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Research Article

Cancer Immunology Research

Baseline Cytokine Profiles of Tuberculin-Specific CD4$^+$ T Cells in Non–Muscle-Invasive Bladder Cancer May Predict Outcomes of BCG Immunotherapy

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Abstract

Intravesical Bacillus Calmette-Guérin (BCG) immunotherapy preserves the bladder after resection of high-risk non–muscle-invasive bladder cancer (NMIBC). About 30% of patients experience treatment failure, which cannot be predicted *a priori* and carries a high risk of disease progression. We examined the *in vitro* tuberculin responsiveness of CD4$^+$ T cells before BCG immunotherapy in 42 patients with high-risk NMIBC. The frequencies and functionalities of cytokine-expressing CD4$^+$ T cells immediately before and after BCG immunotherapy induction were assessed by flow cytometry after overnight tuberculin stimulation. Tuberculin-induced secreted mediators were measured by electrochemiluminescence. We correlated the results with recurrence-free patient survival 6 months after induction. A tuberculin-induced, secreted, IL2 concentration > 250 pg/mL was the best predictor of recurrence-free survival, providing 79% sensitivity, 86% specificity (AUC = 0.852, $P = 0.000$), and overall correct classification in 78.6% of cases. In 50% of patients later experiencing recurrence, but not in any of the recurrence-free survivors, IL2 secretion was < 120 pg/mL. Other parameters predicting recurrence-free survival included secreted IFNγ (AUC = 0.796, $P = 0.002$) and the frequencies of TNF-producing (TNF$^+$) CD4$^+$ T cells (AUC = 0.745, $P = 0.010$). "Polyfunctional" CD4$^+$ T cells (IFNγ$^+$/IL2$^+$/TNF$^+$) were significantly associated with recurrence-free survival (AUC = 0.801, $P = 0.002$). Thus, the amount of IL2 secretion from CD4$^+$ T cells after overnight *in vitro* incubation with tuberculin predicted the outcome of BCG immunotherapy. As many as half of potential BCG failures could be identified before induction therapy is begun, enabling better choices regarding treatment. *Cancer Immunol Res; 6(10); 1212–9. ©2018 AACR.*

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 reduces the otherwise high recurrence rates (2–5). Nonresponders to BCG immunotherapy risk disease progression and death (6, 7). Reliable predictors of treatment outcome prior to BCG immunotherapy or early into therapy have not been identified yet.

BCG has been administered intradermally as a tuberculosis (TB) vaccine for almost 100 years (8). BCG prevents some 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 cells producing IFNγ, is believed to explain the protective effect of the vaccine (10); however, the immunologic correlates of protection from TB are poorly understood (11). The mechanisms by which antimycobacterial immunity induced by BCG might help contain bladder cancer are even less understood. Features of BCG-induced immune responsiveness, such as production of urinary cytokines, natural killer cell activity, or changes in anti-*Mycobacterium bovis* HSP60 (12–19), have been associated with treatment success for NMIBC. Intravesical BCG increased the numbers of tuberculin-specific CD4$^+$ T cells detectable in blood (20); however, prediction of the outcome of BCG immunotherapy in this small study ($n = 18$) with variable pathology was unsuccessful. Our study 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 and colleagues (20), we found associations between tuberculin-induced CD4$^+$ T-cell responsiveness and the absence of tumor recurrence. Our results suggest that a functional test based on T-cell responses could predict BCG immunotherapy

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Note: Supplementary data for this article are available at Cancer Immunology Research Online (http://cancerimmunolres.aacrjournals.org/).

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outcome. Such a test should enable individuals with only a small chance of success from BCG therapy 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 the Urology Department of Brighton and Sussex University Hospital Trust with high-risk NMIBC initially underwent transurethral resection of the tumor (TURBT). Those eligible for intravesical BCG treatment were invited to participate in the study. All patients followed a routine BCG treatment schedule (12.5 mg intravesical OncoTICE, Organon, N.V.) 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 Fig. S1). A cystoscopy with biopsies was carried out under general anesthesia 6 weeks after each treatment course. Recurrence was confirmed by the presence of 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. Twenty-four patients had a history of BCG vaccination and/or showed a BCG vaccination scar. The remaining 18 patients had no known history of BCG vaccination and no vaccination scar.

