ImmunoHorizons
RESEARCH ARTICLE | SEPTEMBER 01 2022

# MARCH1 Controls an Exhaustion-like Program of Effector CD4+ T Cells Promoting Allergic Airway Inflammation

Carlos A. Castellanos; ... et. al

*Immunohorizons* (2022) 6 (9): 684–692.
https://doi.org/10.4049/immunohorizons.2200056

ImmunoHorizons

RESEARCH ARTICLE

Adaptive Immunity

# MARCH1 Controls an Exhaustion-like Program of Effector CD4$^+$ T Cells Promoting Allergic Airway Inflammation

**Carlos A. Castellanos,*[,1] Kamir J. Hiam-Galvez,[†] Satoshi Ishido,[‡] Ansuman T. Satpathy,[†] and Jeoung-Sook Shin***

*Department of Microbiology and Immunology, Sandler Asthma Basic Research Center, University of California, San Francisco, San Francisco, CA; [†]Department of Pathology, Stanford University School of Medicine, Stanford, CA; and [‡]Department of Microbiology, Hyogo College of Medicine, Nishinomiya, Japan



## ABSTRACT

Persistent antigenic signaling leads to T cell exhaustion, a dysfunctional state arising in many chronic infections and cancers. Little is known concerning mechanisms limiting exhaustion in immune-stimulatory diseases such as asthma. We report that membrane-associated RING-CH1 (MARCH1), the ubiquitin ligase that mediates surface turnover of MHC class II (MHCII) and CD86 in professional APCs, plays an essential role in restraining an exhaustion-like program of effector CD4$^+$ T cells in a mouse model of asthma. Mice lacking MARCH1 or the ubiquitin acceptor sites of MHCII and CD86 exhibited increased MHCII and CD86 surface expression on lung APCs, and this increase promoted enhanced expression of immune-inhibitory receptors by effector CD4$^+$ T cells and inhibited their proliferation. Remarkably, ablation of MARCH1 in mice with established asthma reduced airway infiltration of eosinophils and Th2 cells. Thus, MARCH1 controls an exhaustion-like program of effector CD4$^+$ T cells during allergic airway inflammation and may serve as a therapeutic target for asthma. *ImmunoHorizons*, 2022, 6: 684–692.

## INTRODUCTION

Professional APCs contribute to allergic airway inflammation by sustaining Th cell activation and proliferation (1). Dendritic cells (DCs) capture inhaled allergens and present them to naïve CD4$^+$ T cells in the mediastinal lymph node, inducing development of type 2 Th (Th2) cells (2). However, full development of Th2 cells is not completed in the lymph node (3, 4). The tissue of allergen exposure constitutes an important site for terminal differentiation and effectuation of Th2 cells (5). Tissue conditioning enables T cell production of IL-5 and IL-13 in high levels, which drives recruitment of eosinophils to the airway, establishing inflammation (6). Previous studies have shown that tissue DCs and macrophages are important contributors to effector inflammatory responses in allergic asthma. A human cohort study has shown that asthma characterized by high amounts of Th2 cytokines and eosinophils in the airway is accompanied by accumulation of DCs in the airway (7, 8). A mouse asthma model study has shown that depletion of airway DCs during allergen challenge abolished Th2 cell and eosinophilic inflammation, goblet cell hyperplasia, and bronchial hyperreactivity, identifying DCs as key immune cells necessary for Th2 cell stimulation during ongoing airway inflammation (9). Another mouse study demonstrated that depletion of macrophages impaired local Th2 cell homing and activation in the inflamed lung during IL-13–dependent airway inflammation (10). However, the molecular mechanisms by which DCs and macrophages mediate continuous Th2 cell activation in the airway remain poorly understood.

Received for publication July 26, 2022. Accepted for publication August 18, 2022.

**Address correspondence and reprint requests to:** Dr. Carlos A. Castellanos, Immunobiology Laboratory, The Francis Crick Institute, 1 Midland Road, NW204, London NW1 1AT, U.K. E-mail address: carlos.castellanos@crick.ac.uk

ORCIDs: 0000-0003-3615-2009 (C.A.C.); 0000-0001-5893-3354 (S.I.); 0000-0002-5167-537X (A.T.S.).

[1]Current address: Immunobiology Laboratory, The Francis Crick Institute, London, U.K.

Single-cell RNA sequencing data have been deposited in the Gene Expression Omnibus (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE211511) under accession number GSE211511.

**Abbreviations used in this article:** CTV, CellTrace Violet; DC, dendritic cell; HDM, house dust mite allergen; MARCH1, membrane-associated RING-CH1; MHCII, MHC class II; scRNA-seq, single-cell RNA sequencing; UCSF, University of California, San Francisco; UMAP, Uniform Manifold Approximation and Projection; WT, wild-type.

The online version of this article contains supplemental material.





https://doi.org/10.4049/immunohorizons.2200056

*ImmunoHorizons* is published by The American Association of Immunologists, Inc.

DCs and other APCs use MHC class II (MHCII) Ag-presenting molecule and the costimulatory molecule CD86 to stimulate Ag-specific $CD4^+$ T cells (11). MHCII and CD86 are both posttranslationally modified by a ubiquitin ligase named membrane-associated RING-CH1 (MARCH1) (12–14). MARCH1 attaches ubiquitin chains to the cytosolic lysine residues of MHCII and CD86, inducing endocytosis and subsequent degradation (13, 15). MARCH1-mediated turnover of MHCII promotes thymic DC selection of regulatory T cells and promotes splenic DC activation of naive $CD4^+$ T cells (16–18). Yet the role of MARCH1 in APC function in nonlymphoid tissues such as the airways is not known. Because MARCH1 mediates clearance of MHCII and CD86 from the APC surface, MARCH1 may control activation of tissue effector $CD4^+$ T cells and negatively regulate $CD4^+$ T cell–dependent tissue inflammation. Conversely, MARCH1 may sustain effector $CD4^+$ T cell responses by preventing APCs from providing the cells with excessive and/or prolonged antigenic and costimulatory signals because such strong signals could exhaust T cells resulting in poor proliferation and cytokine production (19). In this study, we investigated how MARCH1 affects lung APC function of driving and maintaining Th2 cell–dependent inflammation in a mouse model of allergic asthma.

## MATERIALS AND METHODS

### *Mice*

Wild-type (WT) C57BL/6J and B6.SJL-PtprcaPepcb/BoyJ ($CD45.1^+$ "BoyJ") mice were purchased from The Jackson Laboratory and maintained in our colony. $MARCH1^{-/-}$, $MARCH1^{fl/fl}$, $CD11c^{Cre}$, $MHCII^{K>R}$, $CD86^{K>R}$, and $UBC^{ERT2\text{-}Cre}$ mice were previously described (14, 16, 17, 20–22). $MARCH1^{fl/fl}CD11c^{Cre}$, $MHCII^{K>R}CD86^{K>R}$, or $MARCH1^{fl/fl}UBC^{ERT2\text{-}Cre}$ mice were generated by breeding $MARCH1^{fl/fl}$ mice with $CD11c^{Cre}$ mice, $MHCII^{K>R}$ mice with $CD86^{K>R}$ mice, or $MARCH1^{fl/fl}$ mice with $UBC^{ERT2\text{-}Cre}$ mice, respectively. Mice were housed in a specific pathogen-free facility in the Laboratory Animal Research Center at University of California, San Francisco (UCSF). Mice of both sexes were used at 5–20 wk of age when initiating an experiment and whenever possible, littermates or mice of distinct genotypes were cohoused. Sample sizes were guided by previous studies. Mouse genotypes were not blinded from the investigator. All experiments conformed to ethical principles and guidelines approved by the Institutional Animal Care and Use Committee at UCSF.

