Cell Therapy Report on Current Guidelines Regarding Advanced Cell Therapy Pre-clinical Safety, Efficacy and Potency Testing

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1. Introduction and Definitions

This report will overview the current guidelines and some of the current *in vitro* technologies used within academia and industry to assess the safety, efficacy and potency of advanced therapies.

There are a number of definitions and acronyms used thought out this report which are summarised and explained below;

ATMP - An advanced therapy medicinal product (ATMP) is a medicinal product and is either:
a gene therapy medicinal product (GTMP) a somatic cell therapy medicinal product (which includes substantial manipulation of cells or non- homologous use) or a tissue engineered product (TEP) (https://www.gov.uk/guidance/advanced-therapy-medicinal-products-regulation-and-licensing)
The definition of ATMPs is found in Directive 2001/83/EC as amended by the ATMP Regulation 1394/2007 and includes combination ATMPs with a medical device.

In the UK, the Medicines Health Regulatory Agency (MHRA) is the competent authority for clinical trial authorisation for all medicinal products, including ATMPs for UK manufacturers or importers of ATMPs.

ATIMPs are ATMPs as defined in Article 2(1) of Regulation 1394/2007 which are tested or used in a clinical trial (in accordance with Article 2(d) of Directive 2001/20/EC).

GTMPs - gene therapy medicinal products. GTMPs are defined in Directive 2001/83/EC, Annex I, Part IV and contain an active substance which consists of a recombinant nucleic acid used for therapy in order to regulate, repair, replace, add or delete a genetic sequence; The GTMP therapeutic, prophylactic or diagnostic effect is related to the recombinant nucleic acid sequence it contains, or the genetic expression (product) of this sequence.

Somatic cell therapy medicinal product is also defined in Directive 2001/83/EC, Annex I, Part IV. it is a biological medicinal product which contains or consists of cells or tissues that have been subject to substantial manipulation i.e. those not listed in Annex I to Regulation (EC) No 1394/2007, so that biological characteristics, physiological functions or structural properties needed for the clinical use have been altered, or of cells or tissues that are not intended to be used for the same essential function(s) in the recipient and the donor.

It is also defined as having properties for, or is used in or administered to human beings with a view to treating, preventing or diagnosing a disease through the pharmacological, immunological or metabolic action of its cells or tissues.

Tissue-engineered medicines contain cells or tissues that have been modified so they can be used to repair, regenerate or replace human tissue (https://www.ema.europa.eu/en/human-regulatory/overview/advanced-therapy-medicinal-products)

EMA - European Medicines Agency (EMA) has been ensuring efficacy and safety of human and veterinary medicines across Europe for over 35 years, promoting research and innovation in the development of medicines and cooperation within the European medicines regulatory network, consisting of the European Commission and the medicines regulatory authorities in the European Economic Area countries. (https://www.ema.europa.eu/)

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ICH - International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use. The purpose of the ICH is the promotion of public health through international harmonisation that contributes to the prevention of unnecessary duplication of clinical trials and post market clinical evaluations; the development and manufacturing of new medicines, the registration and supervision of new medicines, the reduction of unnecessary animal testing without compromising safety and effectiveness. This work of the ICH is accomplished by the development of Technical Guidelines which are implemented by the regulatory authorities. There are 19 members including regulatory bodies such as the European Commission in Europe, the Pharmaceutical and Medical Device Agency (PMDA), the Ministry of Health, Labour, and Welfare (MHLW) in Japan and the Food and Drug Administration (FDA) in the United States.

2. Brief History of ATMPs

Cellular therapies were started in the 1950's with the pioneering work of Thomas and others (1) in the successful initiation of haematopoietic stem cell transplantation for leukaemia (1,2). Initial studies involved cells from the bone marrow of a patient's (autologous transplants) "rescuing" the patient after chemotherapy or radiotherapy with the aim of obliterating residual leukaemia and allowing cancer free cells of the bone marrow to re-populate the haematopoietic system. Allogenic transplants, however, were based on the ability of human leukocyte antigen (HLA) matched bone marrow, peripheral blood or cord blood to eradicate residual disease due to the incoming immune cells (T and natural killer (NK cells)) causing a Graft versus Leukaemia (GvL) or Graft versus Tumour (GvT) effect. Apart from the potential problems of engraftment, graft versus host disease (GvHD), where the incoming T cells attack the skin, gut and liver of the patient was the major cause for concern. To this end, especially for peripheral blood stem cell transplants, a dose escalation protocol was required for the procedure.
These pioneering studies (for recent reviews see 2 and 3) have led the way for chimeric antigen receptor (CAR-T) cell studies where the major concern is still specificity of the response, bystander effects and for up and coming allogenic CAR-T, GvHD. Scientific progress in cellular and molecular biotechnology led to the development of ATMPs.

Due to the novelty, complexity and technical specificity of ATMPs, they are regulated in Europe under an overall framework Regulation (EC) No 1394/2007 (4). The Regulation established the Committee for Advanced Therapies (CAT), a multidisciplinary committee, responsible for assessing the quality, safety and efficacy of ATMPs and to follow scientific developments in the field. From June 2009, the CAT has made scientific recommendations on ATMPs classification (https://www.ema.europa.eu/).

