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Keratinocyte expression of A20/TNFAIP3 controls skin inflammation associated with atopic dermatitis and psoriasis

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Short title: A20 and atopic dermatitis/psoriasis

ABSTRACT

Keratinocytes are key players in chronic inflammatory skin diseases. A20 regulates NF- κ B-dependent expression of pro-inflammatory genes and cell death, but the impact of its expression in keratinocytes on systemic inflammation and skin disorders has not been determined. Comparative transcriptomic analysis of micro-dissected epidermis showed that A20 is downregulated in involved epidermis, but not in dermis, of psoriasis (Pso) and atopic dermatitis (AD) patients suggesting that loss of A20 expression in keratinocytes increases the vulnerability for Pso/AD induction. We have previously shown that epidermis-specific A20 knock-out mice (A20^{EKO}) develop mild epidermal hyperplasia, but no macroscopic skin inflammation. We now show that various cytokines and chemokines are upregulated in A20^{EKO} mouse skin. A20^{EKO} mice also display systemic pro-inflammatory changes, even in the absence of skin immune cell infiltration, and an exacerbated disease severity upon induction of experimental Pso, AD or skin barrier disruption. Keratinocytes showed increased pro-inflammatory gene expression in the absence of A20 in unstimulated and IL-17A-stimulated conditions, in part resulting from uncontrolled MyD88-dependent signaling. Our findings indicate that absence of A20 in keratinocytes leads to systemic inflammation at homeostatic conditions and is sufficient to exacerbate inflammatory skin disorders associated with different immune profiles by increasing cytokine and chemokine expression.

Key words

A20/TNFAIP3/atopic dermatitis/psoriasis/keratinocytes

INTRODUCTION

Psoriasis (Pso) and Atopic Dermatitis (AD) are the two most common inflammatory skin disorders, with different etiology. However, certain specific subtypes of AD tend to have overlapping cytokine profiles and histopathology with psoriasis (Guttman-Yassky and Krueger, 2017). Psoriasis, affecting 2-3% of the global population, is mostly characterized by keratinocyte hyper-proliferation, erythema and ichthyosis (Boehncke and Schon, 2015, Greb et al., 2016). Initiation and maintenance of psoriasis depend on IL-12- and IL-23-producing dendritic cells (DC) (Ganguly et al., 2009, Lande et al., 2007) and T helper (Th) lymphocytes, especially Th17 cells (McGeachy et al., 2009). AD or atopic eczema is a more prevalent chronic skin disease (up to 7% in adults and 25% in children), characterized by impaired skin barrier function, dry pruritic lesions, eosinophilia and elevated IgE production (Brunner et al., 2017, Weidinger and Novak, 2016). While often considered as distinct entities, based on genome-wide comparative analysis (Baurecht et al., 2015, Quaranta et al., 2014) and on displaying specific features in pathogenesis Pso and AD share some important features. Indeed, in addition to keratinocyte hyper-proliferation/epidermal thickening, in chronic phases Th17 and Th22 responses are present in both diseases (Eyerich et al., 2017). Moreover, some patients might simultaneously develop Pso and AD (Eyerich et al., 2011). IFN γ , by decreasing skin long chain fatty acid ceramides, seems to be involved in the development of both diseases (Tawada et al., 2014). A neutrophilic signature is also associated with both diseases (Choy et al., 2012). Finally, deficiency of the TNF superfamily molecule TWEAK (TNFSF12) in mice results in reduced skin inflammation in mouse models of HDM-induced AD and imiquimod (IMQ)-induced psoriasis, respectively due to defective maintenance of AD-specific T helper type 2 (Th2) and psoriasis-specific Th17 cells in the skin (Sidler et al., 2017). So one may hypothesize that the pathogenesis of both Pso and AD

can be driven by common defects in controlling mechanisms in the skin providing anti-inflammatory feedback to prevent chronic skin inflammation.

The transcription factor nuclear factor κ B (NF- κ B) plays a central role in maintaining homeostasis and innate immune regulation in the skin. Numerous studies in mice have shown that both impairment and over-activation of NF- κ B signaling in keratinocytes triggers chronic inflammation (Kumari and Pasparakis, 2017, Pasparakis, 2012). In order to ensure control on NF- κ B signaling, several of its target genes act as negative regulators, such as tumor necrosis factor α -induced protein 3 (TNFAIP3), also named A20. Indeed, A20-deficient mice die prematurely due to multi-organ inflammation and cachexia, as a result of excessive Toll-like receptor (TLR) signaling (Lee et al., 2000, Turer et al., 2008). A20 contains an N-terminal ovarian tumor (OTU) domain and seven zinc-finger (ZF) domains at the C-terminal part (Wertz et al., 2004). It holds both deubiquitinase and ubiquitin ligase activity, however the physiological relevance of its catalytic activities is currently not clear (De et al., 2014, Draber et al., 2015, Lu et al., 2013, Skaug et al., 2011, Tokunaga et al., 2012, Verhelst et al., 2012). Loss of A20 has also been shown to either enhance pro-survival signaling via upregulation of anti-apoptotic proteins or sensitize to cell death, possibly depending on the cellular context (Lee et al., 2000, Onizawa et al., 2015, Opiari et al., 1992, Tavares et al., 2010, Vereecke et al., 2010). Keratinocyte-specific deletion of A20 results in epidermal hyper-proliferation and ectodermal defects, but skin infiltration of immune cells has not been observed (Lippens et al., 2011). GWAS identified TNFAIP3/A20 as a susceptibility locus for psoriasis (Haase et al., 2015, Indhumathi et al., 2015, Li et al., 2014, Nair et al., 2009, Zhang et al., 2015) and its role as a negative regulator in Th2-associated lung inflammation, in particular through its expression by dendritic cells, has been well characterized (Schuijs et al., 2015, Vroman et al., 2017). On the other hand, some cases of A20 haploinsufficiency yield distinct

autoinflammatory and/or autoimmune skin pathology, possibly with skin ulcerations and pustular abscesses (Kadowaki et al., 2017, Takagi et al., 2017, Zhou et al., 2016). In addition, TNFAIP3/A20 single nucleotide polymorphisms (SNPs) correlate with response to TNF blockade in psoriasis patients (Tejasvi et al., 2012). All these data demonstrate a clinically relevant association between mutations in the A20 gene and skin disease.