Blood and urine samples

Fifteen milliliters 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) and frozen at −80°C until analysis.

Table 1. Patient characteristics

Total number	42
Age, median (range)	71.2 years (47–89)
Gender	
Male	34
Female	8
Grade	
G3	37 (88.1%)
Carcinoma *in situ*	18 (42.8%)
Grade G3 + carcinoma *in situ*	13 (30.9%)
Carcinoma *in situ* alone	5 (11.9%)
Stage	
Ta	12 (28.6%)
T1	24 (57.1%)
Lymphovascular invasion	0
Focality	
Single	30
Multiple	12
Follow-up time in months median (range)	16 (15–33)
History of BCG vaccination	24 (57.1%)

Peripheral blood mononuclear cells' preparation and activation

Peripheral blood mononuclear cells (PBMC) were prepared using standard Ficoll-Hypaque (Amersham Pharmacia Biotech) density gradient centrifugation as described elsewhere (21). PBMCs were washed twice and resuspended in RPMI1640 medium containing 10% heat-inactivated FCS, 2 mmol/L L-glutamine, and 100 IU of penicillin/streptomycin (all Biochrome; "complete media") at a concentration of 5×10^6 cells/mL. Tuberculin (purified protein derivative, "PPD," Statens Serum Institute) was dissolved in DMSO, (Thermo Fisher Scientific) to a final concentration of 1 mg/mL. Tuberculin was used instead of BCG to stimulate T cells, because 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) 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 400 μL of cell suspension (1×10^6 PBMCs). The negative control contained an equivalent amount of DMSO only. The final assay volume was 500 μL for the first 2 hours of incubation (standard incubator, humidified 5% CO_2 atmosphere). At 2 hours, 10 μg of Brefeldin A (Sigma-Aldrich) was added in 500 μL of complete media (10 μg/mL final concentration) bringing the final assay volume to 1,000 μL. After 16 hours (total incubation time) in a standard incubator (37°C, humidified 5% CO_2 atmosphere), cells were washed and surface stained with fluorescence-conjugated mAbs for 30 minutes at 4°C. After a further wash, pellets were treated with BD Lyse and BD Perm 2 buffers according to the manufacturer's instructions (BD Biosciences). Cells were then stained intracellularly for 30 minutes at 4°C, washed, and refixed in PBS containing 0.5% paraformaldehyde prior to acquisition on a BD LSR II flow cytometer (BD Biosciences).

mAbs for flow cytometry

The following fluorochrome-conjugated mAbs were used for identifying and enumerating activated T cells: anti-IL2-FITC, anti-IFNγ-Phycoerythrin-Cyanine 7 (PE-Cy7), and anti-TNF-Alexa Fluor 700 (Alexa700; all BD Biosciences); 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 dead cell stain from Invitrogen (Paisley) was used to discriminate between live and dead cells.

Flow cytometry

Fluorescence Minus One 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 Fig. S2. FlowJo v8 and v9 (Treestar) were used for flow cytometry data analysis. T-cell polyfunctionality was visualized using SPICE software (22). Cytometer performance was ascertained daily (CS&T beads, BD Biosciences); rainbow calibration beads (Spherotech) were used to adjust photomultiplier (PMT) voltages before each run to ascertain longitudinal comparability between measurements.

Secreted cytokines after tuberculin stimulation

PBMCs (1×10^6 cells) were stimulated overnight (16 hours) in 500 μL of complete cell culture media with 5 μg tuberculin

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(10 μg/mL end concentration; standard incubator). Tubes were centrifuged for 8 minutes at 400 × *g* and supernatants collected and stored at −80°C. Cytokines were measured using the MSD Quickplex SQ 120 platform (Meso Scale Discovery) including IFNγ, IL2, and TNF, as well as IL1β, IL4, IL6, IL8, IL10, IL12p70, and IL13.

