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Innate and adaptive host responses and their genetic control in tuberculosis : studies in Indonesia, a highly TB endemic setting

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The doctor's room in PPTI:

Glass screen to diminish the MTB transmission

On the wall at the back:

List of patients in openly direct observation drugstake scheme

No privacy but effective

(PACUAN KUDA, designed by dr. Halim Danusantoso)

**Plasma Granulysin Levels and
Cellular Interferon- γ Production Correlate with
Curative Host Responses in Tuberculosis,
while Plasma Interferon- γ Levels Correlate with
Tuberculosis Disease Activity in Adults**

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SUMMARY

Granulysin is a recently identified cytolytic protein which is expressed by human cytotoxic T-lymphocytes and natural killer (NK)-cells, and has broad antimicrobial and tumoricidal activity. Circulating granulysin levels are associated with T- and NK-cell activity, and may thus reflect protection-associated cellular immune responses. In a case-control study in Indonesia, a highly tuberculosis (TB)-endemic country, we therefore determined plasma granulysin levels in adults with active pulmonary TB before, during, and after TB treatment, both in mild/moderate-TB and advanced-TB patients, and compared these to healthy neighbourhood controls.

Adults with active pulmonary TB had significantly lower plasma granulysin levels compared to controls. After 2 months of anti-TB therapy, levels in TB patients had significantly increased, reaching values similar to those in controls. Plasma granulysin levels further increased after completion of TB therapy, being significantly higher than those in controls. Plasma granulysin levels correlated inversely with TB disease activity but not with TB disease severity. In contrast, plasma interferon- γ (IFN- γ) levels were significantly higher in active TB cases than in controls, normalised during treatment and correlated with both TB disease activity and TB disease severity.

At the cellular level, granulysin and IFN- γ expression both correlated inversely with disease activity. Interestingly, granulysin was predominantly expressed by IFN- γ negative T-cells, suggesting that the cellular sources of IFN- γ and granulysin in TB are partly non-overlapping.

The observation that plasma granulysin levels and cellular IFN- γ production correlate with curative host responses in pulmonary tuberculosis points to a potentially important role of granulysin, next to IFN- γ , in host defence against *M. tuberculosis*.

INTRODUCTION

An estimated one-third of the world population is latently infected with *Mycobacterium tuberculosis* (MTB) and from this reservoir the majority of future tuberculosis (TB) cases will arise. Upon infection with MTB, most individuals are able to control infection at an asymptomatic stage. However, 5-10% will progress towards active TB during their lifetime. MTB is an intracellular pathogen that targets host phagocytes, and consequently a coordinate innate and adaptive cellular immune response is required for effective host defense.¹

Protective immunity against MTB involves activated macrophages, antigen specific T-cells and type-1 cytokines such as interleukin (IL)-12, interferon (IFN)- γ and tumor necrosis factor (TNF).^{2,3} IFN- γ is produced by T-cells and natural killer (NK)-cells, and activates bactericidal mechanisms in macrophages.² IFN- γ -gene knockout mice, humans with genetic deficiencies in the type-1 cytokine (IL-12/IL-23/IFN- γ) axis, and individuals with neutralising auto-antibodies against IFN- γ all are highly susceptible to mycobacterial infections, including TB,^{4,7} demonstrating the essential role of this cytokine axis in host defense against mycobacteria.

In a mouse model of chronic TB, CD4⁺T-cells were found to be particularly important in the early stages of infection, whereas CD8⁺T-cells were more important in controlling the later phase of infection.⁸ Similarly, stimulation of human peripheral blood mononuclear cells (PBMCs) with live MTB⁹⁻¹¹ resulted in the expansion of various CD8⁺T-cell subsets, predominantly cytotoxic CD8⁺T-cells that produce IFN- γ , TNF, perforin, granzyme-B, and granulysin, which mediates killing of infected macrophages.¹¹⁻¹³ More recently, it was reported that also CD4⁺T-cells can use granulysin as an effector molecule in antimicrobial defence.¹⁴

Granulysin co-localizes with perforin and granzyme-B in cytolytic T-cell granules,¹⁵ and has a broad antimicrobial spectrum, being able to kill bacteria, fungi and parasites¹⁶ as well as tumor cells *in vitro*.¹⁵ Granulysin is able to kill extracellular MTB directly, and intracellular bacteria in the presence of perforin.¹⁶ Whereas expression of granulysin in CD8⁺T-cells is induced upon activation, it is constitutive in NK-cells.¹⁷

High serum levels of granulysin are associated with cytotoxic T-/NK-cell activity, and may thus represent a soluble biomarker of host-cellular

immune responses.¹⁸ Serum granulysin has been proposed as a biomarker of the Th1/Th2 balance,^{19,20} and at the cellular level, granulysin expression may have favourable prognostic potential in certain types of cancer.²¹ In contrast, very low granulysin serum levels were found in severe immunodeficient patients¹⁸ and reduced granulysin expression in carcinoma patients correlated with tumor progression.²¹⁻²³ In leprosy, granulysin expression was six-fold higher in tuberculoid or high responder leprosy lesions compared to lepromatous or low responder lesions, suggesting that *in situ* granulysin expression plays an important role in host defense against mycobacteria.²⁴ Children with active TB have decreased intracellular granulysin expression and IFN- γ production, which normalised following successful anti-TB therapy, indicating that granulysin and IFN- γ are likely involved in curative (protection-associated) immune responses against MTB.²⁵

We therefore decided to measure granulysin levels in adult pulmonary TB patients in Indonesia, an endemic country harbouring over 5% of the world's TB patients.²⁶ First, we analysed whether plasma granulysin levels correlated with (protection against) TB disease-activity in this highly TB endemic setting where most individuals are likely exposed to MTB. Secondly, we assessed whether granulysin levels were associated with clinical TB severity. Third, we studied whether changes in granulysin levels occurred during the course of successful therapy and whether these correlated with clinical outcome. Finally, we analyzed, both in plasma and intracellularly, whether granulysin levels correlated with the level of cellular IFN- γ production, a widely used indicator of the cellular immune status in TB patients.

