IMMUNE COMPLEXES ISOLATED FROM PATIENTS WITH PULMONARY TUBERCULOSIS MODULATE ACTIVATION AND FUNCTION OF NORMAL GRANULOCYTES

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RUNNING TITLE: Immune complexes and granulocytes in TB
ABSTRACT

Purpose:

Circulating immune complexes (ICs) are associated with the pathogenesis of several diseases. Very little is known about the effect of ICs on host immune response in patients with tuberculosis (TB).

Methods:

The effect of ICs isolated from patients with TB in modulating the release of calcium, cytokines, and granular proteins was studied in normal granulocytes, as were their chemotactic, phagocytic and oxidative burst processes.

Results:

IC from TB patients induced decreased production of cytokines and platelet activating factor (PAF) from normal granulocytes. IC from TB patients also induced enhanced chemotaxis and phagocytosis but caused diminished oxidative burst. This was accompanied by an increased release in intracellular calcium. On the other hand, IC from TB patients induced increased release the granular proteins "human neutrophil peptides 1–3" (HNP1–3).

Conclusion:

Thus, IC from patients with TB exhibits a profound effect on granulocyte function with activation of certain effector mechanisms and dampening of others.

KEY WORDS: pulmonary tuberculosis; immune complexes; granulocytes
INTRODUCTION

Several reports have shown the prevalence of high levels of immune complexes (ICs) in pulmonary tuberculosis (PTB) (32). Mycobacterial antigens, antimycobacterial antibodies, and C3 and C4 components have been demonstrated in ICs isolated from sera of patients with active TB (8). Similarly, mean circulating immune complex (CIC) levels in children with TB were found to be significantly higher when compared with levels in normal children (35). A longitudinal study done by Johnson et al (16) suggested that the levels of CIC are related to disease progression, as elevated CIC levels decreased to normal limits following treatment in patients with active TB.

Apart from circulating immune complexes, those deposited in tissues might also modulate disease pathogenesis in patients with TB, as suggested by previous studies. One of these reported the presence of extravascular immune complexes with high bacterial load and low cell mediated immunity in experimental TB (25). Another concluded that the antigen-antibody ratio within the lesions might be crucial in modulating the balance between tissue destruction and healing (26). Another study in humans, showed that the occurrence of Henoch-Schönlein purpura nephritis in patients with pulmonary tuberculosis was associated with the deposition of circulating immune complexes (27). While the presence of ICs is established in both circulation and in tissues in many inflammatory responses including TB, the triggers and mediators downstream of the IC have been less well studied.

The critical role of humoral immune responses has been relatively less extensively studied than has the T cell response in TB. Antibodies can have a significant impact on host immunity and disease outcome in TB by engagement of Fc gamma (Fcγ) receptors that can influence both Th1 activation and mycobacterial containment. ICs are known to modulate cellular functions by several mechanisms, including induction of activating or inhibitory signals (24). Through Fcγ receptor binding, ICs link the specificity of the adaptive immune system and the powerful effector functions triggered by innate immune effector cells (23). In active infections, including TB, large numbers of ICs are generated...
owing to the priming of antigen-specific B cells. It has been reported that ICs trigger
activation cascades in *Mycobacterium tuberculosis* (MTB) infection that limit
susceptibility to infection (18).

Several lines of evidence support a role for neutrophils in the immune response to MTB.
First, neutrophils are recruited early to the lung of TB-infected mice, and depletion of
neutrophils results in increased bacterial growth. Second, *in vitro* studies suggest that
human neutrophils are capable of inhibiting the growth of MTB. Third, neutrophils are
readily detected in sputum and bronchoalveolar lavage fluid from humans with active
pulmonary tuberculosis (21). Also, a number of studies have shown the critical role
played by granulocytes in mouse models of IC-mediated diseases (39, 40). Phagocytosis
in neutrophils has been shown to be an active process, often dependent on the presence of
functionally active Fcγ (33). In addition, generation of oxidative burst, degranulation, and
changes in cytoplasmic calcium have also been shown to be induced by IC in
granulocytes (1).

