Transition from *in vivo* to *in vitro* immunotoxicity prediction:

methodology, practical insights, and in vivo implementation opportunities

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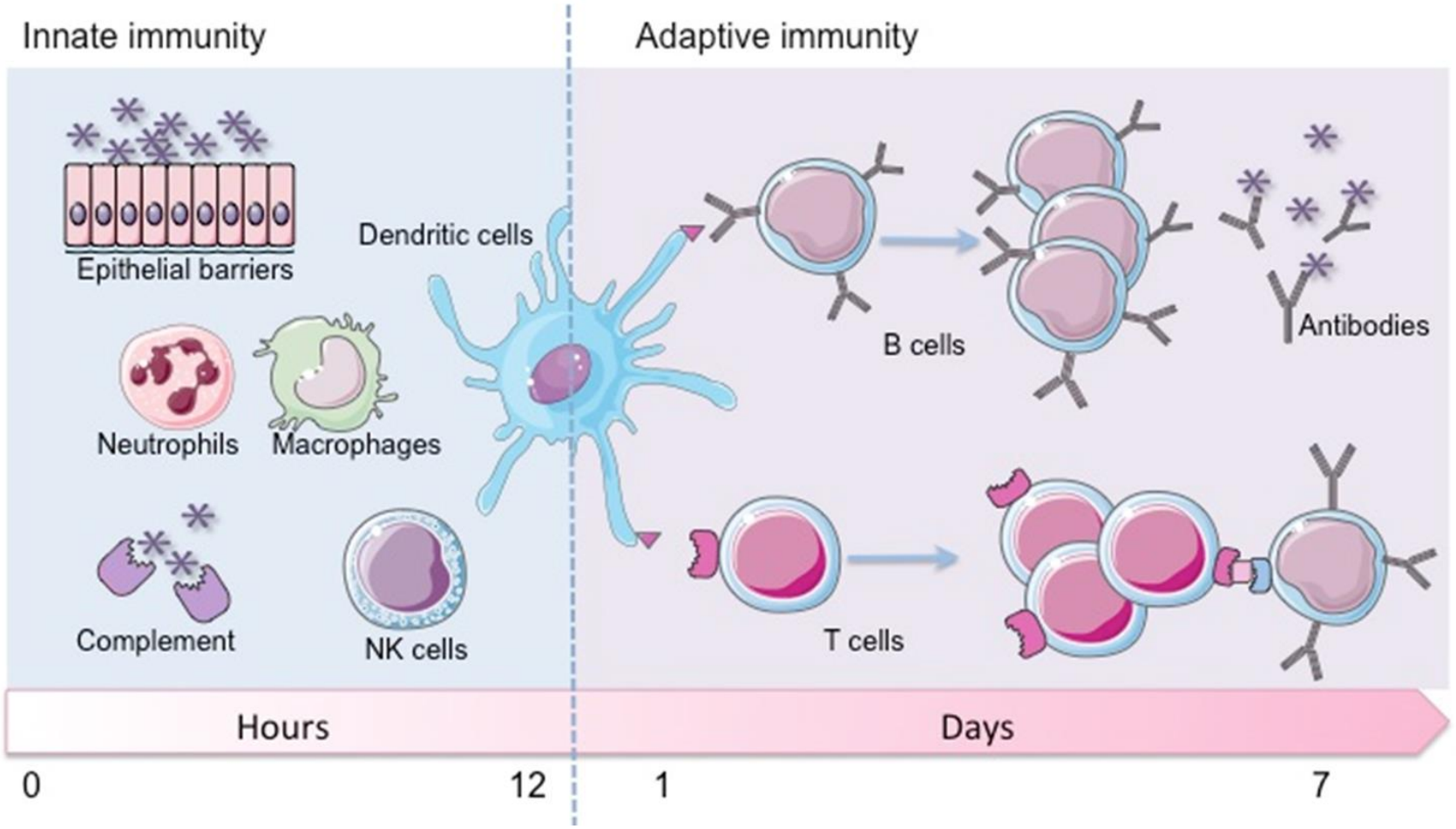
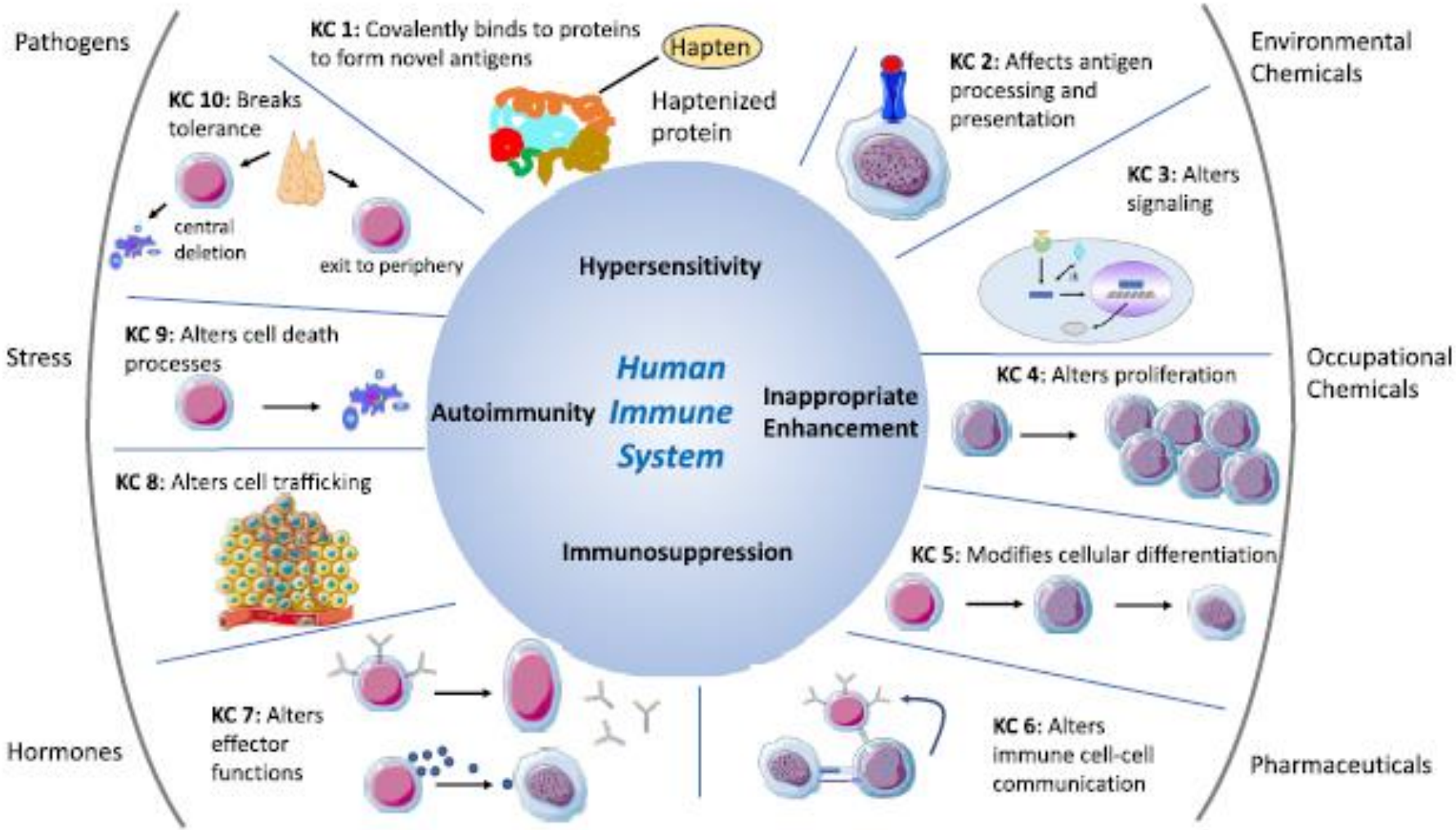
Immunotoxicology and animal studies

