

Letter to the Editor

The expression of cannabinoid receptor 1 is significantly increased in atopic patients

To the Editor:

The human endogenous cannabinoid system (ECS) is a complex signaling network involved in a large number of physiological processes. Recently, 2 articles reported in the *Journal of Allergy and Clinical Immunology* describe that endocannabinoids and cannabinoid receptor (CB) 1 signaling may play a potent inhibitory role in human mast cell (MC) degranulation and activation in the airway mucosa and skin, suggesting that targeting the ECS in these tissues might well represent a novel strategy for the treatment of allergy.^{1,2} In these studies, the pharmacological blockage or gene silencing of CB1 significantly stimulated the degranulation and maturation of MCs from resident progenitors by mechanisms partially depending on the upregulation of stem cell factor production, which was counteracted by the activation of CB1 with specific agonists. Although it is plausible that the ECS may contribute to the regulation of allergic diseases, studies reporting human data are still scarce. Accordingly, we explored the potential effect of the ECS in human allergic diseases by quantifying and comparing the *in vivo* mRNA expression levels of the main components of the ECS (the receptors CB1 and CB2 and the enzymes fatty acid amide hydrolase [FAAH] and monoacylglycerol lipase [MAGL] that are involved in the hydrolysis and inactivation of endocannabinoids) in tonsils and PBMCs of atopic and healthy subjects.

This study has been reviewed and approved by the ethical committee of Satakunta Central Hospital, Pori, Finland, and the ethical committee of the Medical University of Vienna, Vienna, Austria. Detailed methodology is fully described in this article's [Methods](http://www.jacionline.org) section in the Online Repository at www.jacionline.org. We initially explored the *in vivo* mRNA expression levels of main components of the ECS in palatine tonsils directly excised from 35 atopic patients suffering from allergic rhinitis, atopic dermatitis, and/or asthma and 35 nonatopic controls. The main reason for tonsillectomy was tonsillar hypertrophy or recurrent tonsillectomy without significant differences between both groups (nonatopic vs atopic). Tonsils that showed any sign of infection or microabscess were discarded. As shown in [Fig 1, A](#), the mRNA expression levels of CB1 were significantly higher in tonsils ($P = .0002$) from atopic patients than in tonsils from nonatopic subjects. There was no significant difference in the mRNA expression levels of CB2, FAAH, and MAGL. We extended the analysis by subgrouping atopic patients according to allergic diseases. As shown in [Fig 1, B](#), the mRNA expression levels of CB1 were significantly higher in tonsils from atopic patients with allergic rhinitis than in nonatopic subjects without significant differences in the other compared genes. We also compared tonsils from 10 atopic patients suffering from atopic dermatitis with tonsils from 35 nonatopic subjects, and significant upregulation of the mRNA levels of CB1 was also observed in patients with atopic dermatitis than in nonatopic subjects without changes for the other analyzed genes ([Fig 1, C](#)). These data

