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Oral Bacille Calmette-Guérin (BCG) vaccination induces long-term potentiation of memory immune response to Ovalbumin airway challenge in mice.

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ABSTRACT

The Bacille Calmette-Guérin (BCG) is a potent immunomodulator. It was initially used by oral administration, but it is mostly used subcutaneously nowadays. This study shows that oral BCG vaccination modifies the immune response to a second non-related antigen (Ovalbumin) systemic immunization. Airway Ovalbumin challenge six months after the systemic intraperitoneal immunization resulted in a potent $\gamma\delta^+$ T cell response in the lungs biased to IFN-γ and IL-17 production *ex vivo* and a mixed TH1, TH2, and TH17 T cells upon further stimulation with anti-CD3 mAb *in vitro*. Higher percentages of $CD4^+$ T cells accompanied the augmented T cell response in oral BCG vaccinated mice. Also, the proportion of $Foxp3^+$ Tregs was diminished compared to PBS-gavaged and OVA-immunized mice. The anti-OVA-specific antibody response was also influenced by oral exposure to BCG so that these mice produced more IgG2a and less IgE detected in the sera. These results suggest that oral BCG vaccination can modify future immune responses to vaccines and improve immunity to pathogen infections, especially in the mucosal interfaces.

1. Introduction

The mucosal is an attractive surface to induce long-term modifications in immune reactivities at the mucosal level and systemically [1]. For instance, it has been known for a long time that tolerance induced via mucosal antigen administration results in detectable levels of local mucosal immune response and stabilization of systemic immune responses at very low levels [2]. Bacille Calmette-Guérin (BCG), used as a vaccine for tuberculosis, was initially applied orally [3]. However, doubts about its systemic activities were, among other reasons, arguments to change its administration to a subcutaneous route [3]. Recently, it was shown that intravenous, subcutaneous, or mucosal administration of BCG might act on the innate immune cells, producing long-term immune responses towards a more robust natural and adaptative immune response to unrelated antigen immunization, infection, or cancer [4-7]. This long-term effect is called "Trained Immunity" and is now considered an innate immunity memory [8]. Although the mechanisms behind trained immunity are not fully elucidated, epigenetic modifications at the level of innate cell precursors are known to participate [8,9].

Recently, a BCG-adjuvanted COVID-19 vaccine was successfully used to establish sterilizing immunity to SARS-CoV-2 after a single administration, demonstrating its potential as a useful adjuvant and immunomodulator [4]. However, systemic BCG administration does not boost or correct the deficiency in tissue immune responses during senescence [10]. Therefore, safe and effective mucosal adjuvants/immunomodulators are necessary.

Here, we investigated if oral BCG administration could induce long-term modifications of systemic immunization to a non-related antigen (Ovalbumin). In addition, we have studied lung mononuclear cells in response to aerosolized OVA challenge nearly eight months after BGC gavage and systemic OVA immunization.

Abbreviations: Bacille Calmette-Guérin, BCG; Forkhead Box P3, Foxp3; Interferon gamma, IFN-γ.

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This study shows for the first time that a single dose of BCG by oral route can modify future immune responses to unrelated antigens. The modifications include an IFN-γ and IL-17-biased response at distant tissue sites like the lungs upon antigenic challenge and a potential to make mixed TH1, TH2, and IL-17 cytokine responses when memory T cells were further stimulated *in vitro* with anti-CD3 mAb. The potentiated mixed local cytokine response may be interesting in some infectious conditions, allowing the host to contain different pathogens faster 11—13]. We also show that the percentage of lung Tregs decreases substantially after the local OVA challenge. This latter finding may justify the increased presence of a higher frequency of cells spontaneously secreting cytokines and the potentiated local T cell immune response. The functional IFN-γ-biased response is also reflected in the Immunoglobulin isotype pattern produced in response to OVA adsorbed in alum. After months of systemic immunization, augmented production of OVA-specific IgG2a/c in the sera could be demonstrated along with a reduced level of antigen-specific IgE.

These observations suggest that oral administration of BCG preparations may induce trained immunity. Therefore, it could be useful to boost cellular vaccine responses even if a TH2 adjuvant inducer is used to improve the immune response to certain pathogens. Although oral BCG administration diminishes IgE production, the lower frequency of Treg cells and increased numbers of IL-17-secreting T cells in the lungs after the airway challenge may pose cautionary questions in conditions related to respiratory allergies [14–16]. However, other studies suggest a protective role of IL-17, and further studies are required to clarify the issue [17,18].

2. Materials and methods

2.1. Animals

Female C57BL/6 mice were four weeks old at the beginning of the experiments. Mice were maintained under conventional conditions in the Animal Facility of the Petrópolis Medical School, UNIFASE, Petrópolis, RJ. The animal house receives sunlight and therefore is submitted to natural light/dark cycles. Food and water were provided *ad libitum*. All experiments were conducted according to protocols approved by the Institutional Animal Use Ethics Committee (CEUA-FMP-UNIFASE, protocol #P-001/2018).

2.2. Experimental groups, gavages, OVA immunization, and challenge

The experimental groups were: PBS-gavaged, OVA-immunized, and BCG-gavaged, OVA-immunized. BCG gavage was performed once in four-week-old mice. Two hours before BCG or PBS gavage, mice were pre-treated with ranitidine at 8mg/kg (Antak^R, GSK). A total of 1mg of live BCG (Moreau strain, kindly donated by Ataulpho de Paiva Foundation) diluted in PBS was used per animal/gavage. PBS alone was used in control gavaged animals. After one month, mice were immunized with 1μg of OVA (grade V, Sigma Aldrich) diluted in PBS and adsorbed in 1mg of alum via the *i.p.* route. Immunization with OVA in alum was repeated three more times, two weeks apart. Mice were used after six months of the last OVA injection. OVA aerosol challenge was performed using OVA solution (OVA grade III from Sigma-Aldrich, 10mg/ml, diluted in PBS) nebulized for 30 minutes. A commercial ultrasonic nebulization machine coupled to a chamber with enough room space for five mice was used. Mice were killed 48hs after OVA aerosol challenge by an excess sodium pentobarbital *i.p.* injection, perfused with PBS/Heparin as described elsewhere [19].

