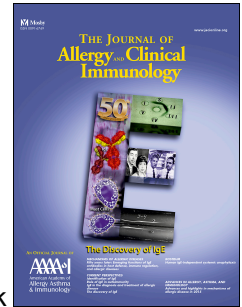


Accepted Manuscript

The Notch Pathway Inhibitor SAHM1 Abrogates the Hallmarks of Allergic Asthma

Alex KleinJan, PhD, Irma Tindemans, MSc, Jeffrey E. Montgomery, Melanie Lukkes, BSc, Marjolein J.W. de Bruijn, BSc, Menno van Nimwegen, BSc, Ingrid Bergen, BSc, Raymond E. Moellering, PhD, Henk C. Hoogsteden, MD PhD, Louis Boon, PhD, Derk Amsen, PhD, R.W. Hendriks, PhD



PII: S0091-6749(17)31658-5

DOI: [10.1016/j.jaci.2017.08.042](https://doi.org/10.1016/j.jaci.2017.08.042)

Reference: YMAI 13079

To appear in: *Journal of Allergy and Clinical Immunology*

Received Date: 19 November 2016

Revised Date: 12 July 2017

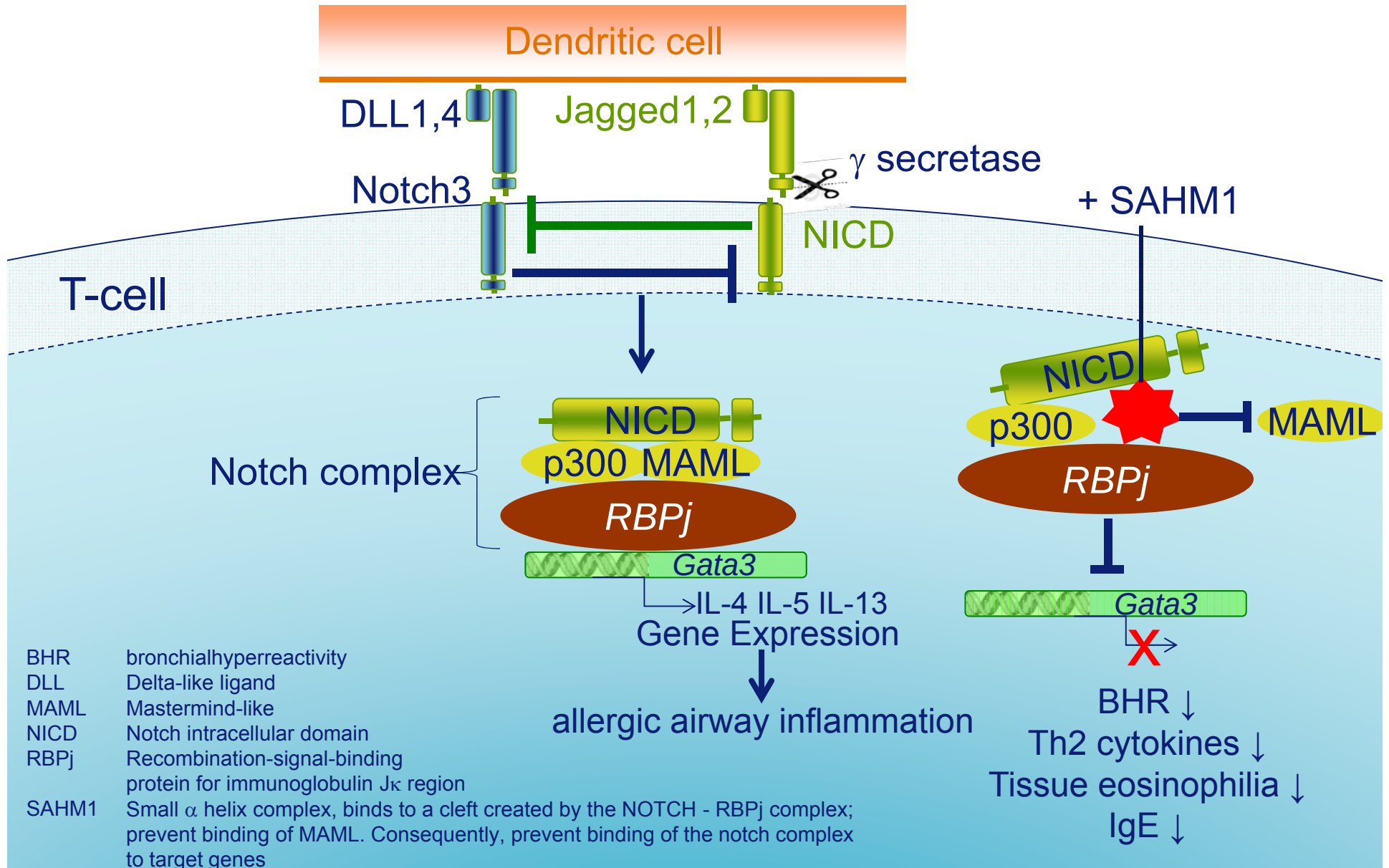
Accepted Date: 24 August 2017

Please cite this article as: KleinJan A, Tindemans I, Montgomery JE, Lukkes M, de Bruijn MJW, van Nimwegen M, Bergen I, Moellering RE, Hoogsteden HC, Boon L, Amsen D, Hendriks RW, The Notch Pathway Inhibitor SAHM1 Abrogates the Hallmarks of Allergic Asthma, *Journal of Allergy and Clinical Immunology* (2017), doi: 10.1016/j.jaci.2017.08.042.

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.



Notch inhibitor SAHM1 abrogates allergic airway inflammation



The Notch Pathway Inhibitor SAHM1 Abrogates the Hallmarks of Allergic Asthma

Alex KleinJan PhD¹, Irma Tindemans MSc¹, Jeffrey E. Montgomery^{2,3}, Melanie Lukkes BSc¹, Marjolein J.W. de Bruijn BSc¹, Menno van Nimwegen BSc¹, Ingrid Bergen BSc¹ Raymond E. Moellering PhD^{2,3}, Henk C. Hoogsteden MD PhD¹, Louis Boon PhD⁴, Derk Amsen PhD⁵ and R.W. Hendriks PhD¹

¹Department of Pulmonary Medicine, Erasmus MC Rotterdam, PO Box 2040, NL 3000CA Rotterdam, The Netherlands; ²Department of Chemistry and ³Institute for Genomics and Systems Biology, University of Chicago, Chicago, IL 60637 USA; ⁴Epirus Biopharmaceuticals Netherlands Yalelaan 46, 3584 CM Utrecht, The Netherlands and ⁵Sanquin, Plesmanlaan 125, 1066 CX Amsterdam, The Netherlands

Correspondence to:

Alex KleinJan PhD, Department of Pulmonary Medicine, Room Ee2251, Erasmus MC Rotterdam, PO Box 2040, NL 3000 CA Rotterdam, The Netherlands,
e-mail: a.kleinjan@erasmusmc.nl, phone: ++31-10-7043698, fax: ++31-10-7044728

Clinical Implications

We show that targeting the Notch transactivation complex with SAHM1, a synthetic cell-permeable peptide, inhibits allergic airway inflammation in mice, indicating that SAHM1 is an interesting novel treatment opportunity in asthma.

Capsule summary

Notch signaling plays a role in the pathophysiology of allergic airway inflammation. Here we show that targeting the Notch transactivation complex with the cell-permeable peptide SAHM1 abrogates house dust mite-driven allergic airway inflammation in mice.

Word count 3571 (max 3500)

Keywords

Allergic asthma, Notch signaling, Gata3, Th2 immunity, House-dust mite, RBPj, SAHM1

Abbreviations

AAI	Allergic airway inflammation
BAL	Bronchoalveolar lavage
CD	Cluster of differentiation
DC	Dendritic cell
DLL	Delta-like ligand
HDM	House-dust mite
Ig	Immunoglobulin
IL	Interleukin
i.n.	Intranasal
i.t.	Intratracheal
IFN	Interferon
MAML1	Mastermind-like 1
MLN	Mediastinal lymph node
PBS	Phosphate buffered saline
RBPj	Recombination-signal-binding protein for immunoglobulin Jk region
SEM	Standard error of the mean
STAT	Signal transducer and activator of transcription
Tbet	T-box-containing protein

Abstract

Background: The Notch signaling pathway has been implicated in the pathogenesis of allergic airway inflammation. Targeting the active Notch transactivation complex by the cell-permeable, hydrocarbon-stapled synthetic peptide SAHM1 results in genome-wide suppression of Notch-activated genes in leukemic cells and other models. However, efficacy of SAHM1 in allergic asthma models has remained unexplored.

Objective: We aimed to investigate therapeutic efficacy of SAHM1 in a house dust mite (HDM)-driven asthma model.

Methods: Topical therapeutic intervention with SAHM1 or a control peptide was performed during sensitization and/or challenge with HDM in mice. Airway inflammation was assessed by multi-color flow cytometry and bronchial hyperreactivity (BHR) was studied. Additionally, SAHM1 therapy was investigated in established asthma and in a model in which we neutralized IFN γ during HDM challenge to support the Th2 response and exacerbate asthma.

Results: SAHM1 treatment during the challenge phase led to a marked reduction of eosinophils and T-cells in bronchoalveolar lavage (BAL), compared with diluent-treated or control peptide-treated mice. Likewise, T-cell cytokine content and BHR were reduced. SAHM1 treatment dampened Th2-inflammation during ongoing HDM challenge and enhanced recovery following established asthma. Additionally, in the presence of anti-IFN γ antibodies, SAHM1 downregulated expression of the key Th2 transcription factor (TF) GATA3 and intracellular IL-4 in BAL T-cells, but expression of the Th17 TF ROR γ t or intracellular IL-17 was not affected. SAHM1 therapy also reduced serum IgE levels.

Conclusions: Therapeutic intervention of Notch signaling by SAHM1 inhibits allergic airway inflammation in mice and is therefore an interesting new topical treatment opportunity in asthma.

Introduction

Allergic asthma is characterized by bronchial hyperreactivity (BHR), eosinophilic airway inflammation and elevated levels of IgE^{1, 2}. The hallmarks of asthma are the direct consequences of enhanced activation of T helper 2 (Th2) cells, producing the cytokines IL-4, IL-5 and IL-13, the expression of which are controlled by the key Th2 transcription factor (TF) Gata3³⁻⁵. Central to the initiation of the differentiation of naïve T-cells into the Th2 direction is IL-4, which induces Gata3 through IL-4R and STAT6.

Intriguingly, the induction of Gata3 by the IL-4/Stat6 axis in differentiating Th2 cells raises the paradox that IL-4 is required for the generation of the cell type that is its major producer. However, in this context it was demonstrated that antigen presenting dendritic cells (DCs) use the Notch signaling pathway to instruct T-cell differentiation, independently of IL-4⁶. In this model, expression of Jagged Notch ligands on DCs constitutes an instructive signal for Th2 differentiation, whereby Notch signaling regulates *Gata3* gene transcription from an upstream promoter as well as *IL-4* gene transcription in parallel with Gata3^{7, 8}. Conversely, expression of the Notch ligands Delta-like (DLL) on DCs, which is induced by stimulation with microbial products, promotes Th1 cell differentiation⁹⁻¹¹. Supporting a critical role for Notch signaling in Th2 differentiation, we recently found that house dust mite (HDM)-driven allergic airway inflammation, Th2 activation and BHR was diminished in mice lacking the canonical Notch signaling mediator recombination signal binding protein for immunoglobulin kappa J region (RBPj) in T-cells¹². However, in this HDM-driven asthma model expression of the Jagged Notch ligands on DCs was dispensable.

Notch signaling also sensitizes T-cells to exogenous cytokines¹³, potentiates T-cell receptor and CD28 signaling and stimulates metabolic reprogramming and IL-2 secretion during priming of naïve T-cells¹⁴. Moreover, Notch is required to maintain Th1 and Th2 programs and controls memory Th cell survival by regulating glucose uptake^{13, 15}. And act as a general amplifier of T cells¹⁶. RBPj in T-cells affected the ability of Th17 cells to adequately respond to IL-23¹⁷.

In addition to its role in T-cell differentiation, Notch is also important during lung organogenesis, alveologenesis and differentiation^{18, 19}. In addition, Notch signaling has

112 been implicated in other immune cells and is e.g. also involved in DC differentiation and
113 maturation²⁰.

114 Ligand binding to the Notch heterodimeric cell-surface receptor initiates its
115 intramolecular cleavage mediated by a γ -secretase complex, resulting in the release of
116 the Notch intracellular domain (NICD), which thereby translocates from the cytosol into
117 the nucleus^{21, 22}. There, NICD forms a transactivation complex with Mastermind-like
118 (MAML) proteins and RBPj, resulting in the activation of target genes. Binding of RBPj
119 to DNA in the absence of NICD prevents target gene transcription by recruiting co-
120 repressors. Interaction of NICD with RBPj removes co-repressors and recruits co-
121 activators, including MAML, which in turn recruit DNA modification enzymes and induce
122 the transcription of Notch target genes.

123 Interestingly, blocking Notch signaling by intranasal administration of γ -secretase
124 inhibitors (GSI) reduced allergic lung inflammation in a mouse asthma model²³.
125 Because GSI are associated with severe, on-target gastrointestinal toxicity, other Notch
126 inhibitors are being developed. For example, it has been demonstrated that therapeutic
127 antibodies blocking Notch signaling prevent immune activation^{24, 25} and activation of
128 AKT downstream of Notch can be inhibited by the phosphatidylinositol 3-kinase inhibitor
129 PI-103²⁶. The assembly of the NICD-MAML-RBPj nuclear complex can be prevented by
130 the synthetic, cell-permeable inhibitor SAHM1 (stapled α -helical peptide derived from
131 MAML1)^{27, 28}. SAHM1 proved to be effective in a murine model of T-cell acute
132 lymphoblastic leukemia caused by inappropriate Notch activation and outside the
133 cancer field²⁸⁻³⁰. Importantly, this inhibitor may be more specific for Notch than the
134 commonly used GSI, which also affect cleavage of many other substrates of this
135 enzyme complex^{28, 31}, or may preferentially affect certain tissues due to pharmacologic
136 differences.

