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Adjunctive immunotherapy with α-crystallin based DNA vaccination reduces Tuberculosis chemotherapy period in chronically infected mice

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By employing modified Cornell model, we have evaluated the potential of adjunctive immunotherapy with DNA vaccines to shorten the tuberculosis chemotherapy period and reduce disease reactivation. We demonstrate that α-crystallin based DNA vaccine (DNAacr) significantly reduced the chemotherapy period from 12 weeks to 8 weeks when compared with the chemotherapy alone. Immunotherapy with SodA based DNA vaccine (DNAsod) reduced the pulmonary bacilli only as much as DNAvec. Both DNAacr and DNAsod, although significantly delayed the reactivation in comparison to the chemotherapy alone, this delay was associated with the immunostimulatory sequences present in the vector backbone and was not antigen specific. Both DNA vaccines resulted in the production of significantly higher number of T_{EM} cells than the chemotherapy alone, however, only in the case of DNAsod, this enhancement was significant over the DNAvec treatment. Overall, our findings emphasize the immunotherapeutic potential of DNAacr in shortening the duration of TB chemotherapy.

Tuberculosis (TB) remains the leading cause of death amongst infectious diseases. Despite the availability of standard chemotherapy regimens, TB claimed 1.45 million lives in 2010. One third of the world's population is estimated to be latently infected with *Mycobacterium tuberculosis*[1,2]. The current TB therapy requires prolonged treatment schedule of 6 months for drug susceptible TB that may extend up to 30 months in the case of drug resistant TB. Non-compliance to such a prolonged regimen often leads to the emergence of multidrug-resistant (MDR) and extensively drug-resistant (XDR) TB along with increased incidence of disease reactivation. Besides, there have been recent reports of totally drug resistant TB which is very difficult and expensive to treat. Moreover, incomplete treatment often leads to increased transmission of the disease. Even if a patient successfully completes chemotherapy, due to inability of the current therapy to impart complete sterilization, there is a 5–10% lifetime risk of reactivation of the latent infection. This risk substantially increases to 10% annual risk in the immuno-compromised individuals[3]. Hence, alternate strategies are urgently required that can shorten the duration of chemotherapy and reduce reactivation of the disease.

DNA vaccines have been evaluated for the control of TB for many years in animal models[4]. In prophylactic mode, several DNA vaccines expressing mycobacterial antigens or cytokines have been found to limit the bacterial growth in mice efficiently[5–8]. Moreover, several candidate TB vaccines and immunomodulatory agents have been evaluated as adjunct to chemotherapy for their ability to enhance the efficacy of the treatment[9,10]. These efforts have resulted in varying degree of success[9,11–15]. The use of hsp65 based DNA vaccine in immunotherapeutic mode after completion of chemotherapy has been shown to effectively prevent the reactivation of TB in mice[16]. Moreover, hsp65 based DNA vaccine has also been shown to be a valuable adjunct to antimycobacterial chemotherapy which not only accelerates the treatment against active TB cases but also improves the treatment of MDR TB[17]. Similarly, the use of Ag85A DNA vaccine either alone or in combination with PstS-3 DNA vaccine as an adjunct to chemotherapy has been shown to markedly prevent reactivation and reinfection of TB besides providing effective treatment of MDR TB in mice[18–20]. In addition, immunotherapy with a combination of DNA vaccines expressing Ag85B, MPT-64 and MPT-83 along with chemotherapy has shown to significantly reduce the duration of chemotherapy[21]. By virtue of their ability to enhance therapeutic efficacy, to reduce the magnitude of disease transmission and to bring down the reactivation of latent TB infections, the importance of DNA vaccines in the control of the disease cannot be over emphasized.

We have previously reported the prophylactic potential of DNA vaccines expressing the *M.tuberculosis* antigens α-crystallin (DNAacr) and superoxide dismutase A (DNAsod) in the guinea pig model of experimental TB[22]. We also showed that DNAacr conferred a superior protection than BCG vaccination when employed either in 'rBCG prime – DNA boost'[23] or 'BCG prime – DNA boost'[24] regimen. In this study, we have evaluated the immunotherapeutic potential of adjunctive immunotherapy with DNAacr and DNAsod in a murine model of latent TB to shorten the duration of chemotherapy and to prevent the reactivation of latent infection.

Results

Establishment of a murine model of latent infection. To establish latent TB infection, we employed a modified Cornell model as described by Ha *et al*[18] with minor modifications (Figure 1A). Briefly, Balb/c mice were infected with 50–100 CFU of virulent *M.tuberculosis* bacilli by aerosol route. After 4 weeks of infection, the bacillary load was 5.40 $\log_{10}$ CFU in the lungs (Figure 1B) and 3.23 $\log_{10}$ CFU in the spleens (Figure 1C). Mice were then treated with isoniazid (INH) and pyrazinamide (PZA) for 12 weeks (0.1 g/L and 8.0 g/L, respectively in drinking water). This regimen resulted in undetectable number of bacilli in the lungs (Figure 1B) as well as in the spleens (Figure 1C) of mice with progressive reduction in gross pathological (Figure 1D) and histopathological damage (Figure 1E). The animals remained free of cultivable bacilli for 8 weeks after the completion of chemotherapy, following which they exhibited spontaneous reactivation (Figure 1B and 1C). These observations demonstrate successful establishment of chemotherapy induced model of latent TB infection in mice.