- Tiered testing approach: 28d+ study, usually rats/mice
- Infection/disease models: rats/mice (Tier 2 models)
- Guinea pig assays for sensitization (incl endpoint)
- Local lymph node assays (LLNA, PLNA) (initiation phase)
  - LLNA for skin sensitization
  - PLNA for systemic drug-induced innate of adaptive immune responses
- Specific animal models for DILI…(?)
- Inflammatory responses (innate immune players, both as initiation/effector mechanisms)

Innate immune system in inflammation

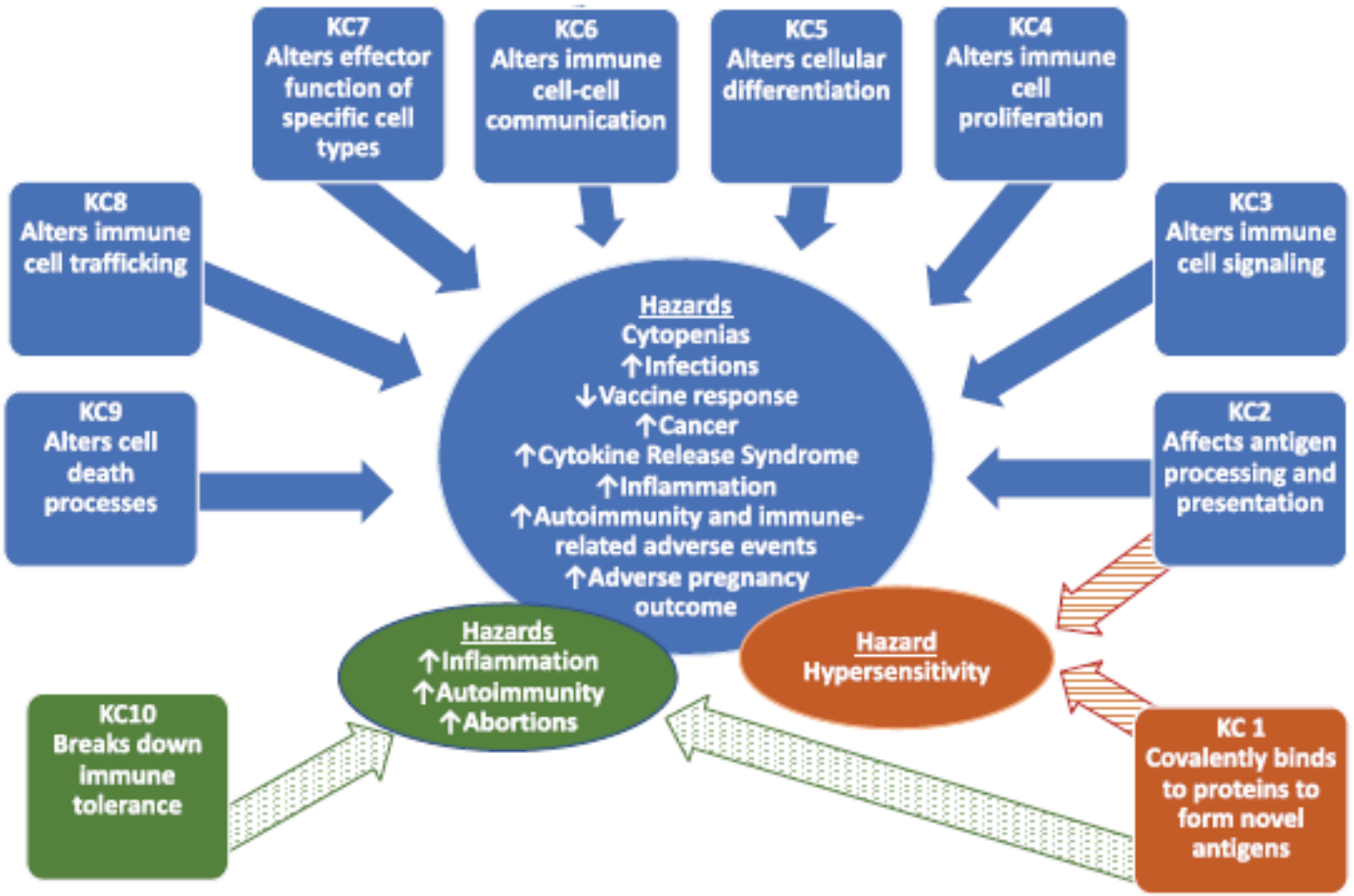


Key Characteristics



Germolic er al, EHP, 2022

Key Characteristics



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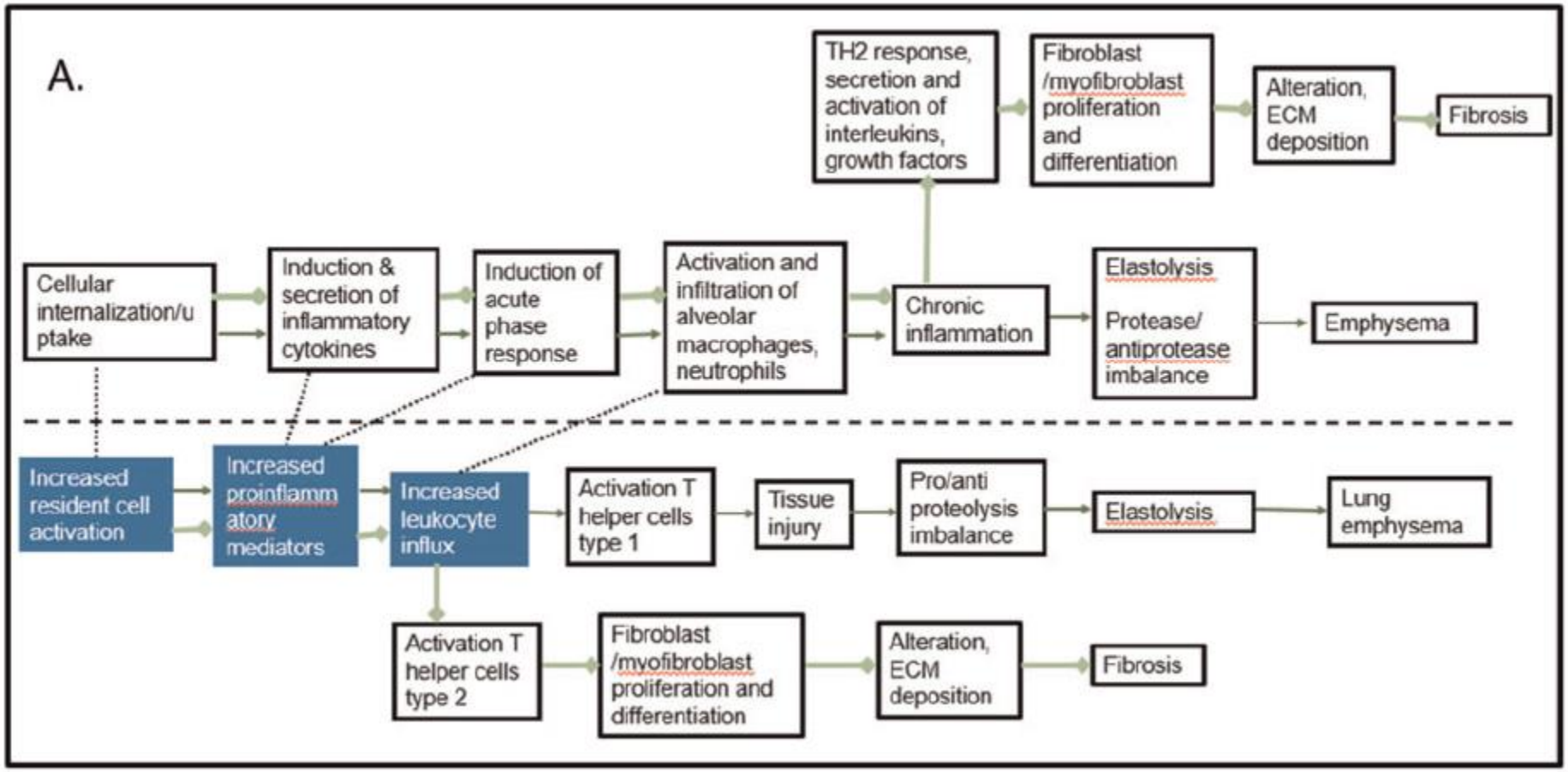
AOP for lung fibroses and emphysema



