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Swarna Bhasma Induces Antigen-Presenting Abilities of Macrophages and Helps Antigen Experienced CD4$^+$ T Cells to Acquire Th1 Phenotypes Against *Leishmania donovani* Antigens

Shashi Saini[1] · Anshul Anand[1] · Abhishek Singh[1] · Baishakhi Mahapatra[1] · Shruti Sirohi[1] · Samer Singh[2] · Rakesh K. Singh[1]

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Abstract

In leishmaniasis, the protective immunity is largely mediated by proinflammatory cytokine producing abilities of T cells and an efficient parasite killing by phagocytic cells. Notwithstanding a substantial progress that has been made during last decades, the mechanisms or factors involved in establishing protective immunity against *Leishmania* are not identified. In ancient Indian literature, metallic "bhasma," particularly that of "swarna" or gold (fine gold particles), is indicated as one of the most prominent metal-based therapeutic medicine, which is known to impart protective and curative properties in various health issues. In this work, we elucidated the potential of swarna bhasma (SB) on the effector properties of phagocytes and antigen-activated CD4$^+$ T cells in augmenting the immunogenicity of *L. donovani* antigens. The characterization of SB revealing its shape, size, composition, and measurement of cytotoxicity established the physiochemical potential for its utilization as an immunomodulator. The activation of macrophages with SB enhanced their capacity to produce nitric oxide and proinflammatory cytokines, which eventually resulted in reduced uptake of parasites and their proliferation in infected cells. Further, in *Leishmania*-infected animals, SB administration reduced the generation of IL-10, an anti-inflammatory cytokine, and enhanced pro-inflammatory cytokine generation by antigen activated CD4$^+$ T cells with increased frequency of double (IFNγ^+/TNFα^+) and triple (IFNγ^+TNFα^+IL-2$^+$) positive cells and abrogated disease pathogeneses at the early days of infection. Our results also suggested that cow-ghee (A2) emulsified preparation of SB, either alone or with yashtimadhu, a known natural immune modulator which enhances the SB's potential in enhancing the immunogenicity of parasitic antigens. These findings suggested a definite potential of SB in enhancing the effector functions of phagocytes and CD4$^+$ T cells against *L. donovani* antigens. Therefore, more studies are needed to elucidate the mechanistic details of SB and its potential in enhancing vaccine-induced immunity.

Keywords Swarna Bhasma · Immunomodulation · Leishmania · Macrophages · T cells

Introduction

Leishmaniasis, caused by parasites of the genus *Leishmania*, is endemic in about 98 countries [1]. Because no prophylactic vaccine is available, leishmaniasis is primarily controlled by nonspecific, limited chemotherapeutic measures [2]. Moreover, the steady emergence of parasites resistant to commonly used drugs, i.e., antimonials, miltefosine, and amphotericin B, warrants the urgent undertaking of studies to identify better therapeutic and preventive measures [3].

Leishmania resides and proliferates in an adverse unfriendly environment of host macrophages by smartly silencing their effector properties, which is characterized by poor production of reactive oxygen species (ROS), nitric oxide (NOx), and pro-inflammatory cytokines such as TNF-α and IL-12 [4, 5]. Additionally, the parasite alters the host's adaptive immune response and initiates anti-inflammatory Th2 type T cells that produce IL-10 and subsequently suppresses Th1 cells functions [5, 6]. Further, it

✉ Rakesh K. Singh
rakesh_bc@bhu.ac.in

[1] Department of Biochemistry, Institute of Science, Banaras Hindu University, Varanasi 221 005, India

[2] Centre of Experimental Medicine and Surgery, Institute of Medical Science, Banaras Hindu University, Varanasi 221 005, India

has been observed that the effective clearance of parasites by macrophages and establishment of Th1-type immunity provide resistance whereas an active IL-10-producing Th2 response leads to disease susceptibility against *Leishmania* parasites [7, 8]. However, due to the inability of parasitic antigens to sufficiently activate naïve T cells, the establishment of Th1 cell–mediated immunity is poor, which favors *Leishmania* persistence and survival in the mammalian host [9]. Eventually, the inhibition of pro-inflammatory cytokine production, poor antigen presentation by phagocytic or antigen-presenting cells (APCs), and altered cellular signaling pathways exacerbates disease pathogenesis [10, 11]. Since the proper activation of APCs is an essential factor for efficient naïve T cell activation and their differentiation in Th1 phenotypes, the identification of modulators of their functions will be of great importance. Recent studies have discovered the mechanisms of phagocytic and T cell dysfunction but the factors to enhance their effector properties, for their protective roles, are not known [12].

Currently, over 80% of the global population relies on herbs and traditional treatments for primary healthcare [13]. The "Ayurveda," or Indian traditional medicine system, has been utilized for thousands of years to treat a wide variety of illnesses [14, 15]. Traditional medicines remain a valuable resource for discovering and identifying treatments and cures for diseases that modern medicine currently considers incurable. For the prevention and treatment of fevers, many ayurvedic formulations are used to reduce fevers of any origin [16]. Immunomodulators are biological or synthetic compounds that can activate, inhibit, or modify any immunity function, including both innate and adaptive immunity [17–19]. These formulations are not specific drugs against any targeted proteins but rather act as generalized "immune modulators" and produce pharmacological effects by balancing both the good and the bad. Ayurveda-based immunomodulators are known to enhance the immunomodulatory properties of vaccine antigen to produce higher levels of protective antibodies against various diseases caused by viruses, bacteria, etc. [14, 20]. Because of their excellent effectiveness, low toxicity, and reduced cost, these immunomodulators are frequently employed and thus require more scientific evidences for their wider acceptability [21].

Gold is one of the most appealing metals in human history for economical and ornamental purposes. Swarna Bhasma (SB) or fine gold particles (incinerated gold) are regarded as one of the most effective metal-based herbomineral therapeutic medicine in Ayurveda, which endowed extraordinary protective and curative potential against various ailments like asthma, rheumatoid arthritis tuberculosis, diabetes mellitus, and nervous disorders [22]. SB is also traditionally thought to boost immunity and augment antioxidant system as well as displays anti-aging properties [23]. Studies have also evidenced that gold can interact with the immune system, in particular with phagocytic (macrophages, dendritic cells) and adaptive immunity, i.e., T & B cells, and helps them to acquire specific functions against antigens [24, 25]. Further, because of their chemical inertness, low toxicity, and high biocompatibility, gold fine particles are also emerging as a good choice for vaccine development against various infectious diseases caused by viruses, bacteria, and parasites [26–28]. However, the immunogenic potential of SB has not been evaluated for its capability to induce effector functions of phagocytic and $CD4^+$ T cells against *Leishmania* antigens. In Ayurveda, more than thousands preparations are described as containing yashtimadhu ("YM," licorice, *Glycyrrhiza glabra*) as one of their constituents with diverse pharmacological applications ranging from antiviral, antibacterial, anti-inflammatory, and antioxidants [29, 30]. Additionally, YM is also considered a potent immune modulator, which is suggested to be a result of a multitude of bioactive constituents such as glycyrrhizin, isoliquiritigenin, flavonoids, formononetin, glabridin, hemileiocarpin, hispaglabridin B, liquiritigenin, and glyciram, although its regulatory roles on immune cells, in leishmaniasis, have not been investigated on the scale of modern research parameters [30–32]. In the current study, we investigated the potential of SB, either alone or in combination with an aqueous extract of YM in inducing the effector properties of macrophages and antigen experienced $CD4^+$ T cells in *L. donovani* infection that cause visceral leishmaniasis in Indian subcontinent. The findings suggested that the SB possess excellent properties to induce antigen-presenting and microbicidal properties of macrophages, and also helps antigen-activated $CD4^+$ T cell to acquire pro-inflammatory cytokine producing abilities against *Leishmania* antigens.