Immunotherapeutic effect of DNAacr and DNAsod in shortening the duration of chemotherapy. We evaluated the immunotherapeutic potential of DNA vaccines expressing either *M.tuberculosis* antigen, α-crystallin (DNAacr) or superoxide dismutase A (DNAsod) as adjunct to chemotherapy to reduce the duration of chemotherapy by comparing the bacillary load and pathological damage in the lungs of animals. For this, in conjunction with chemotherapy, mice were immunized with 3 doses of either DNAacr or DNAsod or DNAvec at 4, 8 and 12 weeks following infection (Figure 2A). The influence of immunotherapy with DNAacr was evident immediately after 4 weeks of treatment, wherein, we observed a significant reduction in the lung bacillary load (by 1.05 $\log_{10}$ CFU, ***$p = 0.0006$) when compared with the chemotherapy alone (Figure 2B). In the case of immunotherapy with DNAsod, we observed a 0.57 $\log_{10}$ CFU reduction in lung bacillary load over the chemotherapy alone, although this reduction was not significant (Figure 2B). After 8 weeks of treatment, we observed that immunotherapy with DNAacr exhibited a significant reduction in the pulmonary bacillary load when compared with the chemotherapy alone [by 0.75 $\log_{10}$ reduction in CFU (***$p = 0.0003$)]. Moreover, adjunctive immunotherapy with DNAacr significantly reduced the bacillary load by 0.26 $\log_{10}$ CFU when compared with DNAvec treatment (*$p = 0.0260$) thus demonstrating the association of immunotherapeutic influence of DNAacr with the antigen specificity (Figure 2C). Besides, the examination of individual animals from various groups revealed that only 30% of the animals belonging to the chemotherapy group exhibited undetectable bacilli in lungs. However, in the case of DNAacr treatment, no bacilli were detected in 100% of the animals thus showing a significant improvement over the chemotherapy treated animals as well as the DNAvec treated animals (Figure 2C, inset). The treatment with DNAsod resulted in undetectable bacilli in 80% of the animals as compared to 60% of the animals in the case of DNAvec treatment; however, this improvement by DNAsod was not significantly different as the bacillary load in the lungs from these two groups of animals was comparable. At the end of 12 weeks of treatment, the bacillary load in the lungs of animals treated with either chemotherapy alone or with DNA vaccines was undetectable (Figure 2D).

The gross pathological damage observed in the lungs of the animals belonging to various groups supported the bacteriological findings. After 8 weeks of therapy, the lungs of untreated mice were enlarged and oedematous with the presence of numerous large tubercles (Figure 3A). The lungs of mice treated with either chemotherapy alone or with DNAvec exhibited a comparable pathological damage with moderate involvement and numerous small tubercles. In contrast, the animals treated with DNAacr displayed a complete restoration of lung architecture in all the animals. The animals treated with DNAsod, however, showed moderate lung involvement in some of the animals (Figure 3A). Further, this was in agreement with the histopathological observations. The untreated mice exhibited an aggravated lung pathology characterized by well circumscribed and organized granulomas and widespread lymphocytic infiltration resulting in the consolidation of lung tissues. Mice treated with chemotherapy alone or with DNAvec exhibited a moderate inflammation in the lungs accompanied by small focal areas of neutrophilic infiltration. In contrast, the animals treated with DNAacr displayed a complete restoration of lung parenchyma with well preserved alveolar and bronchiolar structures in the lungs of all the animals. Immunotherapy with DNAsod resulted in moderate inflammation in some of the animals (Figure 3B). These observations substantiate the immunotherapeutic potential of DNAacr in significantly reducing the duration of chemotherapy.

Influence of adjunctive immunotherapy with DNAacr and DNAsod in reducing the reactivation of tuberculosis. To investigate the immunotherapeutic potential of DNAacr and DNAsod as an adjunct to chemotherapy in preventing the reactivation of latent TB infection, mice were infected with *M.tuberculosis* and treated with either chemotherapy alone or chemotherapy along with DNA vaccines as described in the methods. Treatment was carried out till the bacillary load became undetectable in the lungs and spleens of animals belonging to both the groups. The animals were then maintained on food and water *ad libitum* for the next 16 weeks followed by enumeration of bacillary load in the organs. We observed that mice treated with only chemotherapy displayed the highest bacillary load in lungs (5.28 $\log_{10}$ CFU) indicating spontaneous reactivation of residual infection with age (Figure 4A). However, when DNA vaccines were employed as adjunct to chemotherapy, we observed that adjunctive immunotherapy with DNAacr resulted in 1.58 $\log_{10}$ reduction in CFU (**$p = 0.0036$) while DNAsod resulted in 1.36 $\log_{10}$ reduction in lung CFU (*$p = 0.0203$) in comparison to treatment with the chemotherapy alone (Figure 4A). We did not, however, observe any significant difference in the bacillary load in the lungs of mice treated with either DNA vaccines or DNAvec (Figure 4A). In spleens, it was observed that after the completion of chemotherapy, when the animals were evaluated for bacillary load after 16 weeks, a significant reactivation of the disease has occurred as a bacillary load of 3.75 $\log_{10}$ CFU was recorded in these animals. Immunotherapy with both DNAacr and DNAsod significantly reduced the spleen bacillary load by 2.46 $\log_{10}$ CFU (**$p = 0.0040$) and by 1.68 $\log_{10}$ CFU (*$p = 0.0216$), respectively when compared with the chemotherapy alone, however, the reduction in bacillary load in the case of immunization with DNAvec was also comparable to that observed in the cases of DNA vaccines (Figure 4B). These results demonstrate that immunostimulatory sequences present in the vector backbone of DNA vaccines induced sufficient stimulation of the host immune system to delay the reactivation significantly in comparison to the chemotherapy alone; however, it was apparent that this immune stimulation was not associated with the antigens expressed by these DNA vaccines.