137 Given the prominent role of Notch signaling in type II immunity, here we
138 investigated the capacity of the SAHM1 peptide to mitigate pathology in eosinophilic
139 lung inflammation in a HDM-driven asthma model. We found SAHM1 therapy to be
140 beneficial, because it reduced all hallmarks of asthma, including eosinophilic airway
141 inflammation, Th2 differentiation and BHR.

Material and Methods

Mice

C57bl/6 mice (Envigo Zeist, the Netherlands) and OTII (C57bl/6) mice (Erasmus MC) were kept under specific pathogen free conditions, provided with water and food ad libitum and were used at the age of 6-11 weeks. All experiments were approved by the Erasmus MC Animal Ethics Committee.

House Dust Mite (HDM) allergic airway inflammation model and therapy

To induce airway inflammation on day 0, isoflurane-anesthetized mice were sensitized i.t. using 10 μ g HDM (Greer) extract in 80 μ L PBS or using PBS only³². Ten days later, anesthetized mice were challenged with 10 μ g HDM in 50 μ L PBS intranasally (i.n.) for five consecutive days. In the first set of experiments, treatment with HDM or control PBS was admixed with diluent (DMSO), 0.3, 1.0 or 3 μ g SAMH1 or D1 (stock solution 25 mg/ml in DMSO see supplementary data). The treatment preparation was made just before use. Four days after the last challenge, mice were sacrificed and bronchoalveolar lavage (BAL) was performed by flushing the lungs 3 times with 1 ml PBS containing EDTA (Sigma-Aldrich). The Lungs were snap-frozen in liquid nitrogen and stored at -80°C until further processing for histological analysis. In some mice, lung function was measured following increasing doses of nebulized methacholine (for details: see Supplementary Material and Methods). Innate immune responses were studied with HDM or with IL-33 (for details: see Supplementary Material and Methods).

Flow cytometric analysis and Immunohistochemistry

BAL cells were collected for cellular differentiation by flow cytometry as previously described³². For flow cytometry details: see Supplementary Figure 1. Immunohistochemical stainings were performed in a half-automatic stainer (Sequenza) as previously described³³. Sections were stained with rat-anti-SiglecF (clone E50-2440; BD Biosciences).

Statistical analysis

Reported values are shown as mean $\pm$ SEM. Statistical analyses were performed with SPSS (SPSS Inc., Chicago, IL), using Kruskal Wallis test followed by a Mann-Whitney U-test. Resulting p values less than 0.05(*), 0.01(**) and 0.001(***) are indicated and considered significant. Tests that did not reach significance ($p>0.05$) are not indicated.

Results

SAHM1 abrogates eosinophilic airway inflammation

To evaluate efficacy of SAHM1, we employed an acute airway inflammation model by sensitizing and challenging C56bl/6 mice with HDM, whereby mice were analyzed four days after the last challenge (**Figure 1A**). In this model, mice developed allergic airway inflammation characterized by increased eosinophils, neutrophils, macrophages, DCs, T and B cells and ILC2s in BAL, compared with PBS-sensitized mice (**Figure 1B, 1C**). Treatment with an optimal dose range of 0.3 - 3 μ g SAHM1 during HDM sensitization and challenge abrogated eosinophilic airway inflammation in lung tissue and BAL fluid (**Figure 1A,1B,1C**).

Next, we used intracellular flow cytometry to analyze cytokine-producing T-cells in BAL fluid and observed that although the proportions of Th2-cytokine positive cells were not affected, SAHM1 treatment significantly reduced total numbers of CD4 T-cells positive for IL-5, IL-13, IL-17 and IFN γ , (Suppl. Figure 2) Thus, SAHM1 treatment during both sensitization and challenge reduced Th2 cell differentiation and eosinophilic airway inflammation.

SAHM1 treatment is effective during challenge

Because Notch signaling has been implicated both in induction and in maintenance of Th2 differentiation^{6, 13}, we compared the effects of SAHM1 (1 μ g) treatment given either during sensitization or during challenge (**Figure 1D**). We found that SAHM1 treatment effectively reduced eosinophilic and T-cell accumulation in the BAL fluid only when given during the challenge phase (**Figure 1E**). SAHM1 treatment during sensitization only did not show any effects. IL-4 is not only important for the initiation of Th2 differentiation, but also induces serum IgE (see suppl. M&M), another hallmark of asthma. We observed that the levels of total serum IgE were reduced to control levels when mice were treated with SAHM1 during challenge, showing the effectiveness of SAHM1 treatment.

No effects of SAHM1 treatment were observed in an innate response to a single dose of 100 μ g HDM, regarding BAL differentiation, DC subset cell numbers and CD86 expression (Supplementary Figures 3 - 5), indicating that in this context Notch

signaling is not important for DC activation. Next, when we investigated IL-33-driven alveolar inflammation (Suppl. Figure 6), we noticed that SAHM1 treatment did not impair the IL-33-driven eosinophilic inflammation. Therefore, SAHM1 does not directly affect recruitment and accumulation of eosinophils. Next, we adoptively transfer OVA specific (OTII) T-cells in mice that got SAHM1 therapy or D1 or diluent. No difference in OTII division was seen in the presence of SAHM1 therapy during this primary response. OVA restimulation of the adoptive transferred OVA specific OTII T-cells obtained from the lung draining lymph node (MLN) indicates a reduced level of IFN γ (Suppl figure 7).

SAHM1, but not a mutant control, D1, abrogates eosinophilic airway inflammation

Next, we investigated if a control mutant peptide affected airway inflammation when given during HDM challenge (**Figure 2A**). In contrast to the active SAHM1 inhibitor, the mutant peptide D1²⁸ did not abrogate eosinophilic airway inflammation(**Figure 2B**). When we investigated intracellular cytokine content of CD4 T-cells by flow cytometry, we observed a significant reduction of the total numbers of Th2 CD4 cells producing IL-5 or IL-13 in BAL fluid when HDM SAHM1-treated mice were compared with HDM D1-treated mice (**Figure 2C, 2D**).

SAHM1 targets the nuclear complex downstream of the Notch signaling pathway by preventing the binding of MAML in the RBPj complex, which is important for transcription of Notch target genes. These include Gata3, which has an RBPj-binding site in its upstream promoter^{7,8}. To investigate the effect of SAHM1 on key T-cell subset TFs, intracellular flow cytometric analyses were performed, which showed that SAHM1 treatment induced a significant reduction of the numbers of Gata-3⁺, Ror γ t⁺ and FoxP3⁺ T-cells in BAL fluid (**Figure 3**). For ROR γ t and FoxP3 the reduction was mostly explained by the lower number of T-cells found in SAHM1 treated animals. In contrast, Gata3⁺ CD4 T-cells were also reduced as a proportion of the total T-cells, suggesting that inhibition of Notch specifically affected expression of Gata3, independently from diminishing T-cell numbers. Hereby both the proportions (**Figure 3A**) and the absolute numbers of Gata-3⁺ T-cells (**Figure 3B**) were markedly reduced. In agreement with the limited involvement of Th1 cells in HDM-driven airway inflammation in mice, the key Th1 TF T-bet was not induced and not affected by SAHM1 treatment. Taken together, these

findings demonstrate that SAHM1 is able to reduce the numbers of key inflammatory cells in allergic airway inflammation and particularly the Th2 inflammatory response.

SAHM1 abrogates airway bronchial hyperreactivity

BHR is another hallmark of asthma that we investigated in our HDM-driven asthma model, whereby PBS control mice and HDM-exposed mice were treated only during allergen challenge with either 1 μ g SAHM1 or control diluent (**Figure 4A**). No differences were observed between the four groups of mice in baseline airway responsiveness, as measured by lung resistance. BHR to increasing doses of methacholine (MCh) was significantly increased in HDM-challenged mice treated with diluent, compared with diluent-treated PBS-control mice (**Figure 4B**). Importantly, treatment with SAHM1 was potent enough to abrogate BHR in HDM-challenged mice.

SAHM1 improves recovery from allergic airway inflammation

Because asthma patients only present with clinical symptoms after airway hypersensitivity has already fully developed, we investigated whether SAHM1 treatment would improve airway inflammation in already established asthma. To this end, we investigated five different groups of mice (**Figure 5A**). Two control groups consisted of PBS/diluent or PBS/SAHM1 controls by sensitization and by challenge only. Three other groups were sensitized with HDM, followed on day 10-14 thereafter by five additional HDM challenges. These HDM-exposed mice were then challenged again on five successive days (day 15-19) with either HDM in the presence of diluent (group 3) or PBS, either with diluent or SAHM1 (group 4 and 5, respectively; **Figure 5A**). This set-up allowed us to investigate if SAHM1 enhances the recovery following the established asthma.

When analyzed at day 23, the PBS controls showed no signs of allergic inflammation in the BAL fluid. The group that was HDM-sensitized and then HDM exposed from day 10-19 exhibited significantly increased levels of total cells, eosinophils and T-cells, indicating an allergic response (**Figure 5B**). Importantly when SAHM1 treatment was started 5 days after the HDM challenges, the SAHM1-treated mice (HDM PBS SAHM1, **Figure 5**) still showed a strong reduction in eosinophil and T-cell numbers, compared with the mice receiving diluent (HDM PBS diluent, **Figure 5**).

This showed that SAHM1 treatment was able to enhance the recovery from allergic airway inflammation. Moreover, SAHM1 treatment reduced the numbers of Th2 cytokine producing T-cells, which reached significance for IL-5 and IL-13 (**Figure 5A**), as well as the numbers of neutrophils and DCs in the BAL fluid (data not shown). Therefore SAHM1 treatment improves the recovery of already established allergic airway inflammation in an HDM-driven asthma mouse model.

SAHM1 treatment suppresses α -IFN γ induced asthma exacerbation

A previous study showed that inhibition of Notch resulted in loss of Th2 responses to a helminth parasite, but that Th2 responses could be restored by blocking IFN γ ¹³. This result suggested the possibility that a main function of Notch is to counteract the inhibitory activity of IFN γ towards development of Th2 type responses. The imbalance between Th1 and Th2 type cytokines, in favor of Th2 cytokines, is a major cause of allergic diseases in human patients. If the primary function of Notch is indeed to counteract the inhibitory function of IFN γ on Th2 responses, a prediction would be that neutralization of IFN γ would also obviate the ability of Notch inhibition to mitigate the development of allergic asthma. We therefore tested whether SAHM1 treatment could suppress development of eosinophilic airway inflammation when mice are injected with neutralizing antibodies to IFN γ induced exacerbation. To this end, we investigated eosinophilic airway inflammation in HDM-exposed mice in the presence of α -IFN γ or control rat Ig, treated either with diluent or SAHM1 (**Figure 6A**). On the basis of previous reports showing an increased Th2 response when Th1 activity is suppressed by binding of free IFN γ ^{13, 34}, we expected that the presence of α -IFN γ during the challenge phase would exacerbate the allergic airway inflammation. Indeed, eosinophilic airway inflammation was more severe in the presence of α -IFN γ , as evidenced by increased induction of focal dense infiltrates (**Figure 6B**). Importantly, the inflammation was less severe when mice were treated with SAHM1 (**Figure 6B**). Likewise, numbers of eosinophils, neutrophils, macrophages and T-cells were significantly increased by treatment with α -IFN γ (**Figure 6C**). Exacerbation of asthma by α -IFN γ during HDM challenge was associated with elevated numbers of eosinophils,

neutrophils, macrophages and T-cells in BAL fluid, but additional treatment with SAHM1 restored these numbers to those observed in HDM and control rat Ig exposed, diluent-treated mice (**Figure 6C**).

Intracellular flow cytometry analyses showed that the numbers of Th2 (IL-4), Th1 (IFN γ) and Th17 (IL-17) cells in BAL fluid were significantly increased in the presence of α -IFN γ , compared with mice that received control rat Ig, but only a limited effect of SAHM1 on the relative proportions of cells producing these cytokines content could be detected (**Figure 6D**). Therefore, we investigated TF expression in BAL T helper cells and saw that blocking IFN γ resulted in reduced proportions of Gata3⁺ CD4⁺ T-cells and increased proportions of ROR γ t⁺ CD4⁺ T-cells in BAL (**Figure 6E**). Importantly, in the presence of α -IFN γ SAHM1 treatment still reduced the proportions of Gata3-expressing CD4⁺ T-cells in BAL compared with diluent treatment (**Figure 6E**). This reduction was greater than the reduction of the CD4 T-cell number by SAHM1 (**Figure 6D**), suggesting that Notch inhibition specifically reduced Th2 responses in this model even when IFN γ was neutralized (**Figure 6E**). Quantification of absolute numbers of TF expressing CD4⁺ T-cells in BAL demonstrated that - in the presence of α -IFN γ - SAHM1 treatment reduced Gata3⁺ CD4 T-cells numbers, but did not affect the numbers of ROR γ t⁺ or FoxP3⁺ CD4⁺ T-cells (**Figure 6F**). We observed that total serum IgE levels were increased in HDM-exposed mice, compared with PBS controls, but the effects of α -IFN γ or SAHM1 treatment in HDM-exposed mice were limited (**Figure 6G**). SAHM1-treated HDM/ α -IFN γ -exposed mice showed some reduction of serum IgE, compared with diluent-treated controls, but this did not reach significance (**Figure 6G**).

From these experiments we conclude that SAHM1 treatment reduces allergic airway inflammation, even in the setting of α -IFN γ -induced asthma exacerbation. Furthermore, these findings indicate that Notch signaling in the HDM-mediated airway inflammation model does not only function to counteract inhibitory effects of IFN γ on Th2 differentiation.

