International Immunopharmacology 70 (2019) 408–416

Contents lists available at ScienceDirect

International Immunopharmacology

journal homepage: www.elsevier.com/locate/intimp

Cell wall fraction of *Mycobacterium indicus pranii* shows potential Th1 adjuvant activity

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ARTICLE INFO

Keywords:
Adjuvant
Cell wall
Mycobacterium indicus pranii
Tumor
Vaccine

ABSTRACT

Very few adjuvants inducing Th1 immune response have been developed and are under clinical investigation. Hence, there is the need to find an adjuvant that elicits strong Th1 immune response which should be safe when injected in the host along with vaccines. *Mycobacterium indicus pranii* (MIP), a non-pathogenic vaccine candidate, has shown strong immunomodulatory activity in leprosy/tuberculosis/cancer and in genital warts patients where its administration shifted the host immune response towards Th1 type. These findings prompted us to study the components of MIP in detail for their Th1 inducing property. Since mycobacterial cell wall is very rich in immunostimulatory components and is known to play important role in immune modulation, we investigated the activity of MIP cell wall using Ovalbumin antigen (OVA) as model antigen. 'Whole cell wall' (CW) and 'aqueous soluble cell wall fractions' (ACW) induced significant Th1 immune response while 'cell wall skeleton' (CWS) induced strong Th2 type of immune response. Finally, functional activity of fractions having Th1 inducing activity was evaluated in mouse model of melanoma. CW demonstrated significant anti-tumor activity similar to whole MIP. Anti-tumor activity of CW could be correlated with enhanced tumor antigen specific Th1 immune response observed in tumor draining lymph nodes.

1. Introduction

Adjuvants are incorporated in vaccine formulations in order to enhance, accelerate and prolong the specific immune response towards the desired type (Th1 or Th2). Although, number of vaccine adjuvants are used in research but their application in humans is limited due to their toxic side effects such as allergic and auto immune responses [1,2]. Much of the success regarding the use of adjuvant as an immune potentiator is attributed to alum, which evokes strong humoral immune response with excellent safety profile and has been used worldwide since last 80 years [3,4]. However, till date very few adjuvants inducing strong Th1 response have been developed which are under clinical investigation [5]. Complete Freund's adjuvant (inactivated and dried mycobacteria in mineral oil) is a very strong Th1 promoting adjuvant but its use is limited due to its toxicity [6]. Therefore, there is need to find an adjuvant which is able to promote Th1 immune response and match the safety profile of alum. Mycobacterium contains various components that are immunogenic in nature [7]. Cell wall of mycobacterium which has complex structure comprising of long chain fatty acids, peptidoglycan, arabinoglycan, lipoarabinomannan and various other molecules, plays important role in immune modulation and has been investigated for its adjuvant properties. Cell wall skeleton (CWS) comprising of peptidoglycan, arabinoglycan and mycolic acids has also been shown to be immunogenic in nature [8,9]. CWS isolated from BCG cell wall has been shown to enhance antibody response when used as an adjuvant with known model antigens like OVA [8].

Mycobacterium indicus pranii, "a non-pathogenic mycobacterium" is known to possess immunomodulatory properties. MIP has been shown to induce enhanced Th1 type of immune response in animal models of tuberculosis and tumor. Weekly injections of MIP in tumor bearing mice delayed the growth of melanoma tumor by enhancing the infiltration of T cells and antigen presenting cells in tumor microenvironment

Abbreviations: ACW, aqueous soluble cell wall fraction; CW, whole cell wall fraction; CWS, cell wall skeleton; CFA, Complete Freund adjuvant; FBS, Fetal bovine serum; MIP, *Mycobacterium indicus pranii*; *M.tb*, *Mycobacterium tuberculosis*; MOI, Multiplicity of infection; OVA, Ovalbumin antigen; OCW, oil soluble cell wall fraction; PRRs, Pattern Recognition Receptors; PBS, Phosphate buffered saline; RPMI, Roswell Park Memorial Institute medium; RT, room temperature; TB, tuberculosis; TDB, Trehalose-dibehenate

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https://doi.org/10.1016/j.intimp.2019.02.049
Received 20 August 2018; Received in revised form 16 February 2019; Accepted 27 February 2019
Available online 08 March 2019
1567-5769/

[10,11]. Higher protection against TB was observed in MIP vaccinated group as compared to BCG in animal models of tuberculosis [12,13]. In a recent study, where MIP was used as an immunomodulator with hCG vaccine adsorbed on alhydrogel resulted in significant increase in antibody titer against hCG as compared to antibody titer obtained by using the hCG on alhydrogel alone. MIP genome has been annotated and *in silico* analysis revealed that it possesses higher level of putative antigenic proteins as compared to BCG and other candidate vaccine strains [14]. Such attributes of MIP provide logical reasoning for detailed investigation of adjuvant properties of MIP.

In this study, immunostimulatory/immunoadjuvant activity of different fractions of MIP was evaluated in comparison to whole MIP. These fractions were investigated for their ability to activate macrophages/splenocytes. MIP cell wall was fractionated into aqueous soluble and oil soluble fractions by extraction of cell wall fraction with dichloromethane. Cell wall skeleton was prepared by delipidation and deproteinization of whole cell wall. Whole cell wall fraction, aqueous soluble cell wall fraction and cell wall skeleton demonstrated significant macrophage/splenocytes stimulating activity. These fractions were further evaluated for their immunoadjuvant properties using OVA as model antigen and also for their anti-tumor activity (Th1 immune response) in mouse model of melanoma tumor. Whole cell wall fraction demonstrated substantial Th1 inducing adjuvant activity, while cell wall skeleton induced strong Th2 type of immune response.

2. Materials and methods

2.1. Preparation of different fractions of MIP

For cell wall fraction preparation, method reported previously [30] was modified to get optimum yield. MIP was cultured in Middlebrook 7H9 broth (BD Biosciences) containing 0.05% Tween-80, 0.1% Glycerol and supplemented with 10% Albumin Dextrose Catalase). MIP culture in mid log growth phase was harvested by centrifugation at 3000*g* for 10 min. Pellet was washed twice with PBS, resuspended in cold PBS and passed through French press (Constant Cell Disruption Systems) twice at 40,000 kPa. The suspension of disrupted bacteria was centrifuged at 10,000*g* for 15 min and pellet containing intact bacteria was discarded. Supernatant was further centrifuged at 27,000*g* for 30 min. Both pellet (cell wall) and supernatant (cytosolic fraction) were lyophilized and stored at −20 °C.