Material and Methods

Characterization of Swarna Bhasma

For the evaluation of immune modulatory potential, we used commercially available SB powder available in Indian market over the counter. The size, shape, crystalline phase, and composition of commercial SB were analyzed by various analytical techniques such as X-ray diffraction (XRD), transmission electron microscopy (TEM), scanning electron microscopy (SEM), and energy-dispersive spectroscopy (EDS). The SB particle size and shape were determined by XRD (D8 ADVANCE eco, BRUKER, Germany) and TEM (TECNAI G^2 20 S TWIN, FEI, USA). The topographic nature of SB was measured by SEM (Nova Nano 450, FEI, USA). EDS (EDAX, AMETEK, India) was employed to carry out purity analysis of the

bulk elemental composition in the bhasma samples from different commercial origins available in the market.

Preparation of Emulsified SB and Determination of CC_{50}, EC_{50}, and LD_{50} Values of SB and YM

For the preparation of emulsified SB, we first made oil of ghee using A2 cow ghee. A2 cow ghee was gently melted at 70 °C for 10–15 min and then temperature was elevated above 95 °C for 10–15 min to evaporate moisture, which was finally reduced to 70 °C. The heating of the ghee was further continued to get a brown solid residue. The residue was filtered to obtain a yellowish liquid. A 5% oil-in-ghee emulsion of SB, YM, and *Leishmania* antigens was used to evaluate macrophages and T cell functions. The CC_{50} and EC_{50} values of standard SB and YM were determined on RAW264.7 macrophages, and LD_{50} values of SB were determined in BALB/c animals. The aqueous extract of root and stem of licorice plant was used in this study.

Culture of Parasites and Preparation of Soluble Leishmania Antigen Preparation

Leishmania donovani (MHOM/IN/1983/AG83) promastigotes were used in this study. The parasites were cultured in M199 media (pH 6.8–7.2, Gibco, ThermoFisher, USA) supplemented with 10% FBS (Gibco, ThermoFisher, USA) and antibiotics (1% penicillin/streptomycin, 0.1% hemin, 10 mM adenine, Sigma-Aldrich, USA). Parasite virulence was maintained in BALB/c mice and promastigote forms of parasite were used to infect cells and animals as and when needed. The soluble leishmaial antigens (SLA) were prepared from the stationary phase of promastigotes. Briefly, the promastigotes were washed 3–4 times with FBS free M199 media by centrifuging at 2500 rpm for 10–20 min at 4 °C in order to remove FBS from the medium. Finally, promastigotes (3×10^7 parasites/ml) were suspended into the incomplete M199 media (without FBS) supplemented with antibiotics and incubated under normal promastigote culture conditions (pH 7.2, 26 °C) overnight. After culture, the viability and integrity of parasites were assessed by the Trypan blue dye exclusion method and 99% viable parasites were used to prepare SLA. After wash, parasites were suspended in lysis buffer (50 mM Tris, 5 mM EDTA, 0.5% sodium deoxycholate, 1% Triton-X100, pH 7.0) for 10 min at 4 °C followed by a heat-thaw process 3–4 times using liquid nitrogen. The mixture was centrifuged at 16000 *g* for 15 min at 4 °C and the supernatant containing SLA was collected. SLA was used to activate splenocytes for measurement of cytokine producing abilities of $CD4^+$ T cells as and when needed.

Measurements of Effector Properties of Macrophages

RAW 264.7 macrophages, procured from National Centre for Cell Sciences Pune, India, were used in this study. Cells were cultured in DMEM (Gibco) medium supplemented with FBS (10%) and antibiotics at 37 °C in a humidified mixture of 5% CO_2 and 95% air in a CO_2 incubator. The effector properties of macrophages were quantified in terms of their capacity to produce nitric oxide, pro and anti-inflammatory cytokines, and MHC gene expression in infected and SB-treated cells. Cells (10^6/well) were seeded in 48-well culture plates and kept in CO_2 incubator overnight. The next day, the cells were activated with standardized dose of SB (10 µg/ml), emulsified SB (10 µg/ml), and mixture of the emulsified SB and YM (40 µg/ml) for 6 h. After thorough wash, cells were incubated with parasites in cells to parasites ratio of 1:10 for 6 h. After incubation, cells were washed thoroughly to remove non-internalized parasites. The parasite infectivity was measured at 6 h, and their proliferation was measured at 24 h, 48 h, and 72 h post-infection by counting Giemsa-stained amastigotes in the macrophages under the microscope using 100× objectives. The abilities of SB to induce NO, pro-inflammatory cytokines, and MHC gene expression were quantified at 24 h post-infection as described in the later section.

Measurements of the Parameters of Protective Efficacy and $CD4^+$ T cell Functions

Five to 6-week-old female BALB/c mice were used in this study. This study was approved by the Ethics Committee for Animal Care and Use, Institute of Science, Banaras Hindu University, Varanasi (BHU/DoZ/1802/GO/Re/S/15/CPC5EA/21-22/B01). The animals were infected with *L. donovani* late log phase promastigotes (3×10^6/30 µl) via tail vein and the infectivity of the parasites was confirmed by measuring spleen and liver sizes and counting the splenic parasitic load by serial dilution method [33]. To evaluate the immune modulatory potential of SB, the in vivo experimental design is outlined in Fig. 1.

Isolation of Splenocytes and Measurement of the Parameters of Protective Immunity

The infected animals were administered four doses of SB formulations, i.e., at the time of infection, i.e., at day 0 (D0), D2, D4, and D6. The animals were sacrificed after 14 (D14), 21 (D21), and 56 (D56) post-infection to measure the parameters of protective immunity as depicted in Fig. 1. The splenic parasite load, NO production by splenocytes, and pro- and anti-inflammatory cytokine levels were quantified at D14. The effector function of splenic $CD4^+$

