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Absence of Annexin A1 impairs host adaptive immunity against *Mycobacterium tuberculosis in vivo*

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ABSTRACT

The role of Annexin A1 (ANXA1) in counter-regulating the activities of innate immune cells, such as the migration of neutrophils and monocytes, and the generation of pro-inflammatory mediators in various models of inflammatory and autoimmune diseases is well documented. However, while ANXA1 has been proposed as an important mediator of the adaptive immune response, its involvement in this respect has been less studied. Furthermore, while there have been numerous studies on the role of ANXA1 in inflammatory diseases, less has been reported on its influence in immunity against infection. A recent study reported a link between ANXA1 and tuberculosis, and proposed a model in which *Mycobacterium tuberculosis* exerts its virulence by manipulating the ANXA1-mediated host apoptotic response. This has prompted us to further investigate the role of ANXA1 in the pathogenesis of tuberculosis *in vivo*. Here, we show that ANXA1$^{-/-}$ mice are more susceptible to *M. tuberculosis* infection, as evidenced by a transient increase in the pulmonary bacterial burden, and exacerbated and disorganized granulomatous inflammation. These pathological manifestations correlated with an impaired ability of ANXA1$^{-/-}$ dendritic cells to activate naïve T cells, thereby supporting a role for ANXA1 in shaping the adaptive immunity against *M. tuberculosis*.

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Introduction

Tuberculosis (TB) is the second leading cause of death due to an infectious disease worldwide, after the human immunodeficiency virus (HIV). The World Health Organization (WHO) declared TB a global public health emergency in the mid-1990s, and while mortality and incidence rates have decreased since then, the global burden of TB remains enormous. In 2011, there were an estimated 8.7 million new TB cases (13% of which were co-infected with HIV) and 1.4 million TB-related deaths (WHO, 2012). The emergence of drug-resistant strains (Ghandi et al., 2010) synergistic co-infection with HIV (Pawlowski et al., 2012), and limited efficacy of the existing vaccine, *Mycobacterium bovis* Bacille Calmette-Guérin (BCG) (McShane, 2011) are some of the key challenges presented by the TB epidemic. A deeper understanding of the factors and mechanisms involved in TB pathogenesis and immunity will ultimately pave the way for the development of innovative therapeutic and vaccination strategies to safeguard public health.

Mycobacterium tuberculosis (*Mtb*) is the causative agent of tuberculosis in humans. Infectious bacilli are inhaled as aerosolized droplets into the lungs where they reach the pulmonary alveoli and infect mainly alveolar macrophages (Rohde et al., 2007) as well as dendritic cells (DCs) (Tailleux et al., 2003). Initially, the bacteria are able to subvert the innate bactericidal mechanisms of their macrophage hosts and replicate freely (Nguyen and Pieters, 2005). The transport of bacteria from the primary site of infection, the lungs, to the mediastinal lymph nodes (MLN) where antigen presentation and priming of naïve T cells occurs is required for the initiation of the adaptive immune response (Wolf et al., 2008). Antigen-specific T cells are then recruited to the lungs and aggregate with macrophages to form organized structures known as granulomas, the hallmark histopathological lesions in chronic mycobacterial infection (Cooper, 2009). The natural and

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biological function of granulomas are not fully understood, but at least for the host, the juxtaposition of pathogen-infected antigen-presenting cells and effector T cells allows for immune control of mycobacterial infection that is sufficient to arrest, but not fully eliminate the pathogen (Davis and Ramakrishnan, 2009; Egen et al., 2008; Tsai et al., 2006), resulting in an asymptomatic latent infection characterized by the presence of non-replicating or dormant mycobacteria. A number of soluble factors have been identified as critical modulators of the adaptive immune response to *Mtb* infection, particularly the cytokines IFN-γ, TNF-α and IL-12 (Cooper and Khader, 2008).

Annexin-A1 (ANXA1) is a member of a superfamily of calcium and phospholipid-binding proteins and mediates most of its exogenous effects *via* interaction with formyl peptide receptors (FPRs) (Perretti et al., 2001; Walther et al., 2000). Both receptor and ligand are expressed by a variety of immune cell types, including macrophages, DCs, T cells and neutrophils; however, it has not been detected in B cells (Perretti et al., 2000; Rescher and Gerke, 2004). In resting conditions, human and mouse neutrophils, macrophages and DCs constitutively express high levels of ANXA1 in their cytoplasm which can be mobilized to the cell surface and secreted in a cell-specific response to activation (Perretti et al., 2000; Rescher and Gerke, 2004; Huggins et al., 2009). Naïve T cells express 100-fold less ANXA1 than myeloid cells and negligible levels of FPR2, but upregulate ANXA1-FPR2 pathway following activation and differentiation (D'Acquisto et al., 2007a,b).

