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Safety immunopharmacology: Evaluation of the adverse potential of pharmaceuticals on the immune system

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ABSTRACT

The ICH S6R1 and S8 guidelines define a general framework for the immunotoxicity evaluation of biotechnology-derived pharmaceuticals and human pharmaceuticals, respectively. As severe and unpredicted adverse events dramatically showed in the recent years that the immune system is a critical aspect of drug safety, this framework needs to be revisited to enhance the prediction of nonclinical immune safety evaluation. Safety immunopharmacology is deemed to contribute to this awaited improvement by enabling early screening of the potential for drug candidates to induce unexpected immunosuppressive and immunostimulatory effects as well as nonimmune-mediated hypersensitivity reactions. Dedicated safety immunopharmacology can also generate mechanistic data to determine which relevant additional immunotoxicity studies should be conducted. Immunological assays and models that can be considered for use in the context of safety pharmacology studies are presented as well as perspectives for their timely development.

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1. Introduction

The immune system has so far been very seldom considered as a target for safety pharmacology studies of drug candidates. Thus, the ICH guideline S7A only briefly mentions the immune system as an “example of other organ systems” whose function can be investigated in the context of follow-up or supplemental safety pharmacology studies for human pharmaceuticals (ICH, 2000).

In the recent years, severe and unpredicted immune-related adverse events, however, dramatically showed that the immune system should be considered a critical aspect of drug safety. Illustrative examples of such adverse events include reports of infectious complications, e.g. tuberculosis and a variety of opportunistic infections in patients treated with anti-TNF α drugs (Rychly & DiPiro, 2005), reactivation of the JC virus resulting in progressive multifocal leukoencephalopathy suspected to be a consequence of natalizumab treatment in multiple sclerosis patients (Stüve et al., 2007), and the so-called cytokine storm in healthy volunteers enrolled in a phase I clinical trial of the monoclonal antibody TGN1412 (Suntharalingam et al., 2006). These and other adverse events demonstrate that despite significant progress in nonclinical immune safety evaluation over the last two decades, much remains to be done to improve procedures and strategies in current use.

With the rapid development of novel biologics (biopharmaceuticals), a majority of which target the immune system, the role of safety

immunopharmacology can be expected to increase significantly in the near future. Indeed, two major limitations to the nonclinical safety evaluation of biologics are: (i) immunogenicity, potentially resulting in the generation of neutralizing anti-drug antibodies, which in turn limits the duration and relevance of non-clinical toxicity studies (Ponce et al., 2009), and (ii) the lack of a relevant conventional animal species that can tentatively be replaced by transgenic or diseased animals in which regulatory toxicity studies are faced with many challenges and hurdles (Bussiere et al., 2009). Therefore, safety immunopharmacology studies can be expected to serve as useful add-ons to generate relevant missing data that standard toxicity studies are unable to generate. Moreover, the search for small molecular entities that target the immune system to treat a variety of diseases ranging from cancer to allergic or autoimmune diseases is rapidly expanding (Tsitoura & Tassios, 2006; Erter et al., 2010; Nicholas, Giannetti, Alsanousi, Friede, & Muraro, 2011). Therefore, the need for dedicated exploratory and mechanistic immunopharmacology studies to address potential causes of concern related to immunological safety is not restricted to biologics.

2. Overview of immune-mediated adverse effects of pharmaceuticals

The immune-mediated (immunotoxic) adverse effects of pharmaceuticals should be subdivided into four categories, namely immunosuppression, immunostimulation, hypersensitivity and autoimmunity. It is indeed essential to bear in mind that clinical manifestations as well as modalities for nonclinical as well as clinical safety evaluation are markedly distinct depending on the category of immunotoxic effects being considered.

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2.1. Immunosuppression

Because potent immunosuppressive drugs have been in clinical use for more than 3 decades, adverse events associated with immunosuppression are well characterized. Two major clinical consequences of immunosuppression are infectious complications and virus-associated neoplasia.

