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Inclusion of non-target antigen in vaccination favors generation of OVA specific CD4 memory T cells☆

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ABSTRACT

Inducing long-lived memory T cells by sub-unit vaccines has been a challenge. Subunit vaccines containing single immunogenic target antigen from a given pathogen have been designed with the presumption of mimicking the condition associated with natural infection, but fail to induce quality memory responses. In this study, we have included non-target antigens with vaccine candidate, OVA, in the inoculum containing TLR ligands to suffice the minimal condition of pathogen to provoke immune response. We found that inclusion of immunogenic HEL (hen egg lysozyme) or poorly immunogenic MBP (Myelin Basic protein) non-target antigen enhances the OVA specific CD4 T cell responses. Interestingly, poorly immunogenic MBP was found to strongly favor the generation of OVA specific memory CD4 T cells. MBP not only improves magnitude of T cell response but also promotes the T cells to undergo higher cycles of division, one of the characteristic of central memory T cells. Inclusion of MBP with vaccine targets was also found to promote multiple cytokine producing CD4 T cells. We also found that challenge of host with non-target antigen MBP favors generation of central Memory T cells.

1. Introduction

Generation of long-lived antigen experienced lymphocytes in host is often associated with the protective immunity provoked naturally by pathogens [1]. Although vaccination is done to induce the same, often the immune response generated by subunit/non-live vaccines fail to achieve the desired goal, particularly memory T cell responses [2]. Subunit vaccines are usually the formulation of selected immunogen(s) from the pool of antigens of particular pathogen along with adjuvants and used as vaccine candidate(s). It is expected that the inoculum of the selected Ag mixed with the adjuvant, perceived to mimic natural infection, would provoke desired immune responses in the vaccinated host [3]. Despite considerable success in generating robust immune responses, vast majority of subunit vaccines have been developed without thorough understanding of their requirements to induce memory responses [3]. The question arises whether the subunit vaccine formulation really represents the condition associated with the infection to provoke the desired memory response. In many experimental studies, it is well established that long-lived memory T cells could be generated in hosts encountering pathogens through natural infection [4–6]. In case of pertussis whole organism vaccine protection lasts for 12 years whereas DTaP (combined diphtheria-tetanus-acellular pertussis) vaccine induces protective response lasting for 6 years [7]. The existing subunit vaccine formulation works well for B cell responses [8], but inducing optimal T cell response, particularly memory formation has been a challenge [9,10]. It is critical to understand why induction of memory T cells to immunogenic target is efficient in case of host encountering pathogen through natural infection or whole organism vaccination as opposed to that of subunit vaccine?

Following the onset of an infection host mounts response to few, out of several Ags derived from the pathogen. The frequency of immunogenic antigen (s) (from here onwards termed as target antigens, TAgs) is very low, may be one out of thousands of Ags. PAMPs (pathogen associated molecular patterns) derived from the pathogens have been shown to enhance the immune responses generated against the immunogens. However, PAMPs, used as an adjuvant in the vaccine formulation, alone may not provide all the required help to induce the desired Ag specific response; otherwise all sub-unit vaccines would have been successful in generating long-lived memory response. Even the most potent adjuvants given by best possible route would not

☆ Part of this work is already presented during AAI 2015 Meeting entitled “Generation of central memory T cells by vaccination that mimics natural infection” (LYM5P.704).

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transform weakly immunogenic proteins into potent vaccine antigens independently. Therefore, the multiple antigens themselves might have a very crucial role. It is possible that such TAgs in absence of other antigens (from here onwards termed as non-target antigens, N-TAgs), against which the host may or may not have induced the immune responses, might not suffice the optimum condition of Ag milieu to mimic natural infection to provoke long-lived memory lymphocytes. Use of immunogenic Ag(s) is a sound approach for developing vaccines, but it is likely that immune response to the TAg is influenced by the presence of non-target Ags (N-TAgs) of the pathogen, that are usually ignored in vaccine formulations, such ideas are discussed in our review 'Developing effective vaccines: Cues from Natural infection' published in International Reviews of Immunology [11]. The N-TAgs could be non-immunogen, poorly immunogen, or another immunogen as it may be the case in multivalent vaccines. Furthermore, we often experience that individuals during particular seasons of the year get exposed to multiple infections, or while evoking immune response to one, they encounter another pathogen [12]. It is imperative to understand whether Ags of the secondary infection influence the development of memory T cells to Ag(s) encountered from the first infection.

In this study, we mimicked the pathogen minimally by including N-TAgs with the vaccine targets and designed the experiment to study the role of N-TAgs in modulating CD4 T cell responses generated against vaccine target. C57BL/6 mice were immunized with a model protein Ag, ovalbumin (OVA) mixed with TLR ligands (from bacterial or viral origin) in the presence/absence of immunogenic or poorly immunogenic N-TAg, HEL (Hen egg lysozyme) or MBP (Myelin Basic Protein), respectively, and measured antigen specific CD4 T cell responses to OVA. Memory T cells specific to OVA were characterized based on the expression of phenotypic markers (CD44, CD62L) and reactivity of MHC II-OVA tetramer, and their response to TAg was measured by cell proliferation and intracellular IFN-γ, IL-2 and TNF-α production upon *ex vivo* OVA challenge. We found that inclusion of non-target antigen (HEL or MBP) favors the generation of target Ag specific T cell responses including that of memory T cells. Particularly, the poorly immunogenic MBP promoted the generation of significantly higher frequencies of central memory CD4 T cells against OVA compared to that of immunogenic N-TAg counterpart. Further, the OVA specific CD4 T cells generated in the presence of MBP were also found to be multiple cytokine producing in nature.

2. Materials & methods

2.1. Mice

C57BL/6 mice (6–8 weeks old) were obtained from Zydus Research Centre (Ahmedabad, India) and housed in pathogen free conditions in The Animal facility of Nirma University (Ahmedabad). They were acclimated for a week before studies begun. Food and water were available ad libitum. All experimental protocols were reviewed & approved by Institutional Animal ethics committee (IAEC) of Nirma University and performed in a facility accredited by the IAEC.

2.2. Reagents & antibodies

LPS (tlrl-pelps), Zymosan (tlrl-zym), and Poly I: C (tlrl-pic) were purchased from Invivogen, R848 (SML0196) from Sigma Aldrich and dissolved according to manufacturer's instructions. PPD was a gift from Span Diagnostics. OVA (32467-1GM) and HEL (4403-1GM) were purchased from Merck Millipore, and Myelin Basic Protein (MBP; M1891-5MG) from Sigma-Aldrich. The following mouse monoclonal antibodies were used in different combinations as per the requirement of an experiment. PerCP-conjugated anti-CD3 (145-2C11 clone), PE-Cy7-conjugated anti-CD44 (IM7 clone), V450-conjugated anti-CD62L (MEL-14 clone), V500-conjugated anti-CD4 (RM4-5 clone), APC-conjugated anti-IFN-γ (XMG1.2 clone), FITC-conjugated anti-IL-2 (JES6-5H4 clone), PE-conjugated anti-TNF-α (MP6-XT22clone) (BD Bioscience or BioLegend), respectively. Isotype Controls were also used.

2.3. Immunization

Mice were immunized subcutaneously with TAg with or without TLR agonist in presence or absence of N-TAgs of varying immunogenicity during priming and challenge. While OVA, was used as TAg, PPD and HEL were used as immunogenic, MBP as poorly immunogenic N-TAgs, respectively.

2.3.1. Modulation of target antigen (OVA) specific CD4 T cell response by Non-Target antigens during priming

C57Bl/6 mice were immunized with OVA (100 μg/mouse) in the presence/absence of PPD (0.2 μg/mouse) mixed in IFA or HEL (50 μg/mouse) mixed in PBS with or without TLR ligands LPS (10 μg/mouse) & Zymosan (25 μg/mouse) and sacrificed on Day 15. In the subsequent experiments, C57BL/6 mice were immunized with OVA (100 μg/mouse) in the presence/absence of N-TAgs; HEL (50 μg/mouse) or MBP (50 μg/mouse) mixed in PBS. Mice immunized with HEL or MBP in presence of TLR ligands were used as control groups. Poly I: C (25 μg/mouse) and R848 (25 μg/mouse) were used as PAMPs to mimic viral infection. On day 100 mice were sacrificed and CD4 memory response was measured to OVA.

2.3.2. Modulation of target antigen (OVA) specific CD4 T cell response upon challenge by non-target antigens

C57Bl/6 mice immunized with OVA (100 ug/mouse) along with TLR ligands Poly I: C (50 μg/mouse) and R848 (40 μg/mouse). On day 21 mice were *in vivo* challenged with HEL (50 μg/mouse) or MBP (50 μg/mouse) in presence or absence of OVA (25 μg/mouse) along with TLR ligands as described. On Day 70 mice were sacrificed and CD4 memory response was measured to OVA.

