Heat inactivated mycobacteria, alpha-Gal and zebrafish: Insights gained from experiences with two promising trained immunity inductors and a validated animal model

Ramón Juste, Elisa Ferreras-Colino, José de la Fuente, Mercedes Domínguez, María Risalde, Lucas Domínguez, Alejandro Cabezas Cruz, Christian Gortázar

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REVIEW

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Heat inactivated mycobacteria, alpha-Gal and zebrafish: Insights gained from experiences with two promising trained immunity inductors and a validated animal model

Ramón A. Juste[1,2] | **Elisa Ferreras-Colino**[3] | **José de la Fuente**[3,4] | **Mercedes Domínguez**[5] | **María A. Risalde**[6,7] | **Lucas Domínguez**[8,9] | **Alejandro Cabezas-Cruz**[10] | **Christian Gortázar**[3]

[1]Animal Health Department, NEIKER-Instituto Vasco de Investigación y Desarrollo Agrario, Derio, Bizkaia, Spain

[2]NySA, Servicio Regional de Investigación y Desarrollo Agroalimentario, Villaviciosa, Asturias, Spain

[3]SaBio, Instituto de Investigación en Recursos Cinegéticos IREC-CSIC-UCLM-JCCM, Ciudad Real, Spain

[4]Department of Veterinary Pathobiology, Center for Veterinary Health Sciences, Oklahoma State University, Stillwater, Oklahoma, USA

[5]Unidad de Inmunología Microbiana, Centro Nacional de Microbiología, Instituto de Salud Carlos III, Madrid, Spain

[6]Departamento de Anatomía y Anatomía Patológica Comparadas y Toxicología, Facultad de Veterinaria, Universidad de Córdoba, Córdoba, Spain

[7]CIBERINFEC, ISCIII – CIBER de Enfermedades Infecciosas, Instituto de Salud Carlos III, Madrid, Spain

[8]VISAVET Health Surveillance Centre, Complutense University of Madrid, Madrid, Spain

[9]Department of Animal Health, Faculty of Veterinary Medicine, Complutense University of Madrid, Madrid, Spain

[10]UMR BIPAR, INRAE, ANSES, Ecole Nationale Vétérinaire d'Alfort, Université Paris-Est, Maisons-Alfort, France

Correspondence
Elisa Ferreras-Colino, SaBio, Instituto de Investigación en Recursos Cinegéticos IREC-CSIC-UCLM-JCCM, Ronda de Toledo 12, 13005 Ciudad Real, Spain.
Email: elisa.ferreras@uclm.es

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Abstract

Trained immunity (TRAIM) may be defined as a form of memory where innate immune cells such as monocytes, macrophages, dendritic and natural killer (NK) cells undergo an epigenetic reprogramming that enhances their primary defensive capabilities. Cross-pathogen protective TRAIM can be triggered in different hosts by exposure to live microbes or microbe-derived products such as heat-inactivated *Mycobacterium bovis* or with the glycan α-Gal to elicit protective responses against several pathogens. We review the TRAIM paradigm using two models representing distinct scales of immune sensitization: the whole bacterial cell and one of its building blocks, the polysaccharides or glycans. Observations point out to macrophage lytic capabilities and cytokine regulation as two key components in non-specific innate immune responses against infections. The study of the TRAIM response deserves attention to better characterize the

Abbreviations: AGS, alpha-Gal syndrome; AKR2, akirin-2; BCG, Bacille Bilié Calmette-Guerin; COVID-19, coronavirus disease 2019; GalTG, galactosyltransferase; H3, histone 3; HIMB, heat-inactivated *Mycobacterium bovis*; IL-6, interleukin-6; LncRNA, long non-coding RNA; LPS, lipopolysaccharides; miRNA, microRNA; NFkN, nuclear factor kappa-light-chain-enhancer; NK, natural killer; NLR, nucleotide-binding oligomerization domain-like receptor; PAMP, pathogen-associated molecular pattern; PRR, pattern recognition receptor; SARS-CoV-2, Severe acute respiratory syndrome coronavirus 2; SUB, subolesin; TLR, toll-like receptor; TNF, tumour necrosis factor; TRAIM, trained immunity; α-Gal, Galα1-3Galβ1-(3)4GlcNAc-R.

MYCOTRAINING
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evolution of host–pathogen cooperation both for identifying the aetiology of some diseases and for finding new therapeutic strategies. In this field, the zebrafish provides a convenient and complete biological system that could help to deepen in the knowledge of TRAIM-mediated mechanisms in pathogen–host interactions.

KEYWORDS
cross-protection, glycan alpha-Gal, heat-inactivated *Mycobacterium bovis*, macrophages, trained innate immunity

INTRODUCTION

The development of the classical models of specific immunity revolutionized the field of medicine [1, 2]. They allowed not just to diagnose infectious diseases quickly and specifically, up to then the main causes of human and animal mortality, disability and suffering in general, but contributed to prevent and cure them. These models reversed the impact of evolutionary pressure for resilience brought up by the large and dense social urban way of life and allowed an unprecedented increase in human population. The cumulated knowledge in the field of infectious diseases and immune responses has meanwhile defined a grey area of phenomena that do not fit the specific memory paradigm of adaptive immunity because of involving non-specific immune memory. Trained immunity (TRAIM) is a newly proposed model of immune response that has come to fill in the gap and to explain those immune phenomena that do not fit the classical features of the powerful adaptive immune defence: specificity, memory, clonal selection mechanism and presence only among the vertebrates in the evolutionary tree [3]. Its insights can lead to a better assessment of some inflammatory phenomena as well as to develop better vaccines by understanding the reasons of failure and protection as well as the role of adjuvants.

