Baseline cytokine profiles of tuberculin-specific CD4 T-cells in non-muscle invasive bladder cancer may predict outcomes of BCG immunotherapy within reach?

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Baseline cytokine profiles of tuberculin-specific CD4 T-cells in non-muscle invasive bladder cancer: is predicting the outcome BCG immunotherapy within reach?

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Abstract

Intravesical Bacillus Calmette-Guérin (BCG) immunotherapy is used for preserving the bladder after resection of high-risk non-muscle invasive bladder cancer (NMIBC). Unfortunately, to date, treatment failure, which carries a high risk of disease progression and occurs in about 30% of patients, cannot be predicted a priori with a simple, reliable test. To address this shortcoming, we examined the in vitro tuberculin-responsiveness of CD4 T-cells prior to the first and second rounds of BCG immunotherapy in n=42 patients with high-risk NMIBC. The frequencies of overnight tuberculin-activated cytokine-expressing CD4 T-cells and their polyfunctionality were assessed by flow-cytometry; the levels of overnight tuberculin-induced, secreted mediators were measured by electrochemiluminescence. The primary endpoint was recurrence-free survival at 6 months. Surprisingly, tuberculin-induced, secreted IL-2 before induction therapy performed best. A lower cut-off at 250pg/ml provided 79% sensitivity, 86% specificity (AUC=0.852, p=0.000), and overall correct classification in 78.6% of cases; also, in 50% of patients later experiencing recurrence but none of the recurrence-free survivors it was below 120pg/ml. Several other parameters performed well, too, including secreted IFN-γ (AUC=0.796, p=0.002) and the frequencies of TNF-producing (TNF+) CD4 T-cells (AUC 0.745, p=0.010). Interestingly, higher levels of ‘polyfunctional’ CD4 T-cells (IFN-γ+/IL-2+/TNF+) were significantly associated with recurrence-free survival (AUC 0.801, p=0.002). In conclusion, our work shows that overnight in vitro tuberculin-induced IL-2 secretion has significant potential for predicting the outcome of BCG immunotherapy and may identify 50% of BCG failures with 100% specificity before induction therapy is begun. This should, in future, help doctors and patients make the best treatment choice.
Keywords: Bladder cancer, BCG, Immunotherapy, marker, response.

Synopsis

BCG immunotherapy helps preserving the bladder after removing certain bladder cancers but fails in about 30% of patients. A novel test identifies *a priori* those most likely to fail so they may receive other, more effective treatments. (37 words)
Introduction

Bladder cancer is the ninth most frequently diagnosed cancer worldwide with an estimated 430,000 incident cases and 165,000 deaths in 2012 (1). The incidence is highest in Western and Southern Europe and North America and higher in men than in women. Transitional cell carcinoma (TCC) accounts for over 90% of cases, the majority of which present with early non-muscle invasive bladder cancer (NMIBC) and are treated with transurethral resection. Intravesical Bacille Calmette-Guérin (BCG) immunotherapy (henceforth referred to as ‘BCG immunotherapy’) administered in several cycles over one year is the standard treatment for high-risk NMIBC and significantly reduces the otherwise high recurrence rates (2-5). Non-responders to BCG immunotherapy have a high risk of disease progression and death (6,7), however, reliable predictors of treatment outcome at baseline, i.e. prior to therapy induction, or early into therapy have not been identified yet.