2.4. T cell proliferation and intracellular cytokine assays

On indicated days as mentioned cells from inguinal lymph nodes were harvested and CD4 T cells were challenged *exvivo* with increasing concentration of OVA to measure OVA specific T cell response. The cells harvested from each mouse (n = 6/per group) were used for *invitro* stimulation at varying concentrations of OVA. For each concentration cells were used in triplicates containing 0.2 million cells per well in case of T cell proliferation assay and 0.5 million cells per well in case of Intracellular Cytokine Staining. For *invitro* stimulation, cells were incubated at 37 °C and 5% CO2. Whole protein was used for stimulations. For T cell proliferation assay, CFSE (21888-25MG-F) labeled T cells were stimulated for 72 h and for intracellular cytokine assay, *invitro* stimulation with antigen was given for 18 h with last 8 h in presence of BFA [13,14,15]. BFA was added to each well during incubation without disturbing the wells. During antibody staining for flow cytometry all the three wells of each concentration were pooled. CD4 T cell response was measured by flow-cytometry using fluorescently labeled antibodies CD4-V500, CD44-PE/Cy7 and CD62L-V450 and effector function by APC-IFN-γ, PE-TNF-α and FITC-IL-2 [14]. Cells were prepared with Cytofix kit (BD Biosciences; 554714) in presence of brefeldin A (BD Biosciences; 555029), acquired using a FACS Canto II flow cytometer (BD Biosciences) and analyzed using FlowJo ver.10 software. For T cell proliferation assay, percentage of dividing cells was calculated as no. of cells divided (G1-G5) by the total no. of CD4 T cells (sum of Divided and Undivided cells). Also, we calculated the percentage of dividing cells undergoing number(s) of cycles of division. For intracellular cytokine, we first presented data on the frequency of total cytokine-positive cells expressing IFN-γ, IL-2, or TNF-α (magnitude) and then co-expression profiles for these cytokines and their relationship to the expression of other functions. Lymphocytes were identified based on their scatter patterns. CD4 T cells were then gated for cells positive for the respective

cytokines. Boolean combination gating was then performed (http://www.flowjo.com/v8/html/boolcomb.html) to calculate the frequencies of expression profiles corresponding to the seven different combinations of cytokines by using the FlowJo software. The proportions of the seven different subsets were expressed as percentages of total cytokine-positive cells and the results for mean frequencies of the T-cell subsets positive for the indicated cytokines in each group was represented as pie charts.

2.5. Sample preparation for Tetramer staining

On indicated days cells the from inguinal lymph nodes were harvested and single-cell suspensions were first incubated for 10 min at 4 °C in the presence of Fc Block (prepared in FACS buffer). Tetramer staining was performed at 4 °C for 45 mins-1 h with 3ul (1.3 mg/mL) of MHC II OVA tetramer, I-A (b) HAAHAEINEA PE (38659) provided by NIH Tetramer core facility followed by a 30–35 min surface staining at 4 °C with CD4-V500, CD44-PE/Cy7and CD62L-V450, CD3-PerCP Abs.

2.6. Statistics

Unless otherwise mentioned, the data are presented as the means ± standard errors of the mean (SEM). Statistical analysis was performed in GraphPad Prism 6, and means were compared by two-tailed non-parametric Mann-Whitney *U* test. A P value of < 0.05 was considered statistically significant.

3. Results

3.1. PPD positively modulates CD4 T cell response to OVA

For long CFA (complete freund's adjuvant) having heat killed *Mycobacterium tuberculosis (M.tb)* is in use by immunologists to induce immune response against any given Ag [16]. Although PAMPs associated with *M.tb* is known to potentiate immune response, the role of PPD, the major Ags of *M.tb* in modulating immune response to vaccine candidate Ag is not clear. To study the influence of PPD on OVA specific $CD4^+$ T cell responses we inoculated mice with the emulsion of OVA and agonists of TLR-2 & -4 (Zymosan, in place of *M.tb* LAM, & LPS, in place of *M.tb*. HSP) [17–19] in IFA with/without PPD, and measured the antigen specific T cell response in draining LNs on day 15 post immunization by challenging the lymphocytes *exvivo* with OVA (Fig. 1B). CFA was used as positive control. Mice immunized with OVA alone were found to have lower CD4 T cell responses although the same was amplified *exvivo* to OVA challenge in dose dependent manner (Fig. 1C, D). Under similar conditions but in presence of TLR agonists mice responded better to OVA until 0.5 μg/ml and the response declined at 1 μg/ml. However, challenge with higher concentration of antigen further induced CD4 T cells responses. Interestingly, we found that inclusion of PPD in the formulation of OVA and TLR ligands has boosted 3–4 folds higher T cell responses ($p < 0.05$) (Fig. 1C, D). When PPD was injected to mice along with OVA in absence of TLR ligands CD4 T cells in PO (PPD/OVA) group respond dramatically even at low concentration of in vitro OVA challenge compared to that of mice inoculated with OVA alone. However, the difference in the CD4 T cell responses between OVA and PO group becomes marginal at the higher conc. of OVA stimulation. Nevertheless, response to low OVA stimulation suggest that the OVA specific CD4 T cells generated in the PO group are qualitatively different compared to OVA alone group (Fig. 1C, D). Upon analysis of cell division, we found the results were very intriguing that the presence of PPD in the formulation of vaccine inoculum favors the generation of T cells that undergo multiple (up to five divisions) rounds of cell division in response to Ag challenge (Fig. 1E). It is known that the true memory (Tcm) population undergoes higher number of divisions. Here, the intent of presenting the total division of cells in terms of G1, G2….. G5 cycles is to reflect the role of non-target antigens in Memory T cell formation. Surprisingly, we found that the pattern of cell proliferation among CD4 T cells generated among mice immunized with PPD in presence of TLR ligands was restricted to one or two rounds of division although higher frequencies of T cells had undergone proliferation to Ag challenge (Fig. 1E).

3.2. HEL positively modulates CD4 T cell response to OVA

PPD is a complex mixture of antigens and is known to induce inflammatory response [20,21]. Therefore, we wanted to use a more defined antigen in our subsequent experiments to understand the influence of N-TAgs in modulating CD4 T cell response to TAg. Here we used HEL (Hen Egg Lysozyme) as N-TAg while inducing response to OVA. We injected mice with OVA mixed with HEL in the presence or absence of TLR agonists LPS and Zymosan (Fig. 2A). On day15post immunization, lymphocytes from draining LNs were harvested and T cells were challenged, *exvivo* with OVA to measure antigen specific response. We found that mice injected with OVA alone responded to antigenic challenge in dose dependent manner (Fig. 2B, C) which is consistent with our observations in Fig. 1. Inclusion of TLR agonist was found to boost the generation Ag specific CD4 T cells (Fig. 2B, C). Upon inclusion of HEL in presence of TLR ligands, the response was boosted 2–3 folds higher compared to mice immunized with OVA (Fig. 2B, C). Interestingly, when HEL mixed with OVA was injected to mice in absence of TLR agonists, the T cells responded in a linear dose dependent manner but the OVA specific T cell response was lower (although not significant) when compared to mice immunized with OVA alone. Upon analysis of T cell division, we found that inclusion of HEL with target antigen OVA in presence of TLR ligands promotes higher cycles of division of CD4 T cells, which is very similar to that observed in PPD (Fig. 2D).

3.3. Inclusion of poorly immunogenic N-TAg MBP with vaccine targets favors generation of central memory T cells

HEL is known to be an immunogenic antigen [22,23]. In this study, we wanted to understand how N-TAgs having different level of immunogenicity influence the immune response to TAg, particularly in the formation of memory T cells. Various experimental vaccine models demonstrate that viruses are much better in generation and maintenance of memory T cells [24]. We wanted to test these ideas in a system that would closely mimic viral infection. Hence we used Poly I:C and R848 as PAMPs instead of LPS and Zymosan [25]. Here, we used HEL as immunogenic and MBP as poorly immunogenic N-TAgs [26–28]. C57BL/6 mice were immunized subcutaneously at base of the tail with OVA in presence or absence of HEL or MBP (Fig. 3A). On day 100-post immunization, lymphocytes were harvested from inguinal lymph nodes and challenged *exvivo* with OVA, HEL or MBP to measure the antigen specific response. We used naive mice as negative control, whereas OVA and ORP groups were used as experimental groups. We have also included HRP and MRP as control groups in which mice have not been injected with OVA during immunization. Frequencies of OVA, HEL or MBP specific CD4 T cells before immunization (Naive) and after immunization (OVA immunization group) were determined. Change in basal level of Ag specific effector T cells response upon antigen sensitization as compared to control mice suggests that the population of memory T cells characterized based on phenotypic markers contains antigen-experienced CD4 T cells having memory phenotype (Fig. 3C). We found that mice immunized with OVA in presence of poor immunogen MBP show significantly ($p < 0.01$) higher frequencies of T_{CM} when compared to mice immunized with OVA along with TLR ligands (Fig. 3B). While mice immunized with OVA along with TLR ligands show lowest frequencies of T_{CM}, inclusion of HEL improved the generation of T_{CM} (Fig. 3B). The T_{EM} to T_{CM} ratio in case of stimulated samples is highest on inclusion of MBP (1.74), which indicates that upon second exposure to OVA/Ag experienced central memory cells

