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Granzyme B-expressing neutrophils correlate with bacterial load in granulomas from *Mycobacterium tuberculosis*-infected cynomolgus macaques

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Summary

The role of neutrophils in tuberculosis (TB), and whether neutrophils express granzyme B (grzB), a pro-apoptotic enzyme associated with cytotoxic T cells, is controversial. We examined neutrophils in peripheral blood (PB) and lung granulomas of *Mycobacterium tuberculosis*-infected cynomolgus macaques and humans to determine whether mycobacterial products or pro-inflammatory factors induce neutrophil grzB expression. We found large numbers of grzB-expressing neutrophils in macaque and human granulomas and these cells contained more grzB+ granules than T cells. Higher neutrophil, but not T cell, grzB expression correlated with increased bacterial load. Although unstimulated PB neutrophils lacked grzB expression, grzB expression increased upon exposure to *M. tuberculosis* bacilli, *M. tuberculosis* culture filtrate protein or lipopolysaccharide from *Escherichia coli*. Perforin is required for granzyme-mediated cytotoxicity by T cells, but was not observed in PB or granuloma neutrophils. Nonetheless, stimulated PB neutrophils secreted grzB as determined by enzyme-linked immunospot assays. Purified grzB was not bactericidal or bacteriostatic, suggesting secreted neutrophil grzB acts on extracellular targets, potentially enhancing neutrophil migration through extracellular matrix and regulating apoptosis or activation in other cell types. These data indicate mycobacterial products and the pro-inflammatory environment of granulomas up-regulates neutrophil grzB expression and suggests a previously unappreciated aspect of neutrophil biology in TB.

Introduction

Granzyme B (grzB) is a serine protease associated with cytotoxic T-lymphocyte-mediated defence against intracellular pathogens (Barry and Bleackley, 2002; Afonina *et al.*, 2010)

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Supporting information

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Conflict of interest

The authors declare no competing financial interests.

and cancer (Cullen *et al.*, 2010; Rousalova and Krepela, 2010). GrzB enters target cells through a perforin-dependent mechanism and acts on intracellular substrates including pro-caspase 3 (Trapani and Smyth, 2002), thereby initiating apoptosis and pathogen clearance. Although commonly associated with T cells and natural killer cells, non-lymphoid lineage cells including chondrocytes (Horiuchi *et al.*, 2003), keratinocytes (Hernandez-Pigeon *et al.*, 2006; 2007) and macrophages (Choy *et al.*, 2003; Kim *et al.*, 2007; Vernoooy *et al.*, 2007) can also express grzB. The question of whether neutrophils can express grzB or perforin has been contentious (Grossman and Ley, 2004; Metkar and Froelich, 2004; Wagner *et al.*, 2004; 2008; Martin *et al.*, 2005). In addition to its intracellular activity, grzB secreted into the extracellular milieu can target a range of proteins including von Willebrand factor, cell surface receptors and extracellular matrix (ECM) components including fibronectin, vitronectin, laminin and fibrillin-1 (Hendel *et al.*, 2010). As such, extracellular grzB could act as a modulator of cell activation by cleaving activatory or inhibitory receptors or their ligands (Boivin *et al.*, 2009). Excessive grzB may be pathologic and extracellular grzB has been associated with abdominal aneurysm (Chamberlain *et al.*, 2010), rheumatoid arthritis (Boivin *et al.*, 2009) and chronic obstructive pulmonary disease (COPD) (Ngan *et al.*, 2009).

Tuberculosis (TB) is caused by *Mycobacterium tuberculosis*, a pathogenic intracellular bacterium that causes pulmonary disease characterized by granulomas and associated inflammation. The granuloma represents the functional unit of bacterial containment, and is a multicellular structure where, when successful, lymphoid and myeloid cell lineages mount a coordinated response that maximizes antibacterial responses while minimizing immunopathy to uninvolved peripheral tissues. Protection against TB is strongly correlated with CD8+ T cell-mediated cytotoxicity in murine models (Flynn *et al.*, 1992; Sousa *et al.*, 2000). That said, the role of grzB in TB and the cell types that produce it have been difficult to ascertain. A murine model using the vaccine strain BCG indicates that grzB is up-regulated in lung cells by 14 days post-infection (Aranday-Cortes *et al.*, 2012). Other studies using knockout mice have demonstrated that abrogating perforin expression limits CD8+ T cell-mediated cytotoxicity (Serbina *et al.*, 2000; Woodworth *et al.*, 2008) and impairs bacterial control (Cooper *et al.*, 1997), but eliminating grzB expression has little effect on bacterial control (Cooper *et al.*, 1997). Uninfected and latently infected humans have peripheral blood CD8+ T cells that produce more grzB on a per cell basis than patients with active pulmonary TB (Silva *et al.*, 2014), suggesting that low levels of T cell grzB expression may be a risk factor for disease development. In contrast to the T cell-specific data where intracellular grzB is measured *ex vivo*, higher levels of soluble grzB have been measured in the serum of individuals with cavitary TB, with more grzB being correlated with larger sized cavities (Djoba Siawaya *et al.*, 2008). Similarly, individuals with rapid responses to drug therapy had less soluble grzB in serum than individuals with slower treatment responses (Brahmbhatt *et al.*, 2006). These data suggest that while cytotoxic T cells are important for controlling TB, excessive levels of extracellular grzB may be a negative prognosticative marker in TB. These studies do not, however, address what cell types make grzB in granulomas, what factors induce its up-regulation and how severity of disease and bacterial burden modifies grzB expression.

To better understand the role of grzB in TB, we examined granulomas from *M. tuberculosis*-infected cynomolgus macaques, a non-human primate that accurately models human TB, to determine how grzB is localized and which cells expressed it. To our surprise, we found that neutrophils were the most common grzB-positive cells in granulomas and expressed more grzB on a per cell basis than T cells but did not express perforin. Neutrophil grzB expression could be induced by stimulation with mycobacterial antigens or by whole *M. tuberculosis*, and was secreted despite the lack of perforin expression in neutrophils. Moreover, we found neutrophil grzB expression correlated with bacterial burden suggesting that neutrophil grzB expression in the granuloma may be related to antigen load. These data provide a window into a previously unappreciated aspect of neutrophil biology in TB and suggest that neutrophil-secreted grzB may be a contributing factor underlying poorly controlled TB.

Results

Preliminary experiments examining grzB expression by immunohistochemistry (IHC) indicated that granulomas contain numerous grzB-expressing cells (Fig. 1A), and many of these cells are in regions low in T cells but rich in neutrophils. A closer examination of these cells demonstrated that while CD8+ T cells can be positive for grzB, substantial numbers of the grzB+ cells did not express CD3 (Fig. 1A) or CD8 (Fig. 1B). These cells had segmented nuclei (Fig. 1B) and co-stained for calprotectin, indicating that they are neutrophils (Fig. 1C) (Mattila *et al.*, 2013). Further analysis showed that most grzB signal co-localized in elastase-positive granules, with much less co-localization with cathelicidin- and matrix metalloproteinase 9 (MMP9)- (gelatinase) positive granules (Supplemental Fig. S3), suggesting grzB is present in the azurophilic neutrophil granules (Pham, 2006).

