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Human immunity in vitro — Solving immunogenicity and more ☆

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ABSTRACT

s been widely recognised that the phylogenetic distance between laboratory animals and humans limits the former's predictive value for immunogenicity testing of biopharmaceuticals and nanostructure-based drug delivery and adjuvant systems. 2D in vitro assays have been established in conventional culture plates with little success so far. Here, we detail the status of various 3D approaches to emulate innate immunity in non-lymphoid organs and adaptive immune response in human professional lymphoid immune organs in vitro. We stress the tight relationship between the necessarily changing architecture of professional lymphoid organs at rest and when activated by pathogens, and match it with the immunity identified in vitro. Recommendations for further improvements of lymphoid tissue architecture relevant to the development of a sustainable adaptive immune response in vitro are summarized. In the end, we sketch a forecast of translational innovations in the field to model systemic innate and adaptive immunity in vitro.

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Abbreviations: ADDC, antibody-dependent cell-mediated cytotoxicity; ALN, artificial lymph node; AOP, adverse outcome pathway; APC, antigen-presenting cell; CMV, cytomegalovirus; DC, dendritic cell; DNCB, dinitrochlorobenzene; ECM, extracellular matrix; ELISPOT, enzyme-linked immune spot; FDC, follicular dendritic cell; FRC, fibroblastic reticular cell; GALT, gut-associated lymphoid tissues; GC, germinal centre; HEV, high endothelial venules; HLA, human leucocyte antigen; HuALN, human artificial lymph node; HUVEC, human umbilical vein endothelial cell; Ig, immunoglobulin; IFN, interferon; LN, lymph node; MEMS, micro-electro mechanical system; MHC, major histocompatibility complex; MoA, mode of action; MOC, multi-organ-chip; MRC, marginal reticular cell; NK, natural killer; PBMC, peripheral blood mononuclear cell; PLGA, poly(lactic-co-glycolic acid); QIVIVE, quantitative in vitro – in vivo extrapolation.

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1. Introduction

The recent rapid introduction of biopharmaceuticals, nanostructure-based drug delivery and adjuvant systems [1–4], stem, somatic and immune cell therapies [5–7], and modern wound-healing materials [8] into the pharmaceutical landscape has raised the immunogenicity issue to a historical high [9–14]. Those, in comparison to conventional pharmaceutical agents, contain biological and synthetic entities of a large size and structure open to recognition by the human immune system. AOPs[1] of undesired immunity observed to date were complement activation [15–19], tissue inflammation, leucocyte hypersensitivity [20], and formation of neutralising antibodies associated with their respective clinical disorders [21–24]. It has been widely recognised that the phylogenetic distance between laboratory animals and humans limit the former's predictive value for substance testing [25]. This applies particularly to the evaluation of human immunogenicity due to pronounced species-specific differences in antigen recognition, in immune reactivity of non-lymphoid and lymphoid tissues, and in the systemic orchestration of immunity at organismal level. As a second option to evaluate immunogenicity prior to tests in humans, a broad spectrum of 2D in vitro assays has been established in conventional culture plates based on suspension or matrix-assisted human immune cell cultures to mimic various discrete aspects of human immunity in the past, however, with little success so far.

A prime example of the failure of both laboratory animal tests and human 2D immune cell assays to predict immunological risks of a biopharmaceutical product prior to use in six healthy volunteers was the TGN1214 disaster in 2006. The TGN1214 super-agonist antibody was developed to direct the immune system to treat chronic inflammatory diseases and cancer. It triggered a systemic inflammation syndrome called cytokine storm and multiple organ failure in the six volunteers in phase I clinical testing [26–28]. Non-human primates used in preclinical assessment were not able to predict the human outcome. This demonstrates the shortcomings of experimental animal models, e.g. rodents or primates, independent of their phylogenetic distance to humans. The same case also serves as the most instructive example for the relationship between the localization and degree of a single specific initiating immunological event and its devastating recipient-specific consequences. The MoA[2] on individual organs and adverse systemic outcome among the volunteers differed significantly. This highlights the urgent need for preclinical human immunity test solutions which emulate organ-specific tissue architecture at immune response sites and their crosstalk more closely. Intriguing recent trends to engineer human non-lymphoid organ functionality at a more organotypic 3D level in vitro have already significantly improved the relevance of human liver, skin and respiratory tract models for toxicology read-outs in the past decade. However, components of innate human immunity have rarely been included in such models [29–31]. Tissue engineering of professional lymphoid organs to emulate adaptive human immunity in vitro has been widely ignored so far. The crucial impact of new organotypic 3D test models for human immunity might far exceed adverse immunogenicity responses. It might also strongly increase the contribution to the development of effective designer vaccines, their corresponding delivery systems and novel vaccine adjuvants. These developments are significantly hampered by the restricted knowledge about the most effective human immune response mechanism against a particular pathogen [32] and efficient administration routes, e.g. mucosal [33] or nasal vaccination [34], due to the limited set of preclinical potency assays for vaccines [35–37].

This review provides an up-to-date sketch on the accentuation of the immunogenicity testing dilemma, providing insights into the latest understanding of the relationship between tissue architecture, local microenvironment and essential immune response outcomes in innate and adaptive human immunity. It also touches on the existing 2D and matrix-assisted immune cell assays regarding their equivalence to this complex biology. It details the status of various 3D approaches to emulate innate immunity in non-lymphoid organs exposed to external pathogens, e.g. gut, lung and skin, and immune response in human professional lymphoid immune organs, e.g. LNs and bone-marrow in vitro. We stress the tight relationship between the necessarily changing architecture of professional lymphoid organs at rest and when activated by pathogens. Finally, we sketch a forecast of translational innovations in the field addressing the following three important questions: To what degree can future human in vitro systems emulate the entire human immune response from the initial administration of a potential antigen down to a mature antigen-specific Ig[3]G antibody response? Are these systems practical, and do they solve the immunogenicity testing dilemma?

2. The human immune system — informed crosstalk determines efficiency

The human immune system is designed to protect our body from foreign pathogens, such as bacteria, viruses, parasites, and harmful macromolecules and to eliminate abnormal internally created structures, such as cancer- or virus-infected cells. Therefore, a plethora of immune cells have been selected by evolution to fulfil various specific functions (Table 1).

Neutrophils, NK[4] lymphocytes, eosinophils and monocytes of the blood and tissue-specific mast cells, DCs, macrophages, lymphoid tissue inducer cells, type 2 innate lymphoid cells, γ/δT lymphocytes, lymphoid stromal cells, and antigen-presenting endothelial cells represent the backbone of human innate immunity. α/βT lymphocytes (helper, cytotoxic, regulator), B lymphocytes, and plasma cells enable adaptive immunity leading to antigen-specific cytotoxic or antibody response or target-specific tolerance.

2.1. Innate immunity

Proteins of the complement cascade in blood and the various innate immune cell populations in blood and organs constitute a first line

[1] adverse outcome pathways
[2] mode of action
[3] immunoglobulin
[4] natural killer

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Table 1
Cells of human immunity.

Abbreviation/name	Function	Location	References
Innate immune cells			
Eosinophils	Release of inflammatory substances upon activation	Blood	[38]
Mast cells	Release of inflammatory substances upon activation	Resident in peripheral tissues	[39]
Neutrophils	Bacteria and fungi phagocytosis and killing	All tissues upon inflammation	[40]
DCs	Phagocytosis, antigen presentation and migration	Resident in lymphoid and non-lymphoid organs or migratory (recruited from blood monocytes during tissue inflammation)	[41–47]
Macrophages	Phagocytosis and antigen presentation	Resident in most tissues	[41]
NK lymphocytes	Elimination of tumour- and virus-infected cells	All tissues upon activation	[48,49]
Lymphoid tissue inducer cells	Generating organised lymphoid tissues	Gut other tissues	[50–52]
Type 2 innate lymphoid cells			[53,54]
γ/δ T cells	Pathogen elimination in epithelial barriers (viruses, bacteria)	Resident in mucosa and skin epithelial barriers	[55–57]
Adaptive immune cells			
α/β T lymphocytes	T helper cytotoxic and regulatory functions within adaptive immunity forming antigen-specific T cells or instruction B lymphocytes to develop antigen-specific antibody responses, T memory	Professional lymphoid and peripheral organs	[58]
B lymphocytes	Antibody maturation and class switch memory	Professional lymphoid and peripheral organs	[58]
Plasma cells	Antibody secretion	Professional lymphoid organs	[58]
Immunity supporting cells			
Lymphoid stromal cells (FRCs, MRCs, FDCs)	Architectural scaffolding of professional lymphoid organs, nest formation for recirculation lymphocytes, filtrating antigens and APCs, activation and deletion of cytotoxic T lymphocytes for peripheral tissue-restricted antigens	LNs and other secondary lymphoid organs	[59–68]
Lymphatic endothelial cells	Promote T cell egress from LNs	Afferent and efferent lymphatics	[60]
Blood endothelial cells	Promote T cell recruitment	HEVs	[60]

defence and are responsible for the immediate recognition and elimination of pathogens or abnormal structures. Immune cells of the innate immune system have evolved in a highly specialized way to cover the broad variety of pathogens they have to fight. Eosinophils in blood and mast cells, their resident counterparts in tissues, together with other resident somatic tissue cells provide first signalling about invaders to the other immune cells by immediate histamine and chemokine release. The resulting local inflammation attracts neutrophils, specialized in killing bacteria, fungi and other pathogens, and NK cells, specialized in the elimination of tumour- and virus-infected cells, from blood into the invasion zone. Neutrophils are the largest population of innate immune cells with a daily turnover of 1.5×10^{11} cells and a lifespan of five days [69]. They carry the main first line defence burden at the interface of the human body with the outside world, employing three strategies to eliminate invaders: pathogen uptake, secretion of antimicrobials, and release of neutrophil extracellular traps [70]. In addition, inflammation activates the resident pools of cells of the innate immunity: macrophages and DCs phagocytizing any pathogens left after neutrophil, NK and γ/δT cell attack, and presenting antigenic epitopes in MHC[5] II molecules of their cell surface for further recognition by the adaptive immunity. This orchestrated crosstalk among and between cells of the innate immunity needs a pre-established tissue-specific dynamic 3D network architecture of resident macrophages and DCs in crosstalk with other resident immune cells in the tissues. This ensures the right cell density of sentinel cell distribution and in-depth structure to cope with any invasion. Mechanisms to maintain these networks are still poorly understood but intimate stromal cell contact and crosstalk with the pericyte/endothelial cell network seemed to be mandatory and, therefore, should play an important role in respective models emulating innate immunity in vitro. Innate immunity is most prominently presented in barrier organs between the body and the outside world, such as gut, lung and skin, because these organs present the first invasion site in case of injury. These organs additionally manage to provide a continuous immune privilege to microorganisms leading to a lifelong coexistence of epithelial barrier tissues with their respective physiologically important microbiomes, [71] and [72] for gut; [73] for lung; [74] for skin. Differences in the pathogen-exposed surface (~450 m^2 of gut, ~150 m^2 of lung and ~1.8 m^2 of skin), in the nature and load of the respective microbiome (the largest represented in the gut, followed by the skin microbiome and a relatively small microbiome in the lung), in the physiological epithelial barrier turnover (1–3 days for epithelial barrier of the small intestine, 8 days for lung epithelium and 30 days for skin epidermis), in exposure to mechanical stress and temperature, in wounding frequencies (highest for skin), and in the microenvironment have evolutionarily led to an organ-specific composition and architectural arrangement of the innate immune response networks in these organs. If the first line of defence fails to eliminate invaders, innate immunity of lung and skin crosstalk with the associated LNs induces a proper antigen-specific adaptive immune response, which is then carried out by LNs or the spleen in cellular crosstalk with the bone-marrow.

