CB2 receptors regulate natural killer cells that limit allergic airway inflammation in a murine model of asthma

M.E. Ferrini¹, S. Hong¹, A. Stierle², D. Stierle², N. Stella³, K. Roberts¹, and Z. Jaffar¹

¹Center for Environmental Health Sciences, University of Montana, Missoula, MT, USA
²Biomedical and Pharmaceutical Sciences, College of Health Professions and Biomedical Sciences, University of Montana, Missoula, MT, USA
³Department of Pharmacology, University of Washington School of Medicine, Seattle, WA, USA

Abstract

Background—Allergic asthma is a chronic airway inflammatory disease involving the complementary actions of innate and adaptive immune responses. Endogenously generated cannabinoids, acting via CB2 receptors play important roles in both homeostatic and inflammatory processes. However, the contribution of CB2-acting eicosanoids to the innate events preceding sensitization to the common house dust mite (HDM) allergen, remain to be elucidated. We investigated the role of CB2 activation during allergen-induced pulmonary inflammation and NK cell effector function.

Methods—Lung mucosal responses in CB2-deficient (CB2−/−) mice were examined and compared with wild type littermates following intranasal exposure to HDM allergen.

Results—Mice lacking CB2 receptors exhibited elevated numbers of pulmonary natural killer (NK) cells yet were resistant to the induction of allergic inflammation exemplified by diminished airway eosinophilia, type 2 cytokine production and mucus secretion after allergen inhalation. This phenomenon was corroborated when WT mice were treated with a CB2-specific antagonist that caused a pronounced inhibition of HDM-induced airway inflammation and goblet cell hyperplasia. Unexpectedly, the preponderance of NK cells in the lungs of CB2−/− mice correlated with reduced numbers of group 2 innate lymphoid cells (ILC2s). Depletion of NK cells restored the allergen responsiveness in the lungs and was associated with elevated ILC2 numbers.

Conflict of Interest
The authors declared that they have no conflict of interest.
Conclusions—Collectively, these results reveal that CB2 activation is crucial in regulating pulmonary NK cell function, and suggest that NK cells serve to limit ILC2 activation and subsequent allergic airway inflammation. CB2 inhibition may present an important target to modulate NK cell response during pulmonary inflammation.

Keywords
animal models; asthma; CB2 receptors; innate immunity; NK cells

INTRODUCTION

Airborne allergens, including those of the house dust mite (HDM) Dermatophagoides pteronyssinus (Derp), are the most common trigger of asthma, a disease that currently affects 300 million people worldwide (1). A better understanding of the pathways by which airborne allergens entering the airway elicit immune responses is key to the development of new therapeutic agents. Allergic asthma is a chronic bronchial inflammatory condition, characterized by eosinophilic inflammation, T helper 2 (Th2) cytokine production, mucus hypersecretion, and airway hyperreactivity and remodeling (2, 3). A number of innate cells including airway epithelial cells, dendritic cells (DC), macrophages, natural killer (NK) cells and group 2 innate lymphoid cells (ILC2s) also represent critical components of asthma pathogenesis, although their diverse roles remain to be fully defined. Mature ILC2s, which appear to be enriched in the lung, skin and adipose tissue of mice and humans, release interleukin-13 (IL-13) and IL-5 in response to activation by epithelial cell-derived cytokines IL-25 and IL-33 (4, 5). ILC2s are found in human respiratory tissue and studies from both human and mouse models of asthma demonstrate a role for these cells in promoting eosinophilic inflammation (6–9). NK cells are classically recognized for their ability to identify and kill tumors and virally-infected cells (10). Such reactivity is tightly controlled by an expansive system of activating and inhibiting receptors expressed on the surface of NK cells (11). Notably, NK cells are regulated by a range of endogenously produced eicosanoids including prostaglandin (PG)D$_2$, PGE$_2$ and CB2 cannabinoids (12–16).

