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TST-Negative Individuals Do Not Lack Anti-Mycobacterial Responsiveness

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Abstract: Tuberculin-skin-test (TST) is widely used for the diagnosis of Tuberculosis. Negative TSTs are occasionally found in individuals vaccinated or infected by *Mycobacterium tuberculosis*. We show that false negative results are due to a lack of the Tuberculin-specific immune response as replacing tuberculin by unselected *M. tuberculosis* antigens improved the efficiency of the assay.

Tuberculosis (TB) remains a major global public health concern, with one third of the world population infected, 9 million active cases and 2 million deaths every year [1]. Despite major progress in the development of new strategies for treating TB, the disease remains a great challenge for healthcare workers. Most people infected by *M. Tuberculosis*, the causative agent of TB, remain asymptomatic (latent infection): the bacilli reside within granulomas, the histological hallmark of TB, and only 10% will develop a clinical disease during their life.

The tuberculin skin test (TST) is commonly used to help diagnosis of *Mycobacterium Tuberculosis* infection [2]. Purified protein derivative from *M. Tuberculosis* (PPD), also called Tuberculin, is injected intradermally, and the induction of a positive skin reaction is generally considered to indicate a latent or active TB, depending on the level of the skin reaction. In addition, positive tests are also found in patients after vaccination with Bacille Calmette-Guerin (BCG), or exposure to environmental mycobacteria. Negative test results may yet occur in a proportion of patients with active TB (10 to 25%) [3, 4]. These negative tests may find a biological explanation, as peripheral blood mononuclear cells (PBMC) from TB patients stimulated with Tuberculin (or PPD), release lower levels of IFN-γ and IL-12 compared to PPD responsive (*i.e.* TST-positive) healthy subjects [5]. Qualitative TST responses may vary depending on the clinical presentation of TB, especially in children. Finally, it has been established that there is no relationship between tuberculin skin-reactivity, and protection of vaccinated individuals against the development of active Tuberculosis [6]. Thus, not only the TST does not distinguish between Tuberculosis infection and BCG vaccination, but hence it does not witness a mycobacterial infection (in false negative individuals), and finally does not appear as a correlate of protection either.

Recent advances have improved the specificity of the immune identification of latent TB, using a combination of antigens (early secreted antigenic target 6 (ESAT-6), and culture filtrate protein 10 (CFP-10) encoded by the RD1 locus of *M. Tuberculosis* which is absent from most non-pathogenic mycobacteria, including Bacille-Calmette-Guerin). The sensitivity has also been improved by the ELISPOT (T SPOT-TB assay), an *ex vivo* assay quantifying the γ-Interferon produced by T-cells stimulated with ESAT-6 and CFP-10. On another hand, the performance of the recently proposed Quantiferon tests (based on whole blood interferon-γ release) has been shown to be negatively affected by patient's immune deficiency [7]. These serological and cytokine-based assays have yet to demonstrate an enhanced sensitivity and specificity when compared with the TST [8, 9]. In BCG-vaccinated individuals, false positive results occur with all these tests, and false negative tests due to anergy or immune deficiency are widely recognised [10-12]. Moreover, cost will be a critical factor in determining the global use of these new assays [13].

Pai and colleagues suggest combining the TST and γ-interferon assays to increase the sensitivity of the PPD-test and the specificity of RD1 antigens. This combination was used to confirm that the BCG vaccination gave significant protection against TB in children [14]. Yet, due to the numerous disadvantages of the TST previously discussed, we wondered whether we could find a better antigen than the Tuberculin to be used to give a more specific delayed-type hyper-sensitivity (DTH) reaction that could work in any individual. In this study, we thus investigated the possibility to replace Tuberculin by a more complex mixture of *M. Tuberculosis* (strain $H_{37}Rv$) antigens, obtained by mechanic

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disruption of the bacilli, which could possibly avoid false negative results. We used an *in vitro* model of human granulomas to compare the reactivity of human immunity to PPD and *M. Tuberculosis* antigens. This model reproduces *in vitro* a delayed-type hypersensitivity (DTH) reaction, as does the TST *in vivo*. This reaction is produced by the incubation of PBMC from healthy volunteers with sepharose beads coated with mycobacterial antigens, which produces a granuloma-like structure characteristic of the immune response to *M. Tuberculosis* [15-17].

