COMMENTARY

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RIPping the Skin Apart: Necroptosis Signaling in Toxic Epidermal Necrolysis

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Toxic epidermal necrolysis (TEN) is a rare but potentially fatal drug hypersensitivity reaction. Although a number of pathophysiological hints have been identified over the past decade, details of the effector mechanisms within the skin remain obscure. A novel study by Kim *et al.* now sheds light on its pathophysiology. The investigators demonstrate convincingly that receptor-interacting kinase 3 (RIPK3) levels are upregulated substantially in the lesional skin of patients with TEN and that this is followed by the generation of reactive oxygen species, activation of mixed lineage kinase-like protein, and subsequent necroptotic cell death of keratinocytes. These data suggest that therapies that interfere with RIPK3 activation and necroptosis induction could benefit patients with TEN.

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Toxic epidermal necrolysis (TEN) is a severe adverse drug reaction of the skin and mucous membranes. Its name was first coined by Allan Lyell, (1956). TEN is a rare disease, with incidence rates of about 0.4–1.2 cases per million per year. As TEN has a mortality rate of up to 30%, depending on the extent of skin involvement and comorbidities (Schwartz *et al.*, 2013), the disease can be classified as true dermatological emergency. An efficient treatment for TEN is lacking to date, and randomized clinical trials are difficult to conduct because of its obscure pathophysiology and its overall rare incidence. Thus, additional mechanistic insights into the molecular mechanism leading to the pathology of TEN are imperative and a prerequisite for more effective treatment. Hypotheses for TEN pathophysiology include genetic predisposition combined with HLA determinants (Roujeau *et al.*, 1987). It is based on a direct interaction of specific HLA haplotypes and certain drugs such as carbamazepine, abacavir, or nevirapine (for review see Wei *et al.*, 2012). The initiating phase of cell death is attributed to interactions between HLA determinants, drug epitopes, and T-cell receptors (TCRs) of, as yet, poorly characterized T-cell subsets. This results in unwanted immune cell activation. Ultimately, fulminant keratinocyte cell death and extensive epidermal detachment represent the effector phase and the final consequence of the hypersensitivity response. Cytotoxic immune cells and/or soluble factors such as granulysin, CD95L, annexin A1, and micro RNA 18a-5p regulation have been suggested as causing keratinocyte death (Viard *et al.*, 1998; Chung *et al.*, 2008; Chung and Hung, 2012; Ichihara *et al.*, 2014; Saito *et al.*, 2014). Interestingly, both of the major forms of cell death, apoptosis and necrosis, appear to have roles. Classical apoptosis and the more recently described programed necrosis (in case of involvement of receptor-interacting protein (RIP) kinases (RIPKs) also called necroptosis) have been proposed as relevant cell death pathways responsible for the extensive keratinocyte cell death that is seen in TEN. For example, upregulation of CD95 ligand (CD95L) expression in keratinocytes in TEN has been suggested as causative (Viard *et al.*, 1998). CD95L is one of the key players in the receptor-induced cell death signaling pathway. Stimulation of death receptors (DRs) on the cell surface results in initiation of a proteolytic cascade that culminates in the activation of effector caspases, which are the mediators of apoptosis. In order to avoid uncontrolled apoptosis, a number of antiapoptotic proteins control the cascade at several different levels. The cellular FLICE-like inhibitory protein (cFLIP) is one inhibitor of extrinsic cell death. Epidermal loss of cFLIP was recently suggested as a possible prerequisite for epidermal cell death in TEN, as a mouse model with skin-specific inducible deficiency of cFLIP showed a phenotype resembled typical TEN morphology. This effect was shown to be reduced by blocking tumor necrosis factor (TNF) signaling (Panayotova-Dimitrova *et al.*, 2013), suggesting TNF as an additional player in TEN. In accordance with these data, a recent study reported that early blockade of TNF signaling by TNF receptor 2-Fc resulted in complete healing of 10 TEN patients (Paradisi *et al.*, 2014). These findings suggest an important role for TNF-dependent cell death in TEN, and it suggests TNF inhibitors as potential treatment options.

