Cell Death & Differentiation (2019) 26:395–408
https://doi.org/10.1038/s41418-018-0261-x

Cell Death & Differentiation

REVIEW ARTICLE

To NET or not to NET:current opinions and state of the science regarding the formation of neutrophil extracellular traps

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Received: 18 May 2018 / Revised: 5 November 2018 / Accepted: 30 November 2018 / Published online: 8 January 2019

Abstract

Since the discovery and definition of neutrophil extracellular traps (NETs) 14 years ago, numerous characteristics and physiological functions of NETs have been uncovered. Nowadays, the field continues to expand and novel mechanisms that orchestrate formation of NETs, their previously unknown properties, and novel implications in disease continue to emerge. The abundance of available data has also led to some confusion in the NET research community due to contradictory results and divergent scientific concepts, such as pro- and anti-inflammatory roles in pathologic conditions, demarcation from other forms of cell death, or the origin of the DNA that forms the NET scaffold. Here, we present prevailing concepts and state of the science in NET-related research and elaborate on open questions and areas of dispute.

Facts

- Neutrophil extracellular traps (NETs) are formed as a defense mechanism to immobilize invading microorganisms but also in response to sterile triggers.
- NETs consist of a DNA scaffold decorated with granule-derived proteins, such as enzymatically active proteases and anti-microbial peptides.
- Apart from their function in immune defense, NETs play important detrimental or beneficial roles in inflammation, autoimmunity and other pathophysiological conditions
- NET release can be instigated by many triggers and via a multitude of distinct pathways with often unknown interdependence.

Open questions

- Are NETs primarily formed from nuclear or mitochondrial DNA, or both? Does the source of the DNA depend on the activating stimulus and/or the specific conditions that trigger NET formation? Do NETs composed of nuclear or mitochondrial DNA reflect different pathways that are adapted to distinct physiological needs?
- How can we unambiguously distinguish NETs from the remnants of other forms of cell death?
- Is there a connection between NET formation, neutrophil aggregation and/or neutrophil swarming?

Edited by M. Piacentini

Supplementary information The online version of this article (https://doi.org/10.1038/s41418-018-0261-x) contains supplementary material, which is available to authorized users.

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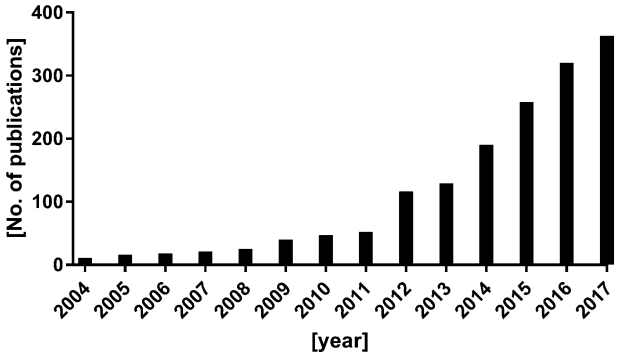

Fig. 1 Number of publications including the term "neutrophil extracellular trap" per year (according to PubMed)

- Is there a link between autophagy, necroptosis, pyroptosis and NET formation?

Introduction

Histones and other nuclear proteins organize DNA in the nucleus of eukaryotic cells into nucleosomes and higher-order chromatin by neutralizing the negative charges on DNA. Thus, protein–DNA interactions constrain the potential energy of DNA to extend into a fibrous polymer and allow it to participate in the complex choreography that defines cellular functions [1]. The uncoiling of DNA represents the release of that potential energy, as can be appreciated during the rupture of a cell, which vastly expands the volume of nuclear DNA.

In 2004, Brinkmann et al. observed that the release of nuclear chromatin can be a regulated process that results in the appearance of what they called neutrophil extracellular traps (NETs) [2]. This insight raised the possibility that the release of nuclear chromatin may have physiologically beneficial consequences by significantly contributing to host defense.

NETs consist of DNA fibers decorated with proteins normally confined to granules, including antimicrobial molecules [2–4]. Extracellular DNA traps have been shown to be able to contribute to the immobilization and neutralization of certain kinds of bacteria [2, 5], fungi [6–8], and even some viruses [9, 10].

NETs form by the release of potential energy contained in the nucleus overall [2], some parts of it [11], or mitochondrial nucleoid DNA [12]. The compact structure of nuclear chromatin may be loosened by several alternative mechanisms: one is the global transcriptional activation that unwinds the inactive chromatin at the majority of loci [13]. A second is the proteolytic degradation of histone termini that assist in folding nuclear DNA [8] and a third is the post-translational modification of positively charged residues in core and linker histones [14, 15]. These three processes may synergize with each other or take precedence under specific conditions.

In the course of the last 14 years, the number of publications involving NETs have virtually exploded (Fig. 1). A search on Pubmed (www.ncbi.nlm.nih.gov/pubmed/) for "neutrophil extracellular traps" yielded 1940 results through the end of October 2018. NETs have been implicated not only in anti-microbial defense but also in a variety of sterile inflammatory and autoimmune conditions [2, 4, 16–37].

A lively discussion is currently ongoing about key aspects of NETs, their contents and morphology [38, 39], how their formation should be precisely named to reflect the different pathways of their generation [40, 41], the fate of the NETing neutrophil [42], by which triggers NET formation can be induced [3, 43], and the implications of NET formation for the host [5, 39, 44, 45]. These reports are partly overlapping, conflicting, or in direct contrast to each other. Specifically, the requirement of certain molecular pathways, the connection between NET formation and cell death, and the source of DNA in NETs are a matter of debate [38, 46, 47] (Fig. 2). The use of different methods of detection and quantification of NETs in vitro, in serum and in tissue [48–56] also impedes interpretation and/or comparison.