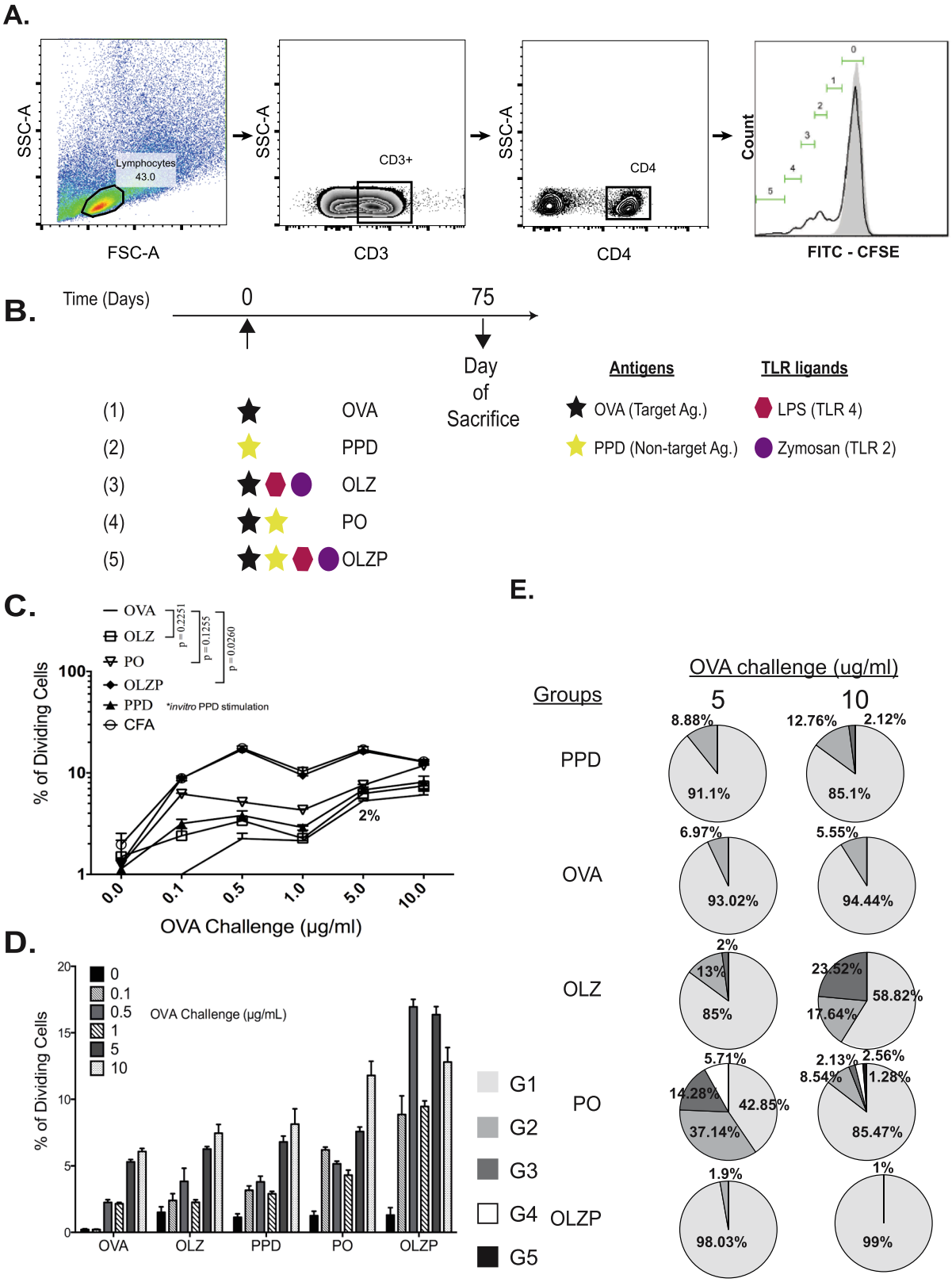


Fig. 1. PPD positively modulates OVA specific CD4 T cell response. (A) Schematics for Gating Strategy used for Flow data analysis to characterize $CD4^+$ T cells by using CD3 and CD4 cell surface marker on gated lymphocytes population. Histograms represent proliferative response of CFSE stained CD4 T cells in DLNs. (B) Groups of C57BL/6 mice (n = 6) were immunized with OVA in presence or absence of PPD with or without TLR ligands LPS and Zymosan. On day 15 post immunization cells were harvested from inguinal lymph nodes and challenged *exvivo* for 72 h with increasing concentration of OVA. T cell proliferation assay was done by CFSE dilution. (C & D) Percentage of dividing cells upon *exvivo* OVA challenge. (E) CD4 T cell divisions upon *exvivo* OVA challenge. Data shown are representative of three independent experiments. Error bars represent standard error of the mean (SEM). $p < 0.05$was considered as statistically significant. (O: OVA, P: PPD, L: LPS, Z: Zymosan).

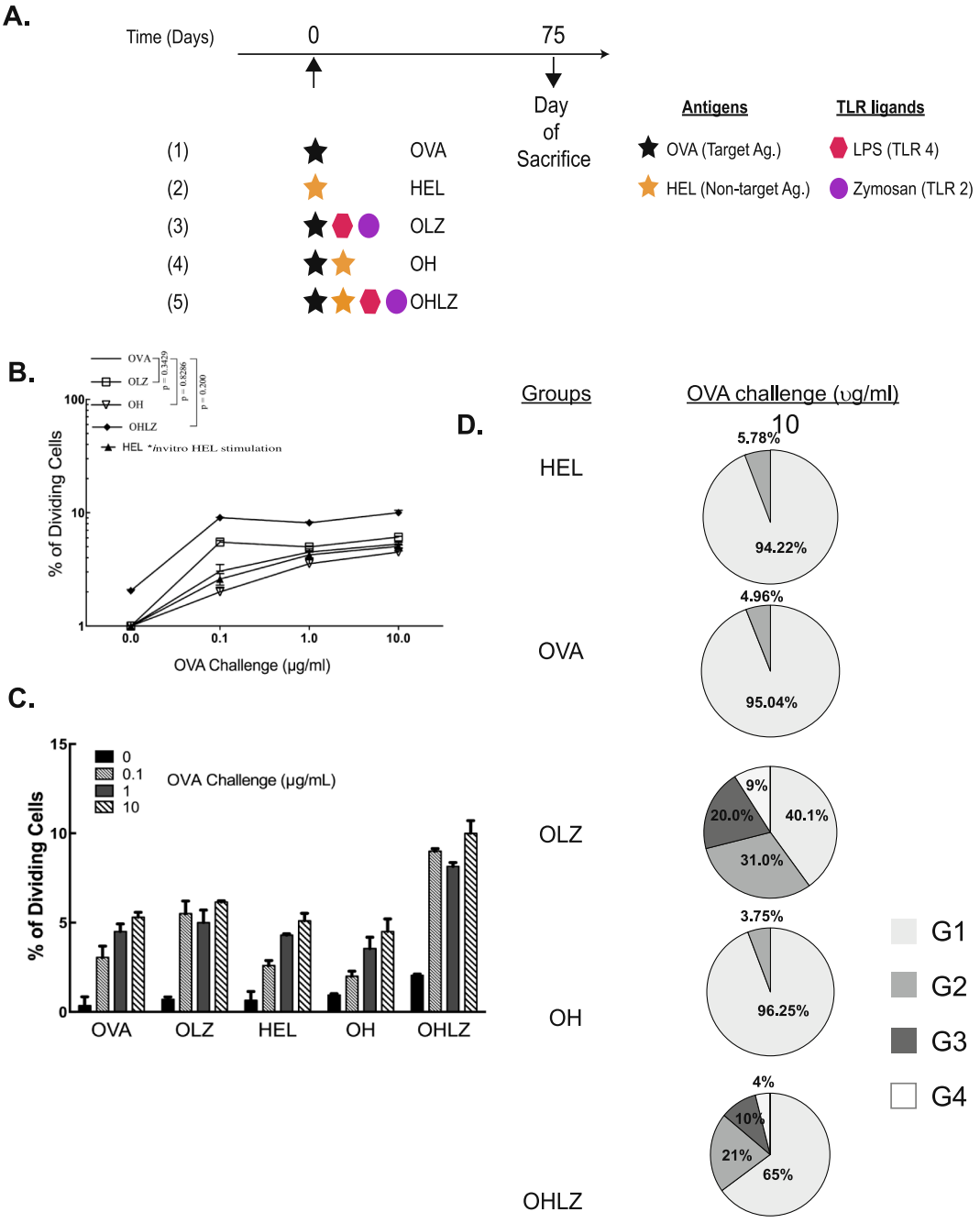


Fig. 2. HEL positively modulates OVA specific CD4 T cell response. (A) Groups of C57BL/6 mice (n = 6) were immunized with OVA in presence or absence of HEL with or without TLR ligands LPS and Zymosan. On day 15 post immunization cells were harvested from inguinal lymph nodes and challenged *exvivo* for 72 h with increasing concentration of OVA. T cell proliferation assay was done by CFSE dilution. Gating Strategy as described in Fig. 1. (B & C) Percentage of dividing cells upon *exvivo* OVA challenge. (D) CD4 T cell divisions upon *ex vivo* OVA challenge. Data shown are representative of three independent experiments. Error bars represent standard error of the mean (SEM). $p < 0.05$ was considered as statistically significant. (O: OVA, H: HEL, L: LPS, Z: Zymosan).

might be formed during primary encounter and may get converted to effector cells.