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

Daniel L. Villeneuve,[*,1] Brigitte Landesmann,[†] Paola Allavena,[‡] Noah Ashley,[§] Anna Bal-Price,[†] Emanuela Corsini,[¶] Sabina Halappanavar,[‖] Tracy Hussell,[|||] Debra Laskin,[||||] Toby Lawrence,[#] David Nikolic-Paterson,[**] Marc Pallardy,[††] Alicia Paini,[†] Raymond Pieters,[a] Robert Roth,[b] and Florianne Tschudi-Monnet[c]

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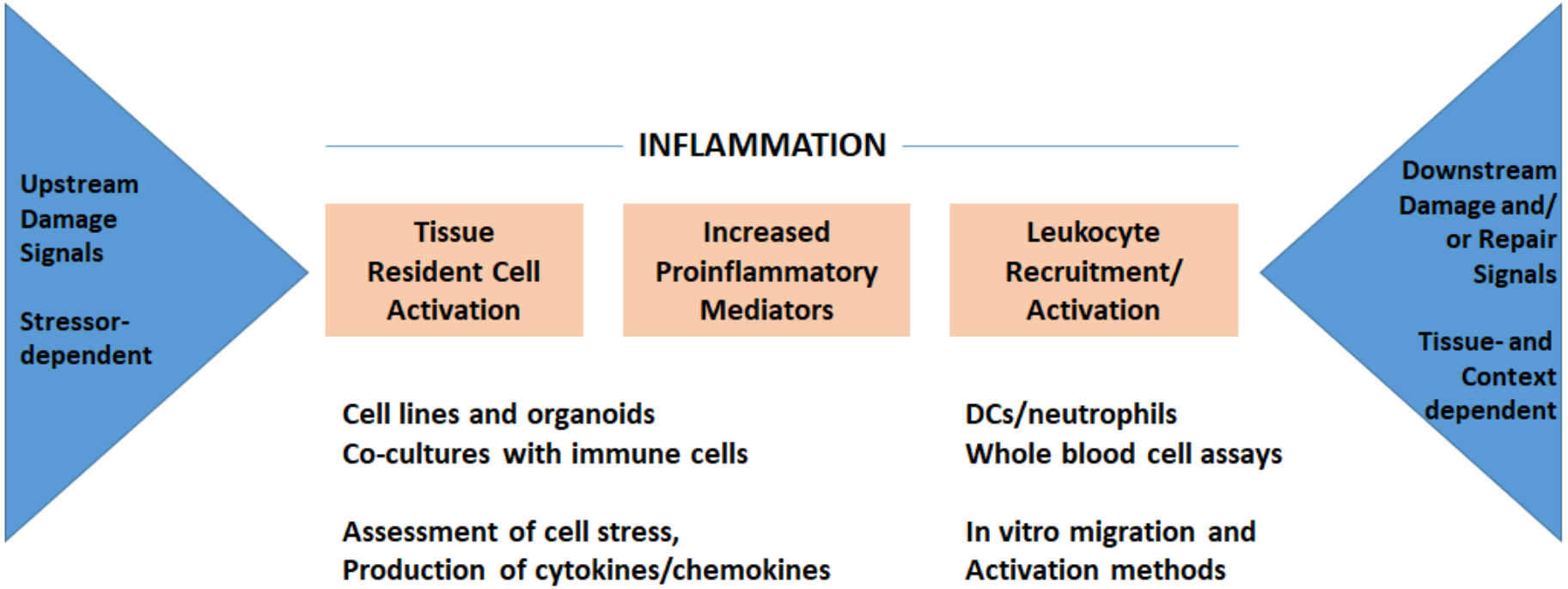
KEY EVENTS CENTRAL TO MANY INFLAMMATORY DISEASE OUTCOMES



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Hub-KEs for inflammation can also be plotted on:

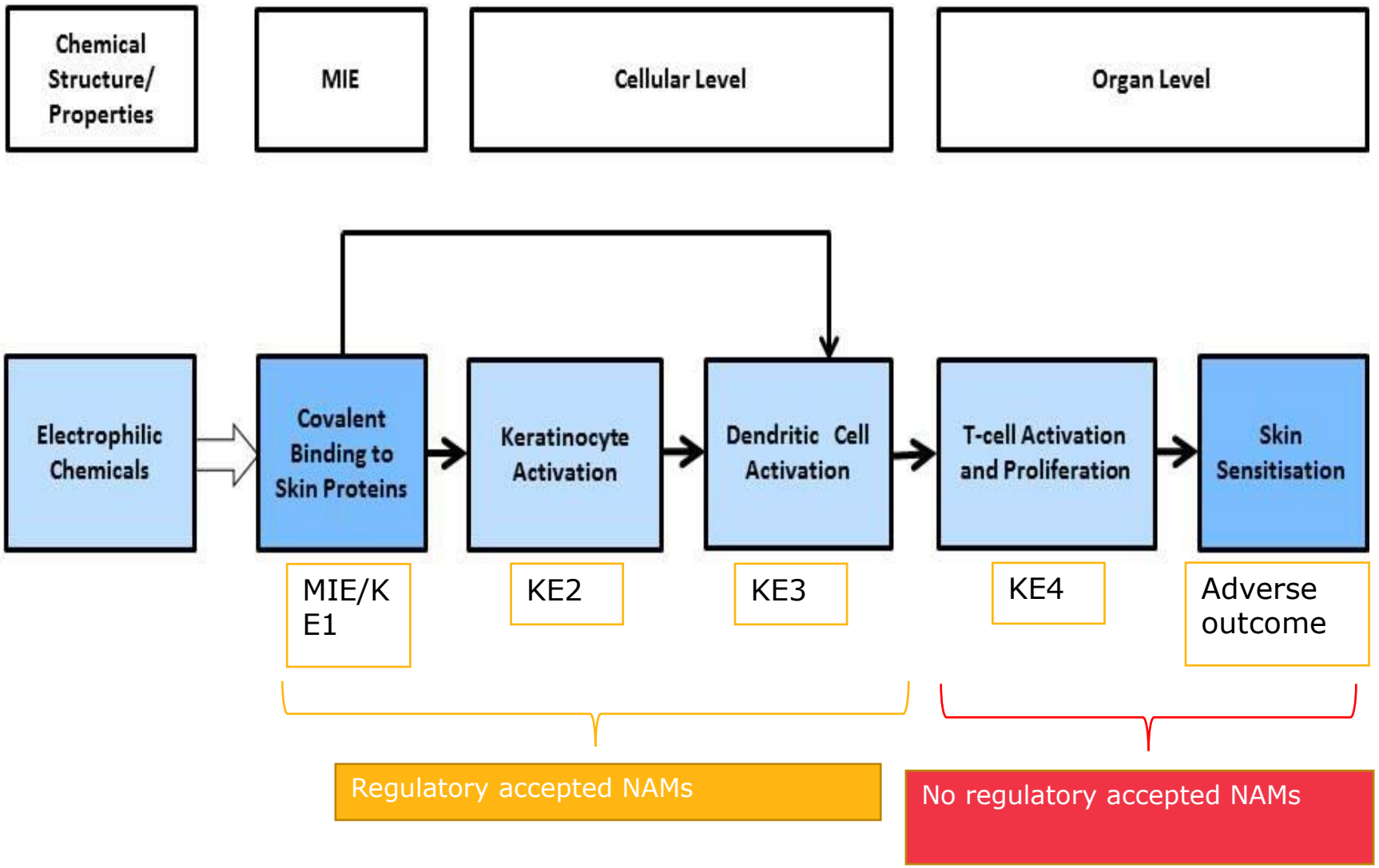
Skin sensitization

Adjuvant-mediated food allergy (NSAIDs, mycotoxin deoxynivalenol (DON) and Cholera Toxin (CT)