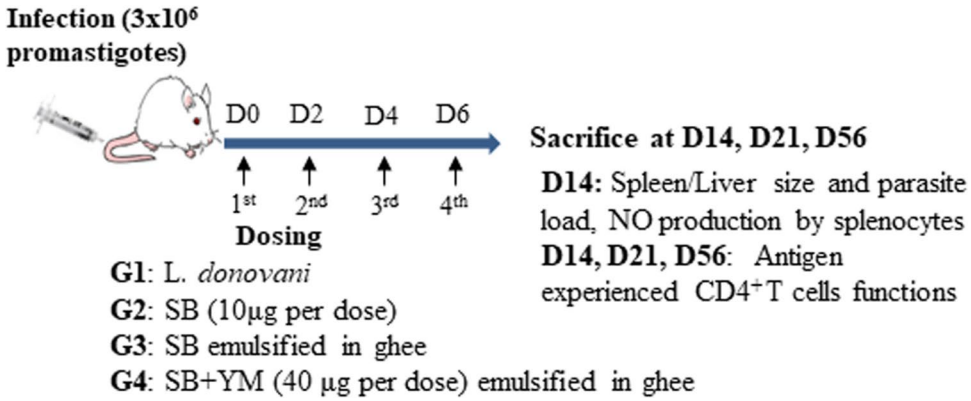

Fig. 1 Experimental design to measure SB immunomodulatory role in parasite infected animals. The study was done on four groups, i.e., infection control (G1), SB treated (G2), emulsified SB treated (G3), and SB &YM treated (G4)

T cells was measured at D14, D21, and D56 post-infection in SB-administered animals. For splenocytes, isolation spleens were washed with 70% ethanol and subsequently macerated with a plunger and the tissue homogenate was passed through a 70-µm cell strainer to isolate splenocytes. Cells were washed three to four times with PBS (1 mM, pH 7.2) by centrifugation at 500 *g* for 5–10 min in incomplete RPMI-1640 medium. The production of nitric oxide and $CD4^+$ T effector cells was measured in SLA-activated splenocytes. The rate of splenocyte proliferation was measured by MTT assay [34].

Measurement of Nitric Oxide

We quantified nitrite (NO_2^-) species in the culture supernatant of macrophages and splenocytes, which was used as an indicator of nitric oxide (NO) production using Griess reagent [35]. In brief, 100 µl of supernatant was mixed with freshly prepared Griess reagent (1% sulfanilamide, and 0.1% naphthyl ethylene diamine in 5% phosphoric acid), and the mixture was incubated for 15–20 min at room temperature. The absorbance of the supernatant was recorded at 540 nm on an ELISA plate reader (Bio-Rad, USA).

Measurement of Gene Expression Levels by qPCR

The total RNA from macrophages and splenic tissues was extracted using Tri® reagent (Sigma-Aldrich, USA) as per the manufacturer's instructions. The cDNA was synthesized by taking 1 µg of total RNA using a cDNA synthesis kit (Applied Biosystems, USA) as per the manufacturer's protocol. The obtained cDNA was amplified using Applied Biosystems 7500 Fast Detection system with SYBR green qPCR master mix as per the manufacturer's instructions (Applied Biosystems, USA). GAPDH was taken as internal control and all the data sets were normalized to the levels of GAPDH expression. Fold changes in gene expression was calculated by the Δ^2CT method, and results were reported as arbitrary units or fold changes. The primers used in this study are listed in Table 1.

Flow Cytometric Analysis: Intracellular and Cell Surface Receptor Staining

The surface and intracellular staining for phenotypic characterization and quantification of cytokine producing abilities of $CD4^+CD44^+$ T effectors (Teff) cells were measured on days 14, 21, and 56 post-infection in the total splenocytes. For cytokine measurement, splenocytes were activated with SLA (10 µg/ml) for 24 h. Before, surface staining cells were blocked with rat α-mouse CD16/32 antibodies (Cat#14-0161-82) (1 µg/10^6 cells) for 20 min prior to surface staining. Splenocytes were stained with anti-CD3-eFluor506 (Cat#69-0032-82), anti-CD4-super bright 702 (Cat#67-0042-82), and anti-CD44-superbright436 (Cat#62-0441-82) antibodies for 2 h at 4 °C or ice. After surface staining, cells were washed

Table 1 List of primers used in this study

S. no	Gene	Forward	Reverse
1	**IFN-γ**	CATGGCTGTTTCTGGCTGTT	TCCTTTTGCCAGTTCCTCCA
2	**IL-12**	TTCCACAACAAGAGGGAGCT	TTGATGGCCTGGAACTCTGT
3	**TNF-α**	TGTCTTTGAGATCCATGCCG	GGCACAGGGTCATCATCAAA
4	**IL-10**	CCTGGGTGAGAAGCTGAAGA	ACTCTTCACCTGCTCCACTG
5	**IL-4**	TCTCGAATGTACCAGGAGCC	ACCTTGGAAGCCCTACAGAC
6	**MHCI**	GAAGTGGATTACGGAGGGGT	TGCTCACTCGAAGGATGTCC
7	**MHCII**	GAAGTGGATTACGGAGGGGT	TGCTCACTCGAAGGATGTCC

and fixed for 20 min at RT for intracellular cytokine staining using cytofix/cytoperm kit (Cat#88-8824-00). After fixing, cells were stained with anti-IFNγ-APC-eFluor 780 (Cat#47-7311-82), anti-TNF-α-PerCP-eFluor 710 (Cat#46-7321-82), anti-IL-2-PE (Cat#12-7021-82), and anti-IL10-Alexa Fluor 700 (Cat#56-7101-82) antibodies for 2 h (on 4 °C or ice). After a thorough wash, cells were suspended in desired amount of acquisition buffer and acquired on Attune NxT Flow cytometer equipped with the required laser lines (VBR) using Attune NxT software. A minimum of 2×10^6 events per tube were acquired and the data were analyzed with FCS Express™ version 7 software (De Novo Software, Los Angeles, CA). All the antibodies and flow consumables were produced from ThermoFisher, USA.

Statistical Analysis

For animal studies, eight mice were used in each group, and in vitro experiments were performed in triplicate and repeated twice or thrice. The Δ^2CT method was used to calculate real-time PCR data and presented as a fold change in gene expression levels. Student's *t*-test was employed to calculate the significance levels between means of groups using Graph Pad Prism 7.0 software. A p-value < 0.05 was considered significant. All data are presented in mean ± standard error of the mean.

Results

Characterization and Determination of CC_{50}, EC_{50}, and LD_{50} Values of Swarna Bhasma

The physical characterization of commercially available SB was done by various techniques and the representative data are presented in Supplementary Fig. 1 (SF1). The XRD data revealed a wide size range of SB particles ranging from 30 to 220 nm and diffraction peaks further attested to the crystalline composition of commercial SB powder (S1A). The TEM analysis also revealed a variable range of SB particles ranging from 20 nm to 1 µm being spherical in nature (SF1B). The SEM analysis confirmed that SB from various sources was consistent in size ranging from 100 nm to 10 µm with a tendency of aggregate formation (SF1C). EDS was carried out to analyze the elemental composition in the bhasma samples from different commercial origins present in the market which indicated that the samples have 100% weight-for-weight pure gold (SF1D).

