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The T-cell-dependent antibody response assay in nonclinical studies of pharmaceuticals and chemicals: Study design, data analysis, interpretation

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ABSTRACT

The T-cell-dependent antibody response (TDAR) assay is a measure of immune function that is dependent upon the effectiveness of multiple immune processes, including antigen uptake and presentation, T cell help, B cell activation, and antibody production. It is used for risk and safety assessments, in conjunction with other toxicologic assessments, by the chemical and pharmaceutical industries, and research and regulatory agencies. It is also employed to evaluate investigational drug efficacy in animal pharmacology studies, provide evidence of biological impact in clinical trials, and evaluate immune function in patients with primary or secondary immunodeficiency diseases. Various immunization schemes, analytical methods, approaches to data analysis, and data interpretations are in use. This manuscript summarizes some recommended practices for the conduct and interpretation of the assay in animal studies.

1. Introduction

The kinetics and magnitude of the antigen-specific antibody response following immunization with a T-dependent antigen – the T-cell-dependent antibody response (TDAR) – is used to assess immune function. Antigens are referred to as T-dependent when B lymphocytes require T cell help in order to elicit an optimal antigen-specific antibody response, in contrast to a T-independent antigen, such as a polysaccharide, that can elicit an antibody response without T cell help. Several days after immunization with a novel (neo) T-dependent antigen, antigen-specific antibodies, primarily of the immunoglobulin (Ig) M isotype, are generated and released into the circulation by B cells and plasma cells, i.e., terminally-differentiated B cells (Ladics, 2007b). Antigen-specific

Abbreviations: APC, antigen presenting cells; AUC, area under the curve; CP, cut point; ECL, electrochemiluminescence; ELISA, enzyme-linked immunosorbent assay; EPA, Environmental Protection Agency; GLP, good laboratory practice; HMW, high molecular weight; HBsAg, Hepatitis B surface antigen; ICH, International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use; Ig, immunoglobulin; KLH, keyhole limpet hemocyanin; NHP, non-human primates; NSB, non-specific binding; PIDD, primary immunodeficiency diseases; PFC, plaque-forming cell; SCID, Severe Combined Immune Deficiencies; SRBC, sheep red blood cell; TDAR, T-cell dependent antibody response; TT, tetanus toxoid; US, United States.

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antibodies of other isotypes (e.g., IgG) and of higher affinity are subsequently released following antibody class switching and somatic hypermutation, respectively (Murphy, 2011). An antigen produces either a primary response (neoantigen) or a secondary response (recall antigen) when the same antigen is used in a second/repeated immunization. The antigen-specific antibody response to a recall antigen is primarily of an isotype other than IgM, is of increased magnitude and occurs with faster kinetics due to immune memory (anamnestic response). Experimental evaluation of antigen-specific antibody-forming cells or circulating antibodies is used to quantify the humoral (antibody) immune response to the antigen.

Multiple immune cell types and functions are required to induce a TDAR (Fig. 1). Immune cell types involved in the TDAR include antigen-presenting cells (APCs; primarily dendritic cells or macrophages), naïve and activated CD4+ T cells and T-follicular helper (T_{FH}) cells, B cells, and plasma cells. Cellular functions required include antigen processing and presentation, differentiation, upregulation of cell surface receptors, secretion of cytokines, somatic hypermutation and immunoglobulin class (isotype) switching. The various immune cells involved must communicate through receptor/ligand and receptor/cytokine interactions (Deenick, 2011). Because an optimal TDAR encompasses coordinated immunological efforts, the TDAR has emerged as a widely used method for assessing immune function in safety assessment studies for environmental chemicals, and both small and large molecule (biologics) pharmaceuticals. It is also employed to evaluate efficacy in nonclinical pharmacology studies and to measure immune function following treatment with pharmaceuticals in the clinical setting (Bingham, 2010; Brodmerkel, 2010; Struijk, 2010), or to evaluate immunodeficiencies (Kuijpers, 1997).

In the late 1980s and early 1990s, the TDAR to sheep red blood cells (SRBC) was extensively evaluated by the National Toxicology Program and was found to be useful for identifying immunosuppressive chemicals in nonclinical studies (Luster, 1992). The assay was based on anti-SRBC antibody (IgM) production by splenocytes (antibody-forming cells) in a semi-solid agar matrix causing the lysis of surrounding SRBC in the presence of complement and was known as the 'plaque-forming cell (PFC) assay', the 'plaque assay' or simply the 'SRBC assay' (Ladics, 2007a,b; White et al., 2010). The predictive value of the same assay was evaluated for the nonclinical safety testing of small molecule pharmaceuticals (Lebrec, 1994) and adapted to evaluate both primary (IgM) and secondary (IgG) responses (Holsapple, 1995). Other T-dependent antigens such as keyhole limpet hemocyanin (KLH) and tetanus toxoid (TT) were subsequently introduced as alternatives to SRBC in the nonclinical testing of xenobiotics.

In response to a recommendation to include the specific evaluation of immunotoxicity as part of the hazard assessment process, in 1998 the United States Environmental Protection Agency (EPA) finalized an immunotoxicity test guideline (OPPTS 870.7800) (US EPA, 1998). In 2007, the assessment of immunotoxicity became a part of the required studies in the revised Toxicology Data Requirements for pesticide registration for food and non-food uses. The intent of the EPA immunotoxicity guidance is to provide information on the ability of a test chemical to suppress the immune system. One of the core requirements under this guideline is the conduct of a TDAR with SRBC as the antigen. More specifically, rats and/or mice are exposed to the test and control substances for at least 28 days and immunized by intravenous or intraperitoneal injection with SRBC approximately 4–6 days prior to the end of the exposure, depending on the immunization route and assay approach (i.e., splenic PFC vs. serum enzyme linked immunosorbent assay [ELISA]) (Ladics, 2007b). At the end of the exposure period, either the PFC assay (Jerne and Nordin, 1963) or an ELISA (Temple, 1993) is performed to determine the effects of the test substance on the primary splenic anti-SRBC IgM response or serum anti-SRBC IgM levels, respectively. Currently the EPA is the only chemicals regulatory agency to have specific immunotoxicity testing requirements although the Organization for Economic Cooperation and

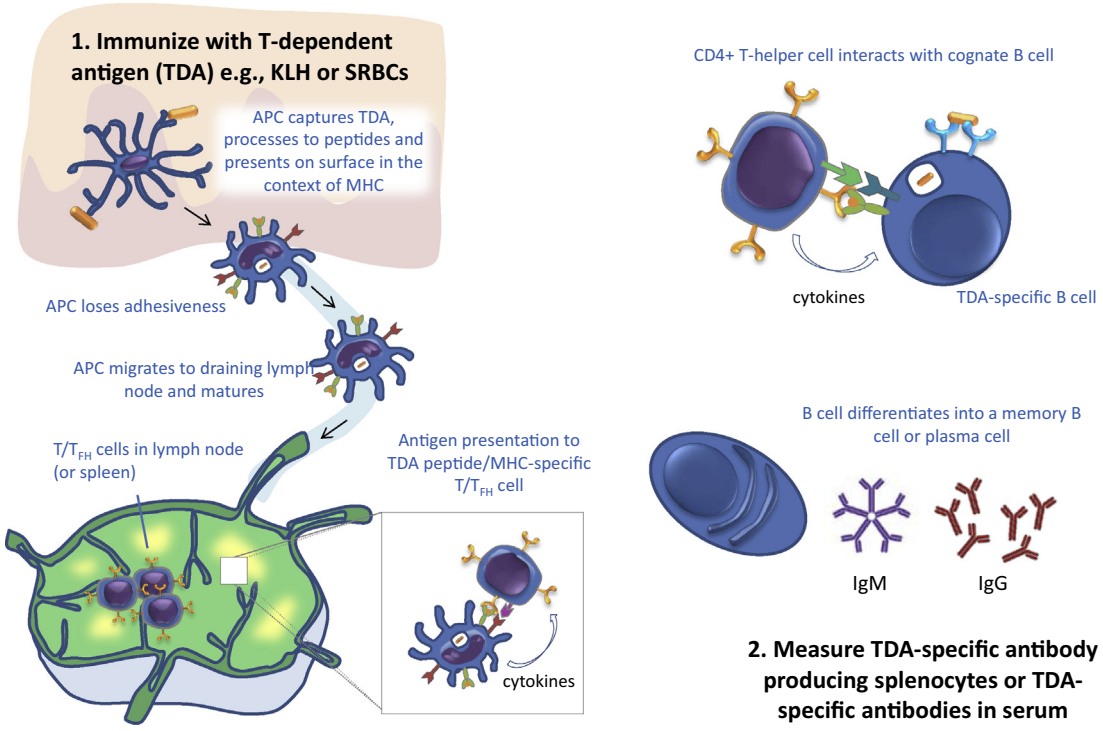

Fig. 1. Schematic representation of the TDAR. APC = antigen presenting cell, TDA = T-dependent antigen, MHC = major histocompatibility complex, T_{FH} = follicular T helper cell.

