDE and CD1 mice. The number of Mϕ, PMN, mast, B and T cells was determined by flow cytometry following staining for F4/80, GR1, c-kit, B220 and CD3 (*=$p<0.05$, NUDE mice vs CD1 mice; n=4-6 mice per group).

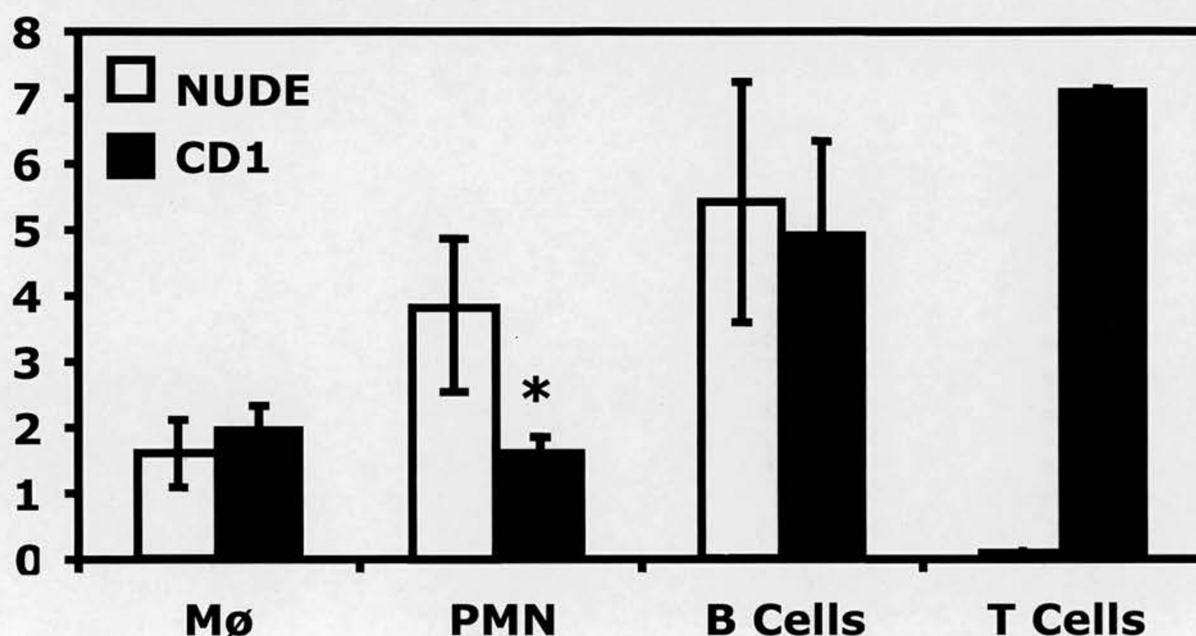


Figure 5.7 – NUDE mice have a trend towards increased circulating PMN numbers compared to control CD1 mice and comparable numbers of circulating monocytes.
Circulating blood leukocyte numbers are compared between non-manipulated NUDE and CD1 mice. The number of Mϕ, PMN, B and T cells was determined by flow cytometry following staining for F4/80, B220, CD3 and GR1 (*=$p>0.09$, NS; NUDE mice vs CD1 mice; n=4 mice per group).

capable of modulating PMN recruitment. In contrast, the monocyte/Mϕ recruitment profile in NUDE mice differs from that observed in RAG-1 KO mice and this suggests that T cells are not critical regulators of Mϕ influx.

5.2.4. The adoptive transfer of T cells to NUDE mice did not normalise PMN influx.

In order to evaluate if leukocyte recruitment in NUDE mice could be normalised, I used the adoptive cell transfer strategy. Approximately 50,000 peritoneal T cells purified by negatively immunoselection from CD1 mice were transferred into NUDE mice (Rescued NUDE). This resulted in reconstituting peritoneal T cell numbers to about 50% of that found in the non-manipulated CD1 peritoneum. In this experiment, approximately 150,000 Mϕ were also transferred with the T cells and this represents a confounding variable as my previous work indicated a prominent role for Mϕ in the recruitment of PMN. However, 150,000 Mϕ represents less than 10% of the number of Mϕ present normally in NUDE or CD1 mice. Peritoneal lavage was performed 8h after the adoptive cell transfer and BTG administration. The numbers of recruited PMN evident in the rescued NUDE mice was comparable to the control group of NUDE mice (Figure 5.8). It should be noted that this experiment was performed with 3 mice per group.

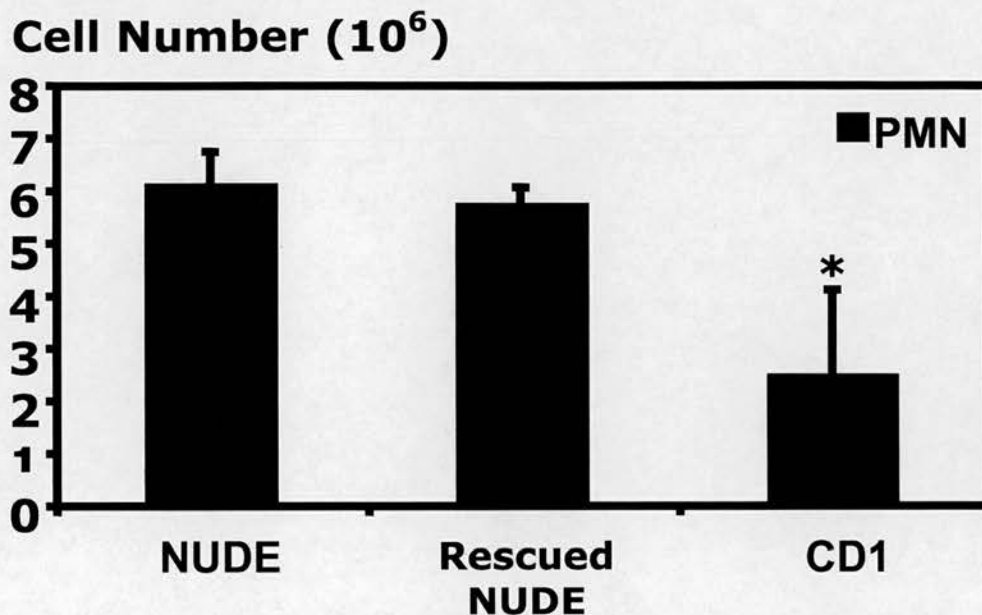


Figure 5.8 – The adoptive transfer of T cells to NUDE mice does not normalise peritoneal PMN influx.
Peritoneal lavages from CD1 were pooled and T cells were negatively selected by immunodepletion. Approximately 50,000 T cells were transferred into NUDE mice at the same time as 3% BTG and peritoneal lavage was perfomed 8h later. Lavaged cells were stained for GR1 and PMN were then counted by flow cytometry (* = $p<0.05$, vs NUDE: n=3 mice per group).

5.2.5. *In vivo* chemokine/cytokine analysis reveals increased levels of KC, MCP-1 and IL-6 at various time points in NUDE mice compared to CD1 mice.

The levels of the chemokines MIP-2, KC and MCP-1 and the cytokines IFN-γ, TNF-α, IL-6 and IL-10 in peritoneal lavage fluid were determined by ELISA at the 1h, 3h, 8h, 24h and 72h time points.

Interestingly, despite the increased PMN recruitment evident in NUDE mice, there were no significant differences in the levels of MIP-2 between NUDE and CD1 mice at any time points (Figure 5.9A). Analysis of the C-X-C chemokine KC levels indicated a significantly higher KC level in NUDE mice at 8h compared to CD1 controls (Figure 5.9B). However, KC levels peaked at the earlier time points of 1h and 3h and the levels present at 8h were very much lower than these peak early values (Figure 5.9). Analysis of the levels of the C-C chemokine MCP-1 revealed similar profiles in both experimental groups with NUDE mice exhibiting significantly higher levels at the 3h time point (Figure 5.10A).

There were no differences in the levels of IFN-γ, TNF-α or IL-10 between experimental groups (data not shown). The only significant difference between groups was that NUDE mice had a higher level of IL-6 at the 8h time point (Figure 5.10B) though comparable peak values were found at 1h and 3h.

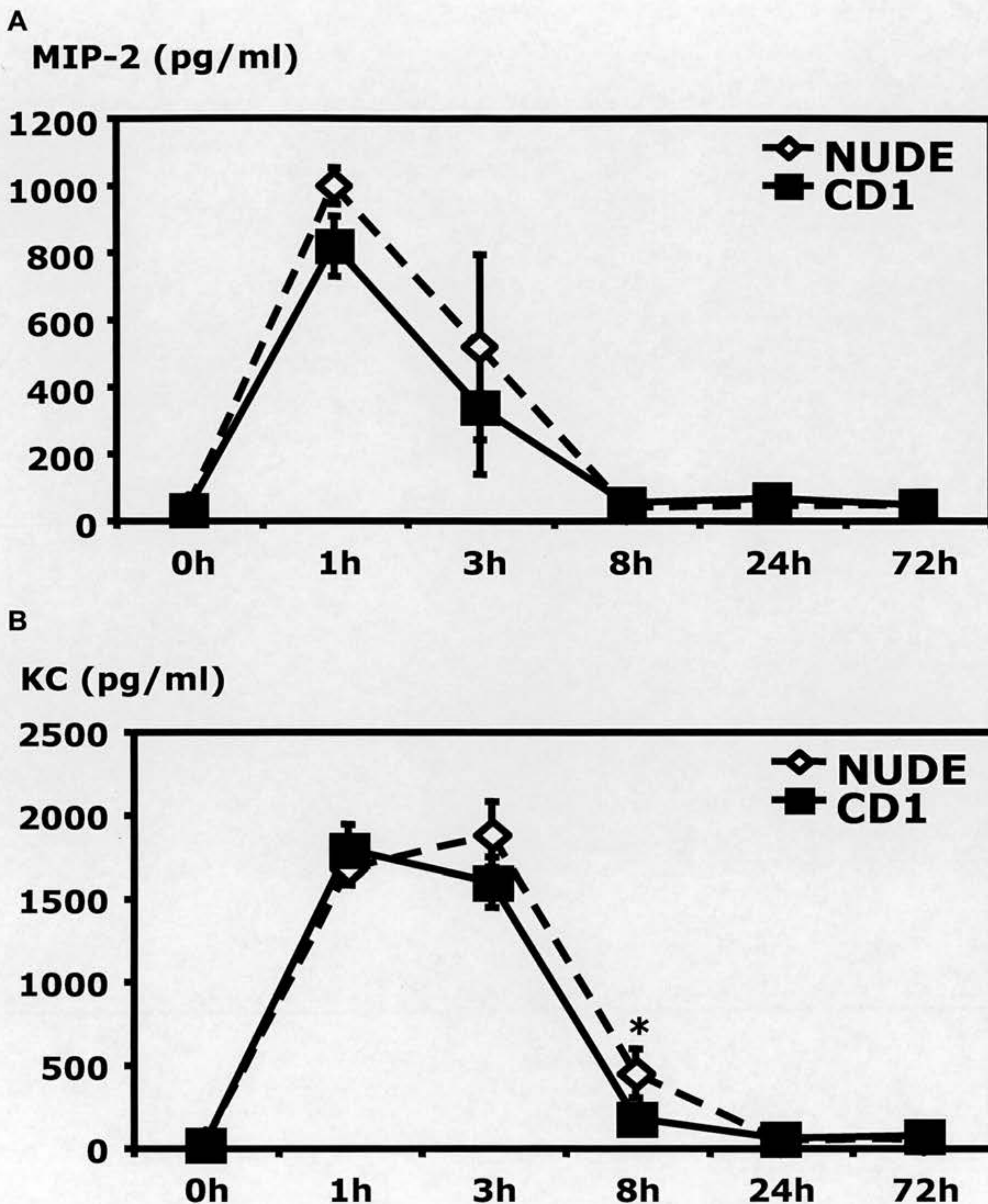
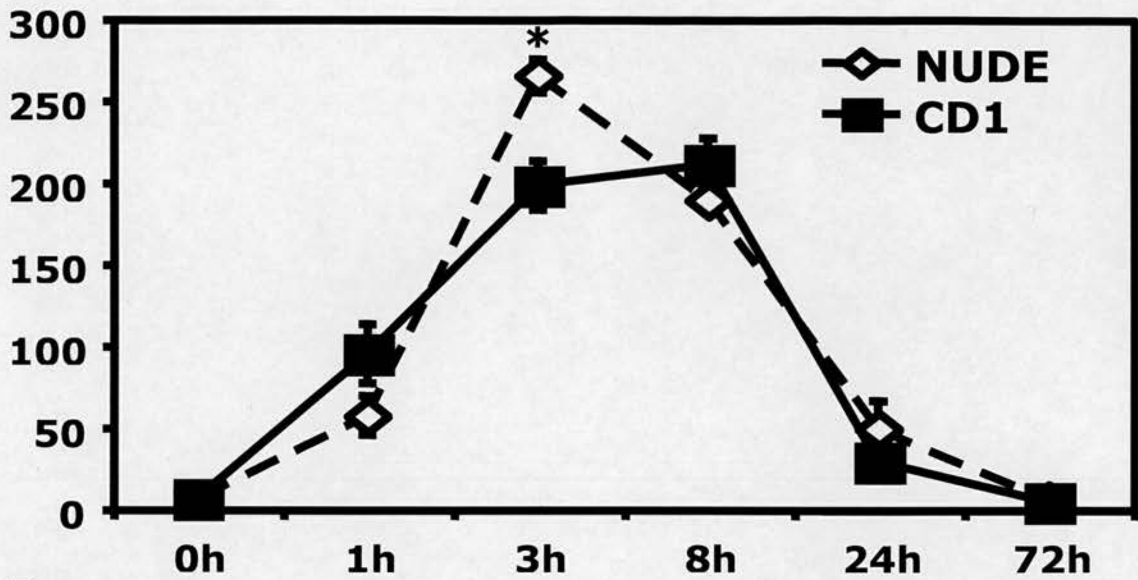


Figure 5.9 - Chemokine levels in NUDE and CD1 mice in BTG peritonitis: no difference in MIP-2 levels with increased KC at the 8h time-point.

NUDE and CD1 mice were injected with 1ml of 3% BTG. Peritoneal lavage was performed 1, 3, 8, 24 and 72 hr after the induction of peritonitis. The level of MIP-2 (A) and KC (B) in the peritoneal lavage supernatant was determined by specific ELISA (*= $p<0.05$ vs CD1 group, n= 6 mice per group per time point).

A



B

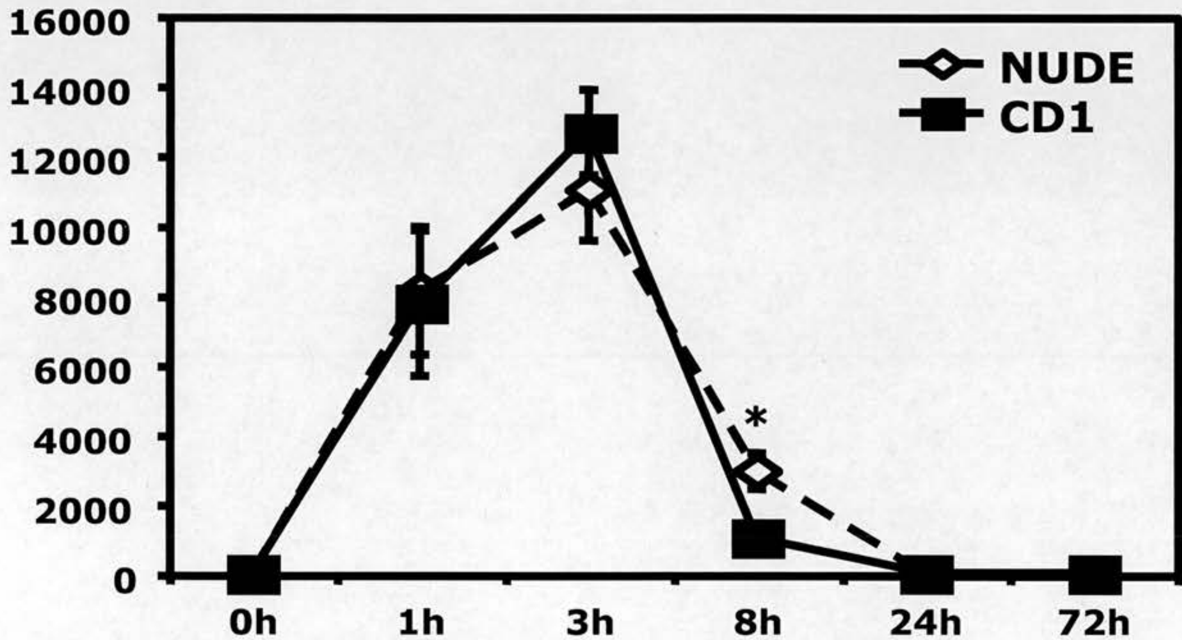


Figure 5.10 - MCP-1 and IL-6 time course in NUDE and CD1 mice in BTG peritonitis: higher levels of MCP-1 and IL-6 at 3h and 8h respectively. NUDE and CD1 mice were injected with 1ml of 3% BTG. Peritoneal lavage was performed 1, 3, 8, 24 and 72 hr after the induction of peritonitis. The level of MCP-1 (A) and IL-6 (B) in the peritoneal lavage supernatant was determined by specific ELISA (*=$p<0.01$ vs CD1 group, n= 6 mice per group per time point).

It is unclear whether the difference in KC level at 8h is sufficient to explain the increased PMN number found in NUDE mice at 8 and 24h after BTG and the tendency of NUDE mice to have more circulating PMN may also be involved. The increased MCP-1 level found in NUDE mice at the 3h time point is somewhat early to explain the difference in monocyte/Mϕ numbers at 24h but it does indicate that NUDE mice are able to produce more MCP-1. The higher IL-6 levels at 8h are consistent with a more prolonged inflammatory state.

5.2.6. Supernatant transfer from NUDE mice did not increase peritoneal PMN recruitment in CD1 mice, but did augment blood-circulating PMN mobilisation.

In the absence of clear cut chemokine differences to explain the different leukocyte kinetics seen in NUDE and CD1 mice, I went on to perform experiments involving the transfer of pro-inflammatory peritoneal supernatant between NUDE and CD1 mice in order to investigate whether there were other PMN chemotactic factors present or other factors that inhibited PMN recruitment. Peritoneal supernatant (SN) was obtained by performing peritoneal lavage on NUDE and CD1 mice 3h after the administration of BTG. This time point was chosen in view of the early increase in MIP-2 and KC levels found at 1h and 3h and the fact that PMN influx was markedly increased at the 8h time point. Pro-inflammatory peritoneal SN transfer was performed in a cross-over fashion with SN from NUDE mice being transferred to both naïve CD1 mice and NUDE mice and vice versa.

The IP injection of SN derived from either NUDE or CD1 mice did induce a mild PMN infiltrate (Figure 5.11A) though this was significantly less than that provoked by BTG at the same time point despite the presence of significant levels of C-X-C chemokines in the transferred supernatants (around 1200 pg/ml of KC and MIP-2 for both groups). This is likely to be secondary to the inevitable dilution of chemokines and cytokines etc. There were no differences in the numbers of peritoneal PMN recruited between NUDE and CD1 mice irrespective of whether they received SN from a NUDE or CD1 mouse (Figure 5.11A). The circulating PMN numbers in blood were also determined 8h after SN transfer. Despite the absence of differences in peritoneal PMN numbers, the transfer of NUDE SN significantly augmented circulating blood PMN numbers in CD1 mice compared to CD1 mice that had received SN derived from CD1 mice. Indeed, circulating PMN numbers were elevated to levels similar to those observed in NUDE mice that had received SN derived from NUDE mice (Figure 5.11B). There was also trend toward a reduction of circulating PMN in NUDE mice receiving CD1 SN compared to NUDE mice administered NUDE SN (Figure 5.11B). Thus, in these experiments it appeared that the number of circulating PMN was determined by the nature and source of the supernatant and not the recipient mouse into which it was administered. Since the number of circulating PMN in NUDE or CD1 mice receiving SN from NUDE mice were greater, this suggested NUDE SN was either 'more chemotactic' or contained a more robust signal for mobilising PMN from the bone marrow. However, the absence of any differences in the actual number of recruited peritoneal PMN highlights the complexity of leukocyte recruitment.

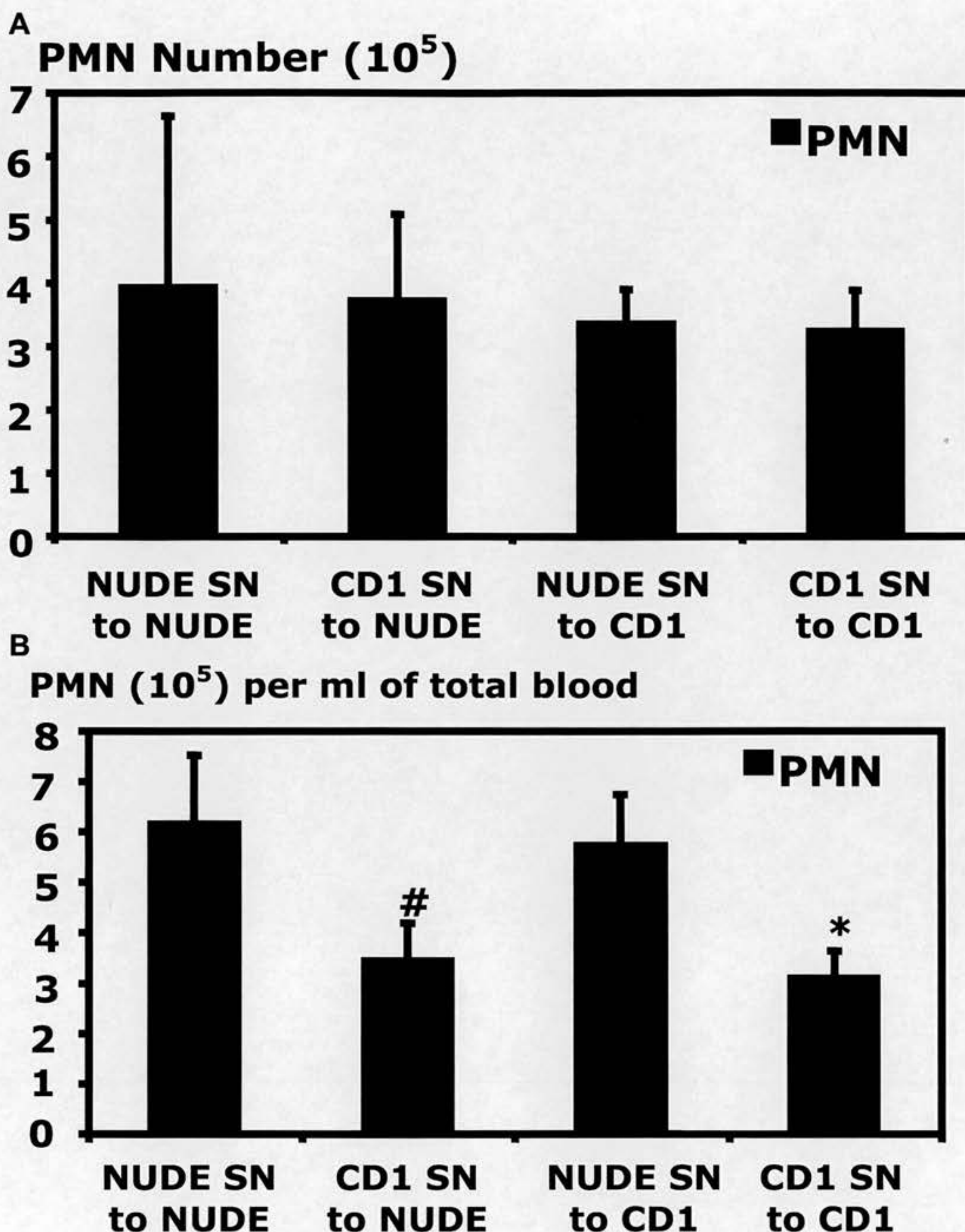


Figure 5.11 – The transfer of peritoneal supernatant from NUDE mice does not influence peritoneal PMN numbers but does increase circulating blood PMN mobilisation in CD1 mice.
NUDE and CD1 mice were injected with BTG and peritoneal lavages were performed after 3h. Peritoneal supernatants from NUDE and CD1 mice were injected back into naïve NUDE and CD1 in a cross-over fashion (see text). After 8h peritoneal cells were obtained by lavage (A) and circulating cells obtained from tail vein blood. Cells were stained for GR1 and counted by flow cytometry (n=4-5 per group; #=p=0.0549, not significant, NUDE SN vs other NUDE group and *=$p<0.05$ vs other CD1 group).