2.2. Preparation of aqueous and oil soluble cell wall fraction

100 mg of cell wall fraction was suspended in 5 ml of PBS. Equal volume of dichloromethane (Sigma-Aldrich) was added to the cell wall suspension and kept on the shaker for 2 h. Aqueous and non-aqueous layers were separated by centrifugation at 4000*g* for 15 min. Non-aqueous layer was re-extracted with equal volume of PBS. Aqueous layer from both the steps was pooled and lyophilized.

2.3. Cell wall skeleton preparation

1 g of dry cell wall of MIP was resuspended in 100 ml of PBS containing 10 mg each of trypsin and chymotrypsin (Sigma-Aldrich) and kept on shaker for 24 h at 37 °C. Resulting suspension was centrifuged at 27,000*g* for 30 min and pellet was washed twice with PBS. Trypsin and chymotrypsin treatments were repeated twice and cell wall fraction was collected by centrifugation followed by washing with PBS. Cell wall was then re-suspended in 100 ml of 0.1 M Tris-HCL buffer containing 15 mg pronase (Sigma-Aldrich) and maintained in shaking condition for 24 h. The suspension was then centrifuged and the pellet containing cell wall was washed twice with the above mentioned buffer. Pronase treatment was repeated twice and deproteinised cell wall was collected by centrifugation and washed three times with Tris-HCl buffer. Subsequently, it was re-suspended in 1% Triton X-100 (HiChem Life Sciences) and stirred at 60 °C to remove protein digest. Finally, the cell wall was washed twice with distilled water and once with methanol (Sigma-Aldrich). Free lipids were removed from deproteinised cell wall by extraction with 100 ml each of Tetrahydrofuran, Chloroform: Methanol (2:1) and Methanol consecutively. Extraction with each solvent was carried out for 48 h and repeated once. Cell wall skeleton fraction obtained from cell wall after deproteinization and delipidation was dried and stored at −20 °C.

2.4. Macrophages (J774.1) and splenocytes stimulation with MIP or its fractions

Murine macrophage cell line, J774A.1 (ATCC® TIB-67) was cultured in RPMI-1460 medium (HiChem Life Sciences) supplemented with 10% FBS (Cat no. 04-127-1A, Biological Industries). For stimulation experiments, 0.4×10^6 macrophages or 1.5×10^6 splenocytes were plated per well in 24-well cell culture plate. Varying concentration of each of whole cell wall (CW), aqueous soluble cell wall fraction (ACW), oil soluble cell wall fraction (OCW) and cell wall skeleton (CWS) were added to the wells. Culture supernatant was collected after 24 h and 48 h after incubation at 37 °C. Level of different cytokines in the culture supernatant was determined by ELISA. ELISA kit for cytokines TNF-α, (Cat. No: 14-7423-68A) IFN-γ (Cat. No: 14-7313-688). IL-2 (Cat. No: 14-7022-68), IL-12 (Cat. No: 13-7123-81), IL-4 (Cat. No: 13-7042-81) and IL-10 (Cat. No: 14-7101-68A) were purchased from eBiosciences.

2.5. Immunoadjuvant properties of MIP cell wall and its fractions

Whole cell wall (CW), aqueous soluble cell wall fraction (ACW) and cell wall skeleton (CWS) induced substantial secretion of IFN-γ, IL-12, and TNF-α from splenocytes. These fractions were evaluated for their immune-adjuvant activity in Balb/c mice. OVA (Sigma-Aldrich) was taken as a model antigen; alum and complete Freund's adjuvant (Sigma-Aldrich) were used as positive controls for Th2 and Th1 adjuvant activity respectively. Two concentrations of each fraction (which were selected for their ability to induce optimum stimulation in initial standardization experiments) were used along with OVA antigen. All the groups received three injections of OVA antigen along with CW/ACW/CWS, while control group was given plain OVA without any adjuvant.

2.6. Analysis of OVA specific antibodies

Eleven groups each containing five C57BL/6 mice were immunized with three doses of each formulation at two week interval. Mice were injected sub-cutaneously by using a 26 × 1/2 G, 13 mm length needle. All the formulations were prepared in PBS and 100 μl of each fraction was used for immunization. OVA was taken as a model antigen. Two concentrations of each fraction (CW: 100 μg, 200 μg/ACW: 50 μg, 100 μg/CWS: 50 μg, 100 μg) were injected along with 25 μg OVA. Complete Freund's adjuvant and alum were taken as positive control for adjuvant activity. In other group, 5×10^6 bacilli of MIP were injected along with OVA. 'Only OVA' and PBS immunized mice were kept as controls. Absolute quantification of OVA specific IgG, IgG1 and IgG2a in sera was done by indirect ELISA. OVA specific IgG, IgG1 and IgG2a ELISA kits were purchased from BD Biosciences. 96-well ELISA plate was coated with 100 μl OVA antigen/well (25 μg/ml in 50 mM carbonate-bicarbonate buffer, pH 9.6) for 24 h at 4 °C. For standard, each isotype specific rat anti-mouse purified monoclonal antibody was used for coating. Plate was washed thrice with PBST (PBS + 0.05% Tween-20) followed by blocking with 5% BSA in PBS for 1 h at room temperature (RT). After three washings, 100 μl of diluted sera samples were added and incubated for 2 h at RT. Again plate was washed three times with PBST and 100 μl of detection antibody (dilution-1: 10000) was added to the plate and incubated for 1 h at RT. After three washes with PBST, 100 μl streptavidin-HRP (dilution-1: 50000) was added and

incubated for 30 min at RT. In the next step, plate was washed five times and 100 µl/well TMB substrate was added followed by incubation for 20 min. Finally reaction was stopped by adding 50 µl stop solution per well. O.D. was measured by ELISA reader (µQuant, BioTek) at 450 nm.

2.7. OVA specific memory recall response

Memory recall response in different groups was assessed by measuring splenocyte proliferation and cytokine secretion from splenocytes stimulated with OVA antigen. Immunization strategy is described in Section 2.6. However, in each group three mice were taken.

2.8. Proliferation assay

0.2×10^6 splenocytes (isolated from different groups of mice) in RPMI-1640 medium supplemented with 10% FBS, were plated per well in 96-well round bottom plate. These cells were re-stimulated with 12 µg of OVA antigen at 37 °C. After 72 h of incubation, cultures were pulsed with 1 µCi of [^{3}H] thymidine. Cells were further incubated for 18 h, and thymidine uptake was measured by scintillation counter.

2.9. Cytokine secretion analysis

1.5×10^6 splenocytes from different groups of mice were plated per well in 24-well plate and were subsequently stimulated with 12 µg/ml of OVA antigen. Supernatant was collected after 24 and 48 h of incubation and level of cytokines was determined by ELISA.

