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An *in vitro* functional assay to measure the biological activity of TB vaccine candidate H4-IC31

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ABSTRACT

Potency assays for vaccine products are an important regulatory requirement, and are used to assess product quality and consistency prior to lot release for clinical testing. Ideally they measure an established correlate of efficacy or protection. In cases where there is no known correlate of protection, however, a functional assay that measures a biological response to a vaccine can be applied as a potency assay. Here we describe an *in vitro* assay which quantitatively measures human T cell activation as a biological response to the TB vaccine candidate H4-IC31. The Cytokine Secretion Assay (CSA) is based on the ability of peripheral blood mononuclear cells (PBMCs) from a Bacillus Calmette-Guérin (BCG)-vaccinated human donor to process and respond to H4-IC31. The ability of H4-IC31 to stimulate a cellular immune response is measured through the quantification of secreted IFNγ and is reported as relative stimulatory activity (RSA) compared to an in-house reference standard. The CSA is specific to the H4-IC31 vaccine, determines the RSA of H4-IC31 in the range of 50% to 150% of the reference standard, and is stability indicating as it detects differences in RSA between intact and heat treated H4-IC31. Although the CSA does not provide a link to clinical efficacy, it fulfills the critical requirements for a biological potency test to assess TB vaccine candidates and can be used along with biochemical and immunochemical assays to define a product profile during clinical development, while eliminating the use of animals for product testing.

1. Introduction

Vaccine manufacturers engaging in clinical trials to assess novel vaccines face a number of regulatory requirements designed to ensure a high level of quality control is maintained for the manufacturing of clinical vaccine lots. As part of the critical quality measurements, regulatory guidelines require that potency assays be developed and utilized to test each clinical vaccine lot. Potency tests are defined as those tests which quantitatively measure relevant biological activity [4], or the specific ability of the product to effect a given result, which ideally reflects the product's mechanism of action [3].

Traditionally, potency assays testing complex vaccines involve animal tests, often with large numbers of laboratory animals. Although animal tests can measure the biological activity of vaccines with high specificity and sensitivity, they are notorious for having low precision, high variability and poor reproducibility [15]. Therefore while animal models can be highly useful in vaccine discovery and initial development, they are not sufficiently rigorous for routine potency testing in the clinical manufacturing environment. In these instances, manufacturers are in need of *in vitro* tests which can provide consistent and high resolution information on the biological activity of the vaccine product.

Ideally, the biological activity that is measured by the potency assay would be a known correlate of protection against disease (such as antibody titre). The potency assay is not required, however, to be directly linked to or predictive of vaccine efficacy, especially during early development of a novel vaccine. Efficacy

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is evaluated in clinical trial(s) that assess reduction in disease across the entire vaccinated target population. In contrast, a potency assay measures a specific biological activity of the vaccine, i.e. the ability of the product to effect a given result [4,3]. This activity may represent only a portion of the response that can ultimately lead to protection. In this paper we present the development of such a potency test for the TB vaccine candidate H4-IC31.

H4-IC31 consists of a recombinant fusion protein H4 and a novel TLR9 agonist adjuvant IC31® (Valneva Austria GmbH) and has been evaluated in Phase 1 and 2 clinical studies [9]. H4 is a fusion protein consisting of antigens Ag85B and TB10.4, which are both highly expressed by *M.tb* and BCG [2]. IC31® adjuvant consists of the antibacterial peptide KLK and a synthetic immunostimulatory oligodeoxynucleotide ODN1a [12]. The vaccine candidate H4-IC31 aims to boost the cellular immune response in BCG immunized individuals (BCG-primed infants, adolescents, and young adults) [2,18].

Potency testing for the initial trials of H4-IC31 assessed only the biological activity of the H4 component, using the Delayed Type Hypersensitivity (DTH) test in guinea pigs. In order to proceed with further clinical trials of H4-IC31, it was desirable to develop an *in vitro* potency test which could: assess T cell responses to the vaccine in cells from BCG-primed individuals, including the immunostimulatory effect of the adjuvant (which the DTH test was not able to demonstrate); be capable of distinguishing degraded from intact vaccine; and be sufficiently robust and reproducible for testing lot to lot consistency of the product in the future.

Although immune correlates of protection against *M.tb* have not yet been determined, the importance of T-cell immunity to *M.tb* infection is well accepted [1]. The focus of new vaccine development has been on induction of type-1T cell response with emphasis on the CD4 phenotype [23]. Polyfunctional T-cells co-expressing IFNγ, IL-2, and TNFα have been identified as important in the immune response to *M.tb* [2,21], and are induced in BCG-primed individuals immunized with H4-IC31 [7]. The secretion of each of these cytokines was assessed as indicators of biological response in the assay described here.