5.2.7. μMT KO mice exhibit reduced PMN recruitment at 24h and reduced monocyte/Mϕ recruitment at 72h and 120h but no differences in MIP-2, KC or MCP-1 levels compared to C57BL/6 mice.

I next examined the peritoneal response of μMT KO mice following the administration of BTG. μMT KO mice are on the C57BL/6 background and C57BL/6 mice were used as controls. μMT KO mice exhibited a blunted peak in PMN influx and recruited less PMN at the 24h time point (Figure 5.12A). In addition, Mϕ influx was also reduced at 72h and 120h (Figure 5.12B). Importantly, μMT mice had comparable numbers of circulating blood monocytes to C57BL/6, but had significantly less circulating PMN (Figure 5.13). Apart from the profound reduction in peritoneal B cells numbers, the numbers of resident peritoneal Mϕ, MC and T cells were similar in μMT KO and C57BL/6 control mice (data not shown). I was unable to perform experiments involving the short time points of 1h and 3h because of lack of mice but analysis of MIP-2 and KC levels on the peritoneal lavage fluid from available time points did not demonstrate any differences between the groups. In addition, MCP-1 levels were not significantly different between μMT KO and C57BL/6 mice.

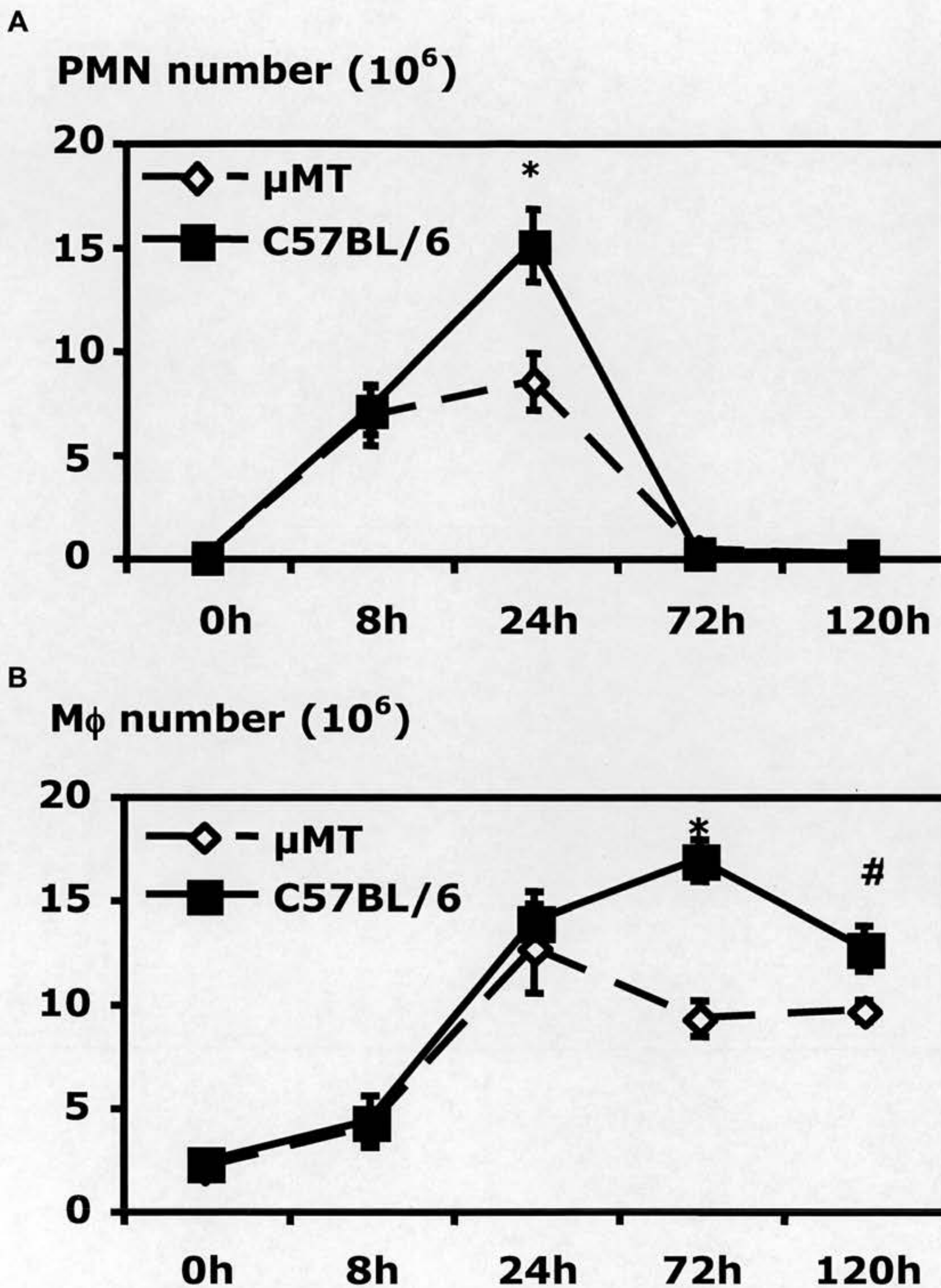


Figure 5.12 - μMT mice exhibit a reduced PMN influx at 24h and a decreased Mϕ influx at 72 and 120h following BTG peritonitis.
1ml of 3% BTG was administered to μMT and C57BL/6 mice. Peritoneal lavage was performed at 0, 8, 24, 72 and 120h following BTG. Lavaged cells were stained for GR1 and F4/80 and counted by flow cytometry. PMN (A) and Mϕ (B) number progression are depicted (* = $p<0.001$ and #=$p<0.05$, μMT mice vs C57BL/6 mice, n=5 mice per group per time point).

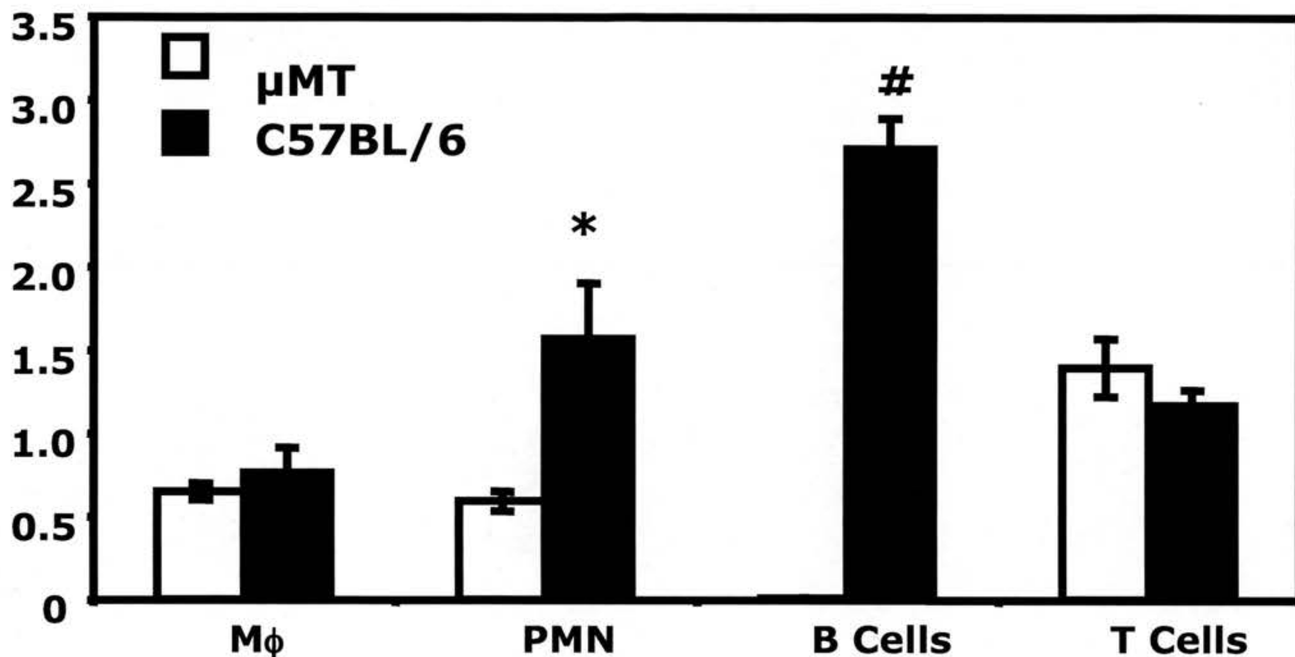


Figure 5.13 – µMT mice have comparable numbers of circulating monocytes but less circulating PMN numbers compared to control C57BL/6 mice.

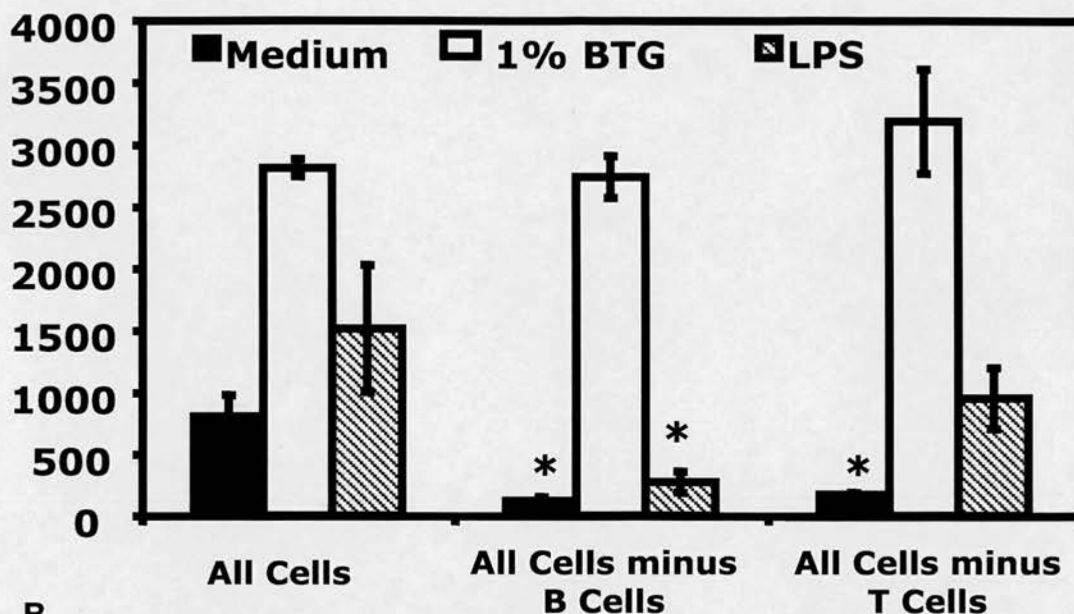
Circulating blood leukocyte numbers are compared in non-manipulated µMT KO and C57BL/6 mice. The number of Mϕ, PMN, B and T cells was determined following staining for F4/80, GR1, B220 and CD3 by flow cytometry (*=p<0.01, #=p<0.0001; µMT KO mice and C57BL/6 mice; n=4-5 mice per group).

5.2.8. *In vitro* assessment of C-X-C chemokine production by peritoneal cell populations following immunodepletion of B and T cells: modulation is stimulus specific.

The data generated by the *in vivo* experiments outlined did not provide any unequivocally clear insights into the role of lymphocytes in peritoneal inflammation as interpretation of the data was confounded by differences in the peritoneal cell populations or circulating leukocyte numbers. I therefore undertook a series of *in vitro* experiments involving the stimulation of peritoneal cells that had been immunodepleted of either B or T cells. Cell populations were then exposed to either normal medium, 1% BTG or LPS (10ng/ml) for 3h and chemokine production determined by ELISA. My previous work outlined in chapter 3 had demonstrated that the production of chemokines in such *in vitro* experiments is entirely dependent upon Mϕ. Therefore the data were normalised for the numbers of Mϕ present in the 'All Cells' group.

The levels of the C-X-C chemokines MIP-2 and KC were compared (Figure 5.14A and 5.14B). The basal production of both MIP-2 and KC was significantly reduced with B cell or T cell depletion. Stimulation with 1% BTG increased the production of MIP-2 and KC with no significant effect of B cell or T cell depletion being evident (Figure 5.14A and 5.14B).

A



B

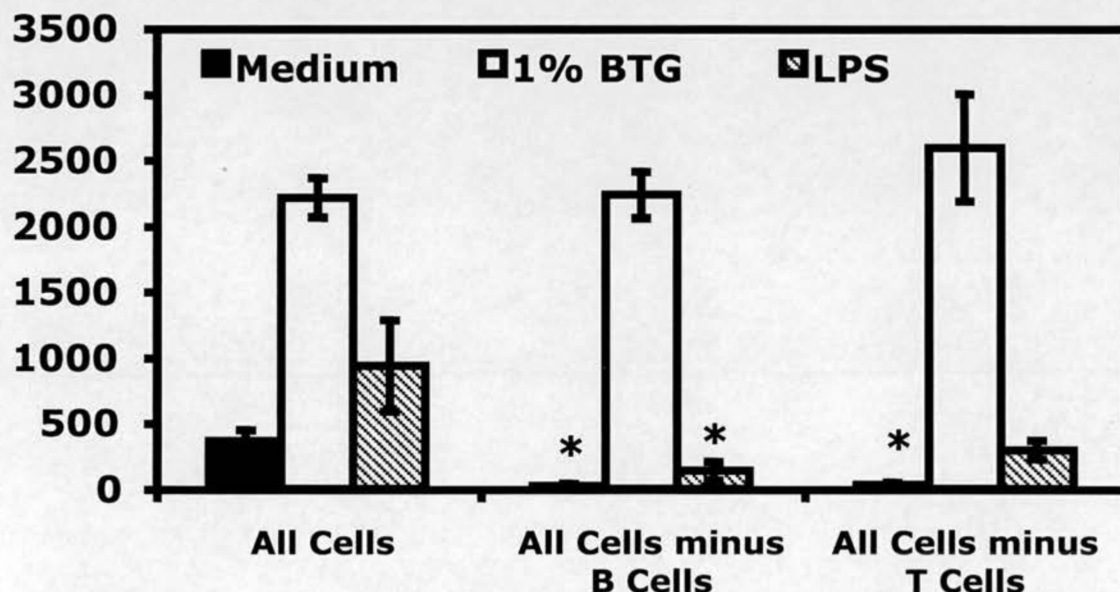


Figure 5.14 - *In vitro* peritoneal cell stimulation resulted in stimulus-dependent MIP-2 and KC production that is modulated by lymphocyte immunodepletion.

Peritoneal lavages were pooled and immunodepleted of either B cell ('All Cells minus B cells') or T cells ('All Cells minus T Cells'). Stimulated total peritoneal cells served as control ('All Cells'). Chemokine levels were normalised according to Mϕ number present in the 'All Cells' population. Cells were incubated in either medium alone, 1% BTG or LPS (10ng/ml) for 6h. Supernatants were harvested and analysed by ELISA for A) MIP-2 (*=p<0.05 vs All cells; n=6) and B) KC (*=p<0.05 vs All cells; n=6).

Stimulation with LPS induced a mild increase in MIP-2 and KC levels. B cell depletion significantly reduced the levels of both MIP-2 and KC after LPS stimulation (Figure 5.14A and 5.14B). Although T cell depletion also exhibited a trend toward reduced chemokine production with LPS stimulation, this was not statistically significant.

5.2.9. *In vitro* cell stimulation demonstrated a reduced production of MCP-1 with B cell depletion and variable effects with T cell depletion.

I then examined the production of MCP-1 by peritoneal cells *in vitro*. Initially, 1% BTG was used to stimulate cells with an incubation period of 6h but this protocol resulted in MCP-1 levels (adjusted for the Mϕ number) that were much lower than those evident *in vivo* (Figure 5.15A). However, preliminary data could demonstrate increased induction of MCP-1 production in the absence of T cells but no difference in the absence of B cells (Figure 5.15A).

LPS (10ng/ml) stimulation of peritoneal cells for 6h did result in significant production of MCP-1. Using LPS as a stimulus, B cell depletion significantly reduced MCP-1 production whereas T cell depletion had no significant effect (Figure 5.15A). I then performed studies with various concentrations of BTG and found that stimulation of peritoneal cells with 0.25% BTG with 10% serum for 6h resulted in significant MCP-1 production. Both B and T cell immunodepletion resulted in a

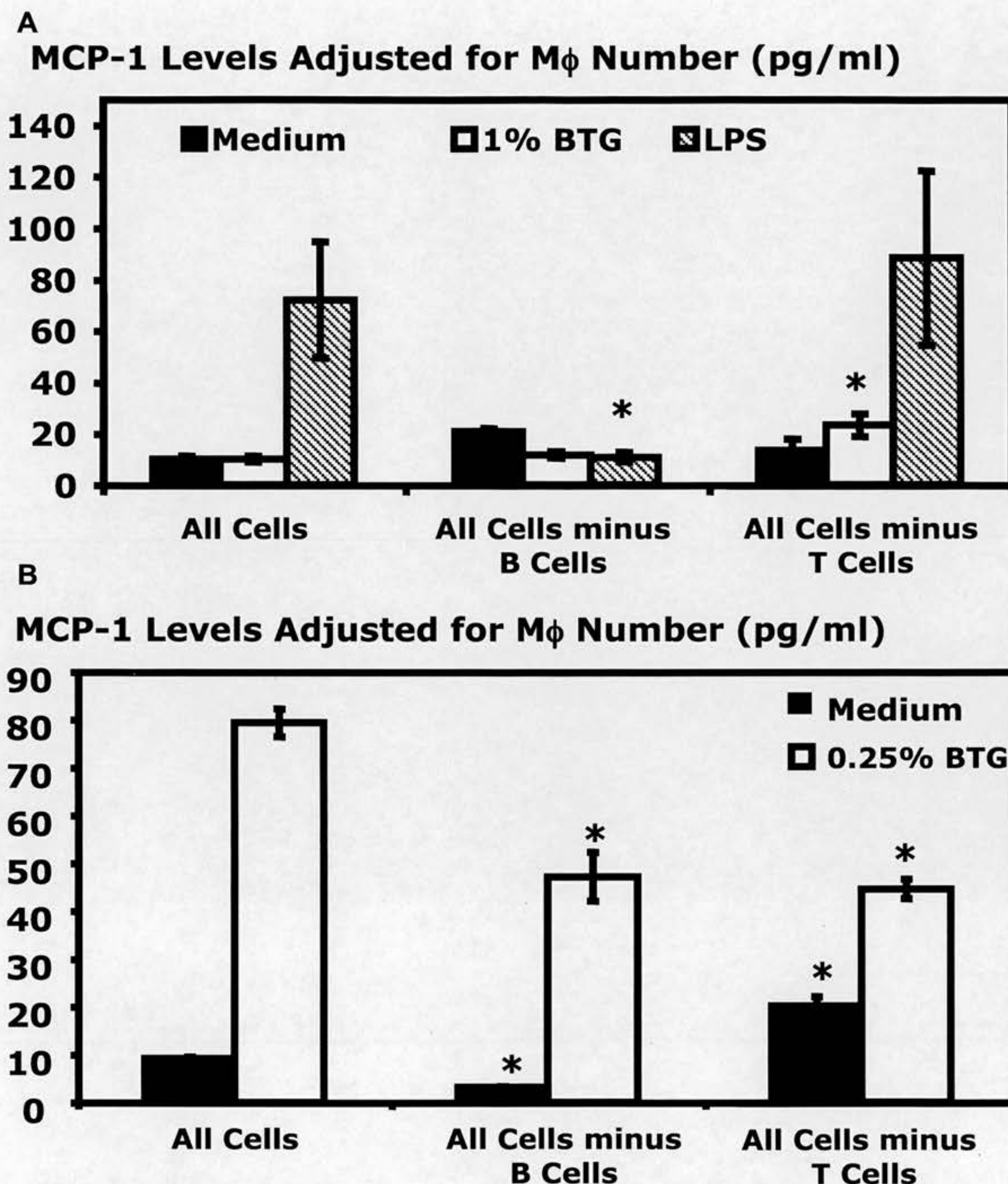


Figure 5.15 - *In vitro* peritoneal cells stimulation resulted in stimuli-dependent MCP-1 production by lymphocyte immunodepletion.
Peritoneal lavages were pooled and immunodepleted of either B cell ('All Cells minus B cells') or T cells ('All Cells minus T Cells'). Stimulated total peritoneal cells served as control ('All Cells'). MCP-1 levels were normalised according to Mϕ number present in the 'All Cells' population. A) Cells were incubated in medium alone, 1% BTG or LPS (10ng/ml) for 6h. Supernatant were harvested and analysed by ELISA for MCP-1 (*=p<0.05 vs All cells; n=6). B) Cells were incubated in medium and 0.25% BTG for 6h and supernatant analysed for MCP-1 (*=p<0.05 vs All cells; n=4).

significant reduction in MCP-1 production (Figure 5.15B). In contrast, the basal secretion of MCP-1 from unstimulated peritoneal cells incubated in medium alone was reduced following B cell depletion and increased following T cell depletion (Figure 5.15B). Again these *in vitro* studies are not conclusive but suggest the following:

(i) The absence of B cells reduces basal MIP-2/KC production, has no effect upon MIP-2/KC levels following 1% BTG stimulation but does decrease MIP-2/KC levels following LPS stimulation.

(ii) The absence of B cells reduces the basal production of MCP-1 and reduces the MCP-1 production induced by LPS and 0.25% BTG.

(iii) The absence of T cells reduces basal MIP-2/KC production but has no significant effect upon MIP-2/KC levels following stimulation with 1% BTG or LPS.

(iv) The absence of T cells increases the basal production of MCP-1, reduces the MCP-1 production induced by 0.25% BTG but has no effect upon LPS induced MCP-1 production.

5.3. Summary

Table 5.1 highlights the major findings outlined in this chapter and table 5.2 compares the populations of peritoneal resident leukocytes in the different strains of mice used in this chapter. RAG-1 KO mice exhibited increased PMN infiltration and blunted monocyte/Mφ infiltration with a trend for a similar recruitment pattern in

Table 5.1-Summary of experimental findings with the various lymphocyte KO

	RAG-1 KO mice	Nude mice	μMT KO mice
Baseline Peritoneal Leukocyte Numbers	More Mϕ, less MC, B and T cells	Less MC, B and T cells	
Circulating Leukocyte Numbers	Less Mϕ, B and T cells	Trend towards more PMN	Less PMN
PMN Recruitment	Increased at 8h and 24h	Increased at 8h and 24h	Reduced at 24h
Mϕ Recruitment	Increased at 8h and reduced thereafter	Increased at 24h	Reduced at 72h and 120h
C-X-C Chemokines	No difference	Increased KC at 8h	No difference
C-C Chemokines	Reduced MCP-1 at 24h	Increased MCP-1 at 3h	No difference

Table 5.2-Comparison of the populations of resident peritoneal leukocytes for each of the mouse strains used.

	Mϕ	PMN	B Cells	T Cells	Mast Cells
RAG-1 KO	$2.5x10^6$ $\pm 0.3x10^6$	$1.2x10^4$ $\pm 0.8x10^4$	$0.8x10^5$ $\pm 0.08x10^5$	$0.5x10^5$ $\pm 0.07x10^4$	$1.7x10^4$ $\pm 0.2x10^4$
NUDE	$1.6x10^6$ $\pm 0.1x10^6$	$3.6x10^4$ $\pm 1.9x10^4$	$0.7x10^6$ $\pm 0.1x10^5$	$0.1x10^5$ $\pm 0.04x10^5$	$0.5x10^5$ $\pm 0.04x10^4$
µMT	$1.8x10^6$ $\pm 0.3x10^6$	$8.0x10^3$ $\pm 1.1x10^3$	$0.9x10^5$ $\pm 0.1x10^5$	$5.7x10^4$ $\pm 0.9x10^4$	$6.1x10^4$ $\pm 0.5x10^4$
C57BL/10	$1.6x10^6$ $\pm 0.3x10^6$	$1.2x10^4$ $\pm 0.8x10^4$	$7.2x10^5$ $\pm 1.3x10^5$	$2.6x10^5$ $\pm 0.4x10^5$	$3.2x10^4$ $\pm 0.3x10^4$
CD1	$2.5x10^6$ $\pm 0.1x10^6$	$3.1x10^4$ $\pm 0.9x10^4$	$1.6x10^6$ $\pm 0.2x10^6$	$1.0x10^5$ $\pm 0.3x10^5$	$1.0x10^5$ $\pm 0.03x10^5$
C57BL/6	$2.2x10^6$ $\pm 0.4x10^6$	$8.6x10^3$ $\pm 4.6x10^3$	$7.4x10^5$ $\pm 1.8x10^5$	$7.5x10^4$ $\pm 1.9x10^4$	$7.1x10^4$ $\pm 0.4x10^4$

zymosan-induced peritonitis. Interpretation of this data is confounded by the fact that RAG-1 KO mice have an increased number of resident peritoneal Mϕ (key players in PMN recruitment) and reduced circulating monocyte numbers. There was no difference evident in MIP-2 or KC levels. In addition to reduced circulating monocyte numbers, RAG-1 KO mice exhibited reduced levels of MCP-1 at the 24h time point and this may also be involved in the impaired peritoneal recruitment of monocyte/Mϕ seen in RAG-1 KO mice.

