Cytokines as effectors and predictors of responses in the treatment of bladder cancer by bacillus Calmette-Guérin

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CYTOKINES AS EFFECTORS AND PREDICTORS OF RESPONSES IN THE TREATMENT OF BLADDER CANCER BY BACILLUS CALMETTE-GUERIN

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Runninghead: **Cytokines as effectors & predictors in BCG therapy**

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ABSTRACT

Purpose: The most effective intravesical treatment of non-muscle-invasive bladder cancer is instillation of live *Mycobacterium bovis* Bacillus Calmette-Guerin (BCG). BCG stimulates the release of cytokines, contributing directly or indirectly to its effectiveness. However, the function of specific cytokines is not well understood.

Methods: We have undertaken a non-systematic review of primary evidence regarding cytokine detection, activation and response in BCG patients.

Results: Cytokines IL-2, IL-8 and TNFα appear to be essential for effective BCG therapy and non-recurrence, whilst IL-10 may have an inhibitory effect on BCG responses. IL-2, IL-8, TRAIL and TNFα are potentially predictive of response to BCG. Alterations in genes encoding cytokines may also affect responses.

Conclusions: There are significant data showing the association of certain cytokines with successful BCG treatment, and which may be useful predictive markers. Isolating those cytokines mediating efficacy may hold the key to ameliorating BCG's side effects and improving efficacy and patient compliance.

INTRODUCTION

In the past few decades BCG intravesical immunotherapy following transurethral resection (TURBT) of non-muscle-invasive bladder cancer (NMIBC) has been established as the most effective adjuvant therapy, significantly reducing tumour progression and local recurrence (1). Intravesical BCG immunotherapy is arguably the most successful immunotherapy modality employed clinically to date. However, since the discovery of its benefits in bladder cancer therapy in the 1970s (2), the mechanisms of its actions have remained unclear.

As the bladder is an enclosed and confined compartment, BCG can be stored at high concentrations, theoretically resulting in a durable and continuous exposure (although the vast majority of BCG is cleared within several hours after instillation (3), some bacteria may persist in the bladder for many weeks or months (4, 5)). Ideally, an intact immune system is also required for successful BCG treatment; however, efficacy and safety have also been demonstrated in some groups of immunologically compromised patients with bladder cancer (6). BCG induces a mass release of cytokines and inflammatory cells into the bladder, and these cytokines have different roles, being anti-neoplastic, inflammatory, or inhibitory.

Despite high clinical efficacy, BCG immunotherapy is associated with significant side effects from local haematuria and dysuria, to life threatening sepsis (7). Such side effects often mean that patients do not complete the full course of induction or maintenance, potentially leading to worse outcomes: although generally considered safe, BCG has local and systemic side effects that lead to treatment cessation in up to 30% of patients, or to a delay or reduction in the number of instillations in 55-83% of patients (8). Therefore, although BCG is effective, it is only suitable for intermediate and high risk NMIBC patients in which current guidelines recommend one immediate instillation of chemotherapy post-TURBT, followed by a minimum of one year of BCG intravesical immunotherapy or further instillations of chemotherapy (7). A better understanding of BCG's mechanism of action

may allow its anti-neoplastic actions to be isolated, potentially improving efficacy and ameliorating side effects. Furthermore, some patients fail to respond to BCG treatment, and identifying these patients at an early stage (when other treatments may be curative) remains difficult. There is evidence to suggest that certain cytokines may be predictive of BCG efficacy, although such cytokine profiles are not yet being used clinically.

For immunotherapy to be effective, three basic steps need to be fulfilled. Firstly, there must be uptake of the therapeutic agent into the tumour cells. In the bladder, fibronectin is responsible for the uptake of BCG (9, 10). Then, an immune response must be induced, either by direct activation in response to microbial products, or by the presentation of antigen by antigen presenting cells (APCs) to effector cells. Finally, effector cells must migrate to the tumour and induce tumour cell killing. This review focuses on the role of individual cytokines as effectors, and their anti-neoplastic actions and prognostic utility in BCG therapy.

METHODS

A non-systematic search was undertaken using the NCBI/NIH library (*PubMe*d) for articles published up to 2013 concerning the involvement of cytokines in BCG treatment for bladder cancer. Keywords used to conduct the search included 'BCG,' 'cytokine,' and 'mechanisms.' As the work progressed, individual cytokines were researched in greater depth (ie. 'BCG and IL-8 mechanism'), as well as 'macrophage response', 'gene variants' and 'cytokine predictor,' all reviewed in conjunction with 'BCG' and 'bladder cancer.'

