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A high ratio of IC31® adjuvant to antigen is necessary for H4 TB vaccine immunomodulation

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Abbreviations: BCG, Bacillus Calmette-Guérin; TB, Tuberculosis; hWB, Human Whole Blood; H4, TB, Antigen; IC31®, Valneva Adjuvant consisting of KLK and ODN1a; KLK, Antimicrobial peptide H-KLKL$_5$KLK-OH; ODN1a, Oligodeoxynucleotide; IFNγ, Interferon gamma; TNFα, Tumor Necrosis Factor alpha; TLR, Toll-like Receptor; TLR4, Toll-like Receptor 4; TLR9, Toll-like Receptor 9; TLR4A, TLR4 Agonist; TLR4-Adjuvant, TLR4A combined with an aluminum salt adjuvant

A tuberculosis (TB) vaccine consisting of a recombinant fusion protein (H4) and a novel TLR9 adjuvant (IC31) is in clinical development. To better understand the H4-IC31 ratio, we measured the binding capacity of IC31 for H4 protein and immunized mice with formulations that contained limiting to excess ratios of IC31 to H4. An immunomodulated H4-specific IFNγ response was only observed when IC31 was present in excess of H4. Since TLR expression is species-specific and the vaccine is intended to boost BCG-primed immunity, we questioned whether data in mice would translate to humans. To address this question, we used the fresh human Whole Blood (hWB) recovered from BCG-vaccinated subjects to screen H4-IC31 formulations. We found IC31 modulation in hWB to be quite distinct from the TLR4-Adjuvant. Unlike TLR4-Adjuvant, IC31 formulations did not induce the pro-inflammatory cytokine TNFα, but modulated a robust H4-specific IFNγ response after 12 d of culture. We then re-stimulated the fresh hWB of 5 BCG-primed subjects with formulations that had excess or limiting IC31 binding for H4 protein and again found that an immunomodulated H4-specific IFNγ response needed an excess of IC31. Finally, we monitored the zeta (ζ) potential of H4-IC31 formulations and found that the overall charge of H4-IC31 particles changes from negative to positive once IC31 is in greater than 9-fold excess. Using two diverse yet mutually supportive approaches, we confirm the need for an excess of IC31 adjuvant in H4 TB vaccine formulations and suggest surface potential may be an important factor.

Several promising TB vaccine candidates utilize a subunit and adjuvant approach to boost immunity induced by previous BCG vaccination.[1-4] One example is a combination of H4 antigen with IC31 adjuvant (H4-IC31). The H4 protein (or HyVac4), is a fusion of 2 *Mycobacterium tuberculosis* antigens: Ag85B and TB10.4,[5] while IC31 is a synthetic cationic adjuvant system, consisting of an antimicrobial polypeptide (KLK) and a phosphodiester-backboned immunostimulatory oligodeoxynucleotide (ODN1a) combined in a KLK:ODN1a molar ratio of 25:1.[5] This novel adjuvant is reported to stimulate potent and sustained antigen-specific Th1 adaptive memory through a TLR9 signaling pathway.[6,7] Non-clinical investigations in a variety of animal models have revealed favorable toxicology profiles.[8] Additionally, H4-IC31 is protective against pulmonary TB when evaluated in animal challenge studies.[5,8,9] Detailed studies in mice have also shed light on the function of IC31. The adjuvant has been shown to form a depot at the injection site where dendritic cells (DCs) and other antigen presenting cells migrate and take up small quantities of the vaccine. The DCs subsequently drain to lymph nodes where they stimulate specific immunity.[10,11]

Recent work in mice suggests protective immunity induced by H4-IC31 is highly sensitive to antigen dose,[12] while a clinical response to a similar vaccine was directly influenced by the adjuvant dose.[13] Understanding the functional relationship between the adjuvant and antigen is critical to define vaccine formulations. Despite the need to understand vaccine-induced immune responses, tools available to assess biological functionality are limited. Approaches to study and screen vaccine candidates, in terms of modulated immunity in a non-clinical setting, rely almost exclusively on animal models, most notably mouse models. However, it is well known that immunogenic and evolutionary differences exist between mice and humans, and