Development (OECD) Technical Guidance Document 443 does recommend that the TDAR be performed when the developmental immunotoxicity option is exercised (OECD, 2011).

For small molecule and biological pharmaceuticals, the TDAR is not required as routine for nonclinical testing. Instead, if the weight-of-evidence review indicates that additional immunotoxicity studies are called for then the TDAR assay can be considered (ICH, 2005). The primary and/or the secondary antibody response may be evaluated depending on the cause for concern that has been identified. Since the rat and the dog are the most common species for nonclinical safety evaluation of small molecule pharmaceuticals, the TDAR may be evaluated in both of these species, albeit the assay is much more commonly performed in the rat. In some cases the TDAR is evaluated in species other than rat or dog, such as the non-human primate (NHP), if it is deemed to be a more appropriate species for a particular small molecule (e.g., similarity of metabolism, better exposure, or immunity-related finding in standard toxicity studies only present in NHP). Although the ICH S8 immunotoxicology guideline explicitly excludes biologics, in the absence of specific guidance related to the immunotoxicologic assessment of biologics, the principles of ICH S8 are often applied. For example, the TDAR might be informative as a functional test when immunotoxicity is a cause for concern. For immunomodulatory biologics, the TDAR is most often used to help characterize the breadth or depth of immunomodulatory potential as well as to provide evidence of bioactivity in the nonclinical species to be used for safety evaluations. Due to the species specificity of biologics, NHPs are the most commonly used species for TDAR testing. However, TDAR testing in rodents (rats or mice) may be conducted if these species are pharmacologically relevant or with proteins homologous to the biologic intended for the clinic.

In safety assessment studies, the TDAR has traditionally been used to explore the possible immunosuppressive effects of a drug or environmental chemical under development. Thus, the methodology of the assay (for example the dose of antigen) has been optimized for detecting immunosuppressive effects. Although not the focus of this review, there are cases where the TDAR assessment has unexpectedly demonstrated potential immunostimulatory effects. The significance of these data remain controversial; however, if enhanced antibody responses are observed it is important to consider these data in the overall weight-of-evidence review (for example, do the enhanced antibody responses correlate with effects on other immune parameters and/or result in adverse outcomes?). More recently there has been increased interest in optimizing the TDAR assay to evaluate intended immunoenhancement. This is primarily due to the rise in the number of biologics in development for the immunotherapy of cancer and viral diseases where the therapeutic goal is to enhance immune-mediated killing of the affected cells. Indeed, an anti-CTLA-4 monoclonal antibody (10D1; ipilimumab), that is now marketed for the treatment of melanoma, was demonstrated to enhance the antibody response to a commercial hepatitis B vaccine in NHPs (Keler, 2003).

Despite the long history, broad utilization and important role of the TDAR assay in both the chemical and pharmaceutical sectors, there is a large diversity in immunization schemes, analytical methods, approaches to data analysis, and data interpretation. This manuscript summarizes some recommended practices for the conduct and interpretation of the assay in the nonclinical setting.

2. Study designs

There are several parameters to consider when discussing TDAR study designs to satisfy regulatory and scientific needs. These include selecting the appropriate antigen, use of adjuvant, animal species/strain, number and sex of animals, immunization route, time of immunization, sampling schedule, and analytes to measure (e.g., all antigen-specific antibodies or a specific isotype of antigen-specific antibodies). TDAR is often evaluated on general efficacy and/or toxicity studies to reduce the use of animals and provide data sets for correlations with other efficacy or toxicity findings.

2.1. Antigens

There are numerous antigens reported to elicit TDAR in mammals. The most commonly used antigens are described below, as are specific concerns or advantages for their usage. Other antigens can be used if well characterized for generally desired attributes: quality attributes (consistency is necessary; limited contaminations by endotoxins which can impact antibody responses), immunogenicity attributes (avoiding adjuvants is preferable per ICH S8), and, for pharmaceuticals, translational attributes (utilization across species, including in humans, is valuable). One issue associated with the use of adjuvants is that they may, in theory, interfere with the assessment of test article effects through their immunomodulatory properties. Before using one given antigen, laboratories should perform validation studies to determine kinetics of the response, dose, schedules and routes of immunization. It should be noted that SRBC were used as the antigen in the original evaluation of the TDAR assay as part of an immunotoxicology testing battery in which the TDAR exhibited the best single assay performance for identification of chemicals with immunotoxic potential (Luster et al., 1988, 1993, 1992). Consequently, SRBC became the only acceptable antigen to generate TDAR data for the testing of pesticides and other toxic substances according to OPPTS 870.7800 (US EPA, 1998). Other antigens may be considered by US EPA for such environmental chemicals when additional data on their sensitivity to toxicants, as compared to SRBC, are generated. However, given the extent of the SRBC IgM TDAR results database at US EPA, the use of SRBC is currently the rule. A greater diversity of antigens described below is used in the pharmaceutical industry.

2.1.1. Sheep red blood cells (SRBC)

A large body of data exists for the SRBC-induced TDAR PFC assay data and serum ELISA data. The number of injected SRBC should be optimized and approaches may differ between species and strains. To induce optimal responses by intravenous immunizations, mice typically receive 0.2 mL of 1×10^9 SRBC/mL while rats typically receive 0.5 mL of a 4×10^8 SRBCs/mL (i.e., 2×10^8 total SRBC for mice or rats) (Ladics, 2007b). As with other antigens, high inter-animal variability can be noted with SRBC induction of a primary IgM response. However, the antigen possesses good sensitivity for the detection of chemical-mediated immunosuppression (Fig. 2B), and a PFC assay (Section 3.1) following immunization with SRBC was shown to be more sensitive to certain immunosuppressive molecules when compared to an ELISA following immunization with KLH (White et al., 2007). Although the IgM response is typically evaluated with this antigen, the IgG response can also be evaluated (Ladics, 2007b). As mentioned above, SRBC have a long history of use in rodents and the associated experience and historical data has established it as the preferred antigen for the assessment of environmental chemical immunotoxic potential (US EPA, 1998). One potential limitation is the need to obtain a reliable source of the SRBC; however, once a source is identified, the antigen can be easily obtained. Other limitations may include the lack of stability of the antigen over time and the ability to store antigen for batch testing across multiple studies (Gore et al., 2004).

SRBC are less commonly utilized to study pharmaceuticals, however few studies using SRBC in NHPs have been reported

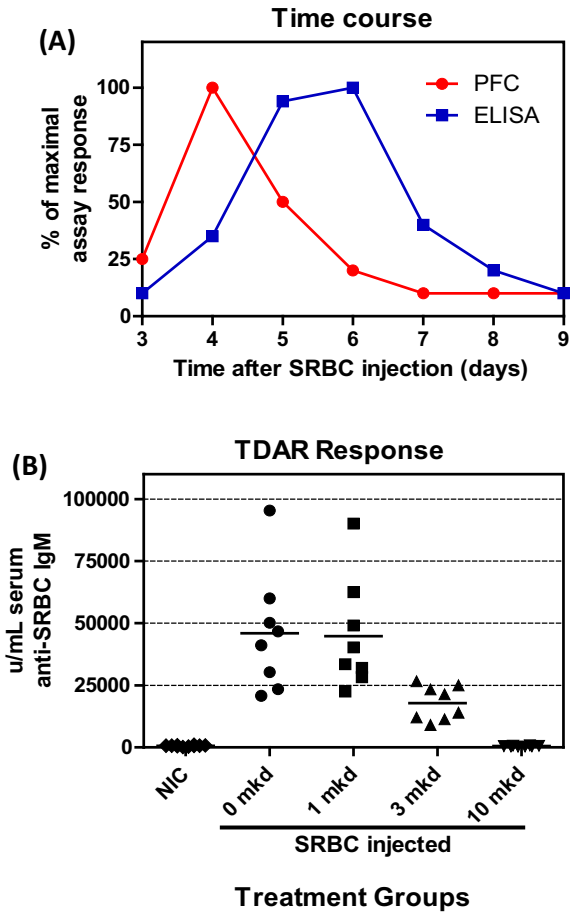

Fig. 2. Temporal response and typical inter-animal variability for the TDAR in rodents. The optimal time point for assessment of the maximal TDAR response differs between the splenic PFC assay and the serum ELISA approach (A). The inter-animal variability in the TDAR can be high but does not preclude the characterization of an immunosuppressive response. The example shown is a dose response into cyclophosphamide after 5 days of exposure as assessed using the serum anti-SRBC IgM ELISA approach (B). (NIC = non immunized controls; MKD = mg/kg/day).

(Srinivas et al., 1996; Tryphonas et al., 2001). Anaphylactic-like responses and complement activation in dogs immunized with SRBC have been observed (Haggerty, 2007). SRBC are thus not recommended for use in dogs.

