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BRIEF COMMUNICATION

Protective effect of DNA vaccine during chemotherapy on reactivation and reinfection of Mycobacterium tuberculosis

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Active disease of tuberculosis (TB) can be developed decades later by either a relapse of the initial infection (endogenous reactivation) or by an entrance of the secondary infection (exogenous reinfection), since the current chemotherapy cannot lead to complete elimination of tuberculosis. Although the immunotherapeutic approaches in conjunction with conventional chemotherapy were tried to prevent TB growth via boosting the immune system, their therapeutic effects are still controversial. Here, we found that TB DNA vaccination completely blocked tuberculosis reactivation and significantly prevented from the secondary infection when chemotherapy was combined simultaneously. In particular, double-gene DNA vaccine composed of Ag85A and PstS-3 genes could reduce bacteria growth better than single-gene DNA vaccine after a secondary reinfection, indicating a correlation between the breadth of Th1 IFN-γ response and the efficacy of the protection from reinfection. Thus, we propose that multigene TB DNA immunotherapy including Ag85A and PstS-3 genes during the period of chemotherapy could benefit patients undergoing TB chemotherapy in prevention from exogenous reinfection as well as endogenous reactivation.

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Tuberculosis (TB) holds the dubious honor of being the leading single-agent infectious disease killer in the world[1,2] and the situation is worsened by the increasing incidence of both multidrug resistant (MDR) stains and combination with AIDS.[1,3] After *Mycobacterium tuberculosis* infection, active disease arises in about 5% of exposed individuals and most of the others will develop a latent infection in which the tubercle bacilli can persist *in vivo* without causing any clinical symptoms. However, active disease may also develop decades later either as a relapse of the initial infection or because of a secondary infection. Although most cases of tuberculosis were once believed to result from a endogenous reactivation acquired in the past,[4] recent studies indicate that one-third of tuberculosis cases are due to recent transmission by exogenous reinfection of multiple *M. tuberculosis* strains,[5–9] implicating that the exogenous reinfection significantly contributes to disease transmission. Therefore, novel immunotherapeutic approaches will be required to prevent reinfection as well as reactivation of *M. tuberculosis* in individuals with latent tuberculosis.

DNA vaccination has become a promising strategy for developing an effective vaccine against TB, since it efficiently induces Th1 immunity, an essential arm of immune system to clear the bacteria. In prophylactic settings, there are several reports that DNA vaccines expressing *M. tuberculosis* antigens are effective for limiting the bacterial growth in mice.[10–13] However, it is still controversial whether DNA vaccines work against TB reactivation in postexposure models.[14–16] For example, the vaccination of plasmid DNA expressing hsp65 after completion of chemotherapy was shown to be effective in preventing the reactivation of intravenously infected *M. tuberculosis*.[17] In addition, we recently demonstrated that Ag85A DNA vaccine, when simultaneously treated with chemotherapy, could be effective in preventing reactivation of aerogenically infected *M. tuberculosis*.[18] In contrast, Hsp60 or Ag85A DNA vaccines, which were administered after chemotherapy, had no effect.[15] The discrepancy may be caused by differences in the route of infection (aerosol *versus* intravenous), antigens used in each vaccine, strain and dose of infected bacteria, and the regimen of antibacterial drug treatment. More importantly, it seems that the timing of DNA vaccination combined with chemotherapy (simultaneously *versus* sequentially) may be one of the major factors for determining vaccine efficacy in a latent model. It was reported that cellular immunity induced by DNA vaccination could enhance pathology in the granulomatous lesions or trigger reactivation of latent bacteria that were already established in the lungs of mice.[15] Thus, we expected that TB DNA vaccination simultaneously given