We developed a novel *in vitro* cell based assay that measures a human cellular immune response to H4-IC31 vaccine. The assay is based on the co-incubation of H4-IC31 vaccine product with PBMC isolated from BCG-vaccinated human donors. The ability of H4-IC31 to stimulate the cellular immune response, as measured through the quantification of secreted cytokine IFNγ, is reported as relative stimulatory activity (RSA) compared to an in-house reference standard formulation. The Cytokine Secretion Assay (CSA) is specific to the H4-IC31 vaccine, is capable of determining the RSA of H4-IC31 in the range of 50% to 150% of the reference standard, and is stability indicating as it shows differences between intact and heat treated H4-IC31 formulations.

2. Materials and methods

2.1. Blood donors

Thirty five healthy volunteers were recruited at Sanofi Pasteur (Toronto, Canada) by registered nurses at the on-site Health Center. Donors self-identified as having received BCG vaccination or not, and were administered the tuberculin skin test if it had not previously been performed. Written consent was obtained from the volunteers by a designated medical doctor prior to recruitment into the study. This study was reviewed and approved by a local research ethics board, the Canadian SHIELD Ethics Review Board.

2.2. PBMC isolation

PBMC from healthy volunteers at Sanofi Pasteur were isolated from freshly drawn whole blood or apheresis samples. Whole blood was diluted 1:1 with wash media [CTL Wash Supplement Medium (CTL #CTLW-010) in RPMI1640 plus glutamine (Gibco #21870)]. Apheresis samples were diluted in wash media supplemented with 36% sodium citrate (Sigma #S1804). Diluted samples were layered onto Ficoll-Paque (GE #17-5442-02) and centrifuged at 880 × g for 15 min at room temperature. PBMC interphase was collected and washed twice with wash media (240 × g for 10 min at room temperature), then frozen at 1×10^7 cells/mL (whole blood) or 3-5 × 10^7 cells/mL (apheresis samples) in IMDM (Gibco #31980) supplemented with 10% DMSO (Sigma #D2650) and 20% heat inactivated human AB serum (Valley Biomedical #HP1022), and stored at −80 °C overnight prior to transfer to liquid nitrogen.

2.3. Vaccine formulation

Experiments were performed using H4-IC31 formulations produced at Sanofi Pasteur, containing 30 μg/mL H4, 1000 nmol/mL KLK and 40 nmol/mL ODN1a. Samples were mixed by gentle inversion and diluted in IMDM medium to the indicated concentration to stimulate PBMCs. H4-IC31 lot 2016–1, considered representative of the manufacturing process, was used as the reference standard in all instances where RSA was calculated. All lots of H4-IC31 were manufactured using the same process and were expected to have similar properties. For experiments comparing responses to H4, IC31®, and H4-IC31, the H4-IC31 formulation was made from the same lots of H4 and IC31® tested singly.

Heat degraded H4-IC31, treated at 45 °C for seven weeks, was used to select donors with PBMC that had a differential response to intact and degraded vaccine. Degradation was monitored by relative quantitation of H4 by SDS-PAGE, with intact H4 determined to be reduced by 80–100% at the seven week time point. To further evaluate the capability of the assay to detect product stability, additional H4-IC31 samples were incubated for up to nine months at 55 °C. Samples stored at 2–8 °C for the same time period were tested for comparison.

2.4. Peptides

Overlapping 15-mer peptides corresponding to various regions in the H4 protein were chosen based on published and predicted immunogenic sequences [5,6,8,10,13,16,22], US patents 6599510 and 6383763). Thirty-three peptides spanning five regions of H4 across Ag85B and TB10.4 were obtained from American Peptide. Peptides were tested individually in the CSA in at final concentration of 1 or 4 μg/mL per peptide.

2.5. Cytokine secretion assay (CSA)

PBMC were thawed, diluted in warm wash media supplemented with 50 U/mL benzonase (Sigma #E8263) and centrifuged at 330 × g for 5 min at room temperature. Cells were washed a second time in wash media and resuspended in IMDM supplemented with 10% human AB serum (IMDM-10) at a concentration of 5×10^6 cells/mL. Cells were added to 96-well U-bottom tissue culture plates (100 μL/well), then serial dilutions of stimulant (peptides, adjuvant, protein, or vaccine) prepared in IMDM were added at 100 μL/well. Plates were incubated for 6–8 days at 37 °C, 5% CO_2, then centrifuged at 365 × g and the supernatant was harvested and stored at −20 °C.

