Unravelling the underlying mechanism of Ritlecitinib's therapeutic actions in alopecia areata

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Background

Alopecia areata (AA) is an autoimmune-mediated inflammatory hair loss disorder in which IFNγ, secreted by CD8+ T-cells, exacerbates peri- and intrafollicular inflammation and induces hair follicle (HF) immune privilege (IP) collapse, characterized by increased expression of MHC class I and II, downregulation of IP guardians and increased expression of pro-inflammatory mediators [1-3]. T-cell activation is regulated by T-cell receptor and IL-2 receptor stimulation and subsequent downstream induction of JAK3 and TEC family kinases (Figure on the right) [4]. The clinical relevance of JAK3/TEC family kinases signalling in AA has been demonstrated by the approval of the JAK3/TEC family kinases inhibitor ritlecitinib for the treatment of severe AA [5].

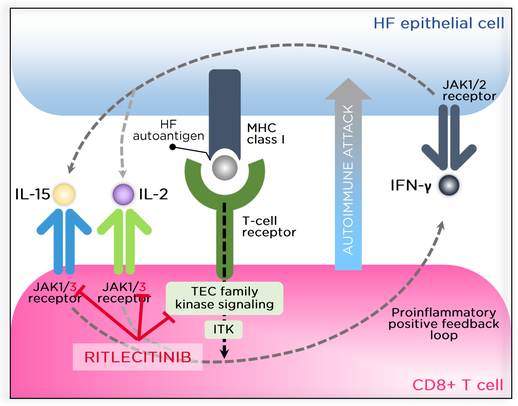


Figure adapted from [9, 10].

Engagement of the T-cell (TCR) and IL-2 (IL2R) receptors induce a positive proinflammatory feedback loop via activation of the interleukin-2-inducible T-cell kinase (ITK) and the JAK1/3 receptors, respectively [3]. This activation enhances the inflammatory milieu in AA scalp. Furthermore, TCR and IL-2R activation regulate proliferation, activation, differentiation and maintenance of CD8+ cells. Cytotoxic CD8+ cells contribute to the IP collapse observed in AA-affected hair follicles. The JAK3/TEC inhibitor Ritlecitinib is approved for the treatment of severe AA [5-8].

Aim of the Study

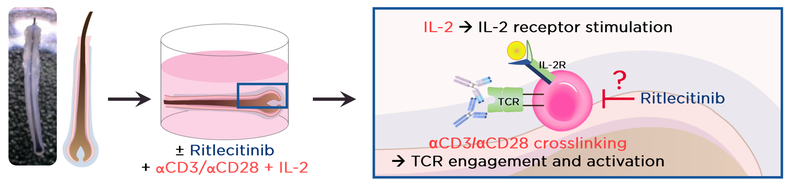
Here we analysed how inhibition of JAK3/TEC family kinases signalling by Ritlecitinib affects alopecia areata pathogenesis in human HFs induced with an alopecia areata-like phenotype by activation of JAK3/TEC family kinases signalling, and in alopecia areata lesional skin *ex vivo*.

Material & Methods

Lesional AA scalp skin *in situ*

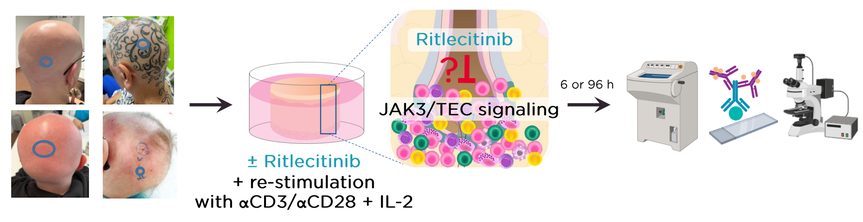
Qualitative immunofluorescence was performed on freshly embedded sections from healthy and chronic lesional AA scalp skin with and without active inflammation to analyse JAK and TEC signalling activity. CD3+pSTAT5+, pSTAT3 and pSTAT6+ cells were assessed as marker for JAK3 signaling and IRF4+ and CD8+GzmB+ cells were assessed as marker for TEC signaling.

