Original article

Endocannabinoids limit excessive mast cell maturation and activation in human skin

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Background: Mast cells (MCs) crucially contribute to many inflammatory diseases. However, the physiological controls preventing excessive activities of MCs in human skin are incompletely understood.
Objective: Since endocannabinoids are important neuroendocrine MC modifiers, we investigated how stimulation/inhibition of cannabinoid 1 (CB1) receptors affect the biology of human skin MCs *in situ*.
Methods: This was investigated in the MC-rich connective tissue sheath of organ-cultured human scalp hair follicles by quantitative (immuno)histomorphometry, ultrastructural, and quantitative PCR techniques with the use of CB1 agonists or antagonists, CB1 knockdown, or CB1 knockout mice.
Results: Kit+ MCs within the connective tissue sheath of human hair follicles express functional CB1 receptors, whose pharmacological blockade or gene silencing significantly stimulated both the degranulation and the maturation of MCs from resident progenitor cells *in situ* (ie, enhanced the number of tryptase+, FcεRIα, or chymase+ connective tissue sheath-MCs). This was, at least in part, stem cell factor–dependent. CB1 agonists counteracted the MC-activating effects of classical MC secretagogues. Similar phenomena were observed in CB1 knockout mice, attesting to the *in vivo* relevance of this novel MC-inhibitory mechanism.
Conclusion: By using human hair follicle organ culture as an unconventional, but clinically relevant model system for studying the biology of MCs *in situ*, we show that normal skin MCs are tightly controlled by the endocannabinoid system. This limits excessive activation and maturation of MCs from resident progenitors via "tonic" CB1 stimulation by locally synthesized endocannabinoids. The excessive numbers and activation of MCs in allergic and other chronic inflammatory skin diseases may partially arise from resident intracutaneous MC progenitors, for example, because of insufficient CB1 stimulation. Therefore, CB1 stimulation is a promising strategy for the future management of allergy and MC-dependent skin diseases. (J Allergy Clin Immunol 2011;■■■:■■■-■■■.)

Key words: *Endocannabinoid, cannabinoid receptor, skin, hair follicle, mast cell, stem cell factor, tryptase*

In many developed countries, the incidence of allergic diseases is increasing to epidemic proportions, affecting up to 30% of the population.[1] Thus, these diseases constitute a considerable burden to affected patients and to health-care providers. Given the crucial role that mast cells (MCs) play in the pathogenesis and clinical phenotype of allergic diseases and many other chronic inflammatory disorders,[2,3] we clearly need a better understanding of how healthy human tissues that are very rich in MCs (such as skin or bronchial mucosa) avoid excessive activities and numbers of MCs under physiological circumstances.[4] This should open new, more effective, and better-tolerated avenues to counteracting the critical input of MCs into allergic and many other chronic inflammatory diseases.

As key protagonists of innate immunity, MCs not only play a pivotal role in anti-infection defense and "danger"-response systems but also regulate inflammation, tissue repair, and tissue remodeling.[2-6] Although it is now understood that MCs are involved in both inciting and limiting inflammation,[3,7-9] the main focus of clinically applied MCs research still is on undesired, excessive activities of MCs and their disease-promoting consequences, for example, in atopic eczema, chronic urticaria, allergic asthma, and allergic rhinitis. Yet, the physiological controls of MCs that prevent an excessive accumulation and activation within normal human tissues have been much less studied and are therefore only very incompletely understood. Thus, it remains a major unmet challenge for translational MC research to identify important endogenous controls that prevent excessive activation and numbers of MCs within healthy human tissues (as opposed to cell culture conditions, where behavior of MCs is generally studied in the—highly artificial—absence of complex regulatory cues that normally emanate from their local tissue environment).

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Abbreviations used
AEA: Anandamide
CB: Cannabinoid
CTS: Connective tissue sheath
ECS: Endocannabinoid system
HF: Hair follicle
KO: Knockout
MC: Mast cell
SCF: Stem cell factor

Therefore, these endogenous controls are best studied under *in situ* conditions. In the human system, they can best be characterized in human skin, since the latter is easily accessible, is very rich in MCs[10] and becomes frequently available during elective plastic surgery. It is important to remember that immature bone marrow–derived MC progenitors are deposited not only in peripheral tissues, such as skin, where they complete their development,[2,3] but mature skin MCs can also be generated *in situ* from resident progenitor cells in the absence of bone marrow, namely, in the stroma of organ-cultured murine and human hair follicles (HFs), the follicular connective tissue sheath (CTS).[11,12] Therefore, we hypothesized that robust mechanisms must be in place to avoid excessive increases in MC numbers by limiting the intracutaneous maturation of MCs from resident progenitor cells within human skin, namely, from resident, CTS-associated MC progenitors.[11,12]

Given the pivotal dependence of MCs on signals from their local tissue milieu,[2,6,9] it is critical to elucidate the behavior of normal primary human MCs within their natural tissue environment. In this context, neuroendocrine controls of MCs are of particular interest since neuromediators regulate multiple human skin MC functions, for example, during innate immune defense, neurogenic inflammation, angiogenesis, wound healing, and hair growth.[9,11,13] Moreover, the maturation of human CTS-MCs from resident precursors and/or their activation are strongly stimulated by prototypic stress-associated mediators, namely, by corticotropin-releasing hormone[12,14] and substance P.[15] Thus, the organ culture of healthy, adult human scalp HFs with their MC-rich CTS[12,15] provides an unconventional, but highly instructive, accessible, and physiologically and clinically relevant human model system for studying primary skin MCs, intracutaneous MC progenitors, and their neuroendocrine controls within a precisely defined mesenchymal compartment *in situ*.

Besides their multiple functions in the nervous system, endocannabinoids are now recognized as important neuroendocrine regulators of MC biology.[16-19] The endocannabinoid system (ECS) consists of cannabinoid (CB) receptors, their endogenous ligands (ie, endocannabinoids, such as anandamide [AEA] and 2-arachidonoylglycerol), and enzymes responsible for endocannabinoid synthesis and degradation.[16,20-23] However, the role of the ECS in the regulation of primary human MCs in general, and of human skin MCs *in situ* in particular, remains unknown. Moreover, there are several conflicting reports on how CB stimulation affects rodent or human MC lines *in vitro* (for details, see this article's introductory section in the Online Repository at www.jacionline.org). Furthermore, it remains to be studied whether the ECS affects MC maturation from human progenitor cells *in situ*.

Therefore, we investigated whether and how CB stimulation/inhibition affects normal, experimentally unmanipulated human skin MCs *in situ*.[24] Specifically, we asked whether resident MCs in the CTS of HFs express functional CB1 and whether the local ECS regulates their activation and/or maturation from resident progenitor cells.

METHODS

HF organ culture

Human scalp HFs in the anagen VI stage of the hair cycle[25,26] were microdissected and organ cultured as described previously.[12,15,24] Human tissue collection and handling were performed according to the Helsinki guidelines, following Institutional Research Ethics approval (University of Lübeck) and informed patient consent. In total, 414 anagen VI HFs were isolated from excess normal occipital and temporal scalp skin obtained from 8 healthy patients (aged 49-72 years, average, 59 years) undergoing routine face-lift surgery. HF organ-culture details are given in this article's Methods section in the Online Repository at www.jacionline.org.

Mast cell histochemistry

Mature human skin MCs were detected with 2 sensitive histochemical staining methods: toluidine blue and Leder's esterase histochemistry.[11]

Quantitative immunohistochemistry

Kit, CB1, tryptase, chymase, and FcεRIα antigens were immunodetected *in situ* by using the highly sensitive tyramide signal amplification technique (Perkin Elmer, Boston, Mass) according to the manufacturer's protocol and were assessed by quantitative immunohistomorphometry with the help of Image J (National Institutes of Health, Bethesda, Md) in precisely defined reference areas (for details, see this article's Methods section in the Online Repository).

CB1 knockdown *in situ*

All reagents required for transfection (human CB1 siRNA [sc-39910], control [scrambled, SCR] siRNA [sc-37007], siRNA transfection reagent [sc-29528], and siRNA transfection medium [sc-36868]) were obtained from Santa Cruz Biotechnology, Inc (Santa Cruz, Calif). HF transfection was performed according to the manufacturer's protocol (for details, see this article's Methods section in the Online Repository).

Statistical analysis

Data were analyzed by using either the Mann-Whitney *U* test or the Student *t* test for unpaired samples, with the use of Prism 4.0 software (GraphPad Prism Program, GraphPad, San Diego, Calif). P values of $<.05$ were regarded as significant. All data in the Figures showing quantitative data are expressed as means ± SEM.

RESULTS

Human CTS-MCs express CB1

Human scalp HFs, including their MC-rich CTS, express CB1 mRNA and protein, but not CB2.[21] Therefore, we first asked whether cells positive for Kit (CD117, a marker that identifies even relatively immature MCs[6,12,27]) within the CTS[12,15] (which does not contain any Kit+ melanocytes) express CB1. By using immunohistology it was found that 75.5% of Kit+ CTS-MCs prominently coexpressed CB1, both in organ-cultured HFs (Fig 1, *A* and *B*) and in intact human scalp skin (Fig 1, *C*). The highly CB1-selective fluorescent ligand Tocrifluor T1117 bound directly to Kit+ CTS-MCs (Fig 1, *D*), demonstrating that these CB1 receptors display functional and specific binding activity.

