Published in final edited form as:

Curr Opin Pharmacol. 2018 August ; 41: 128–136. doi:10.1016/j.coph.2018.05.012.

Exploiting *vita*-PAMPs in vaccines

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Abstract

Live attenuated vaccines elicit stronger protective immunity than dead vaccines. Distinct PAMPs designated as *vita*-PAMPs signify microbial viability to innate immune cells. Two *vita*-PAMPs have been characterized: cyclic-di-adenosine-monophosphate (c-di-AMP) and prokaryotic mRNA. c-di-AMP produced by live Gram-positive bacteria elicits augmented production of STING-dependent type-I interferon, whereas prokaryotic mRNA from live bacteria is detected by TLR8 enabling discrimination of live from dead bacteria. Bacterial mRNA from live Gram-negative bacteria triggers a heightened type-I interferon and NLRP3 inflammasome response. By mobilizing unique viability-associated innate responses, *vita*-PAMPs mobilize adaptive immunity that best elicits protection, including follicular T helper cell and antibody responses. Here, we review the molecular mechanisms that confer the unique adjuvanticity of *vita*-PAMPs and discuss their applications in vaccine design.

Keywords

Vita-PAMP; TLR8; inflammasome; type I interferon; monocyte; phagocyte; vaccine; follicular T helper cell; germinal center; antibody response

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Conflict of interest statement

J.M.B has a patent related to bacterial RNA: PCT/US2012/047087 "Use of Bacterial RNA or Structural Motifs thereof as adjuvants for vaccines".

Introduction

Vaccination is one of the most effective strategies to prevent infectious diseases and pandemics. It involves administration of a live attenuated or dead pathogen, or component thereof, to generate strong and specific immunity that protects against subsequent encounter with that pathogen. Historically, live attenuated pathogens have been used to successfully vaccinate populations, often with a single dose, leading to lasting protective immunity and disease control. The first vaccine involved inoculation with live cowpox to prevent smallpox infection [1]. Other live attenuated vaccines have followed suit including an oral typhoid vaccine consisting of a live attenuated strain of *Salmonella typhi* T21A, a polio virus vaccine (OPV), a rotavirus vaccine (RotaShield, ROTARIX, RIT4237 bovine), a *Shigella flexneri* 2a SC602 vaccine, a live attenuated intranasal influenza vaccine, a live attenuated yellow fever vaccine (YF-17D), as well as vaccines for chickenpox (Varicella), Rabies and tuberculosis (BCG) [2,3].

Despite efficacy, live vaccines face many challenges. In developed countries, fears of vaccination and vaccine refusal, particularly those vaccines based on live attenuated microorganisms, have resulted in poor vaccine coverage and gaps in protection of the general public [4–7]. The preservation and delivery of live vaccines require infrastructure often not available in developing countries. More recent vaccine strategies consist of dead pathogens, novel peptides, and recombinant proteins. While these are more stable and have fewer side effects, they are less effective and generally fail to convey long-lasting immunity requiring multiple boosters [8]. The exact nature of the cellular and molecular mechanisms by which broad-based immunity is elicited is elusive [9], and vaccine design has mostly followed Pasteur's principles: "isolate, inactivate, and inject" [3,10].

Adjuvants increase the immunogenicity of vaccines. Despite the variety of compounds with adjuvant properties, only a handful of adjuvants have been licensed for human use including aluminum salts (used in DTaP vaccines, the pneumococcal conjugate vaccine and hepatitis B vaccines), MF59 (an oil-in-water emulsion of squalene oil used in the influenza vaccine Fludax for the elderly), CpG 1018 (used in the hepatitis B vaccine Heplisav-B), GlaxoSmithKline Biologicals AS03 (an oil-in-water emulsion of D,L- α -tocopherol and squalene with polysorbate 80 emulsifier used in an H5N1 influenza vaccine), AS04 (an aluminum hydroxide with monophosphoryl lipid A (MPL) combination used in the cervical cancer vaccine Cervarix), and AS01B (used in the shingles vaccine Shingrix, comprised of MPL, a purified fat-like substance, and QS-21 from the bark of the *Quillaja Saponaria* tree) [11]. There is a constant need for new adjuvants for safer protective vaccines. A comprehensive understanding of the molecular basis underlying the efficacy of live vaccines will guide the design of new adjuvants that specifically target the relevant immune pathways and induce optimal long-lived protective immunity.

