Netherlands National Committee
for the protection of animals
used for scientific purposes

Ambition statement on animal free innovations in Immunology

The Netherlands National Committee for the protection of animals used for scientific purposes

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To energize the transition within Immunology

Immunological research is aimed at understanding molecular and cellular processes that underlie pathologies of human diseases as well as infections, and to develop interventions to treat or prevent these diseases and infections. This encompasses the fundamental and applied science of immunological processes at the level of molecules, cells, tissues and the organism. Animal models have been, and are still being, used to model many of these processes[1]. However, it is becoming increasingly clear that animal models are indeed models and importantly, they might not even be the best models. The field of immunology moves very fast and new technological and medical advances have led to the development of innovative human models with the potential to replace animal models. These non-animal, or new approach methodologies (NAMs) will allow us to investigate immunological processes in much more detail and with more relevance for human diseases and infections[2].

The aim of the Immunology workgroup was to develop a road map that will energize the transition towards the use of NAMs in immunology. Development and use of NAMs will not only reduce animal use and suffering, but -as stated- also has the inherent potential to better model human immunology and disease than animal models do. These insights form the basis of this Vision, and recommendations are given for the development, improvement and implementation of NAMs to improve science, while at the same time reducing our scientific dependance on animals.

We have approached this by first identifying the types of NAMs that are currently used and/or are being developed in immunology research. We have analyzed the benefits but also the limitations of NAMs and have used this to develop targets for the near future as well as for the not-so-near future. Finally, we have identified the most important barriers for implementation of NAMs and discuss how these can be addressed and overcome.

[1] Zo doende 2020 - Jaaroverzicht dierproeven en proefdieren van de Nederlandse Voedsel- en Warenautoriteit. Nederlandse Voedsel- en Warenautoriteit; 2022.

[2] van der Zalm AJ, Barroso J, Browne P, Casey W, Gordon J, Henry TR, et al. A framework for establishing scientific confidence in new approach methodologies. Arch Toxicol. 2022;96(11):2865-79.

Index

1. Recommendations, an overview

Regulations and decision making

Volunteer or patient studies are becoming more feasible due to the increasing availability of more sensitive (non-invasive and routine) analytical methods. Knowledge about the possibilities for these kinds of studies should be shared and promoted. Most importantly ethical restrictions and bureaucratic **regulatory procedures should be simplified where possible and preparations to conduct direct-in-man and phase 0 studies supported to more directly compete with the use of animal-based approaches**.

Animal usage should be more critically assessed by the institutes as well as by the researchers themselves as more suitable models might exist to answer specific research questions. Initiatives to **facilitate access to information about NAMs** should be stimulated, and access to such information should be easily **available to animal welfare bodies and ethical committees**.

National and Health Organization funding is essential to achieve the transition goals described in this target image. Therefore, more **personal fellowships and grants for young as well as senior scientists that are committed to developing or using NAMs** should be created by funding bodies as well as the funding for large consortia. This should lead to a **career path** in life sciences that is not dependent on animal-based results.

Write and disseminate to researchers a **document with strong clear arguments concerning the physiological relevance of human studies performed without animals but with NAMs**. This document can be used to help the researchers to refute and withstand editorial pressure to conduct animal studies. A standard letter with these arguments signed by people, societies and institutes with authority, should also help researchers.

Education

One of the most important recommendations is to stimulate the **training and education of early career scientists**. Universities and post-doctoral courses should inform and teach on NAMs and on advances in the field (see also Ambition statement on innovation in higher education)[3].

To **help uptake and implementation of the Ambition Statement, cooperation and embedding should be sought** with the Dutch Society for Immunology (NVVI[4]). Young researchers should be made aware that NAMs can be a better alternative than animal studies to answer specific immunological questions, and that new technologies even increase the potential of NAMs over animal studies. Structural attention for NAMs should therefore be embedded within the yearly Dutch Immunology conference.

Sharing and access

Many different immune cell line-based models exist that allow for genetic manipulation and robust analyses. Innovative techniques allow for genetic manipulation and prolonged culturing of primary human immune cells, which makes the use of primary cells in immunological models more attractive and feasible. Therefore, access to human primary immune cells (e.g. blood cells) as well as tissues needs to be **facilitated for every researcher via open access biobank facilities** (for (plastic) surgically removed tissues) **and centralized facilities** (bloodbanks, tissue centers). Shortage of tissue availability hampers the transitions[5].

Innovative analytical techniques and methods provide a plethora on data from patients (genes, proteins, metabolism). **Access and re-use of patient data** will allow other researchers to use this data for their own studies. Already regulations such as Fair Science are in place but further efforts should be made to facilitate access and correct use of these data.

[3] Ambition statement on innovation in higher education using fewer laboratory animals. Universiteiten van Nederland (UNL), Nederlandse Federatie van Universitair Medische Centra (NFU); 2022.