### *House dust mite allergen–induced allergic asthma protocol*

On days 0 and 7–11, mice were anesthetized with isoflurane and given 10 μg house dust mite allergen (HDM) extract (*Dermatophagoides pteronyssinus*; Stallergenes Greer) by oropharyngeal aspiration. On day 14, mice were euthanized, and lungs were collected for flow cytometry. For experiments involving tamoxifen, mice were also challenged on the days of tamoxifen administration with 10 μg HDM extract and euthanized on day 49.

### *Cell preparation for flow cytometry*

Lungs were digested with Collagenase D (Roche) and DNase I (Roche) for 30 min at 37°C and dissociated in C tubes with a gentleMACS Dissociator (Miltenyi Biotec). The digest was stopped with cold FACS buffer (0.5% BSA in PBS) and centrifuged at 500 × *g* for 5 min at 4°C. Cell pellets were resuspended in RBC lysis buffer for 5 min at room temperature and washed with FACS buffer. Cell suspensions were filtered with 70-μm nylon mesh strainers. Cells were centrifuged and resuspended in 1 ml FACS buffer and counted with a Z2 Particle Counter (Beckman Coulter). Cells then were stained for surface and/or intracellular markers with mixtures of fluorescent Abs (Supplemental Table I). In some experiments, cells were stimulated ex vivo with PMA (10 ng/ml; Sigma-Aldrich) plus ionomycin (1 μg/ml; Sigma-Aldrich) in the presence of brefeldin A (10 μg/ml; Sigma-Aldrich) for 3 h in a 37°C $CO_2$ incubator before surface and intracellular staining.

### *Adoptive transfer of HDM-experienced T cells*

T cells were isolated using the EasySep $CD4^+$ T cell Isolation Kit (STEMCELL Technologies) from single-cell suspensions of lungs derived from $CD45.1^+$ donor mice at day 14 of HDM-induced allergic asthma protocol. For each experiment, $CD4^+$ T cells were pooled from multiple donor mice. Purified T cells were labeled with CellTrace Violet (CTV) (Life Technologies) for 20 min at 37°C and quenched with RPMI supplemented with 10% FBS. Cells (~0.5–1 × $10^6$) were transferred i.v. into recipient mice. Recipient mice were administered 10 μg HDMs on days 0–3 posttransfer. On day 4, recipient mice were euthanized, and lungs were collected for determining proliferation of donor T cells by staining cells for surface markers, and CTV dilution was detected by flow cytometry.

### *Tamoxifen-induced ablation of MARCH1*

Tamoxifen was infused in corn oil and injected i.p. at a dose of 50 μg/g.

### *Single-cell RNA sequencing sample and library generation*

Single-cell RNA sequencing (scRNA-seq) libraries were prepared according to the 10× Chromium Next GEM 3′ kit according to the manufacturer's instructions. In brief, FACS-sorted cells were washed once with PBS + 0.04% BSA and resuspended in PBS + 0.04% BSA. After reverse transcription and cell barcoding in droplets, emulsions were broken, and cDNA was purified using Dynabeads MyOne SILANE followed by PCR amplification. For gene expression library construction, amplified cDNA was used for fragmentation, followed by end-repair, double-sided size selection with SPRIselect beads, PCR amplification with sample indexing primers, and double-sided size selection with SPRIselect beads. Sequencing was performed on a MiSeq (Illumina).

### *scRNA-seq library processing and analysis*

Reads from 10× scRNA expression libraries were aligned to the mouse genome assembly GRCm38 (mm10) and quantified using

cellranger *count* (version 3.1.0; 10× Genomics). The filtered feature-barcode matrices containing only cellular barcodes were used for further analysis. Single-cell gene expression matrices were imported into R and analyzed using Seurat (version 4.0). Cells were filtered for high mitochondrial RNA content (keep cells < 5% mitochondrial gene expression) and number of genes captured (keep cells > 800 and < 2500). Each cell was assigned a score for S and G2/M cell-cycle phases based on previously defined gene sets using the CellCycleScoring function. Scaled *Z* scores for each gene were calculated using the ScaleData function and regressed against the number of unique molecular identifiers per cell, S phase score, and G2/M phase score. Scale data were used as input into a principal-component analysis on the basis of variable genes. Dimensionality reduction was performed using Uniform Manifold Approximation and Projection (UMAP) with the first eight principal components. To compare cells with previously described transcriptional states, we computed gene signature scores for each cell with Seurat's AddModuleScore() function using gene signatures from Crawford et al. (23) for exhaustion score and from Tibbitt et al. (4) for Th2 module score. For the exhaustion signature score, inhibitory surface molecules overexpressed in exhausted $CD4^+$ T cells during lymphocytic choriomeningitis virus clone 13 infection compared with naive $CD4^+$ T cells (*Ctla4*, *Pdcd1*, *Lag3*, *Pdcd1lg2*, *Cd274*, *Tigit*, *Cd200*, *Havcr2*) were used. For the Th2 module score, the top 15 genes expressed by bronchoalveolar lavage Th2 cells after HDM challenge (*Igfbp7*, *Il13*, *Il1rl1*, *Plac8*, *Bhlhe40*, *Gata3*, *Nfkb1*, *Rbpj*, *Fgl2*, *Ltb4r1*, *Epas1*, *Il5*, *Zcchc10*, *Hlf*, *Mns1*) were used. scRNA-seq data have been deposited in the Gene Expression Omnibus (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE211511) under accession number GSE211511.

### *Software and statistical analysis*

Flow cytometry data were analyzed by FlowJo (TreeStar). Graphs were drawn by Prism (GraphPad). Unpaired two-tailed Student *t* test was performed to assess the significance for most of the data. For the scRNA-seq exhaustion score and Th2 module score data, statistical significance between groups was calculated by Wilcoxon rank-sum test.