Experimentally induced AA-like phenotype *ex vivo*



Microdissected, full-length, healthy human hair follicles (HFs) were treated with vehicle control or anti-CD3 and anti-CD28 antibodies (αCD3/αCD28) + IL-2 for 5-6 days *ex vivo* to induce T-cell activation on resident peri-follicular T cells via TCR (TEC signalling activation and IL-2 receptor stimulation (JAK3 signalling activation), respectively. 2µM Ritlecitinib was added one day prior to the cytokines. Afterwards, CD3+ T-cell numbers and proliferation, immune privilege status and the release of pro-inflammatory and cytotoxic mediators were assessed.

Lesional AA scalp skin *ex vivo* + Ritlecitinib treatment



Chronic lesional AA scalp skin was obtained from 4 patients. Scalp skin was re-stimulated with αCD3/αCD28 + IL-2 and treated with vehicle control or Ritlecitinib. After 6 or 96 hours of culture, samples were embedded for cryosectioning and quantitative immunofluorescence analysis was performed on activity markers for JAK3 and TEC signaling.

Results

Lesional AA scalp skin *in situ*

JAK3/TEC signaling is active in chronic lesional AA scalp skin

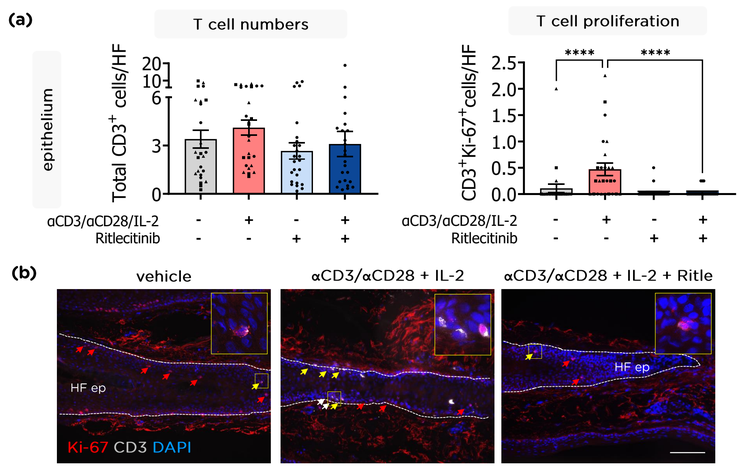
		Healthy biopsies		Typical chronic AA		Chronic AA with active inflammation	
	Cells	HF epi	CTS + surrounding tissue	HF epi	CTS + Pb inf	HF epi	CTS +Pb inf
JAK	CD3+ pSTAT5+	-	-+	-+	+	++	+++
JAK	pSTAT3+	-	+	-+	++	+	+++
JAK	pSTAT6+	+	-+	++	-+	++	+
TEC	IRF4+	+	-+	+	-+ / +	++	-+ / +
TEC	CD8+ GrzmB+	-	-+	-+	+	-	++

-+ very few positive cells + few positive cells ++ positive cells +++ many positive cells -+ / + donor dependent

Table 1. Marker expression of JAK3 and TCR-TEC signalling, as well as cytolytic CD8+GrzmB+ T-cell expression in healthy human skin compared to chronic, lesional AA skin with and without active inflammation. pSTAT3 is induced by various cytokines (e.g. IL-6, IL-10, IFNs, TNF-α...) and growth factors [9], pSTAT5 is induced by, amongst others, IL-2 and IL-15 [10], pSTAT6 is induced by Th2 cytokines, e.g. IL-4 and IL-13 [11], and IRF4 is induced by ITK or IL-2R engagement, inducing CD8+ cell differentiation into cytotoxic GranzymeB+CD8+ cells [6,8]. The table is based on data obtained from n=3 independent donors or patients. AA++ biopsies: biopsies from chronic AA donors with active inflammation, HF epi: hair follicle epithelium, CTS: connective tissue sheath, Pb inf: peribulbar infiltrate.

