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Double Edge Sword: The Role of Neutrophils in Tuberculosis

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1. Introduction

Today, after more than 100 years of tuberculosis (TB) research, which have produced a vaccine and several drugs, TB still remains the most important bacterial infection worldwide. Every year more than 1.5 million people are killed by the infection and around 8 million new cases are reported. The risk of developing the disease is greatly increased by acquired immunodeficiency syndrome (AIDS) and immune-compromising conditions, such as diabetes and malnutrition.

In order to exert a better control of the disease, more effective vaccines and/or chemotherapeutics should be developed. In order to achieve this, better understanding of the immune mechanisms underlying the host-pathogen relationships in TB should be obtained first (Nathan, 2009). Of particular interest is the innate immunity generated by *Mycobacterium tuberculosis* infection, and more precisely the role of neutrophils, since their exact participation in the immunity or pathogenesis of TB is still poorly understood.

Because *M. tuberculosis* is transmitted via aerosols, classically alveolar macrophages, and more recently dendritic cells have been considered to be the first cells to encounter the bacilli in the alveolar sack. This view has just recently started to change dramatically, despite results obtained by Antony *et al.* in 1983, which clearly demonstrated, both *in vitro* and *in vivo*, an active participation of neutrophils in monocyte recruitment, granuloma formation and lung repair (Antony et al., 1983).

Neutrophils are polymorphonuclear cells (PMNs), with abundant granules in their cytoplasm that present large amounts of bactericidal molecules such as antimicrobial peptides and different proteolytic enzymes. Besides this molecular repertoire, PMNs are phagocytic cells and are prone to produce abundant Reactive Oxygen Species (ROS). Usually, PMNs that are part of the tisular inflammatory infiltrate are terminal differentiated

cells and with a short lifespan of 6 to 10 h, a notion that has been challenged recently by Pillay et al. (Pillay et al., 2010).

The goal of this chapter is to describe current knowledge about the role of neutrophils during *M. tuberculosis* infection, and their relationship with other cells. The importance of recent molecular and cellular processes described in neutrophils such as pathogen shuttling, antigen presentation, NET formation and ectosome release will be discussed as well as their contribution to the pathophysiology of TB.

2. Neutrophils

Neutrophils or polymorphonuclear cells (PMN) form the first line of defense of the human innate immune system. They are the most abundant leukocytes in the blood (65% to 75% of all white blood cells) (Nathan, 2006) and are armed with powerful weapons to kill foreign microorganisms. Neutrophils kill by both oxidative (phagocytosis) and non-oxidative (degranulation) mechanisms (Kumar et al., 2010). They are differentiated in the bone marrow from pluripotent hematopoietic progenitor cells into mature neutrophils and have a life span of only a few hours in the bloodstream (Borregaard, 2010). However in a paper published last year, this life span was increase to 5.4 days, ten times longer, forcing to rethink the functions these cells might play in health and disease (Pillay et al., 2010).

Neutrophils possess a multi-lobulated nucleus, abundant storage granules in the cytoplasm (azurophilic or primary, secondary and tertiary granules) (Borregaard et al. 1997), glycogen in the cytosol from which they derive almost all of their energy, and only a few mitochondria. The granules in mature neutrophils contain a variety of proteins that contribute to anti-microbial host defense. Among the proteins in the azurophilic granules are those with direct antimicrobial action (i.e. defensins, bactericidal-permeability-increasing protein, azurocidin), proteases (i.e. elastase, cathepsins), and a peroxidase that is normally expressed only in neutrophils and monocytes (i.e. myeloperoxidase (MPO)) (Borregaard & Cowland, 1997).

The recruitment of neutrophils from the bloodstream to the site of infection is initiated by chemokines and cytokines in a process called extravasation (Kobayashi & DeLeo, 2009). Then, microbes and microbial compounds such as lipopolysaccharide (LPS) activate the neutrophils via transmembrane receptors. Once neutrophils are at the site of infection, they can internalize both opsonized and non-opsonized microbes (Lee et al., 2003). Fc receptors and a subgroup of $\beta 2$ integrins, which are the principal opsonin receptors of neutrophils, bind to immunoglobulin and to complement-coated particles respectively (Witko-Sarsat et al., 2001). The vesicles containing the pathogens, called phagosomes, fuse with neutrophil granules, and the antimicrobial contents are discharged into the lumen of the phagosome, which is then called the phagolysosome (Segal, 2005).

Upon activation, neutrophils are highly effective at generating reactive oxygen species (ROS) by a process known as respiratory burst. In stimulated neutrophils, ROS are generated almost exclusively by NADPH oxidase (Kobayashi & DeLeo, 2009). If phagocytosis is not to occur following pathogen interaction with the neutrophils, the release of granule contents and ROS formation will be directed to the outside of the cell to eradicate extracellular pathogens. Anti-microbial compounds from granules not only kill the bacteria,

but may also act as chemoattractants for T-cells and immature dendritic cells (iDCs), which in turn recruit more neutrophils to the site of infection and also initiate an adaptive immune response (Burg & Pillinger, 2001).