2.3. ELISA assays

Blood from all mice groups was collected before the last OVA aerosol challenge. Blood was collected from the tail vein. Sera were separated and kept frozen at -20°C until used. Standard ELISA procedures assayed anti-ovalbumin antibodies. In short, polystyrene plates (Falcon) were coated overnight at 4°C with 2 μg of OVA (grade V) per well, blocked with 0.25% casein in phosphate-buffered saline (PBS), washed with PBS-0.05% Tween 20, and then incubated with serial dilutions of mouse sera, starting at 1/25. After one hour at 37°C, plates were washed and set for an additional hour at 37°C with a monoclonal antibody to mouse IgG2a/c conjugated to peroxidase (BD Bioscience, clone R19-15). After further washing, H_20_2 and orthophenylenediamine (OPD) were used for detection. The reaction was stopped and read in a Titertek Multiskan at 492nm. Antibody titers are shown as ELISA*, a running sum of O.D. values for ELISA readings from l/25 to l/l2800 serum dilutions. The results are expressed as the mean of ELISA* values [20,21]. The assays were performed in duplicate. Normal sera gave scores below 400. This form of expressing the results was equivalent to showing titration curves or electing the absorbance at one particular serum dilution as representative of the others. For OVA-specific IgE detection, a modified sandwich assay was used. A purified rat anti-mouse IgE monoclonal antibody (BD Bioscience, clone R35-92) diluted in PBS was incubated for 12hs in Maxsorp ELISA plates (Nunc) in a concentration/volume of 10μg/50μl/well. Plates were washed with PBS-0.05% Tween 20 and incubated with different serial diluted sera, starting at 1/2 up to 1/128. Plates were washed and incubated for one additional hour with biotinylated Ovalbumin (prepared in-house). After washing, streptavidin-HRP at appropriate dilution was used for one hour. The assay was revealed and read as described above. The results are expressed as the mean of ELISA* values.

2.4. Preparation of lung mononuclear cells

Perfused lungs, devoided of the tracheas and large bronchus, were sliced into small pieces and incubated in collagenase (Sigma-Aldrich) at a 1 mg/ml diluted in RPMI for 45 min, 37°C. Cell suspension and the remaining tissue were further passed through a metal mesh (70μm pore). The recovered cell suspension was washed in incomplete RPMI, and the pellet was diluted in 40% Percoll (GE Healthcare Biosciences AB, Uppsala, Sweden). A discontinuous Percoll gradient of 40/80% separated mononuclear cells. Cells at the 40/80 interface were recovered, washed three times in incomplete RPMI, and used further. In addition, cells from different individual mice in the same group were used for *in vitro* culture and further analysis. Cell viability evaluated by trypan blue exclusion was above 80%, invariably.

2.5. Cell cultures

Lung mononuclear cells prepared as above were washed and resuspended to determine the number of cells per milliliter. Cells were plated in 24-well culture plates. Each well contained 2.5×10^6 cells/mL, which were incubated in RPMI containing 10% FBS in a 5% CO_2 at 37° C. Cells were left with medium alone or stimulated with anti-CD3 mAb (clone 2C11) at a concentration of 2μg/ml. Brefeldin-A was added 4 hours before the cells were harvested and stained for flow cytometric analysis. After 12 hours in culture, harvested cells were plated in 96-well plates containing $2,5 \times 10^5$ cells, each 100 μL. Subsequently, the cells were centrifuged at 2000 rpm for 3 seconds and stained.

2.6. Flow cytometric analysis

Lung mononuclear cells were resuspended in FACS buffer (PBS, 2% FBS, 2 mg/ml of Protein-A Sepharose purified polyclonal rabbit IgG, and 0,1% sodium azide). Aliquots of the cell suspensions were incubated for 20 minutes with the following mAbs: Anti-CD8 (Clone 53-6.7) PerCP-Cy 5.5 or FITC, anti-CD4 (Clone GK1.5) PerCP-Cy 5.5 or FITC, anti-TCR γδ FITC (Clone GL-3), and anti-CD25 (clone PC-61). The previously stained cells were fixed with 0,1% paraformaldehyde for one hour and washed three times in BD Perm/Wash buffer for intracellular cytokinestaining. The intracellular cytokine staining was performed with anti-IFN-γ

(Clone XMG1.2), anti-IL-4 (clone 11B11), anti-IL-5 (clone TRFK5), and anti-IL-17 (clone TC11-18H10.1). All the anti-cytokine antibodies were PE-labeled.Monoclonal antibodies for cytometry were from Invitrogen, Tonbo Bioscience, Biolegend, or eBioscience. Antibodies were diluted in Perm/Wash buffer, and the cells were incubated for 1 hour. They were washed twice with Perm/Wash and with FACS buffer. Intranuclear staining for foxp3 was performed following the manufacturer's instructions. Anti-foxp3 (clone 150D) PE and buffer set were from Biolegend. A BD FACScan instrument was used for readings. Spleen cells from naïve mice (4-6 weeks old) cultured in medium alone as described for lung mononuclear cellswere stained with each monoclonal antibody combination and further used to establish the fluorescence backgrounds and the manual compensation.

2.7. Gating strategies for the Flow Cytometry analysis

$CD4^+$, $CD8^+$, and $\gamma\delta^+$ T cells were gated in a previous forward versus side scatter (FSC vs. SSC) lymphocyte gate. $CD4^+$, $CD8^+$, or $\gamma\delta^+$ T cells were identified in FL3 or FL1. Then, cytokine-producing cells or foxp3 stainings were studied in FL2. At least100.000 events were collected per sample. Results were analyzed using Flowjo software.

2.8. Statistical analysis

The results are presented as means ± SD. The significance of differences between the experimental and control groups was determined as described in each figure legend. *P* values below 0.05 were considered significant.

3. Results

3.1. Oral BCG administration before OVA systemic immunization reduces tissue-Treg cells upon airway antigenic challenge

Fig. 1A shows that the percentage of $CD4^+$ T cells in the lungs within 48hs after OVA aerosol challenge is higher in animals with BCG before systemic OVA immunization than in PBS-gavaged control mice. The proportion of tissue $CD4^+Foxp\text{-}3^+$ is lower in mice previously gavaged with BCG compared to control mice (Fig. 1B). In addition, the percentage of $CD4^+CD25^+$ $foxp3^+$ T cells is also lower in mice pre-treated with BCG (Fig. 1C). The plot panel illustrates Fig. 1, representing one studied animal of each group.

3.2. Oral treatment with BCG increased the potential of lung-tissue memory $CD4^+$ and $CD8^+$ T cells to produce IL-4 upon polyclonal stimulation in vitro but not after antigenic challenge in vivo

Mice previously gavaged with BCG were immunized with OVA and challenged eight months later with aerosolized Ovalbumin. After 48 hours, lung cells were separated and set for a short period in culture with or without anti-CD3 monoclonal antibodies. Figs. 2A and 2B show lung $CD8^+$ and $CD4^+$ T cells from mice pre-treated with BCG or PBS did not differ in their production of IL-4 after *in vivo* antigenic stimulation. However, the potential of $CD8^+$ and $CD4^+$ T cells from mice pre-treated with BCG to produce IL-4 was enhanced by the previous contact with BCG when these cells were further stimulated *in vitro* with antibodies to CD3 molecules. The flow cytometry plots shown in Fig. 2 illustrate the data in Figs. 2A and 2B, depicting lung mononuclear cells from one studied mouse out of five.

