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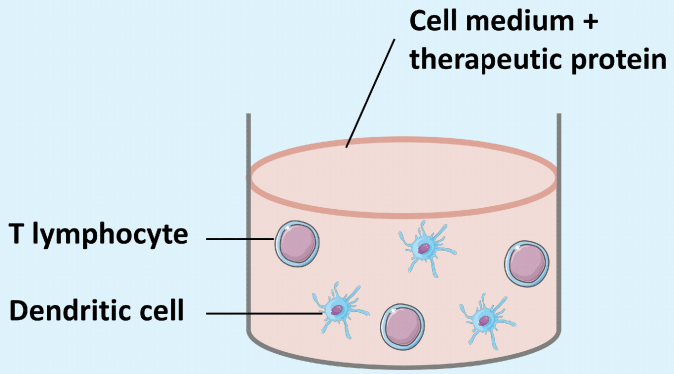
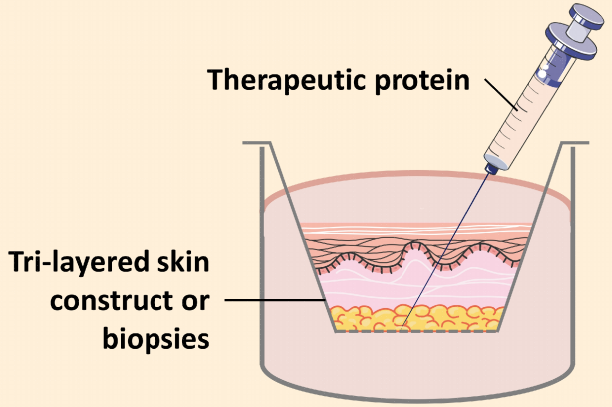
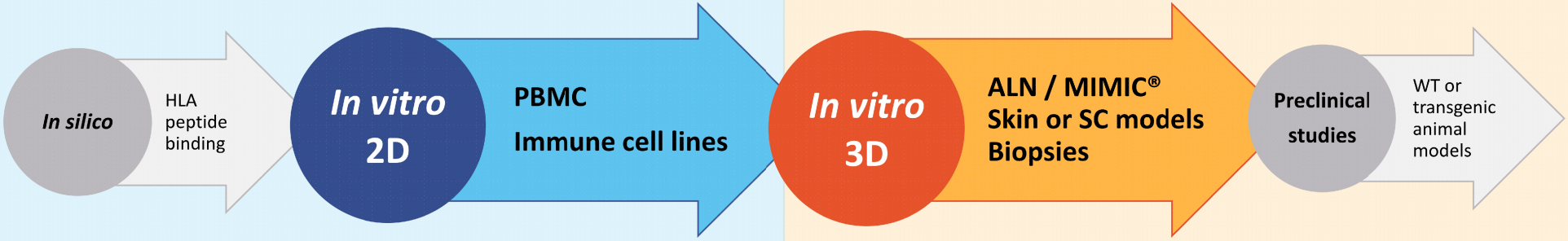
In Vitro Models for Immunogenicity Prediction of Therapeutic Proteins







Review Article

In vitro models for immunogenicity prediction of therapeutic proteins

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Abstract

Immunogenicity assessment of therapeutic proteins is routinely performed through various techniques during the drug development process: (i) *in silico* to design the least immunogenic protein possible, (ii) *in vitro* using mainly classic 2D assays with PBMC-derived cells or immune cell lines to follow protein uptake, immune cell maturation and pro-inflammatory cytokines released, (iii) *in vitro* using 3D models of the human immune lymphatic system or full-thickness skin, (iv) and finally *in vivo* with preclinical and clinical studies. This review focuses primarily on the immunogenicity assessment of therapeutic proteins injected subcutaneously and new *in vitro* models that may be used as specific models of this tissue.

Keywords

Therapeutic protein; immunogenicity assessment; *in vitro* model; subcutaneous injection; injection site reactions.

Abbreviations

ADAs = antidrug antibodies

ALN = artificial lymph node

APCs = antigen presenting cells

ASCs = adipose-derived stem/stromal cells

DCs = dendritic cells

ECL = electrochemiluminescence

ECM = extracellular matrix

ELISA = enzyme-linked immunosorbent assays

EMA = European Medicines Agency

FDA = US Food and Drug Administration

HLA = human leukocyte antigen

ICH = international conference on harmonisation

IL = interleukin

IM = intramuscular

ISRs = injection site reactions

IV = intravenous

LC = Langerhans cells

MDDCs or MoDCs = monocyte-derived dendritic cells

MDM = monocyte-derived macrophages

MHC = major histocompatibility complex

NAbs = neutralizing antibodies

PBMCs = peripheral blood mononuclear cells

Ph. Eur. = European Pharmacopoeia

SC = subcutaneous

SCID = severe combined immunodeficiency

TLRs = toll-like receptors

USP = US Pharmacopoeia

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1. INTRODUCTION

1.1. Immunogenicity definition

Biopharmaceuticals such as therapeutic proteins represent an increasing percentage of new drug approvals. A total of 239 therapeutic peptides and proteins were approved by the US Food and Drug Administration (FDA), formulated in 340 commercialized drug products, among which 116 (35%) are subcutaneously administered [1]. The European Medicines Agency (EMA) approved around 100 biotech-derived medicines over the last 5 years [2]. These therapeutic proteins are targeted treatments for chronic and/or life-threatening diseases such as diabetes, cancer, autoimmune diseases (e.g., multiple sclerosis) and enzyme deficiency replacement therapies. However, the use of biologic therapeutics can lead to – although rare [3] – serious adverse events (AE). One such AE is the stimulation of an immune response that can appear after a single or repeated injections of therapeutic proteins [4].

Conversely to vaccines, immunogenicity of therapeutic proteins is an unwanted effect as it may result in the formation of antidrug antibodies (ADAs), which could negatively impact the efficacy and safety of the treatment. Indeed, ADAs may have a neutralizing activity by binding to the active site of the protein and may induce various degrees of hypersensitivity reactions ranging from injection site reactions (ISRs) to anaphylaxis. This latter type of severe reactions, type I hypersensitivity or allergic reactions, are linked to the production of specific immunoglobulin E (IgE) and the rapid release of histamine, whereas mild type III reactions involve the formation of immune complexes of protein surrounded by ADAs [5]. A classification of all adverse drug reactions encountered following recombinant protein injection was described by Murcada et al.[6], and Corominas et al.[7].

As thoroughly described in the recently revised EMA guideline on the immunogenicity assessment of therapeutic proteins [8], the immunogenic potential of a protein is directly linked to product factors such as the protein structure and peptide sequence, post-translational and chemical modifications, the presence of aggregates and impurities, dose, route and frequency of administration [9, 10]. There are also patient-related factors like disease state, presence of pre-existing antibodies against the therapeutic protein, and genetic background that contributes to the immunogenicity of therapeutic proteins [11-13].

As mentioned above, a protein in its native monomeric form may induce immunological reactions due to non-humanized sequences, HLA binding and T cell epitopes [4, 14]. However, the presence of aggregates is thought to be one major source of enhanced immunogenicity, which may result from the creation of neoepitopes in comparison to the protein in its native state, or due to increase in epitope concentration and their affinity to B cells [15]. Protein aggregates may have various sizes ranging from dimers to visible particles, formed by exogenous interaction with excipients and surfaces, through covalent interactions or not, and be of soluble or insoluble nature [16-18]. These aggregates may be formed during the manufacturing process, transport, storage and (mis)handling. In addition, destabilization due to important changes in the protein's microenvironment during injection may further enhance the creation of aggregates [19]. Factors related to manufacturing and handling can be avoided by optimization of the formulation composition, changes in the manufacturing process, detection using orthogonal characterization methods [20-22], and patient instructions. Formation of aggregates after subcutaneous injection, however, is much more difficult

to predict and identify. The occurrence of immunogenicity of therapeutic proteins in relation to their propensity to form aggregates was detailed in a review by Moussa et al. [23].

1.2. Human immune system

The innate and the adaptive immune system are both implied in the immune response against therapeutic proteins via their recognition and uptake by professional antigen presenting cells (APCs). Immune response against (aggregates of) therapeutic proteins is driven by the interaction with pathogenic pattern recognition receptors (PRRs) of the innate immune system, such as the family of Toll-like receptors (TLRs) expressed on the surface of APCs and epithelial cells. Examples of APCs are macrophages or dendritic cells (DCs) that will phagocytose (or endocytose) and process the protein, to finally present the antigen by virtue of MHC class II proteins at their surface, leading to the activation of CD4+ T cells. Dendritic cells are present as sentinels in all types of tissues, and once matured they are able to migrate to the lymph nodes and lymphoid organs. Immunogenicity can also be raised by a T cell-independent pathway when B cells directly encounter antigenic structures, such as aggregate neoepitopes or post-translational modifications. B cell maturation in plasma cells is induced and will lead to the release of ADAs. For both types of responses, the presence of repetitive neoepitopes induces the breaking of immune tolerance, either the central tolerance linked to B cells, or the peripheral one maintained by specific T lymphocytes, called regulatory T cells or Tregs [24]. The different immune cell activation pathways that may be induced by proteins aggregates are summarized in Figure 1.

