Laser Capture Microdissection Reveals Transcriptional Abnormalities in Alopecia Areata before, during, and after Active Hair Loss

Journal of Investigative Dermatology (2016) **136,** 715–718; doi:10.1016/j.jid.2015.12.003

TO THE EDITOR

Alopecia areata (AA) is a common nonscarring autoimmune hair loss disorder postulated to occur due to hair follicle (HF) immune privilege collapse (Paus and Bertolini, 2013). AA predominantly affects the anagen hair bulb, where a dense infiltrate of T cells is seen in acute disease (Gilhar et al., 2012). The cause of AA is unknown. Recently it was discovered that IFN-IL-15-JAK/Stat cytokine pathways are activated in AA, leading to promising new treatments that are undergoing clinical trials (Xing et al., 2014).

Other potential therapeutic targets may be found in the local chemokine milieu responsible for inflammatory cell recruitment. In a recent microarray analysis, *CCL5*, *CXCL1*, *CXCL10*, and *CX3CL1* were found to be upregulated in lesional compared with nonlesional skin in patients with patchy AA (Subramanya et al., 2010). Other studies in mouse and human AA have shown overexpression of CXCL9, CCL2, CCL17, CCL20, and CXCR3 (Gupta et al., 2006; Ito et al., 2013; McPhee et al., 2012). However, these reports mostly examined a limited selection of chemokines and receptors, and utilized patient serum or bulk tissue rather than targeting the bulb, which is the primary focus of disease activity.

We addressed these issues by collecting skin biopsies from 25 AA volunteers and 23 healthy controls. Biopsies from patients with AA were obtained from (i) active regions of hair loss, (ii) regrown areas of AA remission, and (iii) unaffected areas that had never been affected. Laser capture microdissection was performed on HF cryosections to extract mRNA from anagen bulbs (Figure 1a). The infundibulum, the uppermost HF segment furthest from the bulb, was also collected as a follicle-relevant control, because this region is spared from the core disease process. Quantitative real-time PCR was performed using a panel of primers encompassing all known chemokines and typical chemokine receptors (Zlotnik and Yoshie, 2012), as well as various T-cell- and HF-related markers. Significantly differentially expressed genes were identified (Figure 1b; see Supplementary Materials and Methods online), and immunofluorescent staining of AA cryosections was used to confirm protein expression of selected differentially expressed genes (Figure 2a, b, d, and g).

We found that normal HFs showed distinct patterns of chemokine expression at the bulb and infundibulum (Figure 1c). Our observations differed from previous work by Nagao et al. (2012) in that the most highly expressed chemokine at both sites was *CXCL14*. CXCL14 possesses antimicrobial properties, indicating a probable protective role within the HF (Zlotnik and Yoshie, 2012). Active AA bulbs displayed increased expression of multiple chemokines and chemokine receptors compared with normal HFs (Figure 1b), coinciding with the main site of inflammation. In contrast, at the infundibulum, only a few differentially expressed genes were found (Figure 1b). This observation was as expected, as AA activity is predominantly peribulbar, and confirmed the ability of laser capture microdissection to isolate mRNA from specific regions of interest.

If the abnormal chemokine expression in active AA bulbs attracted immune cells, one would expect a correlation between the upregulated chemokines and their corresponding receptors. We observed strong correlations in the expression levels of certain chemokine-chemokine receptor pairs, particularly *CCL19-CCR7*, *CXCL10-CXCR3*, *CCL1-CCR8*, and *CXCL11-CXCR3* (Figure 1d). This result suggested that the chemokines were likely recruiting immune cells bearing the requisite receptors, thus contributing to inflammation at the diseased bulb.

Remarkably, we noted that in regrown AA bulbs, the transcription pattern remained substantially abnormal, albeit attenuated compared with active AA (Figure 1b). This finding implies that despite recovery of hair growth, a permanent or semipermanent change persists in the previously affected AA HF, which could predispose to future relapse (Li and Sinclair, 2014). One persistently overexpressed gene in regrown AA was *ITGAE* (integrin α_E; Figure 1b), which encodes CD103, a marker for resident memory T cells. Resident memory T cells are CD8+ T cells that stay fixed in the site where they develop, and have been associated with locally recurrent dermatoses such as fixed drug eruption (Gebhardt et al., 2013). We demonstrated numerous peribulbar CD8+ CD103+ T cells on immunostaining of active AA biopsies from multiple patients (Supplementary Figure S1 online; representative image shown in Figure 2b). Furthermore, when we examined serial active and unaffected biopsies from a patient experiencing the first episode of AA, we found that *ITGAE* was initially completely absent, but later became positively expressed only in bulbs from the originally active region (Figure 2c). Although the above

Abbreviations: AA, alopecia areata; HF, hair follicle

Accepted manuscript published online 10 December 2015; corrected proof published online 8 January 2016