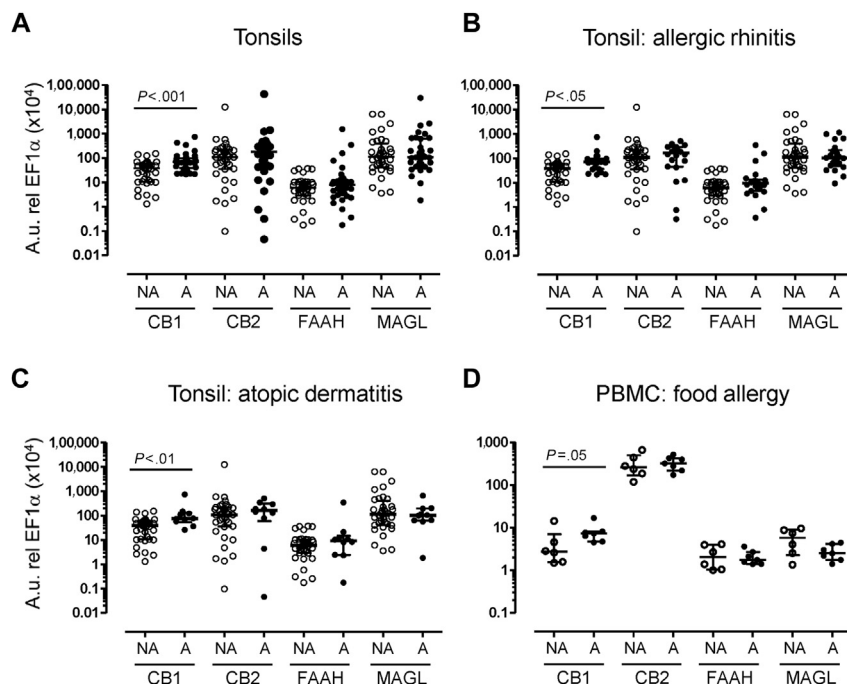


FIG 1. Increased CB1 mRNA expression in tonsils and PBMCs from atopic patients. mRNA expression levels of CB1, CB2, FAAH, and MAGL in tonsils from 35 atopic (A) patients (A), in tonsils from 22 atopic (A) individuals with allergic rhinitis (B), and in tonsils from 10 atopic (A) individuals with atopic dermatitis (C) compared with tonsils from 35 nonatopic (NA) individuals, and PBMCs from 6 healthy (NA) controls compared with 7 highly peanut-allergic (A) individuals (D). Arbitrary units (A.u.) are $2^{-\Delta CT}$ values multiplied by 10^4 , with ΔCT defined as the difference between the cycle threshold value for the gene of interest and EF1 α . Data represent median with interquartile range. Mann-Whitney U test. EF1 α , Elongation factor 1 α .

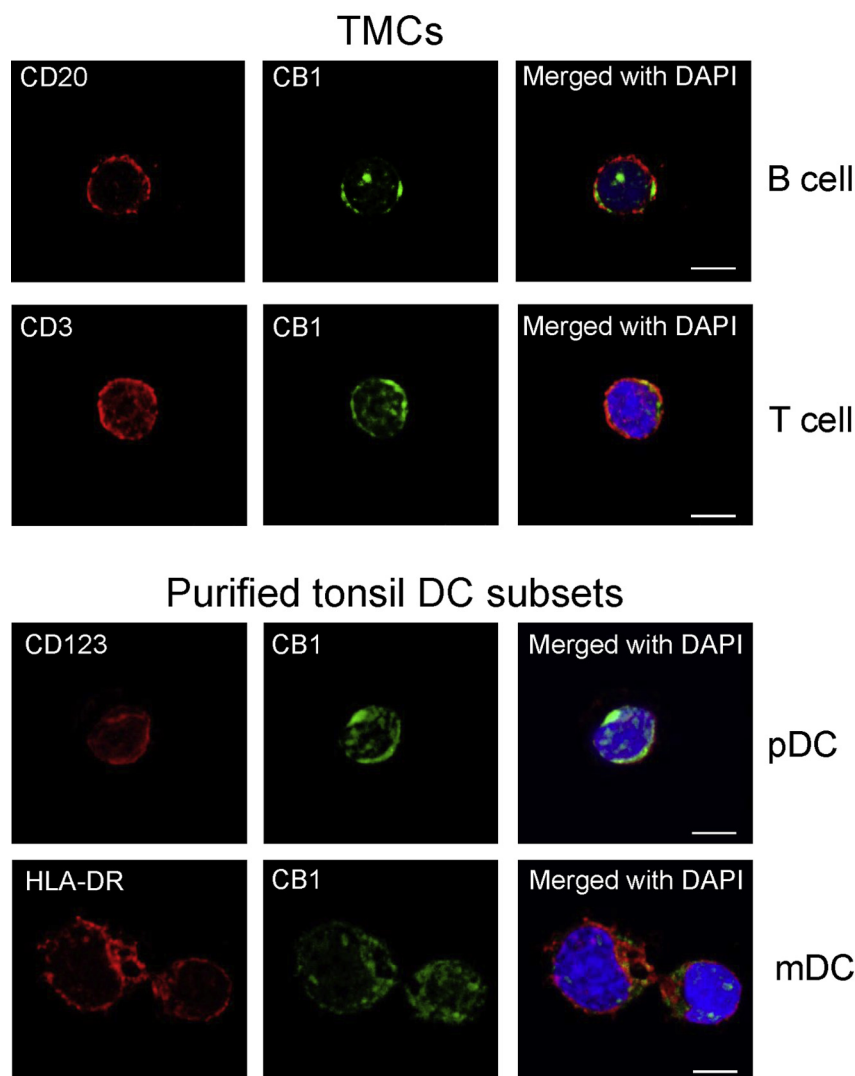


FIG 2. Protein expression of CB1 in tonsil B cells, T cells, and purified pDCs and mDCs from atopic donors. TMCs were stained for CB1 (green), DAPI (blue), and CD20 (red) for B cells or CD3 (red) for T cells. Purified pDCs and mDCs were stained for CD123 (red) and HLA-DR (red), respectively, in combination with CB1 (green) and DAPI (blue). All preparations were analyzed by using confocal microscopy. Data are from 1 of at least 2 different atopic donors with similar results. White bars, 5 μ M. DAPI, 4'-6-Diamidino-2-phenylindole, dihydrochloride.

