CHAPTER ONE: INTRODUCTION

I. The immune system

The environment we live in is far from sterile. Rather, we are constantly exposed to a variety of microorganisms; some are pathogenic and cause diseases in humans. There are five groups of pathogenic microorganisms: viruses, bacteria, fungi, protozoa, and helminthes. To protect from infection, the human body has evolved a multitude of host defenses. The skin and mucous membranes are physical barriers and first line of defense against invading microorganisms. In the respiratory tract, microorganisms reaching the tracheobronchial tree in the respiratory tract can be transported away from the lung by the mucociliary epithelium and removed by coughing. Additionally, in the GI tract, the low pH of the stomach, the antibacterial activity of pancreatic enzymes, bile, and intestinal secretions are mechanisms to kill microorganisms. However, when these physical barriers are breached, our immune system is alerted and it represents a choreographed symphony of host defense against infection. The human immune system has two components: innate immunity and adaptive immunity. Innate immune responses are induced within minutes to hours of microbial infection and play a crucial role in acute eradication of pathogens and induction of inflammatory responses to repair infected host tissues. However, due to the non-specific nature of innate immunity, long-term immunity against similar pathogens cannot be achieved without antigen-specific adaptive responses. In many circumstances, the innate host defense eventually becomes overwhelmed and bypassed, and a more sufficient and antigen-specific adaptive immunity is required to clear pathogens from the body and generate protective memory against subsequent infection. (Janeway’s Immunoiology, 7th Ed., 2007).
A. Innate immunity

The innate immune system is composed of several innate cell types with specialized functions, including macrophages, neutrophils, dendritic cells, eosinophils, basophils, mast cells, Natural Killer T (NKT) cells, NK cells, innate lymphoid cells, and γδ T cells.

Acute response of macrophages and neutrophils to infection

Phagocytes such macrophages and neutrophils are the first responders to infection, owing to the expression of pattern recognition receptors (PRRs), one of the most primitive parts of the immune system, on their cell surface that recognize pathogen-associated molecular patterns (PAMPs) on microorganisms. Resident macrophages in different tissues of the body are the first immune cells to encounter microorganisms in the tissue. They react to infection with two major types of responses. They engulf and kill pathogens and also initiate proper inflammatory responses (Underhill and Goodridge, 2012). Some PRRs are found only on macrophages, including mannose receptors for bacterial carbohydrates and scavenger receptors that bind negatively charged cell wall components of Gram-positive bacteria. In many cases, recognition of pathogens by PRRs results in phagocytosis of pathogens and eventual killing of pathogens within phagosomes after fusion with lysosomes that contain toxic antimicrobial enzymes and proteins. Signaling through some PRRs, such as toll-like receptors (TLRs), in response to bacterial products, induces macrophages to secrete proinflammatory cytokines, such as interleukin-1β (IL-1β), IL-6, and tumor necrosis factor-α (TNFα), CXCL8, and IL-12. These cytokines and chemokines are vehicles for the resident macrophages to initiate local inflammation by accomplishing three tasks: first, activate vascular endothelium and
recruit more immune cells (e.g. IL-1β, TNFα, CXCL8); second, induce local blood clotting to restrict the spread of pathogens to the blood stream (e.g. IL-1β, TNFα); third, activate other cell types such as NK cells and T cells (e.g. IL-1β, IL-6, IL-12) (Savill et al., 2002) (Janeway’s Immunobiology, 7th Ed., 2007).

Neutrophils are also phagocytes. They are short-lived leukocytes abundant in the bone marrow and peripheral blood but scarce in healthy tissues. They are highly phagocytic and able to destroy pathogens quickly without assistance from an adaptive immune response. Neutrophils are typically the first type of immune cells recruited to an infection site, as a result of chemokines such as CXCL8 produced by resident macrophages upon detection of pathogens. However, they undergo apoptosis soon after a round of phagocytosis (DeLeo, 2004; Kennedy and DeLeo, 2009).

**Dendritic Cells**

Dendritic cells (DCs) are a third type of phagocytes that take up particulate matter by phagocytosis. Like macrophages, they are derived from circulating monocytes, but in normal tissues resident macrophages greatly outnumber DCs. The presence of GM-CSF and IL-4 induces monocyte progenitors differentiate into DCs in vitro (Gordon and Taylor, 2005). The main task for DCs, however, is not clearance of pathogens. Although following phagocytosis, as in macrophages, phagosomes in DCs undergo a series of maturation stages and ultimately fuse with lysosomes where the ingested targets are digested, the phagosomal pH in dendritic cells is maintained at relatively alkaline levels, resulting in slower antigen processing and degradation that favors antigen presentation and activation of naïve T cells (Savina et al., 2006). DCs have the capacity to process exogenous antigens for not only MHC II but also MHC I – mediated presentation, thus
providing an important link between innate and adaptive responses. In addition to the conventional presentation of processed microbial antigen on MHC I, DCs can cross-present pathogen-derived antigens taken up from neighboring transformed or infected cells to activate CD8+ T cells. Such a type of cross-presentation is particularly important in tumor immunity as well as the induction of central tolerance and peripheral tolerance of CD8+ T cells to self and environmental agents. Since antigen presentation occurs mainly in peripheral lymphoid organs where DCs mature into professional APCs, DCs are highly migratory. However, in the case of infection, while pathogen-laden DCs migrate from tissues to lymph nodes, not only are T cell activated through antigen presentation, pathogens may be disseminated to lymph nodes in the process (Joffrè et al., 2012; Rescigno and Borrow, 2001).

**Basophils and eosinophils**

Basophils and eosinophils are granulocytes with intracellular granules containing a variety of enzymes and toxic proteins that are released upon activation. They are both thought to play a more prominent role in the defense against helminthes, which are too large to be ingested by macrophages or neutrophils. However, due to their cytotoxic granule contents, excessive recruitment of eosinophils and basophils to the tissue, as seen commonly in allergic inflammation, may pose detrimental effects to the tissue. In addition, basophils have been shown to produce IL-4 and IL-13, two classic Th2 cytokines, during protease-driven mouse Th2-like inflammation to help initiate Th2 response (Sokol and Medzhitov, 2010). Likewise, eosinophils have been reported to produce IL-4 as well and IL-4-producing eosinophils in adipose tissues are important to
sustain the alternatively activated state of adipose macrophages that is pivotal to maintain glucose homeostasis (Wu et al., 2011).

**Natural killer cells**

NK cells are a type of cytotoxic lymphocytes not expressing T cell receptors and functionally analogous to CD8+ cytotoxic T cells, and they are able to recognize stressed cells, such as tumor cells or infected cells, independently of MHC molecules. This unique feature is due to special surface receptors on NK cells that recognize target cells not displaying inhibitory ligands. Upon activation, NK cells release perforin and granzyme to cause lysis or apoptosis of target cells. NK cells also shape immune responses by releasing large amounts of IFNγ and IL-4 (Vivier et al., 2011).

**Natural killer T cells**

NKT cells are lipid antigen (Ag)-reactive CD4+ or CD8+ T cells that express a limited array of αβ T cell receptors (TCRs), which allows the recognition of lipid Ags in the context of CD1d on the surface of antigen-presenting cells (APCs). NKT cells are considered as innate immune cells, since they exhibit no immunological memory and are rapidly activated upon activation of lipid Ags within minutes to hours of antigenic stimulation. A plethora of cytokines, including IL-4, IL-13, and IFNγ, are produced by activated NKT cells, which allows them to modulate immunity in a broad spectrum of diseases, including cancer, autoimmunity, infection, allergy, allograft rejection and graft-versus-host disease (Godfrey and Rossjohn, 2011; Godfrey et al., 2010).
B. Adaptive immunity

Conventional CD4+ T cells, CD8+ T cells (Cytotoxic T lymphocytes, CTLs), and B cells constitute the adaptive immune system.

Cytotoxic T lymphocytes

CTLs recognize specific processed antigens displayed on MHC class I molecule. All nucleated cells express MHC class I. Thus, CTLs can respond to all virally infected nucleated cells. As a type of cytotoxic effector cells like NK cells, activated CTLs secrete proinflammatory cytokines such as IFNγ and release cytotoxins such as perforin and granzymes to induce apoptosis and lysis of target cells. Upon detection of infected cells, CTLs also secrete Fas ligand to bind to Fas on target cells, ultimately leading to Fas-mediated inherent apoptosis of target cells. CTL-induced target cell killing is critical for immunosurveillance, which is supported by the observations of higher rate of spontaneous lymphoma and compromised clearance of tumor in perforin-deficient mice. However, studies have also noted that lytic machinery of CTLs may bring about detrimental effects in the cases of autoimmune diseases and graft-versus-host disease to cause destruction of host tissues. Additionally, CTL targeting of transplanted cells is one of the main causes of graft rejection (Barry and Bleackley, 2002).

Immunological memory

Immunological memory is another key feature of adaptive immunity. Engagement of TCR or BCR with cognate antigens induces T cells and B cells to proliferate and exert their respective effector functions. After the invading pathogen has been cleared, most of antigen-specific T cells and B cells die. However, a small fraction of these cells remain viable and circulate as “memory cells” in the human body, relying on an antigen-
independent mechanism to proliferate. Upon a secondary infection with the same pathogen, these memory T cells and B cells respond swiftly and efficiently to clear the pathogen (Crotty and Ahmed, 2004).

**Immune tolerance**

While it is important for the adaptive immune system to promote pathogen clearance and generate long-lasting immunological memory, it is equally fundamental to prevent unwarranted immune responses to innocuous antigens while responding to harmful antigens. The adaptive immune system achieves this by immunological tolerance. Central tolerance during an early stage of development removes strongly auto-reactive lymphocytes while they are still in the primary lymphoid organs (the thymus for T cells and bone marrow for B cells). For example, lymphocytes reactive to self-antigens are programmed to die by apoptosis and replaced while other lymphocytes with nonreactive TCRs or BCRs are allowed to mature. Those that have escaped clonal deletion may further mature and can be rendered anergic upon exposure to self-antigens (Sakaguchi et al., 2008). Another mechanism to maintain immune tolerance is by induction of T regulatory cells (Tregs).

Naturally occurring CD4+CD25+Foxp3+ Tregs (nTregs) emigrate from the thymus to the periphery to exert their suppressive effects on naïve T cells, effector T cells and APCs, to limit immune response to self and non-self antigens. Additionally, naïve T cells in the periphery have been shown to be able to acquire Foxp3 expression and consequently Treg function in several experimental models. In vitro, antigen stimulation of naïve Tregs in the presence of TGF-β generates induced Tregs (iTregs) that share characteristics with nTregs (Chen et al., 2003). However, it remains controversial
whether iTregs in the periphery are functionally stable in vivo and to what extent they contribute to the peripheral pool of Foxp3+ Tregs (Sakaguchi et al., 2008). The immunosuppressive cytokines secreted by Tregs, IL-10 and TGF-β, are shown to be the mediators of suppression by Tregs in several inflammatory disease models in mice (Venuprasad et al., 2010; von Boehmer, 2005). Additionally, IL-35, another immunosuppressive cytokine, was reported to be secreted by Tregs as well and likely contributes to the suppressive effects of Tregs (Collison et al., 2007). Tregs modulate APC function as well, as revealed by their ability to downregulate dendritic cell co-stimulatory molecules, CD80 and CD86 (von Boehmer, 2005).

CD4 helper T cells

CD4 helper T cells are central to immune protection through a plethora of functions. They instruct B cells to make antibodies, activate macrophages to enhance antimicrobial function, recruit granulocytes to sites of infection and inflammation, and produce cytokines and chemokines to orchestrate immune responses (Zhu and Paul, 2008). They are activated upon recognition of antigen presented in the context of MHC class II molecule. Based on the cytokines they secrete, activated CD4 T cells can differentiate into at least 6 different subsets: Th1, Th2, Th9, Th17, Tregs, and follicular helper T (Tfh) cells. Their fate is determined by the environmental cues, mainly the cytokine milieu, they receive during initial activation by antigen, which promote the development of T helper subsets that secrete specific cytokines and perform distinct functions in regulating immunity (Zhu et al., 2010). For example, IFNγ and IL-12 induces antigen-activated naïve CD4 T cells to become Th1 cells in vitro that secrete IFNγ, but if
IL-4 is present rather than IFNγ, Th2 cells are generated instead to secrete large amounts of IL-4 and IL-13.

Th1 cells secrete IFNγ and TNFα and therefore are poised to activate macrophages and contribute to the clearance of intracellular pathogens such as mycobacteria. The proinflammatory effects of Th1 cells also promote autoimmune diseases (Zhu and Paul, 2008). Th2 cells, through the secretion of IL-4 and IL-13, promote IgE synthesis by B cells and tissue fibrosis, which in excess however contribute to the pathogenesis of allergy and asthma. Th2 cells also mediate the immunity against parasites and give rise to the M2 characteristics of macrophages that are integral for tissue repair and worm expulsion (Zhu and Paul, 2008). Th9 cells are a newly defined T cells subset, characterized by the secretion of IL-9, a pleiotropic cytokine most well-known as a growth factor for T cells and mast cells (Chang et al., 2010; Goswami and Kaplan, 2011). Adoptive transfer of Th9 cells into mice followed by induction of allergic airway disease or colitis showed that Th9 cells exacerbated airway and intestinal inflammation, respectively (Jabeen and Kaplan, 2012). IL-17-secreting Th17 cells are involved in initiation of early response to extracellular bacterial and fungal pathogens, mainly through induction of chemokines to recruit neutrophils to the tissue. Like Th1 cells, excessive Th17 response may promote autoimmunity (Miossec et al., 2009). Tfh cells, mainly producing IL-21, and to a lesser extent, IL-4, are found in B cell follicles and mediate antigen-specific activation of naive or memory B cells to produce antigen-specific antibodies. They promote germinal center formation and differentiation of germinal center B cells into antibody-producing plasma cells or memory B cells. Thus, Tfh cells are essential for the generation of immunological memory (Crotty, 2011).
II. Macrophage biology

A. The origins and development of macrophages

Derived mainly from circulating monocytes in the peripheral blood, macrophages are present in virtually all tissues of a human body. Monocytes originate in the bone marrow from common granulocyte-macrophage progenitors (GMP) and circulate in peripheral blood for several days before migrating to tissues to replenish macrophage populations (Gordon and Taylor, 2005). In the bone marrow, the presence of macrophage colony-stimulating factor (M-CSF), macrophage-granulocyte colony-stimulating factor (GM-CSF) and IL-3, induces GMPs to differentiate into monocytes. Other than cytokines, transcription factors including PU.1 and AML1 are critical for macrophage differentiation and myeloid cell differentiation. Monocytes also have the potential to give rise to DCs. In vitro, this is accomplished by culture in the presence of GM-CSF and IL-4 (Geissmann et al., 2010; Gordon and Taylor, 2005). After migration to the tissue, monocytes increase intracellular content of lysosomal and hydrolytic enzymes, as well as number and size of mitochondria to meet greater energy requirements, to become terminally differentiated resident macrophages (Smith et al., 2011).

Due to unique attributes of macrophages in different tissues, the macrophage populations residing in many tissues are given different names. For example, microglia are specialized macrophages in the central nervous system (CNS) that have “ramified” branches that emerge from the cell body and communicate with surrounding neurons and other glial cells (Saijo and Glass, 2011). Kupffer cells, the specialized macrophages in the liver, function mainly to clear particulate and foreign agents from the portal circulation through phagocytosis. They are efficient scavengers but in general show less active
inflammatory responses, which may in part explain the relative immunological tolerance of the liver for immunogens entering from the portal vein (Laskin et al., 2001). Other examples of macrophages are osteoclasts in the bone, mesangial cells in the kidney, alveolar macrophages in the lung, histiocytes in connective tissues. Despite their heterogeneous features in different tissues, all tissue resident macrophages are similarly long-lived and act as scavengers at the steady state to remove tissue debris and apoptotic cells to maintain tissue homeostasis. They also patrol the tissue microenvironment, and upon sensing danger signals due to tissue stress and microbial invasion, they quickly release chemokines and cytokines to recruit immune cells and initiate acute inflammatory responses necessary to restore a homeostatic state in the tissue. All tissue resident macrophages also exhibit various degrees of phagocytic and antimicrobial activities to combat invading pathogens in the case of infection (Mosser and Edwards, 2008).

In recent years, cells with phenotypes (e.g. F4/80+ CX3CR1+ CD11b+) similar to macrophages were found in the mouse yolk sac prior to definitive hematopoiesis, and these cells populated various tissues and persisted in adults. Development of yolk sac macrophages was shown to be dependent on PU.1 but independent of Myb, a transcription factor absolutely required for the development of hematopoietic stem cells (HSCs) and all BM-derived macrophages (Schulz et al., 2012). This discovery implicates a possible important role for macrophages during yolk sac development.

**B. Monocyte subsets and monocyte recruitment to the tissue**

There are two major subsets of circulating monocytes in mice: CX3CR1low CCR2Ly6C+ and CX3CR1CCR2Ly6Chigh. CX3CR1low CCR2Ly6C+ monocytes are granular and larger than CX3CR1CCR2Ly6Chigh monocytes. They are more responsive
to inflammatory stimuli and migrate quickly to infected or inflamed peripheral tissues. Thus, this subset of monocytes is viewed as inflammatory, corresponding to the CD14$^{\text{hi}}$CD16$^{-}$ inflammatory monocyte subset in humans. In the absence of inflammation, these cells home back to the bone marrow. CX3CR1$^+$CCR2$^+$Ly6C$^{\text{low}}$ monocytes circulate in peripheral blood for longer periods and eventually migrate to peripheral tissues under homeostatic conditions. These cells are postulated to give rise to tissue macrophages and DCs and are referred to as resident monocytes. Human resident monocytes are CD14$^+$CD16$^+$ (Gordon and Taylor, 2005; Serbina et al., 2008).

Monocyte recruitment follows the general paradigm of leukocyte trafficking, which involves rolling, adhesion and transmigration. CC-chemokine ligand 2 (CCL2; also known as MCP1) and CCL7 (MCP3) bind to CCR2 and recruit LY6C$^{\text{hi}}$ monocytes to tissues. Circulating CCL2 is thought to associate with glycosaminoglycans in tissues, which creates a gradient to direct monocyte trafficking towards site of infection or inflammation where high levels of CCL2 are produced by resident cells. Adhesion molecules L-selectin (CD62L), LFA1 and MAC1 on monocytes assist in the adhesion and rolling during monocyte trafficking (Shi and Pamer, 2011). L-selectin has been shown to be critical for recruitment of monocytes during thioglycollate-induced peritonitis as well as migration to lymph nodes (Shi and Pamer, 2011; Tedder et al., 1995). MAC1 is particularly important for adhesion during acute inflammation (Rosen and Gordon, 1987).
C. Macrophage functions

1. Phagocytosis

Phagocytosis is the major route to remove pathogens and cellular debris. It refers to receptor-mediated internalization of extracellular particles (>0.5 \(\mu\)m) by the plasma membrane into a vacuole known as the phagosome. In vivo, such particles are usually microorganisms, dead cells, and tissue debris. Particle internalization is actin-dependent and occurs following the interaction of specific receptors on the surface of the phagocyte with ligands on the surface of the particle. In order to discriminate foreign agents and self, macrophages have evolved a plethora of phagocytic receptors to recognize pathogens. For example, complement receptors on macrophages bind to complements coated on pathogens. Fc receptors allow the capture of pathogens opsonized with specific antibodies. Fc\(\gamma\) receptor, which is specific for IgG opsonized particles, is one of the most studied phagocytic receptor. Dectin 1 can engage \(\beta\)–glucan from yeast cell wall to mediate phagocytosis of yeast cells or yeast components (Underhill and Goodridge, 2012).

After internalization, actins are shed from the nascent phagosome, which however is not yet able to kill the ingested microbe. Despite the diversity in phagocytic receptors, the phagosome undergoes similar maturation stages to acquire antimicrobial activity necessary to kill the ingested microbe, including proteases (e.g. cathepsins) and the phagocyte NADPH oxidase (Kinchen and Ravichandran, 2008). In macrophages, the nascent phagosome undergoes a series of fusions with early and later endosomes that result in proteases and antimicrobial peptides delivered from endosomes. This is followed by fusion of the phagosome with lysosomes to form the mature phagolysosome where
killing and digestion of the microbe occurs. Macrophage phagolysosomes contain hydrolytic enzymes, defensins, antimicrobial peptides, proteases, and a low pH, thus creating a hostile environment to inhibit growth of microbes and promote rapid killing and degradation (Kinchen and Ravichandran, 2008). *M. tuberculosis*, *Legionella pneumophila*, and *Salmonella typhimurium* manipulate phagosome maturation in a manner that favors their intracellular survival (Allen and McCaffrey, 2007; Flannagan et al., 2009; Vazquez-Torres and Fang, 2001).

More recent data demonstrate that phagocytosis results in more than killing and degradation of ingested microbes, evidenced by the production of proinflammatory cytokines and chemokines by phagocytes following engulfment. Phagocytes have evolved mechanisms to sense the nature of ingested targets, process targets to derive antigens for presentation to T cells, and release ligands from targets into the cytosol, which can be detected by various cytosolic pattern recognition receptors to initiate a spectrum of inflammatory responses. For instance, TLR9, an intracellular TLR that localizes to lysosomes, recognizes soluble CpG-containing DNA molecules (Underhill and Goodridge, 2012). Additionally, NOD-like receptors, including NLRP3, NLRC4, and NLRP1B, upon sensing cytosolic danger signals, activate the inflammasome and subsequently caspase1 to promote the release of mature IL-1β and IL-18 from tissue resident macrophages. IL-1β has been shown to promote neutrophil recruitment by increasing expression of adhesion molecules such as VCAMs and selectins on the endothelium, and in the gut promoting a steady-state Th17 response (Shaw et al., 2012). NLRC4 detects cytosolic bacterial products, most notably flagellin, secreted by the
bacterial Type 3 or Type 4 Secretion System from Gram-negative bacteria such as *Legionella pneumophila* and *Salmonella typhimurium* (Franchi et al., 2012).

2. Antigen presentation

Antigen presentation occurs in secondary lymphoid organs where the supportive environment promotes maturation of antigen presenting cells (APCs). It was first recognized in the early 1970s that T lymphocyte activation requires processed antigens in the form of peptides that are presented in the context of MHC class I or class II molecule. Although in vivo and in vitro studies focus more on DCs as APCs to activate naïve T cells, macrophages are also APCs displaying the capability to present antigens. For example, CD169+ macrophages are a subset of in vivo macrophages in mice that have been demonstrated to present antigens to B cells (Carrasco and Batista, 2007), iNKT cells (Barral et al., 2010), and CD8+ T cells (Asano et al., 2011; Martinez-Pomares and Gordon, 2007) in different contexts. CD169 is known as a sialoadhesin that bears ligands for the cysteine-rich domain of mannose receptors. One speculation is that CD169+ macrophages are specialized in processing antigens initially recognized by mannose receptors likely expressed on the same macrophages. Three major populations of CD169+ macrophages are found in the mouse: marginal zone metallophilic (MZF) macrophages in the spleen, and subsapsular sinus and medullary macrophages in lymph nodes. All three populations are posited in regions that are exposed to body fluid, consistent with a role in antigen handling (Martinez-Pomares and Gordon, 2012).
D. Macrophage activation phenotypes

1. Classically activated (M1) macrophages

Classical activation of macrophages is a host defense mechanism in response to microbial infections that results in a macrophage population with an enhanced microbicidal capacity. Two signals, IFNγ and microbial products, most notably TLR ligands, are required to induce classical activation of macrophages. IFNγ can be produced by both innate and adaptive immune cells, but the initial source of IFNγ for classical activation of macrophages is thought to be from innate immune cells. Natural killer (NK) cells are a major cellular source of innate early production of IFNγ in response to microbial infections. While the production of IFNγ by NK cells is rapid, it however is generally transient and thus unable to sustain a population of M1 macrophages that are needed to combat intracellular microbes for an extended period of time. Therefore, induction of an adaptive immune response is critical to provide a continuous source of IFNγ to maintain a population of M1 macrophages. Antigen-specific T helper 1 (Th1) cells typically fulfill this role. Additionally, some TLR ligands can also activate TIR-domain containing adaptor protein inducing IFNβ (TRIF) pathways that signal through IFN-regulatory factor 3 (IRF3) to induce IFNβ production in macrophages (Yamamoto et al., 2003). This endogenous IFNβ has been demonstrated to be able to replace IFNγ from NK and Th1 cells to classically activate macrophages in an autocrine manner.

The enhanced microbicidal capacity of M1 macrophages includes secretion of proinflammatory cytokines (e.g. TNF, IL-6, IL-1, and IL-23) and chemokines (e.g. CCL2, CCL5, and CXCL8) (Yamamoto et al., 2003). M1 macrophages also display increased expression of the phagocyte NADPH oxidase and iNOS and upon activation
give rise to high levels of oxygen and nitrogen radicals that are importantly for efficient killing of intracellular microbes. Uncontrolled classical activation of macrophages however may lead to collateral damage to the host as the oxidative radicals may damage host tissues. The proinflammatory mediators from M1 macrophages can also induce other unnecessary inflammatory responses that are deleterious to the host. For instance, IL-1, IL-6, and IL-23 from M1 macrophages may promote a Th17 response that further recruits neutrophils to the tissue that may further damage the tissue. Thus, classical activation of macrophages must be tightly controlled in order to avoid counter effects. Controlling the availability of IFNγ may be the key, but mechanically it still remains unclear when and how classical activation of macrophages is terminated during microbial infections (Mosser and Edwards, 2008).

2. Alternative activated (M2) macrophages

Alternatively activated macrophages contribute to the defense against parasites, exacerbate allergy pathogenesis, drive fibrosis seen in asthma, and facilitate tissue repair and wound healing (Karp and Murray, 2012). Studies in the recent years have also unfolded extended roles for M2 macrophages in the homeostatic responses to diverse environmental stressors. For instance, M2 macrophages in white adipose tissues are required to maintain insulin sensitivity (Karp and Murray, 2012) and a conversion from M2 to M1 phenotype in white adipose tissues is associated with obesity-induced insulin resistance (Lumeng et al., 2007). Cold stress induces M2 macrophages to produce norepinephrine and trigger adaptive thermogenesis (Nguyen et al., 2011).

Conventionally, alternative activation of macrophages is viewed as a Th2-polarized immune response, since it is potently induced by the signature Th2 cytokines, IL-4 and
IL-13. The initial cellular source of IL-4 responsible for alternative activation of macrophages is also thought to be innate cells. IL-4 and IL-13 can be produced by various innate immune cells, including basophils, mast cells, eosinophils, NKT cells, and even macrophages themselves (Kim et al., 2008; Martinez et al., 2009; Mohrs et al., 2001; Sokol and Medzhitov, 2010; Wu et al., 2011). Similar to classical activation of macrophages, an adaptive immune response, namely Th2 response, is thought to be important for more long-term maintenance of M2 macrophages in vivo.

M2 macrophages display a pattern of gene expression, cell surface molecules, and phenotypic changes that are vastly distinct from M1 macrophages (Stein et al., 1992). Notable markers with significantly increased expression in M2 than M1 macrophages include Arginase1, Ym1 and Ym2, Fizz1, MRC1. IL-4 is more potent than IL-13 in inducing gene expression of Arg1, Ym1, and Fizz1; whereas Mrc1 is induced comparably by IL-4 and IL-13. Macrophages treated with IL-4 or IL-13 in vitro have been shown unable to present antigens to T cells, produce minimal levels of proinflammatory cytokines and much less oxygen and nitrogen radicals than M1 macrophages generated in vitro. As expected, these macrophages were less efficient than M1 macrophages at killing intracellular pathogens (Martinez et al., 2009; Mosser and Edwards, 2008). Therefore, collectively M2 macrophages seem to be more anti-inflammatory than M1 macrophages. While functionally Arg1, Ym1 (a chitinase-like protein), and Fizz1 all promote deposition of extracellular matrix, thus the primary function of M2 macrophages appears to be related to tissue repair and wound healing (Mosser and Edwards, 2008). Notably, arginase from M2 macrophages, which converts arginine to ornithine, a precursor of polyamines and collagen, has been demonstrated to exert a suppressive effect on the
clonal expansion of Th2 cells in vivo (Pesce et al., 2009). Therefore, in addition to their function in tissue repair, M2 macrophages may suppress inflammatory responses by limiting the proliferation of activated T helper cells (Mosser and Edwards, 2008).

**E. Other effects of IL-4 on macrophages**

Although tissue macrophages are generally derived from circulating monocytes released from the bone marrow, in situ proliferation of tissue macrophages has been reported by a few studies. For example, local proliferation of alveolar macrophages was reported by a study that transiently depleted peripheral-blood monocytes (Gordon and Taylor, 2005; Tarling et al., 1987). This study and other studies collectively support that alveolar macrophages can be replenished by recruitment of bone marrow-derived circulating monocytes, which are able to proliferate in situ and inhabit in the lung for a long period (more than one year) (Tarling et al., 1987). Importantly, a recent study on the Th2 response to *L. sigmodontis* (a filarial nematode) revealed self-renewal of peritoneal macrophages that depended on IL-4 production in Th2 cells. Proliferation in situ was proposed as an alternative mechanism of inflammation that allows accumulation of a sufficient number of macrophages to sequester parasites or repair tissues and bypasses the need to recruit more potentially tissue-destructive inflammatory cells (Jenkins et al., 2011).

In recent years, new evidence has emerged and demonstrated that macrophages are able to produce IL-4 as well after stimulation with LPS or infection with *Francisella tularensis* or respiratory syncytial virus (Mukherjee et al., 2009; Pouliot et al., 2005; Shirey et al., 2008; Shirey et al., 2010). Murine alveolar macrophages infected with respiratory syncytial virus were reported to produce IL-4 in vivo, which, in an autocrine
manner, generated M2 macrophages that were important to dampen the inflammatory responses to RSV and facilitate tissue repair (Shirey et al., 2010).

F. Macrophages in human diseases

Given macrophages are dispersed in all organs of the human body, and their versatile functions in immune modulations and metabolism, macrophages are associated with a variety of human diseases.

1. Type 2 diabetes (T2D)

T2D is characterized by insulin resistance and chronic inflammation in adipose tissues, liver and other insulin target tissues. Homeostatically, macrophages residing in white adipose tissue display an M2-like phenotype. PPARγ was shown to be essential to maintain an M2-like phenotype in adipose tissue macrophages, as mice with myeloid cells deficient in PPARγ exhibited impaired alternative activation of macrophages and were predisposed to development of diet-induced obesity, insulin resistance, and glucose intolerance (Odegaard et al., 2007). These M2-like resident adipose tissue macrophages regulate fibrosis and response to acute lipolysis during caloric restriction. Progression of obesity is associated with a switch to an M1 phenotype in adipose macrophages (Lumeng et al., 2007), attributable to NLRP3-dependent sensing of cholesterol crystals that activates macrophage inflammasome response. The accumulated M1 macrophages in adipose tissue produce proinflammatory cytokines and chemokines that establish an insulin-resistant state (Chawla et al., 2011; Duewell et al., 2010).

