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Modulators of the Acute Inflammatory Response: A Dissertation

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MODULATORS OF THE ACUTE INFLAMMATORY RESPONSE

A Dissertation Presented

By

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Dedicated to my Mom, Dad, and Devasheesh

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ABSTRACT

Acute inflammatory response is caused by the rapid recruitment of leukocytes, mainly neutrophils and monocytes, from blood to the tissue site. Diverse agents, including invading pathogens, injured or dead cells, and other irritants, may stimulate this response. In the ensuing inflammatory response, the recruited leukocytes and their secreted molecules help in eliminating or containing the injurious agents and promoting tissue regeneration. But often this response is imprecise and can lead to bystander tissue damage. Unchecked neutrophil activation is implicated in the pathology of many inflammatory conditions. An in-depth understanding of the pathways regulating this response, therefore, becomes critical in identifying therapeutic targets for these diseases. In this study, we investigate the role of intestinal commensal bacteria in regulating the acute inflammatory response. Furthermore, we examine the mechanism by which Interleukin-1 (IL-1) controls the inflammatory response to sterile agents.

Inflammatory responses have been studied in the context of host defense against pathogens. However, we report that the innate immune system needs to be primed by intestinal flora to enable neutrophil recruitment to diverse microbial or sterile inflammatory signals. This priming requires myeloid differentiation primary response gene (88) (MyD88) signaling. In antibiotic-treated mice, which have depleted intestinal flora, we show that neutrophils get released into the blood from the bone marrow, but have a specific defect in migration into the inflammed tissue. This deficiency can be restored by pre-stimulating the mice with a purified MyD88 ligand. Despite having reduced number of infiltrating neutrophils, antibiotic-treated mice make higher levels of

pro-inflammatory cytokines in the tissue, after inflammatory challenge. This suggests that antibiotic-treated mice produce some anti-inflammatory molecule(s) that counteract the effect of the pro-inflammatory cytokines. However, this effect is not due to the overproduction of the anti-inflammatory cytokine, Interleukin-10 (IL-10). In summary, our findings highlight the role of commensals in the development of acute inflammatory responses to microbial and sterile particles.

The inflammatory response to sterile dead cells has been shown to be critically dependent upon IL-1. However, several key aspects of the IL-1 signaling cascade including the source of IL-1 and the cellular target of IL-1 were unresolved. We find that in most cases, the injured cells are not a major contributor of IL-1 that is required to propagate the inflammatory signal. On the contrary, we demonstrate that both the isoforms of IL-1, IL-1α/IL-1β are generated by bone marrow-derived, tissue-resident responding cells, upon sensing the injury. We also sought to determine the identity of the cellular target of IL-1 signaling. Previous studies have shown that for cell death-induced neutrophil recruitment, interleukin-1 receptor (IL-1R) expression is required on parenchymal cells. To identify this parenchymal cell, we are currently in the process of making the conditional knockout mouse of IL-1R. The latter would facilitate the parenchymal tissue-specific deletion of IL-1R. In summary, this study reports our progress in unraveling key aspects of IL-1 signaling during sterile inflammation.

Taken together, we have identified key modulators of the acute inflammatory response and their mechanisms of regulation. These findings would facilitate the development of new therapies for inflammatory diseases triggered by both microbe and sterile agents.

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INTRODUCTION

The central focus of this thesis is elucidating the molecular and cellular entities that regulate neutrophil migration and thus control the acute inflammatory response. Intriguingly, the symptoms of the inflammation response had been first recognized as early as 2000 years ago. However, the progress in identifying the molecular mechanisms underlying this response has happened only in the last twenty years. The findings in the current research add to our understanding of regulation of this response, by revealing the role of intestinal flora in the acute inflammatory response and interleukin-1 (IL-1) in the sterile inflammatory response.

1. 1. OVERVIEW OF THE INFLAMMATORY RESPONSE

1.1.1. The Acute Inflammatory Response

Mammals develop an immediate and non-specific reaction to injury elicited by noxious agents. This reaction is called the acute inflammatory response. Pathologically, it involves a predetermined pattern of events characterized by changes in the microcirculation. This leads to increased passage of fluid out of the microcirculation and emigration of neutrophils into the site of injury. The infiltration of neutrophils occurs within minutes after the injury and is the hallmark of the acute inflammatory response.

1.1.2. Neutrophils: Warriors of the Inflammatory Response

Paul Ehrlich discovered neutrophils in the 19th century[1]. He used hematological staining to distinguish different white blood cells based on their granules. Neutrophils were named so because of their ability to retain neutral dyes. He was also able to identify the lobulated nucleus that is characteristic of neutrophils. This led to the alternate nomenclature of neutrophils as 'polymorphonuclear leukocytes' by Elie Metchnikoff, a contemporary of Paul Ehrlich. Metchnikoff is also credited to be the first to demonstrate the phagocytic function of neutrophils in microbe digestion[2].

Neutrophils are integral to the development of the acute inflammatory response because they are the first cells to accumulate at the site of injury. They are also the most abundant leukocytes in the mouse blood accounting for 60% of the white blood cell count. Like other white blood cells, they are produced and undergo maturation in the bone marrow. Pluripotent hematopoietic stem cells (HSCs) give rise to all types of white blood cells or leukocytes namely granulocytes (neutrophils, basophils, and eosinophils) and agranulocytes (lymphocytes, monocytes, and macrophages) (Figure 1.1). The earliest committed neutrophil progenitor cell is the myelocyte, which differentiates into a metamyelocyte, then a band neutrophil, and finally to a mature segmented neutrophil.

Etiology and Trajectory of neutrophils

The trajectory of neutrophils from the bone marrow to the site of inflammation involves three successive stages, each of which is precisely regulated: *Release of neutrophils*

Figure 1.1

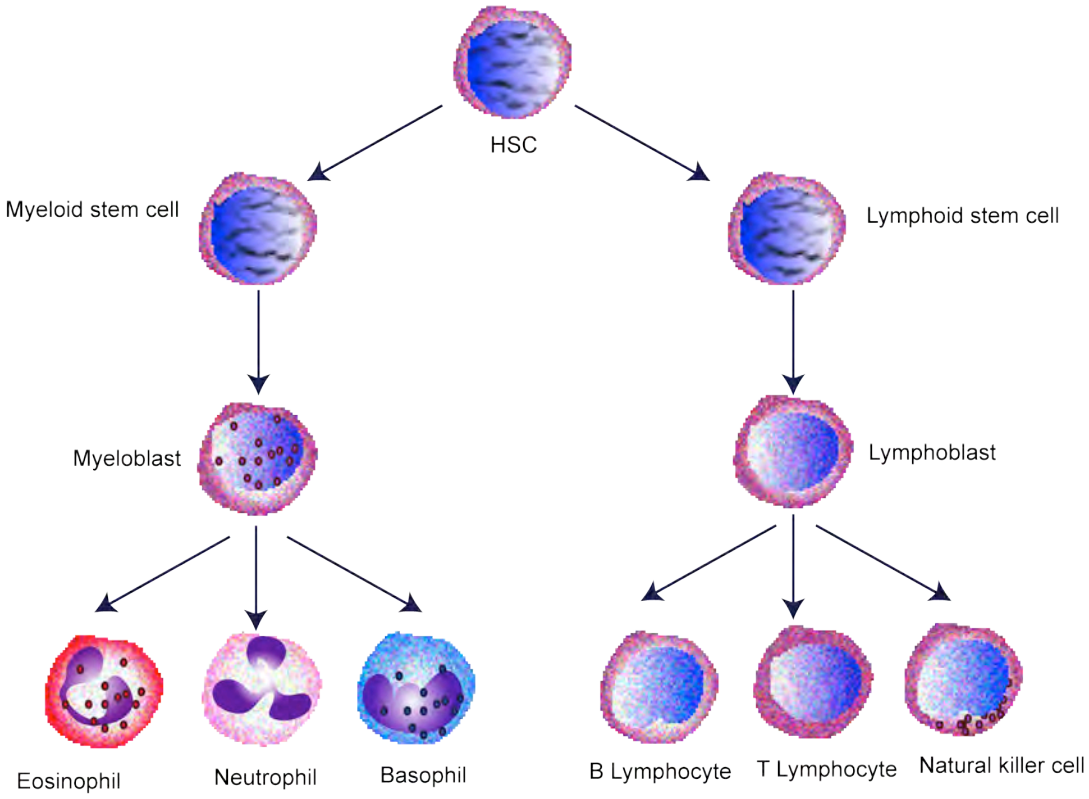


Figure 1.1: Differentiation of Hematopoietic Stem Cells (HSCs) into different types of white blood cells, including neutrophils

from bone marrow into the blood circulation, *Immigration from blood into the tissue site* and *Movement towards the site of inflammation*.

Release of neutrophils from bone marrow into the blood stream: Neutrophils are produced in hematopoietic cords interspersed within the venous sinuses of the bone marrow. HSCs become committed to the granulocytic lineage by their expression of transcription factors like Egr1 and STAT3, proteins like S100A8 and S100A9 and receptors like N-formyl methionyl-leucyl-phenylalanine and granulocyte-macrophage colony-stimulating factor (GM-CSF)[3]. Neutrophils spend most of their lifespan in the bone marrow; the average time lag between being neutrophil-lineage committed to subsequent release into blood stream is 12 days[4,5].

Neutrophil retention in the bone marrow and its exit into the blood stream is conversely regulated by the signaling through the CXC chemokine receptors (CXCR), CXCR2 and CXCR4[6-9] (Figure 1.2). The osteoblast-derived stromal-cell-derived factor (SDF)-1 acts through CXCR4 on neutrophils to retain neutrophils in the bone marrow. This is facilitated by the binding of the integrin, very late antigen (VLA)-4 on neutrophils to vascular cell adhesion molecule-1 (VCAM-1) expressed by bone marrow endothelial and stromal cells. On the other hand, the endothelial cell-derived CXCR2 ligands, KC (CXCL1) and macrophage inflammatory protein (MIP)-2 (CXCL2) mobilize neutrophils from the bone marrow. MIP-2 and KC counteract the effect of CXCR4 signaling via heterologous internalization and desensitization of CXCR4[7]. During the normal course of neutrophil maturation, they progressively down regulate the expression of CXCR4 and VLA-4, favoring their exit from the bone marrow.

Figure 1.2

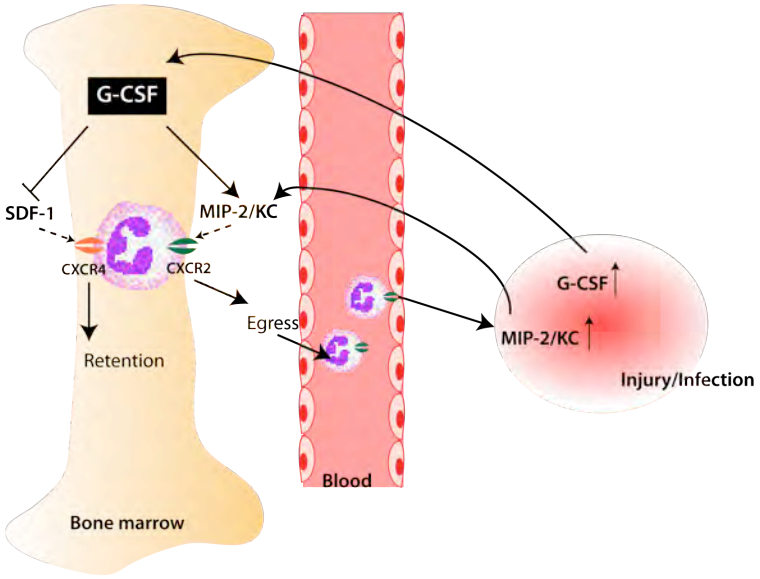


Figure 1.2: Regulation of neutrophil release from the bone marrow into blood
CXCR4 and its ligand SDF-1 promote the retention of neutrophils in bone marrow, while CXCR2 and its ligands, MIP-2/KC stimulate neutrophil egress. G-CSF generated in bone marrow and in inflamed tissue site mobilizes neutrophils by modulating the ratio of CXCR2/CXCR4 ligands.

Granulocyte colony stimulating factor (G-CSF) is a cytokine regulating all aspects of granulocytogenesis, including commitment of HSCs to the myeloid lineage, expansion of neutrophil precursors, facilitating maturation of granulocytes, and their exit into blood[4,5]. G-CSF promotes the release of neutrophils into the bloodstream by downregulating the expression of SDF-1 by stromal cells and also the expression of CXCR4 by granulocytes[6,10].

Once in the blood steam, neutrophils have a very short life span (6-8 h). Neutrophils are destroyed in peripheral tissues like bone marrow, spleen, and liver by apoptosis and consequent phagocytosis by tissue macrophages and dendritic cells[11,12]. The production of neutrophils in the blood is regulated by the rate of apoptosis of neutrophils in these tissues though the IL-23 – IL-17A – G-CSF signaling axis[13,14].

During acute inflammation, cytokines like MIP-2, KC, and G-CSF get generated. These cytokines accelerate the release of neutrophils from the bone marrow into the blood (Figure 1.2). G-CSF as mentioned before, acts by downregulating the CXCR2-SDF-1 retention mechanism. MIP-2 and KC attract neutrophils by generating concentration gradients across the sinus wall of bone marrow sinusoids[15].

Entry of neutrophils from blood into tissues: This process is also called extravasation of neutrophils (Figure 1.3). Tissue injury or infection is recognized by tissue-resident sentinel cells like macrophages leading to activation of intracellular signaling pathways. This leads to the production of mediators like cytokines (TNF-α, IL-1β), neutrophil chemoattractants (MIP-2, KC) and lipid mediators like prostaglandins. All these mediators promote the ‘adhesiveness’ of endothelium. They act by increasing the

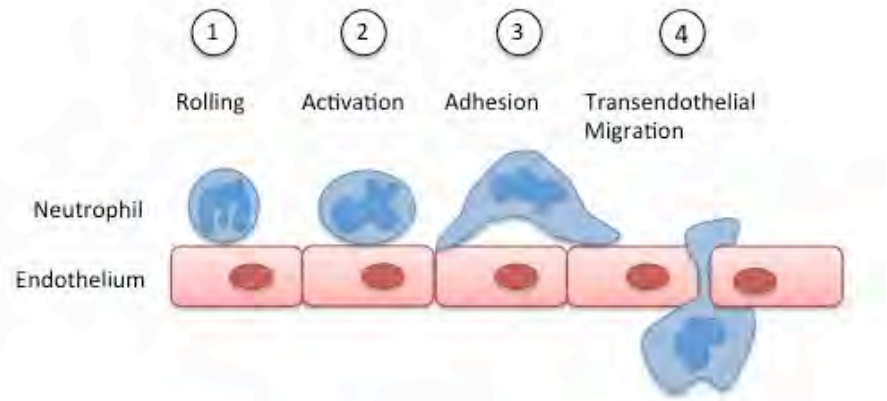
expression of specific cell adhesion molecules on both the endothelium and neutrophils. G-CSF also increases transendothelial migration of neutrophils. The migration of these cells from the endothelium into the tissue occurs primarily in the postcapillary venules in following three steps[16] (Figure 1.3):

- *Rolling:* This event slows blood flow and causes neutrophils in the central column to marginate into the periphery. Here, they transiently stick to the endothelium surface. This process is called 'rolling'. It is mediated by adhesion molecules called selectins, which are present on both neutrophils and the endothelium. Selectins mediate low-affinity binding, which accounts for the loose and transient interactions during rolling. Selectins have an extracellular domain, which binds to sugar moieties. Specific selectins are expressed on specific tissues, which allows for cognate interactions[16]. For e.g., E-selectin (or ELAM-1) is expressed by the endothelium; P-selectin (GMP140) is expressed by both endothelium and platelets and L-selectin is present on neutrophils. P- and E-selectins bind to glycoproteins on neutrophil surface. L-selectin binds to glycoproteins on endothelium. Selectins are expressed at low levels on endothelium but get upregulated during inflammation.

Activation: Neutrophils, which are rolling on the endothelial cell surface are activated by chemokines that are bound to the endothelium by glycoaminoglycans[17]. Integrins are constitutively expressed on neutrophils. However, they require a conformational change to bind to endothelial adhesion molecules, which is facilitated by chemokine activation[18].

- *Adhesion and Transmigration:* Rolling is followed by the next stage of

Figure 1.3



Step / Mediators	Rolling	Activation	Adhesion	Transendothelial Migration
On neutrophils	L- Selectin	IL-8R	Integrin	CXC chemokine receptor
On Endothelium	P-Selectin E-Selectin	IL-8	Ig Superfamily	

Figure 1.3: Extravasation of Neutrophils
Steps involved in the extravasation of neutrophils from blood into the inflamed tissue with the corresponding cell adhesion molecules expressed by neutrophils and the endothelium.

neutrophil trafficking in which they firmly adhere to the endothelial surface and pass via the intracellular junctions through the basement membrane into the interstitial space. This process of transmigration is termed diapedesis. It is facilitated by chemokine-mediated activation of neutrophils. It is mediated by the high-affinity interactions involving adhesion molecules of the immunoglobulin (expressed on endothelium) and integrin (expressed on neutrophils) families. Examples of endothelial adhesion molecules are ICAM-1 (intracellular adhesion molecule 1) and VCAM-1 (vascular cell adhesion molecule 1). ICAM-1 binds to integrins LFA-1 (CD11a/CD18) and Mac-1 (CD11b/CD18) and VCAM-1 binds to VLA-4 on neutrophils[19]. Expression of endothelial adhesion molecules is enhanced by inflammatory stimuli[20]. Transmigration occurs mainly in the venules of the systemic vasculature and also in the capillaries of lungs. To cross the basement membrane, neutrophils secrete collagenase to degrade the membrane.

Movement towards the site of inflammation: Once inside the tissue, neutrophils must make their way towards the site of injury. This is accomplished by spatial and temporal cascades of chemoattractants, which are generated when tissue-resident cells sense the infection or injury. In the mouse models of focal skin injury, two different sets of chemoattractants guide immigrating neutrophils to the site of injury[21]. The movement of neutrophils to about 150μm around the lesion is accomplished by gradients of MIP-2 and KC. Beyond this point, neutrophils move towards a gradient of mitochondrial formyl-peptides released by necrotic cells, by means of their expression of the formyl peptide receptor (FPR)1. The ligation of FPR1 or other chemokine receptors leads to

the activation of the MAPK/ERK pathway. Downstream molecules of the MAPK/ERK pathway promote degranulation and oxidative burst in the activated neutrophils. Neutrophils also express pattern recognition receptors like TLRs (except TLR3), dectin-1, RIG-I, MDA5, and NOD1 constitutively[22-25]. The ligation of these receptors by microbial or cell-derived ligands in the tissue site also promotes oxidative burst. Furthermore, G-CSF has a pivotal role in promoting neutrophil degranulation by simulating mobilization of secretory vesicles[26].

Neutrophil Phagocytosis and Degranulation

The ultimate function of neutrophils is eliminating the noxious agent. This is achieved though the process of phagocytosis and consequent release of their granular contents. Neutrophils have numerous specialized cytoplasmic granules containing acid hydrolases and antimicrobial peptides. There are three major groups of granules containing different molecules and produced at different stages of maturation. Primary granules are developed first and contain myeloperoxidase; secondary granules mature next and contain lactoferrin but no myeloperoxidase. The tertiary granules, which develop in later stages contain gelatinase and surface receptors[27].

Phagocytosis of the microbe or necrotic cell is accompanied by release of granular mediators. In general, these mediators can function in either oxygen-dependent or oxygen-independent mechanisms[28]. The oxygen-dependent mechanism involves the generation of reactive oxygen species (ROS) in a process called ‘respiratory burst’. Following activation, the multimeric NADPH oxidase enzyme complex is activated,

which oxidizes NADPH (nicotinamide adenine dinucleotide phosphate). The activation of the oxidative machinery is a multi-step process often requiring a 'priming' step. For example, neutrophil response to formyl peptides requires priming by LPS. LPS is required for the assembly of the NADPH oxidase complex while formyl peptides stimulate its activation.

The oxidation of NADPH generates superoxide ion. Superoxide gets converted by spontaneous dismutation into hydrogen peroxide (H_2O_2). Neutrophils further convert H_2O_2 into HOCl (hypochlorous radical) by the action of the enzyme myeloperoxidase (MPO). Other reactive oxygen intermediates like singlet oxygen, hydroxyl radical, and ozone are also generated in phagocytes. These are powerful antimicrobial agents, which kill microbes by halogenation or by protein and lipid peroxidation. The dead microbes are further degraded by the action of lysosomal acid hydrolases.

Besides ROS, other proteins present in leukocyte granules are bactericidal too. These molecules account for the oxygen-independent killing mechanisms. They include the bactericidal permeability increasing protein (BPI), lysozyme, lactoferrin, and defensins.

An alternate method by which neutrophils kill microbes is the formation of neutrophil extracellular traps (NETs)[29]. Upon activation by an inflammatory stimulus like TLR ligation, neutrophils die, rupturing the cell membrane. This process releases nucleosomes from unfolded chromatin. NETs comprise of threads of chromatin, which form a web-like structure. Other proteins from the neutrophilic granules may be attached to this structure. NETs can trap gram-positive and gram-negative bacteria and fungi and facilitate their killing by other anti-microbial effectors.

Survival of neutrophils in tissues

In peripheral tissues, neutrophils have a short half-life of about eleven hours, rapidly undergoing apoptosis. Signals like T_H17 cell-derived cytokines (such as IL-17, CXC-chemokine ligand 8 (CXCL8; also known as IL-8), interferon-γ (IFNγ), tumour necrosis factor (TNF) and granulocyte–macrophage colony-stimulating factor (GM-CSF)) greatly influence not only activation and extravasation of neutrophils but also their survival in tissues[30,31]. TNF is interesting in this regard that lower concentrations promote neutrophil longevity while higher concentrations promote apoptosis[32].

Antigenic markers of neutrophil development

During their development in bone marrow and release into blood stream, neutrophils are classified into myeloblasts, promyelocytes, myelocytes, metamyelocytes, bands, and segmented neutrophils based on their morphological characteristics. In normal conditions, mature or segmented neutrophils are released into the blood. Segmented neutrophils are named so because of their characteristic nuclei, which appear segmented into lobes of condensed chromatin connected by filaments. During inflammation, however, both mature and immature neutrophils may be released into the circulation.

The most widely used antigenic marker of neutrophils is Ly6G[33]. These are small glycosyl-phosphatidylinositol (GPI)-linked proteins on the surface of mouse neutrophils. The expression of Ly6G is not constant but is low on immature cells and increases as granulocytes mature. Hence, a number of different markers are used to

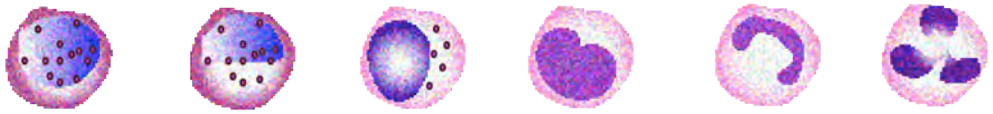
reliably identify neutrophils[34]. The expression profiles of some of these antigenic epitopes based on different phases of maturation of neutrophils has been summarized in Table 1.1. Fully mature neutrophils can be distinguished by their high expression of CD10, CD11b, CD16, and CD45RO[35-40]. On the other hand, they express low to none CD45RA and CD64, which are preferentially expressed on the immature forms of granulocytes[40-43].

Effect of G-CSF and GM-CSF on neutrophil production, maturation, and function

Both granulocyte colony stimulating factor (G-CSF) and granulocyte-macrophage colony-stimulating factor (GM-CSF) have been approved for treatment for neutropenia in human patients[44-46]. Though both these hematopoietic growth factors influence neutrophil production and phenotype, they are differences in their specificity of action on developing and mature neutrophils, and their effects on neutrophil kinetics. A major difference is in the expression of their cognate receptors and hence their specificity of action. G-CSF receptor is expressed almost exclusively on neutrophil progenitors and mature neutrophils[47]. On the other hand, GM-CSF receptors are expressed on neutrophils, monocytes, macrophages, and eosinophils as also non-hematopoietic cell lineage like endothelial cells, myeloid leukemia cells, and a variety of tumor cell lines[48]. G-CSF appears to be more critical than GM-CSF for the development of neutrophils. As mentioned before, G-CSF leads to the differentiation of committed stem cells into granulocytes and also mobilization of mature cells into the circulation[47,49,50].

Table 1.1

Proliferating precursor cells (Immature cell pool) | Non-dividing cells (Mature cell pool)



Stages / Antigen	**Blast**	**Promyelo**	**Myelo**	**Metamyelo**	**Band**	**Seg**
CD10	-	-	-	-	-	++
CD11b	-	-	-	++	++	+
CD11c	-	-	++	++	++	++
CD15	+	+++	+++	+++	+++	++
CD16	-	-	-	+	++	+++
CD33	+++	+++	++	+	+	+
CD44RA	+	+	-	-	-	-
CD44RO	-	-	+	+	++	+++
CD62L	++	++	++	++	++	++
CD64	+	+	++	++	-	-

Table 1.1: **Expression of antigenic surface markers based on the stage of maturation of neutrophils**
Blast, Myeloblast; Promyelo, promyelocyte; Myelo, myelocyte; Metamyelo, metamyelocyte; Seg, segmented neutrophil. +++, strong expression; ++, moderate expression; +, weak expression; -, no expression.

Mice knockout for G-CSF are chronically neutropenic[51]. GM-CSF broadly influences hematopoiesis, stimulating differentiation of committed stem cells into neutrophils, eosinophils, and monocytes[50,52-54]. GM-CSF seems to be dispensable to neutrophil development, since GM-CSF knockout mice are not neutropenic[55].

Both G-CSF and GM-CSF increase peripheral neutrophil numbers by increasing the rate of progenitor cell divisions, reducing maturation time and increasing half-life of mature circulating cells, though G-CSF is more potent than GM-CSF in this regard[5,56,57].

Both G-CSF and GM-CSF are effective in enhancing the phagocytic potential of neutrophils[58-61]. They promote antibody-dependent cellular cytotoxicity, adherence, and microbial killing[59-66]. They also serve as chemoattractants and promote transendothelial migration of neutrophils[67-69]. They increase the expression of cell adhesion molecules by neutrophils[70]. They stimulate respiratory burst; GM-CSF is more effective than G-CSF in this function[71]. They enhance the survival of neutrophils in tissue[13,14,72].

1.1.3. Signaling pathway leading to Neutrophil Migration

Neutrophils are the effector cells of the acute inflammatory response, which is a signaling cascade that is initiated when the body encounters a foreign or an altered self molecule(s). This response comprises of four functional categories of molecules or cells. These are inducers, sensors, mediators, and effectors[73]. Some examples of the signaling cascades elicited by inflammatory particles are given in Table 1.2. They will be described below in detail.

Table 1.2

Inducer	Sensor	Mediator	Effector
Meso-diaminopimelic acid (Peptidoglycan from Gram-negative bacteria)	NOD1	TNFα, IL-6	Neutrophils, hepatocytes, splenocytes
House dust mite allergen	TLR4	Histamine	Endothelial cells, smooth muscle cells
Cholesterol crystals	NALP3	IL-1β	Endothelial cells, smooth muscle cells, macrophages, neutrophils
Collagen (Extracellular matrix)	RAGE	ROS, IL-1β	Endothelial cells, smooth muscle cells, macrophages

Table 1.2: Lineup of the acute inflammatory response with specific examples
The specific type of "inducer" determines the downstream signaling candidates that get activated[73].