RESULTS

The Immune Response

To understand the cytokine response, it is important to clarify the normal T cell lymphocytic response. Cytokines are central to cell-mediated immunity and antibody responses. T lymphocytes have antigen recognition receptors that can bind to antigen and induce immune responses, eventually leading to the destruction of the target cell. The two main subsets of T cells are CD4 helper T cells and CD8 cytotoxic T cells. The identification of such distinct subsets of T helper cells capable of producing different cytokine profiles (differentially polarised from a non-polarised naïve (Th0) precursor cell) led to the conceptualisation of Th1 and Th2 subsets. While the majority of interest has involved $CD4^+$ T helper cells, $CD8^+$ T cells are also polarised to form Tc1 and Tc2 subsets (11, 12). The polarisation of $CD8^+$ T cells to Tc1/Tc2 has similar stimulating factors to the polarisation of $CD4^+$ T cells (13). Subsequently, there have been numerous Th subsets identified, although the two main categories remain Th1 and Th2. Th1 cytokines produce pro-inflammatory responses and the main cytokine secreted is IFNγ, in addition to IL-2, IL-12, TNFα, etc. It is this element of the immune response which is believed to be key to anti-tumour responses (14). In order to protect the body from inflammatory cellular damage, Th2 cytokines counteract the inflammatory response with IL-4, 5, 6, 10, and 13; Th2 cytokines are also involved in antibody reactions (11). IL-17 producing T cells are also noteworthy (e.g. Th17 cells): similarly to Th1 cells, Th17 are pro-inflammatory but are induced under very different conditions to Th1 cells (reviewed extensively elsewhere (15, 16)).

There is a wealth of data regarding response to BCG as an anti-tuberculosis (TB) vaccine, and it is increasingly recognised that IL-17 and the archetypal Th1 cytokine, IFNγ, are closely linked in the response to BCG vaccine. Indeed, protective immunity is at least partially dependant on an effective Th1 response (17), for which it is proposed that IL-17 is also required (18). IL-17 additionally has roles in recruiting neutrophils through the induction of IL-8 (discussed later). IL-8 mediated neutrophil

recruitment has been proposed to be at the heart of the anti-tumour activity of BCG (19, 20), and in murine models IL-17 is required for BCG immunotherapy efficacy (21). Data from BCG vaccine studies have shown that neutrophils are efficacious, but a strong prolonged recruitment is associated with pathology (22); how this relates to the deleterious side effects of BCG immunotherapy is yet to be determined.

It would seem unlikely that anti-tumour T cells are directly activated by BCG; rather, an indirect activation by presentation of tumour antigens in an inflammatory setting (i.e. alongside the response to BCG) could be possible, in which cytokines are essential. Likewise, the killing of tumour cells may be incidental, i.e. they are killed by BCG-specific T cells if infected by BCG. Notwithstanding, the ability of tumour cells to present antigen is associated with response to BCG immunotherapy (23, 24).

Cytokines in BCG

The large number of publications investigating cytokine involvement in BCG immunotherapy is derived from the discovery of elevated urinary levels of macrophages, T cells, NK cells and dendritic cells post BCG instillation (25, 26), which suggest infiltration of lymphocytes into the bladder wall. The internalisation of BCG by tumour cells or normal urothelial cells is likely an early step in this cascade (3), with the tumour/urothelial cells thereafter seemingly functioning like antigen-presenting cells (APCs) to induce cytokine production (27, 28). The rationale for investigating cytokine therapy alone or the administration of cytokines alongside BCG stems from the observation that live BCG creates the side effects, whilst cytokines alone are much better tolerated. Furthermore, it is thought that live BCG is not required to induce the bladder inflammatory cascade (29) (although live BCG is required to induce the APC-like characteristics described above). For example, gamma-irradiated but metabolically active BCG has demonstrated activity in vitro similar

to that of live BCG with respect to tumour growth inhibition and cytokine production (30). Furthermore, therapies utilising cell wall components derived from heat-killed BCG or other mycobacteria have also shown efficacy in vitro and in vivo, with an improved toxicity profile (31, 32). Such studies also suggest potential for using cell wall extracts in patients where BCG has failed (31, 32). Killed BCG and mycobacterial subcomponents can also stimulate the release of TNF-related apoptosis-inducing ligand (TRAIL) from neutrophils (20, 33) (TRAIL is a member of the TNF family that induces apoptosis in cancerous cells (29)). It is feasible that live BCG may only be required for initial BCG priming, and may not be necessary throughout all phases of BCG therapy, potentially improving safety and tolerability (10). In addition, although single cytokine therapy has not yielded promising results (34, 35), combinations of BCG with cytokines have been more successful (29), demonstrating that the cytokine mechanisms are complex and require more investigation.

Many studies have found the presence of a variety of cytokines in urine and serum post BCG instillation (see Supplementary Table 1), including IL-1, IL-2, IL-6, IL-8, IL-10, IL-12, TNFα, and IFNγ (25, 26). These and other cytokines are discussed in more detail below.

Jackson et al. treated 25 patients with carcinoma in situ with six weekly instillations of BCG; they then used immunoenzymatic assays on urine samples for the detection of cytokines (25). They demostrated that, with the exception of IL-6, the cytokines listed above are not detectable in the urine of untreated patients, and that their appearance in urine after treatment was distributed over the treatment period. **Table 1** summarises their findings. The recognition that most cytokines are only present after several instillations was also noted by Kresowik et al. where leukocyte levels peaked after the 6^{th} instillation of BCG (29). This suggests that the immune response in the bladder is a delayed-type hypersensitivity response. Jackson's research found IL-1β, IL-6, IL-8, IL-10 and sICAM1 after the first instillation, but IL-2, TNFα and IFNγ were only found later. This may reflect the source of these cytokines, given that resident macrophages secrete IL-1 and IL-6, but cytokines such as IL-2 and IFNγ are only produced after T cell activation following repeated BCG instillations (25).