Figure 1. Simplified hypothetical pathways for immune cell activation by aggregated proteins are shown. (a) The T cell dependent pathway. (b) The T cell independent pathway. Generation of immune response by the T cell dependent pathway would require presence of both B cell and T cell epitopes. Recognition of the B cell epitope by B cell receptor (BCR) would drive uptake and processing by the B cell and presentation of the T cell epitope in the context of MHC class II molecule on its surface. In parallel, non-specific uptake and processing by professional APCs would lead to the presentation of the T cell epitope on MHC class II to naïve T cells. These activated T cells, on encountering the antigen-primed B cell, deliver the cytokine signal required to cause the B cells to convert to IgG-secreting plasma cells. The T cell independent response occurs as a result of cross-linking of BCRs by repetitive epitopes on the antigen/aggregate. A cytokine signal is required to enable the B cells to mature into plasma cells. Reprints from Kumar et al., 2011 [25].

Therapeutic protein immunogenicity prediction is currently a major issue investigated by academic and industrial research groups, and regulatory organizations. Multiple approaches have been considered to be applied during drug development, preclinical and clinical phases, and post-marketing surveillance. Among them are *in silico* prediction methods, *in vitro* cell-based assays and *in vivo* assessment of ADAs and NAbs from blood samples of animal and human clinical trials. *In silico* and *in vivo* methods will be briefly described in the introduction, before focusing more specifically on *in vitro* approaches and notably the design of 3D cell cultures to investigate subcutaneous immune reactions.

1.3. *In silico* prediction

In silico immunogenicity prediction tools are based on amino acid sequence analysis of new protein drug candidates. Algorithms developed are in perpetual improvement supported by their concomitant use for vaccine design. Many models now offer good estimations of the immunogenic potential of a protein [26], mainly identifying HLA-II peptide epitopes and evaluating their binding affinities. B cell epitope binding remains much more complex to predict as it implies the recognition of nonlinear parts exposed by the three dimensional refolding of the protein [27]. This can be related to neoepitopes formed by aggregates, which involve different conformational structures adopted by the aggregation of protein monomers or by involving excipients (such as polysorbates) and/or impurities and leachables (such as silicone oil) [28-30].

Bryson et al. [27] comprehensively reviewed the available immune epitope databases and software programs. To cite a few more, Epivax Inc. developed an algorithm called EpiMatrix to predict MHC class I and II epitopes [31], Lonza's Epibase® *In Silico* platform allows for high throughput screening of peptides binding to the HLA receptor [32], and the ProSentium™ database was established by ProImmune Ltd. [33].

The (bio-)informatics field is in constant evolution and the most efficient computational tools are now also taking into account the refolding and potential conformational changes of the protein, enabling better prediction. These software and databases are useful during the early discovery phase to select lead candidates, but also to design less immunogenic biotherapeutics. As summarized by Jawa et al. [34], they offer the advantages of high throughput testing at a low cost and have the ability to reduce the search space for further *in vitro* assays. Indeed, the authors have shown that although the exact clinical immunogenicity rate cannot be estimated, EpiMatrix scores allow an accurate ranking of immunogenicity between therapeutic proteins (see example in Table 1 with different variants of a recombinant fusion protein FPX). However, these computational tools still have limitations. As they generally tend to be overpredictive [35], *in vitro* confirmation of epitopes identified are needed and conversely the limited number of HLA class II alleles tested do not completely reflect the important polymorphism found in the human population [36]. Moreover, even though T cell epitope(s) can be predicted, those *in silico* methods still cannot identify which T cell type (T helper, T regulatory) is responding [34].

Table 1. Correlation of algorithm-predicted immunogenicity to clinical immunogenicity rates. EpiMatrix-generated scores associated with each FPX protein and their respective rates of antibody incidence (binding and neutralizing) are shown. An assessment of Tregitope content in each molecule was also performed and scores were adjusted accordingly. FPX 1, for example, had a high rate of clinical immunogenicity and was associated with elevated T-cell epitope content and low Tregitope content, as reflected by its high Z score. FPX 2, 3, and 4 were associated with a low EpiMatrix score, and Tregitope adjustment further reduced the predicted potential for binding. Predictably, FPX 2, 3, and 4 exhibited only minor clinical immunogenicity. Reproduced with permission from Jawa et al., 2013 [34].

1.4. *In vivo* preclinical and clinical assessment (ADAs and Nabs assays)

Later in the protein drug development process, immunogenicity assessment is done by detection of ADAs in blood samples collected during preclinical and clinical phases. A number of *in vivo* models were developed with HLA-transgenic, humanized, and human severe combined immunodeficiency (SCID) mice, using minipigs [37], or non-human primates [38]. Despite tremendous efforts to create better predictive animal models, a poor correlation of immunogenicity prediction was noticed between these animal models and the results of clinical trials for FDA approved therapeutic proteins [39]. Besides the fact that none of them fully reflects the complex functioning of the human immune system, the enforced application of 3R's principles motivates industry to implement new strategies (see paragraphs on *in vitro* assays and 3D models below).

A current approach in immunogenicity assessment during clinical investigation consists of a first row of "ADA screening assays" to determine the presence or absence of circulating ADAs after treatment with the biopharmaceutical, followed by a "confirmatory assay", which if revealed to be positive will be later accompanied by a "characterization assay" defining the neutralizing ability of these ADAs [40, 41]. Various techniques are used for the readout of ADAs assays, the most common being enzyme-linked immunosorbent assays (ELISA). Evolution of immunogenicity assays using different detection methods like "direct, indirect or capture assays, electrochemiluminescence (ECL) assays and antigen-binding tests, such as radioimmunoassays" were recently summarized by Pineda et al. [41]. The authors report on the difficulty in obtaining harmonization between clinical trials and their immunogenicity assessment results due to the inter- and intra-variability of existing assays. Moreover, regulatory authorities and pharmacopeias are now considering a risk-based approach concerning the testing for ADAs. Kinetics of appearance of ADAs should be considered, as well as whether the immune response is transient or persistent and related or not with clinical sequelae. In this way, patient numbers, ADA sample collection time and duration should be carefully planned as well as the design and validation of the ADA assay (format, cut-point, sensitivity, reproducibility, etc.) [40, 42, 43]. Table 2, extracted from Wadhwa et al. [44], compiled the advantages and disadvantages of the most commonly used screening assays for ADA and NAbs detection.

Table 2. Commonly used screening assays for ADA and NAbs detection. Reproduced with permission from Wadhwa et al. [44].

In terms of clinical ADA monitoring, the present common sample collection strategy consists of a systematic collection and ADA testing of all patients in each clinical trial. However, a recent paper [45] by scientists at Amgen and Merck suggests a new "event-driven" strategy for therapeutic proteins at low immunogenicity risk. In this approach, collected samples would only be analyzed by ADA assay in case of safety issues. Alternative strategies, such as a "fit-for-purpose" approach for immunogenicity testing of biotherapeutics, have also been proposed by other contributors [46].

Over the last few years, in addition to regulatory guidelines, many white papers by industry consortia and research articles had convergent interests in the elaboration of ADAs assays and development of novel techniques [47-51]. Refinement of these protocols could have a significant influence on the immunogenicity results obtained [52, 53]. The necessity of having comparable approaches for immunogenicity assessment of biosimilars is frequently mentioned due to the rising number of biosimilar approvals, first in Europe and now followed by the United States [54]. Those papers address problems such as the evaluation and confirmation of cut-points establishing the titer threshold between positive and negative samples in ADAs assays [51], drug-target interferences and their impact on results [47], and impact of the choice of storage buffers used for conjugated reagents

on long-term performance of ADA methods [48]. Advantages of emerging new technologies compared to current ADA assays [50], and comparison of injection site reactions incidence after biosimilar or reference drug administration are also discussed herein [55].

The ABIRISK (Anti-Biopharmaceutical Immunization: prediction and analysis of clinical relevance to minimize the RISK) consortium includes universities, institutes, industrial researchers and clinicians from Europe to focus in particular on the correlation between patient factors, clinical factors and the incidence of immunogenicity [56]. Recent papers from this group show clinical examples of immunogenicity assessment of biopharmaceuticals (infliximab, interferon-beta, natalizumab, rituximab and adalimumab), attempting to establish a link between ADA responses, hypersensitivity reactions and the presence of detectable circulating drug-specific T cells [52, 53, 57-59]. For example, in their study on infliximab, Vultaggio et al. have observed a correlation between the serum ADA levels and the occurrence of hypersensitivity reactions in patients [53]. This results in a new database platform (tranSMART) compiling data of multiple sclerosis cohorts and ADA test results to be compared on a European level [52].

Evaluation of immunogenicity through ADA detection and (semi-) quantification is only one part of the elucidation of the clinical manifestations, but the characterization of their neutralizing ability is also of importance regarding the safety and efficacy of therapeutic proteins. Actually, the subgroup of neutralizing antibodies (NAbs) has a direct impact on the "loss of drug efficacy by blocking the biological activity of a therapeutic product" as stated by Wu et al. [60]. Two different formats exist for these assays, cell-based or non-cell-based assays, and the choice depends on the type of biopharmaceutical tested and whether there is an endogenous counterpart of the therapeutic protein [60]. As for the ADA assays, there is currently a lack of harmonization despite the diverse national and international guidelines edited [8, 42, 43, 61-63]. Although the incidence of immunogenicity is well disclosed in the prescribing information, few report on the impact of immunogenicity on the protein's pharmacokinetic profile [64]. Similarly, Shankar et al. [5] highlighted the fact that current drug package inserts do not clearly inform physicians on how to manage immunogenicity and related adverse events in the clinic.

