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# Bacillus Calmette–Guérin (BCG) vaccine generates immunoregulatory cells in the cervical lymph nodes in guinea pigs injected intra dermally

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ABSTRACT

This work demonstrates the presence of immune regulatory cells in the cervical lymph nodes draining Bacillus Calmette-Guérin (BCG) vaccinated site on the dorsum of the ear in guinea pigs. It is shown that whole cervical lymph node cells did not proliferate *in vitro* in the presence of soluble mycobacterial antigens (PPD or leprosin) despite being responsive to whole mycobacteria. Besides, T cells from these lymph nodes separated as a non-adherent fraction on a nylon wool column, proliferated to PPD in the presence of autologous antigen presenting cells. Interestingly, addition of as low as 20% nylon wool adherent cells to these, sharply decreased the proliferation by 83%. Looking into what cells in the adherent fraction suppressed the proliferation, it was found that neither the T cell nor the macrophage enriched cell fractions of this population individually showed suppressive effect, indicating that their co-presence was necessary for the suppression. Since BCG induced granulomas resolve much faster than granulomas induced by other mycobacteria such as *Mycobacterium leprae* the present experimental findings add to the existing evidence that intradermal BCG vaccination influences subsequent immune responses in the host and may further stress upon its beneficial role seen in Covid-19 patients.

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## 1. Introduction

Regulatory cells play an important role in the control of immune responses. Under optimal conditions, immunocompetent cells help, amplify or suppress the activity of other cells so that any foreign material or invading pathogen are eradicated with minimum damage to the host. Lapses of such regulation could lead to excess detriment to the body or susceptibility of the host to the invader. Normally, under average conditions of antigenic stimulation, the immunostimulatory and suppressor activities are in equilibrium. What conditions decide which of the two mechanisms should choose to occur depends on the host and the occurring infection and are still under investigation. Furthermore, it is now generally accepted that the immune response is a collaborative result involving different populations and subpopulations of cells [1].

Helper function to humoral and cellular immunity has been ascribed to subpopulations of T cells, macrophages, dendritic cells and even B cells [2]. On the other hand, suppression of the two types of immunity has been thought to be regulated by mainly regulatory T cells (former suppressor T cells), certain populations of monocytes/macrophages and B cells [3–5]. In a number of diseases that are associated with low cell mediated immunity, T regulatory (suppressor) cells play an important role, either on their own or in combination with other cells [6–8].

Macrophages may be either stimulatory or inhibitory in immunological reactions and exhibit both pathogenic and protective roles [9–12]. They not only present antigens to mainly T and B cells but also secrete several cytokines which direct the responses of other immunoregulatory cells. The three major functions of macrophages include degradation of non-self or foreign material including apoptotic or necrotic cells, initiation and enhancement of the immunological activation of lymphocytes and, mediation of suppression [13,14]. Macrophages may cause suppression either by helping the generation of other suppressor cells [15,16] or, by releasing immune suppressive factors such as prostaglandins [17,18] and immunoregulatory cytokines such as interleukin-10 These factors in turn, cause the limitation of extensive tissue damage by diminishing the production of inflammatory mediators that cause specific and unspecific immune reactions [19].

B cells may also cause suppression under certain immunological conditions. Involvement of B suppressor cells was demonstrated in delayed type hypersensitivity responses to antigens such as

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ovalbumin, 2,4-dinitro-1-fluorobenzene and Keyhole limpet haemocyanin among others [20–22]. They may act through a negative feedback by specific antibodies or through the induction of suppressor T cells [23]. Their involvement in autoimmune diseases such as multiple sclerosis through memory cell function has also been stressed [24].

The granulomas induced by BCG are very different from those induced by *Mycobacterium leprae* (*M. leprae*) in the guinea pig and have been extensively studied for their immune responsive effects. BCG induces an 'immunological' epithelioid cell granuloma that shows containment, successful killing and degradation. Here the mononuclear phagocyte series take the form of epithelioid cells with extensive rough endoplasmic reticulum. On the other hand, *M. leprae* forms a 'non-immunological' macrophage-type granuloma that shows absence of organization of cells with failure to completely degrade. There is no evidence of epithelioid cell formation but the presence of undifferentiated macrophages that remain loaded with mycobacteria [25]. The BCG vaccine has been used for nearly a century now for protection against tuberculosis but, it also protects against leprosy at a varying magnitude [26]. Recent interest in BCG was triggered because of its relation to the reduction in the severity and the mortality rate of Covid-19 patients that were vaccinated [27–31] and, this has been associated with trained immunity [28,32]. According to some reports BCG may be an option to enhance immunity of at-risk populations such as the elderly and healthcare workers for Covid-19 disease [33–35].

The objective of this work was to investigate the immune regulatory mechanism responsible for the induction of an 'immunological' type granuloma in the draining lymph node after BCG vaccination in guinea pigs and their early resolution in contrast to that observed with another mycobacterium, *M. leprae.*

## 2. Materials and methods

### 2.1. Animals

Outbred Dunkin Hartley strain of guinea pigs of either sex were from David Hall, Newchurch, Staffs UK. They were fed on RGP pelleted diet (C.F. Dixon and sons, Ware, Herts) supplemented with cabbage and hay. Protocols covering the use of animals were followed strictly according to the Legislation for Animal Research of 'The Animal (Scientific Procedures) Act, UK 1986'.