We have made an effort to put together a broad panel of opinion leaders and experts in the field to formulate concepts and raise further questions regarding various aspects of NET formation. The seeds for this effort germinated during a NET consensus meeting held in Erlangen, Germany in September 2016.

This paper revolves around a list of statements that summarize levels of agreements on various NET-related questions (Table 1), accompanied by a commentary that focuses on open questions and areas of scientific dispute. In particular, we list aspects of terminology and mechanisms of NET formation, and of components, triggers, physiological functions and pathological implications of NETs. Furthermore, we elaborate on minimal requirements for proper experimental designs and methodological accuracy of NET-related studies and for quantification and definition of NETs. The article also contains paragraphs penned by individual authors that describe the state-of-the art and ongoing efforts in various areas of NET research in the light of their own research (Supplementary Text). This dual structure of the paper was intended to provide a glimpse into the kaleidoscope of current NET research.

Current consensus and diverging opinions in NET research

To shed light on opinions on NET-related topics, a questionnaire with 140 statements (submitted by the authors of

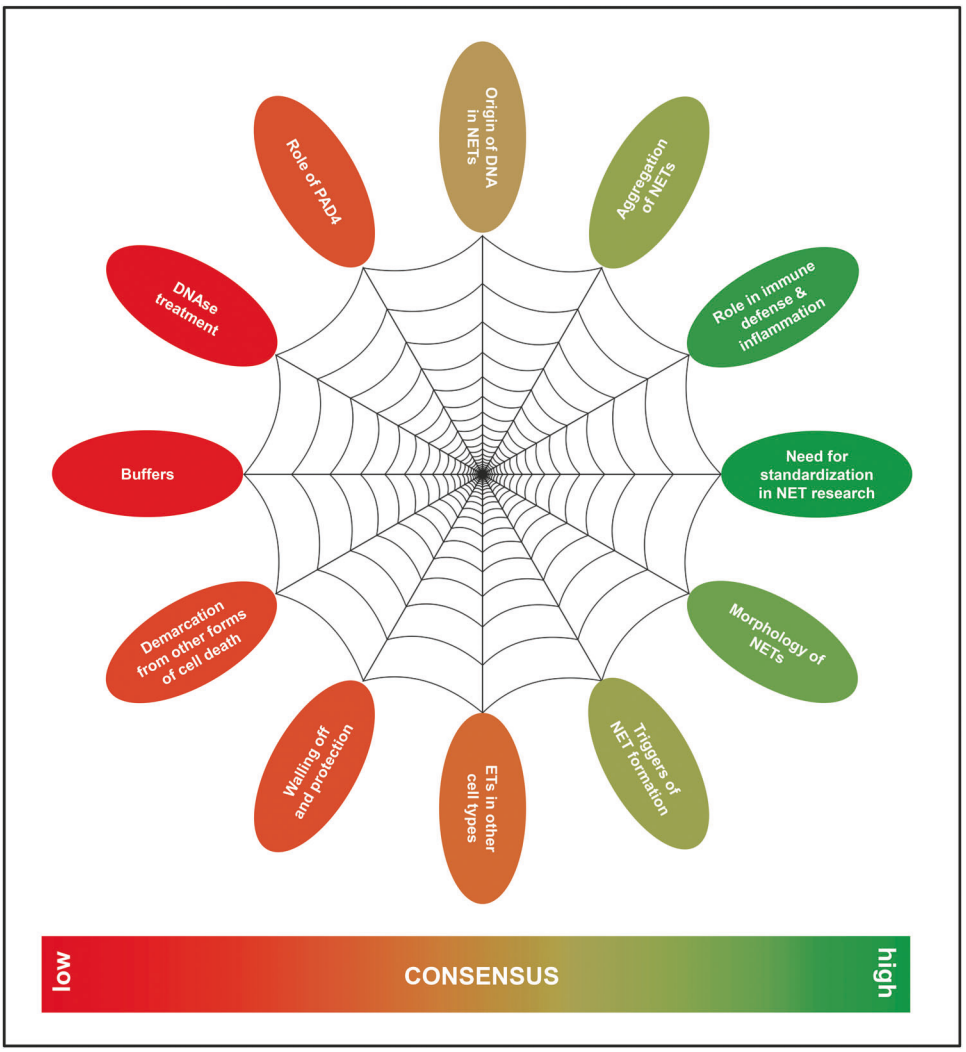

Fig. 2 Current areas of consensus and controversy about neutrophil extracellular traps (NETs)

this paper) was sent out. Every author was to rate the level of agreement with each statement (1, agree; 2, do not agree; 0, undecided). From this list, 85 non-redundant statements with high response rates were chosen (Table 1). This listing illustrates current areas of consensus and dispute.

General statements and terminology

A large majority of the authors of this paper agree that the current literature creates confusion for lack of proper definitions of NETs (*statement 1, st. 1*). In many publications, extracellular DNA derived from different sources and/or after different forms of cell death pathways is collectively and erroneously equated with NETs. This confusion might partly arise due to the use of unspecific bioassays (such as measurement of extracellular DNA, measurement of extracellular elastase activity) that are used as a surrogate for NETs (*st. 2*). Different isolation procedures and neutrophil sources (isolation via density gradient centrifugation or magnetic cell sorting from bone marrow or from peripheral blood) or species differences between mice and humans may further complicate this issue. Also, the term "NETosis" suggests that cell death is an inevitable consequence of extrusion of DNA. Yet it is challenging to determine the exact sequence of events and the fate of the cell in retrospect when analyzing tissue sections. Furthermore, not even all pathways of NET generation elicited under controlled experimental conditions in vitro result in cell death. Therefore, in alignment with the Nomenclature Committee of Cell Death [46] the authors of this paper suggest to avoid the term"NETosis" or use it only in contexts where the demise of the neutrophil is obvious (*st. 3, 4*). In all other cases, we recommend to use the term "NET formation" instead.