Finally, among all clinical studies using ADA and NAb assays to assess immunogenicity, one aspect rarely taken into consideration within the same clinical trial and therefore using exactly the same assay design is the influence of the route of administration [65]. A well-known dogma classified subcutaneous (SC) and intramuscular (IM) injections as being more immunogenic routes than intravenous (IV) infusion [13, 66]. However, there is a common interest of patients, physicians and industry to develop novel subcutaneous formulations to improve compliance via self-administration, ease and rapidity of the medical intervention [67, 68]. Moreover, some IV administered proteins such as trastuzumab and rituximab are now approved for SC administration in Europe. Hamuro et al. [69] recently published perspectives on the SC route of administration as an immunogenicity risk factor for therapeutic proteins. Clinical immunogenicity data of six commercialized products were assembled to compare ADAs and NAb levels obtained after administration by the SC or IV route was performed. Factors affecting the immunogenicity of both routes were listed, such as formulation composition, therapeutic indication and disease state of the patient, mechanism of drug action (immunosuppressive or not), dose and frequency of dosing, concomitant medication, and blood sample timing. In their conclusion, authors highlighted the knowledge gap concerning possible differences between the two parenteral routes of injection in the pathways followed by the immune

reaction. ADA incidence was observed to be similar or up to two-fold increased in SC injection compared to IV, however, formulated protein concentrations tested were not the same.

1.5. Regulatory requirements (FDA/USP – EMA/Ph.Eur.)

As already mentioned above, the regulatory authorities and organizations (EMA, FDA, European and US pharmacopoeias, ICH) publish regularly new or revised guidelines on this immunogenicity issue to help sponsors to fulfill efficacy and safety requirements to be granted marketing authorization and later to perform post-marketing surveillance.

Recommendations by FDA are related to patient and product-specific factors to be considered for a reliable immunogenicity assessment during the clinical phase [11], and to the critical points for ADAs and NAb assay development and validation to investigate immune responses during phase I clinical trials [42]. With respect to preclinical studies, guidance mentions that *in vivo* animal models are not necessarily predictive for immunogenicity in human due to species differences. Still, useful information such as possible consequences of inhibition of an endogenous protein or identification of potential immunogenic aggregate species, may be obtained. Unfortunately, there are no recommendations with regard to the early prediction of immunogenicity during the research and development phase, as well as no (short term) economic incentive, which could encourage the development of new reliable *in vitro* immunogenicity assays, other than assays involving peripheral blood mononuclear cells (PBMCs).

The EMA guidelines on immunogenicity assessment [8, 43, 61] focus on the clinical assessment and factors that may influence the development of an immune response against a therapeutic protein (patient- and disease-related factors, product-related factors). Potential clinical consequences of immunogenicity on efficacy (ADA and NAb assays) and safety (acute, delayed, and/or autoimmune reactions) are comprehensively described. EMA outlines that non-clinical assessment (i.e., preclinical studies) of immunogenicity has limitations and their predictive power in humans is considered to be low. Moreover, it is stated that “non-clinical *in vitro* or *in vivo* studies aiming at predicting immunogenicity in humans are normally not required”, however, “ongoing consideration should be given to the use of emerging technologies […] *in vitro* assays based on innate and adaptive immune cells could be helpful in revealing cell-mediated responses” [8].

The European Pharmacopeia (Ph. Eur.) considers immunogenicity testing only in the context of vaccine products. Only one assay is described for the detection of host-cell proteins contaminants in recombinant therapeutic proteins [70]. In contrast, the USP dedicates two complete sections (<1106> and <1106.1>) to this issue [40, 44, 63]. A consensus appears to exist between regulatory agencies insofar as *in vitro* early assessment of immune response to therapeutic proteins using cell-based predictive assays is not yet sufficiently reliable to be incorporated in their guidelines. This motivates the search for more predictive immunoassays.

1.6. Need for early prediction in the drug development process

In the previous section, the *in silico* tools available to predict immunogenic epitopes and overall immunogenicity potential of a protein from its amino acid sequence were described. However, all results obtained through these methods must still be confirmed by *in vitro* assays before engaging in preclinical studies.

Subsequently in the development process, preclinical studies are classically conducted on animal models of different species (wild type or transgenic rodents, minipigs and non-human primates). Although useful in anticipating severe toxicity problems, prediction of potential immunogenicity issues in humans tends to be over predictive (for most human recombinant proteins) or under predictive [71]. Suitability of various animal models to correctly predict immune response in humans was discussed in detail by Brinks et al. [72]. Without mentioning the important differences between the immune system in human and animals, these studies evaluated protein immunogenicity by means of ADA and NAbs assays. Those assays are similar to the ones performed in clinical trials and were previously shown to be in need of improvement, notably with regard to their reproducibility.

In order to complement *in silico* prediction and avoid over- or underestimation of safety risks in preclinical studies as seen in the example of TGN1412 [71], there is a clear need of an early and better immunogenicity prediction using reliable *in vitro* models. Such models, in addition to allowing for high-throughput screening, are in line with the 3R's principle and may also enable a better understanding of the human immune system in the long term. We will describe in this review the different standard 2D *in vitro* assays currently used for immunogenicity assessment of biopharmaceuticals, their strengths and limitations, and the appearance of new 3D *in vitro* models trying to better representing the human immune system. Special attention is given to the prediction of immunogenicity and clinical sequelae after injection of therapeutic proteins into the subcutaneous tissue.

2. 2D *IN VITRO* ASSAYS

2.1. Primary cell-based assays

Most of the *in vitro* biological assays performed to predict and assess immunogenicity of therapeutic proteins employ human primary immune cells. Isolated from whole blood donations, PBMCs comprise lymphocytes (T cells, B cells, and NK cells), monocytes, and few dendritic cells. They are generally obtained from naïve healthy donors, however, samples from specific subpopulation of patients (antigen-exposed or not) can be of interest to study disease-related immunogenic reactions.

These primary cells can be used as whole PBMCs or be further purified to isolate specific subsets such as CD4+ or CD8+ T cells. Monocytes can be isolated by positive selection using the expression of their specific marker, CD14 in humans. Subsequent differentiation can be induced by addition of targeted cytokines like GM-CSF and IL-4 to obtain monocyte-derived dendritic cells (MDDCs or MoDCs) [73], or M-CSF to obtain monocyte-derived macrophages (MDM) [74]. Finally, different subpopulations derived from PBMCs can be mixed at defined ratios to represent the whole human immune system in one cell culture plate.

As each human donor has their own genetic background, PBMCs will express different major histocompatibility complex (MHC or HLA) proteins on their surface. Therefore, HLA typing is necessary. To reflect immune reactions from the whole population a large number of donors will be required. Usually, results of PBMCs assays from 30 to 50 donors are pooled together to cover around 80-95% of the most frequent human HLA class II haplotypes (HLA-DR, HLA-DQ, HLA-DP) [75-77].

The use of whole PBMCs incubated with the therapeutic protein allows for a complete immune response simulation but is limited to drugs that will not negatively impact cell proliferation (inadequate for immunomodulatory drugs). In this specific situation, a 2-step assay format is preferred to enable first the loading of APCs with the antigen, followed by an interaction between APCs and T cells [78]. Moreover, the therapeutic protein sample concentration has to be optimized in order to not have a deleterious effect on APCs, as well [75].

Concerning immunogenicity assessment of biologics, we can distinguish four types of assays based on the (subsets of) cells used:

- Whole PBMC
- CD4+ or CD8+ T cells
- Monocyte-derived dendritic cell (MoDCs)
- Mixtures of MoDCs and T cells, using different ratios

These primary cells allow performing different types of assays reflecting various steps of the whole pathway of T cell mediated immune response from the antigen uptake by professional APCs to T cell proliferation.

2.1.1. HLA binding assays (class I and II)

In complement to *in silico* identification and evaluation of the binding affinity of HLA-II peptide epitopes, *in vitro* assays may be performed using purified HLA-II peptides (usually 15-mers with an overlapping region) of the protein of interest. Direct quantification of binding affinity and kinetics is realized employing surface plasmon resonance (SPR) or biochemical assays like ELISA. This latter technique used in 384-well plate format and with the assistance of a liquid handling robot allows for high throughput screening of peptide-MHC II binding assays [79].

Despite being less used due to inherent difficulties linked to the high polymorphism of MHC class I molecules and structure/conformation influence on binding affinity, MHC-I peptides binding assay was designed using the same high-throughput approach combined with a technology called luminescent oxygen channeling immunoassay (LOCI or AlphaScreen™) [80], or FACS-based MHC stabilization assay [81].

Several other assays and techniques may be used, like competition assays allowing kinetic measurements and identification of CD4+ T cell epitopes [82], or even cell-based assays where MHC I-peptides bind directly HLA-typed human B-cell lines [83].