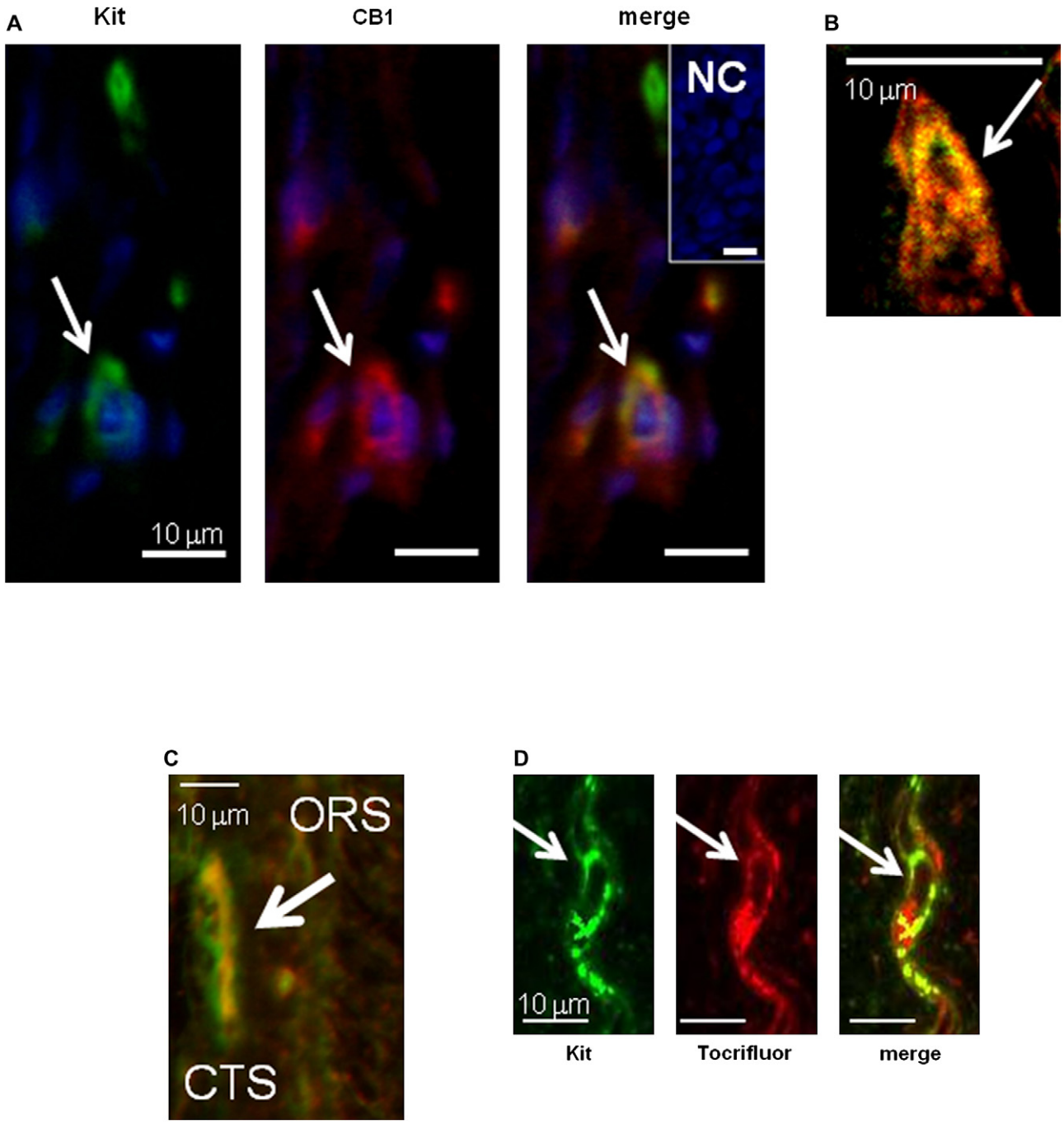

FIG 1. CB1 expression on CTS-MCs. **A,** Kit and CB1 double+ CTS cells within organ-cultured human HFs and isolated scalp skin **(C)**. **B,** A high-magnification image shown in *A* by laser scanning confocal microscopy. **D,** Kit immunostaining with 1-day organ-cultured human HFs with Tocrifluor (1 μM). *Arrow* denotes double+ cell. *NC,* Negative control; *ORS,* outer root sheath.

CB1 inhibition induces the activation of CTS-MCs and increases their number

We then assessed whether treatment with the prototypic endocannabinoid AEA (30 μM)[16,22,24] or with the selective CB1 agonist arachidonyl-2-chloroethylamide (ACEA; 30 μM)[28] altered the total number of histochemically detectable CTS-MCs and/or their activation status (degranulation). Interestingly, this was not the case (Fig 2, *B-E*; see Fig E1, *A* and *B*, in this article's Online Repository at www.jacionline.org).

In contrast, the diarylpyrazole derivative and selective CB1 antagonist/inverse agonist AM251 (1 μM)[16,24] significantly increased both the number of mature CTS-MCs and their degranulation (Fig 2, *B-E*, and Fig E1, *A* and *B*). These effects were completely abrogated by coincubation with AEA or ACEA (Fig 2, *B-E*, and Fig E1, *A* and *B*). High-resolution light microscopy independently confirmed that selectively antagonizing CB1 significantly upregulated the degranulation of CTS-MCs (Fig E1, *D* and *E*). By transmission electron microscopy, we found

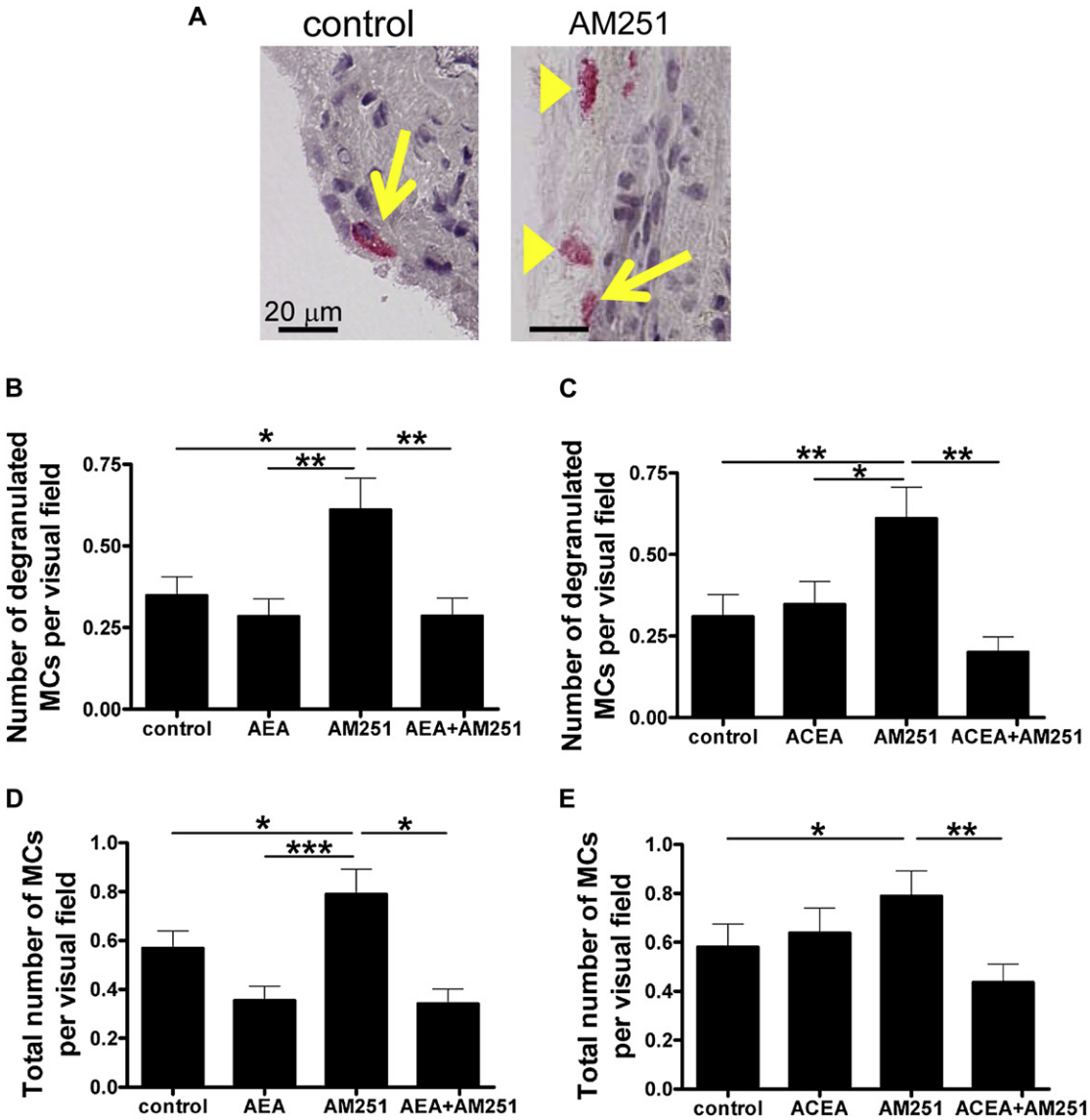

FIG 2. Effect of CB1 signaling on the number and degranulation status of CTS-MCs. **A,** "Degranulated" *(arrowhead)* and "nondegranulated" *(arrow)* CTS-MCs were detected by Leder's esterase histochemistry. The number of degranulated **(B** and **C)** and total CTS-MCs **(D** and **E)** per visual field in 1-day cultured HFs with AEA (30 μM), ACEA (30 μM), and AM251 was analyzed. $*P < .05$, $**P < .01$, and $***P < .001$ denote significant differences for the indicated comparisons. *ACEA*, Arachidonyl-2-chloroethylamide; *AM251*, CB1 receptor antagonist/inverse agonist.

that AM251-treated MCs showed the typical ultrastructural morphology of degranulated human MCs[29] (Fig E1, *F*).

Thus, antagonizing CB1-mediated signaling clearly increases the degranulation of human skin MCs *in situ*. This conflicts with a previous report that the CB1 antagonist AM281 does not affect the degranulation of RBL-2H3 cells by itself.[30]

CB1 inhibition induces the maturation, but not proliferation, of CTS-MCs

Next, we studied the impact of CB1 stimulation/inhibition on the number of Kit+ cells. In human skin mesenchyme, Kit is expressed almost exclusively by MCs, and Kit immunohistology allows visualization of both more immature MCs and mature MC populations than is possible with classical MC histochemistry, which depends on the demonstration of metachromatic granules.[2,12,15] Surprisingly, CB1 inhibition upregulated the number of Kit+ human CTS-MCs *in situ* (Fig 3, *A-C*) (since we cannot exclude that CB1− but Kit+ CTS-MCs were also counted, particularly in the control group, the real effect of CB1 blockade on CTS-MCs may well be even more significant than is apparent from Fig 3, *B* and *C*). Interestingly, however, AM251 altered neither the number of Ki67+/Kit+ cells (Fig 3, *D*) nor of terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling+/Kit+ cells (Fig 3, *F*). Most Kit+ CTS cells were Ki67− in both control and AM251-treated organ-cultured HF (Fig 3, *E*). This suggests that the increased number of Kit+ CTS-MCs seen after CB1 inhibition does not

primarily result from stimulating the proliferation or inhibiting the apoptosis of resident MCs. Although no statistically significant differences in the number of Ki67+/Kit+ cells were seen between test and control groups, it cannot be ruled out that the availability of a larger number of human HFs for study might have revealed a slight, significant difference. However, it is unlikely that this would explain the large differences seen in the total number of histochemically and immunohistologically detectable MCs during such a short HF organ-culture period (24 hours).

To independently validate this concept, we assessed the expression of antigens characteristically found on/in mature MCs, that is, the MC proteases tryptase and chymase and the high-affinity receptor for IgE, FcεRIα.[2,6,27,31] Indeed, multiple cells positive for tryptase, FcεRIα, or chymase were detected in the CTS of organ-cultured HFs, and their number was significantly increased by AM251 treatment (Fig 3, *G-I*).

This suggests that CB1 blockade, rather than affecting the proliferation/apoptosis[4] of resident mature CTS-MCs, first, stimulates the differentiation of resident, highly immature, Kit− MC progenitors into Kit+ MCs, and subsequently promotes their differentiation into fully mature tryptase+/chymase+/FcεRIα+ MCs (note that in our organ-culture assay, MC precursors could not possibly have been recruited from the circulation or bone marrow, even though these resident MC progenitors may well have immigrated from the bone marrow into the HF-CTS *in utero* and/or postnatal life). Thus, constitutive CB1 stimulation is required to avoid the excessive intracutaneous maturation of functional MCs from *resident* progenitor cells within healthy human skin.

CB1 gene knockdown is possible in organ-cultured human HFs

To further probe this novel and provocative concept by experimentally reducing the possibility of endocannabinoids to signal *via* CB1, CB1 gene silencing was attempted by standard siRNA technology. Successful knockdown was demonstrated by a significant downregulation of CB1 immunoreactivity (Fig 4, *A* and *B*; see Fig E2, *A*, in this article's Online Repository at www.jacionline.org) as well as by quantitative PCR (which demonstrated a reduction in the intrafollicular CB1 transcript level; data not shown). Additional functional evidence that CB1 knockdown was successful arose from the MC effects reported below.

Continuous CB1 stimulation by endocannabinoids controls the number and activation of human CTS-MCs *in situ*

CB1 knockdown significantly increased the number of CTS-MCs that were detectable by either histochemistry or immunohistology (Kit+, tryptase+) and increased their degranulation. The CTS of CB1 siRNA-treated human HFs contained greater numbers of mature, degranulated MCs than did the CTS of HFs treated with scrambled oligos (Fig 5, *A-E*, *H* and *I*). Interestingly, Kit immunoreactivity also significantly increased in the CTS of CB1 knockdown HFs (Fig 5, *F*). CB1 knockdown decreased intracellular (see Fig E3, *B*, in this article's Online Repository at www.jacionline.org) but increased intercellular tryptase immunoreactivity (Fig E3, *A* and *C*). This suggests that tryptase was actively secreted after CB1 knockdown. However, CB1 knockdown did not significantly elevate tryptase levels in the culture medium (Fig E3, *D*), possibly because of the well-recognized strong binding of secreted tryptase to collagen.[32] In fact, many extracellularly located, tryptase+ granules were detectable in the collagen-rich CTS, most prominently in CB1 siRNA-treated HFs (Fig E3, *A*). CB1 gene knockdown did not stimulate CTS-MC proliferation *in situ* (Fig 5, *G*).

