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Review Article

Non-specific immunity of BCG vaccine: A perspective of BCG immunotherapy

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ABSTRACT

BCG is a widely used vaccine worldwide for neonates including Pakistan. BCG has more than 90% coverage through the EPI program which was introduced in 1965 in Pakistan. BCG has limited efficacy against the transmissible form of pulmonary tuberculosis in high TB endemic countries. However, BCG vaccination continues in these countries because BCG confers protection against the disseminated form of TB in children. BCG has also shown some protection against leprosy and certain forms of cancers. One reason for such nonspecific protection may be that BCG activates APCs via PAMPS that interacts with TLRs (2, 4 & 8), which initiate the inflammatory cascade thereby recruiting inflammatory cells to the site of infection and providing maturation signals for neutrophils, macrophages and dendritic cells. Such activation may be crucial for restricting the infection at the initial site. Furthermore, activation of the pro-inflammatory cascade also results in expression of adhesion molecules, co-stimulatory molecules as well as MHC class II molecule. MHC class II molecules engage CD4+ cells via the TCR receptor while the adhesion and costimulatory molecules bind to their respective receptors on CD4+ T cells for additional high affinity binding for T cell activation. Although activation of the innate arm may not provide subsequent memory, activation of T cells may introduce a certain level of memory response and therefore, may form a rational basis for BCG immunotherapy. This review, therefore, focuses on the immune activation related to both the innate and adaptive arm of the immune response that has been reported and further explores the utility of BCG immunotherapy related to non TB conditions.

Contents

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1. History of BCG vaccine

BCG is an attenuated strain isolated from *Mycobacterium bovis*, and was first identified by a French scientist Albert Calmette Guerin. BCG was first used in 1921 as a vaccine in humans. It is a widely used vaccine to protect against tuberculosis and leprosy [1,2]. The serial passage of virulent strain of bovine tuberculosis on glycerine bile potato media reduces its virulence while retaining its antigenic properties. The efficacy of BCG against pulmonary tuberculosis is still controversial in several parts of the world with the highest burden of TB [2,3]. The duration of immunity is somewhere between 15 and 60 years post immunization in different trials [4,1]. The variable efficacy of vaccine in different trials has been attributed to variation in different BCG strains, genetic differences in different population [5], prior exposure to non tuberculous mycobacteria [6], and recurrent parasitic infection [7]. However, the reasons for variable protective immunity still remain unclear. To address these issues, a large scale trial was initiated in Chingleput area of South India in 1968 (15 year follow-up was completed in 1987) with the assistance of Indian Council of Medical Research. These results revealed no convincing protection against pulmonary tuberculosis. Irrespective of the finding of the Chingleput trial, BCG vaccination was incorporated in EPI program in 1974 and continued as part of routine vaccination at birth.

Efficacy of BCG in protection against adult tuberculosis is both debatable and controversial. A wide range of efficacy (0–80%) has been reported in case control [8] and cohort studies [2] as well as in clinical trials [9–11]. The reasons for these variable results are not clear; however there is consensus that these are true biological effects and not just due to sampling errors [3]. However, BCG does provide some extent of protection against TB meningitis and miliary disease. This is evidenced in Chingleput trial (initiated in 1968) where no case of TB meningitis or miliary TB was reported for over a 14 year period. The protective effect in randomized controlled trials was 86% (95% CI: 65–95) and in case-control studies was 75% (95% CI: 61–84). The reason of homogenous response is partly due to younger subjects who were less likely to be exposed to atypical mycobacteria. Exposure to atypical mycobacteria presumably obviates protective effect [12]. The role of BCG in infectious diseases was studied thoroughly in both TB and leprosy before observation of its non-specific effects in some cancers. Though BCG was recognized as a vaccine for tuberculosis, widespread BCG coverage has significantly declined the cases of leprosy. It was also evident that BCG vaccinated population in high TB endemic setting had significant impact in declination of leprosy cases [2,13,14]. The efficacy against leprosy ranges between 20 and 80% in controlled trials and observational studies [2,15]. BCG was equally effective against lepromatous as well as a tuberculoid form of leprosy. Given the efficacy of BCG against leprosy, countries like Brazil, Cuba and Venezuela recommended the use of BCG for leprosy contacts. A meta-analysis of 26 studies, that included 7 clinical trials and 19 observational studies (cohort and case control) showed an overall average protection of 26% (96% CI: 14–37%) against leprosy [16]. In observational studies, the protective effect was 61% (95% CI: 51–70%) with significant heterogeneity ($P < 0.00001$). The reason for heterogeneity between studies could be explained by different population, method of exposure and outcome assessment.