### 2.2. Mitogen

Concanavalin A (Con A, Pharmacia Fine Chemicals, Sweden) was dissolved in phosphate buffered saline at a concentration of 1.5 mg/ml, filter sterilized and stored in aliquots at −20 °C until use.

### 2.3. Antigens

PPD (tuberculin purified protein derivative, Central Veterinary Laboratory, Weybridge, UK) – 2 mg/ml was dialyzed against 20 volumes of PBS at 4 °C for 24 h. It was filter sterilized and stored in aliquots of 0.25 ml at −20 °C. Leprosin, a soluble extract of *M. leprae* was obtained from the Clinical Research Center, Harrow, London. Live Bacillus Calmette- Guérin (BCG, Pasteur strain) was obtained from the Pasteur Institute (Paris). In cell cultures, it was used as such, heat killed (60 °C for 60 min) or cobalt irradiated at 2 megarads (co-irr). The *M. leprae* used was always cobalt irradiated (2 megarads) because of legal restrictions on the use of the live form due to its pathogenicity in man.

### 2.4. Immunization

Guinea pigs weighing about 450 g were injected intradermally on the dorsum of the ear with $1 \times 10^7$ BCG, a live attenuated vaccine or $1 \times 10^9$ co-irr *M. leprae* in 0.05 ml saline.

### 2.5. Preparation of peritoneal exudate cells (PECs)

Autologous peritoneal exudate cells were used as antigen presenting accessory cells. Animals were injected intraperitoneally with 20 ml paraffin oil and on the fourth day the washing from the peritoneal cavity was collected and immediately centrifuged at 400*g* for 10 min to remove the oil, washed twice with plain culture medium and suspended in complete medium ($10^6$ viable cells/ml) for further use.

### 2.6. Proliferation of the post auricular and cervical lymph node cell suspensions

The draining post auricular (PA) and cervical (CER) lymph nodes were collected 2 weeks after immunization in the case of BCG (unless otherwise stated) and after 5 weeks in the case of *M. leprae*. Hematoxylin and eosin staining of histological sections and electron microscopy of the lymph nodes were done as described by Narayanan et al. [36]. They were cut into small pieces in Hank's Balanced Salt Solution (HBSS), gently teased to release the cells and the suspension of cells was passed through a steel wire mesh to remove the cell debris. Cells thus obtained were washed three times with HBSS at 400 *g* and re-suspended ($2.5 \times 10^6$ viable cells per ml) in complete medium (RPMI-1640 supplemented with 10% fetal calf serum, 100 IU/ml penicillin, 100 μg/ml streptomycin, 4 mM L-glutamine and 2 mM sodium pyruvate). $5 \times 10^5$ of these cells in 200 μl were cultured in 96-well round-bottom plates with 25 μg/ml PPD, 10 μg/ml leprosin for 66 h or 3 μg/ml Con A for 72 h at 37 °C in the presence of 5% $CO_2$. 1 μCi $^3$H thymidine (Amersham International) was added 24 h before harvesting and the uptake was estimated using liquid scintillation counter (Packard, Berks UK) [37,38]. Cells were also cultured in the presence of live BCG, heat-killed BCG or co-irr BCG for five days, pulsed with thymidine and harvested as above. All cell cultures were done in quadruplicates.

The results are expressed as stimulation index (T/C) defined as counts/min of $^3$H thymidine uptake by proliferated cells divided by the counts/min of unstimulated cells. Thus, T/C expresses the extent of proliferation of sensitized T cells relative to non-sensitized, unstimulated cells when cultured with antigen or mitogen.

Additionally, indomethacin was added in the cell culture to check whether the BCG induced suppression was prostaglandin mediated. Indomethacin (Sigma UK) was dissolved in ethanol at a concentration of 20 mg/ml, the solution was diluted with excess PBS to 500 μg/ml and filter sterilized. When required, 10 μg/ml of indomethacin was added to cell cultures.

### 2.7. Preparation of non-adherent and adherent cells from the cervical lymph nodes of BCG injected animals

To identify which fraction of the cell population of the cervical lymph node was responsible for the observed lack of proliferation, the total cells were separated into adherent and non-adherent fractions using a nylon wool (NW) column, a rapid, single step and cost effective procedure for the separation of high viability T cells that flow out in the non-adherent fraction, from heterogeneous mononuclear cell preparations [35,36,39]. Briefly, $10^8$ cells in 4 ml complete medium were incubated for 45 min in the NW column that was pre-incubated for 1 h with 5% fetal calf serum. The

NW non-adherent cells that are enriched in T cells were eluted with 5% fetal calf serum, washed and suspended in complete medium. Subsequently, the adherent cells were released by teasing the nylon wool and eluting them out. The non-adherent and adherent cells were suspended separately at concentration $5 \times 10^6$ viable cells/ml in complete medium for further use.

### 2.8. Proliferation of mixtures of non-adherent and adherent cell populations of cervical lymph node cells in the presence of autologous accessory cells

The non-adherent and adherent cell populations were mixed at ratios of 0:100, 25:75, 50:50, and 100:0 in 96 cell culture plates to make a total of $5 \times 10^5$ cells per well in 100 µl complete medium. These were cultured in quadruplicates in the presence of 25 µg/ml PPD as the antigen and $5 \times 10^5$ irradiated (1800 rads) autologous PECs as the antigen presenting cells (Section 2.6).