## RESULTS

WT and MARCH1-deficient mice were administered with HDMs as shown in Fig. 1A. On day 14, at the peak of allergic inflammation, lung APCs in MARCH1-deficient mice displayed higher levels of MHCII and CD86 on the surface than those in WT mice, indicating that MARCH1 regulates the surface abundance of MHCII and CD86 in lung APCs (Fig. 1B). Despite higher levels of MHCII and CD86 on lung APCs, the number of effector $CD4^+$ T cells ($CD44^{hi}CD62L^{lo}CD4^+$ cells) was significantly lower in MARCH1-deficient mice compared with WT mice (Fig. 1C, Supplemental Fig. 1A). We hypothesized that the increased MHCII and CD86 on APCs in MARCH1-deficient mice might have induced exhaustion of effector $CD4^+$ T cells, leading to impaired proliferation. To test this possibility, we examined the expression of PD-1, CTLA-4, TIGIT, TIM-3, or LAG-3 immune-inhibitory receptors upregulated during T cell exhaustion (19). The percentage of effector $CD4^+$ T cells upregulating each of these inhibitory receptors was higher in MARCH1-deficient mice than in the WT mice (Fig. 1D, Supplemental Fig. 1B). Expression of multiple inhibitory receptors is a cardinal feature of T cell exhaustion (19). The percentage of effector $CD4^+$ T cells coexpressing multiple inhibitory receptors was also higher in MARCH1-deficient mice than in WT mice (Fig. 1E), implicating greater T cell exhaustion in MARCH1-deficient mice. To determine whether upregulation of these inhibitory receptors is associated with functional exhaustion, we examined proliferative status of the effector $CD4^+$ T cells by determining the expression of Ki-67, a marker indicative of cell-cycle progression. The percentage of $Ki\text{-}67^+$ effector $CD4^+$ T cells was significantly lower in MARCH1-deficient mice than in WT mice (Fig. 1F, Supplemental Fig. 1C). These results strongly suggest that MARCH1 restrains exhaustion of effector $CD4^+$ T cells during allergic airway inflammation.

To determine whether the role of MARCH1 in restraining exhaustion of effector $CD4^+$ T cells is dependent on the expression of this protein in DCs and/or macrophages, but not B cells or other unconventional APCs, we performed similar experiments using $MARCH1^{fl/fl}CD11c^{Cre}$ mice in which Cre recombinase is expressed under the control of the promoter of CD11c (9, 20, 24), a marker of murine DCs and lung macrophages (Supplemental Fig. 2A). As expected, the lung myeloid APCs of $MARCH1^{fl/fl}CD11c^{Cre}$ mice displayed higher levels of MHCII and CD86 on the surface than those of $MARCH1^{fl/fl}$ mice during allergic airway inflammation (Supplemental Fig. 2B). The number of effector $CD4^+$ T cells was lower in $MARCH1^{fl/fl}CD11c^{Cre}$ mice than in $MARCH1^{fl/fl}$ mice (Supplemental Fig. 2C). The percentage of effector $CD4^+$ T cells that express inhibitory receptors such as CTLA-4, TIGIT, and TIM-3 was significantly higher in $MARCH1^{fl/fl}CD11c^{Cre}$ mice than in $MARCH1^{fl/fl}$ mice (Supplemental Fig. 2D). The percentage of effector $CD4^+$ T cells that concurrently express multiple inhibitory receptors was also higher in $MARCH1^{fl/fl}CD11c^{Cre}$ mice than in $MARCH1^{fl/fl}$ mice (Supplemental Fig. 2E), implicating greater T cell exhaustion in $MARCH1^{fl/fl}CD11c^{Cre}$ mice. Lastly, the frequency of Ki-67–expressing effector $CD4^+$ T cells was lower in $MARCH1^{fl/fl}CD11c^{Cre}$ mice than in $MARCH1^{fl/fl}$ mice (Supplemental Fig. 2F). These findings indicate that the role of MARCH1 in restraining exhaustion of effector $CD4^+$ T cells during allergic airway inflammation is largely dependent on the expression of this protein in DCs and/or macrophages, but not B cells or other APCs.

MARCH1 is not expressed in T cells (14); however, MARCH1 deficiency might have caused a defect in the development of effector $CD4^+$ T cells in a T cell–extrinsic manner, and this defect could have resulted in functional abnormality of the cells in MARCH1-deficient mice. To bypass this potential problem, we isolated effector $CD4^+$ T cells from $CD45.1^+$ WT "BoyJ" mice treated with HDMs, labeled the cells with CTV dye, and transferred the cells to either WT or MARCH1-deficient mice that

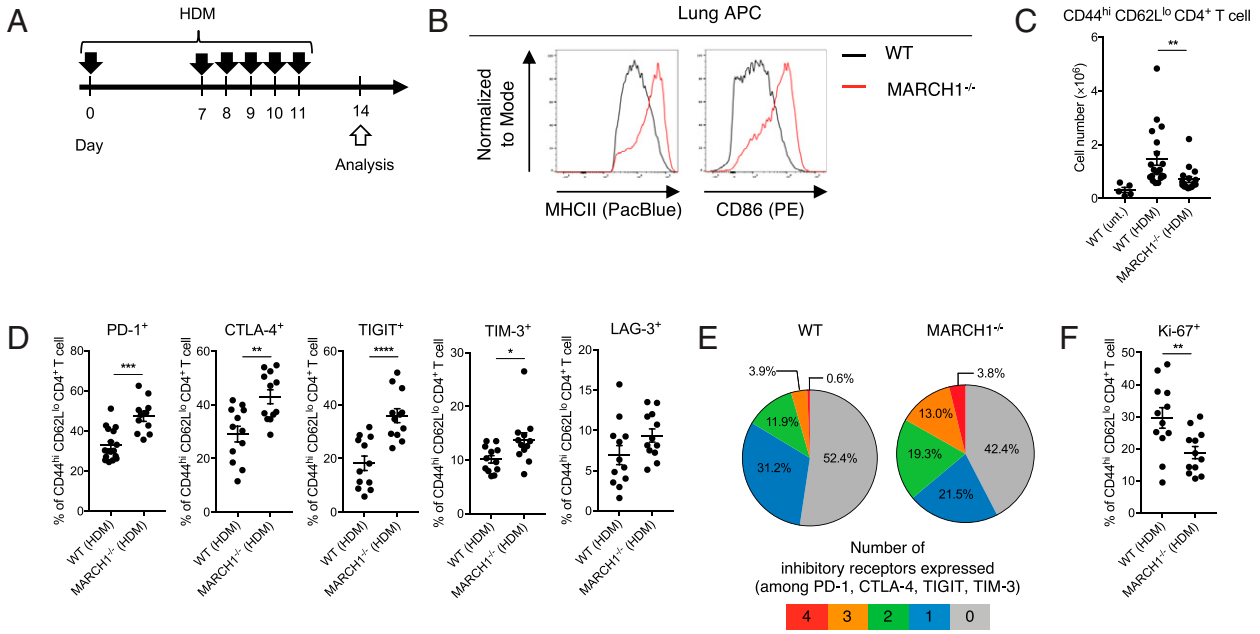


**FIGURE 1. MARCH1 negatively regulates the expression of inhibitory receptors by effector CD4$^+$ T cells and suppresses exhaustion of these T cells during allergic airway inflammation.**

(**A**) Experimental outline of HDM challenge. Solid black arrows indicate oropharyngeal aspiration of 10 μg of HDMs. (**B**) Representative histograms showing MHCII and CD86 surface expression in lung tissue APCs (gated as Siglec-F$^-$B220$^-$CD11c$^+$MHCII$^+$) of WT and MARCH1$^{-/-}$ mice. Data are representative from two experiments with four to five mice per group. (**C**) Total number of CD44$^{hi}$CD62L$^{lo}$ effector CD4$^+$ T cells in the lungs of WT or MARCH1$^{-/-}$ mice untreated (unt.) or treated with HDMs. Data are pooled from three experiments with two to seven mice per group. (**D**) Frequency of effector CD4$^+$ T cells in the lungs expressing PD-1, CTLA-4, TIGIT, TIM-3, or LAG-3. Data are obtained from two to three experiments with five to seven mice per group. (**E**) Composition of effector CD4$^+$ T cells that express single or multiple inhibitory receptors in WT or MARCH1$^{-/-}$ mice. Average frequency of cells expressing 0, 1, 2, 3, or 4 distinct inhibitory receptors is indicated as percentage in the pie charts. (**F**) Frequency of effector CD4$^+$ T cells in the lungs expressing Ki-67. Data are from two experiments with six mice per group. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$ (Student $t$ test). Data are shown as mean ± SEM.

