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PII: S0091-6749(21)00173-1

DOI: <https://doi.org/10.1016/j.jaci.2021.01.026>

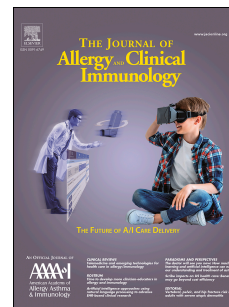
Reference: YMAI 14961

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

Received Date: 17 August 2020

Revised Date: 16 January 2021

Accepted Date: 22 January 2021



Please cite this article as: Branchett WJ, Cook J, Oliver RA, Bruno N, Walker SA, Stölting H, Mack M, O'Garra A, Saglani S, Lloyd CM, Airway macrophage-intrinsic TGF- β 1 regulates pulmonary immunity during early life allergen exposure, *Journal of Allergy and Clinical Immunology* (2021), doi: <https://doi.org/10.1016/j.jaci.2021.01.026>.

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Airway macrophage-intrinsic TGF- β 1 regulates pulmonary immunity during early life allergen exposure

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Conflicts of interest

The authors declare that they have no financial or commercial conflicts of interest.

Funding statement

This work was supported by Wellcome Trust Senior Fellowship 107059/Z/15/Z to C.M.L.

A.O.G. is employed by The Francis Crick Institute, which receives its core funding from

- 33 Cancer Research UK (FC001126), the UK Medical Research Council (FC001126), and the
34 Wellcome Trust (FC001126).

Abstract

Background

Early life represents a major risk window for asthma development. However, the mechanisms controlling the threshold for establishment of allergic airway inflammation in early life are incompletely understood. Airway macrophages (AMs) regulate pulmonary allergic responses and undergo TGF- β -dependent postnatal development, but the role of AM maturation factors such as TGF- β in controlling the threshold for pathogenic immune responses to inhaled allergens remains unclear.

Objective

Test the hypothesis that AM-derived TGF- β 1 regulates pathogenic immunity to inhaled allergen in early life.

Methods

Conditional knockout (*Tgfb1* ^{Δ CD11c}) mice, with TGF- β 1 deficiency in AMs and other CD11c⁺ cells, were analysed throughout early life and following neonatal house dust mite (HDM) inhalation. The roles of specific chemokine receptors were determined using *in vivo* blocking antibodies.

Results

AM-intrinsic TGF- β 1 was redundant for initial population of the neonatal lung with AMs, but AMs from *Tgfb1* ^{Δ CD11c} mice failed to adopt a mature homeostatic AM phenotype in the first weeks of life. Evidence of constitutive TGF- β 1-signalling was also observed in paediatric human AMs. TGF- β 1-deficient AMs expressed enhanced levels of monocyte-attractant chemokines and, accordingly, *Tgfb1* ^{Δ CD11c} mice exposed to HDM throughout early life accumulated CCR2-dependent inflammatory CD11c⁺ mononuclear phagocytes into the airway niche that expressed the pro-allergic chemokine CCL8. *Tgfb1* ^{Δ CD11c} mice displayed augmented Th2, ILC2 and airway remodelling responses to HDM, which were ameliorated by blockade of the CCL8 receptor, CCR8.

Conclusion

62 Our results highlight a causal relationship between AM maturity, chemokines and pathogenic
63 immunity to environmental stimuli in early life and identify TGF- β 1 as a key regulator of this.

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Capsule Summary

TGF- β 1 expression from CD11c⁺ cells governs early life airway macrophage phenotype and restricts inflammatory mononuclear phagocyte and chemokine responses to inhaled house dust mite to limit allergic airway disease in a neonatal mouse model.

Key Messages

- CD11c⁺ MP-derived TGF- β 1 regulates AM maturation and CCR2-dependent inflammatory MP responses to inhaled HDM allergen in early life.
- HDM-treated *Tgfb1* ^{Δ CD11c} mice develop heightened allergic airway disease, dependent on the CCL8 receptor, CCR8.

Keywords

Asthma; type 2 immunity; neonate; TGF- β ; lung immunity; macrophage; monocyte; chemokine

Abbreviations

T2, type 2; AM, airway macrophage; AAD, allergic airway disease; HDM, house dust mite; IM, interstitial macrophage; BAL, bronchoalveolar lavage; MP, mononuclear phagocyte; P, postnatal day; ILC, innate lymphoid cell.

Introduction

The immune system is highly dynamic in early life^{1, 2} and distinct from that in adults. Exposure to airborne pathogens and aeroallergens typically occurs rapidly after birth, yet neonatal immunity is characterised by relatively weak innate responses to infection³ and a type 2 (T2) immune bias⁴. This neonatal period therefore represents a major risk window for allergic sensitisation and development of asthma, a disease that is very common in childhood^{4, 5}. The distinct immunological landscape in early life necessitates greater understanding of how specific features of the developing neonatal lung influence allergic immunity and development of asthma⁵.

The neonatal period is crucial for the seeding and maturation of resident airway macrophages (AMs). AMs are phenotypically distinct lung-resident macrophages, which differentiate from foetal precursors in the first week of life and, once matured, are marked by high expression of CD11c and, in mice, Siglec F, along with pan-macrophage markers such as F4/80 and CD64, but little to no steady state CD11b expression⁶. This profile distinguishes AMs from lung interstitial macrophages (IMs), which express the macrophage markers CD11b⁺, F4/80⁺ and CD64⁺, but lack Siglec F and express little to no CD11c⁷.

AMs are important sentinels of pulmonary homeostasis⁸ and AM depletion in adult mice enhances experimental allergic airway disease (AAD) severity, consistent with an intrinsic capacity of AMs to dampen allergic responses^{9, 10}. However, AMs also produce mediators associated with asthma pathogenesis in patients and experimental AAD¹¹⁻¹⁵, while dysfunctional AMs can drive T2 immunity and pathogenic tissue repair responses to house dust mite (HDM) allergen¹². Although prenatally-derived AMs self-renew in adulthood¹⁶, inflammatory monocytes can differentiate into AMs during allergic inflammation, influenza infection and bleomycin lung injury in adult mice¹⁷⁻¹⁹ and gradually replace prenatally-derived AMs in the steady state with advancing age^{20, 21}. Recruited monocytes can also differentiate into non-AM inflammatory mononuclear phagocyte (MP) populations that are thought to act locally to promote allergic inflammation in the lung^{10, 18, 22}. Recruited MPs, along with resident

AMs, are therefore potentially pivotal cells during AAD, but the factors governing the balance between resident and recruited MPs and their phenotypes in disease are incompletely understood.

Transforming growth factor $\beta 1$ (TGF- $\beta 1$) is a pleiotropic regulator of immune responses, alongside other diverse cellular functions²³. AMs are a major source of pulmonary TGF- $\beta 1$ and TGF- β signalling to AMs is essential throughout their postnatal development²⁴. However, the effects of AM-intrinsic TGF- $\beta 1$ signalling on AM development throughout early life and its implications for immune regulation are not yet clear.

Herein, we examined the role of AM-derived TGF- $\beta 1$ throughout AM development and in the context of neonatal aeroallergen exposure. AM-intrinsic TGF- $\beta 1$ expression was required for maintenance and maturation of murine AMs over the first four weeks of life, but not their initial population of the lung, in contrast to previous reports of mice lacking all TGF- β signalling to AMs²⁴. Evidence of constitutive TGF- $\beta 1$ imprinting of identity was also observed in AMs from children. Mice with TGF- $\beta 1$ deficiency in CD11c⁺ cells displayed dysregulated chemokine production and enhanced inflammatory MP responses to neonatal allergen exposure, along with more severe AAD. This work highlights the importance of tissue- and age-specific factors in imprinting resident macrophage phenotypes to facilitate immune homeostasis during the critical early life window of immune maturation and initial environmental exposures.

Methods

Additional experimental procedures are described in the Supplementary Methods.

Mice

All animal work was performed in accordance with the Imperial College Guide for the Use of Laboratory Animals and the UK Animals (Scientific Procedures) Act 1986, under project license P996A24E1. *Tgfb1*^{ACD11c} conditional knockout mice were bred by mating of *Itgax*-Cre²⁵ and *Tgfb1*^{fl/fl} mice and maintained as Cre hemizygous knockouts and Cre-negative *Tgfb1*^{fl/fl} littermate controls. C57BL/6J mice for bone marrow-derived macrophage generation were purchased from Charles River Laboratories.

Early life allergic airway disease model and chemokine receptor blockade

Mice received three intranasal installations of 20 µg (by total protein) HDM extract (Greer Laboratories) in 10 µl sterile PBS (Gibco, Thermo Fisher), without anaesthesia, from days 7-12 of life. A further six intranasal challenges of 25 µg HDM extract in 15 µl PBS were administered from days 14-27 of life under light isoflurane anaesthesia. HDM challenges were spaced one to two days apart, three times per week and equivalent volumes of PBS were used as controls where applicable. Mice were weaned at 20-23 days of age. In some experiments, rat anti-mouse blocking antibodies to CCR2 (clone MC-21²⁷, Matthias Mack laboratory; 10 µg/dose) or CCR8 (clone SA214G2, BioLegend; 3 µg/dose), or equivalent doses of appropriate IgG2bk isotype control antibodies (clone MC-67, Matthias Mack laboratory and clone RTK4530, BioLegend for CCR2 and CCR8, respectively), were administered by intraperitoneal injection in a volume of 100 µl PBS 24 hours prior to the 7th HDM challenge and then immediately prior to 7th, 8th and 9th HDM challenges. All analysis was performed 24 hours after the final HDM exposure.