In this review, we focused on the analysis of the TRAIM paradigm using two models we use in our research and representing two distinct scales of immune sensitization: the whole bacterial cell and one of its building blocks, the polysaccharides or glycans. While reviewing the subject can help us to better understand our own experience and to overcome the observed problems, we think we can also contribute to the buildup and support of an idea that can have important implications in medicine.

TRAINED IMMUNITY

TRAIM may be defined as a form of memory where innate immune cells such as monocytes, macrophages, dendritic and natural killer (NK) cells undergo an epigenetic reprogramming that enhances their primary defensive capabilities [3–5] (Figure 1). This reprogramming or modification of gene expression control affects immune-related mitochondrial and metabolic functions and is a consequence of exposure to a primary pathogen stimulus. When a homologous or heterologous-related secondary stimulus happens again, the result is an enhanced immune response [6–10]. It has been shown that exposure to live agents such as bacille Bilié Calmette-Guerin (BCG) or microbe-derived products such as heat-inactivated extracts, lipopolysaccharides (LPS), chitosans or β-glucans can reprogram or train the innate immune system for TRAIM-mediated responses to secondary stimuli [7, 11]. The difference between this type of memory and the conventional adaptive one is that, instead of lymphocyte clonal selection, this type of immune memory depends on genetic modification of the cells involved. This takes place through histone 3 (H3) modifications and microRNA (miRNA) release associated with chromatin remodelling and epigenetic reprogramming [6, 12]. A relevant difference in adaptive versus TRAIM response routes is that the former is conformed against exquisitely elaborate singular tridimensional amino-acidic structures, while the latter is driven by larger highly repetitive widespread microbe-associated patterns [3].

Despite the old empiric knowledge that suffering the infection with a certain pathogen can help to fight against others, the first experimental evidence of what today is known as TRAIM dates back to an ex-vivo assay carried out in 1957 by Elberg et al. [13], in which it was shown that contact with *Mycobacterium tuberculosis* (*M. tuberculosis*) induced a higher cell survival to subsequent in vitro challenge with bacteria of the genus *Brucella*. The concept of this cross-protection was further demonstrated a few years later by Mackaness with *Brucella* and *Listeria* [14] and then reviewed in a broader perspective by Allison [15]. This provided a mechanistic basis for the earlier observed non-specific effects of BCG vaccination on child mortality, but was not studied until recently related to this and to other vaccines [16–19].

This enhanced immune resistance seems to have a genetic component related in part to adaptive immune memory due to exposure to different pathogens by

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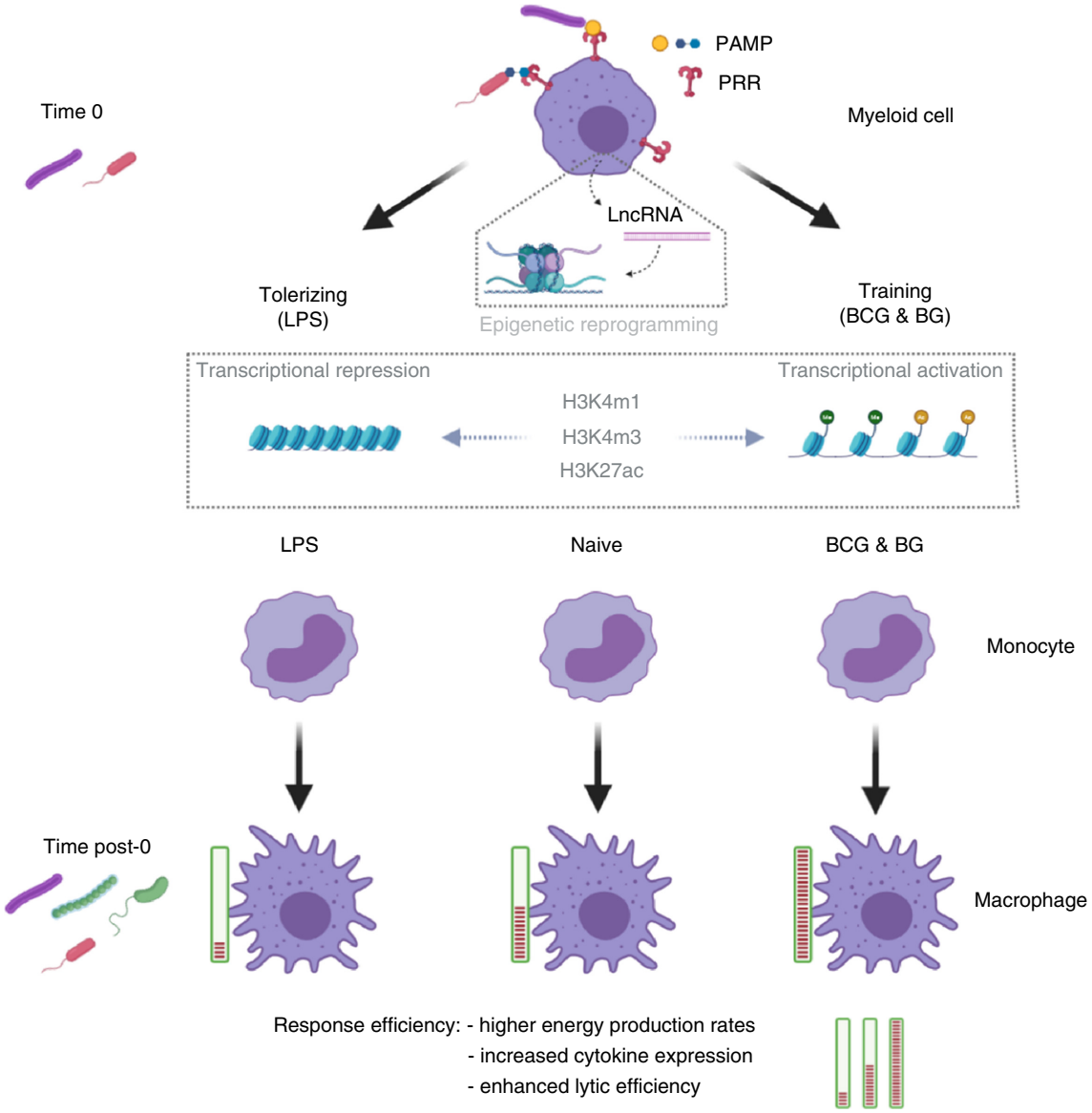