3. The Regulations, the main principles

ATMPs are subject to the same regulatory principles as other types of biotechnology medicinal products although the quality, pre-clinical and clinical data in order to demonstrate the quality, safety and efficacy of the ATMP will be specific to each particular product. Those requirements are described in the Annex I to Directive 2001/83/EC (4) for gene therapy medicinal products and somatic cell therapy medicinal products but need to be established for tissue engineered products.

The regulatory principles also need to be flexible to allow for changes in technology and scientific procedures (4).

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The regulatory framework for ATMPs in Europe and the United States and their similarities and difference has recently been reviewed (5).

In both the EU and the US ATMPs are under the regulatory framework of biological products. In the EU there are four groups as explained above, which include gene therapy, somatic cell therapy, tissue-engineered therapies, and combined advanced therapies. In the US, the classification consists of gene therapy and cellular therapy. The development of advanced therapies within the EU, involves the submission of a clinical trial application to the national competent authorities and for marketing authorization (only by small medium enterprises (SME)) (6) and all ATMPs are evaluated via a centralized validation and scientific evaluation procedure for product approval, carried out by the CAT and the Committee for Medicinal Products for Human Use (CHMP) A recent review describes the process and the history of obtaining manufacturing authorisation within the EU for the current ATIMPs which are commercially available (6). The Guideline on strategies to identify and mitigate risks for first in human and early clinical trials with ATIMPs (7) is designed to aid stakeholders to develop clinical trials from bench to bed-side and identify risks including those associated with tolerability, safety, pharmacokinetics, and pharmacodynamics. This risk-based approach is applied and well documented in order to determine the extent of quality, non-clinical and clinical data to be included in a clinical trial Marketing Authorisation Application (MAA) dossier required for the approval of AITMP. The guideline (7) is applied to all new chemical and biological investigational medicinal products (IMPs) ATMPs, tested or used in accordance with Article 2(d) of Directive 2001/20/EC) are not within the scope of the guidance but some of the principles are relevant depending on the ATMP and on an individual case-by-case basis and especially with regard to risk and patient safety.

It is also recommended under the guidance that the 3Rs principles on animal use (Directive 2010/63/EU), that a scientifically satisfactory test not involving the use of animals can be used wherever possible. The use of *in vitro* studies, especially using human tissues or cells and where the tests or assays have been scientifically validated are encouraged but need to be fully discussed in any supporting clinical trial documentation.

The regulation of cellular therapy products such as cellular immunotherapies, cancer vaccines, autologous and allogenic cells; human-based gene therapy products and devices related to cell and gene therapy in the UK, EU and the United States (US) is the responsibility of the MHRA, EMA and Centre for Biologics Evaluation and Research (CBER) respectively.

In addition to the regulations and guidelines described above, several initiatives have been undertaken at the European level, supported by the European Bone Marrow Transplantation Group (EBMT) and The Joint Accreditation Committee ISCT-Europe & EBMT (JACIE) to assess the safety of novel products and a risk based approach has been developed. This was achieved with European Commission funding and the development of standardized methodologies (EuroGTP II Guide) and an interactive assessment tool (IAT) (http://www.goodtissuepractices.site) as well an online EuroGTP II e-learning course on good practices as applied to tissues and cells (T&C) preparation processes and patient follow-up procedures.

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4. Non-clinical safety assessments

Due to the specific individual characteristics of ATMPs the non-clinical safety data needed before first in man studies, will be by definition different for different ATMPs and will be considered by the regulators on a case-by-case basis taking into account risks and validation data provided.

As a minimum, the following information is required before an ATIMP can be administered into man:

- Proof of concept demonstrated in relevant animal or validated and appropriate *in vitro* models or assays
- Support for the administration route (procedures/devices)
- Support for the selection of safe and biologically effective starting dose with adequate margins for safety and clinical use.
- Appropriate safety data.

ATIMPs can result in activation of both innate and adaptive immune responses and these aspects should be considered during the non-clinical development of the ATMP. They should be part of the overall toxicology assessment and can include, histological analysis of immune activation at administration and can involve the use of appropriate *in vitro* assays. An unwanted immune response due to an administered ATIMP must be addressed before human exposure to prevent adverse cytokine responses, IgE production and potential anaphylaxis responses. Some of these adverse immune responses could also be attributed to GvHD in an allogeneic setting.

Guidelines from the FDA and the EMA were searched for information pertaining to the in-vitro safety assessments of ATMPs and how they correlate with in-vivo patient safety and impact. Overall, no clear guidelines on *in vitro* safety testing for these therapies is currently available, but guidelines on how the experimental design and toxicity assessments should be carried out is illustrated.
The safety guidelines are concerned primarily with the safety of the patient, which also involves assessing the whole quality control process of the ATMP, including that of the materials used in production, process control and a release test of the finished product. The processes which need to be followed relating to the manufacture of ATMPs is not considered here but has recently been reviewed in https://www.theattcnetwork.co.uk/manufacturing-and-preparation-toolkit.

There are several major safety risks associated with autologous ATMPs, such as CAR-T; which include cytokine release syndrome (CRS), neurotoxicity and B-cell depletion. The potential risk of tumorigenesis/tumorigenicity of transgenic cells is also important when considering gene therapy products. Other ATMPs such as antiviral T cell therapies for cytomegalovirus (CMV) and or Epstein barr virus (EBV) could also cause adverse immune responses including bystander tissue cytotoxic effects and cytokine release especially if the specificity, potency and efficacy of the product is not of the highest standard. To date very low levels of adverse immune reactions have been described with anti EBV or anti CMV T cell products with minimal GvHD (8,9).