Seeking for additional commonalities between Pso and AD, we found that, in human, A20 epidermal expression is strongly decreased in both diseases. Therefore, we study the effect of keratinocyte-specific A20 deletion in experimental mouse models relevant to the human pathology (Kabashima and Nomura, 2017). Our findings show that A20 expression in keratinocytes is required to establish a threshold preventing systemic inflammation under homeostatic conditions and to limit inflammatory responses in skin pathologies with distinct immunological programs.

RESULTS

A20/TNFAIP3 is downregulated in human atopic dermatitis and psoriasis

In order to determine common pathways leading to the two most frequent inflammatory skin diseases, Pso and AD, we performed comparative transcriptomic analysis of laser micro-dissected affected epidermis from patients with Pso and AD, as well as healthy epidermis from individuals with no skin diseases. Among transcripts significantly, upregulated in epidermis (at least two-fold), 369 were common to both diseases, while 359 were common among the significantly downregulated transcripts (**Figure 1a**), suggesting that both skin pathologies share common underlying molecular mechanisms in keratinocytes. The common upregulated transcripts included known genes with affected expression in Pso and/or AD, such as S100A family members, KRT16, KRT6A, IL36G, LCE3 family, CNFN, TNC and others. The upregulated genes were enriched for pathways related to the immune system and defense processes and pathways controlling keratinocyte differentiation and keratinization. The most downregulated pathways among common transcripts in Pso and AD were diverse and included epithelial and epidermal development, intracellular signal transduction, negative regulation of response to stimulus, negative regulation of cell communication and regulation of cell death (**Figure 1b**). We found A20/TNFAIP3 among the downregulated genes in involved epidermis of both Pso and AD patients (**Figure 1a**). A20/TNFAIP3 expression was significantly decreased in uninvolved epidermis in Pso patients compared to epidermis from healthy skin, and further decreased in involved epidermis versus uninvolved epidermis in Pso patients. In AD, A20/TNFAIP3 expression was significantly lower in involved epidermis, as compared to epidermis from healthy skin. By contrast, no significant difference in A20/TNFAIP3 expression was observed between healthy dermis and both uninvolved and involved dermis in Pso and AD (**Figure 1c**). These results suggest that downregulation of

A20/TNFAIP3 in involved epidermis in Pso and AD is likely due to a decrease of its expression by keratinocytes, associated with pathogenesis of these diseases.

Keratinocyte-specific A20/TNFAIP3 deficiency in mouse leads to basal systemic inflammation

Mice with keratinocyte-specific A20 deletion ($A20^{EKO}$) do not spontaneously develop macroscopic or histological signs of skin inflammation, as published previously (Lippens et al., 2011). Indeed, no infiltrating immune cells were observed in hematoxylin and eosin-stained sections of skin of $A20^{EKO}$ mice (**Figure S1a**). However, analysis of inflammation-associated gene expression in the skin of these mice by RT-qPCR, revealed a significant increases in TNF, Ccl20, Cxcl1, Lcn2, IL-22, IL-23a and TSLP expression compared to $A20^{Fl/Fl}$ mice (**Figure S1b**). In addition, keratinocyte-specific A20-deficiency led to systemic inflammatory effects. Indeed, $A20^{EKO}$ mice displayed splenomegaly and a lower body weight than their $A20^{Fl/Fl}$ littermates even when housed in SPF conditions (**Figure S1c**). In line, the percentage of splenic neutrophils and Ly6C^{hi} monocytes was increased in $A20^{EKO}$ animals (**Figure S1d**). Although the percentage of splenic CD4⁺ and CD8⁺ T lymphocytes was similar in both genotypes, the percentage of naïve T cells was decreased, while the frequency of effector T cells was increased in $A20^{EKO}$ mice (**Figure S1e**). While a slight increase in frequency of splenic cDC was observed in $A20^{EKO}$ animals, frequency of monocytes, pDC, NK, NKT and $\gamma\delta$ T cells was not altered (**Figure S1f**). Because K14-driven A20 deletion in the thymic epithelium cannot be excluded (Kuraguchi et al., 2006), we analyzed thymic cell populations in $A20^{EKO}$ and $A20^{Fl/Fl}$ mice and found that thymic composition was comparable for both genotypes with only a marginal difference in the proportion of CD25⁺CD5^{hi} CD4⁺T cells (**Figure S2**). These results indicate that A20 deficiency in keratinocytes results in an

inflammatory gene signature in the skin and systemic pro-inflammatory changes in immune cell populations.

Keratinocyte-specific A20/TNFAIP3 deficiency exacerbates experimental psoriasis.

