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ORIGINAL ARTICLE

# Protease- and cell type–specific activation of protease-activated receptor 2 in cutaneous inflammation

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## Abstract

**Background:** Protease-activated receptor 2 (PAR2) signaling controls skin barrier function and inflammation, but the roles of immune cells and PAR2-activating proteases in cutaneous diseases are poorly understood.

**Objective:** To dissect PAR2 signaling contributions to skin inflammation with new genetic and pharmacological tools.

**Methods/Results:** We found markedly increased numbers of PAR2$^+$ infiltrating myeloid cells in skin lesions of allergic contact dermatitis (ACD) patients and in the skin of contact hypersensitivity (CHS) in mice, a murine ACD model for T cell–mediated allergic skin inflammation. Cell type–specific deletion of PAR2 in myeloid immune cells as well as mutation-induced complete PAR2 cleavage insensitivity significantly reduced skin inflammation and hapten-specific Tc1/Th1 cell response. Pharmacological approaches identified individual proteases involved in PAR2 cleavage and demonstrated a pivotal role of tissue factor (TF) and coagulation factor Xa (FXa) as upstream activators of PAR2 in both the induction and effector phase of CHS. PAR2 mutant mouse strains with differential cleavage sensitivity for FXa versus skin epithelial

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cell–expressed proteases furthermore uncovered a time-dependent regulation of CHS development with an important function of FXa-induced PAR2 activation during the late phase of skin inflammation.

**Conclusions:** Myeloid cells and the TF–FXa–PAR2 axis are key mediators and potential therapeutic targets in inflammatory skin diseases.

**KEYWORDS**
allergic contact dermatitis, contact hypersensitivity, factor Xa, protease-activated receptor 2, tissue factor

## 1 | INTRODUCTION

Protease-activated receptors (PARs) constitute a family of G protein–coupled receptors with a protease sensitive extracellular domain that contain a tethered amino terminus acting as activating ligand after proteolytic cleavage.[1] Coagulation proteases are the key activators of PARs in vascular and immune cells.[2–6] However, PAR2 is also linked to multiple aspects of dermatitis such as skin barrier function, cutaneous inflammation, and itch. PAR2 is expressed by different cell types present in the skin, including keratinocytes, and neuronal and immune cells.[7–9] PAR2 is crucial for inflammatory skin diseases with barrier defects, including atopic dermatitis and Netherton syndrome, attributed to functions of PAR2 expressed by keratinocytes.[10–13]

Besides coagulation proteases in the tissue factor (TF) pathway, that is, coagulation factors (F) VIIa and Xa, PAR2 is stimulated by a variety of proteases, including those related to the skin (e.g., matriptase, kallikrein 5 [KLK5], prostasin [CAP1/Prss8]).[14,15] Whereas the TF–FVIIa complex associated with integrin receptors directly cleaves PAR2 for endosomal trafficking,[16–18] the nascent product, coagulation factor Xa in the ternary TF–VIIa–Xa coagulation initiation complex, cleaves and activates PAR2 to induce signaling in innate immune cells.[3–5,19,20] Furthermore, PAR2 exists as a heterodimer with PAR1 and can be activated without proteolytic cleavage by transactivation from thrombin-cleaved PAR1.[16,21]

Allergic contact dermatitis (ACD) is an occupational inflammatory skin disease, affecting 15% of the population worldwide,[22] thereby resulting in significantly reduced quality of life and high socioeconomic costs. Regulated by a Tc1/Th1 T cell response, ACD is comprised of two distinct phases, the induction phase, in which skin-related dendritic cells prime allergen-specific T cells in the regional lymph nodes (LN), and the effector/challenge phase. During the latter, a second encounter with the allergen results in re-activation of hapten-specific T cells in the skin and LN, followed by a massive cutaneous inflammatory reaction with erythema, dry scaly patches, vesicles, and pruritus.[23,24] Previous studies with PAR2 knock-out (KO) mice in the murine contact hypersensitivity (CHS) model of ACD have implicated PAR2 in skin inflammation.[25,26]

Despite these demonstrated roles of PAR2 in cutaneous inflammation, there is only limited information on functional contributions by immune cell–expressed PAR2 and the critical activating proteases in PAR2-dependent inflammatory skin diseases. We here show that myeloid cell–specific PAR2 activation and TF–FXa-induced PAR2 signaling are critical for pronounced skin inflammation and the underlying T cell response in CHS development. Pharmacological approaches furthermore indicate that the TF–FXa–PAR2 axis can be targeted to reduce inflammatory skin diseases.

**Essentials**

- Protease-activated receptor 2 (PAR2) signaling promotes skin inflammation.
- PAR2-activating proteases play effector phase specific roles in contact hypersensitivity (CHS).
- Myeloid cells contribute to the early phase of CHS and express PAR2 in human allergic contact dermatitis.
- The tissue factor (TF)–factor Xa (FXa)–PAR2 axis is a potential therapeutic target in CHS.

## 2 | RESULTS

### 2.1 | PAR2 proteolytic activation is required for skin inflammation in CHS

Coagulation proteases and skin-expressed proteases, including matriptase (ST14), prostasin (CAP1/PRSS8), and KLK5,[27] activate PAR2 by proteolytic cleavage at a canonical cleavage site. However, PAR2 expressed by vascular and immune cells can be cross-activated in a non-proteolytic manner by other receptors.[16,21,28] Given the role of PAR2 in inflammatory skin diseases,[17,32] we addressed whether PAR2-dependent development of skin inflammation was mediated by direct activation of PAR2 or, alternatively, by transactivation from other receptors. Cleavage-resistant PAR2 R38E knock-in mice ($PAR2^{INS}$ mice)[3,4] remain responsive to cross-activation by, for example, thrombin-cleaved PAR1.[16] We analyzed the CHS reaction which resembles ACD in humans[29] in $PAR2^{INS}$ mice[3,4] and strain-matched control mice by applying the contact allergen (hapten) TNCB (2,4,6-trinitro-1-chlorobenzene) on the abdominal skin during the sensitization phase and on the ears 5 days later (challenge; Figure S1).

We observed a significantly reduced ear swelling (Figure 1A, left panel) 8 and 24 h after challenge in PAR2$^{INS}$ mice compared to controls, which was reflected by impaired cutaneous immune cell infiltration visible on histological sections (Figure 1A, right panel, representative images). Analysis of the CHS underlying hapten-specific Tc1/Th1 cell response demonstrated a significantly impaired proliferation of restimulated T cells isolated from the skin-draining lymph nodes of PAR2 cleavage-resistant mice 8 and 24 h after challenge (Figure 1B). In addition, production of the CHS key cytokine interferon (IFN)-γ by *in vitro* restimulated T cells was reduced when cells were isolated in the early (8 h) and late (24 h) challenge phase from PAR2$^{INS}$ mice compared to control animals (Figure 1C). Thus, PAR2 proteolytic cleavage was required for CHS induction and hapten-specific T cell priming and/or re-activation.

We next performed a chronic model of skin inflammation induced by repetitive applications of the contact allergen over a period of 5 weeks. These experiments also demonstrated a significantly reduced CHS reaction in PAR2 cleavage–resistant mice (Figure 1D), confirming the essential role of PAR2 cleavage in this mouse model of CHS.