### 2.9. Estimation of the cell phenotypes in the NW adherent fraction of the cervical lymph node cells from BCG injected guinea pigs

Cell phenotypes present in the NW adherent fraction of the cervical lymph node cells from BCG injected guinea pigs were identified after staining them with respective antibodies. $1 \times 10^6$ NW adherent cells were incubated for 30 min in 100 µl of the following anti guinea pig cell monoclonal antibodies [37,40]: MSgp7, pan T marker antibodies; CT6, putative T suppressor cell marker antibodies (Free University Amsterdam); MSgpM, anti-macrophage antibodies; CT9 (B cell marker). The cells were washed with PBS and incubated for 30 min with fluorescein isothiocyanate labeled anti mouse IgM or IgG (dilution 1:20, Sigma, UK) and the number of positive cells was estimated on a flow cytometer (FACS-1, Becton-Dickinson, Rutherford, N.J., USA).

### 2.10. Proliferation of NW non-adherent cells in the presence of T cells or macrophage enriched population obtained from the NW adherent fraction of the cervical lymph node

To elucidate the relative involvement of either T cells or macrophages (isolated in the NW adherent fraction of the cervical lymph node) in the suppression of cell proliferation, enriched fractions of each of the two populations were prepared by depleting the other using immunomagnetic separation. $10–15 \times 10^6$ cells from the adherent fraction were incubated with 0.5 ml of MSgpM or MSgp7 for 30 min, washed and incubated with magnetic beads coated with sheep anti mouse IgG (for MSgpM) or anti mouse IgM (for MSgp7) (Dynal, Oslo, Norway), for 20 min at 4 °C. The number of beads added was 3–4 per positively labeled cells (Section 2.9). The suspension was diluted 5–10 times with Hank's balanced salt solution containing 1% fetal calf serum and placed on a cobalt-samarium magnet (Magnetic Development, Swindon, Wilts, UK) that attracted the rosetted cells to the sides of the tube (Fig. 1). The unrosetted cells were decanted off and the process was repeated. Therefore, cells in the decanted fraction contained either the T cell enriched or the macrophage enriched fraction which were subsequently added separately to the non-adherent T cells ($2.5 \times 10^5$ cells adherent cells plus $2.5 \times 10^5$ cells non– adherent cells). The final cell mixtures were cultured in quadruplicates with 25 µg/ml PPD in the presence of $1 \times 10^5$ irradiated PECs as antigen presenting cells (Section 2.8).

### 2.11. Statistical analysis

The effects of injected BCG and *M leprae* on the stimulation index (T/C) measured in the post auricular and cervical lymph node cell preparations cultured with PPD, leprosin, concavalin A, or BCG: live, heat killed or co-irr were compared using student's *t*-test for means comparison. Since the BCG and *M. leprae* were injected to different animals the independent samples *t*-test was applied. Depending on the result of the equality of variances Levene's test the appropriate degrees of freedom were used. Differences in the T/C means were considered significant at the $p < 0.05$ level. The measurements are represented as means ± SD. All statistical analyses were conducted using SPSS 20 statistical software (IBM Inc., Chicago, IL, USA, Version 25.0, 2019). For non-linear model fitting and graphical presentations SigmaPlot for Windows 12.5 (Systat Software Inc. San Jose, California, US) was used.

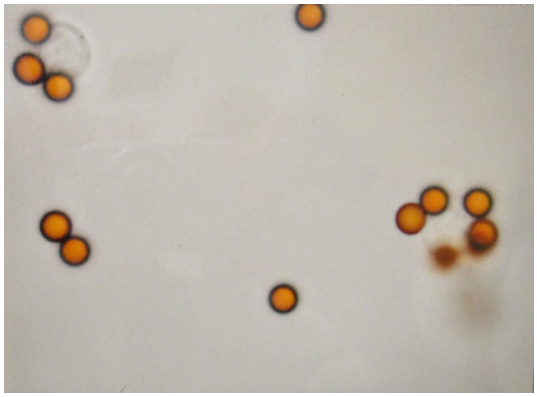

**Fig. 1.** Anti-mouse IgG coated immunobeads rosetting cells labelled with MsgpM (magnification x320).

## 3. Results

In Fig. 2, granulomas in the draining post auricular lymph nodes formed after injection of live BCG or co-irradiated *M. leprae* on the dorsum ear are shown. Histological sections stained with Hematoxylin & Eosin showed maximum infiltration at 2 weeks for BCG and at 5 weeks for *M. leprae.* At these times, the draining cervical lymph nodes showed no granuloma formation but extensive lymphoproliferation. Fig. 2a shows a section of a distinct BCG draining granuloma in the post auricular lymph node consisting of large cells with epithelioid cell morphology surrounded by lymphocytes and fibroblasts. No acid-fast bacilli were detected. Fig. 2b shows an enlarged image of an epithelioid cell in the BCG granuloma with characteristic large nuclei, prominent nucleoli and swollen stacked rough endoplasmic reticulum (arrows). Fig. 2c shows a histological section of an *M leprae* granuloma. The majority of infiltrating cells are phagocytic macrophages. Fig. 2d shows an enlarged image of a macrophage with extensive vacuolation and degraded, undigested remains of *M. leprae* (arrow). The above findings were in accordance with the results of previous works [36,41].