We sampled a series of granulomas from animals with active TB to determine how common neutrophil grzB expression was and to compare the number and frequency of grzB+ neutrophils and T cells in macaque granulomas. Per imaging field, granulomas contained more T cells than neutrophils (Fig. 2A) and neutrophil numbers but not T cell numbers positively correlated with bacterial load [colony-forming unit (CFU)] per granuloma (neutrophils: Spearman $r = 0.4135$, $P = 0.0358$; T cells: $r = 0.1995$, $P = 0.3285$) (Fig. 2B). Of these two cell populations, more neutrophils expressed grzB than T cells (Fig. 2C) and the quantity of grzB per cell, when measured as the number of grzB-stained granules per cell (Fig. 2D), indicated that neutrophils contain significantly more grzB than CD3+ T cells. Because of the relationship between perforin and grzB-mediated cytotoxicity in T cells (Trapani and Smyth, 2002), we examined perforin expression in these cell types. T cell-rich granuloma regions contained numerous perforin-expressing cells whereas neutrophil-rich regions had relatively little perforin staining (Fig. 3A). This relationship was also borne out by a quantitative comparison of T cell and neutrophil perforin expression (Fig. 3B), suggesting that although neutrophils can express grzB, they do not engage in perforin-dependent cytolytic activities.

Human granulomas from TB patients were examined for neutrophil grzB and perforin expression. These granulomas come from individuals who have failed drug treatment and represent complex pathologies associated with repeated cycles of drug therapy and disease relapse, suggesting they may contain large quantities of *M. tuberculosis* antigens. GrzB+

neutrophils were present in these tissues and, as with macaque granulomas, were particularly abundant at the epithelioid macrophage–caseum interface (Fig. 4A). Quantification of the grzB expression (Fig. 4B) and perforin expression (Fig. 4C) indicated that neutrophils in human granulomas are significant contributors to grzB expression but do not express appreciable amounts of perforin.

Identifying neutrophil grzB expression led us to investigate whether neutrophils constitutively express grzB or if expression is induced by activation. Substantial numbers of T cells in unstimulated peripheral blood expressed grzB, but very few grzB-expressing neutrophils were observed (Fig. 5A). Perforin expression by neutrophils in peripheral blood was not observed (data not shown). To determine how activation changes grzB expression by neutrophils, we stimulated cells from red blood cell (RBC)-lysed whole blood with mycobacterial peptides, bacterial ligands and non-specific cell activators [phorbol 12,13 dibutyrate (PDBu) and ionomycin] and measured grzB expression by flow cytometry. PDBu and ionomycin, a chemical cocktail that induces protein kinase C and calcium-dependent signalling pathways (Asehnoune *et al.*, 2005), up-regulated T-cell grzB expression somewhat, but strongly up-regulated neutrophil grzB expression (Fig. 5B). Stimulating cells with ESAT6 and *M. tuberculosis* 38.1 (also known as CFP10), a cocktail of *M. tuberculosis*-specific peptides frequently used to elicit T-cell cytokine responses, did not induce significant up-regulation of grzB by either T cells (data not shown) or neutrophils (Fig. 5B). Conversely, stimulation with *M. tuberculosis* culture filtrate protein (CFP) and *Escherichia coli* lipopolysaccharide (LPS) increased neutrophil grzB expression above basal (unstimulated) and peptide-stimulated expression levels (Fig. 5B). CFP-mediated grzB expression by neutrophils was not significantly different than the expression induced by LPS stimulation (Fig. 5B). T cells did not appear to respond to LPS stimulation by up-regulating grzB, tumour necrosis factor (TNF) or interferon- γ (IFN- γ) (data not shown). These data suggest that pro-inflammatory environments containing bacterial ligands, including mycobacterial products, can stimulate grzB expression by neutrophils in the absence of pro-inflammatory T cell cytokines.

GrzB-mediated T cell cytotoxicity requires perforin (Trapani and Smyth, 2002), but neutrophils do not express perforin, raising the question of whether neutrophils secrete grzB, and if they do, is grzB secretion antigen dependent? To answer these questions, we performed grzB enzyme-linked immunospots (ELISPOTs) on paired samples of neutrophil-depleted (buffy coat) peripheral blood mononuclear cells (PBMCs) and purified neutrophils using three categories of stimulators: T cell-specific stimulators (ESAT6+38.1 peptides), bacterial toll-like receptor (TLR) ligands (mycobacterial CFP or LPS) and non-specific cell activators (PDBu and ionomycin). ELISPOT analysis of grzB secretion identified distinct differences between buffy coat (non-neutrophil) PBMCs and neutrophils (Fig. 6A). Small numbers of buffy coat cells secreted grzB, but there was little evidence of antigen-specific or protein kinase C (PDBu and ionomycin) stimulated grzB expression (Fig. 6B). In contrast, neutrophil grzB secretion was up-regulated by CFP-and PDBu + ionomycin stimulation and LPS-stimulated cells also showed a slight, but not statistically significant, increase in grzB expression (Fig. 6C).

Our data indicate that *M. tuberculosis* antigens can elicit grzB expression from macaque neutrophils. We next sought to determine whether live *M. tuberculosis* can induce grzB expression by neutrophils. Flow cytometry-based experiments identified that exposure to viable *M. tuberculosis* induced grzB expression in neutrophils from RBC-lysed whole blood (Fig. 7A). Likewise, we noted that isolated neutrophils secreted grzB following incubation with *M. tuberculosis* whereas neutrophil-depleted PBMCs did not (Fig. 7B). Granulomas can vary significantly in both bacterial burden (Lin *et al.*, 2014) and neutrophil abundance (Mattila *et al.*, 2013), suggesting that exposure of neutrophils to bacterial products in granulomas varies over a wide range. This led us to investigate whether neutrophils, which are exquisitely sensitive to bacterial products, have grzB expression that is influenced by bacterial burden *in vivo*. To investigate this, we stained granulomas of known bacterial burden (from plating for CFU at necropsy) and correlated the number of bacteria per granuloma with the mean number of T cells and neutrophils in the images for each granuloma, and the number of grzB+ granules per cell (Fig. 7C). We found a trend in the relationship between numbers of bacteria per granuloma and grzB expression by neutrophils (Spearman $r = 0.3468$, $P = 0.0827$), but not for T cells (Spearman $r = -0.1115$, $P = 0.5878$), suggesting that bacterial products can drive neutrophil grzB expression *in vivo*. There was variability in grzB expression by neutrophils and one monkey whose granulomas had normal numbers of granuloma neutrophils and T cells (Fig. 7C, open markers) had very low neutrophil grzB expression, but normal T cell grzB expression. The consequence of this variability is not known.