AM isolation from paediatric human subjects

Children were recruited prospectively with informed consent from parents, between September 2015 and August 2018 at the Royal Brompton Hospital, London, UK. All work with human subjects was approved by Hampstead Research Ethics Committee; reference number 15/LO/1885 and project title: patterns of airway infection and inflammation in children with respiratory symptoms, permitting use of surplus lower airway samples from clinical investigations for research purposes. Children undergoing clinically indicated flexible bronchoscopy as part of investigation for either severe pre-school wheeze or chronic recurrent wet cough were included in the present study. Preschool wheeze was defined as three or more episodes of wheeze, including two or more in the previous six months. Subjects were excluded from the study if diagnosed with cystic fibrosis, primary ciliary dyskinesia, congenital heart disease or structural lung abnormalities and had all been free of symptomatic respiratory infection for at least two weeks prior to bronchoscopy. Further patient details are presented in **Table S1**.

BAL was performed while under general anaesthesia within the right middle lung lobe, using three volumes of 0.9 % saline each at 1 ml/kg bodyweight. BAL differential cell counts, bacterial culture and viral PCR (multiplex respiratory pathogens kit from Fast Track Diagnostics) were performed according to routine diagnostic practice at the Royal Brompton Hospital Pathology Department. BAL was filtered through 70 µm cell strainers and cells pelleted, before brief incubation in ammonium chloride erythrocyte lysis buffer and suspension in cRPMI. Up to 10^7 cells were incubated in MACS buffer (0.5 % foetal bovine serum and 2 mM EDTA in PBS) containing APC/Cy7-conjugated anti-human CD206 antibody (BioLegend, clone 15-2), in the presence of Human TruStain FcX Fc-receptor-blocking reagent (BioLegend), for 10 minutes at 4 °C, before binding of anti-Cy7 microbeads (Miltenyi Biotec) at 4 °C for 15 minutes and magnetic positive selection of labelled cells using LS Separator Columns, a midiMACS Magnetic Separator and MACS Multi-Stand (all Miltenyi Biotec), as per manufacturer's instructions. Purity of positive AM fractions was confirmed to be over 90 % by flow cytometry in all samples.

Upstream regulator analysis of RNA-Seq data

Upstream regulator prediction was performed using Ingenuity Pathway Analysis (IPA, QIAGEN) on the 200 genes with highest median expression by RNA-Seq in AMs from all study subjects. Both direct and indirect relationships were considered, and results limited to cytokines and growth factors. Genes identified as TGF- β 1-regulated were overlaid with TGF- β 1 using the IPA Path Designer tool and reported 'expression' and 'transcription' connections between genes visualised. Literature reporting these connections was then manually inspected and gene symbols colour-coded based on positive, negative, or both positive and negative regulation by TGF- β 1.

Statistical analysis

Statistical testing and graphical representation of non-transcriptomic data was performed using Prism v8. Normally and non-normally distributed data were represented by means and medians, respectively. Normally distributed data were analysed using student's t-tests and ANOVA with Sidak's *post hoc* tests for individual and multiple comparisons, respectively. Non-normally distributed data were analysed using Mann Whitney *U* tests and Kruskal-Wallis with Dunn's *post hoc* tests for individual and multiple comparisons, respectively. Two-tailed tests were used. Figure legends describe the specific tests and results for each experiment. Statistical significance was determined as $P < 0.05$.

Results

TGF- β 1 from CD11c⁺ cells is required for maturation and maintenance of AMs in early life, but not their initial population of the lung

To examine the effects of deficiency in AM-intrinsic TGF- β 1 production on pulmonary MP populations throughout the crucial first weeks of life, lung cells from CD11c-conditional TGF- β 1 knockout (*Tgfb1*^{fl/fl} x CD11c-Cre, hereafter *Tgfb1* ^{Δ CD11c}) mice were analysed by flow cytometry (**Fig. S1A**). CD11c⁺ Siglec F⁺ AMs were present at postnatal day (P)3 in *Tgfb1*^{fl/fl} littermate control mice and their numbers remained similar from P7 to adulthood (8-10 weeks; **Fig. 1A** and **Fig. S1B**). Consistent with previous findings²⁴, AM numbers were decreased by >50 % in *Tgfb1* ^{Δ CD11c} mice at P28 (**Fig 1A**). However, our longitudinal analysis revealed that AM numbers were comparable between *Tgfb1* ^{Δ CD11c} and control mice over the first crucial postnatal weeks from P3-P14 (**Fig. 1A**), suggestive of an age-dependent effect of intrinsic TGF- β 1 deficiency on AM numbers, which was not previously reported.

Despite their equivalent numbers at P3-P14, *Tgfb1* ^{Δ CD11c} AMs displayed an altered phenotype even at P3. While control AMs were uniformly Siglec F^{hi} and lost CD11b expression by P14, *Tgfb1* ^{Δ CD11c} AMs remained Siglec F⁺, but had consistently lower Siglec F staining intensity than age-matched littermate controls (**Fig. 1C-D**) and a proportion retained expression of CD11b, which increased further from P14-28 (**Fig. 1E-F**). Gene expression analysis at P14 confirmed the substantial reduction in *Tgfb1* gene expression in bulk *Tgfb1* ^{Δ CD11c} AMs, accompanied by reduced expression of the key AM transcription factor *Pparg*/PPAR- γ ²⁸, which has been previously shown to require TGF- β 1 signalling for maximal expression (Yu et al., 2017), as well as the hallmark AM gene *Chil3*/Ym1¹⁷ (**Fig. 1G** and **S1H**), consistent with TGF- β 1-deficient AMs failing to fully adopt the homeostatic AM phenotype during this early life window.

AM numbers remained reduced in young adult *Tgfb1*^{ΔCD11c} mice, along with decreased Siglec F expression intensity and an increased proportion of CD11b⁺ AMs. However, these changes were less marked than at P28 (**Fig. S1B-D**), suggesting that the AM deficiency observed in juvenile *Tgfb1*^{ΔCD11c} mice is not stable throughout life. Accordingly, no significant differences in AM number or Siglec F and CD11b expression were observed in 8-10-month-old *Tgfb1*^{ΔCD11c} mice (**Fig. S1E-G**) and no illness was observed throughout the lives of these mice, with lung function and histology comparable to controls when assessed at 7 months of age (data not shown). Importantly, early life defects in the steady-state pulmonary MP compartment of *Tgfb1*^{ΔCD11c} mice were restricted to AMs, with comparable numbers of cDCs, Siglec F⁻ CD11c⁻ CD11b⁺ Ly6C⁻ IMs and Ly6C^{hi} monocytes to *Tgfb1*^{fl/fl} controls at P28, although a small decrease in CD103⁺ cDC1 counts was apparent in young adult knockouts (**Fig. S1I**).

Thus, while redundant for the initial generation of CD11c⁺ Siglec F⁺ AMs in the neonatal lung, TGF-β1 expression in CD11c⁺ cells was required for AM maintenance into late neonatal development and adoption of the classical airway-resident profile, revealing dynamic effects of TGF-β1 during this critical developmental window.

Evidence of constitutive regulation of paediatric human AM identity by TGF-β1

To determine whether TGF-β1 is also likely to imprint human AM identity in early life, we performed RNA-Seq on AMs sorted from bronchoalveolar lavage (BAL) of children under investigation for either chronic recurrent cough or severe preschool wheeze (**Fig. S1J** and **Table S1**). Subjects had no symptomatic respiratory tract infection at the time of sampling, although some had detectable bacteria and/or viruses in BAL (**Table S1**). Ingenuity Upstream Regulator Analysis was performed on the 200 most highly expressed genes across all patients (irrespective of diagnosis), identifying TGF-β1 as the most enriched upstream cytokine or growth factor of highly expressed genes in paediatric AMs, with the

majority (40/60) of the TGF- β 1-regulated genes in this set reported as positively TGF- β 1-regulated (**Fig. 2A-B**). Accordingly, paediatric AMs also expressed high levels of *TGFB1* and TGF- β canonical signalling pathway genes, with *RHOA* showing the highest expression among the canonical TGF- β -induced genes in paediatric AMs (**Fig. 2C**). These data suggest that TGF- β 1 constitutively regulates human, as well as murine, AM gene expression in early life.

***Tgfb1* ^{Δ CD11c} mice accumulate inflammatory mononuclear phagocytes in the lungs following neonatal allergen exposure**

Since AMs are key immune-regulatory cells and *Tgfb1* ^{Δ CD11c} mice showed markedly impaired AM maturation between one to four weeks of life, we hypothesised that this perturbed lung MP state would result in a dysregulated pulmonary MP response to neonatal allergen exposure. We therefore repeatedly administered intranasal HDM to *Tgfb1* ^{Δ CD11c} and littermate *Tgfb1*^{fl/fl} control mice throughout this early life window from P7-P28 (**Fig. 3A**) and analysed MP populations by flow cytometry (**Fig. S1A** and **Fig. 3B**). In contrast to allergen-naïve mice, in which all airway CD11c⁺ F4/80⁺ cells are Siglec F⁺ AMs⁶, two major populations of CD11c⁺ CD64⁺ F4/80⁺ macrophage-like MPs could be distinguished in BAL of P28 mice after three weeks of HDM exposure on the basis of Siglec F expression. One population expressed Siglec F at variable levels and fell into the AM gate defined at steady state, while the other, which was not apparent at steady state, lacked detectable Siglec F expression by flow cytometry (**Fig. 3B**). The large size and AM-like morphology of the Siglec F⁺ population (**Fig. 3C**), along with their expression of the AM marker Siglec F, suggested that this population comprised *bona fide* AMs and/or newly recruited cells adopting an AM-like phenotype, so was termed SigF⁺ AM-like cells. In contrast, the Siglec F⁻ CD11c⁺ population (henceforth SigF⁻ MPs) displayed a macrophage-like morphology but were comparatively smaller than AMs (**Fig. 3C**), suggestive of non-AM recruited MPs.

