

Innate immune memory is associated with increased disease-free survival in bladder cancer patients treated with bacillus Calmette-Guérin

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Abstract

Introduction: While studies suggest that innate immune memory acquired by circulating monocytes may mediate the benefit of bacillus Calmette-Guérin (BCG) in the treatment of patients with high-risk non-muscle-invasive bladder cancer (NMIBC), prospective studies are lacking. Innate immune memory is defined by enhanced release of pro-inflammatory cytokines by innate immune cells following a secondary challenge with pattern recognition receptor (PRR) ligands.

Methods: Peripheral blood monocytes isolated from 33 patients with intermediate- or high-risk NMIBC before and after two or five induction BCG instillations were stimulated with the PRR ligand lipopolysaccharide (LPS). Inflammatory cytokine levels in the culture medium were measured. Extent of innate immune memory acquisition was determined by dividing the levels of cytokines released after BCG instillation by the levels released prior to BCG therapy.

Results: Monocytes secreted variable levels of TNF α , IL-1 β , IL-6, IFN γ , IL-12, and IL-10. Compared with patients with recurrences, the post-BCG:pre-BCG ratio of IL-12 in monocyte cultures from patients without recurrences after five BCG instillations was significantly increased. Patients with no innate immune memory (based on IL-12 ratios) had significantly shorter time to recurrence than patients with innate immune memory ($p < 0.001$). Eighty-four percent (16/19) of patients with innate immune memory vs. only 22% (2/9) of patients without memory had disease-free survival of over 500 days.

Conclusions: Results demonstrate a potential link between BCG-induced innate immune memory peripherally and local anti-tumor

responses. Further validation will increase our understanding of the mode of action of BCG and, therefore, will be used to enhance its effectiveness.

Introduction

A high proportion of patients with non-muscle-invasive bladder cancer (NMIBC) fail to fully respond to immunotherapy with bacillus Calmette-Guérin (BCG), leading to further morbidity and mortality.¹⁻³ Therefore, a better understanding of the mechanism of BCG action in bladder cancer is critical for the development of improved immunotherapeutic approaches.

The current model of BCG action proposes that BCG is internalized upon interaction with fibronectin.⁴ Internalization of BCG results in the release of cytokines from bladder cancer cells,^{5,6} leading to recruitment of lymphocytes, granulocytes, macrophages, and dendritic cells.⁷⁻¹⁴ Most research to date has focused on the role of adaptive immunity in the response to BCG therapy,⁴ and CD4⁺ and CD8⁺ T cells may be critical for BCG-induced responses.^{7,15} In contrast, while innate immune cells, such as macrophages and dendritic cells, are required for effective adaptive responses, surprisingly, little is known regarding the role of innate immunity in the response to BCG.

Studies conducted over the last decade demonstrate evidence of acquisition of innate immune memory (also known as trained immunity) in circulating monocytes from individuals vaccinated with BCG for the prevention of tuberculosis.^{16,17} Innate immune memory is a process by which cells of the innate immune system acquire memory via epigenetic alterations, such as methylation and acetylation of histones associated with inflammatory cytokines, as well as via metabolic reprogramming.¹⁸ Innate immune memory is acquired upon stimulation of pattern recognition receptors (PRRs) on innate immune cells with pathogen-associated molecular patterns (PAMPs), or with danger/damage-asso-

ciated molecular patterns (DAMPs). The end result of innate immune memory acquisition is increased responsiveness of innate immune cells, characterized by an enhanced release of pro-inflammatory cytokines, such as IL-12, IFN γ , and IL-6, following secondary challenge with a similar PAMP or DAMP.¹⁸ Whereas classical adaptive immune memory requires expansion of antigen-specific clones of T and B cells, inflammatory responses resulting from innate immune memory are non-selective, thus leading to non-specific immunity against unrelated pathogens.

