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# NF-κB and inflammation in genetic disease

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*Abbreviations:*
CFTR, cystic fibrosis transmembrane conductance regulator
EDA-ID, anhidrotic ectodermal dysplasia with immunodeficiency
IκB, IkappaB
IKK, IκB kinase
IL-1, interleukin-1
IL-1-R, interleukin-1-receptor
IL-1RacP, IL-1 receptor accessory protein
iNOS, inducible nitric oxide synthase
IP, incontinentia pigmenti
IRAK, IL-1 receptor associated kinase
LPS, lipopolysaccharide
NEMO, NF-κB essential modulator
NF-κB, nuclear factor-kappa B
NOD, nucleotide-binding oligomerization domain
RIP, receptor interacting protein
TAB, TAK1-binding protein
TAK, TGF-β activated kinase
TIR, Toll/IL-1 receptor domain
TIRAP, TIR domain-containing adaptor protein
TLR, Toll-like receptor
TNF, tumor necrosis factor
TNF-R, TNF-receptor

ABSTRACT

By responding to pro-inflammatory cytokines, such as IL-1β and TNF-α, and controlling itself the expression of numerous mediators of inflammation, NF-κB plays a pivotal role in controlling the proper sequence of events characterizing the inflammation process. Although excessive NF-κB activation is often associated with inflammatory signs in many different tissues, impaired NF-κB activation can also generate inflammation. This is the case in humans suffering from the genetic disease incontinentia pigmenti that exhibit severe skin inflammation. Identifying the molecular basis of this pathology, mutations affecting the gene coding for NEMO, has allowed production of mouse models for investigating the disease. Their characterization supports the view that a very tight positive and negative regulation of the NF-κB signaling pathway is required *in vivo* to ensure not only a fine-tuned response to injury or infection but also to maintain tissue homeostasis.



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TRADD, TNF-receptor-associated death domain protein
TRAF, TNF receptor-associated factor
TRAM, TRIF-related adapter molecule
TRIF, TIR-domain-containing adapter-inducing IFN-β

## 1. Introduction: the NEMO-IKK-2 axis in inflammation

Inflammation represents an highly coordinated set of events that allows tissues to respond to injury or infection. It requires the participation of various cell types expressing and reacting to diverse mediators along a very precise sequence [1,2]. Usually, initiation of the inflammation process is achieved through the production of specific cytokines or chemokines and characterized by leukocytes recruitment to the site of damage [3]. Attracted leukocytes exhibit new adhesion properties and produce several mediators that both increase local blood flow and activate phagocytes to eliminate dead cells and tissue debris. Damage is eventually repaired by proliferation of vascular capillary endothelial cells and fibroblasts. At this step, various molecules with anti-inflammatory properties are synthesized to resolve the process.

A wealth of data suggests that the NF-κB signaling pathway plays a crucial role in the initiation, amplification and resolution of inflammation [4–6]. By responding to pro-inflammatory stimuli such as cytokines IL-1β or TNF-α and controlling the expression of dozens of mediators of inflammation, such as IL-1β, IL-6, IL-8, TNF-α or iNOS, NF-κB is at the center of an amplifying loop that requires subtle fine-tuning in order to get properly activated at the right time then turned off. Accordingly, any dysfunction of the NF-κB activation process is prone to generate chronic inflammation [7] or to favor cancer development, especially when tumorigenesis is associated with an inflammatory environment [8].

Over the years, an important field of research has focused on identifying the various components that participate in NF-κB activation and understanding the molecular events involved [9,10]. NF-κB is a dimeric protein formed by various combinations of members of the Rel/NF-κB family of transcription factors. In most cell types, it is kept inactive in the cytoplasm, associated with the inhibitory molecule IκB. Upon stimulation, IκB is phosphorylated by IKK, the IκB kinase, on two Serine residues located within a conserved motif. This modification triggers the ubiquitination of IκB and its recognition and degradation by the 26S proteasome. The resulting free NF-κB translocates into the nucleus, interacts with a specific DNA motif (5′-GGGPuNNPyPyCC-3′, where Pu is purine, Py is pyrimidine and N is any nucleotide) and activates or represses a large collection of target genes.

