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How Flow Cytometry is Changing the Study of TB Immunology and Clinical Diagnosis

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• Abstract

The application of flow cytometry has hugely advanced the field of tuberculosis (TB) across all areas of research ranging from diagnostic tests to understanding the underlying immunology. As cellular responses are understood to be the mainstay of the immune response in the control of TB it is very likely that polychromatic flow will become the tool of choice for assessing the effects of vaccination. Results are particularly encouraging in this area. The development of a new type of diagnostic test, a prototype of which has been reported, may be the spin-off of a broad and systematic approach to understanding and profiling the T-cell response to TB. It is obvious that flow cytometry will not be able to address all research questions in the field of TB. However, its enormous flexibility as a technology will make it the tool of choice in many situations. An ever increasing availability of flow cytometers, even in resource-poor countries, will rapidly change the face of TB research and management in the years ahead. © 2008 International Society for Advancement of Cytometry

• Key terms

T-cells; flow cytometry; intracellular cytokine staining (ICS); interferon-γ-release assays (IGRA)

Tuberculosis (TB) puts an immense burden on global health and severely damages the economy of resource poor areas. Apart from the immense cost of millions of human lives every year it leaves many affected individuals unable to support themselves or their families. While malnutrition and poor living conditions have always been a major challenge, the HIV epidemic has finally destroyed the hope that the global use of the Bacille Calmette-Guerin (BCG) vaccination will eventually contain TB. HIV infection severely damages the cellular immune response required to control active TB and maintain latency.

Cases of active pulmonary TB spread the disease across communities but these only represent a minority of the infected population; latent tuberculosis infection (LTBI) by far represents the biggest reservoir of infection (1). The two primary aims of the strategy to achieve control of this situation, therefore, must be (1) development of an effective protective vaccine (which has given rise to a large number of immunological studies), and (2) the detection and management of active pulmonary TB in the population. Because 10% of all cases of LTBI will turn to cases of active TB in a lifetime (or per year in the HIV positive community) a third aim must be the detection and management of LTBI (2,3).

Although flow cytometry appears to be a mainstay of research in the HIV field, it has not been used to its full potential in the TB field. However, the number of studies using flow cytometry for exploring the immune response against TB has been increasing in recent years. In this short review we will show how some of these studies have produced encouraging results and made a change to our understanding of the immunology of TB. Both vaccine development and the diagnosis of TB will be addressed.

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IMMUNOLOGY AND VACCINES

Understanding the underlying principles of protective immunity after *Mycobacterium tuberculosis* infection will be key to the development of protective vaccines. It is hoped that the characteristics of a protective natural response may serve as a template for responses elicited through vaccination. The advent of multicolor flow cytometry and later polychromatic flow cytometry (more than four colors at the same time, or more than six colors at the same time, respectively), along with the ability to analyze cell function and phenotype in parallel, has resulted in a significant improvement to our ability to characterize complex cell populations (4–6). Researchers have recently begun to apply this technology to exploring the T-cell immune response to TB. The most obvious questions to ask in these initial studies were

what are the protein targets of TB antigen specific T-cell responses?
what is the lineage and the phenotype of the T-cells involved?
what is the functional potential of these cells?

Answering these questions raises several complex issues. One of them is concerned with the immunological compartment from which material is sampled for such measurements. There is very good evidence that TB-specific T-cells are predominantly found in the lung (7), and as a result, studies on lymphocytes derived from the lung should be more relevant than studies on blood. Published flow cytometric data on lung-derived TB-specific T-cells in humans are based on the analysis of broncho-alveolar lavage (BAL) samples (7). In mice, such studies are usually based on tissue samples. Barry et al found that in cases of active TB the frequencies of TB-specific CD4 T-cells in human lung (BAL) were significantly higher than in blood irrespective of whether TB lesions were localized in the lung or at extrapulmonary sites. Such a difference was not found in BCG-vaccinated controls. This finding suggested that TB-specific T-cells home to the lung and that the distribution of TB-specific T-cells between lung and blood had some diagnostic potential (this will be discussed later on). Animal models are very well suited to exploring TB-specific T-cells in tissue sites using flow cytometry. A very recent study illustrates this nicely: Wolf et al. reported a delay in the adaptive immune response to the model antigen, Ag85, following infection of mice with *M. tuberculosis* via the airways (8). This delay was explained by the requirement of live bacteria in local pulmonary lymph nodes for the priming of the CD4 T-cell response. Activation of CD4 T-cells in the mediastinal lymph was, therefore, identified as a limiting step in the initiation of the adaptive immune response. In humans, however, the analysis of lung-seeking T-cells is limited by the fact that BAL is a technically demanding and invasive procedure with limited scope for use in larger studies. In order to avoid this problem some authors have begun analyzing induced sputum samples by flow cytometry as an alternative to BAL fluid. This is an attractive solution but still in its early days (9,10). It also appears to have potential as a diagnostic procedure (9,10) (see later), but may be limited in terms of multicolor flow, due to a lack of sufficient numbers of cells in such samples.