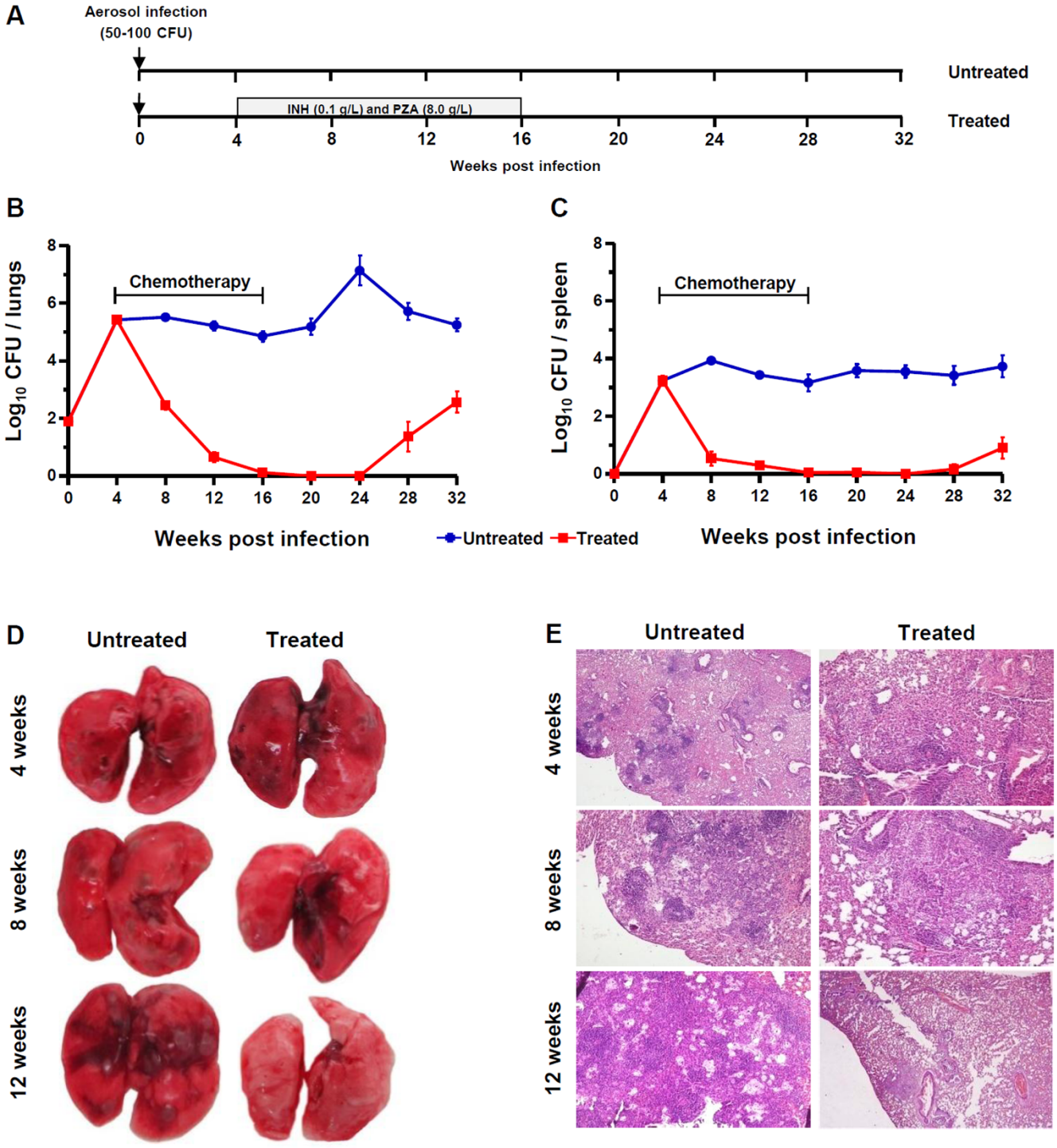

Figure 1 | Establishment of a murine model of latent TB. Mice were infected with 50–100 CFU of *M.tuberculosis*. At 4 weeks post infection, mice were treated with 0.1 g/L isoniazid (INH) and 8.0 g/L pyrazinamide (PZA) for 12 weeks. One group of mice was not treated with chemotherapy. Bacterial enumeration in lungs and spleens of animals belonging to both the groups was carried out at various time points following infection. (A) Treatment and sampling schedule. (B) Bacillary enumeration in lungs. (C) Bacillary enumeration in spleens. The $\log_{10}$ CFU results are pooled values from two experiments expressed as mean ± SE. In each experiment 5 mice were used per group. The data was analyzed by Two-way ANOVA. (D) The figure depicts representative photographs of lungs of infected mice either left untreated or treated with INH and PZA for 4, 8 and 12 weeks. (E) Histopathology of lungs of both untreated and treated mice. The representative photographs of the sections of lung tissues stained with hematoxylin and eosin (magnification = 20×).

Adjunctive immunotherapy with DNAacr and DNAsod induces higher T_{EM} cells. To study the adaptive immune responses elicited by DNA vaccines based immunotherapy, we investigated the phenotype of CD4 T cells isolated from the spleens of mice belonging to various groups at 16 weeks post completion of therapy (Figure 5). Splenocytes were isolated and stimulated with PPD for 6 hrs followed by staining for CD4 T cells as well as IFN-γ, TNF-α and IL-2 in order to measure the intracellular levels of these cytokines. Further, multifunctionality of CD4 T cells was characterized by analyzing the expression of various combinations of IFN-γ, TNF-α

