Original Article

Endocannabinoids Limit Excessive Mast Cell Maturation and Activation in Human Skin

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Abstract

Background-Mast cells (MCs) crucially contribute to many inflammatory diseases. However, the physiological controls preventing excessive MCs activities in human skin are incompletely understood.

Objective-Since endocannabinoids are important neuroendocrine MCs modifiers, we investigated how cannabinoid receptor (CB) 1-stimulation/inhibition affects human skin MCs biology *in situ*.

Methods-This was investigated in the MCs-rich connective tissue sheath (CTS) of organ-cultured human scalp hair follicles (HFs) by quantitative (immuno)histomorphometry, ultstrastructural and qPCR techniques, using CB1 agonists or antagonist, CB1 knock-down, or CB1 knockout mice.

Results-Kit+ MCs within the CTS of human HFs express functional CB1 receptors, whose pharmacological blockade or gene silencing significantly stimulated both, MCs degranulation and maturation from resident progenitor

cells *in situ* (i.e. enhanced the number of tryptase+, FcεRIα or chymase+ CTS-MCs). This was, at least in part, stem cell factor-dependent. CB1 agonists counteracted the MCs-activating effects of classical MCs secretagogues. Similar phenomena were observed in CB1 knock-out mice, attesting to the *in vivo* relevance of this novel MCs-inhibitory mechanism.

Conclusion-Using human HF organ-culture as an unconventional, but clinically relevant model system for studying MCs biology *in situ*, we show that normal skin MCs are tightly controlled by the endocannabinoid system. This limits excessive MCs activation and maturation from resident progenitors via “tonic” CB1 stimulation by locally synthesized endocannabinoids. The excessive MCs numbers and activation in allergic and other chronic inflammatory skin diseases may partially arise from resident intracutaneous MC progenitors, e.g. due to insufficient CB1-stimulation. Therefore, CB1-stimulation is a promising strategy for the future management of allergy and MCs-dependent skin diseases.

Capsule Summary

CB1-stimulation by endocannabinoids is required to limit human and murine skin mast cells activation as well as mast cell maturation from resident progenitors *in situ*. Therefore, mast cell-dependent human skin diseases should profit from CB1 stimulation.

Key Messages

Endocannabinoids control not only human skin mast cell activation but also their maturation from resident progenitor cells *in situ* via 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 mast cell-dependent inflammatory diseases by limiting skin mast cell activation and maturation.

Keywords

endocannabinoid, cannabinoid receptor, skin, hair follicle, mast cell, stem cell factor, tryptase

Abbreviations used

MC mast cell

HF hair follicle

CTS connective tissue sheath

ORS outer root sheath

CB cannabinoid receptor

ECS endocannabinoid system

SCF stem cell factor

KO knockout

INTRODUCTION

In many developed countries, the incidence of allergic diseases is increasing to epidemic proportions, affecting up to 30% of the population[S26]. Thus, these diseases constitute a considerable burden to affected patients and to healthcare 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[1, 2], 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 MCs activities and numbers under physiological circumstances[3]. 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 defence and “danger”-response systems, but also regulate inflammation, tissue repair, and tissue remodelling[1-5]. Though it is now understood that MCs are involved in both inciting and limiting inflammation[2, 6-8], the main focus of clinically applied MCs research still is on undesired, excessive

MCs activities and their disease-promoting consequences, e.g. in atopic eczema, chronic urticaria, allergic asthma and allergic rhinitis. Yet, the physiological controls of MCs that prevent an excessive accumulation and activation of MCs in normal human tissues *in situ* have been much less studied and are therefore only very incompletely understood. Thus, it remains a major unmet challenge for translational MCs research to identify important endogenous controls that prevent excessive MCs activation and numbers within healthy human tissues (as opposed to cell culture conditions, where MC behaviour is generally studied in the – highly artificial - absence of complex regulatory cues that normally emanate from their local tissue environment).

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, very rich in MCs[9] and becomes frequently available during elective plastic surgery. It is important to remember that immature bone marrow-derived MC progenitors not only are deposited in peripheral tissues, such as skin, where they complete their development[1, 2]. 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)[10, 11]. Therefore, we hypothesized that robust mechanisms must be in place to avoid excessive increases in MCs numbers by limiting the intracutaneous maturation of MCs from resident progenitor cells within human skin, namely from resident, CTS-associated MC progenitors[10,11].

Given the pivotal dependence of MCs on signals from their local tissue milieu[1, 5, 8], it is critical to elucidate the behaviour 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 MCs functions, e.g. during innate immune defense, neurogenic inflammation, angiogenesis, wound healing and hair growth[8, 10, 12]. 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[11, 13] and substance P[14]. Thus, the organ culture of healthy, adult human scalp HFs with their MCs-rich CTS[11, 14] provides an unconventional, but highly instructive, accessible, 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[15-18]. The endocannabinoid system (ECS) consists of cannabinoid receptors (CBs), their endogenous ligands (i.e. endocannabinoids, such as anandamide [AEA] and 2-arachidonoylglycerol [2-AG]), and enzymes responsible for endocannabinoid synthesis and degradation[15, 19-22]. 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 impacts on rodent or human MC lines *in vitro* (for details, see Supplementary Introduction in the Online). Furthermore, it remains to be studied whether the ECS affects MC maturation from human progenitor cells *in situ*.

Therefore, we have investigated whether and how CB stimulation/inhibition affects normal, experimentally unmanipulated human skin MCs *in situ*[23]*.* 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[24, 25] were microdissected and organ-cultured as described previously[11, 14, 23]. Human tissue collection and handling was performed according to Helsinki guidelines, after 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 eight healthy patients (aged 49-72, average: 59) undergoing routine face-lift surgery. HF organ culture details are given in the supplementary Online Methods.

Mast cell histochemistry

Mature human skin MCs were detected with two 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* using the highly sensitive tyramide signal amplification (TSA) technique (Perkin

Elmer, Boston, MA) 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 (details: see supplementary Online Methods).

CB1 knock-down *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, CA). HF transfection was performed according to the manufacturer's protocol (details: see supplementary Methods in the Online).

Statistical Analysis

Data were analyzed using either the Mann-Whitney *U*-test or Student's *t*-test for unpaired samples, using Prism 4.0 software (GraphPad Prism Program, GraphPad, San Diego, CA). *p* values <0.05 were regarded as significant. All data in the Figure are expressed as mean + SEM. * P<0.05, ** P<0.01, ***

P<0.001 for the indicated comparisons.

RESULTS

Human CTS-MCs express CB1

Human scalp HFs, including their MCs-rich connective tissue sheath (CTS), express CB1 mRNA and protein, but not CB2[20]. Therefore, we first asked whether cells positive for Kit (CD117, a marker that identifies even relatively immature MCs[5, 11, 26]) within the CTS[11, 14] (which does not contain any Kit+ melanocytes), express CB1. By immunohistology, 75.5% of Kit+ CTS-MCs prominently co-expressed CB1, both in organ-cultured HFs (Figures 1A and B) and in intact human scalp skin (Figure 1C). The highly CB1-selective fluorescent ligand, Tocrifluor T1117, bound directly to Kit+ CTS-MCs (Figure 1D), demonstrating that these CB1 receptors display functional and specific binding activity.

CB1 inhibition induces CTS-MCs activation and increases their number

We then assessed whether treatment with the prototypic endocannabinoid, AEA (30 μM)[15, 21, 23], or with the selective CB1 agonist, ACEA (30 μM)[27] altered the total number of histochemically detectable CTS-MCs and/or their activation

status (degranulation). Interestingly, this was not the case (Figures 2B-E and Figures S1A and B in the Online).

In contrast, the selective CB1 antagonist, AM251 (1 μM)[15, 23], significantly increased both the number of mature CTS-MCs and their degranulation (Figures 2B-E, Figures S1A and B in the Online). These effects were completely abrogated by co-incubation with AEA or ACEA (Figures 2B-E, Figures S1A and B in the Online). High resolution light microscopy independently confirmed that selectively antagonizing CB1 significantly up-regulated CTS-MCs degranulation (Figures S1D and E in the Online). By transmission electron microscopy, AM251-treated MCs showed the typical ultrastructural morphology of degranulated human MCs[S27] (Figure S1F in the Online).

Thus, antagonizing CB1-mediated signaling clearly increases human skin MC degranulation *in situ*. This conflicts with a previous report that the CB1 antagonist, AM281, does not affect the degranulation of RBL-2H3 cells by itself[28].

CB1 inhibition induces CTS-MCs maturation, but not proliferation

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[1, 11, 14]. Surprisingly, CB1 inhibition up-regulated the number of Kit+ human CTS-MCs *in situ* (Figures 3A-C) (since we cannot exclude that CB1-negative, 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 Figure 3B and C). Interestingly, however, AM251 altered neither the number of Ki67+/Kit+ cells (Figure 3D) nor TUNEL+/Kit+ cells (Figure 3F). Most Kit+ CTS cells were Ki67-negative in both control and AM251 treated organ-cultured HF (Figure 3E). 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 excluded that availability of a larger -“*n*”- 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 histochemcially and immunohistologically detectable MCs during such a short HF organ culture period (24 hrs).

To independently validate this concept, we assessed the expression of antigens characteristically found on/in mature MCs, i.e. the MC proteases tryptase and chymase, and the high-affinity receptor for IgE, FcεRIα[1, 5, 26, 29]. 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 (Figures 3G-I).

This suggests that CB1 blockade, rather than affecting the proliferation/apoptosis[3] of resident mature CTS-MCs, first, stimulates the differentiation of resident, highly immature, Kit-negative 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 knock-down was demonstrated by a significant down-regulation of CB1 immunoreactivity (Figures 4A, B and Figure S2A in the Online) as well as by QPCR (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 the CTS of HFs treated with scrambled oligos) (Figures 5A-E, H and I). Interestingly, Kit immunoreactivity also significantly increased in the CTS of CB1-knock-down HFs (Figure 5F). CB1 knockdown decreased intracellular (Figure S3B, Online), but increased intercellular tryptase immunoreactivity (Figures S3A and C, Online). This suggests that tryptase was actively secreted after CB1 knockdown. However, CB1 knockdown did not significantly elevate tryptase levels in the culture medium (Figure S3D, Online), possibly due to the well-recognized strong binding of secreted tryptase to collagen[S28]. In fact, many extracellularly located, tryptase+ granules were detectable in the collagen-rich CTS, most prominently in CB1 siRNA treated HFs (Figure S3A in the Online). CB1 gene knockdown did not stimulate CTS-MC proliferation *in situ* (Figure 5G).

