SNAPSHOT

AOP ID and Title:

AOP 144: Lysosomal damage leading to liver inflammation
Short Title: NM-induced liver inflammation

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Abstract

Hepatotoxicity is known to be an important endpoint of regulatory concern; especially in drug development it has been one of the most frequent reasons for pharmacovigilance safety reports and withdrawal of drugs from the market. Liver inflammation can be both the relevant endpoint itself, or it occurs during development of liver fibrosis, for example, upon repeated exposure. The current AOP links lysosomal disruption to liver inflammation. Lysosomal damage can be caused by multiple initiators: examples are the detergent O-methyl-serine dodecylamide hydrochloride (MSDH), alpha-tocopheryl succinate, naphthazarine [1] [2], 3-aminopropanal [2], the antimalarial agent artesunate (ART) [3] and also nanomaterials (NMs) [4]. Lysosomal rupture by NMs has been described as one of the main causes for their potential to induce cellular damage, which is subsequently linked to an increase of reactive oxygen species (ROS), mitochondrial damage and induction of the inflammatory cascade. Liver inflammation is therefore a local outcome following translocation of NMs to the liver. Uptake and disruption of the lysosome is not a classical MIE, as no "molecular" but rather mechanical processes are involved. However, it is the initiating event for the described AOP.

Summary of the AOP

Stressors

We will add things to here soon

Molecular Initiating Event

Title	Short name	Essentiality
Disruption, Lysosome	Disruption, Lysosome	Strong

Key Events

Title	Short name	Essentiality
Increase, Oxidative Stress	Increase, Oxidative Stress	Moderate
N/A, Mitochondrial dysfunction 1	N/A, Mitochondrial dysfunction 1	Strong
N/A, Cell injury/death	N/A, Cell injury/death	Strong
Release, Cytokine	Release, Cytokine	Strong
Infiltration, Inflammatory cells	Infiltration, Inflammatory cells	Strong

Adverse Outcomes

Title	Short name	Essentiality
Inflammation, Liver	Inflammation, Liver	

Relationships between Key Events

Upstream Event	Relationship Type	Downstream Event
Increase, Oxidative Stress	directly leads to	N/A, Mitochondrial dysfunction 1
N/A, Mitochondrial dysfunction 1	directly leads to	N/A, Cell injury/death
N/A, Cell injury/death	directly leads to	Release, Cytokine
Disruption, Lysosome	directly leads to	Increase, Oxidative Stress
Disruption, Lysosome	directly leads to	N/A, Mitochondrial dysfunction 1
Release, Cytokine	directly leads to	Infiltration, Inflammatory cells
Infiltration, Inflammatory cells	directly leads to	Inflammation, Liver

Life Stage Applicability

Life Stage	Evidence
all life stages	Moderate

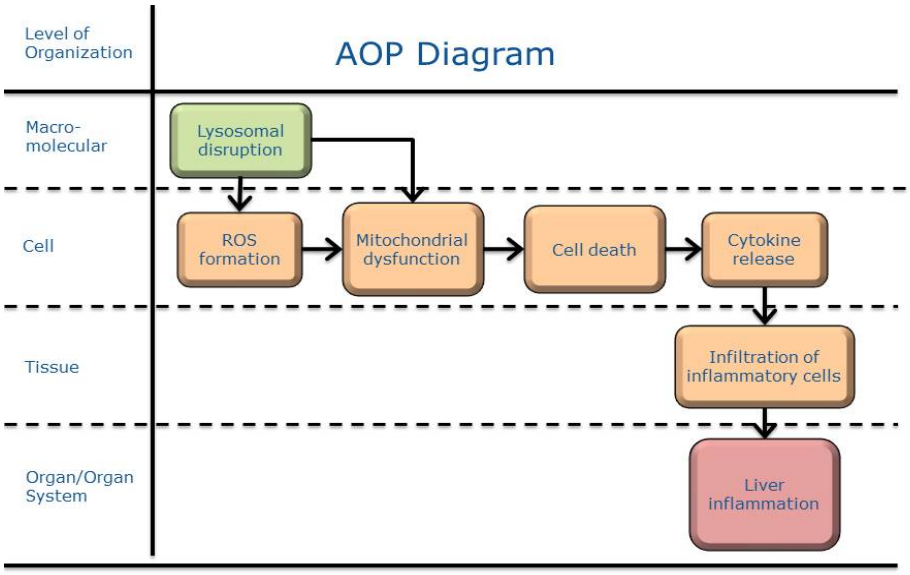
Taxon Applicability

Term	Scientific Term	Evidence
human	Homo sapiens	Strong
mouse	Mus musculus	Strong
rat	Rattus norvegicus	Weak

Sex Applicability

Sex	Evidence
Unspecific	

Graphical Representation



Molecular Initating Event

Event ID and Title

898: Disruption, Lysosome
Short Name: Disruption, Lysosome

Key Event Overview

AOPs Including This Key Event

AOP ID and Name	Event Type	Essentiality
144: Lysosomal damage leading to liver inflammation	MolecularInitiatingEvent	Strong

Stressors

The following are stressors that operate directly through this Event.
Will need to add a loop here once we have chemicals figured out

1. chemical one
2. chemical two

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
human	Homo sapiens	Strong	NCBI
mouse	Mus musculus	Strong	NCBI

Evidence supporting Taxonomic Applicability

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

[14]: murine

[15]: murine, human

[19]: murine, human

[10]: human

[17]: human, murine

[9]: human

Level of Biological Organization

Cellular

Life Stage Applicability

There are no Life Stages associated with this Event

Sex Applicability

There are no Sexes associated with this Event

How this Key Event Works

Lysosomes were first described by de Duve and colleagues in 1955 [1]. 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 [2].

Lysosomes are the terminal organelle of the endocytic pathway, but are also involved in membrane repair and other cellular processes, such as immune responses [3]. The amount of lysosomal enzymes that are released into the cytosol regulates the cell death pathway which is initiated by lysosomal damage: it plays a vital role in the induction of apoptosis, whereas massive lysosomal rupture leads to necrosis [4] [5]. Lysosomes are known to be involved in external as well as internal apoptotic pathways. The external pathway triggers lysosomal destabilization by hydroxyl radicals, p53, caspase 8,through activation of Bax or by ceramide which is converted into sphingosine [6]. The internal apoptotic pathway on the contrary is activated through mitochondrial damage, for example via activation of Bax or Bid, phospholipases, or lysosomal enzymes [6].

The lysosome contains redox-active labile irons which are suggested to be involved in local ROS production via a Fenton-type reaction [7]. 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 [8] [9]. Released enzymes such as phospholipases can further damage the outer membrane of the mitochondrion, leading to a further increased and uncontrolled ROS production, the release of cytochrome c, the activation of the caspase cascade, and subsequent apoptotic cell death [10].

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 [11] [12] [13].

How it is Measured or Detected

Lysosomes are typically analysed microscopically.

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 [14] [15] [16].

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 [17]. This method as well as use of LysoSensor probes has been reported repeatedly elsewhere, for example [9] [16].

More specific staining can be achieved by staining with antibodies against lysosomal membrane proteins [16].

Lysosomal membrane permeabilization can be visualized by immunostaining of lysosomal enzymes such as cathepsin B [18].

References

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Key Events

Event ID and Title

209: Increase, Oxidative Stress
Short Name: Increase, Oxidative Stress

Key Event Overview

AOPs Including This Key Event

AOP ID and Name	Event Type	Essentiality
21: AhR activation leading to embryo toxicity in fish	KeyEvent	
27: Cholestatic Liver Injury induced by Inhibition of the Bile Salt Export Pump (ABCB11)	KeyEvent	
108: Inhibition of pyruvate dehydrogenase kinase leading to hepatocellular adenomas and carcinomas (in mouse and rat)	KeyEvent	
144: Lysosomal damage leading to liver inflammation	KeyEvent	Moderate
149: Oxidative Stress Leading to Hypertension	MolecularInitiatingEvent	Strong

Stressors

The following are stressors that operate directly through this Event.
Will need to add a loop here once we have chemicals figured out

1. chemical one
2. chemical two

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
human	Homo sapiens	Strong	NCBI
rodents	rodents	Strong	NCBI
human and other cells in culture	human and other cells in culture	Strong	NCBI

Level of Biological Organization

Cellular

Life Stage Applicability

There are no Life Stages associated with this Event

Sex Applicability

There are no Sexes associated with this Event

How this Key Event Works

Oxidative stress corresponds to an imbalance between the rate of oxidant production and that of their degradation. The term oxidative stress indicates the outcome of oxidative damage to biologically relevant macromolecules such as nucleic acids, proteins, lipids and carbohydrates. This occurs when oxidative stress-related molecules, generated in the extracellular environment or within the cell, exceed cellular antioxidant defenses. Major reactive oxygen species (ROS), such as hydrogen peroxide (H2O2) and superoxide anion, as well as 4-hydroxy- 2,3-nonenal (HNE) and related 4-hydroxy-2,3-alkenals (HAKs), major aldehydic end-products of lipid peroxidation, can act as potential mediators able to affect signal transduction pathways as well as the proliferative and functional response of target cells. H2O2 and superoxide anion may be also generated as molecular messengers within the cell as part of the cellular response to defined growth factors, cytokines and other mediators. The final consequence at tissue, cellular and molecular level is primarily affected by the steady state concentration of oxidative stress-related molecules. The main biological targets of free radicals are proteins, lipids and DNA.