Taken together, this suggests that, under physiological conditions, continuous CB1 stimulation by endocannabinoids, which are present in substantial quantities within the CTS of normal human scalp HFs,[24] maintains Kit expression and numbers/activation of MCs at a relatively low baseline level. These CB1-silencing data further support the concept that CB1 blockade stimulates the maturation of very immature, resident MC precursors *in situ* that are not yet Kit+. These then differentiate first into Kit+ and subsequently into tryptase+/chymase+/FcεRIα+ mature MCs.

Endocannabinoids inhibit excessive activation of MCs via CB1

Since excessive degranulation and numbers of MCs in human skin play a key role in the pathogenesis and clinical phenotype of several major skin diseases,[2,3,7,9] we asked whether CB1 stimulation counteracts the MC-activating effects of classical MC secretagogues. Quantitative MC histomorphometry *in situ* demonstrated that this is the case: Both the potent nonselectively CB1-stimulating endocannabinoid AEA (30 μM)[21,24] and the CB1-specific agonist, the synthetic cannabinoid ACEA (30 μM)[21,28] inhibited the degranulation-promoting effects of key endogenous and exogenous MC activators: substance P (10^{-10} mol/L), a key mediator of stress-induced, neurogenic skin inflammation[14,33] (Fig 6, *A*), and the standard secretagogue compound 48/80 (10 μg/mL)[34] (Fig 6, *B*). Thus, CB1 stimulation effectively counteracts excessive activation of MCs in normal human skin *in situ*. This suggests that rather than acting on resting MCs (see Fig 2, *B-E*, and Fig E1, *A* and *B*), the ECS of the human skin may primarily tone down *activated* MCs *in situ*.

CB1 stimulation regulates the maturation of human CTS-MCs by controlling stem cell factor expression by the HF epithelium

Human HF epithelium expresses functional CB1[24] and is a major source of stem cell factor (SCF),[12] the key growth factor that drives the maturation of MCs.[3,6] Therefore, we asked whether CB1 stimulation/inhibition may induce the observed effects on the maturation and activation of MCs indirectly through stimulating the intrafollicular expression of SCF by CB1+ HF epithelial cells *in situ*. An intact human scalp skin sample was used as a positive control for SCF immunohistochemistry (see Fig E4 in this article's Online Repository at www.jacionline.org).

Indeed, AM251 (1 μM) significantly upregulated SCF expression in organ-cultured HFs both at the gene level (Fig 7, *C*) and at the protein level (Fig 7, *A* and *B*). This was abrogated by coadministering ACEA (30 μM) (Fig 7, *B* and *C*). Furthermore, AM251 significantly increased SCF secretion into the culture medium of outer root sheath keratinocytes *in vitro* (Fig 7, *D*). After AM251 treatment, compared with the control, SCF immunoreactivity was prominently detectable in proximity to the cell membrane (Fig 7, *E*). Sixty percent of the cells treated with AM251 showed this fluorescence staining pattern, whereas this was observed in

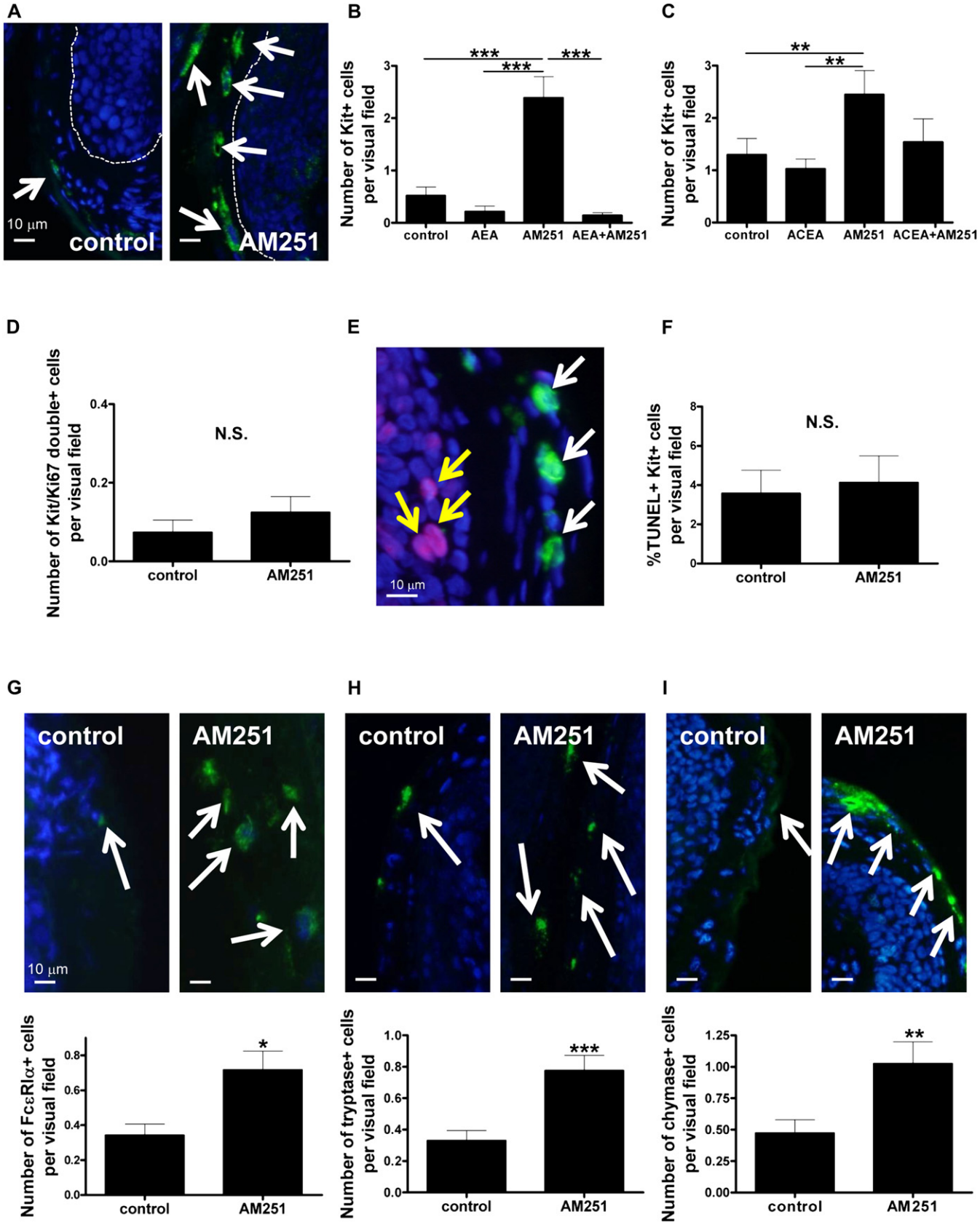

FIG 3. Differentiation and proliferation of CTS-MCs. **A,** Kit immunohistology with organ-cultured HFs. **B** and **C,** Quantitative immunohistomorphometry of Kit+ cells in organ-cultured HFs. **D,** Quantitative immunohistomorphometry of Kit/Ki67 double+ cells. **E,** *Yellow arrow* denotes Ki67+ proliferative hair matrix

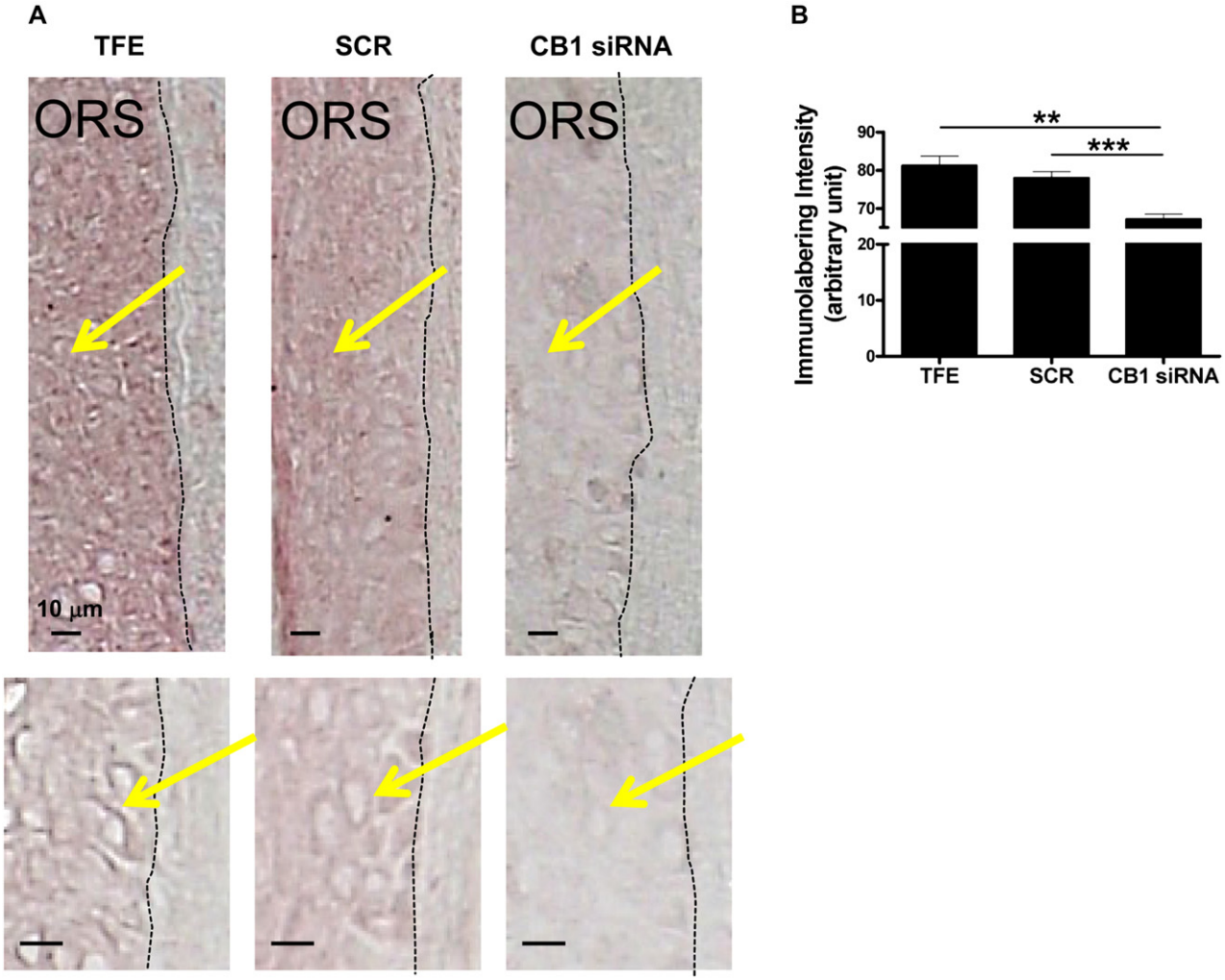

FIG 4. CB1 gene knockdown is possible in human HFs. **A,** Representative images of CB1 immunohistochemistry with TFE, SCR, and CB1 siRNA-treated HFs *(upper panel)*. High-magnification images of CB1+ ORS keratinocytes of each treatment group *(lower panel)*. *Arrows* denote positive immunoreactivity. **B,** Quantitative immunohistomorphometry of CB1 immunohistochemistry with CB1 siRNA-treated human HFs. $^{**}P < .01$ and $^{***}P < .001$ denote significant differences for the indicated comparisons. *ORS*, Outer root sheath; *SCR*, scrambled siRNA treated HFs; *TFE*, transfection reagent treated HFs.

only 36.8% of the cells in the vehicle control group. This further suggests increased SCF secretion after blockade of CB1-mediated signaling and adds additional credence to the concept that SCF production by human HF epithelium is controlled by the ECS *via* CB1 stimulation.

