JAK3/TEC kinase pathways are active in alopecia areata lesions and their inhibition with Ritlecitinib prevents αCD3/αCD28+IL-2 induced immune privilege collapse in healthy human hair follicles ex vivo

Alexandre Lejeune[1], Joana Viola-Söhnlein[2], Thomas Rouillé[2] , Elizabeth Kieras[3], David A Martin[3], Jean-Baptiste Telliez[3], Leslie J Berg[4], Ilaria Piccini[2], Karin Pappelbaum[2], Janin Edelkamp[2], Marta Bertolini[2,*]

1. Pfizer Inc, Paris, France 2. QIMA Life Sciences, QIMA Monasterium GmbH, Münster, Germany 3. Pfizer Inc, Cambridge, USA 4. University of Colorado-Anschutz School of Medicine, Colorado, USA.

* Correspondence to: Marta.Bertolini@qima.com

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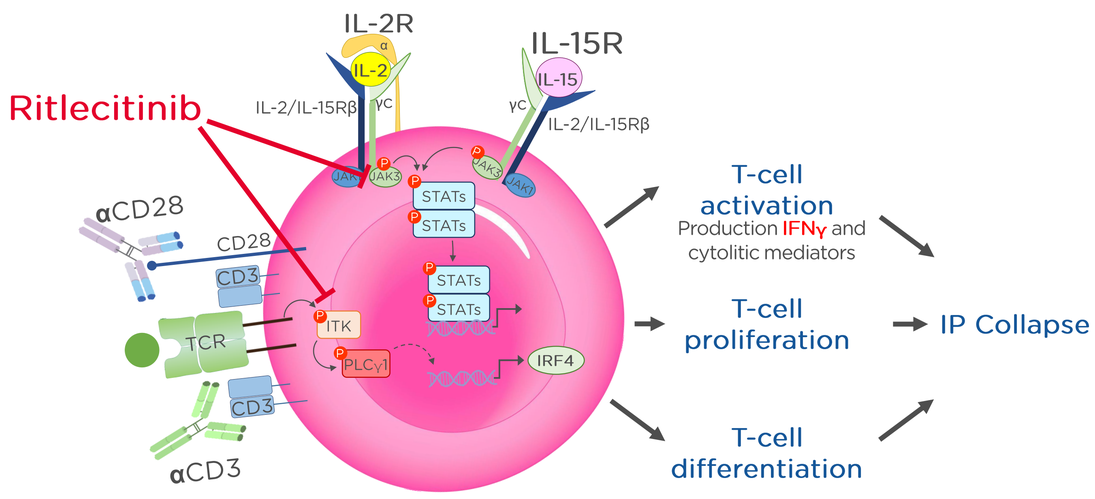
BACKGROUND

Alopecia areata (AA) is an (auto-)inflammatory disorder affecting the hair follicle (HF) bulb in which T-cell-driven inflammation induces premature catagen development and/or HF dystrophy, ultimately resulting into hair loss in affected individuals [1-3]. AA is postulated to be induced by the release pro-inflammatory factors, mainly IFNγ, from CD8+ T-cells. This induces HF immune privilege (IP) collapse, characterized by increased expression of major histocompatibility complex class I and II, downregulation of IP guardians, expression of pro-inflammatory chemoattractants, and exacerbation of the peri- and intrabulbar inflammation [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 below) [4]. The clinical relevance of JAK3/TEC signalling in AA has been demonstrated by the FDA-approval of the JAK3/TEC inhibitor Ritlecitinib (Ritle) for the treatment of severe AA [5].