Immunocompromised patients including those treated with immunosuppressive drugs can be affected by a vast variety of bacterial, viral, fungal and parasitic infections (Bresnihan & Cunnane, 2003; Fishman, 2007). These infections are always more frequent, often more severe and relapsing, and sometimes atypical (“opportunistic”). Overall, most, if not all pathogens can be involved, and infections can develop at any site, even though respiratory, gastrointestinal and skin infections are more common.

Virus-associated neoplasias are also more frequent in immunocompromised subjects (Zafar, Howell, & Gockerman, 2008). Although non-Hodgkin’s lymphomas, especially B lymphomas are usually considered to be another hallmark of severe drug-induced immunosuppression (Everly, Bloom, Tsai, & Trofe, 2007), skin cancers including squamous cell cancers and Kaposi’s sarcomas are actually more frequent. The pathogenesis of neoplasias associated with immunosuppressive drugs is largely thought to be linked to a dormant or subsequent viral infection (e.g. Epstein–Barr virus or human herpes virus), the development of which is facilitated by immunosuppression. Therefore, the major complications of immunosuppression can be viewed globally as the consequences of impaired resistance of the body toward pathogens.

A variety of innate and/or adaptive, often intricate immune mechanisms is involved in the host’s defense against pathogens, so that predicting the potential for a drug candidate to impair resistance toward pathogens should primarily rely on immune functions. Surprisingly, neither the ICH S6R1 guideline (ICH, 2011) nor the ICH S8 guideline (ICH, 2005) requires a systematic investigation of immune functions in treated animals, so that safety immunopharmacology studies are deemed to help generate useful additional information.

2.2. Immunostimulation

Although the adverse effects of immunostimulation have long been known or at least suspected (Descotes, 1985), full confirmation was obtained only after the introduction of potent immunostimulatory drugs, in particular recombinant cytokines and monoclonal antibodies, in the clinic.

The most frequent adverse events associated with immunostimulatory drugs are related to cytokine release (Descotes & Vial, 2007). Depending on the cytokine-releasing potency of the offending drug, these adverse events can manifest as: (i) flu-like reactions with moderate fever, shivering, myalgias and arthralgias; (ii) acute cytokine syndromes with hyperpyrexia, marked shivering, myalgias and arthralgias as well as cardiovascular and/or neurological disturbances; or (iii) “cytokine storm” combining severe clinical manifestations of acute cytokine syndrome with multi-organ failure (especially the severe acute respiratory syndrome or SRAS, and acute renal failure).

Treatments with some, but not all immunostimulatory drugs have been shown to be associated with more frequent autoimmune diseases that are strictly identical to spontaneous autoimmune diseases based on clinical and biological criteria (Descotes, 2004).

The potential for immunostimulatory drugs to induce more frequent hypersensitivity reactions to unrelated allergens has long been recognized, but largely overlooked. Indeed, some patients starting a treatment with a so-called “immunopotentiating drug”, e.g. levamisole, have long been reported to develop or have exacerbation of prior asthma, hay fever or eczema. Importantly, these hypersensitivity reactions are not directed against the immunostimulatory drug, but against unrelated allergens, e.g. microbial or environmental allergens, and should therefore be distinguished from genuine drug-induced allergies. Moreover, it was

unequivocally demonstrated that patients with renal carcinoma when treated with rhIL-2 had a significantly higher incidence of hypersensitivity reactions to radiocontrast media (Zukiwski et al., 1990).

Finally, there is a large body of data to evidence the potential of immunostimulatory drugs for decreasing the expression of several genes involved in the cytochrome P450 system, resulting in significant drug interactions in animals and to some extent in human subjects at therapeutic doses (Descotes, 2004).

2.3. Hypersensitivity

Immune-mediated hypersensitivity reactions are rather frequent adverse events associated with drug treatment (“drug allergies”). The pathogenic mechanisms of antigen-specific immune responses involve the exquisite recognition and memory abilities of the immune system.

Nonimmune-mediated hypersensitivity reactions, which mimic immune-mediated hypersensitivity reactions, at least to some extent, are also described (DeShazo & Kemp, 1997). Importantly, these reactions do not involve a specific immune response, but instead the release of some mediators involved in immune-mediated hypersensitivity reactions via a pharmacological or toxicological mechanism. Examples of such “pseudo-allergic reactions” include direct, i.e. nonIgE-mediated, histamine release or direct complement activation.

