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A Ban on Animal-Derived Antibodies will Stifle European Competitiveness in the Life Sciences.

On 14th May 2020, the EU Reference Laboratory for Alternatives to Animal Testing (EURL ECVAM), released a recommendation on Non-Animal-Derived Antibodies, which recommended that "*animals should no longer be used for the development and production of antibodies for research, regulatory, diagnostic and therapeutic applications*". It added that "*In the EU, the provisions of Directive 2010/63/EC should be respected and EU countries should no longer authorise the development and production of antibodies through animal immunisation, where robust, legitimate scientific justification is lacking*".

The short statement which follows outlines major concerns from the university sector with the EURL ECVAM recommendations. It has been developed by the League of European Research Universities (LERU), an association of 23 leading research-intensive universities in Europe.

LERU agrees that we should avoid the use of animals for antibodies wherever possible and fully supports efforts to develop alternatives. However, LERU believes that it is premature at present to completely abolish the use of animals for generating (monoclonal) antibodies or for (polyclonal) antibody production. This is because animal-derived antibodies have many unique features which cannot be (easily) mimicked by alternative methods. As such, LERU believes that animal and non-animal derived antibodies should in fact be considered as complementary technologies. A ban on animal-derived antibodies would have serious implications, both in terms of therapeutic use, and in terms of research in the life sciences more generally. We therefore recommend that the EC and other authorities:

I. Conclude that it is **premature to ban the use of animal-derived antibodies** at the present time. Indeed, in view of the current pandemic situation, we could argue we need all of the options which we can lay our hands on.

II. Foster a **wider debate amongst scientists** on the use of alternatives to animal-derived antibodies, taking into account the scientific, technical and ethical issues of doing so before making any decisions on such a crucial technology.

III. Ensure that there is a suitable **robust and scientifically-sound method** to guide decisions on authorisation of the use of animals for the generation and production of antibodies. This should be developed in close conjunction with experts[1] to ensure that it is workable and brings no undesired consequences.

There are two sections to this statement:

- Section 1 gives a general outline of the need to maintain animal-derived antibodies;
- Section 2 gives a rebuttal of several points which LERU feels were inaccurate in the EURL ECVAM report.

[1] These should be drawn from universities, academia more widely, regulators and industry.

Section 1 – Why animal-derived antibodies are still needed

The importance of animal-derived antibodies in research and in the clinic

Antibodies have been valuable tools in research and therapy for many decades and they are used in a wide variety of different applications. They offer a 'gold standard' performance in many of today's research, therapeutic and diagnostic applications and are widely trusted by the scientific community as a whole. They constitute unique tools which can be generated to bind with unprecedented specificity to virtually any target molecule. Because of their specificity, scientists can study precisely the role of these target molecules in health and disease. In addition, as they can interfere precisely in disease processes, they have revolutionised the treatment paradigms for millions of patients over the last 30 years, particularly in, but not limited to, oncology and auto-immune diseases.

LERU is surprised that the EURL ECVAM report completely ignores the fact that there are many examples of very-well characterised and validated animal-derived antibodies, especially as approved drugs. Indeed, according to Lu et al, (2020)[2], of the 71 therapeutic monoclonal antibodies approved by the FDA by the end of 2019, only 9 are non-animal derived.

Animal-derived antibodies are still superior for many applications to non-animal derived antibodies

To generate non-animal derived antibodies, antibodies binding to the target molecule are selected from synthetic antibody repertoires under laboratory conditions by the use of a display technology (e.g. phage display). The outcome is typically a limited set of antibodies which do not bind very strongly, and further labour-intensive affinity maturation is needed to improve the binding strength. To generate animal-derived antibodies, an animal (typically a mouse, rat or rabbit) is injected several times with low doses of the target molecules, a procedure causing limited discomfort to the animals, similar to a regular vaccination in humans[3]. The animal's immune system reacts against the injected target molecule by generating antibodies through an extremely efficient process of *in vivo* selection and maturation resulting in outcomes (in terms of diversity and binding strength) that can currently not be mimicked by existing and well-established *in vitro* alternatives.