PATIENTS AND METHODS

Study subjects

In a case-control study, carried out from June 2001 to December 2004, newly diagnosed active pulmonary TB patients ($n = 177$) aged 15-65 years were recruited from the TB clinic "Perkumpulan Pemberantasan Tuberkulosis Indonesia - PPTI" in Jakarta, Indonesia. Patients with HIV-seropositivity ($n = 1$), and incomplete clinical data ($n = 17$) were excluded. TB diagnosis was based on WHO definition²⁶ including the presence of clinical symptoms, chest X-ray examination (CXR), and microscopic detection of acid-fast bacilli in sputum,

or positive culture of MTB. Pulmonary TB-cases were classified according to international classification.²⁷ Mild-TB was defined by the presence of scattered and non-confluent pulmonary infiltrates of slight to moderate density, in one or both lungs with the total volume less than one lung, without cavities. Moderate-TB was defined by scattered and non-confluent pulmonary infiltrates present in one or both lungs, and/or with dense and confluent lesions, not involving more than one third of the volume of one lung, with or without cavities with a total diameter <4 cm. In our study, mild-TB and moderate-TB were grouped together. Advanced-TB was defined by lesions exceeding the above criteria. All CXRs were interpreted by two readers independently. In case of discordance in readings, CXR films were reviewed and final classifications were reached by consensus.

Community control subjects ($n = 213$) were recruited, matched by age ($\pm 10\%$), sex, socio-economic class, and area of residence. Controls were interviewed using the same questionnaire and underwent the same physical, blood and CXR-examinations as cases. Controls with a history of prior anti-TB therapy, with suggestive CXR-data ($n = 7$) or incomplete data ($n = 10$) were excluded. HIV status was not tested for controls, since Indonesia has a low HIV/AIDS prevalence in the general population ($<0.1\%$)²⁶ as also evidenced by the low HIV prevalence among the TB-cases. Written informed consent was obtained from all subjects. The study was approved by the Ethical Committee of the Medical Faculty, University of Indonesia.

ELISA for plasma granulysin measurement

Venous blood was collected from TB patients in lithium heparinised tubes (Vacuette® Greiner) before initiation of TB therapy as well as after two and six months of TB therapy, and from control individuals during recruitment. Plasma was stored at -80°C , and used later for granulysin and IFN- γ measurements in a blinded fashion. Samples of each individual TB patient, which typically consisted of three different time points, were tested in the same plate to rule out any possible inter-plate variability in readings obtained.

A sandwich ELISA was developed by Dieli and co-workers, using the anti-granulysin mAbs DH5 as the capturing Ab and DH4 as the detecting Ab. Generation of anti-granulysin mAbs is described elsewhere.²⁸ Both DH4 and DH5 mAbs recognize the 9- and the 15-KDa forms in human sera, as previously determined by Western blotting analyses on sera from healthy

children and sera from TB patients (data not shown). The available recombinant 9-kDa granulysin, produced as previously described²⁹ was used to set a standard curve.

In brief, ELISA plates (Nunc MaxiSorp) were coated overnight at 4°C with 100 µl of 4 µg/ml purified anti-granulysin DH5 mAb in Tris-buffered saline (TBS). After removing the capture antibody, plates were blocked with 4% (w/v) non-fat dry milk in TBS containing 0.1% Tween 20 (TBST) for 1 hr at 37°C, washed once and incubated for 1 hr at 37°C with 100 µl of plasma samples or recombinant 9-kDa granulysin for assay calibration. After three washes plates were incubated for 1 hr at 37°C with 100 µl of anti-granulysin DH4 biotinylated mAb in TBST (5 µg/ml).²⁸ Plates were washed 3 times, incubated for 45 min at RT with avidine-peroxidase in TBST (1:300) (Sigma), washed 3 more times, and developed colorimetrically with 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS, Sigma), before reading at OD 405 nm with an ELISA reader (Sigma Diagnostics). The ELISA system used had a detection limit of 25 pg granulysin/ml.

The effectively tested numbers of subjects are smaller than the total number of recruited subjects, because plasma samples were not available from all patients for granulysin measurements at each time point. Only patients with plasma samples from three time points (before, during and after therapy) were further analysed.

ELISA for IFN- γ levels in plasma and IFN- γ production in Lymphocyte Stimulation Tests (LST)

The same plasma samples were tested for IFN- γ levels by a standard ELISA according to the manufacturer's protocol (U-CyTech BV, Utrecht, The Netherlands). Again, only patients with three time points (before, during and after therapy) were further analysed. The capacity of PBMCs to produce IFN- γ was measured in a limited set of individuals in a specific time interval during the study. PBMCs were isolated by Ficoll-Hypaque (Pharmacia) density gradient centrifugation, and used for LST or stored in liquid nitrogen. For LST, PBMCs (1.5×10^5 per well) were incubated (in triplicate) with 10 µg/ml MTB H37Rv sonicate (heat killed and ultrasonicated, gift from Dr. D. van Soolingen, RIVM, the Netherlands) at 37°C in a fully humidified incubator (5% CO₂). Supernatants were harvested after 6 days and stored at -20°C before IFN- γ measurement. IFN- γ levels in cell supernatants were measured by a standard

ELISA according to the manufacturer's protocol (U-CyTech BV, Utrecht, The Netherlands). To determine precise concentrations, serial dilutions of the test-samples' were tested. The detection limit of the assay was 30 pg/ml. Detectable values in unstimulated cultures from an individual were subtracted from the values in the stimulated cultures from the same individuals. Because PBMCs could not be isolated from all time points, PBMC stimulation results were collected and analysed cross-sectionally.