In addition, dendritic cell vaccines targeting various cancers or autoimmune disease have also shown low levels of toxicity (10,11).

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5. Safety concerns and the challenges of assessing risk

Safety concerns for ATMPs (12,13) include a discussion of the following risks. Those aspects where *in vitro* human based assays are available or would be of value in assessing adverse immune reactions to the ATMP are shown in bold below. The importance of these assays lies in the fact that they can provide screening tools prior to additional animal models if required, or if animal models are not available.

- Risks to patients related to quality characteristics of the product, in particular:
  - Species of origin and characteristics of cells (and related body fluids, biomaterials, biomolecules) that are used during manufacturing, and the safety testing performed; especially if any animal products are used in the manufacturing although this should be avoided due to the potential to illicit allergic responses
  - Characteristics of vectors for gene therapy medicinal products
  - **Biologically active substances used in manufacturing (e.g. enzymes, antibodies, cytokines, sera, growth factors, antibiotics) – levels can be measured by enzyme linked adsorbant assays (ELISA) , cytokine multiplex assays including flow cytometry or cytokine arrays.**
  - Quality assurance and characteristics of the finished product in terms of defined composition, stability, **biological activity eg measured by T cell activation, cytokine release, IgE production** and purity with reference to non-physiologic proteins and fragments
  - Risk related to transmissible diseases (viral, bacterial, parasitical infections and infestations, but also malignant disease and others)
- Risks to patients related to the storage and distribution of the product, for instance:
  - Risks related to preservation, freezing and thawing
  - Risks of breaking the cold chain or other type of controlled temperature conditions
  - Risks related to stability of the product
- Risks to patients related to administration procedures, for instance:
  - **Biologically active substances used in preparation of the product prior to administration (e.g. enzymes, antibodies, cytokines, sera, growth factors, antibiotics) measured as described above**
  - Risks related to conditioning of the patient
  - Risks of related medical or surgical procedures (such as anaesthesia, infusion, transfusion, implantation, transplantation or other application method.)
  - Risks related to clinical follow-up (immunosuppression as co-medication or as necessary for treatment of complications, diagnostic procedures, hospitalisation)
  - Risks related to mistakes or violations of the standard procedures for administration of the product (e.g. different administration procedures used by different healthcare establishments/healthcare professionals resulting in differing results).
- Risks related to interaction of the product and the patient, for instance:
  - **Unwanted immunogenicity and its consequences (including anaphylaxis, GvHD, graft rejection, hypersensitivity reactions, immune deficiencies, etc) measured with regard to T cell DC activation , IgE production, cytokine assays , histopathological analysis**
  - Risks related to both intended and unintended genetic modification of the patient's cells (apoptosis, change of function, alteration of growth and/or differentiation, malignancy)
  - Early and late consequences of homing, grafting, differentiation, migration and proliferation. Risks related to infection with vectors used in gene therapy medicinal

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products (type of vector, target cells, persistence, potential for latency and reactivation, potential for integration of genetic material into the host genome, prolonged expression of the transgene, altered expression of the host's genes).

- **Risks related to scaffolds, matrices and biomaterials (biodegradation, mechanical factors, etc) methods to assess medical devices potential activation of blood or product components**
- Risks related to persistence of the product in the patient, for instance:
  - Availability of rescue procedures or antidotes and their risks
  - Late complications, particularly malignancies and autoimmunity
  - Considerations on the potential impact of previous, concomitant, or future therapies typical for the diagnosis or treatment of the respective disease on the product, or vice versa impact of the product on those other therapies (e.g., an immunoglobulin treatment later in life could impact on expression of the introduced gene by antibody interaction).
- Risks related to re-administration, for instance:
  - **Immune reactions - anaphylaxis, neutralising antibodies. Measured as described above**
  - Risks related to repeated surgical or administration procedures.
- Risks to close contacts, for instance:
  - Based on the environmental risk assessment, virus shedding and its consequences
- Specific parent-child risks, for instance:
  - Risk of germ line integration of transgene, or other genetic transformation of the germ line
  - Foetal transmission (of vectors, biologically active substances, cells, infectious agents)
  - Transmammary exposure of children in lactating women (to vectors, biologically active substances, cells, infectious agents).

6. Safety testing for cell and gene therapy (CGT) products - An overview.

Genotoxicity, tumorgenicity, reproductive and developmental toxicity and immunotoxicity studies are determined on a case by case basis considering the risks associated with the nature and characteristics of the ATMP and its intended clinical trial use as an ATIMP. In this regard early studies going through the MAA process found several major objections, issues, or concerns with gene therapy products which led to unsuccessful initial applications. These were mainly concerned with clinical efficacy and safety. In particular, for non-clinical assessments these included pharmacodynamics, pharmacokinetics, and toxicology (14).
Repeat-dose toxicity was one reason why some gene therapy products e.g. Advexin for Fraumeni cancer and products using integrating vectors, such as Glybera (AAV vector) for lipoprotein lipase deficiency and Strimvelis (retroviral vector) for Severe combined immunodeficiency due to adenosine deaminase deficiency, were also at the highest risk of tumorigenesis The main reason for failure was the inability to achieve long-term engraftment of transduced cells in mice. Non-clinical development of GTMPs can be supported however by a risk-based approach (RBA) and a strategy to determine the most appropriate date to be included in the Manufacturing Authorisation Application (MAA.).This strategy has been applied since these early studies and together with further addressing toxicology and immunogenicity concerns as well as expertise gained by both the regulators and producers of gene therapies, more successful MAA have been achieved e.g. Glybera, Imlygic for unresectable melanoma and Strimvelis (14).