"**The Inducers"**

These molecules are agents that initiate an acute inflammatory response. They can be broadly divided into following groups and sub-groups:

1) Exogenous agents
    i. *Microbial*
        a. Pathogen-associated molecular patterns (PAMPs)
        b. Virulence factors
    ii. *Non-microbial*
2) Endogenous agents
    i. *Cell injury*

Pathogen-associated molecular patterns (PAMPs):

These are small molecular motifs that are conserved within a class of microbes and are distinct from host-derived molecules. They are recognized by specific receptors expressed by the host immune system called the pattern recognition receptors (PRRs)[74]. The major classes of PRRs and their signaling modules will be discussed later. Common examples of PAMPs are lipopolysaccharide (LPS), which is a component of the bacterial cell wall and zymosan, which is a component of the yeast cell wall. Contrary to what the name suggests, PAMPs are not solely expressed by pathogens. Commensal bacteria that are present on the host tissue surfaces also display PAMPs.

Virulence factors:

The other types of exogenous microbial inflammatory agents are virulence factors. There appears to be an overlap between the definitions of PAMPs and virulence factors as some reviews also cite LPS as a virulence factor[75]. However, unlike PAMPs, most virulence factors are not detected by specific PRRs on host tissues. They perturb the host tissue homeostasis leading to the generation of a downstream effector molecule that is detected by the cognate host sensor. Examples of such virulence factors are the pore-forming exotoxins generated by Gram-positive bacteria[76-78]. There is an efflux of potassium ions through the cell as a result of the channel-forming toxins. Potassium efflux is sensed by the NALP3 (NACHT-, leucine-rich-repeat-and-pyrin-domain containing protein) inflammasome, which activates the inflammatory pathway[79]. The inflammasome pathway has been described later. Alternatively, toxins released by pathogens cause cell death. The secondary effect of endogenous molecules released on account of cell death stimulates inflammation.

Exogenous non-microbial agents:

Examples of such noxious agents include crystals, allergens and other toxic components. Inhalation of silica crystals or asbestos particles causes inflammation in the alveolar space in a NALP3 inflammasome-dependent way[80]. Other examples of exogenous non-microbial particles are certain allergens (though some allergens may be microbe-derived). Allergens may mimic certain host signaling proteins. One such example is the house dust mite allergen Der p 2, which mimics the Toll-like receptor 4

(TLR4) signaling co-factor leading to activation of the TLR4-dependent pro-inflammatory pathway[81].

Cell injury-derived agents:

Non-physiological cell death, tissue damage or stress is inflammatory[82]. Like microbes, which display specific signature molecules, there are certain cell-derived molecules, which stimulate inflammation[83]. These are called damage associated molecular patterns (DAMPs). In normal cells, these molecules are encased in the cellular membrane or sub-cellular compartments and hence are 'hidden' from the host immune system. During necrotic cell death, however, such molecules get released into the environmental milieu. Examples of molecules, which may be putative DAMPs are ATP[84,85], HMGB1 (high-mobility group box 1 protein)[86], heat shock proteins[87-90], double stranded DNA[91-93], thioredoxin[94], and galectins[95]. In other cases, certain molecules are released upon necrotic cell death, which act upon components of the extracellular matrix to generate pro-inflammatory products. For example, various hydrolases and proteases released from the cell cleave collagen[96], heparin sulfate[97], and hyaluronic acid[98,99] to generate pro-inflammatory molecules.

Another class of endogenous inflammatory agents is associated with chronic inflammation during certain diseases. These include crystals like monosodium urate (MSU)[100], cholesterol[101], and also advanced glycation end products (AGEs)[102].

"The Sensors"

As mentioned earlier, certain conserved motifs on microbes and other foreign signals are recognized by "sensors" expressed by host cells, also called pattern recognition receptors (PRRs) (Figure 1.4)[74]. These set of receptors are germ-line encoded and are either present in body fluids, on cell membranes or in the cytoplasm of tissue-resident cells like macrophages, mast cells, fibroblasts and dendritic cells. Activated PRRs oligomerize and assemble into large macromolecular complexes. The latter induce either several extracellular activation cascades like the complement pathways or intracellular signaling pathways. The overall effect of activation of these pathways is the production of chemokines and cytokines, type I interferons, (IFNs), antimicrobial proteins, regulatory proteins of PRR signaling and other uncharacterized proteins and promotion of phagocytosis. Chemokines and cytokines cause vascular changes and promote chemotaxis of leukocytes to the site of infection/injury. PRRs may be present in body fluids (e.g., components of the complement pathway), on cell membrane or other sub-cellular membranes (e.g., TLRs and C-type lectins) or in cytoplasm (e.g., nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs))

Some of the major signaling pathways are described in detail below:

Toll-Like Receptor (TLR) Family:

TLRs are one of the most studied inflammatory signaling pathways. They are characterized by the presence of the extracellular/luminal domain that has leucine rich repeats (LRRs). LRRs function in ligand recognition. The cytoplasmic tail has the

Figure 1.4

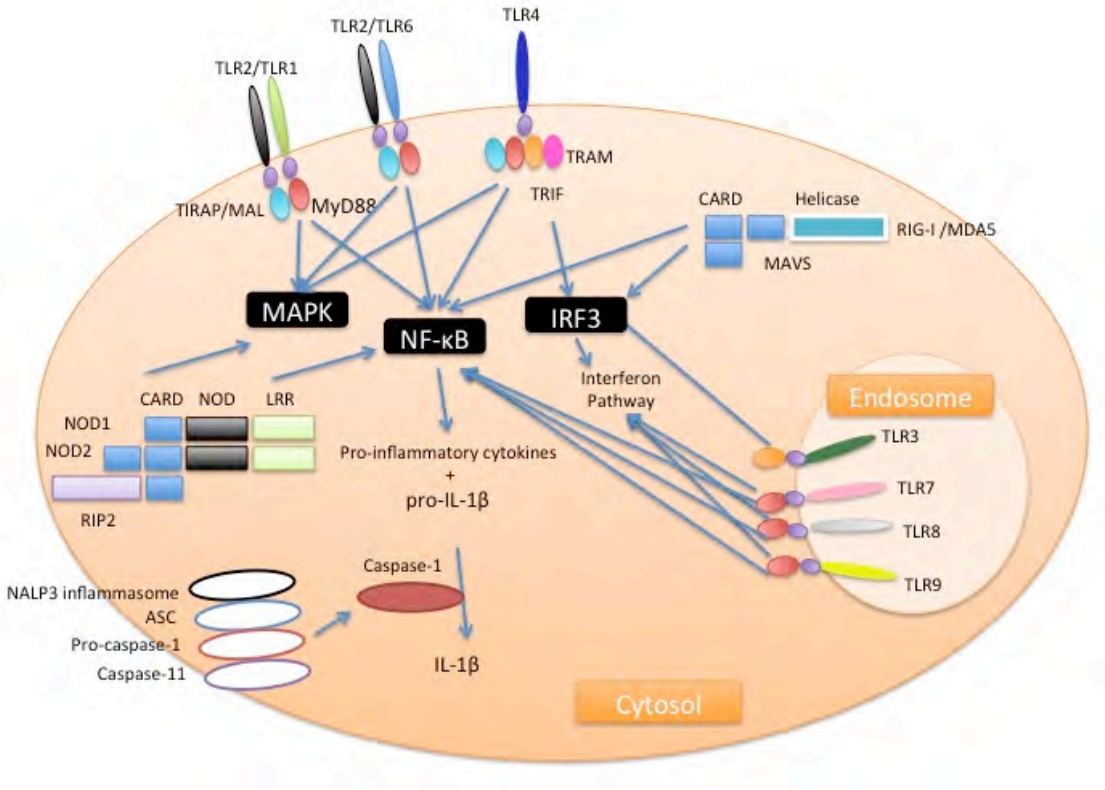


Figure 1.4: Signaling Cascades induced by Pattern Recognition Receptor Ligation
Generation of NF-κB, MAPK and IRF3 by converging intracellular pathways initiated by cell membrane and cytosolic "sensors".

Toll/IL-1R homology (TIR) domain, which functions in downstream signal propagation. The TLRs are examples of Type I integral membrane glycoproteins. They function as homodimers or heterodimers.There are 12 known TLRs in mammals[103]. TLRs respond to diverse ligands associated with pathogens like bacteria, viruses, fungi and parasites as well as certain endogenous ligands.

Distribution of TLRs in tissues: Most human tissues are known to express at least one type of TLR, but innate immune cells exhibit high expression of most TLRs. The distribution of various TLRs differs between innate immune cells[104]. Macrophages or monocytes express all TLRs except TLR3[105]. The expression of TLRs on dendritic cells depends upon the particular dendritic cell subset. Myeloid dendritic cells express (MDCs) express TLR1, 2, 4, 5, and 8, while plasmacytoid dendritic cells (PDCs) express TLR 7 and 9[106-108]. Another type of tissue-resident cells, mast cells express TLR2, 4, 6, and 8[109].

The expression of TLRs on the epithelial cells lining mucosal surfaces is regulated in such a way that it serves the dual purpose of defense against mucosal pathogens but tolerance towards mucosa-associated commensal bacteria[104]. One of the means to accomplish these dual functions is by the specific localization of TLRs on the basolateral surface of epithelial cells and not the apical surface, which is continuously exposed to normal microbiota. For e.g., TLR5 is expressed only on the basolateral surface[110]. Another method for achieving tolerance to commensals is by the low expression of TLR4 on the intestinal epithelium[111,112].

TLR-mediated signaling pathways: Different TLRs recruit different combinations of adaptor molecules, which determine the downstream signaling pathways activated by each TLR. All these adaptor proteins associate with their partner TLRs via TIR domains. TLR ligation leads to the activation of two major signaling pathways. All TLRs activate the transcription factor, nuclear factor-κB (NF-κB) and the Mitogen-activated protein kinases (MAPKs): p38 and c-Jun Kinase (JNK)[113]. In addition, TLR3 and TLR4 activate NF-κB as well as the transcription factor Interferon regulatory factor-3 (IRF3); the latter leads to the activation of Type I interferons[114,115].

All TLRs signal via MyD88 except TLR3. TLR3 and TLR4 can also signal in a MyD88-independent way via IFN-β (TRIF), also known as TICAM-1[114].

RIG-I like receptors (RLRs):

The RLRs are cytosolic PRRs, which detect non-self RNA in the cytoplasm. This family of receptors includes RIG-I, MDA5 and LGP2[116]. RLRs are characterized by a central DExD/H-box helicase domain and a RNA-binding carboxy-terminal regulatory domain (CTD). RIG-I and MDA5 have an amino-terminal caspase activation and recruitment domain (CARDs), which mediate downstream signaling[117]. RIG-I and MDA5 detect and respond to distinct species of viruses[118]. LGP2 positively regulates RIG-I and MDA5 signaling[119]. RIG-I detects 5' triphosphate of double stranded RNA derived either from virus or RNA intermediates generated from cytosolic microbial DNA by the action of RNA polymerase III[120]. RIG-1 binds to its CARD-domain

containing adaptor partner called MAVS[121]. Downstream signaling mediated by ligation of MAVS leads to the activation of IRF3, IRF7, and NF-κB.

NOD-like receptors (NLRs):

As the RLRs, NLRs are also present in the cytoplasm. They are characterized by the presence of LRRs at the carboxy termini, which mediate ligand sensing as well as autoregulation[122-126]. A centrally located NACHT domain mediates self-oligomerization and activation[122,127]. The amino-terminal effector domain, which may be either CARD, pyrin (PYD) or baculovirus IAP repeat (BIR) domain facilitates interaction with adaptor proteins for downstream signaling[128-131]. Based on the nature of the effector domain, NLRs can be divided into subfamilies: NODs, NLRPs, CIITA, IPAF, and NAIPs. NODs, IPAF, and CIITA have the CARD effector domains and NALPs have the PYD effector domains while NAIPs have the BIR domains[132].

- *NOD1 and NOD2:* These are cytoplasmic sensors of microbial cell wall components. NOD1 detects a specific muropeptide present in gram-negative bacteria. NOD2 is a general sensor of peptidoglycan and hence can respond to most bacteria. Ligand recognition by either NOD1 or NOD2 leads to the activation of RICK (also called RIP2). Downstream signaling by RICK results in NF-κB activation[129,133-135].

- *Inflammasomes:* Inflammasomes are multimeric complexes, which recognize diverse microbial and sterile stimuli to activate a pathway that leads to the processing of cytokines like IL-1β and IL-18 to yield mature proteins. Inflammasomes, which belong to the NLR family of receptors contain the NACHT domain, LRRs and either or

both of the CARD/PYD domain[136]. Examples of these inflammasomes include NALP1, NALP2, NALP3, NALP6, NLRC4, and NALP12. Other inflammasomes such as AIM2 and IFI16 have the PYD domain and the HIN200 domain, the latter mediates ligand binding.

Production of functionally active and secreted- IL-β requires a cross talk between TLRs and NALPs[137]. TLR signaling leads to increased transcription of immature pro- IL-β. The cleavage, activation, and subsequent secretion of mature IL-1β require the action of the cysteine protease caspase-1. Caspase-1 itself is synthesized as a non-functional zymogen called pro-caspase-1. Pro-caspase-1 is cleaved by the assembly of the multimeric NALP inflammasome. Inflammasome proteins bind directly to pro-caspase-1 by the CARD domain or indirectly through the PYD domain, via a protein ASC, which contains both ASC and PYD domain.

Ligands activating the NALP3 inflammasome have been extensively researched and include a variety of endogenous, environmental, and microbial stimuli. Some of the examples of NALP3 activators are microbial toxins like maitotoxin and nigericin[79], environmental stimuli like silica and asbestos[80], and endogenous stimuli like monosodium crystals[138]. There have been efforts to understand if all these diverse molecules have a unifying underlying cause of inflammasome activation. Production of ROS[80,139,140], lysosomal rupture resulting from ingestion of crystalline particles[141,142], and formation of the ATP-dependent pannexin-1 membrane pore[143] seem to be events occurring downstream of some of the ligands, purported to cause inflammasome

assembly.

The AIM2[144] and NALP1[145] inflammasome have more specific ligands, double-stranded DNA and muramyl dipeptide respectively.

C-Type Lectin Receptors (CLRs):

Calcium-dependent (C-type) lectins are a group of proteins, which have the high-affinity carbohydrate binding domain[146]. There are 17 known groups of CLRs in vertebrates. They perform diverse functions like capture and uptake of endogenous ligands, microbes, and PAMPs. Examples are collectins, which are serum proteins, which bind pathogens and activate complement. CLRs take up particles either by endocytosis or phagocytosis.

Dectin-1 is the only CLR, which has been conclusively shown to activate expression of innate response genes[147]. Dectin-1 recognizes zymosan, a yeast cell wall particle. Dectin-1 can induce phagocytosis and cytokine production. Dectin-1 has an immunoreceptor tyrosine-based activation (ITAM)-like motif, which mediates intracellular signaling. Responses initiated by Dectin-1 ligation can be Syk kinase-dependent or independent[147,148]. Dectin-1 has been shown to promote the production of inflammatory cytokines by activating NF-κB.

"The Mediators"

"Inducers" of inflammation act on cell-resident "sensors" to trigger the production of chemical "mediators". The vascular and cellular events that lead to the inflammatory

response are a result of these mediators, which are either released from cells or are present in plasma. Plasma-derived factors need to be cleaved by proteases before they are functionally active. Examples of plasma-derived mediators are factors associated with the clotting system, the complement system and the kinins. Cell-derived factors are sequestered in granules and released or generated *de novo* after stimulation. Examples of cell-derived mediators are vasoactive amines (e.g., histamine and serotonin) and products of phospholipids (e.g., platelet-activating factor) and arachidonic acid metabolism (e.g., prostaglandins and leukotrienes), cytokines, nitric oxide and oxygen-derived free radicals and lysosomal constituents. Some of the functions performed by these inflammatory mediators are vasoconstriction and bronchoconstriction, arteriolar vasodilation and increased vascular permeability, increased leukocyte adhesion, chemotaxis and leukocyte activation.

Cytokines and Chemokines:

As described in previous sections, signaling pathways downstream of PRR ligation (except the NALPs, IPAF and NAIPs) lead to the activation of transcription factors NF-κB, AP-1, IRF5, and IRF3 (Figure 1.2). These transcription factors regulate inflammation is several ways, by influencing the transcription of cytokines, chemokines, growth factors, acute - phase proteins, immunoreceptors and adhesion molecules. Cytokines whose expression is regulated by NF-κB, AP-1, and IRF5 include Tumor Necrosis Factor-α (TNF-α), Interleukin-1 (IL-1), IL-1, IL-8, Interferon-β (IFN-β), RANTES, Macrophage chemotactic protein-1 (MCP-1) and Granulocyte colony-

stimulating factor (G-CSF)[149]. IRF3, in conjunction with NF-κB and JNK stimulates the expression of Type I interferons, namely, Interferon-α (IFN-α) and Interferon-β (IFN-β)[150]. While signaling through most PRRs results into increased transcription of cytokines including IL-1, the activation of the NALPs, IPAF and NAIP is important for the post-translational processing of IL-1β and IL-18 to yield mature cytokines[136].

Cytokines like TNF, IL-1, MIP-2, KC, IL-6 play a crucial role in orchestrating the inflammatory response by determining the types of cells that infiltrate into the tissue, their state of activation, and the systemic responses to inflammation. IL-1 is particularly important for the cell-death induced inflammatory response. This function will be further explored in the chapter 3 of this thesis.

"The Effectors"

"Mediators" released during inflammation modulate the functional states of tissues and cells, giving rise to the pathology of the acute inflammatory response. As described before, neutrophils are the key effectors of the acute inflammatory response. Besides neutrophils, there are other tissue-resident and recruited cells that play important roles in this response.

Mast cells: Mast cells are found in almost all tissues[151]. In those tissues, which have fewer numbers of mast cells, their number increases during inflammation. The immediate massive degranulation of mast cells to release inflammatory meditors is associated with IgE-mediated type I hypersensitivity reactions. Apart from the FcεRI, mast cells possess other innate immune receptors like TLRs, complement receptors and

receptors for inflammatory peptides and hormones. Immune activation of mast cells results into an immediate release of the contents of their cytoplasmic granules. Stored in the cytoplasmic granules are inflammatory mediators like histamine, proteoglycans, and other specific proteases as also cytokines like TNF. Also other chemokines and cytokines and some arachidonic acid metabolites are synthesized de novo and released later.

Platelets: Platelets are produced in the bone marrow from where they are released into the circulation[152]. They lack nucleus, hence they appear as irregularly shaped cell fragments. Platelets get activated when they come in contact with fibrillar collagen, which is exposed at the injured endothelium. Also, activation of the coagulation system, which generates thrombin can activate platelets. Once activated, platelets release serotonin and the arachidonic acid metabolite (TxA_2), which cause increased vascular permeability and smooth muscle constriction.

Endothelial cells: As described before, endothelium plays an important role in inflammation by regulating the degree of tissue perfusion[152]. A delicate balance between the production of vasoconstrictors and vasodilators controls the level of vascular permeability. Vasoactive mediators synthesized by endothelial cells in inflammatory conditions include PGI_2, endothelium-derived relaxing factor (EDRF), and endothelin. Besides, endothelial cells control vascular permeability by regulating fibrinolysis.

Monocytes/ Macrophages: Monocytes are generated in the bone marrow[152]. Under physiologic conditions, circulating monocytes have a half-life of about a day. Similar to

chemotactic emigration of neutrophils into the tissue, they infiltrate the inflamed site within 24-48 hours of injury. However, when they enter the tissue, they transform into larger cells specialized in phagocytosis called macrophages. Macrophages can be found in many tissues, where they have specific names like kupffer cells (liver), sinus and lymph nodes (spleen and lymph nodes), microglia (central nervous system), and alveolar macrophages (lungs).

Macrophages have been shown to be the primary cellular sensors for necrotic cell deth-induced damage[153]. Macrophages can also get activated by cytokines IFN-γ and other inflammatory stimuli like LPS. Once activated, macrophages increase in size, express more lysosomal enzymes and become better phagocytes. They also secrete inflammatory mediators like acid and neutral proteases, and plasminogen activator. Though most of the complement components and coagulation factors are synthesized in the liver, macrophages are also able to secrete them locally. Besides, they make ROS, NO, eicosanoids, and cytokines. Upon clearance of the inflammatory agent, macrophages die and are cleared via the lymphatics[154].

1.1.4. Inflammation: A double-edged Sword

Neutrophils, once recruited to the site of infection, are powerful phagocytes that kill microbes and help clear the infection. The importance of neutrophil function is evident in individuals who have a defect in neutrophil chemotaxis or phagocytic functions[155,156]. These individuals are more prone to bacterial infections. In the case of sterile inflammation, effector molecules released from neutrophils and other recruited

cells help eliminate and clear the cell debris and aid in resolution of the wound. However, in some instances, the neutrophil response can be counterproductive to the host. Microbicidal molecules released from activated neutrophils can cause damage to bystander healthy tissue[157]. The consequent cell injury and death can aggravate the inflammatory condition, exacerbating the damage. This situation is particularly relevant when the inciting stimuli are sterile particles like crystals. The accumulation of crystals may not be as harmful to the host as the collateral damage caused by recruited neutrophils. Hence it is essential to study the factors controlling neutrophil migration and activation.

1.2. OVERVIEW OF COMMENSAL MICROBIOTA

1.2.1. Mutualism between Eukaryotes and Bacteria

The term ‘symbiosis’ was coined by the German botanist Heinrich Anton de Bary in 1829 to refer to a close physical association between organisms that extends for a significant period of their life cycles. Based on the outcome of this interaction, symbiotic relationships may further be classified as mutualism, commensalism, amensalism, and parasitism. When both the interacting partners benefit in terms of their growth, survival or procreation, the association is termed as ‘mutualism’, whereas, if only one partner benefits with no effect on the other, it is known as ‘commensalism’. Amensalism and parasitism are interactions, which are detrimental to one partner while being advantageous or neutral to the other[158]. All these types of associations have been powerful forces in determining a species’ fitness during natural selection.

Amongst the most widespread of these symbiotic associations are the ones involving microorganisms and warm-blooded animals like humans. In fact, the extent of colonization by bacteria in humans is such that bacterial mass accounts for 10% of the dry body weight in humans. The large intestine is the organ with the largest numbers and diversity of microbiota, though bacterial colonization occurs at other mucosal surfaces and skin too. The intestinal microbiota aids in crucial functions of metabolism and immunity while deriving a hospitable ecological niche in the gut. A detailed account of the distribution and the diverse functions performed by microbiota with emphasis on their role in immunity will be done in the next two sections.

1.2.2. The human microbiome

The human microbiome (or the human microbiota) is a collective term that refers to all the microorganisms that colonize the different anatomical sites and surfaces of the human body. They are also called 'commensal microflora', though this a misnomer on two counts, firstly this is an association which generally benefits both partners and hence is mutualistic rather than commensal and secondly, the term 'flora' specifically denotes plants. There are over 500 species of microorganisms that include bacteria, fungi and archae. They reside on the skin, conjunctiva, oral cavity, respiratory tract, gastrointestinal tract and urogenital tract. In utero, the fetus is sterile, but microbial colonization initiates during and within 48 hours of birth. Interestingly, the colonization pattern varies with the type of delivery (caesarean vs. natural)[159], kind of feeding (bottle vs. breast feeding)[160] and as adults, individual's genetic makeup, sex, age, hygiene etc. But in general, the microbiota at a specific anatomical site is fairly constant within the species.

Because of the sheer number and diversity of bacterial burden in the intestine tract, gut flora is the most widely studied human microbiota. There are about 100 trillion bacteria over a stretch of $400m^2$ of the intestinal tract[161]. The bacterial density is not uniform along with intestinal tract, with as few as 10^4/g of luminal contents in the stomach to 10^{11}- 10^{14}/g in the large intestine. The identification of intestinal bacteria was a challenge because most of them do not grow in routine culture media. But 16S rRNA sequencing and molecular fingerprinting methods have enabled the identification of more than 40,000 species in the gastrointestinal tract.

The composition of bacterial species varies at different sites of the intestinal tract. In the stomach and the proximal small intestine, there is low bacterial colonization because of the acidic stomach juices, bile, and alkaline pancreatic juices. The acid-resistant lactobacilli, enterococci, oral streptococci, are the most pre-dominant species in the stomach. Most Americans are also colonized by the pathogenic *Helicobacter pylori,* which has been linked to gastric cancer. The proximal small intestine mostly includes lactobacilli and *Enterococcus faecalis*, oral streptococci, and other gram-positive aerobic or facultative anaerobes. The distal small intestine (ileum) represents a transition zone between the proximal small intestine, which is dominated by aerobic species and the large intestine, which primarily houses anaerobes[162]. There are about 10^8/g of bacteria, which include gram-negative microbes like *E. coli* and *Bacteroides*, apart from lactobacilli and enterococci.

The number and variety of enteric flora drastically increases in the large intestine reaching up to 10^{14} cells/g (colon). The microbial composition of colonic contents matches with that of the feces. As mentioned previously, the colon comprises mainly of anaerobes such as *Bacteroides, Porphyromonas, Bifidobacterium, Lactobacillus, and Clostridium.* Of these, *Bacteroides* constitute about 30% of the total gut flora and thus are key anaerobes in health and disease. Intestinal *Bacteroides* consist primarily four species, *B. thetaiotaomicron, B. vulgatus, B. distasonis and B. fragilis*. There are 100-1000 fold more anaerobes to aerobes in the colon[163]. The composition of the colon changes after birth. At birth, the microbiota is predominated by facultative aerobes like streptococcus and *Escherichia coli,* but after weaning there is a shift towards obligate

anerobes, like *Bacteroides* sp[163]. Specific functions in establishing local and systemic immunity has been attributed to some of these bacterial species. These functions are discussed in the next section.

1.2.3. Functions of Gut Flora

Several recent studies have implicated a role for gut flora in diverse functions relating to metabolism, nutrition, tissue maintenance and immunity. These hitherto unknown functions attributed to the gut flora, along with the sheer enormity of their numbers, have prompted researchers to call it 'a forgotten organ'[164]. In fact, the term 'human microbiome' was proposed by Dr. Joshua Lederberg, a Nobel Laureate in 1958, who had the foresight to realize that, the comprehensive knowledge of human genetics and physiology is a composite of both human and microbial genetics[165]. The importance of gut flora in maintaining healthy metabolism was first discovered in "germ-free" rats, which were totally devoid of commensal flora including gut flora[166]. Since then, the use of germ-free animals has been pivotal in elucidating the functions of gut flora in physiology and disease. The functions of microbiota, specifically gut flora, can be broadly divided into two categories, in nutrition and in immunology. The nutritive benefits of gut flora include carbohydrate fermentation and absorption, lipid storage and secretion of vitamins and amino acids and absorption of minerals. The functions of gut flora in the host immunity are discussed below.

Immunological functions of gut flora:

The intestinal epithelium, which is a host for an enormous microbial community, also houses the largest mass of lymphoid tissue in the human body. The bacterial colony is separated from the internal immune tissue by only a single layer of epithelial cells[167]. The most obvious immunological function of the gut flora to the host is forming a natural defense barrier at this site, preventing access to pathogens. Besides, the enormity and diversity of enteric bacteria ensures that invading pathogens face competition in terms of availability of nutrients and receptors for attachment. This is supported by studies in germ free-mice, which show that these mice are more susceptible to infection by enteric pathogens like *Shigella flexneri, Listeria monocytogenes, Clostridium difficile,* and *Salmonella enterica*[168-170]. As would be expected, germ-free mice developed some resistance to these infections, when colonized by specific commensal bacteria.

What was initially assumed to be a mere "competitive role" in immunity against pathogens has now broadened to reveal a more extensive role for commensal microbiota. The past decade has been particularly exciting in the field of gut immunity. More surprising is the fact that gut flora not only influences the local immunity in the gut lymphoid tissue, but also has profound effects on systemic immunity. Efforts have been made in understanding the molecular signaling pathways by which commensal flora influences the various arms of immunity. Studies reveal that commensal bacteria and pathogens use the same signaling machinery in the host cells. This has led to the conundrum, how does host immunity discriminate between 'good' commensal flora and

'harmful' infectious bacteria? What prevents commensal flora from penetrating host tissue and leading to inflammation and sepsis? What are the mechanisms by which the host has developed tolerance to these microbial antigens? The functions of microbiota in stimulating host immunity will be discussed in two parts: i) Role of microbiota in intestinal immunity and ii) Role of microbiota in systemic immunity. The mechanism by which host immunity protects itself from attack by the commensal bacteria will be discussed in part (i).

