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# Title: The toll-like receptor 3 pathway in homeostasis, responses to injury and wound repair

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Abstract: In addition to their established roles in host defence, Toll-like Receptors (TLRs) have emerging roles in control of homeostasis, injury and wound repair. The dsRNA-sensing receptor, TLR3, has been particularly implicated in such processes in several different tissues including the skin, intestine and liver, as well as in the control of reparative mechanisms in the brain, heart and kidneys, following ischemia reperfusion injury. In this review, we provide an overview of TLR3 signalling and functions in inflammation, tissue damage and repair processes, as well as therapeutic opportunities that may arise in the future from knowledge of such pathways.

**Cover Letter**

THE UNIVERSITY OF QUEENSLAND
AUSTRALIA

**Institute for Molecular Bioscience**
A/Prof Matt Sweet
Laboratory Head

Tuesday 16th August 2016

The Editor-in-Chief,
*Seminars in Cell & Developmental Biology*

Dear Professor Davey,

Many thanks for the helpful review of our manuscript "*The toll-like receptor 3 pathway in homeostasis, responses to injury and wound repair*", which we had previously submitted for the special issue on "Innate immune pathways in wound healing and tissue repair" to be published in *Seminars in Cell & Developmental Biology*. We have made the minor revisions requested by the reviewer, and hereby submit our revised manuscript for consideration for this special issue.

All authors concur with this submission and the material within this manuscript has not been previously reported, and is not under consideration for publication elsewhere.

I look forward to the outcome of your consideration.

Yours sincerely,

A/Prof Matthew Sweet (on behalf of the authors)

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**Response to the editor's comments:**

1. Make sure all the three figures are original. If you cite part of the figures which were published, you need to cite the related reference. If your figure(s) is/are copied from other authors, you need to get the written permission and also you should point out the reference(s) at certain place.

**Response:** All the figures in our manuscript are original and have not been published elsewhere.

2. Check the references: All the references you cited in the text must be listed in the "Reference" list, all the references in the list must be cited in the text. Please make a double-check.

**Response:** All the references have been checked to ensure they are cited correctly.

**Response to the reviewer's comments:**

Reviewer #1: This timely review is excellent and nicely highlights the role of immune responses in wound healing with regard to the important role of TLRs.

The review aligns perfectly with the scope of this SCDB Special Issue. Please address the minor comments/suggestions provided below

Minor Suggestions:

1. Introduction: I think within a few words should also mention that inflammation and induction of genes associated with repair that are tissue specific is the response mechanism.

**Response:** A sentence has now been added in the second paragraph of the introduction to highlight this concept.

2. 2.1. Overview of TLR signalling

-Thus implicating this adaptor in responses to multiple TLRs in a tissue dependent manner.

- TRAM

Also, sorry to be a pain, but as a resource article, I think the alternative names for

each should also be mentioned ie TIRAP, TICAM-1, TICAM-2.

**Response:** Thank you for these suggestions. A link between MAL and tissue-specific responses to different TLRs has been included, as have alternate names for the adaptor proteins.

3. 2.3. Role of TLR3 in host defence

   TLR3'..polymorphisms (C1234T, rs1879026 G/T, C13766T) have also been linked to chronic hepatitis B virus (HBV). "

   Is this an increases susceptibility or disease pathology?

   Is this an aspect of failed liver regeneration/wound healing?

   Next page… Collectively, these studies reveal....

**Response:** The association between different TLR3 polymorphisms and susceptibility to HBV infections has now been specified in the text, as requested. Also, the text in the next page has been edited as per the reviewer's suggestion.

4. 3.1 Skin

   Is IRF6 widely expressed or does it have a specific expression pattern. If so, this might be worth pointing out.

   Please include here the recent study naming this protein IL-39 and it's finding in relation to the quoted study.

**Response:** IRF6 expression is restricted to epithelial cells, and this has now been indicated in the text. Also, the very recent literature on the novel cytokine IL-39 has been described, with reference made to implications for wound healing responses in the skin. We have also made a very minor modification to Figure 3, on the basis of these recent publications on IL-39.

5. 3.4 I/R injury in the brain

   I realise this is not I/R however, is it worth mentioning the TLR3 appears to play a role in maintaining blood-brain barrier function as WNV activated TLR3 to allow 'loosening' of the BBB and increase viral dissemination?

   Also if we stick to the theme of brain, what about the role/implications of SARM

or is that just too long a bow?

**Response:** The role of TLR3 in WNV-infection and blood-brain barrier maintenance has now been included in this section of the manuscript. In order to do this, a short section on TLR3 and WNV has also been included in Section 2.3 (role of TLR3 in host defence), so that there is some context for this discussion (we had originally included such a section in our initial draft, but had removed it because of concerns about word limits). In relation to the reviewer's comments about SARM, we do not feel that there is a strong enough connection to make here, and for this reason, we have not included discussion on this point.

# The toll-like receptor 3 pathway in homeostasis, responses to injury and wound repair

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## Abstract

In addition to their established roles in host defence, Toll-like Receptors (TLRs) have emerging roles in control of homeostasis, injury and wound repair. The dsRNA-sensing receptor, TLR3, has been particularly implicated in such processes in several different tissues including the skin, intestine and liver, as well as in the control of reparative mechanisms in the brain, heart and kidneys, following ischemia reperfusion injury. In this review, we provide an overview of TLR3 signalling and functions in inflammation, tissue damage and repair processes, as well as therapeutic opportunities that may arise in the future from knowledge of such pathways.

