FORUM REVIEW ARTICLE

Role of Inflammasome Activation in the Pathophysiology of Vascular Diseases of the Neurovascular Unit

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Abstract

Significance: Inflammation is the standard double-edged defense mechanism that aims at protecting the human physiological homeostasis from devastating threats. Both acute and chronic inflammation have been implicated in the occurrence and progression of vascular diseases. Interference with components of the immune system to improve patient outcome after ischemic injury has been uniformly unsuccessful. There is a need for a deeper understanding of the innate immune response to injury in order to modulate, rather than to block inflammation and improve the outcome for vascular diseases. **Recent Advances:** Nucleotide-binding oligomerization domain-like receptors or NOD-like receptor proteins (NLRPs) can be activated by sterile and microbial inflammation. NLR family plays a major role in activating the inflammasome. **Critical Issues:** The aim of this work is to review recent findings that provided insights into key inflammatory mechanisms and define the place of the inflammasome, a multi-protein complex involved in instigating inflammation in neurovascular diseases, including retinopathy, neurodegenerative diseases, and stroke. **Future Directions:** The significant contribution of NLRP-inflammasome activation to vascular disease of the neurovascular unit in the brain and retina suggests that therapeutic strategies focused on specific targeting of inflammasome components could significantly improve the outcomes of these diseases. *Antioxid. Redox Signal.* 22, 1188–1206.

Inflammation in Vascular Diseases

INFLAMMATION IS THE STANDARD DOUBLE-EDGED defense mechanism that aims at protecting the human physiological homeostasis from devastating threats. Danger signals can be external from intruding pathogens in cases of infection, or from within the different affected tissues themselves in cases of trauma, chemical or biochemical metabolic stress, in an attempt for tissue preservation and/or repair [reviewed in Serhan *et al.* (125)]. Both acute and chronic forms of inflammation have been implicated in the occurrence and progression of vascular diseases. In humans, markers of chronic inflammation have been repeatedly shown to predict increased risk of cardiovascular events (122). Intervention with the anti-inflammatory and cholesterol-reducing statin therapy has shown great benefit in reducing major vascular events (100). The contribution of inflammatory response has been recognized also in retinal microvascular diseases, in-

cluding diabetic retinopathy (DR) (7, 72). In acute vascular events such as myocardial infarction (MI) and stroke, the immune response has been shown to be dynamic and exceedingly complex (20). Ischemic injury ignites a cascading and dramatically time-sensitive immune response that is, on one hand, destructive to surrounding tissue, leading to early recurrence and, on the other hand, an essential element of the repair response. Interference with components of the immune system to improve patient outcome after ischemic injury has been uniformly unsuccessful. Early attempts to block neutrophil infiltration after ischemic stroke in humans resulted in a worsened outcome, potentially due to increased susceptibility to infection (160). In experimental models, impairing the monocyte response can lead to heart failure after MI (103), hemorrhagic transformation after ischemic stroke (41), or potentiate tumor growth (19). There is a need for a deeper understanding of the innate immune response to injury in order to modulate, rather than block, inflammation and lead

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to successful treatment of the most common causes of death and disability in developed nations. The aim of this work is to review recent findings that provide insights into key inflammatory mechanisms and define the place of inflammasome, a multi-protein complex involved in instigating inflammation in neurovascular diseases.

Sterile Inflammation and Pattern Recognition Receptors

Sterile versus microbial inflammation

Inflammation is a cascade of events that includes an initiation step of a massive release of danger signals, chemotactic agents, and/or pro-inflammatory cytokines from tissues in response to an insult; for example: bacterial lipopolysaccharides (LPS), virus, virus-infected cells, or arachidonic acid end products of prostaglandins and leukotrienes. These chemokines are then responsible for alarming and recruiting the first responder cells of innate immunity-like neutrophils and macrophages. In turn, these cells try to clear up the source of inflammation by phagocytosis or endocytosis, while producing additional sources of chemokines and cytokines that lead to the recruitment of more inflammatory cells, the activation of lymphocytes, and the second line of adaptive immune response. Therefore, according to the type of initial insult, inflammation can be generally classified as two main categories: microbial inflammation and sterile inflammation. Inflammation that occurs in response to a chemical, biochemical, or metabolic insult, trauma, or injury in the absence of any microorganism is classically defined as sterile inflammation (14, 68, 93, 118).

Danger signals and pattern recognition receptors: different insults activate similar pathways

In microbial inflammation, pro-inflammatory insults that are directly sensed by the innate immune cells can be collectively called pathogen-associated molecular patterns (PAMPs) (54, 138). PAMPs encompass a major class of conserved structural moieties that are either a metabolic byproduct or cellular fragments of microorganisms. As for sterile inflammation, pro-inflammatory noninfectious materials that result from tissue damage or endogenous molecules released during cellular injury have been known as damage-associated molecular patterns (DAMPs) (91, 138). Sterile inflammation has been implicated as one of the largest roots of many metabolic, cardiovascular, and neurodegenerative diseases. Monosodium urate crystals, cholesterol crystals engulfed by circulating macrophages/foam cells, and β -amyloid plaques are among the most renowned DAMPs responsible for gout, atherosclerosis, and Alzheimer's disease (AD), respectively (14). As depicted in Figure 1, similar to microbial inflammation, sterile inflammation involves the same cellular machinery and receptors that are believed to be responsive to both PAMPs and DAMPs. These receptors are responsible for the subsequent induction of pro-inflammatory responses, which are collectively termed pattern recognition receptors (PRRs). Five major classes of PRRs have been identified to date. As illustrated in Figure 2, PRR can be generally classified based on their cellular location or major structural features into (a) transmembrane Toll-like receptors (TLRs), located at the cell surface or in endosomes; (b) transmembrane C-type lectin receptors (CLRs), characterized with a carbohydrate-binding domain; (c) cytosolic nu-

FIG. 1. Diagram showing that sterile inflammation and microbial infection activate similar inflammatory pathways: PRR. In microbial inflammation, pro-inflammatory insults that are directly sensed by the innate immune cells can be collectively called PAMPs. Sterile inflammation occurs in response to a chemical, biochemical, and metabolic insult; trauma or injury in absence of any microorganism is classically defined as sterile inflammation. In sterile inflammation, pro-inflammatory noninfectious materials that result from tissue damage or endogenous molecules released during cellular injury have been known as DAMPs. Both PAMPs and DAMPs activate the same cellular machinery and receptors that are collectively termed PRRs. Activation of these PRRs leads to induction of pro-inflammatory responses in immune and nonimmune cells. DAMPs, damage-associated molecular patterns; PAMPs, pathogen-associated molecular patterns; PRR, pattern recognition receptor. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

cleotide-binding oligomerization domain-like receptors or NOD-like receptor proteins (NLRs); (d) intracellular retinoic acid-inducible gene-1 (RIG-1)-like receptors (RLRs), which are primarily involved in antiviral responses; and (e) the non-NLRs, known also as absent in melanoma 2 (AIM-2)-like receptors, which are characterized by the presence of a pyrin domain and a DNA-binding HIN domain involved in the detection of intracellular microbial DNA. On their activation with their cognate ligands (PAMPs and/or DAMPs), these receptors mediate the subsequent pro-inflammatory response by activating the major pro-inflammatory and pro-apoptotic pathways, including the nuclear factor kappa-B (NF κ B) and mitogen-activated protein kinases (MAPKs), the major instigators for the pro-inflammatory defensive/detrimental response [for a full review, see Refs. (14, 134, 136, 138)].

