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Minireview

MINIREVIEW

Macrophage form, function, and phenotype in mycobacterial infection: lessons from tuberculosis and other diseases

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One sentence summary: In this review, we highlight the influence of distinct macrophage phenotypes on disease progression in tuberculosis, including similarities to findings about macrophages in cancer biology and atherosclerosis.

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ABSTRACT

Macrophages play a central role in mycobacterial pathogenesis. Recent work has highlighted the importance of diverse macrophage types and phenotypes that depend on local environment and developmental origins. In this review, we highlight how distinct macrophage phenotypes may influence disease progression in tuberculosis. In addition, we draw on work investigating specialized macrophage populations important in cancer biology and atherosclerosis in order to suggest new areas of investigation relevant to mycobacterial pathogenesis. Understanding the mechanisms controlling the repertoire of macrophage phenotypes and behaviors during infection may provide opportunities for novel control of disease through modulation of macrophage form and function.

Keywords: macrophage; granuloma; tuberculosis; mycobacteria

INTRODUCTION

Tuberculosis (TB) is the leading cause of morbidity and mortality associated with a single infectious agent (WHO 2015). *Mycobacterium tuberculosis* (*Mtb*) infection is acquired by inhalation of aerosolized droplets containing infectious bacilli. Bacilli are phagocytosed by alveolar macrophages, tissue-specialized macrophages residing in and acting to clear air spaces of infectious, toxic, or allergenic particles that have evaded the mechanical defenses of the respiratory tract (Rubins 2003). Mycobacteria can also be taken up in the air spaces by recruited monocyte-derived macrophages (Thurnher *et al.* 1997) or by dendritic cells, which can carry the organisms to draining lymph nodes initiating an adaptive response to infection that is abnormally delayed (Henderson, Watkins and Flynn 1997; Marino *et al.* 2004; Wolf *et al.* 2007). Pathogenic mycobacteria have achieved broad evolutionary success through specialization as intracellular pathogens able to achieve persistent infection by manipulating host macrophages in order to reside and replicate within them rather than be destroyed by innate defense mechanisms (Eum *et al.* 2010; Philips and Ernst 2012; Repasy *et al.* 2013). Recruitment of different phagocyte types and subtypes during early infection results in different disease outcomes as diverse phagocyte classes play different roles in advancing or controlling infection (Cadena, Flynn and Fortune 2016). *Mtb*-infected

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macrophages and dendritic cells migrate from alveolar spaces and into the lung interstitium and surrounding tissues, where they are able to either enter the lymphatic or hematogenous systems spreading to other organs and causing primary progressive disease, migrate to draining lymph nodes initiating the adaptive response, or aggregate into characteristic structures called granulomas (Frieden *et al.* 2003). After the initial stages of infection, *Mtb* can be distributed among alveolar macrophages, dendritic cells, and neutrophils. With additional time, dendritic cells and recruited monocyte-derived macrophages become the prominent infected cell types in the airways (Wolf *et al.* 2007; Srivastava, Ernst and Desvignes 2014). As recent reviews have covered the diversity of phagocytic cells that are engaged during mycobacterial infection (Srivastava, Ernst and Desvignes 2014), we will limit our discussion to the specific roles of different macrophage subtypes during mycobacterial infection, how recent understanding of macrophage heterogeneity and subtypes will impact our understanding of mycobacterial infections, and how macrophage diversity may drive infection trajectory.

Granulomas as macrophage-driven structures

Granulomas are compact organized aggregates of mature macrophages that arise in response to *Mtb* infection as well as other inflammatory stimuli and can allow bacilli to persist over time (Russell 2007; Ramakrishnan 2012). Infected macrophages arrange into granulomas characterized by concentric rings of specialized macrophages surrounding a lipid-rich core of central necrosis, referred to as caseum (Russell 2007; Ramakrishnan 2012). Within the granuloma, macrophages can fuse to form multinucleated giant cells (Helming and Gordon 2007b), accumulate lipid, becoming foam cells (Russell *et al.* 2009), transition to epithelioid histiocytes (Adams 1974), or take on diverse chemokine and cytokine expression profiles (Monin and Khader 2014). Cytokines and chemokines produced by granuloma macrophages result in recruitment and aggregation of a diverse cell type complement including neutrophils, dendritic cells, B and T cells, natural killer (NK) cells, and fibroblasts around and within the forming granuloma (Cosma, Sherman and Ramakrishnan 2003; Ehlers and Schaible 2012). There has been growing appreciation for the diverse roles that neutrophils play in driving both early protection as well as mediating detrimental excessive host inflammation and lung damage (Yang *et al.* 2012; Monin and Khader 2014; Dallenga and Schaible 2016). The developing granuloma is dynamic and may have both host-protective and bacteria-beneficial effects, providing a niche in which the bacteria may infect recruited macrophages, grow extracellularly after necrosis of infected macrophages, persist, and disseminate (Davis and Ramakrishnan 2009; Lin *et al.* 2014; Gideon *et al.* 2015). Macrophages can participate in promoting this dissemination by leaving the primary granuloma, while infecting and establishing secondary granulomas at new tissue sites (Davis and Ramakrishnan 2009; Schreiber *et al.* 2011; Welsh *et al.* 2011; Guirado, Schlesinger and Kaplan 2013).

Given the central roles of macrophages during early mycobacterial infection, granuloma development and maintenance, recruitment of diverse cell types, and the eventual dissemination of infection, an understanding of the heterogeneous phenotypes of macrophages at baseline and in response to infection is essential for understanding the mechanisms of mycobacterial infection initiation and progression. In this review, we discuss recent understanding gained about macrophage development, specialization, and activation. We additionally describe how these new insights relate to observations of macrophage diversity during mycobacterial infection and the effect of this diversity on disease progression.

REFINED UNDERSTANDING OF MACROPHAGE DIVERSITY AND FUNCTION

Macrophages are professional phagocytes adapted to kill engulfed microbes but also involved in regulating early organism development and coordinating tissue repair (Tauber 2003; Medzhitov 2010). Initial understandings of macrophage biology are giving way to more nuanced appreciation of the many roles and responsibilities of macrophages during organism homeostasis and disease (Gordon, Pluddemann and Martinez Estrada 2014; Varol, Mildner and Jung 2015). Recent paradigm shifts have changed the dialog around macrophage diversity, opening new pathways of investigation in macrophage biology as a result of a more complex understanding of macrophage heterogeneity and specialization. The importance of macrophage ontogeny, or developmental origin, and specific activation phenotypes are becoming clearer and are relevant to an understanding of the varied roles of macrophages during mycobacterial infection. The baseline heterogeneity of macrophages can help to explain the variation in interaction between pathogenic mycobacteria and macrophages depending on the local microenvironment in which the interaction occurs (Guirado, Schlesinger and Kaplan 2013) and the different macrophage subsets involved in disease (Cambier *et al.* 2014). As has been noted in recent reviews, other infected cell types may also affect the trajectory of disease (Srivastava, Ernst and Desvignes 2014; Cadena, Flynn and Fortune 2016).