**Keywords:** Toll-like receptor 3; wound repair; homeostasis; ischemia reperfusion injury; poly(IC); UV-damage

## List of Abbreviations

BBB = blood-brain barrier, Bcl2 = B-cell CLL/lymphoma 2, CNS = Central nervous system, CXCL = C-X-C motif ligand, EBI3 = Epstein-Barr virus induced gene 3, FADD = Fas-associated death domain, HBV = Hepatitis B virus, HSC = Hepatic stellate cell, HSV = Herpes simplex virus, I/R = Ischemia reperfusion, IAV = Influenza A virus, IEC = Intestinal epithelial cell, IFN = Interferon, IL = Interleukin, IRF = Interferon regulatory factor, MAL = MyD88 adaptor-like, MAPK = Mitogen-activated protein kinase, MyD88 = Myeloid differentiation primary response gene 88, NF-κB = Nuclear factor kappa B, PAMP = Pathogen-associated molecular pattern, Poly(IC) = Polyinosinic-polycytidylic acid, PRR = Pattern recognition receptor, RHIM = Receptor-interacting protein homotypic interaction motif, RIPK = Receptor-interacting protein kinase, TBK-1 = TANK binding kinase 1, TGM1 = Transglutaminase-1, TICAM = TIR-containing adaptor molecule, TIR = Toll interleukin 1 receptor, TIRAP = TIR domain-containing adaptor protein, TLR = Toll-like Receptor, TNF-α = Tumor Necrosis factor alpha, TRAF6 = Tumor Necrosis factor receptor-associated factor-6, TRAM = TRIF-related adaptor molecule, TRIF = TIR domain-containing adaptor-inducing IFN, UV = Ultraviolet, WNV = West Nile virus

## 1. Introduction

Pattern recognition receptors (PRRs) are widely studied in innate immune cells for their roles in host defence. In this context, they are tasked with detecting and responding to conserved pathogen-associated molecular patterns (PAMPs), which are essential for microbial survival and/or pathogenicity [1]. It is now clear that PRRs function more broadly as danger-sensing systems, detecting both pathogen- and host-derived factors that accumulate when homeostasis is perturbed. Indeed, PRRs have emerged as critical regulators of homeostasis and developmental processes. Such roles may relate to PRR functions in non-immune cells such as epithelial cells, where they are also expressed [2].

Of the PRR families, the Toll-like receptors (TLRs) have been most widely studied. These transmembrane receptors, which localize to both the plasma membrane and to endolysosomal compartments, play key roles in development, homeostasis and injury repair. For example, TLR2, which detects bacterial lipopeptides, maintains homeostasis at mucosal surfaces by promoting barrier integrity in intestinal epithelial cells (IECs) [2]. Along with TLR4, which recognizes Gram-negative bacterial lipopolysaccharide, TLR2 has also been implicated as a regulator of cardiovascular functions, thermoregulation and energy metabolism in the autonomic nervous system [3]. Such studies provide examples of roles for innate immune danger-sensors in regulating normal physiological processes to maintain homeostasis. Indeed, the capacity of TLRs to regulate the expression of genes involved in inflammation and repair processes, often in a tissue-specific manner, appears to be critical for maintenance of normal physiological processes [2].

TLR3 is a dsRNA-sensing TLR, first characterized as a regulator of anti-viral responses. However, subsequent studies demonstrated that TLR3 can also detect host-derived RNA, thus enabling it to regulate injury repair processes. In this review, we

provide an overview of TLR3 biology in the context of host defence and inflammation. We particularly focus on TLR3 functions in wound healing and in homeostatic control in the skin, gastrointestinal tract and liver, as well as during ischemia reperfusion (I/R) injury in the brain, heart and kidney (**Fig. 1**). Given that TLR3 agonists and antagonists already exist, manipulation of this pathway to accelerate tissue repair processes may be feasible in some pathophysiological settings.

## 2. TLR3 signal transduction and its role in host defence

### *2.1. Overview of TLR signalling*

A total of thirteen TLRs have been identified in humans and mice; ten in humans (TLR1-10) and twelve in mice (TLR1-9, 11-13) [4]. Each TLR recognizes cognate PAMPs, resulting in activation of distinct but overlapping signalling pathways through the initial recruitment of specific combinations of Toll/Interleukin (IL)-1 Receptor (TIR) domain-containing adaptor proteins. Briefly, upon activation, all TLRs, except for TLR3, recruit the adaptor protein myeloid differentiation primary response gene 88 (MyD88), which contains a C-terminal TIR domain and an N-terminal death domain [5]. In the case of TLR4 and TLR2, MyD88-adaptor-like (MAL; also known as TIR domain-containing adaptor protein, TIRAP) acts as a bridging adaptor between MyD88 and these TLRs. Recently, TLR5 in IECs [6], and TLR7 and TLR9 in macrophages [7], have also been shown to associate with MAL, thus implicating this adaptor in tissue-specific responses to multiple TLRs. MyD88 then relays downstream signalling via the serine/threonine kinase IL-1R-associated kinases, the E3-ubiquitin ligase and scaffolding protein tumour necrosis factor (TNF) receptor-associated factor-6 (TRAF6) and the mitogen-activated protein kinases (MAPKs) [5]. This ultimately enables activation of pro-inflammatory transcription factors such as nuclear factor kappaB (NF-κB) and activator protein-1 to drive inducible expression of pro-inflammatory target genes such as IL-1β, IL-6 and TNF-α (for reviews see [5,8,9]).

TLR3 and TLR4 are both capable of signalling independently of MyD88, via the TIR domain-containing adaptor-inducing interferon (TRIF; also known as TIR-containing adaptor molecule 1, TICAM1) pathway [5]. TLR4 engages both MyD88 and TRIF, whereas TLR3 uses TRIF exclusively. TLR4 initiates the MyD88-dependent pathway at the plasma membrane, whilst signalling switches to TRIF-mediated responses once TLR4 is endocytosed [10]. Endosomal TLR4 signalling via TRIF requires the bridging adaptor TRIF-related adaptor molecule (TRAM; also known as TIR-containing adaptor molecule 2, TICAM2) [10]. TRIF signalling activates the serine/threonine kinase TANK binding kinase-1 (TBK-1), which phosphorylates the transcription factor interferon (IFN) regulating factor 3 (IRF3) [5]. IRF3 phosphorylation enables it to translocate to the nucleus and activate specific pro-inflammatory target genes, for example *IFN-β*, which encodes a type-1 IFN [5].