To further assess this indirect effect of CB1 inhibition by AM251 on CTS-MCs *via* SCF secretion by the HF epithelium,[11,12] we performed additional HF organ culture for 1 day with 1 μM of AM251 in the presence of 1 μg/mL of SCF-neutralizing antibody. When test and control groups were compared with respect to the number of Kit+ CTS-MCs, the increase in the number of Kit+ MCs after pharmacological CB1 blockade was partially, yet significantly, reduced by neutralizing SCF (Fig 7, *F*).

CB1 deletion induces maturation and activation of CTS-MCs *in vitro*

Finally, by examining CB1 knockout (KO) mice,[35,36] we probed whether the novel concepts revealed above in an organ-cultured human skin appendage *in situ* also apply *in vivo*. As expected, c-kit+ CTS-MCs were CB1− in these KO mice (see Fig E5 in this article's Online Repository at www.jacionline.org). Moreover, in line with our human HF organ-culture data, the total

keratinocytes. *White arrow* denotes Kit+/Ki67-cells. **F,** Quantitative immunohistomorphometry of Kit/TUNEL double+ cells. Quantitative immunohistomorphometry of FcεRIα **(G)**, tryptase **(H)**, and chymase **(I)**+ cells. $^{*}P < .05$, $^{**}P < .01$, and $^{***}P < .001$ denote significant differences for the indicated comparisons. *ACEA*, Arachidonyl-2′-chloroethylamide; *AM251*, CB1 receptor antagonist/inverse agonist; *NS*, not significant; *TUNEL*, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling.

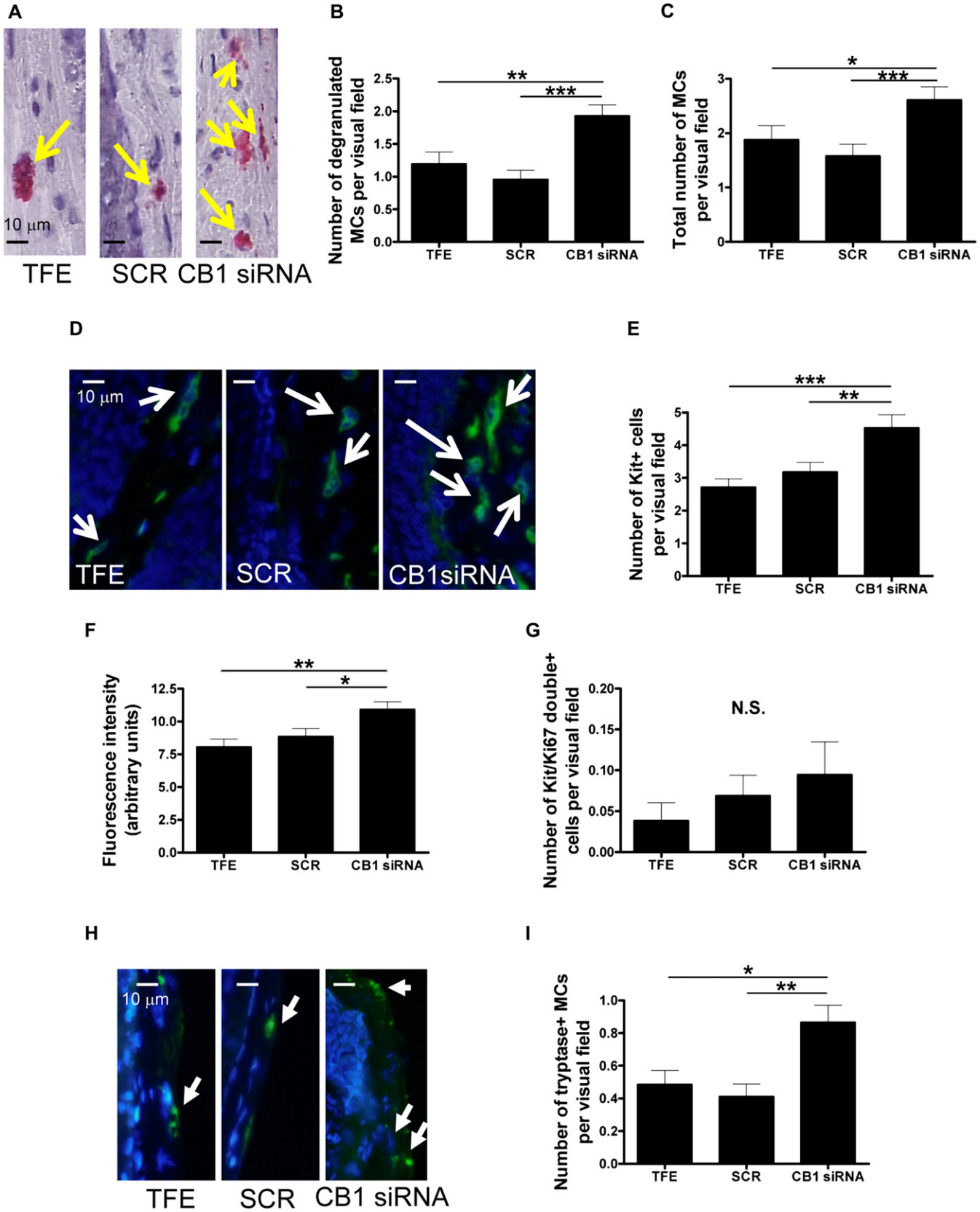

FIG 5. CB1 knockdown increases degranulation and the total number of CTS-MCs *in situ*. **A,** Leder's-esterase histochemistry. **B,** Quantitative histomorphometry of degranulated CTS-MCs. **C,** Quantitative histomorphometry of total CTS-MCs. **D,** Kit immunohistology. **E,** Quantitative immunohistomorphometry of Kit+ cells. **F,** Quantitative analysis of Kit immunoreactivity in Kit+ cells. **G,** Quantitative

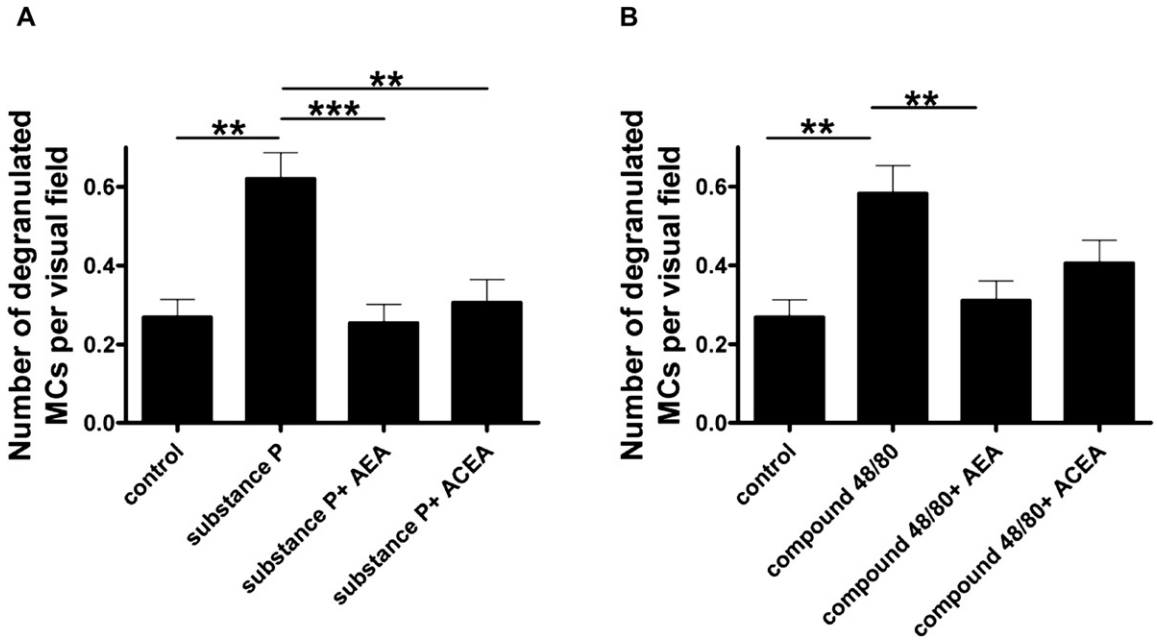

FIG 6. Inhibitory effects of cannabinoids on the degranulation of human skin MCs induced by endogenous or exogenous MC secretagogues. Quantitative histomorphometry of CTS-MCs degranulation detected by Leder's-esterase histochemistry in substance P-treated (10^{-10} mol/L) **(A)** and compound 48/80-treated (10 μg/mL) **(B)** organ-cultured human HFs. $^{**}P < .01$ and $^{***}P < .001$ denote significant differences for the indicated comparisons. *ACEA,* Arachidonyl-2′-chloroethylamide.

number of MCs and c-kit+ cells was significantly increased in the subcutaneous CTS in the skin of CB1 KO mice *in vivo* (Fig 8, *A*, *C*, and *D*). Here, the number of degranulated MCs was also significantly higher than in age- and hair cycle–matched wild-type mice (Fig 8, *B*). There was no significant change in the number of Ki67/Kit double+ CTS-MCs between CB1 KO and wild-type mice (Fig 8, *E*). This suggests that even *in vivo*, CB1 acts primarily on murine skin MC maturation and activation and not at the level of MC proliferation. Taken together, these complementary murine data suggest that constitutive CB1 stimulation is also required *in vivo* to avoid excessive maturation and activation of skin MCs.

DISCUSSION

Collectively, our findings provide the first unequivocal evidence that within their natural tissue habitat, normal human skin MCs utilize CB1-mediated signaling to limit not only their own activation/degranulation but also their maturation from resident progenitor cells *in situ*. We show that CB1 stimulation/blockade has both direct and indirect (ie, SCF-mediated) effects on normal human skin *in situ*.

Previous *in vitro* studies, which had investigated rodent and human MC lines,[17,18,30,37-40] had painted a contradictory picture on the role of the ECS in the activation of MCs (see this article's introductory section in the Online Repository at www.jacionline.org). This may be due to the fact that MC lines of debatable physiological/clinical relevance were studied or that isolated primary MCs were investigated in the absence of crucial physiological cues from their mesenchymal and epithelial microenvironment. Here, we document that under maximally "physiological" *in vitro* conditions, continuous "tonic" stimulation of CB1 expressed on human skin MCs by locally produced endocannabinoids maintains the number and activities of mature MCs at a relatively low baseline level.

The indirect, SCF-mediated effects of CB1 signaling revealed here provide the first indication that tonic CB1 signaling also appears to be essential to avoid excessive SCF secretion in human skin. Since it is very well possible that the SCF-neutralizing antibody we have used here did not completely block all SCF activity (thus explaining why the effect is not completely abrogated), our assay system does not allow one to state with certainty whether all the effects of the CB1 antagonist on MCs are "direct" or "indirect" through SCF. However, our results with Tocrifluor (Fig 1, *D*) as well as with CB1/Kit-double immunofluorescence (Fig 1, *A-C*) make it reasonable to assume that direct, CB1-mediated effects on MCs operate side by side with indirect ones (ie, CB1-regulated secretion of SCF by human HF keratinocytes).