Composition and morphology of NETs

There is a strong consensus that NETs contain enzymatically active neutrophil proteases (*st. 7*) and other antibacterial molecules. The source of DNA in NETs is less

Table 1 Statements about NET-related questions

statement #			yes	no	# of answers	% agreeing
	GENERAL STATEMENTS & TERMINOLOGY					
1	GENERAL	The current NET literature creates confusion for lack of adequate definitions.	42	1	43	97,67
2	GENERAL	The current NET literature creates confusion for overinterpretation of unspecific bioassays.	41	1	42	97,62
3	GENERAL	The use of the term 'NETosis' requires re-consideration.	33	10	43	76,74
4	GENERAL	Cell death via NETosis is an incompletely understood process, there are multiple pathways that can result in the release of NETs, and therefore the use of the term NETosis is misleading and inaccurate.	35	6	41	85,37
	COMPOSITION AND MORPHOLOGY OF NETs					
5	COMPONENTS	NETs may contain mitochondrial and/or nuclear DNA.	34	6	40	85,00
6	COMPONENTS	NETs form from mitochondrial DNA that lacks histones and are organized in a unique manner.	30	9	39	76,92
7	COMPONENTS	NETs are bound by enzymatically active neutrophil proteases.	34	1	35	97,14
8	MORPHOLOGY	NETs *in vitro* can have a cloud-like appearance	33	6	39	84,62
	OCCURRENCE OF NETs IN VIVO					
9	In vivo NETs	NET release that occurs in vivo: bacterial infections	40	0	40	100,00
10	In vivo NETs	NET release that occurs in vivo: fungal infections	34	0	34	100,00
11	In vivo NETs	NET release that occurs in vivo: parasitic infections	28	0	28	100,00
12	In vivo NETs	NET release that occurs in vivo: Thrombus formation in vascular occlusion diseases	33	0	33	100,00
13	In vivo NETs	NET release that occurs in vivo: Tophus formation in gout	36	0	36	100,00
14	In vivo NETs	NET release that occurs in vivo: ductal occlusion in pancreatitis	32	0	32	100,00
	PHYSIOLOGICAL FUNCTIONS OF NETs					
15	ANTIMICROBIAL EFFECTS	As initially proposed and confirmed in a number of studies, NETs serve to immobilize bacteria, viruses and fungi.	36	0	36	100,00
16	ANTIMICROBIAL EFFECTS	NETs are decorated with various anti-microbial compounds and thus provide an environment that is particularly suited for destruction of extracellular pathogens.	40	0	40	100,00
17	ANTIMICROBIAL EFFECTS	NET release is an ancient immune defense mechanism that can be traced to early multicellular organisms.	34	0	34	100,00
18	ANTIMICROBIAL EFFECTS	Certain prokaryotic pathogens have evolved mechanisms to deactivate NETs via nucleases or even exploit NETs for the purpose of constructing biofilms.	34	1	35	97,14
19	INFLAMMATION	Depending on the local site, the trigger and the inflammatory milieu, NETs can have both pro- and anti-inflammatory effects.	38	3	41	92,68
20	INFLAMMATION	NETs contain active components that can degrade or inactivate cytokines and chemokines.	34	1	35	97,14
21	AGGREGATION	Under conditions of high cell density, NETs can form aggregates.	30	0	30	100,00
22	AGGREGATION	Aggregation of activated neutrophils is not related to NET formation.	10	15	25	40,00
23	AGGREGATION	The formation of aggregated NETs can have both detrimental and beneficial effects.	39	0	39	100,00
24	AGGREGATION	Aggregated NETs degrade pro-inflammatory mediators and therefore exert beneficial anti-inflammatory activity.	28	3	31	90,32
25	PLATELETS & COAGULATION	NETs can serve as a stimulus for platelet aggregation.	34	0	34	100,00
26	WALLING OFF & PROTECTION	NETs are preferentially formed in ducts and on the bodies' external and internal surfaces	9	17	26	34,62
27	WALLING OFF & PROTECTION	NETs have a protective function along the body's mucosal surfaces by providing a refractory coating.	15	8	23	65,22
28	WALLING OFF & PROTECTION	Aggregated NETs wall-off sites of tissue damage independently of the presence of pathogens.	17	5	22	77,27
	TRIGGERS OF NET-FORMATION					
29	TRIGGERS	NETs develop in response to bacteria	38	0	38	100,00
30	TRIGGERS	NETs develop after contact to fungal hyphae	39	0	39	100,00
31	TRIGGERS	NETs develop in response to biochemical stimuli	38	0	38	100,00
32	TRIGGERS	An influx of extracellular calcium into the cytoplasm of cells is a strong trigger for NET release.	31	2	33	93,94
33	TRIGGERS	NETs develop in response to immune complexes	34	3	37	91,89
34	TRIGGERS	NETs develop after contact to activated thrombocytes (platelets)	31	1	32	96,88
35	TRIGGERS	Damage associated molecular pattern molecules play an important role in NET release, for example molecules such as HMGB1 and ATP.	26	2	28	92,86
36	TRIGGERS	Lysosomal membrane instability in neutrophils triggers NET formation.	12	9	21	57,14
	PATHWAYS OF NET-FORMATION					
37	PATHWAYS	NET release occurs by distinct and alternative pathways	33	7	40	82,50
38	PATHWAYS	The signaling pathways in NET formation are incompletely understood.	40	2	42	95,24
39	PATHWAYS	NET release is driven by cell-intrinsic pathways that are activated by external stimuli.	35	3	38	92,11
40	REQUIREMENTS FOR NET-FORMATION	NET formation may occur in a NOX2-dependent and independent fashion.	37	1	38	97,37
41	REQUIREMENTS FOR NET-FORMATION	NET formation may occur in the absence of MPO dependent on the stimulus	23	4	27	85,19
42	REQUIREMENTS FOR NET-FORMATION	NET formation may occur in the absence of PAD4 dependent on the stimulus	33	2	35	94,29
43	REQUIREMENTS FOR NET-FORMATION	PAD4 is a calcium-dependent enzyme with a central function in NET formation	26	11	37	70,27
44	REQUIREMENTS FOR NET-FORMATION	PAD4 is not required for chromatin externalization.	20	16	36	55,56