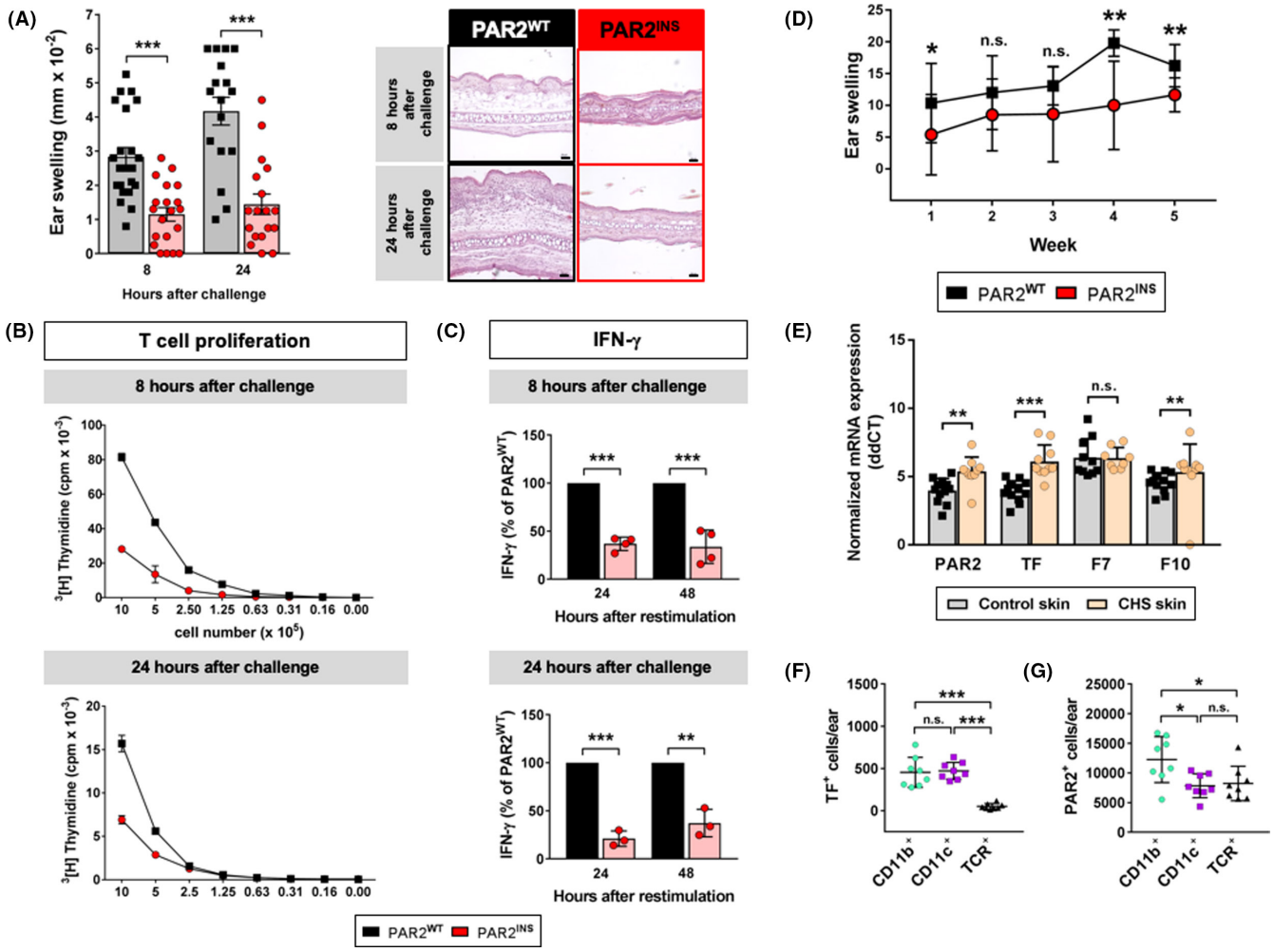


FIGURE 1 Protease-activated receptor 2 (PAR2) cleavage insensitivity abrogates the development of T cell–mediated cutaneous inflammation. A, Representative images of tissue sections of hematoxylin and eosin-stained ear skin (right panel) and quantification of ear swelling data 8 and 24 after challenge (left panel); mean ± standard error of the mean, pooled data of three independent experiments with 5–8 mice/group; unpaired *t*-test. Images, original magnification of 20X, scale bars = 50 μm. B, C, Proliferation and interferon (IFN)-γ production of skin-draining lymph node cells isolated 8 h (upper panels) or 24 h (lower panels) after allergen challenge and hapten-specific restimulation. B, T cell proliferation shown as one representative experiment out of three independent experiments; mean ± standard deviation (SD) of technical replicates, 5–8 mice/group. C, IFN-γ production by T cells 24 or 48 h after hapten-specific restimulation; mean ± SD, normalized to wild-type controls, pooled data of three (8 hours after challenge) or four (24 hours after challenge) experiments; unpaired *t*-test. D, Chronic skin inflammation in PAR2$^{INS}$ and wild-type mice sensitized and challenged five times as assessed by the ear swelling response 24 h after challenge; mean ± SD, $n = 6$, unpaired *t*-test. E, Expression of coagulation factor mRNAs in the skin of untreated and contact hypersensitivity (CHS) wild-type mice 24 h after challenge. Expression of PAR2, tissue factor (TF), factor (F)VIIa, and FXa mRNA was normalized to 18S rRNA; mean ± SD, $n = 8$–12/group, Welch's *t*-test. F, G, Quantification of TF (F) and PAR2 (G) expression in CHS skin 24 h after challenge. Abundance of TF$^{+}$ or PAR2$^{+}$ myeloid cells (CD11b$^{+}$), dendritic cells (CD11c$^{+}$), and T cells (TCR$^{+}$) cells per ear was determined by flow cytometry; mean ± SD; Mann–Whitney test. ns, not significant; $^{*}p < .05$, $^{**}p < .01$, $^{***}p < .001$

Because PAR2 contributes to immune cell migration in inflammation,[30] we first addressed whether the crucial role of PAR2 proteolytic activation in the development of cutaneous skin inflammation might be caused by differences in the recruitment of immune cells to the skin. Flow cytometry of $CD45^+$ cells isolated from the skin after challenge revealed significantly decreased numbers of infiltrating immune cells, including T cells, myeloid cells, and neutrophils, in the skin of $PAR2^{INS}$ mice compared to control animals (Figure S2A,B). Thus, insensitivity to proteolytic PAR2 activation resulted in a reduced Tc1/Th1 T cell activation in the skin-draining lymph nodes and an impaired immune cell recruitment within the skin.

## 2.2 | PAR2 is expressed by myeloid cell in CHS and clinical allergic contact dermatitis

Based on the observation that PAR2 is detectable on innate immune cells in the challenged skin of CHS mice, we compared skin mRNA expression before and after CHS induction. We found that not only PAR2, but also the coagulation factors TF and its ligands FVII and FX were expressed in the skin under homeostatic conditions (Figure 1E). In addition, PAR2, TF, and FX mRNA levels were significantly upregulated after CHS induction in the inflamed skin, suggesting a functional role for the TF–FXa–PAR2 axis in skin inflammation (Figure 1E). Flow cytometry revealed that TF was expressed on $CD11b^+$ myeloid cells and $CD11c^+$ dendritic cells (DC) but not on T cells within the inflamed cutaneous tissue (Figure 1F). $CD11b^+$ myeloid cells were the most abundant PAR2-expressing cell type in the CHS skin, but PAR2 was also present on $CD11c^+$ DC and T cells (Figure 1G). Considering recent data implicating TF–PAR2 signaling not only in cancer immune evasion, but also the development of (auto-)immunity,[3,18] these data suggested a possible contribution of TF and PAR2 signaling in innate immune cells to the development of CHS as prototype of an allergic skin inflammation.

We therefore evaluated whether PAR2 was also upregulated in myeloid cells in the pathology of human skin inflammation. For this purpose, we analyzed PAR2 expression on myeloid immune cell subsets in skin lesions of 15 ACD patients (Table S1) after standardized patch test re-exposure to the previously identified individual contact allergens (Figure 2). Vaseline-treated skin served as negative control. The allergic cutaneous immune response was assessed by clinical symptoms (Figure 2A, Table S1) and the skin tissue was subsequently analyzed by hematoxylin and eosin (HE) staining (Figure 2B) and immunohistochemistry (IHC; Figure 2C–E). We found that the cutaneous cellular infiltrate of ACD patients contained significantly increased numbers of $CD11c^+$ DC, myeloid $CD11b^+/CD14^+$ (monocytes), and $CD68^+$ (macrophages) immune cells (Figure 2C–E). Numbers of $PAR2^+CD11c^+$ DC were not elevated, but in line with our results in mice, IHC staining revealed increased numbers of $PAR2^+CD11b^+$ and $PAR2^+CD14^+$ myeloid cells in ACD (Figure 2D,E). Thus, PAR2 is expressed by myeloid cells both in the CHS mouse model and in ACD, suggesting a conserved functional role of myeloid PAR2 expression in cutaneous inflammation.

## 2.3 | PAR2 signaling in myeloid cells contributes to the early challenge phase of CHS

To define the contributions of myeloid cell-expressed PAR2 to CHS, we analyzed mice with conditional deletion of PAR2 in $LysM^+$ myeloid cells[3] or with a $CD11c^{cre}$ driver line selective for $CD11c^+$ DC.[31] Absence of PAR2 signaling in $CD11c^+$ DC reduced neither ear swelling nor the cutaneous inflammatory infiltrate at 8 and 24 h after challenge compared to control mice (Figure 3A). Accordingly, the hapten-specific CHS Tc1/Th1 cell response in the skin draining lymph node measured by *in vitro* re-stimulated T cell proliferation and IFN-γ production was similar in $PAR2^{flox/flox}/CD11c^{cre}$ mice and $PAR2^{flox/flox}$ littermate controls (Figure 3B,C).

In contrast, the myeloid cell–specific PAR2 KO resulted in a significantly reduced cutaneous inflammation during the early effector phase 8 h after challenge, but the ear swelling was indistinguishable from littermate controls in the late effector phase 24 h after challenge (Figure 3D). In addition, IFN-γ production by hapten-specific T cells, a key feature of the Tc1/Th1 T cell response in CHS, was significantly impaired in $PAR2^{flox/flox}/LysM^{cre}$ mice compared to $PAR2^{flox/flox}$ littermate controls, accompanied by a slightly reduced T cell proliferation (Figure 3E,F). These results indicated a crucial initiating role of myeloid PAR2 signaling in the early CHS effector phase and in control of the Tc1/Th1 T cell response.