TEN: lessons learned from the loss of cell death resistance in the skin

Recent reports have indicated that the alternative death mode, programed necrosis or necroptosis, may contribute to the pathogenesis of TEN. It has been suggested that interaction of soluble annexin 1 with its receptor contributes to TEN (Saito *et al.*, 2014). Both apoptotic and necrotic cell death are regulated in part by overlapping molecular machineries. The Ripoptosome, a

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Clinical Implications

- Toxic epidermal necrolysis (TEN) is a rare but life-threatening drug hypersensitivity reaction with a sudden, and to date largely unexplained, loss of cell death resistance in the epidermis.
- The study by Kim *et al.* reveals that the receptor-interacting kinase 3 (RIPK3) is highly induced in the epidermis of lesional skin of patients with TEN.
- RIPK3 overexpression in TEN correlates with *in situ* activation of the major necroptosis effector, mixed lineage kinase like (MLKL).
- The data suggest that not only effectors of apoptosis such as caspases but also the major effectors of necroptosis contribute to epidermal cell death in TEN.
- Targeting of the necroptosis machinery using RIPK3 inhibitors, currently under preclinical development, may prove beneficial for patients with TEN.

complex described in 2011 (Feoktistova *et al.*, 2011; Tenev *et al.*, 2011), has been shown to participate in both apoptosis and necroptosis. RIPK1 and RIPK3 were described as core molecules in the ripoptosome. RIPK3 is a downstream target of RIPK1, forming a complex, which may or may not contain RIPK1. Activated RIPK3 gains the ability to phosphorylate and activate mixed lineage kinase domain-like protein(MLKL; Murphy *et al.*, 2013). Phosphorylated MLKL molecules form homooligomers, which form pores and disrupt membrane integrity, finally resulting in cell death (Wang *et al.*, 2014) The core molecular complex of RIPK1 and RIPK3 has long been recognized as the necrosome (Vanden Berghe *et al.*, 2014). How to retain the proper quality and quantity of cell death in keratinocytes during the progression of TEN is thus an important question. Most likely the mystery of TEN (and keratinocyte) progression will only be answered by detailed studies and new understanding of the intracellular death cascades in this cell type, but success in this effort may be rewarded by innovative therapeutic options that target the loss of cell death resistance in the skin.

RIPK: a potential target to interfere with the deadly effector phase of TEN?

In the current issue of the *JID*, Kim *et al.* (2015) shed new light on the pathophysiology of TEN. They investigated the molecular machinery governing the execution phase of programed necrosis or more specifically necroptosis. As convincingly shown in the paper, it is the necroptosis effector cascade RIPK3, but not MLKL, that is a potential bottleneck to prevent necroptosis in primary keratinocytes, as evidenced by its strong induction in TEN lesions *in vivo*. At the functional level, the authors show that the BRAF inhibitor Dabrafenib is, surprisingly, a potent inhibitor of RIPK3, based on its full inhibition of MLKL phosphorylation. This indicates a potential therapeutic avenue for RIPK3 inhibitors (Figure 1). Kim *et al.* (2015) first analyzed histologically the expression of two important proteins required for necroptosis in patients withf TEN. Even though their results partially contradict data from another study, which showed variable expression levels of MLKL and RIPK3 in keratinocyte protein lysates isolated from TEN patients (Saito *et al.*, 2014), Kim *et al.* (2015) show convincingly that, in the epidermis of lesional skin, the expression of RIPK3 is strongly and significantly upregulated. Importantly, this upregulation was demonstrated to result in increased levels of phosphorylated MLKL *in situ*. To investigate further the possible role of RIPK3 further, Kim *et al.* (2015) analyzed the potential of this molecule to modulate the sensitivity to cell death signals in different keratinocyte cell lines. In their *in vitro* system Kim *et al.* (2015) choose the nitric oxide (NO) donor sodium nitro prusside (SNP) as a modulator of cell survival and death. It had been demonstrated previously that upregulation of inducible NO is associated with TEN (Viard-Leveugle *et al.*, 2013). Kim *et al.* (2015) now confirmed the significance of RIPK3-dependent necroptosis for SNP-induced keratinocyte cell death. They compared different pharmacological inhibitors of necroptosis, such as necrostatin-1, Dabrafenib, and necrosulfonamide, and they compared these compounds with the "classical" apoptosis inhibitor, the pharmacological caspase inhibitor zVAD-fmk. They showed in conclusive functional experiments that combined pretreatment with these inhibitors resulted in much better protection from SNP-induced cell death when compared with zVAD-fmk alone. Furthermore, when the expression level of RIPK3 was altered by knockdown of RIPK3 or MLKL, they observed inhibition of SNP-induced cell death, accompanied by lowered induction of the stress kinases c-Jun N-terminal kinase (JNK) and p38 activation. Accordingly, RIPK3 overexpressing cells showed increased sensitivity to SNP-mediated cell death, as well as higher levels of JNK and p38 activation. Confirming their *in situ* findings, Kim *et al.* (2015) then also demonstrated that MLKL activation is associated with the RIPK3 expression level. Taken together, the study gives important hints about the significance of necroptotic cell death signaling in the pathogenesis of TEN. The elevated RIPK3 expression in the epidermis of TEN, which is accompanied by the induction of MLKL phosphorylation, gives strong relevance to this pathway during TEN. The study thus highlights that RIPK3 is a potential target for drug development in treating TEN, as RIPK3 inhibitors, as a novel class of kinase inhibitors, are currently under preclinical development (Mandal *et al.*, 2014).