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[23], maintains Kit expression and MC numbers/activation 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 even Kit+ yet. These then differentiate, first, into Kit+, and subsequently into tryptase+/chymase+/ FcεRIα+ mature MCs.

Endocannabinoids inhibit excessive MCs activation via CB1

Since excessive MCs degranulation and numbers in human skin play a key role in the pathogenesis and clinical phenotype of several major skin diseases[1, 2, 6, 8], we asked whether CB1 stimulation counteracts the MCs-activating effects of classical MCs secretagogues. Quantitative MCs histomorphometry *in situ* demonstrated that this is the case: Both, the potent, non-selectively CB1-stimulating endocannabinoid AEA (30 μM)[20, 23] and the CB1-specific agonist, the synthetic cannabinoid ACEA (30 μM)[20, 27], inhibited the degranulation-promoting effects of key endogenous and exogenous MCs activators: substance P (10^{-10} M), a key mediator of stress-induced, neurogenic skin inflammation[30] (Figure 6A), and the standard secretagogue, compound 48/80[31] (10 μg/ml) (Figure 6B).

Thus, CB1 stimulation effectively counteracts excessive MC activation in normal human skin *in situ*. This suggests that, rather than acting on resting MCs (see Figure 2B-E and Figure S1A and B Online), the ECS of human skin may primarily tone-down *activated* MCs *in situ*.

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

Human HF epithelium expresses functional CB1[23] and is a major source of stem cell factor (SCF)[11], the key growth factor that drives MC maturation[2, 5]. Therefore, we asked whether CB1 stimulation/inhibition may induce the observed effects on MCs maturation and activation also indirectly, i.e. through stimulating the intrafollicular expression of SCF by CB1+ HF epithelial cells *in situ*.

Indeed, AM251 (1 μM) significantly up-regulated SCF expression in organ-cultured HFs, both at the gene (Figure 7C) and protein level (Figures 7A and B). This was abrogated by co-administering ACEA (30 μM) (Figures 7B and C). Furthermore, AM251 significantly increased SCF secretion into the culture

355 medium of ORS keratinocytes *in vitro* (Figure 7D). After AM251 treatment,
356 compared to the control, SCF immunoreactivity was prominently detectable in
357 close proximity to the cell membrane (Figure 7E). 60% of the cells treated with
358 AM251 showed this fluorescence staining pattern, while this was observed only
359 in 36.8 % of the cells in vehicle control group. This further suggests increased
360 SCF secretion after blockade of CB1-mediated signaling and adds additional
361 credence to the concept that SCF production by human HF epithelium is
362 controlled by the ECS *via* CB1 stimulation.
363
364 To further assess this indirect effect of CB1 inhibition by AM251 on CTS-MCs
365 *via* SCF secretion by the HF epithelium[10, 11], we performed additional HF organ-
366 culture for 1 day with 1 μM of AM251 in the presence of 1 μg/ml of SCF-
367 neutralizing antibody. When test and control groups were compared with
368 respect to the number of Kit+ CTS-MCs, the increase in the number of Kit+ MCs
369 after pharmacological CB1 blockade was partially, yet significantly reduced by
370 neutralizing SCF (Figure 7F).
371
372 **CB1 deletion induces CTS-MCs maturation and activation *in vitro***

Finally, by examining CB1 knockout mice[32, 33], 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-negative in these knockout mice (Figure S5, Online). Moreover, in line with our human HF organ culture data, the total number of MCs and c-kit+ cells was significantly increased in the subcutaneous CTS in the skin of CB1 knockout mice *in vivo* (Figures 8A, C and D). Here, the number of degranulated MCs was also significantly higher than in age- and hair cycle-matched wild type mice (Figure 8B). There was no significant change in the number of Ki67/Kit double-positive CTS-MCs between CB1 knockout and WT mice (Figure 8E). 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 also is 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 (i.e. SCF-mediated) effects on normal human skin *in situ*.

Previous *in vitro*-studies, which had investigated rodent and human MC lines[16, 17, 28, 34-37], had painted a contradictory picture on the role of the ECS in MC activation (see Supplementary Introduction [Online] for details). Likely, this is explained by 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 required 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 of the effects of CB1 antagonist on MCs are “direct” or “indirect” through SCF. However, our results with Tocrifluor (Figure 1D) as well as CB1/Kit-double immunofluorescence (Figure 1A-C) make it reasonable to assume that direct, CB1-mediated effects on MCs operate side-by-side with indirect ones (i.e., 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[10], but also in healthy human skin[11]. Moreover, we provide the first evidence that the differentiation of these resident precursors into mature, functional MCs

423 is constitutively inhibited by the ECS. In contrast to rapidly proliferating hair
424 matrix keratinocytes[23] or various neuronal cell populations[38], CB1-mediated
425 signaling primarily seems to affect CTS-MC maturation and activation, but not
426 proliferation or cell death. That this also applies to murine skin *in vivo* attests to
427 the physiological relevance of human HF organ culture.
428
429 While the CB1 KO mouse data confirm our human HF organ culture
430 observations with respect to the regulation of skin MCs by CB1, it must be kept
431 in mind that, under *in vivo* conditions, the MC phenomena observed in the skin
432 of CB1 KO mice may reflect more complex mechanisms.
433
434 We are currently testing how CB1 stimulation/blockade affects human mucosal
435 type MCs in organ-cultured human nasal polyp samples, an excellent surrogate
436 tissue for human bronchial mucosa[S29]. Our available pilot observations indicate
437 that CB1 also suppresses the maturation of functional mucosa-type MCs from
438 resident progenitor cells: Kit+ cells in human nasal polyps express CB1 *in situ*,
439 and AM251 increases the total number of tryptase+ and Kit+ MCs without
440 modulating their proliferation. (Sugawara, Hundt, Zákány, and Paus; manuscript

441 in preparation). This encourages one to explore whether CB1-mediated “tonic”
442 inhibition of MC maturation and activation by the ECS is a general principle that
443 also operates in other human MC populations than the ones investigated here
444 in human skin.
445
446 Our study strongly suggests that targeting the ECS for the down-modulation of
447 excessive MC activities in human skin could become an attractive new
448 therapeutic strategy in clinical medicine. Moreover, the current study
449 encourages one to systematically dissect whether allergic diseases and many
450 other disorders characterized by excessive MCs numbers and/or activation (e.g.
451 in bronchial asthma, allergic rhinitis, atopic eczema, prurigo dermatoses,
452 psoriasis, mastocytosis, and chronic urticaria) are associated with defined
453 defects in the ECS, such as insufficient endocannabinoid synthesis, excessive
454 endocannabinoid metabolism, and/or defective CB1-mediated signaling.
455
456 Furthermore, our data call attention to the HF’s CTS as a previously ignored
457 tissue compartment that may play an important role in excessive MC activities
458 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 MCs 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[S8, S30] 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 MCs accumulation and degranulation. Even though such organ culture approaches exclude neural and perfusion-dependent inputs into skin MC biology, these two companion assay systems allow one to dissect the clinically important, but under-explored

neuroendocrine controls of skin MCs[11, 14, 15, 39, 40] under conditions where critical cell-cell (e.g. 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[S31], 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 MCs numbers and/or massive degranulation of skin MCs (e.g. 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[S32].

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”[7] (note that HFs are immunoprivileged mini-organs[S33]). Such an immunoinhibitory role of perifollicular MCs would make it particularly important that excessive, pro-inflammatory 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[3] where this is clinically desired.

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Figure legends

Figure 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. ORS=outer root sheath. NC=negative control.

Figure 2. Effect of CB1 signaling on CTS-MC number and degranulation status

A. “Degranulated” (Arrow head) and “non-degranulated” (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.

Figure 3. CTS-MCs differentiation and proliferation

A. Kit immunohistology with organ-cultured HFs. **B.** and **C.** Quantitative

651 immunohistomorphometry of Kit+ cells in organ-cultured HFs. **D.** Quantitative
652 immunohistomorphometry of Kit/Ki67 double+ cells. **E.** Yellow-arrow denotes
653 Ki67+ proliferative hair matrix keratinocytes. Green-arrow denotes Kit+/Ki67-
654 cells. **F.** Quantitative immunohistomorphometry of Kit/TUNEL double+ cells.
655 Quantitative immunohistomorphometry of FcεRIα (**G**), tryptase (**H**), and
656 chymase (**I**)+ cells.
657
658 **Figure 4. CB1 gene knockdown is possible in human HFs**
659 **A.** (Upper panel) Representative images of CB1 immunohistochemistry with
660 TFE, SCR, and CB1 siRNA treated HFs. (lower panel) High magnification
661 images of CB1+ ORS keratinocytes of each treatment group. Arrows denote
662 positive immunoreactivity. **B.** Quantitative immunohistomorphometry of CB1
663 immunohistochemistry with CB1 siRNA treated human HFs.
664
665 **Figure 5. CB1 knockdown increases degranulated and total CTS-MCs *in***
666 ***situ*.**
667 **A.** Leder's-esterase histochemistry. **B.** Quantitative histomorphometry of
668 degranulated CTS-MCs. **C.** Quantitative histomorphometry of total CTS-MCs. **D.**

669 Kit immunohistology. **E.** Quantitative immunohistomorphometry of Kit+ cells. **F.**
670 Quantitative analysis of Kit immunoreactivity in Kit+ cells. **G.** Quantitative
671 immunohistomorphometry of Kit/Ki67 double+ cells. **H.** Tryptase
672 immunohistology. **I.** Quantitative immunohistomorphometry of tryptase+ cells.
673
674 **Figure 6. Inhibitory effects of cannabinoids on human skin MCs**
675 **degranulation induced by endogenous or exogenous MC secretagogues.**
676 Quantitative histomorphometry of CTS-MCs degranulation detected by leder's-
677 esterase histochemistry in substance P (10^{-10}M) (**A**) and compound 48/80 (10
678 μg/ml) (**B**) treated organ-cultured human HFs.
679
680 **Figure 7. Dependence of CB1-mediated CTS-MC effects on SCF**
681 **A.** SCF immunohistology. **B.** Quantitative analysis of SCF immunoreactivity. **C.**
682 QPCR analysis for *SCF* with 1-day organ-cultured HFs. **D.** SCF measurement
683 in ORS keratinocytes culture medium. **E.** SCF immunocytochemistry. **F.**
684 Quantitative immunohistomorphometry of Kit+ cells in organ-cultured HFs with
685 AM251 (1 μM) or/and SCF neutralizing antibody (1 μg/ml). ORS=outer root
686 sheath.

Figure 8. *In vivo* effects of CB1 knock-out on CTS-MC number, degranulation and proliferation 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.