## 2.4 | TF has dual roles in the induction and effector phase of allergic skin inflammation

PAR2 can be activated by various skin-related proteases,[2] but also by protease complexes formed by TF; that is, the TF–FVIIa and the TF–FVIIa–FXa complex with different signaling specificities.[6] Our data showed a concordant upregulation of TF and PAR2 in challenged skin (Figure 1E) and co-expression of TF and PAR2 in $CD11b^+$ myeloid cells (Figure 1F,G), in line with previous studies implicating TF in skin inflammation.[32,33] We therefore employed a monoclonal antibody to TF with proven inhibitory activity in thrombosis and inflammation models[34,35] as a pharmacological tool to understand the role of TF in the induction and effector phase of CHS (Figure 4A, upper panel).

Functional TF inhibition impaired the inflammatory skin reaction when given in both, the sensitization and challenge phase, as evidenced by a significantly reduced ear swelling and a markedly decreased cutaneous cellular infiltrate compared to control antibody–treated animals (Figure 4B). *In vitro* analysis of T cells isolated from the skin-draining lymph nodes revealed a diminished hapten-specific Tc1/Th1 T cell response as indicated by inhibited T cell proliferation and decreased IFN-γ secretion in anti-TF antibody–treated mice compared to mice receiving isotype matched control antibody (Figure 4C,D).

We next evaluated whether TF displayed a phase-specific function in CHS by applying the anti-TF antibody either prior to sensitization or prior to challenge alone (Figure 4A, middle and lower panels).

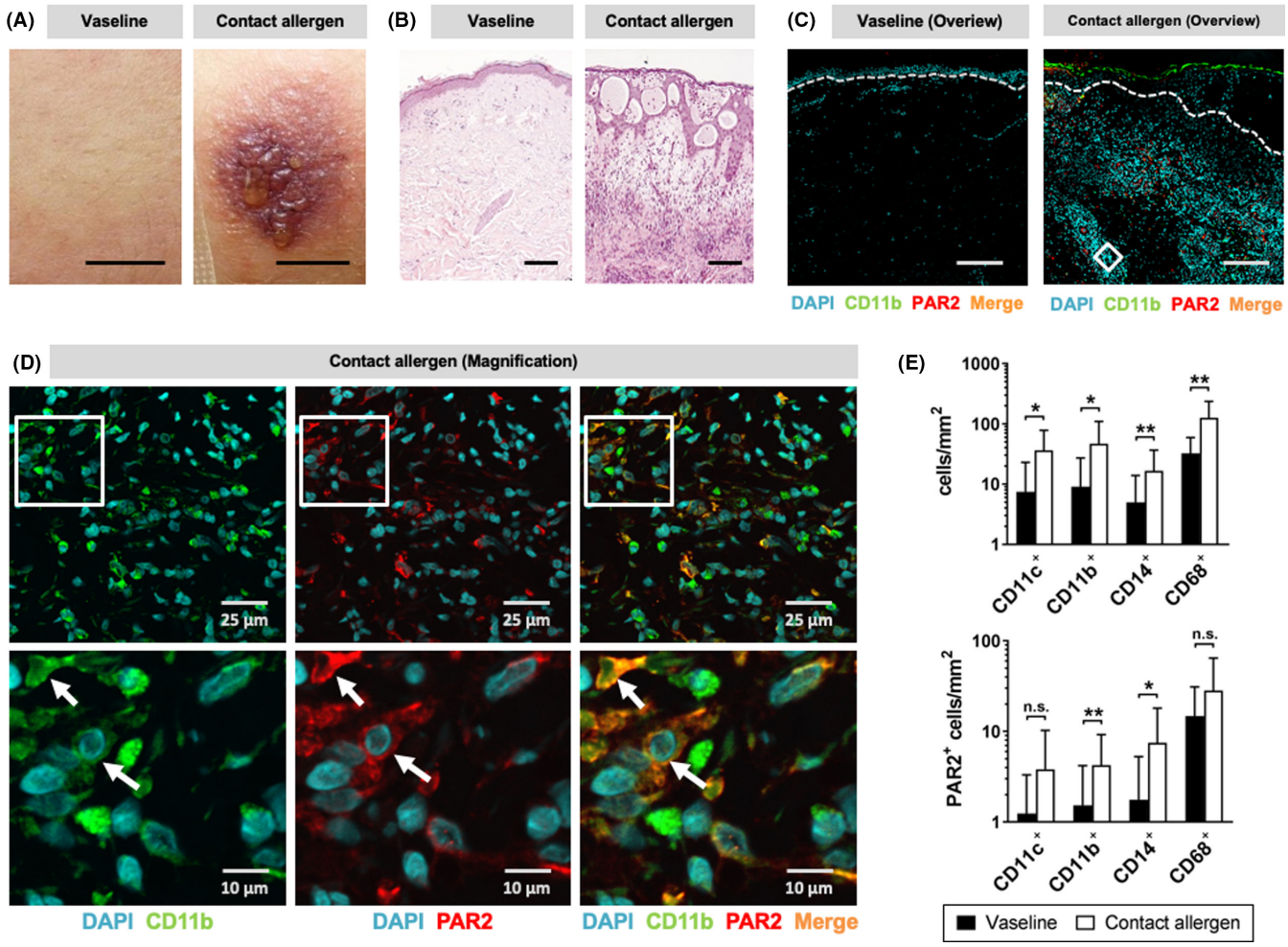


FIGURE 2 Increased protease-activated receptor 2 (PAR2) expression in $CD11b^+/CD14^+$ myeloid cells in human allergic contact dermatitis. A, Photographs of skin exposed to p-phenylenediamine (PPD; right) and vaseline control (left) of a patient with allergic contact dermatitis 72 h after application of the patch test antigen, scale bar = 1 cm. B, Hematoxylin and eosin staining of skin biopsies of the same patient; original magnification 10x, scale bar = 100 µm. C, D, Representative images of immunohistochemistry staining of challenged skin biopsy from a selected patient with contact allergy to PPD versus vaseline-treated skin with overview (C, 20x magnification, scale bar = 250 µm) and close-up views (D) of serial sections stained with DAPI (blue), $CD11b^+$ cells (green), $PAR2^+$ cells (red), and merged with $CD11b^+$ $PAR2^+$ cells appearing in orange. E, Quantification of myeloid immune cell infiltrates (upper panel) and PAR2 expression (lower panel) based on staining for human CD11c, CD11b, CD14, and CD68. Cell numbers/$mm^2$ were enumerated by image quantification; mean ± standard deviation, $n = 15$; paired *t*-tests; ns, not significant; $^*p < .05$, $^{**}p < .01$

Inhibition during either phase significantly diminished ear swelling as a parameter for skin inflammation (Figure 4E). These results demonstrate that TF is required for both the induction and effector phase of the T cell–mediated allergic skin inflammation.

As both TF and myeloid cell–expressed PAR2 played a pivotal role for CHS development, we next addressed whether PAR2 on myeloid cells might be activated by the TF ligand FVIIa, which is known to be expressed in macrophages[32,36,37] and plays a role in skin wound healing.[38] Myeloid cell–specific deletion of FVIIa in $F7^{flox/flox}/LysM^{cre}$ mice did not impair the T cell–mediated CHS response, including ear swelling, cutaneous immune cell infiltration, T cell proliferation, and IFN-γ production (Figure S3A–D). These data show that FVIIa synthesis by myeloid cells is dispensable for CHS and indicate that FVIIa from other sources or extravasation of FVIIa through hyperpermeable endothelium might be sufficient to drive TF-dependent skin inflammation. Of note, very low concentrations of FVIIa are sufficient to drive coagulation as well as TF–FVIIa–FXa–PAR2 mediated cell signaling.[5,17,20]

## 2.5 | Pharmacological inhibition of FXa attenuates the development of skin inflammation

Although direct TF inhibitors are currently not approved in the clinic, oral anticoagulants in clinical use have been shown to influence inflammatory reactions in preclinical models[3] and could provide initial insights into potential roles of FXa in CHS. Direct oral anticoagulants inhibiting FXa are widely used therapeutics in the treatment and prevention of venous thromboembolism and the prevention of stroke in patients with atrial fibrillation.[39,40] To assess FXa contributions