The gut, as well as LNs, has resident lymphoid tissues to initiate an adaptive immune response at an invasion site. Mucosal epithelia cover an area of the human body more than 200 times that of the skin, with the gut mucosa being by far the largest part lifelong, hosting a microbiome the size of 10^{12} bacteria per g (dry weight) of colonic contents [75]. The epithelial cell turnover in the small intestine, the most exposed to external physiological and pathological microorganisms, is 1–2 days (large intestine – 10 days and jejunum – 16 years). Immune cell composition, architecture, and functional crosstalk between the innate and adaptive mucosa-associated immunity have been reviewed recently [76,77]. In brief, mucosal tissue contains special immunocompetent inductive sites where antigens, passing specialized epithelial M-cells, directly enter into mucosa-associated lymphoid tissues, which resemble the fine-tuned 3D architecture of essential LN structures described later in detail, with variable T-cell zones intervening between the B cell follicles containing a variety of APCs, such as DCs[6] and macrophages. This immunity in the gut is called GALT[7] and comprises Peyer's patches and isolated lymphoid follicles for the efficient development of an adaptive immune response primarily resulting in the local secretion of antigen-specific secretory antibodies at effector sites of the gut mucosa. Mucosal immunity plays a crucial role for newborns [78].

[5] major histocompatibility complex

[6] dendritic cells

[7] gut-associated lymphoid tissues

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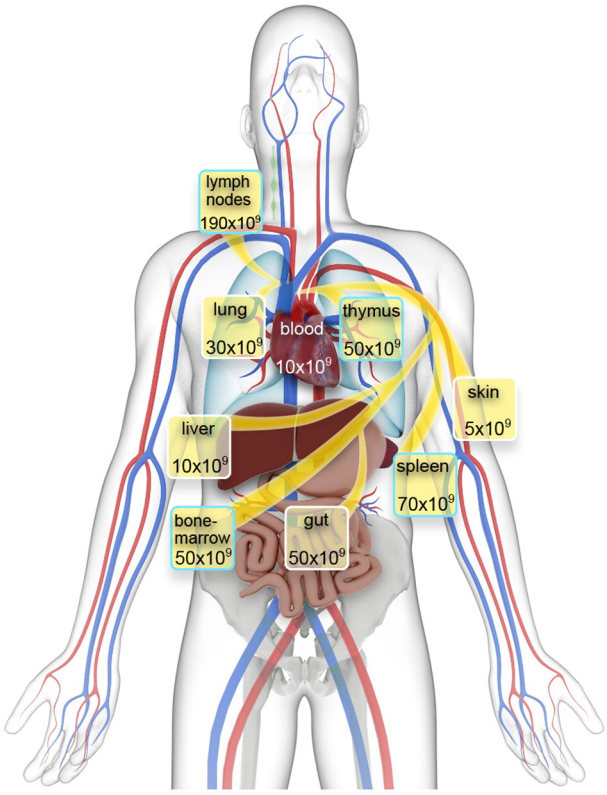

Fig. 1. Lymphocyte distribution and movement in a healthy young adult human at immunological rest A total of approximately 0.5 trillion lymphocytes of the adaptive immunity continuously guard all human tissues supporting a lifelong immunological acceptance of the physiological microbiome of the outside-world contact organs, such as skin, digestive and respiratory tract, highly specifically defending from invading pathogens, whilst simultaneously ensuring the elimination of altered self-structures, such as infected and tumor cells, in a healthy young adult. Approximate distribution of lymphocytes in different body tissues is depicted in the boxes. Adaptive antigen-specific immunity develops and sustains primarily in lymph nodes, spleen (encircled in blue) and mucosa-associated lymphoid tissues in the gut. The 10×10^9 lymphocytes circulated each minute within a volume of 5 L represent only 2% of the total lymphocytes and serve as a surprisingly consistent lymphocyte distribution pool for antigen-exposed organs. Bone-marrow and thymus generate naïve T and B lymphocytes, while liver spleen and thymus are lymphocyte removal sites. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

The human lung consists of airways for air transport and alveoli for gas exchange. Ciliated epithelium in the airways constantly conveys pathogens out of the airways [79–81]. The innate immunity here is composed of a resident 3D transepithelial network of alveolar macrophages – professional phagocytes in the lung – in the epithelial layer and DCs – professional APCs[8] – inside and underneath the epithelium with a steady-state density of 500–750 cells/mm^2 [82,83]. The macrophages share their progenitors with DC cells and possess the ability to switch from one functional phenotype to another depending on environmental signals. Neutrophils transiently transmigrate into lung tissue at activation by microbial or fungal infection and eliminate the pathogens by phagocytosis and associated respiratory burst mechanisms of direct killing of pathogens too large in size for phagocytosis. Details of cellular immune reactions in the lung and involvement of immune cells in lung repair mechanisms have been reviewed recently [79,81,84].

Skin consists of epidermis, dermis, and adipose tissue layers [85]. In the epidermis, a 3D network of macrophages and DCs together with a scarce but even distribution of γ/δT cells is present and in continuous contact with the skin microbiome. Skin microbiome protrudes into skin appendages, such as hair follicles, sebaceous, and sweat glands, but, in a healthy body, never crosses the basal lamina between the last keratinocyte layer and the dermis. The adaptive immunity in skin is presented by T lymphocytes surrounding small diameter venous blood vessels; but the major crosstalk area between antigen presentation of dendritic skin cells and the adaptive immunity are the LNs draining human skin. Therefore, efficient movement of DCs through lymphatics into LNs has been evolutionarily established. B lymphocytes are not present in human skin. The skin is exposed to frequent micro- and macro-injuries throughout life. Therefore, innate immunity in skin supports co-ordinated repair and regeneration [86] and, consequently, requires crosstalk with the local stem cell niches of the epidermis, hair follicles, and sweat glands.

It is important to acknowledge significant differences between innate immunity in experimental animals and humans. Specifically, the rodent gut has distinct pH, distinct mucosal barriers, and many more M-cells and Peyers' patches than those in humans, the skin differs significantly in dendritic and Langerhans cell densities and function and the neutrophils of rodents express very different defencing levels. These are due to the different ecological niches, the different pathogenic challenges, size, and lifespan of rodents in comparison to humans and is often not discussed when interpreting substance testing results.

2.2. Adaptive Immunity

A deep understanding of the informed crosstalk between the innate and adaptive immunity on the systemic organismal level and the local architectural level of each affected organ is necessary to prevent unwanted immunogenicity of products and create effective vaccines. T and B lymphocytes are the backbone of adaptive human immunity. It sustains as both, memory B and T cells and as a plasma cell-based antibody responses. A total of 0.5 trillion lymphocytes (nearly equalling the total liver cell mass) are orchestrated by a fine-tuned crosstalk among lymphoid organs and between non-lymphoid organs with a daily distribution capacity of 370×10^9 lymphocytes through the blood stream and a daily collection capacity of 27×10^9 lymphocytes from tissues through LNs back into the blood. They provide a huge unmatched interface for continuous informed crosstalk with and instruction by the innate immune system on local tissue-specific architecture levels. Fig. 1 schematically represents the absolute tissue distribution and movement of lymphocytes to guard the inner parts of all human tissues behind the basal lamina of the epithelium barrier to the outside world.

The majority of lymphocytes are concentrated in the professional lymphoid immune organs (encircled in blue) constantly checking APCs for fits with antigens. Among them, the 190×10^9 resident lymphocytes of the 500–600 LNs of our body, with clusters found in the armpits, groin, neck, chest, and abdomen, represent the largest architecturally organised 3D interface between innate and adaptive immunity. Resident pools of lymphocytes appear transiently during tissue inflammation primarily in organs with direct contact to the outside world and the liver, the blood gatekeeper. Under such conditions, HEV will develop which then support the entrance of lymphocytes into inflamed tissues. There is a continuous transmigration of lymphocytes from the blood into these aforementioned organ-based reservoirs, guided by a tissue-specific special stromal "address code" in the organs [87,88]. Homeostatic chemokines are involved in the recruitment of naïve T and B cells at rest, leaving the blood vessel system through the so-called HEV[9] — special sites in professional lymphoid organs. In the case of antigen activation, inflammatory chemokines and other mechanisms co-ordinate lymphocyte migration into antigen invasion and presentation areas [89–91]. Lymphocytes with no match to any antigen presented in the LNs or other lymphoid and non-lymphoid antigen-presenting tissues distributed throughout the body return to the blood primarily through lymphatic vessels. Their exit from the lymphoid and peripheral

[8] antigen-presenting cells

[9] high endothelial venules

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tissues to the afferent lymph is not (only) an unselective and passive process [92].

2.3. Interface for innate and adaptive immunity crosstalk

If the innate immunity fails to fully eliminate dangerous targets, structures relevant to further recognition by the adaptive immunity are then presented by APCs (primarily macrophages and DCs, with a growing body of evidence for cases of endothelial and stromal cell antigen presentation) to the T lymphocytes. The latter are capable of developing an antigen-specific long-lasting cellular or antibody-based immune response associated with a memory for immediate antigen recognition and elimination on repeated appearance lifelong. An impressive scan efficiency of 500 lymphocytes per DC per hour in the absence of antigen has been reported in imaging studies in intact mice LNs with hours of individual DC–T lymphocyte interactions and a simultaneous interaction capacity of 10 T cells per DC [93]. This adaptive immunity develops and sustains as a plasma cell-based antigen-specific antibody response or a cytotoxic T-lymphocyte response primarily in the LN,[10] spleen, tonsils, and mucosa-associated lymphoid tissues of the gut. Bone-marrow and thymus provide the background for a continuous maturation of naïve T and B lymphocytes released into the blood to home to the aforementioned immune organs to run through the full-scale adaptive immune response. Remarkably, mature antigen-specific memory lymphocytes and antibody-secreting plasma cells to a certain degree home back into bone-marrow niches. Peripheral naïve and memory T cell pools are regulated by complex homeostatic mechanisms [94]. The adaptive immune response toward molecular structures recognised as foreign antigens described is the reason for immunogenicity of molecular structures of biopharmaceuticals, nanomaterial-based drug delivery and adjuvant systems, cell therapies, and modern wound-healing materials when those exhibit antigen properties. Adaptive immunity is also the inbuilt trigger for successful vaccination of humans.

3. Architecture of professional lymphoid organs — form follows function

Efficient development of an adaptive immune response resulting in the long-lasting formation of antigen-specific antibodies or generation of target-specific cytotoxic T lymphocytes is a time-consuming staged process, well-known to the general public from the developments of colds. Infection, swelling LNs, a week-long fever or feeling under the weather, and, finally, full recovery after two weeks. This corresponds to the first low affinity IgM antibody formation against antigens of the pathogen and a second stage formation of high affinity IgG antibodies and memory lymphocytes supporting fast elimination at repeated contact. LN architecture and transformation capacity in response to activation by antigen are responsible for the reaction described.