2.10. Tumor implantation

B16F10 melanoma cells (ATCC® CRL-6322™) were cultured in RPMI-1640 supplemented with 10% FBS. For tumor implantation, B16F10 cells were harvested and viability was checked by trypan blue staining. If viability was > 90%, 30,000 cells were implanted on the right flank of syngeneic C57BL/6 mice by using a 26 × 1/2 G, 13 mm length needle.

2.11. Treatment of tumor bearing mice with different immunogens (whole cell wall/aqueous soluble cell wall fraction/whole MIP)

Four groups of mice (eight per group) received four immunizations each of whole cell wall fraction (200 µg) or aqueous soluble cell wall fraction (100 µg) or whole MIP (5×10^6 bacteria) or PBS at one week interval. Tumor was implanted after six days of primary immunization. Tumor appeared in about two weeks and its dimensions were measured regularly at two days interval with the help of Vernier Caliper. Volume of tumor was determined by formula $V = 0.5 \times L \times W \times W$ where, L is longer dimension (length) and W is smaller dimension (width) of tumor.

2.12. Tumor specific recall response

Four groups of mice (three mice per group) were immunized with three doses each of whole cell wall fraction (200 µg) or aqueous soluble cell wall fraction (100 µg) or whole MIP (5×10^6 bacteria) or PBS at one week interval. Tumor was implanted 6 days after first immunization by injecting 30000 B16F10 melanoma cells subcutaneously on right flank of syngeneic C57BL6 mice. Two weeks after tumor implantation, spleen and draining lymph nodes were isolated from mice and single cell suspension was prepared by mechanical disruption. RBCs were lysed by Gey's treatment and cells were washed twice with incomplete RPMI. Finally, cells from lymph node and spleen were re-suspended in complete RPMI medium. 1.5×10^6 cells of spleen/lymph node were plated per well in 24-well plate and stimulated with UV irradiated B16F10 melanoma cells. Supernatant was collected after 24/48 h of incubation and cytokines level was measured by ELISA.

Table 1
Yield of different fractions obtained from MIP.

Fractions	Yield (in g) (per 50 g wet cell mass of MIP)
Cell wall	1.0
Cytosolic fraction	0.56
Aqueous soluble cell wall fraction	0.32
Oil soluble cell wall fraction	0.23
Cell wall skeleton	0.21

Table shows the yield of different fractions obtained from 50 g wet cell mass of MIP. The quantities shown here are measured in grams (g).

2.13. Statistical analysis

Data were plotted as the mean ± standard error mean (SEM). Comparison was made among the groups by analysis of variance (ANOVA). p value < 0.05 was considered significant.

3. Results

3.1. Yield of different fractions of MIP after purification

For isolation of cell wall, MIP pellet was re-suspended in PBS and passed through French press, followed by centrifugation of lysate. Pellet (consisting of cell wall) and supernatant (the cytosolic fraction) obtained after centrifugation were lyophilized. Dichloromethane extraction of MIP cell wall resulted into aqueous soluble and oil-soluble cell wall fractions. Cell wall skeleton was isolated after deproteination and delipidation of MIP cell wall. Yield of different fractions obtained from 50 g wet cell mass of MIP is given in Table 1.

3.2. Immunostimulatory activity of different fractions of MIP

Immunostimulatory activity of whole cell wall (CW) and its fractions along with whole MIP was evaluated on J774.1 macrophage cell line. Dose dependent secretion of TNF-α in response to these fractions was observed. TNF-α induction in response to cell wall skeleton was higher as compared to other fractions. Aqueous soluble cell wall fraction had higher activity per unit weight as compared to oil soluble cell wall fraction and whole cell wall (Fig. 1A).

Further, we evaluated the immunostimulatory activity of these fractions on splenocytes. CWS/CW/ACW/OCW treatment resulted in significantly higher secretion of cytokines- IFN-γ (except OCW), TNF-α and IL-12 as compared to whole MIP. As compared to CW or OCW, 4 to 20 fold lower concentrations of CWS/ACW had similar immunostimulatory activity (Fig. 1B).

3.3. OVA specific memory recall response

After two weeks of third immunization, memory recall response was assessed in all the groups by lymphocyte proliferation assay of splenocytes (Fig. 2A) after re-stimulation with OVA antigen and by quantification of cytokines in splenocytes culture supernatant (Fig. 2B). Significantly higher proliferation as well as IFN-γ and IL-2 secretion was observed in OVA + CW and OVA + MIP group as compared to only OVA immunized group. Other Th1 cytokines; IL-12 and TNF-α were also higher in OVA + CW and OVA + MIP group as compared to OVA group but the increase was not significant. Enhanced but not significant Th1 activity was also observed in the group where ACW was administered as an adjuvant along with OVA. In CWS immunized group, OVA specific proliferation and secretion of pro-inflammatory cytokines were similar to 'only OVA' group. On the other hand, Th2 cytokine IL-10 was significantly increased in OVA + CWS. Findings of the study provide

