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Innovative human-specific investigational approaches to autoimmune disease

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Conflict of interest:

The authors declare no conflict of interest.

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Abstract

Autoimmune diseases are exquisitely human diseases with a complex genetic background and variable clinical presentation, of which the underlying pathophysiology is insufficiently understood. Current treatment is mainly empirical with limited efficacy and significant side effects. To develop more effective targeted therapy for personalized treatment, understanding of the human pathophysiology is crucial, implying a high need for *human* investigational disease models. Using the example of anti-neutrophil cytoplasmic antibody (ANCA) autoimmune vasculitis, the concept of building an *in vitro organ-on-chip* type human disease model, consisting of cultured organ-specific vascular tissue in interaction with relevant immune system components (e.g. lymph node and thymus tissue) is presented. This *in vitro* approach makes use of advances in engineering and human stem cell technologies, enabling derivation of pluripotent stem cell lines from patients, differentiation to required cell types, and incorporation in microfluidic chip-based culture systems to optimally mimic *in vivo* disease conditions. Knowledge-based computational disease modeling is introduced as a valuable complementary tool to generate an integral mechanistic picture of the disease. Combining these multidisciplinary developments promises breakthroughs in understanding autoimmune disease and targeted drug development, while simultaneously reducing use of animal models. Current state of the art and issues remaining to be solved are discussed.

A brief introduction to autoimmune disease

The spectrum of autoimmune diseases is wide and clinical disease manifestations are highly variable, ranging from local to systemic disease involving multiple organs, and from acute to a lifelong chronic disease. Different types of immune cells play a role, with a varying role of the innate versus adaptive immune system, and with either a dominant role for a humoral or cellular immunity component. Complex and largely unresolved genetic determinants, e.g certain human leukocyte antigen (HLA) types, but also non-HLA genes, influence the propensity for autoimmune diseases as well as the clinical manifestations (1-4). Breakdown of tolerance of the immune system towards one or more self-antigens is the dominant overall causative factor for any autoimmune disease, and this implies an important pathogenic role for T cells and the adaptive immune response. In general, both hereditary and acquired

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genetic determinants can predispose to an autoimmune reaction. Endogenous factors like cell death associated with tissue damage, or exogenous factors like infections or a chemical substance, can function as trigger for the actual autoimmune reaction and initiation of clinical disease. The responsible self-antigen is probably a major determinant of clinical organ localization and symptomatology. Currently approved treatments for autoimmune diseases are mostly symptomatic to replace the function of the damaged organ, like insulin treatment in the case of autoimmune diabetes, or are based on drugs which interfere with a generic step in immune response, like inflammation or lymphocyte activation. These treatments do not specifically target the root cause of the specific disease and are associated with systemic side effects, for example increased risk at infections. More recently pharma companies have switched towards a personalized medicine approach with development of *targeted drugs* that target the causal factor in the pathophysiology of a specific disease. However, in general, lack of knowledge of the disease-specific human pathophysiology and immune-pathogenesis severely impedes development of targeted drugs for autoimmune diseases.

Investigational approaches in autoimmune disease, from the past to the future

So far, autoimmune diseases have predominantly been studied using a variety of *in vitro* assays and animal models. Cell-based *in vitro* assays are based on relatively simple (co)cultures, for example cell adhesion, migration, antigen presentation, and lymphocyte activation assays (5). As such they lack clinical disease context and can only contribute to a very limited extent to disease understanding. On the other hand, use of animal models for autoimmune disease is intrinsically flawed for several reasons: the animal immune system, especially in rodents, functions crucially different from the human immune system; autoimmune diseases are typically diseases of the elderly while the lifespan of rodents is much shorter; and finally, as said, development of autoimmune disease is influenced by complex underlying genetics - including the many genetic variations determining individual immune system performance (6-10). Together with rapidly increasing knowledge on the complex functioning - and dysfunctioning - of the human immune system, consensus has emerged on limitations inherent to even the best animal models (1, 11,12). This inevitably leads to the conclusion that animal models, while having been very valuable in the past, are not suited to develop novel targeted treatments for human autoimmune diseases. Even mice with a “humanized” immune system lack the complex human genetics underlying

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autoimmune disease, while organ/tissue antigens remain non-human, interfering with recapturing human immune response and tolerance mechanisms (13).

The arguments presented above illustrate the need for representative human disease model systems. Conventional clinical studies involving selected patient cohorts have high value for characterization of disease pathology, subtyping of disease entities, and identification of prognostic or predictive biomarkers or risk factors. However such a patient-oriented research approach in general lacks the power to unravel causal pathogenic relationships, necessary for discovery of reliable new drug targets. In addition, clinical experiments can in principle not be repeated in a reproducible manner and patient heterogeneity can be responsible for extensive experimental noise in clinical studies, especially for diseases that are not well understood and for diseases of the elderly where comorbidity is rule rather than exception. This becomes especially problematic when investigating patient samples with new techniques like next generation sequencing or proteomics which give rise to huge amounts of complex and hard to interpret data. It is inherently difficult to reliably identify relevant disease parameters, and risks at confounding study results are frequently underestimated. Clinical studies on autoimmune diseases typically suffer from the above-mentioned limitations. The "association only" dilemma of clinical studies can be illustrated by the Genome-Wide Association Study (GWAS) approach, frequently used in autoimmune disease (see for examples: ref 14-16). If the study population is large enough and the disease well defined, current GWAS approaches can be powerful in identifying high frequency disease-related genomic variants among the genetic noise, and increasing use of genome sequencing technologies will in principle also enable identification of rare disease-associated gene variants in the future. However, it has proven to be very difficult to assign a mechanistic, causal, role to identified "disease-associated" genome variations (3). Smart use of immune system and cell biology knowledge will be instrumental in defining relevant disease-related genes and potential disease mechanisms; for example when multiple genes within a specific cellular signalling pathway have been identified by GWAS, chances that this pathway is functionally involved in the disease increase (2). In addition, analysis of gene variants shared between different autoimmune diseases may reveal common disease pathways or risk factors (2).

Recently a paradigm shift has been proposed for the study of human diseases to enable improved disease understanding and encompassing intelligent incorporation of human (GWAS) genetics and human pathophysiology information into systems biology models.

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This approach aims for rationally linking causes of disease and disease outcomes by "disease pathways", meaning the mechanistic biochemical and cellular disease processes (17). To be successful, such an approach requires availability of representative *in vitro* human cell culture-based disease models, which can be experimentally queried in a repeatable and reproducible manner to answer questions on disease pathogenesis and pathophysiology. Developments in the form of "organ-on-chip" cell culture systems, combined with advances in human stem cell technology, should be able to turn the utopian idea of *in vitro* "patients-on-a-chip" into a realistic option (18). These new *in vitro* cell culture models are expected to be able to mimic human autoimmune disease in lab-format, including the immune component, and enable *in vitro* real time investigation of human pathogenesis, pathophysiology, immune-pathology, and drug development.

Organ-on-chip disease models

In the organ-on-chip concept cells are cultured inside a so-called "chip", roughly the size of a microscope slide (18-20) (Figure 1). The "chip" provides the basic housing for the cells which will form the tissue or (multiple) organ model. As such it replaces the conventional culture dish, in which culture of multiple cell types together in a three dimensional setting is difficult, while the natural physical microenvironment of the cell *in vivo* cannot be adequately mimicked. The "chip" may contain one or more open or closed small culture compartments (of a variable format) or small channels, in which cells can be seeded and cultured, either two dimensional (2D) or three dimensional (3D). In the chip the cells can multiply like in normal cell culture, or for example be induced to differentiate in a certain direction in case stem cells are seeded. The material of the "chip" is transparent, which makes it possible that using a microscope cells can be real time visualized and their behavior monitored. The surface material to which the cells attach can be chosen to resemble for example the level of tissue stiffness present *in vivo,* or to enable stretching of cells or cultured tissue. The surface can be functionalized by coating with extracellular matrix proteins, by attaching certain growth factors, and it can be patterned to align cells in a certain direction (21). Multiple culture compartments can be connected by microfluidic channels, while microporous membranes can be used to separate two adjacent culture compartments, enabling migration of cells through the pores. To actively flow fluid through the system, microfluidic channels in the chip can be connected to an external pumping system. Continuous refreshment of culture medium has the advantage that the cell culture can remain viable for far longer periods than is possible with

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conventional cell culture. Several human organs and tissue types have already been cultured in an organ-on-chip format, e.g. lung, intestine, kidney, liver, neurons, and blood vessels (22-26).