BCG has been administered intradermally as a tuberculosis (TB) vaccine for almost 100 years (8). It prevents the most severe complications of TB in children (such as meningitis) in about 80% of cases but is less effective in preventing pulmonary TB (9). The induction of CD4 T-cells specific for mycobacterial antigens, in particular those producing IFN-γ, is believed to explain the protective effect of the vaccine (10), however, the immunological correlates of protection from TB are still not well understood (11). The mechanisms by which anti-mycobacterial immunity induced by BCG might help contain bladder cancer are even less well understood. Various features of BCG-induced immune responsiveness, such as urinary cytokines before and at various time points following induction therapy, natural killer cell activity, or changes in anti-Mycobacterium bovis heat shock protein 60 (HSP60) antibody levels (12-19), have in the past been associated with treatment success for NMIBC. Fewer studies, however, have explored specific cellular parameters.
Interestingly, Elsasser et al. showed that intravesical BCG increases the numbers of tuberculin-specific CD4 T cells detectable in blood, however, their attempt to predict the outcome of BCG immunotherapy based on these changes in a small study (n=18) with variable pathology was unsuccessful (20). The study presented here was larger (n=42), focused on early outcome predictors in patients with high-risk NMIBC, and included tuberculin-induced secreted cytokines in addition. Unlike Elsasser et al, we found strong associations between tuberculin-induced CD4 T-cell responsiveness and the absence of tumor recurrence. Our results suggest that a functional, T-cell response-based test for predicting BCG immunotherapy outcome is within reach and should enable individuals with only a small chance of success to receive more effective treatment both in terms of outcome and cost.
Materials and Methods

Ethical Approval

This study was approved by the UK Research Ethics Service (RES), City Road and Hampstead Research Ethics Committee, reference number 11/LO/2039. All patients gave written informed consent. The study was carried out in agreement with the Declaration of Helsinki.

Participants

Patients presenting at our center with high-risk NMIBC initially underwent transurethral resection of the tumour (TURBT). Those eligible for intravesical BCG treatment were invited to participate in the study. All patients followed a routine BCG (12.5mg intravesical OncoTICE®, Organon, N.V., USA) treatment schedule over 1 year, beginning with a 6 week-long BCG induction course consisting of weekly instillations, then further maintenance boosters of 3 weekly instillations in months 4, 7, and 10 (Supplementary Figure S1). A cystoscopy with biopsies was carried out under general anesthesia 6 weeks after each treatment course. Recurrence was confirmed by the presence of transitional cell carcinoma (TCC) in histology. The presence of TCC at 6 months was considered BCG immunotherapy failure. Patient characteristics are shown in Table 1. Urine samples were collected immediately prior to induction therapy and 4 hours after the 6th instillation. Blood samples were taken immediately prior to the induction and the first maintenance course. N=24 patients had a history of BCG vaccination and/or showed a BCG vaccination scar. The remaining n=18 patients had no known history of BCG vaccination and no vaccination scar.
**Blood and urine samples**

15 ml of venous blood was drawn into sodium heparin-coated collection tubes (BD Vacutainer) and analyzed immediately. Urine samples were collected in plain sterile universal containers (Sarstedt, Germany) and frozen at -80°C until analysis.

**PBMC preparation and activation**

Peripheral blood mononuclear cells (PBMC) were prepared using standard Ficoll-Hypaque (Amersham Pharmacia Biotech, Uppsala, Sweden) density gradient centrifugation as described in detail elsewhere (21). PBMC were washed twice and resuspended in RPMI 1640 media containing 10% heat-inactivated fetal-calf-serum (FCS), 2 mM L-glutamine, and 100 IU of penicillin/streptomycin (all Biochrome) ('complete media') at a concentration of 5 x 10^6 cells per ml. Tuberculin (purified protein derivative, ‘PPD’, Statens Serum Institute, Copenhagen, Denmark) was dissolved in dimethyl-sulfoxide (DMSO, Fisher Scientific, Waltham, MA) to a final concentration of 1 mg/ml. Tuberculin was used instead of BCG to stimulate T-cells, since it is the most widely used mycobacterial antigen preparation for human testing and would be easier to standardize and handle in a routine test setting. Staphylococcus enterotoxin B (SEB, Sigma-Aldrich, St. Louis, MO) was dissolved in DMSO with a final concentration of 0.5 mg/ml. For antigen-specific stimulation, 10 µl of tuberculin solution in 90 µl of complete media or alternatively, 2 µl of SEB solution in 98 µl of complete media (positive stimulation control) was added to 400µl of cell suspension (1 x 10^6 PBMC cells). The negative control contained an equivalent amount of DMSO only. The final assay volume was 500µl for the first 2 hours of incubation (standard incubator, humidified 5% CO₂ atmosphere). At 2 hours, 10µg of Brefeldin A (Sigma), was added in
500µl of complete media (10µg/ml final concentration) bringing the final assay volume to 1000µl. After 16 hours (total incubation time) in a standard incubator (37°C, humidified 5% CO₂ atmosphere), cells were washed and surface-stained with fluorescence-conjugated monoclonal antibodies for 30 minutes at 4°C. After a further wash, pellets were treated with BD Lyse and BD Perm 2 buffers according to the manufacturers instructions (BD Biosciences, Oxford, UK). Cells were then stained intracellularly for 30 minutes at 4°C, washed, and re-fixed in PBS containing 0.5% paraformaldehyde prior to acquisition on a BD LSR II flow-cytometer (BD).