3.1. Lymph node

The LN evolved in evolution to optimise the likelihood that rare lymphocytes with unique antigen specificity encounter their cognate antigen in the appropriate immunogenic or tolerogenic context. Search strategies that maximise the exposure of each individual lymphocyte to the largest possible number of APCs are critical to the success of this system. In vivo imaging studies in mice have revealed spatiotemporal cellular dynamics of DC and T cell interaction and, upon antigen activation, T and B cell interaction in LNs [95–98]. In brief, DCs in LNs are enmeshed in an extensive network and remain in place while actively probing adjacent T cells with their processes, facilitating the interaction with migrating T cells. Within a few hours after entry into LNs, T cells make short-lived contact with the antigen-bearing DCs, each contact lasting an average of 11–12 min and occurring mainly on dendrites. An altered pattern of T cell motility during this early stage of antigen recognition promotes serial engagement with several adjacent DCs. Published estimates for scanning efficiency range from 500 to as many as 5000 T cells that may contact individual DCs per hour [99]. Subsequently, T cell behaviour progresses through long-lived clusters, dynamic swarms and autonomous migration punctuated by cell division. Upon exposure of antigen, B cells migrate toward zones of B cell and T cell interface with a low motility which increases after one day to approximately 9 μm/min. Antigen-engaged B cells pair with antigen-specific helper T cells for 10 to more than 60 min, whereas non-antigen-specific interactions last less than 10 min. B cell–T cell conjugates are highly dynamic and migrate extensively being led by B cells. B cells occasionally contact more than one T cell, whereas T cells are strictly monogamous in their interactions. It is assumed that the major principles of those dynamics follow the same routes in rodents and humans; specific receptor background, molecular composition, microenvironment, and pathways may also differ significantly. To accomplish the complex spatiotemporal cellular dynamics at immunological rest and upon antigen activation, the human LN is divided architecturally into three distinct functional areas (Fig. 2a): paracortex, cortex, and medulla (Fig. 2), which are pervaded by fissures and channels draining the lymph fluid [100,92]. These lymphoid compartments are structured and controlled by stromal cells, e.g. MRCs,[11] FRCs[12], and FDCs[13] [67,68,101–103]. APCs and antigens enter the LN via afferent lymph vessels into the sub-capsular sinus. A system of fissures and channels supports their efficient distribution toward cell contact areas with DCs or T lymphocytes [104,105]. Lymph fluid, lymphocytes and secreted antibodies are drained by the medullar sinus and efferent lymphatic vessels. The conduit system ensures the accelerated and effective passage of soluble antigens directly into deeper areas of the paracortex and the cortex [106,107]. The cortex is dominated by B cells arranged as follicles and GCs. The paracortex is dominated by T cells interacting with antigen-presenting DCs. Naïve lymphocytes circulating in the blood enter the paracortex via HEV. B cells then migrate via the T cell zone and home into the cortical zone attracted by FDCs. T cells swarm through the paracortical zone searching for APCs [108]. During their homing from peripheral organs into LNs, DCs have processed antigen molecules and present peptide fractions via MHC II receptors to T cells. Upon discovery, the T cells are specifically activated by DCs presenting the matching epitope for which they are designated. The central part of the LN, the medulla, contains transient plasma cells, macrophages, and B cells. Antigen-specific activated T cells proliferate and migrate toward the border of the cortical zone and come into contact with B cells located in the primary follicles or with individual peripheral B cells. The B cells are activated by the combination of direct antigen contact, presented as immune complexes called iccosomes on FDCs [109,110] and T cell co-stimulation. The GC[14] reaction is initiated. Here, B cells compete for antigene and for T cell help, and start to proliferate [111–114]. The highly sophisticated interaction with the FDC drives the refinement and class-switching of the pre-defined antibody of the B cell [109]. In the dark zone of the GC, activated B cells differentiate into rapidly dividing centroblasts which produce centrocytes competing for antigen contact on FDCs in the light zone of the GC [115]. Weakly binding centrocytes die by apoptosis. FDC contact to centrocytes facilitates affinity refinements of their encoding antibodies. During the process of refinement, centrocytes may also re-enter the dark zone to become proliferating centroblasts again [116–118]. When successfully refined, the centrocytes differentiate into plasmablasts, antibody-secreting transient plasma cells or B memory cells, which stay in the mantle zone of the

[10] lymph node

[11] marginal reticular cells
[12] fibroblastic reticular cells
[13] follicular dendritic cells
[14] germinal centre

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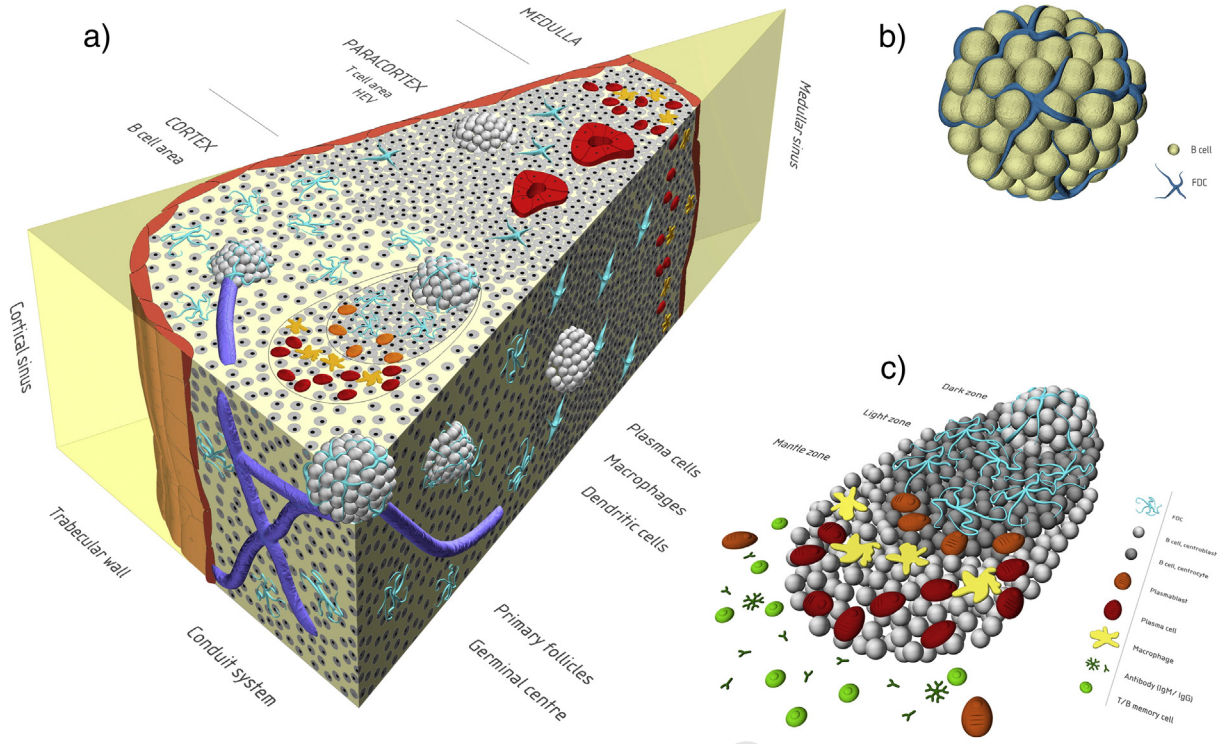

Fig. 2. Lymph node architecture enabling adaptive immunity a) LN tissue is separated into three distinct areas: cortex, paracortex, and medulla. The paracortical zone is dominated by T cells. Primary follicles and germinal centres are found in the cortical zone. Antigens and APCs are transported by lymphatic sinuses and the conduit system. Lymphocytes enter the paracortical zone via HEVs and get in contact with APCs. Activated T cells proliferate and migrate toward the border of the cortical zone. b) Lymphoid follicles, consisting of B cells and FDCs and their border zone represent the architectural units of a lymph node at immunological rest. c) The GC is the core unit of the antibody response upon repeated antigen-activation. In a micro-evolutionary process, B cell-derived centrocytes are competing for the best antigen binding to immune complexes on FDCs, resulting in antibody refinement by affinity maturation and class switch. T and B memory cells and plasmablasts are leaving LNs and homing into bone-marrow.

GC for a while. Plasmablasts and T memory cells leave the mantle zone and home into the bone-marrow via lymphatic drain and blood transport [119]. B memory cells leave the mantle zone but do not home to any particular environment. Continuous circulation through the blood stream and the lymphoid organs support their efficient function as guards against pathogen invasion.

Consequently, ALN models should aim to model the primary follicles and their crosstalk zone with T lymphocytes as the primary functional unit for first antigen contact. Furthermore, they should provide a stable, long-lasting microenvironment for the transformation of such naïve functional units into GCs under initial and repeated antigen exposure. It is important to note that the process of follicle activation and transformation into highly organised GCs is of such importance for antigen-specific antibody formation in humans that these structures sometimes even appear de novo in non-lymphoid tissues associated with sites of chronic pathological inflammation in autoimmune disorders, e.g. rheumatoid arthritis, and are then called tertiary lymphoid organs.

3.2. Bone-marrow

The bone-marrow is divided into red marrow, stroma, and white marrow (Fig. 2b). Haematopoiesis and adaptive immunity niches for T and B memory lymphocytes and plasma cells are located in the red marrow. Re-infection with a known pathogen pushes an antigen-driven recall of memory response and rapid and massive production of highly specific antibodies due to the existence of these niches [120–123]. Stroma controls the homeostasis of bone material and provides the microenvironment for all niches in the bone-marrow. Its role in maintaining bone-marrow immunity has been investigated in-depth [124]. Mechanisms for long-term survival of plasma cells in bone-marrow niches, among others, revealed an important function of eosinophils [125–127]. The white marrow is dominated by fat cells. Bone-marrow is highly compartmentalised to facilitate the massive production of erythrocytes, platelets and leukocytes [128–130] and to provide lifelong niches for stem cell regeneration and immunological memory [131,120]. Haematopoietic stem cells differentiate into continuously proliferating progenitor cells for the supply and renewal of blood cells in distinct functional areas of the red marrow:

- Erythroblasts produce erythrocytes in the erythroblastic area
- Platelets are generated by megakaryoblasts and released into the blood in the thrombopoietic area
- T and B lymphocytes are generated by lymphoblasts in the lymphoblastoid area
- Monocytes are formed by monoblasts in the monocytopoietic area
- Granulocytes (basophils, eosinophils and neutrophils) are generated by myeloblasts in the myeloid area.

The majority of bone-marrow equivalents established in vitro target the emulation of haematopoiesis, mesenchymal stem cell research, and clinical potential for transplantation [132–136]. Here, we focus on systems that emulate human bone-marrow architecture as closely as possible and, consequently, potentially support adaptive immunity functions (plasma cell niches, T and B cell memory niches).

4. Emulating human immunity in vitro

We have divided the in vitro systems emulating human immunity surveyed into two groups according to their level of architectural and, consequently, functional complexity.

1. The first group exemplifies the large quantity of test-relevant 2D and matrix-assisted immune cell assays emulating the response of individual immune cell types to an immunogenic structure in conventional culture plates.

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2. The second group depicts the as yet small number of existing examples of innate non-lymphoid and, even rarer, adaptive lymphoid 3D immunity in vitro; the first emulating innate immune responses of pathogen-exposed tissues, such as gut, lung and skin, integrating immune cells into respective 3D organ equivalents. The latter targeting the organotypic emulation of the adaptive antibody response involving co-ordinated T and B lymphocyte crosstalk in professional lymphoid organs, such as LNs, spleen and bone-marrow.

The majority of assays and systems are operated using primary human immune cells derived from blood and, occasionally, from solid tissue biopsies.

4.1. 2D and matrix-assisted immune cell assays

The assays reviewed in this category have been divided into PBMC, adhesion and migration, DC, ADCC, and ELISPOT, according to their function and cell type.

4.1.1. PBMC[15] assays

The first step of adaptive immunity is an interaction of leucocytes with an antigen, to develop a specific immune response, or a drug, to modulate immunity. The activation and proliferation of T cell populations and induced cytokine secretion, for example, are used to identify drug-related immune interference between 24 h and up to eight days of treatment. The simplest test procedure provides the opportunity to test large cohorts of donor PBMCs with average donor-to-donor variations or to identify certain donor-specific responses (e.g. HLA[16] restriction). Other prime examples are EpiScreen™ for PBMC immunogenicity testing [137,138] and T cell priming for the evaluation of haptenated skin sensitizers in a co-culture model of DCs [139]. Immune responsiveness of IFN β-1a formulations has been tested using T-cell assays [137]. A total of 42 assays using PBMCs in co-culture with HUVECs[17] to estimate the immunotoxic potential of more than 320 chemicals have been included in the US ToxCast™ research programme as of August 10, 2013 (http://www.epa.gov/actor). This programme is currently the most strategic and co-ordinated public sector effort and is aimed at transforming toxicology according to the *Toxicity testing in the 21st century* vision of the US National Research Council [140–142], launched by the US Environmental Protection Agency in 2007 [143]. A very recent improved PBMC assay involving whole blood samples and human endothelial cells finally came up with an in vitro cytokine storm comparable to the in vivo TGN1412 disaster of 2006. Researchers had to perform a short preculture of PBMCs at high densities subsequent to stimulation with soluble TGN1412 to achieve the cytokine release comparable to the in vivo disaster in this artificial in vitro setting [144].

4.1.2. Adhesion and migration assays

Adhesion of blood immune cells to vessel walls and their migration into tissues are the basis of leucocyte homing into non-lymphoid and lymphoid organs and, therefore, initiate any innate or adaptive immune response [145,46]. Consequently, a plethora of adhesion and migration assays on various immune cells were developed in culture plates. Immobilised adhesion molecules or endothelial cells served as attachment surfaces, while semi-solid matrices served as migration tools. Within the last two decades, various cell culture plate modifications have become available to improve the mimicry of blood flow and endothelial cell barrier functions to enable leucocyte rolling, adhesion and transmigration through endothelial cells into another culture compartment within a single cell culture plate. A prime example is the VenaFlux microfluidic cell culture plate of Cellix Ltd., Ireland, which has been successfully applied for antibody-mediated adhesion of malaria-infected erythrocytes to chondroitin sulphate A in placental tissue [146–148]. Higher affinity of surface molecules to some receptors not seen in vivo has been reported using in vivo and in vitro techniques to study the migratory behaviour of T and B lymphocytes [149,150].