Early experiments tested samples in replicate wells on the same assay plates, with IFNγ concentration as the readout. As development progressed to determining relative stimulatory activity, the assay format was modified such that samples were tested using four independently prepared replicates, with each replicate on a

separate assay plate, and individual IFNγ concentration values for each replicate were log transformed prior to analysis for RSA.

2.6. Cytokine quantification

IFNγ, IL-17a, IL-2 and TNFα in culture supernatants were quantified using V-PLEX human cytokine kits from MesoScale Discovery [MSD #K151QOD (IFNγ), #K151AOH (IL-2 and TNFα), #K151RFD (IL-17a)] or human IFNγ Ultra-Sensitive kit (MSD #K151AEC, used for CSAs testing stimulation with peptides only) according to the manufacturer's instructions, except for the use of modified calibration curves which were used at a 4X concentration as advised by the manufacturer to span the required test concentration range. PBMC supernatants were thawed and diluted 1/20 in IMDM prior to testing. MSD plates were analyzed on the MSD sector imager 2400.

2.7. Analysis of relative stimulatory activity

Relative stimulatory activity of the H4-IC31 test samples compared to the H4-IC31 reference standard formulation was calculated by PLA software (Stegmann Systems GmbH, version 2.0) using the four parameter logistic curve fit (full curve fit) with log10-transformed data, with outliers detected using the Dixon test (maximum of one outlier among 4 replicates is masked automatically by PLA software). PLA parameters were set as follows: ANOVA based on pure error separation as hypothesis testing with 95% significance of the regression and deviations from linearity; hypothesis Test (F-Test) to assess significant deviations from parallelism (95% significance); 95% confidence interval for RSA estimation.

3. Results

3.1. Donor screening and selection

PBMC from 35 adult donors, most of whom had received BCG vaccination earlier in life, were assessed for their *in vitro* response to H4-IC31. PBMC from all donors were isolated from freshly drawn whole blood and stimulated *in vitro* for six days with H4, H4-IC31, or IC31® alone, and IFNγ concentration in the culture medium was quantified. Ultimately a panel of ten donors was selected for further analysis, comprising both negative and positive PBMC responses to the stimulation. The positive donors were selected if they were BCG vaccinated, amenable to apheresis blood collection, and met all of the following criteria based on their PBMC IFNγ secretion: (1) a specific response to H4 protein; (2) increased response to H4-IC31 compared to H4 alone; and (3) no response to IC31® adjuvant alone (Fig. 1). The selected donor PBMCs were then stimulated with individual H4 peptides from a library of 33 overlapping 15-mers. Two peptides (P3 and P4) were of particular interest as data from a previous clinical trial showed that BCG-primed, H4-IC31 boosted individuals developed T cell responses mainly directed against these peptides (Supplementary Fig. S1). Responses indicated that eleven of the peptides were immunogenic, and each donor's responses were determined (Table 1).

PBMCs from seven of the selected donors were also stimulated with intact H4-IC31 or H4-IC31 which had been degraded by heat treatment (45 °C for up to seven weeks) in order to determine which donor's PBMCs showed a consistent and significant difference in response to intact vs degraded vaccine. PBMC from four of the donors showed a measurably different response to degraded versus intact H4-IC31. Of these four, PBMC from donor TB04 were selected for further assay development as they: (1) had a strong IFNγ response to H4-IC31; (2) consistently demonstrated discerning responses to intact versus degraded vaccine when tested; and (3) responded to multiple peptides including P3 and P4 (see Table 1 for donor selection summary).

3.2. Selection of indicator cytokine and assay duration

Assay development proceeded from this point using frozen PBMC, which were previously seen to give comparable responses to those with freshly collected PBMC in the CSA (data not shown). IFNγ, IL-2, TNFα, and IL-17a responses were each assessed as possible indicators for recall responses over 1–12 days of stimulation with a fixed dose of H4-IC31 (0.01 μg/mL) (Fig. 2). IL-17a production was found to be very low, short-lived, and did not distinguish between degraded (heat treated) and non-degraded vaccine. IL-2 secretion was observed within 1 day; however the IL-2 levels declined to background levels by day 5. Furthermore, the IL-2 response did not differentiate between intact and degraded vaccine. Secretion of IFNγ and TNFα arose after four days and stayed elevated for up to 12 days of stimulation. Stimulation with degraded vaccine resulted in a reduction of both IFNγ and TNFα secretion compared to intact vaccine, with a greater differentiation seen over a longer time period for IFNγ secretion. Therefore IFNγ was chosen as the indicator cytokine for the CSA assay, with an eight day incubation. The stimulation conditions selected for the CSA were also shown to elicit a relevant functional effect in an *in vitro* granuloma model using *M.tb* (Supplementary Fig. S2). In order to secure an abundant supply of cells for assay development and product testing into the future, a total of two apheresis collections were performed, five months apart, and the two batches were compared in the CSA with equivalent results obtained.

