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Review

Targeting neutrophils for host-directed therapy to treat tuberculosis

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ABSTRACT

M. tuberculosis is one of the prime killers from infectious diseases worldwide. Infections with multidrug-resistant variants counting for almost half a million new cases per year are steadily on the rise. Tuberculosis caused by extensively drug-resistant variants that are even resistant against newly developed or last resort antibiotics have to be considered untreaTable Susceptible tuberculosis already requires a six-months combinational therapy which requires further prolongation to treat drug-resistant infections. Such long treatment schedules are often accompanied by serious adverse effects causing patients to stop therapy. To tackle the global tuberculosis emergency, novel approaches for treatment need to be urgently explored. Host-directed therapies that target components of the defense system represent such a novel approach. In this review, we put a spotlight on neutrophils and neutrophil-associated effectors as promising targets for adjunct host-directed therapies to improve antibiotic efficacy and reduce both, treatment time and long-term pathological sequelae.

1. Introduction

With 1.8 million deaths, tuberculosis is the most common cause of death from infectious disease worldwide (WHO, 2016). Annually, almost half a million people get infected with multi- (MDR) or extensively drug-resistant (XDR) variants of the *M. tuberculosis* complex with an increasing rate. For patients infected with an XDR *M. tuberculosis* variant, the chance to survive is just 28%. Some *M. tuberculosis* variants are even resistant to last resort antibiotics, new variants quickly acquired resistance to newly introduced antibiotics, e.g. Bedaquilin and Delamanid (Andries et al., 2014; Hoffmann et al., 2016; Parida et al., 2015). According to WHO estimations, there have been 45,600 new cases of XDR tuberculosis in 117 countries worldwide in 2015. With over 18% of all new tuberculosis cases, the spread of MDR tuberculosis in Russia, Kazakhstan, Uzbekistan, Kyrgyzstan, Belarus, Ukraine and Moldavia is alarmingly high. Importantly, in absolute numbers of all MDR cases, China and India rank highest with over 50,000. MDR tuberculosis treatment requires extensive chemotherapy for as long as 2 years with second line drugs including injectable ones, namely combinations of kanamycin, streptomycin, para-aminosalicylic acid, ethionamide, cycloserine, moxifloxacin or ofloxacin. Long-term treatment using these second line drugs can cause serious adverse effects including tinnitus, irreversible hearing loss, vomiting, diarrhea, arthritis, liver and renal toxicity, burning sensation in the extremities, epileptic seizures, depression, psychosis, and suicide and often leads patients to discontinue treatment (Torun et al., 2005; Baghaei et al., 2011; Verma and Mahajan, 2007). Moreover, the costs of treatment for MDR are magnitudes higher than for patients infected with susceptible strains. In the US, the cost to treat XDR patients exceeds the one for MDR patients around 3.5 times (Marks et al., 2016; Dheda et al., 2017; Pooran et al., 2013). Finally, tissue damage and cavities due to exacerbated inflammatory responses, such as massive influx of neutrophils and their necrotic cell death, are often irreversible and lead to long-term pathological sequelae and reduced lung function despite successful treatment (Dheda et al., 2017). Taken together, these figures highlight the urgent need for innovative approaches for tuberculosis treatment.

Host-directed therapies adjunct to classical antibiotic treatment represent such an innovative approach. Modulating the host defense system and the interplay of the innate and adaptive immunity may lead to shorter therapies, reduced lung tissue damage and better outcome for the patients. Other HDT targets comprise regulatory immune cell populations such as myeloid suppressor cells (MDSC) or regulatory T cells and their cytokines, including IL-10 as reviewed elsewhere (Schaible et al., 2017, *Front Immunol.*, under review) (Dorhoi and Kaufmann, 2015). Recent studies showed association of neutrophils with active pulmonary tuberculosis, high mycobacterial burden and exacerbated

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inflammation and immunopathology (Dallenga and Schaible, 2016). Thus, neutrophils represent an intriguing target for host-directed therapies.