[4] NVVI [Available from: https://www.nvvi-dsi.nl/.

[5] De beschikbaarheid en toegankelijkheid van menselijk weefsel voor biomedisch onderzoek en onderwijs. Nationaal Comité advies dierproevenbeleid; 2023. Report No.: 23400365.

Research

The use of **animal-derived products as cell culture reagents** (e.g. fetal calf serum, growth factors, enzymes, matrigels, and antibodies) **should be limited as much as possible** for ethical and scientific reasons[6, 7, 8]. Suitable non-animal derived replacements are available, but researchers are reluctant to switch to other models and costs are often higher. Therefore, **centralized efforts and specific funding opportunities** are required.

Stimulate direct side-by-side comparisons between animals and NAMs by bringing together scientists and by **specific funding of parallel studies**. (see NCad Parallelstudies advice)
Institutes should have a NAM core facility besides an animal facility to train and facilitate animal-free studies as well as to further develop NAMs to replace less suitable animal models, or to initiate parallel studies. Close connection with the animal facility would help deciding on the best model and facilitate parallel studies.

Provide support for studies aiming to understand translational gaps between animal models and the human disease that is modelled. If animal models have failed to predict human responses, introduce the researcher to networks focusing on modeling human disease in NAMs such as hDMT[9].

Discourage researchers to use animals as **'black box" models for diseases** for which the causes are not exactly known. **Encourage** researchers (if necessary via ethical committees) to **rewrite research questions towards understanding of human (pathological) mechanisms** instead. NAMs, of varying complexity, should be considered to answer relevant mechanistic research questions leading to pathology.

Stimulate further interdisciplinary collaborations as different fields are required to further develop innovative NAMs. Collaborations with the national human organ and disease model technologies consortium (hDMT) will enable immunologists to work

[6] Sebastian Eggert JW, Jessica Rosolowski & Tilo Weber. Practical Workshop on Replacing Fetal Bovine Serum (FBS) in Life Science Research: From Theory into Practice. ALTEX; 2022.

[7] Kozlowski MT, Crook CJ, Ku HT. Towards organoid culture without Matrigel. Commun Biol. 2021;4(1).

[8] Bradbury ARM, Dübel S, Knappik A, Plückthun A. Animal- versus in vitro-derived antibodies: avoiding the extremes. Mabs-Austin. 2021;13(1).

[9] hDMT [Available from: www.hdmt.tecchnology

together in a multidisciplinary setting with cell biologists, (tissue-)engineers and industry to further develop the models and aid implementation.

High end culture models such as iPSCs, organs-on-a-chip, whole tissue and organoid cultures are extremely important models to address immunological mechanisms and immune pathologies. However, complicated protocols and specialized knowledge prevent widespread use of these models. **Specialized Core Facilities or Centers are required** that train and assist (young) researchers in these innovative models.

2.
Immunology to treat and prevent infections, inflammatory diseases and cancer

The field of immunology studies how we cope with invasive infectious challenges that are continuously present in our surroundings. It also studies how the immune system becomes derailed leading to autoimmune diseases and allergies and how the immune system can be employed to fight diseases such as cancer in an immunotherapy setting.

How does the immune system work? There are multiple mechanisms to protect us from pathogens. Our body barriers, created by the skin, lungs, and gastrointestinal tract make invasion difficult. However, if these barriers are breached, cells belonging to the innate immune system immediately respond to the danger. This is followed by cells from the adaptive immune system becoming activated, maturing, multiplying and most importantly having the ability to specifically remember the danger so that they can immediately respond if exposed to the same danger in the future.

As a general rule, cells of the immune system are present in every organ, and fluids like blood and lymph are used as a means of transport to bring together the different cell types that are necessary to initiate an immune response. Different tasks are delegated to different cell types and different organs, with effective and precise communication being extremely important. Important to note is that every individual is equipped with a genetically unique setup that determines their personal response ('nature'). To make things even more complex, this response is not static and changes during life, depending on age and previous experiences ('nurture').

Animal models in Immunology

Immunologists study the mechanisms by which the immune system develops, which different cell types exist and how these are functionally related. From a more applied perspective, immunologists are trying to harness this knowledge to combat infectious diseases and cancer by developing vaccines, and to prevent or cure auto-immune diseases or allergies by reprogramming the immune system or by restoring the healthy balance. There are many different challenges to investigate human immune responses in health and diseases (see Table 1). Animal models have taught us a tremendous lot about immune mechanisms and disease pathogenesis.