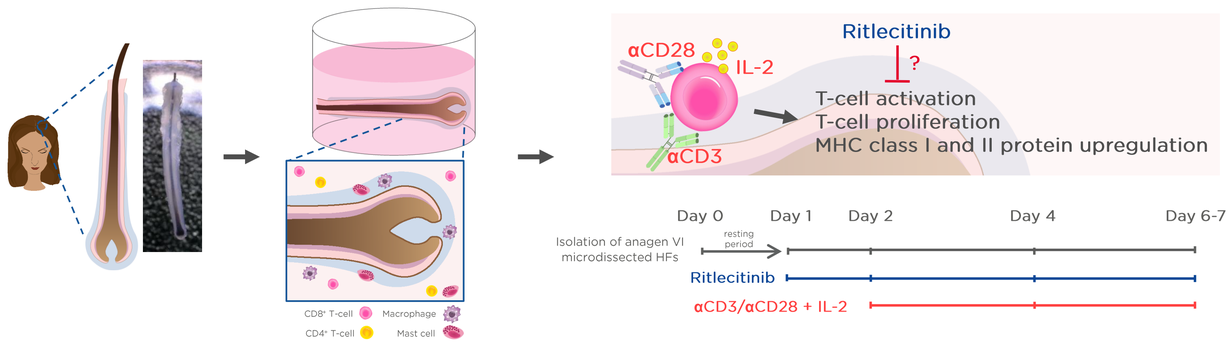
Activation of CD8+ T-cells via T-cell receptor (TCR) and IL-2 receptor (IL2R) engagement and its potential role for immune privilege collapse (IP) in alopecia areata (AA). TEC family kinases are critically involved in T-cell proliferation, and -activation through the TCR complex, and JAK3 signalling is important for the activation and maintenance of T-cells. Binding of cytokines, such as IL-2 or IL-15 to their receptors induces phosphorylation of STATs via JAK3, which is associated with the common-gamma (γc) chain of the receptors. The phosphorylated STATs subsequently translocate into the nucleus where they induce gene transcription. In addition, IL-2R activation can induce the TEC kinase ITK (IL2-inducible T-cell kinase), which enhances TCR signalling. ITK is also, amongst several others, a direct downstream target of activated TCRs, inducing IRF4 expression, which regulates CD8+ cell differentiation into cytotoxic CD8+ cells [6-8]. These CD8+ T-cells might contribute to the hair follicle IP collapse observed in AA. Ritlecitinib (Ritle) inhibits JAK3/TEC kinases and is FDA-approved for the treatment of moderate to severe AA.

AIM OF THE STUDY

Here we analysed how inhibition of JAK3/TEC signalling by Ritlecitinib affects AA pathogenesis in human hair follicles induced with an alopecia areata-like phenotype *ex vivo* and in alopecia areata lesional skin *ex vivo*.

METHODS

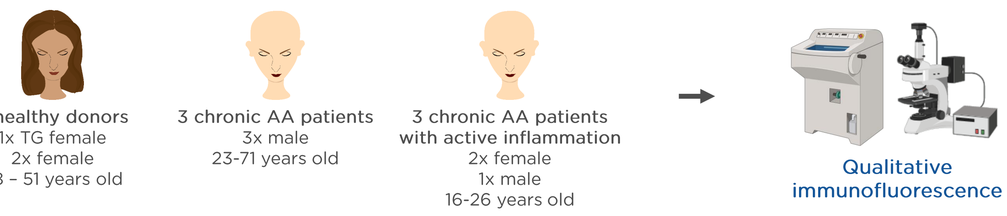
1) To evaluate how JEK3/TEC pathway activation may induce immune privilege collapse by stimulating T-cell (TCR) and IL-2 receptors in human microdissected, full length hair follicles *ex vivo* and evaluate the effect of Ritlecitinib treatment



Few T-cells are present in the perifollicular tissue of microdissected, full-length, healthy human hair follicles (HFs). We treated these HFs, 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 via TCR and IL-2 receptor stimulation, respectively. 2µM Ritlecitinib was added one day prior to the cytokines to mimic a phrophylactic treatment.

Afterwards, **CD3+ T-cell numbers** and **CD3+ T-cell proliferation (Ki-67)**, as well as **immune privilege status (MHC class I and II expression)** were assessed by quantitative (immuno)histomorphometry, and the **release of pro-inflammatory mediators (IFNγ, Granzyme B)** was examined by Legendplex.