Next, we evaluated the toxicity of SB before its use as an immunomodulator as per the objectives of this study. At recommended doses (manufacturer's instruction), SB alone or ghee emulsified was found toxic as we observed significant growth inhibition of activated macrophages as depicted in Supplementary Fig. 2A (SF 2A). Our studies revealed that the cellular toxicity of SB was associated with its size and dose. Therefore, we performed extensive studies to determine the effective size and dose of SB, which is non-toxic to the cells and animals. The findings suggested that a size range from 100 to 300 nm is non-toxic to cells (SF2B) and animals as well. Finally, we determined the CC_{50} value of SB on macrophages, which was found to be in the range of 75–100 µg/ml (depending on the source manufacturer), and the LD_{50} value of SB was found to be 1–5 mg/kg of body weight in BALB/c mice. Next, we determined the EC_{50} values of SB to induce the effector functions of macrophages. The cultured RAW264.7 macrophages were treated with various concentrations of SB (5 µg–100 µg/ml) and their proliferation was measured. The EC_{50} value was determined to be 20–40 µg/ml and at this concentration, the cells were found 100% viable and proliferative even after 72 h of culture. Similarly, the EC_{50} and CC_{50} values of YM were determined which were found to be 40 µg/ml and 200 µg/ml, respectively. For our studies, we used 10 and 40 µg/ml doses of SB and YM, respectively.

SB Administration Induced Effector Functions of Macrophages and Significantly Reduced Parasite Uptake

The number of amastigotes, their proliferation in infected macrophages, and effector molecules at different time points in a different group of cells are presented in Fig. 2. In SB-treated cells, either alone or with YM, the uptake of parasites was found significantly (G1, $p = 0.004$; G2, $p = 0.006$, and G3, $p = 0.011$ vs G1, A) less than the non-treated cells. The rate of parasite proliferation was found non-significant (p = ns, 24 h vs 72 h, B) in SB-treated cells as compared to untreated ($p = 0.034$ 24 vs 72 h, B) cells that were measured up to 72 h. Further, in SB-treated and infected cells, the levels of NO were significantly (G2, $p = 0.003$; G3, $p = 0.007$; G4, $p = 0.001$ vs G1, C) elevated that were measured at 24 h post-infection as compared to infected and non-activated macrophages. The expression levels of pro-inflammatory cytokines, i.e., IFN-γ (G2, $p = 0.038$; G3, $p = 0.034$, D), TNF-α (G2, $p = 0.022$; G3, $p = 0.033$, E), and IL-12 (G2, $p = 0.007$; G3, $p = 0.024$, F), were also found significantly induced in SB-treated cells as compared to untreated cells (G1). In between ghee-emulsified SB (G3) and emulsified SB plus YM groups (G4), the differences between the levels of cytokines expression were non-significant.

Next, we measured the expression levels of antigen-presenting molecules, i.e., MHCI and MHCII in SB-treated macrophages. The treatment of SB significantly enhanced the levels of MHCI (G2, $p = 0.020$; G3, $p = 0.034$, G) and MHCII (G2, $p = 0.022$; G3, $p = 0.004$, H) as compared to infected control, i.e., untreated cells.

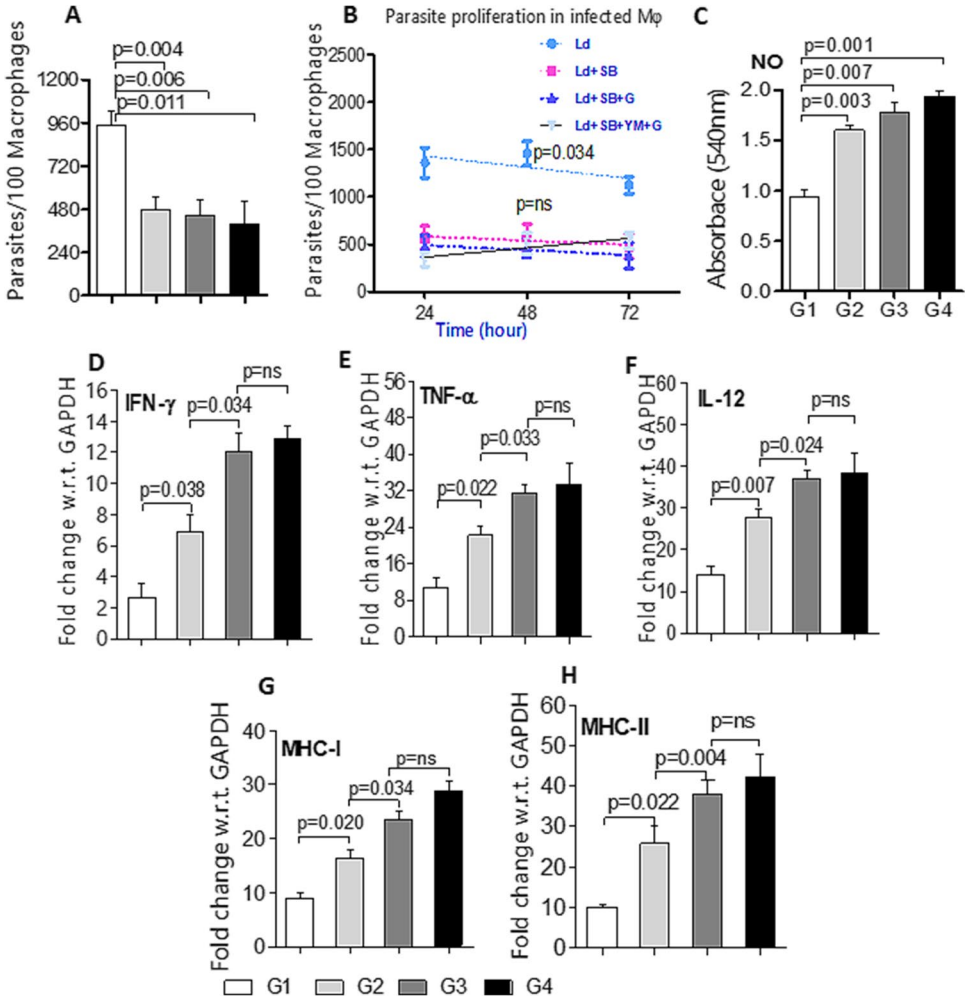

Fig. 2 Parasitic infectivity, proliferation, and effector parameters in SB-treated and untreated parasite-infected macrophages. (A) The parasite uptake was found significantly reduced, measured after 6 h of coculture, in SB-treated cells. (B) Parasite proliferation as count of amastigotes per 100 macrophages was found insignificant, which was measured up to 72 h. (C) SB-treated macrophages were found to produce significantly high NO post-infection. (D–F) The SB treatment also induced the production of IFN-γ, TNF-α, and IL-12 by infected macrophage. (G, H) MHCI/II gene expression level were also found significantly enhanced in SB-treated cells

SB Reduced Spleen/Liver Size, Splenic Parasite Load, and Enhanced Splenocyte Proliferation and Nitric Oxide Production

In the visceral form of disease, hepatomegaly and splenomegaly are the characteristic features; therefore, we measured weight and size of spleen and liver post SB administration in infected animals. The infected animals were treated with SB and its formulation (Fig. 1), as per experimental protocol, and were sacrificed on D14 to measure liver and spleen morphologies, spleen parasites load, proliferation, and nitric oxide producing abilities of splenocytes. The parasite-infected animals were used as control (G1). The findings are presented in Fig. 3. The shape and size of liver (A) and spleen (B) were significantly reduced in animals that were administered with SB either alone or with YM (G2, G3, G4). In SB-administered groups, liver (G2, p=0.036; G3, p=0.023; G4, p=0.023, C) and spleen (G2, p=0.010; G3, p=0.010; G4, p=0.022, C) weights were also significantly reduced as compared to infected control. The spleen size (G2, p=0.029; G3, p=0.027; G4, p=0.033, D) and parasite load (G2, p=0.026; G3, p=0.027; G4, p=0.024, E) were also significantly reduced in SB-treated groups, isolated from SB-treated animals. Further, the SLA-activated splenocytes were also found to produce more nitric oxide (G2, p=0.012; G3, p=0.024; G4, p=0.004, F) and proliferative (G2, p=0.033; G3, p=0.003; G4, p=0.008, G) than those isolated from parasite infected non treated animals.