We induced psoriasis-like inflammation by epicutaneous application of imiquimod (IMQ) (van der Fits et al., 2009) in A20^{EKO} and A20^{FL/FL} mice. Upon IMQ treatment, A20^{EKO} mice displayed an increased disease severity score at the macroscopic level (**Figure 2a, b**) but no sign of psoriatic arthritis (not shown). Despite increased erythema and scaling, epidermal thickening was similar in A20^{EKO} and A20^{FL/FL} mice (**Figure 2c**). In contrast, A20^{EKO} mice showed significantly increased skin infiltration of neutrophils, including their epidermal accumulation, similar to Munro micro-abscesses in human psoriatic epidermis (**Figure 2d**). In keeping with morphological signs of inflammation, skin expression of psoriasis-associated genes involved in skin barrier and stress (Krt16, Lce3c, Lcn2, S100a8) as well as in inflammatory responses (IL-23a, IL-22, Cxcl1) was significantly increased in A20^{EKO} mice upon treatment with IMQ as compared to A20^{FL/FL} mice (**Figure 2e**). Furthermore, the frequency of cDC and macrophages in skin draining lymph nodes, harboring activated cells, was increased in A20^{EKO} mice, while the percentage of Langerhans cells, IL-17-producing CD4⁺ T and $\gamma\delta$ T cells was unchanged, $\alpha\beta$ CD4⁺ T cells were decreased. (**Figure 2f, g**). In contrast A20^{TKO} mice, generated by crossing A20^{FL/FL} and CD4-Cre mice reacted to IMQ in a similar way as A20^{FL/FL} control mice (Figure S3). Because IL-23a expression was increased in skin from A20^{EKO} compared to A20^{FL/FL} mice and IL-23 injection also represents a relevant experimental model for human psoriasis (Kabashima and Nomura, 2017), we intradermally injected IL-23 and observed that A20^{EKO} mice developed exacerbated skin pathology compared to A20^{FL/FL} mice (**Figure S4**). Taken together, these results indicate that A20 deficiency in keratinocytes sensitizes to psoriasis-like inflammation in mice.

Keratinocyte-specific A20/TNFAIP3 deficiency exacerbates experimental atopic dermatitis.

We next evaluated the contribution of A20/TNFAIP3 to the regulation of AD induced upon repeated epicutaneous sensitization with ovalbumin (OVA) in the absence of adjuvant (Spergel et al., 1998). Antigen-sensitized A20^{EKO} mice displayed increased epidermal thickness compared to A20^{F1/F1} animals (**Figure 3a, b**). PBS-treated A20^{EKO} mice also showed, to some extent, epidermal thickening, suggesting that, in addition to the classical allergic response, an increased response to the mechanical stress caused by the repeated sensitization procedure was taking place (**Figure 3a, b**). Surprisingly, the strong eosinophilia associated to Th2 responses induced upon sensitization of A20^{F1/F1} animals was significantly abolished in A20^{EKO} mice (**Fig 3c**). The humoral response, a characteristic of AD, was exacerbated in A20^{EKO} mice with increased concentrations of antigen-specific Ig (**Figure 3d**), associated to both Th2 (IgE and IgG₁) and Th1 immune response (IgG_{2a} and IgG_{2c}). Accordingly, while the percentage of total CD4⁺ T cells in draining lymph nodes remained unchanged, the percentage of CD4⁺ T cells expressing IL-5, IL-13, IL-10 or Foxp3 was increased in both antigen- and vehicle-treated A20^{EKO} mice as compared to A20^{F1/F1} animals (**Figure 3e**). IL-17A⁺ CD4⁺ cells were significantly increased in A20^{EKO} animals compared to A20^{F1/F1} mice only upon OVA sensitization, while an increase in IL-4⁺ CD4⁺ cells only occurred in PBS-treated A20^{EKO} animals (**Figure 3e**). No significant alteration was observed for IFN γ -producing CD4⁺ cells (**Figure 3e**). Taken together, these results show that keratinocyte-specific A20/TNFAIP3 deficiency not only exacerbates the antigen-specific immune response associated to AD, but also the response associated to mechanical skin damage that takes place in vehicle-treated animals.

Skin barrier disruption in A20^{EKO} mice results in inflammation resembling interface dermatitis

Given the observed sensitivity to mechanical stress in the PBS-treated animals in the above-mentioned OVA-based AD model, we next applied an established model of skin barrier disruption, by challenging the skin of A20^{EKO} and A20^{Fl/Fl} mice by repetitive acetone wiping twice a day for five consecutive days. We used both non-treated and saline-treated mice as controls. Histological sections of back skin revealed that A20^{EKO} mice, in contrast to A20^{Fl/Fl} mice, developed epidermal hyperplasia and infiltration of immune cells at the dermo-epidermal junction upon topical treatment with acetone (**Figure 4a, b**). These histological findings are characteristic of interface dermatitis, which is also observed in Keratin5-*IKK2* transgenic mice, a model for NF- κ B overactivation (Page et al., 2010). In addition, we observed increased chemokine (Ccl20, Cxcl1), allergy-related TSLP and stress molecule S100a8 mRNA levels in the skin of acetone-treated A20^{EKO} versus A20^{Fl/Fl} mice, while TNF was not further increased upon acetone treatment (**Figure 4c**). Taken together, these results suggest that physical abrasion is sufficient to induce skin inflammation and innate responses when A20 is deleted in keratinocytes, demonstrating its general anti-inflammatory role in the skin.

A20/TNFAIP3 deficiency increases pro-inflammatory gene expression in mouse keratinocytes

To further study the intrinsic regulation of the keratinocyte inflammatory responses by A20, we isolated keratinocytes from the epidermis of A20^{EKO} mice and A20^{Fl/Fl} littermates. Despite the fact that A20 deficiency sensitizes to TNF-induced cell death in other cell types (Lee et al., 2000, Vereecke et al., 2010), no difference in proliferation and survival was observed in keratinocytes in the absence of A20 whether at resting state or upon TNF stimulation (**Figure**