Role of microbiota in endemic immunity

As mentioned earlier, humans and other warm-blooded animals are colonized by a commensal microbiota at specific anatomical sites like skin, oral cavity, respiratory tract, conjunctiva and the gut. Among these sites, the microbiota is particularly important in stimulating local immunity in the gut and skin.

Establishment of intestinal immunity: At the forefront of intestinal immunity is the intestinal epithelium, which is composed of a single layer of intestinal epithelial cells (IECs)[171,172]. Beneath the epithelium lies the supporting loose connective tissue called the lamina propria, which is composed of stromal cells, and diverse lymphoid and myeloid cell populations. The lymphoid tissue associated with the intestinal lamina propria and epithelium is called the gut associated lymphoid tissue (GALT). Beneath the lamina propria, lies the muscular mucosa (Figure 1.5).

Anatomically, the small intestinal mucosa is organized into millions of projections called villi and intervening invaginations called crypts, each of which is dominated by

Figure 1.5

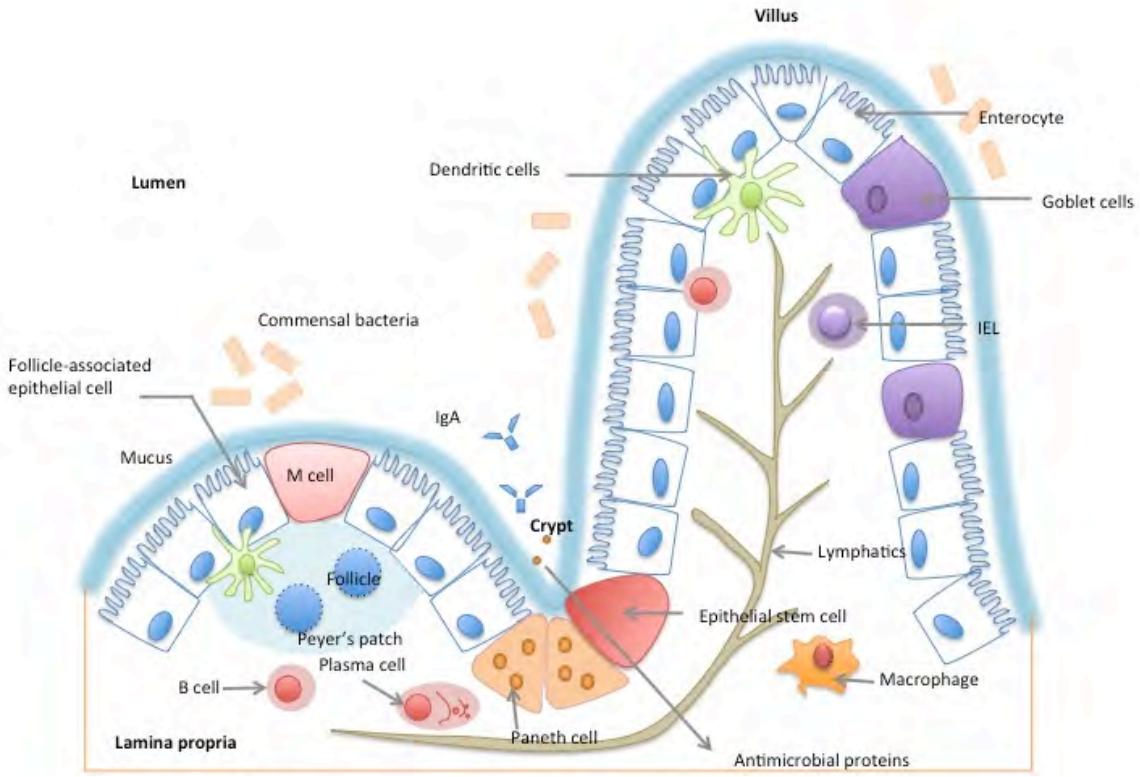


Figure 1.5: The intestinal Immune System

a distinct cell type. The luminal surface of the intestinal epithelium, which comprises of microvilli along with the overlying mucus is called the brush border. IECs are simple columnar and non-ciliated cells that line the intestine. They are interconnected by tight junctions at the basal and apical surfaces, which makes the membrane and the paracellular spaces impenetrable to macromolecules, even 2-kDa molecules. Certain inflammatory conditions cause 'leaky' tight junctions that facilitate the underlying lymphoid tissue to mount a response to dietary antigens and commensal flora[173]. IECs are highly polarized cells with an apical surface facing the intestinal lumen and a basolateral surface facing the lamina propria. IECs are actually composed of four different cell types, all of which are derived from a common stem cell progenitor, the small intestinal epithelial progenitor. Amongst them, goblet cells and paneth cells have functions in immunology; in making mucus and antimicrobial peptides respectively.

Mucus provides a physical barrier that separates the microbiota from the apical side of the epithelium, a process called as non-immune exclusion[174]. Mucus also provides anchorage to gut flora via IgA antibodies.

GALT: Intestinal epithelium plays a critical role in gut 'innate' immunity by mediating non-immune exclusion, i.e., preventing antigen access to the underlying lymphoid tissue and also through production of anti-microbial peptides. But a slower and more specific response to gut antigens, which is characteristic of an adaptive immune response, is enabled by the specialized lymphoid tissue associated with GI tract called GALT[175]. The GALT consists of discrete inductive and effective sites (Figure 1.5). The

inductive sites are organized lymphoid follicles that include the peyer's patches (PP) and mesenteric lymph nodes and other smaller lymphoid follicles, which are scattered throughout the wall of the small and large intestine. At the luminal surface, PPs are surrounded by a distinct type of epithelium called the follicle-associated epithelium (FAE). FAE is adapted to function in immunity. Unlike the intestinal epithelium it has lower absorptive and digestive capacity but is infiltrated by large numbers of B cells, T cells, macrophages and dendritic cells (DCs). In addition to these lymphoid cells, FAE also contains specialized epithelial cells called M (or microfold) cells which actively take up macromolecules and microorganisms from the luminal side and transfer it to antigen presenting cells (APCs) either in the epithelium or the underlying dome region[171].

As in other secondary lymphoid tissue sites, PPs contain 6-12 basally located germinal centers. Germinal centers contain B cells and CD4 T cells, some apoptotic lymphocytes as also APCs such as dendritic cells, macrophages and a specialized stromal cell known as a follicular dendritic cell (FDC). APCs process antigens and present them to T cells, leading to their activation. Mucosal T cells stimulate the differentiation of B cells into plasma cells and their subsequent isotype switching to IgA producing cells. Lymphocytes that are primed in the PPs get drained into the mesenteric lymph nodes (MLNs). They leave the MLN through the efferent lymph and enter the bloodstream through the thoracic duct and eventually accumulate in the mucosa.

Alternatively, the process of antigen-dependent T cell activation might occur in the MLNs when antigen or antigen-loaded DCs in the PPs enter the draining lymph[176].

On activation in the PPs or MLNs, T cells and IgA-producing B cells migrate into the effector sites. Effector sites are scattered throughout the mucosa in the lamina or substantia propria. Effector sites are composed of T cells, which are mostly $CD4^+$, IgA plasma cells with fewer IgG and IgM plasma cells, and few B-cells, dendritic cells, and macrophages. At the effector sites, polymeric IgA is secreted across the mucosal epithelium into the lumen with the help of polymeric IgA receptor, which is expressed in mucosal epithelial cells. Secreted IgA play a role in innate immunity by preventing access of microbial pathogens to the mucosal epithelium. In addition, this antibody provides specific immunity against certain enteric pathogens[177].

Locally resident intra epithelial lymphocytes (IELs) are distinguished from the systemic T cells by their subset composition. TCRγδ+ cells constitute around 50% of murine CD8+ intestinal cells[178]. All IELs are cytolytic and function in a granzyme or Fas dependent fashion and also by secreting T helper 1 (T_H1) cytokines. TCRγδ+ cells promote epithelial tissue repair upon injury and prevent microbial invasion of normal or injured tissue[179].

Gut flora is indispensible in the maintenance of GALT and the intestinal epithelium, so much so that GF animals have poorly developed PPs and isolated lymphoid follicles, fewer plasma cells and IELs with reduced functionality, little production of antimicrobial peptides and IgA[180]. The molecular mechanisms by which microbiota influence these aspects of immune machinery are only partially known. Some of them involve activating the known PRR pathways and will be discussed in the forthcoming sections.

Maintenance of intestinal epithelium integrity under steady state and on injury:

Mature IECs continuously arise from the colonic epithelial progenitors (ColEPs), which are present in the lower half of colonic crypts. Differentiated IECs migrate from the crypt base to the upper regions where they undergo apoptosis and/or exfoliation. It has been shown that TLR signaling by commensal flora is important to maintain a balance of proliferation and differentiation in the colonic crypts under homeostatic conditions. *MyD88*$^{-/-}$ mice have an expanded proliferative zone, which extends to the differentiated crypt orifice and also have an increased number of total proliferating cells[181]. In addition to a defect in basal maintenance of intestinal epithelium, commensal flora induced TLR-MyD88 signaling also has a dramatic role in tissue repair and survival after intestinal injury and inflammation[181].

Development of inductive sites of intestinal immunity: As discussed before, inductive sites in the murine intestinal immune system are comprised of organized tissues like the PPs and MLNs as well as clusters of loosely packed lymphocytes called isolated lymphoid follicles (ILFs). The embryonic genesis of PPs is critically dependent upon lymphotoxin and interleukin-17 (IL-17) signaling, but largely independent upon the presence of gut flora[182]. However, the presence of intestinal flora is essential during post-natal development of PPs. Germ-free mice have smaller and fewer PPs with indistinct germinal centers. This effect is likely to be mediated via the TLR4-MyD88 signaling pathway, as PPs in *MyD88*$^{-/-}$ and *TLR4*$^{-/-}$ mice were significantly smaller than their wild type counterparts[183]. Gut flora is also absolutely critical for the genesis of ILFs[184].

IgA production: Secretory IgA is important as an effector molecule in the adaptive immune response against enteric pathogens. Moreover, mice deficient in production of IgA also show an altered composition of gut microbiota. Interestingly, germ-free mice have lowered IgA numbers indicating that there is a feedback loop between secretion of IgA and symbiotic bacteria. The gut flora directs the expression of inducible nitric oxide synthase in dendritic cells of mucosal tissue. iNOS+ dendritic cells stimulate Ig class switching to IgA in a T-cell-dependent and independent fashion[185].

Production of antimicrobial peptides: Antimicrobial peptides are evolutionary conserved small molecular weight proteins that have broad-spectrum activity against bacteria. As mentioned before, paneth cells are the primary producers of these peptides in the intestine.

Intestinal flora induces the expression of several antimicrobial peptides like REG3γ and REG3β (both of which bind peptidoglycan), CRP-ductin (which targets gram-positive and gram-negative bacteria), RELMβ (which is an inflammatory modulator) and also members of the α-defensin family by isolated paneth cells[186]. Moreover, paneth cells directly sense intestinal flora using the TLR-MyD88 signaling axis leading to a concomitant increase in expression of these peptides.

Interestingly, there exists a negative feedback loop between the presence of intestinal microbiota and the paneth cell-MyD88-dependent expression of antimicrobial peptides[187]. While gut flora stimulates the production of antimicrobial peptides, the latter act to limit bacteria penetration and dissemination across the intestinal epithelium.

Establishment of the T_H17/T_{reg} axis: Commensal bacteria play an important role in the development and maintenance of various pro-inflammatory (T_H1,T_H2, and T_H17) and anti-inflammatory (T_{reg}) subsets of T cells. T_H17 cells are present in the small intestinal lamina propria and important for defense against bacterial and fungal pathogens. Certain species of the microbiota called segmented filamentous bacteria have been shown to induce the production of T_H17 cells[188]. This process is dependent upon the secretion of serum amyloid A in the terminal ileum that acts on the lamina propria dendritic cells. These dendritic cells then produce cytokines that promote development of intestinal T_H17 cells.

Likewise, the differentiation of inducible T_{regs} is critically dependent upon gut flora[189]. Inducible T_{regs} occur in the peripheral tissues like gut instead of the thymus. A single molecule polysachharide A (PSA), which is a zwitterionic capsular polysaccharide of the gut organism *Bacteroides fragilis* is able to restore the production of inducible T_{regs} in the gut in germ-free mice. This process is dependent upon TLR2 signaling. These T_{regs} are CD4+Foxp3+ and inhibit inflammation by their secretion of IL-10. Furthermore, in the experimental mouse models of colitis, monocolonization with *Bacteroides fragilis* is able to protect mice from inflammation and mortality.

Role of microbiota in systemic immunity

Compared to the study of the role of gut flora in intestinal flora, relatively less is known about how intestinal flora affects systemic immunity. Following are some of the pathways influenced by intestinal flora.

Maintaining T_H1/T_H2 balance: Germ free mice have a systemic defect in their effector CD4+ T cells numbers and exhibit a T_H1/T_H2 imbalance[190]. Mazmanian *et al* show that in the absence of intestinal flora, splenic CD4+ T cells make more interleukin-4 (IL-4) and low levels of IFN-, which is characteristic of a T_H2 response. This overproduction of IL-4 is associated with T_H2 mediated thymic pathologies in germ free mice.

PSA, which is the capsular polysaccharide of the gut organism *Bacteroides fragilis* is able to restore T helper cell balances in germ free mice[190]. PSA is phagocytosed and processed by dendritic cells and presented to CD4+ T cells. Monocolonization by *B. fragilis* is sufficient to revert the cytokine profile in germ free mice to that of SPF mice.

Priming neutrophils for opsonophagocytosis: As described earlier, neutrophils kill microbes by oxidative and non-oxidative mechanisms. A recent study has shown that efficient phagocytosis of bacteria like *S. aureus* and *S. pneumonia* by neutrophils requires priming by intestinal flora[191]. Peptidoglycan derived from gut flora translocates through the intestinal epithelium and enters the circulation from where it can diffuse into tissues. Commensal bacteria-derived peptidoglycan then primes bone marrow and blood residing neutrophils via NOD1 signaling. Consequently, neutrophils from NOD1 deficient animals are defective in killing bacteria.

Priming acute inflammation: Souza et al., Fagundes et al., and Amaral et al. have shown that germ free mice have reduced neutrophil accumulation in some models of inflammation like ischemia and reperfusion injury[192-194]. In these studies, defective neutrophil infiltration has been attributed to the overproduction of IL-10 by these animals. However, a study of the role of intestinal flora in regulating the acute

inflammatory response, in the absence of a breach in intestinal mucosa is desired.

1.2.4. Germ free animals

The study of role of microbiome in host physiology is indebted to the breeding strategy developed by Dr. James Reyniers at the University of Notre Dame[166]. Mice pups are derived germ-free by cesarean section. Pups developing in the uterus of a healthy mother are free of bacteria. The uterus is removed aseptically from pregnant mothers, briefly sterilized and the pups are then transferred to germ-free isolators. During the initial development of germ free mice, these pups were nurtured and fed by hand rearing. With the availability of “primer” germ-free mice now, they are fed by lactating germ-free mothers.

The germ-free isolators consist of sterile plastic chambers. Air blown into the chamber is sterilized by filters. The interiors of the chamber are treated with chlorine dioxide. Mouse feeding and other supplies are autoclaved before entry into the chamber.

The sterility of germfree mice is assessed by performing a quantitative PCR for 16S rRNA and also by thioglycolate culturing of the cecal contents[195].

Germ-free animals bred in this fashion, do not have any bacteria in the intestine or on other body surfaces. This is in contrast with specific-pathogen-free (SPF) mice that are only devoid of known mouse pathogens and do contain intestinal bacteria.

1.2.5. Regulation of the Acute Inflammatory Response by Gut flora

The idea that commensal flora could regulate the inflammatory pathway germinated from a conundrum faced in the field of inflammasome activation. In vitro, macrophages generate mature IL-1β upon stimulation with NRLP3 ligands e.g. silica, ATP, nigericin etc. (Signal 2), only when they are pre-stimulated by a TLR agonist (Signal 1)[196]. However, in vivo, an intraperitoneal injection of crystals (e.g. silica) is sufficient to elicit an IL-1β-dependent neutrophil migration[142]. What stimulus serves as "Signal 1" in vivo? We hypothesized that ligands derived from commensal flora constitutively prime macrophages so that they poised to generate IL-1β when stimulated with any NRLP3 ligand.

We sought to test this hypothesis using germ-free mice. Consistent with our hypothesis, germ-free mice showed markedly reduced inflammation to silica, and monosodium crystals. But on further probing, we found that germ-free mice had a generalized defect in mounting an inflammatory response to many ligands like zymosan, dead cells, thioglycollate etc. Since zymosan stimulates neutrophil migration in an IL-1R-independent way, the defect in germ-free mice appeared to be broader, beyond the activation of inflammasomes.

There have been some reports of germ-free mice showing a defect in the acute inflammatory responses to pathogens and sterile injury. However, a thorough mechanistic study of how intestinal bacteria regulate inflammation was desired. As discussed in the before, the acute inflammatory response involves multiple steps of neutrophil activation, chemotaxis and phagocytosis. We sought to examine the precise

step at which gut flora influence the inflammatory pathway. In addition, bacteria activate a variety of known intracellular signaling pathways. We sought to identify the primary signaling pathway employed by commensal flora to prime acute inflammation. The main focus of the second chapter is addressing these questions pertaining to the role of intestinal flora in immunity.

The third and the fourth chapter focus on investigating the mechanism of IL-1 signaling during cell death-induced inflammation. Previous work published in the laboratory has demonstrated the crucial role of IL-1 in cell death-induced neutrophil migration[197]. However, questions regarding the cellular source of IL-1 as well as cellular target of IL-1 signaling remain unresolved. This thesis contributes to the progress achieved in the laboratory in understanding these processes.

CHAPTER 2

ROLE OF INTESTINAL FLORA IN REGULATING THE ACUTE INFLAMMATORY RESPONSE

Attributions and copyright information

This chapter of the thesis will be submitted for a publication.

All experiments in this chapter have been performed by me.

2.1. Introduction

The large intestinal tract of humans and other vertebrates is inhabited by numerous and diverse bacterial populations. The extent of microbial colonization is such that the number of microbial cells outnumbers the total number of cells in the human body by 10-fold. The combined microbial gene set similarly exceeds the human gene complement by about 150-fold[198,199]. As such, intestinal flora plays a vital role in the gut physiology. The mammalian digestive system is limited in its ability to produce all the enzymes, which would be required to metabolize the vast repertoire of energy substrates that are consumed[200]. Gut flora complements the host's digestive system in maximizing their utilization. The nutritive benefits of gut flora extend to carbohydrate fermentation and absorption, lipid storage and secretion of vitamins and amino acids and absorption of minerals[200]. Besides their role in digestion, intestinal flora contributes to intestinal epithelial cell growth and proliferation and development of immunity.

Mice bred in germ-free conditions are more susceptible to pathogens like *Salmonella enterica* , *Listeria monocytogenes* , *Klebsiella pneumonia* and *Candida albicans* [193,201-203]. The role of intestinal flora in preventing enteric infections was attributed to its ability to prevent invasion and colonization by opportunist pathogens in the intestinal niche. However, in recent years it has become increasingly apparent that the host microbiota plays a more active role in the development and functioning of immune system. Germ-free mice have anatomical defects in the gut-associated lymphoid tissue like poorly developed Peyer's patches, isolated lymphoid follicles, plasma cells and fewer intraepithelial lymphocytes[180,184,204,205]. They also produce lower levels of

antimicrobial peptides and IgA[204,206]. Certain species of the microbiota, namely segmented filamentous bacteria have been shown to induce the production of T_H17 cells in the small intestinal lamina propria[188]. Likewise, the gut organism *Bacteroides fragilis* facilitates the production of inducible Tregs in the gut[189]. Hence, commensal microbiota are pivotal for the development of gut-associated immunity.

Recent studies have demonstrated that gut flora have more far-reaching effects on the host innate and adaptive systemic immunity. Germ free mice have a systemic defect in the proliferation of effector CD4+ T cells numbers and exhibit a T_H1/T_H2 imbalance. Mazmanian et al show that in the absence of intestinal flora, splenic CD4+ T cells make more interleukin-4 (IL-4) and low levels of IFN-γ, which is characteristic of a T_H2 response[190]. Commensal flora has been shown to prime bone marrow and blood neutrophils so that they can function effectively in the phagocytosis of *Streptococcus pneumoniae* and *Staphylococcus aureus*[191]. However, a better understanding of how intestinal flora prime innate inflammatory responses *in vivo* is desired. In this study, we sought to determine the contribution of intestinal flora in regulating neutrophil migration during the acute inflammatory response.

Acute inflammatory response is caused by the rapid recruitment of leukocytes, mainly neutrophils, from the blood to the tissue site. Diverse agents including invading pathogens, injured or dead cells and other irritants like crystals may stimulate this response. These inflammatory agents are sensed by tissue-resident cells like macrophages, dendritic cells, and mast cells. The latter, once activated, release inflammatory mediators like histamines, prostaglandins and cytokines like IFN-γ, IL-8,

TNF-α and IL-1. These mediators promote vasodilation and also activate the endothelium, facilitating the chemotactic transmigration of leukocytes, mainly neutrophils into the affected tissue.

Neutrophils, once recruited to the site of infection, are powerful phagocytes that kill microbes by oxidative (e.g., reactive oxygen species) and non-oxidative (e.g., various proteases) mechanisms[207]. The importance of neutrophil function is evident in individuals who have a defect in neutrophil chemotaxis or phagocytic functions[155,156]. These individuals are more prone to bacterial infections. However, in some instances, the neutrophil response can be counterproductive to the host. Microbicidal molecules released from activated neutrophils can cause damage to bystander healthy tissue. The consequent cell injury and death can aggravate the inflammatory condition, exacerbating the damage. This situation is particularly relevant when the inciting stimuli are sterile particles like crystals. The accumulation of crystals may not be as harmful to the host as the collateral damage caused by recruited neutrophils. Hence it is essential to study the factors controlling neutrophil migration and activation.

Using germ-free and antibiotic-treated mice we found that intestinal flora are indispensible to mediate neutrophil and monocyte recruitment to diverse pathogen-derived and sterile particles. In the absence of microbiota, neutrophils can migrate from the bone marrow to the bloodstream but fail to extravasate from blood into the inflamed tissue. Neutrophil infiltration into tissues requires priming by microbiota, which occurs in a MyD88-dependent fashion. For neutrophil extravasation into tissues, gut flora-activated MyD88 signaling is crucial during the development of immune system but

depending upon the ligand, may be dispensable at the time of the actual inflammatory challenge. Pre-treatment of antibiotic-treated mice with a purified MyD88 agonist is sufficient to restore neutrophil migration. Furthermore, after inflammatory stimulation, antibiotic-treated mice are capable of making pro-inflammatory cytokines yet are defective in tissue neutrophil infiltration. Contrary to previous reports[193], the reduced neutrophil infiltration observed in antibiotic-treated mice cannot be attributed to their excessive production of IL-10. In summary, this study provides insight into the precise role of microbiota in mediating acute inflammation.

2.2. Results

Germ free and antibiotic-treated mice have a defect in neutrophilic inflammation.

To investigate the role of commensal microflora in acute inflammation, we examined the recruitment of neutrophils to various stimuli in the peritoneal cavity in mice bred in germ-free conditions. This was done using the peritonitis model of inflammation (Figure 2.1). We found that germ-free (GF) mice showed a dramatic reduction in the number of infiltrating neutrophils as compared to specific pathogen free (SPF) mice in the peritoneum after inflammatory stimulation. This defect in acute inflammation was observed to challenge with microbial components like zymosan, a component of yeast cell wall and thioglycollate (Figure 2.2 A and B) as well as to sterile ligands like silica and monosodium urate crystals (Figure 2.2 C and D). In subsequent experiments we focused on analyzing the responses to zymosan because this agent was easy to administer and gave strong and consistent results.

Figure 2.1

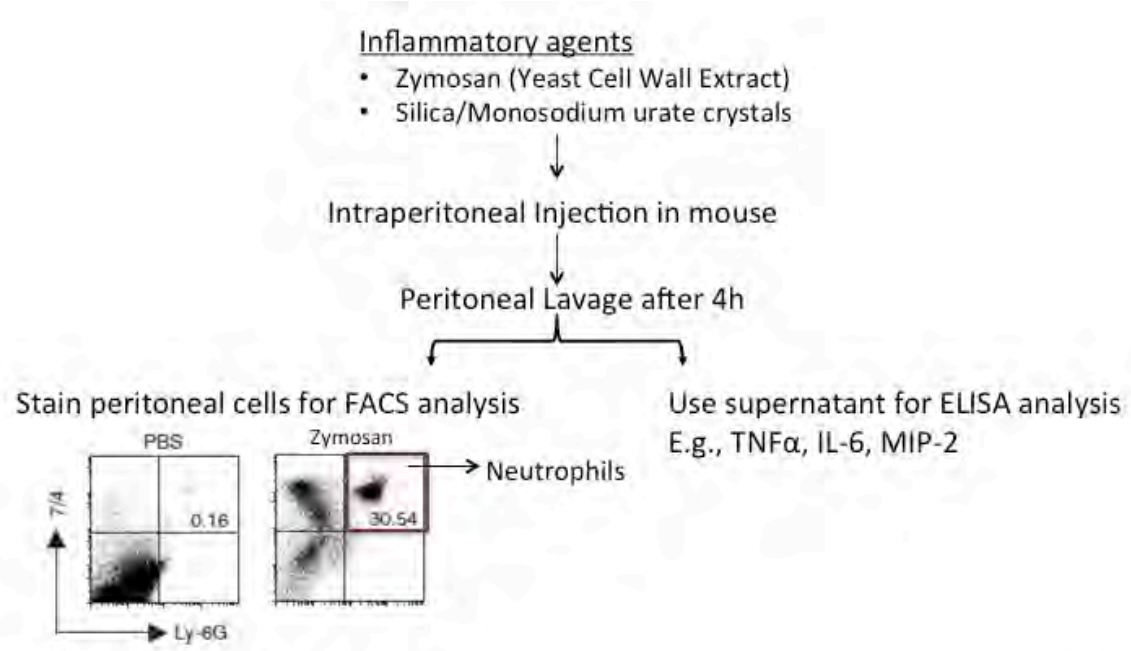


Figure 2.1: Outline of the peritonitis assay used to study the acute inflammatory response

This assay was used to quantify the recruitment of neutrophils and to determine the amount of cytokines produced in the peritoneum after challenge with microbial/sterile stimuli.