Developmental origins and tissue heterogeneity

Macrophage phenotypes can be dramatically influenced by developmental origin, and macrophages of different lineages may contribute to the progression of mycobacterial infection (schematized in Fig. 1). Macrophages were initially considered in the context of differentiated bone marrow-derived blood monocytes exclusively (van Furth *et al.* 1972). However, early work demonstrated that there was a distinct yolk sac-derived population of macrophages seeding the brain in mice (Alliot, Godin and Pessac *et al.* 1999) and that Kupffer cell populations were maintained even after destruction of blood monocytes (Yamada, Naito and Takahashi 1990). In zebrafish, yolk sac-derived macrophages were observed prior to the development of later lineages and shown to distribute throughout body tissues indicating that macrophages were capable of developing from an additional developmental source (Herbomel, Thisse and Thisse 1999). Later, lineage-tracing studies following macrophage sources in animals developing into early adulthood (Ginhoux *et al.* 2010; Schulz *et al.* 2012; Hettinger *et al.* 2013; Jakubzick *et al.* 2013; Yona *et al.* 2013) as well as examination of transcription factor regulatory networks (Lavin *et al.* 2014) demonstrated that most macrophages in adult systems are maintained independent of blood monocytes through self-renewal from precursors established in tissue locations during embryonic development (Hoeffel *et al.* 2012; Guilliams *et al.* 2013; Hashimoto *et al.* 2013; Ginhoux and Jung 2014). Only macrophages resident in the intestine are constantly replenished from blood monocytes (Bain *et al.* 2014), though macrophages and inflammatory dendritic cells (iDCs) can develop in all adult tissues from tissue-infiltrating monocytes (Segura and Amigorena 2013; Yona *et al.* 2013).

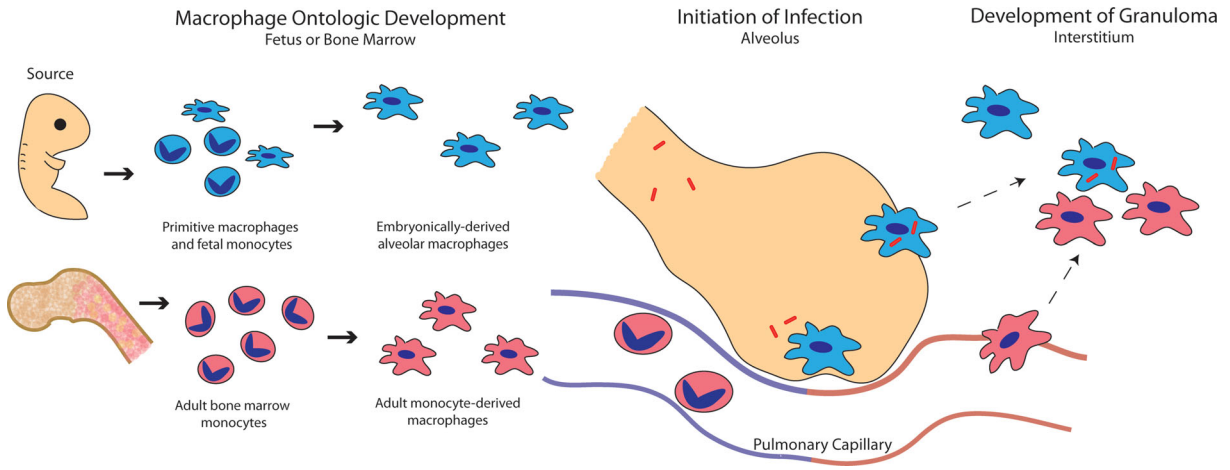

Figure 1. Ontologic sources of macrophages during mycobacterial infection. Macrophages present in adult tissues are derived from embryonic and bone marrow sources. Embryonically derived macrophages establish residence in tissues during early development and are continuously derived from established tissue-specific precursors. Alternatively, adult bone marrow continuously gives rise to monocytes which differentiate into monocyte-derived macrophages in response to stimuli and migrate into tissues. During infection, mycobacteria are phagocytosed by alveolar macrophages, an embryonically derived lineage, shortly after inhalation. Infected alveolar macrophages migrate into the lung interstitum and are joined by recruited monocyte-derived macrophages, which aggregate together in order to form the granuloma structure. In this way, diverse macrophage populations collaborate to respond to infection and develop the granuloma.

Embryonically derived tissue-resident macrophages display remarkable diversity of gene expression (Li *et al.* 2007; Gautier *et al.* 2012) adapting to the specific microenvironment of residence through both their ontological lineage as well as specific chromatin modification enacted through environment-specific enhancer landscapes and maintained in lineages (Lavin *et al.* 2014). Alternatively, monocyte-derived macrophages and iDCs display distinct behaviors and are shorter lived than their embryonically derived counterparts but are able to dynamically respond to specific changing stimuli allowing for on demand activity during times of inflammation or infection regardless of lineage-specific actions (Yona *et al.* 2013). Relatively few studies have specifically addressed the role of monocyte-derived macrophages in the lung compartment (Kopf, Schneider and Nobs 2015). However, recent work has demonstrated that monocyte subsets and their macrophage derivatives are altered in the context of mycobacterial infection (Tung, Ou and Tsai 2013; Lugo-Villarino and Neyrolles 2014; Lastrucci *et al.* 2015). The existence of parallel systems of macrophage derivation allows for the presence of diverse macrophage phenotypes in tissues at baseline or in response to specific stimuli, such as infection, and therefore the possibility of diverse macrophage behavior in response to that stimulus.

Particularly relevant to mycobacterial infections in humans are the identity, diversity and properties of alveolar macrophages. Both alveolar macrophages and dendritic cells have been shown to be organ-specific lineages (Kopf, Schneider and Nobs 2015) which differentiate from fetal monocytes in the very early stages of development under the control of granulocyte/macrophage colony-stimulating factor (GM-CSF), PPAR γ and the lung microenvironment (Guilliams *et al.* 2013; Hussell and Bell 2014; Schneider *et al.* 2014). In contrast to other populations, alveolar macrophages have the ability to self-renew (Hashimoto *et al.* 2013), and have recently been shown to naturally express low levels of MafB and cMaf, transcription factors that generally repress a suite of genes regulating macrophage self-renewal (Soucie *et al.* 2016). In addition, alveolar macrophages appear to undergo tissue imprinting in the lung, and, as a result of tissue environment and context, and in contrast to other tissue-resident macrophages, naturally produce a number of generally immunosuppressive gene products, including transforming growth factor beta (TGFβ), IL-10 and specific prostaglandins (Guirado, Schlesinger and Kaplan 2013; Hussell and Bell 2014). These properties in the context of mycobacterial infections are discussed further below. The unique tissue context and developmental origin of alveolar macrophages help define the properties and outcomes of the initial interactions between infecting mycobacteria and the host.