A study using deep sequencing of immune repertoires showed that humans can generate a quintillion (10^{18}) different antibodies[4]. This is possible due to the unique *in vivo* available gene repertoire, gene rearrangements (V(D)J recombination), nucleotide deletions and insertions at hypervariable junctions during rearrangements, somatic mutation and heavy-light chain pairing. An intricate system of highly regulated clone selection assures the *de novo* generation of high-affinity antibodies against virtually any antigen *in vivo*. In contrast non-animal derived naïve and synthetic antibody repertoires are static, with the best libraries

[2] Lu, R., Hwang, Y., Liu, I. et al. Development of therapeutic antibodies for the treatment of diseases. J Biomed Sci 27, 1 (2020). https://doi.org/10.1186/s12929-019-0592-z

[3] In 2017, around 45,000 mice were used for the routine (commercial) production of antibodies using the ascites method (see https://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:52020DC0016&from=EN.). In the ascites method, a hybridoma cell line is injected into the abdomen of a mouse, where the cell line expands and causes a buildup of fluid in the peritoneal cavity called ascites. Fluid containing the antibody of interest can be collected by drawing the ascites fluid from the animal. This method has been widely criticised on animal welfare grounds and is, to our knowledge, banned in several member states and is not used in academia. As a result, we do not discuss the ascites method further in this note. It cannot be ruled out that ascites antibodies are being used by researchers in European labs, so LERU encourages researchers to be vigilant and check the source of their antibodies when buying from a third party to avoid the use of ascites antibodies.

[4] Briney, B., Inderbitzin, A., Joyce, C. et al. Commonality despite exceptional diversity in the baseline human antibody repertoire. Nature 566, 393–397 (2019). https://doi.org/10.1038/s41586-019-0879-y

having a diversity of 10^{10} to 10^{12} antibody clones. Under optimal conditions, non-immune libraries would therefore, in this example, only capture 1/100,000,000th – 1/1,000,000th part of the potential antibody binding space. Consequently, antibodies from synthetic libraries are initially suboptimal, and so subsequent labour-intensive improvement and affinity maturation is required.

The *in vivo* generation and maturation processes do not only have advantages for the diversity and binding strength, but also have an impact on the biophysical properties of the antibodies (solubility, specificity, stability, …) as outlined by Jain et al 2017[5]. In this study, Jain et al. compared the biophysical properties of all 137 late clinical-stage antibodies (phase 2, 3 or approved antibodies). Using different approaches, Jain et al.[5] defined thresholds to 'flag' problematic behaviour of antibodies (which they call 'red flags'). Their dataset allowed to compare the behaviour of animal-derived antibodies on the one hand (n = 103) and non-animal derived antibodies or engineered antibodies through phage display (n = 34) on the other hand[5]. While around 60% of the antibodies from animal origin did not contain any red flags, only 20% of the antibodies discovered or optimized by phage display did not contain red flags[5]. The main observed issues relate to non-specific binding (with potential toxicity issues and altered pharmacokinetic profiles) and self- and cross-interactions (with potential reduced shelf-life and enhanced immunogenicity)[5]. Indeed, the generation and selection of antibodies in animals selects for antibodies which are amenable for *in vivo* use, weeding out antibodies with unfavourable biochemical and biophysical properties. Synthetic antibodies solely selected and produced in the laboratory lack this selection step requiring time consuming lead-optimisation to improve their developability.

