TRAINED IMMUNITY-BASED VACCINES

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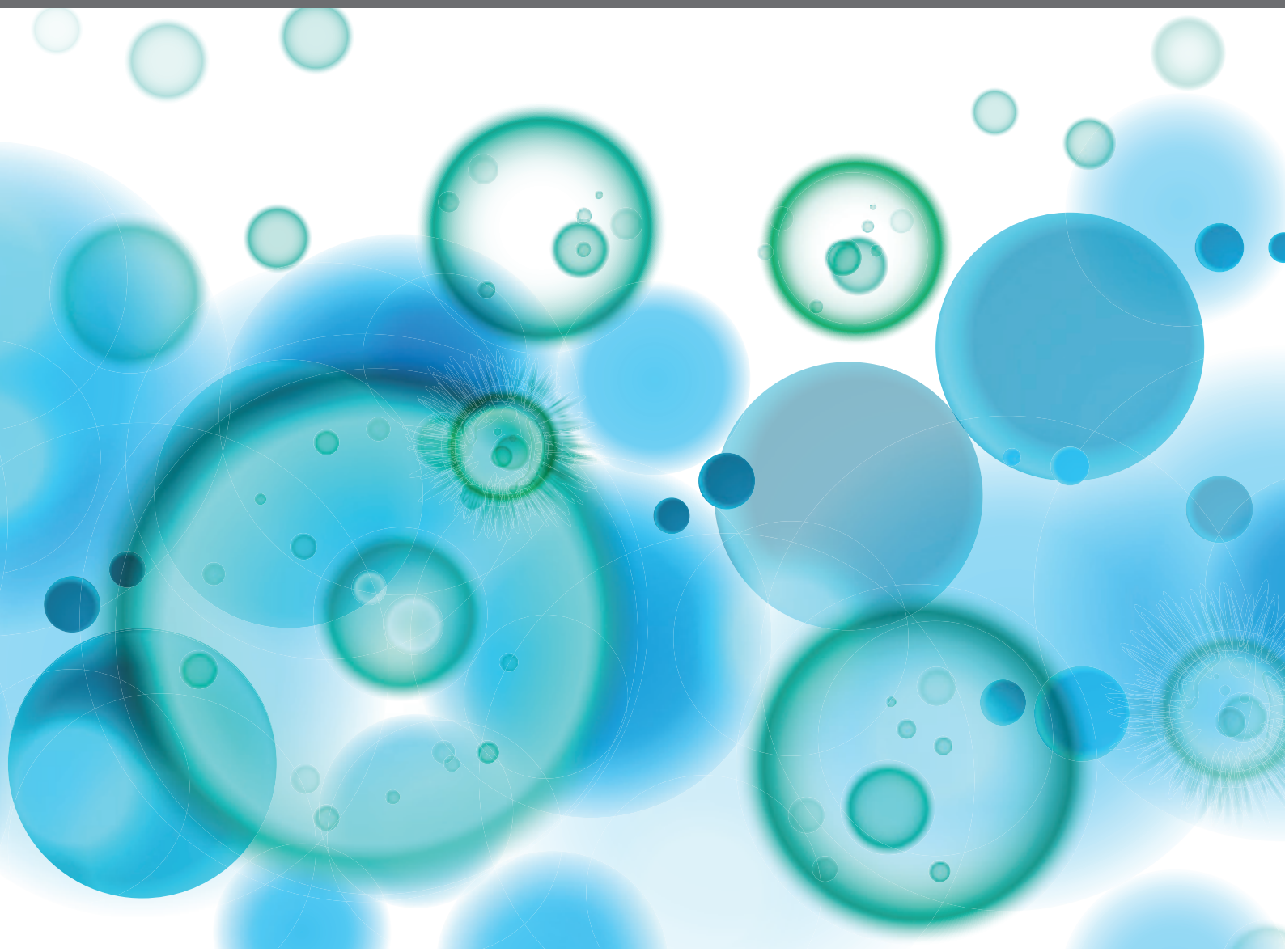

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TRAINED IMMUNITY-BASED VACCINES

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Table of Contents

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EDITORIAL
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Editorial: Trained Immunity-Based Vaccines

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Keywords: trained immunity, vaccine, immunostimulant, adjuvant, infection, TIbV

Editorial on the Research Topic

Trained Immunity-Based Vaccines

Trained immunity is defined as a type of memory of the innate immune system by which innate immune cells undergo a long-term adaptation, largely dependent on persistent epigenetic modifications and metabolic reprogramming of these cells (1). Myeloid cells can be trained with a variety of stimuli (typically of microbial origin) that improve their responsiveness to second stimuli (same or unrelated) in a fairly stable manner. The mechanistic basis of trained immunity and the diversity of trained cells behaviors may have important implications not only for innate but also for adaptive immunity and, therefore, for a response to vaccines or for their design (2).

The emerging concept of trained immunity-based vaccines (TIbV) (3) challenges that of traditional vaccines in several intriguing ways, whose clinical and immunological implications deserve further exploration: i) TIbV may act beyond their antigenic formulation, providing non-specific protection against different pathogens based on trained (innate) immune cells; ii) TIbV have self-adjuvant properties enhancing adaptive immune responses to their own antigens, but also to bystander antigens. Their potential use outside infectious prevention is opening new immunotherapy approaches in other conditions like cancer (4) or allergies (5).

This special issue has gathered a number of latest resonance original research studies and reviews from authors working in different areas related to this topic.

Trained immunity depends on the presence of trained innate cells. Although myeloid cells are considered in general short-lived, trained immunity may last a quite long time (from several months to over a year). This may be due to the presence of long-lived trained macrophages and/or myeloid precursors in the bone marrow. In this issue, Chen and Ozato review how hematopoietic stem/progenitor cells can acquire epigenetic memory upon pathogen exposure and the soluble mediators, e.g. interferons, involved in memory formation within the bone marrow.

The heterologous protection associated with BCG vaccination is one of the best-studied examples linking non-specific protection and trained immunity in a clinical setting. Gonzalez-Perez et al. have reviewed the potential use of BCG vaccine in COVID-19 pandemics. Several clinical trials have been initiated to address whether BCG vaccination confers non-specific protection against SARS-CoV-2, and/or associated infections. This trained immunity-based approach could make it possible to increase the resistance of vulnerable subjects in the context of viral outbreaks

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before a specific vaccine is ready (6). Indeed, even if such a vaccine exists, outbreaks can eventually appear in a vaccinated population by a number of factors reviewed by Connell et al., exemplified by recent outbreaks of mumps in MMR-vaccinated subjects.

The potential of BCG as training inducer can be harnessed to use it together with specific antigens to simultaneously induce specific (adaptive) and non-specific innate immunity. In this line, Covián et al. have reviewed an interesting novel approach using recombinant BCGs expressing antigens from the syncytial virus and metapneumovirus, i.e., as a canonical TIbV. An alternative possibility is to split the TIbV in two separated elements, i.e., the trained immunity-inducer as adjuvant and the nominal antigen (3). This approach has been tested by Paris et al. in veterinary medicine using a pretreatment with β-glucan (from *Euglena gracilis*) to enhance the specific immune response to a rabies vaccine administered at the same time or one month later. Thus, training may be used to optimize vaccine immunization strategies. This has been pointed out in the review by Palgen et al., addressing how myeloid innate cells sense and respond differently to a first and a second dose of vaccine and how trained innate cells can be harnessed to optimize the response to vaccination.

Most described trained immunity-inducers are microbial-derived products that stimulate innate immune cells through different PRRs (pattern recognition receptors). Either the nucleotide-binding oligomerization domain (NOD)-Leucin Rich Repeats (LRR)-containing receptors (NLR) or the C-type lectin receptors (CLRs) have been involved, for example NOD2 (BCG) or Dectin-1 (yeast β-glucans). The involvement of Toll-like receptors (TLRs) as trained-immunity inducers seems less clear (7). TLR ligands, however, are used as adjuvants in different vaccine formulations and shown to confer non-specific anti-infectious protection in different settings. The interplay of TLR agonists with the phenomenon of trained immunity has been reviewed by Owen et al. with an overview of TLR signaling as potential activating mechanisms of trained immunity. Such an interplay may be supported by the fact that the pro-inflammatory cytokine profile (TNF-α, IL-6, and IL-1β), well-known hallmarks of trained immunity, is severely affected in TLR4–/– mice, as described by Sánchez-Tarjuelo et al. This experimental model discloses an impaired innate immune response to a challenge with *S. pneumoniae* indicating the critical involvement of TLR4 in the resistance against these Gram-positive bacteria. On the other hand, Vázquez et al. show how mesenchymal stem cells are able to uptake, process and retain a reservoir of the TLR ligands derived from inactivated bacteria (MV130), which can subsequently be transferred to dendritic cells. MV130 is a mucosal polybacterial preparation that induces trained immunity and prevents viral wheezing attacks in young children (8) and used in a proof of concept study in patients with hematological malignancies, showing beneficial effects in terms of infections incidence (9). The effect of the combination of a similar polybacterial preparation (MV140) used to prevent urinary tract infections with *Candida albicans* (V132) has been studied by Martín-Cruz et al. The authors show how this combination (MV140/V132) promotes metabolic and epigenetic reprogramming in human DCs, which are key molecular mechanisms involved in the induction of trained immunity, while enhancing specific responses to their nominal antigens.

Vaccine formulations based on DCs loaded with recombinant proteins or mRNA derived from *Listeria monocytogenes* Glyceraldehyde-3-Phosphate Dehydrogenase (LM-GAPDH), have been studied by Teran-Navarro et al. In contrast to mRNA, the recombinant proteins induce non-specific DC activation and show higher immunogenicity. The interesting

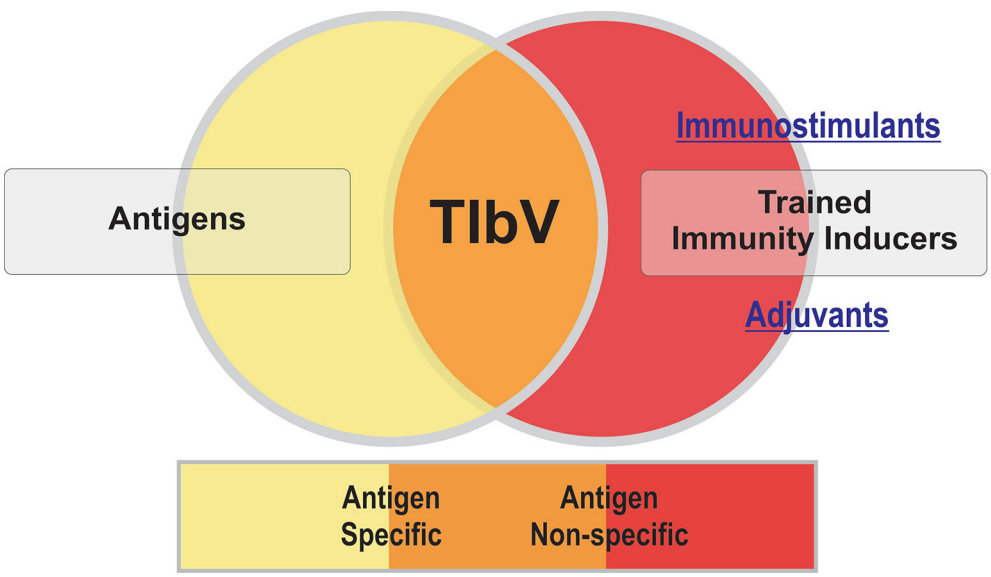

FIGURE 1 | Trained immunity-based vaccines (TIbV) features.

point of this approach is that LM-GADPH is highly cross-reactive with that derived from other pathogens, and vaccinated mice are protected not only to a challenge with *Listeria monocytogenes*, but also to *Mycobacterium marinum* or *Streptococcus pneumoniae*.

In the field of cancer immunotherapy, Zhang et al. have reviewed the functionality of DCs in the tumor microenvironment of ovarian cancer, the role of tumor suppressor signals, and the use of tumor antigen-loaded DCs as cancer vaccines. In their comprehensive review, the authors analyze the different possibilities of these vaccines, alone or in combination with other immunotherapies, as well as possible useful biomarkers.

Finally, Pontigo et al. describe a vaccine candidate inducing an effective protection of Atlantic salmon against *Piscirickettsia salmonis* infection in correlation with the production of Piscirickettsia-specific IgM antibodies and pro-inflammatory cytokines (IL-1β and TNF-α) in contrast to other prototypes that stimulate only innate immunity.

In summary, this timely Research Topic showcases the latest findings and new insights into the potential of trained immunity in vaccine development. TIbV are conceptually novel vaccines that might well confer antigen specific but also antigen non-specific resistance to unrelated pathogens as described for immunostimulants (**Figure 1**). This last aspect highlights the need to review the clinical evaluation of TIbV beyond the specific responses of conventional anti-infectious vaccines. A better understanding of the interaction of different trained immunity inducers, at the molecular and cellular level, could well pave the way for a better design of TIbV and their clinical applications after adequate translational research.

AUTHOR CONTRIBUTIONS

JS wrote the manuscript, together with OP and SS-R. IQ read it and approved it for publication. All authors contributed to the article and approved the submitted version.

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ORIGINAL RESEARCH
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The TLR4-MyD88 Signaling Axis Regulates Lung Monocyte Differentiation Pathways in Response to *Streptococcus pneumoniae*

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Streptococcus pneumoniae is the main cause of bacterial pneumonia, a condition that currently produces significant global morbidity and mortality. The initial immune response to this bacterium occurs when the innate system recognizes common motifs expressed by many pathogens, events driven by pattern recognition receptors like the Toll-like family receptors (TLRs). In this study, lung myeloid-cell populations responsible for the innate immune response (IIR) against *S. pneumoniae,* and their dependence on the TLR4-signaling axis, were analyzed in TLR4$^{-/-}$ and Myeloid-Differentiation factor-88 deficient (MyD88$^{-/-}$) mice. Neutrophils and monocyte-derived cells were recruited in infected mice 3-days post-infection. Compared to wild-type mice, there was an increased bacterial load in both these deficient mouse strains and an altered IIR, although TLR4$^{-/-}$ mice were more susceptible to bacterial infection. These mice also developed fewer alveolar macrophages, weaker neutrophil infiltration, less Ly6C^{high} monocyte differentiation and a disrupted classical and non-classical monocyte profile. The pro-inflammatory cytokine profile (CXCL1, TNF-α, IL-6, and IL-1β) was also severely affected by the lack of TLR4 and no induction of Th1 was observed in these mice. The respiratory burst (ROS production) after infection was profoundly dampened in TLR4$^{-/-}$ and MyD88$^{-/-}$ mice. These data demonstrate the complex dynamics of myeloid populations and a key role of the TLR4-signaling axis in the IIR to *S. pneumoniae*, which involves both the MyD88 and TRIF (Toll/IL-1R domain-containing adaptor-inducing IFN-β) dependent pathways.

Keywords: TLR4, MyD88, *S. pneumoniae*, monocyte differentiation, innate immune responses, ROS

INTRODUCTION

Streptococcus pneumoniae (pneumococcus) is a Gram-positive bacterium that colonizes and invades the respiratory tract. It is the main etiological agent of community acquired pneumonia, accounting for about 90% of all pneumonia deaths, especially in young children and the elderly (1, 2). As a consequence, it is a major cause of morbidity and mortality worldwide (2), although the present pandemic induced by SARS-Cov2 is currently changing the top list of the most dangerous

respiratory pathogens (3). Globally, *S. pneumoniae* causes over 800,000 deaths in children and 13.8 million cases of pneumonia each year. Different vaccines have been generated against the most prevalent *S. pneumoniae* serotypes, of which the conjugate 13-valent (PCV13) and the polysaccharide-based 23-valent (PCV23) are often used (4). The use of antibiotics is becoming compromised by the ever-increasing appearance of antibiotic-resistant strains (5–7). Moreover, as the immune lung response underlying *S. pneumoniae* infection is still not fully understood, a better understanding of this response will be essential to design new effective therapeutic interventions.

It is known that *S. pneumoniae* recognition by lung epithelial cells and by the innate immune system (IIS) involves several pattern recognition receptors (PRRs), receptors that are expressed in different cell lineages and that recognize pathogen associated molecular patterns (PAMPs). Toll-like receptors (TLRs) constitute one of the most important PRR families, playing a critical role in initiating inflammatory responses and promoting adaptive immune responses (8, 9). Of these, both TLR2 and TLR4 may participate in the innate immune response (IIR) against *S. pneumoniae*. TLR2 recognizes lipoteichoic acid (LTA), peptidoglycans and lipopeptides, components of the Gram-positive cell wall (10, 11), although as it appears to play a limited role in the IIR against *S. pneumoniae* other TLRs are likely to be implicated in this process (12). TLR4 plays an essential role in the host's defense against Gram-negative bacteria by recognizing lipopolysaccharide (LPS) (13), although this is not presented by *S. pneumoniae*. Nevertheless, it also recognizes LTA and pneumolysin (Ply), the latter a pneumococcal enzyme that is released by these bacteria. Hence, TLR4 may participate in the response against *S. pneumoniae* (13, 14), and the interaction between Ply and TLR4 during pneumococcal colonization of the nasopharynx may be important for protection (14). After ligand binding, TLR2 and TLR4 depend on the signaling adaptor protein MyD88 (Myeloid-Differentiation factor-88) for activation. In addition, TLR4 can signal through both MyD88- and TRIF-dependent (Toll/IL-1R domain-containing adaptor-inducing IFN-β) pathways (8, 9). Moreover, $MyD88^{-/-}$ mice infected with *S. pneumoniae* generate a stronger bacterial burden and a weaker inflammatory response in the lung (15).

Alveolar macrophages (AMs) are tissue-resident macrophages (Mϕ) that colonize lung tissues during embryogenesis and that are thereafter maintained through self-renewal (16). Alveolar macrophages initiate lung IRs by recognizing and phagocytosing *S. pneumoniae*, and they are essential to control bacterial numbers in the first hours after infection. However, due to the limited number of AMs in the alveoli, the efficacy of AM-mediated immunity against *S. pneumoniae* depends on the magnitude of the bacterial inoculum (16, 17). After antigen-activation, AMs release different cytokines (TNF-α, IFN-γ, GM-CSF, and IL-6) that attract neutrophils (Nϕ) and monocytes, promoting their infiltration into the lung parenchyma (17). Natural killer (NK) cells also play an important role in the immune response against *S. pneumoniae*, producing IFNγ in the early stages of lung infection, and further favoring the activation and recruitment of Nϕ to the lung (18). Nϕ are the most abundant myeloid cell population in the lung and their lung infiltration is fundamental for bacterial clearance, particularly since it is responsible for bacterial phagocytosis and complement activation (19). Furthermore, bone marrow-derived populations of $Ly6C^{hi}$ monocytes migrate to peripheral tissues like the lung or the spleen in response to inflammatory stimuli or to the lesions caused by *S. pneumoniae*, and in a CCR2-dependent manner (20). Once recruited into peripheral tissues, $Ly6C^{hi}$ monocytes can further differentiate into monocyte-derived dendritic cells (moDCs) and monocyte-derived Mϕ (moMϕ), two populations that participate in bacterial removal in conjunction with Nϕ (20, 21).

Mononuclear phagocytic cells are quite heterogeneous and they display significant plasticity, which allow them to acquire specialized functions. In blood, two types of monocytes have been identified with differential phenotype and functions. The classical monocytes (cMO) are $CD11b^{+}Ly6C^{hi}CX3CR1^{lo}CD43^{lo}$, are found in tissues under homeostasis, and during inflammation they are recruited to the damaged tissues and produce proinflammatory cytokines such as TNF-α and IL6, as well as enhancing the inducible nitric oxide synthase (iNOS) available. These classical inflammatory monocytes participate in the local innate immune control of several pathogens, such as *Toxoplasma gondii, Leishmania donivani, Leishmania major* and *Listeria monocytogenes* (22–25). By contrast, the non-classical monocytes (ncMO) monocytes are $CD11b^{+}Ly6C^{lo}CX3CR1^{hi}CD43^{hi}$, they patrol the endothelium and have been shown to have angiogenic activity by secreting vascular endothelial growth factor (VEGF) and IL-10. These ncMO monocytes are also recruited to the damaged tissues and may be involved in tissue repair and wound healing (20, 26–28). In the inflammed tissues, recruited monocytes differentiate into Mϕ. Two phenotypes of Mϕ have been proposed that are related to their functional profile (29–31). Upon activation in response to proinflammatory cytokines like TNF-α and IFN-γ, monocytes differentiate to Mϕ that adopt the M1 phenotype with enhanced microbicidal activity, and secrete pro-inflammatory cytokines (30). By contrast, M2 Mϕ are induced by the Th2-related cytokines (IL-13 and IL-4) and they secrete anti-inflammatory factors like IL-10 and TGF-β. Thus, M2 Mϕ participate in tissue repair and in the phagocytosis of apoptotic cells, limiting inflammation (32). The equilibrium between the M1 and M2 populations is essential to produce an appropriate IR, including its resolution. *S. pneumoniae* infection induces an inflammatory response mediated by M1 Mϕ, killing pathogens and triggering adaptive immunity. Autolysis of *S. pneumoniae* leads to the production of Ply and it induces the production of reactive oxygen species (ROS) by Nϕ (33). Likewise, other bacterial infections like those caused by *Mycobacterium tuberculosis*, induce TLR-activation of Mϕ, bacterial phagocytosis and intense ROS production as the main core of the IIR against such infection (34, 35).

Here we examined the dynamics of the lung IIR against *S. pneumoniae*, using a clinical isolate obtained from peripheral blood and analyzing the myeloid populations that accumulate, their markers of activation and their dependency on the TLR4-MyD88 pathway after intranasal administration. Our data show that 3 days post infection (dpi) there is a local increase in the Nϕ, monocytes, and the moMϕ and moDC monocyte-derived

populations, with a prominent cMO profile. In the absence of TLR4 or MyD88, the production of these myeloid populations was dampened. The ROS produced by different myeloid cell types highlighted the importance of the TLR4-MyD88 axis in the oxidative burst within the myeloid compartment. Moreover, the lung cytokine profile and the production of ROS were less affected by infection in MyD88$^{-/-}$ mice than in TLR4$^{-/-}$ mice, although these responses were weaker than in infected WT mice. Hence, an alternative TRIF pathway appears to participate in the IIR against *S. pneumoniae*. Together, these data indicate that both TLR4-MyD88 and TLR4-TRIF signaling are involved in the recognition of *S. pneumoniae* by the cells generated through the myeloid IIR in the lung, highlighting the role of these pathways in the cytotoxic and regulatory responses that combat this bacterium.

MATERIALS AND METHODS

Mice

Adult (8–10 weeks old) WT (C57BL/6 and C57BL/10), C57BL/10/TLR4$^{-/-}$ and C57BL/6/MyD88$^{-/-}$ mice were bred and maintained at the animal facility of the Centro Nacional de Microbiología-Instituto de Salud Carlos III (CNM-ISCIII, Madrid, Spain). All animal experiments were approved by the Institutional Review Board at the ISCIII, and carried out in strict accordance with EU and National Animal Care guidelines (directive 2010/63/EU and RD 53/2013). The experimental protocol was also approved by the Consejería de Medio Ambiente Comunidad de Madrid (PROEX110/15, PROEX021/18).

Induction of Infection

Mice were lightly anesthetized with an aerosol containing 4% isoflurane (Zoetis, United Kingdom) and they were inoculated intranasally with a suspension (20 μL in Phosphate Buffered Saline –PBS: Bio-Whittaker, Lonza) of the *S. pneumoniae* bacterial strain 1195 (serotype 3 and ST260 genotype). Strain 1195 is a clinical isolate obtained from peripheral blood that was obtained from a septic patient and submitted to the Spanish Pneumococcus Reference Laboratory. This 1195 strain was described previously (referred to as CipR1) (36) and other strains of the ST260 genotype have been used previously in a rabbit model of experimental meningitis (37). The pneumococcal strain was grown to mid-log growth phase in Todd-Hewitt broth supplemented with 0.5% yeast extract. The bacteria were then recovered by centrifugation and suspended in medium with 25% glycerol at a concentration of 10^8 cells/mL, and stored as aliquots at −80°C. For intranasal infection, aliquots were thawed, washed and resuspended in PBS immediately before use. We performed a dose-response study with this strain from 1 to 4 × 10^6 colony-forming units (CFUs) to determine the optimal concentration required to mount an immune response (**Figure 1A**).

Determination of the Colony-Forming Units

The lungs of the mice were dissected out at 1, 2, and 3 dpi, and cell suspensions were prepared by mechanical dissociation in 3 mL of cold (4°C) staining buffer (2.5% Fetal Calf's Serum in PBS). No enzymatic digestion by collagenase treatment was used, since preliminary experiments showed an important reduction in the recovery of myeloid-gated cells and CD11b+ cells after such treatment (**Supplementary Material**). The lung tissue was cut into small pieces and cell suspensions were prepared by mechanical dissociation, disrupting the tissue and filtering through a 40 μm pore cell strainer (BD Biosciences). The filtrate was then centrifuged for 5 min at 110 *g* and 4°C in order to obtain the lung cells. The material recovered was analyzed by flow cytometry (see below), and the *S. pneumoniae* CFUs and cytokines in the supernatants were quantified. Colony-forming units were determined by growing serial dilutions of the lung supernatant on Mueller-Hinton sheep blood agar plates (BD Biosciences, San Jose, CA), counting the colonies formed after a 24 h incubation at 37°C in an atmosphere of 5% CO_2. Dissemination of the bacteria to other organs was determined by CFU quantification in samples from spleen and olfactory bulbs at 3 dpi (50 ± 7 CFUs, $n = 6$ in splenic samples of infected BL10).

Flow Cytometry

Lung cell suspensions obtained from the entire lung were evaluated by flow cytometry, employing a multi-panel strategy to distinguish the different myeloid cell populations in the samples. Cell pellets from lung homogenates were treated for 2 min at room temperature (RT) with 1 mL ACK buffer (Potassium Bicarbonate: Life Technologies) to lyse the erythrocytes. The cells (4 × 10^6/200 μL) were then washed twice with staining buffer, recovered by a 5 min centrifugation at 110 *g* and 4°C, and then prepared in staining buffer. Cell viability was measured by Trypan blue (Merck) dye exclusion and viable cells were counted in a Neubauer's chamber. Non-specific antibody binding was blocked by incubation for 10 min at 4°C with an anti-Fc-Block (clone 2.4G2: BD Biosciences) and the cell populations were then analyzed by Flow Cytometry after staining for 20 min at 4°C using the specific mAbs listed in **Supplementary Table 1**. Dead cells were discharged by staining with Fixable LIVE/DEAD violet-510 kit (Thermo Fisher Scientific) and the cells were fixed for 15 min at RT in 2% paraformaldehyde before acquisition on a LRS Fortessa X-20 cytometer (BD Biosciences). The samples were analyzed using the DIVA v8.0 software package (BD Biosciences) and the cell populations were analyzed after electronic gating on the basis of SSC-A and FSC-A, followed by a gating strategy to rule out doublets and dead cells (**Supplementary Figure 1**). At least 1–3 × 10^5 live cells were analyzed in each sample and the mAbs used in this study allowed the identification of lung myeloid populations, distinguishing the AM (Siglec-F^{hi}CD11b^{lo}), NK (CD3$^-$NK1.1$^+$), Nϕ (Gr1^{hi}CD11b^{hi}), monocytes (Gr1$^-$ CD11b^{hi}F4/80$^+$CD11c^{lo}), moMϕ (Gr1$^-$CD11b^{hi}F4/80^{lo}CD11c$^+$Ly6C$^-$), and moDC (CD11b^{hi}F4/80^{lo}CD11c$^+$Ly6C$^+$) populations. Finally, the differential expression of Ly6C and Ly6G was used to characterize the CD11b$^+$ monocytic populations (22, 25, 27, 28): cMO (CD11b^{hi}Ly6C^{hi}Ly6G$^-$) and ncMO (CD11b^{hi}Ly6C^{lo}Ly6G$^-$). Isotype fluorescence minus one (FMO) controls were performed to rule out non-specific fluorescence and to define gating boundaries (**Supplementary Figure 1**).

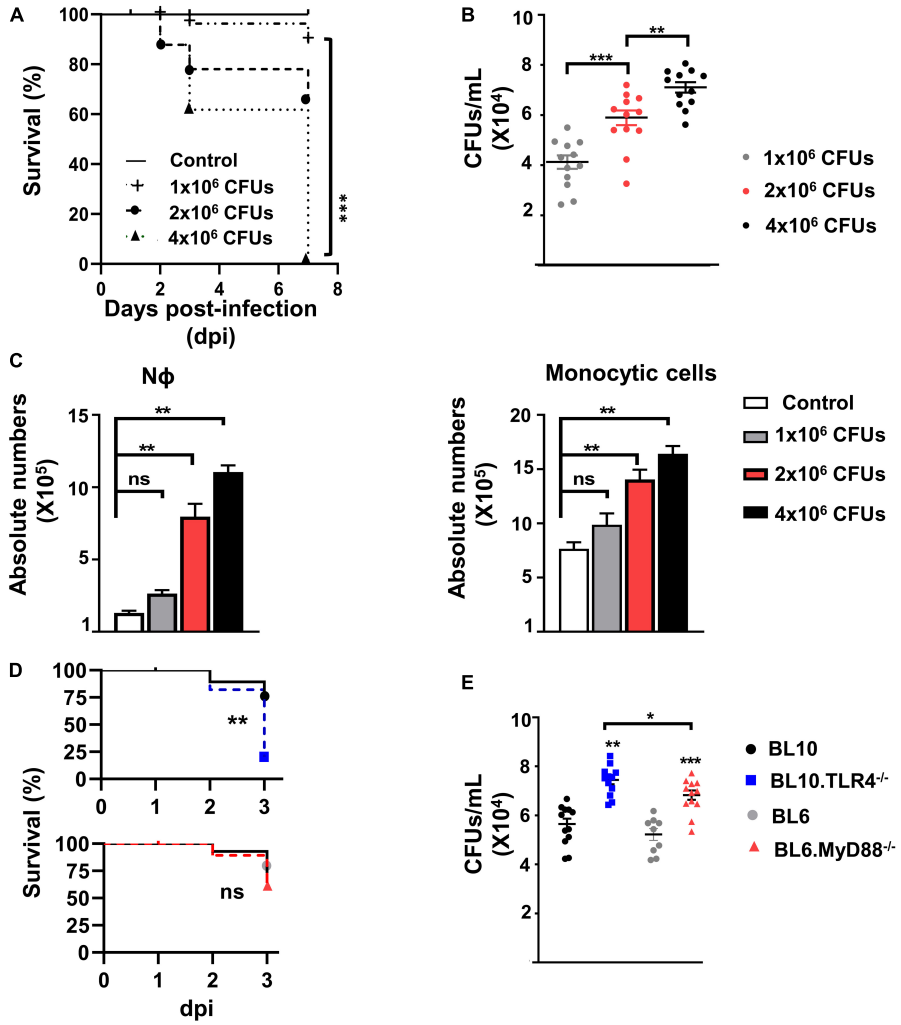

FIGURE 1 | Dose-response study following *S. pneumoniae* bacterial infection of the lungs. **(A)** Survival of WT.BL10 mice after intranasal infection with different concentrations of *S. pneumoniae* CFUs. Survival was determined at 2, 3, and 7 dpi, and the differences between the groups were compared using Kaplan–Meier tests. The data represent measurements from three independent replicates: Control (n = 6); 1 × 10^6 CFUs (n = 8); 2 × 10^6 CFUs (n = 14); 4 × 10^6 CFUs (n = 10). Statistical analysis was performed using a log-rank Mantel–Cox test: ***$p < 0.001$. **(B)** CFUs determined in lung suspensions from infected mice at 72 h pi. Suspensions were plated on sheep blood agar plates and incubated for 24 h at 37°C in 5% CO_2. The data represent the individual measurements of three independent experiments. Shown inside are means ± SEM; n = 12. **(C)** Quantification of Nϕ ($Gr1^{hi}CD11b^{hi}$) and monocyte cell populations ($Gr1^{-}CD11b^{lo/hi}$) in whole lung cell suspensions (without ACK treatment) obtained at 3 dpi following infection with 1 × 10^6, 2 × 10^6 and 4 × 10^6 CFUs (as defined in **Supplementary Figure 1**). Bar graphs show the absolute numbers of each population calculated from the frequencies obtained by flow cytometry and the total number of cells counted in each sample in three independent experiments. Data are means ± SEM; control (n = 6); 1 × 10^6 CFUs (n = 8); 2 × 10^6 CFUs (n = 14); 4 × 10^6 CFUs (n = 10). **(D)** Survival of WT.BL10 (n = 12), $TLR4^{-/-}$ (n = 12), WT.BL6 (n = 10) and $MyD88^{-/-}$ (n = 12) mice after intranasal infection with 2 × 10^6 CFUs. Survival was assessed at 1, 2, and 3 dpi and the differences between the groups were compared using a log-rank Mantel–Cox test. **(E)** Colonies formed from the lung suspensions were counted at 3 dpi as in panel B; WT.BL10 (n = 12), $TLR4^{-/-}$ (n = 12), WT.BL6 (n = 9) and $MyD88^{-/-}$ (n = 12). The data in panels **(B,C,E)** were compared among multiple groups with one-way ANOVA and unpaired two tailed Student's t-test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ns, not significant.

Cytometric Bead Array for Cytokines

Supernatants obtained from fresh lung suspensions (total volume 3 mL) were aliquoted and frozen at −80°C. The aliquots were thawed (only once) and the cytokines present in a 25 μL sample were analyzed (CXCL1, IL-18, IL-23, IL-12p70, IL-6, TNF-α, IL-1β, and IL-12p40) with the LEGENDplex Cytokine Analysis kit (BioLegend), according to the manufacturer's recommendations, and using a FACS Canto I cytometer (BD Biosciences) and the BioLegend LEGENDplex software.

Quantitative Real-Time PCR

Total RNA from lung samples was extracted using NucleoZol (Macherey-Nagel) and reverse transcribed in a final volume of 25 μL with Oligo(dT)-primers, as described previously (38). The cDNA (1 μL) obtained was amplified quantitatively by real-time PCR (qPCR) on a CFX96^{TM} Real-Time System using the SsoFast^{TM}SupermixEvaGreen (Bio-Rad, Hercules, CA), as indicated elsewhere (39). The Bio-Rad CFX Manager software was used to calculate the C_T of each reaction and the specific

amount of cDNA in each sample was determined relative to the expression of the *hypoxanthine phosphoribosyl transferase-1* (HPRT) gene by the $2^{-\Delta Ct}$ method (40). The primers used in this study are listed in **Supplementary Table 2**.

Detection of Intracellular Reactive Oxygen Species

The ROS-sensitive probe 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA, 10 mM stock solution: Invitrogen) was used here on fresh cells surface-labeled as described in the Flow Cytometry section but without fixation procedure. The cells were then labeled for 40 min in the dark at RT with H_2DCFDA (5 μM in PBS), washed twice with PBS and recovering the cells by centrifugation for 5 min at 4°C and 110 *g*, and resuspended them in a final volume of 1 mL. The labeled cells were incubated with 2×10^6 CFUs of heat-inactivated *S. pneumoniae* (60 min, 60°C) for 15, 30, and 60 min at 37°C, and they were then analyzed by flow cytometry to measure the ROS signals of the different myeloid populations. The baseline fluorescence of the cells was determined prior to their exposure to *S. pneumoniae*.

Statistical Analysis

The data are presented as the means ± SEM (Standard error of the mean). Statistical analyses were performed with the Prism 8.0 (Graph Pad) software, after testing the normality of the data distributions with the Kolmogorov–Smirnov and D'Agostino-Pearson tests. The data were compared with one-way ANOVA tests and two-tailed unpaired Student's *t*-tests. Survival curves were obtained using Kaplan–Meier tests and analyzed using a log-rank (Mantel–Cox) test. A value of $p < 0.05$ was considered statistically significant: $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$; ns, not significant.

RESULTS

Innate Immune Responses of Lung Cell After *S. pneumoniae* Infection

We performed initial experiments to establish the optimal dose for *S. pneumoniae* serotype 3 strain 1195 infection and the time required to mount an IIR, parameters that allowed us to study the myeloid cell subpopulations involved, as well as the role of TLR4 and MyD88 expression. Adult WT.BL10 mice were infected intranasally with increasing doses of bacteria (1×10^6, 2×10^6, and 4×10^6 CFUs) and their survival after infection was studied. A dose-dependent decrease in survival was found from 3 dpi, accompanied by an increase in bacterial CFUs in the lungs (**Figures 1A,B**). It is known that Nϕ and monocyte cell infiltration is essential for *S. pneumoniae* lung clearance (12, 41). Enhanced recruitment of Nϕ ($Gr1^{hi}CD11b^{hi}$) and monocytes ($Gr1^{-}CD11b^{lo/hi}$) to the lung was evident at 3 dpi in mice that received 2×10^6 and 4×10^6 CFUs (**Figure 1C**). Thus, we chose the dose of 2×10^6 CFUs to infect and monitor the lung IIR at 3 dpi. Previous studies have shown that pneumococcal LTA and Ply were recognized by TLR2 and TLR4, as the main PRRs required to initiate the immune response (13, 14) and acting through the MyD88-signaling cascade. To analyze the contribution of TLR4 and MyD88 signaling to the IIR, $TLR4^{-/-}$ and $MyD88^{-/-}$ mice were infected intranasally with 2×10^6 CFUs *S. pneumoniae* (**Figures 1D,E**), and their survival and bacterial load was quantified at 1, 2, and 3 dpi (**Figure 1E** and **Supplementary Figure 2**). Since the different genetic backgrounds of the $TLR4^{-/-}$ (BL10) and $MyD88^{-/-}$(BL6) mice may influence their IIR against *S. pneumoniae*, we used both WT.BL10 and WT.BL6 control strains, with no differences in survival and bacterial load detected between them. The $TLR4^{-/-}$and $MyD88^{-/-}$ mice survived less time after infection than their corresponding WT animals (WT.BL10 and WT.BL6, respectively), although the $TLR4^{-/-}$ mice survived worse than the $MyD88^{-/-}$ mice at this CFU dose. Accordingly, the bacterial load was higher in the lungs of infected $TLR4^{-/-}$ than in the $MyD88^{-/-}$ mice, which both exceeded that in the WT infected mice (**Figure 1E** and **Supplementary Figure 2**).

Resident AMs ($Siglec\text{-}F^{hi}CD11b^{lo}$) are essential to control bacterial numbers early after *S. pneumoniae* infection, driving a reduction in AM numbers by inducing their caspase-dependent apoptosis (16, 17, 42, 43). Natural killer ($CD3^{-}NK1.1^{+}$) cells also exert their effector and regulatory functions soon after infection. Accordingly, we found fewer AMs in the lungs of both infected WT strains on days 1–3 pi, along with a transient accumulation of NK cells in the lung at 1 dpi (**Figures 2A**, **3A**). Myeloid-cells in the lung were analyzed by flow cytometry at 3 dpi (**Figures 2B**, **3B**) and as expected, there was significant Nϕ recruitment in the infected lungs, coupled to a decrease in monocytes. Indeed, a decrease in F4/80 (a specific marker of mature Mϕs) and an increase in CD11c was observed, indicating the activation and differentiation of these mature Mϕs. When Ly6C expression was analyzed in the differentiated $CD11c^{hi}F4/80^{lo}$ population, an increase in the absolute numbers of both moMϕ and moDC was seen in infected mice (**Figure 2B** right dot plots, **Figure 3C**). The cMO/ncMO profile was defined on gated $CD11b^{+}$ cells, detecting differential Ly6C and Ly6G expression as described previously. Monocytes in control and infected mice shifted toward a pro-inflammatory cMO/ncMO ratio (BL10 1.12 ± 0.09, $n = 12$; BL6 1.073 ± 0.174, $n = 6$) in the infected lungs when compared to the control non-infected mice (BL10 0.61 ± 0.04, $n = 12$; $p < 0.001$; BL6 0.68 ± 0.05, $n = 6$ $p < 0.05$; **Figures 2C**, **3D**).

Together these data reveal distinct local cell dynamics in the lungs of *S. pneumoniae* infected mice, and both WT.BL10 and WT.BL6 control strains behave similar in terms of myeloid cell recruitment. These changes involved an initial decrease in AM cells during the first 3 dpi, along with the accumulation of NK cells on day 1. Subsequently, there was neutrophil infiltration and monocyte differentiation toward the moMϕ and moDC phenotypes at 3 dpi, with a predominant cMO profile.

Defective Innate Response to *S. pneumoniae* in Infected TLR4 and MyD88 Deficient Mice

The survival of $TLR4^{-/-}$ and $MyD88^{-/-}$ infected mice was worse than that of WT mice, and they had a higher bacterial load, especially the $TLR4^{-/-}$ mice (**Figures 1D,E**). As in

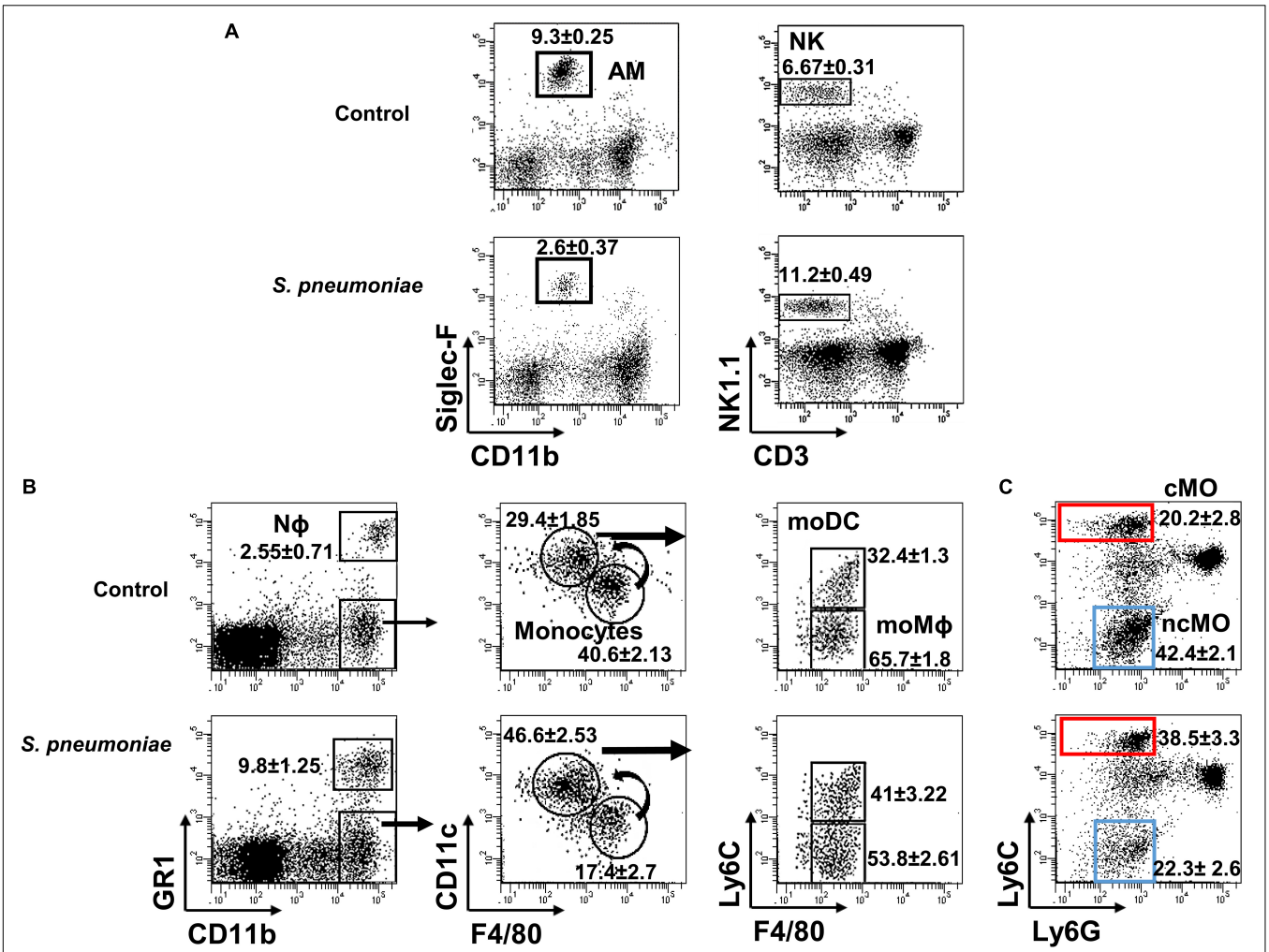

FIGURE 2 | Lung myeloid populations after *S. pneumoniae* infection. Lung suspensions from infected and uninfected WT.BL10 mice were prepared at 1 and 3 dpi, and stained with the indicated mAbs (see section "Materials and Methods") **(A)** Samples obtained at 1 dpi were stained with anti-CD11b and Siglec-F to identify AM (Siglec-F^{hi}CD11b^{lo}) cells in the left dot plots. NK cells were identified using anti-NK1.1 and anti-CD3 antibodies: NK (NK1.1^{+}CD3^{-}) in the right dot plots. **(B)** At 3 dpi, myeloid and granulocyte cell populations were discriminated using the markers CD11b and Gr1, as described in **Supplementary Figure 1** in which the sequential gating strategy to identify distinct monocyte populations is depicted. **(C)** The cMO and ncMO profile of monocytes was defined based on their Ly6C and Ly6G expression: cMO (CD11b^{hi}Ly6C^{hi}Ly6G^{-}, red box) and ncMO (CD11b^{hi}Ly6C^{lo}Ly6G^{-}, blue box). Representative dot plots are shown and the numbers inside are the percentages mean ± SEM of four independent experiments, WT.BL10 (n = 12).

WT.BL10 and WT.BL6 mice, *S. pneumoniae* infection produced an accumulation of NK cells and an important reduction in AM cells in TLR4$^{-/-}$ and MyD88$^{-/-}$ mice (**Figure 3A**), with the decrease in AM cells more pronounced in TLR4$^{-/-}$ infected mice than in either of the WT strains or the MyD88$^{-/-}$ mice (TLR4$^{-/-}$ versus MyD88$^{-/-}$ values $p < 0.001$ and $p < 0.01$ at days 1 and 2 pi, respectively). There was similar recruitment of NK cells to the lungs at 1 dpi in the four strains of mice. By contrast, deficient accumulation of Nϕ was detected by 3 dpi in TLR4$^{-/-}$ mice relative to the WT.BL10 infected mice (**Figure 3B**).

Although the lungs from infected WT.BL10 and WT.BL6 mice have fewer monocytes due to their differentiation toward a moMϕ and moDC populations (**Figure 2B**, right dot plots), there was only a minimal reduction in the monocyte populations in infected TLR4$^{-/-}$ mice (**Figure 3C**), with no increase in their moDC population and a decrease in moMϕ number. By contrast, there was a reduction in monocytes in MyD88$^{-/-}$ infected mice and differentiation toward the moDC lineage was observed, as occurred in WT.BL6 animals, although there was no increase in the moMϕ cell population that remained much lower than in the WT.BL6 mice (**Figure 3C**). Upon infection, and based on the Ly6C expression (20, 22, 26, 28) the cMO/ncMO profile shifted slightly toward a pro-inflammatory profile in TLR4$^{-/-}$ mice (0.64 ± 0.05 uninfected, 0.89 ± 0.079 infected mice, n = 15; $p < 0.05$), whereas this ratio was higher in MyD88$^{-/-}$ mice (0.68 ± 0.04 uninfected mice, 1.003 ± 0.087 infected, n = 15; $p < 0.01$), similar to that found in WT.BL10 and WT.BL6

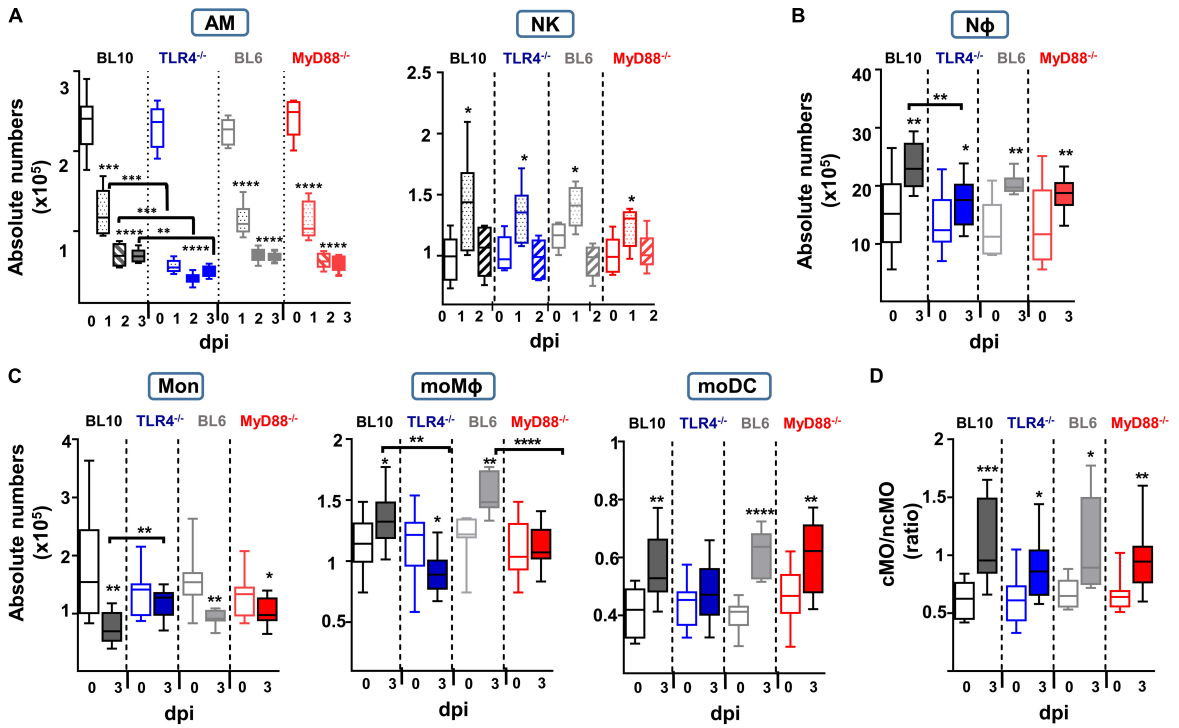

FIGURE 3 | Quantification of the absolute numbers of different lung innate subsets after *S. pneumoniae* (2×10^6 CFUs) instillation to WT.BL10, TLR4$^{-/-}$, WT.BL6 and MyD88$^{-/-}$ mice. **(A)** AM and NK cells were identified and counted at 0, 1, 2, and 3 dpi. Absolute numbers were calculated using the frequencies obtained by cytometry and the total number of cells counted in each sample: WT.BL10 (n = 6), TLR4$^{-/-}$ (n = 6), WT.BL6 (n = 6), and MyD88$^{-/-}$ (n = 6) at 0, 1, 2, and 3 dpi. **(B–D)** At 3 dpi the IIR was analyzed by quantifying the Nϕ cells, monocytes, the moDC and moMϕ cells, and the cMO and ncMO populations from control and infected mice. Box-and-whisker plots show the median values, with the bottom and top of the box indicating the first quartile to the third quartile, with the minimum and maximum values from four independent experiments: WT.BL10 (n = 12), TLR4$^{-/-}$ (n = 15), WT.BL6 (n = 6), and MyD88$^{-/-}$ (n = 15). All the populations were analyzed by one-way ANOVA and with an unpaired two-tailed Student's t-test: *$p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

animals. In parallel, there was an important reduction in adaptive B cell and CD4+ T cell recruitment to the lungs of TLR4$^{-/-}$ and MyD88$^{-/-}$ infected mice at 3 dpi, whereas CD8^{+} cells were recruited normally (manuscript in preparation).

In summary, these findings demonstrate important defects in the local lung IIR in MyD88$^{-/-}$ and TLR4$^{-/-}$ mice, with a more severe phenotype in the latter model. These changes included important defects in AM, NK, and Nϕ cell recruitment, as well as significant defects in monocyte differentiation and the adaptive response.

Differential Impairment of Inflammatory Cytokine Production in TLR4$^{-/-}$ and MyD88$^{-/-}$ Infected Mice

To further understand the susceptibility of the TLR4$^{-/-}$ and MyD88$^{-/-}$ mice to *S. pneumoniae* infection, we assessed the cytokines in the lung homogenates by 3 dpi (**Figure 4A**). There was a significant increase in pro-inflammatory cytokines like CXCL1, TNF-α, IL-6, and IL-1β in WT (BL10 and BL6) mice following *S. pneumoniae* infection, with CXCL1 being greater in infected WT.BL6 compared to WT.BL10 ($p < 0.05$), whereas no such increase in these cytokines was evident in TLR4$^{-/-}$ infected mice. By contrast, there was a mild increase in CXCL1, TNF-α, and IL-6 in MyD88$^{-/-}$ infected mice. The expression of TNF-α and IL-6 transcripts was assessed in lung homogenates from infected mice by qPCR at 3 dpi (**Figure 4B**), and while there was a strong increase in both cytokines in WT.BL10 and MyD88$^{-/-}$ infected mice, this was not the case in TLR4$^{-/-}$mice. An early increase in IL-23 and IL-12p40 has been described 24 h pi with *S. pneumoniae* (44, 45). There was no change in IL-23, IL-18 and IL-12p70 in the lungs of WT (BL10 and BL6), TLR4$^{-/-}$ and MyD88$^{-/-}$ mice at 3 dpi, although IL-12p40 diminished in the WT mice (**Figure 4A**). Thus, in the absence of TLR4 *S. pneumoniae* impaired the pro-inflammatory cytokine cascade in the lung (CXCL1, TNF-α, IL-6, and IL-1β) at 3 dpi. Furthermore, inflammatory cytokines were moderately affected in MyD88$^{-/-}$ infected mice, indicating that the inflammatory response to *S. pneumoniae* is partially dependent on the MyD88 transduction machinery.

In vitro NADPH Oxidase-Activity of Distinct Myeloid Populations After *S. pneumoniae* Challenge

Nϕ and monocytes can activate NAPDH oxidase activity and generate ROS as part of the host's defense against bacteria

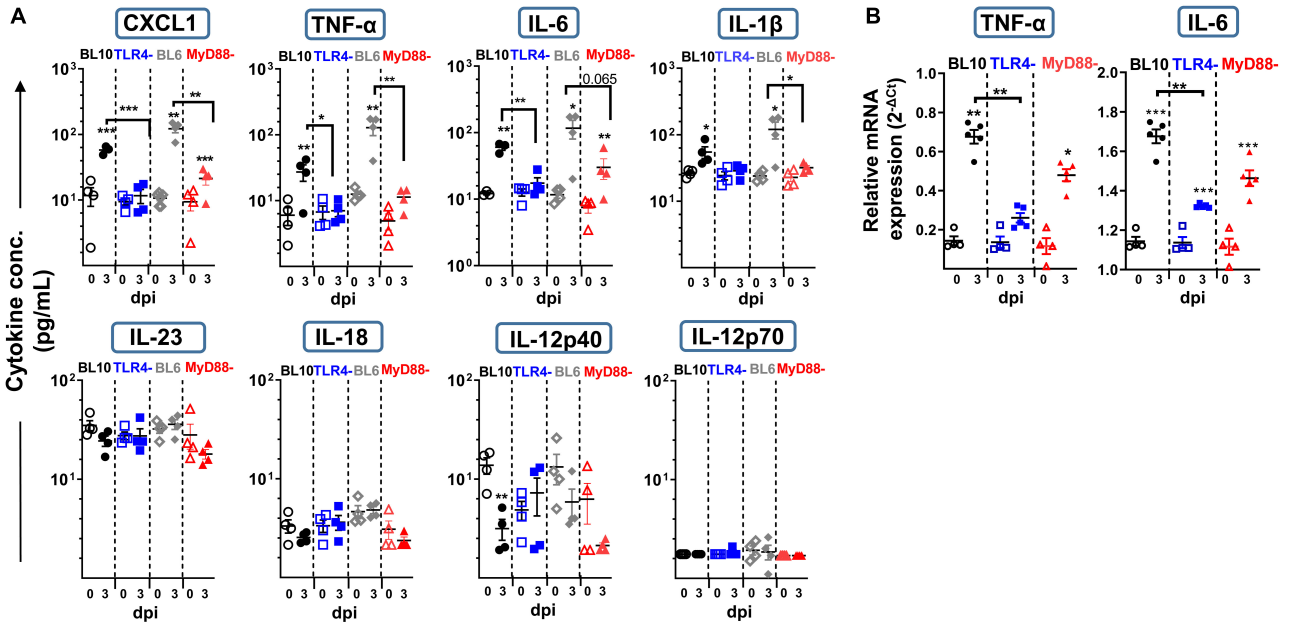

FIGURE 4 | Lung cytokine production at 3 dpi in WT.BL10, TLR4$^{-/-}$, WT.BL6 and MyD88$^{-/-}$ *S. pneumoniae* (2×10^6 CFUs) infected mice. Lung suspensions from control (empty symbols) and infected mice (filled symbols) were prepared and their supernatants analyzed. **(A)** CXCL1, IL-18, IL-23, IL-12p70, IL-6, TNF-α, IL-1β, and IL-12p40 protein levels were measured in LEGENDplex Cytokine Assays. **(B)** TNF-α and IL-6 gene expression in the lungs of WT.BL10, TLR4$^{-/-}$, and MyD88$^{-/-}$ measured by qPCR at 3 dpi using the Bio-Rad CFX Manager software to calculate the C_T for each reaction. The amount of each specific transcript in each cDNA sample was determined and expressed as the $2^{-\Delta CT}$, relative to that of HPRT transcripts. The data are shown as in **Figure 1B**, representing 4-to-6 different samples evaluated in duplicate. The populations were compared with one-way ANOVA and with an unpaired two-tailed Student's *t*-test: $^*p < 0.05$, $^{**}p < 0.01$.

and parasites (46, 47). We studied the *in vitro* production of ROS in lung cell preparations after challenge with heat-killed *S. pneumoniae*, using the ROS-sensitive fluorescence probe H_2DCFDA in Nϕ and in a variety of monocyte cell populations at different time points (**Figure 5A**). As expected, Nϕ rapidly produced large amounts of ROS in WT (BL10 and BL6) mice, with weaker induction and with slower kinetics in moDC, moMϕ and monocytes. The *in vitro* production of ROS in TLR4$^{-/-}$ and MyD88$^{-/-}$ mice differed significantly to that in WT (BL10 and BL6) mice (**Figure 5B**). In the case of TLR4$^{-/-}$ lung cell preparations, ROS production by Nϕ and by the rest of monocyte populations was inhibited after 30 and 60 minutes. Furthermore, while the production of ROS was stronger in MyD88$^{-/-}$ mice than in TLR4$^{-/-}$ mice, in all cell types analyzed except in moMϕ, it remained weaker and with slower kinetics than that detected in WT.BL6 mice.

Regulation of the ROS-NAPDH pathway is mainly due to activation of the NADPH oxidase-2 (Nox2) transcription factor (48) and it is negatively regulated by Nrf2 (49). The transcripts of these two genes were quantified by qPCR in the three mouse models studied and Nox2 induction was evident in all infected mice, although it was weaker in the infected TLR4$^{-/-}$ mice (**Figure 5C**). Furthermore, an important reduction of Nrf2 was evident in lung samples from WT.BL10 and MyD88$^{-/-}$ infected mice but not in those from TLR4$^{-/-}$ infected mice, in which it remained unchanged. Together, maintaining Nrf2 levels and weaker Nox2 induction may contribute to the diminished ROS production in TLR4$^{-/-}$ infected mice.

DISCUSSION

The airway and alveolar epithelia represent the initial barrier for the recognition of respiratory pathogens to initiate a immune response against them. Bacterial airway infections, including those caused by *S. pneumoniae*, are a major cause of worldwide morbidity and mortality. Indeed, pneumonia is the leading cause of mortality in children below 5 years of age, although global child mortality has decreased substantially (50, 51). In addition, the mortality of individuals over 65 years due to pneumonia remains unchanged since 1990, in part because the coverage of pneumococcal vaccines remains low (52, 53). Nasopharyngeal colonization with *S. pneumoniae* in healthy children is common, indicating a role of the host response in avoiding invasive pneumococcal disease.

Identifying mechanisms that can prevent bacterial lung infection is a global health priority, which requires a better understanding of the immune mechanisms involved in response to *S. pneumoniae* infection. The local IIR in the lung is important to combat *S. pneumoniae*, which involves the coordination of multiple cell populations and the activation of effector functions like phagocytosis, cytokine release, the complement cascade and antigen-presentation. Indeed, the IIR against *S. pneumoniae* is initiated through the recognition of PAMPs by TLRs, whose role and that of TLR-adaptor proteins and inflammasome complexes, has been studied using mouse models of infection (54–57). MyD88 and TLR4 are involved in local and systemic bacterial control, as well as in the recruitment of polymorphonuclear

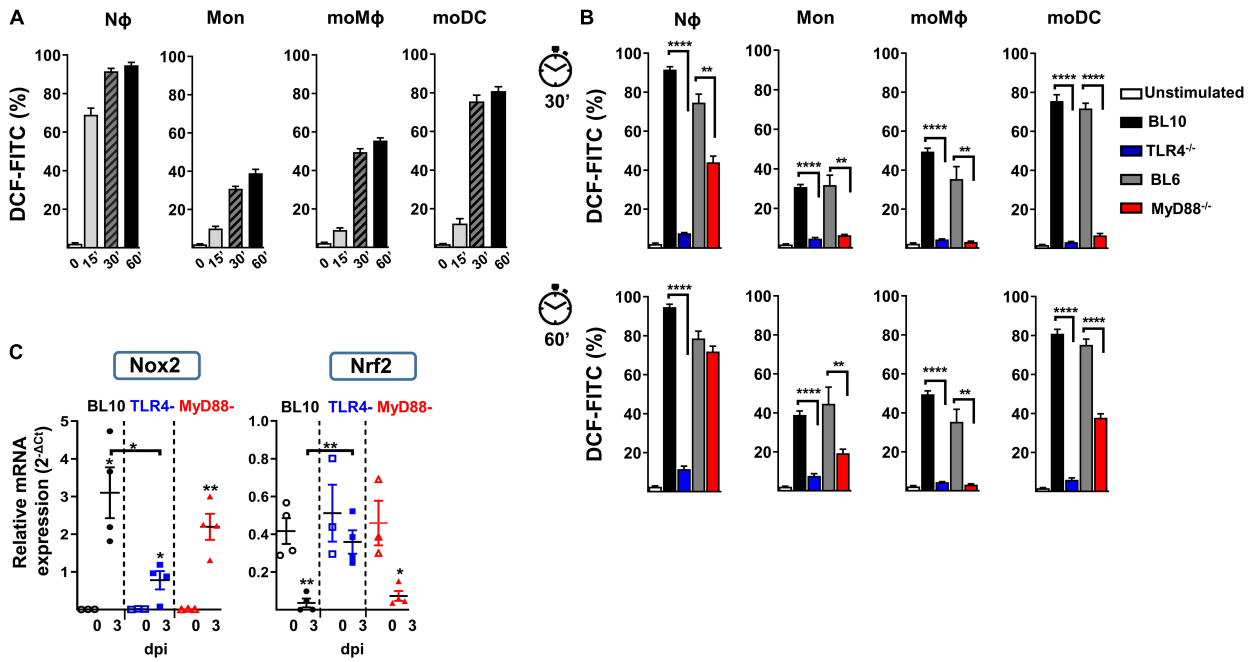

FIGURE 5 | *In vitro* NADPH oxidase activity against heat-inactivated *S. pneumoniae* in lung cells. **(A)** Stained lung cells from WT.BL10 mice were labeled with the H_2DCFDA-ROS sensitive probe (5 μM, DCF-FITC) and incubated with 2 × 10^6 CFUs heat-inactivated *S. pneumoniae* (60 min, 60°C) for 0, 15, 30, and 60 min at 37°C before analyzing them by flow cytometry. **(B)** Detection of ROS in different myeloid populations from naïve mice using heat-inactivated *S. pneumoniae* at 30 and 60 min as in panel **(A)**. The bar graphs show the proportion of H_2DCFDA^+ cells. Unstimulated controls were shown for WT.BL10 animals. The data represents the mean ± SEM: WT.BL10 (n = 7), TLR4$^{-/-}$ (n = 6), WT.BL6 (n = 5), and MyD88$^{-/-}$ (n = 6). **(C)** Nox2 and Nrf2 gene expression by lung cells from WT.BL10, TLR4$^{-/-}$, and MyD88$^{-/-}$ mice measured by qPCR at 3 dpi, as described in **Figure 4**. The differences between the groups were analyzed by one-way ANOVA and with an unpaired two-tailed Student's t-test: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

leukocytes in the lung (54). While TLR2 plays only a modest role in the immune response to *S. pneumoniae* in the respiratory tract, without affecting the overall antibacterial defense during infection (12), TLR9 has been implicated in the early clearance of bacteria, with no significant effect on local cytokine production (55). By contrast, TLR4, which signals both through MyD88-dependent and MyD88-independent (TRIF) pathways, also plays an important role by recognizing *S. pneumoniae* Ply and LTA (13) (**Figure 6A**). The impact of TLR recognition and signaling depends on the bacterial strain, and hence the pneumococcal serotype and the dose used to induce pneumonia may yield different results (54, 57). This study was carried out using a clinical serotype 3 encapsulated *S. pneumoniae* isolate, and by studying myeloid markers of activation and differentiation, we identified the myeloid events in the lung, local inflammatory responses and the ability to induce ROS production in response to infection of TLR4 and MyD88 deficient mice.

Consistent with these previous studies (54, 57), we show here that TLR4$^{-/-}$ and MyD88$^{-/-}$ infected mice have a higher bacterial load than their corresponding WT counterparts. Interestingly, lungs from infected MyD88$^{-/-}$ mice had fewer bacterial CFUs than TLR4$^{-/-}$ mice, which may indicate that the TRIF-dependent TLR4-signaling cascade plays an important role in bacterial clearance (**Figures 6B,C**). It was previously shown that the interaction between Ply and TLR4 during pneumococcal colonization of the nasopharynx is important for protection (14). Indeed, our results suggest an impaired immune response to *S. pneumoniae* in TLR4$^{-/-}$ mice and to a lesser extent in MyD88$^{-/-}$mice, compared to their corresponding WT genetic background (BL10 and BL6, respectively). Infected TLR4$^{-/-}$ mice suffer a stronger reduction in AM cells and defective Nϕ induction. By contrast, the reduction of AM and the induction of Nϕ cells was similar in MyD88$^{-/-}$ and WT.BL6 mice. As reported previously (20, 21), there was rapid differentiation of monocytes to moMϕ and moDC in *S. pneumoniae* infected WT animals. This induction of moMϕ was not observed in MyD88$^{-/-}$ infected mice and it was severely dampened in *S. pneumoniae* infected TLR4$^{-/-}$ mice. By contrast, moDC increased in MyD88$^{-/-}$ infected mice to a similar extent as in the WT.BL6 mice, whereas there was no increase in moDC number in TLR4$^{-/-}$ infected mice. The ratio of the cMO/ncMO populations in *S. pneumoniae*-infected TLR4$^{-/-}$ mice reflected a cMO inflammatory monocyte profile, whereas that found in MyD88$^{-/-}$ and in both the WT strains shifted toward a ncMO phenotype.

All these cellular defects after *S. pneumoniae* inoculation, mostly evident in TLR4$^{-/-}$ mice, may contribute to the incomplete lung cytokine profile in these mice. Firstly, CXCL1 release, together with that of IL-1β and IL-6, plays a central role in Nϕ mobilization from the bone marrow, in Nϕ activation and in the control of bacterial dissemination in the lung after pneumococcal infection (58, 59). At 3 dpi, there was more

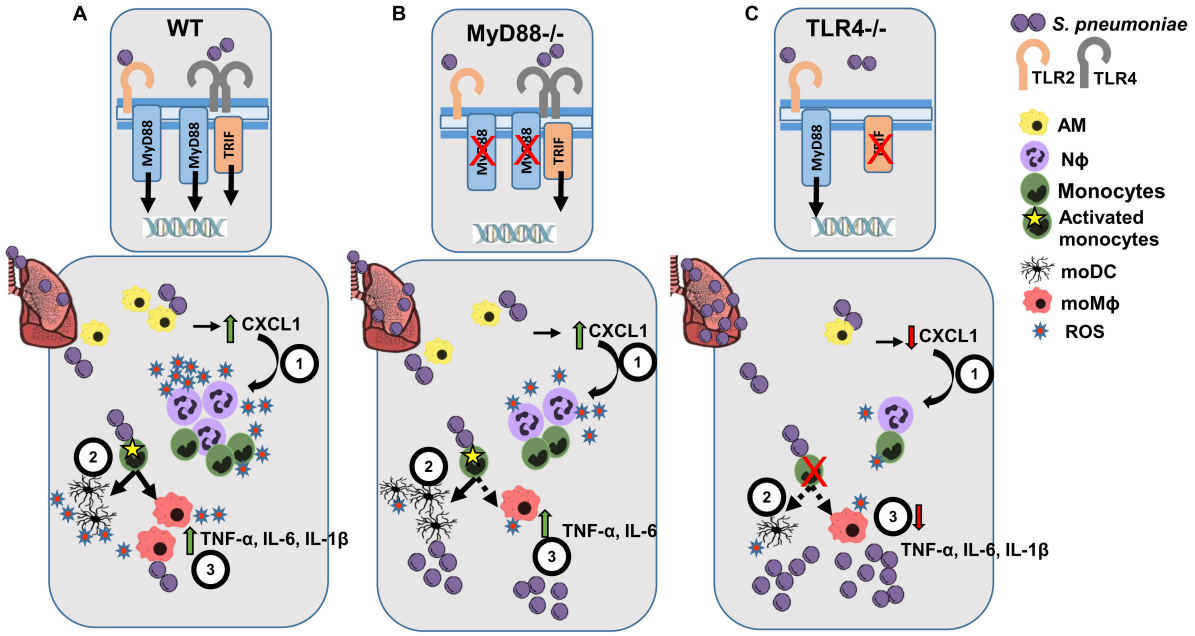

FIGURE 6 | Scheme of the proposed lung IIR against *S. pneumoniae* in WT, MyD88$^{-/-}$ and TLR4$^{-/-}$ mice. **(A)** After infection of WT mice, TLR2 and TLR4 recognizes different PAMPs from *S. pneumoniae* activating the TRIF- and MyD88-dependent transduction pathways. After the initial bacterial encounter with AM cells, CXCL1 (1) is released that favors the early recruitment and activation of Nϕ cells, leading to substantial ROS production. (2) Differentiation of activated monocytes to moDCs and moMϕ helps to amplify the inflammatory response (3) through the secretion of a broad spectrum of cytokines and ROS production. **(B)** Infected MyD88$^{-/-}$ mice can still signal through the TLR4-TRIF-dependent pathway, leading to a weak IIR response while maintaining an inflammatory cytokine environment. **(C)** TLR4$^{-/-}$ infected mice experience impaired Nϕ recruitment and almost a complete blockade of monocyte differentiation, which leads to a very poor cytokine response and the development of a more intense bacterial burden.

CXCL1, TNF-α, IL6, and Il-1β in the lungs of WT (BL10 and BL6) mice, while IL-18 and IL-23 were not enhanced at this time, perhaps due to an earlier action of these cytokines at 24 h (44, 45, 48). Ply is not involved in the pneumococcal induction of IL-12p40 in murine Mϕ (60) and indeed, we detected less IL-12p40 in the lungs of infected WT (BL10 and BL6) mice relative to their uninfected WT controls. These changes may be indicative of a transient Th1 response occurring in the first 24 h pi, and related to the fast action of AM and NK cells as the first innate barrier to impede bacterial growth. In TLR4-defective mice there was no increase in CXCL1, TNF-α, IL6, and Il-1β at 3 dpi, while the increase in these cytokines was only mild in MyD88-deficient mice. Whether this transient inflammatory lung environment could influence the appearance of trained Mϕ cells (resident and bone marrow-derived), with an enhanced capacity to respond to a second PAMP-associated challenge, will be addressed in the near future. As such, an *in vivo* model of trained immunity should be useful in designing novel "trained-induced" based vaccines.

Bacterial phagocytosis and ROS production are essential to control *S. pneumoniae* pathogen-dissemination and overgrowth. Nϕ cells fulfill a central role in removing invading pneumococci through the rapid production of intracellular and/or extracellular ROS in response to PAMPs (61), or after phagocytosis (58). The release of Ply after autolysis of pneumococci activates NADPH oxidase and the intracellular production of ROS by Nϕ (33). Our results following *in vitro* stimulation with heat killed *S. pneumoniae* revealed that Nϕ were the fastest producers of ROS, although moMϕ and moDC can also produce notable amounts of ROS albeit with delayed kinetics and over longer periods. These cells not only play a key role in killing ingested pathogens but also in antigen presentation, cross-presentation and the activation of cytolytic T cells (62). Reactive oxygen species production by Nϕ and by all the monocyte-derived populations was severely inhibited in TLR4$^{-/-}$ and to a lesser extent in MyD88$^{-/-}$ mice, which may at least in part explain the rapid growth of *S. pneumoniae* in these animals. In humans, mitochondrial ROS production by AM cells was dampened in chronic obstructive pulmonary disease (COPD) patients but not in smokers, or in control individuals after *in vitro* challenge with *Haemophilus influenzae* or *S. pneumoniae* (63). Moreover, NADPH defects in humans favor fungal and bacterial infection (61). Likewise, the bactericidal activity of the fluoroquinolones levofloxacin and moxifloxacin against *S. pneumoniae* is related to the production of ROS by the bacteria, associated with transcriptional alterations induced by these fluoroquinolones (64–66). Moreover, oxygen-derived metabolites can also damage infected tissues, regulate immune functions and induce apoptosis (58, 67). The levels of the ROS inducer Nox2 and the antioxidant Nrf2 must be counterbalanced in order to overcome lung infection by *S. pneumoniae*, as demonstrated in WT and MyD88$^{-/-}$ mice but not in TLR4$^{-/-}$ mice. Indeed, targeting mitochondrial regulators of NADPH-oxidase production has been evaluated in leukemia cells (68), ischemic cerebral neurons (69), and human Nϕ cells after LPS activation (70). It is tempting

to speculate that novel therapies of this type might benefit COPD patients, PRR-immunodeficient subjects, aged recurrent-pneumonia and patients with sepsis as promising alternatives to conventional antibiotic treatments. Likewise, such therapies could be useful to combat an important number of multi-resistant bacteria, with important implications for treatment and national healthcare budgets.

In summary, this study involved an in-depth analysis of different resident and recruited innate cell populations in the lung, addressing their cytokine production, their differentiation into distinct phenotypes and NADPH-oxidative metabolism, which in conjunction produces efficient pneumococcal clearance. All these host-mediated responses were severely impaired when TLR4 was absent, demonstrating the critical involvement of this receptor in the defense against the Gram-positive *S. pneumoniae*. In MyD88 deficient mice, a partial effect on the innate cell content, cytokine profile and ROS production was evident, indicating a role for either the TRIF-dependent and MyD88-independent signaling pathways, or alternatively, the implication of other PRRs like TLR9. As a result, our data highlight the importance of the TLR4-MyD88 axis in the recognition and efficient response to *S. pneumoniae* infection in the lung, raising new challenging questions that warrant further attention.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding authors.

ETHICS STATEMENT

This study was carried out in accordance with EU and National Animal Care guidelines (directive 2010/63/EU and RD 53/2013). The procedures were approved by the Institutional Review Board at the ISCIII and the "Consejería de Medio Ambiente Comunidad de Madrid" (PROEX110/15 and PROEX021/18).

AUTHOR CONTRIBUTIONS

RS-T performed the experiments, contributed substantially to the analysis and interpretation of the data, and reviewed the manuscript. IC analyzed the data and reviewed the manuscript. JM, MR, and CR performed the experiments. MP and MA helped with the flow cytometry experiments. EC reviewed the manuscript. MF prepared the bacteria and performed the CFU analysis. AC provided bacteria and reviewed the manuscript. MG designed the experimental procedures, made substantial contributions to the analysis and interpretation of the data, and critically reviewed the manuscript. BA designed the experimental procedures, made substantial contributions to the analysis and interpretation of the data, and drafted and reviewed the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

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frontiers in Immunology

REVIEW
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Mumps Outbreaks in Vaccinated Populations—Is It Time to Re-assess the Clinical Efficacy of Vaccines?

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History illustrates the remarkable public health impact of mass vaccination, by dramatically improving life expectancy and reducing the burden of infectious diseases and co-morbidities worldwide. It has been perceived that if an individual adhered to the MMR vaccine schedule that immunity to mumps virus (MuV) would be lifelong. Recent mumps outbreaks in individuals who had received two doses of the Measles Mumps Rubella (MMR) vaccine has challenged the efficacy of the MMR vaccine. However, clinical symptoms, complications, viral shedding and transmission associated with mumps infection has been shown to be reduced in vaccinated individuals, demonstrating a benefit of this vaccine. Therefore, the question of what constitutes a good mumps vaccine and how its impact is assessed in this modern era remains to be addressed. Epidemiology of the individuals most affected by the outbreaks (predominantly young adults) and variance in the circulating MuV genotype have been well-described alluding to a collection of influences such as vaccine hesitancy, heterogeneous vaccine uptake, primary, and/or secondary vaccine failures. This review aims to discuss in detail the interplay of factors thought to be contributing to the current mumps outbreaks seen in highly vaccinated populations. In addition, how mumps diagnoses has progressed and impacted the understanding of mumps infection since a mumps vaccine was first developed, the limitations of current laboratory tests in confirming protection in vaccinated individuals and how vaccine effectiveness is quantified are also considered. By highlighting knowledge gaps within this area, this state-of-the-art review proposes a change of perspective regarding the impact of a vaccine in a highly vaccinated population from a clinical, diagnostic and public perspective, highlighting a need for a paradigm shift on what is considered vaccine immunity.

Keywords: mumps outbreaks, vaccinated populations, immunity, vaccine efficacy, protection

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INTRODUCTION

Mumps Virus

MuV is an enveloped, non-segmented, negative-sense, single stranded RNA virus that varies between a spherical and pleiomorphic shape of ∼200 nm (85–300 nm) (1, 2). MuV is responsible for an acute viral infection, spread by respiratory droplets (via coughs, sneezes) and urine (3, 4). With an incubation period of 14–25 days, MuV replicates in the nasopharynx and regional lymph nodes, with a secondary viremia occurring late in the incubation period (5, 6). MuV can be detected from saliva up to 7 days prior, and as late as 9 days after clinical onset of parotitis (7).

The MuV genome of seven genes consists of 15,384 nucleotides, and encodes six structural proteins and at least two non-structural proteins; the nucleocapsid protein (NP), V protein (V), phosphoprotein (P), matrix (M) protein, fusion (F) protein, small hydrophobic (SH) protein, hemagglutinin-neuraminidase (HN) protein, and large (L) protein. The role of the I protein is not known (1, 6, 8). The SH gene is the most variable region of the MuV genome; a 2–4% intra-variation and 8–18% inter-variation has been documented (9). This gene is used in molecular phylogeny for genotyping and to identify transmission patterns in populations (6). Despite being serologically monotypic, 12 MuV genotypes (A to L) have been described to date (MuV genotypes E and M are omitted, as the MuV previously assigned to these groups were later re-assigned) (1, 9, 10). The geographic distributions of the MuV genotypes varies worldwide but can co-circulate and thus drive temporal shifts in their distribution. Genotype A was frequently isolated in Europe until the 1990's. Currently genotypes C, D, E, G, and H are prevalent in Europe and the United States of America (USA) whereas genotypes B, F and I are more common in Asian countries (**Table 1**) (10, 18, 86, 87).

Development of the Mumps Vaccine

Since 1946 numerous mumps vaccines have been developed worldwide, varying in efficacy and safety profiles but primarily consisting of an attenuated live MuV without an adjuvant (6, 87–89). Currently in Europe and for the majority of the G20 countries who have a mumps vaccine in their immunization schedule (**Table 1**), the mumps vaccine is included as part of the trivalent measles, mumps rubella (MMR) vaccine, and is primarily administered in two doses (90, 91).

The Jeryl Lynn (JL) vaccine, derived from the genotype A MuV strain was first developed in the USA and has been used extensively in the United Kingdom (UK), Ireland and USA since it was licensed in 1967 (92). Derived from a single clinical sample, and propagated in a chick embryo cell culture, two viral isolates (JL2 and JL5) are present, differing by ∼414 nucleotides and 87 amino acid changes (93–95).

The RIT 4385 mumps vaccine, developed from the dominant viral component (JL5) in the JL vaccine strain appears to have comparative safety and efficacy (seroconversion) profiles to the JL vaccine strain (87, 96–98). However, since no controlled clinical trials of efficacy have been published to compare the two doses of the two vaccines, the clinical significance of this observation is not known.

Despite the integration of the MMR vaccine into childhood immunization programs, cyclical outbreaks [defined as two or more cases linked by place and time (96)] of MuV have been documented in several highly vaccinated populations such as Ireland and the United Kingdom (6, 97–103). Between August 2018–and January 2020, 3,736 mumps cases were notified in Ireland, primarily affecting individuals between the ages of 15–24 years. Of the 32% of cases that stated vaccination status, 72% had received two doses of the MMR vaccine (104). An upsurge of mumps cases has also occurred in 47 states of the United States over the last 2 decades, primarily affecting people between 18 and 24 years in close contact/shared settings (105). In Indiana, 76.9% of mumps cases (84.9% of university affiliated and 52% of community cases) had documented evidence of MMR vaccination (106). This results in a significant resource burden for public health departments to control.

Several reviews, both observational and systematic have demonstrated the clinical benefit of a mumps vaccine (107, 108), the pathogenesis and genomic diversity of the MuV (10, 107, 108) and the epidemiology surrounding the outbreak (1, 10, 82). It is not clear why these mumps outbreaks occur, although it has been alluded to be due to a number of interrelated factors, such as sub-optimal vaccine uptake (1, 109, 110), primary or secondary vaccine failure or failure of the mumps vaccine to protect individuals from infection (vaccine efficacy) (107) (**Figure 1**).

Vaccine Hesitancy: How Public Perception Predominates

History depicts the remarkable public health impact of mass vaccination. Previously inevitable childhood diseases with potentially debilitating or deadly outcomes have seen their rates plummet worldwide or become successfully eradicated. Immunizations of vaccine preventable diseases are estimated to prevent ∼2–3 million deaths per annum and increase life expectancy by ∼29 years (111). More recently there has been a shift in the public and media perception of vaccines to their safety, which has facilitated outbreaks such as mumps (112). Organized opposition to vaccinations has a long history; public outcry and resistance following the introduction of the smallpox vaccine in the nineteenth century led to the introduction in England of the Vaccination Act of 1853 (113).

With one in eight children in the USA under the age of 2 currently thought to be unvaccinated due to parental choice, the WHO now considers vaccine hesitancy as one of the ten threats to global health in 2019 (114). Vaccine hesitancy, defined as a "delay in acceptance or refusal of vaccines despite availability of vaccination services" involves a multitude of social, political, cultural and emotional factors in highly vaccinated, western populations (115, 116). One of the main issues is the parental concerns regarding the perceived risk of a vaccine to their child (such as timing/schedules of vaccines, associated pain of administration, and potential adverse effects) vs. the disease morbidity and mortality associated with the vaccine preventable disease (117, 118). The retracted paper published in the Lancet in 1999 (56) and "anti-vaccination" opinions on social media have also contributed to the persistent and insistent misinformation (116), despite vast follow-up epidemiological studies showing no relationship between the MMR vaccine and autism, or differing cognitive development/intelligence (118–120). However, the resultant reaction of the public led to the uptake of the first MMR vaccine falling sharply from 1999, with uptake falling to below 75% in 2002 (104, 121). The age demographic that are experiencing the most cases of mumps in Ireland during the current ongoing outbreak would have been scheduled to have received the first MMR vaccine between 1997 and 2003. Nevertheless, no deductions can be made, due to the lack of vaccination status information provided with reported cases (104).

TABLE 1 | Comparison of vaccine strain, schedule, and coverage in contrast to the circulating mumps strain and reported cases/year within G20 countries who currently utilize mumps containing vaccines as part of the national vaccination schedule.

Country	Vaccine introduced	Vaccines (strains)	Vaccination schedule	Approximate vaccine coverage (VC)	Circulating strains	Reported cases/Year
Argentina	1997 (11, 12)	Present: JL (A) strain. During outbreaks, JL/JL derived vaccines preferred among adolescents and adults (12, 13)	MMR 1: 12 MThs MMR 2: 5–6 years (catch up at 11 years). From December 2018, the government pays for all vaccinations (12, 14–16)	2013: MMR1: 94%; MMR2: 82% 2014: MMR1: 95%; MMR2: 96% 2015: MMR1: 89%; MMR2: 87% 2016: MMR1: 90%; MMR2: 88% 2017: MMR1: 90%; MMR2: 91% (14)	D (2005) (17) K/94-98 (until 2013) (18)	3772: 2013 87: 2014 156: 2015 74: 2016 4396: 2017 771: 2018 (19)
Australia	1982 (20)	JL (A) (21)	1982: MuCV: 12 MThs 1989: MMR 1: 12 MThs 1996: MMR 2: for adolescents 1998: MMR 1: 12 MThs; MMR 2: 4 years. Catch-up between 4 and 16 years. 2013: MMR 1: 12 MThs. MMR 2 (MMRV): 18 MThs (20, 22)	1998: Proof of immunization/exemption required for welfare benefits. 2016: Immunizations required for Family Tax Benefit "No Jab, No Play" 2017: 93% at 2 years. (22, 23)	J/07-08 (until 2013) G (2015) (18, 24)	216: 2013 187: 2014 633: 2015 800: 2016 806: 2017 634: 2018 (19)
Brazil	1992 (25)	1992: Urabe (B) (MMR campaign) 1997: Urabe (B) and Leningrad–Zagreb (N) (MMR campaign) 2003: RIT 4385 (A) (25–29)	2013: MMR 1: 12 MThs; MMR 2: 4–6 years. Booster 1: 11–19 years. Booster 2: After 20 years. 2016: MMR 1: 12 MThs; MMR 2: 15 MThs. Two additional boosters before 20 years, OR a single dose if over 20 years. (25, 26)	2013: MMR1: 100%; MMR2: 69% 2014: MMR1: 100%; MMR2: 89% 2015: MMR1: 96%; MMR2: 80% 2016: MMR1: 95%; MMR2: 77% 2017: MMR1: 97%; MMR2: 41% (14)	K/07(CAN) and K (until 2013) (18, 30)	2014–2015: 82% increase in reported cases in São Paulo (31)
Canada	1969: MuCV 1972: Trivalent MuCV	Mid-1980's (Urabe Am9 MuCV). Withdrawn late 1980's. 1970's: JL (A). Two different MuCVs are used interchangeably (32)	MMR or MMRV vaccine. MMR 1: 12–15 MThs MMR 2: 18 MThs. No later than around school entry (33)	VC of 2 doses of MuCV in school-aged children has been 90% for the past 10 years. in Toronto schools VC 2017–2018: 7 years: 87.4%; 17 years; 95% (34)	A/88, C/85, 88, 11–13 Imported: D/07, 08, 09, 11; F/11–12, G/05–13; H/07, 08, 11–13; K/07, 09, 12–13 (until 2013) G (18, 33)	216: 2013 187: 2014 633: 2015 800: 2016 806: 2017 634: 2018 (19, 35, 36)
China	1990's: (voluntary) 2008: (NIP)	Since 1990: Monovalent MuCV Imported: MuCV JL(A) Domestic: MuCV, mostly S79 strain derived from JL(A) (37)	Pre-2008: MuCV was voluntary and at own expense. 2008-present: MuCV introduced into NIP. One dose of MuCV at 18–24 MThs (38)	Not Available	F/95, 01–12 (11–12/CAN); J/09 (CHN-HK), G/09–11 (CHN-HK); H/11(CHN-HK) (until 2013) (18) 2013–2015: F (99%), G (1%) (38); K (39)	327759: 2013 187500: 2014 182833: 2015 175001: 2016 252740: 2017 259071: 2018 (19)
France	1983	1983: Monovalent MuCV; Urabe (B) 1986: MMR. Urabe (B) 1992: MMR of Urabe (B) discontinued 1992-Present: JL (A) (40–42)	2005: VC documented at 24 MThs MMR 1: 12 MThs. MMR 2: 16–18 MThs (catch up 6–17 years) (43)	2009–2013: MMR 1: ∼90.4%. MMR 2: 78.2% MuCV compulsory for children born from January 1 2018 (44, 45)	D/89; C/90 (until 2013) (18)	2: 2015 6: 2016 10: 2017 4: 2018 (19)

(Continued)

TABLE 1 | Continued

Country	Vaccine introduced	Vaccines (strains)	Vaccination schedule	Approximate vaccine coverage (VC)	Circulating strains	Reported cases/Year
Germany	Former German Democratic Republic: No MuCV in NIP. Former West Germany (FWG): 1976: (10, 46)	MuCV: JL (A) RIT4385 (A) L-Zagreb (N) [Reviewed in (10, 47)]	FWG: 1976: MuCV at 12 MThs (voluntary); 1980: MuCV in NIP 1991: 2 MMRs. Dose 2 at ⩾5 years 1997: MMR 1: 11–14 MThs 1998: MMR 2: 13 MThs−6 years 2001: MMR 1: 11–14 MThs MMRV: 15–23 MThs. Catch up doses: 2–17 years (10, 46)	2009–2013: MMR 1: ∼97%; MMR 2 /MMRV: 93%. (41, 43, 48, 49)	A/87, 90; C/87, 90, 92, 93; D/77; N/87; G/05, 10 (until 2013) (18)	837: 2014 699: 2015 741: 2016 652: 2017 534: 2018 (19)
Italy	1980's (50)	Pre-2001: Urabe (B), Rubini (A) (41) 2001: JL (A), RIT4385 (A), Urabe (B) (51)	1999: MMR offered free to all children in the second year of life 2005–2007: Two-dose schedule as part of NIP 2017: MMR mandatory for children born from 2001. MMR 1: 13–15 MThs; MMR 2: 6 years (52–54)	2013–2017: MMR 1: ∼88.6%. MMR 2: 84.2% 2018: 94.1% (55)	Genotype G (56)	808: 2013 821: 2014 675: 2015 782: 2016 829: 2017 47: 2018 (57)
Mexico	1998 (58)	Present: Triple Viral SRP (sarampo, parotidite epidémica e rubeola). JL (A)	1998: Two MuCV introduced 2000: MuCV included to NIP Present: MMR 1: 12 MThs; MMR 2: 6 years (59).	2017: MMR1: 79%; MMR2: 62% 2016: MMR1: 97%; MMR2: 98% 2015: MMR1: 100%; MMR2:96% 2014: MMR1: 98%; MMR2: 96% 2013: MMR1: 89%; MMR2: 76% (12, 14, 60)	H (2016) (59)	4142: 2014 3399: 2015 3646: 2016 (19, 59, 61)
Russian Federation	1967 (62)	MuCV used of Russian production, in addition to foreign combination vaccines. Leningrad-3 (Genotype unknown) commonly used (43, 62)	MMR 1: 12 MThs MMR 2: 6 years. (62)	2013–2017: MMR 1 VC: ∼98% MMR 2 VC: ∼97% (63)	N/53; C/94, 02–04; H/02–04 (until 2013) (18) C and H (Novosibirsk) (64)	282: 2013 267: 2014 190: 2015 1106: 2016 4443: 2017 2027: 2018 (19)
Saudi Arabia	1991	Urabe (B) JL (A) (41, 65)	1991: MMR 1: 12 MThs 1993: MMR provided as a part of EPI. Required for birth certificate 1998–2000: MMR school campaign. 2002–present: a 3-dose schedule Measles-containing vaccine: Nine MThs MMR 1: 12 MThs; MMR 2: 4–6 years. (66–69)	1998–2000 Campaign: 96.4% 2000: School Campaign 96.6% 2006: ∼99% of children received MMR vaccine in Keddah. Delays in vaccination have been observed 2014: MMR Campaign for children in 1st grade (6/7 years) (67–69)	Not Available	3: 2015 14: 2016 47: 2017 118: 2018 (19)
South Korea	1981	1981–1997: Urabe AM9 (B) 1997–2000: Rubini (A) 2000-present: JL (A)	1980: MuCV introduced 1985: MMR vaccine included in NIP 1997: MMR 1: 12–15 MThs; MMR 2: 4–6 years. 2001: MMR mandatory for school entrance (70)	Two-dose MMR VC more than 95% among pre-school children in Korea (70). Increase in mumps cases attributed to Rubini strain (71, 72)	I/97-01; H/98-01, 07-10, F/07-10 (until 2013) (18), H and I (71)	17022: 2013 1121: 2017 19237: 2018 (19)

(Continued)

TABLE 1 | Continued

Country	Vaccine introduced	Vaccines (strains)	Vaccination schedule	Approximate vaccine coverage (VC)	Circulating strains	Reported cases/Year
Turkey	1970's	MMR (Kizamik Kizamikçik Kabakulak (KKK): JL (A) (73, 74)	1970's-1987: As part of NIP. MMR dose 1: Eight MThs; MMR 2: 15 MThs 1987–1998: MMR 1: Nine MThs 2006–present: MMR 1: 9–12 MThs MMR 2: 6 years (compulsory, free) (73, 74)	MMR used to eliminate Measles and rubella. 2013–2016: VC for MMR 1: ∼97% VC for MMR 2: 90.5% (75)	Genotype H (2006–2007 winter season) (76) H/05-07 (until 2013) (18)	597: 2013 457: 2014 322: 2015 544: 2016 419: 2017 464: 2018 (19)
United Kingdom	1988 (77)	1988–1992: Urabe (B) (withdrawn) 1992–1998: JL (A) 1998-present: RIT-4385 (A) (77).	MMR 1: 12–13 MThs MMR 2: From 40 MThs (78)	2013–2017: MMR 1: ∼92.6%. MMR 2: 88.6% (75, 79) 2017–2018 (at 5 years): MuCV 1: 94.9%; MuCV 2: 87.2% 2019 (at 5 years): MuCV 1: 94.5%; MuCV 2: 86.4% (80)	B/89, 90; C/75, 80s, 90, 98-00, 04, 06; D/96, 97, 99, 01-04; F/99; G/96-13; H/88, 95-96, 98, 00-04; K/99, 02; J/97, 03-06 (until 2013) (18, 81)	4718: 2013 2958: 2014 1008: 2015 974: 2016 2360: 2017 1398: 2018 (19)
United States	1967	JL (A) (82)	1967: MuCV introduced 1977: MuCV advised for >12 MThs 1989: Second MMR at 4–6 years. Current MMR/MMRV: MMR 1: 12–15 MThs MMR 2: 4–6 years. (82, 83)	2013–2017: VC for ≥1 dose MMR: ∼91.9%. (19–35 MThs) 2017–2018: VC for two doses MuCV 3 6/7 years: ∼ 94.3%. However, MuCV exemption increased to 2.2% (84, 85)	A/45, 50, 63-91; C/08-10; D/09; G/06-10; K/70s, 07, 08, 10; H/88, 06–10 (up until 2013) (18)	584: 2013 1223: 2014 1308: 2015 6369: 2016 6109: 2017 (19)

NIP, National immunization program; EPI, Extended Program of Immunization; VC, Vaccine Coverage; MuCV, mumps containing vaccine; MMR1, measles mumps rubella dose 1; MMR2, measles mumps rubella dose 2; JL, Jeryl Lynn (Genotype A).