In BTG-peritonitis, NUDE mice exhibited increased PMN infiltration at 8h and 24h and increased monocyte/Mϕ infiltration at 24 hr. NUDE mice exhibited increased levels of KC at 8h and increased levels of MCP-1 at 3h together with elevated levels of IL-6 at the 8h time point. Despite the absence of clear chemokine/cytokine differences to explain the observed findings, the SN transfer experiments between NUDE and CD1 mice may shed some light on a possible explanation.

Although SN transfer between NUDE and CD1 mice did not affect peritoneal PMN numbers, analysis of circulating blood PMN numbers revealed that the transfer of NUDE SN increased the number of circulating PMN of CD1 mice compared to CD1 mice that had received SN derived from CD1 mice. Furthermore, the transfer of SN from CD1 mice into NUDE mice resulted in a reduction in the number of circulating blood PMN in NUDE mice compared to the circulating PMN numbers of NUDE mice that had received SN from NUDE mice. The circulating number of PMN was similar amongst mice that had received NUDE SN suggesting the possible

presence of a stronger signal for bone marrow mobilisation of PMN in this SN. There may be increased chemotactic agents or the absence of an inhibitor of PMN mobilisation (or decoy cytokine receptor) in NUDE mice that would enhance the systemic inflammatory response and leukocyte mobilisation.

The lack of increased peritoneal PMN recruitment despite the elevation in circulating PMN numbers highlights the complexity of leukocyte recruitment. One explanation could be that NUDE mice may have a different peritoneal vasculature structure that would facilitate leukocyte transmigration or they may have endothelial cells in an enhanced state of activation (increased selectins or ICAMs at their surface) that would promote rolling of circulating leukocytes thereby facilitating transmigration. It is also possible that the PMN from NUDE and CD1 mice may behave differently to chemotactic stimuli as a result of different leukocyte surface receptors/molecules that would affect emigration from the blood. A full understanding of the effect of T cells on the modulation of peritoneal leukocyte recruitment is still lacking and further work is required.

The μMT KO mice exhibited decreased PMN influx and a reduced Mϕ influx. Unlike RAG-1 KO mice and NUDE mice there were no differences in chemokine levels evident in μMT KO mice. The *in vitro* experiments involving analysis of chemokine production by peritoneal cell populations in the presence or absence of B cells and T cells suggested that lymphocytes may modulate the generation of chemokines by Mϕ but that this is complex. For example, the experimental findings differed according to the stimulus used.

However, the overall impression derived from this body of work suggests that T cells may have the capacity to downregulate leukocyte recruitment with a lack of T cells facilitating increased PMN and monocyte/Mϕ recruitment as in the NUDE mice. B cells may play a role in promoting later Mϕ recruitment as both RAG-1 KO mice and μMT KO mice exhibited reduced monocyte/Mϕ recruitment. However, the exact mechanisms of lymphocyte action remain elusive.

Chapter 6: Discussion

6.1. Discussion

In the experiments described in this thesis, a conditional Mϕ ablation strategy was used to dissect the role of the resident peritoneal and pleural Mϕ in the initiation of serosal inflammation and the orchestration of PMN recruitment in two well established inflammatory models i.e. BTG induced peritonitis and carrageenan induced pleurisy.

Previous work indicated that leukotrienes derived from resident peritoneal Mϕ are involved in the development of early vascular permeability in sterile peritonitis (Kolaczkowska et al., 2002). Although early work in rat models of peritonitis implicated the resident peritoneal Mϕ in the orchestration of PMN recruitment (de Souza and Ferreira, 1985) (Souza et al., 1988) (Barja-Fidalgo et al., 1992), more recent studies have produced conflicting results (Ajuebor et al., 1999; Knudsen et al., 2002). Indeed, studies by Ajuebor *et al* demonstrated that resident Mϕ depletion inhibits PMN influx in LPS induced inflammation, has no effect in BTG peritonitis and augments PMN influx in zymosan peritonitis (Ajuebor et al., 1999). In the latter model, it was proposed that Mϕ-derived IL-10 inhibits PMN recruitment. Conversely, work by Knudsen *et al* using clodronate-induced depletion of peritoneal Mϕ in a rat model of sterile peritonitis demonstrated that PMN infiltration was Mϕ dependent (Knudsen et al., 2002).

In the pleural space, carrageenan induces prominent inflammatory responses that are likely to be involved in human disease. In addition, I examined the effect of Mϕ ablation prior to the administration of fixed *Staphylococcus aureus* bacteria as a model with more direct clinical relevance. Although the resident pleural Mϕ is eminently capable of secreting chemokines and cytokines, its role in pleurisy is currently unclear. It has been demonstrated by multiple investigators that pleural mesothelial cells have the capacity to secrete various chemokines (Antony et al., 1995; Jonjic et al., 1992; Loghmani et al., 2002; Mohammed et al., 1999; Pace et al., 1999; Park et al., 2003). In addition, some studies have identified resident pleural Mϕ-derived pro-inflammatory cytokines such as TNF-α that are essential for the secretion of C-X-C and C-C chemokines from pleural mesothelial cells (Antony et al., 1995; Cuzzocrea et al., 1999b; Frode et al., 2001; Goodman et al., 1992; Mohammed et al., 1998b; Pace et al., 1999; Park et al., 2003) thereby suggesting important cross talk between different cells within the pleural cavity.

In my studies, the administration of 25ng/g body weight of DT to CD11b-DTR transgenic mice resulted in the rapid and effective ablation of resident serosal Mϕ with 98% Mϕ ablation in the peritoneum and greater than 96% ablation in the pleural space with resident Mϕ undergoing rapid apoptosis following administration of DT. This dose was used because of its potency compared to lower doses and it was well tolerated in the short time scale of these studies. Mϕ ablation dramatically blunted PMN infiltration both in the peritoneal and pleural cavities thereby indicating a key role for the serosal resident Mϕ in the initiation of acute serosal inflammation in these experimental models. It is important to note that the reduced

PMN infiltration in DT treated mice was not attributable to a systemic neutropenia as circulating PMN were not sensitive to DT such that the number of circulating PMN in DT treated mice was not reduced compared to PBS treated mice at 24 hours (the timing of BTG administration). Similarly, recruited peritoneal PMN were resistant to DT thereby excluding this as a potential cause for diminished PMN infiltration of the peritoneal cavity. It is unclear why CD11b positive PMN are resistant to DT but this may be a reflection of their lower level of CD11b protein synthesis or the intracytoplasmic localisation of the DTR receptor when PMN are not activated.

The importance of the resident Mϕ was reinforced by experiments involving the adoptive transfer of DT insensitive non-transgenic Mϕ following DT-mediated Mϕ ablation and prior to the initiation of peritonitis or concurrent with the induction of pleurisy. The protocol for adoptive transfer evolved over the course of my studies but studies involving the depletion of Mϕ by adhesion or the purification of Mϕ by immunomagnetic selection yielded similar results. Since the data regarding PMN recruitment differed somewhat between the peritoneal and the pleural adoptive transfer experiments they will be discussed separately.

The adoptive transfer of peritoneal Mϕ was successful with $7.9 \times 10^5 \pm 2.7 \times 10^5$ non-transgenic Mϕ being injected into the peritoneal cavities of Mϕ ablated CD11b-DTR mice. This restored the number of peritoneal Mϕ to approximately 90% of the Mϕ number normally present in the peritoneal cavities of FvB/N control mice. The presence of Mϕ in the transferred peritoneal cell population correlated with the restoration of the PMN influx. The absence of Mϕ in the adoptively transferred cell

population resulted in no significant PMN recruitment thereby suggesting that the peritoneal Mϕ exerts a critical role in this process. PMN recruitment following the adoptive transfer of Mϕ was not secondary to any intrinsic inflammatory nature of the cell transfer since mice receiving the Mϕ-rich peritoneal cell transfer without any BTG did not exhibit significant PMN recruitment.

The adoptive transfer of pleural cell populations that had been effectively depleted of Mϕ by magnetic immunodepletion had no significant effect upon PMN recruitment in the carrageenan induced pleurisy model with PMN numbers being comparable to those in control Mϕ-depleted mice. In contrast, the adoptive transfer of pleural cell populations containing Mϕ or a population of purified Mϕ significantly increased pleural PMN infiltration thereby reinforcing the key role of the resident pleural Mϕ. In these experiments $1.5x10^5 \pm 0.3x10^5$ Mϕ were transferred but this adoptive transfer was unable to restore Mϕ numbers to the normal values found in non-manipulated mice; reconstituted mice had about 45% of the total Mϕ number present in FvB/N control mice. This differs significantly from the peritoneal model and is a likely explanation for the partial restoration of PMN infiltration compared to DT treated FvB/N control mice in the carrageenan model. However, the strong correlation between the number of pleural Mϕ present at the initiation of inflammation and the number of infiltrating PMN 6h after the administration of carrageenan strongly supports a key pro-inflammatory role for the resident pleural Mϕ.

It is also possible that the model of carrageenan pleurisy may be partially dependent upon the pro-inflammatory actions of recruited monocytes, unlike experimental peritonitis where acute PMN infiltration is monocyte independent, as DT treatment markedly reduces the number of circulating monocytes. It should be noted, however, that the presented results indicate a profound effect of Mϕ depletion upon PMN recruitment at the early time point of 6hr and since monocyte recruitment occurs significantly later in the carrageenan model it is likely that monocyte recruitment will be very limited at this early time point. Thus, a reduction in monocyte recruitment in DT-treated CD11b-DTR mice is unlikely to be involved in the very early reduction in PMN infiltration evident in these studies although recruited monocytes may play a role in PMN infiltration at later time points.

Although defective PMN migration consequent upon exposure to DT is an alternative explanation for these findings, it should be noted that work in experimental peritonitis indicated that reconstitution of Mϕ depleted mice with non-transgenic Mϕ was able to fully restore PMN infiltration in response to BTG.

In these experiments, the administration of DT and the subsequent induction of widespread Mϕ death did affect the numbers of B cells and MC within both the peritoneal and pleural cavity. However, despite this potentially confounding issue, there are several factors that support the prominent role of the serosal Mϕ in the BTG and carrageenan models. First, the adoptive transfer of Mϕ-rich peritoneal cells restored PMN recruitment induced by BTG, whereas Mϕ-depleted peritoneal cells did not. Similar findings were found in the pleurisy model, but PMN influx was only

partially restored. The Mϕ-depleted serosal cell populations used in these studies comprised B cells, T cells and MC but they did not induce a significant PMN influx following the administration of BTG or carrageenan. In contrast, the adoptive transfer of Mϕ-rich serosal cells or purified pleural Mϕ isolated by negative selection significantly increased PMN infiltration in response to inflammatory stimuli. Second, data from *in vitro* experiments indicated a dramatic reduction in the production of chemokines and cytokines following the depletion of Mϕ from both resident peritoneal and pleural cell populations. It should be noted that in these studies, peritoneal and pleural cells were labelled with a PE conjugated antibody to the specific Mϕ marker F4/80 prior to immunomagnetic depletion and F4/80 is not expressed by B cells or MC. Lastly, previous work suggested that MC do not play a significant role in BTG-induced peritonitis (Ajuebor et al., 1999) or in the carrageenan model of pleural inflammation (Horakova et al., 1980; Takeshita et al., 2003).

The magnitude of the Mϕ depletion may explain the apparent discrepancy between these results and the study by Ajuebor *et al* (Ajuebor et al., 1999). Administration of a single dose of DT induced 98% Mϕ ablation of peritoneal Mϕ whilst 3 doses of liposomal clodronate resulted in '>85%' Mϕ depletion in their study. Mϕ are a very potent source of chemokines and cytokines and it may be the case that in certain circumstances a relatively small population of residual Mϕ may exert significant biological effects. Although the administration of liposomal clodronate may have marked biological effects despite depletion of only around 80% of Mϕ (Jose et al., 2003), it may be necessary to deplete almost all peritoneal Mϕ in

order to delineate their roles as sentinel cells. PMN recruitment after Mϕ depletion in the pleural space has been less studied.

Although peritoneal Mϕ may produce myriad mediators capable of recruiting PMN (Desouza et al., 2002; Souza et al., 1997), the effect of Mϕ ablation upon the level of C-X-C chemokines was examined in this model. The data suggest that the initiation of PMN infiltration is mediated by resident peritoneal Mϕ dependent production of chemokines such as MIP-2 and KC previously documented to play a role in orchestrating PMN recruitment in BTG peritonitis (Call et al., 2001; Remick et al., 2001) and in other inflammatory situations (Goncalves and Appelberg, 2002; McColl and Clark-Lewis, 1999; Walley et al., 1997). Although MC are also a rich source of pro-inflammatory and vasoactive mediators and have been documented to play an important role in PMN recruitment during inflammation of the peritoneum (Ajuebor et al., 1999) as well as other sites such as the skin (Chen et al., 2002) my studies do not suggest a prominent role for MC.

It was found that ablation of resident peritoneal Mϕ markedly reduced the peak level of MIP-2 and significantly blunted the level of KC at 1 hr. However, the partial inhibition (around 50%) of KC production at 1 hour and the persistent elevation of KC at 3 hours in Mϕ depleted mice suggested that KC may be produced by other cells within the peritoneum. The persistent elevation of KC also suggests that Mϕ may play a role in the negative regulation of KC production by non-Mϕ cells within the peritoneum although this may also result from the dysregulated PMN recruitment consequent to Mϕ ablation. In this context it is pertinent that the *in vitro*

data indicate that both KC and MIP-2 production by peritoneal cells obtained by peritoneal lavage is almost entirely dependent upon Mϕ since peritoneal cells depleted of Mϕ produced minimal levels of chemokines. These findings suggest that peritoneal cells retrievable by peritoneal lavage are not the source of KC detected in the *in vivo* study and that non-lavageable peritoneal cells represent a significant additional source of KC. This is consistent with recent work in a wound model of inflammation (Armstrong et al., 2004). This work demonstrated MIP-2 expression by inflammatory cells whilst KC was predominantly expressed by resident tissue cells such as endothelial cells and fibroblasts. Peritoneal mesothelial cells undoubtedly participate in peritoneal inflammation and can produce chemokines and cytokines (Robson et al., 2001; Topley et al., 1993a; Topley et al., 1993b) and it may be the case that mesothelial cells contribute to the KC production evident in this study. Despite this, however, PMN infiltration is still markedly blunted despite the persistent presence of KC at 3 hours. Peritoneal Mϕ depletion also affected production of C-C chemokines such as MIP-1α and MCP-1; MIP-1α levels were reduced by 80% percent whereas MCP-1 levels were only slightly affected with a 55% reduction at 8h. However, MCP-1 levels at 24h were higher in the Mϕ ablated CD11b-DTR mice suggesting that peritoneal Mϕ might be involved in the control of MCP-1 production but that other cells are involved in its secretion. In contrast to studies of C-X-C chemokine production *in vitro*, MCP-1 production *in vitro* resulted in levels that were much less than those observed *in vivo*. Again, this suggests an important role for a cellular MCP-1 source not recoverable by peritoneal lavage such as the peritoneal MeC.

Study of cytokine levels indicated that TNF-α levels at 1h were reduced by 60% by Mφ depletion. This supports an important role for resident peritoneal Mφ in the secretion of this key pro-inflammatory cytokine (Topley et al., 1993a; Topley et al., 1993b). Therefore, peritoneal resident Mφ would be important in the initiation of secretion of C-X-C chemokines and cytokines by other resident cells such as the MeC (Topley et al., 1993a; Topley et al., 1993b; Topley et al., 1993c). There were no observed differences for any of the other cytokines (IL–6, IL-10, IL12p70 and IFN-γ) measured at any of the time points studied. However, the studies were performed at relatively late time points with 8h being the shortest. In addition, there is also the dilution issue as peritoneal lavages were realised with 5ml of PBS and thus low but biologically significant levels of cytokine might have been missed. The study of earlier time points such as 1 and 3h would be informative.

Mφ depletion also resulted in a profound reduction in TGF-β levels in the peritoneal lavages at 24h. Intracellular staining revealed that mature Mφ produced TGF-β whereas newly recruited monocytes did not. It has been shown that Mφ-driven phagocytosis of apoptotic cells is an important source of TGF-β (Fadok et al., 1998; McDonald et al., 1999) and therefore the observed reduced levels could be explained by the marked reduction in PMN infiltration at earlier time points in the Mφ ablated CD11b-DTR mice combined with the relative absence of Mφ to phagocytose apoptotic PMN. However, the importance of these TGF-β differences in the resolution phase of inflammation and repair are unknown since no peritoneal biopsies were undertaken to assess whether there was evidence of peritoneal fibrosis or injury.

The effect of resident pleural Mϕ ablation upon the level of C-X-C chemokines was also examined in the carrageenan induced pleurisy. Resident pleural Mϕ ablation resulted in a marked reduction in MIP-2 levels in the pleural exudates but had a lesser, albeit significant, inhibitory effect upon KC levels. The *in vitro* study of Mϕ-rich or Mϕ-depleted pleural cell populations indicated that Mϕ are a key source of chemokines since pleural cell populations depleted of Mϕ produced minimal amounts of the chemokines MIP-2 and KC. It is of interest, however, that these *in vitro* studies demonstrated comparable production of MIP-2 and KC whereas the analysis of pleural lavage fluid indicated that the levels of KC were approximately 2-3 fold higher than MIP-2 *in vivo*. These data are similar to what was found in the peritonitis model suggesting that other cells within the pleural cavity such as pleural mesothelial cells may be an important source of KC production *in vivo*. It should be stressed, however, that marked inhibition of PMN recruitment was evident at early time points in the presence of relatively preserved KC levels suggesting that MIP-2 is more important *in vivo* in this model.

It was found that pleural cell populations stimulated with carrageenan *in vitro* produced relatively low levels of the C-C chemokine MCP-1 compared to the levels found *in vivo*. Again this suggests a prominent role for pleural mesothelial cells in the production of MCP-1 *in vivo* and the subsequent recruitment of mononuclear cells. These data are in accordance with previous reports highlighting the importance of the pleural mesothelial cells in the secretion of these chemokines (Antony et al., 1995; Hill et al., 2003; Mohammed et al., 1999).

Mϕ ablation in other experimental models has suggested a key role for Mϕ in inflammation. For example, Mϕ ablation reduces pancreatic inflammation and is associated with reduced levels of IL-6, IL-10 and IL-12 (Shifrin et al., 2005). Also, clodronate pre-treatment reduced the levels of IL-1β, TNF-α, IL-6, IL-10 and IL-12 produced in the liver after LPS administration, suggesting that Mϕ are an important source of these chemokines *in vivo* (Salkowski et al., 1995). The presented data in this thesis also indicate that resident pleural Mϕ are critically involved in the generation of cytokines at inflamed sites as the levels of TNF-α, IL-10 and IL-6 in the pleural exudates were significantly reduced in CD11b-DTR mice treated with DT. Also, *in vitro* studies of carrageenan stimulated pleural cell populations demonstrated a significant reduction in cytokine levels following magnetic immunodepletion of pleural Mϕ.

These studies do raise the question as to why there was no significant PMN infiltration in response to the significant KC production evident *in vivo*. In this regard, it is pertinent to note the results of previous work studying the effect of function blocking antibodies to either MIP-2 or KC in BTG peritonitis. These studies indicate that inhibition of either chemokine individually results in marked (>70%) inhibition of PMN infiltration with inhibition of both chemokines giving little additional effect (Call et al., 2001). The chemotactic activity of pleural lavage fluid from Mϕ-depleted and control mice with pleurisy was not assessed using *in vitro* PMN chemotaxis assays as the preparation of a pure population of non-activated murine neutrophils is problematic. In addition, previous studies and my studies (see

later) indicate that experiments involving the adoptive transfer of lavage fluid are confounded by the resultant dilution of chemokines, cytokines etc. However, it may also be the case that the dramatic reduction in the levels of intrapleural cytokines in Mϕ depleted mice may contribute to the defective PMN infiltration evident in these mice via modulation of local endothelial cell expression of adhesion molecules involved in PMN diapedesis. Although many mediators including cytokines, nitric oxide, complement proteins and prostaglandins are involved in acute inflammatory processes and leukocyte recruitment, the presented findings indicate that both peritoneal and pleural resident Mϕ play a critically important role in orchestrating PMN influx in the BTG model of peritonitis and in the carrageenan model of pleural inflammation.

However, biological generalisation of the findings was sought by the study of 2 clinically relevant models involving different recognition pathways: zymosan peritonitis and the intrapleural administration of killed *Staphylococcus aureus* bacteria. Depletion of resident peritoneal Mϕ significantly reduced PMN infiltration in zymosan peritonitis, thereby suggesting that the 'sensing' function of the peritoneal resident Mϕ may be stimulus independent. In addition, limited experiments performed in mice administered killed *Staphylococcus aureus* indicated that Mϕ depletion markedly reduces staphylococcal induced PMN infiltration. Also, prominent Mϕ ingestion of *Staphylococcus aureus* was evident in control DT treated FvB/N mice and this finding reinforces the key role for resident Mϕ as sentinel cells that act to recognize and clear pro-inflammatory pathogens and particulate material.

Since MC have been involved in the initiation of inflammation (Ajuebor et al., 1999; Chen et al., 2002), potential interactions between peritoneal Mϕ and MC were examined by performing *in vitro* studies of peritoneal cells that had been depleted of Mϕ or MC prior to stimulation with BTG. Interestingly, depletion of MC had no significant effect upon the production of KC and MIP-2 following BTG stimulation suggesting that chemokine production in this model was Mϕ dependent and MC independent. Since previous work suggests that MC do not play a significant role in the carrageenan model of pleural inflammation (Horakova et al., 1980; Takeshita et al., 2003), no *in vitro* experiments with MC depletion were done with pleural resident cells.

These studies have used a transgenic model of conditional Mϕ ablation to dissect the role of the resident peritoneal and pleural Mϕ in the initiation of acute serosal inflammation. The data indicate a key role for the resident Mϕ in sensing peritoneal and pleural irritation and orchestrating PMN infiltration in BTG and zymosan peritonitis as well as in carrageenan- and killed *Staphylococcus aureus* induced pleural inflammation. This pro-inflammatory function is predominantly mediated by production of the potent PMN C-X-C chemokine MIP-2 and, to a lesser extent, KC by peritoneal resident Mϕ as well as Mϕ dependent TNF-α production that can initiate production of such chemokines as well as promoting conditions that favour PMN infiltration. In addition, resident pleural Mϕ are involved in the production of the potent PMN C-X-C chemokine MIP-2 and pro-inflammatory cytokines such as TNF-α and IL-6 that can promote the production of the PMN C-X-C chemokine KC by mesothelial cells. Although previous work has implicated the

involvement of other cells such as peritoneal MC, this study suggests that peritoneal resident Mϕ are critically important producers of C-X-C PMN chemokines and act to orchestrate PMN recruitment in murine BTG peritonitis. The data also suggest that Mϕ-derived products such as TNF-α might be important in the initiation of pleurisy and stimulation of additional MeC-dependent chemokine production. This work therefore suggests that resident Mϕ are critically important producers of PMN chemokines and pro-inflammatory cytokines and act to orchestrate PMN recruitment in murine BTG-induced peritonitis and carrageenan-induced pleurisy.

Consequently, it is important to understand the crucial role of serosal resident Mϕ in the initiation of inflammation. Previous studies of pleural and peritoneal inflammation have suggested that the main role of the Mϕ is to stimulate other resident cells such as MeC or fibroblasts by producing cytokine such as TNF-α (Antony et al., 1995; Cuzzocrea et al., 1999b; Frode et al., 2001; Goodman et al., 1992; Liberek et al., 1996; Mohammed et al., 1998b; Pace et al., 1999; Park et al., 2003; Topley et al., 1994; Witowski et al., 2001). The work presented in this thesis, however, suggests that the Mϕ plays a significantly more important role since resident Mϕ respond to inflammatory stimuli by generating cytokines such as TNF-α that can activate other resident Mϕ (Danis et al., 1991; Kos, 1989; Kumaratilake et al., 1990; Leibovich et al., 1987) thereby inducing the production of significant amounts of chemokines such as MIP-2 that contribute to leukocyte recruitment. The Mϕ also responds very rapidly as MIP-2 production was already present at 1h. In contrast the production of the chemokine KC by other resident cells was significantly slower in the absence of Mϕ dependent activation. Therefore, it is very important

that future work in this area should examine more than just MeC or other single cells in isolation, but try to reproduce a more complex microenvironment with resident Mϕ and/or other resident leukocytes in order to include all the secreted pro-inflammatory products and not just only a few 'chosen' cytokines.