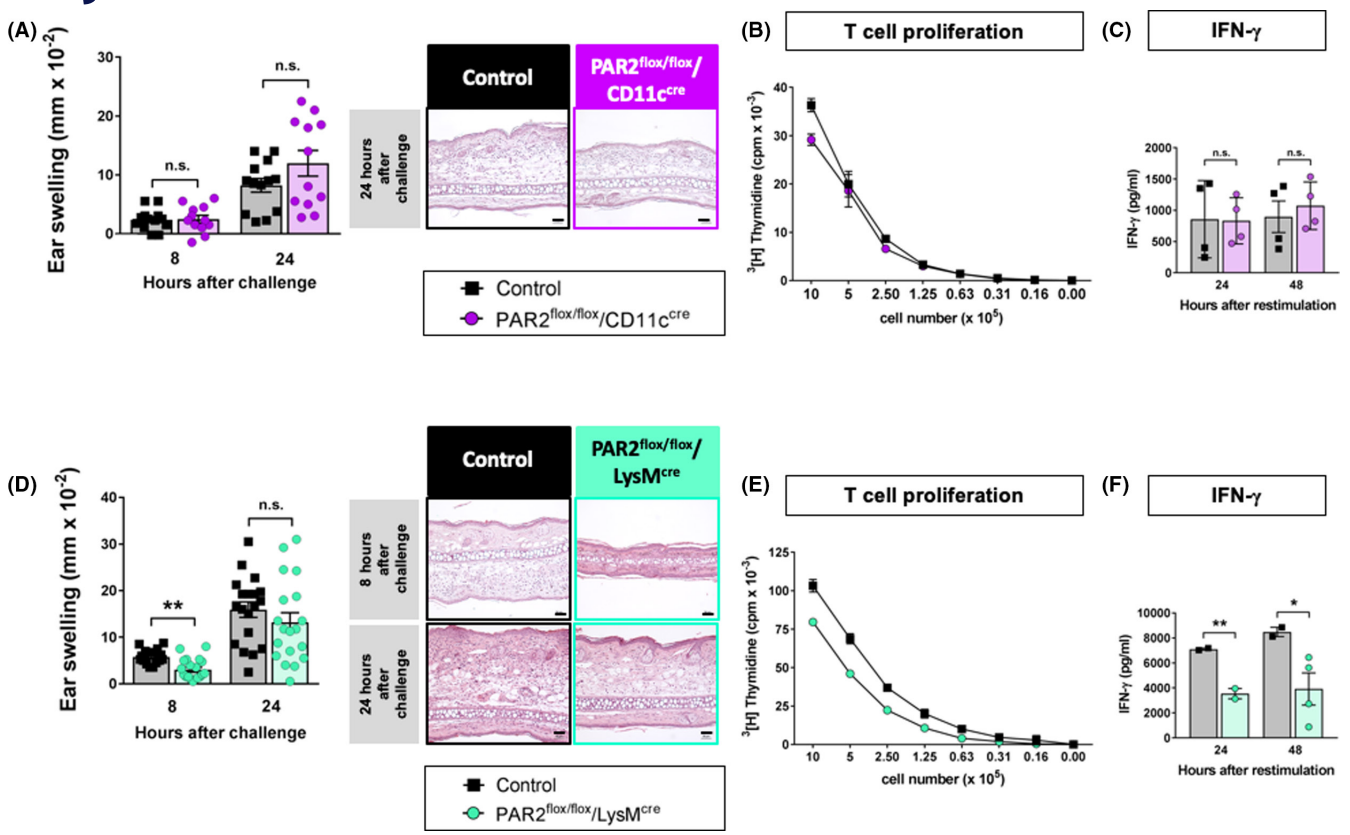


FIGURE 3 Protease-activated receptor 2 (PAR2) deletion in myeloid cells attenuates skin inflammation in contact hypersensitivity (CHS). A–C, CHS in PAR2$^{flox/flox}$/CD11c$^{cre}$ mice versus littermate PAR2$^{flox/flox}$ controls. A, Ear swelling and corresponding representative skin histology; mean ± standard error of the mean (SEM), $n = 12$; pooled data of four independent experiments, unpaired *t*-test; hematoxylin and eosin (H&E) magnification 20x, scale bar = 50 μm. B, T cell proliferation representative of three independent experiments; mean ± standard deviation (SD) of technical replicates, pooled data of 6–7 mice/group. C, Interferon (IFN)-γ production by T cells 24 or 48 h after hapten restimulation; mean ± SD; pooled data of four experiments; unpaired *t*-test. D-F, CHS in PAR2$^{flox/flox}$/LysM$^{cre}$ and littermate control mice. D, Ear swelling and corresponding representative skin histology 8 and 24 h after challenge; mean ± SEM, $n = 18$; pooled data of seven independent experiments, unpaired *t*-test. H&E magnification 20x, scale bar = 50 μm. E, Hapten-specific T cell proliferation 24 h after challenge; mean of technical triplicates representative of two independent experiments with 6–7 mice per group. F, Hapten-specific IFN-γ production by T cells isolated from draining lymph nodes 24 h after challenge was determined 24 and 48 h after restimulation *in vitro*; means ± SEM; pooled data of four independent experiments; unpaired *t*-test; ns, not significant; $^{*}p < .05$, $^{**}p < .01$

to skin inflammation, we treated mice orally with the FXa inhibitor rivaroxaban formulated in the chow to achieve therapeutic plasma levels[3] during the entire CHS protocol (sensitization and challenge), or either during the induction or challenge phase alone (Figure 5A).[5] Ear swelling was significantly decreased with all three treatment protocols, indicating that, in line with data of TF inhibition, FXa contributes to both the T cell priming (sensitization/induction phase) and the development of the allergen-specific skin inflammation (challenge/effector phase) in CHS (Figure 5B). These data support the involvement of the TF–FXa–PAR2 axis in the pathogenesis of skin inflammation.

## 2.6 | PAR2 protease resistant mutant mice implicate FXa in cutaneous inflammation

Completely cleavage-resistant PAR2 mutant mice were protected from CHS throughout the early and late challenge phase (Figure 1A–D), whereas myeloid cell–specific PAR2-deficient mice only displayed reduced ear swelling early during elicitation. We therefore hypothesized that different PAR2 activating proteases contribute to edema formation and immune cell infiltration during different phases of skin inflammation. In addition to FVIIa and FXa,[2,5,20] the skin proteases matriptase, prostasin, and KLK5 are potent activators of PAR2 signaling,[41,42] and KLK5 and prostasin specifically contribute to atopic-like dermatitis through thymic stromal lymphopoietin expression in Netherton syndrome.[11,14] Based on modeling and mutagenesis of PAR2 recognition by FVIIa,[43] we had generated PAR2 mutant mouse strains with restricted protease specificity preventing activation by FXa while preserving FVIIa cleavage.[3] We extended this analysis to skin-expressed proteases in the established cell surface cleavage assay with transiently transfected tagged human PAR2 mutants and recombinant proteases[19,20,43] (Figure 6A,B). In this assay, surface levels of the amino-terminal expression tag are measured after exposure to protease relative to untreated controls and cleavage insensitivity is indicated by 100% residual PAR2 detection on the cell surface.