### *2.2. TLR3 signalling*

Although TLR3 signalling has been extensively characterized, some new players and regulatory mechanisms have recently emerged (**Fig. 2**). TLR3 is assembled in the endoplasmic reticulum, from where it is recruited to endosomes by the transmembrane protein UNC93B1 [11]. It is the only TLR that directly recruits TRIF to its TIR domain to initiate signalling. This may relate to the fact that the conserved proline residue present in the BB-loop of most TLR TIR domains is an alanine in TLR3. Indeed, mutation of Ala795 in TLR3 to a proline resulted in MyD88-biased signalling [12]. As with TLR4 signalling, TRIF recruitment to TLR3 leads to the activation of the serine/threonine kinase TBK-1, which in turn phosphorylates IRF3 [13,14]. Phosphorylation occurs at multiple residues (e.g. Ser385, Ser386) in the C-terminal region of IRF3, enabling dimerization, nuclear translocation and transcription of *IFN-β* [15,16]. IFN-β signals in an autocrine fashion to activate the transcription factors signal transducer and activator of transcription (STAT) 1 and 2, resulting in the activation of

type-1 IFN target genes and subsequent anti-viral responses [17]. Although IRF3 is the primary transcription factor driving IFN-β transcription during TLR3 signalling, other IRFs also function downstream of TLR3 to impart cell-specific signalling responses. For example, IRF6 is an epithelial cell-specific transcription factor that lies downstream of TLR3 signalling in keratinocytes. Specifically, it inhibits poly(IC)-inducible *IFN-β* expression, while promoting poly(IC)-inducible *IL-23p19* expression in primary human keratinocytes [18].

In addition to activating IRF3, TLR3 signalling via TRIF also activates NF-κB [19,20]. The C-terminal region of TRIF contains a receptor-interacting protein homotypic interaction motif (RHIM), which is essential for its interaction with the serine/threonine kinase receptor-interacting protein kinase (RIPK)1 [21,22]. TLR3- and TLR4-mediated NF-κB activation is impaired in the absence of RIPK1 [21]. RIPK1 undergoes K63-linked polyubiquitination and interacts with TRAF6 and transforming growth factor-beta activated kinase 1 (TAK1), resulting in NF-κB activation [23]. The TRIF-RIPK1 axis is a central control point in cell survival/death pathways, since RIPK1 also associates with Fas-associated death domain (FADD), via a death domain interaction [22]. This subsequently leads to the assembly of a death-inducing signalling complex that contains caspase-8 [22]. Homodimerized caspase-8 undergoes autocatalytic processing and activation, leading to RIPK1 cleavage and inactivation, followed by apoptotic cell death [22]. However, if caspase-8 heterodimerizes with a non-catalytically active homologue of caspase-8, FLICE-like inhibitory protein, it is partially activated [22]. This complex is not able to cleave RIPK1 adequately to cause apoptosis, and therefore mediates cell survival [22]. Furthermore, if caspase-8 activity is compromised, RIPK1 cleavage is completely prevented, thereby allowing it to interact with RIPK3 to form a necrosome, leading to necroptotic cell death [24,25]. Thus,

RIPK1 acts as a central signalling hub in dictating whether TLR3 signalling promotes survival, apoptotic cell death or necroptotic cell death.

### *2.3. Role of TLR3 in host defence*

The role of TLR3 in host defence was first dissected through gene knock-out studies in mice, revealing a role in dsRNA recognition and anti-viral responses. In macrophages from *Tlr3*$^{-/-}$ mice, the production of the inflammatory cytokines IL-6, IL-12 and TNF-α in response to the synthetic dsRNA analogue poly(IC), but not other TLR agonists, was impaired [26]. Additionally, *Tlr3*$^{-/-}$ mice are highly susceptible to infection by many RNA viruses, including rhinovirus, influenza A virus (IAV) and respiratory syncytial virus [27]. Genetic association studies in humans have also linked the TLR3 pathway to host defence against IAV. A patient with IAV-induced encephalopathy showed a missense mutation (F303S) in the *TLR3* gene [28]. Additionally, children with a particular TLR3 polymorphism (rs5743313/CT) have an increased risk of pneumonia caused by the pandemic IAV/H1N1 (2009) [29]. Complex roles for TLR3 in host protection versus immunopathology have also reported in other viral infection models, for example West Nile virus (WNV) [30,31]. An initial study showed that *Tlr3*$^{-/-}$ mice were protected from WNV-induced encephalitis, because of reduced viral entry into the central nervous system [30]. This study also linked TLR3 to a TNF-α-mediated reduction in blood-brain barrier (BBB) integrity upon WNV infection. However, a subsequent study reported that TLR3 deficiency resulted in enhanced WNV replication in primary cortical neurons, and that viral replication was actually increased in the brain and spinal chord of *Tlr3*$^{-/-}$ mice [31]. The contrasting conclusions from these studies [30,31] may reflect differences in viral doses and/or routes of administration used. These factors would likely affect the magnitude of the initial inflammatory response upon WNV challenge, potentially leading to differential effects on BBB integrity in the two models.

Although these studies provide links between TLR3 and host responses to RNA viruses, most genetic studies in humans actually posit the TLR3-TRIF-IRF3 axis in responses against DNA viruses. The first evidence for this was the demonstration that TLR3 has a host protective function against the dsDNA virus, herpes simplex virus (HSV)-1 [32]. Patients with mutations in *TLR3* and *UNC93B1* are susceptible to HSV-1 encephalitis [32-34]. Recognition by TLR3 of intermediate dsRNA produced by HSV-1 during its replicative cycle likely explains this phenomenon [32]. These findings were supported by subsequent analysis of patients with defects in genes encoding the TLR3 signalling components *TRIF*, *TBK-1* and *IRF3* [35-37]. An impaired TLR3-inducible type-1 IFN response to HSV-1 in neurons and oligodendrocytes in the central nervous system (CNS) was subsequently linked with this condition [32-37]. These findings are also consistent with studies in mouse models of DNA virus infections. *Tlr3*$^{-/-}$ mice displayed enhanced replication of HSV-1 in the CNS [38], and an increased susceptibility to HSV-2 infection in the CNS after vaginal inoculation [39]. In humans, *TLR3* polymorphisms (C1234T, rs1879026 G/T) have also been linked to increased risk to chronic hepatitis B virus (HBV) infection and HBV-induced hepatocellular carcinoma [40,41], while the C13766T *TLR3* polymorphism was suggested to be protective against HBV infection and HBV-related liver disease [42]. A study analysing Saudi Arabian patients with HBV infections reported a *TLR3* single nucleotide polymorphism (rs1879026 G/T) that was found more frequently in HBV-infected patients [40]. Peripheral blood mononuclear cells from HBV-infected patients were also reported to have increased TLR3 levels in active chronic HBV infection and related liver failure [43]. Collectively, these studies reveal a significant role for TLR3 signalling in responses to a wide range of viral pathogens.