3.3. Dose range and RSA

A dose range for H4-IC31 stimulation was identified which covered the range of IFNγ responses of PBMC stimulated with H4-IC31 prepared at 1.5X and 0.5X normal concentrations, representing super-potent and sub-potent lots of vaccine, respectively (Fig. 3A). The dose response curves were assessed for parallelism using a 4-PL curve fit with the F-test for parallelism (PLA v2.0), and RSA was determined for the 0.5X and 1.5X preparations (Fig. 3B). The RSA for 0.5X and 1.5X preparations were close to expected, i.e. 0.5 and 1.6, respectively.

3.4. Stability indication

The CSA was used to assess heat degraded material in order to confirm its ability to monitor product stability. H4-IC31 vaccine was subjected to heat treatment at 55 °C for up to nine months and then tested in the assay (Fig. 4). RSA of the vaccine was shown to be reduced to 0.6 after eight weeks and < 0.1 after nine months of heat treatment, while the RSA remained at 1 when vaccine was stored under the preferred 2–8 °C condition for the same time period.

4. Discussion

In order to assess the biological activity of vaccine lots being released for clinical trials, an *in vitro* cytokine secretion assay (CSA) has been developed which uses cryogenically preserved PBMC from a BCG-vaccinated human donor with a broad peptide response to TB vaccine candidate H4-IC31. Vaccine is co-incubated with the PBMCs, and IFNγ production is quantified and compared to an in-house reference vaccine with relative stimulatory activity as the assay readout.