2. Rationale of BCG use as adjuvants

The first observation for the use of BCG in enhancement of immune response was evidenced by use of mycobacteria in preparation of Freund's adjuvant in the late 1950s [17]. Complete Freund's adjuvant (CFA) contains heat killed mycobacteria or BCG and trehalose 6,6' dimycolate (TDM) that activates some innate receptors including TLR2, 4 and 9. CFA stimulates a delayed type hypersensitivity reaction at the site which is skewed towards Th1 immunity. In contrast, incomplete Freund's adjuvant (IFA) which lacks mycobacterial component induces Th2 or antibody mediated immunity [18,19]. This is the first observation regarding use of BCG for activation of immune system. The findings of CFA induced arthritis and autoimmune reaction in experimental animals also provide us a rationale of BCG therapy in non-communicable diseases [20,21] . Historically, it was also shown that parasitic, viral and bacterial infection resulted in regression of tumors [22]. The hypothesis of concomitant or cross over immunity due to the presence of cross reactive antigen (parasite's egg) also provides an indirect evidence of protection against reinfection in intermediate host [23,24]. It is a well established fact that parasitic infection induced Th2 immunity [25] and therefore, a switch from Th1 to Th2 immunity is thought to be involved in tumor regression [26,27]. This host parasite relationship was first brought into concept of tumor immunology by Dr. William Coley in 1898. Coley administered his vaccine directly into tumor and observed tumor regression. Coley's toxin or vaccine comprised of a mixture of killed bacteria, (Serratia marcescens and S. pyogenes), which was effective against inoperable sarcomas. In case of Coley's vaccine, an infection precedes spontaneous regression of tumor, supports the idea of non-specific innate immunity in regression rather than adaptive immune response. This idea was further translated into BCG adjuvant therapy in melanoma and bladder cancers. The immune mechanism related to BCG is largely unknown. In this review, we try to shed some light on non-specific immunity conferred by BCG in melanoma and bladder cancers.

2.1 BCG immunotherapy in melanoma cancer

Cutaneous melanoma is the most common skin cancer in United States with annual adjusted incidence of 22/100,000 population in men and 14/100,000 population in women of all races [28]. The incidence is lower in black and Asian Pacific Islander (API) compared to whites with predominance of males affected by this disease [29]. Approximately 15% of primary melanomas develop into distant metastases; the five year survival rate for metastatic melanoma is still less than 5% despite of advancement in treatment and diagnosis. Melanoma is one of the cancers that show regression spontaneously or due to intervention with adjuvant immunotherapy presumably because of infiltration of immune cells at the tumor site [30,31]. The adjuvant immune therapy with BCG has also shown promising results for patients after surgical resection in advanced malignant melanoma [31].

Prior vaccination with BCG or vaccinia in childhood had significant effect in case reduction compared to the non-vaccinated group. A multicenter case control trail of Febrile infection and melanoma (FEBIM), evaluated risk of melanoma and vaccination status in child hood for either BCG or vaccinia or both in six European countries and Israel. This study also addressed the effect of severe infection and risk of melanoma compared to subjects either vaccinated with BCG or vaccinia. The odds ratio for individuals not vaccinated for any of the vaccine were compared to BCG [OR 0.23;(95% CI: 0.05–0.91)] and vaccinia [OR 0.33;(CI:0.10–1.06)] in persons below 50 years of age, signifies the effect of severe infection on reducing risk of melanoma [32]. Prior immunization with BCG or vaccinia vaccine was further evaluated on survival of melanoma patients during a five year follow up. A hazard ratio of 0.69 (95% CI: 0.49–0.98) with BCG compared to 0.52 (95% CI: 0.34–0.79) with vaccinia vaccine for development of melanoma suggests an immune surveillance mechanism of BCG for melanoma skin cancer [33]. Human endogenous retroviral genes (HERV–K) encoded envelop protein is expressed in many cases of melanoma [34] which cause malignant transformation by altering intracellular redox potential [35]. The immune surveillance mechanism of BCG, vaccinia or contact with other infectious agents was described by the presence of

analogs of HERV K MEL epitope by these infectious agents, that generate cross reactive T cell for elimination of malignant cells [35].

