Cannabinoid receptor 1 controls human mucosal-type mast cell degranulation and maturation *in situ*

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Background: Because many chronic inflammatory and allergic disorders are intimately linked to excessive mast cell (MC) numbers and activation, it is clinically important to understand the physiologic mechanisms preventing excess MC accumulation/degranulation in normal human tissues.
Objective: Because endocannabinoids are increasingly recognized as neuroendocrine regulators of MC biology, we investigated how cannabinoid receptor (CB) 1 signaling affects human mucosal-type mast cells (hMMCs).
Methods: Using organ-cultured nasal polyps as a surrogate tissue for human bronchial mucosa, we investigated how CB1 stimulation, inhibition, or knockdown affects hMMC biology using quantitative (immuno)histomorphometry and electron microscopy.
Results: Kit$^+$ hMMCs express functional CB1 *in situ*. Blockade of CB1 signaling (with the specific CB1 antagonist N-(piperidin-1-yl)-1-(2,4-dichlorophenyl)-5-(4-chlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide [AM251] or CB1 gene knockdown) enhanced hMMC degranulation and increased total numbers without affecting their proliferation *in situ*. This suggests that inhibiting CB1 signaling induces hMMC maturation from resident progenitor cells within human mucosal stroma. hMMC maturation was induced at least in part through upregulating stem cell factor production. Both the prototypic endocannabinoid anandamide and the CB1-selective agonist arachidonyl-2-chloroethylamide effectively counteracted secretagogue-triggered excessive hMMC degranulation.
Conclusions: The current serum-free nasal polyp organ culture model allows physiologically and clinically relevant insights into the biology and pharmacologic responses of primary hMMCs *in situ*. In human airway mucosa hMMC activation and maturation are subject to a potent inhibitory endocannabinoid tone through CB1 stimulation. This invites one to target the endocannabinoid system in human airway mucosa as a novel strategy in the future management of allergic diseases.
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Key words: *Nasal polyp, mucosa, organ culture, endocannabinoid, cannabinoid receptor, mast cell, stem cell factor, c-Kit, tryptase, chymase*

Although the central role of human mucosal-type mast cells (hMMCs) in patients with respiratory diseases, including allergic rhinitis and asthma, is well appreciated,[1,2] most research on hMMCs relies on isolated cells obtained during bronchial lavage or cell lines.[3,4] Studies that examine and manipulate primary hMMCs within their natural tissue habitat, the respiratory tract mucosa, are scarce.[5-7] However, it is crucial to study hMMCs *in situ* rather than in isolated cell cultures because mast cell (MC) functions (including activation, degranulation, maturation, proliferation, and apoptosis) are critically influenced by their immediate tissue environment and MCs greatly affect the tissue they reside in, namely the airways.[8-12] For such *in situ* studies of hMMCs, nasal polyps (NPs) have long offered a very attractive yet still regrettably underappreciated assay option for clinically relevant *in situ* MC research in the human system.[5,6]

NPs represent polypoidal masses that arise mainly from nasal and paranasal mucous membranes and are frequently associated with allergic rhinitis and other "atopic" diseases.[13,14] Because the nasal mucosa forms one functional continuum with the upper respiratory tract mucosa,[15,16] organ culture of NPs can serve as an easily accessible, well-defined, and abundantly available surrogate tissue for the much less readily obtainable bronchial mucosa and its hMMC populations.[5,6] Moreover, hMMCs also play an important role in the pathogenesis of NP formation, as such,[2,5,17-19] so that MC research in organ-cultured NPs simultaneously allows one to investigate one of the most common clinical problems of upper respiratory tract care.

Previous human NP organ culture models[5,6,20] are limited by relatively rapid NP decay, the presence of bovine serum in the culture medium, or both; require special matrix support systems; and/or have not systematically addressed key MC biology questions, such as the physiologic controls of hMMC maturation and activation *in situ*. Therefore we aimed to develop a very simple, serum-free organ culture system that (1) prolongs NP tissue viability *in vitro*, (2) permits the quantitative study of key MC research parameters *in situ*, and (3) permits instructive functional and mechanistic studies, including pharmacologic manipulation and gene knockdown, of primary hMMCs *in situ*.

As a potentially interesting system that might control hMMC functions, we turned to the endocannabinoid system (ECS). The ECS is composed of cannabinoid receptors (CBs), their endogenous ligands, and enzymes responsible for endocannabinoid

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Abbreviations used
ACEA: Arachidonyl-2-chloroethylamide
AEA: N-arachidonoylethanolamide (anandamide)
AM251: N-(piperidin-1-yl)-1-(2,4-dichlorophenyl)-5-(4-chlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide
CB: Cannabinoid receptor
CRH: Corticotropin-releasing hormone
ECS: Endocannabinoid system
hMMC: Human mucosal-type mast cell
LDH: Lactate dehydrogenase
MC: Mast cell
NP: Nasal polyp
SCF: Stem cell factor
TUNEL: Terminal dUTP nick-end labeling

synthesis and degradation.[21-25] Components of the ECS are increasingly recognized as important neuroendocrine regulators of MC biology.[21,26-28] Notably, we have recently reported in this journal that the ECS limits excessive human skin MC activation and maturation through CB1-mediated signaling *in situ*.[29] However, the role of CB-mediated signaling in hMMCs still remains largely unknown (see Supplementary Text S1 in this article's Online Repository at www.jacionline.org). Therefore we investigated the effects of CB1 stimulation/blockade on hMMC biology within organ-cultured human nasal mucosa.

METHODS

Human NP organ culture

Human NP samples were obtained from 7 male and 3 female subjects (age, 23-80 years; average, 39.5 years) undergoing elective surgery for nasal obstruction (polypectomy). Human tissue collection and handling was performed according to Declaration of Helsinki guidelines, with institutional research ethics committee approval (University of Lübeck) and written informed consent. Freshly isolated NPs were cut into small pieces ($6 \times 6 \times 6 - 10 \times 10 \times 10$ mm) and maintained in supplemented serum-free William E medium.[29-32] NPs 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 substance P, compound 48/80, and corticotropin-releasing hormone (CRH), NPs were first treated with N-arachidonoylethanolamide (anandamide [AEA]; 30 μmol/L) or arachidonyl-2-chloroethylamide (ACEA; 30 μmol/L) for 1 day after the overnight incubation. Then the NPs were treated with substance P (10^{-10} mol/L), compound 48/80 (10 μg/mL),[29] or CRH (10^{-7} mol/L)[31] in combination with AEA or ACEA for an additional 1 day. After NP organ culture for the indicated time, tissue was processed for cryosectioning or paraffinized sectioning and histochemistry, immunohistochemistry, or transmission electron microscopy. Data from test and control groups within one set of experiments were generated by using only NPs from the same patient.

CB1 knockdown

CB1 silencing in organ-cultured human NPs was performed by using a previously reported method.[29]

Immunohistochemistry/immunofluorescence microscopy

For the detection of CB1, stem cell factor (SCF), Kit, FcεRIα, tryptase, and chymase immunohistochemistry, paraffin-embedded sections were used. For the immunofluorescence study for Kit, CB1, and SCF, cryoembedded sections were also used. For further details, see the Methods section in this article's Online Repository at www.jacionline.org.

hMMCs were defined as degranulated when more than 5 histochemically or immunohistologically detectable MC granules could clearly be observed outside the MC membrane (see representative degranulated MCs in Fig E1, *A*, in this article's Online Repository at www.jacionline.org).[29,31]

Quantitative immunohistomorphometry and transmission electron microscopy

Quantitative immunohistomorphometry of the observed immunoreactivity patterns in defined reference areas were assessed according to previously described principles[29-32] by using ImageJ software (National Institutes of Health, Bethesda, Md). Transmission electron microscopy was performed as previously reported.[29]

Statistical analysis

Data were analyzed by using either the Mann-Whitney *U* test for unpaired samples or 1-way ANOVA (Bonferroni multiple comparison test) with Prism 4.0 software (GraphPad Prism; GraphPad Software, San Diego, Calif). *P* values of less than .05 were regarded as significant. All data in the figures are expressed as means ± SEMs.

RESULTS

Human NPs can be organ cultured for at least 7 days

First, we adapted the long-term organ culture of human skin and hair follicles[29-32] to the organ culture of human NPs using supplemented, serum-free William E medium. This simple and cost-efficient assay preserved NP architecture for at least 7 days (later time points were not examined; Fig 1, *A* and *B*). Because hematoxylin stains viable cells,[33-35] the large number of hematoxylin-positive cells still visible in all NP compartments even after 7 days of organ culture suggests reasonably good general tissue viability over time. Although the cellularity in the lamina propria, but not in the epithelium, was significantly decreased (Fig 1, *C* and *D*), the NP tissue architecture was reasonably well preserved, and even mucosal cilia in the NP epithelium, as well as blood vessels and collagen fibers of the NP stroma (lamina propria), were partially conserved (Fig 1, *A* and *B*).

After a temporary decrease subsequent to the trauma of tissue dissection, at day 7, cell proliferation in the NP epithelium quickly recovered and reached initial day 0 levels (Fig 1, *E*). In contrast, cell proliferation within the NP lamina propria remained stable throughout the examined organ culture period (Fig 1, *F*). Although intermittently increased stromal cell apoptosis was observed at day 3 (Fig 1, *G*), general tissue decay parameters, such as percentage of apoptotic (terminal dUTP nick-end labeling [TUNEL]–positive) cells and lactate dehydrogenase (LDH) release, also had stabilized by day 7 (Fig 1, *G* and *H*, and see Fig E1, *B*). However, as previously reported,[5] with increasing organ culture time, the NP stroma became slowly more edematous (Fig 1, *A*). In addition to the reduction of cellularity in the lamina propria at day 7, this needs to be accounted for when calculating cell numbers per reference area in NP organ culture.

hMMCs are detectable histochemically and immunohistochemically in NP organ culture

Multiple fully granulated (Fig 2, *A*) or degranulated (Fig 2, *B*) hMMCs could be detected histochemically at all examined time points of NP organ culture. For this, Leder esterase (Fig 2, *A*) or Giemsa (Fig 2, *A*) or alkaline Giemsa (see Fig E1, *C*) histochemistry provided optimal cell visualization and morphologic details.