However, those previous approaches are biased by the presentation of all potential MHC II-peptides extracted from the protein sequence, without considering the natural enzymatic processing of the protein inside APCs. Thus, another approach consists in extracting and differentiating immature

MoDCs from PBMCs and incubating them with the whole antigen. After this interaction with the protein of interest, maturated MoDCs are harvested and HLA-peptides are purified and analyzed by mass spectroscopy sequencing [84].

2.1.2. T cell activation and proliferation

Following therapeutic protein uptake and processing by APCs, the specific MHC-II peptides and co-stimulatory molecules expressed at the APC surface are available for recognition by T cells receptors (TCR) inducing the proliferation of T cells. In *in vitro* PBMC assays, the antigen priming of CD4+ T cells by mature MoDCs leads to the proliferation of CD4+ T cells. T cell proliferation is followed by radioactive labeling with tritiated thymidine pulsation and scintillation counter [75, 77, 85], or by using more sensitive labeling with fluorochromes like carboxyfluorescein succinimidyl ester (CFSE) and 5-ethynyl-2'-deoxyuridine (EdU) in combination with flow cytometry [86, 87].

MoDC and T cell activation are also commonly recorded via flow cytometry analysis of surface markers whose expression is down- or up-regulated. MoDCs exposition to antigens, such as aggregates of therapeutic proteins, may induce an up-regulation of the activation and maturation markers CD40, CD80, CD83, CD86, CD209, HLA-DR and a change in morphology [88, 89]. Phenotypic changes of T cells are registered through modification of expression of co-stimulatory CD40-ligands, CD25, CD46 and CD69 [90].

A recent publication by Schultz et al. [85] describes a novel *in vitro* T cell assay combining CD4+ T cells purified from PBMCs and the remaining PBMCs, which were irradiated. Irradiation inhibits cell division and guarantees that proliferation and cytokines released were exclusively linked to CD4+ T cells. Four commercialized therapeutic monoclonal antibodies (infliximab, rituximab, adalimumab, natalizumab) showing CD4+ T cell-dependent immunogenicity in the clinic were used to validate this optimized PBMC:T cell assay. As concluded by the authors, *in vitro* T cell assays hardly predict clinical immunogenicity but they may be used to help to select the lead candidate during drug development by picking a variant with a low T cell response. A direct correlation between these PBMC:T cell assay results and the ADA response observed in the clinic cannot be drawn due to: (i) the negative impact of anti-TNFα (infliximab and adalimumab) on APCs and T cells that could create false results, (ii) the absence of knowledge of the long term immunogenicity of natalimumab, (iii) the difference in formulation and route of administration of these four biotherapeutics, and (iv) the important variation in numbers of positive ADA responses measured depending on the indications and between clinical studies [75, 85, 91].

2.1.3. Cytokine release

Consecutive to the co-activation of DCs and T cells, pro-inflammatory cytokines such as TNFα, IFNɣ, IL-2, IL-4, IL-6, IL-8 and IL-10 are released. The detection and quantification of these cytokines is carried out by testing cell culture supernatants by ELISA [92], by cytometric bead array (Luminex® Multiplex Assay) [75, 89], or by incubation of antigen-stimulated cells using enzyme-linked immunospot (ELISPOT) plates [76, 77, 85]. The secretion of pro-inflammatory cytokines is involved in

the induction of (allergic) immune reactions to foreign proteins at the injection site [92]. For example, interferon β used for the treatment of multiple sclerosis and known to cause frequently inflammatory injection site reactions was tested on primary adult human dermal microvascular endothelial cells (HDMEC), primary human keratinocytes (HKC) and primary human dermal fibroblasts (HDFB). Cell culture supernatants were tested with ELISA experiments for the following cytokines CXCL10, CCL2, CCL5 and CXCL8. Authors observed a strong CXCL 10 expression by the primary cells, which is known to attract T cells [92]. Different formulations of interferon beta-1a were also applied to PBMCs and T cells proliferation induced by secretion of IL-2 and IFN-γ was followed by ELISPOT.

Early phase secretion of cytokines IL-1α, IL-1β, IL-1ra, IL-6, IL-8, IL-10, MCP-1, MIP-1α, MIP-1β, TNF-α, and TNF-β and late phase secretion of cytokines IFN-γ, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12p40, IL-12p70, IL-13, and TNF-α were assessed by Multiplex assay after incubation of PBMCs with various mAbs, some of them already commercially available (e.g., Herceptin, Campath, Xolair, Erbitux, Avastin, Rituxan, Remicade, and Humira [75]). Similar experiments were performed for rituximab and trastuzumab following secretion of IL-1β, IL-6, IL-8, IL-10 and IL-12 by PBMCs using Multiplex CBA analysis [89]. Induction of IL-2 secretion by other therapeutic mAbs (infliximab, rituximab, adalimumab and natalizumab) from PBMCs was measured by IL-2 ELISPOT [85]. Wullner et al. tested two biotherapeutics of known clinical immunogenicity on PBMCs using IFN-γ ELISPOT to follow their ability to induce antigen specific secreting T cells [76].

To conclude, *in vitro* immunogenicity prediction assays like PBMC assays offer advantages to design high-throughput assays testing a part or the whole process of T cell dependent immune response, with more than one assay condition, and to confirm peptide-HLA complexes identified *in silico*. However, to develop reliable assays it remains challenging to optimize the protein concentration and number of challenges necessary to induce T cell proliferation, as well as the number of cells and ratio between T cells and DCs [76]. Usage of whole PBMC amongst the other 2D assays may lead to a non-specific answer due to the presence of cells, which are able to release IFN-γ but do not activate T cell receptor through HLA binding [76]. Moreover, it requires pooling PBMCs from a large number of donors, which is expensive and requests time-consuming standardized procedures for sampling, extraction, cell counting, freezing and quality controls. Finally, the donor-to-donor variability, even though they are selected to reflect the haplotypes frequency in the population, may affect PBMC assays consistency and results. This may explain why some specialized companies offer now PBMC primary cells differentiated into DCs (Poietics™, Lonza), or to provide services of immunogenicity prediction with their own *in vitro* assays like ImmunXperts SA, Antitope Ltd (EpiScreen™), Lonza (EpiBase™), and ProImmune Ltd (REVEAL®) [85].

2.2. Immune cell lines

Challenges associated with the procurement and handling of primary cells encouraged some research groups to find a more reliable and readily available source to perform immune prediction experiments. Moreover, since the new European Union regulation (1223/2009) is abrogating animal testing for safety assessment of cosmetic products and due to the rising interest in nanomaterials [93], industry has now turned to a variety of immune cell lines to identify skin sensitizers, to develop

DC vaccines for cancer immunotherapy, or to detect immunogenicity of impurities in therapeutic proteins.

Most of these immune cell lines are (myelo-)monocytic cell lines obtained from patients suffering from acute or chronic leukemia. Following exposure to cytokines or other signals (PMA, DMSO, 1,25-dihydroxy-vitamin D3), they are able to differentiate into monocytes, DCs, macrophages, and granulocytes. Properties and phenotypes of THP-1, KG-1, HL-60, Mono Mac 6 (MM6), K562 and MUTZ-3 cell lines were described [94], and studied by Santegoets et al. [95] seeking for human DC cell line differentiation models to study DC vaccination. The majority of those DC models display phagocytosis and other phenotypic and functional DC characteristics. Among them MUTZ-3, which stands out by its cytokine-dependence, can be differentiated either into epidermal DCs also called Langerhans cells (LC), or into interstitial DCs (IDC) found in the dermis as well as throughout the body. MUTZ-3 cells exhibit a specific DC phenotype and are able to mature, while upregulating the expression of costimulatory molecules and maturation markers mentioned earlier (CD40, CD80, CD83, CD86 and HLA-DR).

One DC cell line model, U-937, can be distinguished by its histiocytic lymphoma origin. U-937 cells display monoblastic morphology and are not capable of phagocytosis. However, these cells can be differentiated into DCs or macrophages, and were successfully used to study skin sensitizers [94]. In the field of chemical sensitizers, THP-1 and MUTZ-3 cell lines were compared to primary monocyte-derived DCs (MoDCs) in terms of biomarker expression and cytokine release [96]. The authors showed that CD86 DC maturation marker was expressed by all cells after stimulation with contact allergens, and concluded that dendritic cell line models “mimic primary DCs in many aspects”. Nevertheless, their use should remain a “case-by-case decision” depending on the selected biomarker measured. Another study comparing THP-1, HL-60 and MUTZ-3 human DC cell line models revealed that, with its ability to take up and present antigens through expression of MHC class I and II molecules, and to mature and adopt a migratory phenotype, MUTZ-3 derived DCs were the ones which “most closely resemble primary DCs” [97].

One main argument against the use of cell lines instead of primary cells would be their inability to bind diverse HLA I and II peptides. However, MUTZ-3 cell line was proven to be positive for antigens HLA-A2, HLA-A3, HLA-B44, HLA-DR10, HLA-DR11, HLA-DR52, HLADQ5, and HLA-DQ7 [98]. More information on studies using the MUTZ-3 cell line model for detection of skin sensitizers [99-104], vaccine development [105], or to induce antitumor T cell immunity can be found in literature [106].