SB Helped Antigen Experienced $CD4^+$ T cells to Acquire Th1 Phenotypes in Leishmania-Infected Animals

Th1 phenotype of $CD4^+$ T cells is characterized by their abilities to produce pro-inflammatory cytokines such as IFN-γ and TNF-α, which play an important role in providing protection to host against parasites. Therefore, we measured the impact of SB administration on the effector properties of antigen experienced $CD4^+$ T cells, i.e., $CD4^+CD44^+$ T cells in SB-treated groups at D14, D21, and D56 post-infection. The splenocytes were cultured in presence of SLA (10μg/ml) and after 48h, the

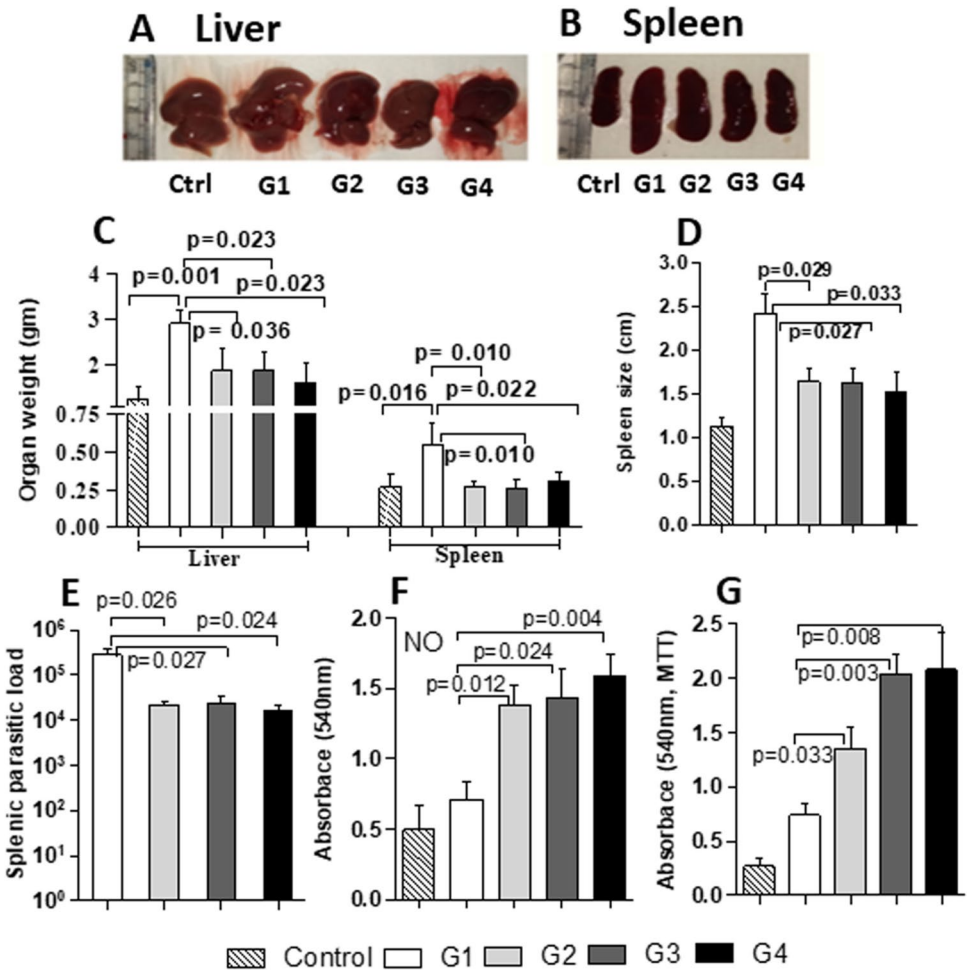

Fig. 3 Liver and spleen weights and sizes, spleen parasite load, proliferation, and nitric oxide production by splenocyte in SB-administered *L. donovani*–infected animals. (A, B) Liver and spleen morphologies isolated from various groups. (C) In SB-administered animals, both liver and spleen weights were significantly reduced. (D) In all SB-treated groups, spleen size was significantly reduced. (E) The SB treatment also reduced spleen parasites burden in infected animals. (F) The splenocytes isolated from SB-treated animals were found to produce more NO, after SLA exposure being highly significant for the G4 (vs G1) group

$CD4^+CD44^+$ T cell's effector function, i.e., their abilities to produce pro- and anti-inflammatory cytokines, were measured by flow cytometry. The findings are depicted in Fig. 4 of which the A–D depicts the gating strategy to select $CD4^+CD44^+$ T cells on splenocytes and $CD3^+$ T cells. In SB-treated animals, the percentage of IFN-γ (G2, p=0.020; D14, p=0.051; D2, p=0.034; D56, E) and TNFα (G2, p=0.048; D14, p=0.019; D21, p=0.04; D56, F) $CD4^+CD44^+$ T cells was significantly higher as compared to untreated animals. Since multiple cytokine producing abilities of T cells are a hallmark of their better activation and proliferation, we also measured double positives, i.e., $IFN\gamma^+TNF\alpha^+CD4^+CD44^+$ T cells (G2, p=0.023; D14, p=0.0021; D21, p=0.005; D56, G) and triple positives, i.e., $IFN\gamma^+TNF\alpha^+IL\text{-}2^+CD4^+CD44^+$ T cells (G2, p=0.031; D14, p=0.082; D21, p=0.048; D56, H) which were found high in SB-treated animals as compared to non-infected cells. Further, the frequencies of these cytokines positive cells were found relatively high in animals who received ghee-emulsified SB containing YM, which suggested a potential of these formulations in augmenting the antigenicity of parasitic antigens.

The production of IL-10 by $CD4^+$ T cells is linked with disease susceptibility and pathogenesis. Thus, we were also interested to know the impact of IL-10 production by $CD4^+CD44^+$ T cells post SB administration in *L. donovani*–infected animals. The percentage of IL-10-positive cells was found significantly less (G2, $p=0.027$; D14, $p=0.009$; D21, $p=0.008$; D56, I) in the SB-treated group, which further suggested an important role of SB in activation and differentiation of antigen-specific $CD4^+$ T cells against *L. donovani* antigens.