**Monoclonal antibodies for flow-cytometry**

The following fluorochrome-conjugated monoclonal antibodies were used for identifying and enumerating activated T-cells; anti-IL-2-Fluorescin isothiocyanate (FITC), anti-IFN-γ-Phycoerythrin-Cyanine 7 (PE-Cy7) and anti-TNF-Alexa Fluor 700 (Alexa700) (all BD Biosciences, San Jose, CA, USA); anti-CD40L-Brilliant violet 421 (BV421), anti-CD3-Brilliant violet 571 (BV571), anti-IL17A-Alexa Fluor 647 (AF647), anti-CD4-Peridinin-Chlorophyll-protein (PerCP) (all Biolegend). Fixable Aqua stain from Invitrogen (Paisley, UK) was used to discriminate between live and dead cells.

**Flow-cytometry**

Fluorescence Minus One (FMO) controls were used to validate panels. Positive responses (SEB, tuberculin) were corrected for cytokine-producing cells in negative control samples (subtraction, subset by subset). The gating strategy is shown in Supplementary Figure S2. FlowJo v8 and v9 (Treestar, OR, USA) were used for flowcytometry data analysis. T-cell polyfunctionality was visualized using SPICE software (22). Cytometer performance was
ascertained daily (CS&T beads, BD), rainbow calibration beads (Spherotech, Illinois, USA) were used to adjust photomultiplier (PMT) voltages before each run to ascertain longitudinal comparability between measurements.

**Secreted cytokines post tuberculin stimulation**

PBMCs (1 x 10^6 cells) were stimulated overnight (16h) in 500 µl of complete cell culture media with 5µg tuberculin (10 µg/ml end concentration) (standard incubator). Tubes were centrifuged briefly and supernatants collected and stored at -80°C. Cytokines were measured using the MSD Quickplex SQ 120 platform (Meso-Scale Discovery, Maryland, USA) including IFN-γ, IL-2 and TNF, as well as IL-1β, IL-4, IL-6, IL-8, IL-10, IL-12p70 and IL-13.

**Statistical analysis**

Statistical analysis was performed using the SPSS 23 (IBM, New York, USA) software package. Non-parametric tests were used to compare cell subset frequencies between groups. Where appropriate, non-normally distributed variables were log-transformed to improve normality for logistic regression analysis. The significance threshold was set at p =0.05. Multiple end-point correction (Bonferroni) was applied by adjusting the significance threshold 0.05/n where n is the number of tested end-points.
Results

Forty-two patients were recruited to the study, all of which completed BCG induction treatment. Six weeks after the first maintenance course, i.e. 6 months into therapy, recurrence was detected by histology in n=12 patients. A majority of these patients had already shown recurrence at the previous biopsy (6 weeks after the induction course) but completed another cycle of immunotherapy (Supplementary Fig. S3A). Two of these patients, however, underwent cystectomy (their histology had shown G3pT1 and multifocal G2pTa, “high grade” tumours). No recurrences occurred later than 6 months; the follow-up time ranged from 15 to 33 months (median 23 months). Since recurrence at 6 months is frequently used to identify BCG failure (23), patients were divided into two groups, identified by recurrence-free survival at 6 months (n=28, referred to as ‘no recurrence’ in figures/tables), or presence of tumor recurrence at 6 month (n=14, referred to as ‘recurrence’). There were no significant differences between the two groups in regards to tumor stage, presence of carcinoma in situ (CIS), disease focality, or BCG vaccination history (Table 2).