FIG. 2. The five major classes of PRRs and their distribution at the cellular level. A simplified model that depicts the five major classes of PRRs that are identified for sensing PAMPs and DAMPs for the subsequent stimulation of proinflammatory responses. TLRs, located at the cell surface or in endosomes; CLRs, cell surface receptors that recognize carbohydrate structures; NLRs, present in the cytoplasm; RLRs, also located intracellularly; and the non-NLRs, present in the cytoplasm to recognize double-stranded DNA. TLR activation recruits multiple adaptor proteins, including TRAF6 activation to trigger NF κ B translocation to the nucleus as well as MAPK cascades, leading to the activation of the transcription factor AP-1. Both transcription factors drive expression of cytokine genes. CLR signaling leads to activation of SYK, and eventually activates NF κ B. In addition, CLR activation leads to phosphorylation of Ras/RAF kinase in an SYK-independent manner that can also modulate NF κ B activation to increase the transcription of cytokine genes. RLRs include RIG-I; MDA5 and LGP2 recognize double-stranded RNA viruses. They include an RNA helicase-DEAD box motifs and a CARD and signal *via* activation of NF κ B to increase the transcription of cytokines. The signaling of NLRs, including NLRP1, NLRP3, and NLRC4, requires the initial NF κ B activation to upregulate inflammasome expression in the NLRs as well as cytokine precursors such as pro-IL-1 β or pro-IL-18. NLR-inflammasome assembly leads to caspase-1 activation, which results in processing and secretion of cytokines IL-1 β and IL-18. Non-NLRs, known also as AIM-2, contain a HIN200 (hematopoietic interferon-inducible nuclear antigens) domain that senses and binds foreign cytoplasmic double-stranded DNA viruses. AIM-2 signals *via* activation of cleavage and release of active caspase-1 to process and mature IL-1 β and IL-18 in response to DNA-virus. AIM-2, absent in melanoma 2; CARD, caspase recruitment domain; CLRs, C-type lectin receptors; MAPK, mitogen-activated protein kinase; NF κ B, nuclear factor kappa-B; NLRs, NOD-like receptors; RIG-I, retinoic acid-inducible gene-1; RLRs, retinoic acid-inducible gene-1-like receptors; SYK, spleen tyrosine kinase; TLRs, Toll-like receptors; TRAF6, TNF receptor-associated factor 6. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

NLR family and inflammasome activation

Unlike the many other PRRs, NLRs have not been restricted to a specific ligand or a typical cognate molecular pattern (PAMP or DAMP) (134, 136). NLRs are generally composed of three separate domains: (a) the N-terminal domain, which contains a pyrin domain, a caspase recruitment domain (CARD), or a baculovirus inhibitory repeat domain and has been used as a structural sub-classification for the NLR family. (b) The central NBD or NACHT (nucleotide-binding domain or NAIP, CIITA, HET-E, and TP1) domain, which is responsible for dNTPase activity and oligomerization in the presence of nucleotides, primarily ATP. (c) A leucine-rich repeat (LRR) domain at the C terminus of NLR proteins (23). Among all the PRRs, the NLR family has been one the most extensively studied PRRs due to its major role in activating the inflammasome, the large multi-protein complex involved in instigating inflammation.

The inflammasome is a multi-molecular protein complex that is composed of: the receptor; the NLR protein, an adaptor protein; apoptosis-associated speck-like (ASC) and the effector enzyme; caspase-1. On activation by different PAMPs

(whole pathogens or bacterial pore-forming toxins) or DAMPs (extracellular ATP, glucose, monosodium urate, β -amyloid, silica, or asbestos), the NLR protein oligomerizes with the ASC adaptor protein, which then recruits procaspase-1, enabling its autocleavage and activation. Activated caspase-1 enzyme, in turn, cleaves upregulated premature proinflammatory cytokines, interleukin-1 (IL-1) and interleukin-18 (IL-18), and causes their release (44, 145). Several NLRs have the capability to activate the inflammasome *in vitro*, including NLRP1, NLRP2, NLRP3, NLRP6, NLRP12, NLRC4, and NOD-2. However, the physiological characterization of inflammasome activation has been established only for a handful of prominent NLRs, including NLRP1, NLRP3, NLRC4, and NAIP5 (23, 145).

NLRP3-Inflammasome Activation

NLRP3: the prominent NLR

As a sensor for metabolic danger, the NLRP3 inflammasome has been highlighted as one of the most established multi-protein complexes responsible for instigating metabolic,

cardiovascular, and neurodegenerative disease-associated inflammation (56, 74, 78, 124, 131, 132, 147). However, the exact molecular mechanisms involved in NLRP3-inflammasome activation are still far from clear. A wide variety of key pathological insults have been well established as direct activators of the NLRP3 inflammasome. These can range from host-derived biochemical factors or DAMPs such as ATP or hyaluronic acid derivatives (in case of cellular injury), hyperglycemia (in case of diabetes), or β -amyloid deposits (in case of AD) on one side, up to other physical and environmental factors such as UVB radiation, silica, or chemical irritants on the other side. Therefore, due to such diversity, a direct specific interaction between the receptor; NLRP3 and each inflammasome activator can be rationally excluded. Instead, other theories and supporting findings that can model the NLRP3-inflammasome activation as a response to disturbance in cellular homeostasis due to activating insults become more accredited (57, 151).

The two-step model of NLRP3-inflammasome activation

A commonly accepted model for NLRP3-inflammasome activation, especially in hematopoietic/myeloid cell types, is a two-step model that is required for NLRP3 inflammasome activation: step 1: priming and step 2: activation.

Step 1: priming. Except for ASC, IL-18, neither NLRP3 nor IL-1 β is constitutively expressed in sufficient amounts for assembling or activating the NLRP3-inflammasome. Moreover, basal levels of NLRP3 can be kept in an inactive but responsive ubiquitinated state and sequestered by ubiquitin ligase-associated protein SGT1 and heat-shock protein 90 (HSP90). Therefore, an initial step of priming that involves either increasing both NLRP3 and pro-IL-1 β protein levels or a post-transcriptional modification of existing NLRP3 levels is required before NLRP3 can assemble and oligomerize with other inflammasome components. NF κ B pathway has been found to be central for driving the gene expression and denovo protein synthesis of NLRP3. Several pro-inflammatory receptors, including interleukin 1 receptor 1 (IL-1R1), TLRs, NLRs, and the cytokine receptors tumor necrosis factor receptor 1 and 2 (TNFR1 and TNFR2), converge on activation the NF κ B transcription factor. MyD88 along with IL-1R-associated kinase family members (IRAK1 and 4) and TIR domain-containing adapter-inducing interferon- β (TRIF) have been implicated in both NF κ B-dependent transcriptional priming and transcription-independent priming pathways (57, 151). Transcription-independent priming pathways involve an interaction between NLRP3, SGT1, HSP90, and the deubiquitinase enzyme BRCC3. Deubiquitination of the LRR domain of NLRP3 by BRCC3 and dissociation of HSP90 and SGT1 from NLRP3 is important for relieving its auto-inhibition and increasing the availability of NLRP3 for self-oligomerization as the first step for subsequent inflammasome activation. Together, both priming pathways function as a regulatory or control check point to protect against aberrant immune response that might be initiated by the inflammasome activation.

Step 2: activation. Due to the diverse natures of different NLRP3-inflammasome activators, it is very hard to reach a common consensus that can reconcile the different aspects or

specific pathways involved. Nonetheless, there is a general agreement on a few distinct signaling events and related subcellular organelles as the major pathways directly involved in inflammasome activation. These include (a) extracellular and intracellular cationic fluxes, (b) oxidative stress, (c) mitochondria, (d) lysosomal rupture and autophagy, and (e) endoplasmic reticulum (ER) stress (135, 151).

- (a) Potassium (K⁺) efflux has been majorly attributed to the ATP-gated cation channel P2X7R and pore-forming toxins such as nigericin. Furthermore, ATP stimulation can also result in increased levels of intracellular calcium (Ca²⁺). ATP stimulation can activate the phospholipase C (PLC)/inositol triphosphate pathway, which results in rapid release of Ca²⁺ from the ER; the largest intracellular Ca²⁺ store. Increased intracellular Ca²⁺ levels subsequently results in increased extracellular Ca²⁺ influx through the store-operated Ca²⁺ entry (SOCE). On the other hand, elevated Ca²⁺ levels can also contribute to NLRP3-inflammasome activation through instigating ER stress and the release of the mitochondrial reactive oxygen species (mROS) or mitochondrial DNA (mtDNA) (101, 151).
- (b) Oxidative stress is a term generally used to describe changes in the cellular redox status, either due to increased ROS production or due to decreased antioxidant defense mechanisms. Several lines of evidence support increased ROS generation as an essential signaling component required for NLRP3-inflammasome activation. There are multiple subcellular sources for increased ROS production, including NADPH oxidases, mROS, xanthine oxidase, peroxisome oxidases, and uncoupling of cytochrome p450 and nitric oxide synthases. Of these, NADPH oxidases and mROS are the most heavily studied sources of ROS in NLRP3-inflammasome activation [reviewed in Refs. (44, 57, 86)].

