

# **THE INFLUENCE OF HOUSING ENVIRONMENT ON THE MURINE INFLAMMATORY IMMUNE RESPONSE**

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A thesis submitted in partial fulfilment of the requirements for the degree of  
Doctor of philosophy

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12 March 2017

## **Details of Collaborations and Publications**

Dr Mark Kristiansen (Genomics Facility, Department of Child Health, University College London) provided the initial microarray service, with analysis carried out by Dr Masahiro Ono (Faculty of Natural Sciences, Department of Life Sciences, Imperial College London). Dr's Thomas Gobbetti and Beatrice Gittens provided their expertise in the planning and performance of the CLP procedure.

During the course of this PhD I have been co-author of two research papers, lead author of a review article and lead author of two original research papers currently under review.

## **Acknowledgements**

Let me attempt brevity, for once.

I consider myself extremely privileged to have been allowed (and paid!) to dedicate the past three years of my life to the pursuit of finding answers to my own questions. I am very aware that a wide variety of people were required to help make this a possibility and while I don't have the energy to credit them all I would like to take this opportunity to especially thank:

### **The Medical Research Council and Queen Mary University of London**

For the money

### **Tony Price and the rest of the BSU team**

For putting up with all my weird requests

### **Martin Goss**

For doing an often thankless job

### **Catherine Conway**

For giving me the freedom to choose my own path

### **Amanda Goodfellow**

For her infinite patience, calming presence and exquisite proofreading

### **Dr Robert Grange**

For his steadfast friendship

### **Malvina Begalli**

For helping me through a difficult time

### **Professor Mauro Perretti**

For being that quintessentially cool second supervisor

### **Professor Fulvio D'Acquisto**

For all that follows

**In Memory of my Father**

**Nick Brod**

I guess that was one way to avoid the proofreading

## Abbreviations

Abbreviation	Term		
-	Nil/Negative (Facs Staining)	KC	Keratinocyte Chemoattractant
Ach	Acetylcholine	LFA1	Lymphocyte Function-Associated Antigen 1
ACKR	Atypical Chemokine Receptors	LPC	Lysophosphatidylcholine
ACTH	Adrenocorticotrophic Hormone	LPS	Lipopolysaccharide
ANXA1	Annexin A1	LTB <sub>4</sub>	Leukotriene B <sub>4</sub>
Apcs	Antigen Presenting Cells	LXA4	Lipoxin A4
ARG1	Arginase-1	Ly6C	Lymphoid Marker Lymphocyte
Bcl	B-Cell Lymphoma/Leukaemia (Protein)	Mac1	Macrophage Receptor 1
CARD	Caspase Recruitment Domain-Containing Protein	MALT	Mucosa-Associated Lymphoid Tissue Lymphoma Translocation Protein
CASP	Colon Ascendens Stent Peritonitis	MCP	Monocyte/Macrophage Chemotactic Protein
CCL	Cc-Chemokine Ligand	MCP-1	Monocyte Chemoattractant Protein 1
CCR	Cc-Chemokine Receptor	MDD	Major Depressive Disorder
CD	Cluster of Differentiation	MDMs	Monocyte Derived Macrophages
CD62L	L-Selectin	MDSC	Myeloid Derived Suppressor Cells
CFU	Colony Forming Units	MHC	Major Histocompatibility Complex
CNS	Central Nervous System	MIF	Macrophage Inhibition Factor
CRH	Corticotrophin Releasing Hormone	MIP	Monocyte Inflammatory Protein
CRP	C-Reactive Protein	MMP	Matrix-Metalloprotease
CSF-1	Colony Stimulating Factor 1	MPO	Myeloperoxidase
C <sub>t</sub>	Threshold Cycle	MYD88	Myeloid Differentiation Primary Response 88
Cx <sub>3</sub> CL1	Fractalkine	NADPH	Nicotinamide Adenine Dinucleotide Phosphate
CXC	C-X-C Motif Chemokine	NB	Nutrient Broth
DAMPS	Damage Associated Molecular Patterns	NBF	Neutral Buffered Formalin
DARC	Duffy Antigen Receptor for Chemokines	NETs	Neutrophil Extracellular Traps
DC	Dendritic Cell	NF-κB	Nuclear Factor Kappa-Light-Chain-Enhancer of Activated B Cells
DSS	Dextran Sulfate Sodium	NLCR	Neutrophil to Lymphocyte Count Ratios
EAE	Experimental Autoimmune Encephalomyelitis	OFT	Open Field Test
ECM	Extra-Cellular Matrix	PAF	Platelet Activation Factor
EDTA	Ethylenediaminetetraacetic Acid	PAF	Platelet Activating Factor
EE	Environmental Enrichment	PAMPS	Pathogen Associated Molecular Patterns
ELR	Glutamic Acid-Leucine-Arginine	PBS	Phosphate Buffered Saline
EPM	Elevated Plus Maze	PCR	Polymerase Chain Reaction
EtoH	Ethyl-Alcohol / Ethanol	PDGF	Platelet Derived Growth Factor
FBGCs	Foreign Body Giant Cells	PECAM-1	Platelet Endothelial Cell Adhesion Molecule
FPR2	Formyl Peptide Receptor 2	PLF	Peritoneal Lavage Fluid
FSC	Forward Scatter	PMA	Phorbol Myristate Acetate
GAPDH	Glyceraldehyde 3-Phosphate Dehydrogenase	PNS	Parasympathetic Nervous System
GMCSF	Granulocyte Macrophage Colony-Stimulating Factor	PRR	Pattern Recognition Receptor
H&E	Haematoxylin and Eosin (Stain)	PS	Phosphatidylserine
H <sub>i</sub>	High (Facs Staining)	PSGL1	P-Selectin Glycoprotein Ligand 1
HOCL	Hypochlorous Acid	PVA	Polyvinyl Alcohol
HPA	Hypothalamic–Pituitary–Adrenal	RA	Rheumatoid Arthritis
IAA	Iodoacetamide	RANTES	Regulated On Activation, Normal T Cell Expressed And
ICAM-1	Intercellular Adhesion Molecule-1		
IFN-γ	Interferon Gamma		
IL	Interleukin		
iNOS	Inducible Nitric Oxide Synthase		
Ip	Intraperitoneal		
IRAK	Interleukin-1 Receptor-Associated Kinase		

	Secreted		Beta
RAGE	The Receptor For Advanced Glycation End-products	Tim4	T-Cell Membrane Protein 4
ROI	Reactive Oxygen Intermediate	TLR	Toll Like Receptor
ROS	Reactive Oxygen Species	TNF- $\alpha$	Tumour Necrosis Factor Alpha
RPMI	Roswell Park Memorial Institute Medium	TRAF	TNF Receptor Associated Factor
RSD	Repeat Social Defeat	T <sub>reg</sub>	T-Regulatory Cells
S1P	Sphingosine 1-Phosphate	TSST	Trier Social Stress Test
SDF	Stromal Cell-Derived Factor	VEGF	Vascular Endothelial Growth Factor
SM-IL-6	Skeletal Muscle Derived IL-6	VLA4	Very Late Antigen 4
SNS	Sympathetic Nervous System	WAS	Water Avoidance Stress
Src	Proto-Oncogene Tyrosine-Protein Kinase	WHRI	William Harvey Research Institute
SRF	Serum Response Factor	Wnt	The Int/Wingless Family
SSc	Side Scatter	ZIP	Zymosan Induced Peritonitis
Syk	Spleen Tyrosine Kinase	$\Omega$ -3 PUFA	$\Omega$ -3 Fatty Polyunsaturated Fatty Acid
TFA	Trans-Fatty Acid		
TGF-B	Transforming Growth Factor		

## Abstract

Studies have demonstrated the immune system to be significantly more plastic than previously believed. Multiple external factors have been shown to influence the immune response including alterations to the host's external environment and psychological status.

This thesis details an investigation of this influence; exposing male CD1 mice to a two-week environmental enrichment paradigm then subjecting them to one of a range of inflammatory disease models chosen to assess a specific aspect of their immune function.

Enriched animals were found to possess significantly higher numbers of circulating innate leukocytes compared to those animals housed in a standard lab environment.

This leukocytosis was found to persist when animals were subject to a model of zymosan-induced peritonitis, where enriched animals presented an enhanced neutrophil and macrophage influx into their peritoneal cavity. Similar results were observed in a model of sepsis induced by caecal ligation and puncture where enriched animals were also found possess an enhanced capacity for systemic bacterial clearance. Across both experiments no changes in inflammatory cytokine expression were observed between enriched and standard environment animals.

Genomic and proteomic profiling supported these findings, revealing the increased expression of immune-modulatory genes associated with a heightened immune and moderated inflammatory response.

*Ex vivo* analysis of leukocytes extracted from enriched animals showed they also possessed enhanced phagocytic function and an accompanying reduction in gene expression associated with heightened cytotoxic function.

When subject to a model of persistent inflammation induced by sponge implantation, enriched animals again presented heightened leukocyte infiltration to the point of immune insult. This was accompanied with a reduction in the release of pro-inflammatory cytokines and the heightened expression of genes associated with a pro-resolving, wound healing phenotype.

This study provides novel insights into the mechanisms by which environmental modulation may influence the immune response and of the potentially immune-protective influence of environmental enrichment.



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# Chapter 1 Introduction

## 1.1 Inflammation

Classically defined as a response to infection or physical injury and more recently described as a biological reaction to disrupted tissue homeostasis [Medzhitov, 2008], inflammation is an integral component of the immune response. Distinct from other homeostatic mechanisms in that damage to self is an unavoidable and indeed necessary part of the process, inflammation instigates and propagates a series of events culminating in the recruitment of blood derived factors including; plasma proteins, signalling factors and leukocytes that act together to neutralise the disrupting factor and repair perturbed tissue [Ashley et al., 2012].

The characteristics of inflammation are alluded to by its name. First clinically described over two millennia ago by Aulus Cornelius Celsus (A Roman encyclopaedist and medical writer) the four cardinal signs of inflammation are; *calore* – heat, *rubor* – redness, *tumor* – swelling and *dolore* – pain [Celsus and Spencer, 1935]. This definition was later appended by the Greek physician Galen of Pergamon<sup>1</sup> (AD 129 – c. 200/c. 216) with a fifth cardinal sign, *function laesa* – loss of function [Rather, 1971].

Inflammation has a long evolutionary pedigree. Its action has been identified in the early stages of metazoan evolution, demonstrated by the typical inflammatory wound response presented by invertebrates such as starfish [Natoli et al., 2011]. Further, several of the key molecules (and molecular domains) involved in the inflammatory process have their lineage traced to the point of animal and vegetal cell divergence roughly two billion years ago [Kimbrell and Beutler, 2001]. An additional point of interest is the recent discovery that such inflammatory mediators and responses are evolving rapidly. Genes encoding scavenger receptors and chemo-attractants display an unusually high rate of deletions and duplications across species [Ponting, 2008]. This high rate of variance has been postulated to reflect the diversity of environmental challenges different animals are exposed to [Natoli et al., 2011].

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<sup>1</sup> This attribution to Galen has been disputed, with medical historians variously attributing the term to the physicians Thomas Sydenham or Rudolf Virchow [Heidland et al., 2006, Rather, 1971]

With its deep and fluctuating lineage, it is perhaps unsurprising that both the aetiology and pathology of inflammation is highly variable. Multiple contexts such as the nature and intensity of stimuli and individual health status can affect the inflammatory process. Regardless the process of inflammation serves as the initiator and mediator of a variety of homeostatic processes, such as; pathogen and foreign body removal, wound healing and the isolation of irresolvable immune insults through the formation of granulomas. The foundation for each of these vital biological mechanisms is acute inflammation.

## 1.2 Acute Inflammation

Acute inflammation consists of a tightly regulated cascade of physiological and behavioural processes and coordinated immunological signalling molecules such as cytokines and chemokines. As summarised in Table 1 the defining features of acute inflammation are a rapid onset, accompanied by a notable local and systemic indications of the response (*calore, dolore, rubor* and *tumor*). The key cellular presence are neutrophils and overall damage is usually mild and rapidly repaired [Kumar et al., 2012].

**Table 1 Key features of chronic and acute inflammation**

Feature	Acute	Chronic
<b>Onset</b>	Rapid: Minutes or hours	Slow: Days
<b>Key Cellular Infiltrate</b>	Neutrophils	Monocytes/Macrophages & Lymphocytes
<b>Tissue damage, fibrosis</b>	Mild, self-limited	Often severe and progressive
<b>Local and systemic signs</b>	Prominent	Initially less prominent; subtle
Table adapted from Robbins Basic Pathology, 9th Ed. [Kumar et al., 2012]		

### 1.2.1 Stimuli for Acute Inflammation

Initiation of the inflammatory cascade is classically triggered by two key associated factors. Pathogen Associated Molecular Patterns (PAMPs) or Damage Associated Molecular Patterns (DAMPs) which are generated by the presence of pathogenic organisms in the host or tissue damage respectively. PAMPs are typically specific, conserved motifs of pathogen expressed molecules, such as the lipopolysaccharides (LPS) that form the outer membrane of gram negative bacteria or the double stranded RNA of specific viruses. DAMPs (or alarmins), are host derived molecules generated through cellular or tissue damage caused by necrosis, ischemia, trauma or infection. DAMPs are often intrinsic cell components, only released into the external environment through lysis or necrosis [Abbas et al., 2014, Murphy, 2011].

More recently, a broader set of inflammation inducing stimuli has been put forward by Ruslan Medzhitov, roughly divided into two categories: 'Exogenous inducers' include the microbial PAMPS previously mentioned as well as allergens, foreign bodies and toxic compounds. The category 'endogenous inducers' includes a wide variety of cell, plasma and extracellular matrix derived factors all associated with dead, damaged or aberrant cells. This more inclusive set of stimuli reflects the hypothesis that inflammation is a response to disrupted homeostatic function as opposed to a specific attempt to clear infection [Medzhitov, 2008].

PAMPs and DAMPs are bound by Pattern Recognition Receptors (PRRs). PRRs are expressed by a broad spectrum of cell types including macrophages, dendritic and endothelial cells [Takeuchi and Akira, 2010]. PRR expression has been observed in multiple cellular compartments including the plasma membrane, endosomes, lysosomes and endolysosomes [Akira et al., 2006]. The apparent ubiquity of these receptor types is believed to be required for efficient ligand accessibility as well as maintenance of tolerance to self-molecules [Kawai and Akira, 2010]. Currently PRRs are divided into four families categorised by structural homology: C-type lectin receptors, nuclear oligomerisation receptors, RIG-1-like receptors and toll-like receptors (TLRs) [Kawai and Akira, 2010, Akira et al., 2006]. While each receptor typically binds a specific molecular pattern (e.g. TLR-4 binds LPS [Lu et al., 2008]) it is interesting to note that several are cross-reactive. TLR-9 is activated by binding to either bacterial DNA (a PAMP) or eukaryotic mitochondrial DNA (a DAMP). Mitochondria are popularly hypothesised to be the ancestors of prokaryotic organisms that entered an endosymbiotic relationship with a proto-eukaryote cell roughly two billion years ago [Emelyanov, 2001]. Such DAMP-PAMP cross-binding is believed indicative of the relationship between prokaria/eukaria evolution and the immune response [Calfee and Matthay, 2010].

Upon PAMP or DAMP binding PRRs stimulate the rapid release of preformed inflammatory mediators as well as upregulating transcription of genes involved in the inflammatory response. These genes encode numerous pro-inflammatory cytokines, chemokines, type-1 interferons, adhesion factors and matrix-metalloproteases (MMPs). Which act in concert to instigate

localised vascular and cellular changes, intended to address the offending damage, infection, or other disruptors of homeostasis [Murphy, 2011].

Following recognition of the appropriate stimuli, acute inflammation can be broadly divided in to two phases; vascular and cellular<sup>2</sup>.

### **1.2.2 The Vascular Response**

During the initial stages of inflammation, affected tissues become reddened and swell, due to vasodilation increasing blood flow and oedema. The vascular events of the acute inflammatory response have two established mechanisms of initiation and involve three core processes.

#### **1.2.2.1 PAMP Mediated Immune Activation**

PAMP recognition by tissue resident or patrolling immune cells, most prominently mast cells and macrophages, stimulates release of various vasoactive molecules. Most notably histamines and bradykinins produced by degranulating mast cells and nitric oxide (NO) released by both macrophages and endothelial cells. Both molecules are potent vasodilators, increasing blood flow to local capillary beds. This net increase in blood causes the redness and heat typically associated with inflammation. In addition to vasodilation, histamine plays several other important roles in inflammation. It binds nociceptors heightening sensitivity to pain (the cause of inflammatory *dolore*) [Cannon et al., 2007]. Additionally histamine induces the permeabilisation of endothelial cells via phosphorylation induced separation of cell to cell (adherins) junctions. [Andriopoulou et al., 1999]. A consequence of this vascular permeation is the influx of fluid – rich in inflammatory factors - from the vessels into surrounding tissue. The osmotic pressure this exudate exerts generates the characteristic swelling of inflammation [Kumar et al., 2012].

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<sup>2</sup> While typically presented as a canonical process for ease of description, the multipartite nature of inflammation and the fact many of its processes feedback on one another means it would perhaps be better envisioned as a series of connected, parallel - as opposed to sequential - processes.

#### **1.2.2.2 Lipid Mediated Immune Activation**

Alternatively, or additionally damage to cellular membranes caused by trauma, infection (or inflammation) leads to the generation of multiple lipid inflammatory mediators. Membrane injury induces activation of lipid bilayer bound platelet activation factor (PAF) a potent inducer of vascular permeability, leukocyte activation and chemotaxis, degranulation and platelet aggregation [Zimmerman et al., 2002]. Following damage, or immune-activation arachidonic acids within the lipid bi-layer of leukocytes are converted into various prostaglandins, including prostacyclin - which induces vasodilation - and leukotriene factors which again elicit a range of inflammatory functions, most notably leukotriene-C4 which increases vascular permeability [Dahlen et al., 1981].

#### **1.2.3 The Cellular Response:**

The culmination of the vascular response is an outpouring of protein rich exudate into the extracellular tissues producing an overall decrease in the rate of local blood flow and an increase in its viscosity. Together this leads to slowing and accumulation (or stasis) of cells in the inflamed area. Which in turn permits the movement (or margination) of leukocytes along the endothelium and into the inflamed tissue [Fedosov and Gompper, 2014].

##### **1.2.3.1 Extravasation**

Leukocyte recruitment to and extravasation into the site of injury can be divided into three steps:

Margination and Rolling Adhesion. Cytokines; Interleukin-1 (IL-1) and Tumour Necrosis Factor alpha (TNF- $\alpha$ ) generated by activated tissue-resident macrophages bind their concordant receptors on the endothelium, inducing surface expression of cellular adhesion molecules; E-selectin and P-selectin, [Albelda et al., 1994, Abbas et al., 2014] Leukocytes (typically neutrophils initially) localised towards the site of inflammation by taxis along chemokine gradients interact with these adhesion molecules through corresponding receptors upon their surface, slowing the immune cell down. These interactions cause the cell to roll along the epithelial surface as weak molecular bonds between the adhesion molecules are made and broken [Muller, 2013].



Tight Adhesion and Transmigration. A second set of epithelial adhesion molecules, integrins, activated by the presence of inflammatory chemokines also bind the leukocyte. ICAM-1 (Intercellular adhesion molecule-1) binds firmly to the leukocyte surface through interaction with L-selectin (CD62-L), an intercellular adhesion molecule found on the surface of multiple leukocyte populations locking it into place. Following immobilisation, the immune cell undergoes cytoskeletal re-arrangement, spreading out over the endothelia and extending pseudopodia into the gaps between cells. Transmigration occurs by binding a second integrin, PECAM-1 (Platelet endothelial cell adhesion molecule), the cell effectively using it to drag itself through the cell endothelium [Muller, 2013]. The process by which the leukocyte passes through the endothelial basement membrane remains a cause for debate, though it's likely achieved through MMP mediated digestion of the membrane, physical force, or a combination of both [Sorokin, 2010].

Chemotaxis. Once within the tissue instersitium leukocytes migrate up chemotactic gradients to an inflammatory focus (differing cell types respond to specific chemotactic factors, these are listed in the proceeding sections), where they will carry out a variety of population specific effector functions.

Inflammation is a nuanced process involving a diversity of different cell types whose presence and predominance depends on the initiator and duration of inflammation. However typically three leukocyte cell types lay at the foundation of the acute inflammatory process; neutrophils, monocytes and macrophages.

#### **1.2.4 Neutrophils**

Neutrophils are the most abundant leukocyte subset found within mammals, making up between 40-75% of circulating leukocytes depending on the animal [Mayadas et al., 2014]. They tend to have a rounded morphology (in circulation) and are readily identified by visible intracellular granules and a multi-lobed nucleus (Table 3).

Neutrophils are generated within the bone marrow from myoblast stem cells primarily in response to granulocyte colony stimulating factor (G-CSF) which also controls their steady release into circulation [Borregaard, 2010]. The overall number of neutrophils within the body varies depending on their rate of production and egress from the bone marrow as well as their survival and clearance by other cells, during inflammation neutrophil production can increase rapidly [Borregaard, 2010].

Neutrophils are typically short lived cells, with the average life span in circulation for humans and mice reported between one to four days (depending on the method of observation), [Tak et al., 2013]. However due to their high numbers and constant presence in circulation they are typically the first leukocyte to encounter an inflammatory/immune activating stimuli. Neutrophils migrate towards sites of infection in response to a variety of chemoattractant factors, the most prominent of which are IL-8 (in mice referred to as keratinocyte chemoattractant (KC)) [Gerszten et al., 1999], IFN- $\gamma$ , Leukotriene B4 (LTB4) and the compliment proteins C3a and C5a [Yadav et al., 2010]. Release of further neutrophils from the bone marrow is regulated by G-CSF and the chemokine, stromal cell-derived factor 1 (SDF1) (also known as C-X-C motif chemokine 12 (CXCL12)) by activated immune and endothelial cells [Eash et al., 2009]. Cells then migrate to sites of inflammation up aforementioned chemokine gradients.

Neutrophils serve as the primary effector cell during acute inflammation, normally reaching an inflammatory focus within minutes to hours of onset [Cash et al., 2009]. During this initial chemotactic response, interaction with chemokines, PAMPs, compliment proteins or bioactive lipids brings the neutrophil into a state of partial activation. Neutrophils are relatively unresponsive to a single inflammatory stimulus, however exposure to one primes the production of various cytokines, chemokines and cytotoxic factors allowing for a more rapid and efficient response once fully activated [Doerfler et al., 1989, Swain et al., 2002]. Following extravasation into inflamed tissues (described in 1.2.3) the neutrophil enters an environment with a high concentration of inflammatory stimuli and become fully activated, allowing the leukocytes to perform a variety of anti-microbial, pro-inflammatory effector functions:

#### 1.2.4.1.1 Pathogen Killing by Neutrophils

##### Phagocytosis


A neutrophils' primary method for neutralising an inflammatory instigator is by phagocytosis of pathogens and other foreign objects. Neutrophils and other phagocytes express PRR's and opsonic receptors such as FcγRs and C-type lectin receptors, capable of binding various PAMPs (or the Fc/C3b motifs of opsonised objects). Upon binding cytoskeletal re-arrangements induce the engulfment and endocytosis of the bound object by the phagocytes [Segal et al., 1980]. Once internalised this phagocytic vesicle is fused with preformed vesicles (referred to as granules) within the cells cytoplasm to form a phagosome. These granules contain various hydrolytic enzymes and nicotinamide adenine dinucleotide phosphate (NADPH) oxidase subunits that initiate destruction of the engulfed particles through generation of reactive oxygen species (ROS) and super oxide radicals, followed by proteasomal degradation [Vieira et al., 2002] (Table 2). The primary generators of ROS are the enzymes myeloperoxidase (MPO) and inducible nitric oxide synthase (iNOS).

MPO catalyses the formation of hypochlorous acid (HOCL) through reaction of chloride ions with hydrogen peroxide [Klebanoff, 2005, Williams, 2006]. HOCL has a potent microbicidal activity, when exposed to the acid *Escherichia coli* lose viability in less than 0.1 seconds [Rakita et al., 1990]. The means by which HOCL achieves this killing is a matter of study with current theories postulating the molecule; inhibits glucose oxidation (preventing respiration), depletes adenine nucleotides (inhibiting ATP metabolism), inhibits of DNA replication, or causes mass decarboxylation, deamination, or peroxidation of proteins and lipids (inducing structural breakdown) [Williams, 2006]. Regardless of its action, the presence of MPO is critical for host defence, demonstrated by the chronic granulomatous disease suffered by individuals lacking proper MPO function [Williams, 2006]. iNOS in turn catalyses the generation of nitric oxide, a short lived free radical that reacts with DNA, lipids causing damage through oxidation. iNOS production is induced during neutrophil priming by TNF-α, IL-1 and IFN-γ [Lambeth, 2004].

## Degranulation

Neutrophils also secrete a variety of other proteinases and antimicrobial peptides by release of their granules into the extracellular milieu [Fauschou and Borregaard, 2003]. Neutrophils possess discrete sets of granules, each containing a different complement of immune mediator's dependent on the inflammatory microenvironment and their overall level of inflammation (Table 2).

**Table 2 Core types and constituents of neutrophil granules**

	Primary/ Azurophil granules	Secondary/ Gelatinase granules	Tertiary/ Specific granules	Secretory Granules
<b>Point of release</b>	Extravascular tissues	Extravascular tissues	Penetration of ECM	PMN-Endothelial cell interaction
<b>Receptors and membrane-bound proteins</b>	CD63, CD68	FPR, $\beta_2$ -integrins, laminin receptor, CD66, CD67	FPR, $\beta_2$ -integrins, TNF receptor	FPR, CD14, CD16 $\beta_2$ - integrins, proteinase- 3
<b>Proteins released into the surrounding</b>	Azurocidin, HNP1- 3, cathepsin G, elastase, MPO, proteinase-3	Collagenase, LL-37, lysozyme, lactoferrin	MMP-9, lysozyme, arginase, Leukolysin	Azurocidin, albumin
Increasing tendency for release 				

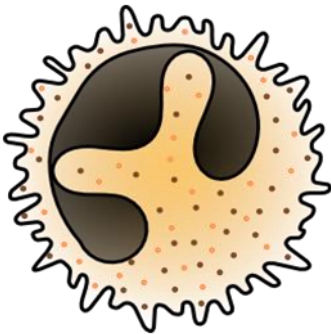
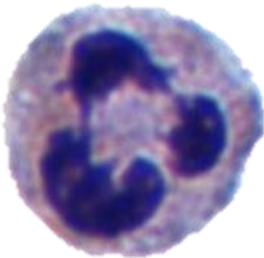
## NETosis

More recently neutrophils have been discovered to possess a third mechanism of microbial clearance; the extracellular release of web like structures capable of immobilising and killing bacteria. Comprised of fibres of chromatin studded with granule-derived antimicrobial peptides and enzymes such as neutrophil elastase and MPO, these Neutrophil Extracellular Traps (NETs) are observed to be released following neutrophil activation [Brinkmann et al., 2004]. NETs function by trapping and aggregating pathogens within an adhesive matrix of chromatin and exposing them to a variety of highly concentrated antimicrobial peptides and enzymes. In addition to MPO and serine proteases NETs also contain high levels of cathelicidin (which destroys lipoprotein membranes of microbes) and lactoferrin (which sequesters free iron, an essential substrate required for bacterial growth) [Papayannopoulos and Zychlinsky, 2009]. The process of NET release, (or NETosis) is a distinct form of cell death in which the breakdown of both nuclear and granule membranes results in the release of decondensed chromatin and granule constituents into the extracellular space. NETosed neutrophils are distinct from other apoptotic cells lacking the markers on their cell surface that signal and coordinate their phagocytosis. As a result the lysed neutrophil and accompanying NET remains in the extracellular space for a protracted period of time before the chromatin matrix that forms the majority of the NET structure is disassembled by nucleases [Fuchs et al., 2007]. NETosis is induced by a variety of inflammatory mediators (e.g. KC, TNF- $\alpha$ , complement immune complexes) and PAMPs from a wide range of bacteria, fungi, and protozoa [Byrd et al., 2013]. The stimuli that promote NETosis as opposed to apoptosis are not yet fully elucidated. However, similar to other immune effector functions, the scale of NETosis likely varies with the inflammatory micro-environment the neutrophil acts within [Mayadas et al., 2014].

Throughout the inflammatory process neutrophils also release various cytokines, chemokines, chemotactic factors and other inflammatory mediators that perpetuate inflammation until its source is neutralised, (Table 3) [Borregaard, 2010]. Of these several of the granule proteins released by neutrophils (most prominently azurocidin and cathelicidin) induce the chemotaxis, adhesion and recruitment of monocytes to a site of inflammation [Soehnlein et al., 2009], while MIP-2 serves as a potent chemotactic factor for macrophages. In this manner neutrophils both

limit the spread of microbial infection and prepare the way for the recruitment and action of further effector cells [Soehnlein et al., 2009, Mayadas et al., 2014].

**Table 3 Defining features of neutrophils**

<b>Neutrophils</b>	<b>Diagram</b>	<b>Microscopic Image</b>
		
<b>Morphology</b>	Rounded, uniform, cells 12-20µm in diameter. Granular cytoplasm. Nucleus subdivided into 2-5 lobes	
<b>Origin</b>	Generated from myoblast stem cells in the bone marrow	
<b>Function in Inflammation</b>	Rapidly migrate towards site of inflammation attacking micro-organisms via: phagocytosis, degranulation (release of soluble and generation of neutrophil extracellular traps (NETs)).	
<b>Key mediators of activation/ chemotaxis</b>	Keratinocyte chemoattractant (KC), (in humans -interleukin-8 MIP-2 (macrophage inflammatory peotion 2) .IL-8 (interleukin 8, interferon gamma (IFN-γ), C3a, C5a, Leukotriene B4 and Tumour necrosis factor alpha (TNF-α), GMCSF	
<b>Key immune regulatory factors released</b>	TNF-α. Matrix metalloproteinases (MMPs) 2,8,9 &12, KC, MIP-2	
<b>Key markers</b>	Gr-1, CD11b , CD31	
<b>References</b>	[Borregaard, 2010, Kim et al., 2010, Lacy et al., 2015, Mayadas et al., 2014, Rose et al., 2012]	

### 1.2.5 Monocytes

Monocytes are large granulated leukocytes with a variably uniform or amoeboid shape, typified by a unilobed, ellipsoidal (or 'bean shaped') nucleus. They typically form 5-10% of the circulating leukocyte population in mice and humans [Nichols et al., 1971] (Table 4).

Like neutrophils, mouse monocytes originate from the bone marrow from precursor stem cells – monoblasts and in mice differentiate into two primary monocyte subsets distinguished by their

expression of the cell surface lymphoid marker, lymphocyte antigen six complex (Ly6C). Monocyte populations expressing high levels of Ly6C are pro-inflammatory in function and rapidly recruited to sites of infection and inflammation [Serbina et al., 2008]. A second population expressing low levels of Ly6C have been observed to play a more defensive role, patrolling the vascular endothelium and contributing to tissue repair [Geissmann et al., 2003]. While distinct, it should be noted that these two populations are not mutually exclusive with studies suggesting that Ly6C<sup>high</sup> monocytes may differentiate into their Ly6C<sup>low</sup> counterparts in the absence of inflammation [Shi and Pamer, 2011].

Like neutrophils monocytes are recruited out of the bone marrow to a point of inflammation by specific chemokines. CC-chemokine ligand 2 (CCL2, also known as Monocyte chemoattractant protein one, MCP1) and CC-chemokine ligand 7 (CCL7, also known as Monocyte chemoattractant protein 3, MCP3) are the two key factors known to mediate inflammatory monocyte recruitment, both binding to CC-chemokine receptor 2 (CCR2) [Tsou et al., 2007]. CCL2 is produced by most nucleated cell types, either constitutively or following induction by oxidative stress, cytokines, or growth factors involved in inflammation [Tsou et al., 2007]. CCL7 is also produced by multiple cell types in response to bacterial infection [Jia et al., 2008]. While the specific roles of these two chemokines in monocyte recruitment is unclear (they may have overlapping, sequential or parallel effects) it has been demonstrated that deletion of either *mcp1* or *mcp3* results in a roughly 40-50% decrease in monocyte recruitment during infection and inflammation [Jia et al., 2008].

Recruitment of Ly6C<sup>high</sup> inflammatory monocytes to a site of inflammation is mediated by two key receptors; CCR1 and CCR5. Both are capable of binding a wide variety of chemokines most pertinently CCL3 (also known as MIP1 $\alpha$ ) and CCL5 (also known as regulated on activation, normal T-cell expressed and secreted, RANTES) [Kaufmann et al., 2001]. While both receptors support monocyte chemotaxis towards CCL5, transmigration assays have established each have further specific functions. CCR1 has been shown to mediate monocyte arrest while CCR5 supports the process of 'spreading' across the endothelial surface, two actions that constitute the initial steps of monocyte extravasation into inflamed tissues [Weber et al., 2001].

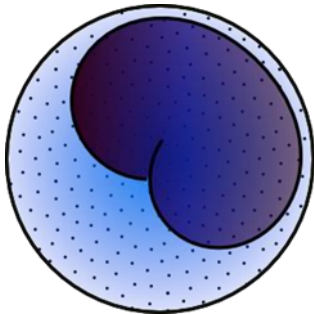
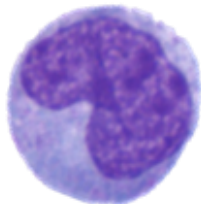
Ly6C<sup>low</sup> monocytes are also dependent in part on the receptors and affiliate ligands listed above. However their comparative expression of CCL2 is lower than that of their Ly6C<sup>high</sup> counterparts [Auffray et al., 2009]. This characteristic that has been suggested to contribute to Ly6C<sup>low</sup> cells slower recruitment to a site of inflammation than Ly6C<sup>high</sup> [Shi and Pamer, 2011]. Ly6C<sup>low</sup> cells respond to an additional ligand, CX3C-chemokine ligand1 (CX3CL1; also known as fractalkine), a membrane-bound chemokine expressed predominantly in the spleen [Auffray et al., 2007]. Deletion of CX3CR1 was demonstrated to diminish patrolling by Ly6C<sup>low</sup> monocytes and reduce their overall survival under both steady-state and inflammatory conditions [Auffray et al., 2009].

Monocyte recruitment follows the same core process as that of neutrophils (rolling adhesion and transmigration). Mouse monocytes express L-selectin (also known as CD62L), P-selectin glycoprotein ligand-1 (PSGL1), macrophage receptor 1 (MAC1, also known as integrin  $\alpha$ M $\beta$ 2), platelet endothelial cell adhesion molecule (PECAM1) lymphocyte function-associated antigen 1 (LFA1; also known as  $\alpha$ L $\beta$ 2 integrin), and very late antigen 4 (VLA4; also known as integrin  $\alpha$ 4 $\beta$ 1), all of which contribute to leukocyte adhesion and migration [Ley et al., 2007]. Recent studies have also highlighted a specific role for neutrophils in chaperoning the recruitment of monocytes from circulation [Wantha et al., 2013]. During inflammation neutrophils that have entered inflamed tissues release cathelicidin a small antimicrobial peptide that binds the luminal surface of the arterial endothelium. Inflammatory monocytes are able to bind cathelicidin via formyl peptide receptor 2 (FPR2). This interaction activates MAC1 and VLA4 allowing endothelial adhesion through binding of ICAM1 and VCAM1 respectively [Wantha et al., 2013]. Upon entry to a site of immune activity monocytes are capable of performing a range of antimicrobial and homeostatic functions. They possess a large array of scavenger receptors capable of recognising and inducing phagocytosis of microorganisms, potentially toxic compounds and dying cells [Serbina et al., 2008]. Like neutrophils they also secrete a variety of effector molecules involved in inflammation and the wider immune response such as reactive oxygen species, nitric oxide, type one interferons, IL-1 $\beta$  and TNF- $\alpha$  [Serbina et al., 2008].



Perhaps the most prominent role for monocytes during inflammation is of an accessory cell linking the primary, innate phase of the response to secondary more adaptive responses. This is achieved primarily through their conditional differentiation into macrophages.

**Table 4 Defining features of monocytes**

<b>Monocytes</b>	<b>Diagram</b>	<b>Microscopic Image</b>
		
<b>Morphology</b>	Large, variable shaped cells 15-20µm, granular, unilobed 'bean shaped nucleus	
<b>Origin</b>	Bone marrow, monoblast stem cells	
<b>Sub-populations</b>	<b>Ly6C-High</b>	<b>Ly6C-Low</b>
<b>Function in Inflammation</b>	Pro-inflammatory, antimicrobials roles	Tissue patrolling and repair
<b>Key mediators of activation/ chemotaxis</b>	CCL2, CCL5	CXCL1, CCL5
<b>Key immune regulatory factors released</b>	TNF-α, IL-1β, ROS	IL-10
<b>Key markers</b>	CD11b <sup>+</sup> CD115 <sup>+</sup> Ly6C <sup>high</sup>	CD11b <sup>+</sup> CD115 <sup>+</sup> Ly6C <sup>Low</sup>
<b>References</b>	[Geissmann et al., 2003, Ginhoux and Jung, 2014, Italiani and Boraschi, 2015]	

### 1.2.6 Macrophages

Macrophages are large, mono-nucleate leukocytes with a diverse morphology dependent on their position within the body (Table 5). The capacity of macrophages to specialise their function to a particular microenvironment makes them a highly heterogeneous cell population taking on different names according to their tissue location. Despite this variety of macrophage classes each carries out the same key functions: Overseeing tissue development and remodelling, surveying and enacting tissue homeostasis (through phagocytosis of apoptotic or senescent cells and reshaping tissue structure), and arbitrating the immune response to pathogens by perpetuation or resolution of the inflammatory response [Italiani and Boraschi, 2015]. We will focus on this final role.

As mentioned previously the origins and ontogeny of macrophage subsets in mice and humans remain a subject of study and debate. Up until recently monocytes were regarded as the systemic reservoir for the generation and renewal of tissue resident macrophages (and dendritic cells) [van Furth and Cohn, 1968]. However, growing evidence has discovered multiple macrophage subpopulations are derived independently of monocytes, with three independent points of origin identified in mice. The first source being the yolk sac in the murine embryo, which gives rise to progenitor cells that seed tissues with macrophages expressing high levels of marker F4/80. Later in the development of the foetus, generation of such tissue resident F4/80<sup>high</sup> macrophages shifts to the foetal liver (so far only confirmed to contribute to skin resident macrophages otherwise known as Langerhans cells) [Epelman et al., 2014].

Development of macrophages from both these sources is dependent on the cytokines; colony stimulating factor one (CSF-1) and IL-34 [Ma et al., 2012]. The third (or classical) source is the bone marrow which gives rise to the formation of monocyte derived F4/80<sup>low</sup> macrophages [Geissmann et al., 2010] through stimulation with CSF-1 under steady state conditions and granulocyte macrophage colony stimulating factor (GMCSF) during inflammation [Burgess and

Metcalf, 1980]. Such F4/80<sup>low</sup> cells are capable of migrating into tissue where they become F4/80<sup>high</sup> tissue resident macrophages and or TNF/iNOS-producing dendritic cells (TIP DCs) <sup>3</sup>. In steady state conditions tissue resident macrophages (here-on resident macrophages) proliferate at low levels. This proliferation increases strongly following macrophage depletion or inflammation [Hashimoto et al., 2013]. During the early stages of the inflammatory response the rapid influx of neutrophils and monocytes is accompanied by a concomitant and radical loss of resident macrophages. The reasons behind this phenomena – referred to as the “the macrophage disappearance reaction”-; tissue adherence, emigration or apoptosis, remain uncertain. Though it has been hypothesised that these cells may emigrate into lymph nodes and stimulate lymphocyte responses [Barth et al., 1995]. This deficit in macrophages is rapidly filled by infiltrating monocytes that ultimately differentiate into tissue resident macrophages [Auffray et al., 2007].

Once inside tissue such monocyte derived macrophages (MDMs) are capable of responding to cues in the microenvironment (e.g. cytokines, cell fragments, PAMPs) to determine whether they should contribute to the continuation of the inflammatory response or its resolution [Lech et al., 2012]. These two functional phenotypes are referred to as M1 and M2 type macrophages respectively. M1 macrophages function in a primarily antimicrobial, killing or inhibitory capacity and are characterised by the ability to metabolise the amino acid arginine into free radical nitric oxide, which as previously mentioned is a powerful effector molecule with microbicidal activity [Mills, 2012]. M2 macrophages act to promote inflammatory resolution and healing, they are capable of metabolising arginine into ornithine, which promotes cell proliferation and repair through polyamine and collagen synthesis, as well as fibrosis [Mills, 2012, Pesce et al., 2009]. *In vitro* studies of rapidly infiltrating MDMs show they are activated towards an M1 phenotype by pathogen associated molecules such as LPS or pro-inflammatory cytokines such as TNF- $\alpha$  or

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<sup>3</sup> The differences between monocyte derived TIP-DC's and tissue resident macrophages are highly blurred. The original functions that distinguished DC's from macrophages (naïve T-cell stimulation for the former and cytotoxicity for the latter) have recently been found to be performed by both cell subsets. There is also currently no known cell surface marker that is not shared between them. As such it seems these two populations are likely to be one and the same. At least, from a functional perspective. [Ferenbach and Hughes [2008], Italiani and Boraschi, 2015].

IFN- $\gamma$  (this will typically be the environment they enter into during inflammation) [Ginhoux and Jung, 2014]. In addition to phagocytosing pathogens and releasing ROS and NO, M1 macrophages release inflammatory cytokines IL-1 $\beta$ , TNF- $\alpha$  and IL-6, polarising a Th1 type lymphocyte response. Conversely M2 polarization has been observed in response to Th2 type cytokines IL-4 and IL-13 and other anti-inflammatory mediators such as IL-10, tumour growth factor beta (TGF- $\beta$ ) and glucocorticoids [Röszer, 2015]. M2 Macrophages release IL-10 and express high levels of scavenger, mannose, and galactose-type receptors [Weisser et al., 2013]. These macrophages participate in; Th2 polarising responses, dampening inflammation, tissue remodelling and angiogenesis [Röszer, 2015]. During the progression of inflammation, a switch from an M1 to M2 type response is indicative of movement to a resolution, or repair stage of the immune process [Ortega-Gómez et al., 2013].

The mechanism of this switch from M1 to M2 responses is currently a matter of study, with the question raised as to whether M1 and M2 macrophages are truly distinct subpopulations, or the same cells that shift from one phenotype to another in response to the microenvironment signals [Crane et al., 2014, Guilliams et al., 2014].

A popular hypothesis is that Ly6C<sup>high</sup> monocytes and/or monocyte-derived macrophages in the tissue become M1 macrophages, while Ly6C<sup>low</sup> monocytes and/or tissue-resident macrophages become M2 macrophages. While elegant, this theory has been disputed, with *in vitro* observations of the trans-differentiation from Ly6C<sup>high</sup> M1 cells to Ly6C<sup>low</sup> M2 cells [Arnold et al., 2007, Crane et al., 2014].


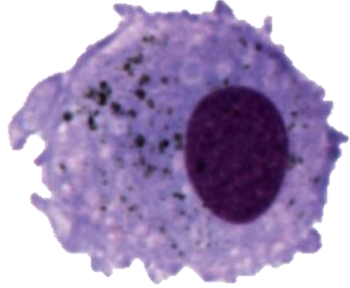
A second hypothesis suggests that monocytes recruited into tissues at sequential time points will encounter varying microenvironments, polarising them towards M1 during early phases and M2 in late phases [Crane et al., 2014]. Accordingly in this scenario cytokines and other such signals play a key role in determining the different functional phenotypes of macrophages. Such a hypotheses would make Ly6C<sup>high</sup> monocytes more likely to take on a M1 phenotype due to their rapid migrating into tissues during the initial phase of an inflammatory response.

A third hypothesis puts forward that polarized macrophage populations can switch from one to the other in response to changing environmental conditions. *In vitro* studies have demonstrated

M1 macrophages are capable of converting into M2 macrophages and vice versa through exposure in culture to IFN- $\gamma$ , IL-12 and LPS or IL-4, or IL-10 [Mylonas et al., 2009, Stout et al., 2005].

Accordingly, it is likely that the M1/M2 macrophage phenotype paradigm is an artificial one, serving as a means of conceptualising a continuum of diverse functional states and overlapping roles between these cells. Accordingly, M1 and M2 activation states should not be regarded as mutually exclusive functional states but the extremes of a single scale. In light of the reflexivity and adaptability of immune cells involved in the inflammatory process it stands that different inflammatory inducers will activate different cellular responses. These are well established in various models of inflammation discussed in section 1.3.

**Table 5 Defining features of macrophages**

<b>Macrophages</b>	<b>Diagram</b>	<b>Microscopic Image</b>
		
<b>Morphology</b>	Large, variably shaped cells 15-20µm, granular, unilobed nucleus	
<b>Subpopulation</b>	<b>Tissue resident</b>	<b>Monocyte derived</b>
<b>Origin</b>	Foetal yolk sack/ liver	Bone marrow, monocytes
<b>Key marker</b>	F4/80 High	F4/80 Negative
<b>Sub-population (ii)</b>	<b>M1 (Inflammatory)</b>	<b>M2 (Tissue Homeostasis)</b>
<b>Function in inflammation</b>	Pro-inflammatory, antimicrobials roles	Inflammatory resolution, tissue repair. Fungal or helminth infection
<b>Key mediators of activation/ chemotaxis</b>	IFN-gamma LPS GM-CSF	IL-4 IL-13, IL-10
<b>Key immune regulatory factors released</b>	TNF IL-1 beta IL-6 IL-12 IL-23	IL-10 TGF-beta IL-1ra
<b>Key markers</b>	MHCII, CD40, CD80, CD25, CD127. CXCL9, CXCL10, CXCL11, NOS2	MHCII, CD206, CD209, Dectin 1. Mrc1, tgm2, Fizz1, Ym1/2, Arg1
<b>References</b>	[Epelman et al., 2014, Ginhoux and Jung, 2014, Guilliams et al., 2014]	

### **1.2.7 Resolution of Inflammation**

For many years resolution was essentially considered the cessation of inflammation, a passive process associated with the fading of inflammatory stimuli, dilution of chemokine and cytokine gradients and the prevention of any further leukocyte recruitment.

However, more recent research has demonstrated resolution to be both an active and dynamic process, facilitated by a variety of mediators that specifically act to; (i) counteract inflammatory cytokine and chemokine signalling, (ii) inhibit blood leukocyte adherence and extravasation, (iii) promote leukocyte apoptosis and clearance of these apoptotic cells, (iv) promote macrophage reprogramming to an anti-inflammatory 'pro-resolving' phenotype [Sugimoto et al., 2016] (Figure 1-1).

Again it should be noted that resolution is not a self-contained phase in the progression of the host immune response. Many of the pro-resolving pathways that come to the fore during resolution are initially activated during acute inflammation, additionally leukocytes involved in the active phase of inflammation undergo a functional transition in order to contribute to the process of resolution and wound healing [Serhan and Savill, 2005]. As such resolution should be regarded as an overlapping and contiguous process to inflammation (and wound healing), with - as it has been put "the beginning programming the end" [Serhan and Savill, 2005]. With this concept in mind it seems fitting that neutrophils – the prime instigators of the inflammatory response are also one of the primary initial players in the process of its resolution.

#### **1.2.7.1 Chemokine Moderation and Neutrophil Cessation**

As previously discussed a successful inflammatory response is dependent on neutrophil recruitment to the point of immune insult (1.2.3). Abrogation of such an influx is a key initial prerequisite for inflammatory resolution and is believed to be brought about through the reduction and inhibition of chemotactic gradients by chemokine sequestration and/or proteolytic cleavage [Headland and Norling, 2015].

Classically associated with remodelling of the extra-cellular matrix (ECM) MMPs have been demonstrated to regulate the immune response through modulating the activity of various bioactive molecules such as TNF- $\alpha$  [Tam et al., 2004],  $\alpha$ -defensin [Wilson et al., 1999], and mannose binding lectin [Butler et al., 2002]. Further and most saliently such MMPs have been reported to act upon inflammatory chemokines. Macrophage specific MMP-12 has been demonstrated to cleave CXC chemokines, removing the ELR (glutamic acid-leucine-arginine) motif, that is vital for receptor binding [Dean et al., 2008]. MMP directed chemokine cleavage has also been shown to block function of neutrophil and monocyte specific CC-chemokines, for example MMP cleaved CCL-7 is still able to bind its cognate receptors (CCR1,2 and 3) but is unable to elicit downstream signalling effectively transforming the once chemotactic factor into an inhibitor [Bonecchi et al., 2004].

Chemokines can also be recognised by a family of proteins referred to as atypical chemokine receptors (ACKRs) (previously termed decoys, scavenger receptors or chemokine binding proteins). Present on the surface of cells and free floating, binding of a chemokine to its respective ACKR fails to induce a migratory response due to the inability of ACKRs to initiate the requisite G-protein coupled signalling pathways [Nibbs and Graham, 2013]. As such, ACKRs sequester chemokines from the environment blocking both their dispersal and function. A key member of this ACKR family involved in neutrophil regulation is Duffy antigen receptor for chemokines (DARC). Predominantly expressed by endothelial cells at sites of inflammation and leukocyte extravasation DARC is able to sequester both CC and CXC pro-inflammatory cytokines, restricting neutrophil influx [Bonecchi et al., 2004].

Additionally, such decoy receptors can also be generated from classical chemokine receptors. For example, in the context of acute murine peritonitis, release of IL-10 has been shown to inhibit downregulation of CCR5, while also blocking activation of the receptor once bound by its requisite chemokine, effectively serving as a trap for the molecule [D'Amico et al., 2000].



### **1.2.7.2 The Role of Neutrophils in Resolution**

While the cessation of neutrophil movement is a key step in the process of resolution, recent discoveries have highlighted the important role these cells play in the attenuation of inflammation. For example, the proteases neutrophils release are capable of degrading pro-inflammatory cytokines such as IL- $\beta$  and TNF- $\alpha$  but not IL-10 [Van Der Meer et al., 2009]. Further, a recent paper suggests that at high densities NETs may be able to build aggregates capable of trapping chemokines and other inflammatory mediators and degrade them again through the action of neutrophil released serine proteases. However, as yet this phenomenon has only been reported *in vitro* [Schauer et al., 2014].

An additional, novel pre-resolving mechanism focused around neutrophils involves generation and release of membrane-bound compartments referred to as microvesicles. Studies have revealed these microvesicles to contain a variety of anti-inflammatory and pro-resolving mediators the most significant of which being annexin A1 (ANXA1) [Dalli et al., 2008]. Able to interact with other cells via its micro-vesicle delivery system ANXA1 interacts with formyl peptide receptor 2 (FPR2) to reduce leukocyte adhesion and migration as well as promoting neutrophil apoptosis and clearance of dead cells [Gastardelo et al., 2009, Perretti et al., 1996].

### **1.2.7.3 Apoptosis and Efferocytosis**

Following the abortion of migration any remaining neutrophils within the now resolving site of inflammation typically apoptose and are cleared by macrophages via efferocytosis. Apoptotic leukocytes induce their own clearance through secretion of factors that attract phagocytic cells, (such factors include; lysophosphatidylcholine (LPC) [Lauber et al., 2003], sphingosine 1-phosphate (S1P) [Gude et al., 2008] and fractalkine (Cx3CL1) [Truman et al., 2008]) and expression of surface markers (such as ANXA1 derived peptide fragments [Blume et al., 2012] and phosphatidylserine (PS) [Ravichandran, 2011]) that permit identification and also ease engulfment of the dying cells.

#### **1.2.7.4 A Functional Macrophage Switch Initiates the Return To Normal Homeostasis**

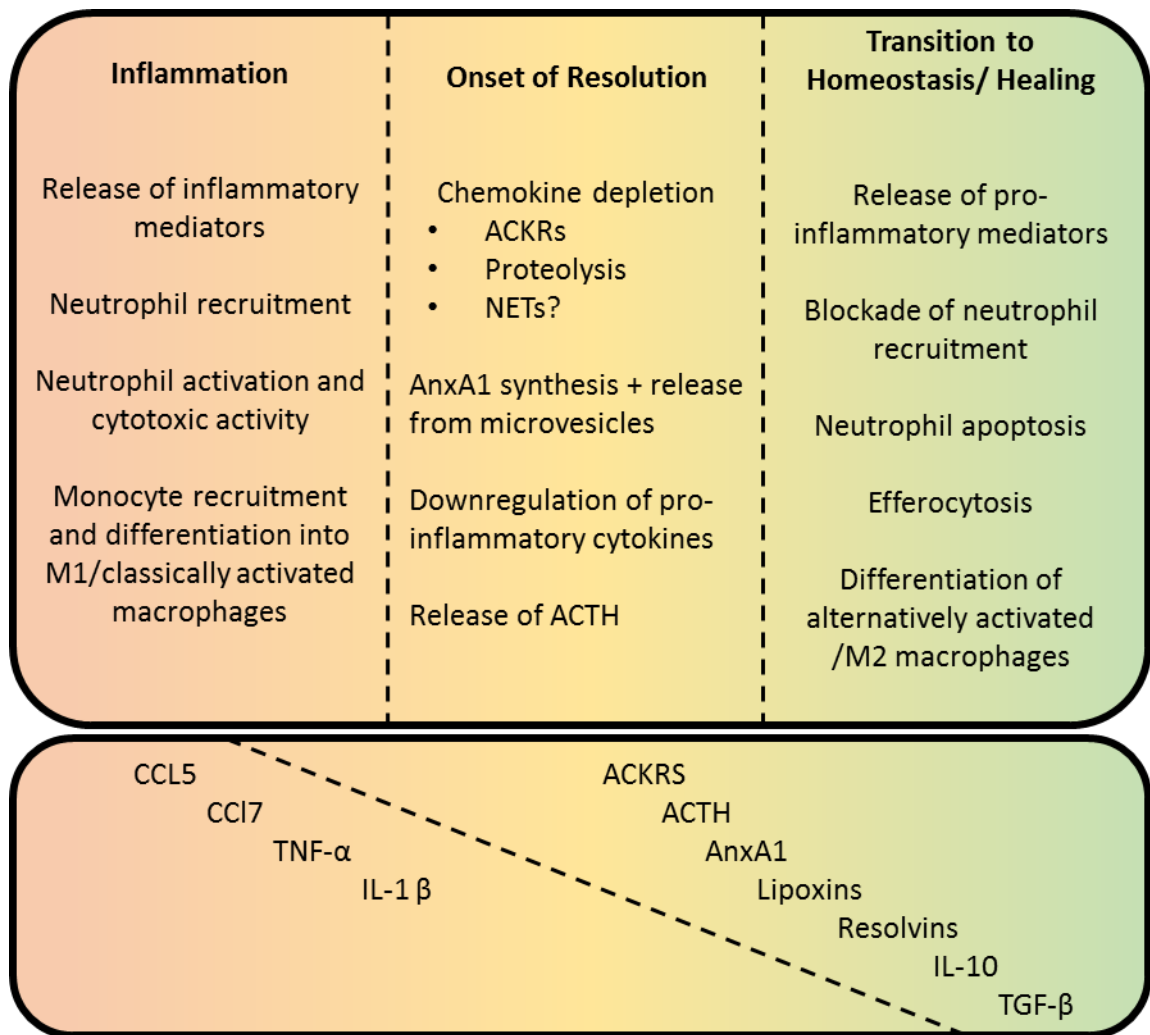
Following efferocytosis of apoptotic cells, macrophages undergo a functional switch from a pro to anti-inflammatory phenotype. This switch is typified by the increased release of TGF- $\beta$ , IL-10 and T-cell membrane protein 4 (TIM4), each key mediators of inflammatory cell clearance and tissue homeostasis [Fadok et al., 1998]. These macrophages also switch to an arginine based metabolism (previously mentioned in 1.2.6) promoting synthesis of pro-resolutionary lipid mediators including lipoxins, and resolvins [Serhan et al., 2008]. Of these lipoxins one of the best characterised is lipoxin A4 (LXA4) which has been shown to stimulate phagocytic activity independently of the release of pro-inflammatory mediators as well as inhibiting neutrophil entry to tissues while promoting monocyte migration [Maddox et al., 1997]. Resolvin E1 has in turn been demonstrated to interact with monocytes, macrophages and dendritic cells, moderating their activation by TNF- $\alpha$  and hence serving as an anti-inflammatory signalling molecule [Arita et al., 2007].

#### **1.2.7.5 The Role of Suppressor Cells in Resolution**

While neutrophils and macrophages have typically been regarded as the dominant cell types involved in the process of resolution recent evidence suggests that myeloid derived suppressor cells (MDSC) also play an important role through production of arginase-1 (ARG1) and inducible nitric oxidase synthase (iNOS) both of which initiates the release of NO and ROS involved in programmed cells death and several other immunosuppressive mechanisms [Ribechini et al., 2010].

T-regulatory cells (T-regs), a subset of CD4<sup>+</sup> T-cells have been shown to support resolution through release of TGF- $\beta$  and IL-10, their presence appears especially important in the context of extended or chronic periods of inflammation [Ortega-Gómez et al., 2013].

Following on from (and overlapping) resolution comes the process of prolonged tissue remodelling and recovery of functionality lost during the detrimental process of inflammation that composes wound healing.



**Figure 1-1 The key processes and mediators governing inflammation and its resolution.** During the initial phase of inflammation release of various inflammatory factors promotes leukocyte; accumulation, survival and differentiation at the site of inflammation. Upon activation, neutrophils release microparticles containing pro-resolving mediators that limit granulocyte ingress activate various pro-resolutionary pathways. As the inflammatory response progresses further processes prepare the environment for the onset of resolution via; remediation of chemokine gradients, activation of feedback loops limiting pro-inflammatory and upregulating anti-inflammatory cytokines and cessation of neutrophil influx. Acetylcholine (ACh) and Annexin-1 (AnxA1) released early in inflammation play a key role in mediating this switch. Further resolutionary mediators downregulate survival pathways and activate apoptosis of granulocytes, prompting the release of further pro-resolving mediators that upon efferocytosis of apoptotic cells promote differentiation of M2- type macrophages. These macrophages mediate the re-establishment of tissue homeostasis. Figure adapted from [Sugimoto et al., 2016]

### 1.2.8 Wound Healing

Like the inflammatory response it proceeds, wound healing is a complex multifaceted process the mechanistic of which are influenced by a myriad of factors. While a comprehensive review of wound healing goes beyond the scope of this thesis, a brief overview is provided in the context of its overlap with the inflammatory process.

Wound healing is stratified into a series of overlapping and intercalated phases determined by the cell lineages and physiological responses involved. As with the process of inflammation and resolution exact determination of these phases is a subject of debate. Typically wound healing is divided into four phases: the immediate response, inflammatory response and proliferatory, migratory and resolutionary phase

#### **1.2.8.1 The Immediate Response**

One of the earliest responses to injury originates from damage caused to local blood vessels. To prevent local haemorrhage a clot of insoluble fibrin fibres formed by activated and aggregated platelets is generated within minutes of vascular injury. In addition to patching the gap in the blood vessel the clot acts as an initial scaffold onto which various growth factors can bind and effector cells infiltrate into [Nurden et al., 2007]. Platelets themselves serve as a source for these growth and chemotactic factors including; platelet derived growth factor (PDGF), CXCL4, vascular endothelial growth factor (VEGF), RANTES and TGF- $\beta$  [Bahou and Gnatenko, 2004, Chai and Tarnawski, 2002]. Each prompting various aspects of the healing response.

In addition to platelet derived factors the clotted blood contains a variety of interleukins, chemokines, colony stimulating factors, tumour necrosis factors  $\alpha$  and  $\beta$  and IFN- $\gamma$  which contribute to the induction and release of serum response factor (SRF) by surrounding cells. SRF is a master transcription factor that activates multiple genes known to contribute to the healing response [Chai and Tarnawski, 2002].

#### **1.2.8.2 The Inflammatory Response**

The inflammatory response has been described in detail previously (1.2). Although the issue of whether the presence of inflammatory cells is essential for wound healing remains a matter of research, it is clear that these cell populations have a strong influence on other cells within the wound and surrounding tissue. The expression profile of neutrophils recruited to sites of healing suggest they may influence several aspects of repair, such as the breakdown of the initially

formed fibrin clot, promotion of angiogenesis and the process of re-epithelialisation [Theilgaard-Mönch et al., 2004].

Monocyte derived macrophages are also regarded as playing a key role in wound healing, present throughout the repair process M2 macrophages have been shown to express a variety of anti-inflammatory, pro-angiogenic factors as well as multiple matrix metalloproteinases required for tissue remodelling [Deonarine et al., 2007].

#### **1.2.8.3 Angiogenesis and Lymphogenesis**

Integral to successful wound repair angiogenesis involves the formation of capillaries from around the wound edge, followed by the infiltration into the site of damage. Typically, a microvascular network will viably permeate the wound several days post injury, this network provides nutrition and oxygen to growing tissues supporting the generation of a more sophisticated wound healing scaffold referred to as granulation tissue [Tonnesen et al., 2000]. Angiogenesis is a stringently regulated process mediated by a plethora of growth factors, the most important of which being vascular endothelial growth factor (VEGF). While far less studied than angiogenesis, lymphogenesis (repair and re-generation of lymph vessels) is also believed to be mediated under the tight regulation of multiple factors, some overlapping those of angiogenesis and some unique, such as; podoplanin VEGF-C, hyaluronic acid and ephrin-B2 [Stacker et al., 2014].

#### **1.2.8.4 Proliferation (Migration and Contraction)**

The clot (and/or scab depending on the location of the wound) that forms during the immediate response serves as a temporary means for restoring the function of skin or subdermal tissue as a protective barrier. During this phase of the healing process this scab retracts in sync with the regeneration of tissue lost during the injury. Re-epithelization of a wound is achieved through the migration and proliferation of keratinocytes in the region of the wound [Matoltsy and Viziám,

1970]<sup>4</sup>. Keratinocytes are able to migrate through collagen and fibronectin that constitute the scab via production of a variety of MMPs [Pilcher et al., 1999] and physically pulling themselves through the matrix using a series of actin mediated cytoplasmic projections in a process referred to as 'keratinocyte crawling' [Mitchison and Cramer, 1996]. In this way keratinocytes are able to bore a pathway to the interface of scab and viable tissue and proliferate.

In addition to the epidermis the underlying dermis undergoes a separate healing process. Fibroblast cells surrounding the site of the wound initially respond by generating a matrix of actin bundles referred to as stress fibres which are capable of facilitating some limited connective tissue contraction [Hinz, 2007]. Around these fibres further fibroblasts are driven to differentiate into smooth muscle myofibrils (myofibrilblasts) through the action of growth factors such as TGF- $\beta$ , and mechanical stress [Hinz, 2007]. Together these wound fibroblasts and myofibrilblasts knit the wound closed and facilitate the synthesis and position of collagen fibres, the primary constituent of scar tissue.

#### **1.2.8.5 Resolution**

In this final and sometimes un-ending phase of repair the wounded tissue attempts to restore full functionality and normal appearance. Migrating and proliferating keratinocytes at the wound's edge eventually meet one another, cease proliferation and re-stratify (the means by which this stop phase is regulated is still under study, the Wnt pathway appears integral [Cooper et al., 2004]). In order for complete healing to occur epidermal appendages such as hair follicles and sebaceous glands need to regenerate. This regeneration often fails to happen in excessive scar tissue although it has been shown that inflammation at the site of the wound may help promote this regrowth process (again through Wnt signalling [Ito et al., 2007]) [Osaka et al., 2007]. During this phase vasculature within the scar tissue becomes more defined and diffuse to form a more functional network, and dense sections of the ECM may be remodelled to restore normal architecture.

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<sup>4</sup> It is currently unknown whether these cells initially migrate from other parts of the body or are generated *in situ*. It has been postulated that stem cells resident in the epidermis and at the base of hair follicles may be the source of these cells [Eming et al., 2014].

The inflammatory response to wound healing also resolves once healing is complete. This appears to be mediated by a number of factors shown to be active in the resolution phase of inflammation such as resolvins [Schwab et al., 2007] and annexins [Perretti and Gavins, 2003] (Figure 1-1). Neutrophils are cleared from the site typically through apoptosis and phagocytosis by macrophages [Haslett, 1992]. Macrophages in turn are thought to de-activate through a combination of anti-inflammatory and resolution factors, such as galectins and glucocorticosteroids. Upon which they may migrate out of the tissue, become phagocytosed by other macrophages or revert into tissue resident cells [Ma et al., 2003].

While this process initiates within hours of injury the time required for complete healing is highly variable and dependent on multiple factors such as; wound size, source of injury, age and health. Imperfect regulation of wound resolution can result in hyper-proliferation of various cells, chronic inflammation and consequential excessive tissue formation all of which contribute to a range of pathologies. Imperfect wound healing and persistent wounds have become a leading cause of morbidity and mortality in the western world fuelled in part by the increasingly aged population and a growing risk of nosocomial infection with antibiotic resistant bacteria [Stacker et al., 2014].

### **1.2.9 Chronic Inflammation**

If the underlying cause cannot be resolved the inflammatory process enters into a long term, chronic phase. Chronic inflammation retains many of the features of acute; increased blood flow, vascular permeability and cellular influx. However, the composition of this influx changes. In place of short lived neutrophils chronic inflammation is typified by a predominance of monocytes, macrophages and lymphocytes [Abbas et al., 2014] (Table 1 Key features of chronic and acute inflammation) which have a lifespan of months or even years dependent on subtype [Takahashi, 2001]. The presence of such cells in a continually activated pro-inflammatory state leads to the constitutive generation of multiple cytokines, enzymes, growth factors and metabolites that cause both tissue injury and fibrosis (the thickening or scarring of connective tissue) [Mosser, 2003]. It is this persistent cycle of cellular influx, tissue damage and

remodelling that generates the pathologies commonly associated with inflammatory disease, one such example of such a pathology is discussed in 1.3.3.



## 1.3 Models of Inflammation

### 1.3.1 Zymosan Induced Peritonitis; A Model of Resolving, Localised Inflammation

Injection of pathogens and other irritants into the peritoneal cavity has been a recognised model for studying the inflammatory immune response for well over a century [Brown, 1995]. As the largest naturally occurring serosal space in the vertebrate body and also located close to the skin surface, the peritoneal cavity serves as both an easy target for the administration of immune-activating reagents and as an efficient reservoir for the collection of cells and soluble effectors released into it.

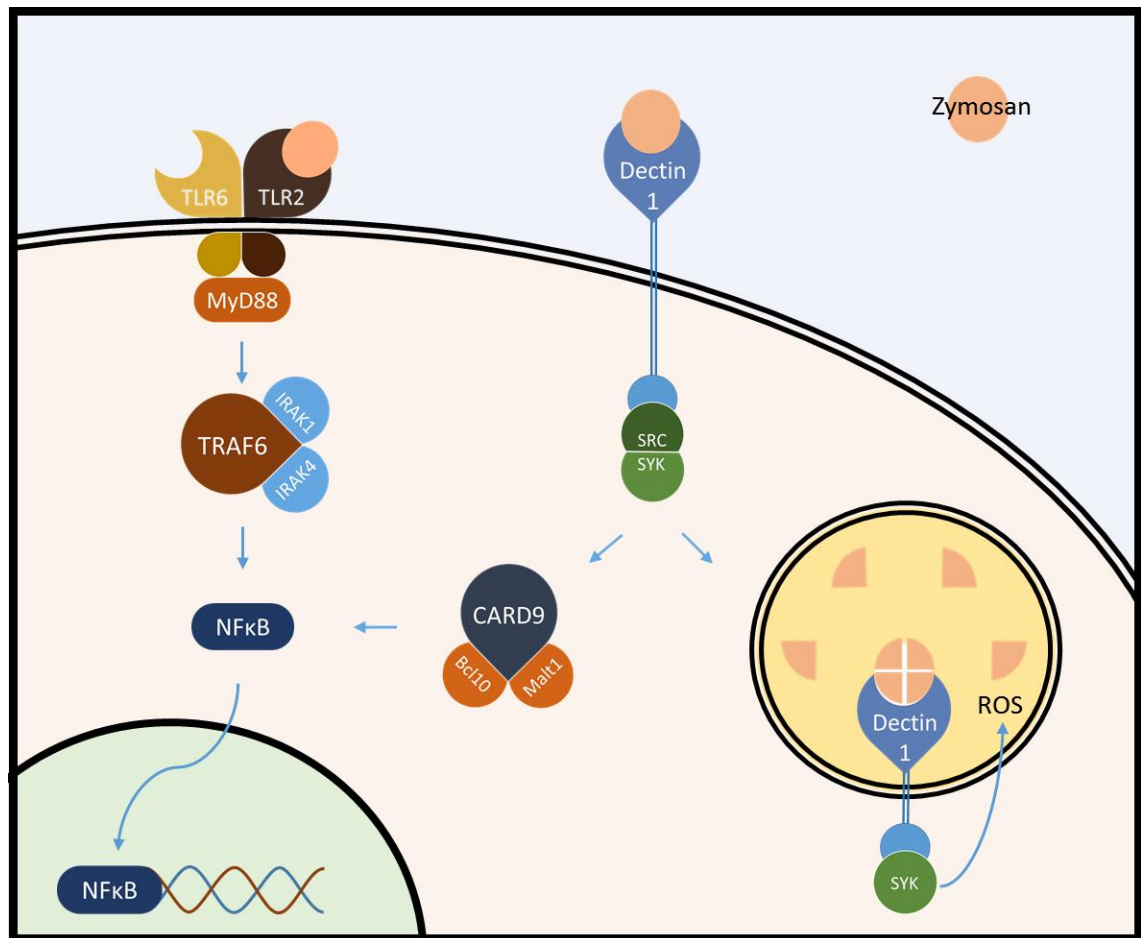
A large variety of immune-activating molecules, compounds and organisms have been used over the years to induce peritonitis (e.g. IL-1 [Song et al., 2013], LPS [Steinmüller et al., 2006] and live *E.coli* [Wells and Johnstone, 1907]). Each instigating its own variation of the inflammatory response. One of the most widely used effectors is zymosan, an insoluble glucan polysaccharide derived from the cell surface of the yeast *Saccharomyces cerevisiae*. Initially used over thirty years ago [Doherty et al., 1985] and to date, still regularly employed [Monroe et al., 2016] zymosan's popularity as reagent stems from its ease of preparation and capacity to induce a consistent, self-resolving model of sterile inflammation [Rao et al., 1994, Perretti et al., 1992].

#### 1.3.1.1 Mechanism of Action

Produced through boiling, trypsin treatment and drying (or lyophilisation) of *S.cerevisiae*, zymosan is administered in the form of homogenised particles suspended in a endotoxin free medium (typically PBS or sterile water). Phagocytes such as monocytes, macrophages and dendritic cells have been shown to express a variety of receptors such as the mannose, complement [Underhill, 2003] and  $\beta$ -glucan receptors - most significantly the beta glucan receptor dectin-1 [Brown et al., 2002] that mediate their recognition and internalisation of zymosan particles (Figure 1-2).

Further inflammatory responses to zymosan are initiated through binding with the heterodimeric complex of toll-like receptors 2 and 6 [Underhill et al., 1999]. Activation of TLR2/6 elicits the

activation of transcription factor NF- $\kappa$ B through a Myeloid differentiation primary response 88 protein (MYD88) dependent pathway, leading to the generation of multiple pro-inflammatory mediators, such as IL-6, CCL2 and KC [Underhill et al., 1999] (Figure 1-2).



**Figure 1-2 Mechanisms of leukocyte activation by zymosan.** Binding of zymosan (and other  $\beta$ -glucans) by the dectin-1 receptor triggers phagocytosis of its bound ligand and the recruitment and activation of Src followed by Syk tyrosine kinases in the cytoplasm. Syk, in turn, induces the CARD9-Bcl10-Malt1 immune signalling complex leading to the production of reactive oxygen species (ROS), activation of NF- $\kappa$ B and subsequent release of pro-inflammatory cytokines. Zymosan can also be bound by the TLR 6/2 receptor complex resulting in activation of signalling protein MyD88 which in turn initiates formation of the IRAK-IRAK4-TRAF6 signalling complex which also up-regulates transcription of NF- $\kappa$ B. Syk -Spleen tyrosine kinase, Src - Proto-oncogene tyrosine-protein kinase Src, TRAF - TNF receptor associated factor, IRAK - interleukin-1 receptor-associated kinase, CARD - Caspase recruitment domain-containing protein, Bcl - B-cell lymphoma/leukaemia protein, MALT - Mucosa-associated lymphoid tissue lymphoma translocation protein. TLR – Toll like receptor.