As said above, activation of NF-κB is dependent upon phosphorylation of IκBα by IKK. This kinase complex is composed of three distinct subunits: IKK-1/IKK-α, IKK-2/IKK-β and NEMO/IKK-γ [10]. IKK-1 and IKK-2 represent the two structure-related catalytic subunits whereas NEMO represents the regulatory one. NEMO is supposed to provide interfaces for various molecules involved in pathways activating NF-κB.

Among the major signaling pathways targeting IKK are the ones used by the two major pro-inflammatory cytokines, IL-1β and TNF-α and the ones used to detect various bacterial or viral products (TLR pathways). An intense area of investigation has recently allowed a better understanding of how these pathways act on IKK [11] (Fig. 1). In the case of the IL-1β pathway, it has been shown that following interaction with the complex formed by IL-1R and IL-1RacP proteins, IL-1β induces the recruitment of Myd88, through TIR/TIR homotypic interaction. Then, IRAK-1 and its adaptor Tollip interact with Myd88 and gets hyperphosphorylated by IRAK-4. This event induces the release of IRAK-1, its interaction with TRAF6, and the activation of the TAK-1/TAB-1/TAB-2/3 kinase complex. TAK-1 is supposed to activate IKK through IKK-1/IKK-2 phosphorylation. TLR4, which is the sensor for Gram-bacteria-derived LPS, uses the same Myd88-dependent mechanism of IKK activation as IL-1R but can also activate NF-κB through a Myd88-independent pathway involving TRAM and TRIF adaptor molecules. In the TNF-α pathway, binding of TNFα to its receptor, TNF-R1, induces recruitment of adaptor TRADD and provides a platform for RIP and TRAF2 binding. Each of them plays a specific role in IKK activation, RIP being able to directly interact with NEMO and to recruit IKK, whereas TRAF2 activates the TAK1 complex that in turn induces IKK-1/IKK-2 phosphorylation. Remarkably, it has been shown that TAK1/IKK activation by TRAFs involves a poly-ubiquitination step that does not result in protein degradation [12]. Indeed, whereas most ubiquitination events that tag substrates for proteasome degradation require internal lysine 48 (K48) of Ubiquitin to synthesize K48-linked poly-ubiquitin chains, TAK1/IKK activation requires internal lysine 63 (K63) of ubiquitin to synthesize K63-linked poly-ubiquitin chains. This step has been shown to be under the negative control of K63-specific deubiquitinase CYLD [13].

In all the pathways described above, it has been demonstrated that NEMO and IKK-2 subunits of IKK are required to activate NF-κB, establishing the NEMO/IKK-2 couple as a key component of the inflammation process. In contrast, IKK-1 appears to play a less important role in inflammation, since cells devoid of this protein are still able to efficiently respond to IL-1 or TNF. It has been shown recently that IKK-1 participates instead in p52/relB activation, through the so-called non-canonical pathway of NF-κB activation that is critically required for lymphoid organogenesis [11].

Although inflammation is most often caused by increased activity of NF-κB, several *in vivo* situations have been identified in which impaired NF-κB activation is the primary trigger of inflammation, demonstrating the complex role of this