Intracellular IFN- γ and granulysin levels in T-cells

Cryopreserved PBMCs from patients (before, during and after therapy) and controls were thawed and stimulated (at 2×10^6 per ml) for 48 hours with the following antigens: 2 μ g/ml SEB (Sigma), 10 μ g/ml MTB H37Rv sonicate, BCG MOI 10, and during the last 16 hours of incubation in the presence of 3 μ g/ml BrefeldinA (Sigma). Cells were then washed and surface stained with CD3 PerCP-CY5.5 (Becton Dickinson (BD) Biosciences) for 30 min at 4°C. The cells were then fixed and permeabilised according to manufacturer's protocol (Intrastain, Dako) before staining them with rabbit anti-granulysin (kind gift from Prof. Alan Krensky, Stanford University School of Medicine, Palo Alto, CA, USA) for 30 min at RT. Cells were then washed again, after which goat-anti-rabbit IgG-FITC (BD Biosciences) was added as second antibody, together with anti-IFN- γ Alexa Fluor 700 (BD Biosciences), and incubated for 30 min at RT. The cells were washed again, and fixed with 1% paraformaldehyde before analysis on a LSR II flow cytometer using FACS Diva™ software (BD Biosciences). Detectable values in unstimulated cultures were subtracted from the values in the stimulated cultures from the same individuals.

Statistical analyses

Unpaired and paired Student's *t*-tests were performed for clinical characteristics. For granulysin and IFN- γ measurements, the Kruskal-Wallis non-parametric analysis and Mann-Whitney *U*-test were used to compare the differences between responses among the groups and the Wilcoxon signed-ranks test to compare median values between paired groups. All analyses were 2-sided and *P* values < 0.05 were considered as significant (SPSS Inc., version 12.0).

RESULTS

Clinical classification of TB patients

Active pulmonary TB patients ($n = 159$) were classified into mild/moderate-TB ($n = 71$), or advanced-TB ($n = 88$). Included healthy controls from the neighbourhood ($n = 196$) had either no abnormalities on their CXR ($n = 145$, 74%) or had minor calcifications ($n = 51$, 26%). The latter group was not excluded since none of these individuals had any clinical symptoms suggestive of TB or had ever taken anti-TB drugs. Clinical characteristics of active pulmonary TB and control subjects are compared in Table 1.

No tuberculin skin-tests were performed in our study. BCG status was determined by anamnesis and/or by the presence of a BCG-scar. Interestingly, the mild/moderate-TB group had a higher percentage of BCG-scarring ($n = 30$; 42.3%) than the group of patients with advanced-TB ($n = 25$; 28.4%), and this difference was statistically significant ($P = 0.04$). There was no such difference between the mild/moderate-TB and the healthy control group (Table 1).

Generally, laboratory parameters such as erythrocyte sedimentation rates (ESR), C-reactive protein (CRP), white blood cell indices (WBC) and granulocyte numbers were significantly increased in TB patients compared to controls, and were higher among advanced-TB compared to mild/moderate-TB. Anemia (corrected for gender, Hb <12 g/dl for females or <13 g/dl for males) was also more frequent among advanced-TB (75.9%) than mild/moderate-TB (55.4%; $P = 0.004$).

Indonesia has a relatively high level (98%) of direct observed therapy coverage.²⁶ After completion of a 6-month course of anti-TB therapy, most patients ($n = 152$, 95.6%) had been cured based on conversion to sputum negativity by microscopic examination, as well as clinical improvement, reduced lesion sizes in CXR and improved laboratory parameters. Four patients had missing sputum data (2.5%) but had completed therapy and showed improved clinical appearance. Three patients (1.9%) had microscopically positive sputa at the end of therapy, indicating treatment failure. In general, laboratory parameters of successfully treated patients improved, approaching values for controls (i.e. decreased ESR, CRP, WBC, and granulocyte numbers; increased Hb and lymphocyte counts (data not shown)).

Table 1.
Characteristics of pulmonary TB patients in relation to TB-disease severity compared to healthy community controls

	Pulmonary TB		Community Controls <i>n</i> = 196
	Mild/moderate-TB <i>n</i> = 71	Advanced-TB <i>n</i> = 88	
Number of male [%]	34 (47.9 %)	49 (55.7 %)	96 (49.0 %)
Median age [range] (years)	29 [17 - 56]	32 [16 - 58]	31[15 - 58]
Number of individuals with BCG-scar [%]	30 (42.3 %)	25 (28.4 %) [#]	88 (44.9 %)
Mean duration of symptoms ± SE (weeks)	13.5 ± 1.5	19.5 ± 1.7	
Mean BMI ± SE (kg/m ²)	18.9 ± 0.4	17.8 ± 0.3	22.1 ± 0.3**
Mean ESR ± SE (mm/h)	83.1 ± 4.1	97.5 ± 3.1*	18.5 ± 1.0**
Mean CRP ± SE (mg/l)	49.8 ± 4.8	73.4 ± 4.8*	7.5 ± 1.5**
Mean Hb ± SE (g/dl)	12.0 ± 0.2	11.5 ± 0.2	14.1 ± 0.1**
Mean WBC ± SE (k/μl)	10.8 ± 0.4	13.0 ± 0.5*	8.2 ± 0.2**
Mean lymphocytes ± SE (k/μl)	2.3 ± 0.1	2.3 ± 0.1	2.8 ± 0.1**
Mean granulocytes ± SE (k/μl)	7.9 ± 0.4	10.1 ± 0.5*	5.2 ± 0.3**