As mentioned above, there are very few data on the functional role played by ICs in
patients with TB. We hypothesized that one mechanism by which ICs could potentially
influence disease activity in TB is by modulating granulocyte activation and function. To
test our hypothesis, we examined the effect of ICs from patients with TB on granulocyte
function. We demonstrate that ICs from TB-infected individuals modulate granulocyte
activation and effector function, a process that could potentially augment pathology or
contribute to protection by activating innate immune effectors.

**MATERIALS AND METHODS**

**Study subjects**

Serum samples were obtained from ten normal healthy Indian subjects (hereafter,
UNINF; mean age ± SD: 35 ± 12.3; 7 males and 3 females) and ten patients with active
TB (hereafter, INF; mean age ± SD: 30 ± 7.3; 7 males and 3 females).
granulocytes were obtained from a separate set of ten healthy, uninfected volunteers. All
INF individuals were culture positive for MTB. Patients with active tuberculosis were
excluded if they had concomitant diabetes mellitus, autoimmunity or immunodeficiency,
pregnancy or any other acute or chronic infections by thorough medical history and
clinical examination. Subjects were enrolled prior to commencement of anti-TB therapy.
The patients included were all newly diagnosed for PTB, and none had extrapulmonary
involvement. The healthy volunteers were matched based on ethnicity and geographic
location. On the basis of a detailed clinical history, controls included in the study did not
have a history of TB or current signs or symptoms consistent with TB. None of the
subjects included in the study had evidence of HIV infection. The study was approved by
the Institutional Ethical Committee of the Tuberculosis Research Center, and informed
written consent was obtained from all participants.

Isolation of serum

Blood samples were collected, left for 1 h at room temperature to clot, and then subjected
to centrifugation at 2200g for 20 min at 4°C. The serum devoid of the clot was then
transferred to serum storage vials and stored at –80°C.

CIC purification

Serum (50 μl) was incubated with an equal volume of 5% PEG-6000 (polyethylene
glycol; final concentration 2.5% in phosphate buffered saline) at 4°C overnight. The
serum was centrifuged at 2,000 rpm for 30 min at 4°C. The precipitate was washed twice
with PBS and suspended in in 500 μl of PBS (pH 7.4). The precipitate was undisturbed
for 30 min at room temperature. The absorbance of the precipitate was read at 280 nm
using a spectrophotometer. CIC levels were determined by interpolation from a standard
curve plotted using aggregated human gamma globulins as a standard. The isolated ICs
was diluted to the initial serum volume in sterile PBS and were used at a concentration of
10% v/v in in vitro culture assays.
**Whole-blood culture and granulocyte isolation**

Granulocytes were isolated as described previously (3). In brief, the anti-coagulated whole blood was treated with ficoll hypaque which allowed the separation of peripheral blood mononuclear cells and the granulocytes layer over the erythrocytes. After removing the PBMC and the dextran layers, dextran was added to the granulocytes and the erythrocyte layers, which was left undisturbed for 45 minutes at room temperature. Once the erythrocytes were removed, the granulocytes were sedimented using centrifugation, washed with RPMI and then used for analysis. Flow cytometric analysis was performed to assess the purity of the isolated granulocytes. Granulocytes were first gated using forward and side scatter and then by selecting CD15+ cells. The purity of granulocytes within the sorted CD15 cell population was typically >95% (S. Figure 1).

**In vitro culture**

Either whole blood or isolated granulocytes used as the responder cells to study the effect of immune complex admixture were obtained from ten healthy volunteers. The whole blood or granulocytes from each healthy volunteer were stimulated with ICs from one UNINF and one INF individually. Either whole blood or granulocytes were cultured with RPMI 1640 in the presence of PEG-precipitated ICs (10% v/v) isolated from INF and UNINF in 24-well tissue culture plates (Corning, Corning, NY, USA) using $2 \times 10^6$ cells per well (isolated granulocytes). PEG precipitates isolated for cell culture assays were prepared fresh and used immediately without freezing or thawing. Unstimulated cells without the presence of IC (as negative controls) from both the study groups and cells stimulated with fMLP (as positive controls) were also included for all the ten healthy volunteers.

