AOP ID and Title:

SNAPSHOT

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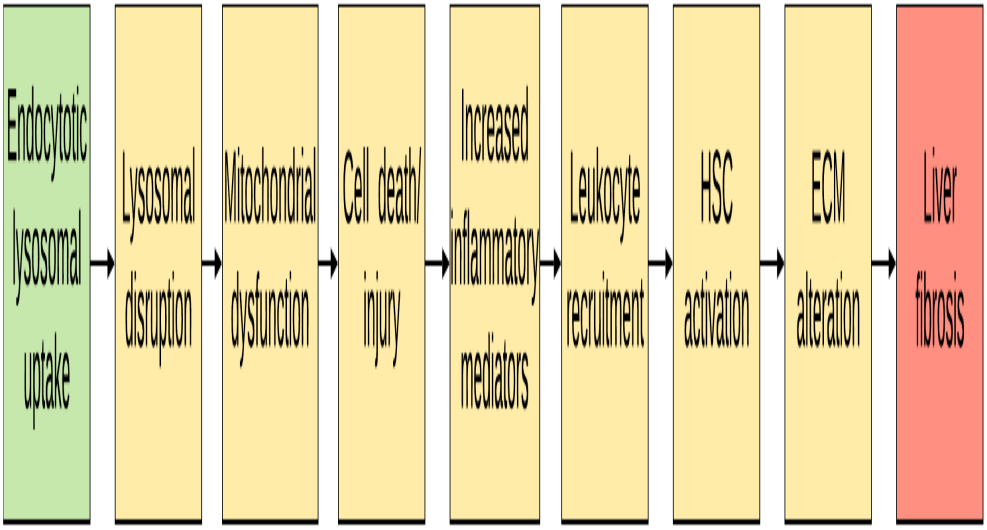
AOP 144: Endocytic lysosomal uptake leading to liver fibrosis

Short Title: lysosomal uptake induced liver fibrosis

Graphical Representation



Add non-adjacent KERs

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Abstract

Hepatotoxicity is known to be an important endpoint of regulatory concern; it has been one of the frequent reasons for pharmacovigilance safety reports and human health risk assessments. Liver fibrosis in particular is a health problem resulting from chronic or repeated-dose chemical exposure and it is considered as an adverse outcome of regulatory interest. Liver fibrosis is a long and complex process involving various hepatic cell types, molecular mediators, receptors and signalling pathways. It occurs as a result of imbalance between collagen deposition and destruction, but also changes in the extracellular matrix composition (ECM). Due to this complexity appropriate cell model is currently unavailable.

The current AOP links endocytic lysosomal uptake and the formation of liver fibrosis. The molecular initiating event (MIE) is endocytic lysosomal uptake of chemical, leading to lysosomal disruption, the first key event (KE). Lysosomal disruption induces mitochondrial dysfunction, which leads to cell injury and both apoptosis and necrosis. Lysosomal disruption, mitochondrial dysfunction and cell injury/death present KEs on the cellular level. Cell death releases damage-associated molecular patterns (DAMPs) which lead to increased inflammatory mediators presence, the next KE along the path. Inflammatory mediators attract and activate leukocytes, which present the next KE. Activated leukocytes through molecular mediators activate hepatic stellate cells, which increases α-SMA in them. This KE increases amount of collagen I and III, which causes its accumulation. Collagen accumulation presents KE at the tissue level and leads to adverse outcome (AO) - liver fibrosis, which changes normal functioning of the whole organ. There is also important on-going process present throughout the pathway, which is connected with different KEs- oxidative stress. It is not classified as individual KE, but described in KEs and KERs related to it.

The value of this AOP is that it might support chemical risk assessment by identifying upstream biomarkers for adverse outcome, even though the adequate cell model is not available. This systematic organization of existed knowledge, but also of present uncertainties can facilitate regulatory processes, but also indicate the need for the new testing methods.

The AOP endocytic lysosomal uptake to liver fibrosis has high biological plausibility, supported with empirical evidence. However, quantitative data and temporal sequences between KEs are currently lacking and further efforts are necessary in their provision.

Background

Liver fibrosis is currently an important health problem potentially leading in its progressive form to cirrhosis and as such present a significant economic burden (Lim and Kim, 2008). The only therapy for chronic liver failure is liver transplantation, with 5.500 liver transplantations in Europe on a yearly basis, costing up to €100.000 the first year (Safadi and Friedman, 2002). There are constant research attempts for new therapeutic strategies, but so far without success. AOP concept presents an alternative approach which organizing mechanistic toxicological knowledge can lead to prevention of liver fibrosis.

The use and possible applications of nanomaterials (NMs) are in constant increase, for example in food, food-related products or cosmetics. Therefore, the safety of NMs systemically taken up in the body needs to be ensured. The liver is known to be one of the main target organs for ingested NMs, but inhaled particles can also reach the liver upon clearance from the lung (Johnston et al., 2010; Cui et al., 2011; Geraets et al., 2012). In vivo experiments on gavaged or injected (intraperitoneal or intravenously) TiO_2 suggest a wide range of adverse effects on the liver: an increase in general serum markers for liver damage such as Alanine Aminotransferase or Aspartate Aminotransferase (Liu et al., 2009; Duan et al., 2010), an increase in inflammatory markers such as pro-inflammatory cytokines and/or infiltration of inflammatory cells (Ma et al., 2009; Cui et al., 2011; Kermanizadeh, 2012), an increase of markers for oxidative stress (Liu et al., 2010; Soliman et al., 2013), apoptosis, necrosis and also fibrosis (Chen et al., 2009; Alarifi et al., 2013). Oral NM administration appeared to induce overall milder adverse effects than systemic administration, most likely due to the typically limited absorption of NMs in the GI tract. Thus, it is important to keep in mind that the route of exposure and the size of the NM, play important role whether these reach the liver, and to which extent they're accumulated (Kermanizadeh, 2014).

Once a chemical is taken up by a cell, it is transported into the lysosome. In the lysosome, acidic environment can enhance the solubility of a NM, or it remains in initial nano form. Both situations can induce toxicity, causing lysosomal swelling, followed by lysosomal damage and the release of pro-apoptotic proteins (Wang et al., 2013; Cho et al., 2011; Cho et al., 2012). But not only NMs cause lysosomal damage: fluoroquinolones (Ouedraogo et al., 2000), lysosomotropic detergents such as o-methyl-serine dodecylamide hydrochloride (Villamil Giraldo et al., 2016), artesunate (Yang et al., 2014), chloroquine (Ashoor et al., 2013) can do the same, and Reactive Oxygen Species (ROS) such as H_2O_2 can amplify this effect (Repnik et al., 2012). The amount of lysosomal enzymes released into the cytosol regulates the cell death pathway: controlled increased permeability of lysosomal membrane, caused by limited level of stress, plays a vital role in the induction of apoptosis, whereas massive lysosomal rupture, caused by high stress levels, leads to necrosis (Bursch, 2001; Guicciardi et al., 2004). Lysosomes are known to trigger mitochondrial mediated cell death by the release of cathepsins into the cytosol (Repnik et al., 2012). At the same time however, lysosomes themselves are a source of ROS, which can lead to damage of the mitochondrial membrane (Wang et al., 2013; Kubota et al., 2010). Cell death further on leads to inflammation (Faouzi et al., 2001), which activates hepatic stellate cells and induce them to secrete collagen (Casini et al., 1997). It is established that collagen accumulation is a prestage of liver fibrosis (Bataller and Brenner, 2005; Lee and Friedman, 2011).

Overall, the connection between lysosomal and mitochondrial damage with liver inflammation and further on with fibrosis, is well known, regardless if triggered by chemicals, proteins or NMs (reviewed in Malhi and Gores, 2008; Kong et al., 2014). Therefore, due to its high importance it is described extensively in the current AOP.

Summary of the AOP

Events

Molecular Initiating Events (MIE), Key Events (KE), Adverse Outcomes (AO)

Sequence	Type	Event ID	Title	Short name
1	MIE	1539	Endocytotic lysosomal uptake (https://aopwiki.org/events/1539)	endocytosis
2	KE	898	Disruption, Lysosome (https://aopwiki.org/events/898)	Disruption, Lysosome
3	KE	177	N/A, Mitochondrial dysfunction 1 (https://aopwiki.org/events/177)	N/A, Mitochondrial dysfunction 1
4	KE	55	N/A, Cell injury/death (https://aopwiki.org/events/55)	N/A, Cell injury/death
5	KE	1493	Increased Pro-inflammatory mediators (https://aopwiki.org/events/1493)	Increased pro-inflammatory mediators
6	KE	1494	Leukocyte recruitment/activation (https://aopwiki.org/events/1494)	Leukocyte recruitment/activation
7	KE	265	Activation, Stellate cells (https://aopwiki.org/events/265)	Activation, Stellate cells
8	KE	68	Accumulation, Collagen (https://aopwiki.org/events/68)	Accumulation, Collagen
9	AO	344	N/A, Liver fibrosis (https://aopwiki.org/events/344)	N/A, Liver fibrosis

Key Event Relationships

Upstream Event	Relationship Type	Downstream Event	Evidence	Quantitative Understanding
Endocytotic lysosomal uptake (https://aopwiki.org/relationships/1775)	adjacent	Disruption, Lysosome	High	
Disruption, Lysosome (https://aopwiki.org/relationships/993)	adjacent	N/A, Mitochondrial dysfunction 1	High	
N/A, Mitochondrial dysfunction 1 (https://aopwiki.org/relationships/363)	adjacent	N/A, Cell injury/death	Moderate	Low
N/A, Cell injury/death (https://aopwiki.org/relationships/1776)	adjacent	Increased Pro-inflammatory mediators	High	
Increased Pro-inflammatory mediators (https://aopwiki.org/relationships/1777)	adjacent	Leukocyte recruitment/activation	High	
Leukocyte recruitment/activation (https://aopwiki.org/relationships/1778)	adjacent	Activation, Stellate cells	High	
Activation, Stellate cells (https://aopwiki.org/relationships/295)	adjacent	Accumulation, Collagen	High	
Accumulation, Collagen (https://aopwiki.org/relationships/82)	adjacent	N/A, Liver fibrosis	High	

Stressors

Name	Evidence
nanoparticles	

Name	Evidence
ROS	
o-methyl-serine dodecylamide hydrochloride (MSDH)	
3-aminopropanal	
artesunate	
Chloroquine bis(phosphate)	
Norfloxacin	
Ciprofloxacin	

Overall Assessment of the AOP

Assessment of the Weight-of-Evidence supporting the AOP

Concordance of dose-response relationships

The present AOP is a purely qualitative description of the pathway. The present literature does not provide quantitative information on dose-response relationships.

Temporal concordance among the key events and adverse outcome

There is empirical evidence to support that a change in KEup leads to a change in the respective KEdown, leading to the AO.

Strength, consistency, and specificity of association of adverse outcome and initiating event

There is strong empirical evidence on the link between MIE and AO that has been described.

Biological plausibility, coherence, and consistency of the experimental evidence

There is high biological plausibility in the description of the AOP and its components. The available data describing the AOP are logic, coherent and consistent with current biological knowledge.

Uncertainties, inconsistencies and data gaps

The present AOP description is plausible, but entirely qualitative, and the addition of quantitative data on dose-response relationship and temporal scale would improve its applicability. Also, though there is strong empirical evidence supporting this AOP, at certain KERs we found some inconsistencies that need to be further investigated. Existing uncertainties and inconsistencies are stated in each KER description, but here we will describe the most important ones.

Repnik and colleagues detected that after exposure to LLOMe, cathepsins remain in lysosomes and are being degraded there which is in contradiction with most of the previous studies (Repnik et al., 2017). There is strong empirical evidence that incubation of cathepsin B with mitochondria and cytosolic factors increase mitochondrial permeabilization, but in some studies pharmacological inhibition of cathepsins or knockout of genes coding for cathepsins failed to prevent mitochondrial membrane permeabilizatio and cell death, suggesting that other lysosomal proteases might be responsible for Bid cleavage (Reiners et al., 2002; Boya et al., 2003).

The histochemical analysis of hippocampus of rats treated with domoic acid for 15 days has revealed no presence of apoptotic bodies and no Fluoro-Jade B positive cells (Schwarz et al., 2014).

The inflammatory role of HMGB-1 is still not completely clear. There are many studies that confirm its pro-inflammatory activity, but in some experiments highly purified HMGB-1 had little pro-inflammatory activity (Rouhiainen et al., 2007), while in another injection of recombinant HMGB-1 into infarcted heart muscle in vivo stimulated regeneration and repair (Limana et al., 2005).

Lloyd and colleagues found that several chemokines can stimulate the adherence of peripheral blood lymphocytes to ICAM-1 coated slides (Loyd et al., 1996). However, by using a parallel plate flow chamber, other study failed to observe such an effect (Carr et al., 1996).

Domain of Applicability

Life Stage Applicability

Life Stage	Evidence
Not Otherwise Specified	

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
human	Homo sapiens	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)
mouse	Mus musculus	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10090)
rat	Rattus norvegicus	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10116)

Sex Applicability

Sex	Evidence
Unspecific	

Life Stage Applicability

The described AOP is a general mechanism, that furthermore can be considered as not being limited to only the liver as the target organ. Also, to current knowledge, this AOP is not limited to a specific life stage.

Taxonomic Applicability

Most of the work performed to elaborate parts of this AOP was done using murine or human cells and cell lines, human blood samples or tissues, or mouse models. Examples include

mouse: Narumi et al., 1992; Fahy et al., 2001; Kagedal et al., 2001; Faouzi et al., 2001; Werneburg et al., 2002; Chen et al., 2007; Seki et al., 2007; Leung et al., 2008; Dalton et al., 2009; Lee et al., 2009 Gäbele et al., 2009; Nan et al., 2013; Pradere et al., 2013; McHedlidze et al., 2014; Chang et al., 2014;

rat: Rockey et al., 1992;George et al., 1999; Reeves et al., 2000; Luckey and Petersen, 2001; Duffield et al., 2005; Imamura et al., 2005; Natajaran et al., 2006; Liedtke et al., 2011; Li et al., 2012; Jung et al., 2015

human: Bleul et al., 1996; Miyamoto et al., 2000; Andersson et al., 2000; Yamada et al., 2001; Scaffidi et al., 2002; Safadi and Friedman, 2002; Boya et al., 2003; Cirman et al., 2004; Bataller and Brenner, 2005; Bell et al., 2006; Rockey and Friedman, 2006; Friedman, 2008; Lim and Kim, 2008; Winklhofer and Haass, 2010; Clarke et al., 2010; Hamacher-Brady et al., 2011; Santibañez et al., 2011; Lee and Friedman, 2011; Wang et al., 2013; Loos et al., 2014; Decaris et al., 2015; Sun et al., 2015;

Sex Applicability

As described above, the AOP is widely applicable, therefore no specific sex applicability is known at this point.

Essentiality of the Key Events

The essentiality of almost all of the KEs in this AOP was rated high as there is much experimental evidence that the blocking of one KE prevents the next downstream KE and therefore the whole AOP. The essentiality of KE Mitochondrial dysfunction was rated as moderate, as there are two pathways to apoptosis (the next downstream KE), intrinsic and extrinsic, and only intrinsic pathway includes mitochondrial dysfunction (reviewed in Elmore, 2007).

Some of the evidence for the essentiality of KEs is listed below.

Exposure of cells to ammonium chloride prior to exposure to sphingosine resulted in formation of NH_3, which entered into lysosomes and became protonated and increased the pH of the organelle. This exposure prevented the accumulation of sphingosine in the lysosome and provided protection against its lysosomolytic and apoptosis-inducing effects (Kagedal et al., 2001).

Inhibition of lysosomal membrane permeabilization (LMP) with Baf A1 – that inhibits lysosomal vacuolar H^+ ATP-ase, prevented mitochondrial membrane permeabilization (MMP) - the next downstream KE, while inhibition of MMP in Bax/Bak double knocks out cells didn't prevent LMP (Boya et al., 2003).

Faouzi and colleagues showed that the inhibition of apoptosis causes inhibition of the inflammatory response (Faouzi et al., 2001), while other study showed that an inhibitor of apoptosis is blocking the release of HMGB-1 mediator specifically (Bell et al., 2006).

A human CXC chemokine antagonist, growth-related oncogene GROα(8-73), inhibited calcium mobilization induced by MIP-2, and the pretreatment of mice with this antagonist inhibited, in a dose-dependent manner, the influx of neutrophils induced by MIP-2, TNF-α, LPS and IL-1β (McColl and Clark-Lewis, 1999).

There is strong evidence that the blockade of TGF-β alone is sufficient to completely block experimental fibrogenesis in liver (reviewed in Gressner et al., 2002). The activation of HSCs can be partially blocked by anti-TGF-β antibodies (Zimmermann et al., 2010) and blocked by overexpression of Smad7, a natural antagonist of TGF-β signalling (Dooley et al., 2003). Macrophage depletion led to a significant reduction in the number of HSCs (Duffield et al., 2005).

Weight of Evidence Summary

Summary Table

Provide an overall summary of the weight of evidence based on the evaluations of the individual linkages from the Key Event Relationship pages.

Support for essentiality of KEs	
MIE Endocytic lysosomal uptake	Essentiality of the MIE is high. Exposure of cells to NH_3 prevented the accumulation of sphingosine, known lysosomotropic agent, in the lysosomes and provided protection against its lysosomolytic and apoptosic effects (Kagedal et al., 2001).
KE1 Lysosomal disruption	Essentiality of the KE1 is high. Inhibition of LMP prevented MMP, the next downstream KE, and the entire pathway (Boya et al., 2003). The treatment of the cells with cathepsins inhibitors decreased MMP and prevented apoptosis (Roberg et al., 1999; Kagedal et al., 2001).
KE2 Mitochondrial dysfunction	Essentiality of the KE2 is moderate. There are two apoptotic pathways, intrinsic and extrinsic, and only intrinsic pathway includes mitochondrial dysfunction (reviewed in Elmore, 2007).
KE3 Cell death/injury	Essentiality of the KE3 is high. The inhibition of apoptosis causes blockage of release of inflammatory mediators and inhibition of the inflammatory response (Faouzi et al., 2001; Bell et al., 2006).
KE4 Increased inflammatory mediators	Essentiality of the KE4 is high. Chemokine antagonist can prevent influx of neutrophils (McColl and Clark-Lewis, 1999).
KE5 Leukocyte recruitment/ activation	Essentiality of the KE5 is high. Macrophage depletion leads to a significant reduction in the number of HSCs, the next downstream KE (Duffield et al., 2005). After bile duct ligation, Kupffer cell–depleted mice showed almost complete suppression of HSC activation and fibrosis (Seki et al., 2007).
KE6 HSC activation	Essentiality of the KE6 is high. Experimental inhibition of HSC activation prevents fibrosis (Friedman, 2002; Anan et al., 2006; Kisseleva and Brenner, 2008; Son et al., 2009).
KE7 Collagen accumulation	Essentiality of the KE7 is high. Continuing imbalance between the deposition and degradation of ECM is a pre-requisite of liver fibrosis (Lee and Friedman, 2011).
AO Liver fibrosis	It is generally accepted that any chronic and repeated liver injury can result in myofibroblast activation, leading to hepatic fibrosis and cirrhosis (Jaeschke, 2002; Lee William, 2003; Ramachandran and Kakar, 2009).
Support for Biological Plausibility of KERs	

MIE->KE1	Biological plausibility of the KER between MIE and KE1 is high. Trapping of lysosomotropic agents accumulates substances inside of the lysosomes, increases volume of these organelles, and big lysosomes are more prone to rupture (Ono et al., 2003).
KE1->KE2	Biological plausibility of the KER between KE1 and KE2 is high. In the last decade there is a growing body of evidences about the strong functional link between lysosomes and mitochondria that play an important role in physiology and pathology. The evidences also showed link between lysosomal and mitochondrial damage, and that lysosomal damage precedes mitochondrial injury (e.g. Droga-Mazovec et al., 2008; Ghosh et al., 2011).
KE2->KE3	Biological plausibility of the KER between KE2 and KE3 is high. There is functional mechanistic understanding supporting this relationship between KE2 and KE3, involving ROS formation, ATP depletion and apoptogenic factors (Richter et al., 1996; Leist et al., 1997; Nicotera et al., 1998; Brenner and Mak, 2009; Lu et al., 2014; Zhou et al., 2015).
KE3->KE4	Biological plasubility of the KER between KE3 and KE4 is high. The dead cells can secrete inflammatory mediators that trigger infiltration of immune cells. However, if this becomes chronic it has the potential to enhance tissue damage and ultimately induce fibrosis (Jaeschke, 2002; Cullen et al., 2013).
KE4->KE5	Biological plausibility of the KER between KE4 and KE5 is high. There is much evidence that application of chemokines attract leukocytes to specific site in different species (Beck et al., 1997; Lee et al., 2000; Fahy et al., 2001; Nikiforou et al., 2016).
KE5->KE6	Biological plausibility of the KER between KE5 and KE6 is high. The recruitment of immune cells from the circulation into the injured tissue is the key mechanism during fibrogenesis in the liver (Heymann and Tacke, 2016).
KE6->KE7	Biological plausibility of the KER between KE6 and KE7 is high. The functional relationship between stellate cells activation and collagen accumulation is consistent with established biological knowledge (Milani et al., 1994; Benyon and Arthur; 2001; Safadi and Friedman, 2002; Kershenobich Stalnikowitz and Weisssbrod , 2003; Bataller and Brenner, 2005; Kolios et al., 2006; ; Guo and Friedman, 2007; Li, Jing-Ting et al., 2008; López-Novoa and Nieto, 2009; Lee und Friedman 2011).
KE7->AO	Biological plausibility of the KER between KE7 and AO is high. By definition, liver fibrosis is the excessive accumulation of ECM proteins that are produced by HSCs. The KER between this KE and the AO is undisputed (Poynard et al., 1997; Bataller and Brenner, 2005; Rockey and Friedman, 2006; Brancatelli et al., 2009; Lee and Friedman, 2011).