S5). However, RT-qPCR analysis revealed that basal expression of inflammation-associated genes is higher in the absence of A20, including target genes of IL-17A (Ccl20, Cxcl1, Csf3) (**Figure 5a**). While we confirmed that A20 restricted IL-17A-dependent NF- κ B and MAPK activation in MEF cells (**Figure S6a**) (Garg et al., 2013), we were not able to observe differences in I κ B- α degradation and JNK-, Erk- and p38-phosphorylation after stimulation with IL-17A of keratinocytes isolated from A20^{EKO} mice and A20^{Fl/Fl} littermates (**Figure S6b**). In line, two-way ANOVA analysis of IL-17A-induced chemokine expression (Ccl20, Cxcl1, Csf3) in keratinocytes revealed a non-significant interaction between the effects of A20 expression and IL-17A stimulation (**Figure 5b**). In addition, loss of A20 did not influence IL17A-induced expression of the transcriptional co-activator I κ B- ζ (nfkbiz) (**Figure 5c**), another IL-17A responsive gene important in Pso (Johansen, 2016). Taken together, these observations ~~tend to argue against~~ demonstrate that A20 is ~~not being~~ a negative regulator of IL-17A signaling in keratinocytes and raises the possibility that a different signaling pathway being sensitized in the absence of A20 might cross-talk with IL17A-induced gene expression. As MyD88-dependent signals underlie the lethal phenotype in full A20 KO mice (Turer et al., 2008), we knocked-down MyD88 in A20^{EKO} and A20^{Fl/Fl} keratinocytes and observed a reduced expression of chemokines (Ccl20, Cxcl1, Csf3) both in unstimulated and IL-17A-stimulated conditions (**Figure 5d**), showing that increased sensitivity upon loss of A20 in keratinocytes is, at least in part, dependent on MyD88 signaling. In contrast to MyD88 knockdown, knockdown of TNFR1 did not result in a similar reduction of Cxcl1 and Csf3 expression while Ccl20 levels were increased (**Figure S7**). We were unable to explain the latter observation.. Inactivating Nfkbiz almost completely abrogated Ccl20, Cxc11 and Csf3 expression both in unstimulated and IL-17A-stimulated conditions (**Figure 5e**), evidencing that IL-17A-induced chemokine expression is mostly dependent on the induction of the transcription co-activator I κ B- ζ . Taken together, these data indicate that loss of A20 in

keratinocytes leads to enhanced cytokine and chemokine production partly as a result of uncontrolled MyD88 signaling, which is further augmented through IL-17A-mediated I κ B- ζ expression in an A20-independent manner.

DISCUSSION

Based on comparative transcriptomics on human biopsies and the use of *in vivo* mouse models, we found that specific downregulation of A20 in keratinocytes, but not in T-cells, determines sensitivity to both AD and/or Pso. As keratinocytes show increased pro-inflammatory gene expression upon loss of A20, probably as a result of cross-talk between different signaling pathways, we provide a mechanism by which A20 expression in keratinocytes is able to control inflammation in various skin immune disorders.

First, transcriptome analysis of laser micro-dissected epidermis, but not dermis, from Pso and AD patients showed downregulation of A20/TNFAIP3 in affected skin compared to healthy controls. In addition, down-regulation of A20 mRNA expression could also be observed in non-involved skin of psoriatic patients. Second, using the best available and most relevant mouse models (Kabashima and Nomura, 2017), we show that AD and Pso severity was increased in A20^{EKO} mice upon repeated OVA sensitization, IMQ application or IL-23-injection. While epidermal thickening was not significantly different in WT and A20^{EKO} mice upon IMQ-induced psoriasis-like skin inflammation, we found significant increase in several important parameters relevant for the human disease: disease severity score, neutrophilia with neutrophilic abscesses (the latter having never been reported in mice to the best of our knowledge), expression of key immune genes, such as IL-22 and IL-23, expression of stress molecule S100A8, as well as expression of structural proteins Krt16 Lce3c, and Lcn2. In the AD model, A20^{EKO} mice, compared to WT animals, displayed exacerbation of the key disease features except eosinophilia which was barely induced by sensitization. This is possibly due to the higher number of IL-17A-positive CD4⁺ T cells as an inverse correlation between Th17 and eosinophilia has been reported in AD (Hayashida et al., 2011). Furthermore, we also found that keratinocytes show increased pro-inflammatory gene expression upon loss of A20, probably as a result of cross-talk between different signaling pathways, providing a

mechanism by which A20 expression in keratinocytes is able to control inflammation in various skin immune disorders.

Unlike K5-IKK2 transgenic mice or keratinocyte-specific deletion of the NF- κ B inhibitor I κ B α , a model for NF- κ B over-activation in keratinocytes, A20^{EKO} mice do not show spontaneous dermal and/or epidermal immune cell infiltration (Lippens et al., 2011, Page et al., 2010, Rebholz et al., 2007). Indeed, A20 is an NF- κ B responsive gene that needs to be induced to prevent persistent trigger-induced NF- κ B activation, so A20 might be redundant for skin immune homeostasis in the absence of pathogen- or stress-inducing stimuli. In contrast, keratinocyte-specific deletion of I κ B α or constitutive IKK2 activation in K5-IKK2 transgenic epidermis results in continuous NF- κ B overactivation and skin inflammation, without the need for an inflammatory trigger (Page et al., 2010). In support for this hypothesis, we found that disrupting the *stratum corneum* barrier in A20^{EKO} mice by acetone treatment, which enables the penetration of skin commensals that can be recognized by pattern recognition receptors, results in an inflammatory skin condition resembling the interface dermatitis observed in K5-IKK2 mice (Page et al., 2010). This argues that in patients expressing lower A20 levels in the epidermis, physical abrasion could lie at the basis of skin inflammation. Remarkably, absence of IKK2 in keratinocytes (IKK2^{EKO}) also results in TNF-dependent spontaneous severe skin inflammation (Pasparakis et al., 2002). While unclear, this might either results from the fact that IKK2 can act as a negative regulator of RIPK1 (Dondelinger et al., 2015), thereby leading to an hypersensitivity towards TNF-induced RIPK1-dependent cell death, and/or from the fact that NF- κ B is no longer activated in keratinocytes causing RIPK1-dependent TNF-induced cell death (Grinberg-Bleyer et al., 2015, Xu et al., 2018).