Figure 2.2

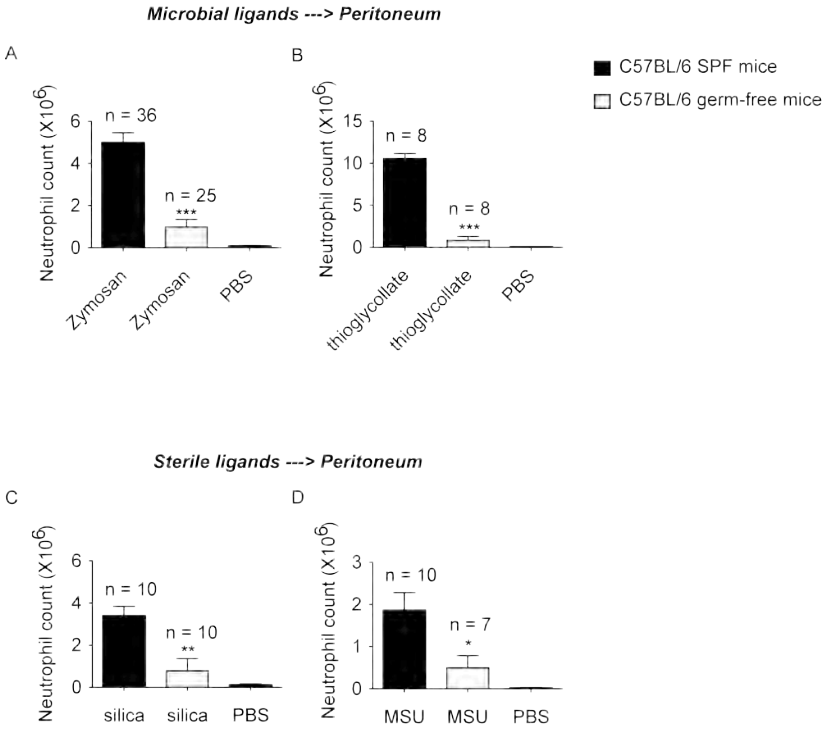


Figure 2.2: Mice lacking intestinal flora are defective in neutrophil recruitment to diverse inflammatory stimuli

Total neutrophil numbers in the peritoneum of SPF C57BL/6 and germ-free (GF) C57BL/6 mice 4h after i.p. injection of (A) 0.2mg zymosan, (B) 1mL 3% thioglycollate (C) 0.5mg silica crystals, and (D) 0.5mg monosodium urate (MSU) crystals. Numbers of neutrophils were determined by multiplying total cell number by percentage of Ly-$6G^+$ $7/4^+$. All data are combined results of three or more experiments and represented as mean ± SEM. n = Total number of mice used per datum group. In all panels PBS group represents SPF C57BL/6 mice challenged with PBS and lavaged after 4h. * $P < 0.0001$, ** $P = 0.0025$, ***$P = 0.04$ versus SPF animals

This phenotype of reduced inflammation observed in germ-free animals was replicated in mice treated with a cocktail of broad-spectrum antibiotics from birth to the time they were used in experiments (Day 0 to Day 45) (Figure 2.3 A); microbial 16S ribosomal RNA was undetectable by PCR in these animals indicating that had severely reduced microbial flora, as has been described by others (Figure 2.4) [208]. Because of the limited availability of germ-free mice, most subsequent experiments were done using antibiotic-treated mice.

The lowered numbers of neutrophils observed in the peritoneum in antibiotic-treated mice after 4 hours was not due to delayed migration of neutrophils, because these mice exhibited defective neutrophil migration even after 16 hours after inflammatory challenge (Figure 2.3 B**)**. We sought to examine the precise step at which microbiota regulate neutrophil activation and migration. Neutrophils originate and mature in the bone marrow[207]. Mature neutrophils subsequently leave the bone marrow and circulate in the blood stream. Cytokines generated at the site of inflammation stimulate an increase in production of neutrophils in the bone marrow and their release into the blood stream and chemotactic factors promote their subsequent migration into the inflamed area.

Figure 2.3

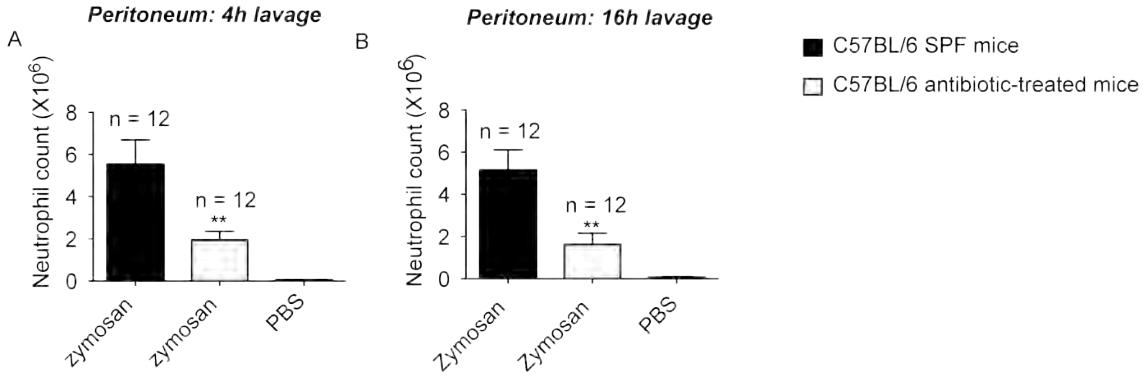


Figure 2.3: Mice having reduced intestinal flora (antibiotic-treated mice) are defective in neutrophil recruitment to inflammatory stimuli

(A, B) Total neutrophil numbers in the peritoneum of SPF C57BL/6 and antibiotic-treated C57BL/6 mice after i.p. injection of 0.2mg zymosan and lavaged after (A) 4h. (B) 16h after injection. Antibiotic-treated mice were subjected a cocktail of antibiotics (ampicillin, neomycin, vancomycin and metronidazole) from birth to day of experimental use. All data are combined results of two or more experiments and represented as mean ± SEM. In both panels PBS group represents SPF C57BL/6 mice challenged with PBS and lavaged after 4h or 16h. * P =0.008 versus SPF animals.

Figure 2.4

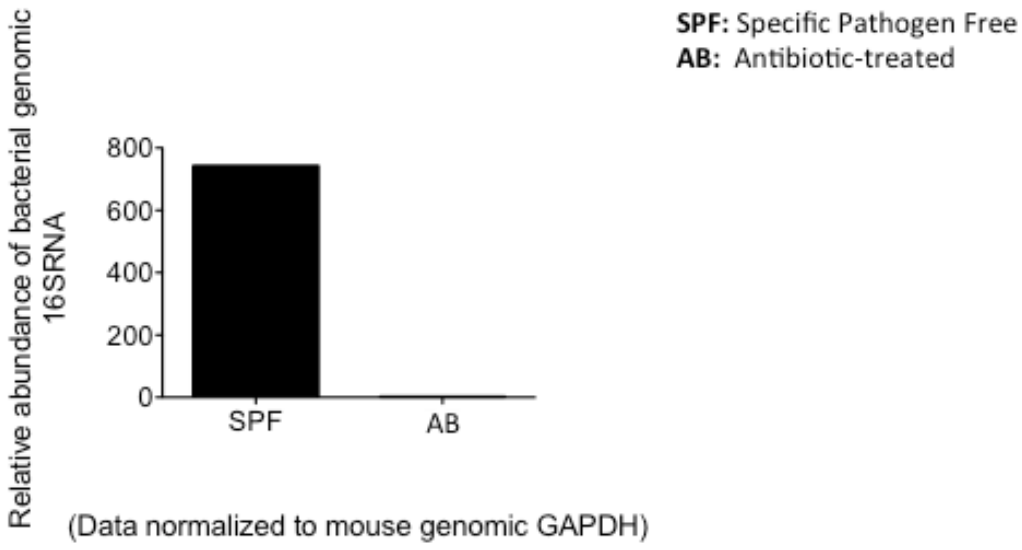


Figure 2.4: qPCR to determine the efficiency of antibiotic treatment

The bacterial load in the colonic contents of specific pathogen free (SPF) vs antibiotic-treated (AB) was quantified by determining the number of copies of bacterial 16S RNA by qPCR.

We observed that antibiotic-treated mice have a substantial reduction in the basal number of neutrophils both in the bone marrow and in the blood stream in the absence of any stimulation (Figure 2.5 A and B). However, when antibiotic-treated mice were challenged with zymosan, the total blood count of neutrophils was substantially higher than that of their SPF counterparts (Figure 2.5 C). Since this change in neutrophil number occurs rapidly, these data indicate that there is not actually a defect in the number of neutrophils present in the periphery of antibiotic-treated mice. Presumably there is a larger pool of marginated cells in the antibiotic-treated mice compared to control mice, which is then rapidly mobilized upon challenge with zymosan. Antibiotic-treated mice had similar percentages of mature neutrophils in the bone marrow and blood (before and after administration of zymosan) as SPF controls as assessed by their expression of Ly-6G and CD11b (Figure 2.6 A, B, and C). This suggested a role for intestinal flora in influencing the extravasation of neutrophils from the blood stream into the inflamed tissue site.

Neutrophil extravasation through blood vessels into tissues is facilitated by cell adhesion molecules expressed by neutrophils and the endothelium. Neutrophils in blood of antibiotic-treated animals showed similar or (higher) percentages and mean fluorescence intensity of cell adhesion molecules like CD44, CD62L, and the chemokine receptor, CXCR2 (Figure 2.7 A-F*).* We next examined if antibiotic-treated mice were able to recruit neutrophils when treated with macrophage inflammatory protein-2 (MIP-2), a chemotactic factor for neutrophils.

Figure 2.5

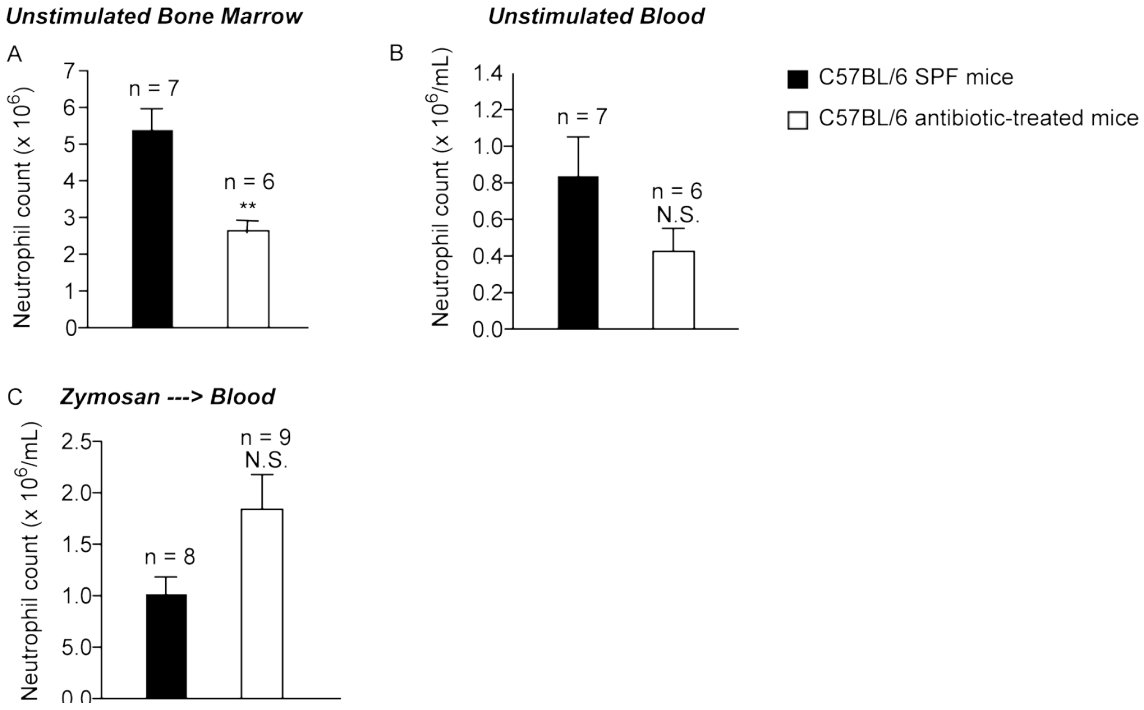


Figure 2.5: Antibiotic-treated mice appear to have a specific defect in the recruitment of neutrophils from the blood into the inflamed tissue

(A, B) Total number of neutrophils in the (A) bone marrow and (B) 1 mL blood in unstimulated SPF C57BL/6 and antibiotic-treated C57BL/6 mice. (C) Total number of neutrophils in 1 mL blood after 4 h of zymosan challenge. (D) Total number of neutrophils in the peritoneum of SPF C57BL/6 and antibiotic-treated C57BL/6 mice after intraperitoneal challenge with 5 ng of recombinant murine MIP-2. All data are combined results of three or more experiments and represented as mean ± SEM. n = Total number of mice used per datum group. ** P = 0.0022, Not significant (N.S.) versus SPF animals.

Figure 2.6

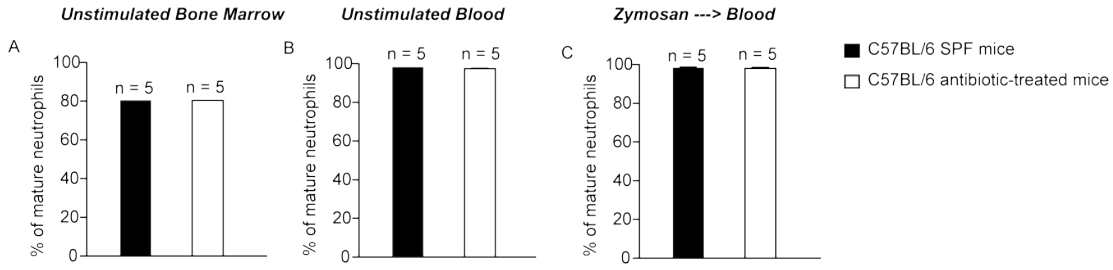


Figure 2.6: Antibiotic-treated mice do not have a defect in maturation of neutrophils in the bone marrow

(A, B, C) % of mature neutrophils in SPF C57BL/6 and antibiotic-treated C57BL/6 mice as assessed by their expression of Ly6G and CD11b by FACS analysis in (A) bone marrow of unstimulated mice (B) peripheral blood of unstimulated mice (C) peripheral blood of mice stimulated in 0.2mg zymosan. The % of mature neutrophils was calculated as total number of CD11b+Ly6G+ cells/total number of CD11b+Ly6G- and CD11b+Ly6G+ cells X 100. All data are combined results of two or more experiments and represented as mean ± SEM.

Figure 2.7

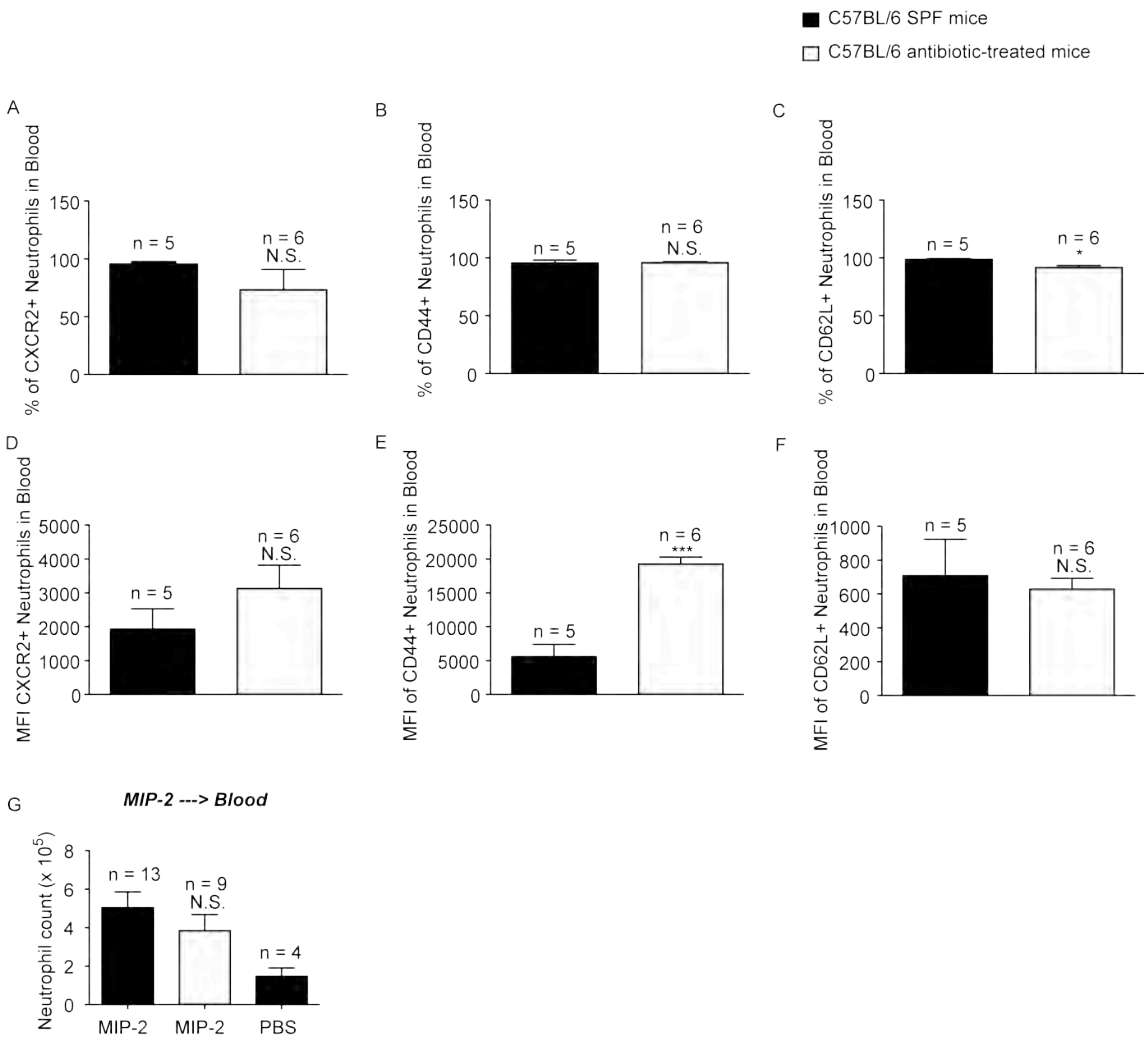


Figure 2.7: Neutrophils in antibiotic-treated mice are not defective in their expression of cell adhesion molecules and can extravasate in response to an intraperitoneal challenge with MIP-2

(A- C) % of neutrophils in blood from zymosan-stimulated SPF or antibiotic-treated C57BL/6 mice expressing (A) CXCR2 (B) CD44 (C) CD62L. (D- E) Mean

fluorescence intensity (MFI) of neutrophils in blood from zymosan-stimulated SPF or antibiotic-treated C57BL/6 mice for their expression of (D) CXCR2 (E) CD44 (F) CD62L. (G) Total number of neutrophils in the peritoneum of SPF C57BL/6 and antibiotic-treated C57BL/6 mice after intraperitoneal challenge with 5 ng of recombinant murine MIP-2. All data are combined results of three or more experiments and represented as mean ± SEM. n = Total number of mice used per datum group. *P= 0.0143, *** P = 0.0002, Not significant (N.S.) versus SPF animals.

We challenged the mice with purified recombinant MIP-2 protein in the amount that is produced in the peritoneum after zymosan challenge. We found these mice were able to mount a neutrophil response in the peritoneum as well as the SPF mice (Figure 2.7 G). Our data showing normal neutrophil migration to MIP-2 in antibiotic-treated mice is consistent with the observation that neutrophils from these mice had normal levels of CXCR2, since CXCR2 is the physiological receptor for MIP-2. This showed that mice lacking microbial flora do not have a generalized defect in the endothelial vasculature and also that neutrophils from these mice are functionally capable of migrating to the inflamed tissue upon receiving the appropriate signals.

The production of cytokines in antibiotic-treated mice.

In the peritoneum, resident macrophages have been shown to sense proinflammatory stimuli and produce cytokines that initiate inflammation[153]. Therefore, we quantified the number of resident macrophages (CD11b +, F4/80 + cells) in the peritoneum and found that they were similar in both SPF and antibiotic-treated mice (Figure 2.8 A). Moreover, peritoneal cells from antibiotic-treated mice were as efficient as those from SPF mice in their phagocytosis of zymosan (Figure 2.8 B), which was consistent with previous reports[209].

We next quantified the cytokines produced in the peritoneum in SPF and antibiotic-treated mice after they were injected with zymosan. Surprisingly, the peritoneal levels of the pro-inflammatory cytokines TNF-α and IL-6 as well as the neutrophil-recruiting chemokines MIP-2 and KC were significantly higher in zymosan-injected antibiotic-

treated mice as compared to their SPF controls (Figure 2.9 A-D). This showed that antibiotic-treated mice were capable of sensing zymosan and causing local activation of cells to produce inflammatory cytokines and that there was no apparent defect in this process. Even more surprising was the observation that when antibiotic-treated mice were injected an equivalent amount of purified MIP-2 as was made in situ, their neutrophil responses were equivalent to wild type mice. Taken together these results suggest upon injecting proinflammatory stimuli into antibiotic-treated mice, there is something that inhibits responses to the proinflammatory cytokines that are produced.

An earlier report had suggested that germ-free mice overproduced the inhibitory cytokine IL-10 and that this could inhibit immune responses[193]. Consistent with that study we found that antibiotic treated animals make 2-fold more IL-10 than SPF controls (Figure 2.10 A). To test whether the IL-10 was responsible for the reduced responses in the antibiotic-treated mice we pre-treated antibiotic treated mice with the Interleukin-10 receptor (IL-10R) neutralizing antibody. This treatment augmented responses in both control and antibiotic-treated mice, indicating that the antibody blocked the effect of IL-10 receptor signaling. However, blocking IL-10 receptor signaling in antibiotic-treated mice failed to restore neutrophilic recruitment to the level of IL-10R antibody-treated SPF controls after zymosan challenge (Figure 2.10 B). This shows that inhibiting IL-10R in general enhances neutrophil numbers on an inflammatory challenge, even in SPF controls, but that production of IL-10 does not fully account for the reduced inflammation phenotype observed in antibiotic-treated mice.

Figure 2.8

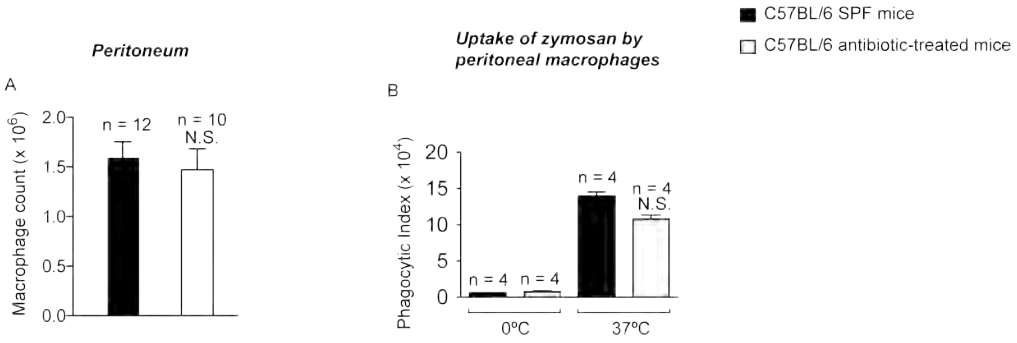


Figure 2.8: Antibiotic-treated mice do not have a defect in either the numbers of resident peritoneal macrophages or their capacity to phagocytose zymosan

(A) Total number of resident peritoneal macrophages in unstimulated SPF C57BL/6 and antibiotic-treated C57BL/6 mice. Numbers of resident macrophages was determined by multiplying total cell number by percentage of $CD11b^+$ $F4/80^+$ cells. (B) Efficiency of phagocytosis of zymosan by resident peritoneal macrophages isolated from unstimulated SPF C57BL/6 and antibiotic-treated C57BL/6 mice. Peritoneal cells were incubated with FITC-conjugated zymosan solution for 45 min at 37°C. The reaction was terminated by transferring the plate to 4°C. In the control group, cells were incubated with zymosan at 0°C throughout the incubation. After washing unbound zymosan, cells were scraped off the plate and resuspended in tryphan blue to quench cell surface-bound zymosan. The efficiency of phagocytosis was termed as 'phagocytosis index,' which was calculated as % of macrophages that have taken up zymosan X MFI of macrophages. n = Total number of mice used per datum group. All data are combined results of two or more experiments and represented as mean ± SEM.

Figure 2.9

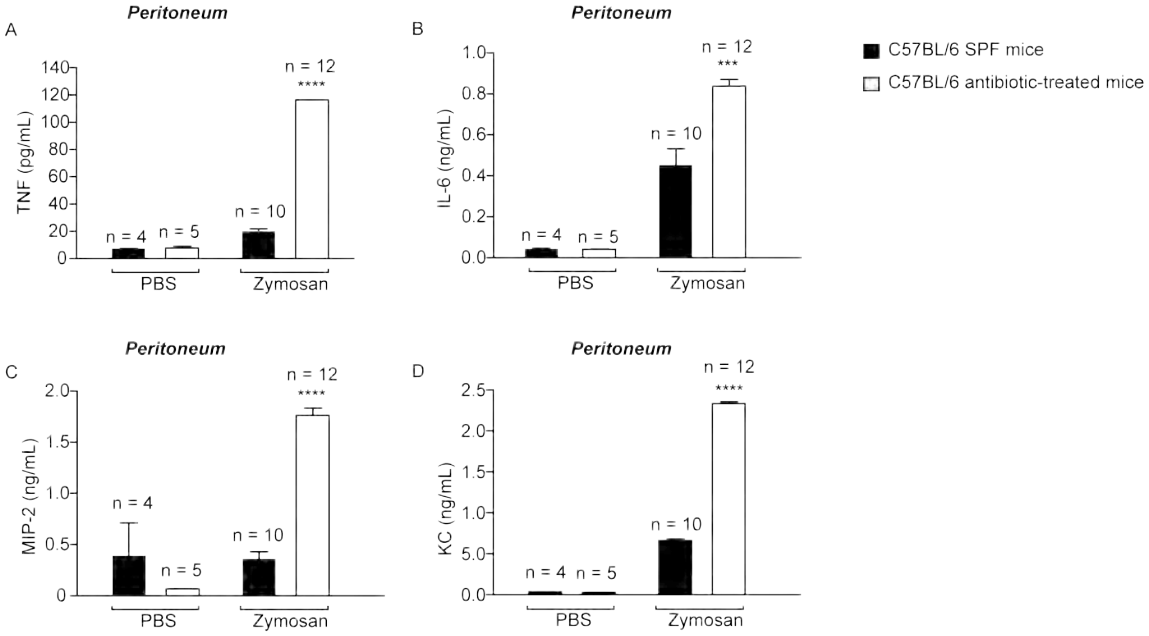


Figure 2.9: Reduced neutrophil recruitment in antibiotic-treated mice is not due to lower production of pro-inflammatory cytokines in the peritoneum

(A - D) SPF C57BL/6 and antibiotic-treated C57BL/6 mice were challenged with zymosan intraperitoneally and 4h post-injection lavaged with 2ml buffer. The concentrations of (A) TNF-α (B) IL-6 (C) MIP-2 (D) KC in the lavage buffer was quantified by ELISA. All data are combined results of three or more experiments and represented as mean ± SEM. * $P < 0.0001$, ** $P = 0.0006$ versus SPF animals.

Figure 2.10

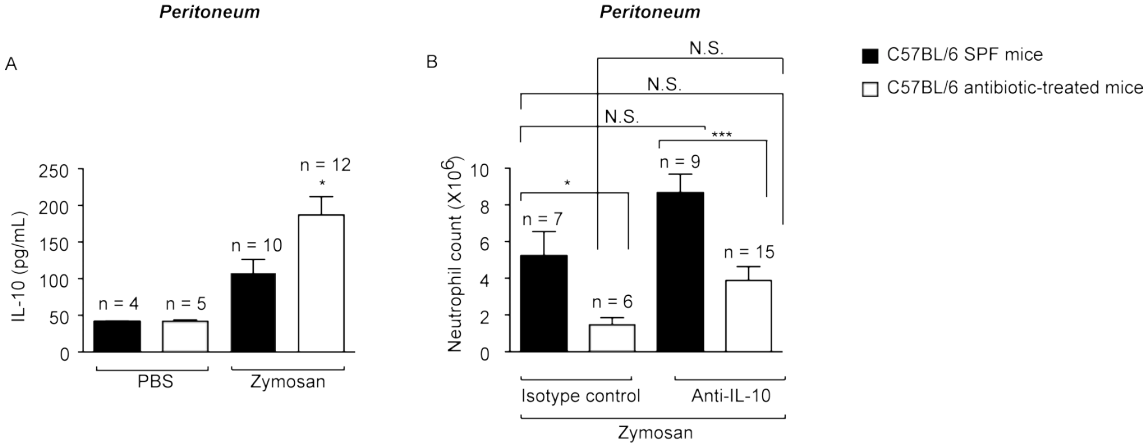


Figure 2.10: **Reduced neutrophil recruitment in antibiotic-treated mice is not due to higher production of IL-10 in the peritoneum**

(A) SPF C57BL/6 and antibiotic-treated C57BL/6 mice were challenged with zymosan intraperitoneally and 4h post-injection lavaged with 2ml buffer. The concentration of IL-10 in the lavage buffer was quantified by ELISA. (B) Mice were pre-treated twice with either IL-10 receptor neutralizing antibody or control antibody before challenging them intraperitoneally with 0.2mg of zymosan. Mice were lavaged after 4h and the numbers of neutrophils in the peritoneum was quantified by flow cytometry. All data are combined results of two or more experiments and represented as mean ± SEM. * P =0.04, ** P = 0.04, *** P = 0.001, Not significant (N.S.) versus SPF animals.