As in lungs of naïve P28 mice (**Fig. 1A**), AM numbers were reduced in BAL and lung tissue of P28 *Tgfb1*^{ΔCD11c} mice after three weeks of PBS mock treatment (**Fig. 3D** and **S2A**). However, HDM-treated *Tgfb1*^{ΔCD11c} mice displayed comparable numbers of SigF⁺ AM-like cells to *Tgfb1*^{fl/fl} littermates (**Fig. 3D** and **S2A**), suggesting that HDM exposure drove either persistence of TGF-β1-deficient resident AMs or recruitment of replacement AM-like cells to the lung. Furthermore, *Tgfb1*^{ΔCD11c} mice displayed enhanced accumulation of SigF⁺ MPs in BAL and lungs compared to *Tgfb1*^{fl/fl} controls, resulting in an increase in number of total pulmonary CD11c⁺ CD64⁺ F4/80⁺ MPs in *Tgfb1*^{ΔCD11c} mice, but not littermate controls, following repeated HDM exposure (**Fig. 3D** and **S2A**). Sorted SigF⁺ AM-like cells and SigF⁺ MPs (**Fig S2B**) had diminished *Tgfb1* mRNA expression, consistent with Cre-mediated *Tgfb1* deletion being maintained within these CD11c⁺ populations during allergic inflammation, concomitant with decreased *Pparg* expression (**Fig. 3E**). In contrast to CD11c⁺ MPs, no changes were observed in numbers of Ly6C^{hi} monocytes or IMs (**Fig. S2A**).

Despite their equivalent numbers in HDM-treated *Tgfb1*^{fl/fl} and *Tgfb1*^{ΔCD11c} mice, the SigF⁺ AM-like cells from *Tgfb1*^{ΔCD11c} mice displayed a distinct, more pro-inflammatory, phenotype. While the SigF⁺ AM-like cells from HDM-treated mice of both genotypes had a reduced Siglec F staining intensity as compared to the steady state AMs from their respective PBS-treated controls, SigF⁺ AM-like cells from HDM-treated *Tgfb1*^{ΔCD11c} mice displayed the lowest expression of all the groups (**Fig. 3B&F**). Expression of CD11b, a marker of activation of mature AMs²⁹, was also more highly expressed on SigF⁺ AM-like cells from HDM-treated *Tgfb1*^{ΔCD11c} than *Tgfb1*^{fl/fl} mice, with nearly 100 % expressing CD11b in *Tgfb1*^{ΔCD11c} mice (**Fig. 3G** and **S2C-D**). In contrast, SigF⁺ MPs were almost all CD11b⁺ with comparable expression between *Tgfb1*^{ΔCD11c} and *Tgfb1*^{fl/fl} mice (**Fig. 3G** and **S2C-D**). Similarly, a greater proportion of SigF⁺ AM-like cells were MHC-II⁺ in *Tgfb1*^{ΔCD11c} than *Tgfb1*^{fl/fl} mice, while MHC-II was expressed by 40-80 % of SigF⁺ MPs in both groups (**Fig. S2D**). Consistent with their definition as AM-like, nearly all SigF⁺ AM-like cells expressed the pan-macrophage marker

MerTK, which was absent from Ly6C^{hi} monocytes (**Fig. 3H** and **Fig. S2E**). However, only a subset of SigF⁻ MPs were MerTK⁺ (**Fig. 3H** and **Fig. S2E**), suggestive of a heterogeneous inflammatory population that includes a large proportion of *bona fide* macrophages. SigF⁺ AM-like cells from HDM-treated *Tgfb1*^{ΔCD11c} mice also exhibited decreased expression of the homeostatic AM marker CD169³⁰ compared to littermate controls, but increased expression of programmed cell death 1 ligand 2 (PD-L2), which is associated with inflammatory monocyte origin of macrophages during IL-4-dependent type 2 immunity³¹ (**Fig. 3H** and **S2E-F**). In contrast, expression of these proteins by was comparable on SigF⁻ MPs from HDM-treated *Tgfb1*^{ΔCD11c} and *Tgfb1*^{fl/fl} mice and was much lower on Ly6C^{hi} monocytes (**Fig. 3H** and **Fig. S2E**).

Both SigF⁺ AM-like cells and SigF⁻ MPs from *Tgfb1*^{ΔCD11c} mice, but not Ly6C^{hi} monocytes, expressed readily detectable mRNA for the classically activated macrophage marker gene *Nos2*/iNOS and high levels of the alternatively activated macrophage marker gene *Chil3* (**Fig. S2G**). *Nos2* expression was increased in SigF⁺ AM-like cells from *Tgfb1*^{ΔCD11c} mice as compared to those from *Tgfb1*^{fl/fl} controls, with a trend towards higher *Chil3* expression also observed (**Fig S2G**). Thus, the inflammatory CD11c⁺ MPs accumulating in HDM-treated *Tgfb1*^{ΔCD11c} mice display features of both classically and alternatively activated macrophages, as observed previously with wild type AMs from an adult AAD mouse model¹⁴.

Collectively, these results reveal a markedly dysregulated pulmonary MP response to early life HDM inhalation in the context of TGF-β1 deficiency in CD11c⁺ cells.

Inflammatory mononuclear phagocyte accumulation in HDM-treated *Tgfb1*^{ΔCD11c} mice is CCR2-dependent

In contrast to the increase in total CD11c⁺ CD64⁺ F4/80⁺ inflammatory MPs in HDM-treated *Tgfb1*^{ΔCD11c} mice (**Fig. 3D** and **S2A**), pulmonary Ly6C^{hi} monocyte numbers were not increased following HDM treatment (**Fig. S2A**), suggesting that infiltrating Ly6C^{hi} monocytes

in this model rapidly differentiate into inflammatory monocyte-derived cells. We therefore hypothesised that the inflammatory CD11c⁺ MPs that accumulate in the lungs of HDM-treated *Tgfb1*^{ΔCD11c} mice derive from recruited inflammatory monocytes. To test this, we therapeutically administered an antibody to CCR2 (αCCR2)²⁷, an essential receptor for inflammatory monocyte egress from bone marrow and mediator of their entry into tissues³², to *Tgfb1*^{ΔCD11c} and *Tgfb1*^{fl/fl} mice throughout the final week of HDM exposures, to systemically deplete Ly6C^{hi} monocytes (**Fig. 4A**). As expected, this αCCR2 regimen substantially depleted circulating Ly6C^{hi} monocytes (**Fig. 4B**). Therapeutic αCCR2 modestly reduced total airway cellularity, but markedly decreased numbers of SigF⁺ AM-like cells and SigF⁻ MPs in BAL and lung tissue of *Tgfb1*^{ΔCD11c}, but not *Tgfb1*^{fl/fl} control, mice (**Fig. 4C-D** and **Fig. S3A**). Neither population was completely ablated by αCCR2 (**Fig. 4D** and **Fig. S3A**), possibly due to incomplete reduction of pulmonary Ly6C^{hi} monocyte numbers in *Tgfb1*^{ΔCD11c} mice (**Fig. S3A**), which might allow CCR2-independent recruitment of residual circulating Ly6C^{hi} monocytes in αCCR2-treated *Tgfb1*^{ΔCD11c} mice. However, these results are consistent with the inflammatory CD11c⁺ MPs that accumulate following early life HDM exposure in the context of TGF-β1 deficiency in CD11c⁺ cells requiring a substantial contribution from CCR2-dependent inflammatory monocytes.

***Tgfb1*^{ΔCD11c} mice display increased monocyte chemokine production during allergic inflammation and at steady state**

Having demonstrated dependency of inflammatory CD11c⁺ MP accumulation in HDM-treated *Tgfb1*^{ΔCD11c} mice on CCR2-dependent monocytes, we next sought evidence for dysregulated monocyte-attractant chemokine production in these mice. Since CCR1 and CCR2 are both required for maximal Ly6C^{hi} monocyte numbers in mouse lungs at the steady state³² and both of these receptors can mediate monocyte accumulation during inflammation³³, we first quantified ligands of these receptors in HDM-treated *Tgfb1*^{ΔCD11c} and

$Tgfb1^{fl/fl}$ control mice. The CCR1 agonists CCL6 and MIP-1 γ (CCL9/10) were significantly more concentrated in BAL and lung tissue of HDM-treated $Tgfb1^{\Delta CD11c}$ mice than $Tgfb1^{fl/fl}$ controls (**Fig. 5A** and **Fig. S3B**), while the CCR2 ligands CCL12 and CCL2 were more concentrated in lung tissue of $Tgfb1^{\Delta CD11c}$ mice, with a trend towards increased BAL CCL2 in knockouts (**Fig. 5A-B**). Notably, inflammatory CD11c⁺ MPs were identified as likely contributors to increased CCR1 ligand levels in $Tgfb1^{\Delta CD11c}$ mice. *Ccl6* and *Ccl9* genes were both highly expressed by SigF⁺ AM-like cells and SigF⁻ MPs from HDM-treated knockout and control mice, as compared to Ly6C^{hi} monocytes, with SigF⁺ AM-like cells from $Tgfb1^{\Delta CD11c}$ mice exhibiting higher *Ccl9* mRNA expression than those in littermate controls (**Fig. 5C**). Expression of the CCR2 ligands *Ccl2*, *Ccl7* and *Ccl12* was relatively low in SigF⁺ AM-like cells (**Fig. 5C** and **Fig. S3C**), whereas SigF⁻ MPs showed relatively high expression of *Ccl2* mRNA (**Fig. 5C**), suggesting that these cells contribute to elevated pulmonary CCL2 levels in HDM-treated $Tgfb1^{\Delta CD11c}$ mice, in which they are highly abundant (**Fig. 3D** and **Fig. S2A**).