Cannabinoid compounds are derived from the plant Cannabis sativa (marijuana), with the major psychoactive constituent being Δ$^9$ tetrahydrocannabinol, but endocannabinoids are also produced endogenously in the brain and immune cells (17–21). To date, two types of receptors have been identified that mediate the biological actions of cannabinoids, CB1 and CB2, both coupled to G proteins (20, 22–24). While CB1 receptors are primarily found in the brain and neuronal tissue, CB2 receptors are highly expressed by NK cells and other immune cells (16, 22–25). Notably, the endogenous ligands for these receptors include arachidonyl ethanolamide, 2-arachidonoyl-glycerol (2-AG) (18, 19, 26) and these endocannabinoids are eicosanoids derived from arachidonic acid (22). Eicosanoids, including prostaglandins and cysteinyl leukotrienes, are potent locally acting arachidonic acid-derived lipid mediators that regulate diverse homeostatic and inflammatory processes linked to various diseases and allergic conditions such as asthma (27). We have previously shown that the eicosanoid PGI$_2$ regulates lung mucosal innate immunity and allergic inflammation (28). Intriguingly, CB2-acting eicosanoids play important roles in the modulation of inflammatory and immune responses including cytokine production,
apoptosis and migration of NK cells and other immune cells (16, 21, 29), however, their contribution to allergic airway sensitization and inflammatory responses to the common airborne HDM allergen remain to be comprehensively elucidated. To this end, lung mucosal responses in CB2-deficient (CB2\(^{-/-}\)) mice were investigated and compared with wild type (WT) littermates following allergen exposure by intranasal HDM instillation into the airways. Remarkably, CB2\(^{-/-}\) mice displayed elevated numbers of NK cells but had impaired allergic airway inflammation compared to WT mice. The preponderance of IFN-\(\gamma\) -producing NK cells in the lungs of CB2\(^{-/-}\) mice was inversely associated with the number of ILC2s. Adoptive transfer of CB2\(^{-/-}\) NK cells into WT hosts suppressed the lung inflammatory response to inhaled HDM and, conversely, depletion of NK cells restored allergen responsiveness in the airways of CB2\(^{-/-}\) mice and was associated with elevated ILC2 numbers in the lungs. Our results reveal a hitherto unsuspected role for CB2 activation in modulating lung inflammation in response to HDM allergen by regulating NK cells, and suggest that pulmonary NK cells serve to limit ILC2 responses during allergic airway inflammation.

**Methods**

Additional details on the methods are provided in the Supporting Information (Data S1).

**Animals, allergen challenge and pulmonary inflammation**

Female and male C57BL/6 mice (Jackson Laboratory, ME) and CB2\(^{-/-}\) mice were bred under pathogen free conditions in a barrier facility and used throughout the study. CB2\(^{-/-}\) and C57BL/6 WT mice (approximately 6 mice per experimental group) were exposed to HDM allergen by lightly anesthetizing with isofluorane to allow intranasal instillation of 30\(\mu\)l solution of HDM allergen extract (Derp, Greer Laboratories, Lenoir, NC) in sterile PBS, or administration of PBS alone (control) over a period of 3 weeks. Briefly, mice were sensitized to HDM by intranasal instillation of 100\(\mu\)g of the allergen on Day 0 and then challenged with 50\(\mu\)g of the allergen on Days 7, 14, 17 and 20. Forty-eight hours after the last exposure (Day 22), the level of lung inflammation was analyzed. Bronchoalveolar lavage fluid (BALF) was collected for determination of cell-associated eosinophil peroxidase (EPO) levels and cell differential percentages. Lung tissue was collected for histological analysis of peribronchial inflammation and mucus production (28).

**CB2 antagonist AM630 treatment**

To inhibit CB2 endocannabinoid action *in vivo*, a CB2 receptor selective inverse agonist/antagonist AM630 (Cayman Chemical, Ann Arbor, MI) was used. C57BL/6 mice were treated intranasally with 30\(\mu\)l solution of AM630 (1mg/kg) or vehicle (2% DMSO in PBS) for 3 days prior to HDM allergen challenge, then on alternate days over a 3-week allergen exposure period. Control mice did not inhale HDM but were exposed to PBS and either untreated or treated with AM630.

**NK cell depletion and transfer**

To deplete NK cells *in vivo*, CB2\(^{-/-}\) or WT mice were treated twice weekly for 2 weeks with either monoclonal IgG antibody OKT3 (control) or anti-NK1.1 depleting antibody (PK136,
250μg by i.p. injection 24h prior (Day −1) to HDM exposure and throughout an acute period of challenge with intranasal HDM allergen or PBS (control). Conversely, purified splenic NK cells from CB2−/− mice were isolated (by negative selection using MagCellect mouse NK cell isolation kit, R&D Systems, Minneapolis, MN) and the enriched CD3−CD19−NK1.1+ NK cells (5x10^5/mouse, 87–95% purity and lacking CD3+NK1.1+NKT cells, as determined by flow cytometry) were adoptively transferred by oropharyngeal instillation into WT mice 24h after the start of an acute intranasal HDM allergen challenge period.