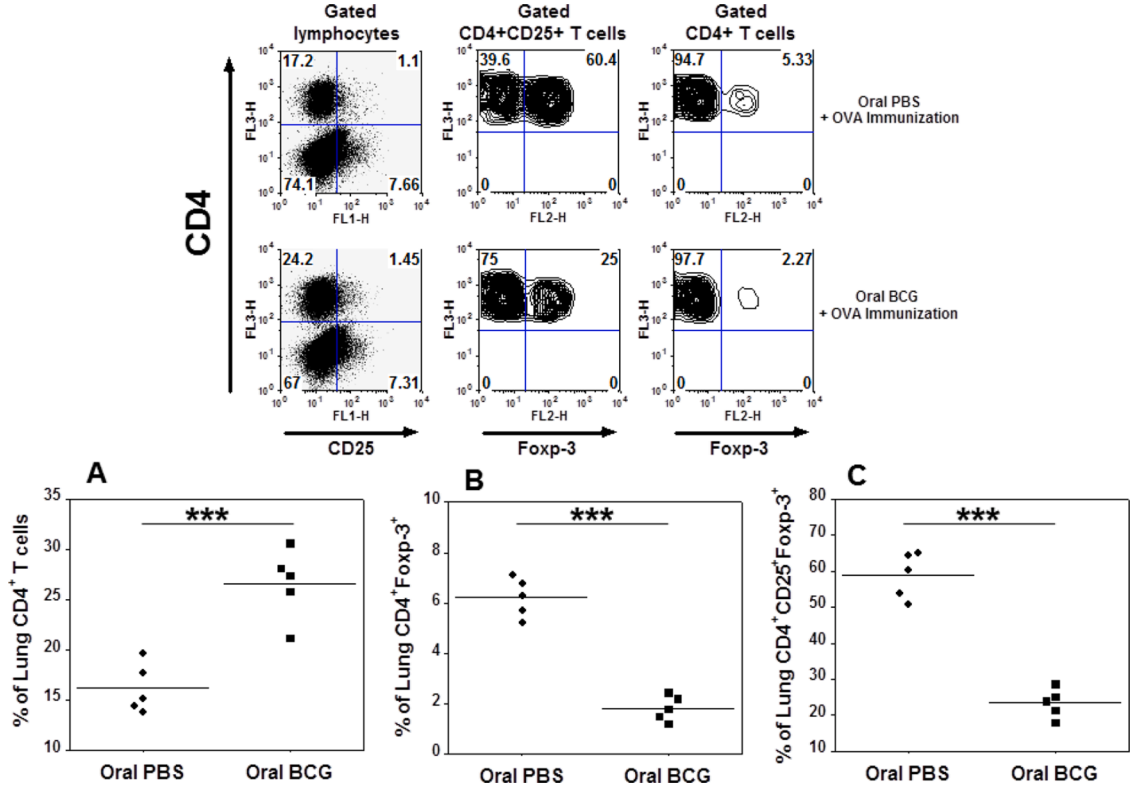

Fig. 1. Oral BCG administration before OVA systemic immunization reduces tissue Treg cells upon airway antigenic challenge. Lung mononuclear cells were studied by cytometry. Five different animals from oral BCG vaccinated and PBS-gavaged groups were analyzed. Cells were stained with anti-CD4, anti-CD25, and anti-foxp3. In Fig.-1A, the frequencies of lung $CD4^+$ T cells are shown. Figs. 1B and 1C show the frequencies of $CD4^+Foxp3^+$ and $CD4^+CD25^+Foxp3^+$ T cells. FACS plots in the upper panel show one representative mouse of each experimental group. Different gates are specified on the top of the plots. Experiments were repeated three times on different occasions with similar results. Kruskal-Wallis test was used to compare different groups of mice (***$P \leq 0.001$, n=5).

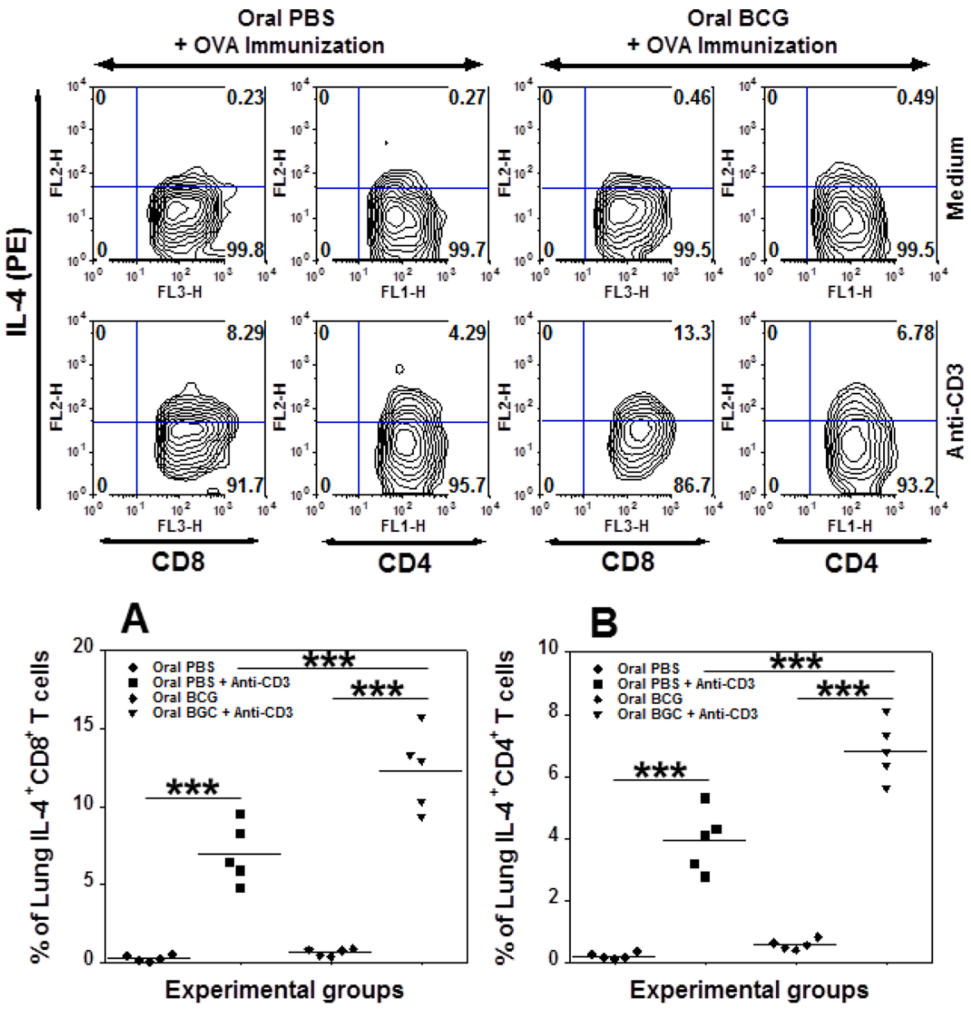

Fig. 2. Oral treatment with BCG increased the potential of lung-tissue memory CD4$^+$ and CD8$^+$ T cells to produce IL-4 upon polyclonal stimulation *in vitro* but not after antigenic challenge *in vivo*. Fig. 2 shows the frequencies of IL-4$^+$CD8$^+$ (A) and IL-4$^+$CD4$^+$ (B) T cells in lung mononuclear cells from mice orally vaccinated with BCG or gavaged with PBS followed by systemic OVA immunization. Lung mononuclear cells were obtained 48 hours after the aerosolized OVA challenge. Cells were either left in the medium alone or stimulated with anti-CD3 mAb *in vitro*. The Material and Methods section described cell culture conditions. ANOVA, Bonferroni post-tests compared groups, (***P<0.001, n=5). FACS plots in the upper panel show one representative mouse of each experimental group. Different groups are specified on the top of the plots. Experiments were repeated twice with similar results.