## 3. TLR3 functions in homeostasis and wound healing

### *3.1. Skin*

Human skin is constantly exposed to microbes. Not surprisingly then, keratinocytes express TLRs, responding to PAMPs with inflammatory cytokine production. These cells are especially responsive to TLR3 agonists [18,44-47], and an initial study on human keratinocytes revealed an unexpected role for this PRR in detecting RNA released from damaged cells [48]. More specifically, UVB-damaged keratinocytes release U1 spliceosomal non-coding RNA that activates TLR3 in non-irradiated cells, resulting in the release of inflammatory cytokines, such as TNF-α and IL-6 [49]. These effects are consistent with findings from earlier studies, showing that UV-radiation activated NF-κB [50], triggered the pro-inflammatory MAPK p38 and c-Jun N-terminal kinase signalling arms [51-53], and promoted IL-6 and TNF-α release [54,55]. In light of subsequent studies on U1 RNA-mediated TLR3 activation, it seems likely that this pathway is involved in initiating these UVB-triggered inflammatory responses. Poly(IC)-mediated TLR3 activation also directly upregulated the expression of several genes encoding proteins associated with maintenance of epidermal structure, for example transglutaminase-1 (TGM1) [56]. In oral keratinocytes, IRF6 regulates Grainyhead-like 3 expression [57,58], which has been reported to directly regulate *TGM1* transcription [59]. Thus, it is conceivable that TLR3-inducible *TGM1* expression occurs via the IRF6-dependent pathway [18]. Finally, the importance of such responses in wound repair is highlighted by the observation that *Tlr3*$^{-/-}$ mice exhibit chronic wounds and impaired re-epithelialization following acute UVB-irradiation [56].

There is also evidence that TLR3 regulates host responses during physical injury of the skin. TLR3 mRNA and protein levels were upregulated in full excisional wounded skin; moreover, TLR3 was required for TNF-α and IL-6 production at wound edges, as well as optimal wound closure [48,60]. Impaired wound closure in *Tlr3*$^{-/-}$ mice may be linked to reduced secretion of the chemokine C-X-C motif ligand (CXCL)-2, leading to defective recruitment of neutrophils and macrophages to the injury site [60,61].

Collectively, these studies demonstrate that TLR3 has a critical role in detecting cell damage in the skin, initiating inflammatory processes that are required for wound healing and directly promoting the expression of genes in keratinocytes required for barrier function (**Fig. 3**).

Mechanistically, IRF6, an epithelial cell-specific transcription factor that is required for keratinocyte differentiation, may be involved in some of the above processes. IRF6 suppressed poly(IC)-inducible *IFN-β* mRNA, while promoting inducible *IL-23p19* mRNA expression in human keratinocytes [18]. IFN-β has been shown to inhibit proliferation and promote terminal differentiation of keratinocytes [62,63]. Therefore, IRF6-mediated IFN-β inhibition may enable a proliferative and reparative response in the skin. Intriguingly, we recently showed that poly(IC) selectively upregulates the expression of the IL-12 family genes IL-23p19 (α sub-unit) and Epstein-Barr virus induced 3 (EBI3) (β sub-unit) in keratinocytes, and that these two sub-units interact when co-expressed in cells [18]. This suggests that IL-23p19 and EBI3 may function as a novel IL-12 family cytokine. Indeed, these two subunits were very recently reported to form IL-39, which was shown to promote neutrophil expansion in lupus-prone mice [64,65]. Therefore, IL-39 may play a role downstream of TLR3 in wound healing responses in the skin by promoting neutrophil-mediated responses at the site of injury. Interestingly, IRF6 itself has been suggested to have a role in wound healing. Patients with *IRF6* mutation-associated cleft lip/palate disorders were more likely to have wound complications after corrective surgery (47% of patients), compared to patients with other forms of cleft lip/palate (19% of patients) [66]. This conclusion is supported by an *in vitro* study showing that *IRF6* mRNA expression was upregulated in normal keratinocytes during scratch wounding [67]. Thus, there may be a functional connection between TLR3 and IRF6 in promoting wound healing responses in the skin, possibly involving co-expressed IL-23p19 and EBI3.

### *3.2. Intestinal Tract*

IECs express functional TLRs and can initiate pro-inflammatory signalling upon pathogen recognition [2]. Emerging evidence also implicates epithelial-expressed TLRs in intestinal homeostasis and prevention of dysregulated mucosal inflammation [68]. Consistent with its pro-wound healing role in the skin, a tissue-protective role for TLR3 in the intestine was observed during dextran sodium sulphate-induced colitis [69]. Subcutaneous poly(IC) pre-treatment protected mice from colitis, an effect not observed in *Tlr3*$^{-/-}$ mice [69]. Levels of inducible nitric oxide synthase, cyclooxygenase-2, serum CXCL1/KC (derived from keratinocytes) and amyloid A were also reduced in poly(IC)-treated mice [69]. However, other studies actually implicate TLR3 signalling in inducing acute injury in gut epithelia [70-72]. Intraperitoneal injection of rotavirus dsRNA resulted in severe injury in the small intestine as characterized by thinning of the intestinal wall, erosion of villi and the mucus membrane, and weight loss; *Tlr3*$^{-/-}$ mice were protected from such effects [70-72]. Poly(IC) administration also up-regulated serum levels of several cytokines including IL-15 and IFN-γ [70]. IL-15 was shown to have a non-redundant role in driving TLR3-mediated injury, whereas IFN-γ had a protective effect [70]. Interestingly, this IL-15-dependent mechanism of intestinal damage has previously been reported in human patients with celiac disease, where the activation of intraepithelial cytotoxic T lymphocytes by IL-15 led to uncontrolled cell destruction and atrophy [73-76]. Poly(IC), acting via TLR3, TRIF and caspase-8, also induced epithelial cell apoptosis and altered the structure and function of the intestine [71], which may relate to host-protective functions against viral infections. Indeed, a subsequent study showed that epithelial-specific deletion of caspase-8 resulted in TRIF-dependent TLR3-mediated necroptosis and a more severe inflammatory phenotype [72]. Finally, poly(IC) has also been reported to regulate gut permeability and induce oxidative stress in an epithelial cell line [77,78]. Thus, TLR3 signalling in the

epithelium seems to contribute both to host-protective epithelial cell shedding during pathogen challenge, as well as cell damage and injury that can be detrimental to the host.