Intestinal flora prime neutrophilic inflammation via MyD88 signaling pathway.

We hypothesized that microbiota mediate their effects on inflammatory response by activating pattern recognition receptor (PRR)-signaling pathways. To test this hypothesis, we analyzed mice deficient in the known PRR pathways and examined their ability to mount neutrophil response to an intraperitoneal zymosan challenge. Receptor interacting protein-2 (RIP-2) knockout mice, which are defective in nucleotide-binding, oligomerisation domain-containing protein-1 (NOD1) or NOD2 signaling, were able to respond normally to zymosan (Figure 2.11 A). Similar results were obtained in mitochondrial antiviral signaling (MAVS) knockout mice, which were defective in retinoic acid-inducible gene-I (RIG-I) and melanoma differentiation-associated gene 5 (MDA5) signaling. Similarly, no defects were found in mice lacking caspase 1, NLRP3, and ASC knockout mice, which are defective in inflammasome activation (Figure 2.11 A). However, myeloid differentiation primary response gene (MYD88) knockout mice showed a markedly reduced recruitment of neutrophils following zymosan stimulation (Figure 2.11 B), similar to that observed in the antibiotic-treated mice. Similar to antibiotic-treated animals, MyD88 knockout mice had a basal impairment in the number of neutrophils in the bone marrow and blood (Figure 2.12 A, B**).** Furthermore, on challenge with zymosan, neutrophils in the bloodstream of *Myd88*$^{-/-}$ mice outnumbered that in wild-type mice (Figure 2.12 C). This showed that MyD88 knockout mice had a possible defect in the specific passage of neutrophils from the blood to the site of injury.

Figure 2.11

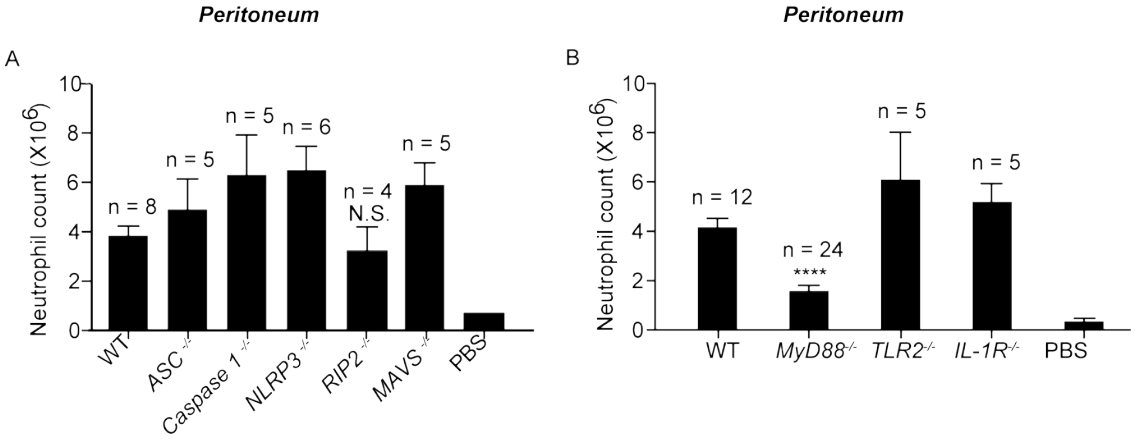


Figure 2.11: Zymosan-induced neutrophil recruitment requires MyD88 signaling but is independent of TLR2/4 or IL-1R signaling

(A, B) Total neutrophil numbers in the peritoneum of (**A**) SPF C57BL/6 (WT), *ASC*$^{-/-}$, *Caspase 1*$^{-/-}$, *NLRP3*$^{-/-}$, *RIP2*$^{-/-}$, and *MAVS*$^{-/-}$ mice and (B) SPF C57BL/6 (WT), *MyD88*$^{-/-}$, *TLR2*$^{-/-}$, and *IL-1R*$^{-/-}$ mice. Mice were injected with 0.2mg zymosan and lavaged after 4 h. Numbers of neutrophils was determined by multiplying total cell number by percentage of Ly-6G^{+} 7/4^{+}. All data are combined results of two or more experiments and represented as mean ± SEM. n = Total number of mice used per datum group. The PBS group represents SPF C57BL/6 mice challenged with PBS and lavaged after 4h. * $P < 0.0001$, Not significant (N.S.) versus SPF animals.

Figure 2.12

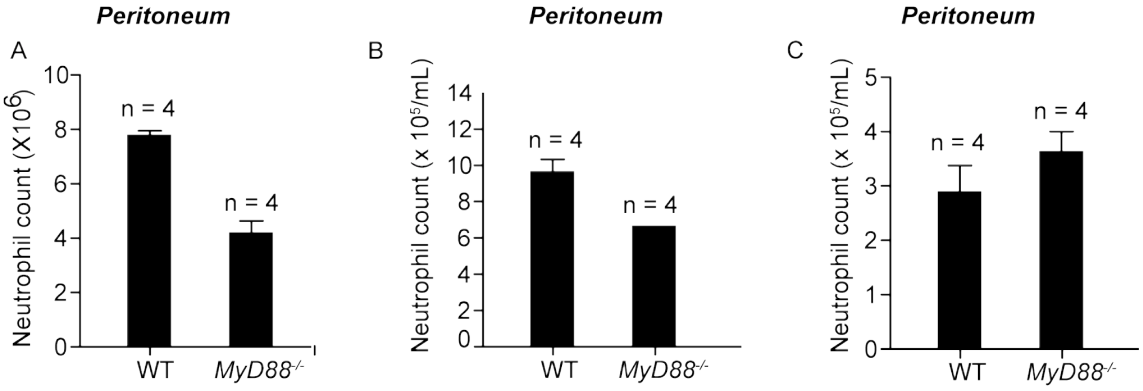


Figure 2.12: *MyD88*$^{-/-}$ mice have a specific defect in extravasation of neutrophils from the bloodstream to the site of injury

(A, B) Total number of neutrophils in the (A) bone marrow and (B) 100µL blood in unstimulated wild type (WT) and *MyD88*$^{-/-}$ mice. (C) Total number of neutrophils in 100µL blood after 4h of zymosan challenge. All data are combined results of two or more experiments and represented as mean ± SEM.

MyD88 is an adaptor protein for TLRs and some cytokine receptors, most notably the IL-1R. TLR2 has been reported to be one of the receptors for zymosan and it was possible that this was why MyD88 was required for the inflammatory response to this agent[210]. However, we found that TLR2-deficient mice had a normal zymosan-induced infiltration of neutrophils in the peritoneum (Figure 2.11 B). IL-1 is a proinflammatory cytokine that plays a key role in recruiting neutrophils to sites of inflammation in response to some inflammatory stimuli. However, we had previously shown that the neutrophilic inflammatory response to zymosan does not require the IL-1R and we confirmed again that this was the case (Figure 2.11 B). Therefore the requirement for Myd88 in the response to zymosan was not as an adaptor protein for TLR2 or IL-1R signaling.

We next sought to determine whether MyD88 was needed during the elicitation of the inflammatory response or was needed earlier to somehow condition the innate immune response so as to be responsive to the proinflammatory stimulus. We observed that intestinal flora influences acute inflammation during the initial development of mouse immune system because adult 6-week old mice treated with antibiotics did not show a defect in neutrophil migration (data not shown), unlike animals treated with antibiotics right from birth. Hence, we hypothesized that the expression of MyD88 in tissues is essential during immune development for commensal flora-induced priming but the presence of MyD88 is dispensable during the actual inflammatory challenge. To test this hypothesis, we used the *MyD88* $^{flox/-}$ *ROSA26-Cre/Esr*$^{+/-}$ (cKO) mice [211] to conditionally eliminate MyD88 just before challenge with zymosan. In these mice, one

allele of the gene had been deleted from the germline while the other could be inducibly deleted globally by the administration of tamoxifen. Mice were treated with tamoxifen for three alternate days and challenged with zymosan a week after the last tamoxifen injection. Therefore, in these mice *MyD88* was reduced at the time of zymosan injection, but present during the maturation of the immune system. Upon administration of tamoxifen, *MyD88* was deleted as assessed by QPCR, as described previously [212] (Table 2.1). cKO mice treated with tamoxifen (one week before zymosan challenge) responded as well as untreated mice to zymosan-induced peritonitis (Figure 2.13 A). These results showed that the presence of MyD88 is not essential for the signaling initiated by zymosan. While the deletion of *MyD88* was partial in these animals, they showed reduced neutrophil recruitment to LPS, confirming the role of the TLR4-MyD88 pathway in detecting LPS and also validating that the deletion was sufficient to impair responses (Figure 2.13 B). In contrast, tamoxifen treatment of wild type mice did not impair responses (data not shown). On the other hand, when cKO mice when treated with tamoxifen from Day 0 of birth, these mice exhibited reduced neutrophil recruitment to zymosan as compared to untreated mice (Figure 2.13 C). These results supported our hypothesis that for inflammatory ligands like zymosan, MyD88 is required during the pre-challenge phase for activation of immune cells but is dispensable during the actual inflammatory challenge.

Table 2.1

Tamoxifen Treatment	Tissue	ΔΔCt	Residual myd88^{flox}	% Deletion
Starting from birth	Blood	1.625	0.324	67.57
	Peritoneal cells	1.835	0.280	71.97
Starting from a week before zymosan challenge	Blood	0.97	0.510	48.94
	Peritoneal cells	0.923	0.527	47.25

Table 2.1: Deletion efficiency in *Myd88$^{flox/-}$ Rosa26-Cre/ESR$^{+/-}$* mice (cKO)
The amount of residual floxed MyD88 after treatment with tamoxifen was determined by qPCR.

Figure 2.13

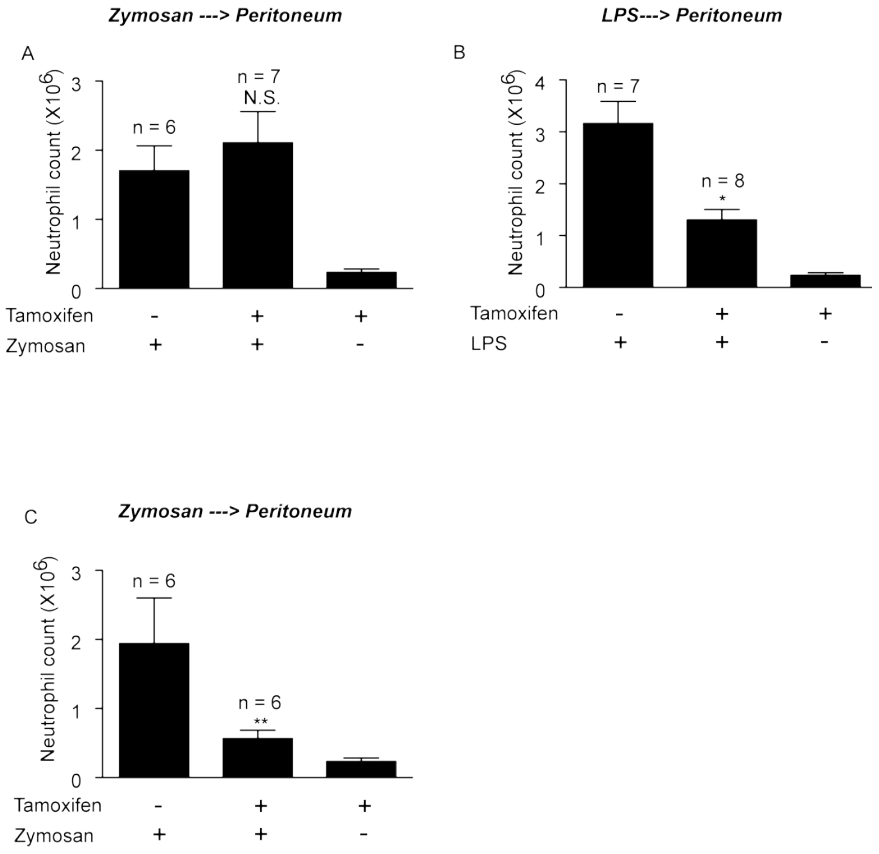


Figure 2.13: MyD88 is required during the conditioning of the immune system for a subsequent zymosan challenge rather than during the actual zymosan challenge

(A- B) Total neutrophil numbers in the peritoneum of *MyD88*$^{-/flox}$ *Rosa26-Cre/ESR*$^{+/-}$ (cKO) which have been treated with tamoxifen or corn oil (vehicle control) one week before challenge with (A) 0.2 mg zymosan and (B) 100 ng LPS. (C) Total neutrophil numbers in the peritoneum of *MyD88*$^{-/flox}$ *Rosa26-Cre/ESR*$^{+/-}$ (cKO) which have been treated with tamoxifen or corn oil (vehicle control) starting from birth and challenged with 0.2 mg zymosan at 7 weeks of age. Mice were lavaged at 4 h after injection with

zymosan or LPS and the numbers of neutrophils in the peritoneum was quantified by flow cytometry. All data are combined results of two experiments and represented as mean ± SEM. n = Total number of mice used per datum group. The PBS group represents cKO mice treated with tamoxifen and challenged with PBS and lavaged after 4 h. * P = 0.04, ** P = 0.004, Not significant (N.S.) versus cKO animals that were treated with vehicle control and injected with zymosan or LPS.

We hypothesized that microbial flora was functioning in our system as a source of PAMPs that stimulated the TLR-MyD88-pathway in ways that made the host responsive

to the proinflammatory stimuli. This argument was supported by our observation that when mice were treated with antibiotics starting from birth for 45 days, they had lowered neutrophil migration, but 6-week old mice treated with antibiotics for the same duration (45 days) did not show a similar defect in neutrophil migration (data not shown). This finding suggested that initial exposure to microbes or microbial ligands might be sufficient to prime neutrophil responses. To test this hypothesis, we sought to determine if MyD88 activation by a purified microbial ligand is sufficient to restore neutrophilic inflammation to zymosan in antibiotic-treated mice. We added pure lipopolysaccharide (LPS) from *E.coli* into the drinking water of mice from 3-5 weeks of age in addition to the antibiotic cocktail. We found that antibiotic-treated mice, which received LPS for 2 weeks, were able to respond to zymosan as well as their SPF counterparts (Figure 2.14). On the other hand, antibiotic-treated MyD88 knockout mice did not show this restoration in inflammation on LPS administration (Figure 2.14). This shows that MyD88 is required for the downstream signaling initiated by LPS, which enables acute inflammation.

Figure 2.14

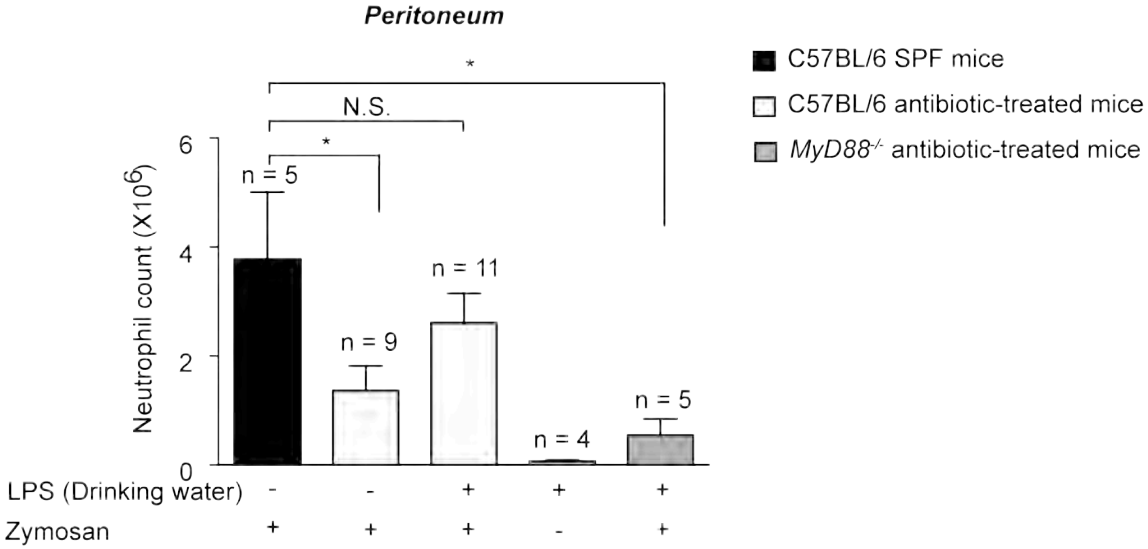


Figure 2.14: LPS pre-treatment in antibiotic-treated mice prior to zymosan challenge restores neutrophilic inflammation in process dependent upon MyD88-signaling

Total neutrophil numbers after zymosan injection in the peritoneum of SPF C57BL/6 and antibiotic-treated C57BL/6 mice and antibiotic-treated *MyD88*$^{-/-}$ mice in the presence of absence of LPS pre-stimulation. LPS from *Escherichia coli* was administered in the drinking water of mice at a concentration of 33 mg/L from 3-5 weeks of age. At 7 weeks of age, mice injected with 0.2mg zymosan and lavaged after 4h. In one group, antibiotic-treated mice were pre-treated with LPS in the drinking water but did not receive zymosan injection. All data are combined results of two or more experiments and represented as mean ± SEM. * P =0.03, Not significant (N.S.) versus SPF animals.

2.3. Discussion

One of the major findings of this study is that for neutrophil-mediated acute inflammation to several proinflammatory agents, the immune system needs to be previously stimulated by intestinal flora in a MyD88-dependent fashion (Figure 2.15). This stimulation enables the host to mount a neutrophil response to future inflammatory insults. We have shown that germ-free and antibiotic-treated mice are defective in neutrophil migration to a number of different microbial and sterile inflammatory ligands. This defect can be corrected by supplementing the drinking water with LPS, a TLR4-MyD88 agonist, prior to challenge with the inflammatory agent. Furthermore, pre-treatment of antibiotic-treated MyD88 knockout mice with LPS failed to restore neutrophilic infiltration, showing that LPS specifically acts through MyD88 to prime the immune system.

A previous report published by our laboratory had shown that MyD88 knockout mice do not show a defect in zymosan-induced neutrophil migration[197]. The basis for this discrepancy is unclear. One likely possibility is that the mice used in the previous study were inadvertently exposed to some TLR stimulation, such as LPS, e.g. in their housing environment, drinking water or chow; as we show here, exposure to LPS and presumably other TLR agonists p.o. for a short period is sufficient to reverse the phenotype of antibiotic-treated mice that of conventional mice. It is also possible that this difference was due to the extent of backcrossing of the MyD88-deficient mice; the mice in the present study were fully backcrossed onto the B6 background whereas those in the earlier study were not.

Figure 2.15

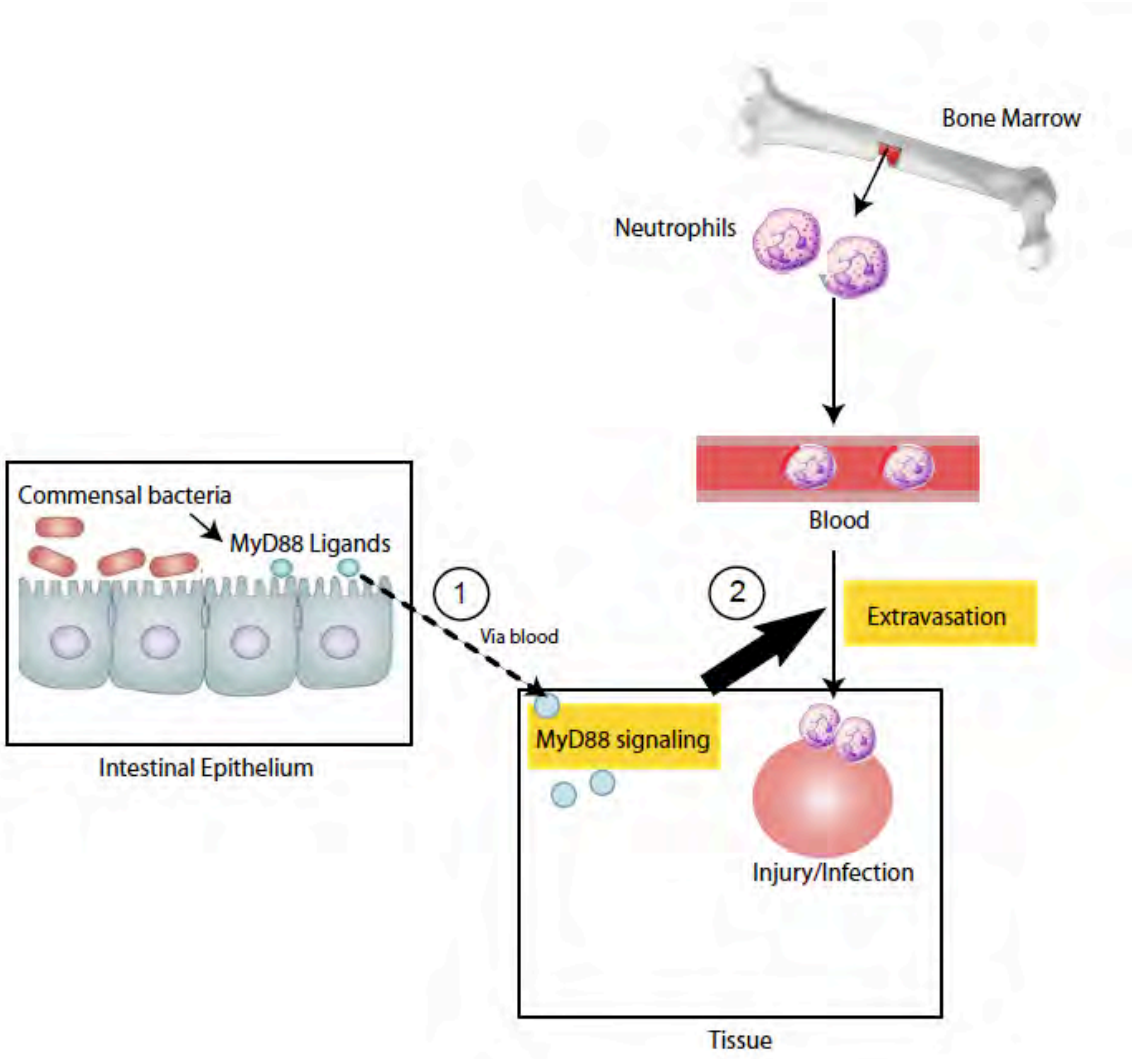


Figure 2. 15: Model depicting the role of gut flora in regulating the acute inflammatory response
(1) Bacterial ligands derived from intestinal flora enter tissues via the bloodstream, where they prime tissue-resident cells through MyD88 signaling
(2) This MyD88-dependent priming enables the extravasation of neutrophils into the tissue upon receiving an inflammatory signal.

While there is a clear role for MyD88 in the ability of conventional mice to mount neutrophilic inflammation to zymosan, we found that several other innate immune signaling pathways were not required for this response. Although Clarke et al. have reported that commensal bacteria prime neutrophils via NOD1 signaling in ways that enhance their phagocytic potential to various bacteria[191], we found that RIP2 knockout mice did not show reduced inflammation to zymosan. Since RIP2 is required for NOD1/2 signaling, this finding argued against a role for either NOD1 or NOD2 in mediating gut flora-induced priming[134]. Therefore, NOD1/2 signaling may be important for phagocytosis but is not needed for neutrophilic inflammation to this agent. Similarly, we found little contribution of the inflammasome components (Nalp3/Asc/caspase-1) or the RNA-sensing RIG-I Like Receptors (RLRs) in mediating zymosan-induced inflammation. Hence, we show that intestinal flora prime the immune system, specifically via MyD88 pathway.

Recent studies by Ganal *et al.* and Abt *et al.* also show that intestinal flora prime tissue-resident cells so that they are better equipped to mount a future immune response[213,214]. By employing a genome-wide transcriptional profile, Abt *et al.* reported that peritoneal macrophages from untreated antibiotic-treated mice show lower expression of genes involved in antiviral immunity as compared to those from SPF mice[213]. Ganal *et al*. further show that in TLR-stimulated splenic DCs isolated from germ free mice, production and nuclear translocation of NF-κB and IRF3 was unimpaired, but they failed to bind to their respective cytokine promoters[214]. Consequently, the germ free mice-derived splenic DCs made less IFN-I than their SPF counterparts after poly

(I:C)/LPS stimulation. Although our study supports the general notion of gut flora priming the systemic immunity, we found that antibiotic-treated mice are not impaired in their production of inflammatory cytokines (like TNFα, MIP-2, and KC) in the peritoneum after zymosan challenge. In fact, we found significantly higher amounts of proinflammatory cytokines including MIP-2 and KC in the peritoneal fluid in the antibiotic-treated mice as compared to the SPF mice.

The finding that the antibiotic-treated mice were producing even higher levels of MIP-2 (and other proinflammatory cytokines) than control mice, yet were defective in inflammation, was surprising because these animals showed normal inflammatory responses to injected MIP-2. This indicated that upon zymosan challenge something was interfering with the ability of the antibiotic-treated mice to respond to the cytokines they were producing. This is reminiscent of some earlier studies by Souza et al., Fagundes et al., and Amaral et al. that found that germ free mice overproduced IL-10, which could inhibit immune responses[192-194]. They show that neutralizing IL-10 with antibodies could restore responses in germ free mice to levels of conventional mice. However, in our system, while anti-IL-10 did increase responses in the antibiotic-treated mice, it did not restore their responses to the level of control mice treated with anti-IL10. Therefore, our data indicate that there must be other anti-inflammatory factors that are inhibiting responses in the absence of microbial stimulation. This anti-inflammatory activity affects inflammation by multiple stimuli including ones that are IL-1-dependent (e.g. silica) and IL-1-independent (zymosan). It will be of interest in future studies to define the nature of this activity.

An implication of our study is that the set point of the unperturbed innate immune system maybe anti-inflammatory for many stimuli. However, in conventionally reared mice the immune system is perturbed by exposure to microbial flora. As part of this process MyD88-dependent pattern recognition receptor signaling by microbial flora appears to alter this set point in ways that promote inflammatory responses.

In summary, we postulate that TLR ligands derived from the intestinal flora constitutively enter the blood and into tissues. Here, they prime tissue-resident cells via MyD88-signaling, so that they provide appropriate stimulatory signals for neutrophil migration to future inflammatory insults. This study provides insights on how intestinal flora affects systemic immunity and specifically innate responses against pathogens and sterile agents.

