2
Review of Literature

2.1 Abstract:

Extracellular traps are formed by immune cells to counter infection (Brinkmann, Reichard et al. 2004). But poor regulation of trap formation and clearance may lead to various pathologic conditions (Brinkmann, Reichard et al. 2004, Apel, Zychlinsky et al. 2018). Pathogens and many other stimuli induce the formation of traps some of which are generated inside our body, leading to inflammation and disease (Brinkmann, Reichard et al. 2004, Apel, Zychlinsky et al. 2018, Daniel, Leppkes et al. 2019). Different stimuli induce ETs in different immune cells following different mechanisms (Simon, Hoesli et al. 2011, Doster, Rogers et al. 2018, Neumann, Brogden et al. 2020). Understanding stimuli dependent ETs formation is needed to regulate ETs formation in case of inflammation and disease.

2.2 CELLS FORMING ETs AND STIMULI:

ETs have been studied extensively in last 15 years with respect to multiple stimuli and in different cell types (Table 2.1) (Daniel, Leppkes et al. 2019). However, the pathway underlying ETs formation has just begun to unfold. In this section I will discuss different stimuli involved in induction of neutrophil ETs along with the known mechanism. Considering the extensive literature on neutrophil ETs I will focus on cells, other than neutrophils, reported to form ETs when induced with different stimuli.

Table 2.1 : Cells and stimuli inducing extracellular traps.

Cells	Inducer	Reference
Eosinophils	• Lipopolysaccharides • IgG • IgA • Phorbol myristate acetate • A23187 (Calcium ionophore) • Platelet activating factor • Thymic stromal lymphopoietin Pathogen • *Staphylococcus aureus*	(Yousefi, Gold et al. 2008),(Ueki, Melo et al. 2013),(Morshed, Yousefi et al. 2012)
Mast cells	• IL-1β • IL-23 Pathogens • *Streptococcus pyogenes*	(von Köckritz-Blickwede, Goldmann et al. 2008) (Abel, Goldmann et al. 2011, Scheb-Wetzel, Rohde et al. 2014, Lopes,

	• *Pseudomonas aeruginosa* • *Staphylococcus aureus* • *Enterococcus fecalis* • *Streptococcus pyogenes* • *Listeria monocytogenes* • *Candida albicans* • *Leishmania donovani* • *Leishmania tropica* • *Mycobacterium tuberculosis*	Stylianou et al. 2015, Möllerherm, von Köckritz-Blickwede et al. 2016, Campillo-Navarro, Leyva-Paredes et al. 2017, Naqvi, Ahuja et al. 2017, Campillo-Navarro, Leyva-Paredes et al. 2018) (Lin, Rubin et al. 2011)
Macrophages/ Monocytes	• Mevastatin • Phorbol myristate acetate • Fosfomycin • TNF-α • Glucose oxidase • Interferon-γ Pathogens • *Candida albicans* • *Escherichia coli* • *Mycobacterium massiliense* • *Histophilus somni* • Nontypeable *Haemophilus influenza* • *Mannheimia haemolytica* • *Klebsiella pneumoniae* • *Staphylococcus aureus*	(Chow, von Köckritz-Blickwede et al. 2010) (Liu, Wu et al. 2014) (Webster, Daigneault et al. 2010, Aulik, Hellenbrand et al. 2012, Hellenbrand, Forsythe et al. 2013, King, Sharma et al. 2015, Halder, Abdelfatah et al. 2017)
Basophils	• Monosodium urate crystals • Anti-FcεRIa • C5a • Eotaxin • Thymic stromal lymphopoietin • Platelet-activating factor • Lipopolysaccharides • Lipoteichoic acid Pathogens • *Escherichia coli*	(Schorn, Janko et al. 2012) (Morshed, Hlushchuk et al. 2014) (Yousefi, Morshed et al. 2015)
Neutrophils	• Phorbol myristate acetate • Simvastatin • Fluvastatin • Mevastatin • Monosodium urate crystals • Lipopolysaccharides • formyl-Met-Leu-Phe • IL-8 • Peptidoglycan • Birinapant/z-VAD-fmk • Immobilized immune complexes	(Itakura and McCarty 2013) (Hazeldine, Harris et al. 2014) (Germic, Stojkov et al. 2017) (Xu, Zhang et al. 2017) (Metzler, Goosmann et al. 2014) (Warnatsch, Ioannou et al. 2015, Hosseinzadeh, Thompson et al. 2016)

	• Oxidized low density lipoprotein • Anti-ribonucleoprotein antibodies from SLE patients • C5a • H_2O_2 • Nicotine • Cholesterol crystals • Ionomycin • A23187 Pathogens • *Leishmania amazonensis* • *Leishmania donovani* • *Candida albicans* • *Staphylococcus aureus* • *Salmonella typhimurium* • *Shigella flexneri*	(Holmes, Shim et al. 2019) (Neeli and Radic 2013) (D'Cruz, Speir et al. 2018) (DeSouza-Vieira, Guimarães-Costa et al. 2016) (Gabriel, McMaster et al. 2010) (Brinkmann, Reichard et al. 2004) (Chen, Nishi et al. 2012) (Awasthi, Nagarkoti et al. 2016) (Garcia-Romo, Caielli et al. 2011)

In 2008 Yousefi et al. reported the induction of ETs in human eosinophils when primed with Interleukin-5 (IL-5) or interferon-γ and stimulated with lipopolysaccharide (LPS) (Yousefi, Gold et al. 2008). Eosinophil extracellular traps (EETs) were composed of mitochondrial DNA, confirmed by PCR with mitochondrial DNA specific primers, instead of nuclear DNA as compared to NETs. EETs were found to be embedded with eosinophil cationic protein (ECP) and major basic protein (MBP) by immunofluorescence. In contrast to neutrophils, eosinophils did not immediately die after formation of traps (Yousefi, Gold et al. 2008). Soon after, formation of traps were also reported by lytic eosinophils by Ueki et al. (Ueki, Melo et al. 2013). The authors reported the formation of EETs by primary human eosinophils in response to immunoglobulin IgG, IgA, PMA (phorbol 12-myristate 13-acetate) and a calcium ionophore A23187. The results were confirmed by immunofluorescence and electron microscopy (Ueki, Melo et al. 2013). Platelet activating factor in combination with IL-5 or granulocyte monocyte colony stimulating factor (GM-CSF) induced lytic EETs formation. Either GM-CSF or IL-5 alone not sufficient to induce EETs formation (Ueki, Melo et al. 2013). Anti-histone staining confirmed the nuclear origin of EETs, contradicting the report of Yousefi et al. (Yousefi, Gold et al. 2008, Ueki, Melo et al. 2013). The result pointed out, and it was evident in many further studies, that the composition and origin of EETs are cell and stimuli dependent (Mukherjee, Lacy et al. 2018).

Gevaert et al. immunostained nasal polyp tissue (Gevaert, Zhang et al. 2017) – from patients with chronic rhinosinusitis with nasal polyps (CRSwNP) – with MBP and confirmed the presence of EETs. The authors observed the induction of EETs in these tissues in response to *Staphylococcus aureus*. IL-5 and periostin were high in CRSwNP patients and was crucial in the formation of EETs. EETs trapped *S. aureus* and eosinophils were migrating towards entrapped bacteria (Gevaert, Zhang et al. 2017). However, these studies did not confirm whether the traps were of nuclear or mitochondrial origin or whether the mechanism involved was lytic or non lytic. Morshed et al. also reported the generation of EETs in response to *S. aureus* and thymic stromal lymphopoietin (TSLP) (Morshed, Yousefi et al. 2012). TSLP levels are elevated in diseases like atopic dermatitis, bronchial asthma and allergic rhinitis. TSLP and *S. aureus* was sufficient to induce traps in human eosinophils. *Staphylococcus epidermidis* was unable to induce EETs, however traps formed after TSLP stimulation trapped and inhibited the growth of the bacteria. EETs were composed of mitochondrial DNA and were formed independent of cell death (Morshed, Yousefi et al. 2012). Inhibition of ROS in TSLP treated eosinophils inhibited the generation of EETs in this study (Morshed, Yousefi et al. 2012). It may be possible that some

stimuli may adopt multiple pathways for ET generation as needed. Though it needs to be further investigated. EETs formation have been reported in asthma, ectoparasitosis, hypereosinophilic syndrome, allergic contact dermatitis, Wells syndrome, bullous pemphigoid, dermatitis herpetiformis, pemphigus foliaceus, eosinophilic esophagitis (Simon, Hoesli et al. 2011, Cunha, Porto et al. 2014, Simon, Radonjic-Hösli et al. 2015, Mukherjee, Bulir et al. 2018, Mukherjee, Lacy et al. 2018). Insights from these studies are critical in understanding disease pathology and EETs formation.

Ko¨ckritz-Blickwede et al. first time reported the formation of ETs by mast cells through immunofluorescence and scanning electron microscopy (von Köckritz-Blickwede, Goldmann et al. 2008). Mast cell extracellular traps (MCETs) generated in response to *Streptococcus pyogenes* were composed of nuclear DNA and was decorated with histones, tryptase and cathelicidin LL-37 (von Köckritz-Blickwede, Goldmann et al. 2008). Since then induction of MCETs were reported by pathogens such as *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Enterococcus fecalis*, *Streptococcus pyogenes, Listeria monocytogenes*, fungi *Candida albicans* and parasite *Leishmania donovani* and *Leishmania tropica* (Abel, Goldmann et al. 2011, Scheb-Wetzel, Rohde et al. 2014, Lopes, Stylianou et al. 2015, Möllerherm, von Köckritz-Blickwede et al. 2016, Campillo-Navarro, Leyva-Paredes et al. 2017, Naqvi, Ahuja et al. 2017). Pathogens have also evolved strategies to escape ETs. Recently Campillo-Navarro reported induction of MCETs by heat killed *Mycobacterium tuberculosis* but not by alive *M. tuberculosis* (Campillo-Navarro, Leyva-Paredes et al. 2018). The formation of traps was inhibited by catalase produce by live bacteria (Campillo-Navarro, Leyva-Paredes et al. 2018). Along with pathogen interactions, inflammation plays critical role in ETs generation. MCETs formation was observed in papillary dermis of human psoriasis plaques by Lin et al. (Lin, Rubin et al. 2011). Interestingly stimulation of normal skin biopsies with IL-1β and IL-23 for 3 days also lead to induction of MCETs embedded with chymase and tryptase (Lin, Rubin et al. 2011). ETs are inflammatory in nature. Inflammation induced ETs may worsen the pathology of diseases and needs more study.