2.1.2. Keyhole limpet hemocyanin (KLH)

Hemocyanins are multimeric oxygen transporter proteins (extracted from hemolymph of arthropods, mollusks) and are immunogenic in mammals. TDARs to KLH have been studied in both nonclinical species (e.g., mouse, rat, NHP, dog) and in the clinic (White et al., 2007; Gore et al., 2004; Piccotti et al., 2005; Haggerty, 2007; Kuijpers et al., 1997; Miller et al., 2005; Plitnick and Herzyk, 2010). Native KLH is comprised of high molecular weight (HMW; 4–8 MDa) assemblies of KLH subunit dimers (>600–800 kDa). It was demonstrated that inter-animal variability for anti-KLH IgM and IgG responses in the rat was lower with HMW KLH than with subunit KLH (when utilizing a same dose of 0.1 mg) (Lebrec et al, 2013). HMW KLH has been the preferred KLH form for nonclinical studies while KLH subunit (available as Good Manufacturing Practice material) has often been used for clinical studies. Common immunization dosages range from 0.1 to 2 mg for rodents and 1–10 mg for NHPs (Lebrec et al., 2011; Kim et al., 2007) and do not require adjuvant. It should be emphasized that different sensitizing dose levels of KLH were associated with different degrees of sensitivity to immunosuppressive effects of cyclophosphamide, azathioprine or cyclosporine A when measuring primary IgM responses in the mouse and rat. Greater inhibitory effects of the above mentioned compounds on TDAR were observed when animals were immunized with a 2 mg dose of KLH as compared to a 100 μg dose of KLH (White et al., 2007).

Although KLH is a common antigen used for TDAR evaluation in the pharmaceutical industry as it elicits a robust response in most mammals, is commercially available and both primary and secondary responses can be studied, some NHPs may have circulating KLH cross-reactive antibodies prior to KLH exposure (Fig. 3A). A possible source of pre-existing KLH cross-reactive antibodies is the presence of an anti-parasite immune response: antibodies against a *Schistosoma mansoni* carbohydrate antigen can cross-react with KLH (Grzych et al., 1987) because KLH and *S. mansoni* share immunogenic carbohydrate epitopes (Kantelhardt et al., 2002; Geyer et al., 2005). Given that interpretation of TDAR data rely on comparisons between groups of KLH-naïve animals, inclusion of animals with pre-existing KLH-cross-reactive immunoglobulin has been previously identified as an important factor to be taken into consideration when interpreting TDAR results (Piccotti et al., 2005; Caldwell et al., 2007). Since animals with pre-existing KLH-cross-reactive antibodies may skew the data following immunization and therefore impact the interpretation of the data (Fig. 3B), some investigators screen NHPs pre-study for the presence of KLH-cross-reactive antibodies and either select only animals without positive titers for the study or randomize animals with low positive titers across groups. Of note, it was observed that the incidence of pre-existing KLH-cross-reactive antibodies in cynomolgus monkeys of Mauritius origin may apparently be lower than in cynomolgus monkeys of Asian commercial origin (Fig. 4).

2.1.3. Tetanus toxoid (TT)

Evaluation of TDAR to TT in nonclinical safety testing is advantageous as it is clinically relevant, albeit not as a neoantigen as most humans are vaccinated with TT. Moreover, NHPs may be vaccinated with TT prior to shipment from vendors. It is important to confirm NHP vaccination status prior to using TT as a neoantigen. TT is more commonly used as a recall antigen. Immunization with 5 Lf (flocculation units) elicits a robust TT-specific IgG response within 8 days of immunization in cynomolgus monkeys with previous exposure to TT (Wang et al., 2013). This response persists for at least 6 weeks post-immunization. It should be noted that most commercially available TT vaccines (e.g., Td adsorbed®, Sanofi Pasteur) contain adjuvant, which may aid in eliciting the TDAR responses; however, ICH S8 states that adjuvants should not be used without justification and that Alum might be considered acceptable for use only in non-human primate studies.

2.1.4. Hepatitis B surface antigen (HBsAg)

As with TT, commercially available clinically utilized vaccines containing adjuvant are available to evaluate TDAR to HBsAg (e.g., Engerix B®, GlaxoSmithKline). These vaccines are available in pre-loaded syringes in both pediatric (10 μg) and adult human (20 μg) doses. Both dosages elicit measurable IgM responses two weeks post immunization in HBsAg naive NHPs. HBsAg-specific IgG responses are detectable 2 weeks post immunization; with a peak response at approximately 4 weeks post immunization. A robust IgG response persists for at least 6 weeks post immunization (Fig. 5) (Wang et al., 2013). In healthy adult cynomolgus monkeys, the adult dose of Engerix B® was shown to elicit a higher and less variable response, which was suppressed with a single dose of the T cell inhibitor abatacept at 10 mg/kg administered on the same day as the HBsAg (Wang et al., 2013).

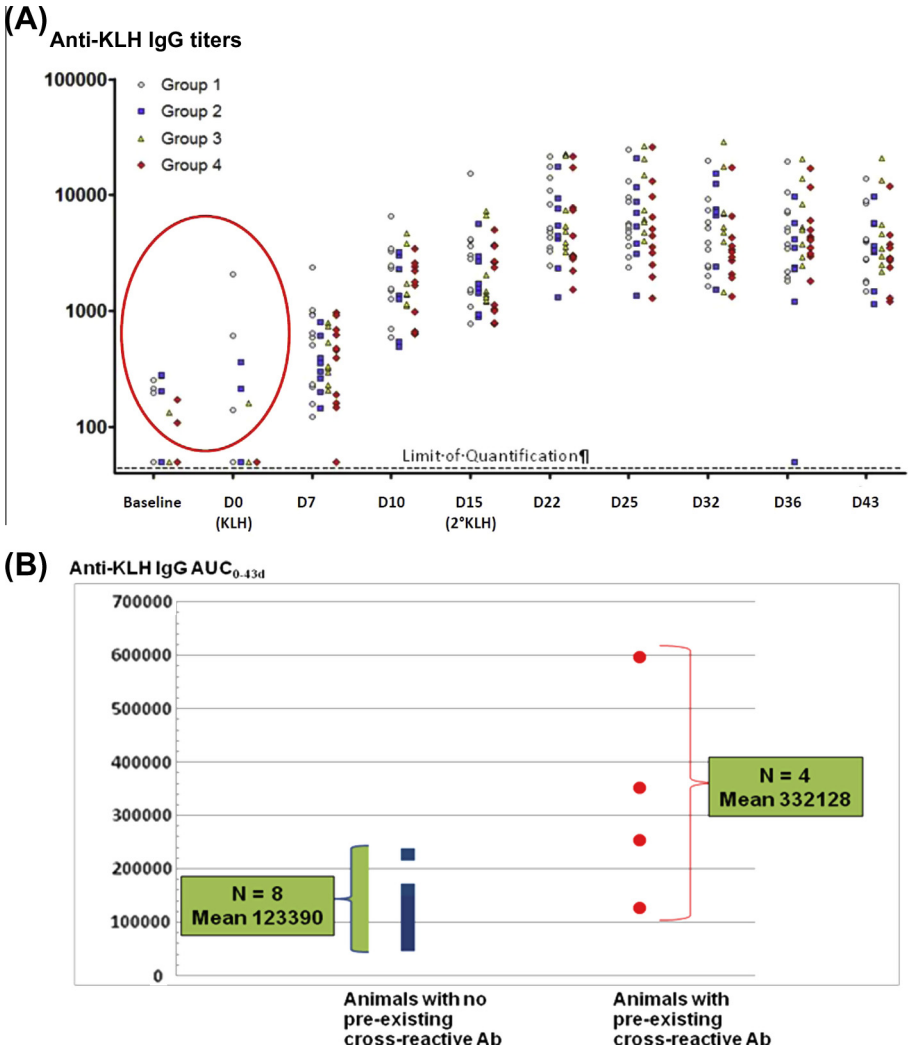

Fig. 3. Pre-existing cross-reactive antibodies and distribution of anti-KLH responses in NHPs. In the context of a 6-month repeat-dose toxicology study for a small molecule investigational product, male and female cynomolgus monkeys (N = 5/sex/group, 4 groups) were immunized subcutaneously with 1 mg KLH on two occasions (study days 90 and 105, immunization days 0 and 15). Serum samples were collected pre-study, on the day of first immunization (labeled D0, sample collected prior to immunization), 7, 10, 15 days after first immunization (sample collected prior to second immunization), 7 (D22), 10 (D25), 17 (D32), 21 (D36), and 28 (D43) days after the second immunization. Serum samples were analyzed for IgM (not shown) and IgG anti-KLH antibodies by ELISA with an interpolated cut-point titer limit of quantitation of 100. In several dose groups, individual animals with pre-existing KLH-reactive IgGs were identified (A). In vehicle-treated animals (shown here, B), the mean global anti-KLH IgG $AUC_{0–43}$ in animals with pre-existing KLH-reactive IgGs was approximately 3-fold greater than in animals without pre-existing KLH-reactive IgGs.