3.3. Upon ovalbumin aerosol challenge, BCG oral administration followed by systemic OVA immunization does not increase IL-5 production by lung-tissue T cells. Still, oral BCG augments the frequency of IL-5$^+$CD8$^+$ T cells after stimulation with anti-CD3 mAb

Fig. 3 shows that previous BCG oral administration does not change the production of IL-5 by memory lung tissue CD8$^+$ (Fig. 3A) or CD4$^+$ (Fig. 3B) T cells. Mononuclear cells were prepared from the lungs after 48 hours of OVA challenge in the airways of mice previously gavaged with BCG or PBS and immunized with OVA. Cells were left in culture for an additional 12 hours in the presence of medium or anti-CD3 mAb. Figs. 3A and 3B show lung CD8$^+$ and CD4$^+$ T cells from mice pre-treated with BCG or PBS did not differ in their production of IL-5 after *in vivo* antigenic stimulation. However, a short *in vitro* stimulation with anti-CD3 mAb induced higher frequencies of IL-5$^\pm$CD8$^\pm$ but not IL-5$^\pm$CD4$^\pm$ T cells in mice gavaged with BCG. The flow cytometry plots shown in Fig. 3 illustrate the data in Figs. 3A and 3B, depicting lung mononuclear cells from one studied mouse out of five.

3.4. Upon aerosolized OVA challenge, the IFN-γ recall response is greatly enhanced by previous exposure to BCG by oral route

Next, we analyzed the production of IFN-γ by lung CD4$^+$ and CD8$^+$ T cells 48hs after aerosolized OVA. Fig. 4 shows that the frequencies of IFN-γ$^+$CD8$^+$ (4A), but not IFN-γ$^+$CD4$^+$ (4B) T cells, increased in animals exposed to BCG by oral gavage compared to controls. Differences were further potentiated by a short *in vitro* stimulation with anti-CD3 mAb where mice gavaged with BCG had higher frequencies of T cells producing IFN-γ. The flow cytometry plots shown in Fig. 4 illustrate the data in Figs. 4A and 4B, depicting lung mononuclear cells from one studied mouse out of five.

3.5. The percentage of TH17 cells in the lungs of mice challenged with aerosolized OVA was increased by previous mucosal exposure to BCG

The frequencies of TH17 lymphocytes among mononuclear lung cells of mice pre-exposed to mucosal BCG and then immunized and challenged with aerosolized OVA were greatly increased compared to control PBS-gavaged mice. A short stimulation of anti-CD3 mAb further reveals an augmented percentage of potential IL-17 secreting CD4$^+$ T cells among mononuclear cells infiltrating the lungs upon local antigenic stimulation. These results are shown in the graph in Fig. 5. The flow cytometry plots illustrate these results, showing one studied mouse out of five.

3.6. The frequency of lung-infiltrating γδ T cells spontaneously secreting Th2 cytokines was not elevated by previous BCG mucosal contact despite an increased percentage of IL-5$^+$ γδ T cells upon stimulation with anti-CD3 mAb in vitro

γδ T cells secreting IL-4 (Fig. 6A) and IL-5 (Fig. 6B) spontaneously were not increased in mononuclear cells, infiltrating the lungs after 48hs of OVA aerosolized challenge in oral BCG vaccinated animals when compared to control mice. In addition, stimulation with anti-CD3 mAb *in*