It has recently been proposed that trained immunity could be an important mechanism mediating BCG immunotherapy.¹⁹ However, while innate immune memory may indeed contribute to the efficacy of BCG in bladder cancer therapy, an association between innate immune memory and improved patient outcomes has not been determined in prospective, longitudinal studies. In the present study, we tested the hypothesis that innate immune memory acquisition in circulating monocytes collected from BCG-treated patients with NMIBC is associated with prolonged disease-free survival.

Methods

Patients

Thirty-three patients with intermediate- or high-risk NMIBC starting induction BCG therapy between June 2017 and June 2019 were recruited after informed consent and approval by the Queen's University Health Sciences and Affiliated Hospitals Research Ethics Board. American Urological Association (AUA) guidelines informed the risk stratification, surveillance, adjuvant therapies, and definitions of recurrence in our cohort.²⁰ Exclusion criteria included exposure to BCG within the previous three years, as such exposure reduces the likelihood of a response to BCG and complicates the assessment of immunological responses in the BCG-naive setting.²¹ Patient characteristics are described in Table 1. Patients were treated and monitored as per standard of care, with a specific assessment of BCG responsiveness in the cohort, including BCG refractory or early relapsing disease. Patients with high-grade T1 disease were re-resected to ensure accurate staging.

Twenty mL of blood was drawn just prior to initiation of BCG induction therapy (week 1; pre-BCG) and immediately before the third (week 3; after two BCG instillations) or sixth (week 6; after five BCG instillations) BCG instillations (Fig. 1). Peripheral blood samples were collected from 17 patients on weeks 1, 3, and 6; however, due to delays of instillations and other patient compliance factors, five additional patients had blood taken only on weeks 1 and 3 (before and after two BCG instillations), and 11 additional patients had blood

Table 1. Characteristics of patients with non-muscle-invasive bladder starting BCG induction therapy (n=33)

Age at diagnosis	
Mean	75.5
Median	77
Range	55–89
Sex	
Male	28
Female	5
Smokers ¹	27
Initial diagnosis: stage and grade	
Ta, low grade	3
Ta, high grade	19
T1, high grade	8
Tis, CIS	3
AUA risk score	
Intermediate	7
High	26
Recurrence	
<1 year	9 (8 males, 1 female)
>1 year	2 (males)
Median followup (range)	25 months (17–41 months)

¹Current or previous: 22 males, 5 females. AUA: American Urological Association. BCG: bacillus Calmette-Guérin; CIS: carcinoma in situ.

taken only on weeks 1 and 6 (before and after five BCG instillations). Peripheral blood mononuclear cells (PBMCs) were isolated using Lymphoprep (StemCell Technologies), suspended in 8% dimethylsulfoxide in fetal bovine serum, and stored in liquid nitrogen. This cryopreservation approach resulted in less than 1% cell death upon thawing.

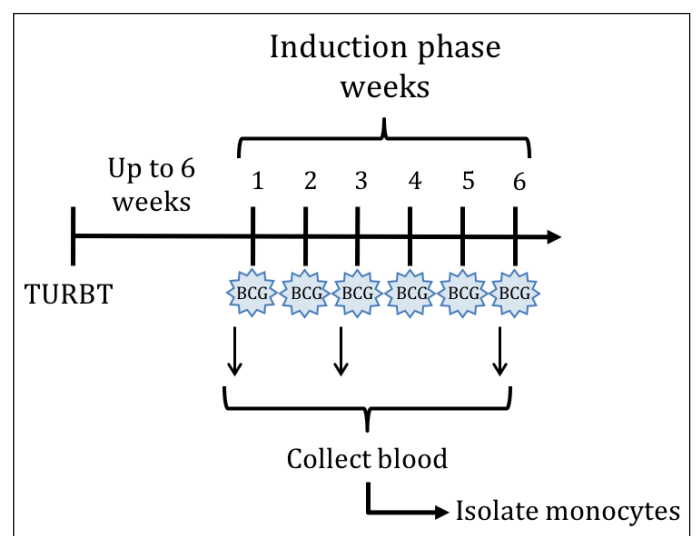


Fig. 1. Monocyte isolation timeline. Blood (20 mL) was collected immediately prior to initiation of BCG induction therapy (week 1), as well as immediately prior to the third (week 3) and fifth (week 6) BCG instillations. TURBT: transurethral resection of bladder tumor; BCG: bacillus Calmette-Guérin.