Discussion

Neutrophils are among the first cells to respond to injury or infection and can use a formidable range of effectors for host defence, including phagocytosis and the ability to generate reactive oxygen intermediates and antimicrobial peptides (Nathan and Shiloh, 2000; Winterbourn and Kettle, 2013; Mayadas *et al.*, 2014). Additionally, neutrophils contribute to cytokine, chemokine and growth factor production (Kemp *et al.*, 2005; Doz *et al.*, 2013; Wang *et al.*, 2013); transport microbes to lymph nodes (Abadie *et al.*, 2005); and participate in T cell priming (Morel *et al.*, 2008; Blomgran and Ernst, 2011; Blomgran *et al.*, 2012), but are generally not considered to engage in T cell-like cytotoxic activity. Although neutrophils can be abundant in tuberculous granulomas, we know very little about what they do (Lowe *et al.*, 2012). Evidence from zebrafish infected with *M. marinum* suggests that neutrophils play a protective role and can restrict bacterial growth during early infection by killing bacteria in apoptotic macrophages (Yang *et al.*, 2012). A rat-based model of early *M. tuberculosis* infection where recruiting neutrophils to the lungs via inhaled LPS leads to decreased viable bacterial viability suggests that neutrophils may have a similar function in mammalian lungs (Sugawara *et al.*, 2004). In contrast, data from murine and non-human primate models, and from human TB patients at later stages of infection, associate large numbers of neutrophils with poorly controlled disease (Ozaki *et al.*, 1992; Law *et al.*, 1996; Ashitani *et al.*, 2002; Brahmhatt *et al.*, 2006; Lin *et al.*, 2010; Nandi and Behar, 2011). In humans, a neutrophil-derived interferon signature is one of the first indicators of active TB (Berry *et al.*, 2010), and neutrophils are the most common *M. tuberculosis*-infected cell type in human airways and can contain viable, replicating bacteria (Eum *et al.*, 2010). These data

imply that neutrophils may not be protective beyond the earliest stages of infection, but instead may engage in activities that modify the granuloma microenvironment in favour of mycobacterial survival, or even provide an alternative replicative niche for *M. tuberculosis*. Considering these disparate observations, neutrophils may play different roles at different phases of infection, being beneficial in very early stages of infection but becoming progressively more pathologic in situations of dysregulated cytokine production, excessive inflammation or higher bacterial burdens. These contradictions beg for further exploration into what neutrophils are doing in granulomas, how these activities may influence granuloma homeostasis and bacterial containment, and whether neutrophils can be manipulated in ways that promote bacterial control.

Like the role of neutrophils in TB, grzB and perforin expression by neutrophils is controversial. Wagner *et al.* (2004) were among the first to identify constitutive neutrophil grzB expression with some variability among different individuals. Metkar and Froelich (2004) dispute this and were unable to identify grzB expression, attributing Wagner *et al.*'s results to methodological errors in their antibody-based assays and lymphocyte contamination in reverse transcription polymerase chain reaction, Western blots and biochemical assays. Grossman and Ley (2004) and Martin *et al.* (2005) corroborated this lack of grzB expression in mice and human volunteers. Although these studies come to different conclusions, it should be noted that the neutrophils used by these groups were obtained from healthy human donors except for Martin *et al.*'s study (2005), which included lymphoma and leukaemia patients. Cells from individuals with inflammatory conditions or infections (such as TB), or from tissues, may give different results. Wagner *et al.* noted that neutrophil grzB expression could be up-regulated by co-incubation with purified IFN- γ (2004), suggesting the inflammatory milieu may contribute to grzB expression. In subsequent work, this group described grzB expression not only in mature neutrophils but also in myeloid origin cell lines and stem cell-derived neutrophils (Wagner *et al.*, 2008). GrzB has been identified in a wide variety of non-lymphocyte cell types in recent years, indicating grzB expression is more broadly distributed than previously thought (Hendel *et al.*, 2010). Perforin expression by neutrophils has been equally contentious (Metkar and Froelich, 2004; Wagner *et al.*, 2004; Martin *et al.*, 2005), and although we looked for perforin-expressing neutrophils, our data suggest that in macaque and human granulomas, or in stimulated macaque blood, neutrophils do not express appreciable amounts of perforin.

Our data demonstrate that large numbers of neutrophils in cynomolgus macaque and human granulomas express grzB. Moreover, granulomas with greater bacterial burdens contained more neutrophils, and more of those neutrophils express grzB, than granulomas with lower bacterial burdens, suggesting a dose-dependent relationship between neutrophil grzB expression and *M. tuberculosis in vivo*. Neutrophil grzB expression was induced by mycobacterial CFP, which is an undefined mixture of secreted proteins and lipids, in a manner that was very similar to what we observed with *E. coli* LPS, a known TLR4 agonist that signals through protein kinase C (Yang *et al.*, 2007), suggesting an analogous mechanism of activation may be occurring with CFP. Unstimulated peripheral blood neutrophils are grzB negative, but TB usually presents as a highly localized disease and the granuloma microenvironment is rich in mycobacterial antigens that appear to stimulate a

robust, but localized, neutrophil grzB response. Moreover, our *in vitro* experiments did not find that CFP or LPS up-regulated IFN- γ or TNF expression by neutrophils or T cells, suggesting that neutrophil grzB expression can be independent of these pro-inflammatory T cell cytokines. Our ELISPOT data using T cell-depleted neutrophil populations further suggest that T cell-produced cytokines or T cell: neutrophil contact is not required for neutrophil grzB expression. Immunohistochemical data from macaque granulomas indicate that neutrophils in granulomas are negative for phosphorylated STAT1 and STAT3 (unpublished) indicators of interferon or IL-6 and IL-10 signalling, respectively, implying that cytokines using these signalling pathways are not likely to be involved in neutrophil grzB expression.