For research in immunology, animals or animal-derived products are being used for different purposes. Animal-derived products as antibodies, serum, enzymes and matrigels can be found in every immunological laboratory as useful tools. In addition, many different animal models have been developed to (a) identify or study genes that are involved in the induction and regulation of immune responses, to (b) study drugs that aim to modulate or attenuate responses, and to aid (c) basic and (d) applied research as well as (e) pre-clinical studies. Such studies are important in our understanding of infections, vaccination, cancer and inflammatory diseases. Animal models also facilitate studies on organ-specific immune processes and how these are influenced by systemic or organ-specific interventions. Moreover, several models have been developed to study (f) safety and (g) efficacy (correlates of infection) of clinical interventions.

Benefits and Limitations of animal models

The benefits of animal models are numerous. Animals possess a complete immune system allowing studies on systemic responses (all different cell types involved, antibodies) over time, they can be genetically manipulated, specific imaging has been developed, the costs are relatively low when compared to human studies, and on top of this the results are often very reproducible as most studies are performed in animals that are genetically identical. There are however also severe limitations. Results need to be extrapolated or translated to humans which is not always straightforward as certain genetic components may differ or may even be completely absent, many pathogens specifically infect humans or primates only (HIV-1, SARS-CoVs, Ebola), and most of the tumors that are used are laboratory adapted. It is now generally accepted that the immune system of animals e.g. mice but also primates, differs considerably from that of humans. For example, to investigate genetic drift of human tumors and tumor immunology immune deficient mice are required; some primate species will overreact to HIV whereas other species do not even develop the disease when infected. Last but not least, the fact that most studies are performed in animals that are genetically identical is a major limitation. As the recent COVID-19 pandemic has strongly reminded us of, individual responses are highly variable and determine pathogenesis as well as disease outcome.

Need for human models for human Immunology

In addition to animal models, *in vitro* models have been very important to immunological research. Immune cells can be isolated with relatively ease from blood to investigate their responses to pathogens or the environment. Particularly in the last decade, innovative techniques have been developed that allow for a very detailed investigation of immune cells in patients or cohort studies. It is to be expected that these technologies will further improve allowing us to investigate the function of the immune system in volunteers or patients. This will provide exciting new models and enable us to investigate human immunology in humans. In parallel, there have been huge advances in the development of next generation *in vitro* models. Human cell-based models, ranging from simple single cell type culture in a dish to complex organoid culture in custom designed bioreactors mimicking the physiological environment of the organ in the human body, are important drivers in the transition towards the use of NAMs[10, 11, 12]. “Big data” is also becoming more and more important. *In silico* and computational models are collecting data derived that is from human studies or from previously performed animal studies and are filling the gaps with new *in vitro* derived data. This helps to predict effects of drugs and substances to which humans are exposed and also to identify novel drug targets and, in turn, drugs.

To make a road map[13, 14], we have made a logical, non-exhaustive inventory of available models and NAMs (see below and Table 2). Where possible and applicable, we provide recommendations. We then continue by analyzing what is necessary to energize transition, including analysis of the, sometimes global, barriers that exist. Finally, we provide an outlook on immunology research and innovations in NAMs. Together, this encompasses a ‘target image’ for the field of immunology.

[10] Anklam E, Bahl MI, Ball R, Beger RD, Cohen J, Fitzpatrick S, et al. Emerging technologies and their impact on regulatory science. Exp Biol Med. 2022;247(1):1-75.

[11] Marx U, Akabane T, Andersson TB, Baker E, Beilmann M, Beken S, et al. Biology- Inspired Microphysiological Systems to Advance Patient Benefit and Animal Welfare in Drug Development. Altex-Altern Anim Ex. 2020;37(3):365-94.

[12] Franzen N, van Harten WH, Retèl VP, Loskill P, van den Eijnden-van Raaij J, IJzerman M. Impact of organ-on-a-chip technology on pharmaceutical R&D costs. Drug Discov Today. 2019;24(9):1720-4.

[13] Mastrangeli M, Millet S, Mummery C, Loskill P, Braeken D, Eberle W, et al. Building Blocks for a European Organ-on-Chip Roadmap. Altex-Altern Anim Ex. 2019;36(3):481-92.

[14] Mastrangeli M, Millet S, Braeken D, Eberle W, Fernandez L, Gidrol X, et al. Organ-on-Chip in Development: Towards a Roadmap for Organs-on-Chip. Altex-Altern Anim Ex. 2019;36(4):650-68.

Table 1. *Challenges when modeling human immune responses*

Complexity of the System[1]	
Different cell types	Macrophages, Monocytes, Dendritic cells, Local antigen presenting cells, T cells, B cells, granulocytes, NK cells
Different organs	Blood, Lymph nodes, Thymus, Bone marrow, all other organs
Different processes	Migration, extravasation, phagocytosis, antigen presentation, differentiation, maturation, immune responses
Long timelines	Full immune responses take up to 6 weeks to fully develop
Complex pathology	Compensatory mechanisms, imbalances, damage and repair
Complexity of the Population	
Different genetics ('nature')	Large donor-donor variability with strong impact on immunity
Different circumstances ('nurture')	Ageing, gender, microbiota, environment

[1]Animal and NAMs need to take the complexity of human immune responses into account. While animal models perform relatively good to model "Complexity of the System" (albeit that many differences exist between animal and human immune responses), they perform poorly to model the "Complexity of the Population". In contrast, current NAMs perform poorly on complexity of the system but are capable of incorporating complexity of the population due to donor variations and genetic differences. Both animal studies and NAMs require translation and extrapolation to the living human individual.