Determination of innate immune memory

Monocytes were isolated from thawed PBMCs using a human-specific, magnetic, bead-based, negative selection kit (StemCell Technologies). An enrichment of approximately 84% CD14⁺ classical monocytes was confirmed using flow cytometry (Supplementary Fig. 1; available at [cuaj.ca](#)). Monocytes (1×10^5) were plated in 96-well, flat-bottom plates and stimulated with 10 ng/ml lipopolysaccharide (LPS, from *E. coli* O111:B4; Sigma-Aldrich) for 24 hours. Levels of various cytokines in the culture medium were measured using a Human Cytokine Array Proinflammatory Focused 13-plex assay (Eve Technologies, Calgary, AB, Canada). This targeted, multiplex panel included cytokines associated with innate immune memory acquisition, mainly TNF α , IL-1 β , IFN γ , IL-6, IL-12, and IL-10. Extent of innate immune memory acquisition was determined by dividing the levels of cytokines released after BCG instillation by the levels released prior to BCG therapy (post-BCG:pre-BCG ratio).

To determine potential associations between circulating levels of cytokines and chemokines and clinical response to BCG therapy, or with acquisition of innate immune memory, plasma levels of IL-1 β , IL-2, IL-4, IL-6, IL-8, IL-10, IL-12, TNF α , IFN γ , MCP-1, and GM-CSF were also measured pre-BCG and post-BCG using the 13-plex assay (Eve Technologies).

Data analysis

The Fisher's exact test was used to compare whether the distribution of patients with high-risk and intermediate-risk NMIBC that recurred, differed significantly from patients with high-risk and intermediate-risk NMIBC who did not experience recurrence. Cytokine ratios (post-BCG:pre-BCG) were calculated by dividing concentrations in cultures of monocytes isolated after two or five BCG instillations (week 3 or 6, respectively) by concentrations in cultures of monocytes isolated prior to BCG instillations (week 1). Differences in the median ratio of cytokines release by monocytes from patients with no recurrences vs. patients with early recurrences were determined using the Mann-Whitney test. Thresholds of cytokine release ratios that best predicted recurrence (based on optimal sensitivity and specificity) were determined using receiver operator curves (ROC) and were used to define innate immune memory acquisition. Such thresholds were used to generate Kaplan-Meier curves of time to recurrence. Differences in time-to-recurrence curves of BCG-trained monocytes vs. BCG-non-trained monocytes were evaluated using a Mantel-Cox log-rank test. Differences in the change in plasma cytokines (week 6 – week 1) were calculated, and the Mann-Whitney test was used to compare patients with no recurrences to those with early recurrences. All data and graphs were analyzed using Prism 8 (GraphPad, San Diego, CA, U.S.).

Results

Patient characteristics

Thirty-three patients (28 males, 5 females; mean age 75 years [range 55–89]) were followed up for at least 17 months to ensure complete assessment of initial BCG response (Table 1). Seven patients in the cohort had AUA-defined intermediate-risk disease and only two of these had multifocal and large low-grade disease. As per guideline-informed institutional protocol, these intermediate-risk patients would only receive one year of maintenance BCG therapy. The median followup time for all patients combined was 25 months (range 16.6–40.2 months from date of consent) (Supplementary Tables 1–4).

At time of assessment, 11 patients (33%) had suffered a high-grade recurrence, with a median time to recurrence of nine months (range 2.4–13.7 months). In this relatively small cohort, there were no significant differences in the distribution of patients with high-risk and intermediate-risk NMIBC that recurred compared to those that did not suffer recurrence (18 high-risk and four intermediate-risk patients in the non-recurrence group vs. four high-risk and three intermediate-risk patients in the recurrence group, $p=0.66$; Fisher's Exact test). Four of the patients that suffered recurrences did so within six months of completion of BCG induction. The rest of the recurrences were documented between six and 14 months. The median followup of those that did not have any recurrence was 23 months (range 17–41 months) (Supplementary Tables 1 and 3; available at [cuaj.ca](#)).