Serine proteases are important components of the neutrophil armamentarium that can have diverse functions. These proteases assist neutrophil migration through tissues, regulate activation of other cells by cleaving cell-surface receptors, proteolytically activate cytokines and antimicrobial peptides, and have their own intrinsic antimicrobial activity in extracellular and intraphagosomal environments (Raptis and Pham, 2005; Pham, 2006; 2008; Standish and Weiser, 2009; Omoto *et al.*, 2010). In murine TB models, containment of *M. tuberculosis* requires serine protease activity (Reece *et al.*, 2010). GrzB is a serine protease, and although best known for its targeted perforin-dependent cytotoxicity, grzB in the extracellular milieu is implicated in proteolytic activation of the pro-inflammatory cytokines IL-1 α (Afonina *et al.*, 2011) and IL-18 (Omoto *et al.*, 2010), and anti-thrombotic activity (Buzza *et al.*, 2008). GrzB can attack a variety of ECM components including fibronectin, fibrinogen, vitronectin, laminin, fibrillin-1 and collagen IV (Boivin *et al.*, 2009; Cullen *et al.*, 2010; Hendel *et al.*, 2010; Hiebert and Granville, 2012). This can result in release of ECM-bound growth factors and cytokines (Boivin *et al.*, 2009; Hiebert and Granville, 2012), production of chemotactic peptide fragments or increased expression of pro-inflammatory cytokines such as IL-6, IL-8 and TNF (Birdsall *et al.*, 2004; Kuhns *et al.*, 2007; Boivin *et al.*, 2009; Cullen *et al.*, 2010; Hendel *et al.*, 2010; Hiebert and Granville, 2012). Moreover, loss of adhesion to the ECM can lead to apoptosis (anoikis) in contact-dependent cells (Choy *et al.*, 2004). We predict that neutrophils in the granuloma can secrete grzB into the extracellular environment, but we have not observed perforin expression, a molecule required for T cell-mediated cytotoxicity (Trapani and Smyth, 2002), suggesting that neutrophil-expressed grzB may be capable of inducing anoikis in nearby cells. Cathepsin G, a serine protease in the same granule as grzB, has been implicated in neutrophil-mediated cardiac myocyte anoikis (Rafiq *et al.*, 2006; 2008). It is not known whether this happens in the granuloma, but an accumulation of grzB-secreting neutrophils in the region adjacent to caseum conceivably could be sufficient to degrade cell: cell adhesions between epithelioid macrophages, thereby inducing anoikis in these cells.

Dys-regulated protease expression, and secretion into the extracellular environment, can have maladaptive consequences that lead to pathology. Although neutrophil serine proteases may play protective roles in some models of mycobacterial disease (Reece *et al.*, 2010; Jena *et al.*, 2012; Steinwede *et al.*, 2012), neutrophil proteases are also associated with pathology including acute lung injury (Zhou *et al.*, 2012), COPD (Owen, 2008; Hoenderdos and Condliffe, 2013), emphysema (Wright and Churg, 2007) and *M. avium* infection (Yamazaki

et al., 1998). Secreted (extracellular) grzB is associated with conditions that include pathologic tissue remodelling including rheumatoid arthritis (Boivin *et al.*, 2009), aortic aneurysm (Chamberlain *et al.*, 2010) and COPD (Ngan *et al.*, 2009). In addition to directly causing pathology, cleavage of ECM, generation of chemotactic factors and cytokine activation, grzB may lead to an excessive inflammatory response that no longer helps contain *M. tuberculosis*, but promotes dissemination instead. Moreover, *in vitro* assays demonstrated that recombinant human grzB is not bactericidal for *M. tuberculosis* nor does it alter bacterial growth kinetics (Supplemental Fig. S4), suggesting that secreted grzB does not have direct bactericidal or bacteriostatic properties. Taken together, these factors suggest that neutrophil grzB production may not be associated with bacterial killing, but may function to facilitate neutrophil migration through the granuloma's dense cellular network.

Historically, neutrophils have been considered to be short-lived phagocytes that are critically important for protection against acute infections, but have less involvement in chronic disease. More recent data have suggested these cells are considerably more dynamic than previously thought, and may have surprising and unexpected functions that can be consequential in a wide range of conditions. The relationship between neutrophils and pathology in TB continues to be complex. Identification of grzB as an additional factor produced by neutrophils presents another possible target for therapeutic intervention that may increase the options for treating TB.

Experimental procedures

Animal husbandry and ethics assurance

Cynomolgus macaques (*Macaca fascicularis*) were housed and maintained by the University of Pittsburgh's Department of Laboratory Animals. All procedures were performed in accordance with protocols approved by the University of Pittsburgh's Institutional Animal Care and Use committee. Experiments were performed on samples from *M. tuberculosis*-infected animals enrolled as untreated controls in unrelated ongoing studies. These animals were not vaccinated, undergoing anti-mycobacterial treatments or experiencing immunomodulatory therapies. For human samples, anonymized lung tissue containing granulomas were collected from patients with treatment refractory TB during therapeutic lung resection surgery at National Masan Hospital. The collection was approved by the hospital's institutional review board, an exemption from National Institutes of Health and with written consent of the subjects.

IHC

Multiparameter IHC was performed on formalin-fixed paraffin-embedded (FFPE) tissue sections from granuloma-containing lung samples as previously described (Mattila *et al.*, 2013). Purified antibodies for IHC were anti-human grzB (clone Grb7; DAKO), anti-human CD8 (clone 1A5; Leica Microsystems), anti-human perforin (clone Ab-2; ThermoFisher), anti-human CD3 (rabbit polyclonal; DAKO), anti-human cathelicidin (rabbit polyclonal; Abcam), anti-human MMP9 (rabbit polyclonal; Abcam) and anti-human neutrophil elastase (rabbit polyclonal; Abcam). A rabbit polyclonal anti-grzB antibody (Abcam) was used for staining human tissue sections. Anti-human calprotectin (clone MAC387; ThermoFisher)

was used to identify neutrophils (Mattila *et al.*, 2013). For detection, antibodies were stained with fluorochrome-labelled secondary antibodies purchased either from Life Technologies or Jackson ImmunoResearch. In applications where two mouse antibodies were needed to stain antigens in the same tissue section (calprotectin and grzB), the least abundant antigen (grzB) was stained using indirect immunofluorescence while and the more abundant antigen (calprotectin) was stained using antibodies labelled with Zenon antibody-labelling reagents (Life Technology). CD8 staining was identified by tyramide amplification (Perkin Elmer) and performed as per the manufacturer's protocol. After staining, coverslips were mounted over the tissue sections with Prolong Gold Antifade Mounting Medium with DAPI (Life Technologies) and the tissue sections were imaged with an Olympus Fluoview laser scanning confocal microscope. For granulomas that were too large to be imaged in a single 20× field, multiple overlapping images were acquired and assembled into a single montage using Photoshop CS4 (Adobe Systems).