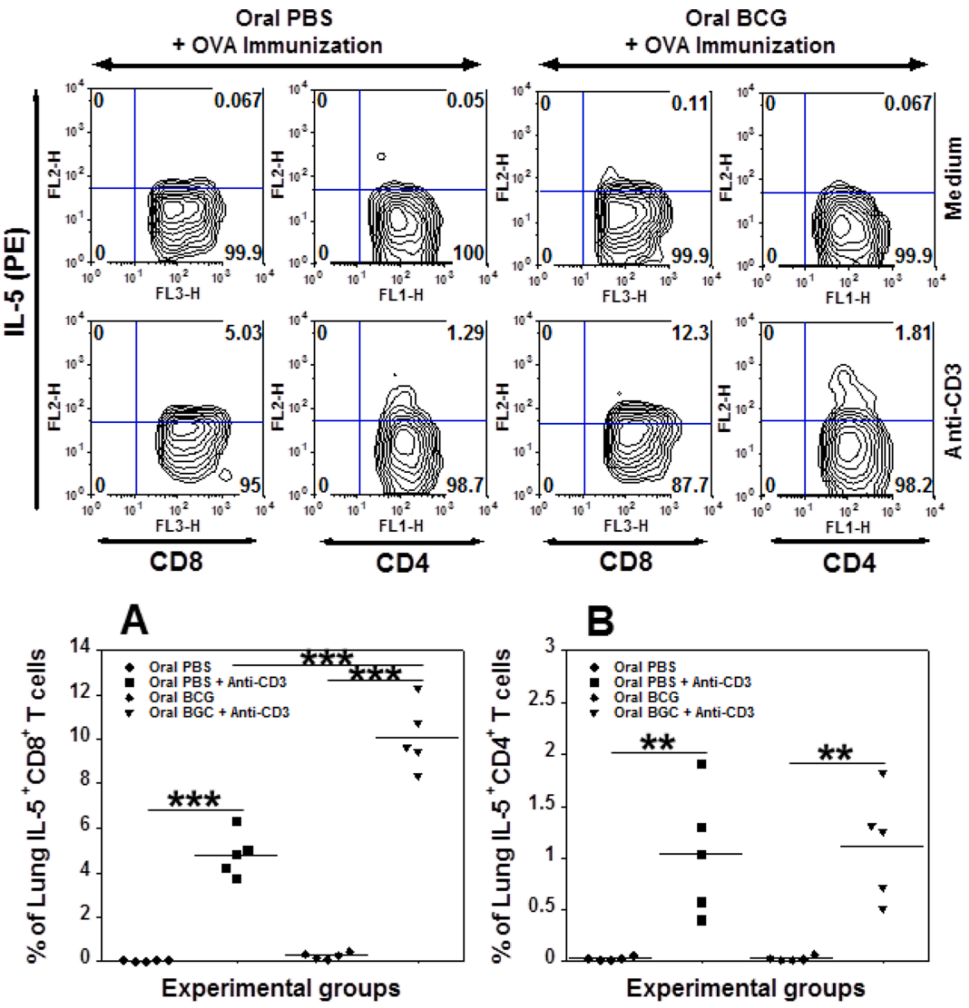

Fig. 3. BCG oral administration followed by systemic OVA immunization does not increase IL-5 production by lung-tissue T cells upon ovalbumin aerosol challenge. Still, it augments the frequency of IL-5$^+$CD8$^+$ T cells after stimulation with anti-CD3 mAb. Fig. 3 shows the frequencies of IL-5$^+$CD8$^+$ (A) and IL-5$^+$CD4$^+$ (B) T cells in lung mononuclear cells from mice orally vaccinated with BCG or gavaged with PBS followed by systemic OVA immunization. Lung mononuclear cells were obtained 48 hours after the aerosolized OVA challenge. Cells were either left in the medium alone or stimulated with anti-CD3 mAb *in vitro*. The Material and Methods section described cell culture conditions. ANOVA, Bonferroni post-tests compared groups, (**P<0.01, ***P<0.001, n=5). FACS plots in the upper panel show one representative mouse of each experimental group. Different groups are specified on the top of the plots. Experiments were repeated twice with similar results.

vitro increased the percentages of IL-5$^+$ and IL-4$^+$ γδ$^+$ T cells. However, after anti-CD3 stimulation, only the frequency of IL5$^+$γδ$^+$ T cells was higher in oral BCG vaccinated mice. Flow cytometry plots in Fig. 6 illustrate these results by showing one representative mouse out of five for each experimental group.

3.7. Previous mucosal contact with BCG significantly increases the percentage of lung IFN-γ$^+$ but not IL-17$^+$ γδ T cells after in vivo restimulation with OVA in the airways

Fig. 7A shows that gavage with BCG before OVA immunization improves the spontaneous production of IFN-γ by γδ T cells in the lungs after aerosolized OVA challenge. A brief stimulation with anti-CD3 mAb greatly potentiates the production of IFN-γ, demonstrating that most infiltrating γδ T cells in the lungs of these animals were INF-γ producers. The comparison was made with animals gavaged with PBS as the control group. We have also studied the production of IL-17 by γδ$^+$ T cells in the lungs of animals pre-treated with BCG or PBS as control and challenged with aerosolized OVA. Fig. 7B shows that the frequency of spontaneous IL-17 lung γδ$^+$ T cell-producers from mice previously gavaged with BCG was not different from PBS-treated controls. However, *in vitro*, anti-CD3 mAb stimulation increased the percentage of γδ$^+$ T cells producing IL-17 in lung mononuclear cells from animals pre-treated with BCG.

3.8. BCG gavaged mice produced more OVA-specific IgG2a/c and less IgE antibodies upon OVA/Alum systemic immunization

Fig. 8A shows that mice pre-treated with BCG orallyincreased antigen-specific IgG2a/c isotype six months after the last OVA systemic challenge. In addition, the levels of OVA-specific IgE were reduced in BCG pre-treated mice compared to control mice, gavaged with PBS before OVA immunization (Fig. 8B). We could not observe significant differences in total IgG to OVA at this time, but there was a trend towards a higher production in BCG-treated animals (data not shown).

4. Discussion

Oral BCG vaccination was used in humans for decades [3]. However, its benefits or the extent of collateral biological effects were not carefully evaluated. Recently there was a renewing interest in oral BCG vaccinations because there was evidence of effective protection against experimental and natural-transmitted *Mycobacterium bovis* infection in cattle and badgers [22–24]. Another important point to be established is whether the oral BCG vaccination induced the so-called trained immunity as its subcutaneous version [9] [5]. There is evidence that the application of BCG in the urinary bladder mucosa may cause trained immunity in humans when this type of immunotherapy is used for non-invasive bladder cancer [7,9]. One study has demonstrated that antigen-specific effector CD4$^+$ T cells remain in the lungs and mice's spleen for long after oral administration of BCG [25]. The same study has shown increased numbers of lung CD4$^+$ T cells producing IFN-γ and