Resident Mϕ are therefore crucial for the initiation of inflammation. However, they can be also essential to promote the resolution of inflammation and promote repair since, depending upon the timing of Mϕ depletion, the initiation, progression or resolution of inflammation can be affected (Cailhier et al., 2005; Cailhier et al., 2006; Duffield et al., 2005a; Kotter et al., 2001; Kotter et al., 2005). Thus Mϕ ablation needs to be targeted to the appropriate stage of inflammation to be beneficial as current data would suggest that Mϕ ablation during the resolution of inflammatory processes may be detrimental.

Chapters 3 and 4 of this thesis focused on the role of the resident serosal Mϕ in the initiation of inflammation. In chapter 5, the involvement of lymphocytes was assessed. The literature regarding the importance of lymphocytes in leukocyte recruitment using mice exhibiting various deficiencies in lymphocyte populations is varied and controversial. Some studies have shown a reduction in leukocyte recruitment (either PMN or Mϕ) (Appelberg, 1992; Hall et al., 1999; Hancock et al., 2002; Zwacka et al., 1997) whereas others did not observe any significant difference (Burne et al., 2001; Burne-Taney et al., 2003; Chen et al., 2002; Kopf et al., 2002; Morrison et al., 2006) between various lymphocyte deficient animals and control

animals. T cells have been shown to be essential for accelerated allograft rejection and leukocyte recruitment whereas B cells were not key (Hancock et al., 2002). Obviously, the immunologic nature of the stimuli might be important and affect the data generated but prior to undertaking my studies there was no clear evidence as to whether lack of B or T lymphocytes will increase or decrease leukocyte recruitment.

The data presented in chapter 5 suggested that lymphocytes may be involved in the regulation of PMN recruitment and monocyte/Mϕ recruitment although a variety of confounding factors were encountered. RAG-1 KO mice, which have no mature lymphocytes, developed increased early PMN recruitment and (apart from a slight increase in Mϕ number at 8h) a failure of monocyte/Mϕ recruitment at late time points. This was found in the model of BTG peritonitis but there was also a similar trend with the zymosan peritonitis model; only a small number of mice were available for the latter experiment. Chemokine analysis could not delineate any differences in the levels of the C-X-C chemokines MIP-2 and KC between RAG-1 KO mice and control mice. However, MCP-1 levels were higher in the C57BL/10 control mice at 24h, an observation, which may partly account for the observed differences in peritoneal monocyte/Mϕ number at later time points. There were no differences in the levels of the other C-C chemokine MIP-1α.

Increased PMN recruitment coupled with reduced Mϕ recruitment could be explained by reduced IFN-γ secretion. IFN-γ has been shown to reduce MeC production of pro-inflammatory C-X-C chemokines such as KC, both *in vitro* and *in vivo*, but increases MCP-1 secretion thereby favouring Mϕ influx and resolution of

inflammation (McLoughlin et al., 2003; Mohammed et al., 1999; Robson et al., 2001; Schnyder-Candrian et al., 1995). Experiments with IFN-γ KO mice in a lung *Mycobacterium avium* model highlighted the increased influx of PMN, compatible with a down-regulatory function of IFN-γ. Similar findings were made in a model of constrictive pericarditis (Afanasyeva et al., 2004). In the lung *Mycobacterium avium* model, IFN-γ mRNA levels were reduced in RAG-1 KO mice (Ehlers et al., 2001). Studies in IFN-γ KO mice demonstrated that this inhibitory effect was only possible when IL-6 signalling is normal (McLoughlin et al., 2003). Therefore, the lack of IFN-γ secretion secondary to an absence of lymphocytes would result in the loss of the inhibitory signal for PMN recruitment and would also impede Mϕ recruitment by loss of the stimulatory signal for MCP-1 production. However, NK cell activity, presumably via IFN-γ production, has been shown to be enhanced in RAG-1 KO (Shultz et al., 2000). Moreover, I did not find any significant levels of IFN-γ in RAG-1 KO or in their controls. There is also evidence that IL-6 signalling with the formation of sIL-6R/IL-6 complex can downregulate production of C-X-C chemokines and stimulate C-C chemokines production (Hurst et al., 2001). Disturbance of this pathway would result in leukocyte recruitment and MCP-1 production similar to that found in RAG-1 KO mice. However, it is not clear how IL-6 signalling would be affected in RAG-1 KO mice.

A study in the CCR2 (receptor for MCP-1) KO mice revealed that after IP BTG administration, these mice did not recruit Mϕ above the baseline level whereas PMN recruitment was increased at early time points (Kuziel et al., 1997). This recruitment pattern is similar to that seen in RAG-1 KO mice. IFN-γ, TNF-α, IL-1

and LPS can decrease CCR2 gene transcription and IL-2 treatment of T cells and monocytes can augment CCR2 expression (Kuziel et al., 1997). CCR2 expression at the cell surface of leukocytes was not evaluated in RAG-1 KO mice and there is no evidence that absence of lymphocytes would modulate such protein expression, but the similarities between the 2 models with BTG peritonitis are intriguing.

These observed differences in the peritoneal leukocyte recruitment between RAG-1 KO mice and their controls cannot be explained by a higher number of PMN in the blood since analysis of circulating PMN numbers revealed that there were no differences between the two groups. However, circulating monocyte numbers were reduced in the RAG-1 KO mice and this could partly explain why there was a lower number of recruited Mϕ at later time points. Nevertheless, the absence of Mϕ recruitment (no elevation of the Mϕ number in the peritoneal cavity after 8h) highlighted a difference in the pattern of leukocyte recruitment in the RAG-1 KO mice.

The specific involvement of either B or T cells cannot be discerned using RAG-l KO mice as these mice lack both cell types and therefore studies were undertaken in NUDE mice. Studies with NUDE mice, lacking T cells, revealed a marked increase in PMN recruitment at the 8 and 24h time points and increased Mϕ numbers at 24h in BTG peritonitis. Here, however, a trend toward an increased level of circulating PMN numbers in the NUDE mice could partly explain the observed difference in recruited PMN numbers. However, the increased peritoneal Mϕ

numbers at 24h could not be explained by any difference in the circulating monocyte number.

Dysregulation in leukocyte apoptosis has been described in IFN-γ KO mice (McLoughlin et al., 2003). The levels of apoptosis were not determined in NUDE mice so it is impossible to tell if the increased PMN and Mϕ numbers were due to reduced levels of apoptosis. However, NK cells and Mϕ from NUDE mice have been shown to exhibit increased cytotoxic activity (Budzynski and Radzikowski, 1994) and IFN-γ production by NK cells in NUDE mice has been demonstrated in a model of sepsis (Seki et al., 1998). Therefore, the lack of IFN-γ production in NUDE mice and loss of its modulating role on C-X-C and C-C chemokines cannot be supported.

In order to assess if the observed phenotype in the NUDE mice could be corrected, I performed adoptive transfer studies of peritoneal T cells from CD1 control mice. The restoration of the initial T cell number was partial since just a little more than 50% of the T cell number present in the WT could be transferred in NUDE mice. Furthermore, a significant number of resident peritoneal Mϕ were also transferred and they may have had a confounding effect as they are involved in PMN recruitment as described previously. Chemokine and cytokine analysis revealed no significant differences in MIP-2 production though there was a small albeit statistically significant increased KC production at the 8h time point. These differences may be involved in the increased PMN recruitment found in NUDE mice but the magnitude of the differences suggests that they are unlikely to be the entire

explanation. IL-6 levels analysis revealed a 2.5-fold persistent increase present at 8h after BTG stimulation in NUDE compared to WT mice and may reflect a sustained pro-inflammatory reaction resulting from the increased leukocyte recruitment.

In order to further investigate the elevated PMN recruitment seen in NUDE mice, I undertook experiments involving the transfer of peritoneal SN between NUDE and control mice. Transfer of SN from NUDE into CD1 control mice failed to increase peritoneal PMN recruitment at the studied time point of 8h. Also, SN derived from CD1 mice and transferred into NUDE mice failed to normalise PMN recruitment such that no significant difference in peritoneal PMN numbers were evident between groups. Interestingly, however, analysis of circulating PMN numbers revealed that the transfer of SN derived from NUDE mice increased the number of circulating blood PMN in CD1 mice compared to control CD1 mice that had received SN derived from CD1 mice. Furthermore, CD1 SN transfer into NUDE mice reduced the number of circulating PMN in NUDE mice compared to NUDE mice that had received SN derived from NUDE i.e. circulating PMN number appeared to be determined by the source of the SN and not the recipient mouse into which it was injected. Circulating PMN numbers in NUDE or CD1 mice that had received SN from NUDE mice were similar and greater than those of mice that had received CD1 SN. This suggested that the SN from NUDE mice contained a more robust 'signal' for PMN mobilisation from the bone marrow compared to that in SN from CD1 mice. There may be a factor in NUDE SN that enhances the chemoattractant response of leukocytes. In the absence of any striking differences in peritoneal C-X-C chemokine levels between NUDE and CD1 it may be that the SN

from NUDE mice might contain a factor that results in increased recruitment of PMN e.g. either an unknown cytokine/chemokine or a decoy cytokine receptor. IL-6/sIL-6R can upregulate production of other C-X-C chemokines such as CXCL5 and CXCL6. Although the exact function of these chemokines in PMN recruitment is unclear, they could be present at higher concentrations in the SN from NUDE mice since NUDE mice had higher levels of IL-6 at 8h (McLoughlin et al., 2004).

Decoy cytokine receptors exhibit a high affinity and specificity for their ligand, but are unable to mediate signalling. They constitute molecular traps for cytokines or chemokines, thereby controlling the intensity of the inflammation (Mantovani et al., 2001). IL-1RII is an important decoy receptor that acts as a negative regulator of IL-1β and is not involved in signal transduction (Matsuki et al., 2005). IL-1RII is expressed on monocytes, T cells and PMN but it can also be cleaved and act as a soluble receptor (Mantovani et al., 2001). Chemokine decoy receptors such as the Duffy antigen receptor for chemokines (DARC) and D6 silence C-X-C and C-C chemokine actions respectively (Locati et al., 2005). There are also other ways to impede the action of a cytokine/chemokine such as functional decoy receptors. For example, IL-1-Ra is a regulating antagonistic protein for IL-1β, and binds to the active IL-1RI thereby preventing its activation (Matsuki et al., 2005). It synergises with IL-1RII to inhibit IL-1β signalling (Mantovani et al., 2001). Any of these antagonistic molecules could be present at reduced levels in the SN of NUDE mice thereby resulting in a more pro-inflammatory milieu.

A pro-inflammatory environment could also be generated in NUDE mice by anomalous expression of the suppressors of cytokine signalling (SOCS). SOCS are intracellular proteins that act as key regulators of cytokine responses (Alexander and Hilton, 2004). Anomalies in SOCS result in uncontrolled inflammation (Chong et al., 2005). However, to my knowledge, there is no evidence to support abnormal SOCS expression in NUDE or RAG-1 KO mice.

The lack of increased peritoneal PMN recruitment in CD1 mice that have received SN from NUDE mice despite higher circulating PMN numbers highlights the complexity of leukocyte recruitment. A potential explanation is that NUDE mice have a different peritoneal vasculature that would more easily facilitate leukocyte transmigration or the presence of endothelial cells in an enhanced state of activation (increased selectins or ICAMs at their surface) that would promote rolling of circulating leukocytes thereby facilitating transmigration. Alternatively, the leukocytes of NUDE mice may have subtle differences in surface receptors or integrins that would enhance emigration from the blood into tissues. This would explain why peritoneal PMN recruitment could not be enhanced in CD1 mice administered SN from NUDE mice as the circulating leukocytes would differ between the mice.

Despite this work, a full understanding of the effect of T cells on the modulation of peritoneal leukocyte recruitment is still lacking. An additional confounding factor is that NUDE mice had significantly less resting peritoneal B cell numbers number compared to CD1 control mice. So, all of the above discussion

regarding the role of T cells needs to take account of the fact that B cells could also be implicated in the altered leukocyte recruitment found in NUDE mice.

Experiments in RAG-1 KO mice and NUDE mice suggest that T cells might be involved in a form of negative feedback loop controlling PMN influx such that an absence of T cells would result in loss of an inhibitory influence. According to previous studies in γδ T cell KO mice, the phenotype of a heightened inflammatory reaction in NUDE mice could be compatible specifically with the absence of γδ T cells (Skeen et al., 2001; Zachariadis et al., 2006). However, the markedly impaired monocyte/Mϕ recruitment seen in RAG-1 KO would not be compatible with a lack of γδ T cells (Zachariadis et al., 2006).

An alternative explanation is that the leukocytes of NUDE mice are more responsive to chemotactic signals such that they would exhibit increased leukocyte extravasation than control mice when confronted with an identical chemotactic gradient. Such a phenotype has been reported in leukocyte-specific protein (LSP)-1 KO mice. LSP-1 is an F-actin binding protein expressed in lymphocytes, Mϕ and PMN in mice and humans and is involved in leukocyte migration. BTG peritonitis in LSP-1 KO mice results in an increased recruitment of PMN and Mϕ. LSP-1 KO PMN have an increased chemotactic response to fMLP and a trend toward a similar response for KC (Jongstra-Bilen et al., 2000). In the absence of clear cut differences in chemokine levels in the NUDE and RAG-1 KO mice, modulation of LSP-1 expression at the leukocyte cell surface may explain the recruitment kinetics. However, there is no data regarding LSP-1 expression in NUDE or RAG-1 KO mice

and the fact that PMN mobilisation was increased with SN transfer suggests a pro-chemotactic signal.

It was obviously important to examine the role of B cells since NUDE mice exhibited a reduced number of resting peritoneal B cells. PMN recruitment was significantly reduced in μMT KO mice compared to C57BL/6 control mice at the 24h time point. However, μMT KO have a reduced number of circulating PMN at baseline. Impaired monocyte/Mϕ recruitment was found in μMT KO mice at the later time points of 72 and 120h despite similar circulating monocyte numbers in μMT KO mice and C57BL/6 control mice. The pattern of Mϕ recruitment is similar to that found in the RAG-1 KO mice. However, despite this similarity in phenotype of leukocyte kinetics, there were no differences observed in MCP-1 levels between μMT KO mice and C57BL/6 control mice; unlike that found in the RAG-1 KO mice experiment. C-X-C chemokine levels were also similar in both groups. As stated in Chapter 5, I was unable to perform additional experiments at shorter time points (1h and 3h) because of the limited number of mice available. A difference at these early time points could explain the differences in monocyte/Mϕ recruitment. It is of interest that the 'combination' of leukocyte recruitment kinetic curves from both NUDE and μMT KO mice produce a kinetic profile that is very similar to that observed in the RAG-1 KO mice i.e. increased early PMN influx and diminished monocyte/Mϕ recruitment at later time points.

These data suggest that B cells may play a role in promoting monocyte/Mϕ recruitment. The B cell compartment of the peritoneal space is characterized by the

important presence of B1 cells (Hayakawa et al., 1985), which differ from the B cells found in the blood. B1 cells can produce natural antibodies and these antibodies or IgM may be involved in monocyte/Mϕ recruitment since immune complexes derived from interactions between C1q and IgM have been shown to promote MCP-1 production by endothelial cells (van den Berg et al., 1998). Therefore, the lack of IgM due to the absence of B cells could reduce the formation of theses immune complexes and prevent MCP-1 production by resident peritoneal cells such as MeC.

MCP-1 production can also be modified by other factors. For example, IL-10 was shown to play an important role in inducing MCP-1 production in Mϕ and monocytes (Seitz et al., 1995; Yano et al., 1996). Therefore, any modifications of IL-10 production, either by (i) a reduction of phagocytosis of apoptotic cells (Voll et al., 1997) due to the absence of IgM (a known promoter of engulfment of apoptotic cells (Boes et al., 2000)), or (ii) the absence of B cells which can also produce IL-10 (Popi et al., 2004), would result in a reduced MCP-1 production and thus less monocyte/Mϕ recruitment. However, despite the difference in MCP-1 levels seen in RAG-1 KO there were no significant differences in the chemokine/cytokine profile of μMT KO mice and thus no mechanistic insights could be drawn from the *in vivo* studies involving μMT KO mice.

The 3% BTG that I have used has been widely used by other investigators (Ajuebor et al., 1999; Baron and Proctor, 1982; Call et al., 2001; Hanayama et al., 2002; Henderson et al., 2003). I did not undertake additional experiments to define dose response curves for reasons of time, cost and the numbers of mice that would be

required for such studies. However, there is evidence that the induced inflammation might be dose dependent since the administration of 0.3% BTG results in a 10-fold reduction in PMN recruitment and IL-6 production (Call et al., 2001). Therefore, the intensity of the inflammation elicited by 3% BTG might have precluded the demonstration of subtle differences beside leukocyte numbers in the experiments performed.

I then attempted to use an *in vitro* approach with the selective depletion of either B or T lymphocytes to dissect the interaction between lymphocytes and Mϕ in order to find a modulating role for the peritoneal lymphocytes in chemokine production. Important elements that are overlooked by such an approach is the potential involvement of recruited lymphocytes in chemokine/cytokine production within the peritoneum and of course the exclusion of the MeC – the most abundant cell within the peritoneum. As indicated previously, MeC are important producers of chemokines and cytokines with the Mϕ depletion studies suggesting a prominent role for MeC in the production of KC and MCP-1.

C-X-C chemokine analysis after 1% BTG stimulation failed to show any impact of B lymphocyte depletion. The intensity of the stimulation might have been maximal thereby precluding any modulation. In contrast, B cell depletion prior to LPS stimulation resulted in a reduced production of both MIP-2 and KC. Extrapolation of this finding to inflammation *in vivo* would result in a reduction in PMN recruitment as evident in the BTG model in μMT KO mice. T cell depletion had no effect upon the production of MIP-2 and KC after BTG or LPS stimulation.

The initial *in vitro* experiments were performed with 1% BTG and a 6h incubation period but this protocol resulted in very low MCP-1 levels compared to the levels found *in vivo*. However, T cell depletion did augment MCP-1 production following BTG stimulation whilst B cell depletion had no effect. LPS stimulation was also used and B cell depletion reduced MCP-1 production; T cell depletion had no significant effect. The use of 0.25% BTG in these assays resulted in increased MCP-1 production – although still much lower levels than the levels observed *in vivo*. In response to 0.25% BTG, both B and T cell immunodepletion reduced MCP-1 production. Although these *in vitro* data are of interest, these experiments only provide partial answers to the as yet unanswered questions regarding the role of lymphocytes in the regulation of peritoneal leukocyte recruitment.

6.2. Future Work:

The CD11b-DTR mice provided a useful resource to ask key questions regarding the interactions between leukocytes *in situ* and the Mϕ ablation strategy was complemented by the experimental approach involving the adoptive transfer of peritoneal cells. Using these tools, I have demonstrated that resident Mϕ are important in the initiation of serosal inflammation and that lymphocytes play a role in the regulation of leukocyte influx. However, the exact mechanisms whereby lymphocytes modulate these processes remain elusive. There are several issues that would be interesting to address in future studies.

1) Can the Mϕ gate-keeping role be modulated?

I have demonstrated that resident Mϕ can sense perturbation of their local milieu generated by different stimuli. These stimuli are sensed by the different Mϕ receptors such as TLRs and RAGE. Is it possible that the ability of Mϕ to sense these 'irritants' can be modulated. Phagocytosis of apoptotic cells is thought to be an important part of resolution of inflammation and it has been demonstrated that instillation of apoptotic cells into an inflamed peritoneum can induce TGF-β production and reduce pro-inflammatory cytokines (Fadok et al., 1998). However, it is not known if Mϕ that have ingested apoptotic cells will be less responsive to pro-inflammatory agents *in vivo*. Resident peritoneal Mϕ from FvB/N mice could be co-incubated with apoptotic cells to allow phagocytosis and the phagocytic Mϕ would be resuspended into BTG or zymosan and administered into Mϕ-ablated CD11b-DTR mice. Peritoneal lavage at 8h would provide good evidence of any change in the 'sensitivity' to pro-inflammatory signals *in vivo* following apoptotic cell ingestion. Furthermore, siRNA for different surface receptors or other proteins such as cytokines or chemokines could be used in order to investigate which receptors are involved in sensing different inflammatory stimuli and which cytokines/chemokines are important.

2) Experiments in NUDE mice in order to establish the role of T cells in regulating leukocyte influx.

It would be prudent to repeat certain experiments such as the T cell repletion using sufficient 'donor' CD1 control mice to fully restore the resident T cell population in NUDE mice. The measurement of PMN number after the induction of peritonitis would indicate whether T cells regulated PMN influx. If so, the supernatants from peritoneal lavages of inflamed NUDE mice and CD1 control mice could be screened for other cytokines, chemokines, eicosanoids or complement proteins. In an attempt to dissect the factors responsible for modulating PMN recruitment, other mediators must be looked at; potential molecules of interest include C-X-C chemokines such as CXCL5 since it was modulated by IL-6 *in vivo* (McLoughlin et al., 2004) as well as decoy cytokine receptors and antagonistic molecules. The use of technology such as proteomic analysis and mass spectrometry could be used in order to compare the NUDE and CD1 derived supernatants to identify differences.

Since peritoneal recruitment was not enhanced despite higher blood PMN mobilisation, there might be structural and/or functional differences in the peritoneum/vasculature in the NUDE. Histology evaluation of the vasculature and lymphoid structure could be performed in fixed tissue after induction of experimental peritonitis. Functional assessment could be done by evaluation of the expression of

adhesion molecules on the vasculature or omental lymphoid organ, either by fluorescent microscopy or molecular biological techniques.

3) How can B cells modulate Mϕ recruitment?

Studies from the RAG-1 KO mice and *in vitro* experiments involving B cell depletion suggest that B cells might be involved in modulating Mϕ recruitment but the exact mechanism remains ill defined. MCP-1 production *in vivo* was reduced in the RAG-1 KO mice and was also diminished *in vitro* after B and T cell depletion. However, it is not clear how B cells could modulate this. *In vivo* experiments with adoptive transfer of B cells into the peritonea of μMT mice would be of interest to see if this normalises Mϕ recruitment. SN transfer experiments could also be undertaken in order to examine the effect upon both peritoneal leukocyte recruitment and circulating blood PMN numbers. A more extensive cytokine and chemokine analysis could also be performed and analysis of earlier time points in the peritonitis model could be done with CBA analysis of the SN as this would provide useful information regarding the early secretion of cytokines such as IL-10 which can promote MCP-1 production. Also, the levels of additional C-X-C and C-C chemokines such as CXCL5 and MIP-1α could be determined.

Since IgM and C1q might be important in regulating MCP-1 production, i*n vitro* stimulation of resident leukocytes could be done with serum from different KO animals (C1q KO, IgM KO and μMT KO mice) in order to assess the respective role of C1q, IgM and B cells in the modulation of MCP-1 production.

6.3. Conclusion

To conclude, I have drafted two figures to illustrate the complex interactions between the different cytokines and chemokines as suggested by the results presented in this thesis(Figure 6.1 and6.2).

In this thesis, it was demonstrated that:

1) Peritoneal and pleural resident Mϕ play a crucial role in the initiation of inflammation and promoting PMN recruitment.
2) Serosal resident Mϕ are key producers of TNF-α, IL-6 and IL-10 *in vivo*. This cytokine production promotes Mϕ-dependent MIP-2 production, which is a key C-X-C chemokine in both BTG peritonitis and carrageenan induced pleurisy.
3) *In vitro* stimulation of serosal cells can generate C-X-C chemokines but these studies highlighted the Mϕ-independent KC production by other resident cells such as the MeC that occurs *in vivo*.
4) Using mice exhibiting deficiencies in various lymphocyte populations, it was demonstrated that T cells negatively regulate PMN influx and that B cells are probably involved in enhancing monocyte/Mϕ recruitment.
5) MCP-1 levels were reduced by 50% at 24h in the RAG-1 KO mice and this correlated with impaired monocyte/Mϕ recruitment.

6) The *in vitro* experiments involving immunodepletion of B and T cells suggested the possible modulation of C-X-C chemokine and MCP-1 production by lymphocytes.