2.4. Auto-immunity

Beside more frequent autoimmune diseases observed in patients treated with some immunostimulatory drugs, drug-induced autoimmune reactions have also been reported. These reactions can be systemic, and then affect many different targets in the body (e.g. drug lupus syndrome), or organ-specific and then can be characterized by a highly specific autoantibody response (e.g. drug-induced myasthenia or autoimmune hemolytic anemia). Globally, these reactions are rare, in particular organ-specific reactions. One given drug is typically associated with one given type of autoimmune reaction, and the underlying mechanism is not elucidated (Descotes, 2004).

3. Current requirements for the nonclinical immunotoxicity evaluation of pharmaceuticals

Regulatory requirements for the nonclinical immunotoxicity evaluation of pharmaceuticals began to emerge at the very end of the 20th century. As the early guidance documents released by EMA (European Medicines Agency, 2000) and the US FDA (U.S. Food and Drug Administration, 2002) included overtly conflicting recommendations, harmonization was needed and actually achieved by the ICH S8 guideline (ICH, 2005), which provides the core of current requirements for the conduct of immunotoxicity studies for human pharmaceuticals. The revised ICH S6 guideline (ICH S6R1) offers only fairly broad recommendations on the immunotoxicity evaluation of biotechnology-derived pharmaceuticals (ICH, 2011).

One major requirement of the ICH S8 guideline is the systematic incorporation of immunotoxicity evaluation into standard drug development. This evaluation comprises a weight of evidence review based on: (i) the results of standard toxicity studies including clinical signs (in particular infections, or tumors when no other cause can be identified), hematological anomalies (e.g. lymphopenia/lymphocytosis), and histological changes in the main lymphoid organs (bone marrow, thymus, spleen, lymph nodes, and mucosa-associated lymphoid tissue or MALT); (ii) the pharmacological properties of the drug candidate (on- and off-target effects on the immune system); (iii) the intended patient population (e.g. immunocompromised patients); (iv) possible structural similarities with known immunotoxicants; and (v) drug disposition (in particular, high concentrations of the test article in immune cells) (ICH, 2005).

When the weight of evidence review concludes that no cause for concern has been identified, no additional immunotoxicity studies are required. In contrast, when a cause for concern (either one marked finding or at least two milder findings among the items listed above) has been identified, additional immunotoxicity studies should be conducted. Additional immunotoxicity studies typically consist of repeated-dose (at least 4-weeks) toxicity studies conducted, mostly in rodents, in the course of which selected immune functions are investigated. A T-dependent antibody response (TDAR) assay is widely considered the first-line assay as it can globally and simultaneously assess the effect of a drug candidate on antigen presentation, helper T lymphocyte function and B lymphocyte-dependent antibody production. Immunotoxicologists have long used the plaque-forming cell (PFC) assay in rodents (Ladics, 2007). Over the last decade, the anti-KLH antibody response (measured by ELISA) has emerged as the preferred assay (Plitnick & Herzyk, 2010). Indeed, the anti-KLH assay can be used in rodents as well as non-rodents in sharp contrast to the PFC assay, which can only be used in rodents. KLH (keyhole limpet hemocyanin) is a better standardized antigen than sheep erythrocytes, the antigens utilized in the PFC assay. The anti-KLH assay is also less time consuming and the reproducibility of results seemingly better.

Other (second-line) immune function assays include lymphocyte subset immunotyping (even though this is not an immune function assay strictly speaking), measurement of NK cell activity using either the gold standard ^{51}Cr release assay *in vitro/ex vivo* or a flow cytometry technique, cell-mediated immunity models such as the lymphocyte proliferation assay *in vitro* or delayed-type (DTH) hypersensitivity models, and neutrophil function (e.g. chemotaxis and phagocytosis) assays (Dieter, 2010).