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7. An overview of the FDA Guidelines

7.1 The FDA Guidelines

The US guidance for industry pertaining to the preclinical assessment of investigational cellular and gene therapy products is provided by the US Department of Health and Human Services, the CBER and the FDA. CBER (15) is the Center within FDA that regulates biological products for human use under applicable federal laws, including the Public Health Service Act and the Federal Food, Drug and Cosmetic Act. CBER protects and advances the public health by ensuring that biological products are safe and effective and available to those who need them. CBER also provides the public with information to promote the safe and appropriate use of biological products.

These US guidelines suggest that before an investigational pharmaceutical agent is administered into a clinical trial, the sponsor organisation must provide adequate information regarding the pharmacological and toxicological studies. The sponsor has the responsibility to determine that the product is safe for the proposed clinical investigations.

Safety and efficacy risk management of ATMPs is covered in the US guidelines (13,15) and includes the conduct of toxicology studies.

The US guidelines suggest that the preclinical program for a cell or gene therapy (CGT) product should consider and include the following considerations or objectives:

- Establish biological plausibility
- Identify the biologically active dose levels
- Select the starting dose, dose-escalation schedule and dosing regimen for clinical trials
- Establish safety of the investigation's proposed clinical route of administration.
- Patient eligibility criteria
- Identification of physiologic parameters that can guide clinical monitoring
- Identification of potential public health risks.

An acceptable risk-benefit ratio needs to be considered before conducting a proposed clinical trial. Preclinical assessment of the safety of an investigational CGT product contributes to this. Toxicology study design should consider the following:

- The proposed clinical indication.
- The amount if there is quality published preclinical or clinical safety information for the specific CGT (i.e. current known toxicities or adverse effects).
- The amount and quality of existing pharmacology (*in vitro/ in vivo*) or point of care (POC) data for specific CGT product.
- Previous clinical/preclinical experience with the proposed clinical delivery device/delivery procedure with any related device/procedure.
- The biological responsiveness of the animal species to the CGT.
- The putative mode of action (MOA) of the CGT.
- The intrinsic properties of the CGT.
- The pathophysiology of the animal disease/injury model, if one is used.

The guidelines for industry (13) suggest the consideration of additional toxicology parameters with respect to the investigational CGT product's effect on the intended patient population. Considerations might include product-specific parameters such as humoral or cellular immune responses, vector biodistribution, CT product fate, behavioural testing, neurological exams,

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ophthalmic examinations, cardiac assessments, imaging (such as MRI, ultrasound or radiography), presence of abnormal/ectopic growths (such as hyperplasia, tumours), putative biomarkers and specialised histopathology (such as immunohistochemistry). Data collected should include morphological and functional assessment to determine whether an association between non-terminal and terminal findings exist. If there is reversibility in these data, that should also be reported.

7.2 Recommendations for investigational cell therapy (CT) products

The guidelines (13) cover recommendations for studies involving CT products, such as study design, animal models to use, CT product fate post administration, considerations relating to CT products with implantable scaffolds, (regulated as Tissue Engineered Products or Combined Products in the EU) , as well as safety concerns.

Safety concerns for CT products include a discussion of the acute and long-term *in vivo* safety of the product, requiring mandatory clinical follow up.

- Local toxicities may be a result of the product components with the tissue or related to the degradation of the product components at the site of administration (such as tumorigenicity, altered tissue function at injection site, abnormal cellular differentiation or inflammatory substrates).
- Cell migration outside the site of administration might lead to systemic toxicities such as ectopic tissue formation and tumorigenicity.
- The immunogenic potential of the construct (the scaffold or the cells themselves) could also cause toxicity.

7.3 Recommendations for investigational gene therapy GT products.

The guidelines (13) cover recommendations for investigational GT products pertaining to animal studies and study design. The overall considerations for GT products include the following:

- Toxicities due to the components of the final formulation (e.g., liposomes and various excipients/contaminants).
- Toxicities due to the route of administration (ROA) used.
- Aberrant localization to non-target cells/tissues.
- Level and persistence of vector and expressed transgene.
- Level of viral replication in non-target cells/tissues.
- Immune activation or suppression.
- Immune response directed against the vector.
- Phenotype/activation state of target cell(s).
- Potential for insertional mutagenesis or oncogenicity.
- Potential for germline transmission.
- Potential horizontal transmission of replication competent vectors from the patient to family members and health care providers (i.e., shedding).

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8. An overview of the European guidelines

Safety concerns for ATMPs in Europe are covered by the European Medicines Agency (EMA) (16). According to the EMA, ATMPs are developed to provide new avenues for the treatment of patients by restoring, correcting or modifying physiological functions. The EMA recognises that the novelty of these new therapeutics may bring challenges in the form of new, unexplored risks to the patient or public health at large.

The EMA state that the rules outlined in the guidelines should facilitate the early detection of risks and provide an effective means for mitigation of their consequences to the patient or public at large. The risk management plan for a particular ATMP should provide comprehensive scientific consideration to the important identified or potential risks and to any potential missing information. The need for flexibility and creativity in the development of any plan is also acknowledged owing to the likely differences between different ATMP therapies developed with differing underlying biological actions and mode of action (MOA).