Major consequences of reaction of ROS, HAKs and NO with biologically relevant macromolecules that can mediate pathophysiological effects:

ROS: DNA: oxidation, strand breaks, genotoxicity Proteins: oxidation, fragmentation, formation of carbonyls Lipids: lipid peroxidation and degradation

HAKs: DNA: adducts (low doses), strand breaks, genotoxicity (high doses) Proteins: adducts (Michael type reactions on Lys, Cys

and His residues)

NO: DNA: oxidation, strand breaks Proteins: oxidation, nitrosation, nitration (nytrosylation of tyrosine) Lipids: lipid peroxidation and degradation

Continued oxidative stress can lead to chronic inflammation. Oxidative stress can activate a variety of transcription factors including NF-κB, AP-1, p53, HIF-1α, PPAR-γ, β-catenin/Wnt, and Nrf2. Activation of these transcription factors can lead to the expression of over 500 different genes, including those for growth factors, inflammatory cytokines and chemokines, which can activate inflammatory pathways. [1] [2] [3]

How it is Measured or Detected

measuring oxidative stress

*Agents for **ROS detection** are primarily fluorescence based, but recently luminescent based detections have been introduced. The biggest difficulty reported with much of the cellular ROS research has been with the lack of reporter agents specific for discrete molecules. ROS moieties by their nature are reactive with a number of different molecules; as such designing reporter agents has been difficult. With more specific chemistries, particularly for hydrogen peroxide, the specific mechanisms for regulation will be elucidated.*

***Reduced glutathione** (GSH) is regenerated from its oxidized form (GSSH) by the action of an NADPH dependent reductase GSSH + NADPH + H+ à 2 GSH + NADP+ Due to the rapid nature of the reduction of GSSH relative to its synthesis or secretion, the ratio of GSH to GSSH is a good indicator of oxidative stress within cells. GSH and GSSH levels can be determined by HPLC, capillary electrophoresis, or biochemically in microplates. Several different assays have been designed to measure glutathione in samples. By using a luciferin derivative in conjunction with glutathione S-transferase enzyme the amount of GSH would be proportional to the luminescent signal generated when luciferase is added in a subsequent step. Total glutathione can be determined colorimetrically by reacting GSH with DTNB (Ellman's reagent) in the presence of glutathione reductase. Glutathione reductase reduces GSSH to GSH, which then reacts with DTNB to produce a yellow colored 5-thio-2-nitrobenzoic acid (TNB), which absorbs at 412 nm.*

***Lipid peroxidation** is one of the most widely used indicators of free radical formation, a key indicator of oxidative stress. Measurement of lipid peroxidation has historically relied on the detection of thiobarbituric acid (TBA) reactive compounds such as malondialdehyde generated from the decomposition of lipid peroxidation products. While this method is controversial in that it is quite sensitive, but not necessarily specific to MDA, it remains the most widely used means to determine lipid peroxidation. This reaction, which takes place under acidic conditions at 90-100ºC, results in an adduct that can be measured colorimetrically at 532 nm or by fluorescence using a 530 nm excitation wavelength and a 550 nm emission wavelength. 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 specific for lipid peroxidation. Unlike the TBA assay, measurement of IsoP appears to be specific to lipid peroxides, they are stable and are not produced by any enzymatic pathway making interpretation easier. There have been a number of commercial ELISA kits developed for IsoPs, but interfering agents in samples requires partial purification of samples prior to running the assay. The only reliable means for detection is through the use of GC/MS, which makes it expensive and limits throughput.*

***Superoxide detection** is based on the interaction of superoxide with some other compound to create a measurable result. The reduction of ferricytochrome c to ferrocytochrome c has been used in a number of situations to assess the rate of superoxide formation. While not completely specific for superoxide this reaction can be monitored colorimetrically at 550 nm. Chemiluminescent reactions have been used for their potential increase in sensitivity over absorbance-based detection methods. The most widely used chemiluminescent substrate is Lucigenin, but this compound has a propensity for redox cycling, which has raised doubts about its use in determining quantitative rates of superoxide production. Coelenterazine has also been used as a chemiluminescent substrate. Hydrocyanine dyes are fluorogenic sensors for superoxide and hydroxyl radical. These dyes are synthesized by reducing the iminium cation of the cyanine (Cy) dyes with sodium borohydride. While weakly fluorescent, upon oxidation their fluorescence intensity increases 100 fold. In addition to being fluorescent, oxidation also converts the molecule from being membrane permeable to an ionic impermeable moiety. The most characterized of these probes are Hydro-Cy3 and Hydro-Cy5.*

***Hydrogen peroxide** (H2O2) is the most important ROS in regards to mitogenic stimulation or cell cycle regulation. 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. The more commonly used substrates include diacetyldichloro-fluorescein, homovanillic acid, and Amplex® Red. In these examples, increasing amounts of H2O2 form increasing amounts of fluorescent product.*

***Nitric Oxide** The free radical nitric oxide (•NO) is produced by a number of different cell types with a variety of biological functions. Regardless of the source or role, the free radical •NO has a very short half life (t½= 4 seconds), reacting with several different*

molecules normally present to form either nitrate (NO3-) or nitrite (NO2-) A commonly used method for the indirect determination of •NO is the determination of its composition products nitrate and nitrite colorimetrically. This reaction requires that nitrate (NO3) first be reduced to nitrite (NO2), typically by the action of nitrate reductase. Subsequent determination of nitrite by a two step process provides information on the "total" of nitrate and nitrite. In the presence of hydrogen ions nitrite forms nitrous acid, which reacts with sulfanilamide to produce a diazonium ion. This then coupled to N-(1-napthyl) ethylenediamine to form the chromophore which absorbs at 543 nm. Nitrite only determinations can then be made in a parallel assay where the samples were not reduced prior to the colorimetric assay. Actual nitrate levels are then calculated by the subtraction of nitrite levels from the total. [4]

References

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Event ID and Title

177: N/A, Mitochondrial dysfunction 1
Short Name: N/A, Mitochondrial dysfunction 1

Key Event Overview

AOPs Including This Key Event

AOP ID and Name	Event Type	Essentiality
48: Binding of agonists to ionotropic glutamate receptors in adult brain causes excitotoxicity that mediates neuronal cell death, contributing to learning and memory impairment.	KeyEvent	Strong
77: Nicotinic acetylcholine receptor activation contributes to abnormal foraging and leads to colony loss/failure 1	KeyEvent	Strong
78: Nicotinic acetylcholine receptor activation contributes to abnormal roll change within the worker bee caste leading to colony loss/failure 1	KeyEvent	
79: Nicotinic acetylcholine receptor activation contributes to impaired hive thermoregulation and leads to colony loss/failure	KeyEvent	
80: Nicotinic acetylcholine receptor activation contributes to accumulation of damaged mitochondrial DNA and leads to colony loss/failure	KeyEvent	
87: Nicotinic acetylcholine receptor activation contributes to abnormal foraging and leads to colony loss/failure 2	KeyEvent	
3: Inhibition of the mitochondrial complex I of nigra-striatal neurons leads to parkinsonian motor deficits	KeyEvent	Strong
144: Lysosomal damage leading to liver inflammation	KeyEvent	Strong
178: Nicotinic acetylcholine receptor activation contributes to mitochondrial dysfunction and leads to colony loss/failure	KeyEvent	
200: Estrogen receptor activation leading to breast cancer	KeyEvent	Strong

Stressors

The following are stressors that operate directly through this Event.
Will need to add a loop here once we have chemicals figured out

1. chemical one
2. chemical two

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
human	Homo sapiens	Strong	NCBI
mouse	Mus musculus	Strong	NCBI
rat	Rattus norvegicus	Strong	NCBI

Evidence supporting Taxonomic Applicability

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).

Level of Biological Organization

Cellular

Life Stage Applicability

There are no Life Stages associated with this Event

Sex Applicability

There are no Sexes associated with this Event

How this Key Event Works

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 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), tetramethylrhodamine, methyl 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 determined 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 (e.g. Sigma, http://www.abcam.com/atp-assay-kit-colorimetricfluorometric-ab83355.html), 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). 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 teh 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 sit 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 (H2O2) 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 H2O2 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

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Event ID and Title

55: N/A, Cell injury/death

Short Name: N/A, Cell injury/death

Key Event Overview

AOPs Including This Key Event

AOP ID and Name	Event Type	Essentiality
48: Binding of agonists to ionotropic glutamate receptors in adult brain causes excitotoxicity that mediates neuronal cell death, contributing to learning and memory impairment.	KeyEvent	Strong
13: Chronic binding of antagonist to N-methyl-D-aspartate receptors (NMDARs) during brain development induces impairment of learning and memory abilities	KeyEvent	Strong
38: Protein Alkylation leading to Liver Fibrosis	KeyEvent	Strong
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	KeyEvent	Strong
144: Lysosomal damage leading to liver inflammation	KeyEvent	Strong

Stressors

The following are stressors that operate directly through this Event.
Will need to add a loop here once we have chemicals figured out

1. chemical one
2. chemical two

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
human	Homo sapiens	Strong	NCBI
human and other cells in culture	human and other cells in culture	Strong	NCBI
Rattus norvegicus	Rattus norvegicus	Strong	NCBI
mouse	Mus musculus	Strong	NCBI

Evidence supporting Taxonomic Applicability

Cell death is an universal event occurring in cells of any species. [11]

Level of Biological Organization

Cellular

Life Stage Applicability

There are no Life Stages associated with this Event

Sex Applicability

There are no Sexes associated with this Event

How this Key Event Works

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 [1]. 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 [1][2]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 [1], [2] [3][4]

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. [5]

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 [6].