Similarly, inclusion of MBP in the presence of TLR ligands promoted generation of higher frequencies of CD4 T cells compared to that of immunogenic N-TAg HEL as evident from cell proliferation (Fig. 3D). Interestingly, we found that presence of MBP in the formulation of vaccine inoculum favors the generation of T cells that undergo multiple (up to five divisions) rounds of CD4 T cell division, a characteristics feature of long-lived memory (Supplementary Fig. 1B).

3.4. Inclusion of N-TAg MBP with vaccine targets promotes generation of multi-cytokine producing CD4 T cells

Here we have looked for the frequencies of memory T cells based on surface expression of CD44 and CD62L, which might not represent all the T cells specific to OVA. Therefore, antigen recall response in terms of IFN-γ, TNF-α and IL-2 synthesis to *ex vivo* OVA challenge was measured [13]. The gating strategies used for all cytokine response were based on cytokine isotypes and FMO controls. We had used AntiCD3 as

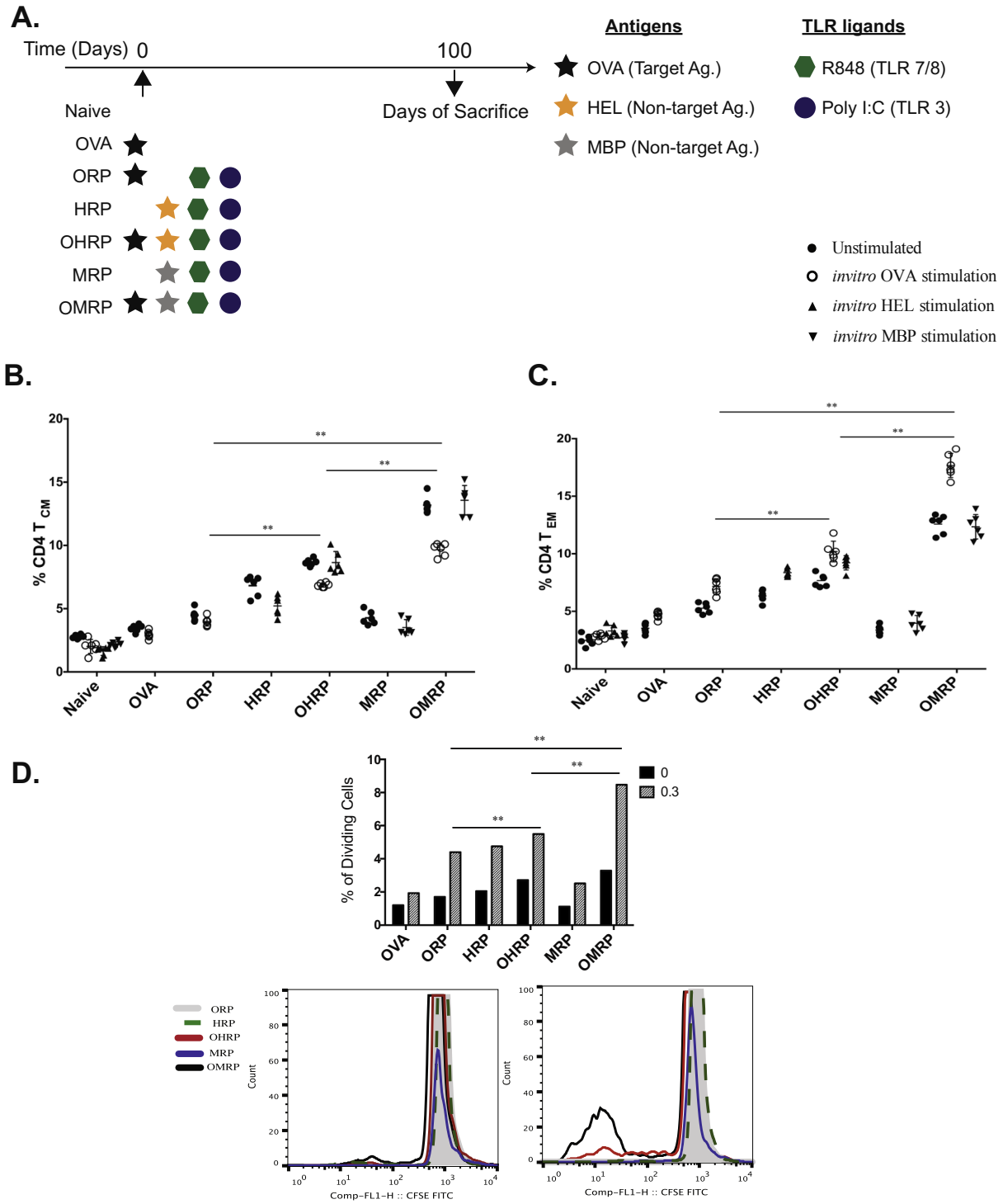


Fig. 3. Poorly immunogenic Non-Target Antigen MBP modulates the generation of T_{CM} & T_{EM} against OVA during priming. (A) Groups of C57BL/6 mice (n = 6) were immunized subcutaneously at base of the tail with OVA in presence/absence of N-TAgs; HEL or MBP along with Poly I: C and R848. On day 100 post priming lymphocytes were harvested from inguinal LNs (DLNs) and CD4 T cells were challenged *exvivo* to measure OVA specific response. Cell surface staining with memory markers (CD44 and CD62 L) and intracellular cytokine staining was performed for the characterization of T cells as per protocol. (B) Frequencies of memory CD4 T cells, T_{CM} ($CD44^{hi}CD62L^{hi}$). (C) Frequencies of memory CD4 T cells, T_{EM} ($CD44^{hi}CD62L^{lo}$). (D) CD4 T cell proliferation upon *ex vivo* OVA stimulation. Data shown are representative of three independent experiments. The data were analyzed with non-parametric Mann- Whitney *U* test. $p < 0.05$ was considered as statistically significant. (O: OVA, H: HEL, M: MBP, R: R848, P: Poly I: C).

a positive control to measure cytokine responses. Intracellular cytokine response was found significantly higher in mice immunized with OVA in the presence of N-TAgs compared to mice immunized with OVA along with TLR ligands. While the difference of IFN-γ, TNF-α or IL-2 response to OVA between mice immunized with OVA alone vs OVA/HEL was significant (IFN-γ, ($p < 0.01$); TNF-α, ($p < 0.01$); IL-2, ($p < 0.01$)), the same, in mice immunized with poorly immunogenic N-TAg MBP was clearly higher than that of OVA alone group ($p < 0.001$) (Fig. 4A–C). The detailed analysis layouts of these groups along with the *invivo* control groups, Naive, OVA, HRP, MRP are given