FIGURE 1 Proposed mechanisms of TRAIM. Upon effective contact of a pathogen-associated molecular pattern (PAMP) with the myeloid cell pattern recognition receptors (PRRs), epigenetic reprogramming (through long non-coding RNA: LncRNA) is induced by modifications in the histone monomer H3 that loosens the DNA packaging making it more accessible for transcription or not depending on the type of stimulus (tolerance [LPS] or training]β-glucan or BCG]). As a result, macrophages encountering the same patterns, no matter on which specific agent, are able to mount a response that might be less efficient if the priming was of a tolerizing type or more efficient if the priming was of the training type. Naïve individuals will respond at the basic level of efficiency. TRAIM, trained immunity.

crowded life in sedentary populations [20] and their accompanying domestic animals. This response is likely to be caused by a primitive mechanism that is present in the whole evolutionary scale, but that will better compare with social insects and that was recently recognized to be an important player also in vertebrates [3]. Social insects like ants and bees are under heavy infectious pressure given their clonic genetic population composition and crowded type of life. In addition to mechanic and behavioural defence activities, epigenetic reprogramming is a widely used mechanism for social insect polyphenism [21] that has been applied to defence against microorganisms allowing transmission of strengthened responses against pathogen recognition patterns [22–25]. Such successful mechanisms of improving immune fitness would have been conserved along the evolutionary chain, but not explicitly recognized nor studied until the recent enunciation of the TRAIM concept [3]. Although there are different cells capable of speeding up offending agent elimination as it occurs in invertebrates and plants [26], the hallmark of active innate immune responses in vertebrates is an efficient lytic activity and phagocytosis by macrophages [3, 27–29].

Epigenetic reprogramming and its metabolism

Gene expression is regulated by promoters (proximal transcription elements) and enhancers (distal transcription elements). The action of these elements can be regulated without DNA sequence modification through three main processes that have been shown to be used by TRAIM: DNA methylation [30, 31], histone proteins configuration [32] and non-coding RNAs [6, 33]. Balance between these mechanisms and reversion of their changes are what determine the degree of enhancement of the response and its duration in time. For instance, it has been shown that trimethylation of lysine 4 at histone 3 marks active promoters while its monomethylation mark enhancers [34]. These marks, as well as the DNA methylation changes lead to the unfolding of the chromatin thus speeding up transcription and expression of pro-inflammatory factors. Since these changes are only partially reversed when the triggering cause disappears, the secondary challenge initiates a quicker and stronger gene expression. How these mechanisms are used in the context of TRAIM has been studied in a series of studies by the Radboud Institute for Molecular Life Sciences [12, 32, 35], who also demonstrated the involvement of two specific enzymes in β-glucan training, the KDM5 family of histone demethylases that is inhibited and thus favours persistence of training [36] and Set7 that writes a histone 3 lysine 4 monomethylation [37] that accelerate the Tricarboxilic acids cycle and the oxidative phosphorylation pathways that are used to fuel the trained immune responses.