Endocannabinoids Limit Excessive Mast Cell Maturation and Activation in Human Skin

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Supplementary Information

Contents:

Supplementary Introduction

There are conflicting reports on how CB stimulation impacts on 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[1]. Methanandamide reportedly also inhibited IgE-mediated MC degranulation in primary murine bone marrow-derived MCs *in vitro*[2]. In RBL-2H3 cells, endocannabinoid, palmitoylethanolamide, produced a small, but significant reduction in antigen-stimulated serotonin release at high concentrations, whereas anandamide was without effect. In contrast, the endocannabinoid, 2-arachidonoylglycerol (2-AG) and methanandimide both increased the antigen-stimulated MC degranulation[3]. Palmitoylethanolamide, but not anandamide downmodulated MC activation via CB2 in same cell line[4]. Furthermore, the phytocannabinoid compound, cannabidiol triggered RBL-2H3 cell degranulation[5]. 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

735 and their analogues neither induced histamine secretion, nor promoted
736 compound 48/80-indued degranulation[6]. Concerning human MCs, it has been
737 reported that supernatants from SW756 cervical carcinoma cells stimulated
738 degranulation of the human MC line, LAD2, which was inhibited by CB2
739 stimulation[7].

740 This leaves us with a confusing and contradictory picture of the role that CB1
741 versus CB2 stimulation may play in the control of MC activation. Moreover, it
742 remains completely unknown how the ECS impacts on primary human MCs,
743 and under clinically relevant conditions, e.g. on human skin MCs *in situ*.

Supplementary Methods

Methods

Reagents

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

HF Organ Culture

Isolated HFs were maintained in supplemented serum-free William's E medium[8-11]. HFs were first incubated overnight to adapt to culture conditions after which the medium was replaced and vehicle or test substances was 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} M) or compound 48/80 (10 μg/ml) in the combination with AEA or ACEA for additional 1 day. Following culturing for the time indicated, HFs were then cryoembedded and prepared for histology and immunohistochemistry.

763

764 **Immunohistochemistry**

765 For the detection of Kit, CB1, tryptase, chymase and FcεRIα, the highly
766 sensitive tyramide signal amplification (TSA) technique (Perkin Elmer, Boston,
767 MA) was applied. Cryosections were incubated overnight at 4°C with primary
768 antibodies, either rabbit anti-human CD117 (Cell Marque Corp., Rocklin, CA,
769 USA) at 1:1000, rat anti-mouse CD117 (BD Biosciences, San Jose, CA, USA)
770 at 1:5000, rabbit anti-human CB1 (Cayman Chemical, Michigan, USA, or Santa
771 Cruz, CA, USA) at 1:400, or mouse anti-human FcεRIα (Acris GmbH,
772 Hiddenhausen, Germany) at 1:1000, or mouse anti-human chymase (Abcam
773 plc) at 1:1000, or mouse anti-human tryptase (Abcam plc, Cambridge, UK) at
774 1:5000 diluted in TNB (Tris, NaOH, Blocking reagent, TSA kit; Perkin-Elmer).
775 Thereafter, the cryosections were incubated with goat biotinylated antibodies
776 against rabbit or mouse IgG (Jackson Immunoresearch Laboratories, West
777 Grove, PA) at 1:200 in TNB for 45 min at room temperature (RT). The TSA
778 method was applied according to the manufacturer's protocol.

779

780 Double-immunostaining for Kit and CB1 was performed by 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 min, RT). Sections were then incubated with streptavidin-conjugated horseradish peroxidase (1:100, 30 min, TSA kit) and were finally incubated with fluorescein isothiocyanate (FITC) conjugated tyramide (1:50, TSA kit). After careful washing with TNT wash buffer (0.1 M Trizma hydrochloride, 0.15 M 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 min, RT). After incubating with streptavidine-conjugated horseradish peroxidase (1:100, 30 min, TSA kit) sections were incubated with tetramer rhodamine conjugated tyramide (1:50, TSA kit).

To study the proliferation of the Kit+ cells, double-immunostaining for Ki-67 and Kit was performed. Briefly, after the staining for Kit 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 phosphate-buffered saline (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 min) at RT.

To evaluate the apoptosis of Kit+ cells, Kit immunostaining and terminal dUTP nick-end labeling (TUNEL) 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, MA) in the presence of terminal deoxynucleotidyl transferase (60 min) at 37°C. After the incubation with Stop/Wash buffer (ApopTag kit) (10 min, RT) and the additional wash with PBS, TUNEL-positive cells were visualized by an antidigoxigenin fluorescein isothiocyanate-conjugated antibody (ApopTag kit) (30 min, 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

817 visualized using the peroxidase-based avidin-biotin complex method (Vectastain
818 Elite ABC kit; Vector Laboratories, Burlingame, CA). Frozen sections were fixed
819 in cold acetone and rinsed with PBS, and endogenous peroxidase activity was
820 saturated with 0.3 % H_2O_2 in PBS for 15 min. After the incubation with 5 % of
821 normal goat serum, sections were incubated with rabbit anti-human CB1
822 antibody (Cayman chemical) (1:40 in PBS) at 4°C overnight. After incubation
823 with a biotinylated goat anti-rabbit antibody (Jackson Immunoresearch
824 Laboratories) (45 min, at RT), sections were treated with Vectastain ABC
825 reagent (Vector laboratories) and visualized with AEC (3-amino-9ethylcarbazol)
826 (Vector laboratories). As negative controls, the appropriate primary antibodies
827 were omitted from the procedure. The specificity of CB1 immunostaining was
828 measured on intact human scalp skin sections (Figure S2B in the Online)
829 mouse brain sections (positive control) which clearly demonstrated positive CB1
830 immunoreactivity in the expected areas (data not shown).
831 For detecting SCF in organ cultured human HFs as well as isolated human
832 ORS keratinocytes, indirect immunofluorescence method was applied using
833 anti-human SCF (Acris GmbH) at 1:20 in PBS as a primary antibody and FITC
834 (Rhodamine for ORS keratinocytes) conjugated goat anti-mouse IgG at 1:200 in

PBS as a secondary antibody. Intact human scalp skin sample was used as a positive control (supplementary Figure S4).

The immunoreactivity of CB1, Kit, tryptase and SCF in defined reference areas was assessed by quantitative immunohistomorphometry[9, 11-13] using the ImageJ software (National Institutes of Health, Bethesda, MD).

For counting MCs, MCs were classified as “degranulated” when five or more extracellularly located metachromatic granules could be detected histochemically at high magnification (x400) 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 (Figures S1A and B in the Online). This is in line with previously reported data[9]. 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 MCs degranulation. MCs in freshly isolated skin also

displayed a steady-state level of degranulation (Figure S1C in the Online).

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 (HRLM) and Transmission electron microscopy (TEM)

Organ cultured human scalp HFs were immersed in a mixture containing 2% paraformaldehyde, 2.5% glutaraldehyde, and 0.025% CaCl2 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). 1 μm of thick sections were prepared for an alkaline-Giemsa histochemistry[14]. MCs were defined as degranulated according to the previous article[14]. Thin sections were stained with uranyl acetate and lead citrate and observed with an electron microscope (JEM-1200EXII, JEOL, Tokyo,

871 Japan).

872

873 **Quantitative PCR**

874 Expressions of specific mRNA transcripts of SCF were analyzed by quantitative
875 real-time PCR performed on an ABI PRISM 7000 Sequence Detection System
876 (Applied Biosystems, Foster City, CA, USA) as described before[12, 13] using
877 TaqMan primers and probes (Assay ID: Hs00241497_m1 for human SCF).
878 Three different internal housekeeping genes, glyceraldehyde 3-phosphate
879 dehydrogenase (GAPDH), β-actin (ACTB), cyclophilin A (PPIA) were assessed
880 (Assay ID: Hs99999905_m1 for GAPDH, Hs99999903_m1 for ACTB, and
881 Hs99999904_m1 for PPIA). The amount of SCF transcripts was normalized to
882 those of the control genes as previously reported[12, 13].

883

884 **CB1 knock-down *in situ***

885 All reagents required for transfection (human CB1 siRNA (sc-39910), control
886 (scrambled, SCR) siRNA (sc-37007), siRNA transfection reagent (sc-29528)
887 and siRNA transfection Medium (sc-36868)) were obtained from Santa Cruz. HF
888 transfection was performed according to the manufacturer's protocol. Briefly,

889 freshly isolated human HFs were kept in cold William's E medium right before
890 the transfection. During transfection CB1 specific siRNA or control siRNA (2.5
891 μl) and siRNA transfection reagent (2 μl) was mixed in transfection medium
892 (500 μl) per well (24 well-plate). After the careful wash, HFs were applied to
893 each well (3 HFs per well) and incubated at 37 °C in a CO_2 incubator for 6
894 hours after which the medium was replaced with supplemented William's E
895 medium. HFs were cryo-embedded 24 hrs following transfection.
896 TFE=transfection reagent treated HFs, SCR=scrambled siRNA treated HFs,
897 CB1 siRNA=CB1 siRNA treated HFs.
898
899 **CB1 knock-out mice**
900 Targeted disruption of the CB1 receptor gene was performed by replacing the
901 CB1 coding sequence with a non-receptor sequence by homologous
902 recombination in MPI2 embryonic stem cells. Mutant mice have been crossed to
903 C57BL/GJ animals for more than 13 generations and are therefore considered
904 to be congenic for this genetic background. Homozygous CB1−/− mice and
905 wild-type (CB1+/+) animals were generated by matings of heterozygous
906 (CB1+/−) mice[15, 16].

907

908 **Tryptase immunoassay**

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

918

919 **Isolation and culture of ORS keratinocytes**

920 Isolation and culture of human ORS keratinocytes were performed according to
921 our established protocol[17]. Briefly, ORS keratinocytes were isolated by an
922 enzymatic digestion (0.2 % trypsin, 0.1 % ethylenediaminetetraacetic acid
923 (EDTA) in calcium and magnesium free phosphate buffered saline (CMF-PBS)
924 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 min) and resuspended in ORS keratinocyte culturing medium. It comprises 3:1 mixture of Dulbecco's modified Eagle medium (DMEM; supplemented with L- glutamine, Na-pyruvate, 4.5 g/L glucose) and Ham's F12 (both from Invitrogen), supplemented with 10% Fetal Clone II (Hyclone) 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 supernates of human ORS keratinocytes culture were collected and freezed at -80 °C until the assay was performed. Samples were analyzed their SCF levels by Quantikine Human SCF ELISA Kit (R&D Systems).

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.