To create an *in vitro* disease model as "a patient-on-a-chip", the different cell types making up the model, including immune cells, in principle should have the same genetic background and HLA status. The recent availability of human stem cells, both induced pluripotent stem (iPS) cells and adult stem cells, combined with rapid developments in differentiating them to all kinds of cell types, makes this possible (27-29). iPS cell lines can be made from every healthy or diseased individual, while adult stem cells can be cultured and passaged like stem cell lines, using "organoid culture" techniques, directly from human tissue biopsies (28-30). Use of such cell sources makes it possible to develop *in vitro* disease models in a reproducible manner, in principle enabling unlimited repeating of experiments on identical disease copies, something that is not possible using a primary cell culture, let alone studies in a real patient.

The ideal culture-based disease model needs to be able to correctly and reproducibly represent the pathophysiology of the human disease. In autoimmune diseases, the key cells, tissue and organ components that determine pathophysiology and clinical symptomatology are the interacting innate and adaptive immune system, the affected and inflamed "target" organ(s) or tissue(s), and connecting blood vessels: all these modules should be integrated in the organ-on-chip disease model. In addition to conventional assays performed on the cultured tissue and cells, (time lapse) microscopy is the prime technology to real time monitor cell behavior in the organ-on-chip device (Figure 2b). 3D fluorescence microscopy is in advanced stage of development and will enable visualization of fluorescent reporter gene activity in the cultured tissue (31). Results from controlled perturbations or interventions will provide mechanistic information on biochemical and cellular mechanisms ("disease pathways") involved in the disease. However, in general, due to experimental constraints, changing one variable at a time in the experimental model in combination with a specific readout to assess the consequence of the perturbation will provide the answer to only one (small) question regarding disease pathophysiology. To enable development of an integral picture of the disease, we propose to add computational disease modeling as an additional, and very promising, investigational tool.

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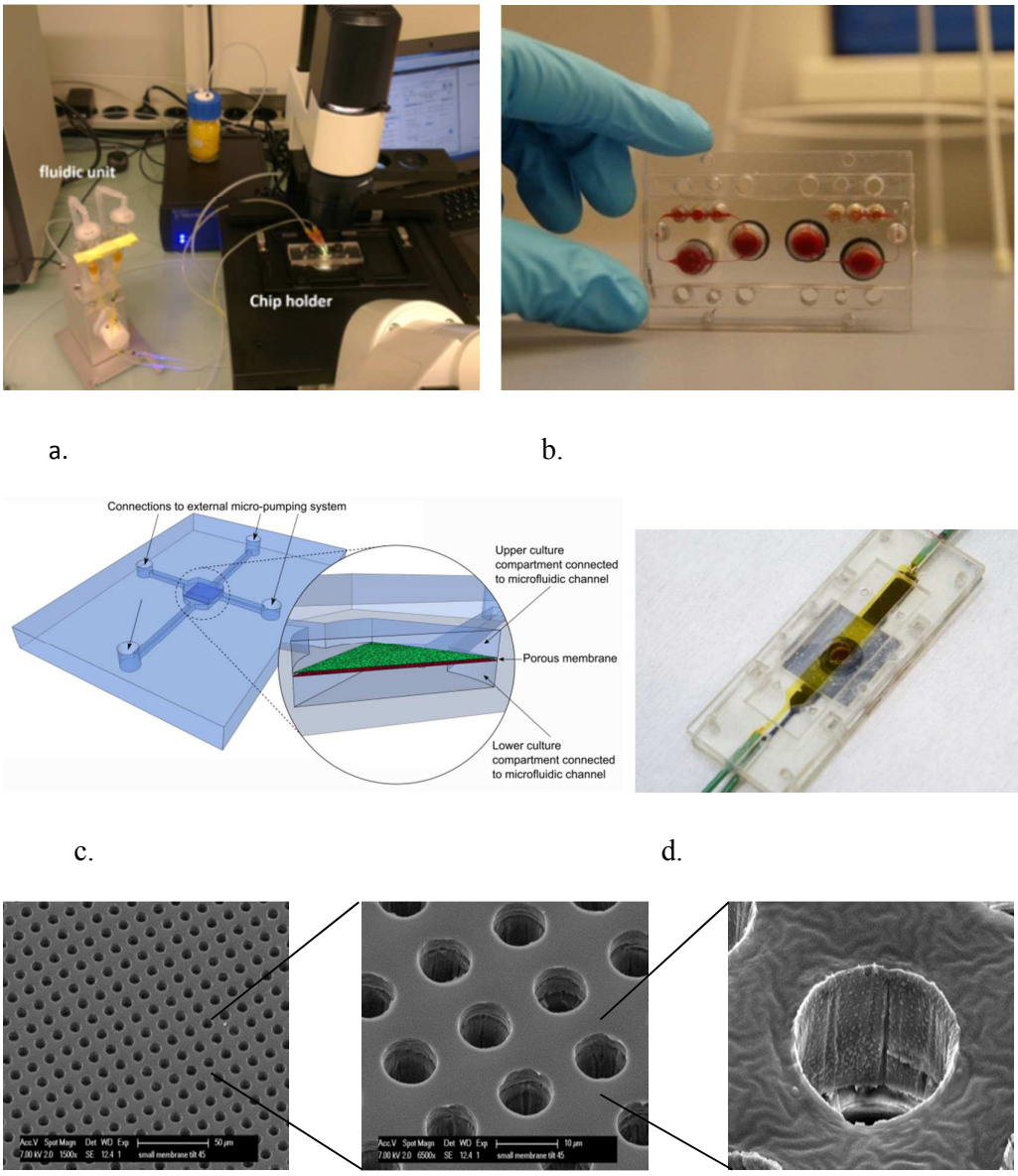

Figure 1. Organ-on-chip devices for advanced cell culture. a. Example experimental set-up of a microfluidic organ-on-chip system (courtesy Youssef Norouzi, Philips Research/Technical University Eindhoven, The Netherlands). b. Multi-organ-on-chip device, which hosts two independent micro-circuits, each with two individual cell/tissue culture compartments connected by a microfluidic channel system; micropumps can be connected to control fluid flow through the culture compartments. With permission, Uwe Marx (Technical University Berlin, Germany) and adapted from Ref. 18; c. A microfluidic chip design with two culture compartments separated by a porous membrane which allows cell migration between compartments (courtesy Tom van Gijsel, Philips

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Research, The Netherlands); d. Dual compartment microfluidic chip device (courtesy Lambert Bergers, Philips Research/Free University Amsterdam, The Netherlands); e. Microfabricated porous membrane enabling cell migration between culture compartments, pore size 8 micron, membrane thickness 10 micron (unpublished results and courtesy Berend van Meer and Ronald Dekker, Philips Research/Technical University Delft).

Knowledge-based computational disease models

The past decades, many computational approaches have been used to create *in silico* systems biology networks to study cell behaviour and disease pathways, mostly based on data-driven statistical modelling methods. Such approaches tend to fail when the number of variables is much larger than the number of samples, when the noise level of the data is high and in the absence of functional mechanistic data, and they are prone to overfitting. Instead, a knowledge-based computational approach based on probabilistic Bayesian principles, which uses solid biological knowledge with known causal relationships to create a high confident systems biology framework can handle noise-containing data in a far more effective and robust manner (32,33). The core structure and parameters making up such a Bayesian model can be derived from qualitative rather than quantitative prior knowledge from literature (32-34). Despite the fact that autoimmune diseases are complex and not well understood, for parts of the disease process the mechanism is known and this can be built into such an initial model framework. A time variable can be incorporated to create a dynamic network model and enable monitoring of a cellular process or disease characteristic over time. Additional knowledge and calibrated computational parameters can be introduced using data obtained from patients as well as experimental results from an *in vitro* (or *in vivo*) model system in which variables can be manipulated in a controlled manner. Quantitative inference in the form of a probability score can be used for example to predict with a certain probability the outcome of an experimental or disease intervention (32). Especially when used in combination with *in vitro* organ-on-chip models this knowledge-based *in silico* modelling approach is expected to be very valuable to create a more complete understanding of autoimmune disease pathogenesis and pathophysiology.

ANCA -associated vasculitis (AAV) as an example autoimmune disease

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Clinical vasculitis presents as many different clinical variants, categorized by the size of the affected blood vessels into large, medium and small vessel vasculitis, and characterized by the presence of inflammatory leukocytes in blood vessel walls. The vascular inflammatory process gives rise to obstruction of the lumen, associated with downstream ischemia, tissue necrosis, and bleeding through the damaged vessel wall. Within the category of small vessel vasculitis a number of diseases have been grouped together because affected patients share the presence of anti-neutrophil cytoplasmic antibodies (ANCA's), the so-called ANCA-Associated Vasculitides (AAV's) (For a review, see ref 35). AAV is a severe chronic systemic disease, which can affect multiple organ locations, with a dominant role for kidney and lung. Current AAV diagnosis and clinical classification is empirically based on symptomatology, serology and histo-/immunopathology analysis. Therapeutic options are similarly empirical and often unsatisfactory (36).

