A novel assay to measure B cell responses to keyhole limpet haemocyanin vaccination in healthy volunteers and subjects with systemic lupus erythematosus

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The aim of the study was to characterize performance of a complementary set of assays to measure antigen-specific immune responses in subjects immunized with a neoantigen. Healthy volunteers (HV) ($n = 8$) and patients with systemic lupus erythematosus (SLE) ($n = 6$) were immunized with keyhole limpet haemocyanin (KLH) on days 1 and 29. Serum antibodies were detected using a flow cytometric bead array (CBA) that multiplexed the KLH response alongside pre-existing anti-tetanus antibodies. Peripheral blood mononuclear cells were studied by B cell ELISPOT. These assays were built upon precedent assay development in cynomolgus monkeys, which pointed towards their utility in humans. Primary anti-KLH IgG responses rose to a mean of 65–93-fold above baseline for HV and SLE patients, respectively, and secondary responses rose to a mean of 260–170-fold above baseline. High levels of anti-tetanus IgG were detected in pre-immunization samples and their levels did not change over the course of study. Anti-KLH IgG1-4 subclasses were characterized by a predominant IgG1 response, with no significant differences in subclass magnitude or distribution between HV and SLE subjects. Anti-KLH IgM levels were detectable, although the overall response was lower. IgM was not detected in two SLE subjects who did generate an IgG response. All subjects responded to KLH by B cell ELISPOT, with no significant differences observed between HV and SLE subjects. The CBA and B cell ELISPOT assays reliably measured anti-KLH B cell responses, supporting use of this approach and these assays to assess the pharmacodynamic and potential safety impact of marketed/investigational immune-therapeutics.

Introduction

Growth in the number of investigational and marketed immuno-therapeutics and breadth of targeted pathways for chronic inflammatory conditions and autoimmune diseases [1–6] is cause for optimism among patients and the

physicians who treat them. Correspondingly, there is a growing need to develop tools to assess the *in vivo* pharmacologic effects of these agents, particularly of novel agents in early clinical development, in relevant populations. Such tools would help define the magnitude and scope of impacts on the immune system during drug

development as well as to provide data to understand better the safety risks. Immunosuppression is a reluctantly accepted risk of effective therapies [7, 8], reflecting the imprecision by which an overactive immune system is quelled. Infection rate serves as a clinically relevant indicator of immune suppression but it is an indirect measure typically requiring large numbers of patients and long treatment duration to characterize. From this perspective, assays that can provide reliable quantitative information regarding the status of the human immune system in a limited number of subjects would be highly valuable.

One approach is to measure serum antibody responses to vaccination in the setting of treatment with an immune-therapeutic [9]. Depending on the nature of the intervention, a variety of immune responses can be interrogated. For instance, responses to T-cell dependent (e.g. KLH or bacteriophage) vs. T-cell independent (e.g. *Streptococcus pneumoniae*) antigens may be studied. Additionally, existing memory B cell responses can be assessed by measuring the level of antibodies to a booster vaccine (e.g. tetanus) or primary responses may be studied by use of a neoantigen.

We sought to characterize and optimize a suite of assays to move beyond single-plex ELISA assays and report an outgrowth of our efforts here. Primary and secondary antibody responses to the KLH neoantigen were studied in healthy volunteers and subjects with systemic lupus erythematosus (SLE) in the context of pre-existing anti-tetanus antibody responses. We adapted a flow cytometric bead immunoassay (CBA) to perform multiplexed semi-quantitative measurements of KLH and tetanus serum antibody levels. Initial experiments were performed in animal models to gain an understanding of assay performance prior to translation of the assay in samples from human subjects immunized with KLH. In these latter studies, detector antibodies with varied specificities were used to allow for detection of anti-KLH IgG, IgG subclasses and IgM. The cytometric bead array (CBA) data generated from human samples was studied alongside B cell ELISPOT assays, where the frequency of KLH-specific B cells in blood was in accord with the relative serum antibody concentrations.

Methods

Cynomolgus monkeys

Data contained in this report are a subset of a larger KLH immunization study of purpose-bred Chinese cynomolgus monkeys (*Macaca fascicularis*) contracted to the Sierra division of Charles Rivers Laboratories (CRL; Sparks, NV, USA), whose results will be reported elsewhere. All animals in this larger study had serum samples tested for anti-KLH antibodies by ELISA (Alta Analytical Laboratory Inc., San Diego, CA, USA), which provided evidence for a typical anti-KLH antibody response. Although all but

four animals (195F, 211F, 126 M, 135 M; Figure 2) received a proprietary experimental therapeutic (Amgen Inc., Thousand Oaks CA, USA), we nonetheless elected to perform an assay-to-assay comparison of samples between the ELISA and CBA to eliminate the need to utilize additional animals for the sole purpose of the assay qualification experiment presented here. Treatment of the animals was in accordance with CRL's standard operating procedures, which adhere to the regulations outlined in the USDA Animal Welfare Act (9 CFR, Parts 1, 2 and 3) and the conditions specified in the Guide for the Care and Use of Laboratory Animals [10]. The study protocol was approved by the CRL IACUC.