Thus, increased accumulation of inflammatory, largely CCR2-dependent, CD11c⁺ MPs following HDM treatment of neonatal mice with TGF- β 1 deletion in CD11c⁺ cells is accompanied by increased monocyte-attractant chemokine production.

Since accumulation of inflammatory monocyte-derived cells in the lung is thought to be an important early driver of HDM-elicited AAD^{10, 22}, we hypothesised that increased inflammatory CD11c⁺ MP accumulation in HDM-treated $Tgfb1^{\Delta CD11c}$ mice would be associated with dysregulation of monocyte-attractant chemokine production during the early life window of AM dysfunction in these mice, such that these mice are primed to overproduce CCR1 and CCR2 ligands prior to any allergen exposure. Correspondingly, in allergen-naïve P14 mice, CCL6, MIP-1 γ and CCL2 concentrations were significantly increased in BAL of naïve P14 $Tgfb1^{\Delta CD11c}$ mice compared to $Tgfb1^{fl/fl}$ controls (**Fig. 5D**). CCL12 was not detectable in these samples (data not shown). Increased chemokine concentrations were at least partially due to dysregulated production by TGF- β 1-deficient AMs, since *Ccl2*, *Ccl6* and

Ccl9 gene expression was elevated in *Tgfb1*^{ΔCD11c} AMs (**Fig. 5E**). *Ccl7* and *Ccl12* gene expression was also higher in *Tgfb1*^{ΔCD11c} AMs than *Tgfb1*^{fl/fl} controls (**Fig. S3D**), but at much lower levels than *Ccl2*, *Ccl6* and *Ccl9*. Thus, TGF-β1-deficient AMs are primed to produce monocyte-attractant chemokines in early life, prior to exposure to exogenous inflammatory stimuli, possibly reflecting a loss of autocrine regulation via TGF-β1.

Consistent with a role for direct TGF-β1-mediated suppression of constitutive chemokine expression by macrophages, exogenous TGF-β1 suppressed constitutive production of CCL6 and MIP-1γ, but not CCL2, by cultured mouse macrophages (**Fig. 5F**), presenting a mechanism by which autocrine TGF-β1 signalling may limit constitutive CCR1 ligand production by AMs in early life to maintain pulmonary immune homeostasis.

***Tgfb1*^{ΔCD11c} mice display enhanced allergic airway disease following early life HDM exposure**

Recruited monocyte-derived cells are thought to act locally to promote type 2 immune responses in the allergic lung^{10, 22}. We therefore hypothesised that the increased inflammatory CD11c⁺ MP accumulation observed in *Tgfb1*^{ΔCD11c} mice exposed to HDM throughout early life would be accompanied by enhanced AAD.

Notably, the predominantly C57BL/6 background strain of the *Tgfb1*^{fl/fl} mice makes them relatively refractory to HDM-driven AAD compared to the more allergy-prone BALB/c strain. Consequently, HDM treatment induced allergic pulmonary inflammation in *Tgfb1*^{fl/fl} mice, as determined by increased total leukocyte and eosinophil counts in BAL and lung tissue and peribronchiolar inflammation as visible on H&E sections (**Fig 6A-C**), but these mice showed a minimal increase in numbers of IL-13- or IL-5-producing Th2 cells and group 2 innate lymphoid cells (ILC2s) in lung tissue and little to no detectable IL-13 in BAL fluid or lung homogenates (**Fig. 6D-F** and **Fig. S4A**). BAL and lung total cell and eosinophil counts were significantly elevated in HDM-treated *Tgfb1*^{ΔCD11c} mice compared to littermate controls (**Fig.**

6A-B), along with Th2 and ILC2 numbers and IL-13 concentrations (**Fig. 6D-F**), indicating that TGF- β 1 deficiency in CD11c⁺ cells lowers the threshold for mounting a substantial type 2 immune response to neonatal HDM exposure in these mice. HDM did not induce substantial neutrophilia in either knockout or control animals (**Fig. S4B**) and lung CD4 T cells producing IFN- γ or IL-17A were several-fold less abundant than Th2 cells after HDM treatment and numbers did not differ between *Tgfb1* ^{Δ CD11c} and control mice (data not shown). Increased Th2 cell and ILC2 numbers in the lung were accompanied by increased *Il4* and *Il5* gene expression in lung tissue (**Fig. S4C**). The enhanced effector CD4 T cell response in HDM-treated *Tgfb1* ^{Δ CD11c} mice was confirmed in the absence of ex vivo stimulation for cytokine production by showing increased numbers of antigen-experienced CD44⁺ FoxP3⁻ CD4 T cells, whereas numbers of FoxP3⁺ regulatory T cells and CD4 T cells producing IL-10 were comparable in *Tgfb1* ^{Δ CD11c} and control mice (**Fig. S4D**), suggesting that deficiency in regulatory T cell populations was not responsible for the augmented T2 lymphocyte response. IL-13⁺ CD4 T cells were also more abundant in lung-draining mediastinal lymph nodes (mLNs) of HDM-treated *Tgfb1* ^{Δ CD11c} mice (**Fig. S4E**).

While HDM treatment of both *Tgfb1* ^{Δ CD11c} and *Tgfb1*^{fl/fl} resulted in airway hyperresponsiveness to inhaled methacholine, relative to PBS-treated controls, the heightened type 2 immune response in HDM-treated *Tgfb1* ^{Δ CD11c} mice was not accompanied by further increased airway hyperresponsiveness (**Fig. S4F**). However, concentrations of circulating total and HDM-specific IgE and IgG1 were markedly enhanced in HDM-treated *Tgfb1* ^{Δ CD11c} mice (**Fig. 6G-H** and **S4G**). Comparable mLN germinal centre B cell and T follicular helper cell frequencies were observed in HDM-treated *Tgfb1* ^{Δ CD11c} and *Tgfb1*^{fl/fl} mice (**Fig. S4H-I**), suggesting that the increased immunoglobulin titres resulted from the overall increased magnitude of T2 immunity, rather than specific germinal centre dysregulation.

Concomitant with increased T2 immunity, sub-epithelial peribronchiolar reticulin thickness, akin to the early reticular basement membrane remodelling observed in preschool wheeze

and difficult asthma in school age children³⁴⁻³⁶, was more substantially increased by HDM treatment in *Tgfb1*^{ΔCD11c} mice than *Tgfb1*^{fl/fl} controls (**Fig. 6I**), indicating more established airway wall remodelling in these mice. Accordingly, soluble, newly synthesised, collagen was also more abundant in lungs of these mice (**Fig. 6J**). Although a greater proportion of *Tgfb1*^{ΔCD11c} than control mice displayed high levels of airway mucus staining following HDM treatment, this was inconsistent and therefore not statistically significant (**Fig. S4J**).

Thus, inflammatory MP accumulation elicited by HDM exposure in the context of early life TGF-β1 deficiency in CD11c⁺ cells is accompanied by enhanced pulmonary T2 immunity and early airway remodelling.

Dysregulated production of the pro-allergic chemokine CCL8 in HDM-treated *Tgfb1*^{ΔCD11c} mice

Given that pulmonary CD11c⁺ cDC2s are crucial drivers of T2 immunity to HDM²², we reasoned that enhanced AAD in *Tgfb1*^{ΔCD11c} mice might be due to Cre-mediated DC-intrinsic *Tgfb1* deletion resulting in cDC dysfunction. However, cDC1 and cDC2 abundance and expression of the maturation markers CD86 and MHC-II were comparable in lungs and mLNs of HDM-treated *Tgfb1*^{ΔCD11c} and *Tgfb1*^{fl/fl} control mice (**Fig. S5**), thus presenting no evidence that pulmonary cDC number, maturation or accumulation in mLNs was responsible for heightened HDM-driven AAD in *Tgfb1*^{ΔCD11c} mice.

We next hypothesised that dysregulated SigF⁺ AM-like cells and SigF⁺ MPs were drivers of enhanced T2 immunity to HDM in *Tgfb1*^{ΔCD11c} mice. In support of a contribution of inflammatory CD11c⁺ MPs to heightened T2 immunity in these mice, αCCR2 treatment, which reduced numbers of these cells in *Tgfb1*^{ΔCD11c} mice (**Fig. 4**), also partially reduced pulmonary Th2 cell and eosinophil numbers, such that numbers were no longer significantly elevated above levels in *Tgfb1*^{fl/fl} controls, and significantly diminished BAL eosinophil counts (**Fig. S6A-B**). Reduced eosinophilia did not appear to be a direct effect of blockade of CCR2

signalling to eosinophils, since no CCR2 expression was detected on these cells during AAD (Fig. S6C).