Phagocytosis

Phagocytosis as a relevant process in tissue defence against infections was defined by Metchnikoff [38]. Phagocytosis can be found in unicellular organisms where it is part of the normal cell metabolism, in invertebrates with specialized and non-specialized cells, and also in vertebrates where it is essentially carried out by cells of the myeloid lineage that mature into specialized mature macrophages whose evolution can further progress to dendritic, epithelioid and multinucleated cells [39]. No specific stimuli seem to be needed to trigger phagocytosis, but complex mechanisms are responsible for the degradation of the pathogens in the lysosomes [40]. This gives the macrophages a plasticity that is a key factor in both innate and adaptive immune defence and can be activated by two main routes: the classical one, stimulated by microbial toll-like receptors (TLRs) ligands and interferon-gamma (IFNγ), and the alternative one, triggered by interleukin (IL)-4/IL-13. These routes cause a polarization of the macrophage functionality into differentiated phenotypes defined as M1 and M2, respectively [41–43]. The M1 is characterized by the release of pro-inflammatory cytokines and high amounts of reactive oxygen and nitrogen intermediates associated with the promotion of a Th1 response with strong anti-microbial and inflammatory activity [42, 44, 45]. The M2, on the contrary, shows an anti-inflammatory profile, tissue remodelling, tumour growth and immunoregulatory functions [28, 38, 46]. From these cellular mechanisms, a wide range of positive consequences, such as pathogen clearing and tissue repair in successful mode, and negative consequences such as pathogen persistence and chronic inflammation in tolerizing mode, emerge depending on external (type of pathogen or intensity of exposure) and internal (genetics or activation route) factors [47]. The former consequences are those associated with the M1 phenotype and constitute the genuine goals of TRAIM. The latter, linked to the M2 phenotype, would be the collateral damages driven by factors like insulin and obesity that confer TRAIM mechanisms a central role not only in infectious agents defence, but also in some of the current most common chronic inflammatory diseases of metabolic origin [34, 42].

Cytokines

Other factors relevant for innate immunity that may be involved in TRAIM include complement component 3 (C3) and akirin-2 (AKR2) [48–50]. Complement receptor 3 mediates activation of innate immune cells in response to β-glucans (a specific type of glycans composed only by monosaccharides) and triggers the macrophage tumour necrosis factor (TNF) and IL-6 response to induce TRAIM [51]. Another possible mechanism of TRAIM includes the stimulation of the immune system through pattern-recognition TLRs [52]. In this sense, when monocytes and NK cells from BCG-vaccinated individuals are compared to non-vaccinated controls, they display higher TLR and cytokine expression levels in response to various pathogens (e.g., *Bacillus anthracis*, *Brucella suis*, *Staphylococcus aureus*, *Pasteurella pestis*, *Listeria monocytogenes*, *Klebsiella pneumonia*, etc.) and their products (β-glucans, lipoproteins, LPS, flagellin, muramyl dipeptide, etc.) [7, 53]. Standardizing methods to measure TRAIM is urgently needed to continue research in this area. In this sense, simple M1/M2 polarization balance macrophage characterization could be a first approach that, further developed with ex-vivo phagocytosis assays, would lead to simplified TRAIM marker assays. Three interesting candidates recently appearing to

have a potential in a killed mycobacterial model could be iNOS, IL10 and MIP-1β [54] that, in an ex-vivo macrophage paratuberculosis vaccination goat model, showed significant increases (iNOS and IL10) or decreases (MIP-1β) relative to controls, but no differences after secondary ex-vivo macrophage challenge with *M. avium* subsp. *paratuberculosis*.

TRAIM INDUCTORS

As pointed out above, TRAIM is a primitive mechanism of eukaryotes that has been maintained throughout the whole evolutionary tree up to its highest branches. It probably appeared as an advantage to respond to the loss of fitness caused by other organisms evolving to parasitism and trying to steal oneself resources. Therefore, it had to adapt pre-existing metabolic mechanisms to recognition of general patterns of those varied range parasitic organisms. Therefore, primitive organism had to be able in the first place to respond to the challenge by increasing energy and materials production at the cellular level through glycolysis oxidative phosphorylation, tricarboxylic acids cycle, pentose phosphate pathway, fatty acid oxidation, fatty acid synthesis and aminoacid metabolism in the fight to destroy the invading parasite. If successful, the next step to keep the gained evolutionary advantage would be to maintain such readiness until the next encounter with the parasite or even, if possible, to any other with similar structural characteristics. This would lead to a sharing of similar response mechanisms after exposure to molecular triggers present in different biological agents. Bacterial and fungal cells and their components (LPS, β-glucan and chitin), virus and parasites are the best-known exogenous ones, but there are other endogenous ones like oxidized low-density lipoprotein, apolipoprotein(a), aldosterone or adrenaline [34]. Since training or tolerizing effects are observed, it must be underscored that TRAIM is a complex delicately balanced mechanism that is only beginning to be understood, and that the same compound, depending on time and concentration might cause opposite effects [53, 55]. In this review, we will focus on two examples: on one side, a whole bacterial cell, *Mycobacterium bovis* inactivated vaccine which would be the closest approach to a natural challenge without its risks, and on the other, one structural component of those types of organism found in bacterial cell wall a broadly known as glycan, an encompassing term grouping large glycosidically bound saccharide polymers including those linked to lipids or proteins, that probably represents the type of true molecular effector of TRAIM present in the former in more complex forms.