Statistical analysis

Statistical analysis was performed using the SPSS 23 (IBM) software package. Nonparametric 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 endpoint correction (Bonferroni) was applied by adjusting the significance threshold 0.05/n where n is the number of tested endpoints.

Results

All of the patients recruited to this study ($n = 42$) completed BCG induction treatment. Six weeks after the first maintenance course, that is, 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" tumors). No recurrences occurred later than 6 months; the follow-up time ranged from 15 to 33 months (median 23 months). Because 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 months ($n = 14$, referred to as "recurrence"). There were no significant differences between the two groups with respect to tumor stage, presence of carcinoma *in situ* (CIS), disease focality, or BCG vaccination history (Table 2).

Tuberculin-inducible cytokines at baseline predict recurrence-free survival at 6 months

As a first line of investigation, we compared tuberculin-induced *in vitro* expression of the T-cell cytokines, IFNγ, IL2, and TNF, between the "recurrence" and "no recurrence" groups, because the expression profiles of these cytokines in $CD4^+$ T cells have been linked to protective antimycobacterial immunity (24, 25). After overnight incubation of $CD4^+$ T cells 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 IFNγ-producing ($IFN\gamma^+$) $CD4^+$ T cells and more secreted IFNγ prior to induction treatment. With respect to discriminating between the two groups, secreted IFNγ (AUC = 0.796, $P = 0.002$, 95% CI for AUC: 0.654–0.938) performed better than the percentage of $IFN\gamma^+$ $CD4^+$ T cells (AUC = 0.714, $P = 0.025$, 95% CI for AUC: 0.555–0.874). Although the frequencies of IL2-producing ($IL2^+$) $CD4^+$ T cells (AUC = 0.721, $P = 0.021$, 95% CI for AUC: 0.520–0.922) performed less well as a discriminatory parameter than the frequencies of $IFN\gamma^+$ $CD4^+$ T cells, secreted IL2 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 IL17 or upregulating 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.

Outcome prediction by the same parameters did not improve after induction therapy. At 8 weeks after induction therapy, the frequencies of tuberculin-inducible $IFN\gamma^+$ $CD4^+$ T cells were higher in some patients but lower in others compared with baseline, although differences between the recurrence and non-recurrence groups remained statistically significant (Fig. 2A). However, neither tuberculin-induced secreted IFNγ, nor any of the other measured parameters (secreted or cellular) were significantly different between the groups at this time point. The groups had no statistically significant differences with regard to changes in any of the measured parameters between baseline and 8 weeks after induction therapy.

Table 2. Patient outcomes at 6 months by tumor characteristics

	No recurrence		Recurrence		*P*
Number	28		14		
Age, mean (±STD)	71 (±10.7)		74 (±9.2)		n.s.
Male:Female	23:5		11:3		n.s.
Original histology (Grade)					
G3	24	82.7%	13	92.9%	n.s.
CIS	14	50.0%	4	28.6%	n.s.
Stage					
Ta	9	32.1%	3	21.4%	n.s.
T1	14	50.0%	10	71.4%	n.s.
Multifocal	5	17.8%	7	50.0%	n.s.
History of BCG vaccination	16	57.1%	8	57.1%	n.s.

Age was not significantly associated with the outcome of BCG immunotherapy

Although patient age has been reported to be positively associated with recurrences after BCG therapy (26), we were unable to confirm a link with age in our study. Receiver operator characteristics (ROC) analysis indicated that age was not a good discriminator between the outcome groups (Fig. 2B). There was a weak, negative correlation between age and secreted IL2 (not statistically significant). Age and secreted IFNγ were negatively correlated ($P = 0.022$; Fig. 2C), indicating that age might contribute to recurrence via reduced cytokine secretion. To determine more precisely the effects of age and secreted cytokines on recurrence-free survival, we performed linear regression analysis. Because tuberculin-induced, secreted IFNγ, and IL2 were highly correlated ($R_S = 0.743$, $P = 0.000$), secreted IL2 provided better discrimination between the outcome groups, and secreted IL2 and patient age were included as covariates in the model (Table 3). The model showed a significant effect of secreted IL2 but not patient age on recurrence-free survival and provided correct patient classification in 78.6% of cases.