Discussion

The Notch signaling pathway has been implicated in the pathogenesis of asthma. Here we show that the cell-permeable, hydrocarbon-stapled peptide SAHM1 is effective in a HDM-driven model for allergic airway inflammation in mice. SAHM1 treatment reduced functional airway abnormalities: HDM-exposed mice showed all signs of allergic inflammation, including BHR to methacholine and eosinophilia. But when they received SAHM1 therapy, they showed less signs of BHR and eosinophilic airway inflammation.

Notch signaling in CD4⁺ T-cells induces the formation of a nuclear complex containing NICD, RBPj and MAML. When RBPj is deleted in CD4⁺ T-cells this complex cannot be formed and the Notch pathway is blunted^{6, 35}. SAHM1 binds this nuclear complex at the MAML interface and thereby functions as a competitor. Since an RBPj-binding site is present in the Gata3 upstream promoter (exon 1a) and we found that expression of Gata3 is reduced by SAHM1, it is conceivable that this drug dampens allergic airway inflammation by inhibiting the expression of Gata3 from the upstream promoter. Direct therapeutic targeting of Gata3 is challenging, although recently improvement of both early and late asthmatic responses after allergen provocation were demonstrated in patients after treatment with a DNzyme that specifically targets Gata3 mRNA³⁶. Intriguingly, there are differences in the regulation of *Il4* gene transcription (which is partly Notch dependent) and *Il5* and *Il13* gene transcription (which is Gata3 and IL-4R/STAT6-dependent)^{6, 37}. This may imply that when Notch signaling is inhibited in a strong asthma protocol, initial production of IL-4 can still induce IL-4R/Stat6-dependent transcription of Gata3 from the exon 1b promoter and thus transcription of the *Il5* and *Il13* genes.

GSIs, which act as Notch inhibitors, were successfully used in models for allergic airway inflammation, illustrating the importance of the Notch pathway in IL-4 producing CD4⁺ T-cells³⁸⁻⁴⁰. The finding that Notch inhibition is effective both during the primary and secondary immune response illustrated the ongoing need of the Notch pathway for maintenance of Th2 responses^{38, 39}. Unfortunately, GSI show significant side effects and are not specific. Here, we demonstrate that in HDM-driven allergic airway inflammation, which is an acute and short T-cell-dependent asthma model, Notch signaling is important during allergen challenge, which is in agreement with earlier findings that

Notch is required for maintaining the Th2 program¹³. Several features of SAHM1 are of significant interest in the context of its application as a new treatment strategy in asthma patients. We observed that SAHM1 is effective in dampening HDM-induced allergic airway inflammation and α -IFN γ -induced exacerbation of allergic inflammation. Moreover, SAHM1 was potent to reduce inflammation in established allergic airway inflammation and to enhance and accelerate recovery. Furthermore, these findings indicate that Notch signaling in the HDM-mediated airway inflammation model does not only function to counteract inhibitory effects of IFN γ on Th2 differentiation. In addition, the Notch signaling pathway is not only critical for T-cells, but also for other cells involved in allergic airway inflammation, including epithelial cells and DCs⁴¹. Because in endothelial cells the *IL33* gene is a Notch target⁴², SAHM1 treatment may result in impaired IL-33 responses. Of note, IL-33 production by airway epithelial cells plays an important role in allergic airway inflammation⁴³. Notch is a common differentiation signal for T-cell-priming CD11b⁺ DC subsets in the spleen and intestine⁴⁴ and deletion of RBP-J resulted in a reduced capacity of DCs to activate T-cells⁴⁵. Human bronchial epithelial cells treated with diesel exhaust particles generate oxidative stress and upregulate Jagged1 and OX40 ligand in mDCs via TSLP, which resulted in mDC-driven Th2 responses⁴⁶. These findings indicate that environmental factors including air pollution influence Notch signaling. Whereas Notch activation in epithelial cells is sufficient to induce mucous metaplasia, which is a hallmark of asthma, the lack of Notch signaling seems to be beneficial⁴⁷. In this context, Notch and Stat6 signaling operate in parallel and independent pathways to regulate mucous metaplasia⁴⁸. Pharmacological targeting of Notch signaling in epithelial cells, e.g. by SAHM1, may thus be considered in airway diseases associated with mucous metaplasia⁴⁸. It is very well possible that in our HDM-driven allergic airway inflammation model the topical intratracheal application of SAHM1 also beneficially targeted epithelial or endothelial cells. Neutralization of Notch2 reverses established goblet cell formation in an IL-13-induced mouse model of mucus hypersecretion^{41, 49}. Taken together, these effects on airway epithelium could be seen as a bonus of SAHM1 therapy topical in the airways targeting inflammatory cells. Nevertheless, our previous finding that HDM-driven airway inflammation is diminished

in mice with conditional deletion of RBPj exclusively in the T cell lineage demonstrated the critical role of Notch signaling in T cells¹².

Our study has implications for the therapeutic use of peptidomimetic compounds in general. While stapled peptides have been used *in vivo* and several of these are in human clinical testing, this is the first report of topical delivery. This suggests the potential to use this class of inhibitor for localized delivery in asthma and other indications. In conclusion, here we demonstrate that the Notch pathway is important for an *in vivo* type 2 immune response. Importantly, targeting the NICD-MAML-RBPj complex by SAHM1 reduced Th2 inflammation during ongoing HDM challenge and enhanced recovery following established asthma. Therefore SAHM1 may represent a novel therapeutic opportunity to abrogate both airway inflammation and BHR in allergic asthma.

Acknowledgements

The authors like to thank the Erasmus MC Animal Facility (EDC) staff for their assistance. This study was partly supported (R.E.M.) by the V Foundation for Cancer Research and the National Institutes of Health (R00CA175399); (I.T. and M.L.) by the Lung Foundation Netherlands Grants 3.2.12.087 and 3.2.12.067.

References

1. Barnes PJ. Immunology of asthma and chronic obstructive pulmonary disease. *Nat Rev Immunol* 2008; 8:183-92.
2. Lambrecht BN, Hammad H. The immunology of asthma. *Nat Immunol* 2015; 16:45-56.
3. Zhang DH, Cohn L, Ray P, Bottomly K, Ray A. Transcription factor GATA-3 is differentially expressed in murine Th1 and Th2 cells and controls Th2-specific expression of the interleukin-5 gene. *J Biol Chem* 1997; 272:21597-603.
4. Zheng W, Flavell RA. The transcription factor GATA-3 is necessary and sufficient for Th2 cytokine gene expression in CD4 T cells. *Cell* 1997; 89:587-96.
5. Tindemans I, Serafini N, Di Santo JP, Hendriks RW. GATA-3 function in innate and adaptive immunity. *Immunity* 2014; 41:191-206.
6. Amsen D, Blander JM, Lee GR, Tanigaki K, Honjo T, Flavell RA. Instruction of distinct CD4 T helper cell fates by different notch ligands on antigen-presenting cells. *Cell* 2004; 117:515-26.
7. Amsen D, Antov A, Jankovic D, Sher A, Radtke F, Souabni A, et al. Direct regulation of Gata3 expression determines the T helper differentiation potential of Notch. *Immunity* 2007; 27:89-99.

8. Fang TC, Yashiro-Ohtani Y, Del Bianco C, Knoblock DM, Blacklow SC, Pear WS. Notch directly regulates Gata3 expression during T helper 2 cell differentiation. *Immunity* 2007; 27:100-10.
9. Maekawa Y, Tsukumo S, Chiba S, Hirai H, Hayashi Y, Okada H, et al. Delta1-Notch3 interactions bias the functional differentiation of activated CD4+ T cells. *Immunity* 2003; 19:549-59.
10. Napolitani G, Rinaldi A, Bertoni F, Sallusto F, Lanzavecchia A. Selected Toll-like receptor agonist combinations synergistically trigger a T helper type 1-polarizing program in dendritic cells. *Nat Immunol* 2005; 6:769-76.
11. Skokos D, Nussenzweig MC. CD8- DCs induce IL-12-independent Th1 differentiation through Delta 4 Notch-like ligand in response to bacterial LPS. *J Exp Med* 2007; 204:1525-31.
12. Tindemans I, Lukkes M, de Bruijn MJ, Li BW, van Nimwegen M, Amsen D, et al. Notch signaling in T cells is essential for allergic airway inflammation, but expression of the Notch ligands Jagged 1 and Jagged 2 on dendritic cells is dispensable. *J Allergy Clin Immunol* 2017.
13. Bailis W, Yashiro-Ohtani Y, Fang TC, Hatton RD, Weaver CT, Artis D, et al. Notch simultaneously orchestrates multiple helper T cell programs independently of cytokine signals. *Immunity* 2013; 39:148-59.
14. Laky K, Evans S, Perez-Diez A, Fowlkes BJ. Notch Signaling Regulates Antigen Sensitivity of Naive CD4(+) T Cells by Tuning Co-stimulation. *Immunity* 2015; 42:80-94.
15. Maekawa Y, Ishifune C, Tsukumo SI, Hozumi K, Yagita H, Yasutomo K. Notch controls the survival of memory CD4(+) T cells by regulating glucose uptake. *Nature Medicine* 2015; 21:55-61.
16. South AP, Cho RJ, Aster JC. The double-edged sword of Notch signaling in cancer. *Semin Cell Dev Biol* 2012; 23:458-64.
17. Meyer Zu Horste G, Wu C, Wang C, Cong L, Pawlak M, Lee Y, et al. RBPJ Controls Development of Pathogenic Th17 Cells by Regulating IL-23 Receptor Expression. *Cell Rep* 2016; 16:392-404.
18. Tsao PN, Matsuoka C, Wei SC, Sato A, Sato S, Hasegawa K, et al. Epithelial Notch signaling regulates lung alveolar morphogenesis and airway epithelial integrity. *Proc Natl Acad Sci U S A* 2016; 113:8242-7.
19. Gomi K, Staudt MR, Salit J, Kaner RJ, Heldrich J, Rogalski AM, et al. JAG1-Mediated Notch Signaling Regulates Secretory Cell Differentiation of the Human Airway Epithelium. *Stem Cell Rev* 2016; 12:454-63.
20. Cheng P, Zhou J, Gabrilovich D. Regulation of dendritic cell differentiation and function by Notch and Wnt pathways. *Immunological Reviews* 2010; 234:105-19.
21. Radtke F, MacDonald HR, Tacchini-Cottier F. Regulation of innate and adaptive immunity by Notch. *Nat Rev Immunol* 2013; 13:427-37.
22. Amsen D, Helbig C, Backer RA. Notch in T Cell Differentiation: All Things Considered. *Trends Immunol* 2015; 36:802-14.
23. Kang JH, Kim BS, Uhm TG, Lee SH, Lee GR, Park CS, et al. Gamma-secretase inhibitor reduces allergic pulmonary inflammation by modulating Th1 and Th2 responses. *Am J Respir Crit Care Med* 2009; 179:875-82.
24. Lafkas D, Shelton A, Chiu C, de Leon Boenig G, Chen Y, Stawicki SS, et al. Therapeutic antibodies reveal Notch control of transdifferentiation in the adult lung. *Nature* 2015; 528:127-31.
25. Tran IT, Sandy AR, Carulli AJ, Ebens C, Chung J, Shan GT, et al. Blockade of individual Notch ligands and receptors controls graft-versus-host disease. *J Clin Invest* 2013.
26. Guest RV, Boulter L, Dwyer BJ, Kendall TJ, Man TY, Minnis-Lyons SE, et al. Notch3 drives development and progression of cholangiocarcinoma. *Proc Natl Acad Sci U S A* 2016; 113:12250-5.
27. Verdine GL, Hilinski GJ. All-hydrocarbon stapled peptides as Synthetic Cell-Accessible Mini-Proteins. *Drug Discov Today Technol* 2012; 9:e41-e7.