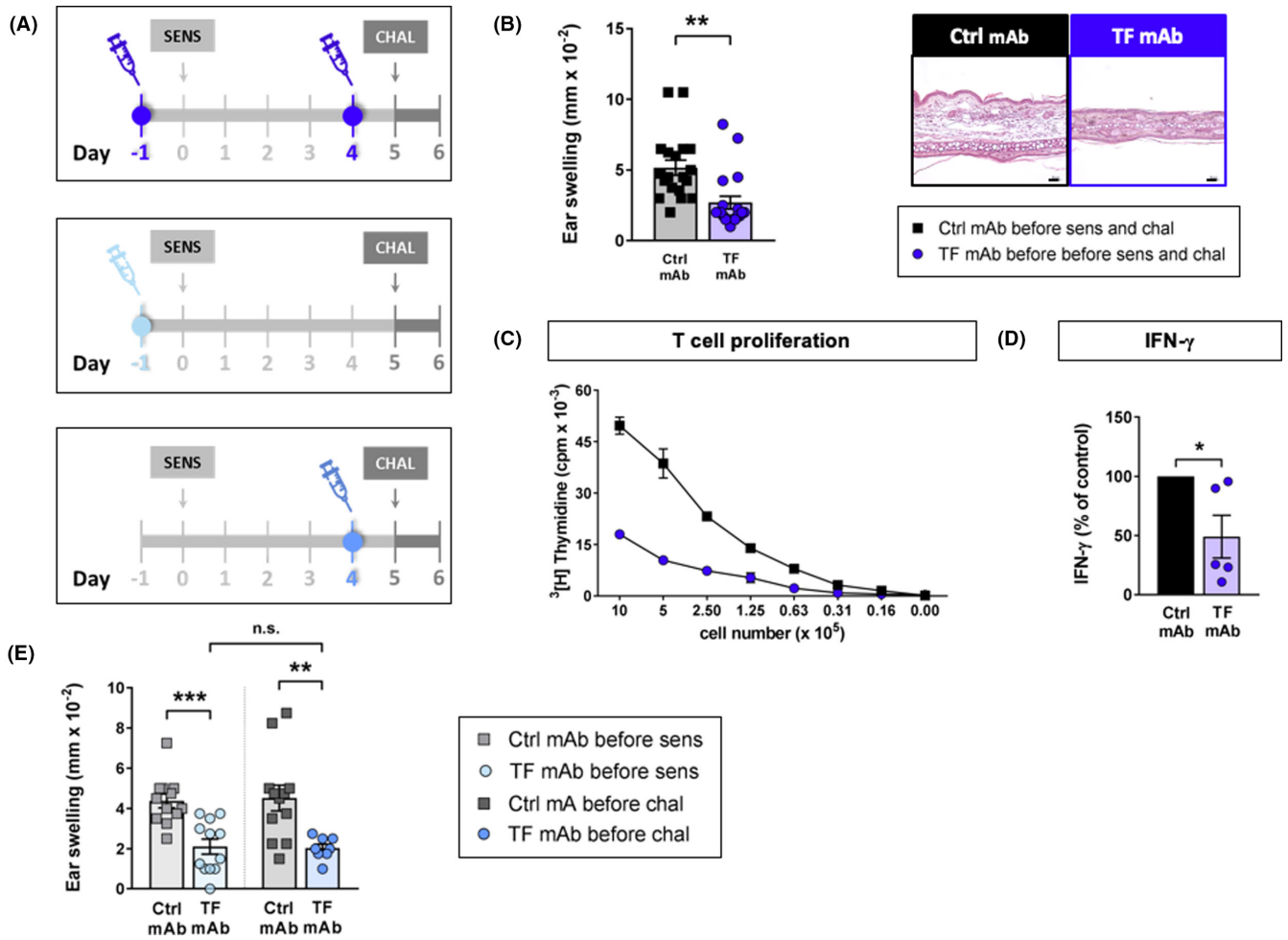


FIGURE 4 Tissue factor (TF) function is required for the induction and effector phase of skin inflammation. A, Treatment schemes with anti-TF mAb 21E10 or an isotype-matched control antibody given 24 h prior to both sensitization and challenge (dark blue; B–D), 24 h prior to sensitization (light blue), or 24 h prior to challenge (medium blue) (E, F). B, Ear swelling and corresponding representative hematoxylin and eosin skin histology 24 h after challenge in wild-type mice receiving antibody throughout the experiment; mean ± standard error of the mean (SEM), pooled data of three independent experiments with 6–8 mice/group; unpaired *t*-test. Tissue sections of the ear skin 20x magnification, scale bar = 50 μm. C, T cell proliferation representative of three independent experiments with pooled data of 6–7 mice/group; mean ± standard deviation of technical replicates. D, Interferon (IFN)-γ release by T cells after hapten-specific restimulation *in vitro*; mean ± SEM; pooled data of three independent experiments; unpaired *t*-test. E, Ear swelling 24 h after challenge; mean ± SEM; pooled data of two independent experiments with 6 mice/group; unpaired *t*-test. ns, not significant; $^{*}p < .05$, $^{**}p < .01$, $^{***}p < .001$

Mutation of the canonical cleavage site Arg[38] (numbering is based on the mouse PAR2 sequence) to Glu in PAR2$^{INS}$ (PAR$^{R38E}$) rendered PAR2 resistant not only to FXa, but also the representative skin-expressed proteases tested in the cleavage assay (Figure 6A,B). Mutation of the adjacent residue Gly to Ile was used in the FXa-resistant PAR2$^{G37I}$ mutant mouse,[3,34] rendering PAR2 insensitive to cleavage by FXa, matriptase, and prostasin, but not by KLK5 (Figure 6A,B). Based on available data on substrate recognition by matriptase,[44] we hypothesized that PAR2$^{K36E}$ could discriminate between FXa and matriptase cleavage. The cleavage assay confirmed that PAR2$^{K36E}$ remained sensitive to Fxa but was resistant to cleavage by all skin-expressed serine proteases available for testing (Figure 6A,B). We therefore generated PAR2$^{K36E}$ mice by introducing this point mutation into the PAR2 coding sequence using targeting of C57BL/6N oocytes by CRISPR/Cas technology[45] and sequence confirmation of generated homozygous PAR2$^{K36E}$ mice.

We analyzed these PAR2 mutant mice with protease selectivity in the CHS model and quantified skin inflammation in the early and late challenge phase. PAR2$^{G37I}$ mice exhibited a significantly decreased ear swelling and cellular cutaneous infiltrate 8 h and 24 h after challenge, indicating that KLK5 and possibly other KLKs are not important PAR2 activators in this model of cutaneous inflammation (Figure 6C,D). In contrast, PAR2$^{K36E}$ mice permissive for FXa activation showed no impairment in skin inflammation 24 h after challenge (Figure 6C,D), implying that FXa-dependent PAR2 signaling plays a critical role in the late phase of immune cell infiltration in CHS. These data were in line with the results from pharmacological FXa blockade. However, the early phase ear swelling was reduced in KLK5-sensitive PAR2$^{G37I}$ as well as in FXa-sensitive PAR2$^{K36E}$ mice. The reduced edema early in

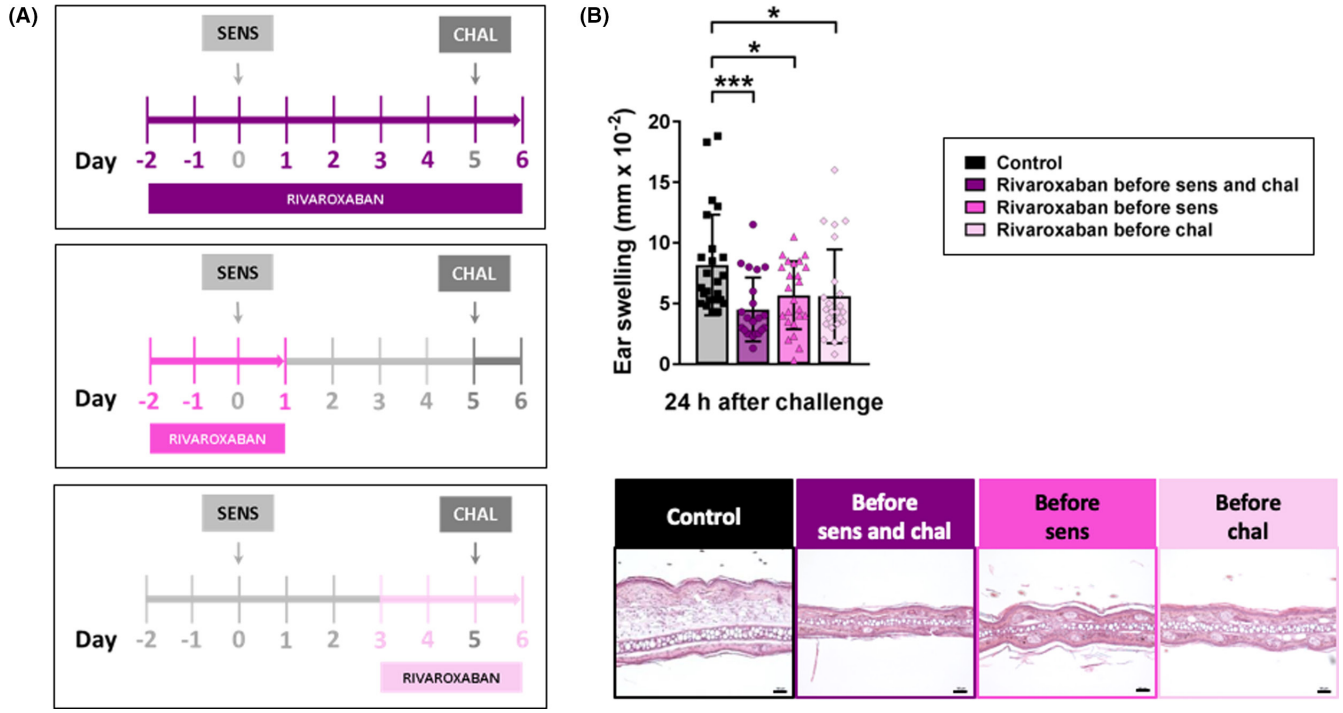


FIGURE 5 Factor Xa (FXa) inhibition with rivaroxaban attenuates cutaneous inflammation in mice. A, Wild-type C57BL/6N mice were treated with the FXa inhibitor rivaroxaban during sensitization and challenge (violet), sensitization alone (pink), or challenge alone (rose). B, Ear swelling was measured 24 h after challenge; mean ± standard deviation, pooled data of four independent experiments; unpaired *t*-test, $^{*}p < .05$, $^{***}p < .001$. Tissue sections of the ear skin stained with hematoxylin and eosin of the experiments in (B); magnification 20x, scale bars = 50 μm

FXa-sensitive PAR2$^{K36E}$ mice appeared to be inconsistent with the inhibition seen in mice treated with the FXa inhibitor rivaroxaban. Of note, FXa can also activate matriptase to indirectly induce epithelial cell PAR2 cleavage,[17,46] which would be prevented by the resistance of PAR2$^{G37I}$ as well as PAR2$^{K36E}$ mice to matriptase activation. Thus, early hyperpermeability and cellular infiltration, which is prevented by oral FXa inhibitors, may be dependent on FXa-dependent matriptase activation, which in turn cleaves PAR2, whereas late inflammation is dependent on direct PAR2 cleavage by FXa.