4. Which pharmacology studies in evaluating the immunological safety of pharmaceuticals?

Although safety pharmacology studies are generally performed by single dose administration, this is impossible when prior sensitization of the animals is required. Several assays and models, however, are available that allow for short-term (≤ 7 days) repeated administrations. Other assays only require a single dose administration and can be performed in the context of dedicated safety immunopharmacology studies or short-term studies (e.g. 7-day studies with endpoint measurement after the first and the last administration, or the last administration). In any case, all assays and models proposed below (see Table 1) can be straightforwardly included into safety immunopharmacology studies.

4.1. Immunosuppression

In the context of immune function evaluation from an immunotoxicology or safety pharmacology point of view, TDAR assays are pivotal early screens. One obvious drawback of the anti-KLH assay as compared to the PFC assay is that the standard procedure comprises a minimal duration of 14–21 days for the anti-KLH assay vs. 4–5 days for the PFC assay for measuring the humoral response. However, White, Sheth, and Peachee (2007) proposed a short-term anti-KLH IgM screening assay in the mouse as an alternative to the PFC assay. Mice were injected once with 2 mg KLH/mouse via the intravenous route, and sera were collected on day + 5 to measure anti-KLH IgM by ELISA. The reference immunosuppressive drugs cyclophosphamide (range: 5–60 mg/kg/day), azathioprine (25–200 mg/kg/day) and cyclosporine (25–200 mg/kg/day) induced a significantly dose-dependent decrease in anti-KLH IgM levels. Using the same dose range, a very similar trend was observed in the PFC assay although the latter assay was slightly more sensitive than the anti-KLH IgM assay. The high level of predictability of the PFC assay has long been demonstrated with a wide panel of immunosuppressive agents (Luster et al., 1992) and it is noteworthy that the immunosuppressive potential of cyclosporine was serendipitously discovered thanks to the systematic use of the PFC assay (Borel, Feurer, Gubler, & Stähelin, 1976). That White et al. (2007) found similar effects in the short-term anti-KLH IgM mouse screening assay with 3 prototypic immunosuppressive drugs whose effects are well-characterized in humans support the conclusion that this assay is likely to be as predictive as the PFC assay. In addition, this screening assay offers the advantage of using the same T-dependent antigen, namely KLH, than the antigen increasingly used in TDAR assays that can be included in dedicated immunotoxicity studies at a later stage of development.

Cell-mediated immunity can be straightforwardly assessed using either a DTH model in mice or rats, or a contact hypersensitivity model in mice with 5–7 days between sensitization and challenge. In rodents, DTH is typically measured from the increase in footpad thickness (footpad assay) induced by an eliciting injection of the selected antigen (e.g. SRBC, bovine serum albumin, ovalbumin...) into one hind footpad of animals previously sensitized by a subcutaneous injection of the same antigen into the back or abdomen. Contact hypersensitivity is measured from the increase in ear thickness (ear assay) induced by an eliciting application of a potent contact sensitizer (e.g. oxazolone, dinitrofluorobenzene or picryl chloride) on both sides of one ear in animals previously sensitized by a prior topical application of the same sensitizer on both sides of the contralateral ear. In keeping with their well-known clinical activity in humans, immunosuppressive drugs have been shown to induce a

Table 1
Selected assays and models to be considered for inclusion in dedicated safety immunopharmacology studies.

Assays/models	Species	Measured immune function/adverse effect	Conditions of exposure
Anti-KLH IgM screening assay (ELISA)	Mice	Humoral immunity (immunosuppression/immunostimulation)	Repeated (5-day) dose administration
Plaque-forming cell (PFC) assay	Rodents	Humoral immunity (immunosuppression/immunostimulation)	Repeated (5-day) dose administration
Delayed-type hypersensitivity (DTH) model	Rodents	Cellular immunity (immunosuppression)	Repeated (7-day) dose administration
Contact hypersensitivity model	Mice	Cellular immunity (immunosuppression)	Repeated (7-day) dose administration
Phagocytosis, oxidative burst, chemotaxis (flow cytometry)	Rodents Dogs Monkeys Humans	Neutrophil functions	Single dose administration <i>In vitro</i>
Experimental model of respiratory allergy	Mice	Adverse consequences of immunostimulation	Repeated (7-day) dose administration
Direct histamine release	Dogs Monkeys Humans	Pseudo-allergic reactions	Single dose administration
Complement activation (C3a, C5a)	Rats Mini-pigs Dogs Humans	Pseudo-allergic reactions	Single dose administration <i>In vitro</i>
Basophil activation (flow cytometry)	Monkeys Humans	Pseudo-allergic reactions	Single dose administration <i>In vitro</i>

reduced increase in footpad or ear thickness, which supports the conclusion that these models can be suitable early predictors of the unexpected or unintended immunosuppressive potential of drug candidates (Descotes, Tedone, & Evreux, 1985; Smith & White, 2010).