Significant differences ($P < 0.05$), were found between pulmonary mild/moderate-TB and advanced-TB as indicated by the symbol * for *t*-test or by the symbol [#] for χ^2 -test; and between all active pulmonary TB and healthy control subjects as indicated by the symbol ** for *t*-test. TB, tuberculosis; SE, standard error means; BMI, body mass index; ESR, erythrocyte sedimentation rate; CRP, C-reactive protein; Hb, hemoglobin; WBC, white blood counts

Plasma granulysin levels correlate inversely with active pulmonary TB

TB patients with available plasma samples from three time points ($n = 127$) and controls ($n = 196$) were included for measurements of circulating granulysin. Compared to controls, granulysin levels before the initiation of TB treatment were significantly lower in TB patients (both $P < 0.0001$); median granulysin levels in mild/moderate-TB ($n = 58$) and advanced-TB ($n = 69$) were 1.1 ng/ml [range 0.1 - 11.2] and 0.9 ng/ml [range 0.1 - 12.4], respectively, whereas controls had median values of 2.6 ng/ml [range 0.2 - 44.6] (Fig. 1(A)). At 2 months of anti-TB therapy, granulysin levels were significantly increased to median values of 2.7 ng/ml [range 0.3 - 8.5] and 2.4 ng/ml [range 0.3 - 14.5] in

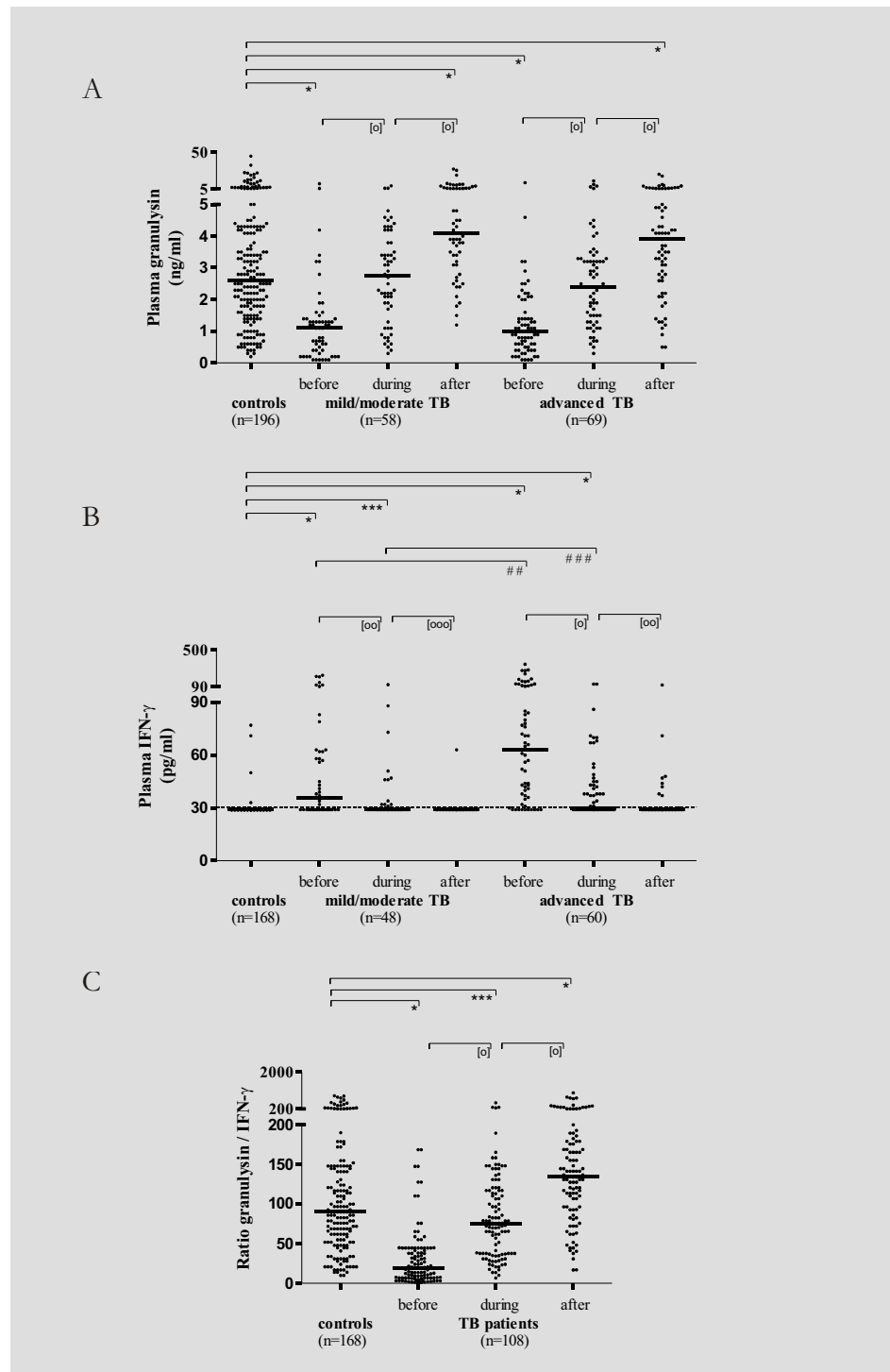
mild/moderate- and advanced-TB, respectively, and these values were not significantly different from the values observed in controls. Granulysin levels in TB patients were further increased after completion of therapy, with median values of 4.1 ng/ml [range 1.1 - 28.8] and 3.8 ng/ml [range 0.5 - 22.5], respectively, all being significantly higher than controls ($P < 0.0001$ and $= 0.001$).