Human blood samples were collected with informed consent from volunteers who had been BCG-vaccinated in infancy, and tested for their reactivity to PPD. For this purpose, PBMC from 100 volunteers (age 35-58, M/F: 61/39), including 85 PPD-positive individuals (Tubertest® 5U, 0.1ml) and 15 PPD-negative individuals, were isolated, incubated with beads coated with PPD, or *M. Tuberculosis* extracts (prepared as described by Puissegur and colleagues [16]), glycin-coated beads serving as negative control for non specific reaction. The ability of the different individuals to develop a granulomatous reaction around the different beads was followed by optical microscopy (data not shown) and scanning electron microscopy (Fig. **1**).

As expected, granulomas were found for PPD-responders (TST > 5mm), both around beads coated with PPD (Fig. **1A**), and beads covered with *M. Tuberculosis* extracts (Fig. **1B**). In the 15 PPD-nonresponders (TST <5mm), no granulomas were ever found after incubation with PPD-coated beads (Fig. **1D**), but important granulomatous reaction developed in response to *M. Tuberculosis* extracts (Fig. **1E**).

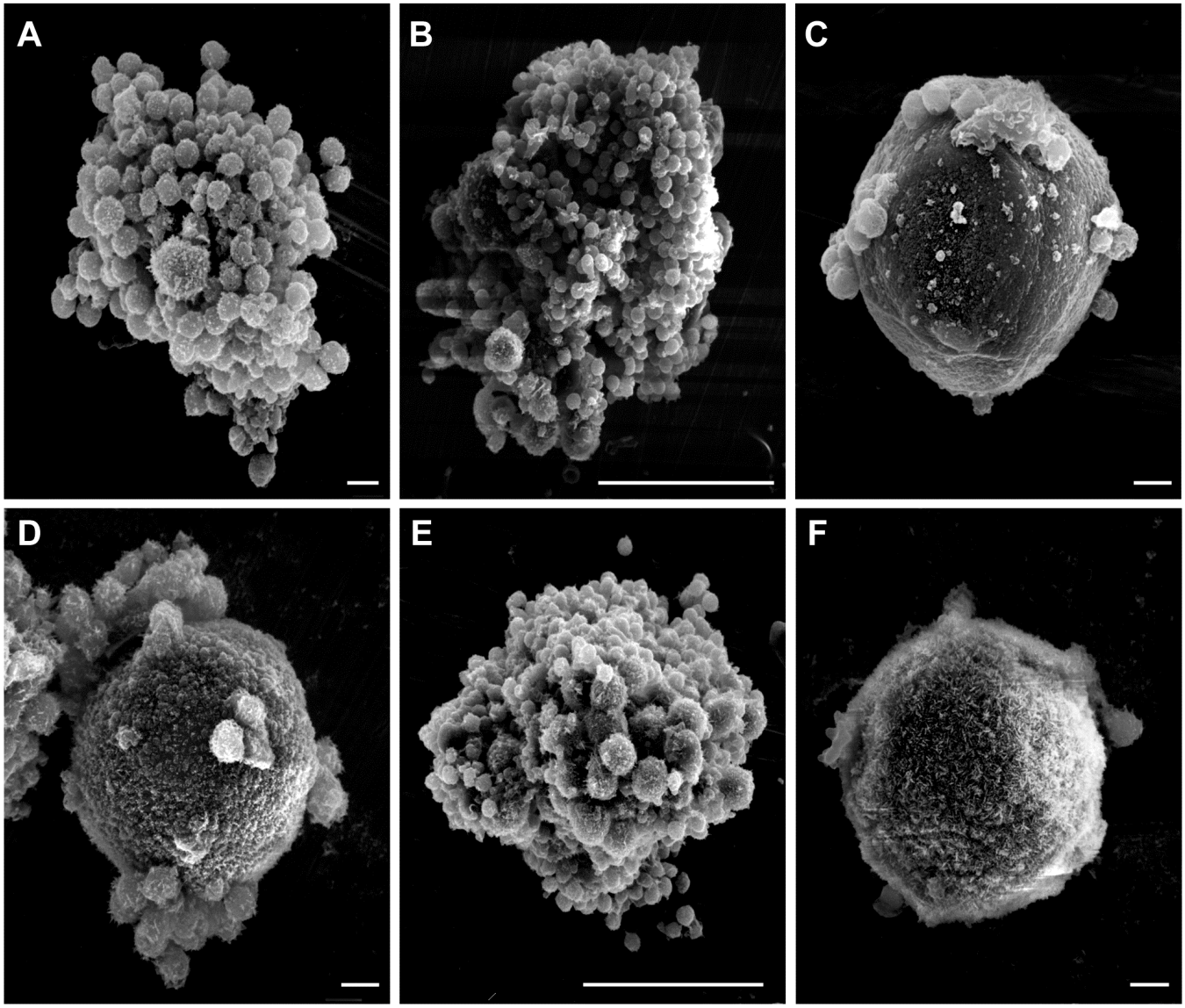

Fig. (1). Scanning electron micrographs of $H_{37}Rv$ surface extract-induced or PPD-induced granulomas. Beads covered with PPD (**A**,**D**) or surface extract from $H_{37}Rv$ (**B**,**E**) were incubated with PBMC isolated from TST positive (**A**,**B**,**C**) or negatives (**D**,**E**,**F**) volunteers, all BCG vaccinated in infancy. Beads covered with Glycine (**C**,**F**) represent the negative control for non specific fixation. After 11 days of culture, granulomas were prepared and observed under a scanning electron microscope. Bar represent 5µm (**A**,**C**,**D**,**F**) or 50µm (**B**,**E**). All the analyses herein presented were performed according to the principles expressed in the Helsinki Declaration, with an informed consent of all volunteers.