Human volunteers

Healthy volunteers (HV, $n = 8$) and SLE subjects ($n = 6$) were enrolled at Hammersmith Medicines Research (London, UK) after receiving Institutional Ethics Committee (IEC) approval. The criteria for all subjects included males and non-pregnant, non-nursing females between 18 and 60 years of age. All subjects must have received a full tetanus series prior to enrolment by history. Healthy volunteers lacked chronic medical diseases or conditions and did not take prescription medications or over the counter medications within 7 days of enrolment. For the SLE group, the subjects had a clinical diagnosis of SLE as defined by ACR guidelines [11] with disease duration of at least 1 year. The patients were clinically stable, defined as no change in SLE therapy within the previous 2 months. Subjects of either group were excluded if they were previously immunized to KLH or had known allergies to shellfish; had received any type of vaccination within 30 days of enrolment, had a positive serology for HIV antibodies, hepatitis B surface antigen, or hepatitis C antibodies. Subjects with SLE were excluded if they received cyclophosphamide (or any other alkylating agent), ciclosporin, tacrolimus or sirolimus, or $\geq 100 \text{ mg day}^{-1}$ prednisone or equivalent in the 6 months prior to randomization, had $>10 \text{ mg day}^{-1}$ oral systemic corticosteroids (prednisone or equivalent) within 30 days of enrolment, prior administration of rituximab or any other investigational molecule that primarily targets the immune system, had renal disease as defined as $>1+$ proteinuria per dipstick and spot urine protein : creatinine ratio ≥ 1.0 – or a calculated GFR $<60 \text{ ml min}^{-1}$, had a total WBC $<3000 \times 10^6 \text{ l}^{-1}$, platelet count $<100\,000 \times 10^6 \text{ l}^{-1}$ or evidence of liver disease (serum ALT or AST $>2\times$ upper limit of normal). Any subject with neuropsychiatric SLE (NPSLE) or a documented history of any non-SLE immune system abnormality was also excluded from study.

KLH immunization

For cynomolgus monkeys, the KLH immunogen, (Pierce, Rockford, IL, USA) was reconstituted with sterile water for injection to yield a 10 mg ml^{-1} stock solution. The stock solution was diluted with sterile water to yield a 1 mg ml^{-1}

dosing solution. The prepared antigen was subcutaneously administered, where the dose volume of KLH antigen was injected in approximately equal amounts across two to four separate dose sites in the skin of the dorsal thoracolumbar region. For humans, BCI-ImmuneActivator™ (KLH) (Intracel, Maryland) was administered intradermally (1 mg) on day 1 and day 29 to each subject in the forearm.

Schedule of assessments

For cynomolgus monkeys, two samples from each of 25 animals were obtained for evaluation in the CBA. The animals were vaccinated with KLH on day 0 (after a baseline blood collection) and boosted on day 37. The post-vaccination blood sample for each animal was taken at day 52. For the human studies, subjects returned to the study centre on days 1, 8, 15, 29, 36, 43, 57, 85, 113, 141, 169 and 197. Assessment of safety and tolerability, including safety laboratory tests and ECGs were conducted throughout the study for all subjects. For SLE subjects, disease activity was evaluated monthly using the SLEDAI [12] and BILAG [13] indices. Serum was collected for serum antibody assessments (CBA) on days 1, 8, 15, 29, 36 and 43. Blood was collected for PBMC isolation (anti-KLH IgG ELISPOT) on days 1, 29, 36 and 43.

ELISA assay

As described above, anti-KLH antibodies were measured in the cynomolgus monkeys by ELISA (performed by Alta Analytical Laboratory Inc., San Diego, CA, USA) in addition to the CBA (as described below). A cutpoint titration method was used, where samples were incubated with KLH immobilized to an ELISA plate as well as an uncoated plate (to determine specificity). After incubation, the plates are washed, and the bound antibodies are detected with goat anti-monkey IgG-HRP or IgM-HRP, and then visualized with TMB (tetramethylbenzidine).

