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Title:

Animal models for the study of nucleic acid immunity: novel tools and new perspectives

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Bullet Points:

- Cytosolic DNA sensing relies on diverse cellular sensors, which contribute to triggering a tightly regulated interferon response.
- Type I interferon may be beneficial or harmful to human health.
- *In vivo* models are essential to study the mechanisms and physiology of interferon-related disorders.
- Zebrafish and organoids are promising tools to refine and reduce animal experimentation and improve the current drug screening strategies.
- Omics approaches provide insights into nucleic acid immunity regulation.

Summary:

Unresolved inflammation fosters and supports a wide range of human pathologies. There is growing evidence for a role played by cytosolic nucleic acids in initiating and supporting pathological chronic inflammation. In particular, the cGAS-STING pathway has emerged as central to the mounting of nucleic acid-dependent type I interferon (IFN) responses, leading to the identification of small molecule modulators of STING that have raised clinical interest. However, several new challenges have emerged, representing potential obstacles to efficient clinical translation. Indeed, the current literature underscores that nucleic acid-induced inflammatory responses are subjected to several layers of regulation, further suggesting complex coordination at the cell-type, tissue or organism level. Untangling the underlying processes is paramount to the identification of specific therapeutic strategies targeting deleterious inflammation.

Herein, we present an overview of human pathologies presenting with deregulated IFN levels and with accumulation of cytosolic nucleic acids. We focus on the central role of the STING adaptor protein in these pathologies and discuss how *in vivo* models have forged our current understanding of nucleic acid immunity. We present our opinion on the advantages and limitations of zebrafish and mice models to highlight their complementarity for the study of inflammatory human pathologies and the development of therapeutics. Finally, we discuss high throughput screening strategies that generate multi-parametric datasets that allow integrative analysis of heterogeneous information (imaging and omics approaches). These approaches are likely to structure the future of screening strategies for the treatment of human pathologies.

Key Words:

Innate immunity, STING, interferon signalling, drug screening, inflammatory models

Introduction:

Dysregulations of inflammatory responses underlie a wide range of human pathologies, including cancer, infectious or autoimmune disorders. “Inflammation” is operationally described as the physical manifestations of a local immune response to injury or infections, including tissue swelling, pain, redness and elevated temperature. These symptoms result from cell-mediated responses to either invading pathogens or local injuries after detection of damage-associated molecular patterns. This localized response, when controlled, is beneficial because it facilitates the recruitment of effector cells and enhances their circulation towards lymph nodes, activating the adaptive immune system.

Under physiological conditions, this process is self-limiting and inflammation is resolved as the infection is cleared or the injury repaired. The correct orchestration of the steps composing these cellular and humoral responses, from its initiation to its resolution, is therefore crucial to restore homeostasis. Indeed, chronic unresolved inflammation causes cell- and tissue-damage while potentially impacting hematopoiesis and causing hematological disorders [1]. Common symptoms of chronic inflammation include fatigue, discomfort, pain and weight loss. However, specific additional symptoms may arise, depending on the condition associated with, or resulting from, chronic inflammation such as joint pain and a limited range of motion experienced by rheumatoid arthritis patients. In addition, chronic inflammation triggered by autoimmune diseases or by modern diet and lifestyle, is associated with cardiovascular, muscular, bone and neurodegenerative diseases as well as cancer [2]. In most cases, it is however difficult to comprehend whether chronic inflammation is the cause or consequence of a specific pathology.

Research performed during the past decades shows that nucleic acid sensing defects are frequently associated with the onset of chronic disease-promoting, or disease-promoted, inflammatory signaling. The first evidence for the induction of nucleic acid-promoted cytokine production dates from the early sixties [3]. Double stranded RNA (dsRNA) and poly(I:C) were shown to induce the production of type I Interferon (IFN), a potent antiviral cytokine. It took

several decades for the immune-stimulatory nature of DNA to be demonstrated [4]. An even more recent notion is the immune-stimulatory potential of endogenous, mitochondrial or nucleus-derived, nucleic acid species [5].

Since then, the presence of endogenous inflammatory nucleic acids, including ssDNA, dsDNA and RNA:DNA hybrids, has been associated with several chronic inflammatory pathologies [6]. Despite the multifactorial origin of the nucleic acid accumulation, it induces a common dysregulation of cytokine production that culminates in chronic inflammation [7]. Genetic disorders, persistent infections and cancers have thus been related to inflammatory diseases resulting from nucleic acids accumulation [8].

Cytosolic nucleic acid detection pathways have been vastly explored *in vitro* (in immune and non-immune cell types) and *in vivo*, mostly focusing on murine models. However, recent work underscores the existence of cell-type-dependent and species-specific detection mechanisms, urging for re-evaluation of nucleic acid sensing in regards to both the spatial distribution of nucleic acid sensors (subcellular, cell and tissue localization) and the evaluation of crosstalk between co-existing signalling pathways. Herein, we summarize the current challenges in the nucleic acid immunity field, focusing on the cGAS-STING pathway, involved in the detection of cytosolic self and non-self dsDNA. In this light, we will discuss the complementarity and limits of murine and zebrafish models for the study of nucleic acid-mediated inflammatory responses and the development of high-content therapeutic screening strategies. Comparative analyses of the species-specificities has allowed the identification of therapeutic targets conserved between human, mouse and zebrafish models [9] and facilitated the development of drug screening approaches to treat inflammatory pathologies of different origins. Zebrafish model present advantages (optical transparency of the embryos, small size and high conservation of human genes) that can be exploited to promote the development of cost-effective therapeutic screening approaches. However, this model faces several limitations such as differences in adaptive immunity, lack of inbred strains and the duplication of its genome (human genes can have multiple copies in zebrafish

genome) [10]. These parameters are crucial to take into account and underscore that Zebrafish rather complement existing murine models. Furthermore, in vivo animal models have recently been challenged by organoid cultures that provide perspectives in the development of precision medicine. This will be discussed, alongside other recent breakthrough technical approaches may open new perspective in the monitoring of regionalized immune responses.

I- DNA sensing in inflammatory pathologies

I.1. Type I Interferon production in health and disease

Interferons (IFNs) belong to the class II helical cytokine family of signaling molecules, encoded by an intron-less multigene family. In humans, the type I IFN family includes at least 13 IFN α , in addition to IFN β , IFN ϵ , IFN κ , IFN ω . They all signal through binding to the virtually ubiquitous heterodimeric interferon- α/β receptor (IFNAR) [11]. In the mouse genome, 14 IFN α and single IFN β , IFN ϵ and IFN κ genes have been identified. Murine Limitin (also known as IFN ζ) was not found in the human genome, while no orthologue of human IFN ω has been identified in mice [12]. In zebrafish, four genes encoding proteins bearing structural similarities with mammalian type I IFNs have been identified (IFN ϕ 1 to ϕ 4), despite low gene sequences identity [13, 14]. These IFNs signal through binding to membrane anchored IFN receptors composed of heteroduplexes of cytokine receptor family B (CRFB) that correspond to mammalian IFNAR receptors [13]. The functionality of IFN ϕ 1, 2 and 3 was demonstrated experimentally while IFN ϕ 4 is suggested to be a pseudogene [15]. In mammals, it is suggested that all type I IFN genes have diverged from the IFN β gene. Consequently, IFN β is usually viewed as the prototypical type I IFN cytokine.