Reasoning that dysregulated inflammatory CD11c⁺ MPs in HDM-treated *Tgfb1*^{ΔCD11c} mice could facilitate HDM-driven AAD via increased local pro-inflammatory cytokine production, we examined expression of potentially MP-derived cytokine genes in lung tissue. Of these genes, only *Il23a*, encoding the alpha subunit of IL-23, was more highly expressed in lungs of *Tgfb1*^{ΔCD11c} mice than littermate controls after HDM treatment (Fig. S7A). However, expression of *Il12b*, encoding the second subunit of both IL-23 and IL-12, was decreased in HDM-treated *Tgfb1*^{ΔCD11c} mice (Fig. S7A), indicating that there was not an overall skewing towards IL-23 production in these mice. Expression of *Il1b* was also decreased in *Tgfb1*^{ΔCD11c} mice compared to *Tgfb1*^{fl/fl} littermates after HDM treatment, with a trend towards decrease in *Il12a* expression (Fig. S7A). These results thus do not support increased pro-inflammatory cytokine production as a correlate of the more severe AAD observed in *Tgfb1*^{ΔCD11c} mice exposed to HDM during early life.

Since both AMs and Siglec F⁺ CD11c⁺ MHC-II⁺ CD64⁺ inflammatory MPs, likely to encompass some of the SigF⁺ MPs observed in our model, produce pro-allergic chemokines during HDM-driven AAD^{14, 15, 22}, we proposed that inflammatory CD11c⁺ MPs could promote elevated T2 immunity in HDM-treated *Tgfb1*^{ΔCD11c} mice by chemokine production. To identify elevated pro-allergic chemokines in the airways of our knockout mice, we screened BAL of HDM-treated *Tgfb1*^{ΔCD11c} and control mice using a commercially available immunoblot array. This approach verified the previously observed increases in airway CCL6 and MIP-1γ concentrations in *Tgfb1*^{ΔCD11c} mice (Fig. 5A), but also highlighted a marked increase in CCL8 concentrations in BAL, which was confirmed by ELISA in both in BAL and lung tissue (Fig. 7A-B and S7B-C).

Among other pro-allergic chemokines, the eosinophil chemoattractant CCL24/eotaxin 2 was also more concentrated in lung tissue of HDM-treated *Tgfb1*^{ΔCD11c} than *Tgfb1*^{fl/fl} mice, while

BAL CCL24 concentrations were highly variable but with a trend towards increase in knockouts (**Fig. S7D**), suggesting that this chemokine might contribute to the elevated eosinophilia observed in these mice. In contrast, only a weak BAL CCL11/eotaxin signal was detectable by chemokine array (**Fig. 7A**) and lung CCL11 concentrations did not differ between HDM-treated *Tgfb1*^{ΔCD11c} and control mice (**Fig. S7D**). Concentrations of the Th2 cell chemokine CCL17 did not significantly differ in BAL or lung tissue of *Tgfb1*^{ΔCD11c} and *Tgfb1*^{fl/fl} mice after HDM treatment (**Fig. S7E**). CCL1, which shares the receptor CCR8 with CCL8 and can promote ILC2 proliferation and effector function³⁷, was more concentrated in BAL and lung tissue of HDM-treated *Tgfb1*^{ΔCD11c} mice than controls, but at around 100-fold lower levels than CCL8 (**Fig. S7F**), indicating that CCL8 is the most abundant CCR8 ligand in this context.

Since we have previously demonstrated increased CCL8 expression by AMs during allergic airway inflammation driven by HDM or IL-33^{14, 15, 38} and others have also reported high relative CCL8 expression by CD11c⁺ Siglec F⁻ CD64⁺ inflammatory MPs that accumulate in HDM treated mice²², we postulated that the inflammatory CD11c⁺ MPs that accumulate in HDM-treated *Tgfb1*^{ΔCD11c} mice were a source of the elevated CCL8. Accordingly, *Ccl8* gene expression was detected in SigF⁺ AM-like cells, SigF⁻ MPs and Ly6C^{hi} monocytes from HDM treated mice and showed significantly greater expression in SigF⁺ AM-like cells from *Tgfb1*^{ΔCD11c} mice than controls (**Fig. 7C**).

No *Ccl8* gene expression was detected in steady state AMs from P14 *Tgfb1*^{ΔCD11c} or *Tgfb1*^{fl/fl} mice (data not shown), suggesting that, unlike CCL6 and MIP-1γ, TGF-β1-deficient AMs do not constitutively overproduce CCL8 in the steady state and that elevated CCL8 production by TGF-β1-deficient MPs may be reliant on additional inflammatory signals in the context of allergic airway disease. Accordingly, cultured mouse macrophages did not produce CCL8 with or without exogenous TGF-β1 in the absence of inflammatory stimuli. However, when activated with a moderate dose of LPS, plus IL-4 and IL-13, to mimic the inflammatory allergic airway environment, exogenous TGF-β1 restricted CCL8 production (**Fig. 7D**).

The CCL8 receptor CCR8 is required for enhanced type 2 lymphoid cell and airway remodelling responses to HDM in *Tgfb1*^{ΔCD11c} mice

Since CCL8 can drive T2 lymphoid cell responses via its receptor CCR8^{38, 39}, we reasoned that the elevated CCL8 production in *Tgfb1*^{ΔCD11c} mice treated with HDM during early life might drive augmented T2 immunity via a CCL8-CCR8 axis. CCR8 expression was detected on total and IL-13⁺ CD4 T cells and ILCs in HDM-treated *Tgfb1*^{ΔCD11c} and control mice, but not MPs or eosinophils (**Fig. 7E** and **Fig S8A**), consistent with a role in T2 lymphoid cell function. Accordingly, anti-CCR8 (αCCR8) administration throughout the final week of HDM exposures (**Fig. 7F**) reduced pulmonary Th2 cell and ILC2 numbers in *Tgfb1*^{ΔCD11c} mice to near *Tgfb1*^{fl/fl} control levels (**Fig. 7G**). Decreased IL-13⁺ ILC2 numbers with αCCR8 were not solely due to a reduction in IL-13 production, since αCCR8 also reduced total GATA3⁺ CD90.2⁺ Lineage⁻ ILC numbers in lungs of knockout mice (**Fig. S8B**).

Therapeutic αCCR8 also significantly reduced BAL and lung IL-13 concentrations and ameliorated the increased peribronchiolar reticulin thickness in HDM-treated *Tgfb1*^{ΔCD11c} mice (**Fig. 7H-I** and **Fig. S8C**), suggesting that a pathogenic relationship exists between the CCL8-CCR8 axis, T2 lymphocytes and airway remodelling in these mice. However, αCCR8 did not affect airway eosinophilia or circulating IgE and IgG1 concentrations (**Fig. S8D-E**). SigF⁺ AM-like cell numbers in BAL were unaffected by αCCR8 treatment, whereas SigF⁻ MP numbers in *Tgfb1*^{ΔCD11c} mice were significantly reduced (**Fig. S7F**). However, since neither SigF⁻ MPs nor Ly6C^{hi} monocytes expressed CCR8 (**Fig. 7E**) and αCCR8 therapy did not affect pulmonary or circulating Ly6C^{hi} monocyte frequency (**Fig. S8G-H**), reduced Sig F⁻ MP numbers appeared to be downstream of the dampened T2 immune response following αCCR8 treatment.

534 Thus, *Tgfb1*^{ΔCD11c} mice produce elevated levels of pulmonary CCL8 following neonatal HDM
535 exposure, derived in part from accumulating inflammatory CD11c⁺ MPs, and the CCL8
536 receptor CCR8 mediates heightened T2 immunity and airway wall remodelling in these mice.

Discussion

The distinct immunological environment of the neonatal lung influences responses to pathogens and aeroallergens^{3, 40-42}, meaning that age is a major factor governing asthma susceptibility and phenotype^{4, 5}. However, the mechanisms of development and regulation of the neonatal immune system in the face of initial exposures to pathogens and allergens are incompletely understood. The particular role of AMs in AAD remains unclear, with both protective regulatory and pathogenic pro-inflammatory and pro-remodelling roles proposed⁹⁻¹⁴. We show here that TGF- β 1 deficiency in CD11c⁺ MPs results in AM dysfunction in the crucial first weeks of life, precipitating dysregulated chemokine and inflammatory MP responses to inhaled HDM allergen, concomitant with more severe allergic airway inflammation and remodelling.

We identified an age-specific effect of CD11c⁺ cell-restricted TGF- β 1 deficiency on neonatal AM development, with surface marker and gene expression profiles suggestive of immature, dysfunctional AMs preceding the decrease in AM numbers in *Tgfb1* ^{Δ CD11c} mice, which was previously reported at a single time-point in late postnatal lung development at P28²⁴. Our results necessitate reinterpretation of the conclusions of Yu *et al.*, who suggested that TGF- β 1 action throughout AM development was entirely autocrine, since mice with conditional TGF- β receptor knockout in AMs displayed reduced AM numbers as early as P3²⁴. We now demonstrate that TGF- β 1 expression by AMs only becomes necessary for maintenance of AM numbers in late postnatal lung development, suggesting that partially compensatory TGF- β 1 sources exist in the first weeks of life. Our longitudinal analysis therefore allowed us to study a window during the crucial first postnatal weeks in *Tgfb1* ^{Δ CD11c} mice where AMs were present but dysfunctional and incompletely matured, facilitating an augmented inflammatory response to HDM.