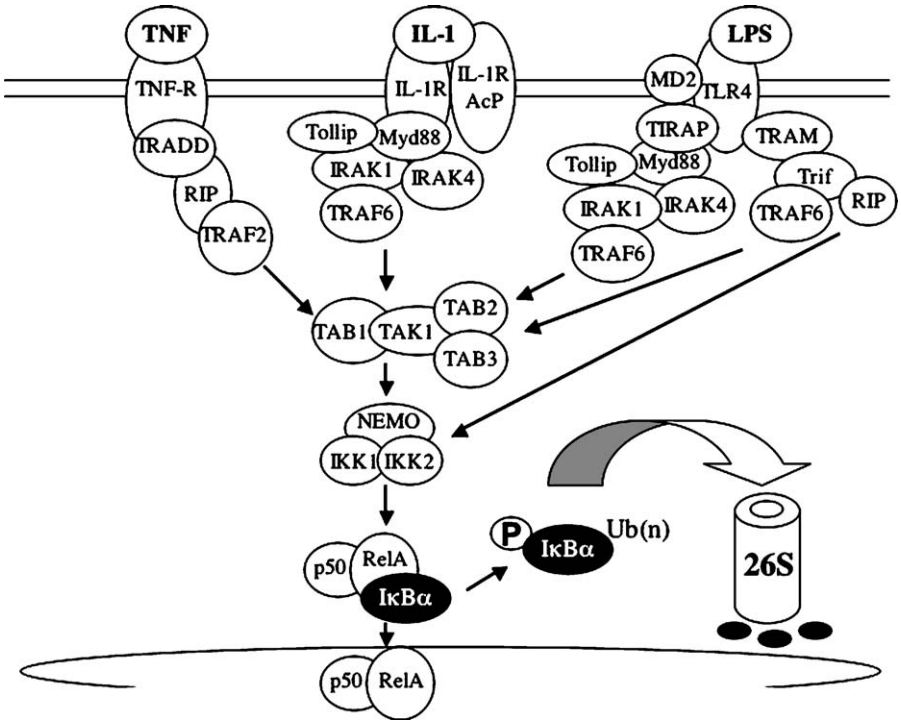


**Fig. 1 – NF-κB activation pathways in inflammation. The major molecules involved in IL-1β, TNF-α and TLR4 signaling pathways and participating in NF-κB activation are presented (see text for more detail). Black labeled proteins are negative regulators. Ub (n): poly-ubiquitin chain; P: phosphate; 26S: proteasome complex.**

transcription factor. One of the most striking example of inflammation resulting from defective NF-κB activation is provided by the first genetic disease that has been associated with NF-κB dysfunction, incontinentia pigmenti.

## 2. Incontinentia pigmenti: major features of the disease

Incontinentia pigmenti (IP) is a severe X-linked genodermatosis (incidence of between 1/10,000 and 1/100,000) exhibiting early lethality in males [14,15]. In affected females the disorder is highly variable in presentation but is always associated with skin problems that start neonatally. Typically, IP dermatosis begins within 2 weeks after birth with blisters and a severe inflammatory response, accompanied by a massive eosinophilic granulocytes infiltration into the epidermis (Vesicular Stage). Then, verrucous hyperkeratotic lesions develop (Verrucous Stage) and disappear over time, leaving behind areas of hyperpigmentation due to melanin accumulation (Hyperpigmented Stage). These areas, that follow the lines of Blaschko, generally disappear by the second decade (Atrophic Stage) but adults may still show areas of dermal scarring with lack of hair follicles. Interestingly, very rare patients can reinitiate a similar sequence of events during their teenage years or adulthood (see below).

In addition to manifestations at the epidermis level, IP patients can also exhibit ophtalmologic (abnormalities of the developing retinal vessels), odontological (missing or deformed teeth) and, in rare cases, neurological (convulsive disorders, motor or mental retardation) problems.

A very characteristic feature of IP pathology is the extensive X-inactivation skewing that is observed in peripheral blood cells of most female patients. This skewing reflects an efficient mechanism of counter-selection affecting cells expressing the mutated X chromosome. As will be explained below, such extensive skewing does not occur in the antenatal epidermis, causing IP dermatosis at birth.

The gene responsible for IP was originally mapped to an interval of about 2 Mb at Xq28, a region containing *NEMO*. This localization as well as the high sensitivity to apoptosis of cells derived from IP patients suggested that *NEMO* mutations might be responsible for this pathology. PCR and Southern blot analysis of the *NEMO* locus have confirmed this hypothesis. Remarkably, 85% of the IP families analyzed so far carry the same complex rearrangement of *NEMO* [16]. This rearrangement involves excision of the region between two MER67B repeated sequences located upstream of exon 4 and downstream of exon 10, respectively, therefore resulting in the synthesis of a truncated 133 amino acid NEMO molecule (corresponding to exons 1–3), which is devoid of activity. Accordingly, IP cells carrying this rearrangement do not show any degradation of IκB molecules when stimulated, resulting in a complete lack of NF-κB activation and an exquisite sensitivity to TNF-α-induced apoptosis. Most of the remaining *NEMO* mutations causing IP are nonsense or frameshift mutations generating more or less severe truncations of NEMO protein [16–20] (Fig. 2). Substitutions of a nucleotide in the splice donor domain at the 3′ ends of exons 3 and 4 have also been reported. Finally, several missense mutations of *NEMO* have been identified [16,19]. It remains to be understood how exactly they affect NF-κB activation and cause IP. One of