**Multiplex cytokine ELISA**

Normal granulocytes were stimulated with isolated ICs along with appropriate culture controls. After a stimulation period of 18 h, the culture supernatants were used for...
analysis of cytokines using the Bioplex multiplex cytokine assay system (Bio-Rad). The cytokines analyzed were IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12, IL-13, IL-17, GM-CSF, IFN-γ, and TNF-α.

**HNP1–3 and platelet-activating factor ELISA**

Supernatants from whole blood stimulated with or without isolated ICs for 4 h at 37°C were collected and stored at –80°C. The levels of HNP1–3 (Hycult Biotech, Canton, MA, USA) and PAF (Uscn Life Science Inc., Wuhan, China) were assessed using commercially available ELISA kits.

**Chemotaxis**

Migration of granulocytes was assessed using the MIGRATEST™ kit (Orpegen Pharma, Heidelberg, Germany). Determination of the number of granulocytes from whole blood stimulated with or without ICs (10 min at 37°C) from UNINF and INF that migrated toward the chemotactic peptide, fMLP was assessed.

**Phagocytosis**

Whole blood was assayed for phagocytic activity by flow cytometry using the Phagotest™ kit (Orpegen Pharma). *Escherichia coli* commercially labeled with fluorescein isothiocyanate (Orpegen Pharma), was added to aliquots of heparinized blood in the presence or absence of ICs (from UNINF and INF) and incubated for 10 min at 37°C. The percentage of cells having phagocytosed the fluorescein isothiocyanate-labeled bacteria was then determined by flow cytometry using a BD FACSCalibur instrument (BD Biosciences).

**Oxidative burst**
Whole blood was assayed for oxidative burst by flow cytometry using a BD FACSCalibur instrument (BD Biosciences) based on the cleavage of dihydrorhodamine 123, by oxidative species, to rhodamine using a Bursttest™ kit (Orpegen Pharma) according to the manufacturer's instructions.

**Calcium levels**

The effect of IC admixture on intracellular calcium mobilization was assessed using the Screen Quest™ Fluo-8 no-wash calcium assay kit (AAT Bioquest, Inc., Sunnyvale, CA, USA). Isolated granulocytes were incubated with 100 μl of Fluo-8 NW dye-loading solution. The cells were incubated in a 5% CO2 incubator for 30 min and then at room temperature for another 30 min. Fluorescence was recorded for 2 min at 490 nm, at which point immune complexes were added (10μl). Fluorescence was then recorded for an additional 5 min. The data are presented as percentages of non-treated control for which calcium-associated fluorescence was measured in parallel cultures at the same time points. Cell counts were performed and densities adjusted for each culture before dye loading to ensure exactly the same cell densities for all study conditions.

**Nitric oxide (NO) levels**

Normal granulocytes were stimulated with isolated ICs along with appropriate controls. After a stimulation period of 18 h, the culture supernatants were collected and used to measure NO using a colorimetric non-enzymatic assay for NO (Oxford Biomedical Research, Rochester Hills, MI, USA) exactly as described by the manufacturer. Briefly, 200 μl of supernatants were incubated at room temperature overnight with 0.5 g of dry granulated cadmium while being agitated. Following a 5-min incubation at room temperature with the colorimetric reagents provided, absorbance was read at 540 nm.

**Statistical analysis**
Geometric mean (GM) was used as the measure of central tendency. Comparisons were made using the nonparametric Mann Whitney U test. P values less than 0.05 were taken to indicate statistical significance. All statistics were performed using GraphPad Prism (V5.0 for Windows; GraphPad Software, Inc., San Diego, CA, USA).