2.1 Neutrophil Extracellular Traps (NETs) are induced by *M. tuberculosis*

Another recently described microbicidal mechanism of neutrophils is the release of structures called neutrophil extracellular traps (NETs) which can trap and kill microbes. These structures are composed of nuclear chromatin or mitochondrial DNA, associated mainly with nuclear histones and granular antimicrobial proteins (Brinkmann et al., 2004; Yousefi et al., 2009). NETs are formed in response to a variety of pro-inflammatory stimuli such as LPS, IL-8, TNF α and PMA (Brinkmann et al., 2004), as well as by fungal (Urban et al., 2006; McCormick et al., 2010), bacterial (Beiter et al., 2006; Brinkmann et al., 2004; Ermert et al., 2009; Ramos-Kichik et al., 2009), or protozoal (Baker et al., 2008; Guimaraes-Costa et al., 2009) strains and species both *in vivo* and *ex vivo*. The formation of NETs has been demonstrated in many non-infectious pathophysiological conditions in mice, cows and humans (i.e. pre-eclampsia, Crohn's disease, systemic Lupus erithematosus and cystic fibrosis) (Gupta et al., 2005; Hakkim et al., 2010; Marcos et al., 2010; Yousefi et al., 2008).

There is much evidence indicating that NETs are released in the context of a cell death different from apoptosis or necrosis. Other granular cell types, such as eosinophils (Yousefi et al., 2008) and mast cells (Kockritz-Blickwede et al., 2008), but not basophils, also release extracellular traps. Therefore, Wartha et al., introduced the term ETosis as a more generalized term to name the process of extracellular trap release by dying cells (Wartha et al., 2008). During ETosis, the lobulated nuclear morphology of neutrophils is lost. Later, both nuclear and granular membranes disintegrate, but plasma integrity is maintained, allowing the antimicrobial granular proteins to mix with nuclear components. Finally, NETs emerge from the cells as the cytoplasmic membrane breaks (Fuchs et al., 2007). No morphological signs of apoptosis are observed, such as membrane blebbing, nuclear chromatin condensation, phosphatidyl serine (PS) exposure before plasma membrane rupture and internucleosomal DNA cleavage (Fuchs et al., 2007). Caspase activity is only detected during spontaneous neutrophil apoptosis, but not during PMA induced ETosis (Remijsen et al., 2011a). In contrast with necrosis, neutrophils do not stain positive for F-actin after they have undergone ETosis (Marcos et al., 2010; ; Palik et al., 2007; Ramos-Kichik et al., 2009). Although the regulation of subcellular events during ETosis remains unclear, increasing evidence indicates that the collapse of the nuclear envelope during ETosis and concurrent chromatin decondensation are regulated by the interplay between histone citrullination, superoxide production and autophagy (Remijsen, 2011b).

Traditionally, neutrophils are viewed as phagocytes important in the resolution of rapidly growing microorganisms. Thus, they were disregarded in the control of intracellular pathogens responsible for chronic diseases. This is the case of tuberculosis, which today is, after AIDS, the second cause of death from an infectious disease worldwide (Young, 2008). The etiological agent, *M. tuberculosis*, is one of the most successful pathogens at evading the host immune response to establish infection. Its pathology is so complex that it has not been fully understood yet. For several years neutrophils were not believed to have a role in the pathogenesis of tuberculosis due to their short life-span and because their microbicidal

mechanisms, although efficient, were associated with tissue damage and inflammation observed during acute infections. However, evidence has accumulated in the past few years emphasizing the role of neutrophils during *M. tuberculosis* infection. *In vivo* studies have revealed that the earliest immune response during mycobacterial infection is a migration of neutrophils to the site of infection during the acute phase of tuberculosis (Appelberg et al., 1989; Barrios-Payán et al., 2006; Pedroza et al., 2000). Moreover, they are thought to be essential for early granuloma formation during chronic *M. tuberculosis* infection (Seiler et al., 2003).

The question of whether neutrophils play a role in killing *M. tuberculosis* or contribute to the development of the pathology remains controversial, and will be discussed at the end of this chapter.