Another more recent study by Shintani et al. demonstrated that urinary GCSF, IL-1β, and IL-8 levels were significantly higher after the sixth instillation than pre-instillation. However, they did not record significant increases in urinary IFNγ or IL-12, despite being key cytokines in CD4 Th1 responses (26). Böhle et al. showed that IFNγ and IL-12 might be secreted in topical CD4 cells in the bladder wall (36), but this was not reflected in Shintani's results. It may be that IL-12 induced by BCG is produced earlier than other Th1 cytokines; Shintani's monitoring period between 4 and 24 hours may have missed the maximum secretions of IL-12, although the stability of IL-12 (and all cytokines) in the urine may also be problematic. It is also feasible that the levels needed to induce responses in the tumour microenvironment may be undetectable in the urine.

Functions of individual cytokines and their actions stimulated by BCG

IL-1α and IL-1β (IL-1) are pro-inflammatory cytokines, whilst IL-1Ra is anti-inflammatory. These IL-1 derivatives compete for IL-1 receptor binding to regulate immune and inflammatory responses. Higher expression of IL-1 has been associated with tissue damage and aggressive tumours and there is a strong association of IL-1Ra with bladder cancer, but data specific to IL-1Ra and its relationship with BCG is not widely available (37). Böhle et al. proposed that IL-1 may function by inducing IL-2, macrophages and cytotoxic T lymphocytes (36); IL-1 may also interact with IL-2 and INFγ to induce the NK cell killing of cancer cells (38) .

IL-2 is involved in T cell proliferation and differentiation. IL-2 was consistently elevated in urine samples of all patients within 24 hours post-BCG instillation in the study by Böhle et al., with maximum levels after 4 hours (36). Haaff et al. confirmed these findings, demonstrating maximal IL-2 secretion after 4 hours (39). IL-2 is produced by Th1 cells, and it thus appears that BCG effectiveness correlates with preferential induction of Th1 cytokines (38) .

IL-4 is an important cytokine in the activation of B cells, as well as Th2 lymphocyte development, along with IL-6 and IL-10. Sander et al. found a temporary increase in IL-4 levels in the urine within 24 hours post BCG instillation (40), although Agarwal et al. showed reduced IL-4 levels in patients receiving combined immunotherapy (41). Table 1 also shows that Jackson et al. did not detect IL-4 in the urine of BCG patients, and confirmed that this was not due to insensitivity since IL-4 was detected in both lymphocyte tissue culture supernatants and 'spiked' urine (25). The relatively low amount of IL-4 compared to other cytokines suggests that Th2 responses are less dominant in BCG responses, consistent with the evidence above regarding the apparent importance of Th1 responses for BCG efficacy. Jackson et al. found an increase in IL-10 alongside the absence of IL-4, which is somewhat contradictory since they are both Th2 cytokines. However, IL-10 is now recognised not be exclusively produced by Th2 cells, having both regulatory roles and being produced by other cells, including Th1, Tr1 regulatory CD8+ T cells and Treg (15). In addition, IL-10 did not show a negative correlation with IL-2 and IFNγ, even though it acts to inhibit them(25). IL-10 is discussed in more detail later.

IL-6 is one of the key cytokines in the acute phase response, and promotes neutrophil synthesis. It supports B cell growth and antagonises Treg cells. Following binding of BCG fibronectin attachment protein (FAP) to cellular fibronectin, IL-6 and other cytokines are produced by tumour cells, a process requiring BCG to be internalised by α5β1 integrin (10, 42-44) and leading to the necessary subsequent activation of NFkB and AP1 (42). Interestingly, the malignant transformation of urothelial cells may render them more susceptible to uptake of BCG (10, 45). Other mechanisms, such as the production of IL-17 by immune cells, may also contribute to the production of IL-6 (46). Furthermore, macrophages are well known to produce IL-6 in response to BCG (46, 47); as macrophages are present within the tumour stroma (48), these cells may also represent a notable source of IL-6 following intravesical BCG application.

IL-6 is able to influence a number of immune cell types, directly and indirectly through aiding recruitment by inducing expression of a variety of chemokines (49). Activation of signal transducers and activators of transcription (STAT)-3 by IL-6 promotes survival of T cells through up-regulation of Bcl-2 (50); likewise, IL-6 has also been shown to affect NK cell cytotoxicity (51). Conversely IL-6 also promotes tumour cell survival (52). IL-6 is also able to suppress IFNγ production through the induction of the transcription factor suppressor of cytokine signaling-1 (SOCS), while promoting IL-4 (Th2) responses through nuclear factor of activated T cells (NFAT) activation (53). Autocrine signaling by IL-4 subsequently reinforces Th2 differentiation. However, as discussed, current studies do not consistently detect IL-4 following BCG therapy. Similarly, in studies of BCG as a vaccine, IL-10 rather than IL-4 dominates in response to BCG (54). These data suggest BCG produces IL-10+ non-Th2 polarised cells, also consistent with the presence of IL-10 in urine following BCG therapy.