Chemokines function in a coordinated manner to drive allergic inflammation, with some acting upstream of others by recruiting additional chemokine-producing cells⁴³. AMs and

inflammatory CD11c⁺ Siglec F⁻ CD64⁺ MPs have been previously described as local chemokine sources during allergic inflammation^{14, 15, 22, 44}. We demonstrate for the first time a role for TGF-β1 in specifically regulating the MP-derived chemokine repertoire in early life. *Tgfb1*^{ΔCD11c} mice displayed distinct patterns of increased CCR1, CCR2 and CCR8 ligand production at steady state and following neonatal HDM exposure. Moreover, we demonstrate therapeutic effects of CCR2 and CCR8 blockade on components of the augmented immune response of *Tgfb1*^{ΔCD11c} mice to HDM, suggesting that agonists of these receptors act co-ordinately to lower the threshold for pathogenic immune responses to aeroallergen when TGF-β1-dependent AM maturation is impaired.

Expression of the major CCR1 ligands CCL6 and MIP-1γ and the major CCR2 ligand CCL2 was increased in TGF-β1-deficient neonatal AMs, prior to any allergen exposure, suggesting that autocrine TGF-β1 signalling regulates expression of these chemokines in AMs during their postnatal development. Accordingly, we also demonstrated suppression of constitutive *in vitro* CCL6 and MIP-1γ production from macrophages by exogenous TGF-β1. CCR2 and CCR1 are both required to achieve maximal pulmonary classical monocyte numbers at homeostasis³², but also mediate inflammatory monocyte recruitment to the lung³³. We have demonstrated CCR2-dependent accumulation of SigF⁺ AM-like cells, with increased expression of inflammatory monocyte-derived macrophage markers CD11b and PD-L2, and SigF⁻ MPs (CD11c⁺ CD64⁺ F4/80⁺) in *Tgfb1*^{ΔCD11c} mice exposed to HDM over the first weeks of life, in which resident AMs display a dysfunctional phenotype.

The exact identity of the CCR2-dependent SigF⁻ MPs that accumulate in increased numbers in HDM-treated *Tgfb1*^{ΔCD11c} mice is unclear, with variable expression of the macrophage marker MerTK suggesting some heterogeneity within the population. Given the reduction in numbers of both inflammatory CD11c⁺ MP subsets in HDM-treated *Tgfb1*^{ΔCD11c} mice by CCR2 blockade, it can be speculated that the SigF⁻ MP population represents an intermediate state between inflammatory monocytes and CCR2-dependent SigF⁺ AM-like cells in these mice. However, independent CCR2-dependent origins of these populations

also remain possible. Regardless of their precise origin and identity, it was clear that CD11c⁺ MPs with inflammatory phenotypes accumulated in increased numbers following HDM exposure of *Tgfb1*^{ΔCD11c} mice. We therefore propose that increased classical monocyte-attractant chemokine expression by AMs in *Tgfb1*^{ΔCD11c} mice in early life, which might present a mechanism for gradual replacement of dysfunctional AMs by circulating monocytes at steady state, facilitates inflammatory CD11c⁺ MP accumulation in the context of additional signals elicited by HDM inhalation.

The mechanism linking the dysfunctional phenotype and increased chemokine expression of neonatal *Tgfb1*^{ΔCD11c} AMs to TGF-β1 deficiency remains to be determined. It is likely that key regulators of AM homeostatic function are disrupted in the absence of intrinsic TGF-β1 expression, contributing to their dysregulated phenotype. Indeed, we show reduced expression of the gene encoding the essential AM transcription factor PPAR-γ²⁸ in TGF-β1-deficient AMs in early life. Another possible contributor is RhoA, a TGF-β-inducible molecule that was highly expressed in paediatric AMs in our study, which has been implicated in macrophage polarisation and function⁴⁵.

CCL8, a key mediator of Th2 and ILC2 responses^{38, 39}, showed dynamics of expression distinct from those of CCR1 and CCR2 ligands. CCL8 was not expressed by wild type or TGF-β1-deficient neonatal AMs at steady state, but was expressed by inflammatory SigF⁺ AM-like cells and SigF⁻ MPs during established AAD, accompanied by increased pulmonary CCL8 concentrations in HDM-treated *Tgfb1*^{ΔCD11c} mice, suggesting that increased CCL8 production occurs downstream of the initial dysregulated inflammatory MP response to HDM in these mice. Importantly, we showed the CCL8 receptor, CCR8, to be central to AAD pathogenesis in *Tgfb1*^{ΔCD11c} mice. *Tgfb1*^{ΔCD11c} mice displayed augmented Th2 and ILC2 responses to HDM, with increased pulmonary IL-13 concentrations and peribronchiolar reticulin deposition, which were reduced by therapeutic CCR8 blockade.

It remains to be seen whether the increased CCL8 production by SigF⁺ AM-like cells in HDM-treated *Tgfb1*^{ΔCD11c} mice is due to intrinsic TGF-β1 deficiency in these cells. While our data showing TGF-β1 suppression of CCL8 production by *in vitro* stimulated macrophages supports such a direct mechanism, it remains possible that the likely monocyte origin of the majority of SigF⁺ AM-like cells in these mice endows them with greater capacity for CCL8 production, as demonstrated recently for IL-6 in monocyte-derived AMs during influenza infection¹⁹. Both mechanisms could conceivably contribute to the enhanced CCL8 observed in our model. Regardless, we can conclude from our data that the dysregulated immune response to HDM in neonatal mice with TGF-β1 deficiency in CD11c⁺ cells results in production of high levels of CCL8- derived partially from the inflammatory MPs that accumulate in these mice- which drives pathogenic T2 lymphocyte and airway remodelling responses to HDM via CCR8.

A previous study of the tolerogenic function of AMs in mice implicated AM-derived TGF-β as a mediator promoting FoxP3 expression in naïve CD4 T cells⁴⁶. However, this analysis of TGF- β function was performed using an *in vitro* co-culture system and the extent to which such interactions between AMs and naïve CD4 T cells occur during allergic sensitisation *in vivo* is unclear. Notably, augmented early life AAD in *Tgfb1*^{ΔCD11c} mice in our present study was not accompanied by a failure to accumulate FoxP3⁺ CD4 T cells in lung tissue, suggesting that this mechanism was not occurring in our neonatal *in vivo* model.

While our results in mice are complemented by RNA-Seq analysis supporting TGF-β1 as a key regulator of human AM gene expression in early life, it remains to be determined whether TGF-β1 signalling promotes maturation and restricts chemokine expression in these AMs to limit inflammatory responses to environmental stimuli and whether this is disrupted in childhood asthma. AM phenotype is governed by a combination of local tissue signals⁸, so it is likely that several factors control AM phenotype in the complex human setting. Further investigation into factors regulating macrophage maturity and phenotype in the paediatric

airway and their relationship to susceptibility to early life pulmonary inflammation is therefore warranted.

Early life presents a crucial period of vulnerability of the pulmonary immune system to infection and allergic sensitisation, but also a window of opportunity for therapeutic manipulation of immunity⁵. Better understanding of intrinsic and extrinsic regulators of the thresholds for initiation of protective and pathogenic immunity in the neonatal lung is therefore vital. We have identified a pivotal and previously unappreciated temporal role for TGF- β 1-dependent AM maturation in controlling the magnitude of immunological and pathological responses to neonatal HDM exposure. Our findings highlight a combinatorial relationship between TGF- β 1, AM maturation, the pulmonary chemokine repertoire, monocyte-derived cell recruitment and T2 immunity that sets the threshold for mounting pathogenic allergic responses in early life.

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Acknowledgements

We are grateful to Lorraine Lawrence for performing histological preparations and staining, Laura Yates for assistance with preparing photographs of histological sections, Nina Sanghani for assistance with patient recruiting, consenting and sampling, Christine Graham for assistance with RNA sequence library preparation and Aldara Martin Alonso for technical assistance with paediatric human sample analysis. We thank the Imperial College Flow Cytometry Facility and The Francis Crick Institute Advanced Sequencing and Bioinformatics Scientific Technology Platforms for their expert assistance. We thank Adam Byrne for critical reading of this work.

This work was supported by Wellcome Trust Senior Fellowship 107059/Z/15/Z to C.M.L. A.O.G. is employed by The Francis Crick Institute, which receives its core funding from Cancer Research UK (FC001126), the UK Medical Research Council (FC001126), and the Wellcome Trust (FC001126).

Author contributions

Conceptualization, C.M.L.; Methodology, W.J.B., J.C.; Investigation, W.J.B., J.C., R.A.O., S.A.W., N.B., H.S.; Formal Analysis, W.J.B., J.C.; Writing – Original Draft, W.J.B.; Writing – Review & Editing, A.O.G., C.M.L.; Visualization, W.J.B.; Funding Acquisition, C.M.L. Resources, M.M., S.S., A.O.G.; Supervision, A.O.G., S.S. and C.M.L.

Competing interests

The authors declare that they have no financial or commercial conflicts of interest.

Data availability statement

The RNA-Seq data supporting this study have been deposited in the GEO and are available at series GSE144033. The other datasets supporting this study are available from the corresponding author upon request.