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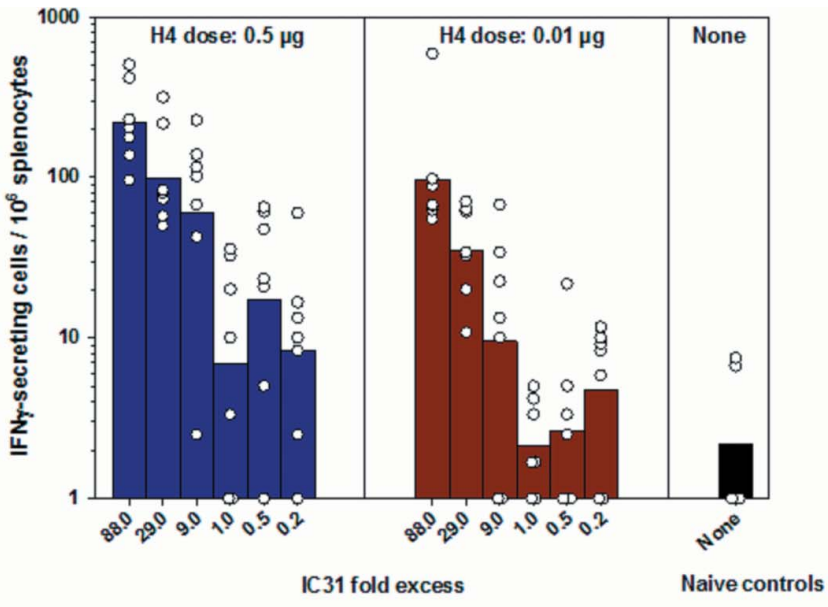
expression and usage of TLR9 is species-specific.[14-16] Although animal models provide a valid representation of the complex *in vivo* environment, data from these studies require translation to humans to ensure more realistic and specific functional evaluations.

The present study provides insight into how binding of H4 to IC31 impacts the immune function of the whole formulation. To determine binding within H4-IC31 formulations, 2 independent adsorption isotherm experiments were performed. Isotherms were analyzed by plotting the amount of H4 adsorbed onto IC31 particles (mg H4/nmol KLK) versus the concentration of H4 in the solution after adsorption. The data for H4 adsorption vs. H4 in solution were plotted according to the linearized Langmuir isotherms (correlation coefficients of 0.998 and 0.993) and the binding capacities were determined using the Langmuir equation. The average H4 adsorptive capacity from the 2 studies (N = 1: 0.00087 mg H4/nmol KLK and N = 2: 0.00089 mg H4/nmol KLK) was determined to be 0.00088 mg H4/nmol KLK. The same lot of IC31 and H4 were used in all further studies.

In order to investigate the connection between H4-IC31 binding and immune function, *in vivo* mouse studies were performed. Previous studies had identified high (0.5 μg) and low (0.01 μg) doses of H4 in a mouse immunogenicity model (inbred CB6F1 female mice).[12] Based on the determined IC31 adsorption capacity measurement, formulations of high and low H4 dose were prepared with limiting, minimal or excess amounts of IC31 for H4 binding, and used to vaccinate groups of 7 naïve mice. Splenocytes were recovered after the second immunization and the frequency of H4-specific IFNγ-secreting cells was determined by ELISPOT assay. An H4-specific cellular response was detectable in the vaccinated groups, with a statistically



IC31 Fold Excess	Formulation (nmol/mL KLK)* H4 dose: 10 μg/mL (0.5 μg)	H4 dose: 0.2 μg/mL (0.01 μg)	IC31 present to adsorb H4†
88	1000	20	Excess
29	334	6.56	Excess
9	100	2	Excess
1	11.36	0.24	Minimal
0.5	5.76	0.08	Limiting
0.2	2.16	0.04	Limiting

* Formulations contain 0.5 or 0.01 μg of H4 combined with the indicated amount of IC31 adjuvant, expressed in molar equivalents of KLK.

† Based on the measured IC31 adsorption capacity, formulations were prepared with limiting, minimal or excess amounts of IC31. 'Excess' refers to doses consisting of IC31 in excess amounts than required for 100% H4 adsorption; 'Minimal' doses consist of the minimum amount of adjuvant required for 100% H4 adsorption; 'Limiting' refers to doses with adjuvant amounts below the amount needed for 100% H4 adsorption.