Using immunohistochemistry, Cardillo et al demonstrated significantly higher levels of IL-6 in bladder tumours (55). Additionally, Zhang et al. investigated the relationship between cAMP production and IL-6 production, and found decreased cAMP and IL-6 production simultaneously in the presence of a specific adenylate cyclase inhibitor (44). This led to the hypothesis that IL-6 may be upregulated by BCG using a cAMP-dependent pathway. However, as illustrated above, this is unlikely to be the only pathway (44).

IL-8 is an early cytokine in the inflammatory response, produced by a variety of immune and epithelial cells in response to bacterial products or other inflammatory cytokines, e.g. IL-17 (as mentioned above). IL-8 has significant chemokine functions, recruiting mainly neutrophils to the site of inflammation, thus driving the early stages of the innate immune response. As such, IL-8 has been shown to be elevated to high levels in the urine within hours of BCG instillation (56) which, as discussed later, may have prognostic value.

IL-10 decreases cytokine production by Th1 cells, cytotoxic T cell generation and antigen presentation (57). It achieves this by blocking MHC-II and the expression of co-stimulatory molecules on APCs, as well as by induction of co-inhibitory molecules (58). It has also been proposed that IL-10 diminishes macrophage activity by reversing the effects of TNFα and IFNγ. Murine studies by Luo et al. using two bladder cancer cell lineages (MBT-2 and MB49, shown to have similar responses to BCG), demonstrated correlations between high IL-10 levels and decreased cytotoxic effector molecules (59). These studies lead to the conclusion that IL-10 could decrease macrophage toxicity against bladder cancer cells. Interestingly, data from BCG vaccine studies also indicate that BCG is capable of inducing IL-10 following chronic exposure (60, 61). It may therefore be possible to promote Th1 responses by IL-10 inhibition, and such approaches have been validated in preclinical animal models (62, 63) as discussed later.

IL-12 immunomodulation has been met with tumour response in many malignancies, including bladder cancer models. It is thought that the anti-tumour effect is driven primarily by $CD8^+$ T cells, and involves an increase of IFN-γ (64). In a study by Riemensberger et al. BCG therapy was ineffective in mice with IL-12 knockout (57). However, despite promising results in mice, trials on humans have been less successful (35). Weiss et al. administered recombinant human IL-12 in patients with recurrent NMIBCs, and this was associated with minimal toxicity, but also poor efficacy (35).

IL-18 is secreted by BCG-activated macrophages, and activates NK cells and cytotoxic T lymphocytes (65, 66). Elevated urinary IL-18 levels are observed after BCG instillation (66, 67), and are associated with significantly longer disease-free survival (66).

TNFα has been linked to many processes in cancer, such as cell transformation, proliferation, survival, invasion, angiogenesis and metastasis (37). Böhle et al. found a large increase in urinary TNFα following BCG instillation when compared to the control group (36), and Jackson et al.'s

studies found that TNFα levels were detected in later instillations (**Table 1**) (25). TNF-related apoptosis-inducing ligand (TRAIL) is a member of the TNF family that induces apoptosis in cancerous cells (29). In a study by Ludwig et al., BCG responders had significantly higher urinary TRAIL levels than non-responders (68); with subsequent BCG instillations, TRAIL was further increased. TRAIL secretion following BCG is neutrophil-dependent, and this same study showed that neutrophils stimulated by BCG were able to kill bladder cancer cells in a TRAIL-dependent manner. TRAIL seems to be unique to the BCG immune response (urine samples from urinary tract infections found lower levels of TRAIL (68)), although the stimulation of TRAIL does not appear to be completely dependent on live BCG: Kemp et al. found that TRAIL can be produced following stimulation with killed BCG and Toll-like receptor 2 and 4 agonists (20). In addition, murine studies have shown that instillations of dead BCG following previous live BCG treatment produce similar cytokine responses to live BCG alone (29). As the side effects of BCG are largely attributed to live BCG, this may be a useful strategy to diminish BCG's adverse effects, although caution would be needed to ensure that full clinical efficacy is maintained; however, clinical trials may be warranted

IFNγ is a pro-inflammatory cytokine. It enhances lymphocyte function, stimulates cell adhesion molecule expression, upregulates MHC expression (37), and has been shown to inhibit the growth of RT4, RT112 and MGH-U1 cell lines in vitro (69). Carriers of the IFNγ +874 A polymorphism are associated with a higher risk of recurrence after BCG immunotherapy (37), possibly as a result of decreased IFNγ production as observed in tuberculosis (70). However, Shintani et al found no significant urinary IFNγ increases between 4 hours and 24 hours even after the 6^{th} instillation of BCG when compared with pre-instillation values (26). According to Böhle et al.'s investigations, IFNγ is a key cytokine in the CD4 response, in conjunction with IL-12 (36), but Shintani's results do not reflect this (26).

Intercellular adhesion molecules (ICAMs) are expressed at a higher level, along with MHC-II, following BCG instillation. They are detected immediately, and levels increase with repeated doses

of BCG, although they are not normally expressed by untreated bladder carcinoma cells (25). In vitro, cytokines such as TNFα, IFNγ and IL-1 can up-regulate the expression of MHC-II and ICAM-1. It is thought that ICAM-1 expression can enhance ligand binding of cytotoxic cells, whilst MHC-II can present antigen to CD4 T cells. This had led to the belief that ICAM-1 expression may predispose tumour cells to cell-mediated cytotoxicity (25).