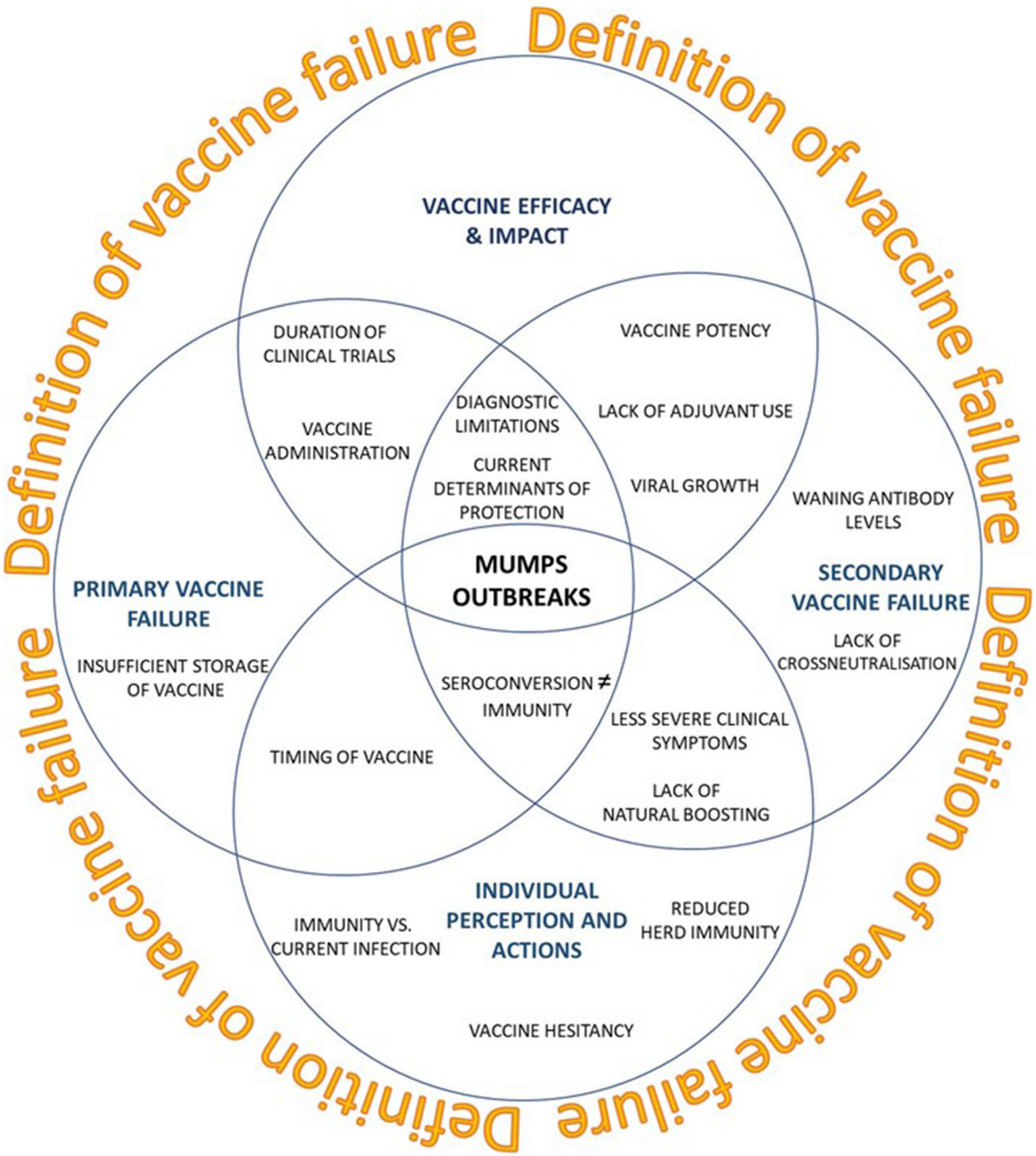

FIGURE 1 | Current perspectives on recent mumps outbreaks seen in vaccinated populations (blue circles). How impactful a vaccine is defined may lead to a paradigm shift in what constitutes an effective vaccine.

Heterogeneity of immunization coverage in specific populations or geographic locations of susceptibility is also becoming an important epidemiological issue in maintaining proficient population immunity for mumps (3, 109, 122). The WHO recommends a >95% MMR vaccine coverage for herd immunity. Maintenance of such coverage is well-demonstrated in Finland, where a country-wide 2-dose MMR vaccination program initiated in the 1980's eliminated measles, mumps and rubella within 25 years (123, 124). Recent publications from around the world indicate that the level of MMR vaccine uptake is far lower than what is recommended [reviewed in Ramanathan et al. (125)] (101, 126–129). Of the G20 nations that implement a mumps vaccine within their vaccination schedule, only 3 countries have maintained vaccine coverage levels of >95% (**Table 1**). However, poor uptake/incomplete vaccination alone may not be the only issue relating to mumps outbreaks. In the

Netherlands, mumps outbreaks still occurred with an overall herd immunity threshold of 86–92%, and where 96 and 93% received the first and second MMR at 14 months and 9 years, respectively (125, 130).

FACTORS FACILITATING CURRENT MUMPS OUTBREAKS IN HIGHLY VACCINATED POPULATIONS

The Changing Criteria of Mumps Diagnosis

The clinical presentation of mumps is pathognomic (bi-lateral parotitis); therefore supporting laboratory diagnosis was rarely employed in the past. As the classical symptoms of mumps are not always typical, there may have been a significant number of individuals in the past who may have been infected but were not identified as such. When mumps vaccination was introduced in 1967, the criteria the vaccine had to meet was the proof that it was clinically effective, i.e., that it reduced the risk of disease in vaccinated individuals in real-world conditions over a set period. Such an example was seen the USA; the reported cases (i.e., diagnosis of clinical symptoms) of mumps declined from >100 cases per 100,000 population before 1967 (pre-vaccine era) to 10 cases per 100,000 population in 1977, a reduction of 99% (105, 110, 126, 131). To note, clinical efficacy was probably based upon the reduction of the "classical bilateral presentation" rather than the milder mumps presentation. Therefore, one could argue that the original vaccine efficacy for clinical manifestations was over estimated.

Currently the laboratory diagnosis of mumps infection in Ireland is based upon two approaches: detection of mumps RNA by reverse transcriptase PCR (RT-PCR) in a buccal swab containing saliva, throat swab or urine specimen, and serological detection of immunoglobulin M (IgM) using a capture assay (132, 133). Both approaches for diagnosis are impacted significantly by the quality and timing of sample collection post-onset of symptoms and also if the subject is mumps naïve or had received mumps containing vaccine (87, 126, 134, 135).

There are challenges in using standard serological laboratory diagnostic methods to reliably confirm mumps re-infection of individuals who had been previously naturally infected or vaccinated (130, 136). Briefly, vaccinated individuals re-infected with MuV may only generate a weak or undetectable IgM response (133). Although a rise in IgG titer may also not occur in vaccinated individuals (87, 137), numerous studies have documented a rapid, variable increase in mumps-specific IgG levels, with neutralization antibody concentrations present up to 10 months post-infection (130, 138, 139).

Therefore, Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) is recommended (133, 140), and was formally introduced in 2015 as the principle diagnostic tool in Ireland to detect mumps in oral fluids (141). RT-PCR can identify current mumps infection more effectively in vaccinated individuals than serological techniques alone as it identifies the presence of the MuV vs. the immunological response (IgG, IgM), and has been previously shown to 100% correlate with viral culture results (140, 141).

The case numbers of more recent mumps outbreaks should always be assessed with this question in mind; are the number of mumps cases increasing, or/and are we better at diagnosing an acute infection? The latter seems to be the most probable, as many individuals who are being tested do not present with classical symptoms. In addition to enhanced surveillance of mumps cases, further optimizations of technologies are also occurring; the utilization of next-generation sequencing demonstrated that by editing one 2-fold degenerate nucleotide in the forward primer and three 2-fold degenerate nucleotides in the probe sequence optimized the fluorescence intensity and clinical sensitivity of the real-time RT-PCR when compared to the CDC-developed and WHO-recommended RT-PCR target [(NP) gene] leading to ∼11% increase in clinical sensitivity (i.e., Ct values that were ∼3.7 cycles lower) (142).

Are Primary and Secondary Vaccine Failures Implicated?

Much is not known about the immunological response to the mumps vaccine strain. However, a number of young adults who were vaccinated as children over the last two decades have demonstrated an increased risk of MuV infection with time, which is assumed to be related to a decline of antibodies to sub-protective levels of immunity (40, 101, 125, 128, 143–146).

Primary Vaccine Failure

Primary vaccine failure is defined as the lack of a sufficient initial antibody response to a vaccine in a recipient resulting in a lack of protective immune responses (6, 147). Although this type of vaccine failure may be because of improper storage/handling or administration of the vaccine, impacting its efficacy, it may also be due to the initial immunological response of an individual to the vaccine, which is usually quantified by the presence of antibodies that should be detectable in the weeks following vaccination. Primary vaccine failure was attributed to primary-school outbreaks of both mumps and measles in Ireland, which subsequently resulted in reducing the age for the second dose of MMR2 vaccine from 10–14 years in 1999 to 4–5 years of age (6). With the cyclical outbreaks occurring, it has been proposed that primary vaccine failure could again be a factor.

How is a response to a vaccine determined? In pre-licensure studies of the JL and Urabe mumps vaccines, high seroconversion and low failure rates were observed in children after the first vaccine dose (>90 and 5.5%, respectively), demonstrating that the vaccine induced an antibody response (148–153). A more recent study by Ong et al. demonstrated that a ≥2-fold increase in mumps antibodies 30-days post-vaccination was considered to be an adequate response of immunity (154). Vaccine effectiveness (i.e., seroconversion post-vaccination) of 2 vaccine doses has only been conducted on the JL strain; 6 studies provided a median vaccine efficacy of 88%. These studies have shown that 2 doses of MMR were more effective (but not statistically significant) than a single MMR dose to combat the incidence of mumps infection (101, 126, 145, 151, 152, 155).

Mumps-specific antibodies have been detected 1–2 years post-vaccination and without substantial decline for 8 years after mumps vaccination, with the immunogenicity and efficacy of the MMR vaccine showing comparable immunogenicity levels to post-vaccination levels at 3 years (148, 156). However, most studies of this vaccine (involving either a mumps-specific vaccine or a combined vaccine) only followed-up to 30–56 days post-vaccination (157–167). Despite few follow-up studies estimating post-vaccination antibody titers specific to the vaccine mumps strain, the evidence of seroconversion post-vaccination in a number of studies indicate that primary vaccine failure does not seem to be a significant contributor to the outbreaks that have been recently observed (118, 149, 150, 152, 158, 168–171).

It has been noted that a small percentage of the population do not seroconvert post-vaccination; <1% who received the MMR vaccine were seronegative 4–9 years after the first dose of MMR ($n = 616$) (143). Poor immune responses to primary vaccination has been shown to be a good indicator of infection susceptibility (172). This is in agreement with the correlation of pre-outbreak JL virus neutralization titres and ELISA results being significantly lower in individuals who became infected compared to non-infected individuals (173). Further studies of these individuals may provide insights of which immunological process are integral to develop immunity.

Secondary Vaccine Failure

The current methods used to determine immunity against mumps cannot discriminate between primary and secondary vaccine failure; only the timing of these tests can assess whether an individual ever mounted an immune response post-vaccination or whether the response is detectable years post-vaccination. Primary vaccine failure encompasses the failure to mount an immune response to a dose of a vaccine, secondary vaccine failure refers to a more gradual loss of immunity after a successful initial response that occurs over a number of years post-vaccination (174). Several factors have been proposed to be implicated with secondary vaccine failure, such as waning immunity, a lack of cross-neutralization, and natural boosting.

Waning Immunity

Waning immunity is defined as a decline in immunological protection proportional to time since vaccination. Potential waning immunity has been documented in the current mumps outbreaks seen in Europe and the USA, mostly affecting young adults within highly vaccinated populations attending tertiary education who have received two doses of the MMR vaccine in early childhood (40, 110, 126, 144, 145, 175–181).

A number of studies from the USA, where a JL vaccine has been used since 1971 have demonstrated waning immunity within the population. The risk of developing clinical mumps was shown to increase by 10–27% for every year post-MMR vaccination (125), with the rates of mumps infection rising from 1.6 cases per 1,000 in those who received the second dose of the vaccine within 2 years of the outbreak, to 11.3 cases per 1,000 in those who received it over 13 years prior. Using a mathematical model with analytical limitations, a recent meta-analysis of six studies estimated that vaccine-derived immune protection to MuV wanes about 27 years post-vaccination (182). Kennedy et al. (183) also demonstrated a decrease of ∼20% in mumps neutralizing antibody titers over 10 years.

In contrast, other studies appear to contradict, these findings, showing no link between mumps protection and time elapsed following administration of mumps vaccine (138, 148, 149, 184, 185). LeBaron et al. (143) and Gothefors et al. (186) demonstrated that 70–99% of individuals still had detectable anti-mumps antibodies ∼10 years after initial vaccination. Cohen et al. (101) also demonstrated minimal antibody level decline after two MMR doses 6–7 years after second vaccination. Neutralizing antibodies against the JL-5 vaccine strain has also been detected in ∼80% for age groups 2–20 years, 67% for age group 24–26 years; and 77% for age group 50+ years (187).

Implementation of a third dose of the MMR vaccine has been shown to be effective as a stop gap measure in limiting disease spread in outbreak settings situations (129). Individuals vaccinated for the third time had a 78% lower risk of contracting mumps, with a decreased attack rate of 6.7 vs. 14.5 cases per 1,000 when compared to those who received a second dose. More than 50% of those who received a third dose of the MMR vaccine showed a 4-fold increase in mumps antibody titers (105, 106, 168, 188). An increase in mumps IgG humoral immunity was also observed post-vaccine administration. However, this immunity boost has been shown to be a transient effect, with mumps antibody titers returning to pre-third dose of mumps-vaccination levels 1 year after vaccination.

Therefore, as waning immunity is thought to be an important factor facilitating mumps outbreaks, the emphasis placed on the quantity/quality of mumps-specific antibodies may need to be re-assessed. It is yet undetermined if the total loss of detectable antibodies correlates to a loss of clinical protection, as the minimal level of neutralizing antibody required for protection against mumps has not yet been defined (184).

Cross-Neutralization

Antigenic variation and thus reduced cross-neutralization between the vaccine and circulating strains of different MuV genotypes have been cited as possible explanations for mumps outbreaks in highly vaccinated populations (125, 184, 189–191). Recent outbreaks in Europe and Northern America (including Ireland) have shown the circulating MuV during the current outbreaks to be genotype G (135, 184, 192, 193). This MuV genotype was first identified in 1996, and has demonstrated intra-genotype diversity of up to 7% (**Table 1**) (6, 134).

The JL vaccine strain (genotype A), differs phylogenetically to the circulating MuV (genotype G) (125). *In vitro* studies of the genotypic distribution and temporal shift of MuV suggest that cross neutralization between wild type and vaccine genotypes may be approximately half the concentration measured against the vaccine strain (130). Pre-infection neutralization titers in mumps positive cases were also significantly lower against genotype G vs. mumps vaccine strain, potentially due to amino acid differences in B-cell epitopes and/or N-linked glycosylation sites on the HN and also within the F protein (194). Santak et al. (195, 196) also demonstrated that conformational changes within the F protein may lead to immunological escape.

Despite the decline/scarcity of cross-neutralizing antibodies, different mumps vaccines used worldwide have been shown to prevent significant clinical mumps infection during outbreaks (101, 197). Dependent on the strain, a 2–16-fold variation of patient sample titers has been shown to be protective in *in vitro* plaque reduction neutralizations (149, 151, 198). Although the sera of one of these studies, was collected only 6 weeks after MMR vaccination, a time point that may not signify the concept of waning immunity and antigenic differences, several other groups have shown that the most divergent strains of MuV can be neutralized *in vitro* with only slight variations in titers, supporting the concept that MuV is serotypically monotypic (184, 190, 195, 198). Epitopes of the MuV that are presented to CD8+ T-cells have been shown to be present in not only the circulating strains of virus but also in a number of vaccine strains (199). In addition, Lewnard et al. (182) also found no evidence that recent mumps outbreaks were due to the emergence of MuV strains escaping vaccine-driven immunological pressure.

Therefore, the limited data does not suggest that antigenic drift of the MuV leading to diminished neutralization capacity of the vaccine strain could fully explain the recent outbreaks (125). Further studies into the cross-neutralizing capacity of the mumps vaccine strain administered 15–20 years previously to the current circulating strain of MuV in countries where outbreaks are being observed will allow better deductions to be made. It is possible that differences in the neutralization capacity of vaccine-induced antibodies against different MuV strains may be more significant when levels of neutralizing antibody are low and become "overwhelmed" when the mumps viral load challenge is high (200).

Natural Boosting

Several prominent MMR/mumps vaccine studies were undertaken at a time when there was still a high prevalence of circulating wild type virus, which enabled sub-clinical boosting to occur in an individual. Such natural boosting is illustrated in Belarus, where a subpopulation of vaccinated individuals only had a small amount of their overall mumps IgG antibody levels specific to the vaccine-strain (201). Neutralization antibodies against Iowa-G/USA06 (the circulating wild type virus) were also present in pre-infection plasma of all mumps cases during a recent outbreak in the US (173). This indicates that the mumps vaccine alone is not solely responsible for the high levels of mumps antibodies (202), and that long-term antibody persistence or protective efficacy data of the vaccines used may not truly reflect the current circumstance of viral transmission/circulating within a highly vaccinated population (99).

Herd immunity increases the chance for natural mumps boosting for an individual is at a minimum, reducing the potential of the frequency of mumps outbreaks (123, 124, 184). With less opportunity for subclinical boosting (asymptomatic response to the circulating virus), the impact of other elements of waning immunity may play an increasingly critical role in the re-emergence of mumps outbreaks (98, 171). Additionally, as the heterogeneous uptake of vaccines in this modern era is leading to susceptible individuals within the community, future work will need to encompass genotyping of circulating MuV to examine how impactful subclinical boosting was on early measures of vaccine efficacy in current populations.

LABORATORY DETERMINANTS OF AN EFFECTIVE IMMUNE RESPONSE TO MUMPS VACCINE

Why Do We Consider Antibodies to Be the Best Measurement of Vaccine Efficacy?

The evolution of an individual's immune response differs between natural infection and vaccination, in particular the difference in the affinity and specificity of an immunological marker such as antibodies (203).

True correlates of mumps immunity after vaccination have been poorly characterized; to date, there are no reliable correlates of protection from either symptomatic mumps infection (clinical immunity), or individuals previously exposed to MuV (204). Therefore, a serological surrogate/ substitute is used (205). Mumps vaccine efficacy is quantified by a single measure, IgG which may not suffice to evaluate the magnitude of the actual humoral response. Borgmann et al. (206) proposed an increase in mumps-specific IgG titer in sera as a diagnostic criteria of mumps reinfection (206). It has been suggested that vaccinated individuals have modified B-cell responses to MuV that allow for the rapid generation of IgG antibodies and a blunted or absent IgM response (207, 208). In addition, emerging data in Simian Immunodeficiency Virus studies suggests that not all antibody responses are equal, and qualitative features of antibodies may be key to defining protective immune profiles (209).

Despite its use, the correlation to mumps-specific IgG concentrations and neutralization titers against the JL virus is poor, suggesting that IgG concentrations do not adequately represent a sufficient surrogate correlate of protection (194). This is demonstrated in Finland; only 24% of vaccinees had no detectable mumps antibodies after 21 years (123, 124). Data from the European Sero-Epidemiology Network (ESEN2) project in 2004 reported that MMR immunization uptake in Ireland in 2004 was 92% (6), however it was also suggested that only 80–85% of 15- to 24-year-olds in Ireland had detectable antibodies to MuV by either natural immunity or immunization (210). In 2011, vaccine coverage of medical students in Germany was reported to be 75.1% (211). In children between the ages of 1–17 years, where 88.8% had been vaccinated with the MMR vaccine at least once, only 76.8% showed prevalence of antibodies (212). However, 7.8% showed a prevalence of antibodies to measles and rubella in the absence of mumps-specific antibodies. Therefore, previous measurement of anti-mumps-specific IgG that represented immunity induced by the mumps vaccine appears to be overestimated (99, 213).

Antibody levels of other components of the MMR vaccine have seen similar trends. Waning rubella antibody titers have been observed, despite the number of acute rubella and congenital rubella syndrome cases not increasing. It has also been shown that college students who received rubella vaccination during childhood and had low/no antibody response were able

to mount a secondary response when challenged with rubella indicating that an individual's low antibody levels are not always indicative of susceptibility to infection (214). Measles antibodies can also be detected for up to a decade post-vaccination, with >90% of individuals still measles IgG positive at 6–7 years of age (144, 215). However, as with mumps and rubella, waning measles antibody titers have been observed (143, 216). Despite this, a recent longitudinal study of up to 10 years demonstrates how effective the MMR vaccine has been in preventing diagnosed measles cases during the 1990's/2000's (217).

Similarly, three doses of the Hepatitis B (HBV) vaccine in a cohort of Alaskan natives showed >95% seroconversion in children and young adult post-vaccination and provided long term and durable protection against chronic HBV infection. Although no increase of HBV prevalence were observed 51% individuals had low to undetectable antibody levels after 30 years.

These observations suggest that an individual's antibody levels do not indicate susceptibility to infection, that either an antibody titer lower than recommended guidelines is still protective, or/and is an ineffective surrogate of protection. This is emphasized in a study by Amanna et al.; (218) responses to non-replicating protein antigens (tetanus and diphtheria) were shown to have approximate antibody half-lives of 11–19 years. In comparison, antibodies following wild type infection were shown to have half-lives of 50 years or more which was thought until recently to confer a more prolonged lifelong protection (214, 218, 219). However, reinfections observed in individuals that were previously naturally infected have demonstrated that the quantitative measurement of antibodies do not indicate sterile immunity (220).

It is also important to stress that seroconversion rates due to immunization/natural infection only reflects a change of antibody status from negative to positive, but not necessarily the intensity of antibody response. In addition, there is no consistency in the timing of sample collected post-vaccination to test vaccine efficacy, and between the serological tests utilized for detecting mumps antibodies. As a result, documented seroconversion rates of the mumps vaccines used vary widely (JL: 74–100%, RIT 4385 strain: 88–98%, Urabe Am 9: 79–100%, Rubini: 35–95%).

This highlights that the assays used to detect immunity to MuV may not always detect an adequate post-vaccination response. Only a small number of serological commercial assays such as the detection of Hepatitis B surface antibody (anti-HBs) (221) and rubella IgG (222) have been designed using WHO reference material as a standard for quantification. However, even utilizing this reference standard demonstrates significant differences in the determined quantification of either anti-HBs or rubella IgG depending on the assays used; although a value for anti-HBs of 10 IU/ml is regarded as protective against significant HBV infection, the detection of this anti-HBs is significantly influenced by which anti-HBs assays is used (223–227). Therefore, it is possible that the current assays/tests mechanisms utilized to measure mumps antibodies are too insensitive/inappropriate/crude to identify nuances in the immune response which could correlate with immunity against mumps. In addition, variation within neutralization epitopes i.e., the quality of the antibody present could be a more important correlate than quantity (190, 198).

Are There Better Correlates of Protection?

Though labor-intensive, neutralizing antibodies are considered to be a better correlate of mumps immunity. Antibodies against the haemagglutinin-neuraminidase protein (HN) and nucleoprotein (NP) have been shown to neutralize MuV, however, repeated attempts to define a titer that provides a protective threshold titer have been inconclusive (203, 228). In older studies, during field evaluations of the JL vaccine, neutralizing antibody titers of 1:2–1:4 in unvaccinated individuals was considered seropositive and protective from mumps infection (149, 151, 152). Using a more contemporary wild-type isolate (Iowa-G/USA06), a 1:8 neutralizing titer cut off was defined between case patients and exposed patients, despite the fact that no cut-off could fully discern between the two groups (173). However, that these results are dependent on the challenge virus strain used in the assay. Rasheed et al. demonstrated a 6-fold lower neutralization titer to the G-genotype when compared to the JL vaccine strain in 18–23 year olds (229). This has also been seen between mumps vaccine strains vs. circulating strains in India and China (47, 197). Despite studies in more highly vaccinated populations demonstrating that HN-inhibiting titers after natural disease were 1:9 compared to 1:5 post-vaccination, neither appeared to prevent reinfection (173, 218–220, 230). There is increasing evidence that the mumps-specific antibody response is broader than neutralization alone (112). Avidity testing for virus-specific IgG has been proposed (3, 220, 229).

Is Lymphoproliferative Immunity a Better Correlate of Protection?

Individuals who lack measurable mumps-specific antibody levels may be susceptible to infection but protected from significant illness as they may be protected by cell-mediated immune memory. Prolonged T-cell responses are reported after other vaccinations; 14–16 years after a single dose of the rubella vaccine RA27/3, a T-cell proliferative response to neutralizing antibody-inducing peptides suggest T helper and B-cell interactions. This indicates that full vaccine effectiveness could be dependent on mounting both an antibody and cell-mediated immune response (214).

Although cell mediated immunity has not been as well-assessed in mumps infection, a lymphoproliferative response was induced in infants vaccinated at 6, 9, or 12 months of age was induced (231) with antigen-specific T-cells reported to appear within 1 month of infection (183). Lymphoproliferative responses to measles and mumps vaccine viruses were shown to persist in two thirds of the population at least 6 years after immunization (232), with T- and B-cell immunity persisting for 10 years post-immunization (202).

Low levels of mumps-specific memory B-cells have also been documented suggesting that mumps infection or vaccination may not generate a robust B-cell memory (136, 233). Two principal mechanisms for maintaining long-term humoral immunity have been proposed and reviewed by Amanna et al. (218): associations between memory B-cell levels and

antibody may reflect an epiphenomenon in which serum antibody levels and memory B-cells are equally stable but independently maintained. If memory B-cells and plasma cells are independently regulated, then multiple re-exposures to antigens may cause divergence between memory B-cell levels and antibody levels (218). Antigens with the highest rates of boosting through vaccination or latent viral infection coincidentally showed the weakest association between memory B-cell titers and antibody titers (234).

Although the role and efficacy of T-cell immunity to mumps infection is unclear, there is a possibility that certain MuV strains may be capable of escaping vaccine induced T-cell responses, which may not be considered of significance until B-cell waning immunity comes into play (198). In individuals who did not respond to vaccination (i.e., had a $\leq$2-fold of mumps antibody titers 30 days post-vaccination), several genes including those implicated in antigen presenting, processing, T-cell response and function showed significantly increased expression, with MHC Class II HLA-DRB3 and HLA-DRA, and CD86 induced when compared to responders 1 day post-MMR vaccination. This may indicate that the stimulation of a rapid adaptive immune response limits antigenic presentation and hence prevent the differentiation of memory B-cells to antibody-producing plasma cells (154).

Differences in predicted B-cell and T-cell epitopes between JL5 vaccine strain and other vaccine strains may also be implicated in the outbreaks witnessed (235). Although, it has also been shown that natural mumps infection or vaccination do not always induce both cellular and humoral immunity. de Wit et al. (199, 236, 237) has shown the presence of Th1-type $CD4^+$ T-cells recognizing a MuV epitope in a HLR-DR restricted manner. In addition, the response of IFN-γ and TNF producing $CD8^+$ T-cells specific to MuV epitopes are lower in vaccinated individuals when compared to individuals who were naturally infected (199, 213, 236–238). Utilizing current knowledge and new technologies may help define a better surrogate correlate of protection and potentially determine a cut-off between the immunity of a vaccinated individual and a secondary mumps infection. This may potentially move the diagnostic preference from serological tests to more comprehensive functional assays.

Why Vaccinate If You Cannot Define Protection?

Despite the large resurgence of mumps outbreaks, there is insurmountable evidence highlighting the benefit of the mumps vaccine (**Table 2**). Routine childhood MMR vaccination has resulted in a dramatic decrease in the incidence of mumps cases, and has shifted the peak age-specific attack rates from a young children (manifesting between 5 and 15 years) to one that affects young adults, in particular those who have close interaction with other young adults (18–24 years) (6, 110). Additionally, clinical manifestations and severity of disease in vaccinated vs. unvaccinated individuals differ (129, 248). Although MuV can be clinically asymptomatic in about 15–30% of those who become infected, the vaccine against mumps confers protection in a dose response manner; unvaccinated individuals saw an attack rate of 31.8–42.9%, whilst one dose and two doses of the JL vaccine were 4–13.6% and 2.2–3.6%, respectively (135, 219, 246).

TABLE 2 | Differences between Mumps vaccinated and unvaccinated persons.

	Vaccinated	Not vaccinated
Symptoms (7, 101, 239, 240)	Milder	Severe
Transmission (197, 241, 242)	Low	High
Mumps viral load and replication (243–245)	Low	High
Mumps isolation rates (135, 239)	Low	High
Duration of viral shedding (244)	Shorter	Lasts Longer
Asymptomatic infection (135, 246, 247)	66%	15–40%

Despite evidence of mumps infection in a vaccinated population, there is evidence to suggest a less severe clinical manifestation of the viral infection.

Based on the reduction seen upon the introduction of a mumps vaccine, it has been proposed that MMR vaccination also prevents the transmission of the virus. There is limited knowledge regarding the shedding and transmission of MuV, but it is thought that close contact and transmission of a certain viral load may induce clinical symptoms (243, 246, 249). Modeling data suggests that infectious MuV shedding decreases rapidly after the onset of symptoms, however 8–15% are patients are thought to still be virally shedding 5 days after the onset of symptoms (244). This could be the reason why the transmission of MuV can be exacerbated by close social situations within a heterogeneously vaccinated population. Outbreaks generally occur in situations of intense contact such as college dormitories, boarding schools, and youth summer camps (191), with up to a third reporting some contact with a mumps case (105).

Evidence of lower levels of viral replication also suggests a clinical benefit of the vaccine (243, 244). Viral load and presence of the mumps vaccine genome in areas of viral replication was lower in vaccinated individuals vs. unvaccinated individuals (243). In addition, patients who contracted mumps but had two doses of MMR have been shown to shed less MuV in their urine, with fewer experiencing bilateral parotitis or orchitis than unvaccinated individuals (239), This suggests that immunity induced by MMR vaccination limits virus transmission and complications (241, 242).

It should be noted also that individuals who received two doses of MMR, and had a positive correlation between viremia, salivary viral loads and systematic clinical mumps infection may have an increased risk of transmitting virus. These individuals also lacked mature functional responses, with low neutralizing antibody titers and avidity indexes (239).

Overall, evidence demonstrates a clinical advantage to receiving a mumps vaccine (**Table 2**). Currently no global consensus exists for the measurement of mumps antibodies, mumps avidity or neutralizing titers that correlate to vaccine response and protection in healthy individuals. If a biomarker is discovered, it could be utilized as an international diagnostic reference standard to allow global harmonization and evaluation of the relative effectiveness of the different vaccination programs worldwide. Such an attempt was conducted by Andrews et al. (250), who reported on the European Sero-Epidemiology Network project which was established to harmonize the

seroepidemiology of five vaccine preventable infections including measles, mumps, and rubella in eight European countries. The study concluded that the development of an international standard for mumps would help in the standardization and comparability of mumps antibodies in the different enzyme immunoassays used in laboratories. However, to date, no international reference standard for mumps has been established.

Can Improvements to Vaccines Be Made?

In response to infection, the human immune system launches a series of immunological responses with the goal of controlling or eliminating the pathogen. If the pathogen circumvents the frontline defense of the innate immune system, an adaptive immune response specific for the pathogen will become activated to respond, with the intention to generate humoral- and cell-mediated immunity. Humoral immunity, represented by antibodies secreted by B-cells are not effective against pathogens that invade host cells. Therefore, cell-mediated immunity instructed by the innate immune system are additionally necessary and consist of B-cells and T-cells. The unique compositions of the B-cell receptor and T-cell receptors specific for the invading pathogen proliferate and gain effector functions based on the antigen fragments presented on antigen presenting cell by MHC class II molecules. The activated Th-cell produces cytokines, resulting in the activation of macrophages (Th1 help), B-cells (Th2 help, called plasma cells), or cytotoxic T-cells. While most plasma cells, produce and secrete large amounts of antibodies, some differentiate into memory cells [reviewed in (251, 252)].

Vaccination aims to stimulate the host immunological process and formation of cell-mediated immunological memory via the use of live-attenuated or of inactivated/subunit vaccine components to promote a cell-mediated immune response. Extensive knowledge gaps significantly hinder improvements to the mumps vaccine and prospects for mumps eradication and maintaining proficient population immunity (3, 122, 187). Few studies have collected data that examines different aspects of mumps immunity and are limited in their predictive value for future outbreaks (253). For example, the importance of T and B-cell responses in protective mumps immunity and how memory/plasma cell numbers are homeostatically maintained post-infection or vaccination is relatively unknown (252). It should be acknowledged that the mechanism of protection of infection may not be the same mechanism of recovery from infection, which may make the identification of a common correlate of protection and recovery difficult (203). Therefore, if a correlate or surrogate correlate is unobtainable to define an individual's protection to mumps, should we re-consider and re-focus efforts on optimizing the vaccine using available historical clinical and trial data?

Administration

It has been suggested that wild-type infection could confer a "better quality," broader and prolonged immuno-activation than vaccine-induced immunity. This is reflected in mean neutralizing antibody titers detected post-mumps vaccination, which were over five times lower than those detected following wild type infection. Similarly, hemagglutination-inhibiting titers after natural disease were 1:9 compared to 1:5 post-vaccination (214, 218, 219).

The use of a live-attenuated virus vaccine is intended to mimic immunological reactions and responses between the host and wild type virus (254). The current live-attenuated MMR vaccine is intramuscularly injected, a route that significantly differs from the natural infection mode of transmission. However, emphasized by differing immunological kinetics between immunized and naturally infected individuals when subjected to wild type pathogens, injectable vaccines are considered not to be the best inducer of antigen-specific mucosal immune responses for mucosal pathogens, especially if the mode of administration is not the natural route (the respiratory tract) (255, 256). Improvements on a broader range of antigen delivery systems will improve vaccination strategies and potentially prolong the effect of a vaccination by producing a localized immunological response in the relevant tissues (257, 258).

Mucosal vaccines such as intra-nasal vaccination have advantages over traditional injectable vaccines as they can induce an effective, more robust immune response without any physical discomfort and more closely replicate the natural route of infection for mumps (255, 259). B-cells induced by the mucosal response are also capable of secreting IgA class of antibodies in the lumen, where the interaction and neutralization of specific antigens form IgA-antigen complexes are easily able to be entrapped in the mucus and eliminated by cilial epithelial cells (259). Activated mucosal lymphocytes can also reach other mucosal sites via the lymphatic system and have the capability to transfer immunity (260).

Such an example is the intranasal immunization of inactivated influenza. With a 70–90% similar efficacy between the injectable and intranasal influenza in healthy individuals this intranasal vaccine can elicit the secretion of haemagglutinin and neuraminidase specific IgA antibodies in the upper respiratory tract, and corresponding IgG antibodies (258). Live, cold adapted attenuated nasal influenza vaccine has been routinely used in Russia for over 50 years (261). Other liquid live-attenuated intranasal vaccines are available; "Nasovac®" in India, and "FluMist®" in the US, UK and New Zealand (258, 259, 262).

Development of Improved Vaccines

Inactivated vaccines consisting of heat/chemical or live-attenuating monovalent or multivalent pathogens in animals/cell lines were developed to protect against disease causing microorganisms (263). Less emphasis was placed on understanding the mechanisms related to conferring immunological memory; the focus lay on the availability, mass production and administration of the vaccine to introduce herd immunity into populations (264).

Currently, the least expensive and time effective method to licensure is the comparison of serologic responses of the new vaccine to an existing licensed vaccine, which can lead to a bias on the development of novel vaccines (222). This methodology also does not account for the fact that each vaccine developed elicits

its own immunological signature and may need to be considered on an individual basis (265).

Raymond et al. (266) has suggested that embryonated chicken egg-based vaccines may induce antibodies that are more preferential to egg adapted strains better than wild type virus. Amino acid substitutions/differences in key antigenic targets due to the passage of the growing virus within this environment may optimize the growth of the virus, but could lead to differences over time that could affect the immunogenicity or potency of the vaccine (172, 222, 267). The JL vaccine contains two isolates of the JL Strain (JL2 and JL5) and whilst no immunological differences have been documented, JL2 grows to higher titers than JL5 in embryonic eggs and also demonstrates significant sequence variability (94, 268). Zost et al. (269) also demonstrated that an egg selected mutation within a glycosylation site in the 2016–2017 influenza vaccine strain led to the production of poorer neutralizing antibodies to the vaccine strain compared to wild type influenza virus.

Vaccine RIT 4385 strain derived from one of the two distinct virus subtypes of the JL vaccine (JL5) showed comparable seroconversion rates despite inducing a significantly lower geometric mean antibody titer when compared to recipients of the JL vaccine, but does not have any longitudinal trials investigating its efficacy, even though there are populations who are currently receiving it (101, 270).

The significant time gap between pathogen emergence and vaccine licensure, could potentially lead to antigenic drift. There is potential that modern biotechnologies could be utilized to design novel vaccine platforms (251, 271, 272). Clinically derived recombinant MuV lacking the expression of the immunomodulatory V or SH protein are currently being investigated (273). In China, a vaccine consisting of the prevalent wildtype virus genotype (F) has recently been produced and is currently undergoing trials (269).

In addition, despite being extremely pleomorphic, utilizing MHC epitopes as potential B-cell and T-cell vaccine candidates are also being investigated (81, 274, 275). Vaccine design has involved the utilization and templating of epitopes that previously induced a B-or T-cell response during natural disease that are considered to be immunogenic enough to induce similar responses if administered in a vaccine. However, the appropriate B-cell and T-cell epitope/peptide candidates to induce a protective immunological response can be difficult to correctly identify and synthesize, as it may differ to the immunodominant epitope and host presentation of that antigen (251, 276). Prediction of MHC-peptide binding and cleavage has demonstrated mismatches in both vaccine T-cell and B-cell epitopes in vaccinated individuals highlighting small number of distinguishing amino acid changes of the JL5 major strain (235). The importance of understanding T- and B-cell responses and how antigen-specific memory cells numbers are homeostatically maintained post-infection is crucial to understand to ensure successful vaccine development (252, 277).

Since the 1990's, significant progress has also been made in developing flexible, amplifiable, scalable, inexpensive, and cold-chain free RNA vaccines, such as synthetic mRNA molecules encoding only the antigen of interest and self-amplifying RNA (sa-RNA) (264). Such examples include an experimental mRNA vaccine candidate (mRNA-1273) which encodes a stable form of the SARS-CoV-2 spike protein and has been accepted as a trial candidate for clinical trials in healthy male and female individuals (278, 279). In addition, sa-RNA viruses as gene delivery and vaccine vectors have also demonstrated therapeutic efficacy in a number of preclinical studies. In the context of influenza, sa-RNA vaccines have shown comparable results of protection at lower doses than mRNA vaccines (272, 280, 281).

Exponential developments in the "OMIC" area has enabled further vaccine development and understanding of the immunological response and challenges surrounding this area (282). Systems vaccinology, which includes immunoformatics, DNA/RNAseq, microarrays, mass spectrometry proteomics, transcriptomics, and metabolomics have all shown huge potential in elucidating differences in vaccine strains, vaccine growth and individual response in depth and on an epigenetic level allowing the identification of new vaccine antigens with increased speed and sensitivity (235, 263, 283–285).

Adjuvants, a group of biological and chemical compounds could also be considered to enhance and improve the longevity of the immune response of a vaccine such as the MMR. Adjuvants have been successful in significantly reducing overall antigen dose in vaccine formulations as well as alter and broaden the host response through epitope spreading and qualitatively shaping the effector function of antibodies through subclass selection (173, 286).

The re-purposing of live-attenuated vaccines as TIbV are also being investigated. Trained Immunity based Vaccines (TIbV) elicit heterologous protective effects by inducing a broader, lasting priming of innate immune cells, in addition to the intended specific immunological response and memory of conventional vaccines [reviewed in (287)]. MMR and BCG vaccines have been considered as potential TIbV in the context of the current coronavirus disease 2019 (COVID-19) pandemic (288), however further research is needed.

Potency of Virus

The mumps component of a vaccine is an unpurified product whose potency is measured through a biological assay for the substance rather than through evaluation of integrity of physical form (quantitative PCR after cell culture) (289). A monovalent mumps vaccine lot is used to characterize the performance of the mumps potency assay with international reference standards. Degradation products are neither identified nor quantified (290). Currently, the minimum potency of the mumps vaccine used varies between brands used [summarized by Su et al. (107)] (291). However, this potency measurement differs to other MMR vaccines strains previously used [reviewed in (10)]. In addition, the maximum required potency is not usually specified. Atrasheuskaya et al. (172) demonstrated that the four out of 14 lots of vaccine associated with six cases of viral transmission post-vaccination to previously vaccinated contacts were in fact twice as potent as the lots that were not associated with viral transmission post-vaccination (172, 292). This may impact the use and efficacy

of specific vaccines. Due to their neurovirulence and increased incidence of aseptic meningitis and mumps cases, the Urabe Am 9 and Rubini mumps vaccine strains were discontinued in many countries (87, 293, 294).

Comparing alternative culturing technologies and defining a viral potency range for vaccines could help reduce variability within the MMR vaccine (292). Ensuring the use of a reference sample that had similar replication rate and composition as the virus to be tested will allow accurate determination of the quantity of virus present per lot of vaccine. Investigating novel vaccine candidates shown to induce a similar quantity but qualitatively different antibodies will help segregate and reveal potential correlates of protection (209). Incorporating more modern technologies such as microarray technology or antibody pattern/profiling (rather than single antibody measures) to investigate biomarkers of neutralizing antibody response and/or correlates of protective immunity, in addition to incorporating what has been accomplished in Finland will allow further understanding of mumps immunity (123, 124, 173, 195, 196, 295).

Are the Current Perceptions of What Is Expected of a Vaccine Skewing the Overall Benefits It Elicits?

The efficacy of a vaccine is defined by disease prevention (sterile immunity, establishment of primary infection and shedding of mature virus particle), or complications associated with infection (orchitis, neurological issues etc.) (203). Despite the well-documented success of the global immunization programs demonstrating how vaccines significantly attenuate disease and onward transmission of infection, they are rarely totally efficacious (demonstrated in pre-licensure clinical trials) or effective (determined by practical use) (99, 173, 296).

Therefore, does "immunity" refer to sterile immunity or solely to protection from symptomatic infection? What defines an effective vaccine, or what constitutes vaccine failure? Does the medical profession and the "pro-vaccine" message contribute to the public skepticism regarding immunization? Is it time to shift the medical and public perception paradigm from "protection of infection following vaccination" to "protection from serious clinical mumps manifestation"?

The lack of definition leads to misinterpretation by health professionals and media of what is truly occurring. Such an example is currently observed with influenza; individuals who have recently being vaccinated against influenza and subsequently become infected with influenza, assume that the vaccine has "failed" even though there is a reduction in symptoms.

The current assertion that vaccines "protect against" or "eliminate" the risk of infection may contribute to the misperception about what level of protection a vaccine actually provides (vaccination efficacy) perpetuated by the witnessing of visible clinical disease and outbreaks despite vaccination (116, 297, 298). Therefore, definition and consensus of what is termed a true "vaccine failure" is required to inform both the clinical and public perception of what the function of a vaccine is. Deciding what the clinical endpoint of a vaccine is i.e., infection with mild clinical symptoms vs. natural infection/disease with its associated complications and assessing the impact of the vaccine in a heterogeneously vaccinated population will allow a better consensus of what is required.

A paradigm shift in what is considered to be a good vaccine i.e. one that provides protection against serious clinical sequalae, in addition to identifying a reliable laboratory marker for this protection is required (203). By focusing on, and acknowledging that vaccines may not prevent infection but will attenuate the clinical complications/consequences that arise from infection in addition to reducing onward transmission will provide a more realistic view of the benefits of vaccination (297). Immunity is therefore beneficial but does not necessarily mean protection.

DISCUSSION

If we can decide whether the end point of a vaccine is either the prevention of infection or protection against serious sequalae of infection, its efficacy and impact can be determined and will have enormous implications on how vaccine failure can be studied, quantified and interpreted. This teasing out of the immunological response to MuV will ultimately provide potential correlates with robust predictive power, suggest directions for further vaccine improvement, and enable the discovery of potential biomarkers to help create a more efficient diagnostic assay that can discern between different infectious diseases and vaccination vs. disease status. The identification and incorporation of a correlate into diagnostic protocols which can be widely accessible may potentially allow global harmonization of criteria defining immunological protection against mumps.

The medical and scientific field needs to inform the public more accurately about what a good vaccine consists of, which may result in a more positive attitude toward vaccines. In the majority of individuals, a vaccine can prevent serious clinical sequalae and associated complications following wild type infections, but also significantly reduce onwards transmission in particular to the cohorts who are not vaccinated due to a contraindication to vaccination. This is the positive and realistic view of vaccination which should be presented rather than the current flawed message of "get the vaccine and be protected from infection." The public deserves, and will appreciate, a more accurate and informed message.

AUTHOR CONTRIBUTIONS

AC, JC, and JH contributed to the conception and design of the review. AC wrote the first draft of the manuscript. JC, TL, and JH contributed to manuscript revision. All authors have read and approved the submitted version.

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β-Glucan as Trained Immunity-Based Adjuvants for Rabies Vaccines in Dogs

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The mechanisms of trained immunity have been extensively described *in vitro* and the beneficial effects are starting to be deciphered in *in vivo* settings. Prototypical compounds inducing trained immunity, such as β-glucans, act through epigenetic reprogramming and metabolic changes of innate immune cells. The recent advances in this field have opened new areas for the development of Trained immunity-based adjuvants (TIbAs). In this study, we assessed in dogs the potential immune training effects of β-glucans as well as their capacity to enhance the adaptive immune response of an inactivated rabies vaccine (Rabisin®). Injection of β-glucan from *Euglena gracilis* was performed 1 month before vaccination with Rabisin® supplemented or not with the same β-glucan used as adjuvant. Trained innate immunity parameters were assessed during the first month of the trial. The second phase of the study was focused on the ability of β-glucan to enhance adaptive immune responses measured by multiple immunological parameters. B and T-cell specific responses were monitored to evaluate the immunogenicity of the rabies vaccine adjuvanted with β-glucan or not. Our preliminary results support that adjuvantation of Rabisin® vaccine with β-glucan elicit a higher B-lymphocyte immune response, the prevailing factor of protection against rabies. β-glucan also tend to stimulate the T cell response as shown by the cytokine secretion profile of PBMCs re-stimulated *ex vivo.* Our data are providing new insights on the impact of trained immunity on the adaptive immune response to vaccines in dogs. The administration of β-glucan, 1 month before or simultaneously to Rabisin® vaccination give promising results for the generation of new TIbA candidates and their potential to provide increased immunogenicity of specific vaccines.

Keywords: trained immunity, rabies (canine), beta-glucan, innate immunity, adjuvants, adaptive immunity, Rabisin®, trained immunity-based vaccines (TIbV)

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INTRODUCTION

Host defense against infections relies on the several parts of immunity, composed of two arms: the innate and the adaptive immunity. The concept of adaptive immune memory is dependent on antigen-specific T and B-lymphocytes that are able to recognize a diverse array of pathogens in a highly specific manner, which is the foundation of vaccine functionality. However, the last years have seen an increasing amount of publications describing features of the memory of the innate immunity (1, 2). This memory enables a heightened response to secondary exposure to homologous as well as heterologous pathogens. Among the cells taking part in trained immunity, monocytes have been largely described while some studies support the role of NK cells and dendritic cells (3, 4). It was recently demonstrated that the monocytes can be trained with pathogens (*Candida albicans*) (5), vaccines (BCG) (6), or prototypical agonists like β-glucans initially purified from *C. albicans* (7), to induce deep epigenetic and metabolic modifications (8, 9). This leads to enhanced inflammatory cytokines (TNF-α, IL-6, IL1-β) secretion when the host encounters pathogens mimicked *in vitro* by LPS or *in vivo* in humans by yellow fever attenuated vaccine (10). We confirmed in an *in vitro* model of training of macrophages that the trained innate immunity is also present in other mammals like dogs with cellular mechanisms similar to those described in mice and humans (11).

The description of trained immunity has set new therapeutic goals, which are starting to be investigated in clinical settings (12, 13). A wide range of applications can be found for trained immunity from the use in fish to increase resistance to infection (14), to adjuvant strategies in human cancer therapy (15). Trained immunity-based protection has been theorized and later assessed in mice, against bacterial infections (16), and in humans with a model of yellow fever vaccination (10), both with conclusive results. Combining TI-based protection with vaccine design would require to refine the kind of adjuvants used to improve, polarize and elongate immune response to vaccine antigens (17, 18). Here, we propose the use of β-glucan to serve as a novel kind of adjuvant for trained-immunity based adjuvantation.

In this study, we assessed the potential of β-glucans to induce innate immune training in dogs, as well as their impact on the adaptive immune response to an inactivated rabies vaccine (Rabisin®). Injection of β-glucan was performed 1 month before vaccination with Rabisin® supplemented or not with the same β-glucan used as adjuvant, i.e., concomitant to rabies vaccination. For this purpose, we selected a β-glucan extracted from *Euglena gracilis* as we confirmed it was the best inducer of trained innate immunity based on the results from the *in vitro* model that we developed in dogs (11). The selected molecule was administered subcutaneously in dogs and then regular blood sampling were performed to isolate PBMCs. The first objective of this research was the evaluation of β-glucan capacity to train dog monocytes *in vivo* as it was demonstrated in other species (19, 20). The second objective was the demonstration of trained immunity-based adjuvantation. For this purpose, and for ethical reasons, we proposed to investigate the immune response after vaccination rather than infection (10). After Rabisin® vaccination, we monitored the immune response to the vaccine and evaluated if the specific responses were modified by innate training.

MATERIALS AND METHODS

Study Design and Sampling

Approval of institutional Animal Care and Use Committee (registered in French Ministry of Research as CEEA N°013 was obtained before conducting the study, ensuring that all experiments are conformed to the relevant regulatory standards (directive EU2010/63) and Corporate Policy on Animal Welfare (029-DCPOL-001). Twenty-four conventional Beagle dogs aged between 4.5 months and 5 months were provided by a commercial supplier and were allocated randomly into 4 groups of 6 animals (Groups A to D). The groups were randomized with 3 males and 3 females each. The statistical analysis revealed no difference given the gender of the dogs (**Figure 1**). Age was close between animals of the different groups and had no impact either on the analyses. On day 28, dogs from group B and D received one subcutaneous injection of the preparation of β-glucan in the inter-scapular space. Injected β-glucans show no inflammation at the site of injection and display a very good tolerance. Groups A and B received no injection at D-28. On day 0, dogs from group A and B received one subcutaneous injection of Rabisin® in the inter-scapular space. Dogs from group C and D received one subcutaneous injection of Rabisin® as well as one subcutaneous injection of the preparation of β-glucan less than 2 cm away from the vaccine injection site in the inter-scapular space as summarized in **Table 1**. Blood samples were collected from all puppies on D-28, D-21, D-14, D0, D7, D14, and D28.

Vaccines and Adjuvants

Rabisin® (Boehringer-Ingelheim, Lyon, France) is an inactivated vaccine against rabies adjuvanted with aluminum hydroxide

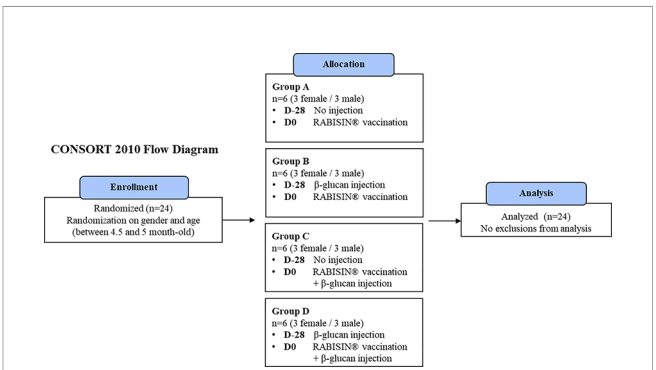

FIGURE 1 | CONSORT 2010 flow diagram of the study design.

TABLE 1 | Clinical settings and group repartition.

Groups	D-28 subcutaneous injection		D0 subcutaneous injection	
	Products	Volume (concentration)	Products	Volume (concentration)
A (n = 6)			Rabisin®	1 ml
B (n = 6)	β-glucan	0.5 ml [5mg/ml]	Rabisin®	1 ml
C (n = 6)			Rabisin®β-glucan	1mL - 0.5mL [5mg/mL]
D (n = 6)	β-glucan	0.5 ml [5mg/ml]	Rabisin®β-glucan	1mL - 0.5mL [5mg/mL]

(21), and represents the reference vaccine (Group A). β-(1-3)-glucan from *Euglena gracilis* was purchased from Sigma-Aldrich (Catalog number: 89862; batch: BCBZ6291). 0.5 ml of the suspension was injected at 5 mg/ml, for a dose of 2.5 mg/animal, a dose that was selected according to a review of several clinical studies performed with β-glucan injection (19, 22–25).

Antibody Response

The antibody response [i.e., serum neutralizing antibodies, total rabies-specific immunoglobulin G concentration (IgG)], concentration of rabies-specific immunoglobulin of IgG1 subclass, and the antibody avidity index) was assessed on D28. Methods used to assess the antibody response are described in Chapat et al. (26).

Serum Neutralizing Antibody Titers

Titration of rabies virus neutralizing antibodies (VNAs) was performed by using the fluorescent antibody virus neutralization (FAVN) test according to the technique described by Cliquet et al. (27) with a positive threshold of 0.5I U/ml. Briefly, serial dilutions of sera and a fixed amount of Challenge Virus Standard (CVS) rabies virus (between 50 and 200 TCID50/well) were incubated for 60 min before adding a volume of 50 µl of a suspension of BHK21 cells (4 × 105/ml) to each well. After incubation for 48 h at 37 ± 2°C in a humidified incubator with 5% CO2, the microplates were then fixed in acetone and the cells were labeled by adding an appropriate dilution of a fluorescein isothiocyanate (FITC)-conjugated anti-rabies monoclonal IgG (FUJIREBIO® Diagnostics, Inc. Malvern, Pennsylvania, United States; dilution of 1:133) to each well. Results were assessed by using a microscope equipped for FITC fluorescence. The well was considered positive if one or more fluorescent cells were observed. The 50% endpoint of the antibody content of test sera was calculated by the method of Spearman and Karber. The serum titers were expressed as IU per ml in comparison with the OIE standard serum adjusted to 0.5 IU/ml.

Measurement of Total Rabies-Specific Immunoglobulin G (IgG) and Rabies-Specific IgG1 Subclass

The concentrations of total rabies-specific IgG antibodies and rabies specific antibodies of the IgG1 subclass were measured using enzyme-linked immunosorbent assays (ELISAs). Briefly, serial dilutions of sera were incubated with rabies antigen (Boehringer Ingelheim Animal Health, Lyon, France) coated onto the wells of a microtitration plate. The incubation lasted at least 10 h at 5°C. After three washes and a blocking step [5% bovine serum albumin (BSA) in carbonate buffer], peroxidase-conjugated antidog IgG [rabbit anti-dog IgG heavy and light chains (H & L); Nordic Laboratories, Tilburg, The Netherlands] was added in order to detect binding of total IgG. To detect binding of IgG1 subclass, anti-dog IgG1 subclass monoclonal antibodies were added and subsequently revealed using a peroxidase-conjugated F(ab')2 rabbit anti-mouse IgG (H & L) antibody (Rockland Antibodies & assays, Gilbertsville, PA, USA). Secondary and tertiary reagents were incubated for 1 h at 37°C. Finally, substrate (tetra methyl benzidine) was added to each well and after an incubation period of 30 min at 21°C, the absorbance in each well was quantified by spectrophotometry. The relative titers of antibody in positive control and test sera were calculated by regression compared with in house control serum and were expressed as $Log_{10}OD_{50}$. The positive control sera for anti-IgG1 and anti-total IgG ELISA were a pool of sera from animals vaccinated with a double dose of RABISIN® vaccine.

Avidity Index

The technique for measuring antibody avidity was based on the detection of anti-rabies total IgG as described above with the inclusion of a dissociation step involving the addition of thiocyanate solution before adding the secondary peroxidase-conjugated anti-dog IgG antibody. In a control experiment, immobilized rabies virus was first exposed to this chaotropic agent to define the concentration and to rule out the possibility that the chaotropic treatment directly stripped the antigen from the ELISA plates. After a washing step, the thiocyanate solution at 0.7 M was added for 14 min at room temperature (RT) in the dark. The effect of thiocyanate enables separation of antigen–antibody complexes with the lowest antibody binding avidity, while preserving the complexes with greatest antibody avidity. The ratio of areas under the curves obtained in anti-rabies total IgG antibody ELISAs performed with or without dissociation gave the avidity index which was proportional to the strength of binding (28, 29). The highest avidity was close to 1, whereas the lowest avidity was close to 0.5–0.6.

Cellular Responses

Methods used to assess the cellular responses are described in Bommier et al. (30).

Isolation of Immune Cells

Canine peripheral blood mononuclear cells (PBMCs) were extracted from whole blood by density-gradient centrifugation over human Pancoll (density 1.077 g/ml, PAN Biotech, D. Dutscher, Issy-les- Moulineaux, France).

For the isolation of monocytes, after extraction from whole blood, PBMCs were seeded at a concentration of 2×10^6cells/well (6.7×10^6cells/cm²) of 96-well culture microplates (CORNING, 353072, Falcon® 96-well Clear Flat Bottom TC-treated Culture Microplate). Cells were incubated at 37± 2°C in a humidified incubator with 5% CO_2 for 2 h. Adherent monocytes were selected by washing out non-adherent cells with warm PBS.

Enzyme-Linked ImmunoSpot (ELIspot) Assays

Rabies specific circulating plasma cells were detected and enumerated by rabies antigen-specific ELIspot assay in blood samples collected respectively on D0 and D7. Briefly, rabies-specific antibody secreting circulating plasma cells were quantified directly within the PBMCs population by rabies specific ELISpot assay. Briefly, PBMCs were added to multiscreen HTS HA ELIspot plates (Merck Millipore, Molsheim, France) coated with canine rabies virus or feline Calicivirus (FCV) antigens (FCV was used as an irrelevant control; Boehringer Ingelheim, Lyon, France) for 24 h. Canine IgG were detected with biotinylated goat anti-dog IgG and horseradish peroxidase-conjugated streptavidin. The spots were counted with a CCD camera system driven by SPOT software (Microvision Instruments, Lisses, France).

Rabies-Specific T-Cell Response

PBMCs extracted from whole blood collected on D14 were stimulated in order to detect rabies-specific T-cell responses induced by vaccination. Briefly, rabies-specific interferon (IFN)-γ-secreting cells (IFN-γ spot-forming cells, SFCs) were quantified by an ELIspot assay (EL781, R&D Systems, Lille, France) after stimulation by rabies antigen or control FCV antigen for 48 h. The rabies antigen used consists in the active ingredient from the Rabisin® vaccine after inactivation and before merthiolate addition, concentrated at 100X by a 30%-PEG precipitation in PBS.

Cytokine Secretion Measurement

Quantification of Cytokines by Enzyme-Linked Immunosorbent Assays (ELISA)

Cytokine levels of Interleukin-1β (IL-1β) were detected using canine DuoSet ELISA kits (R&D Systems, Minneapolis, MN; catalog #DY3747) according to the manufacturer's instructions. The detection limits were as follows: IL-1β, 7.81–500 pg/ml. The optical densities of the peroxidase product were measured by spectrophotometry using a Synergy 2 microplate reader (Biotek, Winooski, VT) at a wavelength of 450 nm. The concentration of interleukin 1β (IL-1β) in the cell-culture supernatant was measured following 72-h stimulation.

Quantification of Cytokines by ProcartaPlex Assays

ProcartaPlex assays use Luminex™ xMAP technology for the multi-analyte detection of secreted proteins. The concentration of multiple canine cytokines (IL-2, IL-6, IL-8, IL-10, TNF-α, IL-12p40, IFN-γ, MCP-1, SCF, β-NGF, VEGFα) released into the cell-culture supernatant was measured using a LUMINEX kit (Affymetrix ProcartaPlex Canine 11-Plex, Ebioscience SAS, Paris, France) according to the manufacturer's recommendations. Sample fluorescence was read on a Bio-Rad Bioplex 200 System and analyzed using Bioplex Manager 6.1 software (Bio-Rad, Hercules, CA).

Immuno-Stimulation and Immune Training Experiments

Immune Training Experiments

After extraction and seeding, monocytes were stimulated with LPS from E. coli O55:B5 for 24 h or left untreated (negative control) Supernatants were then collected and stored at −80°C for further analysis. Cells were cultured in medium (RPMI 1640 (Life Technologies, Villebon sur Yvette, France) supplemented with 10% irradiated fetal calf serum, 1% penicillin-streptomycin (Life Technologies, Villebon sur Yvette, France) and 0.01% β-2-mercaptoethanol (Sigma-Aldrich, Saint-Quentin-Fallavier, France). Finally, cells and supernatants were harvested for characterization of different immunological parameters such as cytokine secretion and cell surface markers.

Flow Cytometry

Antibodies, Instruments, and Analyses

Cells were analyzed using a fluorescence-activated flow cytometer (BD FACSVerse™, BD Biosciences, San Jose, CA, USA) calibrated with BD FACSuite™ CS&T Research Beads; FC Bead 4c, FC Bead 4c+, and FC Bead Violet Research Kits (respective references 650621, 650625, 650626, 650627, BD Biosciences, San Jose, CA, USA). The distribution of doublets was assessed using a side scatter height (SSC-H) vs. side scatter area (SSC-A) density plot followed by a forward scatter height (FSC-H) vs. forward scatter area (FSC-A) plot. Debris and dead cells were assessed both based on forward and side scatter and with viability dyes LIVE/DEAD™ Fixable Yellow Dead Cell Stain Kit, for 405 nm excitation (L349968; Thermo Fisher Scientific Inc., Waltham, MA, USA) before proceeding with the analysis. Acquired events were recorded using BD FACSuite software and analyzed using FlowJo v10.0.7, LLC software. GraphPad Prism software was used for statistical analysis of compiled flow cytometry data. The antibodies against the following antigens were used: CD14 (APC; M5E2; BD Biosciences), CD11b (purified; CA16.3E10; Bio-Rad) coupled with LYNX Rapid APC-Cy7 Antibody Conjugation Kit, CD80 (V450; 16-10A1; BD Biosciences), MHC-II (FITC; CVS20; Bio-Rad), CD369 (Dectin-1; PerCP/Cyanine5.5; 15E2; BioLegend), Arginase-I (PE; 14D2C43; BioLegend).

Statistical Analysis

A total of 14 immune parameters were included in the analysis. For each immune response parameter, the equality of means between groups was compared using a type III ANOVA test at 5% α-risk. If the vaccine group effect is statistically significant, a Tukey's test was performed for pairwise comparisons of means between vaccine groups, with adjustment of p-values for multiple comparisons. The normality assumption of residuals was tested using a Shapiro-Wilk test at 1% α-risk level. If the normality assumption of the ANOVA test was not met, the equality of mean ranks between groups was compared using a Kruskal-Wallis test at 5% α-risk. If the vaccine group effect is statistically significant, a Dunn's test was performed

for pairwise comparisons of mean ranks between vaccine groups, with Bonferroni adjustment of p-values for multiple comparisons. In addition of the parametric ANOVA test, the Kruskal-Wallis test was performed for all immune response parameters. In case of plausible normality assumption, the homoscedasticity assumption of residuals according to the vaccine groups was tested using a Bartlett test at 5% α-risk level. If the homoscedasticity assumption of the ANOVA test was not met, the heteroscedasticity was considered into the model. If the vaccine group effect was statistically significant, a Games-Howell test was performed for pairwise comparisons of means between vaccine groups, with adjustment of p-values for multiple comparisons.

An exploratory factor analysis (EFA) was performed to analyze the covariance structure of the immune response parameters and the age covariate (if treated as a quantitative covariate). The immune response parameters were introduced in the EFA in order to build the factors. The normality of each immune response parameter was checked by Shapiro-Wilk test at 1% α-risk level. When necessary, data transformation [e.g., log10(X), or log10(X + 1) when the measured variables contain some 0 values] was applied to immune parameters, to improve within group normality. As the EFA should produce correlated factors, an Oblimin oblique rotation was applied. The number of factors to retain was determined using two criteria: Velicer's Minimum Average Partial (MAP) test and parallel analysis. The number of factors was concordant with the most clinically meaningful results. The choice of the final model was determined by the factor structure where the factors captured most of the items in a meaningful way, each factor having at least three items with strong loadings (>0.4), and none of the items having strong loadings on more than one factor. For each built factor, the equality of means between groups was compared using a type III ANOVA test at 5% α-risk. If the vaccine group effect was found statistically significant, a Tukey's test was performed for pairwise comparisons of means between vaccine groups, with adjustment of p-values for multiple comparisons.

All data manipulations and factor analyses were carried out using R version 3.6.2.

RESULTS

Evidence for Trained Immunity-Based Adjuvantation With β-Glucan

Using the data generated in our *in vitro* model, we characterized the β-glucan from *Euglena gracilis* as the best training compound for the immune training of canine macrophages. After subcutaneous administration of β-glucan from *Euglena gracilis* in dogs no local reactions or swelling were observed. The follow-up of immune cells numbers did not show any differences between the groups injected and the control groups (**Supplementary Figures 1–3**). We investigated the innate immune parameters described in the scope of trained immunity. Blood-derived macrophages were isolated from blood of dogs one week after the start of the trial, equivalent to D-21. Dogs were separated in two groups, one control (no injection; groups A and C) and one injected with β-glucan (groups B and D). β-glucan injection is considered as a first stimulation, i.e., priming *in vivo*, and, after one week of resting also *in vivo*, cells were extracted and re-stimulated for 24 h *in vitro* with LPS from *E. coli*, mimicking an immune challenge to resume and complete the protocol of trained immunity. We measured the release of pro-inflammatory cytokines, TNF-α, IL-6, and IL-1β, widely described in the trained cytokinic signature (31).

TNF-α secretion upon LPS stimulation is slightly increased with a difference between means of 102.0 ± 88.20 (**Figure 2A**). These results do not pass the significance threshold of the p-value due to few individuals driving a high dispersion of the data. IL-6 and IL-1β secretion profiles behave in the same manner with heightened release in the supernatant of the cells isolated from β-glucan-treated dogs (**Figures 2B, C**). The differences between means are respectively of 197.8 ± 120.2 for IL-6 and of 3725 ± 2025 for IL-1β, indicating a global trend toward increase for the three cytokines. No statistically significant differences can be highlighted, the confidence intervals of these results moderately spilling over negative values: [−69.98 to 465.6] for IL-6 and [−787.4 to 8238] for IL-1β. The increase in all three cytokines, though not significant, tends to confirm an implication of trained immunity mechanisms.

Immune phenotyping was performed using flow cytometry to further examine the macrophage phenotype after *in vitro* LPS stimulation. Results are presented in bar charts **Figures 2D–I** and illustrated in dot plots with their adjunct histograms (**Figures 2J–L**). Phenotypic markers CD14 and CD11b were used to discriminate the macrophage population. CD14 expression was found significantly downregulated on the surface of cells from dogs previously injected with β-glucan (p value < 0.0001; **Figure 2D**) while CD11b expression remain comparable between groups (**Figure 2E**). Then, we assessed activation markers associated with pro-inflammatory macrophages, i.e., MHC-II and CD80 (32) as well as arginase, associated to regulatory functions (33). MHC-II expression dramatically increased after LPS stimulation in the group injected *in vivo* with β-glucan (p value < 0.0001; **Figure 2F**). Upregulation of MHC-II implicates an activation of macrophages (34), consistent with the increase in cytokine levels observed. No differences in the CD80 expression were observed between groups (**Figure 2G**). Intra-cellular staining of arginase shows a significant down-regulation of its expression in the cells isolated from dogs treated *in vivo* with β-glucan (p value < 0.01; **Figure 2I**). Arginase downregulation is associated with a switch in arginine metabolism toward an increase of nitric oxide, which is a marker of pro-inflammatory macrophages (9). Finally, the expression of β-glucan receptor Dectin-1 was enhanced in the group treated with β-glucan compared to control (p value < 0.0001; **Figure 2H**). Overall, macrophages isolated from dogs injected one week before with β-glucan showed a more pro-inflammatory profile upon LPS stimulation *in vitro*, with key markers of trained immunity found upregulated compared to the control group.

β-Glucan Adjuvantation of Rabisin® Tends to Heighten Rabies-Specific Humoral Immune Responses in Vaccinated Dogs

In this study, we used a model of anti-rabies vaccination to characterize the effect of β-glucan to impact the immune response

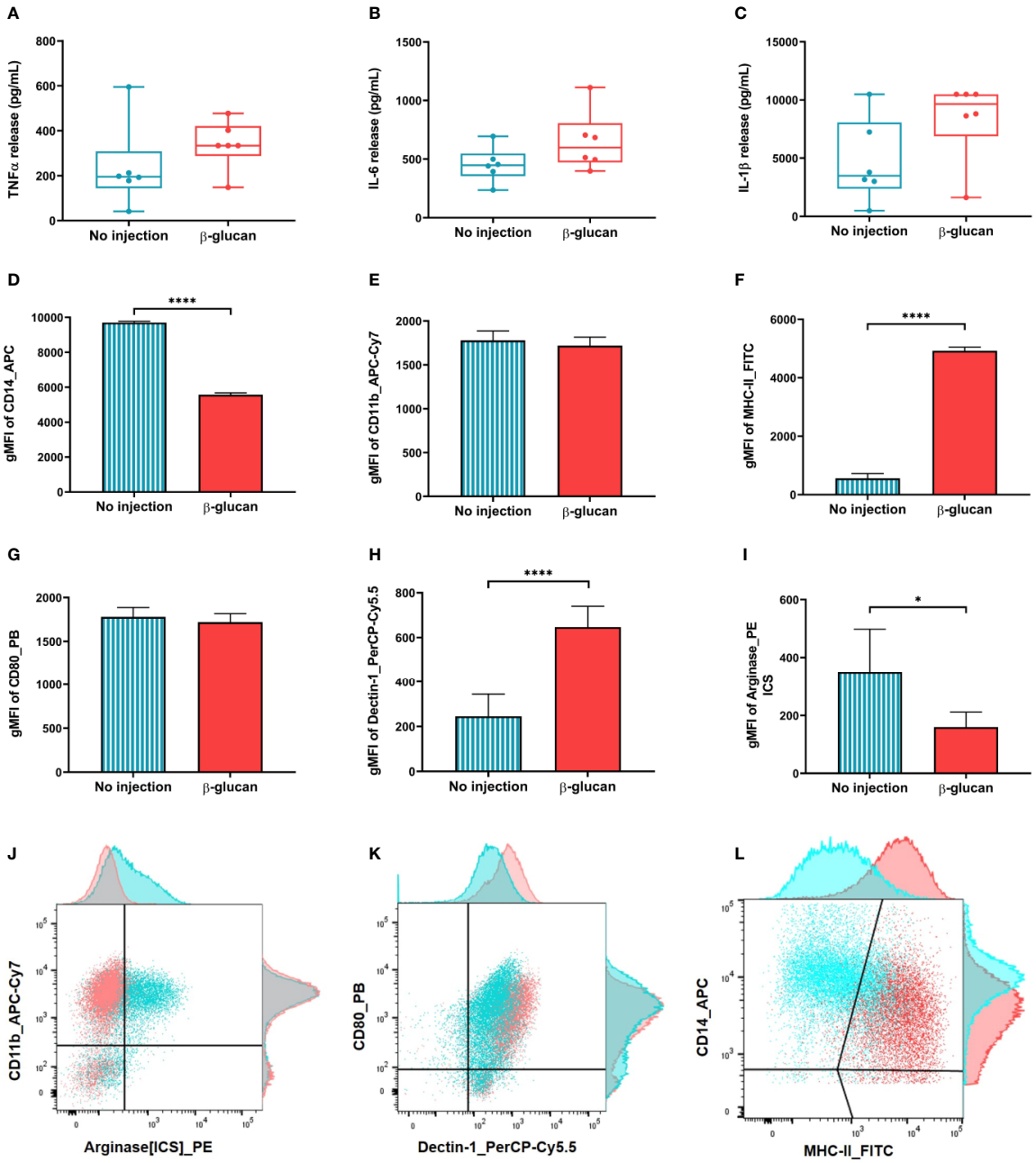

FIGURE 2 | Trained immunity features of macrophages isolated one week after β-glucan injection compared to control. **(A–C)** Cells were stimulated in vitro with LPS for 24 h before quantifying cytokine release in the supernatants. **(D–I)** Immune phenotyping was also performed 24 h post LPS stimulation. **(J–L)** Dot plot illustration of the six markers and their adjunct histograms normalized to mode. *p value < 0.05; ****p value < 0.0001.

of vaccinated dogs. We used a set of 14 well-characterized immune parameters specific to rabies antigen. Altogether, these parameters help drawing a picture of the efficacy of β-glucan adjuvantation compared to standard rabies vaccination. The multivariate analysis of the different immune parameters, i.e., immune fingerprint, was developed to show a representative image of the immune response to rabies vaccination (26, 30).

The medians and range of 14 immunological parameters assessed in this trial are presented in **Table 2**. Protection to rabies infection is mainly achieved through humoral responses, which were assessed with VNA titers (**Supplementary Figure 4**), a pharmacopeia mandatory test for rabies vaccines, IgG antibody concentrations and their avidity index, IgG1 isotype concentrations, and quantification of specific short-lived IgG-plasma cells.

TABLE 2 | Medians and ranges of the 14 immunological parameters included in the exploratory factor analysis, with the addition of VNA titers at D7.

Immune parameters	Day	Indicator	Group A	Group B	Group C	Group D
VNA titers(IU/ml)	D7	Median	0.16	0.72	0.52	0.40
		Range [minimum; maximum]	[0.06; 0.66]	[0.06; 2.62]	[0.29; 3.46]	[0.17; 1.15]
VNA titers(IU/ml)	D28	Median	7.92	12.11	7.50	5.29
		Range [minimum; maximum]	[3.46; 10.45]	[6.01; 41.59]	[4.56; 72.27]	[4.56; 13.77]
Total IgGconcentrations(Log_{10} OD_{50})	D28	Median	2.44	3.18	2.87	2.59
		Range [minimum; maximum]	[2.22; 2.86]	[2.53; 3.61]	[2.49; 3.82]	[2.25; 3.17]
IgG1concentrations(Log_{10} OD_{50})	D28	Median	2.33	3.02	2.62	2.46
		Range [minimum; maximum]	[2.14; 2.81]	[2.52; 3.52]	[2.38; 3.66]	[2.12; 3.11]
Number of IgG-secreting cells/250E3 PBMCs	D7	Median	0.00	13.00	7.00	5.00
		Range [minimum; maximum]	[0.00; 9.00]	[1.00; 26.00]	[0.00; 32.00]	[5.00; 10.00]
Number of IFN-γ-secreting cells/250E3 PBMCs	D14	Median	6.50	2.00	4.50	3.25
		Range [minimum; maximum]	[1.50; 11.00]	[0.00; 8.50]	[1.00; 8.50]	[0.50; 23.00]
IFN-γ release(pg/ml)	D14	Median	37907.68	34282.45	42838.48	28871.47
		Range [minimum; maximum]	[6913.51; 41954.30]	[18670.08; 41089.41]	[27780.47; 50632.98]	[17174.51; 44256.46]
IL-10 release(pg/ml)	D14	Median	3789.61	3945.09	5015.65	2585.72
		Range [minimum; maximum]	[1310.46; 4912.06]	[2277.76; 9633.83]	[2019.95; 7843.86]	[1717.10; 8613.74]
IL-2 release(pg/ml)	D14	Median	443.54	476.93	400.94	380.52
		Range [minimum; maximum]	[397.18; 475.45]	[295.41; 901.93]	[355.40; 695.56]	[276.58; 852.70]
IL-6 release(pg/ml)	D14	Median	798.83	947.07	1062.92	708.50
		Range [minimum; maximum]	[408.38; 920.59]	[471.08; 1743.06]	[571.45; 2616.34]	[451.71; 1640.59]
TNF-α release(pg/ml)	D14	Median	872.02	2015.12	2791.53	855.43
		Range [minimum; maximum]	[129.97; 1528.00]	[617.60; 7465.44]	[682.17; 4419.53]	[255.77; 2314.72]
IL-1β release(pg/ml)	D14	Median	55.94	66.30	60.80	50.90
		Range [minimum; maximum]	[3.52; 90.99]	[3.52; 259.97]	[12.52; 174.09]	[21.86; 80.86]
SCF release(pg/ml)	D14	Median	536.66	864.23	916.98	493.60
		Range [minimum; maximum]	[157.35; 775.92]	[370.80; 1326.26]	[452.33; 1767.22]	[270.69; 1439.44]
β-NGF release(pg/ml)	D14	Median	194.93	209.20	208.48	128.82
		Range [minimum; maximum]	[55.40; 282.51]	[114.01; 1548.47]	[103.93; 637.21]	[64.58; 260.67]
IL12p40 release(pg/ml)	D14	Median	1425.25	1577.44	1774.76	1427.07
		Range [minimum; maximum]	[1154.03; 1735.20]	[699.75; 1835.76]	[1157.24; 2342.68]	[998.80; 2107.06]

Serological parameters, i.e., VNA titers, total IgG and IgG1 concentrations, and avidity indexes were analyzed 28 days after Rabisin® vaccination adjuvanted or not with β-glucan injection. All dogs showed a seroconversion above the protection threshold at 0.5 IU/ml (**Figures 3A, B**). No significant differences in the titers could be highlighted between the four different groups. However, VNA titers observed in group B, β-glucan 1 month prior to Rabisin®, showed a substantial higher median (12.11 [6.01; 41.59]) than group A that underwent the standard protocol of Rabisin® vaccination, with one single dose injected at day 0 (7.92 [3.46; 10.45]). The results observed for group A were very similar to the ones in groups C (7.50 [4.56; 72.27]) and D (5.29 [4.56; 13.77]). Dogs from group B showed a significant increase of total IgG concentrations compared to group A (p-value = 0.337; **Figure 3C**). Dogs from group C, who received concomitant injection of β-glucan and Rabisin®, followed the same trend of increase without passing the statistical threshold of significance (p-value = 0.108). The double injection of β-glucan in group D, 1 month prior and concomitantly to Rabisin® vaccination, showed no difference compared to group A. IgG1 isotype concentrations displayed a very similar profile to total IgG, with a significant difference between groups A and B (*p-value < 0.05) while such difference was not seen with the two other protocols of β-glucan adjuvantation (**Figure 3D**). No differences between groups were seen for avidity indexes on D28 (**Figure 3E**).

Rabies-specific IgG-secreting cells were quantified at day 7 by ELIspot assay (**Figure 3F**). Only two dogs out of 6 in group A had a detectable number of IgG-secreting cells (0.00 [0.00; 9.00]) whereas all dogs from group B displayed higher results, yet with high and low responders (13.00 [1.00; 26.00]). Dogs in group C showed a similar profile as group B but with higher variability

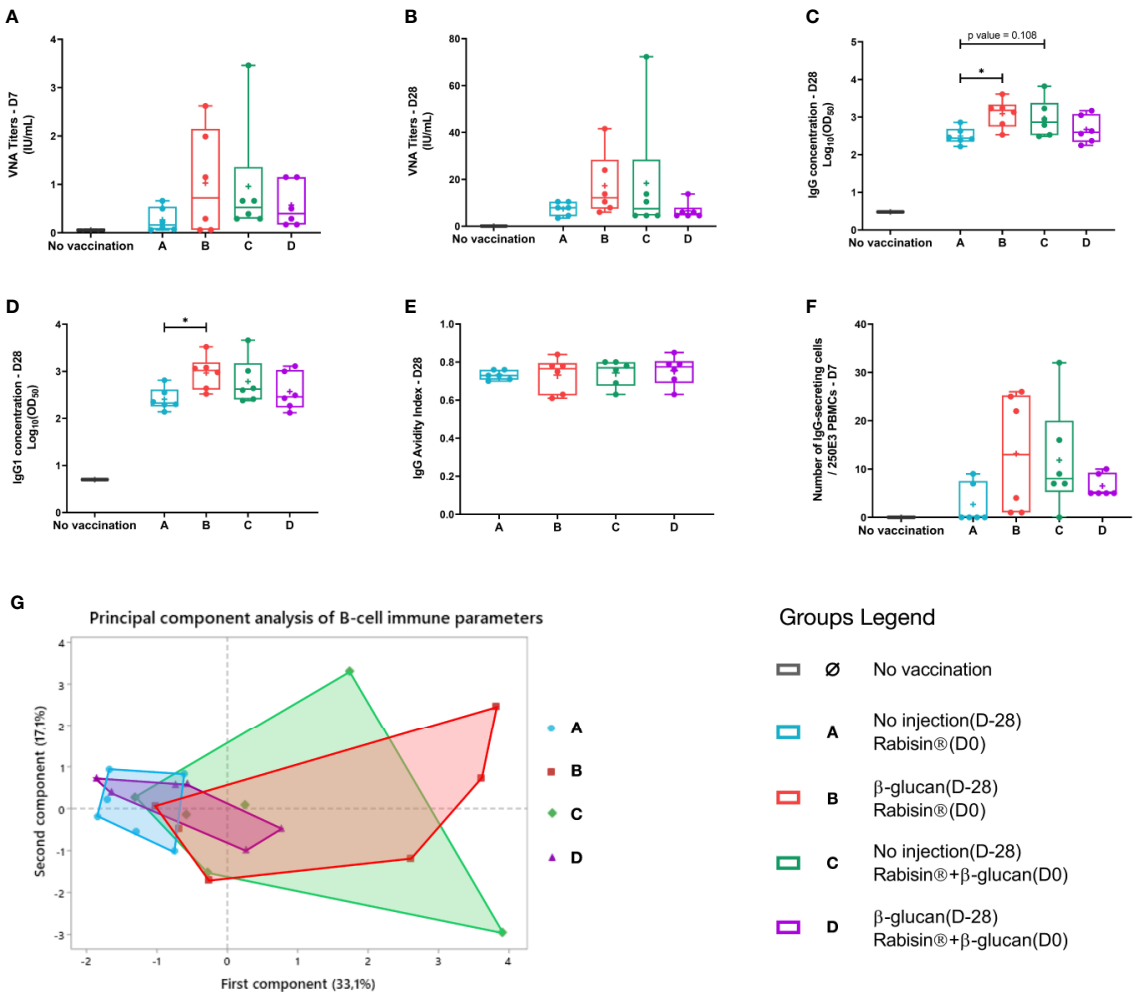

FIGURE 3 | Group comparison of rabies-specific B-cell immune parameters of vaccinated dogs **(A–F)**. Box and whiskers plots of each immunological variables. Kruskal-Wallis tests were performed to compare the values of each immune response parameter between vaccine groups. Dunn pairwise comparisons were performed to evaluate the difference in mean ranks. **(G)** Global image of B-cell immune response using principal component analysis integrating all of the above variables.*p value < 0.05.

and one non-responder (7.00 [0.00; 32.00]). Finally, IgG-secreting cells quantification of dogs from group D presented a more clustered profile with an intermediate median (5.00 [5.00; 10.00]) between groups B and C and group A. No statistically significant differences were observed between groups. These results are very similar to the ones of VNA titers at day 7. B-cell response parameters were combined in a principal component analysis (**Figure 3G**). This highlights the trend of an enhanced B cell response, with almost no overlap of the response between group A and B, despite the lack of statistical significance partly due to the inter-individual variability.

Overall, B-cell responses, which is the main correlate of protection against rabies (26), display similar profiles between groups A (standard Rabisin® vaccination) and D (double injection of β-glucan). Groups B and C, injected once with β-glucan, 1 month prior or at the same time as Rabisin® vaccination respectively, show a wider dispersion of their response toward an increase of all parameters. However, only total IgG and IgG1 isotype concentrations are able to show a significant difference between groups A and B; all other parameters fail to meet the statistical threshold.

Impact of β-Glucan Adjuvantation of Rabisin® on T-Cell Responses Against Rabies

Cellular responses of T lymphocytes were monitored with quantification of IFN-γ-secreting cells and secretion of several cytokines (IFN-γ, IL-10, IL-2, IL-6, TNF-α, and IL-1β) after canine rabies virus antigen stimulation *in vitro*. IL-2, IFN-γ, and TNF-α are associated with multifunctional T-helper 1 (Th1) response (35). Il-1β reinforce the implementation of Th1 responses by stimulating IL-2 synthesis, which act as a T-cell proliferative cytokine (36). IL-6 is known to be a master regulator of inflammation and has also been described to promote B cell maturation along Th17 responses (37). TNF-α, IL-6, and IL-1β are also well described in the cytokine signature of trained immunity (7). IL-10 plays a role in limiting inflammation and regulating the return to homeostasis (38). Interestingly, IL-10 also promotes B-cell survival and antibody production.

IFN-γ production was assessed using two different techniques, ELIspot and ELISA assays, combining both the number of cells producing this cytokine and the amount. The number of specific IFN-γ-secreting cells was quantified on day 14. Group A showed a higher median (6.50 [1.50; 11.00] SFC/250.10^6 PBMCs) than the other groups adjuvanted with β-glucan with no statistical differences (**Figure 4A**). Quantification of the IFN-γ secretion showed similar results between groups, with exception for group C, for which, the median was slightly higher, and the response less dispersed but did not show statistical difference (**Figure 4B**).

No differences in IL-2 release following rabies antigen stimulation *in vitro* were observed though groups B, C and D displayed a higher inter-individual heterogeneity (**Figure 4C**). The secretion of TNF-α from groups A and D followed similar results (medians at 872 and 855 pg/ml, respectively) two-times less than the one observed for group B (2015pg/ml) and almost three-times less than group D (2791pg/ml) (**Figure 4D**). A comparable pattern was obtained with IL-6 secretion, for which both groups B and D have at least three individuals driving higher medians and means than groups A and D (**Figure 4E**). Very low IL-1β quantity is detectable in response to rabies-antigen stimulation *in vitro* with no striking differences between groups (**Figure 4F**). IL-10 release behaved in a similar way to TNF-α and IL-6, means of secretion of groups B and C reaching 1.5 times the ones for groups A and D (**Figure 4G**).

Analysis of the Global Immune Response Against Rabies by a Multivariate Analysis

We then investigated all the immune parameters described above with an EFA in order to generate an integrated view of the whole set of data. This unsupervised statistical analysis has been successfully used to study immunological response to Rabisin® vaccination in dogs (26) and human immune responses to influenza vaccines (39, 40). Every parameter presented in univariate comparison were assimilated to the further described EFA, in a two-factor manner. This model was found best-fitted to the dataset and the separation was relevant with scientific background, each factor recapitulating B- or T-oriented immune response. No correlation was observed between the two factors (coefficient around 0.1). The implication of qualitative covariates (age and gender) was assessed to ensure an unbiased analysis. The representation of the factorial plan is show in **Figure 5A** and the construction of the factors in **Figure 5B**.

The first factor is constituted of all B-related variables described in the first section and also of both IFN-γ-measuring parameters (quantification of secreting cells and of release of IFN-γ) although only VNA titers, total IgG and IgG1 concentrations were highly associated to this factor (coefficient of correlations 1.1 and 0.7, respectively). The second factor was strongly associated with seven variables representing cytokine secretion (IL-2, IL-6, TNF-α, and IL-10) and growth factors secretion (β-NGF, VEGFα, and SCF). The remaining parameters (IL-1β, and IL-12p40 secretion) were poorly associated with this factor (correlation < 0.4). For each factor, there was no statistically significant difference between the groups of vaccination.

The observation of the factorial plan graph highlights the high inter-individual variability, for groups B and C particularly. The area covered by the parameters from group A gather on the lowest scores of both factors with the smallest dispersion. The dataset of group D, showed a very similar profile yet with a larger dispersion. Values of these two groups show really poor discrimination between one another. The immune response of group B was found widely spread in the factorial plan. The factor 1, recapitulating B-cell parameters, exhibited a higher score for this group compared to the reference group A. The scattering of this factor was seemingly attributable to only one individual. The factor 2 was, for its part, very similar in score to the one of group A. Group C also displayed a higher score for the first factor than group A, though not as much as group B. The mean score of the second factor was also higher compared to group A with a noticeable dispersion. However, the statistical significance threshold is not met for any of the two factors in groups B and C compared to group A. Still, the global integrated images of both areas in the factorial plan are from the one of the reference group, indicating a trend in the effect of β-glucan administration.

