The use of recombinant *Mycobacterium bovis* Bacillus Calmette-Guérin in the treatment of non-muscle-invasive bladder cancer.

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Abstract

In the treatment of bladder cancer, Bacillus Calmette-Guérin (BCG) instillation is superior to chemotherapy. However, improving the effect of the therapy remains necessary since even frequent BCG instillation is not fully effective. Currently there are several recombinant BCG vaccines in development to prevent mycobacterium tuberculosis (TB) infections. The question arises, whether these vaccines could also play a role in bladder cancer treatment. The most promising vaccine, VPM1002, results in increased Th1 cytokine production and induces a higher cellular response. This review describes the immune response to bladder cancer and BCG instillation and attempts to answer the question whether the responses of VPM1002 would be safe and beneficial for bladder cancer patients. In addition the response to several of the vaccines in development are described and compared to native BCG.

General introduction

Bacillus Calmette-Guérin (BCG), a vaccine developed to prevent mycobacterium tuberculosis (TB) infections, is prepared from a strain of attenuated *Mycobacterium bovis.* Besides its use in the prevention of TB and leprosy infections, it has also been part of the treatment for non-muscle invasive bladder cancer (NMIBC) ever since its effect was discovered in 1976[1,2]. These malignancies arise from the mucosal urothelium in the bladder wall and constitute 80% of all bladder cancers. They have a recurrence rate ranging from 30-90% within the first 6 to 12 months after resection. In 2008, an estimation on worldwide incidence and deaths by this type of cancer determined 386,300 new patients and 150,200 fatalities[3-5]. Although the treatment of these cancers with additional BCG instillation in the bladder increases survival, there is room for improvement. A possible strategy to improve the BCG treatment is the use of a recombinant BCG. Several attempts were taken to enhance BCG specifically for bladder cancer patients and some were rather promising in animal models however none made it to clinical trials[6]. An alternative approach could be the application of rBCG optimized for the purpose of treating TB. In recent years there has been an increase in the development of new vaccines against this disease, since the current vaccine strain *M. bovis* BCG is not able to give rise to protective immunity, and the problem of multi-drug resistant TB strains is increasing. Several of these vaccines are currently under investigation in clinical trials for the use in TB[7]. Now, the question arises whether these vaccines could also play a role in the treatment of NMIBC. To be able to predict this we need to known the mechanism of action of BCG in bladder cancer. Nowadays, several immunological responses have been identified in response to BCG therapy. The purpose of this review is therefore to investigate if newly developed rBCG might induce these immunological responses needed to clear bladder cancer. We provide an overview of current recombinant BCG vaccines in development, their purposed efficacy over BCG and their potential role in the treatment of NMIBC. In addition, we briefly describe the immune response to NMIBC and give a short overview of tumor immunology.

BCG and cancer

BCG was first linked to cancer on the basis of a paper from 1929, suggesting an antagonism between tuberculosis and cancer. The study, conducted at Johns Hopkins, showed that there were significantly lower numbers of malignancies among patients with tubercular lesions [8,9]. A critical note to this paper was later published and stated the possibility that patients with TB died before developing cancer and thus causing the observed effect. However, additional studies in patients with stomach cancer and research in animal models showed an effect on cancer development when treated with BCG. Furthermore, mice vaccinated with BCG showed to be more resistant to transplanted tumors than non-vaccinated mice[10] and tumor inhibition was observed when infected with live BCG at the site of tumor inoculation [11]. Bladder specific studies later on showed that BCG caused a delayed hypersensitivity reaction and resulted in cellular infiltrates [9,12-14] Recently Biot at al showed that mice but also bladder cancer patients with preexisting immunity to BCG showed improved survival after BCG treatment, compared to patients without preexisting BCG immunity[12]. Current treatment regimens are based on the first clinical trial from 1976 by Morales which resulted in a 12-fold reduction in recurrence of the tumor[2]. During treatment, BCG is instilled in the bladder with 120mg BCG diluted in 50mL saline and retained for two hours. Treatment follows a weekly regimen, for the course of 6 weeks. It has been shown that intravesical BCG is the most efficacious agent for NMIBC since it delayed tumor progression, but also reduces the need for surgical treatment. In addition the five-year survival rate following BCG (70-86%) is similar to what is achieved after immediate cystectomy[15].

BCG treatment is thus effective, however it is associated with a number of side effects, more than occur with intravesical chemotherapy. Side effects such as fever, hematuria and prostatitis, are seen in less than 5% of patients, for which treatment is available. Unfortunately major complications can develop after systemic absorption of the BCG, can occur in immunocompromised patients (immunosuppression and HIV infection) or after errors in BCG administration resulting in physical disruptions in the urothelial blood barrier[1,16].

In addition to bladder cancer, the therapeutic effect of BCG instillation has been studied in melanomas, colon cancer and other malignancies of the urinary and reproductive system (**table 1**). In melanomas, studies done by Morton et al showed that when BCG was injected into the lesions, this caused a reduction of 90% of the lesion. Also, the surrounding lesions decreased in size as well in 20% of the cases. However, consecutive clinical trials were unable to find significant differences between the use of BCG and chemotherapy.[17] In addition, the toxicity of BCG injection was found to be more severe than alternative treatments, which resulted in abandoning of BCG treatment for melanomas[18]. For the treatment of colon cancer, BCG is used as an adjuvant combined with tumor antigens specific for each patient. A review by Deschoolmeester et al showed that the therapy worked best in patients with less severe disease, although there were effects on overall survival and disease free survival. In such cases the vaccine showed a 61% risk reduction for recurrences with a trend towards increased overall survival[19]. Despite the fact that these tumor cell vaccines show to have an effect, no further studies have been attempted due to the fact these personalized vaccines are difficult to optimize and development is time consuming an expensive.[20]

