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Nonclinical evaluation of immunological safety in Göttingen Minipigs: The CONFIRM initiative

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ABSTRACT

There is a growing need to consider non-rodent species for the immunological safety evaluation of drug candidates. The EU Framework-6 RETHINK Project demonstrated that the Göttingen Minipig is a relevant animal model for regulatory toxicology studies. Extensive knowledge on the immune system of domestic pigs is available and fewer differences from humans have been identified as compared to other species, such as mice or non-human primates. Minipig data are too scarce to allow for claiming full immunological comparability with domestic pigs. Another gap limiting minipig use for immunological safety evaluation is the lack of a qualified and validated database. However, available data lend support to the use of minipigs. The need for a COllaborative Network For Immunological safety Research in Minipigs (the CONFIRM Initiative) was obvious. It is intended to trigger immunological safety research in Göttingen Minipigs, to assist and synergize fundamental, translational and regulatory investigative efforts relevant to the immunological safety evaluation of pharmaceuticals and biologics, and to spread current knowledge and new findings to the scientific and regulatory toxicology community.

1. Introduction

From the pioneering interlaboratory validation studies conducted by the US National Toxicology Program in B6C3F1 mice (Luster et al., 1992) and the International Collaborative Immunotoxicity Study Group in rats (ICICIS, 1998) until the most recent guidelines, rodents have usually been recommended as first-line species for the nonclinical immunological safety evaluation of human pharmaceuticals. However, the scene is rapidly changing. Implementation of the ICH S8 guideline (ICH, 2005), which states that *'all new human pharmaceuticals should be evaluated for the potential to produce immunotoxicity'* was a turning point and this statement is in accordance with recommendations of the ICH guideline S6R1 on the preclinical safety evaluation of biologics (ICH, 2011). Indeed, non-rodent toxicity studies are increasingly aimed at improving the translation of immune findings from animals to humans, and thus, the predictability of clinical safety. The dog is a potentially valuable species for immunotoxicity evaluation (Legrand et al., 2013), but it is as yet not fully validated. The use of non-human primates (NHP) dramatically increased over the last few decades with the development of novel biologics despite our still limited understanding on normal and pathological immune responses in NHP. It is also noteworthy that the political and socio-ethical support for using NHP is diminishing, particularly in Europe (SCHEER, 2017), a fact which is likely to limit the future use of NHP. Thus, initiatives to adequately characterize relevant non-rodent species for the immunological safety evaluation of pharmaceuticals and biologics are warranted.

Thanks to RETHINK, a research project funded by the European Commission FP6 Framework Programme, the minipig gained wider acceptance as a relevant animal species for toxicity studies of human pharmaceuticals (Bode et al., 2010) and regulatory acceptability was noted (van der Laan et al., 2010). In addition to their closeness to humans in terms of genetics, genomics and biochemistry, the rationale for

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the use of minipigs for toxicology research is additionally based on anatomic and physiological similarities of the cardiovascular system, skin, kidneys, digestive system or liver. All of these factors add to make the minipig a more appropriate non-rodent species than the dog or even the monkey (Ganderup et al., 2012; Heining and Ruysschaert, 2016), despite remaining concerns in the pharmaceutical industry regarding the characterization and evaluation of the minipig immune system (Colleton et al., 2016).

The CONFIRM Initiative was launched with the objective to demonstrate that the Göttingen Minipig can be a relevant animal species for immunological safety evaluation during the regulatory development of human pharmaceuticals and biologics by bridging gaps in our current knowledge of its normal immune system and immunopathological processes.

2. Immune system of domestic pigs and Göttingen Minipigs

The immune system of the pig is as well characterized as that of the dog or NHP, if even more. Extensive knowledge on the immune system of domestic pigs (swine) has been accumulated over the past 20 years (Haley, 2011; Rubic-Schneider et al., 2016; Saalmüller and Gerner, 2016; Dawson et al., 2017). However, only few data in minipigs are available, thus precluding any detailed immunological comparison with swine. To the best of our knowledge and based on available comparative immunological data, no major differences between swine and minipigs have been reported so far.

2.1. Immunogenetics

Efforts are being made to improve our understanding of the genetic and epigenetic background of pig immune responses (Schroyen and Tuggle, 2015). The genome sequence of the Göttingen Minipig is available, and similarities in gene expression profiles between Göttingen Minipigs and humans have been described (Heckel et al., 2015; Dawson et al., 2017). The antibody repertoire has being extensively described for the adult and fetal pig (Butler et al., 2004, 2005; 2011; Eguchi-Ogawa et al., 2010), and is not expected to differ substantially in the minipig (Heckel et al., 2015). Also, most porcine genes encoding FcγR molecules have been characterized (Qiao et al., 2006; Jie et al., 2009) although their genome annotation is still incomplete (Heckel et al., 2015).

In the last years, a series of genetically modified pigs and/or minipigs have been generated. These include the production of immunodeficient knock-out strains and strains designed to facilitate the transplantation of pig organs into humans. While most of these mutations have been generated in swine, the minipig genome has been shown to be amenable to genetic modification approaches (Jeong et al., 2013; Jakobsen et al., 2016; Shimatsu et al., 2016). These genetically modified systems and further transgenic tools are expected to facilitate immunological safety assessment in Göttingen Minipigs.