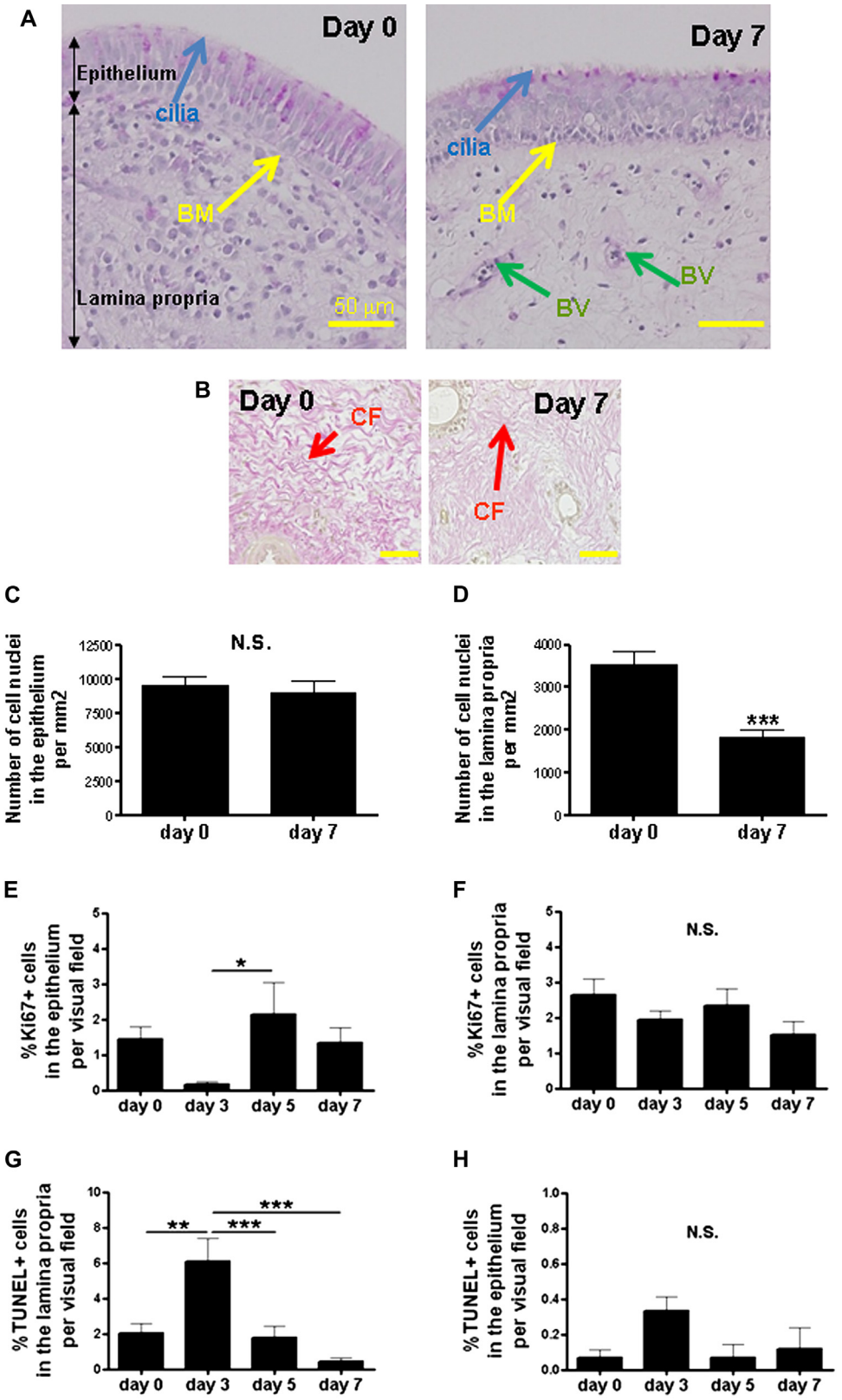

FIG 1. Human NP organ culture. **A** and **B,** Periodic acid–Schiff (Fig 1, *A*) and modified Verhoeff Van Gieson (Fig 1, *B*) histochemistry. **C** and **D,** Cellularity (cells per square millimeter) in the epithelium (Fig 1, *C*) and lamina propria (Fig 1, *D*). **E-H,** Quantitative immunohistomorphometry of Ki67 in the epithelium (Fig 1, *E*) and lamina propria (Fig 1, *F*) and TUNEL in the lamina propria (Fig 1, *G*) and epithelium (Fig 1, *H*). *BM*, Basement membrane; *BV*, blood vessel; *CF*, collagen fibers; *N.S.*, not significant. Data were obtained from 3 subjects. $*P < .05$, $**P < .01$, and $***P < .001$.

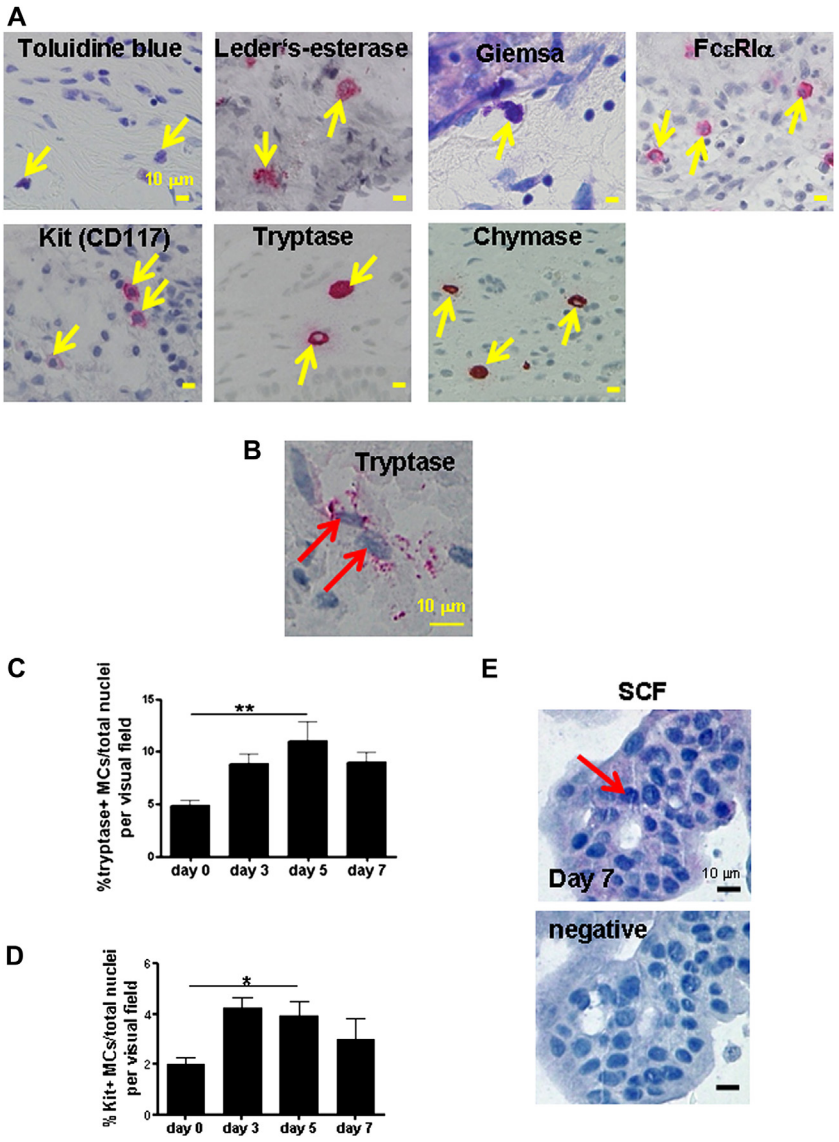

FIG 2. hMMCs in organ-cultured human NPs. **A,** hMMC histochemistry and immunohistochemistry. **B,** Representative image of degranulated tryptase-positive hMMCs. **C** and **D,** Quantitative tryptase-positive (Fig 2, *C*) and Kit$^+$ (Fig 2, *D*) immunohistomorphometry. **E,** SCF immunohistochemistry. *Arrows* in Fig 2, *A* and *B*, indicate positive cells. The *arrow* in Fig 2, *E*, indicates positive immunoreactivity. Data are from 3 subjects. $*P < .05$ and $**P < .01$.

Because FcεRIα$^+$ is not only expressed on hMMCs (Fig 2, *A*) but also, for example, on Langerhans cells, basophils, platelets, eosinophils, monocytes, and dendritic cells,[36,37] FcεRIα$^+$ proved to be a less instructive marker of mature hMMCs in NPs compared with classical MC histochemistry (toluidine blue, Leder esterase, Giemsa, and alkaline Giemsa histochemistry; Fig 2, *A*, and see Fig E1, *C*) on the one hand, and tryptase (Fig 2, *A* and *B*) or chymase (Fig 2, *A*) immunohistochemistry on the other. Most Kit$^+$ cells in human NP stroma clearly represented hMMCs, as indicated by coexpression of FcεRIα, and the majority of hMMCs in NP stroma were of the tryptase-positive/chymase-negative subtype (for details, see Supplementary Text S2 and Fig E1, *D* and *E*, in this article's Online Repository at www.jacionline.org).

Furthermore, tryptase or Kit (CD117)[29,31] immunohistochemistry was performed to assess whether the number of hMMCs within the lamina propria varied during organ culture. The number of tryptase-positive hMMCs in the lamina propria was stable during NP organ culture (see Fig E1, *F*), whereas the number of Kit$^+$ hMMCs increased (see Fig E1, *G*). In view of the slowly progressing NP edema (Fig 1, *A*) and the corresponding reduction of cellularity in the lamina propria (Fig 1, *D*), the number of tryptase-positive hMMCs might actually have increased during organ culture. Therefore the actual increase in the number of Kit$^+$ hMMCs probably even exceeds the absolute values measured here.

The percentage of tryptase-positive cells (which demarcate mature MCs) and Kit$^+$ cells (which demarcate both immature MCs and MC progenitors[29,38,39]) increased until day 7 (Fig 2, *C* and *D*). However, there was no significant increase in Kit$^+$ cell proliferation (Ki67; see Fig E2, *A*, in this article's Online Repository at www.jacionline.org). This suggests that during NP organ culture, new mature hMMCs differentiate *in loco* from resident progenitor cells. Interestingly, immunoreactivity for the Kit

ligand SCF was still detectable in the NP epithelium at day 7 (Fig 2, *E*). The continued presence of this key MC growth factor[1,2,8,29] might explain in part why hMMCs can be preserved in NPs during prolonged organ culture without serum or exogenous growth factors and why there is hMMC maturation from (pre-existing) resident MC progenitors.

hMMCs express functional CB1

Next, we used this simple, optimized, serum-free, clinically relevant NP organ culture assay to investigate whether a new regulatory principle of human MCs that we had previously identified in human connective tissue–type MCs[29] also applies to hMMCs *in situ*. Namely, we had shown that the ECS limits both the degranulation of mature connective tissue–type MCs and the local maturation of these MCs from resident progenitor cells in human skin *in situ* through CB1 stimulation.[29] However, the role of the ECS in hMMC biology *in situ* remains obscure, and whether a similar CB1-mediated "inhibitory tone" is also established in the control of primary hMMC activation and maturation within normal respiratory tract mucosa is completely unknown.

Therefore we first checked whether Kit^+ hMMCs in the NP lamina propria express CB1; this is the case (Fig 3, *A*). Furthermore, stimulation with the highly CB1-selective ligand Tocrifluor T1117 (Tocris Bioscience, Bristol, United Kingdom)[29] demonstrated functional binding activity of these receptor proteins, with CB1-like immunoreactivity for a cognate specific ligand (Fig 3, *B*).