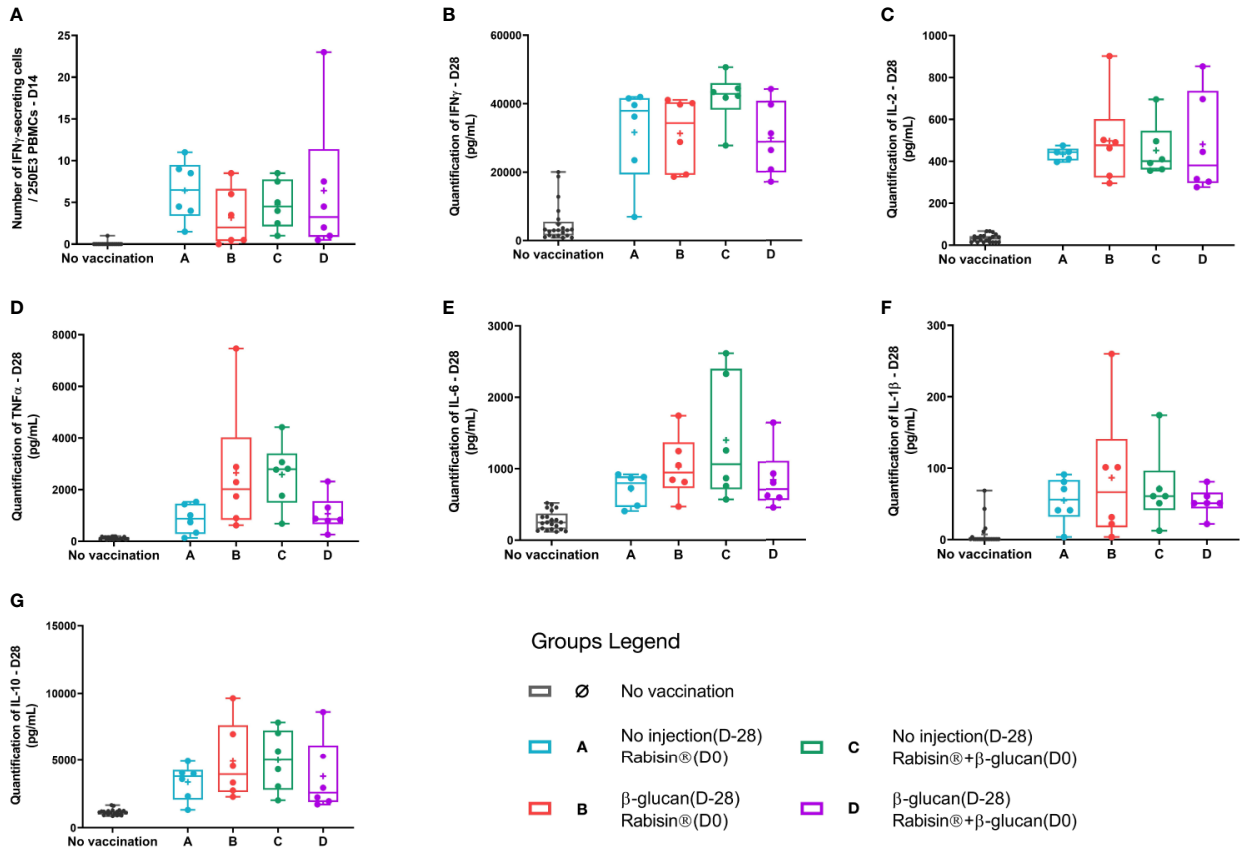

FIGURE 4 | Group comparison of T-cell immune parameters of vaccinated dogs **(A–G)**. Box plots of each immunological variables. Kruskal-Wallis tests were performed to compare the values of each immune response parameter between vaccine groups.

DISCUSSION

One of the hurdles in *One Health* is to increase protection to microbial infections both for human and animal species. Due to the variety of circulating pathogens, development of specific vaccines for all of those is not achievable. One way to tackle this issue would be to rely on the non-specific protection that trained immunity seems to offer and for which data still need to be accumulated (16, 41). The features of trained immunity seem promising for this approach with addition of the development of a new area of trained immunity-based adjuvants (TIbAs) (13).

Several analogies are connecting the signature observed in adaptive immunity after vaccination and the results of early innate immunity stimulation by β-glucan injection. Increase in cytokine secretion levels upon a stimulation mimicking an infectious assault is one of the key markers of trained immunity (7). The general trend of increase that we observe here in TNF-α, IL-6, and IL-1β confirms its implementation in dogs *in vivo*. Further analysis of immune phenotyping of macrophages strengthens this hypothesis. MHC-II upregulation in particular, is an activation marker of antigen-presenting cells and associated to increased antigen uptake and processing (42). Such phenotype is widely described in the context of extracellular pathogen but less so for intracellular ones (43). CD80, another activation marker of macrophages along with its functional equivalent CD86, is implicated in initiation and maintenance of CD4+T-cells responses. The expression of CD80 is documented to be elevated in response to LPS (44), which is consistent with the results showed in this study. Yet no differences are shown between groups, which correlates with the equivalent T-cell adaptive response later observed. Arginase, an enzyme transforming arginine in ornithine, is associated with regulatory macrophages (M2-like types) (32). Arginase forms a match with its counterpart iNOS in arginine metabolism, correlated to the anti- or pro-inflammatory features of macrophages (33). Its downregulation in the macrophages coming from β-glucan-treated dogs is another evidence toward trained immunity implementation *in vivo*. Comparable induction of pro-inflammatory profiles of macrophages by immune training has been reported in a context of protection against leptospirosis in hamster, highlighting the clinical use of trained immunity in pathogen-related diseases (24).

The upregulation of Dectin-1 has already been shown to be inducible by pathogens such as fungi containing β-glucans (5). The upregulation of Dectin-1 can also be linked to increase of

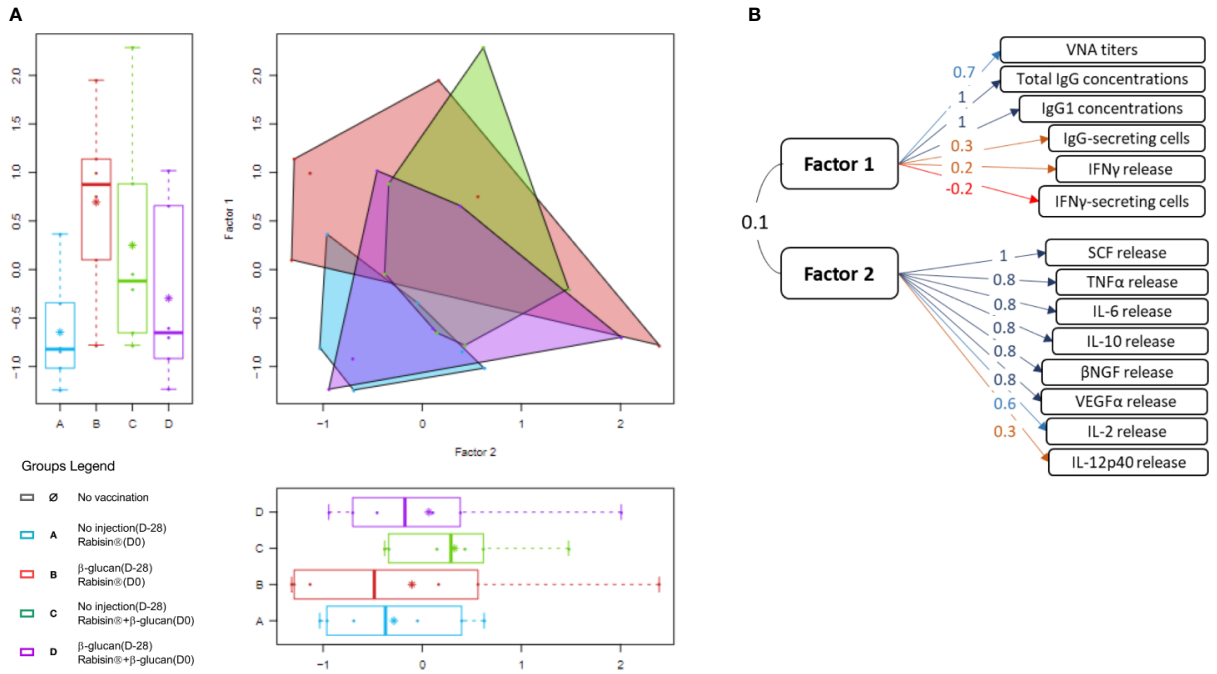

FIGURE 5 | Exploratory factor analysis integrating all immune parameters in the presented study. **(A)** Scatter plot and its adjunct box plots of the exploratory factor analysis recapitulating all parameters. **(B)** Description of the two factors with their coefficients of correlation associated to each variable.

pro-inflammatory profile. Isoform impact in dogs has not yet been described, A and B isoform in humans are known to have distinct cell surface expression and binding capacities (45). Finally, the low expression of CD14 could be associated with special phenotypes such as non-classical monocytes in humans (46), yet no population has been clearly deciphered in dogs. A fair amount of publications suggest that blood-derived macrophages are not the only populations impacted by an innate training *in vivo* (47), suggesting that bone marrow precursors or resident macrophages at the site of injection would be worth investigating. Taken altogether, these results clearly state a first demonstration of *in vivo* β-glucan's effects on the immune system of dogs.

The work presented here is consistent with a recent study showing that trained immunity induced by BCG could provide protection in a vaccination model with a live attenuated vaccine against yellow fever (10). With the objective of investigating the role of trained immunity in dogs, we performed our first clinical trial with a vaccine for which we have a good correlate of protection (VNA titers), and well defined immunological parameters to decipher the immune response in dogs. As shown previously, the immune fingerprint of the immune response to rabies vaccines in dogs differs given the adjuvant associated with the rabies antigen (30). Using the multi-parametric factorial analysis, we were able to evaluate adjuvants to decipher the type of response induced.

In this study, we describe the effects of β-glucan injection at several timings on the adaptive immune response to Rabisin® vaccination. Adjuvanted Rabies antigens induce strong Th2 immune responses with Th1 features (48, 49). The results obtained in this trial with the group A confirmed the strong humoral response well described and monitored upon Rabisin® vaccination. The integrated image of the parameters can then be correlated to the vaccine efficacy (26). The timing of β-glucan injection was set to assess two distinct but equally important types of adjuvantation. The trained immunity-based adjuvantation must be distinguished from sole immune-stimulation, used in classic vaccines. The resting period is crucial to decipher those two types. Indeed, β-glucan's ability to modulate innate responses lies in the description of mechanisms that need time to implement within the cells (metabolic and epigenetic modifications). On the contrary, effects of classic immune-stimulatory compounds as adjuvants are not expected to last several weeks. On the other end of the spectrum, the duration of *in vivo* training effect of β-glucan are still controversial and may depend on the molecules and species (19, 23). Thus, the interval between the first injection, i.e., priming, and the vaccination should be reasonably determined in the same manner as for prime/boost strategies of vaccination. Following the protocol published by Arts et al. (10), we chose an injection schedule 1 month before the vaccination to differentiate between these two adjuvantation methods. Group B, injected with β-glucan 1 month before vaccination, represents the trained-immunity based adjuvantation while group C, injected concomitantly with β-glucan and Rabisin® vaccine, exemplifies a classic way of adjuvantation. Group D collating both injections was used to evaluate of a potential synergistic effect.

Injection of β-glucan 1 month before vaccination showed a consistent increase in all B-cell immune parameters, strongly correlated to protection in rabies. The total rabies-specific IgG antibodies and rabies specific antibodies of the IgG1 subclass are significantly higher than the reference group Rabisin® alone. The other immunological parameters associated with the B cell responses (VNA titers and rabies-specific plasma cells) follow the same trend, with much higher response for group B compared to the control group A.

Interestingly, TNF-α, IL-6, and IL-10 secretions are elevated in response to rabies antigen when β-glucan was injected prior to vaccination. TNF-α is described to modulate adaptive responses by inducing co-stimulatory signals important for both humoral and cellular response maturation (35, 50). IL-6 is required to induce physiological Th1 and Th17 responses and helps to block Treg suppressive effects in synergy with IL-1β (37, 51). IL-10 allows regulation of pro-inflammatory features and help mitigate potential negative impacts (38). Thus, its elevated secretion could show another beneficial effect of β-glucan adjuvantation preventing a deleterious surge in pro-inflammatory mediators. Altogether, these results indicate a heightened immune response to rabies vaccination upon trained immunity-based adjuvantation. This kind of mid-term effects associated with trained immunity-based protection has been documented in humans against yellow fever vaccination (10) and in fish immunity with similar time intervals (19).

The integrated analysis (EFA) distinguishes two factors, one related to B-cell response and one more oriented to T-cell immune parameters. The p-values of both factors are found non-significant, though the first factor is closed to the significance threshold, confirming the results above. The graphical representation clearly shows that groups B and C have an overall increased immune response compared to the reference group A. The underlying reasons of the differences between groups B and C seem to be a stronger Th2 response in group B, shown by factor one, whereas factor 2 increase, Th1-oriented, is observed in group C. It suggests that β-glucan injection 1 month before vaccination tends to increase the immune response without changing its orientation while the concomitant injection does not allow as much elevated B-cell immune parameters but is more effective in increasing T-cell ones. Indeed, simultaneous injection of β-glucan and Rabisin® in group C displays a very similar profile to group B, without passing statistical threshold in the immune parameters associated with humoral response. The implications of these observations can be linked to the necessary interval between the implementation of β-glucan induced trained immunity and its ability to enhance adaptive immune responses (52). Interestingly, the lower values for both B- and T-cell immune parameters are consistently attributed to the same individuals in every group. This clearly favors the hypothesis of non- or low responders to trained immunity. Not only does this increase greatly the dispersion of values but it also reduces the power of the statistical analysis potentially preventing more conclusive results.

Interestingly, the double injection of β-glucan (group D) seems to have a negative impact on the immune response. All parameters measured are diminished in group D compared to groups B and C. Interactions of β-glucan with the antigen uptake and processing are monitored in group C as well, thus the results of group D cannot be attributed to this phenomenon. The most logical cause is a form of immune-modulation due to repeated injections. One hypothesis is the presence of antibodies against β-glucan. Indeed, it has been shown that human serum present high levels of antibodies against several complex sugars from fungal pathogens (53). Although immunoglobulins specific of mannans have been more described, it is now known that anti-β-glucan antibodies are also found (54). The measurement of anti-β-glucan antibodies titers in sera of dogs could be of interest to confirm or disprove their role in trained immunity-based adjuvantation. Cytokine secretion by macrophages after one week of β-glucan injection reflects the early impact of trained immunity. The early results of trained immunity are consistent with the vaccination response of the individuals regarding the amplitude of these responses. Non-responders to BCG vaccines have been described, with insights on discrimination upon changes in DNA methylation (55).

One could consider that the choice of a very potent Rabies vaccine might have limited the full investigation for beta-glucan as a trained-immunity based adjuvant. Rabisin® being a very good vaccine, it is challenging to try to improve it, and even harder to reach statistical significant differences in regards of the immune responses. Furthermore, this vaccine was described to provide non-specific effects in a field study (56). Another challenging choice was made regarding the time frame between the first injection of the β-glucan and vaccination. Although long-lasting effects (from 1 month to several years) were proven to be achievable with attenuated agents like BCG (57). The duration of trained immunity with inert molecules such as β-glucan is still being investigated and discussed in the literature (23). Further studies should aim at evaluating the duration of response *in vivo* with the help of biomarkers of trained immunity in dogs. The concomitant administration to vaccine antigens seems promising nonetheless, with the additional advantage of being convenient for vaccination schedules. Follow-up studies should focus on the trained-immunity based adjuvant role of β-glucan in a context of non-formulated antigens.

In conclusion, β-glucan injection displays a trend of trained immunity-based adjuvantation, theorized from several sources of evidence in this field (1, 12, 13). The present study shows that macrophages presenting an orientation toward a pro-inflammatory profile one week after *in vivo* stimulation are associated with later increased response to Rabisin® vaccination. The link between repolarization of innate immunity modifying, in turn, adaptive immune cells with the use of β-glucan was already documented in the context of tumor-associated macrophages (58). Still the presented work is the first demonstration of such phenomenon in dogs and in a vaccination protocol. This study provides insights for new designs of further investigations with other vaccines candidates, and non-specific protection against infectious agents. Furthermore, this first study in dogs about trained immunity based-adjuvantation gives new insight for the development of adjuvant candidates.

DATA AVAILABILITY STATEMENT

All datasets presented in this study are included in the article/ **Supplementary Material**.

ETHICS STATEMENT

The animal study was reviewed and approved by Approval of institutional Animal Care and Use Committee (registered in French Ministry of Research as CEEA N°013 was obtained before conducting the study, ensuring that all experiments are conformed to the relevant regulatory standards (directive EU2010/63) and Corporate Policy on Animal Welfare (029-DCPOL-001).

AUTHOR CONTRIBUTIONS

SP, PB, LF, HP, and KL designed the clinical trial and the associated research. PB conducted the clinical trial. NM-C and CC delivered all the ELISA tests in Boehringer Ingelheim Clinical Analysis Laboratory and handled the interactions with LD31 in charge of the seroneutralization test. SP, LC, P-YD and LP performed the *ex vivo* experiments and read outs. SP and KL analyzed data and wrote the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.564497/full#supplementary-material

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Involvement of Mesenchymal Stem Cells in Oral Mucosal Bacterial Immunotherapy

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Recent clinical observations indicate that bacterial vaccines induce cross-protection against infections produced by different microorganisms. MV130, a polyvalent bacterial sublingual preparation designed to prevent recurrent respiratory infectious diseases, reduces the infection rate in patients with recurrent respiratory tract infections. On the other hand, mesenchymal stem cells (MSCs) are key cell components that contribute to the maintenance of tissue homeostasis and exert both immunostimulatory and immunosuppressive functions. Herein, we study the effects of MV130 in human MSC functionality as a potential mechanism that contributes to its clinical benefits. We provide evidence that during MV130 sublingual immunization of mice, resident oral mucosa MSCs can take up MV130 components and their numbers remain unchanged after vaccination, in contrast to granulocytes that are recruited from extramucosal tissues. MSCs treated *in vitro* with MV130 show an increased viability without affecting their differentiation potential. In the short-term, MSC treatment with MV130 induces higher leukocyte recruitment and T cell expansion. In contrast, once T-cell activation is initiated, MV130 stimulation induces an up-regulated expression of immunosuppressor factors in MSCs. Accordingly, MV130-primed MSCs reduce T lymphocyte proliferation, induce the differentiation of dendritic cells with immunosuppressive features and favor M2-like macrophage polarization, thus counterbalancing the immune response. In addition, MSCs trained with MV130 undergo functional changes, enhancing their immunomodulatory response to a secondary stimulus. Finally, we show that MSCs are able to uptake, process and retain a reservoir of the TLR ligands derived from MV130 digestion which can be subsequently transferred to dendritic cells, an additional feature that also may be associated to trained immunity.

Keywords: mesenchymal stem cells, sublingual mucosal immunotherapy, polybacterial preparation, vaccine, immunomodulation, pattern recognition receptors, short-term memory

INTRODUCTION

Recurrent respiratory tract infections (RRTIs) are a leading cause of morbidity and mortality in children and adults (1, 2). While many of the RRTIs are of viral origin, antibiotics are often misused in these conditions leading to bacterial resistance and microbiota disruption (3–5). Therefore, implementation of effective strategies to improve their management has become a therapeutic challenge (6). An increasing number of studies are focused on prophylactic and therapeutic interventions that enhance the body's natural defenses against infections and/or downregulate the accompanying harmful inflammatory process (2, 7). Mucosal immunotherapy with bacteria-derived products or whole cell bacteria may play that role. It has been shown that poly-bacterial preparations (PBP) improve RRTIs in both adults and children by reducing the number, duration and severity of the clinical episodes (7–11). Mucosal bacterial immunotherapy induces a broad range of both non-specific and specific immune responses in mucosal and extra-mucosal tissues (7, 12–14). Immune mechanisms include the induction of antimicrobial peptide and antiviral cytokine release, neutrophil and monocyte recruitment and also the modulation of the adaptive response, mainly through its effects on dendritic cells (DCs) (2, 13, 15, 16).

MV130 is a sublingual PBP that contains different species of inactivated whole- cell Gram-positive and negative bacteria (17). MV130 significantly reduces the patient infection rate inducing both a specific T cell immunity against bacteria included in MV130 and an enhancement in T cell responses to unrelated antigens (7, 9, 11, 14). MV130 triggers Toll-like receptors (TLR) and Nod-like receptors (NLR), imprints human DCs with the capacity to generate Th1 and Th17 responses and increases the IL-10 cytokine levels (17). Bacterial-derived products may also act activating non-immune cells such as mucosal epithelial cells and mesenchymal stem cells (MSCs) (18, 19). Although bone marrow, adipose tissue, and umbilical cord blood are the prevailing sources of MSCs used in cell therapy for its greater availability, these cells represent a naturally heterogeneous cell population that can be isolated from a wide range of tissues. Relevant in the context of sublingual delivery of vaccines or PBP, MSCs have been described in human oral soft tissues including oral mucosa proper, gingiva, periodontal ligament, dental follicle and dental pulp (20, 21). In addition, recent studies have reported the presence of numerous MSCs in fetal and adult connective tissue from human major salivary glands, including parotid, sublingual, and submandibular glands (22, 23). These oral MSCs show phenotypical and functional resemblance to MSCs isolated from other tissues (20).

In contrast to the therapeutic role of systemically delivered MSCs, the role of local resident MSCs during infection and the subsequent immune response is unclear at present. Different studies indicate that tissue-resident MSCs could function as early sensors of pathogens when classical immune cells have not been recruited yet, since MSCs are equipped with a wide set of pattern recognition receptors (PRRs), mainly TLRs (24). After TLR-triggering or stimulation with inflammatory cytokines, MSCs acquire a cell autonomous, broad-spectrum antimicrobial effector function directed against clinically relevant bacteria, protozoan parasites and viruses (25–27). Together with these direct effects, MSCs may interact with innate immune cells recruited at the inflammation site, and then, their function would be modulated to establish a fine balance between pathogen clearance and repair processes (28). This balance is essential for controlling inflammation, preserving tissue homeostasis, and preventing organ failure (28–30). Mechanisms triggering a functional switch between pro-inflammatory and anti-inflammatory MSC phenotypes include dose, duration and type of TLR stimuli, as well as expression levels of different autocrine and paracrine pro- and anti-inflammatory cytokines (31–33). MSCs may also act indirectly, driving the polarization of the functional phenotype of immune cells (31–35). In addition, it has been recently suggested that MSCs could be primed by certain pathogens, which would increase their response to a second stimulus, displaying therefore a trained immunity similarly to innate immune cells (18, 36–38). Thus, MSCs could be able not only to dampen the inflammatory response but also to enhance bacterial clearance, as has been demonstrated in preclinical models of sepsis, bacterial pneumonia, and acute respiratory distress syndrome (39–43).

In the present study we report the main effects of MV130 on MSC biology and the impact in their immunoregulatory features. Our data provide first evidence of mechanisms that might be involved in the observed clinical benefits of MV130.

MATERIAL AND METHODS

Culture of Human Mesenchymal Stem Cells (MSCs)

Human bone marrow MSCs from healthy donors (n = 8; Innoprot) were cultured in Mesenpro medium (Thermo Fisher Scientific) supplemented with glutamine and penicillin-streptomycin (Lonza) in a humidified incubator at 37°C with 5% CO_2 until reaching a confluence of 80%. Half of the culture medium was renewed every 3–4 days. MSCs were used between 3rd 7th passages.

MSC Treatments

MV130 (Bactek®, Inmunotek S.L. Spain), is a preparation of whole-cell heat-inactivated bacterial species including 90% Gram-positive bacteria (*Streptococcus pneumoniae*, *Staphylococcus aureus*, *Staphylococcus epidermidis*) and 10% Gram-negative bacteria (*Klebsiella pneumoniae*, *Moraxella catarrhalis*, *Haemophilus influenzae*).

MSCs were treated with MV130 (10^7 bacteria/mL; MV130-MSCs) for different time periods as indicated in each section. Where indicated, other concentrations of MV130 were used. After treatment, culture medium was removed and cells were washed twice with warm PBS to completely remove the unbound bacterial preparation. MSCs under control conditions were treated with the same volume of the excipient of MV130 (CTRL-MSCs).

To analyze if MSCs primed with MV130 during 24 h modify their response to a second challenge, MSCs were gently washed

with warm PBS and cultured for another 3 days. Then, cells were re-stimulated with IFNγ (2 ng/mL; Immunotools) for another 24 h. Supernatants were collected and cells were used to carry out migration assays or to perform co-cultures with T cells (see scheme below).

MV130 Staining With CFSE (CFSE-MV130)

Bacteria from MV130 were stained with CFSE (Biolegend) at 2.5 μM following the manufacturer's instructions, to monitor their presence by immunofluorescence or flow cytometry.

Differentiation Assays

MSCs were cultured at a cell density of 5,000 cells/cm^2 in 24-well culture plates for 6 days. MSCs were treated with MV130 (10^7 bacteria/mL) or excipient and this treatment were repeated once again 24 h later. 72 h after the last dose of MV130, cells were collected, and expression of *NANOG* and *Oct-4* was analyzed by quantitative PCR. In parallel, other cultures were switched to a specific adipogenic or osteogenic conditioning medium, as described previously (44). For osteogenic differentiation, alkaline phosphatase enzyme (ALP) levels were determined as a measure of MSC differentiation into osteoblasts, after 5 days of culture, while for adipogenic differentiation, Oil Red staining (ORO) was performed 10 days after differentiation conditions.

PBMC, Monocyte, and T Cell Isolation

Peripheral blood mononuclear cells (PBMCs) were obtained by density gradient centrifugation using lymphocyte isolation solution (Rafer) from buffy coats of volunteer healthy donors (Centro de Transfusión de la Comunidad de Madrid, Spain). Monocytes were obtained from PBMCs by immunomagnetic isolation using anti-CD14 microbeads and VarioMACS cell separator (Miltenyi Biotec), following the manufacturer's protocol. Non-adherent T lymphocyte-enriched cell suspensions were obtained from PBMCs by nylon wool enrichment and labeled with CFSE (Biolegend) at 2.5 μM following the manufacturer's instructions. The percentage of CD3$^+$ cells were always above 90%.

MSC-Monocyte Co-Cultures

MSCs were seeded in 6-well plates at a concentration of 5×10^5 cells/well in 2 mL. Following overnight adherence, MV130 treatment was added during 24 h, and then MSC cultures were gently washed to remove the unbound bacteria. Monocytes were added at 1:10 MSC/monocyte ratio in the different conditions described below.

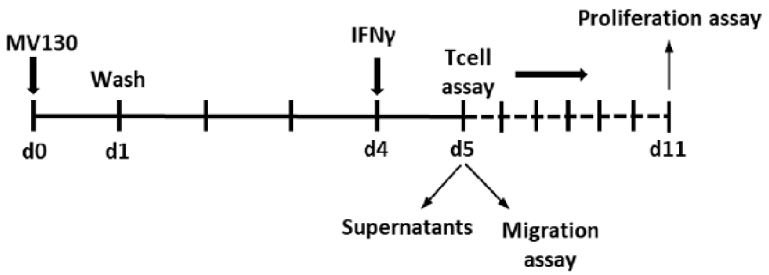
Monocyte-Derived DCs and M1 Macrophages

Monocytes were cultured in RPMI 1640 medium (Lonza) supplemented with 10% fetal bovine serum (Gibco, Thermo Fisher Scientific), 100 U/mL penicillin, 100 μg/mL streptomycin, 2 mM L-glutamine and 1 mM pyruvate (all from Lonza), referred to as complete-RPMI medium, in the presence of GM-CSF (5 ng/mL; Gibco, Thermo Fisher Scientific) to induce M1 macrophages or with GM-CSF (20 ng/mL) and IL- 4 (20 ng/mL; Gibco, Thermo Fisher Scientific) to induce DC differentiation. After 3 days, additional 5 ng/mL GM-CSF was added to macrophage cultures and half of the medium was renewed in DC cultures. At day 6 of co-culture, macrophage and DC phenotypes were analyzed by flow cytometry within CD90$^-$ population.

Macrophages and DCs were stimulated overnight with LPS (Invitrogen, Life Technologies) at 10 ng/mL or 50 ng/mL, respectively. Supernatants were collected and their allostimulatory function was analyzed by culturing in mixed lymphocyte reaction (MLR) with CFSE-labeled T lymphocytes (1:10 Macrophage or DC/T cell ratio). After 5 days of co-culture, T lymphocyte proliferation was analyzed using the CFSE dilution method by flow cytometry in the CD3$^+$ population. Supernatants from different co-cultures were harvested at different times and cytokine secretion was measured.

MSC-T Cell Co-Cultures

2.5×10^3 MSCs were seeded in duplicate and allowed to adhere to 48-well plates for 12 h and after that, primed with MV130 or under control conditions for 24 h. After treatment, MSCs were gently washed and CFSE-T lymphocytes were seeded at 1:25 MSC/T cell ratio in warm complete-RPMI with Dynabeads Human T-Activator CD3/CD28 for T Cell Expansion and Activation (1:4 Bead/T cell ratio; Gibco Thermo Fisher Scientific). Stimulated and non-stimulated T lymphocytes cultured without MSCs was carried out as control. After 3, 4 or 5 days of co-culture, supernatants were collected and proliferation of CFSE-T lymphocytes (CD3$^+$ cells) and CD69 expression were analyzed by flow cytometry.



SCHEME 1 |

Mice

Mice were housed in the animal facility (Registration No. ES280790000183) at CIEMAT (Madrid, Spain). Mice were routinely screened for pathogens in accordance with FELASA procedures and received water and food *ad libitum*. All experimental procedures were carried out according to Spanish and European regulations (Spanish RD 53/2013 and Law 6/2013, European Directive 2010/63/UE). Procedures were approved by the CIEMAT Animal Experimentation Ethical Committee according to approved biosafety and bioethics guidelines.

MV130 Sublingual Administration

Male mice C57 (3 weeks old) were sublingually treated with MV130 (10^9 bacteria/mL) or excipient (control group) in two consecutive doses of 10 µL. This was repeated for 5 consecutive days with a booster two days later and 24 h before sacrifice (see scheme below). Sublingual administration was performed under anesthesia (mixture of 5% isoflurane in oxygen), to ensure proper delivery and prevent swallowing.

Peripheral lymph nodes (P-LN), submaxillary lymph nodes (SM-LN) and oral mucosa (OM) were excised and processed for flow cytometry. Lymph node cell suspensions were obtained by gentle mechanical disruption with a potter homogenizer until completely disaggregated. Oral mucosa cell suspensions were obtained by enzymatic digestion for 1 h with DNase (100 µg/mL), dispase (800 µg/mL), and collagenase (20 µg/mL) (Roche). Cells were stained with CD45, CD3, F4/80, CD29, MHC-II, and CD19 mAbs. Cells were gated based on forward/side scatter characteristics and their ability to exclude propidium iodide. Leukocyte populations were analyzed according to $CD45^+$ expression and, $CD3^+$ for T lymphocytes, $F4/80^+MHC\text{-}II^{lo}CD19^-$ for macrophages and $F4/80^-MHC\text{-}II^+CD19^-$ for dendritic cells. MSCs were identified as $CD45^-CD29^+Sca\text{-}1^+$ cells. For tissue histological analysis, oral mucosa was carefully removed, embedded in OCT compound (Thermo Fisher Scientific) and stored at -80°C until processing. Sections of 10 µm were blocked with 5% normal donkey serum, following the staining with anti-mouse CD45 and Hoechst 33342 (Invitrogen, Life Technologies) for staining the nuclei. For immunofluorescence analysis, preparations were mounted using FluorSave (Millipore) and imaged using a fluorescence microscope (Nikon Eclipse Ci) with a digital camera (Nikon DS-U3) and Nis-Elements D software. Images were assembled using ImageJ software.

LPS-Induced Inflamed Pad Mouse Model

FVB/NJ mice, sedated with isoflurane, received a single injection of 40 µg of LPS in 30 µL of PBS into the right pad. At the same time, 30 µL of PBS were injected into the left pad as control. The baseline measurement was determined by measuring the pad thickness of each mouse with a digital caliper before LPS administration. 24 h after LPS injection, 5×10^5 MSCs, treated as described above, were intravenously infused through the tail vein. To assess the efficacy of the different experimental groups of MSCs, the right pad thickness was measured 24, 48, and 72 h after LPS administration, comparing it to the control pad. At the end of the experiments mice were sacrificed by CO_2 inhalation. Footpads were extracted and processed for histological or flow cytometry analysis. Cell suspensions were obtained by gentle mechanical disruption with a potter homogenizer until completely disaggregated and were stained with CD45, CD3, F4/80 and Gr-1 mAbs to identify T-cells, inflammatory and classic macrophages, and granulocytes (45). For histological analysis, pad samples were fixed in buffered formalin and embedded in paraffin. Sections were stained with Gallego's Trichrome.

Transfer of MV130-Bacteria From MSCs to DCs

The transfer of MV130-bacteria from MSCs to DCs, generated as described above, was studied by flow cytometry and immunofluorescence. 2×10^4 MSCs were seeded in a 12-well plate and primed with MV130, CFSE-stained MV130 or none (control conditions). After 24 h for allowing the uptake of bacteria by MSCs, cultures were extensively washed and 2×10^5 DCs (1:10 MSC/DC ratio) were seeded. MSCs and DCs were co-cultured for 24 h and the presence of CFSE-MV130 in both populations was analyzed by flow cytometry using CD90 and CD1a, as markers for MSCs and DCs, respectively. For the immunofluorescence study, cells were placed on a chamber slide in a 1:10 ratio (7×10^3 MSCs/7×10^4 DCs). After fixing, cells were stained with phalloidin, HLA-DR and Hoechst as described below.

Migration Assays

Migration assays were performed in transwell inserts with 8 µm pore membrane (6.5 mm diameter; Costar). After migration, the upper and/or lower fractions were collected and suspended in the same volume. Quantification of cell number was performed by flow cytometry, acquiring all events gated according to forward/side scatter, for 180 s at a constant low flow rate. The percentage of migrated cells were calculated as follow:

(n° of cells present at the lower chamber/total cell number) x 100

To determine if MV130 priming modifies the migratory capability of MSCs, 10^5 MSCs primed with MV130 (10^6, 10^7 o 10^8 bacteria/mL) or excipient, were seeded in a transwell insert. Cell numbers in each fraction was quantified by flow cytometry after 20 h of culture, as described above.

To assess the possible chemotactic effect of MV130 on MSCs, 10^5 MSCs were seeded in a transwell insert and placed on a well with culture media with MV130 (10^7 bacteria/mL), glycerol or IFNγ (10 ng/mL). After 20 h of culture, the number of cells in the upper and lower fractions was quantified by flow cytometry and the percentage of migrating cells were calculated as described above.

To find out if MV130-MSCs have a chemoattractive effect on different leukocyte populations, 2×10^5 MV130-MSCs or CTRL-MSCs were seeded in 24-well flat-bottom culture plates. 10^6 PBMCs were added into the insert and cultured for 8 h. Migrating cells (present at the lower chamber) were collected and stained for CD14, CD3, CD19, CD56, HLA-DR, and CD90 for flow cytometry analyses. Transwell cultures without MSCs in the lower chamber were used as controls.

Viability Assays

For cell viability studies, 2×10^5 MSCs were cultured in a 12-well plate. The percentage of apoptotic ($Anex^+/IP^-$) and necrotic (IP^+) cells was analyzed by flow cytometry after 24 h of treatment, using annexin V conjugated with DY634 (Immunostep) and propidium iodide (Biolegend).

RNA Extraction and Gene Expression Analysis by qRT-PCR

MSCs were lysed to perform RNA purification using Absolutely RNA Microprep kit (Agilent Technologies) following the manufacturer's protocol. High capacity cDNA Reverse transcription kit (Applied Biosystems. Thermo Fisher Scientific) was used for the synthesis of the cDNA following the manufacturer's instructions. Quantitative real-time PCR (qRT-PCR) was performed using specific predesigned TaqMan Gene expression assays for different genes (Applied Biosystems) (**Supplemental Table S1**). All PCR reactions were set in duplicates using the TaqMan Gene Expression Master Mix (Applied Biosystems). The amplification and detection were performed using a 7.900HT Fast Real-time PCR System (Centro de Genómica, Complutense University of Madrid). ΔCT method was employed using *GNB2L1* as reference gene to normalize gene expression.

Protein Quantification

Production of different cytokines was measured in supernatants from MSC cultures and MSC-Monocyte or MSC-T lymphocyte co-cultures. Levels of TNFα, IL-10 (Biolegend), IFNγ and PGE2 (R&D) were determined by ELISA and levels of IL-6, CXCL8, CCL2, CXCL10 and VEGF-A was determined by Cytometric Bead Array (CBA, BD Bioscience). TGF-β1 production was determined by LegendPlex (Biolegend) according to manufacturer's instructions.

Immunofluorescence Analysis

7×10^3 MSCs were grown and treated with MV130 on chamber slides. After fixation with 4% paraformaldehyde and permeabilized with 0.05% saponin, nonspecific epitopes were blocked with PBS containing 10% donkey serum. Then, cells were sequentially incubated with the primary antibody for 45 minutes at room temperature: Texas Red-conjugated-Phalloidin (Thermo Fisher Scientific); anti-CD63 (46); anti-LAMP2 (Developmental Studies Hybridoma Bank at the University of Iowa); anti-HLA-DR (BD Biosciences); anti-paxillin (Sigma Aldrich) as appropriate. Next, cells were incubated for another 45 minutes with the appropriate secondary antibody: Alexa Fluor 594 conjugated donkey anti-mouse IgG; Alexa Fluor 488 conjugated donkey anti-rabbit IgG (both from Invitrogen, Life Technologies); DyLight 405 conjugated goat anti-mouse IgG (Jackson ImmunoResearch). Finally, a counter-staining of the nuclei was performed with Hoechst 33342 (Invitrogen, Life Technologies) for 10 minutes. After staining, preparations were mounted using FluorSave (Millipore) and imaged using a fluorescence microscope (Nikon Eclipse Ci) with a digital camera (Nikon DS-U3) and Nis-Elements D software. Images were assembled using ImageJ software.

Flow Cytometry

Before staining with specific antibodies, cells were incubated at 4ºC for 5 min with FcR Blocking Reagent (Milteny Biotec) to block nonspecific binding. Then, cells were stained with specific monoclonal antibodies (**Supplemental Table S2**) conjugated with different fluorochromes (Alexa Fluor 488, FITC, PE, PerCP, PE-Cy5, Alexa Fluor 647 or APC).

For the intracellular detection of Bcl-2, Bcl-x_L and Bax proteins, cells were treated with a FACS permeabilizing solution according

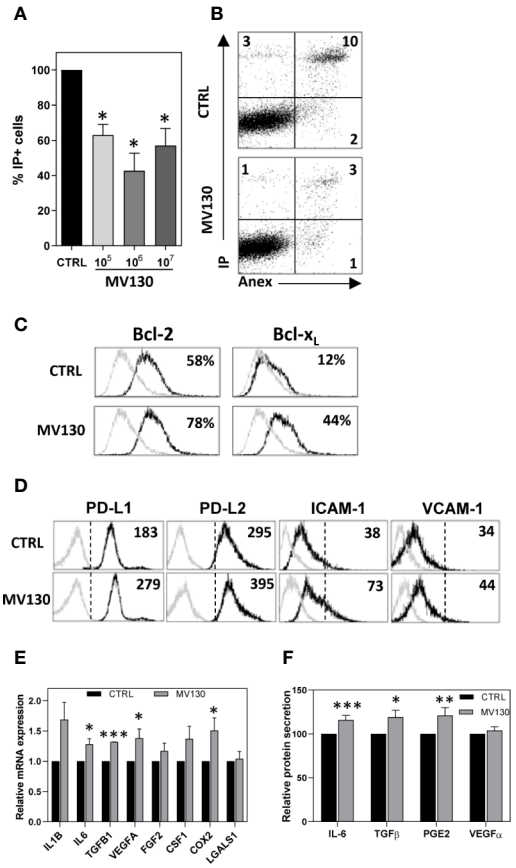


FIGURE 1 | Treatment with MV130 modifies biological properties of MSCs. MSCs were treated with MV130 for 2 h. **(A, B)** After 24 h, MSCs were stained with Annexin V and IP and cell viability was analysed by flow cytometry. Mean ± SEM of 5 independent experiments **(A)** and a representative experiment **(B)** are shown. **(C)** MSCs were treated with MV130 for 2 h. Bcl2 and Bcl-x_L expression in MSCs were analyzed by flow cytometry 24 h after treatment. The percentages of positive cells are indicated in each histogram. Gray filled histograms represent isotype control staining. Data are representative of 4 independent experiments. **(D)** 48 h after treatment, the expression of different surface markers was studied on MSCs by flow cytometry. Representative histograms and MFI values are shown (n = 3-4). Gray histograms represent isotype controls. **(E)** mRNA expression for different immunomodulatory factors was studied on MSCs by qRT-PCR. Data represent mean ± SEM of 8 to 12 independent experiments relative to individual controls. **(F)** Supernatants from MSC cultures were collected 48 h after treatment. Protein secretion relative to individual controls is expressed as mean ± SEM from 15 independent experiments (*$p < 0.05$; **$p < 0.01$***$p < 0.005$ by Wilcoxon test).

to the manufacturer's instructions (BD Biosciences), and stained with anti-human Bcl-2 or anti-human Bcl-x_L Abs (**Supplemental Table S2**) for 30 min. Analysis was performed on a FACSCalibur flow cytometer (BD Biosciences) (Centro de Citometría y Microscopía de Fluorescencia. Complutense University of Madrid) and analyzed with FCS Express V3 software.

Statistical Analysis

All data are expressed as mean ± SEM of the indicated parameter. Data analysis was performed using GraphPad Prism version 8.0.2. Statistical significance was determined by Wilcoxon test. Values of $^*p \leq 0.05$, $^{**}p \leq 0.01$, and $^{***}p \leq 0.005$ were considered to be statistically significant.

RESULTS

Effects of MV130 on MSC Biological Properties

We first analyzed the effect of MV130 on MSC survival. MV130 significantly increased the viability of MSCs (**Figures 1A, B** and **Supplemental Figure S1A**), increasing significantly Bcl-2 and Bcl-X_L antiapoptotic protein expression (**Figure 1C**). **Supplemental Figure S1B** shows that the expression of *NANOG* and *Oct-4* transcription factors, known to be involved in pluripotency and self-renewal of undifferentiated stem cells, were not affected. In addition, no significant differences on the adipogenic and osteogenic differentiation potential were seen when control and MV130-primed MSC cultures were compared (**Supplemental Figures S1C, D**). Thus, while MV130 favors MSC survival, no changes in their stemness and multipotent developmental properties were observed.

MV130 priming of MSCs did not change their expression of *TLR1*, *TLR2*, *TLR3*, *TLR4*, *TLR5*, *TLR6*, and *TLR9* (**Supplemental Figure S1E**), CD86, CD40, or HLA-DR (**Supplemental Figure S1F**) under any of the assayed conditions. CD73, an ectoenzyme with powerful anti-inflammatory properties, was maintained highly expressed in MV130-treated MSCs (**Supplemental Figure S1F**). Relevantly, upon MV130 priming, MSCs significantly upregulated the expression of others immunosuppressive molecules such as PD-L1 and PD-L2, as well as the adhesion protein ICAM-1, key player in MSC-mediated functions (**Figure 1D**). Interestingly, MV130-primed MSCs exhibited enhanced mRNA expression of *IL6, TGF 1, VEGFA* and *COX2* (**Figure 1E**), confirmed at protein level for IL-6, TGF-β1, and for the major COX2 product, PGE2, as detected in MSC culture supernatants (**Figure 1F**).

MV130-Bacteria Are Uptaken by Oral MSCs *In Vivo* and Reduce Their Migration *In Vitro*

To determine whether resident MSCs present in the oral mucosa could be responding to MV130 treatment, mice were administered sublingually with MV130-bacteria labeled with CFSE. Flow cytometry analysis of cell suspensions obtained from the excised oral mucosa indicated that MSCs could uptake MV130-bacteria efficiently *in vivo*. As shown in a representative experiment (**Figure 2A**), the percentage of $CD45^-$ $CD29^+$ $Sca1^+$ MSCs uptaking MV130 (41%) was significantly higher to that seen (28%) in MHC-II^+/F4/

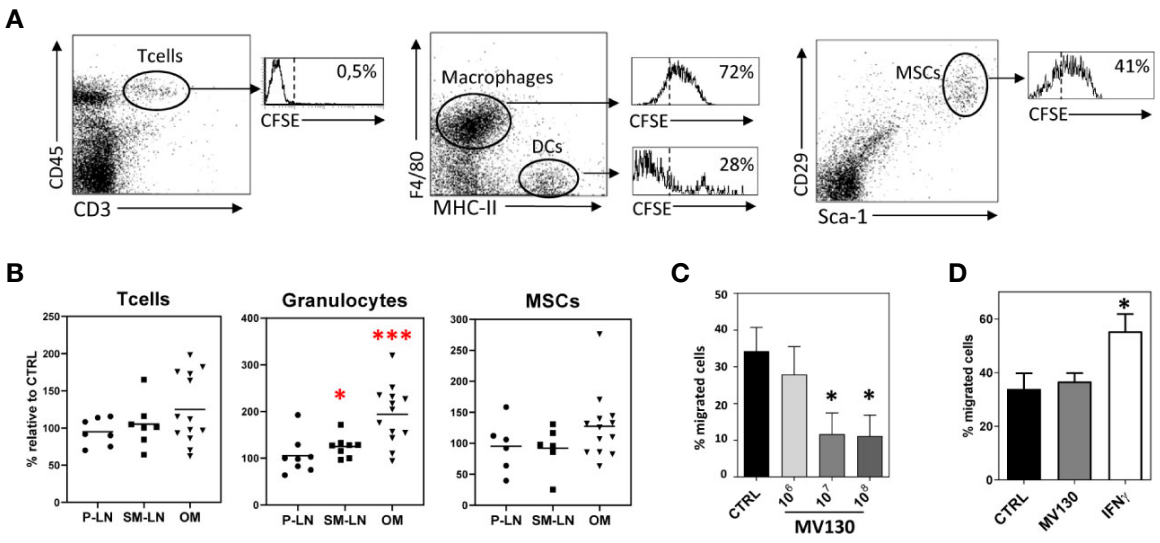


FIGURE 2 | MV130 is uptaken by oral MSCs *in vivo*. **(A)** Flow cytometry analysis of T lymphocytes ($CD45^+CD3^+$), macrophages ($F4/80^+MHC\text{-}II^{lo}$), DCs ($F4/80^-MHC\text{-}II^+$) and MSCs ($CD29^+Sca\text{-}1^+$) present in the oral mucosa from mice after sublingual immunization with MV130-CFSE. Histograms show the percentage of uptake of CFSE-MV130 by each of these populations. Data are representative of 3 independent experiments. **(B)** Percentage of T cells, granulocytes and MSCs present in peripheral lymph nodes (P-LN), submaxillary lymph nodes (SM-LN) and oral mucosa (OM) from mice after sublingual immunization with MV130 respect to control mice (n = 6–13). **(C)** MV130 priming reduces MSC migration capacity. Bar graph shows the percentage of migrating cells in MV130 primed cultures. Results represent the mean ± SEM of 3 independent experiments **(D)** MV130 does not specifically attract MSCs. Bar graph shows the percentage of migrating cells to MV130 or IFNγ. Results represent the mean ± SEM of 4 independent experiments. ($^*p < 0.05$; $^{***}p < 0.005$ by Wilcoxon test).

80^{-}CD19^{-} cells (mostly DCs) but lower than in MHC-II^{lo}/F4/80^{+} macrophages (72%). The relative numbers of MSCs and T cells within the oral mucosa remained unchanged upon MV130 treatment, in contrast to the significant recruitment of granulocytes from extramucosal tissues (**Figure 2B** and **Supplemental Figure S2A**).

As no active recruitment of mucosal MSCs was seen in the *in vivo* mouse model after MV130 treatment, we were prompted to explore the migration features of human MSCs primed *in vitro* with MV130. As shown in **Figure 2C**, the migration ability of MV130-primed MSCs was significantly decreased relative to control MSCs. Also, conventional migration assays showed that MV130 did not represent a chemotactic stimulus for MSCs, unlike IFNγ used as positive control (**Figure 2D**). In consonance with the migration assays, the immunofluorescence study revealed that MV130-primed MSCs showed characteristics of slow-moving cells with sparse and large streak-like focal adhesion complexes at the periphery and a low polymerization degree of F-actin, whereas control MSCs showed small dot-like nascent focal adhesion sites and polymerized actin at the ruffles (**Supplemental Figure 2B**). Thus, the results indicate that MSCs may uptake MV30-bacteria while decreasing their migratory activity.

MSCs Act as Reservoirs of MV130 and Are Able to Transfer it to DCs

Next, we studied the fate of MV130-bacteria uptaken by MSCs. To this end, cells were treated 24 h with CFSE-labeled MV130 and then extensively washed to remove extracellular bacteria. Bacteria-containing MSCs were monitored by flow cytometry and studied by immunofluorescence at different times. After treatment, about 60-85% of MSCs showed fluorescent staining. Although this percentage gradually decreased in the following days, around 50% of the MSCs still showed significant levels of fluorescence 5 days later (**Figure 3A**). As shown under microscopy (**Figure 3B**), MV130-bacteria accumulated in the vesicular compartment near the plasma membrane at the beginning, suggesting their

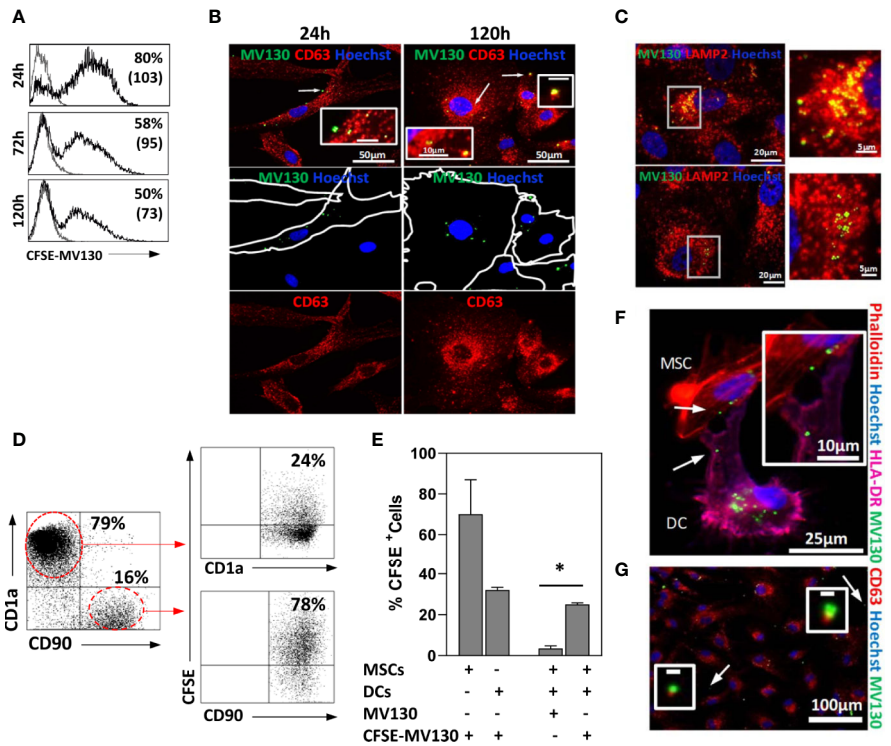


FIGURE 3 | MSCs act as reservoirs of MV130 and are able to transfer it to DCs. MSCs were treated for 24 h with MV130 labeled with CFSE (CFSE-MV130; green) for monitoring. After washing cells to remove the drug, its uptake, processing and transference to DCs were studied. **(A)** Uptake and maintenance of CFSE-MV130. Histograms show the percentage of positive cells and MFI in brackets (n = 6–8 independent experiments). **(B, C)** Spatiotemporal monitoring of MV130 (green) in MSCs. Immunostaining in red for CD63 **(B)** or LAMP2 **(C)** in MV130-MSCs at different times. Inset indicated by arrows in B, scale bar: 10µm (24 h) or 1µm (120 h). Right: Higher magnification of the white square in **(C)** (120 h); examples of LAMP2-positive compartments with CFSE-MV130. Hoechst was used for nucleus staining (blue). Images are representative of 5 independent experiments. **(D–G)** DCs differentiated from monocytes were co-cultured with MSCs, previously treated with MV130 or CFSE-MV130 as described in Material & Methods section, and transfer of MV130 from MSCs to DCs was studied. A representative dot plot **(D)** and the mean ± SEM of five independent experiments **(E)** are shown. DCs and MSCs were gated according to CD1a or CD90 expression, respectively. In **(E)** control MSCs and DCs directly treated with CFSE-MV130 are also shown. **(F)** MV130 transfer from MSCs to DCs studied by immunofluorescence. Hoechst was used for nucleus staining in all cases (blue). Co-cultures were labeled with phalloidin (red) and anti-HLA-DR (magenta). The absence of HLA-DR expression on MSCs allow to distinguish it from DCs (HLA-DR^{+}). White arrows in **(F)** indicate area of insert image magnification. **(G)** MSCs were labeled with anti-CD63 (red) and Hoechst was used for nucleus staining (blue). Co-localization of CD63^{+} extracellular microvesicles with CFSE-MV130 was observed free in the medium. White arrows indicate area of insert image magnification. Representative images of 3 independent experiments (*$p < 0.05$ significance by Wilcoxon test).

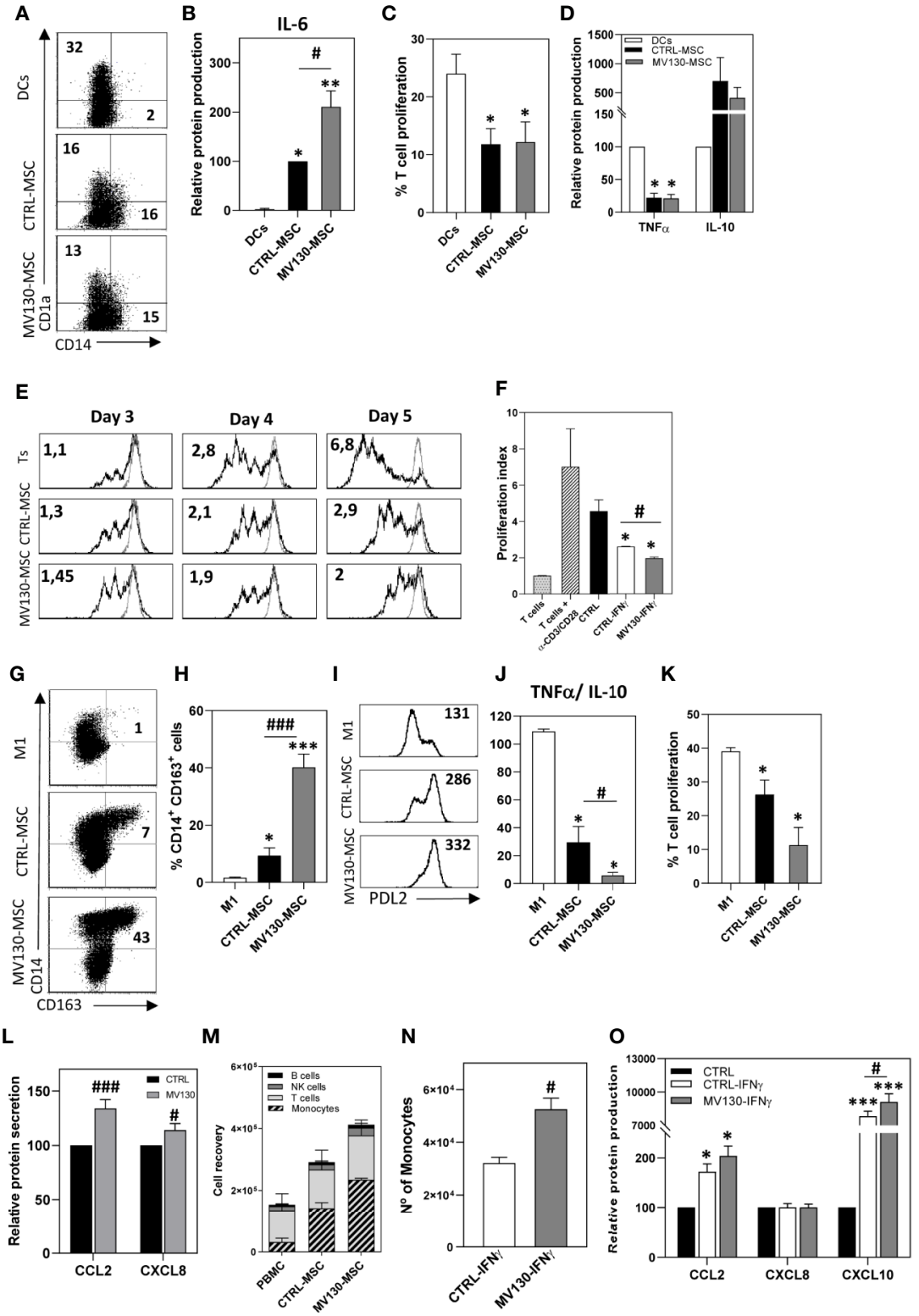


FIGURE 4 | Continued

FIGURE 4 | Immunomodulatory abilities of MSCs after activation with MV130. **(A–D)** Phenotype and function of monocyte-derived DCs differentiated in the presence or absence of CTRL-MSCs or MV130-MSCs. At day 6, CD1a and CD14 expression were analyzed by flow cytometry in the $CD90^-$ population. The percentage of positive cells is shown in each plot **(A)** and IL-6 production was measured in the supernatants **(B)**. Results represent the mean ± SEM (n = 6). **(C, D)** DCs stimulated with LPS were cultured in MLR assays with CFSE-labeled T lymphocytes. After 5 days, the percentage of proliferating T cells was calculated by CFSE dilution method (gated on $CD3^+$ cell population) **(C)** and supernatants from MLR co-cultures were analyzed for TNFα and IL-10 protein secretion **(D)**. Data represent the mean ± SEM (n = 3–5). **(E)** Control or MV130-MSCs were co-cultured with CFSE-labeled T lymphocytes stimulated with CD3/CD28 beads, for different times. Histograms show CFSE staining in proliferating T cells in $CD3^+$ gated cells. Proliferation index referred to unstimulated T lymphocytes (gray line) is indicated. Data are representative from four independent experiments. **(F)** MV130-MSCs re-stimulated with IFNγ, following protocol described in Material and Methods, were co-cultured with CFSE labeled T lymphocytes. Proliferation index is shown. Bar graph shows mean ± SEM (n = 4). **(G–K)** Control and MV130 primed MSCs were co-cultured with monocytes in the presence of GM-CSF to induce M1 macrophage differentiation. Monocytes alone were cultured as M1 control. **(G–I)** After 6 days, CD14, CD163 and PD-L2 expression was determined by flow cytometry in non-MSC population ($CD90^-$ cells) (n = 5–6). A representative experiment **(G)** and mean ± SEM of percentage of $CD14^+CD163^+CD90^-$ cells from five to six independent experiments **(H)** are shown. **(I)** Representative PD-L2 expression on macrophages. MFI is shown in each histogram. **(J)** After 6 days of co-culture, LPS was added and supernatants were analysed for TNFα and IL-10 production. Data represent TNFα/IL-10 ratio production at the different experimental conditions (mean ± SEM; n = 4). **(K)** Macrophages stimulated with LPS were used to carry out MLR cultures with CFSE-labeled T lymphocytes. After 5 days, the percentage of T cell proliferation was measured in the $CD3^+$ cell population. **(L)** CCL2 and CXCL8 protein secretion measured in control and MV130-MSC culture supernatants. Bars represent the mean ± SEM relative to individual controls from 15 independent experiments. **(M)** PBMCs were placed in a transwell insert while MSCs, treated with or without MV130 for the 24 h previous, and seeded in the bottom chamber. After 8 h, migrating PBMCs (present in the lower chamber) were collected and stained for CD14, CD56, CD3, HLA-DR, and CD19, and different leukocyte populations were analyzed by flow cytometry. MSCs were excluded from the analysis by CD90 expression. (mean ± SEM; n = 4) **(N)** PBMCs migrating toward control or MV130 primed MSCs re-stimulated with IFNγ. Monocyte recruitment was analyzed by flow cytometry (mean ± SEM, n = 4). **(O)** Supernatants from MSC cultures following the protocol described in Material & Methods section were analyzed for CCL2, CXCL8, and CXCL10 protein secretion after IFNγ re-stimulation. Results represent mean ± SEM of four to six independent experiments relative to individual controls. ($^*p < 0.05$; $^{**}p < 0.01$, $^{***}p < 0.005$ significances relative to M1-macrophages or DC; $^{\#}p < 0.05$; $^{\#\#\#}p < 0.005$ significances relative to CTRL-MSCs by Wilcoxon test).

internalization into early endosomes. Later (120 h) they appeared translocated to the late endosomal/lysosomal compartments as indicated by the co-localization with CD63 and to a lesser extent with LAMP2 markers (**Figures 3B, C**). These results indicate that MSCs are able to internalize, process and maintain the MV130-bacteria over time.

As it has been shown that MSCs may retain TLR2 ligands and transfer them to immune cells (47), we were prompted if this might be also the case with MV130-bacteria. Thus, MSCs were treated with CFSE-labeled MV130, extensively washed to remove unbound bacteria and co-cultured with monocyte-derived DCs. After 24 h, a notable proportion (around 25%) of DCs co-cultured with MSCs showed significant labelling with CFSE-conjugated MV130, compared to only 2% of background staining (**Figures 3D, E**). Immunofluorescence studies of MSC-DC co-cultures showed that CFSE-labeled bacteria could be transferred from MSCs to neighboring DCs through transient cytoplasmic extensions (**Figures 3F**). In addition, CFSE-MV130 conjugates from MSCs could also reach DCs through extracellular vesicles since in the co-cultures it was possible to observe $CD63^+$ vesicles loaded with CFSE-conjugated MV130 (**Figure 3G**). Interestingly, in control experiments using only DCs, a direct uptake of CFSE-MV130 by these cells was around 35%, whereas it reached to around 70% when only MSCs were used (**Figure 3E**), which underlined the high ability of MSCs to uptake MV130-bacteria.

Priming MSCs With MV130 Promotes Their Immunomodulatory Features Enhancing the Induction of M2-Like Macrophages

Both control and MV130-primed MSCs similarly dampened the generation of $CD14^-$ $CD1a^+$ DCs from $CD14^+$ $CD1a^-$ monocytes (**Figure 4A**). DCs induced in the presence of MV130-primed MSCs showed a slight but constant increase in the expression of HLA-DR, PD-L1 and PD-L2, while decreasing CD86 co-stimulatory molecule expression (**Supplemental Figure S3A**) and inducing a significant increase of IL-6 production (**Figure 4B**). These DCs showed a similarly reduced allostimulation ability in co-cultures with T cells (**Figures 4C, D**).

To assess the effects of the priming of MSCs with MV130 on the adaptive immune response, the ability of MSCs to modulate TCR-triggered activation of T cells was also investigated by proliferation assays. As shown in **Figure 4E**, in the presence of MSCs, T cell proliferation was slightly increased on day 3 while significantly suppressed on day 5. These enhancing and suppressing effects were more evident when MV130-primed MSCs were used, reaching signification on day 5 (**Figure 4E** and **Supplemental Figure S3B**). On the other hand, to test whether this response was transient or could be maintained for a prolonged period of time, MSCs were primed with MV130 for 24 h, extensively washed to remove extracellular bacteria and then stimulated with IFNγ on day 4. **Figure 4F** shows that CD3/CD28-induced T-cell proliferation was inhibited by IFNγ-activated MSCs in a greater extent than non-activated MSCs; yet, such an inhibition was significantly higher using MSCs primed with MV130.

We also addressed whether MV130 priming could influence the effects of MSCs on the repolarization of monocytes toward M2-like macrophages. As shown in **Figures 4G–I**, monocytes undergoing differentiation to macrophages in the presence of MSCs showed a M2-like pattern. This effect was more notable in cultures with MV130-primed MSCs, where the proportion of $CD14^{high}$ $CD163^{high}$ PD-$L2^+$ macrophages generated was significantly increased respect to control MSC cultures (**Figures 4G–I**). After 6 days of differentiation, macrophages generated in the different cultures were activated with LPS and the cytokine production was analyzed. As shown in **Figure 4J**, macrophages differentiated in the presence of MSCs showed a reduced pro-inflammatory TNFα/anti-

inflammatory IL-10 cytokine production ratio when compared with control M1-like macrophages, and this reduction was more pronounced in macrophages generated in the presence of MV130-primed MSCs with significantly increased IL-10 levels ($p<0.05$). In addition, macrophages generated in the presence of MV130-MSCs induced a lower proliferative response of T cells than their control counterparts (**Figure 4K**). As recent studies have demonstrated that MSC-derived CCL2 is required for polarizing IL-10$^+$ tissue macrophages (48–50), the effect of priming MSCs with MV130 on its production was studied. As shown in **Figure 4L**, CCL2 production was upregulated in MV130-primed MSCs at both mRNA and protein levels (**Supplemental Figure S3C**). This was also the case for CXCL8 and CXCL12 (**Figures 4L** and **Supplemental Figure S3C**). Since CCL2, CXCL12 and CXCL8 are the major chemokines driving monocyte extravasation, chemotaxis assays were performed with PBMCs in a transwell system. The results showed that monocytes were the main cell type migrating at higher numbers toward the compartment with MV130-primed MSCs, as compared with untreated MSCs (**Figure 4M** and **Supplemental Figure S3D**). In addition, priming of MSCs with MV130 also modified their capacity to recruit monocytes upon IFNγ re-stimulation (**Figure 4N**). Again, this effect was accompanied by an increase in CCL2, and also CXCL10, chemokine production (**Figure 4O**).

Effects of MV130-Primed MSCs in an *In Vivo* Model of Acute Inflammation

As both MSCs and M2-like macrophages are associated with wound healing and tissue repair, we were prompted to test whether MV130-primed MSCs may have a differential effect in an *in vivo* model of acute inflammation. To this end, mice were injected in the footpad with LPS alone or with control or MV130-primed MSCs 24 h later. Both MSCs significantly reduced the LPS-inflammatory response, measured by footpad thickness, without differences between MV130-primed and control MSCs (**Figure 5A**). However, the histological analysis showed a clear reduction of leukocyte infiltration in those mice co-injected with MSCs primed with MV130 (**Figure 5B**). Flow cytometry analysis of the excised tissues indicated that leukocyte infiltration was markedly decreased by the treatment with MV130-primed MSCs, in comparison to that using control MSCs (**Figure 5C**). As shown, granulocytes and inflammatory macrophages were reduced by 50–60% and 40–50%, respectively (**Figure 5C**).

DISCUSSION

The oral mucosa constitutes an essential body barrier constantly exposed to both potentially harmful and harmless antigens.

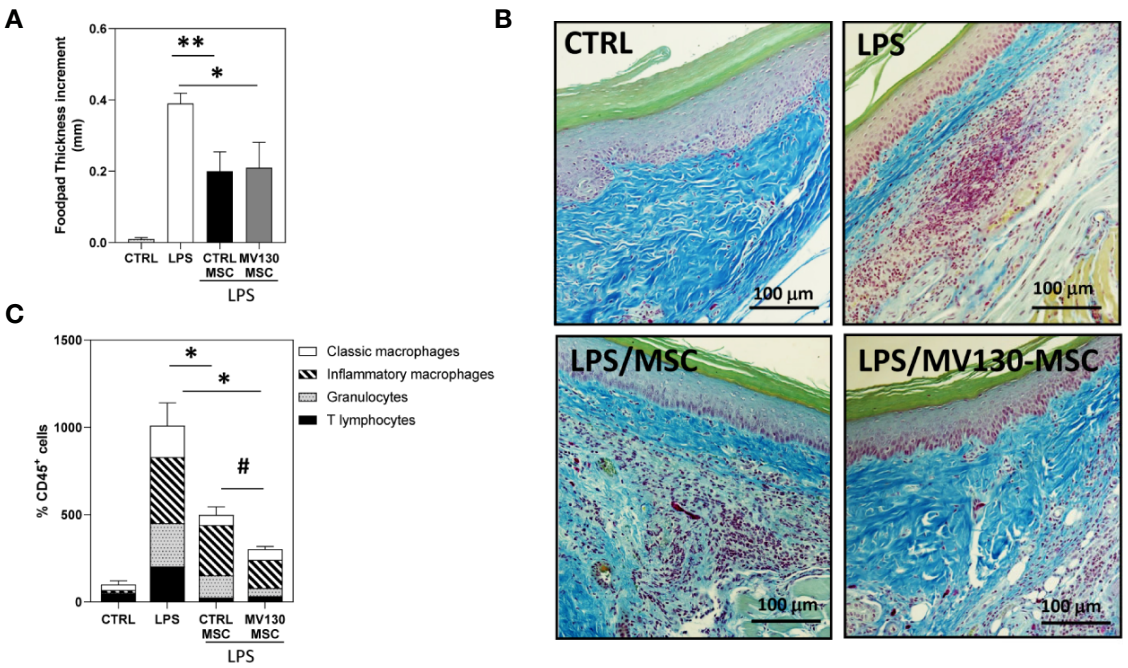


FIGURE 5 | Effects of MV130-MSCs in an *in vivo* model of acute inflammation. FVB/NJ mice were challenged in the footpad with 40µg of LPS and administered with or without control or MV130-primed MSCs 24 h later. **(A)** Footpad thickness increment was determined after 72 h as a measure of the efficacy of the different experimental groups of MSCs. Data shown are mean ± SEM of two independent experiments (three mice per group) **(B)** Images show histological sections of footpad tissue stained with Gallego's Trichrome. Images are representative of 3 mice per group. **(C)** Percentage of CD45$^+$ leukocytes infiltrating footpads in the different mouse groups analyzed by flow cytometry. The distribution of the different leukocyte subpopulations in CD45$^+$ cells is also shown in each experimental group. Results represent increments relative to control animals (2 independent experiments with 3 mice per group) (*$p < 0.05$,**$p < 0.01$ versus LPS alone; $^{\#}p < 0.05$ versus CTRL-MSCs; by Wilcoxon test).

Histologically consists of a stratified squamous epithelium and a connective tissue, the lamina propria, where specialized immune cells function providing protection from pathogens and tolerating commensal microorganisms (51). However, in the last years some studies have pointed out the relevance of oral mucosal non-immune cells, such as MSCs, in the maintenance of tissue homeostasis and also in the immune reactions, producing anti-microbial factors and finely regulating the response to pathogens (20, 26, 27).

Numerous groups have reported that MSCs are equipped with a large variety of TLRs capable to act as sensors of exogenous stimuli, and also endogenous signals related to tissue damage or inflammation (33). In addition, Iwamura et al. have recently demonstrated the NOD1 expression in bone marrow MSCs (52). This arsenal of PRRs may then be essential for MSC recognition of the PAMP ligands from the polibacterial preparation used in the present study, similar to that described for dendritic cells (17). Although it has been previously shown that the TLR expression pattern could be modulated by several environmental conditions, including hypoxia or inflammation (33), our mRNA expression study reveals that MV130 treatment does not modify TLR expression in MSCs, at least in basal conditions. There is currently no consensus on whether TLR activation can affect MSC differentiation capacities, what seems to depend on the type of TLR ligand (53), but an important issue in the context of vaccination with MV130 is the fact that MSCs primed with MV130-bacteria maintain their stemness and multilineage potential. Also, MV130 primed MSCs maintain low immunogenicity due to the lack of HLA class II and co-stimulatory molecule expression after treatment, and in agreement several authors have described that the activation of different TLRs has no significant effect on the immunogenic properties of MSCs (53, 54).

An important proportion of MSCs found in the lamina propria of the oral mucosa contains the polybacterial preparation after sublingual immunization of mice with MV130. How the bacterial preparation reaches MSCs located in the mucosa connective tissue is unknown, but DCs infiltrating the epithelium could be involved in the transfer of bacterial components to MSCs (data not shown) (55). Previous work focused on the distribution and effectiveness of DCs after sublingual vaccination with cholera toxin showed that MHC class II^+ cells were quickly recruited to the sublingual mucosa, where they processed the antigens and immediately transported them *via* afferent lymph to draining lymph nodes (56). Unlike DC behavior, our *in vivo* results point out that, after MV130 administration, oral-resident MSCs remain in the mucosa, in correlation with the *in vitro* assays showing inhibition of MSC migratory abilities after MV130 treatment. To this respect, it has been demonstrated that stimulation of some TLRs could inhibit MSC migration, thus facilitating their local action (57, 58). Our results show that MSCs, apart from capturing the components of the polybacterial preparation, can process and retain a reservoir of the TLR ligands derived from MV130 digestion. Previous work has described the capacity of MSCs to uptake and process proteins although, unlike antigen presenting cells, they do not stimulate directly alloreactive T cells (59). Additionally, Weinstock et al. showed that MSCs were able to retain, in a long-term manner, TLR2 ligands which were subsequently released and transferred to immune cells, inducing a pro-inflammatory response (47). Our data also indicate that MSCs can transfer processed MV130 components to DCs either directly, through cell-to-cell contacts, or indirectly through extracellular vesicles. This fact could contribute significantly to the described beneficial effects of MV130 in the control of recurrent infections, since DCs could be activated more quickly and by a lesser amount of TLR ligands in subsequent infections.

The immunoregulatory capacity of MSCs is largely governed by the local inflammatory intensity, being able to promote an inflammatory response or, conversely, prevent an excessive immunoreaction (60). A complex balance among opposite stimuli guide this striking functional plasticity of MSCs, allowing to adapt MSC responses according to time and course of infection (28). Based on these properties, previous studies have shown that different priming approaches to empower MSCs, including pro-inflammatory cytokines, hypoxia or TLR ligands, modify their immunophenotypic and secretome profiles (61), and as consequence their immunoregulatory effects. In this context, our results show that MV130 treatment of MSCs maintains such plasticity improving each of the immunoregulatory faces of MSCs. Priming with MV130 seems to increase the previously described pro-inflammatory activities of MSCs during early-stage inflammation, initially favoring leukocyte recruitment, primarily monocytes and granulocytes, through the enhanced production of chemokines, as described by Waterman et al. after TLR4 triggering (31). The upregulated expression of adhesion molecules, such as ICAM-1, observed in MV130-primed MSCs could also facilitate close interactions between MSCs and leukocytes, including T cells. MV130 priming of MSCs initially leads to an increased T-cell activation, presumably as a consequence of the higher production of pro-inflammatory cytokines and chemokines (IL-6, CXCL8 and CCL2). TLR4 activation in MSCs has been described to produce similar effects to these reported in our study (31, 62). As proposed by the *licensing* model, the expression levels of inflammatory cytokines, including IFNγ produced by T-cells, determine the immunosuppressive activity of MSCs as shown in different preclinical models (50, 63, 64). In our study, the threshold levels controlling the change in MSC behavior would be reached in the MV130-primed MSC/T cell co-cultures faster than in the control cultures, causing the greater inhibitory effect observed on T cell proliferation. Our data also indicate that MV130-primed MSCs are able to respond more intensively when are subsequently exposed to an inflammatory microenvironment, inhibiting T cell activation. As Liu and colleagues showed, MSCs exhibit short-term memory when are exposed for a second time to danger signals, suggesting that trained immunity could be also carried out by these non-professional immune cells (65). As described for other cells

(14), MV130 treatment imprint an innate immune memory-like in MSCs allowing them to give a better response to a subsequent damage stimulus.

On the other hand, we found that the priming of MSCs with MV130 induces a higher expression of IL-6, a known negative regulator of DC differentiation and function (66, 67). This could explain the changes observed in the phenotype of DCs generated in the presence of MV130-primed MSCs, exhibiting higher levels of PD-L1 and PD-L2 inhibitory molecules. However, these DCs generated in the presence of MV130-treated MSCs show a drastically reduced allostimulatory capacity, very similar to those DCs cultured with control MSCs.

Different preclinical models have described a pivotal role for tissue macrophages as part of the therapeutic response to MSCs, contributing to their anti-inflammatory effects (38, 50, 68). An important result of the present study is the impact of MV130 priming of MSCs in macrophage polarization and function. MV130-primed MSCs altered monocyte differentiation during M1-like polarization, inducing highly immunosuppressive M2-like macrophages associated with reduced TNFα and increased IL-10 production together with a higher expression of PD-L2 inhibitory molecule. Similar to that previously discussed for T cell activation, the production of pro-inflammatory cytokines by monocytes during their M1-like polarization would constitute a stronger activation stimulus for MV130-primed MSCs than for control MSCs, triggering the release of mediators that skew the differentiation of monocytes toward a more anti-inflammatory profile. Mechanistically, the activation of the COX2/PGE2 pathway, as well as the increase of both PD-L2 expression and mainly CCL2 production in MV130-primed MSC cultures, could favor the promoting effects on M2 polarization, as has been described in other systems (69, 70).

In vivo and *in vitro* studies have demonstrated that MSC-derived CCL2, apart from its role in chemotaxis, has a pivotal immunosuppressive function and is required to polarize macrophages toward an IL-10^{+} M2-like phenotype (48–50). MSC-derived MMPs are able to proteolytically process CCL2 to generate an N-terminal-cleaved form with anti-inflammatory functionality (48). Alternatively, CCR2 ligands may heterodimerize and form oligomers which induce MCP-induced proteins (MCPIP1-4), able to specifically promote IL-6 mRNA decay and upregulate M2-associated *c-Maf* gene favoring M2-like macrophage polarization (50, 71, 72). Thus, it could be hypothesized that priming MSCs with MV130 would be advantageous for the resolution of inflammation, also through the enhanced monocyte recruitment and subsequent differentiation to IL-10^{+} anti-inflammatory macrophages. In this sense, in the *in vivo* LPS acute inflammation model used in the current study, the anti-inflammatory effects were accentuated when MSCs had been pretreated with MV130 reducing drastically leukocyte infiltration, mainly inflammatory macrophages and granulocytes. These results suggest that MV130 priming allows MSCs to reach their activation threshold more quickly and thus exert their anti-inflammatory effects more efficiently, limiting macrophage activation to avoid excessive tissue damage.

CONCLUSION

Resident oral mucosa MSCs are able to uptake, process and retain a reservoir of the TLR ligands derived from MV130 digestion. MV130 treatment of MSCs improves each of the immunoregulatory faces of MSCs. Initially during early-stage

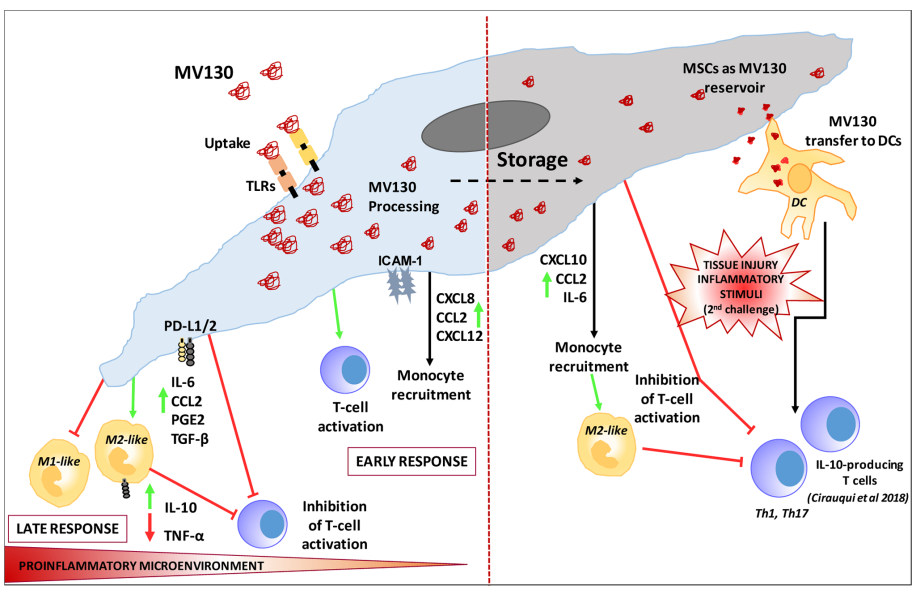


FIGURE 6 | Proposed Model of MSC involvement in oral mucosal bacterial immunotherapy with MV130.

inflammation, MV130-primed MSCs favors leukocyte recruitment and T-cell activation, through the enhanced production of chemokines. Once exposed to sufficient levels of pro-inflammatory cytokines, MV130-primed MSCs respond by adopting an improved immune-suppressive phenotype, to dampen inflammation and avoid excessive tissue damage, reducing T lymphocyte proliferation, and mainly favoring IL-10^+ M2-like macrophage differentiation through upregulation of immune-supressor factors (CCL2, PGE2, TGF-β, and PD-L1/2). MV130-primed MSCs show additional features that can be associated to trained immunity since they modify their response to a secondary inflammatory stimulation, inhibiting more efficiently T cell activation and producing higher levels of CXCL10 and CCL2 chemokines. Furthermore, MSCs can transfer processed MV130 components to DCs allowing them to be activated more quickly and by a lesser amount of TLR ligands in an inflammatory microenvironment. Therefore, we propose that MSCs could be involved in the observed clinical benefits of MV130 (**Figure 6**).

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

All experimental procedures were carried out according to Spanish and European regulations (Spanish RD 53/2013 and Law 6/2013, European Directive 2010/63/UE). Procedures were approved by the CIEMAT Animal Experimentation Ethical Committee according to approved biosafety and bioethics guidelines.

AUTHOR CONTRIBUTIONS

AVá performed research, experimental animal model, data analysis, and wrote manuscript. LF-S performed research. EJ, DP-C and RY performed experimental animal model. JS final writing and editing. AVa designed research, performed data analysis and final writing and editing. JV designed research, supervised the study, performed research, data analysis, and wrote the manuscript. AVi designed research, supervised the study, performed data analysis, and wrote the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.567391/full#supplementary-material

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REVIEW
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Induction of Trained Immunity by Recombinant Vaccines

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Vaccines represent an important strategy to protect humans against a wide variety of pathogens and have even led to eradicating some diseases. Although every vaccine is developed to induce specific protection for a particular pathogen, some vaccine formulations can also promote trained immunity, which is a non-specific memory-like feature developed by the innate immune system. It is thought that trained immunity can protect against a wide variety of pathogens other than those contained in the vaccine formulation. The non-specific memory of the trained immunity-based vaccines (TIbV) seems beneficial for the immunized individual, as it may represent a powerful strategy that contributes to the control of pathogen outbreaks, reducing morbidity and mortality. A wide variety of respiratory viruses, including respiratory syncytial virus (hRSV) and metapneumovirus (hMPV), cause serious illness in children under 5 years old and the elderly. To address this public health problem, we have developed recombinant BCG vaccines that have shown to be safe and immunogenic against hRSV or hMPV. Besides the induction of specific adaptive immunity against the viral antigens, these vaccines could generate trained immunity against other respiratory pathogens. Here, we discuss some of the features of trained immunity induced by BCG and put forward the notion that recombinant BCGs expressing hRSV or hMPV antigens have the capacity to simultaneously induce specific adaptive immunity and non-specific trained immunity. These recombinant BCG vaccines could be considered as TIbV capable of inducing simultaneously the development of specific protection against hRSV or hMPV, as well as non-specific trained-immunity-based protection against other pathogenic viruses.

Keywords: recombinant BCG, trained immunity, unspecific cross-protection, respiratory syncytial virus, metapneumovirus

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INTRODUCTION

Historically, immunological memory development is a characteristic attributed only to the adaptive immune response in an antigen-specific manner. It was recently shown that the innate immune system could develop a type of non-specific immune memory known as "trained immunity" (1). Trained immunity is developed by innate immune cells, such as monocytes, macrophages, and natural killer (NK) cells, after an infection or vaccination (2, 3). Indeed, the development of trained

immunity occurs at the hematopoietic stem cells level in the bone marrow, specifically inducing a trained phenotype in myeloid progenitors (4, 5). Epithelial cells can develop a trained phenotype and show an enhanced inflammatory response when exposed to a secondary pathogen, which has been proposed to be associated with epigenetic regulation (6, 7). Trained immunity is induced by β-glucan (8), *Candida albicans* (9), and live vaccines like *Bacillus* Calmette-Guérin (BCG) (10), among others. The exposure to the infectious agent induces innate immune cells to undergo epigenetic modifications in certain pro-inflammatory genes, leading to a "trained" state, which allows the cell to respond in a faster and stronger way against an infection (**Figure 1**) (3, 10).

Trained immunity confers protection against a wide variety of pathogens, including bacteria (11), fungi (3), viruses (12), and protozoan (8). After developing trained immunity in mice, protection is induced against *Escherichia coli, Listeria monocytogenes, Staphylococcus aureus, Citrobacter rodentium*, and *Pseudomonas aeruginosa* (11). In humans, trained monocytes secrete higher levels of interleukin (IL)-1β, Tumor necrosis factor (TNF)-α, and interferon (IFN)-γ when stimulated with *Mycobacterium tuberculosis, S. aureus*, and *C. albicans* as compared to naïve monocytes (3). BCG vaccination before an experimental viral challenge with yellow fever virus, reduces viremia levels due to the development of trained immunity, with a crucial role for IL-1β (12). Moreover, when stimulated with *Leishmania braziliensis*, trained human macrophages secrete higher amounts of pro-inflammatory cytokines compared to naïve macrophages (8). In mice, the induction of trained immunity is sufficient to protect against the infection with *L. braziliensis*, being IL-1β signaling pathways and IL-32 crucial for this protection (8).

The unspecific immunological memory developed by trained innate immune cells can persist at least three months after vaccination (3). Such an effect on the innate immune system persists one year after vaccination, showing IL-1β and TNF-α production levels significantly higher as compared to non-trained cells after *in vitro* stimulation with LPS (13). Also, the fact that trained immunity is developed at hematopoietic stem cell level supports the notion that it might last for extended times *in vivo* (4, 5). Nevertheless, comprehensive long-term studies are necessary to elucidate how long the trained state persists. Importantly, BCG vaccination increases childhood survival during the first five years of life (14, 15), and the heterologous protection induced by this vaccine has been suggested to persist for several years (16).

TRAINED IMMUNITY INDUCED BY BCG

Vaccines are designed to induce adaptive immunological memory against specific pathogens (17, 18). However, the recent realization that some vaccines can also induce non-specific immunological memory *via* trained immunity suggests that TIbVs could be considered candidates to protect against pathogens with no specific vaccine. Nevertheless, under what circumstances, non-specific TIbVs could be considered a good strategy for promoting immune protection? A good example are the seasonal outbreaks caused by respiratory viruses that lack commercially available effective vaccines. In this scenario, the ability of some vaccines to promote trained immunity and protect against unrelated pathogens would be relevant to induce an innate immune response that readily works in a regulated manner upon exposure to unrelated pathogens.

The BCG vaccine (an attenuated strain of *M. bovis*) has been widely used for over a century to prevent the disease caused by *M. tuberculosis* (19). Since the beginning of its administration to humans, BCG has reduced the mortality of children due to

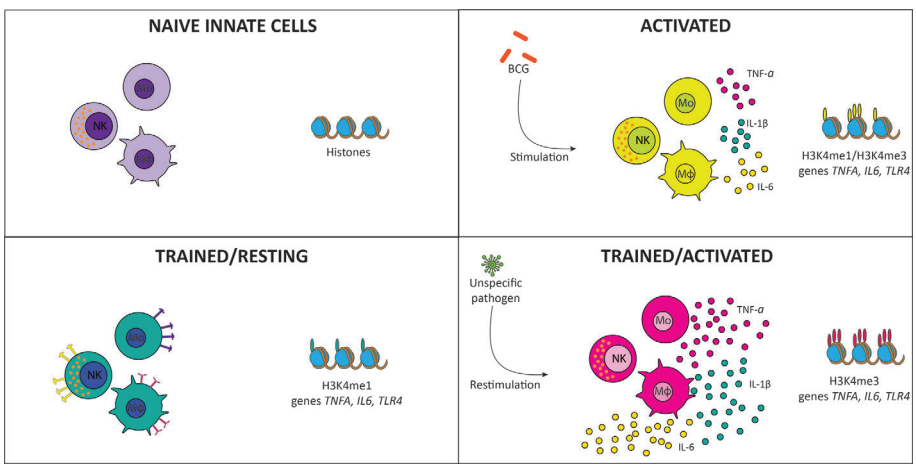

FIGURE 1 | Induction of trained immunity. Naïve monocytes and macrophages (purple) are activated by *Bacillus* Calmette-Guérin (BCG) vaccination, inducing tri-methylation of histones in pro-inflammatory genes and cytokine secretion (yellow). When the infection is resolved, trained cells are maintained in a resting state, with mono-methylation of histones of pro-inflammatory genes and increased expression of membrane receptors (green). When exposed to reinfection, histones are tri-methylated, producing stronger and faster activation and increased cytokine secretion (pink).

causes unrelated to *tuberculosis* disease. Therefore, it was proposed that BCG induces the development of a non-specific immune cross-protection based on the generation of trained immunity in humans (16, 20–22). To date, it is not clear how long does the immune protection induced by BCG vaccination lasts. Some studies suggest that it does not protect longer than ten years, while others suggest between 15 to 20 years, and even up 60 years (23–27).

As mentioned above, the first interaction of BCG with the immune system occurs at site of inoculation (28). This interaction begins with resident epidermal macrophages and dendritic cells (DCs) that recognize and phagocyte the bacterium, initiating the immune response (28, 29). DCs phagocyte bacteria and increase their surface expression of activation, maturation, migration, and antigen-presentation molecules (MHC-II, CD40, CD44, CD54, CD80, CD86) (30). Once stimulated, DCs initiate the immune response by secretion of immunomodulatory components, including cytokines and chemokines, such as TNF, IL-1β, IL-6, IL-4, and IL-10 (31). Bacterium-stimulated DCs express on their surface MHC-II molecules loaded with antigenic peptides (32). They migrate from the immunization site through the lymphatic system to the draining lymph nodes where they present antigens to naïve T cells (32). Additionally, circulating neutrophils enter the inoculation site and contribute to the local inflammatory response (28). The interaction of neutrophils with BCG increases the expression of adhesion markers, such as CD11b and CD18, and receptors, including FcγRs II and III and increase the secretion of cytokines and chemokines (e.g., IL-1α, IL-1β, and TGF-β, IL-8, CCL2, and CCL3) (33). Altogether, the interactions between BCG and these immune cells triggers an innate response that will influence the efficacy of this vaccine. As mentioned above, BCG vaccination induces the development of trained immunity (28), which refers to an acquired phenotype developed by innate immune cells after the exposure to live vaccines, including BCG (34–36), measles (35, 37, 38), oral-polio (39, 40), and smallpox (34). This phenotype is also induced by exposure to other pathogenic stimuli, such as *C. albicans* (9), and β-glucan (8). The trained phenotype allows these cells to have a faster and more effective inflammatory response than non-trained ones (1). As shown in **Figure 1**, BCG vaccination induces in monocytes epigenetic modifications in the promoters of several pro-inflammatory genes (3, 10). When the immune system is activated as a consequence of the immunization, histone H3 is mono- and tri-methylated in lysine 4 (H3K4me and H3K4me3, respectively) in the promoters of the pro-inflammatory genes *TNFA, IL6*, and *TLR4*, increasing the accessibility to the transcription machinery and, in consequence, increasing the expression of these genes (1, 3, 10). When the inflammation is resolved and the innate immune cells are resting, H3K4me is conserved in the histones associated with these genes, developing a "trained" state by which the cells can be activated in a reduced time and more efficiently, as compared to naïve or untrained cells (3, 13, 22, 41). When trained cells are re-exposed to an infection, H3K4me3 increases, augmenting the expression of pro-inflammatory genes as well, thus generating a faster and more robust immune response (1, 3, 10). Through the interaction with hematopoietic stem cells, bacteria induce epigenetic modifications that contribute to the training of the monocyte/macrophage linage associated with long-term protection against infection (42). These monocytes and macrophages express higher amounts of PRRs than non-trained cells and exert an enhanced response when exposed to secondary infections (22).