No cellular recruitment was observed with the negative control glycin-coated beads (Fig. **1C**,**F**). A quantification of the granulomatous response has been possible by the definition of a granuloma index (Fig. **2A**,**B**,**C**). The respective proportions of granulomas typical for each index has been determined for every TST- individual and presented in comparison to the index proportions of a representative TST+ volunteer (Fig. **2D**).

Noteworthy, the granulomatous response observed against *M. Tuberculosis* extracts is specific of the anti-mycobacterial response as no granulomatous response can be observed against *E. Coli* antigens, as previously described [16].

Nowadays, the TST remains the most widely used test worldwide to determine whether an individual has an immunological reactivity against mycobacterial antigens and for the diagnosis of TB. Pai and colleagues [14] suggested a combined use of PPD and a T-cell based ELISpot, using ESAT-6 and CFP-10 as antigens, for the diagnosis of latent TB. Here, we point out that the absence of response to PPD doesn't necessarily mean an energy to *M. Tuberculosis* antigens, but rather a selective defect in the response to PPD of given individuals. In this study, we have shown that PBMC from all subjects who have been vaccinated in childhood, are able to respond *in vitro* to *M. Tuberculosis* complex extracts by the induction of a DTH response, whatever their ability to react to PPD. We could yet not determine the specific antigens from *M. Tuberculosis* complex extracts, responsible for the induction of the DTH response. Our model of *in vitro* granulomas using beads coated with purified antigens from *M. Tuberculosis* such as ESAT-6 and CFP-10, could improve, in combination with the ELISPOT assay, the efficiency and reliability of the diagnosis of tuberculosis.