MNP-induced inflammatory responses

Antibiotic-induced liver disease

Skin sensitization: in vitro models serve well



Source: AOP wiki (https://aopwiki.org/wiki/index.php/Aop:40AOPwiki)

Hub-KEs for inflammation can also be plotted on:

Skin sensitization-
in vivo relevant AOP and matching KE-based in vitro tests

Adjuvant-mediated food allergy (NSAIDs, mycotoxin deoxynivalenol (DON) and Cholera Toxin (CT)
in vitro findings (intestinal cultures, mast cells) reflect part of the in vivo findings and link to AOP (hub-KE)

MNP-induced inflammatory responses-
in vitro findings translate to human effect data

Drug hypersensitivity-DILI

DRUG-INDUCED LIVER INJURY

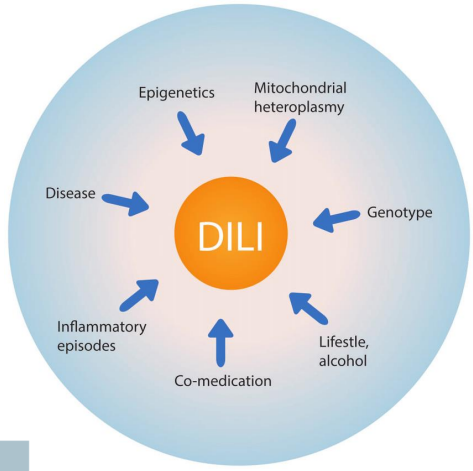
Managing the challenge of drug-induced liver injury: a roadmap for the development and deployment of preclinical predictive models

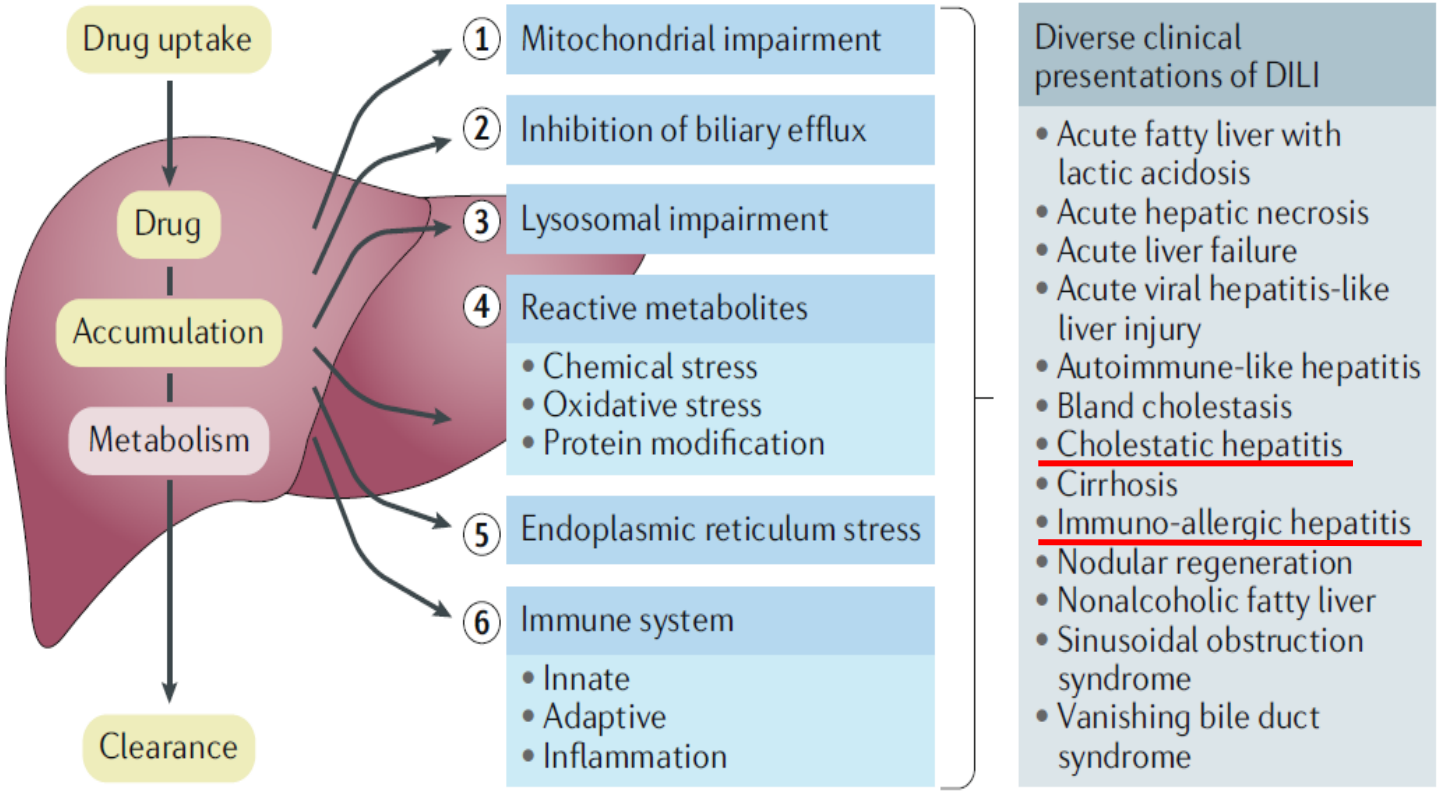
Richard J. Weaver, Eric A. Blomme, Amy E. Chadwick, Ian M. Copple, Helga H. J. Gerets, Christopher E. Goldring, Andre Guillouzo, Philip G. Hewitt, Magnus Ingelman-Sundberg, Klaus Gjervig Jensen, Satu Juhila, Ursula Klingmüller, Gilles Labbe, Michael J. Liguori, Cerys A. Lovatt, Paul Morgan, Dean J. Naisbitt, Raymond H. H. Pieters, Jan Snoeys, Bob van de Water, Dominic P. Williams and B. Kevin Park

Nat Rev Drug Discov. 2020 Feb;19(2):131-148

innovative medicines initiative

MIP-DILI





Diverse clinical presentations of DILI

- Acute fatty liver with lactic acidosis
- Acute hepatic necrosis
- Acute liver failure
- Acute viral hepatitis-like liver injury
- Autoimmune-like hepatitis
- Bland cholestasis
- Cholestatic hepatitis
- Cirrhosis
- Immuno-allergic hepatitis
- Nodular regeneration
- Nonalcoholic fatty liver
- Sinusoidal obstruction syndrome
- Vanishing bile duct syndrome

Table 2. Aetiology of drug induced liver injury.