In 2010 Chow et al. reported the formation of extracellular traps by murine peritoneal macrophages, RAW 264.7 and human neutrophils when treated with mevastatin (3-hydroxy 3-methylglutaryl coenzyme A reductase) and PMA (Chow, von Köckritz-Blickwede et al. 2010). Lovastatin, simvastatin, and fluvastatin also induced NETs in human neutrophils (Chow, von Köckritz-Blickwede et al. 2010). Interestingly Halder et al. saw that simvastatin does not induce ETs in human monocytes while *C. albicans* does ETs (Halder, Abdelfatah et al. 2017). The traps were composed of nuclear DNA and the formation was leading to death of macrophage/monocyte (Halder, Abdelfatah et al. 2017). Liu et al. also saw that macrophage extracellular traps (METs) were induced by *Escherichia coli* and *C. albicans* in J774A.1 murine macrophage cell line and murine peritoneal macrophages (Liu, Wu et al. 2014). The traps induced originated from both mitochondria and nucleus as they contain the mitochondrial genes, *Atp6 and Nds1*, and nuclear genes, *Actinβ and Gapdh* as identified by PCR and fluorescence *in situ* hybridization. *E. coli* (8%), *C. albicans* hyphae (17%) and *C. albicans* (10%) yeast induced different amount of J774A.1 macrophages to form METs (Liu, Wu et al. 2014). Different from the earlier results, the traps originated from some dying macrophages and some of the macrophages remained alive after trap formation. It was clear from the results that formation of ETs varies with both type of stimuli and type of cells (Liu, Wu et al. 2014). Nuclear and mitochondrial origin METs was also reported by Je et al. in PMA differentiated THP-1 cells induced by *Mycobacterium massiliense* (Je, Quan et al. 2016). The authors confirmed it by PCR of the same genes as done by Liu et al. (Liu, Wu et al. 2014, Je, Quan et al. 2016). The authors tested rough strain *M. massiliense* Asan 50594 and smooth strain *M. massiliense* strain CIP 108297 for METs induction and found that rough strain induced METs in 14% of infected cells while smooth strain induced in only 6% of the infected cells (Je, Quan et al. 2016). More METs formation were observed when PMA differentiated THP-1 cells were infected with non sonicated rough strain, i.e. aggregated bacteria, than sonicated bacteria i.e. single bacteria. It is interesting that even the same stimuli in different physical form can affect the formation of ETs. Even the reduction in phagocytosis, by treating the

THP-1 cells with cytochalasin D or by providing less multiplicity of infection (MOI) of *M. massiliense* rough strain, reduced the extent of METs formation (Je, Quan et al. 2016). LPS and hydrogen peroxide, which are known to induce NETs, were not able to induce METs in THP-1 cells, J774A.1 macrophages or murine peritoneal macrophages (Je, Quan et al. 2016). PMA was also unable to induce METs in J774A.1 macrophages or murine peritoneal macrophages, however there are reports of PMA induced METs in murine peritoneal macrophages and others (Chow, von Köckritz-Blickwede et al. 2010, Liu, Wu et al. 2014, Je, Quan et al. 2016). METs are decorated with proteins such as histone, myeloperoxidase, lactoferrin, lysozyme and others (Doster, Rogers et al. 2018).

Basophils also forms basophils extracellular traps (BETs). Schorn et al. reported the formation of BETs, composed of nuclear DNA by human basophils when induced by monosodium urate crystals (MSU) (Schorn, Janko et al. 2012). MSU were inducing BETs and was not getting phagocytosed as checked by flow cytometry. However when incubated with human neutrophils and eosinophils it got phagocytosed and induced ETs (Schorn, Janko et al. 2012). Human basophils when primed with IL-3 and stimulated with anti-FcεRIa (high-affinity IgE receptors (FcεRI)), C5a, eotaxin or thymic stromal lymphopoietin (TSLP) released BETs (Morshed, Hlushchuk et al. 2014). No traps were formed in the absence of IL-3 priming. Platelet-activating factor (PAF), LPS or lipoteichoic acid (LTA) induced BETs without IL-3 priming (Morshed, Hlushchuk et al. 2014). It was again evident that different stimuli are inducing different pathways for ETs formation in different cells. IL-3 primed cells and anti-FcεRIa and C5a induced traps were of mitochondrial origin. It was confirmed by doing quantitative PCR for *COX1* gene specific for mitochondria and *18S rDNA* specific for nucleus. The traps were embedded with basogranulin and not histone (Morshed, Hlushchuk et al. 2014). Yousefi et al. confirmed mitochondrial origin of BETs by stimulating human basophils and Hoxb8 mouse basophils with GFP tagged *E. coli* (Yousefi, Morshed et al. 2015). BETs were being formed in cell death and phagocytosis independent manner as reported earlier (Schorn, Janko et al. 2012, Morshed, Hlushchuk et al. 2014, Yousefi, Morshed et al. 2015).

2.3 MECHANISM

There are two pathways of ETosis. One is suicidal in which the cells expel their decondensed chromatin, which traps and kills pathogens in extracellular space, followed by cell death. Second form is vital ETosis in which the plasma membrane of the cell remains integrated even after the expulsion of DNA and cells keep functioning normally (Papayannopoulos 2018, Daniel, Leppkes et al. 2019, Neubert, Meyer et al. 2020). Suicidal ETosis is the most studied mechanism. Being actively researched, new information and data is continuously shaping the signaling aspect of ETosis.

2.3.1 SUICIDAL ETosis

2.3.1.1 UPSTREAM TO REACTIVE OXYGEN SPECIES

In suicidal NETosis stimuli such as PMA, LPS, calcium ionophores (CAI) and pathogens induce ROS generation through NADPH oxidase (NOX) or mitochondria (Papayannopoulos 2018, Daniel, Leppkes et al. 2019, Neubert, Meyer et al. 2020). Upstream pathways leading to ROS generation are poorly studied and are topic of active research. Hakkim et al. observed that PKC, c-Raf, MEK, ERK pathway works upstream of NOX (Hakkim, Fuchs et al. 2011). Inhibiting any of the components of the pathway inhibited ROS production and subsequent NET formation (Hakkim, Fuchs et al. 2011). Awasthi et al. reported upstream involvement of TLR2 and TLR6, PKC, IRAK, ERK and P38 MAPK in ROS production through NOX and NET formation when

neutrophils were stimulated with Oxidized low density lipoprotein (oxLDL) (Awasthi, Nagarkoti et al. 2016). Interestingly inhibiting MPO with 4-aminobenzoic acid hydrazide (ABAH), a MPO inhibitor, reduced ROS production (Awasthi, Nagarkoti et al. 2016). This effect may be stimuli dependent as many studies argue the production of ROS upstream of MPO activation (Papayannopoulos 2018). PI3K-ERK gets activated upstream of ROS when human neutrophils were treated with parasite *L. amazonensis* (DeSouza-Vieira, Guimarães-Costa et al. 2016). The isoform PI3Kγ follows ROS dependent pathway and its inhibition leads to reduction in NETosis as well as ROS generation, while PI3Kδ is following ROS independent pathway. Inhibiting PKC also inhibited *L. amazonensis* induced NETs, however whether it is also inhibiting ROS generation when induced by parasite was not investigated (DeSouza-Vieira, Guimarães-Costa et al. 2016). Gabriel et al. reported that *Leishmania donovani* induces NETs in human neutrophils independently of ROS (Gabriel, McMaster et al. 2010). The ROS independent formation of NETs in this study may be because of different strain of *Leishmania*, however it must be further studied (Gabriel, McMaster et al. 2010). Immobilized immune complexes (iIC) formed by rabbit IgG induce NETs in human neutrophils (Chen, Nishi et al. 2012). It is well reported that MPO is activated by ROS leading to downstream signaling responsible for NET production (Papayannopoulos, Metzler et al. 2010). Interestingly Behnen et al. reported that ROS produced by MPO plays role in iIC induced NET production in human neutrophils as inhibiting MPO derived ROS by aminopyrine inhibited NETosis (Behnen, Leschczyk et al. 2014). iIC dependent ROS generation was induced by FcγRIIA and FcγRIIIB, both receptors, but only FcγRIIIB was responsible for NET induction. FcγRIIIB lacks cytoplasmic domain so it requires FcγRIIA and β2 integrin Mac-1 as signaling partners. β2 integrin Mac-1 is composed of β2 subunit (CD18) α subunit (CD11b). Blocking of CD18 or CD11b individually or together significantly reduced NETosis. Blocking CD18 had no effect on ROS generation while blocking CD11b inhibited ROS generation, suggesting the involvement of alternate ROS independent pathways for NETosis (Behnen, Leschczyk et al. 2014). However after blocking CD11b the authors only tested the reduction of extracellular superoxide. While this may indicate inhibition of ROS generation, total ROS generation must be calculated for conclusive result. Mac-1 uses Syk dependent downstream signaling. The author saw that activation of Src, Syk, PI3K, Akt, ERK1/2, and p38 MAPK lead to ROS generation and NET induction and inhibiting them inhibits ROS and NETs. Inhibiting ERK1/2 partially inhibited ROS but completely inhibited NETs induction (Behnen, Leschczyk et al. 2014).

Interestingly Akt independent NETosis in PMA stimulated neutrophils and *L. amazonensis* neutrophils have been reported earlier (DeSouza-Vieira, Guimarães-Costa et al. 2016). Douda et al. reported that Akt is required for PMA induced NETosis in human neutrophils, but it plays role downstream of ROS instead of upstream (Douda, Yip et al. 2014). Inhibiting NOX2 with diphenyleneiodonium (DPI) inhibited Akt activation as well as NETosis. However inhibiting Akt with inhibitors M2206 and XI inhibited NETosis but not ROS generation, making Akt essential for NOX2 mediated NETosis (Douda, Yip et al. 2014). Being a well-known inhibitor of apoptosis (Rane and Klein 2009), interestingly Akt was working as switch between NETosis and apoptosis where inhibiting Akt switched NETosis to apoptosis. Preincubation of human neutrophils with Akt inhibitor XI increased the level of cleaved caspase-3 and increased the number of apoptotic cells while decreasing the number NETotic cells in response to PMA (Douda, Yip et al. 2014).