In prostate cancer the injection of BCG turned out to be too dangerous due to the risk of fatal sepsis. But animal models have indeed shown effects in tumor reduction[21]. Regarding renal cancer the results were quite positive, one trial showed an increase of 5-year survival with intradermal BCG treatment and a long-term survival in 10%. And

another study by Mavrichev et al showed increased 8-year survival upon BCG immunotherapy, after nephrectomy[22]. Interestingly, despite the positive results of several clinical trials on BCG immunotherapy, many avenues have been abandoned and focus has been mostly on the therapeutic effects of BCG in bladder cancer.

Table 1. Effect of BCG immunotherapy in malignancies

Type of cancer	Efficacy
Colon cancer	Significant reduction in relapse rate **(61%)**[19]
Melanoma	Regression ranging from **2%-92%**[17, 23]
Prostate	**3-8 month** increased survival in patients with advanced prostate cancer [24]
Renal Cancer	Increase long term survival of 5-8 years **(10-35%)**[22, 25]

Immune response to non-muscle-invasive bladder cancer

The immune response against bladder cancer using BCG instillation is superior to chemotherapy. However, increasing the anti-tumor effect of the therapy remains necessary since treatment consisting of tumor resection with frequent BCG instillation is not fully effective. As was stated previously, several new BCG vaccines are being tested in clinical trials to improve the immune response to TB. However, in order to improve the anti-tumor effect of *M. bovis* BCG it is important to know which factors contribute to the clearing of malignant cells.

The most important cell in the response to tumors is the cytolytic T lymphocyte (CTL). Animal models have shown their role in tumor reduction through the recognition of peptide fragments on MHC-I. These molecules generally present 'self' antigens and in the case of tumor cells, these can be either tumor specific or tumor associated. They are important in the recognition of the malignant cells by the immune system. The specific antigens are not found on normal cells and are formed by the generation of altered proteins in the cell. Tumor associated antigens are regular proteins without alterations caused by mutation, but these are generally not present on normal cells. For instance, these can be proteins that are expressed during fetal development, but no longer in subsequent stages. In addition tumor associated proteins can be proteins that normally have a low expression at the cell surface but are upregulated in malignant cells. MHC class I molecules are often downregulated in on malignant cells, decreasing the availability of tumor antigen presentation and thus evading the immune system.

In NMIBC, the immune response to the tumor cells is not sufficient. Interestingly, a healthy immune system has even been associated with increased risk of bladder cancer development. It has been shown that HIV infected and transplanted patients have a decreased risk of developing bladder cancer. Which points toward a role of the adaptive immune system in cancer development, but a clear mechanism resulting in this effect is unknown[26]. What is known about the immunological response to NMIBC is that the myeloid dendritic cells have are unable to effectively activate T-cells. Dendritic cells (DCs) are antigen-presenting cells, which can activate both the innate and adaptive immune system through natural killer (NK) cell activation and CD4+ and CD8+ T cell activation respectively. After T-cell activation, the subsequent cytokine profile showed a decrease in serum levels of Il-2 and IFN-γ. These are predominantly part of a Th1 cytokine profile, which results in activation of CTLs, NK cells, macrophages and monocytes, all of which play a role in the defense against tumors. In contrast, the Th2 cytokine profile with Il-4, Il-6 and Il-10 shows an increase in serum levels. From this it can be concluded that the tumor cells shift the immune response towards a Th2 profile,

thereby actively evading the immune system. Furthermore, the role of both tumor associated macrophages and tumor-infiltrating dendritic cells shows to be of importance on the basis of a study by Ayari et al., 2013. They found that absence of these cells from malignant tissue was associated with increased tumor recurrence. However, increased presence of these dendritic cells showed a higher risk in progression to muscle-invasive cancer.[27]. In addition to dendritic cells and macrophages, tumor-infiltrating lymphocytes are a highly significant indicator of a favorable prognosis, but in line with the infiltration by DCs, a dense presence of lymphocytes is associated with a more severe stage of bladder cancer[28]. Th17 cells, a subset distinct from Th1 and Th2 in their cytokine production and function, were present in increased numbers in bladder cancer tissue when compared with blood levels of patients and healthy controls. What their role is in the immune response against malignancies is still not fully understood. They have been shown to promote both positive and negative effects within the tumor immunology. In several different types of cancer they stimulate a pro-inflammatory response, which would also increase local inflammation and anti-tumor effect. In contrast to this they increase angiogenesis, which stimulates the growth of tumors. Besides the Th17 cells, there is another cell type which is increased in the blood and the tumor. These are T regulatory cells or Tregs and play a role in tolerance. Unfortunately they also inhibit the secretion of IL-2 and the proliferation of CD4+ T cells, which diminishes the local anti-tumor immune response[29].

Besides the Th1 and Th2 cytokines described previously, there is also a role for Il-18 in bladder cancer. Il-18 is a cytokine important in increasing a Th1 response by stimulating IFN-γ production and decreasing Il-4. Levels of Il-18 found in serum of patients were significantly increased over that of healthy controls[30]. Although there is still a lot left to learn about the immune response to bladder cancer, even these few studies show that there are two effects playing a role in the response. On the one hand there is the infiltration of immune cells in an effort to clear the malignant cells with subsequent cytokine production, at the same time inhibitory cells and cytokines are present shifting the balance to a situation more beneficial for tumor growth. The latter is probably favored and stimulated by the malignant cells themselves, creating the most optimal environment. An overview of these responses is given in table 2.