45	REQUIREMENTS FOR NET-FORMATION	NET release is dependent on an interplay between protein kinase C isoforms.	14	15	29	48,28
46	REQUIREMENTS FOR NET-FORMATION	Autophagy activation is needed to create NETs.	10	16	26	38,46
47	NET FORMATION & CELL DEATH	NET formation in vivo may result in anuclear cytoplasts with a set of retained cellular function (e.g. migration, phagocytosis, ROS production)	26	2	28	92,86
48	NET FORMATION & CELL DEATH	NET formation may or may not involve neutrophil death, therefore, the two processes need to be looked at separately.	26	4	30	86,67
49	NET FORMATION & CELL DEATH	Crystal and nanoparticle-induced NET-formation is accompanied by disruption of the plasma membrane.	30	4	34	88,24
50	NET FORMATION & CELL DEATH	NET formation can occur upon unregulated (passive) or regulated forms of neutrophil necrosis.	11	23	34	32,35
51	NETS & NECROPTOSIS	RIP3/MLKL-mediated signaling may lead to NETs.	27	7	34	79,41
52	NETS & NECROPTOSIS	Not all pathways of NET formation require RIP3/MLKL-mediated signaling.	35	0	35	100,00
53	NETS & NECROPTOSIS	NET release follows an interaction with cell-damaging substances and in that way, NET release is related or analogous to necroptosis.	12	23	35	34,29
54	MITOCHONDRIA	Pathways of NET formation exist that are dependent on mitochondria	35	1	36	97,22
55	MITOCHONDRIA	Pathways of NET formation exist that are independent of mitochondria	31	4	35	88,57
56	MITOCHONDRIA	Nuclear membrane break-down is a hallmark feature of NET formation	33	5	38	86,84
	EXTRACELLULAR TRAPS IN DIFFERENT CELL TYPES					
57	CELL TYPES	Neutrophils release NETs.	43	0	43	100,00
58	CELL TYPES	Eosinophils release EETs.	28	4	32	87,50
59	CELL TYPES	Mast Cells release MCETs.	19	6	25	76,00
60	CELL TYPES	Monocytes release METs.	14	12	26	53,85
	PATHOLOGY AND TREATMENT					
61	PATHOLOGY	NETs may have detrimental or beneficial effects on the host depending on the context.	42	0	42	100,00
62	PATHOLOGY	NETs are central protagonists in autoimmune disease because NET release is coupled to PAD4 activationand deiminated (citrullinated) autoantigens are diagnostic and mechanistic markers in several autoimmune diseases.	25	8	33	75,76
63	PATHOLOGY	NETs represent a potential source of neoantigens in rheumatic diseases	35	0	35	100,00
64	PATHOLOGY	Neutrophils and platelets co-localize and productively interact at sites of vessel injury, hemorrhage and thrombosis.	36	0	36	100,00
65	PATHOLOGY	A consequence of neutrophil-platelet interactions is the change in inflammatory and hemostatic functions.	33	2	35	94,29
66	PATHOLOGY	Activated platelets release signals that influence the ability of neutrophils to generate NETs.	30	0	30	100,00
67	DNAse TREATMENT	In vivo application of DNase removes NETs and decreases collateral damage.	12	9	21	57,14
	MATERIALS AND METHODS					
68	STIMULANTS	NET studies based on application of PMA as a sole inducer are incorrect.	26	15	41	63,41
69	STIMULANTS	More physiologically relevant stimuli are required to study than PMA.	38	4	42	90,48
70	IDENTIFICATION & QUANTIFICATION	Minimal requirements to identify NETs in vitro or in vivo include the co-localization of extracellular DNA, neutrophil elastase and citrullinated histones.	34	8	42	80,95
71	IDENTIFICATION & QUANTIFICATION	Techniques based on intercalating dyes and on immunocytochemistry/immunofluorescence are necessary for a convincing assessment that neutrophils are forming NETs.	39	2	41	95,12
72	IDENTIFICATION & QUANTIFICATION	Techniques based on intercalating dies and on immunocytochemistry/immunofluorescence are not appropriate for a quantitative assessment of the extent of NET formation.	13	26	39	33,33
73	IDENTIFICATION & QUANTIFICATION	NET release can be measured by microscopic image analysis, extracellular DNA quantitation, and co-localization by immunofluorescence reactivity with antibodies to DNA, histones, elastase, or myeloperoxidase	38	0	38	100,00
74	IDENTIFICATION & QUANTIFICATION	NET release can be visualized, with the proper controls, in tissues provided appropriate ex vivo sample preparation is possible.	33	2	35	94,29
75	IDENTIFICATION & QUANTIFICATION	NET release can be observed in tissue sections, by live microscopy, and in fluid secretions.	35	1	36	97,22
76	NEUTROPHIL ISOLATION	Anticoagulation of venous blood with heparin might have unpredictable effects on the ability of neutrophils to generate NETs.	17	11	28	60,71
77	NEUTROPHIL ISOLATION	Neutrophils should not be purified from blood anti-coagulated with heparin.	11	19	30	47,62
78	NEUTROPHIL ISOLATION	NET generation is jeopardized as a consequence of prolonged absence of Mg++ (e.g. from extraction buffers).	13	11	24	54,29
79	NEUTROPHIL ISOLATION	Neutrophils should be purified using Mg++ containing buffers	13	15	28	46,00
80	CULTURE CONDITIONS	The constitution of the culture medium strongly influences the propensity to form NETs	35	2	37	94,59
81	CULTURE CONDITIONS	Standardized buffers are desirable, but hard to enforce.	41	0	41	100,00
82	CULTURE CONDITIONS	NET-forming assays must be performed under controlled CO2/HCO3-/pH balance.	34	0	34	100,00
83	REPORTING	Experiments on NET formation in vitro should exactly specify culture media conditions	42	0	42	100,00
84	REPORTING	Experiments on NET formation should report surface constitution of the culture plate	42	0	42	100,00
85	REPORTING	The company name which provided the biochemical stimulus should be stated in Materials & sMethods.	42	0	42	100,00