RESULTS

Levels of Circulating Immune Complexes
Circulating immune complex levels were significantly higher (P=0.004) in TB patients (GM, 12.8; 95% confidence interval, 9.0-17.8) than normal healthy volunteers (GM, 5.5; 95% confidence interval, 3.6-8.4). The data are shown in Figure 1.

ICs from INF induce decreased release of IL-4, IL-6, GM-CSF, and TNF-α from normal granulocytes

Immune complexes are known to modulate release of cytokines (31, 15). To determine the effect of IC from TB patients, we assessed the production of a panel of pro- and anti-inflammatory cytokines from normal granulocytes stimulated with ICs from INF or UNINF (Figure 2). Expression of cytokines on negative control (unstimulated) and positive control (stimulated with fMLP) was also measured. While the release of cytokines was barely detectable in the unstimulated granulocytes (negative control), stimulation with fMLP resulted in increased production of the cytokines (IL-4, GM, 20 (95% confidence interval, 1.5-30); IL-6, GM, 600 (95% confidence interval, 15-620); GM-CSF, GM, 450, 95% confidence interval, 25-525) and TNF-α, GM, 490, 95% confidence interval, 12-550). The levels of IL-4, INF: GM, 38.67 (95% confidence interval, 31.54-47.40); UNINF: GM, 58.72 (95% confidence interval, 45.81-75.26), IL-6, INF: GM, 253.2 (95% confidence interval, 162.1-395.7); UNINF: GM, 514.0 (95% confidence interval, 423.7-623.7), GM-CSF, INF: GM, 195.0 (95% confidence interval, 164.4-231.3); UNINF: GM, 270.4 (95% confidence interval, 211.6-345.5) and TNF-α, INF: GM, 719.8 (95% confidence interval, 585.0-885.6); UNINF: GM, 923.2 (95% confidence interval, 746.3-1142) were significantly lower in granulocytes that were
stimulated in the presence of ICs from INF compared with those cells stimulated with ICs from UNINF. The levels of IL-2 (GM, 20 (95% confidence interval, 1.5-30), IL-5, IL-10, IL-12, and IL-13 were not significantly different (Table 1), while IL-1β, IL-17, and IFN-γ were not detectable in the supernatants. Thus, IC from TB patients predominantly induced diminished secretion of both pro (IL-6, GM-CSF and TNFα) - and anti (IL-4) – inflammatory cytokines from granulocytes.

**ICs from INF induce increased HNP 1–3 and reduced PAF levels in whole blood**

Because granulocytes produce antimicrobial peptides, and because ICs are known to induce production of these antimicrobial peptides (1), the levels of HNP1–3 upon whole-blood stimulation with ICs were examined. The levels of HNP1–3 were found to be significantly higher in the presence of ICs from INF compared with ICs from UNINF (GM, INF: 1679; UNINF: 1019; P = 0.02) (Figure 3A). Secretion of HNP1-3 by the negative control (unstimulated) and positive control (stimulated with fMLP) was also measured (GM, Negative control: 50 and positive control 1895). PAF has been associated with immune complex mediated diseases (2, 34). The levels of PAF in the supernatants of whole blood stimulated with ICs from INF was significantly lower compared with ICs from UNINF (GM, INF: 125.9; UNINF:149.7; P = 0.001) (Figure 3B). Secretion of PAF by the negative control (unstimulated) and positive control (stimulated with fMLP) was also measured (GM, Negative control: 10 and positive control: 200). On the other hand, there were no significant effects on ICs from either group on the release of NO from granulocytes (GM, 164.0 for INF vs. 164.8 from UNINF) (data not shown).