Table 2. Immune response to NMIBC[27-31]

Type	**Characteristics/function in bladder cancer**
Cellular response	
Dendritic cells	Reduced ability to activate T-cells, decreased expression of surface molecules (CD80, HLA-DR).
Tumor associated macrophages	Reduced presence in malignant tissue is associated with higher recurrence rates.
Tumor infiltrating lymphocytes	Infiltration is associated with improved prognosis, dense infiltration is often seen in higher-grade carcinomas.
Th17	Increased in tumor tissue, function in bladder cancer still unclear.
Tregs	Increased in blood and at malignancy, suppresses anti-tumor immunity.
Cytokine production	
IL-18	Associated with increased susceptibility to bladder cancer.
Il-2, IFN-y (*Th1 cytokines*)	Reduced in serum of NMIBC patients, Il-2 and IFN-y mediate anti-tumor activity.
Il-4, Il-6, Il-10 (*Th2 cytokines*)	Increased in serum of NMIBC patients, Il-6 was associated with increased recurrence rates. Il-4 can inhibit Il-2 and inhibit CTLs.

Immune response to BCG instillation in the bladder.

BCG attachment and internalization

BCG installation in the bladder results in interaction with the bladder wall. The lumen of the bladder is coated with glycosaminoglycans, which are negatively charged and therefore inhibits binding of BCG to the surface. However changes in the cell wall, such as damage after transurethral resection of the tumor (TUR), which is standard practice in treating NMIBC, increases the chance of non-specific binding[32]. Specific binding of BCG to the urothelium requires the participation of the α5 and ß1 integrins [33].

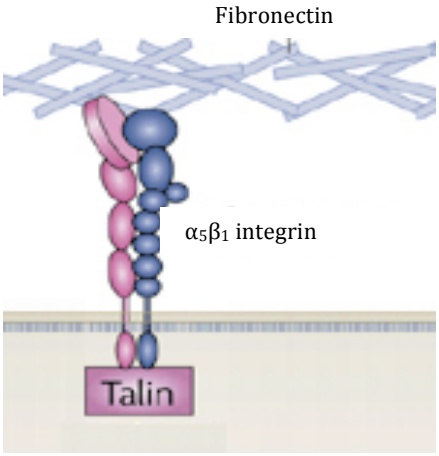

Figure 1; adapted from Morgan et al. Nat. Rev. (2007)

α5ß1 integrins are transmembrane receptors of the urothelial cells that attach to the collagen part of fibronectin (FN) and are involved in internalization of BCG (**figure 1**). Fibronectin is a glycoprotein of the extracellular matrix. Interestingly BCG contains a fibronectin attachment protein (FAP), which is needed for efficient attachment to epithelial cells by binding FN and is also highly conserved among other mycobacterium species[34-36]. To proof the importance of FN binding, several studies show that adding anti-fibronectin antibodies abolishes this attachment and subsequently the anti-tumor response by BCG[37].

In addition to the above-mentioned system, a recent study by Redelman-Sidi et al investigated the ability of BCG infecting different cell lines. They showed that bladder cancer cell lines reacted differently to BCG infection with infection rates ranging from 2-25%. Furthermore, the authors believe that macro-pinocytosis is the entry mechanism for BCG. It is a form of internalization almost all cell types are capable of, and is less specific and not particle dependent. In the case of BCG internalization it is associated with the activation of oncogenic pathways, which could explain why these cells are susceptible to BCG treatment[38].

Antigen presentation and immune response

It has been shown that, a few hours after infection, BCG is able to penetrate several cell layers into the tissue. At that point, innate immune cells like neutrophils followed by monocytes/macrophages are first at the site of infection. These cells produce cytokines, which attract other lymphocyte subsets. In addition, at these sites APCs internalize, process and present antigens from BCG via the endocytic pathway. This results in presentation of antigens on MHC-II molecules at the cell surface. Antigen presentation results in subsequent T cell proliferation via the recognition by the T cell receptor. In addition to this signal, several co-stimulatory signals are needed which are expressed on the cell surface of both antigen presenting and T cells. APCs express B7 and ICAM-1, which bind to LFA-1 and CD28 on the surface of CD4+ T cells. When these T cells are activated, it will result in the formation of T-helper 1 and T-helper 2 subsets. Th1-cells produce cytokines, which result in a pro-inflammatory response, with interferon gamma and IL-2 being the main Th1 cytokines. The Th2-type cytokines such as IL-4, IL-5, IL-6, IL-10 and IL-13 exhibit more anti-inflammatory responses and in addition stimulate B-cell responses and inhibit Th1 responses (**figure 2**). Regulation of the balance between