CB1 inhibition induces hMMC degranulation and increases the number of hMMC

Just as in human skin MCs,[29] the CB1-specific synthetic agonist ACEA[29,40] or the nonselective endocannabinoid CB1 agonist anandamide (AEA)[10,29,32] did not significantly increase the number of tryptase-positive hMMCs in human NPs (counted as the number of tryptase-positive cells per visual field [see Fig E2, *B* and *C*] and as the percentage of positive cells per total number of cell nuclei [Fig 3, *D* and *E*, and see Fig E3 in this article's Online Repository at www.jacionline.org]). hMMC degranulation was also unaffected by these CB1 agonists (Fig 3, *F* and *G*, and see Fig E4 in this article's Online Repository at www.jacionline.org).

In contrast, the specific CB1 antagonist N-(piperidin-1-yl)-1-(2,4-dichlorophenyl)-5-(4-chlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide (AM251)[29,32] significantly increased both the number (see Fig E2, *B* and *C*; Fig 3, *C-E*; and see and Fig E3) and degranulation of tryptase-positive hMMCs *in situ* (Fig 3, *C*, *F*, and *G*, and see Fig E4). This was abrogated by the coadministration with ACEA or AEA (Fig 3, *D-G*, and see Figs E3 and E4). Furthermore, AM251 also increased the number of hMMCs in the deep lamina propria (Fig 3, *H*), suggesting that the increased number of hMMCs by AM251 occurred throughout the entire NP tissue. Increased hMMC degranulation by CB1-specific antagonist was independently confirmed by using high-resolution light microscopy (alkaline Giemsa histochemistry; see Fig E1, *C*, and E2, *D*) and transmission electron microscopy (see Fig E2, *E*).

To further investigate whether the increase in hMMC numbers caused by AM251 was due to the death of other cell types in the tissue, we investigated the cellularity in the epithelium and lamina propria of organ-cultured NPs. AM251 significantly increased cellularity in the epithelium, whereas it significantly decreased cellularity in the lamina propria (Fig 4, *A* and *B*). However, cell proliferation/apoptosis was unaffected by either ACEA or AM251 (see Fig E2, *F-I*). In line with the apoptosis results (see Fig E2, *G* and *I*), LDH measurements in the culture medium of ACEA/AM251-treated NPs did not reveal significant changes between any of the test and control groups (see Fig E5, *A*, in this article's Online Repository at www.jacionline.org). Considering that cellularity reflects tissue volume, AM251 could affect tissue volume of organ-cultured NPs.

Because MC degranulation typically induces mucosal edema[41,42] and MC tryptase is reported as an important protease that can promote tissue edema,[43,44] we next evaluated the intercellular tryptase immunoreactivity in the vicinity of hMMCs. As shown in Fig E5, *B*, AM251 significantly increased intercellular tryptase immunoreactivity (see Fig E5, *B*). Thus, because AM251 can promote tissue edema by stimulating MC degranulation and could thereby alter the NP tissue volume, the current method of evaluating changes in MC numbers (ie, percentage of MCs per number of total nuclei) is more accurate than counting the MC number per area.

CB1 inhibition promotes the maturation of resident hMMC progenitors in NP stroma

Intriguingly, AM251 also significantly increased the numbers and percentages of Kit^+ hMMCs *in situ* (Fig 4, *C*, and see Figs E5, *C*, and E6, left panel, in this article's Online Repository at www.jacionline.org). However, just as in human skin MCs *in situ*,[29] proliferation (see Fig E5, *D*) or apoptosis (Fig 4, *D*) of hMMCs did not change significantly after pharmacologic CB1 blockade. Therefore the increased number of detectable mature hMMCs can only have arisen from resident progenitor cells. Because human nasal mucosa, in addition to tryptase-positive hMMCs (Fig 3, *D* and *E*, and see Fig E3), exhibits many chymase-positive MCs,[45] it is interesting to note that CB1 blockade also increased the numbers and percentages of chymase-positive hMMCs *in situ* (Fig 4, *E*, and see Figs E5, *E*, and E6, right panel).

Importantly, the effect of CB1 stimulation/blockade on hMMC degranulation and numbers *in situ* was highly consistent between distinct NP assays derived from 3 to 4 different patients (see Figs E3, E4 and E6, in this article's Online Repository at www.jacionline.org). For human tissue organ culture standards, in which data variability, even between tissue samples derived from the same patient, can be substantial, the observed CB1 agonists/antagonist effects on hMMC degranulation and on the percentage of Kit^+, tryptase- or chymase-positive cells *in situ* are unusually robust and reproducible. This underscores the usefulness of the current human NP organ culture assay as a novel and clinically relevant model system in preclinical hMMC research.

CB1 blockade increases the number of hMMC immigration into the NP epithelium

hMMCs, which migrate toward and into the NP epithelium, might play an important role in NP pathogenesis.[46,47] Therefore we asked whether CB1 stimulation/blockade also affects the location of hMMCs within the NP. Interestingly, already after a single day of AM251 treatment, the number of tryptase-positive intraepithelial MCs was significantly increased compared with that seen in control subjects (Fig 4, *F* and *G*). This was partially abrogated by coadministration of the CB agonist ACEA (Fig 4, *G*). This suggests that signaling through CB1, which is also expressed on NP epithelial cells (Fig 5, *A*, see untreated skin), affects hMMC immigration into the NP epithelium.

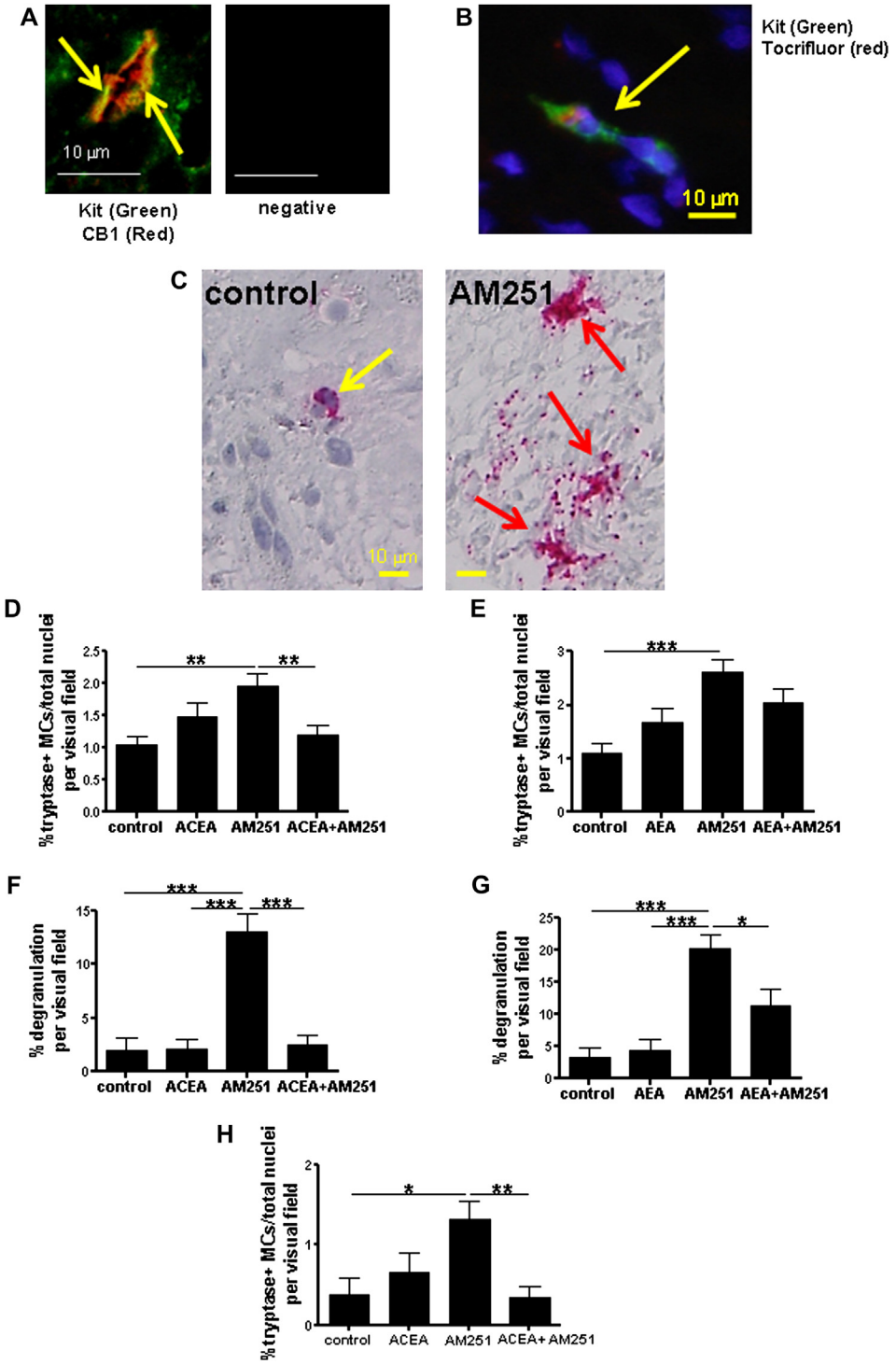

FIG 3. hMMCs express functional CB1 *in situ*. **A,** Kit/CB1 double immunofluorescence. *Yellow arrow*, Double-positive immunoreactivity. **B,** Kit *(green)* immunofluorescence with organ-cultured NPs treated with Tocrifluor *(red)*. **C,** Representative images of tryptase immunohistochemistry. *Yellow arrow*, Nondegranulated hMMCs; *red arrows*, degranulated hMMCs. **D-G,** Quantitative immunohistomorphometry of tryptase (Fig 3, *D*, *E*, and *H*) and percentage of degranulation (Fig 3, *F* and *G*). Data are from 3 to 4 subjects. $*P < .05$, $**P < .01$, and $***P < .001$. There was no significant difference between the control and AEA/ACEA groups.