Empirical Support for KERs	
MIE->KE1	Empirical support of the KER between MIE and KE1 is high. Even the accumulation of substances that are physiologically present in lysosomes, such as pro-cathepsins, can lead towards lysosomal dysfunction (Jung et al., 2015). Sphingosine is a lysosomotropic agent that accumulates within the lysosomes, where it permeabilizes the membrane via a detergent mechanism and provokes release of lysosomal enzymes (Kagedal et al., 2001). NM captured in lysosomes can cause lysosomal swelling, disruption and the release of pro-apoptotic proteins (Cho et al., 2011; Cho et al., 2012; Wang et al., 2013).
KE1->KE2	Empirical support of the KER between KE1 and KE2 is high. LMP is detected couple of hours earlier than MMP, after exposure to ciprofloxacine, norfloxacine and hydroxychloroquine, and cells with MMP are sub-ensmeple of the group of cells with LMP (Boya et al., 2003). When isolated mitochondria are incubated with purified cathepsin B in the presence of cytolic extracts, a release of cytochrome c from mitochondria is detected (Guicciardi et al., 2000). The microinjection of cathepsin D to the cell causes cytochrome c release, caspases activation and apoptosis (Roberg et al., 2002). Bid protein needs to be cleaved in order to cause cytochrome c release (Luo et al., 1998; Gross et al., 1999; Stoka et al., 2001). Incubation of Bax with isolated mitochondria resulted in cytochrome c release while Bcl-xl inhibits this release (Jurgensmeier et al., 1998).
KE2->KE3	Empirical support of the KER between KE2 and KE3 is moderate. Mice injected intraperitoneally with DomA have shown increase of the TUNEL positive cells in the hippocampus, decreased indicators of mitochondria function and elevated ROS levels (Lu et al., 2012, Wu et al., 2012). The incidence of downstream KE (cell death) is higher than the incidence of downstream KE (mitochondrial dysfunction). In vitro studies (Giordano et al., 2007; 2009) suggest that both KEs are affected by the same dose of DomA and that the incidence of KE down (cell death) is higher than the incidence of KE up (mitochondrial dysfunction), with KE up happening earlier than KE down.

KE3->KE4	Empirical support of the KER between KE3 and KE4 is high. During the apoptosis of Jurkat cells treated with various agents, HMGB-1 was released into the media (Bell et al., 2006), which was found to activate leukocytes and stimulate the production of pro-inflammatory mediators in vitro (Li et al., 2004; Zimmermann et al., 2004). ATP can also stimulate the production of pro-inflammatory cytokines from macrophages (Ferrari et al., 1997; Ferrari et al., 2006). Neutralization or genetic deficiency of IL-1 inhibited inflammation responses to injected dead cells (Chen et al., 2007; Kono et al., 2010). Injection into mice of a variety of other dead cell types that genetically lack both IL-1α and IL- 1β stimulated an inflammatory response that was equivalent to that of wildtype necrotic cells (Kono et al., 2010). This implicates that IL-1 that is driving the sterile inflammatory response in many cases is not coming directly from the dead cell but is produced by cells in the host upon recognition of cell death.
KE4->KE5	Empirical support of the KER between KE4 and KE5 is high. The exposure of mice to FliCind strain S. Typhimurium triggered a significant neutrophil influx in the spleen of wild-type mice, but not of Il1b–/–Il18–/– mice (Jorgensen et al., 2016). It was shown that exposure of cells to IL-1β, TNF-α, and IFN-γ resulted in the induction of RANTES mRNA and protein (Ortiz et al., 1996; Miyamoto et al., 2000). Intradermal injection of RANTES induces a potent T-lymphocyte and eosinophils recruitment (Fahy et al., 2001; Beck et al., 1997). Intradermal administration of MIP-1a resulted in accumulation of monocytes, lymphocytes, eosinophils and recruitment of neutrophils (Lee et al., 2000). The number of white blood cells, monocytes, and neutrophils was increased in cord blood after 6 days of IL-1α exposure (Nikiforou et al., 2016).
KE5->KE6	Empirical support of the KER between KE5 and KE6 is moderate. Karlmark et al., 2009 found that intrahepatic CD11bF4/80 monocyte-derived cells and liver resident macrophages produce TGF-β1 and thereby directly activate HSCs. It was proven that the treatment of cultured hepatic cells with TGF-β1 increased type I pro-collagen mRNA levels (Czaja et al., 1989).
KE6->KE7	Empirical support of the KER between KE6 and KE7 is moderate. It is difficult to stimulate sufficient collagen production and its incorporation into a pericellular matrix in vitro. Because of that analytical methods have focused on measurement of pro-collagen secreted into culture medium or measurement of α-smooth muscle actin (α-SMA) expression, a marker of fibroblast activation. In primary culture, HSCs from normal liver begin to express α-SMA coincident with culture-induced activation (Rockey et al., 1992; Chen and Raghunath, 2009).

KE7->AO	Empirical support of the KER between KE7 and AO is high. There is a smooth transition from collagen accumulation to liver fibrosis without a definite threshold with plenty in vivo evidence (Poynard et al., 1997; Bataller and Brenner, 2005; Rockey and Friedman, 2006; Brancatelli et al., 2009; Lee and Friedman, 2011).

Quantitative Consideration

Quantitative understanding of the AOP is low, as there is lack of quantitative data on dose-responce relationship. Further research efforts should be made in this direction to improve the quantitative understanding of the present AOP.

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Appendix 1

List of MIEs in this AOP

Event: 1539: Endocytotic lysosomal uptake (https://aopwiki.org/events/1539)

Short Name: endocytosis

Key Event Component

Process	Object	Action
endocytosis	lysosomal membrane	increased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:144 - Endocytic lysosomal uptake leading to liver fibrosis (https://aopwiki.org/aops/144)	MolecularInitiatingEvent

Stressors

Name
nanoparticles
Ciprofloxacin
Norfloxacin
Chloroquine bis(phosphate)
artesunate
3-aminopropanal
o-methyl-serine dodecylamide hydrochloride (MSDH)
ROS

Biological Context

Level of Biological Organization
Molecular

Cell term

Cell term
eukaryotic cell

Evidence for Perturbation by Stressor

Overview for Molecular Initiating Event

Several well-known drugs have lysosomotropic abilities including chloroquine, the antipsychotics chlorpromazine, thioridazine, aripiprazole, the antidepressants desipramine, imipramine, and clomipramine, as well as fluoroquinolone antibiotics; another substance group are lysosomotropic detergents (Villamil Giraldo et al., 2014; Ouedraogo et al., 2000).

Fluoroquinolones such as lomefloxacin, norfloxacin, BAYy 3118 and ciprofloxacin are lysosomotropic substances because of their Lewis acid–base properties characterized by pKa nearby neutrality (Ouedraogo et al. 2000). The anti-malarial and anti-inflammatory agent chloroquine is a basic lipophilic and therefore lysosomotropic compound that accumulates in lysosomes via pH partitioning (Ashoor et al. 2013). 3-aminopropanal has the structure of a weak lysosomotropic base, concentrates within the acidic vacuolar compartment and causes lysosomal rupture (Yu et al., 2003). Artesunate preferably accumulates in the lysosomes (Yang et al., 2014)

Most nanoscale macromolecules and molecular assemblies are internalized through endocytosis upon contact with the cell membrane. Intracellular trafficking of NPs following endocytosis has been reported to be mediated via the endosomal pathway through early endosomes, late endosomes and then lysosomes (Gilleron et al., 2013; Yang et al., 2013; Ng et al., 2015). Verma and Stellacci showed that 3.4-nm gold NPs were taken up into macrophages via pinocytosis and 24 h after internalization they were found in lysosomes. This endocytic fate has also been observed for iron oxide NPs and fullerenes (Verma and Stellacci, 2010).

Jin et al. investigated the cytotoxicity of Nanotitanium dioxide TiO_2 (an industrial material used as an additive in cosmetics, pharmaceuticals, and food colorants and able to penetrate the skin) in mouse fibroblast (L929) cells. They saw that TiO_2 NPs were phagocytosed and encapsulated in the lysosomes (Jin et al. 2008).

The rate and mechanism of NP uptake are dependent on physiochemical causes related to the properties of the NPs and the cells, but also the local microenvironment (Zhang, 2015). Nanomaterial shape and size contribute significantly to their interaction with cells (Verma and Stellacci, 2010; Oh, 2014). Several reports showing that NPs of 20—50nm are taken up more rapidly than smaller or larger particles (Lu et al., 2009; Iversen et al., 2011; Dykman and Khlebtsov, 2014).

Other variables that could influence the uptake of a NP cargo include orientation, density and steric freedom of targeting ligands and surface groups (Cleal et al., 2013). Most NPs are first coated with serum proteins before they reach cell plasma membranes; endocytosis patterns of aggregated or agglomerated NPs differ from the one of individual NPs (Oh and Park, 2014).

Harush-Frenkel et al. compared the endocytosis into HeLa cells of NPs exposing either a negative or positive charge on their surface and found that the exposed charge significantly affected their ability to internalize as well as the cellular endocytosis mechanism utilized. Negatively charged NPs showed an inferior rate of endocytosis and did not utilize the clathrin-mediated endocytosis pathway, while positively charged NPs internalize rapidly primarily via clathrin-mediated pathways as well as macropinocytosis. When the clathrin-mediated endocytosis pathway is blocked positively charged NPs activate a compensatory endocytosis pathway that results in enhanced accumulation of NPs (Harush-Frenkel et al., 2007). In contrast, a higher uptake of negatively charged quantum dot NPs has been reported in HEK cells by Zhang and Monteiro-Riviere (2009).

Schuetz et al. demonstrated that positively charged SiNPs enter cells largely via dynamin 2-dependent caveolar internalization rather than clathrin-mediated endocytosis and accumulate in lysosomes (Schuetz et al., 2016).

Ng et al. have shown that the uptake of 20 nm size AuNPs in MRC5 lung fibroblasts and Chang liver cells was dependent upon clathrin-mediated endocytosis (Ng et al., 2014). Yang et al. studied the cellular uptake of ultra-small fluorescent gold nanoclusters (AuNCs) by HeLa cells and found that this energy-dependent process involved multiple mechanisms, with clathrin-mediated endocytosis and macropinocytosis appearing to play a significant role, whereas the caveolin-mediated pathway contributing only to a lesser extent (Yang et al., 2013).

Gilleron et al. monitored the uptake of lipid NPs (LNPs) loaded with traceable siRNAs in different cell types in vitro and in mouse liver and found that LNPs enter cells by both constitutive and inducible pathways in a cell type-specific manner using clathrin-mediated endocytosis as well as macropinocytosis (Gilleron et al., 2013).

Saw et al. showed that the major uptake of confeito Au NP of 30 nm was both clathrin and caveolin mediated endocytosis, while for 60, 80 and 100 nm NP was via clathrin mediated pathway. Internalization by both clathrin and caveolin pathways explains higher cellular uptake of 30 nm Au NP compared to other ones (Saw et al., 2018). However, Brandenberger et al. showed that uptake of 30 nm citrate-cappedsphere Au NP was by a macroopinocytosis mechanism (Brandenberger et al., 2010).

It is obvious that not only size of NP, but also the surface property and shape are also important factors in the type of cellular uptake of NPs. As demonstrated earlier cationic NPs favor clathrin mediated endocytosis possibly due to electrostatic interaction with the cell surface receptors. However, not all the studies confirmed this preference of cationic NPs for clathrin mediated internalization. In contrast, caveolin mediated endocytosis occurs by interaction between hydrophobic group of NP with lipid raft on cell surface (Chakraborty and Jana, 2015). There is a connection between the endocytosis mechanism and the subcellular localization of the NPs. Clathrin mediated endocytosis leads to localization of NPs to the lysosome. Also, it has been found that if uptake of NPs occurs via both clathrin and lipid raft- mediated endocytosis, subcellular localization will also be mainly lysosomal. Caveolin mediated uptake of NPs is followed with formation of vesicles with neutral pH and transport to endoplasmic reticulum, Golgi apparatus or even nucleus (Rejman et al., 2005)

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
human	Homo sapiens		NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)

Term	Scientific Term	Evidence	Links
rat	Rattus norvegicus		NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10116)
mouse	Mus musculus		NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10090)

Life Stage Applicability

Life Stage	Evidence
All life stages	

Sex Applicability

Sex	Evidence
Unspecific	

Endocytosis is an universal internalization route in eukaryotes (Dergai et al., 2016). In most animal cells, clathrin-coated pits and vesicles provide an efficient pathway for taking up specific macromolecules from the extracellular fluid (Cooper, 2000) and it is also a mechanism for uptaking extracellular molecules in plant cells (Fan et al., 2015).

The experimental studies have been done on mice in vivo, primary human and rat cells, and human and animal (rat, mouse)-derived cell lines (Harusch Fraenkel et al., 2007; Ng et al., 2015; Nadanaciva et al., 2011; Lu et al., 2017; Xie et al., 2007; Verma and Stellacci, 2010; Zhang and Monteiro-Riviere, 2009).

Key Event Description

Endocytosis was discovered by the Belgian Nobel laureate Christian De Duve in 1963.

Endocytosis is a form of active transport in which molecules are transported into the cell by engulfing them in an energy-using process. In endocytosis, the material to be internalized is surrounded by an area of plasma membrane, which then buds off inside the cell to form a vesicle containing the ingested material. The ingestion of large particles (generally >250 nm in diameter) is termed phagocytosis (cell eating); phagocytosis is actin-dependent and restricted to professional phagocytes. The non-specific receptor-independent process to internalize fluids and solutes is called pinocytosis (cell drinking; via small vesicles of about 100 nm in diameter) and found in all cells (Cooper, 2000; Alberts et al., 2002; Oh and Park, 2014).

Receptor-mediated endocytosis can be clathrin-, and caveolin-dependent; these proteins mediate the invagination of the cell membrane.

The clathrin-mediated endocytotic pathway produces small (approx. 100 nm in diameter) vesicles coated with the cytosolic protein clathrin, forming clathrin-coated pits in the plasma membrane.

Caveolae-mediated endocytosis produces small (approximately. 50 nm in diameter) caveolae, flask-shape pits in the membrane coated with the protein caveolin, derived from lipid rafts (rigid membrane microdomains enriched with phospholipids, sphingolipids, and cholesterol). Clathrin- and caveolae-independent endocytosis is further sub-classified as Arf6- dependent, flotillin-dependent, Cdc42-dependent and RhoA-dependent endocytosis (Cleal et al., 2013; Villamil Giraldo et al., 2014; Iversen et al., 2011; Sahay et al., 2010; Kirkham and Parton, 2005; Mailaender and Landfester, 2009).

Vesicles rapidly lose their coats and fuse to form larger compartments, known as endosomes.

Early endosomes are the first compartment of the endocytic pathway and receive most types of vesicles coming from the cell surface. Endocytosed material is transferred to late endosomes and further to lysosomes, vacuoles of 1-2 µm in diameter containing hydrolytic enzymes in an acid milieu. Their main task is the degradation of ingested material (Villamil Giraldo et al., 2014).

Substances that are taken up selectively into lysosomes are called lysosomotropic agents (De Duve et al., 1974). These agents tend to have both lipophilic or amphiphilic compounds with basic moieties and accumulate in the acidic intracellular compartment, where they become protonated, therefore cannot diffuse back into the cytosol and accumulate within lysosomes, a phenomenon called "acid trapping" (Villamil Giraldo et al., 2014).

Although structurally and pharmacologically diverse, lysosomotropic compounds share certain

physicochemical properties, namely a ClogP > 2 (partition coefficient of the neutral species of a compound between octanol and water) and a basic pKa (the negative base-10 logarithm of the acid dissociation constant of a solution; the lower the pKa value, the stronger the acid) between 6.5 and 11 (Nadanaciva et al., 2010; Lu et al., 2017).

How it is Measured or Detected

- Lysosomal Trapping assay: A fluorescent dye, LysoTracker Red DND-99, which accumulates in lysosomes, is used for detecting

lysosomotropism. There is a gradual decrease in the LysoTracker staining with increasing concentrations of lysosomotropic compounds. This assay is commercially available (Nadanaciva et al., 2011).

- The most widespread method for studying intracellular trafficking involves attaching different fluorescent probes to the protein and nanomaterial that allows analyzing distribution of colour or fluorescence resonance energy transfer (FRET) throughout the cell compartments. The advantage of such approach is that it allows using live cell imaging by confocal microscopy (Sahay et al., 2010).
- Cells can be transfected with constructs containing proteins that reside in specific endocytosis vesicles or intracellular organelles, which are fused with fluorescent proteins, such as the Green Fluorescent Protein (GFP) (Sahay et al., 2010).
- Apart from confocal microscopy, the electron microscopy is also highly useful as it allows visualizing nanomaterials coupled with electron dense labels in different vesicular structures under very high resolution. Atomic Force Microscopy (AFM) has also been used recently to demonstrate the interactions of nanomaterials with the cell membrane (Sahay et al., 2010).
- Exclusion of specific endocytosis mechanisms is a distinct and powerful technique to elucidate endocytosis. This can be achieved, for example, using various pharmacologic inhibitors of endocytosis that include chemical or biological agents or cell mutants (Sahay et al., 2010).
- Co-localization studies of nanomaterials with specific endocytosis markers and structures “pulse-chase” design: proteins, such as transferrin or cholera toxin B (CTB), with known trafficking pathways are exposed to cells simultaneously or before the nanomaterial (“pulse”) and their inclusion or exclusion from the same vesicles is detected at different time points (“chase”) (Sahay et al., 2010).
- Another way of studying co-localization is immunocytochemistry applied to fixed cells. This method allows employing specific antibodies to different proteins present along the endocytic vesicles and organelles (Sahay et al., 2010).
- Transmission electron microscopy (TEM) is an appropriate technique to use for visualizing NPs inside cells, since light microscopy fails to resolve them at a single particle level. TEM is a microscopy technique in which a beam of electrons is transmitted through a specimen to form an image (Brandenberger et al., 2010; Villamil Giraldo et al., 2016).
- Fluorescence Correlation Spectroscopy is a correlation analysis of fluctuation of the fluorescence intensity (Villamil Giraldo et al., 2016).
- Evaluation of NP cellular uptake by confocal laser scanning microscopy (CLSM). CLSM combines high-resolution optical imaging with depth selectivity which allows optical sectioning. The CLSM works by passing a laser beam through a light source aperture which is focused by an objective lens into a small area on the surface of the sample and an image is built up pixel-by-pixel by collecting the emitted photons from the fluorophores in the sample (Harush-Frenkel et al., 2006; Zhang and Monteiro-Riviere, 2009).
- Fluorescence-activated cell sorter (FACS) analysis of NP cellular uptake. FACS is a specialized type of flow cytometry. It provides a method for sorting a heterogeneous mixture of biological based upon the specific light scattering and fluorescent characteristics of each cell (Harush-Frenkel et al., 2006; Zhang and Monteiro-Riviere, 2009).
- Quantitative cellular uptake of AuNPs was performed by inductively coupled plasma mass spectrometry (ICPMS), a type of mass spectrometry capable of detecting metals and several non-metals at very low concentrations. This is achieved by ionizing the sample with inductively coupled plasma and then using a mass spectrometer to separate and quantify those ions (Ng et al., 2015).
- We describe a single-step density gradient subcellular fractionation method combined with fluorescent detection analysis that provides a new tool for characterisation of endocytic traffic of polymer therapeutics for an understanding of intracellular trafficking pathways (Manunta et al., 2007).

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List of Key Events in the AOP

Event: 898: Disruption, Lysosome (https://aopwiki.org/events/898)

Short Name: Disruption, Lysosome

Key Event Component

Process	Object	Action
organelle disassembly	lysosome	occurrence

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:257 - Receptor mediated endocytosis and lysosomal overload leading to kidney toxicity (https://aopwiki.org/aops/257)	KeyEvent
Aop:144 - Endocytic lysosomal uptake leading to liver fibrosis (https://aopwiki.org/aops/144)	KeyEvent

Biological Context

Level of Biological Organization
Cellular

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
human	Homo sapiens	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)
mouse	Mus musculus	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10090)

Life Stage Applicability

Life Stage	Evidence
All life stages	

Sex Applicability

Sex	Evidence
Unspecific	

Typically, human or murine cell lines are used to assess this event. Examples are

murine (Reiners et al., 2002)

murine, human (Li et al., 2000)

murine, human (Ghosh et al., 2011)

human (Loos et al., 2014)

human, murine (Anguissola et al., 2014)

human (Hamacher-Brady et al., 2011)

Key Event Description

Lysosomes were first described in 1955 (de Duve et al., 1955). They are acidic, single-membrane bound organelles that are present in all eukaryotic cells and are filled with more than 50 acid hydrolases to serve their purpose of degrading macromolecules (Johansson et al., 2010).

Lysosomes are the terminal organelle of the endocytic pathway, but are also involved in membrane repair and other cellular processes, such as immune responses (Repnik and Turk, 2010). There are numerous substances that can provoke increased permeability of lysosomal membrane or total lysosomal rupture, and as a consequence release of lysosomal enzymes. Among lysosomal enzymes, one of the major roles has cathepins. There are 11 cysteine cathepins in humans, B, C, F, H, K, L, O, S, V, W and X. Activation of proenzymes usually occurs within the lysosomes (Ishidoh and Kominami, 2002), therefore, the enzymes escaping from the lysosomes are in their active form. The amount of lysosomal enzymes that are released into the cytosol regulates the cell death pathway which is initiated by lysosomal damage: controlled increased permeability of lysosomal membrane, caused by limited level of stress, plays a vital role in the induction of apoptosis, whereas massive lysosomal rupture, caused by high stress levels, leads to necrosis (Bursch, 2001; Guicciardi et al., 2004). Lysosomes are known to be involved in external as well as internal apoptotic pathways. The external pathway triggers lysosomal destabilization by hydroxyl radicals, p53, and caspase 8, through activation of Bax or by ceramide which is converted into sphingosine (Terman et al., 2004). The internal apoptotic pathway on the contrary is activated through mitochondrial damage, for example via activation of Bax or Bid, phospholipases, or lysosomal enzymes (Terman et al., 2004). It has been shown that lack of cathepsin B prevents increased lysosomal membrane permeability in hepatocytes treated with TNF or sphingosine (Werneburg et al., 2002). This indicates that cathepsins can also have a role in initiation of increased lysosomal membrane permeabilization.