Recently, Ramos-Kichik et al., reported that two different genotypes of the *M. tuberculosis* complex with different virulence degrees (*M. tuberculosis* H37Rv and *M. canettii*), induce subcellular changes that led to NETs formation in a time dependent manner, causing the death of infected neutrophils. Although the mechanism by which *M. tuberculosis* induces this process has not been elucidated yet, it is possible that a direct recognition through TLR2/TLR4 of mycobacterial cell-wall pathogen associated molecular patterns (PAMPs) such as lipoarabinomannan (LAM), lipomannans (LM), phosphatidylinositol mannosides (PIM2, PIM6) and/or the 19 kDa lipoprotein, may be involved in NETs induction. In Addition, it was shown that NETs can trap mycobacteria (Ramos-Kichik et al., 2009). The outermost layer of the mycobacterial cell wall may be involved in NETs attachment, since this is an electrodense structure exposing negatively charged groups (Paul et al., 1992, Takade et al., 2003). Despite the ability to bind mycobacteria, NETs were unable to kill any of the *M. tuberculosis* genotypes tested, regardless of their virulence. Neither could intact neutrophils kill *M. tuberculosis* genotypes either. Instead, *M. tuberculosis*-induced NETs were able to kill *Listeria monocytogenes* (a rapid-growing intracellular bacteria) confirming their antimicrobial effect, and therefore establishing that *M. tuberculosis* is resistant to the microbicidal activity of NETs. It seems that the molecular composition and structural features of the mycobacterial cell wall confer an effective permeability barrier, thereby evading the host innate immune response. Accordingly, more studies are needed to dilucidate the strategies used by *M. tuberculosis* to resist and escape from the microbicidal effect of neutrophils.

Since NETs trap but do not kill *M. tuberculosis*, the role of NETs *in vivo* could be relevant in maintaining the infectious focus localized, thus preventing mycobacterial spreading and at the same time setting the basis for granuloma formation. On the other hand, it is also conceivable that NETs could act as a barrier avoiding phagocytosis of mycobacteria by macrophages, which are one of the few cells with known microbicidal properties against mycobacteria.

It would be interesting to clarify whether engulfment of mycobacteria could switch-on different cell death pathways or if there are mechanisms behind neutrophil maturation or environmental conditions regulating which neutrophils undergo apoptosis and which ones undergo autophagy and ETosis. The fact that NETs are induced by *M. tuberculosis* opens another perspective about the possible extracellular role that neutrophils might play during tuberculosis infection.

3. Ectosomes released from *M. tuberculosis* infected neutrophils

The release of vesicles from cell membrane of different eukaryotic cells has been observed in response to chemical stimuli (Allan et al., 1980; Scott & Maercklein, 1979; Scott et al., 1979), complement attack (Hess et al., 1999; Morgan & Campbell, 1985; Morgan et al. 1987) or pro inflammatory agents (Hess et al. 1999; Gasser et al. 2003). The process of vesicle release has been named by Stein and Luzio, ectocytosis and the vesicles released from the cell membrane ectosomes (Ects) (Stein & Luzio, 1991). Ects with size ranging from 50 to 200 nm released from PMNs in response to complement attack or fMLP (formyl-methionyl-leucyl-phenylalanine), a bacterial product with chemo-attractant and pro inflammatory properties have been extensively studied. Ects are cholesterol enriched compared with the cell membrane composition (Stein & Luzio, 1991), express CD35 (complement receptor 1, CR1), a marker abundantly expressed on secretory vesicles present in the PMNs cell cytoplasm (Sengelov et al., 1994), myeloperoxidase and human leukocyte elastase (Hess et al., 1999) (both present in the azurophilic granules of PMNs), proteinase 3 and matrix metalloproteinase 9 (Gasser et al., 2003). Due to the presence of these enzymes, a role for Ects as ecto-organelles with anti microbial activity was initially proposed (Hess et al., 1999). Other markers found on the Ects membrane are MHC I, CD11a, CD11b, L-selectin, CD46, CD16, CD32. Ects bind annexin V, suggesting the presence of phosphatidylserine (PS) in the external side their membrane. Interestingly, Ects released by PMNs bind selectively to endothelial and macrophages but not to red cells. These findings suggest that Ects could play a role in the immune response. (Gasser et al., 2003).

Recently anti-inflammatory properties have been attributed to Ects, this effect is exerted on macrophages after they enter in contact with Ects. The anti-inflammatory effect has been attributed to the presence of PS on the Ects and the concomitant production of TGF β -1 by macrophages. This event could provide a mechanism for the resolution of inflammation (Gasser & Schifferli, 2004). Likewise, it was demonstrated that PS on the Ects membrane can inhibit the maturation of monocyte derived dendritic cells, preventing the expression of co-stimulatory molecules and therefore the proper stimulation of T cells (Eken et al., 2008).

All these interesting findings have been gathered from experiments with Ects obtained from human PMNs stimulated with a fMLP. And until recently there were no reports concerning Ects release in an *in vitro* infection model. For this reason we investigated if Ects could be released after the phagocytosis of *M. tuberculosis* by human neutrophils.

Previously Gasser *et al.* noticed that Ects released by human PMNs after fMLP stimulation did not constitute a homogenous population in size, prompting the author to hypothesize that these different sizes Ects could have different properties (Gasser, et al. 2003). In our *in vitro* infection model we observed that after 10 min of infection with *M. tuberculosis* H37Rv, human PMNs produced Ects. The Ects released constituted a heterogeneous population; we observed small Ects, similar in size to the population previously described (50 - 200 nm) and larger Ects (González-Cano *et al.*, 2010) (0.5 - 0.75 μ m). Both populations differed not only in size, but also in the presence of superoxide anion (O₂⁻), which was clearly visualized in the lumen of the larger Ects.