Image analysis

Cells in immunofluorescently stained FFPE tissue sections were counted manually to determine the frequency of grzB-positive neutrophils and T cells using Photoshop CS4. For studies comparing grzB expression and bacterial burden, FFPE granulomas with known bacterial burden were stained. These granulomas, and their bacterial burdens, were obtained as previously indicated (Lin *et al.*, 2009; 2012; 2014). Briefly, granulomas obtained at necropsy were bisected; half was mechanically homogenized and plated on 7H11 agar plates for bacterial enumeration and the other half fixed in formalin and embedded in paraffin. CellProfiler 2.0 (Carpenter *et al.*, 2006) was used to quantify the area per cell cross-section and number of granules per cell in these granulomas. To quantify these metrics, 40× fields were acquired and individual cells with diameters similar to what we expect for an intact cell's cross section, and not touching other cells, were selected and analysed by CellProfiler. For analysis of granules per cell, 40× fields that contained neutrophils and T cells were selected for analysis while fields that contained caseum or heavily clustered cells were excluded from analysis. Granulomas with autofluorescent artefacts were also excluded from analysis. Granulomas can differ significantly in size and between one and four fields were used included in the final analysis. A CellProfiler pipeline that identified the number of grzB + granules, T cells, neutrophils per image and then overlaid the grzB on top of the masked cells to quantify the number of grzB+ granules per cell was used for analysis.

Neutrophil stimulations and flow cytometry

Erythrocytes in whole blood were lysed using RBC lysing solution (BD Biosciences), and the nucleated cells resuspended in Roswell Park Memorial Institute (RPMI) medium (Sigma Aldrich) containing 10% human AB serum (Gemini Bio-Products), 1% N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulphonic acid] (Sigma) and 1% L-glutamine (Sigma). A total of 10⁶ cells were transferred to sterile polystyrene tubes and incubated 37°C/5% CO₂ in the presence of antigens including mycobacterial ESAT6 and 38.1 (also known as CFP10) (chemically synthesized overlapping peptide pools, 10 µg ml⁻¹ each protein, GenScript USA), mycobacterial CFP (10 µg ml⁻¹; GenScript USA), purified lipopolysaccharide from *E. coli* (LPS) (20 EU ml⁻¹; Sigma) or a cocktail of PDBu and ionomycin (25 nM and 5 µM final concentrations, respectively; Sigma). Recombinant ESAT6 and 38.1 proteins were not

used because they contained small amounts of endotoxin, although preliminary studies indicated there was not a quantitative difference between neutrophil responses to chemically synthesized peptides or *E. coli*-expressed recombinant proteins (Supplemental Fig. S1). For cells incubated with viable *M. tuberculosis*, virulent Erdman strain *M. tuberculosis* were cultured to mid-log growth phase in 7H9 medium and quantified with a spectrophotometer. A volume equivalent to 10^6 bacteria (multiplicity of infection = 1, based on the OD of cultures with known CFU) was centrifuged and the bacteria were resuspended in RPMI before being added to the cells under BSL3 conditions. After 30 min of incubation with stimulators or bacteria, brefeldin A (BD Biosciences) was added at a $1\times$ concentration to the tubes to inhibit protein secretion and the cells were subsequently cultured for another 3.5 h at $37^\circ\text{C}/5\%$ CO_2 before being stained for CD11b (clone ICRF44), CD3 (clone SP34-2), CD4 (clone SP34) and CD8 (clone SK1). As flow cytometry for activated caspase 3 (clone C92-605) indicated that culture with brefeldin A and/or *M. tuberculosis* did not increase the frequency of apoptotic cells (data not shown). Antibodies for flow cytometry were fluorochrome-conjugated antibodies purchased through BD Biosciences. Surface-stained cells were fixed and permeabilized using Fix-Perm/Perm-Wash reagents (BD Biosciences) and stained intracellularly for grzB (clone GB11). After staining, cells were washed, fixed and read on a FACSCalibur (BD Biosciences) or LSRII (BD Biosciences) flow cytometer and data were analysed with FlowJo (Treestar).

PBMC and neutrophil grzB ELISPOT

Percoll (GE Healthcare Biosciences) gradient centrifugation was used to separate plasma-free whole blood into a lymphocyte-rich buffy coat fraction (buffy coat cells) and a neutrophil-enriched erythrocyte pellet. The erythrocyte pellet was subsequently lysed of RBCs, yielding a neutrophil-rich cell population. This procedure yielded cell preparations that were significantly enriched for neutrophils (Supplemental Fig. S2) and was used instead of other multistep procedures to minimize the potential for nonspecific neutrophil activation. For ELISPOT assays, lymphocytes or neutrophils (150 000 cells per well) were added to wells of 0.45 μm MultiScreen plates (Millipore, Billerica, MA) and incubated at $37^\circ\text{C}/5\%$ CO_2 in stimulator- or *M. tuberculosis*-containing medium. Cells were stimulated with Erdman strain *M. tuberculosis*, anti-CD3 antibody (a positive control for T cell responses) ESAT6/CFP10, CFP, LPS or PDBu and ionomycin at the previously indicated concentrations. An 18-h incubation period was chosen because of the short life span associated with neutrophils. With the exception of the incubation period, ELISPOT assays were performed according to manufacturer's instructions and developed with the AEC Development Kit (Vector Laboratories). Spots on plates were read with an ELISPOT reader (CTL) and stimulated cell responses compared against stimulator-free media-only wells.

Statistical analysis

Statistical comparisons were performed with GraphPad Prism 6.0 using the non-parametric Mann–Whitney test for independent data. All pairwise comparisons were tested using the nonparametric Wilcoxon signed-rank test for matched pairs and unadjusted *P*-values were reported. *P*-values were not adjusted to reflect the inflated error rate caused by multiple comparisons. Non-parametric Spearman's correlation was performed to analyse the

correlation between T cells, neutrophils, grzB and bacterial load. The threshold for statistical significance was set at $P < 0.05$.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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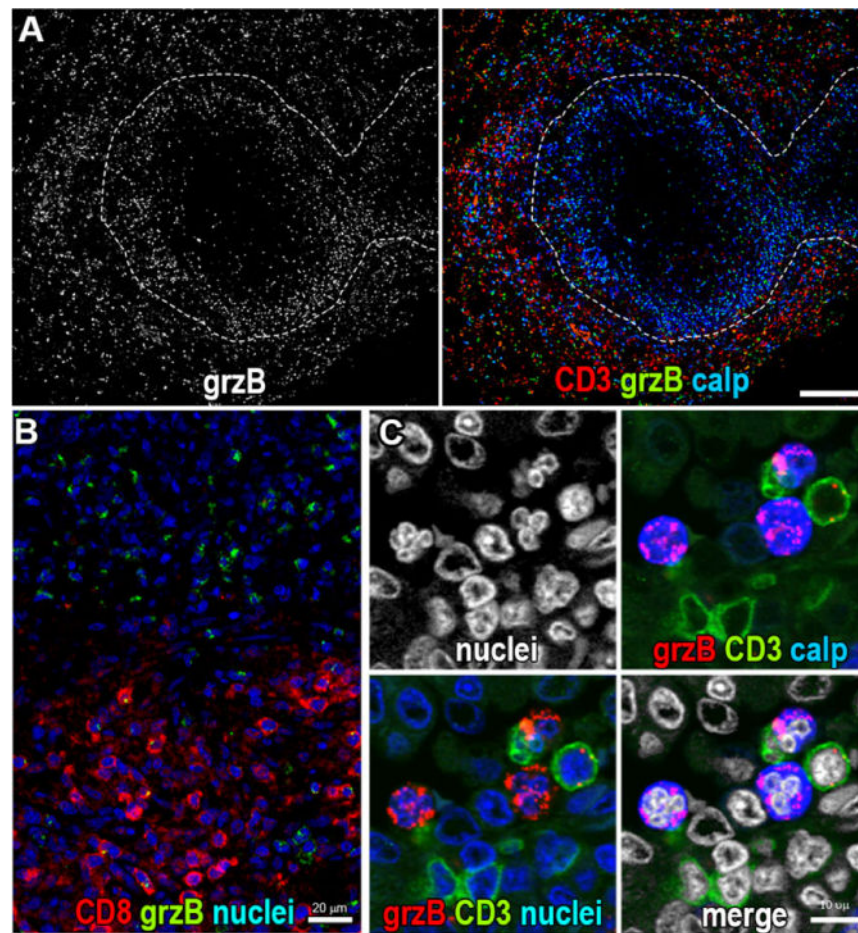