2.4. Material and Methods

Mouse Strains: SPF C57BL/6 mice and *IL-1R*$^{-/-}$ mice were purchased from The Jackson Laboratories (Bar Harbor, ME). Germ-free C57BL/6 mice were obtained from The National Gnotobiotic Rodent Resource Center (P40RR018603 and P30 DK 34987), North Carolina State University Gnotobiotics Unit and Gnotobiotic Research Resource, Medical University of South Carolina. *MyD88*$^{-/-}$ mice were provided by Dr. Shizuo Akira, Osaka University, Osaka, Japan or purchased from The Jackson Laboratories. *RIP2*$^{-/-}$ mice were provided by Dr. Michelle Kelliher and *RIG-I*$^{-/-}$ and *MDA5*$^{-/-}$ mice were provided by Dr. Kate Fitzgerald (University of Massachusetts Medical School, Worcester). *NOD1*$^{-/-}$ mice were provided by Dr. Grace Chen, University of Michigan,

Ann Arbor, MI. For generating the tamoxifen-inducible deletion mutant mice of *MyD88*, we used a strategy similar to the one described previously [211]. *MyD88*$^{-/-}$ mice were crossed to the whole tissue, tamoxifen-inducible Cre transgenic mice *(Rosa26-Cre/ESR*$^{+/+}$*)* (provided by Dr. Roger Davis, University of Massachusetts Medical School, Worcester). The resultant offsprings, *MyD88*$^{+/-}$ *Rosa26-Cre/ESR*$^{+/-}$ mice were crossed to the *MyD88*$^{flox/flox}$ mice (provided by Dr. Robert Finberg, University of Massachusetts Medical School, Worcester) to generate the *MyD88*$^{-/flox}$ *Rosa26-Cre/ESR*$^{+/-}$ (cKO). Animals were housed and handled according to protocols approved by the University of Massachusetts animal care and use committee.

Peritonitis: Mice were injected intraperitoneally with 0.2 mg zymosan (Sigma-Aldrich, St. Louis, MO) or 0.5 mg silica crystals (Sigma-Aldrich, St. Louis, MO) or 0.5 mg MSU crystals or 1 ug recombinant mouse MIP-2 (R&D, Minneapolis, MN) in 0.2 ml PBS. For the thioglycollate injections, 1 ml of 3% thioglycollate (Thermoscientific, Lenexa, KS) was used. MSU crystals were prepared as described before [215]. 4-16 h after the injection, mice were euthanized by exposure to isoflourane. The peritoneum was lavaged with 2 mL DMEM with 2% FCS, 3 mM EDTA, and 10 U/ml heparin. The peritoneal cells were stained with antibodies against mouse Ly-6G, CD11b (both from BD Bioscience, San Jose, CA), 7/4, F4/80 (both from eBioscience, San Diego, CA). The percentage and absolute numbers of different cell types was determined by flow cytometric analysis and cell counting beads (Life Technologies, Grand Island, NY). FACS analysis was performed using a BD Biosciences LSRII Flow cytometer and FlowJo (Tree Star) analysis software. Supernatants from peritoneal lavage were used

for cytokine quantitation by ELISA. ELISAs for the cytokines TNFα, and IL-10 were performed using kits purchased from eBioscience and those for MIP-2 and KC were performed using kit from R&D (Minneapolis, MN). In other experiments, cells from bone marrow and blood were analyzed and quantified by flow cytometry.

Intestinal flora depletion using antibiotics: C57BL/6 and *MyD88*$^{-/-}$ mice were treated with a cocktail of broad- spectrum antibiotics in their drinking water starting from birth to the time they were used in experiments as described before [208]. The antibiotic cocktail consisted of ampicillin 1 g/L, neomycin 1 g/L, metronidazole 1 g/L (Sigma-Aldrich, St. Louis, MO), and vancomycin 0.5 g/L (PhytoTechnology Laboratories, KS). The artificial aspartame sweetener, Equal (Merisant Company, Chicago, IL) was added to the water 5 g/L to make it palatable for the mice to drink. Pups received the antibiotics indirectly via lactating mothers till they were weaned. Drinking water containing the antibiotics was replaced every week.

16S rRNA quantification: DNA was isolated from colonic contents of mice by the DNeasy Blood and Tissue Kit (Quigen, Hilden, Germany). The qPCR primers used to amplify the bacterial 16S V2 region were sense, 5'- AGYGGCGIACGGGTGAGTAA; and antisense, 5'- CYIACTGCTGCCTCCCGTAG. qPCR primers used to amplify the housekeeping gene GAPDH were sense 5'-TGATGGGTGTGAACCACGAG; and antisense 5'- TCAGTGTAGCCCAAGATGCC-3. qPCR reaction was performed using the iQ SYBR Green supermix on the CFX96 Touch Bio-rad machine. The PCR cycling reaction used was 15 min activation step (95°C); 35 cycles of 30 s denaturation (95°C), 30 s annealing (60°), and 30 s extension (72°C).

LPS treatment: LPS from *Escherichia coli,* serotype 026:B6, purified by gel-filtration chromatograph (Sigma Aldrich, St. Louis, MO) was administered in the drinking water of mice at a concentration of 33 mg/L from 3-5 weeks of age.

Antibody neutralizing experiments: Mice were treated with an intraperitoneal injection of IL-10R antibody (Clone 1B1.3A) or control antibody ((HRPN), both purchased from Bio X Cell (New Lebanon, NH)), 16 h and 3 h before zymosan challenge, 0.25 mg antibody per mouse each time. Mice were lavaged 4 h after zymosan injection and analyzed for neutrophil numbers in their peritoneum as described before.

Tamoxifen-induced deletion of MYD88: Tamoxifen (Sigma-Aldrich, St. Louis, MO) solution was prepared in corn oil (Sigma-Aldrich, St. Louis, MO) at 10 mg/mL by incubating at 37ºC for 2 h. To induce deletion of floxed genes in adult mice, tamoxifen (50 mg/kg of body weight) was administered to floxed mice by oral gavage for three alternate days. Mice were used in experiments 7 days after the last administration. For treating pups, lactating mothers were treated intraperitoneally with tamoxifen (200 mg/kg of body weight) from the day of birth for 5 consecutive days.

QPCR to detect deletion of floxed MYD88 gene: The efficiency of deletion of floxed *MyD88* allele was assessed using Taqman PCR using primers and method described previously [212]. The PCR cycling reaction was performed on the C1000 Thermal Cycler (Bio-Rad).

Phagocytosis Assay: Efficiency of phagocytosis by peritoneal cells was assessed as described before [216]. Briefly, FITC-conjugated zymosan (0.8 mg/ml) was prepared in DMEM + 20% FBS. Peritoneal cells plated at 0.5 million cells/well of a 48-well plate

were incubated with 500 µl of the FITC-conjugated zymosan solution for 45 min at 37°C. The reaction was terminated by transferring the plate to 0°C. The uningested zymosan was removed by washing wells with HBSS. Cells were scraped off the plate and resuspended in 2 mg/ml trypan blue to quench cell surface-bound zymosan. In the control group, cells were incubated with zymosan at 0°C throughout the incubation. The efficiency of phagocytosis, 'phagocytosis index,' was calculated as % of F4/80 cells that were FITC+ X MFI of F4/80 cells.

Statistical analyses: Data are reported as means ± SEM. Statistical analysis in each independent experiment was performed with an unpaired, two-tailed Student *t* test.

CHAPTER 3

IDENTIFICATION OF THE CELLULAR SOURCE OF IL-1 IN CELL DEATH-INDUCED INFLAMMATION

Attributions and copyright information

This chapter of the thesis has been published in The Journal of Immunology.

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Author Contributions:

Figure 3.1, 3.4, 3.5 A, B: DK
Figure 3.2, 3.3, 3.4 C, 3.6: HK

3.1. Introduction

Cell death is a potent activator of inflammation[82]. The discovery of cell injury and cell death as triggers of the inflammatory response happened long before that of microbe-stimulated inflammation. However, as compared to pathogen-driven inflammatory responses, very little is known about how cell death triggers inflammation.

Several explanations have been proposed to explain why cell necrosis is a likely inducer of inflammation. Firstly, cell death could be a consequence of an invading pathogen. Some bacteria like *Mycobacterium tuberculosis* and *Shigella flexneri* induce cell death in the host tissue[217,218]. Hence, by recruiting neutrophils to the site of cell death, the host immune system is actually helping in clearing the infection. Secondly, cell death may be an indicator of some "unhealthy" cellular condition like tumor. In all these cases, the inflammatory response helps to limit the extent of prevailing damage by diluting away the injurious agents, removing the noxious stimuli by promoting phagocytosis of the cell debris and/or walling off the problem.

In some cases, the cell death-mediated inflammatory response might prove to be a double-edged sword to the host. In the ensuing inflammatory response, the resident and recruited cells and their secreted molecules help in eliminating or containing the tissue injury and promoting tissue regeneration. But often this response is non-specific,

leading to bystander tissue damage. This is evident from the fact that cell death-induced inflammation contributes to pathogenesis of various diseases like myocardial infarction, and trauma injury[219]. Hence, an in-depth understanding of this process becomes critical in identifying therapeutic targets for these diseases.

The inflammatory response to cell death is mediated by a number of endogenous molecules. These non-microbial, host cell-derived ligands are called Danger Associated Molecular Patterns (DAMPs). Such molecules are normally encased in the plasma membrane, hence hidden from immune surveillance. Some of the known DAMPs have been described in the introduction of this thesis. Different DAMPs may be more or less contributory depending upon the type of injury and the type of tissue involved.

To study how cell death signals the recruitment of neutrophils, mice genetically lacking different receptors and signaling molecules were used[197]. The TLRs were potential candidates for sensing cell death because of their ability to detect a variety of bacteria-derived ligands and their wide tissue expression. Furthermore, they are located either on the cell surface or on the endosomal membrane, which places them in a strategic location to detect injured tissue or phagocytized dead cells. However, the single knockout mice for TLR1, TLR3, TLR6, TLR7, TLR9, and TLR11 were as efficient as wild type mice in recruiting neutrophil migration to dead cells. Mice doubly deficient in

TLR2 and TLR4 showed a modest decrease in dead cell-induced neutrophil infiltration. Therefore, it was found that individual TLRs played only a minor role in dead cell-induced acute inflammation.

Next, the role of the TLR adaptor proteins was assessed by using mice deficient in these proteins. Single knockout mice of TIRAP, TRIF, and TRAM showed no defect in dead cell-elicited inflammation. However, MyD88 was essential for the inflammatory response to dead cells. MyD88 is an adaptor protein for the TLR, IL-1R, and IL-18 signaling pathways. Given the modest contribution of TLRs to dead cell-induced inflammation, the role of IL-1R and IL-18 was examined. It was found that IL-1R deficient animals had a profound reduction in neutrophil recruitment to dead cells. The role of IL-1 signaling was further confirmed by using mice doubly deficient in IL-1α and IL-1β.

The term IL-1 refers to a group of three polypeptides, IL-1α, IL-1β and IL-1 receptor antagonist (IL-1Ra) each of which is encoded by a separate but homologous gene[220,221]. Both IL-1α and IL-1β signal through the same receptor IL-1R1 and have identical biological activities in solution. IL-1Ra is a competitive inhibitor of IL-1α and IL-1β for receptor binding[222]. IL-1α is cell membrane-bound and is believed to act locally. Both IL-1 α and IL-1β have wide tissue expression including hematopoietic cells like macrophages and monocyte and non-hematopoietic cells like keratinocytes and

endothelial cells. IL-1 promotes the initial inflammatory response by triggering the release of histamine from mast cells. In addition, they act as potent endogenous pyrogens by stimulating the synthesis of prostaglandin E2 from the vascular endothelium of hypothalamus. It also stimulates the production of collagenase and PGE2 by synovial cells[223]. The latter has been attributed to the joint damage observed in the chronic inflammatory disease, rheumatoid arthritis[223].

Despite similar functions, there are differences in the expression of IL-1α and IL-1β and how the activity of these molecules is regulated. IL-1β is secreted and circulates systematically. Secondly, IL-1β has an amino terminal pro-domain which needs to be cleaved by the inflammasomes, to generate the functional protein. In contrast, IL-1α has an amino terminal pro-domain, which is cleaved by proteases, but this is not required for its biological activity[221]

IL-1R is expressed on many cell types, including both leukocytes and parenchymal cells such as epithelium, endothelium, fibroblasts, and smooth muscle cells[224]. IL-1α or Il-1β bind to the cytosolic immunoglobulin, ligand-binding domain of the receptor. This leads to the recruitment of a second receptor subunit, the IL-1R accessory protein (IL-1RAcP). The formation of the receptor heterodimer induces signaling, by recruitment of downstream signaling proteins like myeloid differentiation primary response protein (MyD88), through the cytosolic Toll/IL-1 receptor (TIR) domain[225]. Because of its potency and extensive functions, the activity of IL-1α and IL-1β is tightly controlled.

Since IL-1 has a wide tissue expression, further studies to establish the cellular source of IL-1 were needed to unravel the mechanism of cell death-triggered inflammatory response. Experiments described in this section, helped address this question.

3.2. Results

Dying cells are not a significant source of IL-1 during cell-death induced inflammatory response

Both IL-1α and IL-1β are expressed by a variety of tissues[226]. Expression of both the isoforms is enhanced by NF-κB-induced transcriptional upregulation to generate a 31-kDa precursor protein. Pro-IL-1β is further processed by the inflammasome machinery to generate its secreted, functionally active form. Unlike IL-1β, IL-1α exists in three compartments, pro-IL-1α (intracellular), myristoylated form of proIL-1a (membrane-bound), and mature IL-1α (secreted form). All the three forms of IL-1α are biologically active.

Since cells contain intracellular pools of active pro-IL-1α, it is possible that upon cell death they release the cytokine passively. IL-1 α released in this fashion may be sufficient to induce neutrophil migration. This idea was supported by a recent study by Eigenbrod *et al*[227]. The alternate hypothesis is that certain tissue-resident cells detect

certain DAMPs released from the necrotic cells and produce IL-1 after activation. To address the question of whether IL-1 is sourced from the dying cells or the responding host tissue, mice lacking IL-1α or both IL-1α and IL-1β were used either as donors of tissue homogenates or as hosts.

Necrotic tissue homogenates from either wild type or IL-1α deficient hosts were injected intraperitoneally into wild type hosts and the ensuing neutrophilic inflammation was determined (Figure 3.1 A, B, C). There was no reduction in the inflammatory response to the IL-1α deficient homogenates as compared to the wild type tissue homogenates. To eliminate the possibility that this is a tissue-specific effect, homogenates from a variety of tissue origins like heart (Figure 3.1 A), liver (Figure 3.1 B) and brain (Figure 3.1 C) was tested, but in each case, the IL-1α deficient homogenates showed no reduction. Since dying cells could also secrete IL-1β that might contribute to inflammation, we also examined tissues from IL-1αβ double-deficient animals (Figure 3.1 D, E, F). The pro-inflammatory activity of brain (Figure 3.1 D), liver (Figure 3.1 E), and heart (Figure 3.1 F), from *IL-1α*$^{-/-}$*β*$^{-/-}$ was equivalent to wild type tissues, when injected in wild type host.

The above results implied that necrotic tissues were not a significant source of IL-1 during inflammation. The method of inducing death; thermal or mechanical disruption

Figure 3.1

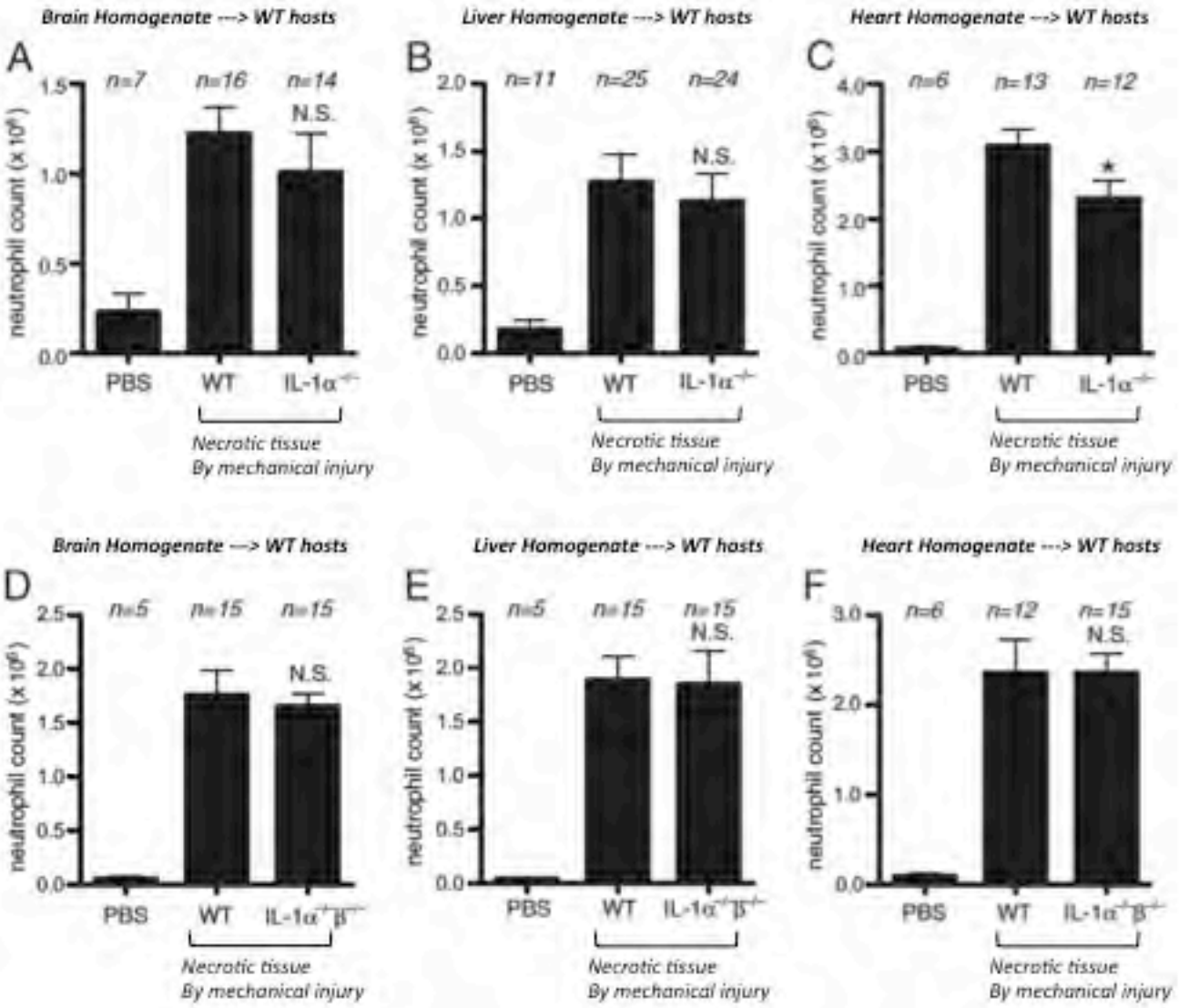

Figure 3.1: Requirement of IL-1 released from dying cells for neutrophil recruitment

A-C, Necrotic brain homogenate (A), liver homogenate (B), or heart homogenate (C) from C57BL/6 (WT) or *IL-1α*$^{-/-}$ mice were injected i.p. into C57BL/6 mice. Total

neutrophil number in the peritoneal cavity was measured 14 h post injection. D–F, Total neutrophil number in the peritoneal cavity of C57BL/6 mice in response to i.p. injected necrotic brain homogenate (D), liver homogenate (E), or heart homogenate (F) derived from C57BL/6 (WT) or *IL-1α*$^{-/-}$*β*$^{-/-}$ mice. All data are combined results of three or more experiments and represented as means ± SEM (n = total number of mice from the multiple experiments for each group). PBS groups, WT mice received i.p. PBS. *$p<0.05$, N.S., not significant, versus WT group.

Figure 3.2

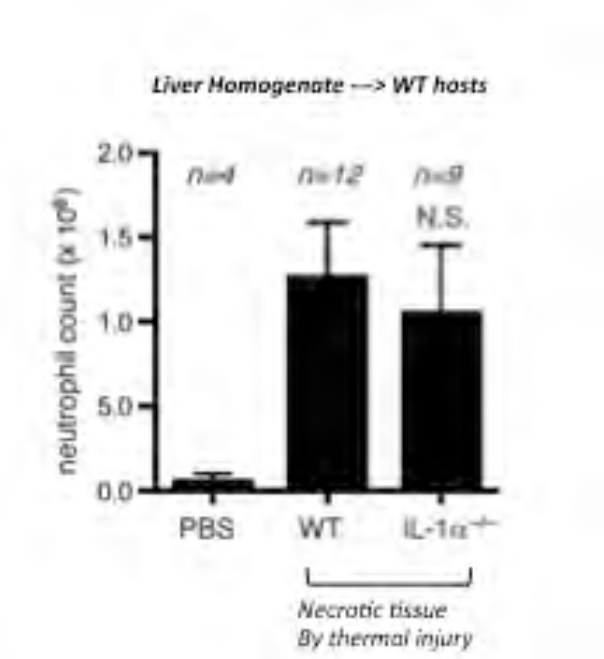

Figure 3.2: Method of inducing cell death has no effect on the requirement of IL-1 from the injured tissue

Total neutrophil number in the peritoneal cavity of C57BL/6 mice after 14 hours of i.p. injection with heat-shocked single cell suspension of liver cells from C57BL/6 (WT) or IL-1$\alpha^{-/-}$ mice. The data are combined results of three experiments and represented as means ± SEM (n = total number of mice from the multiple experiments for each group). PBS groups, WT mice received i.p. PBS. N.S., not significant, versus WT group.

had no effect on the phenotype observed. There was no reduction in inflammation to liver homogenate, which was generated by thermal injury from *IL-1α*$^{-/-}$ mice (Figure 3.2). We next determined if the host cells were the source of IL-1, by using mice that were lacking IL-1α or IL-1β or both. We tested the neutrophilic migration to the secondary cell line, EL4, which had been killed by thermal incubation or nitrogen cavitation (Figure 3.3 A). In line with our hypothesis, the dead cell-mediated inflammation was completely inhibited in host mice lacking both IL-1α/IL-1β. In fact, even single knockout hosts (*IL-1α*$^{-/-}$ or *IL-1β*$^{-/-}$) showed significant reduction in neutrophil migration to dead EL4s. This data stressed the importance of these cytokines in sterile injury as well as confirmed the fact that most of the IL-1 required during inflammation is host-derived. Furthermore, to show that this finding is physiologically relevant, we showed that the response of *IL-1α*$^{-/-}$*β*$^{-/-}$ mice to necrotic liver homogenate is significantly reduced as compared to wildtype hosts (Figure 3.3 B).

How does the above finding reconcile with data shown by Eigenbrod *et al*[227]? Similar to the experiments performed in this paper, we saw that when necrotic *IL-1 α*$^{-/-}$ or *IL-1α*$^{-/-}$*β*$^{-/-}$ dendritic cells were used as the stimuli in wildtype hosts, they had reduced capacity to recruit neutrophils as compared to wildtype necrotic bone marrow dendritic cells (BMDC) (Figure 3.4 A, B). This finding indicated that in certain cell types like

dendritic cells, the necrotic cells themselves could be a source of IL-1. However, even in this case, the host contributes to the IL-1 pool as exemplified by the fact that necrotic dendritic cell-inflammation is partially reduced in *IL-1α*$^{-/-}$*β*$^{-/-}$ hosts (Figure 3.4 C).

Figure 3.3

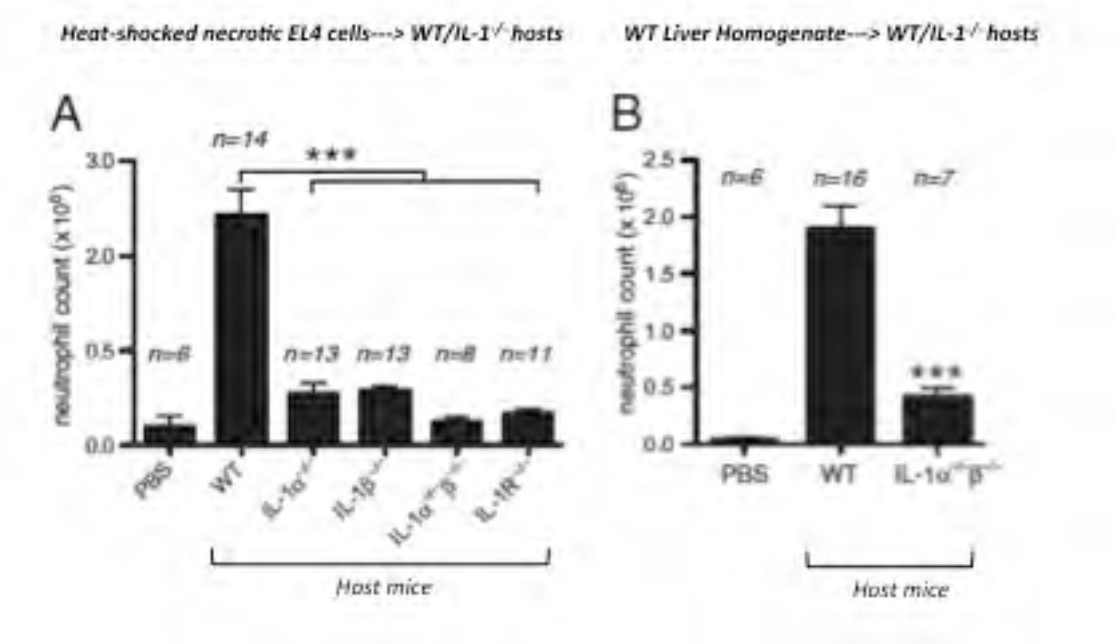

Figure 3.3. Host-derived IL-1 is required for neutrophil recruitment to dead cells

(A) Total neutrophil number of peritoneal cavity 14 h after i.p. injection of heat-shocked necrotic EL4 cells in C57BL/6 WT, *IL-1α*$^{-/-}$, *IL-1β*$^{-/-}$, *IL-1α*$^{-/-}$*β*$^{-/-}$, or *IL-1R*$^{-/-}$. One-way ANOVA and Bonferroni's multiple comparison tests were used to compare all of the groups. There were no significant differences among groups of *IL-1α*$^{-/-}$, *IL-1β*$^{-/-}$, *IL-1α*$^{-/-}$*β*$^{-/-}$, *or IL-1R*$^{-/-}$ (not indicated in the figure). (B) WT C57BL/6-derived liver homogenate was injected i.p. into C57BL/6 or *IL-1α*$^{-/-}$*β*$^{-/-}$mice. The total neutrophil number of peritoneal cavity 14 h after i.p. injection are shown. All of them are combined results of three or more experiments and represented as means ± SEM (n = total number of mice from the multiple experiments for each group). PBS groups, WT mice received i.p. PBS. ***$p < 0.001$, versus WT group.