demonstrated that CB1, CB2, FAAH, and MAGL are expressed in human tonsils and that the atopic patients displayed a significant upregulation of CB1 at the mRNA level. To strengthen our findings in a different population of patients and to determine whether changes in the mRNA expression levels of components of the ECS could also be detected in peripheral blood, we investigated patients who showed clinical anaphylaxis to peanut and an age-matched control group with no allergy. We quantified and compared the mRNA expression levels of the same ECS components in PBMCs from 7 food-allergic and 6 healthy individuals. As shown in Fig 1, D, the mRNA levels of CB1 were also significantly higher in peanut-allergic individuals than in healthy controls without significant differences in the expression levels of CB2, FAAH, and MAGL, demonstrating the same pattern that was observed in the tonsils. To analyze specific cell compartments that might account for differences observed in CB1 mRNA expression, we isolated

tonsil mononuclear cells (TMCs) and purified tonsil B and T cells, plasmacytoid dendritic cells (pDCs), and myeloid dendritic cells (mDCs) (see Fig E1 in this article's Online Repository at www.jacionline.org). Supporting our data, CB1 mRNA levels were significantly higher in TMCs from atopic donors than in TMCs from nonatopic donors. Although significant differences were demonstrated only in purified T cells from atopic patients, the same tendency was also observed when comparing CB1 mRNA expression levels in purified B cells, pDCs, and mDCs from nonatopic and atopic patients, suggesting that the higher CB1 mRNA levels found in allergic patients might be due to a contribution of all these different cell subsets. To further verify our findings, we demonstrated that tonsil B cells, T cells, and purified pDCs and mDCs from atopic donors highly express CB1 at the protein level by using the recently designed specific chemical probes³ that allow the detection of this receptor by confocal microscopy (Fig 2). All these data indicate that not

only MCs^{1,2} but also B cells, T cells, pDCs, and mDCs might play important roles in the regulation of allergic diseases through the ECS. Although future investigations are needed, a potential role of signaling through CB1 inhibiting excessive immune responses in atopic patients or a T_H2-polarizing effect might be possible. We have recently shown that human tonsils are lymphoid organs in which the generation of T-cell tolerance due to the suppressive function of allergen-specific FOXP3⁺ regulatory T cells occurs *in vivo* by mechanisms partially depending on pDCs.⁴ This allergen-specific T-cell tolerance can be broken by activation of TLR4 or TLR8 and proinflammatory cytokines such as IL-1 and IL-6.⁵ The data we report herein demonstrate that differences in mRNA expression levels of CB1 between atopic and nonatopic subjects are detected in tonsils, thus supporting our previous findings showing that tonsils represent peripheral immune responses to allergens in atopic and healthy subjects.⁵ It is important to note here that the tonsil samples used in this study did not show any sign of infection or abscess and they were not subjected to any cell culture procedure before RNA isolation, thus directly mirroring the *in vivo* situation in the tissue.

Alterations in the ECS signaling pathways have been also associated with other diseases including cancer or autoimmune disorders.⁶ In the case of allergic diseases, mouse models demonstrated a beneficial effect of the ECS in allergen-induced airway inflammation.⁷ Interestingly, in other studies, CB2 and endocannabinoids participated in the initiation and maintenance of both the acute and the chronic irreversible acanthosis reactions in mice models of allergic dermatitis.⁸ In addition, the ECS has been shown to play a pivotal role in the placebo analgesia effects because specific antagonists for CB1 block nonopioid placebo analgesic responses without affecting opioid placebo responses.⁹ Whether CB1 might also be involved in the placebo responses observed in allergic patients during different treatments, such as allergen-specific immunotherapy, remain to be investigated.

In conclusion, our data demonstrated for the first time that mRNA expression levels of CB1, a main component of the ECS, are upregulated in tonsils and peripheral blood of patients with 3 different types of allergic diseases: allergic rhinitis, atopic dermatitis, and food allergy. We also show that B cells, T cells, pDCs, and mDCs from atopic donors express high protein levels of CB1, indicating that these cell subsets could also contribute to the regulation of the pathogenesis of allergic diseases mediated by the ECS. Further detailed studies are needed to determine whether the ECS might represent a potential therapeutic target.