The intra-lesional therapy for melanoma cancer with BCG was initiated approximately three decades ago, when Morton et al. reported regression of melanoma using live BCG in 62% of patients. The result of this and other trials reported serious side effects like chills, fever and granuloma formation. Results of few successful trials are given in Table 1 [36–42]. Most of these trials showed a positive response when BCG was mixed with either the Melanoma cell vaccine (MCV) or autologous tumor cell vaccine. These strategies resulted in better disease free survival and overall survival. A newer strategy of ILBCG therapy involves topical treatment of 5% imiquimod (TLR7 agonist) following BCG injections. The topical imiquimod treatment up to 5 months resulted in complete regression in 56% of patients, and 67% remained disease free up to the last follow-up (range 12–58 months). This treatment modality was well tolerated in stage III patients, the major limitation was small sample size of trial [36].

2.2. BCG immunotherapy in bladder cancer

BCG immunotherapy was also described by Morales et al. in 1976. Intravesical BCG is now being used as standard therapy for non-muscle invasive bladder cancer (NMIBC), which resulted in 70–75% complete response rates in CIS (Carcinoma insitu) [43]. The combination of BCG and MMC in nine clinical trials, showed no significant difference between MMC and BCG in ORs of progression in a follow-up of 26 months [44], however maintenance dose with BCG resulted in significant superiority over MMC (OR = 0.66; 95% CI: 0.47–0.94; P = 0.02).

The phenomenon of pre-existing immunity to BCG for better response to therapy requires further investigation in clinical studies. The effect of prior BCG vaccination was also observed in bladder cancer for improved survival in vaccinated or PPD+ patients treated with intravesical BCG therapy [45]. This hypothesis of preexisting immunity to BCG was further tested in orthotropic tumor model implanted with MB49 tumor cells in bladder cancer; results of this study showed that mice receiving BCG subcutaneously before intravesical therapy showed 100% survival up to 70 days post tumor challenge compared to 80% survival up to 50 days with no prior BCG exposure [45]. However, report from Elsaber showed no apparent difference in frequency of IFNγ producing CD4+ T cells in responder vs. non responder to BCG therapy [46], but the presence of multifunctional IFNγ/IL2 positive T cells and higher IFNγ+ve cells in PPD+ve patients represents boosting effect of BCG as observed in TB patients [47]. Recently, concomitant use of vitamin D with BCG has been shown to improve invitro toxicity of BCG induced THP-1 cells on BCa cells [48]. It is well known that BCG antigen activates TLR on macrophages that activates vitamin D signaling pathway and induces expression of antimicrobial peptide Cathelicidin [49]. Cathelicidin also possess immuno-modulatory effect on immune cells and act as chemoattractant for neutrophils and a number of chemokine including IL8 [50]. The use of vitamin D with BCG has been shown to augment the cytotoxic effect of THP-1 cell on BCa cells [48]. The instillation of BCG+vitamin D prolonged the survival up to 70 days in mice compared to BCG alone. Though vitamin D possess anti-inflammatory properties [51,52], it is interesting to know whether or not vitamin D changes the phenotype of pro-inflammatory M1 macrophages into anti-inflammatory M2 phenotype during BCG therapy co administered with vitamin D. Further studies are required to unfold the interaction of BCG and vitamin D for cancer therapy.

The predominant cell types after BCG instillation in bladder cancer are neutrophils [53,54], NK cells [55] and T cells [45]. Within these populations, neutrophils are the most important cell types for BCG mediated local immune response, T cell trafficking and stimulation of chemokine (IL8 and MIP1α,GRO-α) [56].

3. BCG mediated immune modulation

The generalized mechanism of BCG mediated immune surveillance is the release of cytokines and chemokine in micro

Table 1
Clinical studies on adjuvant BCG therapy and their outcome.