It has been shown that BCG vaccination reduces the risk of developing acute lower respiratory tract infections (ALRI) when exposed to respiratory pathogens (43). Indeed, this study suggests a tendency to reduce the risk of respiratory syncytial virus (hRSV)-associated ALRIs in infants that had received BCG at birth (43). Furthermore, BCG-vaccinated mice show fewer cellular lung infiltrates than non-vaccinated animals after a challenge with either hRSV or metapneumovirus (hMPV) (44, 45). Besides, BCG vaccination promotes antibody secretion (46), probably due to the immunogenic capacity of this attenuated bacterium to induce a strong Th-1 profile (47), promoting the survival and subsequent maturation of B cells population into effector plasma cells, with the induction of antibody secretion upon virus exposure (48). Moreover, BCG vaccination at birth reduces hospitalizations due to respiratory infections in children up to 14 years old (16). Importantly, published data suggest that the unspecific protection induced by BCG vaccination may last for a long time, contrary to previously proposed in other studies (13). Moreover, BCG vaccination increases the plasmatic levels of IFN-γ and prevents acute upper respiratory tract infections (AURTI) in the elderly (49). Indeed, a recently published phase III clinical trial showed that BCG vaccination decreases the prevalence of infections, mostly respiratory, in the elderly (50). Thus, due to the induction of trained immunity, BCG may be an excellent candidate to approach outbreaks caused by respiratory pathogens without commercial vaccines available, such as hRSV, hMPV, parainfluenza, adenovirus, rhinovirus, and coronavirus (51–56). The world is currently living a pandemic caused by a novel severe acute respiratory syndrome coronavirus (SARS-CoV-2) that causes millions of coronavirus disease (COVID-19) cases that can lead to the development of pneumonia and even death (57, 58). Up to date, there is no vaccine nor specific treatment for COVID-19, making it even more difficult to control the spread of this disease (59, 60). As mentioned above, the BCG vaccine induces unspecific protection against respiratory infections, suggesting that trained immunity may represent an interesting strategy to contain the pandemic while specific vaccines are being developed (16, 28, 43, 49). Interestingly, those countries where BCG is included in their national vaccination programs at birth have lower mortality rates than those where it is not included (61–65). Based on these observations, we and others have proposed that BCG vaccination may induce protection against SARS-CoV-2 infection due to the development of trained immunity (65, 66).

BCG exerts a wide variety of beneficial non-specific immunological effects through the induction of trained immunity, ranging from protection against non-mycobacterial infection, decreased incidence of allergic diseases, and increased immunity to certain cancers (67). Because of the ability of BCG to induce an effective immune response, it is used since 1976 for the treatment of bladder cancer (68). Several clinical trials have provided support to the notion that BCG could be a beneficial treatment for this pathology, even though the precise underlying mechanism remains to be elucidated (69–72). Some studies suggest that BCG induces locally a non-specific

immune and inflammatory response, contributing to the generation of a localized anti-tumor immunity in patients (73). In a meta-analysis of a randomized clinical trial, BCG was shown to reduce the risk of bladder cancer progression after transurethral resection (74). Also, a topical application of BCG is used as a safe alternative for treating warts in children (75). The immune stimulation in the early years of life induced by BCG vaccination may have a beneficial impact against chronic diseases, such as asthma and allergies (76). Moreover, studies in humans and mice showed that the BCG vaccine offers protection against various viral infections, including herpes and influenza viruses (77). Also, neonatal vaccination protects against sepsis early in life (78). It was recently demonstrated that this latter effect was due to the induction of granulopoiesis by the secretion of Granulocyte colony-stimulating factor (G-CSF), resulting in neutrophil expansion (78). These BCG-induced neutrophils were shown to be necessary and sufficient to induce such protection against sepsis (78). Further, it was proposed that a "trained" innate immune system can direct the adaptive immune response towards a more effective response against different pathogens (77, 79). The enhanced activation of the innate immune system and the secretion of high levels of IL-1β by these cells, when exposed to an infectious agent, may activate more effectively the adaptive immune response (80). Besides, the increased secretion of pro-inflammatory cytokines can accelerate the maturation of DCs, which represent the direct cross-talk between the innate and adaptive immune responses (81–83). Indeed, DCs cooperate with neutrophils after BCG-infection to stimulate T cell responses against these bacteria (84). On the other hand, trained immunity generated by BCG induces heterologous Th1 and Th17 responses, characterized by the secretion of IFN-γ and IL-17, and IL-22, respectively (80). Altogether these characteristics of a trained innate immune response can induce a more effective adaptive immune response against the pathogen. In agreement with this statement, vaccination with BCG before the administration of a trivalent influenza vaccine improves the specific antibody response, inducing a faster seroconversion compared to the administration of the influenza vaccine by itself (85). This finding further supports the notion that trained innate immune cells may activate a more effective adaptive immune response.

Although the non-specific immune effects induced by the BCG vaccine are broadly reported, the molecular mechanisms involved in this phenomenon are only partially understood. Unveiling these mechanisms would be important to design better therapeutic options and vaccination strategies using this attenuated bacterium.

TRAINED IMMUNITY INDUCED BY RECOMBINANT VACCINES

Worldwide, hRSV is considered the most important etiologic agent of acute lower respiratory tract infections (ALRIs) in children under 5 years and adults over 65 years (86), infecting 100% of children at age two (87). In 2015, hRSV caused 33.1 million episodes of ALRIs (bronchiolitis and pneumonia) worldwide, producing 3.2 million hospitalizations and about 120,000 deaths of children under 5 years old (88).

Vaccines consisting of recombinant strains of BCG expressing either the nucleoprotein (N) or the M2 protein of hRSV (rBCG-N-hRSV and rBCG-M2-hRSV, respectively) were evaluated to induce hRSV specific immunity and prevent disease (44, 45, 89–92). These formulations were shown to protect against hRSV in a murine model of infection, reducing the development of clinical symptoms associated with the viral challenge in vaccinated mice (44). The rBCG-N-hRSV and rBCG-M2-hRSV vaccines induce the development of cellular and humoral responses, generating specific T_{H1}/T_{H17} memory cells and antibodies in mice (44, 45, 89, 91). Viral-specific antibodies have neutralizing activity (45), which correlates with diminished viral titers in the lungs of immunized animals (89). rBCG-N-hRSV was developed under cGMP *(Good Manufacturing Practices)* conditions, showing the same protection against hRSV infection mentioned above in animal models (89). Indeed, this vaccine is the only hRSV-vaccine being developed to be administered to newborns, who represent the major risk group for this virus (93). In a phase 1 clinical trial, rBCG-N-hRSV was shown to induce both cellular and humoral immunity against hRSV in humans (90).

HMPV also causes ALRIs and death in children under 5 years old (94). This virus was first described in 2001 by Van Den Hoogen et al. (95), and its incidence has increased every year since then (96, 97). In healthy adults, hMPV infection appears with mild influenza-like symptoms, while in children under 5 years old, elderly, and immunocompromised patients it causes bronchiolitis, pneumonia, and even death (96).

Based on the previous experience with hRSV, recombinant BCG vaccines expressing either the phosphoprotein (P) or the M2.1 protein of hMPV were generated (98). Both, rBCG-P-hMPV and rBCG-M2.1-hMPV, were shown to efficiently protect from hMPV in a murine model of infection, with less cellular infiltrates, lung inflammation, and viral replication in vaccinated mice (45, 98). Mouse vaccination was also effective in the induction of humoral responses against hMPV, with virus-neutralizing antibody production and isotype switching (45).

These recombinant BCG vaccines were based on BCG-Danish 1331, a vaccine that is known to induce trained immunity, suggesting that this type of immunity might be a component of the protection achieved with these vaccines against these two respiratory viruses. Consistently with this notion, vaccination of RAG-deficient mice, which lack T and B cells, with rBCG-N-hRSV induces the secretion of significantly higher IFN-γ levels in bronchioalveolar lavages (BAL) after hRSV challenge as compared to unimmunized mice (91). Surprisingly, this induction reached similar levels of IFN-γ as BCG-WT, suggesting that both could induce a trained immunity phenotype in these immunodeficient mice (91). Furthermore, immunization with BCG-WT reduced cellular lung infiltration and inflammation in murine models for both hRSV and hMPV infections (44). BCG-WT induces specific antibody isotype switching and the production of neutralizing antibodies after hRSV and hMPV infections in mice (45). These data suggest that

non-specific cross-protection induced by BCG may be effective in protecting against these viruses.

During viral infections, the immune system activates the production and secretion of interferons, which mediate the antiviral response, impairing the viral replication by the activation of macrophages and DC (99). As mentioned above, rBCG-N-hRSV and rBCG-P-hMPV vaccination generated an early IFN production soon after the viral challenge in mice, suggesting that these vaccines may induce a non-specific cross-protection against other viral infections (91, 98). The induction of trained immunity-related cytokines, such as IL-6, TNF, and IL-1β by these vaccines still has to be elucidated (3). Besides hRSV and hMPV, there are other important respiratory viruses, as parainfluenza, adenovirus, rhinovirus, and the newly identified SARS-CoV-2, among others (51–56, 100). The unspecific protection mediated by the development of trained immunity may be a good strategy to protect against a broad spectrum of viruses, being recombinant BCGs excellent candidates, since BCG is a safe vaccine used to immunize infants, which constitute a high-risk population (101). Trained immunity protects against yellow fever virus infection in humans (12). In an experimental infection challenge of healthy volunteers, BCG vaccination reduced the viremia after infection compared to non-vaccinated volunteers (12).

Based on the studies mentioned above, vaccination of the risk population with either rBCG-N-hRSV or rBCG-P-hMPV, besides inducing specific protection against the virus for which they are developed, may induce non-specific cross-protection mediated by a trained innate immune system against other viruses to which there is no specific vaccine available (**Figure 2**).

In the context of the pandemic that the world is currently facing, it has been proposed that SARS-CoV-2 exposure in the presence of a trained innate immune system may induce a more robust and effective immune response and, in consequence, a milder manifestation of the disease (65, 102–106). Indeed, those countries where BCG is included in their national vaccination programs at birth showed lower mortality rates due to SARS-CoV-2 infection as compared that do not include it (61, 62, 65, 107, 108), probably by the development of trained immunity. Based on this hypothesis, several clinical trials are being performed in different countries, including Netherlands, South Africa, Australia, United States, Colombia, Egypt, Brazil, Denmark, and France (Clinicaltrials.gov) (104). Interestingly, a recombinant BCG vaccine, VPM1002, is also being tested in a clinical trial to determine the cross-protection against COVID-19 (Clinicaltrials.gov ID: NCT04387409) (104). This recombinant BCG has a *Listeria monocytogenes* gene encoded for listeriolysin instead of the urease C gene (109). This modification increases apoptosis and autophagy, promotes phagolysosome fusion, and improves vaccine efficacy (109).

Even though the induction of trained immunity has not yet been demonstrated for recombinant BCG vaccines, some studies support this notion and suggest an advantageous scenario for the prevention of several infections (28). Positive results deriving from the VPM1002 clinical trial, will suggest a cross-protection due to the induction of trained immunity.

Besides the intramuscular administration of live-attenuated BCG to induce protection against tuberculosis, this bacterium can be inactivated and used as an adjuvant based on its strong immunogenicity (110–112). Also, inactivated BCG induces the

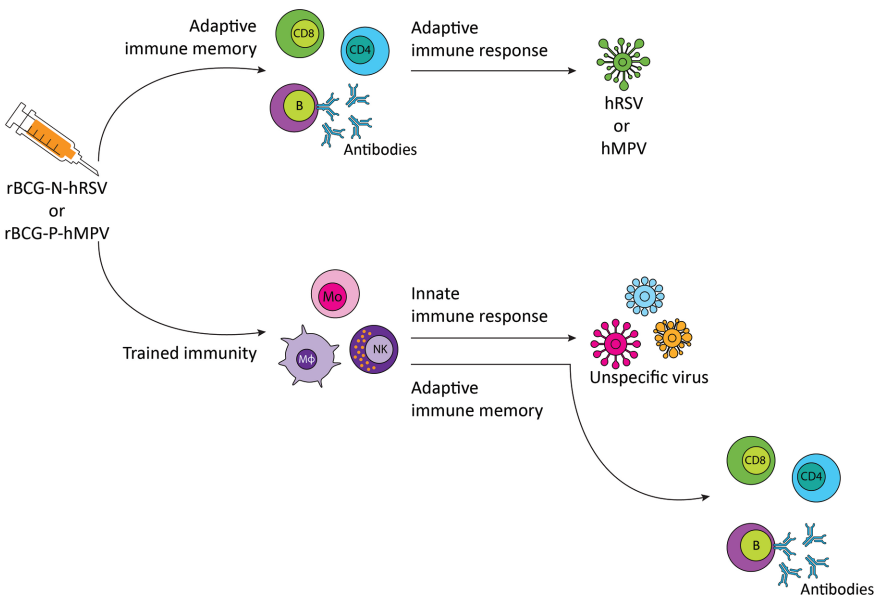

FIGURE 2 | Proposed immune response to respiratory syncytial virus (hRSV) or metapneumovirus (hMPV) and non-specific viruses after rBCG-N-hRSV or rBCG-P-hMPV vaccination, respectively. Immunization with rBCG-N-hRSV or rBCG-P-hMPV induces a specific adaptive immune response against hRSV or hMPV, respectively, that will resolve the infection. Trained immunity induction generates a more robust response against other viruses and orchestrates adaptive immune memory against the virus. CD8, $CD8^+$ T cell; CD4, $CD4^+$ T cell; B, B cell; Mo, monocyte; Mφ, macrophage; NK, natural killer.

development of trained immunity in innate immune cells *in vitro* (10). Based on these findings, a vaccine formulation containing inactivated BCG as an adjuvant could promote the development of a strong specific immune response and induce the development of trained immunity. On the other hand, to prevent and treat respiratory infections, a sublingual vaccine was designed consisting of heat-inactivated bacterial components, called MV130 (113). This vaccine was shown to trigger TLR and NLR signaling pathways on DCs, inducing the production and secretion of trained immunity-related cytokines, such as TNF-α, IL-6, and IL-1β (113). These bacterial preparations could also be considered as good adjuvants for the development of novel vaccines with the capacity to induce trained immunity.

CONCLUDING REMARKS

Vaccines have been used since 1798 to control infections that are potentially harmful to humans (17). A very important feature of vaccines is the specificity of the immunological memory that they induce against a unique pathogen (114). However, what happens when there are no efficient vaccines to protect us against a particular infectious agent? Trained immunity is a non-specific immunological memory mediated by the innate immune system (1). This type of immunological memory protects against a wide variety of pathogens, suggesting that it could be considered an alternative for developing unspecific vaccines (3, 8, 11, 12).

As mentioned above, BCG protects against some respiratory affections, including asthma and upper and lower respiratory tract infections non-related to *M. tuberculosis* (12, 43, 48, 49, 77). These characteristics of BCG are attributed to the induction of trained immunity (1, 28). Similar to BCG, recombinant BCG formulations are expected to induce trained immunity (28). We have developed effective, immunogenic rBCG vaccines against hRSV and hMPV, two common respiratory viruses that can cause serious illness, even death (44, 45, 89, 91, 98). We are exposed to a wide variety of viruses, many of them with no specific vaccine or antiviral drugs available to treat them (100). As proposed in **Figure 2**, an immunogenic vaccine with the ability to induce specific immunity against two of the most relevant respiratory viruses (hRSV or hMPV) and trained immunity to other seasonal respiratory viruses could be an approach to be evaluated for controlling outbreaks and reduce the morbidity and mortality associated to other respiratory viral infections. Up to date, the induction of trained immunity by recombinant BCGs has not been demonstrated. Even though some studies suggest that these vaccines have the same capacity as the parental BCG strain to induce this non-specific innate memory, a formal demonstration would require additional research.

AUTHOR CONTRIBUTIONS

CC, MR, and RB-R wrote the manuscript. AK and SB reviewed the manuscript and approved the version to be published. All authors contributed to the article and approved the submitted version.

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A Combination of Polybacterial MV140 and *Candida albicans* V132 as a Potential Novel Trained Immunity-Based Vaccine for Genitourinary Tract Infections

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Recurrent urinary tract infections (RUTIs) and recurrent vulvovaginal candidiasis (RVVCs) represent major healthcare problems with high socio-economic impact worldwide. Antibiotic and antifungal prophylaxis remain the gold standard treatments for RUTIs and RVVCs, contributing to the massive rise of antimicrobial resistance, microbiota alterations and co-infections. Therefore, the development of novel vaccine strategies for these infections are sorely needed. The sublingual heat-inactivated polyvalent bacterial vaccine MV140 shows clinical efficacy for the prevention of RUTIs and promotes Th1/Th17 and IL-10 immune responses. V132 is a sublingual preparation of heat-inactivated *Candida albicans* developed against RVVCs. A vaccine formulation combining both MV140 and V132 might well represent a suitable approach for concomitant genitourinary tract infections (GUTIs), but detailed mechanistic preclinical studies are still needed. Herein, we showed that the combination of MV140 and V132 imprints human dendritic cells (DCs) with the capacity to polarize potent IFN-γ– and IL-17A–producing T cells and FOXP3$^+$ regulatory T (Treg) cells. MV140/V132 activates mitogen-activated protein kinases (MAPK)-, nuclear factor-κB (NF-κB)- and mammalian target of rapamycin (mTOR)-mediated signaling pathways in human DCs. MV140/V132 also promotes metabolic and epigenetic reprogramming in human DCs, which are key molecular mechanisms involved in the induction of innate trained immunity. Splenocytes from mice sublingually immunized with MV140/V132 display enhanced proliferative responses of CD4$^+$ T cells not only upon *in vitro* stimulation with the related antigens contained in the vaccine formulation but also upon stimulation with phytohaemagglutinin. Additionally, *in vivo* sublingual immunization with MV140/V132 induces the generation of IgG and IgA antibodies against all the components contained in the vaccine formulation. We uncover immunological mechanisms underlying the potential mode of action of a

combination of MV140 and V132 as a novel promising trained immunity-based vaccine (TIbV) for GUTIs.

Keywords: dendritic cells, recurrent urinary tract infections (RUTIs), recurrent vulvovaginal candidiasis (RVVCs), trained immunity-based vaccines (TIbVs), *candida albicans* V132, polybacterial preparation MV140

INTRODUCTION

Bacterial infections represent a major health-care problem and among them, urinary tract infections (UTIs) are one of the most common with a high incidence in women (1–4). UTIs are considered recurrent (RUTIs) with two or more symptomatic infections in less than six months or more than three per year (2, 5). Antibiotic treatment is the current therapy used for RUTIs, but its long-term use increases the risk to develop antibiotic resistance (3, 6). The overuse of antibiotics is also associated with dysregulation of normal vagina and gastrointestinal microbiota, which favor pathogen invasion and subsequent bacterial and fungal infection (1, 3, 7). Due to microbiota alterations, opportunistic pathogens such as *Candida albicans* colonize the vaginal tract generating vulvovaginal candidiasis (VVCs) (8–12). More than 70% of women worldwide suffer from VVCs (with maximum incidence between 20 and 40 years old) and around 5% experience recurrent infections (RVVCs), defined as four or more episodes per year (13–16). RVVCs require antifungal therapy to avoid recurrence and its overuse involves antimycotic resistances (17–19). Both RUTIs and RVVCs markedly diminish quality of life in women, with a negative impact at work and social life (20, 21). Therefore, there is an urgent need to develop novel strategies for concomitant and recurrent genitourinary tract infections (GUTIs), including RUTIs and RVVCs (1, 4, 16–18, 22).

Mucosal bacterial or fungal vaccines have been proposed as a new alternative approach to antibiotics and antifungals, respectively (23–25). These preparations containing soluble antigens or inactivated whole pathogens might well induce both specific immune responses and also non-specific immunomodulation. Sublingual administration of mucosal vaccines based on whole inactivated components has been recently reported as a suitable strategy to prevent and treat recurrent infections (24, 26–28). These preparations proposed as trained immunity-based vaccines (TIbVs) are able to induce long-lasting changes in innate immune cells, including dendritic cells (DCs), that enhance responses and protection against both the pathogens included in the vaccine formulation but also against unrelated pathogens by mechanisms involving immunological, metabolic and epigenetic reprogramming (23, 29–32). DCs survey the mucosal environment using innate pattern recognition receptors (PRRs), and are responsible for linking innate and adaptive immune responses (33, 34). Activation of DCs is linked to metabolic changes coupling the energetic needs with the functional activity (35, 36). Activation of DCs with microbial components promotes Warburg effect, a shift from oxidative phosphorylation to glycolysis and lactic fermentation under normal oxygen conditions (35, 37). This metabolic rewiring in DCs is driven at the molecular level by different mechanisms including activation of the axis protein kinase B (Akt)/mammalian target of rapamycin (mTOR)/hypoxia inducible factor-1α (HIF-1α) (35, 36).

MV140 (Uromune) is a polyvalent bacterial preparation based on whole heat-inactivated components used to prevent RUTIs (38). This sublingual vaccine is composed of bacteria causing the majority of RUTIs in Europe: 75% Gram-negative bacteria (25% *Escherichia coli*, 25% *Proteus vulgaris* and 25% *Klebsiella pneumoniae*) and 25% Gram-positive bacteria (*Enterococcus faecalis*) (26). Clinical data showed that MV140 significantly reduced the infection rates in patients suffering from RUTIs (39–43). MV140 acts directly on human DCs promoting maturation and activation through mechanisms depending on Syk- and MyD88-mediated signaling pathways, supporting the involvement of C-type lectin receptors (CLRs) and toll-like receptors (TLRs). MV140-induced downstream activation of NF-κB and p38 in DCs contribute to Th1/Th17 and IL-10 polarization whereas JNK and ERK are involved in Th1 and IL-10 immune responses, respectively (26). V132 is a preparation of heat-inactivated *C. albicans* that has been developed as a possible treatment of RVVCs. Given the tight relationship between RUTIs and RVVCs, a polymicrobial formulation, *via* combination of MV140 and V132, might well represent a suitable vaccine for both diseases.

In this study, we demonstrated that the combination of MV140 and V132 imprints human DCs with the capacity to generate potent IFN-γ– and IL-17A–producing T cells and FOXP3$^+$ regulatory T (Treg) cells. Mechanistically, we showed that MV140/V132 activates inflammatory and mTOR pathways as well as metabolic and epigenetic reprogramming in human DCs. *In vivo*, sublingual immunization of BALB/c mice with MV140/V132 enhances the proliferation of splenic CD4$^+$ T cells upon *in vitro* stimulation with the antigens contained in the formulation and with phytohaemagglutinin (PHA). Sublingual immunization with MV140/V132 also induces the generation of specific IgG and IgA antibodies against all the components contained in the formulation. Overall, we show for the first time molecular mechanisms that

Abbreviations: Akt, protein kinase B; APC, allophycocyanin; CLRs, C-type lectin receptors; COXII, cyclooxygenase II; DCs, dendritic cells; FITC, fluorescein isothiocyanate; FTU, Formazan Turbidity Units; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GUTIs, genitourinary tract infections; HIF-1α, hypoxia inducible factor-1α; hmoDCs, human monocyte-derived DCs; MAPK, mitogen-activated protein kinases; MTA, 5′-deoxy-5′-(methylthio)adenosine; mTOR, mammalian target of rapamycin; NF-κB, nuclear factor-κB; PBMC, peripheral blood mononuclear cells; PBP, polyvalent bacterial preparation; PE, phycoerythrin; PerCP, peridinin-chlorophyll-protein; PHA, phytohaemagglutinin; RUTIs, recurrent urinary tract infections; RVVCs, recurrent vulvovaginal candidiasis; TIbV, trained immunity-based vaccines; TLRs, toll-like receptors; Treg, regulatory T; UTIs, urinary tract infections; VVCs, vulvovaginal candidiasis.

might be involved in the mode of action of MV140/V132 as a novel promising trained immunity-based vaccine (TIbV) for GUTIs.

MATERIALS AND METHODS

Media and Reagents

RPMI 1640 (Lonza) supplemented with 10% fetal bovine serum, 100 μg/ml normocin, 50 μg/ml penicillin-streptomycin, 1% non-essential amino acids, 1% MEM-vitamins and 1 mM sodium pyruvate. MV140 (Uromune) composed of whole heat-inactivated bacteria (25% *E. coli*, 25% *P. vulgaris*, 25% *K. pneumoniae* and 25% *E. faecalis*), V132 composed of whole heat-inactivated *C. albicans*, MV140/V132 (combination of MV140 and V132) and control excipients (negative control, containing all excipients without bacteria and yeast) were provided by Inmunotek S.L. Inhibitors for histone methyltransferase 5′-deoxy-5′-(methylthio) adenosine (MTA) and histone demethylase (Pargyline) (Sigma-Aldrich) were used for the inhibition experiments.

Generation of Human Monocyte-Derived Dendritic Cells and Purification of Naïve CD4$^+$ T Cells

Peripheral blood mononuclear cells (PBMC) from buffy coats of healthy donors were obtained by using Ficoll density gradient centrifugation (800*g*, 20 min). Monocytes were isolated from total PBMC using anti-CD14 microbeads and cultured for 6 days with RPMI medium containing 100 ng/ml of IL-4 and GM-CSF (PeproTeck) to generate immature human monocyte-derived DCs (hmoDCs). The purity and phenotype of monocytes and generated immature hmoDCs were analyzed by flow cytometry with lineage-specific markers. Peripheral blood naive CD4$^+$ T cells were isolated using the "Naive CD4$^+$ T Cell Isolation Kit" (Miltenyi Biotec), according to manufacturer's protocol.

Cell Cultures

Immature hmoDCs (10^6 cells per ml) were stimulated with control excipients (containing all excipients except the microbial components), V132 (3 FTU, Formazan Turbidity Units, per ml), MV140 (10^7 bact. per ml) or MV140/V132 (MV140, 10^7 bact. per ml, and V132, 3 FTU per ml) for 18 h. Subsequently, cells were collected and centrifuged. Cell pellets were used to analyze their phenotype by flow cytometry and cell-free supernatants were used to quantify IL-6, TNF-α, IL-23, IL-1β, IL-10, and TGF-β1 by ELISA. For inhibition experiments, hmoDCs were preincubated for 1 h with MTA (0.5 mM) or Pargyline (3 μM) (or their vehicle control, DMSO) prior to activation. Then, the cells were stimulated with stimuli for 18 h in the presence of the corresponding inhibitors to quantify TNF-α, IL-23, and IL-10 by ELISA. Cell viability was analyzed in all the cases by trypan blue exclusion with a light microscope.

Flow Cytometry

The following anti-human monoclonal antibodies (mAbs) were used for flow cytometry: fluorescein isothiocyanate (FITC)-conjugated anti-HLA-DR; phycoerythrin (PE)-conjugated anti-CD86; and allophycocyanin (APC)-conjugated anti-CD83 (Miltenyi Biotec). APC-conjugated anti-CD3; and Alexa Fluor 488-conjugated anti-IFN-γ (BD Pharmigen). PE-conjugated anti-IL-10; Alexa Fluor 488-conjugated anti-IL-17A; peridinin-chlorophyll-protein (PerCP)-conjugated anti-CD4; Alexa Fluor 488-conjugated anti-FOXP3; PE-conjugated anti-CD127; and APC-conjugated anti-CD25 (Biolegend). Cells were washed with PBS/EDTA 2 mM/0.5% BSA and stained for 15 min at room temperature in the darkness. For analysis of FOXP3 expression in human T cells primed with hmoDCs, cells were first subjected to surface staining with anti-human CD127-PE, CD4-PerCP, and CD25-APC antibodies. After fixation and permeabilization, cells were stained with anti-human FOXP3-Alexa Fluor 488, according to manufacturer's recommendations. For each staining, the corresponding isotype controls (IgG2A-FITC, IgG1-Alexa Fluor 488, IgG1-PE, IgG2A-PerCP, or IgG1-APC) were also assayed. Flow cytometry analysis was performed in a FACSCalibur in the Cytometry and Fluorescence Microscopy Unit at Complutense University of Madrid.

Cytokine Quantification

The cytokine production of IL-6, TNF-α, IL-1β, IL-10, IFN-γ, IL-5 (BD Biosciences), IL-23 (Invitrogen), IL-17A (Mabtech), and TGF-β1 (R&D System) was quantified by sandwich ELISA in cell-free supernatants following the manufacturer's instructions.

Co-Culture Experiments

HmoDCs were co-cultured with purified allogeneic naïve CD4$^+$ T cells (1:5 DC/T cell) for 3 days in the presence of control excipients, V132 (3 FTU per ml), MV140 (10^7 bact. per ml) or MV140/V132 (MV140, 10^7 bact. per ml, and V132, 3 FTU per ml). IFN-γ, IL-5, IL-17A, and IL-10 were quantified in cell-free supernatants by ELISA. For proliferation assays, purified allogeneic naive CD4$^+$ T cells were labeled with CFSE (Molecular Probes) prior to co-culture with immature hmoDCs. Proliferation was assessed by measuring CFSE dilution on labeled CD4$^+$ T cells by flow cytometry. For intracellular staining, the primed CD4$^+$ T cells were washed and restimulated with 25 ng/ml of PMA plus 1 μg/ml of ionomycin for 6 h and 10 μg/ml of Brefeldin A for the last 4 h. Cells were fixed and permeabilized with Cytofix/Cytoperm (BD Biosciences) according to the manufacturer's instructions. The cells were stained with the combination of fluorochrome-labeled mAbs to IFN-γ, IL-10, and IL-17A.

Western Blot Analysis

HmoDCs (10^6 cells per ml) were treated with control excipients, V132 (3 FTU per ml), MV140 (10^7 bact. per ml) or MV140/V132 (MV140, 10^7 bact. per ml, and V132, 3 FTU per ml). After 30 min at 37°C, cells were harvested and lysed with RIPA buffer (ThermoFisher scientific) in presence of Protease/Phosphatase Inhibitor Cocktail (Cell Signaling) for 30 min at 4°C vortexing every 10 min. Lysates were clarified by centrifugation at 10,000*g* for 15 min at 4°C. Protein quantification was performed with Micro BCA Protein Assay Kit (Pierce) and samples with equal amounts of total protein were resolved in 10% SDS-

polyacrylamide gel electrophoresis (SDS–PAGE). Proteins were then transferred to nitrocellulose membrane (BioRad). The membrane was incubated with the primary antibodies: phospho-ERK1/2 (Thr202/Tyr204), phospho-SAPK/JNK (Thr183/Tyr185), phospho-p38 MAPK (Thr180/Tyr182), phospho-IKKα (Ser176)/IKKβ (Ser177), or phospho-Akt (Ser473) (1:1000, Cell Signaling), phospho-p70 S6 Kinase (Thr389) (1:750; Cell Signaling), and β-actin (1:15000, Sigma-Aldrich); and goat anti-rabbit (1:4000, BioRad) or goat anti-mouse (1:2500, Pierce) conjugate with horseradish peroxidase as a secondary antibody. The signal was developed with Clarity Western ECL Substrate (Bio-Rad) and detected in a Fujifilm LAS-3000 developer.

Metabolic Studies

HmoDCs were stimulated with control excipients, V132 (3 FTU per ml), MV140 (10^7 bact. per ml) or MV140/V132 (MV140, 10^7 bact. per ml, and V132, 3 FTU per ml) for 18 h. Glucose concentration in cell-free supernatants was determined by using the Glucose (GO) Assay Kit (Sigma-Aldrich). The metabolic rate was derived mathematically in percentage of medium without hmoDCs (2 mg/ml). Lactate production in cell-free supernantants was determined by using the colorimetric L-Lactate Assay kit (Sigma-Aldrich). The mitochondrial membrane potential was measured using MitoTracker Red Mitochondrion-Selective Probe kit (ThermoFisher Scientific) following the manufacturer's instructions with minor modifications. Briefly, mitochondrial activation was measured using MitoTracker Red CMXROS (250 nM) for 30 min at 37°C after 18 h of treatment with the corresponding stimulus. The fluorescence of the probe was analyzed using FLUOstar OPTIMA fluorescence reader (BMG LabTech).

RNA Extraction, cDNA Synthesis, and Quantitative Real-time RT-PCR

RNA was isolated from harvested cells using an RNeasy mini kit (Qiagen) and cDNA generated using a PrimeScript RT reagent Kit (Takara) according to manufacturer's instructions. Real-time quantitative PCR was performed on cDNA using FastStart Universal SYBR Green Master (Rox) (Roche). The sequences of the used pair primers were: cyclooxygenase II (COXII) (forward, CTATCCTGCCCGCCATCATC; reverse, GGGATCGTTGACCTCGTCTG), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (forward, CTGCACCACCAACTGC TTAGC; reverse, TCATGTTCTGGAGAGCCCCG), Hypoxia-inducible factor 1α (HIF-1α) (forward, TCGCATCTTGATAA GGCCTCT; reverse, ACAAAACCATCCAAGGCTTTCA), and Elongation Factor 1α (EF1α) (forward, CTGAACCATCCAG GCCAAAT; reverse, GCCGTGTGGCAATCCAAT). Samples were run on a real-time PCR system (ABI Prism 7900 HT; Applied Biosystems). Data were normalized to EF1α and displayed as arbitrary units calculated as $2^{-\Delta CT}$ values multiplied by 10^4. ΔCT was defined as the difference between the cycle threshold value for the gene of interest and EF1α. The mitochondrial/nuclear DNA content was calculated by using the formula: $2^{-\Delta CT}$ mitochondrial COXII/$2^{-\Delta CT}$ nuclear GAPDH.

Sublingual Immunization of Mice With MV140/V132 or Control Excipients: Quantification of CD4$^+$ T Cell Proliferation of *In Vitro* Stimulated Splenocytes With V132, MV140, MV140/V132, PHA, or Control Excipients, and Induced Serum-Specific Antibody Levels

BALB/c mice (6-weeks-old) were immunized four times with 20 µl of MV140/V132, MV140 alone, V132 alone (300 FTU per ml) or control (all excipients without bacteria and yeast) by sublingual administration every 7 days and killed 7 days after the last immunization. Sublingual administration was performed under anaesthesia (ketamine, 100 mg/kg and xylacine, 5 mg/kg) to ensure proper delivery. Spleens were used to prepare single cell suspensions following conventional protocols. Cells were labeled with CFSE (5 mM, Life technologies) and stimulated *in vitro* with V132 (6 FTU per ml), MV140 (6 FTU per ml), MV140/V132 (MV140, 3 FTU per ml, and V132, 3 FTU per ml), phytohaemagglutinin (PHA, 5 µg/ml, Sigma-Aldrich) or control excipients for 5 days. Proliferation of CFSE-labeled CD4$^+$ T cells was monitored by flow cytometry. Serum specific IgG, IgA, IgG1, and IgG2a for *C. albicans*, *K. pneumoniae*, *E. coli*, *E. faecalis*, and *P. vulgaris* induced after sublingually immunization were determined by ELISA. Briefly, 96-well non-tissue culture-treated plates were pretreated with poly-L-lysine (Sigma-Aldrich) for 1 h under UV light and coated with each of the heat-inactivated whole cell bacteria or yeast (300 FTU per ml) overnight at 4°C, and, subsequently, incubated with mouse serum dilutions for 2 h at room temperature. Specific immunoglobulins were detected with biotin rat anti-mouse IgA and IgG (both from Sigma-Aldrich). Signal was developed by incubation with streptavidin-horseradish peroxidase (HRP) (Sigma-Aldrich). Peroxidase activity was revealed by the addition of o-phenylenediamine dihydrochloride (Sigma-Aldrich) and the reaction was stopped with HCl 1 N. Plates were read on an ELISA reader at 490 nm (Triturus Elisa, Grifols). Animals were maintained in Biolab S.L. under the conditions stabilized by the R.D. 53/2013 and in accordance with the international GLP normative, RD 223/83 and ISO 10993-2:2007. The tests are performed in accordance with official methods, European Pharmacopoeia, USP Pharmacopoeia, OECD Guideline, UNE EN-ISO, and specific methodologies developed for R&D.

Statistics

All the data are expressed as means ± s.e.m. of the indicated parameters. Paired or unpaired Student's t-test used for statistical analysis were performed using GraphPad Prism software, version 6.0. Significance was defined as $^*P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$.

RESULTS

MV140/V132-Activated Human Dendritic Cells Display Significantly Higher Levels of CD83, Pro-Inflammatory Cytokines, and IL-10 Than Dendritic Cells Stimulated With the Individual Components

To assess the capacity of the combination of the polyvalent bacteria preparation MV140 and *C. albicans* V132 to modulate the phenotype and function of human DCs, we treated human monocyte-derived DCs (hmoDCs) from healthy donors with V132, MV140, MV140/V132 or control excipients. HmoDCs treated with V132 produced significantly higher levels of IL-6 and TNF-α, and a slight increase in the production of IL-1β and IL-10 than control excipients. IL-23 levels were no detectable in cell-free supernatants from V132-treated hmoDCs (**Figure 1A**). MV140 induced significant production of IL-6, TNF-α, IL-1β, IL-23, and IL-10 compared to control treatment, as previously described (26), and also compared to V132 (**Figure 1A**). MV140/V132-activated hmoDCs induced significantly higher levels of pro-inflammatory cytokines and IL-10 than excipients- or V132-activated hmoDCs (**Figure 1A**). Interestingly, MV140/V132 stimulation induced significantly higher levels of TNF-α, IL-23, and IL-10 than MV140 in hmoDCs (**Figure 1A**). To test whether simultaneous stimulation of hmoDCs with MV140/V132 enhance the production of TNF-α, IL-23, and IL-10 in a synergistic manner, cytokine levels induced by MV140/V132 showed in **Figure 1A** were directly compared to those resulting from the addition of the cytokine levels produced by activation with MV140 and V132 separately (**Figure 1A**). MV140/V132-activated hmoDCs induced significantly higher levels of TNF-α, IL-23, and IL-10 than the corresponding cytokine levels produced by V132- plus MV140-treated hmoDCs, supporting synergistic cooperation in the simultaneous stimulation with the combination of MV140 and V132 (**Figure 1B**). We did not detect significant differences in the levels of TGF-β1 among any of the assayed conditions (**Supplementary Figure 1A**). Although there were no significant differences in the percentage of cells expressing HLA-DR (**Figures 1C, D**), the expression levels (MFI values) were significantly increased after V132, MV140 and MV140/V132 stimulation compared to excipients-treated cells, without significant differences among them (**Figure 1E**). V132,

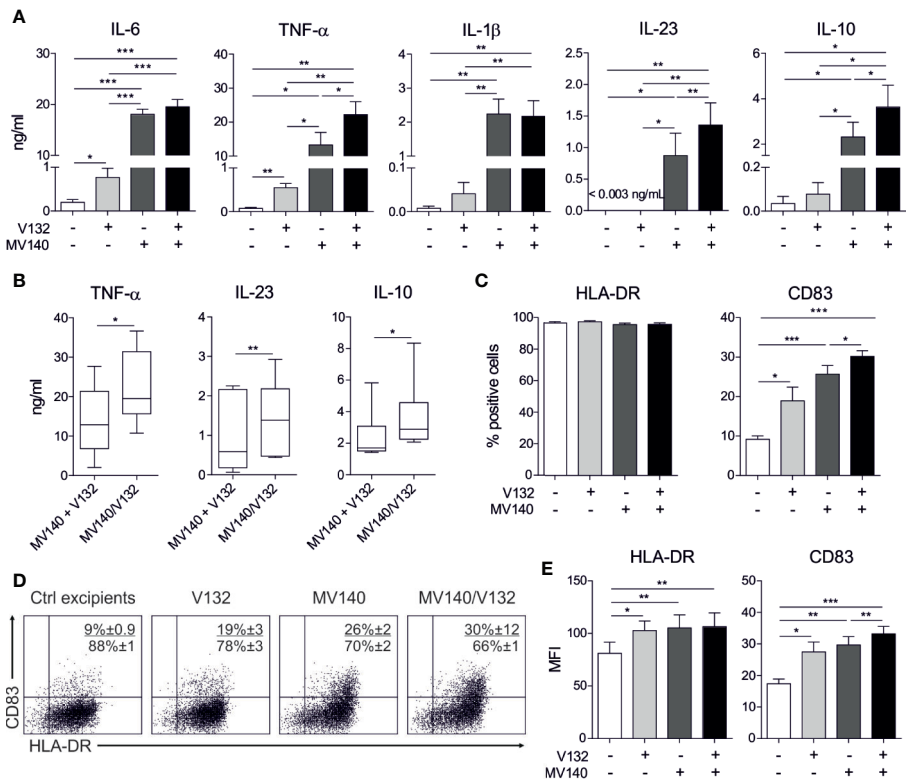

FIGURE 1 | MV140/V132 induces hmoDCs maturation and production of pro-inflammatory cytokines and IL-10. **(A)** Cytokine production after stimulation of hmoDCs with control excipients (containing all excipients except the bacteria and yeast), V132, MV140, and MV140/V132 for 18 h quantified in cell-free supernatants by ELISA. **(B)** Cytokine levels of V132-treated hmoDCs plus cytokine levels of MV140-treated hmoDCs compared with cytokine levels of MV140/V132-stimulated hmoDCs. **(C)** Percentage of CD83- and HLA-DR-positive cells after stimulation with control excipients, V132, MV140 and MV140/V132 for 18 h. **(D)** Flow cytometry representative dot plots for the expression of surface markers CD83 and HLA-DR after stimulation of hmoDCs with the indicated stimulus. **(E)** Mean Fluorescence Intensity (MFI) values for CD83 and HLA-DR. Results are mean ± s.e.m. of 6-8 **(A)**, 6 **(B)**, and 7-8 **(C, E)** independent experiments. Paired Student *t* test, $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

MV140 and MV140/V132 significantly increased the expression of the co-stimulatory molecule CD83 in hmoDCs compared to control excipients (**Figures 1C, D**). MV140 and MV140/V132 induced higher expression of CD83 than V132 without significant differences (**Figures 1C, D**). Both the percentage of positive cells and the expression levels of CD83 were significantly higher in hmoDCs stimulated with MV140/V132 than MV140, indicating that the combination of these stimuli in a single formulation significantly enhance the maturation of hmoDCs (**Figures 1C, E**). The expression of CD86 was significantly higher in hmoDCs stimulated with V132 than the other stimuli, but no differences were observed after MV140 and MV140/V132 stimulation compared to control excipients (**Supplementary Figures 1B–D**).

MV140/V132-Activated Human Dendritic Cells Generate Potent IFN-γ- and IL-17A–Producing T Cells and FOXP3$^+$ Treg Cells

To determine the capacity of human DCs treated with the combination of MV140 and V132 to polarize CD4$^+$ T cell responses, we performed co-culture experiments. HmoDCs stimulated with MV140 and MV140/V132 induced a significantly higher percentage of proliferating allogeneic CD4$^+$ T cells than excipients-treated hmoDCs (**Figure 2A**). V132-activated hmoDCs were also able to stimulate proliferation of CD4$^+$ T cells but significant changes were not detected when comparing with excipients-treated hmoDCs (**Figure 2A**). HmoDCs activated with MV140 or MV140/V132 generated T cells producing significantly higher levels of IFN-γ, IL-17A, and IL-10 than excipients- or V132-stimulated hmoDCs (**Figure 2B**). We did not detect IL-5 production in any of the assayed conditions (**Figure 2B**). Interestingly, MV140/V132-activated hmoDCs generated T cells producing significantly higher levels of IL-17A and IL-10 than MV140-activated hmoDCs (**Figure 2B**). To assess potential synergistic effects, the levels of IL-17A and IL-10 produced by T cells generated by MV140/V132-activated hmoDCs (**Figure 2B**) were compared to those resulting from the addition of these cytokine levels in MV140- and V132-stimulated conditions separately, shown in **Figure 2B**. IL-17A and IL-10 production was significantly higher when T cells were primed by hmoDCs activated with MV140/V132 than the sum of the cytokine levels produced by T cells generated by V132- plus MV140-activated hmoDCs, thus supporting synergistic effects of MV140/V132 at the T cell level (**Figure 2C**). To verify these data at the single cell level, we performed intracellular staining experiments. The percentages of IFN-γ–, IL-10–, and IL-17A–producing CD4$^+$ T cells generated by hmoDCs treated with V132, MV140 or MV140/V132 were significantly higher than those produced by T cells generated by excipients-treated hmoDCs (**Figure 3A**). Supporting our results, MV140/V132-activated hmoDCs generated a significantly higher percentage of IL-10– and IL-17A–producing T cells than MV140-treated cells (**Figure 3A**). To investigate the capacity of activated hmoDCs to induce Treg cells, we also analyzed the generation of FOXP3$^+$ Treg cells in allogeneic co-culture experiments. V132-, MV140-, and MV140/V132-treated hmoDCs induced significantly higher percentages of CD4$^+$CD25^{high}CD127$^-$FOXP3$^+$ Treg cells than excipients-treated hmoDCs (**Figure 3B**). Remarkably, the number of FOXP3$^+$ Treg cells generated by MV140/V132-activated hmoDCs was significantly higher than those induced by hmoDCs stimulated with V132 or MV140 alone (**Figure 3B**).

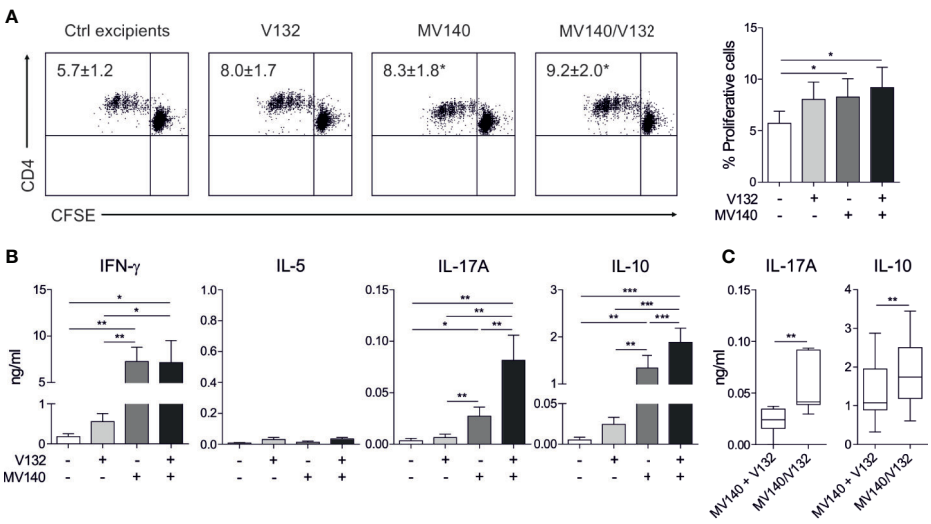

FIGURE 2 | MV140/V132-activated hmoDCs induce T cell proliferation and Th1, Th17, and IL-10 producing T cells. **(A)** Representative dot plots of proliferating CFSE-labeled allogeneic naive CD4$^+$ T cells after 3 days of co-culture with control excipients-, V132-, MV140-, and MV140/V132-stimulated hmoDCs and the graph with the frequency of proliferating cells (right side). **(B)** Cytokines produced by allogeneic naive CD4$^+$ T cells primed by hmoDCs in the presence of the indicated stimulus after 3 days quantified in cell-free supernatants by ELISA. **(C)** Levels of IL-17A and IL-10 for V132- plus MV140-stimulated co-cultures compared to MV140/V132-stimulated co-cultures. Results are mean ± s.e.m. of 5 **(A)**, and 8 to 12 **(B, C)** independent experiments. Paired Student *t* test, $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

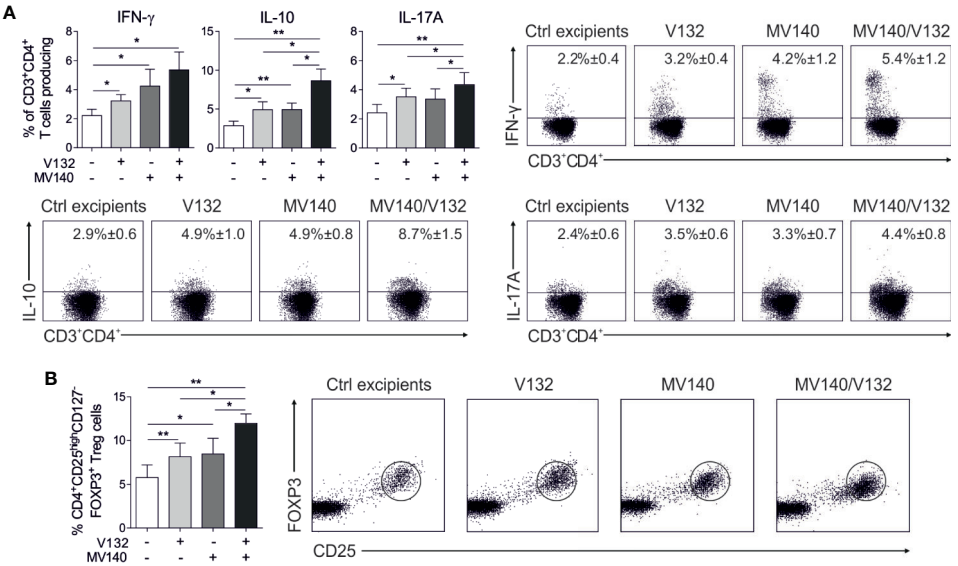

FIGURE 3 | MV140/V132-activated hmoDCs generate IFN-γ-, IL-17A-, and IL-10-producing T cell production as well as FOXP3$^+$ Treg cells. **(A)** Percentage of CD3$^+$CD4$^+$ T cells producing IFN-γ, IL-10 and IL-17A generated after 3 days of co-culture with control excipients-, V132-, MV140-, and MV140/V132-stimulated hmoDCs as determined by intracellular staining and flow cytometry analysis. Representative dot plots are shown for the intracellular staining after flow cytometry analysis. **(B)** Percentage of induced CD4$^+$CD25^{high}CD127$^-$FOXP3$^+$ Treg cells gated on CD4$^+$ T cells after 3 days of co-culture with allogeneic control excipients-, V132-, MV140-, and MV140/V132-activated hmoDCs. Results are mean ± s.e.m. of 7-8 **(A)**, and 5 **(B)** independent experiments. Paired Student *t* test, $^*P < 0.05$, and $^{**}P < 0.01$.

MV140/V132 Stimulation in Human Dendritic Cells Activates Mitogen-Activated Protein Kinases, Nuclear Factor-κB, and mTOR Signaling Pathways

MV140 triggers CLRs and TLRs in human DCs, which culminate in the activation of mitogen-activated protein kinases (MAPKs)- and nuclear factor-κB (NF-κB)-mediated signaling pathways (26). *C. albicans* activates a complex network of PRRs that also lead to the subsequent activation of MAPKs and NF-κB (44). Our data revealed that V132 rapidly induced activation of the MAPK extracellular signal-regulated kinases 1/2 (ERK1/2) (phosphorylation of Thr202/Tyr204) and the MAPK c-Jun NH_2-terminal kinase (JNK) (phosphorylation of Thr183/Tyr185) in hmoDCs without activation of MAPK p38 (phosphorylation of Thr108/Tyr182) (**Figure 4A**). MV140 alone induced a significantly higher phosphorylation of p38 and IKKα/β than V132 alone in hmoDCs without significant changes observed for ERK or JNK compared to V132 alone (**Figure 4A**). V132 alone induced significant phosphorylation of ERK and JNK compared to unstimulated cells without changes in p38 or IKKα/β. Interestingly, MV140/V132 induced a significantly higher phosphorylation of ERK, p38, and IKKα/β than V132 (**Figure 4A**). However, a slight but non-significant increment in the activation of ERK and JNK was observed after MV140/V132 stimulation compared with MV140 alone without differences in the activation of p38 or IKKα/β (**Figure 4A**). Sensing of *C. albicans* β-glucan by dectin-1 induces the activation of mTOR signaling pathway (31), however, the implication of mTOR pathway in the mechanism of action of MV140 remains unknown. V132, MV140 and MV140/V132 induced the activation of the mTOR pathway as determined by the phosphorylation of the upstream activator Akt (Ser473) and the downstream substrate p70S6 kinase (Thr389) (**Figure 4B**). V132 and MV140/V132 stimulation induces a similar and significantly higher phosphorylation of Akt and p70S6K than MV140 in hmoDCs (**Figure 4B**).

MV140/V132 Induces Metabolic and Epigenetic Reprogramming in Human Dendritic Cells

We studied metabolic changes in hmoDCs stimulated with V132, MV140 or the combination of MV140 and V132. A slight non-significant increase in the consumption of glucose and metabolic rate was observed in hmoDCs after V132 stimulation compared to excipients-treated hmoDCs (**Figure 5A**). In contrast, the stimulation of hmoDCs with MV140 or MV140/V132 significantly enhanced the consumption of glucose from the culture medium and, therefore, the metabolic rate compared to excipients- or V132-treated hmoDCs, without significant differences among them (**Figure 5A**). However, MV140/V132-activated hmoDCs significantly increased lactate production relative to MV140-stimulated hmoDCs, suggesting that the combination of MV140 and V132 is able to enhance Warburg effect in human DCs (**Figure 5B**). In the same line, hmoDCs stimulated with MV140/V132 displayed a significant decrease in the mitochondrial membrane potential relative to

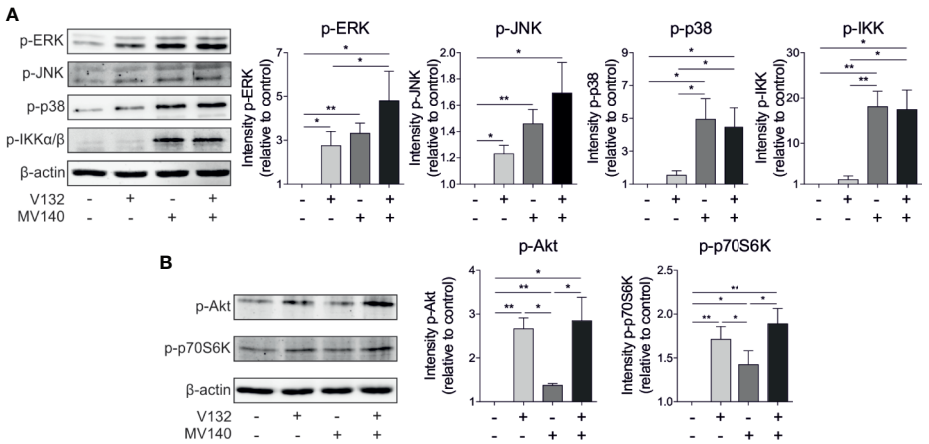

FIGURE 4 | Activation of MAPKs, NF-κB, and mTOR signaling pathways in hmoDCs stimulated with control excipients, V132, MV140, or MV140/V132. **(A, B)** Western blot analysis of protein extracts from hmoDCs stimulated for 30 min in the indicated conditions. Quantification of the reactive phosphorylated bands by scanning densitometry. β-actin was used as a loading control. One representative example is shown. Results are mean ± s.e.m. of four to seven independent experiments. Paired Student *t* test, $^{*}P < 0.05$, and $^{**}P < 0.01$.

stimulation with MV140 alone (**Figure 5C**), supporting that the combination of MV140 and V132 induces a metabolic rewiring that enhances glycolysis and lactic fermentation and reduces mitochondrial activity. Supporting these data, MV140/V132-stimulated hmoDCs showed a significantly lower mitochondrial content than hmoDCs stimulated with MV140 alone (**Figure 5D**) as determined by calculating the ratio of mitochondrial DNA (COXII)/nuclear DNA (GAPDH) as described in (45). Next, we studied the potential implication of the transcription factor HIF-1α as a key regulator of glycolysis and other metabolic processes. No changes in the expression of HIF-1α gene were observed after V132 stimulation compared with control cells. However, both MV140- and MV140/V132-activated hmoDCs expressed significantly higher mRNA levels of HIF-1α than excipients- and V132-treated hmoDCs, without significant differences among them (**Figure 5E**). Metabolic reprogramming has been associated with changes in the epigenome *via* histone methylation and acetylation. To assess whether V132, MV140, and MV140/V132 could induce epigenetic reprogramming in hmoDCs, we performed inhibition experiments using the histone methyltransferase inhibitor 5′-methylthioadenosine (MTA) and the histone demethylase inhibitor pargyline. Interestingly, TNF-α, IL-23, and IL-10 production was significantly impaired in the presence of MTA, but preserved in the presence of pargyline after stimulation of hmoDCs with V132, MV140, or MV140/V132 (**Figure 5F**), suggesting that epigenetic modifications are involved in the increased production of the assayed cytokines. An enhanced IL-10 production was observed in the presence of pargyline compared with stimulated cells without the demethylase inhibitor (**Figure 5F**). Cell viability was not significantly affected in any case and only a slight decrease in cell viability was observed for pargyline after V132 or MV140/V132 stimulation (**Supplementary Figure 2**).

In Vivo Sublingual Immunization of BALB/c Mice With MV140/V132 Enhances T Cell Proliferation and Induces Specific Antibodies Against All the Components in the Formulation

To study the capacity of the different assayed preparations to induce humoral responses *in vivo*, BALB/c mice were sublingually immunized with MV140/V132 or control excipients and the induced T and B cell responses analyzed (**Figure 6A**). Splenic $CD4^+$ T cells from mice immunized with MV140/V132 or control excipients showed significantly higher proliferation rates after *in vitro* stimulation with all the assayed stimuli than after *in vitro* stimulation with control excipients (**Figure 6B**). Remarkably, splenic $CD4^+$ T cells from mice sublingually immunized with MV140/V132 displayed significantly higher proliferation rates than those from control mice after *in vitro* stimulation with V132, MV140, MV140/V132 or with the non-specific T cell mitogen PHA (**Figure 6B**). Mice immunized with MV140/V132 produced significantly higher levels of specific IgG and IgA against all the components of the vaccine formulation including *C. albicans* (V132) and the individual bacterial components of MV140 (*K. pneumoniae*, *E. coli*, *P. vulgaris* and *E. faecalis*) than control mice (**Figures 6C, D**). As shown in **Figure 6D**, the highest specific IgG responses were against the MV140 gram-negative components *E. coli* and *P. vulgaris* whereas for IgA responses the highest levels were observed for *E. faecalis* and *P. vulgaris*. Interestingly, MV140/V132 immunization induced not only specific IgG1 but also IgG2a against all the components of the vaccine formulation (**Supplementary Figure 3**). As shown in this figure, similar specific IgG1 and IgG2a responses were induced upon MV140/V132 immunization, suggesting also the priming of IFN-γ-producing CD4 T cells driving IgG2a isotype switch. Sublingual immunization with MV140/V132 induced higher IgG responses against *E. coli* and *P. vulgaris* than immunization with

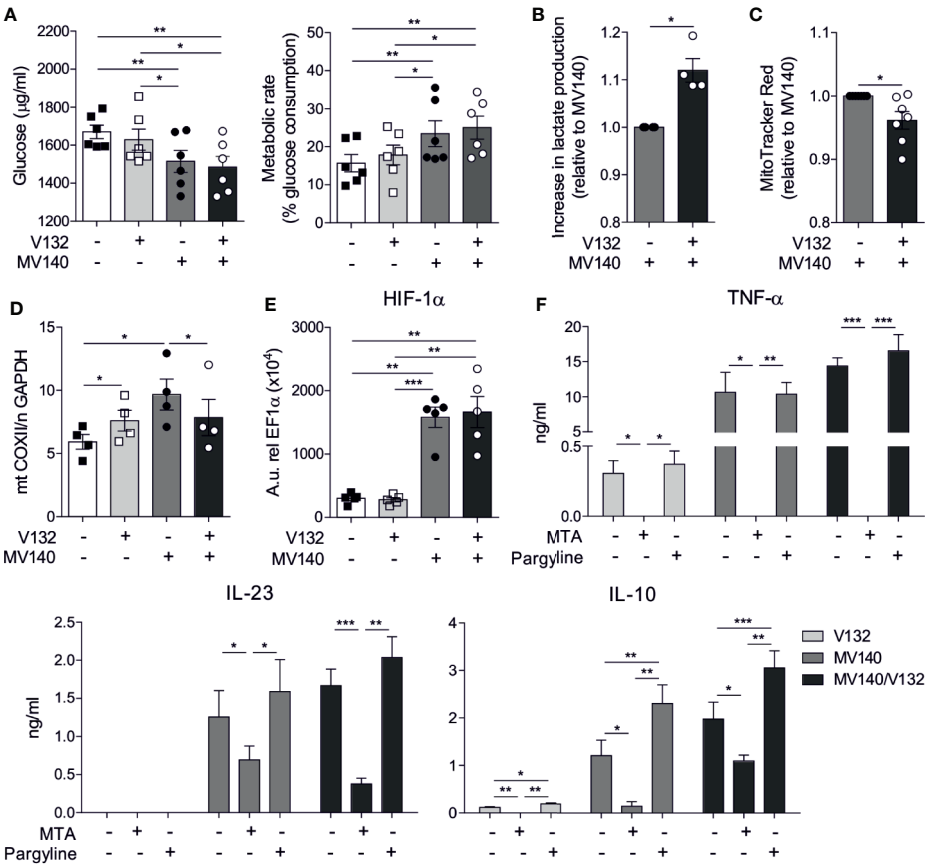

FIGURE 5 | MV140/V132 induces metabolic and epigenetic reprogramming in hmoDCs. **(A)** Glucose consumption by hmoDCs treated with control excipients, V132, MV140 and MV140/V142 after 18 h and calculated metabolic rate. **(B)** Increase in lactate production in cell-free supernatants from MV140/V132-stimulated hmoDCs relative to MV140-activated hmoDCs. **(C)** Relative changes in fluorescence intensity of stimulated hmoDCs stained with MitoTracker Red. **(D)** Mitochondrial mass determination as the ratio of mitochondrial (mt) and nuclear (n) DNA as expressed by mt COXII/n GAPDH. **(E)** Messenger RNA expression levels of HIF-1α gene in hmoDCs stimulated for 18 h. Arbitrary units (A.u.) are $2^{-\Delta CT}$ values multiplied by 10^4, with ΔCT defined as the difference between the cycle threshold value for the gene of interest and EF1α. **(F)** Cytokine production by V132-, MV140-, and MV140/V132-activated hmoDCs in the presence of 5′-methylthioadenosine (MTA) or pargyline as inhibitors of histone methyltransferases and demethylases, respectively. Results are mean ± s.e.m. of 6 **(A)**, 4 **(B)**, 7 **(C)**, 4 **(D)**, 5 **(E)**, and 4-7 **(F)** independent experiments. Paired Student *t* test, $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.01$.

MV140 alone whereas only a slight increase for *K. pneumoniae* and a slight decrease for *E. faecalis* were observed (**Figure 6E**). MV140/V132 induced higher IgA responses for *K. pneumoniae* and *P. vulgaris* than immunization with MV140 alone whereas lower IgA levels were detected for *E. coli* without changes for *E. faecalis* (**Figure 6E**). Interestingly, *in vivo* sublingually immunization with MV140/V132 induced higher specific IgG and IgA responses against *C. albicans* than immunization with V132 alone, which was special marked for IgA, thus indicating that the incorporation of MV140 into V132 preparations might well significantly increase the production of mucosal IgA antibodies specific for *C. albicans* compared to V132 alone (**Figure 6F**).

DISCUSSION

In this study, we demonstrated that the combination of MV140 and V132 at an equal proportion imprints human DCs with the capacity to polarize potent IFN-γ– and IL-17A–producing T cells and $FOXP3^+$ regulatory T (Treg) cells. MV140/V132 induces a more potent maturation of DCs and a higher capacity to produce pro-inflammatory cytokines and IL-10 than MV140 or V132 alone. Mechanistically, we showed that MV140/V132 activates MAPK-, NF-κB- and mTOR-mediated signaling pathways in human DCs compared to unstimulated cells, which might well be connected to the induced metabolic and epigenetic reprogramming as key molecular mechanisms driving trained immunity. In particular, MV140/V132-activated hmoDCs enhance glycolytic capacity and reduce mitochondrial activity. MV140/V132 stimulation also leads to histone methylation that enhances the production of TNF-α, IL-23, and IL-10 cytokines by human DCs. *In vivo* sublingual immunization of BALB/c mice with MV140/V132 resulted in enhanced proliferative responses of splenic $CD4^+$ T cells not only upon *in vitro* stimulation with the related antigens contained in the vaccine formulation but also upon stimulation with phytohaemagglutinin, a non-specific

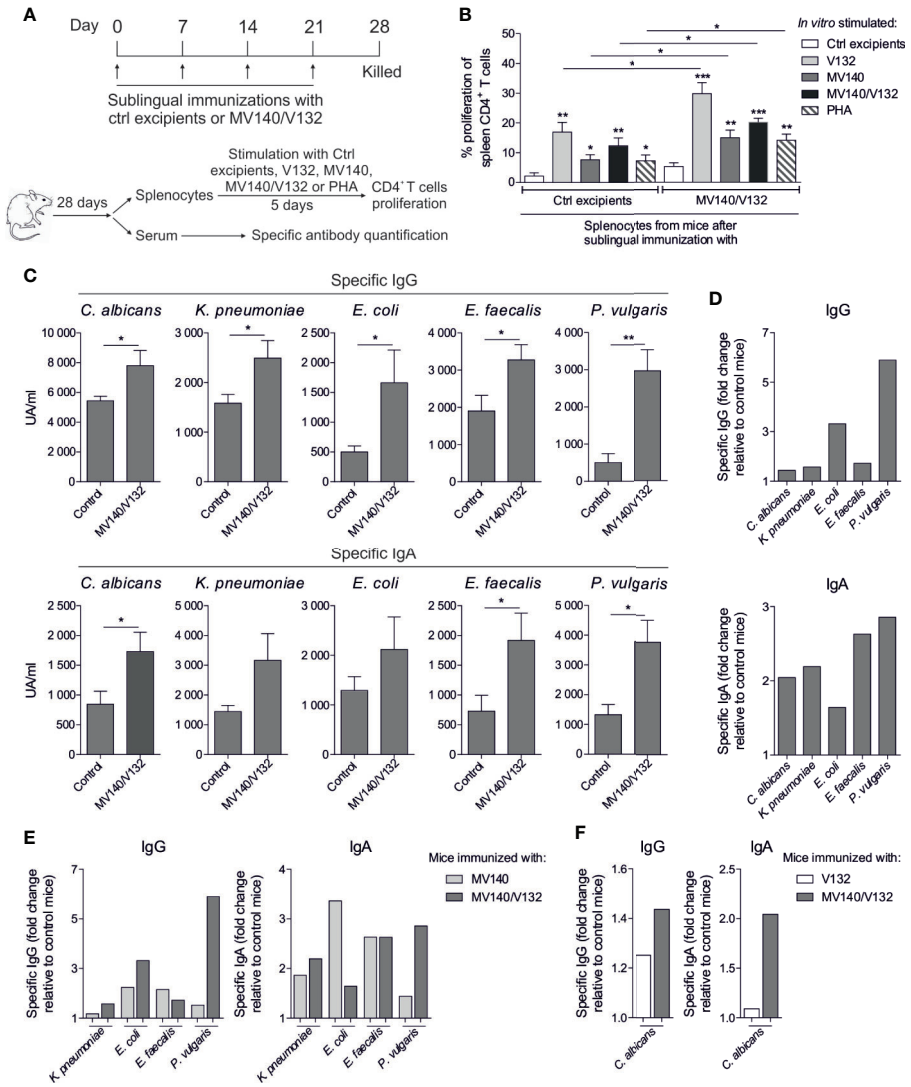

FIGURE 6 | Sublingual immunization of BALB/c mice with MV140/V132 enhances splenic T cell responses and induces specific antibodies against all the components included in the formulation. **(A)** Scheme of the sublingual immunization protocol and analysis of induced systemic responses. **(B)** Proliferation of CFSE-labeled $CD4^+$ T cells from splenocytes isolated from mice immunized sublingually with MV140/V132 or control excipients after *in vitro* stimulation with V132, MV140, V140/V132, phytohaemagglutinin (PHA), or control (containing all excipients except the bacteria and yeast). **(C)** Serum IgG and IgA antibodies specific for **(C)** *albicans*, *K pneumoniae*, *E coli*, *E faecalis* and *P. vulgaris* from mice immunized with MV140/V132 or with control excipients. **(D)** Fold change of specific IgG and IgA antibodies generated in mice immunized with MV140/V132 relative to control mice. **(E)** Fold change of specific IgG and IgA antibodies for *K pneumoniae*, *E coli*, *E faecalis* and *P. vulgaris* generated in mice immunized with MV140/V132 or MV140 alone relative to control mice. **(F)** Fold change of specific IgG and IgA antibodies for *C albicans* generated in mice immunized with MV140/V132 or V132 alone relative to control mice. Results are mean ± s.e.m. of 7-8 **(B)** and 5-6 **(C–F)** individual mice per condition of one single experiment. Unpaired *t* test, $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

T cell mitogen, which suggests the induction of trained immunity mechanisms. Remarkably, *in vivo* sublingual immunization with MV140/V132 induces the generation of specific IgG and IgA antibodies against all the components contained in the vaccine formulation. Overall, we report for the first time immunological mechanisms underlying the potential mode of action of a combination of MV140 and V132 as a novel promising trained immunity-based vaccine for GUTIs.

MV140 (Uromune) is a sublingual vaccine for RUTIs prophylaxis that is able to prime human DCs with the capacity to induce Th1-, Th17-, and IL-10–producing T cells (26). Clinical studies have demonstrated that MV140 significantly prevents RUTIs in 80% to 90% of individuals during the year following vaccination (39–43). The reported clinical benefits indicate that sublingual administration of MV140 also reduces the overuse of antibiotics, which not only might reduce the increase in antibiotic-resistances (40), but also avoid microbiota disruption (1, 7, 9–12). Due to the high incidence of RVVCs and the difficulties for their treatment, mainly based on antifungals, new approaches to combat these infections are also fully

demanded. Among these alternatives, classical anti-*C. albicans* vaccines based on recombinant proteins or cell wall-derived glycans to induce specific protective antibodies have been developed (46–48). However, up to date no safe and effective antifungal vaccine against RVVCs is still available (25). At this regard, V132 is a mucosal heat-inactivated *C. albicans* vaccine that could represent a suitable strategy to improve treatments for RVVCs. A small pilot study performed by our groups showed that sublingual administration of V132 significantly prevented VVCs recurrence, conferring additional protection to RUTIs when combined at 50% with MV140 in the formulation (unpublished data). Supporting these observations, it was previously reported that intranasal immunization with heat-inactivated *C. albicans* generates protection against *C. albicans* infection in mice and, interestingly, the protective role of this fungal vaccine was higher when mice were immunized with *C. albicans* in combination with *E. coli* toxin as a mucosal adjuvant than the single heat-inactivated fungal administration (49). As RUTIs and RVVCs are related to each other, a polymicrobial formulation combining both MV140 and V132 might well be an alternative approach for the treatment of GUTIs that could improve the efficacy of the vaccination within a single preparation. However, prior to definitive translation into clinical practice further preclinical mechanistic studies are needed. The addition of V132 to MV140 would incorporate *C. albicans* antigens into the vaccine, which could induce antigen-specific responses also against the yeast. In addition, our *in vivo* data indicated that mice immunized with MV140/V132 displayed stronger IgG and IgA responses against *C. albicans* than those immunized with V132 alone, suggesting that MV140 might well enhance the capacity of V132 to induce humoral responses. However, how the combination of V132 and MV140 might regulate mucosal DCs' function and their capacity to prime adaptive immune responses at the molecular level remained fully elusive.

Our data revealed that MV140/V132 promotes a potent pro-inflammatory cytokine production (TNF-α and IL-23) and higher levels of IL-10 in human DCs compared with individual MV140 or V132 stimulation (26), which are key cytokines also to polarize T cell responses (50, 51). Human DCs activated with MV140/V132 generate potent IFN-γ– and IL-17A–producing T cells that could contribute to eradicate intracellular and extracellular pathogens, respectively (51). MV140/V132 also induces higher frequencies of IL-10–producing T cells and $FOXP3^+$ Treg cells than individual MV140 and V132, which are essential to control excessive immune responses, to enhance microbiota protection and tissue repair after long-term antibiotic treatments and play a key role in restoration of healthy immune responses in urogenital infections (50, 52–54). Additionally, *in vivo* sublingual immunization with MV140/V132 induced the generation of significant levels of specific IgG and IgA antibodies against all the components contained in the vaccine formulation, which might well confer protection against infections caused by these specific pathogens (33, 55). It is also noteworthy to highlight that splenic $CD4^+$ T cells from mice that were sublingually immunized with MV140/V132 displayed significantly higher proliferation rates than those from control mice not only after *in vitro* stimulation with the vaccine components (either separately or in combination) but also after *in vitro* stimulation with the non-specific T cell mitogen PHA. These data suggest that MV140/V132 immunization might well promote trained immunity mechanisms that enhance both innate and adaptive immune responses against a broad range of pathogens, contained or not in the vaccine formulation, upon new encounters. At this regard, metabolic and epigenetic reprogramming represent well-recognized mechanisms underlying trained immunity in myeloid cells, including DCs (23). The regulation of metabolic rewiring plays an important role in the immunogenic vs tolerogenic properties of DCs (35, 36). For example, the switch to glycolysis and lactic fermentation couple to anabolic metabolism for the biosynthesis needed for cell growth is associated with DCs activation (35, 56). We showed that MV140/V132-activated hmoDCs display enhanced metabolic rate and lactate production while reduced mitochondrial activity, suggesting Warburg effect induction. Supporting this observation MV140/V132 not only induced the rapid activation of immune pathways involved in cytokine and surface marker production such as those mediated by MAPKs or NK-κB but also mTOR-mediated signaling pathways as a central regulator of metabolic reprogramming in hmoDCs (35, 57, 58). mTOR controls numerous cellular processes in DCs to adapt the cellular metabolism to immune functions such as antigen presentation or cytokine production (35, 59). Interestingly, V132 alone and MV140/V132 stimulation induced a higher activation of mTOR signaling pathway than MV140 alone, indicating that V132 significantly contributes to the activation of this key signaling pathway in the induction of innate trained immunity mechanisms. In addition, the activation of the MAPK JNK and p38 are also relevant for the induction of trained immunity in human monocytes (60). Our data show that MV140 is the main driver of p38 and JNK within the MV140/V132 formulation, suggesting also an important contribution of these MV140-induced pathways in the induction of trained immunity. HIF-1α activation is involved in enhanced glycolytic activity and lactate production by the induction of metabolism-related genes (61). MV140 alone and MV140/V132 but not V132 alone enhance HIF-1α mRNA expression levels in hmoDCs. These data suggest that the metabolic reprogramming induced by MV140/V132 might be due to the contribution of both HIF-1α expression, enhanced by bacterial components (MV140) of the preparation (61), and mTOR signaling, induced by *C. albicans* (V132) (31), which together might well drive a more potent glycolytic metabolism. Changes in cellular metabolism could be also accompanied by epigenetic reprogramming, mainly *via* methylation (32, 62). Activation of immune cells induces histone modifications affecting the gene expression patterns (32, 63). The methyltransferase inhibitor MTA but not the histone demethylase inhibitor pargyline abolishes V132-, MV140- and MV140/V132-induced cytokine production in hmoDCs, suggesting that histone methylation is an epigenetic change essential in the mechanism of action of these stimuli (30, 60).

Collectively, MV140/V132-stimulated human DCs activate the Akt/mTOR/HIF-1α axis leading to metabolic and epigenetic

reprogramming, which are defining hallmarks of trained immunity. These mechanistic insights might well support the trained immunity effects observed after sublingually immunization of BALB/c mice with MV140/V132 (23, 29–32, 62, 64). In conclusion, the combination of MV140 and V132 in a single vaccine formulation could represent a novel promising TIbV for RUTIs and RVVCs. In the long run and upon proper clinical translations, this study might well pave the way for the development of novel alternative approaches for GUTIs.

DATA AVAILABILITY STATEMENT

The datasets presented in this article are readily available upon request. Requests to access the datasets should be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Host Institution UCM. Fully anonymous Buffy Coats from healthy donors were provided by the "Centro de Transfusión de la Comunidad de Madrid". UCM is perfectly equipped with the adequate biosecurity infrastructure required to carry out the research presented in this proposal. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements. The animal study was reviewed and approved by Biolab S.L. Animals were maintained in Biolab S.L. under the conditions stabilized by the R.D. 53/2013 and in accordance with the international GLP normative, RD 223/83 and ISO 10993-2:2007. The tests are performed in accordance with official methods, European Pharmacopoeia, USP Pharmacopoeia, OECD Guideline, UNE EN-ISO, and specific methodologies developed for R&D.

AUTHOR CONTRIBUTIONS

OP conceived and designed the study. LM-C, CS-O (human experiments), C B-V (technical support for human experiments), and CD-R (mice experiments) performed the experiments. SS-R, JLS, and OP provided the reagents. LM-C, CS-O, CB-V, C.M. D-R, SS-R, JLS, and OP analyzed and discussed the data. OP and L.M-C wrote the paper. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.612269/full#supplementary-material

SUPPLEMENTARY FIGURE 1 | Production of TGF-β1 and expression of CD86 and HLA-DR in hmoDCs after control excipients, V132, MV140, and MV140/V132 stimulation. **(A)** TGF-β1 production after stimulation of hmoDCs with control excipients, V132, MV140 or MV140/V132 for 18 h quantified in cell-free supernatants by ELISA**. (B)** Flow cytometry representative dot plots for the expression of surface markers HLA-DR and CD86 after stimulation of hmoDCs with control, V132, MV140 and MV140/V132 for 18 h. **(C)** Quantification of the percentage of positive cells and **(D)** Mean Fluorescence Intensity (MFI) values for CD86 after stimulation of hmoDCs. Results are mean ± s.e.m. of 8 **(B, C)** independent experiments. Paired Student *t* test, $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

SUPPLEMENTARY FIGURE 2 | Percentage of viability for each assayed condition respect to each vehicle control by using trypan blue exclusion. Results are mean ± s.e.m. of 3-4 independent experiments.

SUPPLEMENTARY FIGURE 3 | (A) Serum IgG1 and IgG2a antibodies specific for *C. albicans*, *K. pneumoniae*, *E. coli*, *E. faecalis* and *P. vulgaris* from mice immunized with MV140/V132 or with control excipients. **(B)** Fold change of specific IgG1 and IgG2a antibodies generated in mice immunized with MV140/V132 relative to control mice. Results are mean ± s.e.m. of 4-5 individual mice per condition of one independent experiments. Unpaired t test, $^{*}P < 0.05$.

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REVIEW
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Dendritic Cell Vaccines in Ovarian Cancer

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Ovarian cancer (OC) is one of the most lethal malignant gynecologic tumors, characterized by an uncertain presentation and poor outcomes. With or without neoadjuvant chemotherapy, surgery followed by platinum-based chemotherapy and maintenance therapy are the basis for the treatment of ovarian cancer patients, but the outcome is still highly restricted by their advanced stage when diagnosed and high recurrence rate after chemotherapy. To enhance the anti-tumor effect and postpone recurrence, anti-VEGF agents and PARP inhibitors are suggested as maintenance therapy, but the population that can benefit from these treatments is small. Based on the interactions of immune cells in the tumor microenvironment, immunotherapies are being explored for ovarian cancer treatment. Disappointingly, the immune checkpoint inhibitors show relatively low responses in ovarian cancer. As shown in several studies that have uncovered a relationship between DC infiltration and outcome in ovarian cancer patients, dendritic cell (DC)-based treatments might have a potential effect on ovarian cancer. In this review, we summarize the functions of dendritic cells (DCs) in the tumor microenvironment, as well as the responses and drawbacks of existing clinical studies to draw a comprehensive picture of DC vaccine treatment in ovarian cancer and to discuss the promising future of immune biomarkers.

Keywords: dendritic cells (DCs), ovarian cancer (OC), immunotherapy, tumor microenvironment, dendritic cell vaccine

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INTRODUCTION

Ovarian cancer is the most lethal gynecological cancer, with an overall 5-year-survival rate of 48% (US, reported in 2020). Nearly 75% of patients have no symptoms until an advanced stage, which leads to a 29% 5-year-survival (1). First-line treatments include surgery and platinum-based chemotherapy. Although primary treatments show remission effects, approximately 75% of patients suffer from recurrences, followed by eventual drug resistance state.

When confronted with recurrences, platinum-sensitive patients are recommended to accept platinum-based combined chemotherapy followed by targeted therapy according to the NCCN guidance. However, platinum-resistant recurrences have limited effective strategies to choose. The response rates to cytotoxic therapy [ex. topotecan, 20% (2); docetaxel, 22% (3)] and single agents targeted therapy [ex. bevacizumab, 20% (4)] are low, and furthermore, a combination of chemotherapy and bevacizumab increase the median overall survival by only 3.3 months, with

no significant difference between the combination therapy and chemotherapy groups (5). Consequently, studies have focused on maintenance therapy to postpone any recurrence (6). Poly ADP-ribose polymerase (PARP) inhibitors, including olaparib, niraparib, and rucaparib, have manifested inspiring efficacy in maintenance therapy. Olaparib for those with BRCA1/2 mutations has increased the response rate to 36% (7), however, BRCA1/2 mutations exist in only 10% of ovarian cancer patients. Although niraparib and rucaparib extend their indications to those with homogenous repair deficiency (HRD) (8) as well as those with platinum-sensitive recurrent epithelial ovarian cancer (EOC) regardless of BRCA status (9), most patients still do not qualify. Emerging studies aim to elongate recurrence intervals for better survival, and immunotherapy is being considered.

The presence of tumor infiltration lymphocytes is related to a higher 5-year-survival rate (38% vs 4.5%) in ovarian cancer (10), which throws light on immunotherapy. However, the efficacy of immune checkpoint blockers, such as the anti-PD-1 agent pembrolizumab, depend on microsatellite instability-high or mismatch repair-deficient circumstances. The overall response rate to pembrolizumab among PD-L1$^+$ advanced metastatic ovarian cancer patients is only 11.5% (11), and the percentage of PD-L1$^+$ cases of high grade serous ovarian cancer is only 57.4%, and it is 0%–26.7% in other histologic subtypes of ovarian cancer (12). Moreover, adoptive T cell therapy is hindered by a low level of T cell infiltration, poor neoantigen presenting function and immunological tolerance epitopes (6), which suggests to enhance the process of antigen-presenting for amplifying anti-tumor effect.

In the tumor microenvironment, dendritic cells take and process tumor-associated antigens, then present them by MHCI/II molecules to activate T cells. With the aim of enhancing the process of antigen-presenting, DCs are regarded as promising target. The first clinical trial of dendritic cell (DC) vaccine started in 1996. Currently, more than 400 clinical trials of DC-based treatment for tumors have been registered in ClinicalTrials.gov. Up till June 2014, DC-based treatment in only four tumor types had reached phase III clinical trials, including melanoma, prostate cancer, malignant glioma and renal cell cancer (13). During 2014–2017, 43 peer-reviewed publications reported the outcomes of clinical trials on DC vaccines (14) in various cancers. In most clinical trials, some of the patients reached a stable state, and a lower percentage of patients reached a partial response or a complete response (according to RECIST guidelines). During 2017–2019, 34 peer-reviewed papers were published (15) suggesting more strategies to improve the response rate to DC vaccines.

Safety was confirmed in most clinical trials and the response rate to DC-based treatment gradually increased due to improved production strategies. Antigen loading, DC origination and induced maturation strategies are key steps to produce DC vaccines, which stand at the core stage of innovation. In 2010, Sipuleucel-T became the first DC vaccine approved by the FDA, for the treatment of metastatic prostate cancer. In ovarian cancer, various DC vaccines have been tested, showed an increment in progression-free survival (PFS) and overall survival (OS) (16, 17), which inspired further studies.

DIFFERENTIATION, MATURATION, AND FUNCTION OF THE DENDRITIC CELLS

DCs originate from CD34$^+$ hemopoietic stem cells in the bone, differentiate to different subtypes in the peripheral blood and nonlymphoid organs and tissues, and mature in the lymphoid organs (18–21) (**Figure 1**). Immature DCs that express low levels of toll-like receptors (TLRs) MHC molecules, costimulatory molecules, as well as adhesion molecules stay outside of lymphoid tissues and have weak antigen-presenting functions. TLRs are the essential receptors among the sensors of pathogen-associated molecular patterns (PAMPs) and damage associated molecular patterns (DAMPs). PAMPs from bacteria, viruses, or parasites activate DCs to activate the innate immune response (22), which act as a general defense against infectious diseases; in tumors, DCs are activated in response to DAMPs from tumor cells through TLR signaling (23).

The migration of immature DC is induced by the chemokine receptor CCR7 and CCR8, which is up-regulated during the maturation process (24). Stimulated by antigens, immature DCs migrate toward chemokine ligands CCL19 and CCL21 into lymph nodes and gradually develop into a mature state, highly expressing MHC I molecules, MHC II molecules, costimulatory molecules and adhesion molecules (25), then mature DCs active CD4$^+$T cells and CD8$^+$T cells at the tumor site migrate to lymphoid organs to build immune memory.

As robust antigen-presenting cells, DCs exert a key influence in regulating the innate immune response and initiating adaptive immune responses. DCs have more vigorous capability to capture, process and present antigens than other APCs, such as B cells, mononuclear cells, and macrophages (26). DCs take parts in forming the first and second signals to activate T cells. In the process of activating specific T cells, the MHC-antigen peptide-TCR complexes act as the first signal, and the costimulatory factors on the membrane of APCs act as the second signal. Generally, DCs capture and process exogenous antigen peptides into antigen peptide/MHC II molecules in order to activate CD4$^+$ helper T cells, and endogenous antigen into antigen peptide/MHC I molecules for CD8$^+$ T cells (27, 28).

In tumors, different subtypes of DCs play divergent roles. Conventional dendritic cell type 1 (cDC1) and conventional dendritic cell type 2 (cDC2) are two subtypes of cDCs. cDC1 is the main DC subtype that activates CD8$^+$ T cells through the antigen cross-presentation process (29), while cDC2 secretes IL-10, IL-12, IL-23, and TNF-β to stimulate the differentiation of the CD4$^+$T cells (30). It is cDCs that mainly present tumor antigens and promote antitumor effects.

Plasmacytoid DCs (pDCs) may be involved in both tumor protective processes and tumor-suppressive processes. On one side, pDCs mainly secret type I IFN, which is essential antitumor cytokine (31). On the other side, pDCs induce immunosuppressive cells, leading to a poor outcome (25, 32). Due to potentially bidirectional effect, the role of pDCs may be dependent on the tumor microenvironment. pDC is the main subtype of DCs in the tumor sites of ovarian cancer (33), and the infiltration of pDCs in the ovarian cancer microenvironment has a negative association

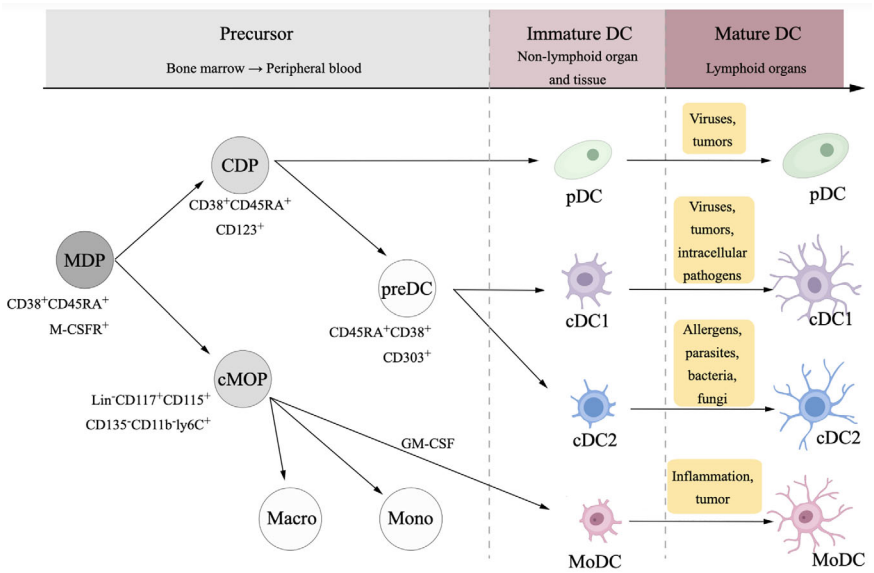

FIGURE 1 | Differentiation and maturation of dendritic cell (DC). DCs and monocytes originate from a common ancestor, namely macrophage dendritic cell progenitor (MDP). MDPs differentiate to common monocyte progenitors (cMOPs) and common DC progenitors (CDPs). cMOPs generally differentiate into monocytes and macrophages, while in some situations with pro-inflammatory context, cMOPs could be stimulated by granulocyte-macrophage colony stimulating factor (GM-CSF) then differentiate to monocyte-derived dendritic cells (MoDCs). CDPs differentiate to two subsets, plasmacytoid DCs (pDCs), and myeloid dendritic DCs, which is usually called conventional DCs (cDCs).

with the prognosis (34), but the pDC in response to TLR could release IFN-α, although such type of function is weaker than that in the peripheral blood (35). The involvement of DCs in antitumor effects may be disturbed in ovarian cancer, which indicates a potential benefit of DC vaccines.

Monocyte-derived dendritic cells (MoDCs) originate from monocytes in the peripheral blood. The differentiation of MoDCs is commonly induced by GM-CSF and IL-4, followed by the maturation of immature MoDCs stimulated by tumor-associated antigens and other agents (36). MoDCs mainly respond to inflammation in the mouse, but human MoDCs are mostly studied *in vitro*, and their function depends on the stimulatory signals in the culture.

DENDRITIC CELL DYSFUNCTION IN THE TUMOR MICROENVIRONMENT

Ovarian cancer lesions have a high degree of DC infiltration, but infiltrated DCs have low efficacy of antigen presentation due to DC tolerance, which is marked by downregulated expression of costimulatory molecules on the surface of DC cells (37), as well as having weaker antigen-presenting ability. DCs also act to assist tumor cells in some situations. In the tumor microenvironment, it has been confirmed that many aspects could induce dysfunction of DCs, as discussed below.

Immune checkpoint signaling may participate in DC dysfunction. The combination of programmed cell death protein 1 (PD-1) on T cells and programmed death-1 ligand (PD-L1) on tumor cells leads to the programmed death of the T cells. Ovarian cancer cells could upregulate PD-L1 in DCs by secreting TGF-β and PGE_2 into the microenvironment (38), enhancing their inhibition of the T cell immune response. PD-1 inhibitors could restore the capacity of DC, thus enhancing their antitumor effect in ovarian cancer (39). Specific DCs interact with immunosuppressive cells to disturb the antitumor effect. Inducible costimulatory molecule (ICOS) is expressed on the immunosuppressive Treg cells, and pDCs in the ovarian cancer microenvironment activate Treg by expressing ICOS ligand, leading to tumor progression (40).

Some metabolic factors could induce DC dysfunction, including dysfunction of amino acid metabolism and lipid metabolism. The overexpression of indoleamine 2,3-dioxygenase (IDO) in DCs plays an immunosuppressive role. IDO is an essential enzyme in amino acid metabolism, which turns tryptophan into kynurenine. TGF-β released by tumor cells can upregulate the expression of IDO in pDC and the secretion of cytokine CCL22, which recruits Tregs into the tumor microenvironment. IDO-expressing DCs reduce the concentration of tryptophan near Tregs and keep Tregs in an immunosuppressive state *via* tryptophan-induced mTORC-Akt signaling (41). Clinical trials have reported that IDO inhibitors lead to a decreased level of the products of IDO in solid tumors (42), which could be used as a combined agent in DC immunotherapy. Additionally, in response to the endoplasmic reticulum stress induced by the byproducts of lipid peroxidation, the transcription factor XBP1 is activated and this leads to lipid body accumulation in tumor-infiltrating DCs, pushing DCs into a tolerant state in the ovarian cancer microenvironment (43).

Insulin-like growth factor (IGF) also has impacts on the DCs in ovarian cancer. The IGF participate in cell proliferation as well as in protein synthesis and growth through the RAS-ERK and PI3K-AKT pathways (44). DCs treated with IGF fail to mature and secret higher levels of IL-10 as well as TNF-α, which are suppressive immune factors in the ovarian cancer microenvironment (45). The insulin-like growth factor type I receptor (IGF1R) is highly expressed in ovarian cancer and is negatively related to the differentiation of DCs towards cDCs (46). IGF1R inhibitors rebuild the DC-mediated antitumor effect (45), which suggests that the IGF axis may induce DCs to enter a dysfunctional state.

In conclusion, immunosuppressive signals in these aspects lead to a dysfunctional state of the DCs in the ovarian cancer microenvironment. Theoretically, infusion of functional DCs into the body could avoid infiltrating in the tumor microenvironment and instead make direct contact with the T cells in the lymph nodes, which compensates for DC dysfunction state. Based on this, DC vaccines could restore the tumor antigen-presenting ability to elicit antitumor effects.

ELEMENTS OF MANUFACTURING DENDRITIC CELL VACCINES FOR OVARIAN CANCER

The common routine of DC vaccine manufacturing includes several elements: (1) obtaining human DC developmental potential cells through apheresis; (2) stimulating autologous immature DCs into a mature state *in vitro*, in which process the DCs are usually activated by a cocktail of various cytokines, Toll-like receptors agonists and other activators; and (3) loading the immature DCs with tumor-associated antigens, namely, DCs being cocultured with antigens in the form of peptides, proteins, tumor cell lysates or tumor cells. After these steps, the mature DCs are gathered and vaccinated back to the patients.