No significant association was found between granulysin levels and clinical severity of pulmonary TB (mild/moderate-TB vs. advanced-TB) as assessed by CXR (Fig. 1(A)). By contrast, other laboratory parameters (Hb, ESR, CRP, WBC and granulocyte numbers) correlated well with clinical severity. However, these laboratory parameters did not correlate with granulysin levels (not shown). Similar analyses on granulysin levels during and after anti-TB therapy also failed to reveal any significant association between disease severity and levels of granulysin in the different groups analysed (data not shown). Granulysin levels were also not associated with the presence or absence of a BCG-scar, age and gender (data not shown).

After TB therapy, granulysin levels remained low (levels < 2.6 ng/ml, the median value in healthy controls, were designated "low" and those > 2.6 ng/ml as "high" granulysin producers) in a proportion of mild/moderate-TB patients (9 of 58 patients, 15.5%) and advanced-TB patients (15 of 69 patients, 21.7%). However, these differences were not statistically significant. Only one patient, which was a granulysin "low" producer, had documented treatment failure, while two other failure cases had normal granulysin levels after 6 six month of therapy.

Together, the results showed that plasma granulysin levels inversely correlate with TB disease activity but not with TB disease severity.

Fig. 1. (A) Plasma granulysin levels, (B) plasma IFN- γ levels and (C) plasma granulysin/IFN- γ ratio in pulmonary TB patients (mild/moderate-TB and advanced-TB) compared to control subjects. Each dot represents one individual. A horizontal bar indicates the median in each group. A dashed line indicates the lower detection limit of the assay. Statistically significant differences are indicated by the symbol * ($P < 0.0001$) or *** ($P < 0.05$) compared to control subjects (Mann-Whitney *U*-test); by the symbol [# #] ($P < 0.005$), or [# # #] ($P < 0.05$) between mild/moderate-TB and advanced-TB (Mann-Whitney *U*-test); and by the symbol [o] ($P < 0.0001$), [oo] ($P < 0.005$), or [ooo] ($P < 0.05$), between median values before, during, and at the end of the therapy (Wilcoxon signed-ranks test).



Plasma IFN- γ levels correlate with active pulmonary TB

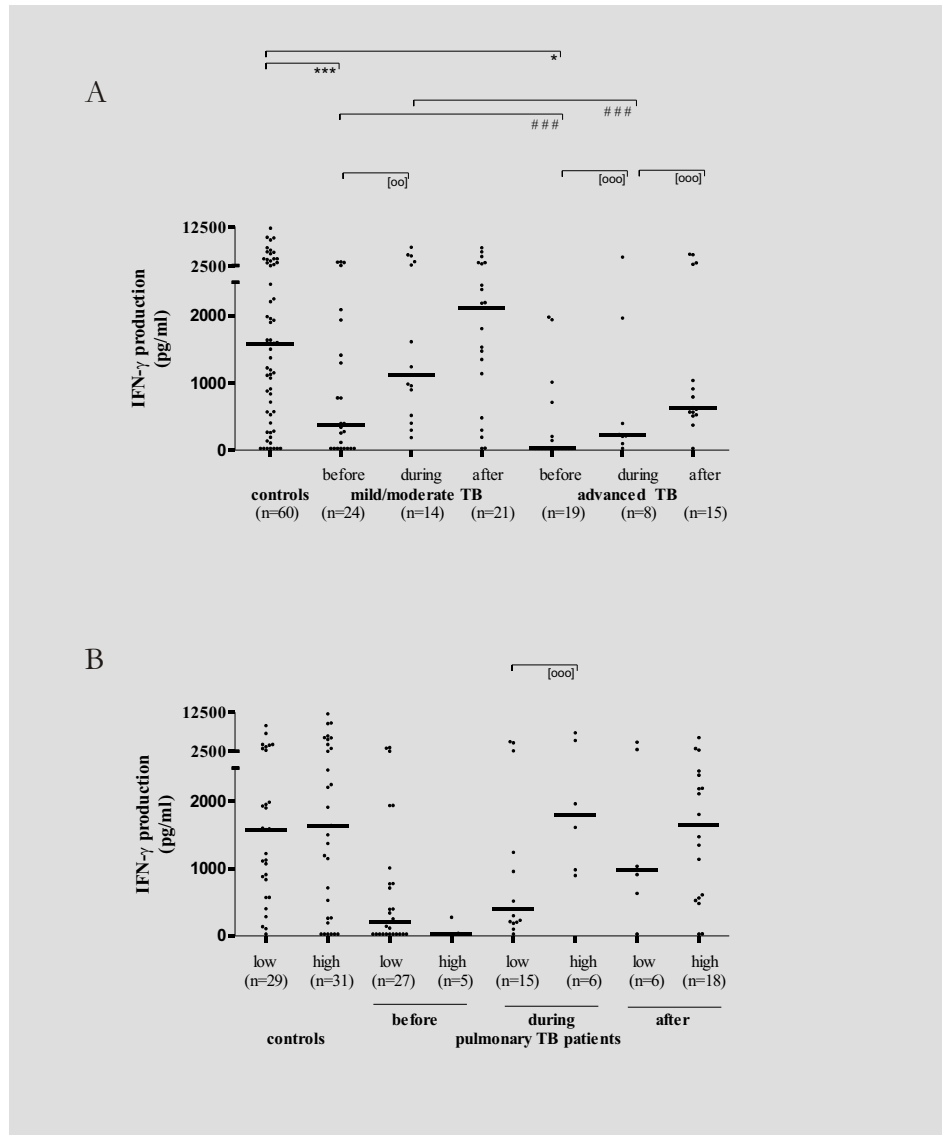
Next, IFN- γ levels were determined in available plasma samples from patients ($n = 108$) and controls ($n = 168$). As shown in Fig. 1(B), low but detectable levels of IFN- γ were present in mild/moderate TB patients ($n = 48$) with a median value of 36 pg/ml (range: undetectable to 279 pg/ml), and advanced TB ($n = 60$) with a median of 63 pg/ml (range: undetectable to 337 pg/ml). These values were all significantly higher than in controls. IFN- γ levels decreased both during therapy (mild/moderate TB, range: undetectable to 110 pg/ml, $P = 0.02$; advanced TB, range: undetectable to 117 pg/ml, $P < 0.0001$) and after therapy (mild/moderate TB, range: undetectable to 63 pg/ml; advanced TB, range: undetectable to 106 pg/ml) (Fig. 1(B)). In only 4 of 168 control individuals, IFN- γ levels were detectable above background; they had normal CXR and no clinical symptoms or any history suggestive of TB. Fig. 1(C) displays the ratios of plasma granulysin over plasma IFN- γ levels in controls and in TB patients before, during or following completion of treatment.