Beyond drug development the study by Kim *et al.* (2015) raises a number of intriguing questions regarding the mechanistic control of RIPK3-dependent cell death in TEN. For example,

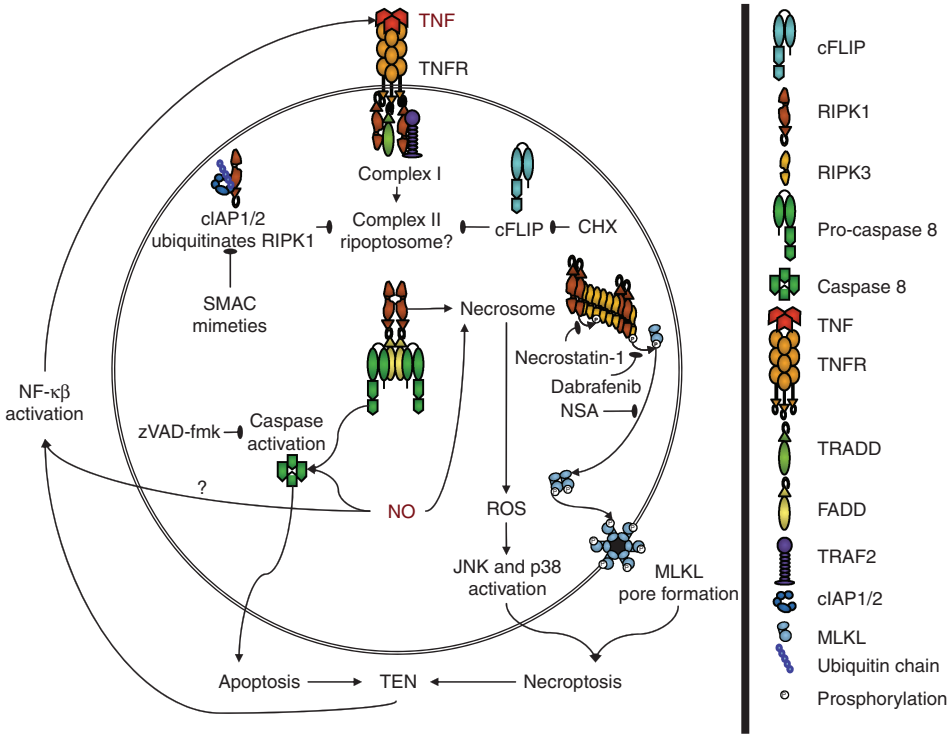

Figure 1. Important effector pathways of cell death in the epidermis and during toxic epidermal necrolysis (TEN). Upon activation of death receptors, such as tumor necrosis factor (TNF-R), a membrane-bound molecular complex (complex I) is formed on the platform of the death domain of TNF-R1. This leads subsequently to the formation of a cytoplasmic complex II (or a Ripoptosome under inhibitor of apoptosis protein (IAP)-depleted conditions). The formation and function of this complex is prevented by cIAP1 and cIAP2. Both IAPs ubiquitinate receptor-interacting kinase 1 (RIPK1). Loss of cIAPs by the use of SMAC mimetics, or loss of cFLIP (a highly CHX sensitive protein), sensitizes keratinocytes to cell death. Abrogation of cell death resistance by loss of these proteins is achieved by several pathways. cFLIP isoforms either partially or completely block complex formation, as well as caspase activation within the TNF complex II. Complex II (or the Ripoptosome) can induce caspase activation (blocked by zVAD-fmk) or can favor formation of the Necrosome, a complex of RIPK1 and RIPK3. The necrosome activates RIPK3 by RIPK1 (blocked by Necrostatin-1) and is followed by mixed lineage kinase-like (MLKL) activation (blocked by Dabrafenib). Activated MLKL lastly oligomerizes and translocates to plasma membranes (blocked by necrosulfonamide (NSA)), ultimately leading to necroptosis. Both apoptosis and necroptosis contribute to TEN as shown by Kim *et al.* (2015). JNK, c-Jun N-terminal kinase.