The CB1 gene can be silenced in organ-cultured human NPs

Next, CB1 gene silencing was attempted using the same protocol we had previously described for human hair follicles.[29] Effective epithelial and mesenchymal CB1 knockdown was demonstrated by a significant downregulation of CB1 immunoreactivity by 40% in the epithelium (Fig 5, *A* and *B*) and on $CB1^+$ stromal cells (Fig 5, *A* and *C*) of organ-cultured NPs. Similar to

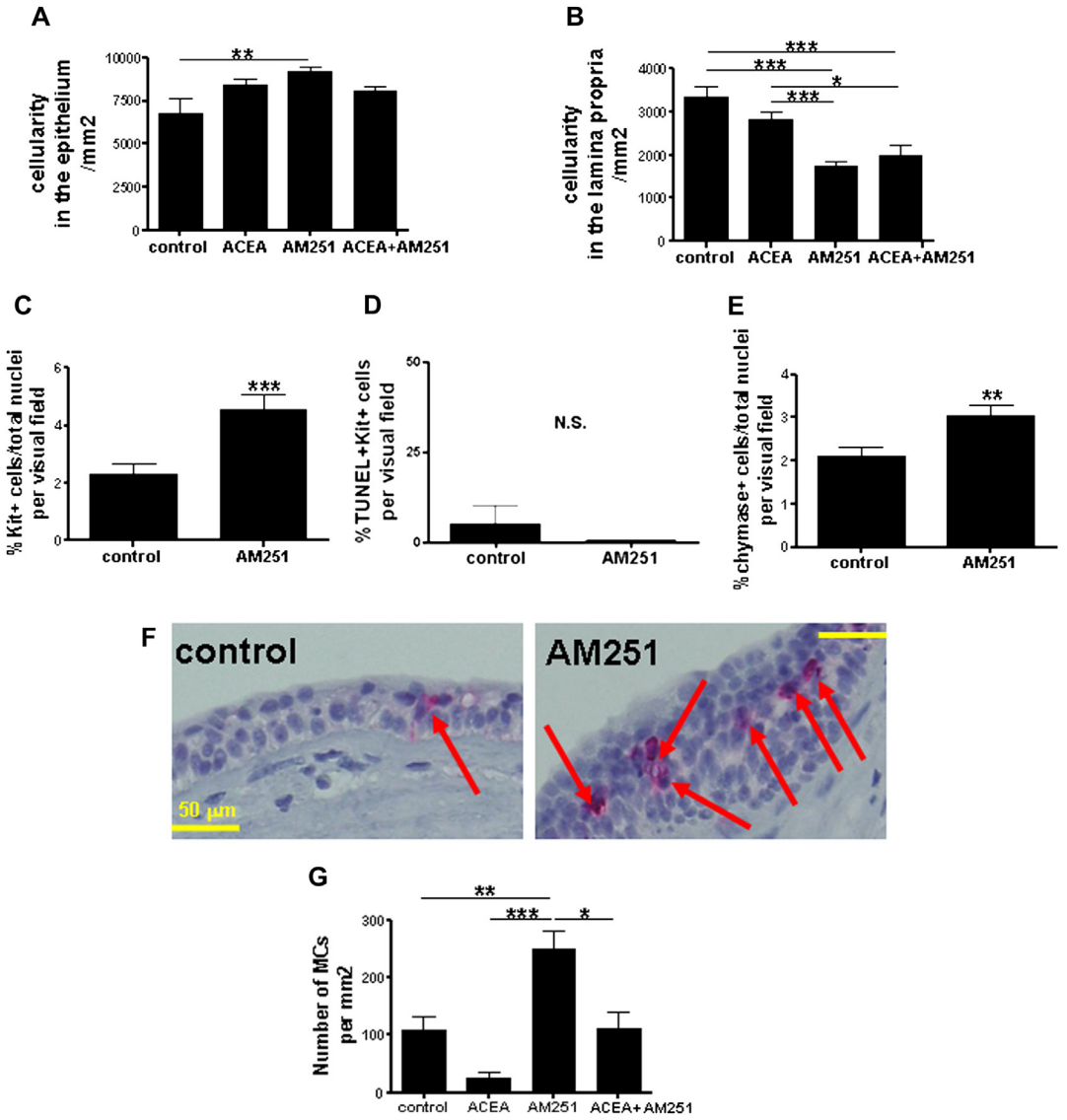

FIG 4. CB1 inhibition induces hMMC maturation but not proliferation. **A** and **B,** Cellularity (cells per square millimeter) of the epithelium (Fig 4, *A*) and lamina propria (Fig 4, *B*). **C,** Quantitative immunohistomorphometry of Kit. **D,** Percentage of $TUNEL^+/Kit^+$ cells in organ-cultured human NPs. **E,** Quantitative immunohistomorphometry of chymase. **F,** Representative images of tryptase-positive hMMCs in the epithelium. *Red arrows* denote tryptase-positive hMMCs. **G,** Quantitative analysis of intraepithelial hMMCs. Data were from 3 subjects. $*P < .05$, $**P < .01$, and $***P < .001$. *N.S.*, Not significant.

the effects of pharmacologic CB1 blockade (Fig 3, *D-G*), CB1 gene silencing significantly increased the percentage of tryptase-positive hMMCs in the lamina propria (Fig 5, *D*) and stimulated their degranulation (Fig 5, *E*). These data independently confirm that CB1 blockade induces hMMC maturation from resident progenitor cells and induces hMMC degranulation *in situ* not only in human skin mesenchyme[29] but also in human respiratory tract mucosa.

Endocannabinoids inhibit excessive activation of hMMCs through CB1

Excessive MC degranulation and numbers in human mucosa play a key role in the pathogenesis and clinical phenotype of major allergic diseases of the respiratory tract.[1,7,36] Therefore we then asked whether CB1 stimulation counteracts the MC-activating effects of 2 classical endogenous MCs secretagogues that play a key role in MC-dependent neurogenic airway inflammation: substance P[29,48-52] and CRH.[31,53] In addition, we examined the exogenous standard MC secretagogue, compound 48/80.[29] Similar to AM251, CRH significantly reduced cellularity in the lamina propria. Although this was not significant, substance P also showed a tendency toward reducing cellularity *in situ* (see Fig E5, *F*). This suggests that these 2 endogenous MC secretagogues can indeed increase tissue edema in our NP assay system, likely via triggering hMMC degranulation.

Quantitative tryptase immunohistomorphometry demonstrated that both the potent endocannabinoid AEA and the CB1-specific agonist ACEA inhibited the degranulation-promoting effects of substance P (Fig 6, *A*), compound 48/80 (Fig 6, *B*), and CRH

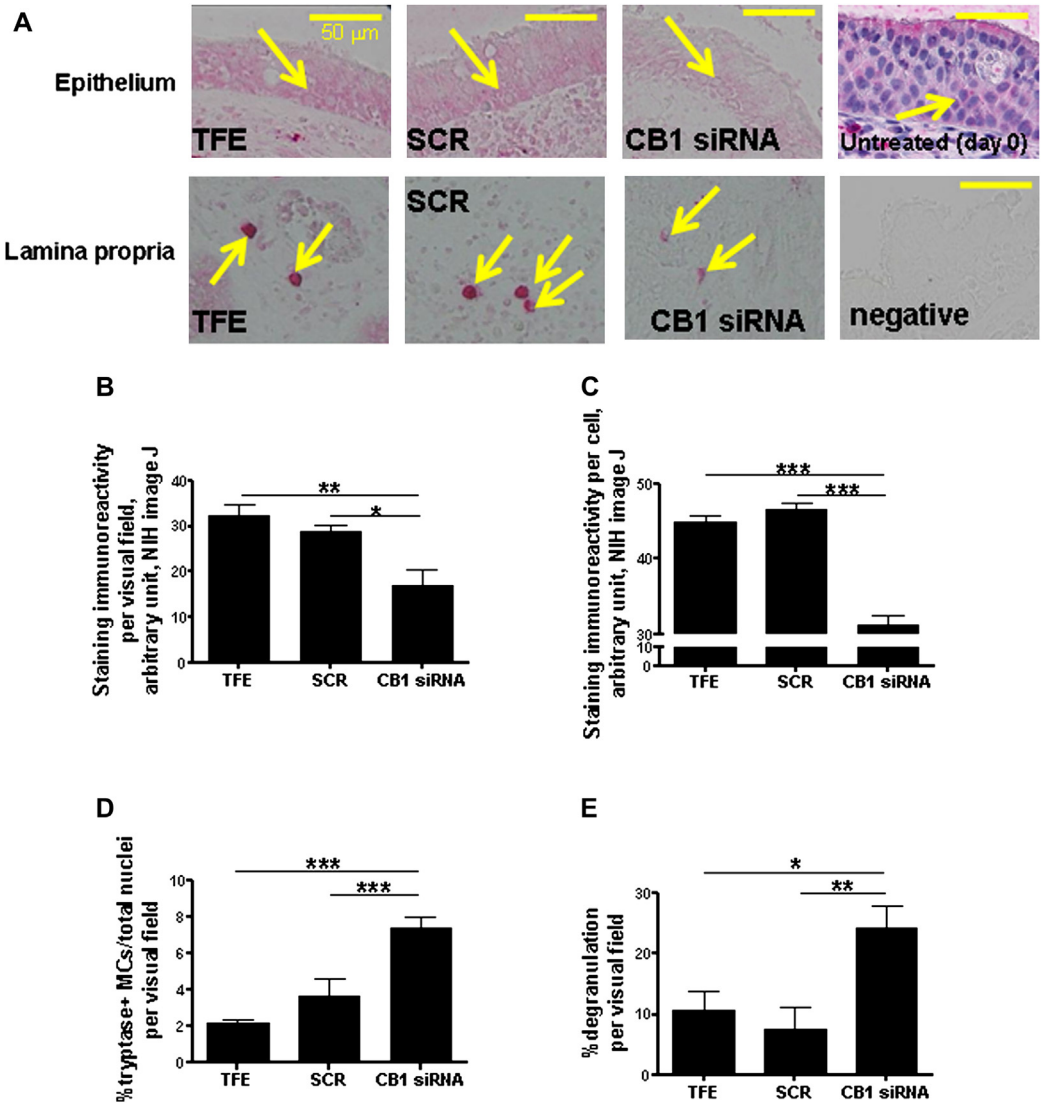

FIG 5. CB1 blockade by gene knockdown increases hMMC numbers and induces degranulation *in situ*. **A,** CB1 immunohistochemistry. *Yellow arrows* indicate positive CB1 immunoreactivity. **B** and **C,** Quantitative analysis of relative CB1 immunoreactivity within the epithelium (Fig 5, *B*) and $CB1^{+}$ mesenchymal cells (Fig 5, *C*). **D** and **E,** Percentages of tryptase-positive (Fig 5, *D*) and degranulated (Fig 5, *E*) hMMCs among TFE-, SCR-, or CB1 siRNA–treated NPs. These experiments were from 2 subjects. $*P < .05$, $**P < .01$, and $***P < .001$. CB1 *siRNA*, CB1 siRNA– treated NPs; *SCR*, scrambled siRNA–treated NPs; *TFE*, transfection reagent.

(Fig 6, *C*). Thus, exactly as in human skin MCs,[29] CB1 stimulation effectively counteracts excessive MC activation in normal human mucosa *in situ*.

CB1 regulates SCF expression by the NP epithelium

Because increased SCF production (along with enhanced number and degranulation of MCs) has been reported in the nasal epithelium of patients with allergy,[54] we asked whether CB1 inhibition also affects SCF expression in human nasal mucosa *in situ*.