### 1.3.1.2 Advantages and Limitations of ZIP

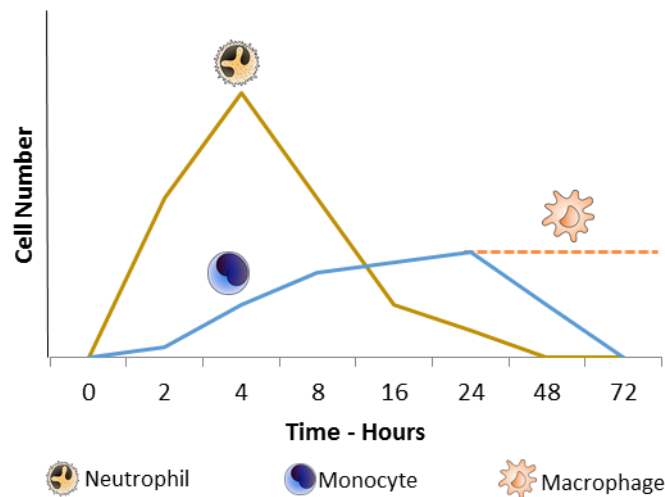
ZIP's key advantages stem from its capacity to induce a relatively consistent mild to moderate inflammatory response that both induces and resolves rapidly. Zymosan has also been shown to produce all the core indicators of acute inflammation (pain, leukocyte infiltration and synthesis of inflammatory mediators) in addition to producing directly analogous results when used *in vitro* upon isolated macrophages [Taylor et al., 2002, Doherty et al., 1985]. Injection into a serosal cavity as opposed to an artificially created one such as an air pouch allows the natural ingress and egress of leukocytes to and from the inflammatory site via draining lymph nodes and the release of quantifiable levels of inflammatory mediators [Bellingan et al., 1996]. Further, the well-established understanding of the events that occur during the model make peritonitis a practical choice for initial investigation into the effects of any immunological/physiological modulator of the inflammatory response (Figure 1-3).

While this model is relatively simple and easily reproducible from a technical standpoint, it does require some practical skill at administering intra-peritoneal injections quickly and consistently. Poor injection technique can lead to leakage of zymosan out of the peritoneal cavity or accidental administration of the reagent subcutaneously or inguinally. Additionally, the high level of vasculature surrounding the peritoneal cavity makes contamination with blood (through needle rupture) a high risk without good injection technique. In both these instances the affected animal will have to be excluded from the study [Cash et al., 2009].

Further it should be noted application of a single antigenic component of a complete pathogen will ultimately generate an artificial response, while ZIP is largely regarded as a powerful and robust model of inflammation it is still only a model. Additionally, as zymosan is an insoluble molecule, care must be taken in its; storage, handling and application for reproducible results.

### 1.3.1.3 Timecourse of ZIP

Intraperitoneal (i.p) injection of zymosan particles elicits a pattern of leukocyte recruitment following that of a typical inflammatory response. Neutrophils are the first readily identifiable cells to enter the peritoneal cavity reaching detectable levels roughly one to two hours post injection, peaking at four to six hours following which numbers begin to fall [Cash et al., 2009]. Monocytes enter the peritoneal cavity roughly four to six hours post injection peaking at around 24-hours. At this point monocyte numbers begin to drop, likely due to a combination of, death, migration out of tissues and differentiation into macrophages [Cash et al., 2009].



**Figure 1-3 Order of leukocyte infiltration during zymosan induced peritonitis.**with a low to moderate dose of zymosan (10 - 500µg) Neutrophils are reported to enter the peritoneal cavity within 1-2 hours of injection. Monocytes typically follow at around the four hour time point with numbers beginning to fall around 24-72 hours post injection as the cells differentiate into macrophages.

### 1.3.1.4 Dose Dependant Effects of Zymosan Administration.

The immune response elicited by zymosan is highly dose dependent, with low doses reported to induce a moderate self-resolving inflammatory reaction while higher doses produce a more intense response that takes a significantly longer time to resolve. Quantities of zymosan injected in studies range across the literature from 10 µg to as much as 1mg per mouse and produce a correspondingly wide range of cellular responses (Table 6). With this variation in dose some concerns have been raised as to whether administration of higher levels of zymosan generate a truly 'resolving' or realistic inflammatory response. It is suggested that high doses at (e.g. 1mg per cavity) induce an excessive and potentially dysregulated inflammatory response

typified by mast cell activation and heightened production of CCL5 and KC, culminating in a huge cellular infiltrate into the peritoneum and an inflammatory response that may never truly resolve [Cash et al., 2009]. Accordingly, care should be taken when using this model to choose a dose of zymosan that meets the needs of the phenomena being investigated.

**Table 6 Referenced doses of zymosan and resulting peritoneal leukocyte numbers**

<b>Study</b>	<b>Dose of zymosan</b>	<b>Leukocyte numbers at four to six hours (x10<sup>6</sup>)</b>
[Cash et al., 2009]	10µg	2-2.5
[Rajakariar et al., 2008]	100 mg	3-7
[Bian et al., 2012]	0.5mg	5-13
[Kolaczowska et al., 2008]	1 mg	16-18
[Ajuebor et al., 1998]	1 mg	8-12

### **1.3.2 Caecal Ligation and Puncture.**

While ZIP portrays the effects a mild to moderate, self-resolving inflammatory response caecal ligation and puncture (CLP) is an attempt to model the uncontrolled inflammatory reaction caused by sepsis.

#### **1.3.2.1 Dynamics of Sepsis**

Sepsis is an acute, systemic reaction to inflammatory activators produced by microbial infection. This infection is typically bacterial but can also be induced by viruses, fungi and eukaryotic parasites. Sepsis progresses in two primary phases, an initial hyper-inflammatory phase; characterised by hyperthermia and leucocytosis, followed by a hypo-inflammatory phase; characterised by hypothermia and leukopenia [Nguyen and Smith, 2007].

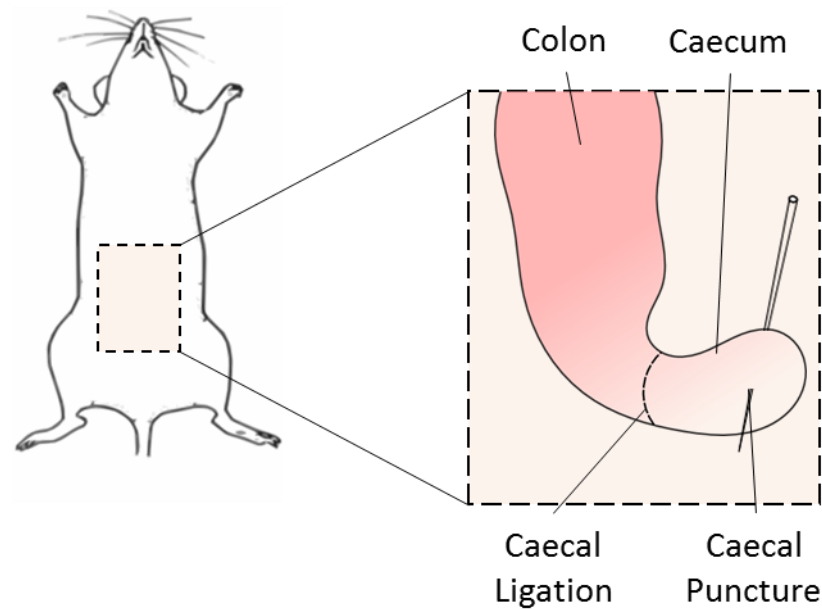
During the hyper-inflammatory phase large numbers of; pro-inflammatory leukocytes, mediators (such as cytokines) and secondary mediators (such as enzymes and ROS) are realised in rapid succession in to circulation. The primary inflammatory cytokines detected during this phase of sepsis are; TNF- $\alpha$ , IL-1, IL-6, IL-12, IFN- $\gamma$ , and macrophage migration inhibitory factor (MIF) [Kelly-Scumpia et al., 2010, Matsukawa et al., 1999, Riedemann et al., 2003, Ward and Bosmann, 2012, Weighardt et al., 2006]. This exacerbated release of pro-inflammatory cytokines has been termed a 'cytokine storm' [Aikawa, 1996]. Responses to this cytokine release include the activation and chemotaxis of leukocytes, enhanced phagocytic and cytotoxic activity, vascular endothelial injury with accompanying oedema, synthesis of acute phase proteins by

the liver and systemic activation of the coagulation and complement systems [Nguyen and Smith, 2007]. More recent research has revealed that concomitant to this pro-inflammatory burst is a near simultaneous release of anti-inflammatory cytokines, including IL-10, transforming growth factor (TGF)- $\beta$ , and IL-4, likely produced as part of a feedback response attempting to restore immunological equilibrium [Junger et al., 1996].

Following this acute hyper-inflammatory response comes a prolonged anti or hypo-inflammatory state typified by the inhibition of multiple leukocyte signalling pathways involved in cell chemotaxis, proliferation and survival [Junger et al., 1996, Muenzer et al., 2010]. This leads to the rapid apoptosis of lymphocytes and dendritic cells, effectively paralysing the adaptive and exhausting innate immune responses [Hotchkiss and Nicholson, 2006]. This shut down of both arms of the immune response renders the sufferer immunocompromised and at high risk of mortality through secondary infection.

#### **1.3.2.2 CLP as a Means of Modelling Sepsis**

Due to its complex, multi-faceted nature, sepsis is challenging to model in experimental animals. One of the most extensively used means to study sepsis over the past thirty years has been the caecal ligation and puncture (CLP) method, which is regarded by many to provide the best representation available of the complexity of human sepsis [Dejager et al., 2011]. CLP consists of three core physiological insults: Tissue trauma caused by laprosectomy, necrosis and inflammation caused by ligation of the caecum and infection caused by leakage of intestinal microbial into the peritoneal cavity and eventually bloodstream (Figure 1-4). It is this leakage that induces the inflammatory response in mice, culminating in septic shock. First signs of sepsis (lethargy, fever, piloerection, diarrhoea, huddling, and malaise) are typically observable in animals 6-12 hours following surgical procedure. Over the following 12-24 hours these symptoms become more severe until animals are unable to move and exhibit a dramatic decrease in body temperature indicative of the hypo-inflammatory phase of the disease [Toscano et al., 2011]. At this point (if not before) animals should be sacrificed to avoid excessive suffering.



**Figure 1-4 Diagram of the caecal ligation and puncture procedure. CLP is induced by the isolation, ligation and puncture of the caecum directly below the ileocaecal valve. The further up the caecum the ligation is performed and the size and number of punctures determine the severity of the disease model. Sham operations are performed by isolation of the caecum without ligation and puncture.**

### 1.3.2.3 Advantages and Limitations of CLP

CLP has several distinct advantages over other models of sepsis. Unlike other procedures that introduce cultured colonies of bacteria into an animal, CLP utilises enteric bacteria that originate from the host, mimicking the injury that typically leads to sepsis in humans. This mimicry is further enhanced by the polymicrobial nature of the immune insult introduced. Supporting this the systemic cytokine profile found in sepsis is very similar to that found in human patients. It is also a relatively simple procedure compared to other models of sepsis such as colon ascendens stent peritonitis (CASP) which requires surgical introduction of a stent into the caecum [Dejager et al., 2011]. Further, as with the ZIP model of peritonitis a variety of cells and soluble analytes can be easily retrieved from the peritoneal lavage and blood of an animal in order to analyse the progression of the inflammatory response.

Despite this relative simplicity, one of the key drawbacks of the CLP model is the variability of the results it produces due to differences in experimental procedures across labs. The outcome of CLP has been found to be highly dependent on a number of variables during the operation:

(i) The fraction of caecum ligated and according amount of necrosis incurred [Ayala et al., 2000], (ii) the size and number of punctures performed on the caecum and the amount of pressure exerted when extruding faecal material [Singleton and Wischmeyer, 2003], (iii) the size of the abdominal incision made, (iv) anaesthesia used, (v) age, sex and strain of the animal used, (vi) the point in the animal's diurnal cycle in which the procedure was performed, (vii) general heterogeneity of immune responses – this is especially evident when using outbred animals [Wichterman et al., 1980, Baker et al., 1983, Dejager et al., 2011].

Despite these drawbacks CLP remains – in balance – the simplest and most realistic means of modelling sepsis in mice, it also serves as an effective way of investigating the immune response in the context of an overwhelming inflammatory and microbial challenge.



### **1.3.3 Sponge Granuloma**

The primary goal of the immune response is the removal of any non-self-objects or molecules from the body and the preparation of the affected tissue for regrowth and repair. However, circumstances in which an immune insult cannot be removed – either due to its size or recalcitrance to immune effector functions – results in a unique chronic immune state termed granulomatous inflammation or, the foreign body reaction [Strauch and Rubenstein, 1984]. In such a reaction offending objects are surrounded with a wall of leukocytes, predominately made up of macrophages that eventually fuse to form a fibrotic capsule referred to as a granuloma. The purpose of this structure is to isolate the antigen from the rest of the body, facilitating its eradication and preventing further dissemination [Strauch and Rubenstein, 1984].

Such foreign body granulomas have been well studied in a clinical context where they are often produced in response to implanted devices (such as pace makers or artificial hips), sutures or other foreign objects that find themselves embedded beneath the skin [Souza et al., 1986].

#### **1.3.3.1 The Foreign Body Response**

The foreign body reaction begins in a similar manner to a classic inflammatory immune reaction. Upon entering the body foreign objects will almost immediately acquire a coat of plasma proteins (including albumin, fibrinogen, complement, fibronectin, vitronectin and  $\gamma$ -globulin [Karsenty and Park, 1995, Majchrzycki et al., 2015] in addition to inducing localised oedema through the physical trauma of entry. Such oedema induces leukocyte recruitment from circulation to the site of injury and their subsequent activation through interaction with the proteins adsorbed to the surface of the foreign object, most particularly those that form part of the complement pathway [Tono-Oka et al., 1996].

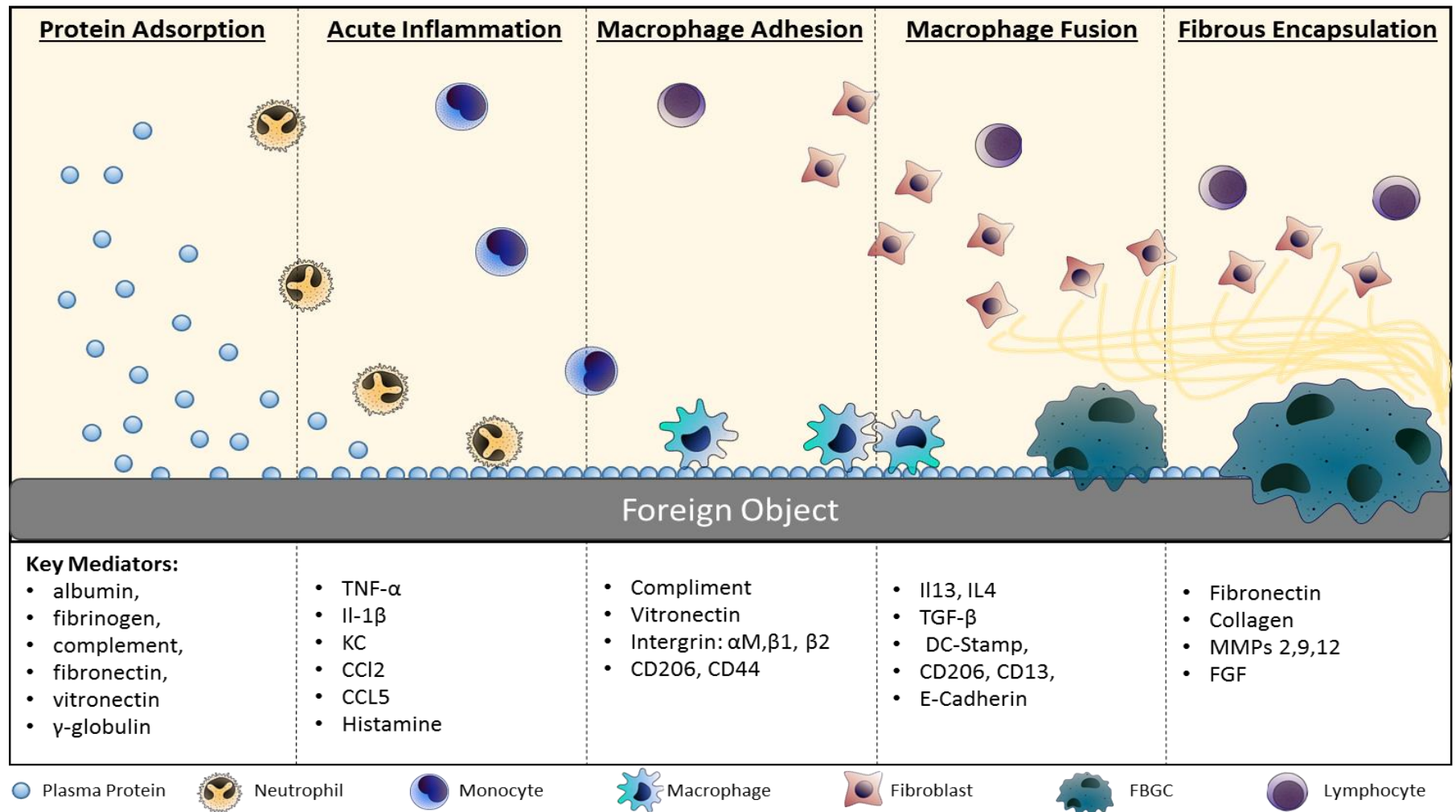
Such binding and activation induces an acute inflammatory response analogous to that previously described, typified by the mass influx of acute phase effector cells such as neutrophils as well as mast cells to the site of injury. Depending on the nature of the object and injury caused [McQuibban et al., 2000] this response is followed several days later by the hallmarks of a chronic inflammatory response characterised by the presence of lymphocytes

and plasma cells at the site of injury, as well as the perpetuation of macrophages and monocytes [Strauch and Rubenstein, 1984].

Unable to phagocytose the offending object macrophages bound to the object's surface will eventually begin to fuse with one another, merging their cytoplasm to form aggregates referred to as foreign body giant cells (FBGCs). Macrophage fusion is a highly orchestrated process, the exact molecular mechanisms of which have not yet been fully characterised [Klopfleisch, 2016]. However, it is known that essential to initiation of this fusion is the presence of the anti-inflammatory cytokine IL-4 [Jia et al., 1996] and its functional homologue IL-13 [Babic et al., 1999]. Binding of these cytokines by macrophages has been observed to upregulate mannose receptor expression by these cells, facilitating macrophage interaction and membrane fusion through a process comparable to phagocytosis [Hicklin and Ellis, 2005]. It is interesting to note that FBGC formation also appears to rely on the presence of CCL2, with CCL2-null mice presenting a high reduction in the level of cell fusion in response to implantation of biomaterial [Assinder et al., 2009]. The fact granuloma initiating events requires a combination of both pro and anti-inflammatory mediators demonstrates the unique immune scenario this response is generated by. If possible FBGCs continue to fuse and multiply until they uniformly cover the object they have adhered to. This effectively seals the object off from the rest of the host, generating an isolated environment between its surface and the FBGC membrane. In a process termed 'frustrated phagocytosis', FBGCs are then able to release multiple effector molecules into the environment targeted at destroying the foreign object. These include reactive oxygen intermediates (ROIs, oxygen free radicals), degradative enzymes, and acid [Opdenakker et al., 2001].

Much like the macrophages they derive from FBGCs are capable of secreting a range of growth, angiogenic and matrix remodelling factors that regulate the process of tissue remodelling and wound healing [Kundu and Patil, 2006]. Concomitant to the formation of FBGCs, macrophages and developing FBGCs release multiple chemotactic factors such as Platelet derived growth factor (PDGF) and LTB<sub>4</sub> which promote migration of fibroblasts to the site of immune activation [Kundu and Patil, 2006]. Upon arrival fibroblasts are activated and deposit multiple (ECM) proteins (predominantly collagens) across the surface of the foreign

object and the FBGCs that surround it [Yokota et al., 2000]. The fibrinogen produced by these cells correspondingly includes macrophages and FBGCs to produce further bone and angiogenic factors [Ramaswamy and Hemler, 1990]. This process of encapsulation, occurring over a period of weeks further isolates the foreign object from the rest of the host environment. While this physical separation ensures the object itself can no longer directly damage the host, the capsule - now a fibrotic ball of scar tissue - risks being detrimental in itself. Often lacking vasculature, the capsule becomes impermeable to cells, slowing healing or preventing its completion entirely, generating a persistent chronic inflammatory response [Strauch and Rubenstein, 1984].



**Figure 1-5 Time course of the foreign object granuloma response.** Immediately following the contact of a foreign object with host plasma it becomes coated with a layer of host proteins. Concomitantly neutrophils enter the site of the foreign body and respond by releasing cytokines, chemokines, reactive oxygen species and other enzymes. Over the course of days, the released neutrophil products recruit circulating monocytes to the site of inflammation which in turn differentiate into macrophages that bind to the surface of the foreign object via the adsorbed plasma protein. In case where the foreign object is too large to be phagocytosed, macrophages will begin to fuse together forming foreign body giant cells (FBGCs) around the surface of the object. At the same time both macrophages and FBGCs release a variety of mediators that attract fibroblasts. Upon arrival fibroblasts release large quantities of collagen, over time forming a dense collagenous fibrotic capsule around the object isolating it physically and physiologically from the host.

### **1.3.3.2 Sponge Implantation as a Model of the Healing and Foreign Body Response**

Owing to its fairly regular occurrence in surgical settings and relationship with the process of wound healing, multiple models of the foreign body/wound healing response have been devised for use in experimental animals. Of these one of the most extensively used are dead space models, which use artificial, porous implants inserted within an animal to study tissue healing and formation as well as the effects of bio-active substances in the wound. Perhaps the most popular and well established model of this type is the sponge implantation model in which one or more sponge disks (typically polyvinyl alcohol - PV(A)) are implanted under the skin for a set period of time and the resultant changes in tissue and cellular physiology and biology assessed [Hynes, 1992, Mosser and Zhang, 2008].

### **1.3.3.3 Advantages and Limitations of the Sponge Implantation Model**

The sponge implantation model possesses a number of advantages over other models of its type. Unlike other materials commonly inserted (e.g. wire meshes) the mechanical properties of PVA sponge (i.e. sponge like) make it very easy to implant and remove with the minimal level of injury to the test animal. The porous nature of these sponges also allows them to capture a range of cells and effector molecules that infiltrate from the surrounding tissues, this sponge infiltrate can be readily recovered and analysed *ex vivo*. Alternatively, the sponge can also be sectioned for histological analysis. Additionally, sponges can also be impregnated with a variety of compounds that may modulate the wound healing or inflammatory response [Chin et al., 2013, Carpenter and Cohen, 1990]. For example injection of carrageenan into the sponge immediately following implantation elicits a chronic, irresolvable inflammatory response culminating in granuloma formation [Yarden and Schlessinger, 1987]. Sponges can also be cannulated permitting repeat sampling of the wound healing/inflammatory microenvironment at successive time points [Smith et al., 2005]. Such additions make this model a highly versatile one.

PVA sponge implantation is also a comparatively rapid means of modelling the foreign body tissue response with inflammation observable within hours of implantation, giant cell formation and angiogenesis detectable within mice within 4-14 days and fibrosis within 14-21 days

(dependent on what the sponge was impregnated with) [Chauhan et al., 1993, Chin et al., 2013].

As with the ZIP model, classification of various cell subsets detected within the sponge infiltrate can provide an insight into the current phase of the inflammatory/foreign body response within an animal [Chin et al., 2013, Qian et al., 2011]. Additionally quantification of various cytokines present within the sponge exudate can be used to determine the biological conditions within the sponge [Al-Alwan et al., 2013]. The granulomatous tissue itself can also be harvested from sacrificed animals and subject to a variety of analyses ranging from gross histology, to RNA microarray analysis.

Many of the shortcomings of the sponge implantation model mirror that of CLP, namely that variations in surgical procedure cause corresponding variation in the animal's response to the implanted sponge. Factors such as the size and dimensions of the sponge, process by which the sponge was sterilised, sponge porosity (which alters on a batch by batch basis ), distance of the sponge from the site of incision, location of the sponge and means by which the sponge was impregnated have all been shown to affect host responses [Opdenakker et al., 1993, Chin et al., 2013, Chauhan et al., 1993]. Accordingly, consistent surgical technique and avoidance of varying the batches of sponge used are vital for consistent data.

It should also be observed that the sponge implantation model is somewhat unique in that it is a near complete imitation of the process it is often used to model – the traumatic implantation of a foreign object under the skin.

## 1.4 Environmental Factors Influencing Inflammation

Influences of inflammatory state are considered both heterogeneous and multifactorial, determined through interaction of both genetic and environmental factors [Jenerowicz et al., 2012, Danese et al., 2004, Kelishadi et al., 2009]. This diversity of influences means inflammatory disorders often possess aetiologies so complex as to be undefinable [Scott et al., 2004, Vodovotz, 2006]. Regardless, multiple lines of research have identified several key inflammatory modulators.

### 1.4.1 Enteric Microorganisms

At the time of writing, the interplay between the immune system and commensal microbiota is a popular field of scientific research. Differential populations of bacteria have been attributed with both instigating and preventing inflammatory responses. Studies of individuals suffering inflammatory bowel diseases have found significantly higher proportions of *Enterobacteriaceae* and *Bacteroides* bacterial species residing in patient intestines compared to healthy individuals. Known to elicit a strong inflammatory immune response these bacteria have been implicated in exacerbating and potentially causing symptoms of the disease. Various bacteria have been implicated in potentiating or instigating; ulcerative colitis, colorectal cancer, Crohn's disease, liver disease and multiple allergic type diseases all through exacerbation of the inflammatory response [Wang et al., 2007, Kleessen et al., 2002].

Conversely, several studies have identified bacterial strains that appear to have a neutralising effect on gut inflammation. Of these the most studied are strains of *Lactobacillus* and *Bifidobacterium* [Isolauri et al., 1991, O'Mahony et al., 2005, Saavedra et al., 1994]. Several methods by which these bacteria prevent inflammation have been suggested: (i) The outcompeting or destruction of inflammation-aggravating bacterial strains; (ii) Enhancing the barrier between the GI tract and circulatory system, reducing efflux of inflammatory molecules; (iii) Direct interaction and nullification of the inflammation driving components of the host immune system [Forsythe and Bienenstock, 2010, Sokol et al., 2008, Hakansson and Molin, 2011].

### 1.4.2 Genetics

Numerous studies have established that specific immunological traits and phenotypes are heritable, many of which have distinct effects on inflammation [Mcdevitt and Benacerraf, 1969]. Arguably the archetypical immuno-genetic determinant is the human lymphocyte antigen (HLA) system. A series of genes located on chromosome six that (amongst numerous other functions) determine immune cell-surface antigen-presenting proteins. Differing alleles of HLA genes (HLA types) have been connected to varying immune responses, including graft rejection, inflammation and risk of autoimmune disease [Thorsby, 1997, Abbas et al., 2014]. More recently a comprehensive immunophenotyping study analysing 78,000 immune traits in 669 female twins identified 23 individual genetic variants within 13 independent loci. Suggesting genetic regulation of the immune and inflammatory response to be both diffuse and wide ranging [Roederer et al., 2015]. Correspondingly research has demonstrated significant variation in cytokine responses, wound healing, recovery time, and development of autoimmune disease across various mouse strains [Kigerl et al., 2006, Ma et al., 2006, Sethi et al., 2004, Shi et al., 1997]<sup>5</sup>.

While the influence of genetics over the immune inflammatory response is undisputed, the scale of this influence remains a subject of debate. In a 2015 study using 210 identical twins scientists at Stanford University suggest human immune variation is primarily driven by non-heritable influences. Immune parameters were shown to become increasingly divergent between twin pairs with age. A single non-heritable factor was demonstrated to alter more than 50% of the cell subsets and serum proteins investigated [Brodin et al., 2015].

### 1.4.3 Ageing

Multiple studies have demonstrated that ageing in both humans and experimental animals is characterised by a state of chronic, low-grade inflammation. A phenomenon popularly referred to as 'inflammaging' [Franceschi et al., 2007]. Inflammaging is characterised by persistent,

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<sup>5</sup> As a corollary, 'strain specific differences' are a commonly cited reason for experimental variation within animal studies [Murphy et al., 2003].



heightened levels of pro-inflammatory cytokines (IL-6, IL-15, IL-8, IL-1 ) [Franceschi et al., 2005, Giacconi et al., 2004, Wikby et al., 2006, Zanni et al., 2003, Hobbs et al., 1993] and other inflammatory indicators such as coagulatory factors, heat shock proteins and C-Reactive Protein (CRP) [Assunção et al., 2012, Coppola et al., 2003]. Expression of these inflammatory factors is often associated with the process of immunosenescence or immune-dysregulation in the elderly and is regarded as a strong predictor of morbidity and mortality [Schmaltz et al., 2005]. However, it should be noted that several studies of human centenarians have demonstrated that such a phenotype is not always indicative of failing health [Franceschi et al., 2007].

#### **1.4.4 Diet**

The increasing prevalence of chronic inflammatory disorders across the western world has stimulated multiple lines of research into the relationships between dietary habits and inflammatory indices. Commonly linked to increased risk of cardiac and inflammatory disease trans-unsaturated fatty acid (TFA) consumption has been positively correlated with increased circulating markers of inflammation [Lopez-Garcia et al., 2005, Mozaffarian et al., 2004]. Conversely numerous human studies have identified an inverse association between levels of circulating inflammatory markers and consumption of  $\omega$ -3 fatty polyunsaturated fatty acids ( $\omega$ -3 PUFAs) [Micallef et al., 2009, He et al., 2009]. Further, diets high in fibrous foods such as fruits, vegetables and unprocessed grain also show an inverse correlation with inflammatory markers [Holt et al., 2009]. Whereas those high in processed and red meats show the reverse [Fung et al., 2005]. As a corollary diets high in fruits, vegetables and  $\omega$ -3 PUFAs, such as the traditional 'Mediterranean' and 'Japanese' diets are typically associated with decreased morbidity, mortality and inflammatory disease burden [Sofi et al., 2008]. Again a diet high in TFAs, processed foods and low in fibre (loosely termed the 'Western diet') presents the converse [Cordain et al., 2005, Tikellis et al., 2008].

#### **1.4.5 Exercise**

The immunomodulatory effects of physical exercise are well studied (if not yet fully elucidated) with an abundance of evidence correlating regular exercise with a decreased risk of developing

numerous chronic diseases each of which has excessive or dysregulated inflammation as part of their aetiology [Booth et al., 2012].

The influences of physical activity on the immune system are at least in part, indirect. Caused by the increased energy expenditure it places upon the body. An inactive lifestyle typically leads to the accumulation of adipose tissue in mammals. With an excess in adipose tissue comes an accompanying infiltration and accumulation of leukocytes, persistent release of cytokines and the development of low grade chronic inflammation [Beavers et al., 2010]. While a discussion of the inflammatory effects of obesity or heightened adiposity goes beyond the scope of this thesis it is noted that a higher level of body fat in mice and humans typically correlates with a higher risk of inflammatory disease [Beavers et al., 2010].

More recently it has been recognised that acute exercise can also actively regulate the inflammatory response by inducing the release of immunomodulatory mediators from skeletal muscle. Such myokines - as they have been termed - are released upon muscular contraction and exert a diversity of effects, some occurring locally within muscle while others act distally upon other organs and tissues throughout the body. Multiple immune-active factors are released from muscle during exercise, including; IL-1 receptor antagonist (IL-1ra), IL-10 and IL-6 [So et al., 2014]. Of these the most well characterised is IL-6, this focus has stemmed from the observation that IL-6 displays the most marked response to exercise stimuli while also possessing a range of immune effector functions [Petersen and Pedersen, 2005].

During acute exercise muscle contraction stimulates the release of IL-6 from skeletal muscle, increasing levels of the cytokine in circulation by up to 100-fold depending on the intensity and duration of the exercise [Leggate et al., 2010]. Skeletal muscle derived IL-6s' (SM-IL-6) role in exercise appears to be as a regulator of energy metabolism, serving to augment glucose release from the liver and fatty acid release from adipose tissue in order to provide sufficient fuel to meet increased metabolic demand [Pedersen, 2012].

Skeletal muscle is unique in being able to release IL-6 independently of TNF- $\alpha$ , instead being stimulated by the increased intracellular calcium levels that accompany increased muscle contraction. In turn, SM-IL-6 effector function appears unique. In contrast to leukocyte or

adipose produced IL-6, SM-IL-6 is predominantly anti-inflammatory in function, purportedly triggering an anti-inflammatory signalling cascade through antagonistic inhibition of TNF- $\alpha$  and IL-1 $\beta$  [Petersen and Pedersen, 2005]. Further, SM-IL-6 release has been demonstrated to induce the release of the anti-inflammatory cytokine IL-10 [Starkie et al., 2003]. Additionally, exercise induces an acute anti-inflammatory effect by stimulating production of adrenaline and cortisol (production of these hormones is believed to be enhanced by IL-6). Both of these signalling molecules, released by the sympathetic nervous system, have been shown to limit production of inflammatory cytokines in monocytes, neutrophils and macrophages [Fischer, 2006].

Acute exercise is also known to influence leukocyte trafficking and function. The nature of these alterations are very much dependent on the nature and duration of the exercise. At a systemic level exercise stimulates bone marrow haematopoiesis transiently increasing circulating numbers of blood cells and neutrophils [Smith et al., 1996]. Transient increases in levels of inflammatory monocytes have been observed after single, acute bouts of intense exercise in both mice and humans [Gleeson et al., 2011], followed by a rapid return to baseline numbers in the hours following the activity. In addition studies have reported increases in neutrophil priming and phagocytic activity following short bouts of intense exercise [Smith et al., 1996, Giraldo et al., 2009].

However, regular exercise appears to reduce the overall proportion of inflammatory monocytes in circulation at resting state [Timmerman et al., 2008] and subjection of mice to a high intensity exercise regimen has been observed to increase circulating numbers of T-Regulatory cells [Wang et al., 2012]. Several studies have also demonstrated that inflammatory cytokine production by monocytes and Th1 but not Th2 T-cells is inhibited following regular bouts of intense exercise [Starkie et al., 2000].

Further studies in mice and humans have shown that the anti-inflammatory effects of repeat high intensity exercise can become so pronounced as to be immunosuppressive, rendering athletes and mice subject to high intensity running programs more susceptible to infection [Gleeson et al., 2012], a scenario referred to as the 'elite athlete's paradox' [Gleeson et al., 2011].

Mechanisms for this biphasic pro or anti-inflammatory response to moderate and intensive exercise regimens are still being elucidated. However, one recent line of study has demonstrated that acute exercise downregulates expression of TLRs 2 and 4 in male humans and mice [Oliveira and Gleeson, 2010]. A later study also found an accompanying reduction in TLR associated monocyte activation factors (MHC II, CD80, CD86) [Lancaster et al., 2005]. This downregulation in TLRs was found to be cumulative over successive exercise bouts and may highlight one potential mechanism by which exercise induces an anti-inflammatory influence over the host immune response [Lancaster et al., 2005].

### **1.4.6 Psychological Status**

#### **1.4.6.1 Negative Mood States**

A growing body of human and animal studies suggest an interplay between emotional and immunological status. Of this research the majority of work has been directed at the negative mood states of stress, anxiety and depression. Multiple lines of evidence suggest that anxiety can induce a pro-inflammatory response in humans and animals. Several cohort studies on stressed or anxious individuals have revealed elevated levels of pro-inflammatory mediators; CRP, IL-6, TNF- $\alpha$  and heightened white blood cell counts [Duivis et al., 2013, Pitsavos et al., 2006, Vogelzangs et al., 2013]. Further studies have identified stress to be a significant instigating or exacerbating factor in multiple inflammatory disorders including: IBD [Mawdsley and Rampton, 2005]; dermatitis [Arndt et al., 2008]; rheumatoid arthritis (RA) [Parker et al., 1995] and asthma [Wright et al., 1998]. Persistent psycho-social stress appears to have a diminishing or deregulating effect on the inflammatory response. In a study comparing rates of wound healing among students, recovery time during the stressful exam period was on average three days slower than during summer vacation. This reduction was accompanied with a reported 68% increase in inflammatory IL- $\beta$  production [Marucha et al., 1998].

In addition, over a third of patients suffering major depressive disorder (MDD) exhibit elevated levels of IL-6, TNF- $\alpha$  and CRP when compared to non-depressed patients. These elevated levels occur in the absence of physical illness or infection [Dinan, 2009, Dowlati et al., 2010, Raison and Miller, 2011, Dantzer et al., 2008]. In turn individuals suffering depression are at

greater risk of suffering disorders with a strong inflammatory aetiology such as atherosclerosis and heart disease [Duivis et al., 2014]. It is interesting to note that these patient often present a depressed cellular and humoral response to infection [Fortes et al., 2002], an overall immune state with parallels to the immunosenescence observed in 'inflammaging'.

Research from animal models of mood and behaviour has helped to further elucidate the relationship between negative emotional states and inflammation. Studies using the repeat social defeat (RSD) behavioural paradigm (a model of social stress in which mice are recurrently attacked and defeated in their home cage by an aggressive conspecific animal) have revealed acute stress to correlate with heightened numbers of myeloid cells (monocytes and granulocytes) in blood circulation [Wohleb et al., 2015]. In turn this increased cycling of myeloid cells in the context of psychological stress has been associated with the development of an inflammatory Ly6C<sup>high</sup> monocyte phenotype in mice [Engler et al., 2004, Powell et al., 2013] (and a synonymous CD14+/CD16- phenotype in human cells [Heidt et al., 2014]).

Accompanying these cellular changes one paper reports the upregulation of inflammatory gene expression in the leukocyte transcriptome [Powell et al., 2013]. Additional studies combining the maternal separation of rat pups (another established stress induction paradigm [Lehmann et al., 1999]) with an OVA-induced model of asthma revealed separated rats present a notably more severe inflammatory response compared to control animals [Kruschinski et al., 2008]

#### **1.4.6.2 Stress Mitigation and Positive Mood States**

Recent estimates by the World Health Organization suggest that by 2030 depression and stress related syndromes will be the most debilitating and widespread health disorders on the planet, closely followed by auto-immune disease and allergy [Mathers et al., 2008]. Such figures have initiated a huge growth in research directed at investigating the effects stress mitigating therapies have on immune state. A study of 50 individuals revealed participants to have lower circulating levels of IL-6 following six weeks of training in compassion meditation, compared to the control group [Pace et al., 2009]. In a further study 49 participants were subject to the Trier Social Stress Test (TSST) (an experimental paradigm intended to induce stress), after which a

local, inflammatory response was induced in each patient's forearm and the resultant exudate extracted. Following this the participants underwent an eight-week regimen of mindfulness-based meditation at the end of which they were subjected to the same TSST and inflammation paradigm once again. Analysis revealed significantly lower levels of inflammatory cytokines IL-8 and TNF- $\alpha$  in the exudates of participants following the eight weeks meditation in addition to lower circulating cortisol levels [Rosenkranz et al., 2013].

More recently several lines of research have begun investigating the influence positive mood states may have on the inflammatory response. Studies have shown that laughing therapy (in which at its most simple subjects are made to watch humorous films) upregulates the expression of genes involved in the NK cell inflammatory immune response, such as granzymes H and B, perforin, cathepsin and granulysin [Hayashi and Murakami, 2009, Hayashi et al., 2007]. Similar effects on NK cells have been described in investigations where laughter was also shown to significantly decrease levels of circulating pro-inflammatory cytokines in patients suffering rheumatoid arthritis [Matsuzaki et al., 2006].

Research modelling the influence of positive emotion on the inflammatory immune state in animals is currently limited. Perhaps due to the questions raised around equivalence of animal-human psychology. Nevertheless, one aspect of animal welfare that has shown a small but consistent outflow of research is that of environmental enrichment and its physiological effects on rodents.

## 1.5 Environmental Enrichment

Environmental enrichment (EE) or behavioural enrichment is an umbrella term for a large range of practises and principles that seek to enhance the quality of life for captive animals by providing environmental stimuli that improve both their physical and psychological wellbeing. Generally the goal of environmental enrichment is to encourage 'normal'/'natural' species specific behaviour while reducing or preventing abnormal behaviours such as stereotypies (e.g. pacing or excessive grooming) [Young, 2003].

The practise of EE has been commonplace in the care of captive animals for close to a century [Mellen and Sevenich MacPhee, 2001], however its use on lab animals only became established in the late 1960's [Mellen and Sevenich MacPhee, 2001]. While its purpose in zoos and similar establishments focuses solely on improving the welfare of the animals, for researchers EE began primarily as an experimental variable in exploring the relationship between an organism's neurological makeup and its external environment [Hutchinson et al., 2005]. Initial work in the field focused on the behavioural effects of enrichment, demonstrating its capacity to enhance cognition and memory in a series of learning based tasks [de Jong et al., 2000]. Later studies further revealed the effects of EE at a cellular level, identifying its effects on gene expression [Rampon et al., 2000a], neuronal development [Kempermann et al., 1997] and synaptic plasticity [Nakamura et al., 1999]. Additional studies have discovered that EE is able to mitigate or facilitate repair of neurological damage caused by chemical exposure [Guilarte et al., 2003], physical trauma [Xerri and Zennou-Azogui, 2003] and neurodegenerative disease [Jankowsky et al., 2005].

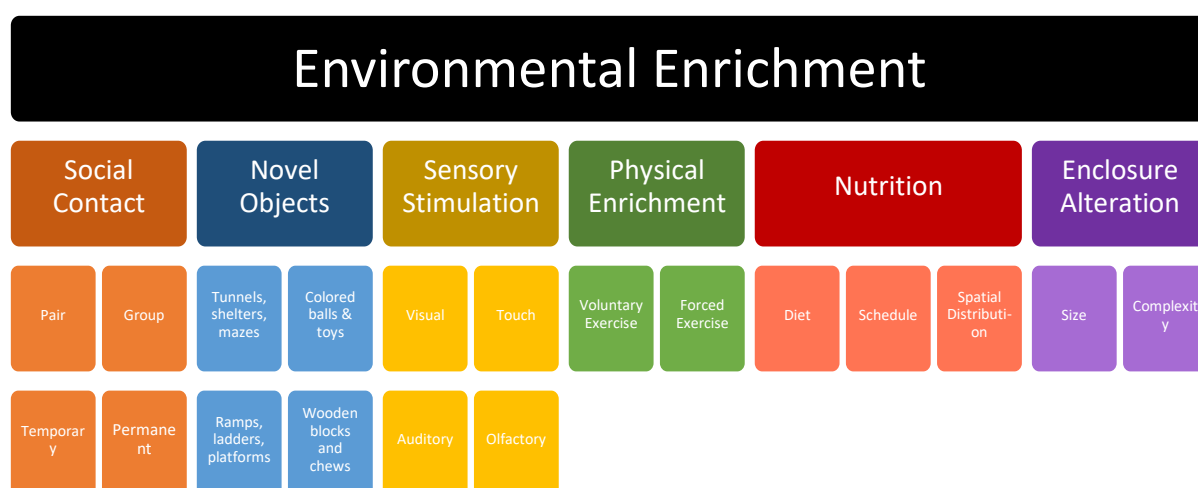
While the majority of EE paradigms use rodents for research a number of other animals such as; birds [Reed et al., 1993], rabbits [Olson et al., 2006], pigs [Beattie et al., 2000], primates [Schneider et al., 1991] and spiders [Bengston et al., 2014] have also been used in EE experiments.

Parallel EE treatment paradigms can be found in clinical studies. While not operationally similar such treatments provide a degree of mental and physical stimulation comparable to that of rodent EE studies. Such treatments include learning of complex tasks, an active social lifestyle

[Wright et al., 1998], cognitive ('brain') training exercises [Valenzuela and Sachdev, 2009], sensory enhancement (e.g. listening to music) [Núñez et al., 2002], food supplementation [Holt et al., 2009] and physical exercise [Siedlik et al., 2015]. These treatments have been shown to produce comparable results to rodent studies, improving cognition and memory suggesting a similar mechanism of action for both humans and animals.

### 1.5.1 Enrichment Methods

While the vast majority of studies have focused on the neurological effects of EE a small number of papers have begun identifying the modulating effects of EE on the immune system utilising a variety of enrichment techniques: Across each of these studies the specific method of EE varies considerably using one or a combination of methods summarised in Figure 1-6 below.



**Figure 1-6 Methods of enriching the environment for rodents in captivity.** EE for rodents in captivity can be achieved by providing them with social contact (pair, group, permanent, temporary), physical activity using running wheel, new larger and complex enclosures, novel objects and accessories, sensory stimulations (visual, auditory, and olfactory), and better nutrition.

#### 1.5.1.1 Novel Objects

Experimental animal's environments can be enriched by inclusion of various novel structures (e.g. mazes, shelters, tubes and additional floors) and accessories (ropes, ladders, chews, ramps, wheels.) To stimulate their attention and engagement with their housing environment. A study on aged rodents housed in an environment enriched with novel objects revealed a significant increase in IL-2 and TNF- $\alpha$  release from leukocytes extracted from the peritoneum of enriched animals. The authors suggested this increased cytokine release could compensate for the age related loss of these cytokines [Arranz et al., 2010]. However, the methodology behind



this study has been questioned. Unlike other EE studies of its kind where the novel objects placed within the environment remained unchanged, this study altered the objects used every 48 hours. It has been suggested that rapid and regular interference with their environment would serve as a source of stress for the mice, confounding the results obtained [Singhal et al., 2014].

In a further study mice that spent four months in a novel object and physically enriched environment displayed a significant reduction in brain IL- $\beta$ , IL-6 and TNF- $\alpha$ , following infection with the influenza type A, when compared to animals housed in a standard environment [Jurgens and Johnson, 2012]. However, it should be noted the study was unable to determine whether the primary source of these immune alterations came from the novel objects, increased physical activity or a combination of the two.

Adolescent rats born of mothers' subject to restraint stress showed a comparative increase in IL-2 and reduction in IL-1 $\beta$  production by activated splenocytes, when housed in the presence of novel objects one-week post birth. The same animals also showed a heightened resistance to the immune-suppressive effects of cyclophosphamide when compared to rats born of stressed pregnancies but housed in a standard environment [Laviola et al., 2004]. Without the addition of a non-stressed, non-enriched control in this study it is difficult to establish the extent of EE's influence on the immune response. Regardless these initial results do provide support to the concept that EE by novel objects may contribute to better immune homeostasis.

#### **1.5.1.2 Physical Exercise**

As previously discussed (1.4.5) multiple studies have reported physical exercise (PE) to have anti-inflammatory effects, most especially in the context of diseases and metabolic disorders associated with chronic low-grade systemic inflammation. Regular voluntary exercise is generally regarded as one of the most consistent means of enhancing the welfare and environment of both humans and captive animals, correspondingly it is the most well studied and utilised method of environmental enrichment used in studies.

### 1.5.1.3 Social Enrichment

Social housing of captive animals is a typical component of most animal research units and scientific studies for reasons of welfare and economy. Mice are characteristically social animals with studies demonstrating that male mice prefer to sleep in close proximity to familiar mice [van Loo et al., 2001] while mice denied social contact show increased anxiety, depression like behaviour and aggression when reintegrated with other animals [Ma et al., 2011].

Behavioural studies of laboratory rodents have revealed their stratification into two key populations within a housed group: 'subordinate' and 'dominant', each with separate behavioural, physiological and immunological profiles [Bartolomucci, 2007]. Submissive animals, especially those of low social rank have been reported to show signs of immunosuppression with a reduction in splenocyte and lymphocyte proliferation, serum antibodies and increased levels of circulating IL-4 and IL-10 [Fleshner et al., 1989, Bartolomucci, 2007]. In a further study two hours of social confrontation led to increased numbers of granulocytes and an accompanying decrease in lymphocyte numbers and elevated CD4/CD8 and T-cell/B-cell ratio in defeated animals [Stefanski and Engler, 1998]. Suggesting that immunosuppression in subordinate animals is caused by the impairment of both humoral and cellular immunity, possibly as a result of prolonged stress. Concomitant to these results dominant male rodents typically show better immune function compared to other members of their cage group while also showing reduced indicators of sickness behaviour when subject to immune challenge [Bartolomucci et al., 2001]. The development of such social hierarchies poses a serious risk of confounding any results obtained from EE paradigms the core goal of which is typically to enhance as opposed to impoverish an animal's external environment<sup>6</sup>. However, it has been reported that mice housed together following the stress of social defeat show improvements in behaviour significantly more rapidly than animals placed in isolation [de Jong et al., 2005]. A similar study in rats revealed that social housing after social defeat reverses the reduction in heart rate, temperature and locomotor activity caused by the

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<sup>6</sup> It could be argued here – and in other incidences where enrichment practises risk increased aggression between captive animals – that such behaviour is typical behaviour within all social groups and as such constitutes the 'natural' behaviours that enrichment regimens seek to evoke. This of course begs the question, how much aggression is too much?

experience [Ruis et al., 1999]. Further it has been shown that when housed together, undisturbed for a long enough period of time incidences of inter-group fighting among mice typically fall to very low levels, most especially if the animals were weaned together [Gurfein et al., 2012b]. Accordingly, while conflict is a definite risk factor when socially housing experimental animals, provided this conflict is kept to a minimum; such social enrichment is likely to be of benefit to them.

#### **1.5.1.4 Sensory Enrichment**

The effects of sensory enrichment on the function of sensory organs (visual, auditory, touch and olfactory) and brain activity has been the subject of much study and review in the field of behavioural biology and captive animal research [Wells, 2009]. To date no studies have specifically investigated the immunomodulatory influence of sensory stimulation in the context of environmental enrichment. However, studies have established that various sensory stimuli can alter immune function. C57/BL6 mice subject to daily stroking for a period of eight days demonstrated a significant nonspecific increase in thymic and splenic T-cells numbers compared to un-stroked animals [Major et al., 2015]. Enhancement of systemic T-cell proliferation has also been observed in response to visible light passing through the eye and hitting the skin [Roberts, 2000]. Randomised trials which subjected adult participants to a slide show portraying images of disease or a series of neutral, control images revealed that the visual perception of disease symptoms is capable of stimulating heightened release of IL-6 into circulation [Schaller et al., 2010].

Several studies report the influence of auditory stimulation on the immune system. Rats played music for five hours a day, for a period of eight days were shown to present an enhanced immune and anti-tumour response compared to control animals and rats subject to auditory stress [Núñez et al., 2002]. Human studies utilising age and gender matched human volunteers has shown that group drumming therapy may enhance cellular immunity by increasing lymphocyte activated NK cell activity [Bittman et al., 2001]. Again the question may be asked if it is the actual process of drumming causing these immune alterations or a neuro-endocrine response to a novel social situation.

Immune mechanisms associated with olfactory stimulation used as an enrichment technique are yet to be studied. However a known relationship exists between olfaction and immunity, with predator odour known to stimulate the Hypothalamic pituitary adrenal (HPA) axis and proceeding immune responses in rodents [Strous and Shoenfeld, 2006]. Instinctual responses to many smells are demonstrated to be mediated by the MHC region of the mammalian genome [Singh et al., 1987]. Accordingly, it seems logical to assume that the olfactory environment humans and animals reside in will have some influence (direct or indirect) on their immune status.

#### **1.5.1.5 Nutritional Enrichment**

While some researchers have advocated the use of food as a means to enrich a captive animals' environment [Sherwin, 2007a, Brown, 2009], in an experimental setting the behavioural and physiological alterations a variation in diet can cause pose a high risk of confounding the effects of the enrichment itself, a brief overview of the effect of diet on inflammatory state is given in section 1.4.4.

## 1.6 Aims and Hypothesis

Currently research investigating the modulating effect EE may have upon the immune response remains in its infancy. A review of those studies that have been carried out (Table 7) presents enriched rodents as possessing heightened innate leukocyte activity coupled with reduced levels of circulating inflammatory cytokines and lymphocytes. Such an unusual immune profile would appear likely to promote an equally unique inflammatory response. However, as of yet no direct research has investigated the effects of environmental enrichment on classical experimental models of inflammation, elucidating these effects is the focus of this thesis.

### Hypothesis:

**Enrichment of a mouse's captive environment will have a distinct and experimentally observable influence on that animal's inflammatory immune response.**

### Aims:

1. To establish a practicable and reproducible enrichment methodology that evokes an altered behavioural and physiological response in mice.
2. To observe any immune alterations enrichment may cause by subjecting animals to established models of disease that evoke specific aspects of the inflammatory response.
3. To investigate any specific cellular/molecular immune alterations enrichment may cause.
4. To determine from the results obtained whether environmental enrichment is of benefit to an organism in the context of its immune-fitness.

**Table 7 Effects of environmental enrichment on cytokine levels and other immune parameters.**

<b>Enrichment Methodology</b>	<b>Strain/Species Used</b>	<b>Immunological Findings</b>	<b>Cortisol level respective to control</b>	<b>Study</b>
Cycled addition of two novel objects to cage every two days. Opaque shelter maintained in cage	Female ICR/CD1 Mice (multiple ages)	↑ Macrophage and NK cell chemotaxis, phagocytosis and ROS production ↑ Increased induced Cellular IL2 and TNF-α production ↓ Lymphocyte TLR2, TLR4 membrane expression (in aged mice)	-	[Arranz et al., 2010]
Addition of ladders, tunnels and running wheels to a large enclosure	Male C3H/eB mice. Four weeks old	↑ NK cell cyto-toxicity	Higher	[Benaroya-Milshtein et al., 2004]
Weekly addition of a plastic hut, compressed cotton nest pad, and two plastic ball	Female Balb/c mice three weeks old	↓ CD4+ and CD8+ thymocytes	Lower	[Hutchinson et al., 2012]
Social interaction (n=5–8 mice in the cage), toys and a set of tunnels, shelters, nesting materials	Male Balb/c Mice six weeks old	↓ Inflammatory cytokine production (IL-6, IL-1 β, TNF-α) in response to influenza infection ↑ Anti-inflammatory factors DNF, CXCL1) ↑ Viral clearance speed	-	[Jurgens and Johnson, 2012]
Swimming training and exercise	Male Sprague Dawley Rats	↓ T-Helper cells ↑ T-regulatory Cells ↑ IgG and IgM production	-	[Kaufman et al., 1994]
Larger enclosure, with multiple novel objects and a wheel	Male/Female Sprague Dawley rats. Three weeks	↑ IL-2 ↓ IL-1β Resistance to CPA-immunosuppression	-	[Laviola et al., 2004]
Standard cages with wooden scaffolding (enrichment) or a spacious glass enclosure with multiple plains, paths, ropes and food locations (super-enrichment)	Congenic strain CS (derived from an inbred ABG strain) Mice	↑ IgG2a ↓ IFN-γ/IL-10 and IL-2/IL-10 ratios	Significantly Higher	[Marashi et al., 2003]
Multiple running wheels, shelters, tunnels and two cotton nestlets. Enrichment was followed by a social stress inducing paradigm	male BALB/cByJ mice, aged six weeks	↓ IL-6 and IL-1 Receptor expression ↓ IL-1 β	Lower	[McQuaid et al., 2013]
A running wheel, tubes, multiple small toys	Adult Male Sprague Dawley Rats	↓ Hippocampal expression of TNF-α, IL-1β ↓ Hippocampal Expression of Cc12, Cc13 and Cxc12	-	[Williamson et al., 2012]
↑ - Increased/upregulated. ↓ - Decreased/down-regulated. BDNF- Brain Derived Neurotropic Factor. Ig – Immuno-globulin				

## Chapter 2 Methods

### 2.1 Animal Breeding and Husbandry

Adult male CD1 mice (acquired at six weeks old, 15-20g body weight) were obtained from Charles River UK Ltd. Animals were kept under standard conditions: in individually ventilated enclosures, food and water provided ad libitum, 12-hour light-dark cycle. All animals were allowed a seven-day acclimatisation period before any experimental procedure was performed upon them. All experiments were approved and performed under the guidelines of the Ethical Committee for the Use of Animals, Bart's and The London School of Medicine and Home Office Regulations [Act 1986]. PPL 70/8714. PIL-30/9411.

### 2.2 Environmental Enrichment

Environmental enrichment (EE) consists of a variety of different principles and methodologies. As can be seen in Table 8 both the recommended forms of enrichment and practised enrichment protocols vary widely across labs and institutions. As an example in one study researchers regularly changed their experimental cage environment as part of their EE protocol. "Rearrangement of toys, tunnels, and wheels was carried out 3–4 times per week to maintain novelty" [Jurgens and Johnson 2012]. Conversely other groups would regard this practise as a definitive way of inducing persistent 'non-natural' stressors into a mouse group. "The cage environment was left unaltered throughout the course to minimise animal stress" [Van de Weerd, Baumans et al. 1994, Baumans 2005, Willner 2005].

While the issues around this lack of methodological consistency and the variability of results have been acknowledged [Toth, Kregel et al. 2011] and attempts have been made to generate a EE 'standard operating procedure' [Sztainberg and Chen 2010], as of yet no single approved method has been agreed upon.

In an attempt to devise an enrichment protocol that would provide both a consistent and measurable psychological and physiological (and ergo immunological) effect, a review was carried out to determine the most consistent EE techniques in use. Table 8 lists those techniques. The most common, effective and practicable cage additions and procedures used across this previous research were chosen for our devised enrichment protocol

**Table 8 Common enrichment techniques**

Type	Action	Study
Social	Forming cage groups with weanlings that know each other	[Sherwin, 2002, Sztainberg and Chen, 2010]
	Same sex groups set up before puberty	[Sherwin, 2007b]
	Avoidance of changing, removing members of social cage groups	[Sztainberg and Chen, 2010]
Human/Animal	Systematic, gentle, daily handling	[NSWAARRP, 2004, van de Weerd and Baumans, 1995]
	‘Gentling’ – mouse is stroked and allowed to explore hand	[NSWAARRP, 2004]
	Allow animals to become used to procedures, involve positive reinforcement	[Hubrecht et al., 1993]
Food	Scatter or hide food to encourage foraging	[Gurfein et al., 2012a, Sztainberg and Chen, 2010, van de Weerd and Baumans, 1995]
	Scatter seed on top of the pelleted food. Animals learn to manipulate the pellets to access the seeds that fall through. Delivery of the seed is random, but foraging is controlled by the mouse	[Hutchinson et al., 2005]
	Provision of hard-shelled nuts (e.g. walnuts, hazelnuts, macadamia nuts), encouraging physical effort to obtain food	[Hutchinson et al., 2005]
	Variation of food type	[Sherwin, 2007b]
Physical Environment	Inclusion of dividers in cage (provision for thigmotaxis).	[NSWAARRP, 2004, Boyd and Love, 1995, Committee, 2004, Sherwin, 2002, Sztainberg and Chen, 2010, van de Weerd and Baumans, 1995]
	Enlarge the floor space by providing levels within the cage or by providing items to climb on or exercise in	[Sherwin, 2007b, van de Weerd and Baumans, 1995]
	Use of a solid bottomed cage (as opposed to a mesh or grate)	[Committee, 2004, Sherwin, 2002, Sherwin, 2007b]
	Provision of a running wheel	[Gurfein et al., 2012a, Sherwin, 2007a, Sztainberg and Chen, 2010]
	Provision of toys – ropes, ladders, balls, chews	[Hutchinson et al., 2005, Sztainberg and Chen, 2010, Smith and Hargaden, 2001]
	White, opaque cage walls	[Sherwin, 2007a]
	Opaque (or transparent red) nest box, with gaps to allow olfaction	[Sherwin, 2007a]
	Provision of multiple exits in any enclosed space	[Gurfein et al., 2012a]



Type	Action	Study
Olfactory Stimulation	Male aggression often follows box cleaning through disruption of scent marking. Placing nesting material from dirty box into clean may reduce this.	[van Loo et al., 2001]
	Avoid presence of strong scents, or predator scents in mouse enclosure	[Sherwin, 2002]
	Addition of shredded paper to reduce olfactory load	[Hubrecht et al., 1993]
Provision for natural Behaviour	Minimise or attempt to block out extraneous noise in mouse enclosure, be aware of potential for high frequency noise through electrical equipment. Ensure correct day night cycle for mice	[Sherwin, 2007b, Sherwin, 2002]
	Provision of nesting material – preferably shredded paper	[NSWAARRP, 2004, Sherwin, 2007a, Sherwin, 2002, Sztainberg and Chen, 2010]
	Provision of nesting boxes	[Gurfein et al., 2012a]
	Provide substrate specifically for defecation – preferably wood shavings or sawdust	[Sherwin, 2007b]
	Provision of a thick layer of substrate to encourage digging	[Sherwin, 2007b, Sherwin, 2002]
	Provision of objects to chew and climb	[Hutchinson et al., 2005, Smith and Hargaden, 2001, Sztainberg and Chen, 2010]

The devised enrichment protocol was as follows:

Same sex, male, CD1 mice aged six weeks and weaned together were given one week to acclimatise to the environment of the animal unit in a standard enclosure used in the unit. Such an enclosure consisted of a 36x20x14cm (523 square cm) cage (Allentown) cage, roughly a 1cm depth of woodchip bedding and roughly 25g of nesting material. Six animals were housed per enclosure. At the end of the week each enclosure group was either placed in a new standard enclosure and designated a standard environment (SE) group or placed in an enrichment enclosure and designated as an environmentally enriched (EE) group.

Enrichment enclosures were 50x38x21cm (1355 square cm, Allentown) (Figure 2-1) and included:

- One wheel, (silent spinner, Kaytee)
- One nest house, (Puzzle playground, Superpet)
- One tunnel
- One chew block
- Ample nesting material (50g)
- Woodchip bedding to a 5cm depth.

Mice from both groups were cleaned out once a week. While cleaning occurred enriched mice were allowed to remain inside their nest house to minimise disturbance <sup>7</sup>. Roughly 20% of soiled bedding was left in the enrichment cage. After cleaning all apparatus was replaced exactly as found. Prior to cleaning/handling/weighing etc. Mice were allowed a period of roughly ten minutes to acclimatise to the presence of the researcher. No other researchers were allowed to handle the mice.

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<sup>7</sup> Upon introduction to the enrichment enclosure, mouse groups would not always choose the nest house as their primary nesting site. This was likely due to the structure being an unfamiliar - and ergo abject - object for these typically neo-phobic animals. It was discovered that placing nesting material from their previous enclosure inside the nest house ensured the mice would take up permanent residence inside it.



**Figure 2-1. Standard (left) vs Enriched (right) enclosures.**

## **2.3 Body Fat and Muscle Analysis**

Following enrichment sacrificed animals were subject to a laparotomy and had their visceral fat pads excised and stored in neutral buffered formalin (NBF). In addition, the left leg of each animal was removed and stored in NBF.

## **2.4 Blood Analysis**

Following terminal anaesthesia through isoflurane inhalation blood samples were taken from mice via cardiac puncture. In this procedure a 1ml syringe, with a ¼ inch 25G needle, pre-filled with 150µl of a 3% solution of sodium citrate was inserted into the ventricle of the heart via the left side of the chest, between the ribs. Application of gentle negative pressure via the syringe allowed for recovery of between 0.25-1ml of blood dependent on the size of the mouse the strength of its heart beat. Following blood collection mice were sacrificed via CO<sub>2</sub> exposure.

Isolated blood was then subject to haematological analysis using a IDEXX ProCyt Dx<sup>®</sup> Haematology Analyser. A tool that utilises laser based flow cytometry, optical fluorescence and measurement of impedance to laminar flow to produce a readout of relative numbers and percentages of monocytes, lymphocytes, neutrophils and eosinophils and erythrocytes as well as large range of other circulating blood factors.

## 2.5 Bone Marrow Cell Extraction

Complete legs (without skin) were removed from sacrificed mice and stored in RPMI media (GIBCO/Invitrogen) + 30% EtOH at 4°C. When ready for processing (a maximum of four hours post sacrifice) a leg was removed from the media using tweezers, muscle and connective tissues was carefully pared off the tibia using a scalpel until the bone was completely exposed, while still being connected to the femur and the foot. Following this the fibula was dissected from the tibia by placing the scalpel between the two bones at the top of the foot paw and drawing it up past the knee joint. If performed correctly this allowed the patella to be carefully peeled from the top of the tibia, cleanly separating the two leg bones and helping ensure a consistent amount of bone was removed from each animal. The foot was then removed from the bottom of the tibia using surgical scissors. Bending of the foot back toward the tibia allowed easier location of the region where cartilage connects the two bone sets. Placing surgical scissors at the apex of this bend and careful manipulation to ensure the blade passes through the regions of least resistance helped ensure consistency in the amount of bone tissue acquired for extraction. Using a gauze, the base of the tibia was gently polished to remove any adhering tissue and then cut ~1mm from the tip with surgical scissors to expose the marrow at both ends. The basal end of the tibia was then gripped gently using locking forceps and held over a small (4cm diameter) bacterial culture plate, with the wider part of the bone facing down. A 2.5ml syringe with a 10mm 26G needle, prefilled with 2ml of sterile PBS (phosphate buffered saline) + 3mM EDTA was inserted into the opening at the narrow end of the tibia and gently pushed roughly 5mm deep using a screwing motion. Gentle pressure was used to flush the PBS EDTA solution through the bone, releasing its contents into the culture plate below. Successful clearance of the bone was identified by a colour shift from pink to white across the tibia's length. The collected cell solution was taken from the plate using a Pasteur pipette and moved to a 15ml falcon tube. The plate was washed with an additional 1ml of PBS EDTA which was also added to the tube. The collate was then centrifuged (1500 RPM, 5 minutes, 4°C). The resulting pellet was reconstituted in 1ml of PBS-3mm EDTA in preparation for further analysis.

## 2.6 Zymosan Induced Peritonitis

Zymosan induced peritonitis (ZIP) is an experimental model that uses application of the eponymous yeast cell wall derived polysaccharide to induce an acute inflammatory response in the peritoneal cavity of a test animal. In our procedure male CD1 mice from both the EE and SE groups were injected intraperitoneally (ip) with 500µl of PBS containing 0.5mg of zymosan-A (Sigma-Aldrich). Using a 500µl, 29G mycojector insulin syringe (Terumo). Following injection animals were observed for sickness behaviour indicative of peritoneal inflammation (lethargy, piloerection, closed eyes). If any animals had displayed sickness symptoms in excess of the observed norms for ZIP (respiratory issues, nasal discharge, vocalisation) they would have immediately been culled by a schedule one procedure. Mice were then left for either 6 or 24 hours, following which they were sacrificed.

## 2.7 Peritoneal Lavage

Placed on their back mice were sprayed liberally with 70% EtOH focused around the abdominal area. Using scissors, a small cut was made in the abdominal skin of the animal just below the diaphragm. The fur either side of the incision was then held firmly and pulled towards the cranial and caudal ends of the mouse, revealing the hypodermis. Using forceps, a region of the peritoneum roughly 10mm below the diaphragm was lifted up and away from the abdominal organs and used as the injection point for 2.5ml of PBS+3mM EDTA at 4°C. (Injection carried out using a 2.5ml syringe fitted with a ½ inch 25G needle). The abdominal cavity of the mouse was gently massaged to ensure suspension of the peritoneal cells within. Again using forceps to lift the peritoneum at its centre point a small lateral incision was made into the cavity using surgical scissors. Through this cut was inserted the tip of a Pasteur pipette which was used to draw the lavage fluid out of the peritoneal cavity. On average around 2ml of fluid was recovered. Samples were then centrifuged (1500 RPM, 5 minutes, 4°C), the supernatant was retained and stored at -80°C. The resultant pellet was reconstituted in 1ml of PBD-3mm EDTA and kept on ice in preparation for further analysis.

On occasion the lavage taken from animals would be contaminated with blood, likely a result of the cardiac puncture they had previously received or inadvertent damage to the organs/tissues during the lavage procedure. Such samples were excluded from any later analysis.

## 2.8 Cell Count

Both bone marrow and peritoneal cell counts were carried out using a haemocytometer (Improved Neubauer). Prior to counting samples were diluted to a factor that permitted visualisation of between 10-100 cells per quadrant of the haemocytometer. In the case of peritoneal cells this necessitated a 10x dilution for the six-hour time point and a 15x for 24 hours. For bone marrow a 25-50x dilution was required.

Following dilution 10µl of cell suspension was added to a further 10µl of trypan blue. 10µl of this mix was pipetted between the haemocytometer and cover-slip. Viewed at 20x magnification under a light microscope cells in four, 4x4 quadrants were counted row by row. Dead cells, evidenced by trypan blue infiltrate into the nucleus were not counted.

The total cell count per ml (and per mouse) was calculated as:

$$\text{Cell count} \times 10^4 \text{ (depth of haemocytometer well)} \times \text{Dilution factor} / \text{four}$$

## 2.9 Cytokine Analysis

Cytokine analysis was carried using a Luminex®- BioPlex Pro® Assay (Life Technologies). A bead based multiplex assay used to quantify levels of KC, IL-1β, TNF-α, IL-6 and MCP-1 in peritoneal lavage fluid. The protocol was carried out in accordance to the manufacturer's guidelines (Invitrogen Manual LCH0001). Briefly, cytokine specific, antibody coated beads were vortexed and added across a 96 well plate. The plate was then inserted into a magnetic cradle that retained the magnetic beads within the plate wells while allowing them to be stringently washed. 50µl of analyte (diluted 20x) was added to the wells including appropriate, blanks, standards and controls. The plate was then sealed, shaken at 1100rpm for 30 seconds before being incubated for 30minutes while shaken at 300rpm. Following three further wash cycles streptavidin was added to each well and the plate was subject to the same process of shaking, incubation and washing once again. 100µl of assay buffer was then added to each well before the plate was read and analysed by the Luminex proprietary Bio-Plex® MAGPIX™ reader system.

## 2.10 Flow Cytometry – Procedures and Analysis

### 2.10.1 General Cell Staining Protocol for Flow Cytometry

Re-suspended peritoneal cell samples were transferred to a series of micro-test tubes (starlabs) fixed into a round bottomed 96 well plate. A small aliquot (50-200µl dependent on the number of stains carried out) was taken from each sample and added to a single tube for use in control stains. The tubes were then centrifuged at 1500rpm (or equivalent) 4°C for five minutes. Maximum acceleration and deceleration. The tubes were then emptied with care taken not to dislodge the pelleted cells and 100µl of Anti-mouse FCGRII/III (Clone 2.4G2) blocking antibody (BD Biosciences) 500x diluted was used to re-suspend each pellet. Samples were then left to incubate for a minimum of ten minutes at 4°C. Following another cycle of centrifugation and removal of supernatant the cell pellets were re-suspended in 100ul of (fluorescence activated cell sorting) FACS buffer and stained with the appropriate antibody mix (listed below). Following a minimum of 30 minutes incubation (in darkness 4°C) each sample was fixed by addition of 100µl 10% paraformaldehyde (PFA) and made up to 400µl with FACS buffer in preparation for analysis. If analysis wasn't carried out immediately samples were wrapped in foil and stored at 4°C to reduce photophore degradation.

### 2.10.2 Cell Differentiation Markers Used

**GR-1 (RB6-8C5) clone, eBioscience**), also known as the myeloid differentiation antigen GR-1 is expressed by cells of the myeloid lineage in a developmentally regulated manner in the bone marrow. Monocytes express GR-1 transiently during bone marrow development, its presence is also found on peripheral neutrophils making it a commonly used marker for these populations. [Daley, Thomay et al. 2008, Wang, Bair et al. 2012].

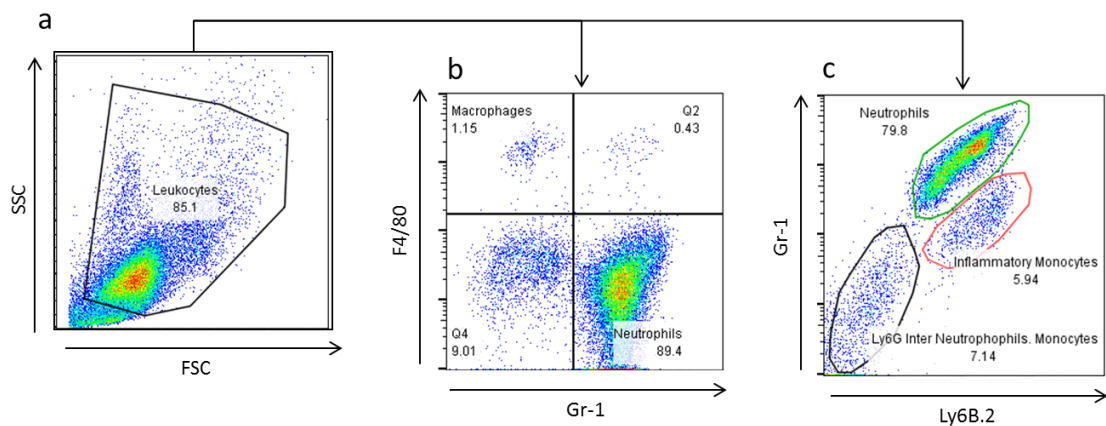
**F4/80 (BM8 clone eEioscience)** antigen is expressed at high levels on the surface of various macrophages: Kupffer cells, splenic red pulp macrophages, microglia, gut lamina propria, and Langerhans cells in the skin. F4/80 is also expressed in the macrophages of the connective tissue, heart, kidney, reproductive, and neuroendocrine systems [Leenen, de Bruijn et al. 1994].

**Ly-6B.2 (7/4 clone eBioscience)** expressed on neutrophils, inflammatory monocytes and some activated macrophages high levels of expression are also seen in bone marrow, spleen, lung and lymph nodes [Rosas, Thomas et al. 2010].