Author	Year	Stage	No. of Patients	Treatment	Outcome	Response to therapy	Reference
Kidner et al.	2012	III		IL BCG + Imquimod	Surgical complete response = 33% Complete response = 66%	Yes	[36]
Hoshimoto et al.	2012	IV	Pre- treatment = 244	Whole cell Melanoma Vaccine, [Canvaxin+BCG] [BCG+placebo]	median follow up 21.9 months pretreatment DFS (HR 1.64, p = 0.002) OS;HR 1.53, p = 0.028)	Yes	[37]
			Duringtreatment = 214		DFS(HR1.91, p = 0.02) OS; (HR 2.57, p = 0.012)		
Morton et al.	2007	III	n = 1160	BCG + Melanoma cell vaccine(MCV)	based on 5 yrs survival	Yes	[38]
		IV	n = 496		42.3% of stage IV 63.4% of stage III		
Haanen et al.	2006	III	n = 25	autologus tumor cell vaccine and BCG	median survival 22.5 months with presence of TAA specific T cells vs 4.5 months for absence of TAA specific TILS inside melanoma tissue. (log rank, p = 0.0094)	Yes	[39]
Agarwal et al	2004	I III	n = 734	cohort 1 BCG vs observation Cohort 2 BCG vs addition of dacarbazine	5 year survival cohort 1 (DFS, p = 0.84) (OS, p = 0.40) cohort 2 (DFS, p = 0.74) (OS, p = 0.81)	No	[40]
Henz et al.	1996	I	no treatment = 22	treatment with two BCG vaccines	median follow up 6 years	Yes	[41]
			BCG RIV = 40 BCG pasteur = 44		DFS with BCG pasteur (p = 0.02)		
Czarnetzki et al.	1993	I	BCG RIV = 108 BCG Pasteur = 109 Follow up only = 110	Different BCG preparation vs no treatment	median follow up 6 years Patients survival (p = 0.82)	No	[42]

DFS: Disease free survival; OS:overall survival; CTC: circulating Tumor cell.

environment that further activates or recruits immune cells at site; these mediators are thought to act in non-specific manner. The predominant cytokines stimulated by BCG are IFNγ, IL2 and TNFα secreted by activated CD4+ T cells [57]. These IFNγ producing CD4+ T cells possess phenotype of CCR7+ CD62L representing the central memory T cell population in *invitro* experiments[57] and in neonates post vaccination at 6 and 27 weeks [58]. A majority of IFNγ producing T cells possess central memory phenotype. However, a smaller subset of T effector cells is also displayed by BCG. To counter balance the IFNγ secretions, IL10 is secreted by T regulatory cells (Tregs). BCG activated CD4+CD25+ Tregs act nonspecifically against inflammation caused by non TB related conditions such as atherosclerosis [59], type 1 diabetes [60] and asthma [61]. BCG vaccine promotes Tregs cells and cytokines such as TGFβ and IL10 [62]. New strategies to suppress Tregs or anti-inflammatory cytokines with BCG vaccine are considered as one of the possibilities to improve BCG vaccination at birth such as use of anti IL10 antibodies. BCG vaccination at birth was shown to be well correlated with Th1/Th17/ Tregs profiling. BCG specific Tregs attenuate BCG response at vaccination due to inhibition of IFNγ [63] and are therefore considered as one of the reasons of failure of BCG vaccine. Exposure to environmental mycobacteria before BCG administration in neonates resulted in increased IL10 or Tregs cells [63]. This indicates that BCG itself derived Tregs in addition with other T cell subsets (Fig. 1). This regulation of Tregs by BCG has implication on counter balancing non-specific inflammation in other non-communicable diseases such as cancers and diabetes.

3.1. Activation of innate arm

The initial immune response to BCG is dependent on complex series of events between immune cells and molecules. The foremost step is the activation of innate immune system via TLRs through PAMPS. The cell wall antigens of BCG mainly activate TLR2 and TLR4 and serves in maturation and activation of macrophages and DCs. The activation of TLRS via these PAMPs activates cascade of events including activation of adaptor molecule (MYD88) which results in recruitment of IRAK (IL1 receptor associated kinase) and TRAF6 (TNF receptor associated factor 6) and MAPK (Mitogen activated protein kinase), which subsequently activates nuclear transcription factor (NFkB), and associated genes for section of pro- inflammatory cytokines such as IL1β, IL12 and TNFα [64]. In case of intralesional injection of BCG, keratinocytes are activated via TLRs which results in production of pro-inflammatory chemokine, antimicrobial peptides, matrix metalloproteinases (MMPs) and up regulate adhesion molecules such as ICAM-1, VCAM-1, E selectins and P selectins, that provides a link between innate and adaptive immune response via NFkB activation pathway [65,66].