Samples were diluted 1:100 in duplicate to determine the presence or absence of antibody. Samples were evaluated relative to the cut point optical density (OD) of the plate, where the cut point OD was defined as the mean OD of the 10 individual normal monkey serums (negative control) at a 1:100 dilution + 1.96 times the SD (of the normal monkey serums at a 1:100 dilution). Samples that generated an OD below the cut point OD were scored negative. Samples that produced an OD greater than or equal to the defined cut point OD were analyzed relative to the corresponding response on the uncoated specificity plates at a 1:100 dilution. If the response was determined to be a true antibody response (by evaluation against the specificity plate), the sample was titred. The antibody titre of samples considered to be reactive based on the criteria for the coated and specificity plates was defined as the reciprocal of the dilution that generated an OD at or above the cut point OD.

Flow cytometric bead array

Serum antibodies were detected using a custom flow CBA prepared by BD Biosciences (San Jose, CA, USA) according to our specifications (Figure 1A). Five beads of distinct and non-overlapping fluorescence intensities (i.e. addresses) were used for this purpose and conjugated as follows. Test beads were covalently coupled with KLH, or tetanus toxoid. Reagent control beads were coupled to human IgM or IgG and were used to verify that the fluorescence emission of the detector antibody (e.g. anti-IgM PE or anti-IgG PE) did not degrade as a function of storage time. An additional negative control bead was utilized, and was conjugated to sperm whale myoglobin. The philosophy and performance of a similar multiplexed assay (to detect serum antibodies to epoetin alfa) has been previously published [14]. Since the anti-IgG PE detection antibody provided in the kit cross-reacted with cynomolgus IgG, the assay could be conducted with cynomolgus or human samples without modification (cynomolgus samples were tested against IgG only).

To run the assay, 50 µl of bead mixture was combined with 50 µl of serum [diluted to a 1% (v/v) concentration], vortexed, and then incubated for 2.5 h at room temperature. Samples were washed by the addition of 1 ml of wash buffer and then centrifuged at 200 g for 5 min. The supernatant was aspirated, and then 50 µl of phycoerythrin detection reagent was added to each tube, vortexed, and then incubated for 30 min at room temperature. Samples were then washed once as described above, resuspended in 300 µl of wash buffer and analyzed on a BD FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA) that was set up as previously described [14].

B cell ELISPOT assay

Blood was drawn from study subjects in a BD Vacutainer® CPT™ Cell Preparation Tube with sodium heparin (Becton Dickinson and Company, Franklin Lakes, NJ, USA). The tubes were centrifuged within 2 h of collection at 1600 relative centrifugal force (RCF) in a horizontal rotor (swing-out head) centrifuge at ambient temperature (18 to 25°C). The plasma and PBMC layer were decanted into a conical tube and centrifuged at 300 RCF. The cells were then washed once with 1 × PBS and centrifuged again at 300 RCF for use in the ELISPOT assay.

To perform the ELISPOT assay, peripheral blood mononuclear cells (PBMCs) were incubated on membrane plates (Millipore Corporation, Billerica, MA, USA) that were coated with KLH (to detect cells producing specific antibodies) or coated with an anti-human antibody (to detect all antibody producing cells). Antibodies are captured onto the coated membrane as they are secreted and therefore produce 'spots' near the antibody-producing B cells. After washing the plates to remove the cells, spots are detected with an antibody to human IgG conjugated to the enzyme alkaline phosphatase (Sigma) and a colorimetric reaction.

Figure 1

Cytometric bead array (CBA) for detection of anti-KLH and anti-tetanus antibodies. (A) This multiplexed assay was designed with five beads of distinct fluorescence intensity that had human IgM, human IgG, keyhole limpet haemocyanin (KLH), sperm whale myoglobin (SWM), or tetanus toxoid (TT) covalently attached to their surfaces by standard amine chemistry. The assay involved incubation with 1% serum, followed by addition of a PE-conjugated fluorescent detection antibody. The detection antibody varied according to the need to measure IgG vs. IgG subclasses vs. IgM (see Methods). Analysis was performed on a standard flow cytometer. The intensity of fluorescence is therefore proportional to the amount of serum antibody captured by each of the respective beads. (B) Example data of an emerging anti-KLH IgG response as measured by the CBA. Specificity of detection is easily visualized, as the only bead address with marked change is that for the bead conjugated to KLH (circled red). It is apparent that the IgG detector was added to the reaction, as the bead conjugated to IgG exhibits maximal intensity (relative to the IgM bead). A high level of anti-tetanus antibodies is also evident in these data, as the bead address fall to the far-right of the x axis

The number of spots is directly proportional to the frequency of antibody-producing B cells.