Early NMIBC recurrence is associated with innate immune memory

Peripheral blood circulating monocytes isolated from patients with NMIBC before and after two or five induction BCG instillations exhibited variable levels of secretion of pro-inflammatory cytokines following incubation with lipopolysaccharide (LPS, 10 ng/mL), including TNF α , IL-1 β , IL-6, IFN γ , IL-12, and IL-10 (Supplementary Tables 5 and 6; available at [cuaj.ca](#)). Acquisition of innate immune memory is characterized by enhanced cytokine release in response to a secondary stimulus. While the concentration ratios (post-BCG:pre-BCG) of cytokines released by monocytes following LPS stimulation were variable in both the recurrence and no recurrence groups before or after BCG instillations (Figs. 2A, 2B) (Supplementary Tables 1–4; available at [cuaj.ca](#)), there was a significant increase in the post-BCG:pre-BCG ratio (week 6:week 1) for IL-12 released by monocytes collected from patients without recurrence compared with patients that suffered early recurrences ($p<0.05$; Mann-Whitney test).

Assessment of various plasma cytokine and chemokine levels (IL-1 β , IL-2, IL-4, IL-6, IL-8, IL-10, IL-12, TNF α , IFN γ ,

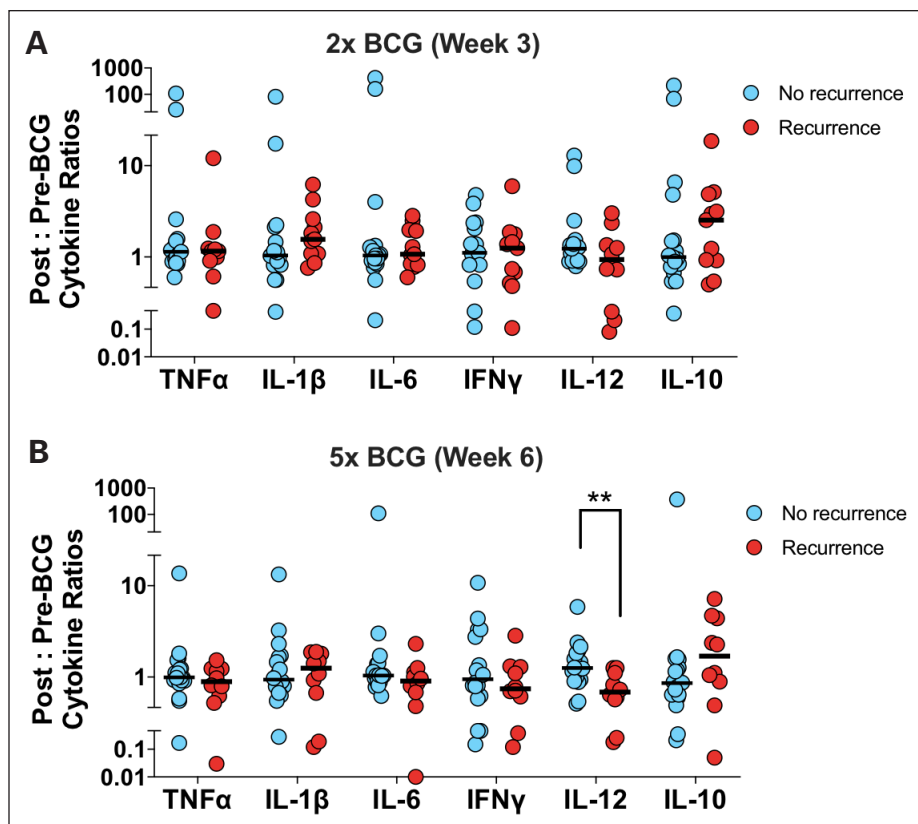


Fig. 2. Post-BCG:pre-BCG cytokine ratios in cultures of lipopolysaccharide (LPS)-stimulated monocytes from (A) patients without recurrences vs. patients with recurrences following two (n=11 recurrences, 17 no recurrences); or (B) five (n=10 recurrences, 18 no recurrences) weekly BCG instillations. Extent of memory is equal to the concentration ratios of cytokines released in vitro by peripheral blood monocytes isolated before and after BCG instillations and following a 24-hour exposure to LPS (10 ng/mL). Bars represent the median ratios. **p<0.01. BCG: bacillus Calmette-Guérin.