Type I IFNs play crucial roles in global homeostasis and their biological impact range from antiviral, antitumor to immune-regulatory functions. Their properties have been exploited in therapeutic approaches in diseases such as multiple sclerosis, hepatitis B and C and cancers, despite important side effects [16]. These side effects may be intrinsically linked to ambiguous roles of type I IFNs that depend on cell or tissue environments and on the global health context of patients [6]. Indeed, type I IFNs can be anti-inflammatory and tissue protective, or to the contrary pro-inflammatory and promote autoimmunity. In agreement, activating type I IFN pathways is beneficial to patients with chronic viral infection, multiple sclerosis and in animal models of arthritis and colitis, while blocking type I IFN responses is beneficial to patients with chronic inflammatory diseases such as systemic lupus erythematosus [17].

Similarly, in cancer, effective tumour suppression relies on the activation and production of type I IFN in tumoral and immune cells. Its anti-tumor effect (inhibition of cancer cell division and stimulation of adaptive immune response) is beneficial upon direct administration to treat leukemias, lymphomas, and myelomas, but limited due to reported short systemic half-life and strong side effects [18]. However, as reported for infectious diseases, chronic activation of type I IFN pathway can be associated to resistance to cancer therapies [19].

Due to interspecies differences in IFN subtypes, functions and cell-type specificity, it is likely that the impact of modulating their production induces different spectra of responses. Furthermore, IFNs signal through conserved signaling pathways, which trigger the expression of a vast array of interferon stimulated genes (ISGs). ISGs mediate the IFN response through their multiple cellular activities. Comparative genomics of ISG repertoires in different species has highlighted the existence of a core set of ancestral ISGs in addition to species-specificities [20]. A better characterization of ISG expression profiles in different species would be instrumental to the identification of the molecular basis of the regulation of type I IFN responses [21] and the improvement of IFN therapies.

Type I IFNs can be secreted by a wide range of immune and non-immune cells in response to various biological stimuli (damage associated molecular patterns (DAMPs) and pathogen associated molecular patterns (PAMPs)) that activate ubiquitous and/or cell type-specific nucleic acid sensors [22, 23]. Interestingly, while there is evidence that IFN α and IFN β may have functional redundancy, evolutionary genetics indicate that they are likely to have specificities [24]. All somatic nucleated cells can produce IFN α , while IFN β is mostly produced by specialized immune cells such as plasmacytoid dendritic cells (pDC) [22]. During viral infections, pDC are the primary source of type I IFN, a transient response, which is further relayed by other cell types depending on virus infection modes and targeted tissues. Indeed, antiviral immune responses involve the orchestration of different cell subtypes (eg: epithelial, fibroblastic or immune cells such as monocytes or tissue resident macrophages) to face viruses targeting mucosa, central nervous system or leading to

systemic infection [25]. Persistence of viral infections, triggering chronic IFN production might evolve towards immunopathologies.

Identification of type I IFN-producing cells in complex biological systems such as biomedical models is thus of interest to evaluate the complexity of regionalized innate immune responses and discover novel biomarkers and therapeutics with broad-range efficacy against inflammatory disorders.

I.2. STING-dependent IFN production

Production of type I IFN in mammalian cells can result from accumulation of cytosolic DNA recognized by specialized receptors. Such receptors notably include DNA-dependent activator of IFN regulatory factors (DAI), cyclic guanosine monophosphate-adenosine monophosphate (cGMP-AMP) synthase (cGAS), and interferon gamma-inducible protein 16 (IFI16) ([26-29]) (Figure 1A). Additional receptors have been described, such as RNA polymerase III (RNA pol III), LRR Binding FLII Interacting Protein 1 (LRRFIP1), DExH-Box Helicases 9 and 36 (DHX9 and DHX36), DEAD-Box Helicase 41 (DDX41), or proteins involved in double strand break repair (MRE11, or Rad50) [30] (Figure 1A and Table I). Most of the human genes encoding these proteins have orthologues in mice and zebrafish genomes (Table I), with the exception of sensors belonging to Pyrin and PYHIN gene family, such as IFI16, which appear to be restricted to mammals (Figure 1A).

Amongst these sensors, cGAS has raised particular interest because of its central role in various pathologies [31-37]. The pathway leading from cGAS-dependent recognition of dsDNA, ssDNA or RNA:DNA hybrids to STING (also known as MITA, ERIS, or MPYS) activation has been well characterized. Indeed, association with nucleic acid substrates leads to the production of cyclic GMP-AMP (cGAMP), a second messenger that triggers the activation of STING through association with its cGAMP-binding pocket [38]. Mouse models of infection show that cGAS is indispensable for the detection of pathogens *in vivo* [34, 39], implying that other reported sensors may be poorly relevant *in vivo*. Activation of cGAS

zebrafish isoforms also triggers synthesis of cGAMP and activation of STING-mediated IFN responses *in vitro* and *in vivo* [40]. These recent data contrast with previously reported DNA sensing mechanisms in zebrafish larvae. Indeed, herpes simplex virus 1 (HSV1) infection triggers STING-dependent IFN pathway through activation of alternative DNA sensors, namely zDHX9 and zDDX41 in a cGAS-independent manner [41].

To have an in-depth study of the diversity of STING proteins, we established a comprehensive phylogenetic tree of STING across the tree of life. Four main kingdoms were identified, namely, mammals, fish, birds and reptiles (Figure 2A). The evolutionary study of STING revealed that even though the evolutionary linkage between human (*Homo Sapiens*), mouse (*Mus Musculus*) and zebrafish (*Danio Rerio*) is very distant, the overall fold of STING is conserved. The sequence alignments of the aforementioned STING proteins (Figure 2B) were used to model the 3D structure of the zebrafish STING. Notwithstanding, in human, murine and Zebrafish models, STING has emerged as a central pivotal molecule in the signaling cascade triggered in response to the presence of immune-stimulatory nucleic acids. Interestingly, the superposed x-ray structures of monomeric human and mouse STING as compared to that of the Zebrafish model (Figure 2B) reveal that all the above-mentioned STING proteins have similar fold when considered as monomers. However, while STING is mostly monomeric in absence of immunological challenge, its association with cGAMP promotes dimerization. Comparative studies of murine and human STING demonstrate species-specificities for their affinity of the DMXAA analogue of cGAMP that results from differences in the dimer conformation [42]. Zebrafish STING follows a similar overall fold to the human and mouse STING proteins. Therefore it is expected that Zebrafish will behave in a likewise pattern where it spends most of its time in monomeric conformation and tends to dimerize upon immunological stress. STING dimerization promotes the assembly of the “STING signalosome”, comprised of the Tank Binding Kinase 1 (TBK1) and transcription factors, including Interferon Response Factor 3 (IRF3) and/or Nuclear Factor kappa B (NF- κ B) [43]. Subsequent phosphorylation of IRF3 and NF- κ B leads to their nuclear translocation