Heterogeneity and the activation spectrum

In addition to ontogeny, recent insights into heterogeneity and activation state will also impact our understanding of TB disease progression. Macrophage 'activation state' has canonically been classified on the basis of the differential effect of interferon γ (IFNγ) and interleukin-4 (IL-4) on *in vitro* cultured macrophage gene expression (Stein *et al.* 1992; Martinez and Gordon 2014). These differential activation or polarization states have been termed classically and alternatively activated or M1- and M2-activated macrophages, respectively. This understanding of macrophage 'polarization' was an effort to understand macrophages as belonging to cell lineages defined by particular markers in the manner which T cells have been classified as Th1 or Th2 (Martinez and Gordon 2014). However, macrophage activation by diverse stimuli is characterized by shifts in the expression of hundreds of genes without any single gene serving as a definitive marker of a particular activation state in contrast to T cells (Xue *et al.* 2014). In order to reflect this difference in mechanism of heterogeneity, recently suggested nomenclature systems present the argument that macrophage diversity is more appropriately classified on the basis of similarity of the expression profile of the macrophage with expression profiles observed during *in vitro* stimulation with IL-4, immune complexes, IL-10, TGFβ, glucocorticoids, lipopolysaccharide (LPS), LPS and IFNγ, and IFNγ alone. These different activation or polarization states exist along a continuum between IFNγ stimulation-type macrophages and IL-4 stimulation-type

Table 1. Macrophage activation spectrum on the basis of stimulation and resulting expression of markers involved in mycobacterial infection. Macrophages are divided into functional subsets on the basis of classic sources of stimulation used in *ex vivo* experimentation. Sources of stimulation are IL-4, immune complexes (Ic), IL-10, transforming growth factor β (TGFβ), glucocorticoids (GC), lipopolysaccharide (LPS), LPS and interferon gamma (IFNγ) and IFNγ alone. Expression of markers relevant to mycobacterial infection is annotated for each stimulation type demonstrating that stimulation types are determined on the basis of expression of sets of markers rather than single markers. This allows for a more nuanced identification of macrophage activation state *in vivo* on the basis of marker repertoire expression or absence and placement along a spectrum. Table is summarized from data collected in Murray *et al.* 2014.

		Stimulation								
		'Alternative' M2 anti-inflammatory							'Classical' M1 pro-inflammatory	
		IL-4	Ic	IL-10	TGFβ	GC	(–)	LPS	LPS + IFNγ	IFNγ
Expression	CD163	+				+				
	IL-10		+	+						
	TGFβ				+					
	Arg1	++++						++	++	
	IL-17				+					
	IL-6		+							
	Nos2		+					+	++++	++++
	IL-4ra			+						
	TNF							+		
	IL-1β							+		
	IL-23								+	
	IL-12								+	

macrophages, defining a spectrum of activation between the previous binary categorization of M1 and M2 macrophages (Table 1) (Murray *et al.* 2014). However, even this graded categorization, while useful, may not be fully adequate to reflect macrophage heterogeneity. It is therefore important that work to define the activation state of macrophages during mycobacterial infection strives to determine the gene expression profile of particular macrophages under study rather than relying on a limited number of markers that have been shown to be expressed at varying levels across the macrophage activation 'spectrum'. In this way, an understanding of macrophages that considers altered expression of genes known to be important to immune activity on the basis of infection or during different stages and locations of infection is particularly relevant during mycobacterial infection.

In vivo complexity

In vivo, macrophage activation states are dynamic, responding to initial stimulation, removal of stimulus, feedback or feed-forward signaling, autocrine production of cytokines, as well as the epigenetic or developmental lineage effects built into the life history of the macrophage (Porta *et al.* 2009; Lawrence and Natoli 2011; Ivashkiv 2013). Identification of macrophage polarization states *in vivo* is more complicated than in *in vitro* systems, as individual macrophages are subjected to complex stimuli, and macrophage phenotypes are often mixed within a particular tissue region or at different times during disease (Sica and Mantovani 2012). As motile cells, macrophages can move through varying stimuli *in vivo* acquiring unique gene expression profiles through sequential alteration. The classically described macrophage phenotypes break down, as macrophages *in vivo* display wide variation in marker expression and mixed phenotypes. Thus, the particular contribution of coexisting cell types, temporal changes in stimuli during disease progression, and the particular molecular landscapes influencing individual macrophage phenotypes during TB infection *in vivo* must be closely dissected to gain understanding of the contributions of different macrophage expression profiles or activation states to disease progression *in vivo*.

Other macrophage forms

Epithelioid histiocytes are macrophages activated to develop characteristics of epithelial cells and form tight interdigitated junctions between their cell membranes (Ramakrishnan 2012). They are a hallmark of granuloma formation and TB disease progression, and were characterized in early reports on the basis of their pathological appearance (Adams 1974; Williams and Williams 1983). It is believed that these epithelioid cells are central to granuloma formation and dynamics. Epithelioid macrophages are not yet able to be fully classified on the basis of their activation state or gene expression profile and relatively little is known about the specific signals that promote their formation during mycobacterial granuloma development and maintenance. Although it was previously believed that macrophages were induced to convert to an epithelioid phenotype through the action of T cells secreting lymphokines, experiments in athymic mice (Epstein *et al.* 1979; Tanaka *et al.* 1982), larval zebrafish void of functional adaptive immunity (Davis *et al.* 2002) and in *in vitro* conditions absent lymphokine stimulation (Feng *et al.* 2014; Huang *et al.* 2015) have demonstrated epithelioid conversion and granuloma formation independent of classical adaptive immunity. Other processes leading to development of epithelioid macrophages remain unknown. Given the importance of epithelioid macrophages to TB progression and granuloma formation and the growing understanding of the diversity of macrophage phenotypes at baseline, it is important to consider both the cytokine influences and the baseline activation or ontological states of macrophages that might influence their conversion to the epithelioid macrophage phenotype during mycobacterial infection.

Turnover and replenishment of epithelioid and other macrophages in the granuloma has important implications for granuloma dynamics, but has been relatively inaccessible to experimental analysis. ^{3}H-thymidine-labeling experiments indicated that granuloma macrophage lifetimes were on the order of days, although the exact turnover rate could not be calculated due to replenishment of granuloma macrophage populations by labeled monocytes (Tsuda *et al.* 1976). In addition, adoptive transfer of bone marrow monocytes in the presence and absence of mycobacterial infection has demonstrated that recruited macrophage and dendritic cell populations were derived

from monocytes (Skold and Behar 2008). These reports, however, do not rule out the involvement of alveolar or tissue-resident macrophages in granuloma formation and their differentiation to epithelioid macrophages.