### *3.3. Liver*

In healthy liver tissue, hepatic stellate cells (HSC) and resident macrophages (Kupffer cells) are in a quiescent state; upon injury, these cells are activated, leading to the release of inflammatory mediators and immune cell recruitment. HSC also undergo transformation into myofibroblasts, leading to increased production of extracellular matrix proteins; this process is a host-protective response, but causes scarring and liver fibrosis when dysregulated [79]. As noted above, TLR3 signalling has been implicated in host responses during HBV infection [40-42]. However, growing evidence suggests that TLR3 also contributes to the inactivation or death of activated HSC and Kupffer cells during liver injury, contributing to protective responses against liver damage [80] and fibrosis [81,82].

Mechanistically, poly(IC) treatment attenuated lipopolysaccharide-induced liver injury by down-regulating TLR4 expression [83]. Poly(IC) also reduced carbon tetrachloride-induced liver fibrosis by activating TLR3 in cytotoxic natural killer cells, resulting in IFN-γ production and apoptosis of activated HSC. This in turn inhibited the progression of inflammation and liver damage [81,82]. Interestingly, in an alcohol-induced liver injury model, TLR3 signalling activated HSC and Kupffer cells to produce the anti-inflammatory cytokine IL-10, which subsequently antagonized TLR4 signalling and minimized liver injury [84]. Another study showed that poly(IC) treatment suppressed inflammation and fat accumulation in the liver of alcohol-injured mice [80]. These effects were not apparent in *Tlr3*$^{-/-}$ or *IL-10*$^{-/-}$ mice, suggesting that TLR3-inducible IL-10 suppresses inflammation and steatosis [80]. Conversely, in a mouse model of autoimmune liver disease, TLR3 was shown to enhance $CD8^+$ T-cell infiltration into the

liver via IFN-γ and chemokines (e.g. CXCL9) [85]. In this case, the TLR3 pathway was linked to pro-inflammatory responses and the progression of liver damage [85]. This finding suggests that TLR3 has a complex role in liver homeostasis, and thus there is a need to more completely understand the mechanisms by which TLR3 exerts protective *versus* pathological effects in liver disease.

### 3.4. *I/R injury in the brain*

I/R injury occurs when there is insufficient blood supply in a tissue leading to oxygen deprivation (anoxia or hypoxia), following which blood supply is restored. In the brain, I/R can occur during stroke, when a blood vessel is blocked, or during cardiac arrest [86]. The cellular and molecular mechanisms of brain I/R injury are poorly understood, however, inflammatory pathways play a critical role in both injury and repair processes [86]. As indicated earlier (see Section 2.3), TLR3 has previously been implicated in mediating loss of BBB integrity and contributing to neuropathology in a mouse model of WNV infection [30]. Nonetheless, recent studies have reported protective effects of poly(IC) treatment in brain I/R injury models [87-89]. This was linked to the capacity of TLR3 signalling in human astrocytes to drive expression of anti-inflammatory cytokines, such as *Ifn-β*, which act as neuroprotective factors [87,88]. More specifically, poly(IC) pre-treatment of mice or primary astrocytes increased cell viability and proliferation, and reduced NF-κB activation and inflammatory cytokine production following I/R injury [87,88]. This treatment regime also triggered Irf3 phosphorylation and upregulated *Ifn-β* expression, suggesting a role for this signalling arm in injury repair [90]. Furthermore, protection against neuronal damage was lost in *Tlr3*$^{-/-}$ mice [90]. *In vitro* experiments also showed that TLR3 interacted with Fas in microglial cells, limiting its interaction with FADD, as well as subsequent Fas/FADD-mediated activation of caspase-3 and 8 and microglial cell apoptosis [90]. Together, these studies

suggest that TLR3 signalling has the capacity to limit neurological damage during I/R injury.

### *3.5. I/R injury in the heart*

I/R injury of the heart can be caused by angioplasty, acute myocardial infarction, exercise, stress-induced ischemia or coronary artery bypass surgery [91]. As opposed to its protective role in brain ischemic injury, TLR3 signalling has been linked to the pathology of myocardial I/R injury. *Tlr3*$^{-/-}$ mice showed significantly attenuated myocardial dysfunction, myocardial apoptosis and reduced infarct size in an I/R injury model [92]. TLR3-mediated NF-κB activation, and subsequent production of inflammatory mediators (e.g. TNF-α and IL-1β) promoted leukocyte infiltration into the heart leading to inflammation and organ dysfunction [92]. *Tlr3* deficiency also reduced the levels of the I/R-inducible pro-apoptotic proteins B-cell CLL/lymphoma 2 (Bcl2)-associated X (Bax) and Bcl2 antagonist/killer (Bak), while increasing Bcl2 expression [92]. Increased cardiac expression of Bcl2 is known to protect mice from I/R injury by preventing apoptotic cell death [93]. Subsequently, another study showed that myocardial I/R injury induces cardiomyocyte necrosis and release of RNA, which activates TLR3 signalling [94]. Moreover, RNase-treated necrotic cardiomyocytes failed to induce inflammatory responses, such as IL-1β production, in macrophages and cardiomyocytes [94]. Similarly, RNase administration *in vivo* reduced inflammation, leading to protective effects [94]. Taken together, these recent studies suggest that RNA released from necrotic cardiomyocytes during I/R injury promotes TLR3 signalling, leading to apoptotic cell death, myocardial injury and pathological sequelae.

### *3.6. I/R injury in the kidney*

I/R in the kidney can induce acute injury or renal failure by triggering inflammation, decreasing microvascular blood flow and causing endothelial dysfunction [95].