2. Neutrophils in tuberculosis

Neutrophils are the most abundant type of white blood cells in the body. These potent phagocytes strongly react to inflammatory stimuli by massive infiltration to the site of infection along an IL-8 gradient that is sensed by the receptors CXCR-1 and -2 (Nathan, 2006). This is also the case in active tuberculosis. Neutrophils represent the most frequent cells in sputum and bronchoalveolar lavage from patients with active pulmonary tuberculosis, and they carry the main mycobacterial load (Eum et al., 2010). Berry et al. identified a blood mRNA profile that is specific for individuals suffering from active tuberculosis (Berry et al., 2010). This profile allowed differentiation between active and latent infection as well as healthy controls. Importantly, the profile was characterized by a neutrophil-associated mRNA signature. An independent study demonstrated that higher numbers of S100A8/9-positive neutrophils in granulomas of patients with active tuberculosis correlated with S100A8/A9 blood concentrations and lung pathology (Gopal et al., 2013). Interestingly, Kimmey et al. linked the autophagy-related protein 5 (Atg5) to pulmonary neutrophil accumulation in the murine tuberculosis model (Kimmey et al., 2015). Atg5 is essential for the formation of autophagosomes and autophagy was thought to exhibit a protective role against *M. tuberculosis* infection on a cellular level. However, when functional autophagy was prevented by *Lysm-cre*-mediated deletion of single genes encoding for *Ulk1*, *Ulk2*, *Atg4b*, *p62*, *Atg14*, *Atg12*, *Atg16*, *Atg7* or *Atg3*, each sufficient to interfere with autophagosome formation, disease outcome was similar to wild type mice after infection with *M. tuberculosis*. These data question if autophagy solely accounts for the protective effect in this context *in vivo*. Surprisingly, *Lysm-cre*-dependent Atg5 deficiency resulted in increased mycobacterial burden, weight loss, lung pathology, and premature death. Histopathological analysis revealed that lung lesions were massively infiltrated with neutrophils, suggesting a negatively regulatory role for Atg5 in neutrophil recruitment. Indeed, treatment of *M. tuberculosis*-infected *Atg5$^{fl/fl}$-Lysm-cre* mice with a neutrophil-depleting antibody against Ly6G (1A8) normalized lung pathology, survival rate and mycobacterial burden to the situation in wild type animals. A well-established effector mechanism against *M. tuberculosis* growth in mice is the nitric oxide synthase-2-mediated synthesis of nitric oxide. Originally, it was thought that nitric oxide directly affects *M. tuberculosis* viability by its DNA damaging property. However, a recent publication revealed that nitric oxide protects mice from tuberculosis by repressing neutrophil influx, whereas neutrophil accumulation appeared to promote mycobacterial growth by forming a nutrient-rich niche (Mishra et al., 2017). Neutrophil recruitment depended on IL-1 and 12/15-lipoxygenase signaling pathways, which were blocked by nitric oxide. The authors observed a positive correlation between levels of small lipid mediator products of 12-lipoxygenase and neutrophil frequencies and mycobacterial burden in patients with cavernous tuberculosis.

Two main host defense effectors, namely autophagy and nitric oxide, thought to directly control *M. tuberculosis* growth, have now been shown to indirectly benefit host defenses through warding off neutrophil infiltration. These reports indicate a detrimental role for neutrophils in promoting lung pathology during active tuberculosis, both in mice and humans, rather than fighting off the infection, thereby, identifying these otherwise potent defense cells as prime targets of host-directed therapies.