Extensive studies comparing animal and non-animal-based approaches in parallel against the same targets are currently missing and would be needed to make firm statements on either of the two approaches. However, multiple LERU members have been exploring both systems in some studies. KU Leuven for example could not find antibody-fragments binding to the required region of a particular cancer target by mining synthetic antibody repertoires, whereas several antibody-fragments could be isolated from animal-derived immune libraries. At the University of Paris Saclay, many screens have been performed to find non-animal derived antibodies specific to tubulin posttranslational modifications, but with little success. In contrast, using the same antigens, they succeeded in raising excellent antibodies in rabbits. These antibodies allowed the research group to make key discoveries, which would otherwise have been impossible. Amsterdam UMC, housing the World Organization for Animal Health and the National Collaborating Centre for Reference and Research on Leptospirosis, produces and provides antibodies to detect which leptospiral serotypes are present within a particular group of humans, animals, or in a geographical region in order to implement public health measures to prevent leptospirosis or reduce the burden of leptospirosis, a deadly disease with over 60,000 casualties annually[6,7,8,9]. Notwithstanding extensive efforts to replace their polyclonal antibodies with non-animal-based alternatives, researchers cannot discriminate between the specific leptospiral serotypes with non-animal-based alternatives[10].

[5] Jain, Tushar, Tingwan Sun, Stéphanie Durand, Amy Hall, Nga Rewa Houston, Juergen H. Nett, Beth Sharkey, et al. "Biophysical Properties of the Clinical-Stage Antibody Landscape." *Proceedings of the National Academy of Sciences* 114, no. 5 (January 31, 2017): 944–49. https://doi.org/10.1073/pnas.1616408114.

[6] World Health Organization. Report of the Second Meeting of the Leptospirosis Burden Epidemiology Reference Group, 2011.

[7] World Health Organization. Report of the First meeting of the Leptospirosis Burden Epidemiology Reference Group., 2010.

[8] Costa F, Hagan JE, Calcagno J, et al. Global Morbidity and Mortality of Leptospirosis: A Systematic Review. PLoS Negl Trop Dis 2015; 9(9): e0003898. https://doi.org/10.1371/journal.pntd.0003898.

[9] Mwachui MA, Crump L, Hartskeerl R, Zinsstag J, Hattendorf J. Environmental and Behavioural Determinants of Leptospirosis Transmission: A Systematic Review. PLoS Negl Trop Dis 2015; 9(9): e0003843. https://doi.org/10.1371/journal.pntd.0003843

[10] Non-animal-based antibodies are however, used for research when the specific serotype is known.

In summary, currently there is insufficient data available to convincingly show that in general non-animal derived antibodies are equal to, or would outperform animal-derived antibodies in different applications.

The implications of restricting or banning the use of animal derived antibodies

Monoclonal and polyclonal antibodies[11] are heavily used research tools in virtually all life sciences laboratories across the EU. Besides research tools, they are at the frontline of many current drug development campaigns and have proven to be indispensable to treat many life-threatening diseases like cancer and autoimmune diseases.

The impact of an immediate ban

An immediate ban would be devastating for *basic research*, *drug supply*, as well as *drug development* campaigns. Indeed:

- For most of the applications, high binding strength and selectivity is key and both of these characteristics are more difficult to achieve with non-animal derived antibodies.
- Drug development requires antibodies with excellent developability and pharmacokinetic properties, characteristics which are more challenging for non-animal derived antibodies.
- The access to high quality non-animal derived antibody repertoires amenable for setting up discovery campaigns is currently severely limited, comes at a high cost, and/or comes with restrictions (e.g. no rights to commercialise or share with collaborators). Significant governmental supported investments would be needed to overcome this and to make it affordable for e.g. academic labs[12].

The impact for patients would be devastating:

- Many polyclonal drugs (e.g. anti-thymocyte-globulins, ATGs) are used for the treatment of critically ill patients (for example those who have received an organ transplant[13] or are being treated for leukaemia). The invariable ban on the use of animals for the production of (polyclonal) antibodies would lead to a *de facto* ban of these therapeutics and cause severe harm to thousands of patients.
- Anti-scorpionic antibodies are produced in animals to obtain polyclonal antibodies with a large spectrum of epitopes reactivity to ensure disposal of neutralising antibodies in countries with low income. Of note, in 2008, it was estimated that more than 1.2 million people suffered scorpion bites, with 3,250 deaths (including 70% children)[14]. The anti-scorpionic polyclonal antibodies are the major tool making it possible to limit deaths and severe illness.