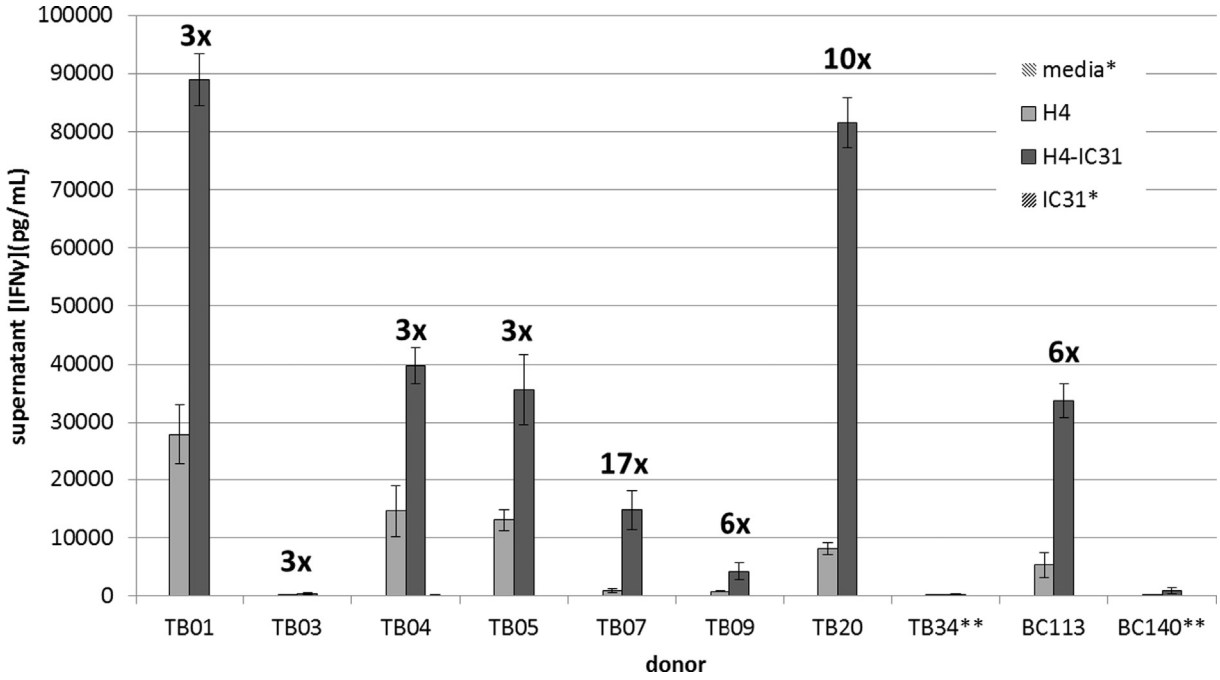


Fig. 1. IFNγ response from PBMC stimulated with H4, H4-IC31, or IC31® alone. PBMC from 10 different TB donors were incubated for 6 days with H4 protein alone (0.1 μg/mL), IC31® alone (3.3 nmol/mL), or H4-IC31 (0.1 μg/mL H4 – 3.3 nmol/mL IC31®). IFNγ concentration in culture supernatant was quantified using MSD; standard deviation of 4 replicate wells from one assay plate is shown. The fold-increase (X) in IFNγ response to incubation with H4-IC31 compared to H4 alone at the same concentration is indicated. *[IFNγ] < 75 pg/mL when stimulated with media or IC31® alone for all donors. **[IFNγ] < 250 pg/mL when stimulated with H4-IC31 for donors TB34 and BC140.

Table 1
Summary of responses from a panel of tests performed on PBMC isolated from freshly drawn whole blood.

Donor	BCG Vaccinated	TB skin test	IFNγ secretion in response to H4-IC31	At least 2-fold increase in IFNγ with IC31®	IFNγ response to H4 peptides: Total # peptides (individual peptide)	Significantly lower IFNγ response to degraded material[1]
TB01	+	+	high	Y	2 (P7, P10)	Yes
TB03	+	–	low	Y	1 (P9)	not tested
TB04	+	+	med	Y	5 (P3, P4, P7, P8, P10)	Yes
TB05	+	+	med	Y	5 (P1, P2, P5, P6, P7)	Yes
TB07	+	+	low	Y	2 (P3, P4)	Yes
TB09	+	–	low	Y	1 (P7)	No
TB20	+	+	high	Y	4 (P3, P4, P7, P10)	marginal
BC113	+	+	med	Y	2 (P4, P11)	marginal
BC140	+	–	negative	N/Ap	0	not tested
TB34	–	–	negative	N/Ap	0	not tested

	Amino acid range	Sequence		Amino acid range	Sequence		Amino acid range	Sequence
P1	008–022	LPVEYLQVPSPSMGR	P5	111–125	LSANRAVKPTGSAAI	P9	308–322	YAGTLQSLGAEIAVE
P2	018–032	PSMGRDIKVQFQSGG	P6	116–130	AVKPTGSAAIGLSMA	P10	313–327	QSLGAEIAVEQAALQ
P3	091–105	KAGCQTYKWETFLTS	P7	288–302	MSQIMYNYPAMLGHA	P11	342–356	QAQWNQAMEDLVRAY
P4	096–110	TYKWETFLTSELPQW	P8	293–307	YNYPAMLGHAGDMAG			

Donors were screened for vaccination status for BCG (verbal) and were given a tuberculin (TB) skin test or self-reported results of previous TB skin test if known. In addition, donor PBMCs were scored for IFNγ secretion. Specifically, IFNγ from culture supernatants was measured after 6 day incubation of PBMCs in the presence of H4-IC31 (0.1 μg/mL H4- 4 nmol/mL IC31®), H4 alone (0.1 μg/mL), IC31® alone (4 nmol/mL), individual H4 peptides (1 or 4 μg/mL), or H4-IC31 formulation (lot 2016–5, 0.01 μg/mL H4-0.33 nmol/mL IC31®) which had or had not been heat treated at 45 °C for 7 weeks ('degraded material') ([1]comparison between response from heat treated and non-heat treated lot 2016–5 stimulation performed by Dunnett Adjustment, $p < 0.05$). Increase with IC31 was calculated by comparing IFNγ secretion following stimulation with H4-IC31 vs H4 alone. Scores for [IFNγ] in culture supernatant: negative (0–20 pg/mL) low (20–1000 pg/mL) medium (1000–10,000 pg/mL) high (>10,000 pg/mL).

The objective of vaccine potency assays is to measure the biological activity of the product and assess the product quality, consistency and stability during the manufacturing process and storage. Currently most licensed vaccines against infectious diseases generate humoral immune responses, and prevention of infection for the vaccine-targeted diseases correlates with the induction of specific antibodies [19,20]. The potency assays for these vaccines are based on *in vivo* models with assessment of serological responses (immunogenicity), or biochemical and immunochemical analyses which measure the preservation of specific vaccine epitopes (antigenicity assays) [14]. In the case of TB, however, it has been a challenge to develop a potency assay due to the absence of correlate(s) of protection for the disease. Therefore, we have focused on developing a potency assay that measures an *in vitro* biological activity of the TB vaccine.