Several mycobacterial components including cell wall fractions (Tx114,mAGP) and DNA up-regulate TRAIL (TNFα ligand superfamily member 10, TNFSF10) and CD62L (L-selectin expressed on B,T and granulocytes, required for endothelial adhesion) from purified neutrophils [67]. Besides this, mycobacterial components such as Lipoprotein, LPS and CpG oligonucleotide are important Toll ligands and activate both extracellular and intracellular TLRs [68,69]. Most commonly activated TLRs by mycobacterial ligands are TLR2, TLR4 and TLR9. TLR2 is important for TNFα, IL1β and IL12 secretion from macrophages [70,71]. It also forms heterodimer with TLR6 and recognizes cell wall glycolipids like LAM, 38kd and 19kd lipoprotein. Intracellular TLR9 is activated by unmethylated CpG motif of bacterial DNA and results in TLR9 dependent dendritic cell activation [69,70]. The glycolipid antigens present in cell wall of mycobacteria activate CD1 restricted T cells which was previously reported in leprosy [72].

3.2. Activation of adaptive arm

Internalization of BCG by professional APCs results in activation of T helper cells. This interaction requires expression of MHC class II molecule recognized by CD4 T cells via TCR. Engagement of TCR

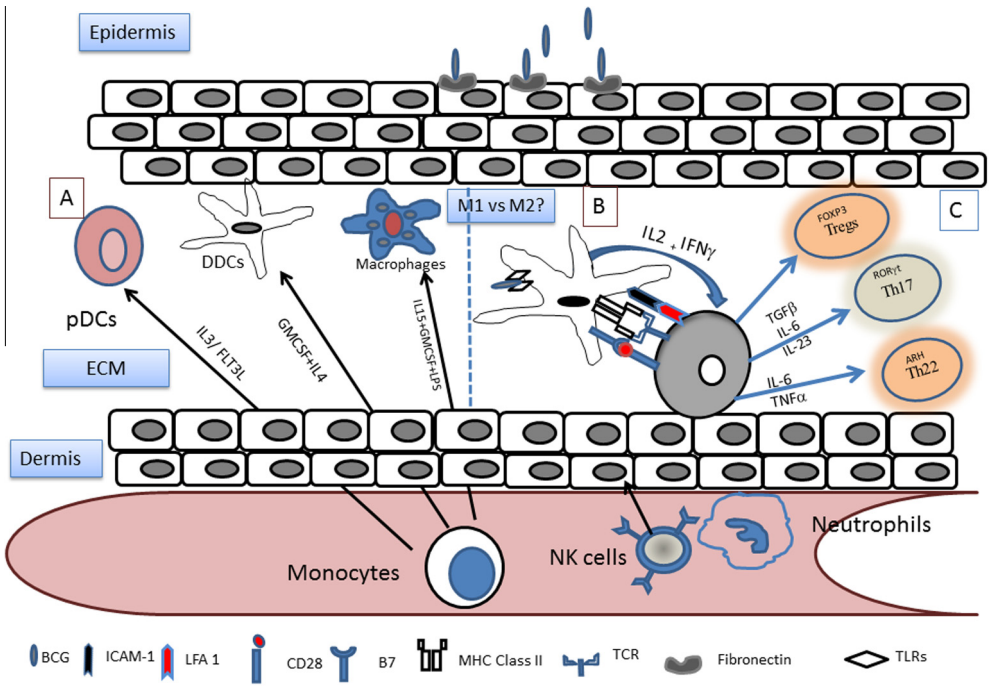

Fig. 1. The battle field between BCG and Immune cells: The mechanism of intralesional BCG therapy in melanoma cancer is shown here. BCG binds to fibronectin receptors for binding to skin tissue. After the uptake of BCG by receptors, monocytes, neutrophils and NK cells migrate from endothelial to dermal layer (A) for initiation of innate immune response. On the basis of cytokine present in the environment, monocytes get differentiated into dermal DCs (DDCs), plasmacytoid DCs (PDCs) or subpopulation of macrophages (M1or M2). In the second event, the BCG is internalized through TLRs on macrophages; BCG antigens are presented on T cell for adaptive immunity (B). T cell co-stimulatory, molecules such as CD28 and LFA 1 bind to B7 and ICAM-1 for activation of T cells. T cells are further differentiated into T cell subsets in response to cytokine milieu (C).

