A novel *ex vivo* model of human hair follicle immune privilege collapse reveals the potential of farudodstat, a DHODH inhibitor, as a therapeutic for alopecia areata treatment

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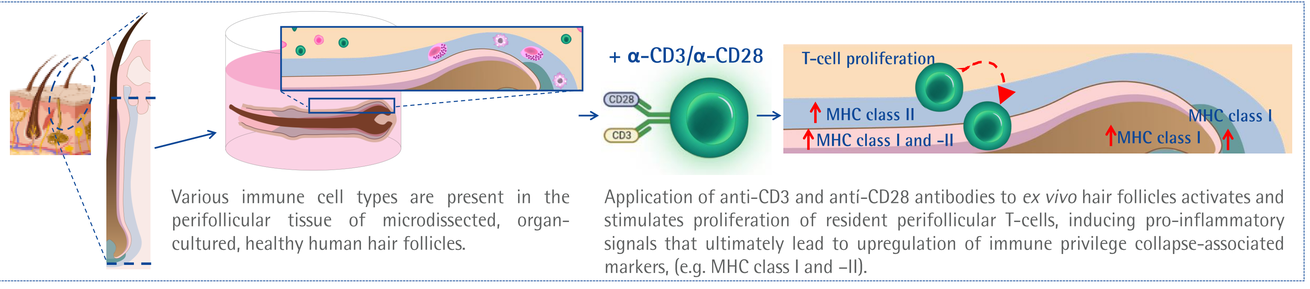
Background & Aim

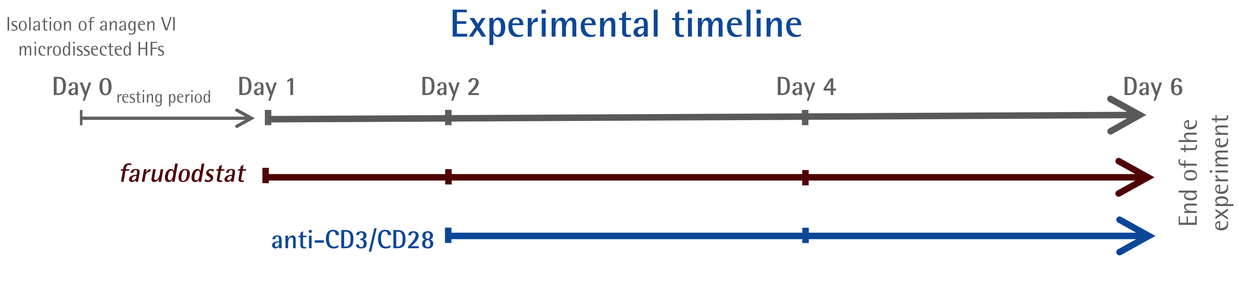
Alopecia areata (AA) is an inflammatory disorder of the hair follicles (HF) characterized by increased IFNγ levels, immune privilege (IP) collapse, and a Th1-mediated inflammatory response towards the hair bulb leading to premature transition to catagen phase, HF dystrophy and hair loss [1]. Dihydroorotate dehydrogenase (DHODH) plays a key role in T-cell proliferation and its inhibition decreases Th1-cell differentiation and IFNγ production [2,3]. Here, we investigated whether *farudodstat*, a DHODH inhibitor, could be beneficial for the treatment of AA by means of an innovative model in which healthy human HFs were stimulated *ex vivo* with anti-CD3/CD28 antibodies to promote perifollicular T-cell activation and proliferation.

Experimental Model & Setup



Various immune cell types are present in the perifollicular tissue of microdissected, organ-cultured, healthy human hair follicles.

Application of anti-CD3 and anti-CD28 antibodies to *ex vivo* hair follicles activates and stimulates proliferation of resident perifollicular T-cells, inducing pro-inflammatory signals that ultimately lead to upregulation of immune privilege collapse-associated markers, (e.g. MHC class I and –II).



Microdissected, full-length healthy human HFs were treated with 25µg/ml anti-CD3 and anti-CD28 antibodies for 4 days *ex vivo* (induction of T-cell activation), in the presence or absence of the DHODH inhibitor *farudodstat* (70nM or 140nM; IC50 = 35). IP collapse-associated markers (MHC class I and –II) and percentage of proliferating T-cells (CD3/Ki-67) were analysed by quantitative (immuno-)histomorphometry. Hair cycle staging and cytotoxicity were evaluated according to [4].