Thus, whereas plasma granulysin levels correlate with curative host responses in TB, plasma IFN- γ levels correlate with TB disease activity and severity.

Cellular IFN- γ production in relation to plasma granulysin levels

The ability of T-cells to produce IFN- γ in response to MTB is considered a minimal requirement for protection. We therefore stimulated PBMCs with MTB sonicate before, during, and after anti-TB therapy in part of the patients and controls enrolled above. In line with previous reports,^{25,30} cellular IFN- γ

Fig. 2. (A) IFN- γ production in PBMC supernatants determined by ELISA of pulmonary TB patients (mild/moderate TB and advanced TB) before, during and after TB therapy compared to control subjects. Each dot represents one individual. A horizontal bar indicates the median in each group. Statistically significant differences are indicated by the symbol * ($P < 0.0001$) or *** ($P < 0.05$) compared to control subjects (Mann-Whitney U -test); by the symbol [# # #] ($P < 0.05$) between mild/moderate-TB and advanced-TB (Mann-Whitney U -test); and by the symbol [oo] ($P < 0.005$), or [ooo] ($P < 0.05$), between median values before, during, and at the end of the therapy (Wilcoxon signed-ranks test). (B) IFN- γ production in PBMC supernatants determined by ELISA of low (< 2.6 ng/ml) and high (> 2.6 ng/ml) granulysin producers in the group of controls subjects and pulmonary TB patients before, during and at the end of the TB therapy. Each dot represents one individual. A horizontal bar indicates the median in each group. Statistically significant differences is indicated by the symbol [ooo] ($P < 0.05$) between low and high granulysin producers (Mann-Whitney U -test).



production at the time of diagnosis were decreased compared to healthy controls (median values 118 pg/ml vs. 1590 pg/ml, $P < 0.0001$). Cellular IFN- γ production, however, increased significantly after 2 months of TB therapy (median 932 pg/ml, $P = 0.006$), and had approached values similar to control subjects at the end of anti-TB therapy (median 1416 pg/ml, $P = 0.78$) (data not shown), confirming that levels of cellular IFN- γ production in pulmonary TB correlated inversely with TB disease activity. Interestingly, cellular IFN- γ