Results

Experiments in cynomolgus monkeys

Before validating performance of the CBA for use in specimens from human volunteers, we tested a set of samples from KLH-immunized cynomolgus monkeys to benchmark assay performance against a traditional ELISA measurement. The need for this exercise reflected the novelty of this bead array, which is reported here for the first time. Utilization of cynomolgus monkeys (rather than humans) for this initial experiment primarily reflected our familiarity with the type of data generated during KLH immunizations of cynomolgus monkeys, which is common to immunotoxicological studies [15].

Our data showed a KLH specific increase in signal 52 days after a primary (KLH) immunization (with a day 37 boost) in 24 of 25 immunized animals, albeit with a baseline KLH signal from some of the animals (grey bars, KLH panel, Figure 2). With respect to the entire data set, the relative concentrations (fluorescence intensities) of IgG anti-KLH antibodies after vaccination as detected in the CBA were well-correlated with results generated by a traditional ELISA ($r^2 = 0.76$, Figure 3). A similar relationship was observed when the post : pre ratio of anti-KLH antibodies measured by CBA (rather than the fluorescence intensity from the post-vaccination sample) was compared against the ELISA ($r^2 = 0.78$, data not shown). The sole animal (130 M, Figure 2) that tested negative for development of anti-KLH antibodies in the CBA also scored negative in the ELISA. An additional animal that scored negative (for development of anti-KLH antibodies) by ELISA gave a weak positive signal in the bead array (lower left data point in Figure 3). As expected, no reactivity against the tetanus bead was detected in any of the samples. The animals were not tetanus immunized.

Experiments in humans

Healthy volunteers and a group of SLE subjects with mild, stable disease were included in these evaluations, with the goal to understand whether antibody responses against the KLH vaccine were appreciably different. Here, we expanded the scope of the CBA to measure IgG subclasses and IgM. An IgG B-cell ELISPOT was also included to establish the relationship between numbers of circulating antigen-specific B cells and IgG serum antibody levels.

Prior to embarking on the clinical effort described below, we assayed a set of 25 banked serum samples from healthy volunteers to understand background reactivity against the bead set from KLH unimmunized persons (Figure 4). This experiment was performed in the same manner as we previously published for a CBA designed to detect antibodies with reactivity against epoetin alfa [14].

In fact, the specimen set used in this effort was identical to the one used in the previous study. Thus, the sera from donor 12 and 63, whose sera reacted with the sperm whale myoglobin bead in our prior study, also showed reactivity here. The serum specimen from donor 12 was particularly promiscuous with respect to its binding profile, where additional low level reactivity against KLH was demonstrated here (and donor 12 sera also reacted to an epoetin alfa bead in our prior report [14]). Serum from donor 63, however, did appear to have specific reactivity to sperm whale myoglobin, and may therefore represent a natural autoantibody [16–18] inasmuch as sperm whale myoglobin and human myoglobin share significant sequence homology [19]. We regard the multiplexed approach superior to single-plex measurements inasmuch as non-specific matrix effects, albeit infrequent, are self apparent as a function of conducting the assay. Conversely, we view the ability to detect and visualize (Figure 1B) the emerging KLH-specific response as value-added.

Subject characteristics and safety of KLH administration

Key subject characteristics are listed in Table 1. The eight healthy volunteers comprised of four males and four females aged 23–45 years old (mean age 31 years). The six SLE subjects comprised of one male and five females aged 31–60 years old (mean age 50 years). The SLEDAI score for those with SLE at entry ranged from 2–4. Most SLE subjects (five of six) were on hydroxychloroquine and two subjects were on azathioprine. Three subjects were on prednisone or prednisolone at a dose of 3–10 mg day⁻¹. Two subjects were hospitalized during the course of this study, with causes deemed unrelated to KLH administration (for ataxia and constipation). There were no significant changes in the vital signs, ECG, laboratory safety tests or physical examinations in all other subjects. All the subjects developed transient erythema and localized swelling after the first and second KLH immunizations but the injections were overall well tolerated. There were no severe flares at any time during the study (data not shown).

Serum IgG responses Consistent with our findings in cynomolgus monkeys, the CBA was a reliable measure of the response to KLH vaccination. These analyses demonstrate KLH-specific responses (Figure 1B), with data depicted in a traditional post : pre vaccine ratio format as well as the primary instrument readout of fluorescence intensity (MFI, Figure 5 and Figure 5 insets, respectively). The latter readout of fluorescence intensity values, which are not normalized to each person's baseline value, reveal low signals for anti-KLH IgG at baseline from some donors that align with our experience with cynomolgus monkey specimens (Figure 2).