and transcription of a set of genes including pro-inflammatory cytokines and type I IFN [43]. This sequence of events has been similarly described in mouse and zebrafish [9]. However, despite conservation of cGAS-STING-IRF3-interferon signalling in vertebrates, in Zebrafish, an extension of the C-terminal domain of STING leads to non-canonical TNF receptor associated factor 6 (TRAF6) recruitment and preponderant NF- κ B activation as compared to in mammalian cells [9]. Additionally, direct activation of signal transducer and activator of transcription 6 (STAT-6) by the STING signalosome has also been reported in mammalian cells, but not in Zebrafish [44].

Dysregulation of STING activation fuels several inflammatory human pathologies, including autoimmune, auto-inflammatory and malignant disorders [6]. The characterization of the engaged molecular mechanisms are therefore paramount to a better understanding and proper implementation of the Zebrafish model as a tool for the study of innate immune responses.

I.3 STING-independent signaling, cross talks and regulatory loops

As stated above, studies using cGAS or STING knockout mice show that the corresponding proteins are indispensable for the detection of DNA viruses *in vivo* [34, 39], establishing the cGAS-STING pathway as the main detector of immune-stimulatory dsDNA. Therefore, the study of other sensors, such as DAI or IFI16 aroused less interest. However, recent work has questioned this paradigm. Indeed, it has been reported that the DNA-PK DNA repair pathway can operate in the detection of cytosolic dsDNA in human cells, while such DNA-PK-dependent IFN production is not witnessed in murine cells [45]. Remarkably, this pathway does not require STING. This work sheds new light on the previous assumption that mouse models are essential for *in vivo* validation of a nucleic acid sensing pathway. This further suggests that alternative *in vivo* models should allow reassessment of the impact of other pathways in a cell-type and/or species specific fashion. The role of such STING-independent

signaling in Zebrafish is, as of today, poorly explored although orthologues of actors of DNA-PK DNA repair pathway are identified (Table I).

There are limited reports of cross talks between known nucleic acid detection pathways. Indeed, it has been reported that detection of RNA by the retinoic acid-inducible gene I (RIG-I) receptor can potentiate cGAS-STING-associated signaling ([46]) *in vitro* in human cells. To the contrary, in murine macrophages and dendritic cells, the Absent In Melanoma 2 (AIM2) inflammasome inhibits the cGAS-STING pathway [47]. Conversely, the cGAS-STING axis can also prevent AIM2 activation in human myeloid cells, favouring activation of the NOD-, LRR- and pyrin domain-containing protein 3 (NLRP3) inflammasome [48]. Recent work shows that the Lysyl-tRNA synthetase (LysRS) inhibits STING in murine fibroblastic cells and *in vivo* in zebrafish larvae [49]. The existence of these cross-talks and regulatory loops, in specific cell types, leave open the question of whether they also operate in additional cell types (Figure 1B). Novel approaches should be designed to integrate both different identified cytosolic receptors and cellular diversity.

II- Cytosolic dsDNA and interferon-related disorders: emerging challenges

Most of our current understanding of nucleic acid detection pathways comes from studies of pathogen-associated inflammatory responses and has been improved through the emergence of models reproducing chronic inflammatory disorders. Below, we discuss some of the most used models of these pathologies to highlight the benefit of combining such divergent systems to decipher nucleic acid immunity and identify potential therapeutic targets common to different pathologies.

II.1 Models of inflammatory disorders

Under homeostatic conditions, cytosolic DNA accumulation is limited by nucleases such as DNases. A growing number of diseases have been characterized as bearing mutations in such genes and associated with dysregulation of IFN pathways. These pathologies are grouped under the name of type I interferonopathies [50] and result from chronic pathological activation of IFN signaling in response to abnormal accumulation or modification of the composition of cytosolic nucleic acid, dysregulation of nucleic acid sensors and/or downstream proteins of the pathways and alteration of regulation loops [50]. Medical studies have been pursued to investigate the potential role of the type I IFN in such diseases.

Several models of Aicardi–Goutières syndrome (AGS) have been developed. AGS is a congenital infection-like syndrome where patients present high plasma levels of type I IFN, leading to neuronal inflammation and encephalopathy. This disease is characterized by an abnormal cytosolic accumulation of nucleic acids that induce cGAS-dependent IFN production. In agreement, mutations in genes encoding proteins involved in nucleic acid catabolism have been shown to be responsible for the onset of AGS, including mutations in TREX1 [51], RNASEH2 endonuclease complex, [52], SAMHD1 [53], ADAR [54] and IFIH1 [55, 56].

Genome editing has been conducted to mutate or invalidate murine orthologues of these human genes and generate AGS models in mice. However, although *Trex1*-null mice present excessive cytosolic DNA accumulation, they do not progress towards AGS-like symptoms,

such as central nervous system inflammation [57]. These mice rather develop myocarditis, an inflammatory cardiomyopathy, with high levels of IFN in the heart. Such abnormalities caused by *Trex1*-deficiency are fully rescued by deletion of *cGas*, *Sting* or *Ifnar1* [58]. Conversely, chronic activation of IFN signaling pathway in *Trex1* D18N mice leads to lupus-like autoimmunity. Intriguingly, *Samhd1*-deficient mice fail to recapitulate any of the AGS associated phenotype so far [59]. Most other models of AGS are embryonic or perinatal lethal promoting the development of inducible alternative models (Cre-loxP technology). In Zebrafish, knockdown of *samhd1* is sufficient to recapitulate the human inflammatory disease and leads to type I IFN induction associated to cerebrovascular abnormalities [60]. Conservation of the *Samhd1* gene from zebrafish to human allows the rescue of zebrafish *samdh1* knockdown by injection of the human *SAMHD1* orthologue, opening perspectives to conduct functional studies of *SAMHD1* mutations. Thus, in combination with transgenic mice, zebrafish may contribute to recapitulate phenotypes of autoimmune disorders, akin to those witnessed in humans. Similarly, zebrafish mutants were described as biomedical models of *rnaseTe2* leukoencephalopathy [61, 62], a genetic disease mimicking a cytomegalovirus brain infection associated to inflammatory pathology as AGS [63].