Our study demonstrates that the CTS provides an important peripheral tissue site of and source for immature MC precursors, not only in mouse vibrissae HFs[11] but also in healthy human skin.[12] Moreover, we provide the first evidence that the differentiation of these resident precursors into mature, functional MCs is constitutively inhibited by the ECS. In contrast to rapidly

immunohistomorphometry of Kit/Ki67 double+ cells. **H,** Tryptase immunohistology. **I,** Quantitative immunohistomorphometry of tryptase+ cells. $^{*}P < .05$, $^{**}P < .01$, and $^{***}P < .001$ denote significant differences for the indicated comparisons. *Arrows* denote positive cells. *NS,* not significant; *SCR,* scrambled siRNA treated HFs; *TFE,* transfection reagent treated HFs.

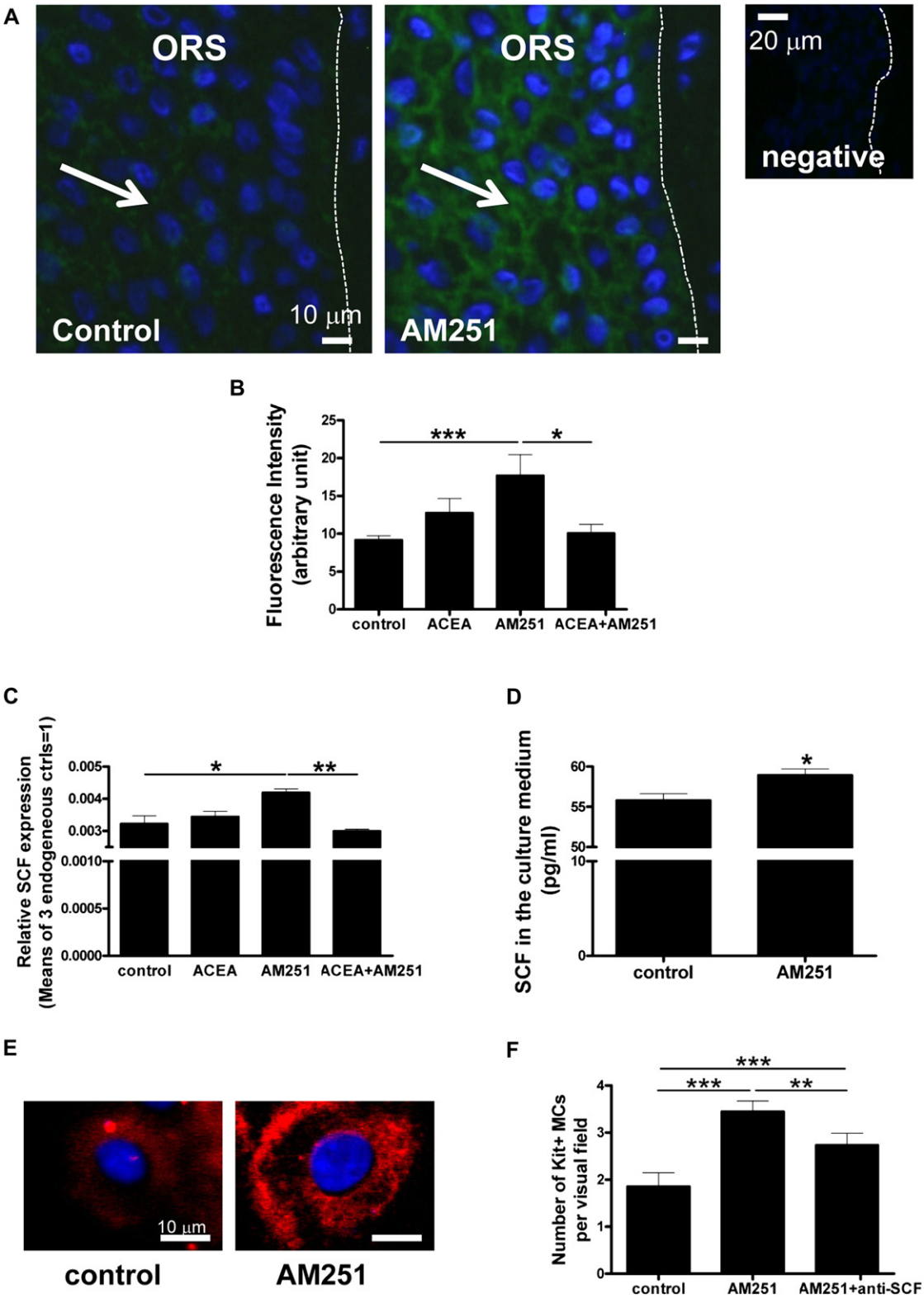

FIG 7. Dependence of CB1-mediated CTS-MC effects on SCF. **A,** SCF immunohistology. *Arrow* denotes SCF positive immunoreactivity. **B,** Quantitative analysis of SCF immunoreactivity. **C,** Quantitative PCR analysis for SCF with 1-day organ-cultured HFs. **D,** SCF measurement in ORS keratinocytes culture medium. **E,** SCF immunocytochemistry. **F,** Quantitative immunohistomorphometry of Kit+ cells in organ-cultured HFs with AM251 (1 μM) or/and SCF-neutralizing antibody (1 μg/mL). $*P < .05$, $**P < .01$, and $***P < .001$ denote significant differences for the indicated comparisons. *ACEA*, Arachidonyl-2′-chloroethylamide; *AM251*, CB1 receptor antagonist/inverse agonist; *ORS*, outer root sheath.

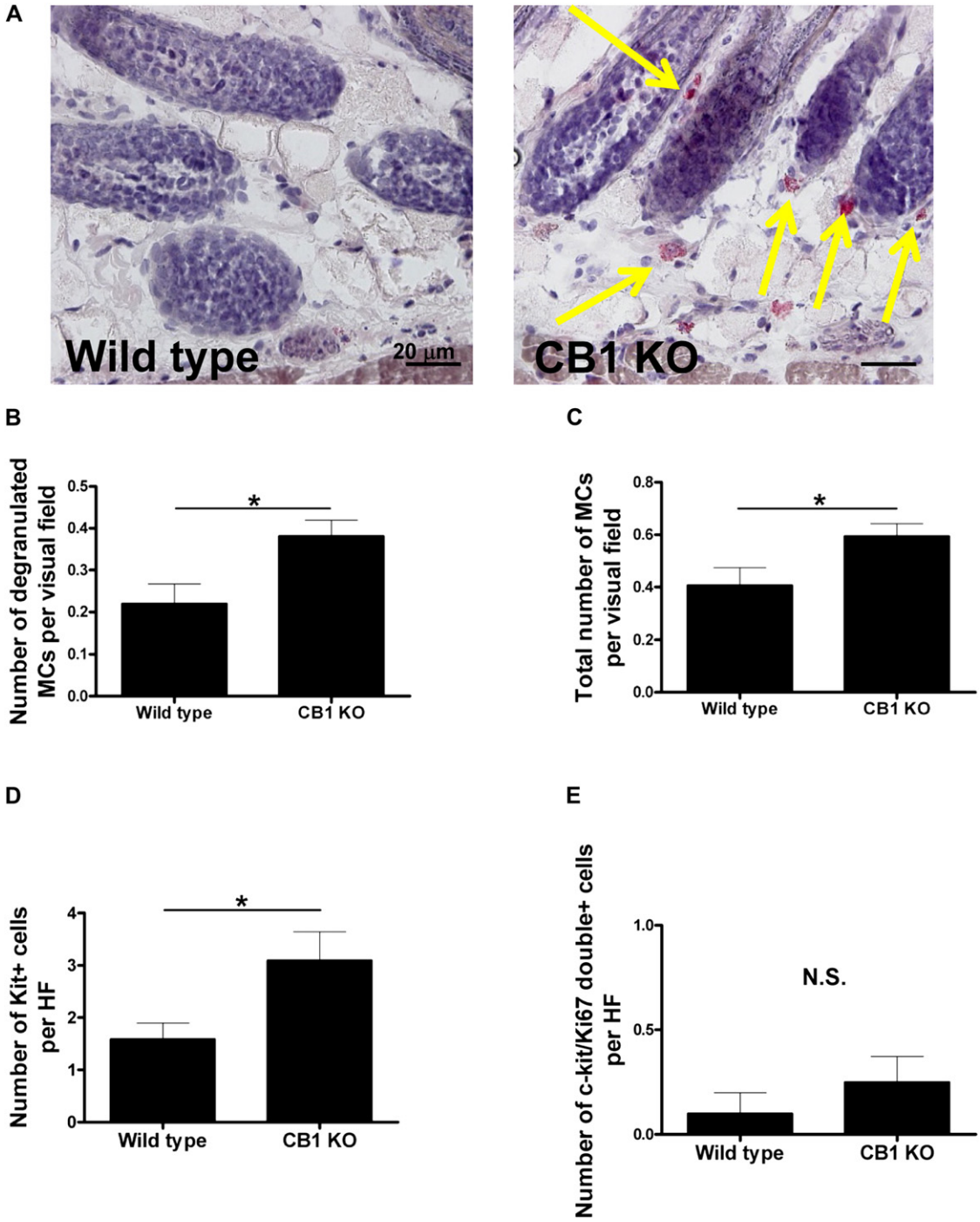

FIG 8. *In vivo* effects of CB1 knockout on number, degranulation, and proliferation of CTS-MCs in mice. **A,** Leder's-esterase histochemistry. *Arrows* denote CTS-MCs. **B,** Quantitative histomorphometry of the number of degranulated CTS-MCs. **C,** Quantitative histomorphometry of the number of total CTS-MCs. **D,** Quantitative immunohistomorphometry of the number of c-kit+ cells. **E,** Quantitative immunohistomorphometry of the number of c-kit/Ki67 double+ cells. $*P < .05$ denotes significant differences for the indicated comparisons. *NS*, Not significant.

proliferating hair matrix keratinocytes[24] or various neuronal cell populations,[41] CB1-mediated signaling primarily seems to affect the maturation and activation of CTS-MC but not their proliferation or cell death. That this also applies to murine skin *in vivo* attests to the physiological relevance of human HF organ-culture.

While the CB1 KO mouse data confirm our human HF organ-culture observations with respect to the regulation of skin MCs by CB1, it must be kept in mind that under *in vivo* conditions, the MC phenomena observed in the skin of CB1 KO mice may reflect more complex mechanisms.

We are currently testing how CB1 stimulation/blockade affects human mucosal-type MCs in organ-cultured human nasal polyp samples, an excellent surrogate tissue for human bronchial mucosa.[42] Our available pilot observations indicate that CB1 also suppresses the maturation of functional mucosa-type MCs from resident progenitor cells: Kit+ cells in human nasal polyps express CB1 *in situ*, and AM251 increases the total number of tryptase+ and Kit+ MCs without modulating their proliferation (K.S., N.Z, T.H., and R.P., manuscript in preparation). This encourages one to explore whether CB1-mediated "tonic" inhibition of the maturation and activation of MCs by the ECS is a general principle that also operates in other human MC populations than the ones investigated here in human skin.

Our study strongly suggests that targeting the ECS for the downmodulation of excessive MC activities in human skin could become an attractive new therapeutic strategy in clinical medicine. Moreover, the current study encourages one to systematically dissect whether allergic diseases and many other disorders characterized by excessive numbers and/or activation of MCs (eg, in bronchial asthma, allergic rhinitis, atopic eczema, prurigo dermatoses, psoriasis, mastocytosis, and chronic urticaria) are associated with defined defects in the ECS, such as insufficient endocannabinoid synthesis, excessive endocannabinoid metabolism, and/or defective CB1-mediated signaling.

Furthermore, our data call attention to the HFs' CTS as a previously ignored tissue compartment that may play an important role in excessive MC activities within inflamed, hair-bearing human skin. (We are currently examining whether similar principles apply to MCs in the stroma of other human skin appendages such as sweat and sebaceous glands.) Methodologically, we show that HF organ culture provides an excellent, clinically relevant new *in situ* model for preclinical MC research in the human system, whose clinical relevance exceeds the traditional analysis of isolated human MCs, MC leukemia lines, or mouse models.