2) To evaluate JAK3/TEC engagement in AA by analysing the expression of pSTAT5, pSTAT3, pSTAT6, IRF4 and granzyme B in biopsies from healthy donors and AA patients



3 healthy donors
1x TG female
2x female
28 – 51 years old

3 chronic AA patients
3x male
23-71 years old

3 chronic AA patients with active inflammation
2x female
1x male
16-26 years old

Qualitative immunofluorescence

Immunofluorescence was performed on **freshly embedded sections from human scalp skin (healthy or AA donors)**. pSTAT3, pSTAT5 and pSTAT6 **(JAK3 signalling)**, IRF4 **(TCR-TEC (IL2-inducible T-cell kinase) signalling)**, and CD8/Granzyme B **(cytolytic T-cells)** stainings were performed. Images were taken from the hair follicle (HF) bulb, of either anagen V/VI (healthy donors) or AA typical HFs. TG, transgender

RESULTS

Ritlecitinib reduces intra- and peri-follicular T-cell numbers and prevents αCD3/αCD28 + IL-2 induced T-cell proliferation

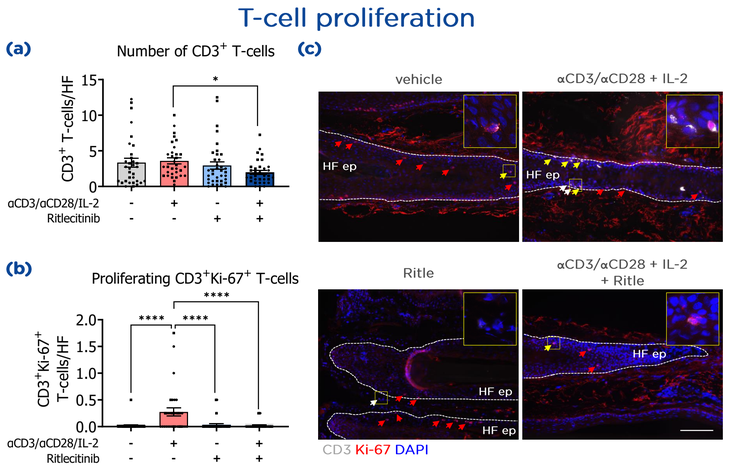


Figure 1. Effects of Ritlecitinib on intra- and peri-follicular T-cell numbers and – proliferation, in the presence of T-cell receptor and IL-2 receptor activation. Quantification of (a) CD3+ T-cell numbers averaged across the HF epithelium and mesenchyme, and (b) proliferating CD3+Ki-67+ T-cell numbers averaged across the HF epithelium and mesenchyme. (c) Representative images for a and b. White arrows: CD3+ cells, yellow arrows: CD3+Ki-67+ double positive cells. Scale bars = 100µm. n = 32-26 anagen+catagen HFs/group from two independent healthy donors. Mean±SEM. D'Agostino & Pearson omnibus normality test, no Gaussian distribution. Kruskal-Wallis test 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

Ritlecitinib significantly prevents the αCD3/αCD28 + IL-2 induced up-regulation of MHC class I and II protein expression