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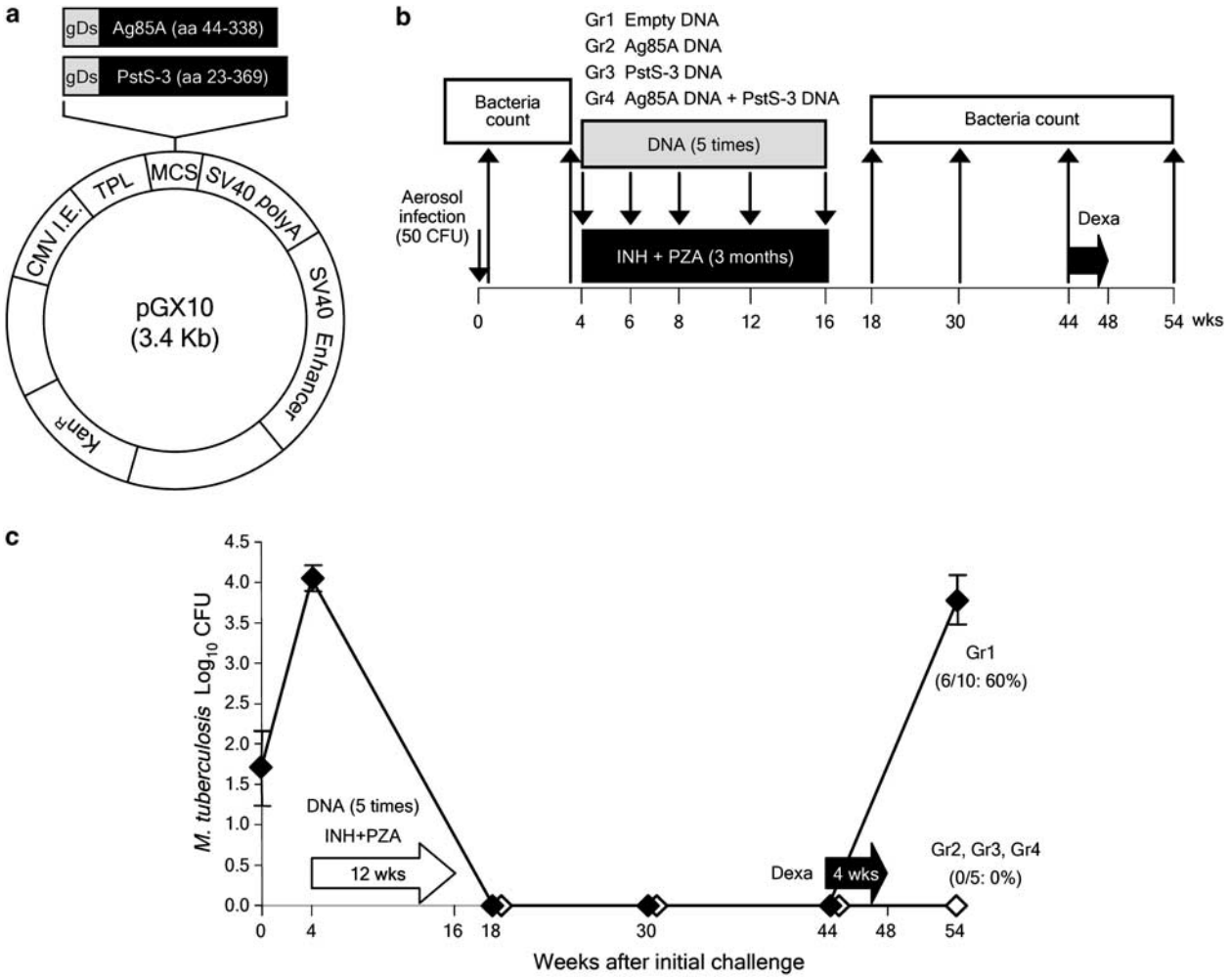
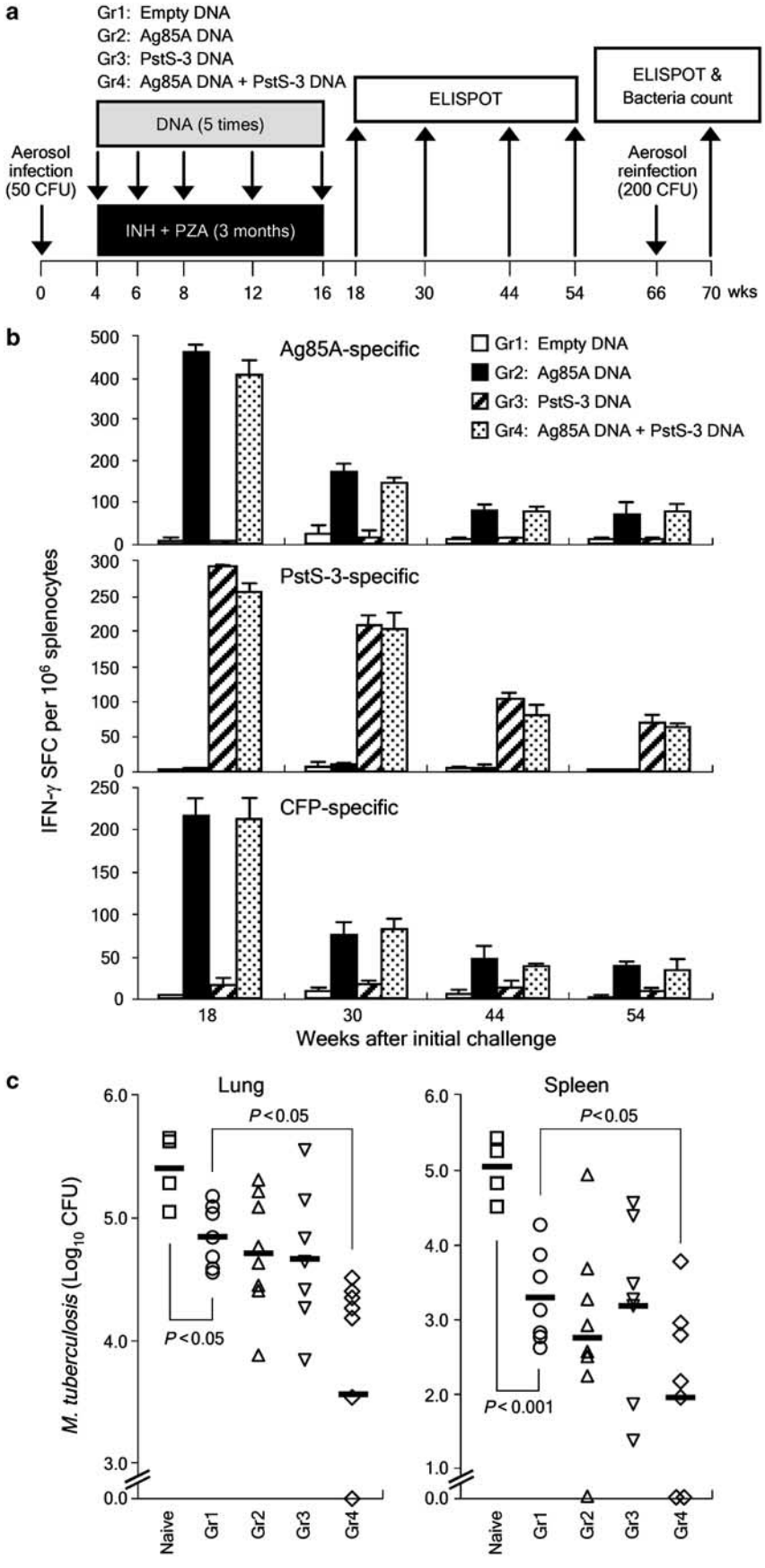