ROS blockade *via* chemical scavengers of ROS, pharmacological and genetic inhibition of NADPH oxidase was shown to suppress NLRP3 activation in response to a wide range of stimuli (57, 86). NOX2 was found to be essential for ATP-mediated NLRP3-inflammasome activation (48, 99), but not for other agonists, including uric acid crystals and silica in macrophages (49). However, other studies have indicated that these effects are mainly dependent on the p22^{phox} common subunit, shared by most of the NOX isoforms, rather than the gp91^{phox}, which is specific only to NOX2 (57, 86). On the contrary, NOX2-mediated NLRP3-inflammasome activation was further evaluated in a different model of hyperhomocysteinemia, a metabolic disease known for increased risk of cardiovascular complications. In a series of elegant studies led by the Pin-Lan Li group, inhibition of gp91^{phox} using siRNA, gp91ds-tat peptide, diphenyleneiodonium, or apocynin abrogated NLRP3-inflammasome activation in cultured mice kidney podocytes in response to treatment of with L-homocysteine. In parallel, similar results were also observed *in vivo* where gp91^{phox} knockout (phox^{-/-}) mice and mice receiving a gp91ds-tat treatment exhibited markedly reduced inflammasome activation, and were protected against hyperhomocysteinemia-induced renal injury (2). Further, in the following study, they were able to dissect the relative contribution of different ROS species toward hyperhomocysteinemia-induced

NLRP3-inflammasome activation. Utilizing the same models, Dr. Li's group elucidated that dismutation of O_2^- by 4-hydroxy-2,2,6,6-tetramethylpiperidine 1-oxyl (Tempol) or decomposition of H_2O_2 by catalase prevented hyperhomocysteinemia-induced activation of NLRP3 inflammasome in cultured mouse podocytes and glomeruli of hyperhomocysteinemic mice along with its associated glomerular injury. However, scavenging of $ONOO^-$ or hydroxyl-free radical (OH^-) had no significant effect (1). Such discrepancy regarding the specificity of different NOX isoforms is a practical example on the complex nature and different signaling pathways involved. This can, in part, be explained by the differences in the nature of cell types studied or their function as professional immune cells *versus* other specialized cell types.

- (c) The mitochondria have been well regarded as a central player in NLRP3-inflammasome activation. This role can be generally explained by the direct role of the mitochondria as a physical platform for NLRP3-inflammasome assembly, or by several indirect mitochondrial-related factors necessary for inflammasome activation. These factors include mROS, mtDNA, and cardiolipin [reviewed in Wen *et al.* (151)]. Earlier studies have indicated that NLRP3-inflammasome activation involves the re-distribution of NLRP3 and ASC adaptor protein from the ER and cytosolic space into the peri-nuclear space, ER, and the mitochondria. Such a process involves a direct association between NLRP3 and mitochondrial antiviral signaling (MAVS) protein and increased generation of mROS, which is dependent on the state of voltage-dependent anion channel (VDAC) isoforms 1 and 3 in the outer mitochondrial membrane (133, 164). In parallel, increased mROS can also result in the dissociation of thioredoxin-interacting protein (TXNIP) from thioredoxin (one of the common antioxidant protein defense systems), and, hence, increased redistribution of TXNIP to the mitochondria and direct association with NLRP3 (83, 164). Another study has also suggested a model where at rest, the ASC adaptor protein is associated with the mitochondria and the nucleus; whereas NLRP3 is mainly localized within the ER and the cytosol. Hence, on activation, mitochondrial dysfunction brings the mitochondria to the peri-nuclear area *via* the microtubule system as a result of decreased nicotinamide adenine dinucleotide (NAD^+) levels, which, in turn, inactivates sirtuin 2 (96). Release of mtDNA from dysfunctional mitochondria in response to ATP and LPS and its oxidation was also reported to induce NLRP3-inflammasome activation (104, 127). Moreover, there was a direct association between NLRP3 and inner mitochondrial membrane-specific lipid; cardiolipin was also observed on NLRP3-inflammasome activation, and its inhibition abrogated inflammasome activation and NLRP3 localization to the mitochondria. However, it is still unclear whether this involves a translocation of cardiolipin or NLRP3 from the inner to the outer mitochondrial membrane or *vice versa* (53, 135).
- (d) Several particulate inflammasome activators such as uric acid crystals, alum, asbestos, can induce phagocytosis. Therefore, NLRP3-inflammasome activation can

occur by means of destabilization of their induced phagosomal or lysosomal compartments and release of their contents. The exact signaling details of how lysosomal rupture and release of its contents into the cytosol can trigger NLRP3-inflammasome activation are still incompletely understood. Some reports have indicated that inhibition of lysosomal protease cathepsin B that is released upon treatment with silica or alum prevents NLRP3-inflammasome activation. Nonetheless, others have also indicated no effect on inflammasome activation in cathepsin-B-deficient mice (30, 31, 43, 44, 49). Therefore, the question whether cathepsin B can act upstream from other major inflammasome activating events such as oxidative stress or whether these events are related to ion flux imbalance remains to be explained (44, 86). In another different, but a cellular recycling-related pathway, inhibition of autophagy has been also closely related to NLRP3-inflammasome activation. Blocking or interfering with different autophagy pathways has been established to increase cellular levels of ROS or dysfunctional ROS-producing mitochondria (in case of dysfunctional mitophagy) and increased NLRP3-inflammasome activation and IL-1 β secretion (43, 135, 150).

- (e) ER stress occurs as a result of increased accumulation of unfolded proteins in the ER compartment or disruption of the ER- Ca^{2+} homeostasis. ER stress can result in stimulation of the unfolded protein response (UPR), which can either result in a proper reparative/survival response or have a detrimental effect on the cell fate if it reaches extreme cellular stress levels or a terminal UPR. Initially, multiple ER stressors were found to induce NLRP3-inflammasome activation, independently from the canonical UPR initiators (PERK, IRE1a, and ATF6) proteins (94). However, two recent studies have suggested TXNIP as a key mediator of ER-stress-induced NLRP3-inflammasome activation (76, 109).

In light of what has been mentioned earlier, it is clear that despite the wide variation in NLRP3-inflammasome activation signaling pathways, a mutual cross-talk can still occur for reciprocal activation. Oxidative stress is among the most commonly activated pathways that can act as a common denominator for signal integration and potentiation of NLRP3-inflammasome activation. However, it is still unclear whether increased ROS production is, indeed, a direct cause and sufficient for NLRP3-inflammasome activation or it is leaning more towards being a facilitating factor that enhances the effect or acts downstream of other activating pathways. Moreover, further clarification and identification of the key signaling molecules that can connect between oxidative stress and sterile inflammation is of great need.

TXNIP: A Possible Link Between Oxidative Stress and Sterile Inflammation

The thioredoxin system

The thioredoxin system is a ubiquitous thiol-reducing system that consists of thioredoxin (Trx), nicotinamide adenine dinucleotide phosphate-oxidase (NADPH), and homodimeric seleno-protein thioredoxin reductase (95). Trx is a

multifunctional protein that acts as a protein disulfide reductase and participates in redox-dependent processes, including protein folding, regulation of apoptosis, and antioxidant protection from oxidative stress (85). Trx has two isoforms, cytosolic/nuclear (Trx-1) and mitochondrial (Trx-2) (107). The activity and expression of Trx is regulated by TXNIP, which tightly controls cellular redox state (112). TXNIP is identical to vitamin D₃ upregulated protein-1 (VDUP-1) and another synonym also is called thioredoxin-binding protein-2 (TBP-2). TXNIP belongs to the α -arrestin family; so, it may serve as an adaptor and a scaffold protein with several interacting domains to activate various signaling pathways [reviewed in Masutani *et al.* (89)].