[11] Polyclonal antibodies are mixtures of antibodies, isolated from serum from immunised animals. The term monoclonal antibody refers to an antibody preparation, produced by a unique cell line (hybridoma or a recombinant antibody production cell line), and contains the genetic information for the production of a single, well-defined antibody.
[12] This is just beginning to happen, for example the University of Geneva is playing a significant role in the development of recombinant antibodies.
[13] ATGs are used as powerful immunosuppressants in solid organ transplantation. In addition, they are used for pre-conditioning of patients before bone-marrow and hematopoietic stem cell transplantation and the treatment of graft-versus-host-disease. More than 3,500 patients in Germany received allogeneic bone-marrow or stem-cells in 2018. On average 30 to 60% of these patients experience a graft-versus-host-disease and the majority of these patients are injected ATGs as therapeutics. So, there must be more than a thousand patients (including solid organ transplants) in Germany alone that will be harmed by a possible loss of ATGs.
[14] Chippaux, Jean-Philippe & Goyffon, Max. (2008). Epidemiology of scorpionism: A global appraisal. Acta tropica. 107. 71-9. https://doi.org/10.1016/j.actatropica.2008.05.021

- Similarly, in 2019, the WHO indicated that an estimated 5 million people are bitten by snakes each year with up to 2.7 million envenomings[15,16]. Around 81,000 to 138,000 people die each year because of snake bites, and around three times as many amputations and other permanent disabilities are caused by snakebites annually[16]. The WHO indicates that "*… Snake antivenom immunoglobulins (antivenoms) are the only specific treatment for envenoming by snakebites. Antivenoms can prevent or reverse most of the snakebite envenomings effects, and play a crucial role in minimising mortality and morbidity*[17]" These antibody preparations are included in the WHO List of Essential Medicines[16] and cannot be generated *in vitro*; the unavailability of animal-derived antibodies would severely hamper the treatment of the patients.
- It is important to note that the method of production of therapeutic polyclonal antibodies is an important part of the marketing approval procedure. A change in the production process (e.g. mixing of monoclonal antibodies to achieve a polyclonal drug) would lead to the loss of the marketing authorisation and a dramatic waste of time which would be a disaster for many patients under therapeutic treatment. Generating and approving mixtures of recombinant antibodies is very complex and a long process. If started now, it is estimated that this will take at least another decade, if indeed it is feasible at all.

Additionally, the EU's *competitiveness* in research would be lost in relation to countries such as the USA, China and Japan who will continue to benefit from the extraordinary resource of animal-derived antibodies used in research and in the clinic. And, importantly, subsequent outsourcing of antibody production outside of the EU could have major detrimental effects on animal welfare because the EU has some of the most *stringent rules* for animal research and use in the world[18].

LERU agrees that wherever possible, antibodies should be used that are derived or produced without the use of animals in line with the requirements of 2010/63/EC.

LERU fully endorses the timely implementation of alternatives to routinely using animals. We also agree that non-animal alternatives are of course desirable, so that whenever possible or available, antibodies should be used that are generated or produced without animals. Several LERU members regularly use novel systems for re-deriving (initially animal-derived) monoclonal antibodies and manipulating them *in vitro*[19]. LERU supports these activities.