2. Cancer

Evidence from clinical and mouse studies showed that macrophages promote cancer initiation, progression into malignancy, and the suppression of antitumor
immunity. By creating an inflammatory environment that is mutagenic and favorable for cell growth, macrophages are thought to facilitate cancer initiation. Tumor-associated macrophages then stimulate angiogenesis and enhance tumor metastasis, in a way reminiscent of wound repair by M2 macrophages (Qian and Pollard, 2010). For instance, in the CNS, glioma cells were reported to secrete IL-4, IL-10, IL-6, TGFβ and prostanglandin E2, thus creating a cytokine milieu favorable for M2 polarization of microglia. These M2-like microglia might have a suppressive effect on tumor-specific CD8+ T cells, and CD4+ Th1 and Th17 cells, as well as promote the function of CD4+ regulatory T cells (Saijo and Glass, 2011).

3. Rheumatoid arthritis

Rheumatoid arthritis (RA) is a common autoimmune chronic inflammatory joint disease that is characterized by increased macrophage and lymphocyte infiltration, proliferation of synovial fibroblasts, and cartilage destruction. In RA synovium, the abundant macrophages show a heightened proinflammatory phenotype, with overexpression of MHC II, cytokines such as IL-1, IL-6, IL-10, IL-13, IL-18, GM-CSF, and chemokines KC (IL-8), MIP-1, and MCP1. The activation state of synovial macrophages is partly attributed to the local inflammatory milieu in the synovium. Interaction with fibroblasts has also been shown to elevate the production of IL-6, GM-CSF, and IL-8, and significantly exacerbate cartilage degradation in vitro (Kinne et al., 2000).

4. Inflammatory bowel disease

Inflammatory bowel disease (IBD) is a group of inflammatory conditions affecting the colon and small intestine. Intestinal macrophages, localized in the submucosal lamina propria, have a phenotype distinct from other tissue macrophages in
that they ingest and may kill microbes but they do not mediate strong pro-inflammatory responses upon microbial recognition. They have been shown ex vivo or in vivo to be anergic to TLR ligands, likely due to IL-10 production by intestinal macrophages in the steady state (Smith et al., 2005; Smythies et al., 2005). Intestinal macrophages are responsible for clearance of apoptotic cells or cell debris in the lamina propria. The tolerance of intestinal macrophages to commensal bacteria is critical to maintain a balance between commensal microbiota and the host in the gut. Perturbation of such a balance is an important contributory factor for the development of IBD. Polymorphisms in genes that regulate microbial recognition and immune pathways, such as Nod 2, and genes that control autophagy (e.g. Atg16L1, IRGM) have been found highly associated with IBD. This supports the importance of the interaction between microbes and gut innate immune cells, particularly macrophages and dendritic cells, in establishing and regulating intestinal immune homeostasis (Abraham and Medzhitov, 2011).

III. Neutrophil biology

Neutrophils (also called polymorphonuclear leukocytes or PMNs) are the most abundant leukocytes in circulation and are particularly important during the acute phase to combat infections, which is underscored by often severe and fatal infections in patients with congenital neutropenia. Neutrophils develop from hematopoietic progenitors in the bone marrow and mature in the bone marrow in a process known as granulopoiesis. As neutrophils mature, they synthesize proteins that are sorted into different granules. There are three types of granules in neutrophils based on resident cargo molecules in each. The key cytokine that stimulates granulopoiesis is granulocyte colony-stimulating factor.
(G-CSF), which is also used in the clinical setting to treat neutropenia (Furze and Rankin, 2008).

**A. Neutrophil mobilization and recruitment**

The bone marrow serves as a reserve of mature neutrophils that can be rapidly mobilized into circulation in response to infection or stress. Agents that induce release of neutrophils include chemoattractants (e.g. KC), leukotrienes, bacterial products (e.g. LPS), cytokines (e.g. G-CSF, IL-17, IL-1β), and inflammatory mediators (e.g. complement factors) (Christopher and Link, 2007). Reports in recent years revealed increased serum G-CSF levels during infections by a number of pathogens in mice, such as *Candida albicans* and *Listeria monocytogenes*, and an important role for G-CSF in regulating stress-induced granulopoiesis. Release of neutrophils from the bone marrow is regulated by stromal derived factor-1 (SDF-1) and its receptor, CXCR4, on neutrophils and hematopoietic progenitors in the bone marrow. The effect of G-CSF on neutrophil mobilization is thought to be a consequence of the disruption of SDF-1/CXCR4 signaling, since G-CSF has been shown to have an inhibitory effect on the expression of SDF-1 (Christopher and Link, 2007; Semerad et al., 2002).

However, the effect of G-CSF on the disruption of SDF-1-mediated retention on neutrophils was shown to result in the mobilization of neutrophils from the bone marrow but was insufficient to elicit recruitment of neutrophils to the infected or inflamed tissue. Local production of chemokines and cytokines in the tissue, such as KC, MIP-2, IL-17 and IL-1β, is integral to promote the infiltration of neutrophils into the tissue (Miller et al., 2007; Wengner et al., 2008). IL-17 and IL-1β promote neutrophil recruitment partly by inducing endothelial cells to upregulate adhesion molecules on their luminal side to
allow tethering and rolling of neutrophils on the endothelium. These adhesion molecules include P-selectins, E-selectins, integrins, and ICAMs (Borregaard, 2010). Neutrophils are relatively short-lived. Mature neutrophils circulate in the peripheral blood for 10-24 h before migrating to tissues where they function for an additional 1-2 days before undergoing apoptosis, followed by macrophage-dependent clearance (Kennedy and DeLeo, 2009).

B. Anti-microbial function of neutrophils

Neutrophils play an important role in the acute host defense against bacterial and fungal infections. Neutrophils combat microbial pathogens with an arsenal of anti-microbial mechanisms: phagocytosis, degranulation, cytokine secretion, and neutrophil extracellular traps (NETs). Phagocytosis in neutrophils is similar to that in macrophages in terms of the phagocytic receptors involved and maturation steps for phagosomes, except for a strong and sudden increase in proteolytic activity very early after engulfment in neutrophils. This is due to high-level production of reactive oxygen species by the NADPH oxidase, causing the alkalization of the phagosomal lumen (to ~pH 7) and activation of neutrophil serine proteases such as cathepsin G (Watts, 2006). In addition, myeloperoxidase, a neutrophil-specific granule protein, can quickly convert hydrogen peroxide generated by the NADPH oxidase in the phagosome to hypochlorous acid (HOCl) that is more deadly for ingested microbes. NETosis is another form of microbial killing by neutrophils. It is an active form of cell death involving the release of decondensed chromatins as well as antimicrobial granular and cytosolic proteins into the extracellular space to create a local and hostile environment to trap and kill microbes. NETs are also thought to be a mechanism to sequester microbes by forming a physical
Given the variety of anti-microbial contents in NETs, mice deficient in specific components of NETs exhibit only a minor impact on the formation of NETs. However, mouse and human neutrophils deficient in the NADPH oxidase, which is found in NETs, are unable to form NETs upon activation, suggesting a critical role for the NADPH oxidase in the formation of NETs (Bianchi et al., 2009; Fuchs et al., 2007). Stimuli that have been demonstrated ex vivo to induce NET formation include phorbol myristate acetate (PMA), *Staphylococcus aureus*, *Candida albicans*, and platelets (Papayannopoulos and Zychlinsky, 2009).

### IV. Natural killer T cell biology

NKT cells are a lineage of CD1d-restricted T cells that express a T cell receptor repertoire that recognizes lipid antigens presented through the CD1d molecule on APCs. They share some surface markers with NK cells, such as NK1.1 (or CD161). NKT cells are regarded as innate immune cells, since upon activation by glycolipids, they rapidly secret copious amounts of a broad range of cytokines, including IFNγ, IL-4, IL-13, IL-17, GM-CSF, and IL-2. The cytokines produced by NKT cells can induce downstream activation of a variety of other cell types, including DCs, NK cells, B cells and conventional T cells. Thus NKT cells may be a crucial in the interplay both innate and adaptive immune responses. In fact, NKT cells have been shown to promote cell-mediated immunity to tumor as well as bacterial and viral infections (Christopher and Link, 2007; Godfrey and Rossjohn, 2011; Godfrey et al., 2010).

NKT cells are developmentally and functionally distinct from conventional CD4+ and CD8+ T cells. These conventional T cells are selected in the thymus by peptide antigens in complex with MHC class I or class II molecules present on the surface of
thymic epithelial cells. In contrast, while NKT cells also originate in the thymus, they develop following selection by self-glycolipids in complex with CD1d presented by CD4+CD8+ double positive thymocytes (Das et al., 2010). CD1d is a surface glycoprotein belonging to the CD1 family, which consists of two groups based on amino acid homology. Group 1 includes CD1a, b, and c; whereas CD1d is the only member of group 2. There are two CD1d molecules in mice: CD1d1 and CD1d2. All CD1 molecules are able to present lipids to a wide variety of T cells, but CD1d molecules present lipids to a specific T cell subpopulation called NKT cells (Brutkiewicz, 2006). Given its important role in the selection of NKT cells during development in the thymus, mice deficient in CD1d lack NKT cells (Van Kaer et al., 2011).

There are two types of NKT cells: Type 1 and Type 2. Type 1 NKT cells or invariant NKT (iNKT) cells express a restricted αβ T cell receptor (TCR) repertoire comprised of the canonical Vα14-Jα18 chain in association with the Vβ2, Vβ7 or Vβ8 chains in mice (equivalent to Vα24-Jα18 and Vβ11 in humans). Through these TCRs, iNKT cells recognize specific lipids presented by CD1d. Known lipid antigens capable of activating iNKT cells include α-glycosylceramides and α–galacturonosylceramide from the cell wall of Gram-negative bacteria Sphingomonas (Kinjo et al., 2005; Mattner et al., 2005; Sriram et al., 2005), and α–galactosyldiacylglycerol from spirochetes Borrelia burgdorferi (Kinjo et al., 2006). Moreover, environmental NKT cell stimuli have also been reported. CD1d-restricted NKT cell antigens are present within common house dust extract (HDE) (Wingender et al., 2011). In addition, α-galactosylceramide (α-GalCer), which was originally isolated from the sea sponge Agelas mauritianus, is a known lipid able to potently activate iNKT cells, and it has been the model NKT cell agonist used in
biomedical research. The use of α-GalCer-loaded CD1d tetramer has greatly benefited the identification of iNKT cells in both mice and humans.

NKT cells also respond to self lipids. The endogenous lysosomal glycosphingolipid, iGb3 (isoglobotrihexosylceramide), was shown to be presented by LPS-stimulated dendritic cells to activate NKT cells following infection with Gram-negative and LPS-positive Salmonella typhimurium (Mattner et al., 2005). A recent study reported that ether-bonded phospholipids generated mainly in peroxisomes are natural ligands that are responsible for the development of a major subpopulation of iNKT cells in the thymus (Facciotti et al., 2012). Other self lipids reported to activate NKT cells include including β-linked glycosphingolipids β-galactosylceramide (β-GalCer), β-glucosylceramide (β-GlcCer), and disialoganglioside, as well as self-phospholipid Ags such as phosphatidylethanolamine, phosphatidylinositol, and phosphatidylcholine (Godfrey and Rossjohn, 2011).

The mechanism by which iNKT cells encounter antigen and become activated in vivo has not been fully investigated. Several studies have suggested that DCs and Kupffer cells play an important role in presenting glycolipids to iNKT cells in spleen and liver. In fact, DCs pulsed in vitro with bacteria such as Sphingomonas and Borrelia or different glycolipids were shown to stimulate activation of iNKT cells in vivo after adoptive transfer (Kinjo et al., 2006; Mattner et al., 2005). A recent study demonstrated that within hours of administration of lipids, subcapsular sinus CD169+ macrophages interacted with lymph node iNKT cells in a CD1d-dependent manner and presented lipid antigen to activate iNKT cells in the lymph nodes (Barral et al., 2010).
V. Efferocytosis

The term “efferocytosis” refers to the phagocytosis of an apoptotic cell. About 200 billion cells are cleared from each human body every day, making efferocytosis the most common type of phagocytosis. In mammals, apoptosis, or self-autonomous cell death, is of fundamental importance during embryonic development (Vaux and Korsmeyer, 1999) and negative selection for T cell development in the thymus. Additionally, proper removal of enucleated erythrocytes, whose life span is about 120 days in humans, is crucial for homeostasis (Vaux and Korsmeyer, 1999). In humans, phagocytes that ingest apoptotic cells are generally categorized in two groups: “professional” phagocytes, namely macrophages and dendritic cells; and “non-professional” phagocytes, which are particularly important in tissues where professional phagocytes cannot infiltrate (Zhou and Yu, 2008). For instance, airway epithelial cells (Walsh et al., 1999), mesenchymal cells (Walsh et al., 1999), microglial cells (Saijo and Glass, 2011) and sertoli cells all exhibit the capacity to engulf apoptotic cells or apoptotic bodies. Apoptosis is associated with retention of plasma membrane integrity, chromatin condensation, cell shrinkage, and cleavage of cytosolic and nuclear proteins in a caspase-dependent manner. In addition, phosphatidylserine (PS), which normally resides on the inner leaflet of the plasma membrane, is oxidized at the early stage of apoptosis and flipped onto the outer leaflet of the plasma membrane when phospholipid asymmetry of the plasma membrane is lost during apoptosis (Fadok et al., 2001; Henson and Hume, 2006).

Defects in clearance of apoptotic cells, as seen in complement deficiency disorders such as systemic lupus erythematosus (SLE) and cystic fibrosis (Vandivier et
al., 2002b), are commonly associated with autoimmunity (Erwig and Henson, 2007; Nagata, 2007). Apoptotic cells are a common source of autoantigens. When clearance is disturbed, apoptotic cells may undergo necrosis and release DNA that subsequently elicits autoimmune responses and production of autoantibodies. SLE is characterized by the presence of autoantibodies that recognize nuclear antigens normally found in apoptotic blebs, such as DNA, RNA and chromatin (Liu et al., 2006). Autoantibodies were found in mice deficient in DAase II, which is localized in lysosomes and is required for degrading DNAs from ingested apoptotic cells in macrophages (Kawane et al., 2006).

A variety of bridge molecules (e.g. C1q, thrombospondin, PS) on apoptotic cells and cell surface receptors (e.g. CD14, CD36, Mac-1) on phagocytes mediate the recognition and engulfment of apoptotic cells (Fadok et al., 1998c; Vandivier et al., 2002a; Vandivier et al., 2002c). Of note, some of these receptors are also engaged in the uptake of microbes. For instance, iC3b opsonized microbes are known to bind to two β2 integrins, complement receptors 3 and 4, without triggering macrophage inflammatory responses (Wright et al., 1983; Wright and Silverstein, 1983). However, the immunosuppressive effect of apoptotic cells has been well documented (Fadok et al., 1998a; Ren et al., 2008; Voll et al., 1997). TGFβ and IL-10 are key anti-inflammatory cytokines reported to be produced by efferocytosing monocytes (Byrne and Reen, 2002; Fadok et al., 1998a; Voll et al., 1997) and macrophages (Fadok et al., 1998b). TGFβ released from efferocytic macrophages has been shown to pose remarkable anti-inflammatory effects on inflamed peritoneum and lungs (Fadok et al., 1998a; Huynh et al., 2002; Savill et al., 2002). Since some phagocytic receptors are engaged in the uptake of microbes and apoptotic cells, elegant mechanisms may exist to allow the
differentiation among different phagocytic prey by phagocytes. PAMPs on microbes may engage stimulatory surface or cytosolic receptors on phagocytes, such as TLRs and NLRs, to elicit proinflammatory responses against microbes. Likewise, apoptotic cells may display different surface ligands or secrete factors to repress proinflammatory responses. The recognition and uptake of apoptotic cells are relatively more characterized in the field of efferocytosis; while the more downstream degradation and processing of ingested apoptotic cells and more importantly the mechanisms underlying the immunosuppressive effects of efferocytosis in vivo remain largely elusive.

VI. Sterile inflammation

Inflammation is an adaptive response commonly triggered by infection and tissue injury, as a mechanism of host defense and tissue repair, respectively. A delicately controlled inflammatory response is beneficial, but it can be detrimental if dysregulated, as seen in chronic infections and autoimmune diseases. Chronic inflammation is also associated with other human diseases that are not caused by infections or tissue injury but rather a homeostatic imbalance of physiological conditions, such as the cases of type 2 diabetes and cardiovascular diseases. Regardless of the cause, inflammation is initiated to restore homeostasis in the affected tissue (Medzhitov, 2008).

Inflammation as a result of trauma, ischemia-repufusion injury, or irritant-induced injury, in the absence of infection, is termed sterile inflammation (Chen and Nunez, 2010). Similar to infection-induced inflammation, sterile inflammation is characterized by acute infiltration of blood granulocytes, typically neutrophils, followed by recruitment of monocytes that mature into inflammatory macrophages in the tissue. The acute inflammatory response to the tissue injury is first executed by resident tissue
cells, which sense the danger signals, known as danger-associated molecular patterns (DAMPs), released from resident damaged cells, such as high mobility group box 1 (HMGB1) proteins, DNAs, RNAs, ATP, and uric acids. DAMPs can be sensed by PRRs expressed in resident cells in the injured tissues, most notably TLRs and NLRs in resident macrophages. PRR signaling result in production of a variety of inflammatory mediators, including chemokines, cytokines, prostaglandins, proteases, and vasoactive amines (Rock et al., 2010). An immediate consequence is localized extravazation and infiltration of neutrophils, as well as delivery of blood plasma to the site of infection or injury. The recruited neutrophils attempt to kill invading pathogens or removing damaged tissue debris by releasing toxic granule contents, including proteinase 3, cathepsins and elastase, as well as reactive oxygen and nitrogen species. While the inflammatory responses in neutrophils are indiscriminative between foreign and host targets, collateral damage to host tissues is minimized by subsequent recruitment of macrophages, which play a crucial role in the transition from inflammation to resolution (Fig. 1) Resolution of inflammation is accomplished when granulocytes in the tissue are eliminated and the tissue mononuclear cell population, namely macrophages and lymphocytes, return to the pre-inflammatory state in terms of numbers and phenotypes (Serhan and Savill, 2005). Unlike the response to microbial infections where antigen-specific adaptive responses by lymphocytes are induced and memory against foreign antigens is formed, sterile inflammation is thought to involve solely innate cells for the purpose of tissue and wound repair.

There are several cases of human diseases involving sterile inflammation (Chen and Nunez, 2010; Rock et al., 2010). Pulmonary interstitial fibrosis is a result of chronic
inhalation of irritants, such as asbestos and silica, which leads to persistent activation of alveolar macrophages. The massive tissue destruction in ischemia-reperfusion injury, commonly resulted from myocardial infarction and stroke, triggers a pronounced acute inflammatory response marked by recruitment of neutrophils and macrophages. Gout is another example of sterile inflammatory disease, where elevated uric acid in the blood crystallizes and these crystals deposit in the joints, resulting in acute neutrophil infiltration and chronic inflammation (Kono et al., 2010). Atherosclerosis is also characterized by infiltration of immune cells, particularly macrophages, which is thought to be a consequence of activation of resident macrophages after engulfment of cholesterol crystals and chemokine production by these cells. Finally, a tumor microenvironment also promotes immune cell infiltration, and these cells secrete factors that in turn influence the grown and progression of cancer (Chen and Nunez, 2010).
Figure 1. Tissue injury – induced sterile inflammation

In response to tissue injury, neutrophils are the first type of leukocytes infiltrating to the injury site where they remove tissue debris but soon undergo apoptosis. Monocytes are recruited at a later time point and differentiating into mature macrophages in the tissue, where they efferocytose apoptotic neutrophils. Efferocytosing macrophages are reported to display an anti-inflammatory phenotype, most notably by releasing anti-inflammatory cytokines such as TGFβ and IL-10 to suppress inflammation (Serhan and Savill, 2005). Ultimately these macrophages migrate to the draining lymph node, resulting in restoration of homeostasis in the tissue.
VII. The phagocyte NADPH oxidase and Chronic Granulomatous Disease

A. The phagocyte NADPH oxidase

The phagocyte NADPH oxidase is responsible for generating reactive oxygen species (ROS) in neutrophils and macrophages in response to microbial infection and therefore contributes to the antimicrobial function of phagocytic leukocytes (Rada et al., 2008; Segal, 2005). Expressed mainly in myeloid cells, the oxidase is a membrane-bound enzyme complex consisting of separate membrane and cytosolic subunits in the resting state. Flavocytochrome b558, a heterodimer of gp91phox (91kD) and p22phox (22kD), is the membrane-bound component of the oxidase and also the focal point for oxidase assembly, which involves translocation of oxidase cytosolic proteins, p40phox, p47phox, p67phox, and a GTPase (Rac1 or Rac2) to the membrane (Dinauer, 2003; Knaus et al., 1991; Nauseef, 2004). Proper assembly of the oxidase is required for electron transfer on flavocytochrome b558, the redox center, to convert molecular oxygen to superoxide molecules (Segal, 2005). Soluble activators of the oxidase, including phorbol esters and chemoattractants, stimulate oxidase assembly on the plasma membrane and lead to extracellular ROS production (Fig. 2). In contrast, phagocytosis of particulate opsonized or non-opsonized bacterial or fungal microbes through phagocytic receptors, such as Fcγ receptors and complement receptors, results in recruitment of the oxidase complex to the phagosome membrane as well. Under these circumstances, most of ROS produced are localized within the lumen of phagosomes and participate in degradation of the trapped microbes (DeLeo et al., 1999; Kennedy and DeLeo, 2009). Various phagocytic receptors are coupled with oxidase activation via common downstream phospholipases, protein kinases, and GTPases that are also engaged in activating oxidase cytosolic subunits to
allow membrane translocation (Groemping and Rittinger, 2005; Hawkins et al., 2007; Kennedy and DeLeo, 2009; Nauseef, 2004). Inappropriate and excessive production of ROS, however, may cause tissue injury (Babior, 2000).

Studies in recent years have demonstrated new mechanisms for phagosomal ROS to participate in host defense. First, phagosomal superoxide molecules generated by the oxidase dismutate to hydrogen peroxide by scavenging protons pumped into the phagosome by the vacuolar ATPase, thereby alkalizing the phagosome pH that favors activation of serine proteases in neutrophils to boost microbial killing (Watts, 2006). However, in dendritic cells, the resulted alkaline milieu in the phagosome lumen reduces activity of cysteine proteases needed for complete degradation, resulting in partial digestion of the ingested particles that favors cross-presentation of microbial antigens to CD8+ T cells (Mantegazza et al., 2008; Savina et al., 2009). Second, formation of NETs has been illustrated to be NADPH oxidase – dependent, as evidenced by the lack of NET formation by neutrophils deficient in the NADPH oxidase (Bianchi et al., 2009). Third, superoxide generated by the NADPH oxidase has been demonstrated to depolarize the phagosomal membrane, resulting in an influx of H+ and K+ into the phagosomes and thereby lowering the pH within the lumen of the phagosomes, which favors activation of lysosomal proteases that are critical for the killing of the ingested microbe (Hultqvid et al., 2009; Sareila et al., 2011). Lastly, phagosomal ROS induced by TLR 2/4 or FcγR signaling are involved in recruiting autophagy components to the phagosome, thereby augmenting phagosome-lysosome fusion for efficient degradation (Huang and Brumell, 2009b; Huang et al., 2009).
In addition, a few mechanisms have been proposed for ROS to affect reactivity of T cells. First, by raising pH and attenuating antigen degradation in DCs that prevents complete digestion and favors antigen presentation (Mantegazza et al., 2008; Savina et al., 2006). Second, macrophages might use the oxidative burst to oxidize T cell membrane proteins during the formation of immunological synapse. T cells from rats and mice bearing an Ncf1 mutant were shown to have more reduced proteins on their cell membrane. Interestingly, chemically reduced T cells were found to survive longer and home to joints and draining lymph nodes during arthritis development (Gelderman et al., 2006; Gelderman et al., 2007). Additionally, intracellular proteins in T cells may be modified and thus alter TCR signaling cascades in T cells following antigen presentation (Hultqvist et al., 2009).

Moreover, new insights into the versatile functions of oxidase-derived ROS came with the identification of mammalian homologs of gp91phox in various non-myeloid cell types, including endothelial cells, epithelial cells, and smooth muscle cells (Bedard and Krause, 2007; Geiszt and Leto, 2004; Lambeth, 2004). These proteins, along with gp91phox, form the NOX (NADPH oxidase) family, and gp91phox is designated as NOX2. These other NOX proteins interact with different binding partners for activation, some of which include homologs of phagocyte oxidase subunits p22phox, p47phox, and p67phox. They are capable of generating superoxide or hydrogen peroxide to various degrees, that likely engage in signal transduction for different cellular functions in these cell types (Nauseef, 2008).
Figure 2. Assembly and activation of phagocyte NADPH oxidase

In resting cells, the membrane-bound flavocytochrome \( b_{558} \) (a heterodomer of gp91\(^{phox} \) and p22\(^{phox} \)) is separated spatially from the cytosolic subunits: Rac, p67\(^{phox} \), p47\(^{phox} \), and p40\(^{phox} \). Upon activation by microbes or inflammatory mediators (e.g. PMA and fMLF), activation of Rac and phosphorylation of p47\(^{phox} \) lead to membrane translocation and association of the cytosolic subunits with flavocytochrome \( b_{558} \) and activation of the NADPH oxidase to generate \( \text{O}_2^- \). Cartoon is adapted from W. Nauseef (Nauseef, 2007).
B. Regulation of the phagocyte NADPH Oxidase by Rac GTPase

The phagocyte NADPH oxidase represents one of the best-characterized Rac-regulated systems (Bokoch, 2005; Diebold and Bokoch, 2005), in which binding of a GTP-bound Rac to the N-terminal tetratricopeptide repeats (TPRs) of p67phox is one of the prerequisites for activation of the oxidase in both intact neutrophils and in cell-free systems (Akasaki et al., 1999; Diekmann et al., 1994; Groemping and Rittinger, 2005). The hematopoietic-specific Rac2 isoform plays a critical role in regulating the oxidase in neutrophils, and Rac1 is more utilized in monocytes and macrophages (Kim and Dinauer, 2001; Knaus et al., 1991; Yamauchi et al., 2004). The essential role of Rac in the activation of phagocyte NADPH oxidase (Fig. 3) is evidenced by reports of significant reduction of ROS production by Rac2−/− neutrophils (Kim and Dinauer, 2001; Roberts et al., 1999) and macrophages (Yamauchi et al., 2004) in response to various stimuli, which is thought to be at least partially responsible for the recurrent life-threatening infections in a patient with a dominant negative Rac2 allele (Ambruso et al., 2000; Williams et al., 2000). Binding of a GTP-bound Rac to p67phox, a downstream effector protein of Rac, is postulated to optimally “position” p67phox on flavocytochrome b558 to allow for electron transfer to oxygen molecules (Akasaki et al., 1999). Derivatives of Rac harboring A27K, G30S, or D38A point mutations in its effector domain cannot associate with p67phox and fail to support NADPH oxidase activity in cell-free assays (Diebold and Bokoch, 2001; Lapouge et al., 2000; Xu et al., 1994).

Emerging evidence in recent years supports a model in which a direct interaction between Rac and gp91phox serves as an additional mechanism whereby Rac regulates activity of the phagocyte NADPH oxidase. In the first study suggesting a possible
physical association between Rac2 and flavocytchrome \( b_{558} \), which used human neutrophils stimulated with phorbol myristate acetate (PMA) to activate the NADPH oxidase, translocation of Rac2 to the plasma membrane was normal in CGD neutrophils lacking either \( p47^{phox} \) or \( p67^{phox} \) but significantly reduced in neutrophils from patients with X-linked CGD (X-CGD) who had mutations in the gene encoding \( gp91^{phox} \) and thus lacked flavocytchrome \( b_{558} \) (Heyworth et al., 1994). In cell free assays using purified proteins, Diebold and Bokoch subsequently demonstrated that the insert domain of Rac (aa 124-135) was integral for both electron transfer from NADPH to FAD, and from reduced FAD to oxygen molecules on flavocytchrome \( b_{558} \), with only the latter dependent on interaction of Rac with \( p67^{phox} \) (Diebold and Bokoch, 2001). They additionally illustrated a direct physical interaction of Rac2 with flavocytchrome \( b_{558} \) using fluorescently labeled Rac2, which also required the insert domain of Rac2. Based on these observations, Diebold and Bokoch proposed a two-step model for regulation of the phagocyte NADPH oxidase by Rac, wherein \( p67^{phox} \) and Rac regulate the first step of electron transfer through their independent interactions with \( gp91^{phox} \), but the second step of electron transfer requires physical interaction between \( p67^{phox} \) and Rac (Fig. 4).

Additional studies showed that recombinant prenylated Rac1 or Rac2 can bind purified flavocytchrome \( b_{558} \), and that a Rac-binding site was present in a cytoplasmic domain of \( gp91^{phox} \) spanning amino acids 419-430, which resides between two regions proposed to interact with intracellular NADPH (Kao et al., 2008). In a transgenic COS7 cell model, mutagenesis studies showed that amino acids Lys-421, Tyr-425, and Lys-426 in \( gp91^{phox} \) were especially critical for PMA-induced activation of the oxidase, while inducing no obvious structural alterations in the C-terminus (Kao et al., 2008) and a 12-
residue peptide derived from gp91\textsuperscript{phox} inhibited NADPH oxidase activity in PMA- or fMLF-stimulated human neutrophils (Kao et al., 2008). Interestingly, homologous Rac-binding sequences are conserved in NOX1 and NOX3, and mutations of this conserved region in NOX1 resulted in loss of Rac-dependent NOX1 activity (Kao et al., 2008). Therefore, parallel interaction between Rac1 and NOX1 or NOX3 may exist in nonhematopoietic cells.

**Figure 3. Chrystal structure of Rac GTPase (Rac 1 or Rac 2)**

The insert domain of Rac binds to the cytoplasmic tail of gp91\textsuperscript{phox}. In a GTP-bound conformation, the effector loop binds to the TPR domain of p67\textsuperscript{phox}. The C-terminus of Rac has basic amino acids that assist in targeting Rac to the membrane.
Figure 4. A two-step model for Rac regulation of the activation of the phagocyte NADPH oxidase and electron transfer

The phagocyte NADPH oxidase contains the binding site for NADPH, FAD, and two heme groups. Activated Rac and p67phox initially interact with Nox2 separately to facilitate electron flow from cytoplasmic NADPH to cytochrome \( b558 \)-bound FAD (Step 1). Then Rac binds to the TPR domain of p67\(^{phox} \) to activate p67\(^{phox} \), this Rac-bound p67\(^{phox} \) can now facilitate electron transfer from FADH\(_2 \) to hemes, and this is coupled with electron transfer to O\(_2 \) to generate O\(_2^- \) (Step 2). This cartoon is adapted from B. Diebold and G. Bokoch (Bokoch and Diebold, 2002).
C. Chronic Granulomatous Disease

Chronic granulomatous disease (CGD), an inherited immunodeficiency found in about 1 out of every 250,000 humans, is caused by absent or reduced generation of phagocyte ROS due to mutations in the subunits of phagocyte NADPH oxidase. CGD patients often suffer from recurrent microbial infections and a higher susceptibility to infections with opportunistic pathogens (Winkelstein et al., 2000). Infection with the fungal pathogen *Aspergillus fumigatus* often leads to pneumonia in CGD patients that could be life-threatening if not treated promptly, and infection with *Aspergillus nidulans* is associated with high incidence of osteomyelitis in CGD patients (Dotis and Roilides, 2004; van den Berg et al., 2009; Winkelstein et al., 2000). Overall, *Aspergillus* infection accounts for one third of all deaths among CGD patients. Other major pathogens responsible for infections in CGD patients are *Staphylococcus aureus* and Gram-negative enteric bacilli including *Burkholderia cepacia* and *Salmonella* species. Other common complications seen in CGD include subcutaneous and liver abscesses, lymphomegaly, and sepsis (Schappi et al., 2008b; van den Berg et al., 2009).