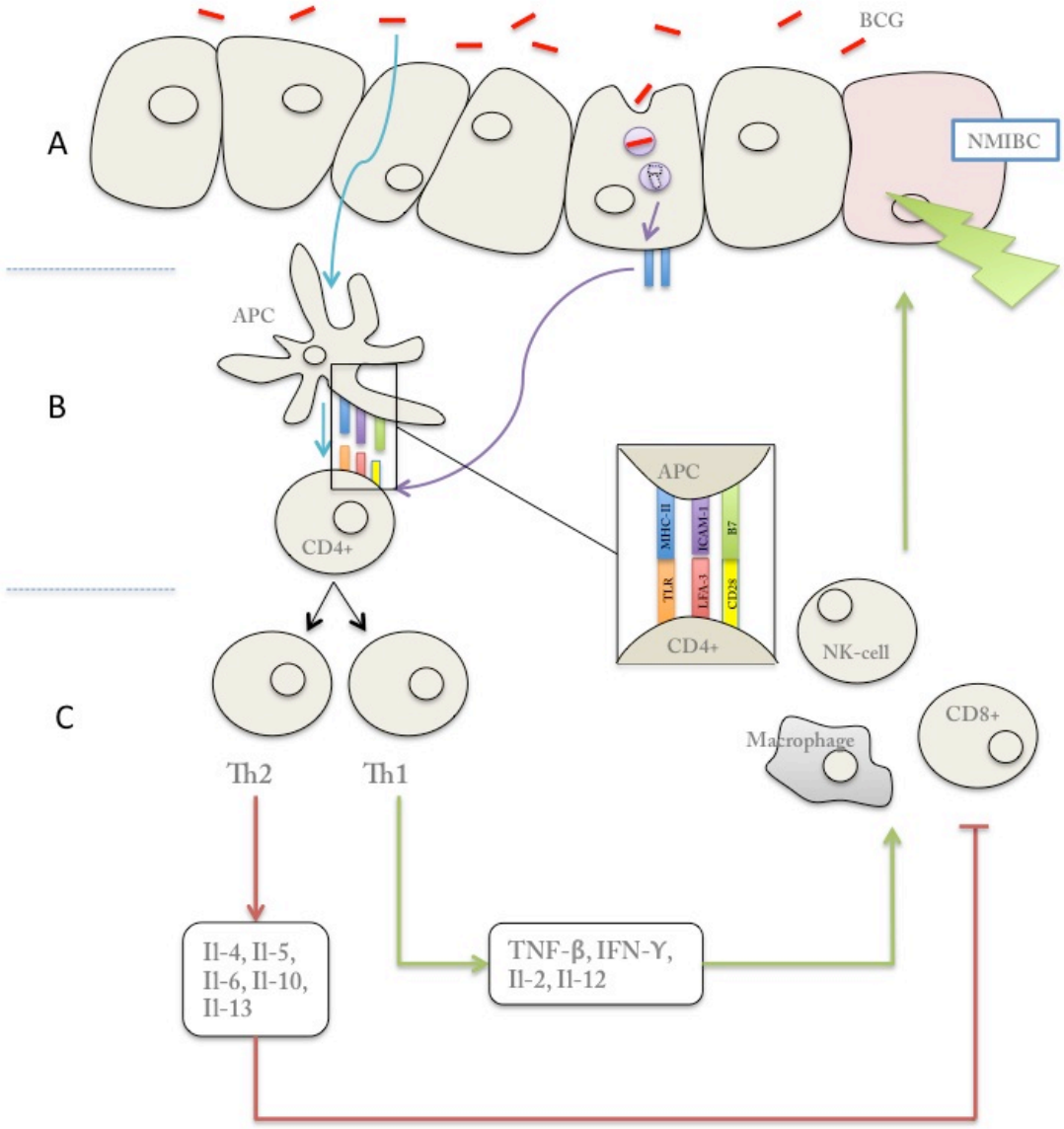

Figure 2; ***Immune response to BCG instillation in the bladder.*** Two routes of activation; via antigen presenting cells (APCs) (*Blue*) and urothelial cells (*Purple*). APCs or urothelial cells take up BCG and antigens are presented on MHC-II (*A*). Both result, in addition to co-stimulatory molecules (*B*), in a Th1 and Th2 response (*C*). Th1 activation results in the recruitment of macrophages, CD8+ T cells and NK cells, which can clear the malignant urothelial cells. (*Green*). These processes are driven by a distinct set of cytokines depicted in the boxes. The additional Th2 response inhibits an effective Th1 response by secreting several cytokines (*Red).*

Th1 and Th2 responses is considered to be important since an increased Th1 production is essential to form an effective antitumor response. Th1 cytokines can stimulate CTLs, NK cells, macrophages and monocytes, which all have the potential of reducing the number of tumor cells. Of these cell types, CTLs and NK cells seem to be the most important cells in decreasing the number of tumor cells during BCG immunotherapy[39]. In contrast to Th1, Th2 cytokines have the ability to diminish the therapeutic effect of BCG.

Although BCG instillation predominantly results in a CD4+ proliferation, this is not the most optimal response to target cancer cells. An additional increase of CD8+ T cells would be preferential since these cells show to be essential in tumor reduction. Also, the presence of preexisting BCG-specific T-cells showed to increase the T-cell response upon BCG instillation in the bladder and could possibly reduce the need for repeated instillations[12].

Effects of BCG uptake on urothelial cancer cells

Urothelial tumor cells are also able to process and present BCG antigens on their cell surface. A study by Chen et al showed that internalization BCG results in cell cycle arrest bladder cancer cell lines as a result of integrin cross-linking. The study also investigated the role of BCG on apoptosis, programmed cell death resulting in organized demise and clearing of cell debris. This process is impaired in many malignancies and BCG infection of the urothelial cancer cells, did not result in the induction of cell death[40] (Chen et al 2007). Other studies do however claim a cytotoxic effect of BCG to the malignant cells, which could be explained by the fact that these studies were in vitro cell lines, different types of tumor cells, and different stages of differentiation of the malignancy[41]. In addition, bladder cancer cell lines incubated with BCG, increase their expression of MHC-II, CD1, CD8 and ICAM-1 on the cell surface, thereby increasing their antigen presenting capability[42]. These functions of the bladder cancer cells show they play an important role in the local immune responses during BCG instillation.

