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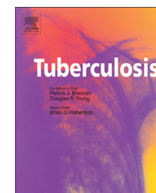

IMMUNOLOGICAL ASPECTS

Increased intra- and extracellular granzyme expression in patients with tuberculosis

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S U M M A R Y

Tuberculosis (TB) is an important cause of morbidity and mortality worldwide. Granzymes (gzms) are proteases mainly found in cytotoxic lymphocytes, but also extracellularly. While the role of gzms in target cell death has been widely characterized, considerable evidence points towards broader roles related to infectious and inflammatory responses. To investigate the expression of the gzms in TB, intracellular gzms A, B and K were measured by flow cytometry in lymphocyte populations from peripheral blood mononuclear cells from 18 TB patients and 12 healthy donors from Bangladesh, and extracellular levels of gzmA and B were measured in serum from 58 TB patients and 31 healthy controls. TB patients showed increased expression of gzmA in $CD8^+$ T, $CD4^+$ T and $CD56^+$ T, but not NK, cells, and of gzmB in $CD8^+$ T cells, when compared to controls. GzmK expression was not altered in TB patients in any lymphocyte subset. The extracellular levels of gzmA and, to a lesser extent, of gzmB, were increased in TB patients, but did not correlate with intracellular gzm expression in lymphocyte subsets. Our results reveal enhanced intra- and extracellular expression of gzmA and B in patients with pulmonary TB, suggesting that gzms are part of the host response to tuberculosis.

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[1] Dr. Hossain passed away during the course of the study.

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1. Introduction

Tuberculosis (TB) is caused by infection with *Mycobacterium* (*M.*) *tuberculosis* and typically affects the lungs. This disease constitutes a serious health problem worldwide, with around one-third of the world's population infected and an estimated 9 million new cases and 1.5 million deaths in 2013 [1]. The majority of TB cases occur in developing countries, with Bangladesh being one of the most affected. Immunity against *M. tuberculosis* depends on a wide range of innate and adaptive immune responses mainly driven by macrophages and different T cell subsets [2].

Granzymes (gzms) are a family of serine proteases found in granules of cytotoxic lymphocytes. In humans five different gzms (A, B, H, K and M) have been identified, of which the expression is generally restricted to cells of the lymphoid lineage [3]. GzmA and gzmB, the most abundant gzms, are expressed constitutively in several cell types including cytotoxic T lymphocytes (CTL), natural killer (NK) cells, NKT cells, and $\gamma\delta$ T cell receptor (TCR) cells [4,5]. GzmK is mainly expressed by CTL [5]. Expression of gzms has also been observed in other cells, including non-lymphoid cells, at least after stimulation [6,7], and even lung parenchymal cells [8]. Multiple studies have described the role of gzms in eliminating infected, neoplasic or foreign cells. While there is a general consensus regarding the role of gzmB in cell death, the physiological relevance of the cytotoxicity produced by gzmA and other gzms remains controversial [3]. Considerable evidence suggests that the effects of gzms are diverse and go beyond cytotoxicity. Plasma levels of gzmA and B have been found elevated in patients with parasitic, viral and bacterial diseases [9,6], severe sepsis [10,11] as well as in induced human endotoxemia [12], whilst induction of gzmA and B secretion has been shown after stimulation of whole blood with bacteria [12]. Extracellular gzms have been implicated in several processes, including induction of proinflammatory cytokine release [7,13]. Studies showing that gzmA and gzmB deficient mice are relatively protected against endotoxin-induced shock further suggest a role for gzms in the regulation of inflammatory responses [14,15].

Some studies have addressed the expression of gzms A and/or B after stimulation with *M. tuberculosis*, while others have studied their extracellular levels or their expression by a specific lymphocyte population in TB patients, hinting at increased gzm expression after stimulation or compared to controls [16–22]. The present study was designed to explore the intracellular expression of gzms A, B and K by diverse lymphocyte populations as well as the extracellular expression of gzms A and B in patients with active pulmonary TB compared to healthy individuals.