Figure 1. Mouse study reveals that excess IC31 is necessary for modulation of the H4 response. H4-specific IFNγ response was measured in Day 36 splenocytes after 2 intramuscular (IM) immunizations in right and left quadriceps (Day 0 "priming" and Day 21 "boost") with H4-IC31 formulations. Formulations were prepared prior to each injection by mixing 2 concentrations of H4 (50 μg/mL or 1μg/mL) with serial dilutions of IC31 in a 1:4 volumetric ratio on a rotator. The total volume of formulations used for each injection was 50 μL. Statistical analysis was performed with the SAS® Software version 9.2 at an α risk of 5% for the main effects and 10% for the interaction effects. Comparison of H4-IC31 formulations with the minimal formulation was performed based on an ANOVA model. A mixed model with IC31 doses (log_{10}) as fixed factors was used with Dunnett adjustment. Bars represent the geometric mean titers of IFNg responses in each group. White circle symbols represent individual IFNg responses in each group.

significant IC31 dose-effect (**Fig. 1**). At both the high and low H4 doses, IC31 in excess modulated statistically higher IFNγ levels than the IC31 limiting doses ($P \leq 0.013$ at 0.5 μg of H4; $P \leq 0.05$ at 0.01 μg of H4). No statistically significant difference was observed between the minimal and limiting doses of IC31, irrespective of the H4 dose tested ($P \geq 0.495$).

The H4-IC31 vaccine is designed to boost the response primed by BCG vaccination. However, stimulating BCG-primed immunity in the mouse model is atypical, since mice need to be rested for several months after vaccination. Also, TLR9 expression is known to be species-specific and it is unclear whether adjuvant immunomodulation will be the same in mice and humans. To support the mouse observations and extend them to humans, we applied a fresh hWB approach using samples donated by BCG-primed subjects, and re-stimulated cultures *ex vivo* with vaccine formulations. A proof of concept (POC) for the hWB approach has already been established using H4-TLR4-Adjuvant formulations.[14] A particular feature of the hWB approach is the ability to demonstrate profound adjuvant-modulated antigen-specific responses.

While a reproducible pro-inflammatory TNFα cytokine response is induced within 24 hours of *in vitro* stimulation with TLR4-Adjuvant stimulation,[14,17] no such response was observed with IC31 (data not shown), confirming results from a previous report.[6] The data demonstrates a distinct *in vitro* functional difference between TLR4-Adjuvant and IC31 activity. Also, when an H4-specific IFNγ memory response was monitored in a 6 d hWB culture, either weak or no IC31 immunomodulation was observed. The data again contrasted with that of a TLR4-Adjuvant modulated H4 response (**Fig. 2A**). Since IC31 is reported to up-regulate activation markers,[7] we hypothesized that the T cell response modulated by IC31 may be more