Supplementary Discussion

Our results show that CB1 blockade effects not only directly on MCs, but also induces SCF secretion by human HF keratinocytes. This suggests that, under physiological conditions, “tonic” CB1 stimulation by the intracutaneous ECS keeps 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[18, 19]. Given the limitations of our human HF organ culture system, which is unsuitable for pruritus research, evidently, we cannot provide any corresponding experimental data with this assay on how the ECS

962 may affect itch (pruritogenic pruritus) and its processing in the central system
963 after inflammation. For this, CB1 KO mice are a more appropriate model.
964 However, a couple of relevant reports on the effects of endocannabinoids on
965 itch already suggest that the ECS may indeed play an important role in itch. For
966 example, topical application of the endocannabinoid, PEA (N-
967 palmitoylethanolamine), to patients with mild to moderate atopic eczema
968 significantly reduced the intensity of erythema, pruritus, excoriation, scaling,
969 lichenification and dryness[20]. Since the inhibition of anandamide (AEA)-
970 degrading enzymes (such as FAAH) increases AEA levels in mice[21, 22], it is
971 interesting to note that FAAH knockout mice or FAAH inhibitor-treated mice
972 show significant reduction in scratching without affecting locomotor behavior[23].
973 Moreover, excessive mast cell activity is well-recognized to play a key role in
974 many itch-associated skin diseases, including allergy and atopic dermatitis, and
975 neurogenic skin inflammation[24-27].
976
977 Therefore, our current data are well in line with the concept that the ECS may
978 also modulate MC-dependent pruritogenic pruritus *via* reducing MC
979 degranulation and *via* avoiding excessive MC maturation from resident

intracutaneous progenitor cells. Moreover, since not only HF and epidermal melanocytes, but also rapidly proliferating hair matrix keratinocytes prominently express Kit on the gene and protein level (see Peters et al.[28]), it is conceivable that CB1-regulated changes in the secretion of the cognate ligand (SCF) could also impact on the growth and remodeling of selected, Kit-expressing epithelial cell populations in the HF, besides direct effects of (endo-)cannabinoid effects on CB1+ HF epithelial cells.

Supplementary Figure legends

Supplementary Figure S1. CB1 blockade significantly increases degranulation of CTS-MCs in situ

A. Percentage of degranulated CTS-MCs in organ cultured human HFs for 1 day with ACEA (30 μM) or/and AM251 (1 μM), and with (**B**) AEA (30 μM) or/and 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**). **F.** TEM images

Supplementary Figure S2. CB1 gene knockdown decreases CB1 expression *in situ*.

A. Representative images of specific CB1 immunofluorescence *in situ*.

B. Representative images of CB1 immunohistochemistry with intact human scalp skin sample (left) and negative staining control (right). Yellow arrow; positive CB1 immunoreactivity in the epidermis and ORS (red arrow).

Supplementary Figure S3. Trypase immunohistology with CB1 siRNA treated HFs and tryptase levels in the culture medium.

A. Representative image of tryptase immunohistology AM251 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.

Supplementary Figure S4. SCF is expressed within the epidermis of human skin.

Indirect SCF immunofluorescence images of intact human scalp skin sample.

Supplementary Figure S5. CB1 expression in c-kit+ MCs of CB1 knockout mice is reduced compared to wild type mice.

Double immunohistology for c-kit and CB1 in both wild type and CB1 knockout mice (postnatal day 32). Scale bar; 5 μm.

Supplementary references

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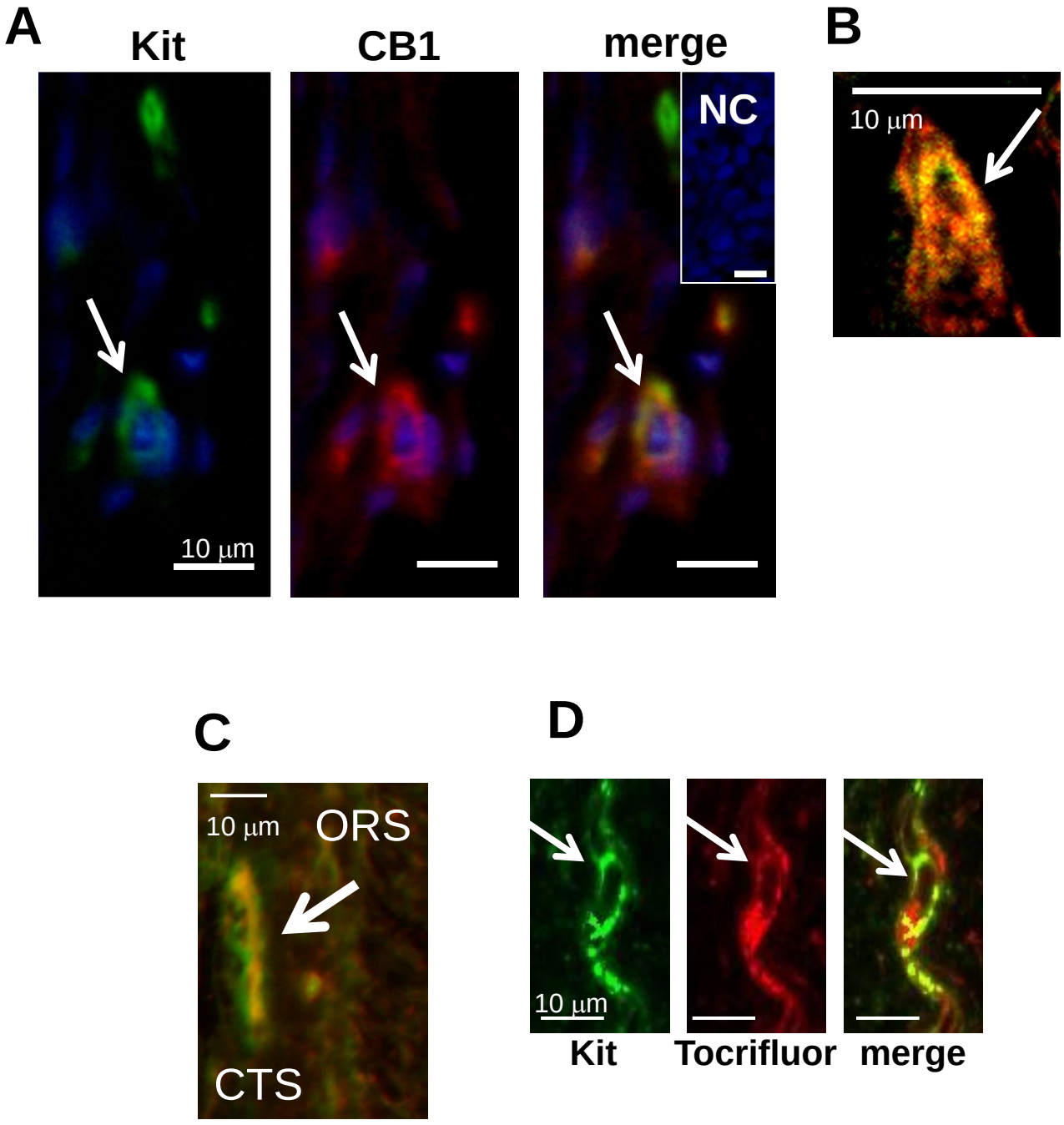

A
Kit
CB1
merge
NC
10 μm
B
10 μm
C
10 μm
ORS
CTS
D
10 μm
Kit
Tocrifluor
merge

Figure 1

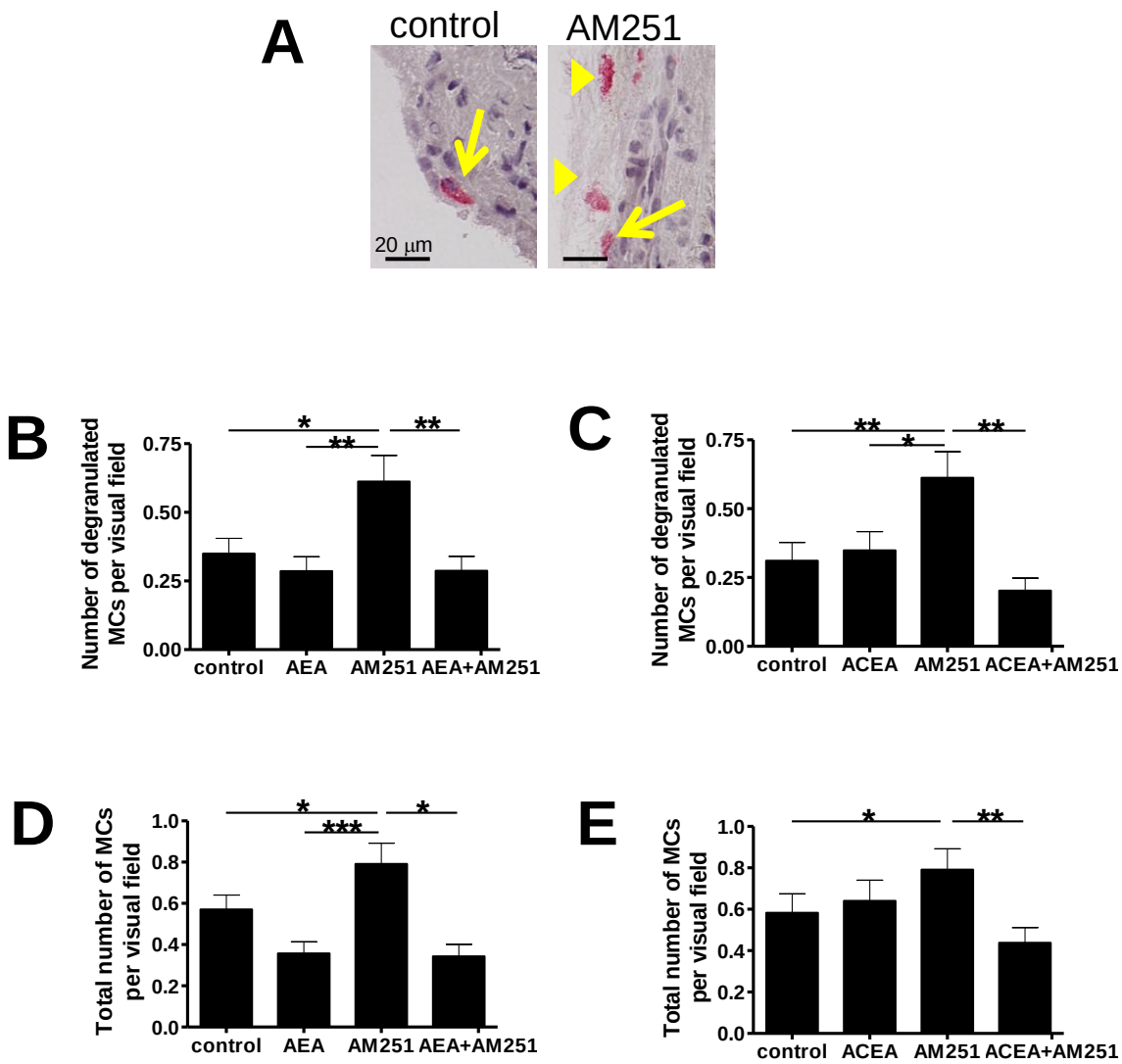

A
control
AM251
20 µm
B
Number of degranulated MCs per visual field
0.00
0.25
0.50
0.75
control
AEA
AM251
AEA+AM251
C
Number of degranulated MCs per visual field
0.00
0.25
0.50
0.75
control
ACEA
AM251
ACEA+AM251
D
Total number of MCs per visual field
0.0
0.2
0.4
0.6
0.8
1.0
control
AEA
AM251
AEA+AM251
E
Total number of MCs per visual field
0.0
0.2
0.4
0.6
0.8
1.0
control
ACEA
AM251
ACEA+AM251