Although relatively little is known of the role of TLR3 signalling during kidney damage, one study demonstrated that serum creatinine levels were significantly reduced in *Tlr3*$^{-/-}$ mice following renal I/R injury [96]. This study also showed that Tlr3 signalling is initiated early in response to acute kidney injury, and that the levels of angiotensin-converting enzyme and inflammatory mediators, as well as cellular apoptosis, necrosis and renal damage, were reduced in *Tlr3*$^{-/-}$ mice [96]. Additional studies are now required to confirm that TLR3-dependent inflammatory responses contribute to kidney damage during renal I/R injury, and to uncover further insights into mechanisms responsible.

## 4. Therapeutic implications

The above studies clearly document non-redundant roles for TLR3 in promoting or impairing tissue repair in different contexts. TLR3 agonists have been widely employed in experimental studies [61,69,87,97], and antagonists of the TLR3 pathway, acting at the level of the receptor [98] or intracellular signalling molecules such as TBK-1 [99], have been reported. Thus, one can envisage manipulation of TLR3 signalling responses to promote wound repair for certain conditions. For example, the demonstration that treatment of wounded skin of humans and mice with poly(IC) significantly reduced recovery time [61] provides proof of concept for such approaches. This may be particularly relevant in situations where wound healing is impaired, as is commonly the case in patients with type II diabetes [100]. The capacity of UVB-radiation to activate TLR3 responses in the skin could also potentially be harnessed for the treatment of certain dermatological conditions [49]. Patients with psoriasis respond well to narrow band UVB-radiation [101], and UVA/UVB-radiation was demonstrated to have beneficial effects in patients with atopic dermatitis [102]. However, given that such approaches can have potential long-term complications, for example skin cancer, alternative strategies to agonise TLR3 may be more applicable.

The literature reviewed above also identifies TLR3 as a candidate target in the context of I/R injury. For example, in a mouse model, poly(IC) treatment following I/R injury improved recovery of brain function, as assessed by neurological behaviour [89]. Beneficial effects were also observed in rats, using a similar approach [88]. These data support the potential for TLR3 agonists in brain I/R injury, such as stroke. Indeed, Hiltonol, a nuclease-resistant and stabilized form of poly(IC), induced IFN-β production in astrocytes and microglia and stabilized the blood-brain-barrier, leading to reduced leukocyte infiltration [103]. Hiltonol has been used to boost IFN production in several clinical trials for many types of cancer, including brain cancer [104,105], but not yet for brain I/R injury. Another synthetic TLR3 ligand, Ampligen, which is composed of poly(IC) with a U mismatch at every $12^{th}$ base of the C-strand, has mainly been studied for activation of Th1 responses [106] and as a treatment for viral infections [107]. The literature reviewed here suggests that there may be merit in investigating Hiltonol, Ampligen and other TLR3 agonists as agents for promoting injury repair in at least some pathological settings, for example stroke and liver injury.

Although TLR3 antagonists have been reported, they have not been extensively assessed as potential therapeutic agents. TLR3 monoclonal antibodies (CNTO4685 and CNTO5429) inhibited poly(IC)-induced NF-κB activation in HEK293T cells expressing TLR3, as well as serum levels of pro-inflammatory cytokines, such as IL-6 and TNF-α, after administration *in vivo* [108]. A small molecule inhibitor of TLR3 (compound 4a), acting at the level of ligand binding, has also been reported [98]. Proof-of-concept studies are now required to determine whether TLR3 antagonism is efficacious in animal models of intestinal inflammation, as well as I/R injury in the heart and/or kidney.

## 5. Conclusions and outlook

TLR3 signalling has a major role not only in immune responses during infection, but also in homeostasis and tissue repair following injury. The molecular mechanisms involved are now being unravelled, such that one can envisage the development of new strategies to control inflammatory responses during injury and to promote tissue repair. However, a major challenge is that the TLR3 pathway has both host-protective and pathological functions in different organs or settings, and this must be given careful attention in considering new therapeutic strategies involving manipulation of this pathway. With this caveat in mind, agonism of TLR3 or specific downstream pathways could be considered in the context of wound healing in the skin, liver fibrosis and brain I/R injury, while TLR3 antagonists may have potential for cardiac and kidney injury, as well as intestinal disease. A more complete understanding of epithelial cell-specific TLR3 signalling events may ultimately guide more targeted approaches for manipulating this pathway to enhance wound healing and injury repair.

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## Figure legends

**Fig. 1. TLR3 signalling in wound repair and homeostasis.**

The TLR3-TRIF signalling pathway exhibits both protective and harmful roles in injury repair processes in different organs.

**Fig. 2. TLR3 signalling and downstream cellular responses.**

TLR3 interacts directly with TRIF to initiate signalling. This may relate to the lack of a conserved proline residue, present in the BB-loop of other TLRs (Alanine 795 in TLR3). TRIF signalling induces *IFN-β* expression via TBK-1 and IRF3, whilst epithelial cell-specific IRF6 inhibits this response. TRIF also interacts with RIPK1 to drive NF-κB activation and inducible expression of inflammatory genes, such as those encoding TNF-α and IL-6. RIPK1 also acts as a signalling hub for control of TLR3-dependent cell survival, apoptosis and necroptosis.

**Fig. 3. TLR3 signalling in the skin.**

TLR3 is activated by U1 spliceosomal non-coding RNA released during cell-damage. TLR3 signalling leads to the production of inflammatory mediators, such as TNF-α and IL-6, which enable recruitment of neutrophils and macrophages to the site of injury. IRF6 promotes epithelial cell-specific TLR3 signalling responses, possibly contributing to wound repair processes in the skin. TLR3 activation also upregulates the expression of genes associated with maintenance of epidermal structure, such as ATP-binding cassette sub-family A member 12 (*ABCA12*), glucocerebrosidase (*GBA*), acid sphingomyelinase (*ASM*) and *TGM1*, which are required for barrier repair.