Tuberculin-induced IL2 secretion identifies half of BCG failures before induction

All patients with tuberculin-inducible IL2 below 120 pg/mL ($n = 7$) experienced recurrence, representing 50% of all BCG failures (Fig. 2D). In contrast to this, all individuals with a

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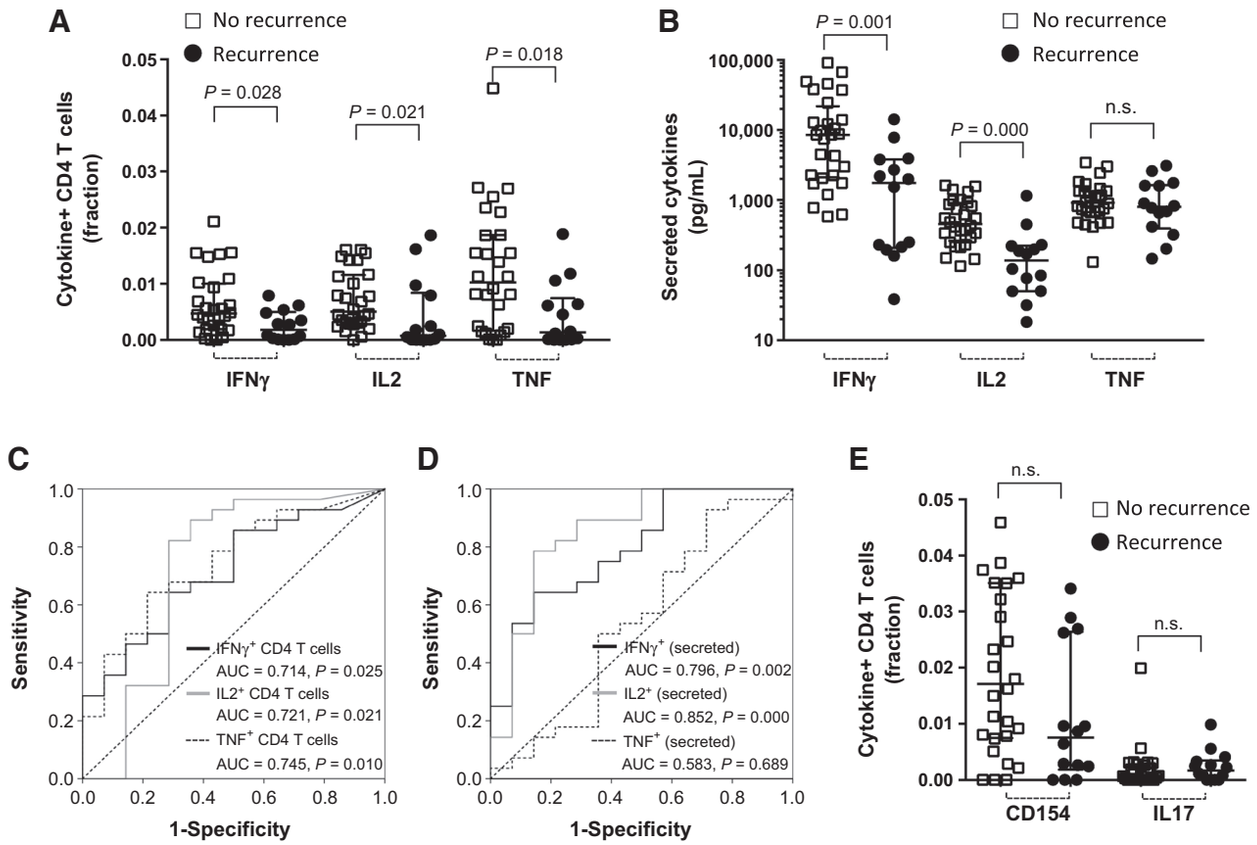

Figure 1.