However, any transition must be made step-by-step and only when the new technology (whichever it is) is broadly and reliably available to the research community and provides an equivalent scientific output as the more commonly applied technology. Thus, only once the replacement of animal-derived antibodies can be introduced systematically and offers the appropriate affinity and specificity, should the use of animals for such purposes be avoided. However, this is far from the current situation. In LERU's view, it would be highly counterproductive to require research labs in the EU to not have the choice to use animals for monoclonal antibody generation or polyclonal antibody production when needed. Up to

[15] An envenoming is a potentially life-threatening disease that typically results from the injection of a mixture of different toxins ("venom") following the bite of a venomous snake. It is considered as a neglected tropical disease.
[16] See https://www.who.int/snakebites/disease/en/#:~:text=The%20World%20Health%20Organization%20(WHO,amputations%20and%20other%20permanent%20disabilities.
[17] https://www.who.int/bloodproducts/animal_sera/en/
[18] Directive 2010/63/EU and national legislations.
[19] For example, existing monoclonal antibodies can be sequenced and the use of recombinant technologies allows to reshape them (e.g. for use in another species), change their isotype or form for use in different assay systems *in vitro* and *in vivo*. This avoids the need for additional animal immunisations to generate these antibodies of differing isotype etc. it also avoids the potential for 'orphan' reagents where hybridoma lines are lost and need to be re-made in mice.

now, animal-based technologies are still the only available approach for some applications and may remain so.

It should be noted that there are still many cases where animal-derived antibodies are still required and we believe that there are sufficient regulations at various levels to ensure that animals are only used in studies where there are no reasonable alternatives available[20]. The protocols that are currently used for the production of monoclonal or polyclonal antibodies include significant refinements introduced to minimise discomfort and pain for animals. All applications for animal research are scrutinised very carefully, and animals are only used as a last resort.

Finally, LERU has significant concerns over the statement in the EURL ECVAM report "…*EU countries should no longer authorize the development and production of antibodies through animal immunization, where robust, legitimate scientific justification is lacking*". LERU is very concerned about where the bar will be set in respect to 'robust, legitimate scientific justification' and who will provide an unbiased assessment.

A wider discussion on the feasibility of replacing animal-derived antibodies for non-animal antibody technologies is crucial before any ban is implemented.

LERU is in favour of replacing animal-derived antibodies where scientifically feasible and is pleased that through its report, the EURL ECVAM has opened the debate into the position of, and further investigations to, possible alternative technologies that could be used.

Given the importance of antibody technologies to the EU research community however, we strongly encourage a much wider consultation on the EURL ECVAM document, to prevent author bias and conflict of interest issues[21]. LERU believes that scientists with expertise in animal-derived antibodies should be included in a larger group of experts. This expanded board should discuss in which areas animals should no longer be used for the generation or production of antibodies, the prerequisites that have to be fulfilled, and the time needed to abandon the use of animals in that context. When this has been put out for a more thorough review and subsequently adapted in line with the conclusions by this expanded board, there should be a discussion with the national authorities on how these recommendations could be enforced. LERU would be delighted to contribute to these discussions.

[20] For instance, in the Netherlands, the use of animals for polyclonal antibodies is critically assessed by the Animal Welfare Body, the Animal Ethics Committee, and the Dutch Scientific Procedures on Animals (CCD).

[21] The composition of the ESAC-working-group is striking: five of the six external experts invited for this position paper are founders, shareholders or employees of companies whose main business is the production of non-animal-derived-antibodies. From that a personal interest of the authors in the recommendation of EURL ECVAM can at least not be excluded. This is also reflected by the literature cited in the publication: the external experts (co-)authored almost half of the publications listed.

Section 2 – Rebuttal of some incorrect arguments in the EURL ECVAM report

In reading through the EURL ECVAM report, LERU members noticed some elements which were inaccurate. In this brief section, we outline the statements LERU believes to be inaccurate in the EURL ECVAM report. We do not claim this to be an exhaustive list, but in outlining these issues, LERU hopes to cast doubt on the validity of some of the claims mentioned in the EURL ECVAM report.