Faint SCF-like immunoreactivity was detected within the NP epithelium at day 7 (Fig 2, *E*). Compared with the vehicle control group (Fig 7, *A*, left), CB1 inhibition by AM251 significantly increased SCF-like immunoreactivity in the NP epithelium after only 1 day of NP organ culture (Fig 7, *A*, right, and Fig 7, *B*). Furthermore, upregulation of the number of tryptase-positive hMMCs by means of pharmacologic CB1 blockade (AM251) was completely abrogated by neutralizing SCF (Fig 7, *C*). This suggests that enhanced hMMC maturation from resident progenitor cells by means of CB1 blockade in NPs *in situ* is mediated at least in part through upregulation of SCF expression and increased SCF secretion by the NP epithelium.

DISCUSSION

Our data provide the first evidence that normal hMMCs use CB1-mediated signaling to limit not only their degranulation but also their maturation from resident progenitor MCs *in situ*. The observed effects of pharmacologic or transcriptional CB1 blockade can be induced not only directly through CB1 receptors on the cell membrane but also indirectly by upregulating SCF production within the NP epithelium *in situ*. These results underscore

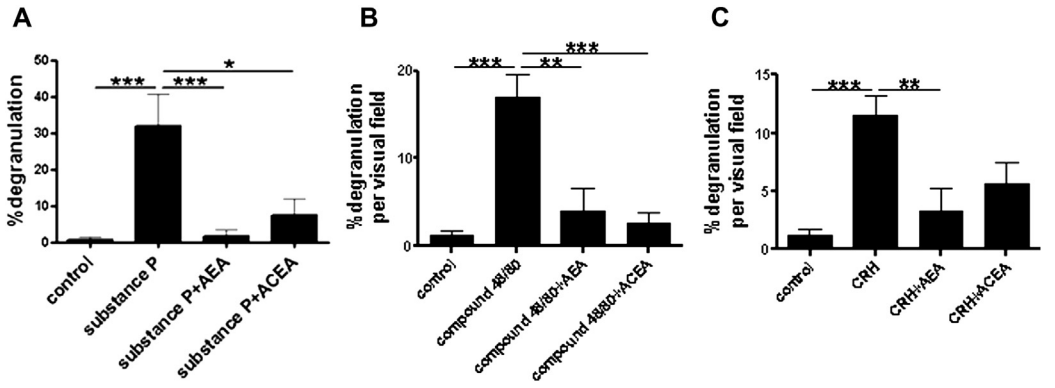

FIG 6. CB1 stimulation inhibits hMMC degranulation by MC secretagogues. Percentages of degranulated tryptase-positive hMMCs in organ-cultured human NPs after stimulation with MC secretagogues, substance P (10^{-10} mol/L; **A**), compound 48/80 (10 μg/mL; **B**), and CRH (10^{-7} mol/L; **C**) in combination with AEA (30 μmol/L) or ACEA (30 μmol/L) are shown. AEA or ACEA clearly inhibited hMMC degranulation by various MC secretagogues. *$P < .05$, **$P < .01$, and ***$P < .001$. Data were from 2 subjects.

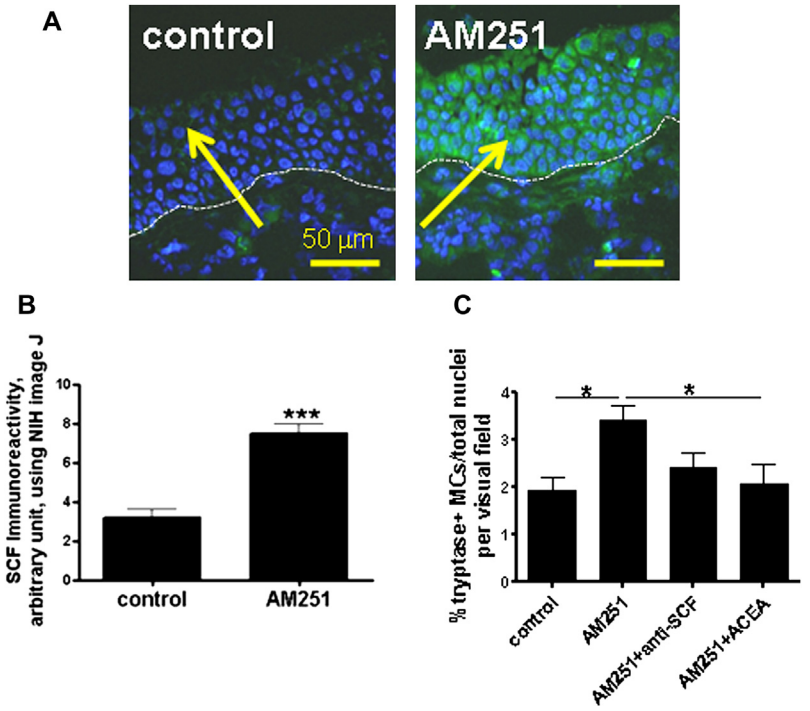

FIG 7. CB1 inhibition increases SCF expression *in situ*. **A,** Representative images of SCF immunofluorescence in NP epithelium. *Arrows* denote SCF^+ immunoreactivity. **B,** Quantitative SCF immunohistomorphometry. **C,** Percentage of tryptase-positive hMMCs in organ-cultured human NPs (1 day). AEA and ACEA, 30 μmol/L; AM251, 1 μmol/L; anti-SCF neutralizing antibody, 1 μg/mL. Data were from 2 subjects. *$P < .05$ and ***$P < .001$.

the key role of the ECS in human MC physiology and confirm the importance of CB1-mediated signaling as an endogenous, "tonic" control system that avoids excessive MC maturation and degranulation, which we had previously identified for human connective tissue–type MCs in human skin.[29]

This clinically and biologically important confirmation of concept in a different tissue system (human airway mucosa versus skin) is complemented by additional novel insights:

1. The current study closes an important gap in the human MC biology literature by clarifying the effect of CB1-mediated signaling on normal primary hMMCs *in situ*.
2. CB1 signaling (both directly through affecting the MC membrane receptor and indirectly through controlling epithelial SCF secretion) has a universal regulatory effect not only on human connective tissue–type MCs but also on hMMCs *in situ* and is no peculiarity of the hair follicle–associated MCs we had investigated before.[29]
3. Our demonstration that CB1 blockade increases the number of intraepithelial MCs in NPs (Fig 4, *F* and *G*) shows that CB1 signaling also regulates hMMC location and migration.
4. We show that serum-free human NP organ culture provides a very instructive, clinically relevant model system that

generates robust and well-reproducible data for investigating the biology of hMMCs and their response to pharmacologic manipulation *in situ.* However, both apoptosis and increased hMMC maturation from resident progenitor cells do occur in this *ex vivo* system. Therefore even though adult primary hMMCs do operate within their natural tissue habitat in this assay, NP organ culture, as such, affects MC biology *in situ* and cannot be entirely equated with the physiologic *in vivo* situation. Yet the translational relevance of human NP organ culture for clinical respiratory medicine surely exceeds that of mainstream mucosal MC research that relies on mouse *in vivo* models and human cell lines.

5. Our assay system also allows instructive studies on how MC secretagogues and MC degranulation inhibitors affect the physiology and pathology of human airway mucosa *in situ.*

Systematic exploitation of this NP assay might also shed new light on the as yet unclear role of hMMCs in NP pathogenesis.[46,47,54] Moreover, our findings raise the possibility that insufficient CB1-mediated signaling (eg, in the epithelium) triggers excessive, SCF-mediated influx of hMMCs into the epithelium of nasal mucosa. This might be relevant not only to NP pathogenesis but also to other hMMC-dependent human airway pathologies. If the ECS indeed operates (eg, through CB1 signaling) as a safeguard system against excessive epithelial SCF production and secretion (to limit undesired MC maturation, activation, and migration into/toward the epithelium), the future management of allergic upper airway diseases could profit from re-establishing effective CB1-mediated signaling (eg, upregulation of intramucosal endocannabinoid levels by inhibition of their degradation by FAAH inhibitors[23-25]).

CB1 blockade induces tissue edema (decreased cellularity plus no modification on cell proliferation/apoptosis), possibly because of increased hMMC degranulation. Surprisingly, CB1 inhibition increased the cellularity within the epithelium without modulating epithelial cell proliferation/apoptosis (Fig 4, *A*, and see Fig E2, *F* and *G*). This needs to be further elucidated. As previously reported by others (eg, Schierhorn et al[5]), there is slowly progressive NP tissue swelling over time, as well as cell apoptosis, in NP organ culture. Therefore it is important to take these phenomena into account as a potentially confounding factor when calculating MC numbers. (We recommend to calculate the percentage of MC/total number of nuclei as the measurement method of choice.) Furthermore, the presence of both tissue edema and apoptosis makes the observed increase in the number of MCs after CB1 blockade even more significant and documents that the total number of MCs increases substantially in organ-cultured NPs when CB1-mediated signaling is inhibited.

We were surprised to find fewer Kit^+ MCs than tryptase-positive or chymase-positive MCs (Fig 2, *C* and *D*, and see Fig E6, patients 1 and 2) because Kit is often interpreted as a universal marker for detecting both immature and mature MCs.[55] However, only 70% to 90% of tryptase-positive cells in human samples from stomach, colon, and breast tissue reportedly are Kit^+.[56] In line with this, the percentage of Kit^+ MCs in the lamina propria of NPs from some patients was lower than that of tryptase-positive or chymase-positive hMMCs (see Figs E3 and E6). Moreover, in addition to MCs, basophils (which are Kit^-[57] yet can be a prominent feature of NP immunopathology[58]) can be tryptase positive.[59] Taken together, this might explain the lower number of Kit^+ MCs compared with that of tryptase-positive or chymase-positive cells in organ-cultured NPs and shows that Kit has inherent limitations as a universal marker for hMMCs also in human NPs.

Fully in line with our previous results obtained from human skin MCs,[29] CB1 blockade significantly increased the number of total Kit^+ hMMCs without significantly modifying their proliferation or apoptosis in organ-cultured NPs (see Fig E5, *D*, and Fig 4, *D*). Although Kit demarcates both immature and mature MCs,[55] there are no selective markers for immature hMMC progenitors, and some tryptase-positive hMMCs do not even express Kit *in situ*.[56] In fact, Kit^- MC progenitors have previously been reported in human peripheral blood,[60] and their existence has also been deduced from morphometric data in the connective tissue sheath of human scalp hair follicles.[29,31] This renders it likely that Kit^- MC progenitors also exist in human NPs.