3.
New Approach Models at different levels of complexity

The complex pathology that is associated with dynamic, systemic responses is perhaps the most important reason for immunologists to prefer the use of animal studies over NAMs. Although studies in animals indeed allow for analysis of unanticipated effects, they also constitute a 'static black-box approach' if we do not aim at understanding the underlying human mechanisms and instead remain focusing on analyzing the 'overall' effect. Where the causative agent of a disease is known (e.g. for infectious diseases), studies in animals can provide rather reliable information on pathobiology and possible protective effects of immune responses. The less is known about the exact cause of the disease (as is e.g. the cause for many human autoimmune diseases), or the longer and more complex the pathology is (e.g. in tumor immunology), the less predictive animal models are and the more dependent results become on the way these models are induced and the strain of mice used. Many animal models for immunological diseases exist, and many of them come with profound reproducibility and translational problems. *We will need to be more critical on the use of animals as relevant models for human diseases and consider whether specific -pathological- processes that play a role in the disease cannot be better modeled using NAMs.* It is therefore, important that institutes have a specialized NAM Core Facility or access to NAM research to facilitate decisions on the best model. Access to animal models via the animal facilities and the lack of access to NAMs increases the use of animal models even though the model could be less suitable to answer the question.

The way forward will be difficult and slow, but inevitable. **Parallel studies** in which researchers that are performing *in vivo* experiments are facilitated to perform, side-by-side, *in vitro* studies for comparative and analytical purposes may allow breakdown of the 'black box' approach. In addition, a better **understanding of the translational gaps** between animal models and the disease that is modelled will allow for a better decision on qualification of the animal model[15, 16]. If animal models have failed to predict human responses, understanding the underlying human mechanisms will aid in the implementation of NAMs.

[15] Pound P, Ram R. Are researchers moving away from animal models as a result of poor clinical translation in the field of stroke? An analysis of opinion papers. BMJ Open Sci. 2020;4(1):e100041.

[16] Roep BO, Atkinson M, von Herrath M. Satisfaction (not) guaranteed: re-evaluating the use of animal models of type 1 diabetes. Nat Rev Immunol. 2004;4(12):989-97.

NAM tools and models should be as simple as possible but as complex as required to answer research questions with valid relevance for humans. Increased complexity is directly related to physiological relevance but also to more complicated culture logistics and to lower throughput (see Table 2).

Table 2: *The New Approach Methods (NAM) tool box*

Type of model (throughput)	Definition	Example	Pros & Cons
In silico (high)	Computational modelling, computer simulations, bioinformatics	• Complex pathway analysis • 3D computer models • Analysis of human genome • Drug discovery: tuberculosis	Enables extensive amount of data from different studies to be incorporated into a single model. Needs data input from *in vitro* and *in vivo* models to develop & validate the model
In vitro cell based assays (high to low throughput with increasing complexity)	Cell based study performed outside of the living organism typically in test tubes, cell culture dishes and bioreactors	• Simple conventional 2D cell culture • 3D spheroid culture with single cell type • 3D organotypic culture with multiple cell types representing a scalable unit of an organ • 3D multi-organ models representing interactions between organs e.g. systemic (immune)toxicity, tumor metastasis, drug biodistribution.	Models need to be as simple as possible but as complex as required to answer research questions. Gives information relevant to human immunology and signalling pathways Needs *in vitro – in vivo* correlations to obtain information relevant to humans
In vivo human studies (low)	Study involving living humans	• Observational study • Intervention study - Part of regular treatment - Additional intervention	Gives information directly relevant to human Study has to be ethically sound, extensive logistics around inclusion and documenting small as well as large patient numbers

In vitro models

To study the different cell types that are implicated in human immune responses, the simplest approach is to use ***in vitro 2D models*** consisting of monocultures of human cell lines or of human primary cells. These are easy to use and manipulate, and in cell lines the reproducibility is very high due to standardized culture conditions and lack of donor-donor variation. However, this donor-donor variation is important as it reflects the responses of individuals as part of the population. In addition, genetic drift of cell lines, associated with prolonged culture times, is a disadvantage. When using primary cells, the natural donor-donor variability is reflected, thus resulting in higher relevance, and providing more insight into the natural variance in immunological processes such as immune activation, innate and adaptive responses. Cell culture models allow for elucidation of molecular mechanisms due to genetic manipulation, screening, and standard readouts.