Figure 1 *Therapeutic effect of DNA vaccines given during chemotherapy on reactivation in a low-dose aerosol infection model. (a) To construct pGX10-Ag85A or pGX10-PstS-3 DNA vaccine, the signal sequences of Ag85A and PstS-3 gene were replaced with that of herpes simplex virus type 1 glycoprotein D (amino acids 1–75) (gD),[30] and the resulting fusion cassettes were inserted into the pGX10 vector.[30] Expression of the Ag85A or PstS-3 gene was confirmed by radio-immunoprecipitation assay following the transfection of pGX10-Ag85A or pGX10-PstS-3 into COS-7 cells (data not shown). pGX10-Ag85A and pGX10-PstS-3 were designed to express Ag85A (aa 44–338) or PstS-3 (aa 23–369) fused with the HSV-1 gDs based on pGX10 vector. CMV I.E.: cytomegalovirus immediate-early enhancer/promoter; TPL: adenovirus tripartite leader sequence; MCS: multicloning site. (b) Each group of 6-week-old C57BL/6J mice was given aerogenic infection with 50 × CFU of M. tuberculosis H37Rv as described previously[18] and maintained under barrier conditions in a BL-3 biohazard animal room. At 4 weeks after postinfection, the infected mice were treated with INH and PZA in diet for a period of 12 weeks as described previously.[18] During chemotherapy, total 40 μg of the following plasmid DNA was intramuscularly administered to each group of mice five times at the indicated weeks. Gr1, pGX10 (40 μg); Gr2, pGX10-Ag85A (20 μg)+pGX10 (20 μg); Gr3, pGX10-PstS-3 (20 μg)+pGX10 (20 μg); Gr4, pGX10-Ag85A (20 μg)+pGX10-PstS-3 (20 μg). At 28 weeks after the completion of chemotherapy, the treated mice received intraperitoneal injections with 200 μl of dexamethasone (Sigma) at 6 mg/kg of body weight every 2 days for 4 weeks (from 44 to 48 weeks postinfection). (c) At specific time points up to 54 weeks postinfection, the number of viable bacilli in the lungs of Gr1 (♦) and Gr2-Gr4 mice (◇) was determined by plating lung homogenates and enumerating CFU to estimate reactivation of bacteria as described.[18] The CFU of Gr1 mice at 54 weeks is the average value from the lungs of reactivated mice only. Data are expressed as means ± s.e.m. in the experiment with five mice per group except Gr1 mice (10 mice per group) at the indicated time points. The rate of reactivation in each group of mice is described in the parenthesis.*