Autophagy also plays role in NETs formation (Remijsen, Vanden Berghe et al. 2011, Papayannopoulos 2018). ROS burst induces autophagy and autophagy is required to sustain efficient ROS burst inside cells (Bhattacharya, Wei et al. 2015, Filomeni, De Zio et al. 2015). Signaling molecule involved in autophagy take part in NETs formation; whether autophagy induced ROS and/or ROS induced autophagy leads to NETosis is still a topic of discussion. Remijsen et al. pretreated human neutrophils with wortmannin, which inhibits autophagy by inhibiting PI3K. Wortmannin inhibited NETs formation in PMA treated neutrophils while it had not effect on PMA induced ROS generation (Remijsen, Vanden Berghe et al. 2011). This results

shows that both ROS and autophagy are required for NETs formation but they may not be dependent on each other. It should be noted that there are studies which shows inhibition of PI3K leads to inhibition of ROS and NETosis (Behnen, Leschczyk et al. 2014, DeSouza-Vieira, Guimarães-Costa et al. 2016). Mammalian target of rapamycin (mTOR) regulates autophagy and also plays role in NETs formation (Itakura and McCarty 2013). mTOR is an inhibitor of autophagy (Mehrpour, Esclatine et al. 2010). Inhibition of mTOR by rapamycin or WYE-354 enhanced histone citrullination and NETosis by bacteria-derived peptide formyl-Met-Leu-Phe (fMLP) (Itakura and McCarty 2013). PMA and fmlp stimulated neutrophils show accumulation of microtubule-associated proteins 1A/1B light chain 3 beta (LC3B), marker for autophagic vesicles, in punctated structure. fmlp was inducing ROS generation in cells and inhibition of ROS inhibited NETosis. This shows the role of fmlp induced ROS and autophagy in NETosis (Itakura and McCarty 2013).

Aging also affects NETs formation and autophagy is one of the mechanisms behind aging related decline in NETs formation. Hazeldine et al. showed that neutrophils from aged humans (mean age 69.89 +/- 5.4 years) form less NETs compared to young humans (mean age 25.54 +/- 4.15 years) when primed with TNF-α and stimulated with IL-8 or LPS (Hazeldine, Harris et al. 2014). Interestingly no difference in NETsosi was observed when the cells were treated with PMA (Hazeldine, Harris et al. 2014). Xu et al. also showed the effect of aging on NETosis and observed the role of TLR2 and autophagy related 5 (*ATG5*) gene in the process (Xu, Zhang et al. 2017). Polymorphonuclear leucocytes (PMN) isolated from aged mice (over 18 months old) formed less NETs compared to young mice when stimulated with peptidoglycan. The expression of ATG5 was significantly lower in the PMNs from aged mice and mTOR was significantly high as compared to young mice (Xu, Zhang et al. 2017). The expression of LC3B increased in cells from younger and aged mice after treatment but the increase in PMNs from aged mice was significantly lower than the younger ones. The use of rapamycin restored the ability of aged PMNs to form NETs. The results clearly establishes the role of autophagy in NETs formation (Xu, Zhang et al. 2017). Contradicting these results Germic et al. showed that autophagy plays no significant role in the ETs formation in neither mice nor human neutrophils nor eosinophils when primed with GM-CSF and treated with C5a or LPS (Germic, Stojkov et al. 2017). Unprimed cells treated with PMA showed similar results. Pharmacological inhibitor of PI3K and autophagy 3-methyladenine (3-MA) and wortmannin does block traps formation. But this is the consequence of ROS inhibition but not autophagy (Germic, Stojkov et al. 2017). Interestingly Pieterse et al. showed that source of LPS and culture condition, such as use of serum free media, affect the pathway of NETosis (Pieterse, Rother et al. 2016). LPS derived from *E. coli O128:B12* and *P. aeruginosa* 10 induced suicidal NETosis in autophagy and ROS dependent, and TLR4 independent manner in a serum and platelet free media. While LPS derived from *Salmonella enterica* (serotype *enteritidis*) did not induce NETosis (Pieterse, Rother et al. 2016). In the media containing platelets, LPS from all sources induced vital NETosis in ROS and autophagy independent manner and required platelet and neutrophil interaction via TL4 and CD62p (Pieterse, Rother et al. 2016). The role of autophagy in NETosis requires further investigation. The evidences does points towards the role of autophagy, however it may be highly stimuli specific. Autophagy is a tightly regulated essential cellular process which helps to maintain homeostasis inside cell by clearing waste products, damaged and senile organelles and detecting and clearing intracellular pathogens. Investigating its role in NETs formation may give insights of NETs role in diseases.

2.3.1.2 DOWNSTREAM TO REACTIVE OXYGEN SPECIES

NETosis pathway downstream to ROS leads to chromatin modification resulting in chromatin decondensation and expulsion. ROS generated by NOX acts on MPO and NE in azurophilic granules (Papayannopoulos, Metzler et al. 2010). Azurophilic granules are found in neutrophils and they contain antimicrobial enzymes such as MPO, NE, proteinase 3 and

cathepsin G. NOX generated ROS stimulates MPO and triggers the activation and translocation of NE to nucleus where it degrades histone H1, H2A and H4 and promotes chromatin decondensation (Papayannopoulos, Metzler et al. 2010). Initially in an in-vitro setting Papayannopoulos et al. observed that MPO was not sufficient to induce significant chromatin decondensation, but it enhanced it. MPO enhanced nuclear decondensation in an enzymatically independent manner. It does not require its substrate H_2O_2 and nuclear decondensation was not inhibited by ABAH, an MPO inhibitor (Papayannopoulos, Metzler et al. 2010). Contradicting insufficiency of MPO for nuclear decondensation Metzler et al., lead by the same group, isolated neutrophils from MPO deficient donors and observed that the neutrophils with complete deficiency of MPO does not produce NETs in response to PMA, while partially MPO deficient neutrophils and healthy neutrophils does (Metzler, Fuchs et al. 2011). Further experiments showed that inhibiting MPO by ABAH in healthy neutrophils does not completely inhibits NETosis but it does delay the process and reduced the number of NETs forming cells. The authors concluded that ABAH does not inhibits MPO completely and even low level of MPO activity was sufficient to drive NETosis (Metzler, Fuchs et al. 2011). The importance of MPO, ROS and H_2O_2 in the NETosis became more evident when Metzler et al. in a different study showed that ROS and MPO are required for the release of NE from the azurophilic granules without disrupting the granules in PMA or C*andida albicans* treated human neutrophils (Metzler, Goosmann et al. 2014). H_2O_2 triggered the MPO dependent release of NE from the granule in a way that does not required release of MPO. MPO also activates NE. In patients with chronic granulomatous disease and patients with MPO deficiency, NE fails to translocate to nucleus. Interestingly released NE was getting attached to F-actin in the cytoplasm. MPO was required to catalytically activate NE so that it can degrade F-actin to translocate to nucleus. In the absence of MPO, NE was not able to cleave H4 also, which is needed for chromatin decondensation (Metzler, Goosmann et al. 2014). Histones can also be cleaved by other proteases such as capase-11. Caspase-11 gets access to DNA with the help of pores formed by gasdermin D and degrades H3 which helps in relaxing chromatin (Chen, Monteleone et al. 2018). However this pathway of NETosis is independent of NE, MPO and chromatin modification by protein arginine deiminase (PAD4) and proceeds through noncanonical activation of NLRP3 inflammasome by Pam3CSK4, TLR1/2 agonist, and cytosolic LPS (Chen, Monteleone et al. 2018). MPO-NE pathway of NETosis is central to many pathogens and stimuli such as crystals (Daniel, Leppkes et al. 2019). NETs formation is deficient in CGD patients and patients with complete deficiency of MPO (Metzler, Fuchs et al. 2011). The MPO-NE axis is reported crucial for NET formation in mice models of pulmonary infection, cancer and sepsis. Mice treated with NE inhibitors or completely lacking NE are also deficient in NET formation (Papayannopoulos 2018, Daniel, Leppkes et al. 2019).

PAD4 plays central role in chromatin decondensation and is downstream to ROS. PAD4 translocate to nucleus and citrullinates arginine, present in histones, by converting amine to ketone (Papayannopoulos 2018, Daniel, Leppkes et al. 2019, Yousefi, Stojkov et al. 2019, Neubert, Meyer et al. 2020). The precise mechanism leading to PAD4 activation and translocation is still being explored but observations point out that the pathway lies downstream to ROS. Initially histone citrullination was considered essential for chromatin decondensation in NETs formation. Later it became clear that the need of citrullination is stimuli dependent. Inhibition of PAD4 blocks nicotine induced NETs formation but does not block cholesterol crystals induced NETs formation (Warnatsch, Ioannou et al. 2015, Hosseinzadeh, Thompson et al. 2016). Holmes et al. reported that PMA, ionomycin, monosodium urate (MSU), and Candida albicans were inducing NETosis, none of the stimuli induced exclusively citrullinated NETs (Holmes, Shim et al. 2019). Induction of different isoforms of PKC by different stimuli is also responsible for differential citrullination (Neeli and Radic 2013). While A23187, a calcium ionophore, induces PKCζ which is an activator of PAD4, PMA induces PKCα which is an inhibitor of PAD4. However PMA still induces citrullination though not as extensive as A23187 (Neeli and Radic 2013). One another reason for less detection of citrullinated histones in PMA induced NETs is the digestion of

citrullinated histones by neutrophil proteases as suggested by Bont et al. (de Bont, Koopman et al. 2018).

Recently D'Cruz et al. showed that PAD4 is activated downstream of receptor-interacting protein kinase-1 (RIPK1) and mixed lineage kinase domain–like (MLKL) in birinapant/z-VAD-fmk, an inducer of necroptosis, treated mouse neutrophils (D'Cruz, Speir et al. 2018). This pathway follows ROS generated from NOX independent source. Interferon-γ (IFN-γ) primed and birinapant/z-VAD-fmk treated cells were undergoing necroptosis and forming NETs. Kinase activity of RIPK1 was required for activation of RIPK3/MLKL and PAD4 (D'Cruz, Speir et al. 2018). Interestingly neutrophils deficient in PAD4 showed nuclear decondensation and loss of nuclear polymorphonuclear architecture but no chromatin externalization. This shows that while PAD4 may not be necessary for nuclear decondensation, it was required for the externalization of NETs (D'Cruz, Speir et al. 2018). Whether PAD4 plays role in chromatin externalization for other stimuli, needs to be investigated.

A chromatin binding protein DEK has also been identified to play role in NETs formation by mouse and human neutrophils (Mor-Vaknin, Saha et al. 2017). NETs formation was defective in *DEK* knock out mouse neutrophils, whereas adding anti DEK aptamers inhibited the formation of NETs by human and mouse neutrophils. The mechanism by which DEK is involved in NETs formation needs further exploration (Mor-Vaknin, Saha et al. 2017).

2.4 NETs IN DISEASES

NETs were discovered as a mechanism to counter pathogens (Brinkmann, Reichard et al. 2004). With studies it became evident that extracellular traps can cause pathology in diseases (Figure 2.1). Since the traps are formed of chromatin embedded with cytoplasmic proteins, they are source of auto-antibody production leading to autoimmunity. Release of DNA and other DAMPs results in inflammation contributing to causes and symptoms of diseases (Papayannopoulos 2018, Daniel, Leppkes et al. 2019, Yousefi, Stojkov et al. 2019). This section will explain the role of extracellular traps in disease pathology.