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 [7] .

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 [8].

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. [9] [10]

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Event ID and Title

87: Release, Cytokine
Short Name: Release, Cytokine

Key Event Overview

AOPs Including This Key Event

AOP ID and Name	Event Type	Essentiality
27: Cholestatic Liver Injury induced by Inhibition of the Bile Salt Export Pump (ABCB11)	KeyEvent	
144: Lysosomal damage leading to liver inflammation	KeyEvent	Strong

Stressors

The following are stressors that operate directly through this Event.
Will need to add a loop here once we have chemicals figured out

1. chemical one
2. chemical two

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
human	Homo sapiens	Strong	NCBI
mouse	Mus musculus	Strong	NCBI

Evidence supporting Taxonomic Applicability

[4][6][5]: mouse [3][7]: human

Level of Biological Organization

Cellular

Life Stage Applicability

There are no Life Stages associated with this Event

Sex Applicability

There are no Sexes associated with this Event

How this Key Event Works

Cytokines are small, soluble molecules secreted by cells to enable intercellular communication. Cytokines may act on the cells that secrete them (autocrine action), on nearby cells (paracrine action), as well as on distant cells (endocrine action). Cytokines can act synergistically or antagonistically, and secretion from one cell can trigger upregulation of a further range of cytokines from the same cell or others [1]. Most cells in the body are able to secrete them, and several subfamilies belong to the group of cytokines, such as chemokines, interferons, interleukins, tumor necrosis factors (TNF), transforming growth factors (TGF) and colony-stimulating factors. They are important players in modulating fundamental biological processes, including body growth, adiposity, lactation, hematopoiesis, and also inflammation and immunity[2]. Damaged cells, such as apoptotic cells, can trigger the upregulation and release of cytokines to induce the inflammatory response. An important receptor responsible for cell death-related cytokine regulation is Fas, a cell surface glycoprotein which belongs to the tumor necrosis factor (TNF) receptor family. The role of Fas in the onset of inflammation by upregulating inflammatory cytokines is increasingly discussed. Fas-activation can trigger the production of MCP-1 and IL-8 and its associated chemotaxis of phagocytes toward apoptotic cells[3].

TNF-α is an inflammatory mediator that can be secreted by many cell types, including hepatocytes and Kupffer cells. TNF-induced cytokines and chemokines, such as IL-6, IL-8, GMCSF, CXCL1, and RANTES, can trigger immune responses by producing acute phase proteins and recruitment of inflammatory cells such as neutrophils, macrophages, and basophils to the site of inflammation. Moreover, an increased production of monocytes/macrophages from bone marrow is triggered[3].

On the other hand, inflammation can be suppressed by cytokines and mediators such as IL-10 and TGF-β. In the liver, TGF-β1 is the most abundant isoform and is secreted by immune cells, stellate cells, and epithelial cells. IL-10 inhibits T cell-, monocyte-, and macrophage-mediated functions and has been detected in several liver cells, in¬cluding hepatocytes, stellate cells, and Kupffer cells [2].

How it is Measured or Detected

Methods that have been previously reviewed and approved by a recognized authority should be included in the Overview section above. All other methods, including those well established in the published literature, should be described here. Consider the following criteria when describing each method: 1. Is the assay fit for purpose? 2. Is the assay directly or indirectly (i.e. a surrogate) related to a key event relevant to the final adverse effect in question? 3. Is the assay repeatable? 4. Is the assay reproducible?

mRNA expression levels of inflammatory cytokines can be determined by using real-time PCR as described in [4]. Equally, In Situ Hybridization of mRNA in liver tissue can be used [5].

Plasma levels of pro-inflammatory cytokines, or levels in cell supernatants can be analysed by enzyme linked immunosorbent assay (ELISA) using commercial kits [6][3]. A more advanced system was described recently by using a multiplex immunoassay platform. In a 96 well plate format the authors describe the analysis of blood, urine and breath samples of human volunteers in a Meso Scale Discovery (MSD) multiplex electrochemiluminescent immunoassay system [7].

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Event ID and Title

901: Infiltration, Inflammatory cells
Short Name: Infiltration, Inflammatory cells

Key Event Overview

AOPs Including This Key Event

AOP ID and Name	Event Type	Essentiality
144: Lysosomal damage leading to liver inflammation	KeyEvent	Strong

Stressors

The following are stressors that operate directly through this Event.
Will need to add a loop here once we have chemicals figured out

1. chemical one
2. chemical two

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
human	Homo sapiens	Strong	NCBI
mouse	Mus musculus	Strong	NCBI

Evidence supporting Taxonomic Applicability

[1]: human (cells); [13]: human (tissue; representative for general application in patients, as liver inflammation is commonly found in patients with DILI)

<[15][14][16]: mouse (nanomaterial-induced)

Level of Biological Organization

Tissue

Life Stage Applicability

There are no Life Stages associated with this Event

Sex Applicability

There are no Sexes associated with this Event

How this Key Event Works

TNF-induced cytokines and chemokines, such as IL-6, IL-8, GMCSF, CXCL1, and RANTES, can instigate and amplify immune responses through triggering the production of acute phase proteins and the recruitment of neutrophils, macrophages, and basophils to the site of inflammation, and by triggering increased production of monocytes/macrophages from bone marrow[1]. Monocytes are the precursors of macrophages and dendritic cells and circulate in the blood for 1-3 days. Upon secretion of chemokines such as CCL2 which is also referred to as monocyte chemoattractant protein 1 (MCP1), they can migrate towards affected tissue. This was nicely demonstrated when depletion of MCP-1 in supernatants of Fas-stimulated cells was sufficient to block almost all THP-1 monocyte chemotaxis. Using an in vivo mouse model, the authors found that Fas stimulation could trigger phagocyte migration by administration of anti-Fas (Jo2) antibody into C57BL/6 mice within 10 h of anti-Fas administration. This correlated with extensive cell death in the thymus and a dramatic increase of CD11b-positive macrophages in the same tissue[1].

Neutrophils, on the other hand, account for about 50 70 % of all blood leukocytes in the human body [2][3]. Upon an inflammatory event, neutrophil production is upregulated, and its lifetime increases as a response to platelet activating factor (PAF), granulocyte-colony stimulating factor (G-CSF) or various pro-inflammatory cytokines, such as interleukin 1ß (IL-1ß) [3]. The crucial role of PMN in the human immune system is long known. In 1968, Baehner and Karnovsky described a link between a reduced PMN activity and the development of chronic granulomatous disease (CGD) [4]. The important peroxidase-mediated bactericidal role of PMN and the formation of superoxide radicals as one of the main bactericial mechanisms was already described more than 30 years ago [5][6]. A strong negative correlation between the chemotactic ability of PMN and patients with increased bacterial sepsis was demonstrated [7], and clinical morbidity from infections is clearly increased with a reduced number of circulating PMN in the blood [8]. The neutrophilic cytosol contains granules that are filled with a variety of proteins, such as defensins, bactericidal-permeability-increasing protein, proteases (e.g. elastase, cathepsins), and myeloperoxidase (MPO) that consumes hydrogen peroxide (H2O2) and generates hypochlorous acid (HOCl), the most bactericidal oxidant that is produced by PMN [8][2]. Activated neutrophils are capable of producing a variety of pro-inflammatory cytokines, e.g. IL-1ß, IL-6, IL-12 and IL-23, and transport internalised pathogens to lymph nodes to support macrophages and dendritic cells in antigen presentation[9]. Also, contact with pathogens results not only in phagocytosis, but also in the so-called oxidative burst, marked by an increased consumption of molecular oxygen and resulting production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) [10].

Deregulation of this response by constant stimulation of PMNs, as could be shown for nanoparticles for example, ultimately leads to the establishment of a (chronic) inflammation. Here, also macrophages play a vital role. Resident alveolar macrophages, such as Kupffer cells in the liver, that usually phagocyte microorgansims or particles will be activated when overwhelmed by the amount of invading pathogens and in turn release inflammatory cytokines and chemokines. Consequently, neutrophils are recruited and activated as described above [11][12].

How it is Measured or Detected

Methods that have been previously reviewed and approved by a recognized authority should be included in the Overview section above. All other methods, including those well established in the published literature, should be described here. Consider the following criteria when describing each method: 1. Is the assay fit for purpose? 2. Is the assay directly or indirectly (i.e. a surrogate) related to a key event relevant to the final adverse effect in question? 3. Is the assay repeatable? 4. Is the assay reproducible?

Chemotaxis assays can be performed in vitro/ex vivo by using Chemotaxis Chambers (for example Neuro Probe Chambers). Supernatants can be added to the bottom well of the chamber and 3–8 mm nitrocellulose filters are placed on top, while the top chamber contains the inflammatory cells (for example neutrophils). After a certain time period, the number of migrated cells towards

the lower chamber can be determined by staining of the cells[1].

Influx of inflammatory cells (mainly neutrophils) can be analysed by tissue staining by using Haematoxylin and eosin [13].

In mice, neutrophil influx can be analysed using a mouse MPO ELISA kit for lysed tissue [14].

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Adverse Outcome

Event ID and Title

902: Inflammation, Liver
Short Name: Inflammation, Liver

Key Event Overview

AOPs Including This Key Event

AOP ID and Name	Event Type
144: Lysosomal damage leading to liver inflammation	AdverseOutcome

Stressors

The following are stressors that operate directly through this Event.