Preclinical and clinical studies are exploring various alternatives of each element in the manufacture of DC vaccines to achieve a better efficacy in the treatment of ovarian cancer. These elements are discussed separately below.

Selecting Appropriate Dendritic Cell Subtypes for Vaccination

The subtypes of autologous DC chosen for vaccine manufacture show various antigen presenting potential, which might affect the efficacy of DC vaccines. In preclinical and clinical studies of the DC vaccines in tumors, DC subtypes selected from peripheral blood cells through apheresis include MoDCs, cDCs and Langerhans cell-type DCs (13, 30). The DC subtypes targeted to improve antitumor immune responses in clinical studies of the vaccines targeting DC *in vivo* and ex vivo are distinct and might be dependent on the cancer types (31). The vaccines targeting DCs *in vivo* do not need apheresis to gather autologous DCs for vaccine manufacturing, and instead, specific antigens targeting receptors on DCs *in vivo* are injected into the body, such as the vaccine CDX-1401 targeting DEC205$^+$cDC1s in multiple tumors including ovarian cancer, which contains the DEC205 antibody fused with NY-ESO-1 and a TLR agonist (47).

The vaccines targeting DCs ex vivo are based on peripheral blood cells gathered from apheresis. Among all subtypes, MoDCs are most frequently used for targeting DCs ex vivo, mainly because the count of DCs in peripheral blood cells is not sufficient to produce a vaccine, but the count of monocytes is higher, and the monocytes cultured *in vitro* provide relatively abundant DCs relative to other origins. However, MoDCs show an unsatisfying effect in eliciting CTL responses compared to Langerhans cells in the treatment of melanoma (13, 48). cDCs used for vaccines are also confirmed to superior to MoDCs in eliciting systemic and long-lasting immune responses. Additionally, cDCs could enhance the efficacy of immune check point blockers (49). Flow cytometry and immune bead sorting have made it possible to select specific DC subtypes to induce specific CTL activation. However, there still a lack of evidence to confirm which subtype of DCs is the best choice.

cDC1, cDC2, and pDC are found in ovarian cancer, with a lower rate of both cDC and pDC in the peripheral blood compared with healthy control (33, 35). The ratio of cDC and pDC varies between peripheral blood, ascites and tumor sites. According to The most prominent subsets of DCs is pDC in ascites (50) and tumor sites (34), while cDC is more than pDC in the peripheral blood (35), which suggests that peripheral blood could be a proper resource of the DCs for manufacturing.

However, the counts of cDCs is hardly sufficient for vaccine manufacturing, in most clinical studies on DC vaccines in ovarian cancer, DCs used for vaccine manufacture are MoDCs. Mononuclear cells are isolated from peripheral blood through apheresis and are cultured *in vitro* with GM-CSF and IL-4 for several days. To monitor the cell components of the DC vaccine, the expression of the markers on DCs are analyzed, mainly including CD11c$^+$, HLA-DR$^+$, HLA-ABC$^+$, CD40$^+$, CD80$^+$, CD83$^+$, CD86$^+$, and CCR7$^+$ (17, 51). Notably, these markers are not sufficient to distinguish MoDCs from other subtypes of DCs, and the final DC vaccine is a mixture of DC and a small fraction of other peripheral blood cells.

To conclude, MoDCs have been most frequently used for manufacturing DC vaccines in the current clinical study on ovarian cancer, and it is unclear if other subtypes of DCs would be more beneficial.

Loading Tumor-Associated Antigens

To induce DCs to recognize and present specific tumor antigens, several potential methods are tested, including pulsing DCs with tumor-associated antigens, inducing tumor cells and DCs into fusion cells, and mRNA transferring. The tumor-associated antigens are most frequently used in the clinical trials, while the other two are limited to case reports or clinical studies of a small population. The antigens to load DCs determine the specificity of the antitumor effect, production costs, and side effects of the DC vaccine, which makes it an essential step. Among current DC vaccine research on various cancers, immature DCs are loaded with various forms of tumor-associated antigens, including peptides, proteins, and whole

tumor lysates. Published studies of different types of DC vaccines in ovarian cancer are listed in **Table 1**.

For targeting antigens expressed on the ovarian cancer cells, DCs are loaded with one or more peptides/proteins. Proto-oncogene HER-2/neu-derived peptides are used to load DC, such as E75 (epitope recognized by cytotoxic T lymphocytes, amino acids 369-377) (52, 63), GP2 (transmembrane part, amino acids 654-662) (52, 64), and recombinant fusion antigen BA7072, which contains both intracellular and extracellular domain of HER-2/neu (65). Wilms tumor 1 (WT-1) is an intracellular protein that overexpressed in many solid tumors including ovarian cancer, therefore it is targeted by specific cytotoxic T lymphocytes when presented by MHC molecules (66). DC incubated with a MHC class I-restricted modified WT-1 derived peptide [HLA-A*2402-restricted, amino acids 235-243 (51), or HLA-A*0201/0206-restricted (55)] successfully induce WT-1 specific CTL effect, with the assist of a streptococcal primer OK-432 (51). Epithelial mucin 1 (MUC1) is a membrane glycoprotein, which is expressed in 90% of ovarian cancer samples (52, 67, 68). Other peptides for pulsing DCs are selected based on the expression rate in the ovarian cancer, including human telomerase reverse transcriptase (hTERT) (53), pan-DR epitope peptides (PADRE) (53), and p53 peptide (16). Most of the DC vaccines loaded with peptides/proteins have induced peptide/protein-specific IFN-γ secreting T cells proliferation after doses of the vaccines. The overall clinical response rate was approximately 26% (16, 51–53, 55, 65) and the disease stabilization period ranged from several weeks to months, but the clinical responses were limited to stable disease, followed by progressive disease.

To load DCs with antigens that contain a wider epitope rather than single epitope of derivative peptides, fusion peptides have also been tested. For example, MUC1 fusion peptides was conjugated to mannan for synthesizing mannosylated mucin 1 fusion peptide (M-FP) (54, 69). In a phase 2 single-arm study, 21 patients received at least three doses of vaccine DCs loaded with M-FP. Monitored by serum CA-125, two patients had a major response during the study, and the response duration was 57 and 71 weeks, respectively. However, the response rate was only 19%, and the IFN-γ releasing immune response was weak to moderate (54).

To further improve the immune and clinical response rate, tumor cell lysates were considered to be more effective cancer-specific antigens. These whole ovarian cancer cell antigens could be obtained from SKOV3 ovarian cancer cell lines (58), fresh tumor biopsy samples (17, 59), or paraffin block allogenic tumor sample (60). For enhanced immunogenicity of tumor antigens, tumor cells were induced to necrosis by repeating freeze-thaw cycles, or induced to apoptosis by irradiation, as well as oxidized by hypochlorous acid (HOCl). Preclinical study has compared the efficacy of DCs pulsed with different tumor lysates. DC vaccines pulsed with tumor lysates that were prepared through HOCl oxidization followed by freeze-thaw cycles induced higher levels of IFN-γ secreting T cells compared with that prepared through irradiation followed by freeze-thaw cycles, or simply freeze-thaw cycles (58). In clinical trials, the frequency of IFN-γ secreting T cells increased significantly after DC treatment in most of these studies, and the increase of tumor-reactive T cells was associated with clinical benefits (17, 59).

As well as peptide-, protein-, and whole tumor lysate-loaded DCs, the use of mRNA transfected DCs have also been reported as a case report (56, 70). Additionally, tumor-DC fusion cell vaccines in ovarian cancer treatment have been tested (71, 72), but lack sufficient clinical trial data.

TABLE 1 | The antigen of dendritic cell (DC) vaccines used in the clinicial trials in ovarian cancer.

Antigen loaded	Clinical effect (Survival period)	Published Year
Her-2/neu or MUC1 peptide	–	2000 (52)
	In arms 1,2: estimated 3-year PFS: 40% vs 80%; estimated 3-year OS: 80% vs 100%	2012 (53)
	–	2014 (54)
p53 peptide	For arms 1/2: median PFS: 4.2 months vs 8.7 months; median OS: 40.8 months vs 29.6 months	2012 (16)
MHC class I-restricted Wilms tumor 1 (WT1) peptide	Median OS: 14.5 months	2014 (55)
	Median OS: 13.1 months	2019 (51)
	PFS: 0, 2 months OS: 70, 64 months	2013 (56)
Neoantigen peptides	OS since the 1st dose: 15 months	2020 (57)
Hypochlorous acid (HOCl)-oxidized autologous tumor lysate	PFS: 1 patient 36 months, 1 patient 44 months	2013 (58)
Autologous tumor cell lysate	–	2013 (59)
	Median PFS: 176 days median OS: 198 days	2014 (60)
	In cohort 2, median OS: 11 months; In cohort 3: median OS > 25 months;	2018 (17)
Keyhole limpet haemocyanin (KLH) and autologous tumor cell lysate	–	2002 (61)
	Median PFS: 19.2 months; median OS: 43.8 months OS: 64.95 ± 7.62 months	2015 (62)

PFS, progression free survival; OS, overall survival.

In addition to ovarian cancer-associated antigens, nonspecific antigens are also involved in DC vaccine trials. Keyhole limpet hemocyanin (KLH) is a foreign helper protein, which could enhance antitumor immunity by stimulating the IFN-γ production of T cells (73). DC vaccines have been pulsed with KLH and tumor lysate simultaneously; however, some patients developed KLH-specific T cell proliferation but failed to develop tumor antigen-specific T cell proliferation (61). KLH has also been added as a surrogate indicator of a DC vaccine (62), but controlled studies are needed to confirm the association between KLH-specific immune responses and the efficacy of DC vaccines.

Personalized Dendritic Cell Vaccines Based on Next-Generation Sequencing

Traditionally, DC vaccines are loaded with the tumor-associated antigens mentioned above, but their antitumor effect might be relatively narrow. For the pursuit of a broader antitumor effect, emerging personalized DC vaccines are being developed to target patient-specific neoantigens, namely, tumor-specific antigens that are derived from individual nonsynonymous single nucleotide variations. To validate the individual neoantigens, whole exome sequencing and bioinformatic analysis (e.g., fetchGWI, NETMHC) are combined, complemented or not by high throughput qPCR essays and mass spectrometry (74). To manufacture personalized DC vaccines, DCs are loaded with these candidate individual neoantigens through neoantigen gene-encoding peptides stimulation (75) or mRNA transfection (76, 77).

As a recent cohort study reported, autologous DCs loaded with autologous tumor cell lysate also successfully elicited a personalized neoepitope-specific T cell reaction as predicted (17). In this study, whole-exome sequencing and bioinformatic algorithms were used to predict individual neoantigens, and T cell clones targeted to these neoantigens are amplified after DC treatment. However, the paucity of the tumor sample attained from surgery might be an obstacle, and it is unclear whether the whole tumor lysate would induce DCs to a dysfunctional state as tumor cells do in the microenvironment. A protocol has been published to compare personalized DC vaccines pulsed either with private peptides or with whole tumor lysates (78), which hopefully will provide further evidence about the production of personalized DC vaccines in ovarian cancer.

CLINICAL STUDIES ON DENDRITIC CELL VACCINES IN OVARIAN CANCER

The safety and efficacy of DC vaccines in the treatment of ovarian cancer has been reported by over 20 studies, including case reports, pilot studies and clinical trials (**Table 2**). These studies either contain multitumors including ovarian cancer (shown with a star mark in **Table 2**), or simply focus on ovarian cancer. Currently, there are 20 registered clinical trials on ClinicalTrials.gov (searched by "ovarian cancer" and "dendritic cell vaccine"). Eleven clinical trials have been completed, three clinical trials are active or recruiting, and two clinical trials are not yet recruiting (**Table 3**). It is important to point out most of the current clinical trials have stagnated before phase II. Recently, a phase-III multicenter, randomized, double-blind, placebo-controlled trial has been registered but is not yet recruiting (NCT03905902), which might provide evidence for the usage of DC vaccines in relapsed platinum-sensitive ovarian cancer patients in the future.

Safety of Dendritic Cell Vaccines

The safety of DC vaccines has drawn great attention for the reason that it might alter the level of immune cells, cytokines, and chemokines *in vivo*. Fortunately, most of the DC vaccines have been well-tolerated by ovarian cancer patients involved in clinical studies. According to the Common Terminology Criteria for Adverse Events, each symptom of side effects is graded by the degree of severity. As listed in **Table 1**, most of the reported side effects are grade 1 or 2, and common ones are local skin reactions, fatigue, pain, flu-like symptoms, myalgia, fever, nausea, and vomiting (17, 51, 53–55, 59–62, 79).

There are several studies reporting serious toxicity of DC vaccines, especially those studies using a combination therapy. In a two-arm, phase II trial of the p53 peptide cancer vaccine and DC vaccine (16), all 21 patients reported a local skin reaction. In the arm that received a combination of DC vaccine loaded with p53 peptide, lymphopenia and fatigue were reported by at least 3 patients. Other reported grade III/IV vaccine-related toxicities are elevated levels of ALT and AST, fever, hypocalcemia, memory loss and rigors. Notably, according to the subgroup analysis in this study, significant toxicity was ascribed to the IL-2 administration. In a phase I trial of DC vaccine in the maintenance therapy for ovarian cancer (17), Tanyi et al. reported that more adverse events emerged in the patients who received a combination of DC vaccine, bevacizumab, and cyclophosphamide. There were grade 3 or 4 toxicities reported by 1 patient each: vasovagal disorder, arthralgia, hip replacement, small intestinal obstruction, anemia, cardiac arrhythmia, and decreased lymphocyte count, and 2 patients reported hypertension. Because these adverse symptoms are also common among ovarian cancer patients following chemotherapy, more evidence is needed to confirm whether these grade 3 or 4 toxicities are related to DC vaccines.

To conclude, DC vaccines are well tolerated in most cases, but a combination therapy of DC vaccines and chemotherapy or immunotherapy should be undertaken only with caution.

The Efficacy of Dendritic Cell Vaccines in Maintenance Therapy

During the past 20 years, DC vaccines for the treatment of ovarian cancer have been most frequently tested during the maintenance therapy (16, 17, 51, 53, 55, 58, 59, 65), with or without other drugs (**Figure 2**). Recurrent ovarian cancer patients are involved in these clinical trials, including chemotherapy-sensitive and chemotherapy-resistant recurrences. Cheryl Lai-Lai Chiang and Lana E. Kandalaft et al. reported a pilot study on a DC vaccine pulsed with HOCl-oxidized tumor lysate in five ovarian cancer patients with recurrence (58). After receiving five doses of DC vaccines

TABLE 2 | The clinical trials of DC vaccines in ovarian cancer.

Published Year	Multiple arms of the trial	NO.	Phase of study	Clinical effect	
				Response of DC treatment	Survival period
2000 (52)	Single arm	3*	I/II	2 SD and 1 PD after 3 doses	–
2012 (53)	Arm1 (n=5): DC vaccine; Arm2 (n=6): Cyclophosphamide + DC vaccine	11	I/II	6 NED; 3 recurrence at 6-26 months; 2 recurrence during vaccination	In arms 1,2: estimated 3-year PFS: 40% vs 80%; estimated 3-year OS: 80% vs 100%
2012 (16)	Arm1 (n=14): wild type p53 peptide; Arm2 (n=7): DC vaccines loaded with p53 peptide	21	II	Arm1: 2 NED, 9 RD; Arm2: 2 NED, 5 RD	For arms 1/2: median PFS: 4.2 months vs 8.7 months; median OS: 40.8 months vs 29.6 months
2014 (55)	–	56	retrospective study	1 PR, 7SD, 42 PD, 7 NE	Median OS: 14.5 months
2014 (54)	–	28	II	1 CR, 1 PR, 2 SD, 24 PD	–
2019 (51)	–	3*	I/II	2 SD, 1 PD	Median OS: 13.1 months
2020	–	1	Case report	–	OS since the 1st dose: 15 months
2013 (58)	–	5	I	2PD, 2SD, 1 mixed response	PFS: 1 patient 36 months, 1 patient 44 months
2013 (59)	UPCC 11807 (n=6): DC vaccine + bev + cyclophosphamide; UPCC 10808 (n=3): DC vaccine + lymphodepletion + autologous vaccine-primed T cells	9	I	UPCC 11807: 2 PR, 2 SD, 1 NED, 1 PD then PR; UPCC 11808: 1 CR, 1PD, 1 SD	–
2014 (60)	–	7*	II	1 PR, 2 SD, 4 PD	Median PFS: 176 days median OS: 198 days
2018 (17)	Cohort 1 (n=5): DC vaccine; Cohort 2 (n=10): DC vaccine + Bev; Cohort 3 (n=10): DC vaccine + Bev + cyclophosphamide	25	I	Cohort 1: 3 SD, 2 PD; Cohort 2: 1 PR, 4 SD, 5 PD; Cohort 3: 1 PR, 5 SD, 4 PD	In cohort 2, median OS: 11 months; In cohort 3: median OS > 25 months;
2002 (61)	–	6*	I	4 SD for 14-45 weeks; 2 PD after 4-8 doses	–
2015 (62)	14 consecutive IL-2 injections	10	I/II	5 CR, 2 SD, 3 PD	Median PFS: 19.2 months; median OS: 43.8 months OS: 64.95 ± 7.62 months
2006 (71)	–	4*	I/II	2-9 months treatment period	–
2007 (70)	–	1	Case report	PR	–
2013 (56)	–	2	Case report	2 PD	PFS: 0, 2 months OS: 70, 64 months

OC, ovarian cancer; SD, stable disease; PD, progressive disease; RD, recurrent disease; doses, doses of DC vaccinations; NED, no evidence of disease; PFS, progression free survival; OS, overall survival; TILs, tumor infiltrating lymphocytes; Bev, bevacizumab; Treg, regulatory T cells.

**Only the data of ovarian cancer patients are shown here, these clinical studies include more than one type of cancer disease.*

injected into the inguinal lymph nodes, two chemotherapy-sensitive recurrent subjects reached stable disease, with progression-free intervals of 36 months and 44 months, respectively. Notably, the PFS responses to DC vaccines of these two patients are longer than the PFS after their previous chemotherapy, which indicates an encouraging effect of elongating remission intervals and a need for further clinical trials with a larger population. Wen Zhang et al. reported a phase I/II study on DC vaccines pulsed with WT1 peptide in three ovarian cancer patients (51). All of these patients were resistant to conventional surgery or chemotherapy, and three chemotherapy-resistant recurrent ovarian cancer patients were involved in this study. Only one patient who had received 10 cycles of chemotherapy responded to the DC vaccines, reaching a state of stable disease and had an improved quality of life. Notwithstanding, it is not fair to conclude that DC vaccines have no effect in chemotherapy-resistant recurrent patients. Compared with the responsive patient in this study, the other two ovarian cancer patients with progressive disease in this study received more cycles of chemotherapy before the trial, namely, they received DC vaccines at a relatively late time. Although the samples involved in these two studies remain limited, it might be more beneficial for ovarian cancer patients to receive DC vaccines at a relatively early time. Due to different study designs, the other clinical studies did not report detailed data or a separate analysis of chemotherapy-sensitive recurrent populations and chemotherapy-resistant recurrent populations, but some of them completed follow-up and survival analysis (**Table 1**).

To evaluate the efficacy of DC vaccines that are received at a relatively early time, namely, before recurrence, Masanori Kobayashi et al. reported a retrospective study on 56 primary

TABLE 3 | Clinical trials of dendritic cell (DC) vaccine in ovarian cancer registered on ClinicalTrials.gov.

Status (up to 2020.2)	NCT number	Treatment	Number enrolled
Not yet recruiting	NCT03735589	DCV, autologous NK cell-like CTLs	18
	NCT03905902	DCV	678
Active/Recruiting	NCT00799110	DCV+GM-CSF, DCV+ GM-CSF+ Imiquimod	23
	NCT02033616	DCV+GM-CSF, autologous monocytes+GM-CSF	99
	NCT02111941	DCV	19
	NCT00703105	DCV	36
Completed	NCT01617629	DCV	9
	NCT01068509	DCV	63
	NCT00478452	DCV, DCV+ Cyclophosphamide	14
	NCT00683241	DCV	36
	NCT01132014	DCV	67
	NCT01522820	DCV+ Sirolimus	18
	NCT00844506	DCV+ Cyclophosphamide	19
	NCT00648102	DCV	36
	NCT00019084	DCV, DCV+ autologous lymphocytes	70
	NCT00004604	DCV	24
	NCT00027534	DCV	14

DCV, dendritic cell vaccine; GM-CSF, granulocyte-macrophage colony stimulating factor; NK cell, natural killer cell.

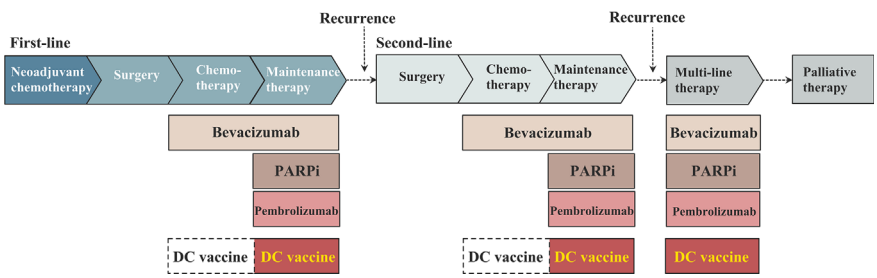

FIGURE 2 | The schema of dendritic cell (DC)-based vaccine in the maintenance treatment in ovarian cancer.

ovarian cancer patients who received DC treatment as maintenance therapy following the initial chemotherapy, and 48% of the patients involved in this study continuously received platinum-based chemotherapy during DC vaccination (55). All of the patients were injected with 5–7 doses of DC vaccines, and the median survival times from diagnosis and the first dose were 30.4 months and 14.5 months, respectively. These data show that the first PFS after initial treatment was elongated by DC vaccines. An increased level of tumor-antigen specific T cells were also detected in some subjects, but subjects with immune responses did not obtain a significantly better survival than those without immune responses. However, as a retrospective study with a relatively small sample size, the evidence it provided is not sufficiently strong. Despite this, it provides evidence for the use of DC vaccines as the initial maintenance therapy and confirms that the patient's nutritional state could affect the DC treatment efficacy, which is useful for the design of future studies.

Combination Therapy With Dendritic Cell Vaccines

In addition to serving as a mono-drug therapy, DC vaccines are also tested in combination with other therapies, including chemotherapies, targeted therapies, immunotherapies, and nonspecific immune-enhancing agents.

Potential synergistic effects of different chemotherapy drugs in ovarian cancer combined with DC vaccines are distinct (80). Paclitaxel is one of the frequently used drugs in chemotherapy for ovarian cancer. Paclitaxel enhances the maturation of early DCs in mice, and DC precursors exposed to a low dose of paclitaxel express higher levels of CD40, MHC-II molecule, and CD86 in response to antigens (81), suggesting a stronger antigen-presenting function. However, most of the patients involved in DC vaccine trials have already completed primary chemotherapy, and only limited evidence is available to help analyzing the effect of DC treatment and chemotherapy at the same time.

Cyclophosphamide is a nonspecific cell phase agent that prevents cell division by suppressing DNA synthesis, although monotherapy with cyclophosphamide acts poorly against ovarian cancer, and combination therapy with it may strengthen the antitumor effect. The synergistic effects of cyclophosphamide are divergent in two clinical studies. In one study, the administration of cyclophosphamide before DC vaccines did not provide additional survival benefits compared with just DC vaccines (53). However, another study suggested cyclophosphamide might strengthen the

effect of DC vaccines. The 2-year overall survival rates of patients with or without cyclophosphamide prior to receiving DC vaccines are 80% and 30%, respectively, and the immune response rate to DC treatment was higher in the cyclophosphamide cohort (17). These two studies differ in many aspects, and one of them is that the latter study also used bevacizumab as a combination drug. Bevacizumab targets vascular endothelial growth factor (VEGF) to suppress tumor angiogenesis while cyclophosphamide decreases Treg as well as MDSC (82), indicating a synergistic antitumor effect with immunotherapy in the tumor microenvironment. Unfortunately, there is no DC vaccines clinical study that provides contrasting groups with or without bevacizumab, which needs further attention.

DC-based therapy might be enhanced by other immunotherapies that have synergistic immune effect, including the immune checkpoint blockers and T cell transfer. It has been demonstrated that immune checkpoint blockers such as anti-PD-1/PD-L1 could theoretically enhance the antitumor effect of DC vaccines (38, 39). However, the expression of PD-L1 on DCs in ovarian cancer patients is moderate compared with on normal ovarian tissues (83), and currently, there is no clinical trial testing the combination of anti-PD-1/PD-L1 and DC vaccines.

Combination therapy with autologous DC and T cells transfer might be beneficial, based on the mechanism that DCs present antigens to T cells. Kandalaft et al. reported a clinical study on DC treatment followed by autologous T cell transfer (59). These T cells were obtained through apheresis after DC treatment and underwent expansion *in vitro*. Seven recurrent advanced-stage ovarian cancer patients received DC vaccines, and three of them that reached PD or PR after DC vaccination were finally enrolled into a T cell infusion group. One of them achieved a partial response (PR) after DC vaccines treatment and later successfully achieved a complete response (CR) after autologous T cell transfer. The second patient who reached a PR after DC vaccination had disease progression, while the third patient had stable disease after DC vaccination and T cell transfer. Moreover, tumor-reactive T cells were detected before T cell transfusion in the peripheral blood of the CR patient but not in the disease progression patient, which suggests that reconstitution of tumor-reactive T cells depends on the immune response to DC vaccines.

Additionally, as a subgroup of T cells, NK cell-like T cells recognize antigens presented by DCs in a CD1c-restricted manner and suppress MDSC in the microenvironment (84), which could help to enhance the efficacy of DC vaccines. A clinical trial of dendritic cell vaccines combined with autologous NK cell-like CTLs for treating ovarian cancer patients (NCT03735589) is carrying out.

Other nonspecific immune-enhancing agents have also been tested as combination agents, including IL-2, IL-12, OK-432, and sirolimus. As Soyoung Baek et al. reported, it was safe to use IL-2 simultaneously with DC vaccines (62). However, another clinical trial reported a combination of DC and IL-2 caused grade 3 or 4 side effects and induced Treg expansion (16). Notably, the administration of IL-2 in these two studies differs in dosage and injection sites, which may account for the divergent results. Recombinant human interleukin-12 (rhIL-12) was used as a combination agent with DC vaccines in various tumors, but limited data reveal the effect of rhIL-12 in clinical trials (71). OK-432 is a streptococcal immunological adjuvant, which is injected simultaneously with DC vaccines but has no significant association with the survival of the patients (51, 55).

The Administration Scheme of Dendritic Cell Vaccines

There is no consensus on the administration scheme of DC vaccines in the clinical context. DC vaccines tested in ovarian cancer patients are administered intradermally (54, 55, 59, 61), subcutaneously (52, 53, 62, 71), intranodally (17, 58) or intravenously (16, 60). The injection route of vaccination might affect the migration of DCs to lymph nodes, as well as the contact between DCs and T cells. Intranodal vaccination came into the spotlight in recent years. It is reported that far more DCs reach the T-cell areas of the lymph nodes in the melanoma patients administered DC vaccines intranodally, notwithstanding, the immune responses were similar between the two groups (85). But the areas of drainage lymph nodes vary a lot in different cancers, limited data is available to confirm the strengths and weaknesses of different injection routes in the ovarian cancer patients, which left a unrevealed answer for further clinical studies.

The vaccination schemes vary greatly between different studies. In some trials, patients receive a fixed number of DC vaccines at fixed intervals, such as two doses at a 4-week interval (62) or four doses at a 3-week interval (53). In other clinical trials, several doses of DC vaccine are administered to prime the immune response, and residual doses are administered at a longer time interval, for example, five doses every 3 weeks and residual doses per month (17). However, there is no consensus on how to arrange vaccination and examination schedules, which should be carefully considered with when designing clinical trials.

In addition to injection routes, limited evidence is available to confirm the best intervals of injection, the total number of doses to receive, and the administration pathway. Further studies are needed to confirm the administration scheme that most effectively promotes the functional process of DCs *in vivo*.

BIOMARKERS TO MONITOR AND PREDICT THE EFFICACY OF DENDRITIC CELL VACCINES

Although PFS and OS are considered as the most reliable assessment criteria, survival analysis may take several years to complete. Therefore, sensitive immune markers will be the cornerstone for monitoring and predicting the responses to DC vaccines. Some of the preclinical and clinical studies of DC vaccines have made exploration on two key issues: how to assess the immune responses and how to predict the clinical responses (58, 59, 62), an alteration of immune cells, especially T cells, are in the spotlight of the stage.

Immune Biomarkers to Predict the Effect of Dendritic Cell Vaccines

Studies on DC vaccines in different types of cancer are exploring biomarkers to predict the clinical response of DC-based treatment. Several types of biomarkers have been reported, including immune cells, cytokines, chemokines, membrane proteins and genes. These immune biomarkers to monitor and predict the effect of DC vaccines in ovarian cancer and other tumors are discussed below.

Immune Biomarkers Based on Peripheral Blood Samples

T cell reactivity is the cornerstone of DC vaccine immunoreactivity. In clinical studies of DC vaccines in ovarian cancer patients, alterations of immune cells in the peripheral blood sample after DCs infusion have been demonstrated, including the activation of specific antigen-induced IFN-γ secreting $CD8^+$ T cells (58, 59, 62), an increased count of $CD4^+$T cells (16) and Th1 polarization (58, 86). These could be regarded as basic indicators, but a monitoring scheme of a comprehensive immune cell profile following DC vaccines has not been established. It should based on both the counts and the functions of immune cells.

The cytokines and chemokines secreted by immune cells are tested as functional indicators. There are significantly increased levels of Th1-polarizing chemokines and cytokines such as IL-12, IL-1Rα, TNF-α after DC vaccine treatment of ovarian cancer patients, while Th2-priming cytokines IL-4, IL-5, and IL13 are at low levels suggesting that DC vaccines elicit a Th-1 antitumor effect (58). A recent clinical study confirmed that DC vaccine-primed $CD4^+$T cells to mainly secret TNF-α and IL-2, while $CD8^+$T cells to mainly secret IFN-γ and TNF-α (17).

The array of T-cell receptor (TCR) sequences present can be detected by next-generation deep sequencing, namely, the TCR repertoire, which could be used to evaluate the immune response of DC vaccines. According to a cohort study in ovarian cancer patients, there is no overlap of the TCR repertoire between peripheral blood T cells pre- and post- DC vaccines, suggesting that DC vaccines have primed a novel T cell immune response. Moreover, novel T cells manifest high avidity due to high-affinity TCR clones, which benefits DC vaccine-induced antitumor effects (17).

To conclude, the immune markers in the peripheral blood that are altered after DC treatment might be proper indicators of the immune response.

Immune Biomarkers in the Ovarian Cancer Microenvironment

Beyond surface markers and the secreting function of T cells in the peripheral blood, characteristics of the ovarian cancer microenvironment might be predictors as well. In the clinical context, a tumor sample could only be obtained during biopsy or surgery, and thus the markers on tumor samples could be utilized to detect infiltration of a sensitive population rather than evaluation indicators of the vaccines.

As a clinical study in glioma patients reported, there was a higher overlap of TCR repertoires between T cells from both peripheral blood and tumor sites, and an increased overlap after DC treatment predicts improved immune and clinical responses to DC vaccines (87).

The molecules on the membrane of T cells can also be taken into consideration. The count of $PD\text{-}1^+$lymphocytes and the percentage of $PD\text{-}1^+CD8^+$T cells are negative prognostic indicators for overall survival and progression-free survival among glioblastoma patients received autologous DCs but not in the control group, suggesting that $PD\text{-}1^+$T cells infiltrating might be a biomarker for DC treatment (88). In contrast, lower expression of B7-H4, a member of the B7 family, is associated with a better response to DC vaccines in glioblastoma (89). However, neither $PD\text{-}1^+CD8^+$T cells nor B7 family molecules has been tested in clinical trials of DC vaccines in ovarian cancer. Further studies are needed to describe the potential of T cells in response to DC vaccination.

One of the limitations in these clinical trials is the lack of the comparison of tumor samples before and after DC treatment, but in the mouse model, such change of ovarian cancer microenvironment has been illustrated, DC vaccination promotes the proliferation of $CD4^+$T cells and $CD8^+$T cells and decreases the level of MDSCs, Tregs and tumor-associated macrophages (90).

The localization of immune cells infiltrating in tumor sites may has potential impact. Ovarian cancer used to be regarded as "immune desert" due to low level of infiltrating immune cells, but studies have reported the existence of tertiary lymphoid structures (TLSs) in tumor sites, which harbor B cells, T cells and $DC\text{-}LAMP^+$ dendritic cells (91). The infiltration of $DC\text{-}LAMP^+$ dendritic cells is associated with better prognosis in ovarian cancer patients (92), but whether DC infusion promotes the build of TLS remains to be explored in the future.

Based on the markers discussed above, an ideal immune response-predicting biomarker should satisfy several points: a strong association with the treatment response or prognosis, a quick examination method to monitor, and a relatively high sensitivity and specificity. However, due to a relatively low response rate to DC vaccines in ovarian cancer compared with other cancers, some biomarkers tested in ovarian cancer but limited progress has been achieved. To explore biomarkers for DC-based treatment responses in ovarian cancer, the basic immune status of the patient, immune characteristics of ovarian cancer and potential drug targets should be taken into consideration. Additional well-designed clinical trials are needed to promote this field forward.

Association Between Immune Responses and Clinical Responses in Ovarian Cancer

An increasing number of studies have focused on the association between immunoreactivity and the clinical responsiveness of tumor immunotherapy. If specific immune markers can be determined to predict an individual's immune reactivity to the vaccine, and the long-term clinical benefit of DC vaccines can be assessed by monitoring changes in immune marker levels, it would help to adjust the vaccine dosage and determine treatment endpoints.

The immunoreactivity of DC vaccines in ovarian cancer is mainly described by the alteration of the following immune cells: $CD8^+$T cells, $CD4^+$T cells, Tregs and NK cells. As a pilot study with 5 recurrent ovarian cancer patients reported, in the patients

with a T cell immune response to DC vaccines, the second PFS following DC treatment was longer than the first PFS before the DC vaccine (58). In some studies that have monitored both immune and clinical responses, T cell reactivity is related to clinical benefit, such as a partial tumor response (PR), disease stabilization, and prolonged survival without progression (59, 62), however, these associations between DC-activated T cells and clinical outcome were not stable. A decrease in Tregs after DC treatment could be an immune response indicator, but not a single biomarker to predict clinical response, because a reduction in Tregs has been detected in both stable disease and progressive disease patients (58, 59). Increased NK cell activity after DC treatment was found in ovarian cancer patients in a pilot study. More than half of the enrolled patients presented with increased NK cell activity, but this change was not significantly correlated with clinical prognosis (62). Other immune cells are potential predictors, such as myeloid-derived suppressor cells (MDSCs), which have an immuno-suppressive effect on immunotherapy. Immune responses to DC vaccines are significantly associated with fewer MDSCs (51), which calls for further follow-up to confirm its association with clinical outcomes.

Additionally, serum antibodies IgG and IgM reflect a basic state of the immune environment, which could also bridge the immune and clinical responses of DC vaccines. Patients show weak antibody responses based on IgG and IgM induced by ovarian cancer-specific antigens prior to DC treatment, suggesting a suppressive immune environment in ovarian cancer (54). After DC treatment, the serum IgG and IgM are higher than the baseline in some ovarian cancer patients, indicating a priming immune response *in vivo* (59). However, how long this alteration is maintained and whether it could benefit survival remain to be illustrated.

Currently, studies on DC vaccines differ from each other in aspects of the scale of sampling, vaccine production, and vaccination schemes, which underscores the difficulty in drawing consistent conclusions. Future large-scale cohort studies with a complete follow-up will add additional power to reveal the link between immune responses and clinical responses.

Timepoints to Monitor Immune Markers

Immunotherapy such as DC vaccines may cause short-term and long-term effects. Immune responses may occur within hours to days or potentially lead to long-term changes in the components of the immune microenvironment. It is essential to capture the alterations of immune reactivity at the correct time, especially in clinical trials where it is unethical to conduct invasive examinations too frequently. In clinical trials of DC vaccines for the treatment of ovarian cancer, there was a significant difference in T-cell reactivity before and after receiving DC (17). The monitoring time points vary from days to weeks (**Table 1**).

To evaluate the immune response to DC vaccines, immune markers should be monitored at least pre- and postvaccination. Examinations at later time points might reflect how long the effect would be maintained. As Christina et al. reported, to evaluate the immune response, examinations were performed at the time of leukocyte apheresis, after the second and fourth doses of vaccine, and at 4, 5, 6, 9, 12 months after the first dose of vaccine. It was observed that antigen-specific effector T cell levels were stable in most patients, but the patients with decreasing T-cell levels showed disease progression (53).

FUTURE STUDIES

In the ovarian cancer microenvironment, tumor immuno-suppressive signals induce dendritic cells into a dysfunctional state by affecting the immune function and metabolism of DCs, resulting in difficulty in performing antigen-presenting functions and even promotion of tumor progression. The dendritic cell vaccine provides functional dendritic cells to ovarian cancer patients; thus, it may be a safe and effective immunotherapy for ovarian cancer.

Among the existing preclinical studies and clinical studies, there are huge differences in the preparation process of dendritic cell vaccines, especially the types of tumor-associated antigens used to load dendritic cell vaccines. With the wide application of next-generation sequencing and bioinformatics analysis in various research fields, personalized dendritic cell vaccines have become a hot topic. Because personalized dendritic cell vaccines can activate T cell cloning targets of patient-specific tumor antigens, they can present a more effective antitumor effect. Although the advantages are obvious, there are still some barriers that need to be overcome for personalized vaccines, such as the complicated preparation process, limited amount of tumor samples from surgery, and difficulty in the accurate selection of tumor antigens. Future studies should pay more attention to these challenges.

Clinical trials in ovarian cancer patients have confirmed the safety of dendritic cell vaccines, but the efficacy of dendritic cell vaccines varies with different preparation methods and trial protocols. Most studies have shown that dendritic cell vaccines can prolong tumor progression-free survival, but the effect on overall survival is not significant. The best evidence will need to be provided by prospective cohort studies with large samples in the future. Although some studies have shown a survival benefit from combination therapy with a vaccine containing dendritic cells, adverse reactions are increased, and this approach should be applied with caution. In addition, although no health economics analysis is available, the cost burden of combination therapy is expected to be greater.

Biomarkers to monitor and predict the efficacy of dendritic cell vaccines will significantly push the research field forward, but none of the biomarkers in current ovarian cancer studies perform well. The ideal biomarker should reflect not only the immune response induced by the vaccine but also the prognosis. Immune cells, cytokines, and chemokines are important parts of the immune response, which are candidate markers. The immune character of tumor microenvironment should also be taken into consideration. According to the current studies, no single indicator can meet the requirements, but a combination of biomarkers may be able to reflect the efficacy of dendritic cell vaccines more comprehensively. Future studies should test different marker groups, making full use of the multilevel information available at the gene, protein, and cell level.

To sum up, dendritic cell vaccines have been shown to be effective in immunotherapy for ovarian cancer, but there is still untapped potential that needs to be explored by a combination of new technologies, new cohort studies and new biomarkers.

AUTHOR CONTRIBUTIONS

XZ was the major contributor in writing the manuscript and design the figures; TH contributed greatly to build the structure of the review; YL contributed to build the structure of the review and gave suggestions on the manuscript; LC provided suggestions on analyzing the clinical trials on dendritic cells; HL and YW gave suggestions on the structure of the review and the manuscript; HG was the corresponding author of this review, contributed greatly to edit and review the manuscript. All authors contributed to the article and approved the submitted version.

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Protein-Based Vaccine Protect Against *Piscirickettsia salmonis* in Atlantic Salmon (*Salmo salar*)

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An effective and economical vaccine against the *Piscirickettsia salmonis* pathogen is needed for sustainable salmon farming and to reduce disease-related economic losses. Consequently, the aquaculture industry urgently needs to investigate efficient prophylactic measures. Three protein-based vaccine prototypes against *Piscirickettsia salmonis* were prepared from a highly pathogenic Chilean isolate. Only one vaccine effectively protected Atlantic salmon (*Salmo salar*), in correlation with the induction of Piscirickettsia-specific IgM antibodies and a high induction of transcripts encoding pro-inflammatory cytokines (i.e., Il-1β and TNF-α). In addition, we studied the proteome fraction protein of *P. salmonis* strain Austral-005 using multidimensional protein identification technology. The analyzes identified 87 proteins of different subcellular origins, such as the cytoplasmic and membrane compartment, where many of them have virulence functions. The other two prototypes activated only the innate immune responses, but did not protect *Salmo salar* against *P. salmonis*. These results suggest that the knowledge of the formulation of vaccines based on *P. salmonis* proteins is useful as an effective therapy, this demonstrates the importance of the different research tools to improve the study of the different immune responses, resistance to diseases in the Atlantic salmon. We suggest that this vaccine can help prevent widespread infection by *P. salmonis*, in addition to being able to be used as a booster after a primary vaccine to maintain high levels of circulating protective antibodies, greatly helping to reduce the economic losses caused by the pathogen.

Keywords: ***Piscirickettsia salmonis*, Atlantic salmon, innate immunity, vaccine, proteome, IgM, IL-1b**

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INTRODUCTION

The aquaculture industry is constantly under threat from infectious diseases, and although antibiotics and chemical treatments have proven to be useful, they present major environmental and economic concerns. Therefore, immunoprophylactic therapies, such as vaccines, need to be developed and applied to prevent and control disease (1, 2). Vaccination has become one of the most important prophylactic tools for disease control in modern industrial aquaculture (3).

Piscirickettsia salmonis is the etiological agent of salmonid rickettsial septicaemia (SRS) or *Piscirickettsiosis*. This bacterium was first isolated from Coho salmon, and the piscirickettsial organism recognized as a fish pathogen in 1989, was designated the LF-89 type strain (4–6). *P. salmonis* has been confirmed as the agent responsible for this disease in various salmonids grown on the Atlantic and Pacific coasts of the United States and Canada, as well as in Ireland, Norway, Scotland, and Tasmania (4, 7–10). In Chile, SRS primarily affects cultured salmonids, such as Coho salmon (*Oncorhynchus kisutch*), Atlantic salmon (*Salmo salar*), and rainbow trout (*Oncorhynchus mykiss*) (11). In addition, *P. salmonis* has been confirmed as a pathogen of other species, such as European sea bass [*Dicentrarchus labrax* (L.)] in Greece (12, 13) and the white sea bass [*Atractoscion nobilis* (Ayres)] in the United States (14, 15).

P. salmonis is a Gram-negative, non-motile, non-encapsulated, facultative intracellular, pleomorphic bacterium with a predominantly coccoid shape measuring 0.5–1.5 μm in diameter. Molecular phylogenetic analyses, based on 16S rRNA gene sequencing, have categorized *P. salmonis* as a γ-proteobacteria in the *Piscirickettsiae* class, with relationship to *Coxiella*, *Francisella*, and *Legionella* (4). This bacterium produces a systemic infection characterized by colonization of the kidney, liver, spleen, intestine, brain, ovary, and gills. However, the mechanisms of bacterial virulence and pathogenesis remain poorly understood (6, 16). *Piscirickettsia salmonis*, being an intracellular bacterium, has been described as using macrophages as an infection strategy, in addition to replicating in cytoplasmic vacuoles as a mechanism for evasion of the host's innate immunity (17). Currently, antimicrobial agents are ineffective, and the high mortality rate causes annual losses in excess of US$700 million in Chile (7, 17, 18).

In the Chilean salmon industry, the management strategy has focused primarily on SRS control through vaccination and antimicrobial therapies. While antibiotics can prophylactically inhibit pathogen growth, these have had little success in stopping new disease outbreaks (19). Furthermore, the use of antimicrobials in the Chilean aquaculture industry has steadily increased in correlation with intensified salmonid production. Related to this, data from the National Fisheries and Aquaculture Service of Chile (SERNAPESCA) confirm that Atlantic salmon cultures receive the largest amount of antibiotics, with respect to the other species in cultivation. Of this amount, *Piscirickettsiosis* garners the most attention, with a 98.3% total of antibiotics administered in the control of SRS mainly oxytetracycline and florfenicol in seawater (20). However, reduced sensitivity to florfenicol and oxytetracycline has been reported in salmon farms, in addition to increased resistances to other antibiotics such as penicillin, streptomycin, oxolinic acid, and oxytetracycline (20).

The lack of effective control treatments for SRS highlights the need for different options, such as new, non-bacterin types of vaccines. Vaccines based on inactivated bacteria can successfully control diseases (2), but currently existing preparations based on *P. salmonis* provide low or variable protection against SRS (6, 7, 21). Outcome differences may be related to variations in epitopes caused by inactivation treatments. Furthermore, different vaccination protocols are intensively used by the Chilean salmon industry, including whole bacterium, inactivated, and adjuvanted vaccines for primary intraperitoneal immunization, which in some cases can be followed by an oral boost (22). However, the efficacy of each of the vaccine formulations is not completely effective, mainly due to the contradictory results obtained with protocols based on bacterins, in addition to the complete ignorance of whether vaccination will grant humoral immunity, most of the time opsonized by professional phagocytes, without obtaining the desired effect of long-term protection (22).

This current study analyzes the effectivity in Atlantic salmon of three SRS vaccine formulations based on proteins isolated from *P. salmonis*. All formulation induced an innate immune response, however, protection of fish against a lethal-dose challenge of *P. salmonis* occurred only when high levels of IgM were produced. This protection was only induced with one of the formulations against *P. salmonis*, this observation suggests that one of the most important issues for induction is a high amount of anti-*P. salmonis* specific IgM. In addition, during this study it was possible to characterize some proteins involved in virulence through the multidimensional protein identification technology (MudPIT) found from one of the formulations of the vaccine from the Austral-SRS 005 strain.

In summary, this study is an approach to the complexities that are present in the host-pathogen interaction, related to the immune response and various characterized virulence factors, observing that diverse integral technologies for the development of effective vaccines against *Piscirickettsia salmonis*.

MATERIALS AND METHODS

Fish Maintenance

First, 1,260 Atlantic salmon (*Salmo salar,* 35 ± 3,8 g) were obtained from the Los Fiordos salmon farm and subjected to health check analyses, using accredited laboratories, to verify pathogen free status. All subsequent bioassays were conducted at the Quillaipe Experimental Station (Fundación Chile). Animals were divided into five tanks (1 m^3), four of which contained 300 fish per tank and one of which held 60 reserve fish in case of mortalities (**Figure 1**). All fish were fed *ad-libitum* for 24 h with the commercial Transfer 50® (EWOS) diet. The experiment was reviewed by an internal animal welfare committee of the Foundation of Chile (PPT256-01). In addition, the study adhered to animal welfare procedures and was approved by the bioethical committees of the Universidad Austral de Chile and the National

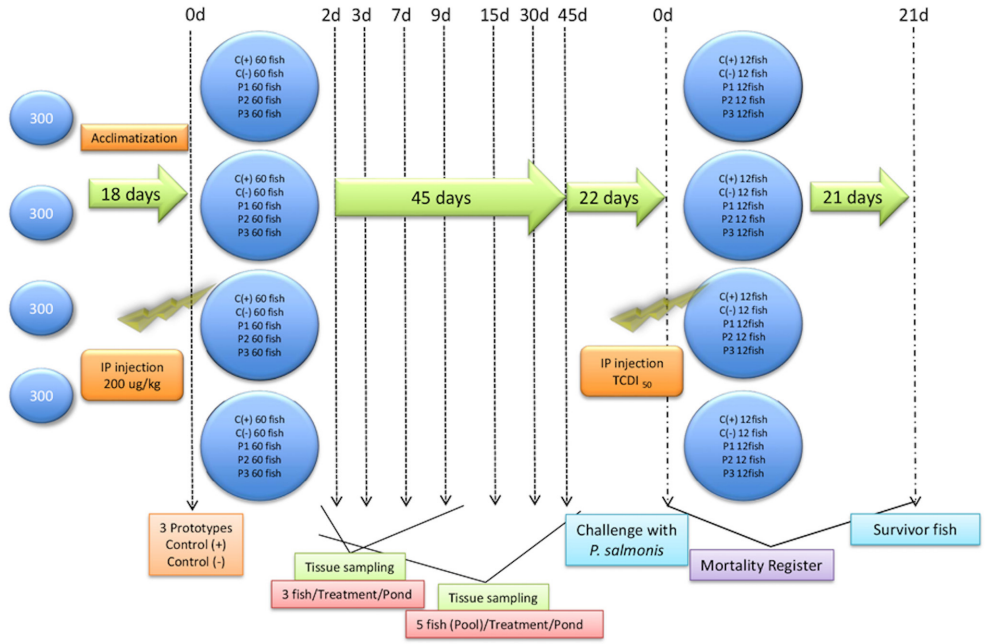

FIGURE 1 | General trial outline. General trial outline showing fish entry, acclimatization days, injection formulations, and sampling days. Prototype 1 (P1), Prototype 2 (P2), and Prototype 3 (P3); Positive controls (C+) commercial *P. salmonis* vaccine, and negative controls (C-) used PBS mixed with a non-mineral oil adjuvant.

Commission for Scientific and Technological Research (ANID) of the Chilean government.

Production of *Piscirickettsia salmonis* Protein

Bacterial suspensions of AUSTRAL-005 strain were prepared in sterile Austral-broth (10), and 15 mL aliquots were adjusted to 1.0 absorbance at 600 nm, as measured in a spectrophotometer. The aliquots were washed with 1 × phosphate buffered saline [(PBS), pH buffer] by centrifugation at 6,000 × *g* for 5 min at 4°C, according to the protocol of Oliver et al. and Yañez et al. (23, 24). The samples were further centrifuged at 13,000 × *g* for 10 min at 4°C In presences of protease inhibitor to obtain the supernatant (24), the samples were subsequently disrupted at 80 W for intervals of 20 s, for 2 min. Centrifuge at 5,500 × *g* at 4° C collecting the supernatant (proteins for the vaccine prototype one formulation). The supernatant is ultracentrifuged at 40,000 × *g* for 2 h at 4°C, the pellet corresponds to Prototype 2 (P2) and the supernatant to Prototype 3 (P3). Later it was carried out for an SDS-PAGE electrophoretic mobility assay (**Figure 2**).

Fish Sampling

Three hundred fish from each tank were anesthetized with Here pond-S® (17 mg/L). After 5 min, the passive integrated transponder (PIT) tags were recorded using a magnetic reader (Trovan®). Blood samples (500 μL) were taken from the tail vein, left to clot for 30 min at room temperature, and centrifuged (1,500 × *g* for 5 min) to obtain 100 μL of serum. The fish were pre-bleed previously. Following blood sampling, head kidney and liver samples were frozen in liquid nitrogen and stored at −80°C.

Vaccination Protocols

Three experimental formulations of different highly immunogenic protein fractions [Prototype 1 (P1), Prototype 2 (P2), and Prototype 3 (P3)] from *Piscirickettsia salmonis* were prepared. Each protein mixtures were emulsified with one volume Montanide™ ISA 763 AVG® adjuvant to obtain a 1:1 oil-in-PBS preparation. Three groups of fish were injected intraperitoneally (IP) with 0.1 mL of each vaccine preparation containing 200 μg/kg of each *P. salmonis* protein fraction. For positive controls (C+) commercial, broad spectrum *P. salmonis* vaccine, while negative controls (C-) used PBS mixed with adjuvant (Montanide™ ISA VG® 763 A). Vaccinated fish were fed *ad-libitum,* moved to a room with four bio-secure tanks (1 m^3), distributed at a density of 60 fish/tank, and kept in seawater previously filtered and treated with ultraviolet.

A lethal dose (LD50) of bacteria was calculated, and fish were challenged with 0.1 mL of $10^{8.5}$ $TCID_{50}$/mL of live bacteria. Serial dilutions were prepared from semi-purified *P. salmonis*. The control group (C-) was injected with saline solution. Injected fish from each group were distributed into two tanks and maintained at 14°C under controlled conditions. Mortalities were recorded every 12 h for 21 d to determine the relative percent survival (RPS) and define vaccine efficiencies and powers (**Figure 1**).

$$\text{RPS} = \left(1 - \frac{\%\text{Vaccinated fish mortality}}{\%\text{Control fish mortality}}\right) \times 100$$

Additionally, visual inspection of all fish and necropsies were performed after any mortality since lesions would indicate if a fish died due to *P. salmonis*. SRS was confirmed through histopathological and PCR analyses (25) of head kidney and liver samples taken from dead fish (data not shown).

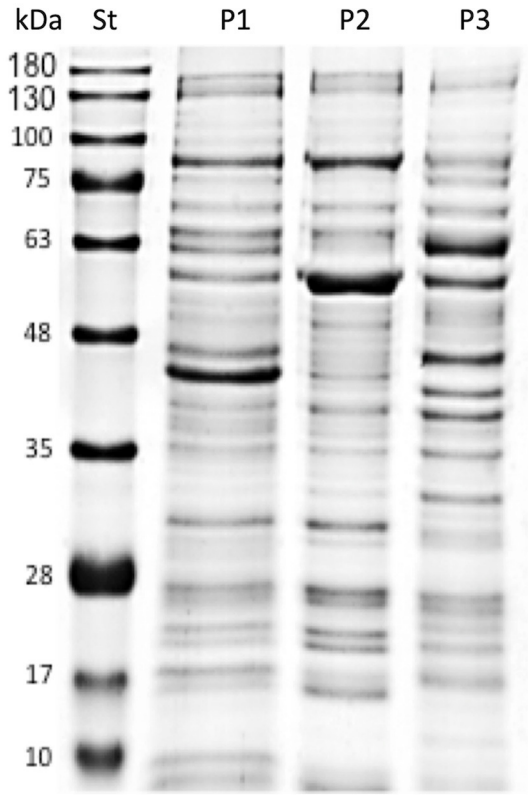

FIGURE 2 | SDS-PAGE analysis of protein *P. salmonis* vaccine formulations. Standard molecular weight markers (St), Prototype 1 (P1), Prototype 2 (P2), and Prototype 3 (P3) indicate the different protein profiles of the *P. salmonis* vaccine prototypes.

ELISA

The ELISA assay was performed as described by Birkbeck et al. and Sotomayor-Garding et al. (26, 27) with same modifications. Firstly, MaxiSorp® (Nunc™ 439454) ELISA plates were incubated overnight at 4°C with 100 μL/well of total proteins mixed fraction of *P. salmonis* antigens in PBS, containing proportional amount of P1, P2, and P3 (1.0 μg/μL). The internal positive plasma control used for the normalization of the ELISA readings on the plates was produced from the pre-bleeding of the fish prior to the challenge tests. Absorbed antigens were blocked with 100 μL of blocking solution [1% bovine serum albumin (BSA)-PBS] for 2 h at 20°C. After washing with 100 μL of wash solution (PBS- 0.05% Tween 20), 100 μL of 1/50 PBS-diluted *S. salar* serum was added and incubated for 1 h at 20°C. The plates were washed and incubated with monoclonal mouse anti-salmon IgM, isotype IgG1 (BiosChile, IGSA, Chile) for 1 h at 20°C. The plates were washed again and incubated with horseradish peroxidase-conjugated goat anti-mouse IgG (Santa Cruz Biotechnology). Serum antibody titers were determined using 3,3,5,5-tetramethylbenzidine (Sigma-Aldrich) as a chromogenic substrate, with H_2SO_4 used to stop the reaction. Values were obtained by measuring absorbance at 450 nm.

Gene Expression Analysis

Head kidney samples were used to assess changes in IL-1β and TNF-α expression. Total RNA was extracted with the TRIzol reagent (Invitrogen) following the manufacturer's instructions, and the obtained samples were treated with amplification grade DNase I (1 U μg^1 RNA, Invitrogen). The SuperScript III RNase H ReverseTranscriptase platform (Invitrogen) synthesized first-strand cDNA from 1 μg of total RNA using the oligo-dT18-22 primer at 50°C for 60 min. Total cDNA was then used as a template for real-time PCR reactions using 7.5 μL Brillant SYBR® Green II (qPCR Master Mix, Stratagene®) and 50 nM of specific primers (for genes IL-1β, TNF-α), and 1 μL of each template (1:10 dilution, in triplicate) in a total volume of 15 μL. The following reaction conditions were used for the Stratagene p3000X® real-time PCR thermocycler: 95°C for 30 s, 58°C for 30 s, and 72°C for 15 s in 45 cycles. The mRNA gene expressions were normalized to the Atlantic salmon β-actin using the comparative ΔΔCt method (28). The oligonucleotides used were: IL-1β: forward *5′-ccacctgctcaacttgc-3′* and reverse *5′-gcagctccatagcctcactc-3′*; TNF-α: forward *5′-cgtggtgtcagcatggaaga-3′* and reverse *5′-agtatctccagttgaggctccatt-3′*; y β-actin (housekeeping gene): forward *5′-gacaacgcatccggtatgtgc-3′* and reverse *5′-cagctcgttgtagaaggtg-3′* and 18s (housekeeping gene) described by Pontigo et al. (29). In all cases, each qPCR was performed with triplicate samples and repeated with at least two independent samples.

Sample Preparation for Proteomics Analysis

AUSTRAL-005 strain were incubated in lysis buffer (50 mM Tris–HCl, pH 7.5; 150 mM NaCl; 1% NP-40; 0.5% sodium deoxycholate; and 1% SDS) for 1 h at 4°C. Finally, the solution was sonicated for10 min at 4°C at a frequency of 20 kHz, lyophilized and stored at −20°C until use. All samples were analyzed by SDS-PAGE.

The proteins of P1 prototypes were subjected to precipitation using 5: 1 v / v cold acetone 100% v/v and incubated overnight at −20°C, then they were centrifuged at 15,000 × *g* for 10 min, the supernatant was discarded, and the pellet was washed three times with acetone at 90% v / v, later the proteins were dried in a rotary concentrator at 4°C, and finally they were resuspended in 8 M urea with 25 mM of ammonium bicarbonate pH 8. Subsequently, proteins were reduced at room temperature for 30 min with 2 mM dithiothreitol and alkylated in the dark at room temperature for 30 min with 10 mM iodoacetamide. The reaction was diluted eight times with 25 mM NH_4HCO_3, pH 7.5; 2 μL of 0.1 ng/mL modified trypsin (Promega, Madison, WI, USA) was added, and the reaction was incubated at 37°C for 16 h. The reaction was stopped by adding acetic acid, pH 2.0.

Protein Identification by MudPIT

Tryptic peptides were concentrated on a CentriVap Concentrator (Labconco, USA) to a final volume of 20 μL and loaded on a 350 μm ID fused silica 2D high-performance liquid chromatography triphasic peptide trap column packed *in-house* with 3 cm of a reverse-phase desalting C18 (100 Å, 5 μm

Magic C18 particles; Michrom Bioresources, Auburn, CA,USA), 3 cm of a strong cation exchange column (300 Å, 5 μm, PolySULFOETHYL A; PolyLC Inc., Columbia, MD, USA), and, finally, 3 cm of reversed phase resolving C18. The peptide trap was mounted on the loop of a Dionex Ultimate 3000 nano series (Thermo Scientific, USA). Following a wash with 0.1% formic acid for 30 min at 0.5 μL/min, the efflux of the peptide trap column was directed to a 10 cm resolving reversed-phase column (100 Å, 5 μm Magic C18 particles, Michrom Bioresources), which was mounted on the electrospray stage of a Velos Pro mass spectrometer (LTQ, Thermo Scientific). The peptides was separated on-line using 15 salt steps (0, 10, 30, 50, 100,150, 200, 250, 300, 350, 400, 500, 1,000, 1,500, and 2,000 mM. NH_4CH_3OO) followed by a 0–35% acetonitrile gradient for 120 min at a flow rate of 350 nL/min. An electrospray voltage of 1.9 kV was used, with the ion transfer temperature set to 250°C. The mass spectrometer was controlled by the Xcalibur software, which continuously performed mass-scan analysis of the LTQ and, subsequently, of the six most intense ions during MS/MS scans of the ion traps. For this, one repeat scan of the same ion was dynamically excluded, using a 30 s repeat duration and 90 s exclusion duration. Normalized collision energy for the MS/MS was set to 35%.

Data Analysis Using Database Search Algorithm

All tandem mass spectra MS/MS samples were analyzed using SEQUEST (v1.4.0.288; Thermo Fisher Scientific, San Jose, CA, USA) and X! Tandem (vCYCLONE 2010.12.01.1; The GPM, thegpm.org). SEQUEST searched the National Center for Biotechnology Information (NCBI) *Piscirickettsia salmonis* 12-21-2015.fasta database (10,012 entries) assuming digestion of the enzyme trypsin. X! Tandem searched a subset of the *Piscirickettsia salmonis* NCBI 11-03-2016 database, also assuming trypsin digestion. SEQUEST and X! Tandem were searched with a fragment ion mass tolerance of 0.80 Da and a parent ion tolerance of 2.5 Da. Carbamidomethyl-cysteine was a fixed modification in SEQUEST and X! Tandem. In SEQUEST, asparagine and glutamine deamidation and methionine oxidation were variable modifications. In X! Tandem, Glu->pyro-Glu of the N-terminus, ammonia-loss of the n-terminus, gln->pyro-Glu of the N-terminus, asparagine and glutamine deamidation, and methionine oxidation were variable modifications.

Criteria for Protein Identification

Scaffold (v.4.5.0; Proteome Software Inc., Portland, OR, USA) was used to validate MS/MS-based peptide and protein identifications. Peptide identifications were accepted if the Peptide Prophet algorithm, with Scaffold delta-mass correction established a > 95.0% probability (30). Protein identifications were accepted if presenting a > 99.9% probability as assigned by the Protein Prophet algorithm, and containing at least two identified peptides (31). Proteins containing similar peptides that could not be differentiated based on MS/MS analysis alone were grouped.

Statistical Analysis

Assumptions of both variance normality and homogeneity were tested. For each vaccine variable, one- or two-way ANOVA general linear models were used. A two-way ANOVA was performed for each immune variable, with the factors being vaccinated fish and time. A Tukey's *post-hoc* test identified significantly different groups ($\alpha = 0.05$).

RESULTS

Identification of *Piscirickettsia salmonis* Protein Profiles for Vaccine Candidates

A previously developed liquid medium for cultivating large amounts of *P. salmonis* (10) was used to mainly culture the AUSTRAL-005 strain. From this, and according to what was described in materials and methods (Production of *Piscirickettsia salmonis* protein), the different protein fractions corresponding to each vaccine prototype (P1, P2, and P3) were visualized by SDS-PAGE (**Figure 2**). Where later prototype 1 was characterized with more than 86 proteins, where some have high immunogenic capacity (7, 10, 17, 18, 21). Which we were able to analyze by having in previous studies the draft of the genome sequence and the gene sequences of the virulent strain AUSTRAL-005 are known (32), and against *P. salmonis* vaccine prototypes were developed by using these data. Focus was given to proteins involved in host-pathogen interactions or to immunoreactive antigens secreted or located on the surface of other known pathogens (7, 32, 33). The protein fractions of the AUSTRAL-005 SRS strain, which is very infectious and produces a high cytopathic effect (24, 34), due to this the existence of different proteins is suggested which can be observed clear differences in the electrophoretic protein profile del prototype 1 with respect to prototype 2 and prototype 3 (**Figure 2**).

Prototypes Vaccine Against *Piscirickettsia salmonis* Induces Innate Immune Responses in Atlantic Salmon

In order to determine the inflammatory immune response, we analyzed the expression of the transcripts of IL-1β and TNF-α. Both cytokines showed high gene expression in the head kidney as a result of immunization (**Figures 3**, **4**). Three groups of fish were injected intraperitoneally with each *P. salmonis* protein-fraction vaccine prototype (i.e., P1, P2, and P3). These groups were compared to a commercial *P. salmonis* vaccine (C+) and negative controls [C- (PBS immunized plus adjuvant)], previously described. Non-injected fish were used as an unstimulated control. Head kidney samples for each group were analyzed at 2, 3, 7, 9, 15, 30, and 45 days. A significant increase ($p < 0.05$) in IL-1β transcript was detected 3, 9, 30, and 45 days after P1 vaccination as compared to the other formulations (P2 and P3) (**Figure 3**). Real-time qPCR data, meant to detected *P. salmonis* DNA over time (35), showed an absence of *P. salmonis* genome for 2–45 days within the bioassay samples (data not shown). Evaluation of TNF-α mRNA expression showed significant changes 30 and 45 days after

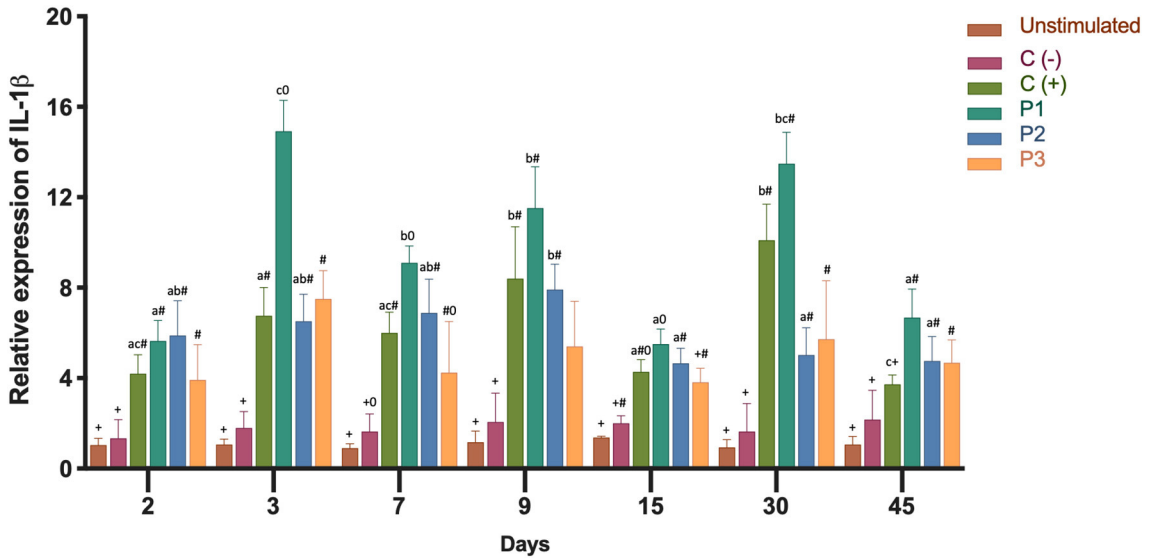

FIGURE 3 | Relative expression of IL-1β mRNA transcription levels in head kidney 2, 3, 7, 9, 15, 30, 45 days after immunization. Fish from each treatment group were sampled at each time point. The mRNA level was measured by the means of real time qPCR, and the mRNA level in the stimulated cells was related to the mRNA level in control cells of unvaccinated fish. Prototype 1 (P1), Prototype 2 (P2), and Prototype 3 (P3); Positive controls (C+) commercial *P. salmonis* vaccine, and negative controls (C-) used PBS mixed with a non-mineral oil adjuvant. Values are mean + SEM. Notations above the bars indicate significant differences among days of experiment. Symbols indicate significant differences between the control and infested fish group (nested ANOVA, *post-hoc* Tukey-Test, $P < 0.05$).

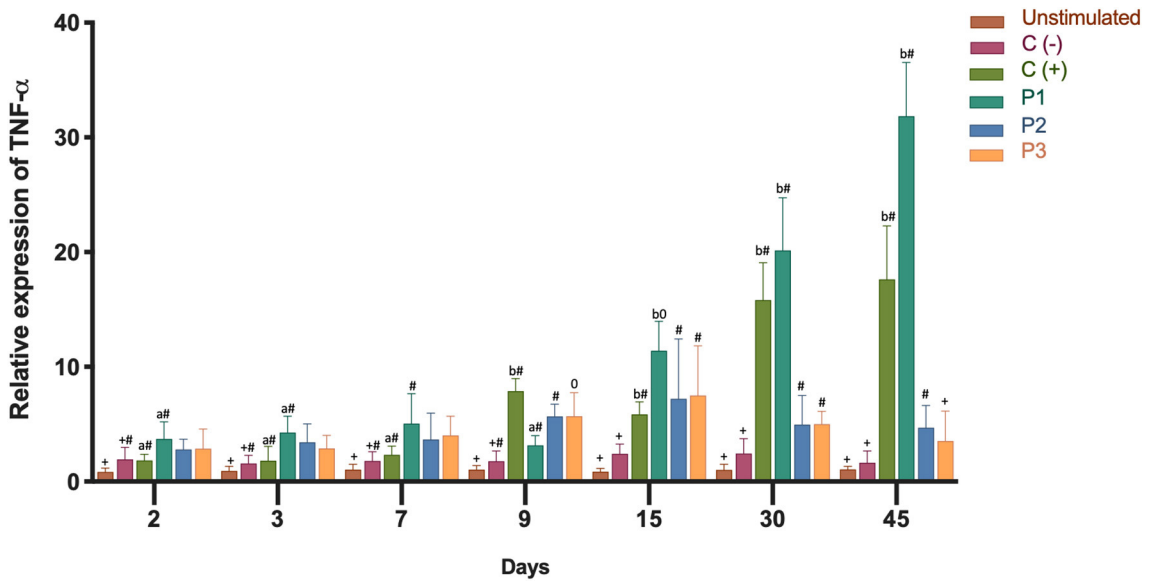

FIGURE 4 | Relative expression of TNF-α mRNA transcription levels in head kidney 2, 3, 7, 9, 15, 30, 45 days after immunization. Fish from each treatment group were sampled at each time point. The mRNA level was measured by the means of real time qPCR, and the mRNA level in the stimulated cells was related to the mRNA level in control cells unvaccinated fish. Prototype 1 (P1), Prototype 2 (P2), and Prototype 3 (P3); Positive controls (C+) commercial *P. salmonis* vaccine, and negative controls (C-) used PBS mixed with a non-mineral oil adjuvant. Values are mean + SEM. Notations above the bars indicate significant differences among days of experiment. Symbols indicate significant differences between the control and infested fish group (nested ANOVA, *post-hoc* Tukey-Test, $P < 0.05$).

vaccination with the prototype P1 v/s the negative control (C-) ($P > 0.05$) (**Figure 4**). These results suggest that administration of the P1 against *P. salmonis* vaccine is able to induce an innate immune response early in mRNA expression for IL-1β and, far later, for increased TNF-α transcript in comparison with the other two formulations.

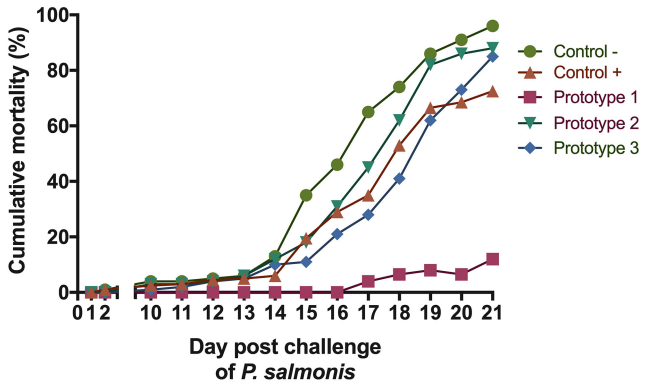

FIGURE 5 | Challenge experiment with *P. salmonis*. Percentage cumulative mortality of *S. salar* vaccinated with the three formulations evaluated in the bioassay, post-ip challenge with *P. salmonis*.

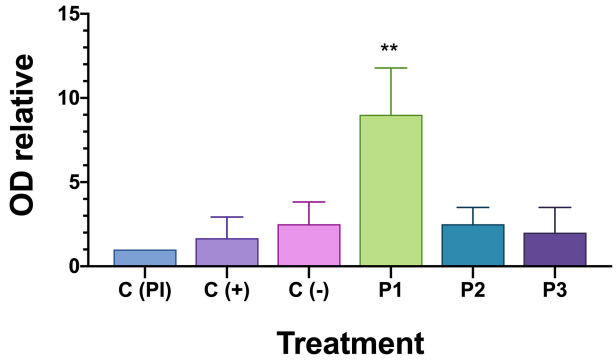

FIGURE 6 | IgM in serum of fish challenged with *P. salmonis* by indirect ELISA. OD relative is measured optical density (OD) at 450 nm for each fish is divided on the optical density of the positive pre-immune control [C(PI)] on each plate. Asterisk indicates statistical difference by one-way ANOVA ($P < 0.05$).

One of the Prototype Vaccines Effectively Protects Against *Piscirickettsia salmonis* Challenge

A large majority of fish in the PBS-injected control group died 21 days after the introduction of *P. salmonis*. Septicaemia symptoms was observed in the liver or (head and posterior) kidney of diseased fish following injection and the infection was confirmed by PCR (25) (data not shown). To evaluate the protective capacity of the three prototype vaccines, fish were given lethal intraperitoneal injections of *P. salmonis* (Austral-005 strain), and daily fish mortalities were monitored (**Figure 5**). The relative percent survival (RPS) of the P1 vaccinated fish (89.6%) was higher than P3 (11.46%) and P2 (8.33%) vaccinated fish, as well as the positive control (C+, 26.1%) 21 days after exposure. These results directly correlated with the recorded specific IgM response (**Figure 6**). The prototype P1 increased both innate and specific adaptive immunity through the expression of pro-inflammatory cytokines and a strong *P. salmonis* specific IgM-response. These data strongly suggest that the prototype P1 effectively induces antibodies production that can protect against this pathogen (**Figures 5**, **6**).

Prototype P1 of Vaccine Induces Immunity in Fish Vaccinated and Challenged With *Piscirickettsia salmonis*

Specific IgM anti-*P. salmonis* production in the serum of surviving fish was evaluated to assess if the vaccine induces against bacteria specific antibodies after Austral-005 strain exposure. Anti-*P. salmonis* antibody were detected using an indirect ELISA assay (**Figure 6**). Only vaccine prototype P1 induced a stronger immune response through the generation of antibody titers in the serum 21 days after the *P. salmonis* challenge. These results could suggest acquired immunity since *S. salar* responded faster and more accurately in generating IgM against *P. salmonis* antigens, suggesting immunological memory is necessary to activated in the case of this intracellular bacteria.

Proteome Analysis of the *Piscirickettsia salmonis* Protein-Fraction Vaccine Prototype 1

In order to identify the proteins components the proteome of the prototype 1, was performed. The purified protein of prototype 1 from *P. salmonis* Austral-005 showed a total of 87 unique associated proteins were identified. The 28 most-abundant proteins from the purified of the protein fraction are listed in **Table 1**. The subcellular localization of the proteins identified, classified by the PSORTb v.3.0 program (36). This classification was organized into six groups: (1) cytoplasmic membrane proteins, (2) cytoplasmic proteins, (3) periplasmic proteins, (4) outer membrane proteins, (5) extracellular proteins, (6) proteins of unknown location. Of these 87 proteins identified from the total fraction of *P. salmonis*, 36 (41%) were cytoplasmic, 11 (12%) of the cytoplasmic membrane, 7 (8%) of the outer membrane, 2 (2%) of the periplasm, 1 (1%) extracellular, and 30 (34%) were of unknown location (**Figure 7A**). The composition of this protein localization of the *P. salmonis* protein-fraction vaccine prototype1 was mainly cytoplasmic and cytoplasmic membrane, therefore a varied composition of various compartments in the formulation of the SRS vaccine is observed.

A functional classification was made of the proteins identified in the proteome of the *P. salmonis* protein-fraction vaccine prototype 1 according to the orthologs groups (COG). Where they were identified in the cell wall, membrane and envelope (13 proteins). In addition, nine proteins in post-transcriptional modification, protein turnover, chaperones, seven proteins in transport and metabolism of inorganic ions, six proteins in production and conversion of energy, five proteins in replication, five protein in cell motility, five proteins in intracellular traffic, four proteins transcription, three proteins in the control of the cell cycle, three proteins in carbohydrate transport, three proteins in signal transduction mechanism, three proteins in defense mechanism, 10 proteins in translation, two proteins in mobilome, two proteins in extracellular structures (**Figure 7B**). The identification of diverse functions but mainly in the

TABLE 1 | Classification of virulence-related proteins identified from total fraction proteins to *Piscirickettsia salmonis*.

Classification	Accession number NCBI	VFDB Gene name	VFDB Description
Adherence	ERL63261	*cadF*	Fibronectin-binding protein
	WP_052104618	*pilA*	Type IV pilin
	ALA23777	*pilC*	Type IV pilus biogenesis protein PilC
	ALB21306	*cadF*	Outer membrane fibronectin-binding protein
	WP_036771893	*omp89*	Outer membrane protein
	ALB23929	*pilJ*	Twitching motility protein PilJ
	WP_016210084	*IlpA*	Immunogenic lipoprotein A
Efflux pump	WP_016209619	*adeF*	RND efflux transporter
	ERL61562	*nrpX*	MFS family transporter
Elongation factor	WP_016209251	*tuf*	Elongation factor Tu
	WP_032126260	*tuf*	Elongation factor Tu
Enzyme	WP_032126147	*katA*	Catalase
	WP_017376766	*mip*	Macrophage infectivity potentiator
Flagellar	WP_016210447	*fliE*	Flagellar hook-basal body complex protein
Iron metabolism	WP_016209255	*fur*	Transcriptional repressor of iron-responsive genes (Fur family)
	WP_032126547	*hasF*	Outer membrane channel protein
Secretion system	ALA25850	*icmG/dotF*	Dot/Icm type IV secretion system core complex protein IcmG/DotF
	WP_017378270	*icmE/dotG*	Dot/Icm type IV secretion system core complex protein IcmE/DotG
	AOS36969	*trwE*	TrwE protein
	WP_016210039	*ssaN*	Type III secretion system ATPase
	WP_016209722	*virB9-1*	Type IV secretion system protein VirB9
Stress protein	KLV35114	*sodCI*	Gifsy-2 prophage: superoxide dismutase precursor (Cu-Zn)
	ALA24403	*ahpC*	Alkyl hydroperoxide reductase subunit C, AhpC (alkyl hydroperoxidase C)
Other		*hasF*	Outer membrane channel protein
	KLV35288	*eno*	Enolase, putative
	WP_016210800	*bcfH*	Thiol-disulfide isomerase
	WP_016209655	*ML1683*	Histone-like protein
	WP_016209645	*tig/ropA*	Trigger factor

The table contain the protein classification, accession number of NCBI Not Redundant database, VFDB gene name and VFDB description. The Virulence Factor data were extracted from the Virulence Factor Database.

biogenesis of the cell wall, the membrane and the proteins associated with post-transitional modifications and chaperones.

Proteins Associated With Virulence Contained in the *Piscirickettsia salmonis* Protein-Fraction Vaccine Prototype 1

The annotations and classification regarding the virulence factors of the most abundant proteins of the *P. salmonis* protein-fraction vaccine prototype 1, was performed using the DIAMOND software (37), considering the cutoff of 1e-10. The analysis showed that 28 proteins of the total fraction, have a high prognostics of bacterial virulence, of these were associated with adherence as: *cad*F, *pil*A, *pil*C, *pil*J, *Il*Pa, *omp*89, besides being some of them associated with biogenesis of pilus type IV (38, 39). In addition proteins were found associated with efflux pump as *ade*F, *nrp*X. Important also are the proteins associated with the conformation of the flagellar motor as it is *fli*E, which has been described previously as a structural part of the flagellar motor, however, transcriptomic analyzes performed under different bacterial culture conditions, the genetic non-expression of *fli*E has been determined (40), which will suggest that the characteristics in the protein expression of the AUSTRAL-005 strain they are different and which can lead to an expression of virulence factors, and addition to the proteins associated with different secretion systems such as: *icm*G/*dot*F and *vir*B9-1 (secretion system type IV), ssaN (type III secretion system), and various external membrane proteins such as: *has*F, *cad*F, and *amp*89, among other proteins (**Table 1**). Also proteins involved in the regulation of iron uptake (fur), which has been described as protein modulates the genes involved in iron uptake, such as those related to siderophore biosynthesis, receptors and transporters. This transcription factor also limits excess iron entry into the bacterium (41).

DISCUSSION

Internationally, infectious diseases are a serious factor affecting aquaculture development. In Chile, SRS is considered the main cause of mortality among cultured salmonids (17) Unfortunately, antibiotic use has been unsuccessful in controlling outbreaks. The need for antibiotic treatments could be significantly reduced through the use of vaccination, which could effectively prevent infectious diseases. Although vaccines against bacterial diseases have great potential in aquaculture, vaccine development is extremely complex (42). More than 33 vaccines exist in the

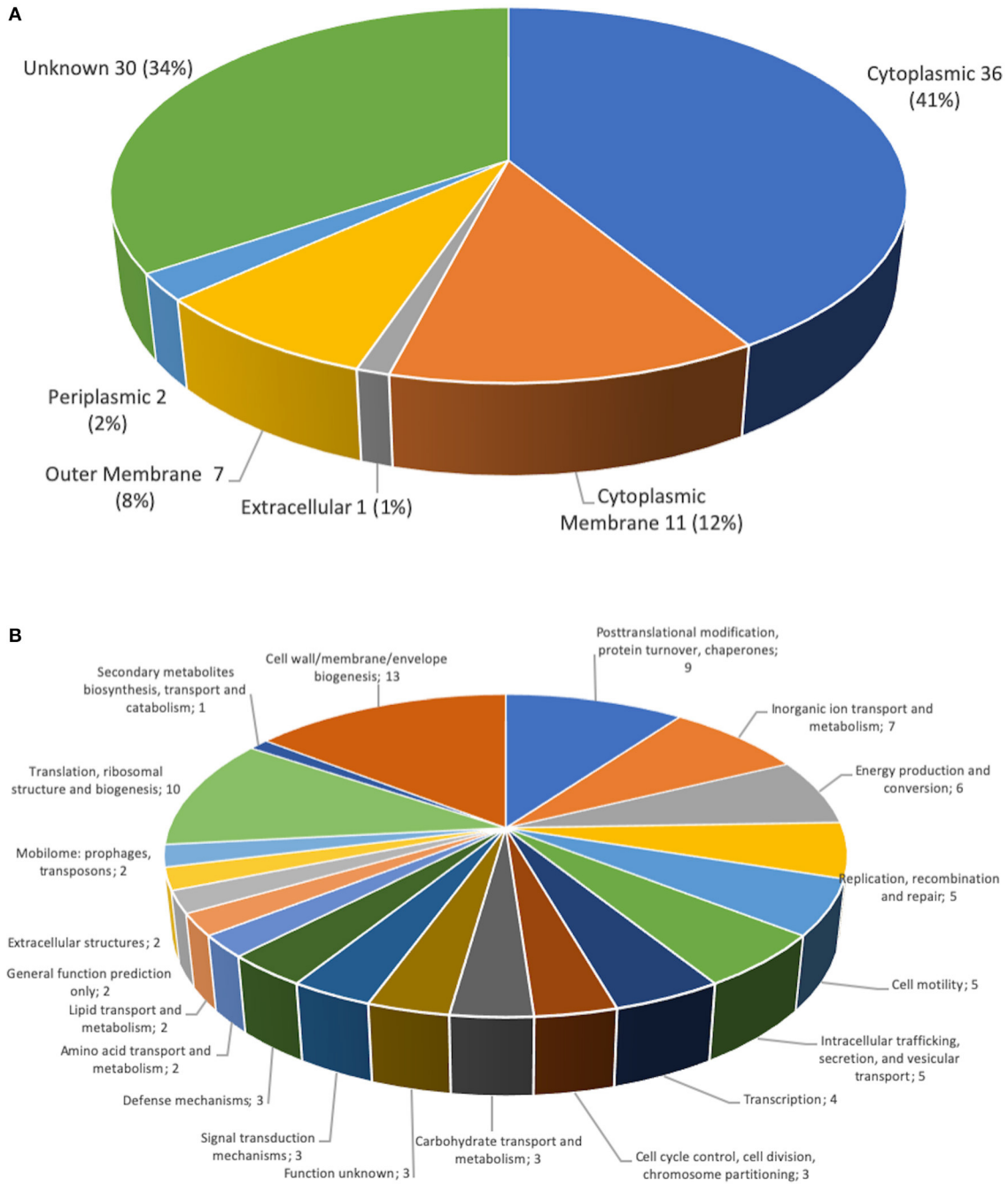

FIGURE 7 | Classification of *P. salmonis* 005-Austral SRS total fraction proteins. **(A)** Subcellular locations of total fraction proteins identified by MudPIT. Predicted subcellular locations of the 87 total fraction proteins identified using PSORT3b. **(B)** Functional classification of *P. salmonis* 005-Austral SRS total fraction proteins. The 87 proteins identified by MudPIT were sorted according to the indicated clusters of orthologous groups.

Chilean market against SRS (17), but these have had variable results and poorly documented efficacies (26, 43, 44). Current vaccine development against *P. salmonis* is hindered by the various virulence factors and pathogenic mechanisms presented by this bacterium. Since transcription could be the key for fully understanding the host-pathogen interaction, the current report developed the P1 vaccine prototype based on the AUSTRAL-005 strain due to an availability of genomic data and transcriptome analyses (24). For this reason, in this work we try to perform a complete analysis with different omics tools such as the proteomic analysis of the protein fraction (P1, vaccine prototype 1) that offers greater protection against

the pathogen during field trials, in order to understand the effectiveness of this vaccine against *P. salmonis*. The developed vaccine included highly expressed major antigenic proteins, with results supporting the administration of microbial antigens as immunocomplexes, which improved innate and acquired immune responses to a highly pathogenic *P. salmonis* strain (32, 45).

The innate immune response, a central fish defense mechanism, is important for activating an acquired immune response (46). Previous studies have demonstrated the feasibility of stimulating an acquired immune response to *P. salmonis* antigens (7, 9). Nevertheless, these studies focus on evaluating antibody levels and RPS after a challenge, without evaluating specific immune responses. This lack of evaluation has left open for debate the antigens to best stimulate the acquired immune system, as well as the exact nature of their effect (47). This study developed a *S. salar* immunization treatment tested via a challenge with *P. salmonis* that included evaluations of various innate immunity parameters, including the expression of transcripts encoding major cytokines (IL-1β and TNF-α). Furthermore, this study evaluated the effect of immune complexes on the acquired humoral immune response, allowing comparisons to be made with the antibody levels in challenged fish.

Cytokines have crucial roles in regulating the immune response (48) and in mediating the effector phase of both innate and adaptive immunity (49). One key, early response, pleiotropic cytokine is IL-1β, which is secreted when pathogens enter circulation. The IL-1β receptor is expressed in all Atlantic salmon tissues (50). One function of Il-1β is to stimulate the vascular endothelium and secrete IL-6, thereby initiating protein synthesis and the acute phase response (51). IL-1β was found highly expressed in the 1st days after immunization, with significant expression in fish injected with the P1 vaccine (**Figure 3**). The P2 and P3 vaccines also increased IL-1β expression when compared to the control. TNF-α, secreted by leukocytes another pleiotropic cytokine, exerts a pro-inflammatory effector mechanism. This cytokine acts as an important factor in the activation of macrophages, resulting in respiratory bursts and phagocytosis (52). In rainbow trout head kidney leucocytes, TNF-α increase phagocytosis and chemotaxis to induce IL-1β and IL-8 expression (48). TNF-α transcript expression was found to be significantly up-regulated in the present study, especially when fish were stimulated with the P1 vaccine. The greatest expression was achieved in the final days of the test period (**Figure 4**). Analyses of IL-1β and TNF-α mRNA expressions showed significant stimulation of the specific immune response by the P1 formulation in comparison with the stimulation observed by P2 and P3 prototypes (**Figures 3**, **4**). *In vivo* measurements of IL-1β and TNF-α mRNA expressions in the bioassay correlated with previous *in vitro* studies that showed increases in both cytokines after cell incubation with SHK-1 protein-based vaccine prototypes (1, 2, and 3) of *P. salmonis* (53).

Three months after vaccination, fish were challenged with $10^{8,5}$ $TCID_{50}$/mL formulations of *P. salmonis.* The cumulative mortality of negative control (C-) fish was 96% (**Figure 5**), supporting the high pathogenicity of the *P. salmonis* strain used, where in addition we obtained protection with a relative survival percentage of (RPS) 89.6% as compared to the control group, which also demonstrated high antibody titers against both proteins in the inoculated salmon serum 3 months after vaccination.

Three different immunoglobulin (Ig) isotypes can be found in teleost fish, IgM, IgD, and the teleost-specific IgT. IgM is considered to have a systemic activity, and IgT is attributed a mucosal role, similar to mammalian IgA. In most teleostean species, the basal expression of *IgM* is dominant, followed by *IgT*. The highest levels of Ig expression are in head kidney, generally followed by spleen (22, 54). Regarding disease prevention and control,

IgM is an important immunoglobulin class for the fish farming industry (55). IgM is very important in phylogenetic research since it is often the only class of immunoglobulin described in fish, in addition to being important in an ontogenetic context as the largest primary antibody among higher vertebrates (56), for effective vaccination against *P. salmonis,* it must be based on the ability to stimulate adaptive immunity and long-term memory responses. This study examined the levels of serum IgM in fish surviving a challenge with *P. salmonis*. A specific antibody titer was found, suggesting that increased antibody titers correlated with post-challenge survival rates. Challenging vaccinated fish with a particular pathogen appears to be an effective direct method for evaluating vaccine potential (**Figure 6**).

In relation to the differential origins, the proteins identified in prototype 1 of the *P. salmonis* vaccine presented various functions. By means of the COG definition, cell wall, membrane and envelope (13 proteins) were classified (**Figure 7**). In addition, nine proteins in posttranscriptional modification, protein turnover, chaperones, seven proteins in transport and metabolism of inorganic ions, six proteins in production and conversion of energy, five proteins in replication, five protein in cell motility, five proteins in intracellular traffic, four proteins Transcription, three proteins in the control of the cell cycle, three proteins in carbohydrate transport, three proteins in signal transduction mechanism, three proteins in defense mechanism, 10 proteins in translation, two proteins in mobilome, two proteins in extracellular structures, some proteins such as: VirB9, VirB10, TraF, IcmG / DotF are required as a component of the type IV secretion system (57), which has been shown to induce humoral and immunity (38). Also associated were proteins that are related peptidoglycan-associated (lipo) proteins and Outer membrane protein as OmpA, Omp89, TolC, in addition to ABC-type metal ion transport components, and periplasmatic component. Several proteins related to antibiotic resistance have been identified in this proteome analyzed by our group, where they include: TolC, AcrA, MFS, and members of multidrug effusion pumps type RND (58). Additionally, several proteins involved transcriptional repressor of iron-responsive genes (Fur family) were identified (41). In turn, a protein wrapped in Flagellar hook-basal body complex protein has been found, such as FliE, which had not previously been described its genetic expression in other culture conditions (40). Within the sequenced proteome, we have been able to identify different

proteins classified with adhesion properties such as: cadF, pilA, pilC, pilJ, omp89, IlpA (**Table 1**), where they belong to the type IV pilus is a filamentous structure existent on the surface of various pathogenic bacteria, such as *Legionella pneumophila* (59), *Pseudomonas aeruginosa* (60), and the fish pathogens *Aeromonas salmonicida* subsp. *Salmonicida* (61), *Vibrio anguillarum* (62), and *Piscirickettsa salmonis* type strain LF-89 (39). Which makes us presume that this first contact that the bacteria should have with the host is of great importance to trigger the immunogenic response and in this same way they are excellent candidates to consider for the formulation of future therapies.

The high amount of proteins in the prototype 1 vaccine involved in the survival of the pathogen, suggests that these mechanisms have a high immunogenicity rate of enveloped proteins, which causes greater protection than the other vaccine prototypes. We sought to divide the analysis in two main mechanisms that the bacteria could use to interact with the host. In particular, we analyzed virulence factors such as adherence, efflux pump, elongation factor, enzyme, flagellar, iron metabolism secretion system stress protein, as described previously.