Redox-dependent functions of TXNIP

Traditionally, TXNIP has been known for its redox-dependent signaling, which involves the suppression of the antioxidant defense mechanism and increased unopposed levels of cellular ROS by limiting the availability of free sulfhydryl (thiol) group of Trx (17, 58). Trx also exerts anti-apoptotic effects by binding and inhibiting the pro-apoptotic protein apoptosis signal-regulating kinase 1 (ASK-1). Thus, under stress conditions, Trx can dissociate from ASK-1 and gain kinase activity to activate c-jun-N-terminal kinase (JNK) and p38 MAPK signaling pathway, leading to apoptosis. TXNIP is a stress sensor and its expression can be induced to a various number of exogenous and endogenous stimuli, including inflammation, metabolic stress, changes in calcium levels, as well as changes in oxygen levels (15, 32, 35, 50, 114, 128). In addition to modulating cellular redox state, TXNIP plays a critical role in stress-induced cellular apoptosis, as it binds reduced Trx and inhibits its activity, resulting in activation of the pro-apoptotic protein ASK-1 pathways (8, 9).

Redox-independent functions of TXNIP

On the other hand, TXNIP has been also identified as a member of the alpha arrestin protein family, highlighting the other major roles of TXNIP as a scaffolding protein in redox-dependent and -independent ways (116). TXNIP shuttling and targeting of proteins into different subcellular compartments, including the plasma membrane, mitochondria, and the nucleus, provided a new paradigm in TXNIP signaling both inside and outside the neurovascular unit (NVU) [reviewed in Refs. (75, 129)]. In the retinal NVU, redox-dependent signaling cascades that include changes in activation of tyrosine receptor activity due to oxidative inhibition of the redox-sensitive phospho-tyrosine phosphatases (PTP) have been reported. In particular, silencing or genetic deletion of TXNIP expression impaired vascular endothelial growth factor receptor-2 (VEGFR2) signaling, with major implications in angiogenesis and ischemic retinopathy pathophysiology (3, 4). TXNIP can also mediate the translocation of Trx-1 to the plasma membrane under increased ROS levels, which was found to be also essential for VEGFR2 signaling in endothelial cells (154). In addition, TXNIP can shuttle from the nucleus to bind the mitochondrial Trx-2 and mediate the internalization of glucose transporter-1 (GLUT1) from the plasma membrane to inhibit glucose uptake in response to increased ROS and high glucose levels in cultured beta cells and hepatocytes, respectively (121, 155). An essential role of enhanced TXNIP in induction of inflam-

mation and proinflammatory cytokine expression has been demonstrated in models of DR and retinal neurotoxicity *in vivo* and in cells (9, 35, 97, 114, 115, 157).

Proinflammatory role of TXNIP

The contribution of TXNIP to inflammatory cytokine production was first demonstrated at the transcription level. Forced expression of TXNIP in isolated microvascular endothelial cells resulted in nuclear translocation and direct activation of the canonical NF κ B pathway (114). Knocking down TXNIP expression completely abolishes NF κ B binding to the cyclooxygenase-2 (Cox2) promoter, suggesting that TXNIP induces inflammatory gene expression by increasing transcription factor accessibility to gene promoters. The same study demonstrated evidence that the mechanism involves remodeling of histone H3 *via* activation of p38 MAPK. Later, studies demonstrated that TXNIP induces the expression of other proinflammatory cytokines and enzymes, including IL-1 β , ICAM-1, TNF- α , VEGF-A, and Cox2 (9, 114, 115). As illustrated in Figure 3(A, B), enhanced TXNIP expression has been shown to contribute to inflammation at two levels. At the transcription level, TXNIP causes activation of NF κ B, resulting in expression of proinflammatory cytokines and inflammasome components. In addition, TXNIP is also proposed as a direct activator of NLRP3 inflammasome, resulting in activation of caspase-1, which, in turn, causes maturation and cleavage of pro-IL-1 β or pro-IL-18 to IL-1 β and IL-18, respectively. In the next section, we will discuss the evidence on the contribution of TXNIP as a direct activator of NLRP3-inflammasome.

TXNIP and NLRP3-inflammasome activation: facts and controversy

The role of TXNIP as a redox-sensitive switch that links between increased cellular ROS levels and induction of the proinflammatory gene expression has been extended beyond the transcriptional factor level. Recent advances suggest the direct protein-protein interaction of TXNIP with the NLRP3 inflammasome, one of the executional machineries responsible for the maturation and release of mature pro-inflammatory cytokines IL-1 β and IL-18. Nevertheless, the emerging role of TXNIP as a direct activator of the NLRP3 inflammasome has been quiet controversial. In February 2010, Zhou *et al.* (163) was the first to identify TXNIP as a direct activator of the NLRP3 inflammasome in an ROS-dependent manner. In this study, different NLRP3-inflammasome activators, including monosodium urate crystals, ATP, and silica, were able to induce increases in cellular ROS levels in cultured macrophages, enabling the dissociation of TXNIP from Trx and its increased binding to the inflammasome receptor, NLRP3. TXNIP inhibition or deletion using TXNIP knock-out (TKO) mice and their isolated macrophages and pancreatic islet tissue had impaired immunogenic response in terms of neutrophil flux and IL-1 β production, in response to intraperitoneal injections of urate crystals *in vivo*, and increased ROS and glucose levels *in vitro*, respectively. However, these observations were challenged by another report in the same year in October 2010, which indicated that they could not replicate the same results utilizing isolated macrophages from TKO mice. These negative results came in line with a previous report in 2008 from mice lacking

FIG. 3. The major upstream activators of TXNIP expression and their proposed role as direct activators of NLRP3 inflammasome. (A) TXNIP expression can be upregulated in response to changes in glucose or Ca^{2+} levels due to activation of NMDA receptors, changes in oxygen level, as well as ischemia. More recently, palmitate has been shown to induce TXNIP expression potentially *via* activation of TLR and ER-stress. Enhanced TXNIP expression has been shown to contribute to inflammation at two levels. At the transcription level, TXNIP causes activation of NFkB *via* chromatin modification, resulting in increased expression of proinflammatory cytokines and inflammasome components. (B) TXNIP is also proposed as a direct activator of NLRP3 inflammasome, resulting in activation of caspase-1, which, in turn, causes maturation and cleavage of pro-IL-1 β or pro-IL-18 to IL-1 β and IL-18, respectively. IL-1 β can activate its receptor and sustain its own expression *via* auto-inflammation loop. ER-stress, endoplasmic reticulum-stress; NMDA, *N*-methyl-D-aspartate; TXNIP, thioredoxin-interacting protein. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

gp91^{phox} subunit of NADPH oxidase cytochrome *b* (one of the major sources for superoxide anion production in macrophages) (87). Another report in early 2011 has also claimed that TXNIP contribution to the increase of IL-1 β production in cultured adipocytes was mainly through the induction of the IL-1 β expression in response to increased glucose levels rather than the direct activation of the NLRP3 inflammasome. This was based on their observation that TXNIP knockdown using siRNA did not affect caspase-1 activity under regular or increased glucose levels (66).

Despite the initial controversy, accumulated literature support the role of TXNIP as one of the direct activators for the NLRP3 inflammasome as illustrated in Figure 3B. In cultured macrophages, Zhou *et al.* (164) reported that ROS-induced activation of the NLRP3 inflammasome was associated with increased localization of TXNIP into the mitochondria. Another report showed that increased ROS levels in the mitochondria resulted in oxidation of Trx, liberation of TXNIP and increased its interaction with NLRP3 that was associated with a conformational change in the NLRP3 protein pyrin domain, as predicted by molecular modeling (83). Increased S-nitrosylation of NLRP3 in macrophages of TKO mice compared with wild-type controls was also suggested to be responsible for the associated decrease in IL-1 β production, as a result of increased levels of nitric oxide and inducible nitric oxide synthase (iNOS), in a model of LPS-induced endotoxic shock (111). Furthermore, TXNIP-mediated activation of the NLRP3 inflammasome has been extensively supported in other nonimmune cell types, rather than macrophages only. In lung endothelial cells, NADPH-

derived ROS promoted the direct association between TXNIP and NLRP3 and, hence, NLRP3-inflammasome activation (156). In pancreatic beta cells, TXNIP has been proposed as an effective link that couples ER stress and inflammation *via* NLRP3-inflammasome activation. Terminal levels of ER-stress known as the UPR can induce rapid increases in TXNIP expression as a result of the increased activity of the dsRNA-activated protein kinase-like ER kinase (PERK) and inositol-requiring 1 (IRE1) pathways, which, in turn, can increase TXNIP mRNA stability by reducing levels of a TXNIP destabilizing microRNA, miR-17 (76, 109). Finally, pharmacological inhibition of TXNIP utilizing pleiotropic agents such as quercetin (a natural antioxidant of flavonoid origin), allopurinol (a uric acid synthesis inhibitor), hemin (an inducer for the antioxidant protein heme oxygenase-1), or rosuvastatin (a lipid-lowering drug from the 3-hydroxy-3-methyl-glutaryl-CoA reductase inhibitor class) has been reported to inhibit the TXNIP-NLRP3-IL-1 β axis and ameliorate the pro-inflammatory insult in different disease models of high fructose diet-induced hypothalamic insulin resistance (161), nonalcoholic fatty liver disease (149), acute liver failure (62), and diabetic cardiomyopathy (84), respectively.