These larger Ects were characterized by the presence of CD35, Rab5, Rab7, Ps and gp91^{phox} (a component of the NADPH oxidase) and the presence of O₂⁻ (figure 1). Human PMNs were infected with *M. tuberculosis* for 10 min, stained with the lyophilic dye CellVue® Jade and

the presence of O_2^- was demonstrated with diaminobenzidine (Kobayashi et al., 1998). Fluorescence microscopy showed that Ects are membranous compartments (fig. 1A) containing O_2^- (fig 1B). In other experiments we confirmed that the release of these larger Ects was not an exclusive event related to *M. tuberculosis* infection, but a general event induced by Gram positive and negative bacteria, as well as by an intracellular parasite. Therefore Ects release could reflect a mechanism of response of PMNs upon invasion (Gonzalez-Cano et al., 2010). More work needs to be done in order to assess the effect that this Ects may have on the anti-microbial capacity of macrophages.

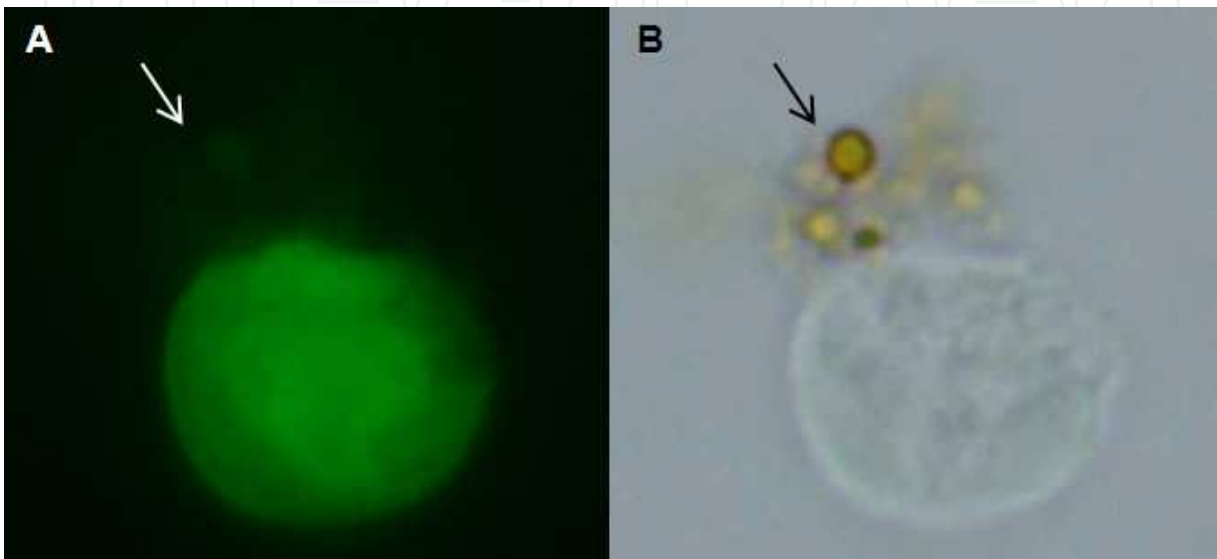


Fig. 1. Ectosomes released by human PMNs infected with *M. tuberculosis* H37Rv showing O_2^- in their lumen. Jade dye staining demonstrates that Ects are membranous compartment (arrow in A), containing O_2^- in their lumen (arrow in B).

Since neutrophils and macrophages “work in a concert” as described by Manuel T. Silva (Silva, 2010a, 2010b) it is essential to analyze the effect that Ects released by neutrophils may exert on the anti-microbial activity of macrophages.

4. Antigen presentation

PMNs were originally described as short lived and terminally differentiated phagocytes that contribute only to the innate immune response. During the last years they have also been considered to be intimately associated with the establishment of acquired immunity. In resting neutrophils, major histocompatibility complex class (MHC) class I molecules are expressed, while MHC class II and costimulatory molecules are not detected on the cell surface. However, these surface molecules exist intracellularly and some studies indicate that human neutrophils express MHC Class II, CD80 and CD86 molecules on the cell surface, either following *in vitro* activation via CD11b (Sandilands et al., 2005), with IFN γ , IL-3 and GM-CSF (Fanger et al., 1997; Gosselin et al., 1993; Radsak et al., 2000) or IL-4 (Abdel-Salam, 2011).