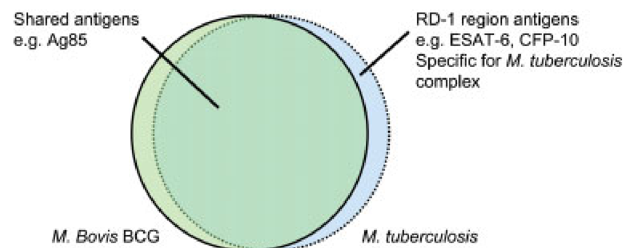

Figure 1. The antigenic overlap between different preparations of mycobacterial antigens is illustrated by the overlapping circles. Some antigens are contained in *M. tuberculosis* complex strains and *M. bovis* BCG. T-cell responses to these antigens cannot discriminate between exposure and vaccination. Responses to antigens contained exclusively in *M. tuberculosis* complex strains but not in *M. bovis* BCG can only be explained by exposure to TB. This is the basis for the use of commercial IGRAs to identify cases of LTBI. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

The other critical issue in regards of analyzing TB-specific T-cells is the choice of TB antigen used for T-cell stimulation. Most flow-cytometric studies of TB-specific T-cells will depend on ex-vivo T-cell stimulation. Intracellular cytokine production is used as the standard method of identifying these cells, but only a few studies have used direct MHC-multimer staining of specific T-cells. In terms of stimulation the most obvious antigens to use are tuberculins (or PPDs = purified protein derivatives). These are crude mixtures of proteins derived from heat-killed, virulent strains of *M. tuberculosis*. PPDs contain many different mycobacterial proteins (11,12), but responses are not strictly specific for the agents pertaining to the *M. tuberculosis* complex, i.e. those causing the clinical picture of TB (13). Rather, the proteomes of *M. bovis* BCG and other mycobacterial strains overlap with that of *M. tuberculosis* complex agents to such a degree that responses to tuberculin and *M. tuberculosis* can only be discriminated if very specific antigens are used which are contained in *M. tuberculosis* complex agents but not in BCG or environmental strains (Fig. 1). The so-called RD-1 antigens ("region of difference-1") antigens, including ESAT-6 and CFP-10, for example, were deleted in *M. bovis* BCG because the RD-1 region, a 9.5-kb DNA segment, is important for the virulence of *M. tuberculosis* complex strains (14). In theory, responses to these antigens should only be acquired by exposure to virulent strains (15,16). Specificity is not 100% though, because these antigens are found in some non-*M. tuberculosis* complex strains as well (17).

Drawbacks of using these subunit antigens are demonstrated by observations that response levels in TB patients may be similar to those of community controls and lower than in contacts (18). Also, responses to subunit antigens if detected are much smaller than PPD specific responses (19,20). This is a problem for multiparameter analysis because the numbers of activated cells may be smaller than the number of subsets potentially arising from the staining panel.

So far, most multiparameter flow-cytometric studies in this area have used more complex antigen preparations such as BCG or standardized human tuberculin, because they were designed to explore the effects of the BCG vaccination or alternative vaccinations.

Of note, protein antigens, whether protein lysates or recombinant proteins, are typically processed and presented by the external pathway of antigen processing and presentation and thus are presented in the context of class-II MHC molecules. This means that they will mainly stimulate CD4 T-cells. The degree to which CD8 T-cell responses elicited, with PPD or BCG for example, may underestimate the size of this response or reflect the actual quality of the CD8 T-cell response to TB is unclear. CD8 T-cell responses can be induced by peptides representing TB antigens, however, given the multitude of TB proteins it is not possible to predict which peptide/proteins should be used.