The lysosome contains redox-active labile irons which are suggested to be involved in local ROS production via a Fenton-type reaction (Kubota et al., 2010). It has been shown that lysosomal membrane disruption induced by lysosomotropic detergents causes early induction of lysosomal cathepsin B and D and induction of ferritin, together with an increase of cellular ROS and concomitant reduction of the antioxidants MnSOD (manganese superoxide dismutase) and GSH (glutathione), possibly due to the release of free iron into the cytosol (Ghosh et al., 2011; Hamacher-Brady et al., 2011).

The list of agents able to destabilize lysosomal membrane includes L-Leucyl-L-leucinemethyl ester (LLOMe) (Goldman and Kaplan, 1973; Uchimoto et al. 1999; Droga-Mazovec et al., 2008), N-dodecylimidazole (NDI) (Wilson et al., 1987), sphingosine (Kagedal et al., 2001), detergent MSDH (Li et al., 2000), siramesine (Ostenfeld et al., 2005), the quinolone antibiotics ciprofloxacin and norfloxacin (Boya et al., 2003a), hydroxychloroquine (Boya et al., 2003b) and NPs (Wang et al., 2018).

Cirman et al. showed that the lysosomotropic agent LLOMe inducing the disruption of lysosomes results in translocation of lysosomal proteases to the cytosol and induce apoptosis through a caspase dependent mechanism (Cirman et al., 2004). However, it has been proven that partially increased permeabilization of lysosome leads to apoptosis, while complete breakdown of the lysosome with release of high concentrations of the enzymes into the cytosol results in necrosis (Bursch, 2001; Kurz et al., 2008).

The short-term exposure to low concentrations of H_2O_2 induce lysosomal rupture by activation of phospholipase A2, which cause a progressive destabilization of the membranes of intracellular organelles degrading the membrane phospholipids (Zhao et al., 2001). Sumoza-Toledo and Penner showed that ROS activate lysosomal Ca^{2+} channels and contribute to increased lysosomal permeability (Sumoza-Toledo and Penner, 2011).

Considering nanomaterials (NMs) as a trigger for lysosomal damage, recent studies underpinned the importance of lysosomal NM uptake for NM-induced toxicity. Once the material is taken up by a cell and transported to the lysosome by autophagy, the acidic milieu herein can either enhance solubility of a NM, or the material remains in its initial nano form. Both situations can induce toxicity, causing lysosomal swelling, followed by lysosomal disruption and the release of pro-apoptotic proteins (Wang et al., 2013; Cho et al., 2011; Cho et al., 2012). Wang et al. showed that the exposure of cells to NH_2-PS NPs results in increased lysosomal membrane permeability and release of lysosomal proteasis (cathepsin B and cathepsin D) into cytosol (Wang et al., 2018).

How it is Measured or Detected

Lysosomes are typically analysed microscopically, usually with fluorescence microscopy (Kagedal et al., 2001).

Changes in morphology can be observed by using acridine orange (AO), a weak base that accumulates in the acidic compartment of the cell mainly composed of lysosomes. Red fluorescence is exhibited when it is highly concentrated in acidic vesicles, while green fluorescence is exhibited when it's less concentrated in other parts of the cell (Li et al., 2000; Reiners et al., 2002; Kroemer and Jäättelä, 2005). This is followed by flow cytometry (Zhao et al., 2001), static cytofluometry or flow cytofluometry (Antunes et al., 2001).

Lysotracker green (200 nM) is regularly used to assess lysosomal acidification; Anguissola and colleagues reported that it was excited through a 475+/240 nm band pass filter and fluorescence emission was collected through a 515+/220 nm band pass filter. Analysis is performed using microscopical methods such as High Content Analysis (Anguissola et al., 2014). This method as well as use of LysoSensor probes has been reported repeatedly elsewhere, for example (Wang et al., 2013; Kroemer and Jäättelä, 2005).

More specific staining can be achieved by staining with antibodies against lysosomal membrane proteins (Kroemer and Jäättelä, 2005).

Lysosomal membrane permeabilization can be visualized by immunostaining of lysosomal enzymes such as cathepsin B (Boya et al. 2003a).

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Event: 177: N/A, Mitochondrial dysfunction 1 (https://aopwiki.org/events/177)

Short Name: N/A, Mitochondrial dysfunction 1

Key Event Component

Process	Object	Action
	mitochondrion	functional change

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:48 - Binding of agonists to ionotropic glutamate receptors in adult brain causes excitotoxicity that mediates neuronal cell death, contributing to learning and memory impairment. (https://aopwiki.org/aops/48)	KeyEvent
Aop:77 - Nicotinic acetylcholine receptor activation contributes to abnormal foraging and leads to colony death/failure 1 (https://aopwiki.org/aops/77)	KeyEvent
Aop:78 - Nicotinic acetylcholine receptor activation contributes to abnormal role change within the worker bee caste leading to colony death failure 1 (https://aopwiki.org/aops/78)	KeyEvent

AOP ID and Name	Event Type
Aop:79 - Nicotinic acetylcholine receptor activation contributes to impaired hive thermoregulation and leads to colony loss/failure (https://aopwiki.org/aops/79)	KeyEvent
Aop:80 - Nicotinic acetylcholine receptor activation contributes to accumulation of damaged mitochondrial DNA and leads to colony loss/failure (https://aopwiki.org/aops/80)	KeyEvent
Aop:87 - Nicotinic acetylcholine receptor activation contributes to abnormal foraging and leads to colony loss/failure (https://aopwiki.org/aops/87)	KeyEvent
Aop:3 - Inhibition of the mitochondrial complex I of nigro-striatal neurons leads to parkinsonian motor deficits (https://aopwiki.org/aops/3)	KeyEvent
Aop:144 - Endocytic lysosomal uptake leading to liver fibrosis (https://aopwiki.org/aops/144)	KeyEvent
Aop:178 - Nicotinic acetylcholine receptor activation contributes to mitochondrial dysfunction and leads to colony loss/failure (https://aopwiki.org/aops/178)	KeyEvent
Aop:200 - Estrogen receptor activation leading to breast cancer (https://aopwiki.org/aops/200)	KeyEvent

Biological Context

Level of Biological Organization
Cellular

Cell term

Cell term
eukaryotic cell

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
human	Homo sapiens	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)
mouse	Mus musculus	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10090)
rat	Rattus norvegicus	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10116)

Life Stage Applicability

Life Stage	Evidence
All life stages	

Sex Applicability

Sex	Evidence
Unspecific	

Mitochondrial dysfunction is a universal event occurring in cells of any species (Farooqui and Farooqui, 2012). Many invertebrate species (drosophila, C, elegans) are considered as potential models to study mitochondrial function. New data on marine invertebrates, such as molluscs and crustaceans and non-Drosophila species, are emerging (Martinez-Cruz et al., 2012). Mitochondrial dysfunction can be measured in animal models used for toxicity testing (Winklhofer and Haass, 2010; Waerzeggers et al., 2010) as well as in humans (Winklhofer and Haass, 2010).

Key Event Description

Mitochondrial dysfunction is a consequence of inhibition of the respiratory chain leading to oxidative stress.

Mitochondria can be found in all cells and are considered the most important cellular consumers of oxygen. Furthermore, mitochondria possess numerous redox enzymes capable of transferring single electrons to oxygen, generating the superoxide (O2-). Some mitochondrial enzymes that are involved in reactive oxygen species (ROS) generation include the electron-transport chain (ETC) complexes I, II and III; pyruvate dehydrogenase (PDH) and glycerol-3-phosphate dehydrogenase (GPDH). The transfer of electrons to oxygen, generating superoxide, happens mainly when these redox carriers are charged enough with electrons and the potential energy for transfer is elevated, like in the case of high mitochondrial membrane potential. In contrast, ROS generation is decreased if there are not enough electrons and the potential energy for the transfer is not sufficient (reviewed in Lin and Beal, 2006).

Cells are also able to detoxify the generated ROS due to an extensive antioxidant defence system that includes superoxide dismutases, glutathione peroxidases, catalase, thioredoxins, and peroxiredoxins in various cell organelles (reviewed in Lin and Beal, 2006). It is worth mentioning that, as in the case of ROS generation, antioxidant defences are also closely related to the redox and energetic status of mitochondria. If mitochondria are structurally and functionally healthy, an antioxidant defence mechanism balances ROS generation, and there is not much available ROS production. However, in case of mitochondrial damage, the antioxidant defence capacity drops and ROS generation takes over. Once this happens, a vicious cycle starts and ROS can further damage mitochondria, leading to more free-radical generation and further loss of antioxidant capacity. During mitochondrial dysfunction the availability of ATP also decreases, which is considered necessary for repair mechanisms after ROS generation.

A number of proteins bound to the mitochondria or endoplasmic reticulum (ER), especially in the mitochondria-associated ER membrane (MAM), are playing an important role of communicators between these two organelles (reviewed Mei et al., 2013). ER stress induces mitochondrial dysfunction through regulation of Ca2+ signaling and ROS production (reviewed Mei et al., 2013). Prolonged ER stress leads to release of Ca2+ at the MAM and increased Ca2+ uptake into the mitochondrial matrix, which induces Ca2+-dependent mitochondrial outer membrane permeabilization and apoptosis. At the same, ROS are produced by proteins in the ER oxidoreductin 1 (ERO1) family. ER stress activates ERO1 and leads to excessive production of ROS, which, in turn, inactivates SERCA and activates inositol-1,4,5- trisphosphate receptors (IP3R) via oxidation, resulting in elevated levels of cytosolic Ca2+, increased mitochondrial uptake of Ca2+, and ultimately mitochondrial dysfunction. Just as ER stress can lead to mitochondrial dysfunction, mitochondrial dysfunction also induces ER Stress (reviewed Mei et al., 2013). For example, nitric oxide disrupts the mitochondrial respiratory chain and causes changes in mitochondrial Ca2+ flux which induce ER stress. Increased Ca2+ flux triggers loss of mitochondrial membrane potential (MMP), opening of mitochondrial permeability transition pore (MPTP), release of cytochrome c and apoptosis inducing factor (AIF), decreasing ATP synthesis and rendering the cells more vulnerable to both apoptosis and necrosis (Wang and Qin, 2010).

Summing up: Mitochondria play a pivotal role in cell survival and cell death because they are regulators of both energy metabolism and apoptotic/necrotic pathways (Fiskum, 2000; Wieloch, 2001; Friberg and Wieloch, 2002). The production of ATP via oxidative phosphorylation is a vital mitochondrial function (Kann and Kovács, 2007; Nunnari and Suomalainen, 2012). The ATP is continuously required for signalling processes (e.g. Ca2+ signalling), maintenance of ionic gradients across membranes, and biosynthetic processes (e.g. protein synthesis, heme synthesis or lipid and phospholipid metabolism) (Kang and Pervaiz, 2012), and (Green, 1998; McBride et al., 2006). Inhibition of mitochondrial respiration contributes to various cellular stress responses, such as deregulation of cellular Ca2+ homeostasis (Graier et al., 2007) and ROS production (Nunnari and Suomalainen, 2012; reviewed Mei et al., 2013).). It is well established in the existing literature that mitochondrial dysfunction may result in: (a) an increased ROS production and a decreased ATP level, (b) the loss of mitochondrial protein import and protein biosynthesis, (c) the reduced activities of enzymes of the mitochondrial respiratory chain and the Krebs cycle, (d) the loss of the mitochondrial membrane potential, (e) the loss of mitochondrial motility, causing a failure to re-localize to the sites with increased energy demands (f) the destruction of the mitochondrial network, and (g) increased mitochondrial Ca2+ uptake, causing Ca2+ overload (reviewed in Lin and Beal, 2006; Graier et al., 2007), (h) the rupture of the mitochondrial inner and outer membranes, leading to (i) the release of mitochondrial pro-death factors, including cytochrome c (Cyt. c), apoptosis-inducing factor, or endonuclease G (Braun, 2012; Martin, 2011; Correia et al., 2012; Cozzolino et al., 2013), which eventually leads to apoptotic, necrotic or autophagic cell death (Wang and Qin, 2010). Due to their structural and functional complexity, mitochondria present multiple targets for various compounds.

How it is Measured or Detected

Mitochondrial dysfunction can be detected using isolated mitochondria, intact cells or cells in culture as well as in vivo studies. Such assessment can be performed with a large range of methods (revised by Brand and Nicholls, 2011) for which some important examples are given. All approaches to assess mitochondrial dysfunction fall into two main categories: the first assesses the consequences of a loss-of-function, i.e. impaired functioning of the respiratory chain and processes linked to it. Some assay to assess this have been described for KE1, with the limitation that they are not specific for complex I. In the context of overall mitochondrial dysfunction, the same assays provide useful information, when performed under slightly different assay conditions (e.g. without addition of complex III and IV inhibitors). The second approach assesses a ‘non-desirable gain-of-function’, i.e. processes that are usually only present to a very small degree in healthy cells, and that are triggered in a cell, in which mitochondria fail.

I. Mitochondrial dysfunction assays assessing a loss-of function.

1. Cellular oxygen consumption.

See KE1 for details of oxygen consumption assays. The oxygen consumption parameter can be combined with other endpoints to derive more specific information on the efficacy of mitochondrial function. One approach measures the ADP-to-O ratio (the number of ADP molecules phosphorylated per oxygen atom reduced (Hinkle, 1995 and Hafner et al., 1990). The related P/O ratio is calculated from the amount of ADP added, divided by the amount of O_2 consumed while phosphorylating the added ADP (Ciapaite et al., 2005; Diepart et al., 2010; Hynes et al., 2006; James et al., 1995; von Heimburg et al., 2005).

2. Mitochondrial membrane potential (Δψm).

The mitochondrial membrane potential (Δψm) is the electric potential difference across the inner mitochondrial membrane. It requires a functioning respiratory chain in the absence of mechanisms that dissipate the proton gradient without coupling it to ATP production. The classical, and still most quantitative method uses a tetraphenylphosphonium ion (TPP+)-sensitive electrode on suspensions of isolated mitochondria. The Δψm can also be measured in live cells by fluorimetric methods. These are based on dyes which accumulate in mitochochondria because of Δψm. Frequently used are tetramethylrhodamineethylester (TMRE), tetramethylrhodaminemethyl ester (TMRM) (Petronilli et al., 1999) or 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazole carbocyanide iodide (JC-1). Mitochondria with intact membrane potential concentrate JC-1, so that it forms red fluorescent aggregates, whereas de-energized mitochondria cannot concentrate JC-1 and the dilute dye fluoresces green (Barrientos et al., 1999). Assays using TMRE or TMRM measure only at one wavelength (red fluorescence), and depending on the assay setup, de-energized mitochondria become either less fluorescent (loss of the dye) or more fluorescent (attenuated dye quenching).

3. Enzymatic activity of the electron transport system (ETS).

Determination of ETS activity can be dene following Owens and King's assay (1975). The technique is based on a cell-free homogenate that is incubated with NADH to saturate the mitochondrial ETS and an artificial electron acceptor [I - (4 -iodophenyl) -3 - (4 -nitrophenyl) -5-phenylte trazolium chloride (INT)] to register the electron transmission rate. The oxygen consumption rate is calculated from the molar production rate of INT-formazan which is determined spectrophotometrically (Cammen et al., 1990).

4. ATP content.

For the evaluation of ATP levels, various commercially-available ATP assay kits are offered based on luciferin and luciferase activity. For isolated mitochondria various methods are available to continuously measure ATP with electrodes (Laudet 2005), with luminometric methods, or for obtaining more information on different nucleotide phosphate pools (e.g. Ciapaite et al., (2005).

II. Mitochondrial dysfunction assays assessing a gain-of function.

1. Mitochondrial permeability transition pore opening (PTP).

The opening of the PTP is associated with a permeabilization of mitochondrial membranes, so that different compounds and cellular constituents can change intracellular localization. This can be measured by assessment of the translocation of cytochrome c, adenylate kinase or AIF from mitochondria to the cytosol or nucleus. The translocation can be assessed biochemically in cell fractions, by imaging approaches in fixed cells or tissues or by life-cell imaging of GFP fusion proteins (Single 1998; Modjtahedi 2006). An alternative approach is to measure the accessibility of cobalt to the mitochondrial matrix in a calcein fluorescence quenching assay in live permeabilized cells (Petronilli et al., 1999).

2. mtDNA damage as a biomarker of mitochondrial dysfunction.

Various quantitative polymerase chain reaction (QPCR)-based assays have been developed to detect changes of DNA structure and sequence in the mitochondrial genome. mtDNA damage can be detected in blood after low-level rotenone exposure, and the damage persists even after CI activity has returned to normal. With a more sustained rotenone exposure, mtDNA damage is also detected in skeletal muscle. These data support the idea that mtDNA damage in peripheral tissues in the rotenone model may provide a biomarker of past or ongoing mitochondrial toxin exposure (Sanders et al., 2014a and 2014b).

3. Generation of ROS and resultant oxidative stress.

a. General approach. Electrons from the mitochondrial ETS may be transferred ‘erroneously’ to molecular oxygen to form superoxide anions. This type of side reaction can be strongly enhanced upon mitochondrial damage. As superoxide may form hydrogen peroxide, hydroxyl radicals or other reactive oxygen species, a large number of direct ROS assays and assays assessing the effects of ROS (indirect ROS assays) are available (Adam-Vizi, 2005; Fan and Li 2014). Direct assays are based on the chemical modification of fluorescent or luminescent reporters by ROS species. Indirect assays assess cellular metabolites, the concentration of which is changed in the presence of ROS (e.g. glutathione, malonaldehyde, isoprostanes,etc.) At the animal level the effects of oxidative stress are measured from biomarkers in the blood or urine.

b. Measurement of the cellular glutathione (GSH) status. GSH is regenerated from its oxidized form (GSSH) by the action of an NADPH dependent reductase (GSSH + NADPH + H+ à 2 GSH + NADP+). The ratio of GSH/GSSG is therefore a good indicator for the cellular NADH+/NADPH ratio (i.e. the redox potential). GSH and GSSH levels can be determined by HPLC, capillary electrophoresis, or biochemically with DTNB (Ellman’s reagent). As excess GSSG is rapidly exported from most cells to maintain a constant GSH/GSSG ratio, a reduction of total glutathione (GSH/GSSG) is often a good surrogate measure for oxidative stress.

c. Quantification of lipid peroxidation. Measurement of lipid peroxidation has historically relied on the detection of thiobarbituric acid (TBA)-reactive compounds such as malondialdehyde generated from the decomposition of cellular membrane lipid under oxidative stress (Pryor et al., 1976). This method is quite sensitive, but not highly specific. A number of commercial assay kits are available for this assay using absorbance or fluorescence detection technologies. The formation of F2-like prostanoid derivatives of arachidonic acid, termed F2-isoprostanes (IsoP) has been shown to be more specific for lipid peroxidation. A number of commercial ELISA kits have been developed for IsoPs, but interfering agents in samples requires partial purification before analysis. Alternatively, GC/MS may be used, as robust (specific) and sensitive method.

d. Detection of superoxide production. Generation of superoxide by inhibition of complex I and the methods for its detection are described by Grivennikova and Vinogradov (2014). A range of different methods is also described by BioTek (http://www.biotek.com/resources/articles/reactive-oxygen-species.html (http://www.biotek.com/resources/articles/reactive-oxygen-species.html)). The reduction of ferricytochrome c to ferrocytochrome c may be used to assess the rate of superoxide formation (McCord, 1968). Like in other superoxide assays, specificity can only be obtained by measurements in the absence and presence of superoxide dismutase. Chemiluminescent reactions have been used for their increased sensitivity. The most widely used chemiluminescent substrate is lucigenin. Coelenterazine has also been used as a chemiluminescent substrate. Hydrocyanine dyes are fluorogenic sensors for superoxide and hydroxyl radical, and they become membrane impermeable after

oxidation (trapping at site of formation). The best characterized of these probes are Hydro-Cy3 and Hydro-Cy5. generation of superoxide in mitochondria can be visualized using fluorescence microscopy with MitoSOX™ Red reagent (Life Technologies). MitoSOX™ Red reagent is a cationic derivative of dihydroethidium that permeates live cells and accumulates in mitochondria.

e. Detection of hydrogen peroxide (H_2O_2) production. There are a number of fluorogenic substrates, which serve as hydrogen donors that have been used in conjunction with horseradish peroxidase (HRP) enzyme to produce intensely fluorescent products in the presence of hydrogen peroxide (Zhou et al., 1997: Ruch et al., 1983). The more commonly used substrates include diacetyldichloro-fluorescein, homovanillic acid, and Amplex® Red. In these examples, increasing amounts of H_2O_2 form increasing amounts of fluorescent product (Tarpley et al., 2004).