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METHODS

Study groups

This study has been reviewed and approved by the ethical committee of Satakunta Central Hospital, Pori, Finland, and the ethical committee of the Medical University of Vienna, Vienna, Austria. Noninflamed tonsil samples from well-characterized nonatopic and atopic patients undergoing tonsillectomy were taken from Satakunta Central Hospital. Allergic rhinitis and atopic dermatitis status of the patients was determined by a physician according to a well-defined clinical history as previously described.⁵ Peripheral blood was drawn from individuals with confirmed anaphylaxis to peanut but a negative history for atopic dermatitis and nonatopic healthy controls according to proper guidelines for the diagnosis and management of food allergy.^{E1}

Isolation of PBMCs and purification of TMCs and tonsil pDCs and mDCs

PBMC were isolated by using Ficoll (Biochrom, Berlin, Germany) density gradient centrifugation from heparinized peripheral venous blood and storage in liquid nitrogen. TMCs and tonsil DC subsets (pDCs and mDCs) were purified following exactly the same protocol previously described by Palomares et al.⁴

RNA extraction, cDNA synthesis, and quantitative real-time RT-PCR

To isolate total RNA directly from palatine tonsil, tissues (that had been previously stabilized in RNAlater) were homogenized in grinding tubes containing CK28 ceramic balls (Bertin, Montigny-le-Brettonneux, France) by using a Precellys 24 homogenizer (Bertin) at 6000 rpm $\times$ 2 $\times$ 50 seconds. Total RNA from tonsils after tissue homogenization and from PBMCs was isolated by using the RNeasy mini kit (Qiagen, Hombrechtikon, Switzerland).

Reverse transcription was performed with the Revert Aid M-MuLV Reverse Transcriptase (Fermentas, Nunningen, Switzerland) using random hexamer primers according to the manufacturer's protocol. Gene expression was analyzed by quantitative real-time PCR using iTaq SYBR Green Supermix with ROX (Bio-Rad, Basel, Switzerland) on a 7900HT Fast Real-Time PCR instrument (Applied Biosystems, Foster City, Calif). The sequences of the primers used for PCR were as follows: Elongation factor 1 α (forward, CTGAACCATCCAAT; reverse, GCCGTGTGGCAATCCAAT), CB1 (forward, AAGGTGACATGGCATCCAAAT; reverse, AGGACGAGA GAGACTTGTGTGTA), CB2 (forward, CACTGATCCCAATGACTAC; reverse, CCACTCCGTAGAGCATAGAT), FAAH (forward, GGCCACACC TTCCTACAGAA; reverse, GTTTGTGCGGTACACCTCGAT), and MAGL (forward, CTGAACCATCCAGGCCAAAT; reverse, GCCGTGTGGCAAT CCAAT).

PCR conditions were 10 minutes at 95°C followed by 40 cycles of 15 seconds at 90°C and 1 minute at 60°C. Arbitrary units show the $2^{-(\Delta CT)}$ values multiplied by 10^4 , where ΔCT corresponds to the difference between the CT value for the gene of interest and the elongation factor 1 α .

Synthesis of chemical probes for cannabinoid receptors and binding assays

High-affinity ligands for cannabinoid receptors HU210 and HU308 were synthesized according to previously described methods.^{E2,E3} The biotinylated derivative of HU210 (HU210-biotin) was prepared starting from (–)-[(6aR,10aR)-3-(1,1-dimethylheptyl)-1-hydroxy-6,6-dimethyl-6a,7,10,10a-tetrahydro-6H-benzo[c]chromen-9-yl]methyl pivalate^{E2} and

commercially available *N*-(+)-biotinyl-6-aminoheptanoic acid (Sigma-Aldrich, Madrid, Spain). The chemical structure was unequivocally assigned by using high-resolution mass spectrometry and nuclear magnetic resonance. Purity was higher than 95% by elemental analysis. CB1 and CB2 receptor binding assays were performed as previously described.³ Radioligand competition experiments revealed that HU210-biotin displayed a comparable affinity for human CB1 and CB2 receptors as HU210^{E4,E5}—HU210: K_i (CB1) = 0.061 nM, K_i (CB2) = 0.52 nM; HU210-biotin: K_i (CB1) = 1.6 ± 0.5 nM, K_i (CB2) = 0.36 ± 0.02 nM. For the selective labeling of CB1 receptor, cells were incubated with the chemical probe HU210-biotin in the presence of an excess of the high-affinity ligand of the CB2 receptor HU308: K_i (CB1) >10,000 nM, K_i (CB2) = 22.7 nM.^{E3}