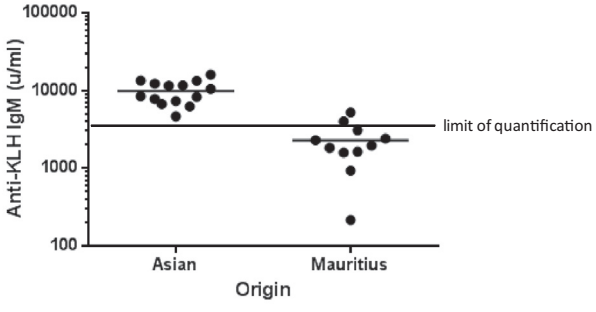

Fig. 4. Pre-existing antibodies cross-reactive to KLH in cynomolgus monkeys of different geographic origin. During testing of an anti-KLH antibody kit for use in Asian origin monkeys, a high number of samples with pre-existing (cross-reactive) anti-KLH antibodies were noted. The kit was initially optimized using serum from Mauritian cynomolgus monkeys. Optimization and validation was completed using serum from monkeys of Asian origin to achieve acceptable assay specificity. Anti-KLH antibody measurements performed using commercial ELISA kit (Life Diagnostics Inc., West Chester, PA). u/mL = units/mL.

Although in NHPs HBsAg is often a neo-antigen upon primary immunization, this is often not the case in the clinic as HBsAg vaccination is now common. Study designs with HBsAg immunizations 2 weeks apart in NHPs have been performed (Wang et al., 2013).

2.1.5. Bacteriophage

Bacteriophage ΦX174 (phi X174) is a T-dependent neoantigen that has been used since the 1960's to measure antibody responses in non-mammalian vertebrates (Uhr et al., 1962b; Ching and Wedgwood, 1967), in mammalian (non-human) vertebrates (Uhr et al., 1962a; Ochs et al., 1983; Ochs et al., 1974; Bue et al., 1986; Hamilton and Ochs, 1989; Kay et al., 1997; Andrews et al., 1997; Felsburg et al., 1997; Miao et al., 2006; Peng et al., 2009; Meyer-Bahlburg et al., 2012) and in the clinical studies with immunomodulatory agents or patients with primary or secondary immunodeficiencies (Ochs et al., 1971; Lopez et al., 1975; Jackson et al., 1979; Ochs et al., 1980; Witherspoon et al., 1981; Ochs et al., 1992; Zhu et al., 1997; Abrams et al., 1999; Fogelman et al., 2000;

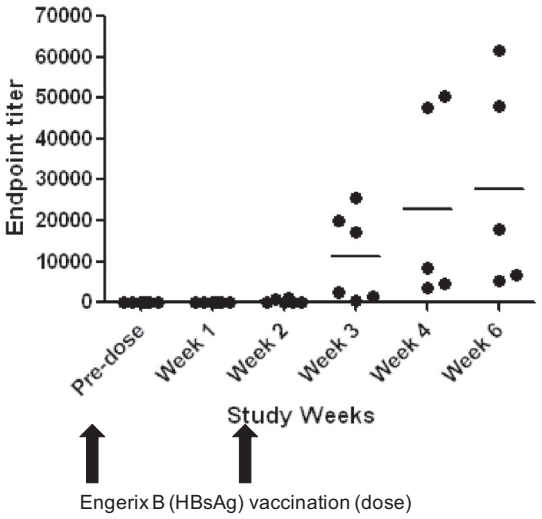

Fig. 5. Evaluation of TDAR to Hepatitis B surface antigen. Engerix B® (HBsAg, 20 μg) was administered subcutaneously to 6 cynomolgus monkeys (3/sex) following a pre-immunization (pre-dose) blood collection. Blood was also collected and processed to serum weekly for 6 weeks post immunization (1 sample not available at week 4 and 6). HBsAg-specific immunoglobulins (G/M/A isotypes) were assessed using an ELISA format. Endpoint titer was defined as the reciprocal of the interpolated dilution that is equal to five times the mean plate background.

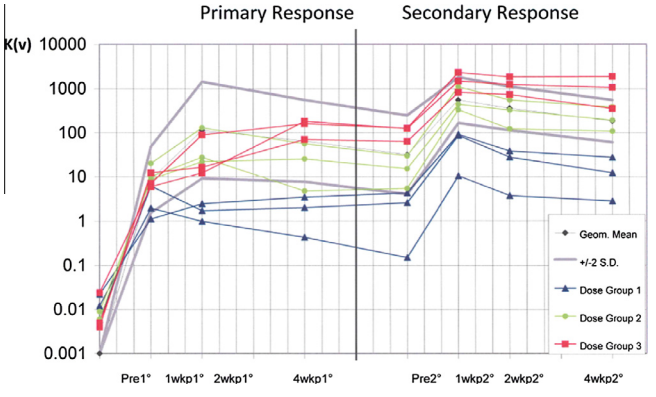

Fig. 6. Cynomolgus monkey TDAR following immunization with different doses of bacteriophage. Nine experimentally naive female cynomolgus monkeys ($N = 3$/group) were immunized on days 1 and 43 with 3 different dose levels of bacteriophage. Blood samples were collected for phage-neutralizing antibody titers (kV) and for phage-specific antibody of the immunoglobulin G (IgG) isotype (% IgG) at various time points. Group 1 received 4×10^9 PFU/kg/immunization, which corresponded to 2 times (2×) the standard human dose. Group 2 received 2×10^{10} PFU/kg/immunization, which corresponded to 10 times (10×) the standard human dose. Group 3 received 1×10^{11} PFU/kg/immunization, which corresponded to 50 times (50×) the standard human dose. All three Group 3 animals responded strongly with antibody titers that were above the geometric mean of the normal human controls. Only approximately 50% of the antibody was of the IgG isotype, suggesting a persistent, strong IgM response following the second dose of phage. The average% IgG of the normal human controls is approximately 50–56%.

Aiuti et al., 2002; Krueger, 2008; Shearer et al., 2009; Pescovitz et al., 2011). A typical bacteriophage-specific IgM response after primary immunization is elicited in all mammals studied to date, including humans and NHPs. Following secondary immunization, the response is characterized by amplification of the antibody titer and class switch from IgM to IgG; in humans, approximately 50% of the antibody generated following secondary immunization is of the IgG isotype. Following tertiary immunization, the antibody produced is predominantly of the IgG isotype. Immunization with bacteriophage ΦX174 is generally 1×10^{10} PFU/kg in rodents. In cynomolgus monkeys, a response comparable to the response in humans can be achieved after immunization with 2×10^{10} PFU/kg (Fig. 6), a dose 10-fold higher than a standard dose for testing in humans (2×10^9 PFU/kg). It should be noted that bacteriophage ΦX174 is not commercially available, has an IND number, and can be only obtained through Seattle Children's Research Institute. Bacteriophage is described in this manuscript since extensive research has been conducted using this neoantigen to document antibody responses associated with different types of immunodeficiencies or immunomodulation (see Section 6). It is, however, currently not generally utilized as a primary choice for nonclinical studies.

2.2. Route and timing of immunization

In rodents, antigens are typically administered subcutaneously (KLH), intravenously (KLH, SRBC, bacteriophage) or intraperitoneally (SRBC, bacteriophage). In nonrodent species, when applicable, KLH is administered subcutaneously or intramuscularly, TT and HBsAg are administered intramuscularly, and bacteriophage and SRBC are administered intravenously. The timing of immunization relative to test article exposure should be driven by the pharmacokinetics of the molecule and, when known, after a period of time in which correlative toxicities (e.g., changes in lymphocyte counts, lymphoid organ weight or histology) have been observed. Immunization can be conducted in animals utilized to analyze standard toxicology endpoints (Boverhof et al., 2014; Ladics et al., 1998; Ladics et al., 1995). In the case of chemical evaluations, the EPA Guideline specifies that the test material be administered for at least 28 days and that animals be immunized by intravenous or intraperitoneal injection with SRBC at the appropriate time prior to the end of the exposure (see Section 2.3 below). Study designs that include primary and secondary immunizations with the same antigen to test immunoglobulin class switching and/or recall response allow for complete assessment of TDAR responses. For example, in CD40L deficient patients (X-linked hyper-IgM syndrome) secondary IgG responses are impacted while primary IgM responses are not (Winkelstein et al., 2003). An appropriate amount of time (generally a minimum of two weeks) should separate primary and secondary immunizations, when applicable. Finally, study designs in which the antigen has been administered during a recovery (test article-free) period have been reported to evaluate reversal of suppression of TDAR (Haggerty and Proctor, 2012).

2.3. Sample collection

It is well accepted that the antibody response to SRBC has a temporal component with the maximal primary IgM response being observed several days after immunization (Ladics, 2007b). Importantly, the optimal time point for evaluation of the antibody response in rats and mice varies based on the methods used in the measurement (Fig. 2A) of the antibody response. For EPA guidance studies either PFCs or antibody titers may be assessed; if the splenic PFC assay is used, the optimal time point for a maximal primary antibody response is typically 4 days post-immunization by the intravenous route (Temple et al., 1993) (Fig. 2A). In the case of the anti-SRBC IgM ELISA assay, the optimal time point for a maximal response is typically 5–6 days post-immunization by the intravenous route, depending on whether mice or rats (respectively) are used as the test species. EPA recommends confirming the optimum time point for each species/strain and analysis approach prior to test chemical evaluations. When the TDAR is performed in rodents for pharmaceuticals, a generally accepted study design is a 28 day study with consecutive daily dosing. While ICH S8 does not prescribe a sample collection schedule, it may be designed with

Table 1
Example of immunization and sample collection schedule.