In the NPs of some patients, the number of Kit^+ MCs was even lower than that of tryptase-positive or chymase-positive MCs (see Fig E3 and Fig E6). Therefore the number of MC progenitors in NPs cannot be reliably estimated by simply subtracting the number of tryptase-positive MCs from the number of Kit^+ MCs. However, the percentage of Kit^+ MCs increased significantly during organ culture (Fig 2, *D*). Moreover, there were no indications of significant CB1 antagonist–induced proliferation of Kit^+ progenitor cells. Instead, the number of mature hMMCs (ie, hMMCs expressing the differentiation-associated proteins tryptase or chymase) increased significantly. Theoretically, these numeric changes might also be an artifact resulting from substantial MC migration from one NP compartment to another. Because there is no definite marker for assessing human MC migration *in situ*, we therefore also calculated hMMC numbers in the deep part of the lamina propria. The fact that CB1 blockade significantly increased here as well (Fig 3, *H*) suggests that the overall number of hMMCs per NP truly increased, rather than reflecting a shift in intra-NP MC trafficking.

Because the isolated NP samples had no connection to the bone marrow or vasculature, these unequivocal findings can only be explained by the differentiation of at least some resident MC progenitor cells into mature MCs. That coadministration of CB1 agonists counteracted the CB1 blockade–induced excessive hMMC maturation (Figs 3, *D* and *E*, and 4, *C* and *E*) supports a key role for CB1 in controlling not only human MC degranulation *in situ* but also MC maturation from resident progenitors. Although there was a slight tendency toward increased MC numbers and degranulation on CB1 agonist stimulation (Fig 3, *D-H*), this was not significant. Furthermore, MC secretagogues–induced hMMC degranulation was greatly reduced by CB1 agonists (Fig 6, *A-C*). This supports the general concept that "tonic" CB1 stimulation is required to prevent excessive degranulation and maturation not only of human skin MCs[29] but also of hMMCs *in situ*.

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Key messages

- **The degranulation of primary adult mast cells in human airway mucosa and their maturation from resident progenitor cells *in situ* is controlled by CB1-mediated signaling.**
- **CB1 stimulation is a promising novel strategy for inhibiting excessive mast cell activation and numbers in human airway mucosa.**

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METHODS

Reagents

AEA, ACEA, AM251, substance P, compound 48/80, and CRH were purchased from Sigma-Aldrich (Taufkirchen, Germany), whereas 5-carboxytetramethylrhodamine (5-TAMRA)–conjugated AM251 (Tocrifluor) was from Tocris Bioscience (Bristol, United Kingdom). SCF-neutralizing antibody was from R&D Systems (Minneapolis, Minn). The sources of the primary antibodies used for immunohistochemistry/immunofluorescence are indicated below.

Histochemistry

Histochemistry for collagen fibers was performed according to the manufacturer's protocol (modified Verhoeff Van Gieson stain, Elastic stain Kit, Sigma). For periodic acid–Schiff staining, deparaffinized sections were incubated with 1% periodic acid at room temperature for 10 minutes. After a wash, the sections were incubated with Schiff reagent (Merck, Darmstadt, Germany) at room temperature for 15 minutes, followed by a wash with tap water. Then the sections were counterstained with hematoxylin. Leder esterase histochemistry was performed with cryoembedded sections, as previously reported.[E1]

We define an MC as degranulated when 5 or more histochemically or immunohistologically identified MC granules are seen to be located clearly outside the cell membrane. Although a cutoff set at 5 extracellular MC granules is evidently arbitrary and likely only captures MCs that are undergoing anaphylactic MC degranulation,[E2] this simple but pragmatic and easily reproducible morphometric technique has proved very instructive and sensitive in multiple previous MC *in situ* studies that we and others have published in murine and human skin.

Immunohistochemistry/immunofluorescence microscopy

For the detection of Kit, tryptase, and chymase, deparaffinized sections were antigen retrieved by using a microwave oven (650 W for 20 minutes). After preincubation with either 5% normal goat serum plus 1% BSA in 0.05 mol/L Tris-HCl–buffered saline (at room temperature for 60 minutes for Kit) or 0.5% triton-X in 6% BSA in Tris-HCl–buffered saline (at room temperature for 30 minutes for chymase, tryptase, and FcεRIα), sections were incubated overnight at 4°C with primary antibodies, rabbit anti-human CD117 (Dako, Hamburg, Germany) at 1:100, mouse anti-human chymase (Abcam, Cambridge, United Kingdom) at 1:100, mouse anti-FcεRIα (Acris Antibodies GmbH, Herford, Germany) at 1:100, or mouse anti-human tryptase (Abcam) at 1:500 diluted in antibody diluent (DCS Innovative Diagnostik-Systeme, Hamburg, Germany). Thereafter, the sections were incubated with goat biotinylated antibodies against rabbit or mouse IgG (Jackson Immunoresearch Laboratories, West Grove, Pa) at 1:200 in antibody diluents (DCS Innovative Diagnostik-Systeme) for 45 minutes at room temperature. Sections were then treated with the alkaline phosphatase–based avidin-biotin complex (Vectastain Elite ABC kit; Vector Laboratories, Burlingame, Calif), and the expression of these antigens was visualized with Fast Red (Sigma).

For the detection of CB1, after deparaffinization and antigen retrieval, the sections were pretreated with avidin and biotin (Blocking Kit, Vector Laboratories) to block the endogenous avidin and biotin. After overnight incubation with the primary anti-CB1 antibody (1:50 in antibody diluents; Santa Cruz Biotechnology, Santa Cruz, Calif), the sections were treated by using the alkaline phosphatase–based labeled streptavidin-biotin method (DCS Innovative Diagnostik-Systeme), and CB1 expression was visualized with Fast Red (Sigma).

Double immunostaining for Kit and CB1 was performed by using the Tyramide Signal Amplification technique (PerkinElmer, Boston, Mass).[E1]

For double immunohistochemistry for Kit and FcεRIα, the sections were first deparaffinized and antigen retrieved by using a microwave oven. After overnight incubation with mouse anti-FcεRIα at 1:100 (at 4°C), slides were incubated with biotin-conjugated goat anti-mouse IgG at 1:200 for 45 minutes at room temperature. Sections were then treated with horseradish peroxidase–based avidin-biotin complex (Vectastain Elite ABC kit, Vector Laboratories), and FcεRIα expression was visualized with DAB (Dako). After careful washing, the sections were then incubated overnight with rabbit anti-CD117 antibody at 1:100 (at 4°C). Thereafter, the sections were incubated with goat biotinylated antibodies against rabbit IgG (Jackson Immunoresearch Laboratories) at 1:200 for 45 minutes at room temperature. Sections were then treated with the alkaline phosphatase–based avidin-biotin complex (Vectastain Elite ABC kit, Vector Laboratories), and the expression of these antigens was visualized with Fast Red (Sigma).

Double immunostaining for Ki67 and Kit or Kit immunostaining and TUNEL was performed on the same cryoembedded sections, as previously shown,[E1] to study the proliferation or apoptosis of the Kit^+ cells.

For detecting SCF in cryoembedded organ-cultured human NPs, an indirect immunofluorescence method was applied,[E1] whereas the alkaline phosphatase–based avidin-biotin method was performed on paraffin-embedded sections using rabbit anti-human SCF antibody at 1:50 (Santa Cruz Biotechnology), and the expression of the antigen was visualized with Fast Red (Sigma).

For detecting double-positive cells for tryptase and chymase within human NPs, double immunostaining could not be successfully performed because the primary antibodies for tryptase and chymase were from the same species. Therefore we performed immunostaining separately with a pair of "mirror-image" sections, so that we could take advantage of the possible detection of 2 different staining patterns in exactly the same cell within the tissue.[E3]

Quantitative immunohistomorphometry

Antigen expression was quantified by assessing immunoreactivity in defined reference areas, which was assessed by means of quantitative immunohistomorphometry[E1] with ImageJ software (National Institutes of Health, Bethesda, Md).

High-resolution light microscopy and transmission electron microscopy

Thin and thick sections of organ-cultured human NPs were generated, as previously reported.[E1] One-micrometer-thick sections were prepared for alkaline Giemsa histochemistry,[E1] whereas thin sections were stained with uranyl acetate and lead citrate and observed with an electron microscope (JEM-1200EXII; JEOL, Tokyo, Japan).[E1]

LDH measurement

LDH activity in culture medium was measured according to the manufacturer's instructions (Cytotoxicity Detection Kit; Roche, Mannheim, Germany) as a biochemical indicator of tissue viability.[E4] The absorbance of the samples was measured at 490 nm with an ELISA reader.

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. Transfection (6 hours) with isolated human NPs was performed, as described previously.[E1]

SUPPLEMENTARY TEXT S1

Giannini et al[E5] reported that a nonselective CB1/CB2 agonist (CP55,940) prevented antigen-induced asthma-like reactions in guinea pigs and that this effect was abrogated by treatment with either CB1 or CB2 antagonists. Interestingly, the degranulation-protective effect of CP55,940 on guinea pig lung MCs was abrogated by coadministration of the CB1-specific antagonist AM251 but not by a CB2-specific antagonist. Yet the authors concluded that both receptors might be important in inhibiting guinea pig lung MC degranulation.[E5]

SUPPLEMENTARY TEXT S2

Although MCs represent by far the largest $c\text{-}Kit^+$ cell population in human and murine skin mesenchyme, it was unclear

whether this also applies to human NPs. However, FcεRIα and Kit double immunohistochemistry (see Fig E1, *D*) revealed that 71.3% of Kit^+ cells coexpressed FcεRIα in freshly microdissected human NP samples (n = 3). Therefore most Kit^+ cells in human NP stroma clearly represent hMMCs. hMMCs have been reported to express tryptase but not chymase.[E6,E7] However, human tissues display mixed MC subtypes and MC distributions, which are not as clearly demarcated as in rodents.[E8] Indeed, in freshly isolated human NP samples, the ratio of MCT (tryptase-positive, chymase-negative MCs; which likely represent T-cell–dependent mucosal MCs[E9]) versus MCTC (tryptase-positive, chymase-positive MCs) was 76.87%/23.13% in the epithelium, whereas that in the lamina propria was 66.85%/33.15%, as detected by tryptase or chymase immunohistochemistry with mirror-image sections (from 2 subjects; Fig E1, *E*).