- The remit of the working group was to review the literature on the state of the art of monoclonal, non-therapeutic antibodies. However, EURL ECVAM included polyclonal therapeutic antibodies in its recommendation as well. From a scientific point of view this is highly problematic, as the recommendation lacks – at least in this part – supporting scientific evidence.
- In some cases, and as stated in the EURL ECVAM report "*certain scientific limitations that are common to both animal-derived and non-animal-derived affinity reagents exist due to the complexity of the natural antibody-antigen binding relationship*" (page 35, section 6.5). Unfortunately, these limitations are not represented in the EURL ECVAM recommendation at all.
- It is incorrectly stated that sequence optimisation (affinity maturation, Fc-engineering (isotope/species switching, effector knock-outs…) and antibody-protein/tag fusions is almost exclusively limited towards non-animal derived antibodies (page 29, paragraph 3; page 32, section 6.2; page 36, table 1; page 46, section 9; page 47, paragraph 2). Most of the therapeutic antibodies on the market have been engineered from monoclonal antibodies obtained from animal immunisation (see footnote 2).
- The paper claims that substantial efforts are needed to elucidate the sequence of animal-derived antibodies (page 39, section 7.1) and non-animal derived antibodies are better because their sequence is known (page 2, section 2). Synthetic antibodies and naïve antibodies also need to be sequenced. Although sequencing used to be labour intensive in the past, more and more labs are implementing sequencing as part of their antibody discovery campaign, irrespective of their origin. The current cost to outsource sequencing of the antibody gene of a hybridoma is less than € 1,000 and hence quite affordable.
- There is no evidence that there is lower intrinsic immunogenicity for non-animal derived antibodies (page 36, table 1), and in fact it may even be higher as they are not selected in an animal but *in vitro* instead. To support this claim, larger numbers of non-animal derived antibodies should be tested in the clinic. This 'immunogenicity' claim is often made based on sequence homology towards human antibodies, however immunogenicity not only depends on sequence homology, but also largely on biophysical behaviour of the antibodies or parameters unrelated to the fact that the antibodies were generated *in vivo* in animals or *in vitro* (dose, route of injection, number of injections, presence of idiotopes, antigenic target, acidic/basic forms, …). A major study comparing multiple clinical-stage antibodies[22] clearly indicates inferior biophysical behaviour for phage-derived antibodies.
- The EURL ECVAM report contains several erroneous theoretical calculations and philosophical considerations. E.g. it is claimed that *in silico* libraries are at least as diverse as the potential animal derived immune response, suggesting similar

[22] Jain, Tushar, Tingwan Sun, Stéphanie Durand, Amy Hall, Nga Rewa Houston, Juergen H. Nett, Beth Sharkey, et al. "Biophysical Properties of the Clinical-Stage Antibody Landscape." *Proceedings of the National Academy of Sciences* 114, no. 5 (January 31, 2017): 944–49. https://doi.org/10.1073/pnas.1616408114.

performance (page 16, last paragraph; page 26, section 5.1; page 27, first paragraph; page 33, section 6.3). As discussed in Briney (2019)[23] and in this LERU statement (section 1, page 2), this is clearly not the case.

- Three antibody derived drugs are mentioned in Appendix 2 (page 26, section 5.1) of the EURL ECVAM report as examples of drugs from phage display libraries and suggesting these were non-animal derived. However, all three antibodies are derived from monoclonal antibodies developed using hybridoma technology, and thus animal-derived:
    - Blinatumomab is a phage display optimized T-cell engager, however, the antigen binding regions were derived from murine hybridomas[24];
    - Ranibizumab is a Fab fragment antibody derived from the full IgG monoclonal humanized antibody Bevacizumab, which was generated using hybridoma technology[25];
    - Certolizumab pegol is a PEGylated Fab fragment derived from an antibody generated using hybridoma technology[26].

[23] Briney, B., Inderbitzin, A., Joyce, C. et al. Commonality despite exceptional diversity in the baseline human antibody repertoire. Nature 566, 393–397 (2019). https://doi.org/10.1038/s41586-019-0879-y
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