Multinucleate giant cells (MGCs) form as a result of the fusion of macrophages recruited to the site of granulomatous inflammation such as during mycobacterial infection (Chambers and Spector 1982). The role of MGCs during granuloma formation and mycobacterial infection remains incompletely explored (Helming and Gordon 2007b). In the context of mycobacterial infection, MGCs generally contain only a few bacteria, if any (Ramakrishnan 2012), and it has been reported that they are deficient in the ability to phagocytose infecting bacilli (Lay *et al.* 2007). Formation of MGCs *in vitro* has been accomplished by exposing macrophages to IL-4 (McInnes and Rennick 1988; McNally and Anderson 1995) or IL-13 (DeFife *et al.* 1997). IL-4 has also been shown to be important for the formation of MGCs during granulomatous schistosomiasis infection (Chensue *et al.* 1992). However, IL-4 alone does not induce MGC formation, but rather it has been demonstrated that macrophages respond to IL-4 stimulation differentially on the basis of their endogenous inflammatory status and the presence of bacterial signals (Helming and Gordon 2007a,b). While the function of this prominent cell type in mycobacterial infection is not well understood, recent work has suggested that MGCs have specialized capacity to phagocytose large and complement-opsonized particles via activation of CR3 during the process of macrophage fusion (Milde *et al.* 2015).

Foam cells or foamy macrophages are lipid-loaded macrophages which form as low density lipoproteins are taken up by LDL receptors and the scavenger receptors SRA and CD36 following stimulation of Toll-like receptors (TLRs) by mycobacterial-derived agonists (Russell *et al.* 2009; Mahajan *et al.* 2012, Singh *et al.* 2012). Macrophages also accumulate lipids by phagocytosis of lipid-loaded platelets (Feng *et al.* 2014). Once internalized, LDL and lipid-loaded platelets are broken down into their constituent parts including phospholipids, triacylglycerides and cholesterol. The triacylglycerides and phospholipids are mainly metabolized by the macrophage, but the cholesterol is esterified and retained by the macrophages stored in lipid droplets (LDs) (Walther and Farese 2012). Under normal conditions, macrophages function to export retained cholesterol through the action of ATP-binding cassette (ABC) transporters secreting high-density lipoproteins (HDL) to the serum in the process (Cuchel and Rader 2006). During mycobacterial infection, either as a result of inflammatory stimulus or through direction of infecting bacilli, cholesterol accumulates within macrophages (Kondo and Kanai 1976). The presence of foamy macrophages has been demonstrated to modulate the inflammatory response through production of prostaglandin E2 and leukotrienes (D'Avila *et al.* 2006; Baldan *et al.* 2008; D'Avila, Maya-Monteiro and Bozza 2008; Silva *et al.* 2009). Mycobacteria associate with LDs within the foamy macrophages, utilizing these lipids as nutritional sources (Peyron *et al.* 2008). This source of cholesterol has been demonstrated to be required for bacterial growth during the chronic phase of infection (Pandey and Sassetti 2008), and the lipid-rich substances observed in the caseous centers of necrotic granulomas, where rich extracellular growth occurs, are thought to derive from the accumulated lipids within foam cells involved in granuloma formation (Russell *et al.* 2009). The many important roles of foam cells in mycobacterial infection make a more detailed understanding of their development during mycobacterial infection, the specific macrophage subsets from which they develop, and the ways in which they are sustained or degrade during infection important.

MACROPHAGE DIVERSITY IN MYCOBACTERIAL INFECTION

During mycobacterial infection, distinct macrophage subsets are recruited to infection, exist within the granuloma and carry out diverse roles within the organism (Guirado, Schlesinger and Kaplan 2013). Recent work has shown that diverse macrophage subsets (and indeed diverse phagocytes e.g. Srivastava, Ernst and Desvignes 2014) are important in initiation of infection, development of the granuloma and cytokine expression and response. Additional environmental influences such as hypoxia (Galagan *et al.* 2013) and lipid environment (Russell *et al.* 2009) are also now understood to direct macrophage phenotype alteration and to affect the course of mycobacterial infection. These studies highlight the importance of macrophage heterogeneity in management and response to diverse stimuli, as well as the ability of infecting mycobacteria to exploit the endogenous heterogeneity of macrophage populations. Mycobacteria have developed strategies to preferentially recruit and infect macrophages more amenable to their growth, to alter the activation state of recruited macrophages, and to influence cytokine production by macrophages. With this understanding, the underlying heterogeneity of macrophage populations becomes increasingly important in an understanding of mycobacterial infection processes.

Heterogeneous macrophage recruitment and infection

Some of the earliest cells infected during initiation of TB are macrophages residing in the air spaces. Alveolar macrophages are classically described in *ex vivo* experiments as most similar to IL-4-stimulated-type macrophages that express high levels of the pattern recognition receptors mannose receptor, scavenger receptor-A and the β-glucan receptor, while also constitutively secreting lysozyme into the extracellular milieu, where it acts non-specifically to damage bacterial cell walls (Gordon 2003). *In vitro* studies have demonstrated that AMs respond to *Mtb* exposure by producing TNF and other pro-inflammatory cytokines (Keane *et al.* 1997). However, these studies have been limited in their ability to fully classify *in vivo* alveolar macrophage cytokine expression on the basis of their *ex vivo* nature.

In the context of human mycobacterial infection, circulating monocytes are skewed significantly toward the non-classical or $CD16^+$ phenotype as compared to uninfected individuals, where greater than 90% of circulating monocytes are of the 'classical' or $CD16^-$ phenotype (Balboa *et al.* 2011). The $CD16^+$ subset of monocytes gives rise to a subset of macrophages that are atypical in secreting elevated levels of TNFα, IL-1 and IL-6 but reduced IL-10 (Frankenberger *et al.* 1996; Aguilar-Ruiz *et al.* 2011), while also displaying increased pro-angiogenic behavior and motility (Wong *et al.* 2012). Specifically, monocytes isolated during TB infection demonstrate elevated cell surface expression of CD11b, TLR2, TLR5, CCR1, CCR2 and CCR5, are deficient in their ability to differentiate into DCs, and demonstrate reduced T-cell activation (Balboa *et al.* 2011). Macrophages differentiated from these monocytes have further been demonstrated to be skewed toward classical markers of 'alternative' or M2-like activation with elevated expression of CD163, MerTK, and STAT3 (Lastrucci *et al.* 2015).