Other macrophages or DC cell line models exist as shown in a recent paper by Haile et al. [107]. Some of them were used to detect product and process impurities present in therapeutic protein formulations that could induce innate immune response. The authors, employees of FDA, used murine macrophages (RAW 264.7), human embryonic kidney cells (HEK293) transfected with toll-like receptors (TLRs), and MM6 and THP-1 models instead of highly variable PBMCs, to detect host-cell impurities activating innate immune response present into biotherapeutics (even with immunomodulatory effect). HEK-BLUE expressing human TLRs 2, 4, 5, 7 and 9 were used to compare level of sensitivity to PBMCs and identify receptor-specific impurities. Then, monocyte or macrophage cell lines with different readouts were used to screen impurities without knowledge of their nature and TLR activation. Later their sensitivities to known impurities were compared to PBMCs, and similar sensitivity was observed when the three human cell lines were combined, except for two ligands of TLR3 (Poly I:C) and MDP-NOD2 (MDP).

The outcomes obtained so far should encourage other research groups to investigate the use of these readily available and constant cell line models during drug development. They might also serve for quality assurance purposes at later stages.

2.3. Triple co-culture

As described in the two previous sections, primary cells and immune cell line models are used in 2D *in vitro* assays to predict immunogenicity of biotherapeutics and other chemical products. Another type of assay, which has a supplementary degree of complexity, is the triple co-culture of cells of different origins to recreate immune function of specific human parts, organs or epithelia. For instance, a 3D model of the human airway epithelium was designed as a triple cell co-culture system combining lung and bronchial epithelial cell lines (A549 and 16HBE14o-) and primary cells from PBMCs (MDDC and MDM) [108]. This model allows the investigation of the interaction between the different cells, as well as the cellular immune response upon xenobiotic stimulation.

Another triple co-culture model was used by Saalbach et al. [109] to study T cell-mediated immune response in the dermis, where primary fibroblasts obtained from skin biopsies were cultured with PBMC-derived DCs and T cells. In order to model another inflamed epithelium to study safety of nanomaterials, Susewind et al. [110] combined intestinal colon-colorectal carcinoma (Caco-2) cells with THP-1 and MUTZ-3 cells, which were embedded into type I collagen on an insert well.

Those co-cultures are often grown on a microporous membrane, using insert wells to define a two-chamber system, and follow the migration of the immune cells after exposure to “foreign” particles by fluorescence labeling. These 2D *in vitro* systems are more suitable to mimic the physiological reality thanks to interactions between the different co-cultured cell types. However, they cannot replace information obtained from *in vivo* assays.

In our opinion, these triple co-culture models may be used instead or complementing 2D PBMCs assays to refine the immunogenicity assessment and bridge to successive animal studies.

Major 2D and 3D *in vitro* models described in the previous and following sections, and their common read-outs are illustrated and summarized in Figure 2.

Figure 2. Processes of therapeutic protein immunogenicity assessment classified by increasing complexity. Schematic representation of *in vitro* standard 2D assays with PBMC-derived DC or immune cell line-derived DC suspensions, major *in vitro* 3D models, and their common read-out technologies described in this review. Artificial lymph node (ALN) bioreactor drawing is adapted from Giese et al. [111]. PBMC = peripheral blood mononuclear cells; WT = wild type.

3. 3D *IN VITRO* MODELS

At the interface between 2D *in vitro* assays and animal models, 3D models are being developed. They serve to either mimic the lymphatic system, which allows migration of immune cells like DCs and T cells, or to mimic skin and subcutaneous models more specifically of interest to therapeutic protein immunogenicity prediction.

3.1. Artificial lymph nodes

The need for relevant human 3D models allowing to reduce animal studies and better mimic physiological conditions than 2D cell cultures, notably in the immunotoxicology field, has brought the development of organotypic tissues such as artificial lymph node (ALN) to the fore [112]. The main difference between these ALN and the previously described co-culture resides in the addition of a microfluidic system in the (mini-) bioreactors to control nutrient and oxygen supply, temperature and pH. This simulates more closely the physiological environment and gradients of stimuli. The fluidic circulation may also induce important variations in the cell phenotype, due to mechanical forces applied [113].

A research group of ProBioGen AG (Berlin, Germany) developed an *in vitro* human lymphatic micro-organoid model in order to perform immunological substance testing, including vaccines [111]. Their model is a combination of a co-culture model and PBMCs based cell material with a microfluidic system. In practice, PBMC derived DCs and T cells are embedded in an agarose matrix while B cells are maintained in suspension in a continuous cycling allowing their interaction with mature DCs and primed T cells. Two different bioreactors were developed, allowing micro-organoid formation after 7 days, and cell maintenance over 14 to 30 days, as well as sampling for cytokine analysis and *in situ* imaging via two-photon microscopy. This human artificial lymph node model was shown to "physically reflect the immunological effects of vaccines and virus preparations and immune-modulating substances" such as dexamethasone [111]. Finally, the authors suggested that their model may be improved by the addition of human cells (from human lymph nodes or bone marrow biopsy), fibroblasts or animal stromal cells. This has been realized and published recently in a paper by Sardi et al. [114] where a network of mesenchymal stem cells (MSC)-derived stromal cells was added and appeared to attract PBMCs and enhance the secretion of pro-inflammatory cytokines.

Another *in vitro* model designed to test immunogenicity of vaccines is the MIMIC® (Modular IMmune In vitro Construct) system well-based format assay [115]. The system is composed of four successive different steps, where the first one is to collect and conserve PBMCs from blood samples of healthy donors, and the second step is to mimic part of the innate immune system through the preparation of a Peripheral Tissue Equivalent (PTE). The PTE is composed of Human Umbilical Vein Endothelial Cells (HUVEC) seeded on top of a collagen matrix and upon which PBMC derived DCs are added. The media sampling and measurement of pro-inflammatory cytokine release (IL-1α, IL-1β, IL-6, IL-8, IL-10, TNFα) allow the quantification of the innate immune response. This PTE module was compared to classical PBMC assays and was shown to produce two-fold to 100-fold higher levels of cytokine secretion, thus increasing the assay sensitivity [116]. The third step consists of the simulation of the adaptive immune response via the elaboration of a Lymphoid Tissue Equivalent (LTE). This latter is similar to an ALN using DCs, follicular DCs, T and B cells but applied in a sequential order to mimic the *in vivo* series of events taking place in the lymphoid tissues. Finally, functional assays are performed

to assess whether the *in vitro* immune response corresponds to the one observed *in vivo*. This MIMIC® model was used to test the immune response to a tetanus vaccine *in vitro* through proliferation of tetanus-toxin specific antibody-secreting cells before and after vaccination. Profiles obtained were similar to the *in vivo* immune response measured in the same donors, providing evidence of the suitability of this model to predict vaccine immunogenicity [115].

These 3D tissue equivalents may be automated allowing for high-throughput testing of biopharmaceuticals. However, intrinsic inflammation or background noise could be a problem for immunogenicity testing [111], and ALN essentially mimic the human immune system without taking into account the specificity of the route of administration and the injection-recipient tissue characteristics (e.g., tissue composition after IM or SC injection).

3.2. (Full-thickness) skin models

Increase in the number of subcutaneously injected therapeutic proteins requires a closer examination of the composition, organization and functioning of the subcutaneous tissue. Human skin is composed of three layers: epidermis, dermis, and hypodermis. While the first two have a barrier function against the environment, the role of the subcutaneous tissue is to insulate, to provide energy and absorb physical shock. More than just composed of adipocytes, the subcutis contains also nerves, a network of lymphatic and blood capillaries, few fibroblasts secreting extracellular matrix (ECM) components, and sentinel immune cells (macrophages and DCs) [117, 118]. Moreover, proximity of the dermis may allow migration of dermal dendritic cells when pro-inflammatory cytokines (IL-8 and IL-6) are secreted by the subcutaneous adipose tissue after minimal trauma, such as SC injection [119]. Indeed, defense mechanisms of the skin against pathogen infections include an array of immune cells: LC and epidermal T cells in the epidermis, and different populations of myeloid and lymphoid immune cells, including three DC subsets, which either reside in or traffic through the dermis [118]. These cells are in constant interaction with the commensal flora, establishing a balance between pro-inflammatory and anti-inflammatory mechanisms resulting in a protective skin immune signature unique to each human [120].

Human SC tissue is organized in lobules containing adipocytes, which are separated by septa of loose connective tissue in which fibroblasts reside. Its structure is slightly different across species, notably among rodents, which possess a loose connective tissue organized in numerous layers, and a specific striated muscle, called *panniculus carnosus*, located close to the dermis [121]. These structural differences, which impact the spreading behavior of therapeutic protein solution after SC injection, could also influence their bioavailability, limiting the predictability of animal models. Conversely, porcine skin seems much closer to human skin in its constitution and, more importantly, with regard to immunological responses [122, 123].