Fig. 1. Neutrophils in granulomas can express grzB. Immunohistochemistry was performed on formalin-fixed paraffin-embedded granulomas to assess the localization and expression of grzB, CD3+ T cells, CD8+ lymphocytes and calprotectin-expressing neutrophils.

A. GrzB (green) localizes with T cells (red) and neutrophils (blue) in a necrotic (caseous) granuloma. The dashed line bisects the epithelioid macrophage region, highlighting a neutrophil-rich, T-cell-poor region adjacent to the caseum with an abundance of grzB expression. Scale bar represents 100 μ m.

B. CD3-grzB+ cells in granulomas do not express CD8, suggesting they are not natural killer cells or $\gamma\delta$ T cells. Scale bar represents 20 μ m.

C. GrzB+ calprotectin+ cells have multilobate nuclei indicating they are neutrophils. Scale bar represents 10 μ m.

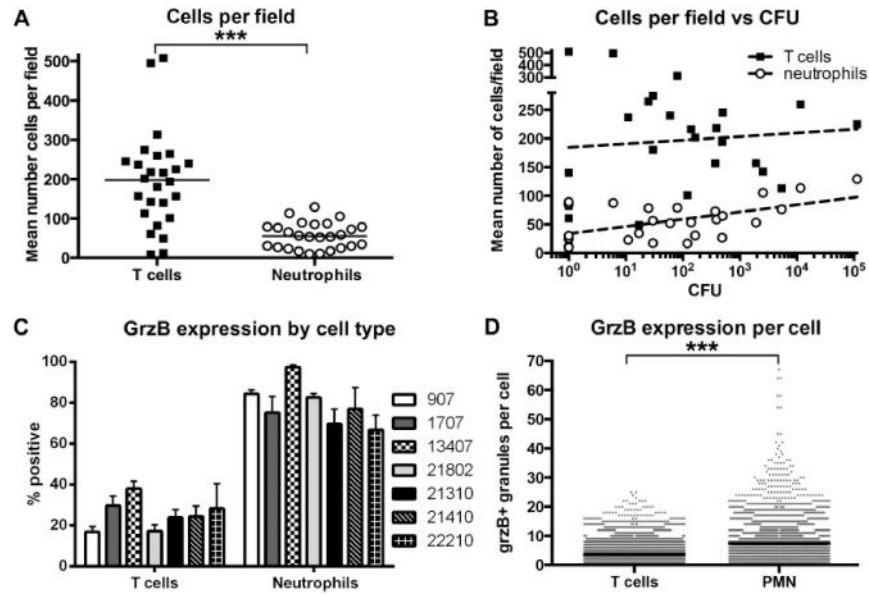


Fig. 2.

Frequency and relative quantification of grzB expression by T cells and neutrophils.

A. Granuloma fields imaged at 40× contained significantly more T cells than neutrophils. Each point represents one granuloma, $n = 12$ monkeys, *** $P < 0.0001$; Mann–Whitney test, bar represents the median.

B. Numbers of neutrophils in granulomas correlate with bacterial load per granuloma, while T-cell numbers do not. Each point represents one granuloma, $n = 12$ monkeys.

C. As a percent of the total population, more neutrophils than T cells express grzB in granulomas. Bars represent the mean with SEM of images from two to four granulomas per animal (monkey ID numbers indicated to the right of the graph).

D. On a per cell basis, granuloma neutrophils contain more grzB+ granules per cell than T cells. $n = 4627$ neutrophils and 10 722 T cells, *** $P < 0.0001$, Mann–Whitney test, bar represents the median.

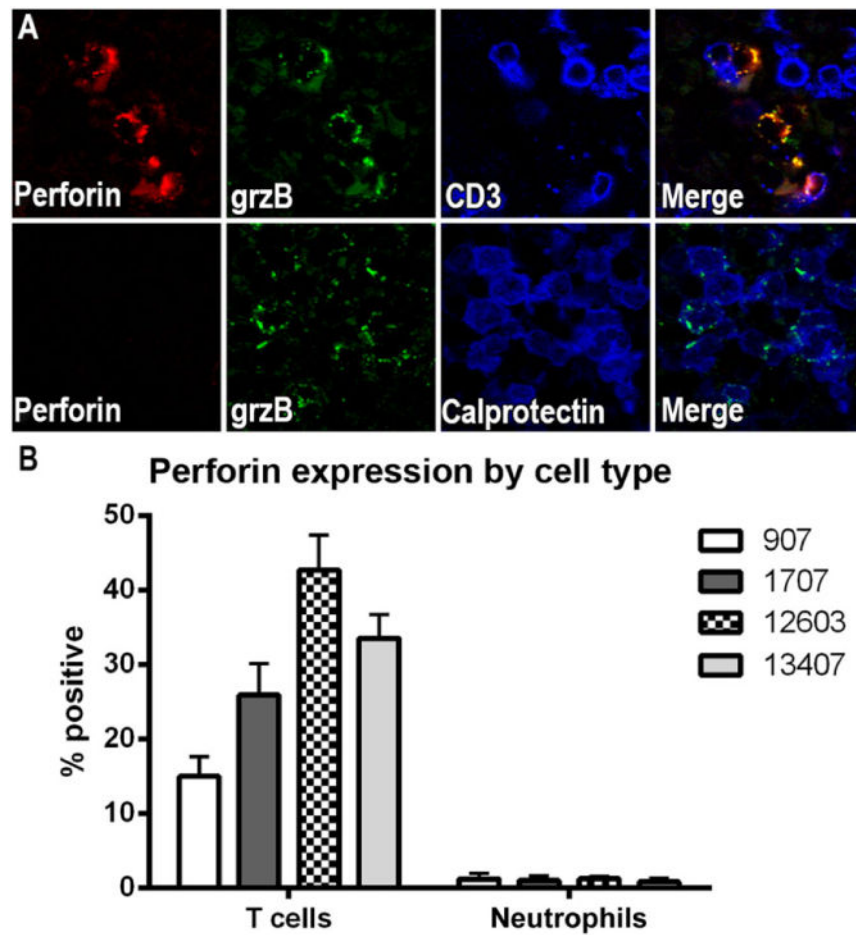


Fig. 3. Neutrophils do not express perforin in granulomas. Images of perforin-stained granulomas were examined for perforin expression.
 A. Perforin co-localizes with grzB in CD3+ T cells but is not observed in calprotectin-stained neutrophils.
 B. Quantification of perforin-expressing T cells and neutrophils in images of perforin-stained granulomas.