We will briefly review current AAV disease knowledge and main issues to be solved. Antibodies against proteinase 3 (PR3-ANCA), a serine proteinase present in neutrophil azurophilic granules, are found in the serum of most patients with *granulomatosis with polyangiitis* (Wegener's granulomatosis), abbreviated as GPA. This disease is characterized by granuloma formation in the lungs and renal failure due to a *necrotizing and crescentic glomerulonephritis* (NCGN) with characteristically scarse immune deposits (pauci-immune NCGN), and involvement of the skin, upper respiratory tract and eyes. Antibodies against the neutrophil enzyme myeloperoxidase (MPO-ANCA) are detectable in the majority of patients with *microscopic polyangiitis*, MPA. In MPA most patients have pauci-immune NCGN, while pulmonary disease is less prominent than in GPA. MPO-ANCA is also present in patients with isolated pauci-immune NCGN, a form of MPA confined to the kidneys. *Eosinophilic granulomatosis with polyangiitis* (EGPA), also referred to as Churg-Strauss syndrome, is a rare disease characterized by eosinophilia and pulmonary, cardiac and cutaneous vasculitis; only about half of these patients have a positive MPO-ANCA test. Although still controversial, a third ANCA antigen, lysosomal membrane protein-2 (LAMP-2) may be present in patients with NCGN (37-39).

AAV shows a distinct geographical distribution with respect to symptoms, presence of specific ANCAs and genetics (e.g. predisposing HLA alleles differ between African-Americans and Caucasians), suggesting that both genetic and environmental factors play a role in the pathophysiological process (40). With respect to genetic factors, a recent genome wide association study (GWAS) comprising Caucasian GPA and MPA patients identified the HLA-DP locus, the PRTN3 gene (coding for the PR3 self-antigen), and the SERPINA1 gene

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(coding for the PR3 inhibitor alpha-antitrypsin) as significantly correlated with PR3-positive AAV vasculitis, while the HLA-DQ locus was associated with MPO-positive AAV vasculitis (4). Interestingly and important, no associations were found with clinical disease as defined according to the current AAV disease classification. In support of the GWAS results, neutrophil PR3 expression seems to be indeed genetically determined and associated with more severe renal disease (41). Several environmental factors, e.g. staphylococcal superantigen in nasal carriers of staphylococcus aureus, the gram-negative bacterial adhesin FimH; use of drugs like propylthiouracil, exposure to silica, may enhance susceptibility to AAV; the pathogenic mechanism however remains largely unexplained, including the actual initiating event (42-44).

Although pathogenic mechanisms leading to organ-specific vascular damage and clinical symptomatology to a large extent remain to be elucidated, including the role of the self-antigens, it is evident that activation of neutrophils is at the core of the disease. Based on results using animal models, neutrophil activation is typically thought to occur in two steps. First the neutrophil becomes pre-activated, most frequently probably by an infection-associated cytokine signal like Tumor Necrosis Factor-alpha (TNF-alpha), lipopolysaccaride (LPS) or interleukin-1 (IL-1), and releases and binds the ANCA target antigens on its cell surface. Subsequently circulating ANCA binds directly to its antigen, which may result in crosslinking antigen-ANCA complexes to Fc receptors on the neutrophil membrane and in full activation of the neutrophil (45-48). This is associated with adhesion to endothelial cells and release of reactive oxygen species and proteolytic enzymes causing endothelial cell damage. Self-antigens may also be expressed on the cell membrane of activated endothelial cells, or become deposited in the endothelial wall upon release from neutrophils, further directing the auto-immune inflammatory reaction towards the vessel wall (49-51). This is the essential event leading to clinical vasculitis (52-54). The mechanism behind the organ-specificity of the vascular endothelial damage in this process remains largely unclear.

Multiple mechanisms contributing to loss of tolerance to the self-antigens have been described for patients with AAV (35). The question as to the role of ANCA antibodies versus T cell-mediated responses to the different AAV diseases remains controversial. In general the disease seems to be dominated by a humoral immune response with a role for CD4+ T-helper cells in recognizing local antigen epitopes within the HLA-II complex of macrophages or other antigen-presenting cells (35,51). Whether ANCA's play a causal role in AAV or are simply an epiphenomenon (with potential value for monitoring of disease activity, like for PR3-ANCA) has remained a controversial issue to date due to contrasting

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evidence derived from both preclinical and clinical studies (55-59). The fact that ANCA antibodies are sometimes not detectable in AAV patients may be due to failure of the routine diagnostic ANCA test to detect LAMP-2 antibodies and some circulating forms of PR3 and MPO antibodies (37,59). On the other hand ANCA antibodies can be present in low levels in healthy individuals as well, although affinity for a specific epitope of the antigen (not routinely assessed in patients) may make them uniquely pathogenic in AAV patients (38,51,60). The role of specific types of ANCAs, IgG, IgM or IgA, of immune complexes, and of associated (alternative) complement pathway activation has been difficult to relate to clinical manifestations in human patients (61-65).

Standard treatment consists of non-specific glucocorticoid and cyclophosphamide therapy, which is effective in obtaining initial remission in the majority of patients. However, the disease frequently relapses, underscoring the high need for more effective treatment. A vast array of experimental drugs has been -and is still being- evaluated in clinical studies such as alemtuzumab which depletes CD52-positive immune cells, abatacept which inhibits T-lymphocyte activation, rituximab which depletes or functionally inhibits B-lymphocytes, CCX 168 which blocks the C5a complement receptor on neutrophils, and a number of TNFalpha-blocking agents (e.g. etanercept) (36; https://clinicaltrials.gov/). All these drugs affect in a more or less generic manner the immune response and are not directed towards specific AAV drug targets. Not unexpectedly therefore, use of most of these drugs carries a high risk at infections. Even more importantly, many clinical trials have been terminated due to inefficacy or too few eligible patients; despite all efforts only rituximab has been approved by the FDA for treatment of GPA and MPA (http://www.fda.gov/drugs/developmentapprovalprocess/howdrugsaredevelopedandapproved/approvalapplications/therapeuticbiologicapplications/ucm093345.htm).

Both lack of understanding of the human disease, which can be contributed at least in part to inadequate preclinical model systems, and the relative rarity of AAV, hamper progress towards development of targeted therapies and reliable (companion) diagnostics (36).

The role of animal models and remaining questions regarding AAV

More animal studies will probably not answer the many remaining questions regarding AAV, nor suffice to discover AAV drug targets. Animal models have been very valuable for investigating AAV in the past, like for identifying the role of the neutrophil and its priming

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factors and some end-organ pathogenic effects of ANCAs. However, they can only represent aspects of AAV disease, and obtained results often appeared to be not translatable to human disease (For a review, including list with animal models, see ref. 66; for human immunology models, see ref. 12). Human-specific histo- and immunopathology findings appeared hard or impossible to replicate in animal models. With the exception of a rare spontaneous NCGN disease model in mice, artificial interventions are needed to mimic at least some histopathology features in the animal, like planting the self-antigen in the organ vasculature in combination with transfer of a reactive T-cell clone (51,61). Due to their inherent limitations, animal models failed in resolving the role of genetics in AAV, in understanding the pathogenic and immunopathology differences between AAV subtypes and the interplay between innate and adaptive immune response resulting in loss of tolerance, and finally in predicting therapy efficacy in humans (35). Anti-TNFalpha drugs function as an example case to illustrate the discrepancy between promising results in an animal model and actual clinical trial results in human patients. While in a murine model for anti-MPO antibody-induced NCGN, selective blocking of TNFalpha attenuated disease severity, convincing clinical evidence for a therapeutic role of TNF-alpha blockade in humans could not been obtained (45,67).

The most important high priority disease questions that need to be answered to enable development of more effective (targeted) therapies are listed in Table 1, and center around elucidation of mechanisms underlying pathogenesis and pathophysiology (68).

Table 1. Some questions on AAV which remain to be answered, and where the described human model systems are expected to be instrumental.

1. Pathogenesis and pathophysiology:
    a. Which genetic variations in combination with environmental factors influence clinical and histopathology presentation, including organ location, progression and response to therapy?
    b. What causes chronic and relapsing disease: repeated external disease triggers, or an internal self-propagating inflammatory process?
    c. What are the autoimmune mechanisms that cause loss of tolerance?
    d. What determines the specific organ localizations of the autoimmune reaction?
    e. What is the mechanism behind endothelial inflammation?