Simple co-culture models allow for studies into cell-cell interactions and, combined with further experimental manipulations, provide opportunities to investigate mechanisms underlying cell-cell communications, such as the initiation of adaptive immune responses and cytolytic responses. These models require access to primary tissue (cells, tissue) involving ethics approval and biobanking. More complex models are required to investigate intercellular communications in different organs.

Ex vivo tissue culture models consist of living whole tissues and organoid slices allowing the study of immunological processes during infections and inflammation. Even more than primary cells, the use of tissues requires access and approval by ethics committees and is more feasible in an UMC than in a university or institute. Vital Tissue (www.vitaltissue.nl) is a new initiative aiming to bridge the gap between the peripheral hospitals and the researchers in order to supply surgical rest material to research laboratories. This will guarantee a regular supply of ex vivo tissue to the research labs who can then isolate the different primary cell types required in NAMs.

Organoid cultures are even more sophisticated culture models and require additional technical knowledge and specific materials but also provide opportunities to study immune cell interactions, such as migration within 3D cultured **miniature organs** (**organoids**). These advanced cultures often include primary cells and stem cells seeded into a scaffold which represents the extracellular tissue environment of the organ and can remain viable for many months. For example, cell culture in 96 well plates containing different microenvironments with subsequent transfer of cell populations to new microenvironments can theoretically be used to mimic human immune responses from 96 different donors. **Organs-on-a-chip** currently provide the most complex and advanced form of cell culture. Organoids are cultured in bioreactors which mimic the environment of the human body. Microfluidics mimic blood supplies, oxygen levels and pressure gradients, and integrated electrical circuits and sensors (chip) simulate organ activities and mechanics enabling the organoid to represent human physiology.

In vivo studies in humans

Human in vivo studies allow us to study the actual disease, and the immunological processes involved, directly in the living human. They are extremely useful to understand inflammation, to investigate immune responses to e.g. infections, and to test novel interventions (e.g. vaccination strategies). However, such studies require medical support as well as stringent ethical evaluation, and manipulation options are limited. Notably, innovative technical advances in analytical analysis methods are currently allowing for ever more extensive and sensitive analyses at the level of genes, proteins and metabolome, and it is expected that the numbers of human *in vivo* studies will increase. An important issue is determining the safety of a drug or treatment. Traditionally these are being done in animal studies. However, other options are available. Although manipulation options are currently limited, these will also be further improved over time as shown by the phase 0 or micro-dosing trials. These trials allow for the application of minute amounts of drugs and analysis of their effects *in vivo*. As a result, safety studies for e.g. antibody-based biologicals or biosimilars in animals can be replaced by micro-dosing studies in humans instead. Moreover, human volunteer studies such as vaccination or challenge studies with viruses or allergens are very powerful in studying specific interventions or effects and have excellent predictive power.

Finally, *in silico* studies using data from cohorts or patient data combined with data from *in vitro/ex vivo* studies, are extremely valuable and can provide important immunological information about immunopathogenesis, treatment efficacy, safety, as well as molecular mechanisms (by studying for example gene polymorphisms).

4. Innovations in NAMs in the near future

In vitro models

Innovation of *in vitro* models will partly be feasible because of advances in technological possibilities but also because of advances in knowledge on culture methods in general, also with regard to primary cell culture methods. Special attention should also be given to the use of animal-derived products in cell culture practice.

Animal-derived products

Many NAMs rely on the use of products that are derived or harvested from animals. The widely spread use of fetal bovine and calf serum as well as that of matrigels comes with severe ethical and scientific disadvantages (see Table 3)[17, 18]. There are many replacements for these animal products but extensive testing and visibility of results means that uptake and implementation is very slow. Researchers are hesitant to change working models and implementation will take time and money, and replacement products are often more expensive. A clear promotion of non-animal products and specific funding opportunities or programs to side-by-side compare products would certainly help implementation and accelerate the replacement of animal products from cell culture practices. National and international regulations provide more guidance for use of non-animal products as well as non-animal methods [ESAC opinion cientific validity of replacements for animal-derived antibodies][19, 20]A specific subcategory of animal-derived products is formed by antibodies. These molecular tools are not only used in immunological research but are pivotal for many cell biological applications across all fields of the life sciences. Moreover, many innovative treatments use biologicals that are modified antibodies, which target specific host factors which cause disease. The current standard, and most straightforward protocol to obtain antibodies against a specific antigen is to inject animals with this antigen formulated in an adjuvant, a protocol very

[17] Sebastian Eggert JW, Jessica Rosolowski & Tilo Weber. Practical Workshop on Replacing Fetal Bovine Serum (FBS) in Life Science Research: From Theory into Practice. ALTEX; 2022.