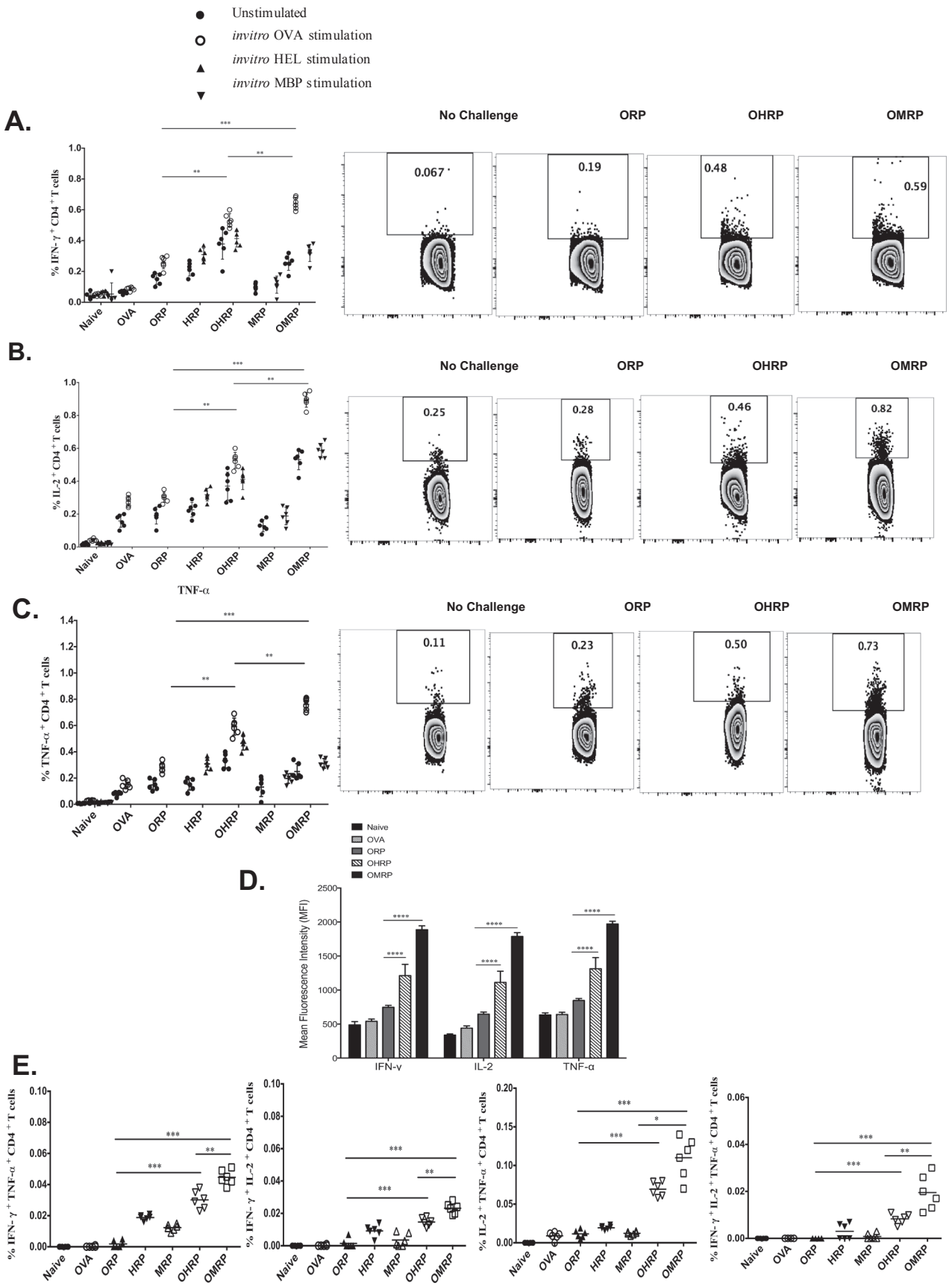


Fig. 4. Inclusion of *N*-TAg MBP with vaccine targets promotes generation of multi-cytokine producing OVA specific CD4 T cells. Frequencies of CD4 T cells producing IFN-γ(A), IL-2 (B) and TNF-α (C) in response to *invitro* OVA challenge. (D) The expression of IFN-γ, IL-2 and TNF-α measured as mean fluorescent intensity (MFI). (E) Frequency of double and triple positive cytokine producing cells. Data shown are representative of three independent experiments. The data were analyzed with non-parametric Mann- Whitney *U* test. $p < 0.05$ was considered as statistically significant. (O: OVA, H: HEL, M: MBP, R: R848, P: Poly I: C).

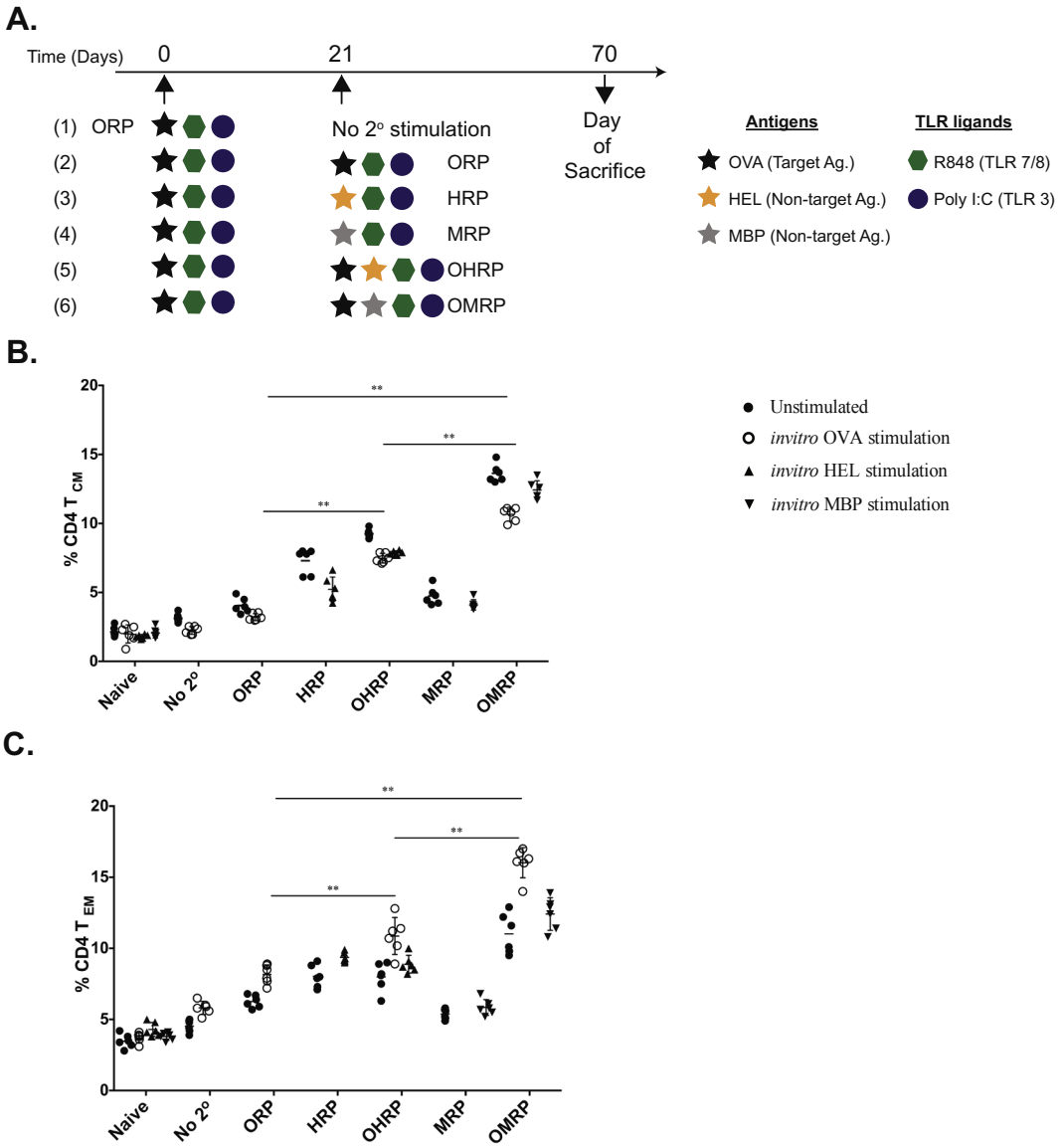


Fig. 5. Non-Target antigen MBP modulates the generation of T_{CM} & T_{EM} against OVA upon *invivo* antigenic challenge. (A) Groups of C57BL/6 mice (n = 6) were immunized subcutaneously with OVA along with Poly I: C and R848. On day 21-post priming mice were *invivo* challenged with HEL or MBP in presence or absence of OVA. Seventy days later, cells were harvested from inguinal lymph nodes and challenged *exvivo* with OVA to measure OVA specific CD4 T cell memory response. Cell surface staining with memory markers (CD44 and CD62L) and intracellular cytokine staining was performed for the characterization of T cells as per protocol. (B) Frequencies of memory CD4 T cells, T_{CM} ($CD44^{hi}CD62L^{hi}$). (C) Frequencies of memory CD4 T cells, T_{EM} ($CD44^{hi}CD62L^{lo}$). Data shown are representative of three independent experiments. The data were analyzed with non-parametric Mann- Whitney *U* test. $p < 0.05$ was considered as statistically significant. (O: OVA, H: HEL, M: MBP, R: R848, P: Poly I: C).

in the Supplementary Fig. 2B–D. We categorized cytokine-positive cells into seven different subsets I, L, T, LT, IT, IL, ILT (I: IFN-γ, I: IL-2, T: TNF-α) consisting of triple producers, double producers, and single producers (Supplementary Fig. 2A). Inclusion of N-TAg MBP with vaccine targets was found to induce significantly higher ($p < 0.001$) percentage of double and triple positive cytokine producing $CD4^+$ T cells as compared to OVA along with TLR ligands group (Fig. 4E). The proportion of triple producers was highest in OMRP group followed by OHRP and ORP group (Supplementary Fig. 2E).

3.5. Challenge of host with poorly immunogenic antigen MBP favors generation of central memory

Often people recovering from one infection encounter another infection during pandemic, and the memory T cell pool developed following first infection might be influenced by the subsequent infection of related or unrelated pathogens [29,30]. We wanted to address this issue in our model from the angle of influence of Ag by challenging the OVA immunized hosts with N-TAgs. C57BL/6 mice were primed with OVA mixed with Poly I: C and R848, and challenged on day 21 with MBP or HEL in presence or absence of OVA (Fig. 5A). Seventy days later, lymphocytes were harvested from inguinal lymph nodes, memory T cell formation was determined by phenotypic characterization, and Ag specific response was measured to *exvivo* OVA challenge. Memory T cells were characterized based on phenotypic markers (CD44, CD62L), and OVA specific response was determined by intracellular IFN-γ, TNF-α, IL-2 as a measure of recall.