express CD45.2. These recipient mice were subsequently challenged with HDMs, and proliferative responses of transferred T cells were examined by determining CTV dilution (Fig. 2A). We found the extent of CTV dilution was significantly lower in MARCH1-deficient mice than in WT mice (Fig. 2B, 2C). When the same experiment was performed using MARCH1$^{fl/fl}$CD11c$^{Cre}$ mice and MARCH1$^{fl/fl}$ mice, the transferred T cells proliferated less in MARCH1$^{fl/fl}$CD11c$^{Cre}$ mice than in MARCH1$^{fl/fl}$ mice (Fig. 2D). These findings demonstrate that MARCH1 in myeloid APCs significantly contributes to promoting tissue proliferation of effector CD4$^+$ T cells during allergic airway inflammation.

Then, we considered whether the adoptively transferred cells in the MARCH1-deficient mice exhibited an exhaustion signature as we had observed with endogenous effector CD4$^+$ T cells during allergic airway inflammation (Fig. 1D, 1E). To address this, we investigated the gene expression profile of the proliferating transferred cells in WT or MARCH1-deficient mice, which were sorted and subjected to scRNA-seq. HDM-reactive transferred cells in the WT or in the MARCH1-deficient recipients were visualized by dimensionality reduction in a UMAP plot (Fig. 3A). Expression of genes such as *Ctla4*, *Pdcd1*, and *Lag3* encoding for the inhibitory receptors CTLA-4, PD-1, and LAG-3, respectively, was higher in the transferred cells in MARCH1-deficient mice than in WT mice (Fig. 3B, 3C). We computed an exhaustion signature score based on inhibitory surface molecules overexpressed in exhausted CD4$^+$ T cells during lymphocytic choriomeningitis virus clone 13 infection (23) and extrapolating this to the HDM-reactive cells showed that the transferred cells in the MARCH1-deficient mice exhibited a higher exhaustion score than in the WT recipients (Fig. 3D). The transferred cells in the MARCH1-deficient recipients were also expressing less *Il13*, *Il4*, and *Il5* encoding for effector cytokines IL-13, IL-4, and IL-5, respectively (Fig. 3E). We calculated a Th2 module score based on top genes expressed in Th2 cells during HDM challenge (4), and the Th2 module score was lower for the transferred cells in the MARCH1-deficient mice (Fig. 3F), suggesting loss of effector function. These findings corroborate a role for MARCH1 in suppressing exhaustion of effector CD4$^+$ T cells, which coincides with a vigorous proliferative and effector response of these cells during allergic inflammation.

Next, we sought to identify the molecular mechanisms by which MARCH1 suppresses exhaustion of effector CD4$^+$ T cells and promotes proliferation of the cells. Our hypothesis was that

https://doi.org/10.4049/immunohorizons.2200056

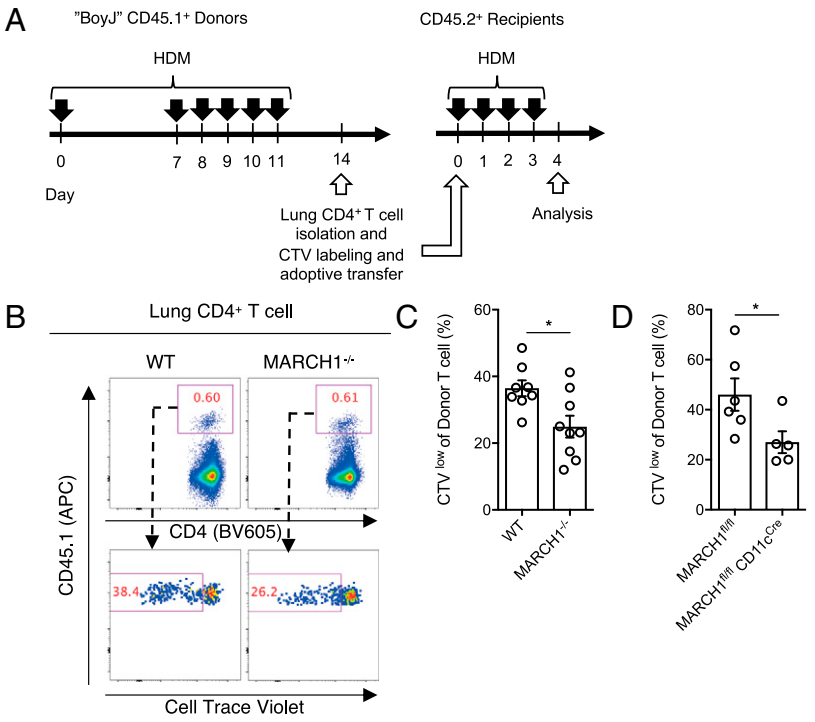


**FIGURE 2. MARCH1 promotes expansion of effector CD4$^+$ T cells during allergic airway inflammation.**

(**A**) Experimental protocol for adoptive transfer of HDM-experienced CD4$^+$ T cells. (**B**) Representative dot plots indicating proliferation of transferred CD4$^+$ T cells (CD4$^+$ CD45.1$^+$) in WT or MARCH1$^{-/-}$ recipient mice. Numbers indicate percentages of cells in the gate. (**C**) Summary of data from (B) showing the percentage of transferred T cells proliferating in WT or MARCH1$^{-/-}$ recipient mice. Data are pooled from two experiments with four to five mice per group. (**D**) The percentage of transferred T cells proliferating in MARCH1$^{fl/fl}$ or MARCH1$^{fl/fl}$ CD11c$^{Cre}$ recipient mice. Data are obtained from an experiment with five to seven mice per group. *$p < 0.05$ (Student $t$ test). Data are shown as mean ± SEM.