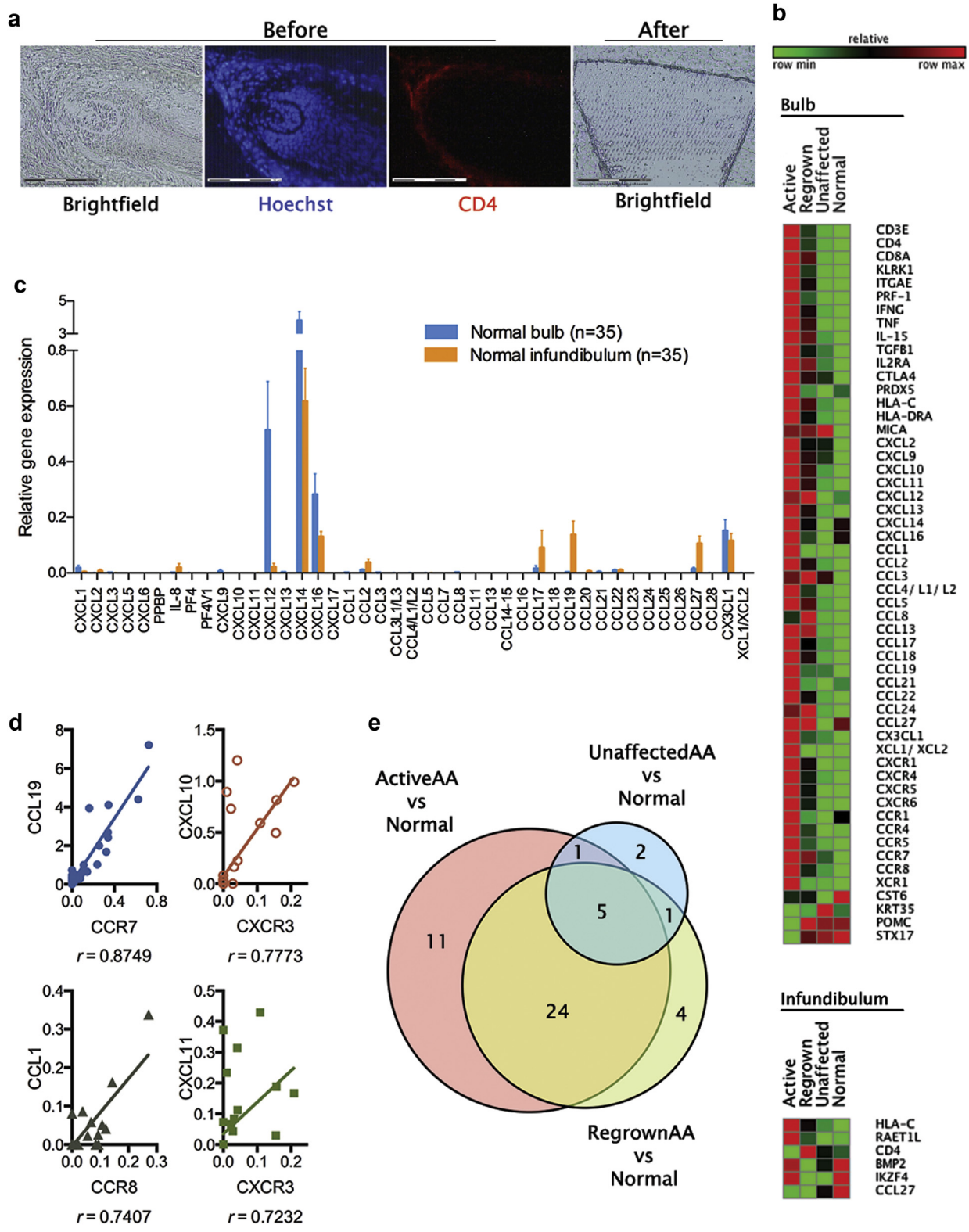

Figure 1. Laser capture microdissection of hair follicles reveals abnormal gene expression in alopecia areata (AA). Healthy and AA bulbs and infundibulums were microdissected and mRNA extracted for PCR. Data obtained were normalized to housekeeping gene expression. (**a**) Representative active AA bulb laser capture microdissection. Scale bars = 75 μm. (**b**) Heatmap of significantly differentially expressed genes (DEGs) in bulbs and infundibulums after pairwise comparison of normal and active, regrown and unaffected AA groups (n = 24–44 bulbs/infundibulums). DEGs were determined by the Mann-Whitney *U*-test with multiple testing correction using the Benjamini-Hochberg method ($P < 0.05$). A red-green color scale shows relative gene expression (green = low, red = high). Heatmap generated using GENE-E software (http://www.broadinstitute.org/cancer/software/GENE-E/index.html). (**c**) Chemokine expression profile in normal hair follicles. Data presented as mean ± SEM. (**d**) Normalized gene expression levels of selected chemokine-chemokine receptor pairs in active AA bulbs. Spearman's *r* as shown (n = 37; all $P < 0.01$). (**e**) Venn diagram showing overlap of DEGs between normal bulbs and bulbs from active, regrown, and unaffected AA (n = 34–44 bulbs).