Contributions of FXa–PAR2 signaling to CHS were further supported by analysis of Tc1/Th1 T cell responses. As also seen with completely PAR2 cleavage resistant mice (Figure 1A–D), IFN-γ production and T cell proliferation were markedly reduced in PAR2$^{G37I}$ mice in both the early and late phase of skin inflammation (Figure 7A,B). In contrast, PAR2$^{K36E}$ mice with preserved direct PAR2 activation by FXa showed an unaffected Tc1/Th1 T cell response as seen in PAR2 wild-type (WT) controls during the early and late phase of the CHS reaction (Figure 7C,D). These data indicate that FXa-mediated PAR2 signaling crucially contributes to the hapten-specific T cell priming and/or re-activation in skin-draining lymph nodes of sensitized CHS animals.

We furthermore evaluated immune cell activation by multiplex analysis of the supernatant from hapten-restimulated immune cells obtained from skin-draining lymph nodes of WT and PAR2 mutant mouse strains 24 h after challenge. Consistent with the data presented above, IFN-γ levels were reduced in mice with attenuated late-stage skin inflammation, that is, PAR2$^{INS}$ and KLK5-sensitive PAR2$^{G37I}$ mice, but not in PAR2$^{K36E}$ mice with unaltered PAR2 cleavage by FXa (Figure 7E). In line with profound suppression of the CHS reaction in PAR2$^{INS}$ and PAR2$^{G37I}$ mice, pro-inflammatory cytokines tumor necrosis factor alpha (TNF-α), interleukin (IL)-1β and IL-6 as well as several macrophage- and monocyte-derived or -attracting chemokines (MIP-1α, MIP-1β, MIP-2, RANTES, GM-CSF, M-CSF) were also reduced after allergen-specific activation of immune cells obtained from the late phase of CHS (Figure 7E). In contrast, PAR2 sensitivity to FXa proteolytic activity in PAR2$^{K36E}$ mice resulted in a pattern of cytokines, chemokines, and colony-stimulating factors expression very similar to WT during the late challenge phase of skin inflammation (Figure 7E), supporting the important function of FXa in PAR2-mediated skin inflammation.

## 3 | DISCUSSION

Our study reveals that myeloid cells exhibit increased PAR2 expression in the mouse and human allergic contact dermatitis and that myeloid cell–specific PAR2 signaling is crucial for the development of T cell–mediated inflammatory skin diseases. PAR2 activation is prevented by blockade of TF and FXa during either sensitization or challenge and experiments with mutant mice of defined PAR2 insensitivity demonstrate a pivotal role for FXa in the late phase of skin inflammation, providing evidence that the PAR2–TF–FXa axis is crucial for the development of cutaneous inflammatory disorders.

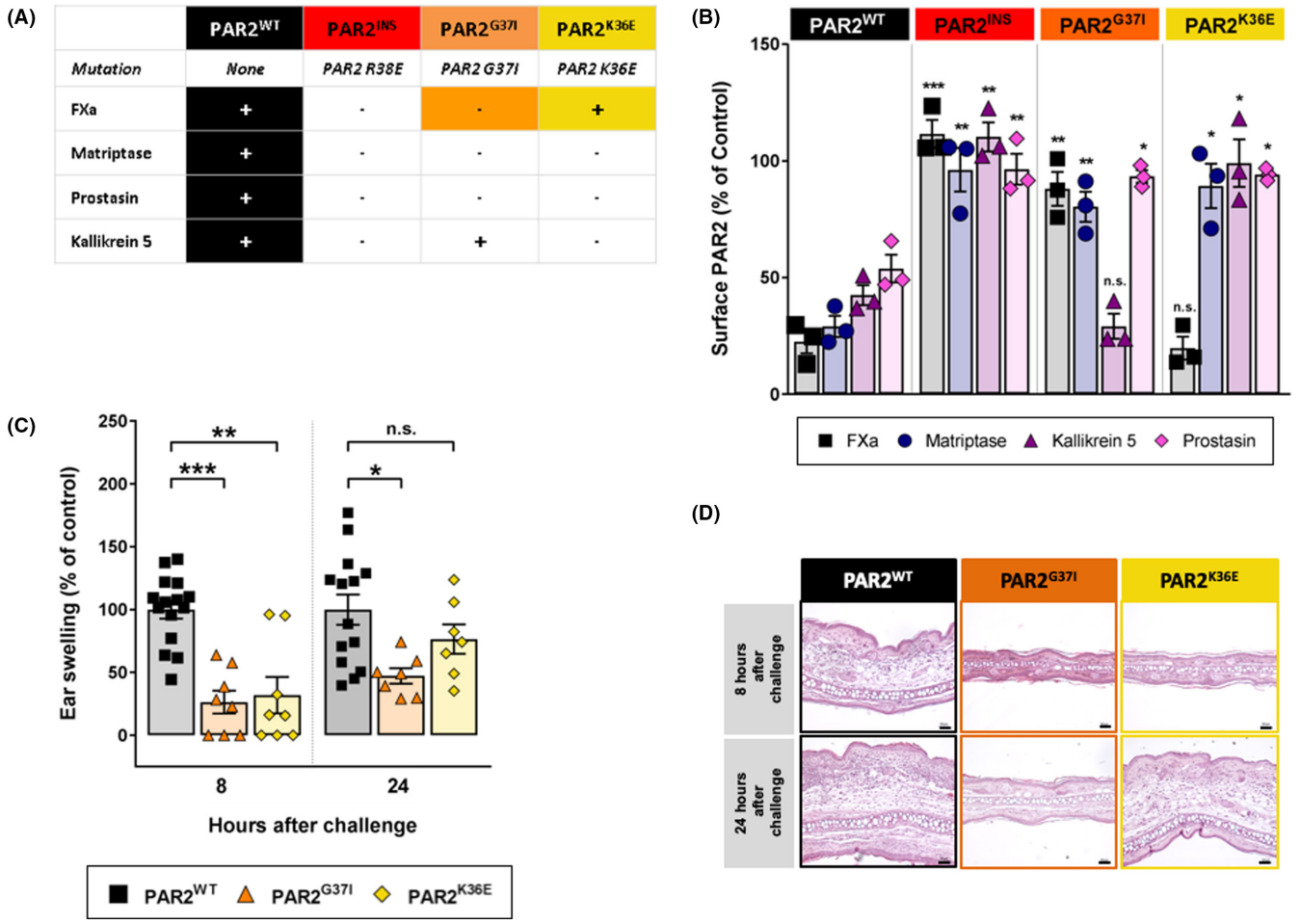


FIGURE 6 Factor Xa (FXa)-mediated protease-activated receptor 2 (PAR2) cleavage is crucial in the late contact hypersensitivity effector phase. A, Summary of experimentally determined FXa and skin protease sensitivity of PAR2 mutants. B, PAR2 cleavage assay with cells transiently transfected with Flag-tagged human PAR2 wild-type (black), $PAR2^{INS}$ (red), $PAR2^{G37I}$ (orange), or $PAR2^{K36E}$ (yellow); numbering corresponds to the residues in the mouse PAR2 sequence. Cells were exposed to 1 nM FVIIa/50 nM FX to produce FXa, 5 nM matriptase, or 25 nM prostasin and kallikrein 5 for 1 h followed by detection of residual PAR2 surface expression by cell ELISA (see Supplementary Methods); mean ± standard deviation; Welch's *t*-test. C, Ear swelling 8 or 24 h after hapten challenge of sensitized $PAR2^{G37I}$ mice (orange), $PAR2^{K36E}$ mice (yellow), and strain-matched wild-type controls (black); mean ± standard error of the mean, pooled data of 4 independent experiments, unpaired *t*-test. D, Hematoxylin and eosin–stained tissue sections of ear skin of the same experimental groups 8 and 24 h after challenge; magnification 20x, scale bars = 50 µm. For all panels, ns, not significant; $^{*}p < .05$, $^{**}p < .01$, and $^{***}p < .001$

PAR2 is expressed by various cells of the skin, especially keratinocytes, endothelial cells, and sensory nerves;[8,26,47,48] and regulates keratinocyte proliferation and differentiation,[49] the maintenance of the epidermal barrier,[50] and the development of itch.[48,51] Previous studies demonstrated that expression of PAR2 is associated with skin barrier defects in cutaneous disorders such as atopic dermatitis,[13,52–55] Netherton syndrome,[11,56] and ichthyosis,[14] including a role in Th2-skewed immune responses.[11–13,15,55] These observations suggested that protease-mediated PAR2 cleavage in skin cells is sufficient to trigger inflammatory skin responses, but overlooked the crucial role of immune cells in inflammatory skin diseases and the sensitization and triggering of cutaneous allergies.[23,24]

CHS mimicking ACD in humans is an allergen specific Tc1/Th1 T cell reaction, which is induced and elicited by epicutaneous exposure to contact allergens.[23,24] In addition to the critical role of T cells in inflammatory allergic skin diseases, several studies have demonstrated that the development of CHS is controlled by myeloid cells and DC as players of the innate immune system that critically contribute to T cell priming, migration, and re-activation.[23,24] A decade ago, Ramelli et al.[57] showed that PAR2 deficiency affects DC lymph node trafficking and subsequent T cell activation in CHS. However, due to the use of general PAR2 knockouts, the observed effects of lacking PAR2 expression could not be attributed to a specific cell type. Our study demonstrates that PAR2 is highly expressed on myeloid cells in the re-challenged skin of patients with ACD and skin lesions of CHS. Importantly, use of cell-specific PAR2 knockouts reveals that the cell-specific absence of PAR2 on myeloid cells, but not on DC, critically regulates CHS development.