with MHC II further requires binding of co stimulatory molecules CD28 to B7-1 on T cells, and up regulation of adhesion molecules such as lymphocytes function associated antigens- 1 (LFA-1, major T cell integrin) that binds with intracellular adhesion molecule-1 (ICAM-1) on macrophages [73]. The T cell co stimulation is essential for T cell activation and anti-tumor responses [74,75]. The key cytokines released after T cell activation are IL2 and IFNγ [76] (Fig. 1). The local secretion of IFNγ is important for expression of ICAM-1. Expression of ICAM-1 by IFNγ also correlates with killing of tumor cell line [77]. Induction of IFNγ by BCG is also important for IL32 secretion. IL32 is a newly discovered pro-inflammatory cytokine that acts via caspase and IL18 dependent pathway, when APCs are stimulated specifically with BCG [78] Differentiation of T cells into Th17 and Th22 is also dependent on cytokine milieu. IL17 producing Th17 cells differentiate from naïve T cells in the presence of TGFβ, IL6 and IL23. On the other hand, IL22 producing Th22 cells require IL6 and TNFα [79]. Whether BCG changes local environment for differentiation of T cells or BCG components drive this differentiation is still not clear. For tumor cell killing, expression of FAS ligand and tumor necrosis factor related apoptosis–inducing ligand (TRAIL) on macrophages and neutrophils are also involved in apoptosis of tumor cells [53,54]. BCG also generates human peripheral blood mononuclear cells into special population of BCG activated killer cells (BAK) [80]. Phenotype of these cells are CD8+, CD56+ and lymphocyte activated killer cells (LAK) for killing target cells [80,81]. Th1 cytokines, such as IL2 and IFNγ are shown to activate BAK cells for cytotoxicity [82].

For BCG to impart its role as powerful vaccine or therapeutic agent, antigen presentation to APCs is the first step for an effective immune response. BCG inhibits inflammsome activation via Zn metalloproteases [83]. The zmp1 deletion mutant of BCG showed enhanced immunogenicity in terms of antigen presentation, DTH and increased IFNγ production [84]. Like pathogenic strain of M.tb, if BCG also interferes in phagosome maturation then it presumably affects the overall efficacy of BCG vaccine in subversion of host immune system by reducing antigen presentation.

4. Conclusion

BCG vaccine, despite its limited efficacy in tuberculosis is still considered as a useful vaccine for both vaccination and treatment purpose in melanoma and bladder cancer. The efficacy of BCG as vaccine or as an immunotherapeutic agent is highly variable in Pakistan. The available information from invitro and clinical studies are sparse, and therefore results are inconclusive for its favorable use. The result of BCG intravesical therapy in bladder cancer has equivocal findings due to high rate of BCG vaccination or exposure to non-tuberculous mycobacteria. One of the complications associated with intravesical therapy is Gran-P (Granulomatous Prostatitis) reported in a case report [85]. The other report from Pakistan (AFIU), showed an overall decrease in recurrence rate (65% to 12%) and tumor progression (26% to 6%) in 16 patients who received BCG therapy for 2 weeks after transurethral resection [86]. In line with these findings, the use of BCG therapy for breast cancer treatment has shown promising results in experimental rat's model in reversion of chemically induced malignancy [87].

New strategies in BCG immunotherapy may involve use of vitamin D analogs that might reduce the exacerbated inflammatory process and untoward side effect of BCG in vaccinated population in high TB endemic setting. Similarly the use of dual toll ligand (CpG ODN or imiquimod) [88] along BCG intralesional therapy are promising for treating melanoma cancer. The role of plasmacytoid DCs in BCG mediated immune surveillance should be further investigated using experimental models or clinical studies. Further studies for non TB use of BCG in type 1 diabetes, arthritis and other autoimmune conditions are needed to explore the benefits of vaccine in non TB related conditions.

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