A more comprehensive way of addressing CD8 T-cell responses is via stimulation with infected macrophages. A very elegant study published in 2005 (21) "visualized" degranulating T-lymphocytes as they were confronted with *M. tuberculosis*-infected macrophages. The versatility of the approach enabled T-cell lineage, chemokine expression, and granulysin content to be explored simultaneously. It was one of the major findings of this study that concurrent expression of CCL5, perforin and granzymes in CD8 T-cells represented an effective response to this challenge. In a less comprehensive manner, the CD8 T-cell responses to a single HLA-A2-restricted peptide was analyzed using an MHC-tetramer for detection, in combination with peptide stimulation (22). TCR Vbeta usage and cytokine secretion patterns in this peptide specific population could thus be analyzed in parallel. It was found that the CD8 T-cells recognizing this peptide exhibited a Tc2 cytokine secretion pattern, producing IL-4 and GM-CSF. This was an interesting discovery greatly facilitated by the application of flow cytometry. Despite their more limited scope in regards of antigen specificity, the use of MHC-multimers is of considerable interest, because no activation of T-cells is required for their detection, so the phenotype is not changed by in vitro manipulation. Additionally, MHC-multimers can be used to examine T-cell receptor binding avidity which has been implicated in response efficacy (23). A disadvantage, from a technical point of view, is that in order to be able to manufacture MHC-multimers, epitopes must already be known. Results obtained with such MHC-multimers should be generalized only very cautiously.

Studies using the most generic antigens like tuberculin or BCG have enhanced our understanding of host defense against TB. For example, the effect of the BCG vaccine on the development of tuberculin and/or BCG-specific T-cells in children was recently analyzed by multiparameter flow cytometry (24,25). These studies looked both at the phenotype of the responding T-cells and functional parameters including cytokine production and degranulation. Although no significant level of degranulation was detected, this study confirmed that BCG vaccination induces a "robust" CD8 T-cell response (apart from a CD4 T-cell response) that may contribute to vaccination-induced protection against TB (24). While CD4 T-cell responses (median) were in the order of 0.1% of CD4 T-cells for IFN-γ, IL-2, and TNF-α, CD8 responses were slightly smaller and decreasing in the order of the cytokines listed.

The induction of "T-cells with a complex pattern of cytokine expression and phenotypes" (25) could not have been revealed other than by flow cytometry. Importantly, it was also found that measuring IFN-γ production alone underestimates not only the magnitude but also the complexity of the immune response to this vaccination. In other words, IFN-γ can probably not stand alone as a read out to test the efficacy of BCG or other, novel, TB vaccines. The application of multiparameter functional flow cytometry in order to explore the effects of different vaccines will likely be key to understanding the differences between them and the effect of potential adjuvants. At this time, however, the available data does not explain if the characterized responses are protective or not. In the future it may be possible to associate the outcomes of the vaccinated children with the characteristics of their response to BCG. Another recent study confirmed the existence of Th17 T-cells as part of the immune response to TB antigens by direct intracellular staining of IL-17 and IL-22 (26). Th17 cells are known to have a role in the regulation of inflammation and the accumulation of IFN-γ producing memory T-cells in *M. tuberculosis* infected tissue (27,28). CD4 T-cells expressing IL-17 and/or IL-22 were identified both in healthy BCG vaccinees and in TB patients. In healthy donors a median of $\sim$ 0.25% of CD4 T-cells produced IL-17 compared with 0.5% of these cells producing IL-22. Unlike recently reported in a mouse model (29), however, only very few cells produced both cytokines at the same time. Both subsets made a sizeable contribution to the overall activated CD4 T-cell population and displayed a phenotype characterized by an early antigen-dependent differentiation stage (mostly CD45RA-negative/CCR7-positive), retaining the ability to migrate into lymphatic tissues (30). Interestingly, these cells were less frequent in the peripheral blood of patients with active TB than in uninfected controls, which would suggest they have a protective role. A second, somewhat similar study analyzed the properties of CD4 T-cell responses induced by a prime-boost vaccination using BCG-MVA85A, a modified vaccinia virus, Ankara-expressing Antigen 85A (a secreted protein contained in many mycobacterial species, including *M. tuberculosis* and *M. bovis* BCG) (Fig. 2), in adults (31). Ag85A specific CD4 T-cells were found to be of a 'relatively immature' phenotype (CD45RO-pos./CD27-intermediate/CD57-neg.) (30), expressing multiple cytokines (IFN-γ, TNF-α, IL-2, and MIP-1beta). This study concluded that the use of BCG-MVA85A was supported by these results, based on the currently widespread perception that polyfunctional T-cells are instrumental in protection from infection. It should be noted, however, that this perception does not stem from observations made during *M. tuberculosis* infection, but is an extrapolation from findings in HIV infected patients (32).