2.4.1 AUTOIMMUNE DISEASES

2.4.1.1 SYSTEMIC LUPUS ERYTHEMATOSUS

Systemic Lupus Erythematosus (SLE) is an autoimmune disease characterized by antibody development against DNA, RNA, histones and DNA-proteins complex (Pisetsky and Jiang 2006, Kienhöfer, Hahn et al. 2017). Initially dying cells were considered to be the source of extracellular DNA present in SLE, against which the antibodies were developed (Pisetsky and Jiang 2006, Pisetsky and Fairhurst 2007). Now it is known that NETs are also central source of auto-antigens in SLE. Immune complexes composed of auto-antibodies and auto-antigens gets deposited in different parts of the body inducing inflammation (Villanueva, Yalavarthi et al. 2011, Fousert, Toes et al. 2020). Persistence of NETs also results in prolonged inflammation. NETs are degraded by serum DNase I or DNaseIL3 (Hakkim, Fürnrohr et al. 2010, Al-Mayouf, Sunker et al. 2011). Degradation of NETs by serum DNase I in SLE patients is impaired. Elevated level of anti-NETs antibodies prevents access of DNase I to traps and presence of DNase I inhibitors inhibits NETs degradation (Hakkim, Fürnrohr et al. 2010). Low-density granulocytes (LDGs), which are prone to NETs formation and spontaneously produce NETs, are increased in SLE patients (Denny, Yalavarthi et al. 2010, Garcia-Romo, Caielli et al. 2011, Gupta and Kaplan 2016). LDGs also synthesize more type I interferon which plays role in reducing tolerance and increasing the symptoms of disease. Type I interferon primed normal density neutrophils form

NETs in response to sera or purified anti-ribonucleoprotein antibodies from SLE patients (Garcia-Romo, Caielli et al. 2011). NETs present in SLE patients' sera are decorated with many anti-microbial peptides including LL37. LL37 present on NETs activates human memory B cells directly or via activation of plasmacytoid dendritic cells (pDCs). Activation of memory B-cells leads to auto-antibody production against LL-37 and aggravates disease pathology (Garcia-Romo, Caielli et al. 2011).

Ribonucleoprotein immune complexes cause mitochondrial hyperpolarization, elevate ROS level and induce release of oxidized mitochondrial DNA in neutrophils (Lood, Blanco et al. 2016). They induce NETs formation in LDGs from SLE patients via ROS dependent pathway.

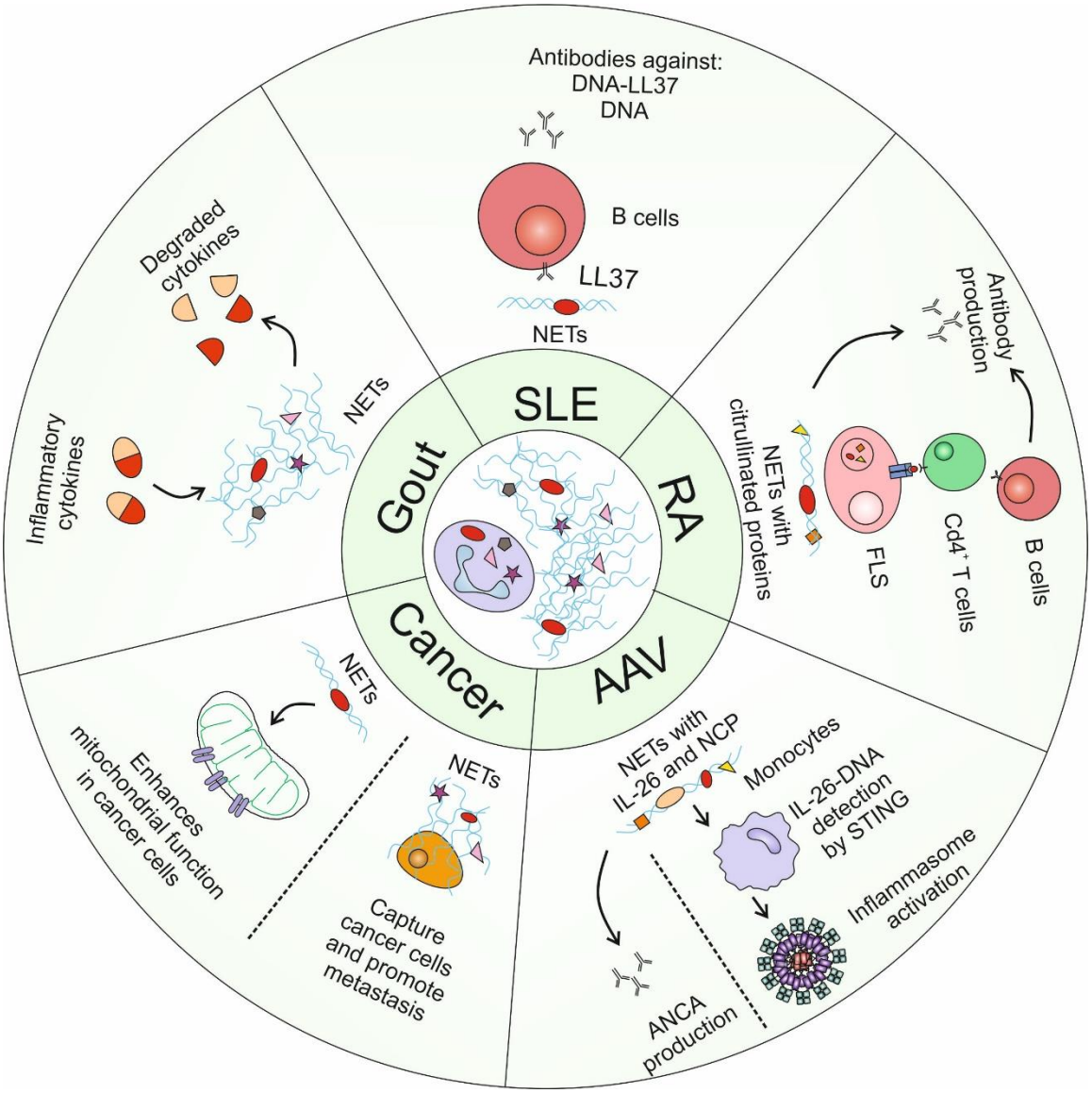

Figure 2.1 : *NETs in diseases: NETs play central role in many diseases.* In systemic lupus erythematosus autoantibodies are produced against the DNA and the complex of DNA-proteins like LL37-DNA complex. In rheumatoid arthritis autoantibodies are produced against the citrullinated histones and other citrullinated proteins present on NETs. Citrullinated proteins are also phagocytosed by fibroblast-like synoviocytes (FLS) which presented then to helper T cells. This further results in the production of antibodies against the proteins. In anti-neutrophil cytoplasmic autoantibody (ANCA) associated vasculitis (AAV) ANCA produced causes inflammation and damages blood vessels. IL-26-DNA complex is detected by monocytes through STING which activates inflammasome and aggravates inflammation. In cancer protein-DNA complex enhances mitochondrial functions which may play role in cancer progression. NETs also capture circulating cancer cells which promotes cancer metastasis. In case of gout NETs have been shown to reduce inflammation. Proteases present on NETs cleave the inflammatory cytokines which results in curbing inflammation.

Inhibiting mitochondrial ROS in MRL/lpr mouse model of SLE reduces type I interferon level, renal complement deposition and albuminuria. Released NETs, oxidized mitochondrial DNA and formed immune complexes induces inflammation and contributes to the pathology of SLE (Lood, Blanco et al. 2016). Renal deposition of immune complexes is one of the most common manifestations of SLE resulting in lupus nephritis. NETs formation is reported to be higher in patients with SLE and lupus nephritis (Daniel, Leppkes et al. 2019). It has also been observed that SLE patients with impaired NETs degradation has higher chance of developing lupus nephritis compared to the patients with normal NETs clearance. Inhibited degradation of NETs in SLE patients along with higher rate of NETs formation leads to accumulation of traps in tissues and plays crucial inflammatory role in SLE (Hakkim, Fürnrohr et al. 2010, Garcia-Romo, Caielli et al. 2011). However there are contradictory studies about role of NETs in lupus nephritis. In animal models of lupus nephritis, chemical inhibitors of PAD4 inhibits albuminuria, type I interferon and glomerular IgG deposition (Campbell, Kashgarian et al. 2012, Knight, Zhao et al. 2013, Knight Jason, Luo et al. 2014, Knight, Subramanian et al. 2015). However mice deficient of PAD4 and NOX2 have impaired NETs formation but lacked the evidence to prove the role of traps in disease (Gordon, Herter et al. 2017, Kienhöfer, Hahn et al. 2017). Interestingly pristane-induced lupus was aggravated in mice lacking PAD4 or NOX2 (Kienhöfer, Hahn et al. 2017). It is possible that NOX2 knockout mice show aggravated symptoms as NETs production induced by ribonucleoprotein immune complexes is mediated by mitochondrial ROS (Lood, Blanco et al. 2016). While the results may be depend on the mouse model used for the study, it cannot be ignored that NETs may also play beneficial role in SLE by degrading pro-inflammatory cytokines. Complement factor C1q also plays role in NETs related SLE symptoms but its role is still controversial. While NETs bound C1q inhibits NETs degradation, C1q has also been found to increase DNase I activity (Gaipl, Beyer et al. 2004, Leffler, Martin et al. 2012). It is clear that some SLE patients have increased NETs formation and inhibited NETs degradation. But role of NETs in SLE needs more investigation so that it can be regulated as therapeutic intervention.

2.4.1.2 RHEUMATOID ARTHRITIS

Rheumatoid arthritis (RA) is an auto-inflammatory disorder that affects bones and joints. It is characterized by presence of auto-antibodies which leads to inflammation of synovial joints and bone destruction (Apel, Zychlinsky et al. 2018). High amount of anti-citrullinated protein antibodies (ACPAs) is a diagnostic marker for RA (Wegner, Lundberg et al. 2010, Khandpur, Carmona-Rivera et al. 2013, Corsiero, Pratesi et al. 2016). PADs play central role in formation of NETs via citrullination of histones. Citrullinated histones and other citrullinated proteins present on NETs are considered as major source of ACPAs in RA. Proteins such as vimentin, fibrinogen and type 2 collagen can also be targeted by ACPA after getting citrullinated by PADs (Corsiero, Pratesi et al. 2016, Apel, Zychlinsky et al. 2018). High level of NETs have also been detected in synovial fluid and sera of RA patients. Spontaneous release of NETs by LDGs, though lesser than SLE, have also been detected in RA (Khandpur, Carmona-Rivera et al. 2013, Wright, Makki et al. 2017). NETs embedded with citrullinated proteins are phagocytosed by fibroblast-like synoviocytes in TLR-9 and receptor for advanced glycation end-products (RAGE) dependent (Carmona-Rivera, Carlucci et al. 2017). They are presented to $CD4^+$ T cells by major histocompatibility complex (MHC) II resulting in ACPAs production by B cells and further inflammation (Carmona-Rivera, Carlucci et al. 2017). The studied in mouse model of RA also shows some contradictory results for role of NETs in RA. Neutrophil depletion in K/BxN mice model of RA reduces disease symptoms (Wipke and Allen 2001, Christianson, Corr et al. 2012). However PAD4 depletion in this mice model failed to abrogate the disease (Rohrbach, Hemmers

et al. 2012). Whereas PAD4 deficiency did reduced the symptoms in collagen induced arthritis mice model (Papadaki, Kambas et al. 2016). Increasing evidences points towards the role NETs in RA development and progression, studies are needed to clarify how important role NETs play in disease and through which pathway.