Potter and Harding demonstrated murine neutrophil Class I restricted antigen presentation and additionally showed that neutrophils processed phagocytosed bacteria via an alternate MHC Class I antigen-processing pathway. Such neutrophils may ‘regurgitate’ processed

peptide into the extracellular space, this peptide may then bind MHC Class I on neighboring macrophages or dendritic cell for presentation to CD8 cells. Hypothetically, neutrophils may directly present peptide to effector T cells *in vivo* at sites of inflammation, inducing cytokine production, whereas dendritic cells in contact with neutrophil-derived antigenic peptides may migrate to lymphoid organs to initiate T cell responses (Potter & Harding 2001). Additionally, another study demonstrated that murine neutrophils present MHC II-restricted peptides and induced T cell proliferation (Culshaw et al., 2008). These evidences suggested that PMNs may communicate with T cells through direct cell contact.

Since neutrophils have a short life-span and are highly susceptible to apoptosis, their role in antigen presentation has been questioned. However, various pro-inflammatory cytokines, such as IL-1, IL-6, TNF- α , produced at the site of inflammation activate neutrophils and suppress apoptosis (Cowburn et al., 2004; McNamee et al., 2005) and as described previously when PMN are cultures in the presence of IFN- γ , GM-CSF or IL-4, these cells show enhanced expression of cell surface molecules and become as competent as dendritic cells or macrophages in their ability to present antigen.

Abi Abdallah *et al.* demonstrated that mouse neutrophils express MHC class II molecules that directly present antigenic peptides, induce T-cell proliferation and promote generation of Th17 effector cells. MHC class II molecules were not constitutively expressed by neutrophils, but instead up-regulation of these proteins required contact with T cells. Most importantly this group showed that ovalbumin-pulsed neutrophils are programmed to induce Th17 differentiation even without addition of exogenous cytokines. This would appear to be an important, and possibly unique, property of PMN, since other antigen presenting cells (APC), such as dendritic cells, typically require addition of recombinant cytokines to mediate optimal T-lymphocyte subset differentiation during cell culture (Abi Abdallah et al., 2011). This is not a recent suggestion, since 1997 PMNs have been demonstrated to act as required accessory cells during T-cell activation with staphylococcal enterotoxin, a superantigen that does not require intracellular processing prior to presentation (Fanger et al., 1997).

Further studies of surface marker expression present additional evidence for the ability of PMNs to differentiate. CD83, a traditional dendritic cell marker, was shown to be expressed on the surface of PMNs stimulated with IFN- γ (Iking-Konert et al., 2001; Iking-Konert et al., 2002; Yamashiro et al., 2000). To assess whether dendritic-like PMN are also generated *in vivo*, cells of patients with acute bacterial infections were tested, the results showed that over half of the patients tested had circulating PMNs expressing CD83. This fact indicates that this phenomenon was not simply the result of unlikely *in vitro* cytokine cocktails, but that the function of CD83 on PMN is still elusive (Iking-Konert et al., 2002). In *ex vivo* experiments Aleman et al. demonstrated that neutrophils in tuberculous pleural effusions, neutrophils expressed CD86, CD83, and major histocompatibility complex class II antigens, acquiring dendritic cell (DC) characteristics. Confirming the fact that the cytokine environment in the pleural space influence the activation of neutrophils allowing them to acquire DC characteristics that in turn influence the immune response against *M. tuberculosis* (Aleman et al., 2005).

PMNs are professional phagocytes that play important roles in many infections, and abundant neutrophils are observed in the bronchoalveolar lavage fluid of patients with

pulmonary tuberculosis (TB) and more intracellular bacilli were found in neutrophils than macrophages in sputum, in bronchoalveolar lavage fluid and in cavities (Eum et al., 2010). The interaction of neutrophils with *M. tuberculosis* induces apoptosis of these cells (Alemán et al., 2002, 2004). Alemán et al. demonstrated that *M. tuberculosis* triggers the maturation of DC while it is impaired by the presence of apoptotic PMN, which abrogate Mtb-induced expression of costimulatory and HLA class II molecules, reducing IL-12 and IFN γ release by DC and partially inhibiting Mtb-driven lymphocyte proliferation (Alemán et al., 2007). Other experiments have shown that phagocytosis of apoptotic neutrophils by macrophages results in the decreased viability of intracellular *M. tuberculosis* suggesting a cooperative role of neutrophils in the host's defensive strategy against *M. tuberculosis* infection (Tan et al., 2006). All these experiments provide evidence about the complex interactions between neutrophils and *M. tuberculosis*, supporting the idea that there is still much to learn about the immune mechanisms involved in this disease.