Summing up, mitochondrial dysfunction can be measured by: • ROS production: superoxide (O2-), and hydroxyl radicals (OH–) • Nitrosative radical formation such as ONOO– or directly by: • Loss of mitochondrial membrane potential (MMP) • Opening of mitochondrial permeability transition pores (MPTP) • ATP synthesis • Increase in mitochondrial Ca2+ • Cytochrome c release • AIF (apoptosis inducing factor) release from mitochondria • Mitochondrial Complexes enzyme activity • Measurements of mitochondrial oxygen consumption • Ultrastructure of mitochondria using electron microscope and mitochondrial fragmentation measured by labelling with DsRed-Mito expression (Knott et al, 2008) Mitochondrial dysfunction-induced oxidative stress can be measured by: • Reactive carbonyls formations (proteins oxidation) • Increased 8-oxo-dG immunoreactivity (DNA oxidation) • Lipid peroxidation (formation of malondialdehyde (MDA) and 4- hydroxynonenal (HNE) • 3-nitrotyrosine (3-NT) formation, marker of protein nitration • Translocation of Bid and Bax to mitochondria • Measurement of intracellular free calcium concentration ([Ca2+]i): Cells are loaded with 4 μM fura-2/AM). • Ratio between reduced and oxidized form of glutathione (GSH depletion) (Promega assay, TB369; Radkowsky et al., 1986) • Neuronal nitric oxide synthase (nNOS) activation that is Ca2+-dependent. All above measurements can be performed as the assays for each readout are well established in the existing literature (e.g. Bal-Price and Brown, 2000; Bal-Price et al., 2002; Fujikawa, 2015; Walker et al., 1995). See also KE Oxidative Stress, Increase (https://aopwiki.org/wiki/index.php/Event:209)

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Event: 55: N/A, Cell injury/death (https://aopwiki.org/events/55)

Short Name: N/A, Cell injury/death

Key Event Component

Process	Object	Action
cell death		increased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:48 - Binding of agonists to ionotropic glutamate receptors in adult brain causes excitotoxicity that mediates neuronal cell death, contributing to learning and memory impairment. (https://aopwiki.org/aops/48)	KeyEvent
Aop:13 - Chronic binding of antagonist to N-methyl-D-aspartate receptors (NMDARs) during brain development induces impairment of learning and memory abilities (https://aopwiki.org/aops/13)	KeyEvent
Aop:38 - Protein Alkylation leading to Liver Fibrosis (https://aopwiki.org/aops/38)	KeyEvent
Aop:12 - Chronic binding of antagonist to N-methyl-D-aspartate receptors (NMDARs) during brain development leads to neurodegeneration with impairment in learning and memory in aging (https://aopwiki.org/aops/12)	KeyEvent
Aop:144 - Endocytic lysosomal uptake leading to liver fibrosis (https://aopwiki.org/aops/144)	KeyEvent
Aop:17 - Binding of electrophilic chemicals to SH(thiol)-group of proteins and /or to seleno-proteins involved in protection against oxidative stress during brain development leads to impairment of learning and memory (https://aopwiki.org/aops/17)	KeyEvent
Aop:278 - IKK complex inhibition leading to liver injury (https://aopwiki.org/aops/278)	KeyEvent

Biological Context

Level of Biological Organization
Cellular

Cell term

Cell term
eukaryotic cell

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
human	Homo sapiens	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)
human and other cells in culture	human and other cells in culture	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=0)
Rattus norvegicus	Rattus norvegicus	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10116)
mouse	Mus musculus	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10090)

Life Stage Applicability

Life Stage	Evidence
All life stages	

Sex Applicability

Sex	Evidence
Unspecific	

Cell death is an universal event occurring in cells of any species (Fink and Cookson,2005).

Key Event Description

Two types of cell death can be distinguished by morphological features, although it is likely that these are two ends of a spectrum with possible intermediate forms. Apoptosis involves shrinkage, nuclear disassembly, and fragmentation of the cell into discrete bodies with intact plasma membranes. These are rapidly phagocytosed by neighbouring cells. An important feature of apoptosis is the requirement for adenosine triphosphate (ATP) to initiate the execution phase. In contrast, necrotic cell death is characterized by cell swelling and lysis. This is usually a consequence of profound loss of mitochondrial function and resultant ATP depletion, leading to loss of ion homeostasis, including volume regulation, and increased Ca2+. The latter activates a number of nonspecific hydrolases (i.e., proteases, nucleases, and phospholipases) as well as calcium dependent kinases. Activation of calpain I, the Ca2+-dependent cysteine protease cleaves the death-promoting Bcl-2 family members Bid and Bax which translocate to mitochondrial membranes, resulting in release of truncated apoptosis-inducing factor (tAIF), cytochrome c and endonuclease in the case of Bid and cytocrome c in the case of Bax. tAIF translocates to cell nuclei, and together with cyclophilin A and phosphorylated histone H2AX (γH2AX) is responsible for DNA cleavage, a feature of programmed necrosis. Activated calpain I has also been shown to cleave the plasma membrane Na+–Ca2+ exchanger, which leads to build-up of intracellular Ca2+, which is the source of additional increased intracellular Ca2+. Cytochrome c in cellular apoptosis is a component of the apoptosome.

DNA damage activates nuclear poly(ADP-ribose) polymerase-1(PARP-1), a DNA repair enzyme. PARP-1 forms poly(ADP-ribose) polymers, to repair DNA, but when DNA damage is extensive, PAR accumulates, exits cell nuclei and travels to mitochondrial membranes, where it, like calpain I, is involved in AIF release from mitochondria. A fundamental distinction between necrosis and apoptosis is the loss of plasma membrane integrity; this is integral to the former but not the latter. As a consequence, lytic release of cellular constituents promotes a local inflammatory reaction, whereas the rapid removal of apoptotic bodies minimizes such a reaction. The distinction between the two modes of death is easily accomplished in vitro but not in vivo. Thus, although claims that certain drugs induce apoptosis have been made, these are relatively unconvincing. DNA fragmentation can occur in necrosis, leading to positive TUNEL staining. Conversely, when apoptosis is massive, it can exceed the capacity for rapid phagocytosis, resulting in the eventual appearance of secondary necrosis.

Two alternative pathways - either extrinsic (receptor-mediated) or intrinsic (mitochondria-mediated) - lead to apoptotic cell death. The initiation of cell death begins either at the plasma membrane with the binding of TNF or FasL to their cognate receptors or within the cell. The latter is due to

the occurrence of intracellular stress in the form of biochemical events such as oxidative stress, redox changes, covalent binding, lipid peroxidation, and consequent functional effects on mitochondria, endoplasmic reticulum, microtubules, cytoskeleton, or DNA. The intrinsic mitochondrial pathway involves the initiator, caspase-9, which, when activated, forms an “apoptosome” in the cytosol, together with cytochrome c, which translocates from mitochondria, Apaf-1 and dATP. The apoptosome activates caspase-3, the central effector caspase, which in turn activates downstream factors that are responsible for the apoptotic death of a cell (Fujikawa, 2015). Intracellular stress either directly affects mitochondria or can lead to effects on other organelles, which then send signals to the mitochondria to recruit participation in the death process (Fujikawa, 2015; Malhi et al., 2010). Constitutively expressed nitric oxide synthase (nNOS) is a Ca2+-dependent cytosolic enzyme that forms nitric oxide (NO) from L-arginine, and NO reacts with the free radical such as superoxide (O2–) to form the very toxic free radical peroxynitrite (ONOO–). Free radicals such as ONOO–, O2 – and hydroxyl radical (OH–) damage cellular membranes and intracellular proteins, enzymes and DNA (Fujikawa, 2015; Malhi et al., 2010; Kaplowitz, 2002; Kroemer et al., 2009).

How it is Measured or Detected

Necrosis:

LDH is a soluble cytoplasmic enzyme that is present in almost all cells and is released into extracellular space when the plasma membrane is damaged. To detect the leakage of LDH into cell culture medium, a tetrazolium salt is used in this assay. In the first step, LDH produces reduced nicotinamide adenine dinucleotide (NADH) when it catalyzes the oxidation of lactate to pyruvate. In the second step, a tetrazolium salt is converted to a colored formazan product using newly synthesized NADH in the presence of an electron acceptor. The amount of formazan product can be colorimetrically quantified by standard spectroscopy. Because of the linearity of the assay, it can be used to enumerate the percentage of necrotic cells in a sample (Chan et al., 2013).

The MTT assay is a colorimetric assay for assessing cell viability. NAD(P)H-dependent cellular oxidoreductase enzymes may reflect the number of viable cells present. These enzymes are capable of reducing the tetrazolium dye MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide to its insoluble formazan, which has a purple color. Other closely related tetrazolium dyes including XTT, MTS and the WSTs. Tetrazolium dye assays can also be used to measure cytotoxicity (loss of viable cells) or cytostatic activity (shift from proliferation to quiescence) of potential medicinal agents and toxic materials. MTT assays are usually done in the dark since the MTT reagent is sensitive to light (Berridgeet al.,2005).

Propidium iodide (PI) is an intercalating agent and a fluorescent molecule used to stain necrotic cells. It is cell membrane impermeant so it stains only those cells where the cell membrane is destroyed. When PI is bound to nucleic acids, the fluorescence excitation maximum is 535 nm and the emission maximum is 617 nm (Moore et al.,1998)

Alamar Blue (resazurin) fluorescent dye. The oxidized blue non fluorescent Alamar blue is reduced to a pink fluorescent dye in the medium by cell activity (O'Brien et al., 2000) (12).

Neutral red uptake, which is based on the ability of viable cells to incorporate and bind the supravital dye neutral red in lysosomes (Repetto et al., 2008)(13).

ATP assay: Quantification of ATP, signaling the presence of metabolically active cells (CellTiter-Glo; Promega).

Apoptosis:

TUNEL is a common method for detecting DNA fragmentation that results from apoptotic signalling cascades. The assay relies on the presence of nicks in the DNA which can be identified by terminal deoxynucleotidyl transferase or TdT, an enzyme that will catalyze the addition of dUTPs that are secondarily labeled with a marker. It may also label cells that have suffered severe DNA damage.

Caspase activity assays measured by fluorescence. During apoptosis, mainly caspase-3 and -7 cleave PARP to yield an 85 kDa and a 25 kDa fragment. PARP cleavage is considered to be one of the classical characteristics of apoptosis. Antibodies to the 85 kDa fragment of cleaved PARP or to caspase-3 both serve as markers for apoptotic cells that can be monitored using immunofluorescence (Li, Peng et al., 2004).

Hoechst 33342 staining: Hoechst dyes are cell-permeable and bind to DNA in live or fixed cells. Therefore, these stains are often called supravital, which means that cells survive a treatment with these compounds. The stained, condensed or fragmented DNA is a marker of apoptosis (Loo, 2002; Kubbies and Rabinovitch, 1983).

Acridine Orange/Ethidium Bromide staining is used to visualize nuclear changes and apoptotic body formation that are characteristic of apoptosis. Cells are viewed under a fluorescence microscope and counted to quantify apoptosis.

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Event: 1493: Increased Pro-inflammatory mediators (https://aopwiki.org/events/1493)

Short Name: Increased pro-inflammatory mediators

Key Event Component

Process	Object	Action
acute inflammatory response		increased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:17 - Binding of electrophilic chemicals to SH(thiol)-group of proteins and /or to seleno-proteins involved in protection against oxidative stress during brain development leads to impairment of learning and memory (https://aopwiki.org/aops/17)	KeyEvent
Aop:38 - Protein Alkylation leading to Liver Fibrosis (https://aopwiki.org/aops/38)	KeyEvent
Aop:144 - Endocytic lysosomal uptake leading to liver fibrosis (https://aopwiki.org/aops/144)	KeyEvent

Biological Context

Level of Biological Organization
Tissue

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
human	Homo sapiens		NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)
Vertebrates	Vertebrates		NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=0)

Life Stage Applicability

Life Stage	Evidence
All life stages	

Sex Applicability

Sex	Evidence
Unspecific	

LIVER:

Human [Santibañez et al., 2011]

Rat [Luckey and Petersen, 2001]

Mouse [Nan et al., 2013]

Key Event Description

Inflammatory mediators are soluble, diffusible molecules that act locally at the site of tissue damage and infection, and at more distant sites. They can be divided into exogenous and endogenous mediators.

Exogenous mediators of inflammation are bacterial products or toxins like endotoxin or LPS. Endogenous mediators of inflammation are produced from within the (innate and adaptive) immune system itself, as well as other systems. They can be derived from molecules that are normally present in the plasma in an inactive form, such as peptide fragments of some components of complement, coagulation, and kinin systems. Or they can be released at the site of injury by a number of cell types that either contain them as preformed molecules within storage granules, e.g. histamine, or which can rapidly switch on the machinery required to synthesize the mediators.

Table1: a non-exhaustive list of examples for pro-inflammatory mediators

Classes of inflammatory mediators	Examples
Pro-inflammatory cytokines	TNF-a, Interleukins (IL-1, IL-6, IL-8), Interferons (IFN-g), chemokines (CXCL, CCL, GRO-α, MCP-1), GM-CSF
Prostaglandins	PGE2
Bradykinin	
Vasoactive amines	histamine, serotonin
Reactive oxygen species (ROS)	O^{2-}, H_2O_2
Reactive nitrogen species (RNS)	NO, iNOS

The increased production of pro-inflammatory mediators can have negative consequences on the parenchymal cells leading even to cell death, as described for TNF-a or peroxynitrite on neurons (Chao et al., 1995; Brown and Bal-Price, 2003). In addition, via a feedback loop, they can act on the reactive resident cells thus maintaining or exacerbating their reactive state; and by modifying elements of their signalling pathways, they can favour the M1 phenotypic polarization and the chronicity of the inflammatory process (Taetzsch et al., 2015).

Basically, this event occurs equally in various tissues and does not require tissue-specific descriptions. Nevertheless, there are some specificities such as the release of glutamate by brain reactive glial cells (Brown and Bal-Price, 2003; Vesce et al., 2007).The differences may rather reside in the type of insult favouring the increased expression and/or release of a specific class of inflammatory mediators, as well the time after the insult reflecting different stages of the inflammatory process. For these reasons, the analyses of the changes of a battery of inflammatory mediators rather than of a single one is a more adequate measurement of this KE.

LIVER:

When activated, resident macrophages (Kupffer cells) release inflammatory mediators including cytokines, chemokines, lysosomal, and proteolytic enzymes and are a main source of TGF-β1 - the most potent pro-fibrogenic cytokine. Following the role of TGF-β is described in more detail.

Transforming growth factor β (TGF-β) is a pleiotropic cytokine with potent regulatory and

inflammatory activity [Sanjabi et al., 2009; Li and Flavell, 2008a;2008b]. The multi-faceted effects of TGF-β on numerous immune functions are cellular and environmental context dependent [Li et al., 2006]. TGF-β binds to TGF-β receptor II (TGF-βRII) triggering the kinase activity of the cytoplasmic domain that in turn activates TGF-βRI. The activated receptor complex leads to nuclear translocation of Smad molecules,

and transcription of target genes [Li et al., 2006a]. The role of TGF-β as an immune modulator of T cell activity is best exemplified by the similarities between TGF-β1 knockout and T cell specific

TGF-β receptor II knockout mice [Li et al., 2006b; Marie et al., 2006;Shull et al., 1992]. The animals in both of these models develop severe multi-organ autoimmunity and succumb to death within a few weeks after birth [Li et al., 2006b; Marie et al., 2006; Shull et al., 1992]. In addition, in mice where TGF-β signaling is blocked specifically in T cells, the development of natural killer T (NKT) cells, natural regulatory T (nTreg) cells, and CD8+ T cells was shown to be dependent on TGF-β signaling in the thymus [Li et al., 2006b; Marie et al., 2006].

TGF-β plays a major role under inflammatory conditions. TGF-β in the presence of IL-6 drives the differentiation of T helper 17 (Th17) cells, which can promote further inflammation and augment autoimmune conditions [Korn et al., 2009]. TGF-β orchestrates the differentiation of both Treg and Th17 cells in a concentration-dependent manner [Korn et al., 2008]. In addition, TGF-β in combination with IL-4, promotes the differentiation of IL-9- and IL-10-producing T cells, which lack

suppressive function and also promote tissue inflammation [Dardalhon et al., 2008; Veldhoen et al., 2008]. The biological effects of TGF-β under inflammatory conditions on effector and memory CD8+ T cells are much less understood. In a recent study, it was shown that TGF-β has a drastically opposing role on naïve compared to antigen-experienced/memory CD8+ T cells [Filippi et al., 2008]. When cultured *in vitro*, TGF-β suppressed naïve CD8+ T cell activation and IFN-γ production, whereas TGF-β enhanced survival of memory CD8+ T cells and increased the production of IL-17 and IFN-γ [Filippi et al., 2008]. TGF-β also plays an important role in suppressing the cells of the innate immune system.

The transforming growth factor beta (TGF-β) family of cytokines are ubiquitous, multifunctional, and essential to survival. They play important

roles in growth and development, inflammation and repair, and host immunity. The mammalian TGF-β isoforms (TGF-β1, β2 and β3) are secreted as latent precursors and have multiple cell surface receptors of which at least two mediate signal transduction. Autocrine and paracrine effects of TGF-βs can be modified by extracellular matrix, neighbouring cells and other cytokines. The vital role of the TGF-β family is illustrated by the fact that approximately 50% of TGF-1 gene knockout mice die in utero and the remainder succumb to uncontrolled inflammation after birth. The role of TGF-β in homeostatic and pathogenic processes suggests numerous applications in the diagnosis and treatment of various diseases characterised by inflammation and fibrosis. [Clark and Coker, 1998; Santibañez et al., 2011; Pohlers et al., 2009] Abnormal TGF-β regulation and function are implicated in a growing number of fibrotic and inflammatory pathologies, including pulmonary fibrosis, liver cirrhosis, glomerulonephritis and diabetic nephropathy, congestive heart failure, rheumatoid arthritis, Marfan syndrome, hypertrophic scars, systemic sclerosis, myocarditis, and Crohn's disease. [Gordon and Globe,2008] TGF-β1 is a polypeptide member of the TGF-β superfamily of cytokines. TGF-β is synthesized as a non-active pro-form, forms a complex with two latent associated proteins latency-associated protein (LAP) and latent TGF- β binding protein (LTBP) and undergoes protolithic cleavage by the endopeptidase furin to generate the mature TGF-β dimer. Among the TGF-βs, six distinct isoforms have been discovered although only the TGF-β1, TGF-β2 and TGF-β3 isoforms are expressed in mammals, and their human genes are located on chromosomes 19q13, 1q41 and 14q24, respectively. Out of the three TGF-β isoforms (β1, β2 and β3) only TGF-β1 was linked to fibrogenesis and is the most potent fibrogenic factor for hepatic stellate cells. [Roberts, 1998; Govinden and Bhoola, 2003]. During fibrogenesis, tissue and blood levels of active TGF-β are elevated and overexpression of TGF-β1 in transgenic mice can induce fibrosis. Additionally, experimental fibrosis can be inhibited by anti-TGF-β treatments with neutralizing antibodies or soluble TGF-β receptors [Qi et al.; 1999; Shek and Benyon , 2004; De Gouville et al., 2005; Chng et al., 2009]. TGF-β1 induces its own mRNA to sustain high levels in local sites of injury. The effects of TGF-β1 are classically mediated by intracellular signalling via Smad proteins. Smads 2 and 3 are stimulatory whereas Smad 7 is inhibitory. [Parsons et al., 2013; Friedman, 2008; Kubiczkova et al., 2012] Smad1/5/8, MAP kinase (mitogen-activated protein) and PI3 kinase are further signalling pathways in different cell types for TGF-β1 effects.

TGF-β is found in all tissues, but is particularly abundant in bone, lung, kidney and placental tissue. TGF-β is produced by many, but not all parenchymal cell types, and is also produced or released by infiltrating cells such as lymphocytes, monocytes/macrophages, and platelets. Following wounding or inflammation, all these cells are potential sources of TGF-β. In general, the release and activation of TGF-β stimulates the production of various extracellular matrix proteins and inhibits the degradation of these matrix proteins. [Branton and Kopp, 1999]

TGF-β 1 is produced by every leukocyte lineage, including lymphocytes, macrophages, and dendritic cells, and its expression serves in both autocrine and paracrine modes to control the differentiation, proliferation, and state of activation of these immune cells. [Letterio and Roberts; 1998]

In the liver TGF-β1 is released by activated Kupffer cells, liver sinusoidal endothelial cells, and platelets; in the further course of events also activated hepatic stellate cells express TGF-β1. Hepatocytes do not produce TGF-β1 but are implicated in intracellular activation of latent TGF-β1. [Roth et al., 1998; Kisseleva and Brenner, 2007; Kisseleva and Brenner, 2008; Poli, 2000; Liu et al., 2006]

TGF-β1 is the most established mediator and regulator of epithelial-mesenchymal-transition (EMT) which further contributes to the production of extracellular matrix. It has been shown that TGF-β1 mediates EMT by inducing snail-1 transcription factor and tyrosine phosphorylation of Smad2/3 with subsequent recruitment of Smad4. [Kolios et al., 2006; Bataller and Brenner, 2005; Guo and Friedman,2007; Brenner,2009; Kaimorei et al., 2007; Gressner et al., 2002; Kershenobich Stalnikowitz and Weisssbrod, 2003; Li et al., 2008; Matsuoka and Tsukamoto, 1990; Kisseleva and Brenner, 2008; Poli, 200; Parsons et al., 2007; Friedman 2008; Liu et al., 2006]

TGF-β1 induces apoptosis and angiogenesis in vitro and in vivo through the activation of vascular endothelial growth factor (VEGF) High levels of VEGF and TGF-β1 are present in many tumors. Crosstalk between the signalling pathways activated by these growth factors controls endothelial cell apoptosis and angiogenesis. [Clark and Coker; 1998]

How it is Measured or Detected

The specific type of measurement(s) might vary with tissue, environment and context and will need to be described for different tissue contexts as used within different AOP descriptions.