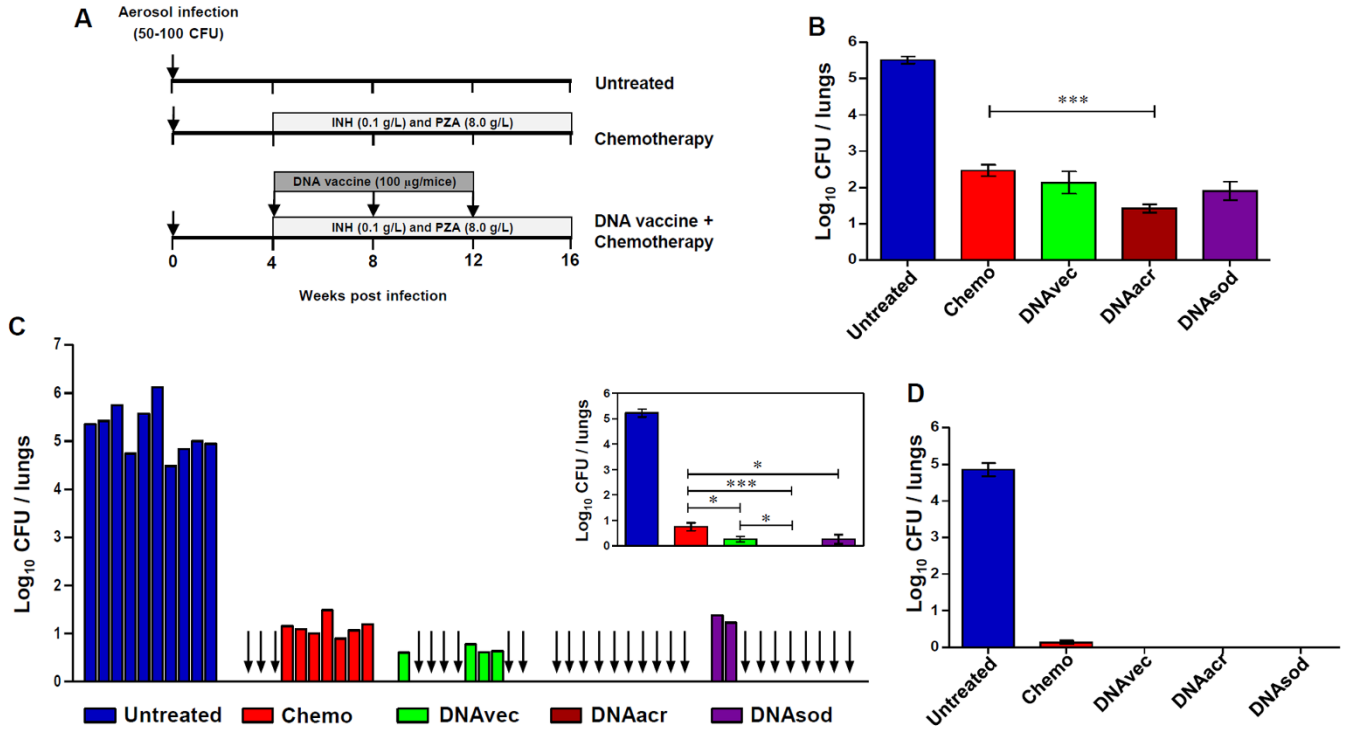

Figure 2 | Influence of DNA vaccines based immunotherapy on the duration of chemotherapy. Mice were infected with 50–100 CFU of *M.tuberculosis*. Four weeks after the infection, chemotherapy was initiated as described in figure 1 and was continued for 12 weeks. At 4, 8 and 12 weeks after infection, the mice were also immunized intramuscularly with 100 μg of either of the following: DNAacr, DNAsod or DNAvec in conjunction with chemotherapy. One group of mice was treated with the chemotherapy alone. Number of viable bacilli in lungs was determined after 4 weeks, 8 weeks and 12 weeks of treatment by plating the lung homogenates on MB7H11 agar. (A) Treatment and sampling schedule. (B) Bacillary enumeration after 4 weeks of treatment. (C, inset) Bacillary enumeration after 8 weeks of treatment. (D) Bacillary enumeration after 12 weeks of treatment. The $\log_{10}$ CFU results are pooled values from two experiments expressed as mean ± SE. In each experiment 5 mice were used per group. The data was analyzed by student's unpaired *t*-test, two-tailed (***$p < 0.001$ and *$p < 0.05$). Arrows indicate the number of animals with undetectable bacilli in lungs.

and IL-2 as described by Seder *et al*[25]. On analyzing the multifunctionality of CD4 T cells, we observed that mice belonging to all the groups exhibited a comparable frequency of CD4 T cells secreting various combinations of TNF-α, IL-2 and IFN-γ. However, we observed a significant increase in terminally differentiated CD4 T cells producing only IFN-γ when compared with the chemotherapy alone (***$p < 0.0001$ for DNAacr and **$p = 0.0033$ for DNAsod, respectively) (Figure 5A). Immunization with DNAvec, however, also resulted in a comparable frequency of IFN-γ^+ CD4 T cells (***$p = 0.0001$) as was observed in the case of DNA vaccines indicating thereby that the observed enhancement of IFN-γ resulting from the immunization with DNA vaccines was not specifically associated with the antigens expressed by these vaccines (Figure 5A). Hence, we further evaluated effector (T_{EM}; $CD4^+CD44^{hi}CD62L^{lo}$) and central memory cell responses (T_{CM}; $CD4^+CD44^{hi}CD62L^{hi}$) generated by these DNA vaccines by measuring the expression of cell surface markers such as, CD44 and CD62L on CD4 T cells by PPD stimulated splenocytes as described by Henao-Tamao *et al*[26] (Figure 5B). We observed that chemotherapy alone or with DNAvec induced a comparable enhancement in T_{EM} response with no significant distinction from each other. In comparison to chemotherapy, when the animals were