The mortality in CGD patients, typically consequential to overwhelming pneumonia and sepsis, has substantially declined over the past two decades due to advances in diagnosis and treatment. Nonetheless, the overall annual mortality is still relatively high, ranging from 2% to 5% (Schappi et al., 2008a). Common treatment regimens for CGD include prophylactic and therapeutic antibiotics and interferon γ, which have been effective in reducing infection rates in CGD patients (van den Berg et al., 2009; Winkelstein et al., 2000). Since the phagocyte NADPH oxidase is primarily expressed in myeloid cells, for CGD patients with available HLA-matched donors, stem
cell transplantation is a curative option, although there is a concern about graft-versus-host disease (GVHD) post-transplantation (Seger et al., 2002; Stein et al., 2006). In addition, the involvement of only a single mutated gene encoding one of the oxidase subunits CGD makes it an appealing candidate for gene therapy. Indeed, especially with the availability of gp91phox−/− (Pollock et al., 1995) and p47phox−/− (Jackson et al., 1995) mouse models and the feasibility of purifying human bone marrow CD34+ progenitors, gene transfer into hematopoietic stem cells to correct CGD has been an subject of active research in the past two decades (Becker et al., 1998; Dinauer et al., 2001; Goebel and Dinauer, 2002; Li et al., 1994; Ott et al., 2006; Stein et al., 2006).

D. Dysregulated inflammation in CGD patients

Given the impairment in microbial killing by CGD neutrophils and macrophages, hyperinflammation in CGD is conceivable. In addition, a few observations suggest that the hyperinflammation in CGD may be partially caused by dysregulation of inflammation in physiologic conditions. One distinctive feature of CGD is formation of inflammatory granulomas in various organs, including the lung, skin, liver, lymph node, or gastrointestinal tracts, where in many cases no microorganisms are recovered from the lesions (Schappi et al., 2008b; Winkelstein et al., 2000). These granulomatous lesions are responsive to immunomodulators such as glucocorticoids but irresponsible to antibiotics. Histological studies of such lesions from GI tracts, liver and lymph nodes revealed the presence of a small number of neutrophils, some eosinophils, abundant nuclear debris and macrophages (Schappi et al., 2008a; Schappi et al., 2003). Together, these observations suggest that incomplete degradation of debris and/or delayed resolution of inflammation may be partly responsible for formation of granulomatous lesions in CGD.
A recent report showed markedly elevated levels of anti-microbial antibodies in CGD patients that do not correlate with a history of colitis but rather reflect a specific defect in innate immunity likely as a result of chronic antigenic stimulation (Yu et al., 2011). X-CGD female carriers possess two distinct phagocytic leukocyte populations: oxidase-positive and oxidase-negative (Schappi et al., 2008a). Although reduced, oxidant production by the oxidase-positive population to a great extent protects these carriers from microbial infections frequently seen in CGD patients. However, there is a higher incidence of discoid lupus erythematosus – like skin lesions in X-CGD carriers, and the incidence of autoantibody production is significantly higher in X-CGD carriers than in non-carrier relatives (Cale et al., 2007; De Ravin et al., 2008). These observations raised the possibility of a form of autoimmunity in CGD (De Ravin et al., 2008). In fact, neutrophil cytosolic factor 2 (Ncf2, which encodes the oxidase subunit p67phox) was recently identified as an important risk factor for system lupus erythematosus (SLE). NCF2 H389Q mutation reduces the binding efficiency of NCF2 with guanine nucleotide exchange factor Vav1, thereby reducing ROS production during FcRγ− elicited activation of the NADPH oxidase in neutrophils. H389Q mutation in NCF2 is strongly associated with SLE (Jacob et al., 2012).

E. Dysregulated inflammation in CGD mice

A previous study reported that intratracheal instillation of sterile hyphae, preparation of Aspergillus fumigatus cell wall, led to pulmonary inflammatory lesions in X-CGD mice that persisted for at least 6 weeks, whereas only mild pulmonary inflammation was observed in WT mice that disappeared within a week (Morgenstern et al., 1997). Moreover, after 24 hours of hyphae instillation, compared with WT mice there
were significantly higher numbers of neutrophils infiltrating to X-CGD lungs and increased expression of the proinflammatory cytokines, TNFα and IL-1β (Morgenstern et al., 1997). Increased neutrophil infiltration to peritoneal cavity at the early phase of inflammation was also found in both the X-CGD (Pollock et al., 1995) and p47phox−/− (Jackson et al., 1995) mouse models during thioglycollate-induced peritoneal inflammation, which resolved at a slower rate compared with WT mice. In a more recent study, exaggerated and prolonged skin inflammation was described in CGD mice that received subcutaneous injection of a specific fungal cell wall component, branched beta2 glucan, which was also present Aspergillus hyphae, was used in the study noted above. Remarkably, in these CGD mice abundant apoptotic and necrotic cells were found, the delayed removal of which in the absence of ROS in by phagocytes was postulated to substantially deteriorate the inflammation in these mice (Schappi et al., 2008a).

In addition, a hypomorphic variant of p47phox (Ncf1) subunit of the phagocyte NADPH oxidase, which results in partial production of ROS by phagocytes, has been reported to be strongly associated with the development of arthritis in rat models (Hultqvist et al., 2009; Olofsson et al., 2003). Furthermore, X-CGD mice crossed to the lupus-prone MRL.FasLPF genetic background exhibited markedly exacerbated lupus and elevated auto-antibody production (Campbell et al., 2012). Collectively, these observations suggest that the phagocyte NADPH oxidase may act to inhibit the pathogenesis of SLE, and dysregulated activated of the NADPH oxidase may contribute to the autoimmune characteristic in CGD. However, the underlying mechanism remains elusive.
VIII. Research goals

A major goal of our research was to delineate cellular mechanisms promoting macrophage-dependent resolution of sterile inflammation from the perspective of macrophages. The contribution of macrophages to suppress sterile injury-induced inflammation and promote tissue repair has been well documented, and clearance of local apoptotic neutrophils by macrophages is a known conduit for the acquisition of anti-inflammatory phenotype of macrophages. However, efferocytosis-induced events leading to the resolution of sterile inflammation and contribution of other innate cell types are not clearly defined in an in vivo setting.

A well-described function of M2 macrophages is to promote tissue repair. Given the potency of IL-4 to induce alternative activation of macrophages, IL-4 is likely present at an early stage following tissue injury and responsible for the M2 phenotype of macrophages recruited to repair tissue. However, it remains unclear what the cellular source of IL-4 is in this setting. Macrophages have been demonstrated to be able to produce IL-4 in several contexts. Given that macrophages are among the first immune cells recruited to an injured tissue, and one of the main tasks for these recruited macrophages is to clear apoptotic neutrophils, we hypothesized that macrophages are an early cellular source of IL-4 following tissue injury and induction of IL-4 may be associated with efferocytosis.

A related goal that is also central to our study was to investigate the roles of the phagocyte NADPH oxidase in the resolution of sterile inflammation. Both CGD patients and mice appear to be hyper-reactive to sterile stimuli and exhibit markedly prolonged inflammation, reflecting a regulatory role for the phagocyte NADPH oxidase in
inflammatory response. We set to first determine whether the phagocyte NADPH oxidase is activated in macrophages undergoing efferocytosis and whether it is associated in the cellular mechanisms delineated in the work to address the first goal described above, to identify underlying mechanisms contributing to the chronic and prolonged inflammation observed in CGD patients and mice.

Additionally, we also aimed to define the function of the interaction between gp91<sub>phox</sub> and Rac GTPase in the activity of the phagocyte NADPH oxidase by characterizing soluble agonist or phagocytosis – induced activity of the phagocyte NADPH oxidase in neutrophils expressing a gp91<sub>phox</sub> mutant lacking the Rac-binding site.
CHAPTER TWO: MATERIALS AND METHODS

I. Antibodies and reagents

Monoclonal antibodies 54.1 (Baniulis et al., 2005) and NS2 (Taylor et al., 2004) against human gp91\textsubscript{phox} and p22\textsubscript{phox}, respectively, were gifts from Dr. Jesaitis (Montana State University, Bozeman, MT). The 7D5 monoclonal antibody (mAb) (Yamauchi et al., 2001) against gp91\textsubscript{phox} for FACS was collected from hybridoma cells kindly provided by Dr. M. Nakamura (Nagasaki University, Japan). A polyclonal antibody against human Rac2 for western blotting and immunofluorescence staining was provided by Dr. G. Bokoch (Scripps Research Institute, CA). A polyclonal anti p47\textsubscript{phox} was a gift from Dr. J.D. Lambeth and Dr. D. Uhlinger (Emory University, GA). APC-conjugated and PBS-57-loaded CD1d-tetramer was obtained from the NIH Tetramer Core Facilities (Emory University, Atlanta, GA). The following antibodies were purchased: a monoclonal anti-Rac1 and Rac2 (BD Biosciences, Franklin Lakes, NJ), F4/80 antibody (Invitrogen, Carlsbad, CA), FITC-conjugated anti-MPO (Abcam, Cambridge, MA). Anti-IL-4 and isotype for flow cytometry were from Biolegend (San Diego, CA), and anti-CD115 (eBioscience, San Diego, CA). Alexa Fluor labeled secondary antibodies for immunofluorescence staining were from Invitrogen. All other antibodies for flow cytometry and ELISA were from BD Biosciences.

3.3µm latex beads were from Bangs Laboratory (Fishers, IN). Phosphate buffered saline (PBS, pH 7.2), penicillin/streptomycin, neomycin, trypsin/ethylene diamine tetra-acetate (EDTA), Dulbecco’s modified Eagle’s medium (DMEM) with low glucose (for LMTK cells), RPMI 1640 (for neutrophils and PLB cells), and IMDM (for macrophages) were purchased from Gibco. Lipofectamine 2000 was purchased from Invitrogen Life
Technologies. Fetal calf serum (FCS) and were purchased from Atlanta Biologicals (Norcross, GA). Chamber slides were purchase from Nunc (Rochester, NY). ACK Lysis Buffer was purchased from Lonza (Walkersville, MD). All other reagents were from Sigma-Aldrich (St. Louis, MO) unless otherwise specified.

II. Mice

WT and Il4^{-/-} C57B/6 mice, and WT and Il4^{Het/Het} BALB/c mice were purchased from the Jackson Laboratory (Bar Harbor, MA). gp91^{phox/-} (also called X-CGD) mice were generated by gene targeting and a neo cassette was placed into exon 3 of Cybb, the gene encoding gp91^{phox} (Pollock et al., 1995). Cd1d^{-/-} (Mendiratta et al., 1997) mice were obtained from Randy Brutkiewicz’s laboratory (Indiana University School of Medicine) that were previously kindly provided by L. Van Kaer (Vanderbilt University, Nashville, TN, USA). Jα18^{-/-} (Cui et al., 1997) mice were also obtained from Randy Brutkiewicz’s laboratory that were previously kindly provided by M. Tanuchi, RIKEN, Chiba University (Chiba, JAPAN). Il4^{L/-}LysM^{Cre} BALB/c mice and littermates (Herbert et al., 2004) were provided by Dr. Thomas A. Wynn (NIH) and Dr. Frank Brombacher (University of Cape Town, South Africa). Irf4^{LysM^{Cre+}} or Irf4^{LysM^{Cre-}} mice were kindly provided by Dr. Jie Sun (Indiana University School of Medicine). Most mice used in our experiments were between 8 to 12-week of age.

III. Cell lines

A. Generation of PLB-985 cell lines

PLB-985 X-CGD cells were transduced with retroviral supernatant that was made by transfecting HEK 293 cells with a retroviral vector under an MFG promotor (Suh et al., 2006) expressing either WT gp91^{phox}, gp91^{phox} K421A, gp91^{phox} Y425A, or gp91^{phox} K421A.
K421A/Y425A. The transduced cells were then FACS sorted for gp91\textsuperscript{phox} using the 7D5 anti-human gp91 mAb. To generate p67-YFP expressing cell lines, PLB-985 WT gp91\textsuperscript{phox}, PLB-985 gp91Y425A and PLB-985 X-CGD were transduced with MSCV-p67-YFP. All PLB-985 cells were grown and differentiated into neutrophil-like cells using dimethylformamide as previously described (Suh et al., 2006).

**B. Generation of COS7 cell lines**

To generate COS7 cell lines for Rac1Q61 rescue assay, COS7 cells were transfected with pcDNA-p22\textsuperscript{phox}, pcDNA-p47\textsuperscript{phox}, and pcDNA-p67\textsuperscript{phox} using Lipofectamine 2000 (Invitrogen) and grown in selective media containing 0.2 mg/mL hygromycin, 0.8 mg/mL neomycin, and 1 µg/mL puromycin, respectively. The resulted cell line, named COS\textsuperscript{22/47/67}, was transduced with MFG-WT gp91\textsuperscript{phox} or MGF-gp91\textsuperscript{phox} Y425A to generate COS\textsuperscript{22/47/67/WTgp91} or COS\textsuperscript{22/47/67/gp91Y425A}, respectively, which were then FACS sorted for gp91\textsuperscript{phox} using the 7D5 antibody. For phagocytosis of hIgG opsonized latex beads, COS7 cells expressing transgenic human FcγIIA receptor and p22\textsuperscript{phox} were transduced with MFG-WT gp91\textsuperscript{phox} or MGF-gp91\textsuperscript{phox} Y425A and sorted as described above to generate COS\textsuperscript{FcγIIA/22/WTgp91} or COS\textsuperscript{FcγIIA/22/gp91Y425A}.

**IV. NADPH oxidase activity**

**A. Isoluminol or luminol chemiluminescence assay for superoxide generation by neutrophils**

3.3-µm latex beads were opsonized with human IgG as described (Suh et al., 2006) and human serum opsonized zymosan particles (Casbon et al., 2009) at 2mg/ml. NADPH oxidase activity stimulated by PMA, fMLF, IgG opsonized latex beads, or SOZ was assayed using chemiluminescence enhanced by luminol or isoluminol as previously
reported (Suh et al., 2006). Specifically, $2.5 \times 10^5$ differentiated PLB-985 cells were plated into a well of a 96-well plate (COSTAR, Corning, NY) in PBSG (PBS plus 0.9 mM CaCl$_2$, 0.5 mM MgCl$_2$, 20 mM dextrose) in the presence of 50 µM isoluminol or 50 µM luminol, without or with superoxide dismutase (SOD; final concentration: 75 µg/ml) and followed by pre-incubation at 37 °C for 10 minutes prior to assay. Horseradish peroxidase (HRP; final concentration: 20 U/ml) was added for luminol assays. PMA and fMLF were used at final concentrations of 300 ng/mL and 10µM, respectively. Relative light units (RLU) were monitored at 60-second to 90-second intervals for up to 1 hour by the Long Kinetic module in an Lmax microplate luminometer (Molecular Devices, Sunnyvale, CA). Integrated RLU values were calculated by SOFTmax software (Molecular Devices). The SOD added into the wells dismutes extracellular superoxide and results in measurement of intracellular superoxide only. Without SOD, total superoxide generated is measured. Luminol and isoluminol are chemicals that, in the presence of oxidizing agents, exhibit chemiluminescence that can be detected by a luminometer. Isoluminol is hydrophobic and remains outside of the cell. It is used to measured extracellular superoxide generation. However, luminol is able to penetrate the plasma membrane and allows the detection of either total (without SOD) or intracellular (in the presence of SOD) generation of superoxide.

**B. Luminol chemiluminescence assay for superoxide generation by macrophages**

The day before luminol assay, $0.5 \times 10^5$ day 3 peritoneal exudate macrophages harvested from mice challenged with sodium periodate were plated into a 96-well plate and non-adherent cells were removed 2 h later, with adherent cells further cultured overnight. The next day, apoptotic neutrophils were washed and added to each well at
2.5x10^5 cells per well. Luminol, HRP, and SOD were added as described above. Since unlike neutrophils, macrophages have little endogenous peroxidase, and HRP does not penetrate the plasma membrane, the luminol assay is usually unable to detect intracellular superoxide generation in macrophages. However, we were able to detect intracellular superoxide generation in macrophages in response to stimulation by apoptotic neutrophils. We speculated that this could be due to the myeloperoxidase from apoptotic neutrophils inside the efferosome.

C. **Nitroblue tetrazolium assay**

1.5x10^5 peritoneal exudate macrophages were plated in 8-well glass chamber slides for 2h and non-adherent cells were removed. The adherent cells were further cultured overnight to detect superoxide production upon stimulation with apoptotic cells or zymosan using Nitroblue tetrazolium (NBT) as described previously (Ochs and Igo, 1973). NBT diluted in sterile IMDM media with no FCS, was added to cells and incubated for 15 or 30 min at 37 °C. Cells were then placed on ice, washed twice with cold PBS, fixed with cold methanol for 1 min, and dried overnight at room temperature. Cell were then counter-stained with 0.2% safrin. NBT is reduced in the presence of superoxide and forms an insoluble purple formazan deposit, which can be identified by light microscopy.

D. **Translocation of oxidase cytosolic components to the plasma membrane**

PMA stimulated translocation of p47^{phox}, p67^{phox}, and Rac2 to the plasma membrane was studied as detailed in a previous study (Akasaki et al., 1999). Briefly, 10^8 differentiated PLB-985 cells were stimulated with PMA (final conc. 500ng/ml) or vehicle control (dimethyl sulfoxide) at 37 °C for 10 min and followed by sonication and
ultracentrifugation to separate the membrane and cytosol. Equal cell equivalents of membrane (40x10^6 cells) and cytosol (10x10^6 cells) from each cell line were loaded for SDS-PAGE. Immunoblotting for membrane protein Rap1b was performed as loading control for membrane proteins. Translocation of oxidase components to the plasma membrane was detected by immunoblotting, and densitometric analysis was done by Image J (National Institutes of Health, Bethesda, MD).

E. INT reduction in permeabilized PLB-985 cells

Permeabilization of differentiated PLB-985 cells and INT reduction were performed as previously described (Pessach et al., 2001). 10^6 permeabilized cells were immediately plated into a 96-well plate in the presence of 1 mM INT and 0.5 µg/ml rotenone with or without 6.5 µM diphenylene iodonium (DPI). INT reduction, as indicated by an increase in absorbance at 495nm, was initiated by adding PMA (final conc. 300 ng/ml) to each well. The plate was read by a Thermomax microplate reader (Molecular Devices) for 10 min over a 30-s interval, and INT reduction was adjusted to e^{-}/10^6 cells/min (Pessach et al., 2001). In parallel, cytochrome c reduction was measured in intact differentiated PLB-985 cells (Price et al., 2002).

V. Confocal microscopy

A. Live cell confocal videomicroscopy

Phagocytosis by neutrophil-differentiated PLB-985 cells expressing p67-YFP was filmed using a PerkinElmer spinning disk confocal microscope equipped with an Ixon air-cooled EMCCD camera (Andor Technology, South Windsor, CT) and 100x/1.4 NA objective. Living differentiated PLB-985 neutrophils loaded onto 35mm glass-bottom microwell dishes (MatTek corporation, Ashland, MA) were mounted on the microscope
and warmed for 5 mins at 37 °C using a stage incubator (Warner Instruments, Hamden, CT). After that, serum opsonized zymosan was added into the dishes. Images of ingesting cells were collected with 488nm excitation, 0.3 second exposure, and a time lapse of 5 seconds for about 10 mins. Translocation of p67-YFP during phagocytosis of SOZ was assessed by Image J as previously described (Birukova et al., 2008).

B. Immunofluorescence microscopy

A day prior to immunofluorescence (IF) assay, differentiated PLB-985 neutrophils were plated onto fetal bovine serum (FBS) coated glass coverslips and cultured overnight. The next day, cells were stimulated with SOZ or IgG opsonized latex beads for 10 minutes in a synchronized phagocytosis as described (Tian et al., 2008). Cells were fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100 and 0.05% BSA, and blocked with 10% goat serum. For IF of Rac2, cells were incubated with anti-human Rac2 polyclonal antibody at 4 °C overnight and then with an Alexa-547 goat anti-rabbit IgG secondary antibody for 1hr. For IF of gp91phox, cells were incubated with the 54.1 anti-gp91 mAb for 1 hr and then with an Alexa-488 goat anti-mouse IgG1 secondary antibody for 1hr. For IF of total Rac (Rac1 and Rac2), a monoclonal anti-Rac antibody was used. COS7-derived cell lines were plated on glass coverslips a day prior to IF. On the next day, a synchronized phagocytosis assay was performed using IgG opsonized latex beads for 30 minutes. The cells were fixed and permeabilized as described above for PLB-985 cells, but prior to permeabilization, non-ingested latex beads were labeled with an Alexa-647 conjugated goat anti-human IgG antibody on ice for 5 minutes. The cells were co-immunostained for Rac1 and gp91phox by sequential incubations with the anti-Rac mAb and the 54.1 anti-gp91 mAb. Then cells were
incubated at the same time with secondary antibodies, Alexa-555 goat anti-Rabbit IgG (Rac1) and Alexa-488 goat anti-mouse IgG (gp91<sub>phox</sub>) for 1hr at 4°C. Nuclei were stained with 5g/mL DAPI for 5 minutes. Slides were imaged on a laser scanning Zeiss LSM-510 confocal microscope (Carl Zeiss, Jena, Germany) using a 100X/1.4 NA oil-immersion objective. Images were analyzed and handled using Zeiss LSM software (Carl Zeiss).

VI. NKT cell activity

A. In vitro NKT activation assay

5x10^4 NKT hybridoma N38-2C12 (Gui et al., 2001) or N37-1A12 (Burdin et al., 1998) cells, both kindly provided by Dr. Kyoko Hayakawa (Fox Chase Cancer Center, PA), in 100 µl IMDM media and 5% FBS were co-cultured with 1x 10^5 macrophages for 24 h at 37°C (Roberts et al., 2002). Supernatants were collected, and IL-2 was measured by ELISA. For activation of splenic NKT cells, 7.5x10^4 splenic NKT cells in 100 µl of IMDM media and 5% FBS were co-cultured with 1x 10^5 mouse CD1d-expressing LMTK cells (Chen and Paul, 1997) for 48 h. Supernatants were collected, and IL-4 and IFNγ were measured by ELISA.

B. Purification and adoptive transfer of NKT cells

Splenic NKT cells from naïve WT mice were purified by staining splenocytes with APC-conjugated PBS-57-loaded mCD1d tetramer and isolation using anti-APC microbeads (Miltenyi Biotec). Isolated cells were stained with anti-CD4, anti-TCRβ, and 7AAD, and then FACS sorted for CD4+TCRβ+CD1d tetermer+7AAD-cells. Sorted NKT cells (15x10^4 in 0.3 ml PBS) or PBS alone were adoptively transferred via IP injection into mice that had been challenged with sodium periodate for 66 h. To measure cytokine
production of transferred NKT cells from recipient mice, 30h later peritoneal CD4 T cells were isolated and ex vivo stimulated as described above. All FACS sorting was done by either FACS Aria (Becton Dickinson) or Reflection sorter (iCyt, Champaign, IL).

PBS-57 is an analog of α-galactosylceramide (α-Gal-Cer) developed by Dr. Paul Savage and colleagues (Brigham Young University, Provo, UT, USA). Activity of PBS-57 has been shown to be indistinguishable from α-galactosylceramide.

VII. Efferocytosis

A. Sodium periodate-induced peritoneal inflammation

Sodium periodate (Sigma, Cat. S1878) was made fresh immediately before injection in PBS (Gibco, Cat. 20012-027) to get a final concentration of 5mM and warmed up at 37 °C for 10 min before injection. Each mouse was injected with 1mL of 5mM sodium periodate intraperitoneally. On day 3 or day 4, peritoneal cells were harvested by lavage with 10 mL of PBS twice. Macrophages were isolated by culturing peritoneal cells in a tissue culture dish with IMDM media plus 20% heat-inactivated FBS for 2h and removing non-adherent cells.

B. Isolation of neutrophils and induction of apoptosis

Human peripheral venous blood was drawn from healthy donors with consents as approved by the institutional review board of the Indiana University School of Medicine. Neutrophils were isolated with Polymorphprep (Axis-Shield PoC AS, Norway; U. S. distributor: Accurate Chemical & Scientific Corp., Westbury, NY) according to manufacturer’s protocol. Specifically, 5 mL of human blood was layered on top of 5 mL of Polymorphprep in a 15 mL conical tube, followed by centrifugation with the brake off at room temperature at 500x g for 30 min. Two layers cells were visible after that on top
of red blood cells (RBCs) that are at the bottom of the tube, with the top layer being mononuclear cells and the second layer being polymorphnuclear cells (PMNs). PMNs were washed twice with PBS, and RBCs were lyzed with ACK Lysing Buffer (Lonza, Cat. 10-548E) for 5-7 min at room temperature, and cells were washed with PBS twice.

To induce apoptosis, human neutrophils were cultured ex vivo with RPMI 1640 and 5% heat-inactivated FBS in a petri dish for 20h. To isolate mouse neutrophils, peritoneal cells were harvested 18h post IP injection of 5mM sodium periodate, neutrophils were purified by Percoll gradient as previously described (Kim and Dinauer, 2001). To induce apoptosis, the purified mouse neutrophils were ex vivo cultured with RPMI 1640 and 10% heat-inactivated fetal bovine serum (FBS) for 12h. Apoptosis of both human and mouse neutrophils was confirmed by cytospin as well as Annexin V and 7AAD staining.

C. In vitro efferocytosis assays

Peritoneal exudate cells were harvested from day3 periodate-elicited mouse peritoneum by lavage with PBS as described above. Cells were cultured in a 24-well tissue culture plate ex vivo for 2h in culture media (IMDM media plus 20% heat-inactivated fetal bovine serum), and non-adherent cells were removed, and the remaining adherent macrophages were further cultured overnight. The next day, apoptotic human neutrophils or mouse neutrophils were added to each well at 5:1 and 2:1 (PMN: macs) ratios, respectively. Cells were co-cultured for various periods of time for different assays. Uptake of neutrophils by macrophages was determined by first labeling apoptotic neutrophils with PKH-26 (Invitrogen) and a 30min co-culture. PKH-26 and F4/80 double positive cells represented macrophages that had ingested neutrophils. Additionally,
ingestion was confirmed by MPO dianinobenzidine histochemistry as previously described (Newman et al., 1982).

**D. Diaminobenzidine histochemistry (DAB) for myeloperoxide (MPO)**

Immediately before assay, the cocktail solution for DAB was prepared by adding 1.25 mg/mL dimethoxybenzidine, ddH₂O, 0.025% BSA in 1x HBSS, 0.1M H₂O₂, and 0.05% sodium phosphate (pH 6.2) into ddH₂O. Macrophages grown in an 8-well chamber slide were washed with PBS, fixed with 4% paraformaldehyde for 10 min at room temperature, and washed with PBS one time. Then 200 μL of DAB cocktail solution was added to each well and incubated at room temperature for 15 min. MPO present in the fixed cells reacts with H₂O₂ in the solution and results in insoluble brown precipitates visible under a light microscope. The slide can later be counter-stained with Diff-Quik for Giemsa to visualize the nucleus and cytosol of the cells.

**E. Intracellular staining for cytokines in macrophages**

Adherent macrophages on wells (in vitro assays) or non-adherent macrophages from peritoneal lavages were first labeled with CD115 or F4/80 at 1:200 dilution in FACS buffer (2% BSA and 0.01%NaN₃ in PBS) at 4 °C for 10 min, fixed with 2% paraformaldehyde and washed once with permeabilization buffer (2%BSA, 0.1% saponin, and 0.01%NaN₃ in PBS) at 4 °C. To stain for intracellular IL-4 and IFNγ, the washed cells were suspended in 200 μl of permeabilization buffer and incubated with anti-IL4 (Biolegend), anti-IFNγ (Biolegend or BD Pharmingen), or isotypes (both at 1 μl of antibody per 1 million cells) for 45 min at 4 °C. Gating strategy to analyze intracellular staining of IL-4 in peritoneal macrophages ex vivo is shown below. Specifically, granulocytes, which were forward scatter (FSC) low and side scatter (SSC)
high were gated out first, and the gated cells (all cells other than granulocytes) were then assessed for CD115 expression. CD115-positive cells, which were macrophages, were then analyzed for intracellular IL-4 staining. Comparison to cells stained with isotype control by a histogram (right panel) allowed the identification of IL-4-producing CD115+ macrophages.

We gated out granulocytes because in our experience, peritoneal granulocytes (mainly eosinophils) showed very bright staining even when stained with the isotype control, likely due to the granule proteins in these cells that may render them more “sticky” for the antibody.

**F. Intracellular staining for cytokines in CD4+ T cells**

To stain intracellular cytokines in CD4+ T cells, first, mouse peritoneal cells were cultured ex vivo with RPMI 1640 media plus 10% heat-inactivated FBS in a tissue-culture dish for 2 h to deplete the adherent macrophages that were also CD4+. CD4+ T cells from the non-adherent cell population were isolated by MACS CD4 beads (Miltenyi
Biotec, Auburn, CA), and ex vivo stimulated with PMA and ionomycin for 6h, or plate-bound anti-CD3 (4 µg/ml, 145-2C11) and soluble anti-CD28 (1 µg/ml; BD PharMingen) for 24h, with monensin or Golgi Plug (BD Biosciences) added to cells for the final 2 h, respectively. Cells were labeled with anti-CD4 (1:200 dilution), APC-conjugated PBS-57 loaded CD1d tetramer (1:1000 dilution), and anti-TCRβ (1:200 dilution) for 20 min at 4 °C, fixed with 2% paraformaldehyde, permeabilized with permeabilization buffer, and stained with anti-IL4 and anti-IFNγ as described above for ICS with macrophages. Cells were analyzed by flow cytometry with either FACSCalibur or LSR II (Becton Dickinson) and results were analyzed with FlowJo (Ashland, OR). If flow cytometry was not performed immediately following ICS, cells were kept in 2% paraformaldehyde with FACS buffer overnight.

VIII. Quantitative RT-PCR

RNAs of macrophages were isolated using Trizol (Invitrogen) and reverse transcribed using SuperScript II Reverse Transcriptase (Invitrogen) to make cDNA. For qPCR, cDNA (2 µl), TaqMan Fast Universal Master Mix (5 µl) and commercially available primers were used for Arg1, Fizz1, Ym1, Il4, Gata3, Irf4, and Nos2 (Applied Biosystems, 0.5 µl each). DEPC water was added to a final reaction volume of 10 µl. RNA expression was normalized to the expression of β actin. The relative expression was calculated by the change-in-threshold (−ΔΔC_T) method.