### 3.1. Proliferative responses of post auricular and cervical lymph node whole cell populations to antigens after BCG and M. leprae injection

In Fig. 3A plots of the extent of proliferation of whole lymph node cells expressed as stimulation index (T/C) are shown for BCG (bars a, b in each group) and *M. leprae* (bars c, d) draining lymph nodes in the presence of the two soluble mycobacterial antigens PPD and leprosin. In Fig. 3B corresponding bar plots are shown in the presence of a non-specific mitogen Con A. From Fig. 3A it appears that except for PA lymph node cells with PPD, T/C is much lower for animals injected with BCG than *M. leprae* (compare bars b with d in both groups and a with c in the second). In other words, the cells from BCG injected animals responded to a lesser extent to specific antigens compared with *M. leprae*. This is

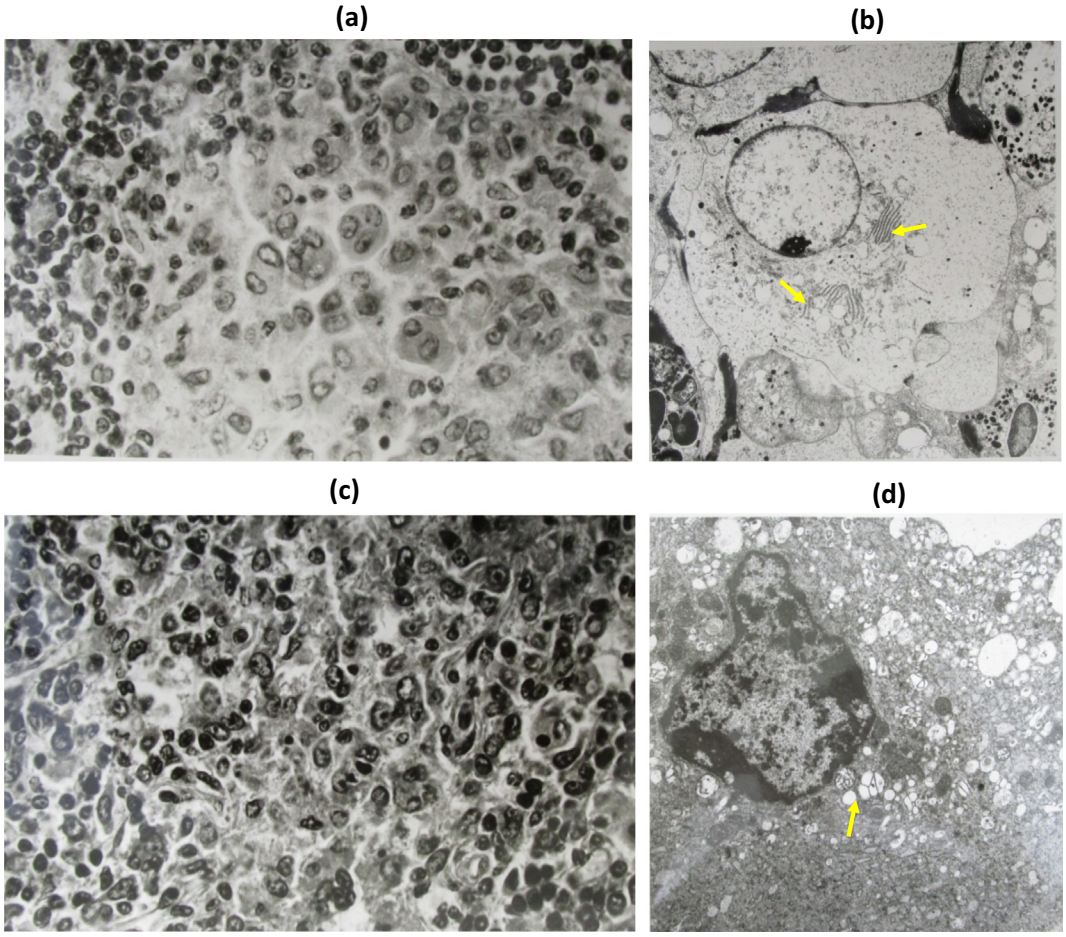


**Fig. 2.** (a) Optical microscopy image (x320) of histological section of BCG draining granuloma in the post auricular (PA) lymph node and (b) corresponding single epithelioid cell (transmission electron microscope x6000, arrow indicates stacked rough endoplasmic reticulum); (c) Optical microscopy image (x320) of histological section of M. leprae draining granuloma in the PA lymph node and (d) corresponding TEM image of a single macrophage (x10000, arrow indicates vacuoles with undigested M. leprae).

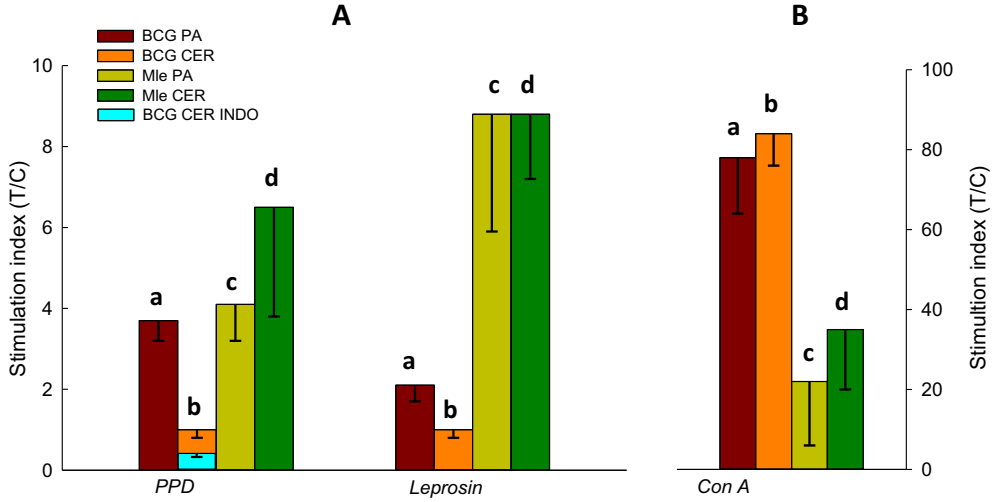


**Fig. 3.** A. Proliferative responses expressed as stimulation index of post auricular and cervical lymph node cell preparations from BCG or *M. leprae* injected guinea pigs in the presence of PPD or leprosin. B. Proliferative responses in the presence of concavalin A.