8.1 Efficacy concerns

Due to the complex nature of ATMPs and the characteristics of the diseases they target, limited efficacy data may be available at the end of pre-authorisation clinical trials. It is therefore now mandatory that full efficacy assessment is carried out for several years of clinical follow-up. This longitudinal follow-up can be illustrated by the decade of information available for CAR-T cell therapy for example, with persistent functional CAR-T cells 10 years after administration (17).

Efficacy may be related to the fact that many ATMPs have genetic modifications. The ATMP may therefore be subject to changing characteristics after their administration to the patient over long periods of time (7). These changes may have biological consequences for the patient with respect to increased efficacy (e.g. overexpression of a gene of interest) or decrease of efficacy of the ATMP. This could be related to the time needed for the target tissue to be altered and fully functional as a result of the ATMP. In the case of an ATMP expected to be a once in a life-time treatment, the sustainability of efficacy over time can only be assessed by long-term (longitudinal) follow up studies. Taking these considerations into account, the efficacy of many ATMPs is highly dependent on the quality of the administration procedure adopted including the conditioning of the patient, surgery and clinical follow-up. Some cell therapy products may have a limited life-span and follow up will be needed to assess efficacy. Findings from such studies will aid in determining the potential for re-application of the product in clinical practice.

8.2 Safety specifications - additional EU requirements

According to the “Guideline on safety and efficacy follow up – Risk management of ATMPs” (12), additional safety considerations and documentation/report requirements include:- a Flow-Chart of the logistics of the therapy (for instance, harvesting, transport, controls, manipulation, conditioning, administration, clinical follow-up) and risks to healthcare professionals, care givers, offspring and other close contacts with the product or its components, or with patients together with an environmental risk assessment.

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9. A Summary of safety specifications

For many ATMPs, the following examples are likely to represent the most important safety concerns:

- The legal requirement to assess transmission of infectious agents to the patient and to close contacts
- Graft dysfunction and/or rejection
- Induction of autoimmunity or immunogenic reactions
- Induction of malignancies
- Impossibility of discontinuing or removal of the product
- Potential of the vector for latency and reactivation, integration of genetic material into host genome, prolonged expression of the transgene, altered expression of the host's genes, potential for germline integration.

10. The ICH Guidelines– human pharmaceuticals

Currently there are no finalised ICH guidelines for the *in vitro* testing of cellular therapies although a set of draft guidelines are being prepared. The current guidelines available for the general assessment of immunotoxicity studies for human pharmaceuticals and biotechnology-derived pharmaceuticals is summarised in the following section. In addition, the draft ICH guidelines on the non-clinical biodistribution considerations for gene therapy products (S12) was endorsed in June 2021 and is currently under public consultation. This guideline (18) covers the use of animal models immunogenicity, *ex vivo* assays and tests to monitor biodistribution of a vector and/or the expression products. These can include enzyme-linked immunosorbent assays (ELISA) immunohistochemistry (IHC), western blot, *in situ* hybridisation (ISH), digital PCR, flow cytometry and *in vivo* and *ex vivo* imaging techniques. A comprehensive description of the methodology and justification for the use of the technique as well as performance parameters of the method are required.

Based on the 2005 ICH guidelines for immunotoxicity studies for use in testing human pharmaceuticals and also the later S8 guidelines (16) *in vivo* methods such as a T cell dependent antibody response (TDAR) assays in mice are recommended. There are no *in vitro* methods for cell mediated immunity which were suggested in the 2005 ICH guidelines. Moving forward *in vitro* assays for measuring cell mediated immune responses are available such as activation of dendritic cells and subsequent T cell proliferation and cytokine release and all can be measured using human peripheral blood lymphocytes.

For investigating the specificity of a CAR-T cell for example, specialist T cell specificity assays are needed to demonstrate that the majority of the T cells were reacting only to the chimeric antigen of choice. This can be accomplished by the use of T cell specificity assays and assessing both the phenotype of the reacting cells as well as cytokine release and in some cases *in vitro* tissue damage from the bystander effects of the cytokines. The ICH S8 guidelines (16) contain recommendations on the non-clinical testing of compounds that might be immunotoxic. It also considers whether additional studies may be needed if the assays fail the risk management plan.