Vita-PAMPs: Molecular signatures of microbial viability

The innate immune system can not only discriminate self from non-self *via* recognition of pathogen associated molecular patterns (PAMPs) [12,13], but it can also discriminate between live and dead microorganisms through a distinct set of PAMPs called *vita*-PAMPs

associated uniquely with live microorganisms [14,15]. *Vita*-PAMPs are detected in combination with classic PAMPs such as lipopolysaccharide (LPS) and DNA, which are shared between live and dead microorganisms [14,15]. Detection of PAMPs in the absence of *vita*-PAMPs signifies a dead microbe, whereas detection of a *vita*-PAMP alongside a PAMP signifies live microbes and mediates a heightened immune response not warranted to dead microorganisms [15]. The activity of virulence factors associated with live pathogens elicits a vigorous immune response, but continued expression of virulence factors becomes unnecessary once a pathogen has gained access to sterile tissues. Targeting microbial viability *per se* independently of virulence factors is a far better strategy for the immune system to eliminate the infectious threat and protect tissue integrity. Two *vita*-PAMPs, bacterial mRNA in the context of a response to live Gram-negative bacteria [14,16,17], and c-di-AMP in the context of a response to live Gram-positive bacteria [18] have been identified so far. A broad array of *vita*-PAMPs likely exists, and much like PAMPs, *vita*-PAMPs may be unique to different classes of microorganisms according to the specific cellular processes conducted by each class.

***Vita*-PAMPs mobilize distinct cell-autonomous innate immune responses**

Detection of live avirulent bacteria by antigen presenting cells (APC), macrophages or dendritic cells (DC), elicits augmented levels of type-I interferon (IFN) production compared to dead bacteria, and this is dependent on bacterial mRNA for Gram-negative bacteria and cyclic-di-adenosine monophosphate (c-di-AMP) for Gram-positive bacteria [14,18] (Figure 1). Murine APC also secrete IL-1 β in response to avirulent live but not dead Gram-negative bacteria, but they do not do so to avirulent live Gram-positive bacteria [14,18] (Figure 1). Similarly, detection of live avirulent Gram-negative *Escherichia coli* by human CD14⁺CD16⁻ monocytes elicits a distinct transcriptional response compared to killed *E. coli*, including *IL12B* and *TNF* [17]. An IFN-inducible gene, IFIT2 is differentially transcribed in response to live *E. coli*, and so are several genes positively regulated by IFN- γ [17]. IL-1 β , IL-12 and TNF α are produced by human monocytes uniquely in response to live but not dead *E. coli*, *Bacillus subtilis* and *Mycobacterium bovis* BCG, as well as killed *E. coli* supplemented with bacterial RNA or CL075, an agonist for the single-stranded RNA receptors Toll-like receptor (TLR)7 and TLR8 [17].

Dissection of the innate signaling pathways mobilized in APC upon detection of *vita*-PAMPs during phagocytosis of bacteria has provided a mechanistic basis for the differential production of inflammatory cytokines to live *versus* dead bacteria. During the innate response of murine APC to Gram-positive bacteria, direct detection of c-di-AMP by the receptor stimulator of interferon genes (STING) culminates in a TBK1 and IRF3 dependent type-I IFN response [18]. STING detection of c-di-AMP triggers an elaborate cell-autonomous stress response that begins with rapid phosphorylation of effectors of the endoplasmic reticulum (ER) stress response, PERK and IRE-1 α , and subsequent inactivation of the mechanistic target of rapamycin mTORC1 [18]. These events in turn precipitate autophagy of the ER (reticulophagy or ER-phagy), which translocates STING from the ER to autophagosomes as a prerequisite to its ability to initiate type-I IFN signaling [18] (Figure 1). On the other hand, murine APC detection of bacterial mRNA released during phagosomal degradation of internalized live Gram-negative bacteria elicits an

augmented IRF3-mediated IFN- β response dependent on the TLR adaptor TIR-domain-containing adapter-inducing interferon- β (TRIF) [14] (Figure 1). Despite the differences in the innate signaling pathways mobilized downstream of live Gram-positive *versus* Gram-negative bacteria, detection of *vita*-PAMPs optimizes protection against infection in mice [14,16,18].