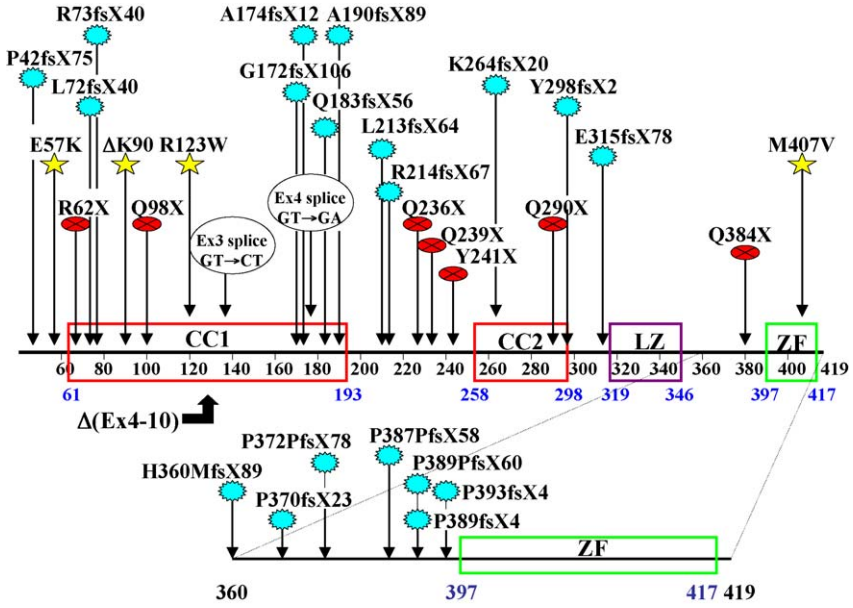


**Fig. 2 – *NEMO* mutations in IP patients. The various mutations reported in the litterature [16–20] and their location within NEMO is presented. The recurrent *NEMO* deletion observed in 85% of IP patients (Δ(Ex4-10)) is indicated by a thick arrow. The other mutations are nonsense mutations (red label) and fs mutations (blue label), which result in more or less severe truncations of NEMO, or missense mutations (yellow label). The last category of mutations, located in splice donors 3′ end domains of exons 3 and 4 (open circles), are responsible for aberrant splicing of *NEMO* transcript. The number after X indicates the number of irrelevant amino acid added in fs mutants. CC1: coiled-coil 1; CC2: coiled-coil 2; LZ: leucine zipper; ZF: zinc finger; Ex: exon; fs: frameshift.**

them, ΔK90 mutation, appears to be associated with a specific reduced interaction with IKK-2 subunit [19].

## 3. Mouse models of incontinentia pigmenti

As said above, IP dermatosis is a very complex process combining inflammation, keratinocyte hyperproliferation and apoptosis. Several mouse models have been developped in order to get more insights into the molecular and cellular events characterizing it.

### 3.1. *Nemo* K.O.

*Nemo* K.O. mice have been engineered by several groups and their phenotype appears very similar to the one of IP patients [21–23]. Male mice die very early during embryogenesis, at embryonic day 12 (E12), from massive liver apoptosis. A similar phenotype has also been observed for *RelA* and *Ikk-2* K.O. mice but death occurs a bit later, at E15.5 and E14.5, respectively [24,25]. Importantly, liver apoptosis has been demonstrated to be triggered by TNF-α. Indeed, double K.O. mice (*RelA*(−/−)/*tnf-α*(−/−) [26] and *ikk-2*(−/−)/*tnf-r1*(−/−) [27]) can survive till birth but die later on from bacterial infections. It remains to be determined whether liver apoptosis is also responsible for male lethality in IP.

In contrast to males, *nemo* K.O. female mice develop normally but, soon after birth, exhibit a transient dermatosis, characterized by patchy skin lesions with massive granulocyte infiltration, hyperproliferation and increased apoptosis of keratinocytes, reminiscent of what is observed in human IP. Hyperpigmentation, a hallmark of IP due to the presence in the dermis of phagocytes containing melanosome complexes, is also observed in skin sections from *nemo* +/− mice. In addition, the extensive X-inactivation skewing in blood leukocytes, which is a major feature of IP patient, is also operant in *nemo* K.O. mice [23] and reflects the essential role that NEMO plays in lymphocyte survival at the periphery. The only difference that is observed between IP pathology and *nemo* K.O. is the high level of mortality that occurs in female mice within 6–10 days after birth, something that is never observed in humans.