In response to BCG instillation in the bladder, tumor cells additionally secrete several cytokines, which interestingly favor both a Th1 and Th2 response (**table 3**). From this, it could be concluded that besides the tendency to favor a pro-tumor microenvironment, BCG instillation in the bladder could possibly shift this balance towards a Th1 response and forcing the malignant cells to act as antigen presenting cells. The following table gives a brief overview of several factors involved in the response to BCG instillation in the bladder.

Table 3. Overview of immune response to BCG instillation

Type	Mechanism of action
Cellular response	
APC	Presentation of BCG antigens to CD4+, production Il-12 stimulating Th1 response.
CD4+ T helper 1 Cells	Stimulates cell-mediated inflammatory reactions. Predominant response needed for clearing tumor cells.
CD4+ T helper 2 Cells	Decreased upon BCG instillation in bladder, increased in bladder cancer.
NMIBC cells	Present antigens on MHC-I/II and CD1 to CD4+ T cells and CTLs.
Macrophages	Cytotoxic function with regard to tumor cells; however also produces Il-10, which diminishes cytotoxic effect.
CD8+ Cytotoxic T cells (CTL)	Recognition of mycobacterial lipids on CD1 molecules at surface. Resulting in target cell death.
NK cells	Recognize 'self-antigens' on MHC-I. BCG results in glycoprotein and lipoprotein presentation on MHC-I, recognition results in target cell death.
Cytokine production	
Th1 cytokines	
Il-2	Induces growth, differentiation and survival of CD4+ T cells and CD8+ T cells, most potent predictive urine marker of BCG response
Il-12	Reduces inhibitory effect of Il-4 on IFN- γ, increases cytotoxicity of NK cells and CTLs.
IFN- γ	Key cytokine resulting in tumor cell phenotypic changes and antigen presentation by cancer cell. Increases ICAM-I expression. Promotes shift to Th1.
TNF- β	Induction of adhesion molecule (ICAM-1) expression. Inhibited by Il-10.
Th2 cytokines	
Il-4	Terminate cellular immune response, absent in urine during BCG therapy.
Il-6	Initiates maturation of Th2. High concentrations block generation of

	Th1 cells
Il-10	Inhibits Th1 cytokine secretion by Th1 cells
Tumor cell cytokines	
Il-6	Determines balance Th1/Th2, promotes Th2 response, inhibition Th1. Found increased in urine.
Il-8	Pro-inflammatory cytokine; attracts T cells and neutrophils. Increased in urine.
Il-10	Inhibits TH1 cytokine secretion by Th1 cells
GM-CSF	Possibly produced by macrophages instead of tumor cells. Augments Th1 response.
TNF-α	Possibly produced by macrophages instead of tumor cells
IFN- α	Induce tumor necrosis factor-related apoptosis-inducing ligand (TRAIL). Direct anti-proliferative activity, reduces angiogenesis, increased cytokine production and tumor associated antigen expression.

Improving efficacy of immunotherapy using BCG in bladder cancer.

Recombinant BCG vaccines

The current *M. bovis* BCG vaccine is capable of producing a sufficient immune response upon instillation in the bladder, resulting in increased tumor clearance. The response results in the production of a predominant Th1 milieu, mainly activated via MHC-II presentation. The recombinant BCG vaccines developed for the use in TB strife to induce an additional response of CD8+ cells and B cells via several ways. The only recombinant vaccine still in production aims to do this by additional mechanisms to evade the lysosomal compartment and increasing the antigen presentation on MHC-I molecules. The currently used BCG is phagocytosed and digested in the phagolysosome resulting in mostly MHC-II presentation. Possible additional MHC-I presentation activates a whole different cascade of effector cells. MHC-I is identified by cytotoxic T lymphocytes and presents 'self-antigens'. Recognition of non-self peptides on MHC-I will therefore result in an additional pathway of tumor cell death.

Recombinant BCG Secreting Lysteriolysin VPM1002 (rBCG ΔureC hly⁺)

Of all recombinant BCG vaccines produced in the last decade, development of VPM1002 is still ongoing in clinical trials and is currently being tested for safety in neonates[43]. This vaccine was developed on the basis that *M. tuberculosis* is able to induce apoptosis in infected cells, resulting in generation of apoptotic vesicles that can be taken up by APCs and also include mycobacterial antigens. This phenomenon, called cross-priming, increases MHC-I presentation and in turn CD8 T cell activation[44]. The current BCG strain stimulates this pathway suboptimal; researchers have therefore developed a recombinant strain that secretes lysteriolysin (Hly), taken from *Listeria monocytogens.* This pathogen uses this pore forming protein to evade phagosomes of the cells it has infected. However it works best at a pH of 5.8, whereas BCG remains in the phagosomes at a pH 6.5[45]. This could possibly affect the efficacy of the Hly, therefore the BCG strain was adapted by making it urease C-deficient (UreC-deficient). UreC plays a vital role in the keeping the pH of the phagosome neutral. A combination of the UreC deficiency and Hly addition in BCG resulted in a strain able to manipulate the phagosome and translocate its antigens into the cytoplasm[46]. Since formation of this rBCG strain, VPM1002 has been tested in several studies[43,47,48]. The first studies in mice showed a significant and fast increase in cytokine production in the rBCG vaccinated group. IFN-γ,