5. Interaction of neutrophils with other cells

Neutrophils are viewed as important cellular elements for the control of bacterial infections due to its phagocytic ability and their potential to produce several effector molecules. However, little is known about their role in the regulation of the immune response and the interaction with other cellular elements. One of the first interactions described were between apoptotic neutrophils and macrophages that lead to the removal of apoptotic bodies in a 'silencing' manner because there is not induction of the inflammatory process (Fadok et al., 2000; Newman et al., 1982). So, it is not surprising that neutrophils isolated from patients with active TB are prone to apoptosis either spontaneously or when activated with the bacilli (Alemán et al., 2002). However, apoptotic bodies from Mtb infected neutrophils do not induce an anti-inflammatory state in macrophages, as reflected by the induction of TNF α and IL-1 β (Sawant et al., 2010). Moreover, it has been shown that residues of apoptotic neutrophils that are phagocytosed by infected macrophages can co-localize in early endosomes with engulfed mycobacteria inducing a decrease in their viability (Tan et al., 2006). Furthermore, the relationship between neutrophils and macrophages has been described *in vivo* through a pleural tuberculosis model. In this model an early recruitment of neutrophils that first ingest bacteria and later undergo apoptosis was observed, a phenomenon that was influenced by the pleural environment (Alemán et al., 2005). The apoptotic bodies, which contained bacteria, were taken by macrophages and these were in turn stimulated to produce suppressor molecules of the inflammatory process such as PGE $_2$ and TGF β 1, which could be detrimental for bacilli elimination (D'Ávila et al., 2008) and consequently neutrophils would behave as "Trojan horses" a phenomenon described for other infections caused by intracellular microorganisms (van Zandbergen et al., 2004).

A second cellular element that can interact with neutrophils is the dendritic cell (DCs), which represent an essential element for the induction of T cell responses during mycobacterial infections (Tian et al., 2005). DCs are present as immature cells in different tissues, but when these cells sense a microorganism or an inflammatory response they fully mature and migrate to draining lymph nodes, where they are responsible for the selection and activation of antigen specific naïve T cells. *M. tuberculosis* is capable of inducing this maturation process, however, in the presence of apoptotic bodies derived from neutrophils this process is inhibited. Interestingly cross-presentation is not blocked by this process

allowing antigen presentation to T cells (Aleman et al., 2007). A more recent work showed that *in vivo* neutrophils are important for DCs migration from the lung to mediastinal lymph nodes facilitating the induction of CD4⁺ response. The authors suggest that neutrophils deliver *M. tuberculosis* to DCs and this process promotes the migration of DC's, making this more efficient and favoring the T cell response (Blomgran & Ernst, 2011).

Although much work has focused on apoptotic neutrophils and their relation with other cell populations, there are other ways by which neutrophil may interact with other cell types. One example of this is TNF α production by *M. tuberculosis* infected neutrophils that is able to activate alveolar macrophages as reflected by an increase in TNF α , IL-1 β and hydrogen peroxide production (Sawant & Murray, 2007). Another possible interaction of neutrophils is with elements of the adaptive immune response, for example a recent report described that neutrophils from patients with active TB have shown increased expression of PDL-1 on the cell surface (McNab et al., 2011), a molecule which has been involved in exhaustion of CD8⁺ T cells during chronic viral infections (Barber et al., 2006) and has been associated with the inhibition of T cell effector functions during human tuberculosis (Jurado et al., 2008).

A different way of interaction among different cells of the immune system could be through ectosomes (Ect) or neutrophil extracellular traps (NETs), which as mentioned before, are released by *M. tuberculosis* infected neutrophils (González-Cano et al. 2010; Ramos-Kichik et al., 2009). The effect of these (*i.e.* Ect and NETs) *in vivo* in tuberculosis, is still under investigation.

6. Participation of neutrophils in the tissue damage of *M. tuberculosis* infection

Tuberculosis can be considered as the prototype of chronic infectious diseases in which the most important pathogenic factor is the balance between protection and tissue damage mediated by the immune response (Rook & Hernandez-Pando, 1996). Historically the first antecedent of tissue damage mediated by the immune response in tuberculosis was described by Robert Koch in 1891 and was called Koch phenomenon (Anderson, 1891). Koch demonstrated that the intradermal challenge of guinea pigs with whole organisms or culture filtrate, four to six weeks after the establishment of infection, resulted in necrosis at both the inoculation site and the original tuberculous lesion site. A similar phenomenon occurs in persons with active TB, in whom the PPD test site may become necrotic. Koch tried to exploit this phenomenon for the treatment of TB and found that subcutaneous injections of large quantities of *M. tuberculosis* culture filtrate (old tuberculin) into TB patients evoked necrosis in their tuberculous lesions. In fact, this treatment was shown to have extremely severe consequences associated with extensive tissue necrosis and was discontinued (Anderson, 1891). Still today, the task for those working in this field is to understand the differences between protective immunity and progressive disease, including the Koch phenomenon (Rook & Hernandez-Pando, 1996).

It seems that the severity of the Koch phenomenon depends on the dose of antigen, as lower doses induce Th-1 response with high production of IFN γ and macrophage activation which altogether produce the classic delayed type hypersensitivity response. High antigen loads produce local necrosis in which a high Th-2 cytokine production like IL-4 has been founded

(Hernandez-Pando et al., 1997). Interestingly, besides necrotic tissue with macrophage and lymphocytes infiltration there is an increased neutrophils influx (Moreira et al., 2002; Taylor et al., 2003; Turner et al., 2000).