Like in IP patients, the phenotype of *nemo* +/− mice is difficult to assess, due to the mosaic nature of their skin. For some unknown reasons, the X-inactivation skewing that is observed in many tissues is less complete in the skin and selective elimination of cells bearing a mutated X chromosome only starts at birth. IP dermatosis is supposed to result from the co-existence at birth of cells carrying a normal copy of *NEMO* and those carrying a defective one and from a complex interplay occuring between them.

In order to eliminate the mosaicism status of *nemo* −/+ mice that complicates the analysis two other mouse models have been engineered. In both cases, a selective inactivation in the epidermis of *ikk-2* or *nemo* has been carried out [28,29].

### 3.2. *Ikk-2 and nemo K.O. in the epidermis*

As said above, IKK-2 and NEMO operate together inside the IKK complex and respond to identical stimuli. Since *ikk-2* is not X-linked its inactivation can offer an alternative to the inactivation of *nemo*. Nevertheless, an *ikk-2* K.O. in the

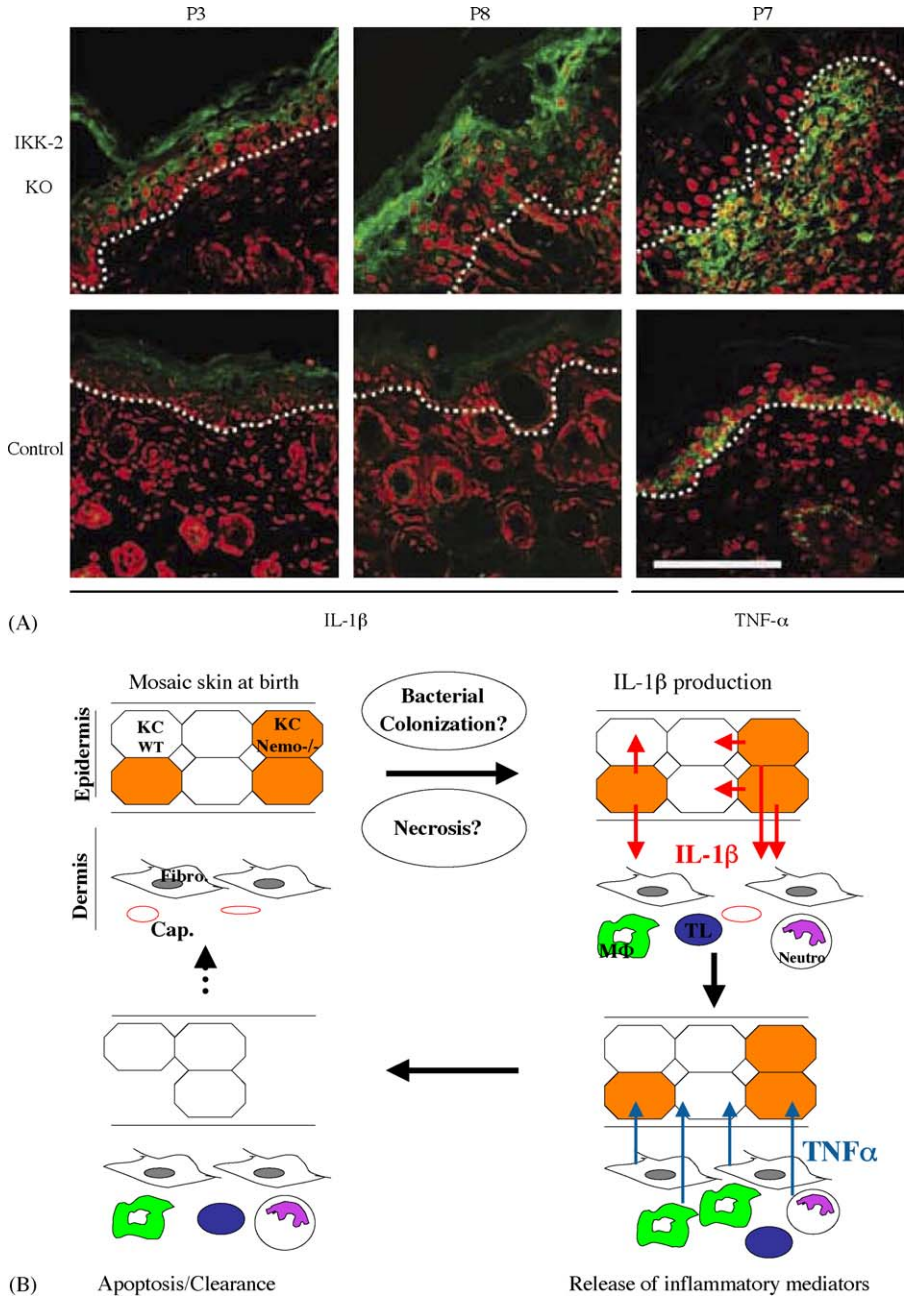


**Fig. 3 – Molecular and cellular events associated with IP dermatosis. (A) Immunohistochemistry analysis of *IKK-2* skin K.O. mice. IL-1β and TNF-α expression is shown at post-natal days 3 (P3), 7 (P7) and 8 (P8) [28]. The dotted line indicates the epidermis/dermis boundary. Ct: wild-type mouse, mt: *IKK-2* skin K.O. mouse. (B) A putative model of IP dermatosis. See text for more details. KC: keratinocyte; Fibro: fibroblast; Cap.: capillary vessel; MΦ; macrophage; TL: T lymphocyte; Neutro: neutrophil granulocyte.**

whole organism generates embryonic lethality at E14.5. In order to avoid such problem a selective *ikk-2* ablation in the epidermis has been carried out [28]. In these animals, epidermis development proceeds normally till birth. At P4–P5 their skin starts becoming hard and inflexible and by day P7–P8 a widespread scaling is observed that preceeds death. At P7, mice exhibit a thickened epidermis and this hyperproliferation is accompanied with cell infiltration into the dermis and severe inflammation. Importantly, purified keratinocytes do not show any hyperproliferation on their own indicating that the defect is not cell-autonomous.