Even though skin from unchallenged A20^{EKO} mice is devoid of infiltrating immune cells, we found an increase in pro-inflammatory gene expression in the skin of these mice, suggesting that A20 represses the skin inflammatory program even under homeostatic conditions. Furthermore, A20^{EKO} mice show signs of basal systemic inflammation, indicating an impact of keratinocyte-specific A20 deficiency extending beyond skin. Such findings might in part explain (distal) articular manifestations found in psoriatic arthritis (Ritchlin et al., 2017), which is also associated with A20/TNFAIP3 gene polymorphisms but is not present in our mouse model probably due to the very short duration of the experimental protocol or to cell-specific A20 deletion.

Given the strong downregulation of A20 transcripts in epidermis, but not dermis from Pso and AD patients compared to healthy controls, we suggest that the decrease in A20 expression occurred in keratinocytes. Although the mechanism controlling a decrease of A20 expression in epidermis in Pso and AD is not clear, the reduced homeostatic capacity of keratinocytes to counterbalance inflammatory signals and/or their increased production of pro-inflammatory factors, in the absence of A20, appears to control skin inflammatory susceptibility to a wide range of stimuli. As the development of AD and Pso depends on crosstalk between immune cells and keratinocytes (Lowe et al., 2014), A20 expression in keratinocytes appears to play an important role in limiting skin immuno-inflammatory responses in these pathologies, as also suggested by the link between hypomorphic A20 expression and psoriasis susceptibility (Aki et al., 2017). As keratinocyte responses to T-cell derived cytokines are paramount to both pathologies (Guttman-Yassky and Krueger, 2017), a decrease of A20 expression in keratinocytes, by not yet established factor(s), seems to be a key trigger of pathogenic circuits in both diseases. Interestingly, the A20-binding partner TNIP1 is also linked to Pso by GWAS (Heyninck et al., 1999, Nair et al., 2009) and epidermis-specific deletion of TNIP1 sensitizes

mice to experimental Pso (Ippagunta et al., 2016). Like A20, TNIP1 also provides negative feedback regulation in TLR pathways (Zhou et al., 2011). So regarding inflammatory skin diseases, it is tempting to speculate that A20 and TNIP act together to repress excessive inflammatory signaling.

Paradoxically, upon anti-TNF treatment of inflammatory bowel disease and rheumatoid arthritis psoriasis-like syndrome can occur. A recent study suggested this was due to reduced levels of TNF-induced A20 allowing p38-MAPK and PKC-driven IL-17A production in CD4⁺ T cells (Urbano et al., 2017). Since A20 was previously reported to negatively regulate IL-17 receptor signaling in the ST2 stromal cell line and in embryonic fibroblasts (Garg et al., 2013), a similar mechanism involving IL-17R could in part account for the inverse correlation between A20 expression in keratinocytes and Pso/AD severity. We found that A20-deficient cultured keratinocytes showed upregulation of pro-inflammatory genes already in unstimulated conditions, including the IL-17A-target chemokines Ccl20, Cxcl1 and Csf3. Inactivating MyD88, a crucial adaptor protein for all TLR's, except TLR3, and IL-1R, in A20-deficient keratinocytes significantly decreased chemokine expression. However, A20 had no significant impact on IL-17A-mediated induction of Ccl20, Cxc1 and Csf3 in keratinocytes. These data suggest that A20 has no direct influence on IL-17 receptor signaling in keratinocytes, in contrast to embryonic fibroblasts (Garg et al., 2013), suggesting some cell specificity in this regulatory process. It also indicates that other signaling pathway(s) are activated in the absence of A20 and cross-talk with IL-17A-induced gene expression. Cross-talk between IL-17 signaling and other signaling pathways, such as TNF and IL-1, are mediated by communication between NF- κ B and its co-activator I κ B- ζ , which acts as a transcription enhancer primarily through chromatin remodeling, resulting in increased mRNA expression from their target promoters (Johansen, 2016). A20 had no impact on basal and IL-

17A-induced I κ B- ζ expression, again showing that, as such, A20 does not influence IL-17R signaling in keratinocytes. However, we found that knocking down I κ B- ζ abolished chemokine expression both in unstimulated and IL-17A-stimulated conditions. Interestingly, I κ B- ζ has been shown to be a key driver in the development of psoriasis in different mouse models (Johansen, 2016, Johansen et al., 2015). Taken together, we propose that, at homeostasis, loss of A20 sensitizes keratinocytes to signaling events in part induced by MyD88-dependent environmental triggers that amplify IL-17A-induced cytokine production resulting in exacerbation of skin inflammation in Pso and AD.

In summary, our findings show that subclinical skin inflammation caused by loss of A20 in keratinocytes is able to drive systemic inflammation under homeostatic conditions. In turn, this sensitivity primes or potentiates disease severity of distinct skin pathologies. How A20 expression could be restored/increased in keratinocytes will be an interesting challenge for novel and better therapies for psoriasis, atopic dermatitis and potentially other inflammatory conditions of the skin.

MATERIALS & METHODS

Patients. Skin biopsies from involved and non-involved areas were obtained from 9 patients with psoriasis, 5 patients with atopic dermatitis and 5 healthy volunteers (see supplementary material).

Mice. Epidermis-specific (A20^{EKO}) and CD4⁺ T cell-specific (A20^{TKO}) A20-deficient mice were generated on a C57BL/6J background as previously described (Drennan et al., 2016, Lippens et al., 2011) (see supplementary material).

***In vivo* skin inflammation models.** Experimental models for psoriasis (IMQ, IL23 injection), atopic dermatitis (OVA) and barrier disruption (acetone) were induced as previously described (Desmet et al., 2014, Hoste et al., 2013, Staumont-Salle et al., 2008, van der Fits et al., 2009) (see supplementary material).

Microarray analysis. Epidermis and dermis were obtained from human skin biopsies by laser capture microdissection using Arcturus XT (MDS Analytical Technologies) system (Chinetti-Gbaguidi et al., 2011), followed by RNA isolation with Arcturus Picopure RNA isolation Kit.

