

FORUM

Representing the Process of Inflammation as Key Events in Adverse Outcome Pathways

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ABSTRACT

Inflammation is an important biological process involved in many target organ toxicities. However, there has been little consensus on how to represent inflammatory processes using the adverse outcome pathway (AOP) framework. In particular, there were concerns that inflammation was not being represented in a way that it would be recognized as a highly connected, central node within the global AOP network. The consideration of salient features common to the inflammatory process across tissues was used as a basis to propose 3 hub key events (KEs) for use in AOP network development. Each event, “tissue resident cell activation”, “increased pro-inflammatory mediators”, and “leukocyte recruitment/activation,” is viewed as a hallmark of inflammation, independent of tissue, and can be independently measured. Using these proposed hub KEs, it was possible to link together a series of AOPs that previously had no shared KEs. Significant challenges remain with regard to accurate prediction of inflammation-related toxicological outcomes even

if a broader and more connected network of inflammation-centered AOPs is developed. Nonetheless, the current proposal addresses one of the major hurdles associated with representation of inflammation in AOPs and may aid fit-for-purpose evaluations of other AOPs operating in a network context.

Key words: networks; cell activation; damage repair; knowledge management; adverse outcome pathway.

Framing the Problem

The adverse outcome pathway (AOP) framework was developed to help organize existing knowledge concerning the linkage between stressor-induced perturbation of biological pathways and adverse outcomes considered relevant to risk assessment and regulation (Ankley *et al.*, 2010). As such, AOPs are envisioned as an important component of a new toxicity testing paradigm that is expected to increasingly rely on mechanistic data, generally measured at low levels of biological organization using high throughput approaches, as a foundation for chemical safety assessment (Kleinstreuer *et al.*, 2016). Likewise, AOPs are envisioned to have application in chemical category formation, design of integrated approaches to testing and assessment, and development of computational toxicity prediction models (Edwards *et al.*, 2016; Wittwehr *et al.*, 2017). An international AOP development program, coordinated through the Organization for Economic Cooperation and Development (OECD) and facilitated through description and dissemination of AOPs via an open access knowledgebase (aopwiki.org; aopkb.org; last accessed February 28, 2018) has emerged to support these efforts (Edwards *et al.*, 2016; <http://www.oecd.org/chemicalsafety/testing/adverse-outcome-pathways-molecular-screening-and-toxicogenomics.htm>; last accessed February 28, 2018).

The AOP framework, implemented via the AOP knowledgebase, employs a modular structure in which information is organized concerning measurements of biological state (key events; KEs) that reflect progression from the initial perturbation toward the adverse outcome (AO) and scientific evidence supporting the linkage from 1 KE to the next in a causal and predictive sequence (KE relationships, KERs; Villeneuve *et al.*, 2014a). This modular structure is employed so that descriptions of common elements (KEs or KERs) can be shared among multiple AOPs in order to increase the efficiency of development and to broaden the scope of their application by allowing for de facto construction of AOP networks from independently described AOPs. An AOP network is defined as a system of 2 or more AOPs that share 1 or more KEs (Knapen *et al.*, 2018). Because it is expected that real-world exposures will often involve multiple AOPs, the ability to visualize and evaluate AOPs in a network context is important for many potential applications (Knapen *et al.*, 2018; Villeneuve *et al.*, 2014a). For example, one of the most obvious applications is in the assessment of mixtures where some chemicals may act on different MIEs that contribute jointly to common downstream effects, whereas others may have opposing effects on KEs pertaining to the same biological object (ie, agonism and antagonism of the same receptor).

Inflammation is a common response to a variety of stressors, including xenobiotics. Under normal conditions, inflammation is a healthy and adaptive process that both combats infection and is involved in repairing damage to tissues. However, xenobiotics can elicit prolonged, severe, and/or inappropriate inflammatory responses that play a causal role in the progression of biological events linking a molecular initiating

event (MIE) to an AO. This is reflected in the observation that inflammation has long been known to play a prominent role in human disease and a variety of target organ toxicities.