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f. What is the mechanism behind granulomatous inflammation?
g. Which pathogenic role play self-antigens MPO, PR3 and LAMP and are there other relevant self-antigens?
h. What is the mechanism behind presence of self-antigens in the endothelial wall?
i. Is the type of ANCA (IgG, IgM, IgA) important?
j. What is the role of immune complexes and complement activation?

2. Diagnosis: Can a better diagnostic classification be developed based on genetics and immunopathology?
3. Disease monitoring: Which biomarkers can be used to reliably monitor disease status and therapy response?
4. Drug development: Can disease-specific drug targets be found for drug development, for example to induce local resolution of the auto-inflammatory process?

Towards new human investigational approaches for ANCA

To overcome the limitations of currently used animal and clinical patient investigational tools, we propose a switch towards developing human cell culture-based AAV models-on-chip. Depending on the research question to be answered a relatively simple or more complex model can be built and used. Investigation of mechanisms involved in end-organ damage caused by circulating autoantibodies may require a relative simple model consisting of a blood vessel on-a-chip which can for example be perfused with blood derived neutrophils and ANCAs. In contrast, a question regarding the pathogenesis of an AAV disease will require more complex integration of immune system tissue modules on the chip.

Ideally models for the various clinical forms of AAV vasculitis should contain surrogate microvascular blood vessel endothelium on a chip representative for the small blood vessels that are involved in the respective disease variant, like renal mesangial endothelium or lung alveolar endothelium, and include pericytes and macrophages surrounding the endothelial vessel structures (69,70). If necessary and possible, the endothelial cell culture can be combined with the relevant organ-specific cells, either in 2D or 3D culture. Human iPS cells have already been successfully differentiated to endothelial cells and associated pericytes and used to construct a blood vessel in a microfluidic channel (26,71) (Figure 2). Similarly, many relevant organ cell types can already be generated from iPS cells, like lung alveolar and

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kidney glomerular epithelial cells (72,73). A model to investigate end-organ disease could thus be built on the basis of an iPS cell line derived from a patient with a form of AAV vasculitis. For example a dual compartment microfluidic chip can be used to culture properly differentiated endothelial cells and pericytes in one compartment on a micropore-containing membrane separating the two compartments, while organ tissue cells can be grown on the other side of the membrane in the second culture compartment (24) (Figure 1d). Specific physical factors necessary to more closely mimic endothelial and organ-specific cell function *in vivo* can be introduced in the device, e.g. continuous fluid flow-induced shear stress, the appropriate stiffness of the membrane substrate, and for example repeated stretching in the case of alveolar endothelium (23,24,74). Inflammatory blood cells like neutrophils and monocytes can be either obtained from patient blood or generated by using the proper *in vitro* differentiation protocols described for iPS cells (75). Relevant cells in combination with selected circulating factors, for example ANCAs, complement and inflammatory cytokines, can be added to the perfusion fluid of the endothelial compartment to mimic a specific disease situation. The porous membrane allows transendothelial migration of adhering inflammatory cells into the other culture compartment (24, and Philips Research unpublished results) (Figure 1e). To validate the disease model as being representative for the AAV disease, histo- and immunopathology analysis and comparison with *in vivo* histopathology is required. Such a long-term culture model could be used to investigate cause-effect relationships with respect to clinical manifestations of the disease, including effects of genetic variations and inflammatory disease triggers. Multiple variables can be manipulated over time, for example perfusion with neutrophil priming factors, one-time or repeated exposure to ANCA antibody, a specific gene knock-down, or addition of drugs. In addition to conventional analyses, results of such manipulations can be investigated using real time readout technologies used in organ-on-chip studies, like 3D fluorescent imaging, time lapse microscopy, sampling and analysis of the microfluidic channel contents (18).

For more complex models to more in depth investigate AAV disease pathogenesis, blood vessel structures will need to be connected by microchannels to a functional lymph node structure, enabling on the one hand circulation of relevant lymphocyte populations, monocytic cells and neutrophils, and on the other hand transport of macrophages to the lymph node structure. In addition, in case no primary immune cells from patients are used in the model, it will be essential to create a bone marrow stem cell niche as well as a functional thymus tissue equivalent on the chip. Efforts to *in vitro* differentiate pluripotent stem cells to

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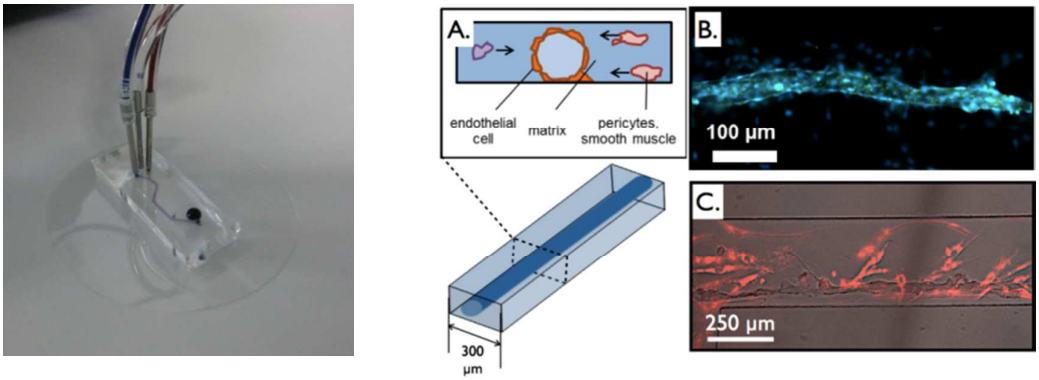
hematopoietic progenitor cells, and subsequently into the relevant white blood cell types (monocytes/macrophages, B- and T lymphocytes, natural killer cells) that are needed to generate such tissues have already been successful (75-80).

Figure 2



a. b.

Figure 2: Culture of a blood vessel on-a-chip. a. An example of a microfluidic chip with a single channel suited for endothelial cell culture with connections to a fluidic pump system (courtesy Andries van der Meer, Wyss Institute, Boston, US); b. Human iPS-derived in vitro model system for a blood vessel with live cell imaging of endothelial-pericyte interactions in a microfluidic channel: A, schematic cross-section of the microfluidic channel; B and C, live imaging of the 3D endothelial blood vessel structure cultured within the microfluidic channel, consisting of human iPS derived endothelial cells (B, blue) surrounded by pericytes (C, red) (unpublished results, courtesy Valeria Orlova and Christine Mummery, Leiden University Medical Center, The Netherlands).

Building immune tissue on-a-chip

To culture a miniature lymph node structure on a chip to mimic functional interaction between the innate and adaptive immune system, antigen presenting cells, T and B cells need to be present in the right cellular, biochemical and physical microenvironment. Giese at al. recently wrote a comprehensive review on how to culture human immune-competent non-lymphoid 3D organ tissue and "professional" lymphoid 3D immune organs on a chip (5). They have developed *in vitro* human 3D lymph node structures, showing functional dendritic cell – B/T lymphocyte interaction. The "lymph node" consists of a 3D hydrogel matrix containing dendritic cells, which is continuously perfused through oxygen-permeable

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microchannels, mimicking blood flow through a lymph node (81,82). B and T cells continuously cycle and migrate to contact the dendritic cells that are embedded in the matrix. Using this system, follicle-like structures developed upon antigen exposure, associated with proliferation of activated immune cells that produced IgM type antibodies, however IgG class switching was not yet observed. Envisioned improvements aim at more closely mimicking the biochemical and physical environment of the immune cells in their 3D matrix, and controlling the cell-containing compartments such that B and T cells can form proper germinal centers (5). Second, proper lymph node endothelium should be introduced into the culture system, and preferably sensors to monitor local metabolic conditions, like pO2, pH, electrolytes.

An initial 3D cell culture model for bone marrow has also been described, where (mouse) bone marrow-derived cells in suspension are flown through a special 3D porous synthetic matrix in a small bioreactor to allow them to attach and form a functional co-culture between mesenchymal stromal and hematopoetic cells, including multipotent progenitor cells (83). Also a functional hematopoietic human bone marrow model-on-chip was developed in which a bone matrix was first engineered *in vivo*, and subsequently transplanted to function as bone marrow niche in an organ-on-chip device (84). More recently functional thymus epithelial cells were generated *in vitro* by direct reprogramming of fibroblasts using forced expression of the FOXN1 transcription factor. These thymus epithelial cells successfully differentiated to thymocytes *in vitro*, and were capable of forming fully functional thymus tissue when grafted *in vivo* in mice, providing important proof of principle for the creation of functioning human thymus tissue on a chip (85).