There were several subjects in both groups with minimal to no apparent primary anti-KLH IgG response, but upon secondary challenge samples from all subjects provided unambiguous signal in their responses.

Figure 2

Characterization of background binding and anti-KLH IgG responses in cynomolgus serum with the cytometric bead array (CBA). In this experiment, sera from 25 animals without prior vaccination with KLH were tested to determine the background binding to each bead (grey bars) and the magnitude of the KLH antibody response 17 days after KLH vaccination (black bars). Although some animals have evidence for pre-existing antibodies that cross react with KLH (elevating the baseline signal from the KLH bead), the response to vaccination is evident (by a further increase in signal). Excluding the sole animal (130 M) that failed to respond to the vaccine, the post : pre vaccine anti-KLH IgG response ranged from 2–350 fold (median = 51 fold). Animal 130 M also scored negative for an anti-KLH IgG vaccine response by traditional ELISA (Figure 3)

Figure 3

Correlation between anti-KLH antibodies detected by the cytometric bead array (CBA) vs. a traditional ELISA measurement. This Figure simply depicts the fluorescence intensity value from the CBA (y axis) against an endpoint titer obtained by standard ELISA (x axis). The correlation between these two readouts is high ($r^2 = 0.76$). It is noteworthy that the correlation analysis is limited by the categorical endpoint of the ELISA, where the reported titre+ is an estimate whose accuracy depends upon the distance between each of the serum serial dilutions. The CBA is a continuous measurement with large dynamic range

Immunosuppressive and/or immunomodulatory agent use in the SLE group (Table 1) did not appear to impact on the magnitude of the IgG antibody responses (Figure 5 insets). An ANOVA analysis of the anti-KLH responses on days 29 and 43 between healthy volunteers and SLE subjects showed no significant differences between these two groups (supporting information Table S1).

The IgG antibody response was also analyzed in terms of component subclasses by using detection antibodies with specificity for each subclass (Figure 6). Day 8 data was excluded from these analyses because the antibody titres were of insufficient magnitude for characterization. For healthy volunteers, IgG1 was the predominant IgG subclass after both primary (day 1) and secondary (day 29) immunizations. In contrast, a predominance of IgG2 followed by accentuation of IgG1 after the secondary immunization characterized the antibody response in persons with SLE. As expected, no change over time was observed for the relative concentration of anti-tetanus antibodies. However, the IgG4 subclass was approximately 4-fold higher on average in specimens from persons with SLE relative to the healthy volunteers.

Finally, IgM responses were examined by using a detector reagent with specificity for IgM (Figure 7). KLH-specific

signal was orders of magnitude lower than the correlate IgG responses for both healthy volunteers and persons with SLE. Moreover, a marginal increase in variability from the control beads was observed. Nonetheless, signal from the KLH bead was qualitatively discernable from background for all healthy volunteers but the signal from SLE donors was less apparent. In this latter case (SLE donors), however, the root cause was not increased background reactivity per se but rather lack of signal from the KLH bead.

B cell ELISPOT responses The presence of anti-KLH serum antibodies in all vaccinated subjects (Table 2) indicates that KLH-specific antibody secreting cells should be found in the peripheral blood. In the B cell ELISPOT assay, the spot count is an estimate of the frequency of antigen-specific plasma B cells present in the PBMC sample. The response to KLH at day 29 ranged from 0–30 spots/ 10^6 PBMC. Maximal levels were reached 7 days after the booster vaccination (day 36), where 56–576 spots/ 10^6 PBMC were enumerated. Spot count waned at day 43, 0–68 spots/ 10^6 PBMC, reflecting the relatively short half-life of circulating plasma cells. As was the case with serum antibody levels measured by CBA, there were no appreciable differences in the magnitude or kinetics of the B cell ELISPOT responses between healthy volunteers and persons with SLE.