Figure 2

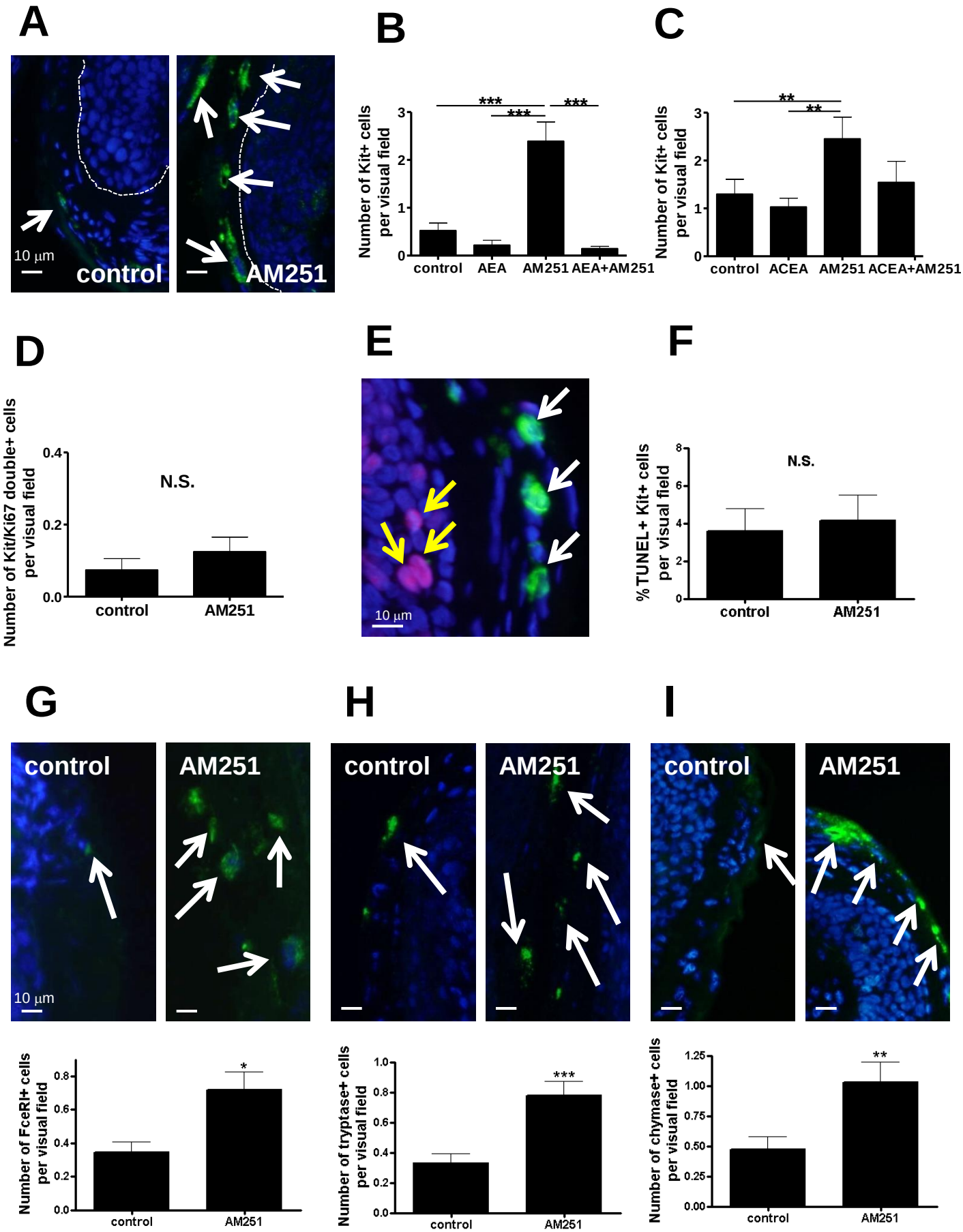
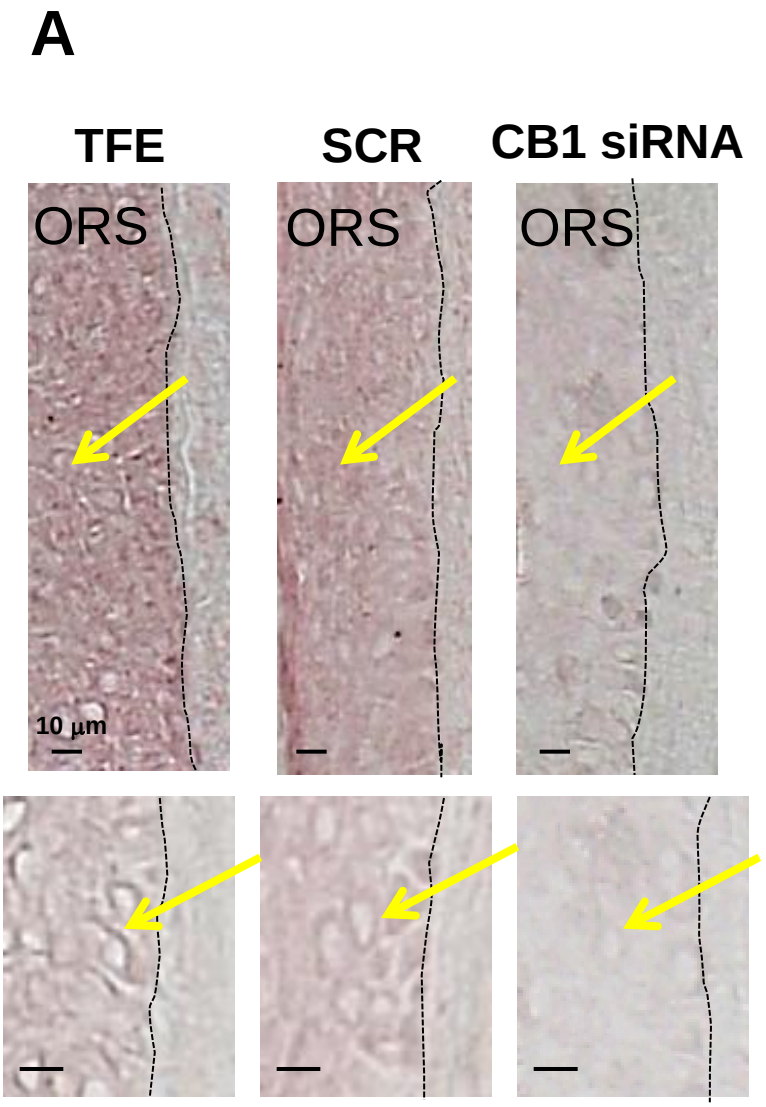

A
10 μm
control
AM251
B
Number of Kit+ cells per visual field
***
***
***
control
AEA
AM251
AEA+AM251
C
Number of Kit+ cells per visual field
**
**
control
ACEA
AM251
ACEA+AM251
D
Number of Kit/Ki67 double+ cells per visual field
N.S.
control
AM251
E
10 μm
F
% TUNEL+ Kit+ cells per visual field
N.S.
control
AM251
G
control
AM251
10 μm
Number of FceRI+ cells per visual field
*
control
AM251
H
control
AM251
Number of tryptase+ cells per visual field
***
control
AM251
I
control
AM251
Number of chymase+ cells per visual field
**
control
AM251

Figure 3


A
TFE
SCR
CB1 siRNA
ORS
ORS
ORS
10 µm

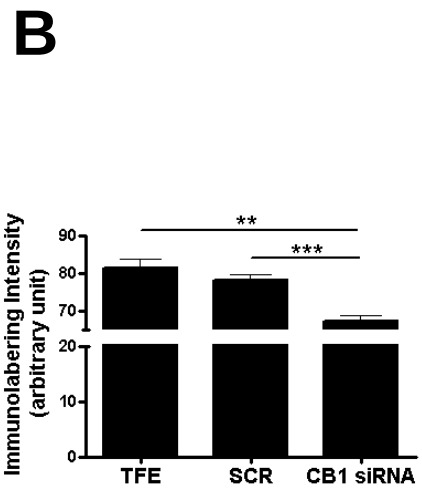

B
Immunolabering Intensity
(arbitrary unit)
**
***
90
80
70
20
10
0
TFE
SCR
CB1 siRNA