Probabilistic Bayesian network models for AAV

In addition to the *in vitro* organ-on-chip AAV models, knowledge-based Bayesian computational models as described earlier can be built for the different forms of AAV disease by creating a high confident basic model framework incorporating qualitative and quantitative parameters describing well established causal immunopathology relationships, like for example disease triggers and ANCA antibodies that are well known to elicit neutrophil activation (Figure 3). For disease-associated variables, like the gene variant SERPINA-1, of which the pathogenic role is not well understood the causal disease relationship needs to be experimentally established prior to integration of the information into

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the computational model. This can be obtained for example by gene-targeting in iPS cells to either introduce or correct the SERPINA-1 gene variant, enabling characterization of its pathogenic role using an organ-on-chip model for AAV disease (86, 87). One can go back and forth between *in vitro* and *in silico* (computational) models, adding new knowledge to the computational model in the form of "nodes" and associated parameters obtained by experimental interventions in AAV on-a-chip models, and re-calibrating the model on the organ-on-chip model system and if possible also on actual patient data. The Bayesian network model can integrate such relatively stand-alone experimentally defined relationships to evolve towards a more complete AAV disease model, which can be interrogated to identify new disease relationships, or used to enter experimental or patient data, for example to investigate the therapeutic effect of a drug. The simple Bayesian network model for AAV disease (figure 3) can already be used to illustrate why efficacy of a drug like anti-TNFalpha in an experimental animal model for kidney vasculitis is unlikely to reliably predict efficacy in human disease, given the complex and largely unknown influence of the human immune system on the disease.

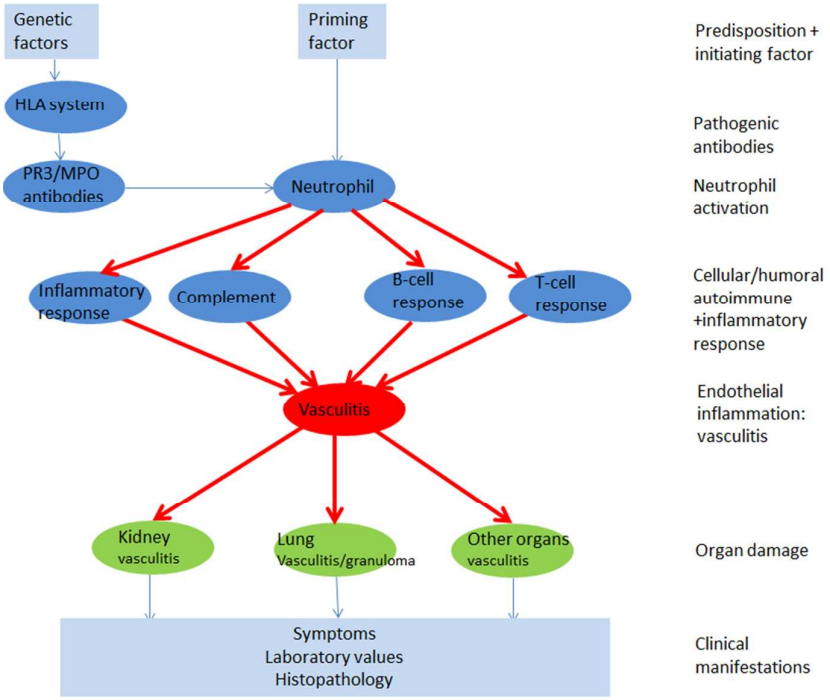

Figure 3. A conceptual (necessarily simplified) example for a probabilistic Bayesian network for ANCA vasculitis, built on qualitative causal relationships. Light blue boxes contain examples of measurable (qualitative or quantitative) variables obtained from patients. Blue "knowledge" nodes represent potential contributions of the immune system to the vasculitis process. Green nodes represent vasculitis-induced organ disease, causing the measured patient variables in the box below.

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The central red vasculitis node will provide the probability of a form of ANCA vasculitis based on integration of blue and green node information. Red arrows indicate causal relationships where knowledge is still lacking (also see Table 1). Parameters for quantitative inference should be added based on validated disease knowledge, for example the quantitative distribution of a specific biomarker measurement in blood in healthy and diseased individuals, or obtained from preclinical experimental data, e.g. from experiments in an organ-on-chip disease model. Additional nodes with computational parameters and variables (substructures) can be added. The text on the right side summarizes the vasculitis process that is represented by the nodes on the left.

Combining in vitro and in silico human AAV disease models for drug development

In addition to providing models systems for discovery of human AAV drug targets and diagnostic biomarkers, the organ-on-chip approach offers the possibility to test drug compounds (and companion diagnostic tests for patient stratification) in a human disease model prior to starting a clinical trial with real patients. In the case of AAV, designated as orphan disease, commercial benefits of developing an AAV drug do not balance the large investments required for conventional drug development. In addition the very limited and heterogeneous patient population interferes with recruiting sufficient numbers of patients for clinical trials. The organ-on-chip approach will enable a "(pre-)clinical trial-on-chip" with as many "on-chip" patients as necessary, allowing unrestricted efficacy testing of drug compounds, including "off-label" drugs already approved and on the market for other diseases than AAV. Integration of both organ-on-chip and relevant patient data into a computational AAV disease model is envisioned to further improve prediction of drug efficacy in real human patients. This approach is expected to lead to more successful clinical trials, reduce costs and accelerate the process towards regulatory approval for novel AAV treatments.

Conclusion

We have discussed AAV vasculitis as an example autoimmune disease where novel human investigational techniques, in the form of *in vitro* organ-on-chip AAV disease models combined with knowledge-based Bayesian network approaches, carry the promise to fill in

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currently missing information on disease mechanisms and pathogenesis as "disease pathways". The "disease pathway" concept is in line with similar developments in toxicology research towards defining and characterizing toxin-associated *adverse outcome pathways* (AOPs) (88,89). Close collaboration between scientists from different disciplines, like engineering, physics, experimental and clinical immunology, stem cell technology, mathematics and bioinformatics, and finally clinical medicine, is a *sine qua non* to successfully design and develop these complex human models for auto-immune diseases, both *in vitro* and *in silico*. However the reward is high and we believe that their use will result in both identification of human drug targets and disease biomarkers for improved classification and diagnostics of patients; in addition "pre-clinical trials-on- chip" for drug efficacy testing will become possible. For development of novel therapies for rare autoimmune diseases like AAV this is expected to provide a unique benefit; all complemented by a sizeable decrease in the use of animal models.

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References

1. Zenewicz LA, Abraham C, Flavell RA, Cho JH. Unraveling the genetics of autoimmunity. *Cell.* 2010;140(6):791-7.
2. Khor B, Gardet A, Xavier RJ. Genetics and pathogenesis of inflammatory bowel disease. *Nature*. 2011;474(7351):307-17.
3. Visscher PM, Brown MA, McCarthy MI, Yang J. Five years of GWAS discovery. *Am J Hum Genet*. 2012 Jan 13;90(1):7-24.
4. Lyons PA, Rayner TF, Trivedi S, Holle JU, Watts RA, Jayne DR, Baslund B, Brenchley P, Bruchfeld A, Chaudhry AN, Cohen Tervaert JW, Deloukas P, Feighery C, Gross WL, Guillevin L, Gunnarsson I, Harper L, Hrušková Z, Little MA, Martorana D, Neumann T, Ohlsson S, Padmanabhan S, Pusey CD, Salama AD, Sanders JS, Savage CO, Segelmark M, Stegeman CA, Tesař V, Vaglio A, Wieczorek S, Wilde B, Zwerina J, Rees AJ, Clayton DG, Smith KG. Genetically distinct subsets within ANCA-associated vasculitis. *N Engl J Med*. 2012;367(3):214-23.
5. Giese C, Marx U. Human immunity in vitro - solving immunogenicity and more. *Adv Drug Deliv Rev*. 2014;69-70:103-22.