Tuberculin-inducible IFNγ and IL2 may predict outcome of intravesical BCG therapy in about two thirds of patients. Freshly isolated PBMCs were stimulated *in vitro* with tuberculin for 16 hours. IFNγ, IL2, and TNF were measured by flow cytometry using intracellular cytokine staining. Alternatively, secreted cytokines were measured in the supernatant by electrochemiluminescence. $CD4^+$ 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 IFNγ and IL2 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, $IFN\gamma^+$: 0.555–0.874, $IL2^+$: 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 IFNγ: 0.654–0.938, secreted IL2: 0.715–0.989, and secreted TNF: 0.336–0.741. **E,** Frequencies of $CD154^+$ and $IL17^+$ $CD4^+$ T cells in individuals with and without recurrence at 6 months. **A**, **B**, and **E,** Error bars show median and interquartile range.

tuberculin-inducible IFNγ greater than 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. A cutoff for IL2 secretion at 250 pg/mL would correctly classify 21 of 28 recurrence-free survivors (75%) and 12 of 14 cases with recurrence (86%), providing an overall correct classification in 33 of 42 patients (78.6%), in agreement with the logistic regression analysis above (Fig. 2D; Supplementary Fig. S3B).

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

There was no difference in outcome between participants who had or had not been vaccinated previously with BCG (Table 2). Amounts of secreted cytokines and frequencies of cytokine-positive cells following tuberculin stimulation also did not differ by status of prior BCG vaccination. There was a slight but nonsignificant trend suggesting that those with a history of BCG vaccination showed higher tuberculin-inducible levels of secreted IFNγ and $IFN\gamma^+$ $CD4^+$ 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). On the basis of IFNγ, IL2, 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 IFNγ, IL2, and TNF were significantly increased in patients who were recurrence-free at 6 months ($P = 0.001$). Additional subsets also discriminated between recurrence and nonrecurrence groups (Fig. 3A). These

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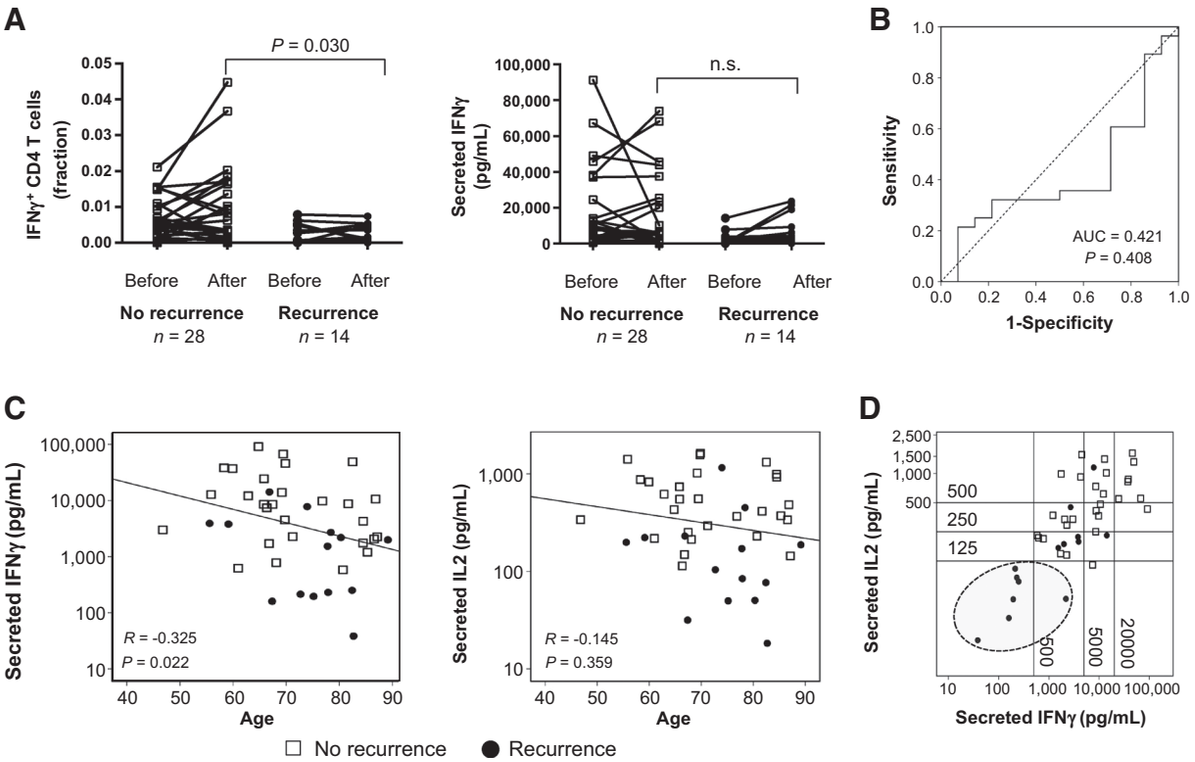