The inflammation process that affects the skin of epidermis *ikk-2* K.O. mice shares many similarities with usual inflammatory reactions, including increased numbers of macrophages, granulocytes and CD4-positive T cells in the dermis, but also presents interesting features concerning the putative mediators involved. For instance, IL-1β expression is detected early in the epidermis, at P2/P3, which means before the onset of the pathology (Fig. 3A). This is a quite unexpected

observation since IL-1β is supposed to be under NF-κB control and the compartment into which it is over-expressed exhibits impaired NF-κB signaling. Worth noting, this IL1-β over-expression is not observed with purified keratinocytes, indicating that it is dependent on the surrounding environment. Moreover, a strong production of TNF-α is detected in the dermis, at later time (P4/P7) (Fig. 3A). The crucial involvement of this cytokine in the development of the pathology appears essential since crossing *ikk-2* skin K.O. mice with *tnf-r1* K.O. mice eliminates all its signs [28].

More recently, a specific invalidation of *nemo* in the epidermis of mice has also been reported [29]. The phenotype is quite similar to the one resulting from inactivating *ikk-2* but one major difference is noteworthy. In addition to the severe inflammation and keratinocyte hyperproliferation that affect the epidermis a majority of cells exhibits apoptosis. This is due to complete abolition of the NF-κB signaling pathway resulting from *NEMO* inactivation, something that is not fully achieved upon *ikk-2* inactivation.

From the analysis of the mouse models, an hypothetical sequence of events can be proposed to occur during IP dermatosis (Fig. 3B). As said above, epidermis of IP patients at birth is a mosaic of cells, at least keratinocytes, expressing either wild type or mutated NEMO protein. At this stage, cells expressing mutated NEMO and exhibiting a defect in NF-κB activation start to produce large quantities of IL-1β. This well known ''alert cytokine'' of the epidermis is likely to act, with possibly other molecules, on neighbouring cells. As a response, TNF-α is synthesized and acts back on *NEMO* mutated cells, inducing their apoptosis. Sensitivity of *NEMO* mutated cells to apoptosis is therefore an important component of the IP disease since it directly participates in clearance of the lesions.