Figure 2 *Preventive effect of DNA vaccines given during chemotherapy on reinfection of M. tuberculosis. (a) Each group of mice was infected and treated with chemotherapy and DNA vaccine as described in Figure 1 legend. The IFN-γ ELISPOT assay was performed at the indicated weeks. Reinfection with 200 CFU of M. tuberculosis H37Rv and bacteria counting was performed at 66 and 70 weeks postprimary infection, respectively. (b) Splenocytes from three to four mice were used in the ELISPOT assay at the indicated weeks to determine the number of cells secreting IFN-γ in response to Ag85A peptide pool, PstS-3 peptide pool (1 μg/ml for each peptide), and CFP (10 μg/ml) after incubation for 20 h. Total 28 and 33 peptides spanning mature forms of Ag85A and PstS-3 of M. tuberculosis, respectively, were synthesized as 20-mer peptides overlapping by 10 amino acids. The number of responsive cells was calculated by subtracting the mean number of spots induced in the absence of peptide pool from that in the presence of peptide pool as described before.[31] IFN-γ ELISPOT responses to medium controls were consistently <10% of the response to peptide stimulation (data not shown). The number of IFN-γ-producing cells per 10^6 splenocytes was represented as the average spot forming cells (SFC) ± s.d.s in triplicate wells. (c) The immunized mice as well as age-matched naïve mice were aerogenically challenged with high dose of M. tuberculosis H37Rv (200 CFU per lung) used in a primary infection. Bacteria counts in the lungs and spleens from infected mice were determined 70 weeks postprimary infection (4 weeks postsecondary infection). The $\log_{10}$ CFU obtained from six to eight mice, except control mice (4 mice per group), was represented. The mean values are shown as the bar. Statistical significance was determined using the Student's t-test and a P-value of <0.05 was considered significant.*