2.4.1.3 ANTI-NEUTROPHIL CYTOPLASMIC AUTOANTIBODY ASSOCIATED VASCULITIS

Anti-neutrophil cytoplasmic autoantibody (ANCA) associated vasculitis (AAV) is an autoimmune disorder leading to the inflammation and damage of blood vessels, majorly involving small blood vessels (Apel, Zychlinsky et al. 2018). Dead neutrophils present on the walls of damaged blood vessels are and autoantibodies against MPO and proteinase 3 (PR3) are hallmark of the disease (Falk and Jennette 1988, Söderberg and Segelmark 2016, Apel, Zychlinsky et al. 2018). About 90% of AAV patients generate antibodies against neutrophil cytoplasmic proteins (Söderberg and Segelmark 2016). ANCA induces ROS generation and degranulation in neutrophils (Falk, Terrell et al. 1990). Stimulation of TNF induced neutrophils with ANCA results in NETs formation (Kessenbrock, Krumbholz et al. 2009). Neutrophils and LDGs from AAV patients spontaneously produce NETs with PR3 and MPO (Grayson, Carmona-Rivera et al. 2015). In some AAV patients NETs are also found in their glomeruli causing glomerulonephritis, one of the common manifestations of AAV (Daniel, Leppkes et al. 2019). Recently high level of IL-26 and IL-26-DNA complexes were found in AAV patients where they were causing inflammation by activating inflammasomes in monocytes through stimulator of interferon genes (STING) (Poli, Augusto et al. 2017). Interestingly a drug induced AAV have been found in some patients with cocaine and levamisole abuse (Lood and Hughes 2017). The ANCAs generated in case of drug abuse were specific to neutrophil elastase. These anti-NE ANCAs have high affinity for NETs and may further promote vasculitis (Lood and Hughes 2017). PAD inhibitor reduces the level of anti-MPO antibodies in MPO-ANCA associated vasculitis mouse model further suggesting the role of NETs (Kusunoki, Nakazawa et al. 2016). Accumulation of NETs and vascular occlusion was also observed in mouse deficient with both DNase 1 and DNase 1-like 3 (Jiménez-Alcázar and Rangaswamy 2017). The role of NETs as a source of ANCA in AAV is evident and NETs can be used as diagnostic marker for AAV. However, mores studies are required for therapeutic interventions.

2.4.2 STERILE INFLAMMATION

NETs regulated inflammatory cytokine production which aggravates pathology of diseases. Cholesterol crystals generated in early atherosclerosis can induce NETs. These NETs can induce transcription of IL-6 and IL-1β in macrophages via TLR2 and TLR4 (Warnatsch, Ioannou et al. 2015). These cytokines cause inflammation, differentiation of Th 17 cells and recruitment of myeloid cells to atherosclerotic lesions (Warnatsch, Ioannou et al. 2015). In mice model of atherosclerosis DNase administration reduced IL-1β concentration in plasma whereas the concentration was not affected in mice that cannot form NETs (Warnatsch, Ioannou et al. 2015). NETs increase inflammation and liver damage in ischaemia–reperfusion injury mouse model. The role of NETs in inflammation and liver damage was confirmed as damage was significantly reduced by PAD4 inhibitors and DNase (Savchenko, Borissoff et al. 2014, Huang, Tohme et al. 2015). NETs can also be induced by histones and HMGB1, released in cell damage, via TLR4 and TLR9 (Huang, Tohme et al. 2015). Adoptive transfer of TLR4 or TLR9 knockout neutrophils in neutrophil depleted mice significantly reduced NETs formation and liver damage (Huang, Tohme et al. 2015). NETs also delay wound healing in diabetic patients and neutrophils from diabetic patients have higher propensity to from NETs (Wong, Demers et al. 2015). However the role of high glucose level in NETs formation is still controversial (Joshi, Lad et al. 2013). The

precise mechanism of NETs formation in skin injury is still being studied, but neutrophil infiltration and NETs deposition in skin injury have been observed which reduces wound healing rate in diabetic mice. PAD4 deficiency restores the wound healing rate confirming the role of NETs (Wong, Demers et al. 2015). The mechanism of reduced wound healing by NETs is not clear. It needs to be studied whether NETs directly or indirectly regulates inflammation and tissue repair mechanisms in wound healing. Neutrophils and NETs are also present in 3xTg-AD and 5xFAD mice models of Alzheimer's disease and also in Alzheimer's patients (Zenaro, Pietronigro et al. 2015). The number of MPO positive cells were high in Alzheimer's patients with respect to control individuals. MPO positive neutrophils forming NETs were identified in the parenchyma, vasculature of patients and in the close proximity of Amyloid β plaques. Neutrophils also produced higher IL-17 and inhibiting neutrophil recruitment or depleting neutrophils significantly improved cognitive ability in mice models (Zenaro, Pietronigro et al. 2015). How NETs are playing role in the pathology of Alzheimer's disease needs further investigation.

2.4.3 CANCER AND METASTASIS

Recently NETs have also been implicated to play central role in cancer progression and metastasis. In 2012 Demers et al. observed that neutrophils from mice models of chronic myelogenous leukemia (CML), lung and breast cancer have higher propensity of forming NETs with respect to the neutrophils isolated from wild type mice (Demers, Krause et al. 2012). At the later stage of disease high amount of plasma DNA and citrullinated histone H3 was present in these mice suggesting NETs formation. G-CSF produced by tumors may prime neutrophils for NETs formation. NETs were also inducing cancer associated thrombosis in the lungs of mice (Demers, Krause et al. 2012). Cancer associated thrombosis due to NETs formation have also been reported in pancreatic cancer mice models and patients (Boone, Murthy et al. 2018). In 2013 Berger-Achituv et al. analyzed the tissue sample of 8 patients with pediatric Ewing sarcoma and observed that in two of the patients, tumor associated neutrophils are forming NETs (Berger-Achituv, Brinkmann et al. 2013). Cancer metastasized in both of the patients and with early relapse (Berger-Achituv, Brinkmann et al. 2013). Interestingly NETs produced in MMTV-PyMT and in RIP1-Tag2 mouse, mouse models for mammary carcinoma and insulinoma respectively, were found to impair heart and kidney vascular functions (Cedervall, Zhang et al. 2015). This study highlighted the systemic effect of NETs on the organs not directly associated with the cancer. Neutrophil-platelet complex population significantly increased in kidneys of mice. NETs accumulation correlated with the upregulation of VCAM-1, ICAM-1, and E-selectin, IL-1β, IL-6 and CXCL1. Neutrophil depletion, DNase or anti G-CSF treatment restored the function of renal vasculature further suggesting the role of NETs in cancer associated organ damage (Cedervall, Zhang et al. 2015). NETs have been proposed to support tumor growth and the underlying mechanisms are still being studied. Recently Yazdani et al. showed that NETs promote tumor cell growth by enhancing their mitochondrial function (Yazdani, Roy et al. 2019). The authors observed increased amount of NETs in tumor tissues of metastatic colorectal cancer patients and increased MPO-DNA in patients' serum. MC38 murine colon cancer cells grew slowly when implanted subcutaneously in PAD4 knockout mice. Human colorectal cancer cell line HCT116 growth was also reduced in nu/nu mice treated with DNase (Yazdani, Roy et al. 2019). The expression of MFN-2 and DRP-1, mitochondrial fusion and fission proteins, and Parkin and PINK1, proteins associated with mitophagy were enhanced by NETs. The expression of these proteins, mitochondrial density and other mitochondrial biogenesis associated proteins NRF-1, TFAM, and PGC1α were reduced in PAD4 knockout tumors. Mitochondrial biogenesis was increased through stimulation of TLR4 by released NE (Yazdani, Roy et al. 2019). This study points towards the diversity of mechanisms NETs can influence to support tumor growth.

Park et al. showed the role of NETs in tumor metastasis (Park, Wysocki et al. 2016). Metastatic breast cancer cells 4T1 injected in mice recruited neutrophils to the lungs in CXCL1

dependent manner and induced NETs. NETs were also present in primary breast cancer tissues of patients, along with highest amount of NETs in triple negative tumors, and in metastatic lung lesions of patients (Park, Wysocki et al. 2016). Interestingly co-culture of neutrophils with 4T1 cells induced NETs and increased the invasion of 4T1 cells. Neutrophil induced increased invasion capacity of 4T1 cells were lost after treatment with DNase I suggesting the role of NETs in invasion. Treatment with DNase I coated nano particles enhanced the persistence of DNase I in plasma of mice and prevented lung metastasis (Park, Wysocki et al. 2016). Recently it has also been observed that NETs can promote tumor metastasis by capturing circulating cancer cells (Najmeh, Cools-Lartigue et al. 2017). A549 lung cancer cell lines adhered to NETs in vivo and in vitro with the help of β1 integrin expressed on both NETs and on cancer cells. The authors showed that β1 integrin was upregulated on NETs in case of systemic inflammation and inhibiting its expression reduced the adhesion of A549 cells to hepatic sinusoids. DNase treatment also abrogated cancer cell adhesion to NETs (Najmeh, Cools-Lartigue et al. 2017). An interesting role of NETs in awakening of dormant cancer cells have recently been reported (Albrengues, Shields et al. 2018). Sustained inflammation caused by tobacco smoke and LPS in mice induced formation of NETs in lungs and converted dormant breast cancer MCF-7 and D2.0R cells to aggressive metastatic cancer cells. Inhibiting NETs formation by inhibiting PAD4 or DNase I treatment reduced or prevented the awakening of dormant cells (Albrengues, Shields et al. 2018). This was caused by the digestion and remodeling of extracellular matrix protein laminin by NE and MMP9 bound to NETs. Integrin activation and FAK/ERK/MLCK/YAP signaling was involved in the awakening of dormant cancer cells which was stimulated by laminin remodeling (Albrengues, Shields et al. 2018). This mechanism again highlights the potential of NETs role in cancer progression and metastasis. NETs can be utilized as prospective target in cancer management. With further studies NETs and associated proteins may also be utilized as biomarkers of cancer metastasis in future.