My work has demonstrated that resident Mϕ are essential for the initiation of inflammation and has highlighted their role as gate-keeping or sentinel cells. I would like to end this thesis by a quote from a microbiologist:

> "But it is not microorganisms only which set up the inflammatory reaction accompanied by the emigration and accumulation of leukocytes. The introduction of inert bodies and of aseptic fluid brings about the same results. The macrophages are, as a matter of fact, endowed with a special susceptibility which enable them to perceive exceedingly small changes in the chemical or physical composition in the medium that surrounds them." (Metchnikoff, 1968).

His name was Ilya Ilyich Mechnikov also known as Eli Metchnikoff and he wrote that in 1905…

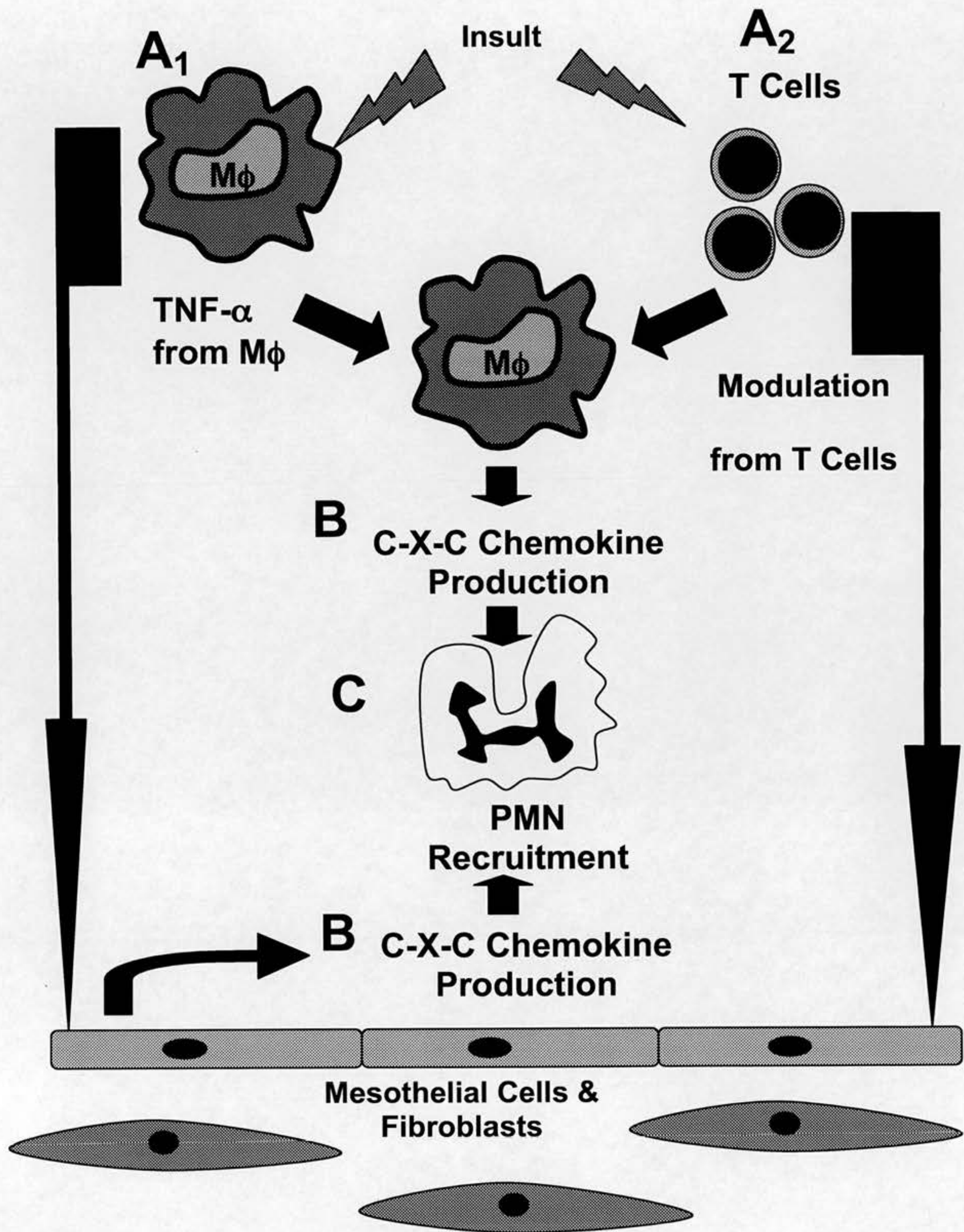


Figure 6.1- PMN recruitment interactions:
A_1) Upon an inflammatory insult, TNF-α would be secreted by resident Mϕ and A_2) resident T cells would modulate this inflammatory response by an ill defined mechanism and B) this would induce C-X-C chemokine production by Mϕ, MeC and fibroblasts C) This chemokine secretion would promote PMN recruitment.

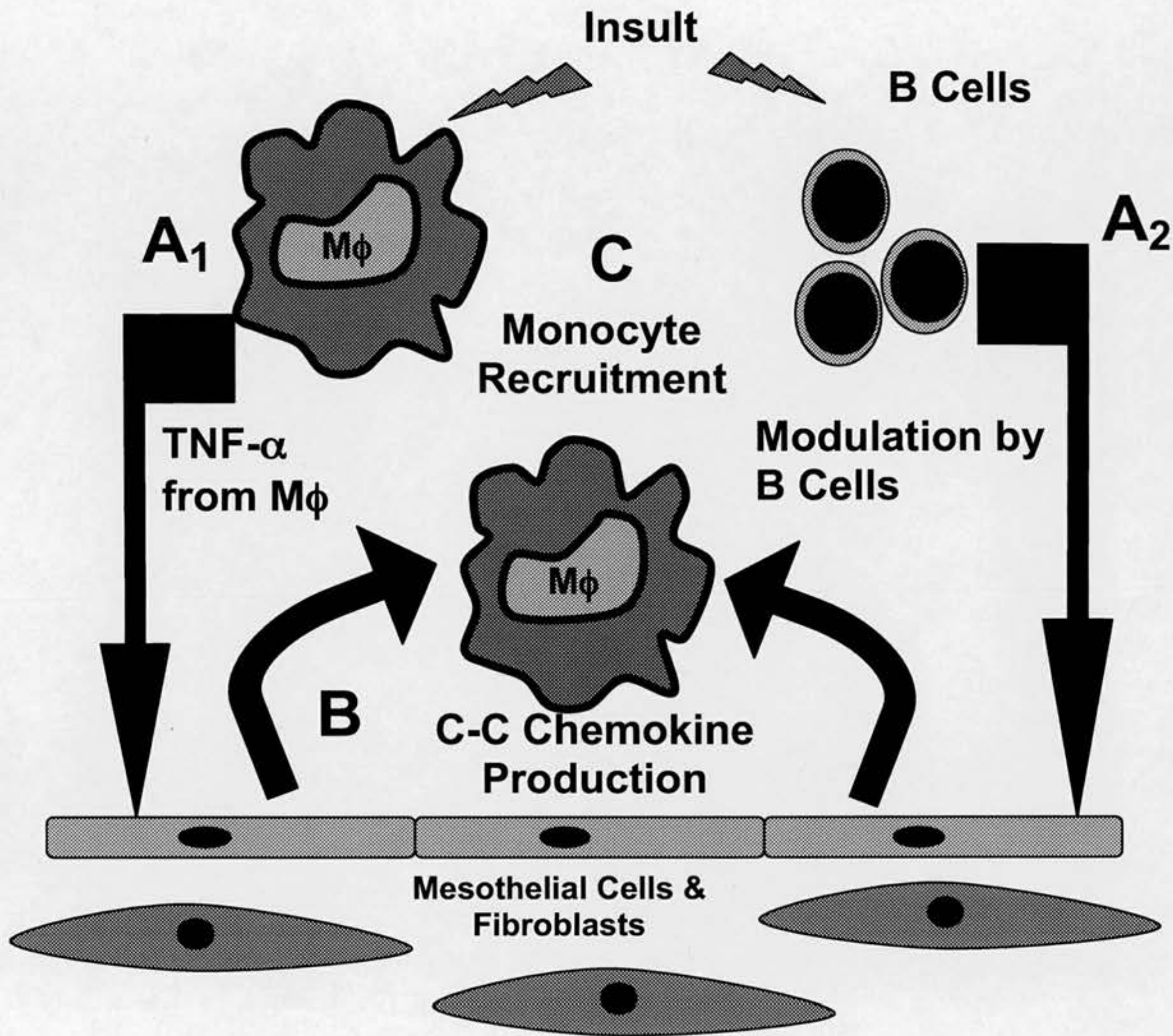


Figure 6.2- Mϕ recruitment interactions:

A_1) Upon an inflammatory insult, TNF-α would be secreted by resident Mϕ and A_2) resident B cells would modulate this inflammatory response by an ill defined mechanism and B) this would induce C-C chemokine production by MeC and fibroblasts C) This chemokine secretion would promote Mϕ recruitment

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Appendix I: Published Papers resulting from work from this thesis:

1) **Conditional Macrophage Ablation Demonstrates That Resident Macrophages Initiate Acute Peritoneal Inflammation.** Jean Francois Cailhier, Marina Partolina, Srilatha Vuthoori, Shengji Wu, Kyung Ko, Simon Watson, John Savill, Jeremy Hughes and Richard A. Lang. *The Journal of Immunology*, 2005, 174: 2336-2342.
   URL: http://www.jimmunol.org/cgi/content/full/174/4/2336.

2) **Resident Pleural Macrophages Are Key Orchestrators of Neutrophil Recruitment in Pleural Inflammation.** Jean François Cailhier, Deborah A. Sawatzky, Tiina Kipari, Kris Houlberg, Dave Walbaum, Simon Watson, Richard A. Lang, Spike Clay, David Kluth, John Savill and Jeremy Hughes. *American Journal of Respiratory and Critical Care Medicine.* Vol 173. pp. 540-547, (2006).
   URL: http://ajrccm.atsjournals.org/cgi/content/full/173/5/540.

Both manuscripts are included at the end of this thesis.

Conditional Macrophage Ablation Demonstrates That Resident Macrophages Initiate Acute Peritoneal Inflammation[1]

Jean Francois Cailhier,[2]* Marina Partolina,[2,3†] Srilatha Vuthoori,[2,4†] Shengji Wu,[2†] Kyung Ko,[5†] Simon Watson,* John Savill,* Jeremy Hughes,[2,6]* and Richard A. Lang[2†]

The role played by resident macrophages (Mφ) in the initiation of peritoneal inflammation is currently unclear. We have used a conditional Mφ ablation strategy to determine the role of resident peritoneal Mφ in the regulation of neutrophil (PMN) recruitment in experimental peritonitis. We developed a novel conditional Mφ ablation transgenic mouse (designated CD11bDTR) based upon CD11b promoter-mediated expression of the human diphtheria toxin (DT) receptor. The murine DT receptor binds DT poorly such that expression of the human receptor confers toxin sensitivity. Intraperitoneal injection of minute (nanogram) doses of DT results in rapid and marked ablation of F4/80-positive Mφ populations in the peritoneum as well as the kidney, and ovary. In experimental peritonitis, resident Mφ ablation resulted in a dramatic attenuation of PMN infiltration that was rescued by the adoptive transfer of resident nontransgenic Mφ. Attenuation of PMN infiltration was associated with diminished CXC chemokine production at 1 h. These studies indicate a key role for resident peritoneal Mφ in sensing perturbation to the peritoneal microenvironment and regulating PMN infiltration. ***The Journal of Immunology,* 2005, 174: 2336–2342.**

Macrophages (Mφ)[7] are dispersed throughout the tissues and have an important role in innate immunity (1), apoptotic cell clearance (2), development (3), and morphogenesis (4, 5). Resident tissue Mφ and dendritic cells are regarded as sentinels of the innate immune system. Various strategies, such as Mφ depletion induced by administration of liposomal clodronate (6), have been used to examine Mφ function in vivo. However, previous work using this method to study the role of resident peritoneal Mφ in experimental peritonitis has produced conflicting results because they have suggested a key role (7), an inhibitory role (8), or no role at all (8). In the current study, we have used a conditional ablation strategy to clarify the role of resident Mφ in the sensing of peritoneal injury and the regulation of neutrophil (PMN) infiltration.

A conditional Mφ ablation strategy has advantages over the available naturally occurring and induced Mφ-deficient mutant mice because the timing of Mφ elimination can be chosen. Despite their limitations, nonconditional Mφ-deficient mice have proven valuable for analysis. For example, the *Csfm^op/Csfm^op* (osteopetrosis) mouse is a naturally occurring mutant of the *CSF-1* gene and exhibits a Mφ deficiency at a level that permits viability in homozygotes (9). These mice have provided insight into Mφ function during development (3). In addition, mice targeted at the *PU.1* locus exhibit multiple defects in development of hemopoietic lineages including a complete absence of tissue Mφ (10, 11). Although *PU.1* mutation results in perinatal lethality, these mice have been used to demonstrate that mesenchymal cells are able to clear apoptotic cells during embryonic regression of interdigital tissues (12).

Previous strategies used to eliminate specific cell types in a living organism include the generation of transgenic lines that express diphtheria toxin A-chain (13, 14) or the ricin polypeptide (15). However, even low levels of unanticipated transgene expression can give unpredictable consequences (13). The alternative ablation strategy of killing thymidine kinase-expressing cells with gancyclovir (16, 17) only permits the elimination of proliferating cells. More recently, conditional Mφ ablation has been achieved using transgenic expression of Fas under the control of the c-*fms* promoter coupled with drug-inducible Fas dimerization to induce cell death (18). Identification of the human receptor for diphtheria toxin (DT) (also known as heparin-binding epidermal growth factor (hbEGF) (19)) created an opportunity for a unique ablation strategy. The murine form of hbEGF binds DT poorly, but mouse cells can be rendered sensitive through transgenic expression of human hbEGF. In transgenic mice expressing human hbEGF lineage specifically, cell ablation results following toxin injection. In addition, DT is a protein synthesis inhibitor and kills both mitotic and terminally differentiated cells. This strategy has recently been used to generate transgenic mice in which hepatocytes (20) or dendritic cells (21) may be conditionally ablated. In the current

*Phagocyte Laboratory, Medical Research Council Center for Inflammation Research, University of Edinburgh, Edinburgh, United Kingdom; and †Dr. Richard A. Lang Division of Developmental Biology and Department of Ophthalmology, Children's Hospital Research Foundation, Cincinnati, OH 45229

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[2] J.-F.C., M.P., S.V., S.W., J.H., and R.A.L. contributed equally to this manuscript.

[3] Current address: Departments of Radiation Oncology and Cell Biology, New York University School of Medicine, 540 First Avenue, New York, NY 10016.

[4] Current address: Regeneron Pharmaceuticals, Tarrytown, NY 10591.

[5] Current address: Molecular Pathogenesis Program, Skirball Institute for Biomolecular Medicine, 540 First Avenue, New York, NY 10016.

[6] Address correspondence and reprint requests to Dr. Jeremy Hughes, Phagocyte Laboratory, Medical Research Council Center for Inflammation Research, University of Edinburgh, Teviot Place, Edinburgh, U.K., EH8 9AG. E-mail address: jeremy.hughes@ed.ac.uk

[7] Abbreviations used in this paper: Mφ, macrophage; PMN, neutrophil; DT, diphtheria toxin; hbEGF, heparin-binding epidermal growth factor; BTG, Brewer's thioglycolate; WT, wild type; PI, propidium iodide; KC, keratinocyte-derived chemokine; MC, mast cell.

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report, we describe how we have used this strategy to generate a conditional Mϕ ablation mouse to assess the role of resident peritoneal Mϕ in the initiation of acute inflammation in experimental sterile peritonitis induced by Brewer's thioglycolate (BTG). We demonstrate that resident peritoneal Mϕ are essential for PMN recruitment through Mϕ-dependent CXC chemokine production.

Materials and Methods

Transgenic construct

The CD11b promoter from coordinates −1704 to + 83 (22) was used to drive expression of the human hbEGF cDNA (19). Splicing and polyadenylation signals were provided by a region of the human growth hormone gene that had previously worked effectively with the CD11b promoter (22). The fusion protein between hbEGF and GFP was generated by continuing the open reading frame from the final residue of hbEGF with the first residue of GFP.

Transgenic mice

The CD11b-DTR construct was used to generate transgenic mice on the FVB/N background (23) using conventional techniques; transgene expression was detected using an RT-PCR assay. The primer sequences used were 5′-AAGATCCGCCACAACATCG for the forward primer and 5′-GCAGCTCTAGGTTGGATTTCTG for the reverse primer. Because the reverse primer sequence was taken from the base pairs flanking intron III of hGH, no PCR product could be amplified from genomic DNA.

Flow cytometry analysis

Bone marrow-derived Mϕ were prepared as previously described (4). Resident peritoneal cells were isolated by peritoneal lavage. Elicited peritoneal Mϕ were lavaged from the peritoneal cavity 4 days after i.p. injection of 1 ml of 3% BTG (Difco). For flow cytometric analysis, $\sim 2 \times 10^6$ cells were incubated for 10 min in FACS buffer containing rat anti-mouse FcR blocker CD16/CD32 (FcγRIII/II). FITC- or PE-conjugated Abs at concentrations suggested by the supplier were then added, and the mixture was incubated at 4°C for 45–60 min. The cells were then washed and fixed in 2% formaldehyde before undergoing flow cytometry analysis. A fixed volume of whole blood was obtained by tail vein bleed into 3.9% sodium citrate. Blood was aliquoted into flow cytometry tubes, blocked, and stained as for peritoneal lavage cells. One milliliter of FACSLysis buffer (BD Biosciences) was then added to lyse erythrocytes, and samples were spun and processed by flow cytometry. The Abs used were anti-CD11b FITC conjugate (eBiosciences), anti-GR1 PE (eBiosciences), anti-B220 (mouse CD45R) FITC and PE conjugates (BD Pharmingen), mouse anti-CD3 PE conjugates (BD Pharmingen), and F4/80 allophycocyanin, FITC, and PE conjugates (Caltag and Serotec). Annexin V^{FITC} conjugate (US Biological) and propidium iodide (PI; Sigma-Aldrich) were used to determine the levels of apoptosis and necrosis. A known amount of fluorescent Flow-Check fluorospheres (Beckman Coulter) was added to peritoneal lavage samples before analysis, and the ratio of cells to beads was used to calculate the absolute number of any cell type in peritoneal lavage fluid. Analyses were performed using a FACScan or FACSCalibur instrument.

Immunolabeling of tissues

Organs were fixed in 4% formaldehyde in PBS at 4°C overnight, and paraffin-embedded tissue sections were prepared according to conventional methods. Rehydrated sections were immersed in acetone for 10 min and rinsed in PBS, and a limited trypsin (Sigma-Aldrich) digestion was performed for 20 min. Sections were then washed in PBS, and endogenous peroxidase activity was quenched with a 30-min incubation in 0.3% H_2O_2 in methanol. Sections were then incubated with F4/80 mAb (Caltag Laboratories) at a 1/100 dilution. Further steps were performed according to the recommendations of the manufacturer of the VECTASTAIN Elite ABC (avidin/biotin complex) system used in the labeling (Vector Laboratories). Sections were then washed in water, lightly counterstained with Mayer's hematoxylin, dehydrated, and mounted.

Experimental peritonitis

Peritonitis was induced by i.p. injection of 1 ml of 3% BTG. Resident Mϕ ablation was induced in transgenic mice by i.p. injection of DT (25 ng/g body weight) 24 h before the administration of BTG, with DT-treated FVB/N wild-type (WT) mice serving as control. Mice underwent peritoneal lavage at various time points following BTG injection. In some experiments, peritonitis was induced by the injection of 0.2 mg of zymosan (Sigma-Aldrich) with peritoneal lavage being performed at 8 h. All experiments were performed in accordance with institutional and U.K. Home Office guidelines.

Adoptive transfer of peritoneal cells

Peritoneal lavage samples from groups of FVB/N WT mice were pooled, spun, and either resuspended in 1 ml of sterile PBS (Mϕ-rich peritoneal cells) or plated on tissue culture plastic for 2 h to deplete Mϕ by adhesion (Mϕ-depleted peritoneal cells). A total of 97 ± 2.8% of Mϕ was removed from the cell suspension. Four hours before induction of BTG peritonitis, Mϕ-rich or Mϕ-depleted peritoneal cells suspended in 1 ml of PBS were injected i.p. Experimental groups consisted of 1) CD11b-DTR transgenic mice depleted of resident peritoneal Mϕ by prior administration of DT, 2) DT-treated FVB/N WT mice, 3) Mϕ-depleted mice reconstituted with Mϕ-rich peritoneal cells, and 4) Mϕ-depleted mice reconstituted with Mϕ-depleted peritoneal cells. Groups 1 and 2 were injected with 1 ml of PBS 4 h before BTG administration as an injection control for the adoptive cell transfer procedure. Animals were sacrificed 8 h following the initiation of peritonitis.

Chemokine studies

Mice underwent peritoneal lavage at 1 and 3 h following the i.p. injection of BTG. Lavage fluid was centrifuged, aliquoted, and stored at −80°C until analyzed by ELISA for MIP-2 and keratinocyte-derived chemokine (KC) (R&D Systems). Chemokine production by peritoneal cell populations that had been depleted of either Mϕ or mast cells (MC) was also determined in vitro. Peritoneal cells were incubated with PE-conjugated anti-F4/80 Ab or an Ab to the MC marker c-*kit* (CD117) to stain Mϕ or MC, respectively. Peritoneal cells were then incubated with anti-PE-conjugated MACS magnetic beads, and Mϕ or MC were removed by passing the cells over a magnetic MACS column (Miltenyi Biotec). As a control, total peritoneal cells were incubated with an isotype control Ab followed by magnetic beads and subsequently passed over the magnetic MACS column. This method resulted in >97% depletion of Mϕ or MC. Control peritoneal cells and Mϕ- or MC-depleted peritoneal cells were then plated in 48-well plates (5×10^5 cells per well) and exposed to 1% BTG for 3 h. Peritoneal cell-conditioned supernatants were harvested, spun, and stored at −80°C until analyzed by specific ELISA for MIP-2 and KC (R&D Systems).

Statistical analysis

The Student *t* test with a tailed distribution or ANOVA was used to analyze data. A value of $p < 0.05$ was deemed statistically significant. Data are presented as mean ± SE.

Results

Generation of transgenic mice

Conditional ablation transgenic mice were generated using an established strategy (21) and a construct (designated CD11b-DTR) that used the CD11b promoter (22) to provide Mϕ expression specificity. CD11b-DTR expresses the DT receptor (alternatively named hbEGF (19)) (Fig. 1*A*) as a GFP fusion protein. The hbEGF-GFP construct conferred sensitivity to DT in transiently transfected murine cells indicating that it was functional (data not shown).

Six transgenic lines were produced with the CD11b-DTR construct. Although the fluorescence signal from the hbEGF-GFP fusion protein was insufficient to permit detection of transgene expression by FACS analysis, a RT-PCR assay indicated that four lines exhibited detectable transgene expression in peritoneal cells and spleen with line 34 exhibiting high expression (Fig. 1, *B* and *C*). WT and CD11b-DTR-34 bone marrow-derived Mϕ expressed the Mϕ-specific gene *F4/80* as expected (Fig. 1*D*), indicating that expression of the DT receptor was unlikely to have disrupted normal Mϕ differentiation. Transgene expression was also observed in day 4 and 8 CD11b-DTR-34 bone marrow-derived Mϕ (Fig. 1*E*). Because line CD11b-DTR line 34 showed the highest levels of transgene expression, further analysis was restricted to this line.

Transgenic Mϕ are killed by DT in vitro and in vivo

Treatment of BTG-elicited peritoneal Mϕ from CD11b-DTR-34 mice with concentrations of DT between 1 ng/ml to 1 mg/ml over

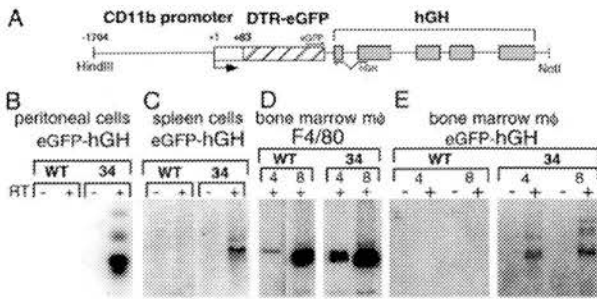

FIGURE 1. Structure and expression of the CD11b-DTR transgene. *A*, Schematic of the CD11b-DTR construct. The transcription start is indicated by the right-facing arrow and exons by shaded boxes. The DTR-eGFP fusion cDNA is inserted between the human CD11b promoter and the human growth hormone (hGH) sequences that provides splicing and polyadenylation signals. Oligonucleotides eGFP and hGH used for RT-PCR transcript detection are indicated by small arrows. *B–E*, RT-PCR expression analysis performed on BTG-elicited peritoneal cells (*B*), spleen cells (*C*), and bone-marrow derived Mϕ (*D* and *E*). Transgene mRNA amplification products were not evident in samples from WT mice or when reverse transcriptase was omitted but was detected in line 34 peritoneal and spleen cells (*B* and *C*). Line 34 bone-marrow derived Mϕ also exhibited normal expression of the Mϕ marker F4/80 (*D*) and persistent transgene expression (*E*).