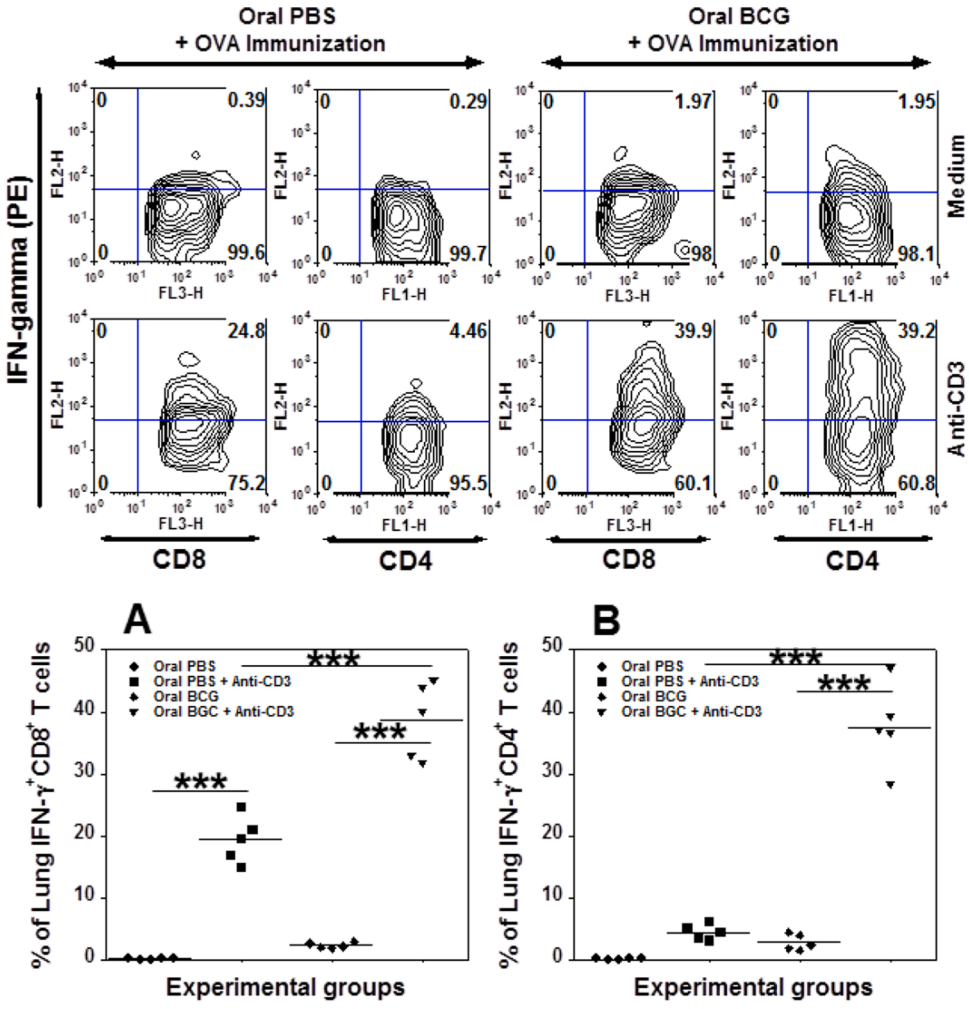

Fig. 4. Upon aerosolized OVA challenge, the IFN-γ recall response is greatly enhanced by previous exposure to BCG by oral route. Fig. 4 shows the frequencies of IFN-γ^+CD8^+ (A) and IFN-γ^+CD4^+ (B) T cells in lung mononuclear cells from mice orally vaccinated with BCG or gavaged with PBS followed by systemic OVA immunization. Lung mononuclear cells were obtained 48 hours after the aerosolized OVA challenge. Cells were either left in the medium alone or stimulated with anti-CD3 mAb *in vitro*. The Material and Methods section described cell culture conditions. ANOVA, Bonferroni post-tests compared groups, (***P<0.001, n=5). FACS plots in the upper panel show the frequencies of IFN-$\gamma^{\pm}$ T cells in $CD8^+$ or $CD4^+$ subpopulations. Plots represent one mouse of each experimental group. Different groups are specified on the top of the plots. Experiments were repeated three times with similar results.

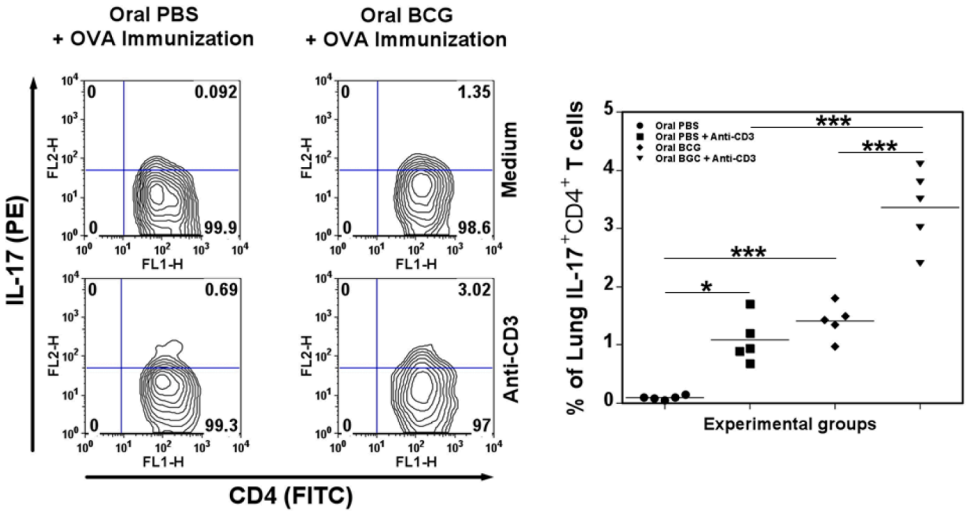

Fig. 5. The percentage of TH17 cells in the lungs of mice challenged with aerosolized OVA was increased by previous mucosal exposure to BCG. Fig. 5 (graph on the right) shows the frequencies of IL-17^+CD4^+ T cells in lung mononuclear cells from mice orally vaccinated with BCG or gavaged with PBS followed by systemic OVA immunization. Lung mononuclear cells were obtained 48 hours after the aerosolized OVA challenge. Cells were either left in the medium alone or stimulated with anti-CD3 mAb *in vitro*. The Material and Methods section described stimulated versus non-stimulated cell culture conditions. ANOVA, Bonferroni post-tests compared groups, (*P<0.05, ***P<0.001, n=5). FACS plots on the left side of Fig. 5 show the frequency of TH17 cells in one representative mouse of each experimental group. Different groups are specified on the top of the FACS plots. Experiments were repeated three times with similar results.

other cytokines simultaneously, characterizing multifunctional $CD4^+$ T cells [25]. These studies suggest that oral BCG vaccination is a strong inducer of systemic and mucosal T cell memory specific to BCG and other pathogens as a non-specific effect. The present study evaluated the effects of oral BCG administration in mice further submitted to an unrelated systemic immunization with Ovalbumin adsorbed in alum adjuvant and challenged *in vivo* with soluble OVA in the airways. Our results agreed with previous observations showing an increased percentage of $CD4^+$ T cells in the lungs upon the OVA challenge (Fig. 1A). In addition, we have demonstrated that the proportion of $CD4^+$ $foxp3^+$ Treg cells in the lungs decreased significantly in mice previously oral vaccinated with BCG (Figs. 1B and 1C). These results are the first