2.4.4 GOUT

Gout is a form of arthritis characterized by the presence of monosodium urate (MSU) crystals in joints (Dalbeth, Merriman et al. 2016). MSU deposition in joints and kidneys results in inflammation. MSU activates macrophages and dendritic cell present in the synovium which results in neutrophils recruitment in joints (Dalbeth, Merriman et al. 2016). The role of neutrophils in gout is still being studied. Neutrophils isolated from gout patients spontaneously produce NETs (Mitroulis, Kambas et al. 2011). Serum from gout patients also induce NETs in neutrophils from healthy patients (Mitroulis, Kambas et al. 2011). Interestingly it has been observed that NETs play beneficial role in controlling inflammation in gout. Proteases present on aggregated NETs degraded inflammatory cytokines such as IL-1β, TNF, IL-6, and macrophage inflammatory protein 1α (MIP1α or CCL3) (Schauer, Janko et al. 2014). Gouty arthritis was enhanced in ROS-deficient mice unable to produce NETs and the symptoms were reduced when injected with in vitro produced NETs. Degradation of cytokines also inhibited further recruitment and activation of neutrophils resolving neutrophil driven inflammation (Schauer, Janko et al. 2014). Whereas the evidence shows beneficial role of NETs in resolving inflammation in gout, it needs further investigation.

2.5 MICROGLIA

In 1913, over a century ago Santiago Ramón y Cajal described cells other than neurons in CNS calling them 'third element' of the CNS. Soon after Pío Del Río Hortega phenotypically identified microglia (Pérez-Cerdá, Sánchez-Gómez et al. 2015, Li and Barres 2018). A long time after their discovery microglia and other glial cells were considered as glue working to keep

neurons together. Now it is known that glial cells comprise about 90% of the cells of CNS (Allen and Barres 2009). They serve various central functions such as protecting CNS from infections and insults, myelinating neurons, providing nutrition, helping in neurodevelopment, maintaining ionic and chemical balance in brain parenchyma and maintaining the blood brain barrier (Allen and Barres 2009, Norris and Kipnis 2018).

Microglia are the principle immune cells of the CNS (Allen and Barres 2009, Salter and Stevens 2017, Norris and Kipnis 2018). They originate from yolk sack and migrate to brain early in development (Ginhoux, Greter et al. 2010). Microglia comprise of 5-10% of brain cells (Aguzzi, Barres et al. 2013, Li and Barres 2018). The population renews itself throughout life and helps in proper functioning of CNS. As the resident macrophage of the CNS microglia performs range of functions such as, but not limited to, surveying the brain parenchyma for debris and pathogens, synaptic pruning during neurodevelopment and regulating synaptic plasticity (Salter and Stevens 2017, Li and Barres 2018, Norris and Kipnis 2018). Along with maintaining homeostasis and immunity in brain microglia are central to pathology of CNS diseases including *Glioblastoma multiforme* (GBM) (Hambardzumyan, Gutmann et al. 2016, Gutmann and Kettenmann 2019). Microglia are also source of inflammatory cytokines and play significant role in insult induce neuroinflammation as well as sterile neuroinflammation (Salter and Stevens 2017, Li and Barres 2018, Norris and Kipnis 2018). Following section will focus on the role of microglia in neurodegenerative disease and in GBM.

2.5.1 MICROGLIA IN *GLIOBLASTOMA MULTIFORME*

Microglia are the only macrophage population residing in healthy CNS. In case of insult, like the GBM, blood brain barrier gets compromised and CNS gets flooded with peripheral monocytes and macrophages (Hambardzumyan, Gutmann et al. 2016). Studies have shown that microglia and infiltrating macrophages (glioma associated macrophages, GAMs) play central role in regulation of GBM formation and propagation. About 30-50% of cells in brain tumors are microglia of macrophages (Morantz, Wood et al. 1979, Rossi, Hughes et al. 1987, Hambardzumyan, Gutmann et al. 2016). However whether they play positive or negative role is still a topic of debate. Glioma recruits GAMs by secreting chemoattractants such as monocyte chemoattractant protein-1 (MCP-1) or CCL2, CXCL12 (SDF-1), glial cell–derived neurotrophic factor (GDNF), granulocyte macrophage colony-stimulating factor (GM-CSF) and CSF-1 (Platten, Kretz et al. 2003, Coniglio, Eugenin et al. 2012, Wang, Hong et al. 2012, Ku, Wolf et al. 2013). Increased population of $Iba1^{+}$ positive microglia have been reported to prolong the survival of GBM patients whereas the accumulation of $CD204^{+}$ GAMs increased the malignancy of tumor and reduced the survival of patients (Sørensen and Dahlrot 2018). Similarly presence of $CD206^{+}$, $CD68^{+}$ and $CD163^{+}$ GAMs in *IDH1*^{R132H} non-mutant GBM correlates with prolonged survival of patients (Zeiner, Preusse et al. 2019). Depleting microglia in experimental models of high grade glioma inhibits tumor growth (Hambardzumyan, Gutmann et al. 2016).

GAMs secrete Il-1β, IL-6, TGF-β, stress-inducible protein 1 (STI-1) and epidermal growth factor which can promote tumor growth (Saederup, Cardona et al. 2010, Carvalho da Fonseca, Wang et al. 2014, Feng, Szulzewsky et al. 2015, Gutmann and Kettenmann 2019). Microglia can also induce expression of platelet-derived growth factor receptor in tumor cells which may increase tumor malignancy (Wallmann, Zhang et al. 2018). In the murine model of low grade optic glioma harboring mutation in Neurofibromatosis type 1 (*Nf1)* gene, GAMs infiltrate tumor with majority of them being $CD11b^{high}$ $CD45^{low}$ microglia (Pong, Higer et al. 2013). In $Nf1^{+/-}$ mice models GAMs are reported to produce stroma-derived factor-1 (SDF-1) and CCL5 which promotes glioma survival and growth. Inhibition of CXCR4 inhibited tumor growth as SDF-1 acts through CXCR4 (Warrington, Woerner et al. 2007, Warrington, Gianino et al. 2010). Inhibition of microglial activation by minocycline or JNK inhibitor also inhibited the growth of optic glioma,

and reduced expression CX3CR1 delayed tumor formation in this model (Daginakatte, Gianino et al. 2008, Simmons, Pong et al. 2011).

In case of high grade glioma M1/M2 polarization of GAMs have been reported to play role in glioma progression (Zeiner, Preusse et al. 2015). RNA microarray analysis of microglia from healthy mouse and GAMs from high grade glioma mouse revealed upregulation or downregulation of about 1000 genes. Some of the gene among then were genetic signature of M1 and M2 macrophages (Szulzewsky, Pelz et al. 2015). CD74, a M1 marker, is expressed by GAMs and positively correlates patients' survival (Zeiner, Preusse et al. 2015). M-CSF derived from glioma induces M2 polarization in microglia and macrophages and supports tumor growth (Pyonteck, Akkari et al. 2013). Blocking of mTOR or CSF1R also reduced M2 GAMs phenotype. mTOR inhibition reduced in vitro proliferation of glioma cells whereas CSF1R inhibition impaired tumor formation and increased the survival of tumor bearing mice (Pyonteck, Akkari et al. 2013, Lisi, Laudati et al. 2014). Dopamine have also been reported to induce M1 polarization in GAMs, inhibit tumor growth and prolong the survival of orthotopic C6 glioma rats. Dopamine also decreased hypoxia-inducible factor-1α (HIF-1α) and microvessel density which also played role in tumor inhibition (Qin, Wang et al. 2015). However in some studies M1 specific marker such as IL-1β have been shown to promote tumor growth (Feng, Szulzewsky et al. 2015).

Evidence suggests that the communication between glioma and GAMs is crucial for glioma growth in mouse models as well as in humans (Hambardzumyan, Gutmann et al. 2016, Gutmann and Kettenmann 2019). But there are many unanswered questions. The lack of glioma specific macrophage and microglia marker restricts our ability to study GAMs contribution in glioma progression leading to conflicting claims. It is still unclear which pathways and molecules are responsible for effective glioma-GAMs interaction and GAMs polarization (Wei, Gabrusiewicz et al. 2013). More studies are needed for utilizing microglia based therapies in GBM.

2.5.2 MICROGLIA IN NEURODEGENERATIVE DISEASES

Microglia activation is crucial for development and progression of neurodegenerative diseases such as Alzheimer's disease (AD), Parkinson's disease (PD) and amyotrophic lateral sclerosis (ALS) (Colonna and Butovsky 2017, Li and Barres 2018). Accumulation of amyloid β (Aβ) plagues are hallmark of AD (Leng and Edison 2020). Microglia can detect to Aβ via receptors such as TLR2/4/6, CD36 and NLRP3 which may lead to prolonged inflammation (El Khoury, Moore et al. 2003, Heneka, Kummer et al. 2013, Heneka, Golenbock et al. 2015). While phagocytosis and degradation of Aβ by microglia can have beneficial effect, chronic activation of microglia and prolonged inflammation can be deleterious. Large-scale genome-wide association studies (GWAS) have identified many loci linked to AD exclusive to microglia or myeloid cells (Bertram, Lange et al. 2008). TREM2 present in microglia plays critical role in AD. Individuals heterozygous for TREM2 variant $TREM2^{R47H}$ possess significant risk for developing AD (Colonna and Wang 2016). An increase in the level of soluble TREM2 have been detected in cerebrospinal fluid of patients in early stages of AD (Suárez-Calvet, Kleinberger et al. 2016). TREM2 is involved in phagocytosis and inflammatory pathways in microglia. But there exact role in AD is still being studied (Colonna 2003, Colonna and Wang 2016). Recently apolipoprotein E (APOE) has also been reported as one of the ligands of TREM2 (Atagi, Liu et al. 2015, Yeh, Wang et al. 2016). CD33, another molecule enriched in microglia in AD, plays role in inflammation and Aβ clearance (Bertram, Lange et al. 2008, Griciuc, Serrano-Pozo et al. 2013). Complement proteins, specifically C1q and C3, are also upregulated in AD and are considered to be crucial for microglia mediated synapse loss in disease. Released C1q can also activate astrocytes and which further causes neurotoxicity (Hong, Beja-Glasser et al. 2016). Microglia are also suggested to spread tau, another hallmark protein of AD, across different brain regions via exosomes (Asai, Ikezu et al. 2015).