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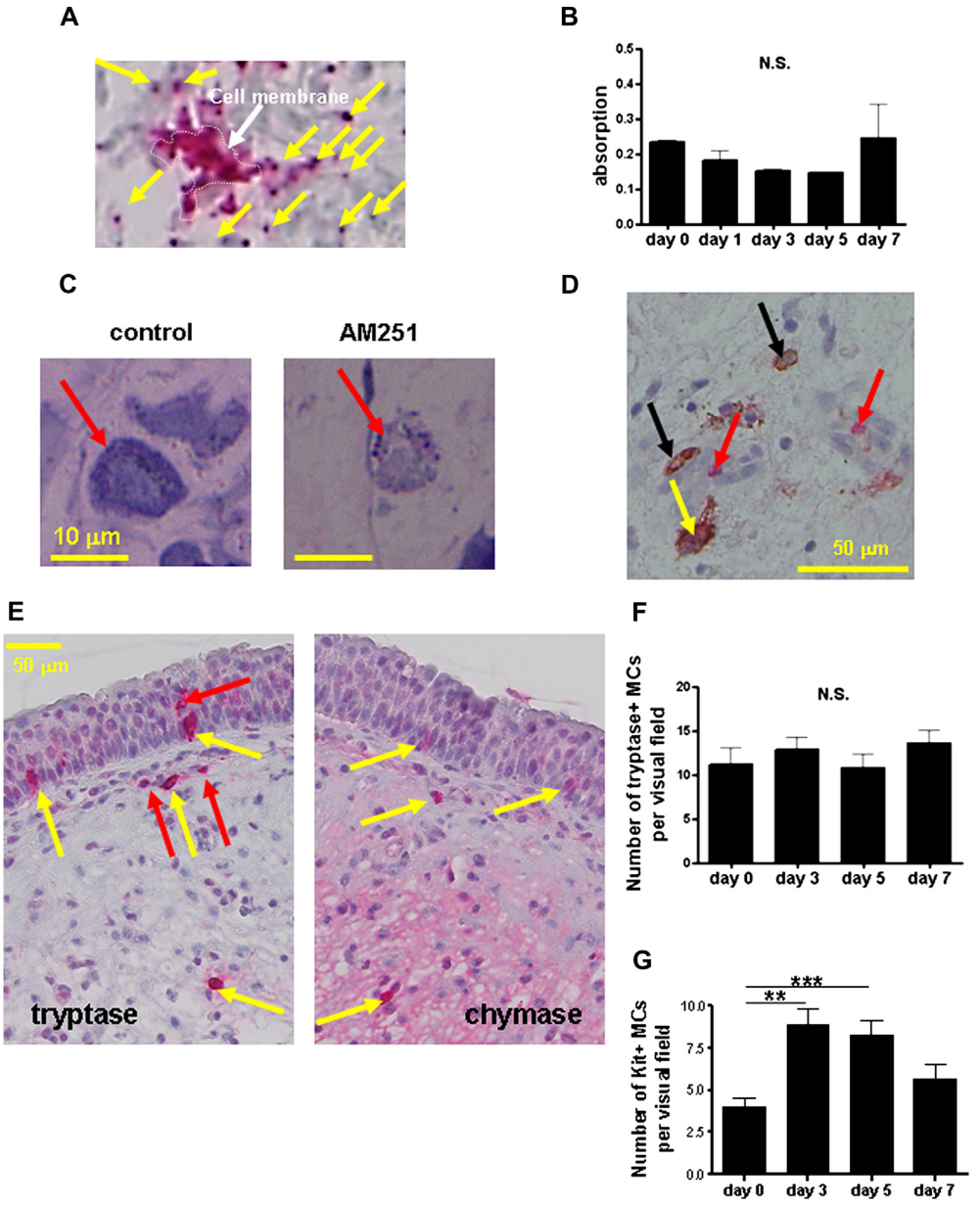

FIG E1. hMMCs are detectable by using MC immunohistochemistry during organ culture. **A,** Tryptase-positive degranulated MC. *Yellow arrows*, MC granules outside the cell membrane. **B,** LDH assay. **C,** hMMCs (alkaline Giemsa histochemistry). **D,** Kit/FcεRIα immunohistochemistry. *Black arrows*, FcεRIα$^+$ cells; *red arrows*, Kit$^+$ cells; *yellow arrow*, double-positive cell. **E,** Tryptase/chymase "mirror-image" immunohistochemistry. *Red arrows*, Tryptase-positive, chymase-negative cells; *yellow arrows*, double-positive cells. **F** and **G,** Quantitative tryptase (Fig E1, *F*) and Kit (Fig E1, *G*) immunohistomorphometry. Data are from 3 subjects. $^{**}P < .01$ and $^{***}P < .001$. *N.S.*, Not significant.

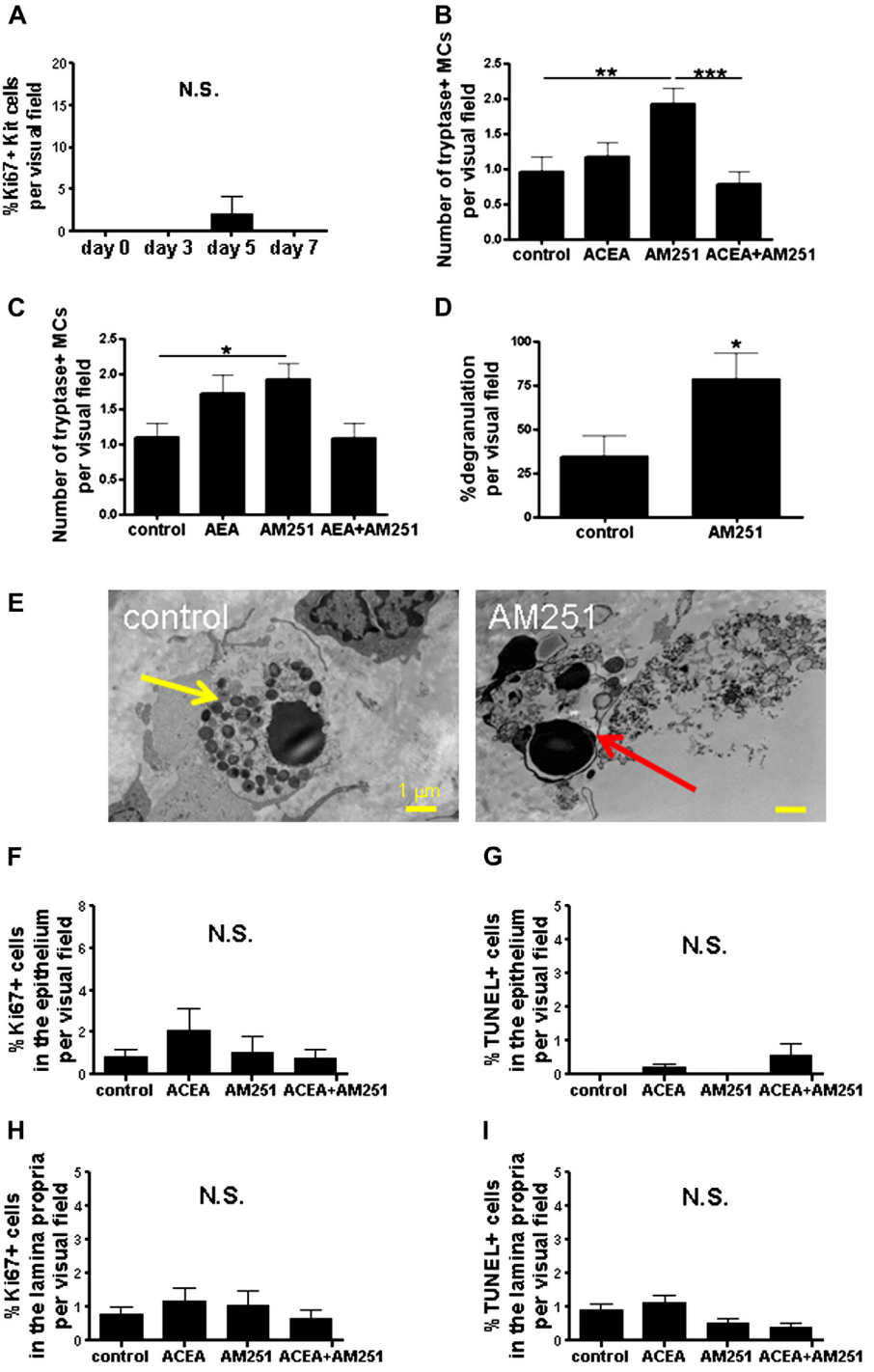

FIG E2. CB1 inhibition promotes the degranulation of hMMCs in NPs. **A,** Quantitative Ki67/Kit immunohistomorphometry. **B** and **C,** Quantitative tryptase immunohistomorphometry. **D,** Quantitative alkaline Giemsa histomorphometry of hMMCs. **E,** Transmission electron microscopy of hMMCs. *Yellow arrow*, Nondegranulated hMMC; *red arrow*, degranulated hMMC. **F** and **H,** Quantitative Ki67 immunohistomorphometry in the epithelium (Fig E2, *F*) and lamina propria (Fig E2, *H*). **G** and **I,** Quantitative TUNEL immunohistomorphometry in the epithelium (Fig E2, *G*) and lamina propria (Fig E2, *I*). Data were from 2 to 3 subjects. $*P < .05$, $**P < .01$, and $***P < .001$. *N.S.*, Not significant.

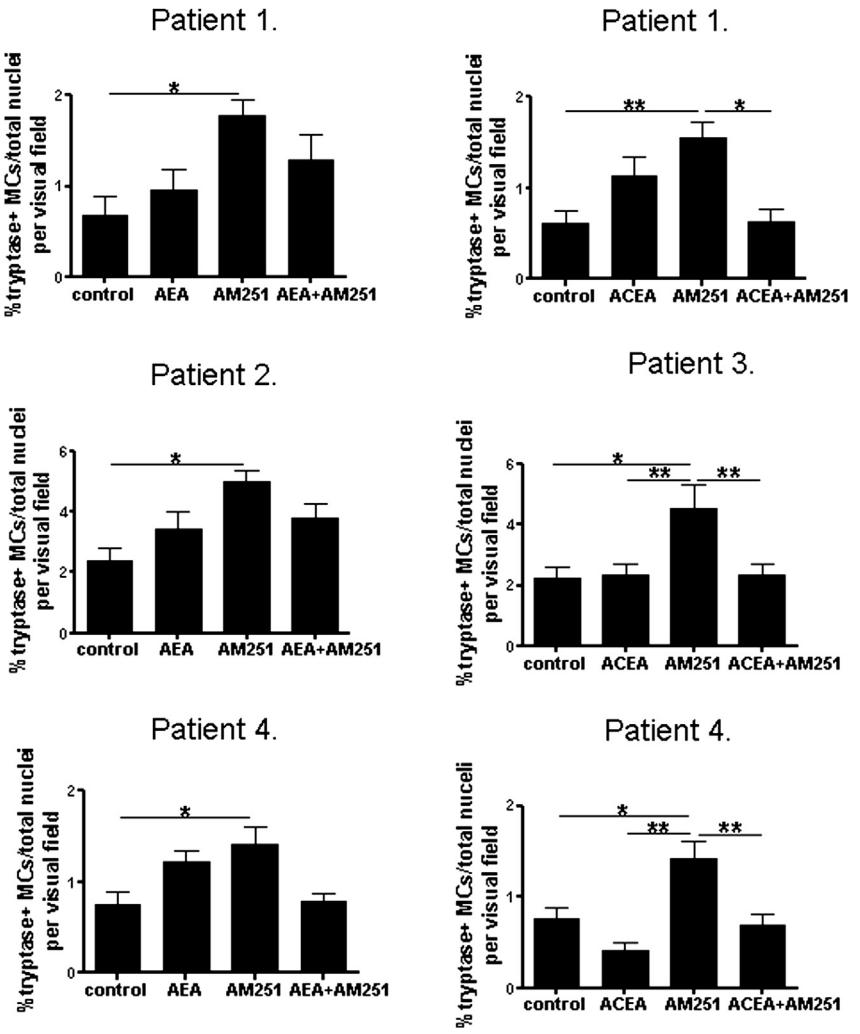

FIG E3. CB1 inhibition increased the percentage of tryptase-positive hMMCs in all samples examined: tryptase immunohistomorphometry. *$P < .05$ and **$P < .01$.