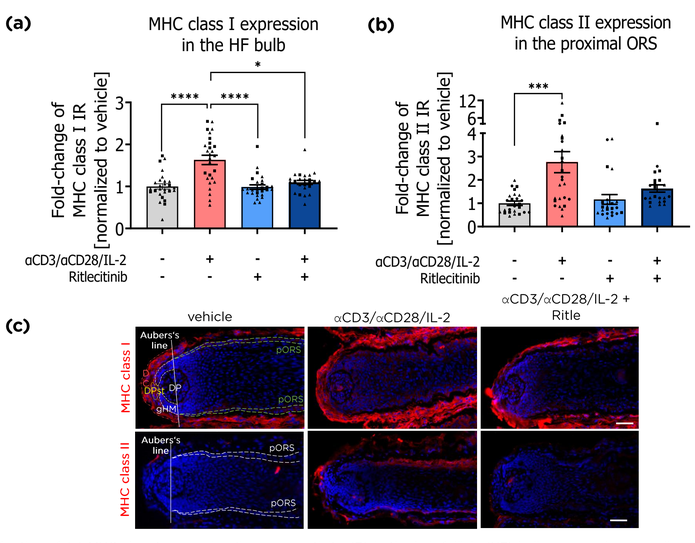
Experimentally induced AA-like phenotype *ex vivo*

Ritlecitinib reduces intra- and peri-follicular T-cell numbers and prevents T-cell proliferation



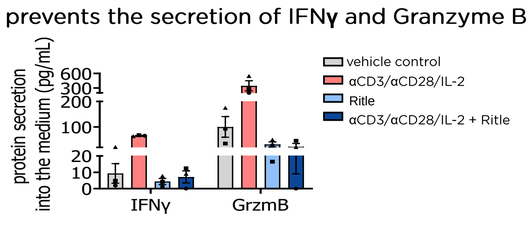
Quantification of (a) CD3+ T-cell numbers and proliferating CD3+Ki-67+ T-cell numbers averaged across the HF epithelium. Similar results were found when mesenchymal HF compartments were assessed. (b) Representative images. Scale bars = 100µm. n = 48-52 anagen+catagen HFs/group from 3 independent donors . Mean±SEM. Kruskal-Wallis with Dunn's multiple comparisons test *p<0.05, ****p<0.0001. Dots of different shape represent HFs from different donors. HF ep: hair follicle epithelium. Of note, stimulation with αCD3/αCD28/IL-2 also increased numbers of CD3+CD69+CD103+ as well as CD3+IRF4+ cells in both epithelium and mesenchyme, and treatment with Ritlecitinib tended to reduced these numbers.

Ritlecitinib significantly prevents the up-regulation of MHC class I and II protein expression



Quantification of (a) MHC class I expression (immunoreactivity; IR) in the hair follicle (HF) bulb, averaged across all analysed HF compartments (proximal outer root sheath, germinative hair matrix, dermal papilla, dermal cup and dermal papilla stalk) and (b) MHC class II expression (IR) in the proximal outer root sheath (ORS). n = 22-27 anagen+catagen HFs/group from 3 independent healthy donors (c) Representative images of MHC class I and II expression. Scale bars = 100µm. Mean±SEM. D'Agostino & Pearson omnibus normality test, Kruskal-Wallis test with Dunn's multiple comparisons test *p<0.05, ***p<0.001, ****p<0.0001. Dots of different shape represent HFs from different donors. DC: dermal cup, DP dermal papilla, DPst: dermal papilla stalk, gHM: germinative hair matrix