Mycobacteria

The BCG vaccine, an attenuated *M. bovis* strain, was first introduced in humans in 1921 and is still the only registered vaccine to prevent tuberculosis (TB) [56]. Both epidemiological and experimental studies concerning BCG suggested the first glimpses of TRAIM. Beyond its specific protective effect against disseminated forms of TB in infants, extensive to leprosy [57, 58], attention has been recently drawn to early and reiterated epidemiological studies that show a decrease of overall mortality rate in BCG-vaccinated children that is larger than that attributable to TB itself [59]. This reduction in infant mortality is mainly associated to BCG-induced cross-protection against unrelated pathogens, especially sepsis and respiratory infections [60, 61]. Furthermore, BCG also provided the first experimental evidence of TRAIM [62], and has become one of the most studied TRAIM inducers [18, 53, 63–71]. Over the last decades, several experimental studies have reported that BCG stimulation induces protection upon a secondary encounter with unrelated pathogens such as herpes simplex virus [72, 73], influenza virus [72, 74, 75], *Staphilococcus aureus* (*S. aureus*) [64, 76], *Salmonella enteritidis* [62], *Leishmania major* [77], *Plasmodium* spp. [78], *Trypanosoma cruzi* [79], *Babesia microti* [80] and *Candida albicans* (*C. albicans*) [64, 81] in murine models, as well as yellow fever virus [82], human papillomavirus [83] and *Plasmodium* spp. [84] in humans. Although adaptative immunity is also likely to participate [85], the speed at which responses appear (few days to 1 week after vaccination), and the particularities of the infant immune system (e.g., adaptative immunity not being fully mature), strongly support the hypothesis of the innate immune system playing a major role in the observed non-specific effects following BCG vaccination [60]. In this respect, after immunization with BCG and challenge with unrelated pathogens, circulating monocytes/macrophages and NK cells display an increased capacity of secreting pro-inflammatory cytokines in a lymphocyte-independent manner [64, 81]. Two molecular mechanisms seem to be involved in the induction of a trained phenotype in innate cells after stimulation with BCG. First, epigenetic reprogramming through histone modifications, concretely TRAIM methylation of lysine 4 in histone 3 (H3K4me3), which is associated with gene transcription [86], occurs at the promoters of genes encoding immunological markers [81]. These epigenetic modifications are dependent on the NOD2 receptor, which is present in monocytes [81]. Second, cell metabolism shifts from oxidative phosphorylation to aerobic glycolysis, a.k.a. Warburg effect, in trained monocytes/macrophages and NK cells [87]. In fact, fumarate and mevalonate, which are metabolites derived from glutaminolysis and cholesterol synthesis,

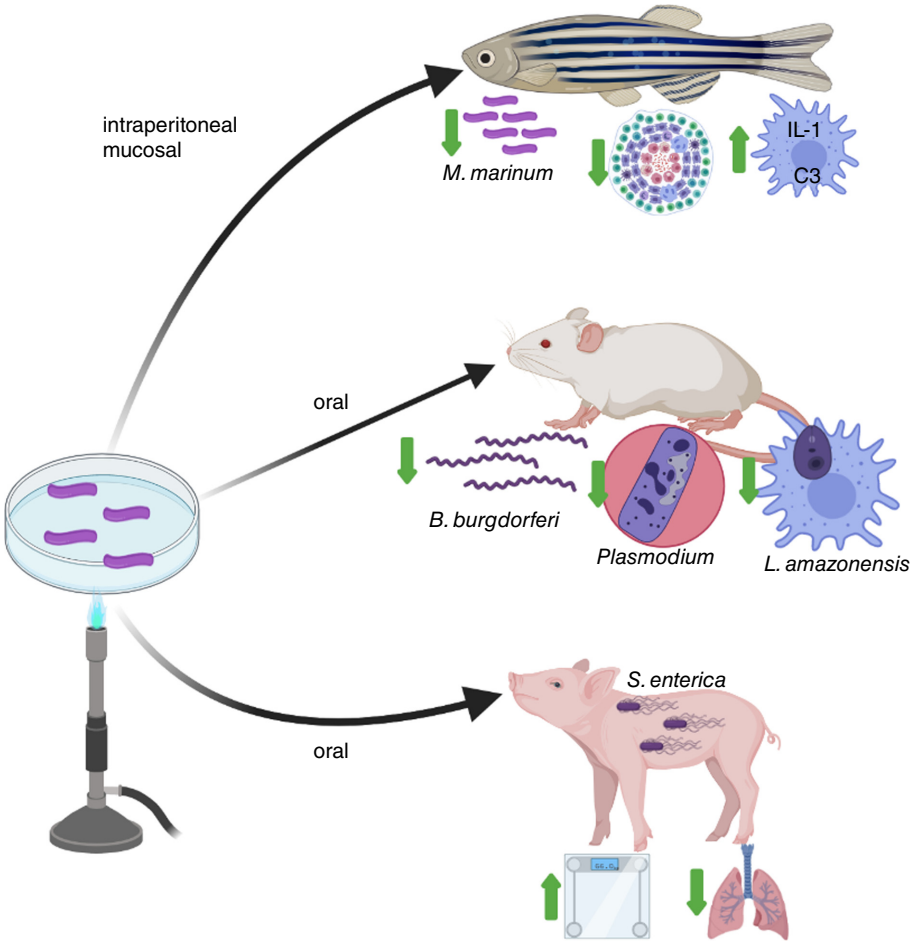

FIGURE 2 Induction by HIMB immunostimulant of protective responses to multiple pathogens. HIMB significantly decreased the number of mycobacteria per granuloma and the number of granuloma, as well as increased the expression of C3 and IL-1b in zebrafish vaccinated intraperitoneally or by immersion and challenged with *Mycobacterium marinum* (*M. marinum*) [99, 104]; significantly reduced bacteria/parasite burden in mice vaccinated orally and challenged with *Borrelia burgdonferi*, *Plasmodium* sp. and *Leishmania amazonensis* in mice, and significantly increased weight and reduced clinical signs and lesions in pigs vaccinated orally and challenged with *Salmonella enterica* [105]. HIMB, heat-inactivated *M. bovis*.

respectively, induce enrichment of H3K4 at the promoters of several cytokine-encoding genes. Thus, epigenetic regulation and metabolic pathways seem to operate jointly [18].