Predictive Cytokines

The study of cytokines as predictors of response to BCG immunotherapy is also highly relevant. The cytokines observed to have the most promising predictive utility for BCG efficacy are IL-2, IL-8, TNFα, TRAIL, and possibly IL-18 (65, 71). Urinary levels of these cytokines may be essential for the success of BCG, or may be indicative of the magnitude or quality of the immune response. Such cytokines are not currently used as predictors of response in clinical practice, nor do we precisely understand the factors which determine their elevation.

In particular, IL-2 and IL-8 are the most widely studied (see **Table 2**). Numerous studies have identified a significant association between higher IL-8 secretion and BCG responses (66, 72-74). For example, De Boer et al. suggest that IL-8 can be used as an indicator of efficacy 6 hours after instillation (56)**,** and Shintani et al. found higher levels of IL-8 in the non-recurrence group within 4 hours after the 6^{th} instillation of BCG (26). However, there are a number of other studies which have failed to demonstrate this relationship (26, 75), including Sagnak et al. who, in contrast to the other studies, demonstrated that patients with lower IL-8 showed improved outcomes (76). Additional studies have shown IL-2 to also be predictive of response. For example, Watanabe et al. found higher levels of IL-2 in later instillations to be a strong predictive factor for a positive response to BCG therapy (72). However, they also found that IL-2 concentrations are variable depending upon the storage method of the urine samples: cytokine concentrations in urine samples before and after

freezing were different, and storage temperature caused variability. Indeed, the small sample size and differences in sampling make interpretation of these data difficult. Despite this, the suggestion that IL-2 is a predictive factor for BCG is supported by other studies (72, 75, 77, 78). Interestingly, Kaempfer et al. showed IL-2 gene expression in peripheral blood to be predictive of response (79); it would be of great interest to assess whether this relationship exists in a larger cohort of patients and using current methodologies.

Urinary TRAIL appears in increased levels in BCG-responsive patients compared non-responders (20, 68). As mentioned above, heat-killed BCG is also able to elicit comparable TRAIL/Apo-2L release from neutrophils as viable BCG (20). The potential of altering TRAIL expression to enhance BCG effect has also been proposed, for example by using a combination therapy of BCG and IFN-α, or even by direct intravesical recombinant TRAIL instillation (68). As well as increasing efficacy, it may permit a reduced BCG dose to achieve the same effects, thereby decreasing the potential for adverse effects.

FUTURE PERSPECTIVE

A full understanding of BCG's mechanism of action in the treatment of bladder cancer remains elusive (10): IL-2, TNFα and INFγ levels appear to be much higher in urine post BCG, which suggests that the BCG reaction is predominantly Th1 mediated, yet the cellular origins of the cytokines do not appear to be divided into classical Th1 and Th2 sources, as demonstrated by contradicting levels of IL-10 and IL-4. In addition, the time lag between the appearance of different cytokines in different studies suggests variability in both individual cytokines and patients. See **Figure 1**.

The future development of BCG immunotherapy for bladder cancer should therefore be directed towards three objectives:

- Identifying patients most likely to benefit from treatment;

- Increasing efficacy using promotion and blockade of specific cytokines;
- Reducing side effects and improving tolerability.

Cytokines with possible predictive value have the potential to act as a screening method for patients who may or may not succeed with BCG treatment: IL-2, IL-8, TRAIL and TNFα appear to have a predictive relationship with BCG efficacy, with significantly higher IL-2 and IL-8 levels in responders compared to non-responders (Table 2). These cytokines appear within 6 hours post-instillation, and have strong positive correlations to successful BCG treatment and non-recurrence. However, these data are not consistent and so have not yet reached clinical practice. More recently, the IL-6:IL-10 ratio has also demonstrated predictive utility (80). This area of research would benefit from further clarification and confirmatory studies since it could lead to efficient tests to identify the subgroup of patients who reap no benefit from BCG but whom suffer from side effects, in addition to reducing the delay to efficacious treatment (and reducing cost).

The physiochemistry of the molecules being studied also needs to be considered and results interpreted carefully - cytokines can be unstable in biological fluids (78) (although IL-8 appears to be stable in urine for over 48 hours (73)), and the immunoassays performed may be affected by ionic strength, pH(25), protease activity, and soluble binding proteins. Uniform or standard units of measurement would also aid the interpretation and comparison of studies. Assessing the profiles of multiple cytokines is also costly, which is why the studies reviewed above rarely surpass 30 individuals, or only a few cytokines are assessed in each study. Moreover, study patients are usually heterogeneous with regard to gender and ethnicity. Recent evidence demonstrates that existing BCG-specific responses (from vaccination, for example) may improve the BCG immunotherapy response in bladder cancer (81); since BCG vaccine efficacy has a significant ethnic bias (82, 83), it should be considered whether this may occur in the setting of BCG immunotherapy for bladder cancer. Additional complexity is provided by the seemingly differential induction of immune

responses and efficacy of the commonly-used BCG strains in both immunisation and NMIBC treatment (84, 85). For example, in vitro, Russian and Connaught strains induce significantly higher cytokine production (IL-6 and IL-8) and inhibition of tumour cell proliferation than Glaxo strain (85), and in a randomised controlled trial treatment with BCG Connaught conferred significantly greater 5-yr recurrence-free survival compared with treatment with BCG Tice (86). In mice, BCG Connaught induces stronger Th1-biased responses, greater priming of BCG-specific $CD8^+$ T cells, and more robust T-cell recruitment to the bladder than BCG Tice (86). Furthermore, different BCG vaccine strains elicit different T-cell responses in human in vitro assays when healthy BCG-vaccinated individuals are tested (84).