MARCH1-dependent ubiquitination of MHCII and CD86 allows APCs to express MHCII and CD86 in a regulated fashion, which in turn allows the effector CD4$^+$ T cells to express inhibitory receptors in a controlled manner eliciting robust proliferative responses. To test this hypothesis, we generated MHCII$^{K>R}$ CD86$^{K>R}$ mice where all the ubiquitin-accepting lysines (K) of MHCII and CD86 were mutated to arginines (R) (16, 21). MHCII$^{K>R}$CD86$^{K>R}$ mice and WT mice were treated with HDMs (Fig. 4A). We found that lung APCs in MHCII$^{K>R}$ CD86$^{K>R}$ mice displayed increased abundance of MHCII and CD86 on the surface compared with those in WT mice, which confirms that surface abundance of these two molecules is indeed regulated by ubiquitination (Fig. 4B). Remarkably, the number of effector CD4$^+$ T cells was significantly low in MHCII$^{K>R}$ CD86$^{K>R}$ mice compared with WT mice (Fig. 4C). The frequency of effector CD4$^+$ T cells that express PD-1, CTLA-4, TIGIT, or TIM-3 was higher in MHCII$^{K>R}$CD86$^{K>R}$ mice than in WT mice (Fig. 4D). The percentage of the effector CD4$^+$ T cells that concurrently express multiple inhibitory receptors was also higher in MHCII$^{K>R}$CD86$^{K>R}$ mice than in WT mice (Fig. 4E). The frequency of Ki-67$^+$ effector CD4$^+$ T cells was low in MHCII$^{K>R}$CD86$^{K>R}$ mice compared with WT mice (Fig. 4F). MHCII$^{K>R}$CD86$^{K>R}$ mice were also inferior in supporting proliferation of HDM-experienced CD4$^+$ T cells compared with WT mice (Fig. 4G, 4H). This poor proliferation was accompanied by increased expression of the inhibitory molecules PD-1 and TIM-3 by the T cells in the MHCII$^{K>R}$CD86$^{K>R}$ mice (Fig. 4I). Taken together, ubiquitination of MHCII and CD86 plays a significant role in restraining exhaustion of effector CD4$^+$ T cells during allergic airway inflammation.

The findings suggesting that the ubiquitin ligase activity of MARCH1 promotes the proliferation of effector CD4$^+$ T cells during allergic airway inflammation led us to hypothesize that inhibiting MARCH1 may exert a therapeutic benefit in allergic asthma by ameliorating inflammation driven by Th2 cells, the pathologic effector CD4$^+$ T cells in this disease (6). We conceived a proof-of-principle experiment that uses a mouse strain (MARCH1$^{fl/fl}$UBC$^{ERT2\text{-}Cre}$) where the expression of MARCH1 can be abolished in an inducible manner with tamoxifen. MARCH1$^{fl/fl}$UBC$^{ERT2\text{-}Cre}$ mice and the control UBC$^{ERT2\text{-}Cre}$ mice were administered for 2 wk with HDMs alone, which would induce allergic asthma to both groups of mice. From the third week, the mice were administered with HDMs and tamoxifen, which would disable the expression of MARCH1 selectively in MARCH1$^{fl/fl}$UBC$^{ERT2\text{-}Cre}$ mice. After 7 wk of HDM treatment in total (Fig. 5A), we found that the surface level of MHCII and CD86 was markedly higher in APCs of MARCH1$^{fl/fl}$UBC$^{ERT2\text{-}Cre}$ mice than that of UBC$^{ERT2\text{-}Cre}$ mice, which confirms the ablation of MARCH1 in MARCH1$^{fl/fl}$ UBC$^{ERT2\text{-}Cre}$ mice (Supplemental Fig. 3A). We also found that the effector CD4$^+$ T cells in MARCH1$^{fl/fl}$UBC$^{ERT2\text{-}Cre}$ mice expressed PD-1, TIM-3, and TIGIT to a higher frequency than those in UBC$^{ERT2\text{-}Cre}$ mice (Supplemental Fig. 3B). The frequency of effector CD4$^+$ T cells that concurrently express multiple inhibitory receptors was also higher in MARCH1$^{fl/fl}$ UBC$^{ERT2\text{-}Cre}$ mice (Supplemental Fig. 3C). This finding is consistent with the role of MARCH1 in restraining the exhaustion of effector CD4$^+$ T cells. Next, we examined the frequency of IL-4–, IL-13–, and IL-5–producing Th2 cells, which drive inflammation in allergic asthma. We also determined