Hepatocellular	Cholestatic	Mixed
Antibiotics/antifungals		
Isoniazid	Co-amoxiclav	Clindamycin
Pyrizinamide	Flucloxacillin	Nitrofurantoin
Rifampicin	Erthromycin	Sulphonomides
Tetracycline	Terbinafine	Co-trimoxazole
Trovafloxacin		
Ketoconazole		
Cardiovascular		
Amioderone	Irebesartan	Enalapril
Lisinopril	Fosinopril	Captopril
Losartan	Clopidogrel	Varapamil
Statins		
NSAIDs		
Diclofenac	Sulindac	Ibuprofen
Bromofenac		
CNS drugs		
Sertraline	Chlorpromazine	Carbamazipine
Valproic acid	Phenothiazines	Phenobarbital
Paroxetine	Tricyclics	Trazodone
Bupropion	Mirtazapine	Phenytoin
Trazodone		Cyproheptadine
Fluoxetine		
Rispiradone		
Nefazodone		
HAART		
Neveripine		
Ritonavir		
Others		
Omeprazole	Azathioprine	Azathioprine
Acarbose	Anabolic steroids	Flutamide
Troglitazone	Oral contraceptives	
Allopurinol	Cytarabine	

Drugs that can cause liver injury classified by indications for use (or class) and type of liver injury. Note that some drugs can cause more than one type of liver injury.

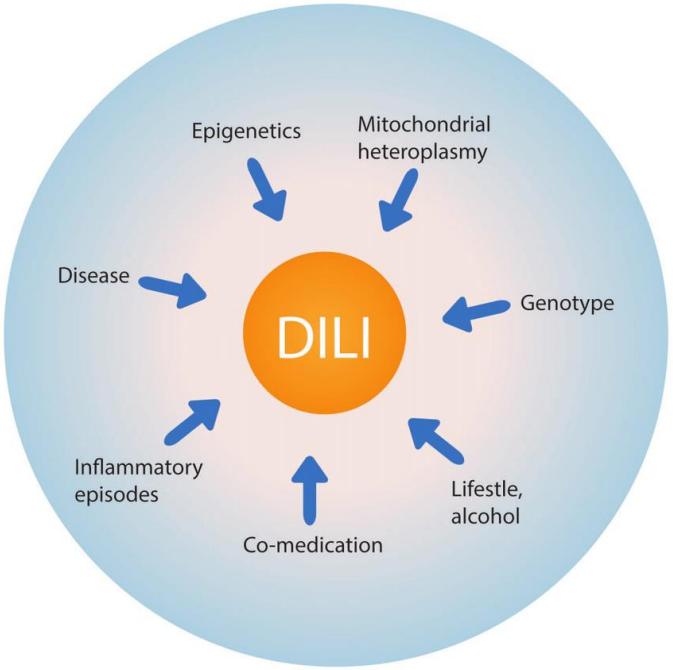


Table 1. Factors That Cause Predisposition to Idiosyncratic DILI

Nongenetic factors	Genetic variability
Age	Phase 1 enzymes
	CYP 2C8
Sex	CYP 2C9
	CYP 2C19
Daily dose	CYP 2D6
	CYP 2E1
Metabolism profile	Phase 2 and detoxifying enzymes
	NAT2
Drug interactions	GSTM1 and T1
	MnSOD
Alcohol	UGT2B7
Underlying comorbidities (pre-existing liver disease, HIV infection, diabetes)	Drug transporters
	BSEP (ABCB11)
	MRP2 (ABCC2)
	MDR3 (ABCB4)
	Immunologic
	HLA class antigen
	Cytokines (IL-10, IL-4, tumor necrosis factor-α)
	Mitochondrial DNA mutations (POLG)

GST, glutathione S-transferase.

Trovafloxacin and liver injury

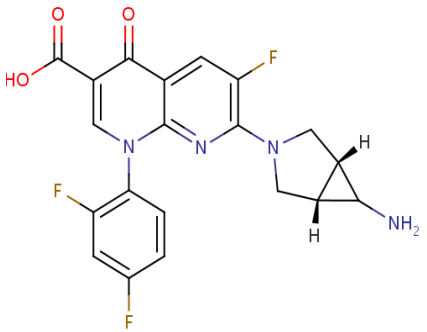
Fluoroquinolone

Pharmacological mechanism of action: Inhibition of DNA gyrase and topoisomerase IV

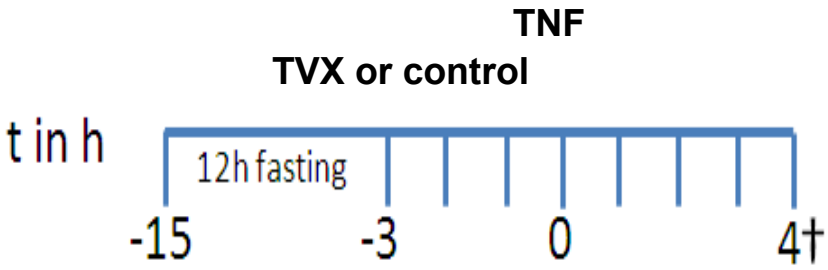
Potential toxic mechanisms:

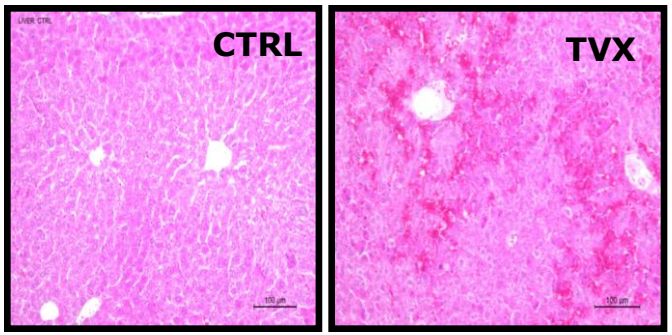
- Highest affinity for eukaryotic topoisomerase II