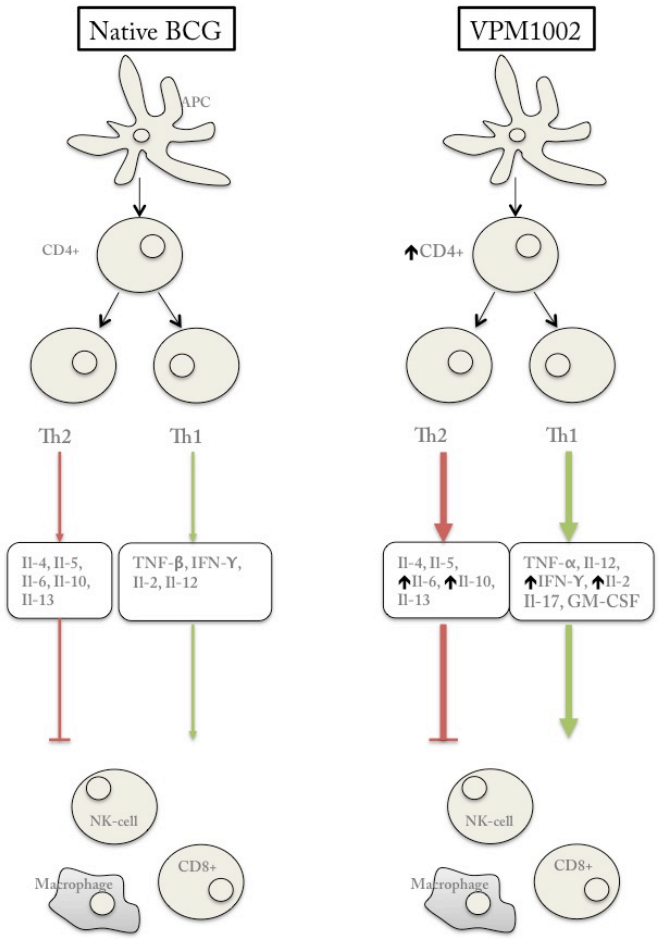

Figure 3 shows the comparison between native BCG and VPM1002 regarding the cytokine production and cellular response.

Il-2, Il-6, Il-17 and GM-CSF were significantly higher in rBCG, pointing towards an induction of a Th1 and Th17 milieu. These cytokines were increased in lung tissue upon aerosol infection with *M. tuberculosis.* Furthermore the tuberculosis bacterial load in the lung was significantly lower in the rBCG group, 90 days after infection. Interestingly, there is a vast difference in the immune response between lung and spleen tissue, when looking at the cytokine production.[47] In addition to increased cytokine production in certain tissues, cellular responses also differ between the parental BCG (pBCG) and rBCG. rBCG induced a significant increase in TNF-alpha and IFN-gamma upon CD4+ T-cell activation. Activation of CD8 T cells showed a significant increase of TNF-alpha[48].

These mice studies showed a promising efficacy increase of the newly developed rBCG vaccine. One of the latest publications by Kaufmann et al from 2012 describes the first clinical trial proving the safety of the vaccine in human volunteers. In addition to the safety, immunogenicity was determined on the basis of cytokine T-cell responses[43]. IFN-γ immediately after encountering a pathogen is crucial in activating phagocytes and decreasing mycobacterium growth. When looking at the IFN- γ response in naïve patients, who have not encountered BCG before, the production is not comparable with parental BCG. IFN-γ production increases over native BCG only after 180 days with stimulation by PPD (purified protein derivative), at earlier time points (day 29 and 57) these are lower. Stimulation with mycobacterium protein Ag85B results in similar IFN-gamma responses at all time points. Taking into account that dosage of rBCG and pBCG is similar. In contrast, BCG-immune patients show a quick decline in IFN- γ production when vaccinated with rBCG, whereas subjects administered with pBCG levels show constant levels higher than rBCG. This could point towards decreased efficacy of the new vaccine in pre-immunized patients[43]. In addition there is also no significant difference of CD8+ T-cell responses between pBCG and rBCG, which was the predominant goal for the new vaccine. The figure above (Figure 3) shows a comparison between the native (paternal) BCG and the new recombinant vaccine. In addition, the table below shows an overview of the immune response to VPM1002 **(Table 4).**

Table 4. Immune response to VPM1002		
Cellular response	**Mice**	**Human[43]**
CD4+ T cells	Elevated cytokine production IFN-γ and TNF-aplha.[48]	Similar or slightly increased numbers compared to pBCG (depends on stimulation)
CD8+ T cells	Increased production TNF-alpha.[48]	Shows trend of increased activation. Not significant
Cytokine production		
Il-2, Il-17, GM-CSF, TNF-alpha, IFN-y (*Th1 cytokines*)	Increased in lung tissue upon TB infection compared to pBCG-vaccinated mice. [47]	Variable IFN-γ production compared to pBCG.
Il-10, Il-23, Il-6 (*Th2 cytokines)*	Increased upon stimulation of DCs by apoptotic vesicles. [48]	--
Apoptosis	Increased apoptosis[46]	--

BCG with RD1 region (BCG::RD1)

Another strategy to enable phagosomal evasion by BCG is the addition of a region of difference-1 (RD-1) locus to a BCG strain. This locus is present in all pathogenic Mycobacteria and encodes for virulence factors ESAT6 and CFP10. These are essential for translocation to the cytosol.[49] The immune response to this recombinant strain of BCG showed protective efficacy in guinea pigs and mice however it increased BCG persistence and pathogenicity in SCID mice. Therefore rBCG was found to be too dangerous to use and was abandoned[50]. A possibility to still use this strain is attenuation by dependence on an additive in an approach similar as what is done by Jacobs et al on M. tuberculosis strains[51]. In the case of rBCG, this should inhibit proliferation in its absence but still ensure an increased immune response due to the inserted RD1 region. This strategy could prove effective since this strain did show the most promising stimulation of the immune system[7,52].