Recent work in both zebrafish and mice has shown that mycobacteria are able to preferentially recruit and infect macrophages more amenable to their intracellular growth by masking pathogen-associated molecular patterns through the action of the cell-surface-associated virulence-associated lipid phthiocerol dimycocerosate (PDIM). This action results in

mycobacteria evading MyD88-dependent macrophages that would recognize their cell surface antigens through the action of TLRs. Instead, mycobacteria are able to recruit MyD88-independent permissive macrophages through a CCL2-mediated mechanism, in which the mycobacterial phenolic glycolipids (PGL) allow mycobacteria to be preferentially taken up by these macrophages. MyD88-independent macrophages were shown to produce less inducible nitric oxide synthase (iNOS) compared to MyD88-dependent macrophages making them more permissive for mycobacterial growth (Cambier *et al.* 2014). This reduced iNOS phenotype makes these macrophages similar to IL-4-stimulated-type macrophages in this regard, indicating that mycobacteria are able to preferentially recruit these 'alternatively activated' alveolar macrophage types and survive within (Gordon 2003; Mosser 2003). The mycobacterially induced skew of monocyte-derived macrophages toward a phenotype expressing CCR2 and displaying an 'alternatively activated' like phenotype indicates that mycobacteria may also be able to selectively recruit these derived macrophages and thrive within them. These findings indicate that even at the earliest stages of infection there are different populations of macrophages circulating within an organism or particular tissue at baseline, and these different populations play different roles in progression or control of mycobacterial infection.

Macrophage diversity within the granuloma

Work has divided macrophage populations within the granuloma into generalized regions on the basis of studies in human tissue and non-human primates. In mature granulomas, the macrophage aggregates are surrounded by a lymphocyte cuff composed of B cells, T cells and plasma cells (Ramakrishnan 2012). Granuloma macrophages have been shown to have both pro- and anti-inflammatory phenotypes (Gideon and Flynn 2011; Duell *et al.* 2012; Cilfone *et al.* 2013; Marakalala *et al.* 2016) and have been shown to take on different activation states or accumulate on the basis of activation states within different granuloma microenvironments (Mattila *et al.* 2013; Marino *et al.* 2015). The most peripheral macrophages, closest to the lymphocyte cuff, have been generally shown to be more similar to IL-4-stimulated or M2 macrophages with elevated expression of CD163 and arginase (Mattila *et al.* 2013) and generally anti-inflammatory eicosanoid profiles (Marakalala *et al.* 2016) (Fig. 2). The intermediate region of the granuloma can largely be thought of as containing mainly epithelioid macrophages with the occasional MGC. These macrophages have been shown to have low CD163 expression and elevated CD11c and iNOS expression, a phenotype more similar to IFNγ-simulated or M1 macrophages (Mattila *et al.* 2013). Macrophages within the most central region in non-necrotic granulomas or just adjacent to the central necrotic core are frequently foam cells as identified by Oil Red O-staining (Peyron *et al.* 2008; Russell *et al.* 2009) and show elevated HAM56 staining, a reported marker for foam cell formation (Ihling *et al.* 1996; Cummings *et al.* 2001; Mattila *et al.* 2013). The caseous granuloma center has also been demonstrated to accumulate pro-inflammatory regulators including TNF and the eicosanoid biosynthetic enzyme LTA4H (Marakalala *et al.* 2016).

Other studies of human tissue have demonstrated an increased ratio of IL-4-type macrophages within granulomas as compared to normal tissue but coexistence of both IL-4- and IFNγ-type macrophages (Huang *et al.* 2015). These two macrophage types compete for the same substrate, L-arginine, metabolized respectively through the action of arginase-1 or Nos2 and influencing macrophage polarization toward the IL-4 or IFNγ type. The availability of each enzyme locally therefore affects the availability of substrate directing macrophage polarization to follow the spatial type (Duque-Correa *et al.* 2014) and indicating a mechanism for possible variation between granulomas and patients in macrophage activation states. Although C57BL/6 mice generally form disorganized granulomas, recent work has demonstrated that *Nos2* knockout mice are able to form necrotic granulomas and that these granulomas demonstrate increased macrophage arginase-1 expression that is associated with necrosis, suggesting an important role for IL-4-type macrophages (Reece *et al.* 2010; Duque-Correa *et al.* 2014). *In vitro* models of granuloma development have identified a shift from the IFNγ-stimulated-type to IL-4-stimulated-type macrophages over time following mycobacterial infection and implicating temporal variation as an important component of macrophage diversity during mycobacterial infection (Huang *et al.* 2015). These *in vitro* observations have also been noted in murine models and human samples where more IL-4 positive cells and more IL-4-type lung macrophages have been observed during late infection stages (Hernandez-Pando *et al.* 1996; Kahnert *et al.* 2006).

These results highlight the diversity of macrophages on the basis of activation state within the granuloma and during mycobacterial infection. To date, studies have been limited to identification of macrophage activation states in particular regions of the granuloma or areas of tissue rather than being able to determine the precise phenotype of individual cells and cell types within the granuloma though multiple activation phenotypes are known to exist simultaneously. An understanding of potentially different ontological sources of macrophages present in the granuloma has also not been determined but might be relevant to understanding disease progression as different macrophage sources might give rise to diverse activation phenotypes under control of similar stimuli.

Diverse cytokine expression and response profiles

The central role of cytokine production and signaling by and to macrophages and the particular effects of a set of key cytokines on macrophage phenotype during mycobacterial infection has been well established (Cooper and Khader 2008). Studies have demonstrated that bone marrow-derived macrophages, necessarily of the monocyte-derived lineage, develop functional phenotypes following initial cytokine exposure that are not altered on exposure to subsequent cytokines (Erwig *et al.* 1998). Other work in both peripheral blood mononuclear cells and tissue-resident macrophages has suggested that macrophages are able to continuously alter their activation phenotype as their environment and the cytokine exposures in the environment change (Stout and Suttles 2004; Porcheray *et al.* 2005; Murray and Wynn 2011). This work highlights the importance of understanding the ontological contributions to macrophage populations in both early and granuloma stage infection and the effects that ontogeny may have on development of activation state in response to stimuli. The contributions of different macrophage subsets to cytokine production demonstrate the important role of macrophages in autocrine and paracrine signaling during mycobacterial infection.

One of the most central cytokines in mycobacterial infection is IFNγ, normally produced in response to IL-12p70 stimulation and central to control of mycobacterial infection (Bustamante *et al.* 2014). IFNγ is produced largely by CD4+ and CD8+ T cells and secondarily by restricted or invariant receptor repertoire innate lymphocytes, $\gamma\delta$ T cells,