Given its prominent role in target organ toxicities, it was anticipated that inflammation would emerge as a highly connected node within an AOP network. However, as inflammation is a complex, multi-stage process, it was not clear what would serve as the appropriate level of abstraction for describing 1 or more KEs that capture the most salient, measurable hallmarks of inflammation, while also accommodating linkage to the wide range of upstream causes and downstream effects that are associated with the process. In particular, there was no consensus on whether inflammation should be represented as a single KE, and if so, how it would be measured, or whether it should be divided into a series of KEs. Likewise, it was unclear whether the inflammatory process was unique to every tissue or whether there were common features that could reasonably be generalized across tissues. Finally, if inflammation was divided into more than 1 event, it was unclear whether those events would occur in sequence (ie, following the linear construction typical of most AOPs), or whether the events would be concurrent and so inextricably linked that they would need to be represented as parallel or interconnecting branches or through introduction of an “and” type logic gate (ie, A and B must happen in order to cause C).

Approach and Objectives

To address on-going uncertainties about how to best represent inflammation using the AOP framework, experts with different backgrounds in research, toxicology, and medicine, specialized in immunology, immune toxicology, cancer research, inflammatory diseases of different organs (liver, lung, kidney, and brain) and endocrine-immune interactions convened at the Joint Research Center (Ispra, Italy), on September 27–28, 2017 to discuss this challenge and provide some recommendations on how inflammatory processes might be represented using the AOP framework. Discussions focused on a number of key topics and questions. First and foremost was an emphasis on the biology and identifying the most salient and indicative features of inflammation. Inflammatory processes in different target organs were discussed and compared to identify the extent to which the process generalizes across various tissues and organs and what aspects of the biology are context-specific. The common understanding of the biology was then used as a foundation for discussion of an appropriate level of resolution/abstraction with which to describe inflammation using the AOP framework. Furthermore, important aspects of the quantitative understanding of KERs that underlie the process were considered. This included discussion of whether there were measurements or indicators that distinguish appropriate, healthy, inflammatory responses from adverse inflammatory responses. Likewise, the need to identify important modulating factors that are known to either influence the severity of an inflammatory response or alter an organism's susceptibility to develop an

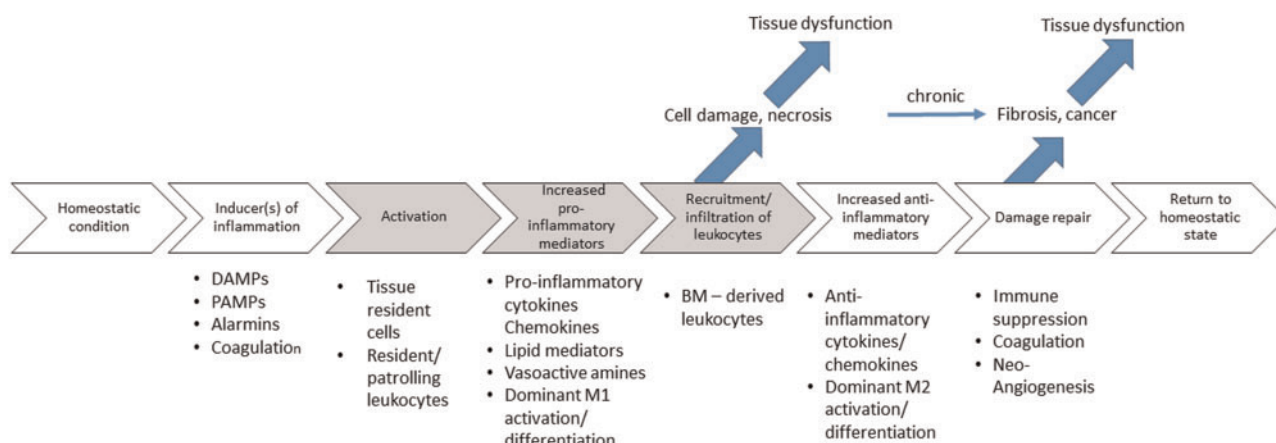


Figure 1. Generalized model of sequential steps involved in inflammatory response in a tissue. Sequence of chevrons indicates anticipated adaptive/protective response to a damage signal. Arrows indicate trajectory diverging from an adaptive homeostatic state to a maladaptive/adverse response. DAMPs, damage-associated molecular patterns; PAMPs, pathogen-associated molecular patterns; BM, bone marrow.