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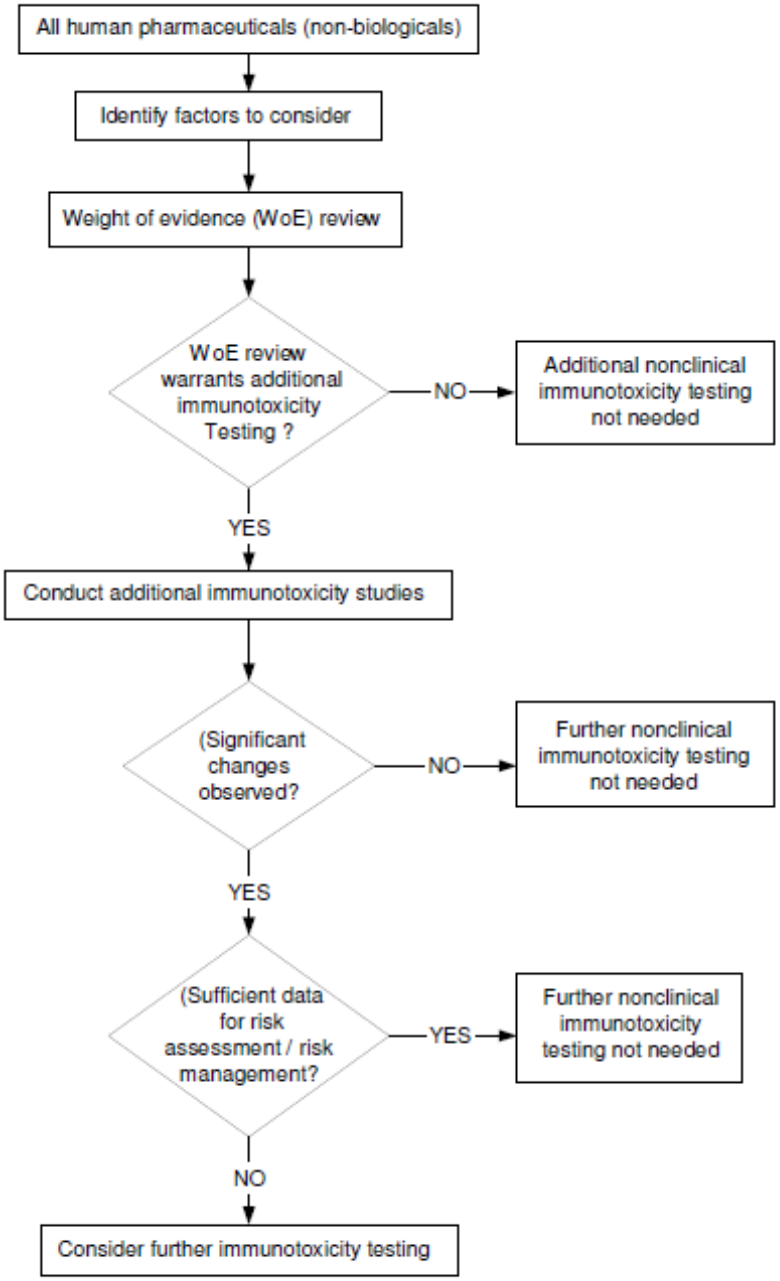

Figure 1: Flow diagram for recommended immunotoxicity evaluation taken from 16

The ICH S8 document also outlines a structured approach including firstly standard toxicity studies (with associated haematology, immune system organ weights, and histopathology data). Secondly functional assays, such as cytotoxic T lymphocyte (CTL) assays, natural killer (NK) cell assays, respiratory burst, phagocytosis, and T-cell-dependent antibody response (TDAR) assays. Finally, host resistance assays. Host resistance assays are considered the optimal test in immunotoxicity testing as they provide a full overview of the extent to which innate, adaptive, and homeostatic regulatory

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immune functions operate together to protect the host. This type of approach/methodology as shown in Figure 1 for evaluating pharmaceutical immunogenicity, can be adapted for ATMP assessment of immunogenicity (16,19).

These guidelines suggest additional immunotoxicity assays should be conducted where required and the tests that can be used need not have been fully validated but a scientific or mechanistic basis for the use of the assay should be demonstrated. Relevant positive controls will be needed to inform the data analysis. Examples of assays which can be used in this context include:

- T-cell dependant antibody response (TDAR)
- Immunophenotyping
- Natural killer cell activity assays
- Host resistance assays
- Macrophage/neutrophil function
- Assays to measure cell-mediated immunity

Similar recommendations are made in the ICH guidelines S6 (R1) (19) which cover biotechnology-derived pharmaceuticals. Although not specifically related to ATMPs these guidelines cover general preclinical safety evaluation guides covering immunogenicity and immunotoxicity.

10.1 The ICH Guidelines– ATMPs

The European guidelines have been updated from the EMA 2008 guidelines discussed previously to cover those recommendations as part of the EMA Science Medicines Health Committee for Medicinal Products for Human Use (CHMP). The current draft document was published in February 2018 (20). This document provides guidelines for the safety and efficacy follow-up and risk management for ATMPs and is the first revision of the original guidelines (19).

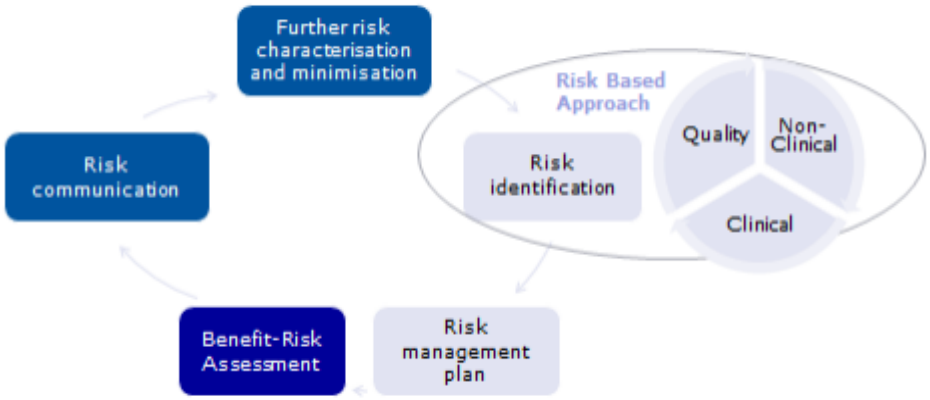

Figure 2: Risk assessment approach: safety and efficacy assessment that should be considered through a risk management plan to be agreed as part of marketing authorisation of a product. Taken from (20).