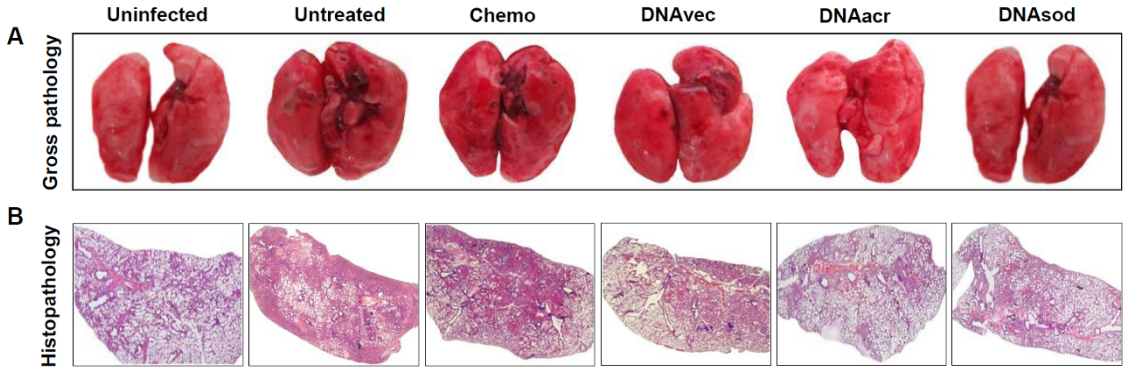

Figure 3 | Pulmonary pathology in the lungs of animals after 8 weeks of treatment. Mice were infected with *M.tuberculosis* and then treated with either chemotherapy alone or chemotherapy along with the DNA vaccines as described in figure 2A. Mice were euthanized after 8 weeks of treatment and their lungs were examined for gross pathological damage and histopathological damage. (A) Representative photographs of lungs of mice belonging to various groups. (B) Representative photographs of the sections of lung tissues stained with hematoxylin and eosin (magnification = 10×).

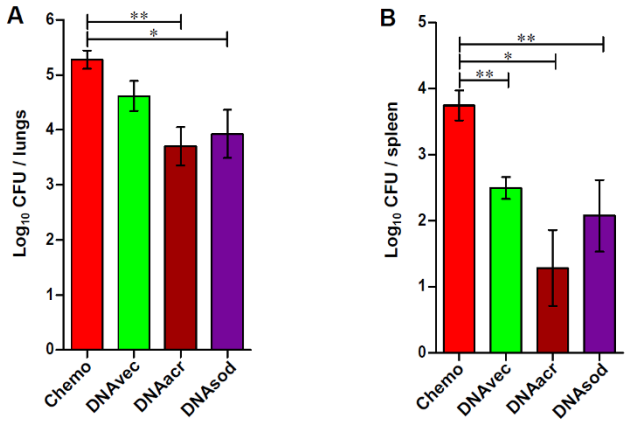

Figure 4 | Influence of DNA vaccines based immunotherapy on the reactivation of latent TB infection. Mice were infected with *M.tuberculosis*. Following 4 weeks post infection, mice were treated with either chemotherapy alone or chemotherapy along with DNA vaccines till the bacillary load in the lungs and spleens of the animals became negligible. After the treatment, mice were maintained on food and water *ad libitum* for the next 16 weeks followed by bacillary enumeration in lungs and spleens. (A) Bacillary enumeration in lungs. (B) Bacillary enumeration in spleen. The $\log_{10}$ CFU results are pooled values from two experiments expressed as mean ± SE. In each experiment 5 mice were used per group. The data was analyzed by student's unpaired *t*-test, two-tailed (**$p < 0.01$ and *$p < 0.05$).

treated with DNA vaccines along with chemotherapy, a significant enhancement in T_{EM} memory cells (*$p = 0.0124$ in case of DNAacr and **$p = 0.0022$ in case of DNAsod) (Figure 5C) was observed. However, we observed that only DNAsod elicited significantly higher levels of T_{EM} response as compared to the chemotherapy along with DNAvec (*$p = 0.0324$) (Figure 5C). The frequency of T_{CM} remained comparable among all the groups and no significant influence of immunotherapy was observed with either of the DNA vaccines (Figure 5D).

Discussion

Reduction in the period of TB chemotherapy as well as reactivation of latent infection can have a positive influence on breaking the chain of transmission and thus on the epidemiologic scenario of the disease. In this study, we have evaluated the potential of adjunctive immunotherapy with DNA vaccines to reduce the duration of chemotherapy as well as reactivation of the latent infection.

Over the past few years, several DNA vaccines have shown promising results against tuberculosis[27–29]. DNA vaccines are known to efficiently induce cellular immune responses including generation of cytotoxic T lymphocytes (CTLs) and Th1 mediated responses, an essential arm of immune system required to clear the bacilli[4,8,21,30]. In this study, we have employed two DNA vaccines expressing the *M.tuberculosis* antigens, α-crystallin (Acr) and superoxide dismutase A (SodA). We chose Acr as it represents one of the most abundantly produced proteins during hypoxic conditions, nutrient starvation and transition of actively dividing bacilli to a dormant state[31,32]. Latently infected individuals (healthy PPD+ and household contacts) exhibit increased lympho-proliferative and IFN-γ response to Acr as compared to the patients with active TB[33]. These observations signify a crucial role of Acr in the elicitation of protective immune responses and maintenance of disease free state in these subjects. This antigen possesses both conformational as well as linear epitopes. The presence of antibodies against Acr in ~85% of the TB patients provides the immunological evidence for the presence of conformational B cell epitopes in this immunodominant protein[34,35]. Moreover, there are several permissively recognized T cell epitopes in Acr which generate an efficient HLA-DR restricted $CD4^+$ immune response[36]. There are experimental evidences that support that the T cell epitope, with peptide sequence of 91–110, is the most immunogenic among them all[36–38]. This immunodominant nature of Acr supports it as a robust candidate for vaccination strategies. A blast search of protein databases also highlighted that *M.tuberculosis* Acr homologs are absent from most of the environmental mycobacterial species including *M.avium* complex (*M.avium* paratuberculosis, *M.avium* and *M.intracellulare*), which further enhances its potential for eliciting efficient and specific immune responses. These attributes make Acr an important antigen for its use in adjunctive immunotherapy.