In general, quantification of inflammatory markers can be done by:

- PCR (mRNA expression)
- ELISA
- Immunocytochemistry
- Immunoblotting

For descriptions of techniques, see Falsig 2004; Lund 2006 ; Kuegler 2010; Monnet-Tschudi et al., 2011; Sandström et al., 2014; von Tobel et al., 2014

LIVER:

There are several assays for TGB-β1 measurement available.

e.g. Human TGF-β1 ELISA Kit. The Human TGF-β 1 ELISA (Enzyme –Linked Immunosorbent Assay) kit is an in vitro enzyme-linked immunosorbent assay for the quantitative measurement of human TGF-β1 in serum, plasma, cell culture supernatants, and urine. This assay employs an antibody specific for human TGF-β1 coated on a 96-well plate. Standards and samples are pipetted into the wells and TGF-β1 present in a sample is bound to the wells by the immobilized antibody. The wells are washed and biotinylated anti-human TGF-β1 antibody is added. After washing away unbound biotinylated antibody, HRP- conjugated streptavidin is pipetted to the wells. The wells are again washed, a TMB substrate solution is added to the wells and colour develops in proportion to the amount of TGF-β1 bound. The StopSolution changes the colour from blue to yellow, and the intensity of the colour is measured at 450 nm [Mazzieri et al., 2000]

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Event: 1494: Leukocyte recruitment/activation (https://aopwiki.org/events/1494)

Short Name: Leukocyte recruitment/activation

Key Event Component

Process	Object	Action
cell activation involved in immune response	leukocyte	increased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:144 - Endocytic lysosomal uptake leading to liver fibrosis (https://aopwiki.org/aops/144)	KeyEvent

Biological Context

Level of Biological Organization
Cellular

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
human	Homo sapiens		NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)
Vertebrates	Vertebrates		NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=0)

Life Stage Applicability

Life Stage	Evidence
All life stages	

Sex Applicability

Sex	Evidence
Unspecific	

Key Event Description

The inflammatory response is the cornerstone of the body's defense mechanism against bacterial and viral pathogens, as well as physical-, chemical- and environmental-mediated tissue and organ damage. Leucocyte recruitment at the site of pathogen evasion or sterile tissue injury is a critical adaptation for the preservation of tissue integrity. Neutrophils are the cell population that acutely responds to the alterations of inflammatory micro-environment. Neutrophil infiltration takes place within 6-8 hours from the initiation of the inflammatory process and is followed by the recruitment of other cell populations, like monocytes, lymphocytes, and eosinophils, which either promote or drive the resolution of inflammation. Leukocyte infiltration into sites of infection or sterile inflammation is a tightly regulated process that follows a sequence of adhesive events, termed as leukocyte adhesion cascade. One can broadly generalize that most leukocytes follow a similar multi-step cascade in the peripheral (non-lymphoid) vasculature with some exceptions. Accordingly, an updated adhesion cascade in postcapillary venules involves free-flowing leukocytes initial attachment or tethering and slow velocity rolling (step 1),stable adhesion (arrest) on endothelial cells (step 2), leukocyte flattening (step 3), and subsequent crawling on the vascular endothelium, transendothelial cell migration (TEM) between (paracellular route) or through (transcellular) the vascular endothelium (step 4), and uropod elongation to complete transmigration of postcapillary venules (step 5). The initial attachment and rolling steps are initiated by interactions of endothelial E- and P-selectins and their counterreceptors on leukocytes L-selectin (Leick et al., 2014).

Each of these steps is necessary for effective leukocyte recruitment; these steps are not phases of inflammation, but represent the sequence of events from the perspective of each leukocyte. At any given moment they all happen in parallel, involving different leukocytes in the same microvessels.

From the initial selectin-dependent leukocyte tethering to endothelial cells to the final migration of leukocytes into the sub-endothelium, this process depends on the interplay between leukocyte receptors and endothelial cell counter-receptors, as well as on the presence of endogenous inhibitors of leukocyte adhesion enabling the targeted recruitment of leukocytes to inflamed tissues.

To enable the infiltration of leukocytes at the site of inflammation, a series of alterations in endothelial cells and leukocytes takes place:

- regulation of the expression of adhesion molecules in leukocytes
- increased secretion of chemokines by endothelial cells
- increased expression of adhesion molecules in the luminal surface of endothelial cells

(Kourtzelis and Mitroulis, 2015) (Robbins and Cotran: Pathologic Basis of Disease 2010).

After recruitment, activation includes phenotype modification with morphologic alterations, changes in marker proteins (MHC, adhesion molecules, co-stimulatory signal), expression of mediators, enzymes, and pro-inflammatory proteins/lipids. Recruited monocytes recruited mature into macrophages with phagocytic activity and elaboration of a myriad of mediators of inflammation. The macrophage can replicate within tissues or die, including by apoptosis.

How it is Measured or Detected

in vivo imaging:

- Flow cytometry (FC/FACS),
- immunhistochemistry
- two photon-intravital microscopy (TP-IVM) (van Grinsven et al., 2017)
- Spinning Disk Confocal Microscopy-IVM (Jenne et al., 2011)

- Histology, increased cell numbers and altered composition

In vitro

- transwell Migration Assay (Justus et al., 2014)
- T-Lymphocyte & Innate Immune Cell Activation Assays
- Leukocyte Surface Markers (Monoclonal Antibodies to Leukocyte Surface Markers)
- Markers of leukocyte activation – protease release, ROS/RNS, NADPH oxidase (NOX), defense response - expression of anti-oxidants.
- organs-on-a-chip (Bnam et al., 2016; Ribas et al., 2017; Wufuer et al. 2016)

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Wufuer M, Lee G, Hur W, Jeon B, Kim BJ, Choi TH, Lee SH, Skin-on-a-chip model simulating inflammation, edema and drug-based treatment, Nature Scientific Reports 6, Article number: 37471 (2016) doi:10.1038/srep37471

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Event: 265: Activation, Stellate cells (https://aopwiki.org/events/265)

Short Name: Activation, Stellate cells

Key Event Component

Process	Object	Action
hepatic stellate cell activation	hepatic stellate cell	increased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:38 - Protein Alkylation leading to Liver Fibrosis (https://aopwiki.org/aops/38)	KeyEvent
Aop:144 - Endocytic lysosomal uptake leading to liver fibrosis (https://aopwiki.org/aops/144)	KeyEvent

Biological Context

Level of Biological Organization
Cellular

Cell term

Cell term
hepatic stellate cell

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
human	Homo sapiens	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)
human and other cells in culture	human and other cells in culture	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=0)
Rattus norvegicus	Rattus norvegicus	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10116)
mouse	Mus musculus	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10090)
pigs	Sus scrofa	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9823)

Life Stage Applicability

Life Stage	Evidence
All life stages	

Sex Applicability

Sex	Evidence
Unspecific	

Human: Friedman, 2008

Rat: George et al.,1999

Mouse: Chang et al., 2014

Pig: Costa et al., 2001

Key Event Description

Stellate cell activation means a transdifferentiation from a quiescent vitamin A–storing cell to a proliferative and contractile myofibroblast. Multiple cells and cytokines play a part in the regulation of hepatic stellate cell (HSC) activation that consists of discrete phenotype responses, mainly proliferation, contractility, fibrogenesis, matrix degradation, chemotaxis, and retinoid loss.

HSCs undergo activation through a two-phase process. The first step, the initiation phase, is triggered by injured hepatocytes, reactive oxygen speecies (ROS) and paracrine stimulation from neighbouring cell types (Kupffer cells (KCs), Liver sinusoidal endothelial cells (LSECs), and platelets) and make HSCs sensitized to activation by up-regulating various receptors. The perpetuation phase refers to the maintenance of HSC activation, which is a dynamic process including the secretion of autocrine and paracrine growth factors (such as TGF-β1), chemokines, and the up-regulation of collagen synthesis (mainly type I collagen). In response to growth factors (including Platelet-derived Growth Factor (PDGF) and Vascular Endothelial Growth Factor (VEGF)) HSCs proliferate. Increased contractility (Endothelin-1 and NO are the key opposing counter-regulators that control HSC contractility, in addition to angiotensinogen II, and others) leads to increased portal resistance. Driven by chemoattractants their accumulation in areas of injury is enhanced. TGF-β1 synthesis promotes activation of neighbouring quiescent hepatic stellate cells, whereas the release of HGF (hepatocyte growth factor) stimulates regeneration of adjacent hepatocytes. The release of chemoattractants (monocyte chemoattractant protein-1(MCP-1) and colony-stimulating factors (CSFs)) amplifies inflammation (Lee and Friedman; 2011; Friedman, 2010; 2008; 2000; Bataller and Brenner, 2005; ↑ Lotersztain et al., 2005; Poli, 2000). Activated HSCs (myofibroblasts) are the primary collagen producing cell, the key cellular mediators of fibrosis and a nexus for converging inflammatory pathways leading to fibrosis. Experimental inhibition of stellate cell activation prevents fibrosis (Li, Jing-Ting et al.,2008; George et al. (1999).

How it is Measured or Detected

Alpha-smooth muscle actin (α-SMA) is a well-known marker of hepatic stellate cells activation. Anti-alpha smooth muscle Actin [1A4] monoclonal antibody reacts with the alpha smooth muscle isoform of actin.

Gene expression profiling confirmed early changes for known genes related to HSC activation such as alpha smooth muscle actin (Acta2), lysyl oxidase (Lox) and collagen, type I, alpha 1 (Col1a1). Insulin-like growth factor binding protein 3 (Igfbp3) was identified as a gene strongly affected and as marker for culture-activated HSCs and plays a role in HSC migration (Morini et al., 2005; Mannaerts et al., 2013).

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Event: 68: Accumulation, Collagen (https://aopwiki.org/events/68)

Short Name: Accumulation, Collagen

Key Event Component

Process	Object	Action
collagen biosynthetic process	collagen	increased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:38 - Protein Alkylation leading to Liver Fibrosis (https://aopwiki.org/aops/38)	KeyEvent
Aop:241 - Latent Transforming Growth Factor beta1 activation leads to pulmonary fibrosis (https://aopwiki.org/aops/241)	KeyEvent
Aop:144 - Endocytic lysosomal uptake leading to liver fibrosis (https://aopwiki.org/aops/144)	KeyEvent

Biological Context

Level of Biological Organization
Tissue

Organ term

Organ term
connective tissue

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
human	Homo sapiens	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)
Rattus norvegicus	Rattus norvegicus	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10116)
mouse	Mus musculus	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10090)

Life Stage Applicability

Life Stage	Evidence
All life stages	

Sex Applicability

Sex	Evidence
Unspecific	

Humans: Bataller and Brenner, 2005; Decaris et al., 2015.

Mice: Dalton et al., 2009; Leung et al., 2008; Nan et al., 2013.

Rats: Hamdy and El-Demerdash, 2012; Li, Li et al., 2012; Natajaran et al., 2006; Luckey and Petersen, 2001.

Key Event Description

Collagen is mostly found in fibrous tissues such as tendons, ligaments and skin. It is also abundant in corneas, cartilage, bones, blood vessels, the gut, intervertebral discs, and the dentin in teeth. In muscle tissue, it serves as a major component of the endomysium. Collagen is the main structural protein in the extracellular space in the various connective tissues, making up from 25% to 35% of the whole-body protein content. In normal tissues, collagen provides strength, integrity, and structure. When tissues are disrupted following injury, collagen is needed to repair the defect. If too much collagen is deposited, normal anatomical structure is lost, function is compromised, and fibrosis results.

The fibroblast is the most common collagen producing cell.Collagen-producing cells may also arise from the process of transition of differentiated epithelial cells into mesenchymal cells (EMT). This has been observed e.g. during renal fibrosis (transformation of tubular epithelial cells into fibroblasts) and in liver injury (transdifferentiation of hepatocytes and cholangiocytes into fibroblasts) (Henderson and Iredale, 2007).

There are close to 20 different types of collagen found with the predominant form being type I collagen. This fibrillar form of collagen represents over 90 percent of our total collagen and is composed of three very long protein chains which are wrapped around each other to form a triple helical structure called a collagen monomer. Collagen is produced initially as a larger precursor molecule called procollagen. As the procollagen is secreted from the cell, procollagen proteinases remove the extension peptides from the ends of the molecule. The processed molecule is referred to as collagen and is involved in fiber formation. In the extracellular spaces the triple helical collagen molecules line up and begin to form fibrils and then fibers. Formation of stable crosslinks within and between the molecules is promoted by the enzyme lysyl oxidase and gives the collagen fibers tremendous strength (Diegelmann,2001). The overall amount of collagen deposited by fibroblasts is a regulated balance between collagen synthesis and collagen catabolism. Disturbance of this balance leads to changes in the amount and composition of collagen. Changes in the composition of the extracellular matrix initiate positive feedback pathways that increase collagen production.

Normally, collagen in connective tissues has a slow turn over; degradating enzymes are collagenases, belonging to the family of matrix metalloproteinases (MMPs).Other cells that can synthesize and release collagenase are macrophages, neutrophils, osteoclasts, and tumor cells (Di Lullo et al., 2001; Prockop and Kivirikko, 1995; Miller and Gay, 1987;Kivirikko and Risteli, 1976).

How it is Measured or Detected

Determination of the amount of collagen produced in vitro can be done in a variety of ways ranging from simple colorimetric assays to elaborate chromatographic procedures using radioactive and non-radioactive material. What most of these procedures have in common is the need to destroy the cell layer to obtain solubilized collagen from the pericellular matrix. Rishikof et al describe several methods to assess the in vitro

production of type I collagen: Western immunoblotting of intact alpha1(I) collagen using antibodies directed to alpha1(I) collagen amino and carboxyl propeptides, the measurement of alpha1(I) collagen mRNA levels using real-time polymerase chain reaction, and methods to determine the transcriptional regulation of alpha1(I) collagen using a nuclear run-on assay (Rishikof et al., (2005) .

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List of Adverse Outcomes in this AOP

Event: 344: N/A, Liver fibrosis (https://aopwiki.org/events/344)

Short Name: N/A, Liver fibrosis

Key Event Component

Process	Object	Action
liver fibrosis	liver	occurrence

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:38 - Protein Alkylation leading to Liver Fibrosis (https://aopwiki.org/aops/38)	AdverseOutcome
Aop:144 - Endocytic lysosomal uptake leading to liver fibrosis (https://aopwiki.org/aops/144)	AdverseOutcome

Biological Context

Level of Biological Organization
Organ

Organ term

Organ term
liver

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
human	Homo sapiens	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)
Rattus norvegicus	Rattus norvegicus	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10116)
mouse	Mus musculus	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10090)

Life Stage Applicability

Life Stage	Evidence
All life stages	

Sex Applicability

Sex	Evidence
Unspecific	

Human: Bataller and Brenner, 2005;Merck Manual, 2015; Blachier et al., 2013.

Rat, mouse: Liedtke et al., 2013

Key Event Description

Liver fibrosis results from perpetuation of the normal wound healing response, as a result of repeated cycles of hepatocyte injury and repair and is a dynamic process, characterised by an excessive deposition of ECM (extracellular matrix) proteins including glycoproteins, collagens, and proteoglycans. It is usually secondary to hepatic injury and inflammation, and progresses at different rates depending on the aetiology of liver disease and is also influenced by environmental and genetic factors. If fibrosis continues, it disrupts the normal architecture of the liver, altering the normal function of the organ and ultimately leading to liver damage. Cirrhosis represents the final stage of fibrosis. It is characterised by fibrous septa which divide the parenchyma into regenerative nodules which leads to vascular modifications and portal hypertension with its complications of variceal bleeding, hepatic encephalopathy, ascites, and hepatorenal syndrome. In addition, this condition is largely associated with hepatocellular carcinoma with a further increase in the relative mortality rate (Bataller and Brenner, 2005; Merck Manual,2015)

Liver fibrosis is an important health issue with clear regulatory relevance. The burden of disease attributable to liver fibrosis is quite high; progressive hepatic fibrosis, ultimately leading to cirrhosis, is a significant contributor to global health burden (Lim and Kim, 2008). In the European Union, 0.1 % of the population is affected by cirrhosis, the most advanced stage of liver fibrosis with full architectural disturbances (Blachier et al., 2013). Besides the epidemiological relevance, liver fibrosis also imposes a considerable economic burden on society. Indeed, the only curative therapy for chronic liver failure is liver transplantation. More than 5.500 orthotopic liver transplantations are currently performed in Europe on a yearly basis, costing up to €100.000 the first year and €10.000 yearly thereafter (Van Agthoven et al., 2001).

How it is Measured or Detected

Liver biopsy is an important part of the evaluation of patients with a variety of liver diseases. Besides establishing the diagnosis, the biopsy is often used to assess the severity of the disease. Until recently it has been assumed that fibrosis is an irreversible process, so most grading and staging systems have relatively few stages and are not very sensitive for describing changes in fibrosis. In all systems, the stages are determined by both the quantity and location of the fibrosis, with the formation of septa and nodules as major factors in the transition from one stage to the next. The absolute amount of fibrous tissue is variable within each stage, and there is considerable overlap between stages. Commonly used systems are the Knodell score with 4 stages - no fibrosis (score 0) to fibrous portal expansion (score 2) to bridging fibrosis (score 3) and Cirrhosis (score 4) – and the more sensitive Ishak fibrosis score with six stages - from no fibrosis (stage 0) over increasing fibrous expansion on portal areas (stages 1-2), bridging fibrosis (stages 3-4), and nodules (stage 5) to cirrhosis (stage 6) (Goodman, 2007). Liver biopsy is an invasive test with many possible complications and the potential for sampling error. Noninvasive tests become increasingly precise in identifying the amount of liver fibrosis through computer-assisted image analysis. Standard liver tests are of limited value in assessing the degree

of fibrosis. Direct serologic markers of fibrosis include those associated with matrix deposition — e.g.procollagen type III amino-terminal peptide (P3NP), type I and IV collagens, laminin, hyaluronic acid, and chondrex. P3NP is the most widely studied marker of hepatic fibrosis. Other direct markers of fibrosis are those associated with matrix degradation, ie, matrix metalloproteinases 2 and 3 (MMP-2, MMP- 3) and tissue inhibitors of metalloproteinases 1 and 2 (TIMP-1, TIMP-2).These tests are not commercially available, and the components are not readily available in most clinical laboratories. Some indirect markers that combine several parameters are available but not very reliable. Conventional imaging studies (ultrasonography and computed tomography) are not sensitive for fibrosis. Hepatic elastography, a method for estimating liver stiffness, is a recent development in the noninvasive measurement of hepatic fibrosis. Currently, elastography can be accomplished by ultrasound or magnetic resonance. Liver biopsy is still needed if laboratory testing and imaging studies are inconclusive (Carey, 2010; Germani et al., 2011) .

Regulatory Significance of the AO

From the OECD - GUIDANCE DOCUMENT ON DEVELOPING AND ASSESSING ADVERSE OUTCOME PATHWAYS - Series on Testing and Assessment 18: "...an adverse effect that is of regulatory interest (e.g. repeated dose liver fibrosis)"

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Appendix 2

List of Key Event Relationships in the AOP

List of Adjacent Key Event Relationships

Relationship: 1775: endocytosis leads to Disruption, Lysosome (https://aopwiki.org/relationships/1775)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Endocytic lysosomal uptake leading to liver fibrosis (https://aopwiki.org/aops/144)	**adjacent**	**High**	

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
human	Homo sapiens		NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)
mouse	Mus musculus		NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10090)

Term	Scientific Term	Evidence	Links
rat	Rattus norvegicus		NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10116)
Hamster	Hamster		NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=0)

Life Stage Applicability

Life Stage	Evidence
All life stages	

Sex Applicability

Sex	Evidence
Unspecific	

Mouse (Werneburg et al., 2002; Kagedal et al., 2001)

Rat (Jung et al., 2015)

Hamster (Hayashi et al., 2008)

Human (Wang et al., 2013)

Key Event Relationship Description

Different substances can be uptaken by endocytosis and localized in lysosomes, while some of them can cause lysosomal disruption. Lysosomotropic agents are mostly weak, lipophilic bases that diffuse across lysosomal membrane, get protonated in the acidic milieu of lysosome and therefore get trapped inside (de Duve et al., 1974). They accumulate and cause the destabilization of lysosomal membranes by acting as surfactants, incorporating its hydrophobic tail in the membrane and with the hydrophilic head facing the interior of the lysosome (de Duve et al.,1974; Firestone et al.,1979). Their accumulation increase the intralysosomal pH, which has many consequences, including the prevention of the further uptake of lysosomotropic compounds, an increase in size and number of lysosomes and the overloading of lysosomes with non-digestible materials.

There are different mechanisms how lysosomotropic agents can disrupt lysosomal membrane. However, not all lysosomotropic agents disrupt lysosomes- for example ammonia salts, methylamine and related hydrophilic weak bases cause swelling of the lysosomes, but do not increase permeability of the membrane. Usually in order to do that, agent requires a certain degree of lipid solubility. The amine will accumulate in the lysosomes until its concentration is high enough to solubilize the lysosomal membrane (Dubowchik et al., 1995)

It has been demonstrated that as a result of protonated agents in lysosomes, there will be accumulation of non-permeable charged substances which will result in inflow of water by increased osmolarity (Bandyopadhyay et al., 2014). Inflow of water results in increase of size and can cause the rupture of lysosome.

Also, oxidative stress can cause destabilization of the lysosomal membrane and for this process, intra-lysosomal ferric ions are essential. They catalyse the formation of oxygen radicals from hydrogen peroxide (Zdoslek et al., 1993).

Evidence Supporting this KER

Biological Plausibility
Trapping of lysosomotropic agents accumulates substances inside of the lysosomes, increases volume of these organelles, and big lysosomes are more prone to rupture (Ono et al., 2003). However, there are many mechanisms for lysosomotropic substances to provoke lysosomal disruption, but their prior uptake by lysosomes is essential.