Figure legends

Figure 1. CD11c⁺ cell-derived TGF- β 1 is required for airway macrophage maturation and maintenance in early life, but not their initial appearance in the lung

A-F) Lung cells from *Tgfb1* ^{Δ CD11c} and *Tgfb1*^{fl/fl} mice were analysed at the indicated ages by flow cytometry. **A)** AM numbers. **B)** Representative plots showing CD11c⁺ Siglec F⁺ AMs gated from CD45⁺ lineage⁻ (non-B/T/ILC/NK) cells. **C)** Geometric mean fluorescence intensity of Siglec F staining on AMs shown relative to *Tgfb1*^{fl/fl} controls at each time-point. **D)** Representative histograms showing Siglec F staining on AMs. **E)** Percentages of CD11b⁺ AMs. **F)** Representative plots showing CD11b expression on AMs. **G)** Expression of the indicated genes by qPCR in AMs isolated from P14 *Tgfb1* ^{Δ CD11c} and *Tgfb1*^{fl/fl} mice. Data are pooled from analysis of a minimum of two litters and are presented as means with either standard errors or all individual replicates. In **(A)**, **(C)** and **(E)**, *Tgfb1*^{fl/fl} points show data from 8 (P3), 16 (P7), 18 (P14) and 10 (P28) mice and *Tgfb1* ^{Δ CD11c} points show data from 8 (P3), 11 (P7), 7 (P14) and 7 (P28) mice. Statistical results shown are from student's unpaired t-tests: **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

Figure 2. TGF- β 1-induced genes are highly expressed in human paediatric AMs

RNA-Seq analysis of CD206⁺ AMs from bronchoalveolar lavage of eight paediatric subjects under investigation for either severe preschool wheeze (N=3) or chronic cough (N=5). **A)** Cytokine and growth factor regulators upstream of the 200 most highly expressed genes in all subjects predicted using Ingenuity Pathway Analysis. Fisher's exact *P* values are shown. **B)** Gene interaction network showing TGF- β 1-regulated genes from **(A)**, colour coded by reported positive and/or negative regulation by TGF- β 1 in the Ingenuity Knowledge Base. Arrows denote direction of effects on expression. **C)** Normalised RNA-Seq expression values of the Broad Institute MSigDB 'HALLMARK_TGF_BETA_SIGNALING' gene set in paediatric AM samples. Symbol shapes indicate patient diagnosis. Means and individual replicate values are shown.

Figure 3. Inflammatory CD11c⁺ mononuclear phagocytes accumulate following neonatal HDM exposure in *Tgfb1*^{ΔCD11c} mice

A) Neonatal AAD model schematic. **B-H)** Flow cytometry analysis of pulmonary MP populations. **B)** Representative plots showing subpopulations of CD64⁺ F4/80⁺ MPs in BAL after HDM treatment, distinguished based on Siglec F expression. **C)** Representative images of cells from populations in **(B)**. **D)** Numbers of SigF⁺ AM-like cells, SigF⁻ MPs and combined populations in BAL. **E)** Relative qPCR gene expression in sorted BAL MP populations. **F)** Geometric mean fluorescence intensity of Siglec F staining on AMs/SigF⁺ AM-like cells, relative to the *Tgfb1*^{fl/fl}/PBS group. **G)** CD11b staining on BAL CD11c⁺ MP populations. **H)** Percentages of BAL MP populations with positive staining for the indicated surface markers compared to lung Ly6C^{hi} monocytes. Scatter/bar plots show means or medians of data pooled from 2-4 individual experiments, with all individual replicates. Statistical results shown are from one-way ANOVA with Sidak's *post hoc* test for multiple comparisons and either student's unpaired t-tests or Mann-Whitney U tests for single comparisons. *, *P* < 0.05; **, *P* < 0.01; ***, *P* < 0.001; ****, *P* < 0.0001; ns, non-significant.

Figure 4. Inflammatory CD11c⁺ mononuclear phagocyte accumulation in HDM-treated *Tgfb1*^{ΔCD11c} mice is CCR2-dependent

A) CCR2 blocking (αCCR2) experiment schematic. **B)** Proportion of Ly6C^{hi} monocytes in peripheral blood as determined by flow cytometry. **C)** Total cell counts in BAL. **D)** Numbers of SigF⁺ AM-like cells and SigF⁻ MPs in BAL as determined by flow cytometry. Scatter/bar plots show data pooled from two individual experiments and show means or medians with all individual replicates. Statistical results shown are from one-way ANOVA with Sidak's *post hoc* test or Kruskal-Wallis with Dunn's *post hoc* test. *, *P* < 0.05; **, *P* < 0.01; ***, *P* < 0.0001; ns, non-significant.

Figure 5. Dysregulated production of monocyte-attractant chemokines in *Tgfb1*^{ACD11c}**mice**

A-B) Concentrations of the indicated chemokines in BAL (**A**) or lung tissue (**B**) as determined by ELISA. Dashed line indicates limit of detection. **C)** Relative qPCR gene expression of the indicated chemokine genes in sorted BAL MP populations. **D-E)** BAL was obtained from naïve P14 mice and chemokine concentration measured in BAL fluid by ELISA (**D**) and gene expression determined in adhesion-purified AMs by qPCR (**E**). **F)** Chemokine concentrations in supernatants of bone marrow-derived macrophages cultured $\pm$ TGF- β 1, determined by ELISA. (**A**) shows results from a single assay using BAL fluid pooled from three mice per group. Scatter/bar plots in (**A-E**) show means or medians and all individual replicates. (**F**) shows results from one of two experiments with similar results. Statistical results shown are from either student's unpaired t-tests or Mann-Whitney U tests. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

Figure 6. *Tgfb1*^{ACD11c} mice have more severe allergic airway disease after neonatal HDM exposure

A) Total BAL and lung tissue cell counts. **B)** BAL and lung eosinophil counts determined by flow cytometry. **C)** Representative images of H&E-stained lung sections. Scale bar = 200 μ m. **D)** Representative plots showing intracellular cytokine staining in lung CD4 T cells. **E)** Numbers of cytokine-positive lung CD4 T cells and ILCs. **F)** Concentrations of IL-13 in BAL and lung tissue. Dashed lines show ELISA limit of detection. **G)** Concentrations of total IgE and IgG1 in serum. **H)** Area under curve analysis of HDM-specific IgE and IgG1 serum ELISAs. **I)** Representative images and quantification of peribronchiolar reticulin black fibres). Scale bar = 75 μ m. **J)** Concentration of soluble collagen in lungs. Scatter/bar plots show means or medians and individual replicate values and are pooled from 2-4 experiments, except for **(E)**, which shows one of two representative experiments. Statistical results shown are from either student's unpaired t-tests or Mann-Whitney U tests. *, $P < 0.05$; **, $P < 0.01$ and ***, $P < 0.001$.

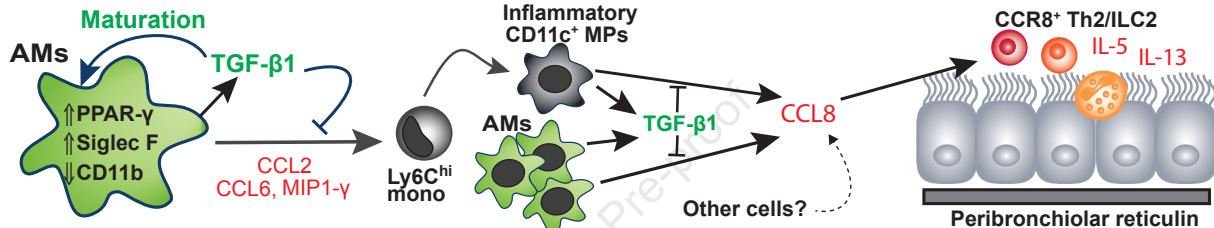
Figure 7. CCR8 signalling mediates enhanced inflammatory phenotypes in HDM-treated *Tgfb1*^{ΔCD11c} mice

A) Quantification of chemokine immunoblot on BAL of HDM-treated mice. **B)** BAL CCL8 measured by ELISA. **C)** Relative qPCR gene expression of *Ccl8* in sorted BAL MP populations. **D)** CCL8 concentrations in supernatants of bone marrow-derived macrophages cultured $\pm$ TGF- β 1 and IL-4, IL-13 and LPS. **E)** Representative plots showing CCR8 surface staining compared to fluorescence minus one controls. Plots are representative of 13 *Tgfb1*^{fl/fl} and 9 *Tgfb1*^{ΔCD11c} mice from two experiments. **F)** CCR8 blocking (α CCR8) experiment schematic. **G)** Numbers of IL-13⁺ and IL-5⁺ CD4 T cells and ILCs in lung tissue. **H)** IL-13 concentrations in BAL. **I)** Quantification of peribronchiolar reticulin. **(A)** shows results from a single assay pooling BAL from three mice per group. Data in **(D)** are from one of two experiments with similar results. Remaining data are pooled from two experiments. Plots show means or medians and individual replicates. Statistical results shown are from one-way ANOVA with Sidak's *post hoc* test for multiple comparisons and either student's unpaired t-tests or Mann-Whitney U tests for single comparisons. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$; ns, non-significant.