Discussion

The goal of this study was to develop and characterize assays for assessment of immune response in persons treated with immune-therapeutic agents. We evaluated the utility of the CBA both in healthy subjects and in patients with SLE alongside assay development efforts in cynomolgus monkeys. This is the second such assay of this type developed by our group for use with clinical specimens, with the initial assay designed to assess anti-drug antibodies in persons treated with erythropoietic agents [14]. Performance of the current assay is consistent with our growing experience with the CBA platform. A detailed manuscript that describes the validation parameters will be presented and published elsewhere. These future reports notwithstanding, this CBA has been characterized with a lower limit of reliable detection of 250 ng ml^{-1} (relative to an anti-KLH monoclonal IgG standard), intra-assay precision $<30\%$, no observable hook-effects and a high degree of specificity (data not shown). Although the overall applicability of this assay is a function of the experimental question at hand (e.g. use of this assay in persons treated with B cell depleting agents would no doubt demonstrate the lack of response), we have demonstrated that the assay can substitute for a traditional ELISA in preclinical experiments and are also successfully using this analytical approach in an ongoing clinical trial (data not shown). We believe that the results of these efforts will provide a useful

Figure 4

Characterization of background binding of human serum IgG in the cytometric bead array (CBA). In this experiment, sera from 25 persons without prior exposure to the KLH vaccine were tested with an IgG detector to establish the expected profile of sera from KLH unvaccinated humans. The blue, pink, and black bars represent data from three independent experiments. Collectively, this experiment shows strong reactivity against the IgG coated bead (positive control bead for the IgG and IgG subclass detectors) and tetanus (reflecting prior tetanus vaccination). Low signal was measured from the IgM coated bead (positive control for the IgM detector), the KLH bead (test bead) and the sperm whale myoglobin bead (negative control). A minority of donors appeared to exhibit low level reactivity against KLH (i.e. donors 12, 15 and 69) and sperm whale myoglobin (i.e. donors 12 and 63), which is consistent with our prior experience in development of a CBA for anti-epoetin alfa antibodies [14]

and feasible means to assess the degree of pharmacologic activity and potential infectious risk in the evaluation of investigational or established immuno-therapeutics, as appropriate.

Generation of serum antibodies to KLH could be readily detected by the CBA in both monkeys and humans. Experiments in cynomolgus monkeys demonstrated the comparability of the anti-KLH CBA measurement with a

Table 1

Subject characteristics

Group	Number	Gender	Race	Age	SLEDAI at entry	Select medications
Healthy	1	Male	White	40	NA	NA
	2	Female	White	26	NA	NA
	3	Male	White	23	NA	NA
	4	Female	White	29	NA	NA
	5	Female	White	31	NA	NA
	6	Male	White	25	NA	NA
	7	Female	White	45	NA	NA
	8	Male	White	28	NA	NA
SLE	1	Female	Black	55	4	Hydroxychloroquine 200 mg twice daily
	2	Female	Black	54	2	Hydroxychloroquine 200 mg twice daily
	3	Female	White	60	4	Prednisone 10 mg once daily Azathioprine 50 mg twice daily
	4	Female	White	31	4	Prednisolone 10 mg once daily Azathioprine 50 mg twice daily
	5	Male	White	55	4	Hydroxychloroquine 400 mg once daily Prednisolone 3–5 mg once daily
	6	Female	White	44	3	Hydroxychloroquine 200 mg once daily Hydroxychloroquine 400 mg once daily

single-plex ELISA. Experiments in humans were expanded to include detection of IgG subclasses and IgM by CBA and IgG ELISPOT assays. We recognize contemporary guidance that regards KLH as a relevant immunization antigen when immune function studies are incorporated into immunotoxicity evaluations [20]. However, pre-existing anti-KLH antibodies in cynomolgus monkey serum may raise concerns regarding the fitness of using KLH for measurement of primary immune responses and point to the possibility of utilizing tetanus (or another antigen) as the representative neoantigen in preclinical experiments. By contrast, since most humans have received a tetanus shot, tetanus then becomes a useful indicator of a memory response that can be concurrently monitored alongside the response to a representative neoantigen (i.e. KLH). Thus in humans the primary and recall responses can be efficiently monitored using a combination of recall and neoantigens whereas it would require additional time to establish experimentally the memory response in animal studies.

Collectively, there is an extensive body of literature describing the use of KLH as a neoantigen for immunization. Immune responses to KLH have been conducted in humans for over 40 years [21] in addition to the experience in monkeys [22], rodents [23, 24] and dogs [25]. The intradermal administration of KLH in human subjects as conducted in this study is a precedented, safe and generally well tolerated procedure [26]. The erythema, induration and discomfort seen with intradermal administration were consistent with information provided by the manufacturer. Intradermal administration should optimize the magnitude of the immune response for the given dose of antigen

[27]. While the intradermal route requires more skill in administering the immunization correctly and may lead to more subject discomfort, it may obviate the need for the use of adjuvant in cases, such as in SLE patients, where induction of disease flare is a potential concern. Studies of SLE subjects show that immunizations (with a number of vaccines and immunogens) may lead to the production of auto-antibodies. However, well controlled studies using a variety of immunogens have shown that immunizations are well tolerated by SLE patients and are not associated with disease exacerbations though auto-antibodies may be transiently formed [28–32].