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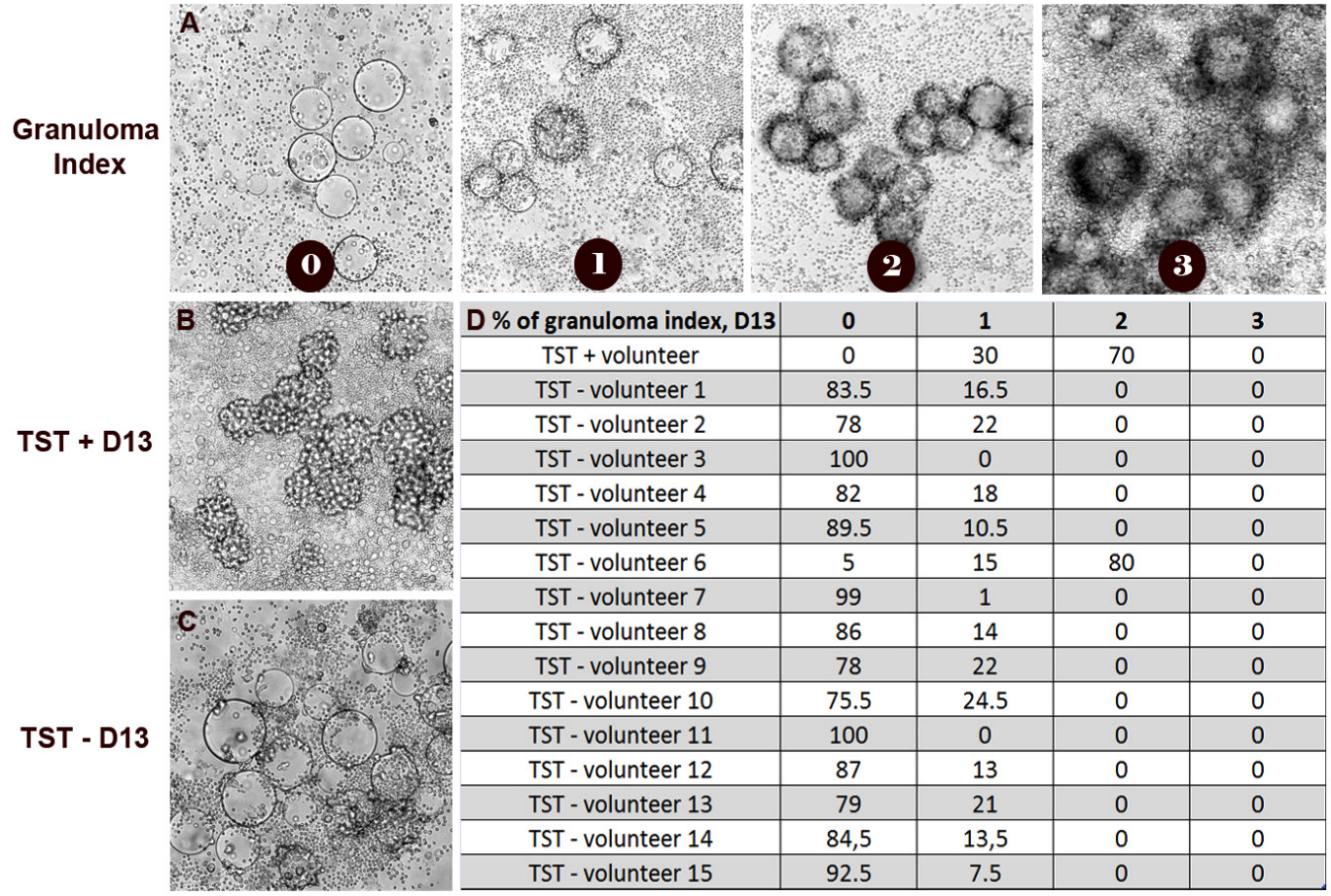

D % of granuloma index, D13	0	1	2	3
TST + volunteer	0	30	70	0
TST - volunteer 1	83.5	16.5	0	0
TST - volunteer 2	78	22	0	0
TST - volunteer 3	100	0	0	0
TST - volunteer 4	82	18	0	0
TST - volunteer 5	89.5	10.5	0	0
TST - volunteer 6	5	15	80	0
TST - volunteer 7	99	1	0	0
TST - volunteer 8	86	14	0	0
TST - volunteer 9	78	22	0	0
TST - volunteer 10	75.5	24.5	0	0
TST - volunteer 11	100	0	0	0
TST - volunteer 12	87	13	0	0
TST - volunteer 13	79	21	0	0
TST - volunteer 14	84,5	13,5	0	0
TST - volunteer 15	92.5	7.5	0	0

Fig. (2). Characterization of the granuloma index of TST negative individuals in response to PPD-beads. Beads covered with PPD were incubated with PPMCs from both TST negative (TST-) and TST positive (TST+) individuals. Pictures representing typical granuloma differentiation stages (index) are presented (**A**), magnification x200. Index 0: no cell recruitment around the beads. Index 1: several cells found on the beads up to a mono-layer cell recruitment with no cellular differentiation. Index 2: One to two cell layers around the beads, no sign of differentiation. Index 3: multi-layer cell recruitment, with strong macrophage differentiation, not visible on this picture. (**B**) Picture representative of granulomas found for TST+ individuals after 13 days of reaction (D13), magnification x200. (**C**) Picture representative of granulomas found for TST- individuals after 13 days of reaction (D13), magnification x200. (**D**) the proportions of the different granuloma index found after 13 days of reaction for the 15 TST- individuals and one TST+ representative individual, are presented.

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