### 2.10.3 FACS Acquisition

FACS acquisition was carried out using a BD LSRFortessa II™. 20,000 events were acquired per sample (where possible). For each staining experiment a forward versus side scatter plot (FSC-SSC) was initially gated to remove all events at the extremes of each axis, FSC-Area versus FSC height plots were also used in order to exclude irregular shaped material and cell aggregates from the final data set (Figure 2-2). Single stain controls were used both for compensation and to determine relative marker expression levels (positive, negative. Low, high). Posthumous analysis was carried out using the FlowJo™ software package. Cell population counts within a cavity (bone or peritoneum) were calculated by multiplying:

Percentage cell in a given gate x total cell count = Relative number of gated cells in cavity



**Figure 2-2.** Basic gating logic used in FACS analysis. (a) SSC-FSC gating, (b) Differential gating of F4/80<sup>hi</sup>, GR-1<sup>-</sup> populations, demarcated as macrophages and F4/80<sup>-</sup> GR-1<sup>hi</sup> populations demarcated as neutrophils. (c) Differential gating of GR-1<sup>hi</sup>, Ly6B.2<sup>+</sup> populations demarcated as neutrophils and GR-1<sup>+</sup>, Ly6B.2<sup>+</sup> populations demarcated as activated/inflammatory monocytes. Gating logic based upon [Davies et al., 2013].



## 2.11 Animal Behavioural Assessments

### 2.11.1 Open Field Test

The open field test (OFT) is an ethologically based paradigm that provides objective measures of exploratory behaviour as well as a useful initial screen for anxiety-related behaviour in rodents. The apparatus used consisted of a white PVC arena (50cm × 30cm × 20cm) divided into 15, 10cm × 10cm squares. The three central squares were designated the “centre” region. Experimental animals were placed in the bottom left corner square (relative to the experimenter), facing the wall and observed and filmed for three minutes. The walls and floor of the arena were thoroughly cleaned between each trial using 70% ethanol and then dried. In order to ensure parity test subjects were alternated between SE and EE groups (e.g. mouse S (standard) 1 was recorded first, followed by E (enriched) 1, then S2, E2 etc). This test was performed in accordance to a published methodology previously used by the lab [Gallo et al., 2014].

Three key parameters were recorded: The total number of squares crossed (delineated by all four paws in a square); total number of rears (defined as both front paws off the ground, excluding grooming) and the number of centre crossings.

Following observation and tabulation, the integrity of results was verified via blind analysis of recordings by another member of the lab.



**Figure 2-3. Open field test. Anxiety-like and exploratory behaviour are tested through observation of the number of: Times the mouse rears (pictured), squares it crosses in total and the number of centre squares crossed in a three-minute period.**

### 2.11.2 Elevated Plus Maze

A widely used test of measuring anxiety like behaviour in mice, the elevated plus maze (EPM) utilises the natural aversion of mice to open and elevated areas. The apparatus consisted of a 50cm x 50cm cross shaped platform, set 50cm above the floor. Two arms of the maze were walled on both sides while the other two were left open. At the test's onset animals were placed at the junction of the open and closed arms, facing the open arm opposite to the experimenter and recorded for five minutes. The walls and floor of the maze were thoroughly cleaned between each trial using 70% ethanol and then dried. In order to ensure parity test subjects were alternated between SE and EE groups (e.g. mouse S (standard) 1 was recorded first, followed by E (enriched) 1, then S2, E2 etc). This test was performed in accordance to a previously published methodology [Walf and Frye 2007].

Five variables were measured: The number of entries made by the rodent onto the open and closed arms; the time spent on the open arm and closed arm and latency to enter the closed arm. Following observation and tabulation, the integrity of results was verified via blind analysis of recordings by another member of the lab.

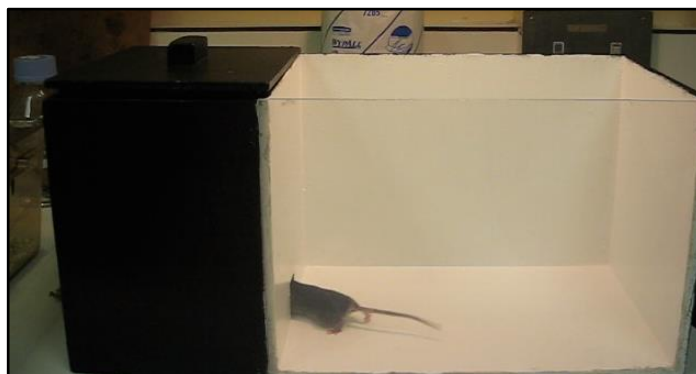


**Figure 2-4. Elevated plus maze. Anxiety-like and exploratory behaviour are tested through observation of the number of: entries made by the rodent onto the open and closed arms the time spent on the open arm and closed arm and latency to enter the closed arm over a five-minute period.**

### 2.11.3 Light / Dark Box Test

This test uses rodent preference for darkened areas and corresponding aversivity to illuminated ones as a means of observing relative levels of anxiety like behaviour. The test apparatus consisted of a 45cm x 20cm x21cm box, divided into two distinct compartments: One third (15 cm long) painted black, with a black lid on top. The remainder of the box was painted white and open at the top. A 2.5cm x 2.5 cm opening connected the two compartments. One wall of the light compartment was formed of transparent Perspex to enable observation. The aversiveness of this compartment was enhanced by illumination from a 50 Watt lamp positioned perpendicular to the centre of the box floor. At the test's onset each animal was placed in the light compartment, facing away from the opening and recorded for five minutes. The walls and floor of the box were thoroughly cleaned between each trial using 70% ethanol and then dried. In order to ensure parity test subjects were alternated between SE and EE groups (e.g. mouse S (standard) 1 was recorded first, followed by E (enriched) 1, then S2, E2 etc). This test was performed in accordance to a published methodology previously used by the lab [Gallo, Rattazzi et al. 2014].

Three dependent variables were measured: The time spent in the light area: latency to cross to the dark area (delineated by all four paws in) and the total number of transitions between compartments. Following observation and tabulation, the integrity of results was verified via blind analysis of recordings by another member of the lab.



**Figure 2-5. Light dark box. Anxiety-like and exploratory behaviour are tested through observation of the time spent in the light area: latency to cross to the dark area and the total number of transitions between compartments.**

### 2.11.4 Serum Corticosterone Analysis

Levels of the stress associated glucocorticoid present in the plasma of EE and SE mice were measured using a commercial kit (Corticosterone EIA kit, Enzo Life Sciences) as per manufacturer's instructions. Briefly, plasma samples were isolated from citrulated blood by

centrifugation for ten minutes at 2,000x g using a refrigerated centrifuge. Samples were diluted 1 in 10 and run in duplicate with a 1.5% steroid displacement buffer on a pre-treated 96 well plate. Assay plates included a corticosterone spiked control well and an eight-point standard curve produced by serial dilution of a kit-provided, 200,000 pg mL<sup>-1</sup> corticosterone standard, plates were read at a 405 nM wavelength. Concentrations of samples were calculated by plotting the concentration of standards against their corresponding absorbance value, adding a line of best fit to these data points and determining the equation for the line. Multiplying the values obtained by the absorption values obtained for each sample produced the corresponding concentration of corticosterone.

## **2.12 Caecal Ligation and Puncture**

Caecal ligation and puncture (CLP) is a commonly used animal model of sepsis, (a state of intense, systemic inflammation normally instigated by infection). As the name suggests the CLP model consists of perforation of the caecum allowing release of faecal matter into the peritoneal cavity. Generating an acute, poly-microbial induced immune response.

### **2.12.1 CLP Procedure**

Mice were anaesthetised by i.p injection (1ml syringe 26G needle) of a solution of 1:1 ketamine (75mg/kg. Vetoquinol) and xylazine (15mg/kg. Bayer). Following confirmation of complete anaesthesia by paw pinch animals had their abdomen; shaved, swabbed with 70% EtOH and placed upon a sterile drape. A 1-2cm midline laparotomy was performed in order to expose the caecum and adjoining intestine. The caecum was then ligated using 4.0 silk sutures (PROLENE, Ethicon) just below the ileo-caecal valve. The caecum was then pierced through twice with a 19G needle at a distance of ~0.5mm from its distal end. Such a distance is reported to induce a mid to low grade severity of sepsis [Toscano, Ganea et al. 2011]. Gentle pressure was applied to the caecum in order to extrude a small quantity of faecal matter from the sites of puncture. The caecum was then returned to the peritoneal cavity and the peritoneum closed with 4.0 silk sutures. In turn the skin was then sutured closed. Animals were placed on a heat pad and resuscitated by subcutaneous injection of pre-warmed 0.9% saline solution using a 25G needle. The mice were left to recover from their anaesthesia in an incubation chamber. Recovery time took between 20-60 minutes. Animals were then returned to their cages where

free access to food and water (and hydrogel) was provided. Following 12 hours, each animal was clinically scored for severity of illness and subject to terminal anaesthesia, followed by further processing.



**Figure 2-6. CLP, ligated caecum 12 hours post procedure.**

### **2.12.2 Peritoneal Lavage and Cell Count**

Lavage and cell count was carried out in the same manner as previously described (2.8), with the modification that the laparotomy and peritoneal lavage was carried out through the left side of the mouse, in order to prevent rupture of the sutured peritoneum.

### **2.12.3 Bacterial Colony Count**

Isolated blood and PLF from each animal was subject to a 10-fold serial dilution in sterile PBS down to  $10^{-6}$ . 100 $\mu$ l of each sample dilution ( $10^{-1}$  to  $10^{-6}$ ) was streaked across one quarter of a nutrient broth (nb) agar plate (10cm diameter, 1cm depth) divided into four equal sections. Sterile conditions were maintained throughout this process. Following seeding, the plates were incubated at 37°C for 24 hours. Following incubation, a count of the bacterial colonies growing on each plate was carried out. Colony forming units (CFU) per ml were calculated by selecting the PLF or blood dilution that provided the most accurately quantifiable number of colonies for each sample (i.e. visible colonies were present but not so many that one colony could not be distinguished from another) and multiplying:

$$\text{CFU/ml} = \text{Colony count} \times \text{Dilution factor} \times \text{ten}$$

## **2.13 Immune-Modulatory Gene Microarray Analysis**

### **2.13.1 Whole Blood Total RNA Extraction for Micro-Array**

Blood isolated through cardiac puncture was immediately placed in RNAProtect Animal Blood Tubes (Qiagen) which contain reagents that lyse blood cells while stabilising intracellular RNA to preserve the gene expression profile.

RNA was then extracted using an RNeasy Animal Blood RNA Mini Kit (Qiagen) adhering to the manufacturer's instructions (RNeasy Handbook 06/2012). Briefly, blood samples were washed and pelleted by centrifugation then re-suspended in a buffered solution of proteinase K.

Following digestion of contaminant proteins the samples were homogenised by centrifugation through a polymer matrix and the eluted sample mixed with 90% ethanol to optimise RNA binding conditions. Samples were then centrifuged through a silica membrane binding the freed RNA. The bound RNA was subject to DNAase digestion to remove DNA contamination and then underwent multiple cycles of washing before being eluted in 30µl of RNA free water.

Quality checking of each RNA sample was carried out by spectrophotometric analysis using a Nandrop (ND-1000. Thermo-Scientific) and microfluidic analysis via Agilent 2100 Bioanalyser (Agilent Technologies). Bioanalyser analysis was carried out by the QMUL Barts and the London Genome Centre. Extracted RNA samples were aliquated into two tubes and stored at -80°C in preparation for microarray analysis.

### **2.13.2 Microarray Protocol**

Purified RNA was sent the University College London Genomics facility for processing and microarray analysis. The following procedures and protocols were performed and provided by Dr Mark Kristiansen (Application Specialist, UCL Genomics. London).

Up to 500ng of total RNA was processed and labelled using the Ambion® WT Expression Kit as outlined in the manufacturer's instructions. Briefly, sense strand cDNA was prepared from total RNA ready for fragmentation and labelling using the Genechip® WT Terminal Labelling Kit. Labelled samples were hybridised on Affymetrix GeneChip Mouse Gene v2.0ST arrays for 16 hours at 45°C with constant rotation. (Detailed protocols can be found in the Affymetrix WT Terminal Labelling and Hybridisation User Manual edition 5.) Subsequent washing and scanning of the arrays was performed on an Affymetrix GeneChip Fluidics station 450 and GCS3000 Affymetrix scanner respectively according to the manufacturer's recommendations.

The resulting CEL files were used for downstream analysis. Quality control (QC) of the datasets obtained from the scanned Affymetrix arrays was performed using Expression Console software.

### **2.13.3 Microarray Data Analysis**

Pathway and statistical analysis was carried out by Dr Masahiro Ono (Faculty of Natural Sciences, Department of Life Sciences, Imperial College, London). Relevant genes were filtered by excluding those without an Entrez ID and those with low expression levels less than 100 by non-logged value. T-statistics were applied across the data set using the Bioconductor package Limma, and differentially expressed genes were identified by  $p < 0.05$  (non-adjusted p-value) and fold change  $> 2$ .

## **2.14 Real Time PCR**

Total RNA was purified from the blood of mice (extracted via cardiac puncture) using an RNeasy Protect Animal Blood System (Qiagen) using the same procedure stated in 2.13.1. Extracted RNA was reverse transcribed using 2  $\mu\text{g}$  oligo (dT)15 primer, 10U AMV reverse transcriptase, 40U RNase inhibitor (all from Promega Corporation, Madison, WI, USA) and 1.25 mM of each dNTP (Bioline, London, UK) for 45 minutes at 42 °C. The real-time polymerase chain reaction was carried out using power a SYBR GREEN master mix (Thermofisher scientific) and QuantiTect primers (Qiagen). Cycling conditions were set according to the manufacturer's instructions. The sequence-specific fluorescent signal was detected by 7900HT Fast Real-Time PCR System (Applied Biosystems, Warrington, Cheshire, UK). CT values for each gene were normalized relative to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and then used to calculate expression levels. We used the comparative Ct method to measure the gene transcription in samples. Results are expressed as relative units based on calculation of  $2^{-\Delta\Delta\text{Ct}}$ , which gives the relative number of gene normalized to an endogenous control (GAPDH).

## 2.15 Neutrophil Activation

Activation assays and analysis were carried out using the same protocols stipulated in [Cooper, Norling et al. 2008, Headland, Jones et al. 2014]. Briefly, blood samples were separated into five aliquots and treated respectively with TNF- $\alpha$  (50 ng/ml), Platelet activating factor (PAF) (10–9M) for 15 or 30 minutes at 37°C, or left unstimulated. Samples were washed and stained for the presence of GR-1 APC (RB6-8C5 clone), CD11b FITC (M1/M70 clone) and L-Selectin (CD62L) PE (Mel-14 clone) on ice for 45 minutes (all antibodies were purchased from, eBioscience). Each sample was lysed and fixed using an Immuno-lyze™ reagent kit (Beckman Coulter). Neutrophils were gated according to forward/side scatter characteristics and positive expression of Gr-1 and CD11b. CD11b expression was recorded as units of fluorescence where the median fluorescence intensity for 10,000 cells was measured in the FL1 green channel (548nm). In the case of CD11b antibody, the red FL2 channel was used (590nm). Samples were analysed by a BD LSRFortessa II™ with post hoc analysis carried out using FlowJo™ 7.0 software (Tree Star, Ashland, OR, USA, Oregon Corporation).

## 2.16 Macrophage Phagocytic Function

The following procedure was based on that of [Montero-Melendez et al., 2015]. Animals were injected i.p with 0.5ml of a 4% solution of biogel P-100 Bio-Rad) and left undisturbed for four days. Animals were then sacrificed and subject to peritoneal lavage with 10ml of PBS. Collected cells were washed twice then re-suspended in complete RPMI-1640 media (Sigma-Aldrich). Isolated cells were confirmed as macrophages via microscopic analysis then counted and seeded at  $0.2 \times 10^6$  cells per well in 200 $\mu$ l of warm complete RMPI on a 96 well, black walled, clear bottomed plate (Grenier one) and allowed to adhere for two hours at 37°C (5% CO<sub>2</sub>). Macrophages were then stimulated with BODIPY 576/589 (Invitrogen) conjugated, lyophilised *E.coli* (sigma) added at a final concentration of 1mg/ml to each well. Following one-hour cell activity was halted and any non-phagocytosed *E.coli* removed by washing three times with cold PBS. Fluorescence was read at 570 and 590nm using a multiscan FC plate reader.



## **2.17 Sponge Granuloma**

### **2.17.1 Sponge Implantation Procedure**

The following procedure was based upon that of [Luvone et al., 1994] Mice were deeply anaesthetised by i.p injection of a 1:1 solution of ketamine (75mg/kg; Vetoquinol) and xylazine (15mg/kg; Bayer). The dorsal hair of each animal was shaved and the skin sterilised with 70% ethanol. A roughly 1cm incision was made just below the midline of the back and a small (~2 x 2cm) subcutaneous pouch formed using artery forceps. A sterilised by autoclave) 0.5 x 1 x 2cm polyether–polyurethane sponge was implanted into the pouch and then injected with 100µl of a 0.1% solution of carrageenan. The cavity was then sutured closed (4.0, Ethicon Ltd, U.K.) and the animals placed in a recovery chamber until fully conscious.

72 hours following surgery animals were sacrificed by Co<sub>2</sub>. The granulomatous tissue and sponge was then carefully excised from each animal with surgical scissors. Following separation of the sponge from the tissue encasing it each granuloma was weighed and then split in two, 1 half was immediately used for cellular analysis and the other stored in RNAlater for later use.

### **2.17.2 Sponge Infiltrate Extraction and Analysis**

To examine the content of each of the excised sponges, each sponge was hooked onto a sterilised paperclip, which was in turn attached to rim of a falcon tube and centrifuged at 1500rpm for five minutes to extract the fluid it contained. The recovered fluid was centrifuged again (1500rpm for five minutes) and the resulting pellet re-suspended in 1ml of sterile PBS and the supernatant kept for cytokine profiling. Following quantification by haemocytometer, the isolated cells were subject to flow cytometric leukocyte profiling.

### **2.17.3 Flow Cytometric Leukocyte Profiling**

Cell samples were each stained in 100µl of FACS buffer (PBS containing 5% FCS and 0.02% NaN<sub>2</sub>) containing CD16/CD32 FcR blocking antibody (clone 93; eBioscience, Wembley, UK) for 30 min at 4°C. Thereafter cells were stained with the following FITC or PE-conjugated antibodies (eBioscience, Wembley, UK): GR-1 (clone RB6-8C5), F4/80 (clone BM8) and Ly6B.2 (clone 7/4). Cells were labelled with the appropriate concentration of conjugated antibodies for 40 minutes at 4 °C then washed and analysed. In all experiments, stained cells

were acquired with an LSR FORTRESSA flow cytometer (Becton Dickinson) post hoc analysis was carried out using FlowJo™ 7.0 software (Tree Star, Ashland, OR, USA, Oregon Corporation).

#### **2.17.4 Exudate Cytokine Profiling**

Quantification of cytokines keratinocyte chemoattractant (KC), interleukin 10 (IL-10), macrophage inflammatory protein-one alpha (MIP1- $\alpha$ ) and tumour necrosis factor alpha (TNF- $\alpha$ ), within the exudate supernatant was performed using a Bio-Plex mouse cytokine assay (Bio-Rad, Hercules, CA, USA) according to the manufacturer's directions. Measurements were carried out using a Luminex Bio-Plex 200 system (Bio-Rad) and then analysed with Bio-Plex Manager 6.1 software (Bio-Rad).

#### **2.17.5 Granuloma Dispersal and Flow Cytometric Analysis**

Granuloma samples were finely minced using a scalpel blade and added to a 0.1% solution of collagenase (Sigma-Aldrich) in HBSS (GIBCO) and incubated while swirling at 37°C for 60-90 minutes until a uniform suspension was formed. The solution was then passed through a 150 $\mu$ m strainer and pelleted by centrifugation (1800rpm, 5 minutes). The resulting pellet was re-suspended in 2ml of a 0.5% solution of pronase-A (Sigma Aldrich) in HBSS and incubated while swirling at 37°C for roughly 30 minutes until the preparation was predominately a suspension of single cells. Following washing and re-pelleting the cells were counted by haemocytometer.

#### **2.17.6 RT PCR Profiling of Granuloma Tissue**

Granuloma tissue was homogenised using a Precellys24 homogeniser (Precellys) and total RNA extracted using an RNeasy mini kit (Qiagen) according to the manufacturer's protocol. Extracted RNA was reverse transcribed using 2  $\mu$ g oligo (dT)15 primer, 10U AMV reverse transcriptase, 40U RNase inhibitor (all from Promega Corporation, Madison, WI, US(A) and 1.25 mM each dNTP (bioline, London, UK) for 45 minutes at 42 °C. Following quality assurance and quantification by nanodrop (Thermo Scientific) samples were analysed for gene expression using a RT<sup>2</sup> Profiler PCR Array (Qiagen) according to the manufacturers protocol.

## 2.18 Statistical Analysis

According to the nature of the data obtained a two tailed t-test, or ANOVA (one or two-way) was performed. Time course observations were analysed with multiple t-tests and the Holm-Sidak post-hoc test. Behavioural data was analysed via non parametric analysis using the Mann Whitney U test. All statistical analysis was performed using GraphPad PRISM software (V6.0) with the exception of microarray analysis which was carried out as stated in 2.13.3 using the software package LIMMA (bioconductor).

## 2.19 Experimental Design and Consideration of the Three Rs

Owing to the explorative nature of the research carried out and the wide variability of results obtained in previous studies investigating the influence of enrichment on the immune response (and in our own pilot studies), it proved very difficult to make any reliable assumptions about effect size when planning for the group sizes to be used in each experiment.

In order to work out a suitable sample size we used a cruder (but still effective) resource equation calculation (or E equation).

According to this method a value “E” is measured, with E equal to the total number of animals used in an experiment minus the total number of experimental groups [Charan and Kantharia, 2013].

The E value should lie between 10 and 20. If E is equal to or less than 10 then adding more animals will increase the chance of getting more significant result. If E exceeds 20 then adding more animals should not increase the chance of getting significant results.

Originally treatment groups of 10 mice were planned with two groups; enriched environment and standard environment

Therefore,  $E = (10 \text{ mice} \times 2 \text{ groups}) - 2$                        $E=18$ .

The same number of mice per group has been used for previous enrichment experiments [Nithianantharajah and Hannan, 2006].

Previous experience of the experimental models performed in this thesis suggested a high likelihood that some of the animals would become unsuitable for data collection due to experimental error (e.g. through presence of blood in peritoneal lavage during ZIP and CLP or removal of sutures in the sponge granuloma model). It was accordingly decided to add a further two mice per group to account for this error, producing a final total of 12 in all.

Efforts were made to ensure this project ascribed to the guidelines provided by the NC3Rs (National Centre for Replacement, Refinement and Reduction of Animals in Research) for the requirements for good rodent housing and husbandry [NC3Rs, 2017]. Owing to the nature and purpose of this study the housing conditions for the standard lab enclosure group conformed to the normal set up used for mice in the facility this study was carried out in. These conditions in turn followed the basic housing requirements stipulated by the NC3Rs.

These included:

- The housing of animals in stable, compatible groups - Staff took into account; sex, age, reproductive condition, familiarity, prior group housing experience when grouping the animals. Special care was taken to assess for signs of excessive fighting among groups in the enriched cages.
- The use of enclosures designed to cause minimum disturbance to the animals
- The provision of enough space for exercise and normal behaviour (e.g. grooming and pandiculation).
- The provision of basic environmental enrichment (nesting material) to help reduce the risk of social stress and aggression and allow the animals to fulfil some of their species-specific behaviours.
- Gentle and frequent handling from early in life.

Housing conditions in the enriched environment group (See section 2.2 for a detailed description) were designed using previous research supported by the NC3Rs as means of refining murine housing conditions [Sherwin, 2007].

Throughout the course of each study carried out all animals were subject to regular assessments of their welfare status (every second day). These checks were verified by staff at the animal unit and (on three separate occasions) visits by a home office inspector.

In order to minimise animal numbers efforts were made to generate as much data as possible from each animal used, these included:

- Taking weight, food/water intake and adiposity measurements from every animal.
- Taking blood (serum and plasma), PLF and bone marrow samples from every animal in order to obtain data on multiple aspects of their biological status.
- Pre-emptive storage of these samples as well as further organs and tissues in preparation for any further analysis that may need to be carried out.
- Where possible, subjecting animals to batteries of tests (e.g. behavioural) to reduce the need for repetition.
- Performing pilots of each procedure with one to two animals in order to refine experimental technique and prevent undue expenditure of animal lives.

## 2.20 General Experimental Schema

All experiments followed the same basic program. Following receipt mice were allocated into SE or EE cage groups and allowed one week to acclimatise to the lab environment (day -7 to 0). Following which they spent two weeks in an enrichment or standard enclosure (Days 0 – 14). Animals were then subject to either a battery of behavioural tests or one model of inflammation, listed below (Figure 2-7).

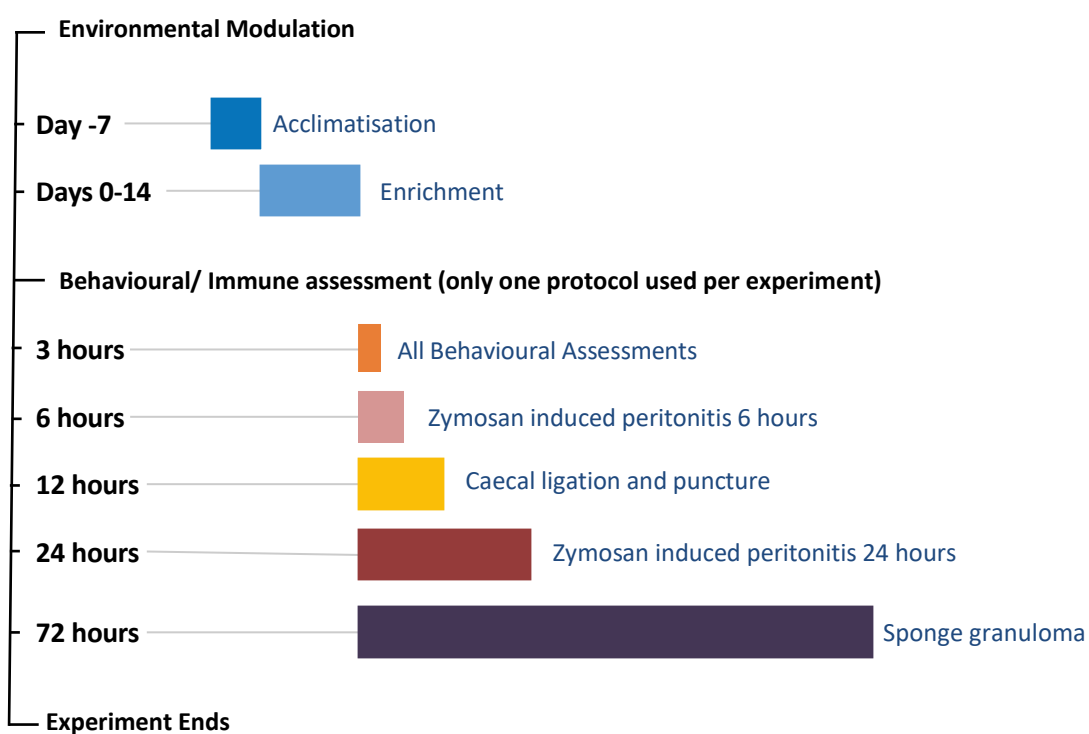


Figure 2-7 Experimental procedures carried out and times each took to complete

## Chapter 3 Results

*N.B Unless otherwise specified all results displayed are representative of one data set chosen from three repeat experiments.*

### 3.1 Physiological Parameters

We recorded the basic physiological parameters (weight, nutritional intake, fat content) of all experimental animals. This data served as a simple, initial means of identifying any gross changes in animal physiology and immune state.

#### 3.1.1 Comparative Weight Gain Over Experimental Period

Over the course of the 14-day enrichment period animals in the environmentally enriched (EE) group demonstrated a significantly slower weight gain than those from the standard environment (SE) (Figure 3-1). At the end of the two weeks SE animals had gained on average one gram of additional weight compared to EE. This pattern of weight change was observable in every experiment these parameters were measured (ten experiments in total.)

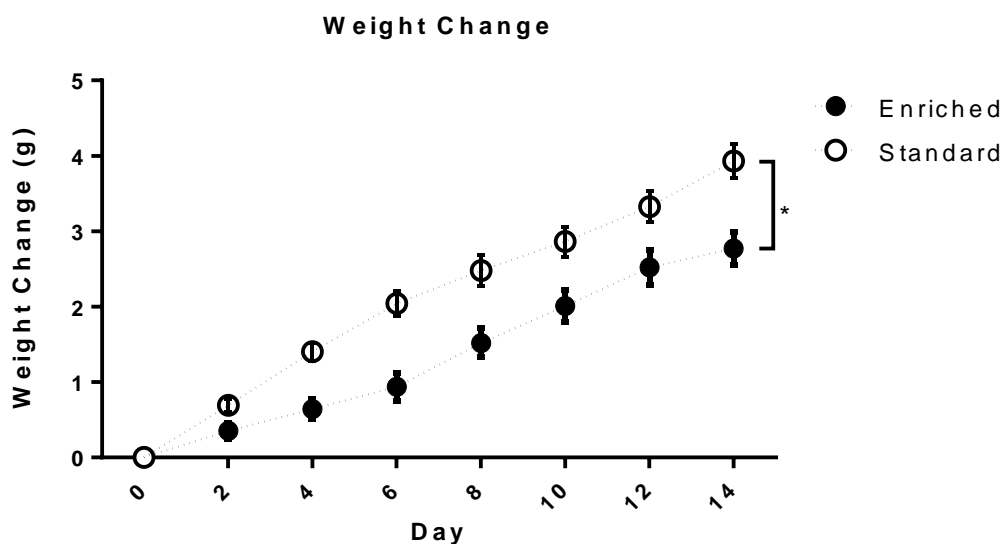
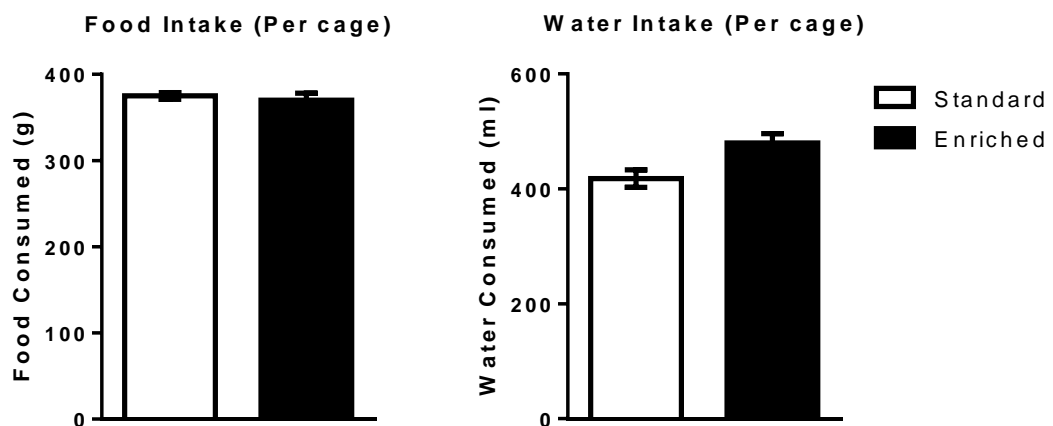


Figure 3-1. Comparative weight change over time. 24 Mice per group, N=12 experiments. Statistical significance determined using multiple t-test analysis, results were normalised using the Holm-Sidak method, with  $\alpha=5.000\%$ .

### 3.1.2 Comparative Nutritional Intake Over the Experimental Period

Animals from both groups maintained roughly the same intake of food (average 27g of chow consumed per cage group, per day). Enriched animals trended towards a higher overall water intake (average SE, 29ml and EE, 35ml of water per group, per day.) (Figure 3-2). In the three separate experiments that were assessed, food intake remained remarkably consistent across test groups and successive days of measurement. The only point in which this trend was perturbed was at the onset of the enrichment period when both groups were moved into new cages. Here food intake would be consistently lower than average at time point one (day one) and higher at time point two (day three). This transient anorexia followed by polyphagia likely indicates the transition from a state of neo-phobia followed by habituation as these animals habituate to a new environment [Misslin et al., 1982].



**Figure 3-2. Total food and water intake per cage group over enrichment period. Results shown are an average of the two cages used for each experimental group N=3 experiments, each with 24 mice per group.**



### 3.1.3 Subcutaneous Fat Levels Following Enrichment

In order to determine the source of this variance in weight change, levels of subcutaneous fat were assessed between groups. The overall weight and size of visceral fat pads excised from animals was consistently lower in EE compared to SE groups (Figure 3-3, Figure 3-4).

Comparatively reduced levels of adiposity were observed throughout the bodies of enriched animals (data not shown).

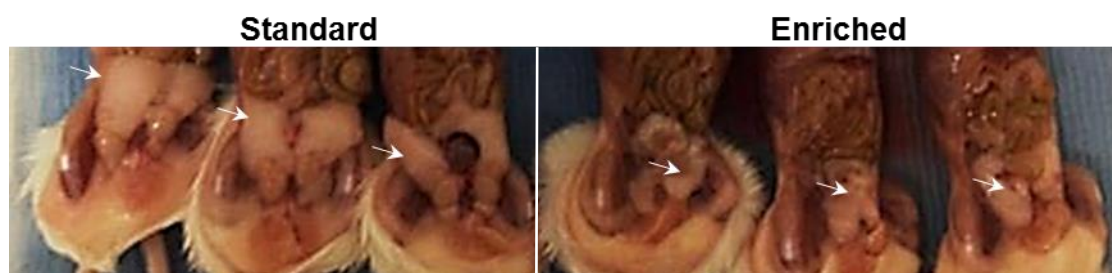


Figure 3-3. Comparative images of visceral fat pads from enriched versus standard environment mice.

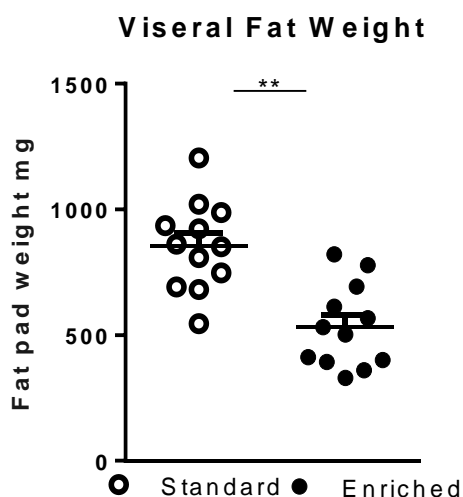


Figure 3-4. Comparative weights of visceral fat pads taken from enriched and standard environment mice . \*\* $p < 0.005$ , t-test. N=1 experiments, 24 mice per group.

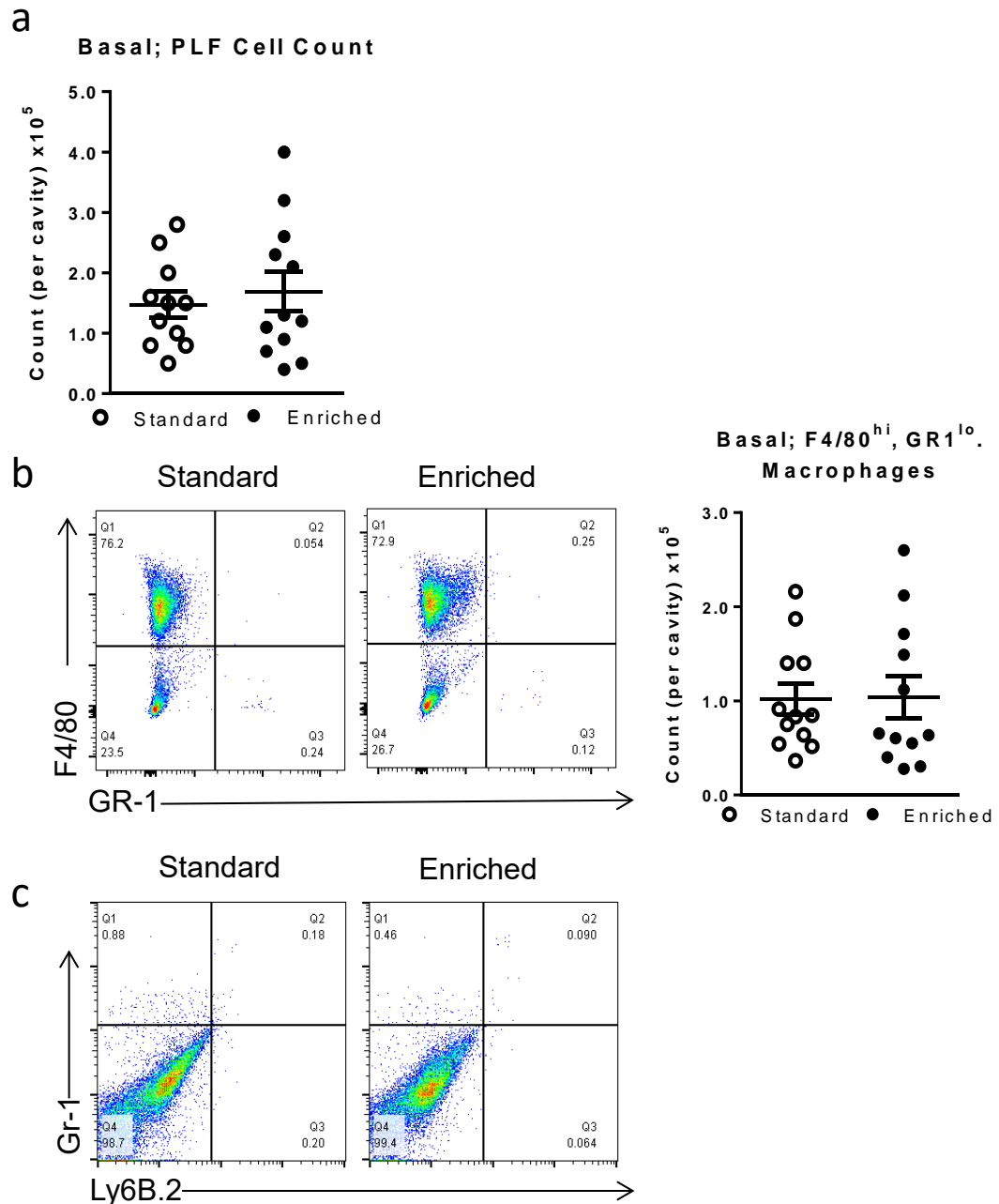
## **3.2 Investigation of Enriched Mice in a Basal Immune State**

Continuing our investigation of the influence of environmental enrichment on murine biology we moved on to focus on its effect on the immune response. As part of this three primary cellular compartments were investigated for signs of immune activity: Bone marrow; the point of origin for most innate immune cells. Blood; the primary route of transit for these cells and the peritoneal cavity; a large serosal space that we chose to use as a focus for inflammation. To determine whether enrichment had any physiological effects on mice in a basal, non-immune activated state we initially chose to investigate enriched mice untreated with any inflammagen or immune-active compound.

### **3.2.1 Basal Immune State: Cellular Profile of the Peritoneal Cavity**

The peritoneum is a large membrane bound and fluid filled cavity found in all mammals, housing the liver, spleen, gastro-intestinal tract and other viscera. Previous studies have identified a variety of immune cells are resident within the peritoneal cavity, including unique macrophage subsets and B-cells [Ray and Dittel, 2010].

Microscopic analysis of lavage fluid extracted from the peritoneal cavity of EE and SE mice revealed the majority of isolated cells to possess a morphology associated with that of monocytes and non-activated macrophages (large size, agranulated, indented nucleus). A cell count of the lavage revealed no differences in overall cell number between EE and SE mice (Figure 3-5a). FACS analysis of this PLF identified a small but distinct population of F4/80 expressing cells in both animal groups, identified previously as peritoneal resident macrophages [Davies et al., 2013], no comparative differences in the number of these cells were found (Figure 3-5b). Additional staining for granulocytic (GR-1) and monocytic (Ly6b.2) markers indicated that the presence of these cell populations was low to nil (Figure 3-5c).



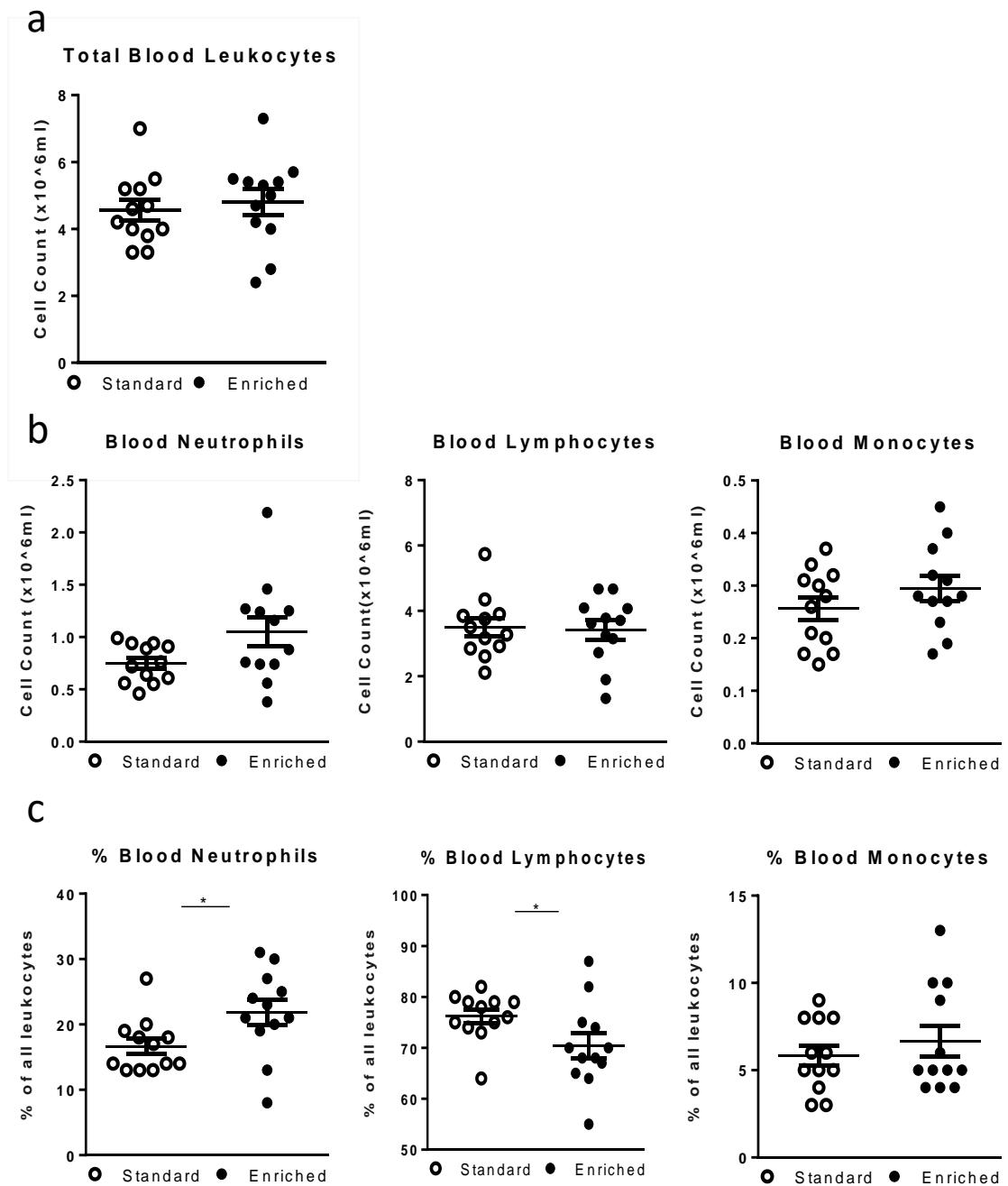
**Figure 3-5.** Peritoneal cell populations in enriched and standard environment mice. (a) Overall peritoneal cell count (b) FACS plot and respective count of high F4/80 expressing cells, attributed as macrophages (c) double negative FACS plot of GR-1 (granulocyte) and Ly6B.2 (monocyte) markers. N=4 experiments, each with 24 mice per group.

### **3.2.2 Basal Immune State: Peritoneal Cytokine Analysis**

Analysis of the extracted PLF by Luminex assay found the presence of inflammatory cytokines in the peritoneal cavity to be below detectable limits (10pg/ml). Such results would be expected for an animal in a basal, non-immune activated state.

### **3.2.3 Basal Immune State: Blood Cellular Profile**

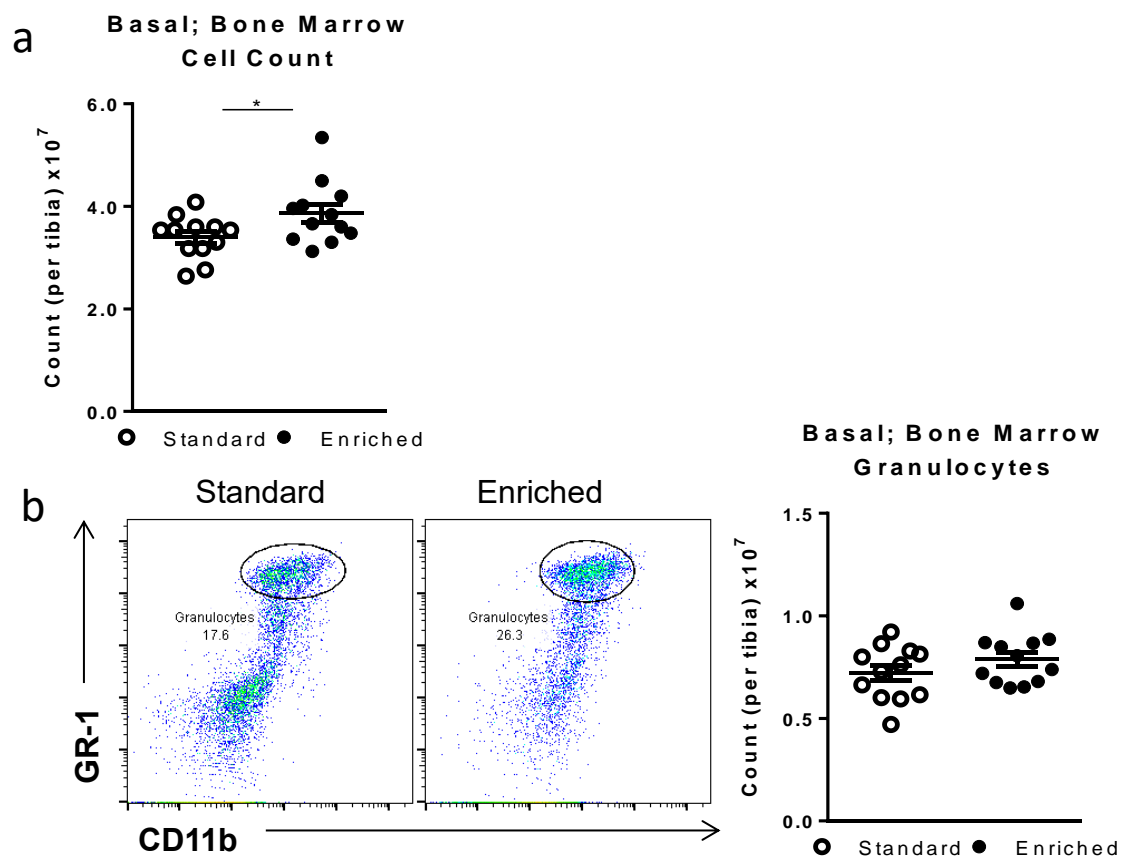
Analysis of extracted cardiac blood (using an IDEXX Haematology Analyser) identified no difference in total circulating leukocytes or individual leukocyte populations (neutrophils, lymphocytes or monocytes) between EE and SE groups (Figure 3-6a and b). However, a significant difference in the relative proportions of circulating cell types was observed, with the blood of EE mice consistently possessing a higher percentage (~10%) of neutrophils and a correspondingly lower percentage (~10%) of lymphocytes when compared to the SE group (Figure 3-6c). Overall lymphocytes were found to be the largest leukocyte population circulating both experimental groups. No significant differences in monocyte numbers were identified.



**Figure 3-6. Circulating blood cell populations in enriched and standard environment mice. (a) Total blood leukocyte count (b) Individual leukocyte population counts (c) Relative percentages of individual leukocyte populations. \* $P = <0.05$  t-test.  $N=4$  experiments, each with 24 mice per group.**

### 3.2.4 Basal Immune State: Bone Marrow Cellular Profile

Microscopic investigation of viable cells extracted from the tibia of EE and SE animals revealed a complex mixture of leukocytes and erythrocytes at various stages of development. While some mature leukocytes could be identified (e.g. neutrophils, erythrocytes), the transitory stage of development most of the cells were within, made distinction between populations difficult. A total count of the extracted tibial cells revealed enriched mice possessed a significantly higher cell number in their bone marrow (Figure 3-7a) compared to those from SE. In an effort to further delineate cell populations within the bone marrow staining for the granulocytic/neutrophilic markers GR-1 and CD11b was carried out. No significant differences were identified between groups for cells expressing these markers (Figure 3-7b). As with the microscopic analysis it was noted that populations appeared to be in a transitional developmental state with plots presenting a continuum of cells with increasing expression of GR-1 and (to a lesser extent) CD11b (Figure 3-7b).



**Figure 3-7. Bone marrow cell population in mice from enriched and standard environments. (a) Total tibial myelocytes and leukocytes. (b) FACS plot and respective count of GR-1 (granulocyte) CD11b (monocyte lineage) positive cells. \*p<0.05, t-test. Representative of N=3 experiments, 24 mice per group.**

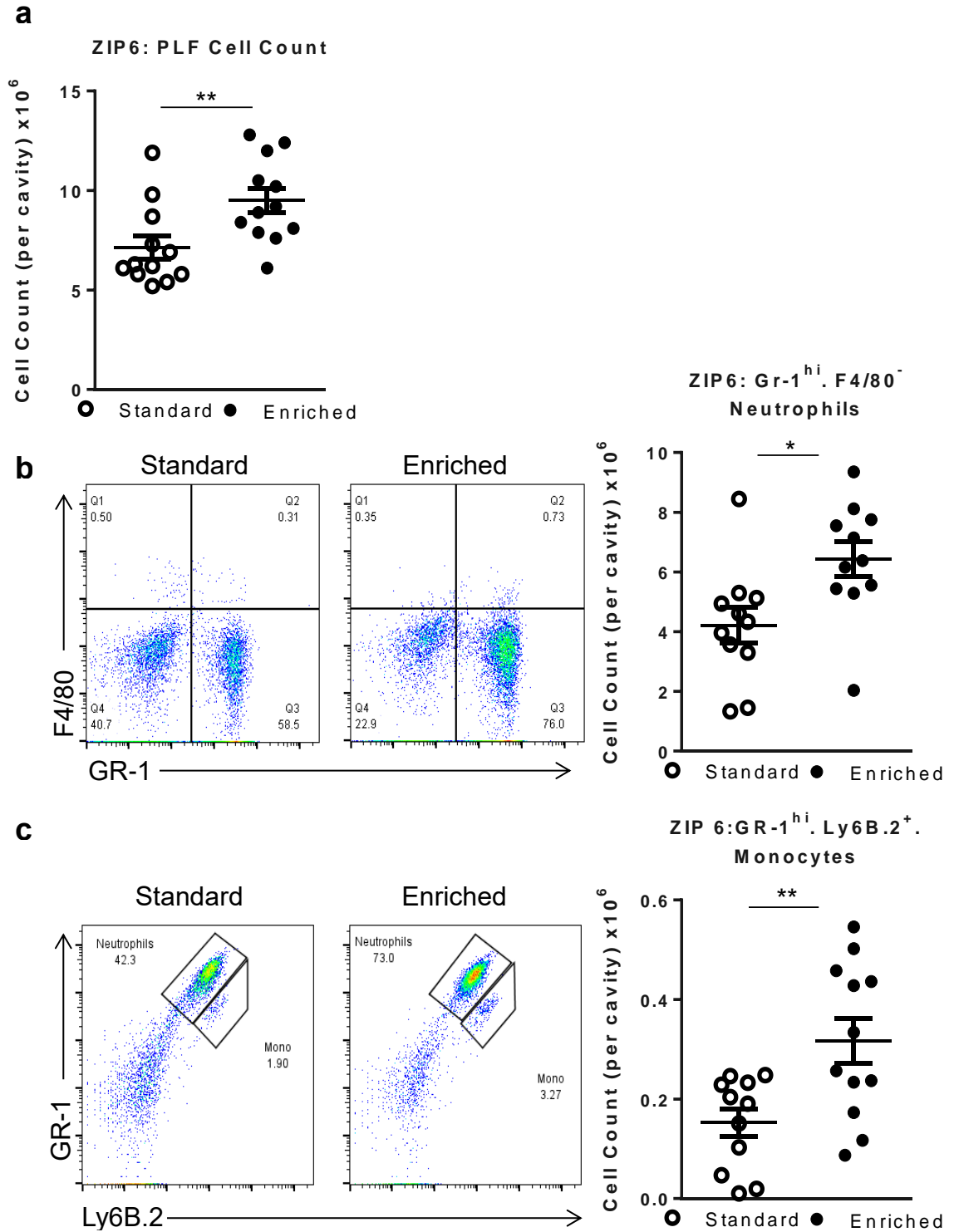
### **3.3 Zymosan Induced Peritonitis: Six Hour Time Point**

We next chose to examine the effects of environmental enrichment on leukocyte recruitment utilising an experimental model of zymosan induced peritonitis (ZIP). Two time points were chosen in which to sacrifice and analyse treated animals. Each time point corresponded to established peaks of innate leukocyte recruitment: Six hours – typified by high numbers of neutrophils and 24 hours – typified by a peak influx of monocytes [Cash et al., 2009].

#### **3.3.1 ZIP 6: Cellular Profile of the Peritoneal Cavity**

Following enrichment and six hours of ZIP analysis of peritoneal lavage revealed a significantly higher number of cells in the peritoneum of EE animals (Figure 3-8a). Concordant with the established time-course of ZIP overall numbers of cells were roughly tenfold higher across both groups when compared to animals that had received no inflammagen [Cash et al., 2009].

Microscopic study revealed a large number of these cells to possess a granular cytoplasm and a tri-lobed nucleus, a morphology typically associated with neutrophils. Flow cytometric analysis confirmed this observation, identifying the dominant cell population in both animal groups to be GR1<sup>hi</sup>, F4/80<sup>-</sup>, ascribed as neutrophils (Figure 3-8b). Smaller populations of GR1<sup>hi</sup>, Ly6B.2<sup>+</sup> cells - attributed as monocytes - were also found (Figure 3-8c). With both sets of markers EE animals typically displayed a higher proportion of positively expressing cell populations compared to SE (~10-20% more neutrophil marker expression and ~1-4% more monocyte marker expression. For both neutrophil and monocyte populations a significantly higher number (~50%) of cells were present in enriched animals compared to those housed in a standard environment.

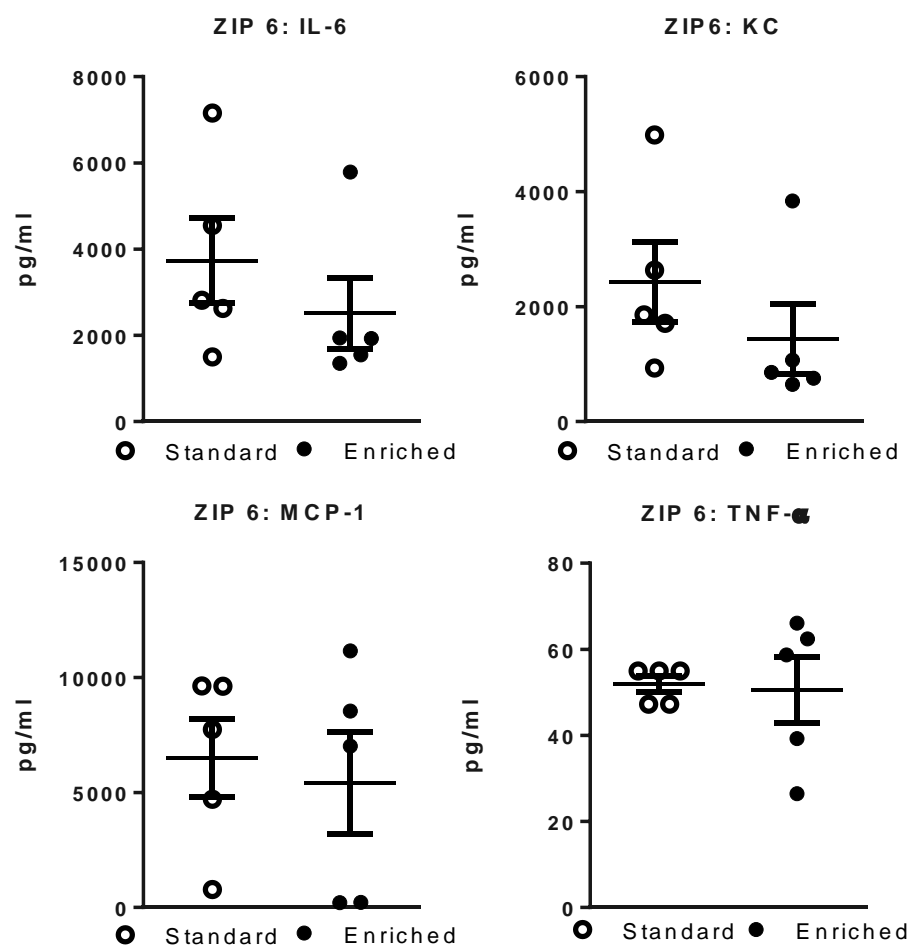


**Figure 3-8.** Peritoneal cell populations in enriched and standard environment mice following six hours of zymosan induced peritonitis. (a) Overall peritoneal cell count. (b) FACS plot and respective count of F4/80<sup>+</sup> GR1<sup>hi</sup> expressing cells attributed as neutrophils (Quadrant 3). (c) FACS plot and respective count of a GR-1<sup>hi</sup>, Ly6B.2<sup>+</sup> cell population attributed as monocytes. \* $p < 0.05$  (\*\* $p < 0.005$ ), t-test. Representative of N=3 experiments, 24 mice per experiment.



### 3.3.2 ZIP 6: Peritoneal Cytokine Levels

Further analysis of the extracted PLF identified heightened levels of pro-inflammatory cytokines in the peritoneal cavity of mice from both groups. These levels were concordant with those found in previous studies using ZIP [Cash et al., 2009]. While no significant differences in peritoneal cytokine levels were found between groups (potentially due to the sample number used) a concurrent trend for reduced IL-6 and KC in enriched mice was observed across all three repeat experiments (Figure 3-9).

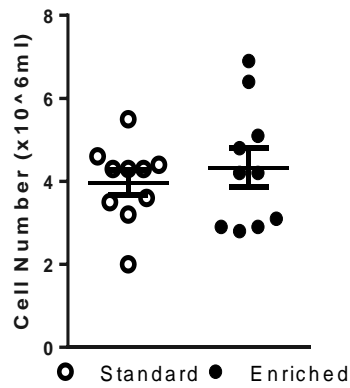


**Figure 3-9. Comparative cytokine levels in the peritoneum of animals subject to six hours ZIP. Comparative levels of (Reading top to bottom left to right): Interleukin six, keratinocyte chemoattractant, macrophage chemoattractant protein 1 and tumour necrosis factor alpha. Results representative of N=3 experiments, 10 mice per experiment.**

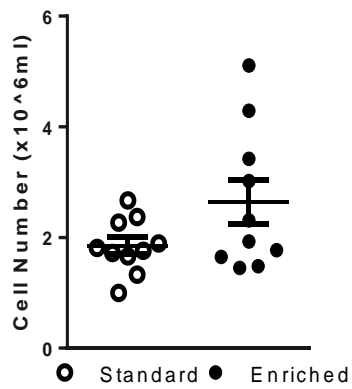
### **3.3.3 ZIP 6: Blood Cellular Profile**

As with the basal readouts blood analysis identified no differences in overall numbers of blood leukocytes between the two experimental groups (Figure 3-10a). Overall numbers of neutrophils were found to have increased roughly twofold across both groups with corresponding decreases in circulating lymphocytes and to a lesser extent: monocytes (Figure 3-10b). However, in contrast to the basal results (3.2.3) circulating lymphocyte numbers were significantly lower in EE animals (~25%) compared to SE and a strong trend was found across all repeat experiments for a higher number of neutrophils (Figure 3-10b). Analysis of relative proportions of leukocytes in circulation revealed an overall increase in the percentage of circulating neutrophils in both animal groups. Such a state is typically associated with an active innate immune response [Liu et al., 2016]. However, within this state of neutrophilia the variation in circulating immune cell proportions identified in the previous basal experiments was amplified. EE mice presented a significantly higher relative percentage of neutrophils (and correspondingly lower percentage lymphocytes in circulation compared to SE mice). Numbers and proportions of monocytes remained unchanged, (Figure 3-10b & c).

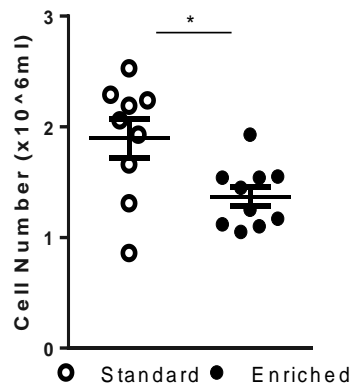
**a ZIP 6:Total Blood Leukocytes**



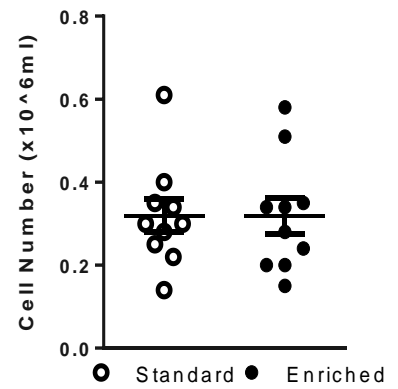
**b Blood Neutrophils**



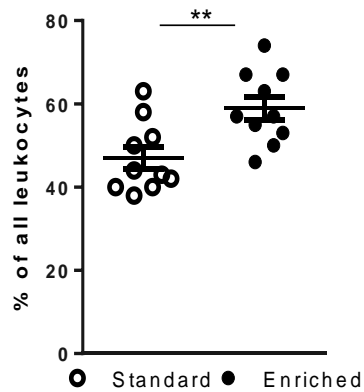
**Blood Lymphocytes**



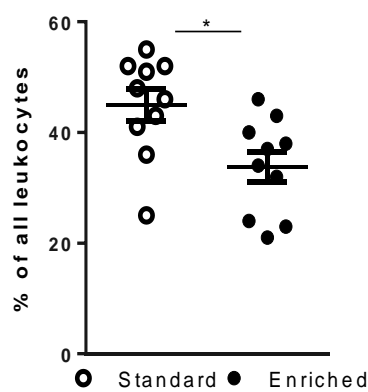
**Blood Monocytes**



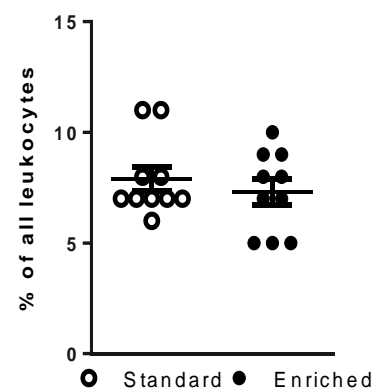
**c % Blood Neutrophils**



**% Blood Lymphocytes**



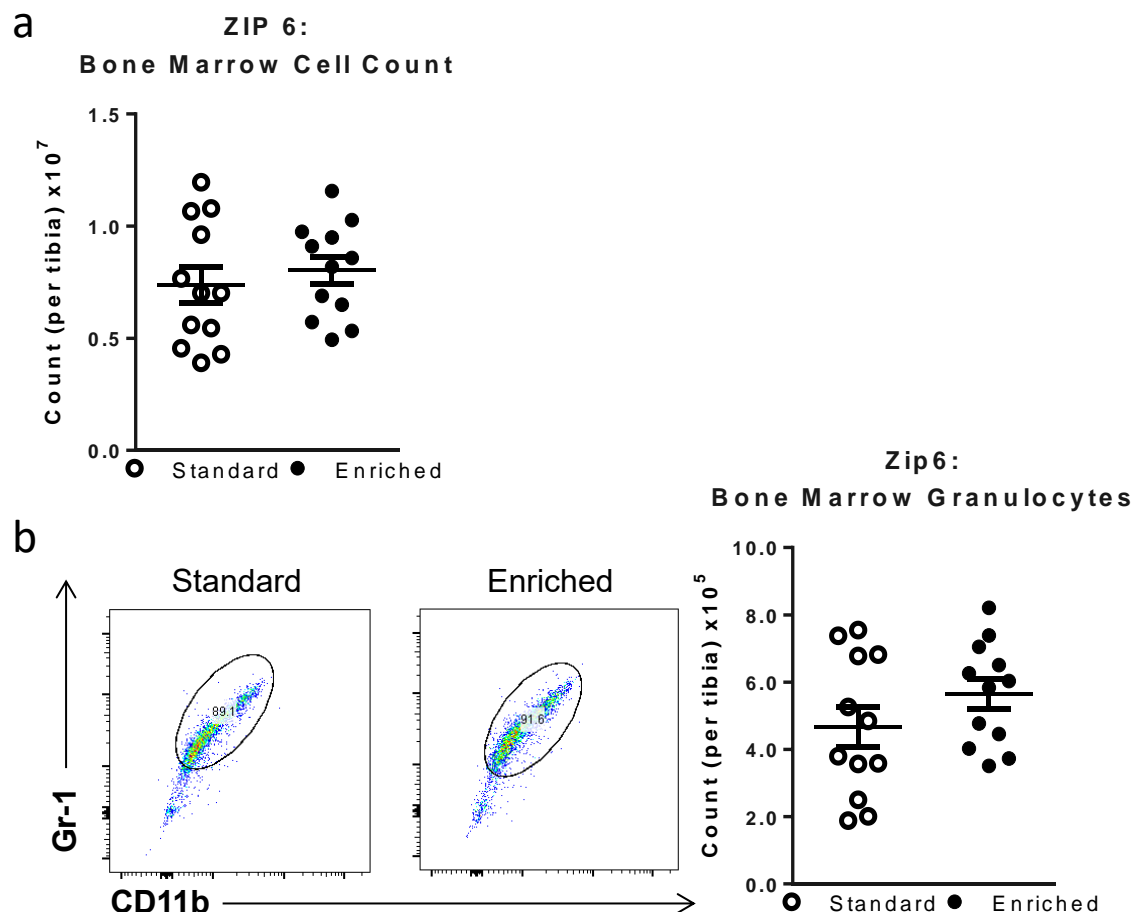
**% Blood Monocytes**



**Figure 3-10. Circulating blood cell populations in enriched and standard environment mice following six hours of zymosan induced peritonitis. (a) Total blood leukocyte count (b) Individual leukocyte population counts (c) Relative percentages of individual leukocyte populations. \* $p < 0.005$  (\*\* $p < 0.005$ ), t-test. Representative of N=4 experiments, 24 mice per experiment.**

### 3.3.4 ZIP 6: Bone Marrow Cellular Profile

Interested to determine the source of this neutrophil influx into the peritoneum and blood circulation we profiled the point of origin for these leukocytes, the bone marrow. Following six hours of ZIP overall cell numbers in the bone marrow were roughly three times lower in both groups compared to basal data (3.2.4) (Appendix 1) such decreases in bone marrow population have been reported as occurring transiently during inflammatory challenge in previous studies [Bian et al., 2012]. No significant difference in cell counts were found between EE and SE animals although a trend for higher cell numbers in the enriched animals was identified across all four experiments (Figure 3 10a). Again a non-significant trend for higher GR1<sup>+</sup>, CD11b<sup>+</sup> cell numbers was identified in the EE group (Figure 3 10b).

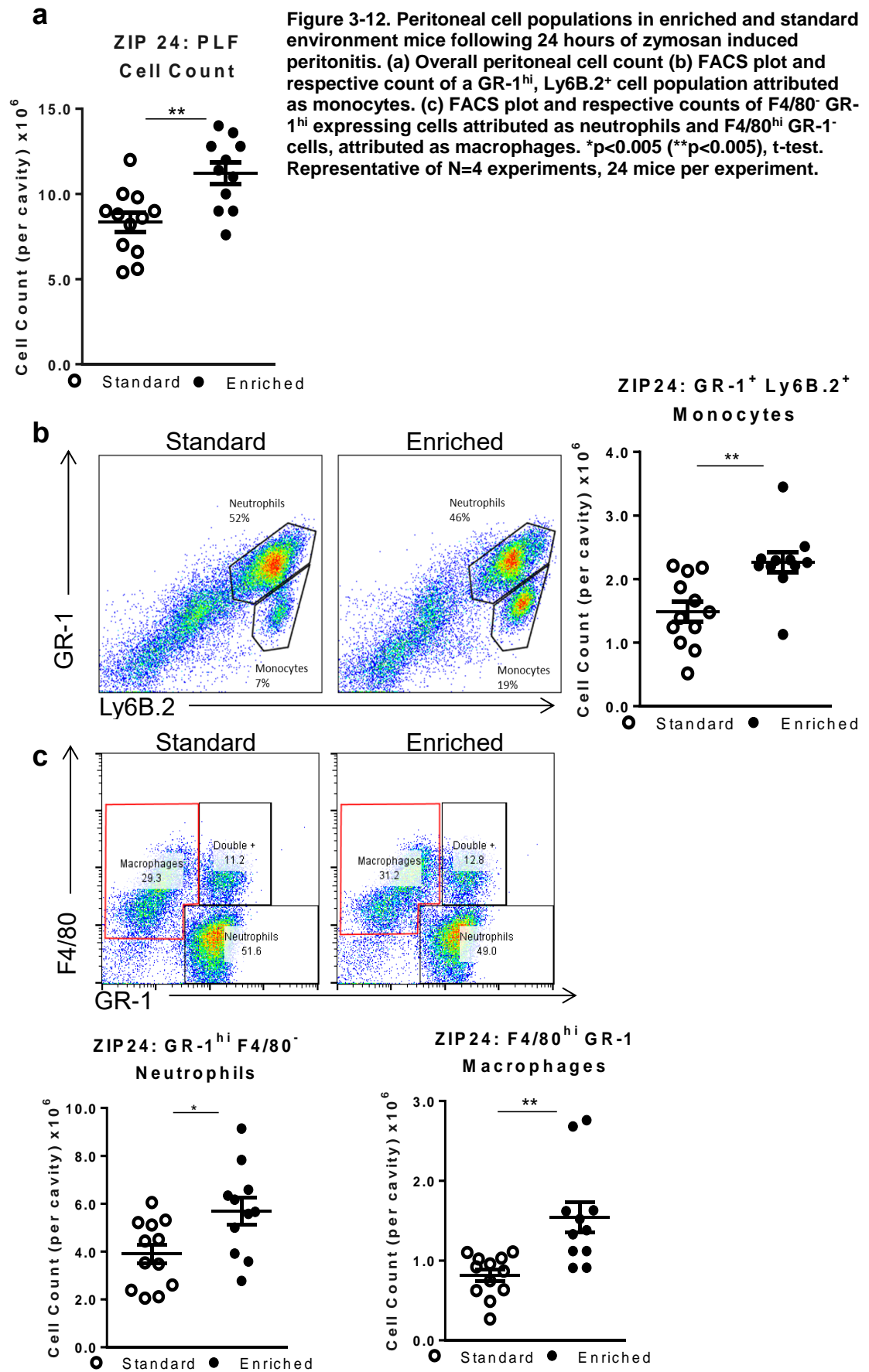


**Figure 3-11. Bone marrow cell populations in mice from enriched and standard environments following six hours of zymosan induced peritonitis. (a) Total tibial myelocytes and leukocytes. (b) FACS plot and respective count of GR-1 (granulocyte) CD11b (monocyte lineage) positive cells. N=4 experiments, 24 mice per experiment.**

### **3.4 Zymosan Induced Peritonitis Twenty-Four Hour Time Point**

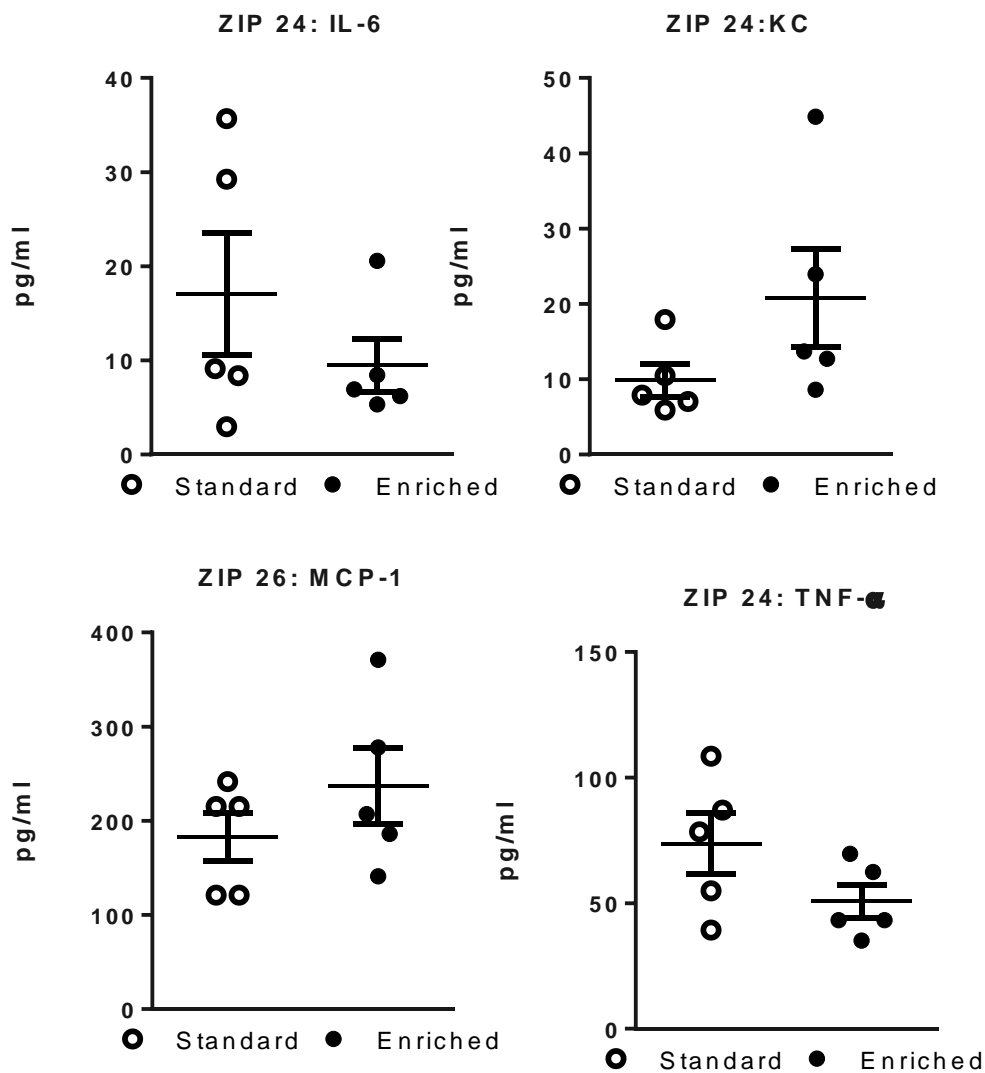
#### **3.4.1 ZIP 24: Cellular Profile of Peritoneal Lavage**

The 24 hour time point of ZIP is typically associated with a transition from a neutrophil focused to a more monocyte/macrophage led immune response [Ajuebor et al., 1998]. Microscopic analysis of lavage fluid extracted at this point supported these findings with cells of a monocyte/macrophage type morphology identified among the neutrophils that dominated the peritoneal lavage at the earlier six hour time point (3.3.1). Enriched animals still possessed a significantly higher number of cells (~20%) compared to those of the standard group (Figure 3-12a). Concordant with the six hour time point FACS analysis identified higher numbers of GR-1<sup>hi</sup>, F4/80<sup>-</sup> neutrophils and GR-1<sup>hi</sup>, Ly6B.2<sup>+</sup> monocytes in the enriched group (Figure 3-12b&c). In contrast to the six-hour time point, monocytes now formed a larger proportion of the overall peritoneal population (24 hours ~20% 6 hours <5%) (Figure 3-12b). An additional F4/80<sup>hi</sup> GR-1<sup>-</sup> population was also identified and ascribed as monocyte derived macrophages (Cash, White et al. 2009). Again numbers of this population were significantly higher in the EE group (Figure 3-12c).