	Cynomolgus monkey study of duration >1 month
Timing of KLH immunization [boost]	Study day 30 (±7 days) [boost on day 45 (primary+15 days)]
Samples for anti-KLH IgM	Pre-immunization, 5-, 7-, 9-, 11-, 13-, and 15-days post-immunization
Samples for anti-KLH IgG	Pre-immunization, 5-, 7-, 9-, 11-, 13-, 15- (pre-boost), 17-, 19-, 21-, 23-, 25-, 35-, and 45-days post-immunization (i.e., 2-, 4-, 6-, 8-, 10-, 20-, 30-days post-boost)

consideration to studying both primary and secondary responses. As discussed below for NHP studies, pre-immunization sample collection may be performed in rodents as well.

For NHP studies, a possible schedule for sample collection to study responses after primary and secondary immunizations is shown in Table 1. Although Table 1 illustrates appropriate timing for TDAR to KLH assessment, a similar schedule would be applicable for most antigens mentioned earlier (with some variation, see HBsAg in Section 2.1). Pre-immunization sample analysis is important to detect pre-existing cross-reactive antibodies to antigens. Although ideal and useful for calculating area under the curve (AUC, mentioned as an option in ICH S8) of the assay response, the multitude of samples indicated in the Table 1 is not always necessary to obtain information on test article effect nor is it always logistically feasible.

2.4. Animal numbers

There is inter-animal variability of TDAR for all antigens. This is consistent with the high degree of variability associated with antibody responses in humans following vaccination (Orange et al., 2012; Tay et al., 2007). This variability is often minimized by optimizing dosages of vaccinations, but even so, it is difficult to limit variability. In order to demonstrate test article effect on TDAR, the studies must be powered adequately for the intended need.

With the use of rats and mice, higher variability is typically noted in outbred strains when compared to inbred stains (Ladics, 2007a; Kawai et al., 2013); typical coefficients of variation range from 15% to 80% in rats and mice. In the case of environmental chemical immunotoxicity evaluations, the EPA guideline recommends a minimum of 8 animals in each test and control group (US EPA, 1998). When conducting stand-alone immunotoxicity evaluations, only a single gender is typically evaluated for environmental chemicals. However, if conducting the evaluation as part of an integrated study design, both males and females are typically used and therefore, if conducted as part of a 90-day subchronic toxicity study, the total number of animals per dose group is increased to 20 (10 males and 10 females) (Ladics et al., 1998). For pharmaceuticals, usually both sexes should be used (ICH, 2005).

For NHPs, which are outbred, the inter-animal variability associated with TDAR responses is also a challenge (Lebrec et al., 2011). A typical percentage of coefficient of variation for the TDAR response is approximately 80%. On that basis, decreases in antigen-specific antibody responses $\geqslant$60% can be detected with adequate statistical power (i.e., approximately 80%) with 10 animals per group (Table 2), while a typical group size for NHP toxicology studies is 6 animals per group (N = 3/sex/group). Because inter-animal variability is a critical issue, TDAR data from male and female animals can be combined, when appropriate. It was demonstrated that control male and female macaques have similar TDAR responses both in terms of magnitude of the response and in terms of inter-animal variability (Lebrec et al., 2011). It is therefore important to consider the combination of male and female data when designing a TDAR study involving both sexes as a way to reduce animal usage. This is only possible if it is demonstrated that exposure to test article is comparable in males and females and that neither sex is particularly sensitive to treatment based on all study endpoints. It is worth noting that ICH S8 (2005) states that immunotoxicology studies in NHP do not have to be conducted in both sexes.

2.5. Use of positive control animals

Under the EPA immunotoxicity guideline, a positive control group with a known immunosuppressant is considered to be useful for interpretation of the results and for verification of the assay sensitivity (US EPA, 1998). The positive control recommended in the EPA guideline is cyclophosphamide, although others should be considered acceptable with appropriate justification. While the use of the positive control is of value for the demonstration of appropriate assay conduct and laboratory proficiency, for animal welfare reasons, laboratories conducting a large number of TDAR assays should consider use of historical positive control data, with re-evaluation periodically as suggested by ICH S8 (2005).

When conducting studies in NHPs with investigational drug substances, including a positive control group is neither necessary nor appropriate for animal welfare considerations if reference can be made to a validation study demonstrating that the design utilized is appropriate.

3. Analytical methods

Methods typically used for detection of antigen-specific antibodies as part of a TDAR evaluation are PFC assay, ELISA, and electrochemiluminesence (ECL) assay. In this section, a brief overview of each method focusing on advantages and disadvantages is provided as well as regulatory considerations, appropriate validation

Table 2
Power analysis: % Power of given sample size per group and %CV for different antibody response (AUC_{0-t}) effect size (2-sided test, significance level = 0.05).

Effect size as ratio (control AUC ÷ test group AUC)		1.25	1.5	1.75	2	2.25	2.5
Effect size as % decrease (100 × [test group − control] ÷ control)		20%	33%	43%	50%	56%	60%
% CV = 80[a]	N = 6/gp	7[b]	14	23	33	43	53
	N = 8/gp	9	18	31	45	57	67
	N = 10/gp	10	23	39	54	68	78
	N = 12/gp	11	27	46	63	77	86

[a] A 80% coefficient of variation is typically observed in NHP TDAR responses.
[b] Values represent the % power associated with different group sizes (N = 6–12) when identifying a certain change in antibody response (20–60% decreases corresponding to 1.25–2.5 control/test group AUC ratio) as being statistically significant (alpha = 0.05). Decreases in antigen-specific antibody responses $\geqslant$60% can be detected with adequate statistical power (i.e., 78%) with 10 animals per group given the % CV typically observed in NHP TDAR (80%).

Table 3
TDAR analytical platforms attributes.

	ELISA	PFC	ECL
Molecular assessment experience	Chemicals/SM/biologics	Chemicals/SM	Biologics
Antigen	KLH/SRBC/TT/other	SRBC	KLH
Matrix	Serum	Spleen	Serum
Throughput	High	Low	High
CRO available	Yes	Yes	Yes[a]
Precision	<20%	20–30%	<20%
Sensitivity	ng/mL	100 PFC/10^6 splenocytes	ng/mL
Detection of IgM and IgG	Yes	No (IgM only)	Yes
Detection of all IgG subclasses	Yes	No	Yes
Titer ready	Yes	No	Yes
Currently applicable species	M, R, D, NHP, H	M, R	R, NHP, H

Abbreviations: AFC = antibody forming cell; CRO = Contract Research Organization; D = dog; ECL = electrochemiluminesence; ELISA = enzyme-linked immunosorbent assay; H = human; KLH = keyhole limpet hemocyanin; M = mouse; NHP = non-human primates; PFC = plaque forming cell; R = rat; SM = small molecules; SRBC = sheep red blood cell; TT = tetanus toxoid.

[a] Specific platform availability varies across CROs.

parameters and key assay parameters and performance considerations (summarized in Table 3).

3.1. PFC Assay

The PFC assay quantifies the number of specific antibody-producing cells in the spleen following immunization, typically when using SRBC as the immunogen. This assay involves mixing splenocytes isolated from the immunized animal with guinea pig complement and SRBC (of same origin as SRBC used for immunization) in an agar-solution and pouring the mixture onto coverslips in Petri dishes. After incubation at 37 °C for at least 3 h, clear areas in the SRBC suspension (plaques) are counted. The plaques that are observed represent B-cells that are producing anti-SRBC antibody that is able to induce complement-mediated lysis of the SRBC which are observed as areas of hemolysis around each antibody producing cell. The full details of this assay are described elsewhere (Ladics, 2007a,b; White et al, 2010; Wilson et al., 1999).

The SRBC PFC assay has been utilized for over 35 years to assess a wide variety of chemicals for immunotoxicity and is considered to be the reference assay for mouse and rat TDAR based on extensive intra- and inter-laboratory validation (White et al., 2010; Luster et al., 1992). In general there are no issues with mid- or high titer cross-reactive antibodies against SRBC; thus, the plaques represent cells producing antibodies specific for the antigen. There are, however, certain disadvantages to the PFC assay: the antibody response is assessed in only one organ (spleen); it is labor-intensive; the requirement for splenic tissue may limit evaluation of splenic histopathology and immunotoxicology in the same animal; and, as it is a terminal procedure, it must be run on the same day as animal necropsy. The assay has historically been used to only measure the primary IgM response, although evaluation of IgG responses can be incorporated (Ladics, 2007a). Technical considerations include the need to characterize each new lot of guinea pig complement prior to use and the difficulty in manual counting of plaques. Many researchers have reported that the PFC may be more sensitive than serum ELISA-based approaches (Loveless et al., 2007; White et al., 2010), however with standardized ELISA methodology, both approaches are generally considered to be of equal sensitivity (Temple et al., 1993; Wilson et al., 1999; Boverhof et al., 2010).