Discussion

Leishmania is a naturally intelligent parasite, which survives in the hostile environment of the host phagocytic cells by dampening their pro-inflammatory cytokines and NO producing abilities [36]. The parasite also suppresses the expression of MHC genes and co-stimulatory receptors, which negatively impact the activation and proliferation of antigen experienced T cells [37–39]. The finding in the study suggested that SB enhances the abilities of macrophages to

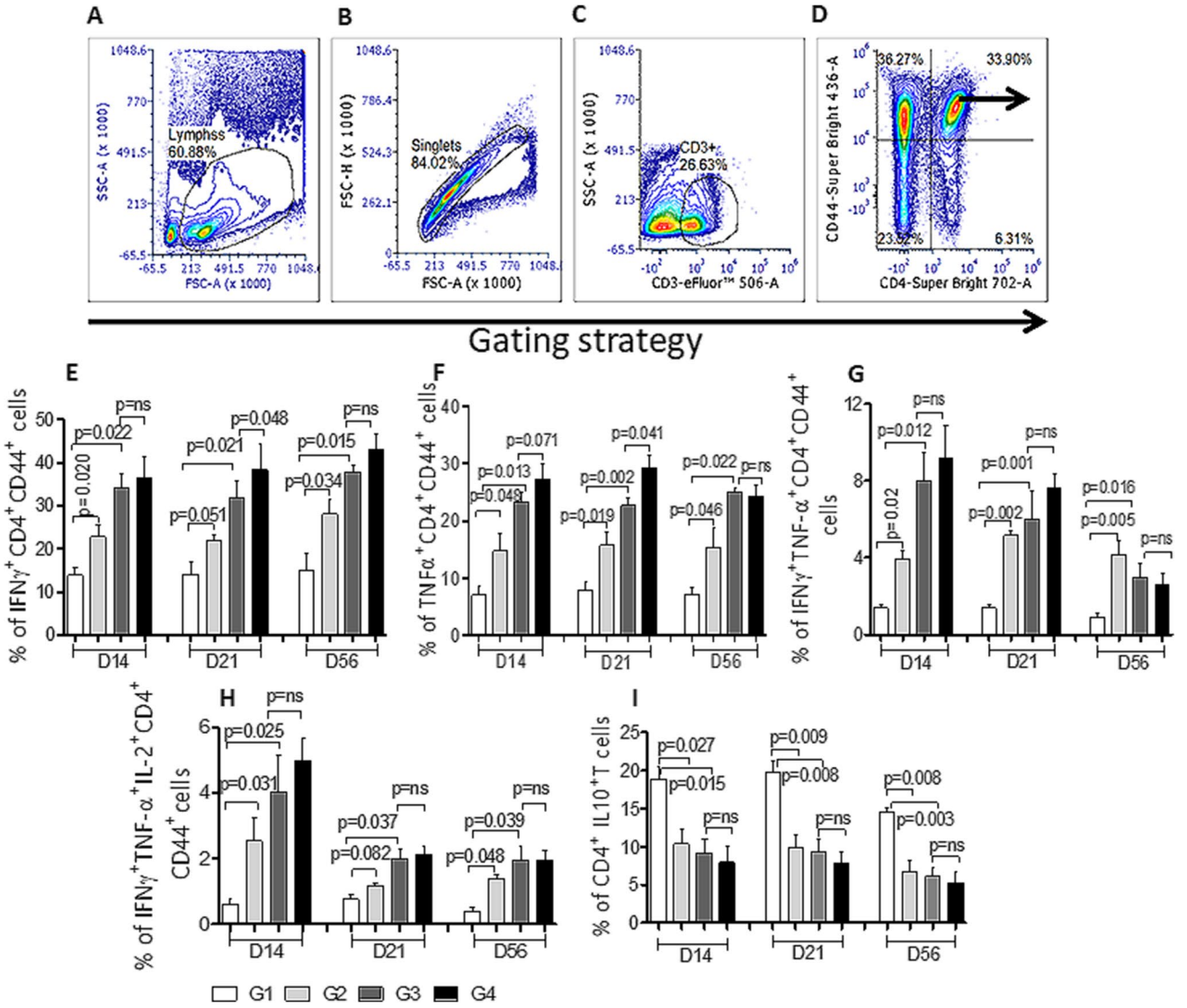

Fig. 4 Percentage of IFN-γ, TNF-α, and IL-2 positives $CD4^+CD44^+$ T cells in SB-treated and untreated parasite-infected groups. (A–D) Flow panels to select $CD4^+CD44^+$ T cells from total lymphocytes and $CD3^+$ T cells. (E, F) The percent of IFN-γ, TNF-α $CD4^+CD44^+$ T cells were significantly higher in the G2 and G3 groups, as compared to GI. In between the G3 and G4 groups, an increase in number of T cells was observed but it was not significant. (G, H) The IFN-γ^+, TNF-α^+ double positive, IFN-γ^+, TNF-α^+, and IL-12^+ triple positive cells were also found significantly more in the G2 and G3 groups as compared to G1. (I) In SB-administered animals, the percentage of IL-10^+ CD 44^+ T cells was significantly less in all treated groups

produce pro-inflammatory cytokines and MHC gene expression against *Leishmania* antigens. The significant induction in levels of nitric oxide, a main leishmanicidal molecule, further suggested the potential of SB in augmenting microbicidal properties of macrophages. We observed reduced parasite proliferation in SB-treated cells, which might be in the impact of induced nitric oxide.

In parasitic infection, the abilities of phagocytes and T cells to produce pro-inflammatory molecules negatively impacted the generation of anti-inflammatory cytokine that facilitate parasites to dominate host immune response [5, 40]. Since generation of microbicidal molecule like NO depends on pro-inflammatory cytokine, the absence of these cytokine eventually leads to parasite supremacy on host immunity [8, 41]. The in vivo observations revealed that in SB-administered animals, the antigen experienced $CD4^+$ T cells acquire more functional nature to produced pro-inflammatory cytokines, which probably in turn helps phagocytic cells to kill parasites more efficiently. Further, hepatomegaly and splenomegaly is the characteristic chemical features of visceral leishmaniasis [42]. The in vivo observation revealed that administration of SB significantly reduced both hepatomegaly and splenomegaly and also reduced spleen parasite load which is a marker of disease progression in infected

animals. Further, the splenocytes from the SB-treated group were found more proliferative and NO producing against parasites, which suggested the abilities of SB to induce microbicidal activity as immunomodulators.

The clinical prognosis of *Leishmania* infection mostly depends on the early clearance of parasites by the phagocytic cells which is mediated by T cell–mediated immune responses [43]. The functions of several T cell subsets, viz., Th1, Th2, Th17, Th22, and Th27, in disease resistance and susceptibility are extensively studied in all forms of leishmaniasis albeit how do parasites regulate T cell differentiation? It is not known, yet [44]. Our findings exhibited that SB potentiates generation of pro-inflammatory cytokines by antigen-activated $CD4^+CD44^+$ T cells with increased frequency of double ($IFN\gamma^+/TNF\alpha^+$) and triple ($IFN\gamma^+TNF\alpha^+IL\text{-}2^+$) positive cells. The $CD4^+CD44^+$ T cells were found to produce significant amount of IL-10, in SB-administered animals, which suggested a possibility that SB maintained homeostasis of pro- and anti-inflammatory cytokines against parasitic antigen, albeit we advocate more studies in this direction. However, in lieu of the compromised immunogenicity of *Leishmania* antigens, SB seems to be as an immunomodulator of choice to enhance and regulate antigen experienced $CD4^+$ T cell function that plays an important role in *L. donovani* control. This provided evidence that SB has potential to regulate antigen experienced $CD4^+$ T cell function and their differentiation. Since T cell immunity plays an important role in *L. donovani* control, and parasite antigens are poor activators of T cells, which are a major hurdle in vaccine development, this finding provided a new data for the validation of the role of SB on increasing the immunogenicity of *Leishmania* antigens.