The levels of tuberculin-inducible cytokines at baseline are a good predictor of recurrence-free survival at 6 months

As a first line of investigation, we compared tuberculin-induced in vitro expression of the classic T-cell cytokines, IFN-γ, IL-2 and TNF, between the two groups, since the expression profiles of these cytokines in CD4 T-cells have been linked to protective anti-mycobacterial immunity (24,25). After overnight incubation with tuberculin, cytokine expression was assessed in two ways, first by enumerating cytokine-producing CD4 T-cells (Fig. 1A), and second, by measuring secreted cytokines in cell-culture supernatants (Fig. 1B). Individuals
who were free from tumor recurrence at 6 months had both higher frequencies of IFN-\(\gamma\)-producing (IFN-\(\gamma^+\)) CD4 T-cells and higher levels of secreted IFN-\(\gamma\) prior to induction treatment. With respect to discriminating the two groups, secreted IFN-\(\gamma\) (AUC= 0.796, p=0.002, 95%CI for AUC: 0.654 – 0.938) performed better that the percentage of IFN-\(\gamma^+\) CD4 T-cells (AUC=0.714, p=0.025, 95%CI for AUC: 0.555 – 0.874). While the frequencies of IL-2 producing (IL-2+) CD4 T-cells (AUC=0.721, p=0.021, 95%CI for AUC: 0.520-0.922) performed less well than those of IFN-\(\gamma^+\) CD4 T-cells, secreted IL-2 performed better than any of the other parameters (AUC=0.852, p=0.000; 95% CI for AUC: 0.715-0.989). TNF expression performed well with respect to cytokine-producing (TNF+) CD4 T-cells (AUC=0.745, p=0.010, 95% CI for AUC: 0.590 – 0.900) but secreted TNF was not a good discriminator between the groups (Fig. 1A-D). The frequencies of tuberculin-inducible CD4 T-cells producing IL-17 or up-regulating CD154 showed no significant differences between the outcome groups (Fig. 1E). None of the other soluble mediators showed a significant difference between the outcome groups.

**Following induction therapy, differences between recurrence-free patients and those with recurrence become blurred**

The frequencies of IFN-\(\gamma^+\) CD4 T-cells showed an increase in some patients but a drop in others compared to baseline but the differences between the groups remained statistically significant (Fig. 2 A). However, neither tuberculin-induced secreted IFN-\(\gamma\), nor any of the other measured parameters (secreted or cellular) were significantly different between the groups at that time. In addition, we found no significant difference between the groups in regards to changes in any of the measured parameters between baseline and 8 weeks after BCG induction therapy.
Age was not significantly associated with the outcome of BCG immunotherapy

While it was previously reported that patient age is positively associated with recurrences after BCG therapy (26), we were unable to confirm a strong link with age in our study. ROC analysis indicated that age was not a good discriminator between the outcome groups (Fig. 2B). Interestingly, there was weak, negative correlation between age and secreted IL-2 (not statistically significant) and a weak to moderate, negative correlation between age and secreted IFN-\(\gamma\) (\(p=0.022\)) (Fig. 2C), indicating that age might indirectly contribute to recurrence via reduced cytokine secretion. In order to determine more precisely the relative, direct effects of age and secreted cytokines on recurrence-free survival, we performed linear regression analysis. Since tuberculin-induced, secreted IFN-\(\gamma\) and IL-2 were highly correlated (\(R_S=0.743, p=0.000\)), but secreted IL-2 provided better discrimination between the outcome groups, secreted IL-2 and patient age were included as covariates in the model (Table 3). The model showed a significant effect of secreted IL-2 but not patient age on recurrence-free survival and provided correct patient classification in 78.6% of cases.