**ICs from INF induce enhanced chemotaxis and phagocytosis of normal granulocytes**

As IC modulation of granulocytes could potentially involve changes in chemotactic and phagocytic activity, the effect of ICs on the chemotaxis as well as phagocytic function of granulocytes was examined. As can be seen in Figure 4A, following stimulation with ICs from INF and UNINF, the number of migrating granulocytes was significantly higher (P
ICs from INF induce reduced oxidative burst of normal granulocytes

Because production of reactive oxygen species by an oxidative burst mechanism is an important function of granulocytes (11), the effect of ICs on the oxidative burst of granulocytes was studied. Granulocytes stimulated with ICs from INF showed significantly reduced oxidative burst compared with those stimulated with ICs from UNINF following PMA (P = 0.009) (Figure 5A) or E. coli (P = 0.0003) (Figure 5B) stimulation. Oxidative burst studies with negative control (MFI, 1.5) using the wash solution alone and positive control using PMA (MFI, 60) and bacteria (MFI, 200) alone in separate tubes were also studied.

ICs from INF induce increased calcium release by normal granulocytes

As calcium release is an early indicator of granulocyte activation by ICs (20), the effect of ICs on calcium release from granulocytes was studied. The ICs from INF showed an increased (P = 0.02) calcium release (INF vs. UNINF, GM: 144.6 vs. 94.9) (Figures 6A and B). Non-treated granulocytes were used as controls and data are presented as percentages of non-treated control for which calcium-associated fluorescence was measured in parallel cultures at the same time points.
DISCUSSION

Although many reports have highlighted the presence of increased CIC levels in TB, very few studies have demonstrated the biologic effects mediated by these complexes. In this study, we assessed the effect of ICs from either patients with TB disease or TB-uninfected healthy controls on the functional activity of granulocytes. Granulocytes are used as responder cells to study the functional effects of the immune complexes since they constitute the bulk of early recruited leukocytes that mediate and elicit responses to MTB. Polymorphonuclear neutrophil activation by ICs is thought to play a significant role in the pathophysiology of a number of inflammatory diseases and in some vasculitides (9, 14). One important mechanism by which neutrophilic inflammation is generated and sustained is through IC formation and/or deposition in tissue (13, 5). Indeed, IC-induced, local generation of chemotactic factors and cytokines promotes neutrophil recruitment and an extracellular inflammatory milieu favoring cell activation (7, 12).

ICs isolated from patients with TB reduced the release of IL-4, IL-6, GM-CSF, TNF-α, from granulocytes compared to cells stimulated with ICs from healthy individuals. IL-4 modulates the production of certain cytokines, induces class switching to IgE (6), upregulating MHCII production (10). IL-6 and GM-CSF are both proto-typical pro-inflammatory cytokines. TNF-α, is an important cytokine involved in systemic inflammation. Our study therefore reveals a global downregulation of cytokine production in normal granulocytes by ICs from patients with TB compared to uninfected individuals. Although previous reports (29) have demonstrated that ICs are potent stimulators of IL-10, we failed to observe any significant differential effect on IL-10 and other cytokines. Since pro-inflammatory cytokines are thought to mediate resistance to TB infection, our results on the decreased production of these cytokines might suggest that ICs might play an immunomodulatory role in TB and promote pathogenesis. This is further reinforced by our finding that the level of PAF (which was reported not to have protective immune response against tuberculosis.) (37, 38) was lower and NO (which
is readily demonstrated in macrophages from patients with inflammatory conditions such as tuberculosis (22) levels were not significantly different upon stimulation with ICs from TB patients. On the other hand, ICs from TB patients do not globally impair granulocyte activations since levels of HNP1-3 (36), which have potent microbicidal, chemotactic, and cytotoxic activity was significantly higher when ICs from INF patients were used to stimulate polymorphonuclear neutrophils.