Risks to safety and efficacy of an ATMP, covered in the ICH guidelines are similar to those described in section 5 and follow the risk assessment approach as described in (12) and Figure 2

An analysis of these risks leads to safety specifications based which could include the following n

- Transmission of infectious agents to the patient and to close contacts.
- Treatment failure (e.g. graft dysfunction and/or rejection), impossibility of re-treatment.
- Harm due to medication errors/maladministration.

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- Induction of autoimmunity or immunogenic reactions.
- Induction of malignancies/tumour formation.
- Impossibility of discontinuing or removal of the product in case of emerging risks.
- Potential of the vector for latency and reactivation, integration of genetic material into host genome, prolonged expression of the transgene, altered expression of the host's genes, activation of oncogenes, potential for germline integration.
- Unwanted tissue formation including abnormal cell proliferation.

The 2018 ICH guidelines have been under review and not fully accepted by the CAT and CHMP. A new document (21) has been published where public consultation ended on 1st August 2019. A date for new recommendations coming into effect is yet to be decided. This document also covers clinical trials and follow up studies and a discussion of this follows.

The new document provides guidance on structure and data collection requirements for a clinical trial application to assess exploratory and confirmatory trials with advanced therapy investigational medicinal products (ATIMPs). Guidelines cover development, manufacturing and quality control as well as non-clinical and clinical development of ATIMPs. The report states that the development of an ATMP should cover the general principles followed for other medicinal products, however, it notes that the distinctive features and characteristics of ATMPs could lead to changes in product development programs.

11. Adventitious agents safety evaluation

Consideration of 'adventitious agents safety evaluation'

- All materials of human or animal origin used in the manufacturing process of either the active substance or the medicinal product needs to be identified and risk assessed with respect to potential contamination with adventitious agents.
- Contamination could originate from the starting raw materials or adventitiously introduced during the manufacturing process.
- Testing for bacteria, fungi and mycoplasma should be competed for the finished product. A viral safety risk assessment should also be completed.

Non-clinical data supporting the safe human use of an ATMP needs to provide an estimation of the safe and biologically effective dose(s) to be used in clinical trials, support the feasibility of administration route and the application procedure. Safety concerns, target organ potential toxicity and identification of safe parameters in clinical trials also need consideration. The guidelines provide a high level frame work designed to endure as new advances in the field occur and further clinical development is dependent on the perceived risks related to the product itself and associated factors.

If the product is expected to be in the body short term, then the risk-based approach can be adapted to identify necessary non-clinical data on a case-by-case basis.

Use of animal models should be considered carefully, and relevant models selected justified and the same is applied for pharmacology studies.

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12. Long term efficacy and safety follow up

These guidelines also refer to long term follow up which is needed to monitor those patients that are treated with an ATIMP which may have a longer mode of action. This information will inform on the activity and efficacy of the product and provide important data for market authorisation application. The appropriate study design should be chosen to maximise information output in order to fully understand the efficacy and mode of action of the AITMP in the longer term..

13. Potency Testing

Potency testing is covered in the EMA guidelines (22) and currently convers cell-based immunotherapy products. Potency is defined as the 'specific ability or capacity of a product to achieve a defined biological effect. Potency is the quantitative measure of biological activity based on the attribute of the product, which is linked to the relevant biological properties.'

Accurately designed potency tests can provide an accurate, reliable and consistent demonstration of the biological activity of the active ingredient at the level of drug substance or drug product. The results of a potency assay should provide assurances that the amount of active ingredient is sufficient to induce a meaningful response and that this amount is consistent between different batches.

Potency of cell-based immunotherapy products can be measured in a number of ways:

- In vivo (animal) potency testing.
- In vitro potency testing (e.g. in vitro lysis of target cells by tumour specific CD8 T cells, in vitro cytokine production by specific cells).
- Viable cell count.
- Autologous cell-based products.
- Reference preparation.
- Adjuvant containing immunotherapy products.

An example of potency testing is assessment of the product pre and post freeze/ thaw where proportions of the cells may be altered due to the thawing process or lack viability. This needs to be caried out over time in order to ensure the activity of the product does not deteriorate as a frozen product over a clinical trial time span.

14. Biologically relevant assays

Biologically relevant assays for predicting adverse immune reactions typically include T cell proliferation assays, cytokine release assays and *in vitro* human tissue-based models for assessment of immunotoxicity.

Examples of assays for the specificity safety and efficacy of CAR T cell-mediated cytotoxicity and other cellular therapies are further described below.