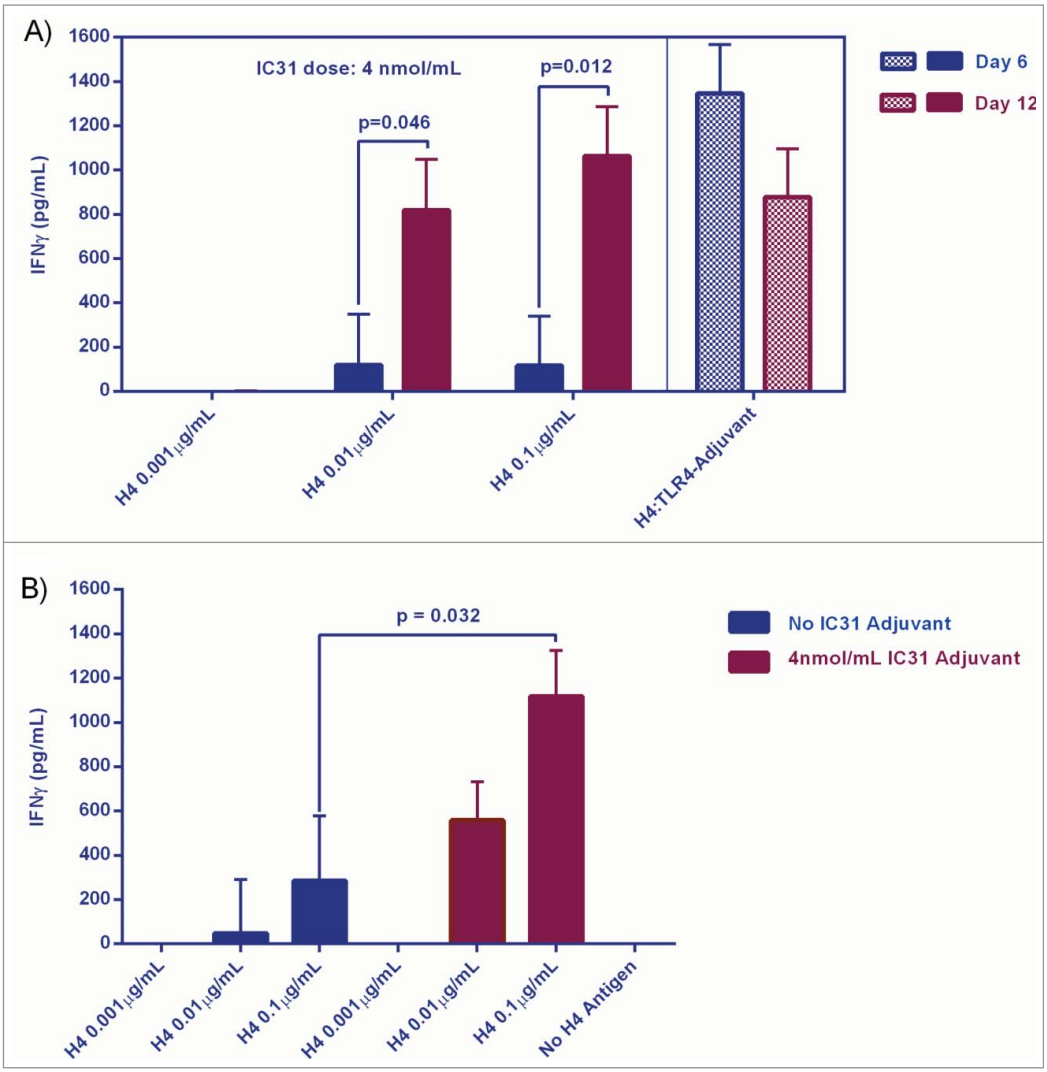
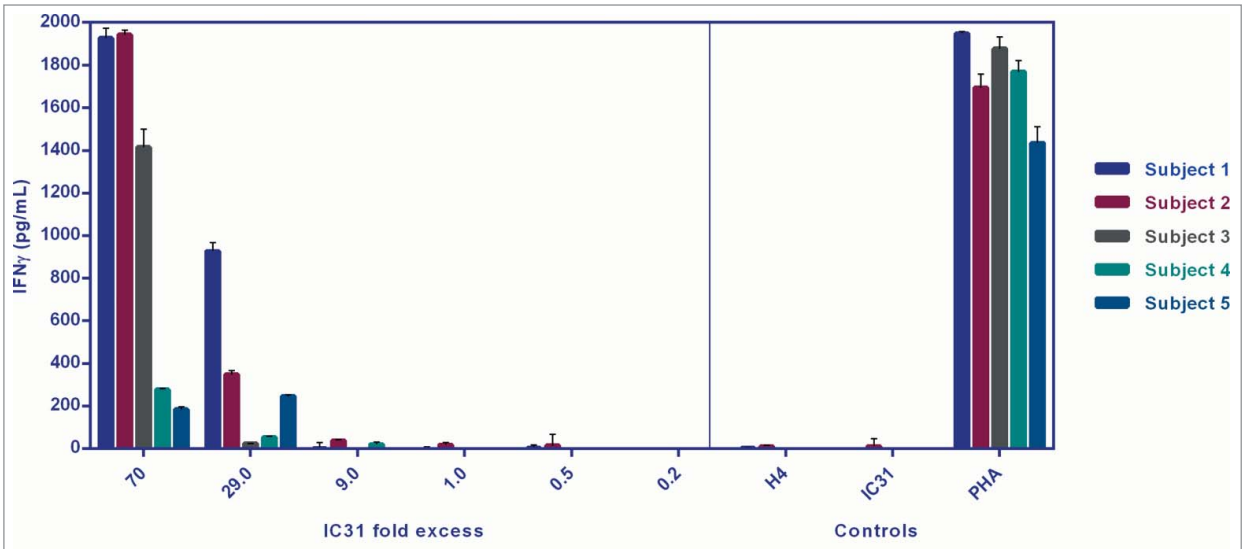