Visualization of cannabinoid receptors at the protein level by confocal microscopy

For labeling experiments, TMCs or purified pDCs and mDCs from tonsils of atopic donors were suspended in PBS, centrifuged onto glass slides (cytospin, 200 rpm, 2 minutes), and fixed in 4% paraformaldehyde for 7 minutes. Then, cells were washed twice with PBS and treated with the endogenous biotin-blocking kit (Invitrogen, Molecular Probe, Basel, Switzerland) following the manufacturer's recommendations. After rinsing with PBS, cells were incubated with the corresponding chemical probe HU210-biotin 0.5 μ M and HU308 100 μ M (for the visualization of CB1 receptor) or HU210 0.5 μ M (for the nonspecific fluorescence) in PBS for 60 minutes. Then, samples were rinsed twice with PBS and incubated with Streptavidin-Alexa Fluor 488 (1:1000, Invitrogen) in PBS for 1 hour. After washing, cells were incubated with the corresponding primary antihuman antibody (CD3 [IgG₁ mouse, inhouse produced], CD20 [IgG rabbit, Epitomics, Burlingame, Calif], CD123 [IgG₁ mouse, eBioscience, Vienna, Austria], or HLA-DR-Alexa647 [IgG2b mouse, Biolegend, Uithoorn, The Netherlands]) or the proper isotype controls in PBS containing 4% normal goat serum for 45 minutes. Next, after washing, CD3-, CD20-, and CD123-binding antibodies were detected by using Alexa Fluor 546-conjugated goat antimouse, antirabbit, and antimouse, respectively. Finally, cells were washed and treated with 1% paraformaldehyde and mounted with ProLong Gold antifade reagent with 4'-6-diamidino-2-phenylindole, dihydrochloride (Invitrogen) for nuclei staining. Images were acquired and analyzed by using the confocal microscope Leica DMI 4000B and the Leica TSC SPE system (Leica Microsystems GmBh, Glatbrugg, Switzerland). All assay and control samples were imaged under the same microscope conditions, and the images shown are representative of 2 independent experiments. There was no significant background in any of the assayed control conditions.

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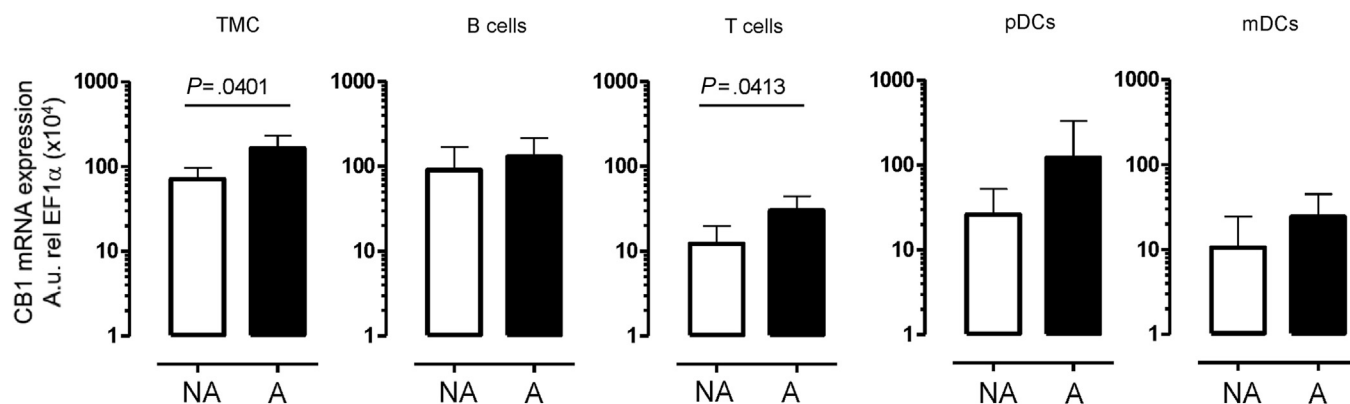


FIG E1. CB1 mRNA expression levels in purified B cells, T cells, pDCs, and mDCs from tonsils of nonatopic and atopic patients. mRNA expression levels of CB1 in TMCs and the indicated purified tonsil cell subsets from nonatopic (NA) and atopic (A) patients. Relative expression was calculated by using the $\Delta\Delta CT$ method using EF1 α as the housekeeping gene. Data represent mean with SD. Unpaired *t* test. A.u., Arbitrary units; EF1 α , elongation factor 1 α .