a
Gr1: Empty DNA
Gr2: Ag85A DNA
Gr3: PstS-3 DNA
Gr4: Ag85A DNA + PstS-3 DNA
DNA (5 times)
ELISPOT
ELISPOT & Bacteria count
Aerosol infection (50 CFU)
INH + PZA (3 months)
Aerosol reinfection (200 CFU)
0 4 6 8 12 16 18 30 44 54 66 70 wks
b
Ag85A-specific
PstS-3-specific
CFP-specific
Gr1: Empty DNA
Gr2: Ag85A DNA
Gr3: PstS-3 DNA
Gr4: Ag85A DNA + PstS-3 DNA
IFN-γ SFC per 10^6 splenocytes
Weeks after initial challenge
c
Lung
Spleen
M. tuberculosis (Log10 CFU)
P<0.05
P<0.001
Naive
Gr1
Gr2
Gr3
Gr4

during chemotherapy may minimize the granuloma formation and/or necrosis induction, because DNA vaccine-induced Th1 and CTL may be working together with chemotherapy to eliminate *M. tuberculosis*.

To investigate the therapeutic effect of DNA vaccines (Figure 1a) on reactivation of primary infection in a latently infected state, mice were aerogenically infected with low-dose of *M. tuberculosis* (50 CFU per lung; Figure 1b and c). At 4 weeks postinfection, the mice were shown to have bacterial numbers that had reached 4.05 ± 0.16 and 3.14 ± 0.60 ($\log_{10}$ CFU) in the lungs and spleens, respectively (Figure 1c). The infected mice were treated with isoniazid (INH) and pyrazinamide (PZA) in the presence or absence of TB DNA vaccines five times from 4 to 16 weeks after infection (Figure 1b). In this study, BCG was not included as a control vaccine because BCG could be rapidly killed by daily chemotherapy. No viable bacilli were detected in the lungs of mice of all groups given chemotherapy regardless of TB DNA vaccination at 2 weeks after completion of chemotherapy, indicating that the bacilli became undetectable by a 12-week course of chemotherapy (Figure 1c). In addition, no spontaneous reactivation occurred in the lungs of mice of all groups up to 44 weeks after primary infection, which is similar to the result from the latent infection model established by the previous reports.[15,19] However, reactivation of bacilli was observed in 60% of Gr1 mice (six out of 10 mice) after the treatment of dexamethasone for 4 weeks and the average bacterial load in the lungs was 5.12 ± 0.30 ($\log_{10}$ CFU) (Figure 1c), indicating that a mode of latent infection was established in a portion of Gr1 control mice. Expectedly, the mice treated with chemotherapy and TB DNA vaccines together (Gr2, Gr3, and Gr4) did not show the reactivation of primarily infected bacilli. These results indicate that Ag85A or/and PstS-3 DNA vaccines combined with conventional chemotherapy could be effective for the prevention of tuberculosis reactivation.

Although it was reported that multi-antigen-specific immune responses are required for protection against intracellular pathogen,[20–24] the exact relationship between broad immune responses and reactivation as well as reinfection of *M. tuberculosis* remains unclear. To evaluate the ability of TB DNA vaccines given during chemotherapy in inducing broad Th1 immune response, we measured the number of T cells expressing IFN-γ from the spleens of the immunized mice (Figure 2a and b). As expected, low number of IFN-γ-producing cells specific for Ag85A and CFP was observed in Gr1 mice, indicating that low-dose infection and subsequent chemotherapy induce a little, but not significant, TB-specific T-cell responses. This result agrees well with the previous reports that the CpG motifs in the empty DNA vaccine may play a role in inducing Ag85A-specific Th1 immune responses.[18] DNA immunization of Ag85A DNA (Gr2) or PstS-3 DNA (Gr3) during chemotherapy could induce strong Ag85A- or PstS-3-specific IFN-γ responses. IFN-γ responses specific for CFP were higher in Gr2 than in Gr3, because Ag85A protein is the major protein component of *M. tuberculosis* CFP.[25] The Gr4 mice showed comparable number of Ag85A-, PstS-3-, or CFP-specific IFN-γ spots compared with Gr2 and Gr3, indicating that coimmunization of DNA plasmids encoding two different antigens did not interfere each other in inducing IFN-γ immune response specific for each antigen. It is worth noting that Ag85A-specific IFN-γ response measured by the ELISPOT assay is originated from $CD4^+$ T cells but not $CD8^+$ T cells, because $CD8^+$ T cells of C57BL/6J mice do not have any Ag85A-specific TCR repertoire.[26,27] In case of PstS-3, the sources of IFN-γ can be both $CD4^+$ and $CD8^+$ T cells, since specific epitopes for both $CD4^+$ and $CD8^+$ T cells were found in the mice.[28] The frequency of IFN-γ-expressing cells specific for Ag85A, PstS-3, and CFP appeared to decrease gradually after the last immunization in all groups of mice over time and the difference of IFN-γ-secreting T-cell frequency among groups was similarly kept, indicating that there are few bacterial antigens *in vivo* enough to stimulate the pre-existing memory T cells.