The presence of low levels of pre-existing anti-KLH antibodies in some animals leads one to question the extent to which KLH can be viewed as a true neoantigen. A well-defined cause of pre-existing antibodies with reactivity to KLH derives from prior schistosome exposure or infection [33] with the Fuc α 1-3GalNAc-motif in N-linked glycans as a primary source of cross-reactive epitopes [34–36]. With respect to animals, it is widely accepted that sourcing cynomolgus monkeys from schistosome-endemic areas (e.g. Asia) creates the opportunity for their exposure to the parasite. Although we did not verify schistosomal infection in the animals used in this study, the animals were of Chinese origin and would be predicted to have been exposed [37], the frequency of reactivity we observed was not outside the bounds of our prior experience (Horner, personal observation), and our assay results aligned well for the set of samples tested by two distinct immunoassays (ELISA vs. CBA) performed in separate laboratories (Figure 3). There is also a case for schistosomal exposure in humans [33, 38], with an estimated 300 million cases of

Figure 5

KLH IgG antibody responses in healthy volunteers and persons with SLE. Fourteen volunteers ($n = 8$ healthy volunteers; $n = 6$ SLE patients) were immunized with KLH on day 1 and day 29. Data in this figure display a baseline-normalized readout, i.e. a post-vaccine/baseline ratio, as well as the absolute readout from the flow cytometer (MFI). Note that the tetanus response data provides ratios of approximately 1 as expressed by post-vaccine : baseline ratio, but that the overall level of anti-tetanus antibody is quite high (as evidenced by the MFI readout)

human infections worldwide and 600 million humans living in environments where infection is a risk [39]. Although *S. mansoni* and *S. haematobium* are the specifically identified shistosomes that elicit cross-reactive antibodies, additional possibilities for exposure to the Fuc α 1-

3GalNAc-motif are made possible on the basis of the numerous additional species of parasitic flatworms (trematodes and flukes), many of which would be harboured as obligate intracellular parasites in commercial sources of shellfish [40].

Figure 6

Anti-KLH IgG subclasses in healthy volunteers and persons with SLE. Here, the sera tested in Figure 5 were re-tested using detector reagents with specificity for IgG1-4. Data are expressed as a percent of the sum total IgG captured on each respective bead. See text for a detailed discussion of the data. ■, Day 15; ■, Day 29; ■, Day 36; ■, Day 43

Despite the potential for this cross reactivity, the antibody response to KLH resembled a prototypical primary and secondary response to immunizations. We found no gross differences in the antibody response to primary and secondary in SLE patients compared with healthy volunteers, although overall variability was qualitatively greater in the SLE group. Some subjects in both groups failed to mount a significant primary anti-KLH IgG response (Figure 5), and the IgM response in some SLE subjects was difficult to detect (Figure 7B). Although we could have indexed IgM in a variety of ways [41] to score a response (e.g. any post-vaccine time point >2-fold above the pre-vaccine signal, maximum ratio against baseline or negative control beads etc.), we avoided such arbitrary calls and prefer to simply point out that the IgM responses observed were weak and that additional work would be required to qualify this readout for a clinical experiment. We say this in part because it is possible that the dose or formulation of vaccine itself might require adjustments to enhance IgM production [23, 41], in addition to any benchwork that could be done to improve upon the resolution sensitivity of the CBA. On the other hand, measurement of the IgG responses was straightforward and unambiguous, and similar to what was reported for prednisone treatment in renal transplant recipients [42], there appeared to be no obvious relationship between immune responses and concurrent low level immunosuppressive medications taken

by our SLE subjects. We do note, however, that SLE subjects in this study had mild to moderate stable disease. We cannot extrapolate these data to patients with more severe disease and/or requiring greater degree of immunosuppression.