particularly obvious for the responses of the cervical lymph nodes cells. The differences were also confirmed by statistical analysis (Table 1). Except for the PA cells with PPD, the responses of BCG and *M leprae* injected animals were significantly different for PA with leprosin ($p$ = 0.025), for CER with PPD ($p$ = 0.032) and for CER with leprosin ($p$ = 0.002). On the other hand, in the presence of Con A (graph 3B), the cells from the BCG injected animals proliferated extensively (Y-axis scale in 3B is x10 larger than in 3A), indicating that otherwise the cells were active and responsive. Furthermore, addition of 10 μg/ml indomethacin did not enhance

**Table 1**
Statistical analysis (means comparison, *t*-test) of the effects of BCG and *M. leprae* injected in the ear on the stimulation index (T/C) of post auricular and cervical lymph node cells in the presence of PPD or leprosin.

| Comparisons | Levene | | 'Student's' *t* test | |
|---|---|---|---|---|
| | F | p -value | t | p - value |
| BCG/PA/PPD – Mle/PA/PPD | 0.874 | 0.386 | −0.771 | 0.470 |
| BCG/CER/PPD – Mle/CER /PPD | 7.631 | 0.033 | −4.125 | **0.025** |
| BCG/PA/LSN – Mle/PA/LSN | 6.182 | 0.047 | −3.728 | **0.032** |
| BCG/CER/LSN – Mle/CER /LSN | 4.405 | 0.081 | −0.9693 | **0.002** |

Abbreviations: BCG, Bacillus Calmette-Guerin Vaccine; PA, post auricular; PPD, purified protein derivative; Mle, *M. leprae*; CER, cervical; LSN, leprosin.

the response of the BCG cervical lymph node cells to PPD (first group, lower of the two stacked bars in b), signifying that the suppression was not prostaglandins mediated. The comparatively elevated responses of *M. leprae* draining lymph nodes to mycobacterial antigens may be an outcome of the excessive inflammatory infiltration observed *in vivo* and was not further analyzed due to reasons explained below. Also, the lower response of these lymph nodes to Con A has been addressed by Gupta et al. [37].

To further examine whether the response of BCG injected animals to antigens changes with harvesting time, the stimulation index (T/C) at two and five weeks after BCG injection ($10^7$) to guinea pigs was compared and the results are presented in Table 2. It can be seen that PA lymph node cells responded to PPD both at two and five weeks after injection (T/C from 3.21 to 4.4, and from 1.17 to 5.35 respectively) and the responses at the two harvesting times were not statistically different (*t*-test, p = 0.768). Additionally, the data in Table 2 show that the responses of the cervical lymph node cells both after two and five weeks remained low (from 0.74 to 1.24, and from 0.88 to 1.28) and not significantly different (*t*-test, p = 0.320).

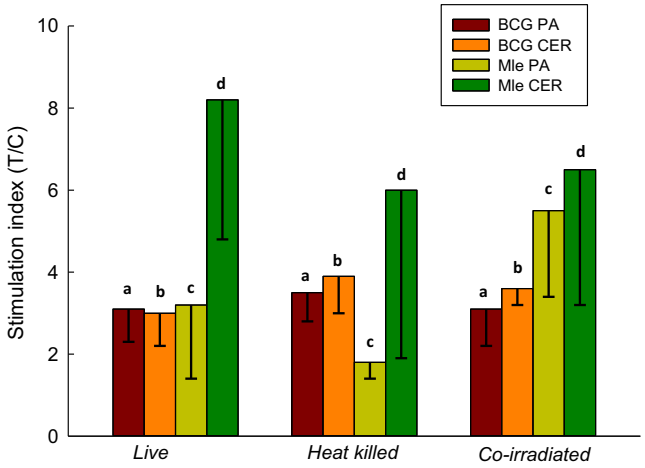


**Fig. 4.** Proliferative responses expressed as stimulation index of post auricular and cervical lymph node cell preparations from BCG or *M. leprae* injected guinea pigs in the presence of 107 whole BCG mycobacteria (live, heat killed or co-irr).