Despite the extensive safety record of BCG vaccination both in humans [88], domestic animals and wildlife species [89], disseminated BCG infection may occur in immunocompromised individuals [90]. In addition, the possibility of excretion into the environment after oral vaccination and the necessity of maintaining the cold chain must be considered when delivering live vaccines [91]. Therefore, immunostimulants based on inactivated mycobacteria displaying BGC-like effects would constitute a more environmentally safe and easily handled approach to induce protection against TB [92, 93], and cross-protection against unrelated pathogens in human and animal populations.

Garrido et al. [93] developed an immunostimulant based on heat-inactivated *M. bovis* (HIMB) which has demonstrated to reduce the mycobacterial load in target tissues and tuberculous lesions in cattle [94], goats [95], pigs [96], wild boar (*Sus scrofa*) [93], red deer (*Cervus elaphus*) [97] and European badger (*Meles meles*) [98]. In the cited experiments, the HIMB formulation, alone or in combination with adjuvants, was administered either via oral or parenteral. The protective capacity of HIMB has been correlated with innate-response markers TLRs, complement factors and pro-inflammatory cytokines [91, 99]. In addition, C3 was proposed as a possible correlate of natural resistance to *M. bovis* infection in wild boar [100]. Furthermore, immunization of calves with HIMB enhanced the capacity of monocyte-derived macrophages to destroy *M. bovis* in vitro, an effect that was independent of cellular or humoral adaptive immune responses and thus coherent with TRAIM [101]. This is consistent with the observation of macrophage-mediated *M. leprae* destruction in TB-infected patients [40]. As a matter of fact, immunization with HIMB has shown protective capacity not only against *Mycobacterium* infection but also against unrelated pathogens such as *Plasmodium* and *Leishmania* in mice [102] and *Salmonella* in swine [103] (Figure 2). Although immunization with HIMB did not reduce *S. enterica* serotype Choleraesuis burden, immunized pigs showed higher levels of pro-inflammatory cytokines such as serum TNFα and lung CCL28, lower levels of lipid oxidation, and higher activity of antioxidant enzymes than non-immunized pigs [103]. In connection with this, pre-exposure to Freund's complete adjuvant containing inactivated *M. bovis* was also

found to be protective against *Pasteurella piscicida* infection in Yellowtail fish [106]. Furthermore, when formulations containing HIMB, alone and in combination with recombinant Subolesin (SUB), the tick ortholog of human AKR2; were orally administered to cattle experimentally infested with cattle ticks *Rhipicephalus microplus*, a significant reduction in the number and fertility of female ticks in cattle immunized with HIMB+SUB compared to HIMB-immunized animals was observed [107]. These findings are consistent with the known utility of *M. bovis* as an immune adjuvant. Indeed, the immunization with HIMB alone also suggested a reductive effect on female tick weight and oviposition, and the analysis of mRNA levels of immune response markers showed upregulation of both innate and adaptive immunity [107]. The innate mechanisms were mediated by TLRs through upregulation of AKR2, IL-1β, C3 and TNFα; while the adaptive response was mediated by anti-SUB antibodies, which is, in fact, the best documented protective effect of tick vaccines [108]. Overall, the abovementioned results suggest that immunization with HIMB, in addition to specific cellular and antibody-mediated adaptive immunity, can also activate innate immune mechanisms and TRAIM to induce not only protection against mycobacteria but also cross-protection against other pathogens [7, 48–50, 53, 109].

Glycans

Contrary to the fine tridimensional singularity lending exquisite species or even lineage specificity to adaptive immune response epitopes of proteins, glycans are highly repetitive patterns shared by many cell structures throughout both pathogenic and non-pathogenic microorganisms. The extensive presence of glycans in pathogens is responsible for the lack of specificity that characterizes the innate immune responses targeting them [53]. Although the role of glycans in innate immunity has been reported, questions remain regarding their role in immune regulation and protection against pathogen infection [110]. β-Glucans in general, and fungal-derived ones, are by far the most studied glycans as innate response modulators. Several decades ago, Di Luzio et al. [111] and Bistoni et al. [112] reported reduced lethality in mice due to *S. aureus* and *C. albicans* after administration of *S. cerevisiae*- or *C. albicans*-derived β-glucans, respectively, through B/T lymphocytes-independent mechanisms [112]. Their findings were subsequently corroborated in recent experiments [113], in which the survival rate to lethal *C. albicans* infection was increased in both wild-type and T/B lymphocytes deficient mice previously infected with low-dose β-glucan. Conversely, this effect was not observed in monocyte deficient mice, suggesting a key role of monocytes/macrophages in the protective immune mechanisms [111, 113, 114]. Furthermore, in vitro production of IL-6 and TNF-α by human peripheral blood mononuclear cells and purified monocytes were enhanced after incubation with *C. albicans* or β-glucans in a dose-dependent manner and up to 2 weeks [113]. Although the molecular mechanisms involved are not fully elucidated, epigenetic programming and metabolic shift also seem to play a crucial role in the innate system training by β-glucans [5, 113, 115].