Many crucial questions remain regarding the initiation and evolution of IP dermatosis. The most puzzling one concerns the true nature of the triggering agent. Although IL-1β production likely represents an early event of the disease it remains unclear why *nemo* mutated keratinocytes start to over-express this cytokine following birth. Two hypotheses can be proposed to explain the cause of this initial defect. The first one involves skin colonization by bacteria. Upon tissue settlement by commensal bacteria such as *Corynebacteria* ssp., *Brevibacterium* ssp., *Staphyloccocus epidermidis* or *Acinetobacter* ssp., a TLR-derived pathway, dependent upon NF-κB, may be turned on to keep skin/bacteria symbiosis under control. This pathway may involve the production of proteins of the defensin family. In case of defective NF-κB activation this mechanism would be blunted and trigger inflammation. The second hypothesis relies upon an undefined cell defect that would result in their death through necrosis [22]. It has been shown that necrotic cells are indeed able to mount an inflammation response, a property that is not shared by apoptotic cells.

Understanding the signal triggering IP dermatosis may benefit from the observation that a similar set of events can also re-occur both in IP patients during their aldulthood [30,31] or when *nemo* or *ikk2* inactivation is carried out in adult mice by using an inducibly expressed cre protein [29,32]. This demonstrates that the origin of IP signs in the epidermis is not due to some developmental skin defect but rather that NF-κB has a critical role in skin homeostasis. Interestingly, in most cases of recurrence in humans an infectious episode has been reported. This indicates that some *NEMO* mutated cells can persist a long time in the epidermis and can be ''reactivated'' upon infection.

## 4. Anhidrotic ectodermal dysplasia with immunodeficiency: *NEMO* mutations without associated inflammation

Another layer of complexity, related to what is described above regarding the NF-κB/inflammation relationship and based on the phenotype of another genetic disease caused by *NEMO* mutations [33–35], must be mentionned at this point. This pathology, anhidrotic ectodermal dysplasia with immunodeficiency (EDA-ID) is characterized by a severe X-linked immunodeficiency associated with an impaired development of skin adnexes (hair, sweat glands, teeth). Describing in detail the phenotype of EDA-ID is beyond the scope of this review but one of its major features desserves comments. In contrast to IP pathology, the affected patients are males whereas females exibit in only rare cases very mild signs of IP. This is due to the nature of the *NEMO* mutations that are not associated with abolished NF-κB activation but allows some residual activation, explaining the survival of males. Very remarkably, although EDA-ID patients express the *NEMO* mutations in 100% of their cells, among them the ones forming the epidermis, no inflammation process occurs. This is unlikely to be due to some NF-κB activation remaining since *ikk-2* mice described above also exhibit residual activation but develop skin inflammation. Most likely, this supports the notion that such process is highly dependent upon the co-existence in tissues of wild type and mutated cells and the complex interplay occuring between them.

## 5. Conclusion

Although NF-κB has been defined early on as a major player in the inflammation process, recent data, among them the ones gathered from the analysis of IP pathology, have recently added further complexity to its function. Depending on the location and the time at which NF-κB activation is impaired different outcomes may result. In particular, through the interplay with other cells, NF-κB defective cells can trigger inflammation, at least in the epidermis. This observation may have significant relevance for other epithelial-related diseases. In Crohn disease, for instance, it is still unclear whether it is an excess or a lack of NF-κB, the major target of mutated *NOD2*, which is responsible for the inflammation process taking place in the gut [36]. In another case, cystic fibrosis, it has been shown that chronic lung infection with *Pseudomonas aeruginosa* constitutes the most severe manifestation of the disease and is due to defects in early clearance of the microbe. This clearance involves epithelial cell ingestion of bacteria, rapid activation of NF-κB and cellular desquamation within minutes of *P. aeruginosa* infection, processes that are deficient in cells with mutant alleles of *CFTR* [37]. Since it has also been proposed that positive NF-κB-driven inflammation may represent an important component of the disease [38] a dual

role of this transcription factor may be at work. All these different facets of NF-κB will have to be taken into account when considering the use of NF-κB inhibitors to treat inflammatory diseases.

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