MCP-1, and GM-CSF) revealed no significant changes in pre-BCG (week 1) vs. post-BCG (week 6) levels (Supplementary Fig. 2; available at cuaj.ca) in either the non-recurrence or recurrence cohorts. Similarly, there were no differences in the post-BCG:pre-BCG plasma cytokine ratios (week 6:week 1), when comparing samples from patients with recurrences vs. those without. Furthermore, no significant correlations were found between levels of plasma cytokine and levels of cytokines secreted by cultured monocytes from the same patients at week 6 of BCG induction therapy.

Innate immune memory is associated with prolonged disease-free survival

We then used the post-BCG:pre-BCG ratios of secreted cytokines and generated ROC curves to determine the cut-off levels of innate immune memory that best predicted recurrence. None of the ROC curves generated using cytokine ratios obtained after only two BCG instillations had significant areas under the curve above 0.5, indicating poor predictive values for recurrence (Supplementary

Fig. 3; available at cuaj.ca). Using ratios obtained after five BCG instillations, IL-12 had an area under the curve of 0.81 ($p < 0.01$) (Supplementary Fig. 4; available at cuaj.ca), indicating excellent predictive value.²² Ratios of other cytokines (TNF, IL-1 β , IL-6, IL-10, and IFN γ) did not yield ROC curves with significant predictive values (Supplementary Fig. 4; available at cuaj.ca). Using the IL-12 ROC curve, a cutoff ratio of 0.85 revealed optimal sensitivity (89%) and specificity (70%). Thus, this ratio was used as a study definition of adequate innate immune memory (monocyte-associated) and to generate a time-to-recurrence curve. Results revealed significantly shorter time to recurrence in patients exhibiting no innate immune memory vs. patients exhibiting innate immune memory ($p=0.0006$; log-rank Mantel-Cox test) (Fig. 3). Patients with no evidence of innate immune memory in monocytes had a median recurrence-free survival of 13 months, whereas patients with innate immune memory had not reached a median time to recurrence (Fig. 3). Furthermore, 84% (16/19) of patients with innate immune memory vs. only 22% (2/9) of patients without innate immune memory had disease-free survival of over 16 months (Fig. 3).

Discussion

This longitudinal, prospective study is the first to demonstrate an association between BCG-induced innate immune memory in peripheral blood monocytes and disease-free survival in patients with NMIBC. Our findings demonstrate that, compared with monocytes from patients with early recurrences, the post-BCG:pre-BCG ratio of IL-12 secretion by monocytes following LPS exposure was significantly higher in monocytes isolated from patients with no recurrences, indicating a higher level of innate immune memory in the latter. Importantly, when innate immune memory was defined based on an optimal ratio of IL-12 released, innate immune memory acquisition appeared to be useful in predicting recurrence. The release of IL-12 following exposure to PRR ligands is typically increased in monocytes that have acquired memory;¹⁸ furthermore, IL-12 production by antigen-presenting cells promotes the development of anti-tumor T_H1 cells.^{23,24} Thus, it is possible that increased IL-12 production by trained monocytes is causally linked to the therapeutic benefit of