Empirical Evidence
Accumulation of different substances in lysosomes is causing severe dysfunction, increased permeability of lysosomal membrane and even their rupture. It was demonstrated that even accumulation of substances that are physiologically find in lysosomes can lead towards lysosomal dysfunction. Use of cathepsins' B and L inhibitors causes abnormal accumulation of pro-cathepsins, enlargement of lysosomes and their severe dysfunction (Jung et al., 2015)

Leu Leu OMe is one of the agents that accumulates in lysosomes, where it is converted to a membranolytic compound and increase permeability of lysosomal membrane (Uchimoto et al., 1999, Thiele and Lipsky, 1990)

Sphingosine is another lysosomotropic agent that accumulates within the lysosomes, where it permeabilizes the membrane via a detergent mechanism and provokes relocation of lysosomal enzymes to the cytosol (Kagedal et al., 2001). It induces lysosomal disruption in primary mouse hepatocytes as well as it permabilize isolated hepatic lysosomes in vitro (Werneburg et al., 2002) Also, exposure of J774 cells to ammonium chloride prior to sphingosine resulted in formation of NH_3, which entered into lysosomes and became protonated and increased the pH of the organelle. This prevented the accumulation of sphingosine in the lysosome and provided protection against its lysosomolytic and apoptosis-inducing effects (Kagedal et al., 2001).

Considering nanomaterials (NMs) as a trigger for lysosomal damage, recent studies underpinned the importance of lysosomal NM uptake for NM-induced toxicity. Once the material is taken up by a cell and transported to the lysosome, the acidic milieu herein can either enhance solubility of

a NM, or the material remains in its initial nano form. Both can induce toxicity, causing lysosomal swelling, followed by lysosomal disruption and the release of pro-apoptotic proteins (Cho et al., 2011; Cho et al., 2012; Wang et al., 2013).

H_2O_2 also changes the permeability of the lysosomal membrane. It reacts with redox-active iron in the lysosomes, and produces hydroxyl radicals in Fenton-type reactions (Kubota et al., 2010). These radicals can destabilize the membrane by lipid peroxidation and damage of lysosomal proteins. Presence of desferrioxamine, that binds iron, is protecting lysosomes from rupture (Brunk et al., 2001).

Hayashi et al. demonstrated the uptake of FITC-crotamine into endosomal compartment of cells, and increased accumulation in a time dependant manner. Only 15 minutes after the treatment it was possible to see morphological changes of the cell, disruption of the lipid bilayer of the lysosomal membrane and subsequent rupture of lysosomes. They presented that crotamine treatment triggered the release of cysteine cathepsins to the cell cytosol (Hayashi et al., 2008).

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Relationship: 993: Disruption, Lysosome leads to N/A, Mitochondrial dysfunction 1 (https://aopwiki.org/relationships/993)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Endocytic lysosomal uptake leading to liver fibrosis (https://aopwiki.org/aops/144)	**adjacent**	**High**	

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
human	Homo sapiens	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)
mouse	Mus musculus	Moderate	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10090)

Life Stage Applicability

Life Stage	Evidence
All life stages	

Sex Applicability

Sex	Evidence
Unspecific	

Murine (Stoka et al., 2001; Zhang et al., 2009; Lindsten et al., 2000)

Human (Boya et al., 2003; Cirman et al., 2004)

Key Event Relationship Description

Disrupted lysosomal membrane release the content of lysosomes including cathepsins. Cathepsins take part in activation of BH3-only proteins, which directly or indirectly activate pro-apoptic Bax and Bak proteins. Once activated Bax and Bak form dimers and higher order oligomers, in order to form pores in outer mitochondrial membrane and cause mitochondrial injury.

Many evidences suggest that lysosomal disruption usually precedes mitochondrial injury (Guicciardi et al., 2000; Brunk et al., 2001; Zhao et al. 2003; Droga-Mazovec et al., 2008), with lysosomal proteases inducing mitochondrial dysfunction.

Zhao and colleagues have also proposed the existence of a positive feed-back mechanism between lysosomal damage and mitochondrial damage, in which early lysosomal rupture causes mitochondrial rupture and leakage of mitochondrial proteins that increase lysosomal damage and consequent apoptosis (Zhao et al., 2000).

The pathway between lysosomal membrane permeabilization (LMP) and mitochondrial membrane permeabilization (MMP) is regulated principally by Bcl-2 family of proteins. The family is subdivided into anti-apoptotic multidomain proteins (such as Bcl-2, Bcl-xl, Bcl-W, Mcl-1 and A1), pro-apoptotic multidomain proteins (Bax and Bak) and pro-apoptotic BH3-only proteins (such as Bid, Puma, Noxa, Bim, Bad, and Bik) (Fletcher and Huang, 2006; Youle and Strasser, 2008).

Cathepsins, released after lysosomal damage, have a role in the cell death through the cleavage of BH3-only proteins, such as Bid, to generate active tBid (truncated Bid) (Blomgran et al., 2007; Cirman et al., 2004; Droga-Mazovec et al., 2008; Houseweart et al., 2003; Stoka et al., 2001) and by degradation of the anti-apoptotic Bcl-2 molecules Bcl-2, Bcl-xl and Mcl-1 (Blomgran et al., 2007; Droga-Mazovec et al., 2008). However it was shown that though Bid is not the only substrate of lysosomal enzymes that induce cytochrome c release, it is the major one (Stoka et al., 2001). Droga-Mazovec and colleagues showed that Bid is cleaved by cathepsins in human liver carcinoma cells (HepG2) (Droga-Mazovec et al., 2008), while other study showed that particularly cathepsin B is active in hepatocytes (Guicciardi et al., 2000).

Bid is also cleaved by caspase 8, which represents a link between extrinsic and intrinsic (mitochondrial) pathway (Li et al., 1998).

Activated BH3-only proteins continue to activate pro-apoptic proteins Bax and Bak. Sarosiek et al. observed that Bid preferentially activates Bak, while Bim activates Bax (Sarosiek et al., 2013). The activation of Bax and Bak occurs after LMP, but before mitochondrial release of cytochrome c and caspase-3 activation (Boya et al., 2003). Currently there are two models describing activation of Bax and Bak proteins and the role of anti-apoptic and pro-apoptic multidomain proteins in it. In the indirect model, Bax and Bak are sequestered and inactivated by anti-apoptotic Bcl-2 proteins. The binding of pro-apoptotic BH3-only proteins to these Bcl-2 proteins triggers the release of Bax and Bak. The direct model proposes that Bax and Bak are activated by direct binding of pro-apoptotic BH3-only proteins, called the activators (Bid, Bim or Puma). However, these activators are normally sequestered by anti-apoptotic Bcl-2 proteins. In order to release the activators, other BH3-only proteins, called senzitizers, neutralize anti-apoptotic Bcl-2 proteins (Brenner and Mak, 2009; Willis et al., 2007).

Bak and Bax go under major conformational changes after binding of BH3 only proteins (as reviewed by Westphal et al., 2014). Once activated Bak or Bax molecules bind reciprocally to form symmetric homodimers. It is thought that homodimers of Bak or Bax must then associate to higher order oligomers to porate the mitochondrial outer membrane (Uren et al., 2017). Heterodimers form only a minor population compared with homodimers (Dewson et al., 2012; Mikhailov et al., 2003).

Bak and Bax shallow insertion into the outer leaflet of mitochondrial membrane (Westphal et al., 2014; Oh KJ et al., 2010) may destabilize the lamellar structure of the bilayer to induce lipidic pores in mitochondrial membrane. This induces release of proteins from the space between inner and outer mitochondrial membrane (Newmeyer et al., 2003).

Evidence Supporting this KER

Biological Plausibility

In the last decade there is a growing body of evidences about the strong functional link between lysosomes and mitochondria that play an important role in physiology and pathology. The evidences also showed link between lysosomal and mitochondrial damage, and that lysosomal damage precedes mitochondrial injury.

Empirical Evidence

LMP after exposure to ciprofloxacine, norfloxacine and hydroxychloroquine is detected couple of hourse earlier that MMP (Boya et al., 2003). The same study proves that the cells with signs of MMP are sub-ensemple of the group of the cells with signs of LMP, not vice-versa. Also inhibition of LMP with Baf A1 – that inhibits lysosomal vacuolar H^+ ATP-ase, prevented MMP, while inhibition of MMP in Bax/Bak double knocks out cells didn't prevent LMP. However, inhibition of MMP prevented LMP to cause manifestations of the cell death. All the evidences from this and other studies (such as Droga-Mazovec et al., 2008; Ghosh et al., 2011) prove that LMP lies upstream from MMP and causes it.

When isolated mitochondria are incubated with purified cathepsin B in the presence of cytolic extracts, a release of cytochrome c from mitochondria is detected (Guicciardi et al., 2000). The microinjection of cathepsin D to the cell causes cytochrome c release, capsases activation and apoptosis (Roberg et al., 2002).

It was shown that mice deficient of stefin B (major intracellular cathepsins inhibitor) developed spontaneous cerebellar apoptosis (Houseweart et al., 2003). Pepstatin A, an inhibitor of cathepsin D, was found to inhibit caspase-3-like proteolytic activity and to prevent apoptosis (Roberg et al., 1999). The treatment of the cells with cathepsin B and cathepsin D inhibitors, pepstatin A and E-64-d, decreased MMP and activation of caspases (Kagedal et al., 2001).

Cathepsin B directly cuts Bid and produces active tBid, while cathepsin B inhibitors z-FA-fmk and E-64-d block Bid activation in cells (Zhang et al., 2009; Blomgran et al., 2007). When cathepsin B silenced HeLa cells were treated with granulysin Bid degradation was blocked, same as cytochrome c and apoptosis inducing factor (AIF) release (Zhang et al., 2009).

Stoka et al. showed that incubation of rat mitochondria to uncleaved Bid resulted only in insignificant levels of cytochrome c release, while exposure to both uncleaved Bid and lysosomal extracts resulted in cytochrome c release (Stoka et al., 2001). Other studies confirmed necessity of tBid for cytochrome c release (Gross et al., 1999; Luo et al., 1998).

Bid knockout mouse embryonic fibroblasts (MEFs) and Bax/Bak deficient MEFs were more resistant to granulysin induced death compared to wild type, and they released less cytochrome c and AIF (Zhang et al., 2009; Lindsten et al., 2000). Bcl-2-overexpressed HeLa cells almost completely blocked the release of cytochrome c and AIF after granulysin treatment (Zhang et al., 2009). Overexpression of Bcl-2 is also suppressing oxidative stress induced apoptosis (Zhao et al., 2000).

Some studies stated that certain cell types such as hepatocytes appear to require a Bid in order to disrupt mitochondrial membrane, release cytochrome c and following steps to execute apoptosis (Korsmeyer et al., 2000). They also proved that BH3 domain of tBid was not required for targeting mitochondrial membrane but it is required for cytochrome c release.

Using specific inhibitors it was demonstrated that cytosolic cathepsin D triggers Bax activation and translocation to mitochondria, resulting release of AIF from mitochondria, and the apoptosis (Bidere et al., 2003).

Incubation of cleaved Bid with mitochondria promotes oligomerization of membrane bound Bak and cytochrome c release (Wei et al., 2000).

Bax/Bak double knockout MEFs didn't show MMP induced by ciprofloxacin, norfloxacin and hydroxychloroquine, while increased LMP was detected (Boya et al., 2003).

Incubation of Bax with isolated mitochondria resulted in cytochrome c release while Bcl-xl inhibits this release (Jurgensmeier et al., 1998)

The length of the core dimer of Bax or Bak is the approximate width of the mitochondrial outer membrane, and the bend observed in the structures may be accommodated by the curved edge of the pore (Uren et al., 2017).

Uncertainties and Inconsistencies

Repnik and colleagues showed that inhibition of cysteine cathepsins by E-64-d had little effect on LDH (cytosolic enzyme lactate dehydrogenase) release in medium in LLOMe treated cells (Repnik et al., 2017). They also detected that after exposure to LLOMe, cathepsins remain in lysosomes and are being degraded there which is in contradiction with most of the previous studies.

As stated earlier there are empirical evidences that incubation of cathepsin B with mitochondria and cytosolic factors increase mitochondrial permeabilization. However, in some studies pharmacological inhibition of cathepsin B, L and D didn't suppress Bid cleavage, suggesting that other lysosomal proteases might be responsible for Bid cleavage (Reiners et al., 2002).

The knockout of genes coding for cathepsins B, D, L and S failed to prevent induced MMP and cell death (Boya et al., 2003).

Housewert et al. showed that the amount of cerebellar granule cell apoptosis in cystatin B-deficient mice did not change when Bid was removed. This indicates that cathepsins can use other mechanisms to initiate apoptosis. They concluded that another molecule may partially substitute for Bid when it is missing (Housewert et al., 2003). Willis and colleagues showed that neither Bim nor Bid are necessary for apoptosis, as their absences didn't stop apoptosis or prevented Bax activation (Willis et al., 2007).

Some reports described Bax/Bak-independent mechanisms of cytochrome c release, involving either cyclosporine A sensitive mitochondrial membrane permeability (Wan et al., 2008) or a serine protease(s)-dependent mechanism (Mizuta et al., 2007).

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Relationship: 363: N/A, Mitochondrial dysfunction 1 leads to N/A, Cell injury/death (https://aopwiki.org/relationships/363)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Binding of agonists to ionotropic glutamate receptors in adult brain causes excitotoxicity that mediates neuronal cell death, contributing to learning and memory impairment. (https://aopwiki.org/aops/48)	**non-adjacent**	**Moderate**	
Endocytic lysosomal uptake leading to liver fibrosis (https://aopwiki.org/aops/144)	**adjacent**	**Moderate**	**Low**

Evidence Supporting Applicability of this Relationship

Life Stage Applicability

Life Stage	Evidence
All life stages	

Sex Applicability

Sex	Evidence
Unspecific	

Neuronal necrosis has been noted in sea lions accidentally exposed to DomA (Silvagni et al., 2005) that correlated well with the histopathological findings previously reported in experimental studies (Tryphonas et al., 1990).

Key Event Relationship Description

ROS generation is known to activate different pathways leading to apoptosis, whereas depletion of energy production induces necrotic cell death.

Evidence Supporting this KER

Biological Plausibility

There is functional mechanistic understanding supporting this relationship between KE3 and KE4.

ROS are known to stimulate a number of events and pathways that lead to apoptosis, triggered by ROS-induced ER stress signalling pathway (Lu et al., 2014), caspase-dependent and -independent apoptosis (Zhou et al., 2015), mitogen-activated protein kinase (MAPK) signal transduction pathways (reviewed in Cuadrado and Nebreda, 2010, Harper and LoGrasso, 2001).

Depletion of cellular ATP is known to cause switching from apoptotic cell death triggered by a variety of stimuli to necrotic cell death (Leist et al., 1997) suggesting that the level of intracellular ATP determines whether the cell dies by apoptosis or necrosis (Nicotera et al., 1998). There is strong proof that apoptosis requires energy, as it is a highly regulated process involving a number of ATP-dependent steps such as caspase activation, enzymatic hydrolysis of macromolecules, chromatin condensation, bleb formation and apoptotic body formation (Richter et al., 1996).

Empirical Evidence

Include consideration of temporal concordance here

In the case of DomA, in vitro studies have shown that oxidative stress and oxidative stress-induced activation of the stress-activated protein kinase/c-jun-N-terminal kinase (SAPK/JNK) pathway is implicated in DomA-mediated apoptosis (Giordano et al., 2007; 2008; 2009; Lu et al., 2010). In vivo findings also show that ROS-mediated cognitive deficits are associated with apoptosis induced by activation of the JNK pathway (Lu et al., 2010; 2011).

- Mice injected intraperitoneally (i.p.) with DomA at a dose of 2 mg/kg once a day for 4 weeks have shown increase (6 fold) of the TUNEL positive cells in the hippocampus . In the same study they have found that indicators of mitochondria function are markedly decreased (1.5-2 fold) and ROS levels are elevated (3.2 fold) (Lu et al., 2012). DomA treatment also significantly decreases the levels of bcl-2, procaspase-3 and procaspase-12 and increases the activation of caspase-3 and caspase-12 in the mouse hippocampus (Lu et al., 2012). The same research group using similar dose but longer exposure (4 weeks), has shown increase of ROS (3 fold) and NOX (2 fold) and elevated (8 fold) mean value of TUNEL-positive cells in the hippocampal CA1 sections as well as increase in the activation of caspase-8 and caspase-3 (Wu et al., 2012). These two in vivo studies (Lu et al., 2012; Wu et al., 2012) suggest that both KEs are affected in response to the same dose of DomA and exposure paradigm and that the incidence of downstream KE (cell death) is higher than the incidence of upstream KE (mitochondrial dysfunction).
- The cell viability has been measured by the MTT reduction assay in mouse cerebellar granule neurons (CGNs) and showed that the IC50 values for DomA are 3.4 μM in Gclm (+/+) neurons and 0.39 μM in Gclm (-/-) neurons (Giordano et al., 2006). This reduction in cell viability has been demonstrated to be concentration dependent after studying a range of concentrations of DomA (0.01 and 10 μM). Giordano et al. 2007 have shown that 100 nM DomA induce apoptotic cell death in mouse CGNs. In a follow-up study, the same research group has performed a dose response evaluation and showed that even 50 nM DomA exposure for 1 h (after washout and additional 23 h incubation) can induce apoptosis in CGNs derived from Gclm (+/+) mice, whereas neurons from Gclm (–/–) mice that have very low levels of glutathione are more sensitive as 10 nM DomA induces a significant increase in apoptotic cell number (Giordano et al., 2009). The maximal apoptosis (5 fold compared to controls) in CGNs from both genotypes has been caused by 100 nM DomA. Interestingly, 1 and 10 μM DomA still cause significant apoptosis in both cell types but to a lesser extent compared to 100 nM DomA. ROS have been measured only at the dose of 100 nM DomA, 30 min after treatment and showed 2.5 fold increase compared to controls in CGNs from Gclm (+/+) mice (Giordano et al., 2009). Caspase 3 activity has also been measured after 12 h with prior 1 h exposure to 100 nM DomA and found to be increased (2.2 fold). In the same study, DomA (100 nM) caused a significant decrease (25%) of Bcl-2 protein levels after 6 h exposure. Again these in vitro studies (Giordano et al., 2007; 2009) suggest that both KEs are affected by the same dose of DomA and that the incidence of KE down (cell death) is higher than the incidence of KE up (mitochondrial dysfunction). Furthermore, KE up (mitochondrial dysfunction) happens earlier (30 min) than KE down (cell death) that takes place 12-24 h later.
- Mixed cortical cultures have been treated with 3, 5, 10, or 50 μM DomA for a variety of exposure durations (10 min, 30 min, 1 h, or 2 h), after which DomA is washed out and the culture medium is replaced with conditioned medium from unexposed sister cultures (Qiu et al., 2006). In all cases neuronal death has been measured 24 h following the beginning of exposure. The results show that DomA-induced neuronal death is determined by both concentration and duration of exposure. After a 10-min exposure, 50 μM DomA produces marked neuronal death of 47.4 %, whereas by 1 h of treatment, the same concentration produces near maximal neuronal death but longer exposures do not increase neuronal death further (Qiu et al., 2006). Regarding time dependence, this study shows that low concentrations of DomA produces more neuronal death if this is measured 22 h after the washout than if measured immediately after DomA treatment, while higher concentrations of DomA (20–100 μM) produces equivalent degrees of neuronal death when measured at these two time points (Qiu et al., 2006). Based on these findings, three EC50 exposure paradigms have been established, which represent weak/prolonged exposure (3 μM/24 h), moderate concentration and duration of exposure (10 μM/2 h), and strong/brief exposure (50 μM/10 min) (Qiu et al., 2006).
- The mean concentration of DomA in rat brain samples obtained at 30 min after intraperitoneal (i.p.) administration of 1 mg/kg DA is 7.2 ng/g (Tsunekawa et al., 2013). These animals have been examined and revealed after histopathological analysis neuronal shrinkage and cell death, including an increase in the percentage of TUNEL positive cells at 24 hours (8.3 %) and after 5 days (19.0 %) compared to the controls (1.7 %) (Tsunekawa et al., 2013). In the same study, indirectly it has been shown that ROS production is associated with these histopathological findings by using the radical scavenger edaravone (Tsunekawa et al., 2013).
- Brain slices from 8-day-old pups have been treated after 2 weeks with 10 μM DomA and assessed with propidium iodine (PI) stain to determine cellular damage (Erin and Billingsley, 2004). A time course has been carried out and viable cultures have been visualized 12, 24, 48 and 92 h after DomA treatment. Changes in PI uptake has been detected after 24 h post-treatment and at 4h the average fold-increase of PI uptake (DomA/control) was 14.5 and 34.5 in cortex and hippocampus, respectively (Erin and Billingsley, 2004). In the same study, incubation of brain slices with DomA induces degradation of α-spectrin to the 120-kDa product after 18 h of treatment but no change has been noted after 12 h incubation, whereas caspase 3 activity results have not been conclusive (Erin and Billingsley, 2004).
- Using observations of neuronal viability and morphology, exposure of cultured murine cortical neurones to DomA for 24 h have shown to induce concentration-dependent neuronal cell death and the EC50 determined to be 75 μM (Larm et al., 1997).