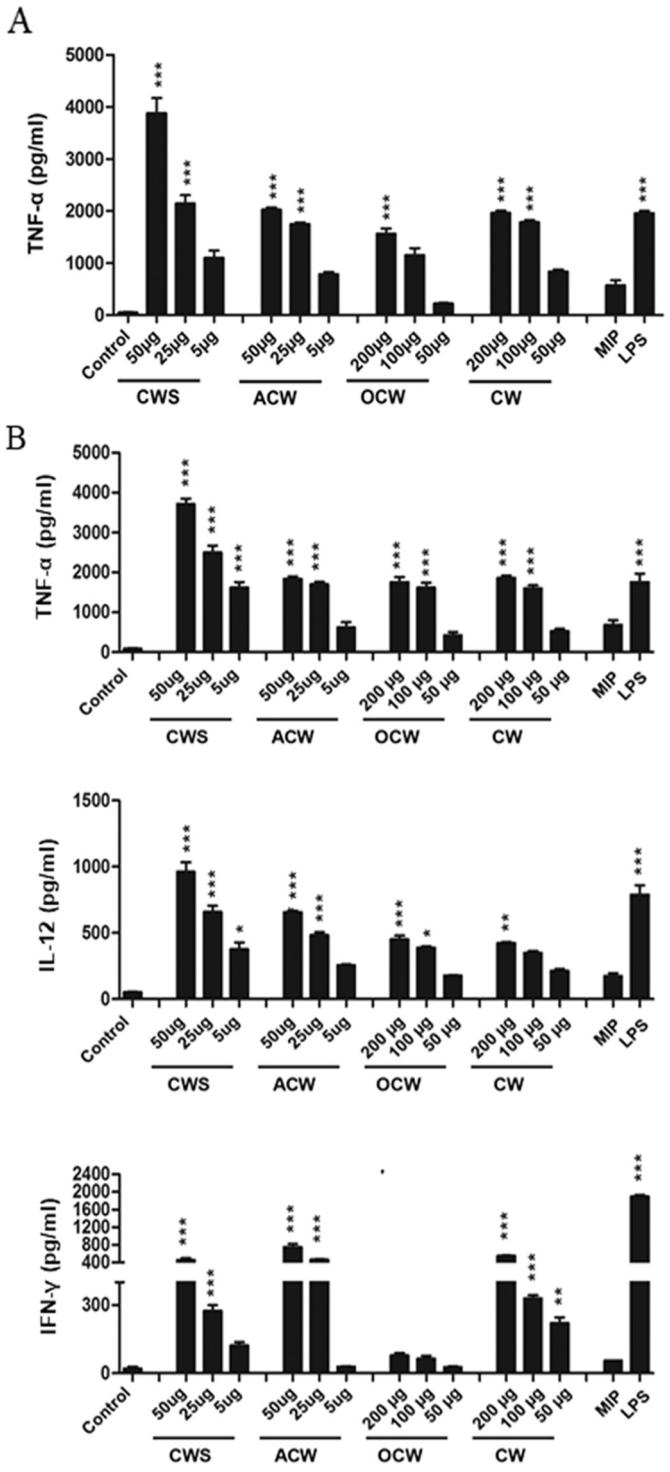

Fig. 1. Stimulation of macrophages (J774.1 cells) and splenocytes with different fractions of cell wall.

Macrophages and splenocytes were stimulated with whole cell wall (CW), aqueous soluble cell wall fraction (ACW), oil soluble cell wall fraction, cell wall skeleton (CWS) and whole MIP for 24 and 48 h. PBS was taken as control for the stimulation of macrophages and splenocytes. Panel (A) and (B) depicts the levels of cytokines in macrophages & splenocytes respectively. TNF-α and IL-12 level in splenocytes was determined by ELISA in culture supernatant collected after 24 h of incubation and IFN-γ level was determined in culture supernatant collected after 48 h of incubation. LPS (1 µg/ml) was used as positive control. MIP was used at MOI of 10. Concentration of each fraction is indicated in the figure. Data represents the mean of three sets of experiments with SEM. The data were statistically analysed by one way ANOVA [$*p \leq 0.05$; $**p \leq 0.01$; $***p \leq 0.001$; comparison were made with MIP].

evidence that whole cell wall (CW), aqueous soluble cell wall fraction (ACW) and whole MIP enhances the OVA specific Th1 response while cell wall skeleton induces Th2 response.

3.4. OVA specific IgG response

Serum was collected after 2 weeks of each immunization and the level of total IgG (Fig. 3) and ratio of IgG1/IgG2A (Table 2) was determined in all the groups. Total IgG level was highest in the group where 'whole cell wall' was used as an adjuvant at all the time points studied. Other groups like OVA + ACW, OVA + CWS and OVA + MIP also had higher total IgG level in comparison to only OVA group at all the time points. IgG1/IgG2A ratio was calculated at 2 weeks after last immunization. In comparison to OVA group, ratio of IgG1/IgG2A was lower in the groups where CW, ACW or MIP was given along with OVA antigen, indicating the Th1 promoting ability of these fractions if they are administered as an adjuvant along with antigen. Strongest Th1 property was observed in OVA + CW group which had lowest IgG1/IgG2A ratio (about 2 fold lower as compared to OVA group) among the groups. On the other hand, OVA + CWS group had about two folds higher IgG1/IgG2A ratio as compared to OVA as well as OVA + Alum group which indicate the ability of CWS in shifting the immune response towards Th2 type.

3.5. Tumor inhibitory property of MIP cell wall and its fractions

Mycobacterium indicus pranii has been shown to reduce growth of B16F10 melanoma tumor in syngeneic C57BL/6 mice by inducing Th1 immune response in tumor microenvironment. MIP cell wall and cell wall based fractions had shown strong immunostimulatory property as compared to whole MIP and in addition to this, cell wall and ACW shifted the OVA specific immune response towards Th1 type. Functional activity of these Th1 promoting CW and ACW was evaluated by determining their tumor inhibiting property. Different groups of mice received four immunizations of CW/ACW/MIP at one week interval. B16F10 melanoma cells were implanted in mice subcutaneously after 6 days of first immunization. Tumor growth was measured regularly at two days interval after the appearance of tumor.

CW, ACW and MIP suppressed the growth of tumor. All the mice of control group had visible tumor within 2 weeks after tumor implantation and tumor volume was very high in this group as compared to other treated groups. Whereas, in the treated groups tumor growth was delayed that is the tumor appeared approximately after 3 weeks of tumor implantation and in 30% of the treated groups of mice, no tumor appeared. Maximum anti-tumor effect was observed in the group treated with CW where average tumor volume was approximately 70% less as compared to that of control group. These results provide evidence of Th1 promoting functional activity of ACW and CW which demonstrated significant anti-tumor response (Fig. 4A, B, C, D & E).

3.6. Tumor specific recall response

Tumor draining lymph nodes and spleen were harvested from all the four groups of mice after two weeks of tumor implantation. Single cell suspension of lymph node and spleen were re-stimulated with irradiated B16F-10 melanoma cells for 24/48 h and cytokines were estimated in culture supernatant.

Significantly high secretion of TNF-α, IL-12 and IFN-γ was observed in splenocytes (Fig. 5A, B & C) as well as lymph node cells (Fig. 5E, F & G) of MIP treated group as compared to control group. Similar trend was observed in the CW treated group; whereas, secretion of these pro-inflammatory cytokines was moderate in ACW treated mice. IL-10 (inhibitory cytokine) secretion was found to be similar in all the groups (Fig. 5D & H). This study provides evidence that immunization with cell wall (CW) induces strong tumor specific immune response of Th1 type.