Color code
Agreement >80% or <20%
Agreement 70-80% or 20-30%
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unambiguous. Simon, Yousefi et al. described extrusion of NETs consisting of mitochondrial DNA together with granule proteins rather than nuclear DNA [57, 58]. This mechanism, which was also shown to occur in eosinophils [12], involves an active reorganization of the cytoskeleton [59] and is ROS-dependent, but not accompanied by cell death. While it is now acknowledged by a majority of authors that NETs can be formed from both nuclear and mitochondrial origin (*st. 5, 6, 54, 55*), a potential mechanistic and/or physiologic demarcation between these processes is still unclear. Simon and Yousefi also claimed that NETs composed of mitochondrial DNA manifest as fibers, while the cloud-like appearance of nuclear DNA often seen after prolonged incubation of neutrophils in vitro with canonical NET instigators such as phorbol 12-myristate 13-acetate (PMA) or bacteria [5] is a result of necrotic cell death [38]. However, according to the opinion of a majority of authors, NETs created in vitro can also have a cloud-like appearance (*st. 8*) and morphological differences might be due to mechanical agitation of culture slides (or the lack thereof).

NET formation in vivo

There is unequivocal consensus that NET formation in response to microbial and sterile agents is a real phenomenon that occurs in vivo (*st. 9–14*). However, it is important to highlight that only a limited number of studies have addressed the direct effect of specific stimuli in the induction of NETs in vivo. Despite these potential limitations, stimuli considered to be inducers of NET formation in vivo are bacteria [2], fungal hyphae [7], biochemical stimuli [2, 34, 41, 60–63], some inflammatory cytokines and chemokines [2, 64, 65], immune complexes [66], and contact with activated platelets [67].

Physiological functions of NETs

An important function of NETs is the defense against bacteria, viruses and fungi (*st. 15*). NETs not only immobilize the opponent [68], but also are equipped with anti-microbial compounds (such as anti-microbial peptides, histones and proteases) (*st. 16*). They are therefore considered able to kill pathogens directly. Extracellular DNA-containing structures have also been described in zebrafish [69], in cats [70], invertebrates [71, 72], and plants [73]. Therefore, the formation of extracellular DNA traps may be considered an ancient, evolutionary conserved defense mechanism [71, 74] (*st. 17*). In alignment with this perspective, bacteria have evolved strategies to avoid killing through NETs, per example via expression of nucleases that degrade NETs, or even to use NETs to their advantage (per example in biofilms) [5, 68, 75] (*st. 18*).

Apart from their multiple enhancing functions in immune defense and autoimmunity, evidence for anti-inflammatory action of NETs is also accumulating [44, 45] (*st. 19*). An important part of the regulatory effect of NETs on inflammation is due to the modulation of cytokine and chemokine activity by NET-related proteases (*st. 20, 24*) [26, 34, 76–79].

NETs have been shown to build larger conglomerates when present in higher densities [34, 77, 80], with both detrimental and beneficial outcome for the host [44] (*st. 21, 23, 24, 25*). A remaining open question is the connection between neutrophil aggregation and NET formation (*st. 22*). The technological progress in two-photon intravital microscopy has enabled the discovery of neutrophil swarming, a phenomenon characterized by highly coordinated series of neutrophil movement, followed by cell accumulation mediated by chemoattractant signals and adhesion molecules [81]. Swarming is observed during infection and sterile inflammation in both mouse and human neutrophils [82]. Interestingly, cell death, both in the inflamed surrounding tissue and within the neutrophil cluster itself, strongly amplifies swarming and fuels immune activation [83]. It is tempting to hypothesize that neutrophil swarming and the formation of NETs might be interdependent processes, but as of now, the connection between these cellular functions remains elusive (*st. 22*).

NET formation has been reported in blood vessels, ductal structures, and surfaces [15, 80, 84], but also in the tissue [34, 85]. NETs constitute an anti-microbial defense mechanism and are therefore likely to be found at places with high microbial burden. Thus, the conclusion that the location of internal and external body surfaces is responsible for the (perceived) enrichment for NETs is controversial (*st. 26*), as is the view that NETs provide a protective coating to mucosal surfaces (*st. 27*).

In a recent publication, a lining of NETs was found adjacent to large necrotic areas [86]. The authors suggested that aggregated NETs wall-off lumps of material with immunostimulatory activity, such as necrotic tissue or monosodium urate crystals, thereby limiting immune reactivity and inflammation to sterile agents (*st. 28*). However, this isolating effect needs to be balanced against the tissue-damaging properties of NETs that have been confirmed in several studies [31, 32, 44, 87].