28. Moellering RE, Cornejo M, Davis TN, Del Bianco C, Aster JC, Blacklow SC, et al. Direct inhibition of the NOTCH transcription factor complex. *Nature* 2009; 462:182-8.
29. Ashley JW, Ahn J, Hankenson KD. Notch signaling promotes osteoclast maturation and resorptive activity. *J Cell Biochem* 2015; 116:2598-609.
30. Mathieu M, Cotta-Grand N, Daudelin JF, Thébault P, Labrecque N. Notch signaling regulates PD-1 expression during CD8+ T-cell activation. *Immunol Cell Biol* 2013; 91:82-8.
31. Wolfe MS, Kopan R. Intramembrane proteolysis: Theme and variations. *Science* 2004; 305:1119-23.
32. KleinJan A, Klein Wolterink RG, Levani Y, de Bruijn MJ, Hoogsteden HC, van Nimwegen M, et al. Enforced expression of Gata3 in T cells and group 2 innate lymphoid cells increases susceptibility to allergic airway inflammation in mice. *J Immunol* 2014; 192:1385-94.
33. KleinJan A, Godthelp T, van Toornenenbergen AW, Fokkens WJ. Allergen binding to specific IgE in the nasal mucosa of allergic patients. *J Allergy Clin Immunol* 1997; 99:515-21.
34. Rzepecka J, Siebeke I, Coltherd JC, Kean DE, Steiger CN, Al-Riyami L, et al. The helminth product, ES-62, protects against airway inflammation by resetting the Th cell phenotype. *Int J Parasitol* 2013; 43:211-23.
35. Tindemans I, Lukkes M, de Bruijn JW, Li WS, van Nimwegen M, Amsen D, et al. Notch signaling in T cells is essential for allergic airway inflammation, but expression of Notch ligands Jagged1 and Jagged2 on dendritic cells is dispensable. *J Allergy Clin Immunol* in press.
36. Krug N, Hohlfeld JM, Kirsten AM, Kornmann O, Beeh KM, Kappeler D, et al. Allergen-induced asthmatic responses modified by a GATA3-specific DNase. *N Engl J Med* 2015; 372:1987-95.
37. Tanaka S, Tsukada J, Suzuki W, Hayashi K, Tanigaki K, Tsuji M, et al. The interleukin-4 enhancer CNS-2 is regulated by Notch signals and controls initial expression in NKT cells and memory-type CD4 T cells. *Immunity* 2006; 24:689-701.
38. Okamoto M, Takeda K, Joetham A, Ohnishi H, Matsuda H, Swasey CH, et al. Essential role of Notch signaling in effector memory CD8+ T cell-mediated airway hyperresponsiveness and inflammation. *J Exp Med* 2008; 205:1087-97.
39. Okamoto M, Matsuda H, Joetham A, Lucas JJ, Domenico J, Yasutomo K, et al. Jagged1 on dendritic cells and Notch on CD4+ T cells initiate lung allergic responsiveness by inducing IL-4 production. *J Immunol* 2009; 183:2995-3003.
40. Gu W, Xu W, Ding T, Guo X. Fringe controls naive CD4(+)T cells differentiation through modulating notch signaling in asthmatic rat models. *PLoS One* 2012; 7:e47288.
41. Danahay H, Pessotti AD, Coote J, Montgomery BE, Xia D, Wilson A, et al. Notch2 is required for inflammatory cytokine-driven goblet cell metaplasia in the lung. *Cell Rep* 2015; 10:239-52.
42. Sundlisaeter E, Edelmann RJ, Hol J, Sponheim J, Kuchler AM, Weiss M, et al. The alarmin IL-33 is a notch target in quiescent endothelial cells. *Am J Pathol* 2012; 181:1099-111.
43. Lloyd CM, Saglani S. Epithelial cytokines and pulmonary allergic inflammation. *Curr Opin Immunol* 2015; 34:52-8.
44. Lewis KL, Caton ML, Bogunovic M, Greter M, Grajkowska LT, Ng D, et al. Notch2 receptor signaling controls functional differentiation of dendritic cells in the spleen and intestine. *Immunity* 2011; 35:780-91.
45. Chen YR, Feng F, Wang L, Qu SY, Zhang ZQ, Liu L, et al. Deletion of RBP-J in dendritic cells compromises TLR-mediated DC activation accompanied by abnormal cytoskeleton reorganization. *Mol Biol Rep* 2013; 40:1531-9.
46. Bleck B, Tse DB, Gordon T, Ahsan MR, Reibman J. Diesel exhaust particle-treated human bronchial epithelial cells upregulate Jagged-1 and OX40 ligand in myeloid dendritic cells via thymic stromal lymphopoietin. *J Immunol* 2010; 185:6636-45.
47. Erle DJ, Sheppard D. The cell biology of asthma. *Journal of Cell Biology* 2014; 205:621-31.

- 526 48. Guseh JS, Bores SA, Stanger BZ, Zhou Q, Anderson WJ, Melton DA, et al. Notch signaling
527 promotes airway mucous metaplasia and inhibits alveolar development. *Development* 2009;
528 136:1751-9.
- 529 49. Coote J, Danahay H, Pessotti A, Xia D, Capodiecì P, Doelemeyer A, et al. Therapeutic Notch2
530 Neutralization Reverses an Established Goblet Cell Formation in an IL-13 Mouse Model of Mucus
531 Hypersecretion. *Am J Respir Crit Care Med* 2016; 193:A5889.

532

Legends to figures

Figure 1. SAHM1 abrogates eosinophilic airway inflammation.

(A) Experimental HDM-driven asthma design, showing intratracheal sensitization (s) and challenge (c) of 10 μ g HDM admixed with diluent, 0.3 and 3 μ g SAHM1 (t) or PBS admixed with diluent or 3 μ g SAHM1 as control. Arrows indicated PBS or HDM treatment and SAHM1 or diluent therapy ($\uparrow$). Analyses (a) were performed 4 days after the last challenge. (B) Immunohistochemical Siglec-F staining to identify eosinophils in the indicated representative lung samples. (C) Quantification of flow cytometric analyses of the indicated populations of BAL cells. (D) Intracellular cytokine expression profiles of gated BAL CD3⁺CD4⁺ T-cells. (E) Quantification of flow cytometric analyses of the indicated populations of BAL CD3⁺CD4⁺ T-cells upon 4hrs of PMA/ionomycin stimulation. The results shown represent one out of two independent experiments with 3-6 animals per group. The results shown are expressed as mean values $\pm$ SEM. * p <0.05, ** p <0.01.

Experimental HDM-driven asthma design, showing SAHM1 treatment either during sensitization (s) or during challenge (c) with 10 μ g HDM admixed with diluent or 1 μ g SAHM1 treatment (t), including PBS exposures admixed with diluent or 1 μ g SAHM1 as controls. Analyses (a) were performed 4 days after the last challenge. (E) Quantification of flow cytometric analyses of the indicated populations of BAL cells. Total serum IgE levels measured by ELISA. Results are from one experiment with 3-6 animals per group and are shown as mean values $\pm$ SEM. * p <0.05, ** p <0.01.

Figure 2. SAHM1, but not the control peptide D1 abrogates eosinophilic airway inflammation.

(A) Experimental HDM-driven asthma design, showing intratracheal sensitization (s) and challenge (c) with 10 μ g HDM admixed with diluents, 3 μ g D1 control peptide, or 3 μ g SAHM1. PBS admixed with diluents, 3 μ g D1, or 3 μ g SAHM1 were included as controls. Analysis (a) were performed 4 days after the last challenge. (B) Quantification of flow cytometric analyses of the indicated populations of BAL cells. (C) Cytokine expression

profiles of gated BAL CD3⁺CD4⁺ T-cells upon 4hrs of PMA/ionomycin stimulation. (D) Quantification of flow cytometric analyses of cytokine positive CD4⁺ T-cells. Results are from one experiment with 3-6 animals per group and are shown as mean values $\pm$ SEM. *p<0.05, **p<0.01.

Figure 3. SAHM1, but not D1, reduces key T-cell subset TF expression.

(A) Transcription factor expression profiles of gated BAL CD3⁺CD4⁺ T-cells. (B) Quantification of intracellular flow cytometric analyses of the indicated populations of BAL T-cells. Results are from one experiment with three (PBS) or six mice (HDM-exposed) per group and are shown as mean values $\pm$ SEM. *p<0.05, **p<0.01.

Figure 4. SAHM1 treatment abrogates airway BHR.

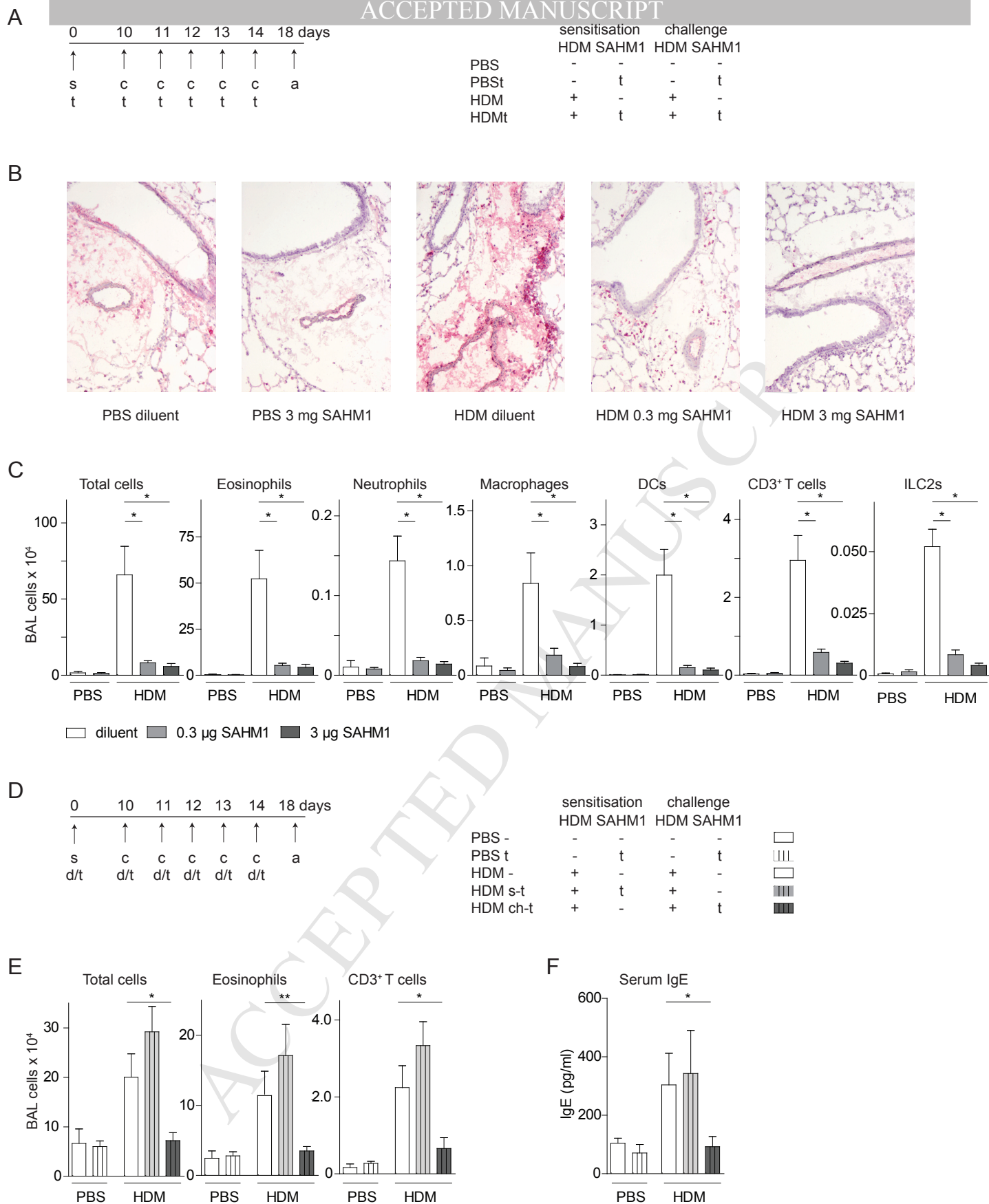
(A) Experimental HDM-driven asthma design, showing intratracheal sensitization (s) and challenge (c) with 10 μ g HDM, admixed with either diluent or 1 μ g SAHM1. PBS admixed with either diluent or 1 μ g SAHM1 served as controls. Analyses (a) were performed four days after the last challenge in the indicated mouse groups. (B) Bronchial hyperresponsiveness measurement (Lr= lung resistance) with Buxco in the indicated mouse groups. Results are from one experiment with four (PBS) or eight mice (HDM-exposed) per group and are expressed as mean values $\pm$ SEM. *p<0.05, **p<0.01.

Figure 5. SAHM1 improves recovery from allergic airway inflammation

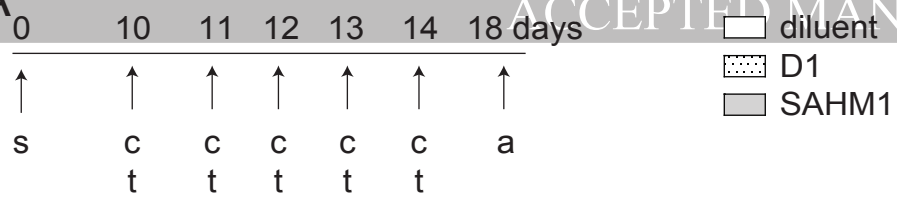
(A) Experimental HDM-driven asthma design, showing intratracheal sensitization (s) and challenge (c) with HDM during the first interval (day 10-14) to induce asthma. During the second interval (day 15-19) HDM was given (ongoing inflammation) or PBS (resolution phase) with either 1 μ g SAHM1 treatment (t) or diluent as a control. Analyses (a) were performed four days after the last challenge in the indicated mouse groups. (B) Quantification of flow cytometric analyses of the indicated populations of BAL cells during ongoing inflammation and during resolution. Results are from one experiment with 3-6 animals per group and are shown as mean values $\pm$ SEM. *p<0.05, **p<0.01.

Figure 6. SAHM1 treatment suppresses α -IFN γ induced asthma exacerbation

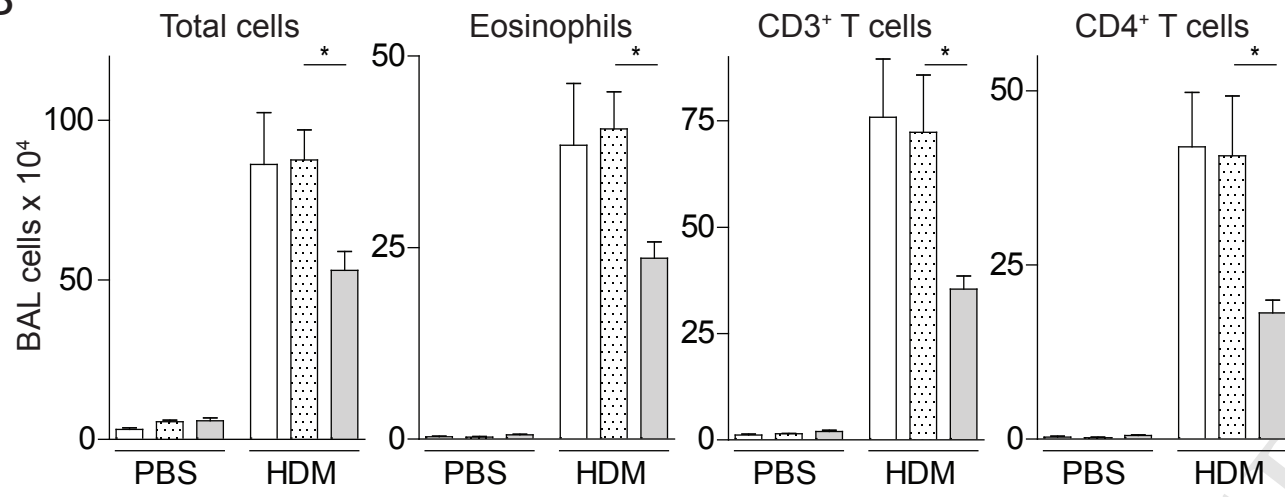
(A) Experimental HDM-driven asthma design, showing intratracheal sensitization (s) and challenge (c). During challenge HDM was admixed either with 150 μ g control rat Ig or α -IFN γ (to exacerbate asthma), and diluent or 3 μ g SAHM1. Analyzes (a) were performed four days after the last challenge in the indicated mouse groups. (B) Immunohistochemical Siglec-F staining to identify eosinophils in the indicated representative lung samples. (C) Quantification of flow cytometric analyses of the indicated populations of BAL fluid. Cells were significant lower for PBS (statistical not indicated in figure). (D) Total CD4⁺ T-cell number in BAL fluid (*left*) and pie charts of CD4⁺ T-cells, classified as Th2 (IL-4), Th1 (IFN γ) and Th17 (IL-17) cells (*right*). (E) Transcription factor expression profiles of gated BAL CD3⁺CD4⁺ T-cells. (F) Quantification (E) of flow cytometric analyses of the indicated populations of TF factor expressing BAL T-cells in the indicated experimental mouse groups. The results shown are from one experiment with 3-6 animals per group and are shown as mean values $\pm$ SEM. *p<0.05, **p<0.01.