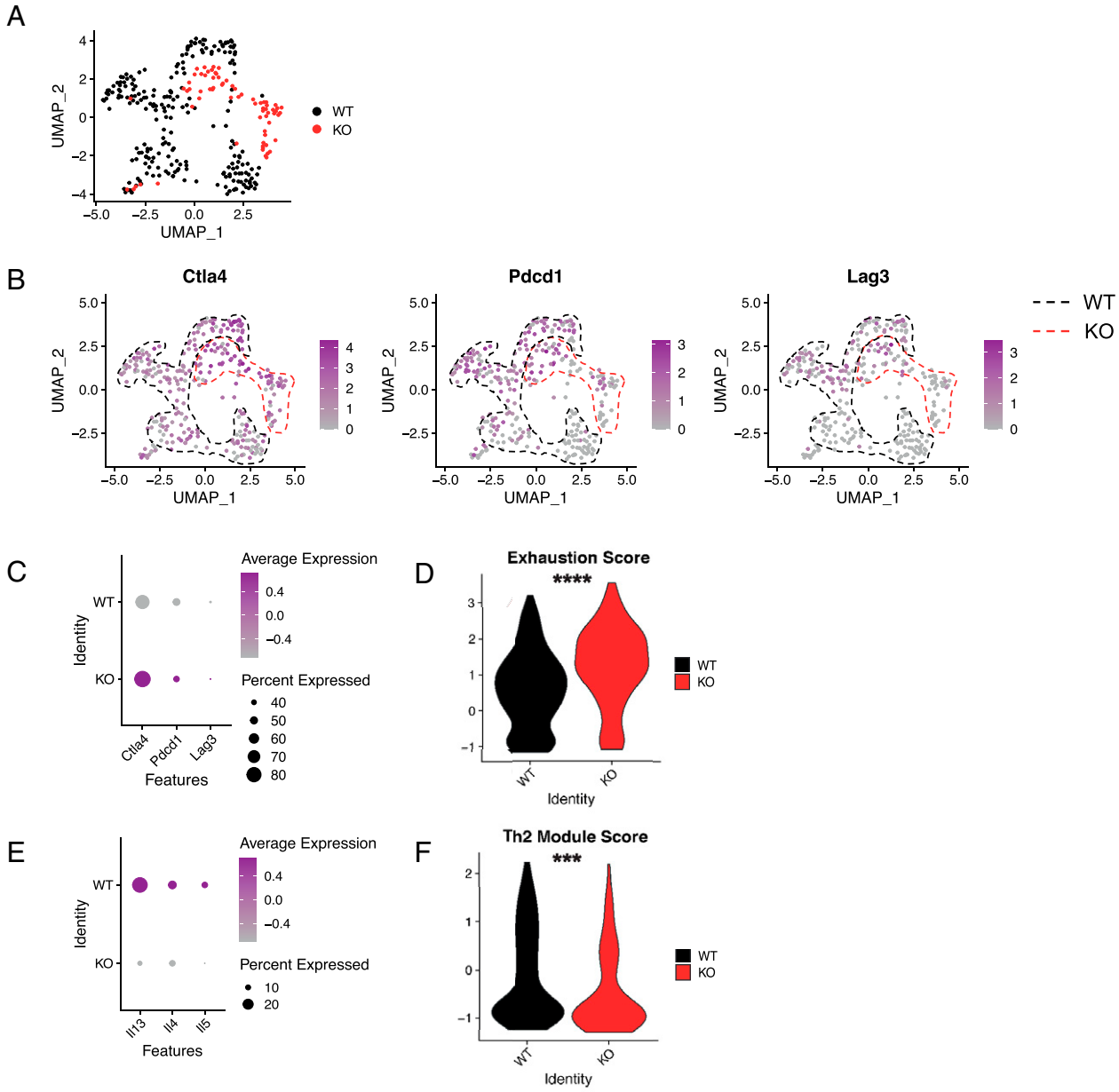


**FIGURE 3. MARCH1 negatively regulates transcriptional exhaustion of effector CD4$^+$ T cells during allergic airway inflammation.**
HDM-experienced CD4$^+$ T cells were CTV labeled and transferred into WT or MARCH1$^{-/-}$ (knockout [KO]) recipients as in Fig. 2A. CTV$^{low}$ donor T cells were sorted at day 4 after transfer and subjected to scRNA-seq. (**A**) UMAP plot shows clustering of donor cells in WT recipients (black dots) and donor cells in KO recipients (red dots). (**B**) UMAP plots show relative expression of *Ctla4*, *Pdcd1*, and *Lag3* transcripts in donor cells. Black outline encompasses most of the cells in the WT recipients, and red outline encompasses most of the cells in the KO recipients. (**C**) Dot plots show average expression of transcripts encoding inhibitory receptors differentially expressed between the cells transferred to WT or KO recipients. (**D**) Exhaustion score for the transferred cells in WT or KO recipients extrapolated from a gene signature of CD4$^+$ T cell exhaustion (23) based on inhibitory surface molecule expression. (**E**) Dot plots show average expression of transcripts encoding Th2 effector cytokines differentially expressed between the cells transferred to WT or KO recipients. (**F**) Th2 module score for the transferred cells in WT or KO recipients extrapolated from the top 20 genes expressed in Th2 cells during HDM challenge (4). Data are obtained from an experiment with three recipient mice per group. ***$p < 0.001$, ****$p < 0.0001$ (Wilcoxon rank-sum test).

the frequency of eosinophils, which also contribute majorly to inflammation in allergic asthma. We found the numbers of Th2 cells and eosinophils were both significantly lower in MARCH1$^{fl/fl}$UBC$^{ERT2\text{-}Cre}$ mice compared with UBC$^{ERT2\text{-}Cre}$ mice (Fig. 5B, 5C). These findings support our hypothesis that inhibiting MARCH1 could be an effective strategy for

https://doi.org/10.4049/immunohorizons.2200056

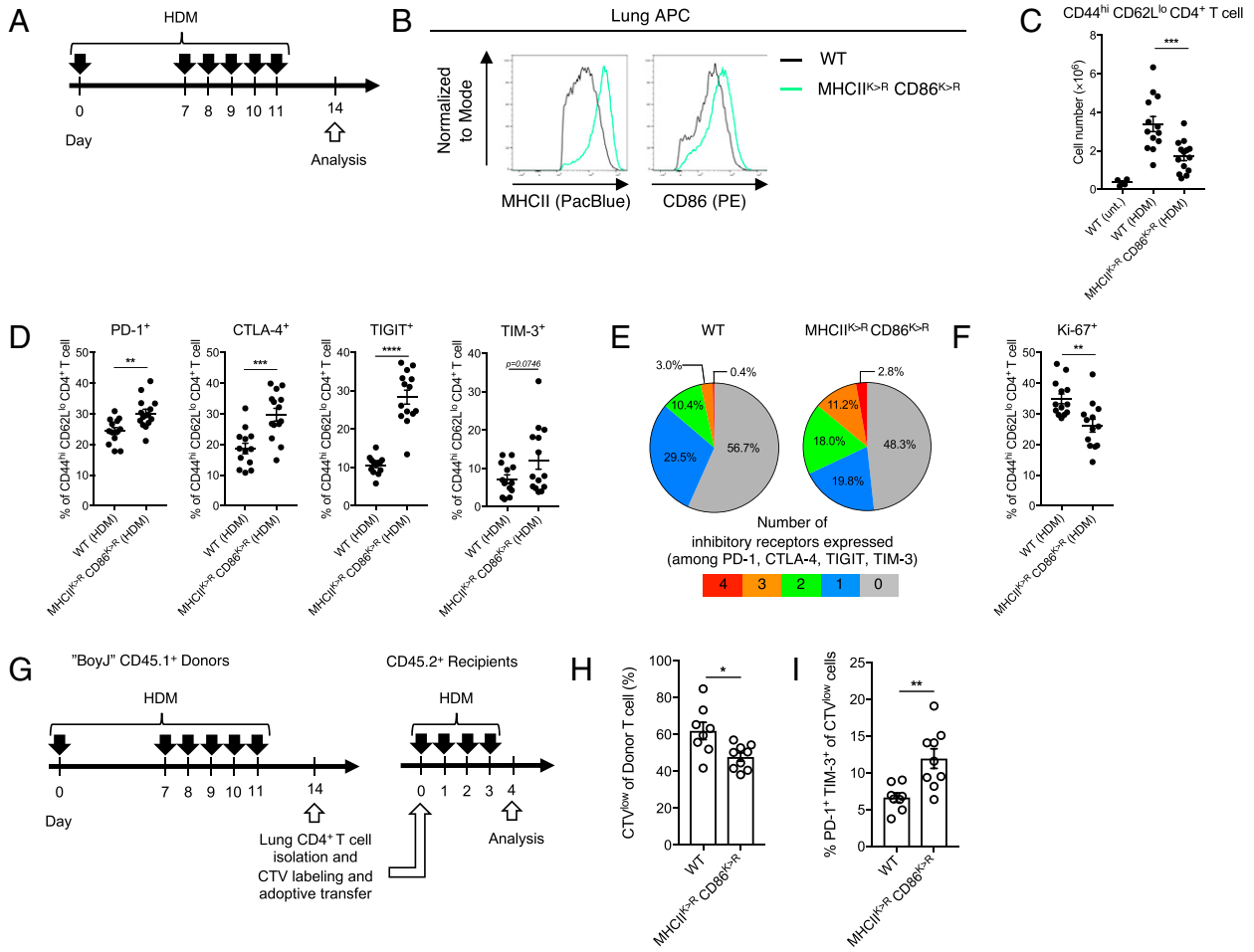


**FIGURE 4. Ubiquitination of MHCII and CD86 negatively regulates the expression of inhibitory receptors by effector CD4$^+$ T cells and suppresses exhaustion of these cells during allergic airway inflammation.**

(**A**) Experimental outline of HDM challenge. (**B**) Representative histograms showing the surface levels of MHCII or CD86 in lung tissue APCs of WT or MHCII$^{K>R}$CD86$^{K>R}$ mice. Data are representative from an experiment with eight mice per group. (**C**) Total number of CD44$^{hi}$ CD62L$^{lo}$ effector CD4$^+$ T cells in the lungs of WT or MHCII$^{K>R}$CD86$^{K>R}$ mice untreated (unt.) or treated with HDM. (**D**) Frequency of effector CD4$^+$ T cells expressing PD-1, CTLA-4, TIGIT, or TIM-3. (**E**) Composition of effector CD4$^+$ T cells that express single or multiple inhibitory receptors in WT or MHCII$^{K>R}$ CD86$^{K>R}$ mice. Average frequency of cells expressing 0, 1, 2, 3, or 4 distinct inhibitory receptors are indicated as percentage in the pie charts. (**F**) Frequency of effector CD4$^+$ T cells expressing Ki-67. Data in (C)–(F) are pooled from two experiments with two to eight mice per group. (**G**) Experimental protocol for adoptive transfer of HDM-experienced CD4$^+$ T cells. (**H**) The percentage of transferred T cells proliferating in WT or MHCII$^{K>R}$CD86$^{K>R}$ mice. (**I**) The percentage of transferred T cells expressing PD-1 and TIM-3 simultaneously in WT or MHCII$^{K>R}$CD86$^{K>R}$ mice. Data in (H) and (I) are from an experiment with eight to nine mice per group. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$ (Student $t$ test). Data are shown as mean ± SEM.

ameliorating airway inflammation associated with allergic asthma.