Mutations in *STING* can also drive type I interferonopathies. Indeed, autosomal dominant gain of-function mutations in *STING* cause *STING*-associated vasculopathy with onset in infancy (SAVI). SAVI patients exhibit early-onset systemic inflammation with a robust type I IFN signature, severe skin vasculopathy and interstitial lung disease resulting in pulmonary fibrosis and respiratory failure [64, 65]. SAVI-associated *STING* mutations lead to spontaneous dimerization and activation of *STING* in the absence of cGAMP [65]. Mouse models of SAVI, harboring the two most common mutations found in patients (N154 and V155), present constitutive activation of *STING* and subsequent systemic inflammation, immune abnormalities and lung inflammation similar to that seen in human patients [66]. Up to now, there is no evidence of SAVI zebrafish model, although zebrafish *Sting* showed 30% of sequence identity with the human protein and a conservation of the N154 and V155

residues. Using conventional homology modeling pipelines, we have established a three dimensional model of the monomer of Sting from Zebrafish (zfSTING) (Figure 2B). Structural superposition of zfSting model to 3D structure of human and mouse STING showed an overall conserved 3D fold arrangement for all proteins. Moreover, the 3D positioning of N154 and V155 residues has also been conserved as they perfectly superposed in human, mouse and zebrafish (Figure 2C). Those two residues are located in the outer surface of a fully exposed to solvent α -helix (Figure 2C). The fact that there is nearby network of α -helices in near proximity (highest overall α -helical content in STING) is indicative of a possible protein interaction site, which makes those residues excellent mutation candidates towards the modelling and elucidation of SAVI..

Beyond diseases with a genetic component, self-DNA and cGAS-STING activation participate to a broader spectrum of diseases. The cGAS-STING pathway is responsible for self-DNA-driven inflammation in myocardial infarction [67], in Parkinson disease [68] or in the development of nonalcoholic fatty liver disease [69]. In these contexts, self-DNA from various sources such as mitochondrial stress, replication stress or engulfed and undigested self-DNA can aberrantly accumulate in the cytosol leading to a state of persistent deleterious inflammation. In addition, activation of the cGAS-STING pathway has also been reported in both familial [70] and sporadic cancer [71], establishing this pathway as a target for therapeutic strategies aiming to manipulate chronic inflammation in cancer, alongside other immunotherapies.

Additional Zebrafish models present robust and progressive IFN induction during the first stage of development [72], together with leukocytes expansion and gross morphological defects. Such induction of IFN relies on STING-TBK1 signalling by increased activity of Class I retro transposons such as the endogenous retrovirus ZFERV (zebrafish endogenous retrovirus). These transgenic lines are thus important tools for screening anti-inflammatory and/or anti-viral molecules, notably in the light of the IFN response being abolished by compounds used in Humans such as inhibitors of TBK1 phosphorylation or inhibitors of

transcriptase activity (eg. Foscarnet) [72].

II.2. Persistent viral infections and dysregulated IFN

Chronic inflammation and viral persistence, leading to sustained type I IFN production are detrimental to innate and humoral responses, as well as T-cell biology. For example, pathogenic Human Immunodeficiency Virus (HIV) infection leads to persistent type I IFN production, which drives significant innate immune dysfunction, ultimately responsible for increased inflammation and immunosuppression, along with reduced antigen presentation [73]. In mice, chronic administration of type I IFN at doses mimicking chronic viral infection, similarly induce immunosuppression via the suppression of specific CD8⁺ T cells responses [74]. Consistently, IFNAR blockade during persistent viral infection restores immune function via a decrease of T-cell apoptosis, hyperactivation and exhaustion [75, 76].

The first demonstration of the role of intracellular DNA sensing pathway in host defense was a report of Sting-knockout mice susceptibility to HSV1 exposure. Indeed, in Sting-knockout mice, HSV1 infection is lethal [39] and a very similar response was observed for cGAS-deficient mice [34]. However, limitations of mouse and zebrafish models to study host pathogen interactions have been encountered for several human viruses such as the Human Immunodeficiency Virus (HIV) that do not replicate in mice [77, 78]. In zebrafish, the establishment of human viral infectious models faces several limitations. Notably, zebrafish can be reared in a limited temperature range (between 25°C and 33°C), which may not be well suited for some viruses [79]. Moreover, efficient viral entry necessitates the expression of zebrafish orthologues of known human viral receptors [79]. In addition to naturally occurring viruses reported in Zebrafish [80-82], experimental infections have been successfully conducted with animal and human viruses [83] recapitulating viral tropisms, pathogenesis and antiviral innate immunity observed in natural hosts [83]. Thus cost effective antiviral screening strategies were described on zebrafish larvae against Herpes Simplex Virus-1, Influenza, Chikungunya and hepatitis viruses [84]. Investigation of antiviral innate

immunity mostly focused on RNA viruses with the exception of HSV-1, whose administration leads to viral replication in the central nervous system and recognition of viral DNA by zDHX9 and zDDX41 cytosolic sensor proteins. This leads to the activation of the STING-TBK1 signalling pathway and type I IFN production. Characterized zebrafish orthologues of mammalian cGAS were dispensable in this context [41], although STING was essential as in humans and mice [34, 39]. These data are contrasted with the ability of zebrafish cGAS orthologues to synthesize cGAMP and activate STING-mediated IFN signalling in response to DNA stimulation [40].

III- Future of Cytosolic NA immunity disorders and therapeutic perspectives

Altogether, the various origins of diseases linked to chronic type I IFN production, and the dichotomous impact of IFN (sometime pro-, sometimes anti-inflammatory) underscores the need for novel approaches to screen for:

- the pathways elicited upon immunological challenge
- the impact of different stimulus in specific microenvironments
- the discovery of novel inflammatory and anti-inflammatory compounds and assessment of their impact

Below, we review the tools and models developed to reach these goals, underlining the importance of integrative and comparative analyses for in depth characterization of human pathologies.

III.1. Models and tools for the study of regionalized innate immune responses

Spatio-temporal analyses of the innate immune responses at cellular resolution *in vivo* is fundamental to uncover the complexity of regionalized inflammatory responses. Historically, this has been mainly achieved through development of IFN-signalling reporter mouse strains and new imaging technologies to investigate cell-type-dependent IFN responses following diverse stimuli (infectious or not). Current models include Mx2-luciferase [85], Mx1-GFP [86], and immunity-related GTPase m1 (*Irgm1*) reporter mice strains (M1Red) [87]. These mouse lines have allowed the identification of cells responding to IFN species at cellular resolution through *ex vivo* analyses (FACS or immunohistochemistry on selected tissues) or at tissular resolution *in vivo* through bioluminescence imaging. These approaches enabled the spatio-temporal analyses of IFN producing/responding cells in naïve or stimulated animals: thymic epithelial cells were showed to produce constitutively high expression level of the IFN- β reporter [88], while splenic plasmacytoid dendritic cells (pDCs), conventional dendritic cells (cDCs) and macrophages are the main type I IFN producers during systemic infection by murine CMV [89]. In the case of respiratory infection (NDV virus), type I IFN is mainly produced by lung macrophages [90].