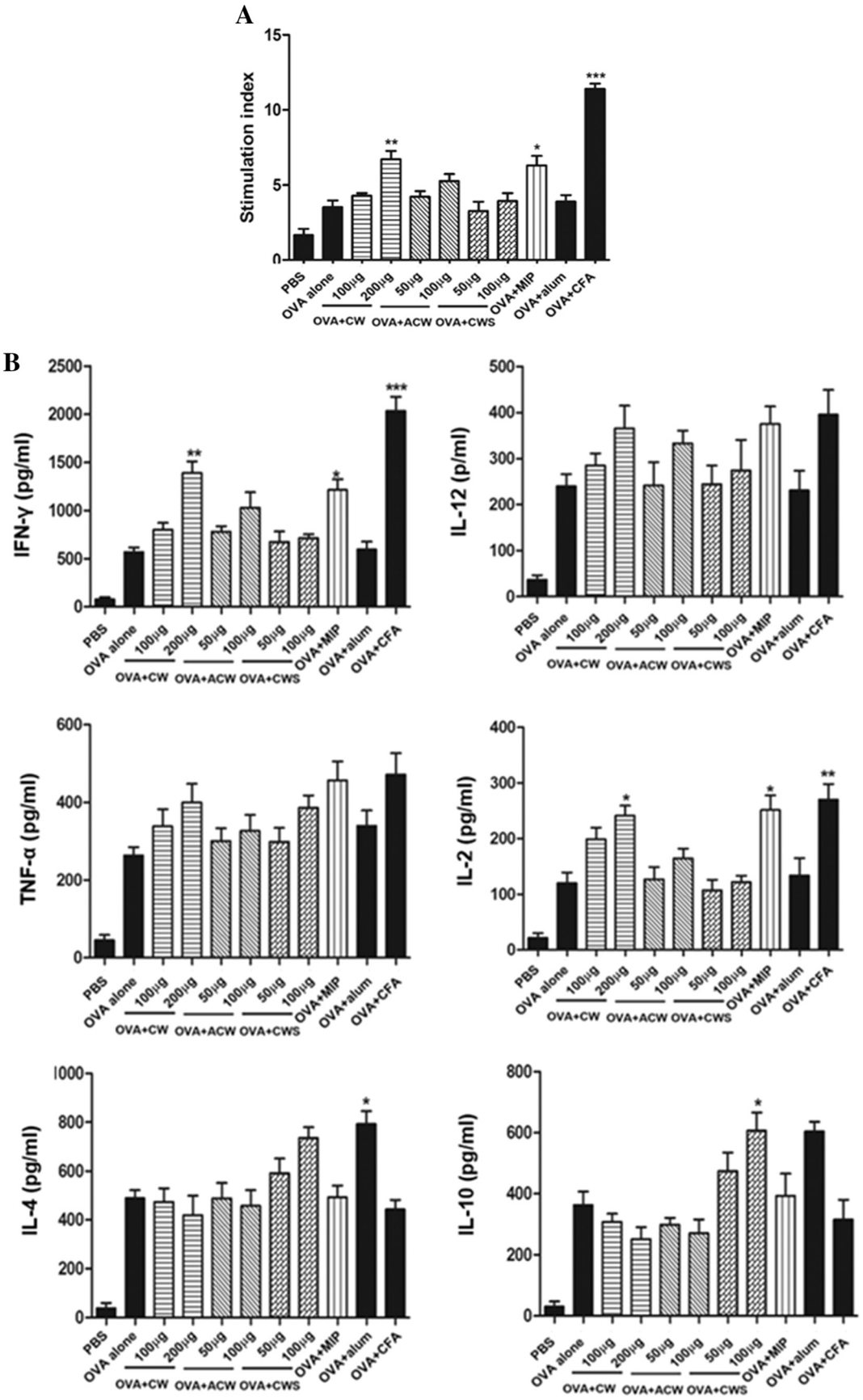

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Fig. 2. (A). Lymphocyte proliferation assay. Splenocytes from three mice of each group were pooled after 2 weeks of third immunization. These were re-stimulated with OVA (12 μg/ml) for 72 h at 37 °C; followed by additional incubation for 16 h with tritiated thymidine (H^3).
(B). Analysis of Cytokines. Splenocytes from three mice of each group were pooled after 2 weeks of third immunization and re-stimulated with OVA for 24 h and 48 h at 37 °C. IL-12, TNF-α, IL-2, IL-4 and IL-10 were quantified by ELISA in supernatant collected after 24 h. Similarly IFN-γ was measured in supernatant collected after 48 h of stimulation with OVA. IFN-γ secretion reaches at its peak at 48 h and for all other mentioned cytokines 24 h time point was optimum. Two concentrations each of MIP cell wall (CW), aqueous soluble cell wall fraction (ACW), cell wall skeleton (CWS) in combination with OVA were injected in mice of respective groups. OVA alone, CFA + OVA and Alum + OVA were taken as control. Data represents the mean of three independent experiments with standard error of mean (SEM). The data were statistically analysed by one way ANOVA [$*p \leq 0.05$; $**p \leq 0.01$; $***p \leq 0.001$; comparisons were made with 'OVA alone' group].

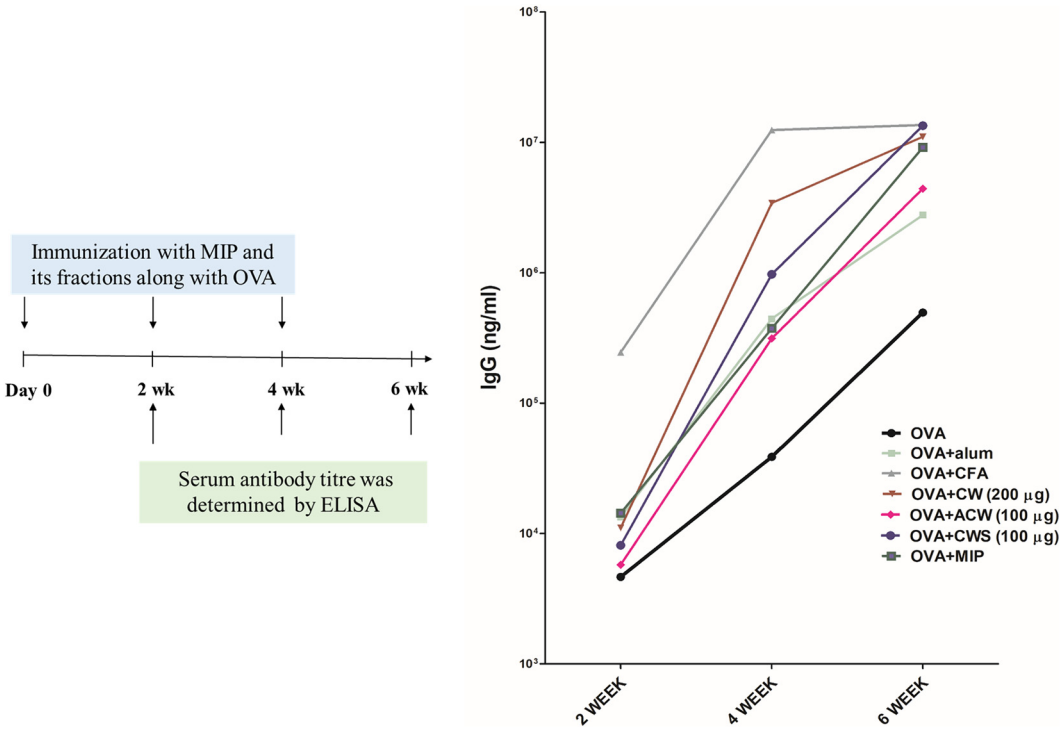

Fig. 3. OVA specific IgG response. Different groups-each containing five mice received three injections of antigen formulations at 2 week interval. Two weeks after each immunization, OVA specific IgG level was determined in the pooled serum of each group. We used two concentrations of each fractions (CW-100 μg, CW-200 μg, ACW-50 μg, ACW-100 μg, CWS-50 μg, and CWS-100 μg), however figure contains the value of fractions at higher concentration. Similar trend were observed at lower concentration of these fractions.