IX. ELISA

Peritoneal CD4 T cells isolated by MACS anti-CD4 microbeads were washed and stimulated (1×10^6 cells/ml) with 4 µg/ml plate-bound anti-CD3 for 24 hours to generate cell-free supernatant. To detect cytokines in supernatants, 2 µg/ml α-IL-4 (11B11, BD
Pharmingen), α-IL-13 (eBio13A, eBioscience), 2 µg/ml α-IL-2 (JES6-1A12, BD Pharmingen), and 4 µg/ml α-IFNγ (R4-6A2, BD Pharmingen) capture antibodies were dissolved in 0.1M NaHCO₃ (pH 9.0) coating buffer. 50 µl of the capture antibodies were coated in each well of 96-well Nunc-immuno plates and were incubated overnight at 4 °C. Plates were washed in ELISA wash buffer (0.1% Tween-20 in PBS) and blocked for 2 hours in FACS/ELISA buffer at room temperature. Supernatants and cytokine standards were added to the plates after FACS/ELISA buffer was removed, and plates were incubated overnight at 4 °C. Biotinylated antibodies (1 µg/ml) [α-IL-4 (BVD6-24G2, BD Pharmingen), α-IL-2 (JES6-5H4, BD Pharmingen), α-IL-13 (eBio1316H, eBioscience), and α-IFNγ (XMG 1.2)] were dissolved in FACS/ELISA buffer and were added to the plates (100 µl/well) after the plates were washed with ELISA wash buffer. Following 2 hours of incubation at room temperature, plates were washed again with ELISA wash buffer and incubated with avidin-alkaline phosphatase (100 µl/well) (1:2000 dilution; Sigma) in FACS/ELISA buffer for 1 hour at room temperature. Cytokine levels were assessed after the addition of Sigma 104 phosphatase substrate (5 mg/ml) dissolved in ELISA substrate buffer (10% diethanolamine, 0.05 mM MgCl₂, 0.02% NaN₃, pH 9.8) by measuring absorbance at 405 and 450 nm to subtract the background noise (Bio-Rad microplate reader model 680).

**X. Statistical analyses**

Data were analyzed using Student’s t-test and shown as means ±SEM. Differences with P < 0.05 were considered statistically significant.
CHAPTER THREE: RESULTS

Part I: Efferocytosis induces an IL-4-dependent macrophage-iNKT cell circuit to suppress sterile inflammation

1. Human peripheral blood neutrophils undergo spontaneous apoptosis ex vivo and are readily ingested by mouse peritoneal exudate macrophages

Given the central role of apoptosis and clearance of neutrophils during the acute response to tissue injury, neutrophils were a logical choice of apoptotic cells to be the “bait” to study macrophage efferocytosis. Technically mouse neutrophils are not the most cost-effective choice to generate apoptotic cells for extensive ex vivo study, due to the difficulty in isolating a large number of mature neutrophils from mouse tissues after inducing local inflammation in the tissue, such as peritonitis. The bone marrow is a reservoir of majority of neutrophils in the steady state, and in our experience typically the femurs and tibia from a mouse yield 15-20 million neutrophils. However, these neutrophils are at various stages of maturation and do not undergo apoptosis as readily as mature tissue neutrophils. Taking into account the drawbacks of using mouse neutrophils, we decided to use human neutrophils isolated from human peripheral blood. A large and pure population of neutrophils can be purified from human peripheral blood readily, and the short life span of these cells in vitro leads to a good yield of apoptotic cells by aging. More importantly, unlike many other cell types that undergo necrosis rapidly once they become apoptotic, the integrity of the plasma membrane of apoptotic neutrophils is maintained for approximately 24 h (Kennedy and DeLeo, 2009), thus offering a larger window of time for experiments.
After ex vivo culture for 20 h in the presence of 5% heat-inactivated serum, cytospin images of human neutrophils showed distinct features of apoptotic cells, such as condensation of nuclei (Fig. 5A). 7AAD and annexin V co-staining showed increased apoptosis over time and that about 80% of human neutrophils were early-apoptotic at 20h (Annexin V+7AAD-), with very few necrotic cells (Annexin V-7AAD+) (Fig. 5B and C). Neutrophils aged by this method were co-cultured with overnight cultured adherent WT day 3 peritoneal exudate macrophages for 30 minutes. When labeled apoptotic neutrophils (ACs) were used, flow cytometry showed ACs in close to 40% of macrophages (Fig. 6A). The efferocytic index was also determined after diaminobenzidine histochemistry for myeloperoxidase (MPO), a neutrophil-specific granule protein. Macrophages with MPO staining in the cytosol (brown) had ingested ACs. About 25% of total macrophages had ingested ACs after 30 min of co-culture (Fig. 6B), consistent with percentages determined by flow cytometry.
Figure 5. Induction of apoptotic human neutrophils

Human neutrophils were purified from peripheral blood and aged in RPMI 1640 with 5% heat-inactivated fetal calf serum for various periods of time. (A) Fresh or neutrophils aged ex vivo for 20h were cytospun to confirm apoptosis by cell morphology. Apoptotic cells were confirmed also co-staining with 7 aminoactinomycin D (7AAD) and FITC-annexin V and flow cytometry (B). 7AAD-Annexin V + cells are considered early apoptotic and 7AAD+Annexin V+ population resperents late apoptotic cells. C. 7AAD+ Annexin V- cells are necrotic cells. Aging kinetics was determined using the flow cytometry method, and the 20-h time point was chosen to obtain apoptotic cells for subsequent efferocytosis studies.
Figure 6. Ingestion of human apoptotic neutrophils by mouse macrophages

(A) Human apoptotic neutrophils (ACs) were labeled with PKH-26, washed 3 times, added to mouse peritoneal exudate macrophages at 5:1 (AC: mac) ratio in serum-free media, and incubated at either 4 °C or 37 °C for 30 min. Then, ACs were washed off and macrophages were labeled with F4/80 and anti-hCD45 on ice for 10 min, washed, fixed, and scraped off the well using a cell scraper. By flow cytometry, percentage of ingestion was defined as the PKH+F4/80+ population, and macrophages with bound neutrophils were excluded by subtracting the F4/80+hCD45+ population. (B) Unlabeled ACs were co-cultured as described in A at 37 for 30 min. ACs were then washed off, and macrophages were fixed and incubated with cocktail solution containing diaminobenzidine that reacts with myeloperoxidase (MPO). Images of cells were taken after Giemsa counter-staining.
2. Efferocytosis of apoptotic neutrophils induce macrophages produce IL-4 in vitro

M2 macrophages (Gordon and Martinez, 2010) and clearance of apoptotic neutrophils are both associated with resolution of inflammation and tissue repair. We first investigated whether efferocytosis induced macrophages to produce IL-4 following stimulation of mouse peritoneal exudate macrophages (PEM) with ACs. After 18 hrs of co-culture with AC, intracellular cytokine staining (ICS) of PEM indicated production of IL-4 in PEMs stimulated with AC not PEMs without AC or PEMs stained with an isotype (Fig. 7A). Consistently, by multiplex, low but detectable amounts of IL-4 (∼5 pg/mL/10⁶ cells) were observed in supernatants with AC (Fig. 7B). In addition, mRNA for Il4 was increased 10-fold (Fig. 1C) in PEM after 9h of co-culture with AC (Fig. 7C). Thus, the data suggested ACs induced macrophages to produce IL-4.

Studies in T cells have demonstrated that GATA3 and IRF4 are transcription factors required for expression of IL-4 in Th2 cells (Zhu et al., 2010). Consistently, mRNA for Gata3 and Irf4 were increased about 2-fold after 9h of co-culture with AC (Fig. 8A). Surface expression of IL4-responsive genes, IL-4Rα and MHC class II (Fig. 8B and C), were both increased, suggesting an autocrine IL-4 signaling to the PEMs. Furthermore, when uptake of ACs was inhibited by treating PEMs with cytochalasin D, we found that IL-4 expression in PEMs was reduced drastically, suggesting ingestion of ACs is a crucial signal to trigger IL-4 expressing in PEMs (Fig. 9A and B). IL-4 was unlikely to be induced by secreted factors or serine protease from ACs, since we still found IL-4 production in PEM with fixed AC or AC treated with diisopropylfluorophosphate (DFP), a neutrophil serine protease inhibitor (Fig. 9C). In addition, efferocytosis of mouse apoptotic neutrophils also resulted in IL-4 production by
PEMs 24 h following co-culture (Fig. 10 A and B) and upregulated surface expression of IL-4Ralpha and MHC class II on PEMs (Fig. 10 C). Collectively, our data supported that the model that efferocytosis of apoptotic neutrophils induce macrophages to produce IL-4 in vitro.

In addition, we also did a pilot experiment using mouse thymocytes that were induced to become apoptotic by UV irradiation as previously described (Fernandez-Boyanapalli et al., 2009). However, we did not detect IL-4 production by ICS for IL-4 in macrophages 24h after these macrophages were co-cultured with apoptotic thymocytes (data not shown). Therefore, IL-4 production by efferocytosing macrophages may be specifically elicited by apoptotic neutrophils, but not other apoptotic cell types. However, further characterization is necessary to determine whether apoptotic thymocytes are able to induce IL-4 production in efferocytosing macrophages and delineate the differential macrophage responses to apoptotic neutrophils and other apoptotic cell types.
Figure 7. Efferocytosing macrophages produce IL-4 in vitro

(A) WT mouse peritoneal exudate macrophages (PEMs) were co-cultured with human apoptotic neutrophils (ACs) at 1:5 (mac:particle) ratio for 18h. IL-4 produced was determined by intracellular staining (ICS) using anti-IL-4 or isotype. F4/80 staining was used to label macrophages. (B) Amounts of IL-4 in supernatants from co-culture in (A) with ACs were measured by multiplex that was performed by Eve Technologies (Calgary, Alberta, Canada). (C) mRNA levels of Il4 in efferocytosing macrophages were measured by quantitative RT-PCR 9h after co-culture with ACs. For both (B and C), macrohages and ACs were cultured at 1:10 ratio in permanox 2-well chamber slides. Specifically, 0.5x10^6 day 3 peritoneal cells were cultured for 2h and then non-adherent cells were removed. The remaining adherent cells were macrophages (about 0.35 x10^6) were cultured overnight before co-culture with 3.5 x10^6 ACs for 18h (B) or 9h (C).
Figure 8. IL-4 signaling in efferocytosing macrophages

(A) mRNA levels of Gata3 and Irf4 in efferocytosing macrophages were measured by quantitative RT-PCR 9h after co-culture with ACs. (B and C) After co-culture of PEMs with ACs for 24h, cell surface expression of IL-4Rα and MHC class II were determined by antibody staining followed by flow cytometry. Data represent at least 3 independent experiments.
Figure 9. IL-4 expression in efferocytosing macrophages

(A) PEMs were treated with 0.5 µM or 1 µM of cytochalasin D for 1 h prior to co-incubation with PKH-26 labeled ACs for 1h. % of uptake was determined by flow cytometry. (B) IL-4 production was determined by ICS for IL-4 18 h post co-culture with or without 1 M of cytochalasin D. Data are representative of 2 independent experiments. (C) IL-4 expression in PEMs were determined by ICS for IL-4 18 hr after co-culture with AC, fixed AC, diisopropylfluorophosphate (DFP) treated AC, or AC aging media. Data are representative of 2 independent experiments. ACs: PEMs ratio was 5:1 in all experiments.
Figure 10. Efferocytosis of mouse apoptotic neutrophils induces macrophages to produce IL-4 in vitro

Day 3 WT PEM were cultured ex vivo overnight, and co-incubated with apoptotic mouse neutrophils for 30 min at 37 °C. Mouse neutrophils were isolated from mouse peritoneum isolated from mice 18h after IP injection of sodium periodate, purified by percoll gradient. Apoptosis was induced by ex vivo culture with 5% heat-inactivated FBS for 10h. (A) Uptake of apoptotic cells was determined by ICS for MPO 3h after co-incubation. (B) IL-4 production was determined by ICS for IL-4 24h after co-incubation. C. Surface expression of IL-4Rα and MHC II were assessed 24h after co-incubation.
3. Efferocytosis of apoptotic neutrophils induce macrophages to produce IL-4 in vivo

We next determined if macrophages undergoing efferocytosis in vivo produced IL-4. As a model of sterile inflammation, irritant-induced peritonitis first elicits rapid neutrophil infiltration, followed by recruitment of macrophages and efferocytosis to remove apoptotic neutrophils (Haslett et al., 1994; Savill et al., 1989) (Fig. 11A). Local inflammation in the peritoneum can be characterized by an increased peritoneal cell number. Consistent with previous reports, in wild-type (WT) C57B/6 mice peritoneal inflammation peaked at day 3 after injection and began to wane thereafter, and the inflammation was resolved by day 6 (Fig. 11B and C). Flow cytometry with lineage cell markers indicated that on day 3, more than 70% of peritoneal exudate cells were macrophages (F4/80+), between 10-15% were B cells (B220+), close to 10% were granulocytes (GR1+), and about 5% were T cells (CD3+). In naïve mice about half of peritoneal cells were resident macrophages (Fig. 11C) but in mice challenged with periodate for 3 days, typically between 70-80% of total peritoneal exudate cells were macrophages (F4/80+ or CD115+).

We used CD115 rather than F4/80 as the marker for macrohages when analyzing intracellular staining of IL-4 in macrophages, since eosinophils also express F4/80 (Wu et al., 2011). CD115, also known as the receptor for M-CSF, is expressed on monocytes, macrophages and osteoclasts (Auffray et al., 2009). Newly recruited monocytes into the tissue are CD115+, and in response to M-CSF in the tissue, the expression of CD115 is increased as these cells differentiate into mature macrophages. On the other hand, tissue resident macrophages, which have resided in the tissue for an extended period of time,
usually display a relatively higher surface expression of CD115 than recently recruited macrophages. Consistently, we also observed a slight higher expression of CD115 on peritoneal resident macrophages from naïve mice than day 3 peritoneal exudate macrophages (Fig. 12B) that were thought to be newly infiltrating cells in response to local inflammation.

To determine when periodate-induced sterile peritonitis led to in vivo efferocytosis, we isolated day 3 PEMs and immediately performed diaminobenzidine (DAB) histochemistry for myeloperoxidase (MPO), which is abundant in neutrophil azurophilic granules but is absent in normal macrophages. We observed a fraction of day 3 PEMs (about 10%) that had MPO (brown staining) that represented PEMs that had ingested apoptotic neutrophils in vivo (Fig. 12A), thus confirming efferocytosis in periodate-elicited peritoneum. Ex vivo staining of day 3 PEM for intracellular MPO using an MPO antibody identified macrophages (CD115+) that had ingested neutrophils, about 15% of total macrophages, whereas MPO-positive macrophages were rare in naïve (day 0) peritoneum (Fig. 12B and C). A similar pattern was observed for IL-4 expression in PEM after ICS for intracellular IL-4 in PEM (CD115+), and staining was not observed in PEMs from Il4−/− mice or PEMs stained with an isotype (Fig. 12B and C, and 13A-C). Importantly, day 3 IL4+ PEM were also positive for MPO, thus suggesting that efferocytosing macrophages produced IL-4 in vivo (Fig. 12D). Consistently, IL-4 was detectable in day 3 but not day 0 peritoneal lavages (Fig. 14A). Additionally, surface expression of IL-4Rα, which is increased by IL-4 signaling in macrophages, was consistently higher on day 3 PEM (Fig. 14B). When day 3 PEM were cultured ex vivo in the presence of anti-IL-4Rα for 48h, the amounts of IL-4 detected in culture supernatants
were increased significantly (Fig. 14C). Thus, IL-4 secreted by efferocytosing macrophages in the supernatant might be at the same time being consumed by these macrophages, thus reflecting an autocrine signaling of IL-4 to efferocytosing macrophages. This may partially account for the low levels of IL-4 detected in the supernatants of day 3 PEM cultures ex vivo.

To confirm that the Il4 gene is activated in macrophages during efferocytosis vivo, we used IL-4 reporter mice (also known as 4get mice) where gfp is knocked in to the Il4 locus such that expression of GFP indicates transcription of Il4 (Mohrs et al., 2001). Consistently, we observed a similar percentage of GFP+ CD115+PEM in day 3 but not day 0 mice (Fig. 15A). We also noticed a population of CD115+ cells that also expressed GFP, which were SSC high and positive for the eosinophil marker CXCR3 (not shown). Thus these CD115-GFP+ cells were eosinophils. However, by ICS for IL-4, we also confirmed that the GFP+CD115+ day 3 PEMs were stained positive for IL-4 (Fig. 15B), indicating not only was the Il4 gene was being expressed, IL-4 protein was produced as well in these cells. Of note, most of the day 3 PEM stained positive for IL-4 by ICS were CD115 positive (Fig. 15B), suggesting that even though the CD115-eosinophils expressed GFP and showed the potential to produce IL-4, they were not actually making IL-4. Importantly, DAB histochemistry for MPO revealed staining in nearly all sorted GFP+CD115+ cells from 4get mice on day 3 but only about 10% of total macrophages (Fig. 15C and D), again confirming these IL-4-producing macrophages had ingested neutrophils. Overall, our data demonstrate the emergence of IL-4-producing efferocytosing macrophages during sterile inflammation.
Figure 11. Sodium periodate – induced inflammation in mouse peritoneum

(A) Kinetic of sodium periodate – induced peritoneal inflammation. Neutrophil infiltration occurs within 24 h after IP injection of sodium periodate, followed by macrophage recruitment and efferocytosis in the next 2 days. The inflammation typically is resolved by day 6. (B) Peritoneal cell count on day 0, 3, 4, or 6 following IP injection of sodium periodate were enumerated and graphed. (C) Fractions of cell types, macrophages (F4/80+), granulocytes (GR1 high), T cells (CD3+) and B cells (B220+) from day 0 and day 3 WT mice. More than 3 mice were used for each time point.
Figure 12. Efferocytosing macrophages produce IL-4 in vivo

(A) Immediately after peritoneal lavage, day3 PEM with ingested neutrophils (indicated by arrows) were identified by cytospin and diaminobenzidine histochemical staining for MPO. (B) Day 0 or day 3 peritoneal cells were first labeled with CD115 and then stained for MPO and IL-4. Granulocytes were excluded by gating out FCS low and SSC high cells as described in Materials and Methods. (C) Percentages of MPO positive or IL-4 positive macrophages were quantified. (D) MPO within CD115+IL-4+, CD115+IL-4-, and CD115-IL-4- populations in day 3 peritoneal cells was measured by anti-MPO and compared by histogram (right panel).
Figure 13. Characterization of intracellular staining for IL-4

(A) PEM from day 3 WT or Il4−/− mice were labeled with CD115 and stained for intracellular IL-4 using anti-IL-4 or isotype. (B) Day 0 or day 3 peritoneal cells were first labeled with anti-CD115 and then stained for intracellular IL-4 or isotype. (C) Gating strategy to assess IL-4 production in peritoneal macrophages by intracellular staining using anti-IL-4 or isotype. Granulocytes were excluded by gating out FCS low and SSC high cells as described in Materials and Methods.
Figure 14. Detection of IL-4 in peritoneal lavage and IL-4 signaling in macrophages

(A) Amounts of IL-4 in peritoneal lavages from naïve mice or day 3 mice were measured by multiplex. 3–4 mice were used for each time point. (B) Surface IL-4Rα chain on day 0 and day 3 macrophages were compared by flow cytometry. (C) Day 3 peritoneal exudate macrophages were cultured ex vivo (10^6/ml) in the presence of anti-IL-4Rα (12 ng/ml) or isotype for 48h. IL-4 in supernatants was measured by ELISA. Data represent results from 2 experiments.
Figure 15. Sodium periodate induced GFP+ PEMs in 4get mice on day 3

(A) % of GFP+ CD115+ (D3) or F4/80+ (D0) cells in 4get mice. (B) Day 3 peritoneal exudate cells were stained for intracellular IL-4. GFP+ cells and IL-4+ cells were analyzed by flow cytometry. (C) Day 3 peritoneal cells from a 4get BALB/c mouse were labeled with CD115. GFP+CD115+ cells were sorted out by FACS sorting and followed by DAB histochemistry for MPO on a microscope slide. (D) The sorted cells were then imaged and brown staining indicated the presence of MPO within the cells.
4. Invariant NKT cells are activated to produce IL-4 to resolve sterile inflammation in the mouse peritoneum

We have demonstrated IL-4 production in peritoneal macrophages in periodate–induced inflamed mouse peritoneum. To examine whether IL-4 from macrophages may modulate T cells responses in the peritoneum, we decided to assess T cell response during resolution of sterile inflammation. We isolated peritoneal CD4 T cells from both day 0 and day 4 mice, followed by ex vivo stimulations with PMA and ionomycin or anti-CD3 and anti-CD28 and analyzed cytokine production. IL-4, IL-13, and IFNγ were produced by day 4 CD4 T cells but not day 0 or day 3 (Fig. 16A and B and data not shown). ICS indicated that more than half of day 4 CD4 T cells produced IL-4. The rapid emergence of an activated population of CD4 T cells producing both Th1 and Th2 cytokines suggested an innate response. Thus, we next determined whether these activated CD4 T cells were natural killer T (NKT) cells, a subset of CD4 T cells known to rapidly produce a variety of cytokines upon activation (Bendelac et al., 2007). Indeed, both day 0 and day 4 CD4 T cells contained close to 50% NKT cells (CD1d tetramer+ TCRβ+). Additionally, IL-4 and IFNγ production were found almost exclusively in the NKT population from day 4 mice; whereas NKT cells from day 0 mice showed little cytokine production (Fig. 16C). Therefore, there are NKT cells in mouse peritoneum, which has been reported by other studies (Naiki et al., 1999). Importantly, after induction of sterile inflammation, these NKT cells are activated to produce large amounts of cytokines and represent the major cytokine-producing CD4 T cells in the resolution phase of inflammation.

80
To determine whether activated NKT cells were required for the resolution of inflammation, we evaluated peritonitis in \( Cdl1d^{-/-} \) mice (Mendiratta et al., 1997). Although peritoneal leukocyte numbers returned to baseline four days after initiation of inflammation in WT mice, these remained elevated in \( Cdl1d^{-/-} \) mice (Fig. 17A). In addition, day 4 \( Cdl1d^{-/-} \) CD4 T cells produced minimal amounts of IL-4 and IL-13 after ex vivo stimulation, and about 25% of the amounts of IFN\( \gamma \) produced by CD4 T cells from day 4 WT mice (Fig. 17B). This result is consistent with NKT cells being the main CD4 T cell population producing these cytokines during this resolution phase of sterile peritoneal inflammation. The absence of cytokines from activated NKT cells likely contributed to the enhanced inflammation in \( Cdl1d^{-/-} \) mice.

Since \( Cdl1d^{-/-} \) mice lack both Type 1 (invariant) and Type 2 (non-invariant) NKT cells, to identify the type of NKT cells important to resolve inflammation in our model, we examined \( J\alpha18^{-/-} \) mice (Cui et al., 1997; Kawano et al., 1997), which specifically lack invariant NKT (iNKT) cells. The inflammation was more profound in day 4 \( J\alpha18^{-/-} \) mice compared to WT controls, as illustrated by increased peritoneal cell count in these mice on day 4 compared to WT controls, thus highlighting the importance of iNKT cells in resolving sterile inflammation (Fig. 17C). Amounts of IFN\( \gamma \) produced by ex vivo stimulated CD4 T cells from day 4 \( J\alpha18^{-/-} \) mice appeared normal. In contrast, IL-4 and IL-13 production by day 4 \( J\alpha18^{-/-} \) CD4 T cells was reduced by almost 70% and ~30%, respectively, relative to WT controls (Fig. 17D). The substantial decrease in IL-4 production by \( J\alpha18^{-/-} \) CD4 T cells suggested that iNKT cells (Type 1) were the major cellular source of this cytokine, and IL-4 appeared to be more important than IFN\( \gamma \) to
suppress inflammation in this model. The diminished IL-4 might contribute to the increased inflammation in these mice.

Our data have demonstrated IL-4 production by both macrophages and NKT cells in periodate-induced inflamed peritoneum. To confirm that the absence of iNKT cells in Jα18−/− mice accounted for the exaggerated inflammation in these mice, and also to determine whether having IL-4-producing NKT cells was sufficient to resolve inflammation, we adoptively transferred 15X10^4 splenic NKT cells isolated from naïve WT mice, or injected PBS as a control, into either WT or Jα18−/− mice at 66h after injection of sodium periodate (Fig. 18A and B). Thirty hours later, peritoneal exudate cell counts from recipient mice indicated that inflammation in Jα18−/− NKT recipients was reduced significantly compared to the Jα18−/− mice that received PBS, and similar to that in WT recipient groups (Fig. 18C). Ex vivo stimulated CD4 T cells from Jα18−/− NKT recipients produced increased levels of IL-4 and IFNγ compared to Jα18−/− PBS recipients (Fig. 18D). A 2-fold increase in cytokines was produced by CD4 cells from WT mice that received WT NKT recipients as compared to WT PBS recipients, likely from the transferred NKT cells (Fig. 18D).
Figure 16. NKT cells are present in mouse peritoneum and are activated following peritonitis

(A) Day 0 or day 4 WT CD4 T cells were ex vivo stimulated by PMA/Ionomycin or anti-CD3 and anti-CD28, and stained for intracellular IL-4 and IFNγ. (B) Following stimulation with anti-CD3 and anti-CD28 for 24h, day 0 or day 4 CD4 T cells were gated into NKT (CD1d tetramer+ TCRβ+) and Non-NKT (CD1d tetramer- TCRβ+) populations. Each population was assessed for intracellular IL-4 and IFNγ. (C) IL-4, IL-13, and IFNγ in supernatants after anti-CD3 and anti-CD28 stimulation were measured by ELISA.
Figure 17. iNKT cells in inflamed peritoneum produced IL-4 and IFNγ during the resolution of inflammation

(A-D) Peritonitis was induced by sodium periodate injection in WT and Cd1d−/− or Jα18−/− mice. Inflammation was assessed by peritoneal cell counts (A and C). IL-4, IL-13, and IFNγ in supernatants of Cd1d−/− (B) or Jα18−/− mice (D) day 4 CD4 T cells after anti-CD3 and anti-CD28 stimulation for 24 h were measured by ELISA. Data are representative of 3 independent experiments.
Figure 18. Jα18−/− mice were rescued by adoptive transfer of WT NKT cells

(A) Experiment overview of adoptive transfer experiment. WT splenic NKT cells (CD1d tetramer+TCRb+CD4+7AAD−) or PBS were adoptively transferred to WT or Jα18−/− mice that were injected with sodium periodate 66 h earlier. 30 h later, recipient mice were sacrificed to assess severity of peritoneal inflammation and cytokine response. (B) Gating strategy to isolate WT splenic NKT cells. Splenic NKT cells were purified by using APC-conjugated, PBS57-loaded CD1d tetramer and anti-APC micro-beads, followed by FACS sorting for CD1d tetramer+TCRb+CD4+ and 7AAD− cells. (C) Peritoneal inflammation was scored by cell counts 30h after adoptive transfer. (D) CD4 T cells from recipient mice in (B) were stimulated ex vivo for 48h and IL-4 and IFNγ were measured by ELISA. Data represent at least 2 independent experiments.
5. Efferocytosing macrophages increased CD1d expression and activate iNKT cells

NKT cells are present in various tissues and display a remarkable heterogeneity in their activation and cytokine production. Activation of NKT cells requires stimulation via CD1d-dependent presentation of lipid antigens (Brutkiewicz, 2006). A previous study reported that differential requirements for co-stimulatory signals from tissue-specific antigen-presenting cells exist among NKT cells from different tissues, and showed a striking difference in IL-4 and IFNγ production by NKT cells from the thymus and the spleen after ex vivo PMA/ionomycin or anti-CD3/anti-CD28 stimulation (Yang et al., 2003). We also found that production of IL-4 by peritoneal NKT cells appeared to be more dependent on activation by tissue-specific APC than thymic NKT cells (Fig. 19A and B). Thymic NKT cells isolated from naïve mice produced large amounts of IL-4 upon stimulation with PMA/Ionomycin (Fig. 19A) or anti-CD3 and anti-CD28 (Fig. 19B). In comparison, peritoneal and liver NKT cells produced only minimal IL-4 when stimulated in the same ways. Thus, different cytokine responses in NKT cells from different tissues following ex vivo stimulation may reflect pre-existing programming of NKT cells by APCs in tissue-specific microenvironments.

As the predominant APC in the inflamed peritoneum, macrophages appeared to be the most likely candidate to activate NKT cells. To test this, we first compared cell surface expression of CD1d on resident macrophages from naïve mice and day 3 and day 4 PEM, and observed increased surface CD1d following inflammation (Fig. 19A and B). Next, to assess whether exudate macrophages were able to activate NKT cells, we co-cultured either resident macrophages or day 4 PEM with either the Type I mouse NKT hybridoma N38-2C12, or the Type 2 mouse NKT hybridoma N37-1A12. Activation of
N38-2C12 NKT cells, as assayed by their production of IL-2, was induced by day 4 PEM but not day 0 macrophages from naïve mice and was dependent on CD1d expression on macrophages (Fig. 20C). In addition, resident macrophages from naïve mice and day 3 PEM displayed a difference in their ability to stimulate IL-4 production by splenic NKT cells (Fig. 20D). This demonstrated that peritoneal inflammatory macrophages were more competent than peritoneal resident macrophages to activate iNKT cells.

To determine whether IL-4 was required for day 4 PEMs to activate iNKT cells, we next tested the ability of Il4−/− or Il4ra−/− macrophages to activate iNKT hybridomas cells. Il4−/− and Il4ra−/− macrophages displayed normal surface expression of CD1d and induction of IL-2 by N38-2C12 NKT cells (Fig. 21A-C), suggesting that IL-4 is dispensable for macrophages to activate iNKT cells.