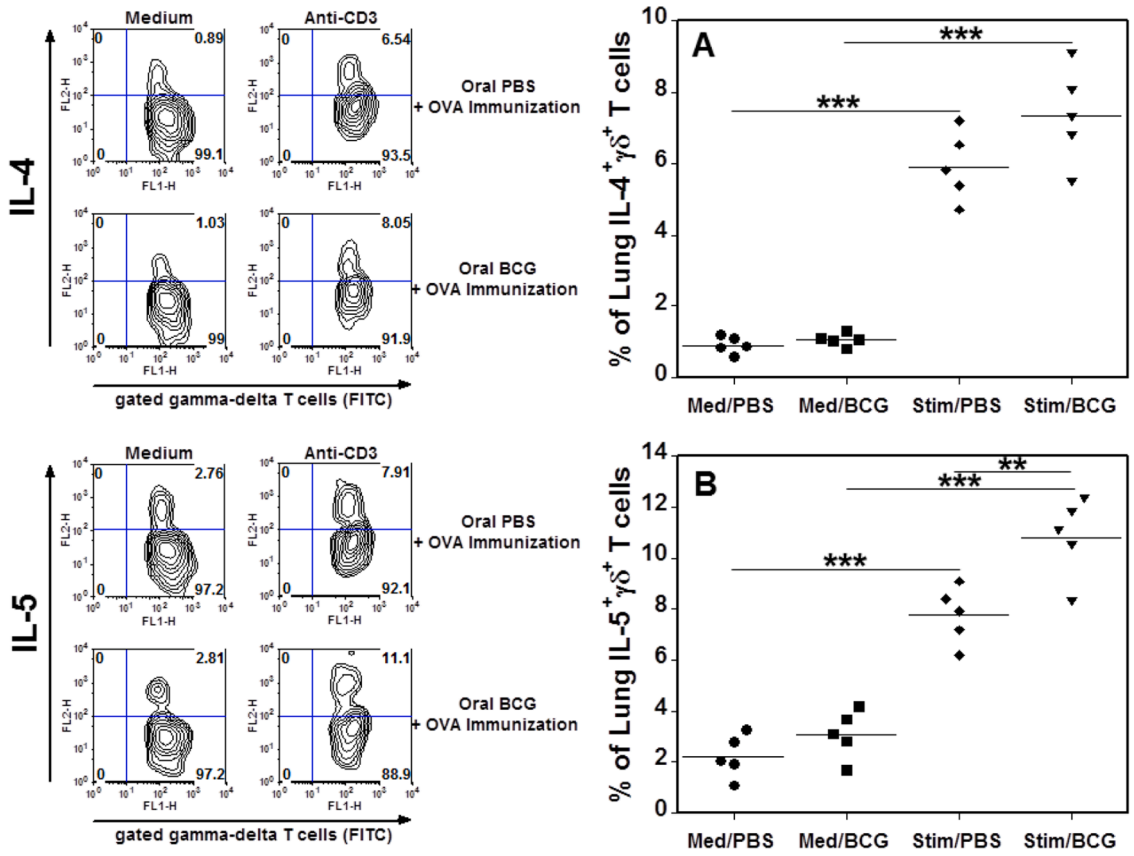

Fig. 6. Oral BCG vaccination increased the potential of lung-tissue γδ± T cells to produce IL-5 upon polyclonal stimulation *in vitro*. Fig. 6 shows the frequencies of IL-4^{+}γδ^{+} (A) and IL-5^{+}γδ^{+} (B) T cells in lung mononuclear cells from mice orally vaccinated with BCG or gavaged with PBS followed by systemic OVA immunization. Lung mononuclear cells were obtained 48 hours after the aerosolized OVA challenge. Cells were either left in the medium alone or stimulated with anti-CD3 mAb *in vitro*. ANOVA, Bonferroni post-tests compared groups, (**P<0.01, ***P<0.001, n=5). FACS plots in the left panel show each experimental group's representative mouse. Different groups are specified in the graphs and FACS plot panels. Experiments were repeated twice with similar results.

description that oral BCG vaccination diminished Treg cells in the lung inflammatory site generated by an unrelated antigenic challenge. Tregs are supposed to control tissue inflammation and functional and proliferative lymphocyte activities [14]. Therefore, a diminished proportion of Tregs versus effector T cells upon antigenic challenge raises concerns about the degree of tissue lesions generated by a BCG-potentiated immune response that could not avoid constant antigenic contacts as for allergens [15]. In addition, some types of allergic conditions are aggravated by IL-17 [17]. Our results show that after the OVA challenge, the oral BCG vaccination promoted higher frequencies of CD4^{+}IL-17^{+} TH17 and potential γδ^{+}IL-17^{+} cells in the lungs (Figs. 5 and 7B). Despite the lower production of OVA-specific serum IgE in animals previously vaccinated with BCG by the oral route (Fig. 8B), the mucosal BCG vaccination strategy deserves more studies before any additional suggestions concerning allergy prevention or treatment could be made. Yet, small clinical trials suggest that subcutaneously BCG vaccination may effectively prevent or treat different types of allergies, and IL-17 is not always associated with allergy aggravation but with protection in some cases [18,26]. The present study has also shown that previous oral BCG administration increased the frequencies of either CD4^{+} or CD8^{+} and γδ^{+} T cells producing IFN-γ upon challenge with aerosolized Ovalbumin (Figs. 4A, 4B, and 7A). Also, the increased production of IgG2a/c specific for OVA indicates a TH1 bias *in vivo* since the IgG2a/c subclass switch is IFN-γ dependent [27]. However, the potential for making TH2-type cytokines (IL-4 and IL-5) in lung-primed T cells remains after a brief activation with anti-CD3 mAb (Figs. 2A, 2B, 3A, 3B, and 6B). Together, these results indicate that oral BCG vaccination provides the environment for a multifunctional immune response to unrelated antigens, that is polarized to IFN-γ and IL-17 but can potentially make TH2 cytokines. We have not directly assessed multifunctional CD4+, CD8+, or γδ+ T cells; therefore, we cannot make any argument on individual T cells in this study. Multifunctional T cells are related to a more protective and long-term effective immune response to different intracellular pathogens, including the Mycobacterium tuberculosis [11,25,28–32]. However, we have demonstrated a multilineage immune response potentiation by previous oral BCG vaccination. This multilineage immune response involves αβ^{+} and γδ^{+} T cells and the increased production of TH1-, TH2-, and TH17-related cytokines and may be part of a more effective response, as previously suggested [30]. The CD4 and CD8 molecules are also expressed in a small subpopulation of mice's innate lymphoid cells (ILC3s and Lti cells) and subpopulations of dendritic cells [33,34]. Therefore it remains possible that part of the cytokines produced by CD4^{+} or CD8^{+} cells is not derived from T cells. The long period between BCG oral priming and the airway OVA challenge (9 months) shows that the immune modifications are long-term, if not permanent. We have analyzed lung mononuclear cells in a few mice from a preliminary study, more than one year after oral BCG exposure, with similar findings, suggesting that oral BCG administration may counteract or prevent senescent immunodeficiency (data not shown) [10]. The long duration of this booster effect in the subsequent OVA immune response provided by oral BCG vaccination could be due to the persistence of the live BCG in the host. Although we have not studied BCG host persistence, previous studies show that BCG may become undetectable in mice within two months of oral vaccination/infection [35,36]. Therefore, the BCG persistence hypothesis can not explain our long-term effect unless the key modification was completely settled during the OVA immunization period. The systemic immunization made with alum adjuvant resulted in a discrete lung immune response upon the OVA challenge