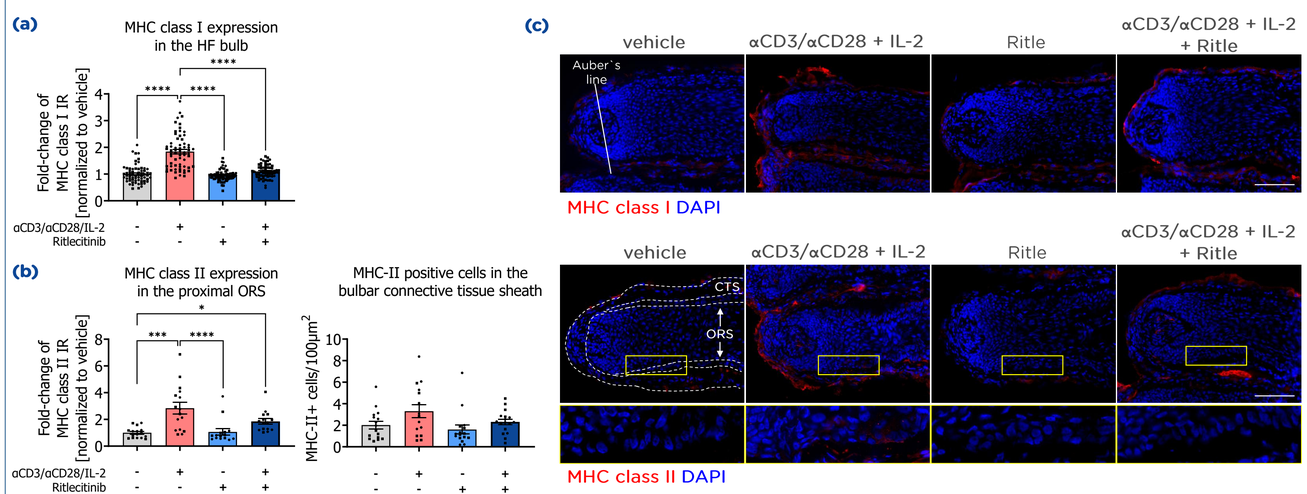


Figure 2. Effects of Ritlecitinib on MHC class I and II expression, induced by T-cell receptor and IL-2 receptor activation. Quantification of (a) MHC class I expression (immunoreactivity; IR) in the hair follicle (HF) bulb, averaged across all analysed HF compartments (proximal outer root sheath, germinative hair matrix, dermal papilla, dermal cup and dermal papilla stalk), n = 63-69 anagen+catagen HFs/group from two independent healthy donors, and (b) MHC class II expression (IR) in the proximal outer root sheath (ORS) and the number of MHC class II+ cells in the bulbar connective tissue sheath (CTS), n = 15-17 anagen+catagen HFs/group from two 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.

Ritlecitinib prevents the αCD3/αCD28 + IL-2 induced secretion of pro-inflammatory mediators

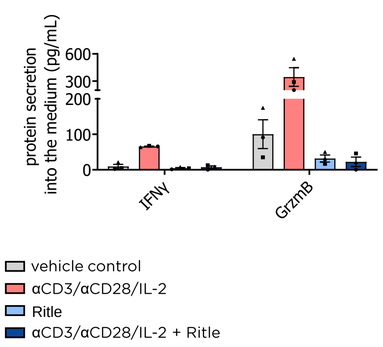


Figure 3. Effects of Ritlecitinib on the release of pro-inflammatory mediators induced by T-cell receptor and IL-2 receptor activation. The quantification of IFNγ and Granzyme B (GrzmB) release into the medium is shown. Supernatant from n = 3 independent healthy donors Mean±SEM. Measured values from 5-fold concentrated samples are shown. Dots of different shape represent different donors.

CD3+pSTAT5+, pSTAT3+, pSTAT6+, IRF4+, and CD8+GranzymeB+ cells are increased in/around HFs of chronic AA lesions with active inflammation