BCG therapy and anti-coagulant drug interactions have also been investigated, but without conclusive results (87). The possibility of warfarin-associated bladder tumour recurrences following intravesical BCG has been suggested, although the underlying mechanism is unclear (88). Similarly, aspirin has been described to decrease recurrences (88, 89). This effect may be explained by local prevention of tumour cell adhesion and implantation to the urothelium (90, 91). Furthermore, COX-2 inhibiton has been shown to have anti-tumoural effects in canine and mice models of bladder cancer (92). There has been evidence of COX-2 expression in CIS and invasive urothelial carcinoma, but not in healthy bladders (92) (the BOXIT trial of celecoxib for reducing recurrence and progression of NMIBC will report findings in 2014/15). Understanding in this area is limited, and certainly not enough to justify exposing patients to the risks of stopping warfarin therapy or changing their regular prescriptions; however, these data may be useful when the mechanism of action of BCG is better understood.

Germline and/or somatic genetic variation is also likely to play a significant role in an individual's response and a tumour's response to BCG. Single nucleotide polymorphisms (SNPs) in IL-10, TGFβ and IL-4 genes are associated with progression despite BCG therapy (29), whilst other polymorphisms are associated with lower recurrence rates. Shintani et al**.** explored the relationship

between recurrence and urinary cytokines and found that Th1 cytokines are associated with longer recurrence-free survival, and Th2 cytokines are associated with BCG failure (26, 37). This suggests that polymorphisms which affect the Th1/Th2 balance have the potential to change the efficacy of BCG treatment. The genetic variability of cytokine expression is an ongoing area of research, and although utilising genetic analysis for determining the suitability of patients for BCG therapy is currently not in clinical use, it may prove beneficial in the future. It is highly feasible, even probable, that modern genomic and epigenomic analytical platforms will permit the stratification of patients into those who are likely to respond to BCG, and those who are not, based upon an initial tumour biopsy. However, until such platforms enter routine clinical practice, the measurement of urinary cytokines as described above appears to demonstrate the most promise in the short to medium-term, notwithstanding issues of reproducibility and timing of measurement.

As described above, there is evidence to suggest certain cytokines either reduce or promote the effects of BCG. For example, identification of the inhibitory actions of IL-10 by Luo et al. suggest that high levels of IL-10 correlate with lower cytotoxic activity (59), and in more recent studies IL-10 blockade using anti-IL-10 neutralising monoclonal antibody and IL-10 receptor blockade has been shown to enhance BCG Th1 responses in preclinical models, with better tumour-free survival rates. These studies also found significantly enhanced levels of Th1 responses, including higher levels of IFN-γ, with the use of anti-IL-10 receptor 1 monoclonal antibody in mice models (62, 63, 93). Translating these promising findings from in vivo preclinical models into early-phase clinical trials should be considered a priority for the field. Mechanisms specific to BCG, such as TRAIL, should also be considered. Therefore, combining BCG with cytokine-specific blockade or promotion may increase effectiveness. However, when altering cytokine activity, consideration should also be given to side effects: increasing efficacy may reduce tolerability, and the two should be considered together since non-compliance due to side effects would be counter-productive.

To reduce adverse effects, alternatives to live BCG have been suggested. Whilst utilising live BCG is standard practice, it produces significant side effects; alternatively, cytokine-only therapy is much better tolerated, although single cytokine therapy has not proved successful. Having identified specific cytokines that are involved in the anti-tumour response, it would be useful to test instillation of a combination of cytokines. It would also be valid to test the differences in efficacy and side effects of dead versus live BCG, given that dead BCG also induces the necessary inflammatory cascade, whilst live BCG is responsible for the side effects. If dead BCG produces a less effective response, it could be feasible to supplement the response with single cytokine therapy or cytokine promotion; alternatively, it may be valid to assess induction with live BCG and maintenance therapy with dead BCG (10).

This review has a number of limitations. Firstly, we have used a non-systematic approach to try to identify the most pertinent studies in the field, but undoubtedly we have not carried out an exhaustive review of all studies in the field. Our non-systematic approach is also a reflection of the heterogeneity of source data and publications, with such data acquired from multiple studies (mostly small in size), each utilising different treatment regimens and procedures for cytokine evaluation and measurement, making direct comparisons difficult. As discussed above, uniformity in such methodology could greatly improve research in this area. Meta-analyses of data regarding the most promising cytokines described above could be appropriate and valuable, but such analyses are beyond the scope of this review. However, it is our opinion that a strategy of co-ordinated early-phase studies in combination with comprehensive laboratory-based analyses is required to progress the field and to optimise the management of patients receiving BCG for NMIBC. Unfortunately, research funding for bladder cancer is poor when compared to other common malignancies (94-96), and this needs to be urgently addressed before such progress can be made.