Triggers of NET formation

While there is a large consensus that microbial agents, biochemical stimuli, calcium influx, immune complexes, and contact with platelets (thrombocytes) and/or damage-associated molecular patterns can trigger NET formation (*st. 29–35*) a direct connection between lysosomal membrane instability and NET formation is still under discussion (*st. 36*). Munoz et al. have reported lysosomal instability and concurrent disintegration of the nuclear morphology in neutrophils upon exposure to nonpolar nanoparticles followed by NADPH oxidase-dependent chromatin externalization [63, 88]. The authors therefore introduced a model where lysosomal leakage triggers a cascade of events involving ROS production and ending in formation of NETs [63]. A direct connection between these phenomena remains, however, yet to be proven.

Pathways of NET formation

In the initially described pathway of NET formation induced by PMA or bacteria and later termed "NETosis" [89], neutrophils release nuclear DNA decorated with proteins into the extracellular milieu via an NADPH oxidase 2 (NOX2)-dependent mechanism involving the death of the neutrophil [2, 90]. Fourteen years later, it has become clear that NET release can occur via multiple distinct pathways with often unknown interdependence (*st. 37, 38*) [3, 43]. Since different stimulators of NET formation induce differential signaling, generalized statements about certain protein requirements should be avoided. A common

denominator is however that NET release is mostly seen as an active process driven by cell-intrinsic pathways that are activated by external stimuli (*st. 39*).

Apart from NOX2, the requirement of neutrophil-specific serine proteases neutrophil elastase (NE) and myeloperoxidase (MPO) has also been described for the later stages of NET formation in association with cell death, more particularly for chromatin decondensation [91, 92]. In particular, NOX2-derived ROS were reported essential for the release of NE and MPO from azurophilic granules [92, 93]. However, it is now clear that some forms of NET formation occur independently of NOX2 and MPO [3] (*st. 40, 41*).

The enzyme PAD4 that is highly expressed in neutrophils mediates conversion of arginine into citrulline, which results in a massive loss of positive charges on arginine residues in histones. This conversion loosens the forces between DNA and histones and thus contributes to chromatin decondensation [94]. The role of PAD4 in NET formation is, however, one of the most controversial aspects in the study of NETs. Inhibition of PAD4 was reported to decrease NET formation in response to certain stimuli and PAD4-deficient mice sometimes display impaired NET formation [15, 26, 84, 95–98]. However, it should be noted that other reports observed normal NET formation in the absence of functional PAD4 [3, 99]. Therefore, this has led to the idea that not all NET release is PAD4-dependent (*st. 42–44*). Some of the discrepancies on the role of PAD4 in NET formation may be explained by our limited understanding of the different functions that PAD4 may have in neutrophil biology. Per example, a recent study linked PAD4 to assembly and activation of NOX2 [100]. Although this function of PAD4 is citrullination-independent, it can be blocked by PAD4 inhibitors. In contrast, conditions in which PAD4 is catalytically active prevented activation of NOX2. These novel findings shed some light on the paradox of why PAD4 is sometimes found to be essential for NET formation under conditions in which citrullination is not detected (e.g., in PMA-induced NETs) [3, 98].

Of note, the presence of citrullinated histones in cell culture or tissue is often regarded as a strong indicator of NET formation having occurred. However, these findings could potentially also be explained by extracellular citrullination of NET-bound histones by the PAD2 enzyme that is released from the cytoplasm upon stimulation with PMA [101], although PAD2 is not directly involved in NET formation triggered by LPS or TNFα [102]. For all the above reasons, caution in extracting conclusions should be exerted when studying PAD4 and citrullination as drivers of NET formation.

Neeli and Radic reported that several pathways of NET formation converge at the level of protein kinase C [41] (*st. 45*). They report that PAD4 activity is dependent on PKCζ activation and that PKCα is a dominant negative repressor of histone citrullination. Still, activation of both isoforms by combinatory treatment with PMA and ionomycin leads to increased NET release without detectable citrullination. It remains unclear, what caused these synergistic effects in the absence of citrullination as a driver of chromatin decondensation and this finding contrasts reports that PAD4 activity is solely dependent on calcium [15, 84].

Over the last few years, evidence has indicated that autophagy might be required for NET formation, although the molecular mechanisms are not clearly defined yet [103]. Remijsen et al. were the first to show that a combination of autophagy and ROS production is necessary for efficient PMA-induced NET formation in human neutrophils [104]. Next, Mitroulis et al. demonstrated that neutrophils from patients with acute gouty arthritis exhibit autophagy-mediated spontaneous NET release [61]. Furthermore, pharmacological inhibition of the mTOR pathway enhanced autophagosome formation along accelerated NET release following neutrophil stimulation with the bacteria-derived peptide fMLP [105]. The first genetic evidence illustrated that silencing of Atg5 in a neutrophil-like human cell line infected with adherent–invasive *Escherichia coli* blocked NET formation [103]. Recently, diminished Atg5 expression due to aging was also shown to reduce the capacity of neutrophils to form NETs [106–108]. In apparent contrast, Atg5-knockout mouse neutrophils had reduced autophagic activity but normal capacity to release extracellular DNA [109]. Of note, pharmacological inhibition of autophagy with PI3 kinase inhibitors such as wortmannin has to be interpreted with caution, because some, but not all, studies have indicated wortmannin to also inhibit ROS production which, consequently, could also block NET formation [104, 110–112]. Treatment with the so-called late-autophagy inhibitors, such as bafilomycin A1 and chloroquine, had no effect on NET formation [109]. Due to these conflicting data, there is yet no consensus on the role of autophagy in NET formation (*st. 46*).