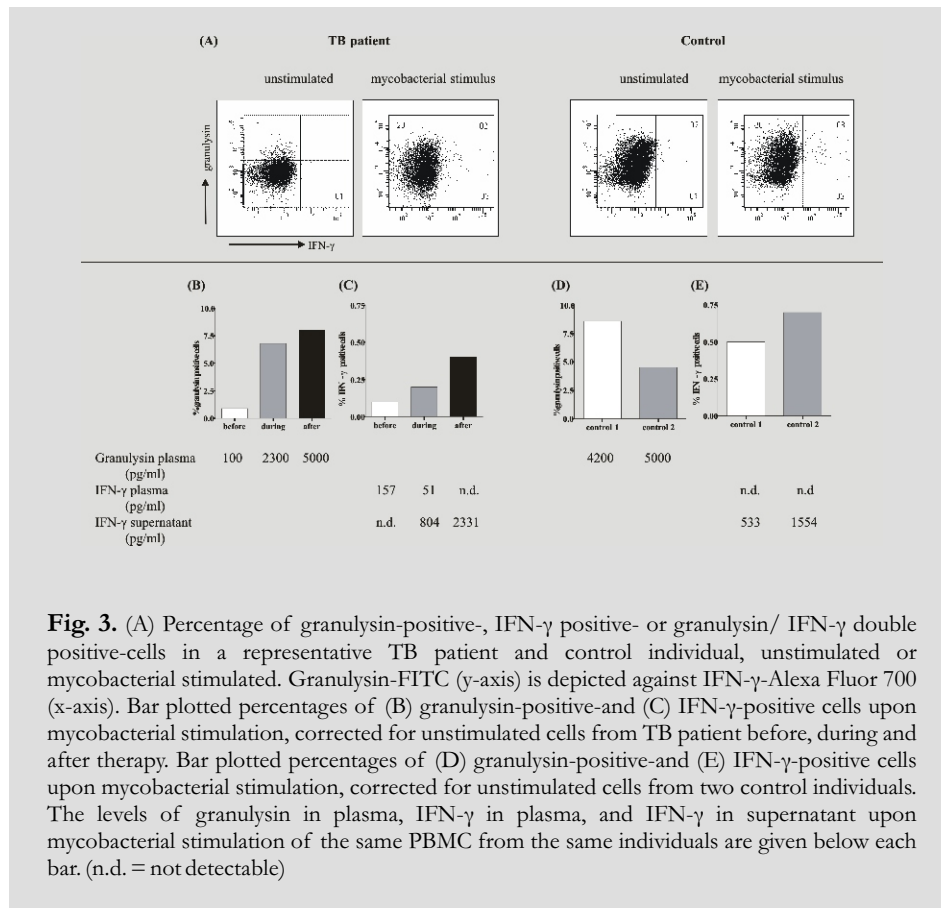


Fig. 3. (A) Percentage of granulysin-positive, IFN- γ positive- or granulysin/ IFN- γ double positive-cells in a representative TB patient and control individual, unstimulated or mycobacterial stimulated. Granulysin-FITC (y-axis) is depicted against IFN- γ -Alexa Fluor 700 (x-axis). Bar plotted percentages of (B) granulysin-positive and (C) IFN- γ -positive cells upon mycobacterial stimulation, corrected for unstimulated cells from TB patient before, during and after therapy. Bar plotted percentages of (D) granulysin-positive and (E) IFN- γ -positive cells upon mycobacterial stimulation, corrected for unstimulated cells from two control individuals. The levels of granulysin in plasma, IFN- γ in plasma, and IFN- γ in supernatant upon mycobacterial stimulation of the same PBMC from the same individuals are given below each bar. (n.d. = not detectable)

production levels in mild/moderate-TB before and during therapy (median 370 pg/ml and 1116 pg/ml) were significantly higher compared to advanced-TB (median 30 pg/ml and 225 pg/ml respectively; $P = 0.035$ and $P = 0.037$) (Fig. 2(A)). The difference between mild/moderate-TB and advanced-TB however, was no longer statistically significant at the end of therapy, though there was a trend (2118 pg/ml and 634 pg/ml, $P = 0.216$). Thus, in contrast with plasma granulysin levels, cellular IFN- γ production levels significantly correlated inversely with TB disease activity and TB disease severity.

Although plasma granulysin levels and *in vitro* cellular IFN- γ production showed a similar trend, i.e. a decrease at the time of diagnosis followed by normalisation during successful 6 months anti-TB therapy, there was no statistically significant correlation between the magnitude of granulysin levels and the levels of PBMC produced IFN- γ ($r = 0.09$, $P = 0.3$)

granulysin levels and the levels of PBMC produced IFN- γ ($r = 0.09$, $P = 0.3$) (data not shown), even though there was a trend towards higher granulysin levels in high IFN- γ producers.

After completion of therapy, some patients continued to produce low granulysin levels. We therefore analysed whether the low granulysin producers were also low cellular IFN- γ producers. Low plasma granulysin levels, however, were found in both low and high cellular IFN- γ producers, both among patients and among controls (Fig. 2(B)), thus confirming the lack of correlation between overall cell mediated IFN- γ production and granulysin levels. Only at one time point (during treatment), there was an association ($P = 0.02$) between cellular IFN- γ production and granulysin levels. Thus, although plasma granulysin and *in vitro* cellular IFN- γ production levels showed similar trends, as both were decreased at the time of diagnosis and normalised during successful treatment, overall there was no consistently significant correlation between these two markers in our study population.

Intracellular granulysin and IFN- γ expression by T-cells

To investigate whether granulysin and IFN- γ expression correlated at the T-cell level and were produced by similar or distinct cellular populations, double staining on CD3 positive T-cells for intracellular granulysin and IFN- γ was performed on unstimulated as well as mycobacterium stimulated T-cells from three TB patients (before, during or after treatment) and two control individuals. Intracellular granulysin was predominantly expressed by IFN- γ negative T-cells (Fig 3(A)). Moreover, a significant proportion of IFN- γ positive T-cells expressed granulysin (Fig. 3(A)). In line with the plasma granulysin levels found above, the total number of granulysin expressing cells increased during and after therapy (Fig. 3(B)). Similarly, the total number of IFN- γ positive cells also increased during and after therapy (Fig. 3(B)), in line with the observation that cellular IFN- γ production correlated inversely with TB disease activity (Fig. 3(C)). Thus, granulysin was predominantly expressed by IFN- γ negative T-cells, while a significant proportion of IFN- γ positive T-cells co-expressed granulysin. This suggests that the T-cell source of IFN- γ and granulysin in TB are largely non-overlapping.