α-Synuclein the causative factor of PD is detected by microglia via receptors such as TL1/2 and Axl (a receptor tyrosine kinase) (Daniele, Béraud et al. 2015, Fourgeaud, Través et al. 2016). High level of Axl expression was observed in the spinal cord microglia of mice overexpressing α-synuclein $SNCA^{A53T}$, a cause of hereditary PD. Lacking Axl and another receptor Mertk prolonged mice survival and partially delayed neurodegeneration (Fourgeaud, Través et al. 2016). Mutations in leucine-rich repeat kinase 2 (LRRK2) are also associated with PD. LRRK2 is expressed in neurons, microglia, monocytes and macrophages. In rat models of PD, myeloid expression LRRK2 correlates with degeneration of dopaminergic neurons (Daher, Volpicelli-Daley et al. 2014). Recently TREM2 variant $TREM2^{R47H}$ has also been identified as a risk factor for PD (Rayaprolu, Mullen et al. 2013). CX3CR1 is also significant for regulating microglial toxicity. Microglia, in mice lacking CX3CR1, are more toxic in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), a dopaminergic neurotoxin, induced PD (Cardona, Pioro et al. 2006).

Progressive loss of motor neurons is characteristic of amyotrophic lateral sclerosis (ALS) and microglia plays critical role in neurodegeneration in the disease. Mutations and aggregation of proteins superoxide dismutase1 (SOD1), TAR-DNA binding protein 43 (TDP-43), C9orf72, fused in sarcoma (FUS), Tau and heterogeneous nuclear ribonucleoproteins (Renton, Chiò et al. 2014, Al-Chalabi, Berg et al. 2017). Removal of Sod1 form myeloid cells in mice inhibited disease progression and prolonged survival (Boillée, Yamanaka et al. 2006). Mutant $SOD1^{G93A}$ expressing mice shows that SOD1 accumulates in neurons in ALS. Released $SOD1^{G93A}$, along with other proteins activates microglia resulting in ROS activation and secretion of proinflammatory cytokines further damaging neurons (Beers, Henkel et al. 2006, Boillée, Yamanaka et al. 2006). Inhibiting microglial activation by inhibition of NF-κB reduced degeneration of motor neurons extending mice survival (Frakes, Ferraiuolo et al. 2014). C9orf72 regulates phagocytosis and lysosome pathways in microglia. Mutated C9orf72 leads to upregulation of inflammatory factors in microglia leading to neurodegeneration in ALS. Accumulation of lysosomes in microglia has been reported in mice lacking *C9orf72* gene causing inflammation and pathology similar to ALS (O'Rourke, Bogdanik et al. 2016).

It is clear that microglia plays central role in the development and pathology of neurodegenerative disease. A better understanding of inflammatory and anti-inflammatory factors regulating microglia in neurodegenerative diseases will help us in utilizing them for better treatment and management of patients.

2.6 DOPAMINE

Dopamine or 3-hydroxytyramine is one of the three, others being epinephrine and norepinephrine, catecholamine neurotransmitters (Klein, Battagello et al. 2019). Avid Carlson in 1958 showed that dopamine plays role as a neurotransmitter in central nervous system; he received Nobel Prize for Physiology or Medicine in 2000 for dopamine discovery (Carlsson, Lindqvist et al. 1958). The role of dopamine in neurological process such as controlling motor functions, learning, cognition, motivation and reward and pleasure is well studied (Klein, Battagello et al. 2019). It is synthesized by a two-step process from amino acid tyrosine. Tyrosine is converted to L-DOPA by tyrosine hydroxylase (TH) and decarboxylation of L-DOPA by aromatic amino acid decarboxylase gives dopamine (Meiser, Weindl et al. 2013). Dopaminergic neurons are concentrated in basal ganglia of humans and mouse. Secreted dopamine communicate to neurons by receptors DR1-DR5 present on them. The receptors are divided into two groups D1-like (DR1, DR5) and D2-like (DR2, DR3, DR4) (Klein, Battagello et al. 2019, Matt and Gaskill 2020). Dopamine is also central to various neurological disease such as Parkinson's disease, Huntington's disease, multiple sclerosis, schizophrenia, attention deficit/hyperactivity disorder addiction and human immunodeficiency virus associated neurological complications (Jakel and Maragos 2000, Dauer and Przedborski 2003, Gaskill, Miller et al. 2017, Klein, Battagello

et al. 2019). It is also reported to be crucial for rheumatoid arthritis, inflammatory bowel disease (Nakano, Yamaoka et al. 2011, Lin, Lin et al. 2016).

Aside from regulating neurological processes, from around 1980s it started to become evident that dopamine also plays immuno-modulatory roles in CNS as well as in periphery (Le Fur, Phan et al. 1980, Cosentino, Marino et al. 1999, Matt and Gaskill 2020). Dopamine receptors subtypes are present on almost all immune cells and many immune cells can synthesize and secret dopamine (Pinoli and Marino 2017, Matt and Gaskill 2020). Dopamine receptors are also present in peripheral organs such as heart, kidney and gastrointestinal tract. Dopamine regulates a variety of functions like blood pressure, adrenal functions, renal functions, sodium balance, cytokine secretion, cytotoxicity, cell adhesion and chemotaxis (Matt and Gaskill 2020). We have just started to explore the immunological functions of dopamine. More understanding of crosstalk between dopamine and immune cells is needed. This may lead to better management of certain diseases.

2.6.1 EFFECT OF DOPAMINE ON IMMUNE CELLS

Dopamine receptors (DRs) are present on T and B lymphocytes. The presence of DRs on T cells were reported in 1980 by Fur et al. (Le Fur, Phan et al. 1980). Since then several studies have reported the presence of DRs on T cells (McKenna, McLaughlin et al. 2002, Matt and Gaskill 2020). The population of all 5 DRs varies on different subtypes of T cells and accordingly there effect and function also varies (Levite 2012). Dopamine activates naïve effector T cells while at the same time it inhibits T cells which have already been activated by antigens, anti-CD3 antibodies or cytokines (Saha, Mondal et al. 2001, Saha, Mondal et al. 2001, Levite 2012). T cells can also synthesize, store and secrete dopamine (Cosentino, Fietta et al. 2007). Activated regulatory T cells are inhibited by dopamine in autocrine and paracrine manner (Cosentino, Fietta et al. 2007). Dopamine regulates migration of $CD8^+$ T cells by inducing their adhesion to endothelium (Strell, Sievers et al. 2009). It has also been seen that activation of DRs present on T cells induces secretion of cytokines. Stimulation of DR3 induces secretion of TNFα while DR2 stimulation induces IL-10 secretion (Besser, Ganor et al. 2005). Interestingly dopamine released by dendritic cells regulates polarization and differentiation of type 2 T helper cells (Nakano, Higashi et al. 2009). Population of DRs present on T cells of patients with multiple sclerosis, systemic lupus erythematosus and rheumatoid arthritis varies from T cells of healthy control (Matt and Gaskill 2020). Though still being studied, it is clear that dopamine and T cell crosstalk plays crucial role in these diseases.

The presence of DR1 on neutrophil was reported in 1999 by Sookhai et al. where the authors reported the induction of apoptosis in neutrophils by dopamine (Sookhai, Wang et al. 1999). Subsequently the presence of all 5 DRs were reported on neutrophils (Chen, Wu et al. 2014). However the functional significance of DRs present on neutrophils are still being studied. Dopamine attenuates expression of CD11b/CD18 on neutrophils. This decreases neutrophils ability to produce ROS and superoxide anions, inhibits phagocytosis, cell migration and endothelium adherence (Pinoli and Marino 2017).

Macrophages and monocytes expresses different subtypes of DRs and are also able store produce and secrete dopamine. Dopamine majorly has inhibitory effect on monocytes and macrophages (Yan, Jiang et al. 2015, Pinoli and Marino 2017). Yan et al. reported that dopamine can inhibit inflammation by inducing ubiquitination and degradation of NLRP3 inflammasome via DR1 in bone marrow derived macrophages (BMDMs) (Yan, Jiang et al. 2015). Dopamine inhibited nigericin induced maturation of caspase-1 and subsequent production of IL-1β and Il-18 in BMDMs. Stimulation of DR1 can induce the production of cyclic AMP (cAMP) and dopamine induced degradation of NLRP3 was cAMP dependent. Dopamine also degraded NLRP3 and inhibited the production of nigericin induced IL-1 β in mice microglia and astrocytes.

LPS induced production of IL-1β and Il-18 was also inhibited by dopamine via DR1 in mice (Yan, Jiang et al. 2015). Human and mouse monocytic cell line U937 and RAW264.7 respectively, express L-DOPA decarboxylase, one of the enzymes needed for dopamine synthesis (Kokkinou, Fragoulis et al. 2009). Interestingly the level of intracellular dopamine rises in RAW264.7 within 48 hours of LPS treatment (Brown, Meyers et al. 2003). Dopamine suppresses the LPS induced production of IL-12p40, a cytokine secreted by antigen presenting cells, in mouse macrophages (Haskó, Szabó et al. 2002). Dopamine has been reported to increase viral replication and the susceptibility of human macrophages towards HIV infection (Gaskill, Calderon et al. 2009). Flupenthixol, dopamine receptor antagonist, inhibited the entry of virus into macrophages (Gaskill, Yano et al. 2014). Methamphetamine also increase the infection of human macrophages by HIV. It further increases the HIV reverse transcriptase activity (Liang, Wang et al. 2008). The infectivity of macrophages to HIV and activation of dopamine induced HIV reverse transcriptase was blocked by SCH23390 and SKF83566, DR1 antagonists (Liang, Wang et al. 2008). These data shows that dopamine plays crucial role in regulation of macrophage and monocyte mediated immunity. The dopamine-monocyte/macrophage axis needs more investigation.

DRs are present on microglia, the innate immune cells of the nervous system, and different microglial cell lines (Mastroeni, Grover et al. 2009). While the population of microglia situated in basal ganglia are more likely to encounter dopamine, not much is known about the immunological effect of dopamine on them. Recently it was observed that dopamine differentially affects resting or activated BV2 microglia (Fan, Chen et al. 2018). In resting cells dopamine increased dopamine increased cell adhesion and spreading, whereas in LPS treated microglia dopamine inhibited cell spreading. Phagocytosis was also inhibited in LPS treated cells. Dopamine further reduced the number of processes in resting BV2 and primary mice microglia and increased the length of vimentin filaments in resting BV2 cells (Fan, Chen et al. 2018). The authors also saw that dopamine does not induce the phosphorylation of ERK1/2 in resting BV2 microglia. However the phosphorylation of ERK1/2 was significantly reduced in LPS treated cells. On the other hand dopamine increased the phosphorylation of p38MAPK in resting BV2 cells and reduced the phosphorylation in LPS activated cells (Fan, Chen et al. 2018). Dopamine is also reported to inhibit the nitric oxide production in BV2 cells through the formation of dopamine quinone (Yoshioka, Sugino et al. 2016). Dopamine is reported to increase chemotaxis of human, mouse and rats microglial cells (Färber, Pannasch et al. 2005, Mastroeni, Grover et al. 2009). Further investigation of immunological effects of dopamine on microglia are needed to understand their role in disease and to utilize dopamine as therapeutic candidate.