To investigate the effect of TB DNA vaccine-induced Th1 immunity on exogenous reinfection, immunized mice and age-matched naïve mice as a control group were aerogenically infected with high dose of *M. tuberculosis* H37Rv (200 CFU) at 66 weeks after primary infection and then bacterial numbers were determined at 4 weeks after reinfection (Figure 2c). The bacterial growth of Gr1 mice in the lungs ($P < 0.05$) and spleens ($P < 0.001$) was decreased at a greater degree compared to that of the naïve mice, which is consistent with the previous result.[14] It is likely that the extent of bacterial growth inhibition in the spleens is more significant than that in the lungs ($P < 0.05$ *versus* $P < 0.001$), suggesting that the dissemination of the lung infection to spleen appears to be effectively controlled in Gr1 mice compared to naïve mice (Figure 2c). This result indicates that pre-existing immunity induced by primary infection, albeit nearly detectable in the IFN-γ ELISPOT assay, may contribute to the reduction of bacteria growth. Compared to Gr1 mice, Gr2 and Gr3 mice further reduced, albeit statistically insignificant, the bacterial growth in the lungs ($P > 0.05$). In the spleens, the number of bacteria in Gr2 mice, albeit statistically insignificant, was lower than that in Gr1 mice (80-fold) (Figure 2c). Interestingly, Gr4 mice immunized with double-gene DNA vaccine expressing both Ag85A and PstS-3, but not Gr2 and Gr3 mice, exhibited significantly reduced bacterial numbers by 100- and 300-fold on average in the lungs and spleens compared to Gr1 mice ($P < 0.05$), respectively. It is worth noting that a complete protection of *M. tuberculosis* was observed from the lungs of one out of seven mice (14%) and from the spleens of two out of seven mice (28%) in Gr 4. These results indicate that a broad range of Th1 IFN-γ immune response is closely related to the efficacy of protection against reinfection, suggesting the potential synergistic effect of multigene TB DNA vaccine.

The major limitation for developing effective TB DNA vaccines is that the protective antigens for TB are not precisely defined. In this study, Ag85A and PstS-3 were chosen on the basis of the following reasons. Firstly, it has been reported that the Ag85A epitopes are frequently expressed on the surface of the infected macrophages during early phase, but downregulated at the late phase, while PstS-3-specific epitopes are more abundantly expressed during late phase.[13] Thus, DNA immunization with combination of these two genes might induce both Ag85A- and PstS-3-specific immune responses required for suppressing bacterial growth during both early and late phases. Secondly, it has also been known that $CD4^+$ T cells play an important role in the protection for the acute

stage of infection, but $CD8^+$ T cells are critical in the control of latent infection.[29] Thus, both $CD4^+$ and $CD8^+$ T-cell responses induced by our double-gene DNA vaccine can lead to an efficient control of bacterial growth during both stages of infection.

In summary, TB DNA vaccination significantly reduced reactivation of *M. tuberculosis* by dexamethasone treatment, enhancing complete elimination of bacteria. In addition, we demonstrated close correlation between broad immunity and inhibition of the bacterial burden after reinfection. To our knowledge, no other experimental vaccine has demonstrated protective activity from exogenous reinfection in TB animal model. Based on our results, we suggest that multigene DNA vaccination combined simultaneously with chemotherapy would be one of the efficient strategies to limit further usage of chemical drugs, and thus to minimize their toxicity and cost as well as to prevent from TB reinfection.

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