Frequencies of OVA, HEL or MBP specific CD4 T cells before immunization and after immunization were determined based on phenotypes. There was change in basal level of Ag specific effector T cell response without immunization, and also upon priming of mice suggesting that the T cell population contains antigen experienced CD4 T

cells having memory phenotype (Fig. 5C). There was increase in frequency of Tcm and Tem with inclusion of MBP to OVA in immunization protocol and more T cell responded to *exvivo* Ag challenge. It is worth noting that, poorly immunogenic antigen MBP was found to significantly favor ($p < 0.01$) the generation of central and effector memory (Fig. 5B, C). While mice immunized with OVA showed lowest frequency of T_{CM}, inclusion of HEL improved the generation of T_{CM}. The TEM to TCM ratio in case of stimulated samples is highest on inclusion of MBP (1.49), which indicates that upon second exposure with OVA/Ag experienced central memory cells might be formed during primary encounter and may get converted to effector cells.

3.6. Challenging the OVA immunized mice with N-TAg promotes generation of multi-cytokine producing CD4 T cells

To understand whether homologous or heterologous Ag challenge influence the quality of memory T cells, we looked for antigen recall response in terms of IFN-γ, TNF-α and IL-2 synthesis to *ex vivo* OVA challenge. Intracellular cytokine response was significantly higher in mice immunized with OVA in the presence of N-TAgs compared to mice immunized with OVA alone. We found that challenging the host *invivo* with N-TAgs (HEL or MBP) modulates the memory T cell response as evident from the results of IFN- γ, IL-2 and TNF–α production. While the difference of IFN-γ, TNF-α or IL-2 response to OVA between mice immunized with OVA alone and OVA/HEL was significant (IFN-γ, ($p < 0.01$); TNF-α, ($p < 0.01$); IL-2, ($p < 0.01$)), the same, in mice immunized with poorly immunogenic N-TAg MBP was clearly higher than that of OVA along with TLR ligands immunized mice ($p < 0.001$) (Fig. 6A–C). All the cytokines have been gated considering the Isotype control. Layouts for antigen specific recall response measured by challenging the T cells *ex vivo* with OVA for intracellular cytokine production are shown (Fig. 6A–C). Inclusion of N-TAg MBP with vaccine targets was found to induce significantly higher ($p < 0.001$) percentage of double and triple positive cytokine producing CD4 T cells as compared to OVA alone-immunized mice (Fig. 6E).

3.7. Inclusion of Non-target Ag(s) promotes generation of OVA specific memory CD4 T cells

We wanted to evaluate the generation of Ag –specific memory CD4 T cell response by using peptide-MHC class II tetramers. To track the OVA-specific CD4 T cells, C57BL/6 mice were immunized subcutaneously at base of the tail with OVA in presence or absence of HEL or MBP (Fig. 3A). On day 75-post immunization, lymphocytes were harvested from inguinal lymph nodes and stained with OVA/MHC class II tetramer (Provided by NIH tetramer core facility) to track OVA specific CD4 T cells. Inclusion of MBP with Target antigen OVA show significantly higher ($p < 0.01$) OVA specific CD4 T cells as compared to ORP group (Fig. 7A). Also significant percentage of CD4 T cells expressed higher levels ($p < 0.01$) of CD44 in OMRP group as compared to ORP group (Fig. 7B). CD44 is a phenotype typically associated with antigen experienced effector and memory CD4 T cells. Total Memory T cells (Sum T central Memory and T effector Memory) was also significantly higher ($p < 0.01$) upon inclusion of MBP with vaccine target OVA (Fig. 7C).

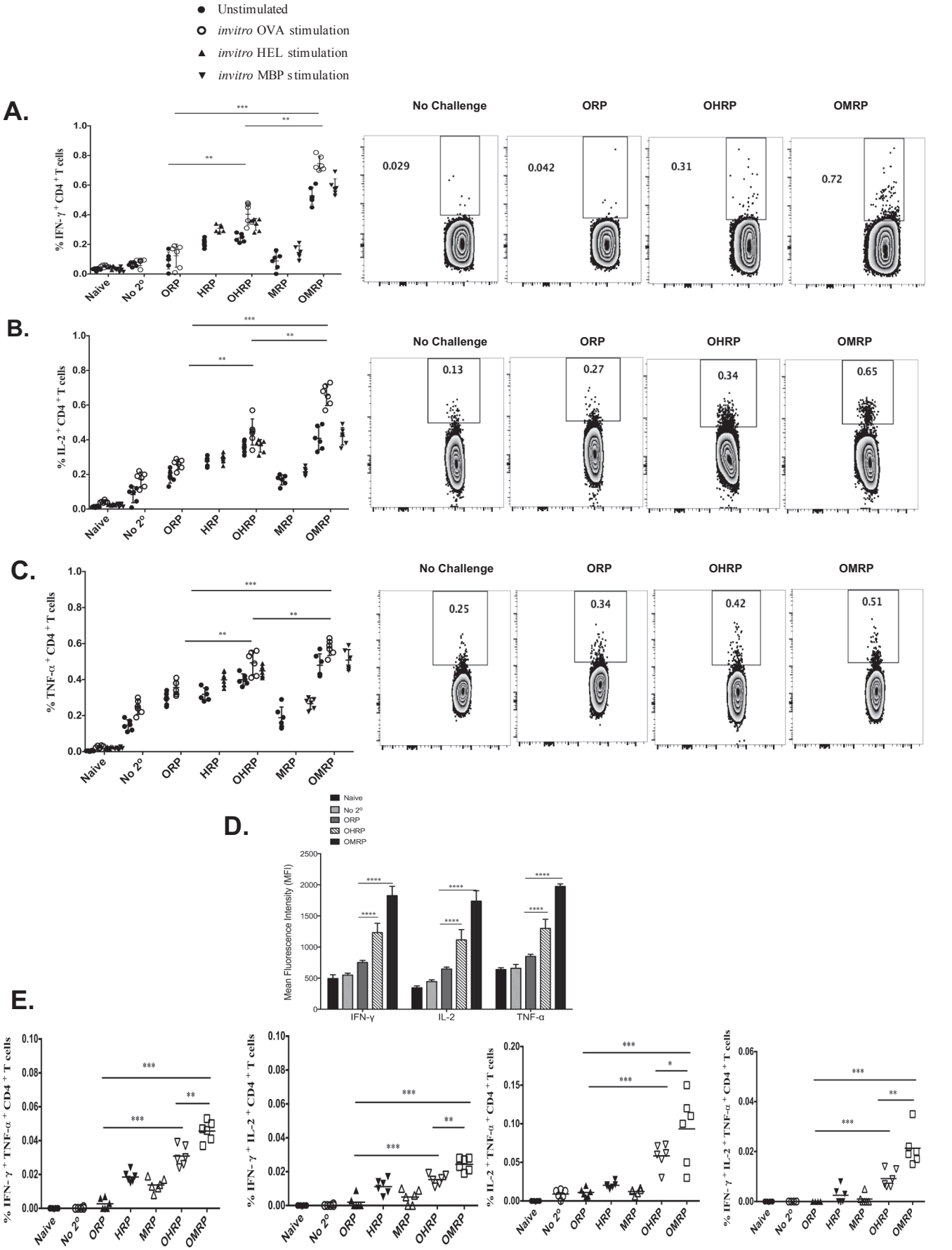
We also observed the change in basal level of Ag specific effector T cells response upon antigen sensitization as compared to control mice suggesting that the population of memory T cells characterized based on phenotypic markers contains antigen-experienced CD4 T cells having memory phenotype, consistent with our earlier observations indicated in Fig. 3 (Fig. 7E). We found that mice immunized with OVA in presence of poor immunogen MBP show significantly ($p < 0.01$) higher frequencies of T_{CM} when compared to mice immunized with OVA along with TLR ligands (Fig. 7D). While mice immunized with OVA along with TLR ligands show lowest frequencies of T_{CM}, inclusion of HEL improved the generation of T_{CM} (Fig. 7D).

When we measured, antigen recall response in terms of IFN-γ, TNF-α and IL-2 synthesis to *ex vivo* OVA challenge, we found similar observations, as indicated in Fig. 4, intracellular cytokine response was found significantly higher in mice immunized with OVA in the presence of N-TAgs compared to mice immunized with OVA along with TLR ligands. While the difference of IFN-γ, TNF-α or IL-2 response to OVA between mice immunized with OVA alone vs OVA/HEL was significant (IFN-γ, ($p < 0.01$); TNF-α, ($p < 0.01$); IL-2, ($p < 0.01$)), the same in mice immunized with poorly immunogenic N-TAg MBP was clearly higher than that of OVA alone group ($p < 0.001$) (Supplementary Fig. 3B–D).

4. Discussion

Natural infection is considered to be the most effective means of inducing protective immunity and memory response [4,31,32]. Often the subunit vaccines might provoke strong effector response but fail to generate appreciable memory [8]. Therefore, we intended to develop an alternative strategy of vaccination to mimic the condition of pathogen, and tested whether the same would generate qualitatively better effector and memory responses. During natural infection host encounters multiple antigens derived from pathogen, but immune response is triggered against a few immunogenic antigens, called target antigens. For subunit vaccines, we usually focus on the immunogenic target (s) and use the same along with PAMPs to induce immune response with the hope to generate long-lived response [33]. Although PAMPs-induced response during natural infection is believed to shape the adaptive immunity including memory, it is possible that Ags other than the target Ags (i.e., N-TAgs) might influence the formation of memory. N-TAgs, which are usually ignored in vaccine formulations, could be non-immunogen, poor immunogen, or immunogen as is the case in multivalent vaccines. Therefore, we tested our idea by vaccinating the host with specific antigen (here, OVA) mixed with N-TAg(s) of varying immunogenicity along with PAMPs in the inoculum. We found that inclusion of N-TAg(s) modulates CD4 T cell responses including the generation of memory T cells to OVA. Although both the categories of N-TAg(s) were found to enhance memory T cell formation, the ability of poorly immunogenic N-TAg, MBP to modulate memory is significantly higher compared to that of immunogenic N-TAg, HEL. These results suggest that immune response generated to a given antigen during the encounter of natural infection would be influenced by antigenic milieu contributed by the pathogens.