Tuberculin-induced IL-2 secretion identifies half of all BCG failures before induction

All patients with tuberculin-inducible IL-2 <120 pg/ml (n=7) experienced recurrence, representing 50% of all BCG failures (Figure 2D). In contrast to this, all individuals with a tuberculin-inducible IFN-\(\gamma\) >20,000 pg/ml (n=7) were recurrence-free at 6 months and for the rest of the follow-up time, corresponding to 25% of all recurrence-free survivors. Alternatively, a cut-off for IL-2 secretion at 250 pg/ml, for example, would have correctly classified 21/28 recurrence-free survivors (75%) and 12/14 cases with recurrence (86%),
providing overall correctly classification in 33/42 patients (78.6%), in agreement with the logistic regression analysis above (Figure 2D and Supplementary Figure S3B).

**BCG vaccination history had no significant effect on outcome or tuberculin response**

There was no difference between BCG-vaccinated and non BCG-vaccinated participants regarding outcome (Table 2). Also, neither the levels of secreted cytokines nor the frequencies of cytokine-positive cells following tuberculin-stimulation were significantly different between the two groups. There was a slight but non-significant trend suggesting that those with a history of BCG vaccination showed higher tuberculin-inducible levels of secreted IFN-γ and IFN-γ+ CD4 T-cells prior to induction therapy (Supplementary Fig. S4).

**Polyfunctional CD4 T-cells are associated with recurrence-free survival**

Using Boolean logic, tuberculin-activated T-cell populations producing several cytokines were divided into defined, non-overlapping functional subsets producing, for example, any one of the cytokines but none of the others, or two specific ones but none of the others, etc. T-cells producing several cytokines simultaneously are often referred to as ‘polyfunctional’, a property that is thought to be relevant for protection in some infection models (27). Based on IFN-γ, IL-2, and TNF seven functional subsets can be defined (an eighth subset has none of the functions and is not considered). The most polyfunctional T-cells expressing IFN-γ, IL-2 and TNF were significantly increased in patients who were recurrence-free at 6 months (p=0.001), however, several additional subsets providing discrimination between the groups were identified (Fig. 3A). Interestingly, these included two effector subsets that were negative for IL-17. Overall the best discrimination was provided by two subsets, one producing IFN-γ, TNF, and IL-2 (AUC= 0.801, p=0.002, 95%CI
for AUC: 0.661 – 0.941), and the other one IFN-γ and TNF, but not IL-17 (AUC= 0.802, p=0.002, 95%CI for AUC: 0.673 – 0.932) (Fig. 3B). All possible subsets based on IFN-γ, TNF, and IL-2 or IL17 are shown in Supplementary Figs. S5 and S6.

Urine cytokine levels and their changes were not predictive of immunotherapy outcome
Prior to treatment, the T-cell cytokines IL-2, IL-4, IFN-γ, and TNF-alpha were undetectable in the vast majority of urine samples. IL-6 and IL-8, by contrast were detected in all but two and all but 1 of the pre-treatment samples, respectively. The remaining cytokines were detected less consistently. Following induction treatment, however, all cytokines were detected in >85% of individuals. However, neither pre-treatment nor post-treatment levels, nor the change between the two time points was statistically different between the outcome groups (Supplementary Table S1).
Discussion

The analysis of CD4 T-cell functions prior to BCG immunotherapy revealed several promising parameters showing significant differences between patients with and without tumor recurrence at 6 months. Because of the importance attributed to IFN-γ in TB infection and its central role in laboratory tests for latent TB (10,28) we hypothesized that this effector cytokine would also be of particular interest for predicting BCG immunotherapy outcome. Indeed, both the number of IFN-γ+ CD4 T cells and the levels of secreted IFN-γ after overnight stimulation with tuberculin were significantly higher in recurrence-free survivors. However, tuberculin-induced IL-2 secretion provided even better discrimination between the groups with an AUC of 0.852 (p=0.000, 95% CI: 0.715-0.989). It is an equally important finding that prior to induction therapy it was below a threshold of 120pg/ml in 50% of patients with recurrence but none of the recurrence-free survivors, since a clinical test based on this threshold could potentially spare half of the patients with predicted BCG failure the experience of undergoing ultimately unsuccessful immunotherapy.