[18] Kozlowski MT, Crook CJ, Ku HT. Towards organoid culture without Matrigel. Commun Biol. 2021;4(1).

[19] Bradbury ARM, Dübel S, Knappik A, Plückthun A. Animal- versus in vitro-derived antibodies: avoiding the extremes. Mabs-Austin. 2021;13(1)

[20] Barroso J. Scientific Validity of Replacements for Animal-Derived Antibodies. Washington, D.C., USA: SACATM Meeting; 2019.

reminiscent of human vaccination. Antibody generation has undergone changes due to innovations but also strict regulations concerning use of Freud's complete adjuvant as well as ascites-derived antibodies. These regulations have decreased the use of these methods and therefore animal suffering. Interestingly, antibody production has not been negatively impacted by these regulations as it forced the field to develop better alternatives. An interesting technical development is the maturation of phage-display technology as replacement option for animal-derived antibodies. A recent report of the European Centre for the Validation of Alternative Methods (ECVAM) has advised the EU to develop initiatives to outphase and ban the use of animal-derived antibodies and to use phage display-derived antibodies instead[21, 22]. However, it is yet unclear whether animal-derived antibodies can be fully replaced by phage display as the latter lacks affinity maturation and has inherently smaller theoretical diversity than animal-derived antibodies. Reliable side-by-side data are currently lacking, and this would greatly facilitate decision making, both by researchers as by ethical committees, regardless of the outcome of the data.

Table 3: *Advantages and disadvantages of using animal-based products for in vitro models*

Animal-derived product	Advantages	Disadvantages	Replacement options
Fetal Calf Serum/ Fetal Bovine Serum	Supports adherence Supports survival Supports proliferation)	Inherently illogical Questionable physiological relevance Associated with animal harm during production Use of product from different species Ill-defined composition Origin often unclear Batch-batch variability Safety issues can hamper clinical application	Chemically defined serum-free culture media (available or under development for culture of many cell types) Supplements (e.g. platelet lysates) Human pool serum
Matrigel (murine derived)	Supports adherence Supports survival Supports maintenance of differentiated cells	Associated with animal harm during production Use of product from different species Batch-batch variability Cost (expensive)	Plant based hydrogels BioSilks Synthetic biomaterials and scaffolds
Animal derived antibodies	Affinity maturation Relatively low costs	Associated with some degree of animal harm during production Long time lines	Phage-display technology, Direct cloning-protocols form human antibodies (for some specific purposes), recombinant techniques

[21] Bradbury ARM, Dübel S, Knappik A, Plückthun A. Animal- versus in vitro-derived antibodies: avoiding the extremes. Mabs-Austin. 2021;13(1).

[22] Luechtefeld T, Hartung T. Computational Approaches to Chemical Hazard Assessment. Altex-Altern Anim Ex. 2017;34(4):459-78.

Furthermore, in immunological research simple co-culture systems are being used to address immune activation, cell differentiation and antigen presentation. The latter depends on MHC compatibility which can be a serious challenge. However, identification of antigen-specific T cell receptors and B cell receptors might allow for genetic manipulation. Indeed, innovative genetic techniques are already available for investigating molecular and cellular processes in sophisticated cell-cultures and tissues and these are expected to be further developed in the near future. This will allow further molecular studies but the issue of whole tissues and systemic responses in immunology will remain challenging.

The big question is: **do we need to mimic an entire living human in detail in a single model?** Or are well-defined and standardized physiological representatives of key (multi-)organs and key events sufficient?[23, 24]These have the additional advantage that they can later be investigated in extreme detail, in contrast to an intact living being where experimental findings are often confounded by many unknown factors.

Several inter-disciplinary collaborations between immunologists, biomedical scientists, technical engineers, clinicians, and industry partners have been developed to develop ***models reflecting human systemic immune events.*** Large consortia projects are being financed to enable realistic goals to be reached within the next 5 years e.g. *The* NWA-ORC programme of the Dutch Research Council *financed projects: the Virtual Human Platform for safety assessment (project NWO 1292.19.272); the LymphChip project which aims to* integrate lymphatics connecting barrier organoids with lymph nodes within organ on chip bioreactors so that the platforms can be applied as a precision tool in the battle against immune-related diseases (NWO; project number 1292.19.019); and the TTW perspective project SMART Organ-on-Chip: Standardized open Modular Approach to Recapitulate Tissues demonstrating functionality by inducing tissue inflammation and testing drugs and the NWO financed project “Body Barriers” which aims to develop an organ-on-chip’ platform that can mimic the functions of our two important body barriers (i.e. mucosa-blood and blood-brain barrier) and to establish Proof of Concept (POC) testing with (dental) medical devices and neuro-inflammatory disorders.