Despite the glare of glucans among glycans, here we will review evidence of the immunological relevance of a less known molecule. The glycan Galα1-3Galβ1-(3)4GlcNAc-R (α-Gal), present in tick salivary glycoproteins and non-catarrhine mammalian cells, has recently been associated with the alpha-Gal syndrome (AGS) that causes delayed IgE-mediated anti-α-Gal anaphylaxis to mammalian meat consumption and immediate anaphylaxis to xenotransplantation, certain drugs such as cetuximab, and tick bites [116, 117]. Humans do not produce α-Gal and natural anti-α-Gal IgM/IgG antibodies are produced in response to gut microbiota with this glycan on bacterial surface [118]. The hypothesis is that humans evolved by losing the capacity to synthesize α-Gal thereby acquiring the capacity to develop a strong antibody response against this glycan that is protective against pathogens containing this modification [119]. As humans, fish and birds do not synthesize α-Gal and display natural antibody levels to this glycan likely in response to bacterial gut microbiota [120, 121]. Accordingly, in the α-Gal-negative galactosyltransferase (GalT)-KO mouse and zebrafish (*Danio rerio*) animal models, immunization with this glycan boosts immune protective mechanisms against multiple pathogens [122]. In experiments conducted in the zebrafish model of TB, immunization with α-Gal followed by experimental infection with α-Gal-positive *M. marinum* resulted in protective responses that could be associated with TRAIM-mediated mechanisms [121]. While α-Gal present in mycobacteria may antagonize TLR2-mediated immune response, immunization with this glycan resulted in antibody-mediated interference with the mycobacterial antagonistic effect to promote TLR/NF-kB/AKR-mediated immune response and upregulation of pro-inflammatory cytokines [121]. One of the questions that arise from these results is whether ticks can induce a TRAIM response in α-Gal-negative hosts. Despite the growing incidence of AGS, only a small fraction of the individuals exposed to tick bites develop this syndrome [123]. Recently, a model for the study of the AGS was established in zebrafish [124]. Although the immune mechanisms associated with AGS

have not been fully elucidated, results in zebrafish treated with tick saliva showed the activation of innate immune responses mediated by upregulation of C3 in fish intestine [124]. If true in humans, the hypothesis is that α-Gal and other unknown tick salivary biomolecules induce TRAIM through C3 and other mechanisms, which may protect against AGS and tick-borne and non-tick-borne pathogens such as mycobacteria. This hypothesis may be addressed by a better characterization of the immune mechanisms induced by tick saliva and in the GalT-KO mouse and zebrafish animal models.

COOPERATION IN PATHOGEN–HOST INTERACTIONS

Tick–host–pathogen interactions evolved as conflict and cooperation [117, 123]. For mycobacteria, TB represents a clear conflict in host–pathogen interactions, but is there any cooperation? The exposure to live BCG vaccine and to HIMB has been shown to train the innate immune system for TRAIM-mediated protection to other pathogens [7]. These results support that *M. tuberculosis* complex bacteria can trigger this type of response. Therefore, it should not be ruled out that natural exposure to these species induces a TRAIM-mediated protection if occurring in the right circumstances. The high rate of latent to clinical TB would be highly suggestive of a cooperative effect of mycobacterial infections that in the long term would be beneficial, at the cost of being clearly conflicting with a few individuals with their TRAIM capabilities genetically or temporarily diminished. The ongoing coronavirus disease 19 (COVID-19) pandemic caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has challenged our knowledge of the host immune response to coronaviruses, showing an unusual immunopathology that has become the cause of this infection lethality [125]. Therefore, the characterization of the immunological mechanisms involved in COVID-19 symptoms and protective response is important to advance in disease prevention and control. Recently, a possible protective effect against SARS-CoV-2 infection elicited by β-glucans and α-Gal glycan has been postulated [10, 126]. Proposed protective mechanisms elicited by these biomolecules include TRAIM [10] and macrophage response, complement system, and upregulation of pro-inflammatory cytokines through the TLR2/NF-kB innate immune pathway [126].

It has also been suggested that BCG vaccination contributes to modulate morbidity and mortality by COVID-19 [67, 123, 127, 128]. Accordingly, despite other factors such as blood group type distribution that affect the protective antibody response to α-Gal [129], the lower prevalence of COVID-19 in most African countries may be due (in addition to under-recording of cases) to BCG-induced TRAIM [18, 130] in conjunction with other host factors that may also reduce COVID-19 symptomatology and mortality [131]. Moreover, it cannot be ignored that TB itself may both induce TRAIM and selectively build up an immunologically stronger population [132]. This selective pressure along with others with a bottleneck effect like plague might have been critical for allowing the dense concentration of individuals of the urban way of life of large human communities [133]. Therefore, the study of the innate immune and TRAIM response to live or inactivated mycobacteria and their components deserves attention to better characterize the evolution of host–pathogen cooperation.