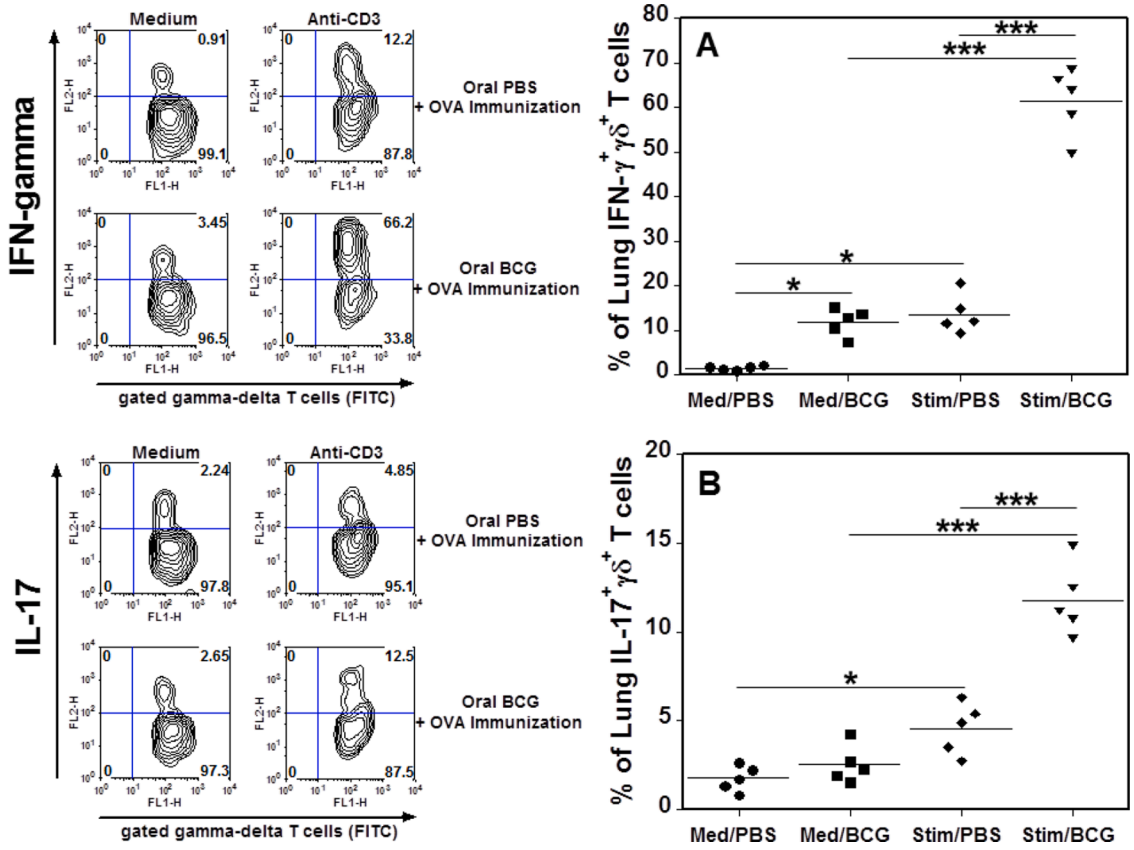

Fig. 7. Previous mucosal contact with BCG increases the percentage of lung IFN-γ^+ but not IL-17^+ γδ T cells after *in vivo* restimulation with OVA in the airways. It potentiates the production of these cytokines upon anti-CD3 mAb stimulation. Fig. 7 shows the frequencies of IFN-$\gamma^+\gamma\delta^+$ (A) and IL-$17^+\gamma\delta^+$ (B) T cells in lung mononuclear cells from mice orally vaccinated with BCG or gavaged with PBS followed by systemic OVA immunization. Lung mononuclear cells were obtained 48 hours after the aerosolized OVA challenge. Cells were either left in the medium alone or stimulated with anti-CD3 mAb *in vitro*. ANOVA, Bonferroni post-tests compared groups, (*P<0.05, ***P<0.001, n=5). FACS plots in the left panel show each experimental group's representative mouse. Different groups are specified in the graphs and FACS plot panels. Experiments were repeated three times with similar results.

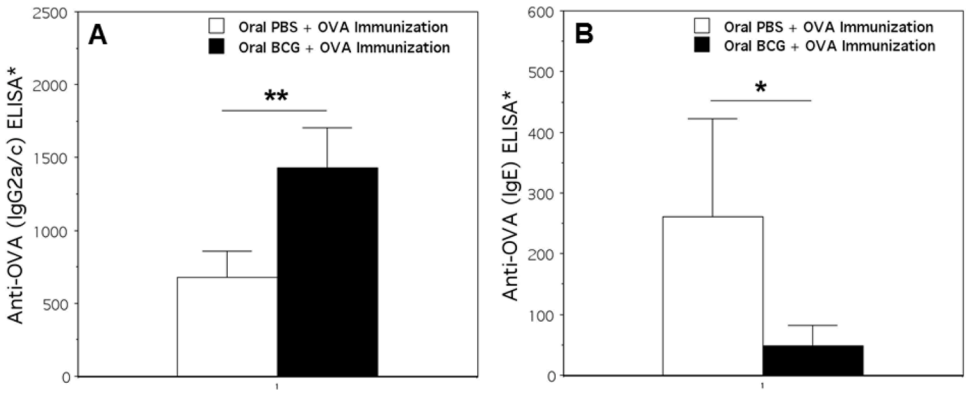

Fig. 8. Oral BCG vaccinated mice produced more OVA-specific IgG2a/c and less IgE antibodies six months after OVA/Alum systemic immunization. Fig. 8 shows the serum levels of anti-OVA IgG2a/c (A) and IgE (B) in PBS gavaged compared to oral BCG vaccinated mice. ELISA* represents a running sum of O.D. values for ELISA readings from l/25 to l/l2800 serum dilutions. ELISA* is proportional to the area under the serum titration curve. Results were compared by the Mann-Whitney test (*P<0.05, **P<0.01, n=5).

compared to animals orally vaccinated with BCG, showing that previous exposure to BCG affects future immunizations with unrelated antigens. In addition, the BCG-potentiated immune response was spreading to the lungs. These observations suggest that oral BCG vaccination may favor effective immune responses to different vaccines and pathogen infections, especially if boosted immunity is required at the mucosal interfaces. It is known that T cell priming at mucosal lymphoid-associated structures like mesenteric, cervical lymph nodes, and Peyer's patches may result in the expression of tissue-specific homing markers and chemokines [37,38]. Therefore, these homing receptors would drive them to major mucosal lymphoid tissues in the body [39]. Indeed, OVA immunization was performed one month after oral BCG vaccination by intraperitoneal route, using very low amounts of antigen. Therefore, it might be possible that some of the mucosal-associated lymphoid structures could be involved in the OVA priming by anatomical proximity and local antigen drainage, generating the associative effect [37] [40]. We can not reinforce or exclude this possibility, but this is important for future investigations as the intraperitoneal route is only used in animal experimentation. Yet another possibility remains: oral BCG vaccination resulted in innate trained immunity, potentiating the OVA adaptative immune response. We have not studied the innate immune system in this model. Still, other studies strongly suggest that trained immunity

induced by oral BCG vaccination may explain our results [25] [41]. However, no evidence shows that trained immunity induced by oral BCG administration can associate different antigens and immunization sites with an integrated long-term mucosal/systemic response.

Together, the results presented in this study suggest that oral BCG vaccination is a viable strategy to increase a multilineage mucosal immune response to unrelated antigenic stimulation, opening up new possibilities to boost vaccines at the level of mucosal sites. In addition, oral BCG vaccination could potentiate non-specifically the mucosal immune response to various pathogens, thus making the BCG a very interesting biological platform for genetic modifications aiming to insert genes coding immunizing proteins.

Declaration of Competing Interests

The authors declare no conflict of interest.

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