Lack of appropriate prophylactic measures and limited therapeutic options has led to an inclination towards traditional medicinal methods for effective therapeutic purposes worldwide. In ancient Indian literature, the role of metallic bhasma, particularly gold nanoparticles, has acquired a prominent place as metal-based therapeutic medicine which can boost vaccines' efficacy [45, 46]. Ayurvedic metal particles, i.e., swarna bhasma, rajata bhasma, tamra bhasma, and yashada bhasma, could be used as novel antipathogenic agents for their anti-inflammatory, immunomodulatory, antiviral, and adjuvant activities [45, 47]. The administration of gold in children is a common practice in Ayurveda in many diseases and even in critical conditions [48, 49]. In line of our findings, other studies have also shown the pharmacological activity of SB in restoring functions of macrophages, dendritic cells, and other immune cells [50, 51]. Further, SB is also known to promote T cells and phagocytic activities in infectious diseases, which is in accordance to our findings [26]. To summarize, we report for the first time that SB enhances the effector functions of antigen presenting and $CD4^+$ T cells, and controls the *Leishmania* growth and proliferation in infected animals. Thus, we advocate more in-depth studies to validate the role of SB adjuvantation in increasing the immunogenicity of vaccine candidature against leishmaniasis, in particular, and against parasitic diseases, in general.

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Author Contribution SS, AA, AS, BM, S Sirohi: designed and performed major experiments like SB characterization, flow cytometry, qPCR, and animal, cell, and parasite culture–related works.

Samer Singh: helped in manuscript writing.

RKS: conceived, designed, directed, wrote, and supervised the complete study.

Data Availability The datasets generated and analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Competing Interests The authors declare no competing interests.

Conflict of Interest The authors declare that the research was conducted without any commercial or financial relationships that could be construed as a potential conflict of interest.

References

1. Alvar J, Velez ID, Bern C et al (2012) Leishmaniasis worldwide and global estimates of its incidence. PLoS One 7(5):e35671. https://doi.org/10.1371/journal.pone.0035671
2. Ghorbani M, Farhoudi R (2018) Leishmaniasis in humans: drug or vaccine therapy? Drug Des Devel Ther 12:25–40. https://doi.org/10.2147/DDDT.S146521
3. Ponte-Sucre A, Gamarro F et al (2017) Drug resistance and treatment failure in leishmaniasis: a 21st- challenge. PLoS Negl Trop Dis 11(12):e0006052. https://doi.org/10.1371/journal.pntd.0006052
4. Reverte M, Snaka T, Fasel N (2022) The dangerous liaisons in the oxidative stress response to Leishmania infection. Pathogens 11(4). https://doi.org/10.3390/pathogens11040409
5. Dayakar A, Chandrasekaran S, Kuchipudi SV (2019) Cytokines: key determinants of resistance or disease progression in visceral leishmaniasis: opportunities for novel diagnostics and immunotherapy. Front Immunol 10:670. https://doi.org/10.3389/fimmu.2019.00670
6. Singh OP, Syn G, Nylen S et al (2021) Anti-interleukin-10 unleashes transcriptional response to leishmanial antigens in visceral leishmaniasis patients. J Infect Dis 223(3):517–521. https://doi.org/10.1093/infdis/jiaa381
7. Bacellar O, D'Oliveira A, Jeronimo S, Carvalho EM (2000) IL-10 and IL-12 are the main regulatory cytokines in visceral