For more than five decades, CFA (complete freund's adjuvant) is in use by immunologists with the purpose of potentiating immune response against the candidate Ag [14]. Although it was considered as one of the most potent adjuvants, its immunopotentiation activity was not fully understood until the discovery of TLRs. Lately, it is shown that adjuvant activity of CFA is mainly contributed by TLR agonists, heat shock proteins (agonists of TLR4), & trehalose dimycolate (TDM) and LAM (agonists of the TLR2)[17–19]. Although CFA would induce innate immune response through the above PAMPs, the question arises whether major mycobacterial protein PPD associated with CFA has any role in potentiating or modulating the generation of T cells. Hence, we wanted to mimic the minimal condition of CFA and tested the role of PPD on OVA specific CD4 T cell responses. We inoculated mice with the emulsion of OVA along with Zymosan (TLR-2 in place of *M.tb* LAM) & LPS (TLR-4 in place of *M.tb*. HSP) in various combinations with/without PPD, and measured the T cell response two weeks post immunization. Our results suggest that PPD in absence of TLR ligands positively modulates CD4 T cell response to target antigen OVA (Fig. 1C, D). PPD not only improved magnitude of CD4 T cell response but also promoted T cells to undergo higher cycles of division, one of the characteristics of memory T cells (Fig. 1E) [34–37]. Interestingly, when TLR ligands were included in the formulation we found that potency of PPD to promote higher divisions among OVA specific CD4 T cells is masked despite higher overall proliferation of CD4 T cells (Fig. 1E). It is possible that



(caption on next page)

Fig. 6. Challenging the OVA immunized mice with *N*-TAg promotes generation of multi-cytokine producing OVA specific CD4 T cells. Frequencies of CD4 T cells producing IFN-γ (A), IL-2 (B) and TNF-α (C) in response to *invitro* OVA challenge. (D) The expression of IFN-γ, IL-2 and TNF-α measured as mean fluorescent intensity (MFI). (E) Frequency of double and triple positive cytokine producing cells. Data shown are representative of three independent experiments. The data were analyzed with non-parametric Mann- Whitney *U* test. $p < 0.05$ was considered as statistically significant. (O: OVA, H: HEL, M: MBP, R: R848, P: Poly I: C).

TLR induced inflammation in presence of PPD might enhance the overall proliferation of T cells resulting in excess secretion of IL-2, which in turn would have induced cell death among the T cells undergoing further expansion [38,39]. We also noticed that CD4 T lymphocytes from mice immunized with target antigen in presence of PPD and TLR ligands respond in a dose dependent manner until 0.5 μg/ml, but there is a sudden decline in response at 1 μg/ml that was further alleviated with increasing the dose of antigen challenge (Fig. 1C, D). Comparing the response of OVA specific T cells generated in mice immunized with PPD in absence of TLR ligands with that in presence of TLR ligands, we speculate that the T cells generated in presence of PPD might possess high affinity Ag specific TCRs. While the high affinity T cells would be undergoing cell death, the low affinity T cells might be proliferating in response to higher antigenic stimulus as supported by our results (Fig. 1) [40]. Thus, PPD is thought to induce heterogeneous T cell population, a characteristic of pathogen-induced immune response [41]. We speculate that PPD might be playing a modulatory role by favoring the T cells bearing high affinity TCRs to the restricted density of MHC-p complexes available on the surface of APCs, thus favoring generation of OVA specific T cells having characteristics of long-lived memory.

PPD is not only a complex mixture of antigens but also known to induce inflammatory response [20,21]. Therefore, we used a more defined N-TAg in our later studies to understand the influence of N-TAgs in modulating CD4 T cell response to OVA. We used HEL as N-TAg while inducing response to OVA. Since HEL and OVA are from the same source, HEL was a natural choice to study its influence on the immune response to OVA. Here, we found that HEL also positively modulates CD4 T cell response to OVA (Fig. 2B). Inclusion of HEL with OVA in presence of TLR ligands was shown to promote higher cycles of division among OVA specific CD4 T cells (Fig. 2D), consistent with our findings of PPD. Although HEL seems to be acting in the same manner as PPD in promoting higher cycles of division among CD4 T cells, its role in modulating T cell response in the presence of PAMPs clearly supports the idea that N-TAgs would positively modulate T cell response. PPD in presence of OVA and TLR ligands induced a mixed response; while the T cells appear to be undergoing higher cycles of divisions, they may be susceptible to death at the same time possibly due to the inflammatory signals induced by TLR ligands as well as by PPD [42,43]. However, HEL depicts a more clear picture; it is a highly immunogenic antigen and under normal inflammatory milieu induced by TLR ligands appear to promote generation of high affinity CD4 T cells (Fig. 2B) [23]. Further, inclusion of immunogenic antigens like HEL could induce T cell response against itself, and cytokines like IL-2 produced by the same T cells would promote expansion of OVA specific T cells adding to frequency of pre-existing antigen specific T cells. The above findings made us curious to test the role of poorly immunogenic N-TAgs in modulating memory T cell responses.

As N-TAgs include both immunogenic and poorly immunogenic antigens, we wanted to test the ability of both types of N-TAgs for their influence in memory T cell formation. Because viruses are known to induce better memory compared to other pathogens, we mimicked viral infection minimally from the point of view of inducing immune response by using TLR ligands Poly I: C and R848 as PAMPs in place of LPS and Zymosan [24]. Here, we used MBP as poorly immunogenic N-TAg. Ideally, we wanted to have a poorly immunogenic antigen from the same source (egg) of HEL and OVA, but due to unavailability of such defined Ags from egg we chose MBP, which shows very poor affinity to IA^b [44].

Here the idea was to mimic the condition favored by pathogen to induce immune response by including N-TAgs with TAgs and PAMPs. In this study, we are also dealing with endogenous T cells, which are usually heterogeneous in nature. Most of the studies dealing with memory T cells have adopted transgenic T cell approaches. Although adoptive T cell transfer approaches are accepted well to study the behavior of T cell clones, in practice broad repertoire of antigen specific T cells are generated when host encounters infection leading to generation of heterogeneous population of memory cells [45]. Therefore, we focused on evaluating the generation of endogenous memory T cells and characterized the same based on their phenotypic markers. We measured antigen specific response by challenging the memory T cells ex vivo with OVA and evaluating the cells by intracellular IFN-γ, TNF-α, IL-2 production. Change in basal level of Ag specific T cells upon priming mice suggests that the population contains antigen experienced CD4 T cells having memory phenotype (Fig. 3C). Inclusion of N-TAg MBP along with TAg OVA shows that more T cells responded upon *exvivo* Ag challenge (Fig. 3B, C). Upon characterization of memory cells, we observed the desired traits of memory T cells and found the overall enhanced memory response to OVA in presence of N-TAgs. These data support our idea that memory T cell response to specific antigen during the course of infection might be modulated by other antigens. Here we have looked for the frequencies of memory T cells based on their surface expression of CD44 andCD62L. Upon antigen stimulation Central memory cells get activated and convert to effector/effector memory pool. Our intention was to look at memory T cell dynamics in terms of conversion of central memory to effector pool.

Based on antigen load and the no. of times antigen is encountered, there occurs a dynamic shift in T cells. Upon primary exposure cells from the naive T cell pool can shift/convert to effector cell pool and also to central memory T cell pool. Whereas upon second exposure with the same antigen it is possible that central memory cells generated during priming would get converted to effector cells. Further, our results also suggest the role of N-TAgs in inducing differentially heightened memory T cell formation as it is demonstrated in our experiment that poorly immunogenic MBP is much better in inducing not only higher frequencies of antigen specific memory CD4 T cells (Fig. 3B, C) but also promoting heterogeneous population as it is reflected in the magnitude and level of IFN-γ, TNF-α and IL-2 response upon recall (Fig. 4A–C).

Memory T cells have the ability to produce cytokines with multiple effector functions. Inclusion of MBP with vaccine targets was found to promote higher percentage of multiple cytokine producing T cells. It is already established that multiple-cytokine-producing Ag specific CD4 T cells are functionally superior to the single-cytokine-producing cells [46,48]. The proportion of triple producers, though lesser in percentage as compared to single producers, was seen higher in case of OMRP group (Supplementary Fig. 2E). In general, the proportion of triple producers was higher upon inclusion of non-target antigens as compared to that of only target antigen, which demonstrates a strong positive association between the cytokine co-production capacity of a antigen-specific CD4 T cell and its other functional characteristics suggesting that poorly immunogenic non-target Ag in vaccines might be promoting T cells that coproduce more than one cytokine [46,48]. Upon analyzing the T cell proliferation, we found both the N-TAgs induced higher division percentage of cell proliferation (Fig. 3D); moreover, poorly immunogenic MBP induced at least 2 more divisions higher than that of HEL (Supplementary Fig. 1B) suggesting poorly immunogenic antigens would favor generation of long-lived memory T cells.