Figure 2. Modulation of H4 response by IC31 in hWB over an extended incubation period. Fresh blood was collected into heparinized 10 mL tubes from selected healthy BCG-vaccinated or Tuberculosis Skin Test positive volunteers. H4-IC31 formulations were prepared by mixing H4 (diluted in serum-free medium to 1 μg/mL, 0.1 μg/mL, or 0.01 μg/mL) with IC31 (diluted in serum-free medium to 10 nmol/mL) in a 1:4 volumetric ratio on a rotator. The mixed product was shown to remain stable for up to 24 hours at room temperature. Upon preparation, formulations were immediately added in 6 replicates on 96-well, U-bottom plates (100 μL/well). hWB (10-fold diluted) was mixed freshly with the formulations on the plates (within 24 hours of plating the formulations), resulting in an additional 2-fold dilution of the blood and formulations. IFNγ responses were measured at time points of Day 6 (D6) or Day 12 (D12) in supernatants of treated hWB cultures. For each formulation containing IC31, 2 observations were made per subject as 2 IC31 lots were tested per formulation. Statistical regressions were performed with JMP Statistical Discovery Software version 7.0.1 using ordinary least squares with a Restricted Maximum Likelihood (REML) mixed model. Differences in immune responses were considered to be significant when *p* values of less than 0.05 were obtained: (**A**) D6 and D12 responses to H4-IC31 formulations containing a constant concentration of IC31 and 3 titrations of H4. H4-TLR4-Adjuvant was used as an internal positive control. The cultures were harvested and replenished on D6 with serum-free medium (n = 4); (**B**) D12 responses to H4 antigen at 3 dosages formulated with or without IC31 adjuvant. The cultures were not harvested or replenished on D6 for the last 3 subjects (n = 7).



IC31 Fold Excess	Formulation (nmol/mL KLK)* H4 dose: 0.05 μg/mL	IC31 present to adsorb H4†
70.0	4.00	Excess
29.0	1.65	Excess
9.0	0.51	Excess
1.0	0.06	Minimal
0.5	0.03	Limiting
0.2	0.01	Limiting

* Formulations contain 0.05 μg/mL of H4 combined with the indicated amount of IC31 adjuvant, expressed in molar equivalents of KLK.
† See Figure 1 legend.

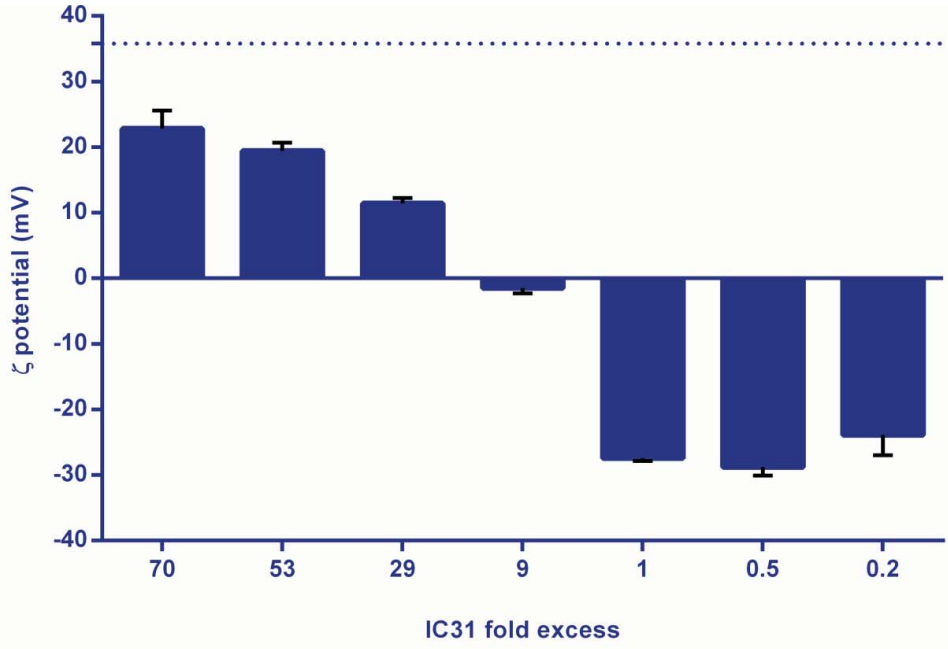
Figure 3. hWBA confirms the importance of excess IC31 for the optimal modulation of H4 response. ELISA analysis of IFNγ responses measured in supernatants of hWB cultures 10 d post-treatment with H4-IC31 formulations containing a constant dose of H4 and various doses of IC31 (n = 5). Formulations were prepared by mixing H4 (5 μg/mL) with serial dilutions of IC31 in a 1:4 volumetric ratio on a rotator. This was followed by a 10-fold dilution of formulations in serum-free medium. Upon preparation, formulations were immediately added in 6 replicates on 96-well, U-bottom plates (100 μL/well). hWB (10-fold diluted) was mixed freshly with the formulations on the plates (within 24 hours of plating the formulations), resulting in an additional 2-fold dilution of the blood and formulations. The cultures were harvested and replenished on D6 with serum-free medium. 'Limiting dose' where adjuvant amount was 0.5 and 0.2-fold below 100% H4 adsorption; 'minimal dose' consisting of minimum amount of adjuvant required for 100% H4 adsorption; and 'excess dose' consisting of IC31 in 9-, 29-, and 70-fold higher than required for 100% H4 adsorption. Controls: H4 (0.05 μg/mL), IC31 (4 nmol/mL), Phytohemagglutinin (PHA, 5 μg/mL).