Results

Treatment with anti-CD3 and anti-CD28 antibodies upregulates immune privilege collapse-associated markers in healthy human HFs *ex vivo*

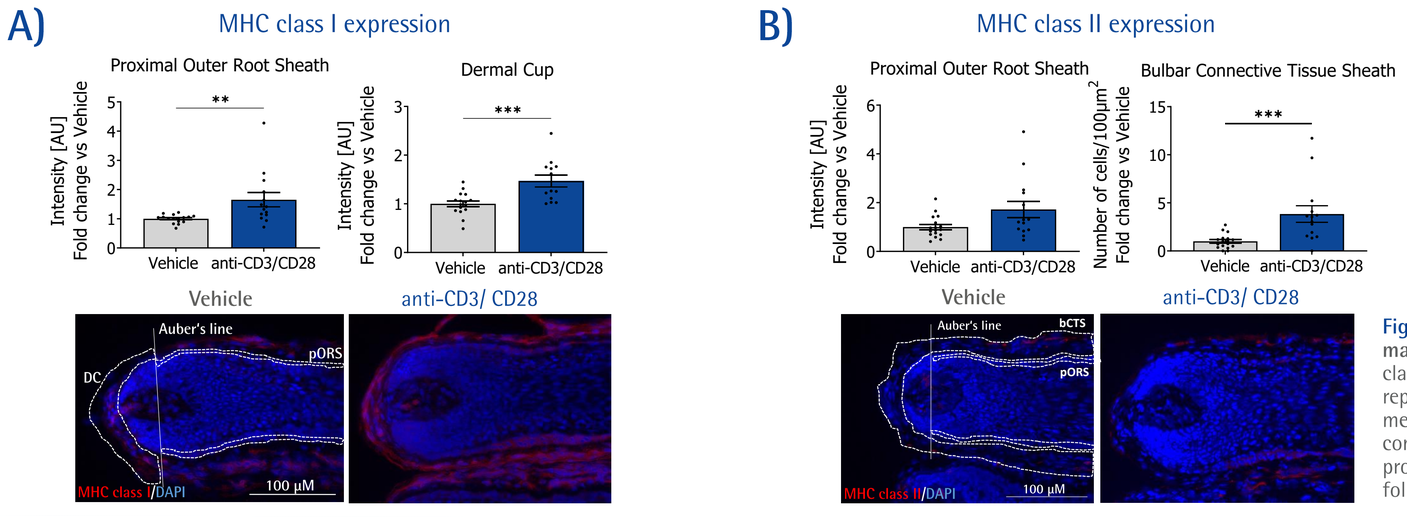
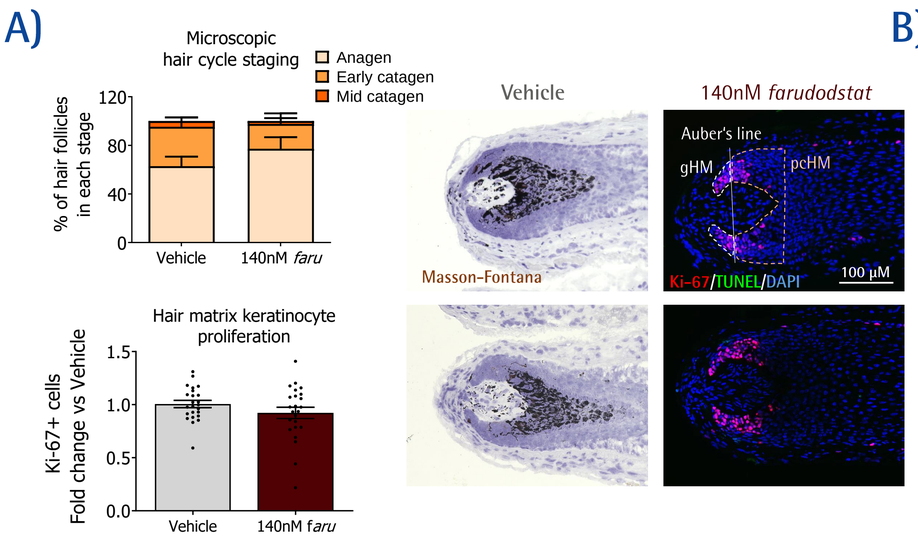