4.1.3. DC assays

DC-based assays have been developed during the last decade to predict skin sensitization and allergic contact dermatitis of chemical compounds, particularly by the cosmetics industry as part of the hazard assessment of cosmetics and consumer care products [151]. This type of assay in combination with in silico modelling could prove to be an alternative to the mouse-based local LN assay currently used [139]. In addition, co-culture assays of DCs with T lymphocytes have been established in culture plates to mimic the antigen presentation process, which is the crosstalk between innate and adaptive immunity. Therefore, DC maturation and priming in vitro have been optimised and T cell activation readouts, such as cytokine release and proliferation, have been qualified [137,138]. The Sens-it-iv programme was launched by the European commission in 2005 (http://www.sens-it-iv.eu/) to develop assays for the identification of skin and lung sensitizers. This programme aimed to identify in vitro mechanisms relevant for in vivo sensitization on the level of skin and lung epithelia, DCs and T lymphocytes and has been extensively reported, including developed assay formats [152]. Non-animal skin sensitization assays have been comprehensively reviewed recently [153].

4.1.4. ADCC[18] assay

The ADCC of an antibody depends upon its specific antigen-binding and Fcγ-receptor-mediated recruitment of NK cells. Therefore, NK cells or cell lines are used in combination with proper target cells, such as tumour cells, in the assays. The most effective antibodies enable aggressive tumour cell killing by NK cells by facilitating their contact [154].

4.1.5. ELISPOT[19] assay

The ELISPOT assay is a cell-based assay to quantify the number of secretory active cells, e.g. antigen-specific activated cells. Only a few assays are available to quantify. It is also a powerful method to test the killing performance of cytotoxic T cells. Antigen-specific T cells are identified by peptide-stimulated IFN[20] secretion and cytotoxic T cells by granzyme B secretion. An immunosorbent multiwell plate is coated with a detection antibody against the secretory target of interest. PBMC, plasma cell or T cell preparations are seeded onto the plate and stimulated by antigen-derived peptides or stimulatory cocktails. During a cultivation time of a few days, secreted target molecules are captured locally by the membrane-bound detection antibody on the plate. Spots are developed using a secondary detection antibody in combination with an enzyme-substrate, and quantified by image analysis [155,154].

4.1.6. Sequential combination of 2D and matrix-assisted assays

The most recent improvement emulating complex crosstalk between innate and first-step adaptive immunity in a multiwell transwell format is the assay platform MIMIC™ [156,157]. This combines three different modules: the peripheral tissue equivalent, the lymphoid tissue equivalent, and the functional assay construct. The peripheral tissue equivalent module mimics a confluent monolayer of HUVECs on a collagen matrix for peripheral drug exposure. Pre-cultivation and treatment are performed in a transwell system. When exposed to the drug, added DCs are activated and transmigrated through the cell layer and matrix construct. The basic idea is that DCs are more effective APCs when exposed to drugs in a skin-like environment and after successful transmigration. The lymphoid tissue equivalent module is based on

[15] peripheral blood mononuclear cell
[16] human leucocyte antigen
[17] human umbilical vein endothelial cells
[18] antibody-dependent cell-mediated cytotoxicity
[19] enzyme-linked immune spot
[20] interferon

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lymphocytes in a 2D culture, as previously described. Co-culture between pre-treated APCs and the lymphocytes is performed. Activation, proliferation and cluster of T cells are used as standard read-outs. Cells can also be used for functional assays post-experimentally. Promising in vitro data have been generated using this system for research on several vaccines and biopharmaceutical drugs and have been compared with the human clinical situation [158–160]. This points out that combining existing assays into an integrated test strategy might support relevant data acquisition and interpretation in industrial settings.

To summarize: Almost all of the regulatory and industry-accepted human immune cell culture assays rely on 2D and matrix-assisted cell culture regimens that provide short antigen or drug exposure time of between a few hours and a few days. Longer cultivation time under such static conditions will usually result in even more artificial cell activation caused by the waste product and cytokine accumulation, cell necrosis or apoptosis, or media starvation. Furthermore, each assay represents a discrete segment of the whole sequence of orchestrated events during a full-scale human immune response, suffers from artificial culture conditions, and supports only single pathogen or antigen exposure. Therefore, these assays are limited to the assessment of perturbations in immunity pathways at the molecular, organelle, and cellular levels only. MoA analyses on a 3D tissue level and AOPs [161] on a systemic organismal level cannot yet be implemented. Furthermore, due to the lack of natural fluid-to-tissue ratios, it is a challenge to integrate data on artificial in vitro exposure with dosimetry and human exposure information. QIVIVE[21]-modelling approaches [162–167] in the pharmaceutical industry, and reference dose, or other exposure guidance values [168–170] in the consumer industry, offer a growing and promising interpretation of data generated by these assays. Finally, there are no assays currently available in a culture plate format to emulate the second important step — B cell maturation into plasma cells and antigen-specific antibody secretion switching from IgM- to IgG-type antibodies. This is an additional critical chasm: It makes it currently impossible to model the entire process of human immunogenicity in vitro. Consequently, stepwise progression of systems, over and above 2D and matrix-assisted immune cell assays, toward 3D immunity is apparent. Any move of in vitro assays toward a higher degree of biology – organ or even organismal level – would eventually permit the transition from more hazard-based prioritisation to fundamental risk assessment.

5. 3D immunity in vitro

Such a move toward organ-level immunity in vitro has been tried via two avenues: the integration of relevant immune cells into established non-lymphoid 3D organ equivalents and the creation of professional lymphoid 3D organ equivalents. Next, we survey the few early successful systems and their respective assay potential.

5.1. Immunocompetent non-lymphoid 3D organ equivalents

3D non-lymphoid organ equivalents have been developed for various uses for at least 100 years [171] with impressive successes, both at conventional culture plate format and, with the use of MEMS[22] at the beginning of this century, miniaturised onto chips to support dynamic fluid flow and parallelized high throughput substance testing [172–176]. The immune cell distribution within their human counterparts has been ignored in non-lymphoid 3D tissue engineering for a long time. Based on registrations, with a current total of 8 million different chemical entities and a total manufacture of 12.2 trillion kilograms in 2006 [177], the variety and potential dosage of chemicals to which a human individual is exposed have both increased exponentially over the last 50 years. Ironically, so have the allergic and immunogenic disorders, such as atopic dermatitis, allergic rhinitis, asthma, hay-fever, food allergies, and idiosyncratic drug-induced liver injuries. The latter have already affected more than 30% of the human population worldwide [178], with alarming allergy growth rates in children. This, in combination with the urgent interest of a broad spectrum of industries to provide safe consumer products and effective drugs, cosmetics and food ingredients, has provoked a shift toward the integration of immunity into non-lymphoid 3D organ equivalents of gatekeeper organs (e.g. gut, lung, skin, and liver) within an organotypic architecture at the beginning of this century. The first remarkable results at a research level have followed. Liver models at a miniature chip-based scale have recently been reviewed elsewhere [29]. We focus on reviewing the prime identified immunocompetent gut, lung and skin models, which are illustrated in Fig. 3. We selected these models from the literature based on two precepts:

1) Static 3D in vitro models using culture formats, applicable to parallelized higher throughput substance testing and operated by periodic medium exchange. They were developed by adding another tissue culture dimension to the advanced 2D and matrix-assisted immune cell assays, described in this review earlier,
2) Dynamic 3D chip-based microfluidic culture formats, which have been developed from scratch to provide continuous media supply to the tissues and mechanical coupling to enable tissue-specific shear stress and, potentially, immune cell circulation.

5.1.1. Immunocompetent 3D in vitro gut

Gut mucosa is the largest interface barrier between the human body and the outside environment and hosts the largest microbiome. It is the major site for the induction of acquired tolerance toward food proteins and microbiome antigens generating the largest pool of regulatory T cells in the human body. It is also the main entrance route (oral administration) for pharmaceuticals and food additives. Inflammatory bowel diseases, such as ulcerative colitis and Crohn's disease, are the most prominent immunological disorders of the gut, with animal models failing to take into account the dysregulation of the innate or adaptive immune system [179]. Human in vitro models of inflamed intestinal mucosa would be of utmost relevance to assess the interference of orally administered drugs with a patient's mucosal immunity. Leonard and colleagues recently developed a 3D inflamed intestinal mucosa equivalent [180,181], based on the co-culture of intestinal epithelial Caco-2 cells with primary, blood-derived macrophages and DCs (seven-day preculture) as components of the intestinal innate immune system (Fig. 4a). The model is established within a static transwell-based tissue culture plate format during a co-culture period of three weeks. This results in a closed Caco-2 cell monolayer covering a collagen layer comprising DCs and macrophages in a 3D architecture, all located on the porous membrane of a transwell insert. Inflammation is induced by a two-day exposure of IL-1β. The model has been proofed by a four-hour exposure of a Budenoside nanoformulation and subsequent four-day culturing to assess relevant responses. Due to the separate access to both the apical and the basolateral compartments in this transwell-based culture plate system, read-out samples can be taken from both sides of the 3D intestinal mucosa equivalent. Budenoside is a standard treatment for moderate and mild inflammatory bowel diseases. In this case, Budesonide solution, its liposome formulation and its combination with PLGA[23] nanocarriers were investigated in the 3D immunocompetent model. Different read-out parameters were used to evaluate the treatment efficacy. The involvement of the immune cells in the inflammatory response resulted in significantly higher inflammatory marker release compared to the respective monocultures. This interplay seems to exist between all three cell types, as only the

[21] Quantitative in vitro — in vivo extrapolation
[22] micro-electro mechanical systems
[23] poly(lactic-co-glycolic acid)

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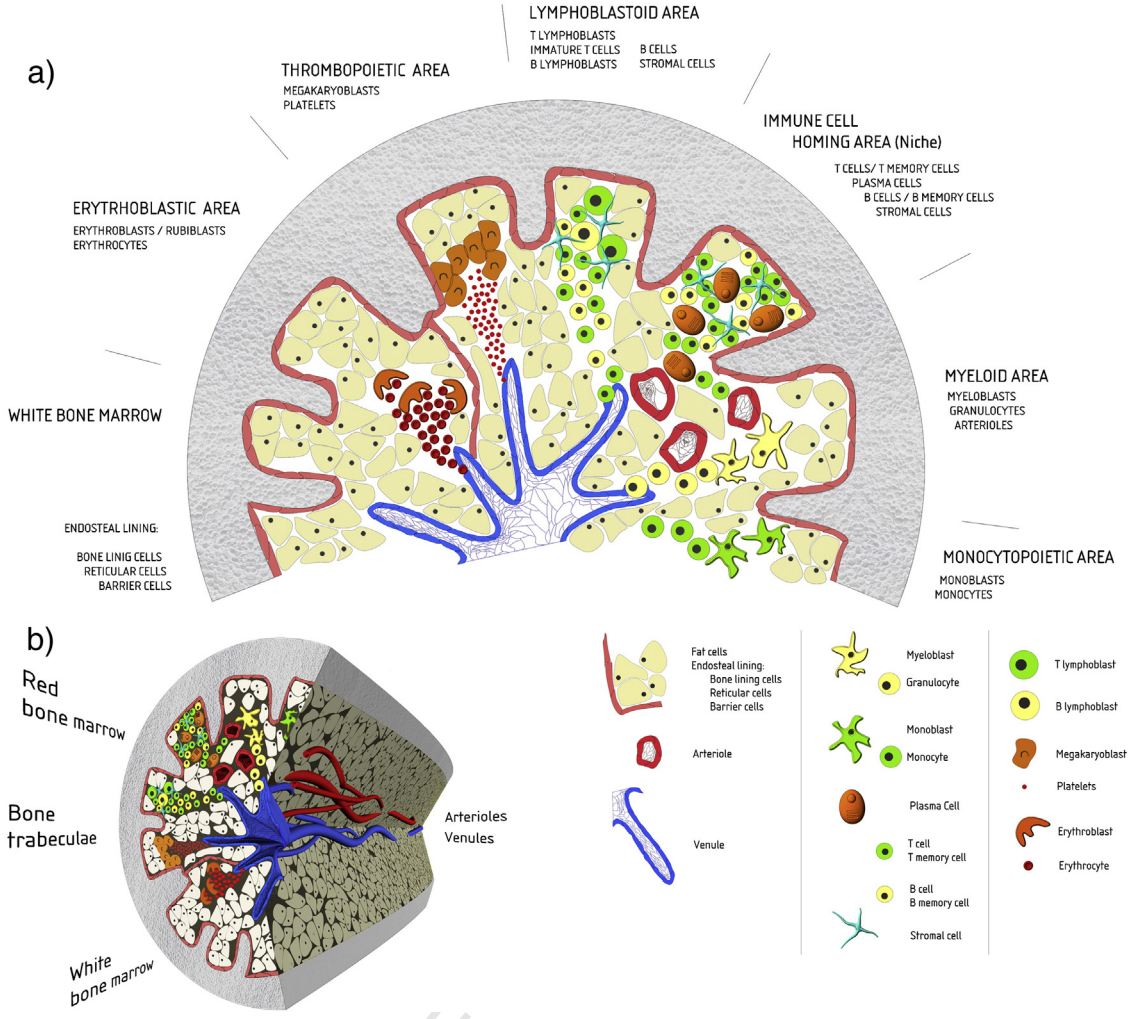