The H4-IC31 vaccine is designed to boost memory responses initiated in early childhood by BCG vaccination. PBMC from BCG immunized people were selected for use in the CSA and assessed for responses by measuring secretion of IL-17a, IFNγ, IL-2, and TNFα following stimulation with H4-IC31. Most BCG-vaccinated

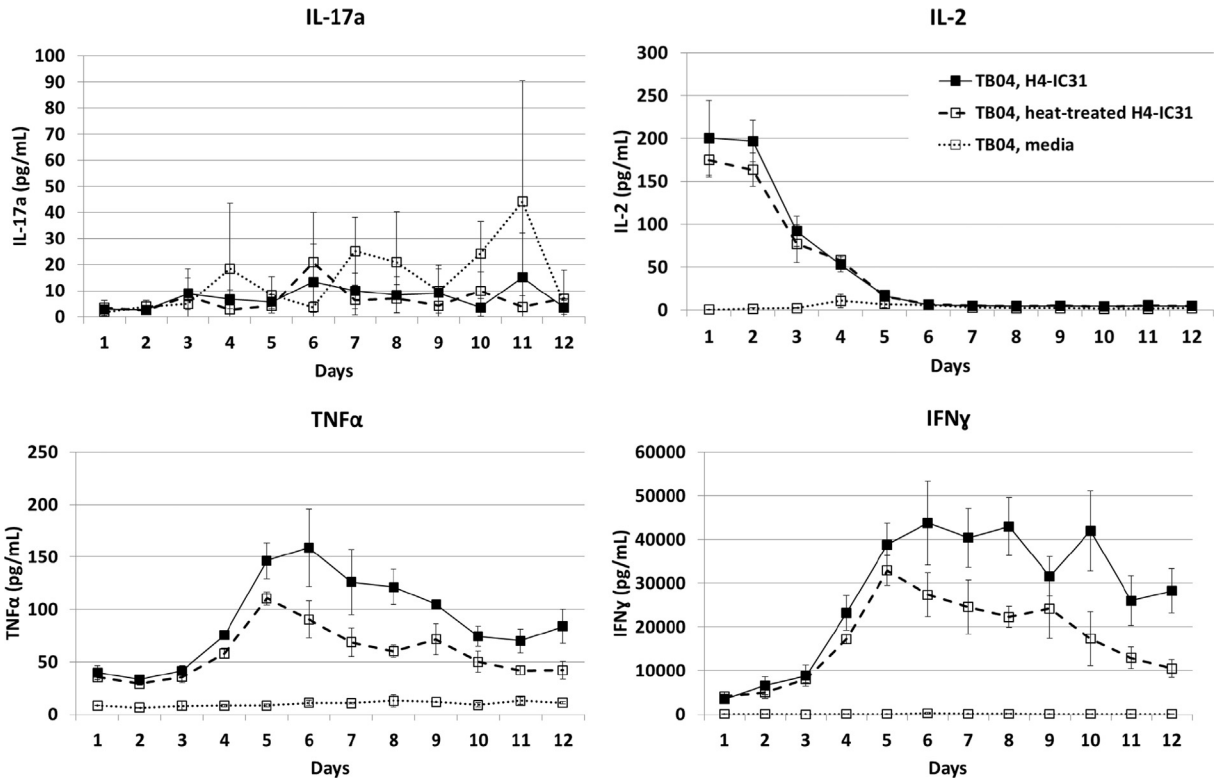
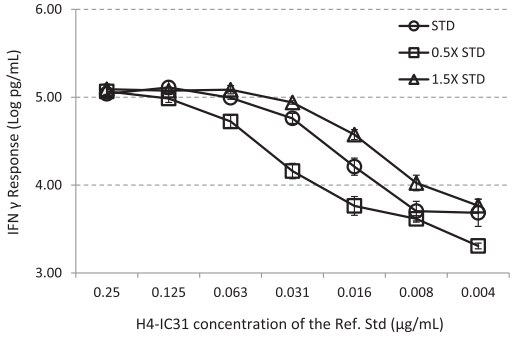


Fig. 2. Cytokine secretion over time: IL-2, IL-17a, TNFα and IFNγ concentration on days 1–12 for PBMC from donor TB04. PBMC were isolated from donor TB04 following apheresis and incubated with H4-IC31 formulation (lot 2016–5, 0.01 μg/mL H4 – 33.3 nmol/mL IC31®) which had or had not been exposed to 45 °C (heat-treated) for seven weeks, or media alone. Culture supernatants were collected each day for 12 days (one assay plate with five replicate wells per sample harvested each day). Concentrations of IL-2, IL-17a, TNFα, and IFNγ in culture were quantified using MSD. Average of five replicate wells per day, on the same plate, shown with standard deviation.