View Article Online
DOI: 10.1039/C4RA15794J

6. Feuk L, Carson AR, Scherer SW. Structural variation in the human genome. *Nat Rev Genet*. 2006;7(2):85-97.
7. Wu YL, Savelli SL, Yang Y, Zhou B, Rovin BH, Birmingham DJ, Nagaraja HN, Hebert LA, Yu CY. Sensitive and specific real-time polymerase chain reaction assays to accurately determine copy number variations (CNVs) of human complement C4A, C4B, C4-long, C4-short, and RCCX modules: elucidation of C4 CNVs in 50 consanguineous subjects with defined HLA genotypes. *J Immunol*. 2007;179(5):3012-25.
8. Orrù V, Steri M, Sole G, Sidore C, Virdis F, Dei M, Lai S, Zoledziewska M, Busonero F, Mulas A, Floris M, Mentzen WI, Urru SA, Olla S, Marongiu M, Piras MG, Lobina M, Maschio A, Pitzalis M, Urru MF, Marcelli M, Cusano R, Deidda F, Serra V, Oppo M, Pilu R, Reinier F, Berutti R, Pireddu L, Zara I, Porcu E, Kwong A, Brennan C, Tarrier B, Lyons R, Kang HM, Uzzau S, Atzeni R, Valentini M, Firinu D, Leoni L, Rotta G, Naitza S, Angius A, Congia M, Whalen MB, Jones CM, Schlessinger D, Abecasis GR, Fiorillo E, Sanna S, Cucca F. Genetic variants regulating immune cell levels in health and disease. *Cell*. 2013;155(1):242-56.
9. Seok J, Warren HS, Cuenca AG, Mindrinos MN, Baker HV, Xu W, Richards DR, McDonald-Smith GP, Gao H, Hennessy L, Finnerty CC, López CM, Honari S, Moore EE, Minei JP, Cuschieri J, Bankey PE, Johnson JL, Sperry J, Nathens AB, Billiar TR, West MA, Jeschke MG, Klein MB, Gamelli RL, Gibran NS, Brownstein BH, Miller-Graziano C, Calvano SE, Mason PH, Cobb JP, Rahme LG, Lowry SF, Maier RV, Moldawer LL, Herndon DN, Davis RW, Xiao W, Tompkins RG; Inflammation and Host Response to Injury, Large Scale Collaborative Research Program. Genomic responses in mouse models poorly mimic human inflammatory diseases. *Proc Natl Acad Sci U S A.* 2013;110(9):3507-12.
10. Lee MN, Ye C, Villani AC, Raj T, Li W, Eisenhaure TM, Imboywa SH, Chipendo PI, Ran FA, Slowikowski K, Ward LD, Raddassi K, McCabe C, Lee MH, Frohlich IY, Hafler DA, Kellis M, Raychaudhuri S, Zhang F, Stranger BE, Benoist CO, De Jager PL, Regev A, Hacohen N. Common genetic variants modulate pathogen-sensing responses in human dendritic cells. *Science*. 2014;343(6175):1246980.
11. Davis MM. A Prescription for Human Immunology. *Immunity*. 2008; 29(6): 835–838
12. Khanna R, Burrows SR. Human immunology: a case for the ascent of non-furry immunology. *Immunol Cell Biol*. 2011; 89(3):330-1.
13. Shultz LD, Brehm MA, Garcia-Martinez JV, Greiner DL. Humanized mice for immune system investigation: progress, promise and challenges. *Nat Rev Immunol.* 2012;12(11):786-98.
14. Li Y, Zhang K, Chen H, Sun F, Xu J, Wu Z, Li P, Zhang L, Du Y, Luan H, Li X, Wu L, Li H, Wu H, Li X, Li X, Zhang X, Gong L, Dai L, Sun L, Zuo X, Xu J, Gong H, Li Z, Tong S, Wu M, Li X, Xiao W, Wang G, Zhu P, Shen M, Liu S, Zhao D, Liu W, Wang Y, Huang C, Jiang Q, Liu G, Liu B, Hu S, Zhang W, Zhang Z, You X, Li M, Hao W, Zhao C, Leng X, Bi L, Wang Y, Zhang F, Shi Q, Qi W, Zhang X, Jia Y, Su J, Li Q, Hou Y, Wu Q, Xu D, Zheng W, Zhang M, Wang Q, Fei Y, Zhang X, Li J, Jiang Y, Tian X, Zhao L, Wang L, Zhou B, Li Y, Zhao Y, Zeng X, Ott J, Wang J, Zhang F.

View Article Online
DOI: 10.1039/C4RA15794J

A genome-wide association study in Han Chinese identifies a susceptibility locus for primary Sjögren's syndrome at 7q11.23. *Nat Genet*. 2013;45(11):1361-5.

15. Okada Y, Wu D, Trynka G, Raj T, Terao C, Ikari K, Kochi Y, Ohmura K, Suzuki A, Yoshida S, Graham RR, Manoharan A, Ortmann W, Bhangale T, Denny JC, Carroll RJ, Eyler AE, Greenberg JD, Kremer JM, Pappas DA, Jiang L, Yin J, Ye L, Su DF, Yang J, Xie G, Keystone E, Westra HJ, Esko T, Metspalu A, Zhou X, Gupta N, Mirel D, Stahl EA, Diogo D, Cui J, Liao K, Guo MH, Myouzen K, Kawaguchi T, Coenen MJ, van Riel PL, van de Laar MA, Guchelaar HJ, Huizinga TW, Dieudé P, Mariette X, Bridges SL Jr, Zhernakova A, Toes RE, Tak PP, Miceli-Richard C, Bang SY, Lee HS, Martin J, Gonzalez-Gay MA, Rodriguez-Rodriguez L, Rantapää-Dahlqvist S, Arlestig L, Choi HK, Kamatani Y, Galan P, Lathrop M; RACI consortium; GARNET consortium, Eyre S, Bowes J, Barton A, de Vries N, Moreland LW, Criswell LA, Karlson EW, Taniguchi A, Yamada R, Kubo M, Liu JS, Bae SC, Worthington J, Padyukov L, Klareskog L, Gregersen PK, Raychaudhuri S, Stranger BE, De Jager PL, Franke L, Visscher PM, Brown MA, Yamanaka H, Mimori T, Takahashi A, Xu H, Behrens TW, Siminovitch KA, Momohara S, Matsuda F, Yamamoto K, Plenge RM. Genetics of rheumatoid arthritis contributes to biology and drug discovery. *Nature*. 2014;506(7488):376-81.
16. Orozco G, Viatte S, Bowes J, Martin P, Wilson AG, Morgan AW, Steer S, Wordsworth P, Hocking LJ; UK Rheumatoid Arthritis Genetics Consortium; Wellcome Trust Case Control Consortium; Biologics in Rheumatoid Arthritis Genetics and Genomics Study Syndicate Consortium, Barton A, Worthington J, Eyre S. Novel rheumatoid arthritis susceptibility locus at 22q12 identified in an extended UK genome-wide association study. *Arthritis Rheumatol*. 2014;66(1):24-30.
17. Langley GR. Considering a new paradigm for Alzheimer's disease research. Drug *Discov Today*. 2014;19(8):1114-1124.
18. Van de Stolpe A, Den Toonder J. Workshop meeting report Organs-on-Chips: human disease models, *Lab Chip*, 2013, 13, 3449–3470.
19. van der Meer AD, van den Berg A. Organs-on-chips: breaking the in vitro impasse. *Integr Biol (Camb)*. 2012;4(5):461-70.
20. Huh D, Kim HJ, Fraser JP, Shea DE, Khan M, Bahinski A, Hamilton GA, Ingber DE. Microfabrication of human organs-on-chips. *Nat Protoc*. 2013;8(11):2135-57.
21. Pakazad Khoshfetrat S, Savov A, Van de Stolpe A, Dekker R. A novel Stretchable Micro-Electrode Array (SMEA) design for directional stretching of cells. *J Micromech Microeng* 24 (3);2014: 034003
22. Sonntag F, Schilling N, Mader K, Gruchow M, Klotzbach U, Lindner G, Horland R, Wagner I, Lauster R, Howitz S, Hoffmann S, Marx U. Design and prototyping of a chip-based multi-micro-organoid culture system for substance testing, predictive to human (substance) exposure. *J Biotechnol*. 2010;148(1):70-5.
23. Mammoto A, Mammoto T, Kanapathipillai M, Wing Yung C, Jiang E, Jiang A, Lofgren K, Gee EP, Ingber DE. Control of lung vascular permeability and endotoxin-induced pulmonary oedema by changes in extracellular matrix mechanics. *Nat Commun*. 2013;4:1759.