Neutrophil functions primarily include chemotaxis and phagocytosis, the latter mainly consisting of bacterial ingestion and oxidative burst. Various techniques are available to measure neutrophil function endpoints either *in vitro* or *in vivo*. Beside many assays used for research purpose, classical techniques using Boyden's chamber or agarose for the evaluation of chemotaxis, bacterial ingestion counting and chemiluminescence for phagocytosis are either time consuming or biased by individual subjectivity, and/or require technical skill and/or expensive equipment. However, flow cytometry techniques can be used to measure chemotaxis and phagocytosis (Lehmann, Sornes, & Halstensen, 2000) and they are considered well suited to safety immunopharmacology studies (Horand, Cretinon, Condevaux, & Descotes, 2003). It is noteworthy that flow cytometry techniques are routinely used in clinical immunology laboratories to test human subjects, especially children suspected of inborn defects, for their capacity to ingest bacteria, or mount oxidative burst or adequate chemotactic response. Although the database with pharmaceuticals using these techniques is still limited, available results suggest they have sufficient predictability (Freebern, Bigwarfe, Price, & Haggerty, submitted for publication).

4.2. Immunostimulation

Only limited experience is available regarding the non-clinical evaluation of immunostimulatory effects. However, data obtained with several drugs, e.g. levamisole and cimetidine in rodents, and anti-CTLA-4 monoclonal antibodies in nonhuman primates, suggest that TDAR assays can also be used to predict the immunostimulatory potential of human pharmaceuticals (Descotes & Piccotti, 2012).

Nowadays, cytokine release is considered to be more reliably predicted by *in vitro* assays using human cells instead of *in vivo* in monkeys, which have been shown to be poorly relevant due to species-specific differences (Eastwood et al., 2010).

Recently, immunostimulatory nanoparticles were reported to enhance ovalbumin sensitization in a well-established experimental model of respiratory allergy in mice with increased respiratory response to ovalbumin (De Haar, Hassing, Bol, Bleumink, & Pieters, 2006). Such a model could be considered to assess the risk for more frequent hypersensitivity reactions toward unrelated allergens in patients treated with an immunostimulatory drug candidate.

4.3. Hypersensitivity

Immune-mediated hypersensitivity reactions are clearly outside the scope of safety immunopharmacology studies in contrast to pseudo-allergic reactions that are due to an inadvertent release of the same mediators via a pharmacological or toxicological mechanism.

Histamine release can be investigated by measuring histamine blood levels following a single dose injection of the test article in dogs, monkeys, and humans (Guedes, Papich, Rude, & Rider, 2007). Interestingly, the histamine-releasing potency of morphine and morphinic derivatives in animal models was found to be correlated with human findings.

Direct, non antigen-specific, activation of the complement cascade can be evidenced by the measurement of C3a levels either *in vitro* or *in vivo* in rats, mini-pigs, dogs or humans (Szebeni, 2005). Interestingly, correlations between measured C3a levels and cardiovascular changes can be studied *in vivo* (Szebeni et al., 2012). Using this model in pigs, it was possible to compare and predict the risk of pseudo-allergic reactions via direct (non-antigen specific) activation of the complement cascade in human patients treated with pegylated or liposomal formulations of the anticancer drug doxorubicin.

Finally, flow cytometry can be used to evidence basophil activation (Ebo et al., 2008) even though this technique has seemingly been very rarely used in animal models (Van Scott et al., 2008).