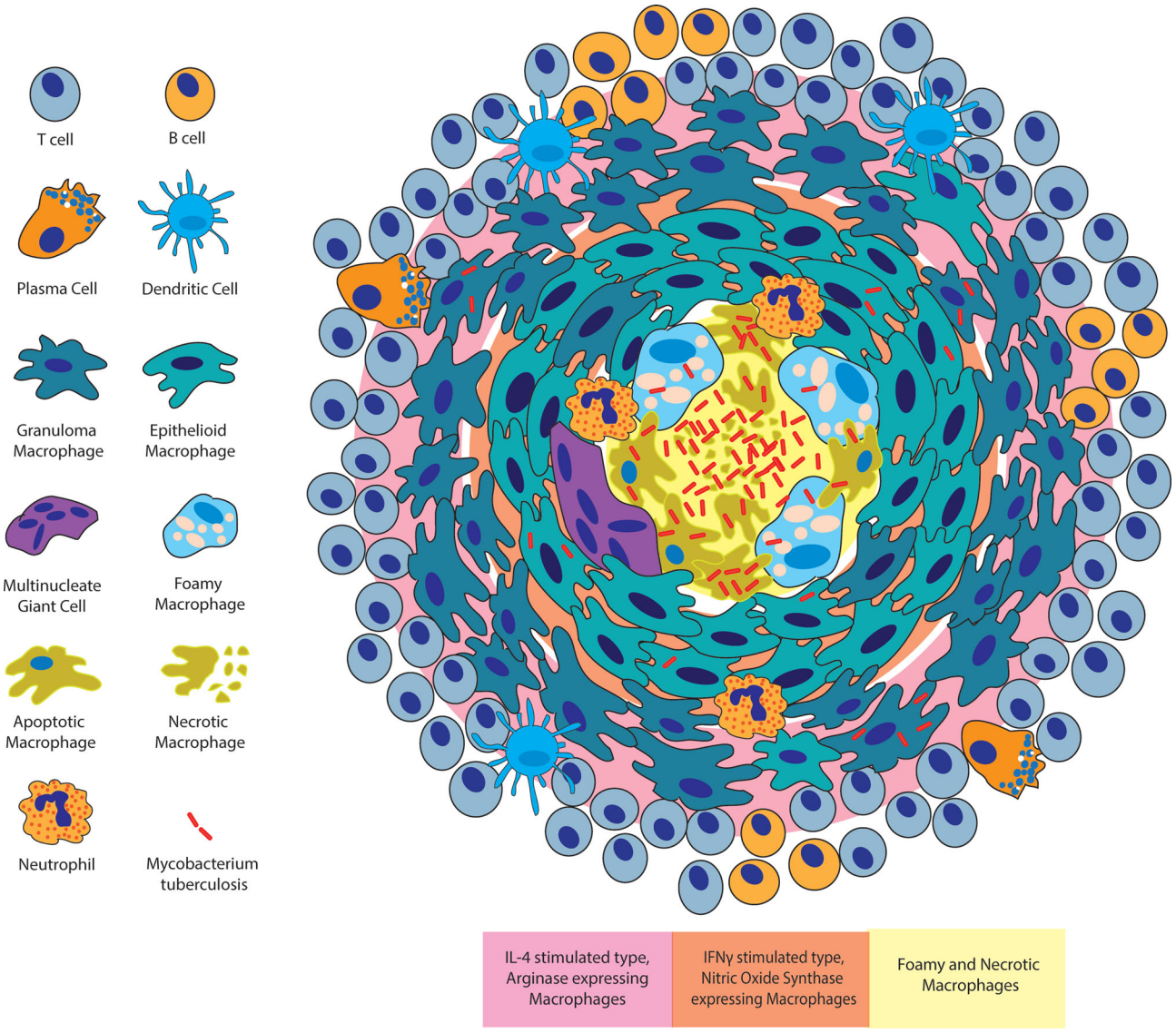

Figure 2. Structure and inflammatory arrangement of the tuberculosis granuloma. The granuloma is an organized aggregate of macrophages surrounded by a cuff of B and T lymphocytes. Plasma cells and dendritic cells are also granuloma associated. Within the granuloma, macrophages differentiate into additional cell types including epithelioid macrophages, MGCs and foamy macrophages. Different cell types are spatially organized within the granuloma with foamy macrophages found surrounding the necrotic lipid-rich core, epithelioid macrophages forming tight junctions around the central core and additional macrophages aggregating at the outer edge of the structure. The activation state of macrophages as well as the presence of pro-inflammatory and anti-inflammatory markers is also spatially arranged with more anti-inflammatory phenotypes at the periphery of the granuloma structure (pink) and more pro-inflammatory phenotypes in central regions (orange).

NK T cells and NK cells, when adaptive responses are restrained or absent (Junqueira-Kipnis *et al.* 2003; Feng *et al.* 2006; Cooper and Khader 2008). IL-23, composed of IL-12p40 and IL-23p19 subunits, is produced by lung macrophages early in infection (Silver *et al.* 2009; Riol-Blanco *et al.* 2010). Differential stimulation of monocytes with either GM-CSF or M-CSF (monocyte colony-stimulating factor) followed by exposure to mycobacteria results in differential expression of IL-23 or IL-10 respectively by these macrophage types (Verreck *et al.* 2004) and therefore differential stimulation of IFNγ production. Short-term production of IFNγ can be accomplished through stimulation by macrophage-produced IL-23, but this production is not sufficient to control bacterial growth in long term (Khader *et al.* 2005). Lung expression of IL-12p70 is low, with major production occurring by cell types in the draining lymph nodes (Cooper *et al.* 2002) and resulting in strong IFNγ production later in infection (Feng *et al.* 2005) IL-23 production by macrophages also induces production of IL-17 by $\gamma\delta$ T cells resulting in induction of adhesion molecule expression and contributing to induction of granuloma formation (Lockhart, Green and Flynn 2006; Umemura *et al.* 2007; Okamoto *et al.* 2008).

IL-10 has been demonstrated to act as a mediator of macrophage phenotype during both early and late mycobacterial infection. Strong autocrine induction of IL-10 during early infection has been shown to reduce protective responses to *Mtb* such as IL-12p40 and TNF production in animal models (Turner *et al.* 2002; Beamer *et al.* 2008) and to be involved in blocking phagosome maturation in human alveolar macrophages (O'Leary, O'Sullivan and Keane 2011). IL-10 is also required for long-term control of inflammation (Higgins *et al.* 2009) and evidence indicates elevated IL-10 signaling in macrophages just interior to the lymphocyte cuff consistent with an increased

prevalence of IL-4-type macrophages and possibly indicative of an important anti-inflammatory role of peripheral granuloma macrophages (Mattila *et al.* 2013). Intriguingly, IL-10 deletion in CBA/J mice results in mature fibrotic granuloma formation (Cyktor *et al.* 2013). The balance of action of macrophages in promoting and controlling inflammation within the granuloma is an active area of research, and the balance of anti-inflammatory and pro-inflammatory cytokines may play a key regulatory role in granuloma organization and trajectory (Gideon *et al.* 2015).