View Article Online
DOI: 10.1039/C4RA15794J

24. Bhatia SN, Ingber DE. Microfluidic organs-on-chips. *Nat Biotechnol*. 2014;32(8):760-72
25. Kanagasabapathi TT, Franco M, Barone RA, Martinoia S, Wadman WJ, Decré MM. Selective pharmacological manipulation of cortical-thalamic co-cultures in a dual-compartment device. *J Neurosci Methods.* 2013;214(1):1-8.
26. Orlova, VV, van der Meer AD , Ten Dijke P, van den Berg A, and Mummery CL. Three-dimensional co-cultures of human endothelial cells and embryonic stem cell-derived pericytes inside a microfluidic device. *Lab Chip* 2013;13(18):3562-8.
27. Takahashi K, Tanabe K, Ohnuki M, Narita M, Ichisaka T, Tomoda K, Yamanaka S. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell.* 2007;131(5):861-72.
28. Sato T, Clevers H. Growing self-organizing mini-guts from a single intestinal stem cell: mechanism and applications. *Science*. 2013;340(6137):1190-4.
29. Mummery C, Van de Stolpe A, Clevers H, Roelen BAJ. Stem Cells. *Scientific Facts and Fiction.* USA: Elsevier ISBN 978-0-12-381535-; 2014
30. Bellin M, Marchiotti C, Gage F, Mummery CL. Induced pluripotent stem cells: the new patient? *Nat Rev Mol Cell Biol.* 2012;13(11):713-26
31. Chen BC, Legant WR, Wang K, Shao L, Milkie DE, Davidson MW, Janetopoulos C, Wu XS, Hammer JA 3rd, Liu Z, English BP, Mimori-Kiyosue Y, Romero DP, Ritter AT, Lippincott-Schwartz J, Fritz-Laylin L, Mullins RD, Mitchell DM, Bembenek JN, Reymann AC, Böhme R, Grill SW, Wang JT, Seydoux G, Tulu US, Kiehart DP, Betzig E. Lattice light-sheet microscopy: imaging molecules to embryos at high spatiotemporal resolution. *Science.* 2014;346(6208):1257998.
32. Chang R, Shoemaker R, Wang W. A novel knowledge-driven systems biology approach for phenotype prediction upon genetic intervention. *IEEE/ACM Trans Comput Biol Bioinform*. 2011;8(5):1170-82.
33. Verhaegh W, Van de Stolpe A, Knowledge-based computational models. *Oncotarget*. 2014;5(14):5196-7.
34. Verhaegh W, Van Ooijen H, Inda MA, Hatzis P, Versteeg R, Smid M, Martens J, Foekens J, Clevers H, Van de Stolpe A. Selection of personalized patient therapy through the use of knowledge-based computational models that identify tumor-driving signal transduction pathways. *Cancer Res* 2014;74(11):2936-45.
35. Jennette JC, Falk RJ Pathogenesis of antineutrophil cytoplasmic autoantibody-mediated disease. *Nat Rev Rheumatol*. 2014;10(8):463-73.
36. Lally L, Spiera R Current Therapies for ANCA-Associated Vasculitis. Annu Rev Med. 2015 (66):227-40.
37. Kain R, Exner M, Brandes R, Ziebermayr R, Cunningham D, Alderson CA, Davidovits A, Raab I, Jahn R, Ashour O, Spitzauer S, Sunder-Plassmann G, Fukuda M, Klemm P, Rees AJ, Kerjaschki D. Molecular mimicry in pauci-immune focal necrotizing glomerulonephritis. *Nat Med*. 2008;14(10):1088-96.
38. Roth AJ, Brown MC, Smith RN, Badhwar AK, Parente O, Chung Hc, Bunch DO, McGregor JG, Hogan SL, Hu Y, Yang JJ, Berg EA, Niles J, Jennette JC, Preston GA, Falk RJ. Anti-LAMP-2 antibodies are not prevalent in patients with antineutrophil cytoplasmic autoantibody glomerulonephritis. *J Am Soc Nephrol.* 2012;23(3):545-55.

View Article Online
DOI: 10.1039/C4RA15794J

39. Peschel A, Basu N, Benharkou A, Brandes R, Brown M, Dieckmann R, Rees AJ, Kain R. Autoantibodies to hLAMP-2 in ANCA-negative pauci-immune focal necrotizing GN. J Am Soc Nephrol. 2014;25(3):455-63.
40. Cao Y, Schmitz JL, Yang J, Hogan SL, Bunch D, Hu Y, Jennette CE, Berg EA, Arnett FC Jr, Jennette JC, Falk RJ, Preston GA. DRB1*15 allele is a risk factor for PR3-ANCA disease in African Americans. *J Am Soc Nephrol*. 2011;22(6):1161-7.
41. Schreiber A, Otto B, Ju X, Zenke M, Goebel U, Luft FC, Kettritz R. Membrane proteinase 3 expression in patients with Wegener's granulomatosis and in human hematopoietic stem cell-derived neutrophils. J Am Soc Nephrol. 2005;16(7):2216-24.
42. Stegeman CA, Tervaert JW, Sluiter WJ, Manson WL, de Jong PE, Kallenberg CG. Association of chronic nasal carriage of Staphylococcus aureus and higher relapse rates in Wegener granulomatosis. *Ann Intern Med*. 1994;120(1):12-7.
43. Tervaert JW, Stegeman CA, Kallenberg CG. Silicon exposure and vasculitis. *Curr Opin Rheumatol*. 1998;10(1):12-7.
44. Slot MC, Links TP, Stegeman CA, Tervaert JW. Occurrence of antineutrophil cytoplasmic antibodies and associated vasculitis in patients with hyperthyroidism treated with antithyroid drugs: A long-term followup study. *Arthritis Rheum*. 2005;53(1):108-13.
45. Huugen D, Xiao H, van Esch A, Falk RJ, Peutz-Kootstra CJ, Buurman WA, Tervaert JW, Jennette JC, Heeringa P. Aggravation of anti-myeloperoxidase antibody-induced glomerulonephritis by bacterial lipopolysaccharide: role of tumor necrosis factor-alpha. *Am J Pathol*. 2005;167(1):47-58.
46. Huugen D, van Esch A, Xiao H, Peutz-Kootstra CJ, Buurman WA, Tervaert JW, Jennette JC, Heeringa P. Inhibition of complement factor C5 protects against anti-myeloperoxidase antibody-mediated glomerulonephritis in mice. *Kidney Int*. 2007;71(7):646-54.
47. Xiao H, Heeringa P, Hu P, Liu Z, Zhao M, Aratani Y, Maeda N, Falk RJ, Jennette JC. Antineutrophil cytoplasmic autoantibodies specific for myeloperoxidase cause glomerulonephritis and vasculitis in mice. *J Clin Invest*. 2002;110(7):955-63.
48. Xiao H, Heeringa P, Liu Z, Huugen D, Hu P, Maeda N, Falk RJ, Jennette JC. The role of neutrophils in the induction of glomerulonephritis by anti-myeloperoxidase antibodies. *Am J Pathol*. 2005;167(1):39-45.
49. Mayet WJ, Csernok E, Szymkowiak C, Gross WL, Meyer zum Büschenfelde KH. Human endothelial cells express proteinase 3, the target antigen of anticytoplasmic antibodies in Wegener's granulomatosis. *Blood.* 1993;82(4):1221-9.
50. Kessenbrock K, Krumbholz M, Schönermarck U, Back W, Gross WL, Werb Z, Gröne HJ, Brinkmann V, Jenne DE. Netting neutrophils in autoimmune small-vessel vasculitis. *Nat Med*. 2009;15(6):623-5.
51. Ooi JD, Chang J, Hickey MJ, Borza DB, Fugger L, Holdsworth SR, Kitching AR. The immunodominant myeloperoxidase T-cell epitope induces local cell-mediated injury in antimyeloperoxidase glomerulonephritis. *Proc Natl Acad Sci U S A*. 2012;109(39):E2615-24.
52. Little MA, Al-Ani B, Ren S, Al-Nuaimi H, Leite M Jr, Alpers CE, Savage CO, Duffield JS. Anti-proteinase 3 anti-neutrophil cytoplasm autoantibodies recapitulate