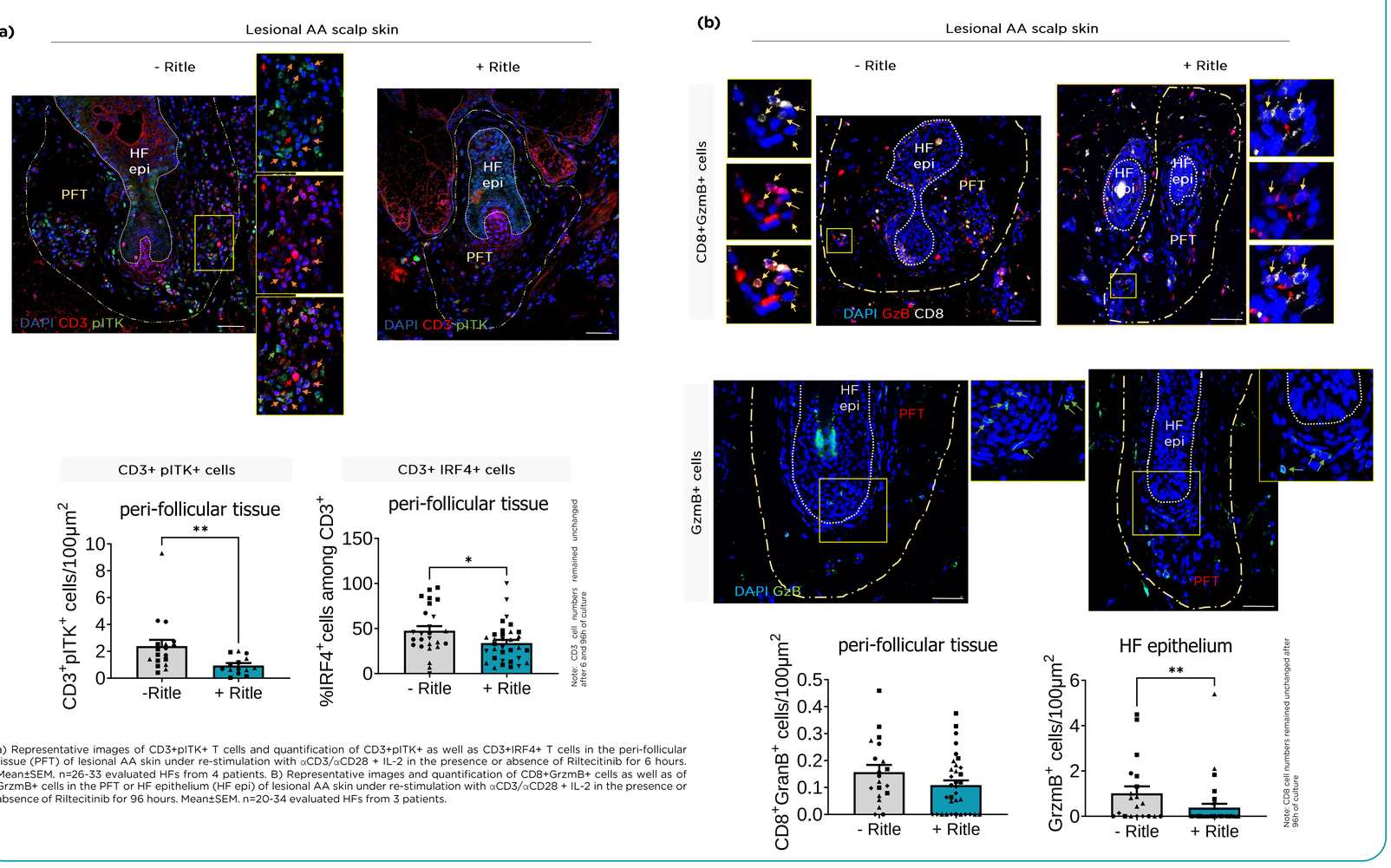
Ritlecitinib prevents the secretion of IFNγ and Granzyme B



Quantification of IFNγ and Granzyme B (GrzmB) release into the medium is shown. Similar results are found for GrzmA, granulysin and FasL Supernatant from n = 3 independent healthy donors. Mean±SEM. Measured values from 5-fold concentrated samples are shown. Dots of different shape represent different donors.

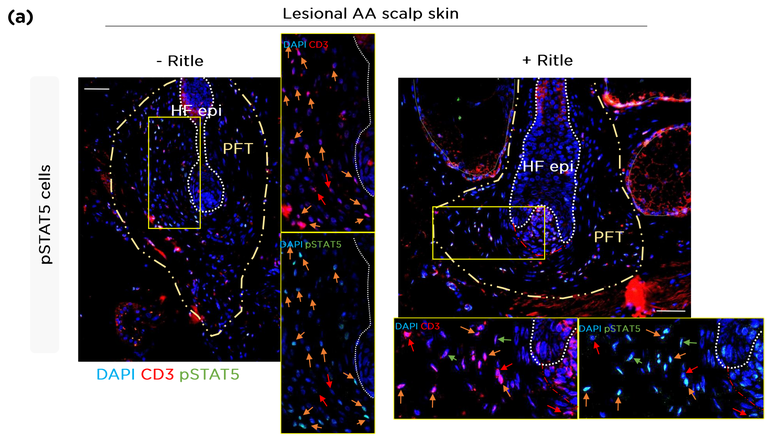
Lesional AA scalp skin *ex vivo* + Ritlecitinib treatment