CONCLUSION

The mechanism of action for BCG is complex and variable, and a full understanding remains elusive. It is likely that many elements of the immune system respond to BCG instillation; however, which of these are necessary for the clinical efficacy of BCG immunotherapy remains to be answered. Likewise, which of these are detrimental in terms of side effects is also unknown. Further research should focus on combinations of BCG and cytokine therapy, as well as indicators of an individual's response to treatment, such as predictive cytokines and genetic variants. Although these areas are unlikely to be fully elucidated or utilised in clinical practice in the immediate future, further research may shed light on determining how we can distinguish between patients who may benefit from BCG treatment, how we can optimise BCG responses, and how we can reduce the side effects that limit the use of BCG for many patients.

EXECUTIVE SUMMARY

Introduction

- Intravesical instillation of Bacillus Calmette-Guerin (BCG) is an effective therapy for non-muscle-invasive bladder cancer.
- Intravesical BCG therapy is associated with significant side effects.
- The precise mechanism of action of BCG remains elusive.
- Understanding the mechanism of action may permit improved efficacy, improved patient selection and a reduction in side effects.

The Immune Response

- The two main subsets of T cells are CD4 helper T cells and CD8 cytotoxic T cells, leading to the concept of Th1 and Th2 subsets.
- Th1 cytokines produce pro-inflammatory responses; Th2 cytokines counteract the inflammatory response and are also involved in antibody reactions.

Cytokines in BCG

- Following intravesical BCG therapy the cytokine milieu of the bladder and urine is complex and variable.
- IL-10 and TRAIL may represent therapeutic targets for improving BCG efficacy.

Predictive cytokines

- IL-2, IL-8 and TRAIL show promise as predictive cytokines for BCG therapeutic responses.

Future Perspective

- Further early-phase studies combined with laboratory-based analyses are required to optimise the management of patients receiving intravesical BCG for NMIBC.

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TABLE & FIGURE LEGENDS

Table 1: Modal week of first appearance of a particular cytokine (from Jackson et al. (25)). Note that some cytokines (IL-6) are readily detected after the first week, whilst for others (IL-2, IFN-γ) several rounds of therapy are first required.

Table 2: Predictive cytokines - Levels of IL-2 and IL-8 and prediction of response to BCG therapy in various studies. The values used to divide responders and non-responders are shown, with the statistical significance of these differences. The range or standard deviation (SD) of the cytokines detected in these groups is also given, where available.

Supplementary Table 1: Summary of cytokine concentrations following final BCG instillation, expressed either as a snapshot concentration (e.g. pg/ml) or as a measurement over a specified time period (e.g. ng/2h where h=hours).

Figure 1: A pictorial representation of the cellular and cytokine mechanisms associated with therapeutic response or failure to intravesical BCG immunotherapy for NMIBC.

740 **Table 1**

	Cytokine appearance in weeks following once weekly BCG instillations								
	IL-1	**IL-2**	**IL-4**	**IL-6**	**IL-8**	**IL-10**	**TNFα**	**IFNγ**	**ICAM1**
Jackson *et al.* (25)	1	4	-	1	1	1	2	3	1

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742

743 **Table 2.**

	Non-responder	Responder	P-value	Patients (Numbers)	Recurrence rate	Median Follow-up (Months)	Reference
IL-2	0.18ng/24h (±0.43)	10.6ng/24h (±12.9)	<0.01	20	30%	46.9	Watanabe et al. (72)
	<27 pg/µmol creatinine	>27 pg/µmol creatinine	0.0009	37	59.5%	29	Saint et al. (77)
	<0.34 U/µmol creatinine	>0.34 U/µmol creatinine	0.003	23	</> 6 months	-	de Reijke et al. (78)
IL-8	<4000 ng/12h (232-8497ng)	>4000 ng/12h (432-8497ng)	<0.05	28	42.9%	66	Thalmann et al. (66)
	<4000 ng/6h (1735.5 ±1596ng)	>4000 ng/6h (6961.4 ±3095ng)	<0.0002	20	50%	36.5	Thalmann et al. (73)
	<400pg/ml @4h (261.82 ±182.66)	>400pg/ml @4h (1099.33 ±708.51)	0.001	26	42.3%	24	Kumar et al. (74)