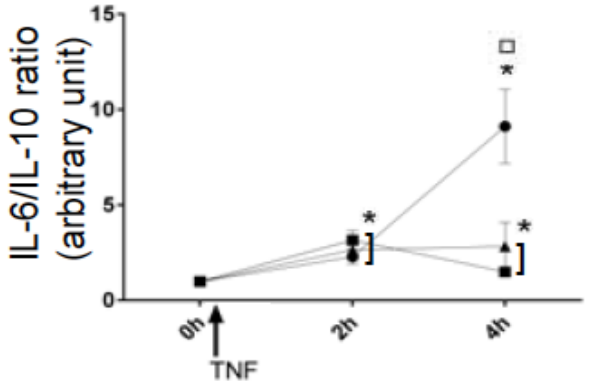
Withdrawn



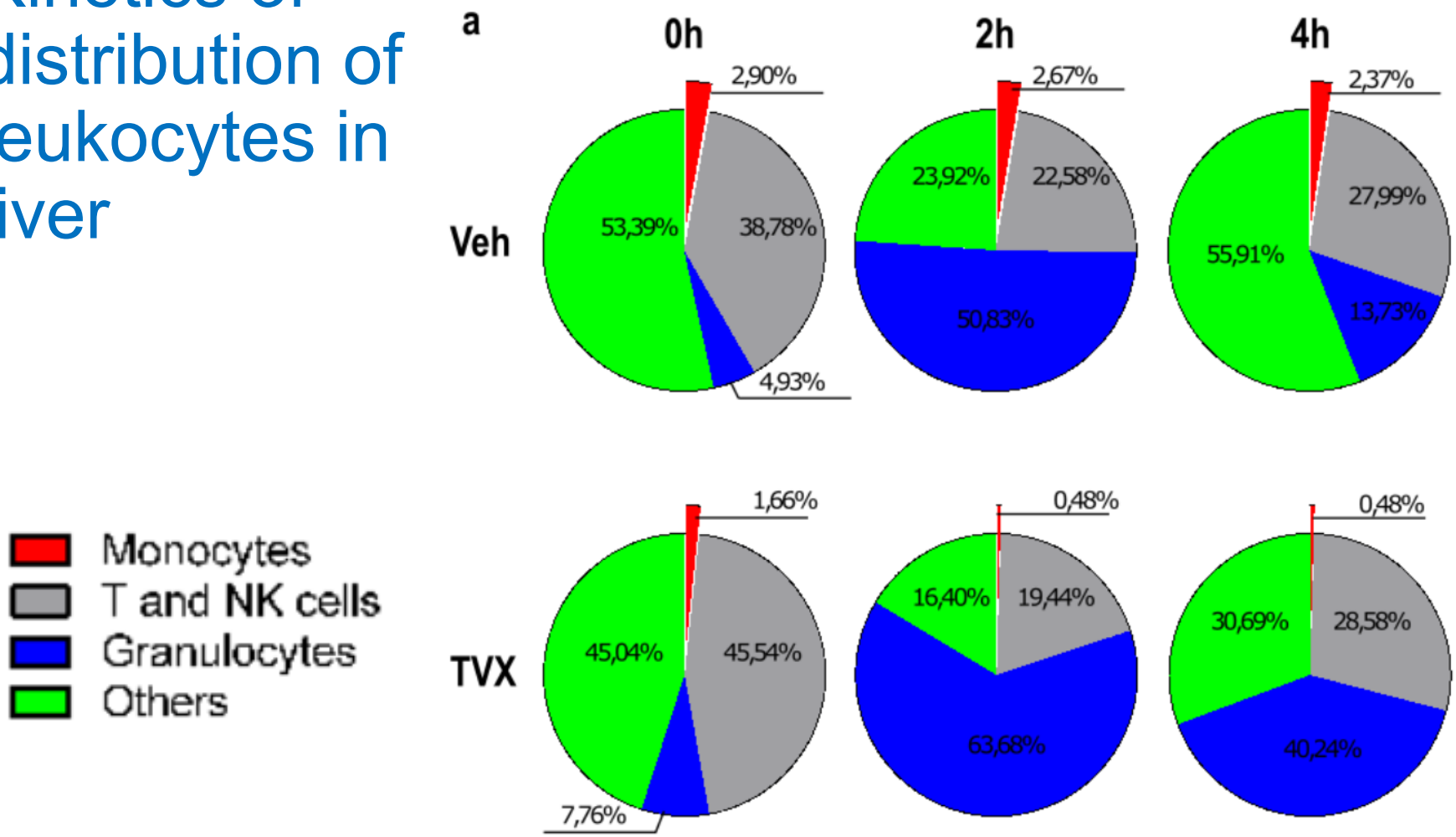
male C57BL/6, fasted for 12 hr







Kinetics of distribution of leukocytes in liver



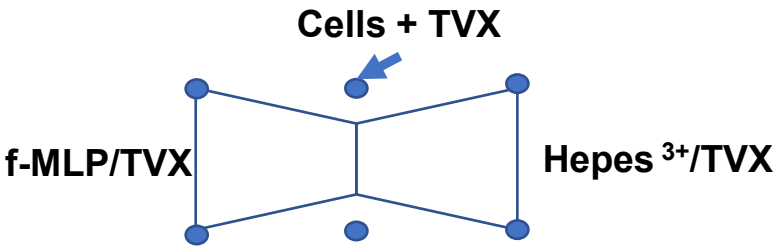
Giustarini G, Kruijssen L, van Roest M, Bleumink R, Weaver RJ, Bol-Schoenmakers M, Smit J, Pieters R. Tissue influx of neutrophils and monocytes is delayed during development of trovafloxacin-induced tumor necrosis factor-dependent liver injury in mice. J Appl Toxicol. 2018 May;38(5):753-765. doi: 10.1002/jat.3585. Epub 2018 Jan 26.

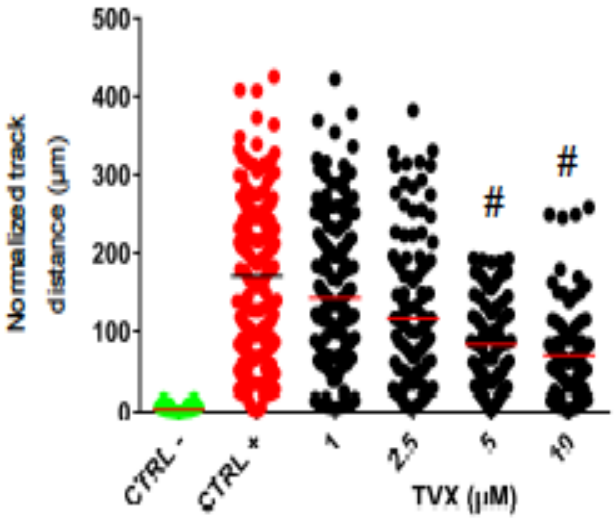
Lessons from TVX studies

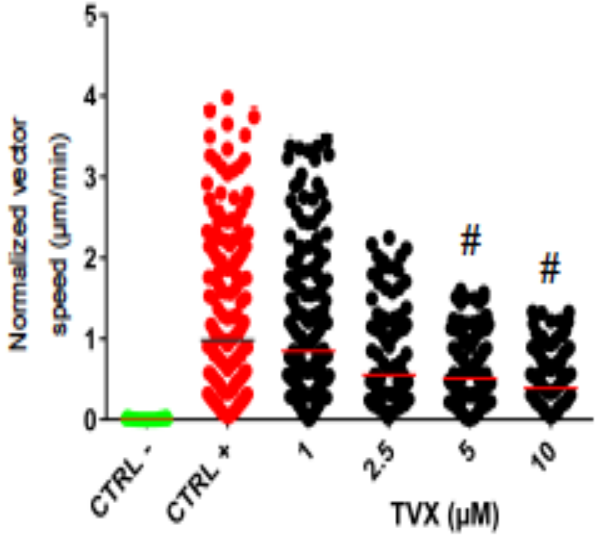
Multitarget:

- topoisomerase II arrest—p21 ↑--- cell cycle arrest-survival
- NFκb signalling ↓ cell activity-interaction (e.g. ICAM1 ↓)
- costimulation by DC ↓ no activation of T cells
- blocks Pannexin 1 channel--- ATP release ↓

Migration of human neutrophils inhibited by TVX







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Lessons from TVX studies

Multitarget:

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- NFκb signalling ↓ cell activity-interaction (e.g. ICAM1 ↓)
- costimulation by DC ↓ no activation of T cells
- blocks Pannexin 1 channel--- ATP release ↓
  - TVX affects attraction and migration of human neutrophil and monocytes (into tissue), and affects also formation of apoptotic bodies

Cholestatic drugs in HepaRG cells

	Drug	IC20 (µM) 24h	Highest concentration used (µM)	Fold xCmax	BC deformation	ROCK MLCK	BSEP Inhibition TCA efflux	MRP2 inhibition DCF efflux	Vesicle membrane[1] BSEP Activity Inhibition	Vesicle membrane[1] MRP2 Activity Inhibition
Clinical Cholestasis	Chlorpromazine	50	50	250	Constriction	ROCK	YES	YES	NO/YES*	NO
	Cyclosporine A	>300	50	44	Constriction	ROCK	YES	YES	YES	YES
	Bosentan	120	10	12	Dilatation	MLCK	YES	YES	YES*	-
	Troglitazone	75	50	8	Constriction	ROCK	YES	YES	YES	A/YES
	Nefazodone	50	75	18	Constriction	ROCK	YES	YES	YES	NO
	Erythromycin	>200	50	12	Dilatation	n.t	YES	YES	YES	NO
	Flucloxacillin	12000	2000	7	Dilatation	ROCK	YES	YES	NO	-
Drugs involved in rare cases of clinical cholestasis	Tolcapone	70	100	4	Dilatation Constriction	ROCK-MLCK	YES	YES	YES*	NO/A
	Entacapone	250	100	26	Dilatation	MLCK	nc.	nc	YES	NO
	Perhexiline	25	20	10	Constriction	ROCK	YES	YES	NO	NO
	Cimetidine	4000	4000	416	Dilatation	MLCK	nc	nc	YES*	NO
	Diclofenac	375	200	25	Dilatation	MLCK	nc	nc	YES*	-
	Metformin	3000	3000	388**	Constriction	ROCK	nc	nc	NO	-
	Tacrolimus	60	50	555	Dilatation	MLCK	YES	YES	YES/NO	YES
	Trovafloxacin	200	20	2	Dilatation	ROCK	YES	YES	-	-
	Levofloxacin	4000	2000	296	Dilatation	MLCK	YES	YES	NO	-
	Fasudil	300	50	17	Dilatation	ROCK	YES	NO	-	-
	Cloxacillin	9000	2000	257***	Dilatation	ROCK	YES	YES	YES	-
	Nafcillin	7000	2000	816**	Dilatation	ROCK	YES	YES	-	-
Drugs not involved in clinical cholestasis	Macitentan (i)	230	100	133	Dilatation	MLCK	YES	YES	-	-
	Sitaxentan	580	600	27	nc	nc	nc	YES	YES	A/YES
	APAP	20 000	25000	179	nc	nc	nc	nc	NO	YES
	Buspirone	>300	100	10000	nc	nc	nc	nc	NO	NO
	Pioglitazone	200	100	24	nc	nc	nc	n.t	YES*	A
	Fialuridine	>300	100	156	nc	nc	nc	n.t	NO	NO
	Ximelagatran	>300	100	333	nc	nc	nc	n.t	NO	NO
	Amiodarone	20	20	25	nc	nc	nc	nc	YES*	NO
	Ampicillin	>24000	24000	1000	nc	nc	nc	nc	-	-
	Amoxicillin	>24000	24000		nc	nc	nc	nc	NO	-
	Ambrisentan	>800	600	187	nc	nc	nc	nc	-	-