Figure 2.

Following induction therapy, differences between recurrence-free patients and those with recurrence were less conspicuous. **A,** Plots show changes between time points one (before induction therapy) and two (after induction therapy) in regards to IFNγ-producing tuberculin-inducible $CD4^+$ T cells (left) and secreted IFNγ (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 IFNγ (left) or IL2 (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 IFNγ and IL2 secretion prior to therapy using the indicated, tentative thresholds for IFNγ and IL2. Open squares indicate recurrence-free survival, filled circles recurrence. A group of seven patients with recurrence whose tuberculin-induced IL2 levels were below 120 pg/mL are highlighted (dotted oval).

included two effector subsets that were negative for IL17. Overall, the best discrimination was provided by two subsets, one producing IFNγ, TNF, and IL2 (AUC = 0.801, $P = 0.002$, 95% CI for AUC: 0.661–0.941), and one producing IFNγ and TNF, but not IL17 (AUC = 0.802, $P = 0.002$, 95% CI for AUC: 0.673–0.932; Fig. 3B). All possible subsets based on IFNγ, TNF, and IL2 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 IL2, IL4, IFNγ, and TNFα were undetectable in 83% to 98% of urine samples. IL6 and IL8, in contrast were detected in all but two and all but 1 of the pretreatment samples, respectively. The remaining cytokines (IL1b, IL10, IL12p70, IL13) were detected less consistently. Following induction treatment, however, all tested cytokines were detected in >85% of individuals. However, pretreatment and posttreatment cytokine content in urine were not predictive of treatment outcome (Supplementary Table S1).

Table 3. The effect of tuberculin-induced secreted IL2 at baseline and patient age on BCG immunotherapy outcome

Parameter	*P*	OR (95% CI)
IL2 secretion (Log_{10})	0.003	0.018 (0.001–0.274)
Age	0.832	1.009 (0.932–1.109)
Constant	0.064	4,144.925

NOTE: A 10-fold increase in IL2 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%.

Discussion

The analysis of $CD4^+$ T-cell functions prior to BCG immunotherapy revealed parameters showing significant differences between patients with and without tumor recurrence at 6 months. Because of the role of IFNγ in TB infection and in laboratory tests for latent TB (10, 28), we hypothesized that IFNγ could also predict BCG immunotherapy outcome. Indeed, both the number of $IFN\gamma^+$ $CD4^+$ T cells and the amount of IFNγ secreted from $CD4^+$ T cells after overnight stimulation with tuberculin were higher in recurrence-free survivors. However, tuberculin-induced IL2 secretion provided even better discrimination between groups with or without disease recurrence with an AUC of 0.852 ($P = 0.000$, 95% CI: 0.715–0.989). Prior to induction therapy, IL2 secretion was below 120 pg/mL in 50% of patients with recurrence, but none of the recurrence-free survivors. A clinical test based on this threshold could potentially spare half of the patients