3.2. ELISA

ELISA for detection of antigen-specific antibodies generally utilizes a sandwich method in which the antigen of interest is coated on a plate. Test samples (serum from animals treated with test article or control), reference standard (if used), and quality controls are incubated followed by a secondary detection antibody conjugated to an enzyme. The addition of a substrate results in a colorimetric detection of captured antibody. For semi-quantitative ELISA, species appropriate reference calibrators are used to generate a standard curve. For endpoint titer assays, the last dilution above the background (or multiple of background) or CP is the titer of the antigen-specific response. Samples are serially diluted with a minimum of 3 dilutions to obtain the appropriate titer value. ELISA methods for anti-KLH, anti-SRBC, and anti-TT antibody determination in serum have been described for mice, rats, dogs, and NHPs respectively (Piccotti et al., 2005; Roman et al., 2004; Ulrich et al., 2004; Finco-Kent and Kawabata, 2005; Plitnick and Herzyk, 2010; Temple et al., 1993; White et al., 2007; Kirk et al., 2008; Ladics, 2007a). Advantages of ELISA assays conducted on serum include the option to store samples for later analysis, assessment of total specific antibody responses (i.e., by spleen, bone marrow and lymph node cells), serial serum sample collection over time to follow the kinetics of antigen-specific IgM and IgG (and potentially IgG subtypes) antibody synthesis, and preservation of the whole spleen for histopathology. Results are reproducible and tend to have less inherent variability compared to manually counted plaques (Ladics, 2007a). Commercial ELISA kits for antigen-specific antibodies are available for the major toxicological species, providing advantages of decreased assay development time, pre-packaged and optimized reagents, and, in some instances, quality controls. Many of these kits include species-specific purified anti-IgM or IgG for the antigen of interest (KLH and SRBC) as calibrator standards providing greater assay specificity. As with any commercial kit, independent evaluation for the intended purpose and assessment of assay performance is strongly recommended (see Section 3.6). Development of an in-house ELISA may be the only option for species without readily available commercial kits, including some non-rodents. For in-house assay development and execution, rat and mouse anti-KLH IgM and IgG reference calibrators are commercially available, while for large animal species (NHPs, dogs, mini-pigs), reference calibrators may be generated by purification of sera from immunized animals. Potential disadvantages of ELISA methods include a lack of commercially available large animal species-specific reference calibrators and/or detection antibodies, differences in calibration of controls (purified vs. positive sera), potential lot-to-lot variability of commercial kits, the potential for large molecules (antibodies in particular) to interfere with the assay, and change in reagents necessitating re-qualification of the assay. Due to its low cost/high throughput capabilities,

broad applicability to different antigens, and translatability to the clinic, the ELISA platform continues to be a lead workhorse to support TDAR.

3.3. ECL

The Meso-Scale Discovery instrument utilizes an ELISA-based format but takes advantage of (ECL) detection (Moxness et al., 2005). It is used extensively for the immunogenicity testing of biologics in both the non-clinical and clinical setting and has been accepted by regulatory authorities. The use of this platform to detect anti-KLH antibodies is relatively new but can provide equivalent to better assay performance compared to the traditional ELISA. The ECL platform has an optimal KLH antigen coating method designed to reduce variability of the IgM response, the use of a broadly reactive secondary reagent to detect all IgG subclasses and has implemented a fixed assay cut-point as well as other normalization features to improve overall IgM and IgG precision (Hock et al., 2011). The instrument is readily available in most laboratories, where it is successfully used to support drug-specific immunogenicity testing.

3.4. Phage-neutralization assay

Bacteriophage φX174 is produced and tested as described in Wedgwood et al. (1975). Phage clearance from the circulation is measured by an agar overlay method using serial dilutions of serum. In mammals, including humans and NHP, phage clearance is completed by day 4 post intravenous injection (Wedgwood et al., 1975). The phage neutralizing antibody response is measured by the rate of phage inactivation by incubating a standard amount of phage with serially diluted test serum. *In vitro* phage inactivation follows the first order kinetics. The antibody isotypes (phage-specific IgM and IgG) are identified by 2-mercaptoethanol (2-ME) treatment: IgM antibody is susceptible to 2-ME treatment, while IgG is resistant (Ochs et al., 1971). While this assay is used for clinical monitoring at Seattle Children's Research Institute for antibody deficiency states and immune reconstitution post-bone marrow transplantation, its use for product development has been limited to testing the effect of therapeutic monoclonal antibodies designed to suppress immune function (Abrams et al., 1999; Krueger et al., 2008; Pescovitz et al., 2011).

3.5. Reagents

Species-specific detection reagents are usually Protein-G purified or affinity-purified polyclonal antibodies with some level of species specificity to the IgM or IgG. Most of these reagents are adequately assessed during validation for species specificity and selectivity with the exception of the NHP reagents. Most detection reagents (anti-IgM, anti-IgG) have been generated by immunization with human IgM and IgG with the assumption that broad cross-reactivity will be achieved against NHP IgM and IgG but may vary across NHP species. The anti-human IgM antibody reagents have a reasonable cross-reactivity to macaque IgM; a more specific reagent (generated from immunization with macaque IgM) is now available (e.g., KPL, Gaithersburg, MD). When considering a commercial assay to use or in development efforts to establish an in-house assay, the detection reagent specificity for NHP IgM and the cross-reactivity to NHP IgGs must be considered. Detection of all IgGs will improve as new polyclonal reagents and monoclonal antibodies with specific cross-reactivity to each NHP IgG subclass become readily available. Until then, one may consider the use of the traditional Protein G or Protein A reagent that has been demonstrated to broadly bind NHP IgG subclasses (Shearer et al., 1999).

3.6. Assay qualification

When assessing TDAR, total IgM and IgG antigen-specific antibody responses are traditionally measured but IgG isotype specific (IgG1–IgG4) antigen-specific responses can also be measured. Optimally, depending on assay format, reference serum, standard curve, and quality control samples are included on each plate and the acceptance criteria met for each plate.

Regardless of the method used to detect antigen-specific antibodies, the assay needs to be appropriately tested and qualified or validated based upon intended use (research, non-Good Laboratory Practice [GLP], GLP). Despite some latitude in validation parameters, one must demonstrate reliability of the assay. For example, studies necessary to predict safety and immunotoxicity are expected to comply with GLP regulations. Parameters typically included in a full assay validation are specificity, selectivity, range of quantification, inter/intra-assay precision and accuracy, dilutional linearity, parallelism (using actual study samples), and stability (assessments should reflect anticipated sample collection, storage, and processing: bench top, short-term, long-term, and freeze–thaw). In-study evaluation over multiple studies is recommended to determine assay robustness, including antigen and reagent lot-to-lot variability. Additional discussion of immunoassay and bioassay development and validation methods can be found in previously published workshop summaries and review papers (Findlay et al., 2000; Miller et al., 2001).

It is generally accepted that optimally the same antigen source used in the immunization be used in the immunoassay to capture the antigen-specific antibodies. Despite this, it is suggested that the different forms of the antigen be tested during development and the one that provides the best specific signal-to-noise or NSB ratio be selected. Since species-specific detection reagents are used, it is recommended that laboratories rely on pooling positive samples, containing antigen-specific IgM and IgG, to use as a positive control. Until species-specific recombinant antibodies to the antigen become available, pooled positive serum provides the most appropriate positive control. Although this may meet the requirements of the positive control for the assay, different positive control lots will have different reactivity and thus it is difficult to compare results in the same species across studies. The use of a negative control (serum from non-immunized animals) is required to be run on each assay plate. A negative control monitors the background NSB of the assay and represents a "true" negative result as it takes into account any matrix effect. Good practice is to run the negative control in duplicate at minimum, but some commercial and in-house assays test multiple "negative" individual serum samples for each species per plate and use the average value as the assay or plate CP. Samples that react above the CP are positive and below are considered negative. All positive samples are further serially diluted to establish their titer which is considered as being the last dilution falling above the defined CP. The titer obtained for each sample is representative of the quantity of anti-KLH antibodies present in the sample. It is recommended to follow the guidance in the non-clinical immunogenicity white paper (Ponce et al., 2009) to establish the CP during assay validation. If this approach is taken and a universal species-specific positive control can be identified, control data across programs can be collected and trending of the controls can provide valuable information about the long-term assay performance and robustness (Barger et al., 2010). As with immunogenicity assays, it is also good practice to identify reagents critical to the overall TDAR assay performance if changes in supplier or lot are anticipated. New reagents such as antigen, secondary detectors and positive controls require a qualification test prior to use, to ensure equivalent results against the current reagent(s). When utilizing the assay in a given animal study, consideration should be given to showing the lack of

interference of the test article present in the serum samples with the analytical method used to measure antigen-specific antibodies.