Figure 1. Effect of treatment with anti-CD3/CD28 antibodies on immune privilege (IP) collapse-associated markers and T-cell proliferation in full-length healthy human hair follicles (HFs). **(A-B)** MHC class I and MHC class II expression after 4 days of treatment with anti-CD3 and anti-CD28 (25µg/ml; upper panel) with representative images below. **(C)** Percentage of Ki-67+/CD3+ (proliferating) T-cells in the HF epithelium and mesenchyme. All data are presented as mean±SEM. n = 13-17 HFs from 2-3 donors. Treatment groups were compared using Mann-Whitney test and *p<0.05, ** p<0.01, scale bars = 100µm. The marked areas represent proximal outer root sheath (pORS), dermal cup (DC), and bulbar connective tissue sheath (bCTS) of anagen VI hair follicles.

Farudodstat alone maintains anagen phase without impacting hair matrix keratinocyte proliferation, cytotoxicity or expression of IP collapse-associated markers in healthy HFs *ex vivo*



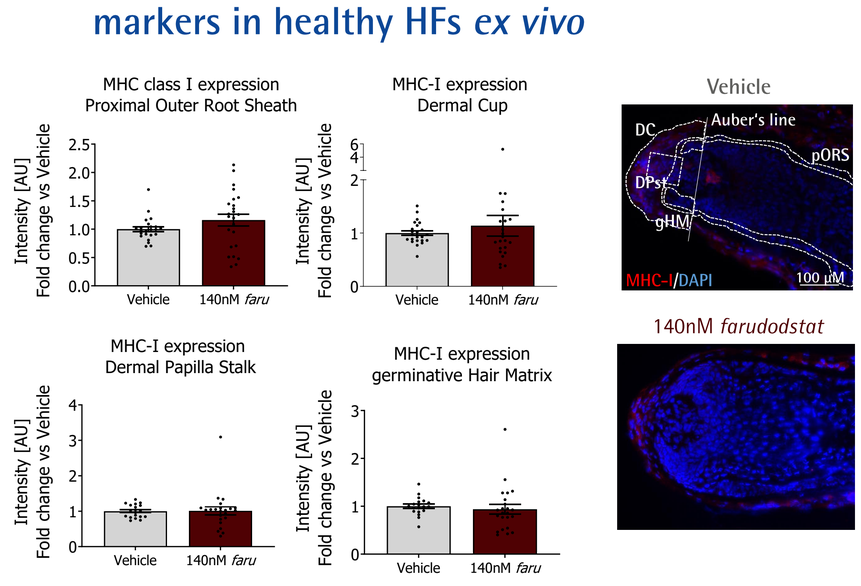
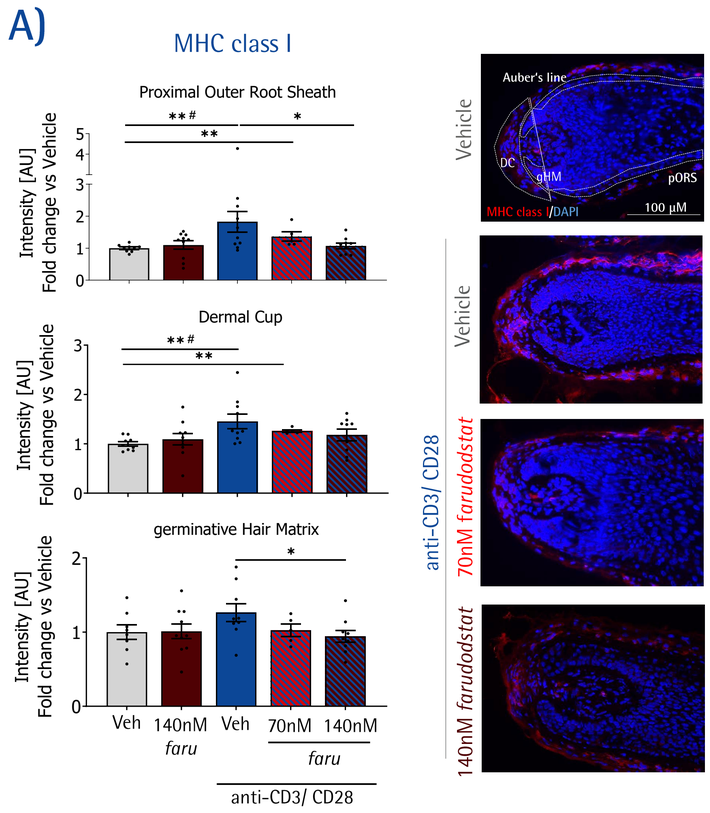
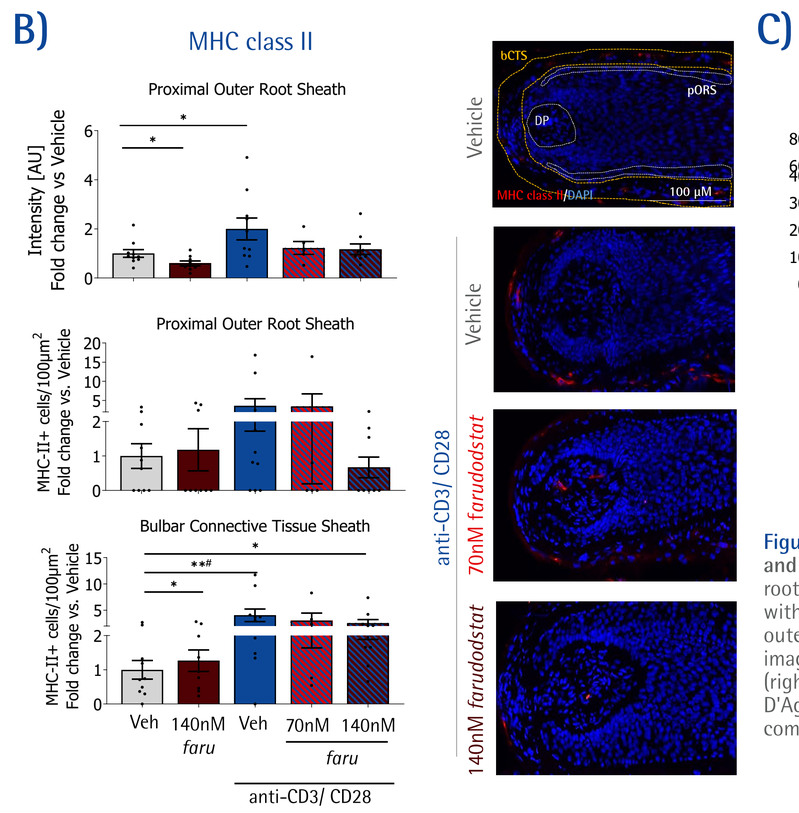