In conclusion, our results show that all three-vaccine prototypes stimulated the innate immune system by increasing the transcript expression of IL-1β and TNF-α, two highly important marker cytokines. These data suggest that there is a correlation between activation of the innate immune system and protection against mortality. Vaccination with the P1 prototype resulted in an elevated survival rate when fish were challenged with *P. salmonis,* and this strongly correlated with a high induction of IgM specific against *P. salmonis*. The exact protective mechanism of the induced IgM antibodies against *P. salmonis* must be studied to understand the role of this immunoglobulin during the early infection stages and secondary multiplication in different tissues. Related studies by our group (34) demonstrate that IgM against the P1 prototype can inhibit bacterial growth, suggesting that circulating anti-*P. salmonis* antibodies may limit the growth and spread of bacteria in fish organs. The present study identified 87 proteins in the prototype 1 of *P. salmonis*, which is an important contribution to the way of generating highly efficient vaccines for control of SRS. Which is value information for future studies to combat this important pathogen that affects the salmon industry. Where we can determine that this vaccine does not produce immunosuppression at the concentrations used in this study, the opposite of what happens with other commercial bacterin formulations that are used today.

We suggest that this new vaccine formulation against *P. salmonis* be used to prevent your widespread infection. Furthermore, a boost may be used after primary vaccination to maintain increased levels of circulating protective antibodies. This protective effect is important in improving vaccine efficacy for full, long-term protection, which would ultimately reduce salmon industry losses caused by this pathogen.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: https://www.ebi.ac.uk/pride/, PXD023109.

ETHICS STATEMENT

The animal study was reviewed and approved by Foundation of Chile and bioethical committees of the Universidad Austral de Chile and the National Commission for Scientific and Technological Research (ANID) of the Chilean government.

AUTHOR CONTRIBUTIONS

JP conceived of and designed the study and drafted the manuscript. CE conceived of and designed the manuscript. GN performed the proteomic analysis. CO performed the protein fractionation. RA-H, JF, and CR contributed in the draft of the manuscript. JT contributed to the experimental design. LV-C contributed in the initial draft of the manuscript and statistical analysis. AY contributed in the initial draft of the manuscript and experimental design. All authors contributed to the article and approved the submitted version.

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REVIEW
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TLR Agonists as Mediators of Trained Immunity: Mechanistic Insight and Immunotherapeutic Potential to Combat Infection

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Despite advances in critical care medicine, infection remains a significant problem that continues to be complicated with the challenge of antibiotic resistance. Immunocompromised patients are highly susceptible to development of severe infection which often progresses to the life-threatening condition of sepsis. Thus, immunotherapies aimed at boosting host immune defenses are highly attractive strategies to ward off infection and protect patients. Recently there has been mounting evidence that activation of the innate immune system can confer long-term functional reprogramming whereby innate leukocytes mount more robust responses upon secondary exposure to a pathogen for more efficient clearance and host protection, termed trained immunity. Toll-like receptor (TLR) agonists are a class of agents which have been shown to trigger the phenomenon of trained immunity through metabolic reprogramming and epigenetic modifications which drive profound augmentation of antimicrobial functions. Immunomodulatory TLR agonists are also highly beneficial as vaccine adjuvants. This review provides an overview on TLR signaling and our current understanding of TLR agonists which show promise as immunotherapeutic agents for combating infection. A brief discussion on our current understanding of underlying mechanisms is also provided. Although an evolving field, TLR agonists hold strong therapeutic potential as immunomodulators and merit further investigation for clinical translation.

Keywords: trained immunity, immunomodulators, Toll-like receptors (TLRs), TLR agonists, nosocomial infections, immunosuppression, antibiotic resistance, vaccine adjuvant

INTRODUCTION

Nosocomial infections, or healthcare associated infections (HCAI), represent a significant cause of global morbidity and mortality, and the United States is no exception. Each year, approximately one out of 25 hospitalized patients in the United States is diagnosed with at least one infection related to hospital care (1). Infection leading to sepsis remains one of the leading causes of death in U.S. hospitals, affecting more than 1.7 million and causing 270,000 deaths annually. Sepsis is also a major contributor to re-hospitalizations and is one of the most expensive conditions treated in U.S. hospitals, costing more than $2 billion per year (2–4). The overall 30-day mortality rate for patients in the intensive care unit (ICU) is approximately 20%, and for patients with sepsis and accompanying organ dysfunction this statistic is 30%–50% (5). Despite advancements in healthcare overall, this clinical outcome has not improved over the past 25 years (6).

Critically ill patients are at a significantly increased risk of infection due to injury- or illness-induced immune dysfunction and pathogen exposure through invasive life-saving procedures in the healthcare setting (7). Further complicating the risk of HCAI is the continuing rise of antibiotic-resistant pathogens. Infections secondary to resistant pathogens are one of the most critical threats to modern medicine, and this situation is being exacerbated by dwindling effective treatment options (8). The United States has more than 2.8 million antibiotic-resistant infections annually, resulting in more than 35,000 deaths (1). Solely focusing on the development of new antibiotics is not a permanent solution as pathogens will continue to evolve and become resistant to new drugs (9). Thus, immunomodulatory therapies that boost host immune responses and protect immunocompromised patients against infections are a highly attractive strategy.

One promising approach to restore immune responses relies on the induction of innate immune memory, also termed trained immunity. Classically, the role of the innate immune system is to recognize a pathogen and mount a broad and rapid response with immunological memory being considered specific to the adaptive immune system. Recent evidence demonstrates that innate immune cells also display long-term adaptive characteristics after initial challenge with pathogens or their products, which results in enhanced capacity to eliminate infections upon subsequent challenge (10, 11). Trained immunity refers to the phenomenon of activating the innate immune system through exposure to pathogen associated molecular patterns (PAMPs), triggering long-term functional reprogramming by which innate leukocytes mount an enhanced antimicrobial response upon exposure to a secondary microbial pathogen (12). This protection is broad whereby the host is resistant to an array of pathogens for weeks to months once the altered functional state of innate immune training is initiated. Important to note, the terminology referring to different adaptive programs of innate immunity has evolved with the field. Although used somewhat interchangeably in the literature, a consensus has recently been made to clearly differentiate between the four different adaptive programs: differentiation, priming, tolerance, and training (13). Trained immunity specifically refers to the phenomenon in which the activation status of innate cells returns to baseline after primary stimulation prior to the secondary stimulation. Nevertheless, this active new field of research is rapidly evolving, an arm of which is aimed at taking advantage of trained immunity as an innovative strategy to combat infection (14).

Emerging evidence suggests that Toll-like receptor (TLR) agonists are a promising class of immunomodulatory agents that confer long-term protection against subsequent infectious challenge *via* enhanced innate immunity (15, 16). TLRs play a crucial role in activation of innate immune responses by recognizing PAMPs which then trigger downstream signaling pathways and ultimately stimulate the production of proinflammatory cytokines and type I interferons. Several TLR agonists are recognized widely for their vaccine adjuvant properties and several are FDA-approved for this use, but their ability to induce trained immunity is becoming more recognized. Here, we review recent progress in our understanding of mechanisms of TLR agonist-mediated trained immunity and its strong potential for clinical translation to protect patients against life-threatening infection.

TOLL-LIKE RECEPTOR SIGNALING PATHWAYS

As the first line of defense against pathogens, the innate immune system utilizes pattern recognition receptors (PRRs) to rapidly detect microbes and deploy antimicrobial responses. TLRs are a well characterized family of PRRs comprised of 10 members in humans (TLR1-TLR10) and 12 members in mice (TLR1-9, TLR11-13) that are expressed in innate immune cells (i.e. dendritic cells, DCs; and macrophages) and non-immune cells (*i.e.* fibroblasts and epithelial cells) (17). These receptors are synthesized in the endoplasmic reticulum (ER), processed in the Golgi apparatus, and transported to the plasma membrane or intracellular compartment depending on the localization of the PAMP they recognize (18, 19). TLRs detect a wide array of PAMPs, including Gram negative and positive bacteria, viruses, flagellin proteins, lipids, nucleic acids, and damage-associated molecular patterns (DAMPs). This is in part accomplished by receptor localization to the cell surface or intracellular compartments (20). TLRs which recognize nucleic acids are localized to intracellular compartments for decreased risk of contact with "self" nucleic acids whereas cell surface TLRs largely recognize microbial membrane compartments and therefore do not require this protective strategy (21).

TLRs are composed of a horseshoe-like leucine-rich repeat (LRR) ectodomain which interacts with the respective PAMP or DAMP, a transmembrane helix domain, and a cytoplasmic Toll/IL-1 receptor (TIR) domain which is involved in activation of downstream signaling (22, 23). Upon ligand binding, TLRs homo- or hetero-dimerize which dictates recruitment of specific TIR containing adaptor proteins for activation of downstream signaling. TLRs signal either by recruiting the

adapter molecule myeloid differentiation primary response differentiation gene 88 (MyD88) or the MyD88-independent Toll/IL-1R (TIR) domain-containing adapter producing interferon-β (TRIF) signaling. Initiation of MyD88- or TRIF-dependent signaling activates mitogen-activated protein kinases (MAPKs) and IkB kinases (IKKs) that then activate transcription factors to regulate the expression of pro-inflammatory cytokines and chemokines as well as type I interferons (**Figure 1**). Therefore, these pathways trigger unique antimicrobial defenses to confer protection against diverse pathogens. MyD88- and TRIF-dependent signaling cascades are important to consider in the understanding and application of TLR-mediated trained immunity.

MyD88-Dependent Signaling Cascades

Upon ligand binding, all TLRs except TLR3 initiate downstream signaling by recruiting adaptor protein MyD88 either directly (TLR9, TLR11, TLR13, TLR7/TLR8, and TLR2/TLR10) or indirectly *via* the sorting adaptor TIR domain containing adaptor protein (TIRAP; also termed MyD88-adaptor-like, MAL; TLR4, TLR5, TLR2/TLR1, and TLR2/TLR6) (18, 24). TIRAP binds with different lipids depending on TLR localization which mediates assembly of kinases (IRAK4 and either IRAK2 or IRAK1) termed the "Myddosome" (17, 25, 26). Through formation of this oligomeric signaling complex, the kinase domains of IL-1 receptor-associated kinases (IRAKs) are phosphorylated. Activated IRAK1 associates with TRAF6 and the TAK1 protein kinase complex which culminates in activation of the transcription factors nuclear factor-κB (NF-κB) and activator protein-1 (AP-1) *via* IKKs and MAPK, respectively. Thus, MyD88-dependent TLR signaling results in translocation of NF-κB and AP-1 to the nucleus for production of pro-inflammatory mediators, playing a critical role in triggering an inflammatory response for defense against an invading pathogen.

Beyond its role in stimulating inflammation, MyD88 is a key regulator of phagocytosis of bacteria by macrophages (27) and DCs (28) *via* IRAK4 and p38 MAPK (29) which lead to the expression of scavenger receptors. This pathway also influences phagocytosis *via* NADPH oxidase assembly and thus superoxide production for bacterial killing (30). Upon TLR4 stimulation by lipopolysaccharide (LPS) in macrophages, it has been found that MyD88 signaling also activates Src tyrosine kinase *via* the cytoplasmic protein EGF receptor pathway substrate number 9 [Eps8 (31)] which causes actin cytoskeleton rearrangement. Although alternate MyD88-independent pathways have been identified [actin-Cdc42/Rac pathway (32); CD14/complement receptor 3 (33)], the MyD88 pathway seems to be the main driver of phagocytosis of *Borrelia burgdorferi* (34). It also plays a role in phagocytosis of fungal pathogens, although this differs depending on the fungal challenge (35). The role of MyD88 signaling in innate immunity is highlighted by high incidence of life-threatening infection in patients with MyD88 and IRAK4 deficiencies (36, 37).

TRIF-Dependent Signaling Cascades

TLR3 exclusively signals through the TRIF-dependent pathway through direct interaction, whereas TLR4 uniquely signals through MyD88 at the cell surface or through TRIF (also referred to as TICAM-1) upon internalization of the receptor complex after ligand binding (20). Trafficking of TLR4 to the endosomal compartment is dependent on CD14 (38, 39). After TLR4 endocytosis, the recruitment of the adaptor protein TRAM (also termed TICAM-2) is coordinated with the release of the TIRAP-MyD88 complex (19, 40). From the endosomal compartment, TLR3 and TLR4 associate with the TRAM-TRIF complex which interact with TNF receptor-associated factor 6 (TRAF6) and lead to activation of NF-κB or AP-1 and downstream production of inflammatory cytokines. Alternatively, interaction with TRAF3 induces interferon regulatory factors 3 (IRF3) or IRF7 and downstream production of type I interferons (17, 40). Briefly, TRAF6 activates the TAK1 complex that subsequently activates NF-κB and MAPKs *via* the IKK family member NF-kB essential modifier (NEMO). On the other hand, TRAF3 recruits TBK1 and IKKi resulting in phosphorylation of IRF3 or IRF7 which dimerize and translocate to the nucleus to induce to transcription of IFNs. Additional intricacies of the TRIF-dependent signaling cascade are reviewed by Ullah et al. (18). Production of type I IFNs is most often associated with defense against double-stranded RNA (dsRNA) viruses; however, they are also important for response to single-stranded RNA (ssRNA) viruses, DNA viruses, and bacteria (41, 42). TRIF signaling seems to play a role in activation of the adaptive immune system *via* T cell stimulation. Importantly, TRIF signaling mediates caspase activation, apoptosis, and necroptosis which may play a role in removing infected cells, thus preventing pathogen dissemination *via* cell death (43–45).

Balance Among MyD88- and TRIF-Dependent-Signaling

Activation of MyD88-dependent and TRIF-dependent signaling cascades allows for immune functional responses specific to the pathogen sensed by TLR ligand binding, as discussed above. Thus, these pathways are subject to several regulatory strategies for the balanced production of inflammatory cytokines and type I IFNs for elimination of pathogens but also to control the magnitude of the response to prevent pathogenic inflammation and autoimmune disease (46). Such regulatory controls of TLR-mediated inflammatory responses include cooperation with coreceptors, post-translational modifications, cellular trafficking, and negative feedback, which are reviewed in detail by Leifer and Medvedev (47). Commonly, regulatory molecules (1) interfere with signaling complex formation *via* TIR domain-containing molecules, (2) prevent association of TRAF6 or TRAF3 with their respective signaling complexes *via* deubiquitinases (48–50), (3) competitively inhibit downstream signaling (51), or (4) provide mRNA stability of signaling molecules regulated by miRNAs (50), or cytokines by RNA-binding proteins (52).

Interestingly, both MyD88-dependent and TRIF-dependent pathways are required for maximal antimicrobial responses upon LPS-activation of TLR4 (53), demonstrating that they work in concert rather than being redundant. As such, TLR4 signaling is tightly controlled by localization, internalization upon ligand binding (thus acting as a temporal regulator), as well as influence of regulatory molecules. In addition to MAPK and NF-κB pathway activation upon TLR stimulation, the PI3K pathway is also activated *via* B-cell adaptor for PI3K (BCAP) in

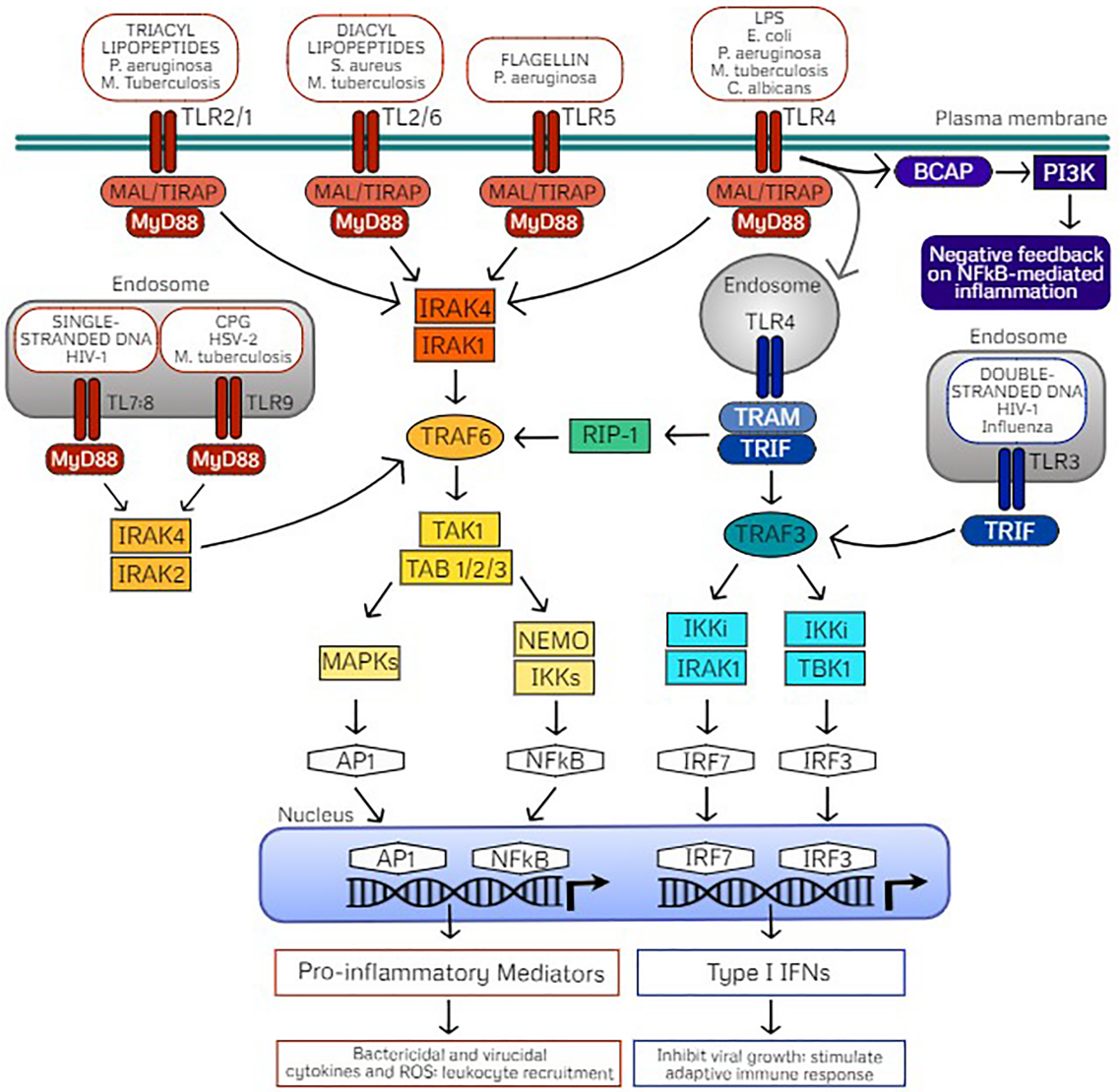

FIGURE 1 | TLR signaling pathways mediated through MyD88- or TRIF-dependent cascades. Cell surface Toll-like receptors (TLRs) include TLR4 and TLR5 which form homodimers upon recognition of their classic ligands lipopolysaccharide (LPS) and flagellin, respectively. TLR2 is also localized on the cell surface which heterodimerizes with TLR1 or TLR6, dependent on ligand recognition with either triacyl or diacyl lipopeptides. TLR recognition of PAMPs on common pathogens are indicated. These cell surface TLRs signal *via* the adaptor protein myeloid differentiation primary response protein (MyD88) through MyD88-adapter-like protein (MAL) also referred to as TIR Domain Containing Adaptor Protein (TIRAP). Intracellular TLRs include TLR9, stimulated by agonist CpG oligodeoxynucleotides (CPG) and the heterodimer TLR7/TLR8, stimulated by single stranded DNA (ssDNA) which signal through direct interaction with MyD88. Activation of MyD88 signaling induces phosphorylation of IL-R-associated kinases (IRAKs) dependent on TLR localization which in turn interacts with TNF receptor-associated factor 6 (TRAF6) and downstream activation of the TAK1 (transforming growth factor β–activated kinase 1)/TAB (TAK1-binding protein) complex. The TAK1/TAB complex activates mitogen-activated protein kinases (MAPKs) or the IkB kinase (IKK) complex of which NF-κB essential modulator (NEMO) is the regulatory subunit. These signaling events activate transcription factors activator protein-1 (AP1) and nuclear factor-κB (NFκB), which translocate into the nucleus for transcription of inflammatory mediators. As a result, pro-inflammatory cytokines and reactive oxygen species drive bacterial killing and limit viral replication as well as stimulate leukocyte recruitment to clear the infectious pathogen. Alternatively, TLR3 recognizes double stranded DNA (dsDNA) and uniquely signals through the adaptor protein Toll/IL-1R (TIR) domain-containing adapter producing interferon-β (TRIF) which interacts with TRAF3. TLR4 also activates TRIF through endocytosis, which activates TRIF signaling *via* the sorting adaptor protein TRIF-related adaptor molecule (TRAM). Activated TRAF3 signals through IKKi/IRAK1 or IKKi/TBK1 which activate the transcription factors interferon-regulatory factor (IRF) 7 and IRF3, respectively, which translocate to the nucleus for transcription of type I interferons (IFNs). Type I interferons act to inhibit viral replication as well as stimulate adaptive immunity. TRIF-dependent TLR4 signaling can also activate TRAF6 *via* receptor interacting protein (RIP)-1 for late NFκB signaling. B-cell adaptor for phosphatidylinositol 3-kinase (PI3K) (BCAP) also seems to be an adaptor protein that confers negative feedback on NFκB-mediated inflammation *via* PI3K as a regulatory mechanism.

macrophages (54, 55) and DCs (56). BCAP/PI3K signaling serves as a negative feedback arm which limits NF-κB induced inflammation and acts as an endogenous regulatory mechanism (57). Additionally, the adaptor TRAF3 has been found to play an inhibitory role on TLR-mediated MAPK activity through preventing the release of the TAK1 signaling complex (58, 59), while peroxiredoxin-1 (PRDX1) attenuates NF-κB activation *via* attenuation of ubiquitin-ligase activity of TRAF6 (60). Understanding these endogenous negative feedback mechanisms will be highly useful while translating TLR-mediated trained immunity for clinical application to protect patients against infection with careful attention as to limit inflammatory responses.

TOLL-LIKE RECEPTOR AGONIST-MEDIATED TRAINED IMMUNITY AND PROTECTION AGAINST INFECTION

Innate Immune Cell Types Which Drive Toll-Like Receptor-Induced Trained Immunity

TLR agonists have exhibited highly attractive immunomodulatory properties whereby they induce augmentation of cell recruitment, antimicrobial effector functions (*i.e.* phagocytosis, respiratory burst, production of proinflammatory cytokines and chemokines), bacterial clearance, attenuate inflammation, and trigger cross-protection to infection with clinically relevant pathogens (61). Importantly, trained immunity is not dependent on T and B lymphocytes as evidenced by preserved protection against several models of infection in transgenic RAG2 knockout mice comparative to survival benefit observed in wild type animals (62, 63).

To date, TLR-mediated innate immune cellular responses have largely been studied in monocytes, macrophages, and natural killer (NK) cells which show long-term functional reprogramming with increased responses to secondary stimulation by bacterial, parasitic, or viral microbes (12, 62, 64) (**Figure 2**). As the 'first responders' to infection, neutrophils play a key role in TLR-mediated resistance to infection *via* increased recruitment and function (65–67). Similarly, activation of macrophages by TLR signaling results in increased antimicrobial effector functions (phagocytic capacity, respiratory burst, altered production of inflammatory mediators) (63). Importantly, both neutrophils and macrophages are required for TLR4-mediated resistance to infection. DCs can be activated by TLR signaling or secondary to TLR-activation of NK cells which 'bridge the gap' between innate and adaptive immunity whereby activated and matured DCs migrate to the lymph nodes and subsequently activate naïve T-cells (15, 68). Thus, immunomodulation of DCs by TLR signaling may prove to be an effective vaccine adjuvant strategy (69). Continued investigation regarding innate immune cell responses to TLR-mediated trained immunity will help refine therapeutic strategies to address specific clinical scenarios.

Immunomodulation *via* Targeting Cell Surface Toll-Like Receptors

Agonists which stimulate cell surface TLRs 2, 4, and 5 have been widely studied for their potential as immunomodulators and conferring host resistance to infection. Such studies are discussed below and are summarized in **Table 1**.

Toll-Like Receptor 2

TLR2 recognizes lipid-containing PAMPs of Gram positive bacteria (*i.e.* lipopeptides, peptidoglycan), as well as viral (*i.e.* HSV glycoproteins) and fungal (*i.e.* zymosan) pathogens, and upon activation it forms heterodimers with TLR1, TLR6, or other cell surface molecules such as Dectin-1 and CD36 (86). Both TLR2/TLR1 and TLR2/TLR6 result in MyD88-dependent signaling *via* MAL/TIRAP; however, the TLR2/TLR1 heterodimer is activated by triacyl lipopeptides whereas the TLR2/TLR6 heterodimer is stimulated by diacyl lipopeptides (87). Several TLR2 agonists have shown promising immunomodulatory effects.

First isolated from *Mycoplasma fermentans* in 1997 (88), macrophage-activating lipopeptide-2 (MALP-2) has become a well-studied immunomodulator which activates TLR2/TLR6 heterodimer. When administered 24 h prior to challenge with *Streptococcus pneumoniae*, MALP-2 treatment reduced bacterial load and enhanced leukocyte migration in the lungs (70). Interestingly, treatment of influenza A virus-infected mice with MALP-2 prior to challenge with *S. pneumoniae* enhanced leukocyte recruitment and reduced bacterial load in the lungs, and was associated with increased survival and improved body condition (71). MALP-2 immunomodulation is presumably driven by its ability to rapidly stimulate neutrophil chemotactic activity followed by induction of monocyte chemoattractant protein-1 (MCP-1) activity in the lungs (89). Further, MALP-2 induces production of proinflammatory cytokines (IL-6, TNF-α) and chemokines (macrophage inflammatory protein-1α and -1β; MIP) (89, 90). Palma et al. also postulated that the microbicidal effect which they observed of MALP-2 on *Mycobacterium tuberculosis* was mediated by nitric oxide (NO) production (72).

As MALP-2 demonstrates attractive immunomodulatory potential, several synthetic analogs of the molecule, termed palmitoylated peptides, have been investigated in the recent decade. The peptide dipalmitoyl-S-glyceryl cysteine (Pam_2Cys) targets TLR2/TLR1, whereas the peptide tripalmitoyl-S-glyceryl cysteine (Pam_3Cys) stimulates TLR2/TLR6. These peptides and their derivatives hold strong potential as vaccine adjuvants, with Pam_2Cys seeming to be more ideal due to increased solubility and potency compared to Pam_3Cys (91, 92). A body of evidence suggests that immunostimulation with Pam_2Cys provides immediate protection against acute infection with influenza virus but also allows for the development of specific immune responses for long-term protection (73). Such protection is mediated *via* activation of DCs, increased leukocyte recruitment, and increased production of inflammatory cytokines (73). Mifsud and colleagues demonstrated that a derivative of Pam_2Cys mediates potent anti-viral activity against influenza infection but also protects against secondary infections with *S. pneumoniae* by reducing bacterial burden and

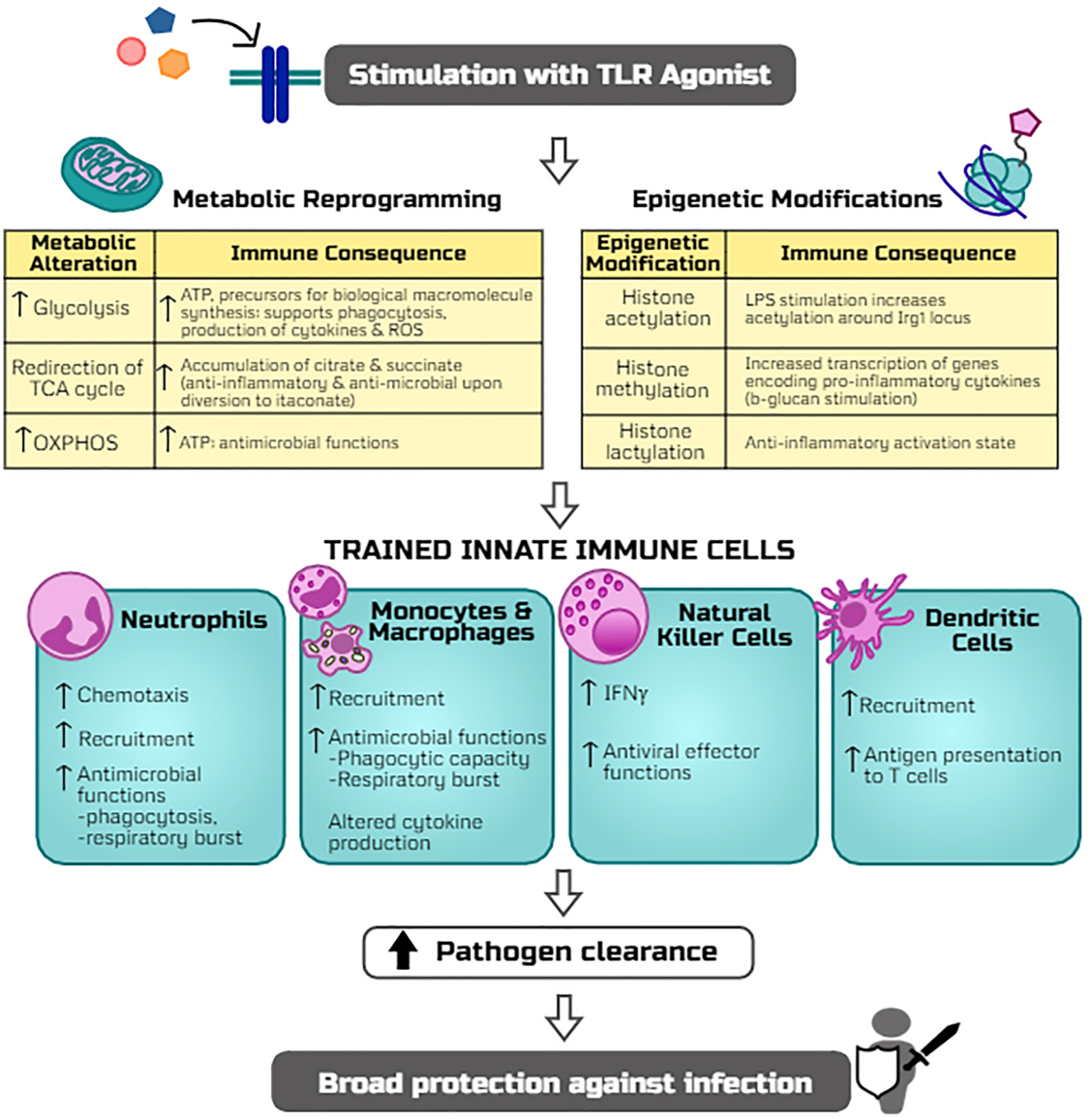

FIGURE 2 | Potential mechanisms by which TLR agonists trigger trained immunity and host resistance to infection. The stimulation of TLR signaling induces metabolic reprogramming (including alterations in glycolysis, TCA cycle, and oxidative phosphorylation, i.e. OXPHOS) and epigenetic modifications (histone acetylation, methylation, and lactylation) which rewire innate leukocytes for more robust antimicrobial functions upon a secondary infectious challenge. Adaptations of innate cell programs thereby allow for more efficient clearance of pathogens and thus protection against a broad array of infections.

inflammation (93). Although these compounds display promise, their synthesis is expensive; thus, recently a novel inexpensive synthesis strategy of an N-acetylated Pam_2Cys analog has been developed, which seems to maintain high potency (94).

The potential application of another TLR2/TLR6 agonist, GSK3277329, was studied in the context of chemotherapy-induced neutropenia which demonstrated that repeated daily injections of the compound for 2 weeks effectively restored neutrophil loss in monkeys given chemotherapy treatment (95). This evidence further illustrates the potential application of TLR agonists in the context of protecting immunocompromised patients from potentially life-threatening infection by boosting innate immunity.

Toll-Like Receptor 4

TLR4 recognizes lipopolysaccharide (LPS) and uniquely signals through both MyD88- and TRIF-dependent pathways. Discovered in 1956 by Landy and Pillemer, mice treated with LPS were resistant to subsequent challenge with Gram negative pathogens (74). Following this work, it became evident that LPS conferred resistance to a broad array of microbes beyond Gram negative bacteria (75, 96) to include Gram positive

TABLE 1 | Agonists which trigger trained immunity *via* cell surface TLRs.

TLR	Agonist	Route of Administration	Infectious Model	Antimicrobial Response	Reference
TLR2	MALP-2	*i.t.*	*S. pneumoniae* (*i.n.*)	↑ Leukocyte recruitment ↓ Bacteremia in lung parenchyma	Reppe et al. (70)
		i.t. prior to *S. pneumoniae* infection	*Influenza A* (*transnasal*) + *S. pneumoniae (i.n.)* superinfection	↓ Pulmonary bacterial load	Reppe et al. (71)
		Assay media of BMDMs	*M. tuberculosis* inoculation of BMDMs	↓ Bacterial growth ↑ Nitric Oxide	Palma et al. (72)
	Pam_2Cys	*i.n.*	Influenza *A* (*i.n.*)	↑ Neutrophil and macrophage recruitment ↑ Pro-inflammatory cytokines	Tan et al. (73)
TLR4	LPS	*i.p.*	*E. coli* (*i.p.*); *P. aeruginosa* (*i.p.*)	↑ Bacterial clearance	Landy & Pillemer (74)
		i.p.	*P. aeruginosa* (*i.p.*)	↑ Bacterial clearance (systemic and lungs) ↓ Pro-inflammatory cytokines (plasma)	Varma et al. (75)
		i.p. and *i.v.*	*S. aureus* (*i.v.*)	↑ Bacterial clearance ↓ Pro-inflammatory cytokines (plasma)	Murphey et al. (61)
	MPLA	*i.p.*	*P. aeruginosa (*topical inoculation of burn wound or *i.p.*)	↑ Bacterial clearance ↑ Leukocyte recruitment	Romero et al. (65)
			Polymicrobial abdominal sepsis (CLP surgical model)	↓ Pro-inflammatory cytokines (plasma)	
		i.p.	*P. aeruginosa (topical inoculation of burn wound)*	↑ Neutrophil mobilization & recruitment to site of infection	Bohannon et al. (67)
		i.p.	*P. aeruginosa (i.p.)*	↑ Neutrophil & macrophage recruitment ↓ Pro-inflammatory cytokines (plasma)	Fensterheim et al. (76)
		i.v.	*S. aureus* (*i.v.*) *C. albicans (i.v.)*	↑ Bacterial clearance ↓ Pro-inflammatory cytokines (plasma) ↓ Organ injury (kidney)	Fensterheim et al. (63)
	PHADs	*i.p.*	*P. aeruginosa (i.p.)*	↑ Bacterial clearance ↑ Leukocyte recruitment	Hernandez et al. (77)
		i.v.	*S. aureus (i.v.)*	↑ Antimicrobial functions Attenuates systemic and local inflammation	
	AGP	*i.n.*	Influenza *A (i.n.)*	↑ Pathogen clearance	Baldridge et al. (78)
		i.v.	*L. monocytogenes* (*i.v.*)		
		i.n.	*F. tularensis* (*inhalation*)	↑ Cytokine & inflammatory responses ↑ Bacterial clearance	Lembo et al. (79)
		i.n.	*Y. pestis* (*i.n.*)	↑ Bacterial clearance (lungs)	Airhart et al. (80)
	fmOMV	*i.n.*	H1N1, PR8, H5N2, H5N1	↑ Type I IFNs ↑ Macrophage recruitment	Bae et al. (81)
	FimH	*i.n.*	Influenza A (i.n.)	↑ Neutrophil recruitment ↓ Organ injury (lung)	Abdul-Careem et al. (82)
		Transurethral instillation	Uropathogenic E. coli (UPEC) or *P. mirabilis*	↑ Bacterial clearance (bladder)	Habibi et al. (83)
TLR5	Flagellin	*i.p.*	Rotavirus (oral inoculation)	↓ Viral load ↓ Viral replication	Zhang et al. (84)
		Sublingual	*S. pneumoniae* (*i.n.*)	↑ Neutrophil recruitment	Munoz-Wolf et al. (85)

i.n., intranasal administration; i.p., intraperitoneal injection; i.t., intrathecal administration; i.v., intravenous injection.

Staphylococcus aureus (61) and fungal pathogens (97) as well as polymicrobial sepsis (98). LPS-mediated resistance to infection is associated with reduced bacterial burden (75, 99), increased leukocyte recruitment (61, 100), and attenuated inflammation (101).

Despite several studies demonstrating its potential therapeutic benefit, the application of LPS as an immunomodulator for translation to the clinical scenario was largely abandoned due to its toxicity (16, 102). More recently, derivatives of LPS as well as synthetic molecules have demonstrated potent induction of trained immunity with significantly reduced toxicity, thus holding strong therapeutic potential. Notably, monophosphoryl lipid A (MPLA) is structurally identical to LPS with the exception of the cleaved C1 phosphate group from lipid A which reduces its toxicity 100-fold (103, 104). Binding of MPLA by TLR4 induces both MyD88- and TRIF-dependent signaling, although MyD88 signaling seems to be predominant (105). Similarly to protection conferred by LPS immunomodulation, administration of MPLA prior to infectious challenge provides a survival benefit to an array of pathogens including Gram negative *P. aeruginosa*, Gram positive *S. aureus*, viral influenza, and fungal *C. albicans* (63, 65, 106). Importantly, MPLA also protects burn-injured mice from wound infection with the clinically relevant pathogen *P. aeruginosa* (67) and in large animals (sheep (107) demonstrating its capacity to induce protection in the immunocompromised host. MPLA-driven survival benefit lasts for at least 10 days following administration (76). Evidence suggests that B and T cells are not required for MPLA-mediated protection to *S. aureus*, neither were recruited monocytes; conversely, depletion of macrophages or neutrophils resulted in loss of MPLA-induced survival benefit (63). MPLA treatment enhances leukocyte recruitment, bacterial clearance, antimicrobial functions, and attenuates inflammation which all likely play a role in resistance to infection (65, 67). It is important to highlight that MPLA similarly enhances human neutrophil responses characterized by increased chemotaxis and bacterial killing (108). Additionally, MPLA stimulates the adaptive immune response whereby it increases antibody titers up to 20-fold (109) and thus is used as an adjuvant in malaria (AS01), human papillomavirus (HPV), and hepatitis B (AS04) vaccines (110, 111).

With their immunostimulatory properties, it is unsurprising that several synthetic TLR4 agonists have been developed with the goal of clinical translation. One promising class of synthetic TLR4 agonists are phosphorylated hexa-acyl disaccharides (PHADs) which are similar in structure to MPLA as they have only one phosphate group (16). PHADs similarly bind TLR4 and activate both MyD88- and TRIF-dependent signaling. Hernandez and colleagues recently showed that treating mice with PHADs confers protection to *P. aeruginosa* and *S. aureus*, both of which are of high clinical relevance (77). They found the survival benefit to be associated with increased bacterial clearance, an effect which was observed up to 10 days after treatment. Further, treatment with PHADs increased leukocyte recruitment and antimicrobial functions while attenuating systemic and local levels of proinflammatory cytokines (77).

Another synthetic lipid A TLR4 agonist, aminoalkyl glucosamine 4-phosphate (AGP), was first found to possess immunostimulatory properties two decades ago (112). Initially studied for their potential as a vaccine adjuvant (80), later studies demonstrated that AGPs confer protection to an otherwise lethal influenza challenge as well as to *Listeria monocytogenes* infection which were associated with increased bacterial clearance (78). Intranasal administration of AGP either before or after infection with the Gram-negative pathogen *Francisella tularensis* resulted in increased survival, and interestingly the survivors were protected against rechallenge with aerosolized *Francisella novicida* (113). Likewise, intranasal administration of AGPs prior to challenge with *Yersinia pestis* also extended time to death which was correlated with cytokine production and decreased bacterial load in the lung (80). Evidence suggests that TLR4 signaling *via* activation by AGPs induces NF-κB and IRF-3 signaling independent of the co-receptor CD14 (114).

Additionally, outer membrane vesicles (OMVs) with low endotoxicity by modification of lipid A of LPS (fmOMV) may increase protective benefit of intranasally administered influenza vaccine (115). Further, fmOMV confers protection against a lethal dose of pandemic viruses (H1N1, PR8, H5N2, and highly pathogenic H5N1) which is dependent on macrophages but independent of neutrophils. Treatment with fmOMV increased macrophage recruitment and production of type I IFNs without observance of adverse effects (81).

Beyond lipid A compounds, other natural and synthetic TLR4 ligands have been investigated. Fimbriae H protein (FimH) is the receptor-recognizing element of the adhesive organelle type 1 fimbriae on uropathogenic *E. coli* (UPEC) (116). Mice which receive FimH intranasally are resistant to influenza infection through increased recruitment of neutrophils and production of proinflammatory cytokines (TNF-α and IL-12) and chemokines (RANTES) in a macrophage-independent manner (82). Fusion of fimH with the MR/P fimbriae protein MrpH from *P. mirabilis* (MrpH.FimH fusion protein) conferred higher protection to UPEC and *P. mirabilis*, associated with reduced bacterial burden in the bladder and kidney as well as increased neutrophil recruitment (83).

Two classes of synthetic small molecule agonists have been studied with modest therapeutic potential. Neoseptin-3 is a more potent TLR4 agonist than LPS despite being structurally unique (117, 118). Neoseptin-3-activation of TLR4 signaling results in MyD88- and TRIF-dependent signaling and downstream activation of NF-κB and IFN-β but not IFN-α (118). Evidence suggests that Neoseptin-3 may hold strong vaccine adjuvant properties whereby mice immunized by ovalbumin (OVA) together with the compound had increased OVA-specific IgG production 21 days later when compared to immunized vehicle controls mice. However, these compounds failed to induce TLR4 signaling in human THP-1 monocytes, thus calling into question their therapeutic potential (118).

Toll-Like Receptor 5

TLR5 recognizes bacterial flagellin (119) and signals through MyD88, culminating in the production of inflammatory

mediators. Studies have shown that bacterial flagellin is indeed an immunomodulatory agent. Mucosal administration of flagellin conferred resistance to *S. pneumoniae* lung infection whereby flagellin treatment increased bacterial clearance which was associated with increased neutrophil mobilization independent of B- and T-cells (120). More recently, this research group showed that sublingual administration of flagellin also effectively protects against pneumonia (85). Demonstrating cross-protection, treatment with bacterial flagellin was able to prevent infection with or cure ongoing infection of rotavirus in mice independent of adaptive immunity (84).

Bacterial flagellin also restores antibiotic-impaired innate immunity (121) and improves efficacy of antibiotics in the treatment of influenza virus or pneumonia (122). Thus, bacterial flagellin may be highly useful in prevention of infection in immunocompetent and immunocompromised individuals, as well as a strategy to combat antibiotic resistant microbes and boost efficacy of current antibiotic drugs.

Immunomodulation *via* Targeting Intracellular Toll-Like Receptors

Numerous TLR agonists which activate intracellular TLRs have also been widely studied for their potential application as immunomodulators that trigger trained immunity, which are summarized in **Table 2**.

Toll-Like Receptor 3

TLR3 is localized to the intracellular compartment and recognizes viral dsRNA, including that produced during replication of ssRNA viruses or self-RNAs released from damaged cells (86, 143). Stimulation of TLR3 results in direct interaction with TRIF for downstream activation of IRF3 and modest activation of NF-κB (144). TLR3/TRIF activation culminates in production of type I IFNs and inflammatory cytokines for killing of invading viruses and has also been found to be important in cross-priming CD8+ T cell responses in a virus-specific manner (21, 145). As TLR3 signaling is MyD88-independent, the safety and immunostimulatory properties of TLR3-specific agonists is unique among other TLR immunomodulators (146).

The TLR3-activating synthetic dsRNA molecule poly-inosinic:poly-cytidylic acid (poly I:C) was first discovered to confer protection against subsequent viral challenge in 1969 (147). More recently, it was found that intraperitoneal administration of poly I:C 3 days prior to challenge with *E. coli* K1 meningitis in neutropenic mice resulted in increased recruitment of NK cells, production of RANTES and IFN-γ, and decreased bacterial burden (126). Pre-treatment with poly I:C resulted in survival benefit of neutropenic but not immunocompetent mice. In another model of infection, poly I:C conferred anti-viral properties (148) which lead to neuroprotection in a mouse model of HSV-1 encephalitis (124). In a unique oyster model, poly I:C injection protected the organism against subsequent environmental infection by mitigating viral replication which persisted for at least 5 months (148). It is suggested that the length of the dsRNA dictates distinct antimicrobial functions dependent on cell type which should be considered during study design and data interpretation (149). It has been posed that the difference in TLR3-mediated protection against viral infection compared to bacterial infection may be due to the production of type I IFNs which impairs bacterial clearance (15). Indeed, intranasal administration of poly I:C prior to challenge with *S. pneumoniae* and methicillin-resistant *S. aureus* increased susceptibility to infection (150).

Since its creation 5 decades ago, analogs of poly I:C have been rapidly developed in effort to reduce toxicity (146). The substitution of a uridylic acid at a molar ratio of 12:1 in the synthesis of the poly C strand results in poly I:C_{12}U which is more rapidly metabolized *in vivo* (151). Pre-treatment with poly I:C_{12}U protects against subsequent viral challenge more effectively than poly I:C (123). Interestingly, a protective benefit has also been observed when the molecule was administered 2 days after viral myocarditis infection (152). In a clinical trial in which poly I:C_{12}U was administered to HIV-infected patients, immune function was restored or stabilized (153); however, clinical investigation did not progress past phase II clinical trials (154). Poly I:C_{12}U also holds promise as a vaccine adjuvant as it increases efficacy of intranasal H5N1 immunization (155) and intradermal HSV-2 immunization, which conferred resistance to subsequent otherwise lethal HSV-2 infectious challenge (156). Demonstrating its safety, this compound has been developed as a therapy for chronic fatigue syndrome (157, 158) that is approved in Argentina and has been approved for early access program in the European Union and Turkey, although it does not currently have FDA approval in the United States (Rintatolimod, tradename Ampligen).

Two other chemically stabilized analogs of poly I:C have demonstrated promising immunostimulatory properties. The first being poly IC : LC (termed Hiltonol) which has been shown to protect rhesus monkeys from several viruses including yellow fever, Rift Valley fever, and rabies (159, 160) and has conferred protection against highly viral strains of H4N1 and influenza in mice (161, 162). Importantly, it was found in 2017 that intranasal administration of poly IC : LC 24 h prior to or 8 h after an otherwise lethal challenge with SARS-CoV conferred survival benefit as well as reduced lung hemorrhage scores and lung viral titers in mice (125). Additionally, this molecule has been investigated for its potential in boosting immunity of HIV-infected patients which induced transient innate immune responses, suggesting application as a vaccine adjuvant may be appropriate (163). This line of investigation is being pursued whereby administration of poly IC : LC alongside an antigen stimulates a 'live virus vaccine equivalent' effect whereby antigen-presenting cells (APCs) are activated, T lymphocyte response is elicited, memory T and B cells are generated, and T_{eff}/T_{reg} ratios are increased (164). Keyhole limpet hemocyanin (KHL) or HPV vaccines elicited significantly elevated antibody responses and Th1 immune responses when administered with poly IC : LC in rhesus Macaques (165).

TABLE 2 | Agonists which trigger trained immunity *via* intracellular TLRs.

TLR	Agonist	Route of Administration	Infectious Model	Antimicrobial Response	Reference
TLR3	Poly I:C & derivatives	*i.p.*	Punta Toro virus (*s.c.*)	↓ Organ injury (liver)	Gowen et al. (123)
		i.p.	HSV-1 (*i.n.*)		Boivin et al. (124)
		i.n.	Mouse-adapted SARS-CoV (*i.n.*)	↓ Organ injury (lungs) ↓ Viral load (lungs)	Kumaki et al. (125)
		i.p.	*E. coli* (*intracranial*)	↓ Viral load (systemic, cerebellum, and spleen) ↑ NK cell recruitment* ↑ INF-γ (brain and spleen)* **In neutropenic but not immunocompetent hosts*	Ribes et al. (126)
	CRL1505	Oral	Respiratory syncytial virus (RSV; *i.n.*)	↓ Viral load Attenuation of Th2 reactions	Chiba et al. (127)
		i.n.	Respiratory syncytial virus (RSV; *i.n.*)	↓ Viral load ↓ Viral replication ↓ Organ injury (lungs)	Tomosada et al. (128)
		i.n.	Influenza A (*i.n.*)	↓ Viral load ↓ Organ injury (lungs) ↑ Lymphocytes and DCs (lungs)	Zelaya et al. (129)
		i.n.	Primary RSV (*i.n.*) + secondary *S. pneumoniae* (*i.n.*)	↑ Alveolar macrophages and T cells (lungs)	Clua et al. (130)
TLR7	1V270 (TMX201)	*i.n.*	*B. anthracis*, Venezuelan equine encephalitis virus, H1N1 virus (*i.n.*)	↑ Local, but not systemic, inflammation	Wu et al. (131)
	Imiquimod	*i.p.*	Polymicrobial sepsis (fecal-induced peritonitis)	↑ Neutrrophil recruitment ↑ Antimicrobial responses (phagocytosis)	Wynn et al. (66)
		i.n.	Influenza A (*i.n.*)	↓ Viral replication ↓ Local inflammation ↓ Organ injury (lungs)	To et al. (132)
	T7-EA	*i.p.*	Hepatitis B (*i.v.*)	↑ HBsAg-specific IgG2a titer & T-cell response	Hu et al. (133)
	CL097	*i.p.*	Hepatitis B transgenic mice	↑ HBsAg-specific T-cells (spleen)	Wang et al. (134)
	GS-9620	Oral	Hepatitis B chronically infected patients	↑ T-cell and NK cell responses	Boni et al. (135)
TLR9	CpG	*i.p.*	*L. major* (*oral*)	Shifts Th2 towards Th1 response	Zimmerman et al. (136)
		Intradermal	*L. amazonensis (intradermal)*	↓ Lesion size ↓ Parasite load	Verthelyi et al. (137)
		Mucosal (genital tract)	HSV-2 (intravaginal)	↓ Viral load (vaginal fluids) T-cell dependent	Harandi et al. (138)
		i.t.	*K. pneumoniae* (*i.t.*)	↑ Bacterial clearance (systemic & lungs) ↑ Neutrophils and lymphocyte recruitment	Deng et al. (139)
		i.p.	*L. monocytogenes* (*i.p.*)	↑ CD4 & CD8 T cells	Ito et al. (140)
		i.n.	New World arenavirus Tacaribe (neurotropic virus: *i.n.*, *i.p.*, or *intracranial*)	↑ Ag-specific antibodies (IgG & IgM)	Pedras-Vasconelos et al. (141)
		i.p.	MRSA (*i.v.*)	↑ Bacterial clearance ↓ Organ injury (lung, kidney, spleen) ↑ Lymphocyte recruitment ↑ Bacterial-reactive antibodies	Kim et al. (142)

i.n., intranasal administration; i.p., intraperitoneal injection; i.t., intrathecal administration; i.v., intravenous injection; s.c. subcutaneous injection.

The second poly I:C stabilized analog is PIKA which has been shown to protect mice against an array of influenza viruses along with decreased viral burden in the lungs and increased recruitment of macrophages, neutrophils, and plasmacytoid DCs (166). However, application of PIKA has mostly focused on its potential as a vaccine adjuvant for H5N1 (167–169), Hepatitis B (HBsAg) (170), and rabies (171), the latter of which underwent phase II clinical trials with moderate success (172).

Beyond the poly I:C class of TLR3-stimulating molecules, oral administration of purified *L. rhamnosus* CRL1505 peptidoglycan confers resistance to RSV infection associated with decreased viral loads in the lungs and augmented cytokine responses (127). Protection against RSV and subsequent secondary infection to pneumococcal pneumonia was found to be dependent on TLR3 (128, 130) and macrophages (173). In an immunocompromised-malnourished model, it was found that immunostimulatory properties of CRL1505 peptidoglycan extended beyond augmentation of innate immunity. Administration of CRL1505 enhanced the Th2 response and recovery of B cells after *S. pneumoniae* infection (174). The investigators also found that intranasal administration of CRL1505 prior to challenge with influenza virus was associated with reduced pulmonary injury and viral loads in the lungs *via* regulation of pro-inflammatory cytokines and increased levels of type I IFNs (129).

Together, TLR3 agonists hold strong promise, especially in protection against viral infections and for vaccine adjuvant strategies. However, careful attention needs to be paid as to the potential propagation of bacterial infections by TLR3-induced production of type I IFNs.

Toll-Like Receptor 7

The endosomally located TLR7 recognizes ssRNA and often plays a role in responding to viral infections through MyD88-dependent signaling. The small molecule 1V270 (also designated TMX201) is a TLR7 ligand conjugated with a phospholipid that has been shown to protect mice from an otherwise lethal infection with *Bacillus anthracis*, Venezuelan equine encephalitis virus, and H1N1 influenza virus (131). 1V270-mediated protection was associated with increased cytokines and chemokines in bronchial alveolar lavage fluids but not in circulation.

The Imidazoquinoline compound Imiquimod is a low molecular weight compound which selectively activates TLR7. Imiquimod is an FDA approved immune response modifier for the topical treatment of genital warts caused by HPV (175). Imiquimod has also been found to be protective against influenza A infection in mice which was associated with reduced viral replication, airway inflammation, proinflammatory cytokine production, and preservation of body weight (132). Neonates were also protected against polymicrobial sepsis when infection was initiated 24 h after treatment (66). It is also important to note that imiquimod may be an effective vaccine adjuvant strategy for influenza (176).

Additionally, several TLR7 agonists have been shown to improve immunity of hepatitis B-infected hosts and are implicated as potential HBV vaccine adjuvants. When administered together with an alum adjuvant and recombinant hepatitis B surface antigen (HBsAg) protein, the novel TLR7 agonist T7-EA induced HBsAg-specific antibody and restored T-cell responses in a murine model (133). Similar improvement of HBsAg-specific T-cell function was observed by immunizing HBV-transgenic mice with a TLR7/TLR8 agonist (CL097)-conjugated HBV protein (134). In a prospective clinical study, it was found that oral administration of the TLR agonist GS-9620 increased T-cell responses to HBV peptides demonstrated by increased cytokine production; however, it failed to reduce serum HBsAg levels (135).

Toll-Like Receptor 9

TLR9 is expressed in DCs, monocytes, macrophages, and B cells and recognizes bacterial and viral DNA. Upon ligand binding, it signals through MyD88 directly; however, it is important to note that signaling depends on intracellular localization as a mechanism to fine-tune the immune response. In resting cells, TLR9 is localized to the ER (177). Recently described in detail by Marongiu et al., trafficking of TLR9 is controlled by the multimembrane protein unc-93 homolog B1 (UNCB1) which is required for the receptor to leave the ER and traffic to the Golgi (178). After delivery to the plasma membrane, the adaptor protein AP-2 is recruited to mediate internalization of the receptor in a clathrin-dependent mechanism (179). In parallel to internalization of TLR9, the ligand must also be endocytosed. TLRs are then localized to early endosomal compartments and the pathway bifurcates to either IRF7 signaling endosomes or NF-κB signaling endosomes (180), which are determined by AP-3 (181). Thus, the localization of TLR9, presence of co-receptors and co-factors, as well as trafficking of the ligand itself all influence downstream signaling and determine whether pro-inflammatory cytokines or type I IFNs are produced. Such intricate control also prevents recognition of self-DNA to prevent autoimmune dysfunction.

CpG oligodeoxynucleotides (CpG ODNs) are synthetic molecules which mimic bacterial DNA and stimulate TLR9. Pre-treatment with CpG has been found to protect mice from *Leishmania major* infection by shifting immune responses from Th2 towards Th1 (136). CpG conferred survival benefit to *Leishmania major* and *F. tularensis* for up to 2 weeks independent of the route of infection (182). Intrathecal administration of CpG 48 h prior to *Klebsiella pneumoniae* infection resulted in increased survival, associated with reduced bacterial burden in the lungs and circulation, increased recruitment of neutrophils, NK cells, γδ-T cells, and augmented inflammatory response (139). Interestingly, treatment of methicillin-resistant *S. aureus* (MRSA)-infected mice with CpG improved survival (142). Beyond protection against bacterial microbes, CpG conferred survival benefit to viral challenge by HSV-2 which was associated with decreased viral replication (138) *via* augmentation of the innate immunity (183). CpG has also conferred protection of neonate mice challenged with *Listeria* infection (140) as well as neurotropic Tacaribe Arenavirus which was associated with decreased viral load, increased antigen-specific antibodies, and NO production *via* NO synthase expression (141). CpG-mediated protection was

preserved in T cell-depleted immunocompromised mice (182). HIV-infected macaques treated with CpG prior to challenge with *Leishmania* exhibited decreased lesion size and parasite load (137). The two latter studies clearly demonstrate the clinical potential of CpG to protect immunocompromised populations against opportunistic infections.

In 2017, the FDA approved the use of CpG 1018 as a vaccine adjuvant in a hepatitis B vaccine (Heplisav-B) which has increased efficacy of the vaccine, thus reducing the prior three-dose strategy to a two-dose strategy (184). CpG 1018 increases antibody concentrations, stimulates helper (CD4+) and cytotoxic (CD8+) T cells, boosts T and B cell memory responses, and shifts T cells towards a Th1 response. Researchers have found that the 2-dose HBV vaccine strategy with CpG as the adjuvant compared to the 3-dose strategy with aluminum hydroxide was more effective in patients aged 60–70 years old with type 2 diabetes mellitus (185), a population which typically demonstrates reduced immunogenicity compared to younger and/or non-diabetic populations. Thus, CpG may be a beneficial agent to boost immune responses in vulnerable patient populations.

Other Pathogen Associated Molecular Patterns Which Trigger Trained Immunity in a Toll-Like Receptor-Associated Mechanism

Bacillus Calmette-Guerin Vaccine

The Bacillus Calmette–Guerin (BCG) tuberculosis vaccine is the most used vaccine globally which has been demonstrated to confer T cell-independent cross-protection against fungal infection with *C. ablicans* or with the parasite *Schistosomiasis mansoni* (186, 187). After adjusting for age and other vaccines, the BCG vaccine is associated with a significantly lower mortality ratio among infants in Ginea-Bissau (188). In animal studies, BCG-mediated non-specific protection lasts for at least 3 months and is independent of T and B cells (189) and there is evidence that protection may last up to a year (190). Although the immunization effect of the BCG vaccine against *M. tuberculosis* requires adaptive immunity, namely T cell activation, the initial response to BCG is through the innate immunity whereby TLR2 and TLR4 and downstream MyD88-dependent signaling are activated (191). The resulting activation of the NF-κB pathway serves as the link between the innate and adaptive response. Therefore, the BCG vaccine does activate the innate immune response and may be responsible for driving broad protection.

β-Glucan

Fungal β-glucans are a promising class of molecules which trigger trained immunity, although the immunostimulatory properties differ depending on the strain from which they were isolated (192). These naturally derived molecules have been found to confer protection against a model of *E. coli* peritonitis (193), *S. aureus* (194), influenza (195), and MRSA (196) and were associated with increased leukocyte recruitment and antimicrobial functions. Treatment of burn-injured mice with glucan phosphate prior to wound infection with *P. aeruginosa* improved survival, attenuated cytokine production, and decreased bacterial load at the burn wound (197).

β-glucans bind their specific PRR dectin-1 which results in downstream inflammasome activation. However, β-glucan-mediated production of inflammatory cytokines and reactive oxygen species (ROS) is dependent on the cooperation between dectin-1 and TLR2 (198). Further, TNF-α production in response to zymosan or live fungi is dependent on MyD88 (199). Thus, β-glucan-mediated trained immunity is dependent on the synergism of dectin-1 and TLR2.

CL429

One research group is investigating the potential immunostimulant properties of CL429, which is a novel chimeric compound that was designed to stimulate both TLR2 and NOD2 by covalently linking the NOD2 ligand Murabutide with the TLR2 ligand Pam_2C (200). Initially studied as a vaccine adjuvant, they went on to find that CL429 confers protection against pneumovirus (PVM) infection associated with attenuated inflammation (201) and against leptospiral infection for up to 3 months *via* increased proinflammatory cytokine and chemokine production (202).

CpG-Oligodeoxynucleotide : AG-OVA Nanoparticles

Similar in approach as the CL429 molecule, the TLR9-activating ligand CpG ODN was crosslinked with the dectin-1/TLR2 stimulating agonist β-glucan-Ovalbumin resulting in CpG-OND : AG-OVA dual-targeting nanoparticles as a vaccine adjuvant strategy (203). Investigators found that the nanoparticles enhanced APC maturation and induced robust Th1 and Th2 responses similar to that triggered by Freund's adjuvant but without the toxicity. Although this novel compound demonstrates promise as a vaccine adjuvant, it would also be of interest to investigate whether it confers broad protection to infection which may be more profound than that mediated by CpG or β-glucan alone, both of which are strong immunomodulators.

Cross-Protection Between Infections

As the BCG vaccine seemingly mediates cross-protection against pathogens besides tuberculosis, evidence suggests that some infections also confer cross-protection which is, at least in part, due to trained immunity (10). For example, administration of an attenuated strain of *C. albicans* conferred host resistance to subsequent challenge with the Gram-positive bacteria *S. aureus*; this phenomenon was found to be independent of T cells but was dependent on macrophages (204). Interestingly, several observations have suggested that viral infections may trigger a similar cross-protection benefit. Barton et al. demonstrated that latent herpesvirus was associated with protection against bacterial *L. monocytogenes* and against bacterial *L. monocytogenes* and *Y. pestis* which was similarly dependent on macrophages (205). This observation was confirmed by others who elucidated that herpesvirus-induced protection against bacterial infection is transient (approximately 5 months) despite stable viral load (206). NK cells are also key mediators of cross-protection whereby they expand during the initial infection and are primed to undergo a second expansion as

well as produce more cytokines upon a secondary infection (207). More recently, a Singapore study of military recruits within a 5-year period showed that men infected with influenza were protected against subsequent infection with adenovirus (79). It should be noted, however, that cross-protection between infections is generally considered to be dependent on both non-specific reprogramming of innate immunity as well as activation of memory T cells.

Toll-Like Receptor Antagonists as Potential Immunomodulatory Strategies for Treatment of Chronic Infectious Diseases

It is clear that TLR agonists hold strong therapeutic potential to mediate host resistance to subsequent infection; however, this is only one of many potential therapeutic applications of TLR immunomodulators. As TLR signaling cascades culminate in robust inflammation, TLR antagonists are under development for the treatment of chronic infectious and inflammatory diseases (208). To date, these compounds are generally designed to bind the TLR, thus preventing the binding of agonists responsible for driving inflammation (209–211). The TLR4 antagonist Eritoran (E5564) reached Phase III clinical trials for the treatment of sepsis. This synthetic lipid A analogue which prevents LPS from activating TLR4, hypothetically preventing propagation of systemic inflammatory response syndrome (SIRS) characteristic of sepsis. Preclinical and early clinical studies with the compound showed promising anti-inflammatory results in response to LPS (212–214), however the study failed to meet its target end-point in phase III (215). Similarly, another TLR4 antagonist designated Resatorvid (TAK-242) was also studied in the treatment of sepsis and reached Phase III of the clinical trials, however it failed to attenuate inflammation in septic patients (216).

Recently, the antimalarial drugs chloroquine and hydroxychloroquine have been under investigation for treatment of COVID-19 with the hypothesis that these drugs will prevent glycosylation of the angiotensin-converting-enzyme 2 (ACE2) as well as inhibit endosomal TLR activation (217, 218). Initial *in vitro* studies showed potent antiviral activity (219). Randomized trials have not shown improved clinical outcomes in the hydroxychloroquine-treated COVID patients (218, 220, 221). In another study, the Bruton tyrosine kinase (BTK) inhibitor acalabrutinib was administered to COVID patients for 10–14 days which seemed to improve patient outcomes as indicated by oxygenation (222).

Although clinical trials using TLR antagonists in the treatment of severe infection have been unsuccessful to date, critical information has been gained from these investigations which provide a strong foundation for future studies. Importantly, this class of compounds is also being widely studied for treatment of chronic inflammatory conditions such as rheumatoid arthritis and autoimmune disorders. One key finding of the clinical trials studying the TLR4 antagonists Eritoran and Resatorvid was that the compounds were well-tolerated (215). Continued drug discovery efforts *via* high throughput screening and alternative approaches such as targeting the transcriptional regulation of TLRs to suppress their expression rather than direct inhibition of the receptor may move the field forward (223). These efforts will be also be supported by continued elucidation of TLR signaling mechanisms and immune responses.

METABOLIC AND EPIGENETIC REPROGRAMMING AS THE BASIS FOR TOLL-LIKE RECEPTOR AGONIST-INDUCED TRAINED IMMUNITY

Upon inflammatory stimulation, innate leukocytes undergo metabolic reprogramming that is characterized by augmentation of glycolysis and mitochondrial oxidative phosphorylation to meet the increased energy demands for combating an infection (14, 224). Previous investigations aimed at deciphering the molecular mechanisms of trained immunity-mediated protection against infections have predominantly used the fungal ligand β-glucan (225, 226). Reviewed in detail by Netea and colleagues, metabolic reprogramming and epigenetic modifications are the key mechanisms of β-glucan-induced trained immunity (226). It has been shown that Akt/mTOR/HIF-1α signaling is critical in β-glucan induced augmentation of glycolysis in monocytes (225). As opposed to the breadth of mechanistic understanding of trained immunity induced by β-glucan, the molecular mechanisms underlying TLR ligand-induced training of leukocytes are an evolving field. Our studies show that treatment with the TLR4 ligand MPLA not only increases glycolysis but also augments mitochondrial oxidative phosphorylation and mitochondrial biogenesis in concert with increased antimicrobial functions of macrophages (63). Further, stimulation of macrophages with the classic TLR4 ligand LPS reprograms mitochondrial metabolism leading to increased accumulation of tricarboxylic citric acid (TCA) cycle metabolites that play an important role in TLR agonist-mediated trained immunity (227, 228).

The finding that a variety of TLR ligands have the ability to mediate protection against infection from a broad array of organisms which activate distinct TLRs, including Gram-positive and Gram-negative bacteria, fungi, and viruses, demonstrates that TLR activation has the ability to offer cross-protection against diverse pathogens. Both MyD88 and TRIF activation have been implicated in facilitating signaling-driven metabolic and epigenetic alterations induced by TLR ligands (229), but little is understood about the roles of these signaling pathways in initiating trained immunity. This raises the question as to whether activation of trained immunity by TLR ligands is mediated through common signaling pathways. Future studies providing insight into these common pathways would pave the way for the discovery of a multitude of potential TLR ligand-based therapeutics to improve resistance to infection. The following sections will provide a succinct overview of leukocyte metabolic reprogramming and epigenetic modifications as the basis for TLR ligand induced trained immunity (**Figure 2**).

Toll-Like Receptor Ligand-Induced Metabolic Reprogramming of Innate Leukocytes

Stimulation of macrophages with LPS increases glucose uptake and glycolytic capacity mediated *via* stabilization and upregulation of hypoxia-inducible factor 1- α (HIF-1α) (230–232). Increased glycolytic capacity serves to rapidly generate ATP and provide essential precursors for synthesis of amino acids, lipids, and nucleotides that are necessary for optimal effector activities and cell viability under stress conditions (233). Our studies have shown that deletion of HIF-1α or inhibition of mammalian target of rapamycin (mTOR; known to stabilize HIF-1α) attenuates MPLA-induced increased glycolysis, abolishing the protective effect of MPLA against infection (63, 76).

Along with augmented glycolysis, reprogramming of mitochondrial metabolism plays a key role in modulating the inflammatory response of innate leukocytes. LPS-induced activation of macrophages introduces 'breaks' in the TCA cycle at the levels of isocitrate dehydrogenase and succinate dehydrogenase (SDH), leading to increased accumulation of citrate and succinate (234). Studies from our laboratory also show that MPLA treatment cause an early reduction in TCA cycle flux between citrate and α-ketoglutarate leading to increased accumulation of citrate (63). Citrate is diverted towards generation of itaconate *via* increased immunoresponsive gene 1 (Irg1) enzyme expression (235). Itaconate is being widely investigated for its direct antimicrobial and anti-inflammatory effects. The direct antimicrobial effect of itaconate is mediated *via* inhibition of the microbial enzyme isocitrate lyase (236). Itaconate has been shown to inhibit the growth of numerous pathogens including *M. tuberculosis, S. aureus*, *Legionella pneumoniae*, *Acinetobacter baumanii*, and *Salmonella enterica* (235, 237, 238). Itaconate can be transported into the phagosome, where it limits microbial growth (239), implying a role for phagolysosomes as a critical site for itaconate's antimicrobial effects. The synthetic itaconate analog, 4-octyl-itaconate (4OI) exerts anti-inflammatory effects and potently activates the NF-E2-related factor 2 (Nrf2) pathway (240) which regulates the expression of cytoprotective proteins and plays a critical role in redox homeostasis (241). A study by Swain et al. shows that endogenous itaconic acid fails to activate Nrf2 as compared to 4OI, and that synthetic itaconate analogs do not recapitulate the effects of endogenous itaconic acid (242). However, endogenous itaconic acid is anti-inflammatory and reduces IL-1β production similar to 4OI (240, 242). In contrast to these findings, treatment with β-glucan does not induce significant levels of Irg1 and itaconate in human monocytes, and pretreatment with 4OI diminishes β-glucan induced trained phenotype (243). However, it is important to note that β-glucan focused studies relied on measuring cytokine responses alone as a key for demonstrating the lack of effect of itaconate on β-glucan-induced trained immunity although cytokine responses to an inflammatory stimuli may not correlate with actual protective response (76).

Beyond the role of citrate in serving as a precursor for itaconate, our studies using MPLA-stimulated macrophages have shown that citrate transported into the cytosol replenishes mitochondrial oxaloacetate pools and fuels a sustained increase in mitochondrial TCA cycle flux (63). Upon TLR4 stimulation of macrophages, inhibition of SDH activity by itaconate and increased TCA cycle flux also results in succinate accumulation (231, 244). Increased succinate levels and inhibition of SDH activity stabilize HIF-1α and increase mitochondrial ROS generation which lead to an enhanced inflammatory response (231, 244). Mitochondrial ROS aid in microbial clearance (245); however, this phenomenon requires further investigation. Importantly, MPLA-induced increase in TCA cycle flux is associated with enhanced macrophage antimicrobial effects and protection against infections (63). Therefore, metabolic reprogramming plays a critical role in TLR ligand induced trained immunity-mediated protection against infections.

Role of Epigenetic Modifications in Toll-Like Receptor Ligand-Induced Trained Immunity

Exposure to inflammatory stimulus or pathogens also causes epigenetic reprogramming in innate leukocytes reflected by alterations in the histone acetylation and methylation status (246). The major histone modifications including acetylation (H3K27ac) and methylation (H3K4me3) in monocytes exposed to β-glucan persist even 7 days after removal of the initial stimulus and are strongly associated with metabolic reprogramming (225). A study by Saeed et al. showed that LPS and β-glucan induce diverse and opposing alterations in the epigenome with β-glucan showing a greater degree of *de novo* H3K27ac modifications in gene loci encoding for inflammatory responses (247). Alternatively, LPS stimulation of human monocytes acutely induces a strong acetylation of H3K27 around Irg1 gene locus within 1 h of stimulation which is associated with increased expression of Irg1 (243).

Metabolic reprogramming and epigenetic modifications are tightly interconnected. Fumarate accumulates in monocytes stimulated with β-glucan *via* glutamine anaplerosis. Increased fumarate levels have been shown to downregulate the activity of histone demethylase KDM5 (248). In turn, decreased KDM5 activity upregulates trimethylation of H3K4 at promoters of genes encoding pro-inflammatory cytokines (248). α-ketoglutarate stimulates the jumonji domain containing family of the lysine demethylase enzyme JMJD33. Further, a high α-ketoglutarate/succinate ratio favors anti-inflammatory phenotype in macrophages (249).

The post-translational modification of succinylation arising from the addition of succinate to the protein lysine residues supports a pro-inflammatory state in macrophages (231). Succinylation is known to occur on histone lysine residues in human cells (250). However, the role of histone succinylation in the context of trained immunity is currently unknown and remains an important question to be addressed in future studies. A study by Zhang et al. showed that lactate can also bind to histone lysine residues (lactylation) and demonstrated 28 distinct histone lactylation sites (251). This study showed that LPS-induced increase in macrophage lactate levels *via* increased glycolysis sets in motion a lactylation epigenetic program which directs the expression of genes involved in alternative anti-

inflammatory activation state in macrophages (251). The influence of accumulated metabolites during trained immunity is just beginning to be explored and needs further characterization.

The traditional school of thought is that treatment with TLR ligands such as LPS induces a state of immune tolerance, while leukocytes exposed of β-glucan produce a heightened response to secondary stimulation (trained phenotype) classically reflected by cytokine production (252). However, as discussed, treatment with clinically applicable TLR agonists protect against a broad array of infections, implying that TLR ligands also induce robust trained immunity. One potential explanation for this discrepancy in the literature regarding whether TLR ligands induce a state of tolerance or training may be due to the reliance on cytokine production upon exposure to a secondary stimuli which has more recently been shown to not be uniformly indicative of antimicrobial immunity (76). Future studies aimed at detailed characterization of TLR agonist-induced epigenetic reprogramming and defining its link with metabolic reprogramming will be critical in elucidating the mechanisms of TLR agonist-induced trained immunity.

TOLL-LIKE RECEPTOR AGONIST-INDUCED TRAINED IMMUNITY: A CLINICAL PERSPECTIVE

Potential Adverse Consequences of Immunostimulation by Toll-Like Receptor Agonists

It is important to thoroughly consider that activation of the immune system may have deleterious consequences and requires careful study to identify the clinical situations in which TLR-mediated immunomodulation are most appropriate. Sepsis and septic shock yield a proinflammatory response that results in organ injury; however, survivors demonstrate an immunosuppressive phenotype that results in secondary infections and increased mortality (253, 254). Likewise, a potentially harmful outcome of TLR agonist treatment is tolerance to subsequent exposure of endotoxin, particularly in the setting of prolonged LPS exposure or treatment, a phenomena also termed as immunoparalysis (255, 256). Aberrant activation of TLR signaling by PAMPs or DAMPs, mutations of TLR signaling molecules, or failure of self-recognition mechanisms are responsible for development of several diseases such as autoimmune, chronic inflammatory, and allergic diseases (257). In the field of oncology research, adverse effects from TLR immunotherapy have been linked to unintended expansion of adaptive leukocytes, such in B-cell lymphoma, where activation of TLR4 MyD88-dependent signaling may exacerbate the disease (258–260). Other adverse effects of treatment with TLR agonists have been described in cardiovascular medicine research where treatment with oxidized low-density lipoprotein and the BCG vaccine yield a dose-dependent response of proinflammatory cytokines. These mediators damage human coronary smooth muscle cells and increase atherosclerosis which was found to be TLR2- and TLR4-dependent (261).

Therapeutic Potential of Toll-Like Receptor Ligands Beyond Infection Resistance

Although there are some potential adverse effects of TLR immunotherapy which require consideration, there are several patient populations which may benefit from novel TLR agonist strategies. The oncology patient population presents a challenge due to immunosuppression. Most cancer-associated antigens are self-antigens and require immunostimulant adjuvants in addition to cancer-targeting strategies (262). In an H22 liver cancer murine model, administration of curdlan sulfate-matured tumor cell lysate-pulsed DCs was associated with an increase in CD80, MHC-1 and MHC-II expression, CD8+T cell infiltration, upregulated TNF-α and INF-γ transcription, and downregulation of TGF-β transcription in tumor tissues, and improved survival (263). In a separate study, the use of the TLR3 agonist Ampligen, a GMP-grade synthetic poly I:C derivative, was shown to mature human monocytes derived from DCs and sustained bioactive IL-12 production, and generate Th1 specific anti-cancer responses in peripheral blood T-cells obtained from cancer patients (262). In a study by Breckpot et al., the zinc finger protein A20 was downregulated in poly I:C treated DCs which led to sustained production of IL-6, IL-10, and IL-12p70, thus making poly I:C a candidate adjuvant for an anti-cancer immunotherapy (264). Likewise, intratumor administration of the TLR7 agonist 1V270 increased the ratio of M1 to M2 tumor-associated macrophages and was associated with improved survival (265). In addition to anti-cancer immunotherapy, TLR agonists have shown promise in reduction of ischemia reperfusion injury in cardiac myocytes (266, 267). Further, TLR therapy has also been studied in progressive diseases such as Alzheimer's disease, where single or repeated treatment has been shown to reduce evidence of disease progression (268, 269).

Aside from innate immune cells, non-immune cells are capable of long-term memory, including hematopoietic, mesenchymal, and epithelial stem cells (270). There is an increasing body of evidence on how the microbiome influences immunity and how probiotic therapy modulates innate immunity (271). Interestingly, trained immunity may be a mechanism of the beneficial effects of probiotics. Probiotics have been found to augment innate immune function *via* receptor antagonism or expression, binding or expression of adaptor molecules, expression of regulatory signaling molecules, induction of micro-RNAs, and secretion of immunomodulatory proteins, lipids, and metabolites (271).

Trained immunity may even play a role in combating the ongoing COVID-19 pandemic. As one of the sequelae of COVID-19 infections include secondary respiratory infections, the BCG vaccine or β-glucan may be adjunct strategies to reduce morbidity and mortality by enhancing immunity (272, 273). Imiquimod, a TLR7 agonist, has also been proposed as a therapeutic adjunct for COVID-19 and related infections (274). In summary, there is a growing body of evidence focusing on trained immunity as a mechanism to enhance immunity against a broad array of infections which are common in critically ill patients, but also for several other patient populations as discussed above. Such

application requires further investigation to elucidate the full potential of TLR agonist-based immunotherapies (**Table 3**).

Clinical Trials Investigating Toll-Like Receptor Immunomodulators

Ongoing clinical trials investigating the adjuvant properties of TLR agonists constitute approximately double of those studying them as therapeutics (275), demonstrating that the immunomodulatory properties of these compounds are largely being harnessed for vaccine development. The application of trained immunity for vaccine development is highly attractive due to its potential to (1) increase nonspecific effector responses of innate immune cells, and (2) to activate DCs to enhance adaptive T cell responses to specific and nonrelated antigens (276). For example, a novel synthetic small molecule TLR7/8 agonist 3M-052 is now in a phase I clinical trial studying the safety and immunogenicity of the HIV-1 BG505 SOSIP.664 gp140 vaccine candidate (NCT04177355). Other TLR agonists currently in clinical trials as vaccine adjuvant strategies for HIV-1 include TLR3 agonist Poly ICLC (NCT02071095) and TLR9 agonists MGN1703 (NCT02443935) and CpG-7909 (NCT00562939). TLR7 compounds are under investigation as vaccine adjuvant strategies for hepatitis B as well, including Vesatolimod (GS-9620; NCT02166047) and R07020531 (NCT02956850). The TLR9 compound SD-101 is being studied as an adjuvant for chronic hepatitis C (NCT00823862).

On the other hand, the TLR9 agonist Lefitolimod in combination with neutralizing antibodies is in phase II trials studying its effectiveness in conferring reservoir reduction in HIV infection (NCT03837756) after phase I demonstrated its safety and effectiveness in improving both innate and adaptive immunity in HIV-1 infected patients (277). The TLR7 compound Imiquimod has completed phase II trials for its efficacy in treating human papillomavirus (HPV) when applied topically (NCT00941811).

In addition to the above listed clinical trials regarding investigation of TLR agonists as vaccine adjuvant strategies or drugs to fight infection, numerous TLR ligands are being investigated as immunomodulators to treat chronic inflammatory diseases, cancer, and autoimmune disorders, which can be found listed in Anwar et al. (275).

Current Challenges to the Clinical Translation of Toll-Like Receptor Immunomodulators

Although there are several potential clinical applications of TLR immunomodulators as stand-alone therapies which are supported by a growing body of strong preclinical evidence, several knowledge gaps hinder progress of clinical translation. Much remains to be elucidated regarding duration of protection mediated by TLR agonists, and whether protection could be continued by repeated treatment once protection wanes. Further, the most effective but feasible route of administration is essential to identify, with oral and intranasal administrations likely most practical. Dosing and efficacy for various patient populations is also essential to understand, such as whether aged patients or those with comorbidities respond similarly to healthy young patients. In this regard, one limiting factor remains the use of healthy young animals for the vast majority of preclinical studies which does not recapitulate the clinical situation and therefore limits the amount of information that could be gained prior to starting clinical trials.

Another limiting factor is the striking lack of reporting of clinical trial data; therefore, scientists do not have all of the tools that otherwise could refine ongoing and future studies. Finally, as TLR agonists initiate inflammatory cytokine pathways, one glaring concern remains the potential of these compounds to trigger inflammatory or autoimmune disease. Thus, it is critical to continue elucidating TLR signaling mechanisms to identify potential therapeutic targets which may circumvent this concern in addition to conducting proper dosing studies aimed at avoiding induction of inflammation. Rapid scientific progress has been made since the discovery of the phenomenon of trained immunity and its potential therapeutic application. As the field drives forward to fill in these knowledge gaps, the goal of clinical translation of TLRs as immunomodulators holds strong promise to be realized.

TABLE 3 | Clinical application of TLR immunomodulators.

Application of TLR Immunomodulator	Examples	Mechanism	Potential Therapeutic Outcome	Potential Adverse Consequences
Resistance to infection	TLR2 agonist Pam_2Cys, TLR4 agonists MPLA & PHAD, TLR3 agonist poly I:C, TLR9 agonist CpG	Increased leukocyte recruitment and antimicrobial functions	Improved survival; reduced risk of nosocomial infections; reduced reliance on antibiotics	Chronic inflammation; autoimmune disease
Vaccine adjuvant	TLR4 agonist MPLA as an approved adjuvant in malaria (AS01), human papillomavirus (HPV), and hepatitis B (AS04) vaccines	Immune stimulation for increased antibody titers	Improved efficacy of vaccines and reduced dosing strategies	Discomfort at injection site; transient malaise
Cancer immunotherapy	TLR3 agonist poly I:C & derivatives; TLR7 agonist 1V270	T-cell activation and DC maturation	Antitumor immunity	Dose-limiting side effects (fatigue, malaise, fever)
Chronic infections & inflammatory diseases	TLR4 antagonist Eritoran to treat sepsis; TLR9 agonist Lefitolimod for reduction of HIV-1 viral reservoir	Antagonize TLR to prevent activation and downstream inflammation	Reduced inflammation and associated organ injury	Immune tolerance

CONCLUDING REMARKS

Unfortunately highlighted during the ongoing SARS-CoV-2 pandemic, immunocompromised patients are highly susceptible to life-threatening infections. Beyond the current healthcare crisis, populations with insufficient immune responses fail to clear pathogens which results in opportunistic infections that are often difficult to combat due to the increasing prevalence of antibiotic resistance. With limited pharmacological tools, it is critical to develop new strategies aimed at combating infection. Here we have reviewed the potential application of TLR agonists as immunotherapies which trigger trained immunity and confer broad protection to microbes. Importantly, since immunomodulation targets the host response rather than the pathogen, development of microbial resistance is unlikely. TLR agonists have also shown promise as adjuvants for cancer-targeting immunotherapies. Moreover, our lab and others have demonstrated that such agonists may be highly useful as stand-alone therapies to protect against infection through boosting antimicrobial responses of innate leukocytes.

With their instrumental role in stimulating innate immunity and in activation of inflammatory responses, TLRs are tightly controlled by localization to the cell surface or endosomal compartment as well as complex downstream signaling pathways *via* MyD88- or TRIF-dependent cascades. Although much remains to be elucidated, TLR-mediated trained immunity seems driven by metabolic reprogramming and epigenetic modifications. It is critical to further elucidate the cell types, signaling pathways, and intracellular mechanisms responsible for conferring the beneficial protective effects of TLR agonists *via* trained immunity. Doing so will aid the translation of TLR-based immunotherapies to protect patients from potentially life-threatening infections.

AUTHOR CONTRIBUTIONS

The manuscript was conceptualized by AO and JB. AO wrote the sections on TLR signaling pathways, trained immunity triggered by TLR agonists, and drafted the figures. JF wrote the introduction and drafted the tables. NP wrote the sections on metabolic and epigenetic modifications, and AH contributed the section on clinical perspectives. JB supervised the drafting and performed the final editing. All authors contributed to the article and approved the submitted version.

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GLOSSARY

ACE2	Angiotensin-converting-enzyme 2
AGP	Aminoalkyl glucosamine 4-phosphate
AP-1	Activator protein-1
APC	Antigen-presenting cell
BCAP	B-cell adaptor for PI3K
BCG vaccine	Bacillus Calmette-Guerin tuberculosis vaccine
BTK	Bruton's tyrosine kinase
CDC	Centers for Disease Control and Prevention
CpG ODN	Cytosine-phosphate-guanine dinucleotides oligodeoxynucleotides
CLP	Cecal ligation and puncture
DAMP	Damage-associated molecular pattern
DCs	Dendritic cells
dsRNA	Double-stranded RNA
DUBA	Deubiquitinating Enzyme A
EGF	Epidermal growth factor
ER	Endoplasmic reticulum
FDA	U.S. Food and Drug Administration
FimH	Fimbriae H protein
fmOMV	Low endotoxicity outer membrane vesicles
H1N1	Influenza A subtype
H5N1	Influenza A subtype
avian flu	bird flu
H5N2	Influenza A subtype
avian flu	bird flu
HBV	Hepatitis B virus
HBsAg	Hepatitis B surface antigen
HCAI	Healthcare associated infections
HIV	Human immunodeficiency virus
HPV	Human papillomavirus
HSV	Herpes simplex virus
ICU	Intensive care unit
Ig	Immunoglobulin
IKK	IkB kinase
IKKi	IKK inducible gene
IRAK	Interleukin-1 receptor-associated kinases
IRF	Interferon regulatory factor
KHL	Keyhole limpet hemocyanin
LPS	Lipopolysaccharide
LRR	Leucine-rich repeat
MAL	MyD88-adaptor-like
MALP	Macrophage-activating lipopeptide
MAPK	Mitogen-activated protein kinase
MCP	Monocyte chemoattractant protein
miRNA	Micro RNA
MPLA	Monophosphoryl lipid A
mRNA	Messenger RNA
MrpH	MR/P fimbriae protein
MRSA	Methicillin-resistant *S. aureus*
MyD88	Myeloid differentiation 88
NADPH	Nicotinamide adenine dinucleotide phosphate
NEMO	NF-kB essential modifier
NF-κB	Nuclear factor-κb
NK cells	Natural killer cells
NO	Nitric oxide
NOD	Nucleotide oligomerization domain
OMV	Outer membrane vesicles
OVA	Ovalbumin
Pam2Cys	Peptide dipalmitoyl-S-glyceryl cysteine
Pam3Cys	Peptide tripalmitoyl-S-glyceryl cysteine
PAMP	Pathogen-associated molecular pattern
PHAD	Phosphorylated hexa-acyl disaccharides
PI3K	Phosphatidylinositol 3-kinase
Poly I:C	Polyinosinic:polycytidylic acid
PR8	Mouse-adapted H1N1
PRDX	Peroxiredoxin
PRR	Pattern recognition receptor
PVM	Pneumovirus
RAG	Recombination-activating gene
RANTES	Regulated upon activation, normal T cell expressed and secreted
RIP	Receptor-interacting protein
RSV	Respiratory syncytial virus
SDH	Succinate dehydrogenase
ssRNA	Single-stranded RNA
TAK	Transforming growth factor β–activated kinase 1
TANK	TRAF-associated NFκB activator
TBK	TANK-binding kinase
TCA cycle	Tricarboxylic citric acid cycle
T_{eff}	Effector T cell
Th	T helper cell
THP	Tamm-Horsfall protein
TICAM	TIR domain-containing adaptor molecule
TIR	Toll/interleukin receptor
TIRAP	TIR domain-containing adaptor protein
TLR	Toll-like receptor
TMX201	Small molecule 1V270
TNF	Tumor necrosis factor
TRAF	TNF receptor-associated factor
TRAM	TRIF-related adaptor molecule
T_{reg}	Regulatory T cell
TRIF	TIR-domain-containing adapter-inducing interferon-B
UNCB1	Unc-93 homolog B1
UPEC	Uropathogenic *E. coli*

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The BCG Vaccine for COVID-19: First Verdict and Future Directions

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Despite of the rapid development of the vaccines against the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), it will take several months to have enough doses and the proper infrastructure to vaccinate a good proportion of the world population. In this interim, the accessibility to the Bacille Calmette-Guerin (BCG) may mitigate the pandemic impact in some countries and the BCG vaccine offers significant advantages and flexibility in the way clinical vaccines are administered. BCG vaccination is a highly cost-effective intervention against tuberculosis (TB) and many low-and lower-middle-income countries would likely have the infrastructure, and health care personnel sufficiently familiar with the conventional TB vaccine to mount full-scale efforts to administer novel BCG-based vaccine for COVID-19. This suggests the potential for BCG to overcome future barriers to vaccine roll-out in the countries where health systems are fragile and where the effects of this new coronavirus could be catastrophic. Many studies have reported cross-protective effects of the BCG vaccine toward non-tuberculosis related diseases. Mechanistically, this cross-protective effect of the BCG vaccine can be explained, in part, by trained immunity, a recently discovered program of innate immune memory, which is characterized by non-permanent epigenetic reprogramming of macrophages that leads to increased inflammatory cytokine production and consequently potent immune responses. In this review, we summarize recent work highlighting the potential use of BCG for the treatment respiratory infectious diseases and ongoing SARS-CoV-2 clinical trials. In situations where no other specific prophylactic tools are available, the BCG vaccine could be used as a potential adjuvant, to decrease sickness of SARS-CoV-2 infection and/or to mitigate the effects of concurrent respiratory infections.

Keywords: Bacille Calmette-Guerin, SARS-CoV-2, vaccination, trained immunity, cross-protection

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INTRODUCTION

Tuberculosis (TB) is a respiratory disease caused by *Mycobacterium tuberculosis* complex (1), a group of bacteria which primarily attacks the lungs along with other body parts such as the kidneys, spine, and brain. This airborne infectious disease can spread from person to person and it was the leading cause of death in the 1900's in Europe. Following 20 years

of research the TB vaccine was developed by Albert Calmette and Camille Guérin, from whose initials takes its name (BCG, Bacillus Calmette-Guerin). Obtaining the vaccine was not an easy procedure. Calmette and Guérin cultivated *Mycobacterium bovis*, one of the possible tubercle bacilli on a glycerin and potato culture medium and subsequently added ox bile to obtain a more homogeneous suspension, which ended up lowering the virulence of the bacterium. The result was an attenuated strain of the bacteria that could be used as a vaccine. They decided to carry out several animal trials (guinea pigs, rabbits, or horses) until in 1921 they first administered BCG to humans at the Charité Hospital, Paris. From that on, 114.000 infants were orally vaccinated without serious complications until 1928 and the BCG vaccination was proved to be safe (2).

It was soon after its administration that it was observed the beneficial effects of BCG vaccination, with multiple studies reporting non-specific cross-protection of the vaccine against other infectious diseases. Carl Näslund introduced the BCG as a prevention of TB in northern Sweden and informed in 1932 that the vaccine was reducing child mortality unrelated to the TB disease, suggesting that "one could evidently be tempted to find an explanation for this lower mortality among vaccinated children in the idea that BCG provokes a non-specific immunity" (3). However, these protective effects were not studied in detail until nearly 70 years after, when Peter Aaby et al. first reported a 45% mortality reduction due to non-specific beneficial effects of BCG vaccination in West Africa. These pioneer studies described that BCG vaccination was associated with lower incidence of neonatal sepsis and respiratory tract infections that reduced child mortality (4, 5). Further, Peter Aaby et al. reported 60% less acute lower respiratory tract infection (ALRI) in girls below 5 years of age, confirming a non-targeted beneficial effect of BCG vaccination on childhood survival (6). Other examples of BCG vaccination mediated non-specific effects include 70% reduction in pneumonia in Japan (7); 80% decrease in acute upper respiratory tract infection (AURTI), including rhino-pharyngo-laryngo-tracheitis, in the elderly (60–75 years old) in Indonesia (8); and 73% lower rate in upper respiratory tract infections in a phase 2 trial conducted in South Africa (CT02075203) (9). More recently, it has been stablished that BCG vaccination protects against a variety of viral infections such as influenza virus, yellow fever virus, herpes simplex viruses, respiratory syncytial virus, and human papilloma virus (10).