Will need to add a loop here once we have chemicals figured out

1. chemical one
2. chemical two

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
mouse	Mus musculus	Strong	NCBI
human	Homo sapiens	Strong	NCBI
rat	Rattus norvegicus	Moderate	NCBI

Evidence supporting Taxonomic Applicability

[7]: human (representative for general application in patients, as liver inflammation is commonly found in patients with DILI)

[8][4][9]: mouse (nanomaterial-induced)

[10]: rat (nanomaterial-induced)

Level of Biological Organization

Organ

Life Stage Applicability

There are no Life Stages associated with this Event

Sex Applicability

There are no Sexes associated with this Event

How this Key Event Works

Approximately 29 million people in the European Union suffer from a chronic liver condition [1]. Inflammation is a crucial link that is related to many of these conditions, with the potential for the development of cirrhosis or primary liver cancer which represent the end-stage of liver pathology and are often associated with mortality: chronic hepatitis (A-E), non-alcoholic steatohepatitis (NASH) which is the progressive form of non-alcoholic fatty liver disease (NAFLD), primary biliary cirrhosis (PBC) or primary sclerosing cholangitis (PSC) [1]. Drug-induced liver injury (DILI) still is a major problem in drug development as its early detection is problematic, and acute liver inflammation is the most common symptom. DILI is the main cause for withdrawal of drugs from the pharmaceutical market [2]. Liver inflammation is marked by an increased influx of neutrophils, following the secretion of signaling factors such as CXC chemokines and macrophage inflammatory protein 2 (MIP-2) from damaged cells [3]. Kupffer cells (KCs), the resident macrophages of the liver and accounting for about 15-20% of total cell numbers in a healthy liver. They are the gatekeepers in the liver, as they monitor the blood that enters this organ [4][5]. Activation of KCs by activation of toll like receptors, for example, leads to the recruitment of further inflammatory cells as well as amplified KC activation. This, in turn, activates Hepatic stellate cells (HSCs) [5] which can link liver inflammation to further severe outcomes such as development of fibrosis

A list of drugs generally known to induce DILI can be found here [6].

How it is Measured or Detected

Methods that have been previously reviewed and approved by a recognized authority should be included in the Overview section above. All other methods, including those well established in the published literature, should be described here. Consider the following criteria when describing each method: 1. Is the assay fit for purpose? 2. Is the assay directly or indirectly (i.e. a surrogate) related to a key event relevant to the final adverse effect in question? 3. Is the assay repeatable? 4. Is the assay reproducible?

Liver inflammation is usually confirmed by analysis of histological features, marked by influx of inflammatory cells (mainly neutrophils) which can be stained by using Haematoxylin and eosin [7].

In mice, neutrophil influx can be analysed using a mouse MPO ELISA kit for lysed tissue [4].

mRNA expression levels of inflammatory cytokines in tissue samples can be determined by using real-time PCR as described in [8].

Plasma levels of pro-inflammatory cytokines can be analysed by enzyme linked immunosorbent assay) ELISA using commercial kits [9].

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Scientific evidence supporting the linkages in the AOP

ID	Upstream Event	Relationship Type	Downstream Event	Evidence	Quantitative Understanding
617	Increase, Oxidative Stress	Directly leads to	N/A, Mitochondrial dysfunction 1	Strong	Weak
618	N/A, Mitochondrial dysfunction 1	Directly leads to	N/A, Cell injury/death	Strong	Weak
619	N/A, Cell injury/death	Directly leads to	Release, Cytokine	Strong	Weak
628	Disruption, Lysosome	Directly leads to	Increase, Oxidative Stress	Moderate	Weak
658	Disruption, Lysosome	Directly leads to	N/A, Mitochondrial dysfunction 1	Strong	Weak
659	Release, Cytokine	Directly leads to	Infiltration, Inflammatory cells	Strong	Moderate
660	Infiltration, Inflammatory cells	Directly leads to	Inflammation, Liver	Strong	

Relationship ID and Title:

144: Increase, Oxidative Stress leads to N/A, Mitochondrial dysfunction 1

How Does This Key Event Relationship Work

The mitochondrion consist of a plethora of antioxidant enzymes to defend against oxidative stress, such as catalases, which has been found in the liver, glutathione peroxidase, and thioredoxin peroxidase [1] [2]. At the same time, the mitochondrion itself is one of the main sources of intracellular ROS formation [3].

NAD(P)H plays a central role in the redox state of the mitochondrion: NADP is reduced, in part, by the activity of the NADH/NADP transhydrogenase that functions as a proton pump [1] and has a reductive effect on glutathione and thioredoxin. This directly links mitochondrial coupling and the membrane potential to the redox potential. As a consequence, an imbalance in the NAD(P) redox status can lead to mitochondrial permeability transition (MPT), a nonselective permeabilization of the inner mitochondrial membrane [4]. An imbalance of the redox state of these pyridine nucleotides and thus condition of oxidative stress can lead to an increased influx of Ca2+, which in turn facilitates activation of the mitochondrial permeability transition pore, leading to apoptosis [5] [6].

Weight of Evidence

Biological Plausibility

Overwhelming the mitochondrial antioxidant defence system and subsequent uncoupling of the respiratory chain leads to MPT, resulting in loss of matrix components, impairment of mitochondrial functionality and substantial induction of apoptosis [4].

Empirical Support for Linkage

Include consideration of temporal concordance here

A direct effect of oxidative stress induction (by using t-butylhydroperoxide TBH) on the opening of the mitochondrial permeability transition pore has been reported using rat liver mitochondria [7]. This was found to lead to an increase in the mitochondrial membrane potential, which could be partly inhibited by addition of the antioxidant GSH [8]. Cell treatment with a lysosomal inhibitor was found to delay the production of ROS that act on mitochondria, thus mitochondria-related cell death was delayed [9]. Superoxide-radical-triggered increase in Ca2+ uptake to the mitochondrion was found to precede loss of mitochondrial membrane potential, which was independent of other oxidants and mitochondrially derived ROS, as determined by using respective inhibitors. This work shows the specific effects of external and not mitochondrially derived ROS on mitochondrial damage [10].

Quatitative Understanding of the Linkage

Is it known how much change in the first event is needed to impact the second? Are there known modulators of the response-response relationships? Are there models or extrapolation approaches that help describe those relationships?

Quantitative understanding of this KER is low. Inhibition of the ROS source could delay mitochondrial damage, and treatment with an antioxidant could partly inhibit the effect on the mitochondrion.

Term	Scientific Term	Evidence	Links
rat	Rattus norvegicus	Moderate	NCBI
mouse	Mus musculus	Moderate	NCBI

Evidence Supporting Taxonomic Applicability

[10]: rat

[7]: rat

[8]: rat

[9]: mouse

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Relationship ID and Title:

144: N/A, Mitochondrial dysfunction 1 leads to N/A, Cell injury/death

How Does This Key Event Relationship Work

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

Weight of Evidence

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 Support for Linkage

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	Lu et al., 2012		Same dose	Incidence of downstream KE (cell death) is higher than the incidence of upstream KE (mitochondrial dysfunction)	

						the mouse hippocampus were increased.					
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	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)	

						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.					
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 or 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).

Quatitative Understanding of the Linkage

Is it known how much change in the first event is needed to impact the second? Are there known modulators of the response-response relationships? Are there models or extrapolation approaches that help describe those relationships?

The experiments describing semi-quantitative effects for this KER is described in the table above.

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Relationship ID and Title:

144: N/A, Cell injury/death leads to Release, Cytokine

How Does This Key Event Relationship Work

Apoptosis is a complex process that regulates whether cell death leads to the induction of inflammation or quiet removal of a damaged cell, for example during development or normal tissue turnover. This most likely depends on the severity of the effect[1]. Additional cell death mechanisms are known to be potent inducers of inflammation, such as necrosis (including secondary necrosis which can follow apoptosis if the removal of apoptotic cells by phagocytic cells fails). Necrosis leads to the release of intracellular contents into the extracellular milieu, thus inducing an inflammatory response[2].

The induction of inflammation by apoptosis is usually linked to infiltration of neutrophils, which are recruited by secreted CXC chemokines. Dying hepatocytes can release intracellular molecules known as damage-associated molecular patterns (DAMPs), which, if persistent, can induce the so-called sterile inflammation. This occurs in the absence of pathogens and is a key factor for the development of (liver) inflammation [3][4]. Mitochondrial DNA (mtDNA) and mitochondria-derived formyl peptides are examples of mitochondria-derived DAMPs which bind to pattern recognition receptors (PPRs) such as toll-like receptors (TLRs). TLRs are found expressed in most liver cells, including hepatocytes, Kupffer cells (KCs) or hepatic stellate cells (HPCs) [3]. Specifically, mtDNA-activated TLR9 has recently been described to play a role in the development of liver inflammation and accompanied induction of the pro-inflammatory cytokine tumor necrosis factor-alpha (TNF-a) [5].

Fas is a cell surface glycoprotein that belongs to the tumor necrosis factor receptor family. It is known that ligation of the Fas receptor promotes the proteolytic cleavage of intracellular caspases and thus leads to the induction of apoptosis[6]. Activation of Fas directly leads to the activation of caspase-3 and induction of a variety of cytokines such as macrophage inflammatory protein-2 (MIP-2)/IL-8, KC, IL-6, MCP-1/CCL2 and sICAM-1. However, when caspase-3 was inhibited, the chemokine-induction was significantly reduced. Faouzi and coworkers could also show that the transcription factor AP-1, and not NF-kB, was involved in the onset of apoptosis-induced liver inflammation[6][7].