Fig. 3. Bone-marrow architecture a) The different zones of haematopoiesis and niches for immunological memory of T and B cells of bone-marrow are highlighted in a schematic cross-section of a bone. Platelets, erythrocytes, lymphocytes, monocytes, and granulocytes are continuously produced in the red marrow and released into the blood. Memory T and B cells home from secondary lymphatic organs into the bone-marrow. Most of the antibodies in humans are produced by plasma cells located in the bone-marrow. b) The extensive vasculature supporting the aforementioned cell movement is accentuated in the 3D sketch of the bone section. The legend describes the different cell types.

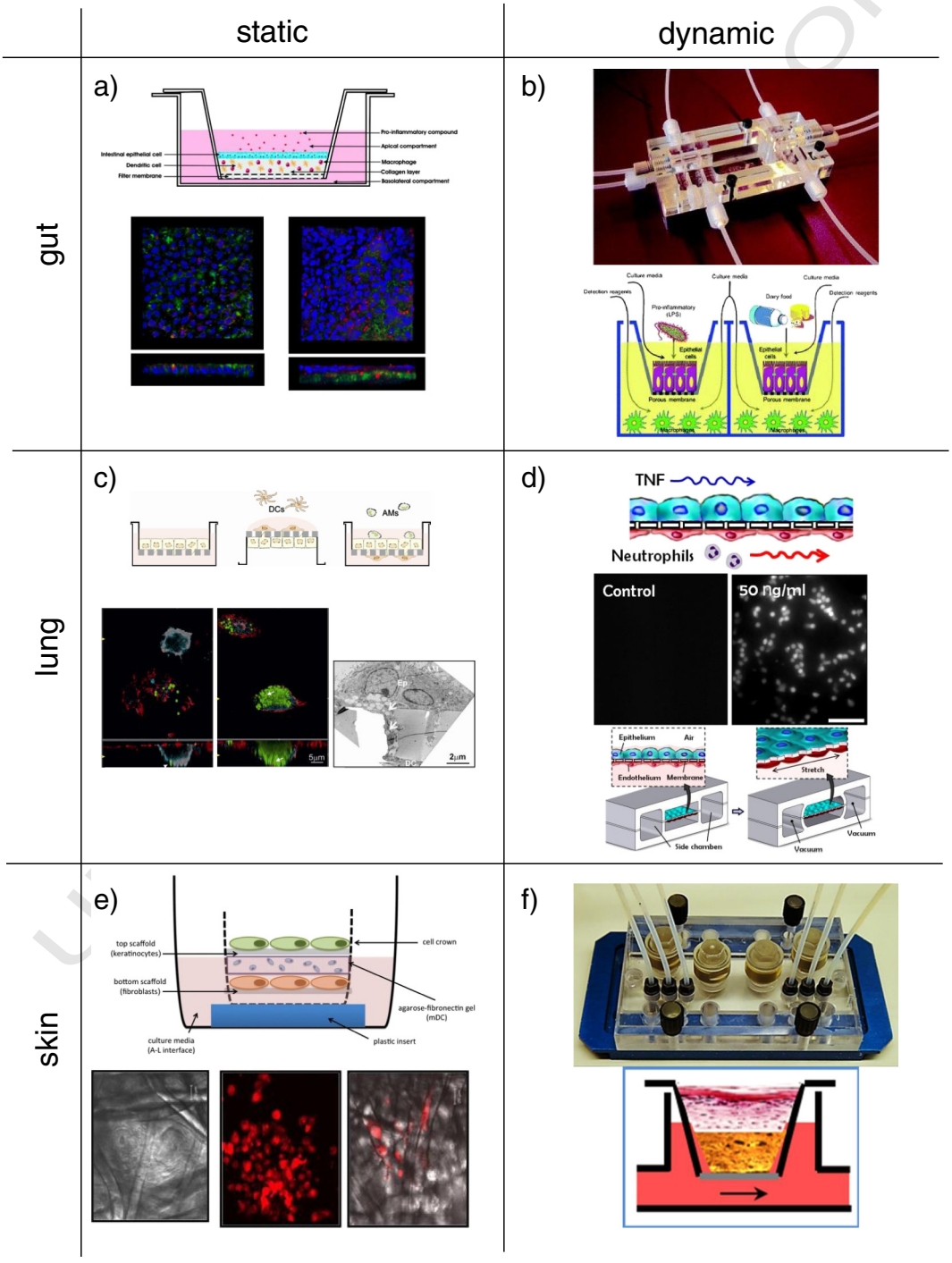
presence of both DCs and macrophages synergistically enhanced the strength of the immune answer. A preferential uptake of nanoparticles into immune cells was observed. Valuable data can be generated in the model as a pre-stage for in vivo animal studies, therefore, reducing the number of animal tests. Several other static cell culture models incorporating immunocompetent cells, such as macrophages or B cells, to stimulate intestinal tissue have been developed [182–184] with the reviewed one being the most advanced both in architecture and, consequently, functional read-outs.

A remarkable dynamic microfluidic system mimicking the immunocompetent mucosal barrier has recently been introduced by Ramadan and colleagues [185]. These researchers, with the aim of providing a platform for nutrition analysis, designed a microfluidic chip translating the transwell insert concept of culturing supporting cell cultures in apical and basolateral compartments divided from each other by a porous membrane (Fig. 4b). The so-called NutriChip system comprises two microfluidic layers sandwiching a polyester membrane with a pore size of 0.4 μm. The apical layer contains a single chamber, which hosts the epithelial cells, and is interfaced by a chamber made in the basolateral layer, which receives the substances transported through the epithelial confluent cell layer grown on the membrane. The human epithelial Caco-2 cells have been used to cover the membrane in the apical layer and the human monocytic cell line U937 has been used as an immunity surrogate in the basolateral layer. It was found that the culture conditions of the epithelial and the monocytic cells were incompatible, therefore, a dedicated culture chamber was created for the monocytes next to the epithelial cell basolateral chamber. The two chambers are separated by a valve, which is normally closed and is opened for the monocyte stimulation process. The very early initial investigations of the system do not allow a conclusive summary of an added value of the system over the aforementioned static culture system, but the principal ability to apply shear stress mimicking that of the gut peristaltic at the apical side, and that of interstitial fluid flow and blood perfusion-assisted substance removal at the basolateral side might increase the longevity of such systems. Another impressive microfluidic gut has been reported by Kim and colleagues [186]. The application of cyclic strain to the epithelial Caco-2 cell layer in this model reduced the formation of a well-defined consistent monolayer from 7 to 21 days down to three days. Furthermore, it supported the co-culture of bacteria (lactobacillus rhamnosus) as a surrogate for the human microbiome. Unfortunately, this dynamic gut model does not yet consist of any human immunity.

5.1.2. Immunocompetent 3D in vitro lung

The total surface area of airways and alveoli in a lung of a healthy human individual is about 120–150 m^2, nearly ten times that of

human skin, but less than half the area of the gut. The most prominent driver of the development of human in vitro lung models is smoking and the increasing morbidity and mortality caused by adverse health effects of ambient particulate air pollution [187,188]. Recent studies indicate a specific toxicological role of inhaled ultrafine particles smaller than 0.1 μm in diameter [189]. In addition, there are progressively more nanoparticles released into the air, water, and soil every year. These particles can cross the air-blood barrier. Therefore, human airway and alveolar epithelial models are commonly used for translocation and toxicity studies [190]. A resident 3D transepithelial network of alveolar macrophages (professional phagocytes in the lung) in the epithelial layer and DCs (professional APCs) inside and underneath the epithelium, with a steady-state density of 500–750 cells/mm^2, is one of the major barriers preventing particles crossing into the blood [82,83]. Among others [191,192], the prime static 3D tissue model of the human respiratory tract identified during our study of the literature has been established by Rothen–Rutishauser and colleagues in a transwell plate format containing epithelial cells, macrophages, and DCs (Fig. 3c [193]). Human epithelial cells, represented by the A549 cell line [194], were seeded at the apical side of a cell culture insert (Fig. 4c) and cultured for seven days. Macrophages and DCs were derived from PBMCs by preculture protocols of seven to ten days. The triple-cell co-culture was established by adding the DCs onto the basolateral side of the insert (2 h incubation for attachment) and the macrophages were applied on top of the epithelial cell monolayer (2 h incubation for attachment). The triple-cell co-cultures were exposed



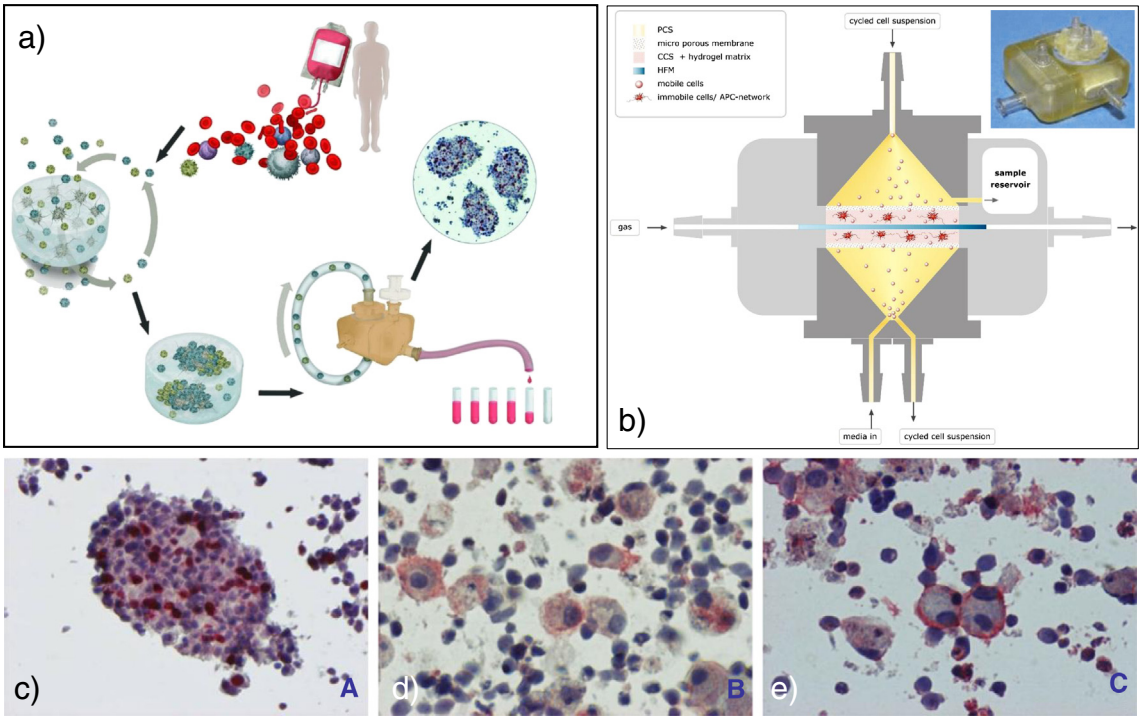


Fig. 5. HuALN — a human 3D lymph node equivalent a) Schematic overview of the HuALN used for substance testing. Different cells of the native immunity are separated from donor leukocytes, differentiated into mature cells, e.g. DCs, seeded into 3D matrices, and, finally, mounted into a bioreactor device (b), where media and cell suspension flow vertically and gas supply perfuses the system horizontally. The oxygenating hollow fibre membranes are encompassed by the immobilised matrix-assisted cell suspension. A photograph of the bioreactor is shown in the upper right corner of b). Follicle-like spheroid formation and proliferation (image c; Ki67; red staining), plasma cell differentiation (image d; CD138; red staining) and antigen-specific binding on plasma cells (image e; biotynilated CMV-lysate; red staining) was confirmed histologically on tissue slices upon tissue culture completion (from [207] reprinted with permission from Elsevier). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

to particles 1 μm in diameter in suspension [193] or on the air–liquid interface by spraying [82] over a period of 24 h. The model has been used for studying the properties of the transepithelial network of macrophages and DCs upon particle stimulation. Important aspects of uptake mechanisms could be elucidated on the level of innate immunity. No other assays or test systems are currently able to provide datasets on that theme.