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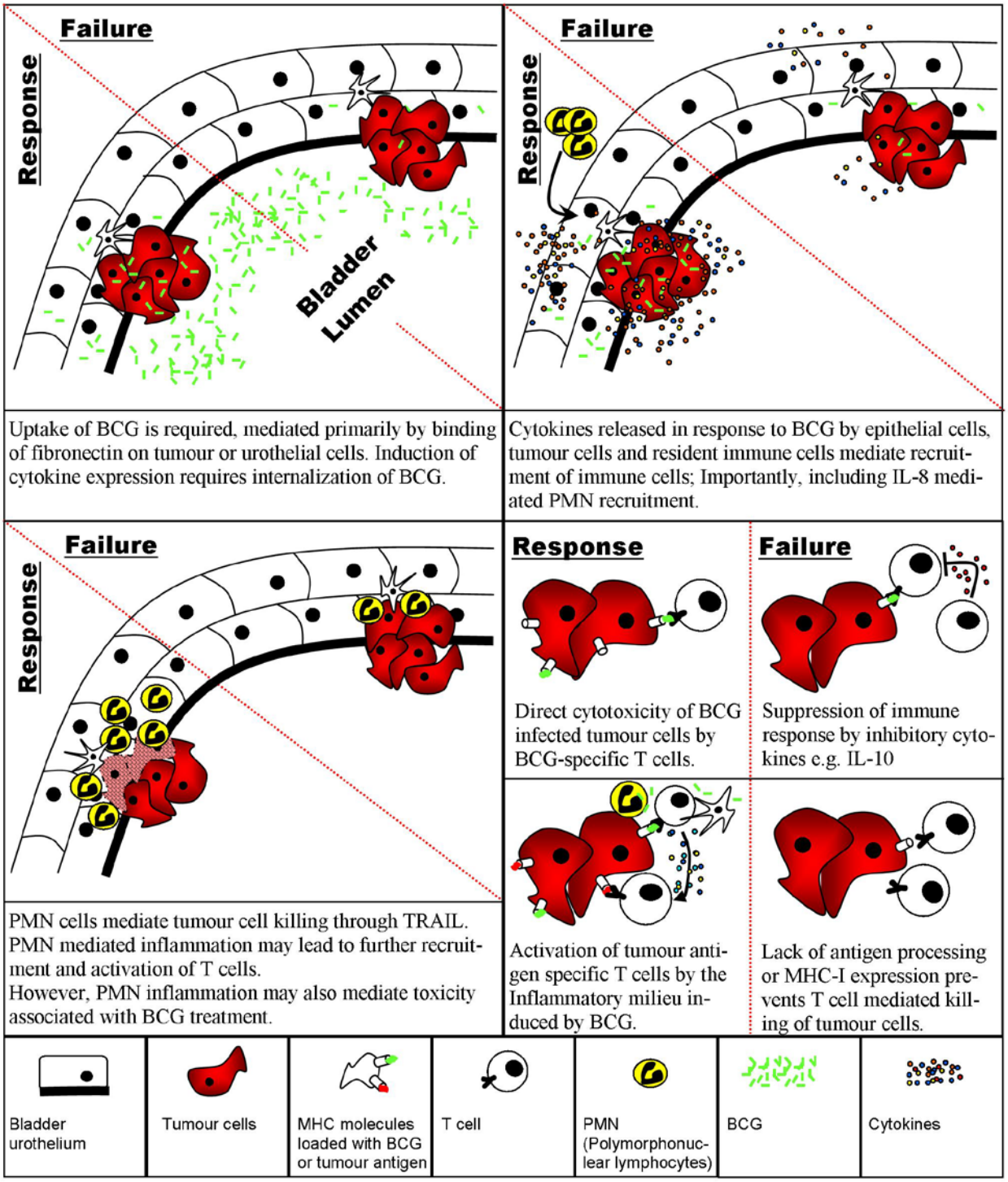
745

746 **Supplementary Table 1**

	Cytokine level following 6th instillation of BCG from various studies							
	0hrs	**2hrs**	**4hrs**	**6hrs**	**8hrs**	**12hrs**	**24hrs**	**Reference**
IL-1	20ng/2h	10ng/2h	85ng/2h	30ng/2h	45ng/2h			Bohle & Brandau (36)
	0.03pg/mL (±0.07)		1.72pg/mL (±1.55)		0.52pg/mL (±0.62)		0.06pg/mL (±0.09)	Shintani et al. (26)
						29.9 pg/12h (2-118)		Jackson et al. (25)
							23.38ng/24h (±61.64)	Watanabe et al. (72)
IL-2	0ng/2h	10ng/2h	300ng/2h	100 ng/2h	20ng/2h			Bohle & Brandau (36)
						74.4 pg/12h (0-666)		Jackson et al. (25)
							7.52ng/24h (±11.75)	Watanabe et al. (72)
IL-6						245 pg/12h (17-747)		Jackson et al. (25)
							100.04ng /24h (±107.31)	Watanabe et al. (72)
IL-8	0.42pg/mL (±1.34)		7.75pg/mL (±13.56)		6.23pg/mL (±10.33)		1.44pg/mL (±2.58)	Shintani et al. (26)
						4.8 mg/12h (0.1-29)		Jackson et al. (25)
							222.27 ng/24h (±144.64)	Watanabe et al. (72)
IL-10						51.3 pg/12h (0-400)		Jackson et al. (25)
							115.77ng/24 h (±191.46)	Watanabe et al. (72)
TNFα	1 ng/2h	8ng/2h	7ng/2h	2ng/2h	3ng/2h			Bohle & Brandau (36)
	0.01pg/mL (±0.02)		5.08pg/mL (±7.89)		0.03pg/mL (±0.05)		0.01pg/mL (±0.02)	Shintani et al. (26)
						80.4 pg/12h (0-363)		Jackson et al. (25)
							488.27 ng/24h (±774.17)	Watanabe et al. (72)
IFNγ	0.01pg/mL (±0.06)		1.47pg/mL (±5.47)		0.35pg/mL (±1.34)		0.02pg/mL (±0.05)	Shintani et al. (26)
						5900 U/12h (0-23000)		Jackson et al. (25)
							134.11 ng/24h (±179.10)	Watanabe et al. (72)

747 **Figure 1**



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