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**Figure 1**

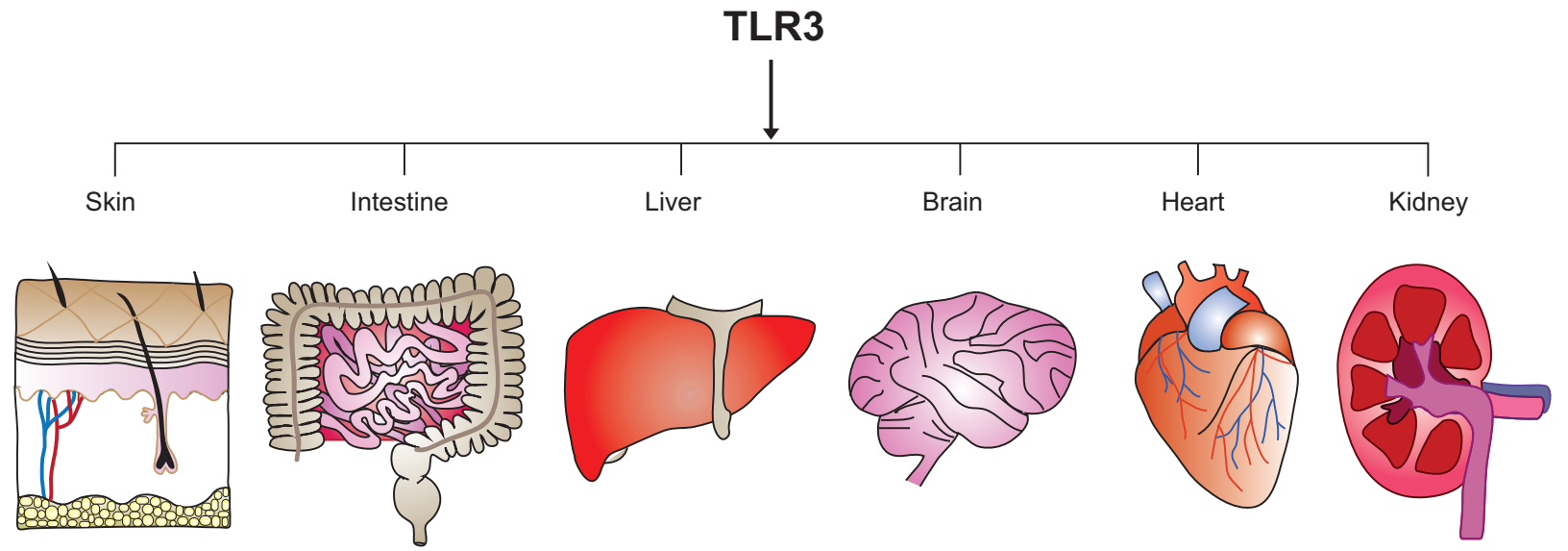

TLR3
Skin
Intestine
Liver
Brain
Heart
Kidney
Inflammatory cytokines and chemokines (e.g. TNF-α, IL-6, CCL5)
Inflammatory cytokines (e.g. TNF-α, IL-6, IL-12, IL-15, type 1 and 2 IFNs)
Anti-inflammatory cytokine release (e.g. IL-10)
Blocks inflammatory cytokines (e.g. TNF-α, IL-6) and increases IFN-β
Inflammatory cytokines (e.g. TNF-α, IL-1β)
Inflammatory cytokines (e.g. TNF-α, IL-1α, IL-17, IL-27)
Wound healing and repair
Promotes inflammation and cell death (apoptosis, pyroptosis and/or necroptosis)
Suppresses inflammation and steatosis
Promotes cell survival
Promotes cell death (apoptosis)
Promotes cell death (apoptosis and necrosis)
Host protective role
Primarily detrimental role
Host protective role
Host protective role
Detrimental role
Detrimental role