In contrast, Zebrafish larvae, which recapitulates the innate immune response to some human infectious diseases [83, 84], were exploited to develop imaging strategies at the level of the whole body, taking advantage of its small size and optical transparency. Hence, transgenic zebrafish lines were engineered, in which the type I IFN promoter drives the expression of fluorescent proteins [91]. Dynamic imaging of these transgenic lines allowed the visualization of the IFN producing cells in response to diverse stimuli. This has fostered innovative imaging approaches to identify, discriminate and track IFN-producing cells (neutrophils and hepatocytes) upon Chikungunya infection, in real time revealing the differential production waves of IFN ϕ 1 and ϕ 3 overtime [91]. Development of novel transgenic lines for the detection of ISGs such as *isg15* and *mx* provide novel tools to decipher the dynamic and distribution of the IFN response [92, 93]. In combination with these imaging approaches, genome publication enables transcriptomic approaches, while proteomic and biochemistry approaches are far less envisaged due to the limited biological material and the lack of available antibodies against Zebrafish antigens.

III. 2. Screening approaches and cellular diversity

Owing to its amenability to high-content screening, zebrafish have been used as a biomedical model for phenotypic-based screening approaches [94]. Existing screening strategies for anti-inflammatory therapeutics relies on endotoxin injections [95], exposure to chemicals [96], tissue damage [97, 98] or challenge of zebrafish mutants with a chronic inflammation phenotype [60]. Efficacy of therapeutic candidates is assessed by combining high-throughput, high-content imaging of zebrafish transgenic lines for immune cells recruitment at the site of inflammation and/or expression of key cytokines involved in the inflammatory response (type I IFN, IL-1 β , IL-6, and TNF- α). Imaging approaches can be combined to transcriptomic analyses at the level of the all larvae or on sorted cell subpopulations.

Technological developments in screening methods have relied on automation of experimental processes, for faster and higher content analyses by novel biostatistics analyses tools. Microfluidic devices have been set up for Zebrafish larvae handling dedicated to high-throughput screening (96-well plates) (Figure 3) [99]. These systems (now commercially available) were combined with automatic stimulation processes such as pathogen microinjections [100] or laser photoablation [99]. High-throughput high-content imaging is assessed by high-speed microscopy (confocal, spinning disk and, recently, lightsheet) allowing fast three dimensional (3D) acquisition of whole larvae, in multi-well plates in less than an hour [99, 101, 102]. Alternatively, microfluidic analyser and sorter enable high content screening of zebrafish larvae based on morphological features and fluorescence signals [103]. These platforms can be extended to sorting of selected experimental clusters for transcriptomic experiments [104]. Currently, the bottleneck of these innovative technologies is the limit of Big data storage, treatment and analyses. Integration of multi-parametric values (imaging, transcriptomic, proteomic) appears now essential to build a global view of the nucleic acid immunity at the level of the individual [104].

While murine models are not amenable to high-throughput screening (Figure 3), the last few years have seen a massive revolution in the field of 3D tissue culture (or organoids). Such self-organizing, multi-cellular *ex vivo* 3D cultures are considered as physiologically relevant representations of organs. These cultures reconstitute architectural properties and part of the biological functions of the original tissues from which they are derived. Recent studies have reported the use of organoids in combination with mouse models or 2D cell culture to describe the role of the IFN in viral infections [105], interferonopathies [106] or support personalized tumor therapies [107]. Because organoids can help reproduce the complex features of inflammatory pathologies, such as cellular heterogeneity, tissue physiology and genetic background, it is now envisioned to generate organoids from patient cells. This would recapitulate the physiopathology of the disease thus allowing to test, adapt and optimize therapies for personalized precision medicine (Figure 3).

The rapid development of single cell RNA sequencing (scRNA-seq) approaches has also contributed to a massive leap forward in our understanding of spatio-temporal organization of immune responses [108]. This, coupled to mass cytometry approaches has been extensively used to characterize immune processes in mice, allowing an unprecedented insight in the ways in which immune responses are orchestrated in complex environments. scRNA-seq data analysis allows insight in the dynamic regulation and activation of immune cell subpopulations in specific contexts, over time. For example it was possible to precisely characterize the expression of antiviral genes in a small subset of Bone marrow-derived dendritic cells (BMDCs) during the early stages of infection, whereas during the late stages of infection, these genes are uniformly expressed by all BMDCs. The 'early responder' BMDCs are responsible for sensing the infection and then signaling to the others BMDCs to act similarly [109]. This exemplifies how single-cell technologies and generated databases have allowed dissecting how immune responses are established at the single cell level, over time. This approach adds up to the already existing tools to dissect networks of immune cells and responses in mice.

IV. Systems-level analysis of nucleic acid immunity: novel insights and old concepts

Previously mentioned innovative technologies and analysis tools that generate and process Big data (omics data) have evolved at a rapid pace over recent year, projecting immunology research into systems-level analysis of immune responses [110].

Systems analysis aims to the integrate information emerging from several hierarchical levels (from cells to organisms or from molecules to tissues) and take into account the physiological context: cellular diversity, inter-cellular communications, tissues and organs microenvironments and species specificity (whole organisms). Therefore, it should combine heterogeneous data obtained from modern omics technologies, that permit the sequencing of full genomes, global transcriptional profiling (from microarray to RNAseq), as well as large-

scale proteomics and metabolomics analyses [111]. Global analyses of such multidimensional big data has led to the development of complex processing, visualization, in-depth analyses and bioinformatic tools. The analysis and storage bottleneck in immuno- and inflammo- bioinformatics is addressed via holistic artificial intelligence pipelines that are mainly cloud based. Innovative biostatistical methodologies are now established in cloud supercomputers to allow management and analysis of such large datasets. Those methodologies revolve around the realms of machine and deep learning as well as data filtering, data mining and autonomous learning. In the post genomics era, integration of omics data can permit elucidation of complex biological mechanisms, assist in efficient diagnosis, while speeding up the discovery and evaluation of novel therapeutics [112].