Flow Cytometry
LMC or BALF cells were stained with combinations of mouse conjugated mAb (detailed in Data S1) and flow cytometric acquisition was performed on a FACSAria II (BD Biosciences) by 6-color analysis using FACSDiva software or Attune NxT flow cytometer (Thermo Fisher Scientific, Waltham, MA). To stain for ILCs, eFluor® 450 anti-CD90.2/Thy1.2 (eBioscience), APC/Cy7 anti-CD45/CLA, and APC anti-IL-33Rα (BioLegend) mAbs were used. Lineage negative (Lin−) cells were CD3−, CD4−, CD8α−, CD5−, CD11c−, CD19−, TCR-β−, TCR-γδ−, Gr1−, NK1.1−, TER-119− (all FITC mAbs used, BioLegend) and Siglec-F− (PE mAb, BD Biosciences).

Statistical analysis
Data were analyzed using GraphPad Prism 5.0. Data are presented as mean ±SEM. Results involving two variables were analyzed by two-way ANOVA with a Bonferroni post-test. Data comparing two groups were analyzed using an unpaired t-test where *P<0.05, **P<0.01, and ***P< 0.001.

Results
Mice lacking CB2 receptors exhibit elevated numbers of pulmonary NK cells and a defect in HDM-induced airway inflammation
To identify the role of CB2 activation in lung mucosal immune responses, we examined the properties of lung resident immune cells in naïve CB2−/− mice. While the CB2−/− mice were not overtly immune compromised, our analysis revealed a marked increase in the number of pulmonary CD3−CD19−NK1.1+DX5+ NK cells in these animals compared with WT counterparts (Fig. 1A). This unique immunological characteristic suggests that CB2 receptors play an important role in lung innate immune responses. Interestingly, both lung and splenic tissue expressed mRNA transcripts for CB2 receptors. Moreover, purified CD3−CD19−NK1.1+ NK cells expressed significantly higher levels of CB2 mRNA compared to other lymphocyte populations (Fig. S1). To assess the impact of the elevated NK cell numbers on lung immunity to an important common aeroallergen, allergic inflammation was induced in the airways of mice by intranasal instillation of HDM allergen extract over a period of 3 weeks. HDM inhalation evoked a pronounced inflammatory response in the lungs of WT mice characterized by a striking elevation in the number of airway eosinophils and lymphocytes, as well as cell-associated eosinophil peroxidase (EPO) levels in the BALF (Fig. 1B). Consistently, flow cytometric analysis revealed a marked rise in both the number of pulmonary Siglec-F+ eosinophils (Fig. 1C) and CD3+CD4+ T cells.
Moreover, allergen inhalation induced a pronounced increase in peribronchial inflammation and mucus production in these mice (Fig. 1E). In striking contrast, CB2−/− mice had severely attenuated allergic inflammation and showed little pulmonary eosinophilia, few airway CD4+ T cells and negligible peribronchial inflammation and mucus secretion after HDM inhalation (Fig. 1B–E). Control WT and CB2−/− mice exposed to PBS did not develop any airway inflammation. Collectively, these results demonstrate that CB2−/− mice are resistant to the induction of pulmonary inflammation in response to allergen inhalation. In order to corroborate this phenomenon evident in CB2−/− mice, we tested the effect of inhibiting the activation of CB2 by endocannabinoids in WT mice using a CB2-specific inverse agonist/antagonist AM630 (30, 31). C57BL/6 mice were challenged with HDM allergen and either treated with vehicle (HDM group) or the antagonist (HDM + AM630 group) and the effect on allergic inflammation examined. The CB2 antagonist caused a pronounced inhibition of HDM-induced eosinophilic inflammation and cell-associated EPO levels (Fig. 2A) and a clear reduction in airway Siglec-F+ eosinophil and CD4+ T cell influx (Fig. 2B), peribronchial inflammation and mucus secretion in these mice (Fig. 2C). Control mice that did not inhale HDM, but inhaled PBS instead or were treated with AM630, had no airway inflammation. In sharp contrast to the antagonist, mice treated with a CB2 selective agonist, HU-308, exacerbated HDM-induced airway eosinophilic inflammation (Fig. S2).

CB2−/− mice exhibit elevated number of IFN-γ-producing NK cells and reduced monocyte-derived DC in the lungs after HDM allergen inhalation