Stressor	Experimental Model	Tested concentrations	Exposure route	Exposure duration	Mitochondrial dysfunction (KE up) (measurements, quantitative if available)	Cell death (KE down) (measurements, quantitative if available)	References	Temporal Relationship	Dose-response relationship	Incidence	Comments
DomA	16-month-old male ICR mice	2 mg/kg	Intraperitoneally (i.p.)	Once a day for 4 weeks	Indicators of mitochondrial function were markedly decreased (1.5-2 fold) and ROS levels were elevated (3.2 fold).	The mean of TUNEL positive cells in the hippocampus was increased (6 fold). The levels of bcl-2, procaspase-3 and procaspase-12 were significantly decreased and the activation of caspase-3 and caspase-12 in the mouse hippocampus were increased.	Lu et al., 2012		Same dose	Incidence of downstream KE (cell death) is higher than the incidence of upstream KE (mitochondrial dysfunction)	
DomA	16-month-old male ICR mice	2 mg/kg	i.p.	Once a day for 4 weeks	ROS levels were increased (3 fold) and NOX (2 fold).	The mean value of TUNEL-positive cells in the hippocampal CA1 sections was elevated (8 fold) and the activation of caspase-8 and caspase-3 was increased.	Wu et al., 2012		Same dose	Incidence of downstream KE (cell death) is higher than the incidence of upstream KE (mitochondrial dysfunction)	
DomA	Mouse cerebellar granule neurons (CGNs) from Gclm (+/+) and Gclm (–/–) mice	0.01 to 10 µM		Time course (15 to 120 min)	DomA caused a significant time- and concentration-dependent increase in ROS production. The higher ROS production (2.5 fold increase) was recorded after 1 h of exposure.	IC50 values for DomA were 3.4 µM in Gclm (+/+) neurons and 0.39 µM in Gclm (-/-) neurons based on MTT assay after 24 h of exposure.	Giordano et al., 2006	KE up (mitochondrial dysfunction) happens earlier than KE down (cell death)	Same doses		
DomA	CGNs from Gclm (+/+) and Gclm (–/–) mice	0.01 to 10 µM		Time course (0 to 180 min)	DomA (0.1µM) caused a 3 fold increase in DHR fluorescence, which accumulates in mitochondria and fluoresces when oxidized by ROS or reactive nitrogen species. This occurred between 1 and 2 h and was higher in CGNs from Gclm (–/–) mice.	0.1µM DomA was maximally effective in inducing apoptosis, while a concentration causing high toxicity (10µM) induced very limited apoptosis, 24 h after exposure.	Giordano et al., 2007	KE up (mitochondrial dysfunction) happens earlier (1-2 h) than KE down (cell death) that occurs after 24 h	Same doses		
DomA	CGNs from Gclm (+/+) and Gclm (–/–) mice	0.01 to 10 µM		For ROS: 30min, Apoptosis: 12-24 h.	ROS levels were measured only at the dose of 100 nM DomA 30 min after treatment in CGNs from Gclm (+/+) mice and showed 2.5 fold increase compared to controls .	A dose response study that showed that even 50 nM DomA exposure for 1 h (after washout and additional 23 h incubation) can induce apoptosis in CGNs from Gclm (+/+) mice, whereas neurons from Gclm (–/–) mice that have very low levels of glutathione were more sensitive as 10 nM DomA induced a significant increase in apoptotic cells number .The maximal apoptosis (5 fold compared to controls) in CGNs from both genotypes was caused by 100 nM DomA. 1 and 10 µM DA caused significant apoptosis in both cell types but to less extend compared to 100 nM DomA. Caspase 3 activity after 12 h with prior 1 h exposure to 100 nM DomA found to be increased (2.2 fold). DomA (100 nM) caused a significant decrease (25%) of Bcl-2 protein levels after 6 h from exposure.	Giordano et al., 2009	KE up (mitochondrial dysfunction) happens earlier (30 min) than KE down (cell death) that take place 12-24 h later	Same dose	Incidence of downstream KE (cell death) is higher than the incidence of upstream KE (mitochondrial dysfunction)	
DomA	Mixed cortical cultures obtained from pregnant Holtzman rats on embryonic day (ED) 16–18	3, 5, 10, or 50 µM		10 min, 30 min, 1 h or 2 h, after which DomA was washed out and the culture medium replaced with conditioned medium from unexposed sister cultures .		EC50 exposure paradigms have been established, which represent weak/prolonged exposure (3 µM/24 h), moderate concentration and duration exposure (10 µM/2 h), and strong/brief exposure (50 µM/10 min) .	Qiu et al., 2006				
DomA	Rat	1 mg/kg DA	i.p.		Indirectly it has been shown that ROS production is associated with these histopathological findings by using the radical scavenger edaravone .	Neuronal shrinkage and cell drop out as well as increase in the percentage of TUNEL positive cells at 24 hours (8.3 %) and 5 days (19.0 %) has been found compared with that of controls (1.7 %) .	Tsunekawa et al., 2013				
DomA	Rat rain slices from 8-day-old pups	10 µM		Time course (12, 24, 48 and 92 h) after DomA treatment.		PI uptake (DomA/control) was 14.5 and 34.5 in cortex and hippocampus, respectively . Degradation of α-spectrin to the 120-kDa product after 18 h of DomA treatment was noted but no change was noted after 12 h incubation, whereas caspase 3 activity results were not conclusive.	Erin and Billingsley, 2004				

DomA	Cultured murine cortical neurones					DomA induces concentration-dependent neuronal cell death and the EC50 determined to be 75 μM .	Larm et al., 1997				

Gap of knowledge: there are no studies showing that GLF induces neuronal cell death through mitochondrial dysfunction.

Uncertainties and Inconsistencies
Rats have been administered with DA at the dose of 1.0 mg/kg for 15 days. The histochemical analysis of hippocampus from these animals has revealed no presence of apoptotic bodies and no Fluoro-Jade B positive cells (Schwarz et al., 2014).

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Relationship: 1776: N/A, Cell injury/death leads to Increased pro-inflammatory mediators

(https://aopwiki.org/relationships/1776)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Endocytic lysosomal uptake leading to liver fibrosis (https://aopwiki.org/aops/144)	**adjacent**	**High**	

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
human	Homo sapiens		NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)
mouse	Mus musculus		NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10090)

Life Stage Applicability

Life Stage	Evidence
All life stages	

Sex Applicability

Sex	Evidence
Unspecific	

Human (Andersson et al., 2000; Scaffidi et al., 2002; Bell et al., 2006; Clarke et al., 2010)

Mouse (Faouzi et al., 2001; Chen et al., 2007)

Key Event Relationship Description

Cell death, including both necrosis and apoptosis can lead toward inflammation. Faouzi and colleagues showed that apoptosis can induce hepatic inflammation equally as necrosis (Faouzi et al., 2001). Some studies indicate that phagocytes can produce inflammatory cytokines upon ingestion of apoptotic bodies (Uchimura et al., 1997).

When cells undergo necrosis they lose the integrity of their plasma membrane and release their intracellular contents, into the extracellular space. The same process can occur when apoptotic cells aren't cleared fast enough and their membrane becomes permeable to macromolecules, which presents secondary necrosis (Majno et al., 1995). There is evidence that the immune system has evolved the capacity to detect the release of intracellular molecules which stimulates the generation of adaptive immune responses to dying cells.

Intracellular content of dying cells that triggers immune response when excreted contains molecules named danger associated molecular patterns (DAMPs). DAMPs include for example HMGB-1, IL-1α, uric acid, DNA fragments, mitochondrial content, and ATP (Eigenbrod et al., 2008; Kono et al., 2010a; Sauter et al., 2000). DAMPs can be molecules that have non-inflammatory functions in living cells (such as HMGB-1, ATP) and acquire immunomodulatory properties when released (Rock and Kono, 2008), or alarmins, molecules that have cytokine-like properties (such as IL-1α, IL-6), which are stored in cells and released after cell lysis and contribute to the inflammatory response (Oppenheim and Yang, 2005; Vanden Berghe et al., 2006).

One of the most investigated DAMPs is HMGB-1 (Lotze et al., 2005). HMGB-1 is a nuclear protein that binds to chromatin and has a role in bending DNA and regulating gene transcription (Landsman et al., 1993). HMGB-1 is released by both necrotic and apoptotic cells (Scaffidi et al., 2002; Bell et al., 2006), but also apoptotic cells activate macrophages that engulf them to secrete HMGB-1 (Qin et al., 2006). This protein induces inflammation, dendritic cells maturation, migration, and T-cell activation (Scaffidi et al., 2002; Messmer et al., 2004; Rovere –Querini et al., 2004; Dumitriu et al., 2005; Yang et al., 2007).

HMGB-1 is a stimulus for tumour necrosis factor (TNF) synthesis and release, but it also significantly activates the synthesis of IL-1 α, IL-1 β, IL-1RA, IL-6, IL-8, MIP-1 a, and MIP-1 (Andersson et al., 2000). It was shown that HMGB-1 released from late apoptotic cells remains bound to nucleosomes and that HMGB1-nucleosome complexes activate antigen-presenting cells (APC) and induce secretion of cytokines by macrophages and expression of co-stimulatory molecules in DCs (Urbonaviciute et al., 2008).

HMGB-1 is not the only pro-inflammatory DAMP released from dying cells. Other DAMPs, S100A8/A9 and S100A12 proteins induce pro-inflammatory cytokine production by macrophages (Hofmann et al., 1999; Yang et al., 2001; Viemann et al., 2004; Ehlerman et al., 2006; Pouliot et al., 2008).

The adjuvant activity of cells was reduced by enzymatic depletion of uric acid, indicating that it is a major DAMP, at least in some cells (Shi et al., 2003). Uric acid is a mediator released from necrotic or apoptotic cells that has immunostimulatory properties in vivo (Gordon et al., 1985; Shi et al., 2003).

Insufficient autophagy of deteriorated mitochondria could lead to massive release of DAMPs such as mtDNA and possibly other mitochondrial proteins (Oka et al., 2012).

Receptors on host cells sense when DAMPs are released and that triggers the inflammatory process. These receptors are pattern-recognition receptors (PRRs) (Chen and Nunez, 2010). PRRs represent proteins by which cells recognize microbial entities, but also some of the host's own molecules and direct an immune response (Piccinini et al., 2010). PRRs can be broadly divided in five subfamilies: Toll-like receptors (TLRs), RIG-1-like receptors (RLRs), NOD like receptors (NLRs), AIM2-like receptors (ALRs) and C-type lectin receptors (CLRs) (Takeuchi and Akira, 2010; Wang et al., 2014). For example, HMGB-1 was reported to stimulate TLR2 and TLR4 (Park et al. 2004) and receptor for advanced glycation end products (RAGE) (Dumitriu et al., 2005), while NLRP3 has been involved in the inflammatory response to mono-sodium urate (MSU) (Martinon et al., 2006). Cellular nucleic acids can stimulate TLR7 and TLR9 on B cells to promote antibody responses (Green and Marshak-Rothstein, 2011; Leadbetter et al., 2002).

TLRs are placed either at the cell surface (TLR1, TLR2, TLR4, TLR5, and TLR6) or in the endolysosomal compartment (TLR3, TLR7, and TLR9) (Barton and Kagan, 2009). Upon binding with the ligand, they undergo a conformational change and initiate a signalling cascade via signal adaptor molecules: myeloid differentiation primary response gene 88 (MyD88), MyD88 adaptor-like protein (MAL, also known as TIR-domain-containing adaptor protein; TIRAP), TIR domain-containing adaptor protein inducing interferon-β (TRIF), and TRIF-related adaptor molecule (TRAM). MyD88 was essential for the inflammatory response to injected dead cells (Chen et al., 2007).

All TLRs, except TLR3, associate with MyD88, and this stimulates a kinase cascade resulting in the activation of mitogen activated protein kinases (MAPKs), c-Jun N-terminal kinases, p38, and extracellular signal–regulated kinases, and nuclear factor NF-kB (Akira and Takeda, 2004; Lee and Kim, 2007). NF-kB is an important transcription factor for IL -1β and NLRP3 (Wang et al., 2004; Bauernfeind et al., 2009).

NF-kB is a central mediator of pro-inflammatory gene induction and functions in both types of immune cells. NF-kB pathway is responsible for transcriptional induction of pro-inflammatory cytokines, chemokines and additional inflammatory mediators, such as NLRP3, pro-IL-1β and pro-IL-18 (Sun et al., 2013; Ghosh and Karin, 2002; Hayden and Ghosh, 2013).

Macrophages must first be ‘primed’ with a stimulus that induces the synthesis of pro-IL -1β and also upregulates the expression of NLRP3 (Bauernfeid et al., 2009; Franchi et al., 2009). The stimuli that can prime macrophages include TLR agonists and cytokines like TNF. When macrophages producing pro-IL -1β are stimulated with ATP or irritant particles, inactive pro-caspase 1 assembles into a molecular complex called the inflammasome and is cleaved into active form (Stutz et al., 2009; Schroder and Tschopp, 2010). Inflammasomes consist of caspase 1, apoptosis-associated speck-like protein containing CARD (ASC) and an NLRP (Schroder and Tschopp, 2010). The catalytically active caspase 1 then cleaves pro-IL-1β to its mature and active form (Stutz et al., 2009). Macrophages lacking any of the inflammasome components don't make mature IL-1 when stimulated in culture with sterile particles (Hornung et al., 2008; Halle et al., 2008). NF-κB signaling pathway is also involved in the regulation of inflammasome (Guo et al., 2015).

Sometimes substantial sterile inflammatory response can be seen in caspase 1-deficient mice (eg. Chen et al., 2007). This contrasts with the much more marked reduction of these responses that is consistently observed in IL-1β -deficient mice. These data imply that there must be a caspase 1-independent pathway for generating mature IL-1β in vivo (Dinarello, 2009).

In the sterile inflammatory response to cell death, the contribution of TNF appears to be more modest than IL-1 (Rock et al., 2011). A possible explanation might be that the IL-1 is being released from the dying cells themselves (Eigenbrod et al., 2008).

After engulfment of apoptotic bodies, Kupffer cells in liver express TNF, TNF-related apoptosis-inducing ligand (TRAIL), and Fas ligand (FasL) (Canbay et al. 2003), which can induce apoptosis in hepatocytes and further aggravate liver inflammation. Engulfment of apoptotic bodies by macrophages also induces FasL expression (Kiener et al., 1997), which is known to exert a pro-inflammatory activity (Chen et al., 1998).

Evidence Supporting this KER

Biological Plausibility

The severity of cell death activation determines the outcome for the cell: inflammation is part of the tissue regeneration process, and intermediate apoptotic stimuli are able to trigger this response. Recruitment of inflammatory cells such as neutrophils is meant as a beneficial process, as for example apoptotic bodies of bacteria-infected cells can be removed. Thus the apoptotic cells can secrete soluble "find-me" factors that trigger infiltration of immune cells. However, if this becomes chronic it has the potential to enhance tissue damage and ultimately induce fibrosis (Jaeschke, 2002; Cullen et al., 2013).

Empirical Evidence

During the apoptosis of Jurkat cells treated with various agents, HMGB-1 was released into the media as assessed by Western blotting. This release was blocked by an inhibitor of apoptosis (Bell et al., 2006).

Some studies reported that purified HMGB-1 activate leukocytes and stimulate the production of pro-inflammatory mediators in vitro (Li et al., 2004; Zimmermann et al., 2004).

Injecting a neutralizing HMGB-1 antibody into animals treated with a hepatotoxic drug, reduced inflammation in the damaged liver (Scaffidi et al., 2002). Other studies showed that anti-HMGB-1 antibodies could also reduce inflammation in livers that had suffered from ischemia-reperfusion injury (Tsung et al., 2005).

In uric acid-depleted mice, the inflammatory responses to cell death were significantly reduced (Kono et al., 2010a).

ATP can stimulate the production of pro-inflammatory cytokines from macrophages (Ferrari et al., 1997; Ferrari et al., 2006). Depletion of ATP or elimination of its receptor inhibited inflammation in vivo in reaction to thermal injury of the liver (McDonald et al., 2010).

Injection of an agonistic anti-FAS antibody into mice causes hepatocytes to undergo apoptosis which stimulates inflammation. If apoptosis is blocked, then this inflammatory response is inhibited (Faouzi et al., 2001).

Chen and colleagues injected dead cells into mice that genetically lacked various TLR. Inflammation was reduced in mice that were doubly-deficient in TLR2 and TLR4, confirming that these receptors play a role in the sterile inflammatory response. However reduction in the response was mild, indicating that there must be other receptors involved in this process (Chen et al., 2007).

IL-1 receptor antagonist (IL-1RA) administration resulted in a significant decrease in IL-6, and a borderline decrease in TNF production. This antagonist does not reduce the number of apoptotic bodies present, indicating that its effect on IL-6 and TNF- levels were due to neutralization of IL-1 (Clarke et al., 2010). Knock out of IL-1 receptor antagonist results in lethal artery inflammation (Nicklin et al., 2000).

The IL-1-dependent inflammatory response to cell death in vivo is significantly reduced in NLRP3-deficient mice (Imaeda et al., 2009; Iyer et al., 2009).

Death-induced neutrophilic inflammation was markedly decreased in mice lacking MyD88. However, this effect was primarily due to a key role for the IL-1 receptor in the recruitment of neutrophils (Chen et al., 2007).

Neutralization of IL-1α or genetic deficiency of IL-1 inhibited inflammation responses to injected dead cells (Kono et al., 2010b; Chen et al., 2007).

Injection into mice of a variety of other dead cell types that genetically lack both IL-1α and IL- 1β stimulated an inflammatory response that was equivalent to that of wildtype necrotic cells (Kono et al., 2010b). This implicates that IL-1 that is driving the sterile inflammatory response in many cases is not coming directly from the dead cell but is produced by cells in the host upon recognition of cell death. That this is the case was formally shown by the loss of inflammatory response to dead cells in mice that genetically lack either IL-1α or IL-1β (Kono et al., 2010b).

Uncertainties and Inconsistencies

The inflammatory role of HMGB-1 is still not completely clear. There are many studies that confirm its pro-inflammatory activity. However, in some experiments highly purified HMGB-1 had little pro-inflammatory activity (Rouhiainen et al., 2007), while in another injection of recombinant HMGB-1 into infarcted heart muscle in vivo stimulated regeneration and repair (Limana et al., 2005).

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Relationship: 1777: Increased pro-inflammatory mediators leads to Leukocyte recruitment/activation (https://aopwiki.org/relationships/1777)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Endocytic lysosomal uptake leading to liver fibrosis (https://aopwiki.org/aops/144)	**adjacent**	**High**	

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
human	Homo sapiens		NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)
mouse	Mus musculus		NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10090)

Life Stage Applicability

Life Stage	Evidence
All life stages	

Sex Applicability

Sex	Evidence
Unspecific	

Human (Bleul et al., 1996; Miyamoto et al., 2000; Yamada et al., 2001; Sun et al., 2015)

Sheep (Nikiforou et al., 2016)

Mouse (Narumi et al., 1992; Fahy et al., 2001; Lee et al., 2009)

Key Event Relationship Description

Circulating blood leukocytes are required to migrate to sites of injury and infection with the aim to eliminate the primary inflammatory trigger and contribute to tissue repair. In this process are involved selectins (expressed both on leukocytes and endothelium) and integrins (expressed on leukocytes) (von Andrian et al., 1991), with the essential role of the vascular endothelium.

Fast activation of the endothelium with inflammatory stimuli such as histamine and PAF (type I) or slow activation with tumor necrosis factor (TNF) or cytokine interleukin-1 β (IL-1β) (type II), makes the surface of endothelium adhesive (Bevilacqua and Gimbrone, 1987; Pober and Sessa, 2007). This transformation is mediated by a transcriptionally regulated program involving the nuclear factor NF-kB dependent pathway triggered by pro-inflammatory cytokines or bacterial endotoxins (reviewed by Collins et al., 1995).

Integrins mediate attachment between cells or to basement membrane. The β2 integrin family is exclusively expressed on leukocytes and is essential for leukocyte arrest on the endothelium and for migration across the endothelium (Ley et al., 2007). In unstimulated leukocytes integrins are usually in a conformation with low binding affinity, until they receive signals from other receptors, such as chemokine receptors (G-protein-coupled receptors), when they change their conformation and display high affinity for ligands (Luo et al., 2007). Chemokines activate β1 or β2 integrins on monocytes, neutrophils, and lymphocytes and as such serve as chemoattractant for these cells during inflammation (Huber et al., 1991; Tanaka et al., 1993; Gunn et al., 1998).

The chemokines are a family of structurally related cytokines that can act as pro-inflammatory agents (Baggiolini et al., 1994; Vaddi et al., 1997). They have the ability to attract leukocyte subsets to specific sites. They recruit neutrophils, monocytes, natural killer cells (NK) and natural killer T (NKT) cells, all of which express inflammatory chemokine receptors and immature dendritic cells (DCs) that provide the link between innate and adaptive immunity (Oo et al., 2010). After antigen-specific activation of lymphocytes by activated DCs, inflammatory chemokines then attract antigen-specific effector T cells to the inflammatory site (Heydtmann and Adams, 2002).

During diapedesis, leukocytes migrate across the endothelium and basement membrane to enter tissue (Ley et al., 2007; Yadav et al., 2003). Once in tissue, the leukocyte follows chemokine gradients to sites of inflammation, using chemokine-mediated changes in the actin cytoskeleton to propel migration. For example, it was demonstrated that chemokines CXCL9, CXCL10 and CXCL11 are important not only in adhesion, but also in transmigration of effectors T lymphocytes through hepatic endothelium (Curbishley et al., 2005; Eksteen et al., 2004). Intracellular actin reorganization is a prerequisite for cell movement, and it has been shown that chemokines such as SDF-1 induce and increase intracellular filamentous actin in lymphocytes (Bleul et al., 1996).

There is essential role of interleukins, but also other factors such as tumor necrosis factor (TNF), interferon (IFN) in leukocyte recruitment and production of chemokines.

Normally, IL-1β binds to IL-1R1 receptor on the surface of target cells. Following ligand binding the adaptor molecule, myeloid differentiation factor-88 (MyD88), interacts with IL-1R1 via its toll interleukin receptor (TIR) domain (O'Neill, 2008). Signal transduction leads to activation of both mitogen-activated protein kinases (MAPKs) and the transcription factor NF-kB, and resulting in pro-inflammatory cytokine expression. For example, chemokine RANTES production requires the transcription factor NF-kB and the activation of mitogen-activated protein kinases (MAPKs) (Genin et al., 2000; Miyamoto et al., 2000; Kujime et al., 2000, Maruoka et al., 2000; Yang et al., 2000).

TNF-α cleavage produces an intracellular domain that translocates to the nucleus and induces pro-inflammatory cytokine signalling, particularly the expression of IL-12 (Friedman et al., 2006). IL-18 induces natural killer and natural killer T cells to produce IFN-γ (Okamura et al., 1998), but it requires IL-12 to induce IFN-γ production by Th1 cells (Nakanishi et al., 2001). There is an essential role of IFN-I in promoting the chronic recruitment of Ly6Chi monocytes. IFN-I production is elicited via a toll like receptor-7 (TLR-7) and MyD88-dependent pathway (Lee et al., 2008).