PAR2 is stimulated by a variety of proteases expressed within the skin[14,15] and by proteases involved in the coagulation cascade. These proteases act in specific cellular contexts and signaling pathways, emphasizing that the identification of functionally relevant

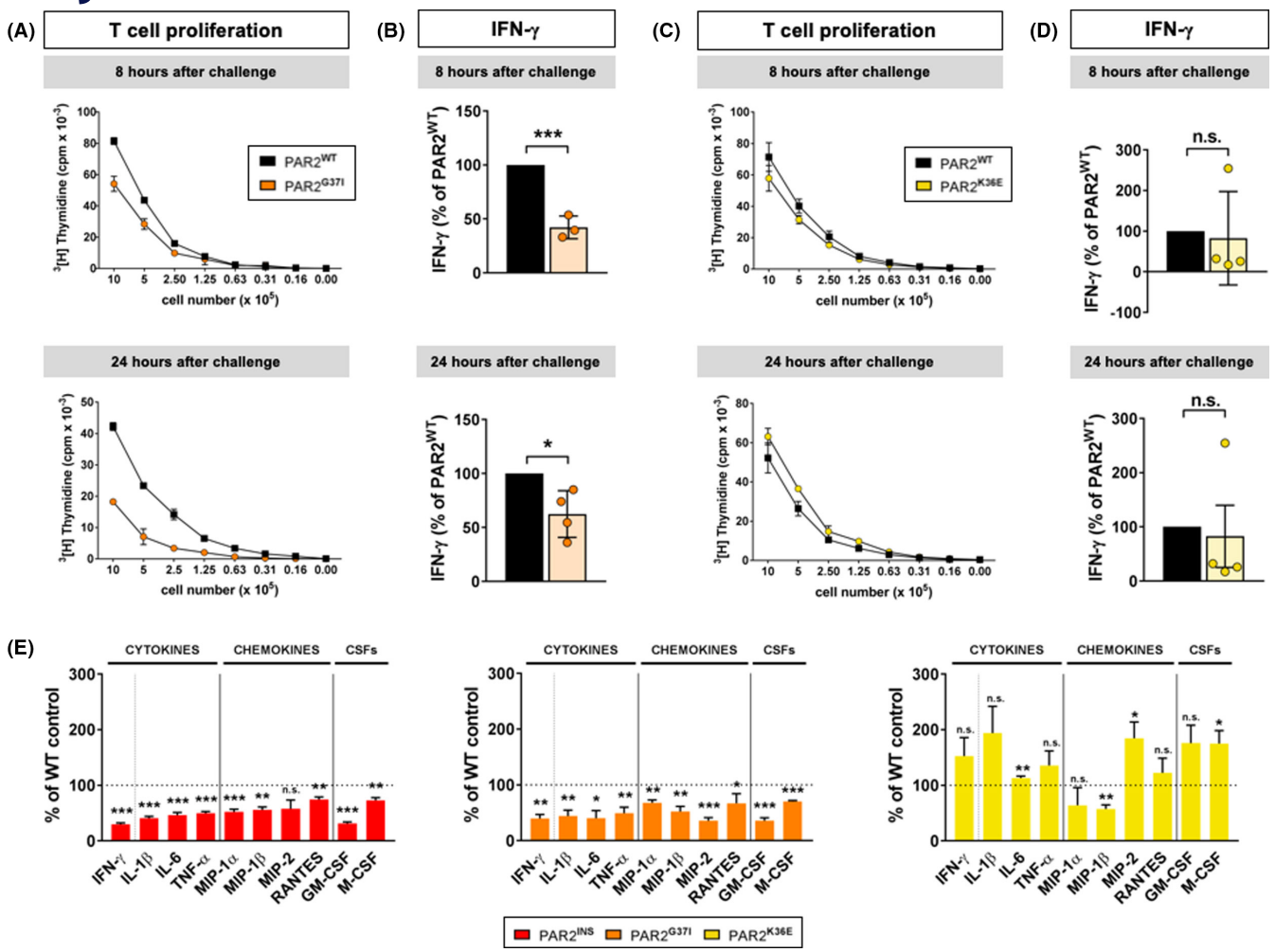


FIGURE 7 Protease-activated receptor 2 (*PAR2*) cleavage selectivity influences the hapten-specific T cell contact hypersensitivity response. A, C, T cell proliferation after restimulation of draining lymph node (LN) cells from PAR2$^{G37I}$ mice (A) or PAR2$^{K36E}$ mice (C) and strain matched wild-type (WT) controls (black). T cells were isolated 8 h (upper panels) and 24 h (lower panels) after challenge; mean ± standard deviation (SD) of technical triplicates, pooled data of 6–7 mice/group; representative data of 2–4 independent experiments. B, D, Release of interferon (IFN)-γ by T cells after hapten-specific restimulation; means ± SD normalized to IFN-γ production by PAR2$^{WT}$ control; pooled data of five to six independent experiments in (B) and of two independent experiments in (D); unpaired *t*-test. E, Multiplex cytokine assays of hapten-specific *in vitro* restimulated skin draining LN cells isolated 24 h after *in vivo* challenge from sensitized PAR2$^{WT}$, PAR2$^{INS}$, PAR2$^{G37I}$, and PAR2$^{K36E}$ mice, pooled data of three (PAR2$^{INS}$, PAR2$^{G37I}$) or four (PAR2$^{K36E}$) experiments with at least 5 mice per group. For each assay, cytokine levels for mutants were normalized to PAR2$^{WT}$ analyzed in parallel and shown as % of WT (dotted line); mean ± standard error of the mean; unpaired *t*-test; ns, not significant; $^{*}p < .05$, $^{**}p < .01$ and $^{***}p < .001$

PAR2-activating proteases may yield new therapeutic targets for the treatment of skin inflammation. To decipher the precise role of proteases in PAR2 activation, we tracked the involvement of all components of the TF–FVIIa–FXa–PAR2 axis over the time course of the allergic skin inflammation. Both the general resistance of PAR2 to proteolytic cleavage and the blockade of TF or FXa, respectively, completely prevented development of the inflammatory skin response. Additional details of the temporal progression in the challenge phase were revealed by PAR2 mutant mice with differential sensitivity to FXa. These experiments pointed to a crucial role of FXa-dependent PAR2 activation in the inflammatory reaction specifically in the late phase of cutaneous inflammation.

In addition to TF, FVIIa, and FXa,[7,14,15] the skin proteases matriptase, prostasin, and KLK5 are potent activators of PAR2 signaling.[41,42] KLK5 activation in the epidermis induces a skin barrier defect associated with atopic dermatitis–like lesions through PAR2-stimulated thymic stromal lymphopoeitin production in SPINK5 KO mice mimicking Netherton syndrome.[11] Overexpression of the serine protease CAP1/PRSS8 (the murine homolog for human prostasin) in keratinocytes causes impaired skin barrier function, itch, and inflammation *in vivo* that are mediated by downstream proteolytic PAR2 activation.[9] However, our observations in PAR2$^{G37I}$ mice showed that KLK5 and possibly other KLKs are not important PAR2 activators in the CHS model, but the PAR2 mutant mice suggest a role for matriptase or a similar protease in the early inflammation phase in rechallenged skin.

Whereas keratinocytes in the basal layer express TF constitutively, endothelial and immune cells typically do not synthesize the

coagulation initiator in the absence of an inflammatory stimulus.[58] We demonstrate that TF, PAR2, and FX are expressed at higher levels in the inflamed skin tissue of CHS mice. Previous studies demonstrated that macrophage-related TF provoked cutaneous delayed-type hypersensitivity reactions of the skin.[32] Our observations extend these findings by showing that TF activity is obligatory for CHS development during both the induction and effector phase and is involved in the induction and activation of the hapten-specific Tc1/Th1 T cell response.