A prominent observation was that the onset of HDM-induced allergic lung inflammation in WT mice was associated with a pronounced increase in the number of pulmonary MHC-IIbrightCD11b+CD11c+ DC, which was lacking in CB2−/− mice (Fig. 3A). The lack of inflammatory DC in HDM-challenged CB2−/− animals was in marked contrast to the number of pulmonary CD3−CD19−NK1.1+ NK cells that were clearly elevated compared to WT counterparts. These lung NK cells were predominantly DX5+ (CD49b) but also expressed Nkp46 (Fig. 3B), NKG2/CD94 and intracellular granzyme A (Fig. S3A) after HDM exposure, and secreted higher levels of IFN-γ in response to in vitro stimulation with anti-NK1.1 mAb (Fig. 3C) yet produced negligible TNF-α or type 2 cytokines (data not shown). These findings led us to examine whether endocannabinoids directly influence cytokine production by NK cells. Lung NK cells from C57BL/6 mice were stimulated (2x10^6 cells/ml) with anti-NK1.1 Ab (20 μg/ml) in the absence (vehicle control) or presence of the cannabinoid 2-AG (2 μM and 10 μM), and the effect on IFN-γ production was determined after culture for 24h. Intriguingly, 2-AG dose-dependently inhibited IFN-γ production by activated NK cells (Fig. 3D), suggesting that CB2-acting endocannabinoids play an important role in suppressing cytokine production by pulmonary NK cells. We next investigated whether CB2−/− mice had elevated levels of Th1 cells (or IFN-γ-expressing T cells) after allergen challenge. However, intracellular cytokine staining revealed negligible numbers of IFN-γ-expressing CD3+CD4+ T cells in the lungs of CB2−/− mice (Fig. S3B), although a slight increase was observed in WT mice following allergen exposure. These results suggest that the attenuated inflammatory response evident in CB2−/− mice is a consequence of elevated numbers of IFN-γ-producing NK cells (rather than an increase in a Th1 response).
CB2<sup>−/−</sup> mice exhibit a defect in type 2 cytokine and CCL2 production after HDM challenge

To investigate events responsible for the attenuated allergic lung inflammatory response in CB2<sup>−/−</sup> mice, we next assessed whether there was an altered cytokine or chemokine production in the airways. Intranasal exposure of WT mice to HDM allergen induced a marked increase in the production of IL-13, IL-4 and IL-5 in the BALF, but little IFN-γ. In sharp contrast, CB2<sup>−/−</sup> mice had negligible levels of airway type 2 cytokines and IFN-γ after allergen challenge (Fig. 4A). Control WT or CB2<sup>−/−</sup> mice exposed to PBS produced no cytokines. Consistent with the depressed Th2 cytokine production, the level of CCL2, shown to significantly contribute to Th2 polarization (32), was strikingly impaired in CB2<sup>−/−</sup> mice compared to WT animals after HDM inhalation (Fig. 4B).

Transfer of CB2<sup>−/−</sup> NK cells into WT hosts suppresses HDM-induced airway inflammation

To ascertain whether the depressed allergic airway inflammation in CB2<sup>−/−</sup> mice was attributable to elevated NK cell numbers in the lungs, CD3<sup>−</sup>CD19<sup>−</sup>NK1.1<sup>+</sup> NK cells (predominantly DX5<sup>+</sup> NK cells) were isolated and purified from spleens of CB2<sup>−/−</sup> mice and adoptively transferred into the airways of WT hosts by oropharyngeal instillation, and their effect on allergic lung inflammation examined. Transfer of CB2<sup>−/−</sup> NK cells caused a pronounced reduction in airway eosinophil and lymphocyte influx, as well as cell-associated EPO levels in the BALF following HDM challenge compared to sham (no transfer) group (Fig. 5A). Control mice that did not inhale HDM, but inhaled PBS instead or received CB2<sup>−/−</sup> NK cells, had no airway eosinophilic inflammation. Flow cytometric analysis revealed a striking reduction in both the number of CD3<sup>+</sup>CD4<sup>+</sup> T cells and monocyte-derived CD11b<sup>+</sup>CD11c<sup>+</sup>MHC-II<sup>bright</sup> DC in the lungs of HDM-challenged mice that received CB2<sup>−/−</sup> NK cells compared to sham (no transfer) group (Fig. 5B,C). Consistently, this was associated with elevated numbers of pulmonary CD3<sup>−</sup>CD19<sup>−</sup>NK1.1<sup>+</sup>DX5<sup>+</sup> NK cells in mice that received CB2<sup>−/−</sup> NK cells (Fig. 5D). These findings confirm a role of NK cells (rather than NKT cells) in the suppression of the lung inflammatory response.