### 3.4.2 ZIP 24: Peritoneal Cytokine Levels

Analysis of the extracted PLF identified levels of IL-6, KC and MCP-1 had fallen strongly in both groups when compared to readouts taken from the six hour time point (3.3.2). TNF- $\alpha$  levels remained roughly consistent. Again, while no significant differences in peritoneal cytokine levels were found between groups a concurrent trend for reduced IL-6 and TNF- $\alpha$  and increased KC and MCP-1 were observed across both repeat experiments (Figure 3-13).



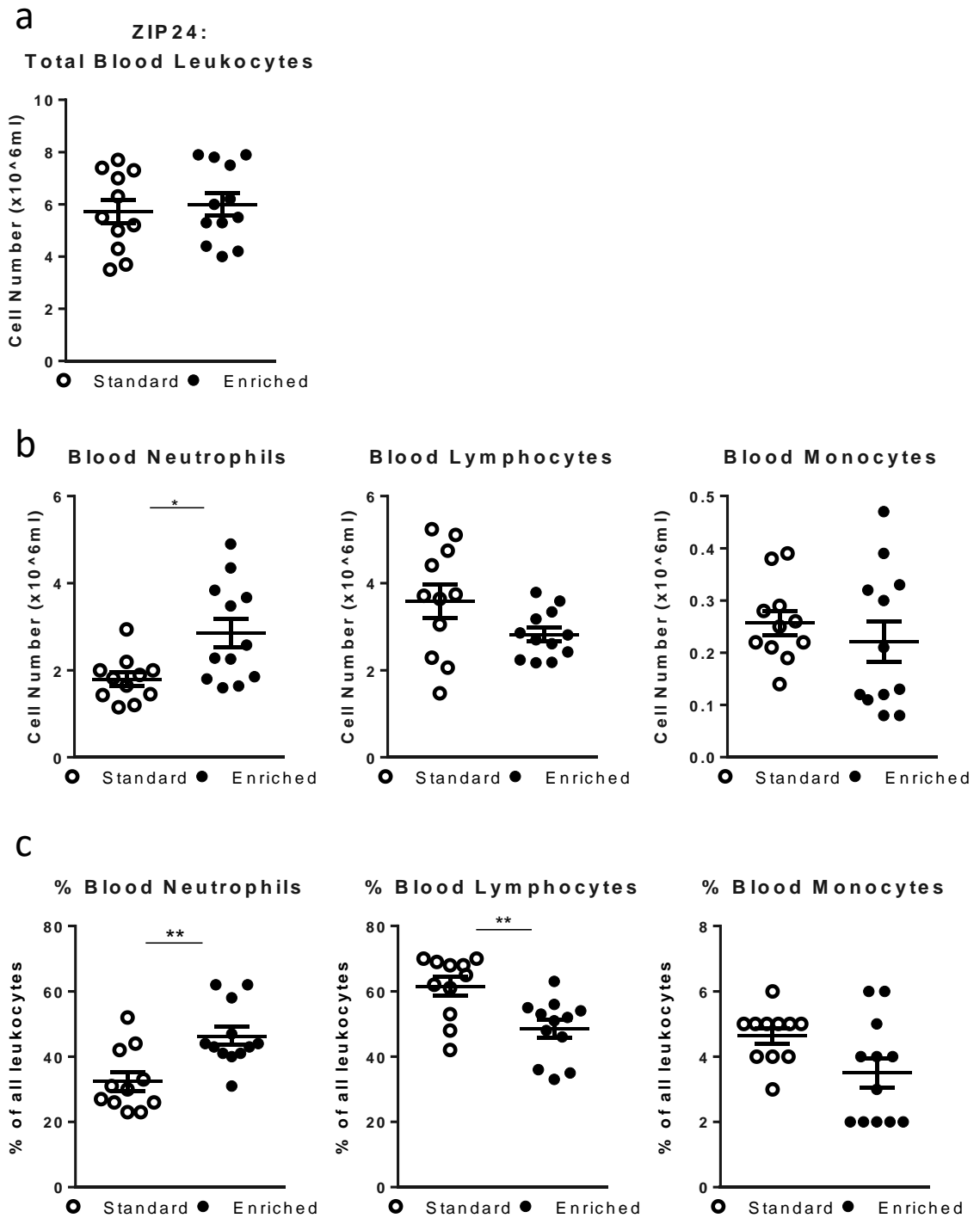
**Figure 3-13. Comparative cytokine levels in the peritoneum of animals subject to 24 hours ZIP. Comparative levels of (reading top to bottom left to right): Interleukin six, keratinocyte chemoattractant, macrophage chemoattractant protein 1 and tumour necrosis factor alpha. Results representative of N=2 experiments, 10 mice per experiment (third repeat experiment failed to yield any data).**

### **3.4.3 ZIP24: Blood Cellular Profile**

Concordant with basal and ZIP six hour analysis no difference in overall numbers of blood leukocytes were identified between the two mouse groups (Figure 3-14a). Mice from the enriched group presented a significantly higher overall number of circulating neutrophils (~25%) compared to SE animals and a correspondingly lower number of circulating lymphocytes (~15%) (Figure 3-14b).

Analysis of relative proportions of leukocytes in circulation revealed that across both groups the overall percentage of neutrophils had dropped by roughly 20% in comparison to results obtained at the six hour time point (3.3.3). Relative lymphocyte populations had increased correspondingly. Despite this shift, enriched animals still maintained a significantly higher proportion of neutrophils (20%) and lower proportion (15%) of lymphocytes in circulation compared to animals housed in a standard environment (Figure 3-14c).

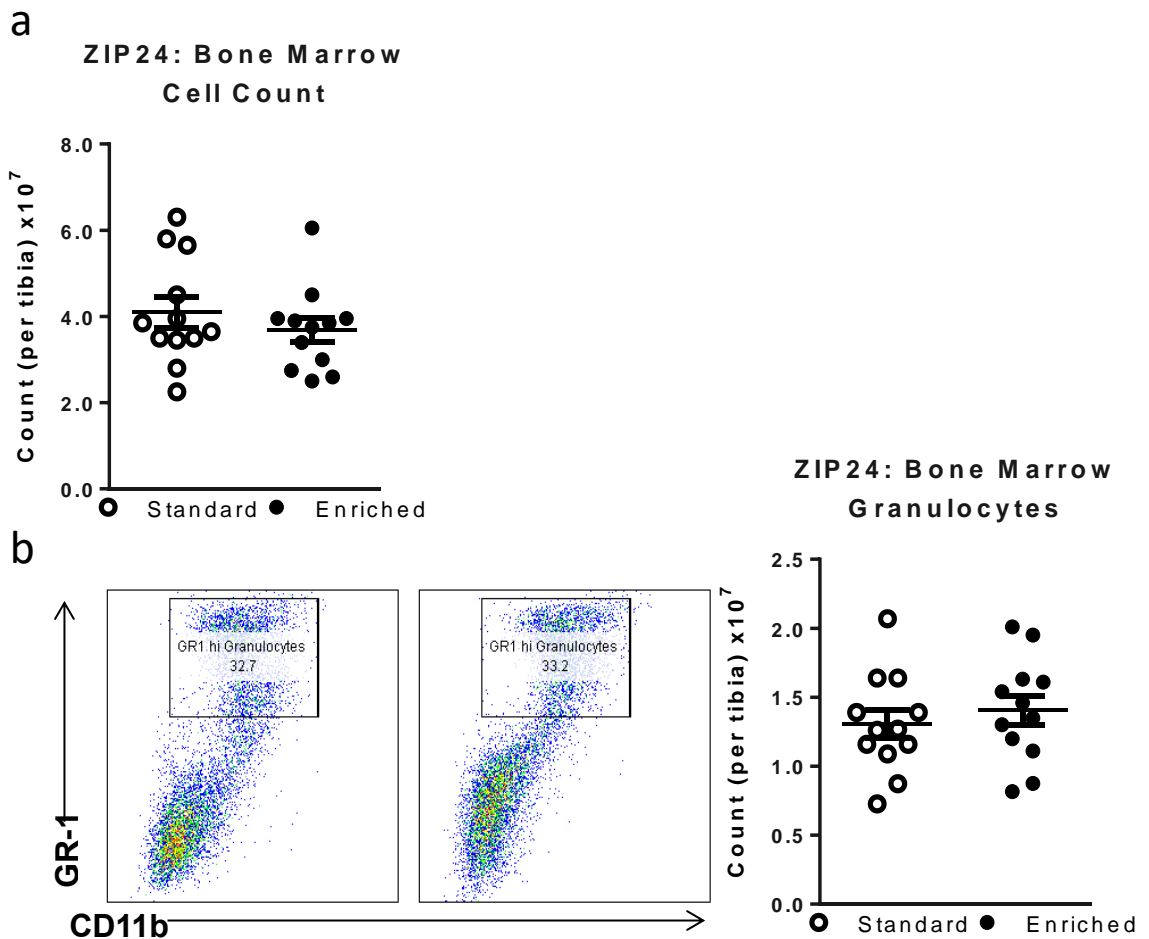




**Figure 3-14. Circulating blood cell populations in enriched and standard environment mice following 24 hours of zymosan induced peritonitis. (a) Total blood leukocyte count (b) Individual leukocyte population counts (c) Relative percentages of individual leukocyte populations.**  
\* $p < 0.005$  (\*\* $p < 0.005$ ), t-test. Representative of N=3 experiments, 24 mice per experiment.

### 3.4.4 ZIP 24: Bone Marrow Cellular Profile

Following 24 hours of ZIP overall cell numbers in the bone marrow had recovered to near basal levels in both groups (3.2.4), transient neutropenia followed by rapid neutrophil re-population such as this has been reported in previous studies [Chervenick et al., 1968] with no significant difference in cell counts (Figure 3-15a). GR-1<sup>+</sup>, CD11b<sup>+</sup> populations had returned to an expression profile similar to that found in basal analysis (3.2.1), accounting for roughly one third of the cells in both groups with no significant difference in numbers (Figure 3-15b).



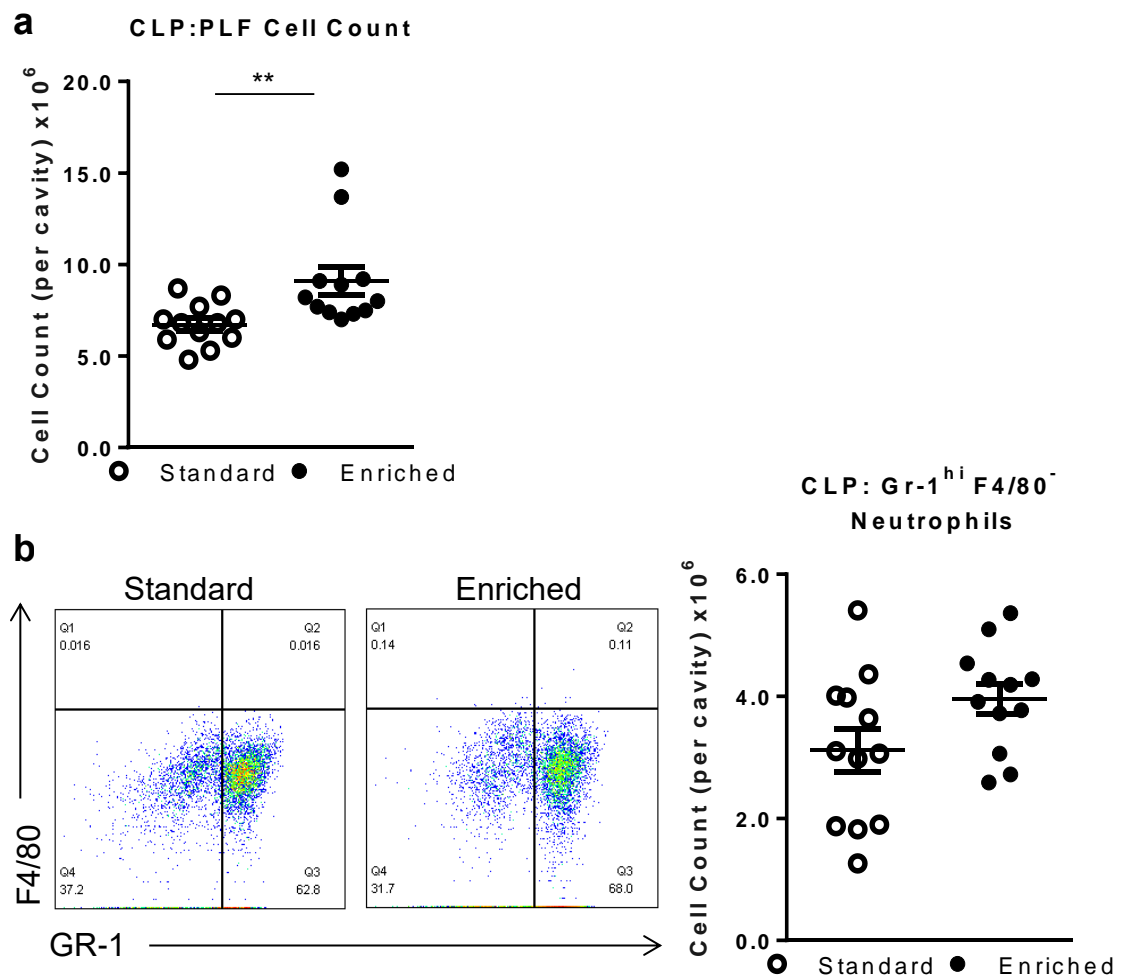
**Figure 3-15. Tibial bone marrow cell population in mice from enriched and standard environments following 24 hours of zymosan induced peritonitis. (a) Total tibial myelocytes and leukocytes. (b) Scatter plot and respective calculated counts of GR-1 (granulocyte) CD11b (monocyte lineage) positive cells. Representative of N=2 experiments, 24 mice per experiment.**

### **3.5 Caecal Ligation and Puncture**

Having established that housing in an enriched environment influences the cellular response to an exogenously administered inflammagen we moved on to explore how enriched animals would respond to a more complex live bacterial model of inflammatory insult. Accordingly, we chose to employ a mouse model of endogenous bacterial sepsis induced by caecal ligation and puncture (CLP). CLP induces a diffuse polymicrobial sepsis originating in the peritoneal cavity and disseminating into circulation usually within hours of the procedure. While CLP is almost invariably fatal to the subject [Dejager et al., 2011], assessment of immunological parameters prior to onset of death provides a valuable means of identifying the strength and direction of the immune-inflammatory response in these animals.

### 3.5.1 CLP: Peritoneal Cell Profile

Similar to ZIP, CLP induces a rapid and acute inflammatory peritonitis, characterised by an influx of innate immune leukocytes into the peritoneal cavity [Dejager et al., 2011]. Twelve hours following the induction of CLP a count of the extracted PLF presented a dramatically heightened number of cells in the peritoneum of both experimental groups compared to baseline data (3.2.1) with microscopic analysis suggesting a large number of these cells to be granulocytic. Comparative cell counts revealed EE mice to possess a significantly higher cell number compared to SE animals (Figure 3-16a). Flow cytometric analysis revealed a corresponding trend (non-significant) for a heightened number of GR-1<sup>hi</sup> F4/80<sup>-</sup> neutrophils in the peritoneal cavity of EE animals (Figure 3-16b).



**Figure 3-16. Peritoneal cell populations in enriched and standard environment mice following 12 hours CLP.** (a) Peritoneal cell count of mice from standard and enriched environments subject to 12 hours of CLP. (b) Representative FACS profiles of F4/80 vs GR-1 expressing cells and comparative plots of total peritoneal populations of GR-1<sup>hi</sup> F4/80<sup>-</sup> neutrophils in SE and EE mice \* $p < 0.005$  (\*\* $p < 0.005$ ), t-test. Representative of N=2 experiments, 24 mice per experiment.

### 3.5.2 CLP: Peritoneal Cytokine Levels

Analysis of extracted PLF (Figure 3-17) and serum (data not shown) identified heightened levels of pro-inflammatory cytokines in both groups. Levels were notably higher than those found at the six hour time point for ZIP, suggesting a far more severe inflammatory response. No significant difference was found in relative levels of cytokine expression between groups.

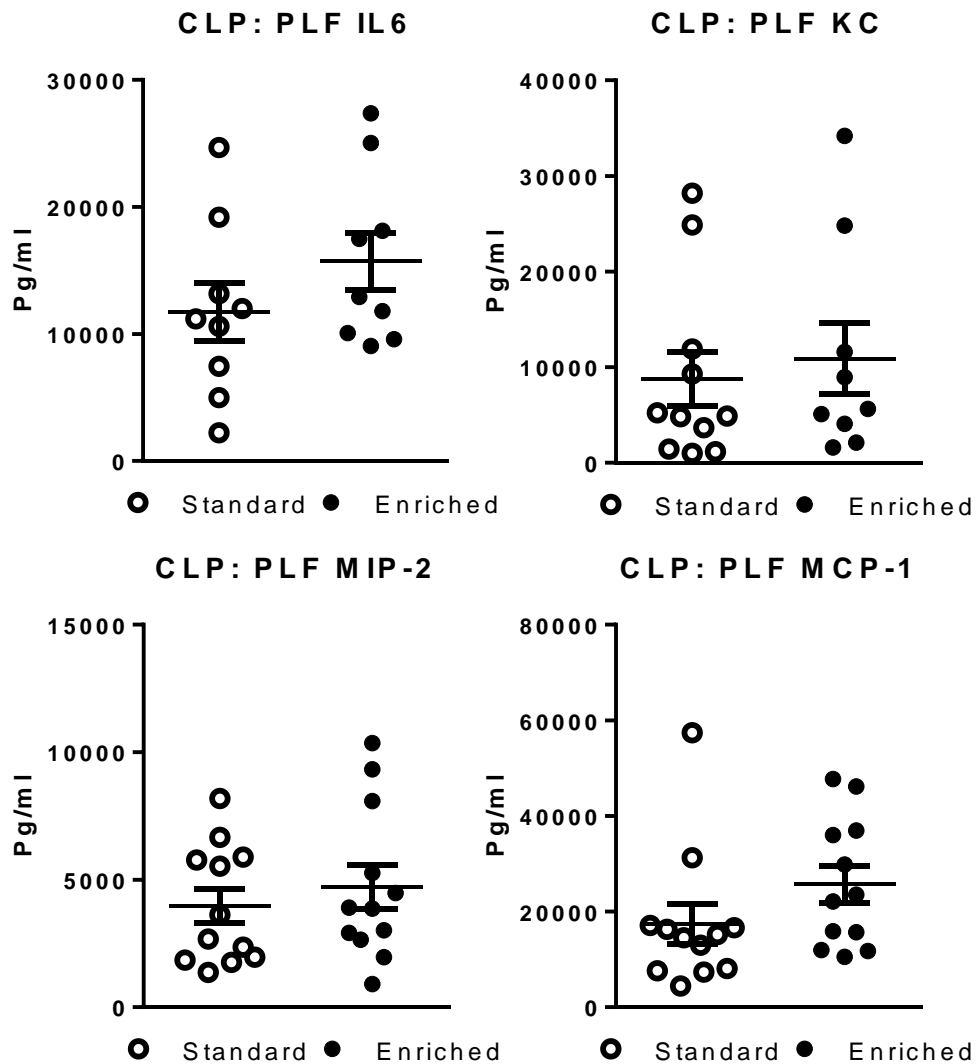
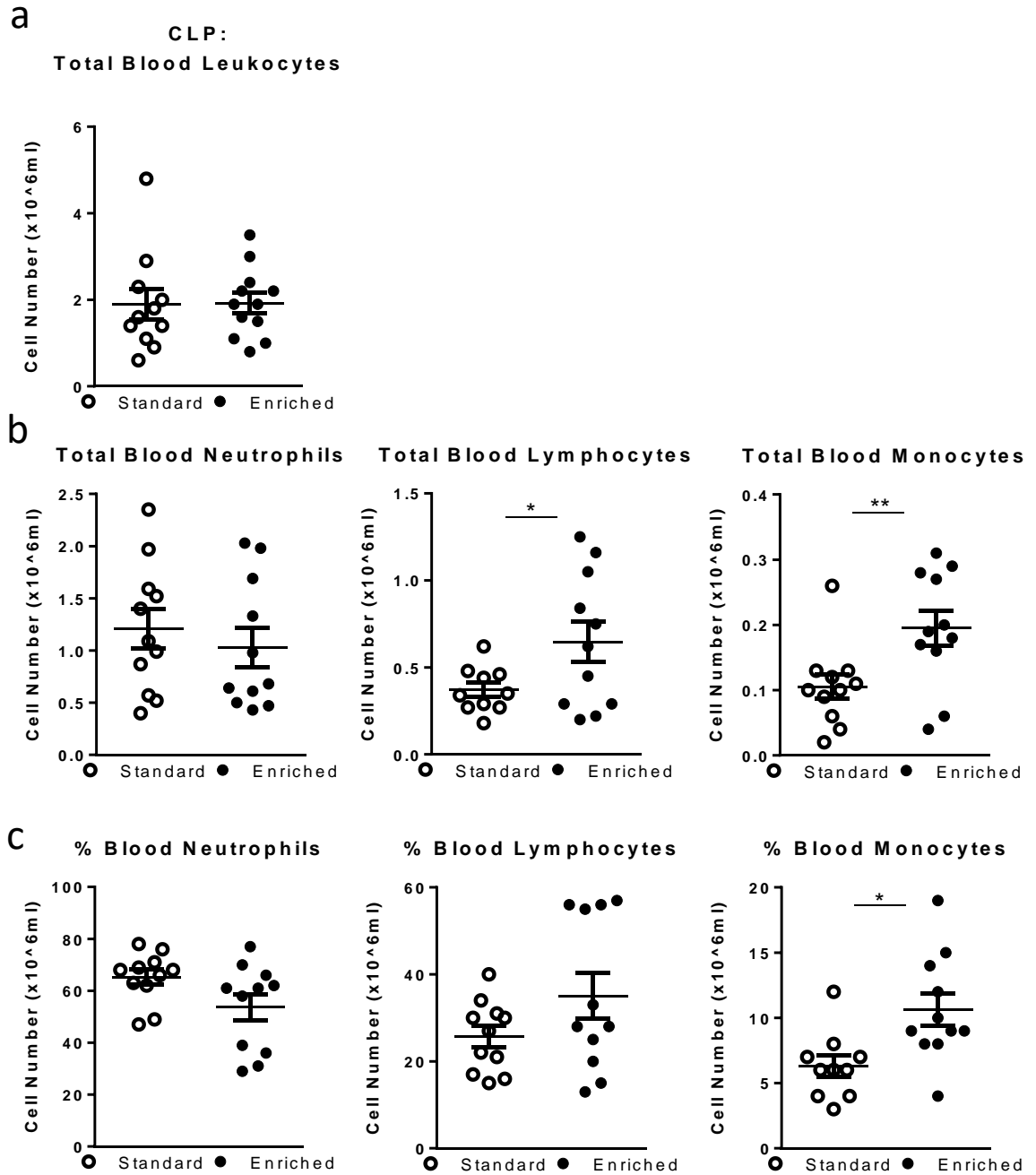


Figure 3-17. Comparative cytokine levels in the peritoneum of mice from and enriched or standard environment subject to 12hours CLP.. Comparative levels of (reading top to bottom left to right): Interleukin six, keratinocyte chemoattractant, macrophage inflammatory protein-2 and macrophage chemotractant protein 1. Representative of N=2 experiments, 24 mice per group.

### **3.5.3 CLP: Blood Cellular Profile**

Blood profiling revealed that both animal groups presented a notably reduced number of circulating leukocytes compared to basal results (3.2.3) (Figure 3-18a). Such a drop in blood leukocytes has been previously reported as a symptom of early stage sepsis [Nguyen and Smith, 2007]. No differences in comparative numbers of total blood leukocytes were found (Figure 3-18a). Significantly higher numbers of circulating lymphocytes and monocytes were identified in EE animals with a concurrent trend towards lower neutrophil numbers when compared to SE mice (Figure 3-18b).

A similar pattern was found in analysis of relative proportions of leukocytes within blood, with a significantly highly percentage of monocytes found in EE animals and a corresponding trend towards increased lymphocytes and reduced neutrophils compared to SE mice (Figure 3-18c). For both groups neutrophils were the dominant leukocyte population in circulation (~40-50%) (Figure 3-18c). This contrasts to animal in a basal state which typically presented a proportion of ~70-80% circulating lymphocytes.



**Figure 3-18. Circulating blood cell populations in mice from a standard or enriched environment subject to 12 hours of CLP. (a) Total blood leukocyte count (b) Individual leukocyte population counts (c) Relative percentages of individual leukocyte populations. \* $p < 0.005$  (\*\* $p < 0.005$ ), t-test. Representative of N=2 experiments, 24 mice per experiment.**

### 3.5.4 Caecal Ligation and Puncture, Bacterial Colony Counts

In order to assess the overall bacterial load of animals subject to CLP, serially diluted samples of extracted blood and PLF were seeded on agar plates and incubated overnight. The following day resulting bacterial colonies found upon the plates were quantified. Colony counts of the PLF showed a trend (non-significant) for a lower overall bacterial count in the peritoneal cavity of enriched animals (Figure 3-19a). Colony counts from blood samples were significantly lower in EE compared to SE mice (Figure 3-19b). Suggesting enriched animals possess a reduced number of bacteria in blood circulation at this 12-hour time point compared to those housed in a standard lab environment.

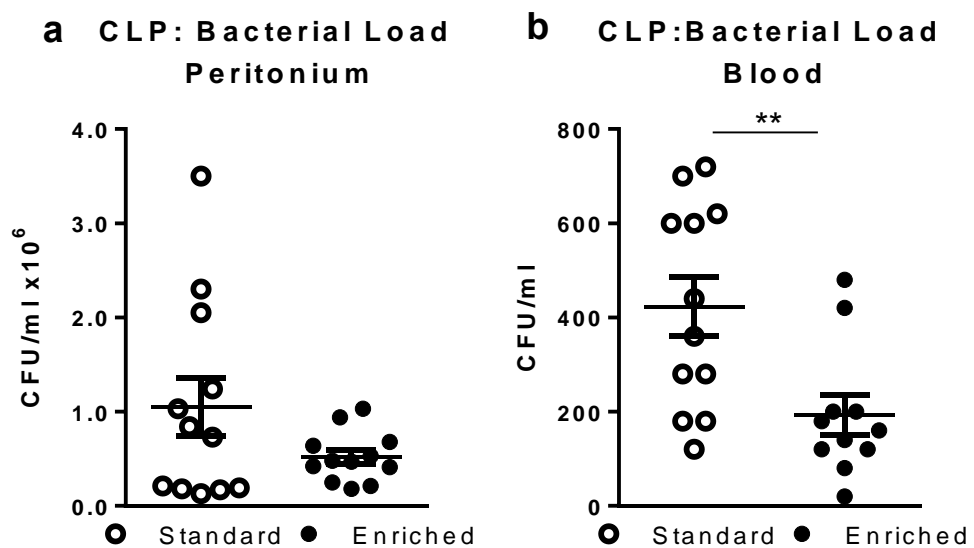
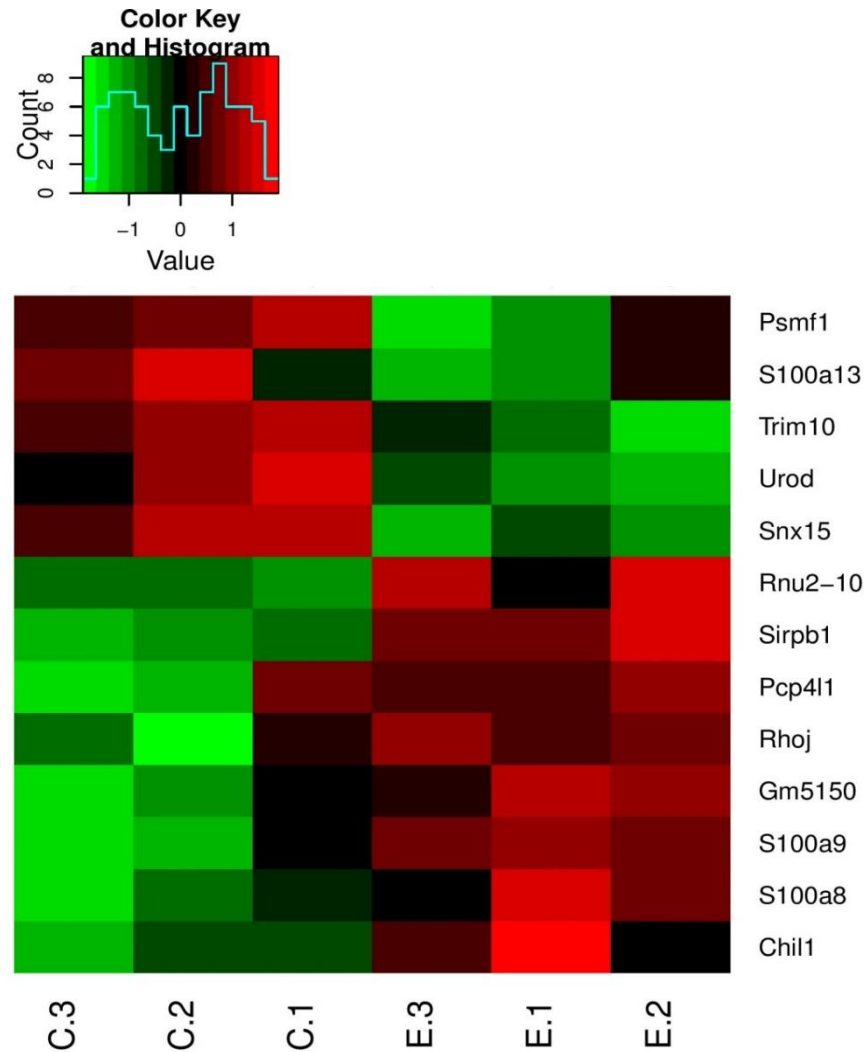


Figure 3-19. Respective bacterial colony counts in (a) peritoneal lavage fluid and (b) blood populations of animals raised in an enriched or standard environment subject to 12 hours of CLP. Values represented a colony forming units per ml. \* $p < 0.005$  (\*\* $p < 0.005$ ), t-test. Representative of N=2 experiments, 24 mice per experiment.



### 3.6 Genetic Profiling of Whole Blood from Environmentally Enriched Mice

Aiming to identify potential mechanisms responsible for the altered cellular immune response in enriched mice we compared the genetic fingerprints of whole blood taken from EE and SE animals in a basal immune state. Using fold change (FC) >2 or <0.5 and non-adjusted p-value <0.05, of the 34,760 probes present on the chip, eight genes were identified as upregulated and five genes downregulated (Figure 3-20), listed in Table 9.



**Figure 3-20. Microarray heatmap of blood RNA extracted from mice raised in an enriched vs standard lab environment. Gene expression across the blood of three mice per experimental group. Colour scale representing most downregulated (bright green) to most upregulated (bright red).**

**Table 9 Differentially expressed genes in the blood RNA of mice raised in an enriched vs standard lab environment**

Gene	Codes	Function (when known)	Immune relevance (if any)
<b>Down regulated genes in enriched animals</b>			
Psmf1	Proteasome inhibitor PI31 subunit	Potentially inhibits the activation of the proteasome by the 11S and 19S regulators [Li et al., 2014]	Inhibit MHCI peptide processing
S100a13	S100 calcium-binding protein A13	Involved in Regulation of Fibroblast Growth Factor-1 and p40 Synaptotagmin-1 Release in Vitro [Carreira et al., 1998]	Implicated in the TH17 differentiation pathway [Nimmo et al., 2012]. Upregulated in response to bacterial activation of neutrophils [McLachlan et al., 2004]. Promotes expression of IL1- $\alpha$ from fibroblast and monocyte cell lines[Mandinova et al., 2003]
Trim10	Tripartite motif containing 10	Erythroid cell terminal differentiation [Blaybel et al., 2008]	-
Urod	Uroporphyrinogen decarboxylase	Key enzyme in heme synthesis pathway [De Verneuil et al., 1983]	-
Snx15	Sorting nexin-15	Transport protein – implicated in furin localisation [Phillips et al., 2001]	-
<b>Up regulated genes in enriched animals</b>			
Rnu2-10	U2 small nuclear RNA 10	-	-
Sirpb1	Signal-regulatory protein beta 1 (SRPb1)	Glycoprotein receptor that recruits and signals via the tyrosine phosphatases SHP-1 and SHP-2 [Alblas et al., 2005].	Macrophage NO production via JAK/STAT [Alblas et al., 2005]. Integrin-Mediated Monocyte Adhesion, Trans-endothelial Migration and Phagocytosis[Liu et al., 2008]
Pcp4l1	Purkinje cell protein 4 like 1	expressed in specific domains of the developing brain [Bulfone et al., 2004]	-
Rhoj	Rho-related GTP-binding protein	Focal adhesion molecule disassembly and angiogenesis[Wilson et al., 2014, Kim et al., 2014]	Upregulated in response to bacterial infection in zebrafish [Salas-Vidal et al., 2005]
Gm5150	predicted gene	-	-
S100a9	S100 calcium-binding protein A9,migration inhibitory factor-related protein 14 (MRP14), Calgranulin B	-Binds S100A8 below to form the neutrophil associated molecule calprotectin	Promotes leukocyte recruitment, neutrophil and macrophage accumulation, macrophage cytokine production, and SMC proliferation[Croce et al., 2009]
S100a8	Calgranulin A	Binds S100A9 above to form calprotectin neutrophil molecule	Regulator of innate inflammation [Ehrchen et al., 2009] leukocyte, (neutrophil) trafficking[Kerkhoff et al., 1998] implicated in colitis [Røseth et al., 1997]
Chil1	Chitinase-3-like protein 1 (CHI3L1)	A member of the family of mammalian chitinase-like enzymes. Has no direct enzymatic function	Secreted by activated macrophages and neutrophils, upregulated during inflammation and the TH2 response [Lee et al., 2008, Lee et al., 2009, Kzhyshkowska et al., 2006]

### 3.6.1 Real Time PCR of Enriched vs Standard Mouse Whole Blood RNA

A literature search was performed for each identified gene and four of particular immunological interest (s100a8, s100a9, chil1, srpb1) were selected for further study. In order to confirm the heightened expression reported by the microarray, real time PCR was carried out using primers specific for each of the four genes. A significantly higher level of expression (~1.5- 2 fold) for three of the selected genes (relative to housekeeping gene GADPH) was identified in EE mice compared to SE (Figure 3-21).

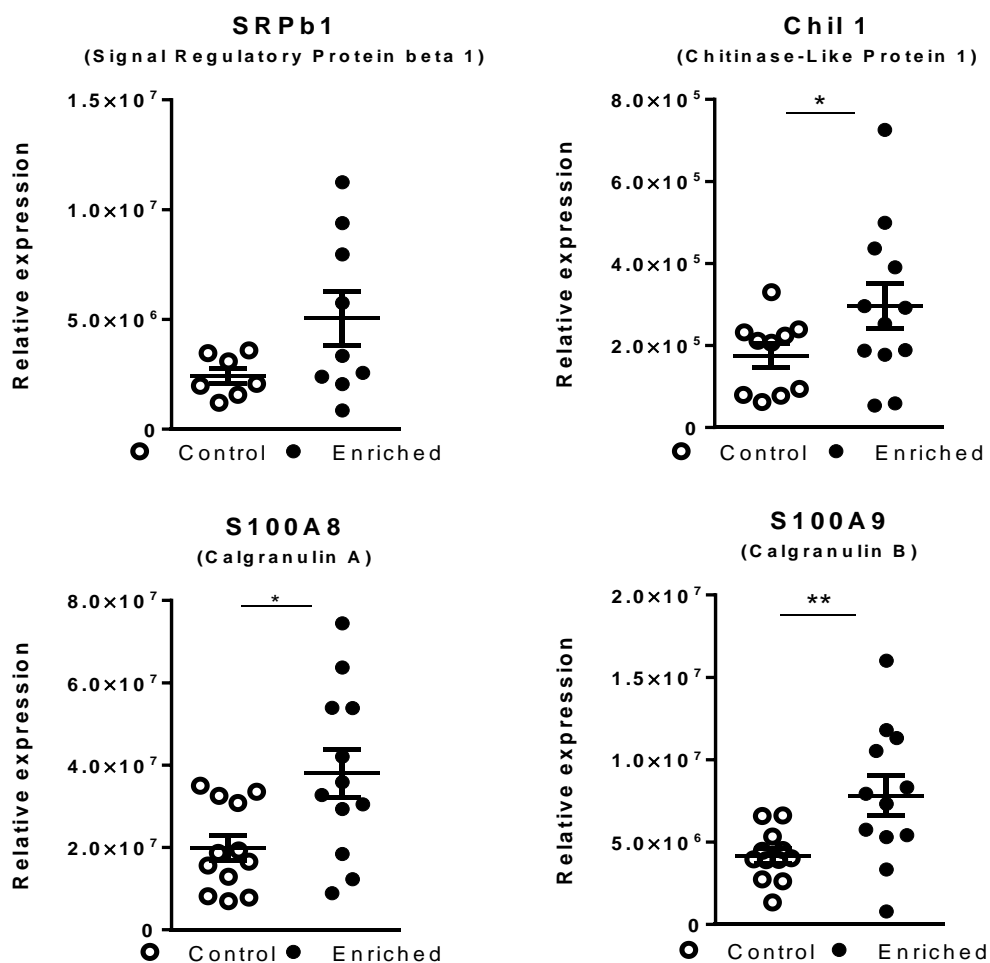


Figure 3-21. Real time PCR analysis of genes found to be differentially expressed in mice raised in an enriched vs standard environment. From top left: SRPb1 – Signal Regulatory Protein Beta One Alpha, Chil-1 – Chitinase like protein one, S100A8 – Calgranulin A, S100A9 – Calgranulin B. \* $p < 0.005$  (\*\* $p < 0.005$ ), t-test. Representative of N=1 experiments, 24 mice per experiment.

### **3.6.2 Analysis of S100A8 and S100A9 Protein Expression**

We then assessed whether this heightened immunomodulatory gene expression program was evident at the translational level. We elected to focus on the protein products of genes S100A8 and S100A9 named calgranulin A and B respectively. In addition to having individual modulatory functions calgranulin A and B are able to form the heterodimeric complex calprotectin that possesses further, distinct antimicrobial properties. Both calprotectin and its constituents are of current interest to clinical research, primarily for their involvement in the regulation of colitis and sepsis [Ehrchen et al., 2009]. For this reason and in light of the commercial availability of reliable antibodies specific to these proteins we chose to focus upon them for protein analysis. Measuring levels of these two proteins in the PLF (Figure 3-22a), blood plasma (Figure 3-22b) and total cell lysate (Figure 3-22c) of EE or SE animals subject to no inflammatory insult revealed significantly higher levels of these two soluble mediators in EE animals. Levels of freely circulating calgranulin A in the PLF and serum of SE mice were so low as to be undetectable, with calgranulin B being found at detectable – but comparatively very low levels in each sample. Both proteins were found at more substantial levels within the cell lysate of SE mice, though still significantly lower compared to samples taken from EE animals (Figure 3-22c). This would suggest that calgranulin A and B is both being produced at higher levels in enriched animals and (unlike SE mice) – both these proteins are being actively released into the blood stream.

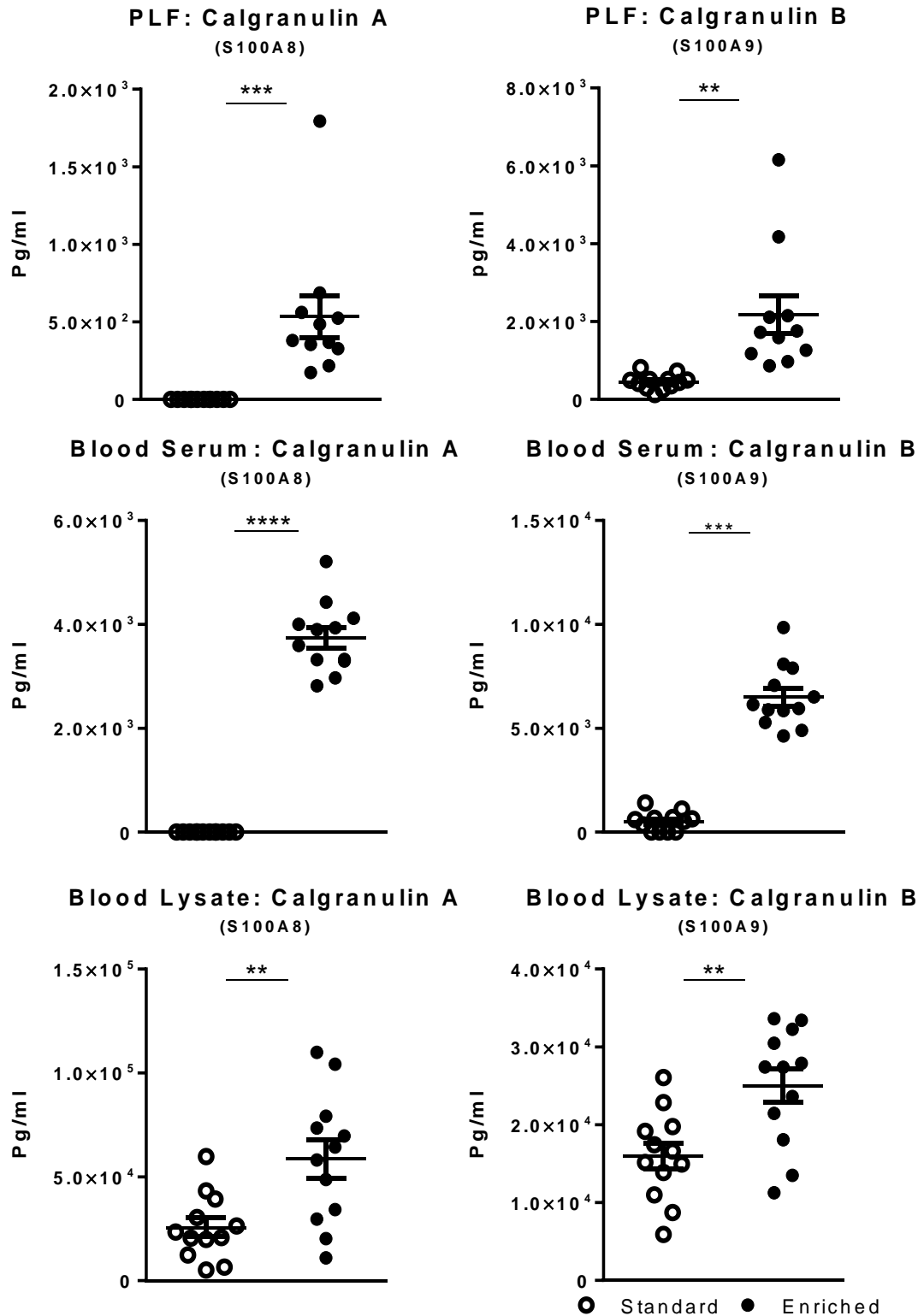


Figure 3-22. Comparative expression of the individual dimers of calprotectin (S100A8 & S100A9) in animals housed in an enriched or standard lab environment. Protein content in: (a) Peritoneal lavage. (b) Blood serum. (c) Blood pellet lysate. Samples were taken from enriched (EE) and standard environment mice (SE) subject to no additional immune modulation. \*p<0.005 (\*\*\*\*p<0.00005), t-test. Representative of N=1 experiments, 24 mice per experiment.

### **3.7 *Ex vivo* Cell Activity Assays**

Having observed its effects at a systemic and genetic level we next chose to investigate if environmental enrichment exerts an influence on the activity of individual leukocytes *ex vivo*. We chose to focus on two cell populations shown to be active in the data acquired from ZIP and CLP; neutrophils and macrophages. Each was subject to a series of assays designed to determine their overall level of immune activation and function.

#### **3.7.1 Neutrophil Activation**

Samples of blood neutrophils from EE and SE animals were stimulated with the leukocyte activating molecules; platelet activating factor (PAF) or Tumour necrosis factor alpha (TNF- $\alpha$ ) for 15 or 30 minutes. Following stimulation, the cell populations were analysed for the expression of activation markers CD11b and CD62L. A quantitative increase in the former and decrease of the latter (shedding) is considered indicative of a state of activation [Cooper et al., 2008]. For each stimuli and respective readout cells from both EE and SE mice demonstrated increased expression of CD11b and shedding of CD62L. However, no significant differences (calculated by 2-way ANOVA) were identified in the relative change of expression between both groups (Figure 3-23).

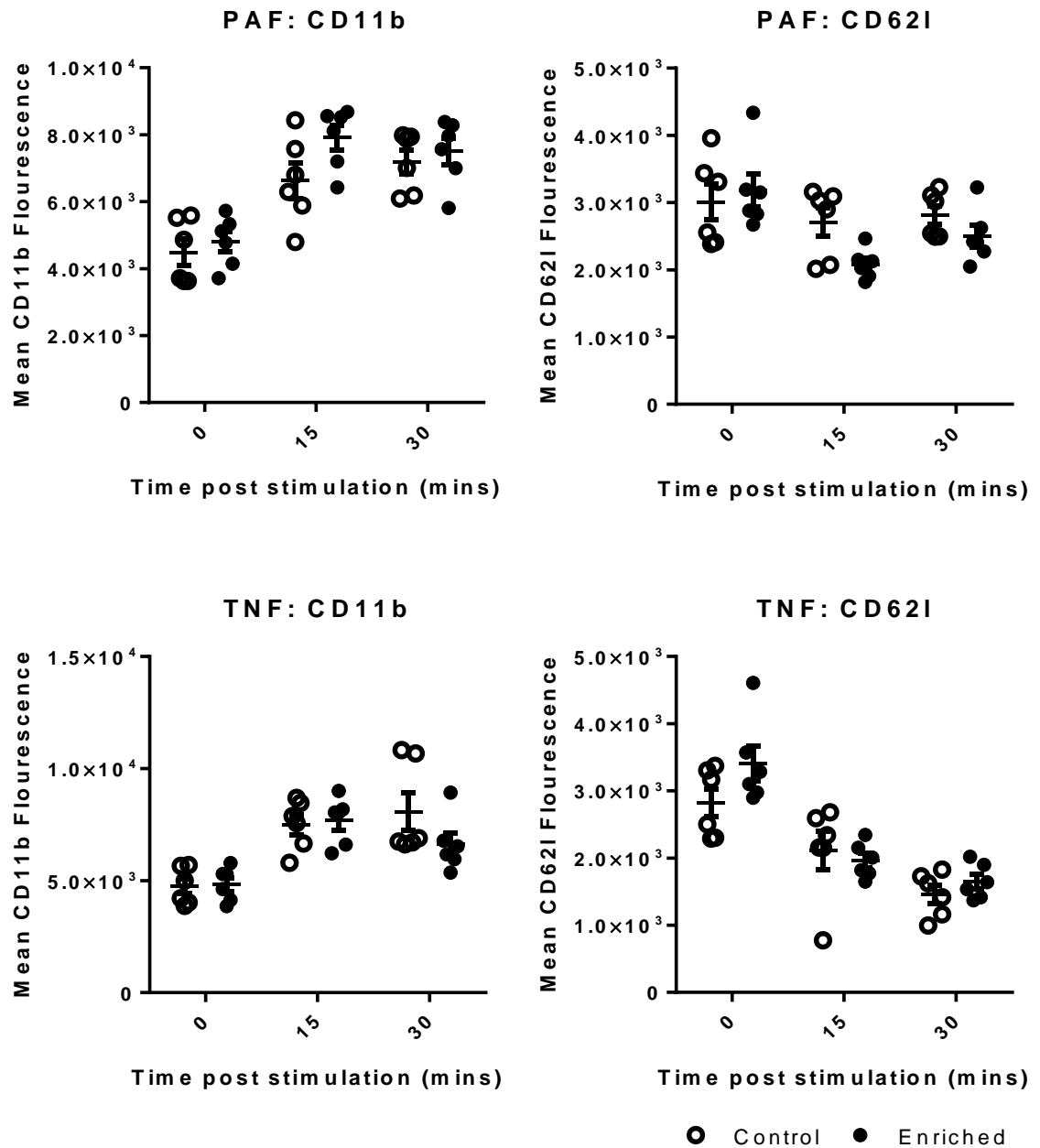


Figure 3-23. Comparative levels of CD11b and CCD62L expression in blood neutrophils extracted from mice housed in an enriched or standard environment. Following stimulation with a TNF- $\alpha$  (50 ng/ml), b Platelet activating factor (PAF) (10-9M) for 15 or 30 minutes. Values are presented as individual data points  $\pm$ s.e.m. of six mice N=2 experiments. Significance calculated (none found) by two-way ANOVA ( $P < 0.05$ ).

### 3.7.2 Macrophage Phagocytosis

Biogel elicited macrophages isolated from the peritoneal cavity of EE and SE and animals had their capacity for phagocytosis assessed by culture with BODIPY-conjugated *E.coli* (dried). Macrophages isolated from enriched mice were found to phagocytose a significantly higher quantity of bacteria over the course of the one hour incubation period than those from animals housed in a standard lab enclosure (Figure 3-24).

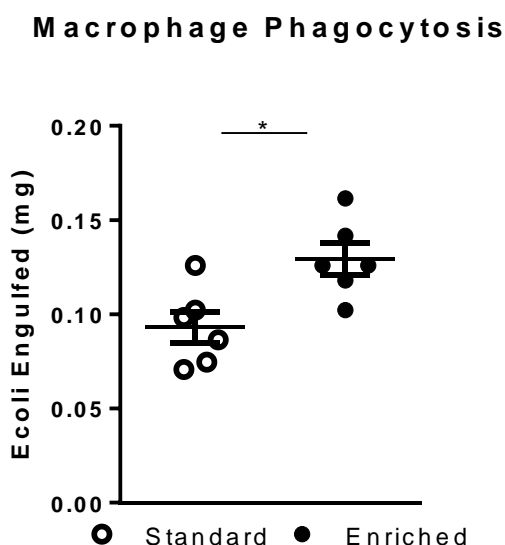
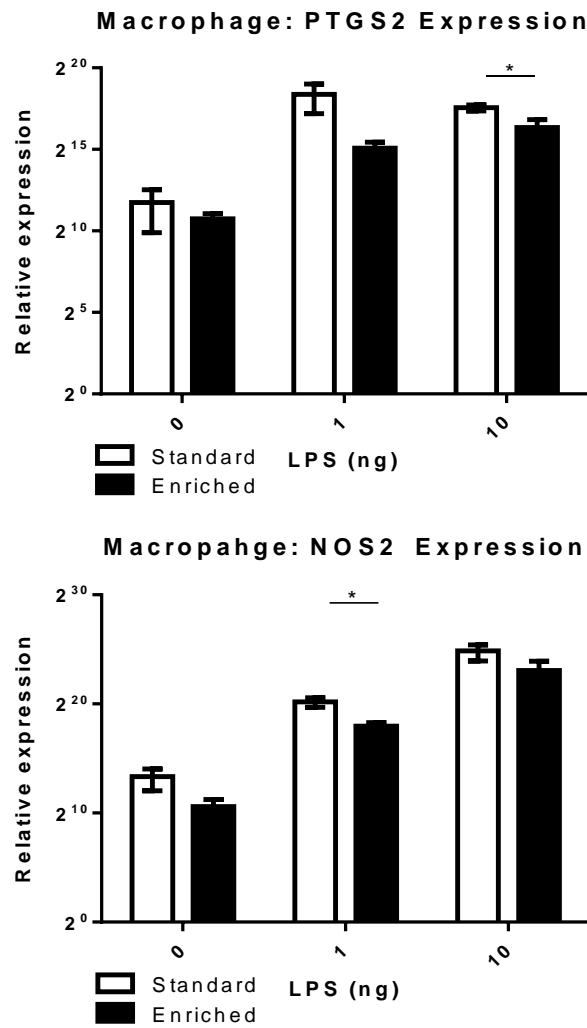


Figure 3-24. Phagocytic capacity of biogel elicited macrophages extracted from animals housed in an enriched or standard environment . Comparative levels of BODIPY linked *E.coli* phagocytosed in one hour. \* $p < 0.005$ , t-test. Representative of N=3 experiments, 12 mice per experiment.



### **3.7.3 RT PCR of Activated Macrophages**

In order to further investigate the activity of EE macrophages. Isolated cells were stimulated with 1 or 10 ng of LPS. The isolated RNA from stimulated cells was subject to RT-PCR to detect expression of two key markers of macrophage activation: PTSG2 which codes for Prostaglandin-endoperoxide synthase 2 (also known as cyclooxygenase-2 – COX-2), an enzyme responsible for the generation of the prostaglandin family of inflammatory mediators. NOS-2, which codes for nitric oxides synthase 2 (iNOS), an enzyme that heightens macrophage cytotoxic function by catalysing the generation of nitric oxide. EE macrophages were found to express lower levels of both genes compared to those isolated from SE animals. These lower expression levels were found to be significant for PTGS2 following stimulation with 1ng of LPS (Figure 3-25a) and for NOS-2 following stimulation with 10ng LPS (Figure 3-25b).



**Figure 3-25. Comparative expression of PTGS2 and NOS2 in LPS stimulated macrophages isolated from animals housed in an enriched or standard environment. (a) Relative PTGS2 expression following overnight stimulation with 0, 1 or 10 ng of LPS. (b) Relative NOS2 expression following overnight stimulation with 0, 1 or 10 ng of LPS. \* $p < 0.005$ , t-test. Representative of N=1 experiments, 24 mice per experiment.**

### 3.8 Sponge granuloma

Having investigated the effects of enrichment in the context of an isolated immune activating ligand and a live pathogenic challenge we decided to investigate the influence of EE in the context of a persistent inflammatory challenge leading to granuloma formation.

#### 3.8.1 Granuloma: Gross Anatomy

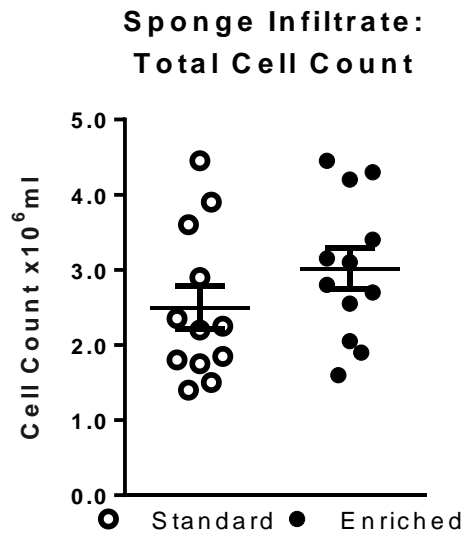
Focusing on the transitionary point between the acute and chronic/resolutionary phases of inflammation [Anderson et al., 2008] animals were sacrificed three days following sponge implantation. Post mortem analysis revealed the sponges to be surrounded by a layer of solid tissue showing some initial signs of angiogenesis (Figure 3-26). At this early stage of the granulation response no signs of fibrosis were identified.



Figure 3-26. Granulomatous tissue and sponge three days post implantation.

#### 3.8.2 Sponge Granuloma: Exudate Cellular Profiling

Due to its positioning at the foci of the granuloma response and capacity to retain infiltrated cell and soluble factors the contents of the excised sponges provide a useful picture of the immediate inflammatory environment of a granuloma. Sponge infiltrate was extracted by centrifugation. A cell count revealed no significant differences in total cell numbers between EE or SE animals (Figure 3-27).



**Figure 3-27. Comparative total cell counts of the extracted infiltrate of sponges implanted in animals from an enriched or standard environment for 72 hours. Representative of N=3 experiments, 24 mice per experiment.**

Further analysis of isolated exudate cell by flow cytometry revealed the presence of two distinct but linked populations of GR-1 expressing cells GR-1<sup>high</sup> and GR-1<sup>+</sup> (Figure 3-28a). No difference in relative numbers of GR-1<sup>hi</sup> cells or total GR-1 expression was found between groups. However, enriched animals possessed a significantly higher number of GR-1<sup>+</sup> cells (Figure 3-28b). Back gating of these populations (Appendix 3) identified both to be of roughly the same size (determined by forward scatter characteristics) but of differing internal complexity (determined by side scatter characteristics). GR-1<sup>hi</sup> cell exhibited a high level of SSC, suggesting them to be granulocytic. In contrast GR-1<sup>+</sup> cells possessed a comparatively low SSC.

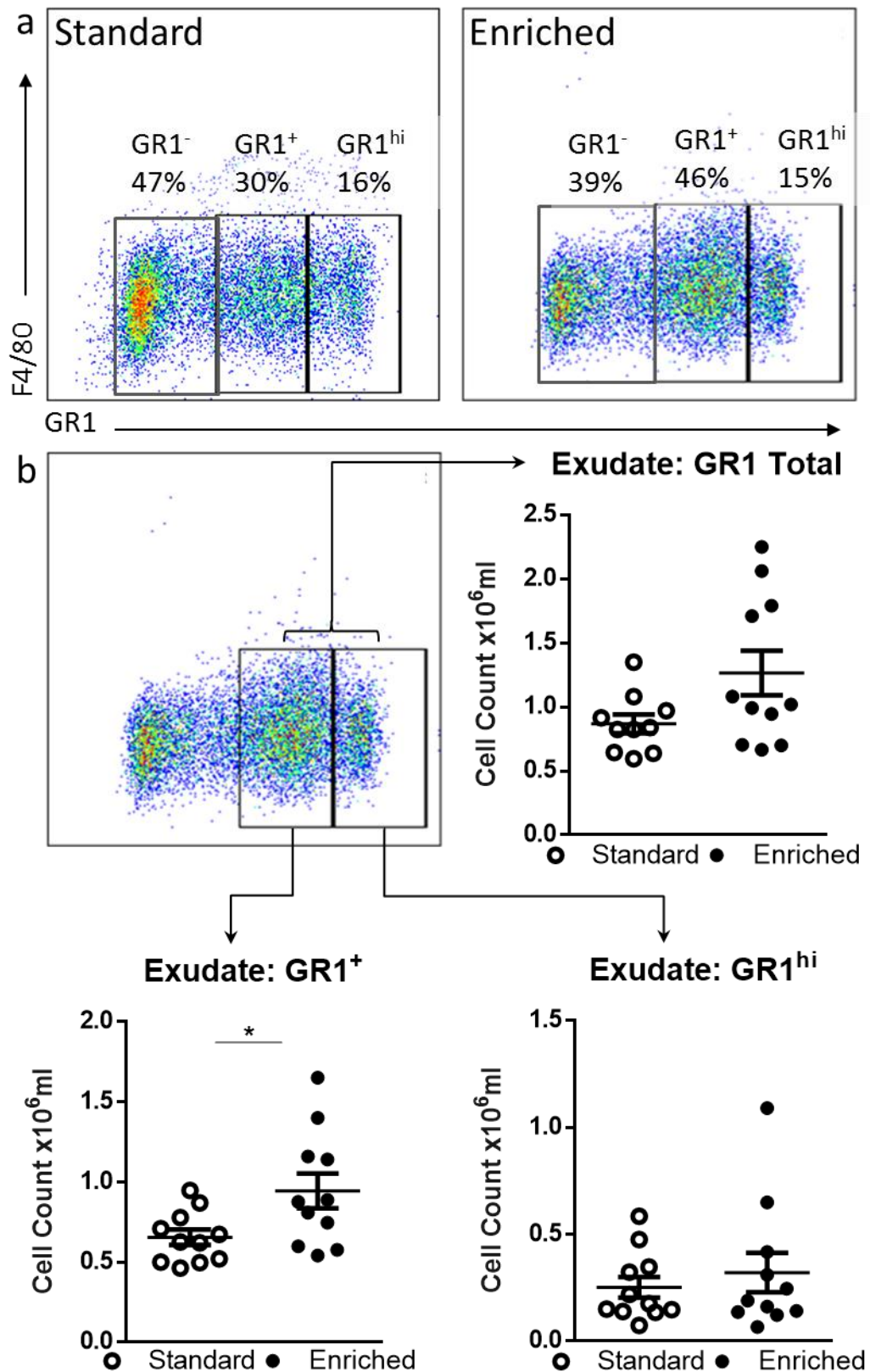
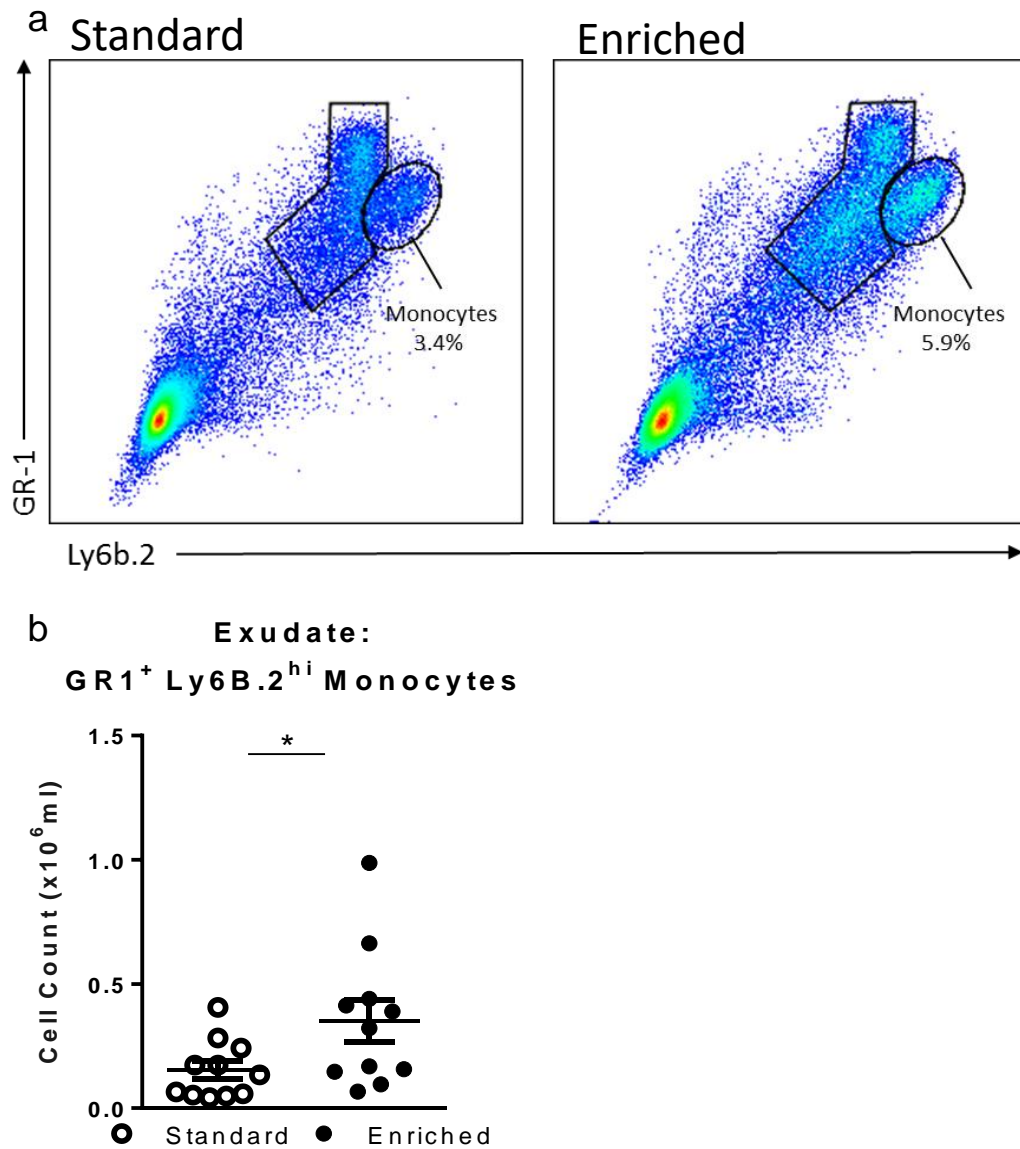


Figure 3-28. Flow cytometric analysis of cellular infiltrate taken from the extracted infiltrate of sponges implanted in animals from an enriched or standard environment for 72 hours. GR-1 vs F4/80 (a) Population stratification into GR-1<sup>-</sup>, GR-1<sup>+</sup> and GR-1<sup>hi</sup> cells. (b) Comparative cell counts of GR1 positive and high populations, individually and combined. \*p<0.005, t-test. Representative of N=3 experiments, 24 mice per experiment.

In an attempt to further delineate the components of this GR-1<sup>+</sup> population, sponge infiltrate cells were also stratified by expression of Ly6b.2 vs GR-1. Analysis revealed a small population (3-10%) of Ly6b.2<sup>hi</sup> GR1-1<sup>+</sup> cells, ascribed as inflammatory monocytes within both mouse groups. Back gating of these cells placed them in the low SSC group described in the previous section (Appendix 2). This cell population was found to be significantly higher in enriched animals (Figure 3 29).



**Figure 3-29.** Flow Cytometric analysis of cellular infiltrate taken from the extracted infiltrate of sponges implanted in animals from an enriched or standard environment for 72 hours. GR-1 vs Ly6b.2 (a) Population stratification into Ly6b.2<sup>+</sup> Gr-1<sup>hi</sup> and GR-1<sup>+</sup> Ly6b.2<sup>hi</sup> populations. (b) Relative population numbers of GR-1<sup>+</sup> Ly6b.2<sup>hi</sup> cells \*p<0.005, t-test. Representative of N=3 experiments, 24 mice per experiment.

### 3.8.3 Granuloma: Sponge Infiltrate Cytokine Analysis

We further investigated the immune milieu within the granuloma by quantifying the presence of key inflammatory cytokines; keratinocyte chemoattractant (KC), tumour necrosis factor alpha (TNF- $\alpha$ ), macrophage inflammatory protein 1 alpha (MIP-1 $\alpha$ ) and anti-inflammatory cytokine Interleukin 10 (IL-10) within the sponge infiltrate. With the exception of IL-10, significantly lower levels of pro-inflammatory, chemotactic cytokines were found in the sponge infiltrate of enriched mice. (Figure 3-30).

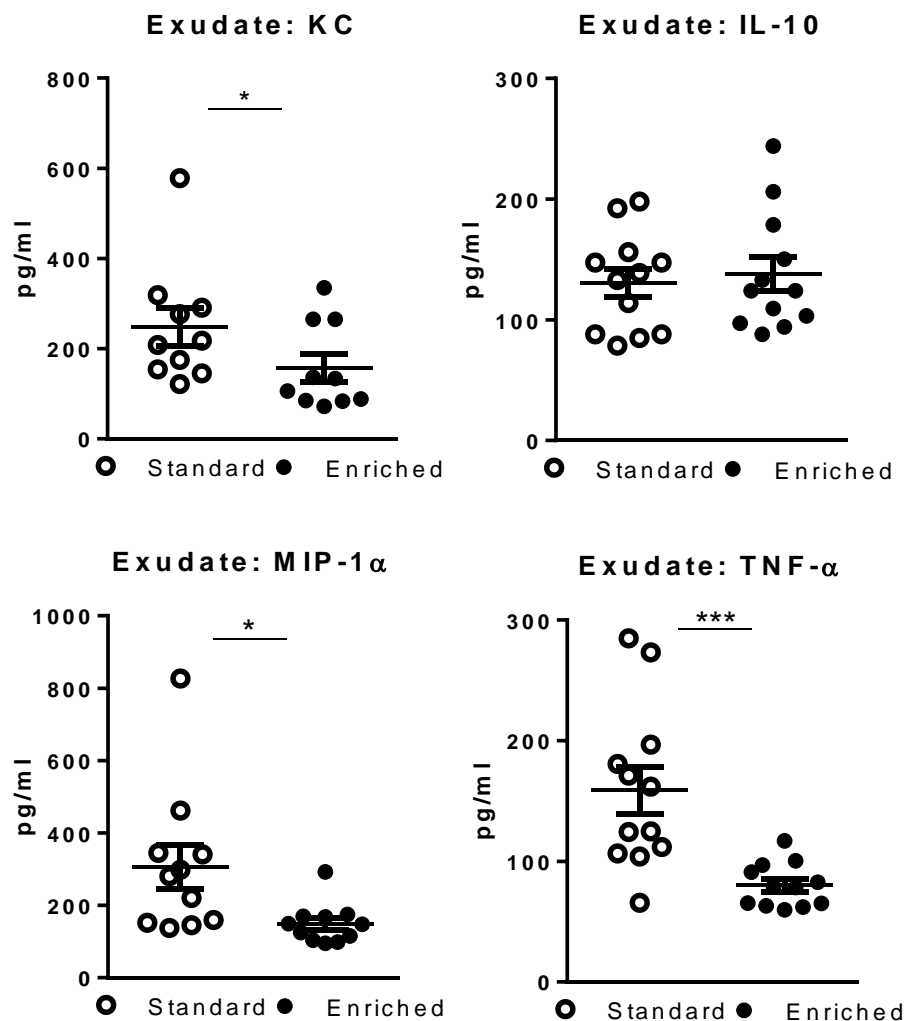
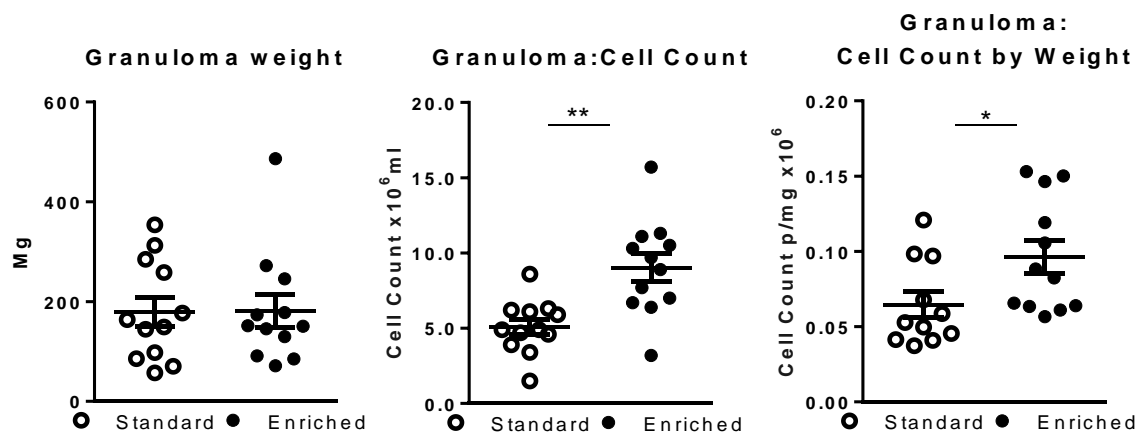


Figure 3-30. Comparative cytokine levels in the sponge infiltrate of animals from an enriched or standard environment subject to 72 hours of sponge implantation. Comparative levels of (reading top to bottom left to right): Keratinocyte chemoattractant, interleukin 10, macrophage inflammatory protein-one alpha and tumour necrosis factor alpha. \* $p < 0.05$  (\*\* $p < 0.005$ , \*\*\* $p < 0.0005$ ). Figure representative of N=3 experiments, 24 mice per experiment.