Various studies have demonstrated that the status of memory T cells generated following an infection could be influenced by another

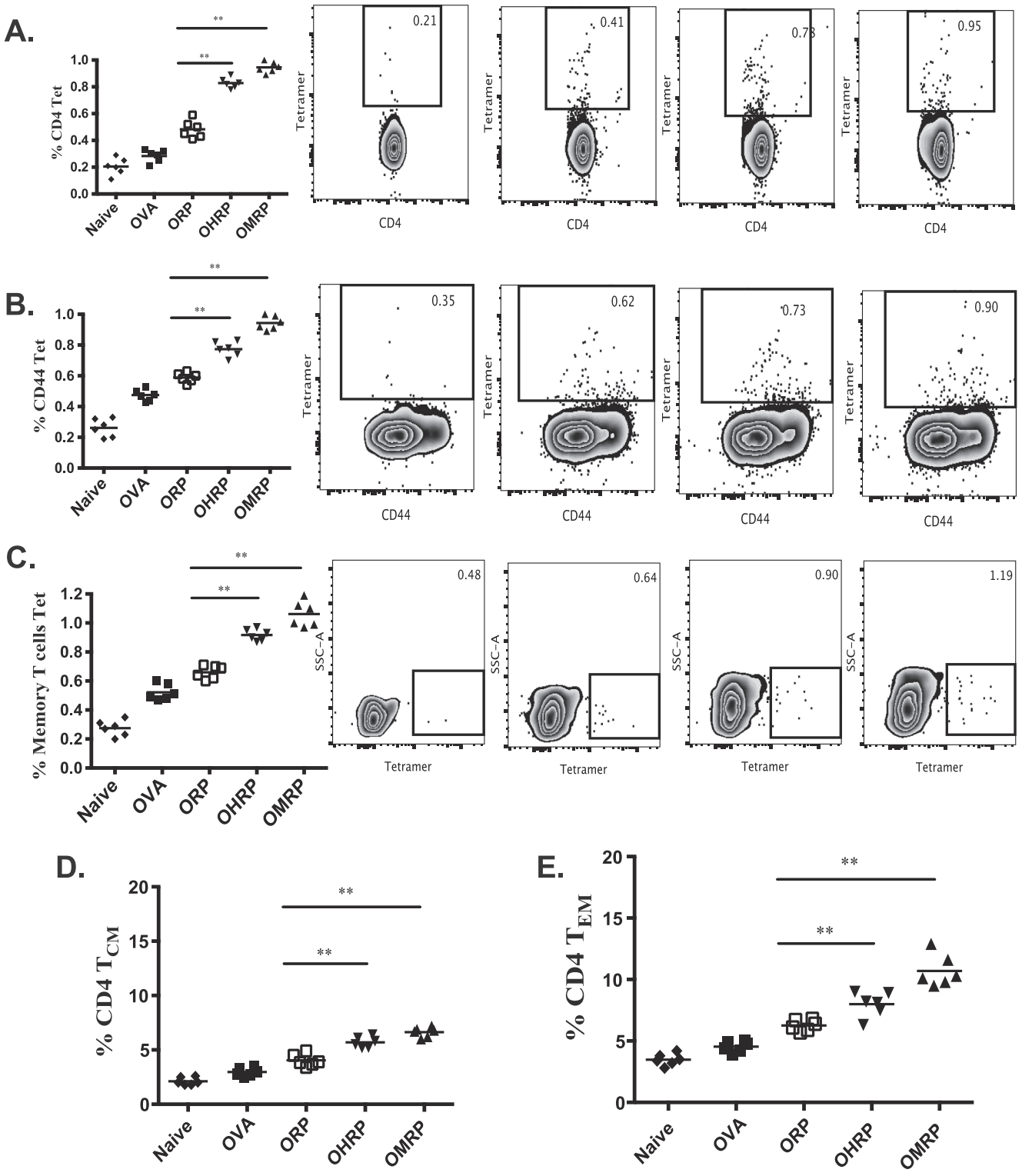


Fig. 7. Inclusion of Non-target Ag(s) promotes generation of OVA specific Memory CD4 T cells. Groups of C57BL/6 mice (n = 6) were immunized subcutaneously at base of the tail with OVA in presence/absence of N-TAgs; HEL or MBP along with Poly I: C and R848. On day 75 post priming lymphocytes were harvested from inguinal LNs (DLNs) and $CD4^+$ T cells were challenged *exvivo* to measure OVA specific response. Tetramer Staining followed by cell surface staining with memory markers (CD44 and CD62 L) and intracellular cytokine staining (Supplementary 3) was performed for the characterization of T cells as per protocol. (A) Frequencies of CD4 T cell producing Tetramer. (B) Frequencies of CD44 T cell producing Tetramer. (C) Frequencies of T central memory (T_{CM}; $CD44^+$ $CD62L^+$) and T effector memory (T_{EM}; $CD44^+$ $CD62L^-$) cell producing Tetramer. (D) Frequencies of memory CD4 T cells, T_{CM} ($CD44^{hi}CD62L^{hi}$). (E) Frequencies of memory CD4 T cells, T_{EM} ($CD44^{hi}CD62L^{lo}$). The data were analyzed with non-parametric Mann- Whitney *U* test. $p < 0.05$ was considered as statistically significant. (O: OVA, H: HEL, M: MBP, R: R848, P: Poly I: C).

infection. During outbreak of virus infection, for example flu in many parts of the world, people do encounter infection from related or unrelated virus, a characteristic of epidemics [29,30]. In addition to history of prior infections, individuals from the areas endemic for multiple pathogens are often co-infected with unrelated organisms. There is mounting evidence from animal and human studies that prior or concurrent infections with unrelated pathogens modulate immune responses including memory [29,30]. The memory T cells developed after an infection can be influenced by the response to subsequent infections with unrelated pathogens. Such heterologous prime boost conditions have been demonstrated to influence the outcome of T cell memory [47]. Similarly, co-infection appears to be relatively common and may be beneficial in boosting protective response. We wanted to understand how concurrent active pathogen challenge from unrelated or related pathogen modifies memory T cell pools to previously encountered pathogen. Immunogenicity of antigen during the challenge might modulate the pre-existing immune response including induction of regulatory pathways [30]. To test the role of antigen in case of staggered infection we primed mice with OVA antigen and *in vivo* challenged with HEL (related) or MBP (unrelated) individually or in combination with OVA. Our results show that MBP in presence of OVA during challenge favors the generation of central memory T cells whereas OVA alone during challenge dampen the memory formation (Fig. 5B). Intracellular cytokine response (IFN-γ, TNF-α, IL-2) was significantly higher in mice immunized with OVA in the presence of MBP compared to mice immunized with OVA along with TLR ligands (Fig. 6A–C). Inclusion of N-TAg MBP with vaccine targets was found to induce higher percentage of double and triple positive cytokine producing CD4 T cells (Fig. 6E). Polyfunctional CD4 T cells simultaneously producing IFN-γ, interleukin (IL)-2 and tumor necrosis factor TNF-α, or a combination thereof, have recently attracted attention as potential correlates of protection against infections like TB [46]. The suggested mechanism underlying the concept that Polyfunctional CD4 T cells are key to protection against intracellular pathogens, including *M. tuberculosis*, is that the simultaneous production of IFN-γ and TNF-α is synergistic in killing intracellular pathogens [46,48] and this effect is further enhanced by IL-2-induced proliferation of these cells.

In the initial set of experiments we measured antigen specific response by challenging the memory T cells *ex vivo* with OVA and measuring the synthesis of IFN-γ, TNF-α, IL-2, markers of effector function. Here, we evaluated the CD4 T cell response by using OVA-MHC class II tetramers to track the OVA-specific CD4 T cells. Upon Inclusion of N-TAg MBP significant higher percentage of CD4 T cells were generated as compared to target antigen alone suggesting that MBP is much better in inducing higher frequencies of antigen specific memory CD4 T cells (Fig. 7).

At present mechanistic explanation of action of N-TAgs in modulating response to TAgs is under investigation. However, we speculate that inclusion of immunogenic N-TAgs antigens like HEL, could induce T cell response against itself and cytokines produced by HEL specific cells would promote expansion of bystander T cells including that of OVA adding to frequency of TAg antigen specific T cells. It is yet to be explored how MBP enhance better memory formation against OVA.

In this study, we have attempted to explore the expanded definition of co-infection and role of N-TAgs in modulating memory T cell formation. Exploitation of the unique role of antigens would help develop novel strategies to generate long-lived protective immunity. Having better insights on these issues will improve our understanding of why natural infection is better alternative of inducing protective immunity and will also pave the path for developing new adjuvants.

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Conflict of interests

We declare that we have no conflict of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cellimm.2018.11.005.

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