5. Role of pharmacology studies in the evaluation of the immunological safety of pharmaceuticals

Despite rare claims that any systemic evaluation of the immunotoxic, in particularly the immunosuppressive potential of drug candidates is not warranted (Snodin, 2004), there is a large body of evidence showing that data obtained in animal studies are fairly well correlated with clinical findings in humans. In fact, when unexpected adverse immune-mediated clinical findings are reported in humans, they are more likely due to inadequate or insufficient immunotoxicity assessment during preclinical studies as suggests the author's experience along more than thirty years of post-marketing drug surveillance and pre-clinical safety activities. To improve the current situation, pharmacology studies are therefore considered to be helpful in the immunological safety evaluation of pharmaceuticals at least for early screening and mechanistic studies.

5.1. Early screening

Standard toxicity studies are deemed to be insufficiently reliable predictors of the immunotoxicity potential of drug candidates due to the lack of systematic investigation of changes in immune function (Germolec et al., 2004). Indeed, no changes in the histology of lymphoid organs can nevertheless be associated with significantly decreased immune responses. Therefore, it can be assumed that an insufficient assessment of the potential for drug candidates to alter immune function may be one of the leading causes of the poor predictability of nonclinical studies.

Even though the ICH S8 guideline requires that the weight of evidence review should be conducted only prior to phase III clinical trials (ICH, 2005), a late discovery of the adverse immune potential of a drug candidate can prove to be problematic (e.g. go/no go decision, or reorientation of further drug development).

As already mentioned, the TDAR assay is considered the best tool for such an early screen as it can globally assess antigen presentation, helper T lymphocyte function and B lymphocyte-dependent antibody production. The PFC or anti-KLH IgM assay can be used for an early prediction of the immunosuppressive potential of drug candidates following short-term administrations (i.e. 4–5 days). Depending on results of the TDAR assay, the mechanism of action of the drug candidate, and the intended future therapeutic indication, other assays and models can be used in early safety immunopharmacology assessment. Indeed, DTH models focus on T cell-mediated immune responses, whereas neutrophil function assays can provide useful information on selective effects on phagocytosis, oxidative burst or chemotaxis depending on the selected assay. As already mentioned, there is a fairly good concordance of results obtained in these assays provided they are actually performed...

5.2. Mechanistic studies

Mechanistic studies are essential to identify causes for concern. The ICH S6R1 requires such mechanistic studies for biologics that are intended to, or inadvertently target the immune system (ICH, 2011). They can also be recommended for any human pharmaceutical known (early screening) or suspected to alter immune responses (mechanism of action, pharmacological properties...). Indeed, mechanistic studies are pivotal to select relevant second-line endpoints/assays case by case beside the first-line TDAR assay that should be included in additional immunotoxicity studies.

Last but not least, mechanistic studies are deemed to be critical to design a translational immune safety evaluation program that is

essential for the extrapolation from animals to humans, and further assessment in clinical trials (Descotes, submitted for publication).

6. Conclusion

This overview is not intended to provide a definitive picture of safety immunopharmacology studies, but instead to propose a starting point and draw perspectives for future development in this timely area of drug safety assessment. The models and assays briefly described above are well established. Nevertheless, they are very seldom included in the preclinical safety assessment of drug candidates although there is a fairly good concordance of results when compared to human findings, most often retrospectively. Safety immunopharmacology studies are believed to be primarily helpful to improve the immune safety assessment of either small-molecular-weight drug candidates with intended or unexpected immunomodulatory activities, or biologics whose immunogenicity precludes the conduct of standard repeated-dose toxicity studies of sufficient duration.

The conclusion was made recently using a large panel of monoclonal antibodies and fusion proteins that neither mice nor nonhuman primates had good predictive value for human adverse effects (Bugelski & Martin, 2011; Martin & Bugelski, 2011). This might be only partially true as no or very limited safety immunopharmacology results have been generated with these biologics. Therefore, this conclusion can be taken as additional evidence that safety immunopharmacology studies should indeed be included more systematically in the preclinical immune safety assessment of novel biologics. It is also obvious that much remains to be done to design and validate adequate assays and models to assess the immune safety of those biologics for which no relevant conventional animal species is available.

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