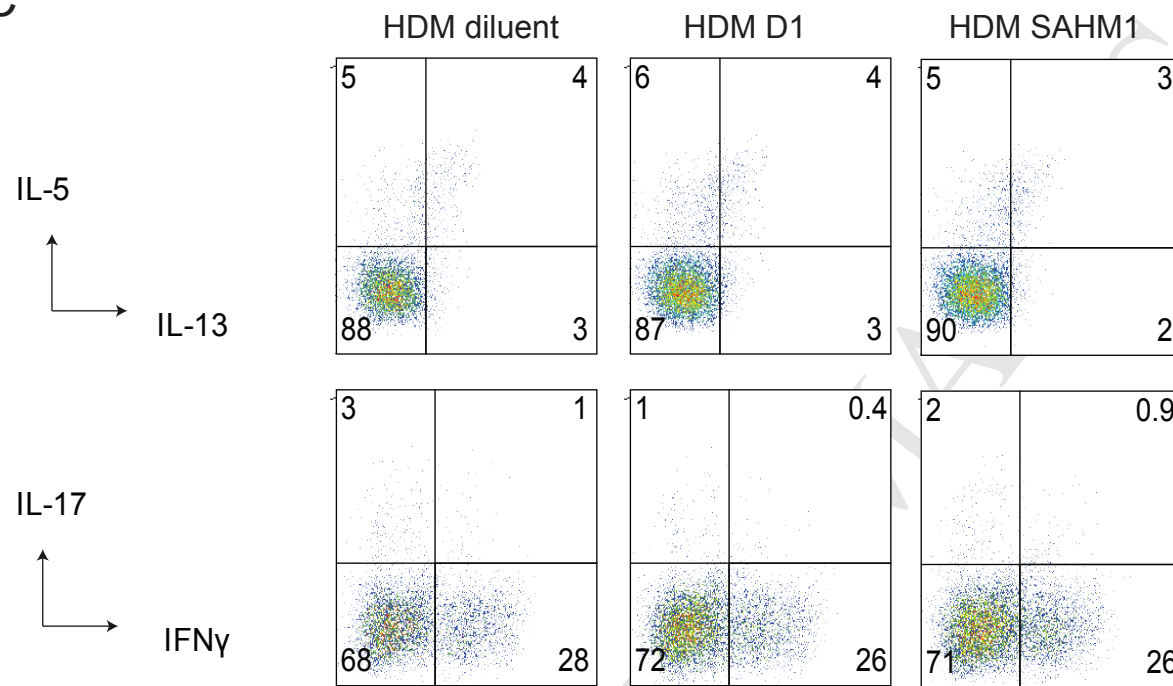
A



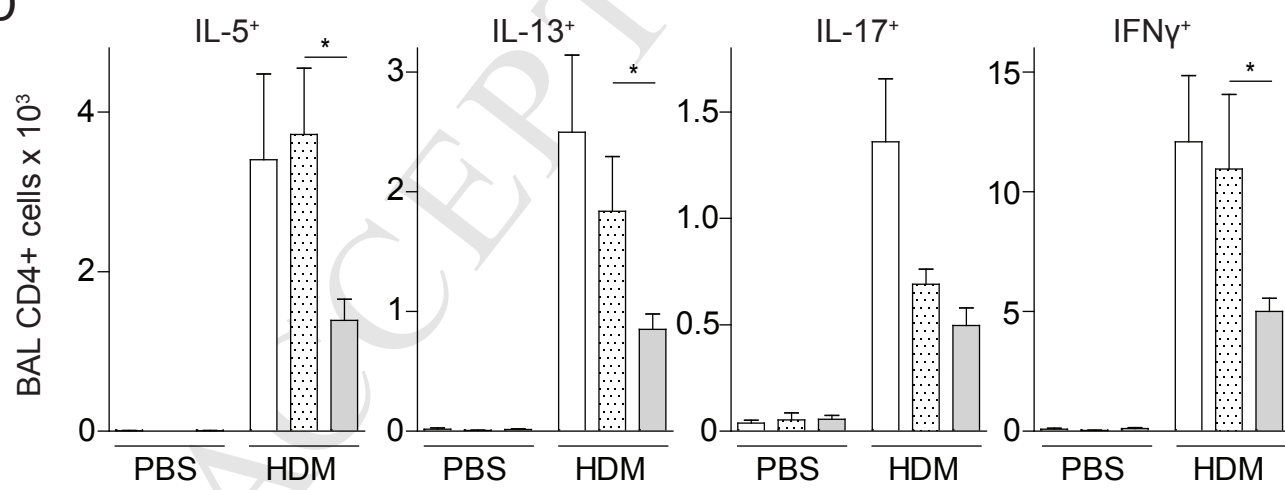
B



C

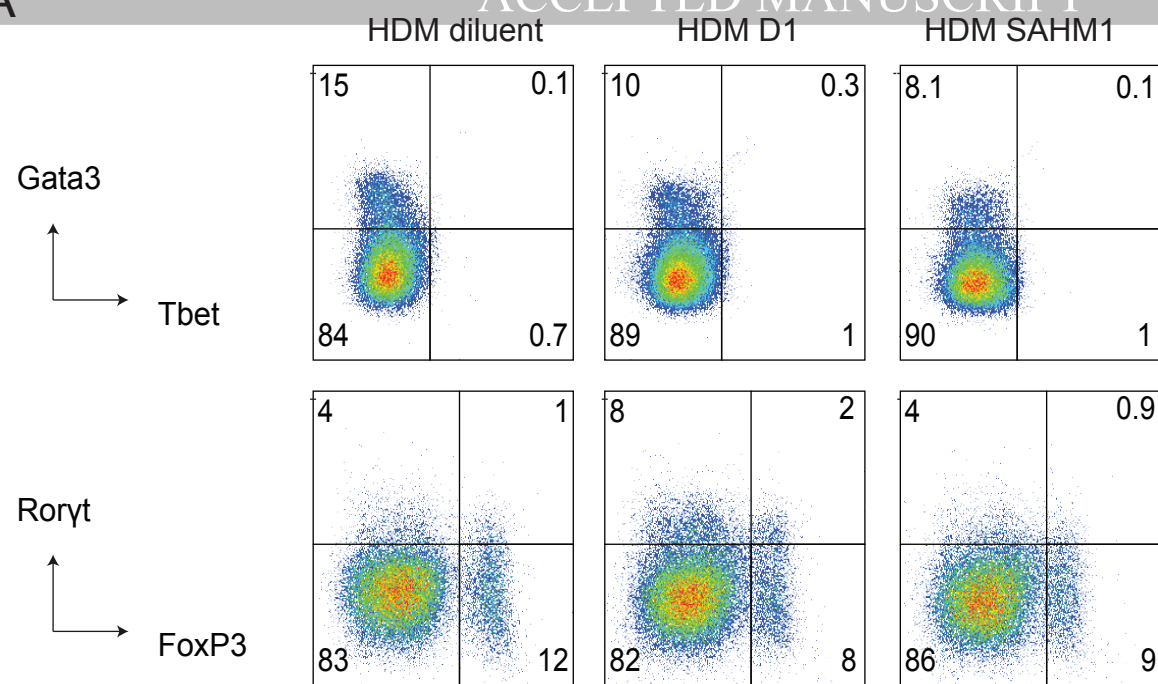


D

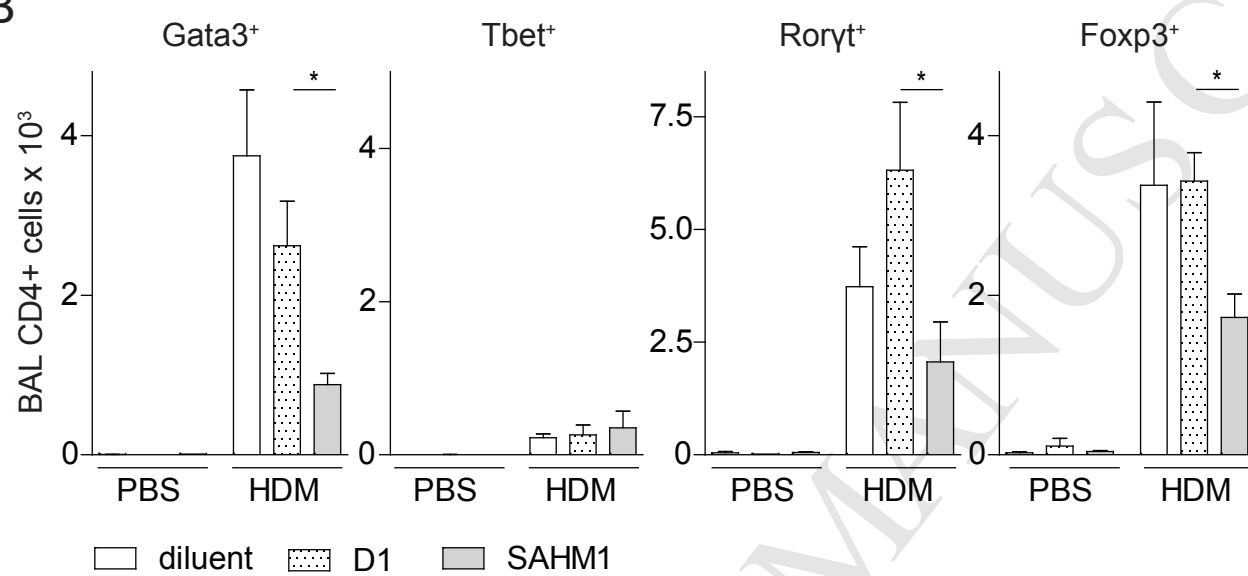


A

ACCEPTED MANUSCRIPT



B

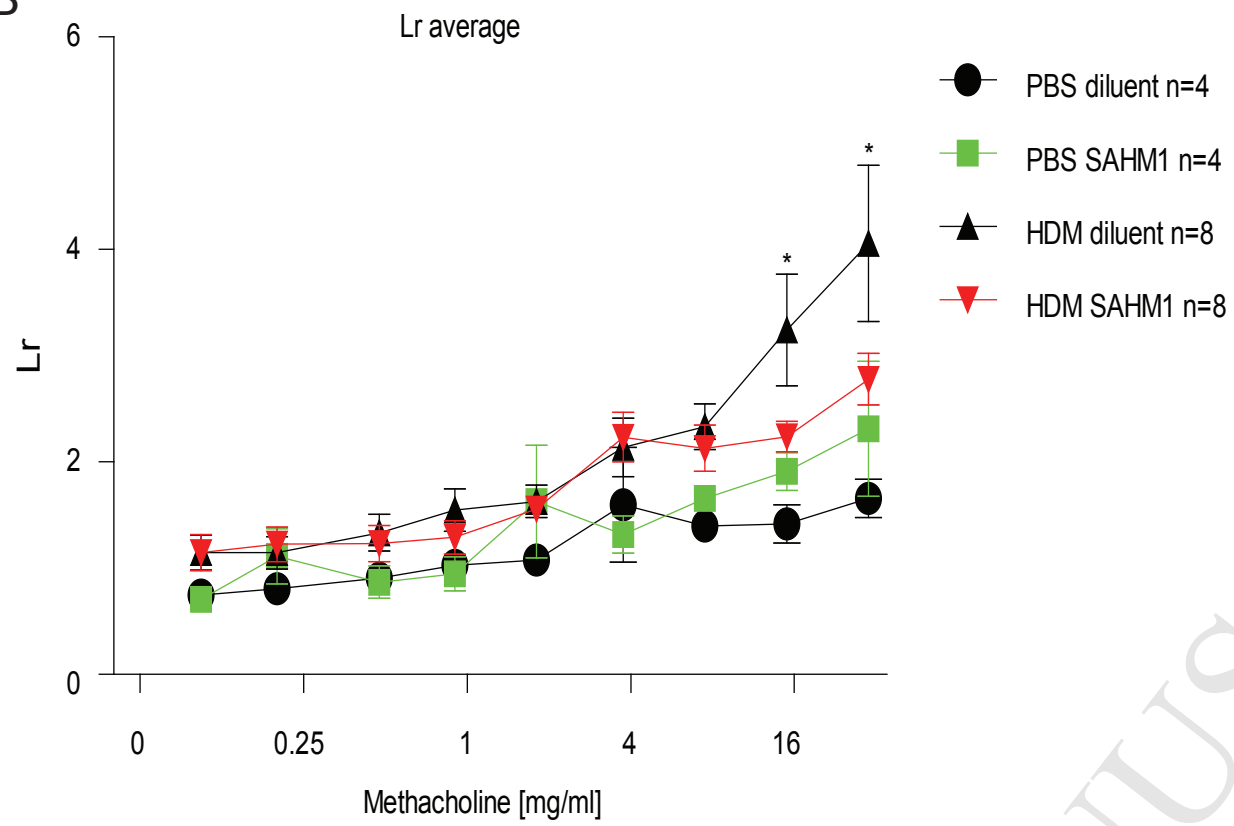


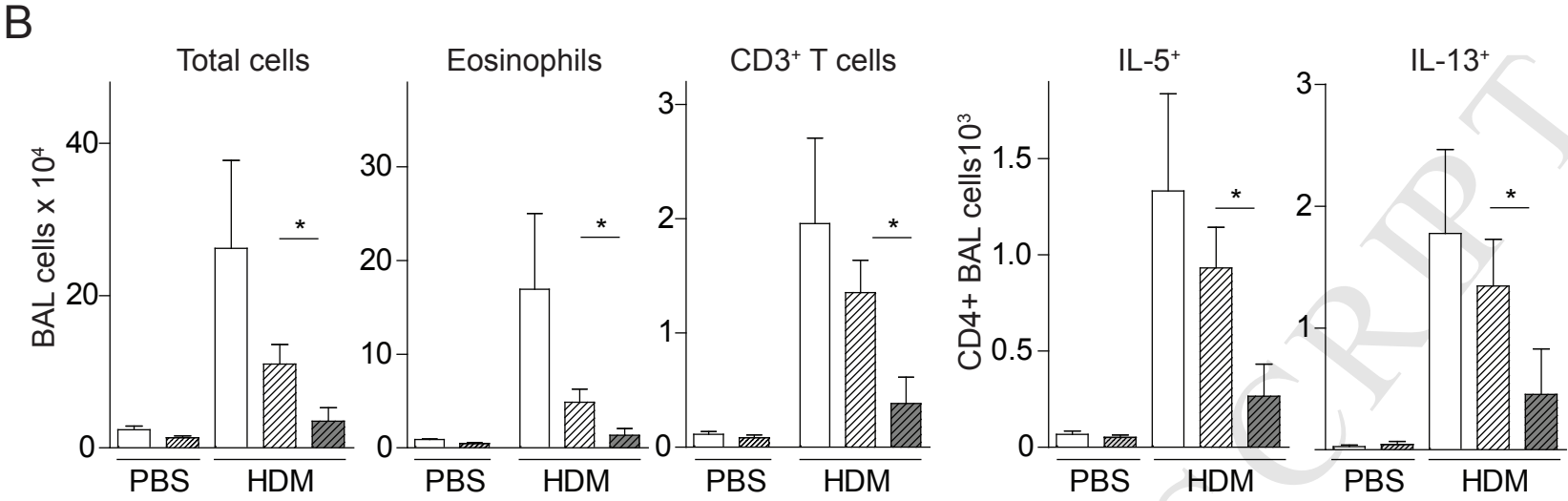
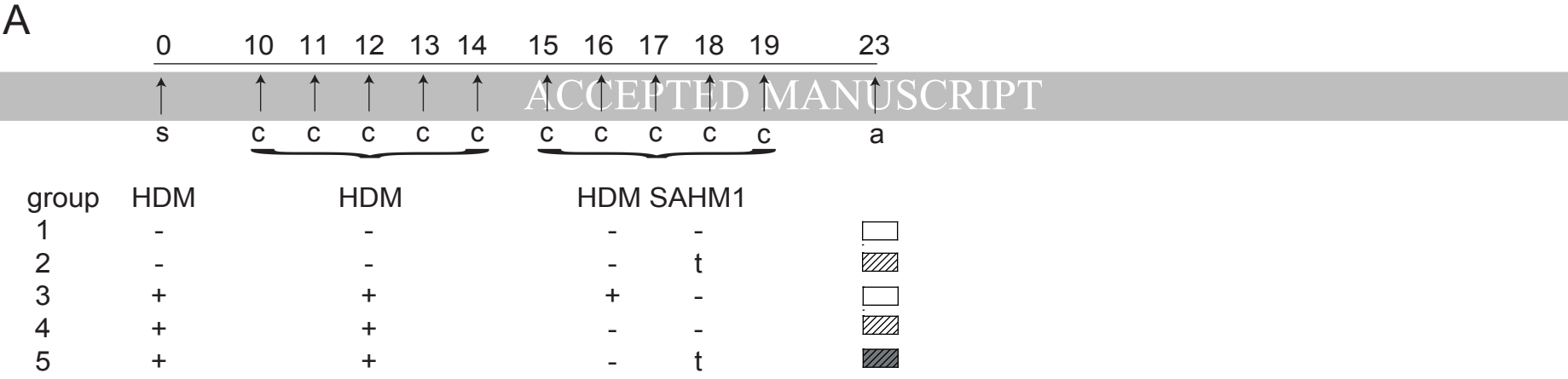
A

0 10 11 12 13 14 18 days
 ↑ ↑ ↑ ↑ ↑ ↑ ↑
 s c c c c c a
 t t t t t t

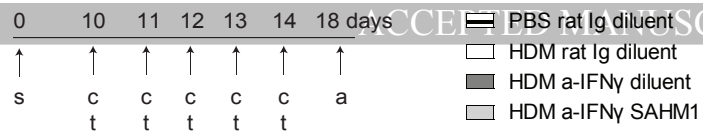
ACCEPTED MANUSCRIPT

B

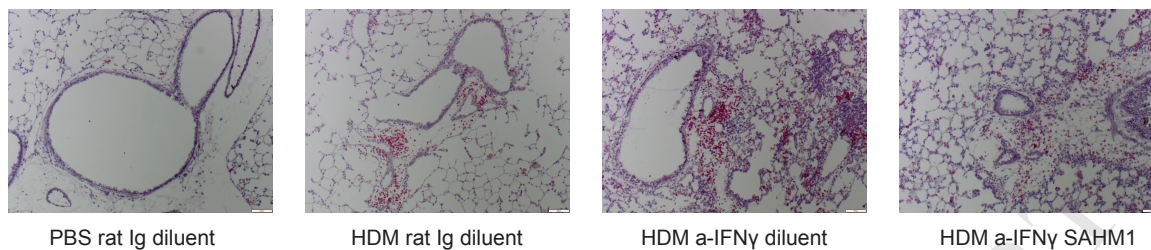




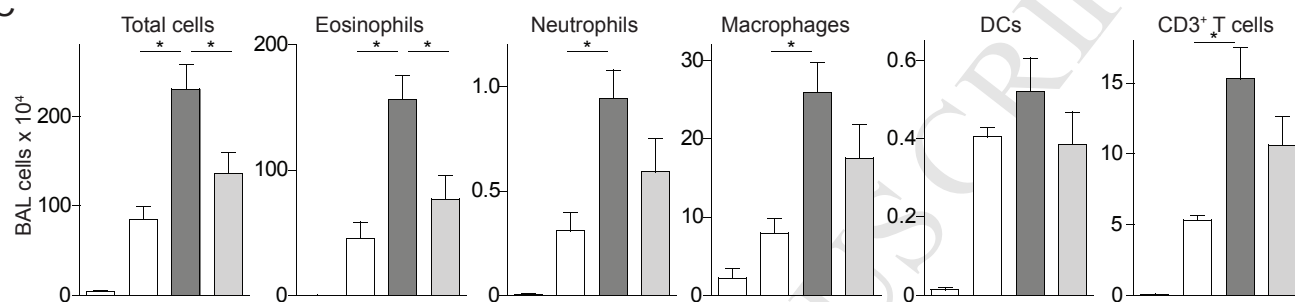
A



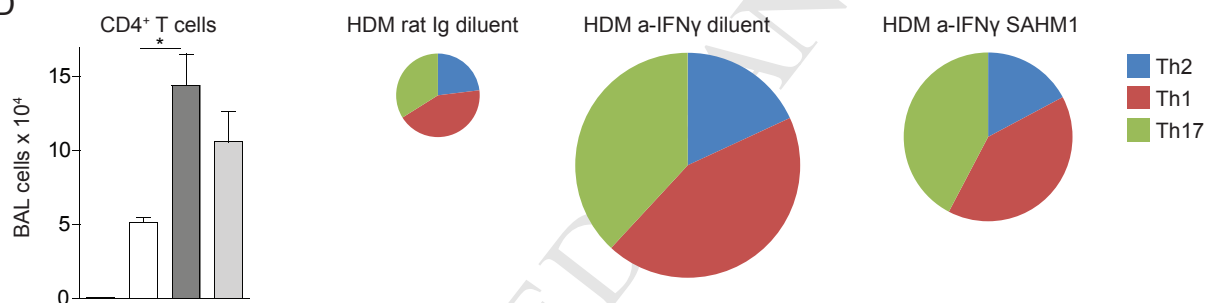
B



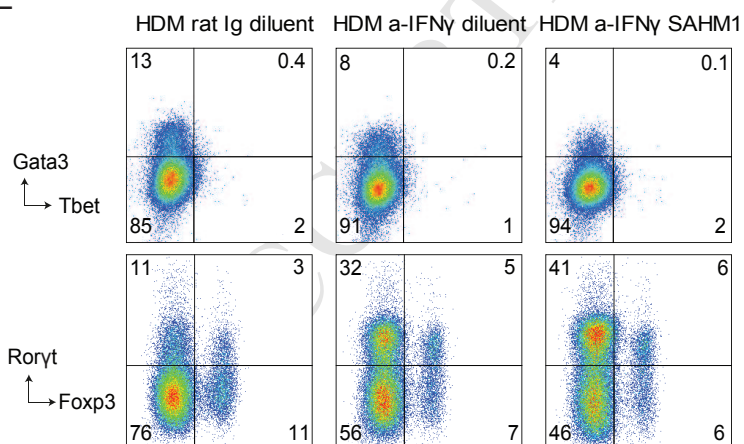
C



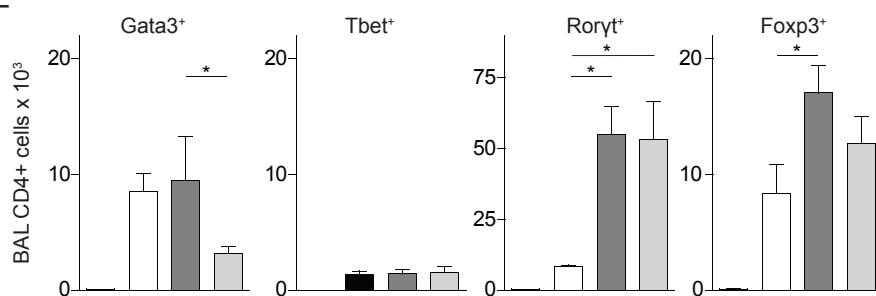
D



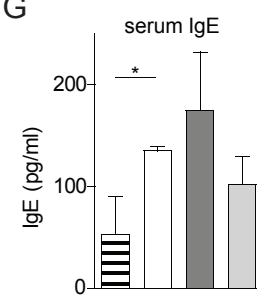
E



F



G



Supplementary Material and Methods and Results

The Notch Pathway Inhibitor SAHM1 Abrogates the Hallmarks of Allergic Asthma

Alex KleinJan PhD¹, Irma Tindemans MSc¹, Jeffrey E. Montgomery^{2,3}, Melanie Lukkes BSc¹, Marjolein J.W. de Bruijn BSc¹, Menno van Nimwegen BSc¹, Ingrid Bergen BSc¹ Raymond E. Moellering PhD^{2,3}, Henk C. Hoogsteden MD PhD¹, Louis Boon PhD⁴, Derk Amsen PhD⁵ and R.W. Hendriks PhD¹

¹Department of Pulmonary Medicine, Erasmus MC Rotterdam, PO Box 2040, NL 3000CA Rotterdam, The Netherlands; ²Department of Chemistry and ³Institute for Genomics and Systems Biology, University of Chicago, Chicago, IL 60637 USA; ⁴Epirus Biopharmaceuticals Netherlands Yalelaan 46, 3584 CM Utrecht, The Netherlands and ⁵Sanquin, Plesmanlaan 125, 1066 CX Amsterdam, The Netherlands

Correspondence to:

Alex KleinJan PhD, Department of Pulmonary Medicine, Room Ee2251, Erasmus MC Rotterdam, PO Box 2040, NL 3000 CA Rotterdam, The Netherlands,
e-mail: a.kleinjan@erasmusmc.nl, phone: ++31-10-7043698, fax: ++31-10-7044728

Supplementary Material and Methods