2. Materials and methods

2.1. Study subjects

This survey was conducted as an additional study of a previous investigation evaluating activation of the coagulation system in patients with pulmonary TB, of which the results have been published [23]. The current study included 61 adult patients with pulmonary TB recruited prospectively in the Tuberculosis Clinic of Chittagong General Hospital and the Chittagong Medical College & Hospital, Chittagong, Bangladesh. Pulmonary TB was considered confirmed when at least two out of three sputum samples collected on two consecutive days, including an early morning sample, tested positive on Ziehl-Neelsen (ZN)-staining. TB-positivity was confirmed by polymerase chain reaction on *M. tuberculosis* (GeneXpert, Cepheid, Solna, Sweden) in the Laboratory of Microbiology in the Academic Medical Center in Amsterdam, the Netherlands. Patients with concomitant disease or a clinical condition which could interfere with the conduct of the study were excluded. Thirty two healthy blood donors were recruited from the Chittagong Medical College & Hospital and served as controls. Controls and patients were recruited between September and November 2010. The study was approved by the National Research Ethics Committee (NREC), Bangladesh Medical Research Council, Bangladesh and the Oxford Tropical Research Ethics Committee, University of Oxford, Oxford, UK (OXTREC 35-09). Written informed consent was obtained from all study subjects or next-of-kin by a native Bengali speaker.

2.2. Assays

Serum and plasma samples were obtained from patients and controls and were kept at -80 °C. Levels of gzmA and B were measured in serum by ELISA using reagents from Sanquin (Amsterdam, The Netherlands) as previously described [24]. Levels of Interleukin (IL)-6, chemokine (C–X–C motif) ligand (CXCL) 8, interferon gamma-induced protein (IP) 10, soluble IL-2 receptor subunit-α (sIL-2Rα), soluble intercellular adhesion molecule (sICAM)-1, soluble tumor necrosis factor receptor (sTNFR)-1 and sTNFR-2, IL-12, interferon (IFN)-γ and tumor necrosis factor (TNF)-α were measured in EDTA plasma by a multiplex assay (Luminex, Austin, TX, USA) using reagents from Bio-Rad (Veenendaal, The Netherlands). C-reactive protein (CRP) was measured in heparinised plasma samples with the C-Reactive Protein Gen.3 test kit (Roche Diagnostics, Mannheim, Germany), an immunoturbidimetric method, on the Hitachi Modular P-800 module (Hitachi, Hitachinaka, Japan).

2.3. Flow cytometry

Peripheral blood mononuclear cells (PBMCs) were isolated from blood harvested in Cell Preparation Tubes (Becton Dickinson, Franklin Lakes, NJ, USA) according to the manufacturer's instructions, from 18 TB patients and 12 healthy controls (of which 15 patients and 11 controls had also serum data) and viable frozen in RPMI 1640 (Life Technologies) medium with 20% FCS (Lonza, Basel, Switzerland) and 20% DMSO with a mr. Frosty freezing container (Thermo Scientific, Waltham, MA, USA), first placed overnight in a -20 °C freezer and subsequently in the gas phase of a liquid nitrogen container. For flow cytometry, cryopreserved cells were carefully thawed and washed, and $2–5 \times 10^5$ cells/well were incubated with monoclonal antibodies against CD3 (AF700), CD4 (PerCP-Cy5.5), CD56 (APC) (all from BD Pharmingen, Breda, The Netherlands) and CD8 (PE-Cy7; Biolegend, San Diego, CA, USA), at 4 °C for 25 min in the dark. For the intracellular staining, cells were fixed for 20 min in Cytofix/Cytoperm (BD Bioscience, San José, CA, USA) at 4 °C in the dark and then washed with Perm/Wash buffer. Subsequently, the cells were resuspended in Perm/wash buffer containing the antibodies against gzmA (PE; BD Pharmingen), gzmB (PE-CF594; BD Horizon) and gzmK (FITC; Santa Cruz Biotechnology, Dallas, TX, USA). The samples were analyzed by flow cytometry with a FACSCanto (BD Bioscience). The FlowJo software (Tree Star Inc, Ashland, OR, USA) was used to analyze the data. Lymphocytes were gated in the forward scatter versus side scatter dot plot. Cells were selected as $CD3^+$ (T cells), or as $CD3^+CD4^+$, $CD3^+CD8^+$, $CD3^+CD56^+$ and $CD3^-CD56^+$ (NK cells), and expression of gzms was analyzed in these populations. The results are expressed as percentage of cells of the specific population expressing the corresponding gzm and as the median intensity of fluorescence (MFI). Alternatively, cells were selected as positive for each gzm and the percentage of the above mentioned lymphocyte populations were analyzed within the gzm^+ lymphocytes.

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