Similarly, SodA was selected as *M.tuberculosis* SodA harbours several immunodominant B and T cell epitopes. The linear peptide with sequence of 160–168 has been reported as the most immunodominant T cell epitope that is largely implicated against HLA-A2 bearing cells thus generating $CD8^+$ responses[39]. A blast search of protein databases showed that *M.tuberculosis* SodA homologs are present in *M.avium* complex and other environmental mycobacteria. However, there is enough experimental evidence to support that several of SodA epitopes (the most immunogenic B-cell epitope is with peptide sequence 7–26) are recognized in PPD positive individuals as well as in tuberculosis (TB) and leprosy patients[40–42]. Moreover, previously in our laboratory, we have shown the potential of DNAsod in imparting protection against *M.tuberculosis* challenge in guinea pigs[22].

In this study, we show that adjunctive immunotherapy with DNA vaccine encoding mycobacterial latency antigen α-crystallin significantly shortens the duration of chemotherapy resulting in a faster clearance of infection when compared with the chemotherapy alone thus imparting stronger therapeutic effect. In the case of reactivation of latent infection, the adjunctive immunotherapy with DNAacr or DNAsod resulted in a significant reduction in the bacillary load in lungs when compared with the chemotherapy alone, suggesting that it can potentially delay the reactivation of the disease. However, since this reduction in bacillary load was not significantly different than in the case of treatment with the DNAvec, it suggested that the observed reduction in the bacillary load and thus the potential of delaying the reactivation of disease may be associated with the immune stimulation caused by the vector alone and may not be attributable to the role of antigens.

Effective antimycobacterial immunity in mice has been associated with the production of multifunctional CD4 T cells[43,44]. Hence, in this study, we evaluated the multi-functionality of T cells based on the production of cytokines such as IFN-γ, TNF-α and IL-2 on a single cell level by multiparametric flow cytometery. We show that adjunctive immunotherapy with DNAacr and DNAsod resulted in a significantly higher frequency of terminally differentiated effector CD4 T cells producing only IFN-γ. There was no prominent production of combination of any of these cytokines. However, immunostimulatory sequences usually present in DNA vaccine backbone are known to induce the production of IFN-γ[45], hence, the observed enhancement in IFN-γ levels in case of both the DNA vaccinations could have resulted similarly in an antigen independent manner. In fact, we observed that the immunization with DNAvec also resulted in a higher level of IFN-γ production that was comparable to those produced by DNA vaccine immunized animals. Therefore, we next measured the impact of parameters associated with memory responses specifically T_{CM} and T_{EM} cells. It was observed that the production of T_{CM} in the animals belonging to all the groups employed in this study was indistinguishable from each other and no significant difference was observed in any particular group. When the levels of T_{EM} cells were analysed in the splenocytes from the animals belonging to various groups, the preponderance of T_{EM} cells was significantly higher in the DNA vaccine treated animals as compared to the animals treated with the chemotherapy alone, however, the