leishmaniasis. Cytokine 12(8):1228–1231. https://doi.org/10.1006/cyto.2000.0694
8. Bittar RC, Nogueira RS et al (2007) T-cell responses associated with resistance to Leishmania infection in individuals from endemic areas for Leishmania (Viannia) braziliensis. Mem Inst Oswaldo Cruz 102(5):625–630. https://doi.org/10.1590/s0074-02762007005000069
9. Reiner SL, Zheng S, Wang ZE et al (1994) Leishmania promastigotes evade interleukin 12 (IL-12) induction by macrophages and stimulate a broad range of cytokines from CD4+ T cells during initiation of infection. J Exp Med 179(2):447–456. https://doi.org/10.1084/jem.179.2.447
10. Rodrigues V, Cordeiro-da-Silva A et al (2016) Regulation of immunity during visceral Leishmania infection. Parasit Vectors 9:118. https://doi.org/10.1186/s13071-016-1412-x
11. Gupta G, Oghumu S, Satoskar AR (2013) Mechanisms of immune evasion in leishmaniasis. Adv Appl Microbiol 82:155–184. https://doi.org/10.1016/B978-0-12-407679-2.00005-3
12. Kapsenberg ML (2003) Dendritic-cell control of pathogen-driven T-cell polarization. Nat Rev Immunol 3(12):984–993. https://doi.org/10.1038/nri1246
13. World Health Organization (2019) WHO establishes the global centre for traditional medicine in India. Available at https://www.who.int/initiatives/who-global-centre-for-traditional-medicine. Updated: 25 March 2022; Accessed 1 Feb 2023
14. Patwardhan B, Vaidya ADB, Chorghade M (2004) Ayurveda and natural products drug discovery. Curr Sci India 86(6):789–799
15. Gadgil VD (2010) Understanding ayurveda. J Ayurveda. Integr Med 1(1):77–80. https://doi.org/10.4103/0975-9476.59836
16. Gacche RN, Dhole NA (2006) Antioxidant and possible anti-inflammatory potential of selected medicinal plants prescribed in the Indian traditional system of medicine. Pharm Biol 44(5):389–395. https://doi.org/10.1080/13880200600751691
17. Jantan I, Ahmad W, Bukhari SNA (2018) Plant-derived immunomodulators: an insight on their preclinical evaluation and clinical trials. Front Plant Sci 6:655. https://doi.org/10.3389/fpls.2018.01178
18. Ortuno-Sahagun D, Zanker K, Rawat AKS et al (2017) Natural immunomodulators. J Immunol Res. Artn 7529408. https://doi.org/10.1155/2017/7529408
19. Nicholls EF, Madera L, Hancock REW (2010) Immunomodulators as adjuvants for vaccines and antimicrobial therapy. Ann Ny Acad Sci 1213:46–61. https://doi.org/10.1111/j.1749-6632.2010.05787.x
20. Singh R, Goel S, Bourgeade P et al (2021) Ayurveda Rasayana as antivirals and immunomodulators: potential applications in COVID-19. Environ Sci Pollut R 28(40):55925–55951. https://doi.org/10.1007/s11356-021-16280-5
21. Gertsch J (2011) Natural immunomodulators - a drug discovery perspective. Planta Med 77(12):1231
22. Patil-Bhole T, Wele A, Gudi R et al (2021) Nanostructured gold in ancient Ayurvedic calcined drug "swarnabhasma." J Ayurveda Integr Med 12(4):640–648. https://doi.org/10.1016/j.jaim.2021.06.017
23. Bhattacharya R, Mukherjee P (2008) Biological properties of "naked" metal nanoparticles. Adv Drug Deliv Rev 60(11):1289–1306. https://doi.org/10.1016/j.addr.2008.03.013
24. Balakrishnan PB, Sweeney EE (2021) Nanoparticles for enhanced adoptive T cell therapies and future perspectives for CNS tumors. Front Immunol 12:600659. https://doi.org/10.3389/fimmu.2021.600659
25. Dykman LA, Khlebtsov NG (2017) Immunological properties of gold nanoparticles. Chem Sci 8(3):1719–1735. https://doi.org/10.1039/c6sc03631g
26. Sengupta A, Azharuddin M, Al-Otaibi N, Hinkula J (2022) Efficacy and immune response elicited by gold nanoparticle-based nanovaccines against infectious diseases. Vaccines (Basel) 10(4). https://doi.org/10.3390/vaccines10040505
27. Dykman LA (2020) Gold nanoparticles for preparation of antibodies and vaccines against infectious diseases. Expert Rev Vaccines 19(5):465–477. https://doi.org/10.1080/14760584.2020.1758070
28. Ferrando RM, Lay L, Polito L (2020) Gold nanoparticle-based platforms for vaccine development. Drug Discov Today Technol 38:57–67. https://doi.org/10.1016/j.ddtec.2021.02.001
29. Bisht D, Mohammad R, Rajeshwar K et al (2022) Revisiting liquorice (Glycyrrhiza glabra L.) as anti-inflammatory, antivirals and immunomodulators: Potential pharmacological applications with mechanistic insight. Phytomedicine Plus 2(1):100206. https://doi.org/10.1016/j.phyplu.2021.100206
30. Committee on Herbal Medicinal Products (HMPC) (2013) Assessment report on Glycyrrhiza glabra L. and/or Glycyrrhiza inflata Bat. and/or Glycyrrhiza uralensis Fisch., radix. European Medicines Agency (EMA). EMA/HMPC/571122/2010. Available at: https://www.ema.europa.eu/en/documents/herbal-report/final-assessment-report-glycyrrhiza-glabra-l/glycyrrhiza-inflata-bat/glycyrrhiza-uralensis-fisch-radix-first-version_en.pdf. Updated 12 March 2013; Accessed 1 Feb 2023
31. Sharma V, Katiyar A, Agrawal RC (2018) Glycyrrhiza glabra: Chemistry and Pharmacological Activity. J.-M. Mérillon, K.G. Ramawat (eds.), Sweeteners, reference series in phytochemistry, Springer International Publishing AG 2018. https://doi.org/10.1007/978-3-319-27027-2_21
32. Asl MN, Hosseinzadeh H (2008) Derivatives, review of pharmacological effects of *Glycyrrhiza* sp. and its bioactive compounds. Phytoher Res 22(6):709–724
33. Titus RG, Marchand M, Boon T et al (1985) A limiting dilution assay for quantifying Leishmania major in tissues of infected mice. Parasite Immunol 7(5):545–555. https://doi.org/10.1111/j.1365-3024.1985.tb00098.x
34. Mosmann T (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods 65(1–2):55–63. https://doi.org/10.1016/0022-1759(83)90303-4
35. Ding AH, Nathan CF, Stuehr DJ (1988) Release of reactive nitrogen intermediates and reactive oxygen intermediates from mouse peritoneal macrophages: comparison of activating cytokines and evidence for independent production. J Immunol 141:2407–2412
36. Martinez-Lopez M, Soto M, Iborra S et al (2018) Leishmania hijacks myeloid cells for immune escape. Front Microbiol 9:883. https://doi.org/10.3389/fmicb.2018.00883
37. Sacks D, Sher A (2002) Evasion of innate immunity by parasitic protozoa. Nat Immunol 3(11):1041–1047. https://doi.org/10.1038/ni1102-1041
38. Shio MT, Hassani K, Isnard A et al (2012) Host cell signalling and leishmania mechanisms of evasion. J Trop Med 819512. https://doi.org/10.1155/2012/819512
39. De Almeida MC, Cardoso SA, Barral-Netto M (2003) Leishmania (Leishmania) chagasi infection alters the expression of cell adhesion and costimulatory molecules on human monocyte and macrophage. Int J Parasitol 33(2):153–162. https://doi.org/10.1016/s0020-7519(02)00266-7
40. Engwerda CR, Murphy ML, Cotterell SE et al (1998) Neutralization of IL-12 demonstrates the existence of discrete organ-specific phases in the control of Leishmania donovani. Eur J Immunol 28(2):669–680. https://doi.org/10.1002/(SICI)1521-4141(199802)28:02%3c669::AID-IMMU669%3e3.0.CO;2-N
41. Perez-Cabezas B, Cecilio P, Gaspar TB et al (2019) Understanding resistance vs. susceptibility in visceral leishmaniasis using mouse models of Leishmania infantum infection. Front Cell Infect Mi 9:30. https://doi.org/10.3389/fcimb.2019.00030

42. Kumari S, Ram VJ (2002) Visceral leishmaniasis: clinical features, pathology, diagnosis, and chemotherapeutic developments. Drug News Perspect 15(7):417–431. https://doi.org/10.1358/dnp.2002.15.7.840077
43. Liu D, Uzonna JE (2012) The early interaction of Leishmania with macrophages and dendritic cells and its influence on the host immune response. Front Cell Infect Microbiol 2:83. https://doi.org/10.3389/fcimb.2012.00083
44. Kumar R, Nylen S (2012) Immunobiology of visceral leishmaniasis. Front Immunol 3:251. https://doi.org/10.3389/fimmu.2012.00251
45. Pal D, Sahu CK, Haldar A (2014) Bhasma: the ancient Indian nanomedicine. J Adv Pharm Technol Res 5(1):4–12. https://doi.org/10.4103/2231-4040.126980
46. Carabineiro SAC (2017) Applications of gold nanoparticles in nanomedicine: recent advances in vaccines. Molecules 22(5). https://doi.org/10.3390/molecules22050857
47. Faa G, Gerosa C, Fanni D, Lachowicz JI (2018) Gold - old drug with new potentials. Curr Med Chem 25(1):75–84. https://doi.org/10.2174/0929867324666170330091438
48. Versiani AF, Andrade LM, Martins EMN et al (2016) Gold nanoparticles and their applications in biomedicine. Future Virol 11(4):293–309. https://doi.org/10.2217/fvl-2015-0010
49. Jyothy KB, Sheshagiri S, Patel KS, Rajagopala S (2014) A critical appraisal on Swarnaprashana in children. Ayu 35(4):361–365. https://doi.org/10.4103/0974-8520.158978
50. Fan LY, Wang WZ, Wang ZH, Zhao MZ (2021) Gold nanoparticles enhance antibody effect through direct cancer cell cytotoxicity by differential regulation of phagocytosis. Nat Commun 12(1):6371. https://doi.org/10.1038/s41467-021-26694-x
51. Dey AK, Gonon A, Pecheur EI (2021) Impact of gold nanoparticles on the functions of macrophages and dendritic cells. Cells-Basel 10(1):96. https://doi.org/10.3390/cells10010096