TNF is a key determinant of susceptibility to mycobacterial infection and has been shown to be robustly induced in granuloma macrophages (Flynn *et al.* 1995; Roach *et al.* 2002; Capuano *et al.* 2003; Clay, Volkman and Ramakrishnan 2008). Recent characterization of human granulomas suggests a pro-inflammatory central milieu associated with necrotic cores and with pro-inflammatory eicosanoids that impact TNF production (Marakalala *et al.* 2016). The eiconsanoids are lipid mediators derived from arachidonic acid and include of prostaglandins, lipoxins and leukotrienes. The balance of these lipid mediators both in cell culture and *in vivo* affects the outcome of infection, via influence on mechanisms of macrophage cell death (Bafica *et al.* 2005; Chen *et al.* 2008; Behar, Divangahi and Remold 2010; Divangahi *et al.* 2010). Necrotic outcomes result in more effective mycobacterial replication. Polymorphisms in human populations at the LTA4H locus, altering production of a key enzyme determining the balance of lipoxin A_4 and leukotriene B_4 production, have been identified and associated with differential disease outcomes in patients (Tobin *et al.* 2010, 2012). In addition, modulation of more upstream components of these arachidonic acid-derived eicosanoids, including targeting of the upstream 5-lipooxygenase and manipulation of PGE_2 levels have strong effects on TB progression in mice, with PGE_2 serving to inhibit the Type I IFN response and increase host resistance to *Mtb* (Mayer-Barber *et al.* 2014; Mayer-Barber and Sher 2015).

ANALOGIES TO MACROPHAGES IN OTHER SYSTEMS

Hypoxia and macrophage heterogeneity: lessons from tumors

Hypoxia has been recognized as a mechanism by which specific types of macrophages are recruited to cancerous tumors and by which macrophages are influenced to take on diverse phenotypes within the tumor environment (Biswas, Sica and Lewis *et al.* 2008; Tripathi *et al.* 2014) The diverse phenotypes of these tumor-associated macrophages (TAMs) have begun to be characterized. Hypoxia has also been identified as an important factor controlling progression during mycobacterial infection. Previous work has focused largely on examining the molecular signatures of the bacteria adapted to hypoxic conditions, attributing changes observed during hypoxia to the influence of mycobacterial products on host processes. A more detailed examination of the influences of local hypoxia on alterations in host immune response would be important for better understanding mycobacterial infection as it relates to this important environmental influence.

Tumors are abundantly populated with macrophages and these TAMs are known to promote tumor initiation, progression and metastasis partly through the production of inflammatory cytokines that generate a chronic inflammatory environment permissive for tumor promotion (Noy and Pollard 2014). Tumor conditions switch infiltrating macrophages from an immunologically active state to a trophic immune-suppressive phenotype that promotes tumor progression and malignancy partly through the action of myeloid-derived suppressor cells (MDSC) (Ostrand-Rosenberg *et al.* 2012). MDSCs have also been reported in TB, where they may play a role in disease severity (du Plessis *et al.* 2013; Knaul *et al.* 2014; Tsiganov *et al.* 2014). Infiltrating macrophages are recruited by hypoxic conditions and take on a variety of phenotypes including a more IL-4-stimulated-type phenotype with low expression of MHC class II (Lin and Pollard 2007). These M2-like cells express growth factors orchestrating the development of angiogenesis into tumors and resulting in promotion of malignancy on the basis of exposure to hypoxia, whereas other macrophages identified in tumors are not involved in this production (Laoui *et al.* 2014). It is believed that most TAMs are monocyte-derived macrophages that are recruited to the developing tumor as a result of identification of danger signals and that these macrophages take on distinct phenotypes on the basis of stimuli at the site of tumor development and as a result of their baseline activation state. This system of macrophage infiltration results in the development of distinct subsets of macrophages in different tumor macrophage microenvironments (Movahedi *et al.* 2010). These microenvironments, including hypoxic conditions and areas of macrophage-directed angiogenesis, are similar to conditions found in TB granulomas.

The necrotic core of the granuloma has long been believed to be a hypoxic environment (Barry *et al.* 2009) and work in representative model organisms has confirmed this suspicion (Via *et al.* 2008; Datta *et al.* 2015; Matty *et al.* 2015; Oehlers *et al.* 2015). Studies have identified characteristic metabolic shifts that occur in infecting mycobacteria during hypoxic conditions (Ohno *et al.* 2003; Kumar *et al.* 2008; Shiloh, Manzanillo and Cox 2008; Galagan *et al.* 2013), and it has been observed that reactivation most commonly occurs in more highly oxygenated locations within the human lung (Meylan, Richman and Kornbluth 1992), indicating both an ability and a need for reactivation to be directed toward well-oxygenated regions. Recent work has also identified host angiogenesis into the early developing granuloma as an important determinant of mycobacterial survival (Oehlers *et al.* 2015). This granuloma angiogenesis is driven by the expression of vascular endothelial growth factor A by granuloma macrophages, indicating a particular role of granuloma macrophages in promoting control of hypoxia and directing increases in mycobacterial burden within developing granulomas as a consequence. Trehalose 6,6′-dimycolate purified from *Mtb* has been demonstrated to induce angiogenesis in experimental models (Saita *et al.* 2000) and VEGF has been shown to be induced during human mycobacterial infections and in granuloma macrophages (Alatas *et al.* 2004; Datta *et al.* 2015). Work in TB has not identified the activation state or otogeny of macrophages involved in induction of angiogenesis. Given the findings that only particular macrophage subsets within tumors are involved in induction of angiogenesis, it is reasonable to assume that a similar subset of macrophages might be responsible for induction of angiogenesis in response to the hypoxic conditions of the granuloma. Identification of this macrophage subset and control of development of this phenotype during mycobacterial infection might be a step toward adjunctive control of disease.

It has been observed that dissemination of mycobacterial infection occurs from the granuloma (Davis and Ramakrishnan 2009) and that iDCs migrate in and out of the granuloma acting to disseminate infection widely while also priming the immune response (Schreiber *et al.* 2011). This indicates a possible specific role for these monocyte-derived macrophage and dendritic cell subsets in mycobacterial infection that is distinct

from the potential roles of tissue-resident embryonically derived macrophage subsets. Given the known role of monocyte-derived macrophages in the hypoxic environment of the tumor, it is possible that this particular macrophage population is also important for both promotion of mycobacterial growth through induction of angiogenesis, and later dissemination of disease when hypoxic conditions can no longer be overcome. The particular role of hypoxia in recruiting monocytes for differentiation into iDCs in the setting of infection is unknown. Investigation of this mechanism would add understanding to the picture of granuloma development and maintenance as it has previously been thought that most macrophages resulting in granuloma development in the lungs were of the alveolar macrophage subset, a population of embryonically derived tissue-resident macrophages. Understanding the mechanisms at play and developing strategies to block the differentiation of monocytes into macrophages and dendritic cells at the sites of granuloma development might be relevant to controlling spread of mycobacterial infection from established granulomas to new infection sites.