Figure 2. Effect of *farudodstat* treatment in healthy human HFs *ex vivo*. **(A)** Hair cycle staging and hair matrix keratinocyte proliferation after 5 days of treatment with 140nM *farudodstat*. Hair cycle staging was performed by calculating the percentage of HFs in each hair cycle phase. Proliferative cells (Ki-67+ cells) were counted in the demarcated area below the Auber's line while apoptotic cells (TUNEL+) were counted in the demarcated areas below and above the Auber's line (respectively germinative hair matrix (gHM) and precortical hair matrix (pcHM)). No apoptotic cells were observed. **(B)** MHC class I expression after 5 days of treatment with 140nM *farudodstat* was evaluated in the proximal outer root sheath (pORS), the germinative hair matrix (gHM), the dermal cup (DC), and the dermal papilla stalk (DPst). All data are presented as mean±SEM. n = 19-38 HFs from n = 6 donors Treatment groups were compared using Mann-Whitney test and no statistical significance was observed, scale bar = 100µm. *faru*: *farudodstat*

Farudodstat protects HFs *ex vivo* from anti-CD3/CD28 induced upregulation of IP collapse-associated markers and T-cell proliferation





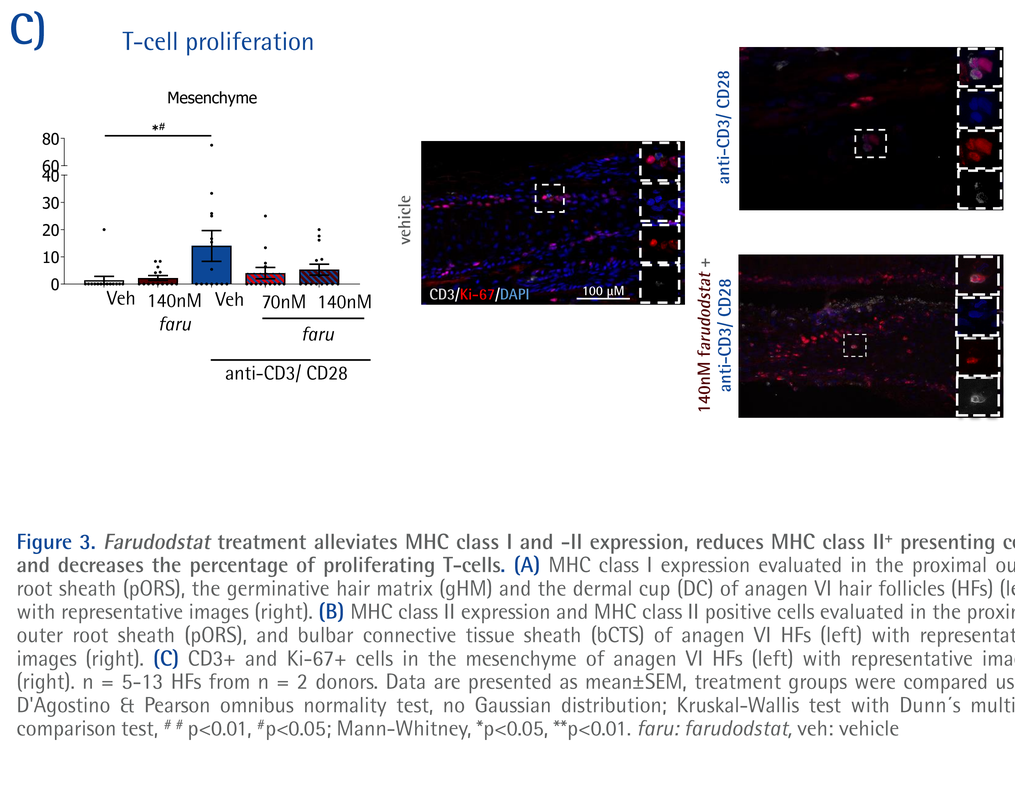


Figure 3. *Farudodstat* treatment alleviates MHC class I and -II expression, reduces MHC class II+ presenting cells and decreases the percentage of proliferating T-cells. **(A)** MHC class I expression evaluated in the proximal outer root sheath (pORS), the germinative hair matrix (gHM) and the dermal cup (DC) of anagen VI hair follicles (HFs) (left) with representative images (right). **(B)** MHC class II expression and MHC class II positive cells evaluated in the proximal outer root sheath (pORS), and bulbar connective tissue sheath (bCTS) of anagen VI HFs (left) with representative images (right). **(C)** CD3+ and Ki-67+ cells in the mesenchyme of anagen VI HFs (left) with representative images (right). n = 5-13 HFs from n = 2 donors. Data are presented as mean±SEM, treatment groups were compared using D'Agostino & Pearson omnibus normality test, no Gaussian distribution; Kruskal-Wallis test with Dunn´s multiple comparison test, ## p<0.01, #p<0.05; Mann-Whitney, *p<0.05, **p<0.01. *faru: farudodstat*, veh: vehicle

Conclusion

Our preliminary results show that T-cell activation via anti-CD3/CD28 treatment in the HF organ-culture model can successfully induce key features of AA, including IP collapse-associated markers and T-cell proliferation. Additionally, our data suggest that the DHODH inhibitor *farudodstat*, at clinically relevant concentrations, may protect HFs from IP collapse, and might offer a novel therapy for AA.

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References: [1] Bertolini et al. Exp Dermatol (2020); [2] Steinert et al. Annu Rev Immunol. (2021); [3] Klotz et al. Sci Transl Med (2019); [4] Langan et al. Exp Dermatol (2015)