[23] Marx U, Akabane T, Andersson TB, Baker E, Beilmann M, Beken S, et al. Biology- Inspired Microphysiological Systems to Advance Patient Benefit and Animal Welfare in Drug Development. Altex-Altern Anim Ex. 2020;37(3):365-94.

[24] Luechtefeld T, Hartung T. Computational Approaches to Chemical Hazard Assessment. Altex-Altern Anim Ex. 2017;34(4):459-78.

Improvements for the near future

- Co-culture models
  - combinations of different (immune-) cells
  - multi-organ combinations
  - integration of blood and lymph vessels containing circulating immune cells
  - prolonged culture lifespan
  - healthy and disease models
- Replacement of animal-derived products in cell culture
- Easier genetic manipulation of primary cells and tissues
- Culture bioreactors / chips to mimic micro-environments and physiological stimuli
- Integration of sensors, real time Imaging, immunofluorescence microscopy, multiplex analysis of small samples

In vivo studies in humans

The living human will always be the best model to investigate and understand immune-mediated human disease. Such studies can take the form of observational studies, using routine diagnostic rest material and being of no additional burden to the patient involved, or of intervention studies which have to comply with very strict ethical and safety guidelines. Currently ethical restrictions and bureaucratic ethical committees are a major hurdle in designing patient or volunteer studies. **A better standardization of clinical protocols and reviewing processes would facilitate the approval.** With enhanced patient involvement, it will also become more important to design more complex patient or volunteer studies. Safety is paramount but often determined with animal models. New methods such as microdosing or validation of in vitro tests for safety will need to be implemented and accepted by the ethical reviewing boards. Alternative non-animal methods for safety testing should become the norm instead of animal models.

Systemic responses are notoriously difficult to model in cell culture models (Ab responses, TCR BCR repertoire, chronic infections/disease). For these purposes cohort studies are very suitable. Advanced innovative sensitive high throughput analyses combined with bioinformatics are crucial to understand *in vivo* immunological processes and to study these. Knowledge on genetic variations makes the study of specific genes in humans more feasible in combination with cohort and volunteer studies as well as in vitro studies.
More sensitive and cheaper high throughput analyses (NGS, metabolomics, proteomics, lipidomics, multiplex assays) will be important in patient cohort studies as well as volunteer studies and in combination with minor-invasion sampling.

Improvements in the near future

- Standardize and simplify clinical protocols and reviewing processes
- Generating more high throughput methods (NGS, proteomics, glycomics, multiplex analysis) for immunological processes in humans (patients, volunteers).
- Non-invasive imaging techniques eg Optical Coherence Tomography (OCT)
- Open access and reusable data
- Further Developments to generate non-animal derived antibodies (e.g. phage display)
- Health RI: Data Driven Health, Connect, Share and Reuse
- Fase-o-studies/micro dosing

5. Transition target

Although challenging, it is feasible to transition to NAMs as the dominant model to study immunology over the next 10 years. The exponential growth of new and innovative NAMs will continue over the next 10 years gaining further importance and implementation.

To reach this target, investments need to be made, some of which will involve a major effort, whereas others are already occurring.

In progress

- Further develop Organoid and iPSC Core facilities paralleled with gradually downsizing animal facilities
- Development of innovative NAMS
- Increasing access to cohorts and patient data.
- Parallel studies to characterize the usefulness of animal models and NAMs. This should encompass parallel studies of NAMs with animal experiments as well as with human clinical research
- Accumulating big data and developing computational models
- Financially “rewarding” projects using NAMs
- Formation of local TPIs to facilitate the transition to NAMs

Regulation and culture changes

- Reducing administrative hurdles and facilitating access to human material for research
- Facilitating match-making websites and workshops (e.g. NVVI shoud play a role in this)
- Requesting clear evidence for relevance of animal studies in proposals by AWB/DEC/CCD and project financers. If necessary request literature reviews or actual data concerning ineffectiveness of NAMs.
- Facilitating local “TPIs” to connect with AWB/DEC/CCD to stimulate awareness and transition

More investments in creating networks and organoid centers should be made to introduce and train scientists to the greatly expanding field of organoid culture. Furthermore, in addition to parallel studies as mentioned below, transition workshops

should be stimulated where scientists currently using animal methods and who want to transition to NAMs are introduced to methods and networks so that they can still carry out their research but without using animals (see www.helpathonhotel.org). This would be particularly relevant for early career scientists (PhD students and postdocs). It is also most important to introduce existing innovative cell biology and immunology methods into the university education system including BSc, MSc and PhD training programs as these young scientists are the future immunologists and biomedical scientists, and to have as standard in the program of NVVI meetings sessions using non animal methods rather than the current bias to present animal studies.