We next directly tested whether efferocytosis induces PEM to be competent for NKT cell activation by examining CD1d expression following efferocytosis by macrophages in vitro. CD1d expression was increased on macrophages co-cultured for 24h with apoptotic mouse neutrophils compared to macrophages not exposed to AC (Fig. 22A). Moreover, only efferocytosing macrophages were able to activate NKT hybridomas (Fig. 22B). This observation suggests that subsequent to efferocytosis, macrophages become capable of activating Type 1 NKT cells, at least in part via increased surface CD1d.
Figure 19. Differential IL-4 production by thymic, liver, and peritoneal NKT cells upon activation ex vivo

Thymic and liver NKT cells were isolated by FACS sorting CD1d-tetramer + cells. Peritoneal NKT cells were isolated as described in Methods and Materials. NKT cells (10^5 cells in 100 uL media) were stimulated with (A) PMA/Ionomycin (6h) or (B) plate-bound anti-CD3 and anti-CD28 (48h). IL-4 in the supernatants was measured by ELISA. Data are representative of 2 independent experiments.
Figure 20. Peritoneal exudate macrophages increased CD1d expression and were able to activate iNKT cells independently of IL-4

(A) Day 0 and day 4 peritoneal macrophages (F4/80+) were assessed for surface expression of CD1d. (B) Mean fluorescence intensity (MFI) was compared. Data represent at least 3 independent experiments. (C) Activation of N38-2C12 or N37-1A12 NKT cells by day 0 or day 4 peritoneal macrophages was determined by measuring IL-2 production by the NKT cells 24h after co-culture. Data represent at least 3 independent experiments. (D) 10^5 resident macrophages from WT naïve mice or peritoneal exudate macrophages from day 3 mice were co-cultured with 7.5x10^4 WT splenic NKT cells for 48h. IL-4 in the supernatants was measured by ELISA. Data represent 2 independent experiments.
Figure 21. IL-4 is not required for peritoneal exudate macrophages to activate iNKT cells

(A and B) Day 4 WT and Il4−/− peritoneal macrophages were assessed for surface expression of CD1d (A) and their ability to activate N38-2C12 NKT cells to produce IL-2 (B). Data represent 2 independent experiments. (C) Day 0 and day 4 WT and Il4ra−/− macrophages were co-cultured with N38-2C12 NKT cells and IL-2 from culture media was measured by ELISA. Data represent 2 independent experiments.
Figure 22. Apoptotic cells induced macrophages to upregulate CD1d and activate iNKT cells

(A) Peritoneal exudate macrophages were co-cultured with apoptotic mouse neutrophils for 24h, and surface expression of CD1d on these macrophages was compared to that on macrophages without AC. (B) Macrophages from (A) were used to co-culture with N38-NKT cells and IL-2 in supernatants was measured by ELISA. Data represent 3 independent experiments.
6. Mice lacking IL-4 displayed impaired resolution of sterile inflammation

So far our data showed IL-4 production in both efferocytosing macrophages and NKT cells following induction of sterile peritoneal inflammation. IL-4 is more known for its role in humoral immunity and allergic inflammation, but its role in sterile inflammation is still unclear. We next assessed whether IL-4 contributed to the resolution of sterile peritoneal inflammation. After periodate challenge, peritoneal leukocytes remained significantly elevated in Il4−/− mice compared to WT mice on days 3 and 4 (Fig. 23A), suggesting an important role for IL-4 to suppress inflammation. Flow cytometry with cell lineage markers illustrated higher numbers of B cells, macrophages, and PMNs in Il4−/− peritoneum on day 3 (Fig. 23B), reflecting an overall enhanced level of inflammation rather than increased infiltration of a particular immune cell type. Additionally, following ex vivo stimulation, CD4 T cells from day 4 Il4−/− mice produced no IL-4, as expected, and minimal levels of IL-13, but higher levels of IFNγ compared to WT controls (Fig. 23C).

To decipher the relative contribution of IL-4 from macrophages and NKT cells to resolve inflammation in this model, we adoptively transferred WT splenic NKT cells isolated from naïve WT mice into Il4−/− mice that had been challenged with periodate. However, adoptive transfer of WT splenic NKT cells into Il4−/− mice did not ameliorate inflammation in these mice, since the inflammation in these mice was still comparable to that in Il4−/− mice that received PBS (Fig. 24A). Thus, the data suggested that NKT cells are not the only relevant IL-4-producing population, and IL-4-producing macrophages also contribute to the resolution of inflammation. In WT mice that received NKT cells, the CD4 T cells produced markedly increased levels of IL-4, IL-13, and IFNγ compared
to controls, likely due to the WT NKT cells transferred into these animals. In contrast, we observed minimal IL-4 or IL-13 production by CD4 T cells isolated from the \( \text{Il4}^{-/-} \) mice that received NKT cells despite more IFN\( \gamma \) production (Fig. 24B). The apparent increase in IFN\( \gamma \) production suggested that the transferred WT NKT cells in \( \text{Il4}^{-/-} \) mice were able to be activated by in vivo \( \text{Il4}^{-/-} \) macrophages, but the transferred NKT cells seemed still unable to produce IL-4 or IL-13. We speculated the absence of IL-4 from macrophages may alter cytokine production from activated NKT cells. This might be one of the mechanisms for IL-4 from macrophages to suppress sterile inflammation.

To test whether IL-4 from macrophages regulates cytokine response in NKT cells, we co-cultured day 3 WT or \( \text{Il4}^{-/-} \) PEM with WT splenic NKT cells isolated from WT naïve mice for 48h and observed less IL-4 but increased IFN\( \gamma \) production (Fig. 25A), supporting that IL-4 from macrophages might in a paracrine manner impact the cytokine response in NKT cells. In addition, 500 pg/mL of exogenous IL-4 added to the co-culture media was able to rescue the ability of \( \text{Il4}^{-/-} \) macrophages to induce IL-4-producing NKT cells (Fig. 25B). Similarly, exogenous IL-4 during anti-CD3 and anti-CD28 stimulation of WT splenic NKT cells resulted in a dose-dependent increase in IL-4 production by the NKT cells (Fig. 25C). Collectively, our data implicated that efferocytosing macrophages activate NKT cells with lipids in a CD1d-dependent manner and at the same time IL-4 secreted from these macrophages may augment type-2 cytokine response in NKT cells and result in increased IL-4 production by NKT cells. This may be an explanation for the failure of transferred WT NKT cells to rescue \( \text{Il4}^{-/-} \) mice, since the in vivo peritoneal exudate macrophages in \( \text{Il4}^{-/-} \) mice still lacked IL-4 production.
Figure 23. Mice lacking IL-4 displayed more exaggerated inflammation following periodate challenge

(A) Inflammation induced by sodium periodate in $Il4^{-/-}$ and WT mice was scored by determining cell counts at day 0, day 3, and day 4. (B) Cell populations were compared between $Il4^{-/-}$ and WT mice on day 3. (C) Peritoneal CD4 T cells isolated from day 4 $Il4^{-/-}$ or WT mice were ex vivo stimulated with anti-CD3 and anti-CD4 for 24h. IL-4, IL-13, and IFNγ in supernatants were measured by ELISA.
Figure 24. Adoptive transfer of WT splenic NKT cells was unable to rescue \textit{Il4}^{-/-} mice

(A) WT splenic NKT cells were adoptively transferred into WT or \textit{Il4}^{-/-} mice 66h post IP injection of sodium periodate and peritoneal cell numbers in recipient mice were enumerated. (B) CD4+ T cells isolated from recipient mice were stimulated ex vivo with anti-CD3 and anti-CD28 for 48h. IL-4, IL-13, and IFN\(\gamma\) in supernatants were measured by ELISA.
Figure 25. IL-4 from macrophages augments type-2 cytokine response in NKT cells

(A) WT or Il4−/− PEM were co-cultured with WT splenic NKT cells for 48h before IL-4 and IFNγ in supernatants were measured by ELISA. (B) 10^5 WT or Il4−/− day 3 PEM were co-cultured with 7.5x10^4 WT splenic NKT cells (100 µl media) from naïve B6 mice with or without 500 pg/ml of mouse recombinant IL-4 48h, and followed by PMA/Ionomycin stimulation in new media and ICS for IL-4 and IFNγ. (C) 7.5x10^4 WT splenic NKT cells (100 µl media) from naïve B6 mice were stimulated ex vivo with anti-CD3 and anti-CD28 with or without 500 pg/ml or 5 ng/ml of mouse recombinant IL-4 for 48h, and followed by PMA/ionomycin stimulation in new media and ICS for IL-4 and IFNγ.
7. IL-4 signaling to macrophages contributed to alternative activation of peritoneal macrophages and was important for the resolution of inflammation

Our results indicated a temporal difference between the emergence of two IL-4-producing cell types in the inflamed peritoneum: efferocytosing macrophages peaking on day 3 and iNKT cells on day 4 (Fig. 26A and B). Since IL-4 induces alternative activation of macrophages, we postulated that the IL-4 induced by these two types of cells in vivo may result in macrophages displaying an M2 phenotype. To test this, we isolated day 3 and day 4 PEMs and measured expression of M2 markers by qPCR. As expected, we observed elevated expression of M2 markers, Arg1, Ym1, and Fizz1 in day 3 PEMs (Fig. 26C). The expression of these M2 markers appeared to coincide with IL-4 production in macrophages more than IL-4 production in NKT cells, thus suggesting that perhaps IL-4 from macrophages contributes more to the acquisition of M2 phenotype of PEMs. To further determine whether the M2 phenotype depended on IL-4, we compared the expression of these 3 M2 markers between WT and Il4−/− day 3 PEMs. All 3 M2 markers were expressed at significantly lower levels in Il4−/− day 3 PEMs than WT controls. On the contrary, the M1 marker, Nos2, was expressed at elevated levels in Il4−/− day 3 PEMs than in controls (Fig. 26D). Overall, our data demonstrated an M2 phenotype in macrophages following sterile inflammation, which is consistent with a role in tissue repair for macrophages. Importantly, our data supported that the acquisition of an M2 phenotype requires IL-4, and IL-4 from macrophages seems to contribute more to alternative activation of macrophages during the response to injury-induced sterile inflammation.
We next wanted to identify cellular targets of IL-4 that mediate the suppression of inflammation in this model. We then utilized $II4ra^{Lo/LysM^{Cre}}$ mice where myeloid cells, mainly macrophages and neutrophils, were unable to respond to IL-4 due to deletion of the IL-4R$\alpha$. Since these mice are on the BALB/c background, we confirmed IL-4 production by efferocytosing macrophages and NKT cells in BALB/c mice, and there was a comparable course and severity of inflammation between WT BALB/c and B6 mice (Fig. 27A-E). We noticed that the percentage of NKT cells within CD4 T cell population in BALB/C mice was overall lower than that in B6 mice where about half of total CD4 T cells were NKT cells (Fig. 16C). Additionally, in BALB/C mice the NKT cells appeared to be recruited after induction of inflammation, as illustrated by an increase in the percentage of NKT cells within CD4 T cell population (Fig. 27D).

Peritoneal inflammation in $II4ra^{Lo/LysM^{Cre}}$ mice was significantly enhanced compared to WT mice (Fig. 28A). Efferocytosing macrophages in $II4ra^{Lo/LysM^{Cre}}$ mice were capable of making IL-4, and MPO$^{+}$ and IL4$^{+}$ peritoneal macrophages in $II4ra^{Lo/LysM^{Cre}}$ mice appeared comparable to WT controls (Fig. 27C and D). Although expression of Arg1 and Fizz1 was not altered by inflammation in WT BALB/c mice (Fig. 27C), induction of Ym1 was attenuated in $II4ra^{Lo/LysM^{Cre}}$ PEM, compared to WT PEM (Fig. 28B), again suggesting that IL-4 signaling to macrophages contributes alternative activation of macrophages. The reduced M2 phenotype may contribute to the enhanced inflammation $II4ra^{Lo/LysM^{Cre}}$ mice. Importantly, activation of NKT cells in $II4ra^{Lo/LysM^{Cre}}$ mice was not affected by the deletion of IL-4 signaling in macrophages, as illustrated by normal cytokine profile of CD4 T cells from these mice after ex vivo stimulation (Fig. 28E). Activated NKT cells were detected on day 3 and day 4 in
BALB/C mice (Fig. 27D) but only detected on day 4 in B6 mice. Together, our data supported the concept that myeloid cells are critical targets for IL-4 to mediate suppression of inflammation.

The elevated inflammation in \(IL4^{LysM^{Cre}}\) mice supported the importance of IL-4 signaling in restraining inflammation, but it did not differentiate the respective contribution of IL-4 from macrophages and NKT cells to resolve inflammation. We additionally did preliminary studies using \(Irf4^{LysM^{Cre}}\) mice in which \(Irf4\) is specifically deleted in myeloid cells. Interferon regulatory factor 4 (IRF4) is a transcription factor required for IL-4 expression in T cells. Naïve T helper cells from mice lacking IRF4 are severely impaired for the production of IL-4 and other Th2 cytokines (Rengarajan et al., 2002). \(Irf4\) was found upregulated in macrophages after coculture with ACs for 9 h (Fig. 6A). Thus, we speculated that IRF4 would be also critical for expression of IL-4 in efferocytosing macrophages. In our pilot experiment, after challenge with sodium periodate, we found increased inflammation in \(Irf4^{LysM^{Cre}}\) mice than \(Irf4^{LysM^{Cre}}\) mice on day 4 (Fig. 29A). However, only Fizz1 was reduced in day 4 \(Irf4^{LysM^{Cre}}\) PEMs than \(Irf4^{LysM^{Cre}}\) PEMs (Fig. 29B). CD4 T cells from day 4 \(Irf4^{LysM^{Cre}}\) mice appeared to produce similar levels of IL-4 compared cells from \(Irf4^{LysM^{Cre}}\) mice (Fig. 29C). However, we need to further characterize whether IRF4 is required for IL-4 expression in macrophages before making conclusions.
Figure 26. IL-4 signaling to macrophages contributed to alternative activation of peritoneal macrophages

(A-B) The number of IL-4-producing macrophages (A) or NKT cells (B) during the course of peritonitis in WT mice was determined by ICS and flow cytometry. At least 3 mice were examined for each time point. (C) Peritoneal macrophages were cultured ex vivo for 48h, and expression of M2 markers Arg1, Ym1 and Fizz1 were quantified by qPCR. Data represent 2 independent experiments. (D) Expression of M2 markers in Day 3 WT and Il4−/− peritoneal macrophages were determined and compared as described in (C). Each dot represents data from one mouse.
Figure 27. Sodium periodate-induced sterile inflammation in WT BALB/C mice

(A) Peritoneal cells were enumerated in naïve BALB/C mice and mice injected with sodium periodate for 3 or 4 days. (B) Peritoneal cells from day 0, day 3, and day 4 mice were labeled with F4/80 and stained for intracellular IL-4. (C) Peritoneal macrophages from day 0, day 3 and day 4 mice were ex vivo cultured for 48h and followed by qPCR to measure expression of Arg1, Fizz1 and Ym1. (D) CD4 T cells isolated from day 0, day 3, and day 4 mice were stained with CD1d-teramer loaded with alpha-GalCer and TCRβ to identify NKT cells. (E) CD4 T cells isolated from day 0, day 3, and day 4 mice were ex vivo stimulated with anti-CD3 and anti-CD28 for 24h. IL-4, IL-13, and IFNγ in supernatants were measured by ELISA.
Figure 28. IL-4 to signaling to myeloid cells was critical to resolve sterile inflammation

(A) Severity of sodium periodate-induced peritonitis in WT or I14raL/− LysMCre mice was scored by enumeration of peritoneal cells at day 0, 3, and 4. (B) Expression of Ym1 in mice from (A) was measured by qPCR. (C and D) Peritoneal macrophages (F4/80+) from day 0, 3, and 4 WT and I14raL/− LysMCre mice were analyzed by ICS for MPO or IL-4. (E) CD4+ T cells isolated from day 0, 3, and 4 WT and I14raL/− LysMCre mice were stimulated ex vivo with anti-CD3 and anti-CD28 for 24h. IL-4, IL-13, and IFNγ in supernatants were measured by ELISA.
Figure 29. IRF4 from myeloid cells was important to resolve sterile inflammation

(A) Severity of sodium periodate-induced peritonitis in Irf4\textsuperscript{ff}LysM\textsuperscript{Cre+} or Irf4\textsuperscript{ff}LysM\textsuperscript{Cre-} mice was scored by enumeration of peritoneal cells at day 4. (B) Expression of M2 markers Arg1, Fizz1 and Ym1 in day 4 PEMs from Irf4\textsuperscript{ff}LysM\textsuperscript{Cre+} or Irf4\textsuperscript{ff}LysM\textsuperscript{Cre-} mice were measured by qPCR after ex vivo culture for 48 h. (C) CD4+ T cells isolated from Irf4\textsuperscript{ff}LysM\textsuperscript{Cre+} or Irf4\textsuperscript{ff}LysM\textsuperscript{Cre-} mice were stimulated ex vivo with anti-CD3 and anti-CD28 for 24 h. IL-4, IL-13, and IFN\gamma in supernatants were measured by ELISA.
Part II: Efferocytosis activates the phagocyte NADPH oxidase in macrophages

1. The NADPH oxidase in macrophages is activated during efferocytosis

Activation of the NADPH oxidase in macrophages is commonly coupled to phagocytosis of microbes, as most activation phagocytic receptors, including complement receptors, FcRγ, dectin 1, leads to activated kinases, phospholipases, and GTPases that are also involved in activating the phagocyte NADPH oxidase. Since efferocytosis also involves similar phagocytic receptors, we hypothesized that the NADPH oxidase may be also activated during efferocytosis. To test that, we first determined whether X-CGD macrophages would be able to ingest ACs as efficiently as WT macrophages, as a previous study suggested X-CGD macrophages were impaired in efferocytosis of ACs (Fernandez-Boyanapalli et al., 2009). However, in contrast to the previous report, we found normal ingestion of ACs by X-CGD PEMs (Fig. 30A and B), likely due to different ways ACs were prepared or different characteristics of macrophages used in our system.

To determine whether the phagocyte NADPH oxidase was activated in efferocytosing macrophages, we first performed nitro-tetrazolium blue (NBT) assay to detect superoxide (O$_2^-$) production in efferocytosing macrophages. NBT present in the media can be taken up with the target during phagocytosis by phagocytes, resulting in NBT in the sealed phagosome later can be reduced by O$_2^-$ produced in the phagosome and turns into purple formazan. 30 minutes after co-culture with apoptotic human neutrophils or zymosan, the NBT assay showed purple formazan within intracellular compartments in WT PEMs that looked like phagosomes, but this was absent in X-CGD PEMs (Fig. 31A). Purple phagosomes were also found in WT PEMs stimulated with
zymosan, a well-known oxidase activator that is reported to bind to dectin 1 and TLR2 (Goodridge et al., 2012). In addition, the absence of NBT positive phagosomes in X-CGD PEMs co-cultured with ACs supported that the NBT positive phagosomes observed in WT PEMs was due to activation of the phagocyte NADPH oxidase in macrophages, not the apoptotic neutrophils. Furthermore, we collected neutrophils from an X-CGD patient whose phagocytic leukocytes lacked gp91<sub>phox</sub>. When we co-culture apoptotic neutrophils from this patient with WT mouse macrophages, by DAB histochemistry for MPO, we found no reduction in the ingestion of X-CGD neutrophils compared to WT neutrophils. Importantly, we noticed NBT-positive purple formazan in WT macrophages that were fed with X-CGD neutrophils (not shown), again suggesting that the superoxide was generated by the macrophages via the NADPH oxidase but not by apoptotic neutrophils. Together the data suggested that the phagocyte NADPH oxidase in macrophages was activated during efferocytosis to generate O<sub>2</sub><sup>-</sup> in efferosomes, or the phagosome containing an AC.

To quantify O<sub>2</sub><sup>-</sup> in efferocytosing macrophages, we did a luminol chemiluminescence assay. Robust intracellular O<sub>2</sub><sup>-</sup> production was observed in WT PEMs cells stimulated with ACs, which peaked at about 8 minutes after ACs were added to the wells (Fig. 31B). On the other hand, X-CGD macrophages stimulated with the same ACs showed only basal levels of O<sub>2</sub><sup>-</sup>. As another way to confirm activation of NOX2, we did immunofluorescence staining for gp91<sub>phox</sub> in efferocytosing macrophages that were co-cultured with labeled ACs for 15 min. The accumulation of gp91<sub>phox</sub> on the efferosome membrane was apparent, suggesting that the phagocyte NADPH oxidase complex was recruited to efferosomes and generated O<sub>2</sub><sup>-</sup> in the lumen of efferosomes.
Collectively, our data demonstrated that efferocytosis leads to activation of the phagocyte NADPH oxidase in macrophages, which subsequently generate $\text{O}_2^-$ in efferosomes.

**Figure 30. Normal ingestion of human apoptotic neutrophils by X-CGD macrophages**

(A) PKH 26-labeled human apoptotic neutrophils (ACs) were added to overnight cultured WT or X-CGD PEMs and incubated at 37 °C for 30 min. The percentage of PEMs with ingested ACs was determined by flow cytometry after labeling macrophages with F4/80. PKH-red positive F480+ PEMs had ingested ACs. (B) The percentage of ingestion by WT and X-CGD PEMs from A. Data represent 7 independent experiments.
Figure 31. Efferocytosis of apoptotic neutrophils activated the NADPH oxidase to generate ROS in macrophages

(A) Zymosan or apoptotic neutrophils were added to overnight cultured PEMs plated in a 8-well chamber slide for nitro blue tetrozolium (NBT) assay. WT and X-CGD PEMs were incubated with either zymosan or ACs for 30 min in the presence of NBT, which forms purple formazan in the the presence of superoxide. PEM to apoptotic cells ratio was 1:4. Black arrows indicate likely X-CGD PEMs with ingested ACs. (B) Intracellular O$_2^-$ production by WT and X-CGD PEMs was measured by luminol in the presence of horseradish peroxidase (HRP) and superoxide dismutase (SOD). SOD cannot penetrate
cell membrane and was used to eliminate extracellular oxidation of luminol. PEM to apoptotic cells ratio was 1:8. Each condition was done in duplicate. (C) WT PEMs were co-cultured with LysoTracker Red-labeled human X-CGD apoptotic neutrophils for 20 minutes and immunostained for gp91<sup>phox</sup> with an anti-gp91 (54.1) that recognized both human and mouse gp91<sup>phox</sup>. Nuclei of both PEMs and ACs were stained with DAPI.
2. Complement receptor 3 and TLR4/MyD88 are both required to activate the phagocyte NADPH oxidase in efferocytosing macrophages

Our next aim was to identify efferocytic receptor(s) involved in efferocytosing that is associated with activation of the phagocyte NADPH oxidase in macrophages. The β2 integrin member complement receptor 3 (CR3) (also known as CD11b/CD18, αMβ2, and Mac1) is a leukocyte adhesion molecule known to bind to a variety of particles, including ICAM-1 (intracellular adhesion molecule-1), iC3b, fibrinogen, and LPS (Mazzone and Ricevuti, 1995). CR3 also serves as a phagocytic receptor that mediates the engulfment of iC3b-opsonized pathogens, which is associated with activation of the NADPH oxidase in phagocytes. Both CR3 and CR4 (CD11c/CD18) have been implicated in the efficient efferocytosis by human macrophages (Mevorach et al., 1998). Therefore, we hypothesized that CR3 may activate the phagocyte NADPH oxidase during CR3-mediated efferocytosis. In addition, a previous study showed increased assembly of the the phagocyte NADPH oxidase to efferosomes in macrophages that required intact MyD88 signaling (Yvan-Charvet et al., 2010). We speculated that TLR4 may also be involved in activation of the phagocyte NADPH oxidase in efferocytosing macrophages. Therefore, we isolated peritoneal exudate macrophages from Cd11b−/− mice and Tlr4−/− mice to characterize their roles in activation of the phagocyte NADPH oxidase during efferocytosis. Both Cd11b−/− and Tlr4−/− PEMs showed normal ingestion of ACs in vitro (data not shown). Given the redundancy of many phagocytic receptors in mediating ingestion of ACs, it is not surprising that deficiency in one particular receptor would not result in an obvious impairment in uptake of ACs. However, by luminol assay, AC-elicited O₂⁻ production in PEMs was almost absent, close to the basal level seen in the
negative control, X-CGD PEMs (Fig. 32A). Since Cd11b^−/− mice lack both CR3, the severely diminished O_2^- production in Cd11b^−/− PEMs in response to stimulation by ACs suggested a critical role for CR3 in activating the phagocyte NADPH oxidase in efferocytosing macrophages. In addition, we only observed partial O_2^- production in Tlr4^−/− PEMs (Fig. 32A) and Myd88^−/− PEMs. However, O_2^- production in response to zymosan was normal in Cd11b^−/−, Tlr4^−/−, and Myd88^−/− PEMs (Fig. 32B and D). Collectively, our data suggested that CR3 or CR4 is the efferocytic receptor associated with activation of the phagocyte NADPH oxidase in efferocytosing macrophages; whereas TLR4, which itself cannot mediate efferocytosis, may be a co-stimulatory molecule leading to MyD88 signaling that potentiates the activation of the phagocyte NADPH oxidase and O_2^- production in efferocytosing macrophages.

We next wanted to identify possible ligands on ACs for CR3 activation during efferocytosis. It should be noted that the apoptotic neutrophils used in our in vitro experiments were aged in RPMI media with 5% heat-inactivated fetal calf serum, in which all the complement labile factors, including the most well-characterized ligand for CR3, iC3b, would have been depleted. However, we still postulated that iC3b may be the ligand on ACs that is recognized by CR3 on PEMs to mediate uptake of ACs and subsequently activation of the phagocyte NADPH oxidase. But the iC3b in this case might be unlikely from the heat-inactivated FBS but rather from the human neutrophils themselves. A previous study demonstrated that both classical and alternative pathways of complement are activated while human neutrophils are aged in vitro, thus ultimately leading to deposition of iC3b on the cell surface of apoptotic neutrophils (Mevorach et al., 1998). To test this, we determined if human iC3b was deposited on the cell surface of
human apoptotic neutrophils using an anti-human iC3b. Following in vitro culture in RPMI media with 5% heat-inactivated FCS, aged human neutrophils showed distinct iC3b expression at 10h, 18h, and 24h (Fig. 33A), suggesting aged human neutrophils likely expressed iC3b on their cell surface as they underwent apoptosis. To see whether the iC3b-expressing aged human neutrophils were in fact apoptotic, we did co-staining for Annexin V on neutrophils aged with or without 5% heat-inactivated FCS. All iC3b+ PMNs were Annexin V positive. Importantly, cells aged without serum still showed strong staining of iC3b (Fig. 33B). Thus, our data suggested that as neutrophils age and undergo apoptosis, they process complement and produce iC3b on their cell surface. However, more work is needed to determine whether iC3b is the ligand on ACs that targets CR3 on macrophages to trigger oxidative burst in macrophages.

CD44 is a transmembrane adhesion molecule. High expression of CD44 isoforms is found in human peripheral blood monocytes (Mackay et al., 1994) and is increased in both monocyte-derived macrophages and inflamed macrophages (Culty et al., 1994; Levesque and Haynes, 1996), where sulfation of CD44 permits interaction with hyaluronan (HA). The importance of CD44 in clearance of apoptotic neutrophils and inflammation resolution has been highlighted in studies showing that following bleomycin- induced lung injury, clearance of apoptotic neutrophils was reduced by about 80% in Cd44−/− mice (Teder et al., 2002). Recently, CD44 was demonstrated as a primary phagocytic receptor directly mediating the internalization of anti-CD44 antibody opsonized or hyaluronan opsonized sheep erythrocytes by mouse macrophages and neutrophils, which required phosphorylated Syk (spleen tyrosine kinase) and the activation of Rac GTPase (Vachon et al., 2006). Most importantly, in this study oxidative
burst was detected in CD44-mediated phagosomes in mouse neutrophils, thus indicating the activation of the NADPH oxidase following CD44-mediated phagocytosis. We wanted to determine whether CD44-mediated efferocytosis would lead to activation of the NADPH oxidase. We found normal generation of superoxide in Cd44−/− macrophages after stimulation with both apoptotic neutrophils and zymosan (Fig. 32B), suggesting that CD44 is dispensable for efferocytosis-mediated activation of the phagocyte NADPH oxidase.
Figure 32. Activation of the phagocyte NADPH oxidase in efferocytosing macrophages requires complement 3 receptor (CR3) and TLR4/MyD88 signaling

(A) Intracellular O$_2^-$ production by WT, $Cd11b^{-/-}$, $Tlr4^{-/-}$, and X-CGD PEMs in response to stimulation by apoptotic neutrophils (1:8 ratio) was measured by luminol in the presence of horseradish peroxidase (HRP) and superoxide dismutase (SOD). Data represent 3 independent experiments. (B) Total O$_2^-$ production by WT, $Cd11b^{-/-}$, $Tlr4^{-/-}$, $Cd44^{-/-}$ and X-CGD PEMs in response to stimulation by ACs (1:8 ratio) and zymosan was measured by luminol assay. O$_2^-$ production by $Cd11b^{-/-}$, $Tlr4^{-/-}$, $Cd44^{-/-}$ and X-CGD PEMs was normalized to O$_2^-$ production by WT. Zymosan was used as a control. Data represent 3 independent experiments. * p = 0.001; **p=0.004; ***p=0.0017; all vs. WT; n=2-3. NS = not significant (C) Total and intracellular O$_2^-$ production by WT, $Myd88^{-/-}$, and X-CGD PEMs in response to stimulation by ACs. (D) Total O$_2^-$ production by WT, $Mdy88^{-/-}$, and X-CGD PEMs in response to stimulation by ACs (1:8 ratio) and zymosan was measured by luminol assay. O$_2^-$ production by $Myd88^{-/-}$ and X-CGD PEMs was normalized to O$_2^-$ production by WT. Zymosan was used as a control. Data represent 3 independent experiments.
Figure 33. Aged apoptotic human neutrophils expressed iC3b on cell surface

(A) Surface iC3b on fresh human neutrophils and human neutrophils that were cultured with 5% heat-inactivated FBS ex vivo for 10h, 18h, and 24h, was determined by flow cytometry using an anti-iC3b. (B) Surface iC3b on fresh human neutrophils and human neutrophils that were cultured with or without 5% heat-inactivated FBS ex vivo for 24h was determined by flow cytometry using an anti-human iC3b (Quidel, San Diego, CA). Co-staining for Annexin V showed apoptotic cells (Annexin V positive).
3. **p40^phox is required for optimal activation of the phagocyte NADPH oxidase in efferocytosing macrophages**

The role of p40^phox in the activity of the phagocyte NADPH oxidase is relatively less understood compared to the other subunits of the phagocyte NADPH oxidase (Matute et al., 2005). Cell-free reconstitution of activity of the phagocyte NADPH oxidase did not require p40^phox (Abo et al., 1992). However, recent studies in p40^phox-deficient cell lines and mice demonstrated that during phagocytosis, the phox-homolog (PX) domain of p40^phox is important for associating phosphatidylinositol 3-phosphate (PI3P), a phosphoinositide that accumulates on phagosomes through the action of class III PtdIns(3)P kinase (Bravo et al., 2001; Ellson et al., 2001; Suh et al., 2006). Mouse neutrophils lacking p40^phox or p40^phox R58A, a PX domain mutant unable to associate with PI3P, showed impaired killing of Staphylococcus aureus due to reduced oxidase activity and intracellular ROS production (Ellson et al., 2006a; Ellson et al., 2006b). Additionally, an R105Q mutation affecting the PX domain of p40^phox was found in a CGD patient presented with granulomatous colitis and contributed to premature loss of p40^phox R105Q from phagosomes, due to weak association with PI3P. Neutrophils from this patient exhibited reduced intracellular ROS response to particulate stimuli (Matute et al., 2009). Thus, evidence from these studies has established an integral role for p40^phox in stabilizing the complex of the phagocyte NADPH oxidase on phagosomes through its association with PI3P, which is important to sustain accumulation of the oxidase on phagosomes to maximize ROS production in phagosomes.

To determine whether p40^phox would be required for activity of the phagocyte NADPH oxidase in efferocytosing macrophages, we measured O_2^- production by p40^phox
R58A PEMs in response to ACs by luminol assay and found about 50% reduction in both total and intracellular $O_2^-$ production compared to WT PEMs (Fig. 34A). However, zymosan-induced $O_2^-$ production in p40\textsuperscript{phox} R58A PEMs was normal (Fig. 34B). Thus, our data suggested that p40\textsuperscript{phox} plays an essential role for the activity of the phagocyte NADPH oxidase in efferocytosing macrophages.