nc = no change n.t = not tested [1]*expressed proteins (IC50)* *- = no information, A=activation, *= medium*

Data on 30 tested drugs indicate that bile duct canilicula-**deformation links with** ROCK/MLCK activation

No false negatives in HepaRG

- **BC Deformation**
- **RhoKinase/Myosin Light Chain kinase pathways**

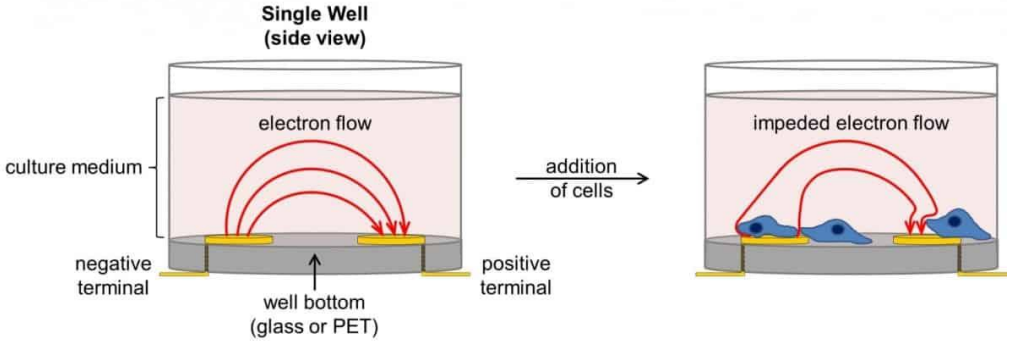
[1] Pedersen *et al* 2013,Morgan *et al* 2010, 2013, Dawson *et al* 2012,

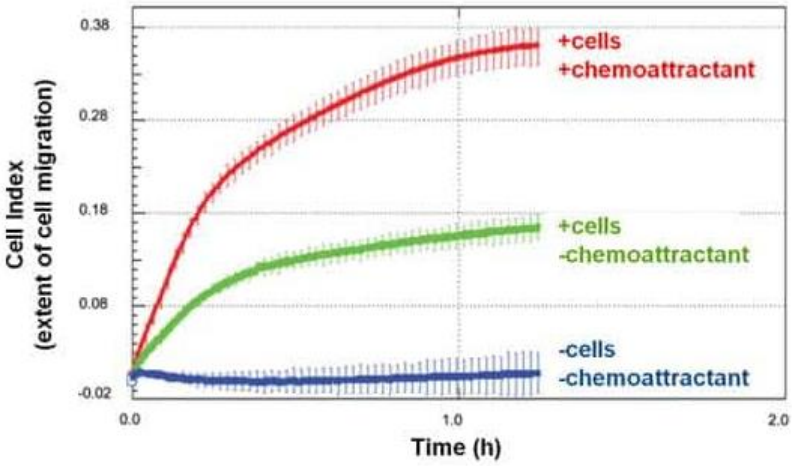
Courtesy of Richard Weaver (Servier)

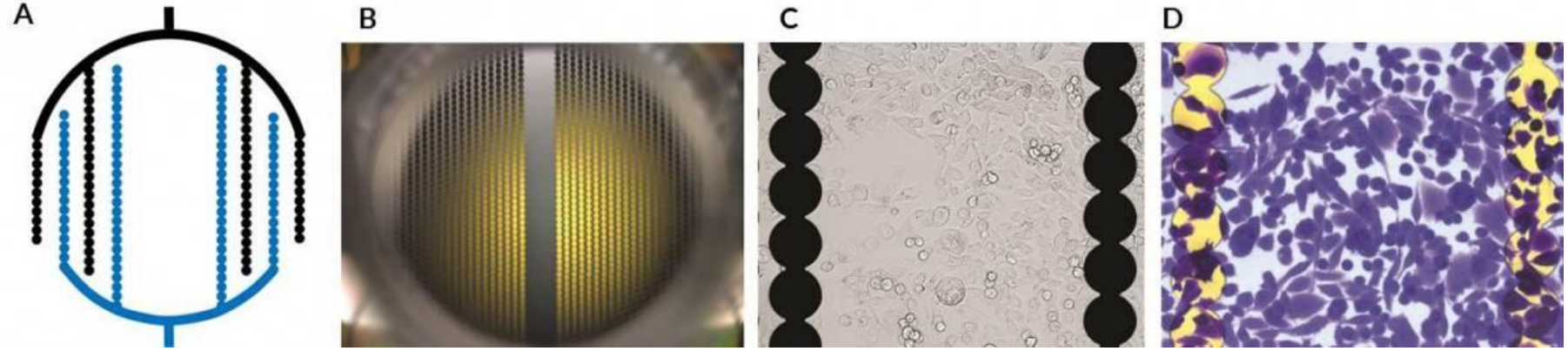
Is cell migration (involving adhesion, dilation and contraction) of cells (bile canucila, immune cells) a common mechanism for different types of DILI?

Do cholestatic and immune-mediated drug-induced liver injuries share common biological targets?

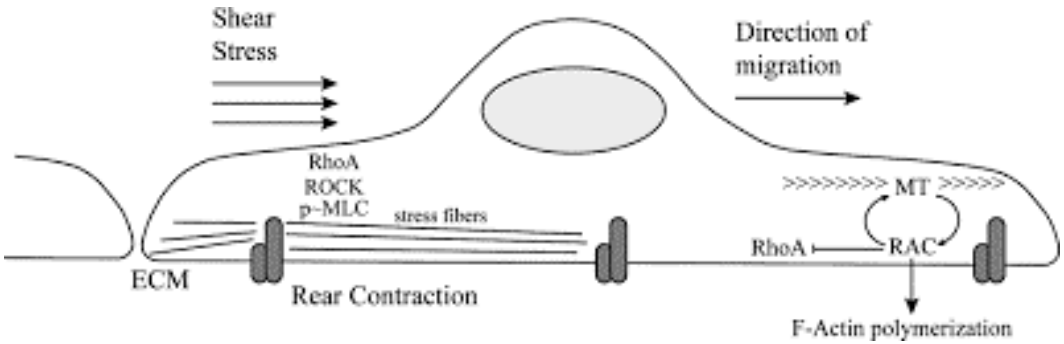
Cell adhesion and spreading on the Xcelligence