Efficient chemotaxis, phagocytosis and the subsequent production of Reactive Oxygen Intermediates (ROIs) are important for the intracellular killing of microorganisms by phagocytes and defects in one or both of these functions may lead to deficient killing of intracellular microorganisms (34), thus predisposing the individual with pulmonary TB to further opportunistic infections. Therefore, the effect of ICs isolated from patients with TB on these granulocyte functions was studied in the present study. Neutrophils from individuals with active pulmonary TB have been shown to have an increased capacity for phagocytosis (28) and respiratory burst (19). Also it was shown in a previous study that granulocytes and monocytes from the TB patients exhibited a significant reduction in their phagocytic capacity. We found that ICs increased the chemotactic and phagocytic ability of granulocytes but diminished the oxidative burst. However, since killing of *M. tuberculosis* by neutrophils occurs via nonoxidative means (17), the deficient respiratory burst activity in patients with TB may not necessarily have an adverse impact on the anti-microbicidal activity of granulocytes. IC stimulation of circulating neutrophils is mediated by Fcγ receptors that cooperate in activating downstream signaling cascades, involving Src kinases, Syk, and PI3 kinase and changes in cytosolic free calcium (30, 4) that, in human neutrophils, generates an oxidative burst and degranulation. Phagocytosis and chemotaxis are granulocyte effector functions that are dependent on early calcium release. Our present study indicates that early calcium release is a characteristic feature of granulocyte activation by ICs from TB-infected patients.

Thus, the results from this study show that ICs can elicit immune responses through activation of granulocytes in PTB. IC-mediated modulation of granulocyte function can
potentially account for a variety of IC-induced inflammatory events in TB. Future studies will examine the signal transduction pathways activated by these circulating ICs in PTB.

ACKNOWLEDGMENTS

This work was supported by the Intramural Research Program of the Division of Intramural Research, National Institute of Allergy and Infectious Diseases, National Institutes of Health. Financial assistance provided in the form of a Research Associate fellowship to P. S. by the Indian Council of Medical Research is gratefully acknowledged. We thank S. Anbalagan for valuable technical assistance with flow cytometry and NIAID intramural editor B. R. Marshall for assistance. Because T. B. N. and S. B. are government employees and this is a government work, the work is in the public domain in the United States. Notwithstanding any other agreements, the NIH reserves the right to provide the work to PubMed Central for display and use by the public, and PubMed Central may tag or modify the work consistent with its customary practices. You can establish rights outside of the U.S. subject to a government use license.

CONFLICT OF INTEREST: The authors declare that they have no conflict of interest.
REFERENCES


Table 1. The release of IL-2, IL-5, IL-10, IL-12 and IL-13 were not significantly altered when granulocytes were treated with PEG-precipitated plasma ICs from patients with TB compared to ICs from UNINF. Granulocytes from normal healthy volunteers (n = 10) were stimulated with PEG precipitates of plasma ICs from UNINF and INF (n = 10 per group) for 18 h, and cytokines were measured by multiplex ELISA. P values were calculated using the Mann-Whitney test. UNINF, uninfected individuals; INF, patients infected with TB; GM, Geometric mean.

<table>
<thead>
<tr>
<th></th>
<th>INF [GM (Range)] (pg/ml)</th>
<th>UNINF [GM (Range)] (pg/ml)</th>
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<tbody>
<tr>
<td>IL-2</td>
<td>141.3 (48.69-409.9)</td>
<td>376.4 (304.6-465.1)</td>
</tr>
<tr>
<td>IL-5</td>
<td>302.0 (224.0-407.1)</td>
<td>341.1 (259.4-448.5)</td>
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<tr>
<td>IL-10</td>
<td>655.7 (425.3-1011)</td>
<td>816.6 (605.8-1100)</td>
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<tr>
<td>IL-12</td>
<td>514.9 (254.6-1042)</td>
<td>913.2 (731.0-1141)</td>
</tr>
<tr>
<td>IL-13</td>
<td>253.8 (377.3-707.3)</td>
<td>516.6 (377.3-707.3)</td>
</tr>
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</table>
Figure 1. Levels of ICs in serum samples from patients with TB and normal healthy volunteers. Serum from UNINF and INF (n = 10 per group) was treated with PEG-6000 and the absorbance of the precipitate was read at 280 nm using a spectrophotometer. CIC levels were determined by interpolation from a standard curve plotted using aggregated human gamma globulins as a standard. P values were calculated using the Mann-Whitney test. UNINF, uninfected individuals; INF, patients infected with TB.