Sterile Inflammation in Vascular Diseases of the NVU

An overview of the NVU

The NVU is a term that is commonly used to describe the elaborate structure of the multicellular interface of endothelial cells, pericytes, glial cells (astrocytes and Müller cells),

and neurons, which serves as the functional building unit of the brain and the retina. The NVU is formed as a result of the immaculate arrangement of small blood capillaries or microvascular tissue composed of endothelial cells immediately ensheathed by pericytes, which are, in turn, surrounded with the astrocytes alongside the end feet processes of Müller glial cells (specialized glia that exist only in the retina) and neurons. This spatial and proximal arrangement of these different but highly related cell types facilitates their direct and mutual interaction, necessary for maintaining central nervous system (CNS) homeostasis in terms of nutritional support, house-keeping, and formation of the blood brain or retinal barrier that protects the CNS against edema or any circulating metabolic or biochemical hazards in the blood stream.

Sterile inflammation: a driving force in neurovascular disorders

Recent findings have provided insights into new key inflammatory mechanisms that may contribute to neuronal death during both acute and chronic neurological diseases [reviewed in Refs. (22, 36, 78)]. Inflammatory processes, including activation of glial cells and subsequent production of pro-inflammatory stimuli, can induce neurovascular barrier dysfunction and amplify neurodegeneration in both brain and retina. Recent evidence has indicated the involvement of the NLRP1- and NLRP3 inflammasome in neurovascular diseases, especially in those related to the NVU of the CNS, including neurodegenerative diseases, stroke, and retinopathy. Therefore, the scope of this review will be focused on the prominent role of the NLRP-inflammasome activation in brain and retina neurovascular disorders. New insights on the specific role of NLRP-inflammasome will be discussed in context with the contribution of each cell type component of the NVU and how this contributes to the development of brain and retina neurovascular pathologies.

Inflammasome Activation in Brain Neurovascular Disorders

NLRP-inflammasome activation in ischemic stroke

Stroke is a complex systemic disease causing severe long-term disability and death worldwide. The postischemic neuroinflammatory response is characterized by microglial and astro-glial activation and increased expression of inflammatory mediators (22, 92, 106). Recent findings have provided insights into a newly discovered inflammatory mechanism that contributes to neuronal and glial cell death in ischemic stroke mediated by inflammasomes (36). Abulafia *et al.* recently found that the inflammasome component proteins (NLRP1, ASC, caspase-1, and IL-1 β) have a role in the inflammatory response after thromboembolic stroke in mice (5). This study also reported the potential of the inflammasome as an anti-inflammatory target by inhibiting inflammasome activation, resulting in reduced cytokine levels in mice treated after ischemia with a neutralizing antibody against NLRP1. Immunofluorescence and cellular localization analysis also revealed inflammasome proteins in neurons, astrocytes, and microglia/macrophages after ischemic stroke in mice (5). Another study also reported that the levels of NLRP1 and NLRP3-inflammasome proteins, and IL-1 β and IL-18 increase in cellular and animal models as well as in a stroke patient's brain tissue (37). Early expression of IL-1 β

in areas of focal neuronal injury suggested that it is the major form of IL-1 contributing to inflammation early after cerebral ischemia (82). Similarly, administration of an IL-1 β -neutralizing antibody or IL-1 receptor antagonist reduced subarachnoid hemorrhagic injury (55). Evidence using IL-1 α/β double KO mice showed that IL-1 production is necessary for subsequent increases in other inflammatory mediators IL-6 and chemokine (C-X-C motif) ligand-1 (CXCL1) in the ischemic hemispheres (120). In caspase-1 KO mice, there was a reduction in mature IL-1 β and IL-18 levels in association with a smaller infarct size (88). Moreover, intravenous immunoglobulin treatment protected neurons in experimental stroke models by a mechanism involving suppression of NLRP1 and NLRP3-inflammasome activity (37).

These findings identified that NLRP1 and NLRP3 inflammasomes play a major role in neuronal cell death and behavioral deficits in stroke, and further suggested a potential clinical benefit of therapeutic interventions that target inflammasome assembly and activity. A recent study showed that milk fat globule-EGF 8 (MFGE8), an endogenous inhibitor of inflammasome-induced IL-1 β production, inhibited ATP-dependent IL-1 β production in macrophages. Further, MFGE8 deficiency was associated with enhanced IL-1 β production and larger infarct size after cerebral ischemia in mice, which was blunted after treatment with an IL-1 receptor antagonist. These findings suggested that MFGE8 regulates innate immunity through inhibition of inflammasome-induced IL-1 β production (26). Recently, Yang *et al.* (158) demonstrated that NLRP3 inhibition ameliorated ischemic injury in cellular and animal models of stroke. The authors showed that NADPH oxidase-mediated NLRP3 signaling contributes to cerebral ischemia injury *via* exacerbation of inflammation and neurovascular damage. In support of the link between TXNIP and NLRP3 inflammasome, our recent work demonstrated that ischemia-induced TXNIP expression can trigger NLRP3 expression, activation of caspase-1, and release of IL-1 β in embolic stroke model (52). TXNIP expression was enhanced within brain vasculature as indicated by its colocalization with CD31, an endothelial cell marker. The activation of NLRP3 inflammasome and IL-1 β levels were significantly mitigated by genetic deletion of TXNIP (TKO mice) or using a pharmacological inhibitor of TXNIP (52). These findings support the multiple levels by which TXNIP can contribute to acute ischemic stroke injury through redox imbalance and inflammasome activation, and suggest that inhibition of TXNIP may provide a new target for therapeutic interventions for stroke.

NLRP-inflammasome activation in AD

AD is a chronic, progressive, and irreversible neurodegenerative disease that is characterized by the deterioration of cognitive function, the formation of β -amyloid peptide plaques, neurofibrillary tangles, and degeneration of cholinergic neurons [reviewed in Refs. (29, 137)]. Although the pathophysiologic mechanism is not fully understood, several recent studies have reinforced the observation that NLRP inflammasome plays a critical role in the pathogenesis of AD (78). IL-1 β is a key player in the innate immune response in AD, and increased expression of IL-1 β was observed in both the brain and the plasma of AD patients (108). Experimental data demonstrated that blocking IL-1 β signaling rescues

cognitive impairment in a mouse model of AD (21). IL-1 β release from microglia was also shown to increase activity of the enzymes responsible for processing of the amyloid precursor protein, ultimately resulting in further amyloid deposition (119). While earlier studies demonstrated high expression of IL-1 β in microglia surrounding β -amyloid deposition in experimental and clinical studies (11, 81), the study by Halle *et al.* provided the first evidence that β -amyloid activates the formation of NLRP3 inflammasome in microglia *in vivo* and *in vitro* (45). In addition, NLRP3 activation by β -amyloid was linked to phagocytosis, as the inhibition of phagocytosis led to a decrease in NLRP3-mediated release of IL-1 β . In support, a recent study in a double transgenic mouse model of AD confirmed the contribution of the NLRP3 inflammasome in the disease pathology, while inhibition of NLRP3 or caspase-1 in mice reduced β -amyloid deposition and improved memory function (47). Likewise, inhibiting the NLRP3 inflammasome reduced the neuritic plaque formation in a transgenic mouse model of AD (126). Another recent study showed that β -amyloid treatment of primary rat glial cultures increases cathepsin activation in the cytosol, formation of the NLRP3 inflammasome, caspase1 activation, and IL-1 β release (102). Altogether, these studies provided insights into the possible roles of NLRP3 inflammasome in AD pathogenesis. Targeting molecular components of the inflammasome signaling pathway appears to be an attractive strategy for neuroprotection and AD therapeutic intervention.