Endogenous and exogenous ANXA1 appear to counter-regulate the activities of innate immune cells such as neutrophils and monocytes, in particular their migration and the generation of pro-inflammatory mediators (Blume et al., 2009; Cote et al., 2010; Perretti and Flower, 2004). ANXA1$^{-/-}$ mice generally display an intensified inflammatory response involving increased leukocyte transmigration (Chatterjee et al., 2005) and increased expression of inflammatory markers (Damazo et al., 2005). In comparison, while ANXA1 has proven to be an important mediator of the adaptive immune response, the mechanisms by which it exerts its influence are not well understood. Exogenously administered recombinant ANXA1 and its derived peptides have been shown to exhibit anti-proliferative activity on mitogen- and antigen-stimulated T cells (Kamal et al., 2001; Gold et al., 1996), and the data suggest that ANXA1 may exert an anti-proliferative effect by specifically interfering with the process of antigen presentation. Several studies reveal inherent complexities in the actions of ANXA1. In a model of antigen-induced arthritis, T cell-expressed ANXA1 was shown to suppress T cell-dependent inflammatory responses such as proliferation and Th1/Th17 cytokine production (Yang et al., 2004). However, the reduction in antigen-specific IgG levels raises the intriguing possibility that ANXA1 may have an opposing effect on B cell function and humoral immunity. Likewise, in an ovalbumin (OVA)-induced model of airway hyper-responsiveness, ANXA1 was associated with an attenuation of allergic inflammation (Ng et al., 2011), whereby exacerbated inflammation was observed in the ANXA1$^{-/-}$ mice. However, antigen-induced activation of MAP kinase and NF-κB pathways in lungs of these animals were reduced. The actions of ANXA1 on T cell activation appear to be subset-specific, promoting Th1 and Th17-mediated responses while restricting Th2 development (D'Acquisto et al., 2007a,b). It is also suggested that the actions of ANXA1 could differ according to the state of T cell activation (D'Acquisto et al., 2007b).

While there have been numerous studies on the role of ANXA1 in inflammatory disease, less has been reported on the role of ANXA1 in immunity against infection (Bist et al., 2013; Huggins et al., 2009; Jorge et al., 2013; Mimura et al., 2012). More specifically, Gan and colleagues were the first to report a link between ANXA1 and TB, proposing a model in which *Mtb* exerts its virulence by manipulating the host apoptotic response *via* ANXA1. Here, using ANXA1$^{-/-}$ mice, we investigated the role of ANXA1 in the pathogenesis of TB *in vivo*.

Materials and methods

All experiments involving the handling of live *Mtb* were performed in a biosafety level 3 (BSL3) facility.

Mycobacterium strains and culture

Mtb CDC1551 wild-type (WT) strain was used. Bacteria were grown in Middlebrook 7H9 medium (Difco, Detroit, MI, USA) supplemented with 10% ADS (50 g bovine fraction V albumin, 20 g D-(+)-glucose, 8.1 g sodium chloride per liter), 0.05% Tween-80 and 0.5% glycerol, or on Middlebrook 7H11 agar supplemented with 10% OADC (BD Bioscience, San Jose, CA, USA), 0.5% glycerol, 20 μg/ml ampicillin and 100 μg/ml cyclohexamide. Cultures were incubated at 37 °C with 5% CO_2. To minimize the number of passages, *Mtb* stocks were prepared in batches as 1 ml aliquots of liquid cultures containing 25% glycerol and were frozen at −80 °C. A new vial was thawed for each infection. Heat-killed mycobacterial suspension was prepared in PBS and incubated at 90 °C for 30 min.

Mice

Specific-pathogen free (SPF) adult Balb/c mice were obtained from the Centre for Animal Resources (CARE) of the National University Of Singapore (NUS). ANXA1$^{-/-}$ Balb/c mice were bred under specific pathogen-free conditions at NUS. All institutional guidelines for animal care and use were strictly followed throughout the experiments and all efforts were made to minimize pain and suffering.

Mouse infection and quantification of bacterial load

Age- and sex-matched 6–8 weeks old Balb/c WT and ANXA1$^{-/-}$ mice were infected by intra-tracheal inoculation with 500 colony-forming units (CFU) of *Mtb* CDC1551 strain. At the indicated time points post-infection, the infected animals were euthanized by CO_2 asphyxiation. For quantification of bacterial load, the lungs and spleens were harvested. Lungs were cut into small pieces and incubated with 1 mg/ml collagenase D (Roche, Penzberg, Germany) for 1 h at 37 °C, under constant agitation. Single cell splenocyte preparations were obtained by gentle mashing of the spleen using the back of a syringe plunger. Splenocytes and collagenase-digested lung tissue were then passed through a 70 μm cell strainer (Fisher Scientific, UK) and neat or 10-fold-diluted single-cell suspensions were plated onto 7H11 agar plates. After 16 days incubation at 37 °C with CO_2, the number of CFU was enumerated.

Immunohistochemistry

Harvested lungs and spleens were immediately fixed in 4% paraformaldehyde solution for 24 h, then paraffin-embedded and processed for histology. Sections (5 μm) were stained with hematoxylin and eosin (H&E) and histological analysis was performed on blinded samples. Representative photographs of sections were taken using a Zeiss Axiovert 40 inverted microscope (Zeiss, Göttingen, Germany).

Allogeneic mixed lymphocyte reaction (MLR)

Bone marrow-derived dendritic cells (BMDCs) from Balb/c WT and ANXA1$^{-/-}$ mice were generated as described by Lutz and colleagues. T cells were isolated from C57BL/6 mouse spleen preparations using a Pan T-cell isolation kit (Miltenyi, Bergisch Gladbach, Germany), and red blood cells were lysed in a buffer

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