Depletion of NK cells restores allergic airway inflammation in CB2<sup>−/−</sup> mice and is associated with elevated ILC2 numbers in the lungs

Pulmonary CD3<sup>+</sup>CD19<sup>−</sup>NK1.1<sup>+</sup> NK cells can be effectively depleted in vivo as previously described (33) with anti-NK1.1 depleting monoclonal antibody (mAb) treatment (>98% reduction in CD3<sup>−</sup>CD19<sup>−</sup>NKp46<sup>+</sup> cells, Fig. S3C). To assess whether the increased number of lung NK cells contribute to the unresponsiveness in CB2<sup>−/−</sup> animals, the mice were treated with the anti-NK1.1 mAb. Mice treated with isotype-matched IgG2a served as controls (control Ig). The effect on airway inflammation in CB2<sup>−/−</sup> and WT mice was examined following an acute HDM challenge. Treatment of CB2<sup>−/−</sup> mice with anti-NK1.1 mAb significantly restored allergen-induced airway eosinophilia and cell-associated EPO expression, IL-13 production (Fig. 6A), peribronchial inflammation and mucus secretion (Fig. 6B) to a level similar to WT animals. A modest increase in airway eosinophil influx was also observed in WT mice following treatment with anti-NK1.1 mAb compared to control Ig (Fig. 6A), presumably reflecting the fewer NK1.1 cells resident in the lungs of these mice. Control WT or CB2<sup>−/−</sup> mice and anti-NK1.1-treated controls that did not inhale HDM, but inhaled PBS instead, had no airway eosinophilic inflammation (data not shown.)
for clarity). These results suggest that the increase in allergic inflammation following NK cell depletion was associated with loss of suppressive effects of NK cells.

Since ILC2s represent a critical source of type 2 cytokines IL-5 and IL-13 and have been proposed to be crucial for orchestrating allergic responses to HDM (5), the effects of NK cell depletion on ILC responses were investigated. ILC2s express IL-33Rα/ST2 and mature ILC subsets can be identified by a lack of known lineage markers associated with T cells, B cells, myeloid cells, or granulocytes, but they share expression of the common leukocyte antigen (CD45) and Thy1 (CD90) markers (reviewed in (5)). Unexpectedly, the number of lineage negative (Lin−) CD45+CD90.2+ ILC2s expressing IL-33Rα was found to be two-fold lower in the lungs of naïve CB2−/− mice compared to WT counterparts (Fig. 6C, the total number of lung ILC2s is 5.91x10^4 in WT vs 2.69x10^4 in CB2−/− mice). A notable observation was that the reduced ILC2 numbers in naïve CB2−/− animals was inversely correlated with the number of pulmonary CD3−CD19−NK1.1+DX5+ NK cells (Fig. 6C,D). Strikingly, treatment of HDM-challenged CB2−/− mice with anti-NK1.1 mAb caused a significant elevation in the total number of lung Lin−CD90.2+IL-33Rα+ ILC2s to a level similar to that of WT animals (Fig. 6E). Importantly, depletion of NK cells and the subsequent increase in allergen responsiveness in the lungs of CB2−/− mice was associated with increased numbers of IL-13-expressing Lin−CD45+CD90.2+ ILC2s (from 1.35% in control Ig-treated to 2.83% in anti-NK1.1-treated CB2−/− mice, Fig. 6F). Collectively, these intriguing observations imply that CB2−/− mice have diminished HDM responsiveness as a consequence of reduced numbers of pulmonary ILC2s. Moreover, depletion of NK cells in CB2−/− mice caused an elevation in IL-13-expressing ILC2 numbers in the lungs and restored the allergen responsiveness. It is thus likely that NK cells play a key role in limiting ILC2 responses and subsequent allergic airway inflammation.

**Discussion**

Asthma is characterized by type 2 inflammatory reactions involving the co-ordination of innate and adaptive immune responses. The role of Th2 cells, an important source of IL-4, IL-5 and IL-13, in adaptive immune responses, is well characterized, but the contribution of innate cells to allergen responses remains to be comprehensively elucidated. A notable immunological characteristic of naïve CB2−/− mice is that their lung tissue contained two-fold more NK cells when compared to WT littermates. The CD3−CD19−NK1.1+ NK cells accumulating in the lungs of CB2−/− mice expressed additional NK cell markers such as DX5, NKG2 and granzyme A and were phenotypically distinct from innate lymphoid cells. NK cells were also found to express high levels of CB2 mRNA. The elevated NK cell pool size in the lungs of CB2−/− mice suggests that CB2 signaling plays a key role in regulating the function of NK cells at this site. In the present study, we demonstrated, for the first time, that CB2 activation is critical in the development of airway inflammation in response to inhaled HDM allergen. Mice lacking CB2 receptors, which displayed a pronounced elevation in the number of NK cells in the lungs, were resistant to the development of allergic airway disease evidenced by severely attenuated pulmonary inflammation, eosinophil and CD4+ T cell infiltration, type 2 cytokine production and mucus secretion. This phenomenon was corroborated in WT mice by inhibiting CB2 activation using a CB2-specific antagonist, AM630. Treatment of C57BL/6 mice with AM630 caused a pronounced
inhibition of HDM-induced peribronchial inflammation, eosinophil and CD4\(^+\) T cell influx into the airways and goblet cell hyperplasia. The elevated number of NK cells observed in CB2\(^{-/-}\) mice could arise from selective expansion of NK cells in the lung tissue or increased recruitment of circulating cells. Pharmacological inhibition of CB2 activation using a CB2 antagonist effectively impacted the development of allergic lung inflammation while a CB2 agonist enhanced the eosinophilic inflammation, indicating that CB2 activation plays a key role in the inflammatory response. These results are in agreement with previous findings of the involvement of CB2 receptors in augmenting allergic inflammation (34, 35).