This last decade, the need for economically viable and standardized full-thickness skin models for cosmetic testing also led to a better understanding of skin biology and skin cancer pathology [124]. It also supported the development of treatment strategies for chronic wounds or large burns [125]. These three-dimensional skin equivalent models could be of interest for immunogenicity prediction of therapeutic proteins. Comprehensive reviews on 3D models of epidermis, full-thickness models

consisting of combinations of epidermal and dermal equivalents, and more complex models with appendages have been published [126-130]. A non-exhaustive list of these models currently available for clinical or research use and their corresponding references is presented in Table 3. Strictly speaking, some of the models cited here belong to the 2D models as defined in this review. Such models include reconstituted human epidermis composed of keratinocytes seeded on a scaffold, like EpiSkin®, SkinEthic®, and EpiDerm™ as predictive models; or autologous keratinocytes in the form of cell sheets or in suspension as Epicel® and Myskin™ for clinical use. For the treatment of burns, various dermal substitutes are commercially available. Some of them consist of allogenic human (AlloDerm®) or xenogenic porcine or bovine acellular dermis (MatriDerm®); other xenogenic and synthetic substitutes use mainly silicone and atelocollagen (Pelnac™). Full thickness skin models, which combine epidermal and dermal equivalents, most generally use a collagen scaffold seeded with fibroblasts, supplemented after a week by seeding of keratinocytes on top. For instance, Apligraf® is commercialized for ulcer wound healing and Phenion® is an *in vitro* full thickness model for safety and efficacy assessment [131].

However, few studies have suggested a three-layer model, i.e. adding subcutaneous fat tissue to existing "full-thickness" skin models. Hypodermis was reconstructed with adipose-derived stem/stromal cells (ASCs) using an adapted self-assembly tissue engineering approach designed by the laboratory of tissue engineering and regenerative medicine (LOEX) at Laval University (Canada) [132], or by embedding these cells into a collagen scaffold [133]. Another technique was used by Bellas et al. [134] to create a three-layered engineered skin. A silk scaffold was seeded with ASCs from abdominoplasty and grown for 14 days before combination with dermal and epidermal construct. Since then, many studies for three-layered skin model construction were performed using mature adipocytes [135], ASCs from rats to decipher their impact on full-thickness skin grafts survival [136], or bone-marrow derived MSCs and ASCs on a human plasma-based hydrogel [137].

Immunocompetent skin equivalent models were also constructed by integration of primary cells from PBMCs [138], or immune cell lines described previously (MUTZ-3) in full-thickness dermo-epidermal models [101, 139, 140]. These models allow to screen potential skin sensitizers and to better understand cell signaling and migration of epidermal DC (Langerhans cells) through the dermis.

As described previously, skin tissue engineering, often called "skingineering", employs scaffolds of different origins (derived from animal tissues, algae, synthetic polymers) and harboring various structural and mechanical properties (pore size, viscoelasticity, biodegradability, biocompatibility). These scaffolds are used as mechanical support for cell growth and sometimes alone for the immediate protection of wounds. They may have an impact on immune cells, naturally present or added to the skin model. It was shown by Park et al. [141] that biomaterials can induce maturation of PBMC-derived DC, as well as pro-inflammatory cytokine secretion, which affect their endocytic ability. Once co-cultured with autologous T cells [142], these PBMC-derived DCs in contact with biomaterial films and antigen (ovalbumin) could lead to different immune responses polarized by the release of specific cytokines and the corresponding stimulated T helper type. Thus, careful selection of hydrogel scaffold for the creation of skin model is of importance in order to provide only a mechanical support without influencing the immune response.

Table 3. Non-exhaustive summary of the tissue engineered skin equivalents commercially available or described in literature. Cells are considered of human origin, unless otherwise mentioned. Pathological models

and complex models containing appendages (hair follicles) are not described. ASCs = adipose-derived stem/stromal cells. BM-MSCs = bone marrow-derived mesenchymal stem cells.

3.3. Subcutaneous tissue models

As mentioned earlier, human SC tissue is mainly composed of adipocytes separated by a connective tissue and few fibroblasts secreting ECM proteins. A newly published review by Kinnunen and Mrsny [19] extensively described the composition of the ECM, as well as its potential interactions with biopharmaceuticals and/or their excipients at the SC injection site. Specific conditions (temperature, pH, interstitial pressure) of this tissue induce important changes in the microenvironment of the injected therapeutic proteins, which are usually formulated in non-physiological buffers for optimal storage stability. The same research group later designed a set-up allowing to mimic SC tissue conditions, using hyaluronic acid and physiological buffer, while following the impact on the injected biopharmaceutical through changes in turbidity, pH and pressure [143]. As claimed in their recent US and European patents [144, 145], the developed method and apparatus for *in vitro* modeling of the SC tissue could be modified to offer sterile conditions and completed by the addition of one or several cell lines.

For the immunogenicity assessment of biotherapeutics it could be of interest (i) to model precisely the SC tissue environment, in particular its mechanical properties leading to distention and mechanical constrains during the injection, (ii) to include the immune cell component reacting to the accumulation of high concentration of therapeutic proteins [146]. In addition, tissue-remodeling signals induced by this depot effect and variations in interstitial pressure will be recognized by resident immune cells, which can then mature and activate a local immune reaction. This can be related to what happens when SC fat tissue is extensively remodeled in obese persons [147]. Increase of fat mass alters metabolic and endocrine functions of adipocytes, inducing secretion of adipokines [148]. Release of these cytokines leads to endothelial cell activation, enhancing diapedesis of blood monocytes [149]. SC tissue infiltration by immune cells (mainly macrophages but also CD8+ T cells [150]) correlated with a chronic low-grade systemic inflammation may also stimulate resident macrophages and DCs [148, 151, 152]. Therefore, immunogenicity of therapeutic proteins may be increased in the specific subpopulation of obese patients. Pathologies related to obesity, such as type 2 diabetes [153], may require daily SC injections of GLP-1 hormone (e.g., Exenatide and Liraglutide) or of recombinant leptin to promote weight loss [154]. This latter was shown to induce an unacceptable incidence of injection site reactions (ISRs), which was more pronounced in the obese population [155]. Obesity today is an increasing major public health concern, thus it is of interest to investigate more specifically the potential immunogenicity of therapeutic proteins used in this subset of patients.

As illustrated before with full thickness skin models, attempts to reconstruct SC tissue using various approaches and techniques were realized these last years and were reviewed elsewhere [156-158]. All of these studies were aiming to regenerate soft tissue in order to provide a cushioning layer for wound healing or to restore volume after resection of tumors, like mastectomy [159-161]. Common basis is the use of synthetic or natural biomaterial(s) as scaffolds, on top of, or into which are seeded

human ASC isolated from (autologous) liposuction. Some of them use decellularized adipose tissue from human or animal sources, to better mimic the composition of the SC fat tissue, transform them (lyophilization, foaming, micronization) and seed with human ASC [160, 162].

Recent advances in bioprinting enable the creation of more complex structures adapted to cell culture conditions (oxygen and nutrients supply) and supporting cell infiltration after *in vivo* implantation [161]. Various types of 3D bioprinting methods and hydrogel-based bioinks (i.e., alginate, hyaluronic acid, collagen, gelatin, fibrinogen, and tissue-derived extracellular matrix) have been developed in the last decade to print a broad range of (vascularized-) soft tissues, as reviewed in detail by Kim et al. [163], and Zhang et al. [164]. These models constitute strong bases and transferable knowledge for the creation of an immunocompetent SC tissue model.

3.4. Full skin biopsies

Another model offering some possibilities to study immunogenicity of therapeutic proteins is full animal or human skin biopsy. Limitations of these explants are mainly (i) maintenance in culture conditions for a sufficient amount of time to enable drug testing and potentially repetitive administration, and (ii) a sufficient supply from different donors to mimic polymorphism of the human population, as for PBMC-derived cells.

Skin explants like Skimune® Pharm/Mab (Alcyomics Ltd), have been used to predict the allergenic potential and immunogenicity of new protein therapeutics or in therapeutic vaccine development (i.e., addition of adjuvants) by performing intradermal injections of drug candidates [165-168].

Another *ex vivo* human skin model, Hyposkin® is currently under development [169]. The three-layer skin biopsy, which contains fat SC tissue in contrast to the models described previously, is maintained in culture and placed on a specific matrix insert. It enables the simulation of SC injections to study compound absorption, catabolism and toxicity of new formulations in the SC tissue.

4. CONCLUSIONS & PERSPECTIVES

In the field of immunogenicity prediction of candidate therapeutic proteins, we can distinguish three kinds of approaches: *in silico*, *in vitro* and *in vivo*. This review focused on the *in vitro* prediction tools currently available in standard 2D cell culture conditions or using novel 3D models. Limitations in terms of primary cell supply and representative polymorphism while using immune cell lines were discussed. In the same way, various scaffolds available commercially for clinical use or to develop research models of subcutaneous compartment or full-thickness skin were presented. Matrices are of interest in order to mimic the structure and mechanical properties of the SC tissue keeping a simple, inert and well-defined environment to seed immune cells. On the other hand, self-assembly systems composed of multiple cell types or biopsies allow the reproduction of physiological complexity and variety of cellular interactions. Three-dimensional *in vitro* models are more complex than 2D assays, aiming to improve predictability. The final objective is to develop a model sufficiently

reliable to be considered by regulatory authorities as an indispensable and valuable step in the immunogenicity assessment of therapeutic proteins.