4. Data Presentation

Proper presentation of TDAR assay results is critical to support data interpretation. In addition to reporting individual animal data, antibody responses for all time points should be presented in a tabular form, including but not limited to the arithmetic mean and/or median antibody titers or concentrations. In addition, titer or concentration AUCs may also be calculated as the area under the curve defined by titers or concentrations (*Y* axis) across study days (*X* axis) (starting from one pre-immunization sample day, e.g., day of administration of immunogen or before) using the linear-log trapezoidal method as AUC is a good representation of the overall antibody response. Depending on the duration of the study and the sampling scheme, it may be possible to either only calculate a global antigen-specific IgM and/or IgG AUC or, preferably, to calculate primary and secondary IgM and/or IgG response AUCs.

Summarizing the descriptive statistics for each group and at each time point and reporting such statistics together with individual assay results is typical. The mean and median can be used to describe the central tendency. The standard deviation, variance, IQR (inter-quartile range) and/or range (minimum to maximum) can be used to describe the variability of the assay results. The number of total observations and missing observations need to be reported as well. Such descriptive statistics allow a quick grasp of the characteristic of the assay response for the study.

While tabulating individual and mean data is critical (for an example, see Table 4), illustrating the antibody response in a graphical format is helpful to illustrate group patterns and inter-animal variability. For example, the box-plots of individual assay responses for all groups can be very beneficial to evaluate the central tendency and variability of the assay results for a group and across groups and to identify extreme observations; the line plot (connecting individual assay responses across time for each subject) helps to see inter-subject variability according to the time profiles and to identify subjects that behave differently from others; the scatter plots (different color or/and symbol for different groups) help to evaluate how individual results are scattered for a group and across groups for a time point (for example Figs. 3A and 7) and one can certainly overlay the scatter plot with a box-plot or a line plot. It is recommended to show the distributions of assay results for all treatment groups and control group of all the time points on the same graph. It does help to visually identify where the assay responses peak and validate whether the animals responded as expected. Other graphical presentations can certainly be used to visualize the pattern and trend of the data.

The log transformation tends to stabilize the variances across groups and time. As such, it is not uncommon to show the assay responses on a log scale in the aforementioned graphs. It is not uncommon to have individual assay results as below the quantification limit of the assay. Such incidences are typically few and as such, these values below the quantification limit of the assay can be replaced by some arbitrary value (e.g., limit of quantification/2).

5. Statistical analyses

The data to be included in statistical analyses may include IgG and IgM values (from multiple groups and multiple time points) and, when available, IgG and IgM AUCs (global, primary and/or secondary depending on the sampling schedule and duration of a study). The primary question to answer is whether the treatment(s) significantly impact antigen-specific immune function as measured by IgG and IgM responses. There is no unique statistical method that is appropriate to answer this question.

Given the repeated measure nature of IgG and IgM titers, it is appropriate to apply a repeated measure analysis of variance model to the IgG and IgM titers, evaluate the treatment effect and examine whether the treatment effect is time dependent. It is acceptable to implement an analysis of variance model to evaluate the treatment effect at each time point or to evaluate the treatment effect when titers of single time point are collected. The pair-wise comparisons are then performed when the treatment effect is statistically significant. Applying multiple comparison adjustment procedures decreases the likelihood to detect changes and may not be desirable.

There are statistical assumptions associated with analysis models such as normality of the distribution for each group at a given time point and equal variances across groups. The residuals from the statistical models should be used for such evaluations. Log transformation is typically effective to re-express the data to be close to normal and achieve equal variance across groups. As such, the residuals shall appear to be close to normal (at least symmet-

Table 4
Example of tabulated TDAR NHP results in vehicle-treated animals with descriptive statistics and excluding animals with pre-existing KLH-reactive IgGs.

Animal ID/sex	Dose level (mg/kg)	Anti-KLH IgG titer										IgG AUC_{0-43}
	0	Pre-study	D0 (KLH)	D7	D10	D15 (KLH)	D22	D25	D32	D36	D43	
1 M	0	50	50	225	1505	1464	5104	5126	5797	5219	3940	141402.300
2 M	0	50	50	509	1267	1533	8379	5664	5171	4813	4082	156029.920
3 M	0	50	50	123	593	776	2460	2366	1645	1941	1818	57859.540
4 M	0	50	50	158	1545	3011	14110	9625	5856	6989	4134	226549.374
5 M	0	50	50	221	1265	1520	3481	2928	2375	2190	1741	78549.578
6F	0	50	50	231	702	1081	3286	3635	3893	3744	2772	96762.386
7F	0	50	50	592	2332	3020	4791	4272	2443	3440	2775	117162.169
8F	0	50	50	644	2466	2830	5151	4779	3410	10575	2810	160807.100
Arithmetic mean		50	50	338	1459	1904	5845	4799	3824	4864	3009	129390.100
Median		50	50	228	1386	1527	4948	4526	3652	4279	2793	129282.234
SD		0.0	0.0	208.4	674.3	906.9	3788.5	2242.4	1637.4	2834.4	960.6	53714.0513
SE		0.0	0.0	73.7	238.4	320.6	1339.4	792.8	578.9	1002.1	339.7	18990.7850
Min		50	50	123	593	776	2460	2366	1645	1941	1741	57859.540
Max		50	50	644	2466	3020	14110	9625	5856	10575	4134	226549.374
N		8	8	8	8	8	8	8	8	8	8	8

Anti-KLH titers were measured by ELISA in samples collected on days 0–43 from male (M) and female (F) NHP immunized on day 0 and day 15 with 1 mg KLH (Imject Maricultur[®] KLH in phosphate buffered saline, Thermo Scientific). Anti-KLH IgG AUC_{0-43} was calculated for each animal using the linear-log trapezoidal method. Arithmetic mean, median, standard deviation (SD), standard error of the mean (SE), minimum and maximum values were reported for each sampling day and for the AUC_{0-43}. Animals with pre-existing KLH-reactive antibodies were excluded from this analysis. Values below the limit of quantitation were replaced by 50 (1/2 of the limit of quantitation).

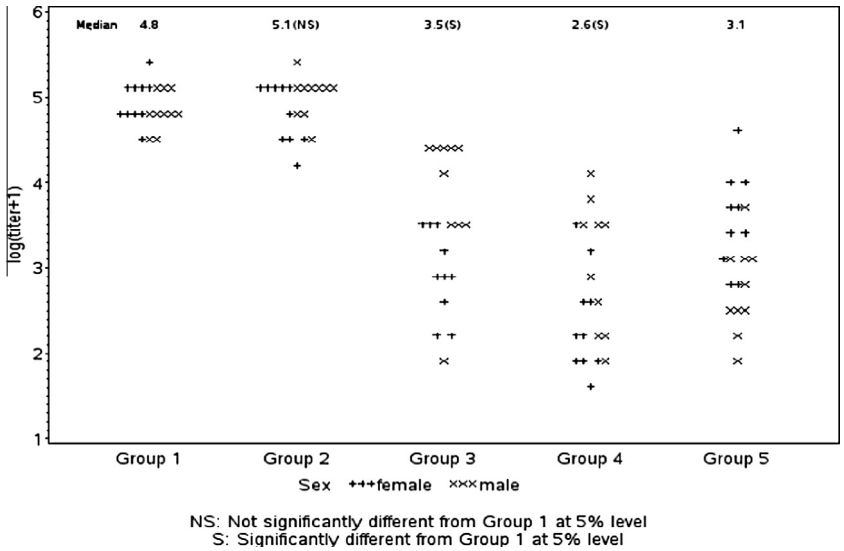

Fig. 7. Example of treatment-related effects in TDAR in rats immunized with KLH. Rats (N = 10/sex/group, 5 groups) were immunized with KLH on Day 15. The TDAR log (titer + 1) primary IgM response was measured by ELISA on Day 20. Group1, neg. control; Groups 2–4, treated group animals; Group 5, positive control (cyclophosphamide) animals.

ric) and have similar variances across groups. Statistical outliers can be detected at this step by applying some outlier detection method (e.g., Tukey's outlier detection method) to the residuals. Although it is possible that the assumptions can be better met by removing these observations, the investigators need to reach consensus about what to do with such outliers and statisticians are encouraged to consider some sensitivity analysis (conduct analysis with or without such observations) to understand the impact of such observations. When the assumption(s) is questionable, other statistical techniques should be considered, such as statistical models that allow comparisons among groups with unequal variances (note that the unequal variance among the groups may represent meaningful information of the treatment itself) or nonparametric methods.

When reporting the statistical analysis results, it is highly recommended to report a p-value for overall treatment effect, p-values for pair-wise comparisons and adjusted p-values if a multiple comparison procedure is applied. It is important to note that a significant p-value does not necessarily mean a meaningful treatment effect and a not significant p-value does not necessarily mean there is no treatment effect. Hence, more important than p-values are the estimates of treatment effects and their corresponding confidence intervals which allow evaluation of the scientific relevance of the findings.

It is recommended to have a pre-specified statistical analysis plan which describes critical components of the statistical analysis, including but not limited to how to detect and handle extreme observations or statistical outliers, the statistical analysis flow, the pairs of groups to be compared, whether and what multiple comparison procedure will be used, and how statistical significance is defined. Such a plan should strive to be clear about the criteria that will be used for decision making during data analysis and interpretation. It requires a close collaboration between the subject matter experts and statisticians to put together a statistical plan that will address the research question and contribute to study conclusions.