An advanced dynamic 3D alveolar model has been proposed by Huh and colleagues. The microfluidic chip-based system allows for media transport above and beneath a barrier, composed of human alveolar epithelial cells on the apical surface of a porous membrane and human endothelial cells on the basolateral surface (Fig. 4d [195]). The chip design supports the establishment of an air–liquid interface and, more importantly, a cyclic strain applicable to the alveolar in vitro barrier. Human neutrophils could be provided to the basolateral surface by the respective microfluidic channel. The establishment of the functional alveolar barrier takes approximately 7–21 days, and subsequent exposure to bacteria, inflammatory cytokines, and nanoparticles allows the investigation of mechanisms of tissue and innate immune response with neutrophil involvement. It could be shown that cyclic mechanical strain accentuates inflammatory responses; this could not have been investigated in static cultures. The universal design of the microfluidic platform recently allowed the establishment of an induced pulmonary oedema model [196]. Drug toxicity-induced pulmonary oedema, observed in human cancer patients treated with interleukin-2 (IL-2), could be reproduced at similar doses and over the same timeframe in this lung-on-a-chip model. Studies using this on-chip disease model revealed that mechanical forces associated with physiological breathing

Fig. 4. Immunocompetent non-lymphoid 3D organ equivalents. The figure presents the prime existing systems of static (left side) and dynamic (right side) 3D in vitro culture systems of human gut (a and b), lung (c and d) and skin (e and f) containing one or another subset of immune cells.

a) The experimental set-up of the static co-culture of an intestinal epithelial cell line with macrophages and DCs is represented above. Deposition of nanoparticles in a non-inflamed state (below left) and after induction of inflammation (below right) is visualized by confocal laser scanning microscopy; blue signal: Caco-2 cell nuclei stained with DAPI; red signal: autofluorescence of immunocompetent cells; green signal: fluorescently labelled nanoformulations (reprinted with permission from [180], Copyright 2010, American Chemical Society and [181]). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

b) A photograph of the microfluidic NutriChip interface unit (above) for the co-culture of gut epithelial cells and macrophages and the underlying tissue culture principle (below) are presented (reproduced from Reference [185] with permission from The Royal Society of Chemistry).

c) Triple static co-culture establishment of lung epithelial cell line, DCs and alveolar macrophages is schematically depicted (above). Immunohistological staining (below) exemplifies particle (green) attachment and uptake by DCs (turquoise). Transition electron microscopy image (below right) illustrates particle uptake by lung epithelial cells (Reprinted with permission of the American Thoracic Society. Copyright © 2013 American Thoracic Society [193]).

d) Fluorescently labelled human neutrophils (white dots; right image in the centre) adhere to the activated endothelium after introduction into the vascular microfluidic channel. The lung mimic device (below) uses compartmentalised microchannels to form the alveolar-capillary barrier, recreates physiological breathing movements by applying vacuum to the side chambers and represents the gold standard for dynamic immunocompetent 3D organ engineering (from [195] reprinted with permission from AAAS).

e) Schematic of the static 3D triple co-culture model of human keratinocytes, fibroblasts and DCs at the air–liquid interface (above). Images (below) depicting the localization of DCs (red) in the three different layers – top layer, middle layer and bottom layer (from left to right) – following stimulation with DNCB, shown by live cell fluorescence imaging (reproduced with permission from [197]).

f) A photograph of the microfluidic MOC platform with built-in micropump and microchannel circuit combining two 3D tissue culture spaces is shown above. The schematic of skin biopsies operated in the culture compartments within a transwell insert is represented below. The platform serves as a reference gold standard for upcoming dynamic multi-organ-chip technologies (reproduced from Reference [203] with permission from The Royal Society of Chemistry).

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motions play a crucial role in the development of increased vascular leakage that leads to pulmonary oedema, and that circulating immune cells are not required for the development of this particular disease. Compounds of resident innate immunity and circulating immune cells were not required for the particular oedema model, but the transepithelial network of macrophages and DCs, described earlier, might be a relevant further addition into the model for a broad coverage of lung diseases.

5.1.3. Immunocompetent 3D in vitro skin

Chau and colleagues [197] recently described an advanced static 3D skin equivalent. This combines human keratinocytes, PBMC-derived human DCs and fibroblasts at a 30:1:10 ratio, each immobilised on their own matrix or scaffold and, subsequently, combined into a three-layer CellGrown™ tissue culture insert (Fig. 4e). A human three-layer skin construct at an air–liquid interface comprising keratinocyte and fibroblast layers at a thickness of 100 μm and 30 μm, respectively, and an agarose-fibronectin layer of monocyte-derived immature DCs sandwiched between the keratinocyte and fibroblast cell layer, has been successfully established. Crucial 3D prerequisites are, therefore, provided for a functional innate immunocompetence: an even distribution of mobile immature DCs, a stromal bed providing de novo ECM[24] compounds and a stratified and closed epithelial barrier layer being in tight crosstalk with the stromal bed and the immature DCs. Furthermore, the pre-culture time for the keratinocyte and fibroblast layers was two days and four days, respectively, while preculture of the mDCs lasted six days. The 3D co-culture of the three layers was performed over seven days. DNCB,[25] an established skin sensitizer with extensive cell response documentation [198–202] was used at 2 μM to stimulate this model for 24 h. The model responded to the topical application of the sensitizer, as evidenced by the upregulation of CD86 and HLA-DR on the mDCs. Intriguingly, these same cells produce a significantly weaker response to DNCB when in isolation. However, none of the cytokines examined (i.e. IL-1α, IL-6 and IL-8) was upregulated in response to DNCB. This is probably due to the more differentiated nature of keratinocytes in this model. This skin equivalent has impressively proven to be suitable for the evaluation of mDC response to the single exposure of sensitizers in the short-term (seven-day) skin-equivalent cultures. However, it still differs in some important aspects from the in vivo immunity of skin, due to the lack of other resident immune cells, e.g. mast cells and γ/δT cells providing other aspects of immunocompetence, and the lack of blood circulation for proper immune cell movement.

Another approach to emulate the human skin composition of both somatic and immune cell populations is the use of skin biopsies in vitro cultures. Those biopsies contain all the immune cell types relevant for the donor at the place of biopsy extraction. Atac and colleagues [203] combined this approach with the use of a MOC[26] platform (Fig. 4f), supporting a dynamic media flow for nutrient supply and mechanical coupling of transwell-based 4–5 mm prepuce skin punch biopsy cultures at the air–liquid interface over 14 days. The comparison of the dynamic cultures of the approximately 500 μm-thick skin biopsies with the respective static control cultures by immunohistology revealed a striking integrity of the epidermis (cytokeratine 10 and 15), dermis, and the basement membrane (collagen IV and tenascine C). It confirms keratinocyte proliferation (Ki67) in the dynamic cultures in comparison to epidermal disruption and tissue reorganisation in static cultures. Immune cell analyses have not yet been carried out on the skin biopsies before and after tissue culture, as the prime focus was the delivery of a long-term skin culture system for cosmetics or drug toxicity evaluation. In this regard, a stable steady-state skin biopsy culture can be achieved with culture periods of at least four weeks and repeated dose substance exposure over seven days are possible [204]. Logically, the use of biopsy material in skin cultures provides all types of immune cells.

The de novo assembly of full skin equivalents and the use of skin biopsies are two equally relevant scientifically grounded approaches to model human skin immunity in vitro. Miniaturised long-term skin culture platforms are urgently required for repeated antigen/sensitizer exposure at relevant test assay throughput for the evaluation of immunogenicity or sensitization hazard of substances or materials administered dermally. Dynamic microfluidic cell culture platforms, such as MOC, seem to be well-suited to support steady-state skin cultures for repeated dermal substance exposure over at least four weeks.

5.2. Professional lymphoid 3D immunity in vitro

LNs, spleen, tonsils, and mucosa-associated lymphoid tissues of the gut are the backbone of adaptive immunity, with the major response burden carried by the LNs. Therefore, the very rare immune organ engineering to emulate adaptive immune responses has been focused on LN equivalents. The discovery of memory T and B lymphocyte and plasma cell niches in the bone-marrow has recently incurred new interest in that aspect of biology. Haematopoiesis, bone formation and mesenchymal stem cell potential are the main drivers for bone-marrow engineering. As no accentuation has been made in the field on modelling human bone-marrow plasma cell and memory lymphocyte niches, we depicted the most advanced in vitro system already providing such niches coincidentally.

5.2.1. The 3D HuALN[27] model

The tremendous challenges and hurdles to tissue engineer artificial human LNs have been recently summarized [205]. HuALN is the prime system with regard to architectural complexity and functional performance. It was developed by Giese and colleagues (Fig. 5, [206]) to emulate the interface between innate and adaptive immunity and the development of an adaptive antibody or T cell response in vitro. The researchers designed a disposable, miniaturised, and membrane-based perfusion bioreactor system. This consists of a matrix-assisted central culture space of about 1 ml providing the area for DC-T cell crosstalk, lymphoid follicle self-assembly and antigen-induced activation. In addition, a peripheral fluidic space of 4 ml for the recycling of suspended lymphocytes mimicking lymph drainage is included. The central culture space is supported by a planar set of perfusable microporous hollow fibres for continuous media and gas supply and exchange, mimicking the nutrient supply of human blood circulation. The positioning and geometry of the hollow fibres within the reactive lymphoid tissue area support constant oxygen gradients, which are known to be crucial for immune cell niche assembly and rearrangement in lymphoid organs. A resident 3D DC network is established and maintained within two perfusable matrix sheets formed by hydrogels, such as agarose, sandwiching the hollow fibres. The matrix sheets are stabilised by macroporous membranes. A quantity of 10^8 suspended T and B lymphocytes continuously move through from the peripheral fluidic space and can pass through the porous stabilising membranes, swarm within the 3D matrices searching for receptor fit with presenting DC cells and, finding no fit, can leave. The bioreactor can function in excess of several weeks, depending on the biological requirements, or ad infinitum. Reproducible adaptive immune responsiveness of the de novo assembled human lymphoid tissues in the HuALN model has been proven by in vitro immunisation of the HuALN-based lymphoid 3D tissues. These tissues were from donors seronegative for Hepatitis A or CMV[28] infection. Immunisation was carried out with cytomegalovirus lysate or Havrix™, a commonly used Hepatitis A vaccine [207]. Immature DCs were prepared in a standard seven-day protocol applying IL-4 and GM-CSF and were then challenged with the respective antigens for

[24] extracellular matrix
[25] dinitrochlorobenzene
[26] multi-organ-chip
[27] human artificial lymph node
[28] cytomegalovirus

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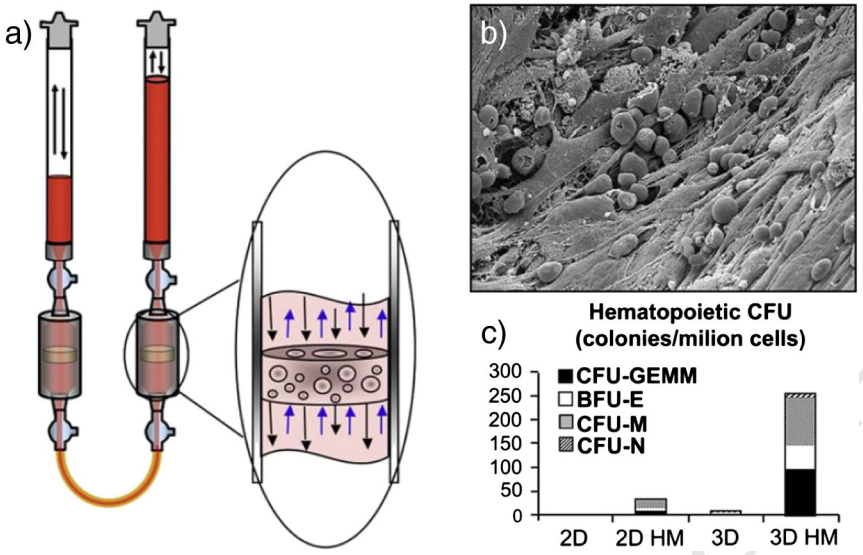

Fig. 6. 3D bone-marrow in vitro a) Schematic representation of the dynamic bone-marrow culture device consisting of two cylinders with the ceramic scaffold-assisted immobilised bone-marrow cells cyclically flushed with medium in alternating directions. b) Scanning electron microscopy image of the 3D network of heterogeneously shaped cells and extracellular matrix after 19 days of dynamic culture. c) Colony-forming unit assay results revealing the strong haematopoietic potential of the dynamic 3D bone-marrow culture (3D HM) in contrast to the 2D and static 3D cultures (from [209] reprinted with permission from Elsevier).