**Figure 34.** p40\textsuperscript{phox} was required for optimal activation of the phagocyte NADPH oxidase in efferocytosing macrophages

(A) Total and intracellular $O_2^-$ production by WT, p40\textsuperscript{phox} \textsuperscript{-/-}, and X-CGD PEMs in response to stimulation by ACs. (B) Total $O_2^-$ production by WT, Myd88 \textsuperscript{-/-}, and X-CGD PEMs in response to stimulation by ACs (1:8 ratio) and zymosan was measured by luminol assay. $O_2^-$ production by p40\textsuperscript{phox} \textsuperscript{-/-} and X-CGD PEMs was normalized to $O_2^-$ production by WT. Zymosan was used as a control. Data represent 3 independent experiments. * $p < 0.05
4. ROS derived from the phagocyte NADPH oxidase are required for efficient degradation of ingested apoptotic cells and suppression of proinflammatory response in macrophages

ROS generated by the phagocyte NADPH oxidase have been demonstrated to modulate phagosome maturation and processing of antigens (Huang and Brumell, 2009a; Savina et al., 2006; Savina et al., 2009). To characterize the function of the phagocyte NADPH oxidase in efferocytosing macrophages, we first wanted to determine whether oxidase-derived ROS may affect the degradation of ingested ACs in macrophages. MPO is a neutrophil-specific granule protein, thus measuring the residual content of MPO in efferocytosing macrophages would show the rate at which the ingested ACs were degraded. We did both diaminobenzidine histchemistry (DAB) for MPO present in the cytosol of PEMs and western blotting for MPO in cell lysates from PEMs. DAB showed that in WT PEMs 6 h after pulse with ACs, most of the MPO was degraded, with light brown MPO staining in most of WT PEMs. In contrast, dark brown MPO staining was apparent in X-CGD PEMs (Fig. 35A), indicating substantial amounts of MPO still present in X-CGD PEMs and reflecting slower degradation in X-CGD PEMs. Consistently, we detected significantly greater amounts of MPO in cell lysates from X-CGD PEMs 6h after pulse with ACs than in cell lysates of WT PEMs, but similar levels of MPO were found in both WT and X-CGD cell lysates 24h after pulse with ACs, when only minimal MPO was detected (Fig. 35B and C). Thus, degradation of ACs appeared to be attenuated in an earlier phase of degradation in X-CGD PEMs.

The luminol assay earlier (Fig. 31B) indicated respiratory burst in WT efferocytosing macrophages within minutes upon stimulation with ACs, indicating
generation rapid generation of ROS in response to ACs in macrophages. ROS generated by the phagocyte NADPH oxidase have been shown to modulate phagosome maturation by accelerating recruitment of the autophagy complex to the phagosomes or altering phagosomal pH to activate lysosomal proteases (Huang and Brumell, 2009b; Huang et al., 2009; Savina et al., 2006; Watts, 2006). Therefore, we next wanted to determine whether the oxidase-derived ROS in efferocytosing macrophages were involved in the maturation of phagosome and fusion with the lysosome. To address that, we first examined degradation of ACs in oxidase-deficient X-CGD PEMs and compared the rate of phagosome-lysosome fusion between WT and X-CGD PEMs following ingestion of ACs. Lamp1 is a lysosomal protein and accumulation of Lamp1 on phagosomes indicates fusion of phagosomes with lysosomes. By immunofluorescence staining for Lamp1 at 15 min and 30 min after pulse with LysoTracker Red – labeled ACs, we observed decreased recruitment of Lamp1 to X-CGD efferosomes at both time points compared to that in WT PEMs (Fig. 36A and B), thus suggesting decreased phagosome-lysosome fusion in X-CGD PEMs, likely due to the absence of ROS.

To further examine whether there were deleterious consequences of deficient activity of the phagocyte NADPH oxidase in X-CGD macrophages in response to ACs, we tried to compare cytokine profile between WT and X-CGD efferocytosing macrophages. We collected co-culture supernatants 24h after PEMs and ACs were co-cultured and measured cytokines in the supernatants using multiplex analysis of a 32-cytokine panel. Most of cytokines quantified were either at low levels or similar levels between WT and X-CGD PEMs. However, we found the most striking difference G-CSF. X-CGD PEMs without ACs already showed increased production of these 4 chemokines
compared to WT controls, and in the presence of ACs further the amounts of these chemokines were elevated in X-CGD PEMs (Fig. 37). In addition, CXCL-10, monocyte chemoattractant protein 1 (MCP1), and macrophage inflammatory protein α (MIPα) were at higher basal levels in X-CGD PEM cultures than WT PEM cultures. In sharp contrast, these 4 chemokines remained at low levels in WT PEMs cultured with or without ACs (Fig. 37). Since PEMs used in this experiments were isolated from the peritoneum of sodium periodate – treated mice for 3 days and ex vivo cultured for 24h before co-culture with ACs. We speculated that the higher levels of these 4 chemokines in X-CGD PEMs may be due to altered response in X-CGD PEMs from the in vivo environment in X-CGD mice.
Figure 35. The phagosome NADPH oxidase was required for efficient degradation of ingested apoptotic cells

(A) Apoptotic human neutrophils aged for 20 h were added into overnight cultured WT or X-CGD PEM cultures. Non-ingested hPMNs were removed 15 minutes later, and PEMs were further cultured in culture media for 6h, and then MPO presence in PEMs was detected by diaminobenzidine histochemistry for MPO. Images were taken with a 100x objective. (B) Apoptotic human neutrophils aged for 20 h were loaded into overnight cultured WT or X-CGD PEM cultures. Non-ingested hPMNs were removed 30
min later, and PEMs were either solubilized to generate cell lysates or further cultured in culture media for 6h or 24h before cell lysates were obtained. The presence of MPO in cell lysates was detected by Western blotting using a polyclonal anti-MPO. Densitometry to measure relative amounts of MPO from Western blots from 3 independent experiments was performed by using Image J. * p <0.05
Figure 36. Delayed recruitment of lysosomal marker Lamp1 to efferosomes in X-CGD PEMs

(A) LysoTracker Red–labeled 20h-aged apoptotic human neutrophils were loaded into overnight cultured WT or X-CGD PEMs and co-cultured in PBS for either 15 or 30 minutes. Then ACs were removed and PEMs were fixed and immunostained for gp91phox (yellow) and Lamp 1 (green). Nuclei were stained with DAPI. Gp91phox expression in ACs were also apparent within efferosomes. Images were taken with a 100X oil-immersion objective. Images were analyzed and handled using Metamorph software (Molecular Devices). White arrows point to areas with accumulated Lamp1. (B) More than 200 efferosomes were scored for each genotype in (A) at each time point from 3 independent experiments. * p <0.01. ** p <0.05.
Figure 37. Apoptotic neutrophils induced increased production of proinflammatory cytokines in X-CGD macrophages

Overnight cultured WT or X-CGD PEMs were co-cultured with apoptotic neutrophils for 24h at 10:1 macrophages: ACs ratio. Cytokines in supernatants, including G-CSF, CXCL-10, MCP-1, and MIP-alpha, were measured by multiplex (Eve Technologies). Data were collected from 3 mice from each genotype at each time point.
Part III: The efferocytosis-induced IL-4-dependent macrophage-iNKT cell circuit is defective in gp91phox-deficient X-CGD mice

1. X-CGD mice exhibited enhanced peritoneal inflammation and delayed resolution

We have demonstrated that efferocytosis of apoptotic neutrophils activates the phagocyte NADPH oxidase in macrophages to generate ROS. Given that the deficiency in activity of the phagocyte NADPH oxidase results in inflammatory complications in CGD patients, we next wanted to determine whether the phagocyte NADPH oxidase was involved in the innate cellular circuit we described in our model of sterile inflammation from Part I. Specifically, we wanted to investigate macrophage efferocytosis or the associated production of IL-4 and activation of iNKT cells during peritoneal inflammation in mice with chronic granulomatous disease. Previous studies reported that instillation of thioglycollate or zymosan into the peritoneum of NADPH oxidase-deficient CGD mice lacking gp91phox or p47phox, respectively, resulted in increased neutrophil infiltration and delayed resolution of inflammation (Fernandez-Boyanapalli et al., 2010; Jackson et al., 1995; Pollock et al., 1995). In the current study, we observed that instillation of sodium periodate also led to significantly increased circulating neutrophils in mice lacking gp91phox, referred to as X-CGD mice, compared to WT mice at 4h following intraperitoneal injection of sterile sodium periodate. The other immune cells, lymphocytes, monocytes, eosinophils, and basophils showed no difference in number before and after challenge with periodate, and no difference was found in these cells between WT and X-CGD mice (Fig. 38A). Mobilization of neutrophils from the bone marrow into the circulation is a typical acute inflammatory response to tissue infection or injury (Eash et al., 2010; Greenbaum and Link, 2011). Our data indicated more
exaggerated acute inflammatory response to periodate–induced tissue injury in the peritoneum in X-CGD mice. Consistently, by eight hours, there was a ≈2-fold increase in the number of neutrophils in the X-CGD peritoneum compared to that in the WT peritoneum (Fig. 38B). The peritoneal inflammation in X-CGD mice was much more exaggerated and remained elevated at day 6 compared to WT mice (Fig. 38C). Three days after initiation of inflammation, the absolute cell numbers of neutrophils and macrophages were increased in X-CGD mice (Fig. 38D), suggesting increased infiltration of these two innate immune cell types and more severe inflammation in X-CGD mice.

There was no difference in the fraction of X-CGD PEM ingesting ACs in vivo, as determined by ICS for MPO, compared to WT PEM (Fig. 39A). Because of the elevated numbers of exudate macrophages in X-CGD mice, there were significantly more MPO-positive and IL-4-producing macrophages recovered from the peritoneum of X-CGD mice compared to WT mice on day 3 (Fig. 39B and C). Together, our data suggested that NOX2 is not required for ingestion of ACs, consistent with our previous observation (Fig. 30A and B), and that IL-4 production in macrophages is not dependent on NOX2. Overall, we demonstrated exaggerated acute inflammation in X-CGD mice following periodate challenge, which was initiated by higher numbers of peritoneal exudate neutrophils early in the response and followed by increased numbers of X-CGD macrophages undergoing efferocytosis and producing IL-4.

Since IL-4 is known to induce fusion of macrophages to form multinucleated giant cells (Helming and Gordon, 2009), which are frequently found in CGD granulomas. We next wanted to determine whether IL-4 produced during efferocytosis may induce giant cell formation in X-CGD macrophages. To test it, we isolated WT and X-CGD
PEMs from mice that were challenged with sodium periodate for 3 days, cultured them ex vivo for 24 h before adding ACs to further culture for another 24h. We observed giant cells in X-CGD PEMs with or without ACs but not in WT-PEMs. The presence of ACs seemed to increase giant cells both by number and size in X-CGD PEMs, with typically more than 10 nuclei in giant cells after stimulation with ACs (Fig. 40A). In addition, we detected significantly higher levels of IL-4 in X-CGD PEMs with or without ACs compared to WT controls (Fig. 40B), which likely contributed to the propensity of X-CGD PEMs to fuse. To see whether giant cell formation in X-CGD PEMs depended on IL-4, we neutralized IL-4 by treating WT and X-CGD PEMs with anti-IL-4 for 48h. The giant cells in X-CGD PEMs were reduced both in both number and size (Fig. 40C), supporting a consistent role for IL-4 in inducing giant cell formation.
Figure 38. Elevated inflammation in X-CGD mice in response to challenge with sodium periodate

(A) Circulating cell counts in the peripheral blood of X-CGD and WT mice 4h post IP injection of sodium periodate were determined by Hemavet (Drew Scientific). (B) Total numbers of neutrophils recruited to the peritoneum were determined 8 h post IP injection. At least 4 mice were used for each genotype at each time point. (C) Total peritoneal cell counts in X-CGD and WT mice were determined at baseline, 8h, day 3, day 4, and day 6 post IP injection of sodium periodate. (D) Differentials were compared between X-CGD and WT mice on day 3.
Figure 39. Increased efferocytosing macrophages and IL-4-producing macrophages in X-CGD mice as a result of elevated inflammation

(A) Day 3 peritoneal exudate cells from WT and X-CGD mice were ex vivo stained for intracellular MPO. Macrophages were labeled with anti-CD115. (B) The numbers of MPO+ (B) or IL-4+ (C) peritoneal macrophages were determined by intracellular staining and compared between X-CGD and WT mice at baseline and day 3. Data represent 3 independent experiments.
A

WT PEMs

WT PEMs + PMN 24h

X-CGD PEMs

X-CGD PEMs + PMN 24h

100X

100X

B

IL4

pg/mL per 10^6 cells

No ACs

18h ACs

WT

X-CGD

* * *

* *

C

X-CGD Mac

X-CGD Mac + αIL4

WT Mac

WT Mac + αIL4

100X

100X

100X

100X

130
Figure 40. Increased IL-4 produced in X-CGD macrophages induced X-CGD macrophages to form giant cells

(A) Day 3 peritoneal exudate X-CGD or WT macrophages were ex vivo cultured in an 8-well chamber slide overnight and co-cultured with apoptotic human neutrophils for 24h. Slides were stained with Wright-Giemsa. (B) WT or X-CGD peritoneal exudate macrophages were ex vivo cultured with human apoptotic neutrophils for 24h. Amounts of IL-4 in supernatants with or without apoptotic neutrophils were measured by multiplex. Data represent 3 independent experiments. (C) Day 3 X-CGD or WT peritoneal exudate macrophages were cultured ex vivo in an 8-well chamber slide overnight. The next day, 10 ng/ml of anti-IL4 antibody was added to some wells and further cultured for another 48h. Slides were then stained with Wright-Giemsa. * p <0.05
2. Activation of peritoneal iNKT cells was impaired in X-CGD mice

Since the production of IL-4 by inflammatory macrophages was not impaired in X-CGD mice, we hypothesized that delayed resolution in X-CGD mice might be in part caused by altered iNKT cell activation. To test this, we first examined intracellular IL-4 and IFN-γ production by day 4 NKT cells isolated from X-CGD mice. The relative percentage of peritoneal NKT cells in the CD4 T cell population in X-CGD mice was similar to that in WT mice (Fig. 41A). However, the fractions of X-CGD NKT cells producing IL-4 alone, or in combination with IFN-γ, were significantly reduced compared to WT NKT cells. In contrast, the percentage of X-CGD NKT cells producing IFN-γ alone was more than two-fold higher than WT NKT cells. Cytokine analysis by ELISA after ex vivo stimulation of peritoneal exudate CD4 T cells was consistent with flow cytometry results, and also revealed decreased IL-13 production (Fig. 41A and B). Since we have demonstrated that activation of peritoneal NKT cells requires peritoneal macrophages, we hypothesized that X-CGD PEMs may be defective in activating NKT cells during sterile inflammation. To test this, we first measured CD1d expression on X-CGD PEMs. CD1d expression on day 4 X-CGD PEM was reduced compared to WT PEM (Fig. 42A - C). To assess the ability of X-CGD day 4 PEM to activate iNKT cells, we used these cells to activate N38-2C12 NKT cells in vitro and observed minimal IL-2 production compared to that induced by WT day 4 PEM (Fig. 42D). Thus, our data suggested a defect in the activation of iNKT cells by X-CGD PEM during sterile inflammation.

We next adoptively transferred WT splenic NKT cells into X-CGD mice but found that inflammation was not ameliorated (Fig. 43A). This was likely due to the impaired ability of X-CGD PEM to activate the transferred WT NKT cells, since there
were only modest changes in IL-4 and IFNγ production by CD4 T cells isolated from X-CGD mice that received NKT cells compared to PBS controls (Fig. 43B). In contrast, adoptive transfer of WT NKT cells led to increased IL-4 and IFNγ production by CD4 T cells from WT recipient mice (Fig. 43B). To delineate whether the defect in NKT cell activation in X-CGD mice was restricted to the macrophage population, or whether there was an intrinsic defect in X-CGD NKT cells that may also contribute to their reduced activation during peritoneal inflammation, we activated either WT or X-CGD splenic NKT cells ex vivo with CD1d-expressing LMTK cells. X-CGD splenic NKT cells produced about 50% and 30% less IL-4 and IFNγ, respectively, compared to WT splenic NKT cells (Fig. 44A and B). Addition of α-GalCer to the co-cultures was still unable to increase IL-4 and IFNγ production by X-CGD splenic NKT cells to the levels seen in WT control, demonstrating a partial intrinsic defect in activation of X-CGD NKT cells.

Therefore, our data supported a role for the NOX2 in the activation of NKT cells by macrophages during sterile inflammation. The defect in X-CGD PEMs to activate NKT cells, perhaps as a result of the absence of NOX2-derived ROS during efferocytosis, and altered cytokine response in X-CGD NKT cells, both may contribute to exaggerated and prolonged inflammation in X-CGD mice.
Figure 41. Impaired cytokine response of day 4 X-CGD NKT cells

(A) NKT (CD1d tetramer+ TCRβ+) or Non-NKT (PBS-57-loaded-CD1d tetramer-TCRβ+) populations from day 4 WT or X-CGD peritoneum were identified. Each population was assessed for intracellular IL-4 and IFNγ. (B) Day 0 or day 4 WT and X-CGD CD4 T cells were ex vivo stimulated with anti-CD3 and anti-CD28 for 24h, and IL-4, IL-13, and IFNγ in supernatants were measured by ELISA.
Figure 42. Day 4 peritoneal exudate macrophages in X-CGD mice displayed reduced CD1d expression and defective activation of iNKT hybridoma cells

(A) Peritoneal macrophages (F4/80+) from day 4 WT and X-CGD mice were assessed for surface expression of CD1d, and (B) CD1d expression was shown by a histogram. (C) MFI of CD1d expression on WT and X-CGD day 0 and day 4 PEMs. Data represent an average of at least 3 independent experiments. (D) Activation of Type 1 mouse NKT hybridoma 2C12 day 4 WT or X-CGD peritoneal macrophages was determined by measuring IL-2 production by the hybridoma cells 24h after co-culture with the macrophages. Data represent 3 independent experiments.
Figure 43. Adoptive transfer of WT splenic NKT cells was unable to rescue X-CGD mice

(A) Adoptive transfer of WT splenic NKT cells into either WT or X-CGD mice after induction of peritonitis was performed as described in Fig. 3. Day 4 peritoneal cell counts were determined. (B) CD4 T cells from recipient mice were ex vivo stimulated as described in Fig. 3, and IL-4 and IFNγ production were measured by ELISA. Data represent 2 independent experiments.
Figure 44. Partial intrinsic defect in activation of X-CGD splenic NKT cells

WT or X-CGD splenic NKT cells isolated by FACS sorting were co-cultured with mCD1d-expressing LMTK cells ex vivo at 1:10 ratio with or without α-GalCer for 48h and IL-4 (A) and IFNγ (B) production were measured by ELISA. Data represent 2 independent experiments.
Part IV. A Rac-gp91\textsuperscript{phox} interaction is important for electron transfer on the phagocyte NADPH oxidase and assembly of the oxidase on phagosome membrane

1. Disrupted interaction with Rac and gp91\textsuperscript{phox} led to defective NADPH oxidase activity in PLB-985 neutrophils

The Rac GTPase is an essential component of the superoxide-generating NADPH oxidase in phagocytic leukocytes. GTP-bound Rac GTPase regulates the catalytic activity of the oxidase through a well-characterized interaction with p67\textsuperscript{phox}, the cytosolic subunit of the oxidase. A direct physical interaction between Rac and a region in the flavoprotein domain of gp91\textsuperscript{phox} were reported by recent studies, and mutations in the Rac binding region in gp91\textsuperscript{phox} disrupted the interaction between Rac and gp91\textsuperscript{phox} (Kao et al., 2008). Amino acids Lys-421 and Tyr-425 within the Rac binding site at the C-terminal cytosolic region of gp91\textsuperscript{phox} were previously demonstrated to modulate PMA-stimulated activation of the NADPH oxidase in a heterologous COS7 model system (Kao et al., 2008). To assess the role of these amino acids in NOX2 activity in neutrophils, we transduced gp91\textsuperscript{phox}–deficient PLB-985 X-CGD cells, a human promyelocytic cell line, with a retroviral vector expressing either WT human gp91\textsuperscript{phox}, gp91\textsuperscript{phox}K421A, gp91\textsuperscript{phox}Y425A, or K421A/Y425A double mutant followed by FACS sorting for gp91\textsuperscript{phox} expression. The expression of vector-encoded gp91\textsuperscript{phox} protein was similar in all lines except for cells expressing the K421A/Y425A double mutant where expression levels were lower (Fig. 45). Consistent with the previous study using COS7 cells and with peptide inhibition studies in human neutrophils (Kao et al., 2008), PLB-985 neutrophils expressing gp91\textsuperscript{phox} derivatives with mutations in the Rac binding domain showed a marked reduction in extracellular superoxide generation in response to soluble stimuli, PMA and fMLF (Fig.
Moreover, phagosome NADPH oxidase activity elicited by either IgG opsonized latex beads or SOZ was similarly affected (Fig. 46C and D). Little if any production of superoxide by cells expressing either gp91\textsuperscript{phox} Y425A or gp91\textsuperscript{phox} K421A/Y425A was detected. However, gp91\textsuperscript{phox} Y421A supported 20-40\% of WT levels of superoxide in response to all stimuli tested except for fMLF, suggesting that amino acid tyrosine 425 may be more essential than lysine 421 for Rac-gp91\textsuperscript{phox} interaction. This result prompted us to focus on the gp91\textsuperscript{phox} Y425A mutant for subsequent experiments for the study.

**Figure 45. Expression of gp91\textsuperscript{phox} mutants in PLB-985 X-CGD cells**

The cDNA of WT human gp91\textsuperscript{phox}, K421A, Y425A, or K421A/Y425A was inserted into an MFG retroviral vector, and used to retrovirally transduce PLB-985 X-CGD to make stable cell lines. FACS sorting based on surface gp91\textsuperscript{phox} expression was done to enrich gp91\textsuperscript{phox} positive cells for each cell line.
Figure 46. Disrupted interaction between Rac and gp91<sup>phox</sup> resulted in defective NADPH oxidase activity in PLB-985 neutrophils

(A and B) Extracellular O<sub>2</sub><sup>-</sup> production stimulated by either PMA (A, n=6) or fMLF (B, n=6) in differentiated PLB-985 cell lines was measured by isoluminol-chemiluminescence assay. (C and D) Both total and intracellular O<sub>2</sub><sup>-</sup> production elicited by either phagocytosis – inducing SOZ (C, n=3) or human IgG opsonized latex beads (D, n=3) were measured by a luminol-chemiluminescence assay.
2. An intact Rac-binding domain in gp91\textsubscript{phox} was not required for assembly of the phagocyte NADPH oxidase on plasma membrane in PMA-stimulated neutrophils

To investigate the mechanism whereby Rac-gp91\textsubscript{phox} interaction regulates the activity of the phagocyte NADPH oxidase, we first examined if this physical interaction was important for membrane translocation of oxidase subunits and assembly of the phagocyte NADPH oxidase upon activation. We first examined oxidase assembly on the plasma membrane in PMA-activated cells using a cell fractionation assay. Translocation of cytosolic subunits, p47\textsubscript{phox}, p67\textsubscript{phox}, and Rac2, to the plasma membrane of gp91\textsubscript{phox} Y425A-expressing PLB-985 cells were comparable to PLB-985 WT cells (Fig. 47A and B). Additionally, consistent with previous reports, translocation of cytosolic oxidase subunits, including Rac2, was undetectable in PMA-stimulated gp91\textsubscript{phox}-deficient PLB-985 X-CGD cells (Fig. 47A and B), suggesting gp91\textsubscript{phox} may indirectly retain Rac2 on the plasma membrane. Thus, although the Y425A mutation in the Rac-binding domain of gp91\textsubscript{phox} markedly impaired PMA-activated superoxide production, it did not appear to affect the recruitment of Rac2 to the plasma membrane and assembly of the oxidase complex on the plasma membrane.
Figure 47. Assembly of NADPH oxidase on the plasma membrane did not require Rac-gp91<sub>phox</sub> interaction

(A) Representative western blots of oxidase subunits in purified cytosol of 10×10⁶ cells and membrane of 40×10⁶ cells 10 min after PMA stimulation. Rap1b was used as a membrane marker for normalization. (B) Densitometric analyses of immunoblots showing translocation of Rac2, p47<sub>phox</sub>, and p67<sub>phox</sub> to the plasma membrane (n=4).
3. Rac-gp91\textsuperscript{phox} interaction was dispensable for recruitment of p67\textsuperscript{phox} to SOZ-phagosomes but was required for efficient Rac accumulation to SOZ or IgG bead – phagosomes

We next investigated whether the Y425A mutation in the Rac-binding domain of gp91\textsuperscript{phox} would affect assembly of the oxidase complex on the phagosome membrane following ingestion of SOZ. Assessment of p67\textsuperscript{phox} translocation during phagocytosis can be effectively monitored using an YFP-tagged derivative and live cell confocal microscopy during phagocytosis. We generated WT, gp91\textsuperscript{phox} Y425A, and X-CGD cell lines expressing an YFP-tagged p67\textsuperscript{phox} (Fig. 48A). Since individual cells were assayed in this experiment, we did not FACS sort to enrich YFP-expressing cells. About 30-40\% of WT and X-CGD cells expressed p67-YFP; whereas less about 10\% of the Y425A mutant cells expressed p67-YFP (not shown), which was reflected by differences in the relative level of p67-YFP in total cell extracts (Fig. 48A). Membrane translocation of p67-YFP was examined at 3 stages during phagocytosis: formation of the phagocytic cup, cup closure, and ~200 sec post internalization. We observed normal translocation of p67-YFP to phagosome membrane at all 3 stages during phagocytosis of SOZ by both WT PLB-985 cells and those expressing gp91\textsuperscript{phox} Y425A. However, no apparent p67-YFP translocation in PLB-985 X-CGD cells was found (Fig. 48 B-D), confirming the importance of an intact flavocytochrome \(b_{558}\) for stable membrane recruitment of oxidase cytosolic subunits (Barker et al., 2009). About 50\% of phagosomes in p67-YFP-expressing WT PLB-985 cells examined showed accumulation of p67-YFP, which was also found in gp91\textsuperscript{phox} Y425A cells expressing p67-YFP (Fig. 48D). Translocation of p67\textsuperscript{phox} to the membrane is known to be dependent on its association with p47\textsuperscript{phox}.
(Nauseef, 2008) Taken together, our data suggested that the absence of Rac-gp91phox interaction does not alter p47phox and p67phox translocation and association with membrane-bound flavocytochrome\textsubscript{b558} on SOZ-phagosomes, and thus a defect in recruitment these two oxidase subunits does not account for the loss of oxidase activity in cells expressing gp91phox Y425A.

We next assessed if the interaction with gp91phox contributed to recruitment of Rac2 to phagosome membranes during phagocytosis by immunofluorescence staining for Rac. Ten min following the addition of human IgG opsonized latex beads or SOZ, Rac2 translocation to phagosomes in WT cells was detected in 76% and 55% of total phagosomes, respectively (Fig. 49). However, in X-CGD cells, only 11.5 % of total phagosomes were Rac2 positive following ingestion of either IgG beads or SOZ (Fig. 49A-C). Interestingly, in gp91phox Y425A cells, only 12 % of IgG bead phagosomes were Rac2 positive (Fig. 49A-C). In response to SOZ, 31% of phagosomes in gp91phox Y425A cells were Rac2 positive, compared to 55% for WT cells. Thus, our data demonstrated a dependence on the interaction with gp91phox for Rac2 accumulation to the phagosome membrane. More importantly, this dependence appeared to be more profound for FcγR-mediated phagocytosis.