6. Data interpretation, relevance and impact

Data interpretation includes the ability to correctly identify treatment-related effects or the lack thereof, and the biological significance of the effect, when present.

The identification of the presence or absence of test article-related effects is made by comparing the response to the Ag in animals dosed with test material to that measured in control animals (including its kinetics, magnitude, and the observation of class-switching when applicable), and using all information available to draw conclusions about the overall effect of the compound. When interpreting TDAR results, potential immunotoxicity can be predicted if effects clearly differ from control responses. One needs to take into consideration the fact that a "normal" immune response varies in magnitude from individual to individual (whether in animal models or human subjects). A statistical signal at an isolated time point should not necessarily be a concern. Biological plausibility must also be considered in the context of the nature of the response, and evidence of overt multisystem toxicity may signify that effects on the immune system are mediated by stress and/or secondary to other effects rather than direct immunotoxicity.

While the TDAR assay is designed to assess functional competency of the immune system, it is also important to note that descriptive immune parameters, such as hematology (leukocyte counts), lymphoid organ weights and histopathology, are routinely evaluated as part of standard toxicity studies. These descriptive data do not evaluate immune function like the TDAR and other functional assays; but may provide an indication of immune system effects. The TDAR assay is a tool which can be used to determine if an anatomic or hematologic finding, particularly lymphoid depletion, has a functional consequence. Ultimately, the need for additional evaluation and the overall interpretation of an immunotoxic effect is made using a weight-of-evidence approach that considers all available toxicity data.

In order to understand the biological significance of different types and degrees of changes in the TDAR, previously described responses to Bacteriophage ΦX174 in patients with different types of primary immunodeficiency diseases (PIDD) can be considered. Quantitatively and qualitatively abnormal immune responses to immunization with ΦX174 have been described, and examples of these abnormal responses have been observed in PIDD patients (Wedgwood et al., 1975), in immunosuppressed (secondary) patients (Witherspoon et al., 1981; Abrams et al., 1999; Fogelman et al., 2000; Krueger et al., 2008; Pescovitz et al., 2011), and in primary and secondary immunosuppressed animals (Bue et al., 1986; Andrews et al., 1997; Felsburg et al., 1997; Kay et al., 1997; Miao

et al., 2009). Because ΦX174 is a T-cell-dependent neoantigen, abnormal antibody responses are observed not only in patients with a prototypic B cell defect (e.g., X-linked Agammaglobulinemia [XLA]), but also in those with a T cell defect (e.g., patients with Hyper IgM syndrome due to CD40 ligand mutations), or in both. Most XLA patients who have markedly reduced circulating B-cells have a prolonged phage clearance of up to five weeks (normal clearance 3–4 days), demonstrating that they are unable to make antibody to this antigen (Ochs et al., 1971; Wedgwood et al., 1975). Some molecularly defined Severe Combined Immune Deficiencies (SCID), including those resulting in inability to rearrange VDJ (e.g., RAG 1 and 2 or Artemis deficiency), also result in prolonged phage clearance and failure to produce specific antibody. In contrast, SCID patients due to common gamma chain, JAK3 or ADA deficiency, are able to clear ΦX174 and produce small amounts of neutralizing antibody. Patients with X-linked Hyper IgM syndrome due to mutations in CD40 ligand have normal phage clearance, and develop a response characterized by lack of amplification and failure to isotype switch, as expected from this T cell defect. On the other hand, patients with common variable immunodeficiency are able to clear phage normally and produce some neutralizing antibody. While some produce only IgM antibody, others can isotype switch to various degrees (Stiehm et al., 2004). Patients with classic Wiskott-Aldrich syndrome (WAS) almost always have mutations that result in complete absence of the WAS protein and produce only small amounts of IgM antibody, whereas those with X-Linked thrombocytopenia (XLT), who generally have missense mutations in the *WAS* gene, are able to express mutated protein and often have a near-normal antibody response to bacteriophage, including immunologic memory and isotype switching (Ochs et al., 2007). Interestingly, patients with early complement component deficiency also have a defective antibody response to ΦX174 (Ochs et al., 1993). This information illustrates how the TDAR can be affected through multiple mechanisms, leading to various types of abnormal responses.

How the results of a positive or negative assay (i.e., that the test article suppresses the TDAR or has no meaningful effect, respectively) will be used by the regulated industry and the government regulatory bodies will depend to a large degree on the nature of the compound and how it is regulated (e.g., environmental chemical or (bio)pharmaceutical as described below), its intended use (e.g., immunomodulation or not), and other factors.

6.1. Environmental chemicals

The EPA immunotoxicity study (OPPTS. 870.7800) is designed to evaluate the immunosuppressive potential of a chemical by measuring TDAR to SRBC in rats or mice with a PFC assay or ELISA. In certain instances, and depending on the outcome of the TDAR assay, it may be necessary to perform quantitative immunophenotyping for major lymphocyte populations and T cell subpopulations by flow cytometry, or to conduct a NK cell activity assay to assess the effects of the test compound on non-specific (innate) immunity.

It is important to note that the EPA Office of Pesticides Programs requires extensive toxicity testing, in addition to immunotoxicity, which evaluates a wide range of toxic effects for conventional pesticides. Immunotoxicity studies are utilized among these other studies to provide the scientific basis for characterizing the potential hazard of pesticide exposure and safety of the product for its intended use. Toxicity studies are conducted in multiple species (mice, rats, rabbits, and dogs) for various durations (acute, sub-chronic and chronic) and evaluate various endpoints (nervous system, developmental, reproduction, subchronic and chronic toxicity, carcinogenicity, mutagenicity, and immunotoxicity). The Agency uses these studies to determine whether adverse effects are caused by different durations of pesticide exposure ranging from short-term (acute) to long-term (chronic) exposure and different routes of exposure (oral, dermal, or inhalation). A no observed adverse effect level is established from the low end of the observed adverse effect and is used as a starting point for conducting a quantitative risk assessment which compares the observed hazards to estimated exposures to determine overall safety. Data generated under the immunotoxicity testing guideline would be used to define the immunotoxic potential of the chemical and any effects observed could be used as part of the quantitative risk assessment process. Importantly, recent retrospective analyses have indicated that pesticides rarely had a primary effect on the TDAR and therefore this endpoint rarely impacted the chemical risk assessment (Gehen, in submission). These findings suggest a triggered approach for conduct of the TDAR, similar to that of the pharmaceutical evaluations, may be more appropriate for pesticide testing requirements.

6.2. Pharmaceuticals

For pharmaceuticals (small molecules or biologics) the response to a positive TDAR result will be highly dependent on the specific details of the drug, including its molecular target, the duration of treatment, the intended patient population and whether the positive TDAR result is a predictable consequence of the intended pharmacology of the drug. At one extreme, it would be expected that a monoclonal antibody that is intended to act as an immunomodulator of B lymphocyte activity would give a positive response in the TDAR. In this instance, as long as the mechanism by which the monoclonal antibody is acting is well understood and the expected result is consistent with the intended treatment, it is possible that additional nonclinical studies would not be required to further characterize the risk of immunosuppression. The TDAR assay is simply providing an understanding of the breadth and depth of the immunologic effect along with other descriptive endpoints and possibly additional functional assays. Clinical studies with this biopharmaceutical however, would likely pay close attention to safety signals indicating immunosuppression and timing of vaccination would have to be considered. In contrast, a positive TDAR result from a drug that is not predicted or intended to affect the immune system may result in a need to better characterize the basis of the perturbation in the immune response. Depending on the patient population to be treated, and the perceived risk–benefit ratio, enhanced clinical monitoring or exclusion of significantly susceptible individuals may be adequate to address these concerns. Unexpected significant adverse effects in a TDAR may be deemed an unacceptable risk for some indications and/or patient populations. In such situations, clear demonstration of reversibility may be useful to justify short-term treatment in a clinical setting with appropriate informed consent and clinical monitoring.

7. Conclusions

In conclusion, this manuscript discusses proper TDAR study design, data analysis, and interpretation for either environmental chemicals or pharmaceuticals based on current information. It is emphasized that only rigorous study design enables proper data interpretation. A proper design includes an appropriate immunization schedule, the utilization of well characterized reagents and of validated analytical methods. The overall methodology used should have been demonstrated to be appropriate for the detection of changes in response to a known immunomodulator. The TDAR assay offers technical challenges that can be met with proper study conduct and an assay not conducted properly can lead to erroneous conclusions. Interpretation of the TDAR and application to human

risk assessment should take into consideration other information known about the molecule being tested, such as mechanism of action and findings in standard toxicity studies. It should be acknowledged that TDAR-related methodologies are evolving. A greater understanding of the pros and cons of different antigens, the development of alternative methods and endpoints, including alternative *in vitro* techniques, should be the focus of future work.

8. Disclaimer

The views expressed in this paper are those of the authors and do not necessarily reflect the views or policies of the U.S. Environmental Protection Agency or the U.S. Food and Drug Administration.

Conflict of interest

None.

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