Data from human experiments were displayed as post : pre vaccination ratios as well as by unadjusted fluorescence intensity values. Post : pre ratios essentially utilize each individual's baseline value as a negative specimen, permitting within-person comparisons that are often heavily relied upon in small scale clinical trials. An important aspect of this data analysis was the finding that the post : pre ratio from all control beads (both positive and negative controls) was remarkably invariant, speaking to the ability to support clinical trials with adequate power even when small cohort sizes are utilized. Conversely, the variability in the absolute magnitude of pre-existing KLH antibodies was apparent and would not be observed if data were solely displayed as post : pre vaccination ratios. Moreover, the magnitude of the anti-tetanus response, which was high, cannot be appreciated from the post : pre-ratio display. However, the lack of variability in this signal is best appreciated when reported as a post : pre ratio. Studying the change in anti-tetanus antibody levels allows assessment of the impact of a therapeutic intervention on a newly emerging immune response in the context of pre-existing (anti-tetanus) antibodies. Although we view this as

Figure 7

KLH IgG antibody responses in healthy volunteers and persons with SLE. Sera tested in Figure 5 were re-tested with a detector reagent with specificity for IgM, with data depicted in the same fashion as Figure 5. See text for a detailed discussion of the data

a value-oriented feature of the assay, these data should not be extended as a potential representation of all pre-existing antibodies, i.e. an overall indicator of immune competence. Beads with a variety of additional antigen specificities, however, could be added to the assay according to the needs of a particular clinical question.

Thus, although the ultimate choice of data representation may be individualized as needed, differences between

raw vs. adjusted datasets should not be overlooked at the risk of losing important information to guide decision-making processes.

Interestingly, differences in the proportion of IgG1 and IgG2 subclasses to primary and secondary KLH immunizations were noted in healthy volunteers vs. SLE patients (Figure 6). The sample sizes in the two groups are too small to draw any steadfast conclusions but they do align with

Table 2

KLH-specific B cell responses (per 10⁶ PBMC)

	Healthy subjects	SLE subjects
Day 1		
n	8	6
Mean	0.13	0.31
SD	0.35	0.54
Day 29		
n	6	5
Mean	13.21	8.65
SD	8.94	12.86
Day 36		
n	8	6
Mean	162.31	240.63
SD	175.67	124.02
Day 43		
n	8	5
Mean	7.98	14.52
SD	9.32	29.96

earlier reports that demonstrate that IgG1 dominates the primary anti-KLH IgG response in accompaniment with lower levels of IgM [43–45]. While factors that determine the relative levels of the IgG subclasses are not well understood, the role of the cytokine milieu [46,47] and temporal factors [48] have been described. Deliberate and careful measurements of Ig responses in terms of IgG subclasses, therefore, may yield insights into disease pathogenesis as well as to provide evidence of pharmacologic activity of a relevant immuno-modulatory agent [49]. Stated another way, the value of IgG subclassing may not be fully appreciated until viewed in the setting of pharmacological intervention.

The B cell ELISPOT is an established and sensitive method to enumerate the number of antigen specific antibody forming cells. Though labour intensive, B cell ELISPOT has been successfully utilized in the SLE clinical trial setting, showing a decrease in anti-dsDNA specific B cells in response to an anti-CD40L antibody in lupus patients [50]. The results in this study demonstrate the expected magnitude after primary and secondary immunizations and the kinetics of response. In particular, the peak response at day 36 which wanes by day 43 is in keeping with the short-lived nature of circulating plasma cells (i.e. as opposed to long lived plasma cells in the bone marrow) [51]. The B cell ELISPOT data confirm and complement the antibody results and provide a cellular perspective on the immune response. Challenges in isolating PBMCs and conducting the assay may limit its widespread use, however.

The development of a standard set of assays using well characterized neoantigens will greatly aid the evaluation and development of immuno-therapeutics in patient populations. KLH and other neoantigens provide an opportunity to delineate clearly primary and secondary

responses and potentially provide benchmarks for comparison of studies by different groups [52–54]. It would certainly be very helpful to extend these studies to include e.g. antigen specific T-cell responses to KLH immunization, intracellular cytokine measurements and inclusion of additional neoantigens such as bacteriophage antigens. We remain committed to expanding the battery of assays that could be used in this setting and we have made progress against many of these additional workstreams. These results demonstrate the validity of this approach for larger scale use and we would encourage the use of the CBA described here by others in the field to move towards a standardized approach to immune status assessments in humans.

Competing Interests

All authors have completed the Unified Competing Interest form at www.icmje.org/coi_disclosure.pdf (available on request from the corresponding author) and declare: S.S.B. reports employment and ownership of stock from Amgen, Inc. during the conduct of the study. M.H. reports personal fees from Amgen, Inc. outside of the submitted work. A.K. reports financial compensation and salary from Amgen Inc. outside of the submitted work. C.B.C reports grants from HMR/AMGEN during the conduct of the study. J.C. and J.F. are full-time paid employees of Amgen, Inc., the sponsor of this study. L.C., M.B., N.M., V.Q., R.J.N. and G.S. have no competing interests to declare.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Table S1

ANOVA Summary of anti-KLH antibody responses at day 29 and day 43*