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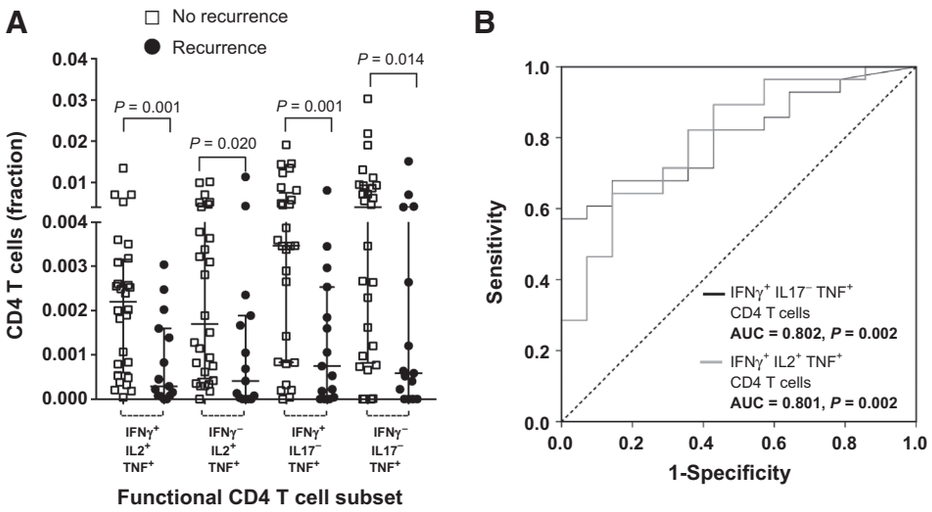

Figure 3.
T-cell polyfunctionality is increased in recurrence-free survivors. **A,** Scatter plots show four functional CD4$^+$ T-cell subsets that were significantly different between recurrence-free survival (empty squares) and recurrence (filled circles). Note that CD4$^+$ T cell frequencies are expressed as fractions (0.01 = 1%). Error bars show median and interquartile range. **B,** ROC curves show the discrimination between the outcome groups based on the two most promising subsets, producing IFNγ and TNF but not IL17 (95% CI for the AUC: 0.673–0.932), or, IFNγ, TNF, and IL2 (95% CI for the AUC: 0.661–941).

with predicted BCG failure the experience of undergoing unsuccessful immunotherapy.

In vitro tuberculin-induced IL2 secretion predicted BCG immunotherapy outcome before induction was begun. To date, no other tests similarly predict BCG immunotherapy outcome before induction therapy (29). With the exception of IFNγ$^+$ CD4$^+$ T cells, the levels of tuberculin-induced secreted mediators or cellular parameters 8 weeks after induction therapy, or their changes compared with baseline, were not useful for predicting BCG immunotherapy outcome. This is in agreement with the results by Elsasser and colleagues (20). However, a test that predicts the outcome after induction therapy has begun 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 but were often attributed to generally augmented immune responsiveness (30, 31).

NK (natural killer) cells, which are part of the innate immune system, are of interest with regard to killing bladder tumor cells (32). Depletion of NK-cell and T-cell populations from BCG-stimulated, human mononuclear cell suspensions indicated that the cytotoxic effects of such suspensions against bladder tumors were mostly explained by NK cells (33). Mice depleted of NK cells or NK-cell–deficient mice had no benefit from BCG immunotherapy in a syngeneic, orthotopic murine bladder cancer model (33). Others have confirmed that NK cells mediate the effects of BCG immunotherapy and suggested that infection of bladder cancer cells with BCG may be essential for this mechanism (34).

However, many publications also pointed to a role of adaptive immunity. For example, lymphocytes in the urine of patients after intravesical BCG are mostly CD4$^+$ T cells (35), and BCG-induced lymphocyte proliferation was higher in recurrence-free survivors compared with individuals with recurrence (n = 10; ref. 36). Mouse models showed that bladder mucosa-infiltrating T cells following intravesical BCG were predominantly CD4$^+$ T cells (37) and that T cells were required for effective BCG immunotherapy (38). Prior parenteral exposure to BCG increased the effect of BCG immunotherapy in mice and patients with a positive tuberculin skin test (TST) response have better recurrence-free survival rates (39).