ZEBRAFISH: A SUITABLE ANIMAL MODEL FOR THE STUDY OF TRAIM

Traditionally, mammalian models and especially mice, have been used in immunology research. In the last few decades, fish models have arisen as an attractive alternative [134–136]. Namely, the zebrafish (*D. rerio*) presents numerous advantages as animal model, such as small size, rapid life cycle and translucency of embryos, as well as sharing high genomic homology with humans [137, 138]. The major counterparts of the mammalian innate immune system, such as macrophages and neutrophiles [139], as well as cytokines, TLRs and nucleotide-binding oligomerization domain-like receptors (NLRs), have been identified in zebrafish [135, 138, 140]. For that matter, the zebrafish model has been consolidated as a unique tool to assess macrophages and neutrophils, which are, indeed, main phagocytic cells of the innate system [141]. In a series of bacterial challenge experiments in zebrafish embryos, Herbomel et al. [142] demonstrated that early macrophages are able to phagocytose *Escherichia coli* and *Bacillus subtilis* in both blood and body cavities, attaining an efficient control of bacterial infection the absence of lymphocytes. As in mammals, the entire macrophage population displayed an activated state, despite the fact that only a fraction migrated to the infection site. Likewise, zebrafish macrophages present pathogen recognition receptors in the cell surface resembling the mannose receptor of mammalian resident macrophage [142].

Over the past few decades, the zebrafish model has been extensively applied in fungal [143], bacterial [144] and viral [145] infection studies. Furthermore, TRAIM has been reported in teleost fishes [7, 146–149], including zebrafish [150]. For instance, haematopoietic stem cell expansion and emergency granulopoiesis induction occurred after infection with *Salmonella* [151] and

Shigella, respectively, in zebrafish [152]. Likewise, zebrafish primed with live or heat-killed *Salmonella typhimurium* survived better to subsequent infection with *Streptococcus iniae* than non-primed and infected fishes [153]. Moreover, correlation between several innate multigene families and phenotype of zebrafish surviving a rhabdovirus infection has been described [154].

In aquaculture, immunostimulants have been administered to fish directly via feed pellets (oral immunization) or indirectly via bath treatment (mucosal immunization) [104, 147]. Even though various immunostimulants have been tested in fish, β-glucans are by far the most used in aquaculture [155]. β-glucans have been demonstrated to induce TRAIM in several fish species, eliciting protection against pathogen infection [7, 156–158]. Indeed, zebrafish primed with β-glucans prior to infection with spring viremia of carp virus (SVCV) [159] or *Salmonella typhimurium* [153] improved survival and increased expression of genes involved in the innate immune response compared with non-primed and infected fishes.

Although not to such a great extent, the protective effect of mycobacteria has also been explored in fish models. For instance, an increased bacteriolytic activity of the serum in Japanese flounder (*Paralichthys olivaceus*) challenged with *Nocardia* was observed upon vaccination with BCG [160]. Moreover, several experiments of immunization with HIMB or α-Gal conducted in zebrafish attributed the protective effect of the immunostimulant against mycobacterial infection to the stimulation of innate response [99, 104, 121, 161]. In fact, protection correlated with the upregulation of innate components involved in immunity against mycobacteria such as the complement component C3 and the pro-inflammatory cytokine IL-1β [162]. This protective innate response was mediated by TLR activation of the Nuclear Factor kappa-light-chain-enhancer of activated B cells (NF-kB)/AKR2 pathway [99, 104, 121] and, thus, revealed immunological mechanisms compatible with TRAIM.

Ultimately, the *M. marinum*-zebrafish model has emerged as a validated tool for the research of TB pathogeny, diagnosis and treatment [163]. Overall, studies administrating live (BCG) or inactivated (HIMB) *M. bovis* to zebrafish via parenteral, oral or mucosal routes support the use of the species as an animal model to deepen in the knowledge of TRAIM-mediated mechanisms in mycobacteria-host interactions [104, 154, 164–167].

CONCLUSIONS AND FUTURE DIRECTIONS

Exposure to live microbes or microbe-derived products such as heat-inactivated cells can train the innate immune system for TRAIM-mediated responses to secondary stimuli. In particular, immunization with HIMB or α-Gal elicits protective responses against several pathogens such as *Mycobacterium*, *Salmonella*, *Plasmodium* and *Leishmania* in different hosts. These observations point out to macrophage lytic capabilities and cytokine regulation as two key components in non-specific innate immune responses against bacterial infections. These mechanisms could be surrogates of TRAIM-mediated protection indicative of host response when antigen-specific immune responses are not effective [59]. Also, the study of the TRAIM response induced by mycobacteria deserves attention to better characterize the evolution of host–pathogen cooperation both for identifying the aetiology of some diseases and for finding new therapeutic strategies. Use of zebrafish provides a convenient complete biological system that could help to carry out this research.

AUTHOR CONTRIBUTIONS

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DATA AVAILABILITY STATEMENT

Data sharing not applicable to this article as no datasets were generated or analysed during the current study.

ORCID

Elisa Ferreras-Colino https://orcid.org/0000-0002-7286-8760

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