Since January 2020, a pandemic situation has emerged worldwide as a result of a novel severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) outbreak. The virus infects the respiratory tract and replicates in cells expressing the ACE2 receptor that are present in the lung parenchyma pneumocytes, the goblet cells along the nasal mucosa and in absorptive enterocytes in the small intestine (11). Scientists around the world are continuously working to develop specific vaccines to lower the incidence of COVID-19 in the population. As there is an urgent need to mitigate the effects of COVID-19, governmental and non-governmental agencies, including the World Health Organization (WHO), urged to test approved therapies that may be used to reduce the consequences of SARS-CoV-2 infection, such as drugs developed for AIDS, Ebola, or Malaria, excluding BCG vaccination, as there was no evidence that the BCG vaccine protected people against infection (12). As of February 2021, several vaccines have been approved for therapeutic use. These include two messenger RNA-based vaccines (Pfizer-Biotec and Moderna) and one adenovirus-based attenuated vaccine (Oxford-AstraZeneca). The three RNA vaccines have been approved by the American Food and Drug Administration (FDA) and the European Medicines Agency (EMA) (13, 14) and are being currently administered to millions of people. In addition, the Russian vaccine Sputnik has been licensed in Russia, Belarus, Hungary, Servia, United Arab Emirates and Argentina, and more than 50 countries in Latin America Europe, Africa, and Asia have purchased the vaccine, while the Chinese vaccines CoronaVac (15) and Sinopharm have been approved for emergency used in China and in the United Arab Emirates, respectively.

In principle, the arrival of SARS-CoV-2 specific vaccines and initial clinical trial results concluding that BCG does not reduce COVID-19 mortality overrides the need to further evaluate the effects of BCG vaccination on SARS-CoV-2 infection. However, the BCG vaccine may represent a therapeutic approach to (i) increase the efficacy of current and future SARS-CoV-2 specific vaccines that aim at developing immunological memory, while (ii) mitigate the effects of potential concurrent infections. BCG vaccination offers certain level of protection against different respiratory viral, bacterial and fungal infections that may coexist with SARS-CoV-2 (16) and therefore, coadministration of BCG and SARS-CoV-2 vaccines may have synergistic protective effects, including increased efficacy and/or duration of the memory response. In addition, since BCG vaccination might be associated with a decrease in the incidence of sickness during the COVID-19 pandemic (17), it may help to reduce hospitalizations dure to SARS-CoV-2 and other respiratory infections. Here, we review some recent data that addresses the potential use of BCG to diminish the magnitude of COVID-19 and other acute respiratory diseases.

BCG, TRAINED IMMUNITY AND CROSSPROTECTION

The ancient innate immune system has evolved to employ multiple defense mechanisms to eliminate infection. In contrast to the adaptive immunity, which relies on the antigen-specificity, additional innate immune cell populations may exhibit heterologous memory responses triggered upon microbial exposure. Indeed, several studies demonstrated that macrophages and natural killer (NK) cells, which have experienced previous pathogen encounter, can be "trained" via epigenetic remodeling to respond to unrelated pathogens (18, 19). Epigenetic marks including as H3K4me1, H3K4me3, and H3K27ac, mediate epigenetic reprogramming of monocytes which results in the opening of chromatin sites at the promoters of pro-inflammatory cytokines such as IL-6, IL-1β, and TNFα (20) (**Figure 1**). It is plausible that a cross-talk between macrophages and NK cells facilitates the fine-tuning of innate immune program during the

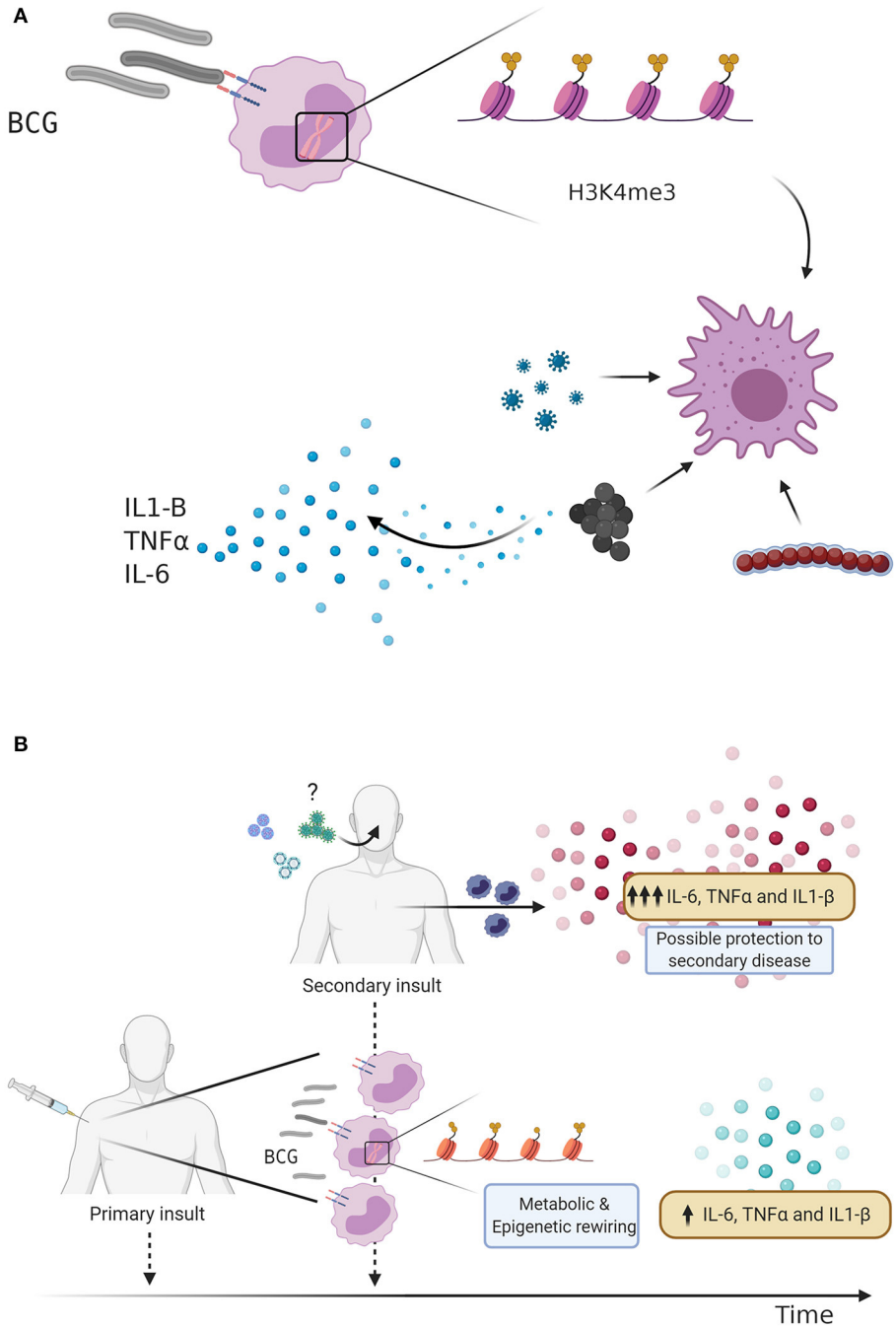

FIGURE 1 | (A) BCG vaccination and Trained immunity. Bacillus Calmette-Guérin (BCG) vaccination induces trained immunity through metabolic changes and epigenetic rewiring. Trimethylation of the H3K4 histone predisposes the innate immune response to a secondary insult, leading to an increased production of pro-inflammatory cytokines. **(B)** Trained immunity as defense mechanism against respiratory infections. BCG vaccination is given as an initial stimulus, leading to metabolic changes and epigenetic rewiring of innate immune cells, increasing the transcription of pro-inflammatory genes and secretion cytokines. BCG vaccinated individuals display an enhanced innate immune response following a secondary challenge, which may lead to protection against subsequent viral infections such as Influenza A virus or Respiratory syncytial virus (RSV). Could BCG vaccination also protect against severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2)?

life-time exposure to microbial insults. In support of this notion, vaccination of healthy volunteers with BCG primes macrophages and NK cells, leading to increased cytokine production after *ex vivo* restimulation (3, 21).

A recent study from Netea et al. demonstrated that the BCG vaccine offers a certain degree of cross-protection against a viral infections through trained immunity related mechanisms. Specifically, the study demonstrated the effects of

BCG vaccination on genome-wide histone modifications induced in trained monocytes, which are associated with reduced levels of yellow fever virus (YFV) viremia due to increased IL-1β production and release (22). This cross-protection effects of BCG against YFV infection confirms the non-specific effects of BCG vaccine described for various viral infections, such as influenza A (H1N1), herpes virus (HSV), respiratory syncytial virus (RSV), and the human papilloma virus (HPV) (10). Since BCG offers protection to TB unrelated viral infections, it was hypothesized that BCG vaccination could also offer protection against SARS-CoV-2 infection in some individuals that have initial defective antiviral responses (23). To explain this, there is another type of immunological mechanism by which BCG could be inducing cross-protection named heterologous immunity. The term heterologous immunity refers to the immunity that can develop to one pathogen after an individual has been exposed to a non-identical pathogen. In this respect, a recent study addressing the homology between SARS-CoV-2 envelope protein and different *Mycobacterium* strains, presents one sequence of 12 amino acids of SARS-CoV-2 envelope protein has high homology to LytR C-terminal domain-containing proteins of *Mycobacterium* sp. (24). BLAST analysis is accompanied by detection of Mycobacteria in formalin and paraffin embedded tissue using immune-based microscopic assays showing a coincidental signal using acid-fast bacillus (AFB) staining and a SARS-CoV-2 envelope-specific antibody. In this manuscript, it is proposed that homology may activate heterologous immunity and a Th1/Th17 response, as previously described between adenovirus vaccine vectors and hepatitis C virus (HCV). Therefore, a combination of trained immunity and heterologous immunity by some BCG epitopes could provide immunity through T-cell cross-reactivity that could be responsible for the beneficial clinical effects of BCG.

BCG STRAINS

In addition to trial design, the heterogeneity of BCG strains may also influence safety and effectiveness of vaccines against respiratory infections. Today, over 14 sub-strains of BCG have evolved and have been used as BCG vaccine in different laboratories around the world (**Figure 2**). It was suggested that the strain variation may contribute to the highly variable protective efficacy of BCG against TB observed in clinical trials. The comparative genomic analysis studies and more recently conducted transcriptional and proteomics profiling experiments have produced a comprehensive map of single nucleotide polymorphisms (SNPs), deletions, insertions, and duplications of genomic regions across 14 BCG strains (25, 26). Intriguingly, BCG strains of the duplication group DU2-IV (BCG Phipps, Frappier, Pasteur, and Tice) exhibited the highest levels of virulence in the mouse infection model, whereas BCG strains of the DU2 group II (BCG Sweden and Birkhaug) were among the least virulent group. The more virulent strains were also more effective in protection against *Mycobacterium tuberculosis* challenge (27). At molecular level, BCG-Japan, -Moreau, and -Glaxo strains are naturally defective in the production of phthiocerol dimycocerosates (PDIMs) and phenolic glycolipids (PGLs), two lipid virulence factors, which could compromise their effectiveness during vaccination (28). To compensate for a potential "over-attenuation," according to the WHO report, a dose of BCG-Japan contains 5-fold more CFU than other distributed BCG vaccines (29). Therefore, selection of the BCG strain, vaccine formulation and route of the administration may ultimately impact the effectiveness of these vaccines against COVID-19 in clinical trials (30).

BCG AND COVID-19

Multiple studies are being carried out around the world in an effort to associate the beneficial effects of BCG vaccination on COVID-19. Initial ecological studies established that in countries with BCG vaccination programs have less COVID-19 cases and deaths per population (31, 32), suggesting that trained immunity inducing vaccines may provide protection to bridge the gap before a COVID-19-specific vaccine is developed (33). On the contrary, other studies concluded that BCG vaccination in childhood does not have a protective effect against COVID-19 in adulthood (34). It is possible that due to the different testing and notification approaches, case and deaths incidences of COVID-19 might not differ in a country with BCG vaccination as it is critical to include different variables of interest such as age structure, income, rurality, and population density. A National Institutes of Health (NIH) study attempted to correct potential confounding variables among countries, such as access to health, education, and stage and size of the COVID-19 epidemic, which observed a strong correlation between BCG vaccination policy and reduction of morbidity and mortality due to COVID-19 in different European countries (10% of the augmentation in the BCG index was associated with a 10.4% mortality reduction from COVID-19) (35). Similar conclusions were reached comparing the same geographical area with similar socioeconomic conditions between Spain and Portugal, with different high mortality rates have been observed in Spain where TB vaccination is no longer part of the official vaccination calendar (36). However, it is important to highlight the importance of including new variants to verify the potential of BCG as a vaccine against COVID-19, such as BCG index (proportion of population of a country vaccinated against BCG), HDI score (number of days since one case per million), population density per Km^2, population >65 years of age, CPI (Corruption Perception Index, government transparency) and percentage of population living in urban areas.

Only ongoing randomized controlled trials (RCTs) will provide answers to whether BCG reduces the incidence and severity of COVID-19 through its cross-protective effects. The phase III randomized clinical trial ACTIVATE (NCT03296423) confirmed that recent vaccination with BCG in elderly (>65 years) protects against new infections. In this trial in which 198 elderly people participated, it was demonstrated the difference between the incidence of new infections after placebo vaccination (42.3%) and BCG vaccination (25.0%), being most of the protection against respiratory tract infections. Furthermore, vaccinated individuals took longer to get infected (16 weeks) than

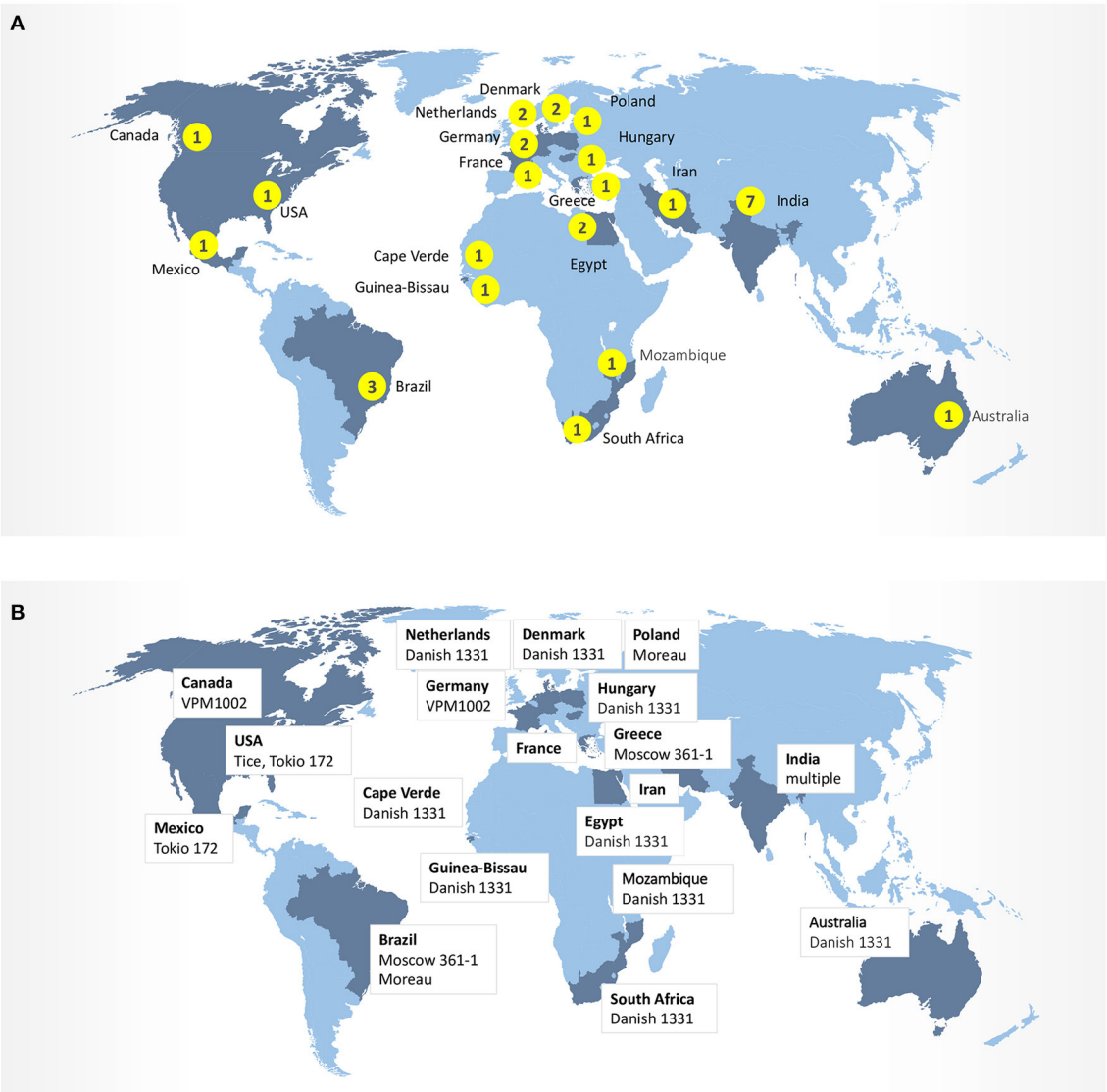

FIGURE 2 | (A) Number of clinical trials BCG-COVID by country. **(B)** BCG strains in ongoing clinical trials.

the ones vaccinated with placebo (11 weeks). Further statistical analysis indicated a 79% decreased on the risk of acquiring at least one new respiratory infection in a 12 months period for BCG vaccinated group. These benefits were suggested to be mediated by pro-inflammatory cytokines, such as IL-10 TNFα and IL1-β, and therefore associated with the induction of trained immunity. Supporting this view, all BCG vaccinated patients showed an increased proinflammatory pattern after a second stimulation of peripheral blood mononuclear cells (PBMCs) with heat-killed *C. albicans* or LPS, although insufficient data was obtained in order to correlate the effect (16). The study concluded that BCG vaccination is safe, as recently reported by the same group (17), and can protect the elderly against infections. The study also suggests that BCG vaccination may be able to protect health workers or vulnerable individuals against SARS-CoV-2 virus infection, although larger and specific studies are needed to assess BCG protection against COVID-19.

Whereas most of the randomized clinical trials are set out to investigate the efficacy in COVID-19 prevention, a BATTLE trial in Brazil (NCT04369794) is designed to test BCG in a therapeutic vaccination setting. Specifically, BATLE trial enrolls patients with confirmed COVID-19 symptoms. This prospective, randomized, double-blind study is testing the potential of BCG to affect (a) clinical evolution of COVID-19, (b) elimination of SARS-CoV-2 at different times and disease phenotypes, and (c) seroconversion rate and titration (anti-SARS-CoV-2 IgA, IgM, and IgG). Such therapeutic vaccination strategy addresses the hypothesis that the induction of both innate and viral-specific immune responses might be beneficial during active SARS-CoV-2 antigenic exposure and provides

further rationale for combining BCG-based vaccines with other approved vaccines. Recent preclinical evidence corroborated this notion: BCG:CoVac, a formulation combining BCG with a stabilized form of the spike (S) protein resulted in the stimulation of SARS-CoV-2-specific antibody and T-cell responses in mice at the levels equivalent to or exceeding responses elicited by current clinical-stage vaccines in the murine models (37). Further demonstrating complex interplay between innate and adaptive immunity, a randomized clinical trial of topical BCG in children having common warts caused by the human papillomavirus, showed 65% complete responses, with no response detected in the control group (38). In conclusion, these preliminary studies suggest that BCG vaccination could enhance vaccine induced immunity against SARS-CoV-2.

NOVEL BCG-BASED APPROACHES

Unlike other vaccination platforms, the robust safety and immunogenicity profile of BCG has rendered it an attractive vector for vaccine development against many viral or bacterial diseases. Antigens expressed by recombinant BCG (rBCG) strains can elicit long-lasting humoral and cellular immunity, including $CD4^+$ and $CD8^+$ T cell responses, to the foreign antigens in animals or humans. Recombinant BCG technology has been studied in the context of vaccination against HIV (39, 40), HCV (41), hMPV (42), RSV (43), rotavirus (44), *Bordetella pertussis* (45), Lyme disease (46), malaria (47), and measles (48). Furthermore, when administered in early life, BCG vaccination can act as an adjuvant enhancing antibody responses to recombinant hepatitis B surface antigen (rHBsAg) both in mice and in human infants (49, 50).

One of the prominent novel approaches is the development of live rBCG engineered to express SARS-CoV-2 antigens to enhance innate and adaptive immune responses and induce sustained antigen presentation. The overall design strategy is based on the hypothesized ability of rBCG-SARS-CoV-2 bacteria to deliver antigens to lymphoid organs, prime a polyfunctional T-cell response and induce long-term systemic and pulmonary protective T-cell immunity. Overall, T-cell responses against the S, N, and M antigens have been reported to be the most dominant and long lasting (51). Accordingly, a priority should be given to the viral antigens or epitopes that are identical to the SARS-CoV-1 and do not include any mutations in the available SARS-CoV-2 sequences, specifically mapped to the S, N, or M structural proteins. Several groups around the world are currently pursuing the construction of such next-generation BCG-based vaccines (52, 53). An earlier-generation genetically modified rBCG-based vaccine, VPM1002 has already entered clinical trials (NCT04387409, NCT04439045) for reducing SARS-CoV-2 infection rate and COVID-19 severity. VPM1002 expresses listeriolysin O (LLO) derived from *Listeria monocytogenes* and deleted Urease C (ureC) gene and has been demonstrated to be safer and more immunogenic in preclinical studies (54).

A different approach by Carlos Martin and colleagues generated a live attenuated *M. tuberculosis* vaccine (MTBVAC) that confers protection against pneumonia through the induction epigenetic and metabolic reprograming of monocytes associated with trained immunity (55). This represents a unique approach that does not use a cow-derived attenuated M. bovis strain but a human-derived *M. tuberculosis* with deletion of phoP and faD25 genes, which is currently under clinical trial.

DISCUSSION

Bacillus Calmette-Guérin (BCG) vaccination has been effective for nearly a century against Tuberculosis (TB), but it has also been described to reduce childhood mortality in a non-specific manner (6, 56, 57). The BCG is a live-attenuated vaccine that represents the most widely used vaccine in the world, with more than 4 billion people vaccinated with BCG worldwide and another 100 million newborn children vaccinated with BCG each year, providing over 50% protection against lung respiratory diseases and over 80% protection against disseminated TB (8). The mechanisms by which BCG induces a cross-protection against other diseases and specifically respiratory tract infections, has been stablished during the last decade and suggests a critical role of the innate immune system (58). The concept of trained immunity brings new insights into the immunological memory concept and described that cells from the innate immune system, such as macrophages, are able to recall a first encounter with a pathogen and produce an enhanced response toward a second assault. As a result, several studies on the cross-protective effects of the BCG vaccines have demonstrated that the positive effects on susceptibility to viral respiratory infections is associated with induction of trained immunity.

Introduction of BCG in developed countries is associated with reduction of mortality that cannot not be explained by a specific disease protection. A recent case cohort study by Aaby et al. reported a better survival rates in Denmark associated to smallpox and tuberculosis vaccination during childhood (59). The study described that individuals who were vaccinated at a young age with both BCG and Vaccinia had a lower mortality rate (adjusted hazard ratio of 0.54) compared to non-vaccinated individuals (adjusted hazard ratio of 1). Interestingly, the study demonstrated the protective role of BCG to lower natural causes of death (adjusted hazard ratio of 0.57) after more than 30 years since BCG vaccination, suggesting that BCG may contribute to lower the mortality long-term. Together with other studies, these conclusions led to further investigations aimed at determining the durability of the BCG vaccine. A similar study suggested that the BCG vaccine remains effective after several decades following vaccination. A retrospective population-based cohort study carried out in Norway revealed that the BCG vaccine effectiveness against pulmonary tuberculosis remains at 40% after 30–40 years (60). Since BCG-derived immunity persist beyond 15 years after vaccination, it is plausible to hypothesize that BCG-induced non-specific protection could also last for decades. Consistent with this view, Mayda and Ishan Gursel hypothesized that countries with continuing BCG immunization programs better contain the spread of SARS-CoV-2 and reported statistical differences between five European countries after 11–22 years since last BCG vaccination and 8 European countries

TABLE 1 | Clinical trials selected from clinicaltrials.gov and additional European studies from the WHO ICTRP.

Trial ID	Source	Acronym	Status	Interventions	Type	Design	Phase	Primary	Enrollment	Age	Locations	Strain
NCT04327206	ClinicalTrials.gov	BRACE	Recruiting	BCG vaccine vs. 0.9%NaCl	Interventional	Randomized	3	Prevention	10,078	≥18 years	Australia	Danish strain 1331
NCT04369794	ClinicalTrials.gov	BATTLE	Recruiting	BCG vaccine vs. placebo	Interventional	Randomized	4	Treatment	1,000	≥18 years	Brazil	N/A
NCT04659941	ClinicalTrials.gov	ProBCG	Recruiting	BCG vaccine	Interventional	Randomized	2	Prevention	1,000	≥18 years	Brazil	N/A
RBR-4kjqtg/U1111-1256–3892	REBEC		Recruiting	BCG vs. placebo	Interventional	Randomized	2	Prevention	800	≥18 years	Brazil	Moscow strain 361–1
NCT04439045	ClinicalTrials.gov	COBRA	Recruiting	VPM1002 vs. placebo	Interventional	Randomized	3	Prevention	3,626	≥18 years	Canada	VPM1002
NCT04641858	ClinicalTrials.gov	EDCTP	Recruiting	BCG-Denmark vs. saline	Interventional	Randomized	4	Prevention	1,050	≥18 years	Cape Verde, Guinea-Bissau, Mozambique	Danish strain 1331
NCT04373291	ClinicalTrials.gov		Recruiting	BCG-Denmark vs. saline	Interventional	Randomized	3	Prevention	1,500	18–100 years	Denmark	Danish strain 1331
NCT04542330	ClinicalTrials.gov		Recruiting	BCG-Denmark vs. saline	Interventional	Randomized	3	Prevention	1,900	65–110 years	Denmark	BCG-1331, AJ Vaccines
NCT04347876	ClinicalTrials.gov		Recruiting	Diagnostic Test: Tuberculin test	Observational	Case-Control			100	12–80 years	Egypt	N/A
NCT04350931	ClinicalTrials.gov		Not yet recruiting	BCG vaccine vs. placebo	Interventional	Randomized	3	Prevention	900	≥18 years	Egypt	Danish strain 1331
NCT04384549	ClinicalTrials.gov	COVID-BCG	Recruiting	BCG vaccine vs. placebo	Interventional	Randomized	3	Prevention	1,120	≥18 years	France	N/A, AJ Vaccine
NCT04387409	ClinicalTrials.gov		Active, not recruiting	VPM1002 vs. placebo	Interventional	Randomized	3	Prevention	59	≥18 years	Germany	VPM1002
NCT04435379	ClinicalTrials.gov		Recruiting	VPM1002 vs. placebo	Interventional	Randomized	3	Prevention	2,038	≥60 years	Germany	VPM1002
NCT04414267	ClinicalTrials.gov	ACTIVATEII	Recruiting	BCG vaccine vs. placebo	Interventional	Randomized	4	Prevention	900	≥50 years	Greece	Moscow strain 361-1
EUCTR2020-001783-28-HU	EU Clinical Trials Register	BACH	Authorized	BCG vs. placebo	Interventional	Randomized	3	Prevention	1,000	≥18 years	Hungary	Danish strain 1331, SSI, Denmark
NCT04475302	ClinicalTrials.gov		Recruiting	BCG vaccine (Freeze-dried)	Interventional	Non-Randomized	3	Prevention	2,175	60–80 years	India	N/A, Serum Institute of India
CTRI/2020/06/025854	CTRI		Not Recruiting	BCG vaccine	Interventional	Non-randomized	N/A	Prevention	1,450	60–95 years	India	BCG-Serum Institute of India
CTRI/2020/04/024833	CTRI		Not Recruiting	BCG vs. placebo	Interventional	Randomized	N/A	Prevention	1,826	18–65 years	India	BCG-Denmark, Green Signal

(Continued)

TABLE 1 | Continued

Trial ID	Source	Acronym	Status	Interventions	Type	Design	Phase	Primary	Enrollment	Age	Locations	Strain
CTRI/2020/05/025013	CTRI		Not Recruiting	BCG plus STANDARD of CARE as suggested by DCGI vs. SALINE plus STANDARD of CARE as suggested by DCGI	Interventional	Non-randomized	2	Treatment	60	20–50 years	India	Tubervac (Serum Institute of India)
CTRI/2020/09/027684	CTRI		Recruiting	BCG vaccine vs. BCG re-vaccination	Interventional	Randomized	N/A	Prevention	400	18–50 years	India	N/A
CTRI/2020/04/024749	CTRI		Not Recruiting	VPM1002 vs. placebo 0.9% saline	Interventional	Randomized	3	Prevention	5,946	18–99 years	India	VPM1002
CTRI/2020/07/026668	CTRI		Not Recruiting	BCG vs. placebo	Interventional	Randomized	3	Prevention	800	18–60 years	India	N/A
IRCT20200411047019N1	IRCT		Recruiting	BCG vs. placebo	Interventional	Randomized	3	Prevention	500	≥18 years	Iran	N/A
NCT04461379	ClinicalTrials.gov		Active, not recruiting	BCG vaccine vs. placebo	Interventional	Randomized	3	Prevention	908	≥18 years	Mexico	Tokio 172
NCT04328441	ClinicalTrials.gov	BCG-CORONA	Active, not recruiting	BCG vaccine vs. placebo	Interventional	Randomized	3	Prevention	1,500	≥18 years	Netherlands	Danish strain 1331
NCT04417335	ClinicalTrials.gov		Active, not recruiting	BCG vaccine vs. placebo	Interventional	Randomized	4	Prevention	2,014	≥60 years	Netherlands	Danish strain 1331
NCT04537663	ClinicalTrials.gov	BCG-PRIME	Recruiting	BCG vaccine vs. placebo	Interventional	Randomized	4	Prevention	5,200	≥60 years	Netherlands	Danish strain 1331
EUCTR2020-002456-21-NL	EU Clinical Trials Register	BCG-PLUS	Not Recruiting	BCG, BCG plus MMR, BCG plus alendronic acid, alendronic acid, placebo	Interventional	Randomized	4	Prevention	100	18–64 years	Netherlands	N/A, AJ VACCINES
NL8547	Netherlands Trial Register	BCG-CORONA-ELDERLY	Recruiting	BCG vs. placebo	Interventional	Randomized	N/A	Prevention	1,600	≥60 years	Netherlands	N/A
NCT04648800	ClinicalTrials.gov		Recruiting	BCG-10 vaccine vs. 0.9% saline	Interventional	Randomized	3	Prevention	1,000	≥25 years	Poland	Moreau
NCT04379336	ClinicalTrials.gov		Recruiting	BCG vaccine vs. placebo	Interventional	Randomized	3	Prevention	500	≥18 years	South Africa	Danish strain 1331
NCT04348370	ClinicalTrials.gov	BADAS	Recruiting	BCG vaccine vs. placebo	Interventional	Randomized	4	Prevention	1,800	18–75 years	United States	Tice BCG
NCT04534803	ClinicalTrials.gov		Not yet recruiting	BCG vaccine vs. placebo	Interventional	Randomized	3	Prevention	2,100	≥70 years	United States	Tokio 172
NCT04632537	ClinicalTrials.gov	NUEVA	Recruiting	Tice BCG vs. saline	Interventional	Randomized	3	Prevention	550	18–64 years	United States	Tice BCG

after 30–45 years. These studies also speculate that BCG re-vaccination represents a valid approach to increase the vaccine effectiveness (31). On the other hand, other studies demonstrated the absence of correlation between a decrease of positive SARS-CoV-2 test results and BCG-vaccinated adults aged 39–41 years vs. non-vaccinated aged 35–37 years, suggesting that the BCG vaccine does not interfere with infection in young adults (61). A recent retrospective observational study carried out in healthcare workers in Los Angeles demonstrated that history of BCG vaccination was associated with an altered seroprevalence and infection with SARS-CoV-2. Specifically, the study indicated that BGC vaccinated healthcare workers were less likely to suffer COVID-19 related symptoms (fatigue, dry cough, and muscle aches), were associated to have a reduced rate of testing positive either with a COVID-19 diagnosis by a medical doctor or a SARS-CoV-2 RT-PCR test and had a significantly lower positive serology against SARS-CoV-2 (IgG) (62). Overall, these finding suggest that BCG vaccination may mitigate sickness associated with COVID-19 infection and ongoing clinical trials are aimed at specifically address this question.

Since the BCG vaccine has proven protection against viral respiratory infections, several laboratories are exploring the possibility that the BCG vaccine could be used alone or synergistically to reduce COVID-19 disease severity. Nowadays, around 20 controlled randomized clinical trials ongoing in the Netherlands, Australia, Germany, Greece, the United States, Egypt, Colombia, Mexico, Brazil, South Africa, Denmark, and France (**Table 1**) to evaluate whether the BCG vaccine decreases the incidence and the severity of COVID-19. The importance of establishing age groups that might be protected against SARS-CoV2 infection by BCG-vaccination is evident, as well as the timing of the vaccine before potential exposure to the virus, as this may determine whether the BCG-vaccine is effective. This represents a critical step to assess the potential of BCG-vaccination to protect the elderly against SARS-CoV-2 and other respiratory infectious agents. However, additional from the above ongoing RCTs are needed to specifically demonstrate the effects of BCG vaccination in the morbidity and mortality of COVID-19 in different scenarios. For example, BCG could be administered in clinics via multiple routes, which potentially include direct mucosal delivery. Whereas current trials with BCG for COVID-19 all rely on the intradermal injections, the oral BCG has been used in Brazil for vaccination against TB until the 1970s. Mimicking the natural mycobacterial infection route by mucosal vaccination with BCG has been known to generate superior protection against TB in animal models. Oral or intranasal BCG vaccination has been shown to induce greater mucosal immune responses and better protection against pulmonary TB compared with subcutaneous vaccination in animals via potent induction of lung parenchyma- and airway-resident memory T cells populations (63, 64). In this respect, immunization with recombinant BCG-N-hRSV protects from hRSV virus associated-lung damage, decreases the infiltration of inflammatory immune cells to the lungs and reduces the virus in the lung tissues when mice were infected with hRSV (65, 66). Based on this evidence, it is possible that mucosal (oral or aerosolized) administration of BCG that engages trained immunity locally may lead to more effective innate immune memory responses in lungs of COVID-19 patients.

We conclude that, in addition to protection from COVID-19 disease, SARS-CoV-2 viral production and death, BCG clinical trials that evaluate (i) alternative routes of administration, (ii) its potential use as adjuvant, and (iii) its potential to prevent concurrent respiratory diseases will be very informative. While some vaccines rely on additional adjuvants in their formulation, live BCG has strong "self-adjuvant" properties that stimulate multiple innate immune sensors or PRRs, including TLR2, TLR4, TLR8, C-type lectin receptors Dectin-1, and Mincle that enhance vaccine induced immunity (67). While the main line of research and therapeutic approaches are focused on the adaptive immune response against SARS-CoV-2, we should not forget the primordial role of the innate immune response against infections. The possibility to stimulate the innate immune system represents a synergistic methodology that should be further explored to shield individuals against SARS-CoV-2 and unknown invading pathogens. Together with BCG, other live attenuated vaccines are currently used to protect against specific infections such as vaccinia (MVA), YFV, measles, mumps, rubella, rotavirus, or attenuated influenza vaccines. It will be therefore interesting to explore the effects of these vaccines that trigger the innate immune response to enhance the basal defenses against coming pathogens, with open questions about their specificity, level of protection, and durability (68).

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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REVIEW
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Optimize Prime/Boost Vaccine Strategies: Trained Immunity as a New Player in the Game

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Most vaccines require multiple doses to induce long-lasting protective immunity in a high frequency of vaccines, and to ensure strong both individual and herd immunity. Repetitive immunogenic stimulations not only increase the intensity and durability of adaptive immunity, but also influence its quality. Several vaccine parameters are known to influence adaptive immune responses, including notably the number of immunizations, the delay between them, and the delivery sequence of different recombinant vaccine vectors. Furthermore, the initial effector innate immune response is key to activate and modulate B and T cell responses. Optimization of homologous and heterologous prime/boost vaccination strategies requires a thorough understanding of how vaccination history affects memory B and T cell characteristics. This requires deeper knowledge of how innate cells respond to multiple vaccine encounters. Here, we review how innate cells, more particularly those of the myeloid lineage, sense and respond differently to a 1st and a 2nd vaccine dose, both in an extrinsic and intrinsic manner. On one hand, the presence of primary specific antibodies and memory T cells, whose critical properties change with time after priming, provides a distinct environment for innate cells at the time of re-vaccination. On the other hand, innate cells themselves can exert enhanced intrinsic antimicrobial functions, long after initial stimulation, which is referred to as trained immunity. We discuss the potential of trained innate cells to be game-changers in prime/boost vaccine strategies. Their increased functionality in antigen uptake, antigen presentation, migration, and as cytokine producers, could indeed improve the restimulation of primary memory B and T cells and their differentiation into potent secondary memory cells in response to the boost. A better understanding of trained immunity mechanisms will be highly valuable for harnessing the full potential of trained innate cells, to optimize immunization strategies.

Keywords: trained immunity, innate immune memory, vaccine, prime/boost vaccine strategies, inflammation, immunization

INTRODUCTION

The goal of vaccination is to elicit long-lasting immune memory, in order to mediate protection from infection, or at least to prevent disease in case of exposure to the pathogen. Multiple immunizations are required for most vaccine strategies, to induce efficient protection. However, there are a few exceptions that elicit life-long protective immunity after a single injection. These vaccines represent the *Grail* for vaccinologists. These include vaccines against yellow fever and smallpox, composed of the yellow fever 17D virus strain (YF17D) and vaccinia virus (VACV), respectively. Even though these are live-attenuated vaccines, what makes them so efficient remains to be completely understood. Mimicking their efficacy is a topic of intense research focus, with the aim to develop new efficient vaccines against other pathogens and diseases.

Repeat vaccinations can be necessary to increase the frequency of responders among vaccinees, and to ensure potent individual and herd immunity. It also enhances and modulates individual immune memory, which is the basis for prime/boost vaccine strategies (see **Boxes 1**, **2**).

Although the mechanisms of differentiation of primary and secondary B and T cells after prime and boost are getting better understood, several outstanding questions remain (6, 7). Less is known about the evolution of innate responses after a primary and secondary vaccine encounter, which has likely been overlooked. Classically, innate immunity provides a first line of defense against invading pathogens and shapes adaptive immunity, which takes more time to develop (8–10). However, innate responses can differ between prime and boost, because (1) specific antibodies (Abs), and memory T cells influence innate cells upon re-exposure, and (2) innate cells themselves can functionally and intrinsically differ. Most textbooks still describe similar innate responses after one or more stimulations, independently of the immunological history, because of the short life span of responding innate cells, and the lack of known immune memory in the innate compartment. However, recent insights have challenged this paradigm (11–13). A better understanding of the principles of memory development, of B and T cells, without excluding innate cells, will certainly be important for optimization of prime/boost strategies and defining which vaccine is best to use first and second in a regime, and how long the delay should be between immunizations.

INNATE RESPONSES IN THE PRESENCE OF SPECIFIC ANTIBODIES AND MEMORY T CELLS

The presence of specific primary Abs and memory T cells at the time of re-vaccination provides a distinct environment to innate cells, which modulates their responses.

Primary Antibodies

Ab concentration and many biophysical and functional features of Abs are determined by the type of vaccine and vaccine strategy used. Features include Ab affinity, isotypes and subclasses, glycosylation profile, and functions like neutralization, and others that depend on Fc-domain interactions with Fc receptors (FcR) (e.g., antibody-dependent cellular cytotoxicity or antibody dependent cellular phagocytosis). These properties also evolve with time and Ag restimulation.

At re-vaccination, innate cells do not sense immunogens of the vaccine as they did at the time of primary vaccination. At first exposure in a naïve host, the vaccine is "free" and detected solely via Pathogen-Associated Molecular Patterns (PAMPs) and Pattern Recognition Receptors (PRRs) expressed by innate cells. Upon re-exposure, vaccine immunogens form immune complexes with primary Abs. They are cleared by FcR-expressing phagocytic cells, they trigger inflammation and it results in the presentation of vaccine-derived epitopes by these innate cells (14, 15).

Consistently, a vaccine-like effect contributing to protection can be observed in the case of Ab-based immunotherapies against infectious diseases. For instance, in a model of retrovirus infection in mice, passive transfer of Abs resulted in long-term protection (16). It required not only Ab neutralizing- but also Fc-functionality, as neutralization alone failed to protect (17). Neutrophils were required. They mediated B cell help and tuned the humoral response (18). Such a vaccine-like effect of Ab infusion has also been observed in non-human primates, where neutralizing Abs induced strong polyfunctional $CD4^+$ T cell response against SIV, mediated by Fc-activated dendritic cells (DCs) (19).

Primary Memory T Cells

Specific memory T cells respond with more strength, are more frequent and react faster, by requiring less activating signals, than their naïve precursor counterparts (20–22). Like Ab responses, T cell responses are modulated by the number of antigen encounters (20), and also evolve over time. Immune memory differentiation is a *continuum*. Primary and secondary memory T cells, as well as early and late memory T cells differ in their frequency, functions (including proliferation, cytokine production and cytotoxicity), and distribution/recirculation. In particular, a subset of memory T cells, called resident memory T cells (TRM), populate barrier tissues (such as the mucosae and skin) and organs. They do not recirculate like other memory T cell subsets, such as central memory and effector memory T cells (23).

TRM are fostered in the tissue where vaccine is delivered, where they act as sentinels. They react more rapidly to secondary vaccine encounter, and participate in the very early local inflammation and modulation of innate cells. The cytokines they produce can catalyze recruitment, or differentially recruit, activate and license innate cells. For example during influenza infection, it was shown that $CD4^+$ memory T cells, can increase the production of innate inflammatory cytokines by antigen-presenting cells (APCs) in the lung upon cell-to-cell contact and cognate antigen (Ag) recognition. This early augmented innate responsiveness likely participates in early control of viral replication (24). Similarly, after immunization with attenuated Listeria monocytogenes, recalled memory T cells rapidly activate innate cells, through an IFN-g/CLL3 dependent mechanism

BOX 1 | First/second vaccine dose and prime/boost.

In the field, one may encounter the term "primary doses," rather than "boosts," particularly when the first vaccine injections are close in time to each other. The very first vaccine dose activates naïve T cells, which undergo proliferation, contraction and a differentiation program to develop into primary memory T cells. As soon as the second vaccine dose is administered, when the primary effector response has started to contract, it can actually be called a boost. It does not always mean that the prime was optimal, and the boost might in fact not only restimulate primary memory T cells, but also prime new naïve T cells, although primary memory T cells have an advantage to respond over naïve T cells.

BOX 2 | Homologous vs. heterologous prime/boost vaccine strategies.

Repeated administrations using the very same vaccine, which are called homologous prime/boost, have proven to be very effective for augmenting humoral responses (1, 2). However, they appeared to be relatively less efficient at enhancing cellular immunity, likely because prior immunity to the vaccine tends to impair robust Ag presentation and the generation of appropriate inflammatory signals for T cells. In contrast, in the 90s, in the context of the development of T cell-based vaccines (e.g., against malaria, *Mycobacterium tuberculosis*, and HIV/AIDS), one strategy to overcome this limitation has been the sequential administration of vaccines using different Ag delivery systems. This approach is called heterologous prime/boost. It has proven to be effective at generating high levels of memory T cells in preclinical studies and clinical trials. However it had never been licensed for humans until very recently with the Gam-COVID-Vac (Sputnik V) aginst COVID-19 (3). It combines recombinant live vectors (such as adenovirus (Ad)- or poxvirus-derived vectors), DNA or RNA vaccines, or adjuvanted subunit vaccines (4).

In addition to the vaccine variables well-known to modulate immunity, such as the nature of the vaccine or adjuvant, its dose and its route of injection for instance, other parameters need to be compared and optimized in the case of prime/boost vaccine strategies (5). They include the number of injections, the delay between them and the combination and order of vaccines for heterologous prime/boost. The exact molecular and cellular mechanisms implicated are not fully understood, preventing a full rationale for optimization of these parameters. Thus, they are defined empirically, and the best parameters out of those tested, neither the absolute nor the individual best parameters, are used.

(25, 26). Furthermore, after cognate or even non-cognate recognition of Ag, TRM trigger an innate alarm, which dampens infection severity by recruiting neutrophils into the lungs (27), or by activating DC and NK cells in mucosae of the female reproductive tract (28).

INNATE CELLS CAN RESPOND INTRINSICALLY BETTER TO STIMULI AFTER BEING TRAINED

In addition to the extrinsic effect provided by specific Abs and recalled memory T cell responses upon Ag re-exposure, innate cells can react differently to restimulation in an intrinsic manner, because of imprinting that might have occurred during a previous inflammatory/infection episode. Innate cells can display memory-like features, brought about by this so-called innate immune training (11).

Concept and Hallmarks of Trained Immunity

Trained immunity features and mechanisms differ from those of B and T cells memory by the involvement of metabolic and epigenetic reprogramming in innate cells. It provides homologous (29, 30) and more strikingly heterologous protection (i.e., against antigenically unrelated pathogens), mediated by trained innate cells that display enhanced innate effector response upon restimulation long after the initial stimulus of training. Trained cells remain present at least 3 months after being induced (31), while the non-specific effects (NSE) of live vaccines on all-cause morbidity and mortality, which is thought to be partly mediated by trained immunity in addition to bystander activation and cross-reactive TCR and Ab, last longer, for several years (32, 33). The mechanisms of trained immunity maintenance, and waning remain to be fully investigated (for open questions on trained immunity see **Box 3**).

BOX 3 | Outstanding questions on trained immunity based prime/boost vaccines.

- Which vaccines and adjuvants are capable of inducing trained immunity?
- Do they stimulate hematopoietic stem or progenitors cells, or a subset, directly or indirectly?
- Are there different mechanisms leading to different flavors of innate memory?
- How long does innate memory take to develop?
- How long does innate memory last?
- Do resting trained cells differ immunophenotypically from their naïve counterparts in addition to their epigenetic marks? Do they represent a distinct subset?
- What are the roles of effector and memory B and T cells, and Abs, in the induction and maintenance of innate memory?
- How to best harness trained immunity to optimize prime/boost vaccine strategies?

Trainable Cells

The first evidence of innate memory in the myeloid compartment was identified in monocytes/macrophages. Trained monocytes and macrophages were described essentially by their ability to more efficiently produce cytokines, especially IL-6 and TNF-a, upon exposure to unrelated stimuli (29, 34–37). Other cells from the myeloid lineage, such as DCs (29, 38, 39) and even neutrophils (40–43), despite their very short life span, were recently reported to display enhanced innate functions

long after the initial stimulation. A burgeoning diversity of neutrophil phenotypes and functionalities are being uncovered, with their capacity to act as APCs a current focus of investigation (44). Innate lymphoid cells (45) and NK cells (46–49) can also "remember" previous infection/inflammation. Ag-specific memory NK cell subsets have been described (50–53). Finally, non-immune cells (such as fibroblast, epithelial stem cells, or interstitial stromal cells) can also be trained, and respond more strongly to tissue stress and damage for instance (54, 55).

Trained Immunity Mechanisms

Trained immunity entails the activation, followed by a long-lasting metabolic rewiring, epigenetic re-programming and changes in gene expression in differentiated myeloid cells, such as monocytes (31), and hematopoietic stem and progenitor cells (HSPCs) from the bone marrow (BM), as demonstrated *in vivo* using Bacillus Calmette-Guérin (BCG), the current live attenuated vaccine made of *Mycobacterium bovis* and used against *Mycobacterium tuberculosis* [both in mice (56) and in humans (57)], and with fungal cell wall component b-glucan (58). The transfer of BM cells from BCG- or b-glucan-trained mice into non-trained animals, led to acquisition of trained immunity features in the transplanted animals. Such an education of the progenitors resulted in a bias toward myelopoiesis and was inherited by the myeloid progeny, because epigenetic modifications of HSPCs were stable and durable throughout differentiation. This explains how innate memory can be long-lasting despite the short life of innate effector cells. Myelopoiesis includes several differentiation and maturation steps, which take time, from HSCs to common, and then more committed, myeloid progenitor cells, through to the terminal differentiation of myeloid cells, i.e., granulocytes, monocytes and DCs. Trained daughter innate myeloid cells remain resting when unchallenged and they display enhanced innate effector functions upon stimulation. Differences in the phenotype of resting trained cells and their naïve counterparts has not been explored thoroughly, with the exception of a few studies that demonstrated differential expression of key surface markers between resting trained vs. naïve innate cells (31, 41) (**Box 3**). In addition, LPS was recently reported to induce long-term cryptic epigenetic changes in *bona fide* hematopoietic stem cells, without modifying their count or gene expression (59). We have previously shown in macaques that the subcutaneous injection of attenuated vaccinia virus, Modified Vaccinia Ankara (MVA), elicited late phenotypic modifications in blood innate myeloid cells resulting in a "defense-ready" phenotype, which was reminiscent of innate training. Monocytes, but also DCs and neutrophils, expressed higher levels of several markers involved in signal transduction (CD45), Ag presentation (HLA-DR), sensing (CD14), binding of immune complexes (CD16, CD32) and complement (CD11b, CD11c), inflammation (IL-10, IP-10, IL-12, IL-8), or migration (CXCR4, CCR5) (41). Admittedly, it remains to be seen whether such phenotypic changes translate into functional innate memory, characterized by an enhanced responsiveness to heterologous stimulation *in vivo*. In any case, our work suggests that MVA imprints different sets of progenitor cells, including downstream of common myeloid progenitors (CMPs)/myeloid-committed granulocyte-monocyte common progenitors (GMPs), because most neutrophils, but only some monocytes and DCs, were modified (**Figure 1**). GMPs are actually heterogeneous, as committed progenitors within GMPs are now being identified and characterized, as well as their downstream precursors (60–63). Thus, depending on the vaccine and the targeted HSPCs, different flavors of trained immunity are likely to be induced (**Box 3**).

The development of trained immunity is associated with major HSPC and monocyte changes related to their glycolysis, tricarboxylic acid (TCA) cycle (also called citric acid cycle or Krebs cycle), glutaminolysis, cholesterol synthesis, and fatty acid synthesis, as shown with BCG and b-glucan. Several metabolites, at the intersection of metabolism and epigenetics, are enriched and play key roles in the development and/or persistence of trained immunity. These include fumarate, which accumulates after glutamine replenishment of the TCA, and mevalonate, a metabolite of the cholesterol biosynthesis pathway (36, 58, 64, 65).

Epigenetic reprogramming of HSPCs and monocytes is mediated by histone modifications and deposition of epigenetic marks, in particular H3K4me3 and H3K27Ac marks, on multiple specific targeted loci, i.e. at the promoters and associated enhancers of immune genes (such as PI3K/AKT and NF-kB pathways, as well as TNF-a and IL-6 promoter regions). Whether other epigenetic marks also participate to the regulation of trained immunity needs to be addressed. In addition to histone modification, a role of DNA methylation in the development of trained immunity has also been reported after BCG immunization (66). Some long non-coding RNAs, called immune priming lncRNAs, also play a key role. They are upregulated by the initial stimulus and they direct epigenetic remodeling enzymes proximal to immune genes, and thus target the deposition of epigenetic marks on specific gene promoters (67).

In addition to HSPCs and circulating myeloid cells, trained immunity can be induced locally, as demonstrated in the instance of alveolar macrophages after intranasal infection with non-replicative human serotype 5 adenovirus (Ad5), independently of monocytes and BM HSPCs (68). The training of these macrophages was dependent on IFN-g, produced by effector $CD8^+$ T cells, and lasted up to 4 months. Increased glycolytic metabolism, modification of transcriptomic profile, and a heightened response to heterologous (bacterial) infection were observed. This work highlights the need to better understand the role of adaptive effector and memory T cells in the induction and maintenance of innate memory (**Box 3**).

TRAINED IMMUNITY-BASED VACCINES

Trained Immunity-Inducing Vaccines

In the last decade, trained immunity has been abundantly reported following BCG vaccination, in humans and mice, and after b-glucan injection in mice (29, 31, 34, 46, 56, 69, 70). Evidenced by epidemiological, pre-clinical and clinical vaccine studies, the occurrence of NSE and/or trained immunity have been witnessed after administration of live-attenuated vaccines

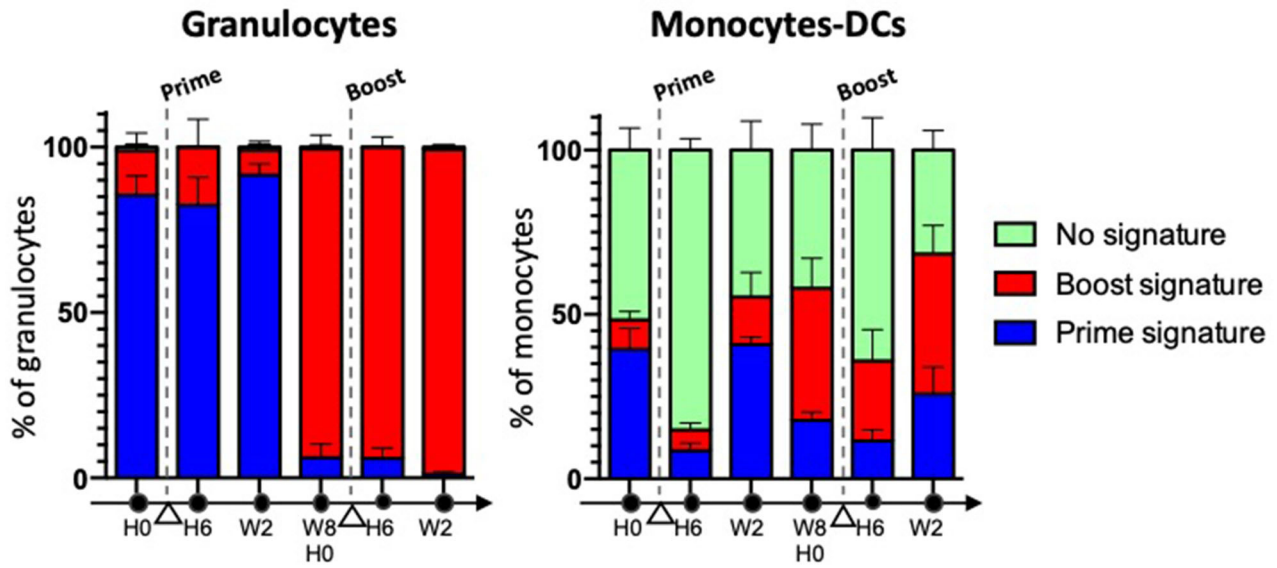

FIGURE 1 | The phenotypic memory of myeloid cells is not restricted to monocytes, and can be more pronounced in granulocytes. Macaques ($n = 5$) were immunized twice, subcutaneously, 2 months apart with a recombinant attenuated vaccinia virus encoding HIV clade B Ags, rec MVA HIV-B (MVA is for Modified Vaccinia Ankara). Blood myeloid cell subsets were analyzed overtime using mass cytometry and a multi-step clustering analysis. They were classified as prime signature (blue), boost signature (red), or non-discriminant "no signature" (green), after a Linear Discriminant Analysis (LDA) performed after Least Absolute Shrinkage and Selection Operator (LASSO). Cell subsets responding to the 2nd immunization differed for the intensity of expression of several markers from those responding to the 1st immunization. They were present prior to vaccine boost, and were induced long after the 1st immunization. They were "better equipped to respond" to restimulation. Most neutrophils were modified, in contrast to some monocytes and DCs (41).

other than BCG, including vaccines against smallpox (vaccinia virus), measles, polio (oral live vaccine, but not the inactivated vaccine), yellow fever, and the new live attenuated *M. tuberculosis* candidate vaccine (*MTB*VAC) (71–76).

What about "non-live" vaccines (such as inactivated or subunit vaccines)? Trivalent influenza vaccination has been reported to elicit imprinting in monocytes and DCs for at least 6 months (77), while gamma-irradiated BCG induced trained monocytes *in vitro,* but failed to do so *in vivo*. In contrast, a long-lasting enhanced anti-inflammatory responsiveness was recently reported after exposure to helminth extracts (78) and a live attenuated anti-pertussis vaccine BPZE1 (79). Finally, diphtheria-tetanus-pertussis (DTP) vaccination, as opposed to BCG, was shown to enhance all-cause morbidity and mortality, more particularly in females (80). Which vaccines/adjuvants can induce trained immunity, and how, is currently one of the hottest topics in the field (**Box 3**). A better understanding of the mechanisms may make it possible in the future, to precisely target the trained immunity metabolic or epigenetic pathways, with pharmacological modulators, to program and tailor immune training, as recently discussed (81). The genetic depletion and pharmacological inhibition of SHIP-1 was shown for instance to improve the b-glucan mediated training of macrophages (82).

Most current licensed vaccines are administered through parenteral routes. They are highly effective for inducing systemic adaptive immune responses, but they are usually poor at eliciting local immunity. In contrast, mucosal vaccines can induce protective specific immunity at the mucosal front line, through which most pathogens enter the body, and to a lower extent systemically (83). Some vaccines, when delivered by mucosal but not parenteral route, have been shown to also induce trained immunity. A recombinant Ad5-based *M. tuberculosis* vaccine expressing the immunodominant *M. tuberculosis* Ag85A, delivered intranasally afforded protection from early stages of pulmonary *M. tuberculosis* infection; it failed to do so when injected intramuscularly. Protection was mediated by trained airway macrophages (both alveolar and interstitial), and independently of the recruitment of blood inflammatory monocytes in lungs (84). Respiratory-mucosal trained immunity-based vaccination may represent a powerful strategy against respiratory infections, such as *M. tuberculosis* and SARS-CoV-2/COVID-19 (85, 86).

BCG, which is injected intradermally in humans, was recently shown, in a prospective double-blind and randomized clinical trial, to protect the elderly from new infections, especially respiratory infections, and increase the responsiveness of their blood cells to unrelated stimuli (87). Several clinical trials to evaluate whether BCG could protect health workers from SARS-CoV2 infection and COVID-19 are ongoing (88). However, a very encouraging retrospective study comparing healthy volunteers vaccinated with BCG in the last 5 years or never before showed that BCG immunization seems to decrease the incidence of sickness (89). In addition, prior BCG vaccination of health workers was associated with a decrease of SARS-CoV2 seroconversion and of incidence of COVID-19 clinical symptoms. In contrast, the history of meningococcal,

pneumococcal, or influenza vaccination did not protect against SARS-CoV-2 infection (90). It is of interest to determine whether BCG delivered to the pulmonary system (by endobronchial instillation) can outperform BCG delivered intradermally, in terms of both systemic and local innate training, as it does in terms of protection against *M. tuberculosis* in non-human primates (91).

The exact stimulus of trained immunity is a matter of great debate. Assuming that vaccine immunogen reaches the BM, HSPCs could be stimulated directly (by detecting vaccine-derived PAMPs), or they could be indirectly stimulated by sensing systemic inflammation signals, including growth factors and cytokines such as GM-CSG, M-CSG, G-CSF, IL-1b, IL-6 (**Box 3**). The route of administration appears to be a key parameter, and not only the nature of the vaccine itself. The vaccine injection site determines vaccine biodistribution, and which are the first immune and non-immune cells sensing the vaccine, and responding to it, and thus the early and transient inflammation. In mice, BCG injected intravenously persisted in BM monocytes (but not in HSPCs) for up to 7 months and trained immunity developed, whereas subcutaneous BCG injection did not lead to the presence of BCG in the BM and failed to elicit training. However, both routes of BCG injection also likely result in different early systemic inflammation. Additionally, early antibiotic treatment showed that the persistence of BCG in BM was actually not required to induce trained immunity, likely at least its initial presence. In non-human primates, BCG injected intravenously was not

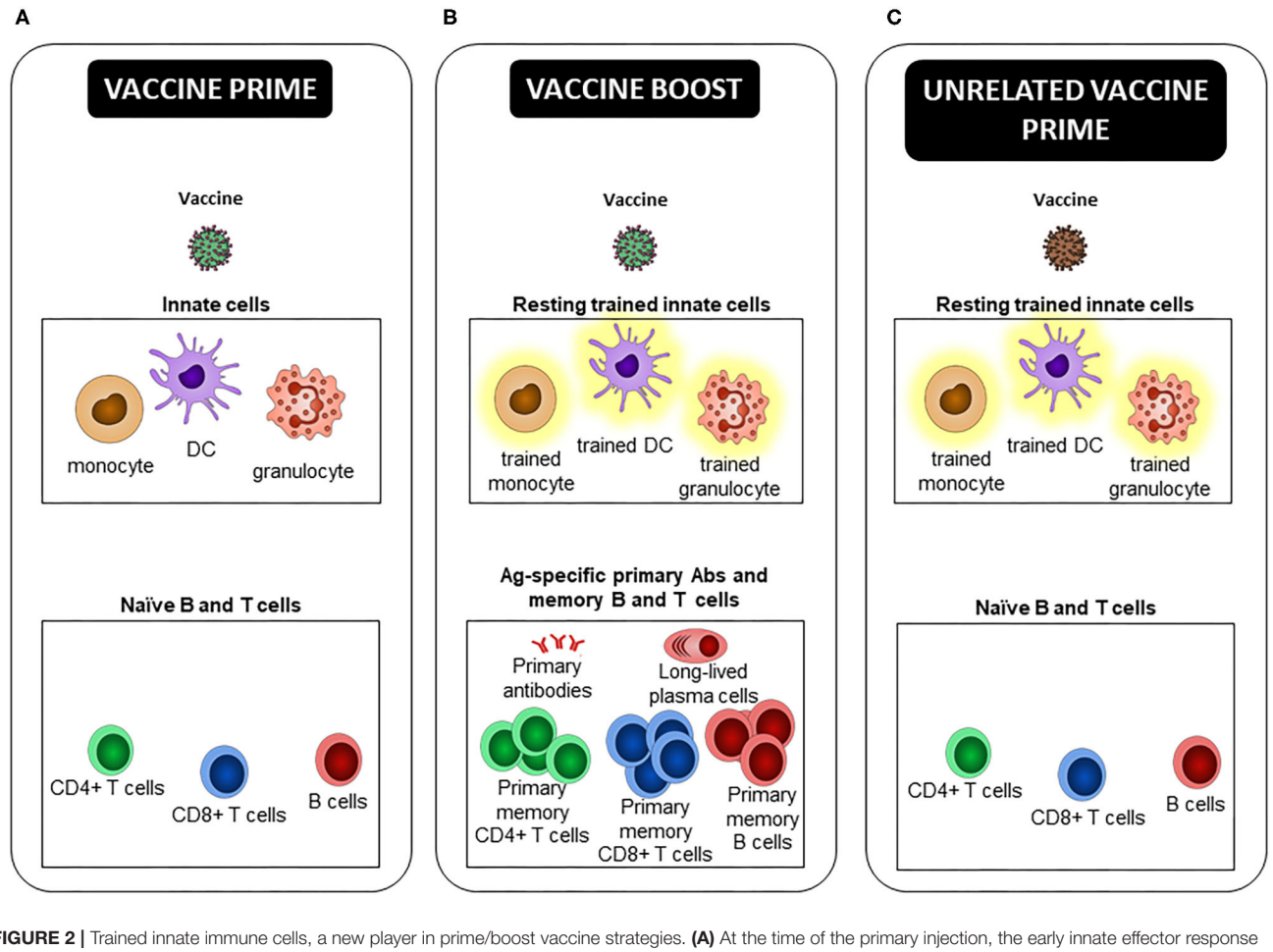

FIGURE 2 | Trained innate immune cells, a new player in prime/boost vaccine strategies. **(A)** At the time of the primary injection, the early innate effector response participates to the activation of Ag specific naive B and T cells, leading to the generation of long-lived plasma cells, primary Abs and memory B and T cells, defined by an Ag-specific heightened effector response upon Ag re-encounter. **(B)** At the time of the vaccine boost, not only "free," but also Ab-bound vaccine and Ag specific primary memory B and T cells, which are triggered by their cognate Ag recognition, activate innate cells. Depending on the type of vaccine, its route of administration, and the delay between immunizations, prime-induced resting trained innate cells can be present and respond better than "naive" ones to restimulation. These extrinsic, and possibly intrinsic, differences can lead to an innate effector response that differs between prime and boost, and differentially shapes the secondary effector and memory adaptive response. If and how primary Abs, and effector and memory B and T cells participate in the induction and maintenance of trained immunity is unclear. **(C)** In the case of a new unrelated vaccination as opposed to a homologous or heterologous boost, only potential trained HSPCs and innate cells induced by the first vaccine, as well as TRM activated after non-cognate Ag stimulation, may modulate the innate effector response and consequently the primary adaptive response to the second vaccine.

found in the BM 1 month later, and, as determined by the production of cytokines in response to heterologous stimulus by PBMCs, there was no evidence of immune training (92). In humans, the intradermal injection of BCG resulted in trained immunity, and no BCG was found in the BM after 90 days (57). In any case, if initial stimulus persists chronically in an anatomical or cellular reservoir, then long-term imprinting of innate cells might not be related to innate memory, but rather reflect a state of chronic stimulation as discussed recently (85). It is also interesting to note that different BCG strains were sub-cultured historically in different laboratories, yielding genetic diversity with differences in virulence, innate activation, immunogenicity and trained immunity-inducing capability (93).

Trained Immunity to Improve Prime/Boost Vaccines

Instead of inducing only classical Ag-specific Ab, B, and T cells, future vaccines could contribute further with induction of both innate and adaptive immune memory as recently highly debated (94–96). Trained immunity-based vaccines could be developed to: (i) increase protection against the targeted infectious agent by relying on both arms of the immune system (innate and adaptive B and T cell responses), (ii) provide heterologous protection against unrelated pathogens, mediated by innate training. More particularly, future vaccines could prevent infection by emerging and old pathogens (such as HIV, RSV, HSV-1/2) for which there remains no potent vaccine. It would benefit more susceptible individuals, such as the newborns and pre-term infants (97), and elderly (87), or patients suffering from immunodeficiency (98). And (iii) pre-condition the innate immune system in order to increase or modulate immune responses after re-vaccination during prime/boost vaccine strategies, or after new unrelated vaccination with a sub-optimal vaccine, or in people less prone to efficiently respond to vaccines, like the elderly (**Figure 2**).

BCG has been shown to provide innate protection against pathogens and diseases not related to *M. tuberculosis*. It can also potentiate and modulate adaptive immune responses to heterologous pathogens and vaccines. For example, concentrations of specific Abs after routine infant immunization were higher in babies whose innate immune system was exposed to BCG at birth (99). Adults, who were immunized with BCG 2 weeks prior to flu vaccine, developed hemagglutination inhibiting Ab responses, faster and to a greater extent (100). Furthermore, prior immunization with BCG was associated with decreased live-attenuated YF17D vaccine viremia. The BCG-induced lower yellow fever vaccine Ag and PAMP doses had no impact on the yellow fever specific neutralizing Ab response though (69). Thus, suggesting an improved priming, or that YF17D vaccine replication is not a key determinant of the magnitude of the humoral response (likely above a certain concentration), as previously proposed (101).

To benefit from trained immunity in the case of re-vaccination or unrelated vaccination, it is yet to be determined which vaccines, recombinant vectors and adjuvants induce trained immunity, and if specific routes of administration are required. Furthermore, the optimal sequence of immunization needs to be defined, given that the long-term NSE induced by different vaccines can augment/inhibit each other, as demonstrated for BCG and tetanus-diphtheria-pertussis inactivated polio vaccine (Tdap) (102). Therefore, vaccine schedules may need to be adapted. The generation of resting trained innate cells, through the reprogrammation of their HSPCs, takes time. We previously demonstrated in MVA-primed/boosted monkeys that intensity and quality of secondary Ab response correlated with the abundance of trained cells in blood at the time of the 2nd vaccine dose. These cells were not present 2 weeks after the 1st vaccine dose, but were enriched 2 months after (103). Thus, delay between immunizations is another likely key parameter.

CONCLUSIONS

Innate memory is changing our view of vaccines and vaccine strategies. It is a challenging and new tool to improve vaccines. It might also contribute to the inter-individual variability of responses to vaccines, depending on the individual inflammation/infection history that needs to be taken into account to personalize vaccines. Innate training might represent the 6th revolution in vaccinology, next to other breakthroughs such as combination vaccines, new adjuvants, systems vaccinology, and vaccines against non-infectious diseases proposed by Stanley Plotkin (104).

AUTHOR CONTRIBUTIONS

J-LP, YF, GD-T, and A-SB wrote the first draft of the manuscript. A-SB oversaw the sections. J-LP and YF designed the figures. CJ, FM, and RL edited the text. All authors discussed the relevant literature and composed the sections of the review article.

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REVIEW
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Innate Immune Memory in Hematopoietic Stem/Progenitor Cells: Myeloid-Biased Differentiation and the Role of Interferon

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Innate immune memory was first described for monocytes and other myeloid cells. This memory is designated *Immune Training*, in which the host animals that had experienced pathogen infection earlier acquire improved resistance to a second infection. Innate immune memory is mediated by an epigenetic mechanism traced to *transcriptional memory* that is conserved throughout evolution and has been selected for the ability to mount an adaptive response to shifting environments. Accumulating evidence shows that not only peripheral myeloid cells but hematopoietic stem/progenitor cells (HSCs/HSPCs) can acquire epigenetic memory upon pathogen exposure. Systemic pathogen infection causes HSCs to exit from quiescence and facilitate myeloid-biased differentiation that leads to efficient host defense. This sequence of events is common in HSC memory generation, which is triggered by different stimuli. Recent studies show that not only pathogens but other stimuli such as metabolic stress can generate memory in HSCs. This review summarizes recent publications relevant to HSC memory. We discuss the current understanding of initial sensors, soluble mediators/cytokines involved in memory formation, including Type I and Type II interferons along with future implications.

Keywords: HSC, myeloid-bias, trained immunity, epigenetic memory, interferon

INTRODUCTION

Epigenetic traits, such as histone modifications and certain gene expression programs are inherited through somatic cell divisions, allowing for the maintenance of phenotypic attributes across cell generations. The inheritance of epigenetic traits is largely attributed to transcriptional memory, an evolutionarily conserved mechanism known from bacteria to plants, and mammals (1). Typically, in transcriptional memory, certain sets of genes that had been expressed earlier in response to external cues, mount a faster and greater transcriptional response when these genes are induced again. Enhanced transcriptional response in turn provides the capacity to adapt to a shifting environment which can improve survival (1–4). Faster and greater response, however, does not represent the entire range of transcriptional memory, as in some cases, a previous induction renders the gene(s) unresponsive to the subsequent stimulus, illustrating a dual feature of memory. Innate immune memory/trained immunity shares these features.

Hematopoietic Stem Cells (HSCs)

Adult hematopoietic stem cells (HSCs) reside in the bone marrow (BM) and hierarchically give rise to all lineages of immune cells, which subsequently migrate into peripheral blood and tissues to perform various physiological functions (5–7). HSCs are heterogeneous with respect to self-renewing and differentiation activity (8, 9). Long-term HSCs (LT-HSCs) are capable of self-renewal and full-range lineage differentiation. Short-term HSCs (ST-HSCs) and multipotent progenitors (MPPs) are generated from LT-HSCs. While they maintain multipotency, these progenitors no longer have the self-renewal capacity. MPPs give rise to downstream progenitors, i.e., common lymphoid progenitors (CLPs), common myeloid progenitors (CMPs), megakaryocyte-erythroid progenitors (MEPs), which generate functional lymphocytes and myeloid cells (10, 11).

When encountered with systemic infection, inflammation, blood loss, or other forms of hematopoietic stress, HSCs exit from a dormant state, undergo proliferation, and then differentiation to facilitate efficient myelopoiesis (12, 13). This process is accompanied by peripheral production of hematopoietic growth factors and cytokines, such as granulocyte-macrophage colony-stimulating factor (GM-CSF), Interleukin-6 (IL-6), IL-1, and Type I and Type II interferons (IFNs), which activates new signaling pathways (14–20). Furthermore, HSCs/HSPCs express pattern recognition receptors (PRRs), such as Toll-like receptors (TLRs), and recognize pathogen components, which could then induce cytokines themselves to facilitate emergency myelopoiesis (21–24).

After acute HSC proliferation and myeloid cell differentiation subside, a new homeostasis is established in HSCs which possess a new chromatin landscape and epigenetic traits. This epigenetic modification is thought to provide a basis of innate immune memory/trained immunity, which typically confers enhanced myelopoiesis and greater pathogen clearance (25). Conversely, in other cases, initial priming causes an unresponsive state, resulting in a reduced response upon secondary stimulus, as typified by bacterial lipopolysaccharides (LPS) from gram-negative bacteria (26–29). In either case, innate immune memory is dependent upon epigenetic mechanisms, and as such differs from the classical immunological memory in B and T lymphocytes, which involves genetic changes in the immunoglobulin, and T cell receptor genes, respectively. Unlike adaptive immune memory, innate immune memory created in peripheral myeloid cells is thought to be short-lived, since these cells are turned over relatively rapidly. However, memory in HSCs/HSPCs, if formed, could persist longer, and produce greater downstream consequences.

PATHOGEN COMPONENT-INDUCED INNATE IMMUNITY

A broad range of microbial infections results in alterations in the BM compartment, involving rapid proliferation and differentiation of HSCs as well as progenitor cells, and the subsequent mobilization to the site of infections (30). *Escherichia coli* infection leads to enhanced granulopoiesis and mobilization of progenitor LK ($Lin^-ckit^+Sca\text{-}1^-$) cells into the peripheral circulation (31). In addition, in the *Pseudomonas aeruginosa* induced sepsis model, the infection causes HSC expansion that permits rapid compensation to cover the loss of mature immune cells (32). Extensive alterations in the HSPCs compartment have also been observed after other forms of systemic infection (33–35). As summarized in **Figure 1**, systemic pathogen exposure can afford improved protection against secondary infection by related or unrelated pathogens (25, 36–40).

Toll-Like Receptor 4 (TLR4) -Induced HSC Activation and Acquisition of Innate Immune Memory

TLRs (10 in humans, and 13 in mice known) detect pathogen-associated molecular patterns (PAMPs) from invading microbes (23, 24). HSCs/HSPCs express a number of TLRs, including TLR1-4, and TLR 6-9 (41), allowing the cells to recognize various forms of PAMPs and to stimulate proliferation and differentiation into myeloid cells. It is reported that HSPCs ($Lin^-IL\text{-}7R\alpha^-ckit^+Sca\text{-}1^+$ (LKS+) cells, LKS^+Flk2^- long-term stem cells (LT-HSCs), $LKS^+Flk\text{-}2^+$ multipotent progenitors (MPPs) are capable of responding to LPS through TLR4 or Pam3CSK 4 *via* TLR2. The downstream adaptor, MyD88 is shown to be required for HSPC activation (21). Another study, on the other hand, reported that LPS induced TLR4 activation depends on TRIF, an alternate adaptor in the TLR signaling cascade (22). Although seemingly inconsistent, these results may not be contradictory, since TLR4 employs both MyD88 and TRIF (23, 24).

Recently, de Laval et al. reported that upon LPS exposure, HSCs undergo expansion and myeloid differentiation and gaining epigenetic memory, which provided an increased protective response to Gram-negative bacteria, *Pseudomonas aeruginosa by* reducing bacterial burden and increasing survival rate (38). Although LT-HSC populations returned to a steady-state (cell number) 4 weeks following LPS priming, the LT-HSCs, conferred protection against p. *aeruginosa* infection when transferred into naïve mice. The LT-HSCs retained the self-renewal and lineage differentiation capacity along with the transcriptome profile of quiescent HSCs, in which LPS induced inflammatory gene expression was transiently seen earlier. LPS induced a number of transcription factors known to promote myelopoiesis, including members of the C/EBP, ATF, and IRF families, which correlated with a sustained change in chromatin accessibility with an increase at PU.1 and RUNX1 motifs. Consistent with this, open chromatin regions correlated with enhancer marks such as H3K3me1 and H3K27ac and are linked to genes involved in myeloid cell development and activity. These observations indicate that LPS induced transcription factors set a new epigenetic mark in chromatin that leads to the establishment of innate immune memory. Accordingly, HSCs without C/EBPβ were unable to alter chromatin

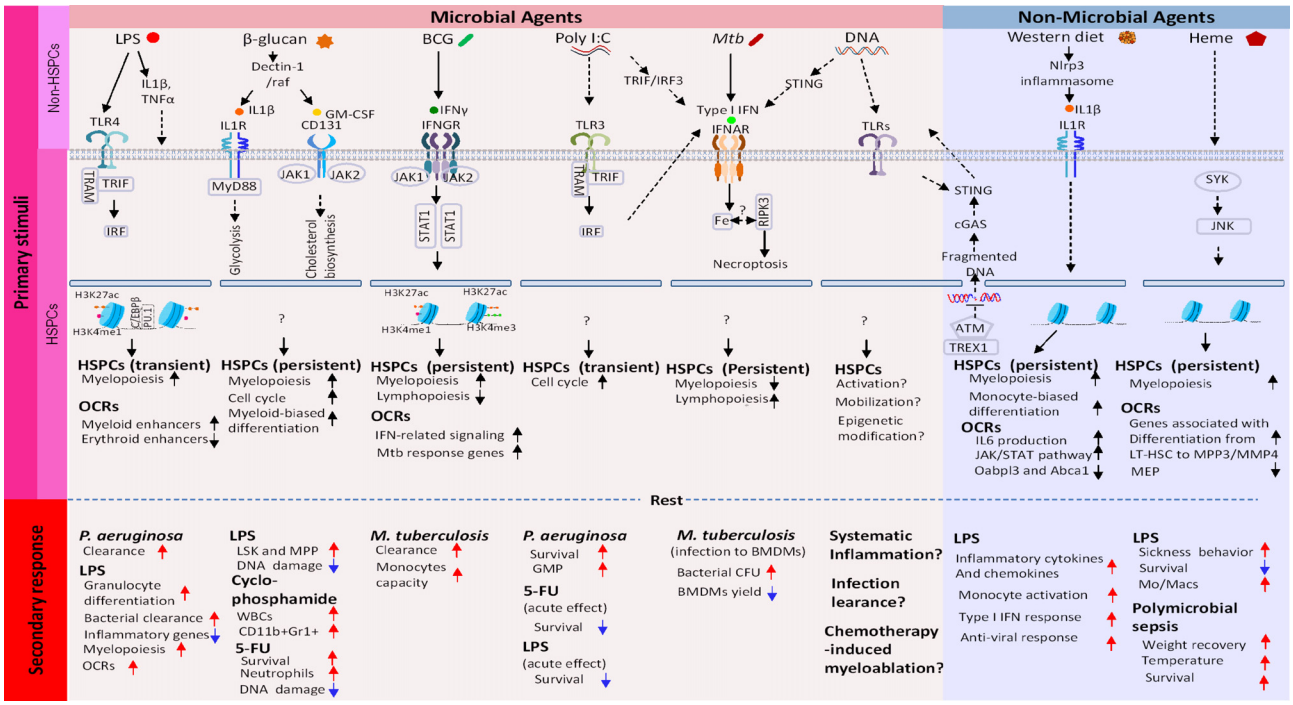

FIGURE 1 | Molecular cascades that create epigenetic memory in HSCs. Top row: Microbial Training Agents and Non-Microbial Training Agents recognized by PRRs and other sensors. Images underneath are subsequent events occurring in descending order. (1): Activation of signaling pathway involving transcription factors and kinases. (2): This then globally alters chromatin accessibility, which leads to building new transcriptome profiles. Open chromatin regions (OCR) can persist longer than transcriptome changes, providing a basis of lasting epigenetic marks. Shown in the bottom two rows are (3): Duration of memory and (4): Phenotypic manifestation of memory. In all cases, HSC memory acquisition involves exit from quiescence, proliferation, and myeloid-biased differentiation of LT-HSC and progenitor cells.

accessibility and failed to provide memory. Other transcription factors expressed in HSCs and regulated by LPS may also modulate these processes (42, 43). Thus, persistent alteration of epigenetic landscape is likely to reflect the state and duration of HSC innate immune memory. In line with this study, another paper reported that LPS priming improved bacterial clearance and survival of mice when challenged with *P. aeruginosa* (37). In addition, increased granulocyte monocyte progenitors (GMP) were also found in an LPS mediated sepsis model (38, 44).

Besides these studies, LPS is known to cause a profound unresponsive state known as LPS tolerance after a single administration (26–28). Thus, LPS tolerance can leave the host more vulnerable to a secondary infection in some cases. Tolerance is the opposite side of innate immune memory/trained immunity, in which many proinflammatory cytokines, including IL-1, TNFα, and IL-6 remain uninduced after a second LPS stimulation as observed *in vivo* and *in vitro* (45, 46).

The Role for TLR3 in HSC Training

Poly (I: C), synthetic ds RNA, used as an RNA virus mimic is a ligand of TLR3 (31–34). de Laval showed that when injected into mice, Poly (I: C), like LPS, led to increased resistance to *P. aeruginosa*, showing that TLR3 signaling activated following RNA virus infection could give rise to trained immunity (38). In addition, Ribes et al. showed that intraperitoneal pre-injection of Poly (I: C) protects mice from the intracranial *E.coli* infection, which is known to cause meningoencephalitis (47). Although this study does not present data for HSCs, it indicates that Poly (I: C) is capable of generating some forms of innate immune memory, as it produced broad effects, including those on NK cell mobility and microglia phagocytic activity. Taken together, given that many RNA viruses are major pathogens that afflict all animals, further investigations are warranted to elucidate TLR3 mediated innate immune training, not only in HSCs but peripheral myeloid cells. In this context, RIG-I and MDA5 that also sense viral dsRNA may also play a role in training (48).

β-glucan Induced Trained Immunity in HSCs

β-glucans are a group of polysaccharides that represent key components of the skeletal cell wall of fungi (such as *Candida albicans*), bacteria, and some plants (such as grain and seaweed) (49, 50). The ability of β-glucans in modulating immune response has been well established. The first evidence for the role of β-glucan in trained immunity was shown in a study of *C. albicans* infection where preinfection with the fungus protected mice from the second, lethal *C. albicans* infection (36). This protection was dependent on monocytes, not lymphocytes,

unraveling a novel training effect in monocyte *in vivo* (36). Cellular response to β-glucans is initiated mostly by the binding to dectin-1, studied extensively in macrophages (50–52). In these studies, the long-term epigenetic reprogramming afforded by *C. albicans* or β-glucans exposure was shown to be mediated by dectin-1 and through the noncanonical Raf-1 pathway (36, 53).

Mitroulis et al. showed that β-glucan, when injected intraperitoneally, induces a dynamic change in the proportion of HSCs and MPPs (39). Gene expression analysis illustrated induction of proliferation and differentiation of LT-HSCs towards myeloid lineage-biased $CD41^+$ LT-HSCs subsets, along with an increase in myeloid-biased MPPs. β-glucan injection also led to an increase in CMP, GMP, and granulocytes ($Gr1^+CD11b^+$). The authors performed adoptive transfer of LT-HSCs from β-glucan injected mice into naïve mice and showed that LT-HCSs from β-glucan injected mice provides sustained myelopoiesis. In addition, β-glucan training afforded a protective response to LPS induced DNA damage in HSCs. Also, β-glucan training led to improved resistance to cytotoxic drugs, 5-fluoracil (5-FU), and cyclophosphamide, resulting in a marked increase in the survival rate (54–56). Importantly, the authors found that IL-1β is produced upon β-glucan injection, and this cytokine is responsible for HSC expansion and myeloid biased progenitor differentiation. IL-1 was also responsible for a metabolic shift towards glycolysis. Verifying these results, pharmacological inhibition of IL-1 by IL1RA (anakinra) abrogated HSC expansion, myelopoiesis, metabolic change and immune training. Presumably relevant to these findings, it is reported that SHIP1 signaling is involved in β-glucan induced myeloid cell training, suggesting the role for the phosphatase in this process (57).

Extending the observations of Mitroulis et al., Moorlag et al. recently demonstrated that β-glucan dependent immune training offers a broad anti-pathogen protection, not only in mice, but in human against virulent *Mycobacterium. tuberculosis* (*M.tb)* (58). Human monocytes pre-exposed to β-glucan *in vitro* followed by *M.tb* infection had lower bacterial load than those without β-glucan preexposure. RNA-seq and ChIP seq analyses showed that some IL-1 family cytokines/chemokines were upregulated in β-glucan trained monocytes, which correlated with increased the H3K27ac mark that indicates enhancers. Preinjection of β-glucan in mice conferred longer survival in mice in response to the secondary *M.tb* infection. As reported by Mitroulis et al, β-glucan increased LT-HSCs and myelopoiesis in an IL-1 dependent manner. Corroborating the critical requirement of IL-1 signaling, IL-1RA treatment increased *M.tb* burden in the lung. These reports provide substantive evidence that β-glucan educates HSCs through IL-1 pathways.

Bacillus Calmette-Guérin (BCG) Induced Trained Immunity in HSCs

BCG vaccine is a live, attenuated strain of *Mycobacterium bovis*, used for protection against *M. tb*. Epidemiological studies on BCG vaccination support its efficacy and the role of innate immunity (59). BCG vaccines are also shown to give cross-protection against different pathogens, even cancers (60). Based on the cross-protective activity of BCG, O'Neill and Netea proposed the possibility that BCG vaccination may be beneficial for boosting host resistance against coronavirus, including Covid-19, pandemic at the time of this writing (61). It is reported that peripheral monocytes acquire trained immunity in volunteers who received BCG vaccine. These monocytes expressed higher levels of proinflammatory cytokines, including IFNγ, TNFα, and IL-6 than those without BCG (62, 63). Also, BCG is reported to provide increased resistance against experimental yellow fever in human monocytes (64), which coincides with a shift towards glycolytic metabolism, important for BCG induced training (65). It is known that HSCs are refractory to direct bacterial infection, including BCG and *Mycobacterium avium*, as HSCs do not take up the bacteria (19, 40, 66, 67).

Kaufmann et al. showed that i.v. injection of BCG in mice causes long term innate immune memory in HSCs, conferring improved resistance to second infection by the virulent *M.tb* (40). The authors found that BCG injection facilitates HSC expansion and development of myeloid lineage dominant multipotent progenitor (MPP3) (19, 40). BM derived macrophages from BCG injected mice gave enhanced protection against *M. tb* compared to those from naïve mice. Moreover, in cell transfer experiments, naive mice given BM HSCs (LKS) from BCG injected mice demonstrated lasting protection against *M.tb*, verifying that memory took place in the HSCs. BCG education of HSCs and enhanced resistance to M. *tb* was dependent on IFNγ (Type II IFN), in which Ifngr-/- mice lacking IFNγ signaling failed to provide anti-*M.tb* protection. Bulk and single-cell (sc) RNA-seq revealed that this memory correlated with changes in the transcriptome programs in HSCs and MPPs. At the epigenome-level, the transcriptome profiles were associated with the appearance of key enhancer elements marked by acetylation of H3K27.

More recently, Khan et al. asked if the virulent *M.tb* strain, H37Rv generates trained immunity and report the results startlingly different from those observed with BCG (67). *M.tb* infection by i.v. injection or aerosol, weakened host's ability to mount resistance against the subsequent *M.tb* infection. The weakened resistance was mediated by Type I IFN signaling, and lasted at least a year. While *M.tb* and BCG both expanded LT-HSCs and MPPs, unlike BCG, *M.tb* suppressed myelopoiesis leading to a dramatic reduction of neutrophils and $Ly6C^{hi}$ monocytes in periphery. RIPK1 dependent necroptosis accounted for the neutrophil deficiency. BM derived macrophages from naïve mice which adoptively received HSCs from *M.tb* infected mice were lower in cell yield and deficient in clearing *M.tb in vitro*. Type I IFN signaling was found critical for the increased susceptibility, as Ifnar1-/- mice (lacking type I IFN receptor), but not Ifngr-/- mice showed better survival after *M.tb* infection than WT mice and displayed reduced phenotypes. The inhibitory role of type I IFNs in *M.tb* infection is partly in line with some of previous clinical/epidemiological studies. Together, *M.tb* trains HSCs somewhat paradoxically to diminish host's own innate resistance.

NON-MICROBIAL AGENTS INDUCED TRAINED IMMUNITY

PRRs recognize not only pathogen-derived molecular patterns, but non-pathogen derived patterns, produced endogenously or exogenously. Some of these can lead to trained immunity with extensive phenotypic changes.

Western Diet-Induced Trained Immunity

Western-style diet (high calorie, high cholesterol), combined with a sedentary lifestyle are prone to cause obesity, type II diabetes, and other health risks. Inflammation in myeloid cells is a major factor for these health problems. Christ et al. reported that Western diet leads to the generation of trained immunity similar to that produced by pathogens described above (68, 69). The authors examined an atherosclerosis model with Ldlr-/- mice, and found that Western diet prompted the expansion of HSPCs and increased myeloid cell outputs as well as the recruitment of myeloid cells to the site of inflammation. In BM, the proportion of HSPCs, MPPs, and GMPs was increased after consumption of the Western diet. Prolonged myeloid prone changes are associated with low-grade inflammation, also seen in aging. In LPS rechallenge experiments, Western diet-fed mice displayed increased monocyte activation and hyper-inflammation, similar to the reported impacts of innate immune training. Analogous effects were previously reported for rabbits fed with cholesterol-rich diets (70). Supporting an epigenetic mechanism, the Western diet altered overall chromatin accessibility with open chromatin regions associated with IL-6 gene expression and the JAK/STAT pathway activity. Moreover, the NLRP3 inflammasome pathway was involved in Western diet-induced trained immunity, in that the diet-induced effects were reversed in *Ldlr*$^{-/-}$*/Nlrp3*$^{-/-}$ double knock-out mice, which exhibited a reduction in systemic inflammation, excessive hematopoiesis, and reprogramming of GMPs. A prolonged Western diet is known to cause cholesterol overloading in HSCs, leading to an increase in the production of growth factors/cytokines, such as GM-CSF and IL-3. Together these studies suggest that prolonged Western diet promotes formation of immune memory in HSCs. However, underlying processes by which Western diet regulates transcription and epigenome programs in HSCs remain to be elucidated.

Heme-Induced Trained Immunity in HSCs

Heme, a key prosthetic group of hemoproteins or enzymes, is composed of protoporphyrin IX and a ferrous ion (71). Free heme can accumulate excessively during sterile and infectious hemolysis, including hemolytic anemias, ischemia-reperfusion, and malaria, once heme scavengers are over-saturated (72, 73). Heme accumulation increases oxidative stress and systemic inflammatory response (72). Somewhat paradoxically, sickle-caused heme accumulation provides protective effects against *Plasmodium* Infection, partially through the NR2 2/heme oxygenase-1 (HO-1) pathway (74). Moreover, heme can induce IL-1β production in LPS primed macrophages through activation of NLRP3 inflammasomes (72, 75).

Jentho et al. reported that heme administration increases myeloid-biased LT-HSCs ($CD41^+$LT-HSC), and myeloid-biased MPPs (MPP3,$Flt3^-CD48^+CD150^-$LSK) with a concomitant decrease in lymphoid-biased MPP4 cells ($Flt3^+CD48^+CD150^-$ LSK) (76). In addition, heme-primed mice were more sensitive to LPS induced acute inflammation, leading to an increase in mortality. Conversely, heme-primed mice showed a protective response to smoldering bacterial sepsis induced by peritoneal contamination and infection. ATAC-seq analysis revealed that heme induces a dramatic change in chromatin accessibility, consistent with myeloid cell-prone development. Heme mediated immune training shared common features with β-glucan driven training, such as upregulated glycolytic metabolism, and enrichment of AP-1 motif in accessible chromatin sites. These findings indicate that labile heme mediates training in LT-HSCs facilitating long-term myelopoiesis with varying outcomes in host defense. It remains to be explored how HSCs sense heme and then reprogram myeloid-biased training *in vivo*.

ROLE OF IFNs IN INNATE IMMUNE MEMORY: IFN ACTION IN LT-HSCs

IL-1 and GM-CSF, cytokines produced by β-glucan priming play a role in HSC immune training (39). IFNs are another class of cytokines that take part in generating innate immune memory in HSCs and peripheral myeloid cells.