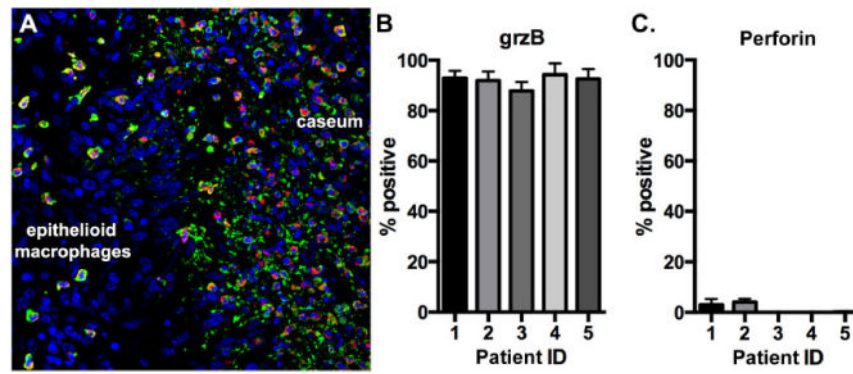


Fig. 4. Neutrophils in human granulomas express grzB but not perforin. Human granulomas were examined for neutrophil grzB and perforin expression.
 A. GrzB (red)-expressing neutrophils (green) preferentially localize at the epithelioid macrophage–caseum interface. Nuclei are indicated in blue.
 B. GrzB expression. C. Perforin expression.

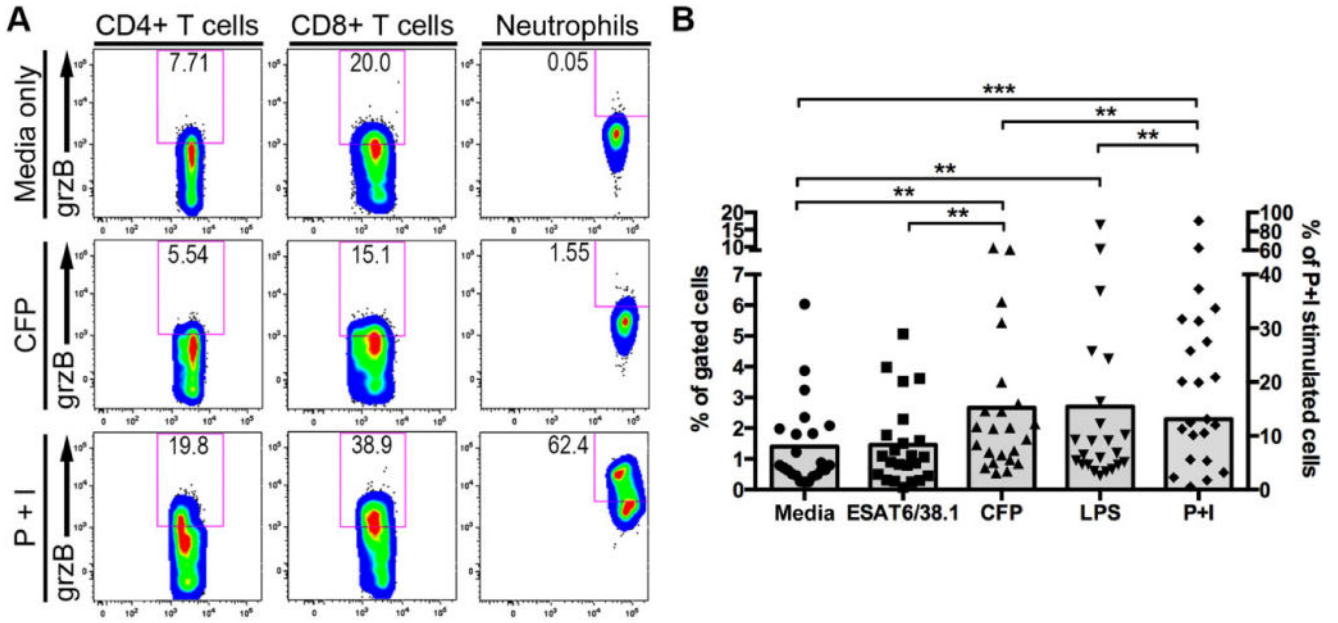


Fig. 5. Stimulating neutrophils with bacterial ligands and pro-inflammatory activators up-regulates grzB expression. PBMCs from RBC-depleted whole blood were stimulated with a cocktail of *M. tuberculosis* ESAT6 and 38.1 peptides, CFP, LPS and P + I and grzB expression was assessed by flow cytometry.

A. Representative FACS plots showing CD4+ and CD8+ T cell and neutrophil activation by CFP and P + I relative to media-only controls.

B. Quantification of grzB expression by neutrophils. Results from PDBu-stimulated cells are represented on the right axis. Each marker represents an individual monkey with $n = 23$ monkeys from five independent experiments depicted, $**P < 0.005$, $***P < 0.0005$, Wilcoxon matched-pairs signed-rank test for pairwise comparison.