AO as a result of an inflammatory state (eg, Maiuri et al., 2015; Roth et al., 2003) and therefore may need to be considered in inflammation-related KERs was discussed.

The objective of the present manuscript is not to provide a comprehensive or critical review of the biology and toxicology of inflammation. Rather the aim is focused on representation of inflammatory processes using the AOP framework. Specifically, we sought to: (1) identify commonalities in the inflammatory process in different tissues and organs; (2) identify 1 or more KEs that could serve as common nodes in AOP networks; (3) agree on a consensus solution for integration of inflammation using the AOP framework; and (4) provide recommendations for how best to represent inflammation in the AOP framework and communicate these recommendations to relevant stakeholders (eg, AOP developers; OECD; scientists studying inflammation). Although it is expected that scientific uncertainty and debate over the specific details of inflammatory toxicology will continue, it is hoped that these recommendations provide a workable path forward for representation of this critical biology within the framework.

SALIENT FEATURES OF THE INFLAMMATORY PROCESS

Regardless of the tissue, the inflammatory process can be organized into a number of sequential steps (Figure 1; Lawrence et al., 2002). Interaction of healthy tissues with a stressor can evoke the release of mediators that initiate inflammation. For example, direct damage to cells/tissues as a result of chemical reactivity leads to the release of molecular signals termed damage-associated molecular pattern molecules (DAMPs), pathogen-associated molecular pattern molecules (PAMPs, released by invading pathogens), or alarmins (Chan et al., 2012; Escamilla-Tilch et al., 2013; Schaefer, 2014). These include a wide range of signal-initiating molecules that vary among cell types and include proteins, RNA or DNA, bacterially derived lipopolysaccharides, small molecule metabolites (eg, metabolic byproducts of bacteria, fungi, plants, etc.), and a variety of carbohydrates, which activate pattern recognition receptors, such as toll-like receptors and others. Certain xenobiotics may also mimic these molecular signals, without direct damage to cells. The tissue- and stressor-specific combinations, concentrations, and durations of these signals are thought to “program” the ensuing inflammatory response.

The diverse upstream signals involved in induction of inflammation subsequently activate resident cells of affected tissues (Davies et al., 2013; Wynn et al., 2013). Activation refers to a phenotypic modification of the resident cells that includes alterations in their secretions, activation of biosynthetic pathways, production of proinflammatory proteins and lipids, changes in the metabolism and sensing of small molecules, and morphological changes (Hussel and Bell, 2014). Although these represent a pleiotropic range of responses that can vary with the tissue, there are a number of common markers or signs of activation that are measurable.

Activation of tissue resident cells promotes the release of proinflammatory mediators, including proinflammatory cytokines, chemokines, vasoactive amines, and lipid mediators. Release of these signals into the tissue, as well as the circulation, promotes the recruitment of bone-marrow-derived leukocytes (eg, neutrophils and monocytes) that differentiate into mature proinflammatory cells, in response to mediators they encounter in the local tissue microenvironment. If persistent and unchecked, the proinflammatory actions of recruited cells can promote tissue injury, for example through the release of reactive oxygen and nitrogen species, which often characterizes acute adverse responses to proinflammatory signaling.

Under normal circumstances, once a sufficient proinflammatory response has been mounted, proinflammatory mediators will be supplanted by the release of anti-inflammatory mediators. These consist of a broad range of molecules that serve to both suppress immune response and promote resolution of inflammation and the repair of the damaged tissue. This phase is associated with differentiation of macrophages to an anti-inflammatory wound repair phenotype (Hussel and Bell, 2014). In cases where damage is on-going (chronic), leading to repeated cycles of damage and repair, fibrosis and other repair associated lesions can form in the affected tissue. These can eventually progress to the point of tissue dysfunction, triggering various diseases (AO). Conditions such as immune suppression, angiogenesis, and cell proliferation associated with repair can create favorable conditions for cancer formation. However, in cases where the damage is relatively acute and adequately repaired, the tissue can return to a “normal” homeostatic state, characterized as a generally quiescent cellular milieu.