NLRP-inflammasome activation in traumatic brain injury

Although the precise mechanisms underlying secondary brain injury after traumatic brain injury (TBI) are complex and poorly understood, a number of studies have suggested that inflammatory responses are likely to be prominent and an early feature in the pathogenesis of TBI (46, 162). The insult triggers an invasion of macrophages and neutrophils into the impact area, producing much of the inflammation and swelling associated with brain damage. Increased production of IL-1 β is well documented, providing clear evidence for a pivotal role of this cytokine in triggering TBI-induced inflammatory process (18, 51, 63). De Rivero Vaccari *et al.*, found that TBI promotes assembly of the NLRP1 inflammasome complex through processing of IL-1 β , activation of caspase-1, and X-linked inhibitor of apoptosis protein cleavage (24). These detrimental effects of TBI were attenuated by acute administration of anti-ASC neutralizing antibodies after traumatic injury in rats. The same group further reported that various components of inflammasome (caspase-1, caspase-11, and the purinergic receptor P2X7) were increased in the cortex at 24 h after TBI (141). In support, *in vitro* results showed significant secretion of caspase-1 into the culture medium and caspase-3 activation after stretch injury in cortical neurons. These findings suggested that the NLRP1-inflammasome is an important contributor to inflammation after TBI. Moreover, a recent clinical study identified increased levels of inflammasome proteins ASC, caspase1, and NALP1 in the cerebral spinal fluid in patients with severe or moderate cranial trauma (6). This study also suggested that NALP1-inflammasome proteins are potential clinical biomarkers to assess TBI outcome, and the secondary injury mechanisms impeding recovery. Most recently, a study showed that TBI induces the assembly of NLRP3-

FIG. 4. Schematic representation showing the contribution of NLRP1- and NLRP3 inflammasome in diseases of the NVU of the brain, including stroke, AD, or TBI. The diagram shows whether findings were obtained from total brain lysate or specific cell types of NVU of the brain. AD, Alzheimer's disease; NVU, neurovascular unit; TBI, traumatic brain injury. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

inflammasome complex, increases expression of ASC, activation of caspase-1, and processing of IL-1 β and IL-18 (77). Taken together, these studies suggest that the NLRP inflammasome constitutes a key component of the innate CNS inflammatory response after TBI and may be a novel therapeutic target for reducing the damaging effects of post-traumatic brain inflammation. Figure 4 provides a summary of the findings that support inflammasome activation in cell and animal models of stroke, AD, and TBI.

Inflammasome Activation in Retina Neurovascular Diseases

Sterile inflammation has been increasingly recognized as a major player in initiating or sustaining retinal neurovascular dysfunction in metabolic disorders such as diabetes or mechanical strain ones such as glaucoma or traumatic injury (7, 72). Retinal neuronal death is a hallmark of multiple retinal diseases, including traumatic optic neuropathy, DR, age-related macular degeneration (AMD), and glaucoma (123, 152). In response to initial neuronal death, macroglial cells (astrocytes and Müller cells) and microglial cells become activated and release cytokines and inflammatory mediators that can adversely affect other retina cell types, including neurons and vasculature. In this section, we will review the contribution of NLRP3-inflammasome activation in various neurovascular diseases and related cell types of the retina NVU. Figure 5 depicts the various cell types of NVU of the retina.

Caspase-1/IL-1 β activations: a landmark of sterile inflammation in DR

Caspase-1 activation and increased IL-1 β levels are well documented in retinal chronic inflammatory conditions such

FIG. 5. Schematic representation illustrating the various cell types of the NVU of the retina, including endothelial cells, pericytes, glial cells (astrocytes and Müller cells), and neurons. The diagram also illustrates published literature discussing the contribution of NLRP3-inflammasome activation to various retinal diseases in each specific cell type of the retina NVU. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

as DR with a crucial proinflammatory role in mediating retinal endothelial cell dysfunction and microvascular degeneration. Clinically, increased IL-1 β levels have been early reported in both the aqueous and vitreous humors and plasma samples of patients with DR *versus* control subjects, and were positively correlated with severity or the progression from mild or moderate nonproliferative diabetic retinopathy (NPDR) into proliferative diabetic retinopathy (PDR) (25, 28, 67). Experimentally, caspase-1 activation in the retina was reported as early as 2 months after onset of streptozotocin (STZ)-induced diabetes, which was persistently higher until 8 months of diabetes and significantly correlated with the severity of diabetes as indicated by percentage of glycated hemoglobin (98). Moreover, intravitreal administration of IL-1 β resulted in massive increases in the number of degenerated retinal acellular capillaries and apoptotic capillary cells labeled with terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) in isolated retinal microvasculature in nondiabetic Wistar rats (69) and pharmacological inhibition of caspase-1 using minocycline (an anti-microbial drug) or deletion of IL-1 receptor suppressed IL-1 β -dependent increases in degenerated acellular capillaries formation in the retinas of diabetic mice (148).

These studies have implicated both endothelial cells and Müller cells as the major possible contributors to retinal caspase-1/IL-1 β activation in experimental models of hyperglycemia. Experimental evidence showed increased caspase-1 activation and IL-1 β production in cultured bovine retinal endothelial cells and retinal Müller cell line rMC-1 (a glia-like cell type) in response to high glucose levels (70, 98, 148, 159). Another study suggested the retinal endothelial tissue to be the initial source of increased IL-1 β production in response to hyperglycemia, after which increasing levels of IL-1 β are sustained *via* its own auto stimulation in endothelial tissue and macroglia (Müller cells and astrocytes). This

scenario was based on the difference in the readily induced caspase-1/IL-1 β activation response to high glucose levels in cultured bovine retinal endothelial cells that was absent in cultured rat Müller cells, astrocytes, and microglia (80). In the same study, the early increase of IL-1 β levels in isolated rat retinal vascular tissue *in vivo* was maintained and coincided with increased markers of glial activation at later time points of hyperglycemia (80). Earlier evidence indicated that the upregulation of IL-1 β production in retinal microglial cells directly caused panretinal apoptosis in a rat model of STZ-induced diabetes (71).

Caspase-1/IL-1 β activation in ischemic retinopathy

In a model of oxygen-induced retinopathy (OIR), upregulation of IL-1 β in retinal microglia cells was linked to increased apoptosis of cultured microvascular endothelial cells under hypoxic conditions. Moreover, it also correlated with the vaso-oblivation/vaso-regression effects of OIR in an indirect manner *via* induction of Semaphorin-3A from retinal ganglion cells (RGC) *in vivo* (117). Nevertheless, the upstream molecular signaling involved in the activation of the NLRP3 inflammasome in endothelial cells, macroglia or Müller cells, and/or other retinal cell types in models of DR or other retinal pathologies remained unexplored in these studies.

NLRP3-inflammasome activation in DR

TXNIP-mediated NLRP3-inflammasome activation in Müller cells, in response to hyperglycemia, was further discussed in models of STZ-induced diabetes *in vivo* (27) and in cultured retinal Müller cell line rMC-1 (143).

In the first study, hyperglycemia in STZ-induced diabetic rats resulted in upregulation of retinal TXNIP and IL-1 β expression along with other proinflammatory gene expression, including iNOS and the PRRs TLR4 and P2X7R at 4 weeks of diabetes. This was associated with increased expression of glial fibrillary acidic protein (GFAP), a marker of glial cell activation. Knockdown of TXNIP by an intravitreal injection of promoter targeted siRNA was able to significantly reduce the IL-1 β and GFAP upregulation. *In vitro*, the authors tried to dissect the temporal regulation of hyperglycemia-induced pro-oxidative and pro-inflammatory response in cultured rMC-1 cells. They observed that despite the sustained upregulation of TXNIP expression in response to sustained high levels of glucose from 4 h till 5 days in culture, which is consistent with the role of TXNIP as a glucose-responsive protein, the associated activation of the NLRP3 inflammasome in terms of pro-IL-1 β , NLRP3 inflammasome, and procaspase-1 levels oscillates at 4 h and day 3 of high glucose exposure. The first early response at 4 h occurs in the absence of ROS release, while the second late response at day 3 occurs under ROS/oxidative stress. These events correlated with an early ER-stress response to the increased glucose metabolism and ATP generation, which is then followed by a later response of hypoxic-like conditions, restriction of ATP, and increased ROS production and induction of autophagic-apoptosis pathway and inflammation. These observations suggested a relationship between high glucose response, oxidative and ER stress in which TXNIP could be an effective link with NLRP3-inflammasome activation (27).