it would be interesting to understand in greater detail the role of MLKL, and whether RIPK3's sole substrate in keratinocytes is MLKL. The interesting finding that RIPK3 expression impacts on the constant activation of JNK and p38 hints to additional targets of RIPK3 beyond MLKL. Understanding this interplay of necroptosis signaling with inflammatory signal transduction pathways, such as JNK and p38, but also inflammasome-controlled signaling pathways, as recently reported (Gurung *et al.*, 2014; Lawlor *et al.*, 2015), will certainly facilitate understanding how the epidermis is killed during TEN (Figure 1). Finally, the experiments shown in the paper raise the hypothesis that NO not only has the ability to induce TNF expression but that it may impact on NF-κB activity in the skin, which is critical during the progression of TEN (Figures 1 and 2). Because TNF is an important player in TEN pathology (Paquet *et al.*, 1994; Panayotova-Dimitrova *et al.*, 2013; Paradisi *et al.*, 2014), additional analyses of the ability of SNP to induce TNF itself should be useful. Experimentally, this may be addressed by experiments that interfere functionally with TNF signaling (e.g., with TNF-R2-Fc fusion proteins or blocking antibodies) whenever NO is induced in keratinocytes.

Altogether, it is obvious that the inability of TEN keratinocytes to maintain intrinsic cell death resistance is regulated in a complex manner. Might there be alternative intracellular cell death inhibitors other than cFLIP that are needed to inhibit keratinocytes from cell death? Other candidates as cell death regulators may be involved in the rapid epidermal destruction that occurs in patients with TEN (Figure 2). For example, the role of inhibitor of apoptosis proteins (IAPs), potent inhibitors of necroptosis, during TEN is unknown. Loss of cIAPs, in line with the data from Kim *et al.* (2015), unleashes the ability of RIP kinases to kill by necroptosis execution. Thus, cIAP modulation, as exemplified by studies with IAP antagonists by Kim *et al.* (2015), may represent an additional strike against cell death resistance in TEN keratinocytes. Furthermore, as NF-κB is a major protector from TNF-mediated cell death, it will also be interesting to determine whether transcriptional NF-κB activity is lost in the skin of patients with TEN. Unquestionably, further detailed experimental studies

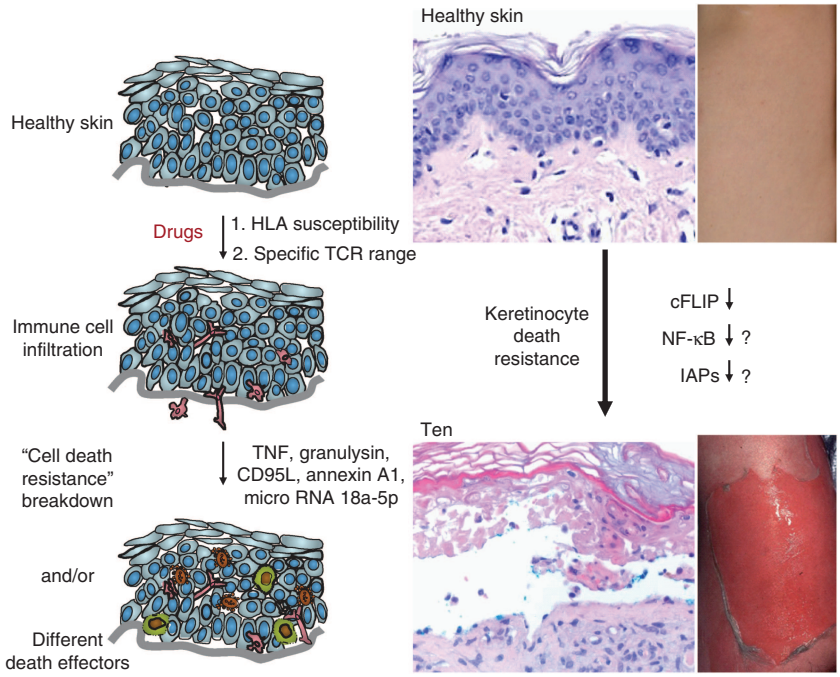

Figure 2. Important roadblocks needed to maintain cell death resistance in keratinocytes *in vivo*. Upon drug intake, in the presence of genetic predisposition and a certain T-cell receptor (TCR) range, a poorly defined immune reaction is activated. Immune cells infiltrate the skin and cell death resistance in epidermal keratinocytes brakes down, resulting in massive cell death and denudation of the epidermis in toxic epidermal necrolysis (TEN) patients. In addition to cFLIP, other important cell death regulators in keratinocytes, such as NF-κB and inhibitor of apoptosis protein (cIAP), may also be regulated during TEN. TNF, tumor necrosis factor.

that investigate the breakdown of cell-intrinsic survival mechanism in TEN keratinocytes will clarify which molecules could be employed for future drug development. Most likely, and as suggested by Kim *et al.* (2015), combination therapies that target not only apoptosis but also necroptotic cell death will achieve better disease control of TEN and could prevent the skin from being "RIPped apart".

CONFLICT OF INTEREST

The authors state no conflict of interest.

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