In addition, using an antibody that detects both Rac1 and Rac2, we noticed Rac accumulation in WT, Y425A, and X-CGD cells 10 min following phagocytosis of IgG beads (Fig. 50), but Rac staining appeared relatively stronger in WT cells. Since Rac2 translocation was reduced in gp91phox Y425A and X-CGD cells (Fig. 48A-C), we speculated that this reflected normal Rac1 translocation in all three cell lines, and Rac2 is a preferred binding partner with gp91phox during FcγR-mediated phagocytosis.
studies using Rac1 or Rac 2–deficient mouse neutrophils showed impairment in O$_2^-$ production by Rac2$^{-/-}$ neutrophils in response to phorbol esters, fMLF, and IgG-opsonized particles. In contrast, Rac1$^{-/-}$ neutrophils showed normal O$_2^-$ production in response to these stimuli, supporting an indispensable role for Rac2 in regulating NADPH oxidase activity in neutrophils (Kim and Dinauer, 2001; Roberts et al., 1999; Williams et al., 2000).
Figure 48. Rac-gp91phox interaction was dispensable for recruitment of p67phox to phagosomes during phagocytosis of SOZ

(A) Expression of transgenic p67-YFP and endogenous p67phox in PLB-985 WT, PLB-985 gp91 Y425A, and PLB-985 X-CGD cell lines after retroviral transduction with MSCV-p67-YFP. (B) Translocation of p67-YFP during phagocytosis of SOZ by the 3 cell lines at three stages: cup forming, phagosome closure, and post internalization. (C) Relative YFP intensity compared to cytosol for at least 10 phagosomes in each cell line was determined at the 3 stages during phagocytosis of SOZ. *p<0.01, **P < 0.005 between X-CGD and gp91 Y425A cells. (D) Percentage of p67YFP positive phagosomes was determined by scoring YFP positive phagosomes from 4 independent experiments. A total of more than 120 SOZ phagosomes were scored for each cell line.
Figure 49. Rac2-gp91phox interaction was required for efficient recruitment of Rac2 to phagosomes

(A) Representative images of cells immunostained for Rac2 after 10 min of synchronized phagocytosis of IgG opsonized latex beads. *= Rac2 negative phagosomes. The percentage of Rac2 positive phagosomes with IgG beads (B) or SOZ (C) was determined by scoring phagosomes with higher Rac2 staining on phagosome membrane than in cytosol from 3 independent experiments. A total of more than 30 phagosomes were scored for each cell line.
Figure 50. Immunostaining for both Rac1 and Rac2 on IgG bead – phagosomes

An antibody against both Rac1 and Rac2 was used to stain for Rac accumulation on phagosomes in WT, Y425A, and X-CGD cells 10 min following phagocytosis of IgG beads. DIC = Differential Interference Contrast.
**4. Rac-gp91phox interaction was sufficient to target Rac1 to phagosome membrane in COS7 cells lacking p67phox**

To further characterize to what extent Rac-gp91phox interaction contributes to recruiting or retaining Rac on phagosome membrane, we utilized a heterologous COS7 model to examine the accumulation of Rac1 on IgG-bead phagosomes. Rac1 is the sole Rac in COS7 cells that has been shown to support oxidase activation in COS cells expressing transgenic oxidase subunits (Price et al., 2002). In PLB-985 neutrophils, the Rac-p67phox interaction remains intact and may also contribute to the recruitment of Rac to phagosomes. Therefore, we generated COS7 cell lines expressing transgenic p22phox and human FcγIIA receptor in the absence or presence of WT or Y425A gp91phox (COSFcγIIA/22, COSFcγIIA/22/WTgp91, and COSFcγIIA/22/gp91Y425A cells), which all expressed endogenous Rac1 but lacked p67phox (Fig. 51). More than 30% of total phagosomes in the positive control COSphoxFcγIIA cells, which expressed all oxidase subunits except p40phox, displayed apparent accumulation of Rac1 on phagosome membrane (Fig. 52A). In COSFcγIIA/22, recruitment of Rac1 to phagosomes was detected only in ≈5% of total phagosomes; whereas in the p67phox-deficient COSFcγIIA/22/gp91WT cells, accumulation of Rac was comparable to that seen in COSphoxFcγIIA cells (Fig. 52 A and B). This indicated that ectopic expression of flavocytochromeb558 even in the absence of p67phox in COS7 cells was sufficient to retain substantial Rac1 on the phagosome membrane. Importantly, in p67phox-deficient COSFcγIIA/22/gp91Y425A cells, where gp91phox harbored a mutation in the Rac binding domain, the percentage of Rac positive phagosomes was reduced to a low level similar to that seen in COSFcγIIA/22 cells (Fig. 52B). Together, these observations supported a role for a physical Rac-gp91phox interaction in mediating Rac1 accumulation.
to phagosomes in the heterologous COS7 cells. In the absence of Rac2 in COS7 cells, Rac1 is the default Rac the oxidase subunits interact with to support oxidase activity. A previous study showed that expression of a constitutively active Rac1 in COS$^{phox/Fc^{-βHA}}$ cells induced translocation of $p47^{phox}$ and $p67^{phox}$ to the membrane and oxidase activation (Price et al., 2002). Collectively, the data supported that the Rac-gp91$^{phox}$ interaction contributes to the retention of Rac on phagosome membrane independently of Rac-p67$^{phox}$ interaction.
Figure 51. Expression of transgenic WT or gp91phox Y425A in COS7 cell lines

Expression of transgenic gp91\textsuperscript{phox}, p67\textsuperscript{phox}, and Rac1 in COS7 cell lines. WT gp91 and gp91 Y425A expressing COS7 cell lines were made from wild-type COS7 cells expressing a transgenic FcγIIA receptor by transducing with MFG-WT or Y425A gp91. COS\textsuperscript{FcγIIA/phox} cells stably express FcγIIA receptor, gp91\textsuperscript{phox}, p67\textsuperscript{phox}, and p47\textsuperscript{phox}. Expression of endogenous Rac1 was used as a loading control. COS\textsuperscript{FcγIIA/phox} and COS\textsuperscript{FcγIIA/p22} cells were used as controls.
Figure 52. Rac-gp91phox interaction was sufficient to target Rac1 to phagosome membrane in COS7 cells lacking p67phox

(A) Representative images of COS7 cells co-immunostained for Rac1 and gp91phox 30 minutes after synchronized phagocytosis of human IgG opsonized latex beads. (B) The percentage of Rac1 positive phagosomes for each cell line was determined by scoring Rac1 positive phagosomes from 3 independent experiments. A total of about 200 phagosomes were analyzed for each cell line.
5. Rac-gp91<sup>phox</sup> interaction partially modulated electron transfer from NADPH to FAD on the phagocyte NADPH oxidase

Proposed by Bokoch and Diebold, a two-step model for Rac regulation of the NADPH oxidase activity notes that Rac participates in both steps of electron transfer on flavocytochrome <i>b</i><sub>558</sub> for generation of superoxide molecules, first from NADPH to FAD, then from reduced FAD to oxygen molecules (Bokoch and Diebold, 2002; Diebold and Bokoch, 2001). Using a cell-free fluorescent-binding assay, Diebold and Bokoch showed that the insert domain of Rac, a postulated binding site for gp91<sup>phox</sup> (Freeman et al., 1996), is required for the initial electron transfer from NADPH to FAD (Diebold and Bokoch, 2001). Subsequent work demonstrated that a peptide encompassing aa 419-430 of gp91<sup>phox</sup> inhibited cell-free NADPH oxidase activity, as measured by both cytochrome c reduction and cell-free iodonitrotetrazolium violet (INT) reduction, with the latter specifically measuring electron transfer from NADPH to FAD (Kao et al., 2008).

Therefore, we sought to examine the initial step of electron transfer in gp91<sup>phox</sup> Y425A-expressing PLB-985 granulocytes. INT reduction measured in permeabilized PLB-985 cells was substantially lower in cells expressing gp91<sup>phox</sup> Y425A than in WT PLB-985 cells (Fig. 53B), supporting a role for Rac in modulating the first step of electron transfer through its interaction with gp91<sup>phox</sup>. Additionally, although reduced, INT reduction in the mutant PLB-985 cells was still higher than in PLB-985 X-CGD cells, consistent with an independent role for p67<sup>phox</sup> in facilitating the first step of electron transfer (Diebold and Bokoch, 2001). The residual INT reduction seen in cells expressing gp91<sup>phox</sup> Y425A was in contrast with the almost complete absence of cytochrome c reduction (Fig. 53A). Cytochrome C is reduced by O<sub>2</sub><sup>-</sup>, the end product of electron flow on the phagocyte.
NADPH oxidase. This also suggests a defect of in the ability of the gp91$^{phox}$ mutant to catalyze the second step in electron transfer from reduced FAD to oxygen molecules. According to the two-step model, Rac participates in the second step of electron transfer by activating p67$^{phox}$ via its binding to the TPR region of p67$^{phox}$ (Bokoch and Diebold, 2002; Diebold and Bokoch, 2001). Therefore, the partial effect on INT reduction compared to the complete absence of cytochrome C reduction seen in PLB-985 cells expressing gp91$^{phox}$ Y425A may suggest a role for Rac-gp91$^{phox}$ binding in the second step of electron flow, possibly by stabilizing Rac to support its association with p67$^{phox}$. However, this remains to be verified.
Figure 53. Electron transfer to FAD was defective in permeabilized PLB-985 gp91 Y425A cells

(A) PMA-stimulated cytochrome C reduction of permeabilized PLB-985 cells (n=6). (B) Electron flow to flavins in PMA-stimulated permeabilized PLB-985 cells was spectrophotometrically measured by INT reduction (n=6). * p<0.05; **p<0.01.
6. RacQ61L could not overcome the loss of Rac-gp91\(^{\text{phox}}\) interaction to activate the NADPH oxidase

A D38A mutation in the Switch I effector domain of Rac ablates Rac-p67\(^{\text{phox}}\) interaction. A Q61L substitution adjacent to Switch II domain of Rac, which inhibits both intrinsic- and GTPase-activating protein (GAP)-stimulated GTP hydrolysis, generates a potent constitutively active form of Rac. Although a D38A mutation in the Switch I effector domain of Rac ablates Rac-p67\(^{\text{phox}}\) interaction and is non-functional in cell-free oxidase assays, a double D38A Q61L mutant is still active (Xu et al., 1994). Based on this, it was speculated that Q61L introduces a conformational change of Rac that enhances the association with the NADPH oxidase via a second site, which overcomes the loss of binding to p67\(^{\text{phox}}\) to support oxidase activation. To determine whether the altered conformation of RacQ61L might rescue the gp91\(^{\text{phox}}\) Y425A mutant to support oxidase activity. We first transiently transfected differentiated PLB-985 cells with Rac2 Q61L, but profound cell death within hours after transfection was observed, likely due to cytotoxicity of the constitutively active Rac mutant that could not be tolerated by neutrophils (data not shown). Therefore, we instead used COS7 cells ectopically expressing the NADPH oxidase subunits, including either WT or Y425A gp91\(^{\text{phox}}\) (Fig. 54A). Transient expression of Rac1Q61L in COS\(^{22/47/67/WT \text{gp91}}\) cells enhanced PMA-stimulated NADPH oxidase activity measured by cytochrome c reduction by \(\approx 2\) fold (Fig. 54B). On the other hand, PMA-stimulated cytochrome c reduction in COS\(^{22/47/67/gp91Y425A}\) cells with or without transfection with Rac1Q61L remained undetectable. Thus, a constitutively active Rac1Q61L was unable to bypass the requirement for an intact Rac-gp91\(^{\text{phox}}\) interaction to support oxidase activation. This
suggests that a physical interaction between Rac and gp91\textsuperscript{phox} is of fundamental importance to activating the oxidase catalytic activity.
Figure 54. Rac1Q61L was unable to overcome the requirement of Rac-gp91\textsuperscript{phox} binding for oxidase activation in COS cells

(A) Representative western blots of gp91\textsuperscript{phox}, Rac1, and p47\textsuperscript{phox} from cell lysates of COS7 cell lines. COS\textsuperscript{22/47/67} cells stably expressing human WT gp91\textsuperscript{phox} or gp91Y425A were transiently transfected with either WT Rac1, Rac1Q61A, or an empty vector (EV).

(B) PMA-stimulated cytochrome C reduction assay 24 hours after transient transfection of either WT Rac1 or Rac1Q61A (n=3).
CHAPTER FOUR: DISCUSSION

Part I: Efferocytosis induces an IL-4-dependent macrophage-iNKT cell circuit to suppress sterile inflammation

Macrophage efferocytosis of apoptotic cells induces many responses to suppress inflammation. In this study, we demonstrate a novel cellular circuit involving three innate cell types required to resolve sterile inflammation. Upon ingesting apoptotic neutrophils, macrophages produce IL-4, which induces an M2 phenotype in an autocrine fashion. Efferocytosing macrophages increase CD1d expression and activate iNKT cells that produce IL-4, and iNKT production of IL-4 can be augmented by IL-4 production from macrophages. IL-4, IL-4Ra on macrophages, and iNKT cells are each required for prompt resolution of sterile inflammation. Thus, efferocytosis of apoptotic neutrophils by macrophages initiates the activation of iNKT cells and IL-4 production that contribute to the resolution of acute sterile inflammation (Fig. 55).
Figure 55. Efferocytosis-induced macrophage-iNKT cell circuit in sterile inflammation

During tissue injury-induced sterile inflammation, ingestion of apoptotic neutrophils induces macrophages to produce IL-4, which in an autocrine manner activates an alternative phenotype in macrophages. Efferocytosing macrophages also upregulate surface expression of CD1d and become able to activate and stimulate iNKT cells to secrete cytokines such as IL-4, IL-13, and IFNγ. IL-4 from efferocytosing macrophages may augment IL-4 production in iNKT cells.
1. Efferocytosing macrophages are an early cellular source of IL-4 to alternatively activate macrophages during the acute response to tissue injury

The crucial role of M2 macrophages in resolution of inflammation, tissue repair, and homeostasis is well documented (Karp and Murray, 2012; Martinez et al., 2009). M2 macrophages display enhanced mannose receptors to scavenge tissue debris, increase fluid-phase pinocytosis and endocytosis, as well as accelerated phagosome proteolysis to repair damaged tissues (Balce et al., 2011; de Keijzer et al., 2011; Gordon and Martinez, 2010; Loke et al., 2007). The signature M2 markers, Arg1, Fizz1, and Ym1 are enzymes that promote deposition of extracellular matrix and collagen synthesis during tissue repair (Mosser and Edwards, 2008). Although alternative activation of macrophages has been largely attributed to IL-4 and IL-13 from Th2 cells, additional sources of IL-4 during acute sterile inflammation have not been investigated. Our observation of IL-4 production in efferocytosing macrophages revealed a novel mechanism of autocrine IL-4 production by macrophages to promote M2 characteristics. IL-4 production by efferocytosing macrophages might provide a mechanism to acutely skew macrophages towards the M2 phenotype to promote digestion of damaged tissue and tissue repair prior to activation of IL-4 production by other cell types. Efferocytosis of apoptotic neutrophils, and the recruitment and induction of M2 macrophages are known crucial early events necessary for wound healing. Our study establishes a link between these two events.

Induction of IL-4 production from macrophages by homeostatic stimuli such as apoptotic cells, and the resultant acquisition of M2 characteristics by these macrophages, may represent a novel mechanism to generate M2 macrophages in homeostatic responses associated with other environmental stressors. For instance, a recent study showed that
cold-stress results in alternative activation of macrophages in adipose tissue that subsequently secrete norepinephrine to drive adaptive thermogenic responses in brown adipose tissue and increase lipolysis in white adipose tissue (Nguyen et al., 2011). M2 macrophages are also important to maintain adipocyte function, insulin sensitivity and glucose tolerance, which can prevent the development of diet-induced obesity and type-2 diabetes (Lumeng et al., 2007; Odegaard et al., 2007). While the cellular sources of IL-4 in these contexts involving tissue stress are still being explored, macrophages should not be overlooked in the efforts to identify cellular sources of IL-4, since tissue stressors might induce cell apoptosis.

It should be noted that in our study we did not extensively characterize IL-4 production in cell types other than macrophages and NKT cells. We did not see mast cells or basophils in the inflamed peritoneum but saw a small fraction of eosinophils (~ 5% of total cells) in day 3 WT B6 mice. Eosinophils may be an innate cellular source as well, since eosinophils have pre-formed IL-4 in their granules (Spencer et al., 2006). Thus, even if these eosinophils show IL-4 staining by ICS, it does not mean that they are activated and releasing IL-4. In addition, from our experience, FACS staining of eosinophils for cell surface markers or intracellular cytokines frequently resulted in non-specific staining. Therefore, FACS analysis may not be the best approach to characterize cytokine production in eosinophils. To determine if these eosinophils are activated to release IL-4 and if IL-4-producing eosinophils also contribute to the resolution of inflammation in our model of sterile inflammation, we will need to isolate peritoneal exudate eosinophils and determine if IL-4 is being secreted from them by ELISA after ex vivo culture. Excessive recruitment of eosinophils is pathogenic in asthma or allergic
inflammation (Wardlaw et al., 2000), but the role of eosinophils in sterile inflammation is unclear. To evaluate the importance of eosinophils in our model of sterile inflammation, we could attempt to deplete eosinophils by using anti-CCR3 (Grimaldi et al., 1999) and assess whether that would result in alteration of the inflammation in our model.

2. IL-4 in clearance of apoptotic cells and immunologic tolerance

Our data showed that IL-4 was not required for the uptake of ACs but rather affected the activation state of macrophages, as illustrated in the reduced M2-ness of Il4−/− peritoneal exudate macrophages. In addition, downstream components of IL-4 signaling in macrophages have been illustrated to play critical roles in the clearance of apoptotic cells. The peroxisome proliferator-activated receptor-γ (PPAR-γ) is a ligand-dependent nuclear receptor that is activated by a range of synthetic and naturally occurring substances, including components of oxidized low-density lipoprotein, such as 13-hydroxyoctadecadienoic acid (13-HODE) and 15-hydroxyeicosatetraenoic acid (15-HETE) (Nagy et al., 1998). A previous study illustrated that 13-HODE and 15-HETE can be generated through 12/15-lipoxygenase (12/15-LO) that is activated by IL-4 signaling in macrophages. Therefore, IL-4 signaling in macrophages leads to increased expression of PPARγ on macrophages by directly activating 12/15-LO (Huang et al., 1999). This study also demonstrated that coordinate induction of PPARγ and 12/15-LO mediates IL-4-dependent transcription of CD36 (Fadok et al., 1998c; Huang et al., 1999), a membrane protein that binds to phosphatidylserine on apoptotic cells to facilitate the recognition and uptake of apoptotic cells (Ravichandran, 2010). In addition, a recent report showed that in mice with thioglycollate-induced peritonitis, uptake of injected apoptotic cells were exclusively found 12/15-LO-expressing M2 resident macrophages, and 12/15-LO-derived
oxidation products blocked uptake of apoptotic cells by freshly recruited inflammatory monocytes. In an experimental murine lupus model where pristane was injected intraperitoneally into mice to induce peritonitis, 12/15-LO-deficient mice displayed a lupus-like autoimmune disease, characterized by production of autoantibodies and the development of glomerulonephritis (Uderhardt et al., 2012). Il4-deficient mice are not more susceptible to experimental autoimmune encephalomyelitis (EAE), likely due to compensatory mechanisms such as redundant function of IL-13 (Liblau et al., 1997). A murine lupus model might be more appropriate for further characterization of the function of IL-4 in clearance of apoptotic cells and induction of immunologic tolerance.

3. Expression of IL-4 in macrophages

IL-4 production by macrophages has been reported in a few contexts, including LPS-stimulated bone marrow-derived macrophages, and macrophages infected with respiratory syncytial virus (RSV) or Francisella tularensis (Mukherjee et al., 2009; Pouliot et al., 2005; Shirey et al., 2008; Shirey et al., 2010). Thus, certain PAMPs might elicit IL-4 production through activation of PRRs on macrophages. Th2 cells are a major cellular source of IL-4 in allergic inflammation or parasitic infection. It has been shown that during in vitro differentiation of naïve CD4 T cells to Th2 cells, stimulation of TCR induces Ca^{2+} influx-mediated calcineurin activity and subsequent NFAT1 binding to the Il4 and Ifng promoters, resulting in small amounts of IL-4 production by naïve CD4 T cells. Under conditions of IFNγ neutralization, the low levels of IL-4 produced by naïve CD4 T cells are sufficient to initiate their own Th2 differentiation, which requires STAT6 and GATA3 (Ansel et al., 2006). Other cells known to produce IL-4 include mast cells, basophils, eosinophils, NK cells, and NKT cells (Ansel et al., 2006; Spencer et al., 2006;
Stetson et al., 2003; Weiss and Brown, 2001). These innate immune cells display high basal levels of *Il4* transcripts and can be triggered rapidly to secrete IL-4 upon stimulation: mast cells and basophils via crosslinking of IgE receptors (Weiss and Brown, 2001), and NKT cells through TCR recognition of glycolipids in the context of CD1d (Stetson et al., 2003). In eosinophils, eotaxin stimulates mobilization of pre-formed IL-4 from eosinophil granules into secretory vesicles (Spencer et al., 2006).

In our in vitro co-culture experiment, *Il4* mRNA in macrophages was increased 10-fold in the presence of apoptotic neutrophils. We also observed increased *Gata3* mRNA in efferocytosing macrophages. Given the low levels of IL-4 detected in the co-culture media, in contrast to the other types of immune cells described above, macrophages unlikely have preformed *Il4* mRNA for rapid release. Rather the *Il4* gene is likely induced in response apoptotic cells. When fixed neutrophils were used in the co-culture, we still observed production of IL-4 in macrophages, suggesting that IL-4 expression in macrophages is not due to secretory factors from neutrophils. Additionally, we noticed a reduction in IL-4 production when uptake of apoptotic cells was inhibited by cytochalasin D, implicating engulfment of apoptotic cells as an important signal to initiate expression of *Il4*. A previous study implicated IL-4 production in macrophages upon recognition of phosphatidylserine exposed on apoptotic cells. It still remains unclear how expression of IL-4 is induced in efferocytosing macrophages (Fernandez-Boyanapalli et al., 2009). Of note, efferocytosis of apoptotic cells induces Ca\(^{2+}\) influx (Gronski et al., 2009), and the NFAT family of transcription factors is expressed in macrophages as well (Hogan et al., 2003). Delineating the mechanism for induction of IL-4 may allow us to manipulate efferocytosis and monitor sterile inflammation.
4. IL-4 producing-efferocytosing macrophages are antigen-presenting cells capable of both activating and promoting a type-2 cytokine response from iNKT cells

Our data also demonstrated an important role for iNKT cells activated by efferocytosing macrophages in resolving sterile inflammation. NKT cells have regulatory functions against viral infections, and can modulate autoimmune diseases and tumor rejection (Berzofsky and Terabe, 2008; Grajewski et al., 2008). We showed that efferocytosis induced higher surface expression of CD1d on macrophages and the ability to stimulate activation of Type 1 but not Type 2 NKT cells. Additionally, the increased inflammation and cytokine profile in Ja18\(^{-/-}\) mice to an extent mirrored the observations in Il4\(^{-/-}\) mice, and collectively the data supported the importance of IL-4 in suppressing the inflammation. Although counter-regulation by Type 1 and Type 2 NKT cells has been observed (Ambrosino et al., 2007; Berzofsky and Terabe, 2009; Halder et al., 2007), the inability of Cd1d\(^{-/-}\) and Jα18\(^{-/-}\) mice to promptly resolve the inflammation supported a more essential role for type I NKT cells in resolving sterile inflammation.

Our data also demonstrated that IL-4 from efferocytosing macrophages or exogenous IL-4 added to cultures enhanced IL-4 production in NKT cells. Activation of NKT cells requires presentation of glycolipid antigens via CD1d by APC, but additional signals are likely necessary to drive Th1-like or Th2-like responses from NKT cells (Gui et al., 2001). For example, IFNγ production by α-GalCer-stimulated NKT cells is mainly dependent on IL-12 from DC (Kitamura et al., 1999). Therefore, IL-4-producing-efferocytosing macrophages may represent a type of in vivo antigen-presenting cells capable of promoting a type-2 cytokine response of iNKT cells. Interestingly, IL-4-producing iNKT cells were recently demonstrated to mediate the anti-inflammatory
effects of apoptotic cells by limiting autoreactive responses by CD1d+ B cells (Wermeling et al., 2010). Our findings suggest IL-4-producing efferocytosing macrophages may link clearance of apoptotic cells and induction of IL-4-producing iNKT cells in this setting to suppress autoimmune responses.

5. CD1d-dependent activation of iNKT cells by efferocytosing macrophages

NKT cells can be activated directly by glycolipids presented by CD1d or indirectly by cytokines such as IL-12 and IL-18 secreted from infected or LPS - treated APC (Van Kaer et al., 2011), which however were not detected when we co-cultured macrophages and AC in vitro. In contrast, CD1d expression on macrophages increased in the presence of AC and is required for iNKT cell activation. However, glycolipids involved to activate iNKT cells in this model are yet to be identified. These glycolipids could be self lipids from efferocytosing macrophages, or possibly from the ingested AC. Interestingly, digestion of AC within macrophages is known to liberate cholesterol and fatty acids from ingested cells (Chaput and Zychlinsky, 2009; Mukundan et al., 2009). Loading of glycolipids onto CD1d occurs in late endocytic compartments (Brutkiewicz et al., 2003; Roberts et al., 2002). The processing of ingested apoptotic cells involves fusion with endosomes (Kinchen et al., 2008; Kinchen and Ravichandran, 2008; Zhou and Yu, 2008), and processed glycolipids from ingested neutrophils may have access to CD1d in late endocytic compartments. Identification of the glycolipids presented by efferocytosing macrophages to activate iNKT cells will be the subject of future studies.
6. The efferocytosing macrophage–iNKT cell circuit as a novel mechanism to resolve sterile inflammation

The activation of iNKT cells by efferocytosing macrophages to produce IL-4 may be one of the suppressive components of AC clearance in the resolution of inflammation induced by tissue injury. The clearance of ACs is not as “silent” as once viewed; rather, it elicits various immune responses. For example, dendritic cells that have engulfed apoptotic tumor cells or virus-infected cells can cross-present antigens from apoptotic cells to cytotoxic T cells (Albert et al., 1998; Bonmort et al., 2008). Infected apoptotic cells were also demonstrated to direct Th17 differentiation by inducing IL-6 production by dendritic cells (Torchinsky et al., 2009). Our findings also raise some additional questions. One is whether infected apoptotic cells are able to induce IL-4 production in macrophages. The study described above showed the production of the proinflammatory cytokine IL-6 after dendritic cells engulfed infected apoptotic cells. Conversely, infected apoptotic cells in the cytosol of macrophages may trigger activation of cytosolic PRRs such as cytosolic TLRs and NLRs, which may results in a more proinflammatory phenotype in these macrophages. As a result, the altered activation state may impact the ability of these efferocytosing macrophages to activate NKT cells. Likewise, if NKT cells are still activated, their cytokine response may as well be influenced by the altered phenotype of efferocytosing macrophages. Therefore, a major question is whether the macrophage-iNKT cell circuit we defined in our model of sterile inflammation can be translated to an inflammatory setting where the apoptotic cells are infected.

In a potentially parallel mechanism to our work, IL-4- producing iNKT cells were recently demonstrated to mediate the anti-inflammatory effects of apoptotic cells by
limiting production of autoantibodies by CD1d+ B cells (Wermeling et al., 2010). Therefore, our demonstration of a link between iNKT cells and efferocytosis by macrophages reveals a possible mechanism for iNKT cell activation to suppress autoreactivity that could otherwise be induced if apoptotic cells underwent necrosis and released DNA content into the microenvironment.

In addition, the immunoregulatory role of iNKT cells has been reported to involve recruitment of other immune cells (Naumov et al., 2001). The activated iNKT cells in our model may also dampen inflammation by either inducing or repressing chemokines for other immune cells. To address this, we could compare chemokines in WT and Cdl1d−/− or Jα18−/− mice after the induction of peritonitis. The chemokines in X-CGD mice could be examined as well, since our data revealed impaired activation of NKT cells in these mice. It is noteworthy that discoid lupus has been reported in X-CGD patients and carriers (De Ravin et al., 2008). Up to date, very little is known about the iNKT cell function in X-CGD patients or mice. Thus, modulating iNKT cell function from the perspective of macrophage efferocytosis could be explored further to address the inflammation and possible enhanced autoimmune response arising in patients with CGD and systemic lupus erythematosus.
Part II: Efferocytosis activates the phagocyte NADPH oxidase in macrophages

The phagocyte NADPH oxidase is activated in complement receptor or FcRγ mediated – phagocytosis of microbes and participates in the anti-microbial function of phagocytes by generating microbicidal ROS. Here, our study demonstrated activation of the phagocyte NADPH oxidase in macrophages during efferocytosis of apoptotic neutrophils, which required complement receptor 3 (CR3, or CD11b/CD18). The TLR4/Myd88 pathway appeared to provide a co-stimulatory signal to synergize with CR3 to activate the phagocyte NADPH oxidase. Notably, we showed iC3b, a well-known ligand for CR3, was expressed on apoptotic neutrophils. In addition, degradation of apoptotic neutrophils in oxidase-deficient X-CGD macrophages was delayed, suggesting that oxidase-derived ROS may be involved in the processing of apoptotic neutrophils (Fig. 56).
Figure 56. Activation of the phagocyte NADPH oxidase in efferocytosing macrophages

CR3-mediated efferocytosis of an apoptotic neutrophils leads to activation of the phagocyte NADPH oxidase and generation of ROS in the efferosome. TLR4 is also activated during efferocytosis and leads to MyD88 signaling, which acts in concert with CR3-mediated signaling to activate the phagocyte NADPH oxidase.
1. CR3 in efferocytosis and activation of the phagocyte NADPH oxidase

Apoptotic cells opsonized with iC3b activate both the classical and alternative complement pathways (Fishelson et al., 2001; Mevorach et al., 1998). The importance of complement-mediated clearance of apoptotic cells is illustrated by development of systemic lupus erythematosus in >90% of homozygous patients with C1q deficiency, in 75% of homozygous patients with C4 deficiency (Walport et al., 1998). Our data showed that Cd11b−/− macrophages exhibited normal ROS production in response to zymosan, but a severely impaired ROS response to apoptotic cells. Zymosan, prepared from fungal cell wall, binds to dectin 1 and TLR2 (Underhill and Goodridge, 2012). Uptake of apoptotic cells by Cd11b−/− macrophages was intact. Thus, the specific defect in the activity of the phagocyte NADPH oxidase in response to apoptotic cells highlighted the importance of CR3 in linking efferocytosis with activation of the phagocyte NADPH oxidase.