### 3.8.4 Granuloma: Granuloma Tissue, Cellular Profiling

We moved on to analyse the granulomatous tissue itself. Superficial investigation revealed no differences in the weight or gross appearance between the excised tissue of EE and SE mice (Figure 3-31a). However, once dispersed a significantly higher cell count was revealed in the tissue of enriched animals (Figure 3-31b). This significance persisted after calculating cellularity relative to the weight of granuloma tissue recovered (Figure 3-31

c). Due to the nature of the tissue dispersal process we were unable to perform flow cytometric analysis on the granuloma cells. In order to gain some insight into their biological state genetic profiling was carried out instead.

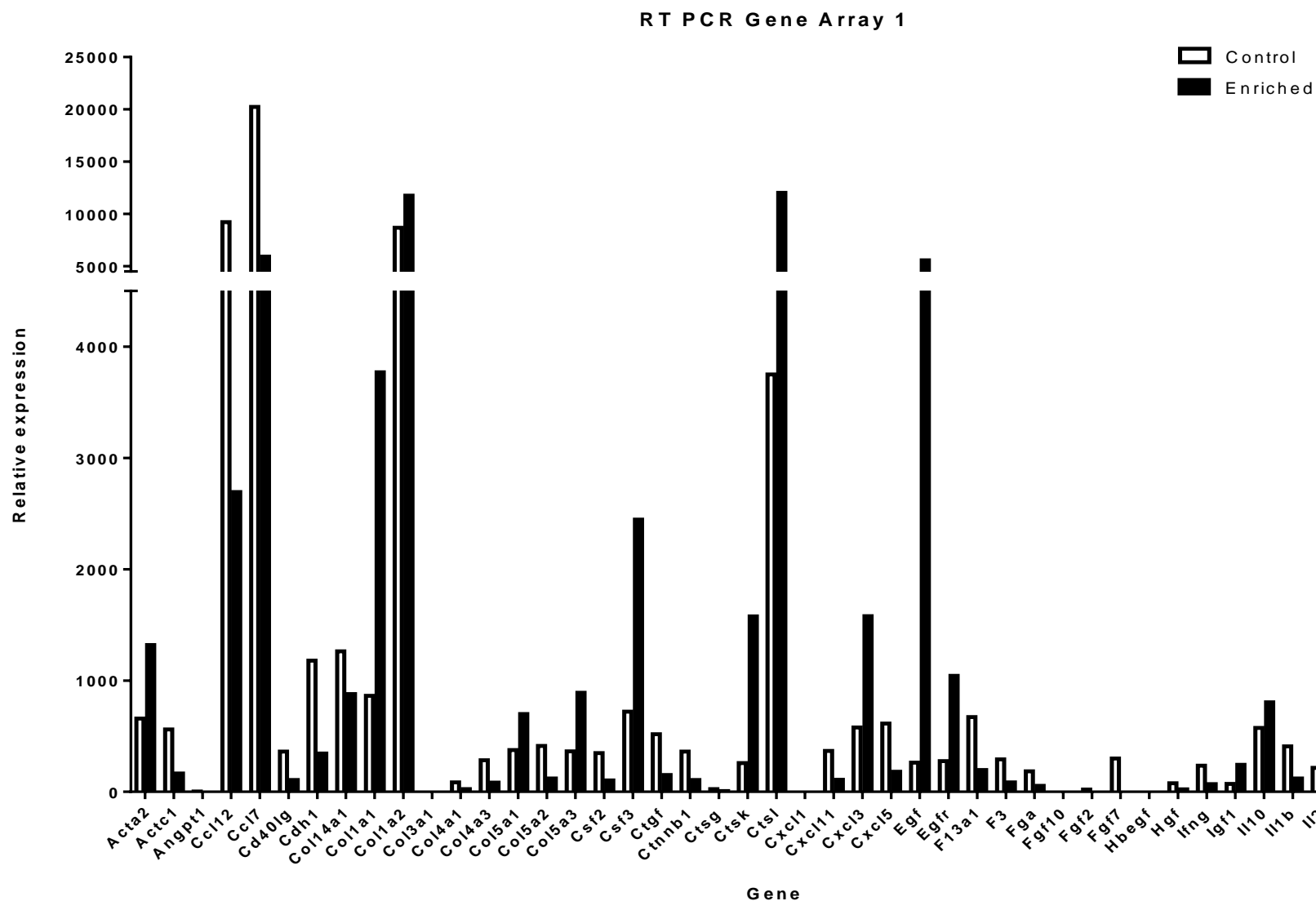


**Figure 3-31. Weight and cellularity of granulomatous tissue extracted from animals from an enriched or standard environment subject to 72 hours of sponge implantation. From left to right figures detail; gross granuloma weights for each experimental group, total cell count recorded for each dispersed granuloma, granuloma cell counts relative to the weight of granulomatous tissue excised. t test. \*p<0.05 (\*\*p<0.005) Figures representative of N=3 experiment, 24 mice per experiment.**

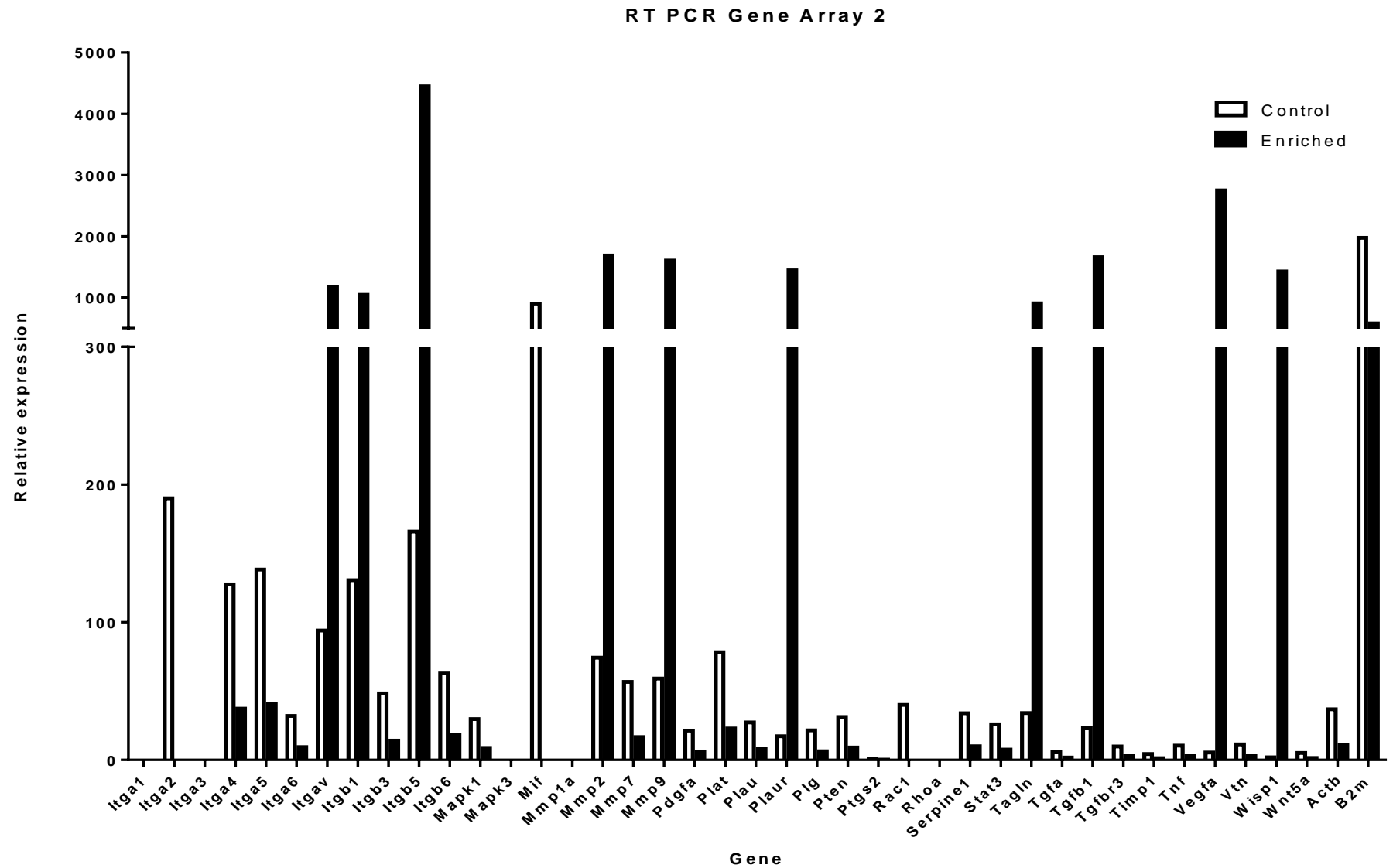
### 3.8.5 Granuloma: Granuloma Tissue, Genetic Profile

Interested to discover whether the altered cellular and cytokine profile in EE animals was modulated at the transcriptional level we analysed extracted granuloma tissues for the relative expression of a panel of 84 genes involved in inflammation, immunity and tissue repair. In this initial profiling only those genes that were at least 1000 times more highly expressed when compared to expression from the other group were selected for further investigation (Figure 3-32 and Figure 3-33). The genes identified suggested an expression pattern that favoured leukocyte mobilisation and chemotaxis in SE and tissue remodelling and repair in EE mice (Table 10).





**Figure 3-32. Comparative real time PCR profiling array of RNA extracted from the granulomatous tissue of animals housed in a standard or enriched environment - 1. Expression detailed relative to that of house keeping gene GADPH. A full list of the genes profiled can be found in Appendix 6 animal used per group.**



**Figure 3-33. Comparative real time PCR profiling array of RNA extracted from the granulomatous tissue of animals housed in a standard or enriched environment - 2. Expression detailed relative to that of house keeping gene GADPH A full list of the genes profiled can be found in Appendix 6. One animal used per group.**

**Table 10 Differentially expressed genes in the granulomatous tissue of animals housed in an enriched or standard environment ;72 hours post implantation**

Symbol	Gene	Role	Reference
<b>Orange = Upregulated in enriched mice</b>			
<b>Blue = Downregulated in enriched mice</b>			
Acta2	Actin, alpha 2, smooth muscle	Alpha actins are a family of proteins involved in cell motility, structure and integrity, forming key constituent of the contractile apparatus. Alpha-smooth muscle actin ( $\alpha$ -SMA) is commonly used as a marker of myofibroblast formation	[Strauch and Rubenstein, 1984]
Ccl12	Chemokine (C-C motif) ligand 12	Also known as monocyte chemotactic protein 5 (MCP-5). CCL12 specifically attracts eosinophils, monocytes and lymphocytes	[Jia et al., 1996]
Ccl7	Chemokine (C-C motif) ligand 7	A monocyte chemotactic factor and regulator of macrophage function. CCL7 has been shown to interact with MMP2.9 (see below)	[McQuibban et al., 2000]
Col14a1	Collagen, type XIV, alpha 1	Interacts with the collagen fibril surface. Involved in the regulation of fibrillogenesis (collagen fibre formation)	[Tono-Oka et al., 1996]
Col1a1	Collagen, type I, alpha 1	The major component of type I collagen, the fibrillar collagen found in most connective tissues, including cartilage	[Majchrzycki et al., 2015]
Col1a2	Collagen, type I, alpha 2	Forms one of the chains for type I collagen, the fibrillar collagen found in most connective tissues	[Karsenty and Park, 1995]
Csf3	Colony stimulating factor 3 (granulocyte)	Stimulates bone marrow production of granulocytes and stem cells and their release into the bloodstream. Regulates the survival, differentiation, and function of neutrophil precursors and mature neutrophils	[Souza et al., 1986]
Ctsk	Cathepsin K	Catabolises elastin, collagen, and gelatin allowing the breakdown of bone and cartilage. Expression is stimulated by inflammatory cytokines released after tissue injury	[Inaoka et al., 1995]
Ctsl	Cathepsin L	A lysosomal cysteine protease that plays a major role in intracellular protein catabolism. Its substrates include collagen and elastin, as well as alpha-1 protease inhibitor, a major controlling element of neutrophil elastase activity	[Chauhan et al., 1993]
Cxcl3	Chemokine (C-X-C motif) ligand 3	A monocyte migration and adhesion factor	[Smith et al., 2005]
Egf	Epidermal growth factor	Stimulates cellular proliferation, differentiation and survival. A key soluble factor in the wound healing process	[Carpenter and Cohen, 1990]

Symbol	Gene	Role	Reference
Egfr	Epidermal growth factor receptor	A cell-surface receptor for members of the epidermal growth factor family (see above)	[Yarden and Schlessinger, 1987]
Il10	Interleukin 10	A key anti-inflammatory cytokine, downregulates the expression of Th-1 cytokines, MHC class II antigens and co-stimulatory molecules on macrophages. It also enhances B-cell proliferation and antibody production	[Mosser and Zhang, 2008]
Itga1	Integrin alpha 1	Forms part of the surface receptor for collagen and laminin. This receptor is involved in cell-cell adhesion and may play a role in inflammation and fibrosis	[Gardner et al., 1999]
Itgav	Integrin alpha V	Receptor for multiple components of the cell matrix	[Bauer et al., 1993]
Itgb1	Integrin beta 1 (fibronectin receptor beta)	Primarily links the actin cytoskeleton with the extracellular matrix. Integrin beta 1 is further involved in cell adhesion and interaction in multiple processes including: embryogenesis, haemostasis, tissue repair and the immune response	[Hynes, 1992]
Itgb5	Integrin beta 5	A receptor for fibronectin, plays a role in binding together components of the extracellular matrix	[Ramaswamy and Hemler, 1990]
Mif	Macrophage migration inhibitory factor	Aids regulating macrophage function in host defence through suppression of the anti-inflammatory effects of glucocorticoids	[Yokota et al., 2000]
Mmp2	Matrix metalloproteinase 2	Degrades type IV collagen, (the major structural component of basement membranes). MMP2 plays a role in tissue reorganisation, regulation of vascularization and the inflammatory response	[Kundu and Patil, 2006]
Mmp9	Matrix metalloproteinase 9	Degrades type IV and V collagens and other extracellular matrix proteins, MMP9 plays several important functions within neutrophil action, angiogenesis and wound repair	[Opdenakker et al., 2001]
Plaur	Plasminogen activator, urokinase receptor	Part of the plasminogen activation system, which is involved in tissue reorganisation events such as mammary gland involution and wound healing	[Kjøller, 2002]
Tagln	Transgelin	A shape change sensitive actin-binding protein and early marker of smooth muscle differentiation	[Assinder et al., 2009]
Vegfa	Vascular endothelial growth factor A	A pluripotent protein involved with: mediating increased vascular permeability, inducing angiogenesis, vasculogenesis and endothelial cell growth, promoting cell migration and inhibiting apoptosis	[Hicklin and Ellis, 2005]
Wisp1	WNT1 inducible signalling pathway protein 1	Part of the WNT signalling pathway, regulates multiple cellular functions, including adhesion, migration, proliferation, differentiation, and survival	[Babic et al., 1999]

### 3.9 Behavioural Analysis

In order to investigate the potential behavioural effects of enrichment, mouse groups were subject to a battery of behavioural tests designed to ascertain if the altered housing paradigm caused observable changes in the animal's anxiety like behaviour.

#### 3.9.1 Open Field Test

The open field test revealed that over the course of the two week enrichment period enriched animals show a small but significant decrease in the overall number of squares crossed over the test period. Suggesting a potential reduction on overall exploratory activity (Figure 3-34). No significant differences were identified in the number of centre crossings or number of rears between each group, though a trend for a reduced number of overall rears by enriched animals was identified in each experiment.

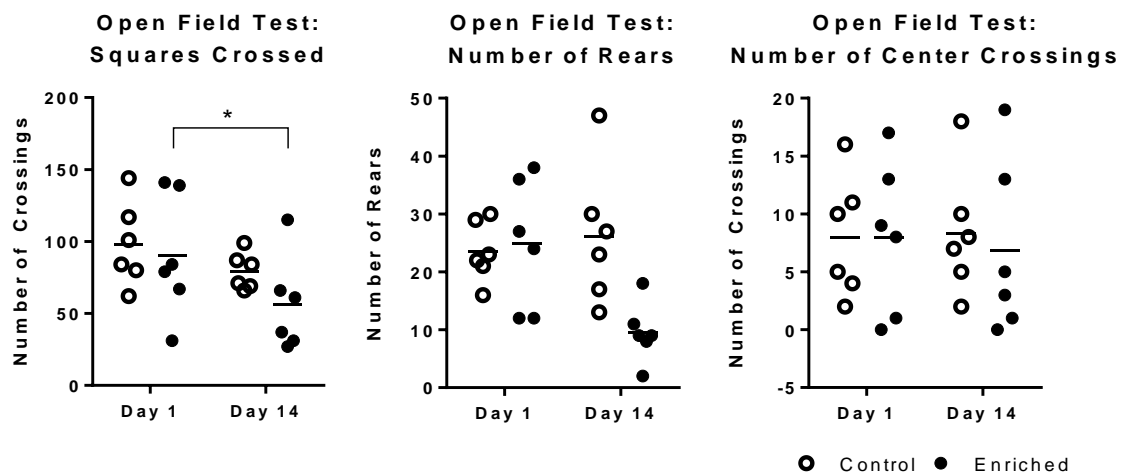


Figure 3-34. Open field test on animals preceding and following two weeks housing in a standard or enriched environment . From left to right total number of: Squares crossed, rears and centre crosses. Experiments representative of N=3 experiments, 12 mice per experiment. P<0.05, Mann-Witney test.

### 3.9.2 Light/Dark Box Test

While no observable differences were noted for overall time spent in light or the number of transitions between zones, light dark box test analysis presented a significantly higher crossing latency in animals subject to the enrichment program (Figure 3-35). Again suggesting a reduced willingness for exploratory activity and potentially heightened anxiety like behaviour in enriched animals.

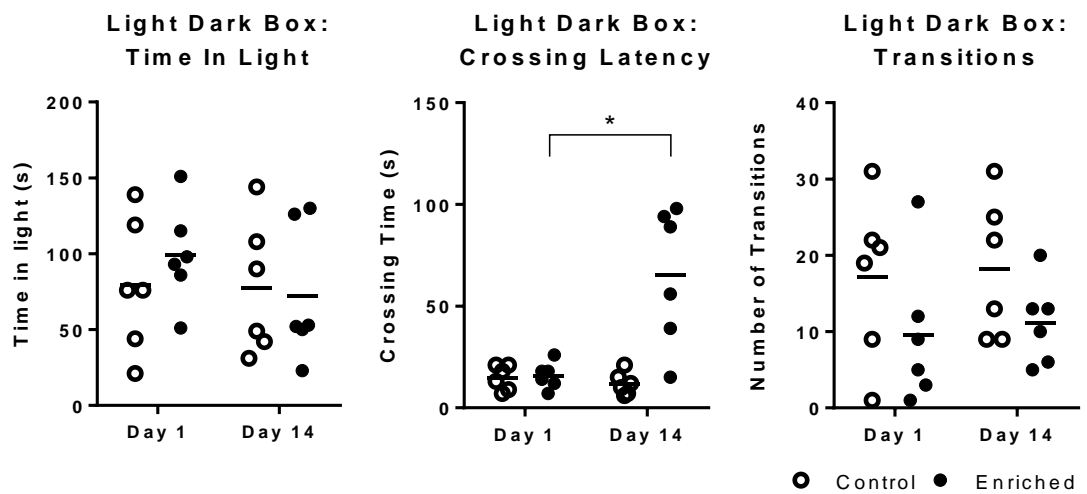


Figure 3-35. Light Dark test analysis on animals preceding and following two weeks housing in a standard or enriched environment. From left to right: Time spent in light, crossing latency, transitions from light to dark region. Figures representative of N=3 experiments, 12 mice per experiment.  $P < 0.05$ , paired t test.

### 3.9.3 Elevated Plus Maze

Elevated plus maze analysis produced no significant differences between groups for each parameter investigated, however as with the previous two behavioural tests a trend was identified for an overall reduction in exploratory activities carried out by enriched animals (Figure 3-36).

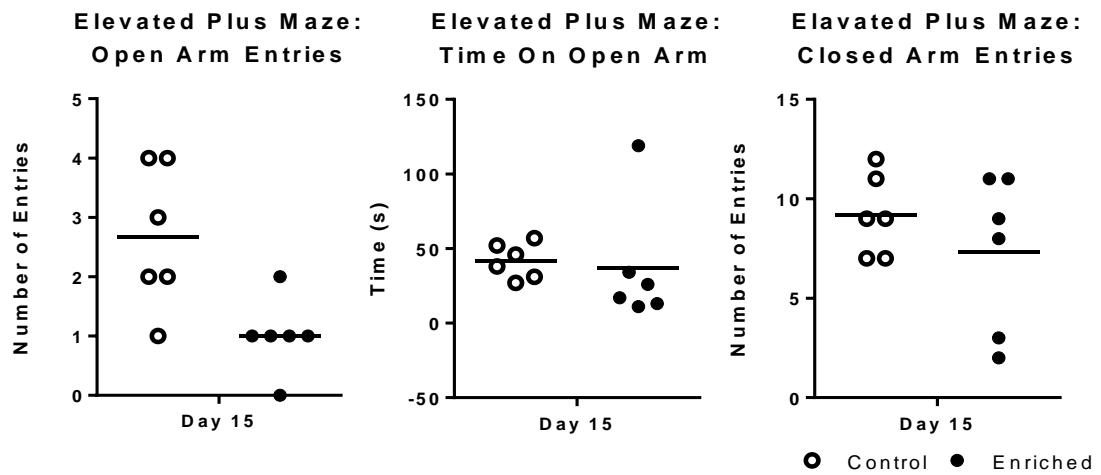
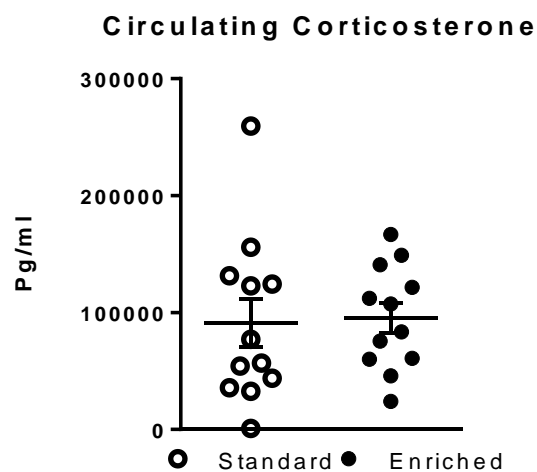


Figure 3-36. Elevated plus maze test analysis on animals following two weeks housing in a standard or enriched environment. From left to right: Number of open arm entries, total time spent on open arm, number of closed arm entries. Figures representative of N=3 experiments, 12 mice per experiment.

### 3.9.4 Circulating Corticosterone Levels

Levels of the stress associated immune regulatory hormone corticosterone were also analysed in the blood serum of EE and SE animals. While no significant alterations in overall corticosterone levels were found between EE and SE groups (

Figure 3-37), a consistent trend for intra-enclosure variation was identified (Appendix 4). Suggesting that corticosterone levels were influenced at a group level.



**Figure 3-37. Relative corticosterone levels in the blood plasma of mice following two weeks in a standard or enriched environment. Figure representative of N=3 experiments, 12 mice per experiment.**



## Chapter 4 Discussion

This study sought to assess whether defined alterations to an animal's housing conditions could have a modulating effect on its immune response. The experimental paradigm of environmental enrichment (EE) was used alongside established models of inflammatory disease to explore these potential modulating effects and investigate the possible underpinning cellular and molecular mechanisms behind them.

Our results show that two weeks environmental enrichment invokes a significant alteration in the immune response of male CD1 mice:

- Enhancing circulating leukocyte numbers and myelopoiesis
- Increasing leukocyte infiltration to foci of inflammation while maintaining circulating inflammatory mediators at comparably safe levels
- Enhancing macrophage phagocytosis (but not neutrophil activation) while moderating oxidative burst
- Enhancing cellular clearance of bacteria in a model of polymicrobial sepsis
- Inducing the upregulation of circulating immune-protective factors at a basal state
- Instigating a genetic program geared towards tissue remodelling and wound repair

Together these results present the picture of environmental enrichment promoting a potentially more efficient and immune-protective phenotype in mice. This raises two key questions: How are such changes brought about? and what are the implications of such enviro-immune influences in an experimental and clinical context?

### 4.1 Enrichment and Leucocytosis

Perhaps the most prevalent finding in across all the models used in this study was the consistently higher number of leukocytes – most predominantly granulocytes – identified in animals subject to environmental enrichment. Analysis of tibial bone marrow suggested that this tissue was the likely source of these cells with significantly higher numbers of granulocytic cells found in EE animals compared to those animals housed in a standard environment (SE).

As EE mice have access to both a running wheel and a larger overall enclosure space, one explanation for this amplified cell number is the increased level of physical exercise they may perform compared to SE animals. Multiple studies have reported heightened haematopoiesis in

response to increased physical exercise in mice [Baker et al., 2011] and humans [Morici et al., 2005, Rehman et al., 2004]. Further, short term exercise has been shown to promote the release of granulocytic factors G-CSF, GM-CSF, M-CSF, IL-8 and MCP-1 [Suzuki et al., 2002]. The similarities between such previous studies and the data acquired in this one make it tempting to ascribe the alterations in bone marrow and blood leukocyte numbers solely to the increased physical activity EE animals likely perform. However, it should be noted that studies have found a high variation in leucocytosis in response to exercise, dependent on multiple factors including the nature and duration of the physical activity itself and the metabolic status of the individuals undertaking the exercise. In both mice and humans acute exercise bouts are typically correlated with an augmented inflammatory state, while prolonged physical activity appears immunosuppressive, inhibiting both lymphocyte and granulocyte function [Smith et al., 1992].

Personal observations of the enrichment enclosures suggested a high level of inter-group variation in wheel use. With typically only one or sometimes two animals running on the wheel for the majority of the time observed. Studies carried out by Vargas-Pérez *et al* have identified the same behaviours, suggesting that dominance hierarchies within cage groups determine resource distribution, with dominant mice running on wheels placed within the cage significantly more than subordinate animals [Vargas-Pérez et al., 2009]. Accordingly, it is probable that levels of physical exercise within enrichment cages vary greatly between enriched animals and so it could logically be assumed that corresponding levels of leucocytosis would be equally variable as well. While there is certainly a relatively high level of variation in leukocyte counts taken from EE animals it can be seen that while leukocyte values are often significantly different between EE and SE groups the level of spread between values remains more or less consistent between them. This suggests that while wheel running may be a contributing factor to heightened haematopoiesis in EE mice it is not the sole one. Preliminary investigations into the effects of housing mice in enrichment cages without an exercise wheel present lends support to this hypothesis, revealing that mice from such cages still display a higher leukocyte count than SE animals (Appendix 7) Further, while these animals possess lower leukocyte numbers than EE animals housed with a wheel the values presented for them have a notably smaller spread.

Raising the possibility that inclusion of a wheel in mouse enclosures may indeed increase variability of results.

It was also observed that enriched animals presented a consistently lower overall level of adiposity than SE animals while maintaining the same average food intake. Suggesting an higher overall level of metabolic activity in enriched animals. Concordant results has been observed in previous enrichment studies [Grippe et al., 2014, Slater and Cao, 2015] with researchers suggesting that housing in a socially enriched environment induces an increase in levels of metabolically thermogenic brown fat [Cao et al., 2011, During et al., 2015]. Enriched animals in our study were observed to be considerably more physically active than their comparatively sedentary SE counterparts, continually exploring and moving around their enclosure when awake (Appendix 5). This increased activity is the probable cause of their reduced adiposity and another potential contributor to increased haematopoiesis and circulating granulocytes in EE animals.

Increased levels of adiposity are in fact also correlated with higher levels of circulating leukocytes [Reyes et al., 2015, Johannsen et al., 2008]. However, this increase is typically associated with the physically detrimental state of chronic low grade inflammation as opposed to the observed immune-protective effects of regular physical exercise. Obesity and a sedentary lifestyle have been linked with increases in circulating inflammatory mediators and pro-inflammatory leukocyte subsets in both mice [Vieira et al., 2009] and humans [Lira et al., 2010] and with this a correspondingly higher risk of disease associated with a dysregulated inflammatory response [Lumeng and Saltiel, 2011]. Of interest, obesity has also been correlated with an exacerbation of sepsis induced inflammation and vascular dysfunction in mice [Vachharajani et al., 2005] as well as a reduced capacity for wound healing in both clinical [Wilson and Clark, 2004] and animal studies [Seitz et al., 2011].

While the effects of adiposity and sedentarism (exemplified by animals housed in a standard lab environment) on circulating leukocyte numbers does not appear as pronounced as those of housing in an enriched environment this influence does raise the question as to whether

animals housed in a standard lab environment – typically used as the control group in a large number of scientific experiments serve a realistic representation of an organism in a ‘normal’ immune state.

One challenge of trying to discern whether the leukogenic effects of environmental enrichment are effected solely by physical activity or other factors is that the large majority of experimental setups used to investigate enrichment on lab animals (or captive animals in zoos) inherently promote increased physical activity as part of their design (be this directly through use of wheels or treadmills or indirectly through increased cage size). One exception to this experimental paradigm is in enrichment studies performed on animals used as livestock. The financial impetus on the livestock industry typically leads to research on improving animal welfare focusing on methods to improve an animal's environment without increasing the floor space of the enclosure. In one study laying hens were housed in standard commercial steel battery cages, augmented with a perch, sandbox and polypropylene string ‘pecking’ bunches. Following 17 weeks, birds in enriched cages were revealed to possess significantly higher levels of circulating heterophils (the avian analogue of neutrophils) compared to those birds housed in a standard unadorned cage, as well as heightened leukocyte activity [Matur et al., 2016]. In a further study juvenile crayfish housed in enriched tanks displayed higher levels of circulating cells in their haemolymph (the invertebrate equivalent of blood) compared to those housed in ‘unadorned tanks’ [Ayub et al., 2011]. Weaning piglets subject to social enrichment have also been found to increase their circulating neutrophil to lymphocyte ratio, however it is uncertain whether this effect is transitory or not [Puppe et al., 1997].

## **4.2 Altered Leukocyte Ratios in Enriched Animals**

Analysis of blood leukocyte populations of mice in a basal, non-immune-activated state revealed EE animals to possess a significantly higher proportion of neutrophils in blood circulation compared to SE. This trend was amplified following six hours of ZIP with neutrophils becoming the dominant leukocyte population in the blood of EE mice. This neutrophilic trend persisted up to 24 hours post zymosan injection, where a heightened proportion of neutrophils was found in EE animals despite lymphocytes returning as the overall dominant blood leukocyte population (Figure 4-1). While no previous studies investigating the immune-modulatory effect

of enrichment on rodents have identified concordant increases in circulating neutrophil numbers<sup>8</sup>, several have observed decreases in blood lymphocyte populations following enrichment [Kaufman et al., 1994, Hutchinson et al., 2012, Arranz et al., 2010]. As can be seen in the results of this thesis and previous studies a decrease in blood lymphocyte population is often accompanied with a con-committant increase in neutrophils [Doeing et al., 2003].

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<sup>8</sup> Possibly because this cell population was not specifically looked for.

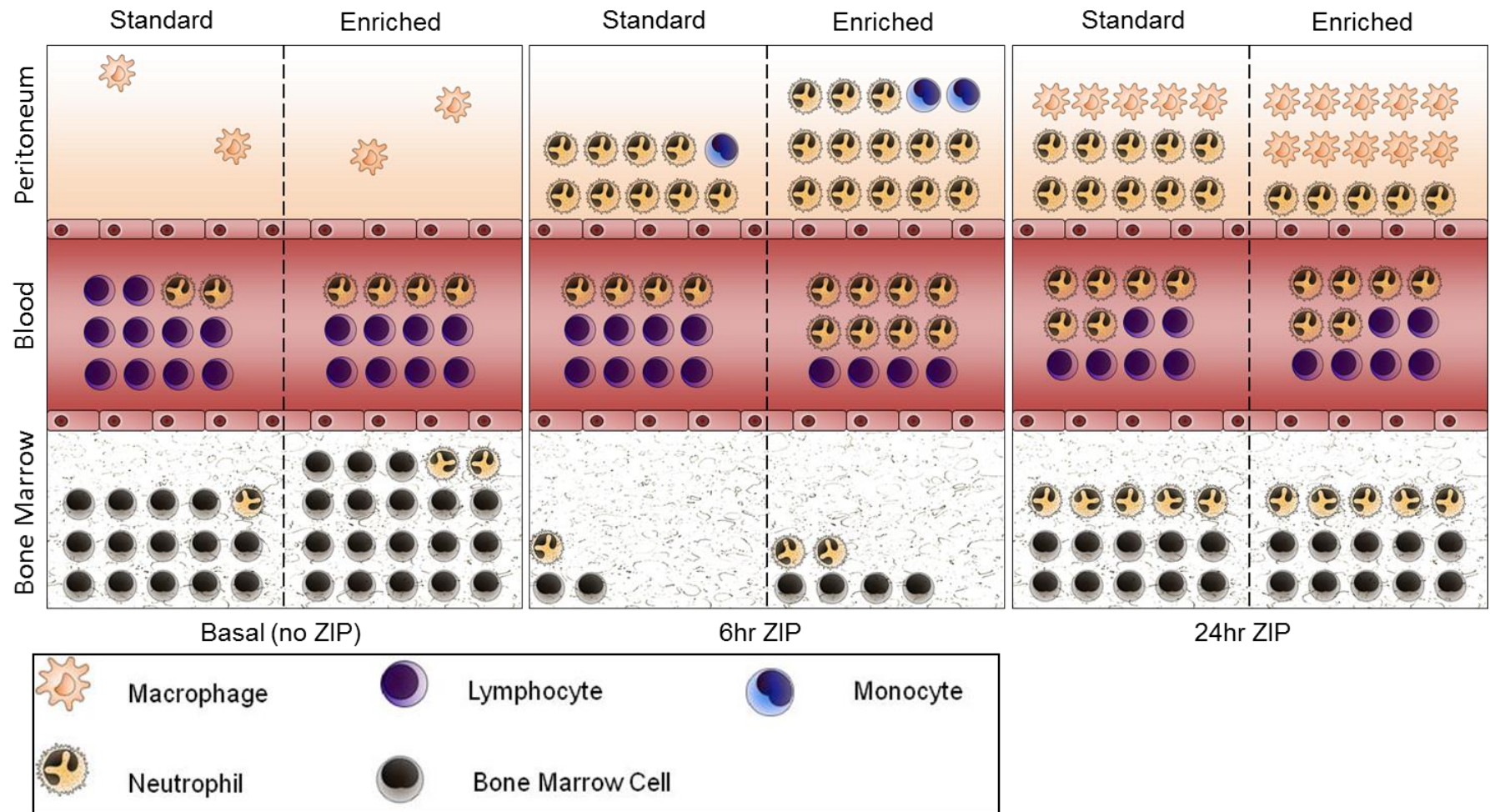


Figure 4-1. Alterations in leukocyte numbers and ratios across three cellular compartments following peritoneal administration of zymosan.

Corresponding to the increased neutrophil to lymphocyte ratio identified in the blood of EE mice circulating neutrophil [McCarthy and Dale, 1988] and NK [Nieman et al., 1990] concentrations have been shown to increase during and persist following repeat bouts of moderate exercise. Circulating lymphocyte concentrations initially increase during exercise and then drop to below pre-exercise levels for several hours or days following physical activity depending on the intensity of the exercise program [Fry et al., 1992, Nielsen and Pedersen, 1997, Smith et al., 1992]. This peak and trough of lymphocyte numbers has been attributed to their rapid tracking out of the lymphatic system and into blood circulation during physical activity and their corresponding succession by granulocyte populations during recovery [Fry et al., 1992]. Again, for the reasons previously stated, while physical activity is likely a contributing factor to the results observed in our study, it is unlikely to be the sole reason for the altered leukocyte ratios observed.

Increased neutrophil to lymphocyte count ratios (NLCR) are a common prognostic marker of blood stream infection [Liu et al., 2016] and has found growing use as an indicator of underlying low level systemic inflammation other chronic inflammatory disease states [Ahbap et al., 2016, van Wolfswinkel et al., 2013, Meng et al., 2016]. In our blood profiling of animals subject to CLP we found reduced cell numbers typically associated with the instigation of the hypo-inflammatory phase of sepsis - leukopenia and lymphopenia [Nguyen and Smith, 2007]. However, in EE mice, the severity of this lymphopenia was reduced with enriched animals maintaining higher overall population of lymphocytes and monocytes compared to SE mice. Studies in both mice [Bommhardt et al., 2004, Hotchkiss et al., 1999] and humans [Okashah et al., 2014] have demonstrated that elevated lymphocyte numbers are correlated with improved survival rates in studies of sepsis. It is interesting to note that the blood profile of EE animals in CLP is the reverse of that found in those subject to ZIP with the former presenting higher lymphocyte numbers relative to SE mice and the latter higher neutrophils, again this could be attributed to our study animals being in a state of immune-stimulation in ZIP and imminent (if not already present) immune-suppression in CLP.

### 4.3 Heightened Cellular Infiltration

Another feature of the EE-evoked cellular response was an enhanced level of cellular infiltration at the point of inflammatory insult (e.g. the peritoneum in ZIP and CLP and surrounding the sponge in sponge granuloma). Recent studies investigating the effects of enrichment on a model of dextran sulphate sodium (DSS) induced inflammatory colitis also reported increased neutrophil infiltration as feature of the EE-immune response. This heightened cellular response was observed to increase the severity of symptoms in enriched animals suggesting enrichment aggravated the disease. However, in the same study the author reported EE had no effect on the cellular inflammatory response to a model of Iodoacetamide (IAA) induced gastritis. Suggesting that the effects of EE on the inflammatory response vary depending on the region an inflammatory response occurs [Reichmann et al., 2013]. It should be noted that this particular study specifically investigated the effects of EE on the immune response of animals that had been subject to water avoidance stress (WAS), a behavioural paradigm designed to induce an acute state of psychological stress that, as will be later discussed is known to have an acute modulatory influence on the immune response.

In a further study where EE and environmentally impoverished (EI) <sup>9</sup> mice were subject to a model of Piry virus induced encephalitis, enriched animals were found to display higher T-cell infiltration into the central nervous system (CNS). Accompanying this enhanced infiltration EE mice presented a more rapid resolution of infection and a reduced level of overall tissue damage compared to EI reared animals [De Sousa et al., 2011]. Additional studies carried out by Kipnis *et al* have identified that EE can spontaneously induce recruitment of T-cells into the brain without any inflammatory insult [Ziv et al., 2006]. While these two studies focus upon different leukocyte subsets than this thesis (and also a region of the body typically regarded as immune-privileged) they still lend some support to the concept that EE may positively influence leukocyte trafficking and infiltration.

Intraperitoneal injection of zymosan induces a rapid accumulation of PMNs into the peritoneal cavity. This immune response presents a consistent time course, made up of an initial influx of neutrophils 2-10 hours post injection followed by the later arrival of monocytes and monocyte-derived macrophages

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<sup>9</sup> It is interesting to note that what is classed as an 'environmentally impoverished' enclosure in this study is essentially the standard environment enclosure used in all the experiments discussed in this thesis (and throughout the five separate animal units I have worked in).



at 10-25 hours [Getting et al., 1997, Cash et al., 2009]. It was due to these well-established leukocyte dynamics that ZIP was chosen to assess the rate and dynamics of the acute inflammatory response in EE animals.

Differential analysis of the cell populations in EE revealed elevated numbers of neutrophils and monocytes compared to SE animals at six hours after challenge. Correspondingly, significantly higher numbers of peritoneal macrophages were found in enriched animals 24 hours post injection. The initially higher influx of neutrophils into the EE animal mirrored the higher proportion we observed in circulation in basal conditions. This raises question as to whether these increased cell numbers are simply a result of the correspondingly higher levels of myeloid derived cell in the bone marrow and blood of EE mice or potentially due to an increased speed of egress to the site of inflammation. The observation that circulating monocyte numbers remained the same in EE and SE mice while monocyte and macrophage (which likely derived from infiltrating monocytes) numbers in the peritoneal cavity were heightened lends some support to this hypothesis. Additionally a previous study which subjected mice to enrichment for up to 18 weeks revealed isolated leukocytes to exhibit heightened proliferation and chemotaxis indicating a common mode of effect for enrichment [Arranz et al., 2010].

It is also interesting to note that at 24 hours the most significantly heightened leukocyte population in the EE mouse peritoneal cavity, (as a number and a proportion of the total cell count) are macrophages. Macrophage influx is typically associated with a more advanced stage of the inflammatory immune response, mediating the shift past the acute phase onto resolution or chronic inflammation [Italiani and Boraschi, 2015]. This raises the possibility that enriched animals not only experience a heightened cellular inflammatory response but a more rapid one also.

EE animals subject to 12 hours of CLP also demonstrated a significantly higher cellular influx of neutrophils into the peritoneal cavity than SE mice. These cells were identified to be predominantly neutrophils. This heightened leukocyte presence during the transitional point between the hyper and hypo-inflammatory phase of the sepsis response is likely a key contributing factor in the reduced bacterial counts identified in enriched animals. Consistent with this finding previous studies have shown that diminished innate leukocyte activity and/or numbers are associated with a high mortality rate in sepsis [Brown et al., 2006, Alves-Filho et al., 2008]. Congruently, supply of the neutrophil attractants CXCL1 and CXCL2 into the peritoneal cavity after inducing polymicrobial sepsis enhances

both neutrophil recruitment and clearance of bacteria, as well as improving survival [Craciun et al., 2010].

Granulocyte and monocyte influx was also found to be enhanced in animals subject to a carrageenan-sponge implantation model of wound healing and granuloma. As with the pathologies emulated by other models in this thesis, heightened neutrophil influx has been shown to promote improved wound healing in previous studies [Devalaraja et al., 2000]. However, it should be noted that efficient cellular profiling proved challenging in this particular model. We were unable to perform direct flow cytometric analysis on the granuloma tissues we excised. This was due to our methodology of proteinase induced cell dispersal having the side effect of cleaving expressed surface receptors from the leukocyte cell surface. Preventing binding by marker antibodies. In turn cells extracted from the implanted sponges typically displayed high levels of apoptosis likely diminishing the accuracy of the data we obtained from them. Future studies would need to devise more effective means of isolating and profiling individual leukocytes with granulomatous tissue or otherwise use alternative methodologies such as histology or fluorescence microscopy.

#### **4.4 The Cytokine Profile of Enriched Animals**

The consensus of previous studies is that environmental enrichment elicits a reduction in circulating pro-inflammatory cytokines IL-6 [Jurgens and Johnson, 2012, McQuaid et al., 2013] , IL-1 $\beta$  [Jurgens and Johnson, 2012, McQuaid et al., 2013, Williamson et al., 2012] TNF- $\alpha$  [Jurgens and Johnson, 2012, Williamson et al., 2012] and IFN- $\gamma$  [Marashi et al., 2003]. While promoting increased expression of anti-inflammatory lymphoproliferative IL-2 [Arranz et al., 2010, Laviola et al., 2004, Marashi et al., 2003] and IL-10 [Marashi et al., 2003].

Analysis of an established panel of four cytokines involved in the progression of ZIP identified no significant differences in inflammatory cytokine levels in the peritoneum of EE and SE animals at both six and 24 hour time points. It is possible that the concentration of zymosan administered in these experiments (0.5mg per mouse) was so high as to cause an exacerbated inflammatory response, with cytokines released at such high levels that more subtle variations in their concentration would become indistinguishable [Cash et al., 2009]. However, fluctuations in these cytokine's overall levels over the two time points were found to match that of previous studies [Cash et al., 2009], elevated at the 6 hour

time point and falling towards normal homeostatic levels at 24 hours, suggesting that cytokine expression in these animals follows a comparatively normal homeostatic response.

Correspondingly no significant differences in expression between EE and SE mice were identified in the panel of chemotactic factors and pro-inflammatory cytokines chosen to investigate mice subject to CLP. (Though as with the ZIP the cytokine levels observed were concordant with previous studies employing this model [Huber-Lang et al., 2014]). This finding suggests a unique scenario in which EE animals present an intensified cellular response without an accompanying increase in pro-inflammatory cytokines.

One could speculate that the increase in circulating anti-inflammatory mediators reported in previous EE studies may serve to balance or mitigate detrimental levels of inflammatory cytokines that infiltrating inflammatory leukocytes typically release. One study lends some support to this concept reporting that EE promotes an anti-inflammatory phenotype in brain microglia through adiponectin dependent suppression of TNF- $\alpha$  release [Chabry et al., 2015].

In contrast to ZIP and CLP significant differences were found in the cytokine levels of EE and SE mice subject to sponge granuloma. Significantly lower levels of pro-inflammatory KC, MIP-1 $\alpha$  and TNF- $\alpha$  were identified in the sponge infiltrate of enriched mice, while levels of anti-inflammatory IL-10 remained the same between SE and EE groups. A reduction in pro-inflammatory factors and presence of anti-inflammatory cytokines such as IL-10 is typically associated with the shift of the immune response into a resolving or reparative phase [Eming et al., 2014, Ortega-Gómez et al., 2013]. Viewed together these cytokine readouts suggest that environmental enrichment may support the mediation of pro-inflammatory signalling factors without incurring detrimental immunosuppressive effects on the cellular response.

## **4.5 Leukocyte Activity in Enriched Animals**

Having established EE increased cell quantity, we moved on to investigate if it also effected cell quality. Neutrophil selectins (L, E and P) are vital components in the process of the cell's extravasation into tissues. Interaction of selectins with their composite ligands expressed by both leukocytes and activated endothelial cells causes the slowdown of flowing leukocytes facilitating their entrance into inflamed tissues. Unlike E- and P-selectins, the cellular mechanisms involved in the regulation of L-

selectin (CD62-L) expression are not fully defined [Gómez-Gavero et al., 2000]. Regardless L-selectin is constitutively expressed on the cell surface of neutrophils and has been shown to be enzymatically cleaved and shed simultaneously with the cell's activation [Gómez-Gavero et al., 2000]

Conversely, CD11b, an integrin also involved in leukocyte adhesion and migration during the inflammatory response is shown to be upregulated on the surface of neutrophils following activation [Fortunati et al., 2009]. Accordingly, measurement of the relative expression levels of these two markers is a popular means of assessing a neutrophil's rate and state of activation *ex vivo*.

Stimulation of blood neutrophils with activating factors PAF and LPS induced an overall decrease in L-selectin and increase in CD11b expression in both EE and SE neutrophils at levels concordant with those reported in previous studies [Cooper et al., 2008]. No differences in expression or shedding were found between EE and SE groups suggesting that EE does not influence the dynamics of neutrophil extravasation. However, such receptor expression assays can only be regarded as indicators of leukocyte activity. In further studies, specific chemotaxis and endocytosis assays (such as flow chamber assays or intravital microscopy) would provide a more comprehensive means of establishing the effects of EE on neutrophil activity<sup>10</sup>.

Phagocytosis is the eponymous effector function of macrophages and an essential component of the immune response to infectious organisms, inflammatory resolution, wound healing and general tissue homeostasis [Ginhoux and Jung, 2014]. Previous enrichment studies carried out on female CD1 mice have identified that macrophages isolated from EE animals proved more efficient at phagocytosing latex beads when compared to control animals [Arranz et al., 2010]. Enhanced phagocytosis of opsonized latex-coated beads by peripheral blood neutrophils has also been reported in beagles subject to a two year long enrichment regimen [Hall et al., 2006]. This thesis' results support these findings, observing that macrophages isolated from enriched animals are significantly more effective at phagocytosing *E.coli* than those from SE mice. However, it should be noted that the previous two papers cited only found results when analysing cells isolated from aged animals. The study by Arranz et al, in fact found no differences in phagocytosis when studying adult mice (44 weeks) observing improved macrophage function (with increasing significance) only at successively older ages (69 and 92 weeks) [Arranz et al., 2010]. The mice used in our studies were between 9-10 weeks old when

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<sup>10</sup> We had planned to investigate the chemotaxis and extravasation of EE leukocytes using intravital microscopy. Unfortunately, loss of the necessary equipment prevented this avenue of research.

studied, an age regarded to correspond with the early phase of adulthood in the mouse lifecycle [Dutta and Sengupta, 2016]. This disparity in results could possibly be attributed to gender differences in the mice used for each study, research has established that phagocytic responses in male and female mice differ (with female mice macrophages typically being regarded as the more effective phagocytes) [Scotland et al., 2011]. Additionally, differences in methodology (e.g. use of opsonised beads vs lyophilised bacteria) could also account for these differences. Regardless, these studies raise an interesting observation found in previous neurological studies utilising EE. Namely that the protective physiological influence of enrichment becomes increasingly pronounced with age. A potentially constructive line of continued research would be to apply the methods employed in this thesis to mice at a range of ages (and both genders).

The enzymes PTGS2 (responsible for the generation of prostaglandins) and iNOS (which is one of three enzymes that generate nitric oxide from the amino acid L-arginine) are both released by macrophages and their heightened expression is typically associated with an activated, pro-inflammatory phenotype [Martinez and Gordon, 2014]. While both integral to proper homeostatic responses, excessive production of these enzymes and their respective products has been associated with a variety of pathologies including cancer [Howe, 2007], chronic inflammation [Hugo et al., 2015] and reduced mortality in sepsis [Hollenberg et al., 2000].

Previous enrichment studies have reported increased reactive oxygen species (ROS) production by macrophages isolated from aged mice stimulated with phorbol myristate acetate (PMA) [Arranz et al., 2010]. These results seemingly conflict with our findings which suggest iNOS gene expression is reduced in EE animals following stimulation with LPS. The age of the animals used and methodological variations may explain these disparate results: In the same study EE mice responses to LPS were found to be age dependent. NK cells extracted from older enriched animals presenting an exacerbated release of inflammatory TNF- $\alpha$  in response to LPS stimulation while cells from younger animals displayed a reduced response compared to the control. As the animals used in our study were considerably younger than those used by Arranz *et al* it is possible that the reduced expression of iNOS observed in EE macrophages is a reflection of this age dependent anti-inflammatory response. Corroborating this hypothesis studies in humans [Gomez et al., 2005] and mice [Godbout et al., 2005] have also observed such an age dependent switch in the inflammatory immune response.

Expression of *ptgs2* was also found to be reduced following stimulation of EE macrophages with LPS. To date no immunological studies have specifically assessed prostaglandin associated gene production in the context of enrichment. However, neurological studies have found that EE leads to the downregulation of PTGS2 and a further downstream enzyme in the prostaglandin synthesis pathway; prostaglandin D2 Synthase PTGDS [Hüttenrauch et al., 2016]. Conversely, a further study found evidence that enrichment indirectly enhanced *ptgs2* gene expression in the brain through inhibition of a microRNA found to in turn inhibit expression of a range of genes including *ptgs2* [Vallès et al., 2014], suggesting the possibility of differential cellular responses to enrichment in circulation and the CNS. Further analyses of leukocyte prostaglandin release (through an ELISA or similar immune-assay) or oxidative burst activity (through myeloperoxidase or cytochrome-C based assays) would provide a better understanding of the potential alterations in effector function in enriched leukocytes.

## **4.6 Gene Profile of Enriched Animals**

### **4.6.1 Gene Expression in A Basal Immune State**

To date no studies have attempted to profile the immune-genotype of an EE animal. To this end we took a hypothesis driven approach performing microarray analysis of RNA extracted from whole blood of EE and SE animals. Most of the genes identified by the array had a specific metabolic or immune regulatory function. Of these, four stood out for their role in inflammation: *Chil1* (Chitinase 3 like protein 1, *CHI3L1*, YKL-40), Signal regulatory protein beta 1 (*SRPB1*), *S100A8* and *S100A9* (*S100* calcium-binding protein 8 and 9). Saliently a trend was found among each of these genes for heterogeneous immunomodulatory properties, promoting an efficient but moderated inflammatory response.

*CHI3L1*, is a member of the family of mammalian chitinase-like enzymes. Produced by a wide variety of cells including neutrophils [Volck et al., 1997], macrophages [Rehli et al., 2003], fibroblasts and endothelial cells [Malinda et al., 1999] clinical studies typically present *CHI3L1* as an acute phase inflammatory protein, with heightened circulating levels detected in patients suffering rheumatoid arthritis [Hua and Recklies, 2004], sepsis [Kornblit et al., 2013] and inflammatory bowel disease [Kamba et al., 2013]. Supporting this *CHI3L1* secretion is increased by leukocytes upon stimulation by IFN- $\gamma$  [Kzhyshkowska et al., 2006] and IL-6 [Nielsen et al., 2011]. Of note *CHI3L1* has also been

shown to downregulate cellular responses to the same cytokines [Hua and Recklies, 2004] and promote release of TGF- $\beta$ . Suggesting that CHI3L1 may be part of a regulatory mechanism to mediate the inflammatory response.

While the exact physiological role of CHI3L1 is under ongoing research, studies have demonstrated its involvement in a range of immunological processes, including; apoptosis [Lee et al., 2009], opsonisation [Johansen, 2006], M2 macrophage differentiation [He et al., 2013], dendritic cell activation [Lee et al., 2011], mitogen-activated protein kinase (MAPK) and Akt signalling [Chen et al., 2011] as well as tissue remodelling and angiogenesis [Francescone et al., 2011]. Additionally a study has found that CHI3L1 is able to augment macrophage bacterial killing by inhibiting macrophage apoptosis while simultaneously enhancing host tolerance by suppressing inflammasome activation [Cruz et al., 2012]. A set of functions that corresponds with the enhanced macrophage activity and moderated release of soluble inflammatory mediators found in our studies. Of interest a recent study has suggested that circulating levels of CHI3L1 decrease in response to acute exercise [Mygind et al., 2016].

Also up regulated was SRPB1, a glycoprotein receptor expressed on human monocytes, granulocytes, macrophages and microglial cells [Seiffert et al., 2001]. Like CHI3L1, SRPB1 has been shown to upregulate phagocytic function, with stimulation of the receptor on murine macrophages enhancing their engulfment of latex beads [Hayashi et al., 2004]. Further studies of SRPB1 expression on mouse microglial cells have revealed that ligation of the receptor not only upregulates microglial phagocytosis of neuronal debris (through re-organisation of the actin cytoskeleton) but also inhibited lipopolysaccharide-induced stimulation of TNF- $\alpha$  and NOS2 (Gaikwad, Larionov et al. 2009). It is tempting to speculate that the action of this protein and the heightened expression of its gene in enriched mice lends further credence to our observation of enhanced phagocytosis and reduced iNOS expression in EE macrophages. A further study has revealed that SRPB1 binding also upregulates neutrophil transendothelial migration [Liu et al., 2008], raising one possible explanation for the heightened leukocyte influx we observed in our CLP and ZIP models.

S100A8 and S100A9 together form the protein complex calprotectin, a molecule found to be highly abundant in myeloid cells, with neutrophils expressing the highest content (calculated to be ~30% of total cytosolic proteins [Sugimoto et al., 2016]). Released by leukocytes upon activation at sites of

infection, calprotectin exerts a direct antimicrobial action by sequestration of the essential bacterial nutrients manganese and zinc [Stříž and Trebichavský, 2004]. In addition, calprotectin stimulates the innate immune system through activation of RAGE and TLR4, resulting in downstream NF- $\kappa$ B activation and secretion of pro-inflammatory cytokines, TNF- $\alpha$  and IL-17 [Riva et al., 2012, Vogl et al., 2007].

Individually the S100A8 and A9 proteins have also been found to act as powerful regulators of the innate immune response, promoting neutrophil and macrophage accumulation, cytokine production, and smooth muscle cell proliferation [Croce et al., 2009, Kerkhoff et al., 1998]. S100A9 deficient neutrophils exhibited a reduced secretion of cytokines (e.g. TNF- $\alpha$  and MCP-1) in response to LPS stimulation while inflammatory cytokine production in dendritic cells was exacerbated by S100A8 deficiency [Averill et al., 2011].

These immune stimulating properties have led many researchers to suggest that calprotectin is an acutely pro-inflammatory protein. This idea is supported by calprotectin's use as a biomarker for inflammatory disease, most especially intestinal inflammation [Konikoff and Denson, 2006] and sepsis [Decembrino et al., 2015]. However, a number of studies have described an anti-inflammatory and immune-protective role for calprotectin (and its constituent proteins). Calprotectin deficiency has been correlated with increased risk of developing chronic, non-healing wounds [Trøstrup et al., 2011]. It has also been demonstrated to have markedly anti-inflammatory and tissue-protective effects in a model of adjuvant-induced arthritis in rats [Brun et al., 1995]. Further, calprotectin suppressed NF- $\kappa$ B expression and pro-inflammatory cytokine release in a mouse model of autoimmune myocarditis [Otsuka et al., 2009]. Pharmacological blockade of calprotectin has correspondingly been found to exacerbate T-cell activation and cardiac allograft rejection [Shimizu et al., 2011].

Pharmacological administration of S100A8 to mice at the onset of a model of LPS induced sepsis has been reported to significantly increase survival rates, reducing tissue damage, inflammation, and oxidative injuries to major organ systems (Sun, Lu et al. 2013). Of note (following a trend displayed by SRPB1 and CHI3L1) calprotectin has been shown to inhibit the oxidative metabolism of LPS-activated PMNs *in vitro*, which has been postulated to help reduce the oxidative organ injury that follows septic shock [Schwartz et al., 2010, Sun et al., 2013].



The seemingly disparate biological functions observed for calprotectin and its subunits suggest that their effects might be concentration dependent or modulated by the cellular and biochemical composition of the local tissue environment [Schiopu and Cotoi, 2013].

In light of its current interest to clinical research and the commercial availability of reliable antibodies specific to the protein we elected to focus on S100A8 and S100A9 for proteomic analysis. The differences in levels of the gene protein products (calgranulin A and B) between experimental groups were striking, with circulating and peritoneal levels of both proteins found to be almost non-existent in SE animals while freely detectable in enriched mice. The presence of the proteins in the cell lysate of both mouse groups suggested that while calgranulin A and B is actively synthesised in EE and SE animals, only EE leukocytes are constitutively releasing it into circulation. It is interesting to note that if subject to clinical analysis these heightened levels of calprotectin in enriched mice would be regarded as indicative of a hyper-inflammatory or disease state, an assumption that does not correspond with the results acquired in our study. Again, it is tempting to speculate that the increased levels of calprotectin protein (and possibly CHI3L1 and SRPB1) are bestowing an immune-protective phenotype upon enriched animals.

#### **4.6.2 Gene Expression in The Granulomatous Tissues of Enriched Mice**

Sponge implantation induced granuloma is a unique disease model encompassing various aspects of the acute inflammatory, chronic inflammatory and wound healing response. We initially chose this model to investigate how EE would modulate the host response to a persistent, irresolvable immune insult. Consistent with previous models we found this response to be typified by an enhanced cellular infiltrate at the point of inflammation and a moderated release of inflammatory mediators. However, in light of several studies that have correlated EE to improved wound healing [Detillion et al., 2004, Ernst et al., 2006, Vitalo et al., 2012] we became interested to learn if our own enriched animals presented any alteration in both their inflammatory and wound healing response.

A 72 hour endpoint was chosen for this model to allow us to explore the cellular and molecular immune environment of EE mice at a transitional point between the acute and chronic/resolutionary phases of inflammation [Anderson et al., 2008]. As previously mentioned the cytokine readouts and cell types identified in the sponge infiltrate suggested the granuloma may have been proceeding more quickly in enriched mice. As we were unable to specifically investigate the cellular content of the granuloma tissue itself we chose instead to compare patterns of gene expression between EE and SE

groups. One benefit this avenue of investigation afforded was that the gene expression identified not only gave insight into the homeostatic state of the granuloma but also allowed some prediction of how the immune response in these tissues may progress.

The largest group of upregulated genes that we observed in EE animals coded for the core structural components of endothelial tissue.

These included:

Three collagens: (1 $\alpha$ 1, 1 $\alpha$ 2 and 14 $\alpha$ 1): Primarily released by fibroblasts, collagens form the major constituent of connective tissues such as cartilage as well as being abundant in other fibrous tissues such as tendons, bone and muscle [Sherman et al., 2015]. It is a vital resource in the wound healing process not only serving as the raw material for many tissues but also assisting: chemotaxis of fibroblasts and leukocytes to the wound site, aiding the generation of haemostatic plugs and serving as a matrix for the deposition of further tissue [Sherman et al., 2015].

Actin alpha 2: A component of smooth muscle and a marker indicating the differentiation of fibroblasts into smooth muscle cells [Strauch and Rubenstein, 1984]

Transgellin: A shape change sensitive protein reported to bind actin and aid in the formation of actin stress fibres (which provide a provisional level of contractility in healing wounds [Hinz, 2007]) and smooth muscle [Assinder et al., 2009].

Three Integrins: ( $\alpha$ 5,  $\beta$ 1 and  $\beta$ 5): A class of transmembrane protein involved in the adhesion of cells to one another and the extracellular matrix, integrins can be regarded as the mortar (and collagen the bricks) that holds mammalian tissues together [Hynes, 1992]. Previous neurological studies in mice have also reported upregulation of integrin gene expression in the brain following 14 days of environmental enrichment [Rampon et al., 2000a].

The second largest group of upregulated genes were found to code for two families of enzymes involved in tissue remodelling and immune regulation:

Two matrix metalloproteinases: (MMPs, 2 and 9): Both able to degrade various collagens, these MMPs are known to play important roles in tissue reorganisation, repair and angiogenesis [Kundu and Patil, 2006]. Of particular interest MMP9 has been shown to be actively released by neutrophils and act as a neutrophil chemotactic factor [Opdenakker et al., 2001]. A recent study has also reported that rats

show a significant upregulation of hippocampal MMP9 protein when subject to EE at a young age [Cao et al., 2014].

Two cathepsins: (K and L): Cysteine proteases that catabolise elastin, collagen, and gelatine allowing the breakdown of bone and cartilage, cathepsins are released by neutrophils and macrophages [Chauhan et al., 1993, Inaoka et al., 1995].

Several downstream regulators of the previously listed genes were also upregulated in EE mice. Each pluripotent regulators of tissue remodelling and repair:

Epidermal growth factor (EGF) and its receptor (EGFR): known to stimulate fibroblast and keratinocyte proliferation and induce both cells to secrete collagenases (such as cathepsins and MMPs) to degrade the matrix during the remodelling phase [Carpenter and Cohen, 1990].

Plasminogen activator urokinase receptor (PLAUR): A component of the plasminogen activation system, an enzymatic cascade involved in the control of fibrin degradation, matrix turnover and cell invasion. Again neurological studies have demonstrated that EE upregulates plasminogen activation in the hippocampus of adult mice [Horii-Hayashi et al., 2011]

WNT Inducible signalling pathway protein 1 (WNT1): Part of the eponymous WNT signalling pathway, regulates multiple cellular functions, including adhesion, migration, proliferation, differentiation and survival (Babic, Chen et al. 1999).

Vascular endothelial growth factor A (VEGFA): A pluripotent protein involved with mediating; increased vascular permeability, inducing angiogenesis, vasculogenesis endothelial cell growth, promoting cell; migration, and inhibiting apoptosis [Hicklin and Ellis, 2005]. Multiple studies have demonstrated increased circulating levels of VEGF in mice and rats following environmental enrichment paradigms with several suggesting that enhanced VEGF expression is one of the key mechanisms by which EE conveys its neuroprotective and reparative properties [Fares et al., 2013, Goshen et al., 2009, Ortuzar et al., 2013] .This hypothesis has been supported by the finding that inhibition of VEGF activity blocks the neuro-regenerative properties of enrichment [Cao et al., 2004].

Concordant with the cytokine data obtained for the sponge granuloma model the IL-10 gene was also found to be upregulated in EE mice. Increased levels of IL-10 are typically associated with the cessation of inflammation and a shift to a later, resolution focused stage of the immune response [Sugimoto et al., 2016]. Lending additional explanation to the increased leukocyte numbers found within the granulomatous tissue of EE mice, expression of colony stimulating factor three (also known

as granulocyte-colony stimulating factor, G-CSF) was also found to be upregulated. G-CSF has been shown to stimulate bone marrow production of granulocytes and stem cells and promote their release into the bloodstream, it also modulates the survival, differentiation and function of neutrophil precursors and mature neutrophils [Roberts, 2005].

While the results of this gene array only serve as a peripheral analysis of the potential immune state of EE mouse granuloma tissue, it gives a strong indication that enriched animals present an altered immune-regulatory gene program geared towards cellular regrowth and repair. However, whether this program would result in an improved granulomatous response or increased fibrosis and chronic inflammation remains to be seen.

## **4.7 The Behavioural Profile of Enriched Mice**

As a large number of studies have correlated environmental enrichment with a variety of neurological and behavioural changes. We were interested to investigate how EE altered the behaviour of our own mice. Investigations of circulating corticosterone levels proved challenging with levels of the hormone found to vary considerably between experiments and groups (Appendix 4). These variations occurred despite measures to keep the measurement protocol as consistent as possible; taking blood samples at the same time of day to avoid circadian variation in cortisol levels [Chung et al., 2011]. Performing the collection one cage at a time in an isolated room to minimise the effect of transport stress on the mice and performing the EIA assay as constantly as possible.

Previous studies present an equally wide spread in the corticosterone levels observed in EE animals with some higher than those from a standard environment [Benaroya-Milshtein et al., 2004] some lower [McQuaid et al., 2013], and some displaying no change at all [Jain et al., 2013]. While this disparity of results has been attributed to variations in EE protocols, mouse strain or gender [Hutchinson et al., 2012] other researchers have suggested that corticosterone release is triggered too readily in response to a variety of minor physiological and psychological stressors (e.g. loud noises, unusual odours, light) to be used as a reliable measure of mouse behavioural status [Bielohuby et al., 2012]. It is possible that unforeseen and unpreventable events occurring in the animal unit our mice were housed in (e.g. fire alarms, entrance of cleaning staff etc.) could account for the inter-cage and intergroup variation seen in our results.

Our behavioural tests provided more constant results, suggesting that EE animals develop a small but significant reduction in exploratory activity by the end of the enrichment period. Such a reduction in activity is typically associated with a heightened state of anxiety.

Again previous research reports a high level in variation in the behavioural change EE induced, with studies utilising the same behavioural paradigms as ours respectively presenting EE as; anxiogenic [Rampon et al., 2000b], anxiolytic [Benaroya-Milshtein et al., 2004], or causing no change in anxiety at all [Wolfer et al., 2004]<sup>11</sup>.

Many of these variations have also been attributed to disparities in methodology, mouse strain and gender used for EE experiments [van de Weerd et al., 1994]. Male mice in particular have been reported to demonstrate significantly higher levels of aggression in enriched cages compared to those of a standard environment [Wolfer et al., 2004]. Increased bouts of fighting were regularly observed in our enrichment cages as were typical displays of dominance behaviour (such as grooming, barbering and territorial marking) and submissive behaviour (avoidance, crouch response, submissive upright posture) [Malatynska and Knapp, 2005]. The increased complexity of social dynamics in EE cages could very likely account for the heightened anxiety related behaviour observed in our animals, raising the possibility that EE may influence the murine immune response by placing animals under an increased burden of psychological stress.

The effect of psychological stress on the immune system has been a subject of growing study for the past 20 years [Okimura et al., 1986]. Psychosocial status has been shown to strongly influence leukocyte numbers with several studies correlating stress to increased circulation of neutrophils and

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<sup>11</sup> It should be noted that use of behavioural assessors such as these is not without its controversy. Many researchers argue that activities that constitute anxiety like behaviour in one; species, strain, gender, environment are not contiguous with anxiety like behaviour in another [Bourin and Hascoët, 2003, Crawley, 1985].

A further personal (and hence subjective) observation: After spending several hundred hours in the company of enriched mice, I found their behaviour when interacting with; each other, their environment and myself to be far more active and engaged compared to animals from a standard environment (Appendix 5). Enriched animals – at the risk of anthropomorphising – seemed more interested in their environment, and standard animals correspondingly, more apathetic. Such apathy, within the paradigm of the light dark box, open field test and elevated plus maze would be classified as a low state of anxiety (and accordingly present EE animals as comparatively more anxious). To me this seems a misleading assessment of these animal's behaviour. However, I am not a behavioural biologist and accept the established wisdom of these long used behavioural assessors.

monocytes [Wohleb et al., 2015, Swan and Hickman, 2014]. Stress has further been shown to induce myelopoiesis, upregulate cell effector activity and promote the release of numerous immunomodulatory factors [Viswanathan and Dhabhar, 2005, Fuertig et al., 2015], results that parallel many of our own. These findings have more recently led to some ecologists and behavioural biologists advocating leukocyte numbers and ratios as a biomarker for stress in animals, although the reliability of such readout is contested [Swan and Hickman, 2014].

Many of these studies have focused on the immune-destabilising effects of chronic or persistent stress. Which have been demonstrated to include leukocyte suppression, dysfunction and a chronic inflammatory phenotype [Padgett et al., 1998, Zhang et al., 2008, Glaser and Kiecolt-Glaser, 2005]. However, an emerging body of research has turned its attention to the effects of mild or sub-acute stress on psychology, neurology and the immune response.

Such studies have found mild stress elicits a number of beneficial effects on the central nervous system, such as reduced anxiety levels [Benaroya-Milshtein et al., 2004, Rampon et al., 2000b] enhanced memory, modulation and upregulation of neurogenesis [Kempermann et al., 1997] neural plasticity [Sale et al., 2009] and most saliently; improved recovery from brain injury and cerebral disorders [Laviola et al., 2004, Kovessdi et al., 2011]. This phenomena has been termed eustress (*eu – good*, Greek) [Le Fevre et al., 2003] or inoculation stress [Crofton et al., 2015]. Studies of eustress have associated beneficial immuo-protective effects with parallels to our own; enhancing leukocyte numbers, cellular effector function and promoting a immune protective, immune-moderated phenotype [Dhabhar, 2000, Dhabhar et al., 2012, Berk et al., 2001].

Such findings present the possibility that environmental enrichment serves as a means of generating eustress in experimental animal and that this stress in turn may contribute to the immune alterations we see in EE animals. This raises many questions for further research, such as; as what point does eustress simply become 'stress'? And would this shift from a positive to negative stress state produce an accompanying alteration in immunity? Initial attempts to answer these questions are made in the proceeding section of this thesis.

## 4.8 Questions, Challenges, Limitations and Conclusions

Perhaps the biggest issue within this of this body of research originates from the methodology itself. As discussed in Hutchinson et al., 2005 and throughout this thesis EE studies display a huge variation in their methodologies and an accompanying large disparity in the results they produce. While attempts at standardising the EE methodology have been made [Gurfein et al., 2012a] variation persists. In addition, the effects of enrichment on mice have been demonstrated to depend on the strain and sex of the animals used, further complicating the search for a single enrichment strategy that can be used effectively for all animals, [Coutellier and Würbel, 2009, Fitchett et al., 2005, Lin et al., 2011, Touma et al., 2003, Wood et al., 2010]. A consequence of this variation is that that several authors have come to regard EE as a confounding variable and accordingly a methodology that should be avoided [Hutchinson et al., 2012].

A possible source of this issue is that as a concept environmental enrichment is ambiguous; its underlying purpose defined by the nature of the research performed and the field it is focused within. For example, in the field of zoo and captive animal studies, where the practise of EE was originally conceived, EE's goal is to improve the psychological and physical welfare of animals with an emphasis on mitigating aggressive or stereotypical behaviours. For livestock research however, welfare is regarded as something of a bi-product of attempts to improve the economy of generating meat, dairy or any other animal products. Neurobiologists typically consider EE as a means of modelling a sensorially and psychologically stimulating environment, seeking to interpret how such external stimuli effect animal and human neurobiological processes. Immunology currently seems to lack a definite purpose behind its use of EE, with some studies describing it as a means of improving welfare [Marashi et al., 2004], inducing stress [Cao et al., 2011], promoting physical exercise [Benaroya-Milshtein et al., 2007], or often giving no reason at all other than that it has been shown to effect immunology in previous literature [Reichmann et al., 2013].