Innate recognition of bacterial RNA varies according to the species and cells studied [19]. Human monocytes sense bacterial RNA through endosomal TLR8 while both human and murine plasmacytoid DC do so through TLR7 [19–23]. TLR8 is recruited to bacteria-carrying phagosomes [20,22], and senses bacterial RNA during infection with Group B *Streptococci* or *Borellia burgdorferi* to induce IL-6, IL-12 and IFN- β [20,21]. Human monocytes uniquely respond to live *E. coli* and bacterial RNA through TLR8 and MyD88 with higher levels of IL-12 and TNF- α production than to killed *E. coli* (Figure 1) [17]. This pathway is conserved in porcine cells [17], but not functional in murine cells where TLR8 is suggested to be non-responsive [24]. MyD88 is also involved in sensing bacterial RNA by murine bone marrow-derived macrophages and DC, but in this case, Unc93B1, which delivers nucleic acid sensing TLRs from the ER to endolysosomes, and TLR13 have been implicated [19]. Both mouse TLR13 and human TLR8 are sensors for bacterial 23S ribosomal RNA [19,22]. Whether Unc93B1 and TLR13 can specifically sense microbial viability has not been tested.

In mice and humans, detection of live but not dead Gram-negative bacteria also elicits IL-1 β secretion [14,17,18] (Figure 1). In murine macrophages and DC, this is dependent on detection of bacterial mRNA and activation of the NLRP3 inflammasome irrespective of virulence factor expression [14]. This feature is unique to Gram-negative and not Gram-positive bacteria [18], and is likely dependent on concomitant cytosolic detection of the Gram-negative PAMP LPS [25], rather than inherent differences between RNA from Gram-positive *versus* Gram-negative bacteria [18,19]. While MyD88 is important for the transcriptional induction of *Il1b* and *Nlrp3*, murine NLRP3 inflammasome activation and IL-1 β secretion by live Gram-negative bacteria and bacterial mRNA is dependent on TRIF [14]. TRIF-dependent IFN- β licenses NLRP3 inflammasome activation through transcriptional induction of pro-caspase-11 [25]. The human NLRP3 inflammasome has also been reported to sense bacterial RNA including ribosomal, messenger, transfer, and small RNA [26], but its involvement in the ability of human APC to sense microbial viability has not been tested.

It is worthwhile noting here the pre-clinical success of mRNA vaccines for cancer and infectious disease [27]. Although mRNA vaccines were initially designed to induce expression and presentation of antigens in APC, they often contain double-stranded RNA contaminants, formed during *in vitro* transcription, that contribute to their adjuvanticity by eliciting type-I IFN production [28]. Recent vaccine formulations have enhanced the adjuvanticity of these mRNA vaccines to induce robust antigen specific protective antibody and cytotoxic T cell responses [29]. A number of ongoing clinical trials for Rabies, influenza virus, and Zika virus use lipid nanoparticles as a delivery tool, and notably interim findings from the mRNA vaccine against H10N8 and H7N9 influenza viruses report antibody titers above the expected protective threshold [29].

Bacterial viability and bacterial RNA elicit T_{FH} responses in mice and humans

Characterization of the innate response to *vita*-PAMPs has set the stage for investigating their use as unique adjuvants that could augment the performance of dead vaccines to the levels achieved by equivalent live vaccines. As proof of principle, addition of bacterial RNA to a dead vaccine comprised of killed *E. coli* elevated serum titers of class-switched antibodies to those induced by a counterpart live vaccine [14]. Identification of the *vita*-PAMP as well as the viability-associated innate pathways and effector cytokines involved enabled a targeted and systematic evaluation of the impact on the ensuing adaptive immune response [16,17]. This led to the discovery that innate detection of bacterial RNA as the *vita*-PAMP signifying microbial viability is a particularly robust signal for differentiation of both murine and human follicular T helper (T_{FH}) cells [16,17]. A comparison of three *E. coli* vaccines in mice – live, dead and a *vita*-PAMP-supplemented dead vaccine (*vita*-vaccine) – showed that only the live and *vita* vaccines induced a T_{FH} response [16]. These vaccines also activated most subsets of B cells including B1, marginal zone and the predominant follicular B2 cells. Vaccination of domestic pigs with the live but not dead form of an attenuated Salmonella vaccine increased the frequency of T_{FH} cells. Human monocytes also promoted T_{FH} cell differentiation in culture after stimulation with live but not dead *E. coli* as well as the TLR8 agonists CL075 and R848 [17]. The T_{FH} response in mice and pigs correlated with augmented germinal center formation, IgG class-switching, and frequencies of plasma cells in response to the live vaccine [16,17], and in mice to the *vita*-vaccine as well [16]. Similarly, human T_{FH} cells induced by *E. coli*-stimulated monocytes promoted plasma cell differentiation of co-cultured B cells and production of IgG [17]. These studies collectively show that prokaryotic RNA as a proxy of bacterial viability comprises a physiological innate trigger for T_{FH} differentiation in both mice and humans (Figure 2). They provide a mechanistic basis for the characteristic ability of live vaccines to elicit higher serum levels of class-switched antibodies [16,17]. They also identify a much-needed pathway for the rational design of vaccines that succeed in eliciting robust T_{FH} responses, which in turn directly promote development of the antibody response.