Conflict of interest

The authors state no conflict of interest

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SUPPLEMENTARY MATERIALS & METHODS

Experimental atopic dermatitis. Atopic dermatitis (AD) was induced by epicutaneous sensitization (Staumont-Salle et al., 2008) with ovalbumin (OVA) (Spergel et al., 1998). Briefly, patches soaked with 25 μ L of 0.2% OVA solution in PBS or with vehicle were applied on abdominal skin 24 hours after shaving and were left on for three 1-week periods (with patch renewal at midweek), with a 2-week interval between applications, on 8-12 week-old female mice with *ad libitum* access to water and food. Animals were sacrificed by cervical dislocation. Mice with fight wounds were excluded from analyses. Skin and inguinal lymph nodes were collected for histological analyses and real-time qPCR after RNA extraction.

Experimental psoriasis. IMQ-induced psoriasis-like inflammation was induced as previously described (van der Fits et al., 2009) on 8-12 week-old male mice with *ad libitum* access to water and food. Mice were treated with a daily topical dose (31 mg) of IMQ cream (Aldara 5%, Meda AB) or control Lanette cream (Fagron, ref. 1289-511), unless stated otherwise, on the shaved abdominal or back skin for 5 days and sacrificed 24 h later. For macroscopical scoring we used a psoriasis disease severity score adapted for mouse models of psoriasis (based on the intensity of erythema, thickness and ichthyosis: 0 – absent, 1 – mild, 2 – moderate, 3 – severe, for each criteria), which was visually estimated daily during treatment with IMQ cream or control “Lanette” cream. At the time of sacrifice, skin samples were directly frozen in liquid nitrogen for subsequent RNA isolation, fixed in Immunohistofix (Gentaur, Paris, France) for histological analysis (Staumont-Salle et al., 2014), or processed for cell isolation. IL-23-induced psoriasis-like inflammation was induced by repeated intradermal injections of human IL-23 (1 μ g) every other day within a period of 8 days into the right ear pinna of 8-12 week-old male and female mice, as previously described (Bloch et al., 2018, Desmet et al., 2014). Recombinant IL-23 was kindly provided by Yehudi Bloch and

Prof. Dr. Savvas Savvides (UGent, Belgium). Control mice received intradermal injections of PBS following the same administration scheme. Ear swelling was monitored with a caliper.

Barrier disruption. Barrier disruption was induced as previously described (Hoste et al., 2013). The dorsal side of 8-12 week-old female mice was shaven 48 hours before the experiment. Acetone or control saline treatment was performed twice daily a day (morning and evening) by means of wiping the skin five times with a paper cloth soaked in acetone or 1 M NaCl respectively. Animals were sacrificed by cervical dislocation. Skin was collected for histological analyses and real-time qPCR after RNA extraction.

Preparation of immune cells from spleen, lymph nodes and thymus and flow cytometry analysis. Cells were isolated from mechanically dissociated spleen, lymph nodes and thymus, washed twice with cold PBS and filtered through a sieve (70 μ m). Spleen and lymph nodes cells were then incubated with RPMI medium with PMA (20 ng/ml), ionomycin (1 μ g/ml) and brefeldin A (1 μ g/ml) for 4 hours in a CO₂ incubator. Intracellular staining was performed using the Cytofix/Cytoperm kit (BD Biosciences) according to the manufacturer's instructions. The following fluorochrome-conjugated antibodies were used (clones are shown in parenthesis): CD3 PE-CF594 (17A2) or APC (145-2C11), Ly6C BV421 (HK1.4) or BV785 (1A8), CD4 BV605 (RM4-5) or BV510 (RM4-5), CD8a APC-Cy7 (53-6.4), CD11c APC-Cy7 (N418) or BV785 (N418), CD115 AF488 (AFS98), TCR δ Percp-Cy5.5 (GL3), F4/80 PE-Cy7 (BM8) or BV785 (BM8), IFN γ AF488 (XMG1.2), IL-22 PE (Poly5164), CD207 PE (4C7), IL-10 PE (JES5-16E3), IL-5 PE (TRFK5), IL-13 PE-Cy7 (eBio13a), IL-4 APC (11B11), FoxP3 APC (FJK-16), CD45 BV510 or BUV737 (30-F11), CD11b BV605 or BUV395 (M1/70), IE/IA AF700 (M5/114.15.2), Ter119 BV785 from Biolegend; CD19 PE-CF594 (1D3) or BV785 (6D5), Gr1 FITC (RB6-8C5), CD25 BUV395 (PC61), CD44 V450 (IM7), TCR β BV711 (H57-597), TCR $\gamma\delta$ PE-CF594 (GL3), CD24 PE (M1/69), CD5 BUV737 (53-7.3) from BD Biosciences; IL-17A APC (eBio17B7), CD8a PE-Cy5 (53-6.7), CD62L

FITC (MEL-14) from eBiosciences. Flow cytometry analysis was performed on BD LSRFortessa™ X-20 flow cytometer (Becton Dickinson). Results were acquired with the Diva software (Becton Dickinson) and analyzed using FlowJo software (Tree Star)

Histology and immunohistochemistry. Tissue biopsy specimens were processed as previously described (Devos et al., 2016, Staumont-Salle et al., 2014). In brief, samples were fixed in ImmunoHistoFix (Gentaur) and embedded in ImmunoHistoWax (Gentaur) at 37°C. 5 µm sections were stained with May-Grünwald Giemsa, hematoxylin/eosin or immunostained with anti-Ly6G mAb (1A8; BD Biosciences). Average epidermal thickness was determined as a mean of 10 measures calculated for each skin sample. Eosinophils, neutrophils and mast cells were enumerated by examining 10 random fields at 400-fold magnification for each section; cell frequency was converted to cells per square millimeter and results were expressed as mean ± SEM. A microscope with Arcturus XT software was used and investigators were unaware of the experimental groups during epidermal thickness measurements and cell enumerations.