### *3.2. Proliferative responses of post auricular and cervical lymph node cell populations to live, heat killed or co-irr BCG mycobacteria after BCG and M. leprae injection*

Following the unresponsiveness of BCG induced cervical lymph nodes to soluble mycobacterial antigens, it was thought worthwhile to check the response of lymph node cells of BCG and *M. leprae* injected animals to whole BCG organisms. Differently processed whole BCG bacteria ($10^7$) (live, heat killed or co-irr) were used, representing three different states. The results of the cell proliferative responses expressed as stimulation indices (T/C) are presented in Fig. 4. In each group, the first two bars (a, b) correspond to BCG and the last two (c, d) to *M. leprae* induced lymph node cells. It is seen that for the BCG injected animals the cells from PA and the CER lymph nodes gave T/C between 3 and 4, i.e. they responded 3–4 times more than the starting population. This is in contrast to the previously observed unresponsiveness to soluble antigens (Fig. 3), signifying the presence in the lymph nodes of cells reactive to whole BCG. No significant differences are seen in Fig. 4 between the responses of BCG draining PA and CER lymph nodes (compare bars a and b in each group). Regarding the response from *M. leprae* injection, with the exception of PA lymph nodes to heat killed mycobacteria, T/C ranged between 3.2 (live, bar c) and 8.1 (live, bar d) indicating greater overall proliferation than BCG.

Statistical analysis (*t*-test) was conducted to identify significant differences between the T/C of PA or CER lymph node cells for BCG and *M. leprae* injected animals with live, heat killed and co-irr BCG mycobacteria. The results in Table 3 show that BCG induced significantly lower response in the CER lymph nodes than *M. Leprae* (p = 0.044) as it was also the case with the response to soluble mycobacterial antigens (Fig. 3). However, BCG induced higher PA lymph node responses compared to *M. leprae* in the heat killed form (Fig. 4) bars a and c in the second group) (p = 0.005) which contradicts the results on soluble antigens, indicating different

**Table 2**
Proliferative responses expressed as stimulation index (T/C, mean ± SD) from the post auricular and cervical lymph node cells of guinea pigs to PPD, two and five weeks after injection with $10^7$ BCG.

| Two weeks after injection | | | Five weeks after injection | | |
|---|---|---|---|---|---|
| Experiment | Cell origin | T/C | Experiment | Cell origin | T/C |
| 1 | PA | 4.40 ± 1.14 | 5 | PA | 5.35 ± 0.50 |
| | CER | 0.99 ± 0.22 | | CER | 1.27 ± 0.28 |
| 2 | PA | 3.21 ± 0.53 | 6 | PA | 2.60 ± 0.13 |
| | CER | 0.98 ± 0.29 | | CER | 1.12 ± 0.24 |
| 3 | PA | 3.64 ± 0.47 | 7 | PA | 1.17 ± 0.03 |
| | CER | 1.24 ± 0.35 | | CER | 1.28 ± 0.01 |
| 4 | PA | 3.60 ± 0.30 | 8 | PA | 4.49 ± 1.55 |
| | CER | 0.74 ± 0.13 | | CER | 0.88 ± 0.21 |

**Table 3**
Statistical analysis (means comparison, *t*-test) of the effects of BCG and *M. leprae* injection on the stimulation index (T/C) of post auricular and cervical lymph node cells in the presence of live, heat killed or co-irr BCG.

| Comparisons | Levene's test | | 'Student's' *t*-test | |
|---|---|---|---|---|
| | F | P -value | t | P - value |
| BCG/PA/LV – Mle/PA/LV | 1.534 | 0.262 | −0.104 | 0.920 |
| BCG/CER/LV – Mle/CER/LV | 2.964 | 0.136 | −2.535 | **0.044** |
| BCG/PA/HK – Mle/PA/HK | 0.474 | 0.517 | 4.298 | **0.005** |
| BCG/CER/HK – Mle/ CER /HK | 7.190 | 0.036 | −1.005 | 0.383 |
| BCG/PA/IR – Mle/PA/IR | 1.483 | 0.269 | −0.165 | 0.874 |
| BCG/CER/IR – Mle/CER /IR | 3.587 | 0.107 | −1.751 | 0.131 |

Abbreviations: BCG, Bacillus Calmette-Guerin Vaccine; PA, post auricular; CER, cervical; LV, live BCG; HK, heat killed BCG; IR, cobalt irradiated BCG.

responses of BCG and *M. leprae* draining lymph nodes to the heat-killed form of BCG mycobacteria. Particularly, this may be due to the inability of proper digestion and presentation in culture *in vitro*. More studies on *M. leprae* induced granulomas were not further pursued because of dearth of supply of this mycobacterium.

### 3.3. Proliferation in co-culture of NW adherent and non-adherent cells from cervical lymph nodes of BCG injected animals

To elucidate which cells of the BCG draining cervical lymph node caused suppression of proliferation and lowering of the stimulation index (T/C) described above, they were separated into two fractions, the nylon wool (NW) adherent and the NW non-adherent. Then, the two fractions were mixed at different ratios and the T/C with PPD was determined using PECs as accessory antigen presenting cells. The results are presented in Fig. 5 where it can be seen that the non-adherent cells proliferated up to a T/C of 16 when cultured without the adherent, but with increasing proportion ($X$) of adherent cells the index decreased exponentially. The relationship is described by Eq. (1) with excellent fitting of the data as indicated by the value of the coefficient of determination ($R^2$ = 0.999).