Cholesterol and macrophage heterogeneity: lessons from atherosclerosis

Macrophage heterogeneity is developing as an important area of inquiry toward understanding the processes of atherosclerosis. Atherosclerosis and the immunological response that ensues results in the development of foamy macrophages, which persist in plaques and are thought to promote disease progression (Moore, Sheedy and Fisher 2013). A second important parallel to TB infection is in the development of foamy macrophages within the granuloma environment. As previously described, foamy macrophages are also considered important cell types in TB, developing in response to infection and persisting or dying in granulomatous tissues providing lipid-rich contents in central granuloma regions, where exuberant extracellular mycobacterial growth can occur. The concentration of cholesterol within foamy macrophages during mouse and guinea pig *Mtb* infections has also been shown to lead to the development of cholesterol crystals during TB (Caceres *et al.* 2009). In mouse models of atherosclerosis, cholesterol crystals can result in lysosomal destabilization and activate the NLRP3 inflammasome and IL-1 secretion (Duewell *et al.* 2010; Sheedy *et al.* 2013). The role of inflammasome activation in mycobacterial infections is complex and the subject of more in-depth treatment in a number of reviews (Briken, Ahlbrand and Shah 2013; Mayer-Barber and Yan 2016). There appears to be ESX-1-dependent activation of the AIM2 and NLRP3 inflammasomes as well as counterregulation via NO, Type I IFN production and additional mechanisms (Mishra *et al.* 2010; Saiga *et al.* 2012; Mishra *et al.* 2013; Shah *et al.* 2013; Wassermann *et al.* 2015; Kupz *et al.* 2016) However, the sufficiency of cholesterol crystals in atherosclerosis to activate the NLRP3 inflammasome may provide additional insights into macrophage phenotypes and activation states during mycobacterial infection. Further work to understand the reason that particular macrophages and not others develop the foamy phenotype would also add value to our understanding of this important disease process.

Atherosclerosis occurs when, following intramural retention of cholesterol-rich lipoproteins in areas of the vascular wall, monocyte-derived phagocytes migrate into the subendothelial space where they take up accumulated lipids at an abnormally elevated rate and transform into cholesterol-laden foamy macrophages. It has been observed that hypercholesterolemia results in an increase in circulating monocytes (Averill, Meagher and Gerrity *et al.* 1989; Feldman *et al.* 1991) particularly of the inflammatory Ly6C^{high} subset (Swirski *et al.* 2007; Tacke *et al.* 2007) due to an increase in hematopoietic stem and progenitor cell proliferation (Yvan-Charvet *et al.* 2010). This results in development of more pro-inflammatory IFNγ-stimulated-type macrophages in atherosclerotic lesions following infiltration of these circulating inflammatory monocytes into developing atherosclerotic lesions (Ley, Miller and Hedrick 2011). It is these monocyte-derived macrophages that go on to develop into foam cells during atherosclerosis (Moore and Freeman 2006; Miller *et al.* 2011) highlighting the possibility that particularly monocyte-derived macrophages are responsible for foam cell development during TB infection as well. Similar to findings in granulomas, atherosclerotic plaques have demonstrated distinct subsets of IL-4-type- and IFNγ-type-activated macrophages that exist simultaneously and are dynamically altered over time and in response to environmental stimuli (Gallardo-Soler *et al.* 2008; Chinetti-Gbaguidi *et al.* 2011). However, the factors that influence differential macrophage activation state *in vivo* during atherosclerosis are incompletely defined.

The scavenger receptors SRA and CD36, known to be expressed on macrophages during both atherosclerosis and mycobacterial infection, have been demonstrated to account for 75%–90% of uptake and breakdown of LDL in atherosclerotic plaques resulting in foam cell formation during atherosclerosis (Kunjathoor *et al.* 2002; Kzhyshkowska, Neyen and Gordon 2012). Excessive cholesterol uptake during atherogenesis results in elevated levels of free cholesterol in cell membranes and defects in the ability of macrophages to process free cholesterol to cholesterol esters for safe intracellular storage. This accumulation results in increased inflammatory signaling from lipid rafts including increased action of TLRs (Yvan-Charvet *et al.* 2008; Zhu *et al.* 2010; Mogilenko *et al.* 2012) and also inhibits controlled apoptosis of lipid-loaded cells (Feng *et al.* 2003). Eventually, the cholesterol accumulation becomes toxic to cells resulting in their death through secondary necrosis and release of cellular contents resulting in formation of a necrotic core within atherosclerotic plaques (Tabas 2005). The development of the necrotic core is thought to make atherosclerotic plaques more dynamic, leading to thinning of the fibrous cap surrounding the lesion and increasing risk of plaque rupture and dissemination of atherosclerotic products into the bloodstream.

The observed processes by which foamy macrophages are killed during atherogenesis, initiate necrosis and ultimately influence instability of the atherosclerotic plaque that might also be relevant to mycobacterial infection, and might represent a pathogen-directed protective mechanism through which mycobacteria are released extracellularly and provided with rich nutritional content for growth, initiating the central necrosis and caseation observed in mature granulomas, and ultimately influencing later dissemination of mycobacteria by inflammatory macrophage and dendritic cell subsets that respond to the development of necrosis. As research has demonstrated a reliance on the cholesterol accumulated within foamy macrophages to sustain mycobacterial infection over the long term, the particular role of monocyte-derived versus tissue-resident macrophage populations in contributing to the development of foamy macrophages in granulomatous regions might prove a fruitful area of investigation in order to understand and abrogate this important pathological process in TB disease progression. Foamy macrophages are found in granulomas of

infectious origin but are rarely reported in sterile or autoimmune granulomas (James 2000). Foamy macrophages are also observed in other chronic intracellular infections including chlamydia (Kalayoglu and Byrne 1998) and toxoplasma (Portugal *et al.* 2008) species and can be observed as part of an inflammatory response to accumulated lipids during endogenous lipoid pneumonia caused by structural lung disease (Harris *et al.* 2011). Future work should lead to improved understanding of the unique triggers that encourage particular macrophage subsets to incorporate into granulomas and, upon arrival or infection, accumulate lipids and adopt this phenotype.

CONCLUSION

Diverse macrophage repertoires *in vivo* contribute to wide variation in macrophage phenotype during homeostasis. Developing understanding of the differential roles of monocyte-derived and tissue-resident macrophage populations in disease processes such as cancer and atherosclerosis are contributing to an improved understanding of the importance of macrophage ontogeny in determining responses to cytokine stimulation and initial as well as ultimate activation state of macrophages. In the context of mycobacterial infection, diverse macrophage phenotypes respond to and influence an array of stimuli allowing dynamic infection to result in control, low-level infection or dissemination of infection. An improved understanding of the particular role of individual macrophages and macrophage subsets, the stimuli which cause their development and recruitment, and their effect on mycobacterial development is essential to the improved understanding of the role of the macrophage in mycobacterial infection. Future work toward understanding this variability may be facilitated by recently developed techniques allowing for analysis of single cells by infection phenotype, RNA-seq expression profile and cytokine production profile (Avraham *et al.* 2015; Xue *et al.* 2015). Ultimately, an understanding of the complex cell-to-cell variation among macrophages during mycobacterial infection will help us to better understand the diversity of response to infection in patients and the processes that occur during TB infection. This understanding may allow for the development of novel approaches to manipulating disease progression via modulation of macrophage phenotype, form and function.

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