Immunoglobulin concentrations. Total and OVA-specific IgE, and OVA-specific IgG1, IgG2a, IgG2c concentrations in serum were measured by ELISA as previously described (Staumont-Salle et al., 2008).

Keratinocyte cultures. Primary keratinocytes were isolated from the skin of adult mice as previously described (Jensen et al., 2010).

RNA analysis by RT-qPCR. Total RNA isolation, cDNA generation and RT-qPCR was performed using standard procedures. All primer sequences are available on request.

Microarray analysis. 10 ng RNA was amplified with ExpressArt Kit and labeled with One-color microarray-based Gene Expression Analysis Low Input Quick Amp WT Labeling (Agilent Technologies) and hybridized with Human Gene Expression Microarray 8x60K chip (Agilent Technologies). Epidermis and dermis from healthy donors (n=5), from lesional skin

from psoriasis (n=9) and atopic dermatitis (n=5) patients was analyzed. Microarray data were background corrected with the normal-exponential convolution model (Silver et al., 2009) and normalized with the quantile approach (Bolstad et al., 2003) using the limma R package (Ritchie et al., 2015). Gene Expression Omnibus (GEO) repository accession numbers are GSE68923, GSE68937, GSE68924, GSE68939.

Antibodies used for Westernblot analysis. p-I κ B- α (9246, Cell Signaling), I κ B- α (sc-371, Santa Cruz), p-JNK (44682G, Invitrogen), JNK (9252, Cell Signaling), p-ERK (9106, Cell Signaling), ERK (9102, Cell Signaling), p-p38 (9211, Cell Signaling), p38 (9212, Cell Signaling).

Statistical analyses of biological data. For a two-group comparison two-sided Student's *t*-test or Mann-Whitney test was used. For more than two groups data were analyzed by repeated measures ANOVA or two-way ANOVA, followed by Tukey's *post-hoc* tests for multiple comparisons. P-values below 0.05 were considered significant. Statistical analyses were performed with Prism 6 (GraphPad Software, Inc.) or R 3.1.3 open source software.

Figure Legends

Figure 1. A20/TNFAIP3 is downregulated in epidermis of psoriasis and atopic dermatitis patients

(a) Significantly deregulated transcripts (Benjamini-Hochberg adjusted p-values less 0.05) in human epidermis isolated by laser capture microdissection from healthy skin (n = 5) or affected skin from patients with psoriasis (n = 6) or atopic dermatitis (n = 5). Some common up- and down-regulated genes are shown. (b) Gene set enrichment analysis of common genes significantly dysregulated in psoriasis and atopic dermatitis samples shown in a. (c) *TNFAIP3* is decreased in human epidermis but not in dermis in psoriasis and atopic dermatitis. Relative expression of *TNFAIP3* from healthy skin (n = 5) or involved and uninvolved skin from patients with psoriasis (n = 9) or atopic dermatitis (n = 5). *P < 0.05, **P < 0.01, ***P < 0.001 by paired and unpaired Mann–Whitney U test. Graphs present mean ± SEM.

Figure 2. A20/TNFAIP3 deficiency in keratinocytes exacerbates IMQ-induced experimental psoriasis

(a) Abdominal skin morphology and histology (MGG, scale bar 50 µm) (b) Disease severity score, (c) Average epidermal thickness, (d) infiltration of neutrophils (brown) in dermis and epidermis detected by Ly6G immunostaining, (e) RT-qPCR analysis of inflammatory genes in whole skin. (f) flow cytometry of classical dendritic cells (cDC), Langerhans cell (LCs), and macrophages in inguinal lymph nodes and (g) flow cytometry of $\gamma\delta$ T cells, $\gamma\delta$ T17, CD4 T cells and Th17 in inguinal lymph nodes, during/after 6 days in the IMQ-induced model of psoriasis on A20^{FL/FL} and A20^{EKO} mice. n = 5-6 mice per group. *P < 0.05, **P < 0.01 ***P < 0.001 by two-way ANOVA with Sidak post-hoc test or two sided t-test. Graphs present mean ± SEM.

Figure 3. A20/TNFAIP3 deficiency in keratinocytes exacerbates OVA-induced experimental atopic dermatitis

(a) Abdominal skin morphology and histology (MGG, scale bar 20 μm), (b) average epidermal thickness, (c) quantification of eosinophils in dermis, (d) total, OVA-specific IgE, IgG1, IgG2a and IgG2c in serum and (e) flow cytometry of T cells in inguinal lymph nodes after the epicutaneous OVA sensitization model of atopic dermatitis on A20^{FL/FL} and A20^{EKO} mice. n = 6-8, *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 by two-way ANOVA with Tukey's post-hoc test. Graphs present mean \pm SEM.

Figure 4. Keratinocyte-specific A20/TNFAIP3 deficiency in combination with skin barrier disruption results in inflammation resembling interface dermatitis

(a) Back skin histology (H&E, scale bar 50 μm), (b) epidermal thickness and (c) RT-qPCR analysis of inflammatory genes in whole back skin of A20^{FL/FL} and A20^{EKO} mice that were left untreated or subjected to repetitive treatment two times daily with saline or acetone wipes for 5 days. The inset magnifying the dermo-epidermal junction in (a) shows the presence of inflammatory cells. n = 3-7, *P < 0.05, **P < 0.01, ***P < 0.001 by two-way ANOVA with Bonferroni post-test. Graphs present mean \pm SEM.