CR3 facilitates efferocytosis by human macrophages (Mevorach et al., 1998). Activation of the GTPase Rap1 regulates activation of CR3 in macrophages in response to various soluble stimuli and particles (Caron et al., 2000), and phosphorylation of Syk, an early event during integrin signaling, is critical for coupling integrin ligation and NADPH oxidase in neutrophils. CR3 is evenly distributed on the plasma membrane of resting macrophage, and increased lateral mobility of activated CR3 in the plasma membrane facilitates its accumulation to the phagocytic cup during phagocytosis (Ross et al., 1992; Vachon et al., 2007). Therefore, examining the accumulation of CR3 to the efferocytic cup by confocal microscopy or detecting the activation of Rap1 and Syk in our system of efferocytosis will provide additional evidence of the engagement of CR3 in efferocytosis and activation of the phagocyte NADPH oxidase.
iC3b is a proteolytically inactive product of the complement cleavage fragment C3b. A previous study showed that iC3b-opsonized apoptotic cells mediate a distinct anti-inflammatory response characterized by decreased IL-1 and increased IL-10 production in macrophages (Amarilyo et al., 2010). Complement proteins are mainly produced by hepatocytes and released into the circulation upon activation. Human monocytes were shown to produce complement proteins (Perlmutter and Colten, 1986). However, it is unclear whether other cell types have an intact complement system to intrinsically produce opsonins. We did not observe surface expression of iC3b on freshly isolated human neutrophils, but iC3b was found on apoptotic neutrophils that were aged overnight ex vivo, even in the absence of serum in the culture media. Thus, our preliminary data suggested that apoptotic neutrophils might have an intrinsic complement system and be able to process C3b to iC3b. We will need to further confirm the expression of C3b or iC3b in apoptotic cells by qPCR or immunoblotting. Our observation of iC3b on apoptotic neutrophils also raised the question of how the complement system in neutrophils is activated as they age ex vivo. One possibility is that during isolation of human neutrophils, material in the reagents or media might activate the complement system. Alternatively, neutrophils may possess an intrinsic mechanism to activate the complement system as they undergo apoptosis. This will be a subject of future investigation. Importantly, it is yet to be determined whether iC3b on apoptotic neutrophils is the ligand that activates CR3 on macrophages to trigger efferocytosis and activation of the phagocyte NADPH oxidase.
2. The phagocyte NADPH oxidase in efferocytosis and processing of ingested apoptotic cells

The phagocyte NADPH oxidase is a key component of the anti-microbial function of phagocytic leukocytes in response to infection. Initial ROS generation by the phagocyte NADPH oxidase is critical to prevent escape of the pathogen from the phagosome, as seen in the infection with *Listeria monocytogenes* (Wu et al., 2009). In TLR2/4 or FcγR mediated phagocytosis, the autophagy component LC3 is recruited to phagosomes in an oxidase dependent manner. ROS derived from the phagocyte NADPH oxidase promote autophagosome formation by facilitating the accumulation of lipidated LC3 (LC3 II) to the phagosome, resulting in accelerated fusion of the autophagosome with lysosomes and faster degradation of the ingested microbe (Huang and Brumell, 2009a; Huang et al., 2009). Here, we have demonstrated that macrophage NADPH oxidase is activated in response to apoptotic neutrophils, in the absence of microbial stimuli. More work will be required to define the function of oxidase-derived ROS in this setting. Our preliminary data showed slower degradation of apoptotic neutrophils in the gp91phox-deficient X-CGD macrophages, suggesting the oxidase-derived ROS may participate in the processing and degradation of apoptotic neutrophils. It is not known whether autophagy is involved in efferocytosis. Since our data suggested that efferocytosis activated TLR4/MyD88 signaling, if autophagy is engaged, oxidase-derived ROS might promote the assembly of the autophagy complex on the efferosome to accelerate fusion with lysosomes. Notably, we observed decreased efferosome-lysosome fusion in X-CGD macrophages by examining the recruitment of the lysosomal marker Lamp1 to the efferosome.
Surface molecules on apoptotic cells such as HMGB1 can be recognized by TLR4. TLR signaling upregulates phosphorylated p38 MAP kinase, which accelerates Rab5 recycling by phosphorylating Rab-associated GDIs (Blander and Medzhitov, 2004, 2006), thus likely expediting the delivery of the complex of the phagocyte NADPH oxidase from endosomes to efferosomes. Phosphorylation of p38 MAP kinase has been shown to lead to phosphorylation of p47phox (Yvan-Charvet et al., 2010). Of note, HMGB1 was demonstrated to be an agonist of the phagocyte NADPH oxidase (Berger et al., 2010). Thus, the TLR4/MyD88 pathway may serve as a co-stimulatory signal in concert with CR3 to activate the phagocyte NADPH oxidase during efferocytosis.
Part III: The efferocytosis-induced IL-4-dependent macrophage-iNKT cell circuit is defective in gp91<sup>phox</sup>-deficient mice with CGD

Defects in the resolution of inflammatory responses in the absence of NADPH oxidase-generated oxidants have been implicated in CGD patients and mice (Dinauer, 2005; Schappi et al., 2008b). In our study, elevated numbers of neutrophils in X-CGD mice led to a prolonged increase in the number of macrophages, including those ingesting neutrophils and producing IL-4 and IL-13. The presence of these cytokine-producing macrophages is insufficient to promote resolution of inflammation in these mice, and their prolonged presence may in fact contribute to sustained inflammation, as seen in chronic Th2-dependent airway inflammation (Bosnjak et al., 2011). We also showed defective activation of iNKT cells in X-CGD mice during sterile inflammation, and delineated two contributory factors; an impairment in CD1d-dependent antigen presentation by X-CGD macrophages, and an intrinsic defect in cytokine production from X-CGD NKT cells (Fig. 57).
Figure 57. Impaired efferocytizing macrophage-iNKT cell circuit in mice with X-CGD

X-CGD macrophages are gp91phox-deficient and unable to generate ROS in the efferosome in response to efferocytosis of an apoptotic neutrophil. However, they are able to produce even more IL-4 than WT macrophages, and IL-4 signaling contributes to giant cell formation in X-CGD macrophages. During sterile inflammation, X-CGD macrophages display reduced surface CD1d and are defective in activating iNKT cells. As a result, the cytokine response by X-CGD iNKT cells is skewed and is characterized by increased IFNγ but minimal IL-4 and IL-13 production.
1. Exaggerated acute infiltration of neutrophils into the inflamed peritoneum of X-CGD mice

The exaggerated infiltration of neutrophils into the X-CGD peritoneum in response to sodium periodate is in line with other reports using different sterile stimuli to induce peritonitis in X-CGD mice (Fernandez-Boyanapalli et al., 2010; Jackson et al., 1995; Pollock et al., 1995). Our data also illustrated elevated mobilization of neutrophils shortly in X-CGD mice 4h after challenge with periodate. However, the cause of increased acute neutrophil infiltration in X-CGD mice remains poorly understood. Peritoneal resident macrophages are among the first immune cells that detect danger signals resulting from periodate-induced tissue injury and stress in the peritoneum. Thus, it would be informative to examine the acute response of peritoneal resident macrophages in X-CGD mice within hours after injection of periodate. Danger signals released from damaged cells in the peritoneum, such as ATP, DNA, and HMGB1, are sensed by PRRs on resident macrophages to elicit inflammatory responses to recruit immune cells (Rock et al., 2010). NLRP3-mediated inflammasome response may be one of the mechanisms for resident macrophages to recruit immune cells, since some of the danger signals (e.g. ATP) can be recognized by NLRP3. The role of NLRP3 in sensing danger signals during sterile inflammations has been established by several studies (Chen and Nunez, 2010; Franchi et al., 2012; Iyer et al., 2009). NLRP3-mediated inflammasome response in macrophages leads to release of mature IL-1β, a proinflammatory cytokine known to upregulate adhesion molecules on the endothelium to facilitate recruitment of neutrophils (Franchi et al., 2012). Interestingly, monocytes from CGD patients have been reported to display an elevated inflammasome response and caspase-1 activity in response to danger
signals that target NLRP3 (Meissner et al., 2010; van de Veerdonk et al., 2010), implicating a possible role for the oxidase-derived ROS in tuning down inflammasome responses or an altered activation state of CGD monocytes.

In addition, from Part II, we found higher basal levels of G-CSF in X-CGD macrophages than WT cells, which were at even higher levels after co-culture with apoptotic neutrophils. G-CSF can be released by tissue macrophages in response to infection or tissue stress to promote emergency granulopoiesis in the bone marrow and recruitment of neutrophils to the infection or inflammation site (Furze and Rankin, 2008; Rankin, 2010). Although it remains to be determined why X-CGD macrophages show higher basal levels of G-CSF, this may be a contributory factor for the increased acute release of neutrophils from the bone marrow in X-CGD mice and elevated infiltration into the peritoneum.

2. Increased IL-4 production by X-CGD efferocytosing macrophages and giant cell formation in X-CGD macrophages

Our results showed X-CGD macrophages were able to ingest apoptotic cells and produce IL-4 efficiently, reflecting that the phagocyte NADPH oxidase is not required for these two processes. Furthermore, our in vitro culture experiment revealed higher basal levels of IL-4 in X-CGD macrophages, and significantly increased IL-4 in the presence of apoptotic cells in X-CGD macrophages compared to controls. Importantly, our data suggested that the increased production of IL-4 by X-CGD efferocytosing macrophages in vitro might be the culprit for giant cell formation in X-CGD macrophages, and provides a mechanistic explanation for the giant cells frequently observed in granulomas from X-CGD patients, a hallmark of CGD.
More work is required to understand why X-CGD efferocytosing macrophages produce more IL-4. Data from Part II revealed slower degradation of ingested apoptotic cells by X-CGD macrophages, perhaps due to the absence of oxidase-derived ROS in efferosomes. We postulate that the prolonged time required to digest apoptotic neutrophils in X-CGD macrophages may sustain the signal for IL-4 expression in X-CGD macrophages. On the other hand, even though IL-4 detected in X-CGD efferocytosing macrophages was 2-fold higher than the WT control, the amount of IL-4 was still minuscule. However, the phenotype of giant cells was striking. This either suggests autocrine signaling of IL-4 as commented in the discussion of Part I, or hyper-sensivity of X-CGD macrophages to IL-4 due to the absence of ROS. Additional in vitro experiments can be carried out to address whether X-CGD macrophages are hyper-sensitive to IL-4, possibly by examining the downstream components, such as phosphorylation of STAT6 and proteins known to be induced by IL-4, such as IL-4Rα, PPARγ, CD36, 12/15-LO. Enlarged granulomas in CGD patients may obstruct blood flow and lead to more complications. Deciphering how or whether IL-4 contributes to giant cell formation by X-CGD efferocytosing macrophages will provide insights into the development of therapeutic approaches to control granulomatous inflammation in CGD patients.

3. The phagocyte NADPH oxidase in processing of apoptotic cells and the macrophage-iNKT cell circuit

ROS derived from the phagocyte NADPH oxidase are linked to phagosome maturation and processing of ingested particles. Cross-presentation via MHC class I by dendritic cells was influenced by derived from the phagocyte NADPH oxidase
(Mantegazza et al., 2008; Savina et al., 2009). Conversely, oxidase-derived ROS may modulate the processing of ingested apoptotic cells in macrophages and subsequent interaction with iNKT cells. Two studies demonstrated that oxidized or native fatty acdis and oxysterols released from ingested apoptotic cells respectively activate PPARδ and LXRα and β, which in turn initiate or repress transcription of target genes to facilitate AC clearance and tolerogenic responses in macrophages. Both pparδ−/− and Lxr double knockout mice showed defective clearance of apoptotic cells, production of autoantibodies, and manifestation of lupus-like autoimmunity (Bensinger and Tontonoz, 2008; Chawla et al., 2001). Thus, oxidation of lipids from apoptotic cells appears to be essential for the immunosuppressive effect of apoptotic cells. ROS generated by derived from the phagocyte NADPH oxidase in efferocytosing macrophages might be involved in modifying the glycolipids presented by macrophages to activate iNKT cells in this model.

The reduced CD1d expression on X-CGD peritoneal exudate macrophages is puzzling. Recycling between the plasma membrane and endosomal compartments is an essential component of CD1d-mediated presentation of lipids to activate NKT cells. CD1d access to endosomal compartments is regulated by two independent pathways: first, by an intrinsic tyrosine-based motif, which governs recycling between the plasma membrane and the endosome; second, by the invariant chain, with which CD1d associates in the endoplasmic reticulum (ER) (Jayawardena-Wolf et al., 2001). Association of proteins in subcellular compartments such as the endosome or ER can be redox-sensitive. There are reports of the phagocyte NADPH oxidase recruited to the endosomes and generating H2O2 that influences the interactions of proteins in the endosomes (Ushio-Fukai, 2006). For example, ROS generated by derived from the
phagocyte NADPH oxidase alter the formation of an active IL-1R complex in the endosomal compartment by directing the H$_2$O$_2$-dependent binding of TRAF6 to the IL-1R1/MyD88 complex (Li et al., 2006). Conversely, trafficking of CD1d from the endosome after lipid loading might be influenced by the redox state in the endosomal compartment, and the absence of a function NADPH oxidase and ROS in X-CGD macrophages may attenuate the trafficking of lipid-loaded CD1d to the plasma membrane. This however will require an extensive study to address.

Although our in vitro NKT activation assay showed impaired activation of iNKT hybridoma cells by day 4 X-CGD peritoneal exudate macrophages, likely partly attributable to the reduced expression of CD1d on these X-CGD macrophages, the X-CGD iNKT cells exhibited skewed rather than an overall diminished cytokine response, as characterized by increased IFN$\gamma$ but reduced IL-4 production. It is unclear whether the skewed cytokine response in X-CGD NKT cells is due to an inherent defect with X-CGD NKT cells, or if it is associated with an altered activation state of X-CGD macrophages.

The intrinsic defect we observed in X-CGD NKT cells is intriguing and implicates possible engagement of the phagocyte NADPH oxidase during activation of NKT cells. Of note, activation of the phagocyte NADPH oxidase was observed after T cell receptor stimulation (Jackson et al., 2004), and dysregulated T cell responses have been reported in X-CGD mice (Romani et al., 2008; Sareila et al., 2011). The difficulty to resolve inflammation in the absence of infection is a hallmark of CGD. Therefore, defective activation of NKT cells during sterile inflammation might contribute to the pathology of CGD.
Part IV: A Rac-gp91\textsuperscript{phox} interaction is important for electron transfer and assembly of the phagocyte NADPH oxidase on phagosome membrane

Rac GTPase is a crucial regulator of the activity of the phagocyte NADPH oxidase. For years it was thought to regulate the activation of the NADPH oxidase solely by binding and activating p67\textsuperscript{phox}. Our study has delineated two mechanisms by which a physical interaction between Rac and gp91\textsuperscript{phox} modulates the activity of the phagocyte NADPH oxidase in neutrophils: it facilitates the first step of electron transfer on the phagocyte NADPH oxidase to generate $O_2^-$ and it is required to retain Rac on the phagosome membrane for sustained phagosome $O_2^-$ production. Disruption of Rac-gp91\textsuperscript{phox} interaction resulted in a nearly complete loss of $O_2^-$ production by neutrophils in response to both soluble and particulate stimuli due to impaired electron flow from cytosolic NADPH to FAD bound on flavocytochrome$b_{558}$, consistent with a two-step model previously proposed by Diebold and Bokoch. Our data additionally demonstrated that a physical interaction with gp91\textsuperscript{phox} is important to recruit and retain Rac2 on phagosome membrane.

1. Differential mechanisms to recruit and retain Rac2 on the plasma membrane and phagosome membrane

While the Y425A mutation in the Rac binding site in gp91\textsuperscript{phox} almost completely abolished $O_2^-$ production in response to both soluble and phagocytosis-inducing particles, and Rac translocation to the plasma membrane was unaffected, its accumulation to phagosome membrane was drastically reduced in cells expressing gp91\textsuperscript{phox} Y425A. Thus, our data suggest differential mechanisms governing translocation of Rac to the plasma membrane and phagosome, with the latter seemingly relying more on its interaction with
gp91phox. Studies using Rac1 or Rac2-deficient mouse neutrophils showed that Rac2−/− neutrophils were impaired in O2− production in response to phorbol esters, fMLF, and IgG-opsonized particles; whereas Rac1−/− neutrophils showed no defect in O2− production in response to these stimuli, supporting an indispensible role for Rac2 in regulating NADPH oxidase activity in neutrophils. Rac2 has a higher affinity than Rac1 for p67phox in yeast two-hybrid studies, and Rac2 is more active than Rac1 in cell-free NADPH oxidase assays using neutrophil cytosol. The distinct functions associated with each Rac are attributable to the different number of basic amino acids in their c-termini (Yamauchi et al., 2005) and different GEFs they associate with during phagocytosis (Hall et al., 2006; Price et al., 2002).

Consistent with previous reports, we observed reduced Rac2 translocation to the plasma membrane in PMA-stimulated PLB-985 X-CGD differentiated neutrophils, reflecting that an intact NADPH oxidase on the plasma membrane is required to recruit Rac2 to the plasma membrane. In differentiated PLB-985 neutrophils expressing gp91phox Y425A mutant, Rac2 translocation to the plasma membrane in response to PMA was unaffected by the loss of interaction with gp91phox due to the Y425A mutation. We also saw normal membrane translocation of p67phox and p47phox in these cells. It is possible that interaction with p67phox is sufficient to recruit and retain Rac2 on the plasma membrane. However, these cells still displayed a severe impairment in O2− production in response to PMA stimulation, thus highlighting the essential role for a physical interaction between Rac and gp91phox to allow electron transfer on the NADPH oxidase to generate O2−.

Both immunofluorescence for Rac2 in differentiated PLB-985 neutrophils and for Rac1 in reconstituted COS7 cells demonstrated the importance of a physical interaction
between Rac and gp91\textsuperscript{phox} in retaining Rac at the phagosome membrane, which was more profound in Fc\textgamma R-mediated phagocytosis. In non-hematopoietic cells, such as the kidney COS7 cells, Rac2 is not expressed, but Rac1 in these cells operates with non-hematopoietic NOX family members such as NOX1 and NOX3 to support NOX activity and O\textsubscript{2}\textsuperscript{\textsuperscript{-}} production (Miyano and Sumimoto, 2007). The assembly and activation of the NADPH oxidase is differentially regulated on the plasma membrane and on the phagosome. This is supported by studies showing that during phagocytosis, the phox-homolog (PX) domain of p40\textsuperscript{phox} facilitates its association with phosphatidylinositol 3-phosphate (PI3P) that is required to retain p40\textsuperscript{phox} on phagosome membrane and activate the phagocyte NADPH oxidase (Bravo et al., 2001; Ellson et al., 2001; Suh et al., 2006). A study in mouse neutrophils expressing p40\textsuperscript{phox} bearing an R58A mutation, which prevents p40\textsuperscript{phox} R58A from binding to PI3P, showed normal extracellular ROS production but defective intracellular ROS response (Ellson et al., 2006a). Premature loss of p40\textsuperscript{phox} from phagosomes was reported in a CGD patient bearing a p40\textsuperscript{phox} with deleted PX domain (Matute et al., 2009). Thus, p40\textsuperscript{phox} relies more on its association with PI3P to remain associated with the phagosome membrane. Conversely, different mechanisms likely dictate how Rac2 is associated with the plasma membrane and phagosome membrane. Association with a membrane-bound protein like gp91\textsuperscript{phox} rather than with p67\textsuperscript{phox}, a cytosolic protein, may contribute more to the retention of Rac2 on phagosome membrane in neutrophils. A previous study reported that Rac binds to p67\textsuperscript{phox} with a low affinity in vitro (Diekmann et al., 1994).
2. Rac-gp91<sub>phox</sub> interaction serves as an additional checkpoint before initiation of electron flow on the NADPH oxidase

Our observation of impaired electron transfer from NADPH to FAD bound on flavocytochrome<sub>b<sub>558</sub></sub> in PLB-985 gp91<sub>phox</sub> Y425A cells confirms that Rac2 acts as an active player in the catalytic function of the oxidase rather than the previously postulated adaptor role. Independent regulation of the first electron transfer on flavocytochrome<sub>b<sub>558</sub></sub> by Rac2 and p67<sub>phox</sub> in neutrophils, with the former accomplished through its interaction with gp91<sub>phox</sub>, may represent two checkpoints to tightly control electron flow to ensure robust oxidation of NADPH occurs only when both requirements are met. Our observations suggest translocation of p67<sub>phox</sub> and Rac to phagosomes can occur independently of each other. Hypothetically, if electron flow from NADPH to FAD were unilaterally controlled by p67<sub>phox</sub>, the reduced form of FAD, FADH<sub>2</sub>, could build up in the cell before Rac2 is recruited to unfold the “activation domain” of p67<sub>phox</sub> (Han et al., 1998) for subsequent electron flow from FADH<sub>2</sub> through hemes on flavocytochrome<sub>b<sub>558</sub></sub> to an oxygen molecule (Nisimoto et al., 1999). Expectedly, untimely accumulation of the unstable FADH<sub>2</sub> could create oxidative stress in the cell. Therefore, the parallel checkpoints imposed for the first step of electron flow might represent a well-coordinated mechanism to ensure that the first step of electron flow is only initiated when the oxidase is properly equipped to execute the second step of electron flow immediately.

In an X-CGD patient whose flavocytochrome<sub>b<sub>558</sub></sub> was defective in transferring electrons from FAD to oxygen molecules due to a mutation in gp91<sub>phox</sub>, normal FAD reduction as confirmed by INT reduction was detected. Importantly, high levels of intracellular oxidants were found in the patient’s neutrophils, which expectedly was due
to the buildup of reduced intracellular FAD (Cross et al., 1995). It is unclear how deleterious high levels of reduced FAD, even transiently, would be for normal cellular functions. During phagocytosis of microbial particles, oxidase-derived ROS are formed either in the extracellular space or the lumen of the phagosome so as to avoid oxidative damage to the cell. Oxidative stress has been commonly seen resulting from oxidative phosphorylation when the electron flow on the mitochondrial oxidase is uncoupled. As for the study of the X-CGD patient mentioned above, much of the study focus was placed on the generation of superoxide by the NADPH oxidase by the neutrophils, as this process is more relevant to the microbicidal ability of neutrophils. However, unwanted aggregation of intracellular oxidants in this patient’s neutrophils may compromise other functions of neutrophils as well. Hence, future studies are warranted to address how transient accumulation of reduced FAD due to disrupted electron flow from reduced FAD to oxygen on flavocytochrome $b_{558}$ may impact the function of neutrophils.
CHAPTER FIVE: FUTURE DIRECTIONS

Part I: Efferocytosis induces an IL-4-dependent macrophage-iNKT cell circuit to suppress sterile inflammation

1. To further delineate the contribution of IL-4 from macrophages to the resolution of sterile inflammation using macrophage-specific IL-4-deficient mice

   Although our experiments with *Il4r<sup>fl/fl</sup>*<sup>LysM<sup>Cre</sup></sup> mice demonstrated the importance of IL-4 signaling to macrophages and possibly other myeloid cells in suppressing the inflammation in our model of sterile inflammation, it however did not directly address the contribution of IL-4 from macrophages. The inability of adoptively transferred WT splenic NKT cells to rescue *Il4<sup>-/-</sup>* mice suggested IL-4-producing macrophages are indispensable, but multiple factors likely contribute to the unsuccessful rescue. The low levels of IL-4 and IL-13 from ex vivo stimulated CD4 T cells recovered from the *Il4<sup>-/-</sup>* recipient mice indicated that the transferred WT NKT cells were not properly activated by in vivo *Il4<sup>-/-</sup>* macrophages in the *Il4<sup>-/-</sup>* recipient mice. Therefore, a more direct approach to delineate the contribution of IL-4 from macrophages to the resolution of sterile inflammation would be utilizing macrophage-specific IL-4-deficient mice. These mice however are not available. To generate these mice, we will need to generate and cross *Il4<sup>fl/fl</sup>* mice with *LysM<sup>Cre</sup>* mice.

   When *Il4<sup>fl/fl</sup>*<sup>LysM<sup>Cre</sup></sup> mice become available, we will induce peritonitis in the mice and littermates to monitor the severity of inflammation, as well as characterize the M2 phenotype of macrophages and cytokine response of NKT cells from these mice. Based on our results, we anticipate more severe inflammation in these mice, and a reduced M2
phenotype in macrophages and an impaired type-2 cytokine response in NKT cells from these mice.

2. To identify glycolipids involved in the activation of iNKT cells by efferocytosing macrophages

We have demonstrated a CD1d-dependent activation of iNKT cells by efferocytosing macrophages, which raises the question as to what glycolipids are being presented to iNKT cells. To address this, we will first need to determine the cellular origin of these lipids, since ingested apoptotic cells in efferocytosing macrophages may liberate lipids into the cytosol of macrophages (Mukundan et al., 2009). Sonicates from fresh neutrophils, apoptotic neutrophils, efferocytosing macrophages, and control macrophages can be prepared (Kinjo et al., 2006) and added separately to co-cultures of iNKT hybridoma+LMTK cells expressing CD1d, and measure IL-2 production by the iNKT hybridoma cells. The sonicates will be added to co-culture of iNKT hybridoma + LMTK cells without CD1d, as negative controls to confirm that any increase in IL-2 production by iNKT cells is CD1d-dependent. Sonicates displaying the ability to activate the iNKT cells will indicate the cellular origin of lipids involved in activating iNKT cells. Then crude lipid extracts from these cells (apoptotic neutrophils, efferocytosing macrophages, or macrophages) can be prepared, and lipids are fractionated as described previously and analyzed by electrospray mass spectrometry (ESMS), nuclear magnetic resonance (NMR), and by gas chromatography-mass spectrometry (GCMS) (Kinjo et al., 2006). A collaborative effort with a biochemist will be needed to complete the biochemical analyses.
Part II: Efferocytosis activates the phagocyte NADPH oxidase in macrophages

1. To further characterize CR3-mediated efferocytosis and activation of the phagocyte NADPH oxidase

We plan to perform antibody blocking assays to further confirm the role of CR3 (CD11b/CD18) in efferocytosis-induced activation of the phagocyte NADPH oxidase. Prior to luminol assay to measure apoptotic cell-stimulated $O_2^-$ production, macrophages will be treated with anti-CD11b. Reduced $O_2^-$ production after blocking CD11b will provide additional evidence that CR3 is required to activate the phagocyte NADPH oxidase in efferocytosing macrophages. Serum-opsonized zymosan, which binds to CR3 to activate the NADPH oxidase, can be used as a control to confirm the efficiency of blocking.

Additionally, prior to the luminol assay, apoptotic cells can be incubated with autologous human serum to enhance surface opsonins. If these apoptotic cells induce higher levels of $O_2^-$ production, it suggests CR3 or CR4 (CD11c/CD18) is linked to the activation of the phagocyte NADPH oxidase. Alternatively, $Cd18^{-/-}$ mice are available and macrophages from these mice will be useful to elucidate the importance of complement receptors in the activation of the phagocyte NADPH oxidase in efferocytosing macrophages.

To prove whether $iC3b$ on apoptotic neutrophils is the ligand that binds and activates CR3 on macrophages, anti-$iC3b$ can be used to block $iC3b$ on apoptotic neutrophils prior to co-culture with macrophages. $O_2^-$ production measured by a luminol assay will indicate whether blocking $iC3b$ on apoptotic neutrophils results in a reduction in the activity of the phagocyte NADPH oxidase.
A separate effort is to study whether neutrophils have an intrinsic complement system, and whether the complement system is activated and results in the deposition of iC3b on their cell surface as they undergo apoptosis. This aspect of investigation is currently carried out in our collaborator, Dr. John Atkinson’s laboratory (Washington University, St. Louis, MO).

2. To define the function of ROS generated by the phagocyte NADPH oxidase in efferosome maturation and processing of apoptotic cells

We already demonstrated slower degradation of apoptotic neutrophils in X-CGD macrophages by diaminobenzidine histochemistry and immunoblotting for MPO. To additionally show that it is not an altered activation state but rather the absence of ROS in X-CGD macrophages that accounts for the delayed degradation in X-CGD macrophages, we will add an NOX2 inhibitor, such as diphenyleneiodonium (DPI), to WT efferocytosing macrophages to determine whether degradation of apoptotic neutrophils is attenuated when ROS are abolished. Alternatively, small amounts of H$_2$O$_2$ could be added to X-CGD efferocytosing macrophages to see if it would improve degradation of apoptotic neutrophils in these macrophages.

Additionally, we are interested to study the mechanisms by which NOX2-derived ROS promote faster efferosome-lysosome fusion. One possibility is by facilitating the assembly of the autophagy complex on efferosomes. A first step to test this is to determine whether the autophagy pathway is involved in efferocytosis. As an early event of autophagy activation in murine macrophages, LC3 is cleaved by Atg4 at its C-terminus to become LC3-I, which is rapidly conjugated with phosphatidylethanolamine (PE) to form the membrane-associated form LC3-II (Ravikumar et al., 2009). Therefore, to determine
if the autophagic response is associated with efferocytosis, we could initially assess the localization of LC3-II to efferosome by confocal microscopy. We could also detect whether there is increased LC3-II in PEMs with ingested ACs by immunoblotting to compare the expression levels of LC3-I and LC3-II in cell lysates of WT PEMs alone and WT PEMs treated with apoptotic neutrophils. After confirming autophagy activation during efferocytosis, we could compare the rate at with LC3-II is recruited to efferosomes between WT and X-CGD efferocytosing macrophages. A slower rate in X-CGD macrophages would suggest ROS derived from the NADPH oxidase might promote efferosome-lysosome fusion by recruiting the autophagy complex to the efferosome.
Part III: The efferocytosis-induced IL-4-dependent macrophage-iNKT cell circuit is defective in CGD mice

1. To characterize NKT cell function in CGD mice and CGD patients

To further confirm whether impaired NKT cell function contributes the chronic inflammation seen in CGD patients or mice, we would like to more thoroughly characterize NKT cell function in CGD mice. First, αGalCer, which induces rapid release of both IL-4 and IFNγ by NKT cells in mice within a few hours after IV injection, can be injected in WT and X-CGD mice and IL-4 and IFNγ in serum can be measured 4 h after injection (Smiley et al., 1997). In addition, splenic and liver NKT cells can be isolated from WT and X-CGD naïve mice can be compared for their cytokine response to stimulation by either WT peritoneal exudate macrophages or LMTK-CD1d cells.

Additionally, if possible, NKT cells can be isolated from the peripheral blood of CGD patients and tested ex vivo for their cytokine response after stimulation. These human NKT cells can be expanded ex vivo to increase the cell number for ex vivo assays (Chang et al., 2005).

2. To study CD1d trafficking in X-CGD macrophages for activation of NKT cells

Our results showed reduced CD1d on the surface of X-CGD peritoneal exudate macrophages, which we speculate to partially account for the impaired activation of iNKT cells by X-CGD macrophages. It is unclear whether the reduced surface expression of CD1d on X-CGD macrophages during sterile inflammation is a direct consequence of the absence of ROS in these cells. To address this, we could isolate day 4 WT and X-CGD peritoneal exudate macrophages and first compare the amounts of total CD1d protein in these cells, by both flow cytometry with permeabilized cells and confocal
microscopy. Confocal microscopy would reveal the localization of cytosolic CD1d, likely in endosomes, and also allow us to see whether the localization of cytosolic CD1d is altered in X-CGD cells. In addition, we could do co-staining for gp91phox in WT macrophages to study whether gp91phox is also localized in endosomes where CD1d is present. The co-localization of gp91phox in the endosome would implicate possible ROS generation in the endosome, which might affect lipids loading and CD1d trafficking to the plasma membrane. The initial characterization of CD1d expression and trafficking in X-CGD macrophages would be helpful for designing more sophisticated approaches, such electron microscopy and measurement of endosomal pH, to delineate the cause of reduced surface expression of CD1d on X-CGD macrophages.
Part IV. A Rac-gp91\textsuperscript{phox} interaction is important for electron transfer and assembly of the phagocyte NADPH oxidase on phagosome membrane

1. To define the dependence of p67\textsuperscript{phox} for Rac2 localization to the plasma membrane and phagosome membrane using p67\textsuperscript{phox}–deficient neutrophils

Our data suggested that Rac localization to the plasma membrane in response to a soluble stimulus, such as PMA, can occur independently of gp91\textsuperscript{phox}. Our speculation is that interaction with p67\textsuperscript{phox} may stabilize Rac2 on the plasma membrane. On the other hand, our results supported an integral role for the physical interaction with gp91\textsuperscript{phox} in recruiting and retaining Rac2 on phagosomes. We generated COS7 cell lines with reconstituted oxidase subunits to examine the gp91\textsuperscript{phox} mutant in the absence of p67\textsuperscript{phox}, and the data suggested the interaction with p67\textsuperscript{phox} is dispensable for the localization of Rac to the phagosome, as it appeared to rely more on its interaction with gp91\textsuperscript{phox}.

As an alternative approach to confirm these implications from our results, we are interested to examine Rac2 translocation to the plasma membrane and phagosome in p67\textsuperscript{phox}–deficient neutrophils. At the time when this study was carried out, p67\textsuperscript{phox}–deficient mice were not available. However, the Dinauer laboratory is currently breeding these mice that were generated using gene targeting of embryonic stem cells. Neutrophils from these mice will be used to investigate the translocation of Rac2 in response to PMA and IgG opsonized beads. If Rac2 translocation to the plasma membrane of PMA-stimulated p67\textsuperscript{phox}–deficient neutrophils is normal, it suggests that the interaction with gp91\textsuperscript{phox} may be able to recruit Rac2 to the plasma membrane in the absence of p67\textsuperscript{phox}. In addition, if Rac2 accumulation on phagosomes is normal in these cells, it confirms that
Rac2 depends on its interaction with gp91\textsubscript{phox} to stay associated with the phagosome membrane.

2. To determine the importance of the interaction between Rac and gp91\textsubscript{phox} in the activity of the phagocyte NADPH oxidase in macrophages

Macrophages have a different machinery to regulate the activity of the phagocyte NADPH oxidase than neutrophils. For instance, Rac2 is the predominant Rac and is indispensable for the oxidase activity in human neutrophils. However, Rac1 is the predominant form of Rac in macrophages. To address the importance of the interaction between Rac and gp91\textsubscript{phox} in the activity of the NADPH oxidase in macrophages, we could retrovirally transduce X-CGD bone marrow progenitors (gp91\textsubscript{phox}–deficient) with WT gp91\textsubscript{phox} or gp91\textsubscript{phox}Y425A, followed by in vitro differentiation into macrophages. NOX2 activity can be tested after stimulation of these transduced macrophages with PMA or IgG opsonized beads. Reduced oxidase activity in macrophages expressing gp91\textsubscript{phox}Y425A would support a critical role for the interaction between Rac and gp91\textsubscript{phox} in regulating activity of the NADPH oxidase in macrophages as well.
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