The consistent presence of neutrophils in the necrotic areas could be mediated by IL-17 (Kolls & Linden, 2004; Miyamoto et al., 2003). In fact, IFN- γ is able to regulate the IL-17 response during BCG infection (Cruz et al., 2006), and in IFN- γ absence in TB granuloma there is an increase in neutrophils (Desvignes & Ernst, 2009). Thus, IL-17 could overcome the apparent IFN- γ mediated regulation and participate in immunopathology.

In recent studies, the overexpression of IL-17 and IL-23 has been related with neutrophils influx in necrotic pulmonary lesions (Khader & Cooper, 2008). IL-17 induces the production of the chemokine MIP-2 α which is an efficient neutrophils chemoattractant molecule. Indeed, the participation of IL-23, IL-17 and MIP-2 α has been recently demonstrated in a model of repetitive BCG vaccination in mice infected with low dose aerosols (Cruz et al., 2010), implying that IL-17, IL-23 and neutrophils are key molecules in the development of tissue necrosis during advanced pulmonary tuberculosis, and a potential adverse mechanism in specific vaccination schemes such as revaccination with BCG (Cruz et al., 2010). Thus, neutrophils can be protective during early TB infection, but when exposed to excess of IL-23 or IL-17, their function is altered and they become more able to mediate tissue damage (Zelante et al., 2007). Indeed, neutrophils are abundant in the sputum and bronchoalveolar lavage of patients with active TB (Eum et al., 2010), and rapid accumulation of neutrophils that are permissive for bacterial growth is a dominant feature in genetically susceptible mice (Eruslanov et al., 2005; Keller et al., 2006).

The restriction of neutrophil accumulation is dependent on the IFN- γ receptor dependent activity of indoleamine-2, 3-dioxygenase by radio-resistant cells in the lung, which results in increased tryptophan catabolic products that apparently inhibit IL-17 producing cells in situ (Desvignes & Ernst, 2009). These results support the detrimental role of neutrophils in TB pathogenesis.

Increased neutrophil apoptosis is observed in patients with active tuberculosis (Aleman et al., 2002) and mycobacteria is phagocytosed and inactivated by neutrophils, then many of these cells rapidly enter apoptosis via an oxygen-dependent pathway (Brown et al., 1987; Perskvist et al., 2002). This is a significant process, which prevents the release of toxic compounds from the intracellular compartments. Apoptotic cells are cleared by macrophages which in general induce an anti-inflammatory response by the secretion of TGF- β and other anti-inflammatory cytokines (Fadok et al., 1998; Hernandez-Pando et al., 2006). The production of these anti-inflammatory mediators suppresses the production of significant protective cytokines such as TNF- α and IFN- γ promoting disease progression (Hernandez-Pando, 2006). Interestingly, recent reports have showed that phagocytosis of apoptotic neutrophils by macrophages can result in a pro-inflammatory activation of macrophage including release of TNF- α (Persson, et al., 2008), and high intracellular expression of heat shock proteins 60 and 72 (Hsp60 and Hsp72) in order to protect the cells from damage. HSPs activate immune cells through interaction with several receptors such as CD91, LOX-1, CD14, TLR-2 and TLR-4 (Binder et al., 2004). Thus, mycobacteria induce apoptosis in neutrophils and these cells also release Hsp72 as a consequence of the stress

mediating an early pro-inflammatory stimulation of macrophages during the elimination of the bacilli that induced apoptotic cells. Although this event has been related to early infection bridging innate with acquired immunity, it is possible that this could also happen during advanced infection producing immunopathology, by the combined presence of TNF α and Th-2 type cytokine which can also produce tissue damage (Hernandez-Pando et al., 2004).

In conclusion, neutrophils are significant cells in the initial protective response of the innate immune response against mycobacterial infection, but during the advanced stage of the disease these cells can also contribute to the tissue damage characteristic of this chronic infectious disease.

7. Conclusions

Because neutrophils are the first inflammatory cells to arrive at sites of infection and present a diverse collection of antimicrobial molecules, they are associated as one of the first lines of defense against all microbes; TB is not an exception, in humans this infection elicits apoptosis of neutrophils, ingestion of these by macrophages triggers a pro-inflammatory response, which may or may not control disease progression.

In recent years the whole role of neutrophils in inflammation and bacterial control has been challenged. Remarkable is the work done by Zhang et al., who showed that coactivation of Syk kinase and MyD88 adaptor protein pathways by mycobacteria (BCG or *M. tuberculosis* H37Rv) promote previously unsuspected regulatory properties in neutrophils. According with their results, in contrast to monocytes and macrophages, murine neutrophils contribute poorly to inflammatory responses, and secrete high amounts of the anti-inflammatory cytokine IL-10. In a murine model they showed that mycobacteria induced the recruitment of neutrophils secreting IL-10. Interestingly, during the acute mycobacterial infection IL-10 producing neutrophils controlled the inflammatory response of DC, monocytes and macrophages in the lung. However, during the chronic phase of infection (high mycobacteria load), neutrophil depletion promoted inflammation and decreased of the mycobacterial load, these effects could be attributed to a reduce amount of IL-10 and increased TNF- α produced by lung cells in neutrophil depleted animals, and to increased amounts of IL-6 and IL-17, but not IFN- γ . The possible explanation given by the authors for these results is the dual role that neutrophils play, having direct antimicrobial activity (killing) counterbalanced by anti-inflammatory properties (IL-10 production). Neutrophils, at least in mice, are the dominant producers of IL-10 in the lung (Zhang et al., 2009).