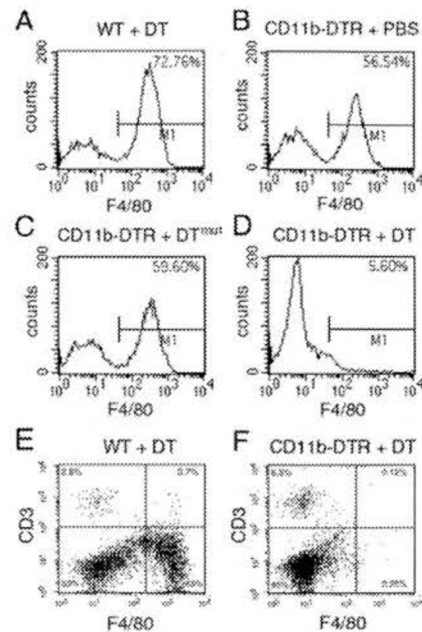

FIGURE 2. Flow cytometric analysis of peritoneal cell Mϕ killing by DT. *A–D*, Cells were removed from the peritoneal cavity by peritoneal lavage and labeled with PE-conjugated F4/80 Ab, and flow cytometric analysis was performed. *A*, WT mice injected with DT (25 ng/g body weight) show a normal percentage of peritoneal Mϕ. *B* and *C*, CD11b-DTR-34 mice receiving either PBS (*B*) or DT^{mut} (*C*) have normal F4/80 profiles. *D*, In contrast, CD11b-DTR-34 mice treated with active DT show complete absence of F4/80-positive cells. *E*, In WT mice, injection of DT at 25 ng/g mouse weight does not affect either the small population of $CD3^+$ T cells (*upper-left quadrant*) or the larger population of $F4/80^+$ Mϕ (*lower-right quadrant*) in the peritoneal cavity. *F*, CD11b-DTR-34 mice receiving DT exhibit elimination of the $F4/80^+$ population, whereas the $CD3^+$ cells remain unaffected.

a period of 48 h induced cell death at concentrations as low as 25 ng/ml. In contrast, Mϕ from WT mice or other transgenic lines were resistant (data not shown). We then asked whether i.p. injection of DT ablated resident peritoneal Mϕ in vivo. DT was injected at 25 ng/g mouse weight, and FACS analysis of peritoneal cells was performed 24 h later. Normal numbers of resident peritoneal Mϕ (F4/80 positive, CD11b positive, and Ly6C/G negative) were evident in WT mice receiving DT (Fig. 2*A*) as well as CD11b-DTR-34 transgenic mice injected with either PBS (Fig. 2*B*) or the inactive form of the toxin DT^{mut} (Fig. 2*C*). However, CD11b-DTR-34 transgenic mice showed an almost complete absence of F4/80-positive peritoneal Mϕ after a single dose of DT (Fig. 2*D*). Administration of DT doses of 6.25 and 12.5 ng/g mouse weight resulted in Mϕ ablation of ~72 and 82%, respectively, whereas lower doses resulted in <50% ablation. We therefore chose to use a dose of 25 ng/g body weight for the in vivo studies.

Time course of Mϕ elimination in vivo

We then examined the kinetics of Mϕ ablation in the peritoneal cavity following administration of a single dose of DT (25 ng/g body weight). The appearance of apoptotic and necrotic cells was monitored using Annexin V^{FITC} and PI staining, respectively. Peritoneal lavages and flow cytometric analyses were conducted on a series of mice 4, 6, 8, and 12 h after DT injection (Fig. 3). After 6 h, 65% of the peritoneal population was annexin V positive, indicating a dramatic increase in early-stage apoptotic cells (Fig. 3*A*). The maximal numbers of PI-positive cells occurred ~2 h later at 8 h after DT injection and represented ~20% of the total peritoneal cells (Fig. 3*B*). The number of F4/80-positive cells was nearly zero at 12 h (Fig. 3*C*), and this corresponded to very low levels of PI-positive and annexin V-positive cells (*A* and *B*). These data suggest that DT induces sensitive cells to undergo apoptosis and that some of these dying cells then undergo secondary necrosis. All F4/80-positive cells were cleared by 12 h. Restoration of the peritoneal Mϕ population occurs by day 4 following a single i.p. dose of DT ($3.94 \times 10^5 \pm 1.7 \times 10^5$ Mϕ vs $5.8 \times 10^5 \pm 1.3 \times 10^5$; day 4 following DT treatment vs day 1 following PBS treatment; $n = 5$ per group, $p > 0.05$).

Specificity of Mϕ elimination in vivo

To test the specificity of Mϕ elimination, we examined $CD3^+$ T cells in spleen and peritoneal cavity and $B220^+$ B cells in the spleen. In this case, we injected two doses of DT at 25 ng/g at 48-h intervals and assessed ablation 24 h later. The F4/80-positive peritoneal population was unaffected in WT mice (Fig. 2*E*, *lower-right quadrant*), but was eliminated in CD11b-DTR-34 animals (*G*, *lower-right quadrant*). Despite complete peritoneal Mϕ elimination in CD11b-DTR-34 animals, the peritoneal $CD3^+$ T cells were present in both WT and transgenic animals injected with DT (Fig. 2, *E* and *F*, *upper-left quadrants*). The relative increase in CD3-positive and double-negative cells in the DT-treated CD11b-DTR-34 animals is due to the plotting of equal numbers of detection events in the FACS analyses. $B220^+$ and $CD3^+$ populations in spleen were unaffected in either WT or CD11b-DTR-34 mice receiving two doses of DT (data not shown).

Because CD11b is expressed on both granulocytes and Mϕ, we asked whether both of these cell types were sensitive to DT. CD11b-DTR-34 mice were injected with BTG. DT (25 ng/g body weight) or PBS was injected 8 h after initiation of peritonitis with peritoneal lavage being performed 12 h later. Despite 90% Mϕ ablation ($0.5 \times 10^6 \pm 0.15 \times 10^6$ vs $4.8 \times 10^6 \pm 0.23 \times 10^6$; DT injection vs PBS; $p < 0.005$), there was no difference in PMN number ($2.3 \times 10^6 \pm 0.22 \times 10^6$ vs $1.6 \times 10^6 \pm 0.39 \times 10^6$; DT injection vs PBS; $p > 0.05$). In addition, flow cytometric analysis of whole blood performed 24 h following DT administration indicated that circulating PMN numbers were unaffected by DT administration ($1.02 \times 10^6 \pm 0.18 \times 10^6$ PMNs/ml whole blood vs

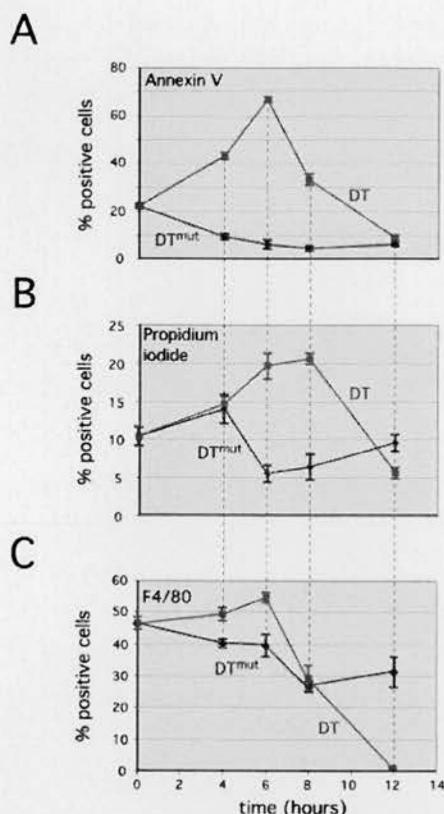

FIGURE 3. Time course of peritoneal Mϕ depletion. Cells were removed from the peritoneal cavity by peritoneal lavage. Mice were either injected with a single dose of DT (gray lines) or DT^{mut} (black lines) at 25 ng/g mouse weight i.p., and the resident peritoneal population was assessed for the appearance of annexin V-positive cells (*A*), for labeling of cells with PI (*B*), and for presence of the Mϕ marker F4/80 (*C*). Flow cytometric analysis identified the labeled proportion of total cells over a 12-h time course following DT injection.

$0.97 \times 10^6 \pm 0.22 \times 10^6$; DT injection vs PBS; $p > 0.05$). In contrast, DT administration induced significant depletion of circulating monocytes ($0.117 \times 10^6 \pm 0.059 \times 10^6$ monocytes/ml whole blood vs $0.52 \times 10^6 \pm 0.073 \times 10^6$; DT injection vs PBS; $p < 0.05$).

Differential deletion of Mϕ populations

We also asked whether DT injection could eliminate Mϕ in distant organs. Two doses of DT (25 ng/g) were administered IP at 48-h intervals, and the presence of F4/80-positive Mϕ in kidney, liver, and lung was analyzed 24 h later and in the ovary 16 h later (Fig. 4). The ovary was examined at 16 h, because there was evidence of some patchy ovarian necrosis present at 20 h. WT mice injected with DT and CD11b-DTR-34 homozygote mice injected with DT^{mut} were unaffected (Fig. 4, *top two rows*). Both kidney and ovary of CD11b-DTR-34 homozygote mice injected with DT exhibited an absence of F4/80$^+$ cells. In the kidney, mesangial and interstitial Mϕ were ablated in the absence of overt renal injury. However, hepatic sinusoidal Mϕ and alveolar Mϕ were unaffected, indicating that not all populations of tissue Mϕ were susceptible. However, the rapid elimination of Mϕ populations in the peritoneal cavity and kidney while leaving other cell populations intact establishes the basic validity of this approach to conditional cell ablation.

Resident Mϕ ablation reduces PMN influx and CXC chemokine responses during experimental peritonitis

We used the conditional ablation strategy to investigate the role of resident tissue peritoneal Mϕ in sensing perturbation of the microenvironment and subsequent initiation of acute peritoneal inflammation and PMN recruitment in experimental peritonitis. Resident Mϕ ablation markedly attenuated PMN infiltration following the administration of 3% BTG (Fig. 5*A*). We also performed Mϕ repletion studies with either Mϕ-rich or Mϕ-depleted peritoneal cells derived from WT mice. Reconstitution of DT-treated CD11b-DTR-34 homozygote mice with Mϕ-rich peritoneal cells 4 h before BTG treatment resulted in complete restoration of peak PMN infiltration at 8 h. In contrast, the administration of Mϕ-depleted peritoneal cells was ineffective (Fig. 5*B*). Previous work suggested that the nature of the inflammatory stimulus may determine the involvement of Mϕ in experimental peritonitis (8), and we therefore performed Mϕ depletion studies in zymosan peritonitis. We also found that depletion of resident peritoneal Mϕ resulted in a significant reduction in PMN infiltration 8 h following the induction of zymosan peritonitis ($2.6 \times 10^6 \pm 8.8 \times 10^5$ PMNs vs $5.4 \times 10^6 \pm 3.7 \times 10^5$; DTR plus DT vs FVB/N WT controls plus DT; $n = 6$ per group; $p < 0.05$).

In this model, we found peak levels of the PMN CXC chemokines MIP-2 and KC at the 1-h time point. Resident peritoneal Mϕ

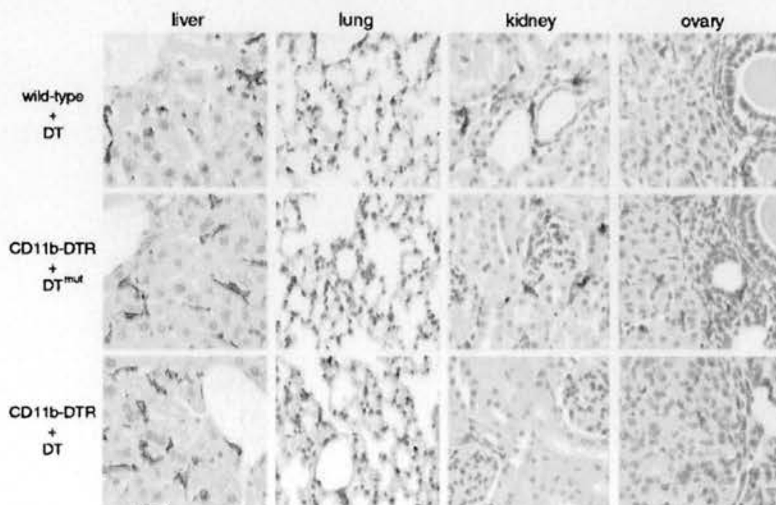

FIGURE 4. Effect of DT on tissue Mϕ populations. Micrographs showing the effect of DT treatment of WT mice (*top row*), DT^{mut} treatment of CD11b-DTR mice (*middle row*), and DT treatment of CD11b-DTR mice (*bottom row*) upon the presence of F4/80-positive Mϕ in liver (*left column*), lung (*center left column*), kidney (*center right column*), and ovary (*right column*). Animals received two doses of DT or DT^{mut} delivered at 48-h intervals. Liver, lung, and kidney were assessed 24 h after the second DT injection, whereas ovary was assessed at 16 h.

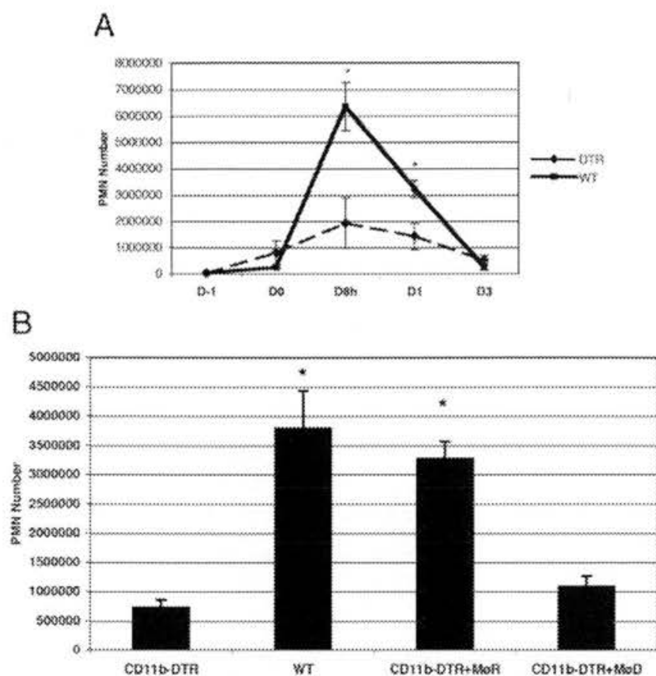

FIGURE 5. Resident Mφ ablation attenuates peritoneal inflammation. WT and CD11b-DTR mice were injected i.p. with DT (25 ng/g body weight). One milliliter of 3% BTG was injected i.p. 24 h later with mice undergoing peritoneal lavage at various time points. *A*, Peritoneal cells were stained for the PMN marker GR1. Resident peritoneal Mφ ablation induced a marked blunting of PMN infiltration of the peritoneal cavity. *B*, WT and three groups of CD11b-DTR mice were injected with DT (25 ng/g body weight). Four hours before i.p. injection of 3% BTG, two groups of Mφ-depleted CD11b-DTR mice were reconstituted with either Mφ-rich peritoneal cells (MφR) or Mφ-depleted peritoneal cells (MφD). Administration of PBS served as control to the remaining groups. Mice underwent peritoneal lavage 8 h following administration of 3% BTG, and peritoneal cells were stained for GR1. *, $p < 0.05$.

ablation before the initiation of BTG peritonitis markedly reduced the elevation in MIP-2 levels (148.5 ± 34.8 vs 1762.1 ± 153.5 pg/ml; Mφ-depleted mice vs nondepleted mice: $p < 0.00001$). There was a slight, albeit statistically significant, difference in the much lower levels of MIP-2 between DT-treated and control mice at 3 h (204 ± 54 vs 74 ± 8 pg/ml; Mφ-depleted mice vs nondepleted mice; $p < 0.05$). This suggests that the production of MIP-2 in vivo is predominantly Mφ dependent. In addition, a 50% reduction in the level of KC was evident in Mφ-depleted mice at the 1-h time point (1408.2 ± 322.5 vs 2467.5 ± 264.9 pg/ml; Mφ-depleted mice vs nondepleted mice; $p < 0.05$). Interestingly, the levels of KC levels at 3 h are higher in Mφ-depleted mice compared with control mice (1477 ± 400 vs 74 ± 8 pg/ml; Mφ-depleted mice vs nondepleted mice; $p < 0.01$), thereby suggesting a source of KC other than resident Mφ.

CXC chemokine responses are Mφ dependent in vitro

Previous studies of peritoneal and dermal inflammation have implicated the MC as playing an important role in the initiation of PMN infiltration (8, 24). We therefore performed in vitro studies to determine the production of PMN chemokines by BTG-stimulated peritoneal cell populations that had been depleted of Mφ or MC. Control peritoneal cells produced significant levels of MIP-2 and KC, which was not affected by MC depletion (Fig. 6). However, chemokine levels were dramatically reduced following the depletion of Mφ, thereby indicating that chemokine production was completely Mφ dependent with no involvement of MC (Fig. 6).

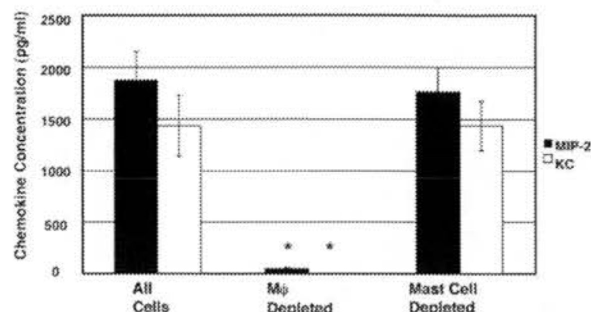

FIGURE 6. CXC chemokine production in response to BTG stimulation is Mφ dependent and MC independent in vitro. Peritoneal cells were depleted of either Mφ or MC by incubation with PE-conjugated anti-F4/80 or anti-c-*kit* (CD117) followed by incubation with anti-PE-conjugated magnetic beads and passage over a magnetic column (>97% depletion of Mφ or MC). Incubation of total peritoneal cells with an isotype control Ab followed by magnetic beads and passage over the magnetic column served as control. Cells were then plated in 48-well plates (5×10^5 cells per well) and exposed to 1% BTG for 3 h. Peritoneal cell-conditioned supernatants were harvested, spun, and analyzed by specific ELISA for MIP-2 and KC. *, $p < 0.005$.

Discussion

Previous analyses (20, 21) and the experiments we describe here show that expression of human hbEGF (19) in mouse cells can confer sensitivity to DT in vivo, and that, as a consequence, injection of DT will kill cells that express hbEGF. Our data indicate that Mφ populations in the peritoneal cavity and kidney can be rapidly killed or eliminated while leaving other cell populations intact, and this establishes the basic validity of this approach to conditional cell ablation. We noted that hepatic and alveolar Mφ populations were unaffected, and it may be the case that a higher dose of DT may have ablated these cells. However, we found that mice could become unwell with doses of DT >25 ng/g body weight, and we therefore did not use doses >25 ng/g body weight in this study. It is pertinent that, despite PMN expression of CD11b, the administration of DT did not induce the death of recruited or circulating PMNs, indicating that PMNs are insensitive to DT, potentially as a result of their lower level of protein synthesis.

We used the conditional ablation strategy to investigate the role of resident peritoneal Mφ in the initiation of acute peritoneal inflammation following the administration of BTG. Previous work has indicated that leukotrienes derived from resident peritoneal Mφ are involved in the development of early vascular permeability in sterile peritonitis (25). Although early work in rat models of peritonitis implicated the resident peritoneal Mφ in the orchestration of PMN recruitment (26–28), more recent studies have produced conflicting results (7, 8). Indeed, studies by Ajuebor et al. (8) suggest that resident Mφ depletion inhibits PMN influx in LPS-induced inflammation, has no effect in BTG peritonitis, and augments PMN influx in zymosan peritonitis. In the latter model, it is proposed that Mφ-derived IL-10 inhibits PMN recruitment. Conversely, work by Knudsen et al. (7) using clodronate-induced depletion of peritoneal Mφ in a rat model of sterile peritonitis demonstrated that PMN infiltration was Mφ dependent.

In this study, administration of DT resulted in a dramatic 98% Mφ ablation that markedly blunted PMN infiltration, thereby indicating a key role for the resident Mφ in the orchestration of acute peritoneal inflammation in this experimental model. It is important to note that the reduced PMN infiltration in DT-treated mice was not attributable to a systemic neutropenia, because PMNs were not sensitive to DT and the number of circulating PMNs in DT-treated

mice was comparable with that of PBS-treated mice at 24 h. The importance of the resident Mϕ was reinforced by experiments involving the adoptive transfer of nontransgenic peritoneal Mϕ following DT-mediated Mϕ ablation and before the initiation of peritonitis. The presence or absence of Mϕ in the transferred peritoneal cell population directly correlated with the restoration of the PMN influx, thereby suggesting that the Mϕ exerts a critical role in this process. In addition, we found that depletion of resident Mϕ also significantly reduced PMN infiltration in zymosan peritonitis, thereby suggesting that the sensing function of the resident Mϕ may be stimulus independent.

The magnitude of the Mϕ depletion may explain the apparent discrepancy between these results and the study by Ajuebor et al. (8). Administration of a single dose of DT induced 98% Mϕ ablation, whereas three doses of liposomal clodronate resulted in >85% Mϕ depletion in the study by Ajuebor et al. (8). Mϕ are a potent source of chemokines and cytokines, and it may be the case that, in certain circumstances, a relatively small population of residual Mϕ may exert significant biological effects. Although administration of liposomal clodronate may exert marked biological effects despite depletion of only ~80% of Mϕ (29), it may be necessary to deplete almost all peritoneal Mϕ to delineate their roles as sentinel cells.

Although peritoneal Mϕ may produce myriad mediators capable of recruiting PMNs (30, 31), we examined the effect of Mϕ ablation upon the level of CXC chemokines in this model. Our data suggest that the initiation of PMN infiltration is mediated by resident peritoneal Mϕ-dependent production of chemokines previously documented to play a role in orchestrating PMN recruitment in BTG peritonitis (32, 33) and in other inflammatory situations (34–36). MC are also a rich source of proinflammatory and vasoactive mediators and have been documented to play an important role in PMN recruitment during inflammation of the peritoneum (8) as well as other sites such as the skin (24).

We found that resident Mϕ ablation markedly reduced the peak level of MIP-2 and significantly blunted the level of KC at 1 h. However, partial inhibition (50%) of KC production at 1 h and the persistent elevation of KC at 3 h in Mϕ-depleted mice suggest that KC may be produced by other cells within the peritoneum. The persistent elevation of KC also suggests that Mϕ may play a role in the negative regulation of KC production by non-Mϕ cells. In this context, it is pertinent that our in vitro data indicate that both KC and MIP-2 production by peritoneal cells obtained by peritoneal lavage is almost entirely dependent upon Mϕ, because peritoneal cells depleted of Mϕ produced minimal levels of chemokines. These findings suggest that peritoneal cells retrievable by peritoneal lavage are not the source of KC detected in our in vivo study. This interpretation of the data is consistent with recent work in a wound model (37) demonstrating MIP-2 expression by inflammatory cells and KC expression by resident tissue cells such as endothelial cells and fibroblasts. Peritoneal mesothelial cells undoubtedly participate in peritoneal inflammation and can produce chemokines and cytokines (38), and it may be the case that mesothelial cells contribute to the KC production evident in this study. Despite this, however, we found that PMN infiltration is still markedly blunted despite the persistent presence of KC at 3 h.

We also examined the potential interaction between Mϕ and MC by performing in vitro studies of peritoneal cells that had been depleted of Mϕ or MC before stimulation with BTG. Interestingly, depletion of MC had no significant effect upon the production of KC and MIP-2 following BTG stimulation, suggesting that chemokine production in this model was Mϕ dependent and MC independent.