So far all studies seem to agree that BCG vaccination (including the BCG-MVA85A prime/boost strategy) induces

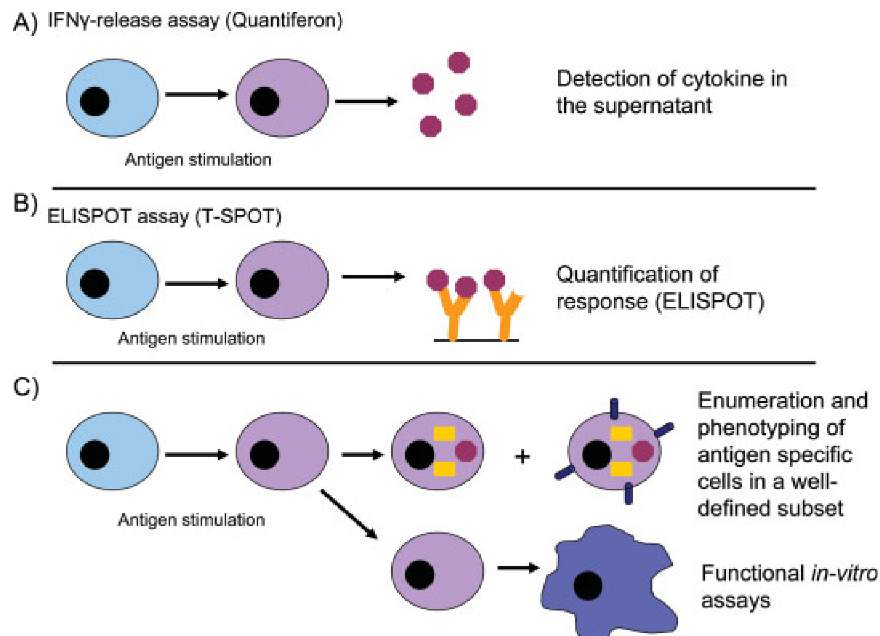

Figure 2. Principle of IFNγ-release assays. Quantiferon (**A**) and T-SPOT.TB (**B**) detect IFN-γ release after stimulation with TB antigens. Both methods are easy to perform as routine tests but can be disturbed by bystander IFN-γ-production (e.g. HIV coinfection). (**C**) TB-specific T-cells can be detected with flow cytometry as well. Intracellular IFN-γ-accumulation and co-staining of surface markers (e.g. CD3 and CD4) helps identify the T-cell subset of interest. ICS can be combined with functional assays (e.g. degranulation). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

the maturation/differentiation of T-cells with the ability to produce multiple cytokines and an intermediate differentiation phenotype. In light of the fact that the BCG vaccine only appears to lend limited protection to vaccinees, additional studies will be required to define correlates of protection in the context of TB. For example, studies addressing potential changes in the T-cell response occurring when LTBI develops into active TB would be of great use. Because timing of this event is unpredictable, such studies are not easy to realize. However, comparing TB-specific T-cell responses in individuals with recent TB reactivation and individuals with latent TB would be easier and could be of similar interest, because the immune system could be considered to have lost control in the former while maintaining it in the latter.

Using enzyme-digested tissue samples from TB infected mice, Lyadova et al. showed for the first time that a majority of T-cells producing INF-γ in response to challenge with mycobacteria in the lung of mice have a CD27-negative phenotype (33). CD27 is a differentiation marker which is gradually lost with increasing effector type differentiation (30). Lyadova et al. also discovered that these CD27-negative T-cells can migrate from peripheral sites to the lung but can also be derived in situ from CD27-positive T-cells (they are infrequently found in lymph nodes) (34). They appear to play an important role in protecting mice challenged with mycobacteria. Work published in 2007 compared T-cell responses in humans with active pulmonary TB and uninfected, BCG vaccinated controls. A striking difference was noted between the two groups in terms of CD27 expression on tuberculin-specific CD4 T-cells. The downregulation of CD27 might occur following infection with TB (20). The same study suggested that the degree of CD27 downregulation (or, more precisely, the number of CD4 T-cells that had lost CD27-expression) in individuals with LTBI was intermediate, not as low as in the uninfected vaccinees and not as high as in the cases with active pulmonary TB. Loss of CD27 might be the reason for, or the result of, LTBI getting out of control when LTBI develops into active TB. The complete loss of CD27 expression in the majority of T-cells suggest a more advanced antigen dependent differentiation than found in response to vaccination and may be linked to lung homing (30,33,34). Additional studies are needed to clarify this issue, but whatever the result may be, this test has potential for the diagnosis of active TB as discussed in more detail later. Table 1 lists the main technical features of recent studies on the phenotype and function of T-cells specific for mycobacterial antigens.