Figure 5. A20/TNFAIP3 deficiency increases pro-inflammatory gene expression in keratinocytes

(a) RT-qPCR analysis of inflammatory genes of primary keratinocytes derived from A20^{FL/FL} and A20^{EKO} mice. n = 5-6, *P < 0.05, **P < 0.01, ***p < 0.001 by two sided t-test. (b, c) RT-qPCR analysis of indicated genes of unstimulated and *in vitro* IL-17A-stimulated (6h, 50 ng/ml) primary keratinocytes derived from A20^{FL/FL} and A20^{EKO} mice. n = 6, p-value of

interaction analyzed by two-way ANOVA is depicted on graph. (d, e) RT-qPCR analysis of indicated genes of primary keratinocytes derived from A20^{FL/FL} and A20^{EKO} mice transfected with indicated siRNA's, unstimulated or *in vitro* stimulated with IL-17A (6h, 50 ng/ml). n = 3, *P < 0.05, **P < 0.01 ***p < 0.001 by two-way ANOVA with Bonferroni post-test. Graphs present mean ± SEM or matched individual measurements.

Figure S1. A20/TNFAIP3 deficiency in keratinocytes results in spontaneous systemic pro-inflammatory phenotype

(a) Hematoxylin and eosin (H&E)-stained sections of A20^{FL/FL} and A20^{EKO} back skin reveals no signs of local skin inflammation in A20^{EKO} skin. Scale bars represent 50 μ m. (b) RT-qPCR analysis of inflammatory genes in whole skin of A20^{EKO} mice versus A20^{FL/FL} controls. (c) Body weight and percentage spleen weight of A20^{EKO} mice versus A20^{FL/FL} controls. (d, e) flow cytometry of neutrophils and Ly6C^{hi} monocytes (d) and T-cells (e) in spleen from naïve mice. (f) Flow cytometry of monocytes, pDC, NK, NKT and $\gamma\delta$ T cells in spleen from naïve A20^{EKO} and A20^{FL/FL} mice. n = 5-7, *P < 0.05, ***P < 0.001 by two sided t-test.

Figure S2. Thymus development is unchanged in A20^{EKO} mice. (a) Total thymocyte cell number. (b) Gating strategy of thymic subpopulations thymocytes shown for a representative wildtype mouse. The names of the gates written inside the plots and headlines on the plot refer to the population shown. (c) Subpopulations of developing thymocytes. DN: Double negative (CD4⁻CD8⁻), TN: Triple negative (CD4⁻CD8a⁻CD3⁻), TN1-TN4: Identified by their expression of CD44 and CD25, DP: Double positive (CD4⁺CD8a⁺), CD8SP CD8 single positive, CD4SP: CD4 single positive, Immature/mature CD8SP and CD4SP are defined by their expression of CD24 and CD62L. Immature: CD24^{hi}CD62L^{lo}, Mature: CD24^{lo}CD62L^{hi}. Regulatory T cells were identified by their expression of CD4, CD25, and CD5 (CD4⁺CD25⁺CD5^{hi}). The innate-like T cell subsets TCR $\gamma\delta$ ⁺, TCR $\gamma\delta$ ⁺CD24 (immature $\gamma\delta$ T cells), and TCR β ⁺DN cells were also evaluated. Lin⁻: Lineage negative defined as cells negative for CD19, CD11c, F4/80, Ly6G, and Ter119. n = 4-5, mice were 9 weeks of age at termination.

Figure S3. A20/TNFAIP3 deficiency in T-cells does not exacerbate IMQ-induced experimental psoriasis

(a) Western blot analysis of A20 expression in lysates of CD4⁺ and CD8⁺ splenocytes isolated from untreated A20^{FL/FL} and A20^{TKO} mice. (b) Skin morphology and histology of A20^{FL/FL} and A20^{TKO} mice after 6 days in the IMQ-induced model of psoriasis. Scale bar 100 μ m. (c) Disease severity score during 6 days of the IMQ-induced mouse model of psoriasis. (d) Average epidermal thickness after 6 days in the IMQ-induced model of psoriasis. (e) RT-qPCR analysis of inflammatory genes in whole skin after 6 days in the IMQ-induced model of psoriasis. n = 4-5, values in (b), (c) and (d) were compared using two-way (ANOVA), p-values of interaction are depicted on graphs. Graphs present mean \pm SEM.

Figure S4. Keratinocyte-specific A20/TNFAIP3 deficiency exacerbates experimental psoriasis induced by intradermal IL-23 injection

A20^{FL/FL} and A20^{EKO} ears were injected intradermally with PBS or hIL-23 (1 μ g) every other day for 8 days and ear thickness was measured. n = 6-8, ***p < 0.001 by two-way ANOVA. Graphs present mean \pm SEM.

Figure S5. Loss of A20 in keratinocytes does not increase TNF-induced cytotoxicity

Keratinocytes derived from A20^{EKO} and A20^{FL/FL} mice were treated with 20 ng/ml TNF and cell death was measured after 24 hours by SytoxGreen positivity (SG⁺). n =4, p-value of interaction analyzed by two-way ANOVA is depicted on graph. Graph presents mean \pm SEM.

Figure S6. Effect of A20 on IL-17A-induced NF- κ B and MAPK signaling in mouse embryonic fibroblasts cells and primary keratinocytes

Western blot analysis of NF- κ B and MAPK activation in lysates of MEF cells derived from wild-type and A20 knock-out mice (A) and primary keratinocytes derived from A20^{FL/FL} and A20^{EKO} mice at different timepoints after IL-17A administration.

Figure S7. Effect of knockdown of TNFR1 on Ccl20, Cxcl and Csf3 expression in unstimulated and IL-17A-stimulated A20^{Fl/Fl} and A20^{EKO} primary keratinocytes

RT-qPCR analysis of indicated genes of primary keratinocytes derived from A20^{FL/FL} and A20^{EKO} mice transfected with indicated siRNA's, unstimulated or *in vitro* stimulated with IL-17A (6h, 50 ng/ml). n = 3, **P < 0.01 ***p < 0.001 by two-way ANOVA with Bonferroni post-test. Graphs present mean \pm SEM for TNFR1 expression and matched individual measurements for Ccl20, Cxc1 and Csf3 expression.

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