Studying primary human MC biology and pathology under clinically relevant *in situ* conditions in human HF organ culture, thus, deserves to be fully discovered by mainstream MC research as a research tool that ideally complements and validates concepts derived from the study of MC lines and murine *in vivo* models. Available human skin organ-culture assays[43,44] can complement such HF organ-culture systems so as to further probe whether selective CB1 agonists can be employed as an adjuvant strategy for the management of allergic and chronic inflammatory skin disorders with excessive accumulation and degranulation of MCs. Even though such organ-culture approaches exclude neural and perfusion-dependent inputs into skin MC biology, these 2 companion assay systems allow one to dissect the clinically important but underexplored neuroendocrine controls of skin MCs[12,15,16,45,46] under conditions where critical cell-cell (eg, MCs-fibroblast and MCs-keratinocyte) as well as cell-matrix interactions are fully preserved.

Since CB1 receptors, *in vitro*, may signal in the absence of ligand,[47] in theory, CB1 receptors might exert "tonic" MC inhibition in human skin even in the absence of endocannabinoids. Also, it deserves to be investigated whether some patients with excessive skin MC numbers and/or massive degranulation of skin MCs (eg, in atopic dermatitis or chronic urticaria) display CB1 receptor mutations or CB1 receptor polymorphisms that incapacitate this "tonic" inhibitory signaling system. Similar effects could be brought about in genetically susceptible individuals by insufficient intracutaneous endocannabinoid synthesis and/or excessive endocannabinoid degradation.[48]

Although Paul Ehrlich himself, the discoverer of MCs, had already noted that skin MCs are found in highest density around blood vessels, nerves, and HFs, their physiological functions in these specific locations remain to be fully explored. In selected peripheral tissue sites, such as HFs, MCs have been proposed to bestow "some low-level immune privilege"[8] (note that HFs are immunoprivileged miniorgans[49]). Such an immunoinhibitory role of perifollicular MCs would make it particularly important that excessive, proinflammatory MCs activation and excessive numbers of mature MCs are strictly avoided in human skin. The constitutive, inhibitory "endocannabinoid tone" revealed here may represent one such mechanism. Moreover, therapeutic stimulation of this inhibitory pathway offers an attractive alternative to, and complementation of, promoting MC apoptosis[4] where this is clinically desired.

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Key messages

- **Endocannabinoids control not only human skin mast cell (MC) activation but also their maturation from resident progenitor cells *in situ* via cannabinoid 1 (CB1) stimulation.**
- **Endocannabinoids also regulate stem cell factor (SCF) expression in human hair follicle epithelium (increased SCF production via CB1 stimulation).**
- **CB1 stimulation is a promising strategy in the future management of allergy and other MC-dependent inflammatory diseases by limiting skin MC activation and maturation.**

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APPENDIX

There are conflicting reports on how CB stimulation affects rodent or human MC lines *in vitro*. For example, the CB1 and 2 agonist CP55940 and the CB1 agonist methanandamide reportedly inhibited IgE-mediated MC degranulation in the RBL-2H3 MC line *in vitro*. These effects were reversed by treatment with the CB1 antagonist AM281; however, administration of this CB1 antagonist alone did not affect MC degranulation.[E1] Methanandamide reportedly also inhibited IgE-mediated MC degranulation in primary murine bone marrow–derived MCs *in vitro*.[E2] In RBL-2H3 cells, the endocannabinoid palmitoylethanolamide produced a small, but significant reduction in antigen-stimulated serotonin release at high concentrations, whereas anandamide (AEA) did not have an effect. In contrast, the endocannabinoid 2-arachidonoylglycerol and methanandimide both increased the antigen-stimulated MC degranulation.[E3] Palmitoylethanolamide, but not AEA, downmodulated MC activation via CB2 in the same cell line.[E4] Furthermore, the phytocannabinoid compound cannabidiol triggered RBL-2H3 cell degranulation.[E5] Δ^9-Tetrahydrocannabidol and Δ^8-tetrahydrocannabidol also induced histamine release from rat peritoneal MCs *in vitro,* apparently in a CB1/CB2-independent manner, while endocannabinoids and their analogues neither induced histamine secretion nor promoted compound 48/80-induced degranulation.[E6] Concerning human MCs, it has been reported that supernatants from SW756 cervical carcinoma cells stimulated the degranulation of the human MC line LAD2, which was inhibited by CB2 stimulation.[E7]

This leaves us with a confusing and contradictory picture of the role that CB1 versus CB2 stimulation may play in the control of MC activation. Moreover, it remains completely unknown how the ECS affects primary human MCs, and under clinically relevant conditions, for example, human skin MCs *in situ*.

METHODS

Reagents

AEA, ACEA, AM251, substance P, and compound 48/80 were purchased from Sigma-Aldrich (Taufkirchen, Germany), whereas 5-carboxytetramethylrhodamine-conjugated AM251 Tocrifluor was purchased from Tocris Bioscience (Bristol, United Kingdom).

HF organ culture

Isolated HFs were maintained in supplemented serum-free William's E medium.[E8-E11] HFs were first incubated overnight to adapt to culture conditions after which the medium was replaced and vehicle or test substances were added. For the organ-culture with MC secretagogues, substance P and compound 48/80, HFs were first treated with AEA (30 μM) or ACEA (30 μM) for 1 day after the overnight incubation. Then, the HFs were treated with either substance P (10^{-10} mol/L) or compound 48/80 (10 μg/mL) in combination with AEA or ACEA for an additional 1 day. Following culturing for the time indicated, HFs were then cryoembedded and prepared for histology and immunohistochemistry.

Immunohistochemistry

For the detection of Kit, CB1, tryptase, chymase, and FcεRIα, the highly sensitive tyramide signal amplification (TSA) technique (Perkin-Elmer, Boston, Mass) was applied. Cryosections were incubated overnight at 4°C with primary antibodies, rabbit anti-human CD117 (Cell Marque Corp, Rocklin, Calif) at 1:1000, rat anti-mouse CD117 (BD Biosciences, San Jose, Calif) at 1:5000, rabbit anti-human CB1 (Cayman Chemical, Ann Arbor, Mich, or Santa Cruz, Calif) at 1:400, mouse anti-human FcεRIα (Acris GmbH, Hiddenhausen, Germany) at 1:1000, mouse anti-human chymase (Abcam plc, Cambridge, United Kingdom) at 1:1000, or mouse anti-human tryptase (Abcam plc) at 1:5000 diluted in TNB (Tris, NaOH, blocking reagent, TSA kit; Perkin-Elmer). Thereafter, the cryosections were incubated with goat biotinylated antibodies against rabbit or mouse IgG (Jackson Immunoresearch Laboratories, West Grove, Pa) at 1:200 in TNB for 45 minutes at room temperature (RT). The TSA method was applied according to the manufacturer's protocol.

Double-immunostaining for Kit and CB1 was performed using the TSA technique. Briefly, cryosections were incubated overnight at 4°C with a primary antibody against Kit followed by biotinylated goat anti-rabbit IgG (Jackson Immunoresearch Laboratories) (1:200 in TNB, 45 minutes, RT). Sections were then incubated with streptavidin-conjugated horseradish peroxidase (1:100, 30 minutes, TSA kit) and were finally incubated with fluorescein isothiocyanate-conjugated tyramide (1:50, TSA kit). After careful washing with TNT wash buffer (0.1 mol/L Trizma hydrochloride, 0.15 mol/L NaCl, and 0.05% Tween 20), sections were then incubated overnight with rabbit anti-human CB1 antibody (Santa Cruz) at 4°C followed by incubation with goat biotinylated antibody against rabbit IgG (Jackson Immunoresearch Laboratories) (1:200 in TNB, 45 minutes, RT). After incubating with streptavidine-conjugated horseradish peroxidase (1:100, 30 minutes, TSA kit), sections were incubated with tetramer rhodamine–conjugated tyramide (1:50, TSA kit).

To study the proliferation of Kit+ cells, double-immunostaining for Ki-67 and Kit was performed. Briefly, after the staining for Kit by using a TSA kit, sections were incubated overnight at 4°C with a mouse anti-human Ki67 antibody (DAKO, Hamburg, Germany) at 1:20 in PBS for detecting human Ki67 or with rat anti-mouse Ki67 antibody (DAKO) at 1:100 in PBS for detecting mouse Ki67+ cells. Sections were then washed with PBS, followed by incubation with rhodamine-conjugated goat anti-mouse IgG or goat anti-rat IgG (Jackson Immunoresearch Laboratories) (1:200 in PBS, 45 minutes) at RT.

To evaluate the apoptosis of Kit+ cells, Kit immunostaining and terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling was performed on the same sections. Briefly, after the immunostaining for Kit, sections were incubated with a digoxigenin-deoxy-UTP (ApopTag fluorescein *in situ* apoptosis detection kit; Millipore Corp, Billerica, Mass) in the presence of terminal deoxynucleotidyl transferase (60 minutes) at 37°C. After the incubation with Stop/Wash buffer (ApopTag kit) (10 minutes, RT) and the additional wash with PBS, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling–positive cells were visualized by an anti-digoxigenin fluorescein isothiocyanate–conjugated antibody (ApopTag kit) (30 minutes, at RT).

To evaluate the immunoreactivity of CB1 in CB1 siRNA-treated HFs as well as in intact human scalp skin sections, the expression of CB1 in the HFs was visualized by using the peroxidase-based avidin-biotin complex method (Vectastain Elite ABC kit; Vector Laboratories, Burlingame, Calif). Frozen sections were fixed in cold acetone and rinsed with PBS, and endogenous peroxidase activity was saturated with 3% H_2O_2 in PBS for 15 minutes. After the incubation with 5% normal goat serum, sections were incubated with rabbit anti-human CB1 antibody (Cayman Chemical) (1:40 in PBS) at 4°C overnight. After incubation with a biotinylated goat anti-rabbit antibody (Jackson Immunoresearch Laboratories) (45 minutes, at RT), sections were treated with Vectastain ABC reagent (Vector Laboratories) and visualized with AEC (3-amino-9-ethylcarbazol) (Vector Laboratories). As negative controls, the appropriate primary antibodies were omitted from the procedure. The specificity of CB1 immunostaining was measured on intact human scalp skin sections (Fig E2, *B*) and mouse brain sections (positive control), which clearly demonstrated positive CB1 immunoreactivity in the expected areas (data not shown).

For detecting SCF in organ-cultured human HFs as well as isolated human outer root sheath (ORS) keratinocytes, indirect immunofluorescence method was applied by using anti-human SCF (Acris GmbH) at 1:20 in PBS as a primary antibody and fluorescein isothiocyanate –conjugated (rhodamine for ORS keratinocytes) goat anti-mouse IgG at 1:200 in PBS as a secondary antibody. An intact human scalp skin sample was used as a positive control (Fig E4).

The immunoreactivity of CB1, Kit, tryptase, and SCF in defined reference areas was assessed by quantitative immunohistomorphometry[E9,E11-E13] by using the Image J software (National Institutes of Health, Bethesda, Md).

For counting MCs, MCs were classified as "degranulated" when 5 or more extracellularly located metachromatic granules could be detected

histochemically at high magnification (×400) by light microscopy (visual field). The number of degranulated and total CTS-MCs around the HF per visual field was counted, and at least 13 visual fields per HF in total were evaluated.

Some degranulated MCs were detected even in the vehicle control group (Fig E1, *A* and *B*). This is in line with previously reported data.[E9] The percentage of degranulated MCs in freshly microdissected HFs evaluated by Leder's-esterase histochemistry was significantly higher than in unmanipulated human skin, suggesting that the trauma of HF microdissection caused some degree of degranulation of MCs. MCs in freshly isolated skin also displayed a steady-state level of degranulation (Fig E1, *C*).