## DISCUSSION

Collectively, we conclude that MARCH1-dependent control of MHCII and CD86 grants tissue APCs an ability to sustain robust effector CD4$^+$ T cell responses by limiting a T cell exhaustion-like program, and that this function plays a significant role in sustaining airway inflammation associated with allergic asthma. Mice deficient in either MARCH1 or the ubiquitin acceptor sites of MHCII and CD86 elicited poor effector CD4$^+$ T cell responses compared with WT mice. The effector CD4$^+$ T cells in these mice upregulated the expression of a broad range of inhibitory receptors and proliferated poorly, which are characteristic of exhausted T cells (19). We propose that MARCH1 limits MHCII and CD86 ligand availability on the APC surface and thus prevents persistent antigenic signaling to responding effector T cells, which restrains the effector cells from becoming exhausted.

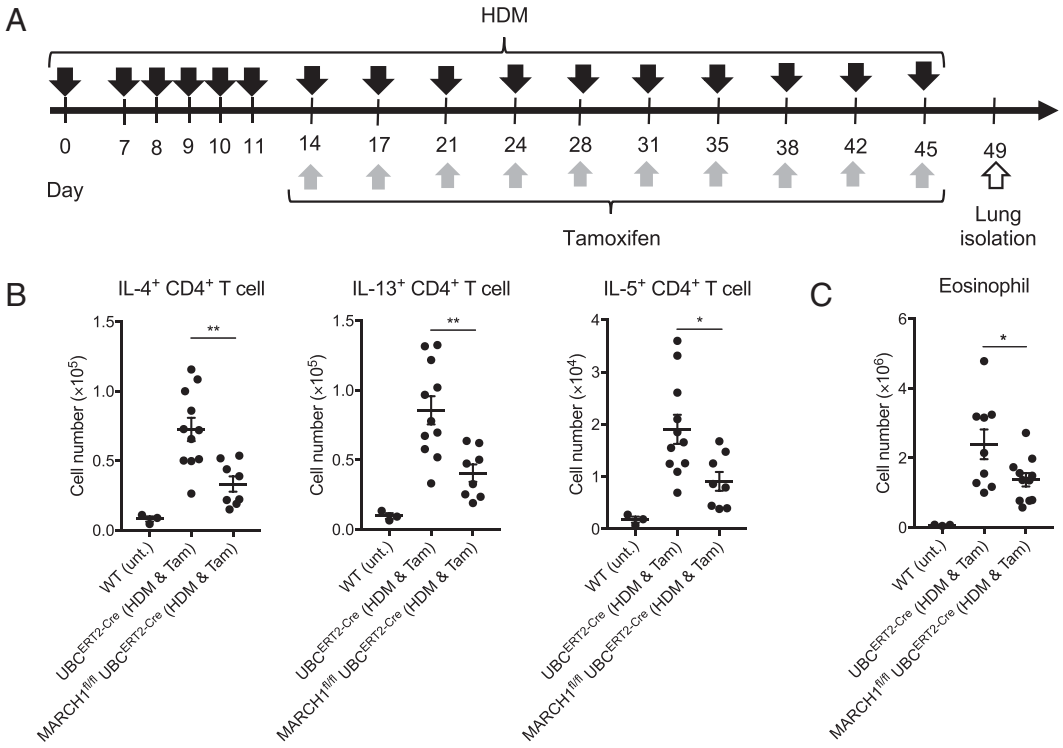


**FIGURE 5. MARCH1 ablation after establishment of allergic airway inflammation reduces Th2 cell effector function and eosinophilia to chronic HDM exposure.**

(**A**) Experimental outline of HDM challenge (black arrows) and tamoxifen injection (gray arrows). (**B** and **C**) Total numbers of IL-4, IL-13, or IL-5 competent $CD4^+$ T cells (B) and eosinophils (C) in lungs of untreated mice (unt.) or $UBC^{ERT2\text{-}Cre}$ or $MARCH1^{fl/fl}UBC^{ERT2\text{-}Cre}$ mice treated with HDMs and tamoxifen (Tam). Data are representative from two experiments with 3–11 mice per group. $*p < 0.05$, $**p < 0.01$ (Student $t$ test). Data are shown as mean ± SEM.

Kim et al. (18) have shown that MARCH1-mediated ubiquitination of MHCII promotes DC function of inducing naive $CD4^+$ T cell proliferation in the spleen, although a role for MARCH1 during an effector $CD4^+$ T cell response was not addressed. We show in this study that MARCH1 ubiquitination of MHCII and CD86 functions in APCs to support effector Th2 cell responses in the lung independent of a primary response. In addition, a previous study has shown that MARCH1-deficient mice exhibited defective Th2 cell responses but competent eosinophilia in the lung (25). The discrepancy of this study from ours may represent inherent differences in the mouse models of asthma. We did not address the distinct or redundant contribution of MARCH1 among myeloid APCs (i.e., DC or macrophage subsets), although our previous work showed that MARCH1 in the lymph node resident DCs plays a critical role for Th2 cell priming and sequential Th2 cell responses (26). This previous work did not exclude a role for MARCH1 among lung APCs (i.e., DC or macrophage subsets), which we have shown in this study could also contribute to effectuation of a Th2 cell response. Although the question of the dependency of MARCH1 among individual lung APC subsets remains to be addressed, we identify MARCH1 as a key player for lung APCs to mediate optimal effector $CD4^+$ T cell responses and propose inhibiting MARCH1 could bring a therapeutic benefit for the control of allergic asthma.

## DISCLOSURES

A.T.S. is a scientific founder of Immunai and founder of Cartography Biosciences and receives research funding from Allogene Therapeutics and Merck Research Laboratories.

## ACKNOWLEDGMENTS

We thank H. K. Li (UCSF) for mouse colony management, S. L. Gonzalez (UCSF) for performing preliminary experiments, and K. M. Ansel (UCSF), R. M. Locksley (UCSF), and R. A. Rahimi (Massachusetts General Hospital, Harvard) for helpful discussion.

## REFERENCES

1. Schuijs, M. J., H. Hammad, and B. N. Lambrecht. 2019. Professional and 'amateur' antigen-presenting cells in type 2 immunity. *Trends Immunol.* 40: 22–34.
2. Lambrecht, B. N., and H. Hammad. 2009. Biology of lung dendritic cells at the origin of asthma. *Immunity* 31: 412–424.