Whether the *vita*-PAMP c-di-AMP similarly augments T_{FH} cell differentiation remains to be assessed. Nonetheless, cyclic-di-nucleotides (CDNs) such as c-di-AMP, c-di-GMP, and c-GAMP all serve as STING agonists and have been highly successful in inducing potent antibody and T cell responses in experimental models of vaccination [30]. Their potent adjuvant activity may stem from their ability to relay microbial viability to the innate immune system. Research and development surrounding STING agonists is presently a top priority for pharmaceutical companies, many of which have several cancer immunotherapy clinical trials already underway using synthetic CDN STING agonists alone or in combination with the checkpoint blockade inhibitor anti-PD1.

How *vita*-PAMPs elicit T_{FH} cell differentiation in mice and humans

T_{FH} cells have common and divergent requirements for differentiation in mice *versus* humans [31]. Detection of the *vita*-PAMP bacterial mRNA by either human or murine APC

fulfills the criteria for instructing T_{FH} differentiation in both species [16,17]. In both mouse and human APC, PAMPs shared by live and dead bacteria elicit similar levels of certain cytokines such as IL-6. However, live and not dead bacteria uniquely induce IL-1 β production by human monocytes [17], and while dead bacteria can elicit IL-12 production [17,32], the levels produced in response to live bacteria are significantly higher than those to dead bacteria [17]. IL-12, and to a lesser extent IL-23, is the best inducer of ICOS, CXCR5, Bcl-6 and IL-21 expression by activated human CD4 T cells [32–34]. TGF- β promotes human T_{FH} differentiation by either IL-12 or IL-23, and the additional presence of IL-1 β , secreted uniquely in response to live bacteria [17], further drives commitment towards the T_{FH} fate [35]. TLR8-MyD88 signaling augments IL-12 production in response to live bacteria [17]. For both human and porcine APC, the TLR8 agonists bacterial RNA, CL075 and R848 trigger the highest levels of IL-12 and T_{FH} differentiation, while silencing monocyte TLR8 and MyD88 expression has the opposite effect [17]. T_{FH} differentiation was unaffected by the presence or absence of IL-6, IL-27, or IFN- β in human monocyte-CD4 T cell co-cultures, and neutralization of IL-1 β partially inhibited T_{FH} cell differentiation [17], consistent with its promoter role in human T_{FH} differentiation [35]. Notably, hypermorphic *TLR8* polymorphism in humans is associated with improved BCG vaccine-mediated protection [17], and IL-12 receptor β 1 deficiency is associated with a lower number of circulating T_{FH} cells and lymph node germinal centers, as well as lower avidity, albeit normal levels, of circulating antibodies [34]. Collectively, these results point to TLR8 agonists as potential T_{FH} cell skewing adjuvants.

In mice, IL-12 induces both *Il21* and *Bcl6* via the transcription factor STAT4 prior to CD4 T cell commitment to either T_H1 or T_{FH} cell fates [36]. The cytokines which play dominant roles in skewing towards mouse T_{FH} cell differentiation are the STAT3 activating cytokines, IL-6, IL-21 and IL-27 [37,38], although T_{FH} differentiation was reduced but not abrogated in the combined absence of IL-6 and IL-21 during an acute infection with lymphocytic choriomeningitis virus (LCMV) [39]. Additional cytokines such as IL-1 β may be important. LCMV and other viruses such as influenza A virus, elicit inflammasome activation and IL-1 β production in mice [40–42]. IL-1 β and IL-1R1 are also essential in the early immune response to *Mycobacterium tuberculosis* and *Candida albicans* [43]. IL-1 β is secreted in response to bacterial viability [14,17], which also triggers T_{FH} cell differentiation in both mice and humans [16,17]. Linking the highly inflammatory and tightly regulated production of IL-1 β to an adaptive T_{FH} response that best drives neutralizing antibody production would be beneficial to the host. Indeed, dissection of the mechanisms behind the higher antibody titers elicited by live vaccines has supported a role for IL-1 β as a critical mediator of T_{FH} differentiation.