Expression and Function of IFNs

There are three types of IFNs, Type I (IFNα/β), Type II (IFNγ), and Type III (IFNλ). Type I and Type II IFNs are shown to be involved in innate immune memory (see below). However, to date, the role of Type III IFN in memory formation has yet to be deciphered. Type I IFNs are encoded by a cluster of related genes (one *Ifnb* gene, many *Ifna* genes), and synthesized mostly in DCs and macrophages in response to PRR signaling, but other non-immune cells such as fibroblasts and epithelial cells also produce Type I IFNs. Type II IFN is encoded by a single gene and synthesized in NK and T cells in response to cytokines such as IL-12 and TCR activation (77, 78). Type I IFNbinds to the surface receptor, IFNAR, and signals through a JAK-STAT pathway, leading to activation of the STA1/STAT2/IRF9 complex. This prompts transcriptional induction of more than 2,000 IFN stimulated genes (ISGs), which collectively confer anti-viral and anti-microbial activity on the host cells (79). Type II IFN binds IFNGR and signals through a similar, but distinct JAK-STAT pathway which activates STAT1 dimers. Type II IFN also induces over 2,000 ISGs, many overlapping with ISGs induced by Type I IFN (79, 80). Together, these IFNs provide innate resistance against all types of pathogens, from viruses (DNA and RNA viruses) to bacteria, fungi, and even parasites, a trait that distinguishes them from other cytokines (81). There is an extensive crosstalk between IFNs and NFκB induced inflammatory responses. For example, IFNβ is activated not only by IRFs but by NFκB, which in turn creates an IFNβ

feedback loop. Thus, ISGs and NFκB-induced factors are often co-expressed during infection and inflammation.

Both Type I and Type II IFNs are involved in regulating HSC activity and play a role in forming innate immune memory (18, 38, 40). It is reported that the injection of IFNα and Poly (I: C), a Type I IFN inducer, prompts LT- HSCs to exit quiescence prompting their proliferation (18). This process is dependent on IFNAR and JAK-STAT1 signaling.

Convincing evidence has been presented for the requirement of IFNγ in BCG mediated HSC immune training: Kaufmann et al. showed that priming mice with BCG trains HSCs to form memory, which provided enhanced protection against *M.tb* and that this training was dependent on IFNGR (40). In addition, IFNγ creates innate immune memory in peripheral myeloid cells (82, 83).

Baldridge et al. showed that injection of recombinant IFNγ activates LT-HSCs, triggering the cell cycle entry, and the subsequent mobilization to the spleen (19, 84). Infection with *Mycobacterium avium* which induces IFNγ also stimulated LT-HSC expansion. A later study by Matatall et al. showed that LCMV infection, also inducing IFNγ, triggered LT-HSC proliferation, and directed myeloid-biased differentiation along with an increased expression of C/EBPβ (85). Furthermore, myeloid-biased HSCs expressed IFNGR at higher levels than lymphoid biased HSCs, thus were more sensitive to IFNγ signaling than lymphoid biased HSCs. The differential IFNGR expression reinforced the selective expansion of myeloid-biased progenitors and their differentiation. Furthermore, IFNγ primed myeloid-biased HSC were preferentially mobilized to periphery upon *Mycobacterium. avium* infection (85). These observations support a significant role of IFNγ for HSC memory and provide a clue to the mechanism of IFNγ action.

IFN Stimulation Creates Classical Transcriptional Memory

In a separate line of approach, our group reported that Type I and Type II IFNs generate transcriptional memory in somatic cells (83). When NIH 3T3 cells, mouse embryonic fibroblasts, and BM macrophages were treated with IFNβ or IFNγ, respectively in advance, the cells mounted a faster and greater ISG response upon the second IFN stimulation, a typical feature of transcriptional memory. Supporting the biological significance of this memory, IFN pretreatment led to improved protection against EMCV viral infection. This memory was inherited through generations, as the memory response was retained after cycles of fibroblast proliferation, another hallmark of transcriptional memory. Transcriptome analysis revealed that memory has a dual quality. While some ISGs exhibited enhanced transcription, other ISGs became unresponsive (or less responsive) to the second IFN stimulation. A similar dual feature has been documented for LPS induced memory, in that LPS pre-administration enhanced expression of some LPS response genes, but repressed other genes (26–28, 38, 45). GO analysis indicated that this duality has a functional meaning, since ISGs showing enhanced expression in the second response were associated with anti-viral, anti-pathogen responses, whereas ISGs with reduced second response were enriched with terms for cell growth, metabolic regulation, etc, unrelated to host defense. The memory response was accounted for by accelerated recruitment of STAT1 and RNA polymerase II to ISGs for ISGs with the enhanced second response. On the other hand, a block in transcriptional elongation was observed for ISGs tolerized in the second response. Epigenome analysis showed that memory coincides with the deposition of the histone H3.3, a conserved histone variant implicated in memory (86). H3.3 and its specific chaperon HIRA, which is responsible for genic H3.3 deposition are expressed highly in murine adult BM HSCs. Our subsequent study with conditional Hira-/- mice demonstrated that HIRA is essential for the generation and maintenance of BM LT-HSCs. In the absence of HIRA, the number of BM LT-HSCs were dramatically reduced, along with the reduction in immediate (MPPs) and downstream progenitors (CMPs, GMPs), leading to a marked paucity in mature, functional myeloid and lymphoid cells. These observations support the possibility that the histone H3.3 and its chaperon HIRA play a substantial role in shaping the development and function of HSCs, and may contribute to their memory formation.

Trained Immunity and DNA Damage in HSCs: Unsolved Questions

Although a limited number of IFN stimulation can generate trained immunity, repeated IFN exposure is shown to exhaust HSC pools by an internally controlled process, not fully understood (84, 87, 88). Transcription factor families including the IRF family appear to play a role (84, 87). HSC attrition is presumably a result of DNA damage that occurs during HSC proliferation and associated replication stress (88). In addition to IFNs, Poly I: C and LPS are shown to cause DNA damage in HSCs even after a single exposure, as evidenced by phosphorylation of H2AX and nuclear foci formation (39, 88). Similarly, chronic exposure to IL-1, a proinflammatory cytokine involved in β-glucan mediated HSC training is shown to exhaust HSC pools (17). Since HSC activation and resultant proliferation leads to DNA damage, it is possible that HSC training is in some way linked to DNA damage. On the other hand, excessive HSC activation/proliferation may have a negative consequence on HSC's self-renewal capacity and lifespan. It remains unclear how HSC exhaustion affects immune training and vice versa.

There is evidence suggesting that DNA damage activates another signaling pathway, STING, and influences innate immune memory in HSCs. STING is a cytoplasmic adaptor for a DNA sensing signaling pathway (89). Canonical STING ligands, cyclic di-GMP/AMP are produced by various pathogens, which activate TBK and IRF3, resulting in Type I IFN and ISG induction (86). The STING pathway is functional in LT-HSC since they are activated and mobilized by a canonical STING ligand (90). It is now evident that not only cyclic di-GMP/AMP, but DNA breaks produced by genotoxic, chemotherapy drugs activate the STING pathway (91). STING is also activated in mice defective in DNA repair (92). DNA damage-induced STING pathway is reported to chronically activate ISGs and NFκB mediated inflammatory cytokines in some cell types (91, 92).

It is noteworthy that chronic ISG expression and STING activation is a hallmark of Aicardi-Goutieres Syndromes (AGS) and related retinal vasculopathy with cerebral leukodystrophy (RVCL), which produce complex inflammatory diseases often involving neurological defects (93, 94). It may be of interest to study how HSC DNA damage and immune training intersect with AGS and related chronic inflammatory disorders.

CONCLUDING REMARKS

Innate immune memory is an emerging concept that opened a radically new perspective on infection and inflammation. Convincing evidence has been presented demonstrating that HSCs form epigenetic memory in response to pathogens and other stress, which confers adaptive responses to the subsequent stress upon the host. HSC memory coincides with the induction of proliferation and myeloid-biased progenitor differentiation, the process driven by IFN, IL-1 and other inflammatory cytokines. Many questions regarding molecular mechanisms, signaling pathways, and epigenome landscapes leading to HSC innate immune memory remain to be elucidated further. In addition, relationships between HSC immune training, DNA damage, and hematopoietic aging are subjects of future investigation.

AUTHOR CONTRIBUTIONS

KO proposed LC to write a minireview on innate immune memory. The two authors discussed the scope of the task, undertook literature surveys, and outlined the structure of the review. Both authors wrote the manuscript and performed editing. All authors contributed to the article and approved the submitted version.

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A Comparison Between Recombinant *Listeria* GAPDH Proteins and GAPDH Encoding mRNA Conjugated to Lipids as Cross-Reactive Vaccines for *Listeria, Mycobacterium*, and *Streptococcus*

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Cross-reactive vaccines recognize common molecular patterns in pathogens and are able to confer broad spectrum protection against different infections. Antigens common to pathogenic bacteria that induce broad immune responses, such as the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) of the genera *Listeria, Mycobacterium*, or *Streptococcus*, whose sequences present more than 95% homology at the N-terminal $GAPDH_{1-22}$ peptide, are putative candidates for universal vaccines. Here, we explore vaccine formulations based on dendritic cells (DC) loaded with two molecular forms of *Listeria monocytogenes* GAPDH (LM-GAPDH), such as mRNA carriers or recombinant proteins, and compare them with the same molecular forms of three other antigens used in experimental vaccines, listeriolysin O of *Listeria monocytogeness*, Ag85A of *Mycobacterium marinum*, and pneumolysin of *Streptococcus pneumoniae*. DC loaded with LM-GAPDH recombinant proteins proved to be the safest and most immunogenic vaccine vectors, followed by mRNA encoding LM-GAPDH conjugated to lipid carriers. In addition, macrophages lacked sufficient safety as vaccines for all LM-GAPDH molecular forms. The ability of DC loaded with LM-GAPDH recombinant proteins to induce non-specific DC activation explains their adjuvant potency and their capacity to trigger strong $CD4^+$ and $CD8^+$ T cell responses explains their high immunogenicity. Moreover, their capacity to confer protection in vaccinated mice against challenges with *L. monocytogenes, M. marinum*, or *S. pneumoniae* validated their efficiency as cross-reactive vaccines. Cross-protection appears to involve the induction of high percentages of $GAPDH_{1-22}$ specific $CD4^+$ and $CD8^+$ T cells stained for intracellular IFN-γ,

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and significant levels of peptide-specific antibodies in vaccinated mice. We concluded that DC vaccines loaded with *L. monocytogenes* GAPDH recombinant proteins are cross-reactive vaccines that seem to be valuable tools in adult vaccination against *Listeria, Mycobacterium*, and *Streptococcus* taxonomic groups.

Keywords: glyceraldehyde-3-phosphate-dehydrogenase, listeriosis, pneumonia, tuberculosis, cross-reactive vaccines, innate immunity

INTRODUCTION

Vaccines for adults is one of the biggest challenges of current vaccinology and several methodologies have been proposed for this purpose such as reverse vaccinology, a genome-based approach to vaccine development (1), or immune algorithm approaches (2–4). One of the main issues regarding vaccines for adults is the possibility to prepare bacterial vaccines that induce cross-protection against infections caused by different pathogens that provide cellular specific immunity, involving both T and B cells, known as cross-reactive vaccines (CRV). However, cross-protection against infections can also be achieved if innate immune cells acquire long functional states such as in trained immunity-based vaccines (TIbV) (5). Dendritic cells (DC) are pivotal cells for conventional, CRV, or TIbV vaccines and serve as efficient vaccine platforms. In this regard, DC based vaccines can recognize non-specific patterns in pathogens and can induce specific immunity (5–7), allowing cross-protection against infections. In fact, the COVID-19 pandemic has highlighted the possibility that vaccines designed for unrelated pathogens such as *Mycobacterium bovis Bacillus Calmette-Guérin* (BCG), could also confer some protection for a coronavirus (8, 9).

Bacterial pathogens such as *Mycobacterium tuberculosis, Listeria monocytogenes*, or *Streptococcus pneumonia* can cause severe meningitis both in the elderly and in adults with immunocompromising conditions, such as cancer patients, in all cases that require long-term antibiotic treatment (10). Opportunistic skin diseases, mild or severe, caused in adults by *Mycobacterium marinum, Mycobacterium chelonae, Mycobacterium fortuitum, Listeria monocytogenes*, or *Streptococcus pyogenes* also require long-term treatment with antibiotics that might contribute to the development of antibiotic resistance (11–13). On the other hand, there are no vaccines available for meningitis or severe skin diseases in the elderly (14). Preparing DC based vaccines that can cross-protect against bacterial genera of *Listeria, Mycobacterium*, or *Streptococcus* might therefore provide relevant tools for adult vaccination.

Poly-bacterial preparations such as MV130 (Bactek®) are composed of heat-inactivated bacteria with 90% gram-positive bacteria (*Streptococcus pneumoniae, Staphylococcus aureus, Staphylococcus epidermidis*) and 10% gram-negative bacteria (*Klebsiella pneumoniae, Haemophilus influenza*, and *Moraxella catarrhalis*) (15). MV130 acts as adjuvant and improves recurrent respiratory tract infections by inducing a specific T cell immunity against bacteria present in the preparation, but also with T cell responses to other different antigens (16, 17). The ability of MV130 to immunomodulate DC, implies the triggering of Toll-like (TLR) and Nod-like receptors (NLR) with the ability to stimulate Th1 and Th17 immune responses and increases the levels of IL-10 (18). Other bacterial adjuvants such as DIO-1, a lipopolysaccharide of *Ochrobactrum intermedium* that acts as a TLR-2/4 agonist, is also able to immunomodulate DC, inducing Th1 immune responses and conferring protection against experimental listeriosis in different vaccine formulations (19–21).

Bacterial ADP-ribosylating enterotoxins such as the heat-labile enterobacterial toxin subunit of *Escherichia coli* (LT), or the cholera toxin (CT) are also used as adjuvants as they promote multifaced antigen-specific responses inducing Th1, Th2, and Th17 patterns. The availability of LT and CT mutants lacking toxicity have allowed these bacterial toxins to be included in vaccine designs, as they retain their adjuvant capacities (22). Other bacterial enzymes with ADP-ribosylating abilities are the glyceraldehyde-3-phosphage dehydrogenases (GAPDH) of gram-positive bacteria, also proposed as universal vaccines against different *Streptococcus* serotypes, since they induce broad spectrum immune responses (23). Our group also described that the GAPDH of *L. monocytogenes* (GAPDH-LM, Lmo 2459), which also presents ADP-ribosylating abilities (24), showed two interesting abilities for vaccine designs—a 22 amino acid peptide at the N-terminal that presented 95–98% sequence homology to GAPDH of *Mycobacterium* and *Streptococcus* and the ability of anti-*Listeria* GAPDH antibodies to recognize *Mycobacterium* or *Streptococcus* spp (25–28).

Messenger RNA (mRNA) is a promising vehicle for vaccination (29), however, naked mRNA suffers a quick degradation by RNases activity and is consequently not internalized efficiently. Several delivery carriers for mRNA vaccines have been developed, mostly based on lipid particulate complexes. Typical examples are the COVID-19 vaccines by Moderna and Pfizer-BioNTech and others such as nanoparticles (30–33). In this regard, cationic lipids commercially available, such as lipofectamine (Invitrogen), can also serve as protective capsules to incorporate nucleic acids into eukaryotic cells. In fact, this is a classical procedure to transfect cDNA or antisense oligonucleotides into cells as well as showing antimicrobial abilities (34–36). In this study, we compare the immune response capacities of mRNA encoding GAPDH encapsulated in lipofectamine (mRNA-GAPDH-LIPO) and GAPDH recombinant proteins with antigens involved in experimental vaccines such as listeriolysin O (LLO) of *L. monocytogenes* (LM), Ag85A antigen of *M. marinum* (MM), or pneumolysin (PLY) of *S. pneumoniae* (SP) (37–42) and

explore their potential as CRV vaccines to confer antigen cross-protection immunity.

MATERIALS AND METHODS

Bacteria, Adjuvants, Cells, Reagents, and Cell Medium

We used *L. monocytogene*s wild type 10403S strain (LMWT) and LLO *L. monocytogenes* deficient mutant (LMΔLLO) derived from the 10403S strain (Prof. D.A. Portnoy, University of California, Berkley, CA, USA). The *Mycobacterium smegmatis* strain was donated by F.J. Sangari and A. Seoane (IBBTEC-University of Cantabria, Santander, Spain) and the *S. pneumoniae* non-pathogenic vaccine strain, 49619-19F, was obtained commercially from ATCC. *Listeria monocytogenes* (LM), *Mycobacterium marinum* (MM), *M. chelonae* (MC), *Mycobacterium avium* (MA), *Mycobacterium tuberculosis* (MTB), *Streptococcus pneumoniae* (SP) (all of them serotype 5), *Streptococcus pyogenes* (SPY), and *Streptococcus agalactiae* (SA) were all clinical isolates of the Microbiology Department at our institution (Hospital Universitario Marqués de Valdecilla, Santander, Spain). DIO-1 is a TLR2/4 targeted molecule that we used as an adjuvant (19–21). Bone-marrow-derived macrophages (DM) or bone-marrow-derived dendritic cells (DCs) were obtained from femurs of 8–12-week-old female mice. DMs or DCs were cultured at 2×10^6 cells/mL in six-well-plates in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 20% fetal calf serum (FCS), 1 mM glutamine, 1 mM non-essential amino acids, 50 μg/mL gentamicin, and 30 μg/mL vancomycin (DMEM complete medium) and 20 ng/mL granulocyte–macrophage colony-stimulating factor (GM-CSF) for DC, was added to the complete medium to obtain differentiated immune cells. On Day 7, the cells were harvested and analyzed by fluorescence-activated cell sorting (FACS) to evaluate cell surface markers and appropriate differentiation of DCs using the following markers: CD11b–fluorescein isothiocyanate (FITC), CD11c–phycoerythrin (PE), IAb–allophycocyanin (APC), F4/80–PE, CD80–FITC, and CD86–V450 (BD Biosciences, Palo Alto, CA). Cells were collected using cell scrapers to detach adherent cells. In certain samples we also used, after detachment, anti-mouse CD11c-coated magnetic beads and MACSTM separation columns (Miltenyi Biotech Inc., Auburn, CA) on day 7 for positive selection, as previously described (34). Lipofectamine was obtained from Invitrogen.

Mice

We used C57BL/6 mice from our animal facilities at the University of Cantabria at 20–24 weeks old, an age that mimics human beings that are 50 years old and older. LD50 of the *L. monocytogenes* strain 10403S in C57BL/6 mice is 2 × 105 CFU/mice (2, 39, 43). LD50 of LM (HUMV-01) was 2-fold higher 4 × 105 CFU/mice. LD50 of *M. marinum* (HUMV-MM01) is 2 × 104 CFU/mice in C57BL/6 mice and LD50 of *S. pneumoniae* (HUMV-SP01) is 5 × 104/mice in C57BL/6 mice. LD50 were evaluated in groups of mice ($n = 10$) i.v infected with 2 × 104 CFU/mice, 5 × 104 CFU/mice or 105 CFU/mice. Mice were examined for death every 12 h and checked for clinical parameters of illness every 24 h.

Bioinformatics Analyses

GAPDH of *L. monocytogenes* (GAPDH-LM) similarity searches were done online using FASTA (available at http://www.ebi.ac.uk/fasta33/) and BLAST (available at http://www.ebi.ac.uk/blast2/ and (http://www.ncbi.nlm.nih.gov/sutils/genom_table.cgi). The analysis of protein domains was based on the Pfam database (available at: https://www.ebi.ac.uk/interpro/) (44). Theoretical 3D predictive models for *L. monocytogenes* GAPDH (GAPDH-LM), *M. tuberculosis* GAPDH (GAPDH-MTB), and *S. pyogenes* GAPDH (GAPDH-SP) were obtained using the Automated Comparative Protein Modeling Server SWISSMODEL (available at https://swissmodel.expasy.org/). Multiple alignment and phylogenetic trees of GAPDH from *L. monocytogenes*, *M. tuberculosis*, *M. marinum*, *M. chelonae*, *S. agalactiae*, *S. pneumoniae*, and *S. pyogenes* were carried out using Clustal Omega, a multiple sequence alignment program that uses seeded guide trees and HMM profile-profile techniques (available at https://www.ebi.ac.uk/Tools/msa/clustalo/). The aligned regions correspond to the InterPro domain IPR020828 that all the proteins have at the beginning of their sequence. The InterPro domain IRP020828 corresponds to the glyceraldehyde 3-phosphate dehydrogenase, NAD(P) binding domain: https//www.ebi.ac.uk/interpro/entry/InterPro/IPR020828/. The consensus symbols of the alignments were taken from https://www.ebi.ac.uk/seqdb/confluence/display/JDSAT/Clustal+Omega+FAQ#ClustalOmegaFAQ-Whatdotheconsensussymbolsmeaninthealignment? Their meaning is the following: an * (asterisk) indicates positions which have a single, fully conserved residue; a: (colon) indicates conservation between groups of strongly similar properties as below—roughly equivalent to scoring >0.5 in the Gonnet PAM 250 matrix: (STA, NEQK, NHQK, NDEQ, QHRK, MILV, MILF, HY, FYW); a. (period) indicates conservation between groups of weakly similar properties as below—roughly equivalent to scoring = < 0.5 and >0 in the Gonnet PAM 250 matrix (CSA, ATV, SAG, STNK, STPA, SGND, SNDEQK, NDEQHK, NEQHRK, FVLIM, HFY). Note that TV is included in the weaker scoring groups despite scoring 0–0 in the PAM 250 matrix, this is because it is a fairly common substitution as they are both beta-branched in fully buried residues, at the cost of a hydrogen bond. In fact, this substitution has been used in the past to make TS mutants (Information courtesy of Toby Gibson).

cDNA Plasmids, *in vitro* Transcription and Recombinant Proteins

cDNA plasmid clones of antigens from *L. monocytogenes* serovar 1/2 (listeriolysin O, LLO, and glyceraldehyde-3-phosphate-dehydrogenase, GAPDH), Ag85A antigen of *M. marinum*, or pneumolysin from *S. pneumoniae* were obtained from Bioclone Inc. Plasmids were first linearized to prepare mRNA by *in vitro* transcription (Qiagen *in vitro* transcription kit) and mRNA transcripts purified with spin columns that contain a silica-based membrane. Purity and concentrations were measured by Nanodrop and further quantification of purity and the size

of transcripts was verified by electrophoresis. *Escherichia coli* strain BL21 bearing plasmids to express large quantities of His-fusion recombinant full-length proteins of LLO (LM-LLOrec or LLOrec) and GAPDH of *L. monocytogenes* (LM-GAPDHrec or GAPDHrec), pneumolysin O (PLYrec) of *S. pneumoniae*, and Ag85A of *M. marinum* (Ag85Arec) were obtained from Bioclone Inc. The expression of large quantities His-fusion proteins was induced with 1 mM IPTG for 5 h at 37°C. His-tagged recombinant proteins were purified with TALON resin, according to the manufacturer's instructions (Clontech). Purification of recombinant proteins was evaluated after SDS-PAGE gels loading 3 μg of protein per lane and Coomasie staining (**Figure 2A**, labeled as His-protein expression in *E. coli*) as previously reported by our group (39). Verification of protein purification was evaluated after cutting the bands from gels, TCA precipitation, and proteomic identification at the Centro Nacional of Biotechnology (Madrid). Protein purification was passed through the ToxinEraserTM kit (Genescript, catalog number L0038) to eliminate traces of endotoxin recombinant purified proteins and traces of endotoxin verified with the Genescript ToxiSensorTM chromogenic Limulus Amebocyte lysate kit (catalog number L0035C). The endotoxin elimination kit consists of columns composed by an affinity matrix of modified polymyxin B. Endotoxin levels in protein purifications were lower than 0.1 EU/mL, according to the manufacturer. All reagents to be incubated with DC were tested for endotoxin traces and confirmed to have <0.1 EU/mL of endotoxin.

Preparation of mRNA Encoding Antigens Conjugated to Lipid Carriers (mRNA-Antigen-LIPO)

We prepared the lipid carriers using lipofectamine 2000 (Invitrogen) (5 μl) which was added to mRNA encoding antigens (GAPDH, LLO, PLY and Ag85A) prepared in the previous section before (100 pmol), in a total volume of 100 μL of Opti-MEM. mRNA encoding antigens and lipofectamine mixtures (mRNA-antigens-LIPO) were incubated for 1 h at RT to allow conjugation to mRNA, followed by 5 min of incubation in a water-bath sonicator to allow for the forming of liposome-like carriers. DC prepared in 6-well-plates (1×10^6/well) were incubated with mRNA encoding antigens-LIPO mixtures in Opti-MEM medium without serum for 4 h. Supernatants were removed and cells were incubated for 12 h in DMEM-1% FCS. Efficiencies of mRNA uptake by DC are shown in **Figure 2A** (DC lysates Coomasie gel of immunoprecipates). Briefly, DC were loaded with 50 μg/mL of mRNA encoded PLY, Ag85A, LLO, or GAPDH conjugated to the lipid carrier, lipofectamine for 16 h. Next, DC were lysed and immunoprecipitated with rabbit anti-Mycobacterium antibody (Colorado University), rabbit anti-PLY (a gift of JR de los Toyos, Oviedo, Spain), and rabbit anti-Listeria monocytogenes GAPDH1-22 antibody (performed by C. Alvarez-Dominguez and M. Fresno at CBMSO facilities using GAPDH1-22 peptide and incomplete Freund's adjuvant) as previously reported (24). Immunoprecipates were stained with Coomasie blue.

Preparation of Murine DC Vaccines and Assays for DC Activation

Bone-marrow derived DC cells obtained from mice femurs were differentiated with GM-CSF (20 ng/mL) for 7 days. Differentiated DC presented a phenotype of 98% $CD11c^+MHC^-II^+CD11b^{-/+}CD40^-CD86^-$ cells. These DC were used *in vivo* for T cell responses or vaccination protocols. For DC activation assays, differentiated DC were treated with different reagents for 16 h: 5 μg/mL of recombinant proteins LM-GAPDHrec or LM-LLOrec or 50 μg/mL of mRNA-LIPO complexes: mRNA-LLO-LIPO and mRNA-GAPDH-LIPO. Two adjuvants were also included as reference controls: LPS (10 ng/mL) and the Th1 adjuvant DIO-1 (10 ng/mL). Cell surface markers of DC activation were explored by flow cytometry. Activated DC presented a phenotype of 90% $CD11c^+IAb^+CD40^+CD86^+$ positive cells. Activation was also measured in DC supernatants after filtration and storage at −80°C to measure cytokine production using a multiparametric CBA kit of BD Biosciences (see Cytokine Measurement section).

Cell Toxicity and Apoptosis Assays on Macrophages and DC Vaccines

Bone-marrow derived macrophages (BM-DM) were obtained, as described above, from mice femurs and differentiated with M-CSF (20 ng/mL) for 7 days. BM-DM and activated DC were treated, or not, with the different recombinant proteins or mRNA encoded antigens conjugated to lipid carriers (50 μg/mL) for 16 h in culture medium, washed, and analyzed for cell toxicity or apoptosis. Cell toxicity was examined with Trypan-blue staining by light microscopy as well as by hemolysis of sheep red blood cells. Apoptosis was examined by flow cytometry using two reported products, annexin-V conjugated to allophycocyanin (APC) fluorochrome and 7-AAD (7-aminoactinomycin D) (BD Biosciences, San Jose, CA, USA). Staining of cells with 7-ADD corresponded to necrotic cell death, whereas staining of cells with annexin-V alone corresponded to apoptotic programmed cell death (mean ± SD). Results are expressed as the % of cell toxicity or as the percentages of apoptotic cells ± SD of triplicate samples, respectively ($P < 0.05$).

Virulence of Bacterial Clinical Isolates *in vitro* and *in vivo*

DC vaccines prepared in mice (1×10^6 cells/mL) were infected at a MOI of 10:1 (bacteria: cells) to evaluate the *in vitro* replication of invasive clinical isolates of LM (HUMV-LM01), MM (HUMV-MM01), and SP (HUMV-SP01) which were calculated as replication indexes (RI) as previously reported (2, 27, 39). RI are calculated by the CFU at 16 h post-infection, divided into CFU at 1 h post-infection. This parameter is considered an indicator of bacterial growth in DC and is comparable to *in vivo* virulence in spleens 72 h post-infection, as we have previously reported for listeriosis (27). We included the following bacteria as controls: LM 10403S strain (LMWT) as the LM basal control, LLO deficient strain, LMΔLLO as non-pathogenic LM, Mycobacterium smegmatis as non-pathogenic mycobacteria control and the vaccine strain 49619-19F of *S. pneumoniae* as

the non-pathogenic SP control (**Figure 1C**). Similarly, to evaluate virulence *in vivo*, we inoculated intravenously 104 CFU of each clinical isolate to be tested. 104 CFU/mice corresponded to a bacterial dose lower than LD50 (see section Mice for LD50 calculations). Spleen homogenates were plated in agar plates to count CFU and results are expressed as CFU/mL. Bacterial controls were the same as those used for *in vitro* virulence assays.

Delayed Type Hypersensitivity (DTH) Reactions Elicited by DC-Vaccines

C57BL/6 mice were immunized *i.p* with LM (HUMV-LM01), MM (HUMV-MM01), or SP (HUMV-SP01) (5×10^3 CFU). Seven days later, mice were inoculated in the left hind footpads using DC vaccines (10^6 cells/mice) pre-loaded with the following reagents: the recombinant proteins of *L. monocytogenes* LLO_{rec}, and $GAPDH_{rec}$, *M. marinum* $Ag85A_{rec}$ or *S. pneumoniae* PLY_{rec}, or the mRNA-Ag-LIPO complexes: mRNA-LLO-LIPO, mRNA-GAPDH-LIPO, mRNA-Ag85A-LIPO, or mRNA-PLY-LIPO. DC vaccines were formulated in the presence of DIO-1 (2 μg/mL) (2). The negative controls were the right hind footpads, since they were not inoculated. After 48 h, we measured the footpad thickness with a caliper; results are expressed in millimeters as the mean of three different experiments. To explore T cell responses in detail, we collected and homogenized the popliteal lymph nodes of mice analyzed for DTH reactions and cell homogenates were passed through cell strainers to analyze $CD4^+$ and $CD8^+$ T cells by flow cytometry. Results are expressed as the percentage of positive cells ± SD.

Vaccination Experiments With DC Vaccines Loaded With *Listeria* Recombinant Proteins or mRNA-LIPO

C57BL/6 female mice were vaccinated ($n = 5$/vaccine), or not ($n = 5$), *via* the lateral tail vain (*i.v*), with one dose of DC-vaccines (10^6 cells/mice) pre-loaded with recombinant *Listeria* proteins as $GAPDH_{rec}$ and LLO_{rec}; mRNA-LIPO complexes such as mRNA-LIPO-LLO and mRNA- LIPO-GAPD-; or empty DC-LIPO. Seven days post-vaccination, mice were challenged *i.v* with 100 μL bacterial suspension of LM (HUMV-LM01), MM (HUMV-MM01), or SP (HUMV-SP01) in saline (1×10^4 CFU/mL). All animals were examined daily and 14 days after the bacterial challenge, the mice were bled and sacrificed to quantify viable CFU/mL in the spleens and the cytokines in mice serum. Results are expressed as the percentages of protection ± SD of CFU/mL of vaccinated vs. non-vaccinated animals, using two controls: empty DC and saline. CFU of non-vaccinated mice are as follows: saline LM (HUMV-LM01) 2.75×10^5 CFU/mL, DC-CONT LM (HUMV-LM01) 2.60×10^5 CFU/mL, saline MM (HUMV-MM01) 1×10^5 CFU/mL, DC-CONT MM (HUMV-MM01) 0.9×10^5 CFU/mL, saline SP (HUMV-SP01) 2.5×10^5 CFU/mL, and DC-CONT SP (HUMV-SP01) 2.49×10^5 CFU/mL.

Intracellular IFN-γ Staining

Spleen cells of vaccinated and non-vaccinated mice were cultured in 96-well plates (5×10^6 cells/mL) and stimulated with *L. monocytogenes* $GAPDH_{1-22}$ peptide (50 μM) for 5 h in the presence of brefeldin A. Cells were surface labeled for CD4 or CD8, fixed, and permeabilized with a cytofix/cytoperm kit to measure IFN-γ (BD Biosciences). After sample acquisition by flow cytometry, data were gated for $CD4^+$ or $CD8^+$ events, and the percentages of these cells expressing IFN-γ were determined. Results were corrected according to the percentages of total $CD4^+$ or $CD8^+$ positive cells. Data were analyzed using FlowJo software (Treestar, Ashland, OR, USA).

Peptide-ELISA Assay to Measure *Listeria monocytogenes* $GAPDH_{1-22}$ Antibody Titers

Ninety-six –well-plates were coated with *L. monocytogenes* $GAPDH_{1-22}$ peptide (50 μg/mL) and coated to 96-well-plates in carbonate buffer (pH 8.0) overnight at 4°C. Plates were washed and incubated with 1 mg/mL of BSA (fraction V) to saturate all sites in the plates. Sera of patients infected with LM, MM, or SP or sera of vaccinated or non-vaccinated mice were 1/10 diluted and peptide coated plates were incubated with diluted sera for 2 h at RT, as previously described (2, 24). Reactions were developed with goat anti-human IgG or goat anti-mouse IgG and absorbances were analyzed at 450 nm. Results are presented as optical density measurements (OD) from mean values ± SD, of triplicate experiments.

Isolation of MoDC From Healthy Donors and *in vitro* Virulence With Clinical Isolates

Leukocytes from whole blood cells were isolated as the interphase of a Ficoll gradient. Leukocytes were incubated with microbeads conjugated to a mouse IgG2a monoclonal anti-CD14 antibody, and passed through MACS™ columns (Miltenyi, Bergisch Gladbach, Germany) to select monocytes (Mo) as $CD14^+$ positive cells. Mo cells were differentiated to monocyte derived DC (MoDC) using standard procedures previously reported (27). In brief, Mo (1×10^6 of cells/mL) are cultured into 6-well-plates (Falcon™) over 7 days using GM-CSF (50 ng/mL) and IL-4 (20 ng/mL) in RPMI-20% FCS medium. Differentiated cells were 98% $CD45^+HLA\text{-}DR^{\pm}CD86^-CD14^-$ positive cells and were used for the *in vitro* virulence analysis.

Adjuvant Effects of Vaccine Vectors on to MoDC From Healthy Donors

MoDC (2×10^6 cells/mL) were incubated with different recombinant proteins (5 μg/mL) or adjuvants (20 ng/mL), LLO_{rec}, $GAPDH_{rec}$, LPS, or DIO-1. After 16 h, supernatants were collected, filtered, and stored at −80°C until use for the cytokine analysis. Cell surface markers were analyzed by flow cytometry to evaluate the percentages of CD45, MHC-II, CD86, and CD14 positive cells to determine an activation phenotype of 99% $CD45^+HLA\text{-}DR^{++}CD40^{++}CD86^{++}$ positive cells.

Cytokine Measurement

Cytokines in mice sera, DC, or MoDC supernatants were quantified using multiparametric CBA kits, either for mice or for human samples (BD Biosciences, San Jose, CA, USA). The human Th1/Th2/Th17 CBA kit (catalog number 560484) was

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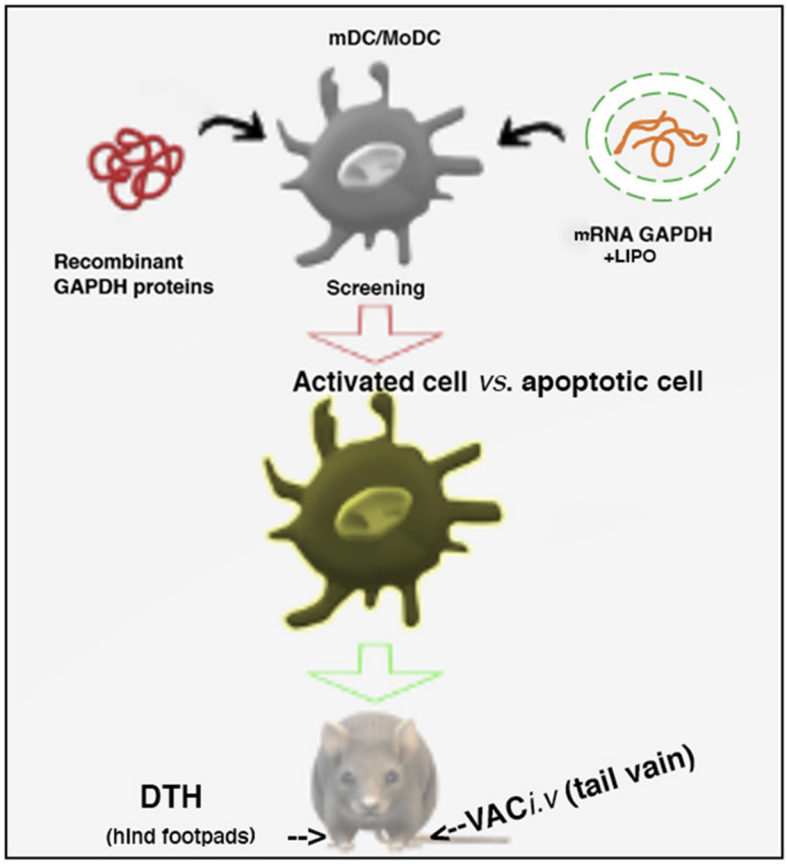

B

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Listeria_monocytogenes_GAPDH_NAD-binding          VKVGINGFGRIGRLAFRRIQNV------EGIEVVAINDLTDAKMLAHLLKYDTTQGRFDG  54
Mycobacterium_tuberculosis_GAPDH_NAD-binding      VRVGINGFGRIGRNFYRALLAQQEQGT-ADVEVVAANDITDNSTLAHLLKFDSILGRLPC  59
Mycobacterium_marinum_GAPDH_NAD-binding           VRVGINGFGRIGRNFYRALLAQQQQGS-ADIEVIAVNDITDNQTLAHLLKFDSILGRLPQ  59
Mycobacterium_chelonae_GAPDH_NAD-binding          VRVGVNGFGRIGRNFFRALDAQKAQGINTDIEIVAVNDLTDNATLAHLLKFDSILGRLPY  60
Streptococcus_agalactiae_GAPDH_NAD-binding        VKVGINGFGRIGRLAFRRIQNV------EGVEVTRINDLTDPNMLAHLLKYDTTQGRFDG  54
Streptococcus_pneumonia_GAPDH_NAD-binding         VKVGINGFGRIGRLAFRRIQNV------EGVEVTRINDLTDPVMLAHLLKYDTTQGRFDG  54
Streptococcus_pyogenes_GAPDH_NAD-binding          VKVGINGFGRIGRLAFRRIQNI------EGVEVTRINDLTDPNMLAHLLKYDTTQGRFDG  54
                                                  *:**:********  :* :          .:*:   **:**   ******:*:  **:

Listeria_monocytogenes_GAPDH_NAD-binding          EVEVHDGFFNVNG--KEVKVLANRNPEELPWGDLGVDIVLECTGFFTAQDKAELHIK-AG  111
Mycobacterium_tuberculosis_GAPDH_NAD-binding      DVGLEGDDTIVVGRAKIKALAVREGPAALPWGDLGVDVVVESTGLFTNAAKAKGHLD-AG  118
Mycobacterium_marinum_GAPDH_NAD-binding           DVSLEGEDTIVVGGVKIKALEVREGPAAMPWGDLGVDVVVESTGLFTNAAKAKGHLD-AG  118
Mycobacterium_chelonae_GAPDH_NAD-binding          DVSLEGDDTIVVGDHKIKALEVREGPAALPWGDLGVDVVVESTGIFTKREKAQGHLD-AG  119
Streptococcus_agalactiae_GAPDH_NAD-binding        TVEVKEGGFEVNG--QFVKVSAEREPANIDWATDGVEIVLEATGFFASKEKAEQHIHENG  112
Streptococcus_pneumonia_GAPDH_NAD-binding         TVEVKEGGFEVNG--KFIKVSAERDPEQIDWATDGVEIVLEATGFFAKKEAAEKHLK-GG  111
Streptococcus_pyogenes_GAPDH_NAD-binding          TVEVKEGGFEVNG--NFIKVSAERDPENIDWATDGVEIVLEATGFFAKKEAAEKHLHANG  112
                                                   * :.     * *  :   : ... *  : *.  **::*:*.**:*:    *: *:.  *

Listeria_monocytogenes_GAPDH_NAD-binding          AKKVVISAPATGDMKTIVYNVNHETLDGTETVISGASC       149
Mycobacterium_tuberculosis_GAPDH_NAD-binding      AKKVIISAPATDEDITIVLGVNDDKYDGSQNIISNASC       156
Mycobacterium_marinum_GAPDH_NAD-binding           AKKVIISAPATDPDLTIVLGVNDDKYDGSQNIISNASC       156
Mycobacterium_chelonae_GAPDH_NAD-binding          AKKVIISAPASDEDITIVLGVNDDKYDGSQNIISNASC       157
Streptococcus_agalactiae_GAPDH_NAD-binding        AKKVVITAPGGNDVKTVVFNTNHDILDGTETVISGASC       150
Streptococcus_pneumonia_GAPDH_NAD-binding         AKKVVITAPGGNDVKTVVFNTNHDVLDGTETVISGASC       149
Streptococcus_pyogenes_GAPDH_NAD-binding          AKKVVITAPGGNDVKTVVFNTNHDILDGTETVISGASC       150
                                                  ****:*:**. .   *:* ..*.:  **::.:**.***
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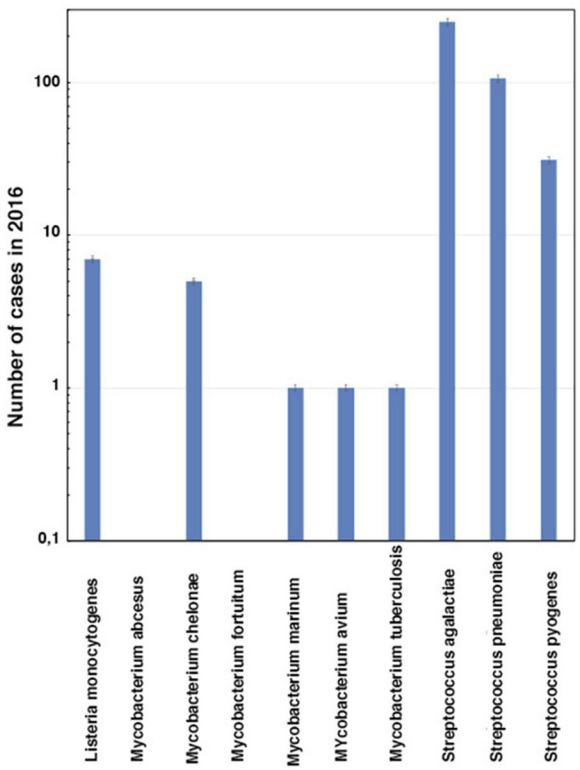
FIGURE 1 | Continued

C

D



Isolate code[a] Year 2016	anti-GAPDH-L1 antibodies[b]	Virulence in MoDC RI (CFU/mL) ± SD[c]
*HUMV-LM01	2.85 ± 0.1	2.9 x 10^4 ± 10
HUMV-LM02	2.31 ± 0.2	2.7 x 10^4 ± 11
HUMV-LM03	2.01 ± 0.1	2.5 x 10^4 ± 12
HUMV-LM04	1.90 ± 0.1	2.2 x 10^4 ± 12
HUMV-LM06	1.94 ± 0.1	2.2 x 10^4 ± 10
HUMV-LM07	1.98 ± 0.2	2.4 x 10^4 ± 11
LM^{WT} (10403S basal control)	**ND**	**2.5 x 10^1 ± 12**
LM-ΔLLO (non-virulent control)	**ND**	**4.2 x 10^{-1} ± 10**
HUMV-MTB01	2.20 ± 0.1	3.9 x 10^3 ± 10
HUMV-MA01	1.82 ± 0.2	3.5 x 10^3 ± 12
*HUMV-MM01	2.15 ± 0.1	3.8 x 10^3 ± 11
HUMV-MC01	1.75 ± 0.2	3.0 x 10^3 ± 10
HUMV-MC02	1.75 ± 0.2	3.0 x 10^3 ± 11
HUMV-MC03	1.71 ± 0.1	3.2 x 10^3 ± 12
HUMV-MC04	1.64 ± 0.2	3.0 x 10^3 ± 11
HUMV-MC05	1.63 ± 0.1	3.1 x 10^3 ± 11
M. smegmatis (non-virulent control)	**ND**	**1.0 x 10^1 ± 8**
*HUMV-SP01	2.21 ± 0.1	4.8 x 10^4 ± 13
HUMV-SP02	1.71 ± 0.1	3.9 x 10^4 ± 16
HUMV-SP03	1.73 ± 0.1	3.8 x 10^4 ± 15
HUMV-SA01	2.13 ± 0.1	4.3 x 10^4 ± 13
HUMV-SA02	2.20 ± 0.1	3.9 x 10^4 ± 12
HUMV-SA03	2.12 ± 0.2	3.8 x 10^4 ± 11
HUMV-SPY01	1.85 ± 0.1	4.1 x 10^4 ± 17
HUMV-SPY02	1.90 ± 0.1	4.0 x 10^4 ± 12
HUMV-SPY03	1.87 ± 0.2	4.2 x 10^4 ± 12
S. pneumoniae 49619-19F (vaccine control)	**0.67 ± 0.1**	**1.2 x 10^1 ± 10**
CONTROL-NI	**0.1 ± 0.2**	**ND**

FIGURE 1 | Scheme of the study and selection of the bacterial antigens for the vaccine vectors. **(A)** Scheme explaining our strategy in this study. First DC are incubated with the different antigen forms: recombinant *L. monocytogenes* GAPDH proteins or mRNA-LIPO-GAPDH carriers for screening of the suitable ones, causing DC activation with minimal apoptosis induction. DC vaccines loaded with the different antigens are tested for DTH responses as a measurement of T cell responses. DC vaccines with the maximal DTH responses are tested for vaccination experiments followed by bacterial challenge. **(B)** Multiple alignment of GAPDH sequences of NAD-binding domains of the following bacteria detected at our Health Institution showing more than 95% homology: *Listeria monocytogenes* (A0A0B8RGN3_LISM), *M. tuberculosis* (A0A045ITJ4_MYCTX), *M. chelonae* (A0A0E3TR96_MYCCH) *M. marinum* (A0A2Z5YDP2_MYCMR), *S. agalactiae* (Q9ALW2_STRAG), *S. pyogenes* (G3P_STRPY), and *S. pneumoniae* (I6L8L9_STREE) protein sequences using CLUSTAL O (1.2.4) multiple sequence alignment. The aligned regions correspond to the InterPro domain IPR020828 that all the proteins have at the beginning of their sequence. The InterPro domain IPR020828 corresponds to the Glyceraldehyde 3-phosphate dehydrogenase, NAD(P) binding domain: https://www.ebi.ac.uk/interpro/entry/InterPro/IPR020828/. The consensus symbols are taken from https://www.ebi.ac.uk/seqdb/confluence/display/JDSAT/Clustal+Omega+FAQ#ClustalOmegaFAQ-Whatdotheconsensussymbolsmeanin thealignment? The symbols meaning is explained in *Materials and Methods section Bioinformatics Analyses*. Colors on protein alignments correspond to residues according to their physicochemical properties: RED corresponds to Small (small $^+$ hydrophobic-including aromatic-Y), BLUE corresponds to acidic, MAGENTA corresponds to basic—H, GREEN corresponds to hydroxyl $^+$ sulfhydryl $^+$ amine $^+$ G and GRAY corresponds to unusual amino/imino acids (see **Supplementary Material** for complete residues description). **(C)** Phylogenetic tree of the seven bacteria species compared in this study. The tree data are the following: (Listeria_monocytogenes_GAPDH_NAD-binding:0.09732, ((Mycobacterium_tuberculosis_GAPDH_NAD-binding:0.04475, Mycobacterium_marinum_GAPDH_NAD-binding:0.05140):0.03060, Mycobacterium_chelonae_GAPDH_NAD-binding:0.07196):0.31226):0.14525, Streptococcus_agalactiae_GAPDH_NAD-binding:0.03596, (Streptococcus_pneumonia_GAPDH_NAD-binding:0.02528, Streptococcus_pyogenes_GAPDH_NAD-binding:0.02170):0.01751); **(D)** Analyses of the clinical cases of bacteria species after the bioinformatic analysis of GAPDH sequences showing 95% homologies in *B* and detected in the year 2016 at the Hospital U. Marqués de Valdecilla (Microbiology Dpt) from a complete study from 2014 to 2019 (graphic on the left). In the Table (on the right), we show sera from patients (HUMV codes) infected with the bacterial strains of *B* and examined for anti-LM-GAPDH$_{1-22}$ antibodies using a peptide ELISA. Sera were collected from patients and storage at −80°C. In the table, we present the antibody titers of patients from a representative 2016 year and with anti-GAPDH-L1 titers higher than 2.0 OD after performing a peptide-specific ELISA. Results are presented as

(Continued)

FIGURE 1 | the mean ± SD of OD units in triplicate experiments ($P < 0.05$). Asterisks and highlighted in yellow correspond to the selected clinical isolates for our further study. Virulence of these clinical isolates into human MoDC (2×10^5/mL) from healthy donors is evaluated *in vitro* after MoDC infection with 2×10^6 CFU/sample of the clinical isolates detailed in the Table. MoDC were lysed at two different times, at 1 h and at 16 h infection and lysates cultured in agar plates to count CFU. *In vitro* virulence is expressed as a replication index (RI) of the ration of CFU at 16 h to CFU at 1-h post-infection. Results expressed as RI numbers ± SD of three different experiments. ELISA and virulence assays *in vitro* using MoDC are performed in triplicate. A Student *t*-Test is applied for statistical analysis ($P \leq 0.05$).

used to measure human cytokines in MoDC supernatants, and the mouse Th1/Th2/Th17 CBA kit (catalog number 560485) was used to measure cytokines in mice sera and DC supernatants. Cytokine concentrations were expressed as the average of three replicates in pg/mL ± SD. ANOVA was applied to these samples according to the manufacturer's instructions. Data were analyzed using the FlowJo software.

FACS Analysis

Cell surface markers of human MoDC, murine DC, or murine spleens were analyzed by FACS using the following antibodies: anti-HLA-DR-FITC, anti-CD45-PerCP, anti-CD14-PE, and anti-CD86-V450 (clone 2331) for human MoDC. For cell surface markers of murine DC, we used, biotin anti-IAb (clone AF6-120-1), anti-CD11c-PE (clone HL3), anti-CD40-APC (monoclonal 3/23 from BD Pharmingen), and anti-CD86-V450 (clone GL-1) and for murine spleens we also used anti-CD4-FITC (clone RPA-T4) and anti-CD8-PE (clone RPA-T8) (BD Biosciences). Data were analyzed using the FlowJo software. ANOVA was applied to these samples according to the manufacturer's instructions.

Statistical Analysis

For statistical analysis, the Student's *t*-test was applied to mice assays infected with bacterial pathogens. For statistical purposes, each group included five mice for all assays reported ($P < 0.5$ was considered significant). ANOVA analysis was applied to the cytokine measurements and flow cytometry analysis as per the manufacturer's recommendations ($P \leq 0.05$ was considered significant). For statistical purposes, each flow cytometry sample was performed in triplicate. GraphPad software was used for generation of all the graphs presented.

Ethics Statement

This study was carried out in accordance with the Guide for the Care and Use of Laboratory Animals of the Spanish Ministry of Science, Research and Innovation. The Committee on the Ethics of Animal Experiments of the University of Cantabria approved the protocol (Permit Number: PI-01-17) that follows the Spanish legislation (RD 1201/2005). All surgeries were performed by cervical dislocation, and all efforts were made to minimize suffering. Similarly, for the use of human data of bacteria clinical isolates, we have an approved project from the Committee of Clinical Ethics of Cantabria (CEm) entitled: "Clinical Development of *Listeria* based vaccines" which includes Informed Consent and General Project Information documents of patients (Permit Acta Number: 29/2014, internal code: 2014.228).

RESULTS AND DISCUSSION

We initiated this study with the hypothesis that bacterial vaccines for adults can benefit from the discovery of antigens that are able to immunodulate DC and drive a wide spectrum immunity that cross-protects against bacterial infectious diseases of the genera *Listeria, Mycobacterium*, and *Streptococcus*. Vaccines inducing cross-protection immunity has recently been suggested for these taxonomic groups as multivalent vaccines (28). They are differentiated from conventional vaccines as they have the capacity of cross-reactive immune responses. For this reason, here, we refer to them as CRV vaccines to differentiate them from other type of vaccines, such as trained-immunity based vaccines (TIbV). CRV and TIbV might share two features: (i) stimulation of non-specific protection against several pathogens that involves innate immune cells and (ii) induction of specific immune responses to the vaccine antigens (5, 6). DC are innate immune cells responsible for antigen presentation and is relevant in all types of vaccines, conventional, CRV, or TIbV. DC are explored here as vaccine platforms to evaluate any bacterial antigen as a candidate for cross-protection vaccination, if the antigens induced minimal DC apoptosis, along with maximal expansion of T cells. In this context, two types of vaccine carriers are explored: an mRNA-encoded antigen conjugated with lipid carriers and recombinant proteins (see scheme of our procedure in **Figure 1A**). The bacterial antigens we use in this study are those reported in experimental vaccines for the above-mentioned bacteria genera: *L. monocytogenes* GAPDH and LLO, *M. marinum* Ag85A and *S. pneumoniae* PLY (37–42). DC and macrophages were selected as vaccine vectors since they are innate immune cells that participate actively in cross-protection immunity (5, 6). We focused our study to *L. monocytogenes* GAPDH antigen (Lmo2459) since it presents similar ADP-ribosylating abilities, immunogenic domains, and cross-immune responses in three bacterial genera of our study, *Listeria, Mycobacterium*, and *Streptococcus* (24–26, 28). These features prompted us to hypothesize that *L. monocytogenes* GAPDH was a candidate for CRV vaccines.

Selection of Antigens and Antigen Forms

We performed two approaches to select the bacterial pathogens for our study: first a bioinformatic analysis we previously reported (28) to search for homologies higher than 80% among GAPDH of most common pathogenic bacteria communicated annually at our Health institution and virulence analysis of clinical isolates. From a 5-year study from 2014 to 2018, we chose year 2016 as representative and detected several bacterial genera with GAPDH homologies higher than 80%, such as *Hemophilus, Klebsiella, Listeria, Mycobacterium, Pseudomonas,*

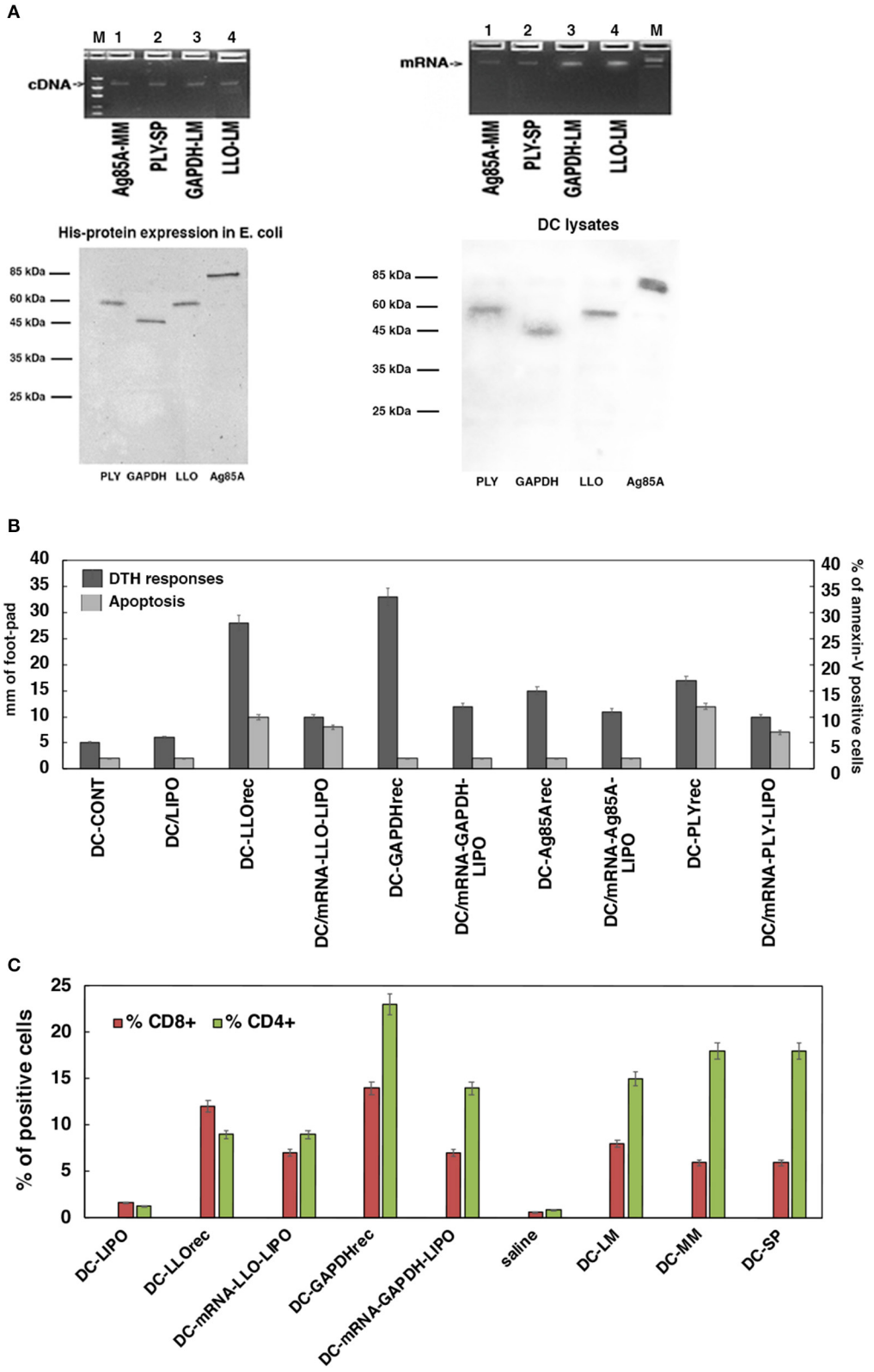


FIGURE 2 | Selection of the antigen forms for the vaccines. **(A)** Upper geles correspond to preparation of the mRNA antigens from cDNA plasmids encoding for Ag85A antigen of *M. marinum* (MM), PLY of *S. pneumoniae* (SP), and GAPDH or LLO of *L. monocytogenes* (LM) after *in vitro* transcription. Gels show the linear plasmids (upper left) and mRNA transcripts (upper right). Concentration of the mRNA preparations and qualities are shown in **Supplementary Figure 1A**. Lower gels

(Continued)

FIGURE 2 | correspond to Coomasie stained gels of purification of His-recombinant proteins (lower left) and DC uptake of prepared mRNA from all antigens (PLY of SP, Ag85A of MM, LLO, or GAPDH of LM) conjugated to the lipid carrier, lipofectamine, and after 16 h DC cells are lysed. Lysates were immunoprecipitated with rabbit anti-LLO antibody (DIATEVA), rabbit anti-Mycobacterium antibody (Colorado University), rabbit anti-PLY (a gift from JR de los Toyos, Oviedo, Spain), and rabbit anti-LM-GAPDH1-22 antibody (C. A-D and M.F obtained at CBMSO) (24). All immunoprecitpates were stained with Coomasie blue. **(B)** DC apoptosis (light gray bars) and DTH responses measured as the footpad swelling (dark gray bars) evaluated after incubation of DC with different antigens: empty DC (DC-CONT), lipofectamine (DC-LIPO), recombinant proteins as PLYrec from SP, LLOrec, and GAPDHrec from LM and antigen Ag85A from MM or mRNA-LIPO complexes of PLY, LLO, GAPDH, or Ag85A. Apoptosis is measured *in vitro* by flow cytometry and results are expressed as the percentages of annexin-V positive cells ± SD of three different experiments. ANOVA test was applied for flow cytometry results ($P \leq 0.05$). DTH responses are measured *in vivo* after inoculation of right hind footpads of C57BL/6 mice with the different DC vaccines ($n = 5$ per DC vaccine). Forty-eight hours after inoculation of DC vaccines, DTH responses are evaluated by the swelling of the hind footpads measured with a caliper. Results are expressed as millimeters ± SD of each group of 5 mice. Student *t*-Test was applied for statistical analysis ($P \leq 0.05$). **(C)** C57BL/6 mice were immunized i.v with 5×10^3 CFU/mice (HUMV-LM01, HUMV-MM01, or HUMV-SP01) and 7 days later, left hind footpads were inoculated with 1×10^6 DC vaccines (pre-loaded with 5 μg/mL of LLOrec, GAPDHrec, or 50 g/mL of mRNA-LIPO-GAPDH or mRNA-LIPO-LLO, or 1×10^6 CFU of LM, MM or SP, LIPO incubated DC, or saline incubated DC). Popliteal lymph nodes are isolated from mice legs and after homogenization, T cells sub-populations are analyzed by flow cytometry. Percentages of $CD4^+$ (green bars) or $CD8^+$ T cells (red bars) are shown. Results are expressed as the percentages of positive cells ± SD of three different experiments. Student *t*-Test was applied for statistical analysis ($P < 0.05$).

TABLE 1 | Main bacteria detected at Hospital Universitario Marqués de Valdecilla yearly with GAPDH showing more than 80% sequence homology at N-terminus.

[a]Bacteria	N° isolates
**Escherichia coli*	3,748
Haemophilus influenzae	122
Haemophilus parainfluenzae	36
Klebsiella pneumoniae	766
Listeria monocytogenes	7
Mycobacterium avium complex	1
Mycobacterium avium	1
Mycobacterium chelonae	5
Mycobacterium marinum	1
Mycobacterium tuberculosis complex	14
Mycobacterium tuberculosis	1
Pseudomonas aeruginosa	746
Staphylococcus aureus	1,012
Staphylococcus epidermidis	363
Streptococcus agalactiae	248
Streptococcus pneumoniae	106
Streptococcus pyogenes	31

[a] *Bacteria genera and species with GAPDH sequence homology higher than 80% detected in clinical isolates of the Microbiology Department at HUMV in the year 2016, a median time of the 5-year analysis we have performed. *Escherichia coli was included as negative control because GAPDH sequence homology was 60% and because it is the most abundant infection at HUMV.*

Staphylococcus, and *Streptococcus* (**Table 1**). However, if we quoted GAPDH homologies to 95% or higher, only clinical isolates of the bacterial taxonomic groups of *L. monocytogenes, Mycobacterium*, or *Streptococcus* fitted this category. The highest GAPDH homologies corresponded to *L. monocytogenes* (LM), *M. chelonae* (MC), *M. tuberculosis* (MTB), *M. marinum* (MM), *S. agalactiae* (SA), *S. pneumoniae* (SP), and *S. pyogenes* (SPY). Next, we focused on the NAD-binding domains of GAPDH from these taxonomic groups, using CLUSTAL 0 (1.24) multiple sequence alignment (**Figure 1B**), and, observed that protein sequences covering amino acids 3–25 displayed the highest identities (asterisks corresponds to 100% identity, colon symbol to 90%, and period symbol to 80% (*detailed analysis is described in Material and Methods, section Bioinformatics Analyses*). Amino acids are shown in a colored codes to distinguish homologies (**Figure 1B**) and color code explanations are provided in the Figure legend and Supplementary Material (**Supplementary Table 1**). These alignments might explain that the peptide-specific anti-*Listeria monocytogenes* $GAPDH_{1-22}$ antibody prepared in rabbits, with the 1-22 amino acid sequence of LM, can also detect MTB, MM, and SP bacterial extracts and surface shapes of the bacteria as previously described by our group (24, 28), suggesting that LM, MTB, MM, and SP shared immunogenic domains, in addition to enzymatic abilities and enzymatic domains. This is especially relevant as the phylogenetic tree relates NAD-binding domains of LM with MTB, MM, and MC. Another branch of the phylogenetic tree relates the NAD-binding domains of LM and SA and a third branch relates NAD-binding domains of LM with SP and SPY (**Figure 1C**), suggesting that GAPDH could be a common virulence factor. To further investigate this issue, we collected sera from all patients reported with infections caused by these eight bacterial species, detected at year 2016 (graph plot in **Figure 1D**), and explored for the presence of antibodies recognizing the LM-$GAPDH_{1-22}$ peptide, using a peptide-ELISA previously described (27). Several patients with infections caused by LM (HUMV-LM01, HUMV-LM02, and HUMV-LM03), MM (HUMV-MM01), MTB (HUMV-MTB01), SP(HUMV-SP01), SA (HUMV-SA01, HUMV-SA02, HUMV-SA03), and SPY (HUMV-SPY02) presented very high levels of antibodies recognizing the LM-$GAPDH_{1-22}$ epitope with O.D. $\geq$ 2.0, (right table in **Figure 1D**, column labeled as anti-GAPDH-L1 antibodies), while the remaining patients presented high levels of antibodies with O.D. $\geq$ 1.5. We concluded that immune responses generated by *Listeria, Mycobacterium*, and *Streptococcus* taxonomic groups are mainly targeted to a common $GAPDH_{1-22}$ epitope, strongly suggesting that GAPDH might be a common virulence factor to these pathogens. Evaluation of the *in vitro* virulence of their clinical isolates also supports our hypothesis. *In vitro* virulence was performed, infecting monocyte derived dendritic cells (MoDC) from healthy donors with the clinical isolates at a MOI of 10:1 and examining the bacteria replication indexes (RI). RI are defined as the ratio of CFU/mL at 16 h post-infection to CFU/mL at 1 h (27). We detected that

those patients with the highest titers of antibodies recognizing the LM-GAPDH$_{1-22}$ epitope, also presented the highest virulent strains of LM, MTB, MM, SP, or SPY, showing at least 100-fold higher replication indexes (RI) than non-virulent strains (right table in **Figure 1D**, column labeled as virulence in MoDC). This methodology was confirmed by the virulence of the clinical isolates *in vivo* using C57BL/6 mice and bacterial doses lower than LD50 [see Material and Methods section Mice and (41–44)], that reported similar results as *in vitro* virulence using MoDC (**Supplementary Table 2**). In brief, these data strongly suggests that GAPDH is a common virulence antigen of *Listeria, Mycobacterium*, and *Streptococcus* that needs to be explored as a candidate for CRV vaccines.

The second approach was to decipher the best antigen form to prepare a T-cell based vaccine vector from using DC loaded with the antigens and inoculation of mice hind footpads to examine a classical delayed-type hypersensitivity assay (DTH), a valid measure of T cell immunity (2). The antigens included in this strategy are commercially available as cDNAs (Bioclone Inc): Ag85A of MM (Ag85-MM), pneumolysin (PLY) of SP (PLY-SP), and GAPDH (GAPDH-LM) and listeriolysin O (LLO) of LM (LLO-LM). We prepared and compared two types of antigen forms, recombinant proteins and mRNA-lipid carrier complexes (mRNA-LIPO) because they can load different antigen processing compartments on DC. While recombinant proteins load the endo-lysosomal compartments relevant for MHC-class II antigen presentation, mRNA-lipid carrier complexes (LIPO) load the cross-presentation compartments relevant for MHC-class I antigen presentation (30, 45, 46). To prepare mRNA-lipid carrier complexes, commercially available DNA plasmids were first linearized (left upper cDNA gel in **Figure 2A** showed cDNA plasmids of each antigen) and mRNA samples were obtained by *in vitro* transcription (right upper mRNA gel in **Figure 2A**). Next, we added a CAP site at the 5′ end and a poly A tail at the 3′ end, following the manufacture's recommendation (see details in Materials and Methods, section cDNA Plasmids, *in vitro* Transcription, and Recombinant Proteins) (concentration and purity of transcripts are shown in **Supplementary Figure 1A**). Next, mRNA samples (100 pmol) were incubated with the lipid carrier, lipofectamine (5 μL), to obtain mRNA-Antigen-lipid carrier complexes (labeled here as mRNA-antigen-LIPO) and offered to DC to evaluate maximal uptake by antigen presenting cells (right lower Coomasie stained gel in **Figure 2A**). To prepare recombinant proteins, commercially available DNA plasmids were expressed in large quantities as His-fusion proteins in *E. coli* strain BL21 to obtain LLO$_{rec}$, Ag85A$_{rec}$, PLY$_{rec,}$ or GAPDH$_{rec}$ (left lower Coomasie stained gel in **Figure 2A**). Toxicities of mRNA-antigen-LIPO complexes and recombinant proteins were examined by hemolysis of sheep red blood cells in macrophages (BM-DM) and DC (**Supplementary Figure 1B**), as well as by Trypan blue staining in DC which reflects cell viabilities (**Supplementary Figure 1C**). Both methods of analyzing toxicities—hemolysis and Trypan blue—are relevant when using cytolysins (LLO or PLY) that are able to lyse red blood cells as LLO or PLY, while not causing significant reductions on cell viabilities. In fact, the high hemolysis detected with both cytolysins in macrophages, either as recombinant proteins or mRNA-antigen-LIPO complexes (**Supplementary Figure 1B**), drove us not to use macrophages for vaccine platforms. None of the antigen forms we used with DC caused hemolysis (**Supplementary Figure 1B**) or reduction of cell viability (**Supplementary Figure 1C**), therefore, we concluded that DC were the most suitable vaccine platform.

Selection of Most Immunogenic Antigens

Once antigen forms were prepared, we examined the DTH responses in C57BL/6 mice previously challenged intravenously (*i.v*) with the pathogens LM (HUMV-LM01), MM (HUMV-MM01), or SP (HUMV-SP01). Seven days post-infection we inoculated the left hind footpads with 10^6 DC pre-loaded with the different bacterial antigens, either recombinant proteins (5 μg/mL) or mRNA-LIPO complexes (50 μg/mL), in solutions with DIO-1 adjuvant to amplify the immune response, per mouse. The DTH response was measured as the swelling on the left hind footpad of each mouse 48 h post-inoculation, compared to the right hind footpad, which acts as the negative control. DC loaded with recombinant LM-GAPDH$_{rec}$ presented the highest DTH responses, followed by DC loaded with recombinant LM-LLO$_{rec}$, next were mRNA-LM-GAPDH-LIPO and mRNA-SP-PLY-LIPO complexes. DC loaded with MM-Ag85A$_{rec}$, mRNA- MM-Ag85A-LIPO complexes (dark gray bars in **Figure 2B**) induced significant DTH responses but lower than LM-GAPDH or LM-LLO antigen forms. DC loaded with SP-PLY$_{rec}$ and mRNA-SP-PLY-LIPO show half the footpad swelling than GAPDH antigen forms, therefore they induce only partial DTH responses. We also explored the abilities of these antigens to induce apoptosis in DC as a measure of the undesired inactivation of DC (≥10% apoptosis) (see Material and Methods in section Cell Toxicity and Apoptosis Assays on Macrophages and DC Vaccines). Whole pathogens, LM, MM, or SP (HUMV-LM01, HUMV-MM01, or HUMV-SP01, respectively) induced high levels of apoptosis (12–17%) as well as recombinant cytolysins like SP-PLY$_{rec}$ and LM-LLO$_{rec}$ (11–18%) or mRNA-LIPO complexes of these cytolysins (10–13%) (light gray bars in **Figure 2B**). All the other molecular forms tested (mRNA-LIPO complexes of LM-GAPDH or MM-Ag85A, and their recombinant proteins) presented apoptosis below 5% and similar to controls: DC loaded with lipofectamine (DC-LIPO) or incubated with saline (DC-CONT) (**Figure 2B**). Therefore, we concluded that the highest immunogenic and less apoptotic antigen forms corresponded to recombinant LM-GAPDH$_{rec}$. mRNA-LIPO complexes of LM-GAPDH show half the lower immunogenic DTH responses than LM-GAPDH$_{rec}$, although we inoculated a 10-fold concentration of mRNA-LIPO complexes compared to recombinant proteins. In brief, we do not consider this antigen form, mRNA-LIPO complexes, as suitable for exploring CRV vaccines. Next, we collected the popliteal lymph nodes of mice with the highest DTH immune responses (LM-GAPDH$_{rec}$, LM-LLO$_{rec}$, mRNA-LIPO complexes of LM-GAPDH or LM-LLO) and cultured them *in vitro* with 1 μg/mL of each antigen for 72 h, and examined the percentages of T cell populations, both $CD4^+$ or $CD8^+$ T cells by flow cytometry. We detected the highest percentages of $CD4^+$

(23%) and $CD8^+$ (14%) T cells in mice inoculated with LM-$GAPDH_{rec}$ (**Figure 2C**). mRNA-LIPO complexes of GAPDH presented significant percentages of $CD4^+$ (15%) T cells, but low percentages of $CD8^+$ (7%) T cells. The molecular forms of LLO, presented low percentages of $CD4^+$ (9%) T cells but significant percentages of $CD8^+$ (12%) T cells. However, mRNA-LIPO complexes of LLO induced low percentages of $CD4^+$ (9%) and $CD8^+$ (7%) T cells. No significant T cell responses were observed in the controls, DC, or in saline. When we compared these results with the DTH responses, we confirmed a correlation between the highest DTH responses (dark gray bars in **Figure 2B**) and the highest percentages of $CD4^+$ and $CD8^+$ T cells induced in the popliteal lymph nodes (**Figure 2C**). We argue that antigens in vaccine platforms that induced high DTH responses reflect the high expansion of T cell responses they induced and explains their high immunogenicity; both features are specific of the antigen.

Adjuvant Abilities of Vaccine Vectors

There is another possible explanation for DC-LM-$GAPDH_{rec}$ vaccines generating high DTH immune responses with induction of $CD8^+$ and $CD4^+$ T cells that is not related to the antigen immunogenicity. Some antigens can also induce DC activation, such as adjuvants or cell-walls of dead bacteria (16–19) and are interesting compounds for vaccine platforms. Here, we tested the possibility that LM-$GAPDH_{rec}$, LM-LLO_{rec}, or mRNA-LIPO complexes of LM-GAPDH or LM-LLO serve as non-specific DC activators. We evaluated two characteristics of activated DC, the cell surface expression of activation markers and the production of cytokines. We treated DC with different reagents, LM (HUMV-LM01), MM (HUMV-MM01), SP (HUMV-SP01), LM-LLO_{rec}, LM-$GAPDH_{rec}$, mRNA-LIPO complexes of LM-GAPDH or LM-LLO for 16 h, to examine activation. Two different adjuvants were also included in the assay, LPS and DIO-1 (14). Classical cell surface activation markers of DC are CD11c, MHC-II, CD40, or CD86, while CD11b is a macrophage-DC marker that, upon DC activation, reduces its surface expression and GR1 is a classical polymorphonuclear leukocyte (PMN) marker. LM (HUMV-LM01), MM (HUMV-MM01), and SP (HUMV-SP01) bacteria clearly induce DC activation, reflected by high percentages of CD11c, MHC-II, CD40, and CD86 positive cells (**Figure 3A**). mRNA-LIPO complexes of LM-GAPDH or LM-LLO did not induce DC activation, as the percentages of positive cells for MHC-II, CD40, or CD86 were similar to non-infected controls (NI). Recombinant LM-$GAPDH_{rec}$ protein was the only antigen form that clearly increased the percentages of all DC activation markers, CD11c, MHC-II, CD40, and CD86. However, LM-$GAPDH_{rec}$ effect was different than the activation pattern induced with LPS that increased only the percentages of the MHC-II activation marker (violet bars in **Figure 3A**) and was also different to the activation pattern induced by the DIO-1 adjuvant that increased the percentages of two activation markers, MHC-II and CD40. Neither LPS (dark blue bars), nor DIO-1 (garnet bars) caused significant effects in the percentages of CD86 positive cells. We conclude that LM-$GAPDH_{rec}$ activation of DC affected the expression of all classical markers of DC activation (light blue bars), suggesting a broader activation pattern. Next, we explored other features of DC activation, after collection of DC supernatants and analysis of cytokines using a Th1-Th2 parametric flow cytometry assay (BD Biosciences). As shown in **Figure 3B**, DC stimulation with adjuvants as LPS released high levels of Th1 (MCP-1, TNF-α, or IFN-α and Th2 (IL-6 and IL-10) cytokines; while stimulation with adjuvants like DIO-1 produced Th1 (MCP-1, TNF- α, or IFN-α), but not Th2 cytokines. DC stimulation with mRNA-LIPO complexes of GAPDH or LLO produced no cytokine at all (undetectable levels) and LLO_{rec} only showed low levels of Th1 cytokines (1–5 pg/mL). DC stimulated with recombinant LM-$GAPDH_{rec}$ released high levels of Th1 cytokines such as MCP-1, TNF-α, IFN-α, and IL-12, while no significant levels of Th2 cytokines such as IL-6 or IL-10 were observed. IL-12 production is associated with the ability to stimulate $CD8^+$ T cells and might explain the effect of DC loaded with recombinant LM-$GAPDH_{rec}$ to promote DTH responses (red bars in **Figure 2C**) after DC activation. We also confirmed that $GAPDH_{rec}$ was able to activate monocyte derived DC (MoDC) from healthy donors, as they induced Th1 cytokines with high levels of IL-12 and very low levels of IL-6 and IL-10 (**Supplementary Table 3**). We conclude that LM-$GAPDH_{rec}$ is a classical pro-inflammatory adjuvant that is able to activate DC in a stronger and broader manner.

Validation of DC-$GAPDH_{rec}$ as CRV Vaccines for *Listeria monocytogenes*, *Mycobacterium marinum*, and *Streptococcus pneumoniae* Infections

Specific DC activation with production of IL-12 have been linked to vaccine efficiency (47), therefore, we tested the vaccine efficiency of DC loaded with the highest immunogenic antigen forms, recombinant proteins LM-$GAPDH_{rec}$ and LM-LLO_{rec} (see **Figure 3C** for vaccination scheme). Five mice per group were inoculated *i.v* with a single dose of DC vaccines (10^6 cells/mice) pre-loaded with 5 μg/mL of LM-LLO_{rec} or LM-$GAPDH_{rec}$ (DC-LM-LLO_{rec} or DC-LM-$GAPDH_{rec}$) for 7 days and was then challenged *i.v* with either LM (HUMV-LM01), MM (HUMV-MM01), or SP (HUMV-SP01) for 14 days. Next, mice were sacrificed and their sera and spleens were collected. CFU were examined in spleens by plating in specific agar plates and results were expressed as the percentages of protection (see Material and Methods, section Vaccination Experiments With DC Vaccines Loaded With Listeria Recombinant Proteins or mRNA-LIPO for the detailed procedure). Only DC vaccines pre-loaded with LM-$GAPDH_{rec}$ conferred good protection against a challenge with LM (HUMV-LM01), MM (HUMV-MM01), or SP (HUMV-SP01) (blue, red and green bars in **Figure 3D**), while DC-LM-LLO_{rec} protected only for LM (HUMV-LM01) infection. Empty DC showed no protection at all against any of the pathogens (bars labeled as DC-CONT in **Figure 3D**).

We also checked specific humoral and cellular immune parameters in vaccinated and non-vaccinated mice reported in

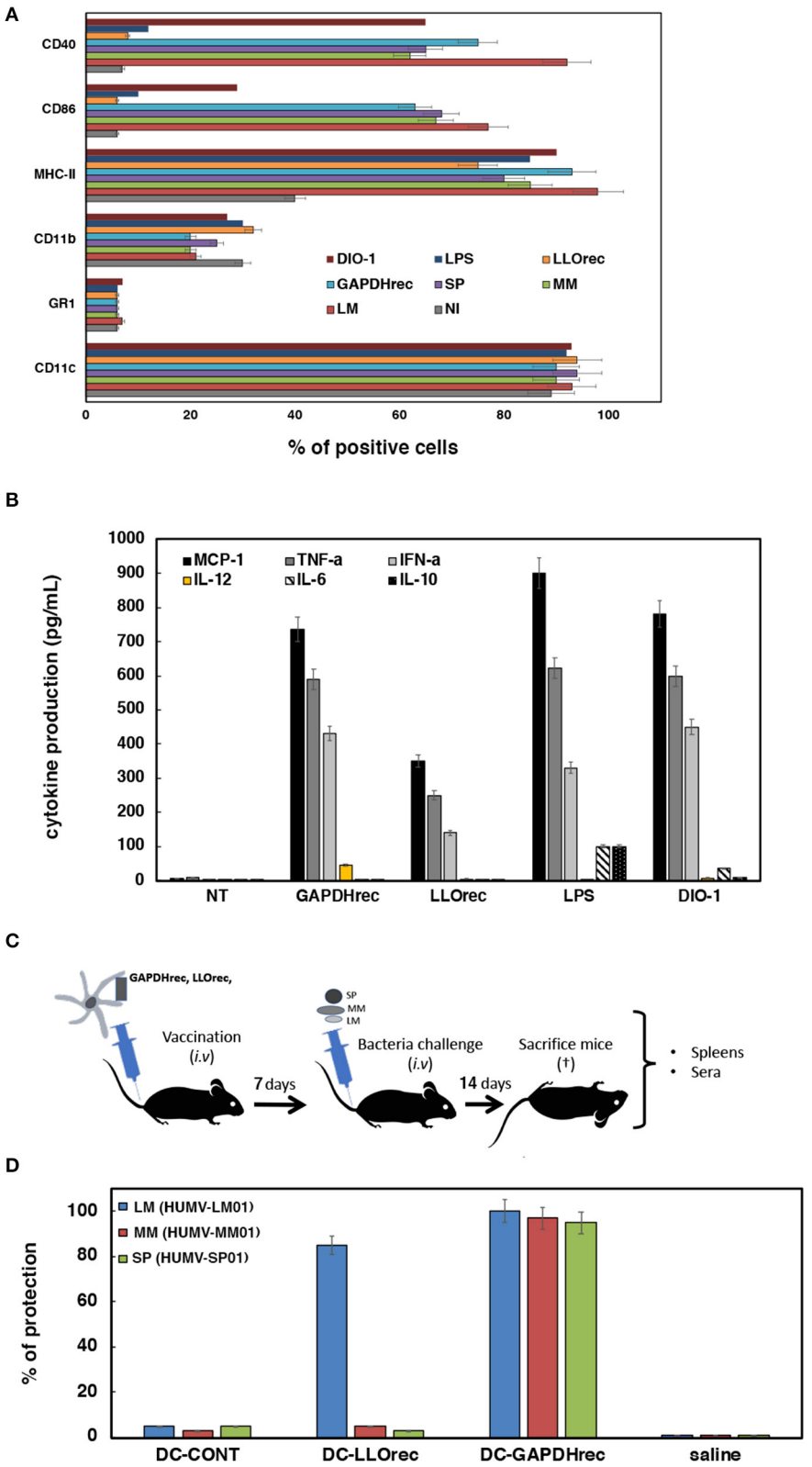


FIGURE 3 | Adjuvant and vaccine abilities of DC vaccine vectors loaded with recombinant or mRNA-LIPO antigens. **(A)** Flow cytometry analysis of DC surface markers after incubation with recombinant proteins LLO_{rec} or $GAPDH_{rec}$ or mRNA-LIPO complexes: mRNA-LIPO-LLO, mRNA-LIPO-GAPDH, bacteria MM

(Continued)

FIGURE 3 | (HUMV-MM01), SP (HUMV-SP01), or LM (HUMV-LM01) or two adjuvants, LPS or DIO-1. Results show the percentages of $CD11c^+$, MHC-II^+, $CD40^+$, or $CD86^+$ positive cells. Results are the mean of three different experiments ± SD. Student *t*-Test was applied for statistical analysis ($P \leq 0.05$). **(B)** Cytokine levels released to the supernatants of DC and measured with a multiparametric CBA kit (BD Biosciences). Results are expressed as pg/mL of each cytokine ± SD of triplicate samples. ANOVA test was applied to the cytokine's concentrations according to the manufacturer recommendation ($P \leq 0.05$). **(C)** Scheme of vaccination model and sample collection to analyze immune responses and protection: spleens and sera. **(D)** Vaccination of C57BL/6 mice with a single dose of DC vaccines. Seven days later, each group of vaccinated mice are divided in 3 sets and challenged i.v with 10^4 CFU/mice of hypervirulent strains of HUMV-LM01, HUMV-MM01, or HUMV-SP01. Next, after 14 days mice are bled, sacrificed and spleens collected. Vaccination results expressed percentages of protection as the mean ± SD of triplicates. Percentages are calculated as the number of CFU/mL counted in spleen homogenates of NV mice (saline) divided by CFU/mL of each set of vaccinated mice. Results are expressed as the mean ± SD of triplicates. Student *t*-Test was applied for statistical analysis ($P \leq 0.05$). CFU of non-vaccinated mice are the following: saline LM (HUMV-LM01) 2.75×10^5 CFU/mL, DC-CONT LM (HUMV-LM01) 2.60×10^5 CFU/mL, saline MM (HUMV-MM01) 1×10^5 CFU/mL, DC-CONT MM (HUMV-MM01) 0.9×10^5 CFU/mL, saline SP (HUMV-SP01) 2.5×10^5 CFU/mL, DC-CONT SP (HUMV-SP01) 2.49×10^5 CFU/mL.

TABLE 2 | Specific immune responses elicited after vaccination of mice with DC-LM-GAPDHrec and challenge with LM (HUMV-LM01), MM (HUMV-MM01), or SP (HUMV-SP01).

Mice vaccination[a]	[c]anti-$GAPDH_{1-22}$ antibodies	[d]$CD4^+$ %$GAPDH_{1-22}$ and IFN-γ	$CD8^+$ % $GAPDH_{1-22}$ and IFN-γ	[e]% Gated dimer CD8/$GAPDH_{1-22}$
[b]HUMV-LM01 (NV)	0.85 ± 0.1	0.8 ± 0.2	0.7 ± 0.3	0.4 ± 0.1
HUMV-MM01 (NV)	0.75 ± 0.1	0.8 ± 0.2	0.4 ± 0.3	0.3 ± 0.1
HUMV-SP01 (NV)	0.67 ± 0.1	0.9 ± 0.2	0.3 ± 0.3	0.4 ± 0.1
DC-GAPDHrec/LM01	2.31 ± 0.2	2.1 ± 0.3	4.2 ± 0.2	4.5 ± 0.3
DC-GAPDHrec/MM01	2.10 ± 0.1	1.9 ± 0.2	3.9 ± 0.3	3.5 ± 0.2
DC-GAPDHrec/SP01	2.01 ± 0.1	2.1 ± 0.3	2.7 ± 0.2	3.5 ± 0.1
CONTROL-NI	**0.1 ± 0.2**	**0.3 ± 0.1**	**0.5 ± 0.1**	**0.2 ± 0.1**

[a] *Female C57BL/6 mice (n = 5) were i.v. vaccinated with DC loaded with $GAPDH_{rec}$ and 7 days later they were challenged i.v with 5×10^3 CFU bacteria from clinical isolates HUMV-LM01, HUMV-MM01, or HUMV-SP01. Fourteen days later, mice were bled, sacrificed and spleens collected.*

[b] *Mice non-vaccinated (NV) and challenged with the different pathogens are examined for anti-$GAPDH_{1-22}$ in sera and peptide specific CD4 or CD8.*

[c] *Sera from mice as in a were examined for anti-$GAPDH_{1-22}$ antibodies by a peptide ELISA. Results are presented as the mean ± SD of OD units in triplicate experiments. Student t-Test was applied for statistical analysis ($P < 0.05$).*

[d] *Spleens of mice vaccinated or not were homogenized and cultured cells were used to measure intracellular IFN-γ after peptide stimulation in the presence of brefeldin A (procedure described in Material and Methods section Intracellular IFN-γ Staining). The percentages of $CD4^+$ or $CD8^+$ expressing IFN-γ were determined according with the manufacturer's recommendations. ANOVA test were applied for statistical analysis ($P \leq 0.05$).*

[e] *Some experiments spleen cells of vaccinated and non-vaccinated mice were incubated with recombinant dimeric H-2Kb:Ig fusion protein loaded with $GAPDH_{1-22}$ peptide. The staining cocktail contained dimeric fusion protein loaded with $GAPDH_{1-22}$ peptide, CD8, and IFN-γ antibodies. $CD8^+$ were gated for anti- IFN-γ staining (%Gated dimer-CD8) to calculate the frequencies of $CD8^+$-$GAPDH_{1-22}$ restricted cells and IFN-γ producers. Results are the mean ± SD of triplicates. ANOVA test were applied for statistical analysis ($P \leq 0.05$).*

experimental listeriosis vaccines (38, 48), such as the presence of antibodies recognizing the LM-$GAPDH_{1-22}$ peptide in sera and the percentages of $CD4^+$ or $CD8^+$ cells specific for LM-$GAPDH_{1-22}$ peptide-specific and IFN-γ producers, as well as verification of high frequencies of $CD8^+$ T cells specific for $GAPDH_{1-22}$ peptide using H2-Kb:Ig dimers (**Table 2**, see procedures in Materials and Methods, section Intracellular IFN-γ *Staining*). We detected high titers of antibodies recognizing LM-$GAPDH_{1-22}$ epitope, and high percentages of $GAPDH_{1-22}$ specific $CD4^+$ and $CD8^+$ and IFN-γ producers after vaccination with DC-LM-$GAPDH_{rec}$ and being challenged with LM (HUMV-LM01), MM (HUMV-MM01), or SP (HUMV-SP01) infections. Moreover, these vaccinated mice presented very high frequencies of $CD8^+$ T cells specific for the $GAPDH_{1-22}$ peptide, while non-vaccinated mice challenged with LM, MM, or SP presented undetectable frequencies. We concluded that DC-LM-$GAPDH_{rec}$ vaccines caused mainly antigen specific DC immune stimulation that confer cross-protection against LM, MM, and SP and induced GAPDH specific immune responses, both in T and B cells. However, we cannot discard non-specific broader DC immune stimulation.

CONCLUSION

Listeria monocytogenes GAPDH in two forms, either as a recombinant protein or as an mRNA-GNP complex, appears to be a safe bacterial antigen that induce significant T cell mediated immune responses when used in DC vaccine vectors. However, only the *Listeria* GAPDH recombinant protein activates DC in a specific and non-specific but broader form, different than adjuvant activation, as it induces all relevant activation markers and high production of Th1 cytokines, including IL-12. Therefore, not only is stimulation of T cell immune responses required for an antigen form to be considered a good candidate for vaccines, but specific DC activation also seems necessary to induce cross-protection against *Listeria, Mycobacterium*, and *Streptococcus* infections. DC vaccines loaded with recombinant LM-GAPDH can be considered not only as CRV vaccines with cross-protection abilities, but also as TIbV vaccines, since they present broad-spectrum protection for the common GAPDH virulence factor of *Listeria, Mycobacterium*, and *Streptococcus* and induces specific GAPDH immune responses. In fact, cross-protection abilities of these vaccines correlate with high levels of

antibodies and high percentages of specific $CD4^+$, $CD8^+$ T cells, and IFN-γ producers, recognizing the N-terminal $GAPDH_{1-22}$ peptide that has 98% homology in *Listeria, Mycobacterium*, and *Streptococcus*. The ability of mRNA-lipid carrier complexes to induce DC activation and strong T cell responses should be improved to include them in vaccine formulations for multivalent vaccines.

We speculate that experimental multivalent vaccines that can protect against *Listeria, Mycobacterium, Streptococcus*, bacterial genera responsible for severe meningitis, and long-lasting cutaneous infections in adults and the elderly, are promising tools for the new generation of human vaccines that are based on cross-reactive immunity, either as multivalent or as trained immunity-based vaccines.

PATENTS

This study is protected by patent number WO200802108A1. https://patents.google.com/patent/WO2008020108A1/fi#patentCitations (accessed March 27, 2021), entitled to Fundación Marqués de Valdecilla and patent number WO2019243647. https://patentscope.wipo.int/search/es/detail.jsf?docId=WO2019243647 (accessed March 27, 2021), entitled to Fundación Instituto de Investigación Marqués de Valdecilla.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Committee of Clinical Ethics of Cantabria. The patients/participants provided their written informed consent to participate in this study. The animal study was reviewed and approved by Universidad de Cantabria Animal Ethical Committee.

AUTHOR CONTRIBUTIONS

CA-D designed the experiments and directed the coordination of the experiments. DS-C and HT-N performed all the experiments and contributed equally to this study. RC-G helped in the performance of the vaccination experiments. RT performed the bioinformatic analyses. JC-M and IP-D provided all the clinical isolates, collected sera from patients, collected the Informed Consents of the patients, and have the custody of them. SY-D provided clinical isolates and clinical histories of patients infected with *M. marinum*, collected the Informed Consents of the patients and have their custody and helped with the mice models of infection. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.632304/full#supplementary-material

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