While CXC-chemokines, e.g. IL-8, act mostly on neutrophils (Springer, 1995), members of the CC-chemokines, e.g. RANTES and macrophage inflammatory protein have been shown to exert function on monocytes, eosinophils and lymphocytes (Baggiolini et al., 1994; Carr et al., 1994). This depends on the receptors that are expressed on leukocytes. Th1 express preferentially CCR5 and CXCR3, while Th2 cells have CCR3, CCR4 and CCR8 on their surface (Syrbe et al., 1999). Monocytes and macrophages express CCR5 and other receptors for RANTES (Weber et al., 2000).

RANTES chemokine is produced by many cells in the extravascular compartment, including fibroblasts, epithelial cells, and tissue-infiltrating lymphocytes and monocytes (MacEwan, 2002; Hehlgans and Männel, 2002; Black et al., 1997). It acts as a potent chemoattractant for monocytes, memory T cells, eosinophils, and basophils (Schall et al., 1988, 1990; Baggiolini and Dahinden, 1994). Elevated levels of RANTES transcripts are detected within hours of exposure to pro-inflammatory stimuli, including IL-1β, TNF-α, IFN-γ, viruses and LPS (Barnes et al., 1996).

Evidence Supporting this KER

Biological Plausibility
There is much evidence that application of chemokines attract leukocytes to specific site in different species (Beck et al., 1997; Lee et al., 2000; Fahy et al., 2001; Nikiforou et al., 2016).

Empirical Evidence
It was shown that a number of chemokines destabilize the rolling of lymphocytes on L-selectin ligands, suggesting that chemokines are capable of regulating the rolling process (Grabovsky et al., 2002).

Jorgensen and colleagues showed that exposure of mice to FliC^{ind} strain S. Typhimurium triggered a significant neutrophil influx in the spleen of wild-type mice, but not Il1b$^{-/-}$Il18$^{-/-}$ mice (Jorgensen et al., 2016).

The expression of chemokines CCL2, CCL7, and CCL12 was reduced dramatically in MyD88-/- mice (Lee et al., 2009).

Miyamoto and colleagues showed that exposure of cells to IL-1β, TNF-α, and IFN-γ resulted in the induction of RANTES mRNA and protein (Ortiz et al., 1996; Miyamoto et al., 2000). The levels of RANTES production by the fibroblasts in the presence of IL-1β or TNF-α were significantly elevated compared with those in the absence of these factors (Yamada et al., 2001).

In the mouse, IFN-γ administration induces high levels of IP-10 expression in liver and kidneys, with lower levels in spleen (Narumi et al., 1992).

CAPE inhibitor of NF-kB blocked partially IL-1β induced expression of chemokines MIP-1a and MIP-1b (Guo et al., 2003).

CCR2 and CCR5 receptors on the CD8 T cells are enriched in the inflamed human liver, and CCR1 is important in the regulation of hepatic inflammation in murine models (Shields et al., 1999; Boisvert et al., 2003).

Intradermal injection of RANTES induces a potent T-lymphocyte and eosinophils recruitment (Fahy et al., 2001; Beck et al., 1997). Intradermal administration of MIP-1a resulted in accumulation of monocytes, lymphocytes, eosinophils and recruitment of neutrophils (Lee et al., 2000).

Direct IL-1α exposure to the gut resulted in increased numbers of CD3+ cells in the fetal sheep ileum when compared with control animals on the first day after the exposure. The number of white blood cells, monocytes, and neutrophils was increased in cord blood after 6 days of IL-1α exposure to the lung and chorioamnion/skin. The number of lymphocytes on the day 6 was increased for the lung. Compared with controls, gut mRNA levels of TNF-α and IL-1 was significantly increased at 6 days after IL-1α exposure to the GI tract (Nikiforou et al., 2016).

IL-1 β induced up-regulation of CXCR4 in certain cancer cells, but in order to do so necessary is that these cells have IL-1R1. Presence of IL-1R antagonist significantly inhibited the up-regulation of CXCR4 induced by IL-1β at both mRNA and protein level (Sun et al., 2015).

SDF-1 is an efficacious chemoattractant and showed a similar dose response for murine lymphocytes and human monocytes, but was not active on human or murine neutrophils. SDF-1 is a highly effective transendothelial chemoattractant (Bleul et al., 1996).

Uncertainties and Inconsistencies
Lloyd and colleagues found that several chemokines can stimulate the adherence of peripheral blood lymphocytes to ICAM-1 coated slides (Loyd et al., 1996). However, by using a parallel plate flow chamber, other study failed to observe such an effect (Carr et al., 1996).

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Relationship: 1778: Leukocyte recruitment/activation leads to Activation, Stellate cells (https://aopwiki.org/relationships/1778)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Endocytic lysosomal uptake leading to liver fibrosis (https://aopwiki.org/aops/144)	**adjacent**	**High**	

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
human	Homo sapiens		NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)
mouse	Mus musculus		NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10090)
rat	Rattus norvegicus		NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10116)

Life Stage Applicability

Life Stage	Evidence
All life stages	

Sex Applicability

Sex	Evidence
Unspecific	

Human (Zimmermann et al., 2010; Liaskou et al., 2013)

Mouse (Seki et al., 2007; Gäbele et al., 2009; Pradere et al., 2013; McHedlidze et al., 2014)

Rat (Reeves et al., 2000; Duffield et al., 2005; Imamura et al., 2005)

Key Event Relationship Description

During hepatic injury quiescent hepatic stellate cells (HSCs) undergo activation which is associated with proliferation, increased contractile activity, fibrogenesis, changes in matrix protease activity, loss of intracellular retinoid storage, production of cytokines, and phenotypic transformation (Friedmann, 2000).

Different inflammatory cells activate HSCs to secrete collagen (Casini et al., 1997). Factors that promote activation of HSC include the cytokines, TNF-α and TGF-β (Bachem et al., 1993), endothelin-l (ET-1) (Rockey and Chung, 1996) and oxidative stress (Lee et al., 1995).

TGF-β is considered the most powerful mediator of HSC activation in vitro and in vivo (Friedmann, 2000; Bataller and Brenner, 2005). TGF-β triggers phenotypical HSC activation by paracrine and autocrine action, and induces collagen I expression and α-smooth muscle actin (α-SMA) stress fiber organization (Gressner et al., 2002; Dooley et al., 2003). TGF-β binds to heteromeric transmembrane receptors, including TβRI and TβRII (Heldin et al., 1997). Binding to TβRII triggers heteromerization with and transphosphorylation of TβRI. The signal is then propagated through phosphorylation of receptor associated Smad2 and Smad3 and their oligomerization with the common mediator Smad4. Complexes of phosphorylated Smad2 or 3 and Smad4 translocate into the nucleus, where they modulate the transcription of target genes, including those encoding extracellular matrix components (ECM) components (Piek et al., 1999; Miyazono et al., 2000; Moustakas et al., 2001). IFN-γ suppresses TGF-β and PDGF-dependent signalling pathways (Fujita et al., 2006).

IL-33 is released by stressed hepatocytes and attracts type 2 innate lymphoid cells (ILC2), which trigger the profibrogenic activation of HSCs via mediators such as IL-13 (McHedlidze et al., 2013; Heymann and Tacke, 2016). The binding of IL-33 to ST2 receptor activates NF-kB and mitogen activated protein kinases (MAPKs) and drives the production of pro-inflammatory and Th2 associated cytokines (Schmitz et al., 2005), which resulted in the stimulation of α-SMA and collagen expression in HSCs (Tan et al., 2018).

IL-13 indirectly activates TGF-β by upregulating the expression of matrix metalloproteinases (MMPs) that cleave the LAP–TGF-β1 complex (Lee et al., 2001; Lanone et al., 2002). Also, IL-13 has been reported to directly induce production of TGF-β1 in HSCs during liver fibrosis (Shimamura et al., 2008). When treating HSCs with IL-13 and performing a time-course analysis, a time-dependent activation of Smad proteins was observed (Liu et al., 2011).

TNF-α stimulated both p38 MAPK and JNK activity in a time-dependent manner, same as ET-1. However, TGF-β had no significant stimulatory effect on either of these MAPKs. The addition of p38 MAPK inhibitor pyridinyl imidazole derivative SB202190 resulted in reduction of α-SMA, indicator of activated HSC (Reeves et al., 2000)

Oxidative stress enhanced activation of HSCs and collagen synthesis in them, whereas antioxidants stopped the stimulatory effect of free radicals (Lee et al., 1995; Svegliati-Baroni et al., 2001). Oxidative stress molecules, such as superoxide, hydrogen peroxide, hydroxyl radicals, may be derived from hepatocytes, activated KCs, other inflammatory cells and HSCs (Natarajan et al., 2006; Kisseleva and Brenner, 2007; Lee and Friedman, 2011).

Liver-infiltrating CD14+ CD16+ monocytes secrete high levels of chemokines (such as CCL1, CCL2, CCL3, CCL5), cytokines (IL-1α, IL-1β, IL-6, IL-13, IL-16, TNF-α and macrophage migration inhibitory factor), growth factors (granulocyte colony-stimulating factor and granulocyte-macrophage colony-stimulating factor) and can efficiently activate primary HSC in vitro (Liaskou et al., 2013; Zimmermann et al., 2010).

Neutrophils may activate HSCs through matrix degradation, secreting compounds like elastase, which then degrades laminin, an extracellular matrix protein in normal liver that is critical for keeping stellate cells in a quiescent state (Friedman et al., 1989). Neutrophil-derived reactive oxygen species (ROS) significantly stimulated procollagen type I accumulation in the HSC culture medium, while the addition of vitamin E or SOD impaired the ROS stimulated stimulation of procollagen I (Casini et al., 1997).

Macrophages are primary source of TGF-β1 in the fibrotic liver (Bataller and Brenner, 2009). Macrophages release several pro-inflammatory cytokines like TNF-α or IL-1 (Tacke and Zimmermann, 2014), which activate the transcription factor NF-kB in HSC and promote the survival of activated HSC (Pradere et al., 2013).

Kupffer cells, liver resident macrophages, after their activation, activate HSC via mechanisms that involve the potent profibrotic cytokines like TGF-β and platelet derived growth factor (PDGF), and ROS (Karlmark et al., 2008; Bataller and Brenner, 2009; Bataller and Lemon, 2012). Apart from directly stimulating matrix-secreting HSC, hepatic macrophages may aggravate scarring by promoting HSC survival via IL-1 and TNF-α induced NF-kB activation (Pradere et al., 2013). Beside resident macrophages, infiltration of macrophages from blood is essential for liver fibrogenesis (Duffield et al., 2005; Imamura et al., 2005).

Th2 cell– derived cytokines, IL-4, IL-5, IL-13, can enhance fibrosis progression by stimulating TGF-β production in macrophages and by direct effects on HSCs (Wynn, 2004).

DCs have a minor contribution to NF-kB activation (Pradere et al., 2013).

NKT cells were also found to promote liver fibrogenesis in vivo, likely by releasing pro-inflammatory cytokines and activating HSCs (Wehr et al., 2013; Syn et al., 2012). However, there are also studies demonstrating that NKT cells may exert antifibrotic actions, because they can, under certain conditions, also kill HSC and produce IFN-γ, like NK cells (Gao et al., 2013; Park et al., 2009).

Signalling pathways for HSC activation include, for example, NF-kB that is involved in HSC activation upon lipopolysaccharide (LPS) or TLR4

stimulation or ATP induced cytosolic Ca^{2+} influx via purinergic signalling receptors (Dranoff et al., 2004). The activation of TLR4 receptor in HSC downregulates TGF-β pseudoreceptor BAMBI and sensitizes these cells for TGF-β, resulting in increased ECM production by HSCs and fibrosis (Seki et al., 2007).

Activated HSCs secrete inflammatory chemokines, express cell adhesion molecules, and modulate the activation of lymphocytes (Vinas et al., 2003). Therefore, a vicious circle in which inflammatory and fibrogenic cells stimulate each other is likely to occur (Maher, 2001).

Evidence Supporting this KER

Biological Plausibility
The recruitment of immune cells from the circulation into the injured tissue is the key mechanism during fibrogenesis in the liver (Heymann and Tacke, 2016).

Empirical Evidence
Upon stimulation, Th1 cells produce interferon-gamma, which is antifibrogenic in liver. C57BL/6 mice contain primarily Th1 cells. Thus, the weak response to CCl_4 in these mice is consistent with an interferon effect. In contrast, BALB/c mice have primarily Th2 lymphocytes. Th2 cells produce IL-4, which induces TGF-β. Both of these cytokines are profibrogenic in liver, which explains why BALB/c mice display enhanced fibrosis in response to CCl_4 in comparison with C57BL/6 mice (Maher, 1999).

In vivo data from Karlmark et al., 2009 suggested that intrahepatic CD11bF4/80 monocyte-derived cells, same as liver resident macrophages, produce TGF-β1 and thereby directly activate HSCs. This was confirmed in an in vitro experiment, in which only intrahepatic recruited monocytes could readily activate HSCs in a TGF-β–dependent manner.

There is strong evidence that the blockade of TGF-β alone is sufficient to completely block experimental fibrogenesis in liver (reviwed in Gressner et al., 2002).

Overexpression of Smad7, a natural antagonist of TGF-β signalling, prevents activation of HSCs and liver fibrosis in rats (Dooley et al., 2003).

The activation of HSC by $CD14^+$ $CD16^+$ monocytes could be partially blocked by anti- TGF-β antibodies (Zimmermann et al., 2010).

In rats receiving CCl_4, HSC activation correlates temporally and spatially with superoxide production (Montosi et al., 1998).

Macrophage depletion by administration of diphtheria toxin intraperitoneally or intravenous led to a significant reduction in the number of HSCs (Duffield et al., 2005).

After bile duct ligation, Kupffer cell–depleted mice showed almost complete suppression of HSC activation and fibrosis (Seki et al., 2007).

Liver injury caused by ischemia and reperfusion, along with TNF-α, CXCL1, and endothelin-A receptor expression, was significantly decreased in HSC-depleted mice compared with controls, with decreased neutrophil infiltration and parenchymal cell death. This suggests that HSCs are involved in hepatic production of CXCL1 and contribute to neutrophil recruitment (Stewart et al., 2014).

Important evidence that HSCs produce chemoattractant is that MCP-1 mRNA was clearly co-distributed with cells expressing α-SMA, a marker of activated HSCs (Marra et al., 1998).

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Relationship: 295: Activation, Stellate cells leads to Accumulation, Collagen (https://aopwiki.org/relationships/295)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Protein Alkylation leading to Liver Fibrosis (https://aopwiki.org/aops/38)	**adjacent**	**High**	
Endocytic lysosomal uptake leading to liver fibrosis (https://aopwiki.org/aops/144)	**adjacent**	**High**	

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
human	Homo sapiens	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)
Rattus norvegicus	Rattus norvegicus	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10116)

Life Stage Applicability

Life Stage	Evidence
All life stages	

Sex Applicability

Sex	Evidence
Unspecific	

Human: Safadi and Friedman, 2002; Bataller and Brenner, 2005; Lee und Friedman 2011.

Rat: Li, Li et al., 2012; Luckey and Petersen, 2001; Rockey et al., 1992

Key Event Relationship Description

Up-regulation of collagen synthesis following hepatic stellate cell (HSC) activation is among the most striking molecular responses of HSCs to injury and is mediated by both transcriptional and post-transcriptional mechanisms. Activated HSCs do not only proliferate and increase cell number, but also increase collagen production per cell. Synthesis of type I collagen is initiated by expression of the col1a1 and col1a2 genes, giving rise to α 1(I) and α 2(I) procollagen mRNAs in a 2:1 ratio. Upon activation of HSCs and other myofibroblast precursors, there is a > 50-fold

increase in α 1(I) procollagen mRNA levels. The half-life of collagen α1(I) mRNA increases 20-fold in activated HSCs compared with quiescent HSCs. Monocytes and macrophages are involved in inflammatory actions by producing large amounts of Nitric oxide (NO) and inflammatory cytokines such as TNF-α which have a direct stimulatory effect on HSC collagen synthesis. Synthesis of TGF-α and TGF-β promotes activation of neighbouring quiescent HSCs, whereas the release of HGF (hepatocyte growth factor) stimulates regeneration of adjacent hepatocytes.

The basement membrane-like matrix is normally comprised of collagens IV and VI, which is progressively replaced by collagens I and III and cellular fibronectin during fibrogenesis. Although multiple extracellular matrix (ECM) components are up-regulated, type I collagen is the most abundant protein. These changes in ECM composition initiate several positive feedback pathways that further amplify collagen production. Increasing matrix stiffness is a stimulus for HSC activation and matrix-provoked signals link to other growth factor receptors through integrin-linked kinase and transduce via membrane-bound guanosine triphosphate binding proteins, in particular Rho67 and Rac, signals to the actin cytoskeleton that promote migration and contraction.

The overall amount of collagen deposited by fibroblasts is a regulated balance between collagen synthesis and collagen catabolism. Down-regulated expression of degrading Matrix metalloproteinases (MMPs) and up-regulation of tissue inhibitors of metalloproteinases (TIMPs), MMP-inhibitors, lead to a net decrease in protease activity, and therefore, matrix accumulation. Chronic inflammation, hypoxia and oxidative stress reactivate epithelial-mesenchymal transition (EMT) developmental programmes that converge in the activation of NF-kB. Cells that may transdifferentiate into fibrogenic myofibroblasts are hepatocytes and cholangiocytes. Additional sources of ECM include bone marrow (which probably gives rise to circulating fibrocytes) and portal fibroblasts (Benyon and Arthur; 2001; Milani et al., 1994; Safadi and Friedman, 2002; Kolios et al.,2006; Bataller and Brenner, 2005; Lee und Friedman 2011; Guo and Friedman, 2007; Li, Jing-Ting et al., 2008; Kershenobich Stalnikowitz and Weisssbrod , 2003; López-Novoa and Nieto, 2009; Friedman, 2010; 2008; Dalton et al., 2009; Leung, et al., 2008; Nan et al., 2013; Hamdy and El-Demerdash, 2012;Li, Li et al., 2012; Natajaran et al., 2006; Luckey and Petersen, 2001; Chen and Raghunath, 2009;Thompson et al., 2011; Henderson and Iredale, 2007).

Evidence Supporting this KER

Biological Plausibility

There is general acceptance that HSCs are collagen producing cells and key actors in fibrogenesis. The functional relationship between these KEs is consistent with biological knowledge (Benyon and Arthur; 2001; Milani et al., 1994; Safadi and Friedman, 2002; Kolios et al.,2006; Bataller and Brenner, 2005; Lee und Friedman 2011; Guo and Friedman, 2007; Li, Jing-Ting et al., 2008; Kershenobich Stalnikowitz and Weisssbrod , 2003; López-Novoa and Nieto, 2009).

Empirical Evidence

It is difficult to stimulate sufficient collagen production and its subsequent incorporation into a pericellular matrix in vitro; therefore analytical methods have focused on measurement of pro-collagen secreted into culture medium or measurement of α-smooth muscle actin (α-SMA) expression, a marker of fibroblast activation. In primary culture, HSCs from normal liver begin to express α-SMA coincident with culture-induced activation (Chen and Raghunath, 2009; Rockey et al.,1992).

Uncertainties and Inconsistencies

no inconsistencies

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Relationship: 82: Accumulation, Collagen leads to N/A, Liver fibrosis (https://aopwiki.org/relationships/82)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Protein Alkylation leading to Liver Fibrosis (https://aopwiki.org/aops/38)	**adjacent**	**High**	
Endocytic lysosomal uptake leading to liver fibrosis (https://aopwiki.org/aops/144)	**adjacent**	**High**	

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
human	Homo sapiens	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)
Rattus norvegicus	Rattus norvegicus	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10116)

Life Stage Applicability

Life Stage	Evidence
All life stages	

Sex Applicability

Sex	Evidence
Unspecific	

Human: Lee and Friedman, 2011; Bataller and Brenner, 2005; Brancatelli et al., 2009; Rockey and Friedman, 2006; Poynard et al., 1997.

Rat :Liedtke et al., 2013.

Key Event Relationship Description

Liver fibrosis is the excessive accumulation of extracellular matrix (ECM) proteins including collagen. Liver fibrosis results from an imbalance between the deposition and degradation of ECM and a change of ECM composition; the latter initiates several positive feedback pathways that further amplify fibrosis. With chronic injury, there is progressive substitution of the liver parenchyma by scar tissue. Deposition of collagen in the liver progressively disrupts the normal hepatic architecture so that the normal relationship between vascular inflow and outflow is destroyed and the normal collagen content around hepatic sinusoids in regenerating nodules becomes modified.Advanced liver fibrosis results in cirrhosis (Lee and Friedman, 2011; Bataller and Brenner, 2005; Pellicoro et al., 2014;Brancatelli et al., 2009; Rockey and Friedman, 2006; Poynard et al., 1997).

Evidence Supporting this KER

Biological Plausibility
By definition, liver fibrosis is the excessive accumulation of ECM proteins that are produced by HSCs. The KER between this KE and the AO is undisputed (Lee and Friedman, 2011; Bataller and Brenner, 2005;Brancatelli et al., 2009; Rockey and Friedman, 2006; Poynard et al., 1997).

Empirical Evidence

There is a smooth transition from ECM accumulation to liver fibrosis without a definite threshold and plenty in vivo evidence exists that ECM accumulation is a pre-stage of liver fibrosis (Lee and Friedman, 2011; Bataller and Brenner, 2005;Brancatelli et al., 2009; Rockey and Friedman, 2006; Poynard et al., 1997).

Uncertainties and Inconsistencies
no inconsistencies

References

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