View Article Online
DOI: 10.1039/C4RA15794J

systemic vasculitis in mice with a humanized immune system. *PLoS One*. 2012;7(1):e28626.
53. Halbwachs L, Lesavre P. Endothelium-neutrophil interactions in ANCA-associated diseases. *J Am Soc Nephrol.* 2012;23(9):1449-61.
54. Xiao H, Ciavatta D, Aylor DL, Hu P, de Villena FP, Falk RJ, Jennette JC. Genetically determined severity of anti-myeloperoxidase glomerulonephritis. Am J Pathol. 2013;182(4):1219-26.
55. Boomsma MM, Stegeman CA, van der Leij MJ, Oost W, Hermans J, Kallenberg CG, Limburg PC, Tervaert JW. Prediction of relapses in Wegener's granulomatosis by measurement of antineutrophil cytoplasmic antibody levels: a prospective study. *Arthritis Rheum.* 2000;43(9):2025-33.
56. Tomasson G, Grayson PC, Mahr AD, Lavalley M, Merkel PA. Value of ANCA measurements during remission to predict a relapse of ANCA-associated vasculitis--a meta-analysis. *Rheumatology (Oxford)*. 2012;51(1):100-9.
57. Guillevin L, Durand-Gasselin B, Cevallos R, Gayraud M, Lhote F, Callard P, Amouroux J, Casassus P, Jarrousse B. Microscopic polyangiitis: clinical and laboratory findings in eighty-five patients. *Arthritis Rheum*. 1999;42(3):421-30.
58. Little MA, Smyth CL, Yadav R, Ambrose L, Cook HT, Nourshargh S, Pusey CD.Antineutrophil cytoplasm antibodies directed against myeloperoxidase augment leukocyte-microvascular interactions in vivo. *Blood.* 2005;106(6):2050-8.
59. Roth AJ, Ooi JD, Hess JJ, van Timmeren MM, Berg EA, Poulton CE, McGregor J, Burkart M, Hogan SL, Hu Y, Winnik W, Nachman PH, Stegeman CA, Niles J, Heeringa P, Kitching AR, Holdsworth S, Jennette JC, Preston GA, Falk RJ. Epitope specificity determines pathogenicity and detectability in ANCA-associated vasculitis. *J Clin Invest*. 2013;123(4):1773-83.
60. Cohen, I. Autoantibody repertoires, natural biomarkers, and system controllers. *Trends in Immunology.* 2013; 34(12):620-5
61. Neumann I, Birck R, Newman M, Schnülle P, Kriz W, Nemoto K, Yard B, Waldherr R, Van Der Woude FJ. SCG/Kinjoh mice: a model of ANCA-associated crescentic glomerulonephritis with immune deposits. Kidney Int. 2003;64(1):140-8.
62. Haas M, Eustace JA. Immune complex deposits in ANCA-associated crescentic glomerulonephritis: a study of 126 cases. *Kidney Int.* 2004;65(6):2145-52.
63. Xiao H, Schreiber A, Heeringa P, Falk RJ, Jennette JC. Alternative complement pathway in the pathogenesis of disease mediated by anti-neutrophil cytoplasmic autoantibodies. *Am J Pathol*. 2007;170(1):52-64.
64. Xing GQ, Chen M, Liu G, Heeringa P, Zhang JJ, Zheng X, E J, Kallenberg CG, Zhao MH. Complement activation is involved in renal damage in human antineutrophil cytoplasmic autoantibody associated pauci-immune vasculitis. *J Clin Immunol.* 2009;29(3):282-91.
65. Gou SJ, Yuan J, Chen M, Yu F, Zhao MH. Circulating complement activation in patients with anti-neutrophil cytoplasmic antibody-associated vasculitis. *Kidney Int.* 2013;83(1):129-37.
66. Coughlan AM, Freeley SJ, Robson MG. Animal models of anti-neutrophil cytoplasmic antibody-associated vasculitis. *Clin Exp Immunol.* 2012;169(3):229-37.

View Article Online
DOI: 10.1039/C4RA15794J

67. Morgan MD, Drayson MT, Savage CO, Harper L. Addition of infliximab to standard therapy for ANCA-associated vasculitis. *Nephron Clin Pract.* 2011;117:c89-97.
68. Millet A, Pederzoli-Ribeil M, Guillevin L, Witko-Sarsat V, Mouthon L. Antineutrophil cytoplasmic antibody-associated vasculitides: is it time to split up the group? *Ann Rheum Dis.* 2013;72(8):1273-9.
69. Aird WC. Phenotypic heterogeneity of the endothelium: I. Structure, function, and mechanisms. *Circ Res.* 2007;100(2):158-73.
70. Aird WC. Phenotypic heterogeneity of the endothelium: II. Representative vascular beds. *Circ Res.* 2007;100(2):174-90.
71. Orlova VV, van den Hil FE, Petrus-Reurer S, Drabsch Y, Ten Dijke P, Mummery CL. Generation, expansion and functional analysis of endothelial cells and pericytes derived from human pluripotent stem cells. *Nat Protoc*. 2014 Jun;9(6):1514-31.
72. Gotoh S, Ito I, Nagasaki T, Yamamoto Y, Konishi S, Korogi Y, Matsumoto H, Muro S, Hirai T, Funato M, Mae S, Toyoda T, Sato-Otsubo A, Ogawa S, Osafune K, Mishima M. Generation of Alveolar Epithelial Spheroids via Isolated Progenitor Cells from Human Pluripotent Stem Cells. *Stem Cell Reports*. 2014;3(3):394-403.
73. Kang M, Han YM. Differentiation of human pluripotent stem cells into nephron progenitor cells in a serum and feeder free system. *PLoS One.* 2014;9(4):e94888.
74. Remuzzi A, Dewey CF Jr, Davies PF, Gimbrone MA Jr. Orientation of endothelial cells in shear fields in vitro. *Biorheology.* 1984;21(4):617-30.
75. Choi KD, Vodyanik MA, Slukvin II. Generation of mature human myelomonocytic cells through expansion and differentiation of pluripotent stem cell-derived lin-CD34+CD43+CD45+ progenitors. *J Clin Invest.* 2009 Sep;119(9):2818-29.
76. Schmitt TM, de Pooter RF, Gronski MA, Cho SK, Ohashi PS, Zúñiga-Pflücker JC. Induction of T cell development and establishment of T cell competence from embryonic stem cells differentiated in vitro. *Nat Immunol.* 2004 Apr;5(4):410-7.
77. Raya A, Rodríguez-Pizà I, Guenechea G, Vassena R, Navarro S, Barrero MJ, Consiglio A, Castellà M, Río P, Sleep E, González F, Tiscornia G, Garreta E, Aasen T, Veiga A, Verma IM, Surrallés J, Bueren J, Izpisúa Belmonte JC. Disease-corrected haematopoietic progenitors from Fanconi anaemia induced pluripotent stem cells. *Nature.* 2009;460(7251):53-9.
78. Carpenter L, Malladi R, Yang CT, French A, Pilkington KJ, Forsey RW, Sloane-Stanley J, Silk KM, Davies TJ, Fairchild PJ, Enver T, Watt SM. Human induced pluripotent stem cells are capable of B-cell lymphopoiesis. *Blood.* 2011;117(15):4008-11.
79. Van Wilgenburg B, Browne C, Vowles J, Cowley SA. Efficient, long term production of monocyte-derived macrophages from human pluripotent stem cells under partly-defined and fully-defined conditions. *PLoS One.* 2013;8(8):e71098.
80. Ni Z, Knorr DA, Kaufman DS. Hematopoietic and nature killer cell development from human pluripotent stem cells. *Methods Mol Biol*. 2013;1029:33-41.
81. Giese C, Demmler CD, Ammer R, Hartmann S, Lubitz A, Miller L, Müller R, Marx U. A human lymph node in vitro-challenges and progress. *Artif Organs*. 2006; 30(10):803-8.

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82. Giese C, Lubitz A, Demmler CD, Reuschel J, Bergner K, Marx U. Immunological substance testing on human lymphatic micro-organoids in vitro. J Biotechnol. 2010; 148(1):38-45.
83. Di Maggio N, Piccinini E, Jaworski M, Trumpp A, Wendt DJ, Martin I. Toward modeling the bone marrow niche using scaffold-based 3D culture systems. *Biomaterials*. 2011;32(2):321-9.
84. Torisawa YS, Spina CS, Mammoto T, Mammoto A, Weaver JC, Tat T, Collins JJ, Ingber DE. Bone marrow-on-a-chip replicates hematopoietic niche physiology in vitro. *Nat Methods*. 2014 Jun;11(6):663-9.
85. Bredenkamp N, Ulyanchenko S, O'Neill KE, Manley NR, Vaidya HJ, Blackburn CC. An organized and functional thymus generated from FOXN1-reprogrammed fibroblasts. *Nat Cell Biol*. 2014;16(9):902-8.
86. Braam SR, Denning C, Mummery CL. Genetic manipulation of human embryonic stem cells in serum and feeder-free media. *Methods Mol Biol.* 2010;584:413-23.
87. Xue H, Wu J, Li S, Rao MS, Liu Y. Genetic Modification in Human Pluripotent Stem Cells by Homologous Recombination and CRISPR/Cas9 System. *Methods Mol Biol.* 2014. [Epub ahead of print]
88. Collins FS. Reengineering translational science: The time is right. *Sci Transl Med* 2011;3(90):90cm17
89. Vinken M. The adverse outcome pathway concept: a pragmatic tool in toxicology. *Toxicology*. 2013;312:158-65.