High-magnification images of Kit and CB1 double+ cell were taken by laser scanning confocal microscopy (Fluoview 300, Olympus, Tokyo, Japan) running Fluoview 2.1 software (Olympus).

High-resolution light microscopy and transmission electron microscopy

Organ-cultured human scalp HFs were immersed in a mixture containing 2% paraformaldehyde, 2.5% glutaraldehyde, and 0.025% $CaCl_2$ in 0.1 mol/L sodium cacodylate buffer, pH 7.4, and fixed. The specimens were then immersed in 1% osmium tetroxide in the same buffer. The samples were dehydrated in a gradient series of ethanol, immersed in propylene oxide, and embedded in plastic resin. Thin and thick sections were generated on a Leica Ultra UCT (Leica, Vienna, Austria). Thick sections (1 μm) were prepared for an alkaline-Giemsa histochemistry.[E14] MCs were defined as degranulated according to the previous article.[E14] Thin sections were stained with uranyl acetate and lead citrate and observed with an electron microscope (JEM-1200EXII, JEOL, Tokyo, Japan).

Quantitative PCR

Expressions of specific mRNA transcripts of SCF were analyzed by quantitative real-time PCR performed on an ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, Calif) as described before[E12,E13] using TaqMan primers and probes (Assay ID: Hs00241497_m1 for human SCF). Three different internal housekeeping genes—glyceraldehyde 3-phosphate dehydrogenase, β-actin, cyclophilin A—were assessed (Assay ID: Hs99999905_m1 for glyceraldehyde 3-phosphate dehydrogenase, Hs99999903_m1 for β-actin, and Hs99999904_m1 for cyclophilin A). The amount of SCF transcripts was normalized to those of the control genes as previously reported.[E12,E13] Data were analyzed using 1-way ANOVA with Bonferroni's post hoc test $*P < .05$, $**P < .01$.

CB1 knockdown *in situ*

All reagents required for transfection (human CB1 siRNA [sc-39910], control (scrambled, SCR) siRNA [sc-37007], siRNA transfection reagent [sc-29528], and siRNA transfection medium [sc-36868]) were obtained from Santa Cruz. HF transfection was performed according to the manufacturer's protocol. Briefly, freshly isolated human HFs were kept in cold William's E medium right before the transfection. During transfection, CB1-specific siRNA or control siRNA (2.5 μL) and siRNA transfection reagent (2 μL) were mixed in the transfection medium (500 μL) per well (24-well plate). After the careful wash, HFs were applied to each well (3 HFs per well) and incubated at 37°C in a CO_2 incubator for 6 hours after which the medium was replaced with supplemented William's E medium. HFs were cryoembedded 24 hours following transfection.

CB1 KO mice

Targeted disruption of the CB1 receptor gene was performed by replacing the CB1 coding sequence with a nonreceptor sequence by homologous recombination in MPI2 embryonic stem cells. Mutant mice have been crossed to C57BL/GJ animals for more than 13 generations and are therefore considered to be congenic for this genetic background. Homozygous $CB1^{-/-}$ mice and wild-type ($CB1^{+/+}$) animals were generated by matings of heterozygous ($CB1^{+/-}$) mice.[E15,E16]

Tryptase immunoassay

Organ-cultured human scalp HFs were treated with CB1 siRNA for 1 day. The culture supernatants of these HFs were collected for the analysis. The level of tryptase was measured by a fluorescent enzyme immunoassay by using a commercial assay from Phadia (ImmunoCap Tryptase, Uppsala, Sweden). The principle of the assay is based on a monoclonal antitryptase capture antibody that specifically binds tryptase. After washing, β-galactosidase-labeled antitryptase antibody is added. Bound complexes are stained by the conversion of 4-methyliumbelliferyl-β-D-galactoside. The fluorescent signal is correlated with the amount of tryptase.

Isolation and culture of ORS keratinocytes

Isolation and culture of human ORS keratinocytes were performed according to our established protocol.[E17] Briefly, ORS keratinocytes were isolated by an enzymatic digestion (0.2% trypsin, 0.1% EDTA in calcium- and magnesium-free PBS for 1 hour at 37°C all from Sigma-Aldrich) and gentle trituration. Following isolation, the single-cell suspension was removed, collected by centrifugation (1000 rpm for 10 minutes), and resuspended in ORS keratinocyte culturing medium. It comprises 3:1 mixture of Dulbecco's modified Eagle medium (supplemented with L-glutamine, Na-pyruvate, 4.5 g/L glucose) and Ham's F12 (both from Invitrogen, Invitrogen Ltd, Paisley, United Kingdom), supplemented with 10% fetal clone II (Hyclone, HyClone Laboratories Inc, Logan, Utah) and 5 μg/mL insulin, 0.4 μg/mL hydrocortisone, 2.43 μg/mL adenin, 2 nM triiodothyronine, 0.1 nM cholera toxin, 10 ng/mL EGF, 1 mM ascorbyl-2-phosphate, 100 U/mL penicillin G, and 25 μg/mL gentamycin (all from Sigma). ORS keratinocytes were seeded and cultured on mitomycin-treated human dermal fibroblast feeder-layer in ORS keratinocyte culturing medium.

SCF immunoassay

The supernatant of human ORS keratinocytes culture were collected and freezed at −80°C until the assay was performed. Samples were analyzed for their SCF levels by using Quantikine Human SCF ELISA Kit (R&D Systems, Abingdon, United Kingdom).

Mouse skin harvesting was performed under an appropriate animal experimentation license obtained by the University of Bonn. Human tissue use was approved by the Ethics Committee, University of Lübeck.

DISCUSSION

Our results show that CB1 blockade affects MCs not only directly but also induces SCF secretion by human HF keratinocytes. This suggests that, under physiological conditions, "tonic" CB1 stimulation by the intracutaneous ECS maintains SCF production by human HF epithelium at a relatively low level and that blocking CB1 releases this endogenous "molecular brake" on SCF production. Increased SCF secretion then serves as a stimulus for the intracutaneous maturation of MCs from resident precursors in the CTS. These data not only provide the first available evidence for a link between CB1 signaling and SCF biology but also underscore the importance of epithelial-mesenchymal interactions in human skin MC biology.

Do ECS affect itching as well as tissue remodeling after inflammation? This important question has been discussed in a number of original reports and reviews, including our own.[E18,E19] Given the limitations of our organ-cultured human HF system, which is unsuitable for pruritus research, evidently, we cannot provide any corresponding experimental data with this assay on how the ECS may affect itch (pruritogenic pruritus) and its processing in the central system after inflammation. For this, CB1 KO mice are a more appropriate model.

However, a couple of relevant reports on the effects of endocannabinoids on itch already suggest that the ECS may indeed play an important role in itch. For example, topical

application of the endocannabinoid *N*-palmitoylethanolamine, to patients with mild to moderate atopic eczema significantly reduced the intensity of erythema, pruritus, excoriation, scaling, lichenification, and dryness.[E20] Since the inhibition of AEA-degrading enzymes (such as fatty acid amide hydrolase [FAAH]) increases AEA levels in mice,[E21,E22] it is interesting to note that FAAH KO mice or FAAH inhibitor-treated mice show significant reduction in scratching without affecting locomotor behavior.[E23] Moreover, excessive mast cell activity is well recognized to play a key role in many itch-associated skin diseases, including allergy and atopic dermatitis, and neurogenic skin inflammation.[E24-E26]

Therefore, our current data are well in line with the concept that the ECS may also modulate MC-dependent pruritogenic pruritus *via* reducing MC degranulation and *via* avoiding excessive MC maturation from resident intracutaneous progenitor cells. Moreover, since not only HF and epidermal melanocytes[E27] but also rapidly proliferating hair matrix keratinocytes prominently express Kit on the gene and protein levels (see Peters et al[E28]), it is conceivable that CB1-regulated changes in the secretion of the cognate ligand (SCF) could also have an on the growth and remodeling of selected, Kit-expressing epithelial cell populations in the HF, besides direct effects of (endo-)cannabinoid on CB1+ HF epithelial cells.

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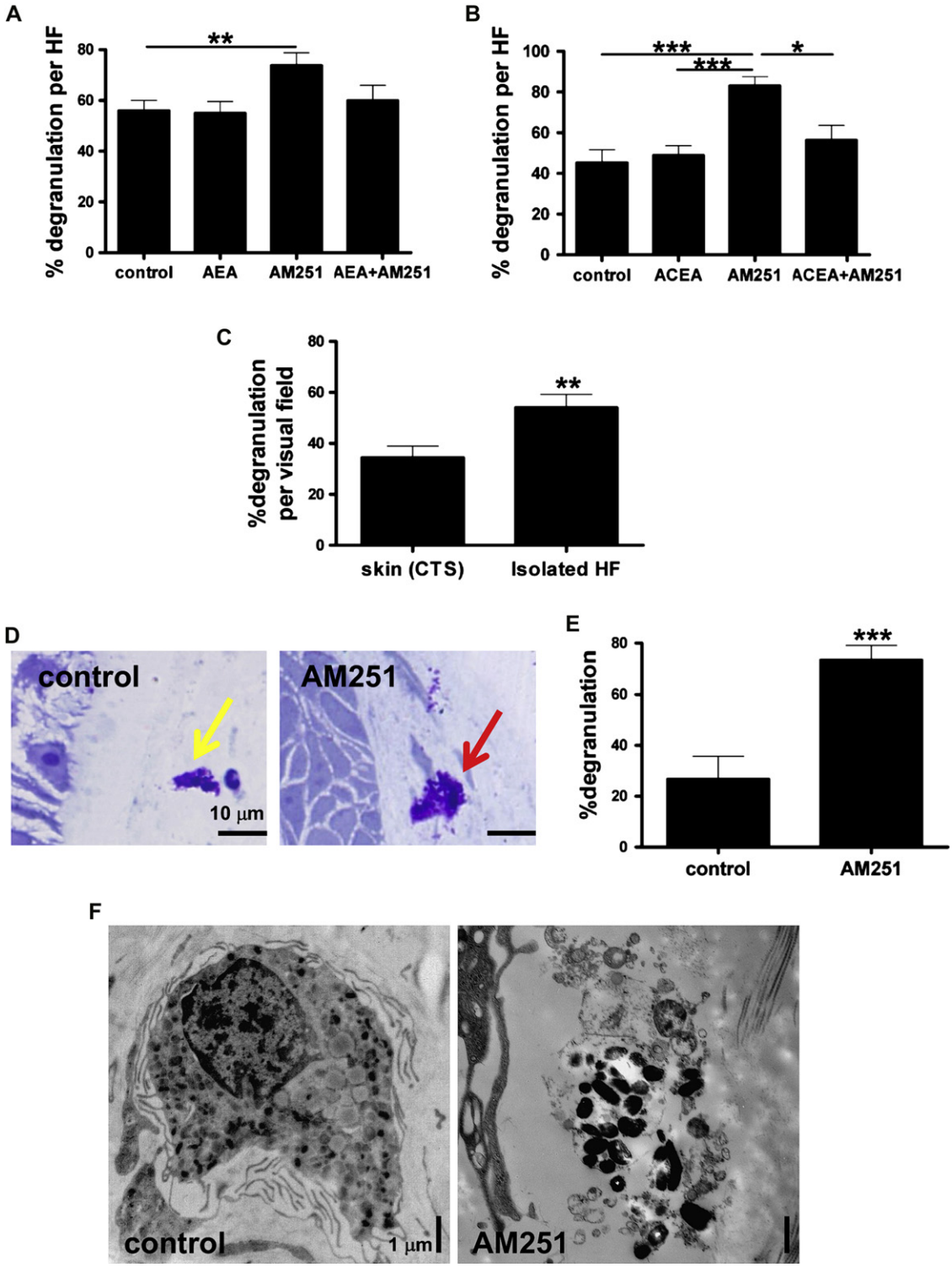

FIG E1. CB1 blockade significantly increases the degranulation of CTS-MCs *in situ*. **A,** Percentage of degranulated CTS-MCs in organ-cultured human HFs for 1 day with ACEA (30 μM) and/or AM251 (1 μM) and with **(B)** AEA (30 μM) and/or AM251. **C,** Percentage of degranulated CTS-MCs within the HFs of intact human scalp skin or isolated human scalp HFs. **D,** High-resolution light microscopy of alkaline-Giemsa histochemistry and statistical analysis **(E)**. *Yellow arrow* denotes non-degranulated MC. *Red arrow* denotes degranulated MC. **F,** TEM images. $^{*}P < .05$, $^{**}P < .01$, and $^{***}P < .001$ denote significant differences for the indicated comparisons. *ACEA*, Arachidonyl-2′-chloroethylamide; *AM251*, CB1 receptor antagonist/inverse agonist; *TEM,* transmission electron microscopy.