Figure 3.4

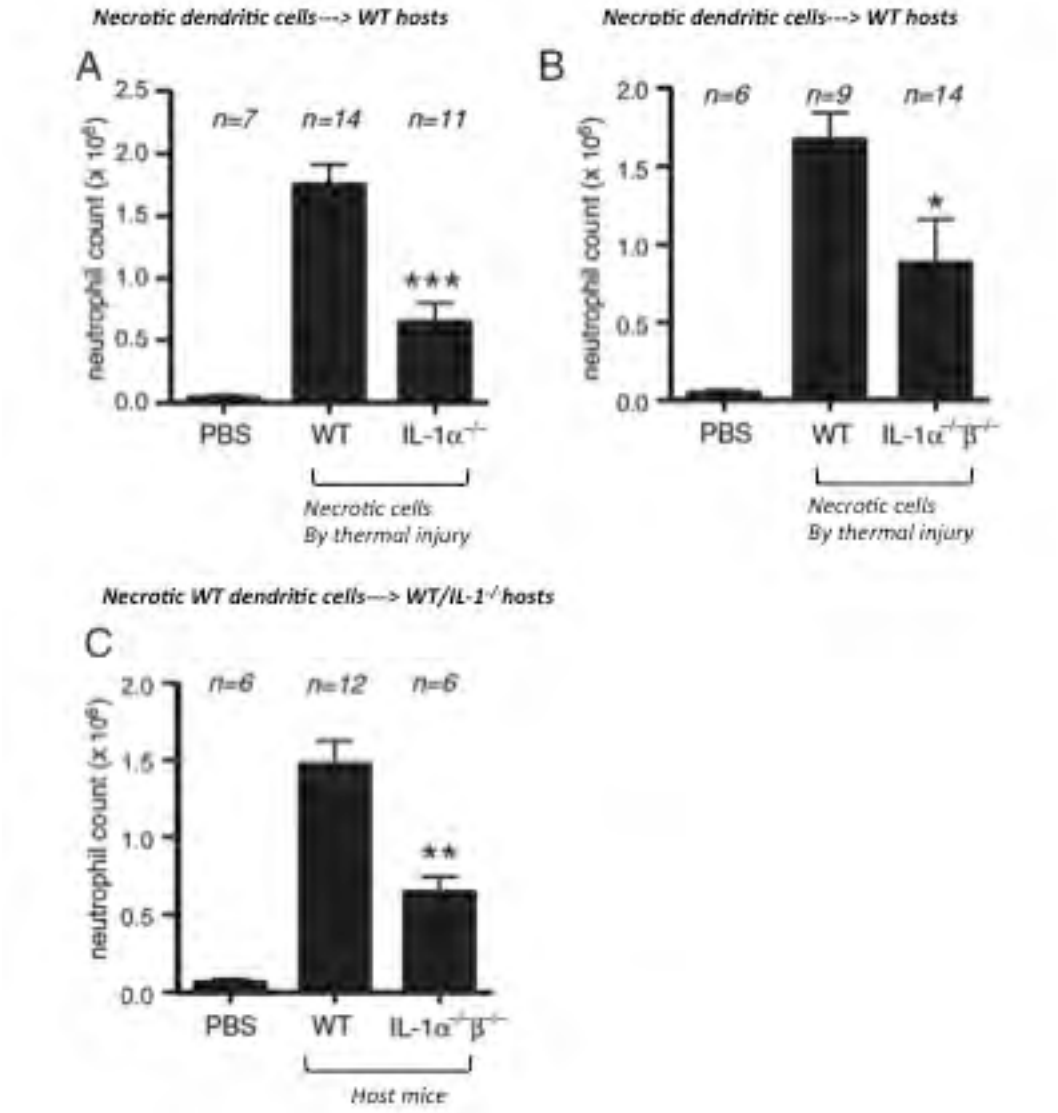

Figure 3.4: Both host-derived and dead cell-derived IL-1 is contributing to neutrophil recruitment to necrotic BMDCs

(A, B) The total neutrophil numbers in peritoneal cavity of WT C57BL/6 mice after 14 h i.p. injection of heat-shocked necrotic BMDCs from WT and *IL-1α*$^{-/-}$ (A) or *IL-1α*$^{-/-}$*β*$^{-/-}$

(B) mice. (C) The total neutrophil numbers in peritoneal cavity of WT C57BL/6 or *IL-1α*$^{-/-}$*β*$^{-/-}$ mice after 14 h i.p. injection of heat-shocked necrotic BMDCs from WT mice. All the data displays are combined results of three or more experiments and represented as means ± SEM (n = total number of mice from the multiple experiments for each group). ***p < 0.001; **p < 0.01; *p < 0.05, NS versus WT group (A–C).

The above data that necrotic dendritic cells are a source of IL-1, suggested that live dendritic cells have preformed IL-1, which gets released upon cell death. This led to the hypothesis that live dendritic cells themselves are inflammatory, by virtue of their existent IL-1 pool. This idea was supported by the finding that live dendritic cells were as inflammatory as necrotic ones (Figure 3.5 A). Furthermore, neutrophil recruitment to live dendritic cells requires the presence of IL-1α and IL-1β (Figure 3.5 B). However, dendritic cells are an exception because in the case of other cells, for e.g., (Figure 3.5 C), live cells fail to elicit inflammation.

Hematopoietic cells release IL-1 in response to cell death

The next step was to examine the nature of this host-derived cell. To broadly characterize if the IL-1 secreting cell was bone marrow- derived or parenchymal in origin, bone marrow chimeras were made. It was seen that the inflammatory response to dead cells was reduced substantially but not completely in radiation chimeras, whose bone marrow lacked both IL-1α and IL-1β. This showed that hematopoietic or bone marrow derived cells are the significant contributors of these cytokines, though non-hematopoietic cells play a minor role (Figure 3.6 A). We sought to confirm this observation by using hosts deficient in IL-1α and/or IL-1β and wildtype animals as bone marrow donors. This experiment demonstrates that IL-1α is entirely derived from bone marrow-derived cells. On the other hand, IL-1β is derived from bone marrow and in a smaller degree from parenchymal cells (Figure 3.6 B).

Figure 3.5

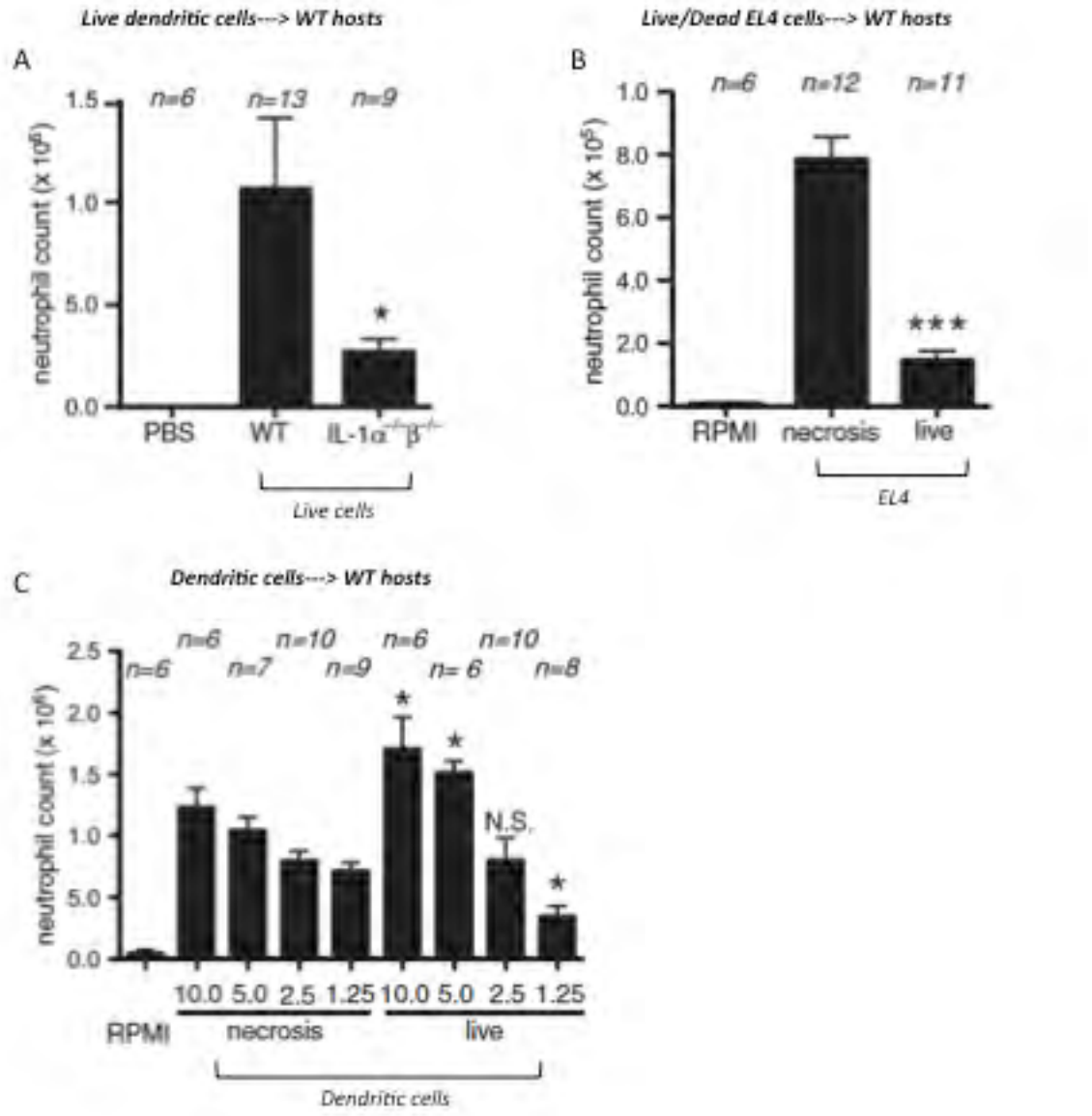

Figure 3.5: Live BMDCs are capable of eliciting neutrophil recruitment in a IL-1α/IL-1β dependent way

(A) The total neutrophil numbers in peritoneal cavity of WT C57BL/6 mice after 4 h

i.p. injection of indicated number (million) of live or heat-shocked necrotic BMDCs from WT mice. (B) The total neutrophil numbers in peritoneal cavity of WT C57BL/6 mice after 4 h i.p. injection of 5 million of live BMDCs from WT or *IL-1α*$^{-/-}$*β*$^{-/-}$ mice. (C) The total neutrophil numbers in peritoneal cavity of WT C57BL/6 mice after 4 h i.p. injection of live or heat-shocked necrotic EL4 cells. The small amount of inflammation seen after injection of live EL4 is presumably due a small number of dead cells in the injected suspension. All the data displays are combined results of three or more experiments and represented as means ± SEM (n = total number of mice from the multiple experiments for each group). ***$p < 0.001$; *$p < 0.05$, NS versus WT group (B) or same amount of necrosis group (A, C).

Figure 3.6

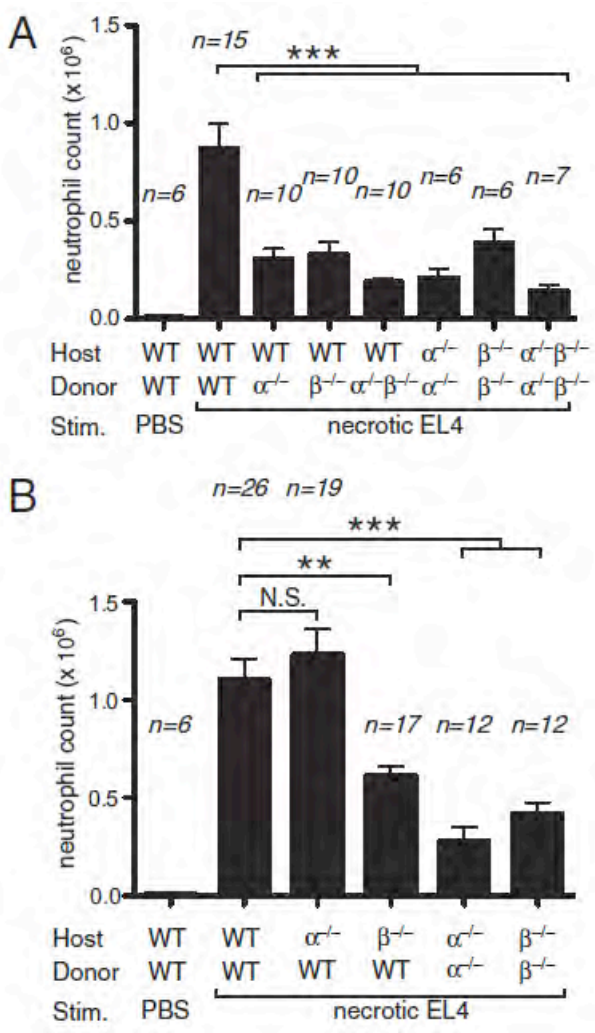

Figure 3.6: Bone marrow-derived cells are the major producer of IL-1 in inflammation to dead cells

(A) Total neutrophil numbers in peritoneal cavity were determined in bone marrow chimeric mice 14 h after i.p. challenge of heat-shocked necrotic EL4 cells. Chimeric mice were generated using C57BL/6 (WT) mice as hosts and the indicated *IL-1*$^{-/-}$ mice

(*IL-1α*$^{-/-}$, *IL-1β*$^{-/-}$ or *IL-1α*$^{-/-}$*β*$^{-/-}$) as donors or using *IL-1*$^{-/-}$ mice as hosts and *IL-1*$^{-/-}$ mice (*IL-1α*$^{-/-}$, *IL-1β*$^{-/-}$ or *IL-1α*$^{-/-}$*β*$^{-/-}$) as donors. (B) Total neutrophil numbers in the peritoneal cavity were determined in bone marrow chimeric mice 14 h after i.p. challenge of heat-shocked necrotic EL4 cells. Chimeric mice were generated by using IL-1$^{-/-}$ mice as hosts and C57BL/6 WT mice as donors. All the data displays are combined results of three or more experiments and represented as means ± SEM (n = total number of mice from the multiple experiments for each group. (A) One-way ANOVA and Bonferroni's multiple comparison test for all of the groups showed a significant difference for WT >WT to all the other groups ($p < 0.001$), but there was no significant difference among the groups of cells from IL-1$^{-/-}$ donors (not shown in the figure). B, Bonferroni's multiple comparison test for all the groups showed a significant difference for WT > *IL-1 α*$^{-/-}$ to WT > *IL-1β*$^{-/-}$, *IL-1 α*$^{-/-}$ > *IL-1 α*$^{-/-}$ or *IL-1β*$^{-/-}$ > *IL-1β*$^{-/-}$ ($p<0.01$, not shown in the figure). ***$p < 0.001$; **$p < 0.01$, NS versus EL4 stimulated WT greater than WT group. stim, stimulated.

3.3. Discussion

Cell-death induced neutrophilic inflammation is important for the clearance of the cell debris and resolution of the wound. On the other hand, in some cases, neutrophil infiltration can actually exacerbate the existing cell injury. Indeed, this is observed in the case of pathological conditions like ischemic damage to the heart, lung, liver, brain, and kidney. Considering the physiological impact of dead cell-induced inflammation, very little is known about the molecular and cellular "messengers" which propagate signals from dying cells in a tissue to neutrophils in the bone marrow and blood. The current study addresses some of these questions.

Previous work in the laboratory had demonstrated the requirement of IL-1 for mediating cell-death induced inflammation. The role of IL-1 in cell injury related neutrophil recruitment was more significant than that of other pro-inflammatory cytokines like TNFα[228]. Furthermore, neutrophilic inflammation to the yeast cell wall component, zymosan was not diminished in *IL-1R*$^{-/-}$ mice. This exemplified the specific role of IL-1 in propagating inflammation triggered by cell injury but not microbes. Further information regarding the source of IL-1 was needed.

An important finding of this study is that in most cases, the injured tissue is not a major contributor of IL-1α that is required to propagate the inflammatory signal. On the

contrary, IL-1α/IL-1β is generated by hematopoietic host cells, in response to sterile injury. Exceptions to this rule may be cells, which make high quantities of IL-1α, even when they are alive. In this study, IL-1α deficient dendritic cells, whether alive or dead showed substantially reduced neutrophilic inflammation as compared to wild-type cells. However, even in this instance, some part of the required IL-1α/IL-1β was sourced from host tissue. This supports the idea that there exist some host responding cells, which relay the inflammatory signal from injured tissues to blood neutrophils, via the secretion of IL-1.

A favored model is that the injured tissue releases DAMPs, which bind to cognate receptors on tissue-resident cells, which have hematopoietic origins. The latter, once activated are the source of IL-1. To identify this bone marrow derived tissue resident sensor of cell injury, a series of cell depletion studies were performed[153]. These studies revealed that the initiation of an acute inflammatory response to sterile cell death requires a $CD11b^{high}$ leukocyte that is almost certainly macrophage. This conclusion was supported by the finding that conditionally depleting CD11b-positive cells in mice inhibited these responses.

In summary, necrotic tissues are not a significant source of the cytokines IL-1α/IL-1β in the context of the inflammatory response. Rather, bone marrow-derived cells, most

likely macrophages are the primary sensors and producers of IL-1α during cell death-induced inflammation.

3.4. Material and Methods

Animal and cell lines: Wild-type (WT) C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME). IL-1α, IL-1β, and IL-1αβ double-deficient mice of C57BL/6 background were described previously[229]. Irradiated bone marrow chimeras was prepared as described[215]. All animal protocols were approved by the University of Massachusetts animal care and use committee. EL4 cells were maintained in RPMI-1640 with 10% FCS and antibiotics. All the cultured cell lines are tested negative for mycoplasma (Lonza, Basel, Switzerland).

Preparation of necrotic cells: Bone marrow-derived dendritic cells (BMDCs) were obtained from cultures of bone marrow cells of C57BL/6 or IL-1–deficient mice with IL-4 (10 ng/ml, Invitrogen, San Diego, CA) and GM-CSF (5 ng/ml, Invitrogen) in Hybridoma culture medium for 7 d. A single-cell suspension of liver was obtained by collagenase type IV treatment as previously described[230]. To induce necrosis from thermal injury, BMDCs, liver or EL4 cells were washed five times with PBS, then resuspended in PBS at 10 million cells/50 μl, and then heat shocked at 45°C for 10 min,

followed by 37°C incubation for 5 h; this resulted in necrosis (7-aminoactinomycin D/propidium iodide positive cells). After thermal injury, there were no viable cells observed by microscopy with trypan blue staining.

To induce mechanical necrosis, brain, liver, or heart from C57BL/6 or IL-1–deficient mice were weighed and added with five times of weight of PBS and subjected to mechanical injury by a motor-driven tissue tearer, followed by nitrogen cavitation for 10 min at 500 pounds per square inch. Similarly, mechanical necrosis was induced in EL4 and BMDCs by nitrogen cavitation for 10 min at 500 pounds per square inch. After mechanical injury, no viable cells were seen by microscopy and when analyzed by flow cytometry typically 99.9% of the cells were disrupted into fragments. The resulting necrotic cell suspensions were used for experiments without any clarification (i.e., containing all released cellular components and debris).

Neutrophil recruitment to peritoneal cavity: Mice were injected i.p. with 5 million necrotic BMDCs in 500 μl PBS, 30 million of necrotic EL4 cells in 150 μl PBS, 150 μl liver homogenate, or 500 μl heart or brain homogenate unless otherwise indicated. The total protein amounts in necrotic cell preparations that were injected were 4.1 mg dendritic cells (DCs), 12.6 mg (EL4), 72.4 mg (brain), 25.4 mg (liver), or 77.0 mg (heart). The amount of the necrotic DCs, brain, liver, and heart preparations that was

injected was one that was determined to induce a non-maximal response so as to be on a sensitive portion of the dose response curve. After 13–15 h of injection, the peritoneum was lavaged with 6 ml PBS with 2% FCS, 3 mM EDTA, and 10 U/ml heparin. The absolute number of neutrophil (Ly-6G^{+} 7/4^{+}) in 100µl lavage was counted using flow cytometer equipped with a high throughput sampler (BD Bioscience).

CHAPTER 4

IDENTIFICATION OF THE CELLULAR TARGET OF IL-1 SIGNALING IN CELL DEATH-INDUCED INFLAMMATION

ATTRIBUTION AND COPYRIGHT INFORMATION

Design and construction of vector, screening of embryonic stem cells, and breeding chimeric mice was done by me. The UMass Transgenic Core Facility performed the electroporation into embryonic stem cells, electroporation with the Cre recombinase and injection and implantation of blastocysts.

4.1. Introduction

Neutrophil recruitment to dead cells is critically dependent upon IL-1 signaling[197]. The role of IL-1 signaling in sterile inflammation was conclusively shown using mice deficient in IL-1R as well as those doubly deficient in IL-1α and IL-1β. The previous chapter describes the experiments undertaken to identify the cellular source of IL-1. Another area of research was to elucidate the downstream events of IL-1 signaling. Previous work in the laboratory showed that non-hematopoietic cells are the cellular targets of IL-1[197]. This was based on results obtained from IL-1R bone marrow chimeric mice. Wild-type (WT) mice that were reconstituted with IL-1R–deficient bone marrow (*Il1r*$^{-/-}$ → WT) did not show any significant reduction in inflammatory responses to necrotic cells. In contrast, IL-1R–deficient mice reconstituted with wild-type bone marrow (WT → *Il1r*$^{-/-}$) had markedly reduced inflammatory responses to dead cells. Therefore, the IL-1R expression was required on radioresistant (non–bone-marrow-derived or parenchymal), but not radiosensitive (bone marrow–derived or hematopoietic) host cells for them to respond to IL-1.

To elucidate the key cellular target of IL-1 in cell death-induced inflammation, we proposed to generate the conditional knock out mouse for IL-1R. This would be achieved by making the *Il-1r* floxed mouse, in which loxP sites flank the protein-encoding exon. The intervening sequence between the loxP sites can be deleted by recombination mediated by the enzyme Cre recombinase. This is enabled by crossing the floxed mice with mice expressing Cre recombinase under the control of promoters specific to parenchymal tissues. Examples of the parenchymal tissues, which are likely

to be targets of IL-1 signaling, are the endothelium, epithelium, fibroblasts, and smooth muscle. Our favored hypothesis is that the endothelium is the major IL-1 target, because it controls the recruitment of neutrophils into tissues. However, it is likely that there are different IL-1 signaling targets in different tissue sites and pathological conditions.

4.2. Research design and methods

We obtained two independent BAC clones containing the chromosomal sequences of the IL-1R of 129Sv origin from the BACPAC Resource Center, Children's Hospital Oakland Research Institute, California, USA (Figure 4.1). Since the genomic sequence of this strain is not available in databases, we sequenced both BAC clones 3 times each. With this information, we amplified the suitable fragments and designed a targeting vector as described below.

Construction of the targeting vector: The strategy used for constructing the targeting vector is based on the design of the vector described previously to generate the straight knock out mice of IL-1R[231]. This strategy has been shown to completely abrogate IL-1R expression and function, without affecting the viability of the mice, hence it has been validated already. It involves targeted Cre-mediated deletion of the loxP site-flanked exon 2 of the *Il-1r* locus. This exon encodes the signal peptide of the mature protein (Figure 4.2) and also contains a possible ATG initiation codon. The deleted mutant after homologous recombination, would not encode a functional protein because the signal peptide is absent and the other potential upstream ATG would be out of

Figure 4.1

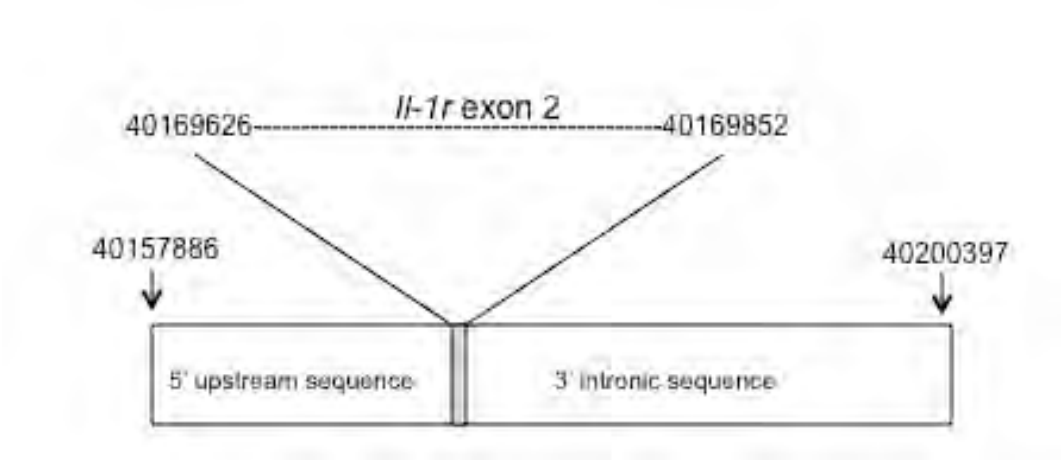

Figure 4.1: Map of the BAC clone used to sequence and amplify the *Il-1r* gene fragments
The numerals denote the position on Chromosome 1. Exon 2, which would be deleted in the knockout mouse and its chromosomal position, is denoted in blue.

Figure 4.2

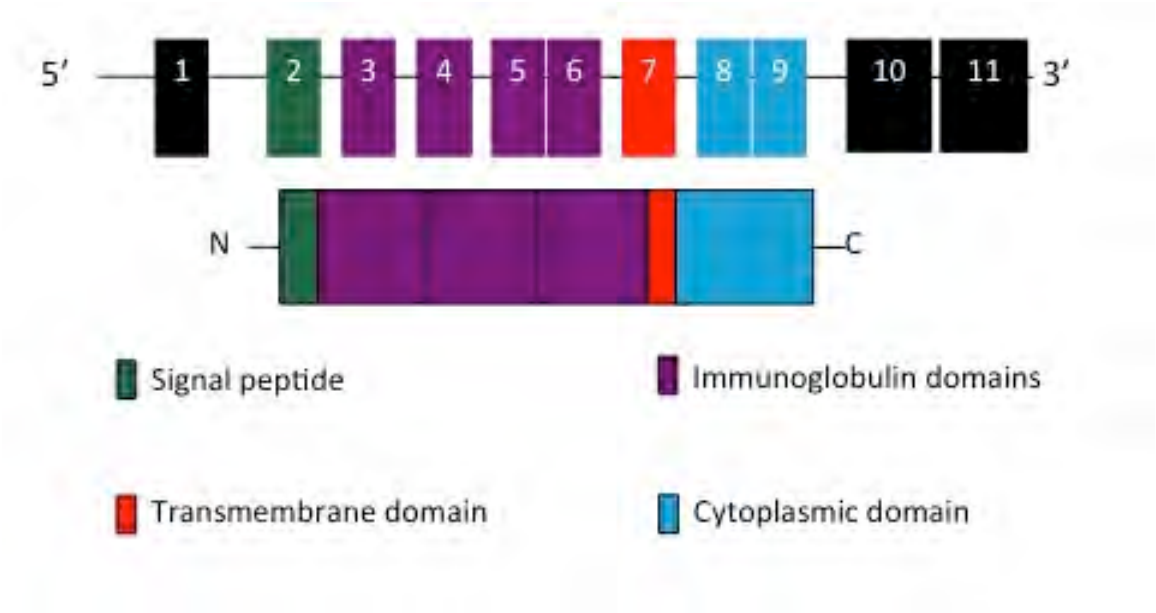

Figure 4.2: The endogenous *Il-1r* gene locus (top) and the corresponding mature protein domain structure (bottom)
Each numbered box in the *Il-1r* genome represents an exon. Each box in the protein structure represents a distinct domain. The color code indicates the different exons encoding distinct domains of the mature protein. The exons in black represent untranslated regions.

frame when fused to downstream exons. The downstream ATG if used, would result into a highly truncated protein without a signal peptide.

The construction of the vector in brief, was as follows (Figure 4.3). The 4.6kb long MscI-XmnI fragment which contained sequence 5' to the targeted Exon 2 was amplified from the BAC clone and cloned in SacII site of the ploxPIII-neo-lTK plasmid (a gift from Dr.Joonsoo Kang, University of Massachusetts Medical School, Worcester). The 3.3kb long amplified XmnI- SwaI fragment, which contained sequence 3' of the targeted Exon 2 was cloned in the PmeI site of the above vector. Finally the 0.4kb XmnI-XmnI fragment encompassing the Exon 2 was cloned in the AscI site, to get the complete targeting vector. The vector already contained the 3-loxP sites and PGK-Neo (encoding the neomycin resistance gene) and the HSV-TK (encoding the thymidine kinase gene, conferring susceptibility to ganciclovir) cassettes, which would be used for positive and negative selection respectively, as described in the following paragraph.