Figure 4

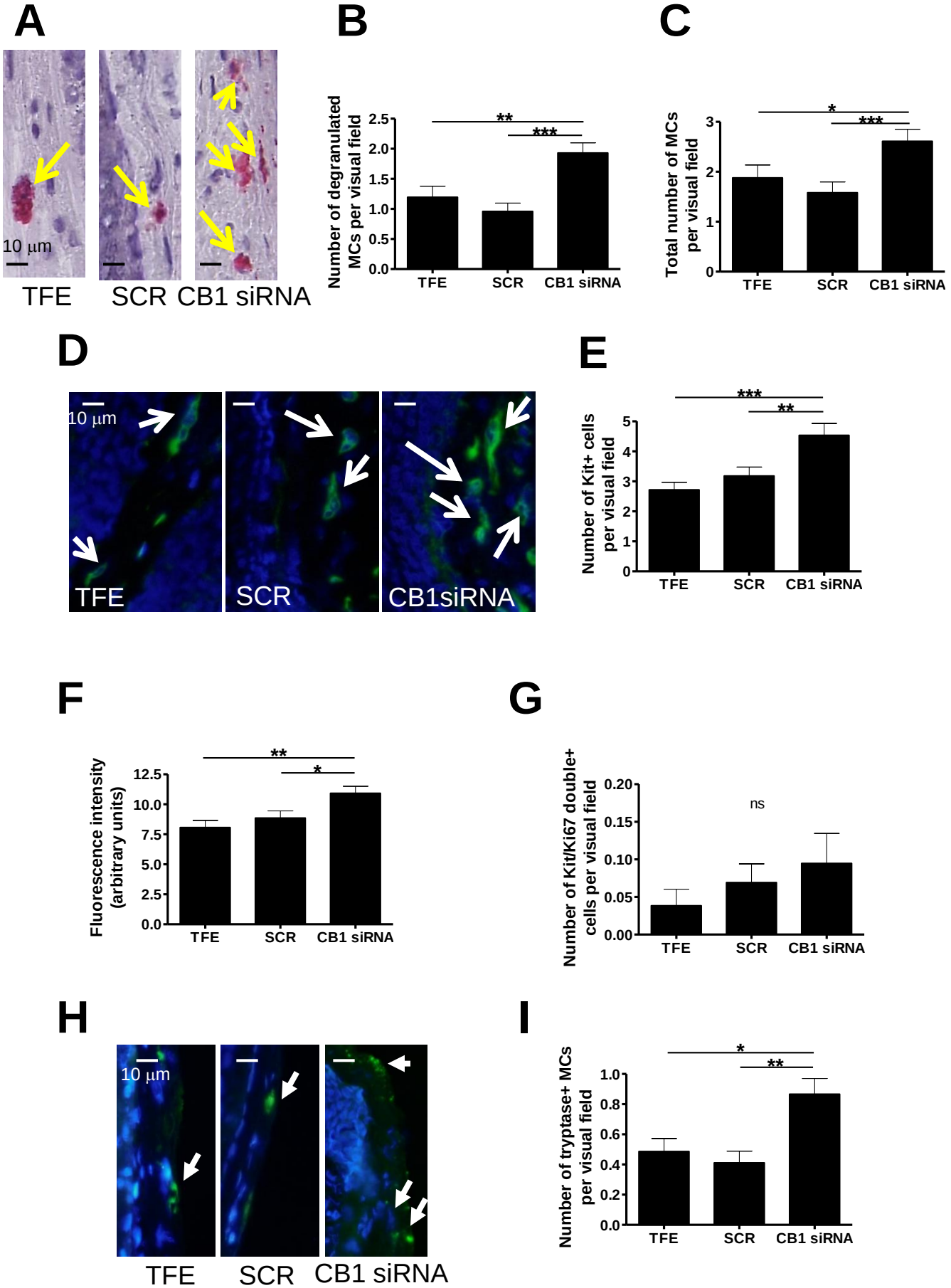

A
10 μm
TFE
SCR
CB1 siRNA
B
Number of degranulated MCs per visual field
2.5
2.0
1.5
1.0
0.5
0.0
**
***
TFE
SCR
CB1 siRNA
C
Total number of MCs per visual field
3
2
1
0
*
***
TFE
SCR
CB1 siRNA
D
10 μm
TFE
SCR
CB1siRNA
E
Number of Kit+ cells per visual field
5
4
3
2
1
0
***
**
TFE
SCR
CB1 siRNA
F
Fluorescence intensity (arbitrary units)
12.5
10.0
7.5
5.0
2.5
0.0
**
*
TFE
SCR
CB1 siRNA
G
Number of Kit/Ki67 double+ cells per visual field
0.20
0.15
0.10
0.05
0.00
ns
TFE
SCR
CB1 siRNA
H
10 μm
TFE
SCR
CB1 siRNA
I
Number of tryptase+ MCs per visual field
1.0
0.8
0.6
0.4
0.2
0.0
*
**
TFE
SCR
CB1 siRNA

Figure 5

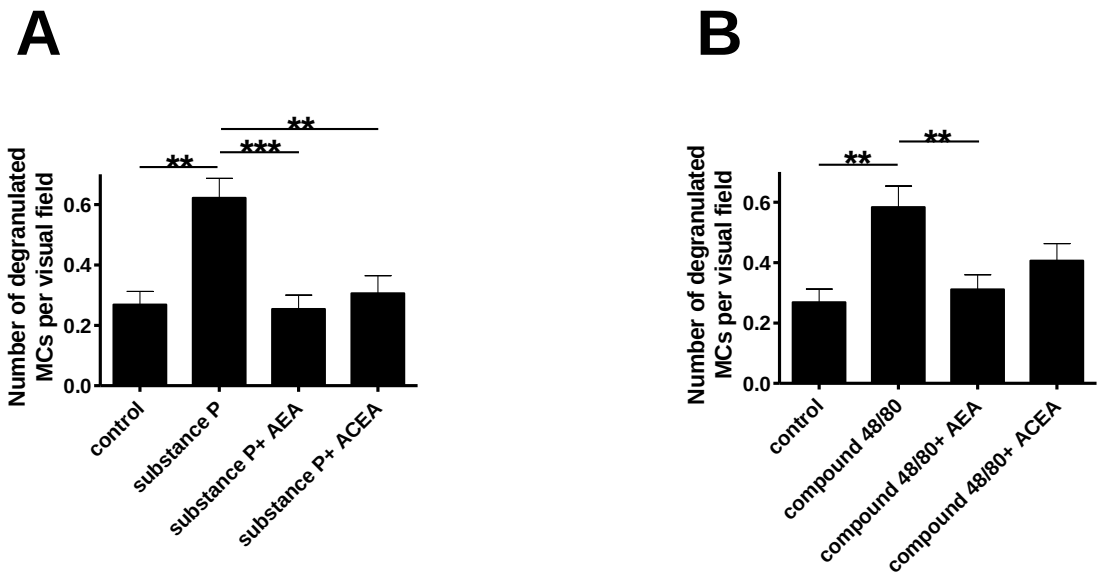

A
B
Number of degranulated MCs per visual field
0.0
0.2
0.4
0.6
**
***
**
control
substance P
substance P+ AEA
substance P+ ACEA
Number of degranulated MCs per visual field
0.0
0.2
0.4
0.6
**
**
control
compound 48/80
compound 48/80+ AEA
compound 48/80+ ACEA

Figure 6

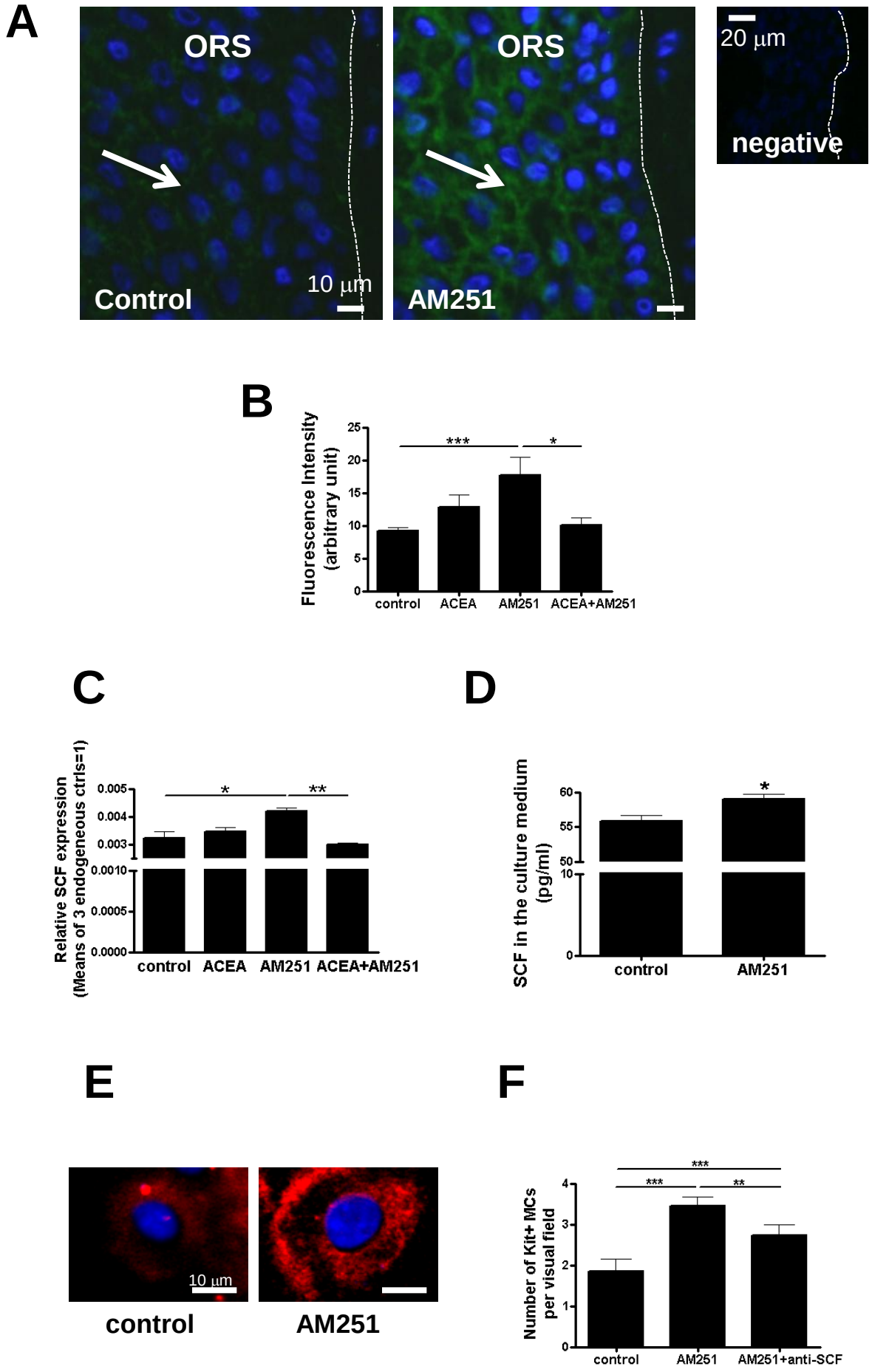

A
ORS
Control
10 μm
ORS
AM251
20 μm
negative
B
Fluorescence Intensity (arbitrary unit)
***
*
control
ACEA
AM251
ACEA+AM251
C
Relative SCF expression (Means of 3 endogeneous ctrls=1)
*
**
control
ACEA
AM251
ACEA+AM251
D
SCF in the culture medium (pg/ml)
*
control
AM251
E
10 μm
control
AM251
F
Number of Kit+ MCs per visual field
***
***
**
control
AM251
AM251+anti-SCF