The complexity of innate immune responses and pathologies resulting from its misregulation calls for the application of such multiscale approaches. This is particularly true because, as discussed throughout this review, all model organisms (and organoids) only partially recapitulate disease spectra observed in humans. In support, a genome wide linkage analysis has allowed the identification of several genes involved in AGS, despite its heterogeneous symptoms and partial overlap with other autoimmune syndromes (systemic lupus erythematosus) or congenital viral infection [113]. This approach has led to the identification of several genes encoding nucleases as responsible for the onset of AGS (TREX1, RNase H2A, RNase H2B, RNase H2C, SAMHD1, ADAR and MDA5). Interdisciplinary researches (clinic, genetic, biology and data processing) has allowed a better understanding of this complex pathology, notably through integration of multi-systems (ie populations, individual patient, single cell to experimental models) and multi- data sets (Exome sequencing,) and has resulted in the selection of IFN-related biomarkers [114].

In systemic lupus erythematosus, integration of multi-omics data (publicly available data from SLE patients combined to various types of biological data (data-driven and knowledge-based approaches) has provided knowledge on regulation of IFN gene expression and its putative roles in SLE pathogenesis [115]. Another example is the integration of assays for

transposase-accessible chromatin using sequencing, RNA-seq and proteomics analyses to identify therapeutics to antagonize type I IFN deleterious effects on pancreatic human beta cells in autoimmune type I diabetes [116]. To go deeper in the characterization of the innate immune response, development of single cell omics analysis technologies now enables the investigation of spatial organization of innate immunity, dynamic clonality of IFN-producing cells and expression of nucleic acid sensor repertoire.

PERSPECTIVES

Despite the pressing urge to reduce, refine and replace (3R), in animal experimentation, studying immunological processes, their complexity and interconnection and how they lead to onset of human diseases heavily relies on animal models. In this context, zebrafish embryos, the second most common animal species used in research appears, are used as an alternative model of choice. However, mice models bear several historical advantages, which increase with time. Indeed, although mice models of pathologies bear several phenotypical differences with human disease spectrum, it has been thoroughly investigated and *in vitro* and *ex vivo* culture systems have allowed the dissection of molecular mechanisms in parallel to those dissected in human cell lines. Murine models are also extensively used in novel omics approaches, allowing unprecedented insight, at the molecular levels, in the complex processes occurring in distinct organs. We believe that comparative analyses between Zebrafish and mice could bring a novel insight in human pathologies and help identify primordial biomarkers and therapeutic targets. Finally, with the emergence of organoids, and their application in drug-screening approaches, questions have emerged concerning the place of animal experimentation in the future. However, it is important to bear in mind that organoids imperfectly replicate an organ, and their function and do not recapitulate what occurs at the whole body level. It is therefore likely that comparative and integrative approaches will in the future determine the way in which we investigate innate immune responses, while taking into account the whole organism.

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Figure legends:

Table 1: Orthology of human DNA cytosolic sensors and adaptor for mouse and zebrafish. Gene symbols are indicated as following Shh(italicized) for mice, SHH(italicized) for humans and shh(italicized) for Zebrafish.

Figure 1: STING-dependent and STING-independent signalling. **A.** Nucleic acid ligands, in particular dsDNA are recognized by a broad array of receptors. Amongst these, in mammalian cells (human, murine), cGAS has been shown to be the major receptor. In addition to dsDNA, cGAS has been shown to be stimulated by ssDNA and RNA:DNA hybrids. Activation of cGAS leads to STING dependent activation of IRF3 and NF-kB. In Zebrafish, cGAS has been shown to be dispensable for STING activation. STING is rather activated by DHX9 and DDX41, leading to activation of NF-kB-dependent cytokine production. **B.** Double stranded DNA (dsDNA) can elicit a STING independent response through recognition by the DNA-PK DNA repair complex. dsDNA can also inhibit STING following recognition by AIM2. In addition, LysRS is activated by RNA:DNA hybrids to inhibit STING. TF: Transcription Factors

Figure 2: Evolutionary and structural study of STING. **A:** Left; A comprehensive phylogenetic tree of STING across the tree of life (Arrows pinpoint Homo Sapiens – HS, Mus Musculus – MM and Danio Rerio – DR, Right; The sequence alignment amongst the human, mouse and the Zebrafish STING sequences (Arrow points at V155 and N154). **B:** Top Left; Molecular modelling of the Zebrafish STING (magenta ribbon) superposed on the human STING (green ribbon). Top Right; Superposition of the Mouse STING (blue ribbon) superposed on the human STING (green ribbon). Bottom; Hydrophobicity surface

representation of the superposed Human, Mouse and Zebrafish STING proteins. The Arrow point the entry point of the STING binding site, which is more hydrophobic. **C:** Superposition of the Human, Mouse and Zebrafish STING proteins with V155 and Q155 showing in ball and stick representation in the insert. The color coding follows the conventions of Fig2:B.

Figure 3: Comparison of experimental models used for discovery of new drugs. The drug development pipeline can take more than 10 years. The figure shows the different steps of drug development. Appropriate use of experimental models for studying nucleic acid immunity is of major importance for selection of lead drug candidates. *In vitro*, *ex vivo* or *in vivo* models can be used. While they are all amenable to genome editing, there are important differences regarding the feasibility of live imaging and high-throughput screening, physiological relevance and immune system complexity. Live imaging in mouse models can be performed at high-resolution to a limited depth using imaging window and 2-photon microscopy or at low resolution using bioluminescence imaging. Complexity and variability of 3D organoids culture has been problematic for establishment of high-throughput screening but different screening strategy has already been well implemented with zebrafish larvae. Relevance of the model for the disease studied has to be examine carefully. Zebrafish larvae are useful to study innate immunity as adaptive immunity is functionally mature at 4 weeks post-fertilization.

Supplementary Information 1: The full, high resolution, phylogenetic tree of STING across the tree of life including species name and accession numbers. The high resolution tree is provided in PDF and TIFF format at 600dpi resolution.