The unresponsiveness of the CB2\(^{-/-}\) mouse to inhaled HDM allergen was found to be a consequence of the action of NK cells. The most notable demonstration of NK cell involvement was that depletion of NK1.1\(^+\) NK cells in CB2\(^{-/-}\) mice restored the allergic inflammatory response. Conversely, transfer of purified CB2\(^{-/-}\) NK cells into WT hosts suppressed the allergic lung inflammation. The observation that NK cells adoptively transferred into the airspaces of mice could suppress the development of HDM-induced allergic response is striking and suggests that these cells not only survive in this environment but their anti-inflammatory effects are long lasting. These findings reveal a pivotal role for NK cells in limiting allergic inflammatory responses in the CB2\(^{-/-}\) mouse that was previously unrecognized. Indeed, NK cells have been shown to prevent the development of T cell responses by several different mechanisms including events that eliminate T cells themselves (10, 36). NK cell numbers or responses are often reduced or suppressed during type 2-dominated immune responses such as human asthma (37, 38). Moreover, our data is consistent with previous findings demonstrating that other eicosanoids, such as PGD\(_2\) and PGE\(_2\), inhibit human NK cells (12–14).

In our study, a crucial finding indicative of the immunoregulatory events operative in CB2\(^{-/-}\) mice was that the preponderance of NK cells in their lungs was correlated with reduced numbers of ILC2s, suggesting that NK cells limit ILC2 responses. This is further supported by the depletion of pulmonary NK cells in these mice, which significantly restored the HDM-induced eosinophilic inflammation, IL-13 production and goblet cell hyperplasia, and was associated with elevated numbers of IL-13-expressing ILC2s. That NK cells suppress lung inflammatory responses to inhaled allergen by inhibiting ILC2 responses is a novel finding. Certainly, mature ILC2s represent a potent and early source of type 2 cytokines in response to allergen or other stimuli and are thought to play a critical role in the initiation of the adaptive immune response in asthma. Production of ILC2-derived IL-5 promotes the accumulation of eosinophils (39). In addition, ILC2-derived IL-13 can act on goblet cells to induce mucus production (40). ILC2s can also promote chronic inflammation by enhancing Th2 responses either indirectly by IL-13–elicited migration of activated DCs to the lung draining lymph node and subsequent Th2 cell priming (41), or directly by MHCII-dependent interactions with CD4\(^+\) T cells (42, 43). Our findings suggest that in the absence of CB2 activation, elevated pulmonary NK cell numbers serve to limit ILC2 activation and subsequent HDM-induced inflammatory response. An important observation was that pulmonary NK cells from CB2\(^{-/-}\) mice produced high levels of IFN-\(\gamma\) when stimulated \textit{in vitro} with anti-NK1.1 antibodies, yet did not secrete TNF-\(\alpha\) or type 2 cytokines. Given that NK cells express high levels of CB2 mRNA, we investigated the effect of 2-AG, a recognized endogenous ligand for CB2 receptors (26), on cytokine production by lung NK
Intriguingly, the cannabinoid 2-AG significantly inhibited IFN-γ production by pulmonary NK cells, suggesting that CB2-acting endocannabinoids play a key role in suppressing cytokine production by NK cells in the lungs. It is likely that pulmonary NK cells in CB2−/− mice restrict CD90.2+IL-33α+ ILC2 numbers through their capacity to produce high levels of IFN-γ. Indeed, IFN-γ has recently been shown to suppress tissue resident ILC2 function in vivo (44). Our data is also in line with previous report that airway epithelial cell-derived IL-25 was significantly upregulated as a consequence of NK cell deficiency and reduced IFN-γ levels in virus-infected mice (revealing a unique inverse relationship between these factors) thus demonstrating that viral-specific type 2 responses and allergic inflammation are suppressed by NK cells and their production of IFN-γ (45). By investigating whether CB2−/− mice had elevated levels of Th1 cells, we examined the number of IFN-γ-expressing CD4+ T cells in the lungs and the levels of IFN-γ in the BALF after allergen inhalation. Our analysis revealed that negligible numbers of IFN-γ-expressing CD4+ T cells were present in the lungs and no IFN-γ was produced in the BALF of CB2−/− mice following allergen exposure. These results demonstrate that the attenuated inflammatory response in CB2−/− mice is influenced by elevated numbers of IFN-γ-producing NK cells, rather than amplification of the Th1 response.