Figure 7

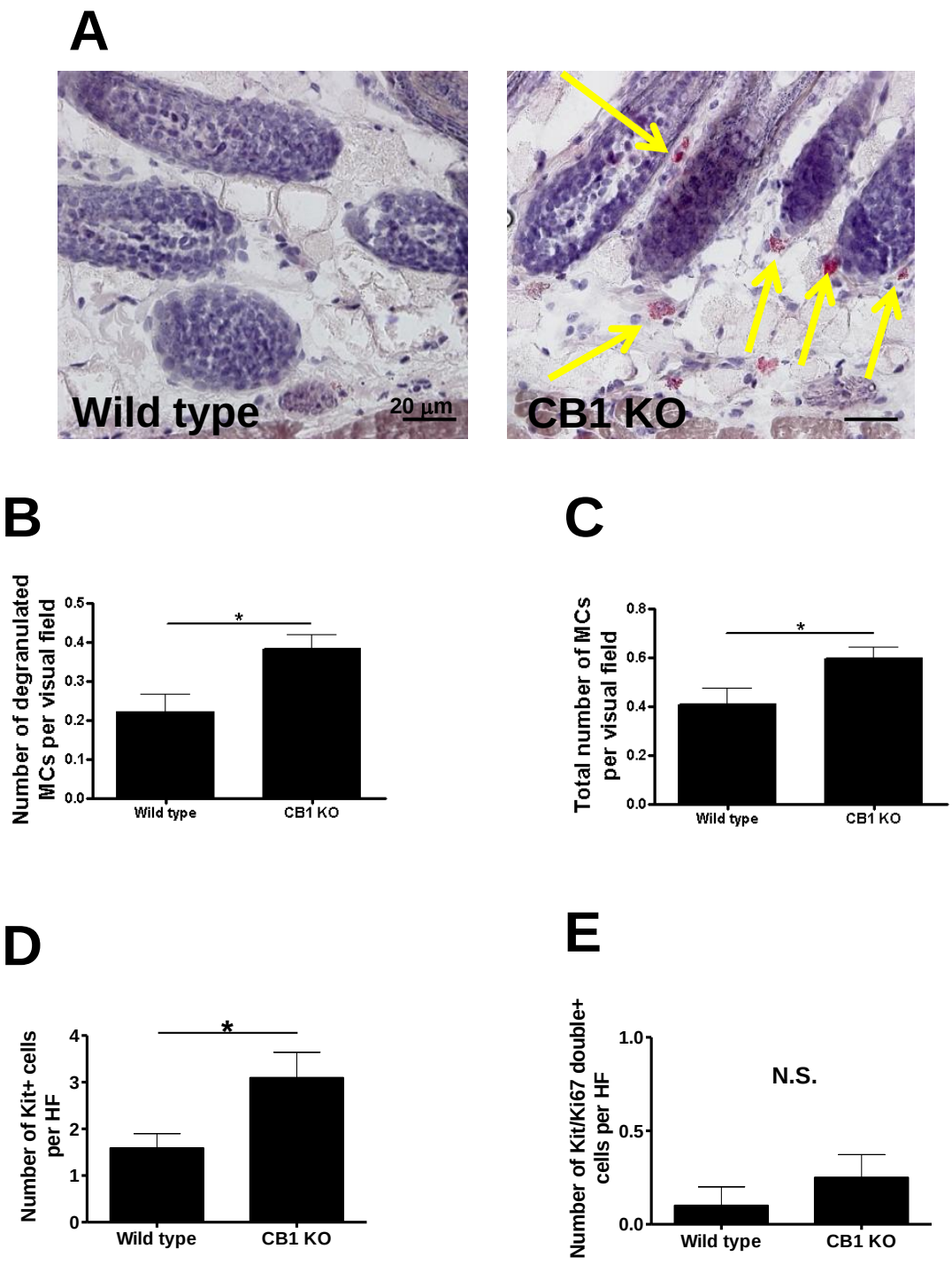
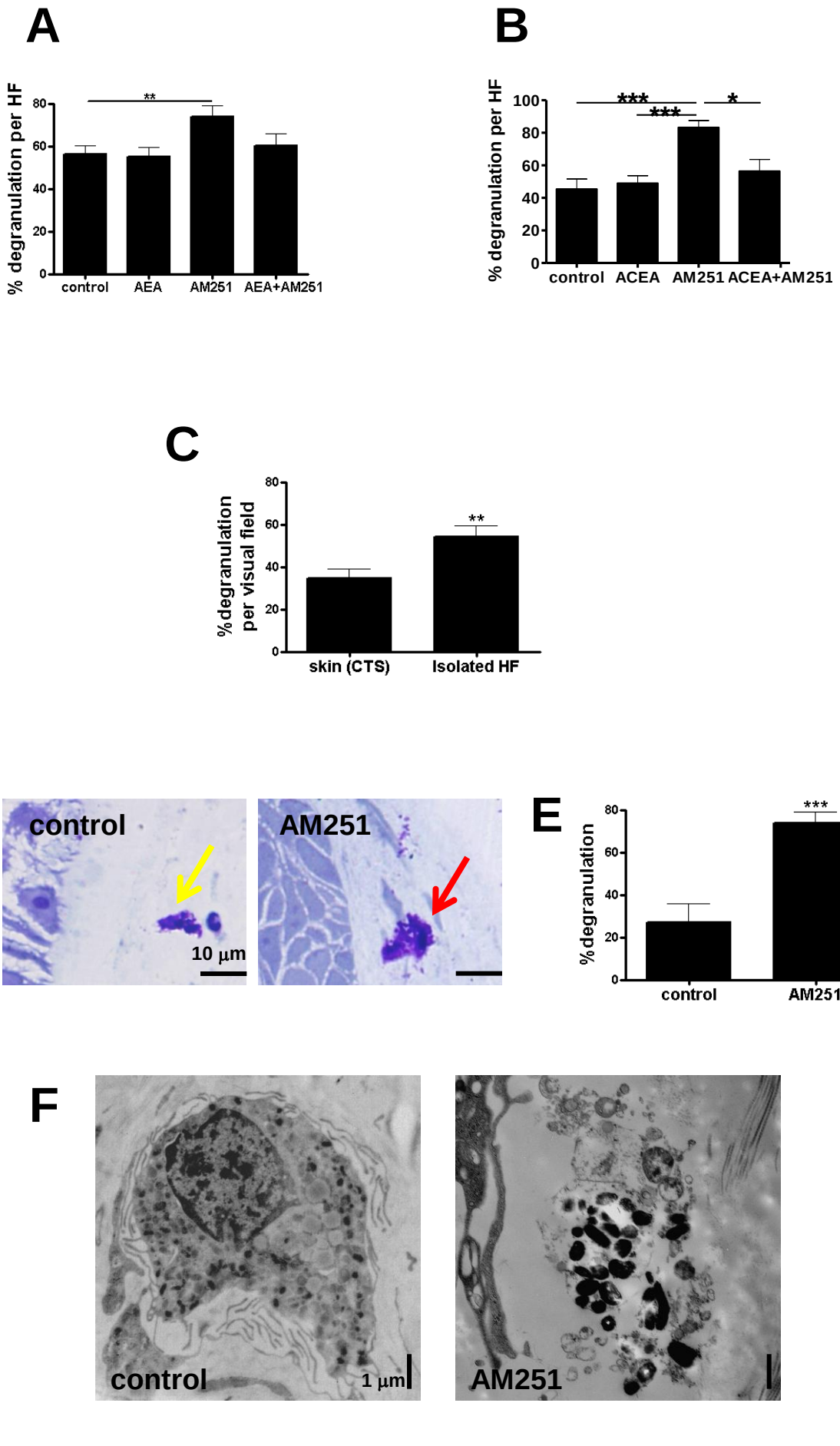
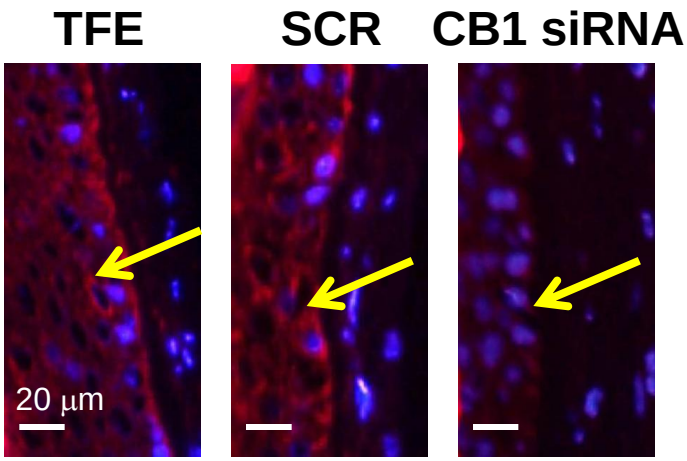

A
Wild type
20 µm
CB1 KO
B
Number of degranulated MCs per visual field
0.5
0.4
0.3
0.2
0.1
0.0
*
Wild type
CB1 KO
C
Total number of MCs per visual field
0.8
0.6
0.4
0.2
0.0
*
Wild type
CB1 KO
D
Number of Kit+ cells per HF
4
3
2
1
0
*
Wild type
CB1 KO
E
Number of Kit/Ki67 double+ cells per HF
1.0
0.5
0.0
N.S.
Wild type
CB1 KO