The diagnostic potential of in vitro tuberculin-induced IL-2 secretion was also impressive in regards to predicting therapeutic success. To date, there is no other test available providing similar sensitivity and specificity in regards to predicting BCG immunotherapy outcome before induction is even begun. The levels of tuberculin-induced secreted mediators or cellular parameters 8 weeks after induction therapy (expect IFN-γ+ CD4 T-cells), or their changes compared to baseline were not useful for predicting BCG immunotherapy outcome. This seems to confirm the results by Elsasser et al.(20). At any rate, a test that predicts the outcome after induction therapy will be less useful than a test that predicts the outcome beforehand.
When BCG immunotherapy became the gold standard treatment for preserving the bladder after resection of high grade NMIBC about three decades ago, the mechanisms of protection were unclear. Its success was often attributed to generally augmented immune responsiveness and in particular the innate immune system (29,30).

NK-cells, which are part of the innate immune system, are of particular interest in regards to killing bladder tumor cells (31). Alternative depletion of NK-cell and T-cell populations from BCG-stimulated, human mononuclear cell suspensions indicated that the substantial cytotoxic effect of such suspensions against bladder tumors was mostly explained by NK-cells (32). Also, mice depleted of NK-cells or NK-cell deficient mice had no benefit from BCG immunotherapy in a syngeneic, orthotopic murine bladder cancer model (32). Others have confirmed a critical role of NK cells in mediating the effects of BCG immunotherapy and suggested that infection of bladder cancer cells with BCG may be essential for this mechanism (33).

However, many publications also pointed to a role of adaptive immunity. For example, it was shown that lymphocytes in the urine of patients after intravesical BCG are for the most part CD4 T-cells (34), and that BCG induced lymphocyte proliferation was higher in recurrence-free survivors compared to individuals with recurrence (n=10) (35). Mouse models showed that bladder mucosa-infiltrating T-cells following intravesical BCG were predominantly CD4 T-cells (36) and that T-cells were required for effective BCG immunotherapy (37). More recently it was reported that prior parenteral exposure to BCG increased the effect of BCG immunotherapy in mice and that patients with a positive tuberculin skin test (TST) response have better recurrence-free survival rates (38).
However, despite their obvious role in the response to BCG immunotherapy, the effect of CD4 T-cells may be indirect, for example via IFN-γ-mediated natural killer(NK)-cell activation. Of note, the cytotoxic potential of BCG-induced NK cells can be enhanced by IL-12 and IFN-γ (39) and CD4 T-cells are a major source of IFN-γ in TB infection. Whereas NK-cells may ultimately kill the tumor cells, memory CD4 T-cells boosted by BCG are likely to facilitate their action. This is one plausible explanation, why an in vitro CD4 T-cell activation assay can predict BCG immunotherapy outcome.

The reason why cytokine responses (both secreted and intracellular) were not significantly higher in participants with a known history of BCG vaccination compared to those without, is probably that a majority of the older individuals in our study were exposed to mycobacterial antigens (other than the BCG vaccine) in the past. This will include exposure to TB during and after World War II but also nontuberculous mycobacteria, which, likewise, will cause tuberculin responsiveness (40)(41). Like the TST, our test measures previous exposure, but provides a better quantitative measure of tuberculin sensitization, and apparently, better prediction of BCG immunotherapy success compared to the TST (38).