Pharmacological inhibition of FXa by rivaroxaban for cardiovascular indications in broad clinical use is accompanied by anti-inflammatory effects of the target selective anticoagulant.[59,60] Preclinical studies in acute lung injury,[61] type 2 diabetes,[62] or experimental autoimmune encephalomyelitis[63] implicate FXa in innate immune mechanisms, specifically neutrophil and myeloid cell adhesion and extravasation.[60–63] In our study, we observed that abrogation of the allergic skin inflammation by rivaroxaban was accompanied by reduced local edema and impaired cutaneous inflammatory cellular infiltrate. In summary, we identify myeloid cell–specific PAR2 activation and the TF–FXa–PAR2 axis as essential components of T cell–mediated inflammatory skin responses, revealing this pathway as a potential target in the topical or systemic treatment of cutaneous inflammatory disorders.

## 4 | METHODS

### 4.1 | Mice

Age- (6–12 weeks) and sex-matched mutant and WT mice of the same genetic background were bred under identical housing and special pathogen-free conditions in the central animal facility of the University Medical Center Mainz. All mice were housed in a controlled room under conventional conditions with 23°C room temperature, 60% humidity, and maintained on a 12:12 h dark:light cycle. Food and water were available ad libitum. Animal studies were approved by the Animal Care Committee of Rhineland-Palatinate (LUA 23177-07/G 12–1-030 and LUA 23177-07/G 15–1-002) and supervised by the Animal Protection Representatives of the University Medical Center Mainz. For inhibitor treatments, female C57BL/6N WT mice were purchased from Jackson Laboratories (Bar Harbor). The following mutant mouse strains were used on a C57BL/6N background and generated, as previously described: completely cleavage-insensitive $PAR2^{INS}$ ($PAR2\text{-}^{R38E}$) mice,[4,16,64] FXa-resistant $PAR2^{G37I}$ mice,[3,34] and $PAR2^{fl/fl}$ mice.[3] $PAR2^{fl/fl}$ mice were crossed with either LysM-cre ($Lyz2^{tm1(cre)Ifo}$) or $Tg(Itgax\text{-}cre)^{1\text{-}1Reiz}$ to generate celltype–specific deletion mutants of PAR2.[3,31] Experiments with conditional deletions employed cre driver–negative littermates of the same sex as controls. The PAR2 protease cleavage selective $PAR2^{K36E}$ was generated by pronuclear injection and CRISPR-mediated targeting to change the *F2rl1* coding sequence from 5′ AGT AAA GGA AGA AGT 3′ (S **K** G R S) to 5′ AGT gAA GGA cGA tcg 3′ (S **E** G R S), which also introduced the diagnostic silent PvuI restriction site. Founders were bred in C57BL/6N mice to homozygosity and the sequence confirmed by genomic sequencing. C57BL6/N were employed as controls for this strain. $F7^{tm1a(EUCOMM)Hmgu}$/H mice from the European Mouse Mutant Archive (EMMA) were crossed with a flp driver line to generate $F7^{tm1c(EUCOMM)Hmgu}$/H and crossed with LysM-cre ($Lyz2^{tm1(cre)Ifo}$) to delete FVII in myeloid cells. All strains were confirmed to carry the undeleted NNT gene typical for C57BL/6N.

### 4.2 | Protocol of CHS and ACD induction and pharmacological treatments

At day 0, mice were ensitized by application of 450 µg TNCB (picrylchloride, 2,4,6-trinitro-1-chlorobenzene; VeZerf Laborsynthesen) dissolved in 15 µl acetone/olive oil (AOO; vol/vol 3:1) onto the shaved abdominal skin. Mice were challenged 5 days later by painting 45 µg TNCB onto the dorsal side of both ears (Figure S1). Ear thickness was measured at baseline and after 8 and/or 24 h with an engineer's micrometer (Oditest, Kroeplin) and the increase in ear thickness calculated in absolute values or percentage of baseline thickness. For additional measurements, mice were sacrificed at 8 or 24 h after challenge; ears were processed for histology or flow cytometric analyses; and auricular, cervical, and inguinal lymph nodes were taken for analysis of the immune response after challenge. Chronic inflammation was induced by weekly sensitizations on the abdomen, followed by a challenge 6 days later on the ear over a period of 5 weeks.

The anti-mouse TF monoclonal antibody 21E10[65] and the corresponding isotype-matched control IgG2a antibody were given intraperitoneally at 20 mg/kg at defined times, as indicated in the figures. In some experiments, mice were fed with a 0.4 mg/g rivaroxaban formulated as described[3] in chow diet with a drug kindly provided by Bayer for different durations as indicated in the figures. The human studies, immunohistochemistry, quantitative image analysis, and RT-PCR are described in the Supplementary Methods.

### 4.3 | Cell culture, proliferation assay, and cytokine/chemokine measurements

For analysis of the hapten-specific T cell responses, LN cells were isolated from skin draining LN after challenge for the indicated times. For assessment of T cell proliferation, pooled LN cells ($10^7$/ml) from the same genotype were restimulated with hapten 10 mM TNBS (picrylsulfonic acid, 2,4,6-trinitro-benzenesulfonic acid; Sigma) in HBSS (Hank's Balanced Salt Solution without $Ca^{++}$, $Mg^{++}$ and Phenol Red; BioWhittaker) for 10 min, washed three times in RPMI-1640. and resuspended in RPMI-complete supplemented with 2% normal mouse serum for 24 h. Cultured cells were then pulsed for 18 h with 1 µCi [$^3$H] thymidine per well (Amersham Biosciences Europe) and incorporated radioactivity was determined by liquid scintillation counting (1205 Betaplate, LKB Wallac).

Results of T cell proliferation are expressed as mean ± standard deviation counts per minute (cpm) calculated from triplicate wells. T cell proliferation of LN cells from hapten-exposed animal without hapten-specific restimulation *in vitro* was consistently low in all experiments (not shown).

For assessment of cytokine production by T cells, LN cells were obtained from CHS animals after challenge for the indicated times. T cells ($5\times10^5$), pooled from several mice of the same geneotype, were purified by T Cell Isolation Kit (MACS© system, Miltenyi Biotec), and cocultured in 200 µl RPMI-1640 supplemented with 2% normal mouse serum at 37°C/5% $CO_2$ with irradiated (3000 rad) control or hapten-exposed LN cells ($5\times10^5$) obtained from untreated animals as antigen-presenting cells. In some experiments, hapten-restimulated LN cells from CHS animals were analyzed for IFN-γ production. Cell-free supernatants were collected at 24 and 48 h and immediately frozen at −80°C for cytokine ELISA for IFN-γ (R&D Systems) at a later time. For multi-analyte protein profiling, unstimulated and hapten-restimulated LN cells ($10^6$/well) from hapten rechallenged mice were incubated in 200 µl RPMI complete supplemented with 2% normal mouse serum for 24, 48, and 72 h at 37°C/5% $CO_2$. IFN-γ, IL-1β, IL-6, TNF-α, MIP-1α, MIP-1β, MIP-2, RANTES, GM-CSF, and M-CSF in the supernatants were measured with a MagPIX (eBioscience; Thermo Fisher Scientific) mouse multiplex panel. Cytokine levels determined for mutant mice were normalized to cytokine levels in WT mice (=100%) measured at the same time point after restimulation *in vitro*.

### 4.4 | Statistical analyses

Statistical analysis was performed on data from biological replicates derived from experiments performed on several occasions. Differences between experimental groups were evaluated by paired or unpaired, two-sided Student *t*-test, Mann–Whitney U test, or Welch's *t*-test using GraphPad Prism version 7.0.3 (GraphPad Software).

## AUTHOR CONTRIBUTIONS

M. I. Fleischer: designing research studies, conducting experiments, acquiring data, analyzing data, creating of figures, writing and critical revision of the manuscript. N. Röhrig: conducting experiments. V. K. Raker: conducting multiplex assays, support in conducting flow cytometry, critical revision of the manuscript. J. Springer: conducting clinical trial, conducting, acquiring, and analyzing of immunohistochemistry. D. Becker: designing and conducting clinical trial. S. Ritz: conducting, acquiring, and analyzing of immunohistochemistry. M. Bros: conducting RT-PCR. H. Stege: conducting RT-PCR. M. Haist: critical revision of the manuscript. S. Grabbe: critical revision of the manuscript. J. Haub: support in flow cytometry conduction. C. Becker: critical revision of the manuscript. S. Reyda: generation and breeding of mouse strains, critical revision of the manuscript. J. Disse: critical revision of the manuscript. T. Schmidt: support in conducting of experiments. K. Mahnke: critical revision of the manuscript. H. Weiler: generation and breeding of mouse strains, critical revision of the manuscript. W. Ruf: providing animals and reagents, designing research studies, analyzing data, critical revision of figures, writing and critical revision of the manuscript. K. Steinbrink: designing research studies, analyzing data, critical revision of figures, writing and critical revision of the manuscript.

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## CONFLICTS OF INTEREST

No relevant conflicts of interest are related to this study.

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## SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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