Figure 2

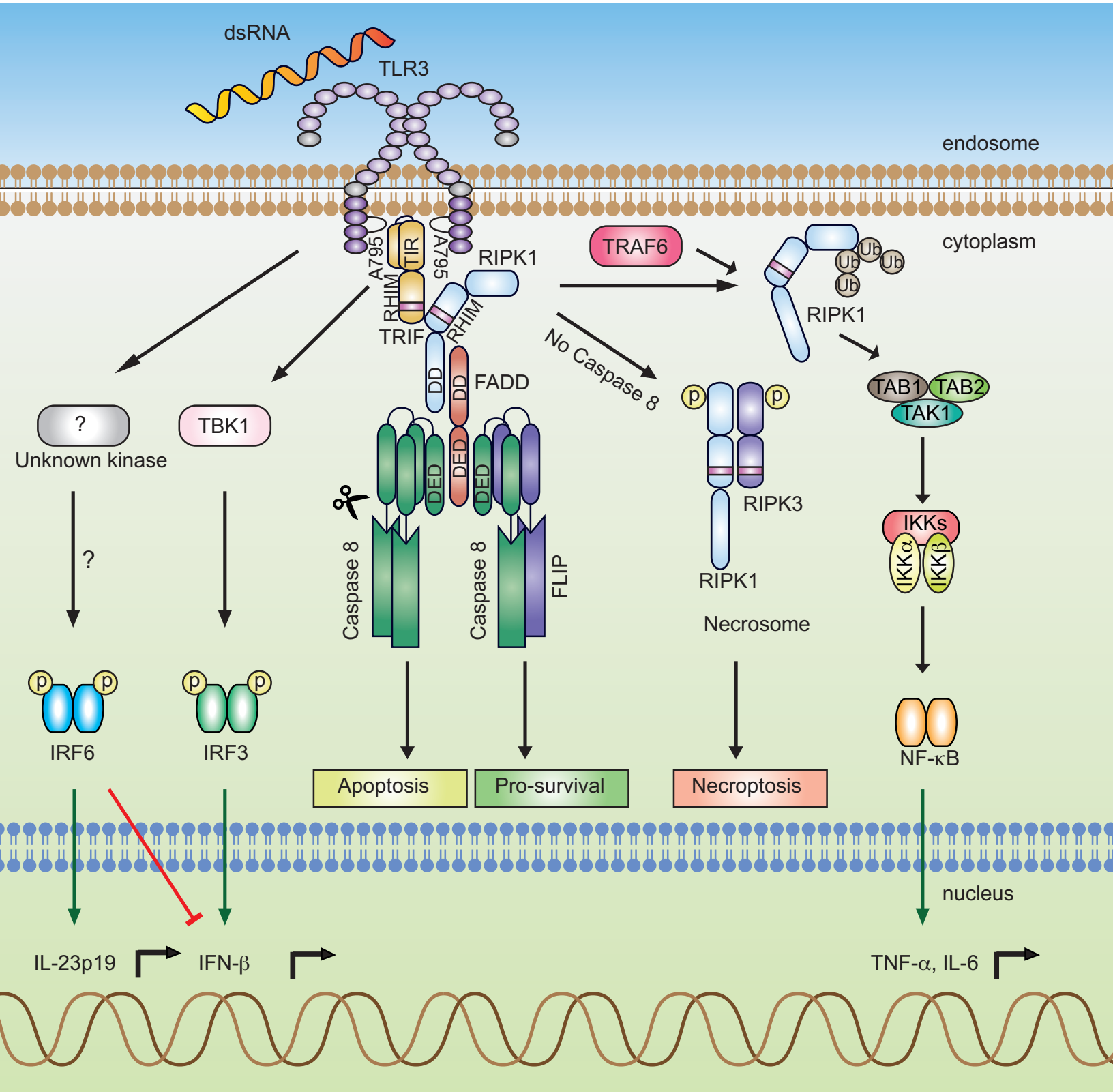
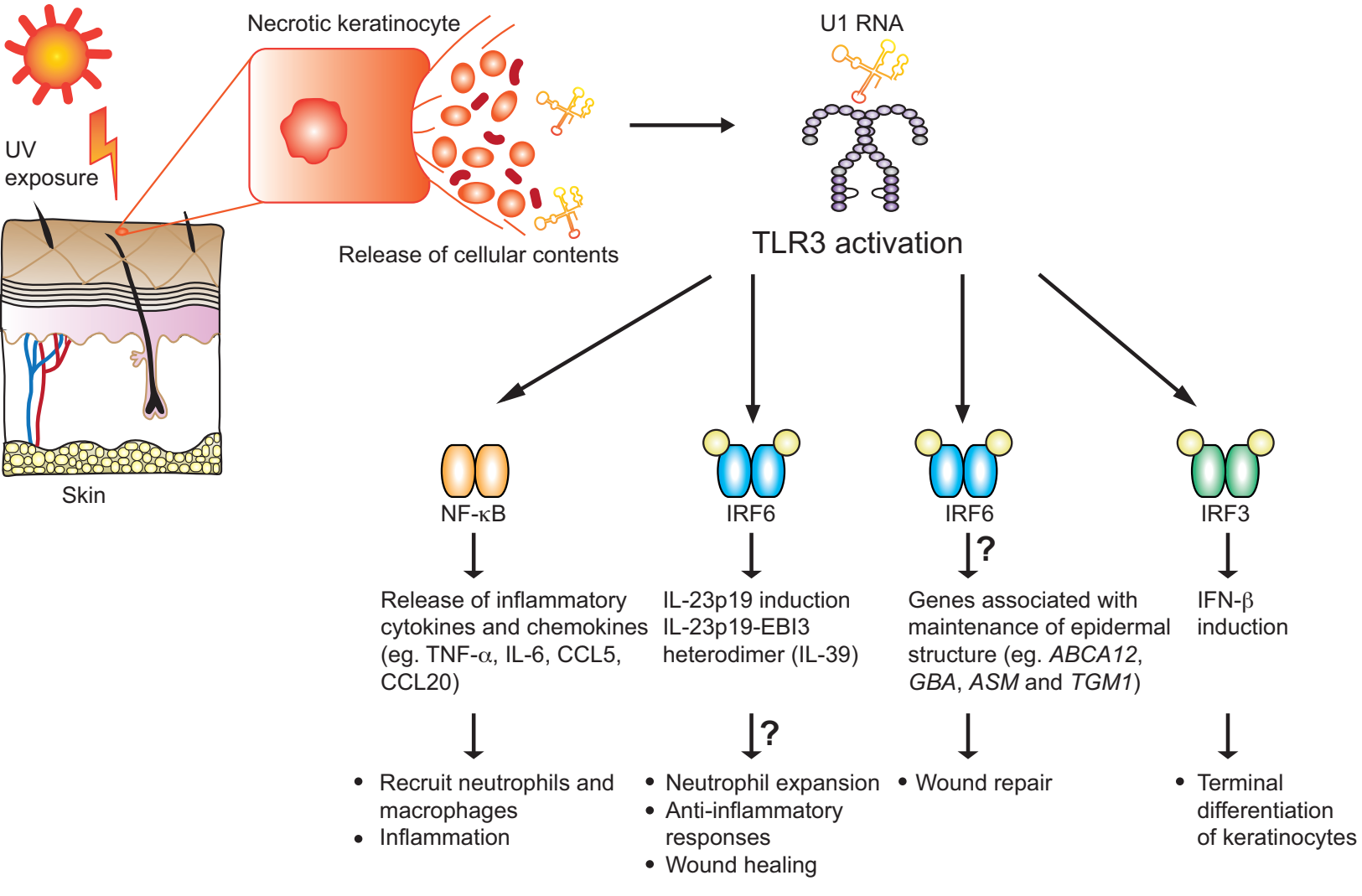

dsRNA
TLR3
endosome
cytoplasm
A795
TIR
A795
RHIM
RHIM
TRIF
RIPK1
TRAF6
Ub
Ub
Ub
Ub
RIPK1
No Caspase 8
DD
DD
FADD
?
Unknown kinase
TBK1
DED
DED
DED
Caspase 8
Caspase 8
FLIP
p
p
RIPK3
RIPK1
Necrosome
TAB1
TAB2
TAK1
IKKs
IKKα
IKKβ
?
p
p
IRF6
p
p
IRF3
NF-κB
Apoptosis
Pro-survival
Necroptosis
nucleus
IL-23p19
IFN-β
TNF-α, IL-6

**Figure 3**


Necrotic keratinocyte
U1 RNA
UV exposure
Release of cellular contents
TLR3 activation
Skin
NF-κB
IRF6
IRF6
IRF3
?
?
Release of inflammatory cytokines and chemokines (eg. TNF-α, IL-6, CCL5, CCL20)
IL-23p19 induction IL-23p19-EBI3 heterodimer (IL-39)
Genes associated with maintenance of epidermal structure (eg. ABCA12, GBA, ASM and TGM1)
IFN-β induction
• Recruit neutrophils and macrophages
• Inflammation
• Neutrophil expansion
• Anti-inflammatory responses
• Wound healing
• Wound repair
• Terminal differentiation of keratinocytes