In creating my own EE protocol my goal, (similar to that of neurobiology) was to attempt to generate an environment that evokes the complex physical and psychosocial events that occur in the lives of non-captive, wild animals and the organisms whose biology we so often try to emulate in scientific studies; humans. A secondary goal was to keep my enrichment set up as simple and reproducible as possible (use of enrichment items typically already available in animal units and of enclosures

compatible with the rack system most animal units employ) with the intention that proceeding research will utilise it again. It is my hope that with further study the purpose of EE in immunology research will become more clarified and its methodologies accordingly more consistent.

Increased variability in results will inherently be a component of techniques such as EE. When you increase the complexity of any system you increase the number of outputs it can produce. Nevertheless, patterns of consistency were found in my results, that have been reproduced by other members of my lab. Suggesting that such consistency is not an impossibility.

It is also hard to ignore the overlap that lays between EE and physical enrichment. Many of the results of EE can be attributed to the effects of physical exercise alone. However, as mentioned previously, studies utilising environmental enrichment without the inclusion of apparatus that invoke overt physical exercise has been shown to have significant effect on the immune response [Arranz et al., 2010, De Sousa et al., 2011, Marashi et al., 2003]. Further, in one study that demonstrated EE limited the growth of pancreatic tumours, it was found that separating the EE methodology utilized into its individual parts greatly lessened its anti-tumorigenic properties:

*“Each single component of EE (inanimate stimulation, social stimulation or physical exercise) was not profound enough to achieve comparative anti-tumor effects as EE.”[Li et al., 2015].*

My own data, though preliminary supports these findings (appendix 7). It also interesting to note that experiments in which groups of mice were subject to equal bouts of forced or voluntary exercise in the context of an experimental model of Alzheimer’s disease showed strong differences in their physiological responses, with the voluntary group presenting a greater reduction in disease symptoms than the forced [Yuede et al., 2009]. This would suggest that there is at the very least a psycho-neurological component in the influence of exercise on the immune system (and logically for EE paradigms as well). Nevertheless, without a more thorough dissection of PE from EE, it is difficult to categorically say what the root causes of these immunological effects are.

While the results our study clearly demonstrate that environmental enrichment increases leukocyte cellularity and infiltration to a site of inflammation, our data can currently only strongly suggest that this results in a more rapid and efficient immune response or inflammatory resolution. It is difficult to be



certain of this effect without allowing the models we chose to continue to completion (i.e. the end of inflammation, complete granuloma formation, or the death/survival of the mouse). This was not possible in the models we employed due to the constraints of our project licence. However, in further studies more moderate, resolving models of inflammation could be used to investigate the process of EE, such as those modelling atopic dermatitis or scratch wound assays.

Further this study elected to focus upon three specific inflammatory cell types to investigate the immunomodulatory effects of EE; neutrophils, monocytes and macrophages. These cells were chosen for their vital role in the instigation and progression of inflammation and also because the majority of other studies on EE and the immune response have – following on from neurological studies – focused on lymphoid leukocyte subsets. However, there are many other leukocyte populations that play a key role in the models we have used (such as dendritic cells and eosinophils for ZIP and sponge granuloma) that should be studied for a more complete picture of the EE effect. As an addendum to this, a more complete flow cytometric cellular profile could be pursued. Which, for example could seek to determine relative levels of pro or anti-inflammatory macrophage subpopulations elected in the EE-immune response<sup>12</sup>.

A final limitation of this study (one that only became truly apparent in its latter stages) is that though the data we have obtained presents a relatively detailed picture of the effects of environmental enrichment on the murine inflammatory response. It provides little incite as to its cause. While some speculation can be made as to the relationship between the effects of enrichment on the psychological, neurological and immunological state of an animal, none of the data generated in this study can provide a definite connection between them.

Nevertheless, this study has demonstrated that enrichment does have a tangible effect on immune homeostasis.

The fact that our external environment evokes such a strong biological response is not a surprising one. The immune response is, after all one of an interconnected series of homeostatic systems designed to respond to alterations in both our internal and external environment [Medzhitov, 2008].

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<sup>12</sup> Preliminary attempts were made to further dissect infiltrating monocyte populations in ZIP, utilising differential Ly6C, F4/80, CD11b and MHCII staining. The results (not shown) proved inconclusive.

However, this study and others of its kind serve to remind the researcher of the huge variability of immunological and clinical outcomes that varied living conditions can generate. With this in mind and the fact that the ultimate purpose of most animal experimentation is to emulate human biological responses as accurately as possible; it seems that the housing environment experimental animals are kept within should be a chief point of consideration in experimental design.

While the results of this study and others like it are framed in such a way as to suggest that environmentally enriched animals possess an improved immune capacity it is equally possible to suggest the obverse; that animals housed in a standard lab environment have diminished immune function. Currently the standard environment most experimental animals are housed within are considered by many researchers to be impoverished; lacking in adequate psychological, social or physical stimulation [Balcombe, 2010, Balcombe, 2006, Benefiel et al., 2005, De Sousa et al., 2011]. As a result animals housed in standard laboratory conditions are demonstrated to be relatively overweight, insulin resistant, hypertensive and more likely to experience premature death than their wild counterparts<sup>13</sup> [Martin et al., 2010].

*“The standard laboratory mouse lives what might be considered a “couch potato” existence... They are kept comfortable with an endless supply of food and water and a few potential playmates. But they don't have much of anything to do.”* [Cao et al., 2011].

As such it could be argued that protocols such as environmental enrichment increase the biological relevance of captive environments for experimental purposes [Newberry, 1995].

Taking a wider view of the implications of this research further consideration should be made of the influence of the psychosocial (not just the physical) environment on human immune responses in a clinical context. There is strong evidence that patient-specific immune states before surgery are highly correlated with recovery [Fragiadakis et al., 2015] and in turn that these immune states are often strongly influenced by the environment they live within [Brod et al., 2014b]. Enrichment procedures such as the one used in this study could be used as a means of stratifying the potential effects of environment on patient immune outcomes and personalising treatment regimens accordingly.

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<sup>13</sup> Excluding, one presumes the risk of mortality from predation wild animals experience.

# Chapter 5 Ongoing Research

## 5.1 Introduction, Stress and Immune Function

The concept of a palpable relationship between psychological and physiological states is an ancient one. As far back as the second century AD roman physician Galen (Aelius Galenus) hypothesised that a “balance of the passions of the soul” was fundamental to good physical health going on to distinguish between disease states induced by organic and emotional means [James, 1997]. Such theories have pervaded western medicine for much of recorded history. Up until the late nineteenth century intense emotional states and responses (such as grief, anger and anxiety) were still considered a leading cause of human morbidity and mortality. More recently, in light of modern analytical techniques this field has received a resurgence in interest. In 1975 seminal studies by Robert Ader and Nicholas Cohen observed that rats fed the immunosuppressive drug cyclophosphamide in a liquid solution containing saccharin would later (after recovery) display the same immunosuppression after being fed saccharin solution alone. The authors suggested this provided early proof of a neurological effect (psychological conditioning) influencing the immune response [Ader and Cohen, 1975]. Later studies would go on to identify the presence of neuropeptide specific receptors (for signalling molecules such as calcitonin gene-related peptide, secretoneurin, vasoactive intestinal peptide (VIP), and substance P (SP) [Kaneider et al., 2003]) on the surface of leukocytes and direct innervation of immune organs such as the thymus and spleen [Ruff et al., 1985]. These findings and others led to the initiation of the interdisciplinary field of psychoneuroimmunology (PNI). Perhaps the greatest contribution made by this burgeoning field so far is through its investigations of the influence of psychological stress on the immune response.

Stress is a nebulous term, its connotation varying across the fields (and individuals) that study it. Dhabar and McEwin provide an integrated definition of the term suggesting stress is a:

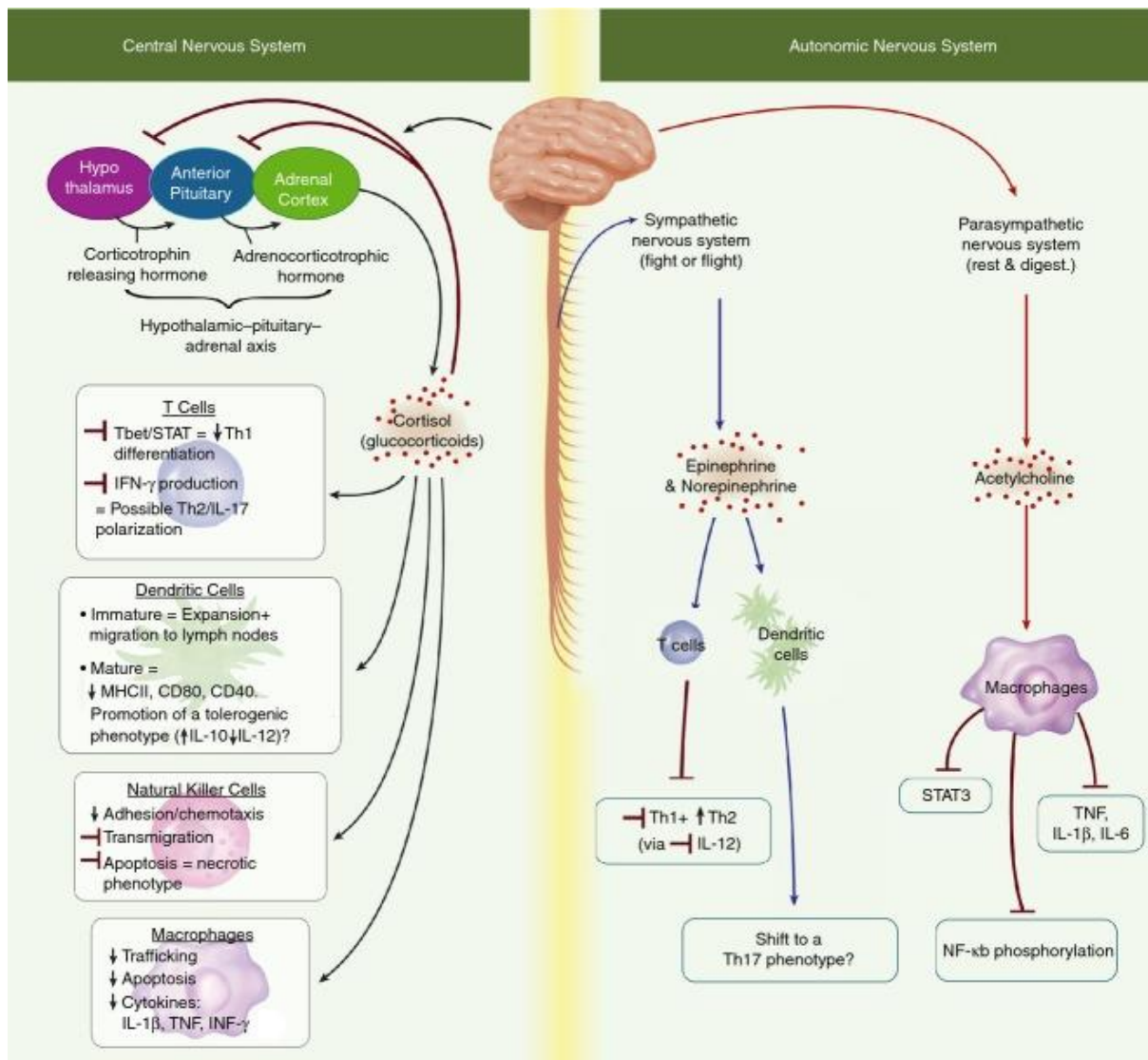
*“Constellation of events, consisting of a stimulus (stressor), that precipitates a reaction in the brain (stress perception) that activates physiological fight or flight systems in the body (stress response).”*

*[Dhabhar and McEwen, 1997].*

Stress has been shown to elicit biological responses through a variety of neurological and endocrinological mechanisms, many of which have not yet been fully elucidated. However, central to

the stress response is set of interconnected regions in the brain referred to as the hypothalamic pituitary adrenal (HPA) axis. Immediately following a perceived stressor, the hypothalamus releases corticotrophin releasing hormone (CRH). CRH in turn stimulates the pituitary gland to secrete adrenocorticotrophic hormone (ACTH), which correspondingly stimulates the adrenal cortex to secrete glucocorticoids (in humans, the primary glucocorticoid is cortisol, in mice corticosterone). Activation of the hypothalamus also activates the sympathetic nervous system (SNS), which in turn stimulates the adrenal gland (specifically the adrenal medulla) to secrete further hormones; epinephrine (also known as adrenalin) and norepinephrine. These hormones heighten metabolic activity and are responsible for the feelings of anxiety that proceed stress [Barnes, 2010]. Additional studies have shown that some time following activation of the HPA and SNS, stress also activates the parasympathetic nervous system (PNS) inducing release of acetylcholine, a pluripotent neurotransmitter that (broadly speaking) counteracts the effects of cortisol and adrenaline returning the body to a resting – non stressed – state [Lehrner et al., 2016].

Each of these stress induced hormones (cortisol, adrenalin and acetylcholine) have been shown to exert specific immune-modulatory effects; inhibiting release of a variety of inflammatory mediators and promoting a tolerogenic or anergic leukocyte state (Figure 5-1). This has led many researchers to suggest that psychological stress is acutely immunosuppressive in effect [Pruett et al., 1993, Segerstrom and Miller, 2004].



**Figure 5-1.** The immune regulatory influence of the nervous system. The central nervous system (CNS) mediates the release of various immune influencing glucocorticoids via activation of a series of connected regions within the brain referred to as the hypothalamic–pituitary–adrenal axis. Research suggests the CNS to be primarily immune-suppressive in action, inhibiting production of pro-inflammatory cytokines, chemotactic factors and limiting migration and activation in several immune cell types. [Barnes, 2010, Moser et al., 1995, van Oosten et al., 2010, Wingett et al., 1996] Additionally the CNS elicits differential immune effects dependent on cell type and stage of development, inducing the expansion and migration of immature dendritic cells while seemingly promoting a tolerogenic phenotype in their mature counterparts [Moser et al., 1995, Roca et al., 2007]. Although less studied, several papers have demonstrated that the sympathetic nervous system is able to modulate immune activity through production of epinephrine and norepinephrine promoting a T helper type 2 (Th2) and Th17 phenotype in T-cells and dendritic cells, respectively [Kim and Jones, 2010, Panina-Bordignon et al., 1997]. Produced by the parasympathetic nervous system, acetylcholine has been shown to interact directly with multiple immune cell subsets through expression of acetylcholine receptors, leading to suppression of a number of pro-inflammatory pathways in macrophages and other immune cells. [Borovikova et al., 2000, de Jonge et al., 2005, Yoshikawa et al., 2006] Abbreviations: IFN-γ, interferon-γ; IL-17, interleukin-17; NF-κB, nuclear factor-κB; STAT, signal transducer and activator of transcription; Th1, T helper type 1; TNF, tumour necrosis factor. Figure taken from [Brod et al., 2014a].

Numerous studies have revealed that subjection to chronic or persistent stress (repeated stress exposures over the course of weeks or months) has a dysregulating or suppressive effect on the immune system. In human and animal studies, chronic stress has been shown to inhibit: leukocyte proliferation [Jiang et al., 1990, Bartrop et al., 1977] antibody production [Fleshner et al., 1989, Edwards and Dean, 1977], NK cell activity [Irwin et al., 1990, Bartrop et al., 1977, Rosenne et al., 2014], Lymphocyte function, macrophages function [Brown and Zwillling, 1994, Bonneau et al., 1991, Bobel et al., 2015], and the overall humoral immune response [Kelley et al., 1982, Weiner 3rd et al., 2012].

Corresponding to these studies, chronic stress has been linked with a number of pathologies associated with immune dysfunction such as cancer [Antoni et al., 2006], improper wound healing [Padgett et al., 1998] and age related acceleration of immuno-senescence [Epel et al., 2004]. Interestingly and in accordance with its purported immune-suppressive effects, chronic stress has been shown to reduce the severity of symptoms in murine models of type II collagen-induced arthritis [Rogers et al., 1980] and experimental autoimmune encephalomyelitis (EAE) [Griffin et al., 1993].

However, as mentioned in the main body of this thesis, acute stress (short-term, intermittent) has conversely been shown to have an activating effect on the immune/inflammatory response. Short-term stress promotes an initial increase and subsequent decrease in blood lymphocyte and monocyte numbers and an accompanying increase in blood neutrophil numbers [Dhabhar et al., 2012]. It has further been demonstrated to enhance leukocyte infiltration into tissues, and upregulate their effector activity [Dhabhar and McEwen, 1996]. This effect is postulated to be driven in part by upregulation of the inflammatory cytokines, IFN- $\gamma$ , TNF- $\alpha$ , MCP-1, IL-1 $\beta$  and IL-6 [Viswanathan et al., 2005, Dhabhar and Viswanathan, 2005].

In contrast to chronic stress, acute stress has been shown to improve anti-tumour activity [Dhabhar et al., 2010]. While correspondingly increasing the severity of symptoms in experimental arthritis [Cutolo and Straub, 2007] and EAE [Franklin et al., 1988].

The seemingly bi-phasic effects of stress are currently a subject of ongoing research, however its effects hint of the presence of a biological feedback mechanism. Supporting this, low-dose corticosterone or epinephrine administration has been found to significantly heighten the inflammatory

immune response, while high-doses of corticosterone are found to be potently anti-inflammatory in effect [Dhabhar and McEwen, 1999]. This suggests that released stress hormones are immune-activating at physiological/acute concentrations and immune suppressive at pharmacologic concentrations [Dhabhar, 2014].

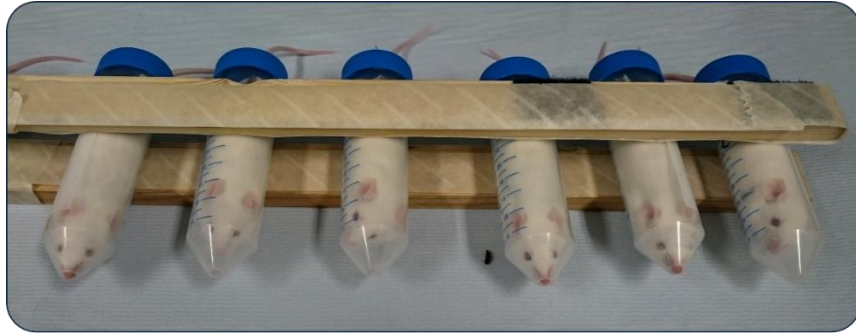
Such findings raise several questions in light of our own data on EE animals, which according to our behavioural analysis are likely in a state of heightened psychological stress. The immune phenotype of EE mice; increased leukocyte numbers, heightened infiltration and activity coupled with a moderated inflammatory cytokine release would appear to sit somewhere between that of chronically and acutely stressed animals: A unique and possibly advantageous physiological state to be in.

In an attempt to begin dissecting the effects of varying form of stress on the immune response and contrast them with previously observed results, we have begun a series of experiments subjecting CD1 mice to the same battery of inflammatory immune models previously described, in the context of an environment of chronic psychological stress. The initial findings follow.

## **5.2 Methodology. Stress Induction**

With the exception of the stress induction procedure, methodologies used in the proceeding experiments were the same as those stated in chapter two. Briefly, 24 age matched male CD1 mice were housed in cages groups of six in a standard lab enclosure set up. Following one week of acclimatisation animals were separated into standard environment (SE) and chronic stress environments (12 mice per experimental group).

Animals in the chronic stress group were subject to two hours of chronic restraint stress for each day of the experimental period. In accordance with established procedures [Roper et al., 2010, Lin et al., 2013] stress was induced by placing animals in 50ml falcon tubes with air and tail holes cut at each end and securing them in place for two hours. Animals were continually observed over the stress period to ensure they experienced no physical harm and were each checked for signs of injury once the stress period was concluded.



**Figure 5-2. Experimental set up for chronic restraint stress.**

At the end of the experimental period animals were either subject to behavioural tests or six hours zymosan induced peritonitis, with accompanying observations of peritoneal and circulating blood populations.



## 5.3 Current Results

### 5.3.1 Comparative Weight Gain

Over the course of the 14 day experimental period animals subject to a high stress environment demonstrated a significantly reduced weight gain than those from the standard environment, with weight gain being observed to plateau for restrained animals at day 10. At the end of the two weeks SE animals had gained on average two grams of additional weight compared to restrained.

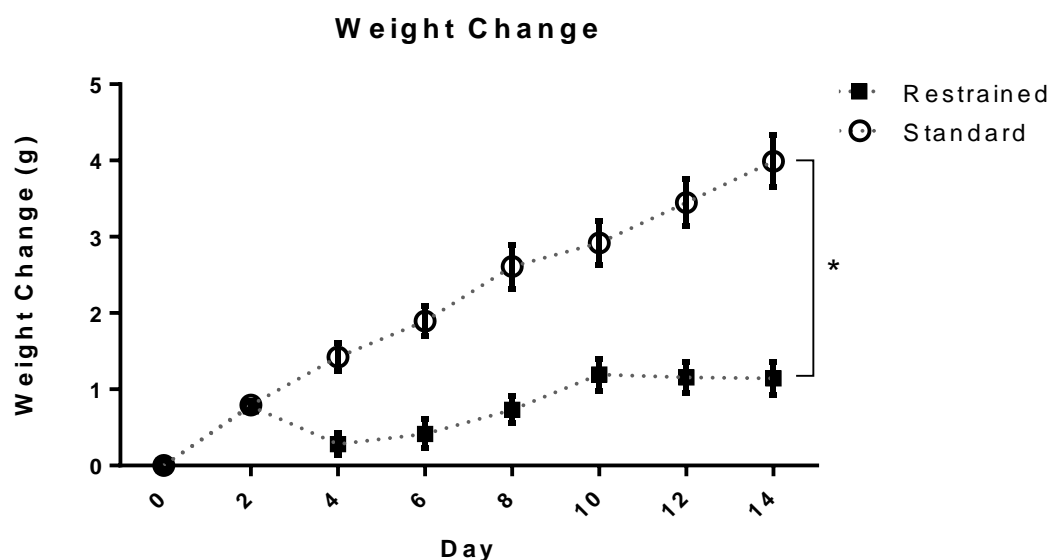


Figure 5-3. Comparative weight change over time. 24 Mice per group, N=3 experiments. Statistical significance determined using multiple t-test analysis, results were normalised the Holm-Sidak method, with alpha=5.000%.

### 5.3.2 Circulating Corticosterone Levels and Initial Behavioural Analysis

At the end of the two week experimental period significantly higher levels of circulating corticosterone were recorded in animal's subject to restraint stress (Figure 5-4a). Corresponding to these findings restrained mice were observed to display significantly heightened anxiety like behaviour, demonstrated through a significant reduction in the overall time spent in light and transitions across the light and dark areas of the test apparatus (Figure 5-4b).

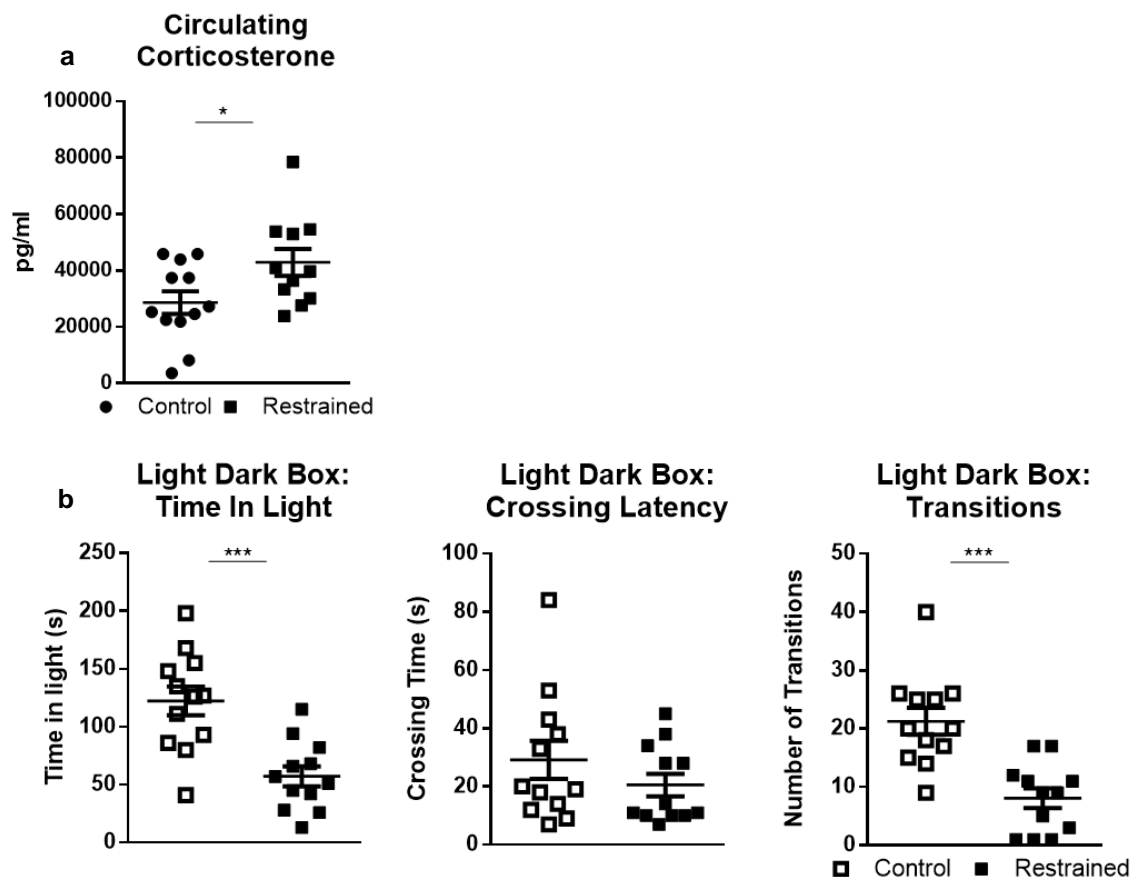
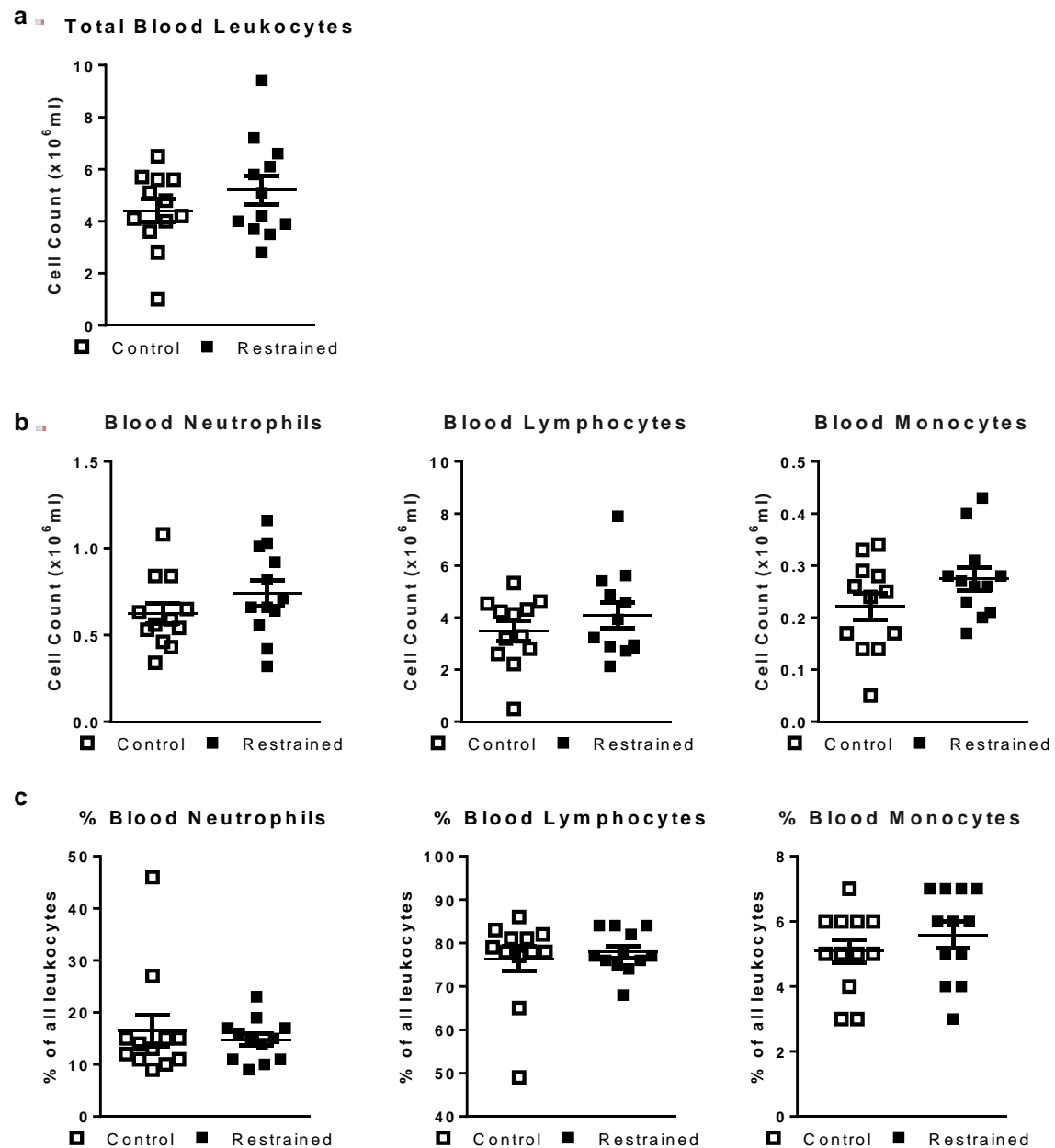


Figure 5-4. Corticosterone and light dark paradigm analysis of animals following two weeks in a standard or chronic stress environment. (a) Comparative levels of blood corticosterone representative of N=2 experiments. (b) Results of light dark box behavioural paradigm, from left to right: Time spent in light, crossing latency, transitions from light to dark region. \* $p < 0.005$  (\*\* $p < 0.0005$ ), t-test. Figures representative of N=1 experiments, 24 mice per experiment.

### 5.3.3 Blood Profile of Chronically Restrained mice

In contrast to animals housed in an enriched environment, stressed animals in a basal immune state showed no significant differences in overall leukocyte numbers or relative proportions of leukocyte population in blood.

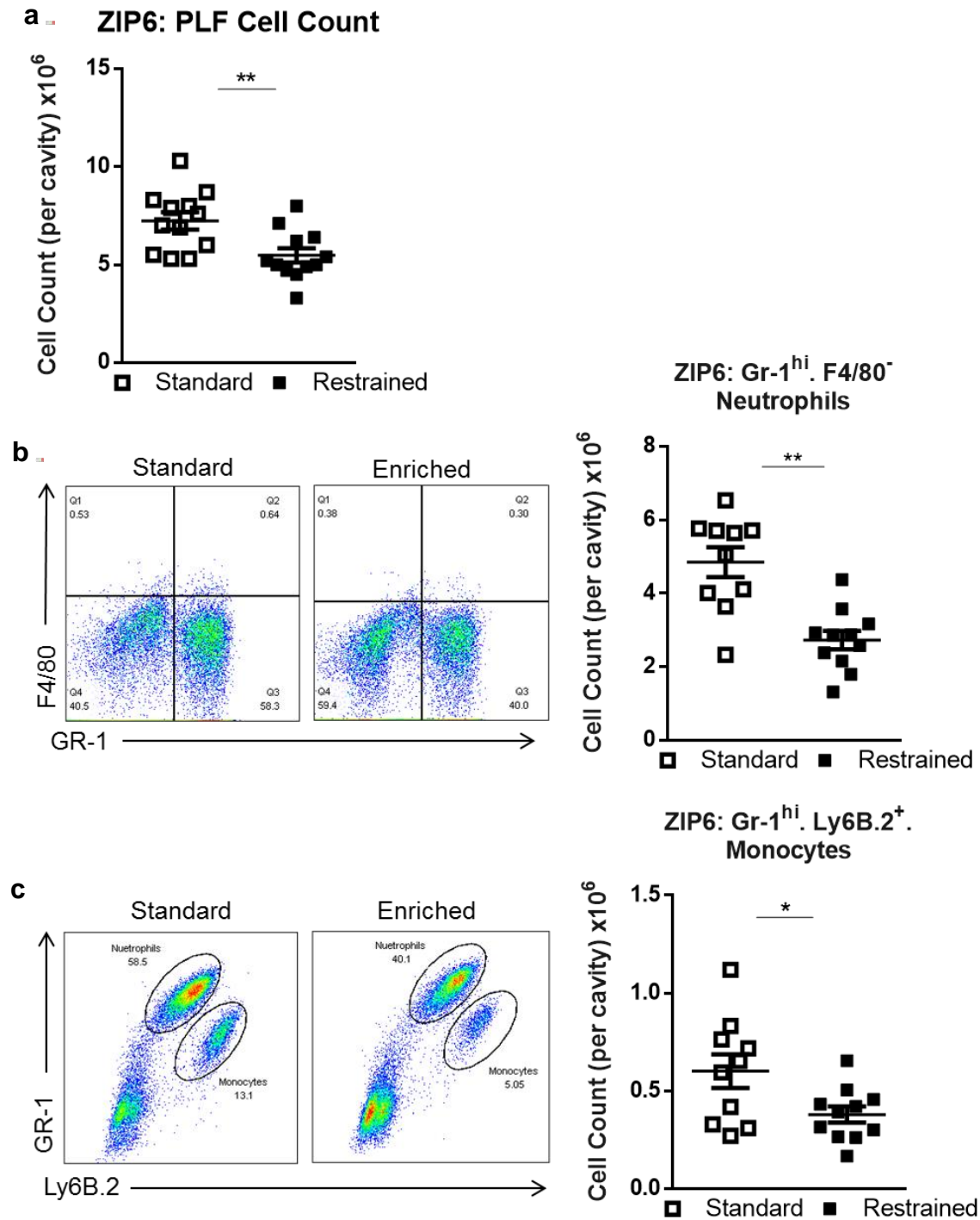


**Figure 5-5. Circulating blood cell populations in mice from and standard or chronic stress environment.** (a) Total blood leukocyte count (b) Individual leukocyte population counts (c) Relative percentages of individual leukocyte populations. t-test. Representative of N=2 experiments, 24 mice per experiment.

#### **5.3.4 Zip6: Cellular Profile of the Peritoneal Cavity**

Following two weeks of chronic stress and six hours ZIP, analysis of peritoneal lavage revealed significant differences between stressed and standard environment animals. In a reversal of the results obtained from enriched animals stressed mice displayed a significantly lower level of leukocyte infiltration into the peritoneal cavity compared to SE.

Flow cytometric analysis revealed the major source of this depleted infiltration to be to be GR1<sup>hi</sup>, F4/80<sup>-</sup>, expressing cells ascribed as neutrophils, with numbers on average half of that found in SE animals. Smaller but significant reductions were also observed for GR1<sup>hi</sup>, Ly6B.2<sup>+</sup> cells suggesting a wholesale reduction in the level of leukocyte infiltration in stressed mice.



**Figure 5-6. Peritoneal cell populations in chronic stress and standard environment mice following six hours of zymosan induced peritonitis.** (a) Overall peritoneal cell count. (b) FACS plot and respective count of F4/80<sup>+</sup> GR1<sup>hi</sup> expressing cells attributed as neutrophils (Quadrant 3). (c) FACS plot and respective count of a Gr1<sup>hi</sup>, Ly6B.2<sup>+</sup> cell population attributed as monocytes. \* $p < 0.05$  (\*\* $p < 0.005$ ), t-test. Representative of N=3 experiments, 24 mice per experiment.

## 5.4 Initial Points of Discussion

These results present the initial findings of a planned series of experiments aimed at comparing and contrasting the differential effects of stress on the inflammatory immune response. While only preliminary these findings suggest that the immune modulating effects of the stress incurred by environmental enrichment are very different to that of chronic stress.

In order to confirm that our animals were indeed under an increased burden of stress we initially subjected them to behavioural analysis and assessed circulating levels of corticosterone. In accordance with previous studies chronic restraint stress invoked a significant increase in blood corticosterone [Kant et al., 1987, Shutt et al., 1988, Gong et al., 2015] and a highly significant increase in anxiety like behaviour [Bourin and Hascoët, 2003, Mineur et al., 2006]. These results contrast to those found in our study of EE animals which were observed to display variable levels of corticosterone and a lower level of anxiety like behaviour compared to restrained animals. This would suggest that although enriched animals are likely to be under some form of stress, its duration and intensity is less than that of animals subject to chronic restraint.

Restrained animals were also found to present drastically reduced levels of weight gain compared to SE animals. Studies on mice have observed variable changes in food consumption in response to stress. With some reporting anorexia [Hosoi et al., 2016, Jeong et al., 2013] and others increased weight gain [Zhang et al., 2014]. Regardless, these results – when viewed in light the data obtained for ZIP – would suggest that reduced adiposity is not a primary contributing factor to the improved cellular response EE mice present.

In contrast to other studies of its type, chronic stress was found to have no significant influence on circulating blood leukocyte populations [Dhabhar and Mcewen, 1997]. However, a significantly impaired cellular responses was observed following administration of zymosan to restrained animals. Reduced or inhibited cellular responses are a frequently reported effect of chronic stress, with several studies implicating heightened circulating levels of immuno-suppressive corticosteroid hormones as the cause [Butcher et al., 2005, Roth, 1985, Barton et al., 1987].

A key source to this reduced cell influx was found to be neutrophils. When contrasted to the results obtained in our analysis of EE animals one could hypothesise that the cause of this reduction might be reduced granulopoiesis in the bone marrow. Indeed reduced myelopoiesis is one of a number of immunosuppressive effects attributed to elevated corticosterone levels [Garvy et al., 1993].

At this date we plan to subject chronically stressed animals to a more complete biological and immunological analysis including; cytokine analysis and a full timecourse for ZIP. Complimentary experiments are also planned to investigate the effects of acute non-persistent stress on the murine immune response.

It has been proposed that an optimum level of stress may exist, a point in which an individual is suitably stimulated (psychologically and physiologically) by their environment without being overwhelmed by it [Le Fevre et al., 2006]. Identification of such a zone of 'optimal stress', would certainly be a great interest to researchers, clinical practitioners and the public.

It is hoped that with this data and more comprehensive behavioural/neuro-endocrinological analysis we may be able to identify where environmental enrichment sits on the currently developing spectrum of stress induced immune responses (Figure 5-7).

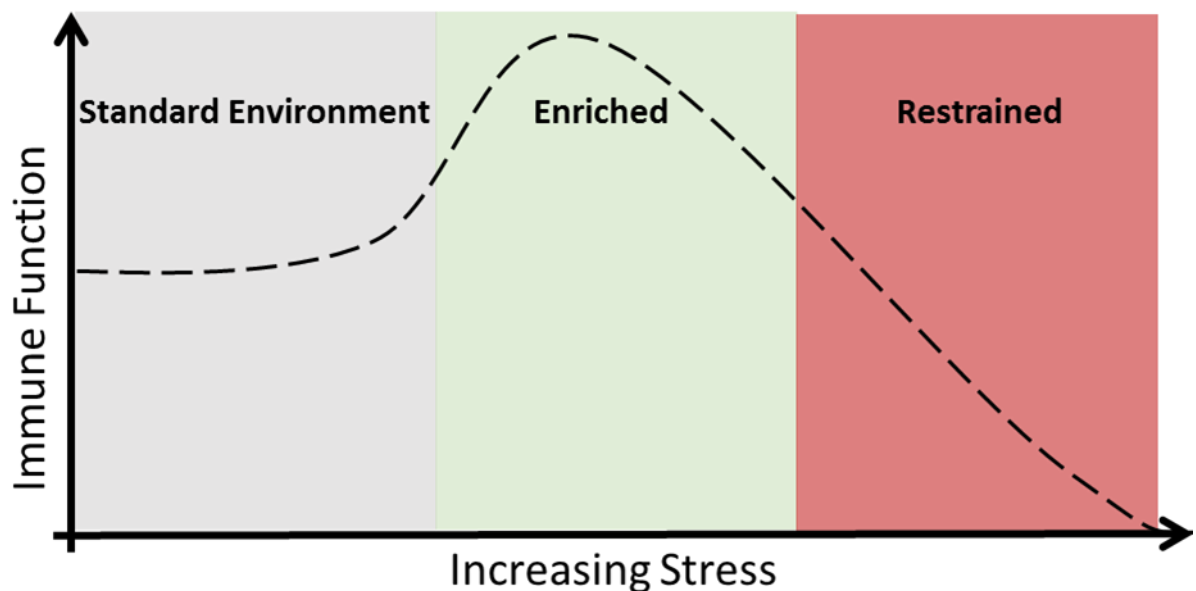


Figure 5-7. Hypothesised differential effects of stress on the immune response.

## Chapter 6 **Afterword**

A great deal of research within my lab and across many other groups is invested in exploring mechanisms that favour or contribute to a more effective immune response and a faster return to a functional homeostatic state. The results of this study provide a new<sup>14</sup> outlook on the potential mechanisms that may facilitate this re-establishment of equilibrium, suggesting that alterations to an individual's external environment (and the improvement or reduction in wellbeing that may cause) can alter both the mode and magnitude of the host immune response.

While environmental enrichment appears to have a tangible effect on the immune response, deeper analysis will be required to ascertain if its effects could be of benefit in the context of disease and more specially disease recovery. In order to derive a practical benefit from these findings concerted research efforts should be made to correlate and stratify clinical and animal studies with one another. Environmental enrichment has already been demonstrated to improve rodent wound healing in response to physical trauma [Xerri and Zennou-Azogui, 2003] and comparable clinical studies have revealed interventions designed to improve the mental wellbeing of patients leads to a marked improvement in overall health [Diener and Chan, 2011] and reduced expression of genes associated with chronic systemic and cellular inflammation [Irwin et al., 2015]. Longitudinal and cross sectional studies are beginning to correlate discrete elements of human life (at the social, psychological and physical level) with alterations in inflammatory gene and biomarker expression [Cole et al., 2007]. It would be interesting (though ambitious) to attempt to reproduce specific aspects of social, home and hospital ward environments in experimental animal enclosures, in order to investigate how such elements may influence murine biology and compare these results to those found in human subjects.

As a corollary, the results of this study are in line with a growing body of literature demonstrating the need to investigate immune responses (and disorders) as dynamic multifaceted systems. Attempts to regulate these systems effectively must account for different factors such as living conditions and psycho-social status [Beura et al., 2016].

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<sup>14</sup> Perhaps 'renewed' might be the more appropriate term. The concept that our external environment may influence our internal, is an ancient one.



A common question posed when presenting the findings of this study is “can environmental enrichment serve as a viable treatment for human immune disease or as a prophylactic against microbial or inflammatory disease?” The honest answer to this question is currently “No”. The results we have obtained cannot be easily translated from mice to humans. Most humans live in an environment significantly more dynamic and sensorially diverse than that of an ‘enriched’ mouse enclosure. Further, the emotional complexity of humans is such that it is often very difficult to categorically define what would be considered an ‘enrichment’ to their environment. Consider for example the differing ways introverted and extroverted personality types may respond to a novel social situation, for one it could be a stimulating and engaging experience while for the other a potentially detrimental stressor<sup>15</sup>.

Put explicitly this studies’ results find that enriching the living conditions of male CD1 mice modulates the expression of a specific set of immune genes that may promote an improved innate cellular response both in the animals and humans. Providing this can be verified, one might use such genes to identify and stratify patients at risk of developing septicaemia or other inflammatory pathologies. Accordingly, a far-reaching ambition for this study could be to pharmacologically modulate the immune genes we have identified to therapeutically treat high-risk patients.

Perhaps the most convincing finding to emerge from this thesis is that we cannot consider biological systems such as experimental animals or indeed human beings in all their diversity as ‘fixed’ in their immune response. Both mice and humans actively respond to alterations in their living conditions, changing multiple aspects of their behaviour and biology to accommodate variations in their environment. Such dynamic adjustments to an altering external and internal environment are often termed allostasis or homeorhesis [Verburg-van Kemenade et al., 2017].

The ultimate purpose of animal experimentation is to emulate human physiological responses as accurately as possible. With the results of this study and others in mind, it is clear that a consideration of the environment we house such animals in [French et al., 2009] and its parallels to human living conditions [Beura et al., 2016] should be an important part of experimental design.

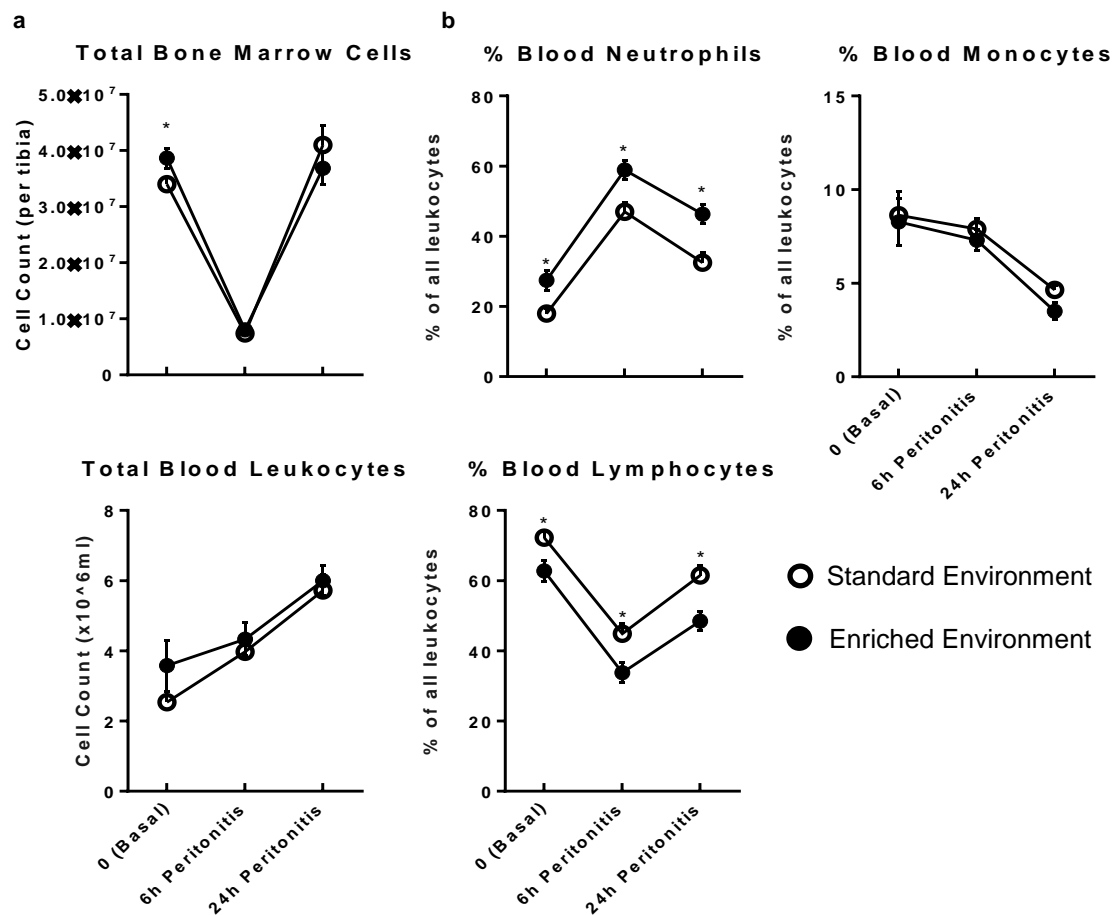
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<sup>15</sup> Note that our understanding of the emotional world experimental animals inhabit remains in its infancy. It is quite possible that personality types such as ‘introverted’ and ‘extroverted’ exist in mice and accordingly it should be remembered that our current classification of what constitutes an ‘enriching element’ in a mouse environment is potentially as crude a designation than any one might attempt to apply to human enrichment.

As corollary of this it is my opinion that the conditions we house many experimental animals under both fail to adequately illustrate the complex biological systems we wish them to emulate and are in fact often physically and psychologically impoverishing for the animal in question. It is my hope that future research and policy around animal research takes these factors into consideration and my personal intent to involve myself in endeavors that will drive these goals forward.

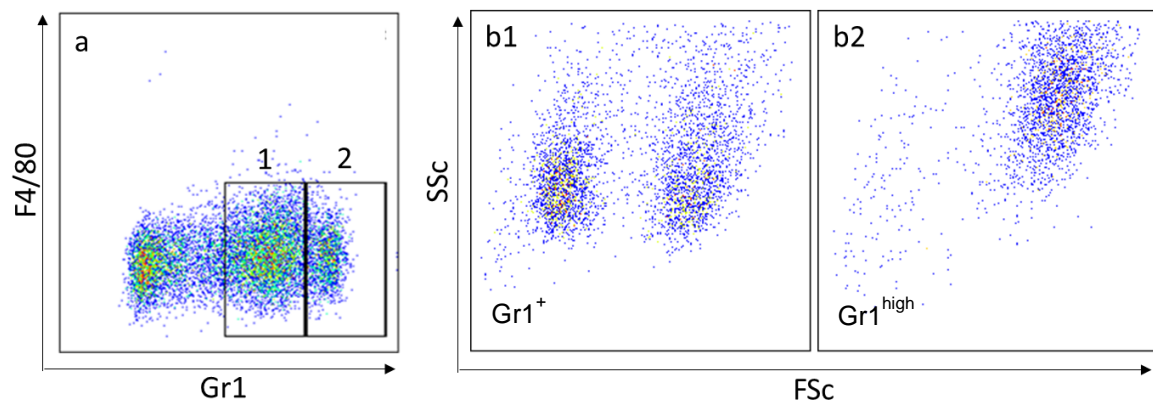
# Chapter 7 Appendix

## 7.1 Variations In Blood And Bone Marrow Leukocyte Populations Over The Timecourse Of Zymosan Induced Peritonitis

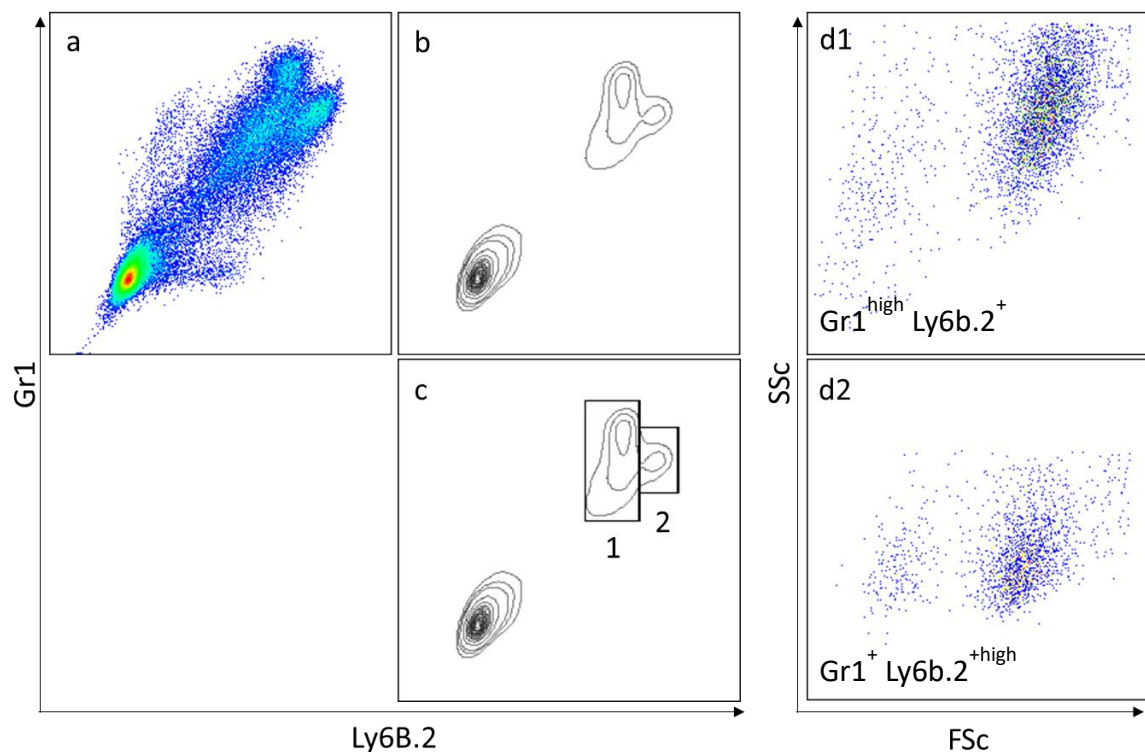


Appendix 1. Comparative cellularity in the (a) Bone marrow and (b) Blood of SE and EE mice following 6, 24 or 0 hours peritonitis (basal, no inflammation induced). Values are presented as individual data points  $\pm$  s.e.m. of 12 mice for each group at each time point. Charts are representative of N=3 experiments. Significance calculated by multiple T-tests, with correction for variance via the Holm Sidak method ( $P < 0.05$ ).

## 7.2 Flow Cytometric Profiling of Sponge Infiltrate Leukocytes

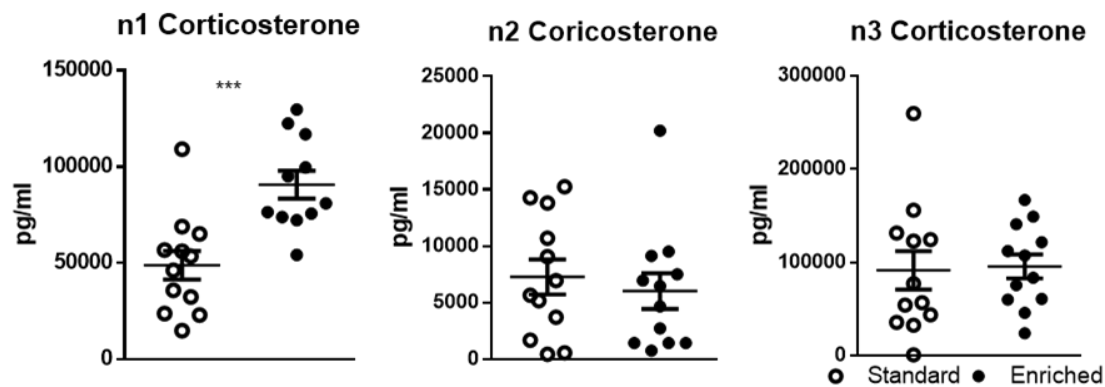


**Appendix 2. Granuloma infiltrate analysis one** (a) Two distinct populations of GR1 expressing cells were identified and gated. GR1<sup>+</sup> = gate 1. GR1<sup>high</sup> = gate 2. (b) Back-gating of these populations revealed differential scatter profiles. The Gr1<sup>+</sup> gate (b1) was found to contain two populations with a roughly consistent SSc (internal complexity) but variable FSc (size). The scatter profile of the higher FSc population is typically associated with that of monocytes. The GR1<sup>high</sup> gate (b2) was found to contain a population of cells with a high FSc and SSc profile typically associated with that of neutrophils.



**Appendix 3. Granuloma infiltrate analysis two.** (a) GR1 vs Ly6B.2 staining of recovered sponge infiltrate cells revealed a forked cell population. (b) Switching to a contour plot allowed easier demarcation of two primary populations expressing varying levels of GR1 and Ly6b.2,(c). (d) back gating of population d1 (GR1<sup>high</sup>,Ly6b.2<sup>+</sup>) revealed an FSc vs SSc profile corresponding to that of neutrophils (as well as gated population 2 (GR1<sup>high</sup>) identified in the F4/80 vs GR1 plot (Appendix 2). Back gating of population d2 (GR1<sup>+</sup>,Ly6b.2<sup>high</sup>) revealed an FSc vs SSc profile corresponding to that of monocytes (as well as gated population 1 (GR1<sup>+</sup>) identified in the F4/80 vs GR1 plot (Appendix 2).

7.3 Corticosterone Readouts Over Successive Experiments



Appendix 4. Differential levels of corticosterone expression recorded over successive enrichment experiments. \*\*\* $p < 0.0005$ , t-test. 24 mice per experiment.

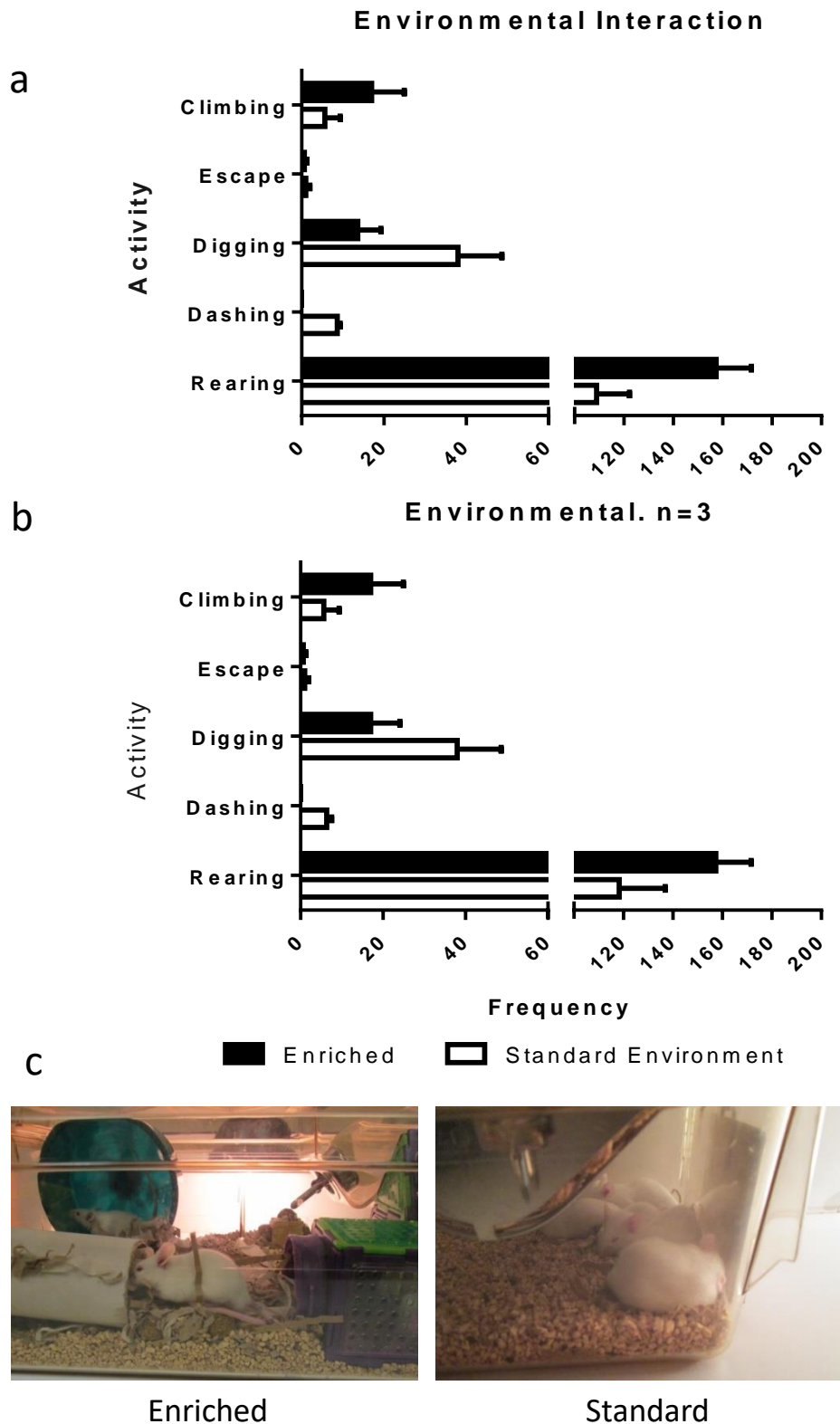
## 7.4 Continued Behavioural Analysis; Ethograms

An ethogram is a catalogue of stereotypical behaviours or actions exhibited by a particular species of animal. These behaviours are broken up into discrete acts or postures referred to as action patterns, which can be used as score-able units for (semi) quantitative analysis of animal behaviour [Sluyter et al., 2013]

Mouse ethograms have typically been used in the field of behavioural genetics to link specific behaviours to the influence of one or more genes [Sluyter et al., 2013]. However there has been some use of this technique to study the effects of environment enrichment [Marashi et al., 2003, van de Weerd et al., 1998, van Loo et al., 2004]. Interested to see how the behaviour of the EE mice used in this study may have altered compared to SE mice I subjected them to my own preliminary ethogram analysis, based upon action patterns used in previous papers utilising an enriched environment [Marashi et al., 2003, van de Weerd et al., 1998, van Loo et al., 2004].

**Protocol** Two groups of six male CD1 mice (enriched, non enriched) were removed from their standard enclosures and placed in an empty cage. These mice were recorded with a digital camera for 30 minutes and a tally of the actions of these animals was made and later corroborated by another member of the lab.

**Results** Tallying and comparing the number of individual actions performed by the two mouse groups revealed a trend towards higher levels of social activity (Appendix 5a) and a higher level of overall activity in enriched animals (Appendix 5a & b). It was of interest to note that ten minutes following return to their respective cages the standard group had settled down to sleep while the enriched group remained in a state of heightened activity (Appendix 5c).



**Appendix 5. Further behavioural analysis of enriched animals. (a) Comparative ethogram of EE and SE group interaction with cage environment. (b) Comparative ethogram of EE and SE group interaction with one another. N=1 experiment, 12 mice per experiment (six per group). (c) Comparative photographs of EE and SE mice 10 minutes post ethogram experiment.**

Ultimately we chose not to continue this line of analysis as it would have become highly labour intensive to produce data of viable quality for publication. However, it did provide some useful insights into the altered behavioural profile of enriched mice



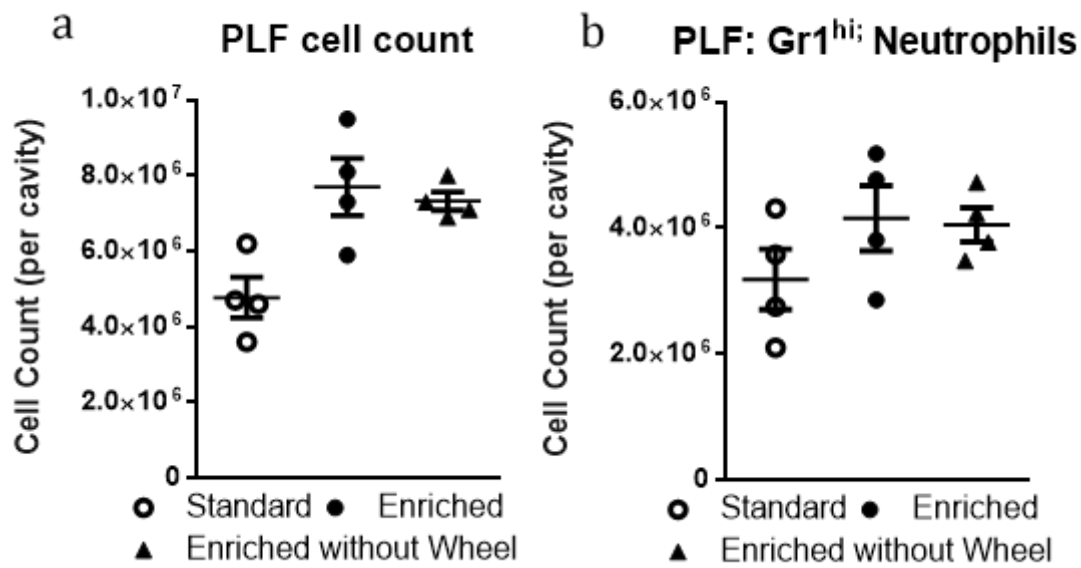
## 7.5 Genes Measured In RT-PCR Profiling Of Granuloma Tissue

**Appendix 6 Complete list of genes measured in RT-PCR profiling of granuloma tissue**

UniGene	GenBank	Symbol	Gene
Mm.213025	NM_007392	Acta2	Actin, alpha 2, smooth muscle, aorta
Mm.686	NM_009608	Actc1	Actin, alpha, cardiac muscle 1
Mm.309336	NM_009640	Angpt1	Angiopietin 1
Mm.867	NM_011331	Ccl12	Chemokine (C-C motif) ligand 12
Mm.341574	NM_013654	Ccl7	Chemokine (C-C motif) ligand 7
Mm.4861	NM_011616	Cd40lg	CD40 ligand
Mm.35605	NM_009864	Cdh1	Cadherin 1
Mm.297859	NM_181277	Col14a1	Collagen, type XIV, alpha 1
Mm.277735	NM_007742	Col1a1	Collagen, type I, alpha 1
Mm.277792	NM_007743	Col1a2	Collagen, type I, alpha 2
Mm.249555	NM_009930	Col3a1	Collagen, type III, alpha 1
Mm.738	NM_009931	Col4a1	Collagen, type IV, alpha 1
Mm.389135	NM_007734	Col4a3	Collagen, type IV, alpha 3
Mm.7281	NM_015734	Col5a1	Collagen, type V, alpha 1
Mm.10299	NM_007737	Col5a2	Collagen, type V, alpha 2
Mm.334994	NM_016919	Col5a3	Collagen, type V, alpha 3
Mm.4922	NM_009969	Csf2	Colony stimulating factor 2 (granulocyte-macrophage)
Mm.1238	NM_009971	Csf3	Colony stimulating factor 3 (granulocyte)
Mm.390287	NM_010217	Ctgf	Connective tissue growth factor
Mm.291928	NM_007614	Ctnnb1	Catenin (cadherin associated protein), beta 1
Mm.4858	NM_007800	Ctsg	Cathepsin G
Mm.272085	NM_007802	Ctsk	Cathepsin K
Mm.930	NM_009984	Ctsl	Cathepsin L
Mm.21013	NM_008176	Cxcl1	Chemokine (C-X-C motif) ligand 1
Mm.131723	NM_019494	Cxcl11	Chemokine (C-X-C motif) ligand 11
Mm.244289	NM_203320	Cxcl3	Chemokine (C-X-C motif) ligand 3
Mm.4660	NM_009141	Cxcl5	Chemokine (C-X-C motif) ligand 5
Mm.252481	NM_010113	Egf	Epidermal growth factor
Mm.8534	NM_007912	Egfr	Epidermal growth factor receptor
Mm.235105	NM_028784	F13a1	Coagulation factor XIII, A1 subunit
Mm.273188	NM_010171	F3	Coagulation factor III
Mm.88793	NM_010196	Fga	Fibrinogen alpha chain
Mm.317323	NM_008002	Fgf10	Fibroblast growth factor 10
Mm.473689	NM_008006	Fgf2	Fibroblast growth factor 2
Mm.330557	NM_008008	Fgf7	Fibroblast growth factor 7
Mm.289681	NM_010415	Hbegf	Heparin-binding EGF-like growth factor
Mm.267078	NM_010427	Hgf	Hepatocyte growth factor
Mm.240327	NM_008337	lfng	Interferon gamma
Mm.268521	NM_010512	lgf1	Insulin-like growth factor 1
Mm.874	NM_010548	Il-10	Interleukin- 10
Mm.222830	NM_008361	Il-1b	Interleukin 1 beta
Mm.14190	NM_008366	Il-2	Interleukin-2
Mm.276360	NM_021283	Il-4	Interleukin-4

Mm.1019	NM_031168	Il-6	Interleukin-6
Mm.4364	NM_010560	Il-6st	Interleukin 6 signal transducer
Mm.482186	NM_001033228	Itga1	Integrin alpha 1
Mm.5007	NM_008396	Itga2	Integrin alpha 2
Mm.57035	NM_013565	Itga3	Integrin alpha 3
Mm.31903	NM_010576	Itga4	Integrin alpha 4
Mm.16234	NM_010577	Itga5	Integrin alpha 5 (fibronectin receptor alpha)
Mm.225096	NM_008397	Itga6	Integrin alpha 6
Mm.227	NM_008402	Itgav	Integrin alpha V
Mm.263396	NM_010578	Itgb1	Integrin beta 1 (fibronectin receptor beta)
Mm.87150	NM_016780	Itgb3	Integrin beta 3
Mm.6424	NM_010580	Itgb5	Integrin beta 5
Mm.98193	NM_021359	Itgb6	Integrin beta 6
Mm.196581	NM_011949	Mapk1	Mitogen-activated protein kinase 1
Mm.8385	NM_011952	Mapk3	Mitogen-activated protein kinase 3
Mm.2326	NM_010798	Mif	Macrophage migration inhibitory factor
Mm.156952	NM_032006	Mmp1a	Matrix metalloproteinase 1a (interstitial collagenase)
Mm.29564	NM_008610	Mmp2	Matrix metalloproteinase 2
Mm.4825	NM_010810	Mmp7	Matrix metalloproteinase 7
Mm.4406	NM_013599	Mmp9	Matrix metalloproteinase 9
Mm.2675	NM_008808	Pdgfa	Platelet derived growth factor, alpha
Mm.154660	NM_008872	Plat	Plasminogen activator, tissue
Mm.4183	NM_008873	Plau	Plasminogen activator, urokinase
Mm.1359	NM_011113	Plaur	Plasminogen activator, urokinase receptor
Mm.971	NM_008877	Plg	Plasminogen
Mm.245395	NM_008960	Pten	Phosphatase and tensin homolog
Mm.292547	NM_011198	Ptgs2	Prostaglandin-endoperoxide synthase 2
Mm.292510	NM_009007	Rac1	RAS-related C3 botulinum substrate 1
Mm.757	NM_016802	Rhoa	Ras homolog gene family, member A
Mm.250422	NM_008871	Serpine1	Serine (or cysteine) peptidase inhibitor, clade E, member 1
Mm.249934	NM_011486	Stat3	Signal transducer and activator of transcription 3
Mm.283283	NM_011526	Tagln	Transgelin
Mm.137222	NM_031199	Tgfa	Transforming growth factor alpha
Mm.248380	NM_011577	Tgfb1	Transforming growth factor, beta 1
Mm.200775	NM_011578	Tgfbr3	Transforming growth factor, beta receptor III
Mm.8245	NM_011593	Timp1	Tissue inhibitor of metalloproteinase 1
Mm.1293	NM_013693	Tnf	Tumor necrosis factor
Mm.282184	NM_009505	Vegfa	Vascular endothelial growth factor A
Mm.3667	NM_011707	Vtn	Vitronectin
Mm.10222	NM_018865	Wisp1	WNT1 inducible signaling pathway protein 1
Mm.287544	NM_009524	Wnt5a	Wingless-related MMTV integration site 5A
Mm.328431	NM_007393	Actb	Actin, beta
Mm.163	NM_009735	B2m	Beta-2 microglobulin
Mm.343110	NM_008084	Gapdh	Glyceraldehyde-3-phosphate dehydrogenase
Mm.3317	NM_010368	Gusb	Glucuronidase, beta
Mm.2180	NM_008302	Hsp90ab1	Heat shock protein 90 alpha (cytosolic), class B member 1

7.6 Peritoneal Leukocyte Profile of Animals Subject To Six Hours Zymosan Peritonitis Using An Enriched Environment With And Without A Running Wheel



Appendix 7. Peritoneal leukocyte profile of animals subject to six hours zymosan peritonitis using an enriched environment with and without a running wheel. N=1 Experiments, 12 mice per experiment (4 per group).