A



B

Sample	H4 conc. Range (μg/mL)	Expected RSA	PLA-Four parameter logistic curve analysis		
			Observed RSA	95% CI	Relative CI
0.5X STD	0.125-0.002	0.5	0.5	0.4-0.5	90%-111% (21%)
1.5X STD	0.375-0.008	1.5	1.6	1.4-1.7	90%-111% (21%)

Fig. 3. IFNγ response of TB04 PBMC following stimulation with H4-IC31 (A) and relative stimulatory activity of the 0.5X and 1.5X preparations of reference standard (B). PBMCs from donor TB04 were stimulated with H4-IC31 reference standard lot 2016–1 (STD) (dose range of 0.25–0.004 μg H4/mL), 0.5X STD (dose range of 0.125–0.002 μg H4/mL) and 1.5X STD (dose range of 0.375–0.008 μg H4/mL). After 8 days of culture, PBMC supernatants were collected and evaluated for IFNγ concentration using MSD. Relative stimulatory activity was calculated using PLA software with 4 parameter logistic curve analysis. Results are representative of several similar *in vitro* cytokine secretion assays performed with independent sample dilutions on three separate plates (triplicate wells, error bars denote standard deviation). STD: standard; Ref. Std: reference standard; Conc.: concentration; RSA: relative stimulatory activity; CI: confidence interval.

donor PBMCs had a measurable IFNγ response to H4-IC31, with at least some increased response in the presence of adjuvant IC31® and decreased response to degraded vaccine. Responses were seen to vary across donors, as expected in a collection of PBMCs from adult individuals immunized in childhood. In order to be used in a potency assay for clinical lot release, however, it was important to identify donor PBMCs which had a sufficiently strong response to be reproducibly measured with statistically significant results, and therefore support the rigour required for product testing. It was also desirable that the response generated include reactivity against peptides that are known to be differentially induced by H4-IC31 boosting of BCG-primed individuals. PBMC from one donor that met all of these requirements were identified and banked. Although the PBMC from this individual are not intended to represent all clinical trial subjects, they are suited for *in vitro* use in a potency assay that can measure the required attributes of biological activity outlined herein.

In the CSA, primary PBMCs from the BCG-immunized donor process, present, and respond to a vaccine over a period of multiple days, reflecting the combined multicellular response of antigen presenting cells and T cells against the H4 protein in the context of IC31® adjuvant. Since antigen and adjuvant together contribute to immunogenicity, it is important that a biological activity test be able to measure the influence of adjuvant in order to assess the functionality of the vaccine product as a whole. The CSA shows that the IFNγ response to H4 is increased in the presence of IC31® adjuvant, and there is no background activity from adjuvant alone.

Exposure to heat is an important means used to assess stability of a vaccine product. In the CSA, vaccine which was heat treated induced a lower response than intact vaccine, with RSA decreasing