Expressing perfingolysin O (BCG ΔureC pfoA)

Recombinant BCG with Perfringolysin O (pfo), a pore-forming toxin, normally secreted by *Clostridium perfingens* was showed to be safe in SCID mice. This is in contrast to the previous vaccine, BCG::RD1. It also showed a significant increase in IFN-γ production upon aerosol challenge with virulent *M. tuberculosis* in mice and guinea pigs. Unfortunately, regarding efficacy of the vaccine, the results were similar between parental BCG when looking at mycobacterial loads in lungs and spleen[53]. Furthermore, it was not deemed as safe as VPM1002, which showed in phase I clinical trials, where it was terminated. [7,54]

rBCG specifically developed for bladder cancer immunotherapy

Besides the above mentioned rBCG vaccines in development for TB and leprosy, research has been done to specifically manipulate *M. bovis* in such a way that it increases its anti tumor effect. Recombinant BCG vaccines are made secreting Il-2, IFN-γ, pertussis toxin and IFN-α. rBCG-IFN-γ was tested in murine bladder cancer cells. Infection resulted in upregulation of MHC-I, increased CD4 T-cell presence, IL-2 and IL-4 expression.[55] Il-2 secreting BCG increased cytotoxicity of the murine cancer cell line and increased expression of Il-12, TNF- α and IFN-γ over parental BCG[56]. The strain producing a detoxified pertussis toxin (rBCG-S1PT) was tested on cancer cells implanted in BALB/c mice. Intravesical immunotherapy resulted in tumor reduction compared to parental BCG. Furthermore TNF- α and IL-10 mRNA levels were shown to be increased in bladder tissue[57]. Most interesting is BCG expressing IFN-α 2B. It has been shown in

clinical trials that addition of recombinant IFN- α to intravesical BCG treatment has been successful in patients who do not respond to BCG alone[6]. Recombinant BCG expressing IFN-alpha increased cytotoxicity by 2 fold compared to parental BCG and also increased IFN-γ and IL-2 production in human cancer cell lines[58] These additional recombinant BCG vaccines show promising effects in vitro, pointing towards their potential usefulness in the treatment of bladder cancer. Additional in vivo studies and clinical trials are needed to determine efficacy. Further details on rBCG vaccines aimed at improving bladder cancer treatment can be found in a comprehensive review by Luo et al from 2011[6].

Discussion

This review describes several key features of the immune response to BCG in the treatment of bladder cancer. Combining the immune response of three components described in this review results in **table 5**. It describes the response to NMIBC, traditional BCG and VPM1002 and gives an overview of the overlap of the responses. However, most importantly, it shows the mismatch between the groups. What becomes clear from the table is the limited information on the effect of the new recombinant vaccine. Since its development there have been studies proving efficacy in animal models and safety in human volunteers. Thus far, the detailed immune response has not been completely investigated, specifically not in the clinical trials. When looking at the second and third column of the table, it becomes clear that instillation of BCG in the bladder, shifts the immune response to a Th1 milieu. This is more favorable to clear BCG, but also more effective in clearing malignant cells. Comparing traditional BCG to VPM1002 (column 3 to 4) shows that rBCG increases cytokine production and cellular responses. These increases are both needed to improve the effect of BCG vaccination for clearing bladder cancers. Increased production of Th1 cytokines has been show to decrease bacterial load and increase mycobacterial clearing[6]. From the table it shows that the new vaccine results in increased cytokine production over the native BCG in all tested cytokines, however, this is also the case for the Th2 cytokines. This last group of cytokines is not beneficial in the immune response and diminishes the Th1 response. From the table it also shows that this Th2 response is already present in bladder cancer and does not decrease upon BCG instillation. Further research is needed to investigate the differences between these responses in clinical trials. The cellular response, after VPM1002 vaccination, also shows a possible increase in CD8+ T cells and a significant increase in CD4+ T cells, is also of importance in effective tumor clearance. However, the clinical trials of the rBCG show a non-significant increase in CD8+ T cell responses whereas he animal studies show a significant and strong increase. Furthermore, the IFN-γ response is also highly variable in the human rBCG trial, where again, the animal studies show a strong and clear increase over native BCG.

This is exactly where a problem arises in the development of these vaccines. Often the animal models in which these vaccines are tested are not optimal to predict the immune response in humans. In addition, infecting the study population with TB cannot be used to test vaccines in humans and therefore limit the predictions that can be made with these studies.

In addition to the difficulty extrapolating data from animal models, these models also bring a feature to light that might be important in BCG instillation in the bladder. The paper by Desel et al 2011 shows a varying immune response in different tissues of VPM1002. In this study the authors show a significant difference in cytokine production in the spleen and lungs of infected mice, where the lungs show a significant increase compared to native BCG vaccination. Spleen tissue only shows this effect in one of the six

tested cytokines.[47] Clinical trials should be done to investigate whether this holds true for the response in different tissues in humans as well. For the use in bladder cancer, it is essential to know whether the response to intravesical BCG treatment shows a response as is seen in lung tissue since it could greatly influence the anti-tumor response. The advantage of the use of a recombinant vaccine in bladder cancer is that it is used at the site of the malignancy and therefore results in an immune response at the site of infection. Although a critical note should be made, since even different bladder cancer cell lines show variation in the ability to take up BCG[38]. This could explain the variation in clinical efficacy in the trials and might not be changed by a new vaccine such as VPM1002, which is not made to improve BCG uptake. Therefore uptake of rBCG should be incorporated in these studies.