Figure 2. PEG-precipitated plasma ICs from patients with TB induce diminished production of cytokines from granulocytes. Granulocytes from normal healthy volunteers (n = 10) were stimulated with PEG precipitates of plasma ICs from UNINF and INF (n = 10 per group) for 18 h, and cytokines were measured by multiplex ELISA. P values were calculated using the Mann-Whitney test. UNINF, uninfected individuals; INF, patients infected with TB. The box plot denotes geometric mean and 95% confidence interval.

Figure 3. PEG-precipitated plasma ICs from patients with TB induce increased release of HNP1-3 and decreased release of PAF from granulocytes. Whole blood from normal healthy volunteers (n = 10) were stimulated with PEG precipitates of plasma ICs from UNINF and INF (n = 10 per group) for 4 h, and the granular proteins (HNP1-3) (A); PAF (B) in the culture supernatants were measured using ELISA. P values were calculated using the Mann-Whitney test. UNINF, uninfected individuals; INF, patients infected with TB.

Figure 4. PEG-precipitated plasma ICs from patients with TB induce enhanced chemotaxis (A) and phagocytosis (B) of normal granulocytes. Whole blood from normal healthy volunteers (n = 10) were stimulated with PEG precipitates of plasma ICs from UNINF and INF (n = 10 per group) for 10 min, and chemotaxis as well as phagocytosis of granulocytes was measured using flow cytometry. P values were calculated using the Mann-Whitney test. UNINF, uninfected individuals; INF, patients infected with TB.
Figure 5. PEG-precipitated plasma ICs from patients with TB induce decreased oxidative burst of granulocytes. Whole blood from normal healthy volunteers (n = 10) were stimulated with PEG precipitates of plasma ICs from UNINF and INF (n = 10 per group) for 10 min, and oxidative burst of granulocytes was measured using flow cytometry in response to PMA (A) or E. coli (B). P values were calculated using the Mann-Whitney test. UNINF, uninfected individuals; INF, patients infected with TB.

Figure 6. PEG-precipitated plasma ICs from patients with TB induce increased release of calcium from granulocytes. Granulocytes from normal healthy volunteers (n = 5) were stimulated with PEG—precipitates of plasma ICs from UNINF and INF (n = 5 per group), and calcium release was assessed using a fluorimeter. Data are presented as a representative histogram (A) showing calcium release in response to ICs from one INF and UNINF individual and as percentages of non-treated control (B) for which calcium-associated fluorescence was measured in parallel cultures. P values were calculated using the Mann-Whitney test. UNINF, uninfected individuals; INF, patients infected with TB.
Figure 1

UN
INF
INF
0
10
20
30
p = 0.004
μg/ml
Cytokine levels (pg/ml)

- IL-2
- IL-4
- IL-5
- IL-6
- IL-10
- IL-12
- IL-13
- GM-CSF
- TNF-α

p-values:
- p=0.008
- p=0.02
- p=0.02
- p=0.05
- p=0.008
- p=0.02
- p=0.02
- p=0.05

Figure 2
Figure 3A

HNP 1-3 levels (pg/ml)

UNINF INF

Figure 3B

PAF levels (pg/ml)

UNINF INF

p=0.02

p=0.001
Figure 4A

Chemotaxis index
(Number of granulocytes migrated)

p = 0.002

Figure 4B

Phagocytic index
(Mean fluorescence intensity)

p = 0.02
Figure 5A

Figure 5B

Phagoburst index, PMA control (Mean fluorescence intensity)

Phagoburst index, Bacteria control (Mean fluorescence intensity)

p = 0.009

p = 0.0003
Figure 6A

[Graph showing calcium release over time]

Figure 6B

[Graph showing calcium associated fluorescence (% of control)]