Finally, lab scale *in vitro* 3D models of various organs are now established and the complexity of the immune system could be even better represented when being integrated in a systemic model, as the "human-on-a-chip" concept elaborated this last decade [170, 171].

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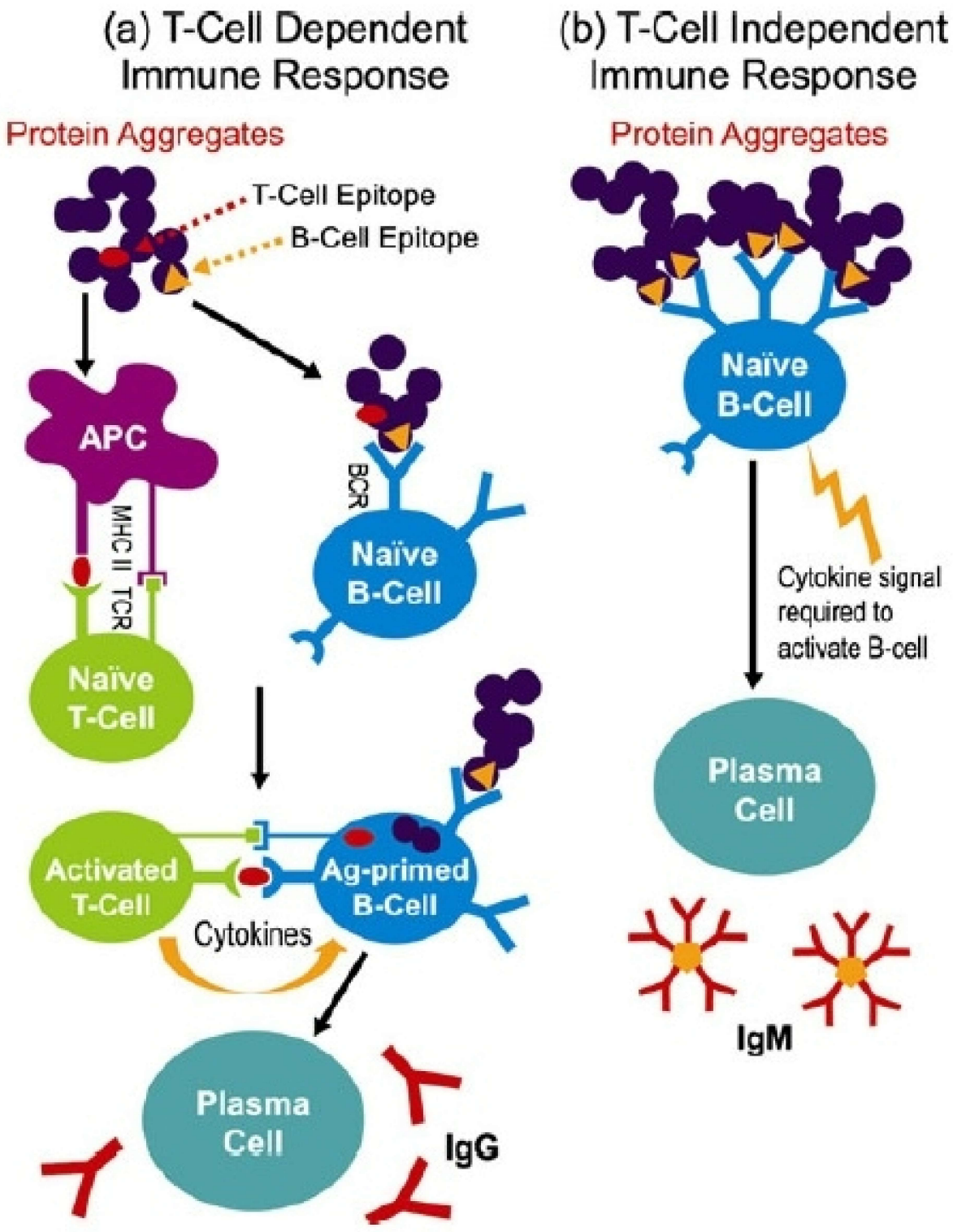
(a) T-Cell Dependent Immune Response
Protein Aggregates
T-Cell Epitope
B-Cell Epitope
APC
MHC II
TCR
Naïve T-Cell
BCR
Naïve B-Cell
Activated T-Cell
Ag-primed B-Cell
Cytokines
Plasma Cell
IgG
(b) T-Cell Independent Immune Response
Protein Aggregates
Naïve B-Cell
Cytokine signal required to activate B-cell
Plasma Cell
IgM

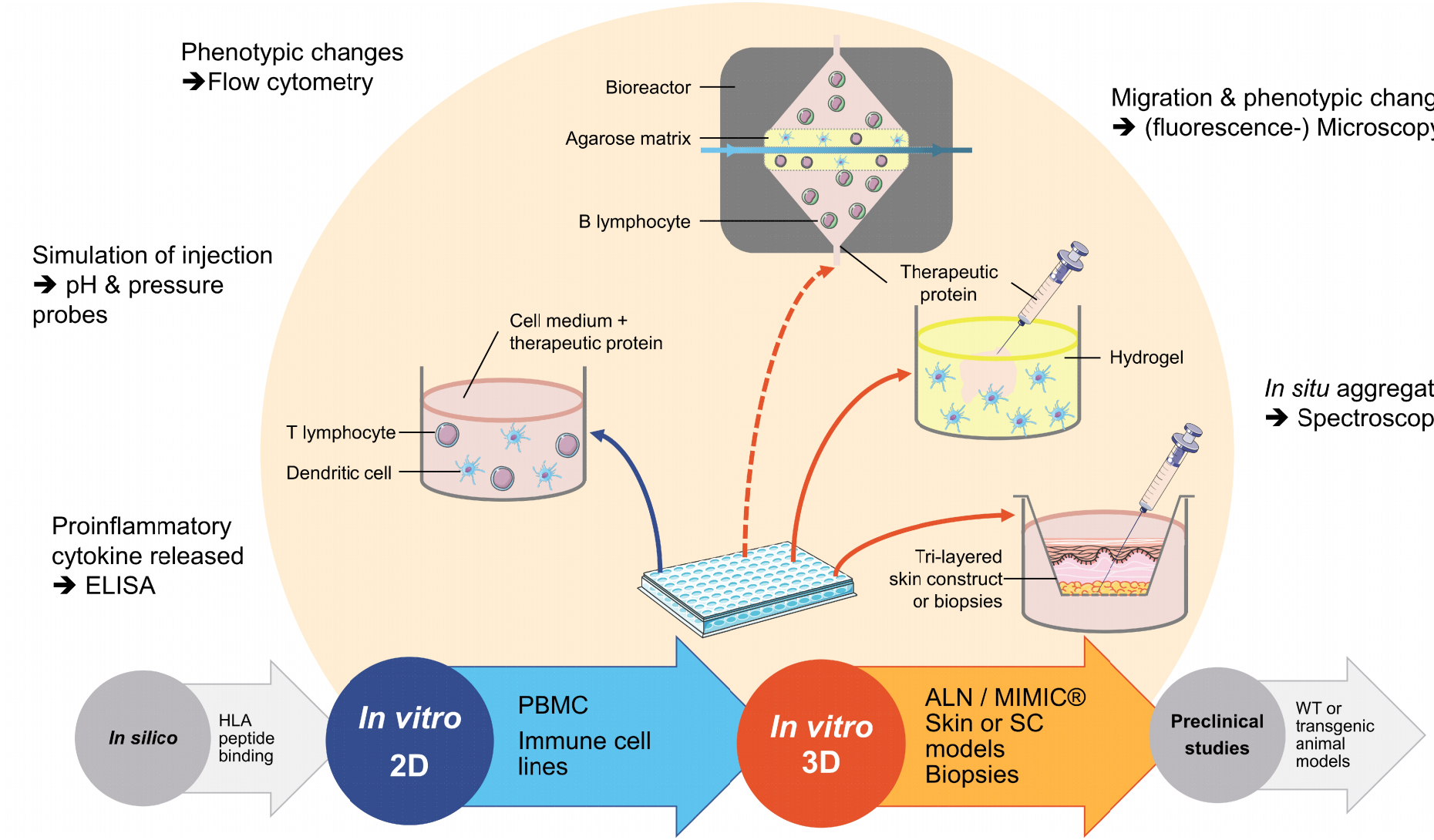
Phenotypic changes
➔Flow cytometry
Bioreactor
Agarose matrix
B lymphocyte
Migration & phenotypic changes
➔ (fluorescence-) Microscopy
Simulation of injection
➔ pH & pressure probes
Therapeutic protein
Cell medium + therapeutic protein
Hydrogel
T lymphocyte
Dendritic cell
In situ aggregation
➔ Spectroscopy
Proinflammatory cytokine released
➔ ELISA
Tri-layered skin construct or biopsies
In silico
HLA peptide binding
In vitro 2D
PBMC
Immune cell lines
In vitro 3D
ALN / MIMIC®
Skin or SC models
Biopsies
Preclinical studies
WT or transgenic animal models

Table 1. Correlation of algorithm-predicted immunogenicity to clinical immunogenicity rates. EpiMatrix-generated scores associated with each FPX protein and their respective rates of antibody incidence (binding and neutralizing) are shown. An assessment of Tregitope content in each molecule was also performed and scores were adjusted accordingly. FPX 1, for example, had a high rate of clinical immunogenicity and was associated with elevated T-cell epitope content and low Tregitope content, as reflected by its high Z score. FPX 2, 3, and 4 were associated with a low EpiMatrix score, and Tregitope adjustment further reduced the predicted potential for binding. Predictably, FPX 2, 3, and 4 exhibited only minor clinical immunogenicity. Reproduced with permission from Jawa et al., 2013 [34].