The use of CAR-T cell therapy has been offset by safety considerations such as cytokine release syndrome, neurotoxicity, and other adverse events. Various companies have developed assays to

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assess adverse events to cellular therapies. Cytotoxic tests include the direct assessment of CAR T cell-mediated cytotoxicity to target cells in a 96-well plate format, such as that provided by Nexcelom Biosciences which uses a fluorescent dye-based assay to image cells looking at effector:target ratios (https://www.nexcelom.com/applications/celigo/virology/car-t-cell-mediated-cytotoxicity/). Creative Biolabs uses normal cells in their assay which can be harmed by the potentially toxic CAR T. The normal cells express tumour associated antigens on the cell surface and the release of cellular toxic products (or cytokines) as a result of tumour cell lysis can be assessed in a multiplex cytokine release assays aiding assessment of on-target or off-target toxicological analysis (https://www.creative-biolabs.com/car-t/cytotoxicity-test.htm). A similar cytokine release-based method was developed by BioAgilytix using three standardised multiplex plates. One consisting of a pro-inflammatory panel, a standard cytokine panel and a chemokine panel (https://www.bioagilytix.com/from-the-stage/toxicities-of-car-t-cell-therapy-and-measurement-of-cytokines-during-therapy/). Contract research organisations, such as Charles River have also developed a suite of assays for the assessment of safety to CAR T including their T cell assays for immunotherapies (https://www.criver.com/products-services/discovery-services/in-vitro-assays/immuno-oncology/t-cell-assays?region=3696).

Alcyomics (www.alcyomics.com) has used their Skimune® technology (23,24) for the assessment of immunogenicity and specificity of cellular products aimed for use in combating various diseases, including haematological disorders, viral infections and auto immune disease. The Skimune® platform has been shown to demonstrate the specificity of T cell clones developed for use in the treatment of leukaemia (23). More recently the same Skimune® platform was used to show the safety and efficacy of anti-cytomegalovirus (CMV) T cells used in the treatment of CMV post haematopoietic stem cell transplantation (24).
In these studies, a skin explant assay is used to test the safety and specificity of the cellular therapy using skin from a third-party donor. The read out of the assay includes cytokine analysis as well as T cell proliferation and histopathological grading of skin tissue.
In addition Alcyomics has recently launched an innervated skin model for the testing of neurotoxicity which can also be adapted for assessment of neurotoxic effects due to cellular therapies eg CAR-T . Alcyomics is currently using this assay to test 2 exemplars, anti SARS COv2 T cells for combatting persistent COVID 19 infections and a tolerogenic dendritic cell product for treating rheumatoid arthritis, within the NAATTC partnership prior to clinical trial. (https://attc-143fd.kxcdn.com/wp-content/uploads/2022/03/DL97_Assessing-the-safety-and-Graft-versus-Host-Disease-GvHD-reactivity-of-ATMPs-v0.3.pdf( )

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15. A comparison of FDA and European guidelines

Although there are some differences between the FDA and EMA guidelines they are both overlapping and do not give any direction for manufacturers regarding the type of assays which they should conduct as well as their number, specificity or validation. Although this may be advantageous as being too explicit may inhibit innovation some indication would support manufacturers in their testing strategies. Alcyomics recently conducted a survey of manufacturers with regard to this issue and the overwhelming conclusion was that further clarification and direction from the regulatory bodies as well as good and poor examples of compliance with the guidelines would be extremely useful especially to those manufacturers new to the field or within academia.

	US Guidelines	**European Guidelines**
Type of advanced therapy covered	US Guidelines consider cellular therapy products as cellular immunotherapies, cancer vaccines, autologous and allogenic cells; human-based gene therapy products and devices related to cell and gene therapy.	European Guidelines cover ATMPs specifically. ICH guidelines also cover ATMPs. Overall, more information than FDA guidelines to aid developers.
Specific risks/approach to evaluate risks pre-clinically that are considered by the guidelines	The US guidance covers preclinical assessment of investigational cellular and gene therapy products. Safety and efficacy require an effective risk-benefit ratio before conducting clinical trials.	The European guidance refers to a risk identification approach with reference to a 'risk management plan' to aid 'early detection of risks'. The assessment of risks is wide-ranging, including environmental.
Specific considerations relating to the advanced therapy or factors that specifically affect the patient	Pre-clinical study should consider the plausibility of the biological activity of the therapy, the dose levels, safety levels (including risks to public health) and parameters that can be used for clinical monitoring well as patient criteria.	Safety concerns cover risks to the patient as a result of the therapy (e.g. biological activity, quality and storage of therapy, unwanted effects when administered).
Specific mention to toxicology	Considerations for a toxicology study output are given. Pre-clinical data from which can used to aid clinical trial design and to establish no-observed-adverse-effect level (NOAEL).	Toxicology study design considerations are discussed.
Specific mention to efficacy studies	Efficacy is mentioned, but not elaborated upon, with the user needed to devise their own strategy with limited suggestions in the guidelines.	Efficacy concerns are directly referred to in the guidelines. There is a comprehensive discussion including consideration of efficacy and safety follow up studies (longitudinal studies).

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Specific mention to potency studies	No consideration is given to potency testing specifically.	Potency testing for cell-based immunotherapies is covered in the ICH and EMA guidelines.

16. Summary

A review of the current guidelines (EU, FDA and ICH) detail safety requirements for clinical trials based on that required for ensuring patent safety and clear risk assessment strategies are well documented. Although immunogenicity risks are clearly defined for pharmaceuticals the types of in vitro testing requirements are less clear and not specified especially with regard to cellular therapies. There is a current consensus that *invitro* testing using human cells or tissues, if validated and has clear indications of predicting clinical outcome should be used in preference to animal model experimentation. In this report we have summaried the risks to patient safety and some of the types of *in vitro* tests especially for immunogenicity which could or are currently being used within the cellular therapy field. More research is needed ,for example to develop assays for the more complex toxicities, such as neurotoxicity which will need collaborations between both and commercial and academic groups in the future.

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