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Figures & Table

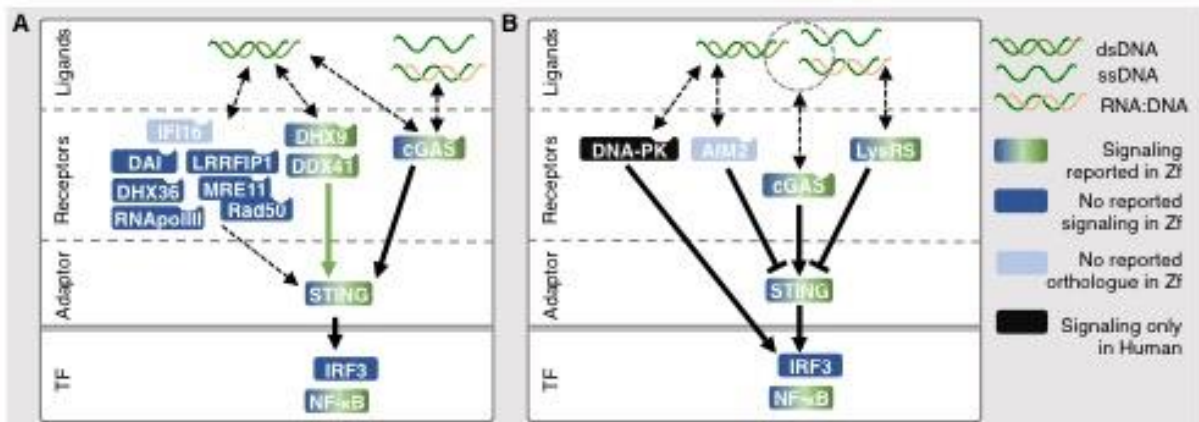


Figure 1: STING-dependent and STING-independent signalling. A. Nucleic acid ligands, in particular dsDNA are recognized by a broad array of receptors. Amongst these, in mammalian cells (human, murine), cGAS has been shown to be the major receptor. In addition to dsDNA, cGAS has been shown to be stimulated by ssDNA and RNA:DNA hybrids. Activation of cGAS leads to STING dependent activation of IRF3 and NF-κB. In Zebrafish, cGAS has been shown to be dispensable for STING activation. STING is rather activated by DHX9 and DDX41, leading to activation of NF-κB-dependent cytokine production. **B.** Double stranded DNA (dsDNA) can elicit a STING independent through recognition by the DNA-PK DNA repair complex. dsDNA can also inhibit STING following recognition by AIM2. In addition, LysRS is activated by RNA:DNA hybrids to inhibit STING. TF: Transcription Factors

		Human	Zebrafish	Mouse
	DNA SENSORS			
name of the Human gene	gene description	ensembl ID	ensembl ID	ensembl ID
ZBP1	DAI: DNA-dependent activator of IFN regulatory factors	ENSG00000124256	none	Zbp1 ENSMUSG000000027514
AIM2	AIM2: Absent-in-melanoma	ENSG00000163568	none	Aim2 ENSMUSG000000037860
POLR3A	RNA polymerase III subunit	ENSG00000148606	polr3a ENSDARG00000102569	Polr3a ENSMUSG000000025280
POLR3E	RNA polymerase III subunit	ENSG00000058600	polr3e ENSDARG00000037358	Polr3e ENSMUSG000000030880
LRRFIP1	Leucine Rich Repeat-1ightless-interacting protein	ENSG00000124831	lrrfip1a ENSDARG00000030012	Lrrfip1 ENSMUSG000000026305
DHX9	DEAH-Box Helicase	ENSG00000135829	dhx9 ENSDARG00000079725	Dhx9 ENSMUSG000000042699
DHX36	DEAH-Box Helicase	ENSG00000174953	dhx36 ENSDARG00000101059	Dhx36 ENSMUSG000000027770
DDX41	DEAD-box helicase	ENSG00000183258	ddx41 ENSDARG00000099739	Ddx41 ENSMUSG000000021494
IFI16	Interferon Gamma Inducible Protein	ENSG00000163565	none	Ifi204 ENSMUSG000000073489
PRKDC	DNA dependent protein kinase	ENSG00000253729	prkdc ENSDARG00000075083	Prkdc ENSMUSG000000022672
MRE11	meiotic recombination 11 homolog	ENSG00000020922	mre11a ENSDARG00000105014	Mre11a ENSMUSG000000031928
RAD50	RAD50: double strand break repair protein	ENSG00000113522	rad50* ENSDARG00000038917*	Rad50* ENSMUSG000000020380
CGAS	cyclic guanosine monophosphate-adenosine monophosphate (cGMP-AMP) synthase	ENSG00000164430	cgasa/cgasb ENSDARG00000021572	Cgas ENSMUSG000000032344
	DNA SENSING ADAPTOR			
TMEM173	Stimulator of Interferon Genes	ENSG00000184584	ENSDARG000000091058	ENSMUSG000000024349

*the human gene is orthologous to multiple genes in the other species.

Table 1: Orthology of human DNA cytosolic sensors and adaptor for mouse and zebrafish.