Protein Therapeutics	**FPX 1**	**FPX 2**	**FPX 3**	**FPX 4**
EpiMatrix Score	21.97	1.76	-0.76	1.63
Tregitope — adjusted EpiMatrix score	21.97	1.62	-1.76	-111.25
Binding Antibodies	37%	7.80%	5.60%	4.50%
Neutralizing Antibodies	40%	0.50%	Not analyzed	0%

Table 2. Commonly used screening assays for ADA and NAbs detection. Reprints from Wadhwa et al. [44].

Type of Assay	Advantages	Disadvantages
Direct/Indirect ELISA	Easy to use and automate High through-put High therapeutic tolerance Inexpensive Generic reagents and instrument	May bind non-specifically High background May fail to detect low-affinity antibodies Requires species specific secondary reagent
Bridging ELISA	Easy to use and automate High through-put Low background, High therapeutic tolerance in solution phase High specificity (dual-arm binding) Generic reagents and instrument	Antigen labelling required May fail to detect low-affinity antibodies Highly susceptible to interference by therapeutic, serum components e.g., anti-human Ig molecules, multivalent targets May not detect IgG4
Electrochemiluminescence	High through-put, large dynamic range Minimally affected by matrix High tolerance to therapeutic in solution phase Detection signal consistent during life of TAG conjugate	May require two antigen conjugates Antigen labelling required Susceptible to interference by therapeutic, serum components e.g., anti-human Ig molecules, multivalent targets May not detect IgG4 Vendor-specific equipment & reagents
Radioimmunoprecipitation assay	Moderate through-put High sensitivity Can be specific Inexpensive	Can be isotype specific May not detect low-affinity antibodies Requires radiolabelled antigen Decay of radio-label may affect antigen stability
Surface plasmon resonance	Automated Determines specificity, isotype, relative binding affinity Enables detection of both 'low-affinity' and high affinity antibodies Detection reagent not required	Antigen immobilization may alter therapeutic Regeneration step may degrade antigen Sensitivity often less than binding assay Expensive vendor-specific equipment & reagents

Table 3. Non-exhaustive summary of the tissue engineered skin equivalents commercially available or described in the literature. Cells are considered of human origin, unless otherwise mentioned. Pathological models and complex models containing appendages (hair follicles) are not described. ASCs = adipose-derived stem/stromal cells. BM-MSCs = bone marrow-derived mesenchymal stem cells.

Layer(s) reconstructed	Scaffold component(s) or presentation	Usage	Cell types	Name/Supplier or Lab	Reference
Epidermal constructs	Cell culture insert	Research use	Keratinocytes	epiCS® /CellSystems, Troisdorf, DE	[109, 126]
	Cell culture insert	Research use	Keratinocytes	EpiDerm™/MatTek Co., Ashland, MA, USA	[109, 127]
	Collagen matrix	Research use	Keratinocytes	EpiSkin® /Episkin, Lyon, FR	[109, 128]
	Cell culture insert	Research use	Keratinocytes	LabCyte EPI-MODEL/Japan Tissue Engineering Co., Gamagori City, Aichi, JP	[129]
	Polycarbonate filter	Research use	Keratinocytes	SkinEthic® RHE/Episkin, Lyon, FR	[109, 128]
	Cell sheet of 2 to 8 layers, 50 cm^2	Clinical use	Autologous keratinocytes, proliferation arrested murine fibroblasts	Epicel® /Vericel Corporation, Cambridge, MA, USA	[110, 113, 130]
	Spray	Clinical use	Autologous keratinocytes	Myskin® /Regenerys Ltd, Sheffield, UK	[131]
	Spray	Clinical use	Autologous cells suspension, wound healing factors	Regenerative Epithelial Suspension™ (RES™)/Avita Medical Europe Ltd, Wimbledon, London, UK	[132]
	Spray	Clinical use	Autologous cells suspension	SkinGun™ and CellMist™ / RenovaCare Inc, New York, NY, USA	[133]
Dermal constructs	Acellular dermal matrix	Clinical use	-	AlloDerm® /BioHorizons, Birmingham, USA	[110, 112, 134]
	Bioabsorbable polyglactin mesh scaffold	Clinical use	Allogeneic neonatal fibroblasts	Dermagraft® /Organogenesis, Inc., Canton, MA, USA	[110, 135]
	Porcine xenograft (aldehyde cross-linked reconstituted dermal	Clinical use	-	E-Z Derm® /Mölnlycke Health Care Ltd, Oldham Lancashire,	[110, 136, 137]

	collagen)			UK	
	Human dermal matrix (pre-meshed)	Clinical use	Decellularized	GRAFTJACKET™/Wright Medical Technology, Inc., Arlington, TN, USA	[110, 138]
	Cross-linked bovine tendon collagen and glycosaminoglycan, polysiloxane (silicone) layer (Meshed and Non-Meshed)	Clinical use	-	Integra® Template/Integra LifeSciences Co., Plainsboro Township, NJ, USA	[110, 139]
	Bovine collagen-elastin matrix	Clinical use	Decellularized	MatriDerm®/MedSkin Solutions, Billerbeck, DE	[112, 140]
	Porcine small intestinal submucosa (SIS) extracellular matrix	Clinical use	Decellularized	OASIS® Wound Matrix and OASIS® Ultra/Cook Biotech, Inc., West Lafayette, IN, USA	[110, 141]
	Silicone layer and atelocollagen matrix from porcine tendon	Clinical use	-	Pelnac™/Eurosurgical Ltd, Guildford, Surrey, UK	[142]
	Porcine dermal collagen implant	Clinical use	Decellularized	Permacol™ Surgical Implant/Medtronic, Minneapolis, MN, USA	[110, 143]
	Human dermal matrix	Clinical use	Decellularized	SureDerm®/HansBiomed Co., Seoul, KR	[110, 144]
Epidermal and dermal constructs	Transport-agar	Research use	Not mentioned	Phenion®/Henkel AG & Co., Düsseldorf, DE	[114, 145]
	Inert polycarbonate filter	Research use	Keratinocytes, fibroblasts	T-Skin™/Episkin, Lyon, FR	[128]
	Type I collagen matrix (bovine)	Clinical use	Allogeneic neonatal keratinocytes and fibroblasts	Apligraf®/Organogenesis, Inc., Canton, MA, USA	[112, 146]
	Type I collagen matrix	Clinical use	Autologous fibroblasts and keratinocytes	denovoSkin™/Cutiss AG, Zurich, CH	[113, 147]
	Collagen-glycosaminoglycan substrate	Clinical use	Autologous fibroblasts and keratinocytes	NovaDerm™/Regenicin Inc., Little Falls, NJ, USA	[113, 148]
Subcutaneous Fat Tissue	No scaffold, self-assembly	Research use	ASCs	Laboratoire d'organogénèse expérimentale (LOEX), Hôpital du St Sacrement, Québec, CA	[115, 149]
	Porcine or bovine acellular dermal	Research use	ASCs	University of Wuerzburg,	[150]

	collagen scaffold			Wuerzburg, DE	
	Human decellularized adipose tissue microporous foams	Research use	ASCs	Queen's University, Kingston, ON, CA	[151]
	Porcine acellular dermal matrix, small intestinal submucosa and gelatin microspheres	Research use	Mouse or rat ASCs, *in vivo* assays on mice	The Fourth Military Medical University, Xi'an, PRC	[152]
	Collagen scaffold (Renoskin)	Research use	Porcine ASCs, *in vivo* assays on pigs	University of Texas, USA and University of Lyon, FR	[116]
Tri-layered skin	Bovine type I collagen or self-assembly	Research use	Keratinocytes, fibroblasts and ASCs	LOEX and Laval University, Québec, CA	[153]
	Type I collagen matrix	Research use	Keratinocytes, fibroblasts and ASCs	Saga Medical School, Saga-City, JP	[154]
	Human plasma, fibrinogen, antifibrinolytic tranexamic acid, $CaCl_2$	Research use	Keratinocytes, fibroblasts and BM-MSCs or ASCs	Fundación Inbiomed and Hospital Donostia, San Sebastián, ES and CIBERNED, Valencia, ES	[120]
	Silk + type I collagen scaffolds	Research use	Keratinocytes, fibroblasts, ASCs and endothelial cells	Tufts University, Medford, MA, USA; and Johnson & Johnson Consumer Companies Inc, Skillman, NJ, USA	[117]
	Type I collagen matrix	Research use	Keratinocytes, fibroblasts and mature adipocytes	IGB and University of Stuttgart, Stuttgart, DE; and School of applied chemistry, Reutlingen, DE	[118]