2.7 INFLAMMASOMES

While looking into the mechanism of extracellular trap formation by DA induced microglia, I found that NLRP3 plays a crucial role. While I am still exploring the mechanisms further, a brief introduction to inflammasomes and their components are detailed here.

Inflammasomes are multimeric protein complexes comprising of a pattern recognition receptor (PRR), an adaptor protein and pro-caspase-1 (Figure 2.2). NLRs detect broad range of signals from different microbial motifs to multiple danger signals from cells. Certain NLRs like NLRP3 require a priming signal such as LPS (Figure 2.2, number 1). Priming leads to transcriptional activation of cells which increases the level of NLRs and other inflammasome proteins (Figure 2.2, number 2-5) (Guo, Callaway et al. 2015, Sharma and Jha 2016). Following priming second stimulation of cells with different pathogen-associated molecular patterns (PAMPs), danger-associated molecular patterns (DAMPs) and irritants results in the formation of inflammasomes and autocatalytic processing of pro-caspase-1 (Figure 2.2, number 6a-9b). Activation of caspase-1 leads to processing and secretion of IL-1β and IL-18 and leads to pyroptosis in the case of gasdermin-D, a protein involved in cell lysis, cleavage (Figure 2.2,

number 10-15) (Martinon, Burns et al. 2002, Broz and Dixit 2016, Dick, Sborgi et al. 2016, Jha, Brickey et al. 2017). ASC plays a significant role in regulation of inflammation. This section will give an overview of the inflammasome signaling in homeostasis and disease.

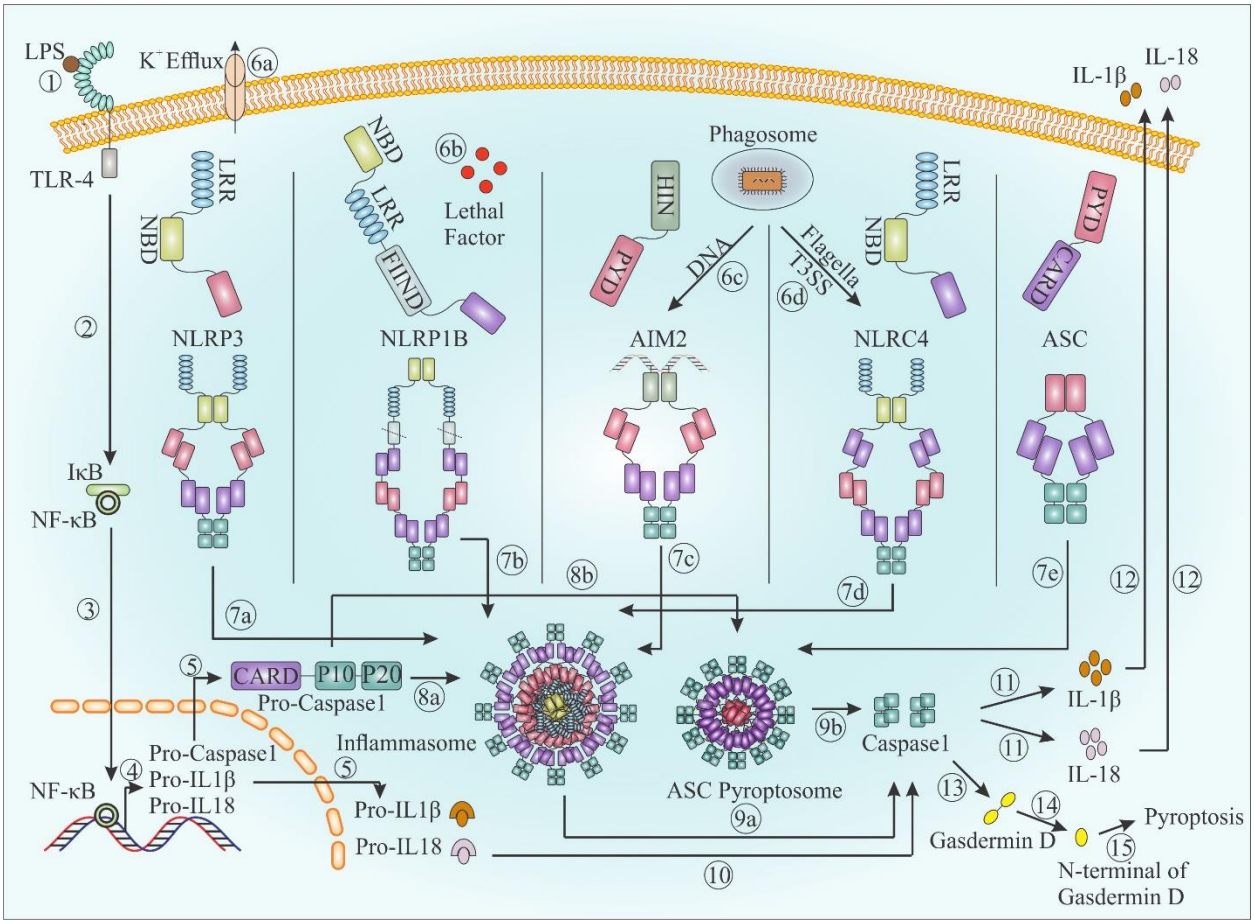

Figure 2.2 : *The inflammasome signaling pathways.* (1) LPS is detected by TLR4. (2, 3) This leads to activation of the NF-κB which further translocates to nucleus. (4, 5) Activation of NF-κB leads to the transcription of procaspase-1, pro-IL-1β, pro-IL-18 and other mRNAs needed for priming of the cell. Different NLRs detect different signals which lead to their activation. (6a) K^+ efflux is detected by NLRP3, (6b) lethal factor from *Bacillus anthracis* is detected by NLRP1B, (6c) DNA from various pathogens is detected by AIM2, and (6d) flagellin and type 3 secretion system proteins are detected by NLRC4. (7a, b, c, d) The detection of various stimuli leads to the formation of multiprotein complex called inflammasome comprising of NLR, ASC and (8a) procaspase-1. All of these stimuli along with the K^+ efflux may also lead to the formation of ASC pyroptosome, besides inflammasome, comprising (7e) ASC and (8b) procaspase-1. (9a, b) The formation of inflammasome and ASC pyroptosome results in the auto-proteolytic cleavage of procaspase-1 into mature caspase-1. (10, 11) Activated caspase-1 cleaves pro-IL-1β and pro-IL-18 into mature IL-1β and IL-18 which is further (12) secreted out of the cell. Caspase-1 cleaves gasdermin-D and the N-terminal of gasdermin-D causes pyroptosis (13, 14, 15).

2.7.1 EXTRACELLULAR SECRETION OF INFLAMMASOME COMPONENTS FOR INFLAMMATORY SIGNALING

In 2014 Franklin et al. reported the presence of functional ASC specks in extracellular space (Franklin, Bossaller et al. 2014). In the cell free supernatant of macrophages and THP1 monocytes, ASC-mCerulean specks were detected after treatment with ATP, nigericin, poly (dA:dT) or anthrax lethal toxin. Disuccinimidyl suberate cross linking of cell free supernatant

revealed the presence of monomers, dimers, trimers and oligomers of ASC. Caspase-1 activity was required for the presence of extracellular specks (Franklin, Bossaller et al. 2014). Interestingly pro-IL-1β was found to be attached with specks from *Casp1*$^{-/-}$ macrophages. The mechanism of this interaction and the functional significance would be interesting to explore. These extracellular ASC specks were perceived as danger signal by surrounding macrophages and were phagocytosed, leading to damaged lysosome and inflammation. Injecting these specks into the ear or peritoneum of mice resulted in recruitment of neutrophils that was partially dependent on *Nlrp3* and fully dependent on *IL-1β* (Franklin, Bossaller et al. 2014). ASC specks were also seen to accumulate in the extracellular space in the mice infected with *P. aeruginosa* and in humans in the BALF of COPD patients confirming its pathological significance. Antibodies were able to opsonize these specks. These opsonized specks were readily phagocytosed by macrophages and enhanced IL-1β production (Franklin, Bossaller et al. 2014).

At the same time Baroja-Mazo et al. independently reported the extracellular presence of NLRP3 inflammasome and oligomeric ASC specks, capable of processing pro-caspase-1 (Baroja-Mazo, Martin-Sanchez et al. 2014). After treating LPS primed mouse macrophages with ATP for 20 minutes, most of the ASC specks were extracellular. They also collected and analyzed serum from cryopyrin-associated periodic syndrome (CAPS) patients and observed significant abundance of oligomeric ASC with respect to healthy donors (Baroja-Mazo, Martin-Sanchez et al. 2014). CAPS is a hereditary disorder resulting from a gain of function mutation in *NLRP3* leading to constitutive activation of inflammasome (Yu and Leslie 2011). There are archived papers which have characterized the genes and proteins exclusive to NETs (Scieszka, Lin et al. 2020). The papers reports the presence of proteins like caspase-6 and BAX which participate in cell death pathways (Scieszka, Lin et al. 2020). It will be further interesting to explore whether inflammasome proteins or associated components are also present on ETs and if they are playing role in regulating ETs formation.

2.8 CONCLUDING REMARKS

ETs produced by different innate immune cells play central role in defense against pathogen as well as in inflammation leading to disease pathology. Intracellular proteins present on the traps serve as auto antigens. Their role is crucial in disease like SLE, rheumatoid arthritis, AAV, cancer, gout and other sterile inflammations. While ETs role is clear in homeostasis and disease, the precise mechanism of trap formation remains largely unknown. Along with the other cells immune system microglia was recently reported to from ETs. Microglia, the resident myeloid cells of CNS, are responsible for maintaining homeostasis in CNS. They are also central to inflammation and diseases of CNS. Their activity is regulated by many factors including hormones like dopamine. Dopamine is one of the catecholamine neurotransmitters which majorly regulates motor functions, pleasure, reward and addiction. The immunological effect of dopamine is being studied actively. Almost all the immune cells including microglia possess dopamine receptors. While various immune-regulatory effects of dopamine have been discovered, it is crucial to explore how dopamine is effecting microglia in diseases as well as in homeostasis. Present study investigated the role of dopamine in inducing ETs in microglia.