Following this line of research, Redford *et al.* found similar results, providing evidence that IL-10 $^{-/-}$ mice showed enhanced control of *M. tuberculosis* infection with significant reduced bacterial load in lungs and spleen, which was maintained over the course of the infection. Again, IL-10 seems to regulate the balance of the immune response between pathogen clearance and immunopathology. The reduction of bacterial load in the absence of IL-10 was preceded by an enhanced cytokine/chemokine production (IFN- γ , CXCL10 (IP-10) and IL-17) and an increased of CD4 $^{+}$ T cells in the lung. Because IL-17 has been shown to promote influx of Th1 cells into the lungs after vaccination against *M. tuberculosis* (Khader et al., 2007), Redford et al. neutralized IL-17, and found a reduction of *M. tuberculosis* load in the spleen, suggesting this cytokine may affect dissemination of mycobacteria, which in turn

may be carried out by neutrophils, since they were also significantly reduced (Redford et al., 2010).

Neutrophils have been shown to be the predominant infected phagocytic cells in the airways of patients with active pulmonary TB (Eum et al., 2010), and in an experimental model neutrophils shuttled live *M. bovis* BCG to draining lymph nodes after intradermal vaccination (Abadie et al., 2005). All together, results from Zhang et al. and Redford et al. (Redford et al., 2010; Zhang et al., 2009) confirm a detrimental role for neutrophils in tuberculosis, and confirms the negative effect that IL-17 have during *M. tuberculosis* infection in humans as shown by Cruz et al. (Cruz et al., 2010).

Neutrophil functions in immunity have been extended thanks to a renewed interest in these neglected cells. Besides having huge amounts of cytokines and effector molecules, they can also produce extracellular traps and ectosomes. In addition, they actively participate in the activation and regulation of both innate and adaptive immune responses (Bratton et al., 2011; Mantovani et al. 2011, Zhang et al., 2009). These newly identified functions might lead to reconsider their role in infectious and non infectious diseases, particularly in tuberculosis, which is a major health concern worldwide.

8. Acknowledgments

JSL*, RCS*, RHP, SEP* and IEG* are fellows of SNI, and *COFAA/EDI. Part of the results presented here were supported by CONACYT grant 105278 and SIP/IPN project 20110369 “Estudio de la interacción de las trampas extracelulares de neutrófilos y células presentadoras de antígenos”.

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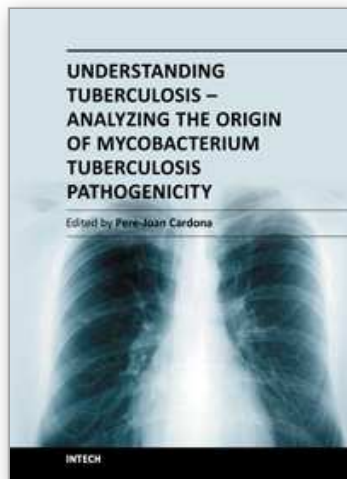
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Understanding Tuberculosis - Analyzing the Origin of Mycobacterium Tuberculosis Pathogenicity

Edited by Dr. Pere-Joan Cardona

ISBN 978-953-307-942-4

Hard cover, 560 pages

Publisher InTech

Published online 24, February, 2012

Published in print edition February, 2012

Mycobacterium tuberculosis in an attempt to understand the extent to which the bacilli has adapted itself to the host and to its final target. On the other hand, there is a section in which other specialists discuss how to manipulate this immune response to obtain innovative prophylactic and therapeutic approaches to truncate the intimal co-evolution between Mycobacterium tuberculosis and the Homo sapiens.

How to reference

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Patricia González-Cano, Rommel Chacón-Salinas, Victoria Ramos-Kichik, Rogelio Hernández-Pando, Jeanet Serafín-López, Georgina Filio-Rodríguez, Sergio Estrada-Parra and Iris Estrada-García (2012). Double Edge Sword: The Role of Neutrophils in Tuberculosis, Understanding Tuberculosis - Analyzing the Origin of Mycobacterium Tuberculosis Pathogenicity, Dr. Pere-Joan Cardona (Ed.), ISBN: 978-953-307-942-4, InTech, Available from: <http://www.intechopen.com/books/understanding-tuberculosis-analyzing-the-origin-of-mycobacterium-tuberculosis-pathogenicity/double-edge-sword-the-role-of-neutrophils-in-tuberculosis>

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