## References

- ABBAS, A. K., LICHTMAN, A. H. & PILLAI, S. 2014. *Cellular and Molecular Immunology: with STUDENT CONSULT Online Access*, Elsevier Health Sciences.
- ADER, R. & COHEN, N. 1975. Behaviorally conditioned immunosuppression. *Psychosomatic Medicine*, 37, 333-340.
- AHBAP, E., SAKACI, T., KARA, E., SAHUTOGLU, T., KOC, Y., BASTURK, T., SEVINC, M., AKGOL, C., KAYALAR, A. O. & UCAR, Z. A. 2016. Neutrophil-to-lymphocyte ratio and platelet-to-lymphocyte ratio in evaluation of inflammation in end-stage renal disease. *Clinical nephrology*, 85, 199-208.
- AIKAWA, N. 1996. Cytokine storm in the pathogenesis of multiple organ dysfunction syndrome associated with surgical insults. *Nihon Geka Gakkai Zasshi*, 97, 771-777.
- AJUEBOR, M. N., FLOWER, R. J., HANNON, R., CHRISTIE, M., BOWERS, K., VERITY, A. & PERRETTI, M. 1998. Endogenous monocyte chemoattractant protein-1 recruits monocytes in the zymosan peritonitis model. *Journal of leukocyte biology*, 63, 108-116.
- AKIRA, S., UEMATSU, S. & TAKEUCHI, O. 2006. Pathogen recognition and innate immunity. *Cell*, 124, 783-801.
- AL-ALWAN, L. A., CHANG, Y., MOGAS, A., HALAYKO, A. J., BAGLOLE, C. J., MARTIN, J. G., ROUSSEAU, S., EIDELMAN, D. H. & HAMID, Q. 2013. Differential roles of CXCL2 and CXCL3 and their receptors in regulating normal and asthmatic airway smooth muscle cell migration. *The Journal of Immunology*, 191, 2731-2741.
- ALBELDA, S. M., SMITH, C. W. & WARD, P. 1994. Adhesion molecules and inflammatory injury. *The FASEB Journal*, 8, 504-512.
- ALBLAS, J., HONING, H., DE LAVALETTE, C. R., BROWN, M. H., DIJKSTRA, C. D. & VAN DEN BERG, T. K. 2005. Signal regulatory protein  $\alpha$  ligation induces macrophage nitric oxide production through JAK/STAT-and phosphatidylinositol 3-kinase/Rac1/NAPDH oxidase/H<sub>2</sub>O<sub>2</sub>-dependent pathways. *Molecular and cellular biology*, 25, 7181-7192.
- ALVES-FILHO, J. C., DE FREITAS, A., SPILLER, F., SOUTO, F. O. & CUNHA, F. Q. 2008. The role of neutrophils in severe sepsis. *Shock*, 30, 3-9.
- ANDERSON, J. M., RODRIGUEZ, A. & CHANG, D. T. Foreign body reaction to biomaterials. *Seminars in immunology*, 2008. Elsevier, 86-100.
- ANDRIOPOULOU, P., NAVARRO, P., ZANETTI, A., LAMPUGNANI, M. G. & DEJANA, E. 1999. Histamine induces tyrosine phosphorylation of endothelial cell-to-cell adherens junctions. *Arteriosclerosis, thrombosis, and vascular biology*, 19, 2286-2297.
- ANTONI, M. H., LUTGENDORF, S. K., COLE, S. W., DHABHAR, F. S., SEPHTON, S. E., MCDONALD, P. G., STEFANEK, M. & SOOD, A. K. 2006. The influence of bio-behavioural factors on tumour biology: pathways and mechanisms. *Nature Reviews Cancer*, 6, 240-248.
- ARITA, M., OHIRA, T., SUN, Y.-P., ELANGOVA, S., CHIANG, N. & SERHAN, C. N. 2007. Resolvin E1 selectively interacts with leukotriene B4 receptor BLT1 and ChemR23 to regulate inflammation. *The Journal of Immunology*, 178, 3912-3917.
- ARNDT, J., SMITH, N. & TAUSK, F. 2008. Stress and atopic dermatitis. *Current allergy and asthma reports*, 8, 312-317.
- ARNOLD, L., HENRY, A., PORON, F., BABA-AMER, Y., VAN ROOIJEN, N., PLONQUET, A., GHERARDI, R. K. & CHAZAUD, B. 2007. Inflammatory monocytes recruited after skeletal muscle injury switch into antiinflammatory macrophages to support myogenesis. *The Journal of experimental medicine*, 204, 1057-1069.
- ARRANZ, L., DE CASTRO, N. M., BAEZA, I., MATÉ, I., VIVEROS, M. P. & DE LA FUENTE, M. 2010. Environmental enrichment improves age-related immune system impairment: long-term exposure since adulthood increases life span in mice. *Rejuvenation research*, 13, 415-428.
- ASHLEY, N. T., WEIL, Z. M. & NELSON, R. J. 2012. Inflammation: mechanisms, costs, and natural variation. *Annual Review of Ecology, Evolution, and Systematics*, 43, 385-406.
- ASSINDER, S. J., STANTON, J.-A. L. & PRASAD, P. D. 2009. Transgelin: an actin-binding protein and tumour suppressor. *The international journal of biochemistry & cell biology*, 41, 482-486.
- ASSUNÇÃO, L., ELOI-SANTOS, S. & PEIXOTO, S. 2012. High sensitivity C-reactive protein distribution in the elderly: the Bambui Cohort Study, Brazil. *Brazilian Journal of Medical and Biological Research*, 45, 1284-1286.
- AUFFRAY, C., FOGG, D., GARFA, M., ELAIN, G., JOIN-LAMBERT, O., KAYAL, S., SARNACKI, S., CUMANO, A., LAUVAU, G. & GEISSMANN, F. 2007. Monitoring of blood vessels and tissues by a population of monocytes with patrolling behavior. *Science*, 317, 666-670.
- AUFFRAY, C., FOGG, D. K., NARNI-MANCINELLI, E., SENECHAL, B., TROUILLET, C., SAEDERUP, N., LEEMPUT, J., BIGOT, K., CAMPISI, L. & ABITBOL, M. 2009. CX3CR1+

- CD115+ CD135+ common macrophage/DC precursors and the role of CX3CR1 in their response to inflammation. *The Journal of experimental medicine*, 206, 595-606.
- AVERILL, M. M., BARNHART, S., BECKER, L., LI, X., HEINECKE, J. W., LEBOEUF, R. C., HAMERMAN, J. A., SORG, C., KERKHOFF, C. & BORNFELDT, K. E. 2011. S100A9 Differentially Modifies Phenotypic States of Neutrophils, Macrophages, and Dendritic Cells Implications for Atherosclerosis and Adipose Tissue Inflammation. *Circulation*, 123, 1216-1226.
- AYALA, A., SONG, G. Y., CHUNG, C.-S., REDMOND, K. M. & CHAUDRY, I. H. 2000. Immune depression in polymicrobial sepsis: the role of necrotic (injured) tissue and endotoxin. *Critical care medicine*, 28, 2949-2955.
- AYUB, N., BENTON, J. L., ZHANG, Y. & BELTZ, B. S. 2011. Environmental enrichment influences neuronal stem cells in the adult crayfish brain. *Developmental neurobiology*, 71, 351-361.
- BABIC, A. M., CHEN, C.-C. & LAU, L. F. 1999. Fisp12/mouse connective tissue growth factor mediates endothelial cell adhesion and migration through integrin  $\alpha\beta 3$ , promotes endothelial cell survival, and induces angiogenesis in vivo. *Molecular and cellular biology*, 19, 2958-2966.
- BAHOU, W. F. & GNATENKO, D. V. Platelet transcriptome: the application of microarray analysis to platelets. *Seminars in thrombosis and hemostasis*, 2004. Copyright© 2004 by Thieme Medical Publishers, Inc., 333 Seventh Avenue, New York, NY 10001, USA., 473-484.
- BAKER, C. C., CHAUDRY, I. H., GAINES, H. O. & BAUE, A. E. 1983. Evaluation of factors affecting mortality rate after sepsis in a murine cecal ligation and puncture model. *Surgery*, 94, 331-335.
- BAKER, J., DE LISIO, M. & PARISE, G. 2011. Endurance exercise training promotes medullary hematopoiesis. *The FASEB Journal*, 25, 4348-4357.
- BALCOMBE, J. 2010. Laboratory rodent welfare: Thinking outside the cage. *Journal of Applied Animal Welfare Science*, 13, 77-88.
- BALCOMBE, J. P. 2006. Laboratory environments and rodents' behavioural needs: a review. *Laboratory animals*, 40, 217-235.
- BARNES, P. J. 2010. Mechanisms and resistance in glucocorticoid control of inflammation. *The Journal of steroid biochemistry and molecular biology*, 120, 76-85.
- BARTH, M., HENDRZAK, J., MELNICOFF, M. & MORAHAN, P. 1995. Review of the macrophage disappearance reaction. *Journal of leukocyte biology*, 57, 361-367.
- BARTOLOMUCCI, A. 2007. Social stress, immune functions and disease in rodents. *Frontiers in neuroendocrinology*, 28, 28-49.
- BARTOLOMUCCI, A., PALANZA, P., GASPANI, L., LIMIROLI, E., PANERAI, A. E., CERESINI, G., POLI, M. D. & PARMIGIANI, S. 2001. Social status in mice: behavioral, endocrine and immune changes are context dependent. *Physiology & behavior*, 73, 401-410.
- BARTON, B. A., SCHRECK, C. B. & BARTON, L. D. 1987. Effects of chronic cortisol administration and daily acute stress on growth, physiological conditions, and stress responses in juvenile rainbow trout. *Dis. Aquat. Org*, 2, 173-185.
- BARTROP, R., LAZARUS, L., LUCKHURST, E., KILOH, L. G. & PENNY, R. 1977. Depressed lymphocyte function after bereavement. *The Lancet*, 309, 834-836.
- BAUER, J. S., VARNER, J., SCHREINER, C., KORNBERG, L., NICHOLAS, R. & JULIANO, R. L. 1993. Functional role of the cytoplasmic domain of the integrin alpha 5 subunit. *The Journal of Cell Biology*, 122, 209-221.
- BEATTIE, V., O'CONNELL, N. & MOSS, B. 2000. Influence of environmental enrichment on the behaviour, performance and meat quality of domestic pigs. *Livestock production science*, 65, 71-79.
- BEAVERS, K. M., BRINKLEY, T. E. & NICKLAS, B. J. 2010. Effect of exercise training on chronic inflammation. *Clinica chimica acta*, 411, 785-793.
- BELLINGAN, G. J., CALDWELL, H., HOWIE, S., DRANSFIELD, I. & HASLETT, C. 1996. In vivo fate of the inflammatory macrophage during the resolution of inflammation: inflammatory macrophages do not die locally, but emigrate to the draining lymph nodes. *The Journal of Immunology*, 157, 2577-2585.
- BENAROYA-MILSHTEIN, N., APTER, A., YANIV, I., KUKULANSKY, T., RAZ, N., HABERMAN, Y., HALPERT, H., PICK, C. G. & HOLLANDER, N. 2007. Environmental enrichment augments the efficacy of idiotype vaccination for B-cell lymphoma. *Journal of immunotherapy*, 30, 517-522.
- BENAROYA - MILSHTEIN, N., HOLLANDER, N., APTER, A., KUKULANSKY, T., RAZ, N., WILF, A., YANIV, I. & PICK, C. 2004. Environmental enrichment in mice decreases anxiety, attenuates stress responses and enhances natural killer cell activity. *European Journal of Neuroscience*, 20, 1341-1347.
- BENEFIEL, A. C., DONG, W. K. & GREENOUGH, W. T. 2005. Mandatory "enriched" housing of laboratory animals: the need for evidence-based evaluation. *ILAR Journal*, 46, 95-105.

- BENGSTON, S. E., PRUITT, J. N. & RIECHERT, S. E. 2014. Differences in environmental enrichment generate contrasting behavioural syndromes in a basal spider lineage. *Animal Behaviour*, 93, 105-110.
- BERK, L. S., FELTEN, D. L., TAN, S. A., BITTMAN, B. B. & WESTENGARD, J. 2001. Modulation of neuroimmune parameters during the eustress of humor-associated mirthful laughter. *Alternative therapies in health and medicine*, 7, 62-76.
- BEURA, L. K., HAMILTON, S. E., BI, K., SCHENKEL, J. M., ODUMADE, O. A., CASEY, K. A., THOMPSON, E. A., FRASER, K. A., ROSATO, P. C., FILALI-MOUHIM, A., SEKALY, R. P., JENKINS, M. K., VEZYS, V., HAINING, W. N., JAMESON, S. C. & MASOPUST, D. 2016. Normalizing the environment recapitulates adult human immune traits in laboratory mice. *Nature*, 532, 512-6.
- BIAN, Z., GUO, Y., HA, B., ZEN, K. & LIU, Y. 2012. Regulation of the inflammatory response: enhancing neutrophil infiltration under chronic inflammatory conditions. *The Journal of Immunology*, 188, 844-853.
- BIELOHUBY, M., POPP, S. & BIDLINGMAIER, M. 2012. A guide for measurement of circulating metabolic hormones in rodents: Pitfalls during the pre-analytical phase. *Molecular metabolism*, 1, 47-60.
- BITTMAN, B. B., BERK, L. S., FELTEN, D. L. & WESTENGARD, J. 2001. Composite effects of group drumming music therapy on modulation of neuroendocrine-immune parameters in normal subjects. *Alternative therapies in health and medicine*, 7, 38.
- BLAYBEL, R., THÉOLEYRE, O., DOUABLIN, A. & BAKLOUTI, F. 2008. Downregulation of the Spi-1/PU. 1 oncogene induces the expression of TRIM10/HERF1, a key factor required for terminal erythroid cell differentiation and survival. *Cell research*, 18, 834-845.
- BLUME, K. E., SOEROES, S., KEPPELER, H., STEVANOVIC, S., KRETSCHMER, D., RAUTENBERG, M., WESSELBORG, S. & LAUBER, K. 2012. Cleavage of annexin A1 by ADAM10 during secondary necrosis generates a monocytic "find-me" signal. *The Journal of Immunology*, 188, 135-145.
- BOBEL, J., DI-LERNIA, M., ABBOTT, J., LONG, M. & WARREN, L. 2015. 43 Influence of oat  $\beta$ -glucan on neutrophil function and lymphocyte subsets in response to stress. *Journal of Equine Veterinary Science*, 35, 402.
- BOMMHARDT, U., CHANG, K. C., SWANSON, P. E., WAGNER, T. H., TINSLEY, K. W., KARL, I. E. & HOTCHKISS, R. S. 2004. Akt decreases lymphocyte apoptosis and improves survival in sepsis. *The Journal of Immunology*, 172, 7583-7591.
- BONECCHI, R., LOCATI, M., GALLIERA, E., VULCANO, M., SIRONI, M., FRA, A. M., GOBBI, M., VECCHI, A., SOZZANI, S. & HARIBABU, B. 2004. Differential recognition and scavenging of native and truncated macrophage-derived chemokine (macrophage-derived chemokine/CC chemokine ligand 22) by the D6 decoy receptor. *The Journal of Immunology*, 172, 4972-4976.
- BONNEAU, R. H., SHERIDAN, J. F., FENG, N. & GLASER, R. 1991. Stress-induced effects on cell-mediated innate and adaptive memory components of the murine immune response to herpes simplex virus infection. *Brain, behavior, and immunity*, 5, 274-295.
- BOOTH, F. W., ROBERTS, C. K. & LAYE, M. J. 2012. Lack of exercise is a major cause of chronic diseases. *Comprehensive Physiology*.
- BOROVIKOVA, L. V., IVANOVA, S., NARDI, D., ZHANG, M., YANG, H., OMBRELLINO, M. & TRACEY, K. J. 2000. Role of vagus nerve signaling in CNI-1493-mediated suppression of acute inflammation. *Autonomic Neuroscience*, 85, 141-147.
- BORREGAARD, N. 2010. Neutrophils, from marrow to microbes. *Immunity*, 33, 657-670.
- BOURIN, M. & HASCOËT, M. 2003. The mouse light/dark box test. *European journal of pharmacology*, 463, 55-65.
- BOYD, J. & LOVE, J. 1995. The effects of dividers on the nesting sites of mice. *Frontiers in Laboratory Animal Science*, 2.
- BRINKMANN, V., REICHARD, U., GOOSMANN, C., FAULER, B., UHLEMANN, Y., WEISS, D. S., WEINRAUCH, Y. & ZYCHLINSKY, A. 2004. Neutrophil extracellular traps kill bacteria. *science*, 303, 1532-1535.
- BROD, S., RATTAZZI, L., PIRAS, G. & D'ACQUISTO, F. 2014a. 'As above, so below' examining the interplay between emotion and the immune system. *Immunology*, 143, 311-318.
- BROD, S., RATTAZZI, L., PIRAS, G. & D'ACQUISTO, F. 2014b. "As above, so below" Examining the interplay between emotion and the Immune System. *Immunology*, n/a-n/a.
- BRODIN, P., JOJIC, V., GAO, T., BHATTACHARYA, S., ANGEL, C. J. L., FURMAN, D., SHEN-ORR, S., DEKKER, C. L., SWAN, G. E. & BUTTE, A. J. 2015. Variation in the Human Immune System Is Largely Driven by Non-Heritable Influences. *Cell*, 160, 37-47.
- BROWN, C. 2009. Novel food items as environmental enrichment for rodents and rabbits. *Lab animal*, 38, 119.

- BROWN, D. H. & ZWILLING, B. S. 1994. Activation of the hypothalamic-pituitary-adrenal axis differentially affects the anti-mycobacterial activity of macrophages from BCG-resistant and susceptible mice. *Journal of neuroimmunology*, 53, 181-187.
- BROWN, G. D., TAYLOR, P. R., REID, D. M., WILLMENT, J. A., WILLIAMS, D. L., MARTINEZ-POMARES, L., WONG, S. Y. & GORDON, S. 2002. Dectin-1 is a major  $\beta$ -glucan receptor on macrophages. *The Journal of experimental medicine*, 196, 407-412.
- BROWN, H. 1995. Ilya Mechnikov and his studies on comparative inflammation. *Experimental Biology and Medicine*, 209, 99-101.
- BROWN, K., BRAIN, S., PEARSON, J., EDGEWORTH, J., LEWIS, S. & TREACHER, D. 2006. Neutrophils in development of multiple organ failure in sepsis. *The Lancet*, 368, 157-169.
- BRUN, J. G., HÅLAND, G., HAGA, H. J., FAGERHOL, M. K. & JONSSON, R. 1995. Effects of calprotectin in avridine - induced arthritis. *Apmis*, 103, 233-240.
- BULFONE, A., CACCIOPOLI, C., PARDINI, C., FAEDO, A., MARTINEZ, S. & BANFI, S. 2004. Pcp411, a novel gene encoding a Pcp4-like polypeptide, is expressed in specific domains of the developing brain. *Gene expression patterns*, 4, 297-301.
- BURGESS, A. W. & METCALF, D. 1980. The nature and action of granulocyte-macrophage colony stimulating factors. *Blood*, 56, 947-958.
- BUTCHER, S. K., KILLAMPALLI, V., LASCELLES, D., WANG, K., ALPAR, E. K. & LORD, J. M. 2005. Raised cortisol: DHEAS ratios in the elderly after injury: potential impact upon neutrophil function and immunity. *Aging cell*, 4, 319-324.
- BUTLER, G. S., SIM, D., TAM, E., DEVINE, D. & OVERALL, C. M. 2002. Mannose-binding Lectin (MBL) Mutants Are Susceptible to Matrix Metalloproteinase Proteolysis POTENTIAL ROLE IN HUMAN MBL DEFICIENCY. *Journal of Biological Chemistry*, 277, 17511-17519.
- BYRD, A. S., O'BRIEN, X. M., JOHNSON, C. M., LAVIGNE, L. M. & REICHNER, J. S. 2013. An extracellular matrix-based mechanism of rapid neutrophil extracellular trap formation in response to *Candida albicans*. *The Journal of Immunology*, 190, 4136-4148.
- CALFEE, C. S. & MATTHAY, M. A. 2010. Clinical immunology: Culprits with evolutionary ties. *Nature*, 464, 41-42.
- CANNON, K. E., CHAZOT, P. L., HANN, V., SHENTON, F., HOUGH, L. B. & RICE, F. L. 2007. Immunohistochemical localization of histamine H<sub>3</sub> receptors in rodent skin, dorsal root ganglia, superior cervical ganglia, and spinal cord: potential antinociceptive targets. *Pain*, 129, 76-92.
- CAO, L., CHOI, E. Y., LIU, X., MARTIN, A., WANG, C., XU, X. & DURING, M. J. 2011. White to brown fat phenotypic switch induced by genetic and environmental activation of a hypothalamic-adipocyte axis. *Cell metabolism*, 14, 324-338.
- CAO, L., JIAO, X., ZUZGA, D. S., LIU, Y., FONG, D. M., YOUNG, D. & DURING, M. J. 2004. VEGF links hippocampal activity with neurogenesis, learning and memory. *Nature genetics*, 36, 827-835.
- CAO, W., DUAN, J., WANG, X., ZHONG, X., HU, Z., HUANG, F., WANG, H., ZHANG, J., LI, F. & ZHANG, J. 2014. Early enriched environment induces an increased conversion of proBDNF to BDNF in the adult rat's hippocampus. *Behavioural brain research*, 265, 76-83.
- CARPENTER, G. & COHEN, S. 1990. Epidermal growth factor. *J Biol Chem*, 265, 7709-7712.
- CARREIRA, C. M., LAVALLEE, T. M., TARANTINI, F., JACKSON, A., LATHROP, J. T., HAMPTON, B., BURGESS, W. H. & MACIAG, T. 1998. S100A13 is involved in the regulation of fibroblast growth factor-1 and p40 synaptotagmin-1 release in vitro. *Journal of Biological Chemistry*, 273, 22224-22231.
- CASH, J. L., WHITE, G. E. & GREAVES, D. R. 2009. Zymosan - Induced Peritonitis as a Simple Experimental System for the Study of Inflammation. *Methods in enzymology*, 461, 379-396.
- CELSUS, A. C. & SPENCER, W. G. 1935. *De Medicina. With an English Translation by WG Spencer*, London; Harvard University Press: Cambridge, Mass.
- CHABRY, J., NICOLAS, S., CAZARETH, J., MURRIS, E., GUYON, A., GLAICHENHAUS, N., HEURTEAUX, C. & PETIT-PAITEL, A. 2015. Enriched environment decreases microglia and brain macrophages inflammatory phenotypes through adiponectin-dependent mechanisms: relevance to depressive-like behavior. *Brain, behavior, and immunity*, 50, 275-287.
- CHAI, J. & TARNAWSKI, A. 2002. Serum response factor: discovery, biochemistry, biological roles and implications for tissue injury healing. *Journal of physiology and pharmacology: an official journal of the Polish Physiological Society*, 53.
- CHARAN, J. & KANTHARIA, N. 2013. How to calculate sample size in animal studies? *Journal of Pharmacology and Pharmacotherapeutics*, 4, 303.
- CHAUHAN, S. S., POPESCU, N., RAY, D., FLEISCHMANN, R., GOTTESMAN, M. & TROEN, B. 1993. Cloning, genomic organization, and chromosomal localization of human cathepsin L. *Journal of Biological Chemistry*, 268, 1039-1045.

- CHEN, C.-C., LLADO, V., EURICH, K., TRAN, H. T. & MIZOGUCHI, E. 2011. Carbohydrate-binding motif in chitinase 3-like 1 (CHI3L1/YKL-40) specifically activates Akt signaling pathway in colonic epithelial cells. *Clinical Immunology*, 140, 268-275.
- CHERVENICK, P., BOGGS, D., MARSH, J., CARTWRIGHT, G. & WINTROBE, M. 1968. *Quantitative studies of blood and bone marrow neutrophils in normal mice*.
- CHIN, Y. K.-Y., HEADEY, S. J., MOHANTY, B., PATIL, R., MCEWAN, P. A., SWARBRICK, J. D., MULHERN, T. D., EMSLEY, J., SIMPSON, J. S. & SCANLON, M. J. 2013. The structure of integrin  $\alpha 11$  domain in complex with a collagen-mimetic peptide. *Journal of Biological Chemistry*, 288, 36796-36809.
- CHUNG, S., SON, G. H. & KIM, K. 2011. Circadian rhythm of adrenal glucocorticoid: its regulation and clinical implications. *Biochimica et Biophysica Acta (BBA)-Molecular Basis of Disease*, 1812, 581-591.
- COMMITTEE, A. R. A. 2004. Enrichment Strategies for Rodents in the Laboratory. In: HEALTH, N. I. O. (ed.). United States.
- COOPER, D., NORLING, L. V. & PERRETTI, M. 2008. Novel insights into the inhibitory effects of Galectin-1 on neutrophil recruitment under flow. *Journal of leukocyte biology*, 83, 1459-1466.
- COOPER, L., JOHNSON, C., BURSLEM, F. & MARTIN, P. 2004. Wound healing and inflammation genes revealed by array analysis of 'macrophageless' PU. 1 null mice. *Genome biology*, 6, 1.
- COPPOLA, R., MARI, D., LATTUADA, A. & FRANCESCHI, C. 2003. Von Willebrand factor in Italian centenarians. *Haematologica*, 88, 39-43.
- CORDAIN, L., EATON, S. B., SEBASTIAN, A., MANN, N., LINDEBERG, S., WATKINS, B. A., O'KEEFE, J. H. & BRAND-MILLER, J. 2005. Origins and evolution of the Western diet: health implications for the 21st century. *The American journal of clinical nutrition*, 81, 341-354.
- COUTELLIER, L. & WÜRBEL, H. 2009. Early environmental cues affect object recognition memory in adult female but not male C57BL/6 mice. *Behavioural brain research*, 203, 312-315.
- CRACIUN, F. L., SCHULLER, E. R. & REMICK, D. G. 2010. Early enhanced local neutrophil recruitment in peritonitis-induced sepsis improves bacterial clearance and survival. *The Journal of Immunology*, 185, 6930-6938.
- CRANE, M. J., DALEY, J. M., VAN HOUTTE, O., BRANCATO, S. K., HENRY JR, W. L. & ALBINA, J. E. 2014. The monocyte to macrophage transition in the murine sterile wound. *PloS one*, 9, e86660.
- CRAWLEY, J. N. 1985. Exploratory behavior models of anxiety in mice. *Neuroscience & Biobehavioral Reviews*, 9, 37-44.
- CROCE, K., GAO, H., WANG, Y., MOOROKA, T., SAKUMA, M., SHI, C., SUKHOVA, G., PACKARD, R. R. S., HOGG, N., LIBBY, P. & SIMON, D. I. 2009. MRP-8/14 is Critical for the Biological Response to Vascular Injury. *Circulation*, 120, 427-436.
- CROFTON, E. J., ZHANG, Y. & GREEN, T. A. 2015. Inoculation stress hypothesis of environmental enrichment. *Neuroscience & Biobehavioral Reviews*, 49, 19-31.
- CRUZ, C. S. D., LIU, W., HE, C. H., JACOBY, A., GORNITZKY, A., MA, B., FLAVELL, R., LEE, C. G. & ELIAS, J. A. 2012. Chitinase 3-like-1 promotes *Streptococcus pneumoniae* killing and augments host tolerance to lung antibacterial responses. *Cell host & microbe*, 12, 34-46.
- CUTOLO, M. & STRAUB, R. H. 2007. Stress as a risk factor in the pathogenesis of rheumatoid arthritis. *Neuroimmunomodulation*, 13, 277-282.
- D'AMICO, G., FRASCAROLI, G., BIANCHI, G., TRANSIDICO, P., DONI, A., VECCHI, A., SOZZANI, S., ALLAVENA, P. & MANTOVANI, A. 2000. Uncoupling of inflammatory chemokine receptors by IL-10: generation of functional decoys. *Nature immunology*, 1, 387-391.
- DAHLEN, S.-E., BJÖRK, J., HEDQVIST, P., ARFORS, K.-E., HAMMARSTRÖM, S., LINDGREN, J.-A. & SAMUELSSON, B. 1981. Leukotrienes promote plasma leakage and leukocyte adhesion in postcapillary venules: in vivo effects with relevance to the acute inflammatory response. *Proceedings of the National Academy of Sciences*, 78, 3887-3891.
- DALLI, J., NORLING, L. V., RENSHAW, D., COOPER, D., LEUNG, K.-Y. & PERRETTI, M. 2008. Annexin 1 mediates the rapid anti-inflammatory effects of neutrophil-derived microparticles. *Blood*, 112, 2512-2519.
- DANESE, S., SANS, M. & FIOCCHI, C. 2004. Inflammatory bowel disease: the role of environmental factors. *Autoimmunity Reviews*, 3, 394-400.
- DANTZER, R., O'CONNOR, J. C., FREUND, G. G., JOHNSON, R. W. & KELLEY, K. W. 2008. From inflammation to sickness and depression: when the immune system subjugates the brain. *Nature reviews neuroscience*, 9, 46-56.
- DAVIES, L. C., ROSAS, M., JENKINS, S. J., LIAO, C.-T., SCURR, M. J., BROMBACHER, F., FRASER, D. J., ALLEN, J. E., JONES, S. A. & TAYLOR, P. R. 2013. Distinct bone marrow-derived and tissue-resident macrophage lineages proliferate at key stages during inflammation. *Nature communications*, 4, 1886.



- DE JONG, I. C., PRELLE, I. T., VAN DE BURG WAL, J. A., LAMBOOIJ, E., KORTE, S. M., BLOKHUIS, H. J. & KOOLHAAS, J. M. 2000. Effects of environmental enrichment on behavioral responses to novelty, learning, and memory, and the circadian rhythm in cortisol in growing pigs. *Physiology & behavior*, 68, 571-578.
- DE JONG, J. G., VAN DER VEGT, B. J., BUWALDA, B. & KOOLHAAS, J. M. 2005. Social environment determines the long-term effects of social defeat. *Physiology & behavior*, 84, 87-95.
- DE JONGE, W. J., VAN DER ZANDEN, E. P., THE, F. O., BIJLSMA, M. F., VAN WESTERLOO, D. J., BENNINK, R. J., BERTHOUD, H.-R., UEMATSU, S., AKIRA, S. & VAN DEN WIJNGAARD, R. M. 2005. Stimulation of the vagus nerve attenuates macrophage activation by activating the Jak2-STAT3 signaling pathway. *Nature immunology*, 6, 844-851.
- DE SOUSA, A. A., REIS, R., BENTO-TORRES, J., TRÉVIA, N., DE ALMEIDA LINS, N. A., PASSOS, A., SANTOS, Z., DINIZ, J. A. P., DA COSTA VASCONCELOS, P. F. & CUNNINGHAM, C. 2011. Influence of enriched environment on viral encephalitis outcomes: behavioral and neuropathological changes in albino Swiss mice. *PloS one*, 6, e15597.
- DE VERNEUIL, H., SASSA, S. & KAPPAS, A. 1983. Purification and properties of uroporphyrinogen decarboxylase from human erythrocytes. A single enzyme catalyzing the four sequential decarboxylations of uroporphyrinogens I and III. *Journal of Biological Chemistry*, 258, 2454-2460.
- DEAN, R. A., COX, J. H., BELLAC, C. L., DOUCET, A., STARR, A. E. & OVERALL, C. M. 2008. Macrophage-specific metalloelastase (MMP-12) truncates and inactivates ELR+ CXC chemokines and generates CCL2,-7,-8, and-13 antagonists: potential role of the macrophage in terminating polymorphonuclear leukocyte influx. *Blood*, 112, 3455-3464.
- DECEMBRINO, L., DE AMICI, M., POZZI, M., DE SILVESTRI, A. & STRONATI, M. 2015. Serum Calprotectin: A Potential Biomarker for Neonatal Sepsis. *Journal of immunology research*, 2015.
- DEJAGER, L., PINHEIRO, I., DEJONCKHEERE, E. & LIBERT, C. 2011. Cecal ligation and puncture: the gold standard model for polymicrobial sepsis? *Trends in microbiology*, 19, 198-208.
- DEONARINE, K., PANELLI, M. C., STASHOWER, M. E., JIN, P., SMITH, K., SLADE, H. B., NORWOOD, C., WANG, E., MARINCOLA, F. M. & STRONCEK, D. F. 2007. Gene expression profiling of cutaneous wound healing. *Journal of translational medicine*, 5, 1.
- DETILLION, C. E., CRAFT, T. K., GLASPER, E. R., PRENDERGAST, B. J. & DEVRIES, A. C. 2004. Social facilitation of wound healing. *Psychoneuroendocrinology*, 29, 1004-1011.
- DEVALARAJA, R. M., NANNY, L. B., QIAN, Q., DU, J., YU, Y., DEVALARAJA, M. N. & RICHMOND, A. 2000. Delayed wound healing in CXCR2 knockout mice. *Journal of Investigative Dermatology*, 115, 234-244.
- DHABHAR, F. S. 2000. Acute stress enhances while chronic stress suppresses skin immunity: the role of stress hormones and leukocyte trafficking. *Annals of the New York Academy of Sciences*, 917, 876-893.
- DHABHAR, F. S. 2014. Effects of stress on immune function: the good, the bad, and the beautiful. *Immunologic research*, 58, 193-210.
- DHABHAR, F. S., MALARKEY, W. B., NERI, E. & MCEWEN, B. S. 2012. Stress-induced redistribution of immune cells—From barracks to boulevards to battlefields: A tale of three hormones—Curt Richter Award Winner. *Psychoneuroendocrinology*, 37, 1345-1368.
- DHABHAR, F. S. & MCEWEN, B. S. 1996. Stress-induced enhancement of antigen-specific cell-mediated immunity. *The Journal of Immunology*, 156, 2608-2615.
- DHABHAR, F. S. & MCEWEN, B. S. 1997. Acute stress enhances while chronic stress suppresses cell-mediated immunity in vivo: A potential role for leukocyte trafficking. *Brain, behavior, and immunity*, 11, 286-306.
- DHABHAR, F. S. & MCEWEN, B. S. 1999. Enhancing versus suppressive effects of stress hormones on skin immune function. *Proceedings of the National Academy of Sciences*, 96, 1059-1064.
- DHABHAR, F. S., SAUL, A. N., DAUGHERTY, C., HOLMES, T. H., BOULEY, D. M. & OBERYSZYN, T. M. 2010. Short-term stress enhances cellular immunity and increases early resistance to squamous cell carcinoma. *Brain, behavior, and immunity*, 24, 127-137.
- DHABHAR, F. S. & VISWANATHAN, K. 2005. Short-term stress experienced at time of immunization induces a long-lasting increase in immunologic memory. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*, 289, R738-R744.
- DIENER, E. & CHAN, M. Y. 2011. Happy people live longer: Subjective well - being contributes to health and longevity. *Applied Psychology: Health and Well - Being*, 3, 1-43.
- DINAN, T. G. 2009. Inflammatory markers in depression. *Current opinion in psychiatry*, 22, 32-36.

- DOEING, D. C., BOROWICZ, J. L. & CROCKETT, E. T. 2003. Gender dimorphism in differential peripheral blood leukocyte counts in mice using cardiac, tail, foot, and saphenous vein puncture methods. *BMC clinical pathology*, 3, 1.
- DOERFLER, M. E., DANNER, R. L., SHELHAMER, J. H. & PARRILLO, J. E. 1989. Bacterial lipopolysaccharides prime human neutrophils for enhanced production of leukotriene B<sub>4</sub>. *Journal of Clinical Investigation*, 83, 970.
- DOHERTY, N., POUBELLE, P., BORGEAT, P., BEAVER, T., WESTRICH, G. & SCHRADER, N. 1985. Intraperitoneal injection of zymosan in mice induces pain, inflammation and the synthesis of peptidoleukotrienes and prostaglandin E<sub>2</sub>. *Prostaglandins*, 30, 769-789.
- DOWLATI, Y., HERRMANN, N., SWARDFAGER, W., LIU, H., SHAM, L., REIM, E. K. & LANCTÔT, K. L. 2010. A meta-analysis of cytokines in major depression. *Biological psychiatry*, 67, 446-457.
- DUIVIS, H. E., DE JONGE, P., PENNINX, B. W., NA, B. Y., COHEN, B. E. & WHOOLEY, M. A. 2014. Depressive symptoms, health behaviors, and subsequent inflammation in patients with coronary heart disease: prospective findings from the heart and soul study.
- DUIVIS, H. E., VOGELZANGS, N., KUPPER, N., DE JONGE, P. & PENNINX, B. W. 2013. Differential association of somatic and cognitive symptoms of depression and anxiety with inflammation: findings from the Netherlands Study of Depression and Anxiety (NESDA). *Psychoneuroendocrinology*, 38, 1573-1585.
- DURING, M. J., LIU, X., HUANG, W., MAGEE, D., SLATER, A., MCMURPHY, T., WANG, C. & CAO, L. 2015. Adipose VEGF links the white-to-brown fat switch with environmental, genetic, and pharmacological stimuli in male mice. *Endocrinology*, 156, 2059-2073.
- DUTTA, S. & SENGUPTA, P. 2016. Men and mice: relating their ages. *Life sciences*, 152, 244-248.
- EASH, K. J., MEANS, J. M., WHITE, D. W. & LINK, D. C. 2009. CXCR4 is a key regulator of neutrophil release from the bone marrow under basal and stress granulopoiesis conditions. *Blood*, 113, 4711-4719.
- EDWARDS, E. A. & DEAN, L. M. 1977. Effects of crowding of mice on humoral antibody formation and protection to lethal antigenic challenge. *Psychosomatic Medicine*, 39, 19-24.
- EHRCHEN, J. M., SUNDERKÖTTER, C., FOELL, D., VOGL, T. & ROTH, J. 2009. The endogenous Toll-like receptor 4 agonist S100A8/S100A9 (calprotectin) as innate amplifier of infection, autoimmunity, and cancer. *Journal of leukocyte biology*, 86, 557-566.
- EMELYANOV, V. V. 2001. Rickettsiaceae, rickettsia-like endosymbionts, and the origin of mitochondria. *Bioscience reports*, 21, 1-17.
- EMING, S. A., MARTIN, P. & TOMIC-CANIC, M. 2014. Wound repair and regeneration: mechanisms, signaling, and translation. *Science translational medicine*, 6, 265sr6-265sr6.
- ENGLER, H., BAILEY, M. T., ENGLER, A. & SHERIDAN, J. F. 2004. Effects of repeated social stress on leukocyte distribution in bone marrow, peripheral blood and spleen. *Journal of neuroimmunology*, 148, 106-115.
- EPEL, E. S., BLACKBURN, E. H., LIN, J., DHABHAR, F. S., ADLER, N. E., MORROW, J. D. & CAWTHON, R. M. 2004. Accelerated telomere shortening in response to life stress. *Proceedings of the National Academy of Sciences of the United States of America*, 101, 17312-17315.
- EPELMAN, S., LAVINE, K. J. & RANDOLPH, G. J. 2014. Origin and functions of tissue macrophages. *Immunity*, 41, 21-35.
- ERNST, K., TUCHSCHERER, M., KANITZ, E., PUPPE, B. & MANTEUFFEL, G. 2006. Effects of attention and rewarded activity on immune parameters and wound healing in pigs. *Physiology & behavior*, 89, 448-456.
- FADOK, V. A., BRATTON, D. L., KONOWAL, A., FREED, P. W., WESTCOTT, J. Y. & HENSON, P. M. 1998. Macrophages that have ingested apoptotic cells in vitro inhibit proinflammatory cytokine production through autocrine/paracrine mechanisms involving TGF-beta, PGE<sub>2</sub>, and PAF. *Journal of Clinical Investigation*, 101, 890.
- FARES, R. P., BELMEGUENAI, A., SANCHEZ, P. E., KOUCHI, H. Y., BODENNEC, J., MORALES, A., GEORGES, B., BONNET, C., BOUVARD, S. & SLOVITER, R. S. 2013. Standardized environmental enrichment supports enhanced brain plasticity in healthy rats and prevents cognitive impairment in epileptic rats. *PLoS One*, 8, e53888.
- FAURSCHOU, M. & BORREGAARD, N. 2003. Neutrophil granules and secretory vesicles in inflammation. *Microbes and Infection*, 5, 1317-1327.
- FEDOSOV, D. A. & GOMPPER, G. 2014. White blood cell margination in microcirculation. *Soft matter*, 10, 2961-2970.
- FERENBACH, D. & HUGHES, J. 2008. Macrophages and dendritic cells: what is the difference? *Kidney international*, 74, 5-7.
- FISCHER, C. P. 2006. Interleukin-6 in acute exercise and training: what is the biological relevance. *Exerc immunol rev*, 12, 41.

- FITCHETT, A. E., COLLINS, S. A., MASON, H., BARNARD, C. J. & CASSADAY, H. J. 2005. Urinary corticosterone measures: effects of strain and social rank in BKW and CD-1 mice. *Behavioural processes*, 70, 168-176.
- FLESHNER, M., LAUDENSLAGER, M. L., SIMONS, L. & MAIER, S. 1989. Reduced serum antibodies associated with social defeat in rats. *Physiology & behavior*, 45, 1183-1187.
- FORSYTHE, P. & BIENENSTOCK, J. 2010. Immunomodulation by commensal and probiotic bacteria. *Immunological investigations*, 39, 429-448.
- FORTES, C., FARCHI, S., FORASTIERE, F., AGABITI, N., PACIFICI, R., ZUCCARO, P., PERUCCI, C. A. & EBRAHIM, S. 2002. Depressive symptoms lead to impaired cellular immune response. *Psychotherapy and psychosomatics*, 72, 253-260.
- FORTUNATI, E., KAZEMIER, K., GRUTTERS, J., KOENDERMAN, L. & VAN DEN BOSCH, V. J. 2009. Human neutrophils switch to an activated phenotype after homing to the lung irrespective of inflammatory disease. *Clinical & Experimental Immunology*, 155, 559-566.
- FRAGIADAKIS, G. K., GAUDILLIÈRE, B., GANIO, E. A., AGHAEPOUR, N., TINGLE, M., NOLAN, G. P. & ANGST, M. S. 2015. Patient-specific immune states before surgery are strong correlates of surgical recovery. *The Journal of the American Society of Anesthesiologists*, 123, 1241-1255.
- FRANCESCHI, C., CAPRI, M., MONTI, D., GIUNTA, S., OLIVIERI, F., SEVINI, F., PANOURGIA, M. P., INVIDIA, L., CELANI, L. & SCURTI, M. 2007. Inflammaging and anti-inflammaging: a systemic perspective on aging and longevity emerged from studies in humans. *Mechanisms of ageing and development*, 128, 92-105.
- FRANCESCHI, C., OLIVIERI, F., MARCHEGANI, F., CARDELLI, M., CAVALLONE, L., CAPRI, M., SALVIOLI, S., VALENSIN, S., DE BENEDICTIS, G. & DI IORIO, A. 2005. Genes involved in immune response/inflammation, IGF1/insulin pathway and response to oxidative stress play a major role in the genetics of human longevity: the lesson of centenarians. *Mechanisms of ageing and development*, 126, 351-361.
- FRANCESCONE, R. A., SCULLY, S., FAIBISH, M., TAYLOR, S. L., OH, D., MORAL, L., YAN, W., BENTLEY, B. & SHAO, R. 2011. Role of YKL-40 in the angiogenesis, radioresistance, and progression of glioblastoma. *Journal of Biological Chemistry*, 286, 15332-15343.
- FRANKLIN, G. M., NELSON, L. M., HEATON, R. K., BURKS, J. S. & THOMPSON, D. S. 1988. Stress and its relationship to acute exacerbations in multiple sclerosis. *Neurorehabilitation and Neural Repair*, 2, 7-11.
- FRENCH, S. S., MOORE, M. C. & DEMAS, G. E. 2009. Ecological immunology: The organism in context. *Integr Comp Biol*, 49, 246-53.
- FRY, R. W., MORTON, A. R., CRAWFORD, G. & KEAST, D. 1992. Cell numbers and in vitro responses of leucocytes and lymphocyte subpopulations following maximal exercise and interval training sessions of different intensities. *European journal of applied physiology and occupational physiology*, 64, 218-227.
- FUCHS, T. A., ABED, U., GOOSMANN, C., HURWITZ, R., SCHULZE, I., WAHN, V., WEINRAUCH, Y., BRINKMANN, V. & ZYCHLINSKY, A. 2007. Novel cell death program leads to neutrophil extracellular traps. *The Journal of cell biology*, 176, 231-241.
- FUERTIG, R., AZZINNARI, D., BERGAMINI, G., CATHOMAS, F., SIGRIST, H., SEIFRITZ, E., VAVASSORI, S., LUIPPOLD, A., HENGERER, B. & CECI, A. 2015. Mouse chronic social stress increases blood and brain kynurenine pathway activity and fear behaviour: Both effects are reversed by inhibition of indoleamine 2, 3-dioxygenase. *Brain, behavior, and immunity*.
- FUNG, T. T., MCCULLOUGH, M. L., NEWBY, P., MANSON, J. E., MEIGS, J. B., RIFAI, N., WILLET, W. C. & HU, F. B. 2005. Diet-quality scores and plasma concentrations of markers of inflammation and endothelial dysfunction. *The American journal of clinical nutrition*, 82, 163-173.
- GALLO, I., RATTAZZI, L., PIRAS, G., GOBBETTI, T., PANZA, E., PERRETTI, M., DALLEY, J. W. & D'ACQUISTO, F. 2014. Formyl Peptide Receptor as a Novel Therapeutic Target for Anxiety-Related Disorders. *PloS one*, 9, e114626.
- GARDNER, H., BROBERG, A., POZZI, A., LAATO, M. & HEINO, J. 1999. Absence of integrin alpha1beta1 in the mouse causes loss of feedback regulation of collagen synthesis in normal and wounded dermis. *Journal of Cell Science*, 112, 263-272.
- GARVY, B., KING, L., TELFORD, W., MORFORD, L. & FRAKER, P. 1993. Chronic elevation of plasma corticosterone causes reductions in the number of cycling cells of the B lineage in murine bone marrow and induces apoptosis. *Immunology*, 80, 587.
- GASTARDELO, T. S., DAMAZO, A. S., DALLI, J., FLOWER, R. J., PERRETTI, M. & OLIANI, S. M. 2009. Functional and ultrastructural analysis of annexin A1 and its receptor in extravasating neutrophils during acute inflammation. *The American journal of pathology*, 174, 177-183.
- GEISSMANN, F., JUNG, S. & LITTMAN, D. R. 2003. Blood monocytes consist of two principal subsets with distinct migratory properties. *Immunity*, 19, 71-82.

- GEISSMANN, F., MANZ, M. G., JUNG, S., SIEWEKE, M. H., MERAD, M. & LEY, K. 2010. Development of monocytes, macrophages, and dendritic cells. *Science*, 327, 656-661.
- GERSZTEN, R. E., GARCIA-ZEPEDA, E. A., LIM, Y.-C., YOSHIDA, M., DING, H. A., GIMBRONE, M. A., LUSTER, A. D., LUSCINSKAS, F. W. & ROSENZWEIG, A. 1999. MCP-1 and IL-8 trigger firm adhesion of monocytes to vascular endothelium under flow conditions. *Nature*, 398, 718-723.
- GETTING, S. J., FLOWER, R. J. & PERRETTI, M. 1997. Inhibition of neutrophil and monocyte recruitment by endogenous and exogenous lipocortin 1. *British journal of pharmacology*, 120, 1075-1082.
- GIACCONI, R., CIPRIANO, C., ALBANESE, F., BOCCOLI, G., SABA, V., OLIVIERI, F., FRANCESCHI, C. & MOCCHEGIANI, E. 2004. The- 174G/C polymorphism of IL-6 is useful to screen old subjects at risk for atherosclerosis or to reach successful ageing. *Experimental gerontology*, 39, 621-628.
- GINHOUX, F. & JUNG, S. 2014. Monocytes and macrophages: developmental pathways and tissue homeostasis. *Nature Reviews Immunology*, 14, 392-404.
- GIRALDO, E., GARCIA, J., HINCHADO, M. & ORTEGA, E. 2009. Exercise intensity-dependent changes in the inflammatory response in sedentary women: role of neuroendocrine parameters in the neutrophil phagocytic process and the pro-/anti-inflammatory cytokine balance. *Neuroimmunomodulation*, 16, 237-244.
- GLASER, R. & KIECOLT-GLASER, J. K. 2005. Stress-induced immune dysfunction: implications for health. *Nature Reviews Immunology*, 5, 243-251.
- GLEESON, M., BISHOP, N., OLIVEIRA, M., MCCAULEY, T., TAULER, P. & MUHAMAD, A. S. 2012. Respiratory infection risk in athletes: association with antigen - stimulated IL - 10 production and salivary IgA secretion. *Scandinavian Journal of Medicine & Science in Sports*, 22, 410-417.
- GLEESON, M., BISHOP, N. C., STENSEL, D. J., LINDLEY, M. R., MASTANA, S. S. & NIMMO, M. A. 2011. The anti-inflammatory effects of exercise: mechanisms and implications for the prevention and treatment of disease. *Nature Reviews Immunology*, 11, 607-615.
- GODBOUT, J., CHEN, J., ABRAHAM, J., RICHWINE, A., BERG, B., KELLEY, K. & JOHNSON, R. 2005. Exaggerated neuroinflammation and sickness behavior in aged mice following activation of the peripheral innate immune system. *The FASEB journal*, 19, 1329-1331.
- GÓMEZ-GAVIRO, M. V., DOMÍNGUEZ-JIMÉNEZ, C., CARRETERO, J. M., SABANDO, P., GONZÁLEZ-ALVARO, I., SÁNCHEZ-MADRID, F. & DÍAZ-GONZÁLEZ, F. 2000. Down-regulation of L-selectin expression in neutrophils by nonsteroidal anti-inflammatory drugs: role of intracellular ATP concentration. *Blood*, 96, 3592-3600.
- GOMEZ, C. R., BOEHMER, E. D. & KOVACS, E. J. 2005. The aging innate immune system. *Current opinion in immunology*, 17, 457-462.
- GONG, S., MIAO, Y.-L., JIAO, G.-Z., SUN, M.-J., LI, H., LIN, J., LUO, M.-J. & TAN, J.-H. 2015. Dynamics and correlation of serum cortisol and corticosterone under different physiological or stressful conditions in mice. *PloS one*, 10, e0117503.
- GOSHEN, I., AVITAL, A., KREISEL, T., LICHT, T., SEGAL, M. & YIRMIYA, R. 2009. Environmental enrichment restores memory functioning in mice with impaired IL-1 signaling via reinstatement of long-term potentiation and spine size enlargement. *The Journal of Neuroscience*, 29, 3395-3403.
- GRIFFIN, A. C., LO, W. D., WOLNY, A. C. & WHITACRE, C. C. 1993. Suppression of experimental autoimmune encephalomyelitis by restraint stress: sex differences. *Journal of neuroimmunology*, 44, 103-116.
- GRIPPO, A. J., IHM, E., WARDWELL, J., MCNEAL, N., SCOTTI, M.-A. L., MOENK, D. A., CHANDLER, D. L., LAROCCA, M. A. & PREIHS, K. 2014. The effects of environmental enrichment on depressive-and anxiety-relevant behaviors in socially isolated prairie voles. *Psychosomatic medicine*, 76, 277.
- GUDE, D. R., ALVAREZ, S. E., PAUGH, S. W., MITRA, P., YU, J., GRIFFITHS, R., BARBOUR, S. E., MILSTIEN, S. & SPIEGEL, S. 2008. Apoptosis induces expression of sphingosine kinase 1 to release sphingosine-1-phosphate as a "come-and-get-me" signal. *The FASEB Journal*, 22, 2629-2638.
- GUILARTE, T. R., TOSCANO, C. D., MCGLOTHAN, J. L. & WEAVER, S. A. 2003. Environmental enrichment reverses cognitive and molecular deficits induced by developmental lead exposure. *Annals of neurology*, 53, 50-56.
- GUILLIAMS, M., GINHOUX, F., JAKUBZICK, C., NAIK, S. H., ONAI, N., SCHRAML, B. U., SEGURA, E., TUSSIWAND, R. & YONA, S. 2014. Dendritic cells, monocytes and macrophages: a unified nomenclature based on ontogeny. *Nature Reviews. Immunology*, 14, 571.

- GURFEIN, B. T., STAMM, A. W., BACCHETTI, P., DALLMAN, M. F., NADKARNI, N. A., MILUSH, J. M., TOUMA, C., PALME, R., DI BORGIO, C. P. & FROMENTIN, G. 2012a. The calm mouse: an animal model of stress reduction. *Molecular Medicine*, 18, 606.
- GURFEIN, B. T., STAMM, A. W., BACCHETTI, P., DALLMAN, M. F., NADKARNI, N. A., MILUSH, J. M., TOUMA, C., PALME, R., DI BORGIO, C. P., FROMENTIN, G. & OTHERS 2012b. The calm mouse: an animal model of stress reduction. *Molecular Medicine*, 18, 606.
- HAKANSSON, A. & MOLIN, G. 2011. Gut microbiota and inflammation. *Nutrients*, 3, 637-682.
- HALL, J. A., PICTON, R. A., FINNERAN, P. S., BIRD, K. E., SKINNER, M. M., JEWELL, D. E. & ZICKER, S. 2006. Dietary antioxidants and behavioral enrichment enhance neutrophil phagocytosis in geriatric Beagles. *Veterinary immunology and immunopathology*, 113, 224-233.
- HASHIMOTO, D., CHOW, A., NOIZAT, C., TEO, P., BEASLEY, M. B., LEBOEUF, M., BECKER, C. D., SEE, P., PRICE, J. & LUCAS, D. 2013. Tissue-resident macrophages self-maintain locally throughout adult life with minimal contribution from circulating monocytes. *Immunity*, 38, 792-804.
- HASLETT, C. 1992. Resolution of acute inflammation and the role of apoptosis in the tissue fate of granulocytes. *Clinical science (London, England: 1979)*, 83, 639-648.
- HAYASHI, A., OHNISHI, H., OKAZAWA, H., NAKAZAWA, S., IKEDA, H., MOTEGI, S.-I., AOKI, N., KIMURA, S., MIKUNI, M. & MATOZAKI, T. 2004. Positive regulation of phagocytosis by SIRP $\beta$  and its signaling mechanism in macrophages. *Journal of Biological Chemistry*, 279, 29450-29460.
- HAYASHI, T. & MURAKAMI, K. 2009. The effects of laughter on post-prandial glucose levels and gene expression in type 2 diabetic patients. *Life Sci*, 85, 185-7.
- HAYASHI, T., TSUJII, S., IBURI, T., TAMANAHA, T., YAMAGAMI, K., ISHIBASHI, R., HORI, M., SAKAMOTO, S., ISHII, H. & MURAKAMI, K. 2007. Laughter up-regulates the genes related to NK cell activity in diabetes. *Biomed Res*, 28, 281-5.
- HE, C. H., LEE, C. G., CRUZ, C. S. D., LEE, C.-M., ZHOU, Y., AHANGARI, F., MA, B., HERZOG, E. L., ROSENBERG, S. A. & LI, Y. 2013. Chitinase 3-like 1 regulates cellular and tissue responses via IL-13 receptor  $\alpha 2$ . *Cell reports*, 4, 830-841.
- HE, K., LIU, K., DAVIGLUS, M. L., JENNY, N. S., MAYER-DAVIS, E., JIANG, R., STEFFEN, L., SISCOVICK, D., TSAI, M. & HERRINGTON, D. 2009. Associations of dietary long-chain n-3 polyunsaturated fatty acids and fish with biomarkers of inflammation and endothelial activation (from the Multi-Ethnic Study of Atherosclerosis [MESA]). *The American journal of cardiology*, 103, 1238-1243.
- HEADLAND, S. E. & NORLING, L. V. The resolution of inflammation: Principles and challenges. *Seminars in immunology*, 2015. Elsevier, 149-160.
- HEIDLAND, A., KLASSEN, A., RUTKOWSKI, P. & BAHNER, U. 2006. The contribution of Rudolf Virchow to the concept of inflammation: what is still of importance? *Journal of nephrology*, 19, S102.
- HEIDT, T., SAGER, H. B., COURTIES, G., DUTTA, P., IWAMOTO, Y., ZALTSMAN, A., VON ZUR MUHLEN, C., BODE, C., FRICCHIONE, G. L. & DENNINGER, J. 2014. Chronic variable stress activates hematopoietic stem cells. *Nature medicine*, 20, 754-758.
- HICKLIN, D. J. & ELLIS, L. M. 2005. Role of the vascular endothelial growth factor pathway in tumor growth and angiogenesis. *Journal of clinical oncology*, 23, 1011-1027.
- HINZ, B. 2007. Formation and function of the myofibroblast during tissue repair. *Journal of Investigative Dermatology*, 127, 526-537.
- HOBBS, M., WEIGLE, W., NOONAN, D., TORBETT, B., MCEVILLY, R., KOCH, R., CARDENAS, G. & ERNST, D. 1993. Patterns of cytokine gene expression by CD4<sup>+</sup> T cells from young and old mice. *The Journal of Immunology*, 150, 3602-3614.
- HOLLENBERG, S. M., BROUSSARD, M., OSMAN, J. & PARRILLO, J. E. 2000. Increased microvascular reactivity and improved mortality in septic mice lacking inducible nitric oxide synthase. *Circulation research*, 86, 774-778.
- HOLT, E. M., STEFFEN, L. M., MORAN, A., BASU, S., STEINBERGER, J., ROSS, J. A., HONG, C.-P. & SINAICO, A. R. 2009. Fruit and vegetable consumption and its relation to markers of inflammation and oxidative stress in adolescents. *Journal of the American Dietetic Association*, 109, 414-421.
- HORII-HAYASHI, N., YOSHIKAWA, M., MATSUSUE, Y., ISHIZAKA, S., NISHI, M. & WANAKA, A. 2011. Environmental stimulation changes tissue-type plasminogen activator activity in the adult mouse hippocampus. *Neurochemistry international*, 58, 1-4.
- HOSOI, T., YAMAWAKI, Y., KIMURA, H. & OZAWA, K. 2016. Immobilization stress-induced anorexia is mediated independent of MyD88. *NeuroReport*, 27, 974-977.

- HOTCHKISS, R., TINSLEY, K., SWANSON, P., CHANG, K., COBB, J., BUCHMAN, T., KORSMEYER, S. & KARL, I. 1999. Prevention of lymphocyte cell death in sepsis improves survival in mice. *Proceedings of the National Academy of Sciences*, 96, 14541-14546.
- HOTCHKISS, R. S. & NICHOLSON, D. W. 2006. Apoptosis and caspases regulate death and inflammation in sepsis. *Nature Reviews Immunology*, 6, 813-822.
- HOWE, L. R. 2007. Inflammation and breast cancer. Cyclooxygenase/prostaglandin signaling and breast cancer. *Breast Cancer Research*, 9, 1.
- HUA, L. & RECKLIES, A. D. 2004. The chitinase 3-like protein human cartilage glycoprotein 39 inhibits cellular responses to the inflammatory cytokines interleukin-1 and tumour necrosis factor- $\alpha$ . *Biochemical Journal*, 380, 651-659.
- HUBER-LANG, M., BARRATT-DUE, A., PISCHKE, S. E., SANDANGER, Ø., NILSSON, P. H., NUNN, M. A., DENK, S., GAUS, W., ESPEVIK, T. & MOLLNES, T. E. 2014. Double blockade of CD14 and complement C5 abolishes the cytokine storm and improves morbidity and survival in polymicrobial sepsis in mice. *The Journal of Immunology*, 192, 5324-5331.
- HUBRECHT, R., HURST, J., MORTON, D., PETERS, A., RAYMOND, R., SALES, G., SHERWIN, C. & WEST, C. 1993. Refining rodent husbandry: the mouse. *Laboratory Animals*, 27, 301-329.
- HUGO, H. J., SAUNDERS, C., RAMSAY, R. & THOMPSON, E. 2015. New insights on COX-2 in chronic inflammation driving breast cancer growth and Metastasis. *Journal of mammary gland biology and neoplasia*, 20, 109-119.
- HUTCHINSON, E., AVERY, A. & VANDEWOUDE, S. 2005. Environmental enrichment for laboratory rodents. *ILAR journal*, 46, 148-161.
- HUTCHINSON, E. K., AVERY, A. C. & VANDEWOUDE, S. 2012. Environmental enrichment during rearing alters corticosterone levels, thymocyte numbers, and aggression in female BALB/c mice. *Journal of the American Association for Laboratory Animal Science: JAALAS*, 51, 18.
- HÜTTENRAUCH, M., SALINAS, G. & WIRTHS, O. 2016. Effects of Long-Term Environmental Enrichment on Anxiety, Memory, Hippocampal Plasticity and Overall Brain Gene Expression in C57BL6 Mice. *Frontiers in Molecular Neuroscience*, 9.
- HYNES, R. O. 1992. Integrins: versatility, modulation, and signaling in cell adhesion. *Cell*, 69, 11-25.
- INAOKA, T., BILBE, G., ISHIBASHI, O., TEZUKA, K.-I., KUMEGAWA, M. & KOKUBO, T. 1995. Molecular cloning of human cDNA for cathepsin K: novel cysteine proteinase predominantly expressed in bone. *Biochemical and biophysical research communications*, 206, 89-96.
- IRWIN, M., PATTERSON, T., SMITH, T. L., CALDWELL, C., BROWN, S. A., GILLIN, J. C. & GRANT, I. 1990. Reduction of immune function in life stress and depression. *Biological psychiatry*, 27, 22-30.
- IRWIN, M. R., OLMSTEAD, R., BREEN, E. C., WITARAMA, T., CARRILLO, C., SADEGHI, N., AREVALO, J. M., MA, J., NICASSIO, P. & BOOTZIN, R. 2015. Cognitive behavioral therapy and tai chi reverse cellular and genomic markers of inflammation in late-life insomnia: a randomized controlled trial. *Biological psychiatry*, 78, 721-729.
- ISOLAURI, E., RAUTANEN, T., JUNTUNEN, M., SILLANAUKEE, P. & KOIVULA, T. 1991. A human Lactobacillus strain (Lactobacillus casei sp strain GG) promotes recovery from acute diarrhea in children. *Pediatrics*, 88, 90-97.
- ITALIANI, P. & BORASCHI, D. 2015. From monocytes to M1/M2 macrophages: phenotypical vs. functional differentiation. *M1/M2 Macrophages: The Arginine Fork in the Road to Health and Disease*, 47.
- ITO, M., YANG, Z., ANDL, T., CUI, C., KIM, N., MILLAR, S. E. & COTSARELIS, G. 2007. Wnt-dependent de novo hair follicle regeneration in adult mouse skin after wounding. *Nature*, 447, 316-320.
- JAIN, V., BAITHARU, I., PRASAD, D. & ILAVAZHAGAN, G. 2013. Enriched environment prevents hypobaric hypoxia induced memory impairment and neurodegeneration: role of BDNF/PI3K/GSK3 $\beta$  pathway coupled with CREB activation. *PLoS One*, 8, e62235.
- JAMES, S. 1997. Passion and action: The emotions in seventeenth-century philosophy.
- JANKOWSKY, J. L., MELNIKOVA, T., FADALE, D. J., XU, G. M., SLUNT, H. H., GONZALES, V., YOUNKIN, L. H., YOUNKIN, S. G., BORCHELT, D. R. & SAVONENKO, A. V. 2005. Environmental enrichment mitigates cognitive deficits in a mouse model of Alzheimer's disease. *The Journal of Neuroscience*, 25, 5217-5224.
- JENEROWICZ, D., SILNY, W., DANCZAK-PAZDROWSKA, A., POLANSKA, A., OSMOLA-MANKOWSKA, A. & OLEK-HRAB, K. 2012. Environmental factors and allergic diseases. *Annals of agricultural and environmental medicine*, 19.
- JEONG, J. Y., LEE, D. H. & KANG, S. S. 2013. Effects of chronic restraint stress on body weight, food intake, and hypothalamic gene expressions in mice. *Endocrinology and metabolism*, 28, 288-296.
- JIA, G.-Q., GONZALO, J. A., LLOYD, C., KREMER, L., LU, L., MARTINEZ-A, C., WERSHIL, B. & GUTIERREZ-RAMOS, J. C. 1996. Distinct expression and function of the novel mouse

- chemokine monocyte chemotactic protein-5 in lung allergic inflammation. *The Journal of experimental medicine*, 184, 1939-1951.
- JIA, T., SERBINA, N. V., BRANDL, K., ZHONG, M. X., LEINER, I. M., CHARO, I. F. & PAMER, E. G. 2008. Additive roles for MCP-1 and MCP-3 in CCR2-mediated recruitment of inflammatory monocytes during *Listeria monocytogenes* infection. *The Journal of Immunology*, 180, 6846-6853.
- JIANG, C. G., MORROW-TESCH, J. L., BELLER, D. I., LEVY, E. M. & BLACK, P. H. 1990. Immunosuppression in mice induced by cold water stress. *Brain, behavior, and immunity*, 4, 278-291.
- JOHANNSEN, N. M., PRIEST, E. L., DIXIT, V. D., EARNEST, C. P., BLAIR, S. N. & CHURCH, T. S. 2008. Association of white blood cell subfraction concentration with fitness and fatness. *British journal of sports medicine*.
- JOHANSEN, J. S. 2006. Studies on serum YKL-40 as a biomarker in diseases with inflammation, tissue remodelling, fibroses and cancer. *Dan Med Bull*, 53, 172-209.
- JUNGER, W. G., HOYT, D. B., LIU, F. C., LOOMIS, W. H. & COIMBRA, R. 1996. Immunosuppression after endotoxin shock: the result of multiple anti-inflammatory factors. *Journal of Trauma and Acute Care Surgery*, 40, 702-709.
- JURGENS, H. A. & JOHNSON, R. W. 2012. Environmental enrichment attenuates hippocampal neuroinflammation and improves cognitive function during influenza infection. *Brain, behavior, and immunity*, 26, 1006-1016.
- KAMBA, A., LEE, I.-A. & MIZOGUCHI, E. 2013. Potential association between TLR4 and chitinase 3-like 1 (CHI3L1/YKL-40) signaling on colonic epithelial cells in inflammatory bowel disease and colitis-associated cancer. *Current molecular medicine*, 13, 1110-1121.
- KANEIDER, N. C., EGGER, P., DJANANI, A. M. & WIEDERMANN, C. J. 2003. Leukocyte motility in response to neuropeptides is heparan sulfate proteoglycan dependent. *Peptides*, 24, 695-700.
- KANT, G. J., LEU, J. R., ANDERSON, S. M. & MOUGEY, E. H. 1987. Effects of chronic stress on plasma corticosterone, ACTH and prolactin. *Physiology & behavior*, 40, 775-779.
- KARSENTY, G. & PARK, R.-W. 1995. Regulation of type I collagen genes expression. *International reviews of immunology*, 12, 177-185.
- KAUFMAN, J. C., HARRIS, T. J., HIGGINS, J. & MAISEL, A. S. 1994. Exercise-induced enhancement of immune function in the rat. *Circulation*, 90, 525-532.
- KAUFMANN, A., SALENTIN, R., GEMSA, D. & SPRENGER, H. 2001. Increase of CCR1 and CCR5 expression and enhanced functional response to MIP-1 $\alpha$  during differentiation of human monocytes to macrophages. *Journal of leukocyte biology*, 69, 248-252.
- KAWAI, T. & AKIRA, S. 2010. The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. *Nature immunology*, 11, 373-384.
- KELISHADI, R., MIRGHAFARI, N., POURSAFA, P. & GIDDING, S. S. 2009. Lifestyle and environmental factors associated with inflammation, oxidative stress and insulin resistance in children. *Atherosclerosis*, 203, 311-319.
- KELLEY, K., GREENFIELD, R., EVERMANN, J., PARISH, S. & PERRYMAN, L. 1982. Delayed-type hypersensitivity, contact sensitivity, and phytohemagglutinin skin-test responses of heat-and cold-stressed calves. *American journal of veterinary research*, 43, 775-779.
- KELLY-SCUMPIA, K. M., SCUMPIA, P. O., DELANO, M. J., WEINSTEIN, J. S., CUENCA, A. G., WYNN, J. L. & MOLDAWER, L. L. 2010. Type I interferon signaling in hematopoietic cells is required for survival in mouse polymicrobial sepsis by regulating CXCL10. *The Journal of experimental medicine*, 207, 319-326.
- KEMPERMANN, G., KUHN, H. G. & GAGE, F. H. 1997. More hippocampal neurons in adult mice living in an enriched environment. *Nature*, 386, 493-495.
- KERKHOFF, C., KLEMPF, M. & SORG, C. 1998. Novel insights into structure and function of MRP8 (S100A8) and MRP14 (S100A9). *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research*, 1448, 200-211.
- KIGERL, K. A., MCGAUGHY, V. M. & POPOVICH, P. G. 2006. Comparative analysis of lesion development and intraspinal inflammation in four strains of mice following spinal contusion injury. *Journal of Comparative Neurology*, 494, 578-594.
- KIM, B.-J. & JONES, H. P. 2010. Epinephrine-primed murine bone marrow-derived dendritic cells facilitate production of IL-17A and IL-4 but not IFN- $\gamma$  by CD4 $^{+}$  T cells. *Brain, behavior, and immunity*, 24, 1126-1136.
- KIM, C., YANG, H., FUKUSHIMA, Y., SAW, P. E., LEE, J., PARK, J.-S., PARK, I., JUNG, J., KATAOKA, H. & LEE, D. 2014. Vascular RhoJ is an effective and selective target for tumor angiogenesis and vascular disruption. *Cancer cell*, 25, 102-117.
- KIM, H.-J., ALONZO, E. S., DOROTHEE, G., POLLARD, J. W. & SANT'ANGELO, D. B. 2010. Selective depletion of eosinophils or neutrophils in mice impacts the efficiency of apoptotic cell clearance in the thymus. *PloS one*, 5, e11439.

- KIMBRELL, D. A. & BEUTLER, B. 2001. The evolution and genetics of innate immunity. *Nature Reviews Genetics*, 2, 256-267.
- KJØLLER, L. 2002. The urokinase plasminogen activator receptor in the regulation of the actin cytoskeleton and cell motility. *Biological chemistry*, 383, 5-19.
- KLEBANOFF, S. J. 2005. Myeloperoxidase: friend and foe. *Journal of leukocyte biology*, 77, 598-625.
- KLEESSEN, B., KROESEN, A., BUHR, H. & BLAUT, M. 2002. Mucosal and invading bacteria in patients with inflammatory bowel disease compared with controls. *Scandinavian journal of gastroenterology*, 37, 1034-1041.
- KLOPFLEISCH, R. 2016. Macrophage reaction against biomaterials in the mouse model—Phenotypes, functions and markers. *Acta Biomaterialia*.
- KOLACZKOWSKA, E., BARTECZKO, M., PLYTYCZ, B. & ARNOLD, B. 2008. Role of lymphocytes in the course of murine zymosan-induced peritonitis. *Inflammation Research*, 57, 272-278.
- KONIKOFF, M. R. & DENSON, L. A. 2006. Role of fecal calprotectin as a biomarker of intestinal inflammation in inflammatory bowel disease. *Inflammatory bowel diseases*, 12, 524-534.
- KORNBLIT, B., HELLEMANN, D., MUNTHE-FOG, L., BONDE, J., STRØM, J. J., MADSEN, H. O., JOHANSEN, J. S. & GARRED, P. 2013. Plasma YKL-40 and CHI3L1 in systemic inflammation and sepsis—Experience from two prospective cohorts. *Immunobiology*, 218, 1227-1234.
- KOVESDI, E., GYORGY, A. B., KWON, S.-K. C., WINGO, D. L., KAMNAKSH, A., LONG, J. B., KASPER, C. E. & AGOSTON, D. V. 2011. The effect of enriched environment on the outcome of traumatic brain injury; a behavioral, proteomics, and histological study. *Frontiers in neuroscience*, 5, 42.
- KRUSCHINSKI, C., SKRIPULETZ, T., BEDOUI, S., RABER, K., STRAUB, R. H., HOFFMANN, T., GROTE, K., JACOBS, R., STEPHAN, M. & PABST, R. 2008. Postnatal life events affect the severity of asthmatic airway inflammation in the adult rat. *The Journal of Immunology*, 180, 3919-3925.
- KUMAR, V., ABBAS, A. K., FAUSTO, N. & MITCHELL, R. N. 2012. *Robbins basic pathology*, Elsevier Health Sciences.
- KUNDU, G. C. & PATIL, D. 2006. MMP2 (matrix metalloproteinase 2 (gelatinase A, 72kDa gelatinase, 72kDa type IV collagenase).
- KZHYSHKOWSKA, J., MAMIDI, S., GRATCHEV, A., KREMMER, E., SCHMUTTERMAIER, C., KRUSELL, L., HAUS, G., UTIKAL, J., SCHLEDZEWSKI, K. & SCHOLTZE, J. 2006. Novel stabilin-1 interacting chitinase-like protein (SI-CLP) is up-regulated in alternatively activated macrophages and secreted via lysosomal pathway. *Blood*, 107, 3221-3228.
- LACY, P., DENKERS, E. & GARG, A. D. 2015. Neutrophil-derived cytokines: facts beyond expression. *Secretion of Cytokines and Chemokines by Innate Immune Cells*, 38.
- LAMBETH, J. D. 2004. NOX enzymes and the biology of reactive oxygen. *Nature Reviews Immunology*, 4, 181-189.
- LANCASTER, G. I., KHAN, Q., DRYSDALE, P., WALLACE, F., JEUKENDRUP, A. E., DRAYSON, M. T. & GLEESON, M. 2005. The physiological regulation of toll - like receptor expression and function in humans. *The Journal of physiology*, 563, 945-955.
- LAUBER, K., BOHN, E., KRÖBER, S. M., XIAO, Y.-J., BLUMENTHAL, S. G., LINDEMANN, R. K., MARINI, P., WIEDIG, C., ZOBYWALSKI, A. & BAKSH, S. 2003. Apoptotic cells induce migration of phagocytes via caspase-3-mediated release of a lipid attraction signal. *Cell*, 113, 717-730.
- LAVIOLA, G., REA, M., MORLEY - FLETCHER, S., DI CARLO, S., BACOSI, A., DE SIMONE, R., BERTINI, M. & PACIFICI, R. 2004. Beneficial effects of enriched environment on adolescent rats from stressed pregnancies. *European Journal of Neuroscience*, 20, 1655-1664.
- LE FEVRE, M., KOLT, G. S. & MATHENY, J. 2006. Eustress, distress and their interpretation in primary and secondary occupational stress management interventions: which way first? *Journal of Managerial Psychology*, 21, 547-565.
- LE FEVRE, M., MATHENY, J. & KOLT, G. S. 2003. Eustress, distress, and interpretation in occupational stress. *Journal of Managerial psychology*, 18, 726-744.
- LECH, M., GRÖBMAYR, R., WEIDENBUSCH, M. & ANDERS, H.-J. 2012. Tissues use resident dendritic cells and macrophages to maintain homeostasis and to regain homeostasis upon tissue injury: the immunoregulatory role of changing tissue environments. *Mediators of inflammation*, 2012.
- LEE, C. G., DA SILVA, C. A., CRUZ, C. S. D., AHANGARI, F., MA, B., KANG, M.-J., HE, C.-H., TAKYAR, S. & ELIAS, J. A. 2011. Role of chitin and chitinase/chitinase-like proteins in inflammation, tissue remodeling, and injury. *Annual review of physiology*, 73.
- LEE, C. G., DA SILVA, C. A., LEE, J.-Y., HARTL, D. & ELIAS, J. A. 2008. Chitin regulation of immune responses: an old molecule with new roles. *Current opinion in immunology*, 20, 684-689.