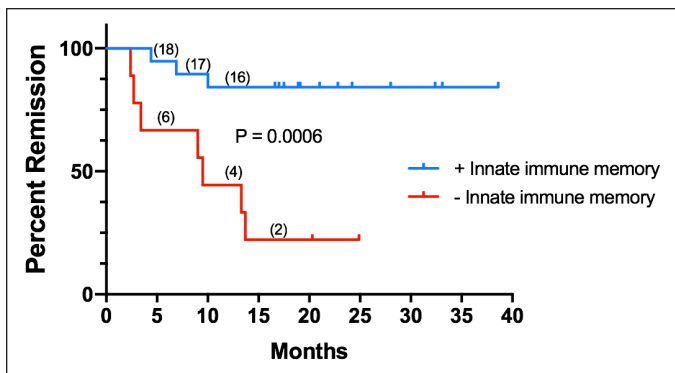


Fig. 3. Innate immune memory assessed after five BCG instillations (week 6) vs. time-to-recurrence in NMIBC patients. A threshold of IL-12 release ratio (i.e., post:pre-BCG therapy) that best predicted remission (based on optimal sensitivity and specificity) was determined using receiver operator characteristic (ROC) curves and was used to define trained immunity. This threshold was 0.85. The area under the ROC curve was 0.81. A log-rank (Mantel-Cox) test was used to calculate p-values for the Kaplan-Meier curves. $n=18$ no recurrences, 10 recurrences. Numbers in brackets indicate the number of patients who were disease-free as of October 11, 2020. BCG: bacillus Calmette-Guérin.

BCG. While the findings of our study are important, they need to be validated in a larger patient cohort.

Despite decades of BCG use and obvious clinical utility for higher-risk NMIBC, our understanding of its mode of action is incomplete.^{4,25,26} Given that BCG vaccination results in non-specific responses against unrelated pathogens, including cancer cells, a role for innate immunity in mediating this positive effect has been proposed.^{27,28} Since the heterologous effects of vaccines appear to be long-lasting,²⁹ it is likely that innate immune memory is involved in the therapeutic benefit of BCG in bladder cancer.

Buffen et al provided retrospective evidence that patients with single nucleotide polymorphisms in the autophagy-related ATG2B gene, resulting in reduced BCG-induced autophagosome formation, had increased risk of recurrence and progression following intravesical BCG therapy.¹⁷ That study also revealed that autophagy is required for the epigenetic reprogramming and innate immune memory induced by BCG in monocytes. Those findings provided indirect evidence for a potential role of autophagy and innate immune memory in the therapeutic effect of BCG. This current prospective study demonstrates, for the first time, a direct association between innate immune memory acquisition and improved patient outcomes following intravesical BCG therapy.

A recent study revealed that, compared with conventional wild-type BCG, a recombinant form of BCG engineered to release the STING agonist c-di-AMP exhibited improved anti-tumor activity in mouse models and enhanced innate immune memory in mouse and human monocytes to a greater extent.³⁰ However, as in previous studies, a causal relationship between enhanced innate immune memory and improved treatment outcomes still needs to be determined.

Conclusions

Although the number of patients in this study was limited to 33, our findings point to a potential prognostic role for innate immune memory in the response to BCG therapy. Furthermore, assessment of innate immune memory was limited mostly to monocytes isolated prior to the first and sixth BCG instillations. Future work is required to validate these preliminary observations in this discovery cohort and should focus on assessing innate immune memory throughout the BCG induction, as well as during and after maintenance dosing. Nonetheless, as acquisition of innate immune memory can be determined relatively simply and non-invasively, future validation studies may allow stratification of patients based on their acquisition of innate immune memory following BCG therapy. Identifying patients with suboptimal early immunological responses to BCG could lead to modifications of BCG schedules (induction and maintenance), dosing, and potentially switching to more effective strategies. Furthermore, if innate immune memory is determined to be critical for BCG anti-cancer responses, maximizing innate immune memory through other interventions could be of great therapeutic potential. For patients who fail to fully acquire BCG-induced innate immune memory, future studies could investigate the role of other PAMPs, alone or in combination therapies, to boost anti-tumor adaptive immune responses (e.g., immune checkpoint blockade) and improve patient outcomes.

Competing interests: The authors do not report any competing personal or financial interests related to this work.

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This paper has been peer-reviewed.

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