Inhibitor studies with SAHM1 and SAHM1-D1 (D1)

SAHM1 peptide was purchased from MerckMillipore, whereas SAHM1-D1 (D1) was synthesized as previously described²⁶. Both compounds were stored at -20°C as a dry powder and resuspended in DMSO (25 mg/ml) and stored until use at 4°C.

Restrained whole body plethysmography (Buxco) invasive measurement airway function (resistance (RL))

To investigate if SAHM1 is effective during challenge to improve lung function, mice were treated during HDM challenge by admixing SAHM1 1 µg (25 mg/ml in DMSO) (4-8 mice/groups). Next mice were anesthetized by intraperitoneal injection of 200 µl 10% Urethane in PBS, followed by another 200 µl i.p. after 10 minutes and animals above 18 grams weight another dose of 100 µl. The animals were ventilated (O₂/air (1:2) 0.6 l/min) at a frequency of 180 strokes/min (TV = 0.18 – 0.20 mL). A trachea cannula was placed in the mice for the measurement of pulmonary resistance (RL). The cannula was connected to a pressure transducer fixed on the box (EMKA Technologies, Paris, France) and transpulmonary pressure was determined by measuring pressure differences in the box. Airflow and tidal volume were determined using a flow transducer fixed to the body box that measured flow differences inside the box. During 20 seconds, increasing doses of methacholine (acetyl-β-methyl-choline chloride, Sigma) (0–30 mg/ml) aerosols were administered by aerosol generated in a nebulizer (EMKA Technologies, Paris, France) connected in between the animal in the body box and the ventilator (EMKA Technologies, Paris, France). First, a baseline pulmonary resistance (RL) was measured for 3 min followed by a saline exposure and measurement and finally increasing methacholine doses and measurements. Data are presented as average RL in cm H₂O/ml/s (4-8 mice/groups).