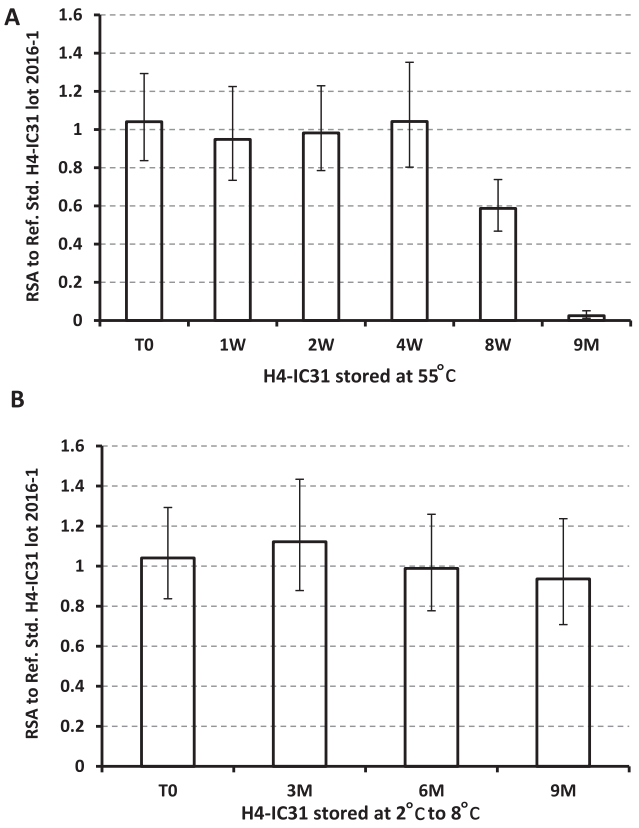


Fig. 4. Relative stimulatory activity of H4-IC31 lot 2016–2 stored for up to 9 months at 55 °C (A) or 2–8 °C (B) as determined by IFNγ secretion assay. Stability samples were kept for zero (T0), one week, 2 W, 4 W, 8 W or 9 months at 55 °C (A) or for T0, 3 M, 6 M or 9 M at 2–8 °C (B). IFNγ secretion assay was performed using PBMCs from donor TB04 stimulated with 0.250–0.004 μg/mL of H4-IC31 stability samples or the reference standard lot 2016–1. After 8 days of culture, IFNγ in PBMCs was quantified using MSD. The relative stimulatory activity was calculated using PLA software with 4 parameter logistic curve analysis. Results represent one *in vitro* cytokine secretion assay performed with independent sample dilutions on three or four separate plates (triplicate or quadruplicate wells). The error bars denote the 95% confidence intervals of the RSA values. W: week; M: month; Ref. Std: reference standard; RSA: relative stimulatory activity.

further with extended heat treatment, confirming that the assay's RSA readout is reflective of stability of the vaccine.

Since the development of the CSA as a potency assay, additional clinical trials of H4-IC31 have been concluded, and show that in the conditions tested, H4-IC31 boosting of BCG-vaccinated individuals did not provide significantly improved protection compared to boosting with BCG alone [17]. This observation serves to illustrate the challenges in determining the complex mechanisms and correlates of protection against *M.tb*, and by extension, the development of an optimally protective vaccine. Nonetheless, researchers must continue to explore and develop improved vaccines for TB, and any new or improved candidate vaccine will face the same requirements for potency testing of clinical trial lots.

The advantages of using a potency assay such as the CSA instead of existing animal models to assess the immune response to vaccines for the purpose of clinical lot release are several. In addition to ethical reasons for reducing routine animal testing for product quality, the use of *in vitro* tests can eliminate the variability associated with animal systems, and the length and cost of *in vivo* immunization studies. *In vitro* tests are also more amenable to validation, which is a requirement for a potency assay to release vaccine product. Importantly, testing using human cells instead of animal cells also ensures that the antigens and epitopes being assessed in the assay are relevant to vaccine performance in humans. In the case of the CSA, the use of primary PBMCs from a BCG-immunized donor reflects the multicellular responses of antigen presenting cells and T cells together to the H4 protein along with the immunomodulating effect of the adjuvant IC31®. The variability associated with the use of primary cells is minimized by the use of apheresis collection of PBMC, where one collection provides sufficient cells for at least five years of routine testing, and subsequent collections could be bridged to ensure consistent results.

In summary, we have developed a novel *in vitro* cell based biological activity assay, the CSA, for testing TB vaccine candidate H4-IC31. The assay is quantitative, can assess the immunostimulatory effect of the adjuvant, and is stability indicating. In addition, the development of this assay is fully aligned with the 3Rs principle to reduce, refine and replace animal testing. While *in vitro* IFNγ secretion by PMBCs alone is not considered to be directly indicative of protection from infection, the CSA represents a potency assay measuring a relevant biological response to H4-IC31. The CSA assay, in conjunction with physicochemical and antigenicity testing, can play a critical role in quality control of drug products to be released for clinical trials.

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Declarations

Marin Ming, Danielle Salha, Lidice Bernardo-Reyes, Kimberley Williams, Leslie G. Chan, Anke Pagnon, Fabienne Piras, Jin Su, Beata Gajewska, and Lucy Gisonni-Lex were employees of, and may hold shares or stocks in, Sanofi Pasteur at the time of this work. At the time of submission, Sanofi Pasteur is a partner in the clinical development of the H4-IC31 vaccine.

The laboratory of Pappachan Kolattukudy and Nidhi Kapoor at the University of Central Florida, Burnett School of Biomedical Science received funding from Sanofi Pasteur to conduct the *M.tb* infection and granuloma research described in this manuscript.

Data statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.vaccine.2019.04.035.

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