While several studies identified significant differences between patients with recurrence and recurrence-free survivors in regards to a number of parameters (cytokines in blood or urine, patient and/or tumor-related characteristics such as age, sex, tumor stage, carcinoma in situ, etc.) these were too subtle to effectively inform treatment choice (26,42-49). Our study was probably too small to confirm any of these differences but conversely, did not have sufficient power to detect their absence.
In this study, the analysis of cytokine levels in urine did not discriminate between the outcome groups, neither at baseline nor after the last dose of induction treatment. However, a promising approach to predicting BCG immunotherapy outcome was recently presented by Kamat et al. who developed an algorithm based on a number of clinical characteristics and in addition the changes of a panel of 12 cytokines measured in urine at 6 weeks (last dose of induction course) and at the time of the third dose of the first maintenance course (50). This approach, which effectively includes a functional in vivo test, provided an AUC of 0.855 (ROC-analysis) for discriminating recurrence from recurrence-free survival and was termed ‘CyPRIT’. Since our cytokine panel included different cytokines and our study was smaller, we did not try and emulate this approach. Of note, overnight tuberculin-induced secretion of IL-2 in our study provided similarly good discrimination between outcome groups (AUC 0.852) as the CyPRIT method. It should be tested in larger studies as soon as possible to see if it is as robust as CyPRIT. Also, tuberculin-induced secreted IL-2 provided good outcome prediction prior to BCG immunotherapy, which in a clinical setting would be a massive advantage over tests not predicting outcome until the end of the induction therapy.

In regards to functional subsets of T-cells, those that produce IFN-γ, TNF, and IL-2 simultaneously (‘polyfunctional’) were previously associated with protection in vaccine models (27) and, interestingly, this subset was significantly increased in recurrence-free survivors prior to BCG immunotherapy. One possible reason for this might be that T-cells producing several cytokines at the same time also produce higher levels of each (21). However, overall, intracellular markers relating to T-cell polyfunctionality showed
considerable promise to be developed into clinical markers, but would be more complex to measure than secreted cytokines or single cytokine-positive CD4 T-cells.

Conclusions

Our pilot study shows that the in vitro tuberculin-responsiveness of CD4 T-cells prior to BCG immunotherapy is a promising outcome predictor. It measures the ability of the adaptive immune system to mobilize an effective immune response to BCG, however, it remains unclear what the involvement of CD4 T-cells is in the actual anti-tumor response. It is an advantage of our approach that T-cell activation assays based on overnight tuberculin stimulation are easy to standardize; similar tests already exist for TB and are based on IFN-γ-release (Interferon-γ release assays or ‘IGRAs’ for short)(10). Our work suggests that in vitro tuberculin-stimulated secretion of IL-2 (effectively an ‘IL-2 release assay’) carries particular potential as a predictor of BCG immunotherapy success. If confirmed in larger studies in future, its apparent ability to identify 50% of non-responders with 100% specificity, even before the beginning of therapy, would be of immediate practical interest.
Acknowledgements

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References


## Tables

### Table 1: Patient characteristics

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Table 2: Patient outcomes at 6 months by tumor characteristics.

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Table 3: The effect of tuberculin-induced secreted IL-2 at baseline and patient age on BCG immunotherapy outcome

<table>
<thead>
<tr>
<th>Parameter</th>
<th>p</th>
<th>OR</th>
<th>95% C.I. for OR</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Lower</td>
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<tr>
<td>IL-2 secretion (Log10)</td>
<td>.003</td>
<td>.018</td>
<td>.001</td>
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<tr>
<td>Age</td>
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<td>1.009</td>
<td>.932</td>
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<tr>
<td>Constant</td>
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<td>4144.925</td>
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A 10-fold increase in IL-2 is associated with a 98.2% reduction in the odds of developing recurrence by 6 months. The model provided correct classification of individuals into remission and recurrence groups of 87.6%.
Figure legends

Figure 1: Tuberculin-inducible IFN-γ and IL-2 correctly may correctly predict outcome of intravesical BCG therapy in about two thirds of patients.