Figure 8

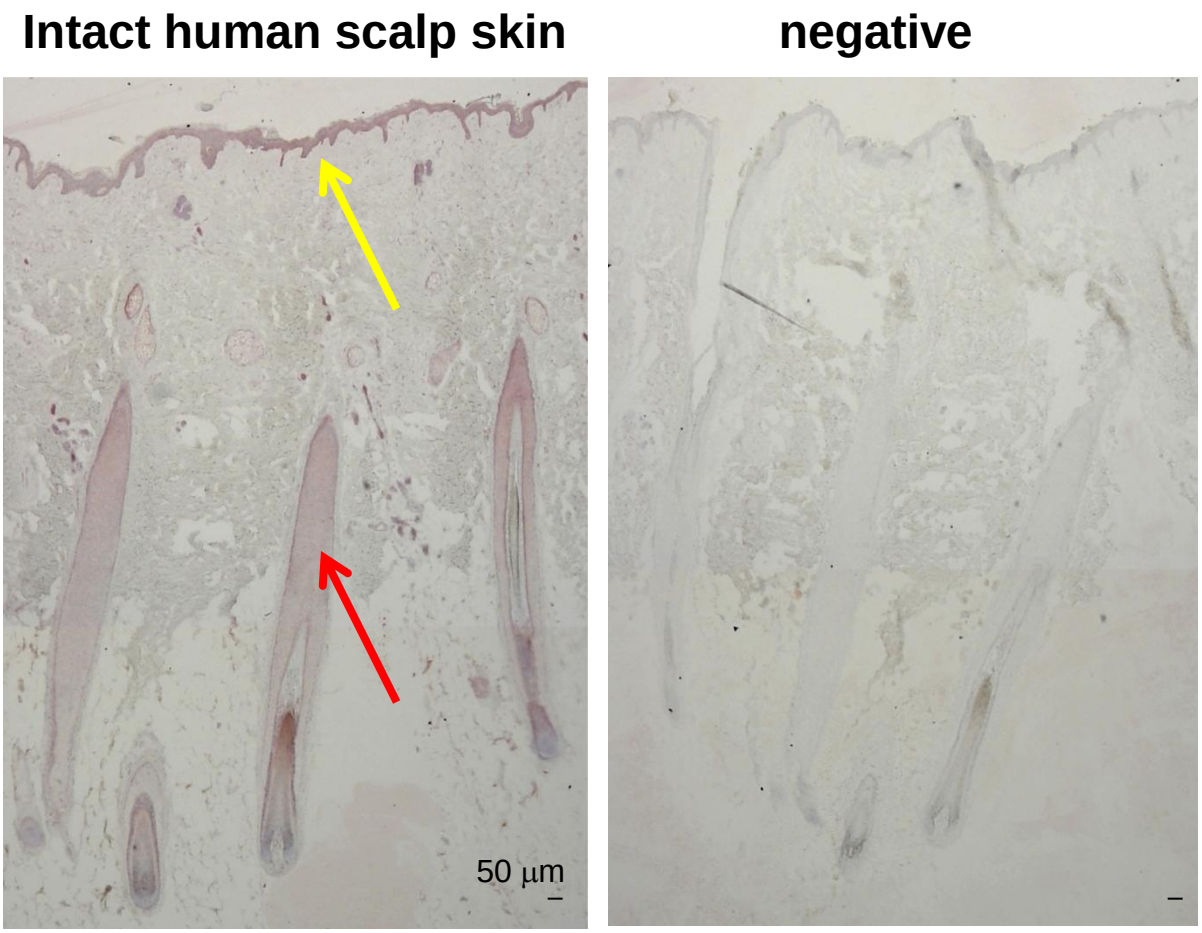


Supplementary Figure S1

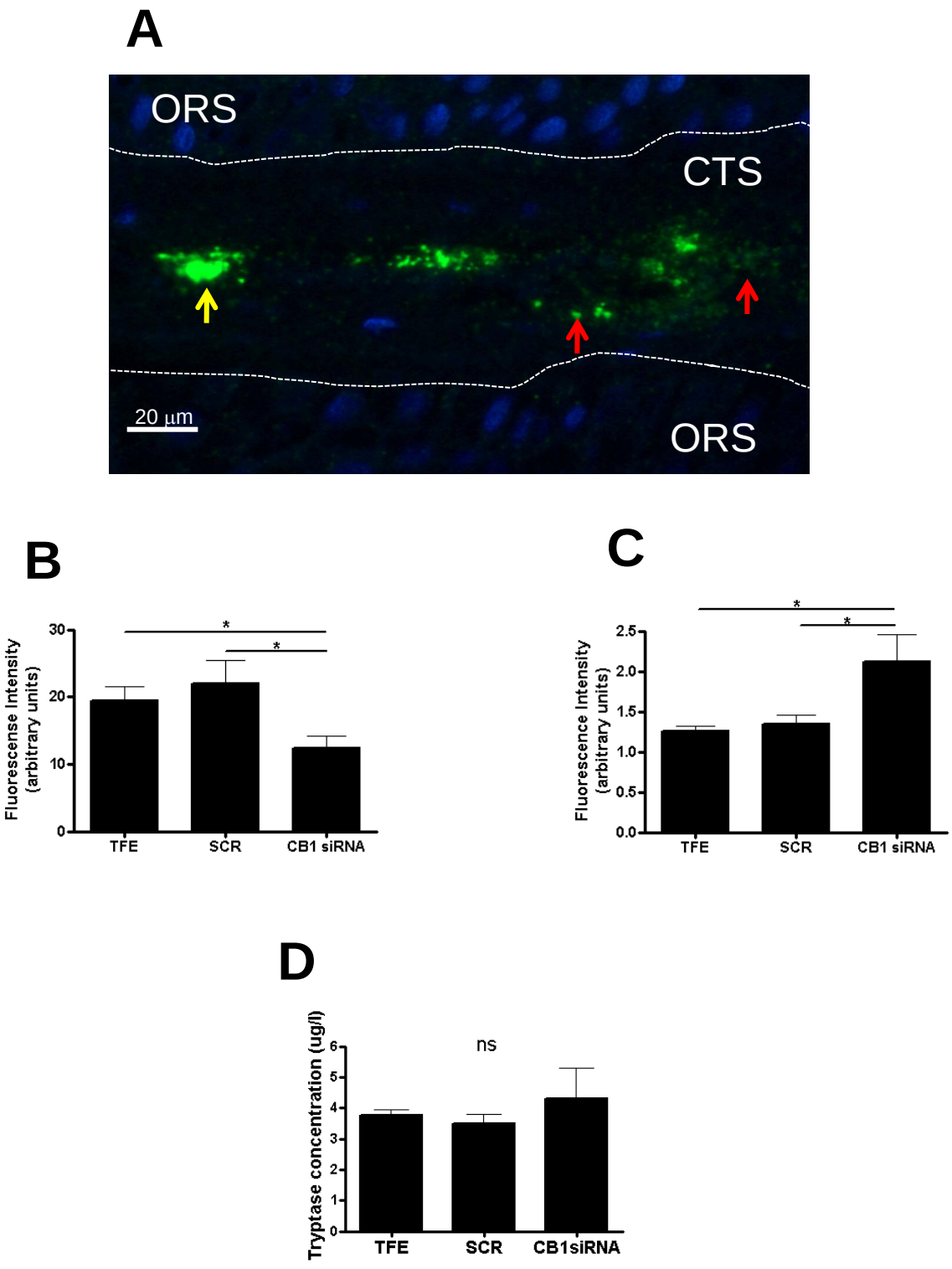
A



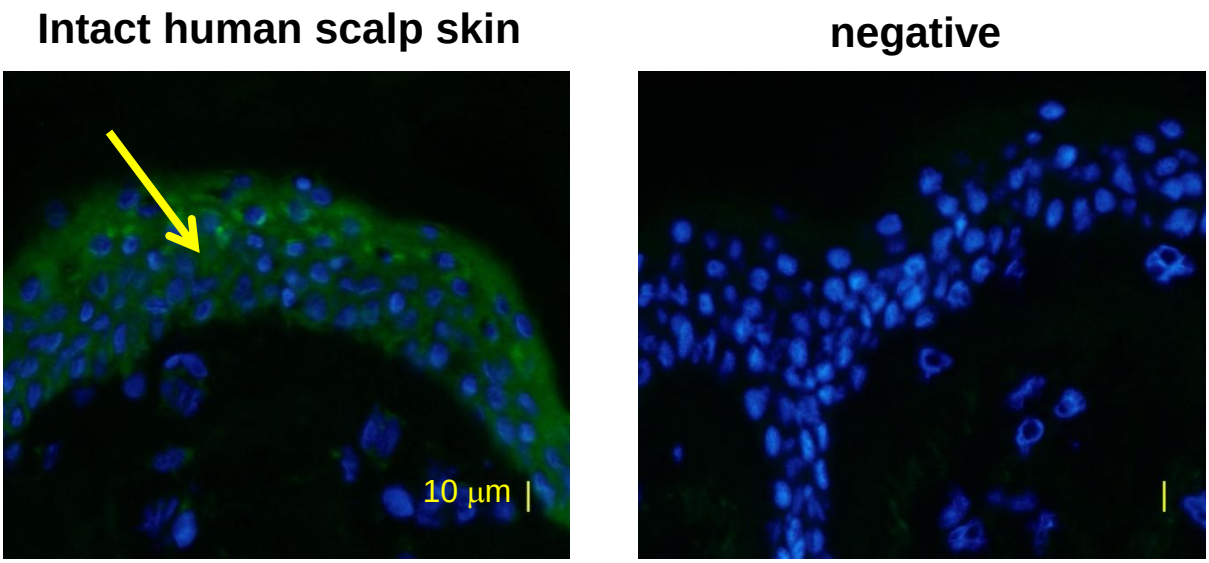
B



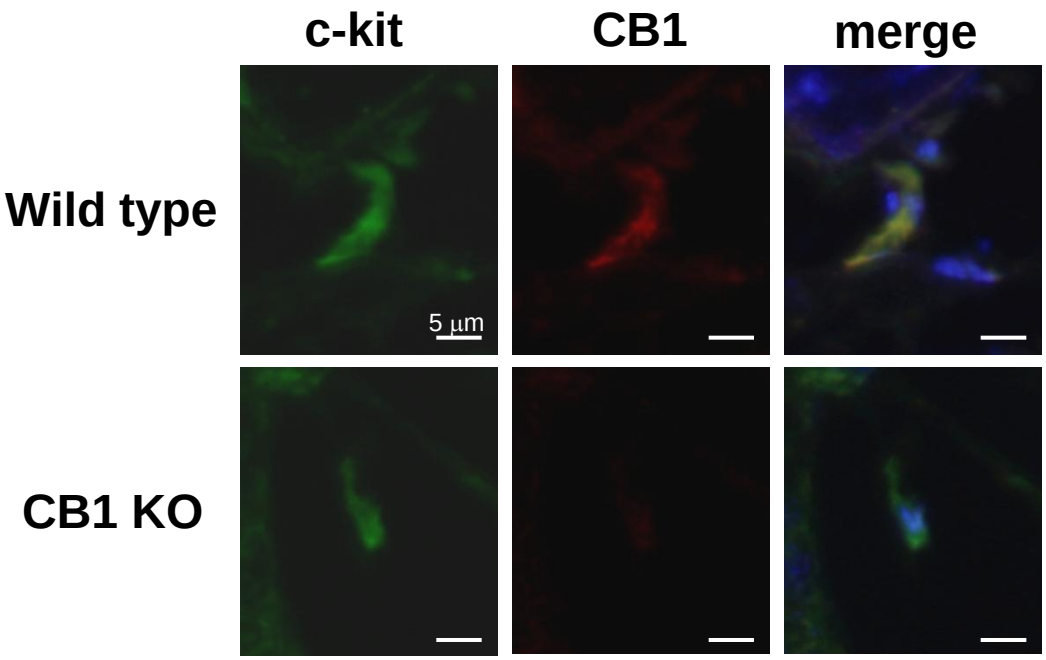
Supplementary Figure S2



Supplementary Figure S3



Supplementary Figure S4



Supplementary Figure S5