24 h. Thereafter, the 3D network was formed in the HuALN model, and T and B lymphocytes were continuously brought into contact with the network at a perfusion rate of 1 ml/day. This first antigen challenge on day one was followed by a second challenge on day six, with a follow up of 14–30 days. Assembly of lymphoid follicle-like tissue structures, immune cell proliferation, and antigen-dependent local cytokine storms could be detected associated with an expected donor variability. Exact surrogates of GCs could not be observed, however, histological and immunological plasma cell differentiation and antigen-binding on plasma cells could be observed. Furthermore, IgM formation progressively increasing during the week after immunisation and decreasing over the second week could be measured, mimicking the typical IgM profile in humans after a mild virus infection. Recognising the crucial role of mesenchymal stromal cells in immune modulation, Seifert and colleagues [208] recently integrated rat mesenchymal stromal cells into the respective professional lymphoid rat ALN[29] model, revealing an active crosstalk between immune and stromal cell populations. Translation of this approach into the human model would further improve the 3D HuALN performance. At the current development stage the model supports immunogenicity and immunotoxicity testing in long-term cultures applying repeated antigen challenges. A transformation of primary lymphoid follicles/organoids into mature GCs and, consequently, class switch toward IgG with respective antibody affinity maturation has not yet been fully accomplished in the system. Equivalents to stromal and vascular beds have to be rationally introduced to facilitate this accomplishment.

5.2.2. A 3D human bone-marrow culture platform

Maggio and colleagues described an advanced 3D scaffold-based perfusion system as a potential model to reconstruct the bone-marrow stem cell niche in vitro (Fig. 6, [209]). The model is based on a human bone-marrow-derived nucleated cell culture, including both stromal and haematopoietic fractions, within 3D porous hydroxyapatite scaffolds, with a perfusion of cell suspension through the scaffolds in alternating directions. This results in efficient and uniform cell-seeding and tissue development over several weeks [210–212]. This system allowed the elimination of the standard 2D expansion phase of stromal cells on plastic. Bone-marrow stromal cells were successfully expanded within the 3D scaffolds and the resulting cellular constructs reproducibly formed bone tissue at high efficiency when implanted ectopically in laboratory animals. Furthermore, the system allows the establishment of a 3D co-culture of mesenchymal and haematopoietic cells, thus resembling a "stromal" tissue bed (Fig. 4b). When medium was supplemented with haematopoietic growth factors, not only mature haematopoietic cells, but also early multipotent progenitors of human immunity could be entrapped in the pores of the scaffold to a higher extent than the corresponding 2D cultures. The crucial impact of synthetic scaffold geometry and chemoattractant composition on marrow-derived stem cell motility along with adhesivity and stiffness has been reviewed elsewhere [213,214]. Cui and colleagues have developed a dynamic microbioreactor platform operating 12 perfused microbioreactors simultaneously to miniaturise in vitro bone-marrow equivalents down to scales supporting larger test throughput. The system presented provides a perfect basis for further implementation of plasma cell or T and B memory cell niches. However, none of the developments reviewed focus on the modelling of immunocompetent bone-marrow niches.

6. Status and bridging the gap

While reviewing the current landscape, we have found two existing levels of complexity of organotypic 3D complexity of immunity in vitro, summarized in Table 2, reflecting the respective levels of the AOP paradigm.

The level A of 3D immunity in vitro was the natural development evolving from the 2D assays, which failed to give a full picture of immunogenicity, immunotoxicity, and immune dysregulation. The development follows the stringent paradigm of "form following function" in human lymphoid tissue microarchitecture [215,113]. At this level, we identified strong development into immunocompetent non-lymphoid 3D organ models, with the primary focus on barrier organs between the body and the outside world. Research at this level is aiming for organotypic 3D architecture advancing from static to dynamic systems with relevant mechanical coupling of the tissue compartments. These systems are the first to support the complex analyses of simultaneous molecular events within human-like microarchitecture involving immune and somatic cell crosstalk at a still rudimentary stage. They have already added significant value to MoA analysis on an organ level in vitro within the framework of integrated testing strategies for skin and lung sensitizers. More importantly, the first systems have been

[29] artificial lymph node

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Table 2
Relationship between levels of architectural complexity and biological function of human immunity in vitro. The prime dynamic professional lymphoid and immunocompetent non-professional models have been chosen as gold standards setting a state-of-the-art threshold. The consequences of a subsequent addition of crucial cell types (+) at each level of architecture for added-value read-outs are proposed.

Level of architecture	Gold Standard + additional cell/tissue types	Structure/biology	Corresponding function and read-outs
Level A Dynamic 3D single organ equivalents	"Lung-on-a-chip" [196] Epithelial layer spike with microbiome/ pathogen surrogate (E. coli) endothelial layer, moving neutrophils	Epithelial–endothelial alveolar barrier with resident bacteria on luminal epithelial surface and attachment surfaces for neutrophils on activated endothelial surface, all operating at cyclic mechanical stretch applied to the barrier	ECM formation between epithelia and endothelia Neutrophil movement, adhesion and transmigration into epithelia and, finally, bacteria phagocytosis
	+ macrophages and DCs	+ resident 3D network of macrophages and DCs on top and beneath the basal membrane	+ phagocytoses-mediated presentation and antigen-activated egress of DCs from the lung tissue
	+ PBMCs	+ circulating suspension of different naïve immune cells supporting homing into or egress from the lung barrier	+ highly effective pathogen-screening and increased steady-state response efficiency at the activation site
	Human artificial lymph node [206,207] Resident DC-macrophage network combined with continuous recirculation of PBMCs or isolated B and T lymphocytes	Lymphoid follicle-like organoid formation; IgM and antigen-specific plasma cell formation	Continuous screening for antigen-specific match
	+ human lymphoid stromal and endothelial cells	+ germinal centre formation	+ immunoglobulin class switch toward IgG and antigen-specific antibody affinity maturation
Level B Dynamic 3D multi-organ equivalents	Multi-Organ-Chip [204] Microfluidic chip platforms combining several tissue culture compartments by microfluidic channels for interconnected long-term culture	Not yet established	- Artificial interaction of compartmentalised cell cultures
	+ combining an immunocompetent non-lymphoid organ equivalent (e.g. skin) with a lymph node equivalent	+ interconnecting channel systems	+ entire skin or lung sensitization pathway; immunogenicity and tolerance formation over mid-term periods (2–4 weeks)
	+ arranging at least a gut, lung, skin, liver, spleen, bone-marrow, and kidney equivalent in a common blood capillary circulation	+ biological capillary network surrogate recirculating blood within an natural arrangement of organ equivalents	Organismal homeostasis with the capability of eliciting entire innate and adaptive immune responses over months

identified which try to mimic functional tissues of professional lymphoid organs at human-like architecture. The following improvements, highlighted in Table 2, might optimise performance on this level A:

- The composition and architecture of the stromal bed and resident immune cells of non-lymphoid and lymphoid organ equivalents should match that of their in vivo counterparts for cell crosstalk, antigen transport, and interstitial migration of immune cells [216,217]. Therefore, either the integration of all immune cell types at relevant ratios or the use of biopsies (e.g. skin and gut), precision-cut organ slices (e.g. lung, spleen, LNs, tonsils) or tissue aspirates (bone-marrow) are equally valid alternatives. Precision-cut tissue slices have been particularly used in short-term in vitro assays, with the restriction of rapidly losing functionality due to being excised from their capillary blood supply and microenvironment [218–221].
- The introduction of each immunocompetent 3D tissue equivalent into a fluid flow, as in the HuALN model, to ensure efficient nutrient supply, establishment of local chemokine and oxygen gradients, and to provide transient immune cell recirculation is of the utmost importance for human-like immune cell crosstalk on this artificial organ level [43,92,222]. This maintains steady-state immune cell homeostasis in the organs at a high rate of immune cell turnover to ensure antigen fit of the designated and fast removal of non-binders.
- Architectural heterogeneity in the tissue culture compartment and gradient-based nutrient supply are important requirements to ensure the strict separation of functional units in each and every organ model. A prime example is the strikingly strict separation of B follicles and the T cell areas in LNs, where B and T cells can migrate vigorously in their respective domains, but rarely trespass into each other's territory [103]. This is the prerequisite for later transformation of these areas into GCs upon antigen activation.
- Improvement of immunocompetent non-lymphoid 3D organ models should further elaborate on the integration of the physiological microbiome into the respective epithelial areas of the models to add the constantly maintained immunoprivilege [223] mechanisms.
- The separation of the 3D lymphoid and immunocompetent lymphoid organ equivalents from fluid flow by endothelial cells, integrating mechanisms of natural transendothelial migration [88,145,224,225] into the models would establish a new quality of human biology at this level A of 3D complexity.

A key read-out parameter for successful emulation of adaptive immunity in 3D LN equivalents would be the de novo transformation of lymphoid follicle-like structures into GC equivalents matching the architecture and, consequently, function of their in vivo counterparts upon repeated antigen stimulation [113].

Integrating datasets derived from the aforementioned level of organotypic 3D immunity in vitro with various existing methods for in silico prediction of immunogenic T cell epitopes using protein database-supported algorithms and T cell epitope mapping tools, such as Episcreen and EpiMatrix [226–228] could reveal the MoA of immunogenic structures at an organ level.

Unfortunately, this level A again does not fully emulate human immunity, differing significantly from human biology in two pivotal aspects: the lack of informed crosstalk among all components and organs in a systemic homeostatic arrangement and the lack of a flexible immune homeostasis, based on an equilibrium of dying immune cells being replaced by a new generation. In order to bridge this gap, we propose a possible approach to step over to the decisive level B, illustrated in Table 2.

7. "Human-on-a-chip" — the ultimate systemic level to emulate human immunity!?

Any systemic in vitro model needs to ultimately emulate human organ and system functions as closely as possible within their interplay. Macro-scale bioreactor systems providing interconnected systemic

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arrangements of different human cell types were proposed back in the early-1990s (European Patent EP0584170B1; [229]). Only the appearance of MEMS technologies at the advent of this century brought these historical approaches into recent tangible reality, leading to a few platform developments capable of co-culturing different crosstalking human cell types or tissue equivalents within a single microfluidic chip. Recent reviews have elaborated on the opportunities and challenges in the field of "organ-on-a-chip" and "human-on-a-chip" research [230–233].