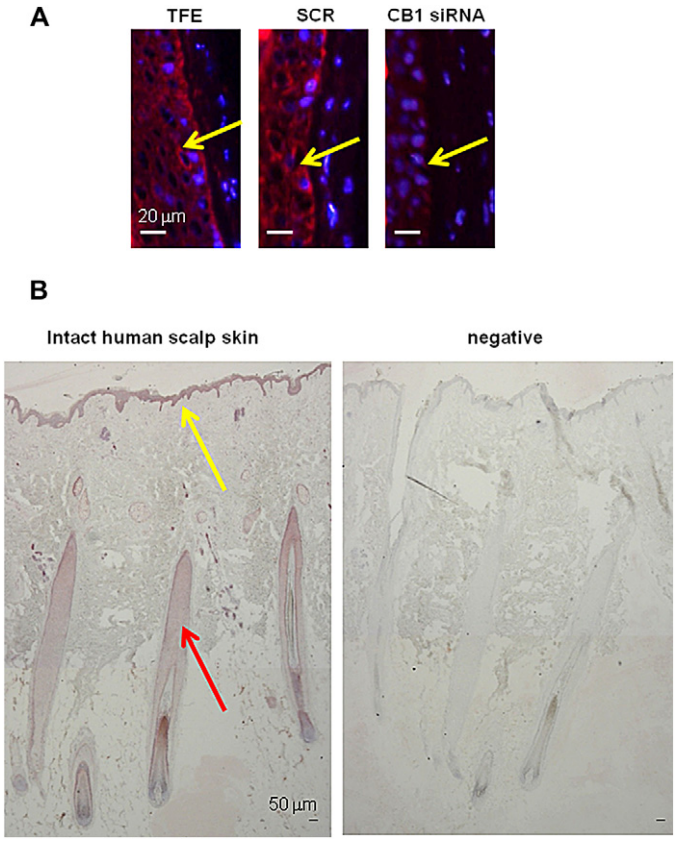

FIG E2. CB1 gene knockdown decreases CB1 expression *in situ*. **A,** Representative images of specific CB1 immunofluorescence *(yellow allow) in situ*. **B,** Representative images of CB1 immunohistochemistry with intact human scalp skin sample *(left)* and negative staining control *(right)*. Positive CB1 immunoreactivity in the epidermis *(yellow arrow)* and ORS *(red arrow)*. *SCR*, Scrambled siRNA treated HFs; *TFE*, transfection reagent treated HFs.

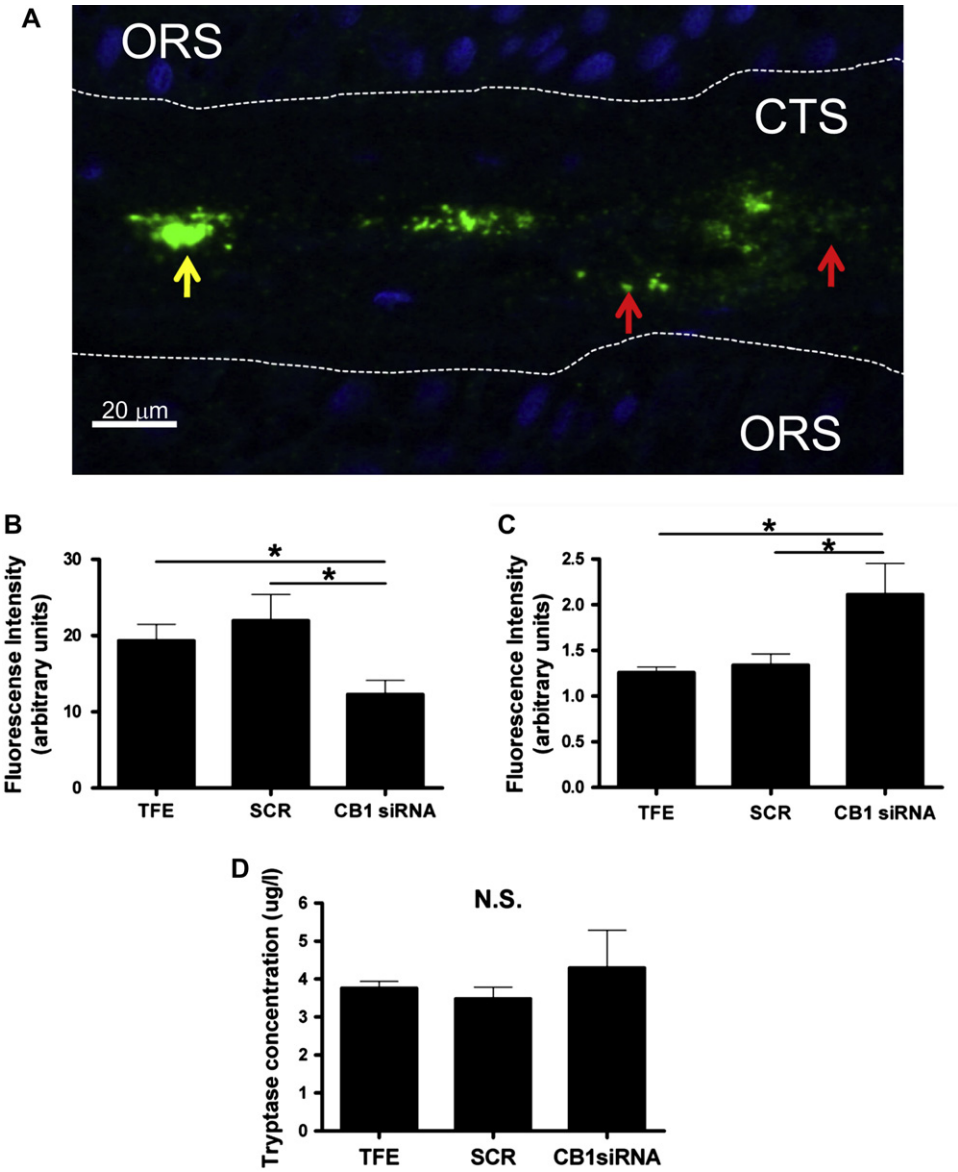

FIG E3. Trypase immunohistology with CB1 siRNA-treated HFs and tryptase levels in the culture medium. **A,** Representative image of tryptase immunohistology with CB1 siRNA-treated HFs. *Yellow arrow*: tryptase+ intracellular immunoreactivity; *red arrows*: intercellular tryptase+ immunoreactivity. **B,** Quantitative immunohistomorphometry of intracellular tryptase immunoreactivity. **C,** Quantitative immunohistomorphometry of intercellular tryptase immunoreactivity. **D,** Statistical analysis of tryptase levels in the HF organ-culture medium. $*P < .05$ denotes significant differences for the indicated comparisons. *NS*, Not significant; *SCR*, scrambled siRNA treated HFs; *TFE*, transfection reagent treated HFs.

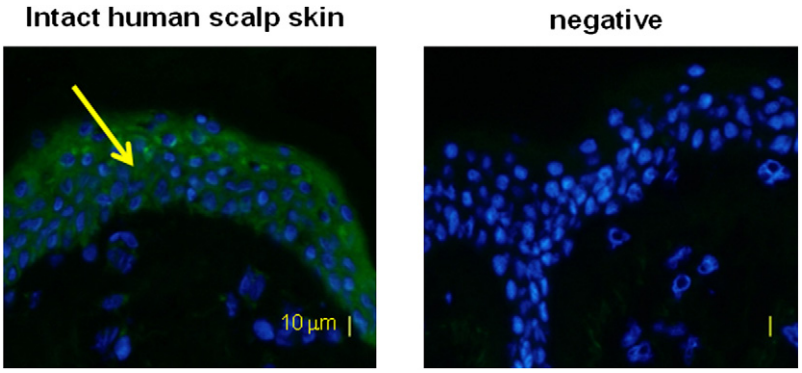

FIG E4. SCF is expressed within the epidermis of human skin. Indirect SCF immunofluorescence images of intact human scalp skin sample.

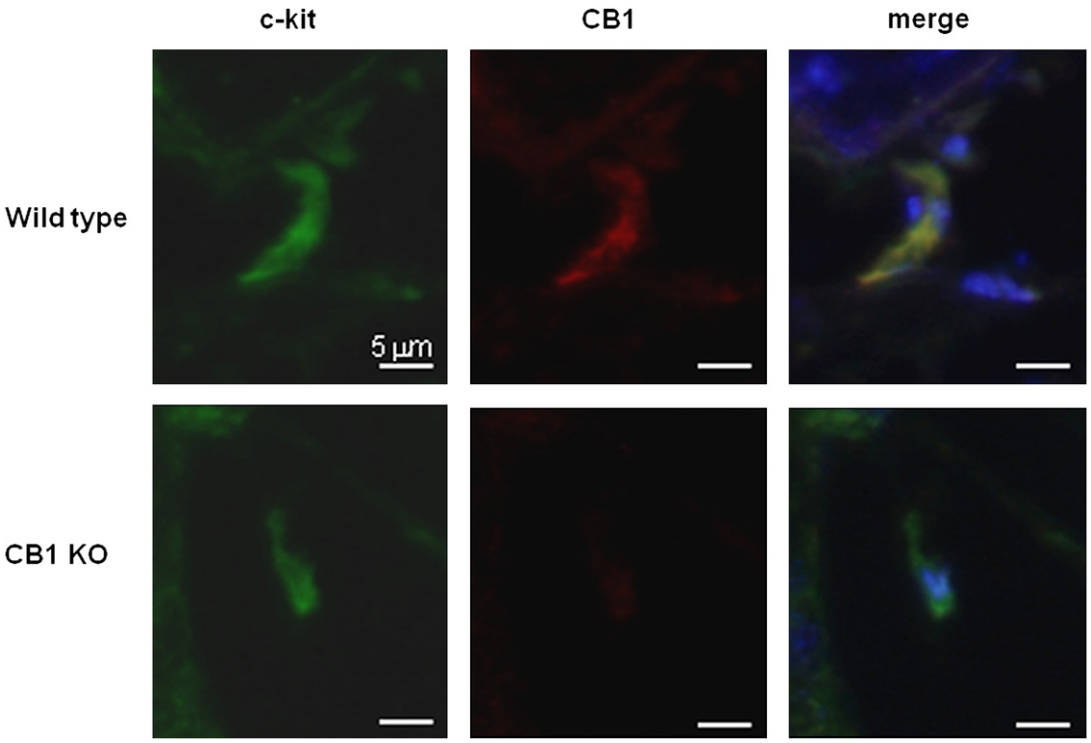

FIG E5. CB1 expression in c-kit+ MCs of CB1 KO mice is reduced compared with that in wild-type mice. Double immunohistology for c-kit and CB1 in both wild-type and CB1 KO mice (postnatal day 32). Scale bar: 5 μm.