gradual. Therefore, in order to optimize the H4-IC31 response for *in vitro* cultures, we extended the hWB culture beyond the typical 6 d, and the H4 antigen was titrated to 0.1, 0.01, and 0.001 μg/mL to demonstrate an H4 antigen dose response with an optimized concentration of the adjuvant (4 nmol/mL KLK). In line with our hypothesis, a significant IC31-modulated, H4-specific dose-dependent response was observed when the cultures were extended for 12 d (**Fig. 2A and B**). Although we did not monitor expected cell death in hWB cultures over the culture periods, no evidence of non-specific effects from IC31 and H4 were observed (**Figs. 2 and 3**). Overall, these results demonstrate that modulation of an H4 -specific IFNγ memory response with IC31 adjuvant can be assessed by extending hWB cultures for up to 12 d. The data also reveal that immunomodulation of IC31 and TLR4 in hWB is quite distinct (**Fig. 2A**).

Once the application of the hWB approach for H4-IC31 formulations was established, further hWB studies were designed to monitor H4 formulations with excess, minimal, and limiting doses of IC31 for H4 binding (**Fig. 3**). An H4 dose of 0.05 μg/mL and an upper target of 4 nmol/mL of KLK were selected as an optimal antigen to adjuvant ratio based on results in **Figure 2**, corresponding to an IC31 fold excess of 77. The remaining H4-IC31 formulations consisted of 0.05 μg/mL of H4 with titrating amounts of IC31 at the same ratios as the mouse study in **Figure 1**. Fresh hWB from 5 BCG-primed subjects were stimulated with H4-IC31 formulations and the IFNγ released into the culture supernatant was measured by

ELISA after 10 d of culture. Consistent with observations from the mouse study, an H4-specific IFNγ response was only seen in cultures stimulated with excess IC31 formulations (**Fig. 3**). For comparison with the mouse immunogenicity study in **Figure 1**, the data in **Figure 3** are normalized as fold excess of IC31.

To investigate a potential mechanism for the effect of higher IC31 ratios, we hypothesized that the overall charge on IC31 particles may be important. It is reported that positive charge facilitates KLK binding to the cell surface and delivery of ODN1a to the cytosol,[6] and the use of cationic polymers for the delivery of oligonucleotides to cells is largely recognized.[18] It has also been reported that phagocytic activity of antigen presenting cells is enhanced by optimizing the surface charge of cationic microparticles.[19] The hypothesis was tested by using phase



IC31 Fold Excess	Formulation (nmol/mL KLK)* H4 dose: 12.5 µg/mL	H4 dose: 100 µg/mL	IC31 present to adsorb H4†
70	1000	-	Excess
53	750	-	Excess
29	412	-	Excess
9	127	-	Excess
1	-	120	Minimal
0.5	-	60.0	Limiting
0.2	-	20.0	Limiting

* Formulations contain 12.5 or 100 µg/mL of H4 combined with the indicated amount of IC31 adjuvant, expressed in molar equivalents of KLK.
† See Figure 1 Legend.