The second study further dissected the role of purinergic signaling (purines are well-known DAMP activators for

the PRR and NLRP3) in NLRP3-inflammasome activation in cultured rMC-1 cells. The study showed that the high glucose-mediated response of increased caspase-1 activation in rMC-1 cells is, in part, due to the autocrine stimulation of ATP-sensing P2 receptors and adenosine-sensing P1 receptors. This was supported by the ability of apyrase, which metabolizes extracellular ATP to AMP, or adenosine deaminase (ADA), which metabolizes extracellular adenosine to inosine, to attenuate the response. They further elaborated that the purinergic signaling involved is mainly due to the P1/P2 receptor-mediated cAMP response, rather than due to P2X7 ATP-gated ion channel receptors. Despite the increased P2X7 mRNA expression in response to high glucose levels (25 mM), neither P2X7 protein nor function was detected in rMC-1 cells. In addition, attenuation of caspase-1 activity under high glucose conditions was achieved by either suramin (a nonselective P2 antagonist) or A2 adenosine receptor antagonists, but not by antagonism of P2X7 ATP-gated ion channel receptors. Lastly, the increase in caspase-1 activity was stimulated by high glucose levels or exogenous addition of ATP, 5'-N-ethylcarboxamido-adenosine, a non-selective P1 receptor agonist, forskolin, an adenylyl cyclase activator that increases intracellular levels of cAMP, or dipyrindamole, which suppresses adenosine reuptake under control glucose levels (5 mM). The increased caspase-1 activity correlated with increased gene expression of caspase-1 and TXNIP. The authors proposed an intricate model where Müller cells exposed to high glucose upregulate NLRP3 *via* NF κ B transcriptional signaling, TXNIP *via* MondoA/MLX transcriptional signaling, and cAMP/PKA-driven increase in the Ets-1 transcription factor, which positively modulates caspase-1 gene expression (143).

NLRP3-inflammasome activation in retina neurotoxicity models

In another model of retinal neurotoxicity, our findings also supported a contributing role for TXNIP-mediated NLRP3-inflammasome activation in retinal Müller cells in facilitating neuroglial activation and death of RGCs (35). Intravitreal injections of *N*-methyl-D-aspartate (NMDA) as a model of neuronal excitotoxicity resulted in a massive increase in RGC death at 1 day after injections, associated with increased oxidative stress, blood retinal barrier breakdown, Müller and glial cell activation, and expression of pro-inflammatory mediators in wild-type (WT) mice, but not in TKO mice. Immunolocalization studies highlighted Müller cells as a major source of the proinflammatory response, evidenced by increased GFAP immunoreactivity in Müller cell filaments, which co-localized with those of IL-1 β and TNF- α in WT mice *versus* TKO mice in response to NMDA injections. Isolated primary Müller cell cultures from both WT and TKO mice confirmed that TXNIP deletion abrogated the NMDA-induced upregulation of NLRP3, cleaved caspase-1, and increased production of IL-1 β and TNF- α in culture media (35). Such initial insults were necessary for maintaining secondary events, including increased microvascular degeneration indicated by the number of acellular capillaries formation, a hallmark of retinal ischemia. These effects were associated with increased death and dysfunctional activity of other neuronal cell types; bipolar and photoreceptor cells which occurred at 2–3 weeks after initial NMDA injections, and

further helped to sustain and aggravate the primary insult of increased RGC death (35). In line with these findings, NLRP3-inflammasome activation was also noted to be directly upregulated in astrocytes (140) and neurons, including RGC (33), which are also critical components of the NVU in other experimental models of glaucoma and transient ischemia reperfusion injury.

NLRP3-inflammasome activation in pre-DR

Our group was the first to show the activation of the retinal NLRP3 inflammasome in rats, utilizing high-fat-diet (HFD)-induced obesity as a model of prediabetes (97). In response to HFD, increased TXNIP expression was co-localized within retinal vasculature and macroglial cells (astrocytes and Müller cell endfeet), surrounding retinal blood vessels, and increased formation of retinal acellular capillaries formation (97). TXNIP-mediated NLRP3-inflammasome activation in retinal endothelial cells was confirmed *in vitro* using cultured human retinal endothelial cells (HRECs) in response to incubation with saturated fatty acid “palmitate” coupled to bovine serum albumin (Pal-BSA). Pal-BSA triggered increased TXNIP expression and an interaction with NLRP3, resulting in activation of caspase-1 and IL-1 β in HRECs. Silencing TXNIP expression abolished Pal-BSA-mediated IL-1 β production in HRECs and cell death, evident by decreases in cleaved caspase-3 expression and the ratio between live and dead cells (97). In the same study, there was an observation of an inverse relationship between accumulation of intracellular cleavage/maturation of IL-1 β and its release. Pal-BSA alone was able to induce both intracellular maturation and release of IL-1 β in HRECs, which was mitigated by silencing TXNIP expression. In contrast, stimulating the cells with exogenous peroxynitrite as a model of oxidative stress resulted in a higher surge of IL-1 β release that was independent of TXNIP inhibition, although it was not able to induce significant changes in its intracellular cleavage/maturation, suggesting accelerated activation and trafficking of IL-1 β . These findings lend further support to previous evidence that the process of caspase-1 activation/processing of pro-IL-1 β by caspase-1 and the release of mature IL-1 β from human monocytes are distinct and separable events (38). TXNIP has been shown to shuffle between different cellular compartments, including the nucleus, mitochondria (75), and plasma membrane (110). This intriguing observation might be attributed to the nature of TXNIP as a member of the alpha arrestin scaffolding proteins, which are believed to play an important role in intracellular cargo trafficking and/or internalization of different proteins (110, 155). Further studies are warranted to fully unfold the role of subcellular localization of TXNIP in response to different insults and how this can cause enhancement or inhibition of mature IL-1 β release and the auto-inflammation process.

High-fat, high-carbohydrate meals and elevated free fatty acid (FFA) plasma levels can directly cause systemic inflammation, oxidative stress, and endothelial dysfunction (39, 40, 142). FFA plasma levels are relatively higher in obesity and are one of the major factors for inducing obesity and metabolic syndrome-associated inflammation and insulin resistance (12, 13, 34, 59). Previous reports have established palmitate, which is one of the most abundant circulating saturated fatty acids in plasma (65), as a direct activator of the

TABLE 1. ILLUSTRATES THE TYPE OF INFLAMMASOME ACTIVATION, CELL TYPE, AND MOLECULAR PATHWAYS ASSOCIATED WITH THE RETINA AND BRAIN DISEASES

<i>Disease model</i>	<i>Cell type studied</i>	<i>Type of inflammasome studied</i>	<i>Molecular pathways involved</i>	<i>References</i>
Diabetic retinopathy	Total retinal tissue Müller cells Total retinal lysates Retinal microvascular tissue Endothelial cells	NLRP3 inflammasome NLRP3 inflammasome	Hyperglycemia/glucose metabolism Mitochondrial ATP generation oxidative stress/ER stress TXNIP upregulation Hyperglycemia/glucose metabolism Purinergic P1/P2 receptor cAMP response TXNIP and caspase-1 upregulation	(27) (143)
Prediabetic retinopathy	Total retinal tissue Retinal microvascular tissue Endothelial cells	NLRP3 inflammasome	High-fat-diet-induced obesity Saturated fatty acid “palmitate” Oxidative stress TXNIP-NLRP3 direct interaction	(97)
Retinal neurotoxicity	Total retinal tissue Retinal microvascular tissue Müller cells	NLRP3 inflammasome	NMDA-excitotoxic insult results in TXNIP-NLRP3 direct interaction	(35)
AMD	Total retinal lysates RPE cells	NLRP3 inflammasome	β -Amyloid (1–40 and 1–42), A2E, Alu RNA transcripts due to DICER1 deficiency or IL-1 α treatment, can directly induce the NLRP3-inflammasome activation	(10, 39, 61, 79, 144)
Stroke	Total brain lysate Neurons Astrocytes Microglia/macrophages Endothelial cell	NLRP1, NLRP3	Cerebral ischemia can directly induce the NLRP1-inflammasome activation; Oxygen–glucose deprivation/ischemia-reperfusion induce the NLRP1 and NLRP3-inflammasome activation; oxidative stress/TXNIP-NLRP3 direct activation; ROS/oxidative stress-mediated NLRP3 direct activation	(5, 37, 52, 158)
AD	Total brain lysate Microglia Neurons Astrocytes	NLRP3	β -Amyloid (1–42) induces IL-1-mediated NLRP3 activation; β -amyloid (1–42) induces cathepsin-dependent NLRP3 activation; NF κ B and NLRP3 activation	(45, 47, 102, 126)
TBI	Total brain lysate Microglia Neurons Astrocytes	NLRP1, NLRP3	Can directly induce the NLRP1-inflammasome activation; Stretch injury activates NLRP1-inflammasome; direct activation of NLRP3 inflammasome	(6, 24, 77, 141)

AD, Alzheimer's disease; AMD, age-related macular degeneration; ATP, adenosine triphosphate; cAMP, cyclic adenosine monophosphate; ER-stress, endoplasmic reticulum-stress; NF κ B, nuclear factor kappa-B; NMDA, *N*-methyl-D-aspartate; ROS, reactive oxygen species; RPE, retinal pigmented epithelium; TBI, traumatic brain injury; TXNIP, thioredoxin-interacting protein.