Given an ethically acceptable supply of the necessary human tissues, "human-on-a-chip" systems that provide unlimited homeostasis and organoid repair capability on the basis of biological vascularisation, physiological blood perfusion and the maintenance of organ-specific stem cell niches could be a translational approach [231]. In brief, it is a prerequisite that human endothelial cell-based blood vasculature needs to form and interconnect several microvascular beds in a common blood circulation. Each of the microvascular beds needs to be integrated into an organ-specific stromal tissue bed, which provides the respective ECM-based microarchitecture for proper organoid assembly and immune cell traffic. Orchestrated organ-specific groups of fully functional organoids should maintain their specific immune functions in concert. The concept is based on the fact that almost all human organs are assembled from multiple, identical and functional self-reliant structural units, such as the primary follicle and GC in resting or responding immune organs, respectively, the skin segment, the lung alveolus, the intestine villus, the liver lobule, or the kidney nephron, each performing the most prominent functions of the particular organ. The multiplication of these structures, called organoids, within a given organ is nature's risk management tool to prevent a total loss of functionality during partial organ damage. The reactivity of organoids to biopharmaceuticals is supposed to be representative of the whole organ because of their distinguished functionality and a high degree of self-reliance within the respective organ. The natural composition of immune cells within each organoid is mandatory to ensure human immunity.

Fig. 7 illustrates an example of this "human-on-a-chip" concept developed to fit the MOC format the area of a standard microscope slide. It is aiming, at this scale, to operate an immunocompetent "human-on-a-chip" 100,000-fold smaller than that of its in vivo counterpart. Therefore, the liver equivalent is designed to host ten human liver lobules. All the other organ compartments are designed to hold a number of their organoids that is proportional to the ten liver lobules. The concept includes the gatekeeper-organs — gut, lung, skin, and liver. Furthermore, it consists of bone-marrow for haematopoiesis, renewal of leukocytes, and provision of memory lymphocyte and plasma blast cell niches. The spleen equivalent within this concept takes over its natural functions, the professional adaptive immune responses, and removal of older erythrocytes [234]. A kidney equivalent is included to ensure metabolic homeostasis of the multi-organ arrangement by removal of toxic metabolic products from the bloodstream. The aforementioned seven organs would be minimally sufficient to model human immunity at the systemic organismal level over long time periods at system homeostasis. The addition of other organ equivalents would add extra biology (e.g. brain, pancreas, testicles, and adipose tissues).

The biological level proposed here is very challenging. The "human-on-a-chip" concept competes with the rapidly growing effort to "humanise" laboratory mice, taking into consideration human biology, economics and ethics. "Humanising" mice is represented by:

i) The development of transgenic mice expressing specific human HLA allotypes and lacking endogenous mouse MHC class II [235] targeting tolerance induction mechanisms and associated with significant technical challenges [22].

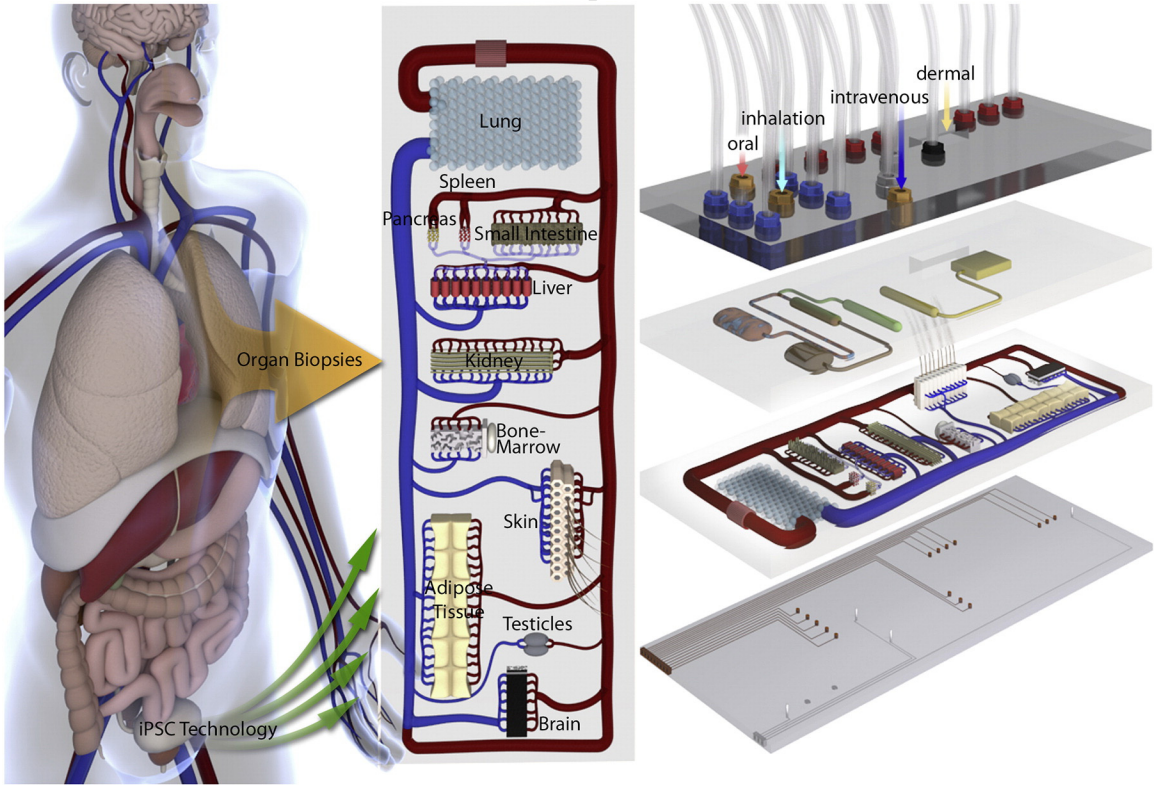

Fig. 7. "Human-on-a-chip" at 0.00001 scale. Here is a possible design for maintaining multiple human organ equivalents including spleen, bone-marrow and pathogen entrance sites, such as skin, lung and small intestine (middle top view) in a common blood vasculature on a chip the area of a standard microscope slide. The microfluidic device (right side) consists of sensors, organ equivalents, antra for nutrition, bile excretion, urine and faeces removal, and actuators providing mechanical cues for heartbeat, peristaltic intestinal movement, lung air-flow, bone compression, arteriolar constriction, and urine and bile removal. Access points for substance exposure through the different routes are highlighted. Finally, organ biopsies or the use of pluripotent stem cell technologies as tissue sources are accentuated on the left (reproduced from Reference [29] with permission from The Royal Society of Chemistry).

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ii) Xenograft transplantation models in immunodeficient mice in which human immune system compounds are transplanted by engraftment of human CD34 stem cells [236,237] or by engraftment of human lymphoid tissues engineered in vitro [238]. Each mouse has to be engrafted on an individual basis and considerable variability in immune responses to factors that stimulate potent responses in humans appear [22].

The logical alternative, the "human-on-a-chip" approach, demands the establishment of complex 3D micro-tissues consisting of the right composition of different cell types. In order to achieve this aim, either building the 3D organ by assembling the exact structure using its individual parts applying the latest tissue engineering tools, such as organ printing [239], or self-assembly and remodelling can be applied. The latter follows the natural routes of tissue formation, repair or regeneration [240–242] by providing the necessary microenvironment and stem cell niches. No matter which way one proceeds, microfluidic platform technologies are the basis for success. They enable long-term steady-state co-culture of different organ equivalents in common media circulation. Reproducible maintenance of a human liver and skin equivalent co-culture over four weeks has already been reported for the MOC platform [204].

Another important success factor would be the establishment of a human vasculature, a prerequisite to establish endothelial capillary beds within the organ equivalents and full endothelial coverage of fluid transport channels to allow whole blood circulation in the systems without bleeding and clotting. Significant progress has been made in the past to establish human vasculature in vitro by pre-vascularization techniques [243,244], re-seeding of decellularized scaffolds with human endothelial cells [245–247], or tissue printing procedures involving the establishment of capillary networks [248,249]. A complete coverage of the entire surface of polymeric microchannels of the MOC system with human endothelial cells operating with pulsatile fluid flow at physiological conditions has been reported recently [250]. Furthermore, any MOC concept demands the provision of actuators and sensors that match the functions of their in vivo counterparts. The actuators should couple a broad range of mechanical stresses differentially into relevant organs at natural degrees. Sensors with exceptionally high sensitivity need to be developed to detect the main parameters of human organismal homeostasis, such as organ viability, tissue temperature, pH, daily fluid balance, intracapillary pressure, blood flow volume, oxygen and nutrient consumption, fluid adsorption and intestinal juice secretion, albumin, immunoglobulin and bile synthesis, urea excretion, ion balances, and osmolality in the minute sample volumes derived from such a miniaturised microfluidic device. Miniature organ sizes and contact-free access to the transparent bottom of the chips allow the use of strong in-process research tools, such as two-photon microscopy for immune tissue imaging, fluorescence ratio imaging for local interstitial pH measurement [251], phosphorescence quenching microscopy for interstitial pO_2, and infrared spectroscopy to detect physiological stresses [252]. System biology approaches for the identification of physiological performance of the immune system at rest and while activated could be applied on samples daily. State-of-the-art and possible avenues to improve the technical layouts of single and multi organ-on-a-chip systems have been recently discussed and reviewed in a workshop [233]. A few years ago, such a target seemed to be pure science fiction, but the unprecedented joint development programme between the NIH, DARPA and the FDA (http://www.ncats.nih.gov/research/reengineering/tissue-chip/tissue-chip.html), initiated in 2012, to develop "human-on-a-chip" systems combining human systems/organs on a single chip is a unique hallmark, indicating the translational impact of such concepts on the drug development and immunogenicity testing pipeline. The forecasted complexity of such systems could offer a platform to fully emulate innate and adaptive human immunity in vitro within a single system and process. Despite the technical hurdles, the major biological question remains: Would the miniaturisation of lymphoid organs associated with the limitation of lymphocyte numbers checking for antigen-match limit the chance of such a match?

Looking beyond immunogenicity, "human-on-a-chip" systems might be able to model immunotoxicity on all levels of lymphoid tissues in the body and immune malfunction in such pandemic disorders such as allergies, resulting, for example, in asthma or atopic eczema, drug-induced liver injury and autoimmune disorders, resulting, for example, in rheumatoid arthritis. Finally, it remains speculative to expect such a "homunculus" (small man) on-a-chip to develop consciousness. One must always keep in mind that the term "human" in "human-on-a-chip" has the meaning of a model: an artificial copy, effigy or image. The uniqueness of a human being is inviolable.

8. Concluding remarks

Systems emulating human immunity in vitro are at a very early stage of development. The recent remarkable efforts and achievements in 3D modelling of immunocompetent human gut, lung and skin reviewed here will further facilitate the integration of the resident elements of innate immunity into these models at organotypic architecture in the near future, providing added value to sensitizing hazard testing of new biological and chemical structures exposed to humans. In contrast to animal models these human immunity models in vitro provide human cell and tissue substrates. This ensures human relevant receptor interactions and signalling pathways. Furthermore immune cell densities and functions relevant to human physiology can already be established by proper architectural tissue engineering of these models. But these models currently are unable to provide solutions for other well-known differences between laboratory animal and human physiology relevant to immune responses, such as the microbiome pattern, pH, and density of M-cells in the gut or the hair follicle density and their immune privilege niches in skin.

The few existing dynamic tissue culture platforms of professional lymphoid 3D organ equivalents – LN and bone-marrow – presented here provide an excellent first basis for further improvements of lymphoid tissue architecture, cellular composition, and the antigen exposure process over timeframes relevant to the development of a sustainable adaptive immune response. On the basis of the HuALN model, robust immune responses leading to the maturation of primary follicles into GCs, resulting in high affinity antibody responses and specific cytotoxic T cell clones are well within reach. Bone-marrow model improvements could support the investigation of antibody secretion in plasma cells and the maintenance of the B and T cell vaccination memory, all in their respective bone-marrow niches.

Unfortunately, none of the existing immunocompetent non-lymphoid or professional lymphoid 3D in vitro systems reviewed here provides a translational alternative to recapitulate the entire adverse immunogenicity pathway in man; neither do the human in silico immunity models and the "humanisation" of the immune system of laboratory mice. The majority of existing approaches to emulate human immunity in vitro followed a trial and error strategy rather than rational design principles. Such principles have to be introduced in the future.

We hypothesise that a decisive level of human immunity in vitro can be finally reached by the "human-on-a-chip" developments outlined. If the latter are successful, the possibility of significantly extending the predictiveness of such systems far beyond human immunogenicity issues will be possible, covering allergies, autoimmunity, and immunity-based malignant malformations.

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