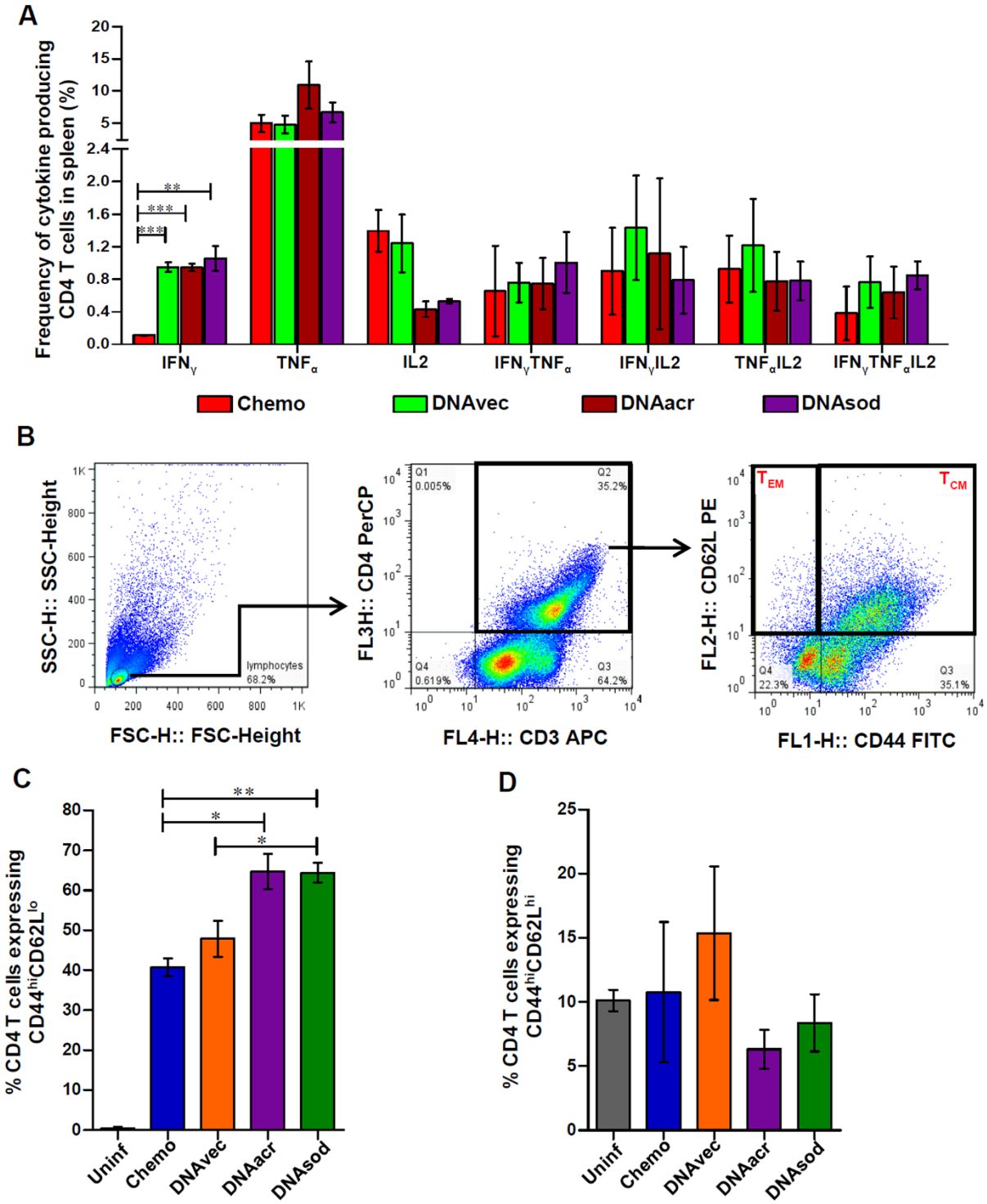

Figure 5 | Influence of DNA vaccines based immunotherapy on the expression of intracellular cytokines and CD44 and CD62L. Single cell suspensions of splenocytes (5×10^6 cells per well) were prepared from mice treated with either chemotherapy alone or chemotherapy along with DNA vaccines as described in methods. Lymphocytes were purified from splenocytes by using T cell enrichment kit and stained for cell surface marker (CD4-PerCP) along with intracellular staining for IFN-γ-(PE), TNF-α-(FITC) and IL-2-(APC). Frequency of IFN-γ, TNF-α and IL-2 producing CD4 T- cells was determined on CD4 T cell gated population. CD4 T cells were also selected by staining with antibodies conjugated with cell surface markers namely CD3 (APC) and CD4 (PerCP) as per the manufacturer's instructions. These CD4 T cells were then treated with monoclonal antibodies against CD44 (FITC) and CD62L (PE). (A) The frequency of cytokine producing CD 4 T cells in spleen. (B) The representative FACS plots to measure T_{EM} and T_{CM} cells. (C) The percentage of CD4 T cells expressing T_{EM} cells ($CD44^{hi}CD62L^{lo}$). (D) The percentage of CD4 T cells expressing T_{CM} cells ($CD44^{hi}CD62L^{hi}$). 10^5 cells were acquired on a FACS Calibur flow-cytometer (by using Cell Quest Pro software) and analyzed by using FlowJo software (Tree Star, USA). For cell stimulation and FACS analyses, 12 mice were used per group. The splenocytes from 4 mice were pooled and 3 such pools (from 12 mice) were subjected to statistical analyses. The values are expressed as mean ± SE. Uninf data belongs to the mice that were uninfected and hence not treated. The data was analyzed by student's unpaired *t*-test, two-tailed (***$p < 0.001$; **$p < 0.01$ and *$p < 0.05$).

comparison of T_{EM} cells production between DNA vaccine groups and DNAvec group revealed that only in the case of adjunctive immunotherapy with DNAsod, there was some significant increase in T_{EM} cells when compared with the DNAvec treatment.

Our laboratory has developed several candidate TB vaccines that have shown a superior protection than BCG in small animal models[22–24,46]. In a previous study, we have reported the prophylactic potential of DNAacr vaccine as a booster to BCG or recombinant BCG expressing the same antigen, in a heterologous prime boost approach. In the present study, we demonstrate the immunotherapeutic potential of DNAacr along with chemotherapy to shorten the duration of chemotherapy. Hence, α-crystallin based DNA vaccine holds a significant promise for its use both as a prophylactic vaccine as shown by us earlier and as a partner in the therapeutic approach as shown in this study.

Methods

Mice. Pathogen-free Balb/c mice of either sex (30–40 g) were purchased from Division of Laboratory Animals, Central Drug Research Institute, Lucknow, India. The animals were maintained in a BSLIII animal facility at University of Delhi South Campus, New Delhi and routinely cared for according to the guidelines of CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals), India. All the experimental protocols included in this study were reviewed and approved by the Institutional Animal Ethics Committee (Ref No. 1/IAEC/AKT/Biochem/UDSC/24.08.10).

Bacteria and DNA vaccines. *M.tuberculosis* (H37Rv strain, ATCC No. 25618, procured from Dr. J. S. Tyagi, AIIMS, New Delhi, India) was grown to mid-log phase in Middlebrook (MB) 7H9 medium supplemented with 1× ADC and 0.05% Tween-80. PBS stocks were prepared and stored at −80°C till further use. The CFU of stocks was enumerated by plating appropriate dilutions on MB7H11 agar (1× OADC and 0.5% glycerol). The DNA vaccines, namely, DNAacr and DNAsod (pAK4acr and pAK4sod, respectively) were prepared as described previously[22]. The vector pAK4 (DNAvec) was employed as a control. pAK4, a eukaryotic expression vector carried the gene for ampicillin resistance that harbours two copies of CpG motifs and tPA signal in addition to CMV promoter, intron A and BGH terminator along with SV 40 ori and pUC ori and Kanamycin resistance gene. For this study, endotoxin free plasmid DNA for vaccination was prepared by using EndoFree plasmid purification DNA kit (Qiagen, USA) and was diluted in 0.9% normal saline solution to a final concentration of 1 mg/ml.

Establishment of latent TB infection in mice. We have employed a modified Cornell model of latent TB infection as described by Ha *et al*[18] with minor modifications as described below.