Because of the central role of TRIF in orchestrating the innate response to live Gram-negative bacteria and bacterial mRNA in mice, vaccine-induced T_{FH} responses are critically dependent on TRIF and also rely on IRF3-mediated IFN- β and caspases 1 and 11 dependent IL-1 β production. Patrolling CX3CR1⁺CCR2[−] monocytes isolated from the spleen produce IL-1 β within a few hours of injecting live *E. coli*, and these monocytes instruct the T_{FH} response [16]. IFN- β and IL-1 β elicited by live bacteria and bacterial RNA play non-redundant roles in promoting T_{FH} cell differentiation [16]. In mice, IL-1 β induces expression of the T_{FH}-specific transcription factor Bcl6 and T_{FH}-specific marker CXCR5

through IL-1R1 on T cells [16], and its injection during immunization significantly increases T_{FH} cell generation [16,44]. On the other hand, TRIF-dependent IFN- β plays two distinct roles in T_{FH} differentiation; to mediate inflammasome activation and innate IL-1 β secretion through type I interferon receptor (IFNAR) engagement on APC [25,45], and to promote T_{FH} production of IL-21 through IFNAR engagement on T cells [16] (Figure 2). Notably, type I IFN induces STAT1 binding to the *Bcl6* locus in activated CD4 T cell cultures to sustain its expression but fails to induce IL-21 production on its own unless combined with IL-6 [46]. In the murine response to vaccination when cytokines such as IL-12 and IL-6 are also present, the specific and hierarchical induction of IFN- β and IL-1 β in response to a live or *vita* vaccine sets the stage for optimal T_{FH} differentiation. TRIF-dependent IFN- β licenses NLRP3 inflammasome and caspase-1 activation by upregulating and activating caspase-11 through IFN- β /IFNAR signaling [25]. As such, IL-1 β cleavage is downstream of and dependent on IFN- β production and availability. Addition of IL-1 β , whose secretion by human monocytes is also significantly higher in response to live bacteria [17], to the combination of IL-12, TGF- β , and IL-6 in activated human CD4 T cell cultures, yields the highest expression of the master T_{FH} transcription factor BCL-6 [35].

RIG-I-dependent type-I IFN produced by dermal human CD14⁺ DC in response to Dengue virus, which initiates infection through a mosquito bite, induces IL-27 expression through IFNAR signaling, and as such enables the CD14⁺ DC to instruct T_{FH} differentiation [47]. Interestingly, Dengue virus RNA replication, a proxy for viral viability, is necessary for production of both type-I IFN and IL-27 and is a critical determinant of the formation of IL-21-producing CXCR5⁺PD1⁺BCL6⁺ human T_{FH} cells [47]. Through IFNAR signaling on both mouse and human APC, type-I IFNs thus play an important role in instructing T_{FH} differentiation by controlling the secretion of additional key cytokines such as IL-1 β and IL-27 that act directly on CD4 T cells to drive their differentiation into T_{FH} cells [16,47,48]. By specifically promoting T_{FH} differentiation at different steps during the response to microbial viability, both the IRF3/IFN- β and inflammasome/IL-1 β pathways impact long-term protection, germinal center formation and IgG class-switched antibody responses in vaccinated mice [16], consistent with observations in other models [45,49]. The inflammasomes and IL-1R1 signals are also necessary for eliciting mucosal IgA and systemic IgG responses during respiratory infection with influenza A virus [50].

The unique adjuvant properties of the *vita*-PAMP bacterial mRNA stem from its ability to induce both type-I IFN and inflammasome pathways. The contribution of inflammasome activation and IL-1 β secretion to the T_{FH} cell response is in line with previous observations in mice including dependence of the potent humoral response elicited by aluminum-containing (alum) adjuvants on NLRP3 inflammasome activation [51,52], the enhancement of antibody production through IL-1 administration during immunization [53–55], and the significant impairment of antibody production noted in IL-1 α/β -deficient mice [56,57]. The attribution of alum adjuvant activity to the NLRP3 inflammasome has been contested, and the oil-in-water emulsion MF59 also works independently of NLRP3 [58–60]. Nonetheless, many particulate adjuvants that induce potent humoral responses are IL-1 β inducers by both mouse and human APC [43]. The collective experimental evidence, at least so far in mice, supports the use of inflammasome activating adjuvants in vaccine design [43]. Whether

NLRP3 inflammasome activation also promotes T_{FH} responses in humans remains to be formally tested.