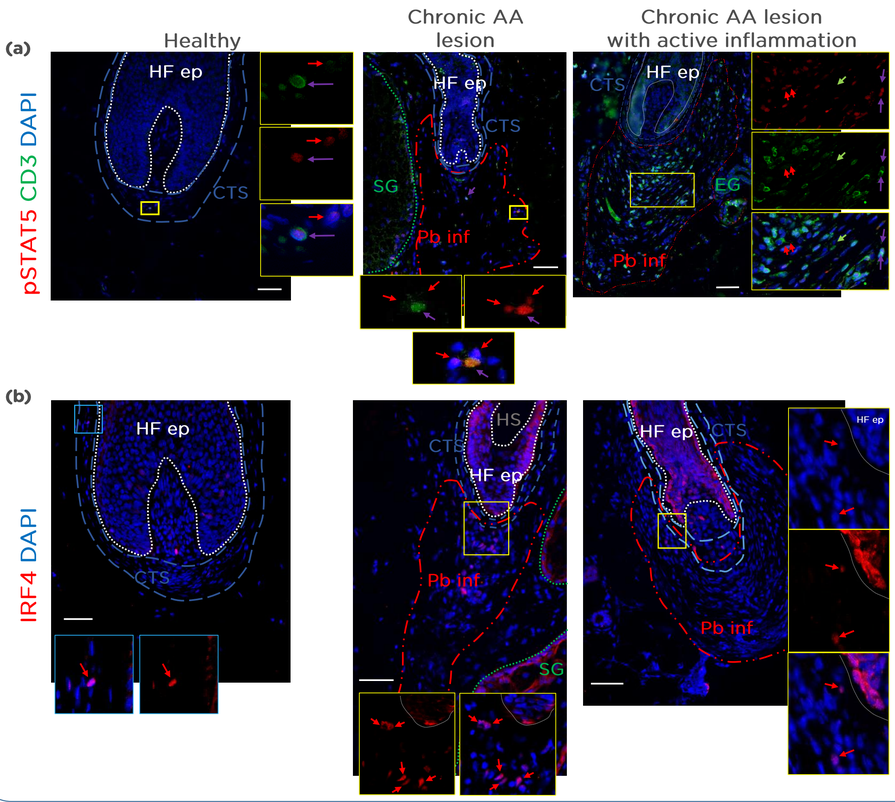


Table 1: Marker expression of JAK3 -, and TCR-TEC (ITK) signalling, as well as cytolytic CD8+ T-cell expression in healthy human hair follicles compared to chronic, lesional AA

	Healthy biopsies		Typical AA		AA++	
Cells	HF epi	CTS + surrounding tissue	HF epi	CTS + Pb inf	HF epi	CTS +Pb inf
CD3+ pSTAT5+	absent	- (few)	- (few)	+	++	+++
pSTAT3+	absent	+ (several)	- (few)	++	+	+++
pSTAT6+	+	-(few)	++	- (few)	++	+
*IRF4+	+	- (few)	+	+/- (D.D.)	++	+/- (D.D.)
CD8+ GzB+	absent	- (few)	-	+	-	++

AA++ biopsies: biopsies from chronic AA donors with active inflammation, D.D. : donor dependent, HF epi: hair follicle epithelium, CTS: connective tissue sheath, Pb inf: peribulbar infiltrate
*Cells without rounded shape were excluded from evaluation as IRF4 signalling is also active in melanocytes [10]

Figure 4. CD3+pSTAT5+, pSTAT3+, pSTAT6+, IRF4+, and CD8+GranzymeB+ cell expression in healthy human hair follicles compared to chronic, lesional AA hair follicles with and without active inflammation. (a,b) Representative images of pSTAT5 and IRF4, two of the marker that indicate activated JAK3 and TEC signalling, respectively, chosen from **Table 1** (above). (a) pSTAT5 expression, which is induced by, amongst others, IL-2 and IL-15 [9]. Red arrows: pSTAT5+ cells, Green arrows: CD3+ cells; purple arrows: CD3+pSTAT5+ cells. Scale bars: 50µm. (b) Qualitative immunofluorescence of IRF4, which acts downstream of TCR-TEC (ITK) signalling [6,8]. Red arrows: IRF4+ cells. Scale bars: 50µm. For each condition representative images of n=3 independent donors are shown. The HF epithelium (HF ep) is noted by a white dotted line and the connective tissue sheath (CTS) by blue colour. When present (AA donors), peribulbar infiltrates (Pb inf) are noted by red dotted lines. SG, sebaceous gland, EC, eccrine gland.

CONCLUSION

JAK3/TEC 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 effectively prevented these effect.

Our data highlight the clinical relevance of targeting JAK3/TEC pathways to combat AA.

DISCLOSURES

This study was sponsored by Pfizer, Inc and QIMA Life Sciences, QIMA Monasterium, Münster, Germany.
AL, DM, and JT are employees of Pfizer, Inc, and may hold stock or stock options in Pfizer, Inc.
JVS, TR, IP, KP, JE, and MB are employee of QIMA Life Sciences, QIMA Monasterium GbmH.

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