Serum IgE measurement with ELISA

Serum IgE levels were determined using commercial ELISA kit for IgE (BD Biosciences).

Supplementary Results

SAHM1 therapy on Innate activation with HDM or with IL-33 treatment

To investigate if SAHM1 is effective during an innate response mice were treated with HDM 100 μ g or with IL-33 (0,25 μ g at days 0, 2 and 3) in case mice Innate lymphoid cells type 2 (ILC2s) were studied and got SAHM1 therapy (Li et al EurJI 2016). SAHM1 therapy 1 μ g (25 mg/ml in DMSO) was given daily intranasal from 2 days before until the mice got 100 μ g HDM(3-4 mice/groups). The effect of SAHM1 on IL-33 dependent inflammation was investigated when mice got SAHM1 1 μ g (25 mg/ml in DMSO) (3-4 mice/groups) therapy. Mice got therapy by intranasal SAHM1 1 μ g from day -1 till day 3. Twenty-four hrs after the last IL-33 dose mice were sacrificed and BAL cells were taken as described before.

SAHM1 is not effective during innate responses

Next we investigated if there is additional effect of SAHM1 on the role of innate cells of the immune system. Upon 100 μ g HDM intratracheal stimulation mice we see a clear influx in neutrophils and CD8 T-cells in the PBS group and a lower number of DCs in the PBS group when compared to HDM treated mice (supplementary figure 2). In addition analyzed BAL-cells, lungs-cells and MLN-cells regarding the presence of DC subsets and their activation status. We observed that there are slightly more DCs in the BAL when mice were treated with SAHM1. In the lung no difference could be observed between different therapies in the HDM treated mice. In the MLN we observed a trend (not significant for oneway anova Kruskal-Wallis test) that there were less DC when mice were treated with SAHM1 when compared to diluent (DMSO or to D1). Next we analyzed the activation status of the DCs and DC subsets by the expression of CD86 and MHCII. We observed a tiny lower CD86 expression on BAL cDCs and on BAL

CD103 DCs and on MLN moDCs. In addition we observed a tiny lower MHCII expression in MLN cDCs and moDCs. Regarding the numbers there are no clear differences the data suggest that DCs are slightly more present in the alveolar space when mice were treated with SAHM1 compared to control treatment. However the MLN DCs numbers tended to be lower. The effects of SAHM1 on DCs during primary immune response seem to be too weak to visualize in either number or the expression of costimulatory molecules as measured by CD86 to have an effect on priming of the immune system (supplementary figure 4 and 5). Next we investigated the effect of SAHM1 on the epithelial derived IL-33 driven alveolar inflammation supplementary figure 5. Here we didn't see any effect of SAHM1 therapy on eosinophilic inflammation driven by IL-33. These experiments suggest that SAHM1 is mainly effective during the adaptive immune response and showed no effect during a primary immune response.

No impaired OVA TCR specific T-cell proliferation by SAHM1

We investigated the effect of SAHM1 on the primary immune response by the use of OVA in the OTII-T-cell response where we analyzed the T-cell response in relation to cell division and restimulation of the MLN-cells with OVA. This experiment could not show any differences between cell proliferation of the OVA specific TCR positive cells in mice that got SAHM1 therapy or control therapy indicating no significant effect on sensitization (data not shown). OVA restimulation of the adoptive transferred OVA specific OTII T-cells obtained from the lung draining lymph node (MLN) indicates a reduced level of IFN γ (Suppl figure 7) when mice were treated with SAHM1 compared to D1 and diluent. Levels of IL4, IL-5 and IL-13 were below detection level (data not shown). This result indicates that during restimulation the Notch signaling in T-cells is important in the context of cell cell contact and maintenance.

Supplementary legends

Figure S1

Gating strategy of BAL cells.

Based on forward and sideward scatter debris was excluded. Next single cells were selected and finally, based on granulation SSC and FSC, granulocytes, lymphocytes and macrophages were gated. The granulocytes gate was subdivided in eosinophils (eo), neutrophils (neutro), and high autofluorescent (Fitc) siglec F positive Alveolar macrophages (AM). Lymphocytes were subdivided in MHC negative CD3 T-cells and MHCII positive cells. T-cells were subdivided in CD4 and CD8 positive cells. MHCII positive lymphocytes were subdivided in CD11c positive dendritic cells (DCs) and CD19 positive B-cells. Alveolar macrophages (AM) are siglec F positive and show high Fitc autofluorescence.

Figure s2

Reduced cytokine expression after SAHM1 therapy in HDM treated mice

Intracellular cytokine expression profiles of gated BAL CD3⁺CD4⁺ T-cells (A) Quantification of flow cytometric analyses of the indicated populations of BAL CD3⁺CD4⁺ T-cells upon 4hrs of PMA/ionomycin stimulation (B). The results shown represent one out of two independent experiments with 3-6 animals per group. The results shown are expressed as mean values $\pm$ SEM. *p<0.05, **p<0.01.

Figure s3

Quantification of innate BAL cells after i.t. HDM treatment

Quantification of flow cytometric analyses of the indicated populations of BAL cells. The results shown represent one experiments with 3-4 mice per group. The results shown are expressed as mean values $\pm$ SEM. *p<0.05.

Figure s4

DC subsets numbers in BAL, Lung and MLN

Quantification of flow cytometric analyses of DC subsets BAL cells. The results shown represent one experiments with 3-4 mice per group. The results shown are expressed as mean values $\pm$ SEM. *p<0.05.

Figure s5

CD86 and MHCII expression by DC subsets in BAL, Lung and MLN

Expression levels of CD86 by DC subsets obtained from BAL, Lung cells and MLN cells. Histograms were performed from concatenate samples of each group with 3-4 mice per group.

Figure s6

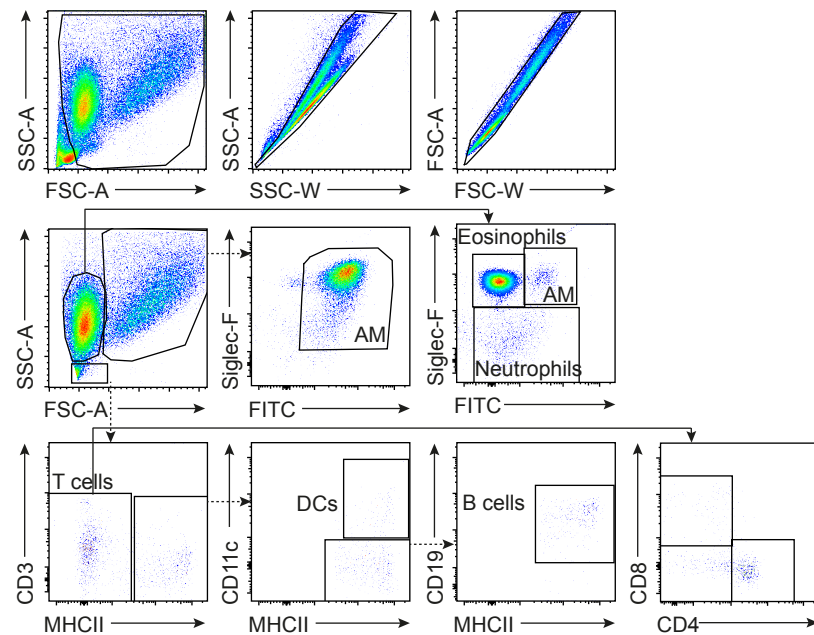
SAHM1 was unable to abrogate IL-33 induced eosinophilic inflammation

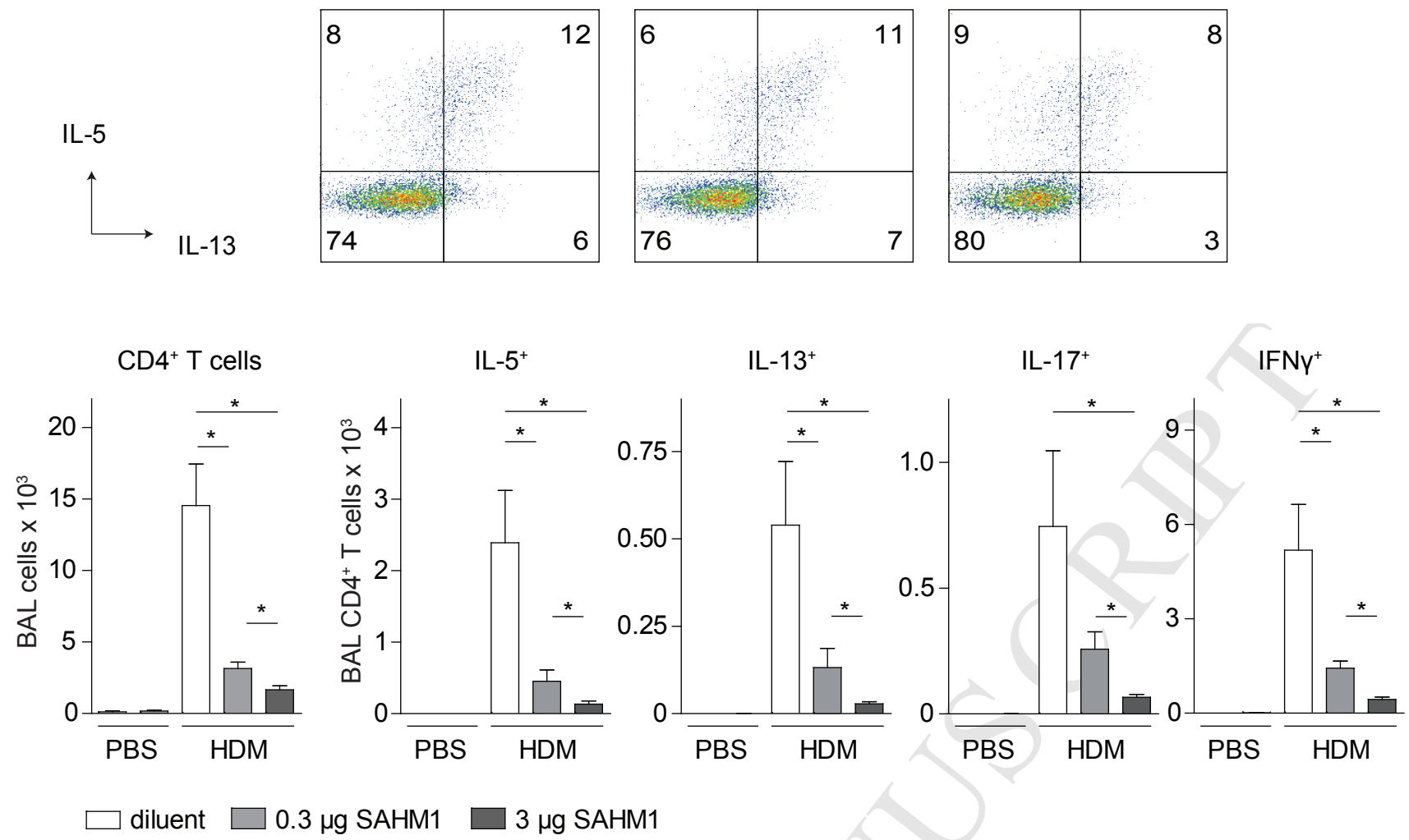
Quantification of the total BAL cells and flow cytometric analyses of BAL eosinophils. The results shown represent one experiments with 4 mice per group. The results shown are expressed as mean values $\pm$ SEM. * $p < 0.05$.

Figure s7

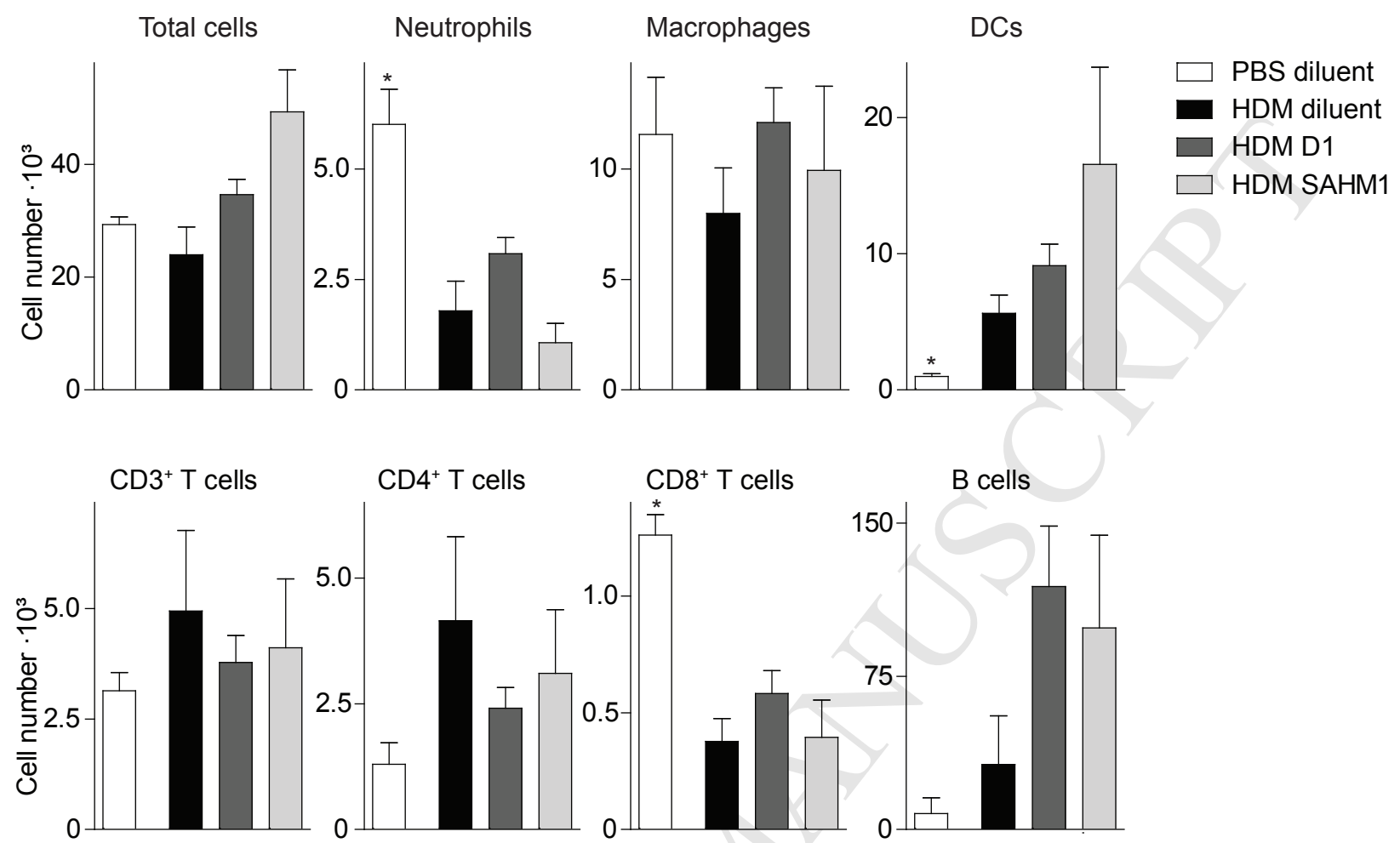
Reduced IFN γ levels in OVA restimulated MLN cells obtained from SAHM1 treated mice.