$$T/C = 3.20 + 12.70e^{(-0.070X)} \quad (1)$$

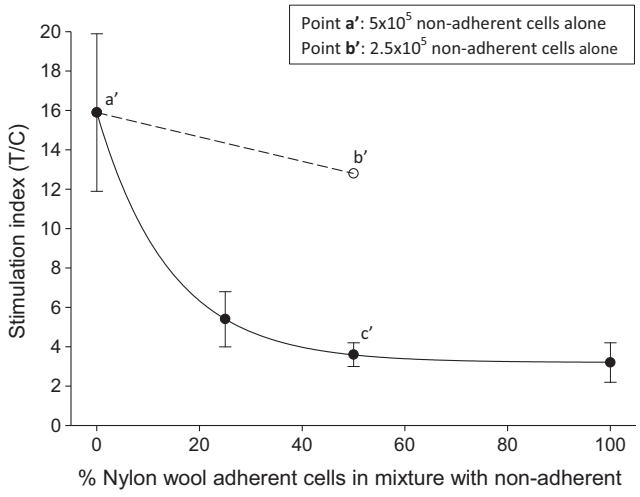


**Fig. 5.** Plot of stimulation index with added percentage of nylon wool adherent cells in mixtures with non-adherent from the cervical lymph nodes of BCG injected guinea pigs in the presence of PPD using PECs as antigen presenting cells (mean ± S. E., n = 4). Dotted line connects T/C values obtained with non-adherent cells only: number of cells at point a'=5 × $10^5$ and point b'=5 × $10^5$.

The sudden drop of proliferation reaching T/C of about 5 at only 20% proportion of adherent cells, ==signifies their important role in suppression. To further demonstrate the strong abrogative effect of the adherent cells an experiment was conducted using of 2.5 × $10^5$ non-adherent cells, the same number as that in the 50% mixture (point c'), but in the absence of adherent cells (b'). As it can be seen from the dotted line in Fig. 5, the T/C obtained was 12.8, which is about 3.5 times higher than the T/C of 3.6 obtained with the same number 2.5 × $10^5$ non-adherent cells but mixed with equal number of adherent (point c'). This result clearly signifies the strong suppressive effect of the NW adherent fraction of the BCG draining cervical lymph nodes cells.

### 3.4. Phenotypic analysis of cells in the NW adherent fraction of the BCG stimulated cervical lymph nodes

To elaborate the cell phenotypes that were present in the NW adherent fraction of the BCG induced cervical lymph nodes they were labeled with monoclonal antibodies specific for major cell populations found in the guinea pig lymph nodes. These included MSgp7 (pan, total T cells); CT6 (T suppressor cells); MSgpM (macrophages); MSgp9 (B cells). The proportions of the cell phenotypes in the NW adherent fraction of cells from the cervical lymph node are presented in Table 4. The majority were macrophages (total 46.6%) and B cells (total 32.8%) followed by T lymphocytes (totals 16.6%) which consisted mostly of the suppressor lineage (14%), as they labelled with CT6, a putative suppressor cell marker. The 4% difference of the total (96%) from 100% can be ascribed to a small population of immature or other cells that did not stain with any of the antibodies. It can be noticed that all pan T cells consisted of suppressor/cytotoxic subtype. Differences in the percentages of MSgp7/CT6 among animals may be ascribed to inter-subject variation.

**Table 4**
Cell phenotypes in the nylon wool adherent fraction of cells from the cervical lymph node.

| Percentage of cell phenotypes | | | |
|---|---|---|---|
| GP No. | MSgp7[a] (CT6[b]) | MSgpM[c] | MSgp9[d] |
| 1 | 14 (114.3%) | 41 | 42 |
| 2 | 13 (92.3%) | 39 | 48 |
| 3 | 15 (100%) | 50 | 28 |
| 4 | 15 (100%) | 48 | 27 |
| 5 | 26 (46.2%) | 55 | 19 |
| Mean ± SD | 16.6 ± 5.3 (14 ± 1.9) | 46.6 ± 6.6 | 32.8 ± 11.9 |

Explanations:
[a] Pan T cells.
[b] Parenthesis gives percentage suppressor/cytotoxic T cells, which are a subpopulation of Pan T cells.
[c] Macrophages.
[d] Pan B cells.

### 3.5. Proliferation of macrophage or T lymphocyte enriched NW adherent cell populations in co-cultures with NW non-adherent cells from BCG stimulated cervical lymph nodes

Since the results of the phenotypic analysis showed that the NW adherent population of cells from BCG stimulated cervical lymph nodes consisted of T cells (16.6%) and macrophages (46.6%), it was of interest to further look into their role in suppression. For this purpose, enriched cell populations of (i) T cell enriched and (ii) macrophage enriched were prepared from the adherent fraction by immunobead enrichment (Section 2.10). These were added to the non-adherent (1:1 ratio) in the final cultures. Thus, the total number of cells in the resulting cultures was $2.5 \times 10^5$ enriched adherent (either T cell enriched or macrophages enriched), $2.5 \times 10^5$ non-adherent, $1 \times 10^5$ PECS and PPD (25 μg/ml).

In Fig. 6 are presented the proliferative responses expressed as counts/min of non-adherent cells alone, non-adherent cells with the T cell enriched fraction from the adherent population and non-adherent cells with the macrophage enriched fraction from the adherent population. Although there is large inter-subject variability, in 4 out of the five experimental animals, it is seen that when the T cells or macrophages were present individually in the culture, the proliferative response was enhanced. This is more pronounced with the macrophages enriched culture. Therefore, T cells or macrophages individually increased proliferation, whereas their co-existence in the NW adherent fraction caused immunosuppression as shown by the reduction of the stimulation index in Fig. 5.

## 4. Discussion

In 1981, Narayanan et al demonstrated that intradermal injection of mycobacteria in the ear of guinea pigs caused the formation of granulomas in the draining PA lymph nodes while the CER lymph nodes showed no granuloma but extensive blastogenesis. The BCG draining granulomas were analogous to tuberculoid (immunological) leprosy lesions and the *M. leprae* draining granulomas analogous to lepromatous (non-immunological) leprosy lesions [42]. Therefore, these became excellent models for studying mycobacteria induced granuloma formation and immune responses in an animal model that was not possible in patients.