NLRP3 inflammasome (90, 113, 150) and an inducer of the pro-inflammatory response in human coronary endothelial cells *versus* other unsaturated fatty acids (73, 130). Together, these observations can provide novel insights into the explanation of the clinical manifestations of increased risk of developing retinopathy (16, 64, 146). It has now become evident that these retinal vascular changes might be markers of the early preclinical stages of these metabolic disorders and might predict the onset of clinical disease. Various components of the metabolic syndrome were associated with retinal microvascular signs: A larger waist circumference was associated with wider venular diameter and retinopathy lesions; a higher blood pressure level was associated with focal arteriolar narrowing, arteriovenous nicking, enhanced arteriolar wall reflex, and narrower arteriolar diameter; and a

higher triglyceride level was associated with enhanced arteriolar wall reflex (60, 153). Population studies have shown that subjects with various components of the metabolic syndrome, including obesity, and dyslipidemia were more likely to have retinal microvascular abnormalities such as focal and generalized retinal arteriolar narrowing and venular dilatation and changes in arteriolar wall reflex observed in obesity independently (42, 105), or in patients with type 1 or type 2 diabetes (105).

NLRP3-inflammasome activation in AMD

Despite the fact that retinal pigmented epithelium (RPE) cells are not considered a part of either the sensory retina or the typical NVU, it is worth mentioning that NLRP3-

inflammasome activation is also attracting increasing attention as a crucial instigator of the proinflammatory response in other retinal diseases such as AMD. Sections from human ocular tissue from patients with geographic atrophy or neovascular AMD were positive for NLRP3 immunostaining when compared with tissues from age-matched controls (144). Of note, RPE cells were found to express all inflammasome components, including ASC, NLRP3, and caspase-1. As an immediate sensor of many DAMPs, several metabolic components of the drusen lesions associated with AMD (extracellular accumulations of a lipoprotein-like material that build up between Bruch's membrane and the RPE) have been recently shown to activate the NLRP3-inflammasome proinflammatory response. Among these, β -amyloid (1–40 and 1–42), N-retinylidene-N-retinylethanolamine, A2E (an essential component for lipofuscin/drusen), Alu RNA transcripts (due to deficiency of DICER1, a ribonuclease type III enzyme), or IL-1 α treatment were able to directly activate the NLRP3 inflammasome. Further, their inhibition was sufficient to prevent RPE degeneration both *in vivo* and in cultured RPE cells *in vitro* (10, 61, 79, 139, 144). Activation of the NLRP3 inflammasome occurs traditionally after “priming,” which involves the upregulation of the inflammasome gene expression *via* various transcriptionally active signaling receptors followed by an “activation” step, leading to activation of caspase-1 and secretion of IL-18 and IL-1 β . While published literature support the significance of IL-1 β , little is known about the role of IL-18. The recent study by Ambati group showed that the activation of NLRP3 inflammasome and release of IL-18 can result in RPE cell death and geographic atrophy of AMD (61). Similar findings were observed in response to β -amyloid insult in RPE cells (79). Together, these findings demonstrate the central role of NLRP3-inflammasome activation in RPE cells and in models of AMD.

Summary and Future Directions

Taken together, in light of all discussed studies, this review lends further credit to the central role that NLRP1 and NLRP3 inflammasomes play in neuronal cell death and behavioral deficits in diseases of the brain NVU, including stroke, AD, and TBI. These findings further suggest a potential clinical benefit of therapeutic interventions that target inflammasome assembly and activity. On the other hand, NLRP3-inflammasome activation plays a major role in mediating the pro-inflammatory response involved in the pathophysiology of the diseases of the retinal NVU. Table 1 provides a summary of the type of inflammasome, cell type, and molecular pathways involved in relation to specific diseases of the NVU of the brain and the retina. These findings will have broader implications in multiple blinding conditions, including diabetic/prediabetic retinopathy, glaucoma, traumatic optic neuropathy, and retinal ischemia. Targeting specific components of the NLRP3 inflammasome will provide a therapeutic strategy to modulate the inflammatory response rather than to completely block it.

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Abbreviations Used

AD = Alzheimer's disease
ADA = adenosine deaminase
AIM-2 = absent in melanoma 2
AMD = age-related macular degeneration
ASC = apoptosis-associated speck like
ASK-1 = apoptosis signal-regulating kinase 1
CARD = caspase recruitment domain
CLR = C-type lectin receptor
Cox2 = cyclooxygenase-2
CNS = central nervous system
CXCL1 = chemokine (C-X-C motif) ligand-1
DAMP = damage-associated molecular pattern
ER-stress = endoplasmic reticulum-stress
FFA = free fatty acid
GFAP = glial fibrillary acidic protein
GLUT1 = glucose transporter-1
HFD = high fat diet
HRECs = human retinal endothelial cells
IL = interleukin
IRE-1 = inositol-requiring 1
iNOS = inducible nitric oxide synthase
JNK = c-jun-N-terminal kinase
KO = knockout
LPS = lipopolysaccharides

LRR = leucine rich repeat
MAPK = mitogen activated protein kinase
mDNA = mitochondrial DNA
MFGE8 = milk fat globule-EGF 8
MI = myocardial infarction
mROS = mitochondrial reactive oxygen species
NADPH = nicotinamide adenine dinucleotide phosphate-oxidase
NATCH = NAIP, CIITA, HET-E and TP1
NBD = nucleotide-binding domain
NFκB = nuclear factor kappa-B
NLR = nucleotide-binding oligomerization domain like receptor
NLRP = NOD-like receptor proteins
NMDA = N-methyl-D-aspartate
NOD = nucleotide-binding oligomerization domain
NPDR = non proliferative diabetic retinopathy
NVU = neurovascular unit
OIR = oxygen-induced retinopathy
Pal-BSA = palmitate coupled to bovine serum albumin
PAMP = pathogen-associated molecular pattern
PDR = proliferative diabetic retinopathy
PERK = dsRNA-activated protein kinase-like ER kinase
PLC = phospho lipase C
PRR = pattern recognition receptor
PTP = phospho-tyrosine phosphatases
P2X7 = purinergic receptor
RGC = retinal ganglion cell
RIG-1 = retinoic acid-inducible gene-1
RLR = retinoic acid-inducible gene-1 like receptor
rMC-1 = retinal Müller cell line-1
ROS = reactive oxygen species
RPE = retinal pigmented epithelium
SOCE = store operated Ca²⁺ entry
STZ = streptozotocin
TBI = traumatic brain injury
TBP-2 = thioredoxin binding protein-2
TKO = TXNIP knockout
TLR = Toll-like receptor
TNFR = tumor necrosis factor receptor
Trx = thioredoxin
Trx-1 = cytosolic/nuclear thioredoxin
Trx-2 = mitochondrial thioredoxin
TUNEL = terminal deoxynucleotidyl transferase dUTP nick end labeling
TXNIP = thioredoxin-interacting protein
UPR = unfolded protein response
VDAC = voltage dependent anion channel
VDUP-1 = vitamin D₃ upregulated protein-1
VEGFR2 = vascular endothelial growth factor receptor-2
WT = wild type