In conclusion, this work has used a novel model of conditional Mϕ ablation to dissect the role of resident peritoneal Mϕ in the initiation of acute peritoneal inflammation. Our data indicate a key role for the resident Mϕ in sensing peritoneal irritation and orchestrating PMN infiltration in BTG and zymosan peritonitis. This proinflammatory function is predominantly mediated by production of the potent PMN CXC chemokine MIP-2 and, to a lesser extent, KC. Although previous work has implicated the involvement of other cells such as MC, our study suggests that resident Mϕ are critically important producers of PMN chemokines and act to orchestrate PMN recruitment in murine BTG peritonitis. We also anticipate that CD11b-DTR transgenic mice will be valuable for studying other Mϕ functions in vivo in a variety of different biological contexts. The option of being able to choose the time and, with local toxin injection, perhaps the locality of ablation offers a number of advantages over other cell ablation systems.

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Resident Pleural Macrophages Are Key Orchestrators of Neutrophil Recruitment in Pleural Inflammation

Jean François Cailhier*, Deborah A. Sawatzky*, Tiina Kipari, Kris Houlberg, Dave Walbaum, Simon Watson, Richard A. Lang, Spike Clay, David Kluth, John Savill, and Jeremy Hughes

Phagocyte Laboratory, MRC Centre for Inflammation Research, University of Edinburgh, Edinburgh, United Kingdom; and Division of Developmental Biology, Department of Ophthalmology, Children's Hospital Research Foundation, Cincinnati, Ohio

Rationale: **The role played by resident pleural macrophages in the initiation of pleural inflammation is currently unclear.**
Objective: **To evaluate the role of resident pleural macrophages in the initiation of inflammation.**
Methods: **We have used a conditional macrophage ablation strategy to determine the role of resident pleural macrophages in the regulation of neutrophil recruitment in a murine model of experimental pleurisy induced by the administration of carrageenan and formalin-fixed *Staphylococcus aureus*.**
Measurements and Main Results: **Conditional macrophage ablation mice express the human diphtheria toxin receptor under the control of the CD11b promoter such that the administration of diphtheria toxin induces ablation of nearly 97% of resident macrophages. Ablation of resident pleural macrophages before the administration of carrageenan or *S. aureus* dramatically reduced neutrophil influx into the pleural cavity. In the carrageenan model, the reduction in neutrophil infiltration was associated with marked early reduction in the level of macrophage inflammatory protein 2 as well as reduced levels of various cytokines, including tumor necrosis factor α, interleukin 6, and interleukin 10. Adoptive transfer of nontransgenic macrophages partially restored neutrophil infiltration. We also stimulated macrophage-depleted and nondepleted pleural cell populations with carrageenan *in vitro* and determined the production of chemokines and cytokines. Chemokine and cytokine production was markedly reduced by macrophage depletion, reinforcing the role of resident pleural macrophages in the generation of mediators that initiate acute inflammation.**
Conclusion: **These studies indicate a critical role for resident pleural macrophages in sensing perturbation to the local microenvironment and orchestrating subsequent neutrophil infiltration.**

Keywords: inflammation; macrophage; pleural diseases

The pleural membranes and associated cells are important because they are metabolically active and act as a barrier to invading pathogens by generating an innate and adaptive immunologic response. The pleural cavity is lined with mesothelium and contains resident macrophages (Mϕ), mast cells, and lymphocytes (1, 2). During pleural inflammation, it has been reported that mesothelial cells are predominantly responsible for the secretion of C-X-C chemokines, such as interleukin 8 (IL-8), and C-C chemokines, such as macrophage inflammatory protein 1α (MIP-1α) and macrophage chemoattractant protein 1 (MCP-1), which act to recruit neutrophils (polymorphonuclear leukocytes [PMNs]) and mononuclear cells (3–6). In addition, a recent study demonstrated that activated pleural fibroblasts may also be a source of C-X-C and C-C chemokine production (7).

Previous work suggested that the initiation of inflammation is dependent on endogenous IL-6 secretion that subsequently stimulates the additional production of tumor necrosis factor α (TNF-α) and IL-1β from resident pleural cells (8). In contrast, increased IL-1β levels have been reported to precede elevated IL-6 levels (9), thereby suggesting that IL-1β might induce IL-6 production. There is no doubt that TNF-α and IL-1β are key cytokines in the development of pleural inflammation because they act to enhance IL-8 and MCP-1 production from mesothelial cells (3, 5, 10–12). In addition, studies using function-blocking antibodies suggest that activated resident Mϕ could be responsible for this TNF-α and IL-1β secretion (10, 12).

Carrageenan-induced pleurisy is a well-established model of acute inflammation (13) and is characterized by a rapid influx of PMNs followed by mononuclear cell infiltration (14, 15). This model is often used to assess the antiinflammatory effects of pharmaceutical agents (16–20) and to assess the *in vivo* importance of established inflammatory mediators (21–23). Although the neutrophil influx evident in this model is generally used as an experimental readout of acute inflammation, there are data indicating that neutrophils are involved in the release of injurious enzymes and modulation of vascular permeability in carrageenan-mediated pleural inflammation (24, 25).

To date, there has been little study of the role of the resident pleural Mϕ in the initiation of inflammation and orchestration of PMN recruitment. Previous work demonstrated a reduced eosinophil influx after administration of LPS to mice that had been previously treated with diphosphonate-containing liposomes to deplete resident pleural Mϕ (26). Although this suggests that resident pleural Mϕ may play a key role in the initiation of pleural inflammatory responses, there are no definitive data available for PMN infiltration and proinflammatory cytokine production.

This study used transgenic mice expressing the human diphtheria toxin receptor (DTR) under the CD11b promoter (designated CD11b-DTR mice) (27) to examine the role of resident pleural Mϕ in carrageenan-induced pleurisy. Administration of diphtheria toxin (DT) to CD11b-DTR mice results in rapid depletion of resident pleural Mϕ. Our data indicate that ablation of resident pleural Mϕ markedly blunted both PMN recruitment and the levels of key chemokines and cytokines. In addition, resident Mϕ ablation markedly reduced the acute PMN infiltration that followed the instillation of fixed, killed *Staphylococcus aureus*. This study demonstrates that resident pleural Mϕ play an essential role in the orchestration of pleural PMN recruitment

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*These authors contributed equally to this article.

Correspondence and requests for reprints should be addressed to Jeremy Hughes, M.D. Ph.D., Phagocyte Laboratory, MRC Center for Inflammation Research, University of Edinburgh, Teviot Place, Edinburgh, UK, EH8 9AG. E-mail: jeremy.hughes@ed.ac.uk

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in pleural inflammation induced by carrageenan and fixed, killed *S. aureus*.

METHODS

Macrophage Ablation and Pleurisy Induction

Mice were housed in the University of Edinburgh animal facilities and experiments were performed in accordance with institutional and U.K. Home Office guidelines. CD11b-DTR transgenic mice were generated as previously described and were on an FVB/N background (27). Resident pleural Mϕ were ablated in homozygous CD11b-DTR mice by intraperitoneal injection of DT (25 ng/g body weight) 24 h before the administration of carrageenan. DT-treated FVB/N wild-type (WT) mice served as control animals. Carrageenan-induced pleurisy was induced as described previously (28). λ-Carrageenan (0.1 ml of a 1% solution) was injected into the pleural cavity. Animals were culled at various time points after pleurisy developed. In addition, 3×10^6 formalin-fixed, fluorescently labeled *S. aureus* (Sigma, Dorset, UK) were injected into the pleural cavity of CD11b-DTR mice and FVB/N WT mice 24 h after administration of DT or phosphate-buffered saline (PBS). Animals were culled 4 h later.

Cell Processing and Analysis

Pleural cavities were washed with 1 ml of 3.15% (weight/volume) sodium citrate (Sigma, Dorset, UK) in saline. We performed flow cytometric analysis of pleural lavage and circulating blood as described previously (27). The antibodies used were anti-CD11b fluorescein isothiocyanate, anti-GR1 phycoerythrin (PE) and anti–c-kit PE (all from eBiosciences, London, UK), anti-B220 (mouse CD45R) PE and mouse anti-CD3 PE (both from Pharmingen, San Diego, CA), and F4/80 allophycocyanin (APC) and F4/80 PE (both from Caltag, Botolph Claydon, UK). Cell number was determined as described previously (27).

Adoptive Transfer of Pleural Cell Populations

Pleural lavages from groups of naive FVB/N WT mice were incubated with PE-conjugated anti-F4/80 antibody to stain Mϕ and then incubated with anti-PE conjugated magnetic cell sorting (MACS) magnetic beads (Miltenyi Biotech Ltd., UK). Mϕ were removed by passing the cells over a magnetic MACS column (27). As a control, pleural cells were incubated with an isotype control antibody and then processed as previously stated. This method removed 98.2 ± 0.7% of the Mϕ. In addition, resident pleural Mϕ were purified by negative selection after incubation of pleural cells with PE-conjugated anti-B220, anti–c-kit, and anti-CD3 antibodies followed by incubation with anti–PE-conjugated MACS magnetic beads and passage through the magnetic MACS column; isolated Mϕ were 90% pure. Purified Mϕ and the Mϕ-depleted and Mϕ nondepleted pleural cell populations were resuspended in 1% carrageenan and administered into the pleural cavity of each mouse. Groups therefore consisted of (*1*) DT-treated CD11b-DTR transgenic mice depleted of resident pleural Mϕ, (*2*) DT-treated FVB/N WT mice, (*3*) Mϕ-depleted mice reconstituted with a nondepleted Mϕ-rich pleural cell population, (*4*) Mϕ-depleted mice reconstituted with a Mϕ-depleted pleural cell population, and (*5*) Mϕ-depleted mice reconstituted with a population of pleural Mϕ purified by negative selection. As a control, the effect of cell transfer alone was assessed by reconstituting Mϕ-depleted mice with either nondepleted Mϕ-rich pleural cells or purified Mϕ alone in the absence of any additional stimulus. Animals were killed 6 h after induction of pleurisy.

Chemokine Studies

Mice underwent pleural lavage at 1, 3, 6, 24, and 72 h after administration of carrageenan. Lavage fluid was centrifuged and stored at −80°C until analyzed by specific ELISA for MIP-2, keratinocyte-derived chemokine (KC), and TNF-α (R&D Systems, Abingdon, UK). Cytometric bead array (BD Biosciences, Oxford, UK) was also used to determine the concentration of IL-6, IL-10, IL-12p70, IFN-γ, and MCP-1, with samples being processed as described previously (29). Chemokine and cytokine production by intact pleural cell populations or Mϕ-depleted pleural cell populations stimulated with carrageenan *in vitro* was also determined: Mϕ depletion was achieved using the MACS magnetic column and resulted in more than 98% Mϕ depletion. Control pleural cells and Mϕ-depleted pleural cells were plated in 48-well plates and exposed to 0.25% carrageenan for 6 h. In control experiments, cell preparations were exposed to medium alone. Pleural cell–conditioned supernatants were analyzed as above. No bioassays were undertaken.

Statistical Analysis

One-way analysis of variance with a Bonferroni multiple comparison *post hoc* test, with a 95% confidence interval, or a Student's *t* test was used as appropriate. Statistical analysis including correlation analysis was performed using GraphPad Prism software (San Diego, CA). The significance level was set at $p < 0.05$. Data are presented as mean ± SEM.

RESULTS

Transgenic Pleural Resident Mϕ Are Ablated by DT *In Vivo*

There was no difference in the number of pleural Mϕ, B cells, T cells, or mast cells between CD11b-DTR and FVB/N control mice (data not shown). Flow cytometric analysis of pleural cells was performed 24 h after the injection of DT (25 ng/g mouse body weight). CD11b-DTR transgenic mice exhibited almost complete ablation (96.1% ± 0.8) of F4/80-positive pleural Mϕ after a single dose of DT (Figure 1). In addition, flow cytometric analysis of whole blood performed 24 h after DT administration indicated a significant 88% reduction in circulating monocyte numbers ($1.17 \times 10^5 \pm 5.9 \times 10^4$ monocytes/ml whole blood vs. $5.23 \times 10^5 \pm 7.3 \times 10^4$, DT injection vs. control; $p < 0.05$). Circulating monocyte and pleural macrophage numbers remained markedly reduced for 48 h after the administration of DT with recovery of monocyte/macrophage numbers evident at 72 h (data not shown). However, no reduction in the number of circulating PMNs was evident 24 h after DT administration ($10.1 \times 10^5 \pm 1.9 \times 10^5$ PMNs/ml whole blood vs. $4.7 \times 10^5 \pm 0.8 \times 10^5$, DT injection vs. control; $p < 0.05$). In addition, no difference in circulating PMN number was evident 6, 48, or 72 h after the administration of DT, indicating an absence of any initial neutropenia or delayed effects (6 h: $7.9 \times 10^5 \pm 1.2 \times 10^5$ PMNs/ml whole blood vs. $5.0 \times 10^5 \pm 1.4 \times 10^5$, DT injection vs. control; $p > 0.05$; 48 h: $4.0 \times 10^5 \pm 0.6 \times 10^5$ PMNs/ml whole blood vs. $4.9 \times 10^5 \pm 0.1 \times 10^5$, DT injection vs. control; $p > 0.05$; 72 h: $2.9 \times 10^5 \pm 1.4 \times 10^5$ PMNs/ml whole blood vs. $4.4 \times 10^5 \pm 0.3 \times 10^5$, DT injection vs. control; $p > 0.05$). We did, however, note a significant reduction in the number of B cells and mast cells within the pleural cavity 24 h after the administration of DT although T-cell numbers were unaffected (B cells: $8.1 \times 10^4 \pm 5.7 \times 10^4$ vs. $32.9 \times 10^4 \pm 8.8 \times 10^4$, DT vs. control; $p < 0.05$; mast cells: $6.1 \times 10^2 \pm 0.1 \times 10^2$ vs. $67.8 \times 10^2 \pm 18.2 \times 10^2$, DT vs. control; $p < 0.05$). Interestingly, the depletion of pleural Mϕ is almost complete at 6 h at which time no significant difference in the number of B lymphocytes or mast cells was evident. The loss of B cells and mast cells may be a consequence of the secondary necrosis of apoptotic macrophages that may occur in the absence of a population of viable macrophages to phagocytose the dying cells. Also, a subset of B lymphocytes and mast cells may express CD11b and this may account for the reduced numbers seen after the administration of DT (30–32).

Pleural Resident Mϕ Ablation Reduces PMN Influx in Carrageenan-induced Pleurisy

We used the conditional Mϕ ablation strategy to investigate the role of resident pleural Mϕ in initiating PMN recruitment after the administration of carrageenan. PMN infiltration after the administration of 1% carrageenan was markedly attenuated at all experimental time points after resident Mϕ ablation (Figure 2). It is particularly noteworthy that the early time points of 6 and 24 h demonstrated a dramatic difference between groups. Although PMN infiltration in CD11b-DTR mice did reach approximately

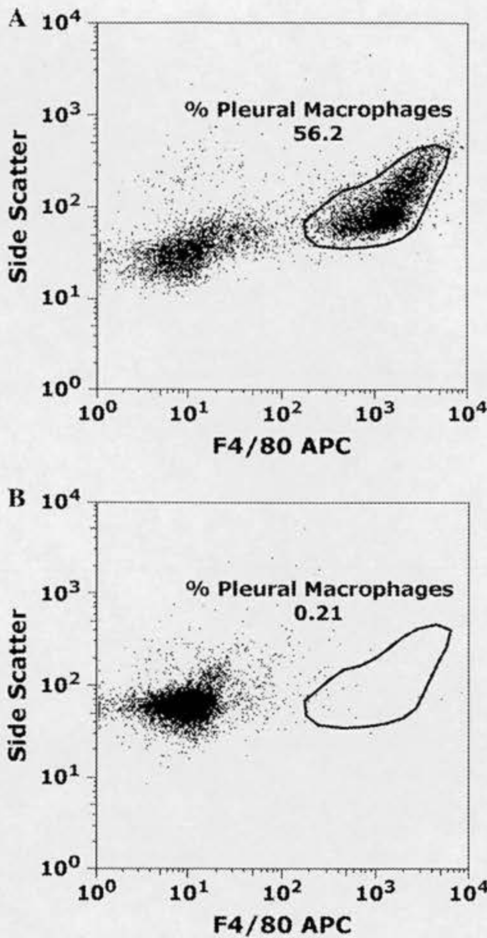

Figure 1. Administration of DT 24 h before performing pleural lavage results in ablation of pleural F4/80-positive macrophages (Mϕ). CD11b-DTR and FVB/N wild-type (WT) mice were treated with diphtheria toxin (DT) intraperitoneally at a dose of 25 ng/g body weight. Pleural lavage was performed 24 h later. Cells were stained for the Mϕ surface marker F4/80 and analyzed by flow cytometry. (*A*) Representative flow cytometry *dot plot* indicating that over 50% of pleural cells retrievable by pleural lavage 24 h after DT administration in FVB/N mice are F4/80 positive. (*B*) Administration of DT results in marked ablation of resident F4/80 positive pleural Mϕ in CD11b-DTR mice. DT administration ablated 96.1 ± 0.8% of the resident Mϕ population compared with baseline Mϕ numbers (n = 9 mice, $p < 0.0001$). APC = allophycocyanin.

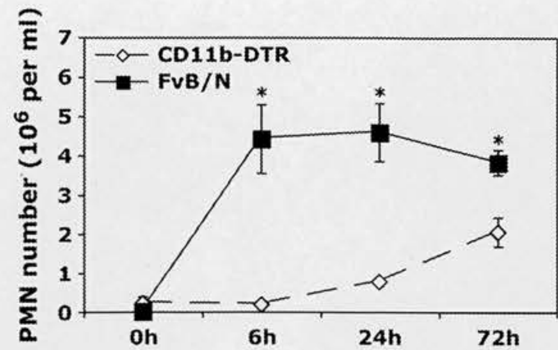

Figure 2. Resident Mϕ ablation 24 h before administration of carrageenan blunts neutrophil (PMN) recruitment. 0.1 ml of 1% carrageenan was administered to CD11b-DTR and FVB/N WT mice 24 h after DT treatment. Pleural lavage was performed at 0, 6, 24, and 72 h after carrageenan administration. Lavaged cells were stained for GR1 and counted by flow cytometry (*$p < 0.05$ vs. CD11b-DTR group; n = 4–5 mice/group).

50% of control levels at the later time points of 72 h, this was still significantly less than DT-treated nontransgenic FVB/N WT mice.

Adoptive Transfer of Nontransgenic Purified Mϕ or Mϕ-rich Pleural Cell Populations Partially Restores PMN Influx in Mϕ-ablated CD11b-DTR Mice after Carrageenan Administration

To further analyze the role of resident pleural Mϕ in the initiation of acute pleural inflammation, we also performed Mϕ repletion studies using the adoptive transfer of either Mϕ-rich or Mϕ-depleted pleural cell populations derived from DT-insensitive nontransgenic FVB/N WT mice. In these experiments, the adoptive transfer of Mϕ-rich pleural cell populations restored Mϕ number to approximately 50% of the Mϕ number normally present in pleural lavage fluid. However, despite the fact that Mϕ reconstitution of DT-treated CD11b-DTR mice was incomplete, the administration of Mϕ-rich pleural cells concurrently with carrageenan significantly increased PMN infiltration at 6 h (Figure 3). The partial restoration of peak PMN infiltration was approximately 35% of levels present in control DT-treated FVB/N WT mice at the same time point. In contrast, administration of Mϕ-depleted pleural cells concurrently with carrageenan made no significant impact on PMN infiltration compared with Mϕ-depleted CD11b-DTR mice (Figure 3). Interestingly, a significant correlation ($R^2 = 0.9979$) was found between the Mϕ number present in the pleural space at the initiation of inflammation and the number of infiltrating PMNs present at 6 h. We also reconstituted DT-treated CD11b-DTR mice with purified Mϕ (90% pure) concurrently with the administration of carrageenan and this resulted in a comparable PMN influx to that evident after reconstitution with Mϕ-rich pleural cells. It should be noted that, although DT-induced Mϕ ablation is associated with a reduction of B-cell and mast cell number, the administration of Mϕ-depleted pleural cells comprising B cells, mast cells, and T cells had no significant impact on PMN infiltration. Last, the adoptive transfer of a control population of Mϕ-rich pleural cells or purified Mϕ was noninflammatory (Figure 3).

Mϕ-dependent Chemokine and Cytokine Responses during Carrageenan-induced Pleurisy

In this model, we found peak levels of the PMN C-X-C chemokines MIP-2 and KC at the 1- and 3-h time points, respectively. Ablation of resident pleural Mϕ before administration of carrageenan markedly reduced MIP-2 levels at both 1 and 3 h (Figure 4A), thereby suggesting that the early production of MIP-2 *in vivo* is predominantly Mϕ dependent. Interestingly, however, Mϕ-ablated mice exhibited a delayed and significantly blunted MIP-2 response. It is of interest that very few Mϕ (< 30,000) are present within the pleural cavity of DT-treated CD11b-DTR mice at the 6-h time points, suggesting that the delayed MIP-2 response may be derived from production by local cells, such as mesothelial cells and others. MIP-2 levels are very low at the 24-h time point and beyond in both experimental groups. In contrast to the MIP-2 data, a very modest, albeit statistically significant, reduction in KC levels was evident in Mϕ-depleted mice at the 1-, 3-, and 6-h time points (Figure 4B), but no differences were evident thereafter, suggesting that cells other than Mϕ may be responsible for production of this chemokine. The fact that ablation of resident pleural Mϕ dramatically

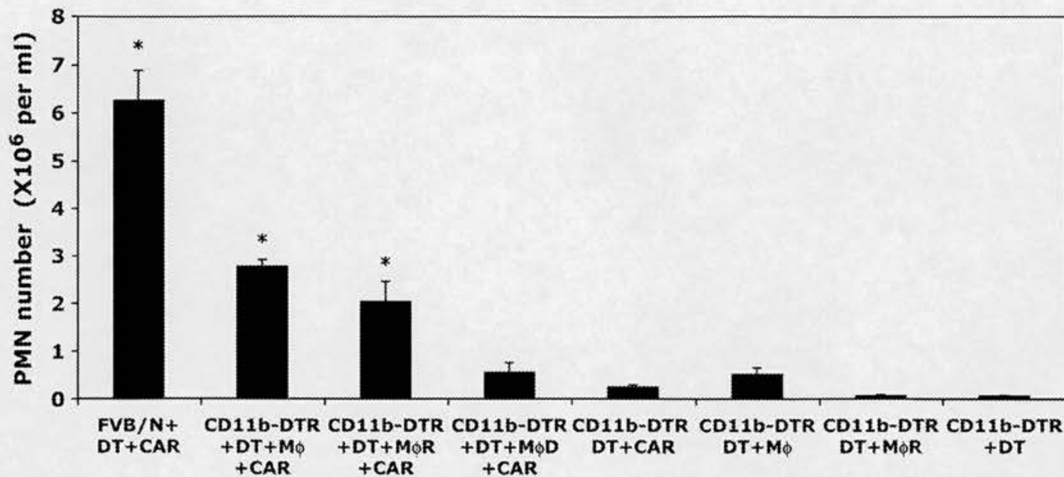

Figure 3. Adoptive transfer of Mϕ-rich pleural cells and purified pleural macrophages partially restores PMN infiltration in carrageenan-induced pleurisy. FVB/N WT and six groups of CD11b-DTR mice were injected with DT (25 ng/g body weight) 24 h before carrageenan injection. Three groups of Mϕ-depleted CD11b-DTR mice were reconstituted with (*1*) purified Mϕ isolated by negative selection (90% pure, designated Mϕ), (*2*) Mϕ-rich pleural cells (designated MϕR), or (*3*) Mϕ-depleted pleural cells (designated MϕD) at the same time as the administration of carrageenan. Mice underwent pleural lavage 6 h after the induction of inflammation. Controls comprised the adoptive transfer of either (*1*) purified Mϕ or (*2*) Mϕ-rich pleural cell populations to DT-treated CD11b-DTR mice in the absence of carrageenan. DT-treated CD11b-DTR mice exhibited a marked reduction in PMN infiltration in response to carrageenan, whereas reconstitution of Mϕ-depleted mice with either purified Mϕ or a Mϕ-rich pleural cell population partially restored PMN infiltration. The adoptive transfer of an Mϕ-depleted pleural cell population did not increase PMN infiltration. The adoptive transfer of either purified Mϕ or an Mϕ-rich pleural cell population alone did not induce significant PMN infiltration compared with DT-treated CD11b-DTR mice (n = 8–10 mice/group; $^*p < 0.05$ vs. DT-treated CD11b-DTR mice that received carrageenan).

blunted PMN infiltration suggests that early PMN influx is very dependent on resident Mϕ production of MIP-2. The ablation of resident pleural Mϕ did not exert marked effects on the production of MCP-1 as levels were only reduced by approximately 36% at the 3-h time point (Figure 4C), suggesting a source other than Mϕ.