Table 2
Levels of IgG1 and IgG2a isotype and IgG1 and IgG2a ratio.

Antigen formulations	IgG1 (ng/ml)	IgG2a (ng/ml)	IgG1/IgG2a ratio
OVA	4.49×10^5	2.09×10^3	214.60
OVA + alum	1.51×10^6	3.924×10^3	386.20
OVA + CFA	1.17×10^7	3.07×10^5	38.22
OVA + CW (100 μg)	1.00×10^6	1.02×10^4	97.96
OVA + CW (200 μg)	4.12×10^6	3.19×10^4	128.80
OVA + ACW (50 μg)	1.25×10^6	8.54×10^3	148.20
OVA + ACW (100 μg)	1.42×10^6	9.73×10^3	146.10
OVA + CWS (50 μg)	7.29×10^6	1.29×10^4	561.00
OVA + CWS (100 μg)	4.55×10^6	9.81×10^3	464.00
OVA + MIP	1.19×10^6	7.30×10^3	164.06

Shown here are the levels of IgG1 and IgG2a isotype and the ratio of IgG1 and IgG2a in mice after injecting various antigen formulations. Different groups, each containing five mice received three injections of antigen formulations at two week interval. 2 weeks after third immunization, OVA specific IgG1and IgG2a antibodies were estimated in pooled serum from each group. Ratio of IgG1/IgG2a was calculated in all the groups.
ACW-*Aqueous soluble cell wall fraction*; CW-*Whole cell wall fraction*; CWS-*Cell wall skeleton*; CFA-*Complete Freund Adjuvant*; OCW-*Oil soluble cell wall fraction*.

4. Discussion

Though there are plenty of adjuvants known to elicit strong immune response but their use is limited due to their adverse effects like toxicity, autoimmune response and allergic reactions [15]. For human use, an adjuvant must have strong safety profile and should generate specific immune response. Vaccines for intracellular pathogens like *M.tb* and Salmonella require adjuvant that promote Th1 immune response. However, for extracellular pathogens, adjuvants that evoke strong humoral immune response are needed [16]. Alum, a Th2 response promoting adjuvant has strong safety profile and has been successfully used with several vaccines since last 80 years [3]. However, an effective adjuvant, inducing strong Th1 immune response with strong safety profile is yet to be found. In relation to this, several new adjuvant formulations have been developed and are currently being examined with recombinant subunit vaccines targeted to intracellular pathogens like *M.tb* and influenza viruses. Adjuvant formulations- AS01 (monophosphoryl lipid A combined with QS21 in liposomes) and AS02 (oil in water emulsion containing same components) have been shown to generate strong Th1 response when given with recombinant fusion protein of *M.tb* (*M.tb* antigen Rv1196 and Rv012) [17,18]. Adjuvants IC31 and CAF01 are under investigation with recombinant vaccines for TB where IC31 has shown high secretion of IFN-γ from CD4-T cells in humans [19] and CAF01 a liposome based formulation, containing a synthetic analog of mycobacterial cord factor (TDB) has shown long lived Th1 and Th17 responses in mice [20,21]. The above mentioned adjuvants have purified compounds which have lower toxicity as compared to their crude source but still they have side effects which cannot be ignored and there is need to find new adjuvants with safety