It is important to note that although the inflammatory process can be organized into this generalized sequence, there is overlap between these states. Some signals ramp up as others

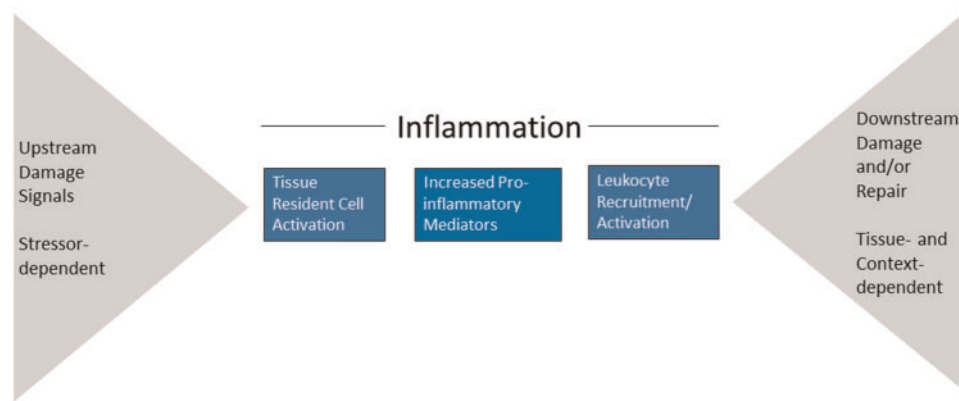


Figure 2. Overview of 3 key events (KEs, boxes) associated with hallmarks of inflammation that were proposed for use as potential hub KEs in inflammation-related adverse outcome pathway (AOP) networks. The 3 events are viewed as points of convergence between a wide range of potential stressor-dependent upstream signals that can induce inflammatory response and points of divergence toward a wide range tissue and context-dependent adverse outcomes. One or all of these KEs may be included in a given AOP, and in some cases the order, particularly of the first 2, may be reversed, consequently no arrows between the boxes are shown. Note “Tissue Resident Cell Activation” may also include activation of resident/patrolling leukocytes.

decline. The cells in the tissue change dynamically in terms of both numbers and phenotypes as the process progresses. Additionally, waves of subsequent damage due to flare-ups of a pathogen, chronic exposures to a chemical stressor, or introduction of another insult (ie, a “second hit”) can restart the process, such that there are overlapping waves occurring throughout different microenvironments in the tissue and organs that are not necessarily in phase with each other.

This all lends to a picture of inflammation as a highly complex process with stochastic components. Although many of the actors, their roles, and the stages they act upon (in terms of tissue-specific milieus) are known, existing knowledge is generally not adequate to predict the specific company of actors that will respond to any particular stressor, their sequence, or the specific outcomes that will ensue. The signals and cell types responding to any given stressor/exposure scenario are defined largely by the type and context of the tissue. Additionally, the factors deemed most relevant are influenced by investigator opinion and what those individual investigators choose to measure (or do not), which in turn shapes our current understanding of inflammation. Although the same could be said for most fields of study, it is noted here in recognition of the daunting challenge we face in drawing quantitative or even semiquantitative inference or predicting outcomes along inflammation-mediated AOPs. Although various markers or hallmarks of inflammation can reasonably be viewed as red flags or indicators of potential or even probable hazard, their application as reliable quantitative predictors of outcome is likely to be limited. With this caveat, we considered how inflammatory response can be represented and described using the AOP framework.

HUB KEs FOR INFLAMMATION-RELATED AOP NETWORKS

Through consideration of the salient features of the inflammatory process, 3 generalized KEs (tissue resident cell activation; increased proinflammatory mediators; leukocyte recruitment/activation) were proposed as candidates for common nodes (hubs) that could be shared by a diversity of AOPs involving inflammation (Figure 2). Each KE corresponds with one of the generalized sequential steps involved in inflammatory response in a tissue (shaded in Figure 1). Each of these broad processes was

Table 1. Illustration of Process/Object/Action Terms (see Ives et al., 2017) That Would Define the Proposed “Hub” Key Event (KE) Titled “Increased Proinflammatory Mediators”

KE Title	Event Components		
	Process	Object	Action
Increased proinflammatory mediators	Inflammation	TNF-alpha	Increase
		IL-1	
		IL-6	
		CXCL2	
		CCL2	
		Histamine	
Prostaglandins			

Note, the object listing is intended to be illustrative, not comprehensive.

viewed as a generalizable hallmark of inflammation, independent of the tissue. Although they are closely linked, each can be measured independent of one another. Together, they are viewed as a minimum set of common denominators reflective in the inflammatory process across tissues.