Diagnosis of TB

It was recently estimated that a quick and accurate diagnostic tool for TB could save over a quarter of $\sim$ 2 million lives claimed by TB each year (35). Currently flow cytometry has no role in the clinical diagnosis of TB, however, several promising developments will be discussed in this section that may well lead to such a test becoming available in the near future.

The diagnosis of active pulmonary TB is normally made based on clinical signs and symptoms, sputum smear, X-ray, and culture. LTBI is classically diagnosed by a positive result in

Table 1. Studies that used multiparameter (polychromatic) flow cytometry to explore mycobacteria specific T cells in humans

STUDY	INDIVIDUALS	STIMULANTS USED	RESPONSES STUDIED	SURFACE MARKERS	CYTOKINES/FUNCTIONAL READOUTS
Scriba et al. (26)	TB patients, healthy BCG vaccinees	Live *M. bovis* BCG, PPD, ESAT-6[a], CFP-10[a]	CD4 T-cells mainly	CD27, CD45RA, CCR7	IFN-γ, TNF-α, IL-2, IL-22, IL-17
Soares et al. (25)	Vaccinated human infants	BCG	CD4 and CD8 T-cells	CD27, CD45RA	IFN-γ, TNF, IL-2
Beveridge et al. (31)	Healthy BCG vaccinees	PPD, Ag85A[a]	CD4 T-cells	CD27, CD45RO, CD57	IFN-γ, IL-2, TNF-α, MIP-1b, Degranulation (CD107a)
Streitz et al. (20)	TB patients, healthy BCG vaccinees	PPD, ESAT-6	CD4 T-cells	CD27, CD28, CD57, CD62L, CCR7	IFN-γ, TNF, IL-2
Murray et al. (24)	Vaccinated human infants	Live M. bovis BCG, H37Rv	CD4 and CD8 T-cells	CD69	Proliferation (BrdU), Degranulation (CD107a/b), IFN-γ

[a] Peptide pool.

the tuberculin skin test in the absence of clinical signs or symptoms of active TB. Results are counted as positive if the response is positive in individuals who were not vaccinated or if it exceeds the response normally seen in BCG vaccines (diameter of area of induration). Interestingly the skin response to PPD has previously been analyzed by flow cytometry using cells extracted from blister fluid (36). Blisters were induced by a slow vacuum pump at different time points after intracutaneous injection of tuberculin and analyzed following restimulation with PPD. Depending on the time point of blistering, up to 30% of CD4 T-cells responded to PPD with IFN-γ production. Remarkably, the peak of T-cell infiltration/proliferation was not reached until about 2 weeks after tuberculin injection, which stands in apparent contrast to the clinical practice of reading the tests after 48 or 72 h. Clonal analysis of both blood derived and infiltrating PPD specific CD4 T-cells revealed that not all clones present in the blood can actually infiltrate, because only between 44 and 85% of the blood clones were present in the lesions.

In as far as ex vivo immunological tests go there has been some confusion relating to the so-called interferon-γ release assays (IGRAs). These are based on in vitro T-cell stimulation with RD-1 antigens (16,37). IFN-γ release above a certain threshold is considered a positive response (in analogy with the skin test, responses in BCG vaccinees are supposed to be below this threshold). IGRAs are currently supplanting the skin test for LTBI because they are considered more reliable and have the advantage that the patient need not return for reading the test (15,17). Figure 2 shows a schematic representation of the IGRAs and the flow cytometry based approach to TB-specific T-cells. The confusion about the purpose of the IGRAs probably results form the fact that some recently published papers on the diagnosis of TB have implicated IGRAs in the diagnosis of both latent and active TB (38,39). However, there is (relative) consensus that the only gold standard for active TB is a positive culture result (38,40). A positive IGRA test indicates exposure to TB and, therefore, that TB might be the cause of a patient's signs and symptoms suggestive of TB, but this is not a reliable way of diagnosing active TB.