3. Neutrophils in the crosshairs

Neutrophils have just recently stepped into the spotlight of tuberculosis research. One reason why neutrophils remained understudied in tuberculosis is because they are rather elusive in the most commonly used murine models for tuberculosis, namely C57BL/6 and BALB/c mice, which are considered resistant to *M. tuberculosis* infection (Keller et al., 2006; Dallenga and Schaible, 2016). Neutrophils have been found only within the first 10 days of infection in the lungs of these mice but were absent from multifocal lung lesions at later time points. These lesions consist of inflammatory infiltrates dominated by macrophages but do not represent necrotic granulomas as seen in human patients (Irwin et al., 2014). Moreover, depletion of neutrophils in these mice did not change disease outcome (Seiler et al., 2000). However, in mouse strains that are highly susceptible to tuberculosis, namely C3HeB/FeJ and I/St mice, neutrophils represent a significant proportion of the cellular infiltrates at the site of infection. In these strains, neutrophils were associated with necrotic tissue damage and premature death of the animals (Yeremeev et al., 2015). After depletion of neutrophils by administration of a Ly6G-specific antibody, *M. tuberculosis*-infected I/St mice showed reduced weight loss, lower mycobacterial burden, and pathology but increased numbers of specific, IFNγ-secreting T cells and improved survival rates (Yeremeev et al., 2015). C3HeB/FeJ mice, which were first described by Igor Kramnik, are hyper susceptible to *M. tuberculosis* infection compared to other conventional mouse strains (Cardona et al., 2003; Kramnik et al., 1998; Medina and North, 1998; Major et al., 2013; Nikonenko et al., 2000; Apt and Kramnik, 2009). *M. tuberculosis* infection in these mice elicit lung pathology with structured granulomas and neutrophil-driven, centrally caseating, necrotizing, and sometimes even cavitary lung lesions similar to those in humans (Pichugin et al., 2009; Harper et al., 2012; Lanoix et al., 2015; Driver et al., 2012). Although the mechanisms behind early mortality of these animals after infection are still elusive, genetic predisposition plays an important role. Kramnik et al. identified an allele in C3HeB/FeJ mice, termed the “super susceptibility to tuberculosis 1” (sst1) locus, that renders those animals hyper susceptible to *M. tuberculosis* infection (Kramnik et al., 2000; Pichugin et al., 2009; Pan et al., 2005). More precisely, within the sst1 locus, expression of the “intracellular pathogen resistance 1” gene was downregulated in C3HeB/FeJ mice, whereas it was found to be upregulated in sst1-resistant macrophages (Pan et al., 2005). Animals that inherited the susceptible version of the sst1 allele develop necrotic lesions, failed to control growth of virulent *M. tuberculosis* and, ultimately, exhibited reduced survival rates (Kramnik et al., 2000; Lanoix et al., 2015; Pan et al., 2005).

We found that human neutrophils, isolated from peripheral blood of healthy donors, quickly succumbed to necrotic cell death after infection with virulent *M. tuberculosis in vitro* (Corleis et al., 2012) (Fig. 1A). In contrast, an ESAT-6-deficient *M. tuberculosis* mutant, lacking the functional type 7 secretion system ESX1, failed to induce neutrophil necrosis (Dallenga et al., 2017, *Cell Host Microbe*, accepted). Upon infection with attenuated mutants, neutrophils rather underwent default apoptosis similar to uninfected ones (Fig. 1B). Notably, *M. tuberculosis*-induced neutrophil necrosis was dependent on the neutrophil’s own generation of reactive oxygen species since pharmacological inhibition of myeloperoxidase prevented necrosis (Corleis et al., 2012). Ramos-Kichik et al. described that *M. tuberculosis* infection triggered NETosis (Ramos-Kichik et al., 2009). Moreover, NETosis induction depends on the generation of reactive oxygen species by myeloperoxidase (Bjornsdottir et al., 2015). Thus, it is possible that the *M. tuberculosis*-induced neutrophil necrosis observed by us is associated with or resembles NETosis, a neutrophil-specific cell death that also features plasma membrane disruption along with DNA and intracellular molecules release into the extracellular space. However, despite observing NET-like structures, which protrude from the *M. tuberculosis*-infected neutrophil into the extracellular space, those formations did not stain with Sytox Green and, thus, are not composed of stainable DNA (Dallenga et al., 2017, *Cell Host Microbe*, accepted). Rather the whole *M. tuberculosis*-infected neutrophil became quickly Sytox Green-positive during necrotic cell death, suggesting a sudden plasma membrane breach with no directed release of DNA into the extracellular space. In a co-culture system of