Analysis of the levels of cytokines in pleural lavage samples indicated a key role for resident Mϕ in the early production of the cytokines TNF-α, IL-6, and IL-10. Mϕ ablation resulted in greater than 90% reduction in TNF-α and IL-6 levels, with a less dramatic but significant inhibitory effect on IL-10 levels (Figure 5). IL-12 levels were also reduced with Mϕ ablation at 24 h (data not shown). Despite these important differences in these cytokines, IFN-γ levels were comparable between DT-treated CD11b-DTR and FVB/N control mice at each time point (data not shown), suggesting a source other than resident Mϕ.

Chemokine and Cytokine Responses of Pleural Cell Populations *In Vitro* Are Mϕ Dependent

Because pleural mesothelial cells may be an important source of chemokines, we performed additional *in vitro* studies to determine the production of chemokines and cytokines by carrageenan-stimulated pleural cell populations that had been depleted of Mϕ. Immunomagnetic Mϕ depletion using antibodies for the Mϕ specific marker F4/80 resulted in 98% depletion of Mϕ from pleural cell populations, whereas B-cell and mast cell numbers were comparable between groups (data not shown). Stimulation of control Mϕ-rich pleural cell populations for 6 h with carrageenan resulted in significant production of MIP-2 and KC (Figure 6). In contrast, no significant chemokine production was evident after stimulation of pleural cell populations depleted of resident Mϕ but containing B cells, T cells, and mast cells, thereby indicating that production of these PMN C-X-C chemokines *in vitro* was completely Mϕ dependent. Limited production of MCP-1 was evident *in vitro* but this was also significantly reduced by depletion of resident Mϕ (25.3 ± 5.3 vs. 7.1 ± 4.7 pg/ml, Mϕ-rich pleural cells vs. Mϕ-depleted pleural cells; $p < 0.05$). Analysis of *in vitro* cytokine production demonstrated that resident Mϕ were key cytokine producers, because Mϕ depletion before carrageenan stimulation resulted in a reduction of 63, 67, and 92% in the production of TNF-α, IL-10, and IL-6, respectively (Figure 7).

Pleural Resident Mϕ Ablation Reduces PMN Influx in Response to *S. aureus*

Although the carrageenan model of pleurisy is a useful model of inflammation and has been used by many investigators to dissect inflammatory pathways, we sought evidence that resident Mϕ were involved in models of inflammation that were more closely related to clinical disease. We initially used the model of intrapleural LPS instillation, but this resulted in a very low level of PMN infiltration compared with carrageenan. We therefore instilled formalin-fixed, fluorescently labeled *S. aureus* into the pleural cavity and this induced a marked PMN infiltrate at the 4-h time point ($> 1.5 \times 10^6$ PMNs). The ablation of resident Mϕ significantly reduced PMN infiltration after the administration of *S. aureus* (Figure 8). We also found comparable PMN infiltration in DT-treated FVB/N WT mice and PBS-treated CD11b-DTR mice, indicating that insertion of the transgene had no significant effect on the generation of acute inflammatory responses (Figure 8A), with comparable findings evident after the administration of carrageenan (data not shown). Cytospin preparations of pleural lavage cells indicated prominent ingestion of *S. aureus* particles by Mϕ in DT-treated FVB/N WT mice (Figure 8B) with very limited uptake by PMNs. In contrast, in the absence of Mϕ, DT-treated CD11b-DTR mice exhibited marked ingestion of *S. aureus* particles by PMNs (Figure 8B).

DISCUSSION

We used a conditional macrophage ablation strategy to dissect the role of the resident pleural Mϕ in the initiation of pleural inflammation and PMN recruitment in carrageenan-induced pleurisy. Carrageenan induces inflammatory responses that are likely to be involved in human disease such as tuberculosis, which is a cause of significant morbidity and mortality. We also examined the effect of Mϕ ablation before the administration of fixed *S. aureus*, a model with direct clinical relevance. Although

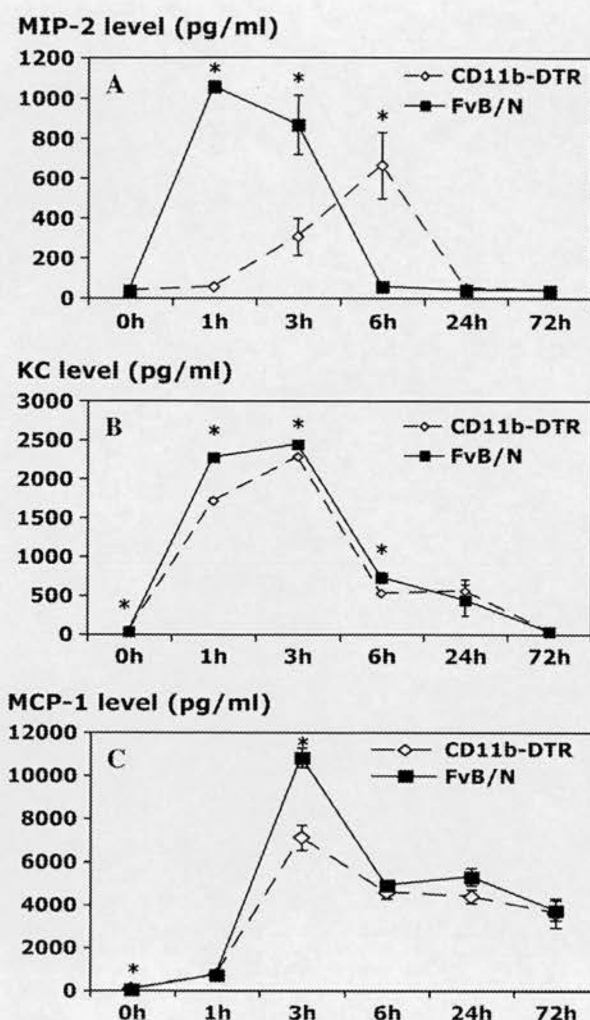

Figure 4. Resident Mϕ ablation attenuates chemokine production in carrageenan-induced pleurisy. CD11b-DTR and FVB/N WT mice were injected with DT (25 ng/g body weight) 24 h before administration of carrageenan. Pleural lavage was performed 1, 3, 6, 24, and 72 h after the induction of pleurisy. The levels of macrophage inflammatory protein 2 (MIP-2; *A*) and keratinocyte-derived chemokine (KC; *B*) were determined in the pleural lavage supernatant by specific ELISA. The level of macrophage chemoattractant protein 1 (MCP-1; *C*) in the pleural lavage supernatant was determined by cytometric bead array (CBA) analysis (*$p < 0.05$ vs. CD11b-DTR group; $n = 5$ mice/group).

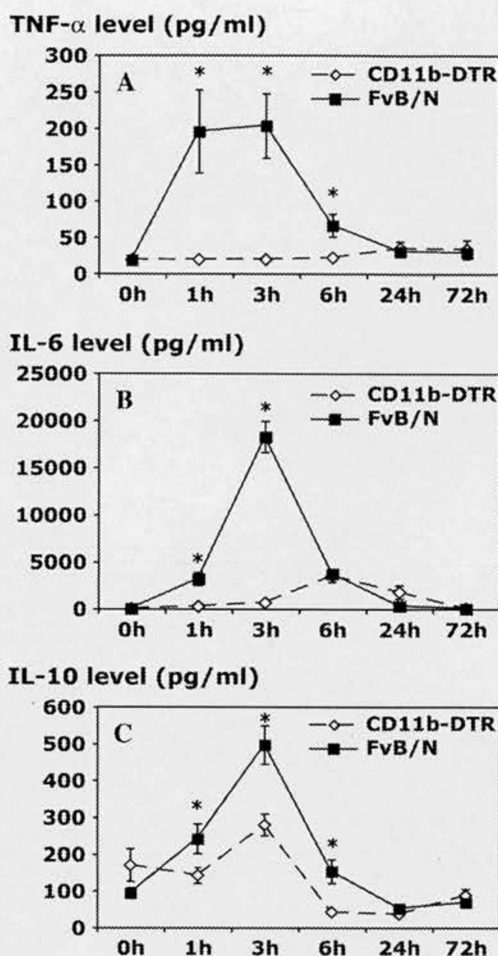

Figure 5. Resident Mϕ ablation attenuates cytokine production in carrageenan-induced pleurisy. CD11b-DTR and FVB/N WT mice were injected with DT (25 ng/g body weight) 24 h before carrageenan injection. Pleural lavage was performed 1, 3, 6, 24, and 72 h after the induction of pleurisy. The level of tumor necrosis factor α (TNF-α; *A*) in the pleural lavage supernatant was determined by specific ELISA, whereas the levels of interleukin 6 (IL-6; *B*) and IL-10 (*C*) were determined by CBA analysis (*$p < 0.05$ vs. CD11b-DTR group; $n = 5$ mice/group).

the resident pleural Mϕ can secrete chemokines and cytokines, their role in pleurisy is currently unclear. Pleural mesothelial cells also have the capacity to secrete various chemokines (3, 6, 7, 12, 33, 34). In addition, some studies have identified resident pleural Mϕ-derived proinflammatory cytokines such as TNF-α that are essential for the secretion of C-X-C and C-C chemokines from pleural mesothelial cells (3, 5, 8, 10–12, 33), suggesting important cross-talk between different pleural cells.

The first major finding of this study is that the administration of DT to CD11b-DTR transgenic mice results in the rapid and effective ablation of resident pleural Mϕ, with greater than 96% of resident pleural Mϕ being depleted 24 h after DT treatment. This is comparable with our previous work studying peritoneal inflammation (27). Interestingly, despite PMN expression of CD11b, the administration of DT did not induce the death of circulating PMNs, indicating that PMNs are insensitive to DT, potentially as a result of their lower level of protein synthesis.

The second major finding of this study is that resident pleural Mϕ ablation dramatically blunted early PMN infiltration into the pleural cavity, indicating an important role for resident pleural Mϕ in initiating acute pleural inflammation. The administration of DT did not affect the numbers of circulating PMNs, thereby excluding this potential cause for diminished PMN infiltration of the pleural cavity. We performed Mϕ repletion studies involving the adoptive transfer of nontransgenic pleural cell populations to Mϕ-depleted mice concurrent with the induction of pleurisy. The adoptive transfer of pleural cell populations depleted of Mϕ by magnetic immunodepletion had no significant effect on PMN recruitment; PMN numbers were comparable to those evident in control Mϕ-depleted mice. In contrast, adoptive transfer of either pleural cell populations containing Mϕ or a population of purified Mϕ significantly increased pleural PMN infiltration, reinforcing the key role of resident pleural Mϕ. Adoptive transfer of pleural cells was unable to restore Mϕ numbers to normal values and this may explain the partial restoration of PMN infiltration compared with DT-treated FVB/N control mice. However, the striking correlation between the

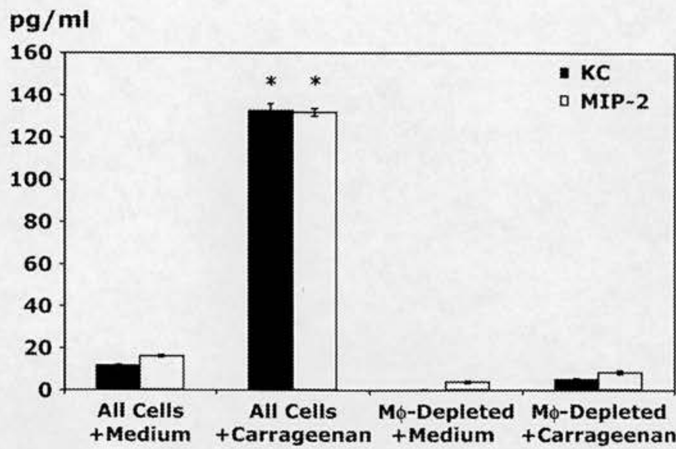

Figure 6. *In vitro* production of MIP-2 and KC after carrageenan stimulation is Mϕ dependent. Resident pleural cells were harvested and immunodepleted of resident pleural Mϕ by passage over a magnetic column. Equivalent numbers of cells were plated and stimulated with 0.25% carrageenan or normal medium for 6 h. Supernatants were harvested and analyzed by specific ELISA for MIP-2 and KC (*$p < 0.05$ vs. all cells with medium; n = 4 wells/condition).

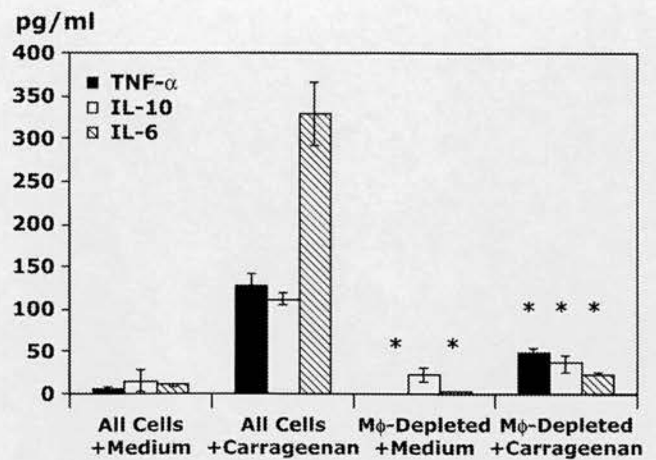

Figure 7. *In vitro* production of the cytokines TNF-α, IL-10, and IL-6 after carrageenan stimulation is Mϕ dependent. Resident pleural cells were harvested and immunodepleted of resident pleural Mϕ by passage over a magnetic column. Equivalent numbers of cells were stimulated with 0.25% carrageenan or normal medium for 6 h. Supernatants were harvested and analyzed by specific ELISA for TNF-α and by CBA for IL-10 and IL-6 (*$p < 0.05$ all cells vs. Mϕ-depleted for their respective condition, i.e., Mϕ with medium or Mϕ with carrageenan). n = 4 wells/condition.

number of pleural Mϕ at the initiation of disease and the number of infiltrating PMNs at 6 h after carrageenan administration strongly supports a key proinflammatory role for resident pleural Mϕ. It is possible that carrageenan pleurisy may be partially dependent on the proinflammatory actions of recruited monocytes, unlike experimental peritonitis where acute PMN infiltration is monocyte independent (27). Our data indicate a profound effect of Mϕ depletion on PMN recruitment at the early time point of 6 h and because monocyte recruitment occurs significantly later in the carrageenan model it is likely that monocyte recruitment will be very limited at this early time point. Thus, a reduction in monocyte recruitment in DT-treated CD11b-DTR mice is unlikely to be involved in the early reduction in PMN infiltration in these studies, although recruited monocytes may play a role in PMN infiltration at later time points.

Although defective PMN migration consequent on exposure to DT is an alternative explanation for these findings, our previous work in experimental peritonitis indicated that reconstitution of Mϕ-depleted mice with nontransgenic Mϕ was able to fully restore PMN infiltration in response to thioglycollate (27). In addition, intrapleural administration of the chemokine MIP-2 to Mϕ-depleted CD11b-DTR mice resulted in significant PMN infiltration ($3.2 \times 10^5 \pm 0.9 \times 10^5$ PMNs/ml at 4 h after the intrapleural administration of 30 ng MIP-2), suggesting that PMN migration is not defective under these experimental conditions.

In these experiments, DT administration and the subsequent induction of widespread Mϕ death did affect the numbers of pleural B cells and mast cells. However, despite this potentially confounding issue, several factors support the prominent role of the pleural Mϕ in the carrageenan model. First, data from *in vitro* experiments indicate a dramatic reduction in chemokine and cytokine production after Mϕ depletion from resident pleural cell populations. In these studies, pleural cells were labeled with a PE-conjugated antibody to the specific Mϕ marker F4/80 before immunomagnetic depletion, and F4/80 is not expressed by B cells or mast cells. Second, adoptive transfer of Mϕ-depleted pleural cells comprising B cells, T cells, and mast cells did not induce significant PMN recruitment after carrageenan administration. In contrast, adoptive transfer of Mϕ-rich pleural cells or purified Mϕ isolated by negative selection significantly increased PMN infiltration in response to carrageenan administration. Last, previous work suggests that mast cells do not play a significant role in the carrageenan pleurisy model (35, 36).

We then examined the effect of resident pleural Mϕ ablation on the level of C-X-C chemokines in this model. Resident pleural Mϕ ablation markedly reduced MIP-2 levels in the pleural exudate but had a lesser, albeit significant, inhibitory effect on KC levels. *In vitro* study of Mϕ-replete or Mϕ-depleted pleural cell populations indicated that Mϕ are a key source of chemokines because Mϕ-depleted pleural cell populations produced minimal amounts of the chemokines MIP-2 and KC. Interestingly, these *in vitro* studies demonstrated comparable production of MIP-2 and KC, whereas analysis of pleural lavage fluid indicated that KC levels were approximately two- to threefold higher than MIP-2 levels *in vivo*. These data are comparable to our previous studies of thioglycollate peritonitis (27) and suggest that other cells within the pleural cavity, such as mesothelial cells, may be an important source of KC *in vivo*. The suggestion that pleural cells, other than those retrievable by pleural lavage, represent a significant source of KC is consistent with recent work in a wound model of inflammation (37) that demonstrated MIP-2 expression by inflammatory cells while KC was predominantly expressed by resident tissue cells, such as endothelial cells and fibroblasts. Pleural mesothelial cells undoubtedly participate in pleural inflammation and our data suggest that mesothelial cells actively contribute to KC production. It should be stressed, however, that marked inhibition of PMN recruitment was evident at early time points in the presence of relatively preserved KC levels suggesting that MIP-2 is more important *in vivo* in this model. Also, pleural cell populations stimulated with carrageenan *in vitro* produced relatively low levels of the C-C chemokine MCP-1 compared with the levels found *in vivo*, suggesting a prominent role for mesothelial cells in MCP-1 production *in vivo* and subsequent mononuclear cell recruitment. Our data are therefore also in accordance with previous reports highlighting the importance of pleural mesothelial cells (3, 6, 38).

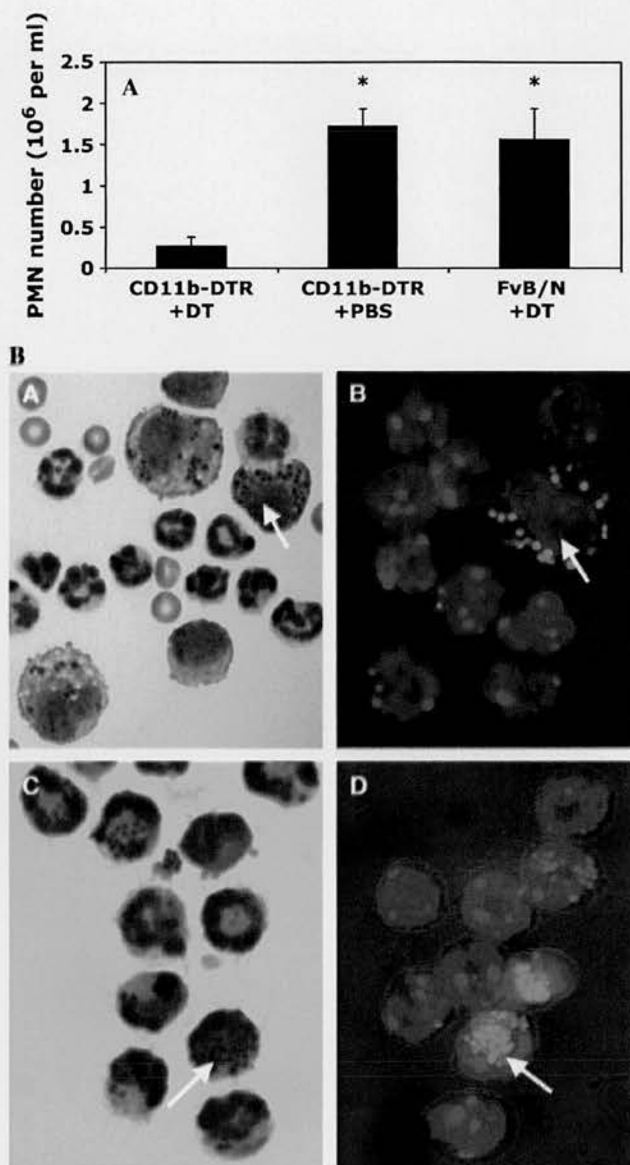

Figure 8. Resident Mϕ ablation 24 h before the administration of formalin-fixed *Staphylococcus aureus* significantly blunts PMN recruitment. (*A*) A total of 3×10^6 formalin-fixed, fluorescently labeled *S. aureus* were instilled into the pleural cavity of CD11b-DTR and FVB/N WT mice 24 h after DT treatment with phosphate buffered saline (PBS)–treated CD11b-DTR serving as an additional control. Pleural lavage was performed at 4 h after the administration of *S. aureus*. Lavaged cells were stained for GR1 and counted by flow cytometry (*$p < 0.05$ vs. DT-treated CD11b-DTR group; n = 4 mice/group). (*B*) *Photomicrographs* of Diffquick-stained (A and C) or Hoechst-stained (B and D) cytospin preparations of pleural lavage cells from either DT-treated FVB/N WT mice (A and B) or DT-treated CD11b-DTR mice (C and D) 4 h after the administration of 3×10^6 formalin-fixed, fluorescently labeled *S. aureus*. PMNs may be readily distinguished from Mϕ by their smaller size and the characteristic lobulated or circular nuclear morphology. Note that in B, the cell indicated with an *arrow* is the only Mϕ present in the field and exhibits a large, rounded nucleus, whereas the remaining smaller PMNs exhibit a polylobular nuclear morphology. There are no Mϕ present in C and D. Prominent ingestion of *S. aureus* particles by Mϕ is evident in control DT-treated FVB/N WT mice (examples shown with *arrows* in A and B), with very limited uptake by PMNs. In contrast, in the absence of Mϕ, DT-treated CD11b-DTR mice exhibit marked ingestion of *S. aureus* particles by PMNs (examples shown with *arrows* in C and D).

Our data also indicate that resident pleural Mϕ are critically involved in the generation of cytokines because TNF-α, IL-10, and IL-6 levels in pleural exudates were significantly reduced in CD11b-DTR mice treated with DT. Also, carrageenan-stimulated pleural cell populations exhibited a significant reduction in cytokine levels *in vitro* after magnetic immunodepletion of pleural Mϕ. Our studies raise the question as to why there was no significant PMN infiltration in response to significant KC production. Pertinent previous work studying the effect of function-blocking antibodies to either MIP-2 or KC in thioglycollate peritonitis indicates that inhibition of either chemokine individually results in marked (> 70%) inhibition of PMN infiltration with inhibition of both chemokines giving little additional effect (39, 40). We did not perform *in vitro* PMN chemotaxis assays to assess the chemotactic activity of pleural lavage fluid from Mϕ-depleted and control mice with pleurisy because the preparation of pure populations of nonactivated murine neutrophils is problematic. In addition, our previous studies indicate that experiments involving the adoptive transfer of lavage fluid are confounded by the resultant dilution of chemokines and cytokines. However, the dramatic reduction in the levels of intrapleural cytokines in Mϕ-depleted mice may contribute to the defective PMN infiltration via modulation of local endothelial cell expression of adhesion molecules involved in PMN diapedesis. Although many mediators, including cytokines, nitric oxide, complement proteins, and prostaglandins, are involved in acute inflammatory processes and leukocyte recruitment, our findings indicate that resident Mϕ play a key role in orchestrating PMN influx in carrageenan pleurisy. In addition, our limited experiments performed in mice administered killed *S. aureus* indicated that Mϕ depletion markedly reduces staphylococcal-induced PMN infiltration. Also, prominent Mϕ ingestion of *S. aureus* was evident in control DT-treated FVB/N mice and this reinforces the key role for resident Mϕ as sentinel cells that act to recognize and clear proinflammatory pathogens and particulate material.

In conclusion, this study used a transgenic model of conditional Mϕ ablation to demonstrate a key role for the resident pleural Mϕ in sensing pleural irritation and orchestrating PMN infiltration in carrageenan-induced pleurisy. This proinflammatory function is predominantly mediated by production of the potent PMN C-X-C chemokine MIP-2 and proinflammatory cytokines such as TNF-α and IL-6 that can promote the production of the PMN C-X-C chemokine KC by mesothelial cells. Our study suggests that resident Mϕ are critically important producers of PMN chemokines and proinflammatory cytokines and act to orchestrate PMN recruitment in murine carrageenan-induced pleurisy.

***Conflict of Interest Statement*:** None of the authors have a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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