3. Van Dyken, S. J., J. C. Nussbaum, J. Lee, A. B. Molofsky, H. E. Liang, J. L. Pollack, R. E. Gate, G. E. Haliburton, C. J. Ye, A. Marson, et al. 2016. A tissue checkpoint regulates type 2 immunity. *Nat. Immunol.* 17: 1381–1387.
4. Tibbitt, C. A., J. M. Stark, L. Martens, J. Ma, J. E. Mold, K. Deswarte, G. Oliynyk, X. Feng, B. N. Lambrecht, P. De Bleser, et al. 2019. Single-cell RNA sequencing of the T helper cell response to house dust mites defines a distinct gene expression signature in airway Th2 cells. *Immunity* 51: 169–184.e5.
5. Hondowicz, B. D., D. An, J. M. Schenkel, K. S. Kim, H. R. Steach, A. T. Krishnamurty, G. J. Keitany, E. N. Garza, K. A. Fraser, J. J. Moon, et al. 2016. Interleukin-2-dependent allergen-specific tissue-resident memory cells drive asthma. *Immunity* 44: 155–166.
6. Locksley, R. M. 2010. Asthma and allergic inflammation. *Cell* 140: 777–783.
7. Greer, A. M., M. A. Matthay, J. Kukreja, N. R. Bhakta, C. P. Nguyen, P. J. Wolters, P. G. Woodruff, J. V. Fahy, and J. S. Shin. 2014. Accumulation of BDCA1$^+$ dendritic cells in interstitial fibrotic lung diseases and Th2-high asthma. *PLoS One* 9: e99084.
8. Woodruff, P. G., B. Modrek, D. F. Choy, G. Jia, A. R. Abbas, A. Ellwanger, L. L. Koth, J. R. Arron, and J. V. Fahy. 2009. T-helper type 2-driven inflammation defines major subphenotypes of asthma. *Am. J. Respir. Crit. Care Med.* 180: 388–395.
9. van Rijt, L. S., S. Jung, A. Kleinjan, N. Vos, M. Willart, C. Duez, H. C. Hoogsteden, and B. N. Lambrecht. 2005. In vivo depletion of lung CD11c+ dendritic cells during allergen challenge abrogates the characteristic features of asthma. *J. Exp. Med.* 201: 981–991.
10. Borthwick, L. A., L. Barron, K. M. Hart, K. M. Vannella, R. W. Thompson, S. Oland, A. Cheever, J. Sciurba, T. R. Ramalingam, A. J. Fisher, and T. A. Wynn. 2016. Macrophages are critical to the maintenance of IL-13-dependent lung inflammation and fibrosis. *Mucosal Immunol.* 9: 38–55.
11. Banchereau, J., and R. M. Steinman. 1998. Dendritic cells and the control of immunity. *Nature* 392: 245–252.
12. De Gassart, A., V. Camosseto, J. Thibodeau, M. Ceppi, N. Catalan, P. Pierre, and E. Gatti. 2008. MHC class II stabilization at the surface of human dendritic cells is the result of maturation-dependent MARCH I down-regulation. *Proc. Natl. Acad. Sci. USA* 105: 3491–3496.
13. Baravalle, G., H. Park, M. McSweeney, M. Ohmura-Hoshino, Y. Matsuki, S. Ishido, and J. S. Shin. 2011. Ubiquitination of CD86 is a key mechanism in regulating antigen presentation by dendritic cells. *J. Immunol.* 187: 2966–2973.
14. Matsuki, Y., M. Ohmura-Hoshino, E. Goto, M. Aoki, M. Mito-Yoshida, M. Uematsu, T. Hasegawa, H. Koseki, O. Ohara, M. Nakayama, et al. 2007. Novel regulation of MHC class II function in B cells. *EMBO J.* 26: 846–854.
15. Shin, J. S., M. Ebersold, M. Pypaert, L. Delamarre, A. Hartley, and I. Mellman. 2006. Surface expression of MHC class II in dendritic cells is controlled by regulated ubiquitination. *Nature* 444: 115–118.
16. Oh, J., N. Wu, G. Baravalle, B. Cohn, J. Ma, B. Lo, I. Mellman, S. Ishido, M. Anderson, and J. S. Shin. 2013. MARCH1-mediated MHCII ubiquitination promotes dendritic cell selection of natural regulatory T cells. *J. Exp. Med.* 210: 1069–1077.
17. Oh, J., J. S. A. Perry, H. Pua, N. Irgens-Möller, S. Ishido, C. S. Hsieh, and J. S. Shin. 2018. MARCH1 protects the lipid raft and tetraspanin web from MHCII proteotoxicity in dendritic cells. *J. Cell Biol.* 217: 1395–1410.
18. Kim, H. J., J. Bandola-Simon, S. Ishido, N. W. Wong, V. N. Koparde, M. Cam, and P. A. Roche. 2021. Ubiquitination of MHC class II by March-I regulates dendritic cell fitness. *J. Immunol.* 206: 494–504.
19. Wherry, E. J., and M. Kurachi. 2015. Molecular and cellular insights into T cell exhaustion. *Nat. Rev. Immunol.* 15: 486–499.
20. Caton, M. L., M. R. Smith-Raska, and B. Reizis. 2007. Notch-RBP-J signaling controls the homeostasis of CD8- dendritic cells in the spleen. *J. Exp. Med.* 204: 1653–1664.
21. Ishikawa, R., M. Kajikawa, and S. Ishido. 2014. Loss of MHC II ubiquitination inhibits the activation and differentiation of CD4 T cells. *Int. Immunol.* 26: 283–289.
22. Ruzankina, Y., C. Pinzon-Guzman, A. Asare, T. Ong, L. Pontano, G. Cotsarelis, V. P. Zediak, M. Velez, A. Bhandoola, and E. J. Brown. 2007. Deletion of the developmentally essential gene ATR in adult mice leads to age-related phenotypes and stem cell loss. *Cell Stem Cell* 1: 113–126.
23. Crawford, A., J. M. Angelosanto, C. Kao, T. A. Doering, P. M. Odorizzi, B. E. Barnett, and E. J. Wherry. 2014. Molecular and transcriptional basis of CD4$^+$ T cell dysfunction during chronic infection. *Immunity* 40: 289–302.
24. von Garnier, C., L. Filgueira, M. Wikstrom, M. Smith, J. A. Thomas, D. H. Strickland, P. G. Holt, and P. A. Stumbles. 2005. Anatomical location determines the distribution and function of dendritic cells and other APCs in the respiratory tract. *J. Immunol.* 175: 1609–1618.
25. Kishta, O. A., A. Sabourin, L. Simon, T. McGovern, M. Raymond, T. Galbas, A. Majdoubi, S. Ishido, J. G. Martin, and J. Thibodeau. 2018. March1 E3 ubiquitin ligase modulates features of allergic asthma in an ovalbumin-induced mouse model of lung inflammation. *J. Immunol. Res.* 2018: 3823910.
26. Castellanos, C. A., X. Ren, S. L. Gonzalez, H. K. Li, A. W. Schroeder, H. E. Liang, B. J. Laidlaw, D. Hu, A. C. Y. Mak, C. Eng, et al. 2021. Lymph node-resident dendritic cells drive $T_H2$ cell development involving MARCH1. *Sci. Immunol.* 6: eabh0707.