- LEE, C. G., HARTL, D., LEE, G. R., KOLLER, B., MATSUURA, H., DA SILVA, C. A., SOHN, M. H., COHN, L., HOMER, R. J. & KOZHICH, A. A. 2009. Role of breast regression protein 39 (BRP-39)/chitinase 3-like-1 in Th2 and IL-13-induced tissue responses and apoptosis. *The Journal of experimental medicine*, 206, 1149-1166.
- LEGGATE, M., NOWELL, M. A., JONES, S. A. & NIMMO, M. A. 2010. The response of interleukin-6 and soluble interleukin-6 receptor isoforms following intermittent high intensity and continuous moderate intensity cycling. *Cell stress and Chaperones*, 15, 827-833.
- LEHMANN, J., PRYCE, C., BETTSCHEN, D. & FELDON, J. 1999. The maternal separation paradigm and adult emotionality and cognition in male and female Wistar rats. *Pharmacology Biochemistry and Behavior*, 64, 705-715.
- LEHRNER, A., DASKALAKIS, N. & YEHUDA, R. 2016. Cortisol and the Hypothalamic–Pituitary–Adrenal Axis in PTSD. *Posttraumatic Stress Disorder: From Neurobiology to Treatment*, 265.
- LEY, K., LAUDANNA, C., CYBULSKY, M. I. & NOURSHARGH, S. 2007. Getting to the site of inflammation: the leukocyte adhesion cascade updated. *Nature Reviews Immunology*, 7, 678-689.
- LI, G., GAN, Y., FAN, Y., WU, Y., LIN, H., SONG, Y., CAI, X., YU, X., PAN, W. & YAO, M. 2015. Enriched environment inhibits mouse pancreatic cancer growth and down-regulates the expression of mitochondria-related genes in cancer cells. *Scientific reports*, 5.
- LI, X., THOMPSON, D., KUMAR, B. & DEMARTINO, G. N. 2014. Molecular and cellular roles of PI31 (PSMF1) protein in regulation of proteasome function. *Journal of Biological Chemistry*, 289, 17392-17405.
- LIN, E.-J. D., CHOI, E., LIU, X., MARTIN, A. & DURING, M. J. 2011. Environmental enrichment exerts sex-specific effects on emotionality in C57BL/6J mice. *Behavioural brain research*, 216, 349-357.
- LIN, Q., WANG, F., YANG, R., ZHENG, X., GAO, H. & ZHANG, P. 2013. Effect of chronic restraint stress on human colorectal carcinoma growth in mice. *PloS one*, 8, e61435.
- LIRA, F. S., ROSA, J. C., PIMENTEL, G. D., SOUZA, H. A., CAPERUTO, E. C., CARNEVALI, L. C., SEELAENDER, M., DAMASO, A. R., OYAMA, L. M. & DE MELLO, M. T. 2010. Endotoxin levels correlate positively with a sedentary lifestyle and negatively with highly trained subjects. *Lipids in health and disease*, 9, 82.
- LIU, D.-Q., LI, L.-M., GUO, Y.-L., BAI, R., WANG, C., BIAN, Z., ZHANG, C.-Y. & ZEN, K. 2008. Signal Regulatory Protein  $\alpha$  Negatively Regulates  $\beta$ 2 Integrin-Mediated Monocyte Adhesion, Transendothelial Migration and Phagocytosis. *PLoS ONE*, 3, e3291.
- LIU, X., SHEN, Y., WANG, H., GE, Q., FEI, A. & PAN, S. 2016. Prognostic Significance of Neutrophil-to-Lymphocyte Ratio in Patients with Sepsis: A Prospective Observational Study. *Mediators of inflammation*, 2016.
- LOPEZ-GARCIA, E., SCHULZE, M. B., MEIGS, J. B., MANSON, J. E., RIFAI, N., STAMPFER, M. J., WILLETT, W. C. & HU, F. B. 2005. Consumption of trans fatty acids is related to plasma biomarkers of inflammation and endothelial dysfunction. *The Journal of nutrition*, 135, 562-566.
- LU, Y.-C., YEH, W.-C. & OHASHI, P. S. 2008. LPS/TLR4 signal transduction pathway. *Cytokine*, 42, 145-151.
- LUMENG, C. N. & SALTIEL, A. R. 2011. Inflammatory links between obesity and metabolic disease. *The Journal of clinical investigation*, 121, 2111-2117.
- LUVONE, T., CARNUCCIO, R. & DI ROSA, M. 1994. Modulation of granuloma formation by endogenous nitric oxide. *European journal of pharmacology*, 265, 89-92.
- MA, B., BLACKBURN, M. R., LEE, C. G., HOMER, R. J., LIU, W., FLAVELL, R. A., BOYDEN, L., LIFTON, R. P., SUN, C.-X. & YOUNG, H. W. 2006. Adenosine metabolism and murine strain-specific IL-4-induced inflammation, emphysema, and fibrosis. *Journal of Clinical Investigation*, 116, 1274.
- MA, J., CHEN, T., MANDELIN, J., CEPONIS, A., MILLER, N., HUKKANEN, M., MA, G. & KONTTINEN, Y. 2003. Regulation of macrophage activation. *Cellular and Molecular Life Sciences CMLS*, 60, 2334-2346.
- MA, X.-C., JIANG, D., JIANG, W.-H., WANG, F., JIA, M., WU, J., HASHIMOTO, K., DANG, Y.-H. & GAO, C.-G. 2011. Social isolation-induced aggression potentiates anxiety and depressive-like behavior in male mice subjected to unpredictable chronic mild stress. *PloS one*, 6, e20955.
- MA, X., LIN, W. Y., CHEN, Y., STAWICKI, S., MUKHYALA, K., WU, Y., MARTIN, F., BAZAN, J. F. & STAROVASNIK, M. A. 2012. Structural basis for the dual recognition of helical cytokines IL-34 and CSF-1 by CSF-1R. *Structure*, 20, 676-687.
- MADDOX, J. F., HACHICHA, M., TAKANO, T., PETASIS, N. A., FOKIN, V. V. & SERHAN, C. N. 1997. Lipoxin A4 stable analogs are potent mimetics that stimulate human monocytes and THP-1 cells via a G-protein-linked lipoxin A4 receptor. *Journal of Biological Chemistry*, 272, 6972-6978.

- MAJCHRZYCKI, M., BARTKOWIAK-WIECZOREK, J., WOLSKI, H., DREWS, K., BOGACZ, A., CZERNY, B., ZAGRODNIK-UŁAN, E. & SEREMAK-MROZIKIEWICZ, A. 2015. Polymorphisms of collagen 1A1 (COL1A1) gene and their relation to bone mineral density in postmenopausal women. *Ginekologia polska*, 86, 907-914.
- MAJOR, B., RATTAZZI, L., BROD, S., PILIPOVIĆ, I., LEPOSAVIĆ, G. & D'ACQUISTO, F. 2015. Massage-like stroking boosts the immune system in mice. *Scientific reports*, 5.
- MALATYNSKA, E. & KNAPP, R. J. 2005. Dominant-submissive behavior as models of mania and depression. *Neuroscience & Biobehavioral Reviews*, 29, 715-737.
- MALINDA, K. M., PONCE, L., KLEINMAN, H. K., SHACKELTON, L. M. & MILLIS, A. J. 1999. Gp38k, a protein synthesized by vascular smooth muscle cells, stimulates directional migration of human umbilical vein endothelial cells. *Experimental cell research*, 250, 168-173.
- MANDINOVA, A., SOLDI, R., GRAZIANI, I., BAGALÁ, C., BELLUM, S., LANDRISCINA, M., TARANTINI, F., PRUDOVSKY, I. & MACIAG, T. 2003. S100A13 mediates the copper-dependent stress-induced release of IL-1 $\alpha$  from both human U937 and murine NIH 3T3 cells. *Journal of cell science*, 116, 2687-2696.
- MARASHI, V., BARNEKOW, A., OSSENDORF, E. & SACHSER, N. 2003. Effects of different forms of environmental enrichment on behavioral, endocrinological, and immunological parameters in male mice. *Hormones and Behavior*, 43, 281-292.
- MARASHI, V., BARNEKOW, A. & SACHSER, N. 2004. Effects of environmental enrichment on males of a docile inbred strain of mice. *Physiology & behavior*, 82, 765-776.
- MARTIN, B., JI, S., MAUDSLEY, S. & MATTSON, M. P. 2010. "Control" laboratory rodents are metabolically morbid: why it matters. *Proceedings of the National Academy of Sciences*, 107, 6127-6133.
- MARTINEZ, F. O. & GORDON, S. 2014. The M1 and M2 paradigm of macrophage activation: time for reassessment. *F1000Prime Rep*, 6, 12703.
- MARUCHA, P. T., KIECOLT-GLASER, J. K. & FAVAGEHI, M. 1998. Mucosal wound healing is impaired by examination stress. *Psychosomatic Medicine*, 60, 362-365.
- MATHERS, C., FAT, D. M. & BOERMA, J. 2008. *The global burden of disease: 2004 update*, WHO Press, 20 Avenue Appia, 1211 Geneva 27, Switzerland, World Health Organization.
- MATOLTSY, A. G. & VIZIAM, C. B. 1970. Further observations on epithelialization of small wounds. *Journal of Investigative Dermatology*, 55, 20-25.
- MATSUKAWA, A., HOGABOAM, C. M., LUKACS, N. W., LINCOLN, P. M., STRIETER, R. M. & KUNKEL, S. L. 1999. Endogenous monocyte chemoattractant protein-1 (MCP-1) protects mice in a model of acute septic peritonitis: cross-talk between MCP-1 and leukotriene B4. *The Journal of Immunology*, 163, 6148-6154.
- MATSUZAKI, T., NAKAJIMA, A., ISHIGAMI, S., TANNO, M. & YOSHINO, S. 2006. Mirthful laughter differentially affects serum pro- and anti-inflammatory cytokine levels depending on the level of disease activity in patients with rheumatoid arthritis. *Rheumatology (Oxford)*, 45, 182-6.
- MATUR, E., AKYAZI, İ., ERASLAN, E., ERGUL EKİZ, E., ESECELI, H., KETEN, M., METINER, K. & AKTARAN BALA, D. 2016. The effects of environmental enrichment and transport stress on the weights of lymphoid organs, cell - mediated immune response, heterophil functions and antibody production in laying hens. *Animal Science Journal*, 87, 284-292.
- MAWDSLEY, J. & RAMPTON, D. 2005. Psychological stress in IBD: new insights into pathogenic and therapeutic implications. *Gut*, 54, 1481-1491.
- MAYADAS, T. N., CULLERE, X. & LOWELL, C. A. 2014. The multifaceted functions of neutrophils. *Annual review of pathology*, 9, 181.
- MCCARTHY, D. & DALE, M. 1988. The leucocytosis of exercise. *Sports medicine*, 6, 333-363.
- MCDEVITT, H. O. & BENACERRAF, B. 1969. Genetic control of specific immune responses. *Advances in immunology*, 11, 31-74.
- MCLACHLAN, J. L., SLOAN, A. J., SMITH, A. J., LANDINI, G. & COOPER, P. R. 2004. S100 and cytokine expression in caries. *Infection and immunity*, 72, 4102-4108.
- MCQUAID, R. J., AUDET, M.-C., JACOBSON-PICK, S. & ANISMAN, H. 2013. Environmental enrichment influences brain cytokine variations elicited by social defeat in mice. *Psychoneuroendocrinology*, 38, 987-996.
- MCQUIBBAN, G. A., GONG, J.-H., TAM, E. M., MCCULLOCH, C. A., CLARK-LEWIS, I. & OVERALL, C. M. 2000. Inflammation dampened by gelatinase A cleavage of monocyte chemoattractant protein-3. *Science*, 289, 1202-1206.
- MEDZHITOV, R. 2008. Origin and physiological roles of inflammation. *Nature*, 454, 428-435.
- MELLEN, J. & SEVENICH MACPHEE, M. 2001. Philosophy of environmental enrichment: past, present, and future. *Zoo Biology*, 20, 211-226.
- MENG, X., WEI, G., CHANG, Q., PENG, R., SHI, G., ZHENG, P., HE, F., WANG, W. & MING, L. 2016. The platelet-to-lymphocyte ratio, superior to the neutrophil-to-lymphocyte ratio,

- correlates with hepatitis C virus infection. *International Journal of Infectious Diseases*, 45, 72-77.
- MICALLEF, M., MUNRO, I. & GARG, M. 2009. An inverse relationship between plasma n-3 fatty acids and C-reactive protein in healthy individuals. *European journal of clinical nutrition*, 63, 1154-1156.
- MILLS, C. 2012. M1 and M2 macrophages: oracles of health and disease. *Critical Reviews™ in Immunology*, 32.
- MINEUR, Y. S., BELZUNG, C. & CRUSIO, W. E. 2006. Effects of unpredictable chronic mild stress on anxiety and depression-like behavior in mice. *Behavioural brain research*, 175, 43-50.
- MISSLIN, R., KEMPF, E., SCHLEEF, C., GOBAILLE, S., ROPARTZ, P. & MANDEL, P. 1982. Responses to novelty in two inbred strains of mice and their F 1 hybrids: Behaviour and brain biogenic amines. *Behavioural processes*, 7, 135-141.
- MITCHISON, T. & CRAMER, L. 1996. Actin-based cell motility and cell locomotion. *Cell*, 84, 371-379.
- MONROE, L. L., ARMSTRONG, M. G., ZHANG, X., HALL, J. V., OZMENT, T. R., LI, C., WILLIAMS, D. L. & HOOVER, D. B. 2016. Zymosan-induced peritonitis: effects on cardiac function, temperature regulation, translocation of bacteria and role of dectin-1. *Shock*.
- MONTERO-MELENDEZ, T., GOBBETTI, T., COORAY, S. N., JONASSEN, T. E. & PERRETTI, M. 2015. Biased agonism as a novel strategy to harness the proresolving properties of melanocortin receptors without eliciting melanogenic effects. *The Journal of Immunology*, 194, 3381-3388.
- MORICI, G., ZANGLA, D., SANTORO, A., PELOSI, E., PETRUCCI, E., GIOIA, M., BONANNO, A., PROFITA, M., BELLIA, V. & TESTA, U. 2005. Supramaximal exercise mobilizes hematopoietic progenitors and reticulocytes in athletes. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*, 289, R1496-R1503.
- MOSER, M., DE SMEDT, T., SORNASSE, T., TIELEMANS, F., CHENTOUFI, A. A., MURAILLE, E., VAN MECHELEN, M., URBAIN, J. & LEO, O. 1995. Glucocorticoids down - regulate dendritic cell function in vitro and in vivo. *European journal of immunology*, 25, 2818-2824.
- MOSSER, D. M. 2003. The many faces of macrophage activation. *Journal of leukocyte biology*, 73, 209-212.
- MOSSER, D. M. & ZHANG, X. 2008. Interleukin - 10: new perspectives on an old cytokine. *Immunological reviews*, 226, 205-218.
- MOZAFFARIAN, D., PISCHON, T., HANKINSON, S. E., RIFAI, N., JOSHIPURA, K., WILLETT, W. C. & RIMM, E. B. 2004. Dietary intake of trans fatty acids and systemic inflammation in women. *The American journal of clinical nutrition*, 79, 606-612.
- MUENZER, J. T., DAVIS, C. G., CHANG, K., SCHMIDT, R. E., DUNNE, W. M., COOPERSMITH, C. M. & HOTCHKISS, R. S. 2010. Characterization and modulation of the immunosuppressive phase of sepsis. *Infection and immunity*, 78, 1582-1592.
- MULLER, W. 2013. Getting leukocytes to the site of inflammation. *Veterinary Pathology Online*, 50, 7-22.
- MURPHY, C. A., LANGRISH, C. L., CHEN, Y., BLUMENSCHN, W., MCCLANAHAN, T., KASTELEIN, R. A., SEDGWICK, J. D. & CUA, D. J. 2003. Divergent pro-and antiinflammatory roles for IL-23 and IL-12 in joint autoimmune inflammation. *The Journal of experimental medicine*, 198, 1951-1957.
- MURPHY, K. M. 2011. *Janeway's immunobiology*, Garland Science.
- MYGIND, N. D., AXELSSON, A., RUWALD, M. H., DALSGAARD, M., STEFFENSEN, R., IVERSEN, K., JOHANSEN, J. S. & KASTRUP, J. 2016. The inflammatory biomarker YKL-40 decreases stepwise after exercise stress test. *Cardiovascular Endocrinology*, 5, 21-27.
- MYLONAS, K. J., NAIR, M. G., PRIETO-LAFUENTE, L., PAAPE, D. & ALLEN, J. E. 2009. Alternatively activated macrophages elicited by helminth infection can be reprogrammed to enable microbial killing. *The Journal of immunology*, 182, 3084-3094.
- NAKAMURA, H., KOBAYASHI, S., OHASHI, Y. & ANDO, S. 1999. Age - changes of brain synapses and synaptic plasticity in response to an enriched environment. *Journal of neuroscience research*, 56, 307-315.
- NATOLI, G., GHISLETTI, S. & BAROZZI, I. 2011. The genomic landscapes of inflammation. *Genes & development*, 25, 101-106.
- NC3RS. 2017. *Housing and Husbandry of Rodents* [Online]. Available: <https://www.nc3rs.org.uk/3rs-resources/housing-and-husbandry/rodents> [Accessed Feb 12 2017].
- NEWBERRY, R. C. 1995. Environmental enrichment: increasing the biological relevance of captive environments. *Applied Animal Behaviour Science*, 44, 229-243.
- NGUYEN, H. B. & SMITH, D. 2007. Sepsis in the 21st century: recent definitions and therapeutic advances. *The American journal of emergency medicine*, 25, 564-571.

- NIBBS, R. J. & GRAHAM, G. J. 2013. Immune regulation by atypical chemokine receptors. *Nature Reviews Immunology*, 13, 815-829.
- NICHOLS, B. A., BANTON, D. F. & FARQUHAR, M. G. 1971. DIFFERENTIATION OF MONOCYTES Origin, Nature, and Fate of Their Azurophil Granules. *The Journal of cell biology*, 50, 498-515.
- NIELSEN, A. R., PLOMGAARD, P., KRABBE, K. S., JOHANSEN, J. S. & PEDERSEN, B. K. 2011. IL-6, but not TNF- $\alpha$ , increases plasma YKL-40 in human subjects. *Cytokine*, 55, 152-155.
- NIELSEN, H. & PEDERSEN, B. 1997. Lymphocyte proliferation in response to exercise. *European journal of applied physiology and occupational physiology*, 75, 375-379.
- NIEMAN, D., NEHLSSEN-CANNARELLA, S., MARKOFF, P., BALK-LAMBERTON, A., YANG, H., CHRITTON, D., LEE, J. & ARABATZIS, K. 1990. The effects of moderate exercise training on natural killer cells and acute upper respiratory tract infections. *International journal of sports medicine*, 11, 467-473.
- NIMMO, E. R., PRENDERGAST, J. G., ALDHOUS, M. C., KENNEDY, N. A., HENDERSON, P., DRUMMOND, H. E., RAMSAHOYE, B. H., WILSON, D. C., SEMPLE, C. A. & SATSANGI, J. 2012. Genome - wide methylation profiling in Crohn's disease identifies altered epigenetic regulation of key host defense mechanisms including the Th17 pathway. *Inflammatory bowel diseases*, 18, 889-899.
- NITHIANANTHARAJAH, J. & HANNAN, A. J. 2006. Enriched environments, experience-dependent plasticity and disorders of the nervous system. *Nature Reviews Neuroscience*, 7, 697-709.
- NSWAARRP 2004. Guidelines for the Housing of Rats in Scientific Institutions. 2 ed. New South Wales Agriculture Animal Research Review Panel
- NÚÑEZ, M. J., MAÑÁ, P., LINARES, D., RIVEIRO, M. A. P., BALBOA, J., SUÁREZ-QUINTANILLA, J., MARACCHI, M., MÉNDEZ, M. R., LÓPEZ, J. M. & FREIRE-GARABAL, M. 2002. Music, immunity and cancer. *Life Sciences*, 71, 1047-1057.
- NURDEN, A. T., NURDEN, P., SANCHEZ, M., ANDIA, I. & ANITUA, E. 2007. Platelets and wound healing. *Frontiers in bioscience: a journal and virtual library*, 13, 3532-3548.
- O'MAHONY, L., MCCARTHY, J., KELLY, P., HURLEY, G., LUO, F., CHEN, K., O'SULLIVAN, G. C., KIELY, B., COLLINS, J. K. & SHANAHAN, F. 2005. Lactobacillus and bifidobacterium in irritable bowel syndrome: symptom responses and relationship to cytokine profiles. *Gastroenterology*, 128, 541-551.
- OKASHAH, A., EL-SAWY, M., BESHAY, B. & ABD EL-RAOUF, A. 2014. Ratio Of Neutrophil To Lymphocyte Counts As A Simple Marker For Sepsis And Severe Sepsis In Intensive Care Unit.
- OKIMURA, T., OGAWA, M. & TOSHIHIKO, Y. 1986. Stress and immune responses. III. Effect of restraint stress on delayed type hypersensitivity (DTH) response, natural killer (NK) activity and phagocytosis in mice. *The Japanese Journal of Pharmacology*, 41, 229-235.
- OLIVEIRA, M. & GLEESON, M. 2010. The influence of prolonged cycling on monocyte Toll-like receptor 2 and 4 expression in healthy men. *European journal of applied physiology*, 109, 251-257.
- OLSON, A. K., EADIE, B. D., ERNST, C. & CHRISTIE, B. R. 2006. Environmental enrichment and voluntary exercise massively increase neurogenesis in the adult hippocampus via dissociable pathways. *Hippocampus*, 16, 250-260.
- OPDENAKKER, G., FROYEN, G., FITEN, P., PROOST, P. & VANDAMME, J. 1993. Human monocyte chemotactic protein-3 (MCP-3): molecular cloning of the cDNA and comparison with other chemokines. *Biochemical and biophysical research communications*, 191, 535-542.
- OPDENAKKER, G., VAN DEN STEEN, P. E., DUBOIS, B., NELISSEN, I., VAN COILLIE, E., MASURE, S., PROOST, P. & VAN DAMME, J. 2001. Gelatinase B functions as regulator and effector in leukocyte biology. *Journal of leukocyte biology*, 69, 851-859.
- ORTEGA - GÓMEZ, A., PERRETTI, M. & SOEHNLEIN, O. 2013. Resolution of inflammation: an integrated view. *EMBO molecular medicine*, 5, 661-674.
- ORTUZAR, N., RICO-BARRIO, I., BENGOTXEA, H., ARGANDOÑA, E. & LAFUENTE, J. 2013. VEGF reverts the cognitive impairment induced by a focal traumatic brain injury during the development of rats raised under environmental enrichment. *Behavioural brain research*, 246, 36-46.
- OSAKA, N., TAKAHASHI, T., MURAKAMI, S., MATSUZAWA, A., NOGUCHI, T., FUJIWARA, T., ABURATANI, H., MORIYAMA, K., TAKEDA, K. & ICHIJO, H. 2007. ASK1-dependent recruitment and activation of macrophages induce hair growth in skin wounds. *The Journal of cell biology*, 176, 903-909.
- OTSUKA, K., TERASAKI, F., IKEMOTO, M., FUJITA, S., TSUKADA, B., KATASHIMA, T., KANZAKI, Y., SOHMIYA, K., KONO, T. & TOKO, H. 2009. Suppression of inflammation in rat autoimmune myocarditis by S100A8/A9 through modulation of the proinflammatory cytokine network. *European journal of heart failure*, 11, 229-237.

- PACE, T. W., NEGI, L. T., ADAME, D. D., COLE, S. P., SIVILLI, T. I., BROWN, T. D., ISSA, M. J. & RAISON, C. L. 2009. Effect of compassion meditation on neuroendocrine, innate immune and behavioral responses to psychosocial stress. *Psychoneuroendocrinology*, 34, 87-98.
- PADGETT, D. A., MARUCHA, P. T. & SHERIDAN, J. F. 1998. Restraint stress slows cutaneous wound healing in mice. *Brain, behavior, and immunity*, 12, 64-73.
- PANINA-BORDIGNON, P., MAZZEO, D., LUCIA, P., D'AMBROSIO, D., LANG, R., FABBRI, L., SELF, C. & SINIGAGLIA, F. 1997. Beta2-agonists prevent Th1 development by selective inhibition of interleukin 12. *Journal of Clinical Investigation*, 100, 1513.
- PAPAYANNOPOULOS, V. & ZYCHLINSKY, A. 2009. NETs: a new strategy for using old weapons. *Trends in immunology*, 30, 513-521.
- PARKER, J. C., SMARR, K. L., BUCKELEW, S. P., STUCKY - ROPP, R. C., HEWETT, J. E., JOHNSON, J. C., WRIGHT, G. E., IRVIN, W. S. & WALKER, S. E. 1995. Effects of stress management on clinical outcomes in rheumatoid arthritis. *Arthritis & Rheumatism*, 38, 1807-1818.
- PEDERSEN, B. K. 2012. Muscular interleukin-6 and its role as an energy sensor. *Medicine and science in sports and exercise*, 44, 392-396.
- PERRETTI, M., CROXTALL, J. D., WHELLER, S. K., GOULDING, N. J., HANNON, R. & FLOWER, R. J. 1996. Mobilizing lipocortin 1 in adherent human leukocytes downregulates their transmigration. *Nature medicine*, 2, 1259-1262.
- PERRETTI, M. & GAVINS, F. N. 2003. Annexin 1: an endogenous anti-inflammatory protein. *Physiology*, 18, 60-64.
- PERRETTI, M., SOLITO, E. & PARENTE, L. 1992. Evidence that endogenous interleukin-1 is involved in leukocyte migration in acute experimental inflammation in rats and mice. *Agents and actions*, 35, 71-78.
- PESCE, J. T., RAMALINGAM, T. R., MENTINK-KANE, M. M., WILSON, M. S., EL KASMI, K. C., SMITH, A. M., THOMPSON, R. W., CHEEVER, A. W., MURRAY, P. J. & WYNN, T. A. 2009. Arginase-1-expressing macrophages suppress Th2 cytokine-driven inflammation and fibrosis. *PLoS Pathog*, 5, e1000371.
- PETERSEN, A. M. W. & PEDERSEN, B. K. 2005. The anti-inflammatory effect of exercise. *Journal of applied physiology*, 98, 1154-1162.
- PHILLIPS, S. A., BARR, V. A., HAFT, D. H., TAYLOR, S. I. & HAFT, C. R. 2001. Identification and characterization of SNX15, a novel sorting nexin involved in protein trafficking. *Journal of Biological Chemistry*, 276, 5074-5084.
- PILCHER, B. K., WANG, M., QIN, X. J., PARKS, W. C., SENIOR, R. M. & WELGUS, H. G. 1999. Role of matrix metalloproteinases and their inhibition in cutaneous wound healing and allergic contact hypersensitivity. *Annals of the New York Academy of Sciences*, 878, 12-24.
- PITSAVOS, C., PANAGIOTAKOS, D. B., PAPAGEORGIOU, C., TSETSEKOU, E., SOLDATOS, C. & STEFANADIS, C. 2006. Anxiety in relation to inflammation and coagulation markers, among healthy adults: the ATTICA study. *Atherosclerosis*, 185, 320-326.
- PONTING, C. P. 2008. The functional repertoires of metazoan genomes. *Nature Reviews Genetics*, 9, 689-698.
- POWELL, N. D., SLOAN, E. K., BAILEY, M. T., AREVALO, J. M., MILLER, G. E., CHEN, E., KOBOR, M. S., READER, B. F., SHERIDAN, J. F. & COLE, S. W. 2013. Social stress up-regulates inflammatory gene expression in the leukocyte transcriptome via  $\beta$ -adrenergic induction of myelopoiesis. *Proceedings of the National Academy of Sciences*, 110, 16574-16579.
- PRUETT, S. B., ENSLEY, D. K. & CRITTENDEN, P. L. 1993. The role of chemical - induced stress responses in immunosuppression: A review of quantitative associations and cause - effect relationships between chemical - induced stress responses and immunosuppression. *Journal of Toxicology and Environmental Health, Part A Current Issues*, 39, 163-192.
- PUPPE, B., TUCHSCHERER, M. & TUCHSCHERER, A. 1997. The effect of housing conditions and social environment immediately after weaning on the agonistic behaviour, neutrophil/lymphocyte ratio, and plasma glucose level in pigs. *Livestock Production Science*, 48, 157-164.
- QIAN, B.-Z., LI, J., ZHANG, H., KITAMURA, T., ZHANG, J., CAMPION, L. R., KAISER, E. A., SNYDER, L. A. & POLLARD, J. W. 2011. CCL2 recruits inflammatory monocytes to facilitate breast-tumour metastasis. *Nature*, 475, 222-225.
- RAISON, C. L. & MILLER, A. H. 2011. Is depression an inflammatory disorder? *Current psychiatry reports*, 13, 467-475.
- RAJAKARIAR, R., LAWRENCE, T., BYSTROM, J., HILLIARD, M., COLVILLE-NASH, P., BELLINGAN, G., FITZGERALD, D., YAQOOB, M. M. & GILROY, D. W. 2008. Novel biphasic role for lymphocytes revealed during resolving inflammation. *Blood*, 111, 4184-4192.

- RAKITA, R. M., MICHEL, B. R. & ROSEN, H. 1990. Differential inactivation of *Escherichia coli* membrane dehydrogenases by a myeloperoxidase-mediated antimicrobial system. *Biochemistry*, 29, 1075-1080.
- RAMASWAMY, H. & HEMLER, M. E. 1990. Cloning, primary structure and properties of a novel human integrin beta subunit. *The EMBO Journal*, 9, 1561.
- RAMPON, C., JIANG, C. H., DONG, H., TANG, Y.-P., LOCKHART, D. J., SCHULTZ, P. G., TSIEN, J. Z. & HU, Y. 2000a. Effects of environmental enrichment on gene expression in the brain. *Proceedings of the National Academy of Sciences*, 97, 12880-12884.
- RAMPON, C., TANG, Y.-P., GOODHOUSE, J., SHIMIZU, E., KYIN, M. & TSIEN, J. Z. 2000b. Enrichment induces structural changes and recovery from nonspatial memory deficits in CA1 NMDAR1-knockout mice. *Nature neuroscience*, 3, 238-244.
- RAO, T. S., CURRIE, J. L., SHAFFER, A. F. & ISAKSON, P. C. 1994. In vivo characterization of zymosan-induced mouse peritoneal inflammation. *Journal of Pharmacology and Experimental Therapeutics*, 269, 917-925.
- RATHER, L. 1971. Disturbance of function (functio laesa): the legendary fifth cardinal sign of inflammation, added by Galen to the four cardinal signs of Celsus. *Bulletin of the New York Academy of Medicine*, 47, 303.
- RAVICHANDRAN, K. S. 2011. Beginnings of a good apoptotic meal: the find-me and eat-me signaling pathways. *Immunity*, 35, 445-455.
- RAY, A. & DITTEL, B. N. 2010. Isolation of mouse peritoneal cavity cells. *JoVE (Journal of Visualized Experiments)*, e1488-e1488.
- REED, H., WILKINS, L., AUSTIN, S. & GREGORY, N. 1993. The effect of environmental enrichment during rearing on fear reactions and depopulation trauma in adult caged hens. *Applied Animal Behaviour Science*, 36, 39-46.
- REHLI, M., NILLER, H.-H., AMMON, C., LANGMANN, S., SCHWARZFISCHER, L., ANDREESSEN, R. & KRAUSE, S. W. 2003. Transcriptional regulation of CHI3L1, a marker gene for late stages of macrophage differentiation. *Journal of Biological Chemistry*, 278, 44058-44067.
- REHMAN, J., LI, J., PARVATHANENI, L., KARLSSON, G., PANCHAL, V. R., TEMM, C. J., MAHENTHIRAN, J. & MARCH, K. L. 2004. Exercise acutely increases circulating endothelial progenitor cells and monocyte-/macrophage-derived angiogenic cells. *Journal of the American College of Cardiology*, 43, 2314-2318.
- REICHMANN, F., PAINSIPP, E. & HOLZER, P. 2013. Environmental enrichment and gut inflammation modify stress-induced c-Fos expression in the mouse corticolimbic system. *PloS one*, 8, e54811.
- REYES, M., QUINTANILLA, C., BURROWS, R., BLANCO, E., CIFUENTES, M. & GAHAGAN, S. 2015. Obesity is associated with acute inflammation in a sample of adolescents. *Pediatric diabetes*, 16, 109-116.
- RIBECHINI, E., GREIFENBERG, V., SANDWICK, S. & LUTZ, M. B. 2010. Subsets, expansion and activation of myeloid-derived suppressor cells. *Medical microbiology and immunology*, 199, 273-281.
- RIEDEMANN, N. C., NEFF, T. A., GUO, R.-F., BERNACKI, K. D., LAUDES, I. J., SARMA, J. V., LAMBRIS, J. D. & WARD, P. A. 2003. Protective effects of IL-6 blockade in sepsis are linked to reduced C5a receptor expression. *The Journal of Immunology*, 170, 503-507.
- RIVA, M., KÄLLBERG, E., BJÖRK, P., HANCZ, D., VOGL, T., ROTH, J., IVARS, F. & LEANDERSON, T. 2012. Induction of nuclear factor -  $\kappa$ B responses by the S100A9 protein is Toll - like receptor - 4 - dependent. *Immunology*, 137, 172-182.
- ROBERTS, A. W. 2005. G-CSF: a key regulator of neutrophil production, but that's not all! *Growth factors*, 23, 33-41.
- ROBERTS, J. E. 2000. Light and immunomodulation. *Annals of the New York Academy of Sciences*, 917, 435-445.
- ROCA, L., DI PAOLO, S., PETRUZZELLI, V., GRANDALIANO, G., RANIERI, E., SCHENA, F. P. & GESUALDO, L. 2007. Dexamethasone modulates interleukin-12 production by inducing monocyte chemoattractant protein-1 in human dendritic cells. *Immunology and cell biology*, 85, 610-616.
- ROEDERER, M., QUAYE, L., MANGINO, M., BEDDALL, M. H., MAHNKE, Y., CHATTOPADHYAY, P., TOSI, I., NAPOLITANO, L., BARBERIO, M. T. & MENNI, C. 2015. The Genetic Architecture of the Human Immune System: A Bioresource for Autoimmunity and Disease Pathogenesis. *Cell*.
- ROGERS, M. P., TRENTHAM, D. E., MCCUNE, W. J., GINSBERG, B. I., RENNKE, H. G., REICH, P. & DAVID, J. R. 1980. Effect of psychological stress on the induction of arthritis in rats. *Arthritis & Rheumatism*, 23, 1337-1342.

- ROPER, J., CRAIGHEAD, M., O'CARROLL, A. M. & LOLAIT, S. 2010. Attenuated Stress Response to Acute Restraint and Forced Swimming Stress in Arginine Vasopressin 1b Receptor Subtype (Avpr1b) Receptor Knockout Mice and Wild - Type Mice Treated with a Novel Avpr1b Receptor Antagonist. *Journal of neuroendocrinology*, 22, 1173-1180.
- ROSE, S., MISHARIN, A. & PERLMAN, H. 2012. A novel Ly6C/Ly6G - based strategy to analyze the mouse splenic myeloid compartment. *Cytometry Part A*, 81, 343-350.
- ROSENKRANZ, M. A., DAVIDSON, R. J., MACCOON, D. G., SHERIDAN, J. F., KALIN, N. H. & LUTZ, A. 2013. A comparison of mindfulness-based stress reduction and an active control in modulation of neurogenic inflammation. *Brain, Behavior, and Immunity*, 27, 174-184.
- ROSENNE, E., SORSKI, L., SHAASHUA, L., NEEMAN, E., MATZNER, P., LEVI, B. & BEN-ELIYAHU, S. 2014. In vivo suppression of NK cell cytotoxicity by stress and surgery: glucocorticoids have a minor role compared to catecholamines and prostaglandins. *Brain, behavior, and immunity*, 37, 207-219.
- RØSETH, A., AADLAND, E., JAHNSEN, J. & RAKNERUD, N. 1997. Assessment of disease activity in ulcerative colitis by faecal calprotectin, a novel granulocyte marker protein. *Digestion*, 58, 176-180.
- RÖSZER, T. 2015. Understanding the mysterious M2 macrophage through activation markers and effector mechanisms. *Mediators of inflammation*, 2015.
- ROTH, J. A. 1985. Cortisol as mediator of stress-associated immunosuppression in cattle. *Animal stress*. Springer.
- RUFF, M., SCHIFFMANN, E., TERRANOVA, V. & PERT, C. B. 1985. Neuropeptides are chemoattractants for human tumor cells and monocytes: a possible mechanism for metastasis. *Clinical immunology and immunopathology*, 37, 387-396.
- RUIS, M., TE BRAKE, J., BUWALDA, B., DE BOER, S., MEERLO, P., KORTE, S., BLOKHUIS, H. & KOOLHAAS, J. 1999. Housing familiar male wildtype rats together reduces the long-term adverse behavioural and physiological effects of social defeat. *Psychoneuroendocrinology*, 24, 285-300.
- SAAVEDRA, J. M., BAUMAN, N., PERMAN, J., YOLKEN, R. & OUNG, I. 1994. Feeding of Bifidobacterium bifidum and Streptococcus thermophilus to infants in hospital for prevention of diarrhoea and shedding of rotavirus. *The Lancet*, 344, 1046-1049.
- SALAS-VIDAL, E., MEIJER, A. H., CHENG, X. & SPAINK, H. P. 2005. Genomic annotation and expression analysis of the zebrafish Rho small GTPase family during development and bacterial infection. *Genomics*, 86, 25-37.
- SALE, A., BERARDI, N. & MAFFEI, L. 2009. Enrich the environment to empower the brain. *Trends in neurosciences*, 32, 233-239.
- SCHALLER, M., MILLER, G. E., GERVAIS, W. M., YAGER, S. & CHEN, E. 2010. Mere visual perception of other people's disease symptoms facilitates a more aggressive immune response. *Psychological Science*.
- SCHAUER, C., JANKO, C., MUNOZ, L. E., ZHAO, Y., KIENHÖFER, D., FREY, B., LELL, M., MANGER, B., RECH, J. & NASCHBERGER, E. 2014. Aggregated neutrophil extracellular traps limit inflammation by degrading cytokines and chemokines. *Nature medicine*, 20, 511-517.
- SCHIOPU, A. & COTOI, O. S. 2013. S100A8 and S100A9: DAMPs at the crossroads between innate immunity, traditional risk factors, and cardiovascular disease. *Mediators of inflammation*, 2013.
- SCHMALTZ, H. N., FRIED, L. P., XUE, Q. L., WALSTON, J., LENG, S. X. & SEMBA, R. D. 2005. Chronic cytomegalovirus infection and inflammation are associated with prevalent frailty in community - dwelling older women. *Journal of the American Geriatrics Society*, 53, 747-754.
- SCHNEIDER, M. L., MOORE, C. F., SUOMI, S. J. & CHAMPOUX, M. 1991. Laboratory assessment of temperament and environmental enrichment in rhesus monkey infants (Macaca mulatta). *American Journal of Primatology*, 25, 137-155.
- SCHWAB, J. M., CHIANG, N., ARITA, M. & SERHAN, C. N. 2007. Resolvin E1 and protectin D1 activate inflammation-resolution programmes. *Nature*, 447, 869-874.
- SCHWARTZ, R., LU, Y., VILLINES, D. & SROUSSI, H. Y. 2010. Effect of human immunodeficiency virus infection on S100A8/A9 inhibition of peripheral neutrophils oxidative metabolism. *Biomedicine & Pharmacotherapy*, 64, 572-575.
- SCOTLAND, R. S., STABLES, M. J., MADALLI, S., WATSON, P. & GILROY, D. W. 2011. Sex differences in resident immune cell phenotype underlie more efficient acute inflammatory responses in female mice. *Blood*, 118, 5918-5927.
- SCOTT, A., KHAN, K., COOK, J. & DURONIO, V. 2004. What is "inflammation"? Are we ready to move beyond Celsus? *British journal of sports medicine*, 38, 248-249.



- SEGAL, A. W., DORLING, J. & COADE, S. 1980. Kinetics of fusion of the cytoplasmic granules with phagocytic vacuoles in human polymorphonuclear leukocytes. *Biochemical and morphological studies. The Journal of cell biology*, 85, 42-59.
- SEGERSTROM, S. C. & MILLER, G. E. 2004. Psychological stress and the human immune system: a meta-analytic study of 30 years of inquiry. *Psychological bulletin*, 130, 601.
- SEIFFERT, M., BROSSART, P., CANT, C., CELLA, M., COLONNA, M., BRUGGER, W., KANZ, L., ULLRICH, A. & BÜHRING, H.-J. 2001. Signal-regulatory protein  $\alpha$  (SIRP $\alpha$ ) but not SIRP $\beta$  is involved in T-cell activation, binds to CD47 with high affinity, and is expressed on immature CD34+ CD38- hematopoietic cells. *Blood*, 97, 2741-2749.
- SEITZ, O., SCHÜRMANN, C., HERMES, N., MÜLLER, E., PFEILSCHIFTER, J., FRANK, S. & GOREN, I. 2011. Wound healing in mice with high-fat diet-or ob gene-induced diabetes-obesity syndromes: a comparative study. *Experimental diabetes research*, 2010.
- SERBINA, N. V., JIA, T., HOHL, T. M. & PAMER, E. G. 2008. Monocyte-mediated defense against microbial pathogens. *Annual review of immunology*, 26, 421.
- SERHAN, C. N., CHIANG, N. & VAN DYKE, T. E. 2008. Resolving inflammation: dual anti-inflammatory and pro-resolution lipid mediators. *Nature Reviews Immunology*, 8, 349-361.
- SERHAN, C. N. & SAVILL, J. 2005. Resolution of inflammation: the beginning programs the end. *Nature immunology*, 6, 1191-1197.
- SETHI, S., WRONA, C., GRANT, B. J. & MURPHY, T. F. 2004. Strain-specific immune response to Haemophilus influenzae in chronic obstructive pulmonary disease. *American journal of respiratory and critical care medicine*, 169, 448-453.
- SHERMAN, V. R., YANG, W. & MEYERS, M. A. 2015. The materials science of collagen. *Journal of the mechanical behavior of biomedical materials*, 52, 22-50.
- SHERWIN, C. 2007a. Validating refinements to laboratory housing: asking the animals. *NCR3RS (database online)*. Available from <http://www.nc3rs.org.uk/downloadaddoc.asp>.
- SHERWIN, C. M. 2002. Comfortable quarters for mice in research institutions. *Comfortable quarters for laboratory animals*, 9, 6-17.
- SHERWIN, C. M. 2007b. Validating refinements to laboratory housing: asking the animals. *National centre for replacement, refinement and reduction of animals in research*.
- SHI, C. & PAMER, E. G. 2011. Monocyte recruitment during infection and inflammation. *Nature Reviews Immunology*, 11, 762-774.
- SHI, Z., WAKIL, A. E. & ROCKEY, D. C. 1997. Strain-specific differences in mouse hepatic wound healing are mediated by divergent T helper cytokine responses. *Proceedings of the National Academy of Sciences*, 94, 10663-10668.
- SHIMIZU, K., LIBBY, P., ROCHA, V. Z., FOLCO, E. J., SHUBIKI, R., GRABIE, N., JANG, S., LICHTMAN, A. H., SHIMIZU, A. & HOGG, N. 2011. Loss of myeloid related protein-8/14 exacerbates cardiac allograft rejection. *Circulation*, 124, 2920-2932.
- SHUTT, D., SMITH, A., WALLACE, C., CONNELL, R. & FELL, L. 1988. Effect of Myiasis and Acute Restraint Stress on Plasma Levels of Immunoreactive,  $\beta$ -Endorphin, Adrenocorticotrophin (ACTH) and Cortisol in the Sheep. *Australian journal of biological sciences*, 41, 297-302.
- SIEDLIK, J. A., DECKERT, J. A., CLOPTON, A. W., GIGLIOTTI, N., CHAN, M. A., BENEDICT, S. H., HERDA, T. J., GALLAGHER, P. M. & VARDIMAN, J. P. 2015. Immunoendocrine alterations following marine corps martial arts training are associated with changes in moral cognitive processes. *Physiology & behavior*.
- SINGH, P. B., BROWN, R. E. & ROSER, B. 1987. MHC antigens in urine as olfactory recognition cues.
- SINGHAL, G., JAEHNE, E. J., CORRIGAN, F. & BAUNE, B. T. 2014. Cellular and molecular mechanisms of immunomodulation in the brain through environmental enrichment. *Frontiers in Cellular Neuroscience*, 8.
- SINGLETON, K. & WISCHMEYER, P. 2003. Distance of cecum ligated influences mortality, tumor necrosis factor-alpha and interleukin-6 expression following cecal ligation and puncture in the rat. *European surgical research*, 35, 486-491.
- SLATER, A. M. & CAO, L. 2015. A protocol for housing mice in an enriched environment. *Journal of visualized experiments: JoVE*.
- SLUYTER, F., GERLAI, R. T. & PIETROPAOLO, S. 2013. Behavioral Genetics of the Mouse. *Autism*, 1, 13.
- SMITH, D. F., GALKINA, E., LEY, K. & HUO, Y. 2005. GRO family chemokines are specialized for monocyte arrest from flow. *American Journal of Physiology-Heart and Circulatory Physiology*, 289, H1976-H1984.
- SMITH, J., MCKENZIE, S., TELFORD, R. & WEIDEMANN, M. 1992. Why does moderate exercise enhance, but intense training depress, immunity. *Behavior and Immunity*, 155-168.
- SMITH, J. A., GRAY, A. B., PYNE, D. B., BAKER, M. S., TELFORD, R. D. & WEIDEMANN, M. J. 1996. Moderate exercise triggers both priming and activation of neutrophil subpopulations.



- American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*, 270, R838-R845.
- SMITH, M. M. & HARGADEN, M. 2001. Developing a rodent enrichment program. *Lab animal*, 30, 36-41.
- SO, B., KIM, H.-J., KIM, J. & SONG, W. 2014. Exercise-induced myokines in health and metabolic diseases. *Integrative Medicine Research*, 3, 172-179.
- SOEHNLEIN, O., LINDBOM, L. & WEBER, C. 2009. Mechanisms underlying neutrophil-mediated monocyte recruitment. *Blood*, 114, 4613-4623.
- SOFI, F., CESARI, F., ABBATE, R., GENSINI, G. F. & CASINI, A. 2008. Adherence to Mediterranean diet and health status: meta-analysis. *Bmj*, 337.
- SOKOL, H., PIGNEUR, B., WATTERLOT, L., LAKHDARI, O., BERMÚDEZ-HUMARÁN, L. G., GRATADOUX, J.-J., BLUGEON, S., BRIDONNEAU, C., FURET, J.-P. & CORTIER, G. 2008. Faecalibacterium prausnitzii is an anti-inflammatory commensal bacterium identified by gut microbiota analysis of Crohn disease patients. *Proceedings of the National Academy of Sciences*, 105, 16731-16736.
- SONG, J., WU, C., ZHANG, X. & SOROKIN, L. M. 2013. In Vivo Processing of CXCL5 (LIX) by Matrix Metalloproteinase (MMP)-2 and MMP-9 Promotes Early Neutrophil Recruitment in IL-1 $\beta$ -Induced Peritonitis. *The Journal of Immunology*, 190, 401-410.
- SOROKIN, L. 2010. The impact of the extracellular matrix on inflammation. *Nature Reviews Immunology*, 10, 712-723.
- SOUZA, L. M., BOONE, T. C., GABRILOVE, J., LAI, P. H., ZSEBO, K. M., MURDOCK, D. C., CHAZIN, V. R., BRUSZEWSKI, J., LU, H. & CHEN, K. K. 1986. Recombinant human granulocyte colony-stimulating factor: effects on normal and leukemic myeloid cells. *Science*, 232, 61-65.
- STACKER, S. A., WILLIAMS, S. P., KARNEZIS, T., SHAYAN, R., FOX, S. B. & ACHEN, M. G. 2014. Lymphangiogenesis and lymphatic vessel remodelling in cancer. *Nature Reviews Cancer*, 14, 159-172.
- STARKIE, R., ANGUS, D., ROLLAND, J., HARGREAVES, M. & FEBBRAIO, M. 2000. Effect of prolonged, submaximal exercise and carbohydrate ingestion on monocyte intracellular cytokine production in humans. *The Journal of physiology*, 528, 647-655.
- STARKIE, R., OSTROWSKI, S. R., JAUFFRED, S., FEBBRAIO, M. & PEDERSEN, B. K. 2003. Exercise and IL-6 infusion inhibit endotoxin-induced TNF- $\alpha$  production in humans. *The FASEB Journal*, 17, 884-886.
- STEFANSKI, V. & ENGLER, H. 1998. Effects of acute and chronic social stress on blood cellular immunity in rats. *Physiology & behavior*, 64, 733-741.
- STEINMÜLLER, M., SRIVASTAVA, M., KUZIEL, W. A., CHRISTMAN, J. W., SEEGER, W., WELTE, T., LOHMEYER, J. & MAUS, U. A. 2006. Endotoxin induced peritonitis elicits monocyte immigration into the lung: implications on alveolar space inflammatory responsiveness. *Respiratory research*, 7, 1.
- STOUT, R. D., JIANG, C., MATTA, B., TIETZEL, I., WATKINS, S. K. & SUTTLES, J. 2005. Macrophages sequentially change their functional phenotype in response to changes in microenvironmental influences. *The Journal of Immunology*, 175, 342-349.
- STRAUCH, A. R. & RUBENSTEIN, P. 1984. A vascular smooth muscle alpha-isoactin biosynthetic intermediate in BC3H1 cells. Identification of acetylcysteine at the NH2 terminus. *Journal of Biological Chemistry*, 259, 7224-7229.
- STRÍŽ, I. & TREBICHAŤSKÝ, I. 2004. Calprotectin—a pleiotropic molecule in acute and chronic inflammation. *Physiol Res*, 53, 245-253.
- STROUS, R. D. & SHOENFELD, Y. 2006. To smell the immune system: olfaction, autoimmunity and brain involvement. *Autoimmunity reviews*, 6, 54-60.
- SUGIMOTO, M. A., SOUSA, L. P., PINHO, V., PERRETTI, M. & TEIXEIRA, M. M. 2016. Resolution of inflammation: what Controls its Onset? *Frontiers in immunology*, 7.
- SUN, Y., LU, Y., ENGELAND, C. G., GORDON, S. C. & SROUSSI, H. Y. 2013. The anti-oxidative, anti-inflammatory, and protective effect of S100A8 in endotoxemic mice. *Molecular immunology*, 53, 443-449.
- SUZUKI, K., NAKAJI, S., YAMADA, M., TOTSUKA, M., SATO, K. & SUGAWARA, K. 2002. Systemic inflammatory response to exhaustive exercise: cytokine kinetics. *Exerc Immunol Rev*, 8, 6-48.
- SWAIN, S. D., ROHN, T. T. & QUINN, M. T. 2002. Neutrophil priming in host defense: role of oxidants as priming agents. *Antioxidants and Redox Signaling*, 4, 69-83.
- SWAN, M. P. & HICKMAN, D. L. 2014. Evaluation of the neutrophil-lymphocyte ratio as a measure of distress in rats. *Lab animal*, 43, 276.
- SZTAINBERG, Y. & CHEN, A. 2010. An environmental enrichment model for mice. *Nature protocols*, 5, 1535-1539.

- TAK, T., TESSELAAR, K., PILLAY, J., BORGHANS, J. A. & KOENDERMAN, L. 2013. What's your age again? Determination of human neutrophil half-lives revisited. *Journal of leukocyte biology*, 94, 595-601.
- TAKAHASHI, K. 2001. Development and Differentiation of Macrophages and Related Cells. *Journal of clinical and experimental hematopathology*, 41, 1-31.
- TAKEUCHI, O. & AKIRA, S. 2010. Pattern recognition receptors and inflammation. *Cell*, 140, 805-820.
- TAM, E. M., MORRISON, C. J., WU, Y. I., STACK, M. S. & OVERALL, C. M. 2004. Membrane protease proteomics: Isotope-coded affinity tag MS identification of undescribed MT1-matrix metalloproteinase substrates. *Proceedings of the National Academy of Sciences of the United States of America*, 101, 6917-6922.
- TAYLOR, P. R., BROWN, G. D., REID, D. M., WILLMENT, J. A., MARTINEZ-POMARES, L., GORDON, S. & WONG, S. Y. 2002. The  $\beta$ -glucan receptor, dectin-1, is predominantly expressed on the surface of cells of the monocyte/macrophage and neutrophil lineages. *The Journal of immunology*, 169, 3876-3882.
- THEILGAARD-MÖNCH, K., KNUDSEN, S., FOLLIN, P. & BORREGAARD, N. 2004. The transcriptional activation program of human neutrophils in skin lesions supports their important role in wound healing. *The Journal of Immunology*, 172, 7684-7693.
- THORSBY, E. 1997. Invited anniversary review: HLA associated diseases. *Human immunology*, 53, 1-11.
- TIKELLIS, C., THOMAS, M. C., HARCOURT, B. E., COUGHLAN, M. T., PETE, J., BIALKOWSKI, K., TAN, A., BIERHAUS, A., COOPER, M. E. & FORBES, J. M. 2008. Cardiac inflammation associated with a Western diet is mediated via activation of RAGE by AGEs. *American Journal of Physiology-Endocrinology and Metabolism*, 295, E323-E330.
- TIMMERMAN, K. L., FLYNN, M. G., COEN, P. M., MARKOFSKI, M. M. & PENCE, B. D. 2008. Exercise training-induced lowering of inflammatory (CD14+ CD16+) monocytes: a role in the anti-inflammatory influence of exercise? *Journal of leukocyte biology*, 84, 1271-1278.
- TONNESEN, M. G., FENG, X. & CLARK, R. A. Angiogenesis in wound healing. *Journal of Investigative Dermatology Symposium Proceedings*, 2000. Nature Publishing Group, 40-46.
- TONO-OKA, S., TANASE, S., MIIKE, T. & TANAKA, H. 1996. Transient expression of collagen type XIV during muscle development and its reappearance after denervation and degeneration. *Journal of Histochemistry & Cytochemistry*, 44, 907-918.
- TOSCANO, M. G., GANEA, D. & GAMERO, A. M. 2011. Cecal ligation puncture procedure. *JoVE (Journal of Visualized Experiments)*, e2860-e2860.
- TOUMA, C., SACHSER, N., MÖSTL, E. & PALME, R. 2003. Effects of sex and time of day on metabolism and excretion of corticosterone in urine and feces of mice. *General and comparative endocrinology*, 130, 267-278.
- TRØSTRUP, H., LUNDQUIST, R., CHRISTENSEN, L., JORGENSEN, L., KARLSMARK, T., HAAB, B. & ÅGREN, M. 2011. S100A8/A9 deficiency in nonhealing venous leg ulcers uncovered by multiplexed antibody microarray profiling. *British Journal of Dermatology*, 165, 292-301.
- TRUMAN, L. A., FORD, C. A., PASIKOWSKA, M., POUND, J. D., WILKINSON, S. J., DUMITRIU, I. E., MELVILLE, L., MELROSE, L. A., OGDEN, C. A. & NIBBS, R. 2008. CX3CL1/fractalkine is released from apoptotic lymphocytes to stimulate macrophage chemotaxis. *Blood*, 112, 5026-5036.
- TSOU, C.-L., PETERS, W., SI, Y., SLAYMAKER, S., ASLANIAN, A. M., WEISBERG, S. P., MACK, M. & CHARO, I. F. 2007. Critical roles for CCR2 and MCP-3 in monocyte mobilization from bone marrow and recruitment to inflammatory sites. *The Journal of clinical investigation*, 117, 902-909.
- UNDERHILL, D. M. 2003. Macrophage recognition of zymosan particles. *Journal of endotoxin research*, 9, 176-180.
- UNDERHILL, D. M., OZINSKY, A., HAJJAR, A. M., STEVENS, A., WILSON, C. B., BASSETTI, M. & ADEREM, A. 1999. The Toll-like receptor 2 is recruited to macrophage phagosomes and discriminates between pathogens. *Nature*, 401, 811-815.
- VACHHARAJANI, V., RUSSELL, J. M., SCOTT, K. L., CONRAD, S., STOKES, K. Y., TALLAM, L., HALL, J. & GRANGER, D. N. 2005. Obesity exacerbates sepsis-induced inflammation and microvascular dysfunction in mouse brain. *Microcirculation*, 12, 183-194.
- VALENZUELA, M. & SACHDEV, P. 2009. Can cognitive exercise prevent the onset of dementia? Systematic review of randomized clinical trials with longitudinal follow-up. *The American Journal of Geriatric Psychiatry*, 17, 179-187.
- VALLÈS, A., MARTENS, G. J., DE WEERD, P., POELMANS, G. & ASCHRAFI, A. 2014. MicroRNA-137 regulates a glucocorticoid receptor-dependent signalling network: implications for the etiology of schizophrenia. *Journal of psychiatry & neuroscience: JPN*, 39, 312.

- VAN DE WEERD, H., BAUMANS, V., KOOLHAAS, J. & VAN ZUTPHEN, L. 1994. Strain specific behavioural response to environmental enrichment in the mouse. *Journal of experimental animal science*, 36, 117-117.
- VAN DE WEERD, H., VAN LOO, P., VAN ZUTPHEN, L., KOOLHAAS, J. & BAUMANS, V. 1998. Strength of preference for nesting material as environmental enrichment for laboratory mice. *Applied Animal Behaviour Science*, 55, 369-382.
- VAN DE WEERD, H. A. & BAUMANS, V. 1995. Environmental enrichment in rodents. *Environmental Enrichment Information Resources for Laboratory Animals. AWIC Resource Series*, 145-9.
- VAN DER MEER, J., NETEA, M. & DINARELLO, C. 2009. Modulation of muramyl dipeptide stimulation of cytokine production by blood components. *Clinical & Experimental Immunology*, 156, 428-433.
- VAN FURTH, R. & COHN, Z. A. 1968. The origin and kinetics of mononuclear phagocytes. *The Journal of experimental medicine*, 128, 415-435.
- VAN LOO, P. L., DE GROOT, A. C., VAN ZUTPHEN, B. F. & BAUMANS, V. 2001. Do male mice prefer or avoid each other's company? Influence of hierarchy, kinship, and familiarity. *Journal of Applied Animal Welfare Science*, 4, 91-103.
- VAN LOO, P. L., VAN DE WEERD, H. A., VAN ZUTPHEN, L. F. & BAUMANS, V. 2004. Preference for social contact versus environmental enrichment in male laboratory mice. *Laboratory Animals* 38, 178-88.
- VAN OOSTEN, M. J., DOLHAIN, R. J., KOPER, J. W., VAN ROSSUM, E. F., EMONTS, M., HAN, K. H., WOUTERS, J. M., HAZES, J. M., LAMBERTS, S. W. & FEELDERS, R. A. 2010. Polymorphisms in the glucocorticoid receptor gene that modulate glucocorticoid sensitivity are associated with rheumatoid arthritis. *Arthritis research & therapy*, 12, 1.
- VAN WOLFSWINKEL, M. E., Vliegenthart-Jongbloed, K., DE MENDONÇA MELO, M., WEVER, P. C., MCCALL, M. B., KOELEWIJN, R., VAN HELLEMOND, J. J. & VAN GENDEREN, P. J. 2013. Predictive value of lymphocytopenia and the neutrophil-lymphocyte count ratio for severe imported malaria. *Malaria journal*, 12, 1.
- VARGAS-PÉREZ, H., SELLINGS, L., GRIEDER, T. & DÍAZ, J.-L. 2009. Social dominance rank influences wheel running behavior in mice. *Neuroscience letters*, 457, 137-140.
- VERBURG-VAN KEMENADE, B. M., COHEN, N. & CHADZINSKA, M. 2017. Neuroendocrine-immune interaction: Evolutionarily conserved mechanisms that maintain allostasis in an ever-changing environment. *Dev Comp Immunol*, 66, 2-23.
- VIEIRA, O. V., BOTELHO, R. J. & GRINSTEIN, S. 2002. Phagosome maturation: aging gracefully. *Biochemical Journal*, 366, 689-704.
- VIEIRA, V. J., VALENTINE, R. J., WILUND, K. R., ANTÃO, N., BAYNARD, T. & WOODS, J. A. 2009. Effects of exercise and low-fat diet on adipose tissue inflammation and metabolic complications in obese mice. *American Journal of Physiology-Endocrinology and Metabolism*, 296, E1164-E1171.
- VISWANATHAN, K., DAUGHERTY, C. & DHABHAR, F. S. 2005. Stress as an endogenous adjuvant: augmentation of the immunization phase of cell-mediated immunity. *International immunology*, 17, 1059-1069.
- VISWANATHAN, K. & DHABHAR, F. S. 2005. Stress-induced enhancement of leukocyte trafficking into sites of surgery or immune activation. *Proceedings of the National Academy of Sciences of the United States of America*, 102, 5808-5813.
- VITALO, A. G., GORANTLA, S., FRICCHIONE, J. G., SCICHLONE, J. M., CAMACHO, J., NIEMI, S. M., DENNINGER, J. W., BENSON, H., YARMUSH, M. L. & LEVINE, J. B. 2012. Environmental enrichment with nesting material accelerates wound healing in isolation-reared rats. *Behavioural brain research*, 226, 606-612.
- VODOVOTZ, Y. 2006. Deciphering the complexity of acute inflammation using mathematical models. *Immunologic research*, 36, 237-245.
- VOGELZANGS, N., BEEKMAN, A., DE JONGE, P. & PENNINX, B. 2013. Anxiety disorders and inflammation in a large adult cohort. *Translational psychiatry*, 3, e249.
- VOGL, T., TENBROCK, K., LUDWIG, S., LEUKERT, N., EHRHARDT, C., VAN ZOELLEN, M. A., NACKEN, W., FOELL, D., VAN DER POLL, T. & SORG, C. 2007. Mrp8 and Mrp14 are endogenous activators of Toll-like receptor 4, promoting lethal, endotoxin-induced shock. *Nature medicine*, 13, 1042-1049.
- VOLCK, B., PRICE, P. A., JOHANSEN, J. S., SØRENSEN, O., BENFIELD, T. L., NIELSEN, H. J., CALAFAT, J. & BORREGAARD, N. 1997. YKL-40, a mammalian member of the chitinase family, is a matrix protein of specific granules in human neutrophils. *Proceedings of the Association of American Physicians*, 110, 351-360.
- WANG, J., SONG, H., TANG, X., YANG, Y., VIEIRA, V., NIU, Y. & MA, Y. 2012. Effect of exercise training intensity on murine T - regulatory cells and vaccination response. *Scandinavian journal of medicine & science in sports*, 22, 643-652.

- WANG, M., MOLIN, G., AHRNÉ, S., ADAWI, D. & JEPSSON, B. 2007. High proportions of proinflammatory bacteria on the colonic mucosa in a young patient with ulcerative colitis as revealed by cloning and sequencing of 16S rRNA genes. *Digestive diseases and sciences*, 52, 620-627.
- WANTHA, S., ALARD, J.-E., MEGENS, R. T., VAN DER DOES, A. M., DÖRING, Y., DRECHSLER, M., PHAM, C. T., WANG, M.-W., WANG, J.-M. & GALLO, R. L. 2013. Neutrophil-derived cathelicidin promotes adhesion of classical monocytes. *Circulation research*, 112, 792-801.
- WARD, P. A. & BOSMANN, M. 2012. A historical perspective on sepsis. *The American journal of pathology*, 181, 2-7.
- WEBER, C., WEBER, K. S., KLIER, C., GU, S., WANK, R., HORUK, R. & NELSON, P. J. 2001. Specialized roles of the chemokine receptors CCR1 and CCR5 in the recruitment of monocytes and TH1-like/CD45RO+ T cells. *Blood*, 97, 1144-1146.
- WEIGHARDT, H., KAISER-MOORE, S., SCHLAUTKÖTTER, S., ROSSMANN-BLOECK, T., SCHLEICHER, U., BOGDAN, C. & HOLZMANN, B. 2006. Type I IFN modulates host defense and late hyperinflammation in septic peritonitis. *The Journal of Immunology*, 177, 5623-5630.
- WEINER 3RD, J., PARIDA, S. K., MAERTZDORF, J., BLACK, G. F., REPSILBER, D., TELAAR, A., MOHNEY, R. P., ARNDT-SULLIVAN, C., GANOZA, C. A. & FAÉ, K. C. 2012. Biomarkers of inflammation, immunosuppression and stress with active disease are revealed by metabolomic profiling of tuberculosis patients. *PloS one*, 7, e40221.
- WEISSER, S. B., MCLARREN, K. W., KURODA, E. & SLY, L. M. 2013. Generation and characterization of murine alternatively activated macrophages. *Basic Cell Culture Protocols*, 225-239.
- WELLS, D. L. 2009. Sensory stimulation as environmental enrichment for captive animals: a review. *Applied Animal Behaviour Science*, 118, 1-11.
- WELLS, H. G. & JOHNSTONE, O. 1907. On the route of absorption of bacteria from the peritoneal cavity. *Journal of Infectious Diseases*, 4, 582-594.
- WICHTERMAN, K. A., BAUE, A. E. & CHAUDRY, I. H. 1980. Sepsis and septic shock—a review of laboratory models and a proposal. *Journal of Surgical Research*, 29, 189-201.
- WIKBY, A., NILSSON, B.-O., FORSEY, R., THOMPSON, J., STRINDHALL, J., LÖFGREN, S., ERNERUDH, J., PAWELEC, G., FERGUSON, F. & JOHANSSON, B. 2006. The immune risk phenotype is associated with IL-6 in the terminal decline stage: findings from the Swedish NINA immune longitudinal study of very late life functioning. *Mechanisms of ageing and development*, 127, 695-704.
- WILLIAMS, R. 2006. Killing controversy. *The Journal of experimental medicine*, 203, 2404.
- WILLIAMSON, L. L., CHAO, A. & BILBO, S. D. 2012. Environmental enrichment alters glial antigen expression and neuroimmune function in the adult rat hippocampus. *Brain, behavior, and immunity*, 26, 500-510.
- WILSON, C. L., OUELLETTE, A. J., SATCHELL, D. P., AYABE, T., LÓPEZ-BOADO, Y. S., STRATMAN, J. L., HULTGREN, S. J., MATRISIAN, L. M. & PARKS, W. C. 1999. Regulation of intestinal  $\alpha$ -defensin activation by the metalloproteinase matrilysin in innate host defense. *Science*, 286, 113-117.
- WILSON, E., LESZCZYNSKA, K., POULTER, N. S., EDELMANN, F., SALISBURY, V. A., NOY, P. J., BACON, A., RAPPOPORT, J. Z., HEATH, J. K. & BICKNELL, R. 2014. RhoJ interacts with the GIT-PIX complex and regulates focal adhesion disassembly. *Journal of cell science*, 127, 3039-3051.
- WILSON, J. A. & CLARK, J. J. 2004. Obesity: impediment to postsurgical wound healing. *Advances in skin & wound care*, 17, 426-432.
- WINGETT, D., FORCIER, K. & NIELSON, C. P. 1996. Glucocorticoid - mediated inhibition of RANTES expression in human T lymphocytes. *FEBS letters*, 398, 308-311.
- WOHLEB, E. S., MCKIM, D. B., SHERIDAN, J. F. & GODBOUT, J. P. 2015. Monocyte trafficking to the brain with stress and inflammation: a novel axis of immune-to-brain communication that influences mood and behavior. *Frontiers in Neuroscience*, 8, 447.
- WOLFER, D. P., LITVIN, O., MORF, S., NITSCH, R. M., LIPP, H.-P. & WÜRBEL, H. 2004. Laboratory animal welfare: cage enrichment and mouse behaviour. *Nature*, 432, 821-822.
- WOOD, N. I., CARTA, V., MILDE, S., SKILLINGS, E. A., MCALLISTER, C. J., ANG, Y. M., DUGUID, A., WIJESURIYA, N., AFZAL, S. M. & FERNANDES, J. X. 2010. Responses to environmental enrichment differ with sex and genotype in a transgenic mouse model of Huntington's disease. *PloS one*, 5, e9077.
- WRIGHT, R. J., RODRIGUEZ, M. & COHEN, S. 1998. Review of psychosocial stress and asthma: an integrated biopsychosocial approach. *Thorax*, 53, 1066-1074.
- XERRI, C. & ZENNOU-AZOGUI, Y. 2003. Influence of the postlesion environment and chronic piracetam treatment on the organization of the somatotopic map in the rat primary somatosensory cortex after focal cortical injury. *Neuroscience*, 118, 161-177.

- YADAV, A., SAINI, V. & ARORA, S. 2010. MCP-1: chemoattractant with a role beyond immunity: a review. *Clinica chimica acta*, 411, 1570-1579.
- YARDEN, Y. & SCHLESSINGER, J. 1987. Epidermal growth factor induces rapid, reversible aggregation of the purified epidermal growth factor receptor. *Biochemistry*, 26, 1443-1451.
- YOKOTA, T., ORITANI, K., TAKAHASHI, I., ISHIKAWA, J., MATSUYAMA, A., OUCHI, N., KIHARA, S., FUNAHASHI, T., TENNER, A. J. & TOMIYAMA, Y. 2000. Adiponectin, a new member of the family of soluble defense collagens, negatively regulates the growth of myelomonocytic progenitors and the functions of macrophages. *Blood*, 96, 1723-1732.
- YOSHIKAWA, H., KUROKAWA, M., OZAKI, N., NARA, K., ATOU, K., TAKADA, E., KAMOCHI, H. & SUZUKI, N. 2006. Nicotine inhibits the production of proinflammatory mediators in human monocytes by suppression of I -  $\kappa$  B phosphorylation and nuclear factor -  $\kappa$  B transcriptional activity through nicotinic acetylcholine receptor  $\alpha 7$ . *Clinical & Experimental Immunology*, 146, 116-123.
- YOUNG, R. 2003. Environmental enrichment for captive animals. Universities Federation for Animal Welfare (UFAW) Series. Oxford: Blackwell Publishing.
- YUEDE, C. M., ZIMMERMAN, S. D., DONG, H., KLING, M. J., BERO, A. W., HOLTZMAN, D. M., TIMSON, B. F. & CSERNANSKY, J. G. 2009. Effects of voluntary and forced exercise on plaque deposition, hippocampal volume, and behavior in the Tg2576 mouse model of Alzheimer's disease. *Neurobiology of disease*, 35, 426-432.
- ZANNI, F., VESCOVINI, R., BIASINI, C., FAGNONI, F., ZANLARI, L., TELERA, A., DI PEDE, P., PASSERI, G., PEDRAZZONI, M. & PASSERI, M. 2003. Marked increase with age of type 1 cytokines within memory and effector/cytotoxic CD8+ T cells in humans: a contribution to understand the relationship between inflammation and immunosenescence. *Experimental gerontology*, 38, 981-987.
- ZHANG, L., LEE, I.-C. J., ENRIQUEZ, R. F., LAU, J., VÄHÄTALO, L. H., BALDOCK, P. A., SAVONTAUS, E. & HERZOG, H. 2014. Stress-and diet-induced fat gain is controlled by NPY in catecholaminergic neurons. *Molecular metabolism*, 3, 581-591.
- ZHANG, Y., ZHANG, Y., MIAO, J., HANLEY, G., STUART, C., SUN, X., CHEN, T. & YIN, D. 2008. Chronic restraint stress promotes immune suppression through toll-like receptor 4-mediated phosphoinositide 3-kinase signaling. *Journal of neuroimmunology*, 204, 13-19.
- ZIMMERMAN, G. A., MCINTYRE, T. M., PRESCOTT, S. M. & STAFFORINI, D. M. 2002. The platelet-activating factor signaling system and its regulators in syndromes of inflammation and thrombosis. *Critical care medicine*, 30, S294-S301.
- ZIV, Y., RON, N., BUTOVSKY, O., LANDA, G., SUDAI, E., GREENBERG, N., COHEN, H., KIPNIS, J. & SCHWARTZ, M. 2006. Immune cells contribute to the maintenance of neurogenesis and spatial learning abilities in adulthood. *Nature neuroscience*, 9, 268-275.

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