Weight of Evidence

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[1][7].

Empirical Support for Linkage

Include consideration of temporal concordance here

A high fat diet increases the amount of plasma mtDNA levels, which were shown to induce TLR9, accompanied by the induction of TNF-a. TLR9 knock-out mice were shown to show less severe symptoms for developing liver inflammation when put on a high fat diet compared to control mice [5].

Induction of apoptosis by using an anti-Fas antibody was found to lead to upregulation and secretion of KC and MIP-2 in liver tissue, while inhibition of caspase-3 significantly reduced chemokine-induction[6].

Uncertainties or Inconsistencies

No dose-response or time dependency is described; proof is presented mainly by using respective inhibitors.

Quatitative Understanding of the Linkage

Is it known how much change in the first event is needed to impact the second? Are there known modulators of the response-response relationships? Are there models or extrapolation approaches that help describe those relationships?

Currently, there is no quantitative understanding of this KER. The use of Fas-inhibitors has led to understanding this mechanism. Interestingly, an increase of the stimulus (increased concentrations of anti-Fas) has led to decreased cytokine formation which is explained by a potential caspase-3-dependent block in chemokine translation[6].

Term	Scientific Term	Evidence	Links
mouse	Mus musculus	Strong	NCBI
human	Homo sapiens	Moderate	NCBI

Evidence Supporting Taxonomic Applicability

[6][5][7]: mouse [7]: human

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Relationship ID and Title:

144: Disruption, Lysosome leads to Increase, Oxidative Stress

How Does This Key Event Relationship Work

The lysosome contains redox-active labile irons which are suggested to be involved in local reactive oxygen species (ROS) formation via a Fenton-type reaction [1]. Many iron containing metallo-proteins are degraded within the lysosomes, leading to an enrichment of this transition metal within this organelle. Iron which is released inside lysosomes due to degradation processes is transported to the cytoplasm and then stored in ferritin, a ubiquitous and highly conserved iron-binding protein [2]. Induction of lysosomal membrane disruption by lysosomotropic detergents has been found to cause an induction of ferritin, together with an increase of cellular ROS and concomitant reduction of the antioxidants MnSOD (manganese superoxide dismutase) and GSH

(glutathione). A suggested explanation for this is the release of free iron into the cytosol [2].

Weight of Evidence

Biological Plausibility

The main lysosomal function is the degradation of macromolecules. To this end, they are filled with more than 50 acid hydrolases [3] and are additionally enriched with iron, as explained above. Via the Fenton-type reaction, iron can catalyse formation of ROS. Thus, damage of the lysosomal membrane can induce cell death mechanisms such as necrosis and apoptosis, depending on the severity of lysosomal damage [4].

Empirical Support for Linkage

Include consideration of temporal concordance here

[5]: By using galactosyl dextran-retinal (GDR) nanogels, the authors demonstrated a negative correlation between ROS production and lysosome function in dendritic cells. Neutralizing the lysosomal pH with NH4Cl partially recovered lysosomal fluorescence but dramatically attenuated GDR-induced ROS after 4h of incubation.

[6]: The authors found that active autophagy is related to basal ROS generation in neuronal cells. Using relevant fluorescent probes, localisation of ROS at lysosomes was found. The decrease of lysosomal ROS by treatment of cells with lysosomal inhibitors delayed the mitochondrial ROS burst and thus cell toxicity.

[2]: The authors suggest that ROS is initially produced due to LMP and release of lysosomal contents, which further promotes mitochondrial membrane permeabilisation (MMP) in apoptosis. This was further supported by experiments with NH4Cl pre-exposure, in which intra-lysosomal trapped NH4+ reduced cellular oxidative stress and apoptotic cell death by blocking lysosomal accumulation of the trigger (O-methyl-serine dodecylamide hydrochloride, MSDH). ROS production was found as early as 3 h and clear reduction of antioxidant enzymes took place from 6 h following exposure, prior to alteration of MMP.

[7]: Using positively charged polystyrene nanoparticles (PS-NH2) as initiators, the authors performed a time-resolved experiment where lysosomal damage was found as the first adverse effect, followed by an increase in reactive oxygen species and subsequent loss in mitochondrial membrane potential. They could show that KEup occurred at earlier time points (3-6 hours) than KEdown (starting after 8 hours).

Uncertainties or Inconsistencies

All studies were performed in varying cell types, either immune or brain cells. Applicability in other cell types such as hepatocytes needs to be determined.

Quatitative Understanding of the Linkage

Is it known how much change in the first event is needed to impact the second? Are there known modulators of the response-response relationships? Are there models or extrapolation approaches that help describe those relationships?

To date, there are no quantitative studies to determine this KER. However, when lysosomal damage is reduced by neutralizing the lysosomal pH with NH4Cl, ROS-induction is strongly decreased [5].

Term	Scientific Term	Evidence	Links
mouse	Mus musculus	Moderate	NCBI
human	Homo sapiens	Moderate	NCBI

Evidence Supporting Taxonomic Applicability

[5]: murine

[6]: murine

[2]: murine, human

[7]: human

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Relationship ID and Title:

144: Disruption, Lysosome leads to N/A, Mitochondrial dysfunction 1

How Does This Key Event Relationship Work

Lysosomes were first described by de Duve and colleagues in 1955 [1]. 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 [2]. Amongst these are cathepsins that are released into the cytosol once lysosomal membrane permeabilisation (LMP) occurs.

The major substrate of cathepsins are Bid, Bcl-2 and Bax. This initiates the subsequent activation of caspase-9 and 3/7, leading to mitochondrial membrane permeabilisation (MMP) and mitochondria-induced apoptosis [3]. The role of cathepsins, especially cathepsin D on mitochondria has been studied in detail: microinjection of cathepsin D into the cytosol induced a subsequent release of cytochrome c, caspase activation and induction of apoptosis. This could be prevented by using inactivated cathepsin D or the cathepsin D inhibitor pepstatin A [4]. The use of this inhibitor and its effect on preventing cytochrome c release or caspase-3 activation, together with an inhibition of apoptosis, has also been reported previously [5].

Moreover, translocation of tBid was observed following LMP induced by Au-ZnO hybrid nanoparticles, which was accompanied by a time-dependent release of cytochrome c and activation of caspase 3 [6].

Another study applied high content imaging to show the occurrence of LMP and MMP at different concentrations in a variety of different cell lines. When determining the IC50/EC50 value, by using positively charged polystyrene particles (PS-NH2) it could be shown that lysosomal damage appeared at lower concentrations than mitochondrial damage in all cells [7].

The importance of MMP in LMP-induced cell death was furthermore confirmed by work of Boya and colleagues: by using the quinolone antibiotics ciprofloxacin (CPX) or norfloxacin (NFX) (with or without UV light) which are known inducers of LMP, treatment of cells resulted in caspase-independent cell death, with hallmarks of apoptosis such as chromatin condensation and phosphatidylserine exposure on the plasma membrane. However, inhibition of the lysosomal accumulation of CPX or NFX suppressed their capacity to induce LMP and to kill cells. Moreover, using Bax/Bak double deficient cells, MMP and subsequent cell death were completely abolished, showing that mitochondria are indispensable for cell death initiated by lysosomal destabilization [8].

Weight of Evidence

Biological Plausibility

Lysosomal permeabilisation has long been known to play a role in necrotic and autophagic cell death. More recently, its role in apoptosis as well as regulation of immune responses has been additionally acknowledged. Release of proteolytic enzymes such as cathepsin D from compromised lysosomes contributes to signalling pathways for apoptosis induction. Thus lysosomes are not only important in mediating cell death, but also play a key role in the induction and regulation of inflammation [9] [10].

Empirical Support for Linkage

Include consideration of temporal concordance here

[6]: Inhibition of cathepsin B decreased subsequent tBid translocation and downstream caspase 3 activation.

[5] [4]: Inactivated cathepsin D or the cathepsin D inhibitor pepstatin A prevented the release of cytochrome c, caspase activation and induction of apoptosis.

[7]: By using high content imaging to show the occurrence of LMP and MMP at different concentrations in a variety of different cell lines, it could be shown that the IC50/EC50 values for the induction of lysosomal damage were lower than those for mitochondrial damage in all tested cells.

[8]: Cathepsin B release occurred before caspace c release (1 vs 6-15h); Lysotracker positive stain was present already after 1 hour, whereas MMP staining was positive only after 6 hours and later.

Uncertainties or Inconsistencies

The available quantitative information relates to LMP and MMP only, without providing information on the mechanism between (such as cathepsin D release). Therefore, involvement of for example ROS as intermediate MMP inducer cannot be ruled out (see KER 921).

Quatitative Understanding of the Linkage

Is it known how much change in the first event is needed to impact the second? Are there known modulators of the response-response relationships? Are there models or extrapolation approaches that help describe those relationships?

The quantitative understanding of this KER is low. However, as described above, the absence of important modulators such as Bax/Bak completely prevents induction of MMP after LMP. Moreover, inhibition of cathepsin D prevented the subsequent effects on the mitochondrion, such as the release of cytochrome c, caspase activation and induction of apoptosis.Also, by using nanoparticles, LMP was induced at lower concentrations as MMP.