Flow cytometry based IGRAs can be set up quite easily (19,41–43) but no commercial test of this format is available at this time. Barry et al., however, made an important discovery when analyzing BAL fluid using a flow cytometry based IGRA (7). The frequencies of PPD-specific CD4 T-cells in BAL fluid observed in TB patients were very high,—comparable to those observed in the skin blister infiltrating population in healthy donors studied by Reed et al. (36)— and more importantly, a large difference in PPD-specific CD4 T-cell frequencies between peripheral blood and BAL fluid was seen exclusively in cases of active TB. This would be the result of an infiltration of the lung by PPD specific CD4 T-cells; however, curiously, this marked difference in frequencies was also observed if TB was extrapulmonary. So, in a way, a rapid flow cytometry based test for active TB already does exist, but is limited by the need to perform BAL. On the other hand, BAL as part of the diagnosis of TB, especially in HIV+ individuals is performed relatively frequently, because in HIV patients,

decreasing CD4 T-cell counts limit the usefulness of IGRAs performed on blood. Because there is a concentration of TB-specific T-cells in the lung, using BAL fluid is clearly more promising (10,44). One of the major advantages of flow cytometry over the commercial IGRAs in this setting is the ability of the former to specify the source of cytokine production. This is, because increased spontaneous IFN-γ production of HIV-specific CD8 T-cells in the lung is likely to produce false positive results, unless the origin of the IFN-γ production can be specified, which is possible using flow cytometry, but not with ELISA or Elispot. This topic was reviewed elsewhere (10).

Regarding the more accessible peripheral blood compartment, Tesfa et al. demonstrated that PPD-induced IFN-γ and IL-2 production identifies similar numbers of responding CD4 T-cells. Hughes et al. demonstrated the enhanced sensitivity which can be achieved when using several different cytokines simultaneously (albeit in different replicates). This underscores the notion that IFN-γ should not be used as a sole read-out for T-cell activation by TB antigens (19).

Despite these obvious advantages, a diagnostic accuracy test comparing a flow cytometry based IGRA with the commercial tests has not yet been performed. However the commercial IGRAs (T-Spot.TB and Quantiferon Gold) are very easy to use. In particular the Quantiferon Gold test can be automated to run on standard pipetting robots available in most hospital labs (45,46).

The loss of CD27-expression on tuberculin reactive CD4 T-cells discussed earlier could provide an interesting diagnostic marker for active TB. It appears to discriminate active smear and/or culture positive TB from smear and/or culture negative TB, latent TB, and status post BCG vaccination. However, the reported results need to be confirmed in a bigger (field) study, in particular the distinction between latent and active TB. The basic principle behind this test is very attractive and may be able to be applied in other clinical situations as well. Rather than subunit antigens like ESAT-6 or CFP-10, the broadest and most unspecific antigen, tuberculin, is used for stimulation of blood cells. This results in a much bigger response which is also found in BCG vaccinees. As expected, the size of this response is not discriminatory, just as the skin test cannot reliably distinguish between the status post vaccination, LTBI, or active TB. However, when the actual phenotype of the response is considered, differences are found. These may result from the fact that following vaccination, only a limited degree of differentiation takes place. Following exposure this response is boosted, and more cells lose CD27 (as CD4 T-cells do when they differentiate following antigen contact) (30,47). This interpretation is in agreement with the analysis of T-cell responses induced by BCG vaccination discussed earlier, indicating that BCG vaccination leads to loss of CD27 on some, but not most, TB-specific T-cells. Thus, the proportion of cells that have lost CD27 would be an indirect measure of TB antigen exposure. This may explain why highly exposed individuals were found to have higher levels of CD27-negative CD4 T-cells than vaccinees. A simple version of this test requires only three fluorescence channels (CD4 for helper T-cells, IFN-γ, CD27) and can be run on very basic flow cytometers.

In conclusion, flow cytometry continues to give answers in many areas of TB research where questions remained open due to a lack of a research tool combining phenotypic and functional analysis of T-cell subsets of interest. While the application of multicolor flow cytometry to vaccine development is logical and merely parallels the development in other fields, in particular HIV infection, the successful development of a clinically applicable flow cytometry based diagnostic test for active TB would be novel. It remains to be seen if the existing data can be corroborated. At any rate, flow cytometry will increasingly complement or replace other methods used in TB research and diagnostics and the field is likely to greatly benefit from this development.

Acknowledgments

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