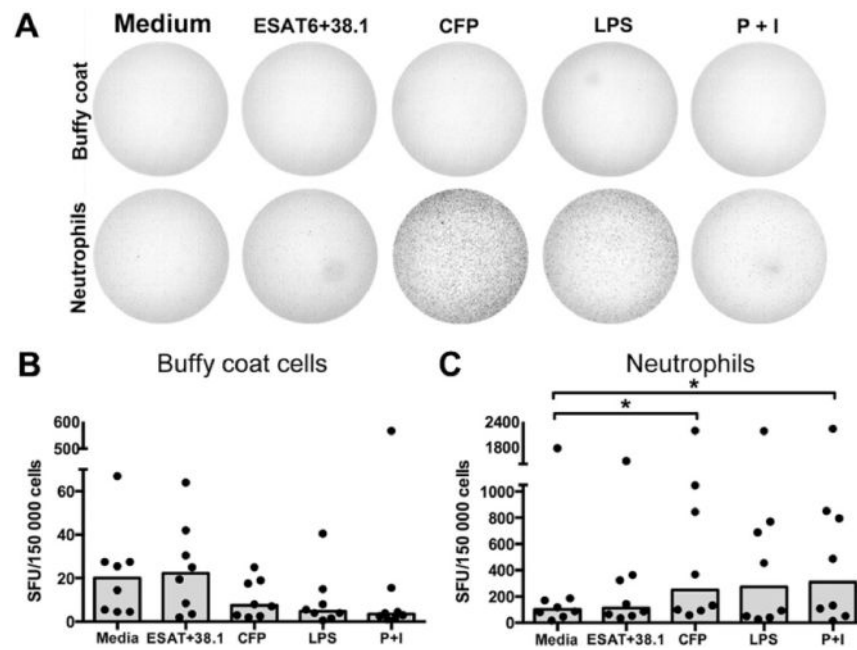


Fig. 6.

Mycobacterial products and pro-inflammatory stimuli elicit grzB secretion by peripheral blood neutrophils. Paired sets of neutrophil-depleted buffy coat PBMCs and purified neutrophils were subject to stimulation in ELISPOT assay and the number of spot-forming units (SFU) were quantified.

A. Representative ELISPOT wells showing the differences between cell fractions and appearance of spots.

B. Quantification of grzB expression. Each spot represents the mean of two wells per cell fraction from each monkey. Bars represent the median of results from two independent experiments, four monkeys per experiment. * $P < 0.05$, Wilcoxon matched-pairs signed-rank test for pairwise comparison between unstimulated and stimulated samples.

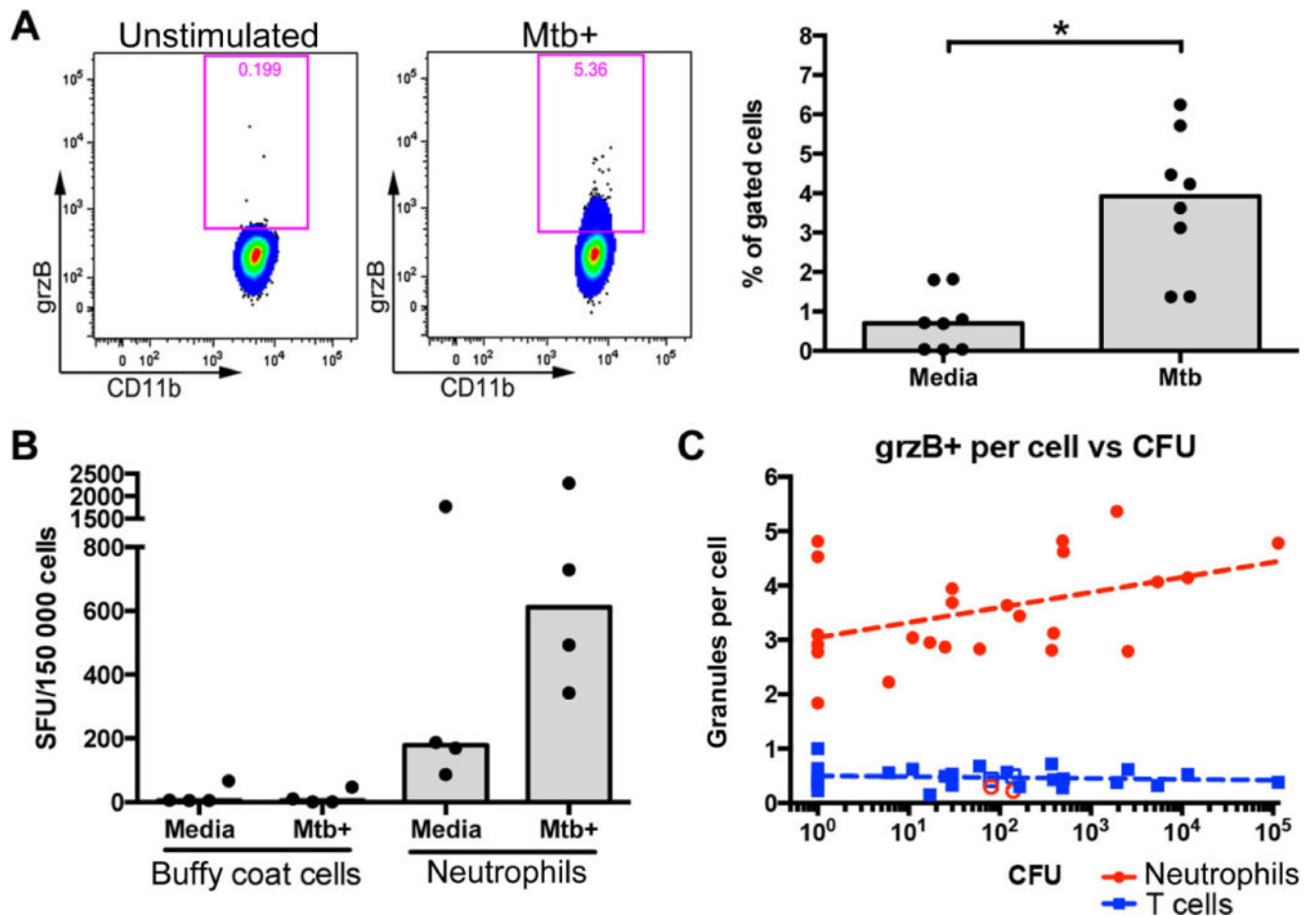


Fig. 7.

GrzB expression is up-regulated by *M. tuberculosis* in an antigen-dependent manner. FACS experiments using whole blood co-cultured with *M. tuberculosis*, ELISPOT assays comparing buffy coat PBMCs with purified neutrophils and immunohistochemistry on tissues with known bacterial burdens demonstrate that *M. tuberculosis* can cause up-regulated grzB expression in neutrophils.

A. FACS experiments using neutrophils from RBC-free PBMCs. Bars represent mean with SEM. * $P < 0.05$; Wilcoxon matched-pairs signed-rank test.

B. ELISPOT assays indicate *M. tuberculosis* does not significantly up-regulate grzB expression by buffy coat PBMCs but does significantly up-regulate grzB expression by neutrophils. * $P < 0.05$; Wilcoxon matched-pairs signed-rank test.

C. Bacterial burdens correlate with higher numbers of grzB+ granules per cell for neutrophils (Spearman $r = 0.3468$, $P = 0.0827$) but not for CD3+ T cells (Spearman $r = -0.1115$, $P = 0.5878$). One monkey (indicated by open markers) had normal numbers of neutrophils and T cells per image, but had neutrophils with very few grzB+ granules per cell, but normal T-cell grzB expression.