IFN γ levels in supernatants from OVA restimulated MLN cells. The results shown represent one experiments with 4 mice per group. The results shown are expressed as mean values $\pm$ SEM. * $p < 0.05$.

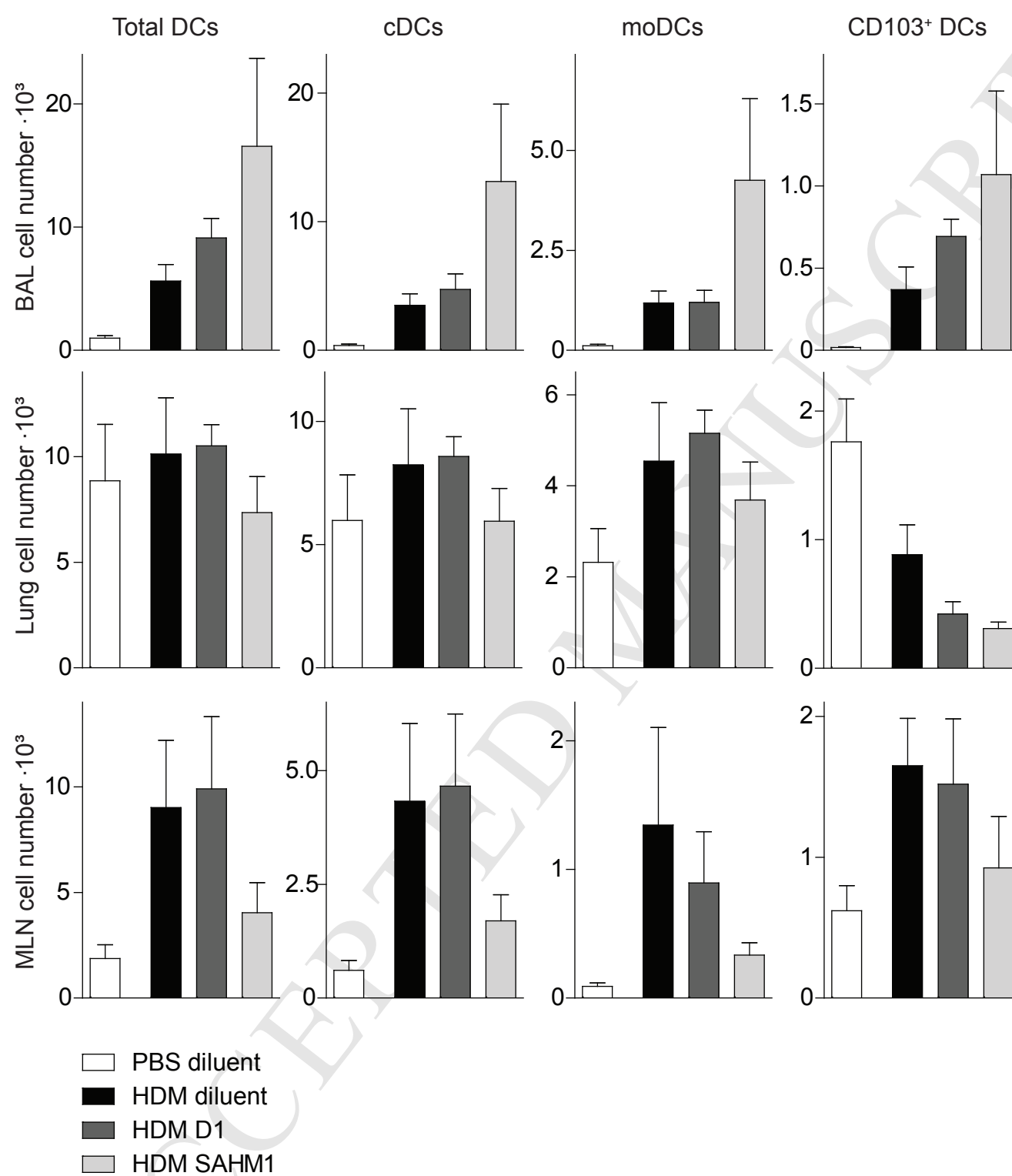
KleinJan *et al* - Supplementary figure 1

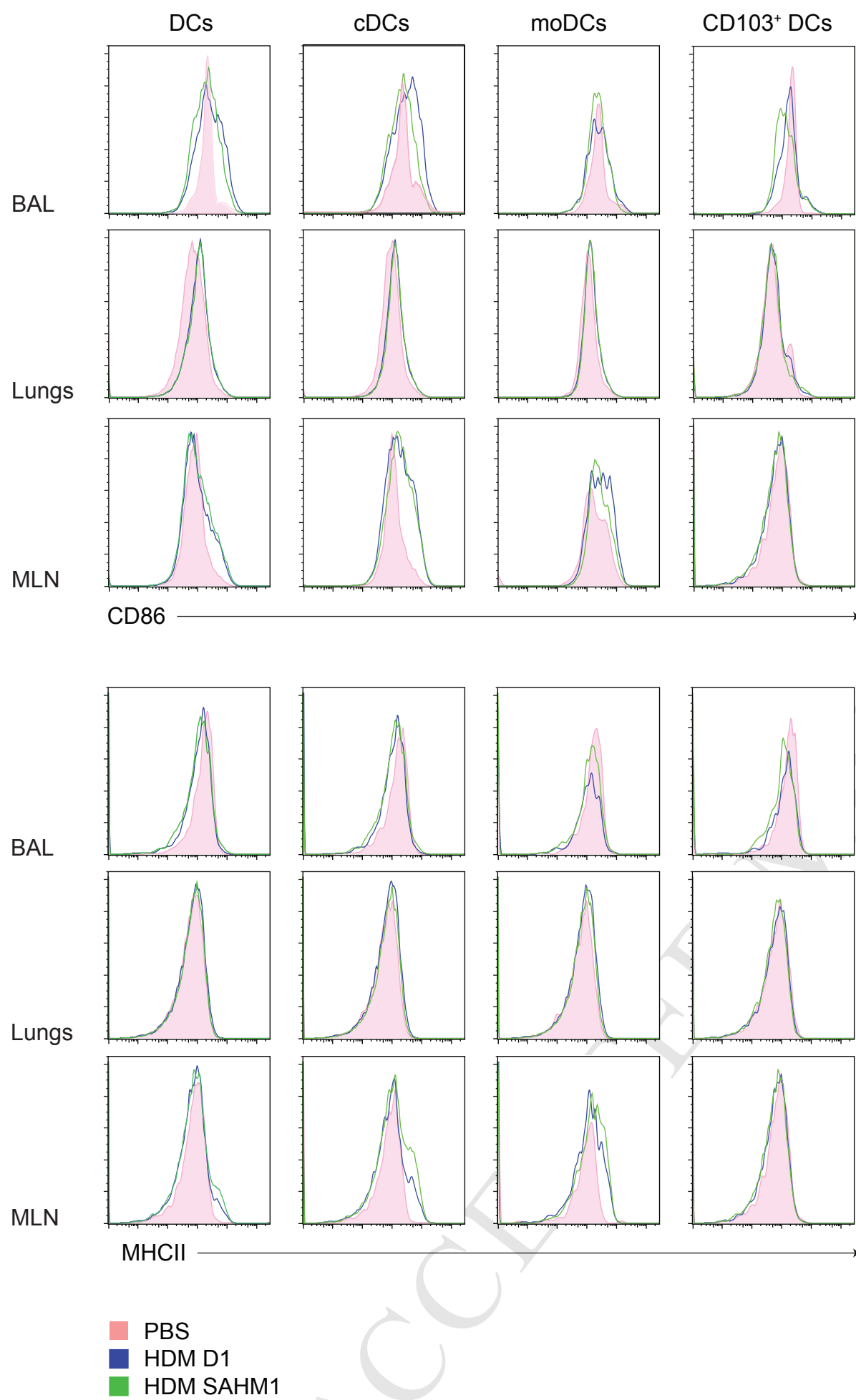


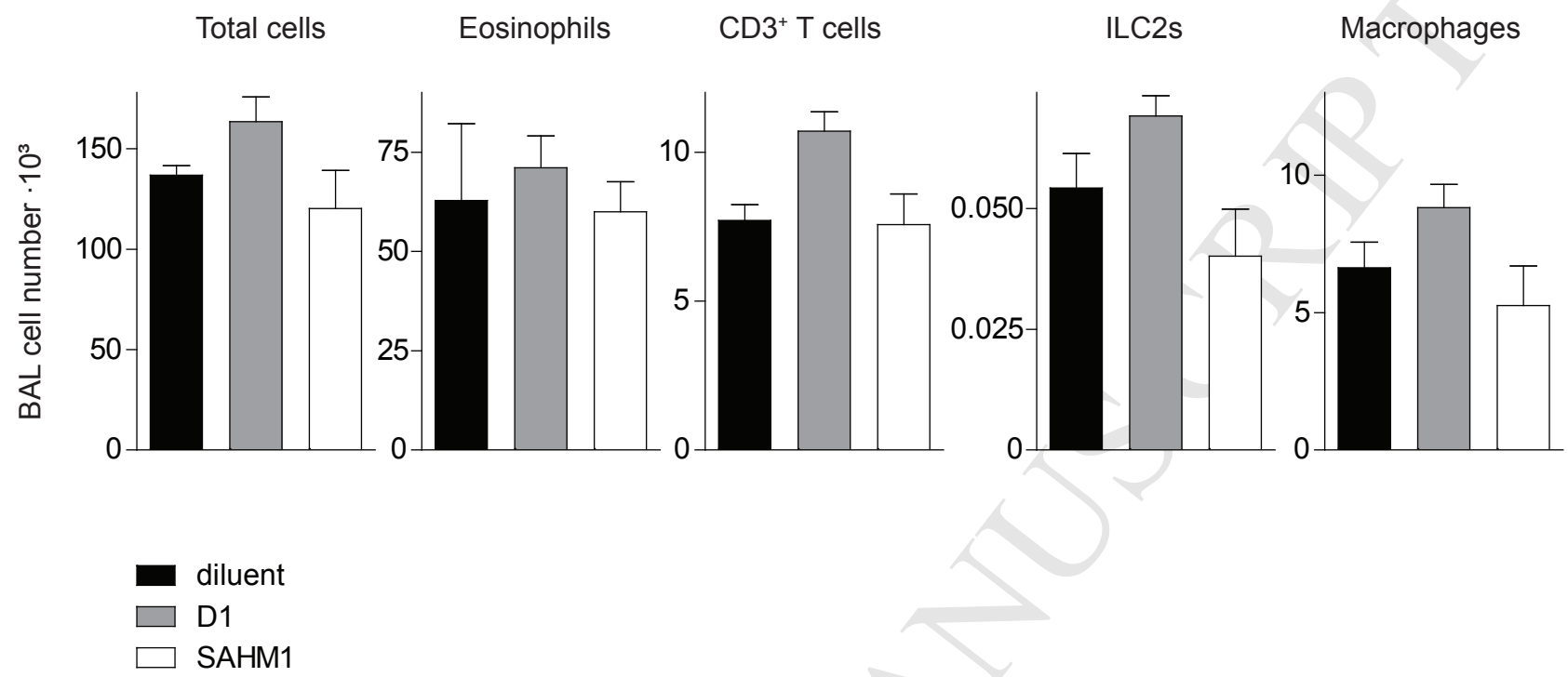
KleinJan *et al* - Supplementary figure 2



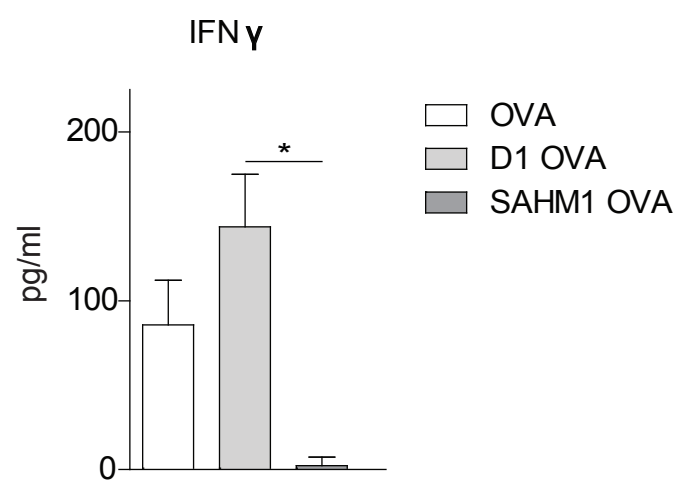
KleinJan et al - Supplementary figure 3







KleinJan et al - Supplementary figure 6



KleinJan et al - Supplementary figure 7