In summary, the present findings reveal a hitherto unsuspected role for CB2 endocannabinoid signaling in modulating lung inflammatory responses to HDM allergen by regulating the function or properties of tissue NK cells, and suggest that NK cells serve to restrict ILC2 responses and subsequent allergic airway inflammation.

**Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.

**Acknowledgments**

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**References**


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CB2<sup>−/−</sup> mice exhibit elevated numbers of pulmonary natural killer (NK) cells and a defect in HDM-induced airway inflammation. (A) Lungs from naïve CB2<sup>−/−</sup> mice or C57BL/6 wild type (WT) animals (6 mice per group) were collected and lung mononuclear cells (LMC) isolated by collagenase dispersion of lung tissue. The number of LMC CD3<sup>−</sup>CD19<sup>−</sup>NK1.1<sup>+</sup> NK cells expressing DX5 was determined using flow cytometry (by gating on CD3<sup>−</sup>CD19<sup>−</sup> cells). Results denote mean ±SEM (n=6) and expressed as total number of lung NK cells per mouse (**p<0.01). (B) Bronchoalveolar lavage fluid (BALF) from CB2<sup>−/−</sup> or wild type (WT) mice that were challenged by intranasal instillation of house dust mite (HDM) allergen or PBS (control) was collected and cell differential counts determined and expressed as absolute cell numbers per mouse of lymphocytes (LYM), macrophages (MAC), eosinophils (EOS), and polymorphonuclear neutrophils (PMN). Cell-associated eosinophil peroxidase (EPO) levels were assessed by colorimetric analysis. Results are mean ±SEM (n=6), **p<0.01 and ***p<0.001, and representative of four independent experiments. (C) Number of CD11b<sup>+</sup>Siglec-F<sup>+</sup>Gr1<sup>−</sup> eosinophils (by gating on CD11b<sup>+</sup> & F4/80<sup>−</sup> cells) and (D) CD3<sup>+</sup>CD4<sup>+</sup> T cells were determined by flow cytometry. (E) Peribronchial inflammation and