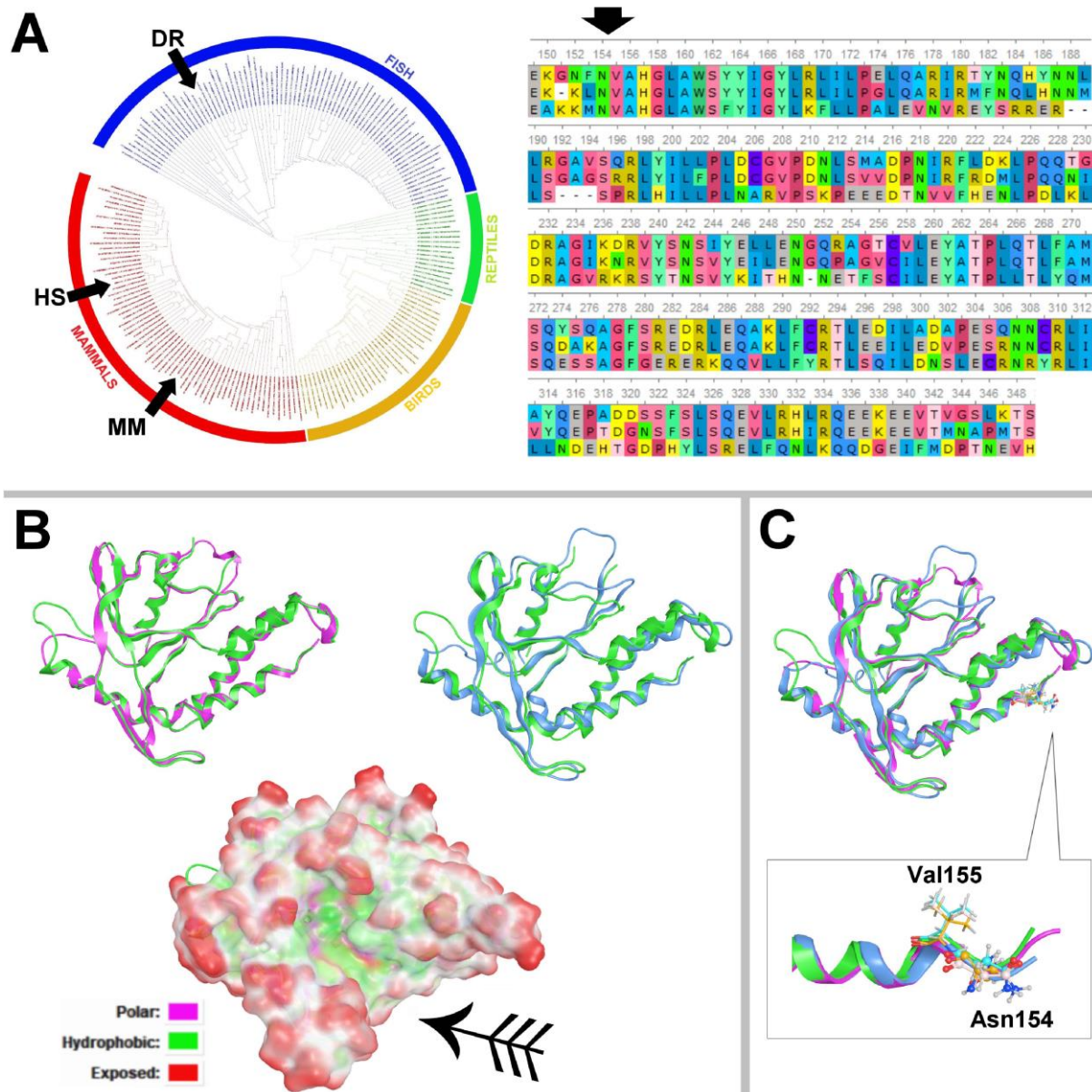


Figure 2: Evolutionary and structural study of STING. **A:** Left; A comprehensive phylogenetic tree of STING across the tree of life (Arrows pinpoint Homo Sapiens – HS, Mus Musculus – MM and Danio Rerio – DR, Right; The sequence alignment amongst the human, mouse and the Zebrafish STING sequences (Arrow points at V155 and N154). **B:** Top Left; Molecular modelling of the Zebrafish STING (magenta ribbon) superposed on the human STING (green ribbon). Top Right; Superposition of the Mouse STING (blue ribbon) superposed on the human STING (green ribbon). Bottom; Hydrophobicity surface representation of the superposed Human, Mouse and Zebrafish STING proteins. The Arrow point the entry point of the STING binding site, which is more hydrophobic. **C:** Superposition of the Human, Mouse and Zebrafish STING proteins with V155 and Q155 showing in ball and stick representation in the insert. The color coding follows the conventions of Fig2:B.

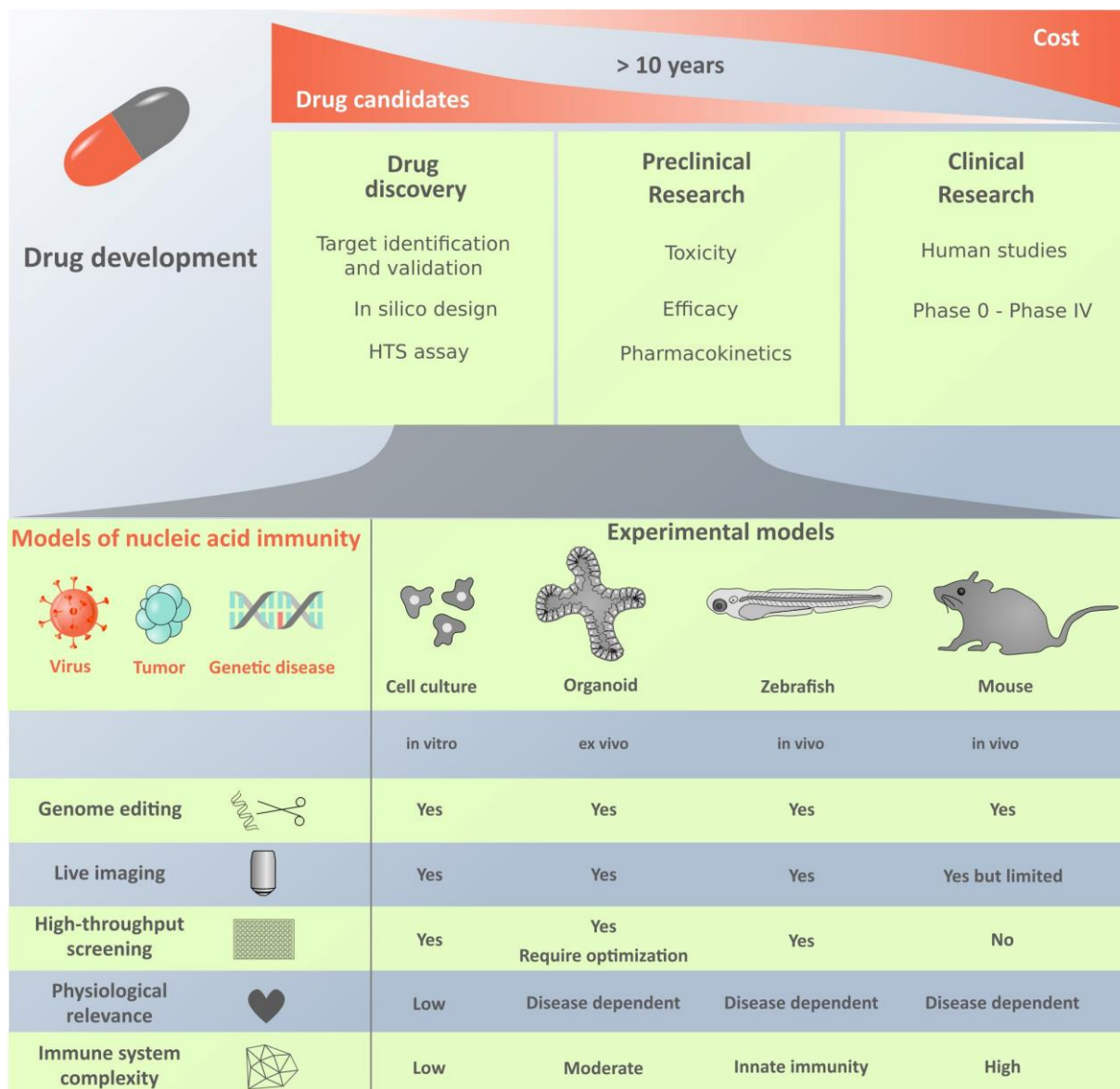


Figure 3: Comparison of experimental models used for discovery of new drugs. The drug development pipeline can take more than 10 years. The figure shows the different steps of drug development. Appropriate use of experimental models for studying nucleic acid immunity is of major importance for selection of lead drug candidates. *In vitro*, *ex vivo* or *in vivo* models can be used. While there are all amenable to genome editing, there are important differences regarding the feasibility of live imaging and high-throughput screening, physiological relevance and immune system complexity. Live imaging in mouse models can be performed at high-resolution to a limited deepness using imaging window and 2-photon microscopy or at low resolution using bioluminescence imaging. Complexity and variability of 3D organoids culture has been problematic for establishment of high-throughput screening but different screening strategy has already been well implemented with zebrafish larvae. Relevance of the model for the disease studied has to be examine carefully. Zebrafish larvae are useful to study innate immunity as adaptive immunity is functionally mature at 4 weeks post-fertilization.

Tree scale: 0.1



Supplementary Figure 1: The full, high resolution, phylogenetic tree of STING across the tree of life including species name and accession numbers.