Non-suicidal pathways of NET formation were described, where the cell remains intact and normal cellular functions of neutrophils, such as chemotaxis and phagocytosis, are still carried out [11, 42, 57, 113] (*st. 47*). These processes seem to occur much more quickly than the canonical NET pathway induced by PMA [90] or other forms of NET release that result in disruption of the plasma membrane [7, 34, 62, 114], per example crystal-induced NET formation (*st. 49*) and thus seem to be mechanistically distinct. Suicidal and live NET formation therefore need to be looked at separately (*st. 48*).

The frequent use of unspecific bioassays, such as the detection of extracellular DNA as a surrogate for the presence of NETs has created confusion, since it is not able to distinguish between NET formation and other forms of cell death with a necrotic morphotype. A distinction that is

followed by the majority of authors of this paper is that NET formation requires an active and regulated process (*st. 39*), while necrosis can also occur in a passive, unregulated way (*st. 50*).

Desai et al. have found that NET formation upon 2 h stimulation with PMA or crystals involves the RIPK1/RIPK3/MLKL-dependent pathway of necroptosis [115, 116] (*st. 51*). They therefore argue that NET formation that involves cell death is a passive process secondary to plasma membrane rupture induced by necroptosis or other forms of necrosis [117] (*st. 53*) This view is opposed by others who have seen RIP3/MLKL-independent extrusion of DNA choosing different experimental conditions [118] (*st. 52*) or who argue that the definition of NET formation includes a highly regulated and coordinated process that is different from both necroptosis and necrosis (*st. 50, 53*). It also needs to be mentioned that two novel studies [119, 120] have demonstrated that the rupture of the plasma membrane during ROS-dependent NET formation is mediated by gasdermin D, thus connecting NETs with pyroptosis [121, 122].

Several recent reports have demonstrated that a considerable fraction of the nucleic acids contained in NETs is of mitochondrial origin [29, 57, 123, 124]. Special caution is, however, required to distinguish NET-derived mitochondrial DNA from mitochondrial nucleic acids expulsed during incomplete neutrophil mitophagy [125]. Due to its pro-inflammatory and interferogenic properties, oxidized mitochondrial DNA has been allotted an important role in the pathogenesis of SLE [29, 124, 125]. Although the mutual interdependence of extrusion of mitochondrial and nuclear DNA in NETs has yet to be confirmed, there seem to exist pathways of NET formation that are dependent and independent of mitochondria (*st. 54, 55*). For the majority of the authors of this paper, however, the breakdown of the nuclear membrane is still a hallmark of NET formation (*st. 56*).

Extracellular traps in different cell types

Extracellular trap release from cells other than neutrophils is understudied, and the mechanisms of chromatin decondensation release remain elusive. While extracellular trap formation in neutrophils and eosinophils [12] is more or less unequivocally accepted (*st. 57, 58*), further research, best performed in genetic models, is needed to understand both prevalence and relevance of extracellular trap release in cell types other than granulocytes (*st. 59, 60*). Extracellular trap release has also been reported for mast cells [126, 127]. Although a necessity for ROS production was observed, definite cellular pathways and further details are still warranted. The caspase-1-dependent release of monocyte extracellular traps following high multiplicities of infection has been reported by Webster et al. [128] and has very recently been described to contribute to the pathogenesis of rhabdomyolysis [129]. Similar to the cell death reported by Webster et al., also pyroptosis relies on caspase-1 activity and also leads to the release of intracellular components [46]. It is therefore unclear, whether extracellular trap formation in monocytes is distinct from pyroptosis.

Pathology and treatment

Owing to the multiple reports of the detrimental effects of NETs, especially in autoimmune diseases such as SLE, RA and vasculitis [4, 130–134], treatment with DNAse has become a promising therapeutic option (***st. 67***). NETs are degraded by endonucleases and DNase I-like proteins in the circulation. Apart from DNase I, also DNase I-like 3 is involved in vivo in the disintegration of NETs [135]. Removal of extracellular DNA by inhalation of recombinant human DNAse I is already a widespread and safe therapeutic option for cystic fibrosis [136]. In lupus, impairment of DNAse I function is associated with nephritis [22] and DNase I activity negatively correlates with disease activity [137]. Missense mutations in nucleases cause lupus-like disease in humans and mice [22, 138–141]. Furthermore, NET-binding proteins, such as antibodies or complement factor C1q, protect them from degradation possibly by inhibiting DNase I [22, 142]. Taken together, this argues for a beneficial role of DNAse in lupus. Similar mechanisms might be at work in other autoimmune diseases with occurrence of autoimmune reactivity to components of the nucleus. However, DNAse removes DNA from any source and its effect is not NET-specific. Furthermore, intravital imaging has revealed that when injected into circulation, DNase I is effective in the removal of DNA and decomposition of the NET-like structure but not necessarily in detachment of other components on NETs, which additionally attach to (glyco)proteins lining endothelium [143] and have potential tissue-damaging properties. Last but not least, other studies have challenged the detrimental effect of NETs on lupus-like autoimmunity and tissue damage [26, 96, 144]. Thus, caution needs to be exercised to identify the precise clinical conditions and developmental stages of diseases that warrant the in vivo use of DNAse or other therapeutic agents that aim at inhibition of NET formation.

Materials and Methods in NET-related research

PMA was initially used as one of the triggers to induce and define NETs [2, 90]. It is therefore often used as a surrogate for other NETs. PMA-induced NET formation is ROS-dependent and results in cell death (formerly called NETosis). Since then, many other pathways have been detected [3, 43], so that nowadays, the sole use of PMA is often considered limiting and the use of additional other more physiologically relevant stimuli is encouraged (*st. 68, 69*).