Each of these events is consistent with the definition of a KE as reflecting a measureable change in biological state that is demonstrably essential to progression along the pathway, but not necessarily sufficient by itself to cause adversity (ie, just because these events are observed, does not mean injury will occur—only that it *could*, based on current scientific understanding and evidence; Villeneuve et al., 2014a). However, these proposed hub KEs run counter to some of the conventions and best practices concerning KE description (Villeneuve et al., 2014b). Most notably, the title of the events does not clearly indicate what biological object (eg, protein, metabolite, cell type, etc.) should be measured. This is because although the function/biological role/toxicodynamics of the event is well conserved across different tissues and organisms, the specific “actors” that are playing these roles, and thus how the measurements are made can vary. For example, in liver measures of “tissue resident cell activation” may focus on Kupffer cells, whereas in the brain they might focus on microglia or in bone osteoclasts (Davies et al. 2013). In terms of the ontological description of KE components (Ives et al., 2017), these KEs are

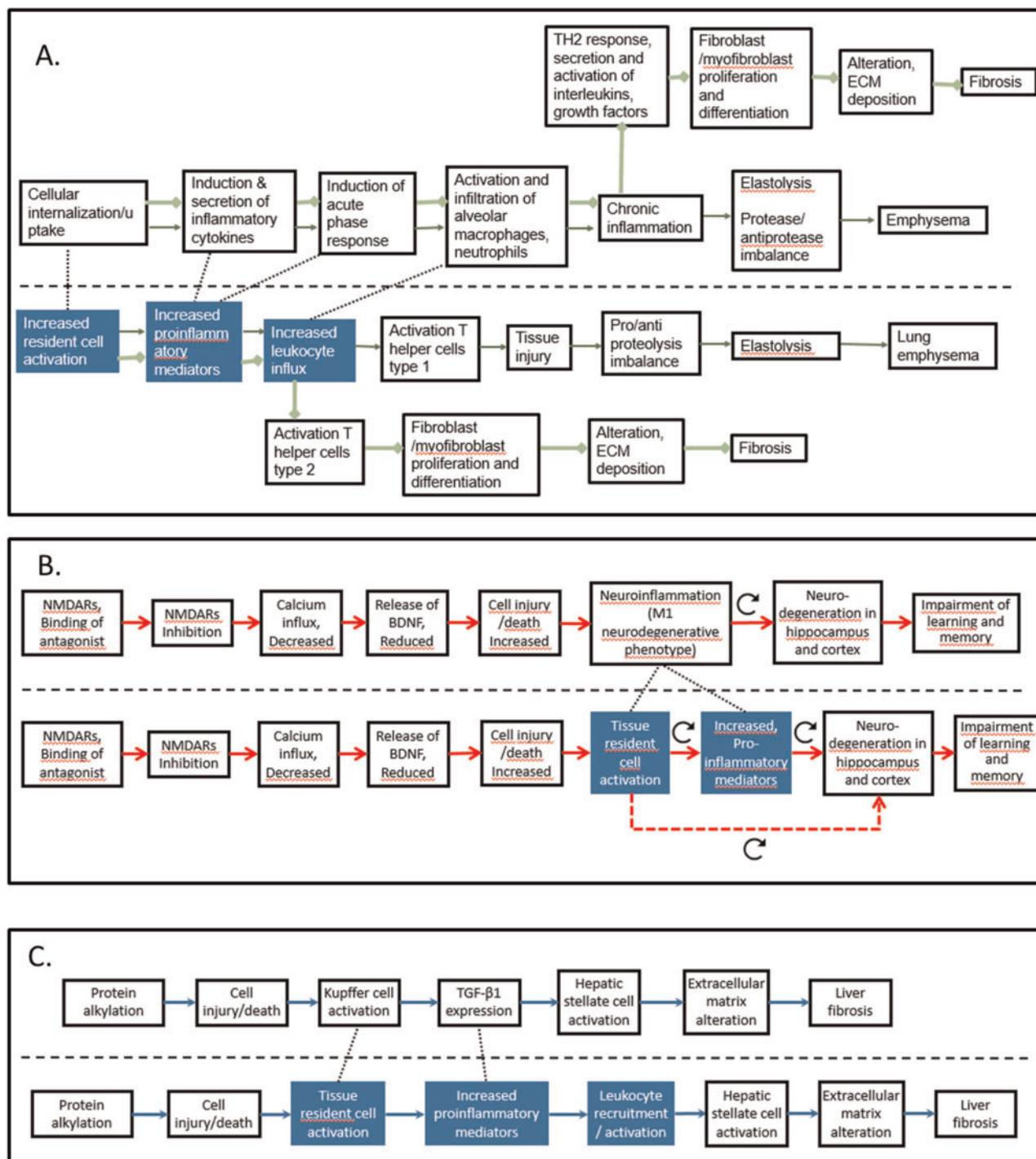


Figure 3. Four AOPs shown before (above the horizontal dashed line) and after (below the horizontal dashed line) incorporation of the proposed hub KEs (shaded). A, A network of 2 AOPs linking induction and secretion of inflammatory cytokines to lung fibrosis (AOP 173; <https://aopwiki.org/aops/173>) or lung emphysema (novel AOP not yet entered in the AOP-Wiki). Revised titles, resident cell activation leading to lung fibrosis or lung emphysema, respectively. B, AOP linking chronic binding of antagonists to N-methyl-D-aspartate receptors (NMDARs) during brain development to neurodegeneration with impairment of learning and memory in aging (AOP 13; <https://aopwiki.org/aops/13>). C, AOP linking protein alkylation to liver fibrosis (AOP 38; <https://aopwiki.org/aops/38>).

viewed as well conserved in their “process” and “action” terms. However, the specific “object” terms can be expected to vary with tissue environment and context and will need to be described for different tissue contexts as each KE description is expanded/revised for use with different AOPs (eg, Table 1).

The 3 proposed hub KEs were viewed as reflecting functional points of convergence in relation to a diverse array of upstream, stressor-specific signals. Likewise, they were functional points of divergence in relation to different tissue- and context-specific outcomes. Thus, they could be viewed as the “knot” in

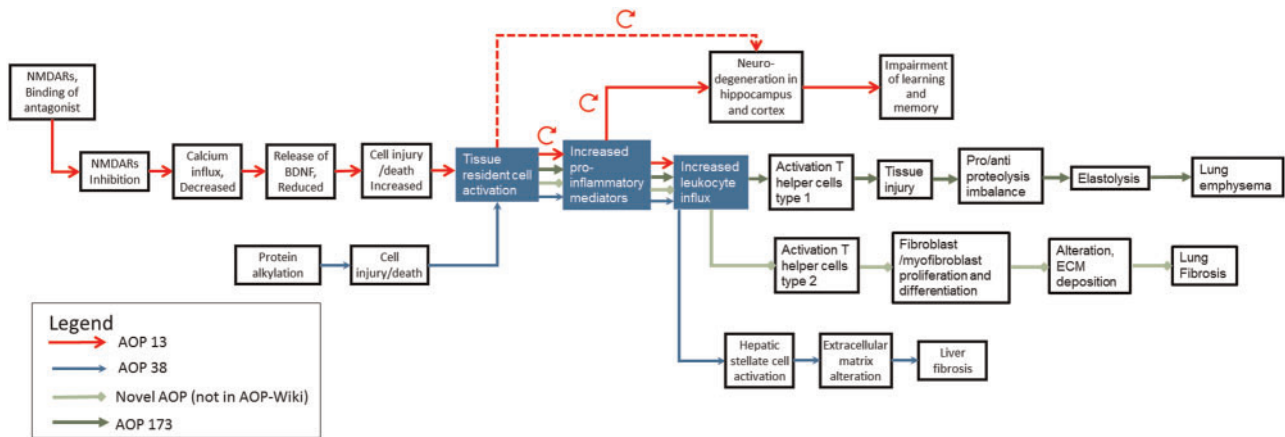


Figure 4. Network composed of 4 AOPs (<https://aopwiki.org/aops>; AOPs 13, 38, 173 and 1 novel AOP not yet in the AOP-Wiki) illustrating how incorporation of the proposed hub KEs facilitates improved connectivity of disparate inflammation-related AOPs within a broader AOP network.

classic bow tie structures in systems biology (Friedlander et al. 2015) and thus are well suited to serve as central, highly connected, nodes within an AOP network (Knapen et al., 2018).