Different strains of the traditional BCG vaccine show no difference in the efficacy on bladder cancer. The question arises whether this could also predict VPM1002 having no increased effect in the treatment of bladder cancer.[59] To get an answer to the question on the efficacy of VPM1002, clinical trails must be performed.
When looking at the recombinant BCG vaccines in development, VPM1002 is the only new vaccine candidate for use against TB and leprosy infections that shows promising results after preliminary trials. Compared to the original BCG vaccines, VPM1002 has several advantages. One of the major improvements it has over the old vaccines is its safety. Performed and ongoing clinical trials have showed the vaccine can be administered with fewer side effects[43]. Increasing the safety of a BCG vaccine is important since the current vaccine is associated with a risk of disseminated infection. This is most common in patients on immunosuppressive treatment or those whom are infected with HIV. Unfortunately this last group is also at high risk of acquiring TB infections. Recombinant vaccines could prove to be safe in these groups as well. An example of this is seen in an attenuated *M. tuberculosis* strain, which was considered save in immunosuppressed simian immunodeficiency virus infected infant macaques[60]. A model with close similarity to human HIV infected patients.
Besides its increased safety, animal models show a higher effect in protection against TB. This could be explained by the MHC-I presentation and subsequent CD8 T cell stimulation. However in human studies this increase in CD8 stimulation was found not to be significantly different when compared to traditional BCG. It would however more closely resemble the actual mechanism of TB protection, since its antigens are able to escape the phagosomes and activate this immune response. This would not only be an effective strategy for TB. Also in the treatment of NMIBC, improving the CD8 T cell response might positively influence the immune response to malignancies; since MHC class I immunity by CD8+ CTLs is important in the reduction of tumor cells[43].
On the basis of the current available data on the immunogenicity, the vaccine is a promising and safe candidate to replace the traditional BCG. There is an increase in the production of Th1 cytokines, CD4+ T cells and a possible increase of CD8+ cells. This last group is of importance since these cells are known to be most effective in the reduction of tumor cells. The increase in immune response in humans might not be as strong as compared to the animal models, but there are improved responses. Increasing the study population, investigating a broader immune response and in the end, testing its effect in bladder cancer models are the future steps that need to be taken to clarify its possible use.

Table 5. Comparison of immune response to non-muscle-invasive bladder cancer, BCG and recombinant BCG

Response type	Immune response to NMIBC	Immune response to BCG	Immune response to VPM1002	Function
Cellular response				
Antigen presenting cells	↓	↑		Activate CD4+, ↑IL-12 ↑Th1 response[32]
Tumor infiltrating lymphocytes	↑			Consist of several types (CD8+, CD4+, NK cells)
CD8+ T cells		↑/-	↑(h)	Mediate tumor cell death.
CD4+ T cells		↑	↑↑(h)	
CD4+ T helper 1 Cells		↑		Production of pro inflammatory response (*see Th1 cytokines*)
CD4+ T helper 2 Cells		↑		Inhibits Th1 response, mediates humoral immunity.
Antigen presentation by NMIBC cells		↑		Present antigens. Activate CD4+ T cells and CTLs
Macrophages	↓	↑		Target cell death ↑IL-10[61]
Cytotoxic T cells (CTL)		↑		Target cell death [61]
NK cells		↑		Target cell death. [61]
Th17	↑			Function in bladder cancer unclear.
Tregs	↑			↓anti-tumor immune response
Cytokines				
Th1 cytokines				
Il-2	↓	↑	↑↑(m)	Stimulates CD4+ T cells, CD8+ T cells
Il-12		↑		↓Il-4 ↑NK and CD8+ cytotoxic T cells. ↓ angiogenesis
IFN- γ	↓	↑	↑↑(h)	↑Shift to Th1.
TNF- β/ LT-α		↑		↑ICAM-1 expression
TNF- α	↓		↑↑(m)	Proinflammatory, [62]
Il-17			↑↑(m)	↑ IFN-γ–producing cells to site of infection
Th2 cytokines				
Il-4	↑	↑	↑	↓cellular immune response [63]
Il-5		↑	↑	
Il-6	↑	↑	↑↑(m)	↑Th2 ↓generation of Th1 cells
Il-10	↑	↑	↑[47]/↑↑(m)[48]	↓ secretion cytokines Th1 cells, macrophages, and dendritic cells.
Il-18	↑			Associated with increased susceptibility to bladder cancer.
Tumor cell cytokines				
Il-6		↑		↑Th2 ↓ Th1[64]
Il-8		↑		Pro-inflammatory, attracts T cells and neutrophils. ↑in urine.
Il-10		↑		↓secretion Th1 cytokines, macrophages, and dendritic cells[65] ↓TNF-β
GM-CSF		↑		↑Th1 response. [66]
IFN- α		↑		↑TRAIL ↓ proliferation ↓angiogenesis ↑cytokine production ↑tumor associated antigen expression.

Shows a comparison of immune responses to bladder cancer, BCG and a recombinant BCG vaccine (VPM1002). The last column describes its function in the immune system. Arrows indicate an increase (↑) or decrease (↓) in cytokine production or cellular response. Double arrows (VPM1002) only indicate an *increased* production or presence of cytokine/cell type compared to BCG. It does not indicate actual doubling of values. (h) And (m) stand for data collected from human and mice studies respectively.

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