DISCUSSION

Successful control of MTB infection is dependent on a variety of immunological effector mechanisms that participate in the killing of MTB-infected host cells.⁶ Helper CD4⁺ T-cells and cytotoxic CD8⁺ T-/NK-cells not only secrete IFN- γ but also can express granulysin which together with perforin and granzyme-B is localised in granules¹⁵ and is released into the immunological synapse following T-/NK-cell activation. Whereas IFN- γ activates infected macrophages, granulysin has a broad range of antimicrobial activities, and can kill bacteria, fungi, and parasites³¹ as well as tumor cells *in vitro*.¹⁵ As discussed above, several studies have suggested the involvement of granulysin in human host-defense.^{18,19,24,31-33} Since granulysin is secreted following cellular activation, levels of circulating granulysin might reflect the activity of cytotoxic T-/NK-cells,^{18,34} and potentially represent a soluble biomarker of (innate and/or adaptive) cellular immune activity.

In the present study, we therefore measured plasma granulysin and IFN- γ levels in Indonesian pulmonary TB patients and healthy controls from the same area. With a case detection rate of 285/100,000 per year and >200 million inhabitants, Indonesia is a highly TB-burdened country.²⁶ The granulysin levels that were found in healthy individuals in our study were comparable to those reported in Japanese individuals.¹⁸ In newly detected TB patients who had received no anti-TB therapy yet, however, plasma granulysin levels were strongly decreased compared to healthy controls. The proportional decrease of granulysin levels in active TB patients was not associated with TB disease severity, nor with any standard laboratory parameters of clinical severity, including Hb levels, ESR, CRP, WBC and granulocyte numbers. Thus, granulysin levels likely reflect a different component in the disease process than these latter classical markers.

During TB therapy granulysin levels normalised, reaching levels that were similar to controls, and increased further after completion of TB therapy. It is interesting to note that levels of circulating granulysin in TB patients at the end of TB therapy had increased significantly above those observed in the control group. It is not clear whether and when these increased levels of circulating granulysin would decrease back to normal values following TB cure. Additional follow-up studies are necessary to obtain better insight into the dynamics of plasma granulysin levels. This will allow us to answer this

question, as well as to determine whether the low granulysin levels observed in active TB patients precede or result from active infection. In addition, it will be of interest to determine whether controls with low granulysin levels have a higher risk of acquiring TB.

In this study, we were unable to determine granulysin levels in relation to treatment failure, due to the low number of failure cases in our cohort ($n = 3$). Also in TB-affected children, increased granulysin levels at the end of therapy have suggested a role for granulysin in the protective immune response against MTB.²⁵ This is in line with the observation that *in situ* granulysin expression levels in leprosy lesions correlate well with host defense in mycobacterial infections.²⁴

The possible function of the increased levels of circulating granulysin remains unknown. We hypothesize that this may reflect increased T-/NK-cell activity against infection during resolution of disease. This increased cellular activity likely extends significantly beyond TB microbiological cure, possibly reflecting a remaining antigen load of killed organisms. It is possible that circulating granulysin participates directly in microbial killing *in vivo*. This may be particularly relevant in targeting extracellular pathogens and indeed such organisms are also found in TB, even though most organisms will reside intracellularly. In such a scenario, the cellular immune system would be able to secrete humoral components with anti-microbial activity that can survey the extracellular milieu for microbial pathogens. Stenger *et al.* reported that recombinant granulysin has direct *in vitro* bactericidal activity against extracellular MTB.¹⁶

The dynamics of plasma granulysin levels in TB patients during curative treatment followed a similar trend as the dynamics of the cellular capacity to produce IFN- γ . In active TB, numbers of circulating T-/NK-cells are known to be decreased, which is considered to be partly responsible for the decreased production of IFN- γ ³⁵ and likely also granulysin.^{25,36} Interestingly, however, we observed low but detectable levels of IFN- γ in the plasma of patients before therapy, which were significantly higher than those during or after therapy, or in endemic controls. Together with the cellular IFN- γ production data, these results are compatible with compartmentalization of Th1 responses:³⁷⁻³⁹ the circulating levels of IFN- γ in the plasma may result from the local production and spill-over of IFN- γ from activated lymphocytes sequestered at the site of MTB infection. Of interest, circulating IFN- γ levels

correlated with disease severity. Thus, IFN- γ and granulysin levels in the peripheral circulation may provide potential biomarker signatures to measure immune status in TB.

Unexpectedly, even though the increase of granulysin and antigen specific induced IFN- γ production during the course of therapy might be anticipated to originate from similar cellular sources (activated T-/NK-cells), only at one time point -i.e. during treatment- a significant association was detectable. There was no overall significant correlation between quantitative *in vitro* IFN- γ and plasma granulysin levels.⁴⁰ We therefore postulate that their production may have different kinetics, or involve secretory pathways or cellular subset sources. This was confirmed by experiments in which intracellular granulysin and IFN- γ expression was determined: granulysin was predominantly expressed by IFN- γ negative T-cells, while a significant proportion of IFN- γ positive T-cells expressed granulysin. In agreement with the plasma granulysin levels, the number of granulysin expressing cells increased during and after therapy. The number of IFN- γ positive T-cells also increased during and after therapy, in line with the increased cellular IFN- γ production during TB cure. The observation that granulysin was predominantly expressed by IFN- γ negative T-cells, while a significant proportion of IFN- γ positive T-cells co-expressed granulysin, suggests that the cellular sources of IFN- γ and granulysin in TB are at least partly non-overlapping.

Finally, levels of granulysin and type-1 cytokines may provide new biomarker signatures of TB disease and curative host responses in TB, and may act in concert or in synergy in controlling MTB.

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