PILOT IMPLEMENTATION

Following the proposal of the hub KEs, corresponding event pages were created in the AOP-Wiki (<https://aopwiki.org/events/1492>; <https://aopwiki.org/events/1493>; <https://aopwiki.org/events/1494>). As a pilot, these events were integrated into a number of existing AOPs (Figure 3) linking different MIEs to distinct inflammation-mediated AOs or to AOPs where inflammation is an essential exacerbating element (<https://aopwiki.org/>; AOP 13; 38; 173; 1 novel AOP not yet in the AOP-Wiki). By introducing these hub KEs, it is now possible to link together AOPs that were previously disconnected (Figure 4) in the overall AOP network represented in the AOP-Wiki. The previously defined AOPs were readily adapted to incorporate the proposed hub KEs. For example, for AOP 38 (Figure 3C) Kupffer cell (resident macrophages) activation aligned with “tissue resident cell activation” and TGF- β 1 expression corresponded with “Increased pro-inflammatory mediators.” The third hub KE “Leukocyte recruitment/activation” had not been displayed as a separate KE in the original version of AOP 38, because that process had been incorporated into the description of the Kupffer cell activation KE. Likewise, in the case of an AOP leading to neurotoxicity (AOP 13; Figure 3B) it was possible to link neuroinflammation—which is considered a neurospecific process relying on specific neural cells—with the postulated hub KEs and their generic description. This allowed interconnectivity with other AOPs unrelated to the brain (Figure 4).

NEXT STEPS AND PERSPECTIVES

Through deliberations on the biology and toxicology of inflammation, a consensus proposal was achieved with regard to an appropriate level of abstraction with which to represent the process of inflammation within a broad AOP network. The proposed level of abstraction resolves inflammation into 3 hallmark KEs that are expected to be functionally equivalent across multiple AOPs. However, the specific objects or actors involved in these functions are expected to vary by AOP according to the tissue specific context. This solves the primary challenge

of representing inflammation in a way that is biologically appropriate yet is generalizable enough to facilitate AOP network connectivity, which was our major aim.

The approach recommended here is somewhat of a departure from current conventions regarding the description of KEs (Villeneuve et al., 2014b). Although the biological function/process each KE represents is well conserved across different tissue contexts, the specific objects (eg, genes, proteins, metabolites) one would measure as appropriate indicators of these KEs are likely to be tissue-dependent. This will require creativity, careful thought, and perhaps some trial and error, in terms of how best to structure the KE descriptions for these events. Likewise, with experience, guidance regarding the minimum set of tissue-specific object changes that represent “triggering” of a given KE may need to be defined. Nonetheless, it should facilitate our ability to represent inflammation in an AOP network context.

We and others (Leist et al., 2017) have noted the potential predictive limitations of inflammation-related AOPs based on the fact that they are inherently dependent on the relative balance of damage and repair and the associated timing and conditions of exposure. The proposed hub KEs do not specifically address that potential limitation to the predictive utility of the AOP framework in such circumstances. However, accepting this limitation, the ability to better represent inflammation in an AOP network is still highly beneficial. Specifically, it may help to more effectively differentiate those AOPs involving or intersecting with an inflammatory component, where more sophisticated models or *in vivo* testing may be required to predict outcomes, from those acting independent of inflammation where predictive confidence may be higher. In this way, the ability to identify intersection with inflammatory KE hubs aids the evaluation of fit-for-purpose of specific AOPs and AOP networks for different types of decision-making. Consequently, consensus around how to best represent inflammation via a set of hub KEs represents a significant step forward in the evolution and application of the AOP framework.

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