The present study showed that contrary to *M. leprae* injected guinea pigs, cells from the cervical lymph nodes draining BCG induced granuloma did not respond *in vitro* to soluble mycobacterial antigens, PPD and leprosin, though they responded to whole BCG organisms. The observed suppression was neither due to prostaglandins (addition of indomethacin did not increase the stimulation index (T/C), Fig. 3) nor early harvesting of cells from the lymph nodes. Interestingly, these cervical lymph nodes did contain PPD reactive T cells as was confirmed when the latter were cultured with the antigen in the presence of autologous accessory antigen presenting cells. Additionally, neither the adherent macrophage fraction nor the adherent T cell fraction individually suppressed the response of the T cells, signifying that their synergistic action was required.

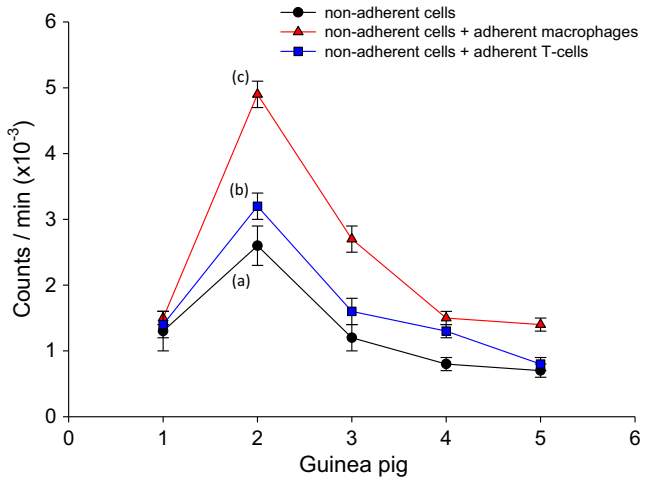


**Fig. 6.** Proliferative responses (counts/min) of (a) nylon wool non-adherent cells alone (circles); (b) non-adherent cells with the T cell enriched fraction from the adherent population (squares); (c) non-adherent cells with the macrophage enriched fraction from the adherent population (triangles) with PPD in the presence of PECS.

In humans, BCG vaccination provides long-term imprinting of suppressor T regulatory phenotypes with low inflammation [43]. Similarly, suppressor macrophages have also been demonstrated in a number of studies involving mycobacteria and other intracellular pathogens [44,45]. Their mode of suppression may be through direct contact with lymphocytes or by releasing suppressive mediators [46,47]. It has also been demonstrated that macrophages and T cells may act together in concert to induce suppression of other cell functions [48–51].

In the present investigation, the *in vitro* suppression observed in the cervical lymph node cells was not due to classical tolerance to the antigen, because cells from the adjacent PA lymph nodes showed marked responses. Therefore, an immune-controlling regulatory mechanism must operate in the cervical lymph nodes of guinea pigs injected with BCG but not with *M. leprae*. And, this must boost the early resolution at 2 weeks post injection along with progressive replacement of the infiltrated areas by fibrosis and decrease in the lymph nodes weight, signs indicating recovery. Similar self-contained lesions are observed in healthy human controls after BCG vaccination and, tuberculoid but not lepromatous leprosy where the lesions are of 'infiltrating' type and wide spread. It has therefore been implied that the suppressor activity in certain infections such as tuberculoid leprosy was generated as part of well-regulated immune response [52]. Thus, it is likely that the suppressor cells in the BCG induced cervical lymph nodes, as seen in this study, participate in the early resolving of the granulomas by controlling excessive proliferation or inflammation.

The immune system is inherently a "double-edged sword" and must be tightly regulated [53]. BCG vaccination offers heterologous protection against unrelated pathogens through altered responses to subsequent stimuli, termed "trained immunity" [54]. Even though it is not clear to what extent guinea pigs can be infected by SARS-COV and SARS-CoV2 [55,56] SARS-CoV-2 has been found to be immunogenic in these animals [57–59]. This has sparked interest in the use of BCG vaccine for potential new therapeutic uses and treatment of autoimmune diseases [52]. In the case of the current Covid-19 pandemic, in countries where BCG vaccination is obligatory, number of deaths due to infection, was reduced [28,29]. Through an experimental model, the present work provides evidence for an induced regulatory mechanism and highlights the role of BCG in the regulation of a granuloma by the induction of immune suppressor cells.

Finally, we show experimentally, the generation of an immunoregulatory mechanism that controls and resolves a granulomatous lymph node condition induced by BCG vaccination. Further characterization of the cell populations involved should be fascinating although it is practically beyond the immediate scope of this investigation.

## 5. Conclusion

In this study, we show that two important cell populations namely, regulator (suppressor) T cells and macrophages function

together in a process that lead to the 'immunological' character and early resolution of a granuloma in guinea pigs vaccinated intra dermally with BCG. For comparison purposes, results from a 'non-immunological' granuloma induced in the same manner by another mycobacterium *M. leprae* are also presented.

## Author contributions

S.V. contributed the *in vivo* and *in vitro* experiments, interpretation of data and writing of the manuscript. I.N. contributed statistical analysis, interpretation of data and writing of the manuscript.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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