Figure 4. The Surface charge of formulations switches from negative to positive when IC31 is greater than 9 fold in excess. The ζ potential of formulations containing different H4-IC31 ratios expressed as IC31 fold excess was measured by phase analysis light scattering on a ZetaPALS (Brookhaven Instruments, Holtsville, NY). The dotted line represents the ζ potential value of IC31 in the absence of H4.

analysis light scattering to determine the overall charge of H4-IC31 formulations containing H4 with limiting, minimal and excess IC31 (**Fig. 4**). For comparison with the results (from **Fig. 1** and **Fig. 3**) the data were normalized as fold excess of IC31. The results show that the overall charge of H4-IC31 particles changes from negative to positive once IC31 is in greater than 9-fold excess for H4 binding. Another major consideration for H4-IC31 function is differences in the vaccine particle size. For IC31 alone and formulations at 1-, 9- and 70- fold IC31 excess, the mean particle size distribution (D 50) was 78, 84, 88 and 86 μm respectively, as determined by laser diffraction analysis (Malvern Mastersizer 2000, Malvern, UK), suggesting that the different H4-IC31 ratios had little to no impact on the particle size.

A number of key findings stem from this study. We found that 2 adjuvants can function quite differently in hWB culture, although, the same pool of H4 specific memory T cells must be involved. One difference included the ability of TLR4-Adjuvant, but not IC31, to stimulate TNFα release from a short-term *in vitro* culture. Whether this and other striking differences translate to *in vivo* function is unclear. Further work to understand possible differences in the characteristics of the T cell populations expanded by different adjuvants is warranted. While replicating the 'whole body' response to a complete vaccine formulation is a major challenge, and *in vivo* models are fraught with issues relating to translation to human, we show that mouse *in vivo* and human *ex vivo* approaches can be mutually complementary and supportive. Rather than attempting to replicate complex mouse *in vivo* immunogenicity, the hWB *in vitro* culture aims to provide additional data, namely the ability to re-stimulate BCG-primed human immunity. However, we recognize that many differences exist between an *in vivo* model and an *in vitro* culture and the antigen presenting cells in hWB may not be identical to those that infiltrate muscle after vaccination. In addition, drainage of specific cell types to lymph nodes and the effects of antigen depot after vaccination cannot be readily replicated *in vitro*. Other factors such as media and vaccine dose-to-cellular dose ratio may also differ. Despite these differences, we believe an ability to take up, process and present the whole vaccine formulations within a hWB culture provides valuable additional information. Furthermore, the combined data may be more meaningful and relevant and could help de-risk clinical progression. For *in vitro* cultures, we fixed the highest concentration to be the optimized dose of 4 nmol/mL KLK. At this adjuvant dose, we show an antigen dependent dose response with 0.1, 0.01 and 0.001 μg/mL of H4. For the study in **Figure 3** we used an antigen dose of 0.05 μg/mL and show an adjuvant dependent dose response starting from 0.01 nmol/mL KLK.

At this time, it is unclear why the hWB approach is so sensitive to adjuvant-modulated, antigen-specific immunity. One possibility may be that innate immunity remains intact.[20] However, our work indicates that there is value in applying the approach to screening experimental vaccine formulations either before or after performing animal immunogenicity studies. The present approach demonstrates a new method for studying formulations, which bridges mouse and human systems for a greater understanding and control over the function. This combined approach supports, and may be applicable to Quality by Design (QbD), enabling improved product characterization and formulation screening.

Our observation that a specific surface charge on H4-IC31 particles may be critical for its function is intriguing. It is likely that a highly negative ζ-potential results from the negatively charged H4 when IC31 is limiting, and the charge is completely reversed by the positively charged KLK when IC31 is in excess. However it must also be noted that IC31 is being titrated and a dose effect may factor into both *in vitro* and *in vivo* observations. Given the added complexity of the *in vivo* mechanisms, one explanation for the loss of activity may also be the development of temporary anergy in the presence of excess H4 antigen.[21]

In conclusion, both hWB and mouse immunogenicity models suggest H4-specific immunomodulation requires an excess of IC31 adjuvant, and an overall positive surface charge on the vaccine may be important for such activity.

Disclosure of Potential Conflicts of Interest

All authors are employees of Sanofi Pasteur.

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