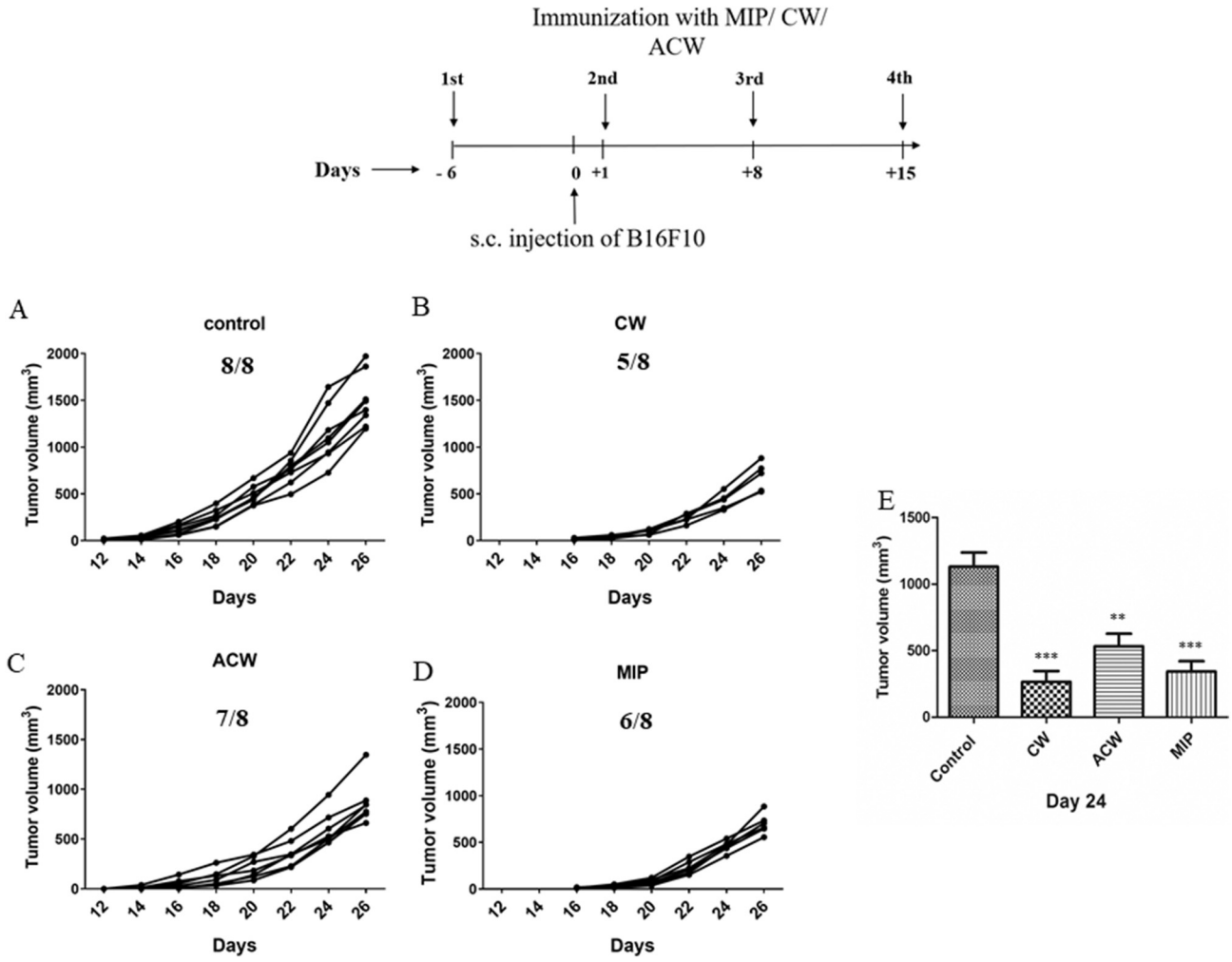

Fig. 4. Anti-tumor activity of MIP cell wall and its fractions. Mice were immunized with four doses each of cell wall (200 μg)/aqueous soluble cell wall fraction (100 μg)/killed MIP (5×10^6) at one week interval. 30,000 B16F10 melanoma cells were implanted on the right flank of each mouse, six days after first immunization. Control mice were given PBS. Tumor growth was measured at two days interval. Ratio (fraction value) indicates the number of tumor bearing mice per group of 8 mice. The graphs depict the tumor volume measured till day 26 (A, B, C & D). At day 24, average tumor volume of different groups is shown in bar graph (E). The data were statistically analysed by one way ANOVA [$*p \leq 0.05$; $**p \leq 0.01$; comparisons were made with saline group].

profiles similar to alum and ability to induce strong Th1 response. For routine childhood vaccines, safety is the biggest concern. Whole MIP has well established safety profile; is approved for human use and is being evaluated in clinical trials. Similarly, no untoward reaction was seen with any of the cell wall fraction when studied *in vivo*.

MIP has shown immune-adjuvant activity in several studies. It induced significant Th1 response in tumor microenvironment; activated the macrophages and dendritic cells which were otherwise in immunosuppressed state and increased the infiltration of T cells in tumor milieu, when given by peritumoral injection in mouse model of melanoma [10]. In tuberculosis also, therapeutic administration of MIP along with chemotherapy resulted in improved Th1 immunity in infected lungs. This shift in immune response towards Th1 type reduced the bacterial burden [11]. In a recent study, where MIP was used as an immunomodulator with hCG vaccine adsorbed on alhydrogel resulted in significant increase of antibody titer against hCG vaccine as compared to the vaccine on alhydrogel alone [22]. These studies have provided sufficient ground for further investigation of MIP to explore its immunoadjuvant/imunostimulatory activity.

Immunostimulatory activity of different fractions of MIP CW/ACW/OCW and CWS was evaluated on macrophages and splenocytes. These cell wall fractions showed strong immunostimulatory activity in macrophages and splenocytes, which was substantially higher than whole MIP except for oil soluble fraction. Very low IFN-γ secretion from splenocytes after treatment with oil soluble fraction could be explained as oil soluble cell wall fraction is very low in protein content and high in lipids. Unlike proteins, lipids are presented through molecules like CD1d receptor and activate NKT cells which also secrete IFN-γ but amount of IFN-γ secreted in this case is very low due to poor activation of other T-cell populations like CD4 and CD8-T cells. So, the low protein content could be the reason behind poor secretion of IFN-γ from splenocytes stimulated with OCW [23,24]. On the other hand, CW, ACW and CWS have complex structure with several immune active components which could be responsible for their immunostimulatory activity on splenocytes. ACW was obtained after DCM extraction of cell wall and is likely to have most of the components of cell wall except lipophilic components that would be in OCW. Another noteworthy observation was that as compared to CW or OCW, 4 to 20 fold lower concentrations of CWS/ACW had similar immunostimulatory activity. It is very unlikely that removal of lipids from cell wall has increased the immunogenicity of resulting fractions; possible explanation could be the increased concentration of proteins per unit weight of ACW after removal of lipids from cell wall. Higher immunostimulatory activity including IFN-γ secretion after treatment with CWS could be due to its complex structure which has several immunostimulatory components *viz*. core-mycolyl arabinogalactan-peptidoglycan (mAGP) complex