Term	Scientific Term	Evidence	Links
human	Homo sapiens	Strong	NCBI
mouse	Mus musculus	Moderate	NCBI

Evidence Supporting Taxonomic Applicability

[6]: human

[4]: human

[7]: human, murine

[8]: human, murine

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Relationship ID and Title:

144: Release, Cytokine leads to Infiltration, Inflammatory cells

How Does This Key Event Relationship Work

Binding of damage- or pathogen-associated molecular patterns (DAMPs or PAMPs) to pattern recognition receptors (PPRs) such as toll-like receptors (TLRs) can lead to the activation of, amongst others, nuclear factor-κB (NF-κB) or the transcription factor AP-1. This leads to an upregulation of chemokines and inflammatory cytokines, such as tumor necrosis factor-α (TNF-α), interleukins or proteases [1][2]. TLRs are found expressed in most cells, including liver cells such as hepatocytes, Kupffer cells (KCs) or hepatic stellate cells (HPCs) [2]. Upon cytokine secretion, polymorphonuclear neutrophils (PMNs) that, amongst others, circulate in the blood, can become attracted. PMNs are potent phagocytes, but they also lead to pathogen destruction upon oxidative bursting and are for their part capable of pro-inflammatory cytokine production as well. Various endothelial adhesion molecules, such as the intercellular adhesion molecule 1 (ICAM-1), mediate neutrophil adhesion to endothelial cells. ICAM-1 expression on the luminal surface of the capillary is increased during inflammation, and interacts with ß2 integrin, which is expressed on the surface of PMN. Subsequent to adhesion, neutrophils begin to migrate across the endothelium and towards the center of inflammation [3][4]. Interleukin-8 (IL-8) is known to be one of the most potent chemoattractants for the recruitment and activation of neutrophils into various organs (e.g. lung, intestine), and binds to the human CXC chemokine receptor 1 (CXCR1) and CXC chemokine receptor 2 (CXCR2) on the surface of the PMN [5][6][7]. But not only IL-8, also macrophage inflammatory protein-2 (MIP-2), growth-regulated oncogenes-α, -β, and -γ, as well as the rodent peptides cytokine-induced neutrophil chemoattractant and KC are all members of the CXC subfamily of chemokines and chemoattractants for inflammatory cells[8]

Cullen and co-workers could further confirm the importance of specific chemokines for chemotaxis of different inflammatory cells. They depleted certain chemokines by using respective antibodies in supernatants of Fas-stimulated HeLa cells (see Relationship:924 for explanation on Fas and its role on cytokine induction) and subsequently assessed the chemotactic activity of immune cells. Only depletion of MCP-1 was sufficient to block almost all THP-1 monocyte chemotaxis. On the other hand, chemotaxis of primary human peripheral blood neutrophils was depending mainly on secreted IL-8. Using an in vivo mouse model, the authors found that Fas stimulation could trigger phagocyte migration by administration of anti-Fas (Jo2) antibody into C57BL/6 mice within 10 h of anti-Fas administration. This correlated with extensive cell death in the thymus and a dramatic increase of CD11b-positive macrophages in the same tissue[9].

Weight of Evidence

Biological Plausibility

Secreted chemokines are signalling proteins that attract immune cells to migrate to the infected or damaged tissue, in order to trigger tissue repair, removal of cell bodies or bacteria.

Empirical Support for Linkage

Include consideration of temporal concordance here

Infiltration of the hepatic parenchyma by neutrophils was found coinciding with chemokine induction. When chemokines have been neutralized by the addition of neutralizing monoclonal antibodies, a study found KC mainly responsible for abrogating an inflammatory response to Fas-induced hepatic inflammation. In this study, chemokine induction in the livers of anti-Fas-treated mice was not associated with activation of NF-kB, but it coincided with nuclear translocation of activator protein-1 (AP-1). AP-1 activation in the liver was detected shortly (1–2 h) after anti-Fas treatment, suggesting a connection to the onset of apoptosis. When apoptosis was prevented by pre-treatment of the mice with a caspase-3 inhibitor, AP-1 activation and hepatic chemokine production were both significantly reduced. Moreover the authors report a reduction of the hepatic inflammation by 70%[8].

Addition of 200ng/ml anti-Fas antibody to HeLa cells resulted in the secretion of about 0.7ng/ml IL-8 into the supernatant. In the same study, they could show that supernatants of cells treated with 250ng/ml anti-Fas induced the strongest infiltration of neutrophils (which was almost abolished when the supernatants were treated with an anti-IL-8 antibody). This infiltration was strongly decreased upon dilution of the supernatants. Thus, this allows for a rough quantification of the IL-8 concentrations that are needed for potent

chemoattraction of neutrophils[9].

A further study on quantification of neutrophil migration in dependence of IL-8 concentration found a biphasic exhibition of migration, with an optimum random neutrophil motility at 3 nM of IL-8 [10].

Uncertainties or Inconsistencies

Studies exist that report an optimal IL-8 concentration for strongest neutrophil motility, so this KER can actually be quantified. However, this is usually performed ex vivo. Isolated neutrophils are very sensitive towards manual handling and need to be treated with care and within a very short time frame. Therefore, results give an indication on necessary concentrations, but need to be carefully considered with regards to direct transferability to the in vivo situation. Moreover, not only IL-8 is responsible for the recruitment of neutrophils, but also other chemokines can contribute to attraction of inflammatory cells. As they play a minor role, they are usually not considered and included in ex vivo studies.

Quatitative Understanding of the Linkage

Is it known how much change in the first event is needed to impact the second? Are there known modulators of the response-response relationships? Are there models or extrapolation approaches that help describe those relationships?

This KER can be described quantitatively, as useful chemotaxis-assays are available that make use of isolated immune cells ex vivo. Those results give an indication on concentrations necessary for cell migration, but need to be carefully considered with regards to direct transferability to the in vivo situation. Moreover, not only IL-8 is responsible for the recruitment of neutrophils, but also other chemokines can contribute to attraction of inflammatory cells. However, additional proof for this KER is provided by the neutralization of chemokines, which prevented a further onset of inflammation.

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Relationship ID and Title:

144: Infiltration, Inflammatory cells leads to Inflammation, Liver

How Does This Key Event Relationship Work

Immune cells such as polymorphonuclear neutrophils (PMNs) or monocytes, circulate in the blood and become attracted towards a gradient of secreted pro-inflammatory cytokines. PMNs have a life span of only 7-12 hours. Therefore, around 1-2 x 1011 PMN are produced daily in the human body. They account for about 50-70 % of all blood leukocytes in the human body [1][2]. Upon an inflammatory event, neutrophil production is upregulated, and its lifetime increases as a response to platelet activating factor (PAF), granulocyte-colony stimulating factor (G-CSF) or various pro-inflammatory cytokines, such as interleukin 1ß (IL-1ß) [2]. In sterile tissue injury, for example as the result of apoptosis, there is no need for PMNs to function as antimicrobial effectors; instead, they

clear debris and initiate the wound-healing process. Released damage-associated molecular patterns (see Relationship:924) stimulate Kupffer cells to produce IL-1ß which leads to intercellular adhesion molecular-1 (ICAM-1) upregulation on sinusoidal endothelial cells [3]. ICAM-1 in turn mediates neutrophil adhesion to endothelial cells, as it interacts with ß2 integrin, which is expressed on the surface of PMNs. Subsequent to adhesion, neutrophils begin to migrate across the endothelium and towards the affected tissue [4][5]. The transition of neutrophils from a resting state, as during circulation in the blood, to an activated state at the site of infection is triggered by an ordered sequence of signals from cytokines[3].

The aberrant activation of neutrophils and their extended lifespan upon an inflammatory stimulus can increase the probability of extracellular damage. PMNs are potent phagocytes, but they also lead to pathogen destruction upon oxidative bursting. The oxidative burst is marked by an increased consumption of molecular oxygen, resulting in the production of reactive oxygen species (ROS) such as H2O2 and OH•, and reactive nitrogen species (RNS)[6]. In general, the acute inflammatory response, as in the liver, is bi-phasic. The initial phase is characterised by a macrophage (Kupffer cell)-mediated phase, with the generation of reactive oxygen species aggravating the organ damage. The activated macrophages and subsequent infiltrating lymphocytes produce additional cytokines that further promote the inflammatory response, leading to a second phase, during which neutrophils become fully activated and secrete ROS, complement components, proteases, CXCL-1 and CXCL-2[3]. The role of IL-1 and IL-17A in neutrophil activation and subsequent induction of inflammation has been confirmed by the use of knock-down models, showing that the absence of these mediators prevent neutrophil infiltration and subsequent onset of inflammation, inhibition of the latter also being shown by direct depletion of neutrophils[7].

Weight of Evidence

Biological Plausibility

The infiltration of immune cells to the infected or damaged tissue is initiated in order to repair the tissue or remove cell bodies or bacteria. However, if the trigger persists, an overstimulation of immune cells such as neutrophils, and the corresponding secretion of ROS can enhance the tissue damage, in turn leading to further infiltration of inflammatory cells and eventually manifest a chronic inflammation.

Empirical Support for Linkage

Include consideration of temporal concordance here

Infiltration of the hepatic parenchyma by neutrophils was found coinciding with chemokine induction. When chemokines have been neutralized by the addition of neutralizing monoclonal antibodies, a study found the chemokine KC mainly responsible for abrogating an inflammatory response to Fas-induced hepatic inflammation. When apoptosis was prevented by pre-treatment of the mice with a caspase-3 inhibitor, AP-1 activation and hepatic chemokine production were both significantly reduced, directly resulting in a reduction of the hepatic inflammation by 70%[8].