2.2. Structure of the lymphoid system

Globally, the lymphoid system structure in pigs is similar to that of other mammalian species including man and only few differences are described (Rothkötter, 2009; Haley, 2011, 2017; Dawson et al., 2017). The structure of the porcine thymus as well as the composition of T cell progenitors are very similar to that of other mammalian species. Thymic subpopulations are defined by the expression of typical CDs, such as CD1, CD3, CD4 or CD8. As in most other mammalian species, approximately 1% of pig thymocytes leave the thymus daily. Thymus increases in size after birth and involutes after 6 months of age. The porcine spleen is highly variable in size, and like in the dog, it appears to function more as a storage and clearance organ than a lymphoid organ. Pig lymph nodes have a unique 'inverted' structure, with reversed cortical and medullary areas and lymph flow, compared to other mammalian species. Migration of lymphocytes out of pig lymph nodes is preferentially via the high-endothelial venules (HEV) rather than the medulla. The functional consequences, if any, of these differences in lymph nodes are unknown. The porcine mucosa-associated lymphoid tissue (MALT) shows a high degree of similarity with other mammalian species including man. However, in pigs, Peyer's patches can be seen in the jejunum and there is one long Peyer's patch in the terminal ileum. Jejunal patches play a similar role compared to other mammals, but the role of ileal Peyer's patches is not elucidated (Butler and Sinkora, 2013).

2.3. Innate immune responses

As in other mammalian species, pattern recognition receptors (PRRs) are expressed on porcine innate immune cells (Mair et al., 2014). Detailed gene family analyses have revealed a large overall pig-human homology of PRRs, and human-like responses to IFN-γ and lipopolysaccharide (LPS) were found by transcriptome analyses using next generation sequencing (Dawson et al., 2017). In that context, it might be mentioned that LPS has been shown to have a surprisingly low potency in Cynomolgus Macaques (Picha et al., 2004). In pigs, including Göttingen Minipigs, major acute phase proteins include C-reactive protein (CRP), major acute phase protein (MAP) and haptoglobulin (Heegaard et al., 2011; Christoffersen et al., 2015). The porcine complement system bears many similarities with that of other mammalian species, and pig models have proven useful in studies of the role of the complement cascade in human diseases. One very illustrative example relevant to the area of immunological safety is the prediction of direct (i.e. not antigen-specific) activation of the complement cascade by pharmaceutical formulations containing Cremophor EL® or liposomes, which can result in severe, life-threatening, pseudo-allergic reactions. The Göttingen Minipig was recently shown to be an animal model very similar to the domestic pig, which is considered to be the gold standard to predict such a risk (Jackman et al., 2016).

Cells of the innate immune system include macrophages, dendritic cells, granulocytes and natural killer (NK) cells on which our current knowledge is rather limited as far as pigs are concerned. Pig-specific markers of NK cells have so far not been fully qualified. NK cells in pigs are typically identified through the rather tricky use of marker combinations, such as CD8α vs. CD16, CD8α vs. CD3, or $CD3^-CD4^-CD8\alpha^+$ (Shekhar and Yang, 2015). $CD3^+NKp46^+$ cells with NK cell characteristics were recently identified (Mair et al., 2016).

One difference of pigs compared to rats or humans is the existence of pulmonary intravascular macrophages (PIMs), the activation of which by microbial pathogens and inflammatory triggers of varied origin, such as endogenous or therapeutic macromolecules can result in acute lung injury (Schneberger et al., 2012).

2.4. Adaptive immune responses

As in the majority of mammals, five immunoglobulin isotypes (IgG, IgM, IgA, IgE and IgD) have been identified including six IgG subclasses, namely $IgG1_a$, $IgG1_b$, IgG2, IgG3, IgG4, $IgG5_a$, $IgG5_b$, $IgG6_a$, $IgG6_b$ (Butler et al., 2017). Although pigs have an IgD gene, the protein does not seem to be expressed. Limited information is so far available on porcine B lymphocytes due to the lack of adequate reagents. Today, the most commonly used surface markers are wCD1, wCD21, SWC7 and CD79. As in many species, two subsets of $CD3^+$ T lymphocytes, namely the $CD3^+$ α/β and γ/δ T cells have been identified in swine. There is a high percentage of double positive $CD4^+/CD8^+$ cells in the circulation, the role of which is not fully elucidated. Null (CD2) lymphocytes account for ≥30% of lymphocytes, the majority of which consists of γ/δ T lymphocytes (up to 15% vs. 3% in mice and 1–5% in humans) (Talker et al., 2013). Only rudimental knowledge is available on the functions of γ/δ T lymphocytes in pigs. Similarly, there is still limited information on the Th1 and Th2 paradigm in pigs. $CD4^+CD25^+FoxP3^+$ regulatory T cells (Treg) account for 1–3% of porcine T lymphocytes (Käser et al.,

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