Parallel studies should be initiated to stimulate researchers using animal methods to collaborate with researchers using and developing NAMs to identify hurdles, determine feasibility and aid the transition of researchers still using animal methods. Parallel studies also allow the researchers using animals to investigate whether the animal models are the best model or whether they can be eplaced with NAMs. This will create awareness and also enable animal researchers to feel part of the solution, rather than as part of the problem. This would also align with EU law that states that animals can only be used in research when there is convincing scientific justification, where the expected benefits outweigh animal suffering and when the objectives cannot be achieved using non-animal alternative methods (See The EU Directive 2010/63/EU amended in 2019 and in the Dutch Experiments on Animals Act (or Wet op de Dierproeven, WoD 2014, based on EU legislation). If the NAM is at least as sufficient, then further research should use this model and technology transfers initiated. Regulators (EMA / FDA) will accept NAMs if their use is scientifically supported and provide data that allows assessment of safety and efficacy. More awareness and guidance should be made on this subject within the universities. Contact with the “scientific advice” offices of EMA / FDA early in the research pipeline should be made to discuss what is needed for future regulatory acceptance and to eliminate potential hurdles created by using NAMs.

Collaborations with the national human healthy and disease model technologies consortium (hDMT[9]) will enable immunologists to work together in a multidisciplinary setting with cell biologists, (tissue-)engineers and industry to further develop the models and aid implementation.

Some areas in Immunology will be faster in transition than others depending on the urgency, requirements, and expertise. Therefore, one goal should be to increase expertise and make expertise for innovative models available to every researcher. Examples are the iPSC hotel and organoid centers which are arising in many universities. A major requirement to succeed is the sharing of technologies and knowledge. Acceptance by the stakeholders including policy makers and the community (journals, animal committees, peers). Acceptance by stakeholders is currently being promoted. Often knowledge concerning alternatives are not easily available to the institutional animal committees and this could be promoted by integrating more with the local “TPI” initiatives within the universities. Also, these local “TPI’s ” should connect directly with the AWB/DEC/CCD, preferably including a DEC member in the management group. In this way awareness and transition will be optimally facilitated.

Animal usage should be more critically assessed by the institutes as well as the researchers. Currently the researcher decides whether the experiment needs to be performed in an animal and the AWB/DEC/CCD assesses the degree of suffering to the animal. AWB/DEC/CCD have also knowledge of NAMs and can help researchers in alternatives. In addition to points already addressed above, it should be made compulsory that the researchers provide actual evidence (literature or data) that the animal experiments are necessary over NAMs. This would enable more critical reviewing of the applications without requesting more time from the AWB/DEC/CCD.

The **Five Year Target** would be to invest in education and training of young researchers by Universities, UMCs and institutes, as this will enable a culture change and speed up the transition. The NVVI can play an important role in this by providing course material as well as workshops and teachers. Further focus needs to be on developing immune competent models, concentrating on single organ and combined two organ models. Also in establishing methods for multi organ models without immune system. To reach this target, the supply of *ex vivo* tissues to the researchers should be facilitated (including blood). Furthermore, investments should be made in gathering patient cohort data from -omics studies and bioinformatics. Therefore, this 1st 5 years can be seen as an investment in the future.

The **Ten Year Target** would be the model implementation phase. This will have a significant impact on the animal use. Models (immune competent multi-organ) should be continued to develop and become more sophisticated. This would enable systemic, chronic, biodistribution, safety studies to significantly replace animal usage in the Netherlands.

Final recommendations for transition

A major hurdle for many academic researchers is the request by many high impact journals and reviewers to include animal experiments to support their NAM data. This is currently a subject of international stakeholder meetings which emphasizes its significance to the field. The research institutes could aid their researchers in writing rebutals by e.g. providing standard letters or phrases on their research institute web sites as to why they have chosen for NAMs. However it remains with each researcher to provide the convincing scientific reasoning for the choice of NAM. Furthermore, this hurdle can be overcome by continuing to encourage scientists to publish open access (by covering publication fees for many journals), decline publication in journals demanding animal experiments and continuing to score scientists by other methods (e.g. scientific and societal impact) rather than H factor and journal impact when submitting grants. This transition has already started by NWO and other funding agencies and will positively impact the use of NAMs.

Financing will always be a main driver in the transition to NAMs, along with a more critical assessment of whether a proposed animal study is the best way to answer a particular research question. National (e.g. NWO, ZonMW, NWA) and Health Organization (samenwerkendegezondheidsfondsen) funding are essential to achieve the transition goals described in this target image. Key to transition is the training and education of early career scientists. Therefore, more personal fellowships and grants should be created by funding bodies as well as the funding for large consortia to create the possibility of career paths.

Finally, we now live in a more critical world concerning research into human diseases and treatments. New focuses arise to improve on health research such as how gender, individuality and age affects human disease and health. These issues become more important and also ask for new approaches that are more difficult to investigate in animal models and require innovative NAMs. This is the era that will boost human research in relevant human models *in vitro, ex vivo, in vivo or in silico*.

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