However, despite their role in the response to BCG immunotherapy, the effect of CD4$^+$ T cells may be indirect, for example, via IFNγ-mediated NK-cell activation. The cytotoxic potential of BCG-induced NK cells can be enhanced by IL12 and IFNγ (40) and CD4$^+$ T cells are a major source of IFNγ in TB infection. Although NK cells may kill the tumor cells, memory CD4$^+$ T-cells boosted by BCG are likely to facilitate NK-cell action. If this is the mechanism, then an *in vitro* CD4$^+$ T-cell activation assay could predict BCG immunotherapy outcome.

The reason why cytokine responses (both secreted and intracellular) were not higher in participants with a history of BCG vaccination compared with 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 to nontuberculous mycobacteria, which, likewise, will cause tuberculin responsiveness (41, 42). Our test measures previous exposure, as does the TST, but provides a better quantitative measure of tuberculin sensitization and better prediction of BCG immunotherapy success (39).

Although, several studies have identified differences between patients with recurrence and recurrence-free survivors with regard 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 differences were too subtle to inform treatment choice (26, 29, 43–49). Our study had insufficient power to either confirm or detect the absence of these differences.

In this study, the analysis of cytokines in urine, either at baseline or after the last dose of induction treatment, did not correlate with outcome. However, an approach to predicting BCG immunotherapy outcome was presented by Kamat and colleagues who developed an algorithm on the basis of a number of clinical characteristics as well as changes in a panel of 12 cytokines measured in urine at 6 weeks (last dose of the induction course) and at the time of the third dose of the first maintenance course (50). This approach, which 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." Because our study included different cytokines and was smaller, we did not try to emulate this approach. Overnight tuberculin-induced secretion of IL2 in our study discriminated between outcome groups

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(AUC = 0.852) as effectively as the CyPRIT method. Our test should be tested in larger studies to see if it is as robust as CyPRIT. Tuberculin-induced secreted IL2 predicted outcomes prior to BCG immunotherapy, which in a clinical setting would be an advantage over tests not predicting outcome until the end of the induction therapy.

With regard to functional subsets of T cells, those that produce IFNγ, TNF, and IL2 simultaneously ("polyfunctional" T cells) were previously associated with protection in vaccine models (27). This subset was increased in recurrence-free survivors prior to BCG immunotherapy, perhaps because 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 promise as clinical markers, but would be more complex to measure than secreted cytokines or $CD4^+$ T cells positive for a single cytokine.

Our pilot study shows that the *in vitro* tuberculin responsiveness of $CD4^+$ T cells prior to BCG immunotherapy may predict its outcome. This predictor measures the ability of the adaptive immune system to mobilize an immune response to BCG. However, it remains unclear what the involvement of $CD4^+$ T cells is in the actual antitumor response. Our T-cell activation assays, based on overnight tuberculin stimulation, are easy to standardize. Similar tests already exist for TB and are based on IFNγ release (IFNγ release assays or "IGRAs" for short; ref. 10). Our work suggests that among the tested parameters, *in vitro* tuberculin-stimulated secretion of IL2 (effectively an IL2 release assay) carries the greatest potential as a predictor of BCG immunotherapy success. If confirmed in future studies, the ability of this IL2 release assay to identify 50% of nonresponders with 100% specificity before the beginning of therapy would be of the most immediate practical interest.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: S. Jallad, P. Thomas, M.J. Newport, F. Kern
Development of methodology: S. Jallad, M.J. Newport, F. Kern
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): S. Jallad
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): S. Jallad, M.J. Newport, F. Kern
Writing, review, and/or revision of the manuscript: S. Jallad, P. Thomas, M.J. Newport, F. Kern
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): S. Jallad, F. Kern
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