Freshly isolated PBMC were stimulated in vitro with tuberculin for 16 hours. IFN-γ, IL-2 and TNF were measured by flow-cytometry using intra-cellular cytokine staining. Alternatively, secreted cytokines were measured in the supernatant by electro-chemiluminescence. CD4 T-cell frequencies are expressed as fractions (0.01=1%) (A) Patients without recurrence of cancer at 6 months show significantly higher numbers of cytokine-producing CD4 T-cells in response to tuberculin stimulation (left). (B) Secreted IFN-γ and IL-2 but not TNF seem to discriminate better between treatment success and recurrence than the percentage of CD4 T-cells displaying the corresponding cytokine under (A). (C) ROC analysis for cytokine-positive CD4 T-cells. The 95% CI for the AUCs were, IFN-γ: 0.555 – 0.874, IL-2: 0.520-0.922, TNF+ CD4 T-cells: 0.590 – 0.900. (D) ROC analysis for secreted cytokines. The 95% CI for the AUCs were, secreted IFN-γ: 0.654 – 0.938, secreted IL-2: 0.715-0.989, and secreted TNF: 0.336 – 0.741. (E) Frequencies of CD154+ and IL-17+ CD4 T-cells in individuals with and without recurrence at 6 months. A,B,E: Error bars show median and interquartile range.
Figure 2: Following induction therapy, differences between recurrence-free patients and those with recurrence are blurred. (A) Plots show changes between time points one (before induction therapy) and two (after induction therapy) in regards to IFN-γ-producing tuberculin-inducible CD4 T-cells (left) and secreted IFN-γ (right). CD4 T-cell frequencies are expressed as fractions (0.01=1%). (B) ROC analysis shows discrimination between the outcome groups by age. (C) Scatterplots show associations between age and secreted IFN-γ (left) or IL-2 (right). Patients with remission are shown as empty squares, patients with recurrence as filled circles. (D) The scatter plot illustrates how recurrence-free patients and those with recurrence may be discriminated based on tuberculin-induced IFN-γ and IL-2 secretion prior to therapy using the indicated, tentative thresholds for IFN-γ and IL-2. Open squares indicate recurrence-free survival, filled circles recurrence. A group of seven patients with recurrence, whose tuberculin-induced IL-2 levels were below 120 pg/ml are highlighted (dotted oval).

Figure 3: T-cell polyfunctionality is increased in recurrence-free survivors. (A) Scatter plots shows four functional CD4 T-cell subsets that were significantly different between recurrence free survival (empty squares) and recurrence (filled circles). Note that CD4 T-cell frequencies are expressed as fractions (0.01=1%). Error bars show median and interquartile range. (B) ROC curves show the discrimination between the outcome groups based the two most promising subsets, producing IFN-γ and TNF but not IL-17 (95% CI for the AUC: 0.673-0.932), or, IFN-γ, TNF and IL-2 (95% CI for the AUC: 0.661-941).
Figure 1

A) Cytokine+ CD4 T-cells (fraction)

B) Secreted cytokines (pg/ml)

C) Sensitivity vs. 1-Specificity

D) Sensitivity vs. 1-Specificity

E) Cytokine+ CD4 T-cells (fraction)

Legend:
- No recurrence
- Recurrence

Statistical significance:
- p = 0.028
- p = 0.021
- p = 0.001
- p = 0.000
- n.s.
Figure 2

A. IFN-γ+ CD4 T-cells (fraction)
- Before: No recurrence (n=28), Recurrence (n=14)
- After: No recurrence (before), Recurrence (after)

P-value: 0.030

B. ROC curve
- Sensitivity vs. 1-Specificity
- AUC = 0.421, p = 0.408

C. Secreted IFN-γ vs. Age
- No recurrence: R = 0.325, p = 0.022
- Recurrence: R = 0.145, p = 0.359

D. Secreted IL-2 vs. Secreted IFN-γ
- Age range: 40 to 90 years
- Secreted IFN-γ range: 10 to 1,000,000 pg/ml

Legend:
- No recurrence
- Recurrence
Figure 3

A

- No recurrence
- Recurrence

CD4 T-cells (fraction)

Functional CD4 T-cell subset

B

Sensitivity

1 - Specificity

IFN-γ+ IL17- TNF+
CD4 T-cells

AUC=0.802, p=0.002

IFN-γ+ IL-2+ TNF+
CD4 T-cells

AUC=0.801, p=0.002