Neutrophils infiltration and subsequent liver inflammation and are drastically attenuated in IL-1R1 deficient mice or by using a neutralizing antibody, and also in the absence of IL-17RA signalling. The same study demonstrated that increased IL-17A was mainly expressed by CD4+ T cells, but also by neutrophils themselves, in the damaged liver, showing that these cells are critical for the further recruitment of circulating immune cells into the tissue. Depletion of neutrophils (by using the neutrophil depleting antibody NIMP-R14) directly resulted in a drastic reduction of the inflammation[7].

A general proof of the importance of infiltrated neutrophils is the fact that liver inflammation is usually clinically confirmed by analysis of histological features, marked by the influx of neutrophils (which can be stained by using Haematoxylin and eosin) [9].

Quatitative Understanding of the Linkage

Is it known how much change in the first event is needed to impact the second? Are there known modulators of the response-response relationships? Are there models or extrapolation approaches that help describe those relationships?

Inhibition of messengers for the infiltration of inflammatory cells leads to a strong reduction of these. Furthermore, direct inhibition of neutrophils prevents the onset of liver inflammation.

Term	Scientific Term	Evidence	Links
mouse	Mus musculus	Strong	NCBI
human	Homo sapiens	Strong	NCBI

Evidence Supporting Taxonomic Applicability

[8][7]: mouse [9]: human

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Overall Assessment of the AOP

Domain of Applicability

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. Lysosomal damage can occur in almost all cell types and all organs. This is shown by several mechanistic studies that used immune or brain cells[36][21][37][38], thus underlining the broad applicability of the MIE/early KE. Therefore, 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, where a specific knock-down could be performed. Examples include

mouse: [1] [2][39][40][36]

human: [2][38][4][40][3][41]

Only some studies analysed specific aspects of the AOP in rat models, for example [42][43]

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

Molecular Initiating Event

Title	Short name	Essentiality
Disruption, Lysosome	Disruption, Lysosome	Strong

Key Events

Title	Short name	Essentiality

Title	Short name	Essentiality
Increase, Oxidative Stress	Increase, Oxidative Stress	Moderate
N/A, Mitochondrial dysfunction 1	N/A, Mitochondrial dysfunction 1	Strong
N/A, Cell injury/death	N/A, Cell injury/death	Strong
Release, Cytokine	Release, Cytokine	Strong
Infiltration, Inflammatory cells	Infiltration, Inflammatory cells	Strong

Molecular Initiating Event Summary, Key Event Summary

Provide an overall assessment of the essentiality for the key events in the AOP. Support calls for individual key events can be included in the molecular initiating event, key event, and adverse outcome tables above.

Depending on the considered trigger, lysosomal disruption can be either the MIE or already an early KE. In the present AOP, it has been identified as the relevant (M)IE, as the lysosomal uptake of NMs can result in simple storage of the material, or in damage to the lysosomal membrane, which is the MIE of the described AOP. This is dependent on the NM's properties, as has been described above. Briefly, the acidic milieu within the lysosome can further enhance solubility of a (soluble) NM, or it 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 [21][23]. Particles of low solubility and toxicity, such as TiO2, may cause inflammation in proportion to their specific surface area[44][45] and their zeta potential[23].

Subsequent to lysosomal disruption, secretion or ROS can be initiated, which then leads to damage to the mitochondrion. As the main role of lysosomes is the degradation of macromolecules, they are filled with more than 50 acid hydrolases to serve this purpose[46]. Amongst these are cathepsins that are released into the cytosol once lysosomal membrane permeabilisation (LMP) occurs. The major substrate of cathepsins are Bid, Bcl-2 and Bax. This initiates the subsequent activation of caspase-9 and 3/7, leading to mitochondrial membrane permeabilisation (MMP) and mitochondria-induced apoptosis[29]. Both pathways of initiating MMP are essential in this AOP. The role of ROS is a complex one: increased ROS lead to the induction of MMP, while MMP again leads to the secretion of ROS, thus initiating a vicious cycle. Overall, the vital role of MMP in LMP-induced cell death was underpinned by work of Boya and colleagues: by using the quinolone antibiotics ciprofloxacin (CPX) or norfloxacin (NFX) (with or without UV light) which are known inducers of LMP, treatment of cells resulted in caspase-independent cell death, with hallmarks of apoptosis such as chromatin condensation and phosphatidylserine exposure on the plasma membrane. However, inhibition of the lysosomal accumulation of CPX or NFX suppressed their capacity to induce LMP and to kill cells. Moreover, using Bax/Bak double deficient cells, MMP and subsequent cell death were completely abolished, showing that mitochondria are indispensable for cell death initiated by lysosomal destabilization[47].

Therefore, cell death is described as the subsequent KE to mitochondrial dysfunction, which can lead to the induction of the apoptotic pathway. Apoptosis is a complex process that regulates whether cell death leads to the development of inflammation or quiet removal of a damaged cell, for example during development or normal tissue turnover. This most likely depends on the severity of the effect[48]. Dying hepatocytes can release intracellular molecules known as damage-associated molecular patterns (DAMPs), which, if persistent, can induce the so-called sterile inflammation. This occurs in the absence of pathogens and is a key factor for the development of (liver) inflammation [49][50]. Mitochondrial DNA (mtDNA) and mitochondria-derived formyl peptides are examples of mitochondria-derived DAMPs which bind to pattern recognition receptors (PPRs) such as toll-like receptors (TLRs). TLRs are found expressed in most liver cells, including hepatocytes, Kupffer cells (KCs) or hepatic stellate cells (HPCs) [49].

Activation of apoptosis leads to the induction of a variety of cytokines such as macrophage inflammatory protein-2 (MIP-2)/IL-8, KC, IL-6, MCP-1/CCL2 and sICAM-1, which is described as the next KE. When apoptosis was blocked by inhibition of caspase-3, the chemokine-induction was significantly reduced. [51][52].

An increase in cytokine release is inevitably linked to subsequent infiltration of inflammatory cells, KE number 5. Specifically neutrophils (PMNs) are recruited towards a chemotactic gradient. This could even be quantified by using chemotaxis assays, which allow for determining relevant chemokine concentrations in order to trigger neutrophil migration [53]. The neutrophilic cytosol contains granules that are filled with a variety of proteins, such as defensins, bactericidal-permeability-increasing protein, proteases (e.g. elastase, cathepsins), and myeloperoxidase (MPO) that consumes hydrogen peroxide (H2O2) and generates hypochlorous acid (HOCl), the most bactericidal oxidant that is produced by PMN [54][55]. Activation of PMNs leads to the production of a variety of pro-inflammatory cytokines, e.g. IL-1ß, IL-6, IL-12 and IL-23, which can further aggravate the resulting inflammation[56].

The outcome of infiltration of neutrophils and other immune cells leads to the development and establishment of inflammation. It could be shown that depletion of neutrophils by using the neutrophil depleting antibody NIMP-R14 directly resulted in a drastic reduction of the resulting liver inflammation[57]. A general proof of the importance of infiltrated neutrophils is the fact that liver inflammation is usually clinically confirmed by analysis of histological features, marked by the influx of neutrophils (which can be stained by using Haematoxylin and eosin) [58].

Weight of Evidence Summary

ID	Upstream Event	Relationship Type	Downstream Event	Evidence	Quantitative Understanding
617	Increase, Oxidative Stress	Directly leads to	N/A, Mitochondrial dysfunction 1	Strong	Weak
618	N/A, Mitochondrial dysfunction 1	Directly leads to	N/A, Cell injury/death	Strong	Weak
619	N/A, Cell injury/death	Directly leads to	Release, Cytokine	Strong	Weak
628	Disruption, Lysosome	Directly leads to	Increase, Oxidative Stress	Moderate	Weak
658	Disruption, Lysosome	Directly leads to	N/A, Mitochondrial dysfunction 1	Strong	Weak
659	Release, Cytokine	Directly leads to	Infiltration, Inflammatory cells	Strong	Moderate
660	Infiltration, Inflammatory cells	Directly leads to	Inflammation, Liver	Strong	

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.