Selection of correctly integrated ES cell clones: The construct was linearized by MluI and electroporated into 129Sv embryonic stem (ES) cells. The actual electroporation and the subsequent blastocyst injections were performed by the UMass Knock out Core Facility. The cells were grown in media containing neomycin and ganciclovir for selection of cells, which have undergone homologous recombination with the correct arm of the targeting construct. Initially the DNA from the individual ES cells was digested with BamHI and analyzed by Southern Blot analysis with a 300bp probe as

Figure 4.3

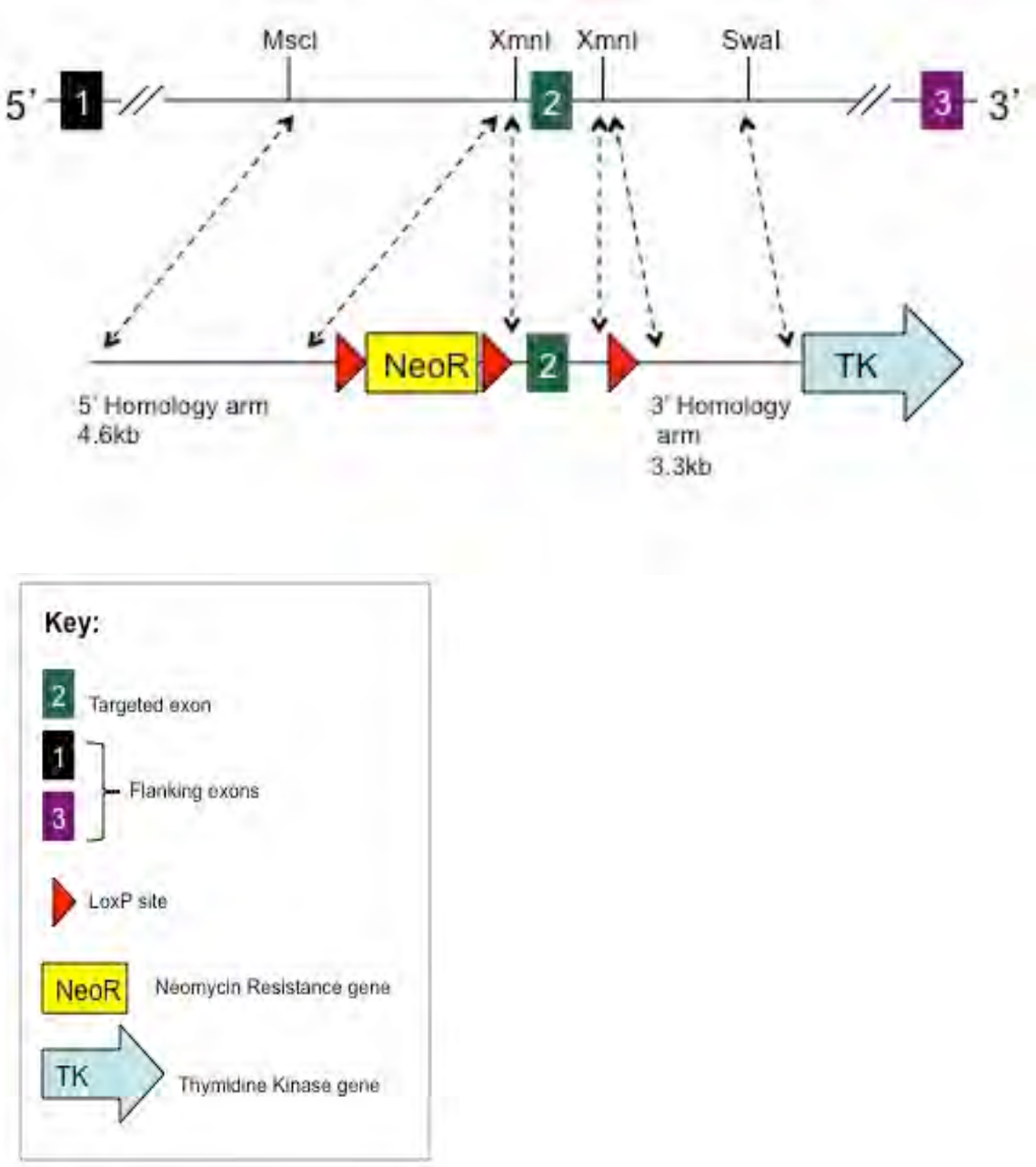

Figure 4.3: Schematic representation of the targeting vector (bottom figure) and the corresponding elements in the endogenous *Il-1r* locus (top figure).
The dotted lines indicate the fragments used in construction of the vector. MscI, XmnI, and SwaI are the restriction sites that were used for cloning.

Figure 4.4

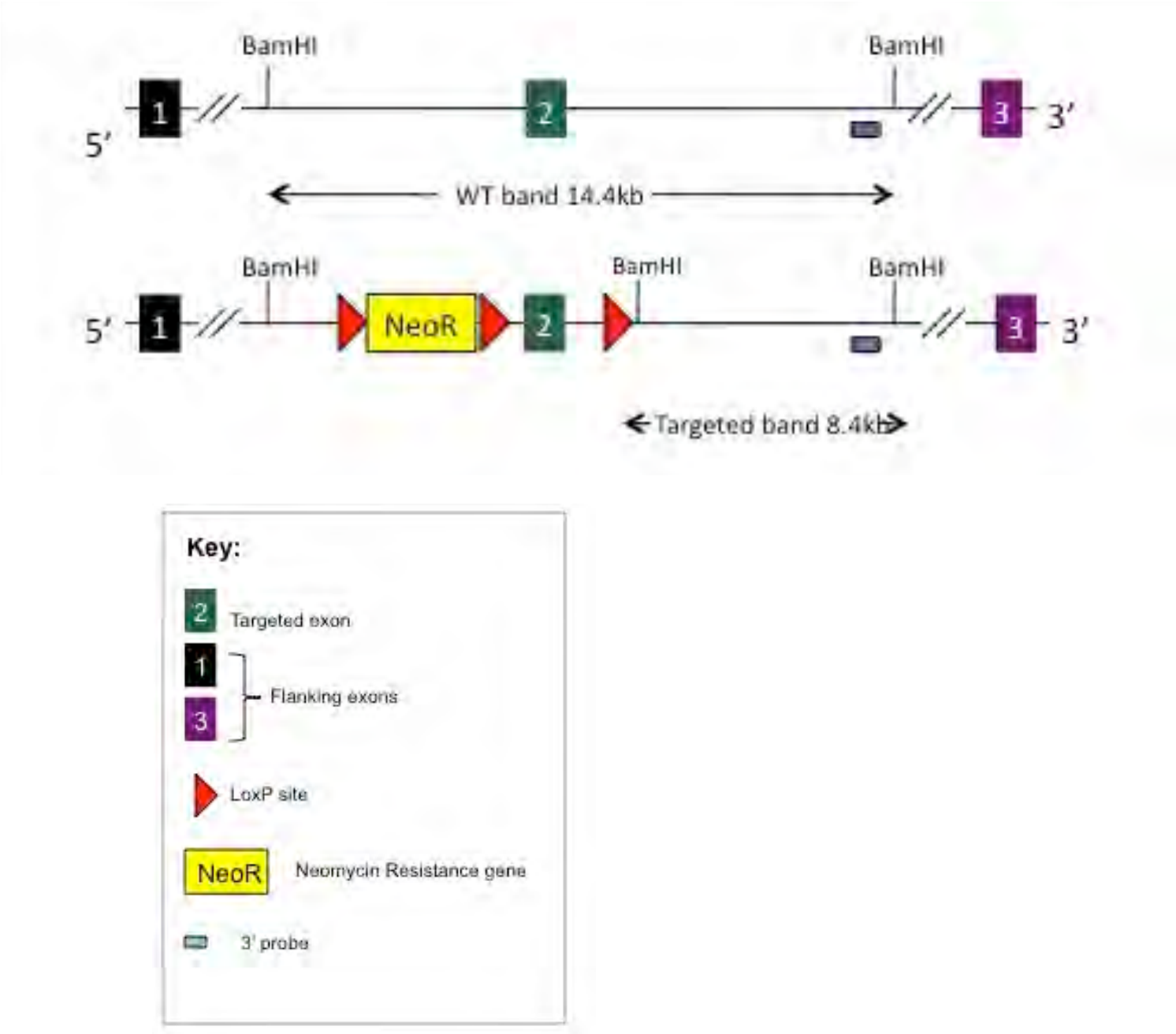

Figure 4.4: Southern Blotting strategy to identify targeted allele
The expected band pattern of the wildtype (WT) (top figure) and the targeted alleles of the *il-1r* locus (bottom figure), after BamH1 digestion and hybridization with 3' probe.

Figure 4.5

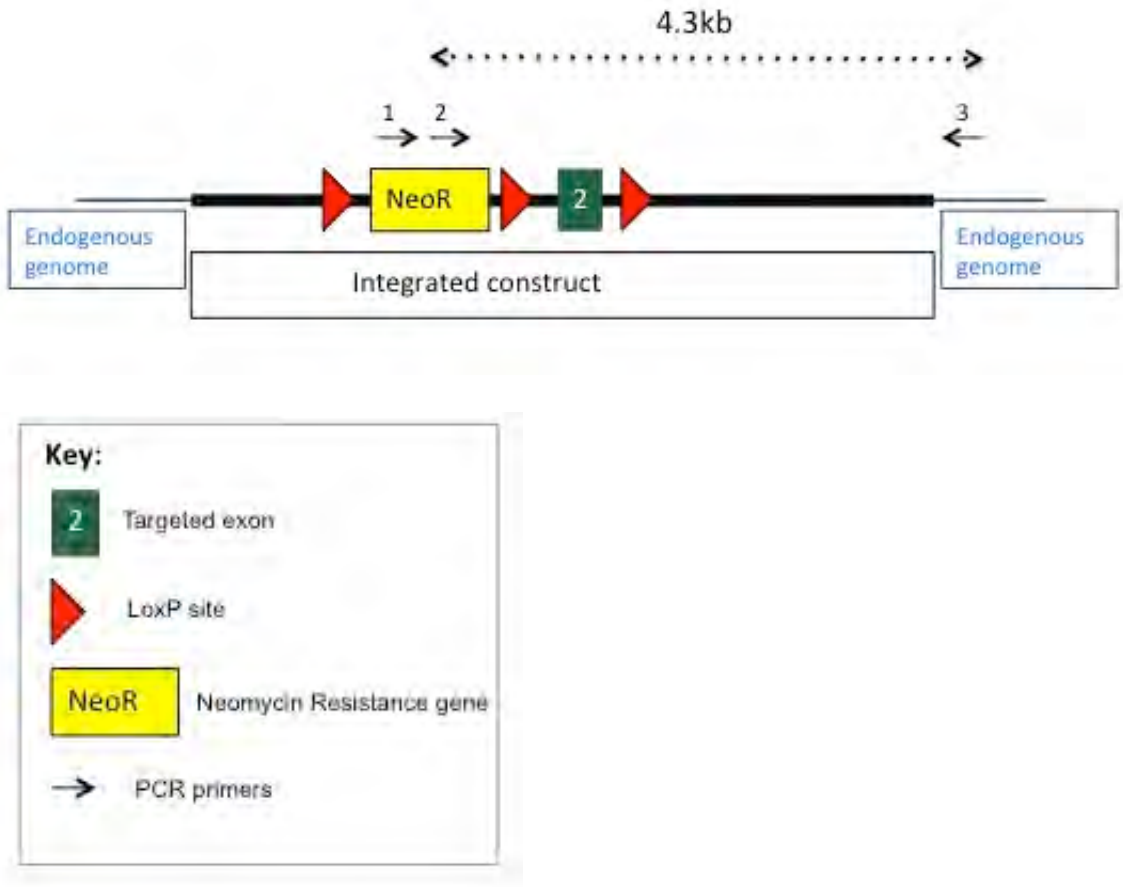

Figure 4.5: Floxed PCR strategy to identify targeted allele
Only the clones carrying the targeted allele would show the expected band (4.3kb) after PCR with the above primers.

shown in the figure (Figure 4.4). The wild type genome was identified as a 14.4kb band. The targeted allele was distinguished by the presence of a shorter 8.4kb band, because of the introduction of a BamHI site by the integrated vector construct. Out of 300 ES cell clones screened, 8 were found to be homologous recombinants for the targeting construct. The presence of the targeted allele in these clones was further confirmed by a nested PCR (Figure 4.5)

Out of these 8 targeted clones, 2 were chosen for subsequent electroporation with the CMV- Cre plasmid (which encodes Cre recombinase gene), with the goal of removing the neo cassette. Since there are three possible outcomes of the in vitro Cre-mediated excision, the clones in which neo cassette has been specifically deleted, while leaving the Exon 2 intact, were screened by PCR (Figure 4.6). Out of the 200-screened clones, consistent with the expected recombination frequency, $1/3^{rd}$ were found to have undergone the correct recombination event. 3 of these correctly recombined clones were used for injection in blastocyst and further implantation into mice to generate the chimeras.

Breeding the chimeric mice: The 129Sv ES cells had the locus for agouti coat color and were injected into C57BL/6 non-agouti blastocysts. These blastocysts were implanted into white, non-agouti C57BL/6 mothers. The resultant pups were chimeric and could be identified by the presence of brown hairs on a white background. Higher degree of chimerism generally indicates a greater chance of germline transmission of the

Figure 4.6

Figure 4.6.1:

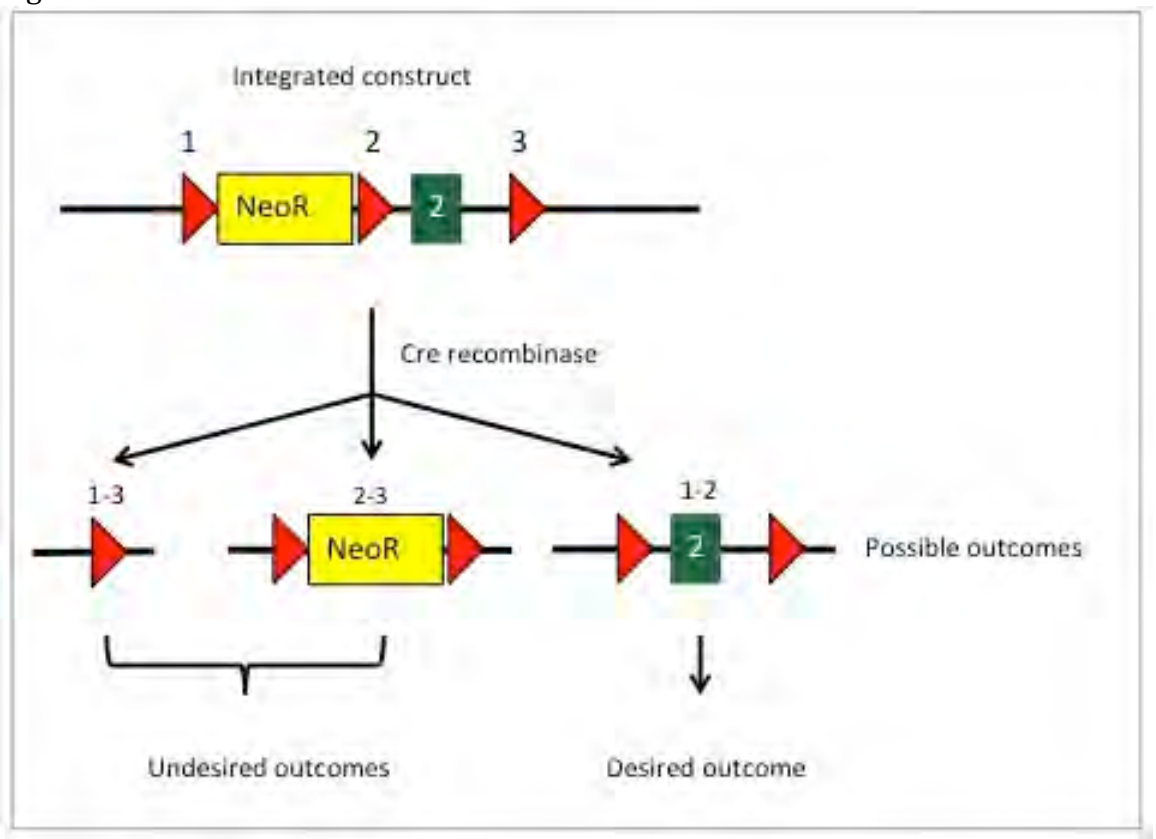

Figure 4.6.2:

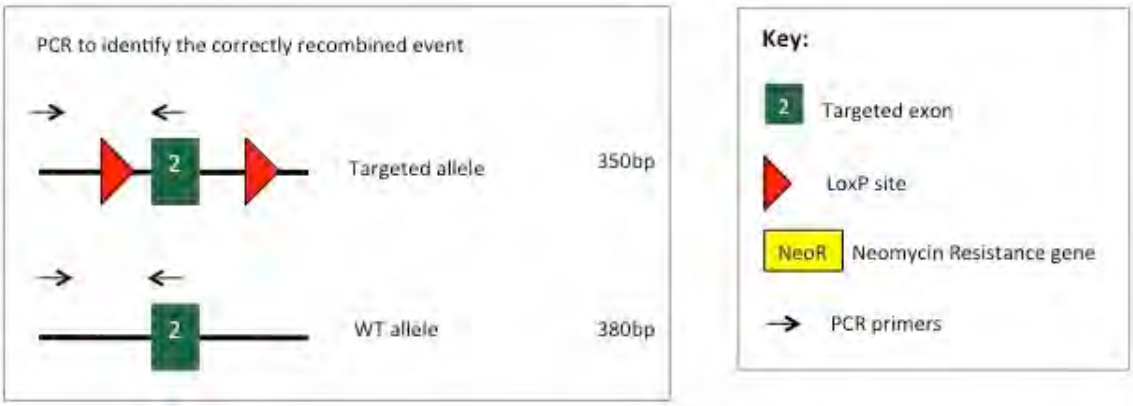

Figure 4.6: PCR strategy to identify the correctly recombined clone
Treatment of targeted clones (From figure 4.5) with Cre recombinase leads to three possible genomic recombination events (Figure 4.6.1). The heterozygous 'desired' recombined allele was identified using PCR (Figure 4.6.2)

transgenic 129Sv cells. We obtained several mice with a high percentage of chimerism (>70%). These mice were used for breeding with black non-agouti C57BL/6 mice. After multiple rounds of breeding, we have been able to obtain agouti pups that have one copy of the transgenic 129Sv genome.

4.3. Future Directions

Breeding the conditional knockout mice: The agouti pups heterozygous for the floxed *il-1r* gene are presently been bred to get homozygous offsprings. The latter, which are on the 129Sv background will be backcrossed 10 generations onto the C57BL/6 background.

The next step would be to generate mice that lack the IL-1R only on their vascular endothelium. This will be accomplished by mating the Floxed-IL-1R mice with transgenic mice expressing the cre recombinase under the control of an endothelial specific promoter. There are several such transgenic mice available, e.g., mice expressing cre under the control of Tie-1, Tie-2 and Flk promoters and have been used successfully to make endothelial-specific knock out mice[232-234]. We plan to use the Tie2-cre mice because they work uniformly in embryonic and adult stages. We will verify the selective excision of the IL-1R by assaying for the expression of the IL-1R in endothelium and other cells by immunohistochemistry and PCR on cells isolated by laser capture microscopy. The cre-transgene will be eliminated through breeding and mice homozygous for the endothelial IL-1R knock out will be bred.

Testing the conditional IL-1R - deficient mice for their neutrophilic inflammation to dead cells: The goal of this project is to test the hypothesis that the vascular endothelium is the parenchymal target of IL-1 that is required for the death-induced acute inflammatory response. To this end, we will compare the inflammatory response between wild type and the endothelial tissue- specific IL-1R knock out mice, in different tissues to cell death. In all these assays, the sterile inflammatory response has been shown to be IL-1R dependent.

It is possible that the endothelium will not be the key target of IL-1 in the cell death-induced inflammatory pathway. If this is what is found in our analysis of the endothelial IL-1R knock out mice, we will investigate other potential parenchymal targets. Specifically we would next investigate the role of the IL-1R in fibroblasts and smooth muscle using tissue-specific cre expressing mice. The rationale for these targets is that the IL-1-dependent death-induced inflammatory response is seen when dead cells are injected s.c. and the principal parenchymal cells in this location are fibroblasts and smooth muscle. Moreover, both of these cell types express IL-1R.

If neutrophilic inflammation were still intact in these mutant animals then we would consider testing other parenchymal targets like epithelial cells using cell type specific cre-mediated deletion. If there is a partial phenotype seen in the tissue-specific knockouts, we will consider creating a double/triple conditional knockout.

5. GENERAL DISCUSSION

The present study about the regulation of inflammation by commensal flora demonstrates for the first time that gut resident bacteria are absolutely essential for the extravasation of neutrophils from blood into the inflamed tissues. Our favored model is that in the absence of intestinal flora (or in the default state), the host immune system makes some anti-inflammatory factor(s) either constitutively or after inflammatory activation. In other words, in the absence of priming by endogenous flora, the host is in a state of inhibited neutrophilic inflammation. On the other hand, in the presence of intestinal flora, this inhibitory "brake" on inflammation is released, facilitating neutrophil migration upon receiving an appropriate stimulus. However, our data does not eliminate the possibility that in the absence of intestinal flora, the host fails to make some essential pro-inflammatory molecule(s). This scenario would also cause the observed generalized defect in neutrophil migration.

The major future goal is to determine this factor, which is either upregulated in germ-free mice (if it is an inhibitory factor) or downregluated in germ-free mice (if it is a pro-inflammatory factor). We aim to perform a microarray study using peritoneal macrophages from zymosan-stimulated SPF or germ-free mice. We propose that macrophages are good candidates for these studies because previous work in the laboratory has shown that these cells are the primary sensors for various inflammatory stimuli[153]. Once this factor is identified, further confirmatory experiments will be done using antibody blocking experiments (similar to the experiment performed to assess the role of IL-10 in acute inflammation).

We have also shown that the intestinal flora-mediated regulation of acute inflammation requires MyD88 signaling. We propose that MyD88 stimulation though any of the TLR agonists released from resident bacteria is sufficient to prime neutrophilic responses. Hence, single knockout mice for each of the TLRs are capable of stimulating neutrophil migration, because these mice can still ligate MyD88 through other TLRs. The systemic release of microbial ligands from resident pathogens appears to be a powerful means by which they can exert widespread effects on host immunity and possibly other aspects of physiology. Indeed, Clarke et al have demonstrated that peptidoglycan released from stably colonized *E. coli* translocates from the gut mucosa into the blood stream[191]. However, additional direct evidence of other bacterial products in the circulation and distal tissues that have originated from endogenous microbes, in the absence of a breach in intestinal epithelium is desired.

Another unresolved question is which MyD88-expressing cell types are the targets of intestinal flora priming. We propose to create bone marrow chimeras to broadly understand if it's the parenchymal/hematopoietic compartment, which requires the expression of MyD88 to propagate zymosan-induced peritonitis. Also, an adoptive transfer of peritoneal cells from wildtype or *MyD88*$^{-/-}$ mice into wildtype or *MyD88*$^{-/-}$ mice would help address the question if it were the peritoneal cells, which require the presence of MyD88. Based on our observation that neutrophils can immigrate to a MIP-2 injection in the peritoneum, we propose that lack of MyD88 activation by resident flora does not affect the neutrophils *per se*. Rather, there is a defect in the activation of some other MyD88-expressing cell which acts upstream of neutrophil migration in the

inflammatory signaling pathway.

Yet another area of investigation would be to identify which species of the intestinal flora is crucial for priming neutrophil migration. Treatment of mice with a single antibiotic (for e.g., ampicillin or neomycin) can help identify if it is a specific class of bacteria (gram-positive or gram-negative bacteria) that is mediating the phenotype. However, our initial experiments using single antibiotic treatments instead of a cocktail, did not implicate a single class of bacteria as the primary mediator of inflammation. Another convenient tool to investigate the role of specific intestinal bacterial residents mice is the process of mono-colonization. Mono-colonization of germ-free animals is the process of reconstituting them with a single bacterial species. Several recent studies have implicated particular resident bacteria in the establishment of a specific aspect of immunity[235]. Germ-free mice colonized with known prominent species of the resident flora will be tested for their inflammatory responses.

There is a possibility that the commensal flora-MyD88 signaling axis is essential for other aspects of neutrophil production and migration. Previous studies have shown that the release of neutrophils from bone marrow into blood stream during inflammatory conditions is independent of MyD88 signaling[236]. However, MyD88 may influence the blood-tissue transit of neutrophils by regulating the expression of certain cell adhesion molecules, which are crucial for neutrophil infiltration into tissues. We have shown that neutrophils from antibiotic-treated mice have similar expression of CXCR2, the receptor of MIP-2 and KC and cell adhesion molecules like CD62L and CD44. But it is possible that the expression of other cell adhesion molecules is affected in antibiotic-

treated mice. This can be determined by a performing a quantitative PCR of putative adhesion molecules from isolated endothelial cells and neutrophils. The commensal flora-MyD88 signaling axis might affect neutrophil homeostasis in terms of its survival in the peripheral tissue. We saw that in antibiotic-treated mice, the numbers of neutrophils in the peritoneum are lower than that in SPF mice even at earlier time points (as early as 45 min after zymosan challenge). Also, the total numbers of dead cells in the peritoneum were not higher in antibiotic-treated mice as compared to SPF mice after zymosan challenge. This data argued against the possibility that neutrophils are being recruited into tissues in antibiotic-treated mice but are dying sooner than in SPF mice.

Another likelihood is that the production of G-CSF and GM-CSF is impaired in the absence of commensal flora. The defect shown in this study appears specifically in the immigration of neutrophils into inflamed tissues. Previous papers have shown that these two cytokines are dispensable for the neutrophil influx into tissues, which suggests that impaired production of G-CSF/GM-CSF is unlikely to be the cause of the observed defect[8]. However, a quantitative estimation of these cytokines in the blood stream of antibiotic-treated mice after inflammation would be more conclusive to rule out the role of G-CSF/GM-CSF in this phenotype.

We observed that in the germ-free mice, there were much higher amounts of pro-inflammatory cytokines (TNFα, MIP-2, and KC) in the peritoneum than in SPF mice, in spite of having fewer neutrophils. This could possibly be because of lack of negative regulation of cytokine expression by infiltrating neutrophils in the peritoneum.

Neutrophils upon immigrating into the site of injury, undergo rapid apoptosis (as early as 2h). The apoptotic neutrophils are then cleared by tissue-resident macrophages, promoting the resolution of inflammation[237]. Shibata *et al* have shown that early apoptotic neutrophils inhibit the production of cytokines like TNFα, IL-6, MIP-2 from macrophages *in vitro*[238]. Also, in an *in vitro* co-culture model, neutrophils have been shown to inhibit the secretion of IL-1β and TNFα from peripheral blood mononuclear cells[239]. The absence of infiltrating neutrophils in the germ-free mice could explain the higher levels of peritoneal cytokines in these mice.

Probiotics is a term given to live organisms, which restore the host intestinal flora balance, replacing the harmful bacteria with “good” ones. Some strains of bacteria have been approved by research for treatment of specific gastrointestinal disorders, based on strong, positive, well conducted and controlled studies[240]. Examples of such disorders include antibiotic and non-antibiotic associated diarrhea and *Clostridium difficile*-associated diarrhea. However, there is no evidence for the application of probiotics for general immune strengthening.

Our research might explain why administration of probiotics is not effective in reversing systemic immune pathologies caused by intestinal flora imbalance. The “good” bacteria in the probiotic cultures might be effective in displacing the unhealthy bacteria restoring intestinal homeostasis. However, the priming effected by virulence factors or other ligands released from the harmful bacteria might still persist in the system, triggering the undesirable immune pathology. This argument was supported by our observation that when mice were treated with antibiotics starting from birth for 45

days, they had lowered neutrophil migration, but 6-week old mice treated with antibiotics for the same duration (45 days) did not show a similar defect in neutrophil migration. This finding suggested that initial exposure to microbes or microbial ligands might be sufficient to prime neutrophil responses. Similarly, a short exposure of antibiotic-treated mice to a MyD88 ligand was sufficient to restore inflammation in these mice, in spite of the fact that there was no MyD88 stimulation at the time of inflammatory challenge. In the same way, effect of the immune priming triggered by pathogens might be long term and irreversible.

In summary, this study demonstrates that the hype regarding the use of probiotic cultures to treat a myriad of immune diseases needs to be viewed with caution. However, modulation of the intestinal flora at a younger age in humans might be more successful in protecting individuals from immune pathologies in adulthood and needs to be assessed in future studies.

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