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mucus production were determined using hematoxylin and eosin (H&E) stain and periodic acid-Schiff (PAS) stain, respectively (20x). Data are representative of two to four independent experiments.
Figure 2.
CB2 antagonist AM630 inhibits allergen-induced airway inflammation. C57BL/6 mice (6 per group) were challenged with house dust mite (HDM) allergen and either untreated (HDM group) or treated intranasally with AM630 (HDM + AM630 group). Control mice did not inhale HDM allergen but were exposed to PBS and either untreated (PBS control) or treated with AM630 (PBS + AM630). (A) Bronchoalveolar lavage fluid (BALF) was collected and cell differential counts expressed as absolute cell numbers per mouse of lymphocytes (LYM), macrophages (MAC), eosinophils (EOS), and polymorphonuclear neutrophils (PMN). Cell-associated eosinophil peroxidase (EPO) levels were assessed by colorimetric analysis. Data are mean ±SEM (n=6), ***p<0.001 and **p<0.01. (B) Number of CD11b^+Siglec-F^+Gr1^- eosinophils (by gating on CD11b^+ & F4/80^- cells) or CD3^+CD4^+ T cells analyzed by flow cytometry and representative of three independent experiments. (C) Peribronchial inflammation and mucus production determined using hematoxylin and eosin (H&E) stain and periodic acid-Schiff (PAS) stain, respectively (20x). Results are representative of three or four independent experiments.
CB2−/− mice exhibit elevated number of IFN-γ-producing pulmonary natural killer (NK) cells but reduced monocyte-derived dendritic cells (DC) numbers after allergen inhalation: effect of cannabinoids on IFN-γ production by NK cells. CB2−/− or wild type (WT) mice (6 per group) were challenged by intranasal instillation of house dust mite (HDM) allergen or PBS (control). (A) Number of CD11b+CD11c+MHC-IIbright DC (by gating on CD11b+ cells) and (B) CD3−CD19−NK1.1+NK cells expressing DX5 or Nkp46 (by gating on CD3−CD19− cells) in the lung mononuclear cells (LMC) prepared by collagenase dispersion of lung tissue was determined by flow cytometry. Results denote mean ±SEM (n=6) and expressed as total number of CD11c+MHC-IIbright DC or CD3−CD19−NK1.1+NK cells per mouse (**p<0.01). (C) IFN-γ production by LMC from WT or CB2+/− mice stimulated in vitro with anti-NK1.1 antibody (PK136, 20 μg/ml) for 24h was determined using ELISA. (D) To examine the effect of the cannabinoid 2-AG on cytokine production by NK cells, LMC from C57BL/6 mice were stimulated in vitro with anti-NK1.1 antibody (20μg/ml) in the absence (vehicle control) or presence of 2-AG (2 μM and 10 μM) for 24h. Following culture, IFN-γ production was determined in the supernatant using ELISA. Results are mean ±SEM (n=6), *p<0.05 and **p<0.01. Data are representative of three or four independent experiments.
CB2−/− mice exhibit impaired type 2 cytokine and CCL2 production after allergen inhalation. CB2−/− or wild type (WT) mice (6 per group) were challenged with either intranasal house dust mite (HDM) allergen or PBS (control). (A) IL-4, IL-5, IL-13 and IFN-γ, and (B) CCL2 levels were measured in the bronchoalveolar lavage fluid (BALF) using ELISA or V-PLEX assay. Results are mean ±SEM (n=6), **p<0.01. Data are representative of three or four independent experiments.
Figure 5.
Transfer of CB2−/− natural killer (NK) cells suppress house dust mite (HDM) allergen-induced airway inflammation. Purified CD3−CD19−NK1.1+ NK cells from CB2−/− mice were adoptively transferred into wild type (WT) hosts (6 mice per group) that were then challenged with intranasal HDM allergen (HDM + CB2−/− NK) or PBS (PBS + CB2−/− NK). Allergic inflammation was compared with HDM-challenged Sham group that did not receive NK cells. (A) Bronchoalveolar lavage fluid (BALF) was collected and cell differential counts were determined and expressed as absolute numbers of lymphocytes (LYM), macrophages (MAC), eosinophils (EOS) and polymorphonuclear neutrophils (PMN). Cell-associated eosinophil peroxidase (EPO) levels were assessed by colorimetric analysis. Results are mean ±SEM (n=6), **p<0.01 and *p<0.05. (B) CD3+CD4+ T cell numbers in the BALF, and (C) CD11b+CD11c+MHCIIBright dendritic cell (DC) numbers, and (D) CD3−CD19−NK1.1+DX5+ NK cell numbers in lung mononuclear cells (LMC) analyzed by flow cytometry. Data are representative of three independent experiments.

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Depletion of natural killer (NK) cells in vivo restores allergen-induced airway inflammation in CB2\(^{-/-}\) mice. CB2\(^{-/-}\) or wild type (WT) mice (6 per group) were challenged to house dust mite (HDM) allergen, and either depleted of NK cells by treatment with anti-NK1.1 depleting antibody (PK136 clone) or treated with isotype IgG2a control (control Ig). (A) Bronchoalveolar lavage fluid (BALF) was collected and cell differential counts were expressed as absolute numbers per mouse of lymphocytes (LYM), macrophages (MAC), eosinophils (EOS) and polymorphonuclear neutrophils (PMN). Cell-associated eosinophil peroxidase (EPO) and cytokine IL-13 levels were measured in the BALF. Results are mean ±SEM (n=6), ***p<0.001 and *p<0.05, (ns = not significant). (B) Peribronchial inflammation and goblet cell hyperplasia were determined using hematoxylin and eosin (H&E) stain and periodic acid-Schiff (PAS) stain, respectively (20x). (C) Inverse correlation between lineage negative (Lin\(^-\)) CD45\(^+\)CD90.2\(^+\) ILC2 numbers expressing IL-33R\(\alpha\) and (D) CD3\(^-\)CD19\(^-\)NK1.1\(^+\)DX5\(^+\) NK cell numbers in lung mononuclear cells (LMC) of naïve CB2\(^{-/-}\) compared to WT mice. (E) Total number of Lin\(^-\)CD45\(^+\)CD90.2\(^+\)IL-33R\(\alpha\)^+ ILC2s in LMC of HDM-challenged WT and CB2\(^{-/-}\) mice that were treated with control Ig or anti-NK1.1 antibody (expressed as cell number per mouse). Results are mean ±SEM (n=6), **p<0.01. (F) Number of Lin\(^-\)CD45\(^+\)CD90.2\(^+\) ILC2 expressing IL-13 in LMC of HDM-challenged WT and CB2\(^{-/-}\) mice that were treated with control Ig or anti-NK1.1 antibody (expressed as cell number per mouse). Results are mean ±SEM (n=6), **p<0.01.
challenged WT or CB2−/− mice that were treated with control Ig or anti-NK1.1 antibody was determined by intracellular staining using flow cytometry. Data are representative of three independent experiments.