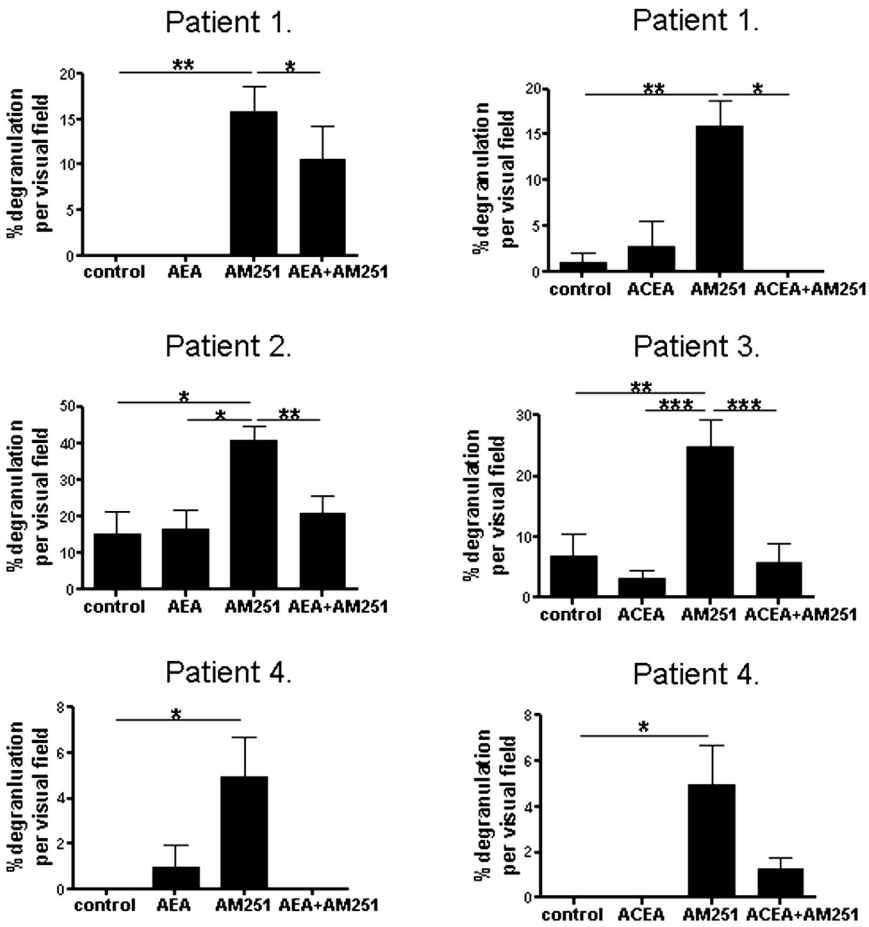

FIG E4. CB1 inhibition induced tryptase-positive hMMC degranulation in all samples examined: tryptase immunohistomorphometry. $*P < .05$, $**P < .01$, and $***P < .001$.

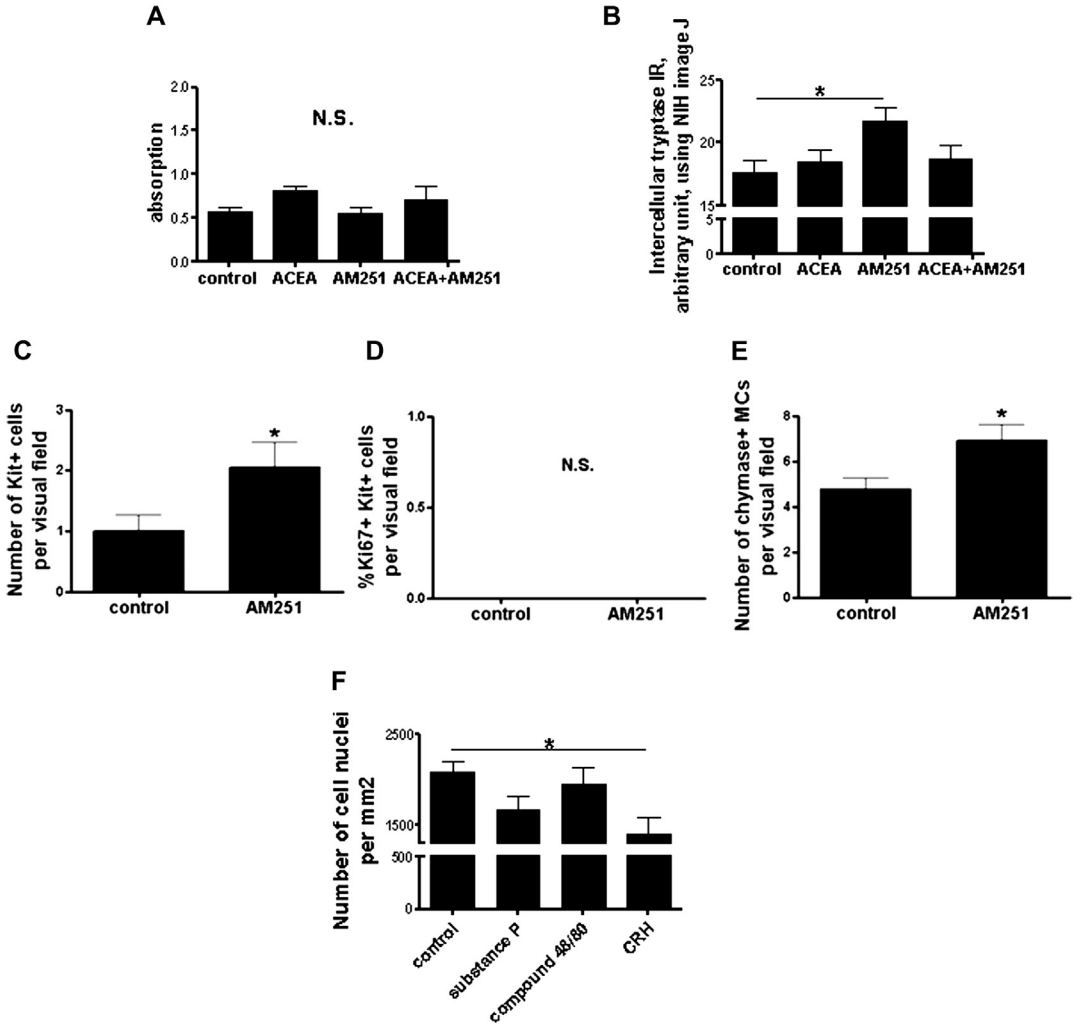

FIG E5. CB1 inhibition increases intercellular tryptase immunoreactivity and induces hMMC maturation. **A,** LDH measurement in the medium of organ-cultured NP samples. **B,** Intercellular tryptase immunoreactivity. **C,** Quantitative Kit immunohistomorphometry. **D,** Percentage of $Ki67^{+}$ Kit^{+} cells in organ-cultured human NPs. **E,** Quantitative chymase immunohistomorphometry. **F,** Cellularity (cells per square millimeter) of the lamina propria. Data were from 2 to 3 subjects. $*P < .05$. *N.S.*, Not significant.

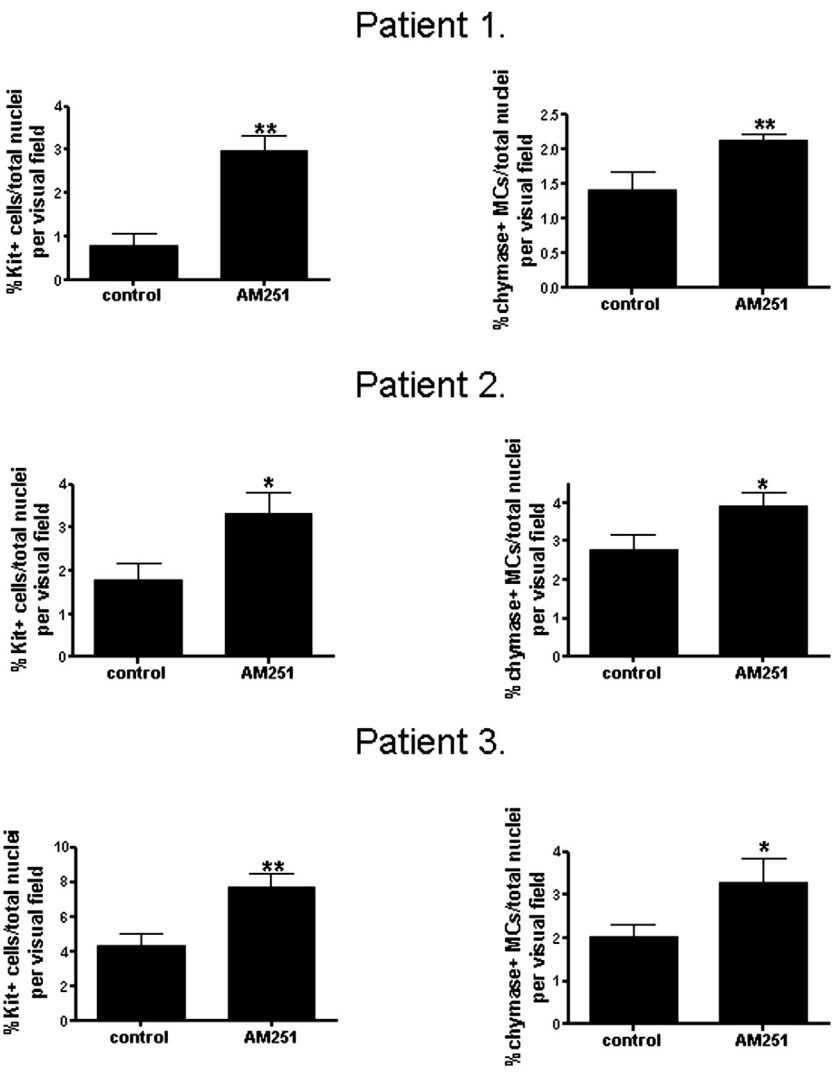

FIG E6. CB1 inhibition increased the percentages of Kit^+ and chymase-positive hMMCs in all samples examined. *Left,* Quantitative Kit immunohistomorphometry of organ-cultured human NP samples treated with either vehicle or AM251 (1 μmol/L). *Right,* Quantitative chymase immunohistomorphometry of organ-cultured human NP samples treated with either vehicle or AM251 (1 μmol/L). $*P < .05$ and $**P < .01$.