CONCLUDING REMARKS

Continued research is necessary to characterize more molecules with *vita*-PAMP properties as well as to better understand the mechanisms through which microbial viability signals are communicated to different cells of the immune system. For example, different APC have been reported to elicit T_{FH} and antibody responses [16,61,62]. Thus, depending on the route of infection and the immunological context of the tissue, different requirements might be necessary to elicit a potent immune response. Elucidating the cellular and molecular events which lead to the robust immunity and long-lived memory that a live vaccine affords will guide the design of new adjuvants. *Vita*-PAMPs hold the promise of such adjuvants. Their incorporation into already existing killed and subunit vaccines would recapitulate the efficacy of live attenuated vaccines. The unparalleled performance expected of *vita*-vaccines would lay in their ability to effectively trigger the same immune pathways that live vaccines would mobilize but without their associated safety risks.

Acknowledgments

Our work on *vita*-PAMPs has been supported by the US National Institutes of Health (NIH) grants AI095245, AI127658 and AI080959, the Searle Scholars Award, and the Burroughs Wellcome Fund to J.M.B. G.B. was supported by the Crohn's and Colitis Foundation. J.M.B. and her laboratory were supported by NIH grants AI123284, AI073899, DK072201, and DK111862.

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HIGHLIGHTS

- *vita*-PAMPs are specific PAMPs expressed only by live microbes
- *vita*-PAMPs induce type I IFNs and/or inflammasome activation
- Bacterial viability and bacterial RNA elicit follicular T helper cell and antibody responses
- *vita*-PAMP supplemented (dead) vaccines induce better protection

Figure 1. The molecular and cellular events subsequent to *vita*-PAMP recognition

In mice, mononuclear phagocytes respond to microbial viability. Live Gram-positive and Gram-negative bacteria contain *vita*-PAMPs, such as bacterial mRNA and c-di-AMP, respectively, which serve as the molecular signatures of bacterial viability. Both *vita*-PAMPs induce type-I interferon through the Toll-like receptor adaptor TRIF for Gram-negative bacteria and through the stimulator of interferon genes (STING) and the kinase TBK1 for Gram-positive bacteria. In the response to phagocytosed Gram-negative bacteria, bacterial mRNA which gains access to the cytosol activates the NLRP3 inflammasome (1a) and IFNAR activation by TRIF-dependent IFN- β (1b) licenses NLRP3 inflammasome activation by promoting Caspase-11 expression (2) and interaction with caspase-1 to mediate IL-1 β cleavage and secretion (3). In the response to phagocytosed Gram-positive bacteria, c-di-AMP gains access to STING on endoplasmic reticulum(ER) membranes to induce ER stress and subsequent ER-stress induced autophagy to orchestrate translocation of STING to autophagosomal membranes, TBK1 activation, and induction of type-I IFN production.

Figure 2. Cellular activation after sensing the *vita*-PAMP bacterial mRNA

Bacterial mRNA recognition by monocytes increases follicular T helper cell differentiation, enhances IgG antibody response through higher follicular T helper cells and higher germinal center formation for mouse, human, and porcine (not shown) models. In mice, T_{FH} cell differentiation by a live vaccine comprised of Gram-negative bacteria or a dead counterpart vaccine supplemented with bacterial mRNA is dependent on TRIF signaling and downstream cytokines IFN-β and IL-1β. Both cytokines act directly on mouse T cells to promote full T_{FH} differentiation. In the human model, activation of the MyD88-dependent TLR8 receptor on monocytes induces the expression of IL-12, the main cytokine promoting human T_{FH} cell differentiation, and induces T_{FH} differentiation of co-cultured activated CD4 T cells. Human IL-1β is also produced in response to live bacteria by a yet to be explored inflammasome pathway and has additive effects on T_{FH} differentiation. Despite the different cytokine requirements for T_{FH} cell differentiation, detection of bacterial viability by either mouse or human monocytes serves as the physiological trigger for T_{FH} cell differentiation and subsequent B cell class-switching and IgG production.