Ritlecitinib decreases TEC signaling in lesional AA scalp skin



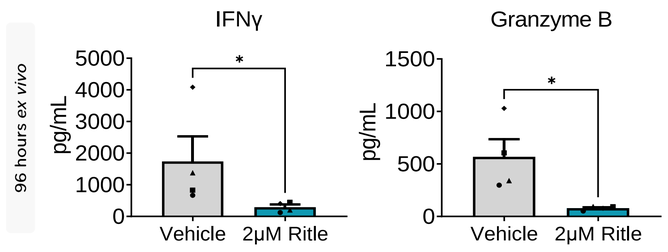
a) Representative images of CD3+pITK+ T cells and quantification of CD3+pITK+ as well as CD3+IRF4+ T cells in the peri-follicular tissue (PFT) of lesional AA skin under re-stimulation with αCD3/αCD28 + IL-2 in the presence or absence of Riltecitinib for 6 hours. Mean±SEM. n=26-33 evaluated HFs from 4 patients. B) Representative images and quantification of CD8+GrzmB+ cells as well as of GrzmB+ cells in the PFT or HF epithelium (HF epi) of lesional AA skin under re-stimulation with αCD3/αCD28 + IL-2 in the presence or absence of Riltecitinib for 96 hours. Mean±SEM. n=20-34 evaluated HFs from 3 patients.

Ritlecitinib decreases JAK3 signaling in lesional AA scalp skin



a) Representative images of pSTAT5+CD3+ T cells and (b) quantification of pSTAT5+CD3+ as well as pSTAT3+ cells in the peri-follicular tissue (PFT) of lesional AA skin under re-stimulation with αCD3/αCD28 + IL-2 in the presence or absence of Riltecitinib for 6 hours. Mean±SEM. n=35-38 evaluated HFs from 4 patients. HF epi: hair follicle epithelium

Ritlecitinib reduces the secretion of IFNγ and cytotoxic effector molecules in lesional AA scalp skin



Quantification of IFNγ and Granzyme B (GrzmB) release into the medium from lesional AA scalp skin under re-stimulation with αCD3/αCD28/IL-2 in the presence of vehicle control of Ritlecitinib for 96 hours. Similar results are found for IL-17A, IL-6, IL-10. TNFa, FasL, Granulysin and GrzmB both after 6 and 96h ex vivo. Supernatant from n = 4 patients. Mean±SEM. Dots of different shape represent different patients.

Conclusion

JAK3/TEC kinases signalling is active in lesional AA skin and its experimental stimulation induces intra- and peri-follicular T-cell expansion and HF immune privilege collapse *ex vivo*. Treatment with Ritlecitinib reduces JAK3 and TEC signaling in lesional AA scalp skin *ex vivo* and modulates key immune mechanisms in AA, including the release of IFNγ and the cytotoxic mediator Granzyme B.

Our data highlight the clinical relevance of targeting JAK3/TEC kinases pathways for the treatment of AA.

References: References: [1] Bertolini M. et al. Exp Dermatol. 2020. [2] Passeron T. et al.. Front. Immunol. 2023. [3] Gilhar A. et al. N Engl J Med. 2012. [4] Marchingo J. M. et al.Science 2014. [5] King B. et al. Lancet. 2022. [6] Nayar R. et al. PNAS. 2012. [7] Shah K. et al. Target Ther. 2021. [8] Kapnick, S.M. et al. J Immunol 2017. [9] Rébé C. et al.. JAKSTAT. 2013. [10] Shepers H. et al. JAKSTAT. 2012. [10] Karpathiou G. et al. Pathol Res Pract. 2021. [11] Dahabreh D, et al. 2023, [12] Divito SJ, Kupper TS. 2014