Mice were challenged with *M.tuberculosis* bacilli by respiratory route in inhalation chamber (Glascol Inc., USA) pre-calibrated to deliver 50–100 bacilli per animal in the lungs. Following four weeks of infection, mice were treated with 0.1 g/L isoniazid (INH) and 8.0 g/L pyrazinamide (PZA) for 12 weeks in drinking water. As a control, one group of mice was not treated with chemotherapy and therefore maintained a chronic *M.tuberculosis* infection. Before initiating the chemotherapy, the infection in the animals was verified by euthanizing a group of animals (n = 5) at 4 weeks after the aerosol challenge followed by pathological observations as well as enumeration of bacilli in the lungs and spleens. Every 4 weeks after initiation of chemotherapy, mice (n = 5) were euthanized by CO_2 asphyxiation and monitored for gross pathological observations, histopathological changes and bacillary load. For bacterial enumeration, mice were dissected and lungs and spleens were aseptically removed and homogenized in 0.9% normal saline. Appropriate dilutions of the homogenates were plated in duplicates onto MB7H11 agar and the plates were incubated at 37°C for 3–4 weeks followed by enumeration of colonies. The results were expressed as $\log_{10}$ CFU per organ.

Evaluation of DNA vaccination as an adjunct to chemotherapy. Mice were infected with 50–100 *M.tuberculosis* bacilli. Four weeks after the infection, chemotherapy was initiated as described above and was continued for 12 weeks (i.e. till 16 weeks post infection). At 4, 8 and 12 weeks after infection, the mice were also immunized intramuscularly with 100 μg of either of the following; DNAacr, DNAsod or DNAvec in conjunction with chemotherapy (Figure 2A). One group of mice was treated with chemotherapy alone. After initiating the chemotherapy, mice (n = 5) were euthanized by CO_2 asphyxiation every 4 weeks till the bacillary load became negligible in the organs of all animals treated with chemotherapy alone or chemotherapy and DNA vaccines. Animals were then maintained on food and water *ad libitum* for the next 16 weeks following which they were evaluated for bacillary load in the lungs and spleens along with the assessment of gross pathological and histopathological changes.

Histopathology. For histopathological analyses, 5 μm thick sections of formalin fixed, paraffin embedded lung tissues were stained with haemotoxylin and eosin (H & E). The tissues were coded and the coded samples were evaluated by a certified pathologist having no knowledge of the treatment groups.

Intracellular cytokines analyses by using flow cytometry. To study the expression of intracellular cytokines, spleen cells were isolated, cultured and stimulated for 6 hrs with PPD (20 mg/ml) (Statens Serum Institut, Denmark). The cells were stained for cell surface marker (CD4) and intracellular cytokines (IFN-γ, IL-2 and TNF-α) as described by Dey and Jain *et al*[23]. Anti-mouse monoclonal antibodies: CD4-PerCP, IFN-γ-PE, TNF-α-FITC and IL-2-APC (BD PharMingen, CA) were employed in this study. 10^5 cells were acquired per sample by using a FACS Calibur flow-cytometer (by employing Cell Quest Pro software) and analyzed by using FlowJo software (Tree Star, USA). For cell stimulation and FACS analysis, 12 mice were used per group. The splenocytes from 4 mice were pooled and 3 such pools (from 12 mice) were subjected to statistical analyses.

Analyses of lymphocyte subpopulations expressing CD44 and CD62L. Single cell suspensions of splenocytes (5×10^6 cells/well) were prepared as described[44] and cultured in RPMI-GlutaMAX™ (containing 10% heat inactivated Fetal Bovine Serum and 1× antibiotic-antimycotic) (Invitrogen, USA). Cells were then stimulated with PPD (20 mg/ml) (Statens Serum Institut, Denmark) for 72 hrs at 37°C in 5% CO_2. Following this, the cells were counted by trypan blue exclusion method and 10^6 cells were stained with specific antibodies against different surface markers as per the manufacturer's recommendations. Briefly, viable lymphocytes were washed twice with 1× PBS containing 2% FCS and 0.1% sodium azide. Subsequently, the cells were stained for cell surface markers, CD3-Allophycocyanin (APC), CD4-Peridinin-Chlorophyll-Protein-Complex (PerCP), CD44-fluorescein isothiocyanate (FITC) and CD62L-phycoerythrin (PE) (BD PharMingen, CA). The cells were incubated at room temperature for 20 min followed by incubation on ice for 20 min. Cells were washed twice with the 1× PBS and finally re-suspended in sheath fluid. 10^5 cells were acquired per sample by using a FACS Calibur flow-cytometer (by employing Cell Quest Pro software) and analyzed by using FlowJo software (Tree Star, USA). Firstly, viable lymphocytes were gated based upon their forward and side scatter characteristics. The lymphocytes were then gated by using CD3- (APC) and CD4- (PerCP) for the selection of $CD3^+CD4^+$ cell population. The gated $CD3^+CD4^+$ cell population was then analysed for $CD44^{hi}CD62L^{lo}$ and $CD44^{hi}CD62L^{hi}$ cell population by using CD44- (FITC) and CD62L- (PE) (BD PharMingen, CA). For cell stimulation and FACS analysis, 12 mice were used per group. The splenocytes from 4 mice were pooled and 3 such pools (from 12 mice) were subjected to statistical analyses.

Statistical analyses. For comparison between the groups, Two-way ANOVA with Bonferroni multiple comparison test was employed. For the calculation of exact p values, student's unpaired *t*-test (two-tailed) was performed. Differences were considered significant, when $p < 0.05$. For statistical analysis and generation of graphs, Prism 5 software (Version 5.01; GraphPad Software Inc., USA) was used.

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Author contributions

P.C., R.J., B.D. and A.K.T. conceived and designed the experiments. P.C. performed the experiments. P.C., R.J. and B.D. analyzed the data. P.C. and A.K.T. wrote the manuscript with inputs from R.J. and B.D. A.K.T. provided overall supervision throughout the study.

Additional information

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