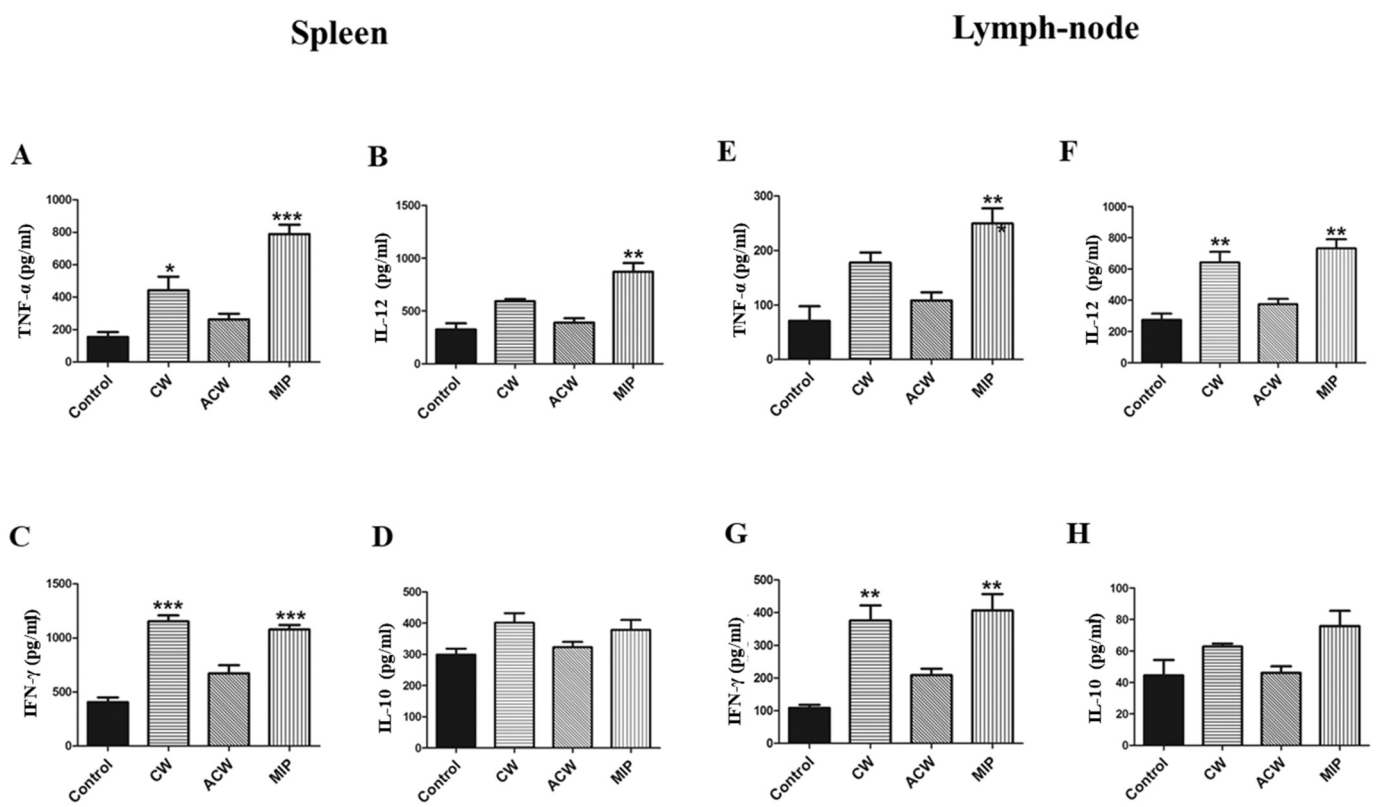

Fig. 5. Tumor specific recall response. Single cell suspension of spleen (A, B, C & D) and tumor draining lymph node (E, F, G & H) from three mice of different control and treated groups were prepared two weeks after tumor (B16F10 melanoma) implantation and re-stimulated *in vitro* with UV irradiated B16F10 melanoma cells for 24/48 h. Level of cytokines was determined by ELISA. TNF-α, IL-12, and IL-10 were measured after 24 h of stimulation; while, IFN-γ was measured in culture supernatant collected at 48 h after stimulation. Data is presented as mean ± SEM of three independent experiments ($n = 3$). The data were statistically analysed by one way ANOVA [$^{*}p \leq 0.05$, $^{**}p \leq 0.01$; comparisons were made with saline group which was taken as control].

[25,26]. Peptidoglycan portion of cell wall is recognized by TLR2 and TLR4 receptors of antigen presenting cells that result in activation of APCs [27]. Presence of mycolic acid in cell wall skeleton probably makes it suitable for presentation *via* CD1d molecules. In addition, PGN recognition proteins and NOD proteins have also been reported in cell wall skeleton [28,29]. Presence of these immunostimulatory molecules in CWS could be responsible for efficient secretion of pro-inflammatory cytokines from splenocytes. Adjuvants under development have diverse mechanism of action. MIP and its cell wall fraction fall in the category of 'immunostimulators' as these induce production of immunomodulatory cytokines. Examples of other immunostimulators which are being evaluated are FCA, MDP and derivatives of Lipid-A but their toxicity is matter of concern.

Aim of this study was to isolate and identify the immunostimulatory fraction/s of MIP which could be explored for immune-adjuvant activity. As substantial immunostimulatory activity of CW, ACW and CWS was observed in macrophage cell line and splenocytes; these fractions along with whole MIP were further evaluated for adjuvant properties. OVA was taken as model antigen. There was an increase in OVA specific IgG level when any of these fraction was administered as an adjuvant along with OVA. In comparison to OVA alone, ratio of IgG1:IgG2a was reduced considerably in cell wall group while moderate reduction was observed in ACW/MIP group providing evidence that CW/ACW/MIP shifts the OVA specific immune response towards Th1 type. On the other hand, immune response shifted towards Th2 type in cell wall skeleton group, as very high ratio of IgG1:IgG2a was observed in this group as compared to 'OVA alone' group.

It is well established that Th1 immune response is crucial for the control of tumor growth. As CW, ACW and MIP modulated the OVA specific immune response towards Th1 type, functional activity of these fractions was evaluated in mouse model of tumor. CW fraction reduced the tumor growth considerably followed by MIP and ACW. Elimination of lipid components in aqueous soluble cell wall fraction (ACW) has probably made it less immunogenic when used *in-vivo*, suggesting that combination of both lipids and proteins make the cell wall more effective adjuvant.

In this study, first time it is shown that cell wall fraction of MIP significantly reduces the tumor growth similar to whole MIP. Mycobacterial cell wall has number of immunostimulatory components like peptidoglycan, arabinogalactan, phenolic lipids, lipoarabinomannan, dimycolyltrehalose (cord factor), phthiocerol dimycocerosate (DIM/PDIM), PIM and sulpholipids (SLs). These components have been shown to play crucial role in immune modulation [7]. Elimination of lipid components in aqueous soluble cell wall fraction has probably made it less immunogenic suggesting that combination of both lipids and proteins make the cell wall more effective adjuvant.

Whole cell wall fraction demonstrated substantial Th1 inducing adjuvant activity while cell wall skeleton induced strong Th2 type of immune response *in vivo*. These two fractions could be evaluated in different formulations *viz*. emulsions, micro particles *etc*. which possibly would further increase their adjuvant activity. CW along with tumor specific antigens would be evaluated in formulations like biodegradable polymer microparticles for targeting to antigen presenting cells macrophages/dendritic cells which possibly would further strengthen its anti-tumor activity.

This is the first study where immunostimulatory/adjuvant activity of different fractions of MIP have been compared. This study provides evidence for potential adjuvant activity of cell wall fractions of MIP which should be further explored.

Acknowledgements

This work was supported by the core research grant of the National Institute of Immunology. This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Conflict of interest statements

The authors declare that they have no competing interests.

Animal ethics

The experiments involving the use of animals were done in accordance with the Institute's Animal Ethics guidelines. IAEC approval number was IAEC#362/14.

Author contributions

Mohd. Saqib: Conception and design, Methodology, data curation, analysis and interpretation of the data, Writing– original draft.

Rahul Khatri: Conception and design, data curation.

Bindu Singh: Formal analysis, Methodology, Writing– review & editing.

Ananya Gupta: Methodology, Writing– review & editing.

Sangeeta Bhaskar: Conceptualization and design of experiments, Analysis and interpretation of the data, Management of resources, Project administration, Supervision, Writing – review & editing.

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