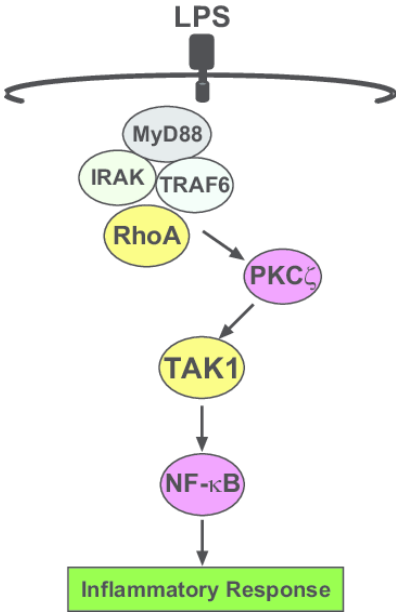






Cell migration as in vitro predictor for drug-induced liver injury



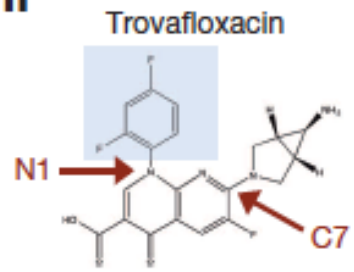


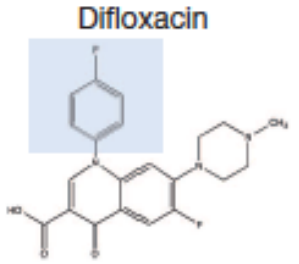
Drug	IC_{20} 24h µM	Highest conc used µM	BC deformation	HL60 Migration	Raw264.7 Migration	Cho cell Adhesion/Spreading	THP1 NFkB activity
Chloropromazine	50	50	Constriction	Not Tested	Strong Inhibition	Decreased	Strong Inhibition
Cyclosporine A	>300	4	Dilatation	Strong Inhibition	Strong Inhibition	Increased	Strong Inhibition
Bosentan	120	10	Dilatation	Not Tested	Weak Inhibition	Increased	No Effect
Troglitazone	75	50	Constriction	Strong Inhibition	Strong Inhibition	Decreased	Mild Inhibition
Flucloxacilline	12000	200	Dilatation	Not Tested	Partial Inhibition	Decreased	No Effect
Diclofenac	375	200	Dilatation	Partial Inhibition	Partial Inhibition	Increased	No Effect
Metformin	3000	3000	Constriction	Partial Inhibition	Partial Inhibition	No Effect	No Effect
Trovafloxacin	200	20	Dilatation	Strong Inhibition	Strong Inhibition	Increased	No Effect
Levofloxacin	4000	2000	Dilatation	No Inhibition	No Inhibition	No Effect	No Effect
Levofloxacin high	4000	2000	Dilatation	Not Tested	Inhibition	No Effect	No Effect
Buspirone	>300	100	No Effect	Not Tested	Partial Inhibition	No Effect	No Effect
Fialuridine	>300	100	No Effect	Not Tested	Strong Inhibition	No Effect	No Effect
Ampicillin	>24000	24000	No Effect	No Inhibition	No Inhibition	No Effect	No Effect

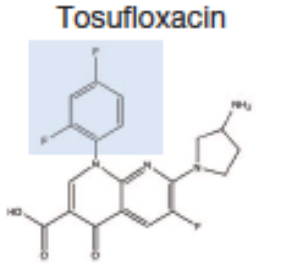
Ten Klooster, unpublished data

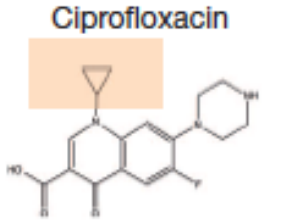
Effects of other fluoroquinolones

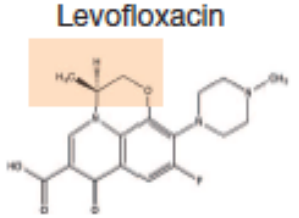
	DNA damage topoisomerase IIa	NFkB apoptotic cytokine	PNX1 migration
Trovafloxacin (1:18000, liver failure) hepatocellular	✓	✓	✓
Difloxacin	**na**	**na**	✓
Tosufloxacin	**na**	**na**	✓
Ciprofloxacin (isolated cases)	✓	**na**	o
Moxifloxacin (isolated cases 1:85M) hepatocellular-cholestatic	✓	**na**	**na**
Levofloxacin (rare, <1:5M)	o	o	o



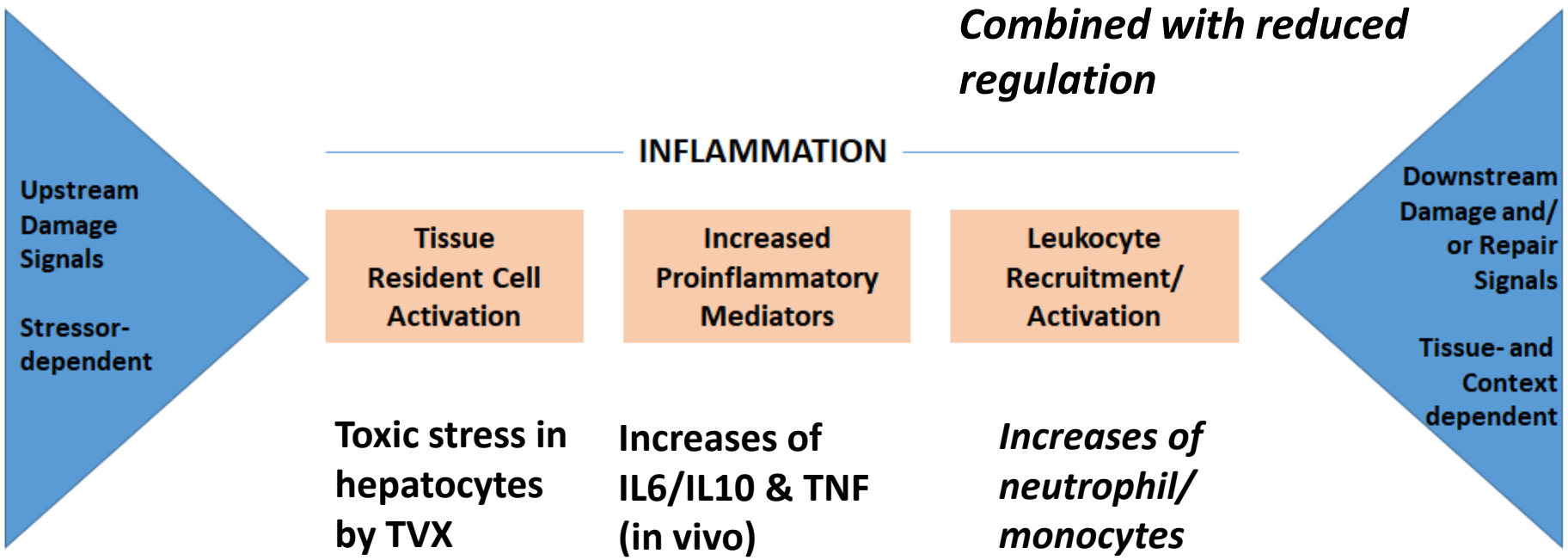








KEY EVENTS AFFECTED BY DILI INDUCING ANTIBIOTIC



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CONCLUSION

Hub key events of inflammation can be plotted on many immunotoxicological AO, including DILI

In case of DILI, it may help to screen new drug entities,

keeping in mind that actual adverse immunotoxicological outcomes are:

-personalized (human studies needed)
-dependent on ongoing immune responses (inflammations, infections, …)
-exposure-route dependent (more advanced in vitro methods needed)

Thanks to:

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Joost Smit
Marianne Bol-Schoenmakers

SERVIER

Richard Weaver

UNIVERSITY OF APPLIED SCIENCES UTRECHT

Jean Paul Ten Klooster

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Nienke Vriesekoop
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