MIE to Oxidative stress	when lysosomal damage is reduced by neutralizing the lysosomal pH with NH4Cl, ROS-induction is strongly decreased	Wang et al., 2016	Direct evidence was found by inhibiting lysosomal damage and applying time-resolved experiments.
	A decrease of lysosomal ROS by treatment of cells with lysosomal inhibitors delayed the mitochondrial ROS burst and thus cell toxicity.	Kubota et al., 2010	
	ROS production as a consequence of LMP was found after 3 h, and clear reduction of antioxidant enzymes took place from 6 h following exposure, prior to alteration of the MMP	Gosh et al., 2011	
	Using positively charged polystyrene nanoparticles (PS-NH2) as initiators, the authors performed a time-resolved experiment where lysosomal damage was found as the first adverse effect, followed by an increase in reactive oxygen species and subsequent loss in mitochondrial membrane potential. They could show that KEup occurred at earlier time points (3-6 hours) than KEdown (starting after 8 hours).	Wang et al., 2013	
Oxidative stress to Mitochondrial dysfunction	Oxidative stress induction (by using t-butylhydroperoxide TBH) directly affects the opening of the mitochondrial permeability transition pore	Halestrap et al., 1997	Inhibition of the ROS source could delay mitochondrial damage, and treatment with an antioxidant could partly inhibit the effect on the mitochondrion.
	Opening of the mitochondrial permeability transition pore was found to lead to an increase in the mitochondrial membrane potential, which could be partly inhibited by addition of the antioxidant GSH	Hüser et al., 1998	
	Cell treatment with a lysosomal inhibitor was found to delay the production of ROS that act on mitochondria, thus mitochondria-related cell death was delayed	Kubota et al., 2010	
	Superoxide-radical-triggered increase in Ca2+ uptake to the mitochondrion was found to precede loss of mitochondrial membrane potential, which was independent of other oxidants and mitochondrially derived ROS, as determined by using respective inhibitors. This work shows the specific effects of external and not mitochondrially derived ROS on mitochondrial damage	Madesh et al., 2005	
	Inhibition of cathepsin B decreased subsequent tBid translocation and downstream caspase 3 activation.	Gao et al., 2014	
	Inactivated cathepsin D or the cathepsin D inhibitor pepstatin A prevented the release of cytochrome c, caspase activation and induction of apoptosis.	Roberg et al., 1999; 2002	

MIE to Mitochondrial dysfunction	By using high content imaging to show the occurrence of LMP and MMP at different concentrations in a variety of different cell lines, it could be shown that the IC50/EC50 values for the induction of lysosomal damage were lower than those for mitochondrial damage in all tested cells	Anguissola et al., 2014	Time-resolved experiments and the use of specific inhibitors confirmed this KER.
	Cathepsin B release occurred before caspase c release (1 vs 6-15h); Lysotracker positive stain was present already after 1 hour, whereas MMP staining was positive only after 6 hours and later.	Boya et al., 2003	
Mitochondrial dysfuntion to Cell death	By using high content imaging to show the occurrence of MMP and cell death markers at different concentrations in a variety of different cell lines, it could be shown that the IC50/EC50 values for the induction of MMP were lower than those for most markers of cell death in all tested cells	Anguissola et al., 2014	Inhibition of the MMP can prevent the onset of apoptosis; MMP is induced at lower concentrations, prior to cell death
	When applying an apoptotic trigger, stabilisation of the MMP can directly inhibit the onset of apoptosis	Marchetti et al., 1996: Mitochondrial Permeability Transition Is a Central Coordinating Event of Apoptosis	
Cell death to Cytokine release	A high fat diet increases the amount of plasma mtDNA levels, which were shown to induce TLR9, accompanied by the induction of TNF-a. TLR9 knock-out mice were shown to show less severe symptoms for developing liver inflammation when put on a high fat diet compared to control mice	Garcia-Martinez et al., 2016	mitochondria-derived DAMPs bind to pattern recognition receptors such as toll-like receptors, which directly upregulates cytokines, as did the induction of the apoptotic pathway. Inhibition of apoptosis prevented upregulation of cytokines.
	Induction of apoptosis by using an anti-Fas antibody was found to lead to upregulation and secretion of KC and MIP-2 in liver tissue, while inhibition of caspase-3 significantly reduced chemokine-induction	Faouzi et al., 2001	
Cytokine release to Infiltration of inflammatory cells	Neutralisation of chemokines leads to abrogating an inflammatory response to Fas-induced hepatic inflammation.	Faouzi et al., 2001	Useful chemotaxis-assays are available that make use of isolated immune cells ex vivo and allow for quantification of this KER. Those results give an indication on concentrations necessary for cell migration, but need to be carefully considered with regards to direct transferability to the in vivo situation. Moreover, not only IL-8 is responsible for the recruitment of neutrophils, but also other chemokines can contribute to attraction of inflammatory cells. However, additional proof for this KER is provided by the neutralization of chemokines, which prevented a further onset of inflammation.
	Quantification of neutrophil migration in dependence of IL-8 concentration showed a biphasic exhibition of migration, with an optimum random neutrophil motility at 3 nM of IL-8	Lin et al., 2004	
	Addition of 200ng/ml anti-Fas antibody to HeLa cells resulted in the secretion of about 0.7ng/ml IL-8 into the supernatant. The same study showed that supernatants of cells treated with 250ng/ml anti-Fas induced the strongest infiltration of neutrophils (which was almost abolished when the supernatants were treated with an anti-IL-8 antibody). This infiltration was strongly decreased upon dilution of the supernatants. Thus, this allows for a rough quantification of the IL-8 concentrations that are needed for potent chemoattraction of neutrophils	Cullen et al., 2013	
	Neutrophil infiltration and subsequent liver inflammation and are drastically attenuated in IL-1R1 deficient mice or by using a neutralizing antibody, and also in the absence of IL-17RA signalling. The same study demonstrated that increased IL-17A was mainly expressed by CD4+ T cells, but also by neutrophils themselves, in the damaged liver,	Tan et al., 2013	

Infiltration of inflammatory cells to Liver inflammation	showing that these cells are critical for the further recruitment of circulating immune cells into the tissue. Depletion of neutrophils (by using the neutrophil depleting antibody NIMP-R14) directly resulted in a drastic reduction of the inflammation		Inhibition of messengers for the infiltration of inflammatory cells leads to a strong reduction of these. Furthermore, direct inhibition of neutrophils prevents the onset of liver inflammation.
	A general proof of the importance of infiltrated neutrophils is the fact that liver inflammation is usually clinically confirmed by analysis of histological features, marked by the influx of neutrophils (which can be stained by using Haematoxylin and eosin)	Huebscher 2006	

Quantitative Consideration

ID	Upstream Event	Relationship Type	Downstream Event	Evidence	Quantitative Understanding
617	Increase, Oxidative Stress	Directly leads to	N/A, Mitochondrial dysfunction 1	Strong	Weak
618	N/A, Mitochondrial dysfunction 1	Directly leads to	N/A, Cell injury/death	Strong	Weak
619	N/A, Cell injury/death	Directly leads to	Release, Cytokine	Strong	Weak
628	Disruption, Lysosome	Directly leads to	Increase, Oxidative Stress	Moderate	Weak
658	Disruption, Lysosome	Directly leads to	N/A, Mitochondrial dysfunction 1	Strong	Weak
659	Release, Cytokine	Directly leads to	Infiltration, Inflammatory cells	Strong	Moderate
660	Infiltration, Inflammatory cells	Directly leads to	Inflammation, Liver	Strong	

Summary Table

Provide an overall discussion of the quantitative information available for this AOP. Support calls for the individual relationships can be included in the Key Event Relationship table above.

Overall, quantitative understanding of the individual KERs is low. A lot is based on established knowledge, particularly late KERs that describe cytokine upregulation and immune cell infiltration during the onset of inflammation. Mostly, this knowledge is supported by experiments using inhibitors, specific activators or neutralising substances (such as antibodies).

The KER from the **MIE** to KE1 (**Oxidative stress**) is based on findings where the lysosomal response was inhibited or reduced; a subsequent induction of ROS could be decreased and further outcomes (such as effects on the mitochondrion) delayed. Temporal concordance was described (LMP was followed by production of ROS, which was followed by alterations of the MMP); however, quantitative understanding is still low.

It is well-established that **Oxidative stress** leads to **Mitochondrial Dysfunction**, although also for this KER, quantitative understanding is low. Inhibition of the ROS source could delay mitochondrial damage, and treatment with an antioxidant could partly inhibit the effect on the mitochondrion. A direct effect of oxidative stress on the opening of the mitochondrial permeability transition pore has been found already in 1997, which was described to lead to an increase in the mitochondrial membrane potential.

The KER from the **MIE** to **Mitochondrial dysfunction** is based on findings in time-resolved experiments and the use of specific inhibitors which confirmed this KER. The prominent role of cathepsins, which are secreted from a compromised lysosome, was repeatedly underpinned. Also here, quantitative understanding is still low.

Mitochondrial dysfuntion leads to **Cell death** by, for example, induction of apoptosis. Stabilising the MMP prior to applying an apoptotic trigger can prevent the onset of apoptosis. MMP is induced at lower concentrations, prior to cell death. However, quantitative understanding is low.

Studies on the role of **Cell death** inducing to **Cytokine release** found that mitochondria-derived DAMPs bind to pattern recognition receptors such as toll-like receptors, which directly upregulates cytokines, as did the induction of the apoptotic pathway. Inhibition of apoptosis prevented upregulation of cytokines.

The KER from **Cytokine release** to **Infiltration of inflammatory cells** can be described quantitatively, as useful chemotaxis-assays are available that make use of isolated immune cells ex vivo. Those results give an indication on concentrations necessary for cell migration, but need to be carefully considered with regards to direct transferability to the in vivo situation. Moreover, not only IL-8 is responsible for the recruitment of neutrophils, but also other chemokines can contribute to attraction of inflammatory cells. However, additional proof for this KER is provided by the neutralization of chemokines, which prevented a further onset of inflammation.

A general proof of the importance of **Infiltration of inflammatory cells** in the development of **Liver inflammation** is found by the fact

that liver inflammation is usually clinically confirmed by analysis of histological features, marked by the influx of neutrophils. Inhibition of messengers for the infiltration of inflammatory cells leads to a strong reduction of these. Furthermore, direct inhibition of neutrophils prevents the onset of liver inflammation.

Applicability of the AOP

Life Stage Applicability

Life Stage	Evidence
all life stages	Moderate

Taxon Applicability

Term	Scientific Term	Evidence
human	Homo sapiens	Strong
mouse	Mus musculus	Strong
rat	Rattus norvegicus	Weak

Sex Applicability

Sex	Evidence
Unspecific	

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