Immunocyto- and immunohistochemistry are the most widely used methods for the detection of NETs. NETs are identified as structures containing extracellular DNA co-localizing with granule-derived proteins, such as neutrophil elastase, and histones [2] (*st. 70–72*). NET formation can be monitored in real time via intravital microscopy [42, 143], live cell imaging [43, 51, 145–147] and with techniques based on DNA-intercalating dyes [11, 148] (*st. 73, 74*). Given the correct sample preparation and the use of proper controls, NETs can be visualized ex vivo in tissue sections and in fluid secretions [50, 149] (*st. 75*), although demarcation from necrosis can be challenging and caution should be taken to avoid overinterpretation of findings. Confirmation of the presence of granule proteins is encouraged also in these settings and even in in vivo settings to identify NETs. NETs can either appear cloud-like or filamentous [5] (*st. 8*).

Regarding neutrophil isolation for NET assays, blood might either be anti-coagulated with heparin or by chelating divalent ions, although it should be noted that a study found inhibition of NET formation by heparin [150] (*st. 76, 77*). Besides, isolation of neutrophils is generally performed in the absence of calcium and magnesium to prevent clumping and adhesion [151, 152] although NET generation and aggregation was reported to be inhibited by agents chelating divalent cations [34, 62] (*st. 78, 79*). The presence of calcium, magnesium and chelators (EGTA and EDTA) should therefore be described in the paper as they may have an impact on NET release.

Also, no real consensus yet exists about which medium to use for the storage of neutrophils prior to or during assessing NET formation in vitro, although the composition of the culture medium strongly influences the propensity to form NETs [153, 154] (*st. 80*). Also, NET assays must be performed under controlled CO_2/HCO_3-/pH balance [155–157] (*st. 81*). For the future, the introduction of standardized buffers to assess NET formation is desirable (*st. 82*).

As of now, the minimal requirements are that experiments on NET formation should exactly specify the culture conditions (*st. 83*). This constitutes the base medium, the use of serum [90, 158] or protein, the absence or presence of platelets [60] and the surface constitution of the cell culture plate [159] (*st. 84*). In addition, independently of the stimulus used, the source or preparation of the inducer should be stated in detail (*st. 85*).

Concluding remarks

Prompted by the excitement that followed the seminal paper by the Zychlinsky group that introduced NETs to the scientific community [2], a large body of data emerged that allotted major roles in defense from pathogenic microorganisms, in inflammation, and multiple pathophysiological conditions to NET formation. This wave of excitement was, and is, accompanied by doubts and criticisms. This paper illustrates current areas of consensus and dispute in the NET field (Fig. 2). The main areas of discussion are 1) the source of the DNA in NETs, 2) that demarcation from other forms of cell death is incomplete because factors that unambiguously distinguish NETs from the remnants of other forms of cell death are still missing, and 3) that NET formation can be mediated by multiple pathways. Therefore, it is unlikely that targeting a single pathway inhibits all NET formation without having a considerable impact on other aspects of cell biology and/or pathophysiology. Finally, 4) certain aspects of experimental procedures are not yet standardized. By highlighting these open questions, this paper aims to instigate further research and contribute to the harmonization of these issues.

Interestingly, defects in the signaling cascades that precipitate NET formation (such as the oxidative burst in chronic granulomatous disease or neutrophil serine proteases in Papillon–Lefèvre syndrome) are associated with pathologies characterized by chronic autoimmunity and inflammation, both of sterile and infectious origin [160–164]. On the other hand, treatment with PAD4 inhibitors that impedes NET formation (along with other cellular pathways) has had promising results for the treatment of autoimmune diseases [27, 165–167]. Thus, NET formation can be considered a major therapeutic target for the management of multiple human disorders. Understanding of the molecular mechanisms and the spatiotemporal dynamics that regulate NET formation and clearance and delineate it from other forms of cell death, will enable to fine-tune therapeutic approaches and minimize the risk of detrimental side effects and adverse outcome.

Acknowledgements SB, JH, LM, MHH, and MH received support from the Collaborative Research Center 1181 of the German Research Foundation (project # CRC1181-C03). HJA, JD, and DN were supported by funding from the German Research Foundation (projects # DFG AN372/14-3 and 24-1). JSK was supported by funding from the National Institutes of Health (R01HL134846) and the Lupus Research Alliance. EK was supported by the National Science Centre of Poland (project # 2014/15/B/NZ6/02519). Research in the Radic lab (MR, IN, and ND) on this topic was funded by the Lupus Research Alliance of New York, NY (USA). AH is supported by Fundación Centro Nacional de Investigaciones Cardiovasculares Carlos III (CNIC). The CNIC is supported by the Ministerio de Ciencia, Innovacion y Universidades (MCIU) and the Pro CNIC Foundation, and is a Severo Ochoa Center of Excellence (MEIC award SEV-2015-0505). PS, IM, and KR were supported by BMBF/GSRT German-Greek Bilateral Research and Innovation grant no. T2DGE-0101. FA was supported by the Jerome L. Greene Foundation and the National Institutes of Health (R01 AR069569).

Authors contributions SB, CM and MHH prepared draft versions of the paper and the table. MHH and MH integrated inputs from all co-authors (listed in alphabetical order). All co-authors filled out the

questionnaire, provided constructive feedback for the preparation of the article, and approved its content.

Compliance with ethical standards

Conflict of interest FA is an inventor on issued patent no. 8,975,033 held by The Johns Hopkins University that covers "Human autoantibodies specific for PAD3 which are cross-reactive with PAD4 and their use in the diagnosis and treatment of rheumatoid arthritis and related diseases" and serves as a consultant for Bristol-Myers Squibb. The other authors declare no competing interests.

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