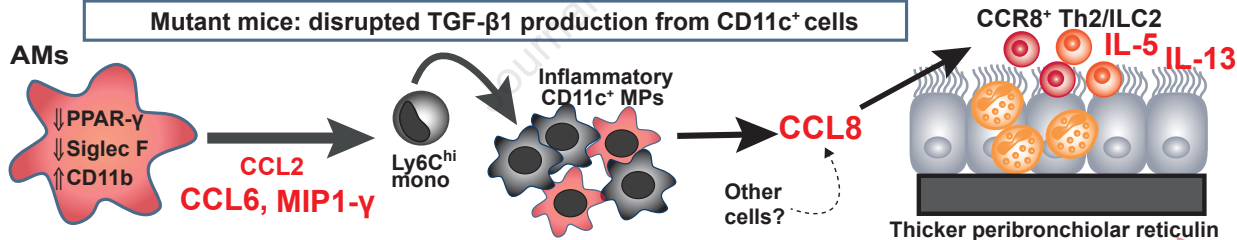


Airway macrophage-intrinsic TGF- β 1 regulates pulmonary immunity during early life allergen exposure

Control mice: intact TGF- β 1 production from CD11c⁺ cells



Mutant mice: disrupted TGF- β 1 production from CD11c⁺ cells



Steady state

Early life house dust mite exposure

Abbreviations:

AM, airway macrophage
MP, mononuclear phagocyte
Mono, monocyte

Key:

Monocyte



Macrophage/
Inflammatory MP



Th2 cell



Group
2 innate
lymphoid cell

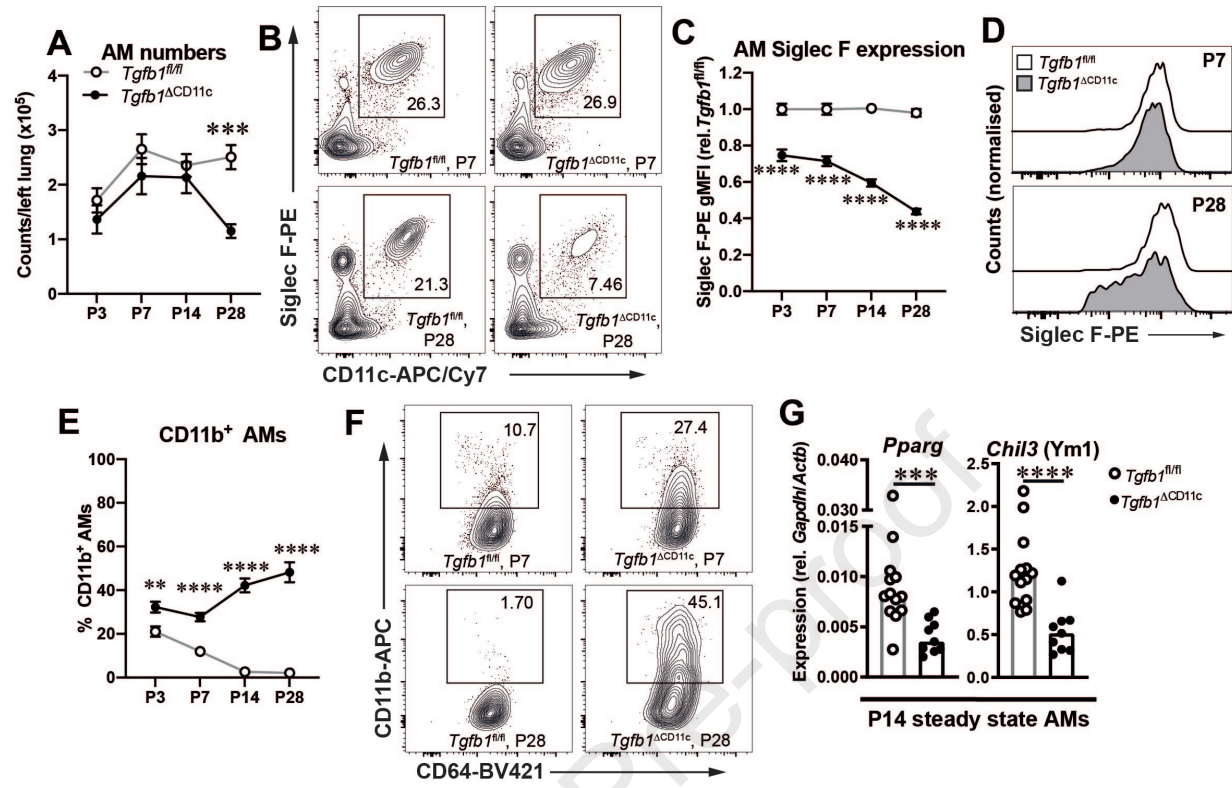


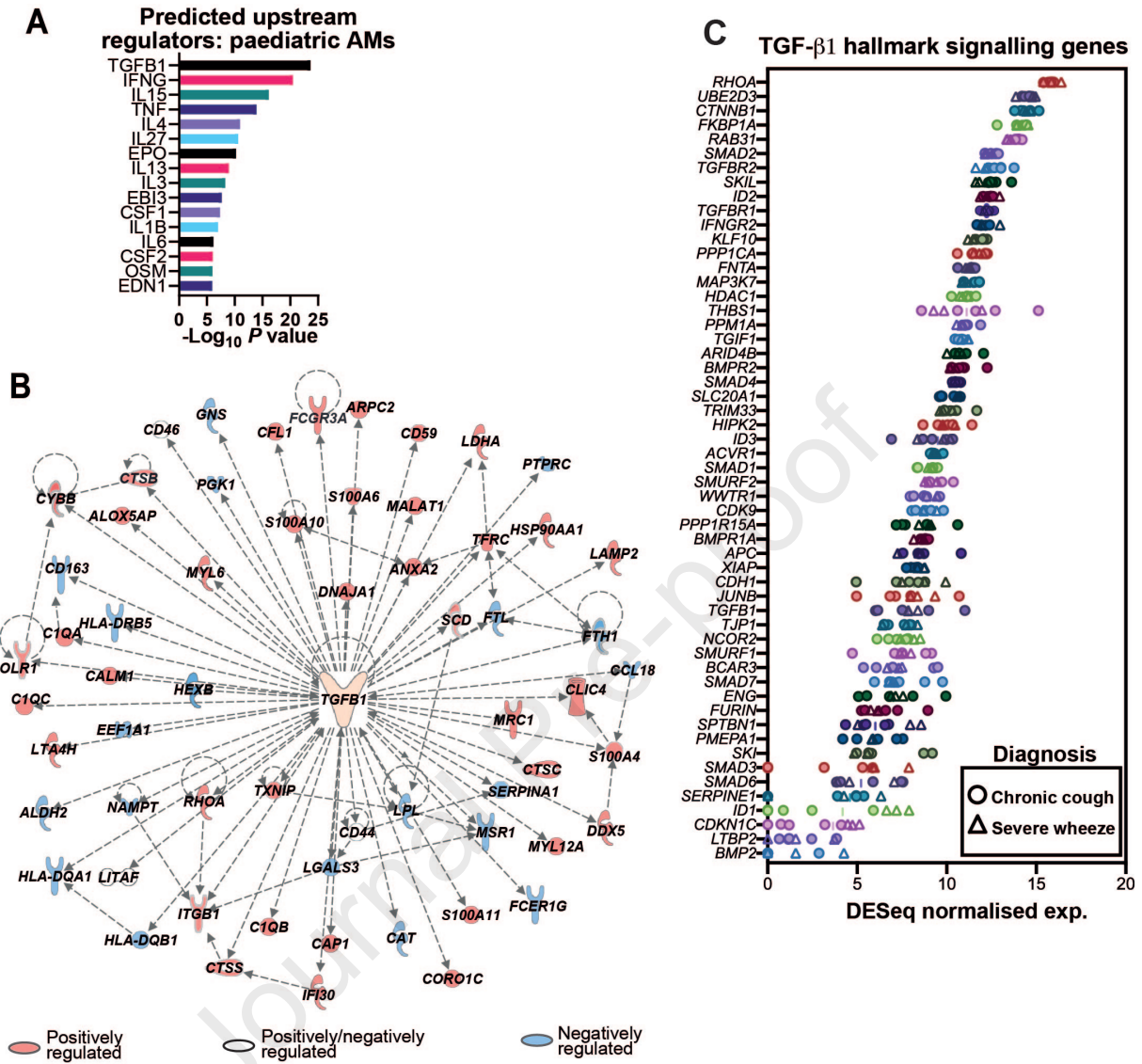
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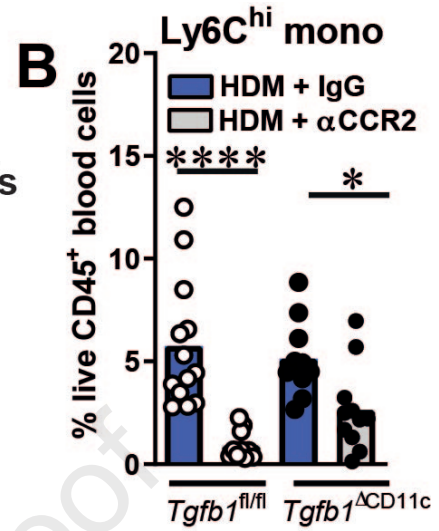
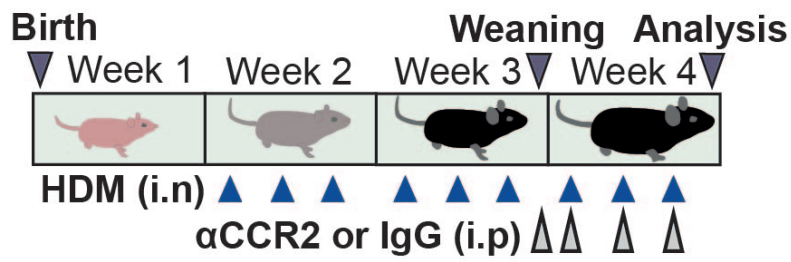
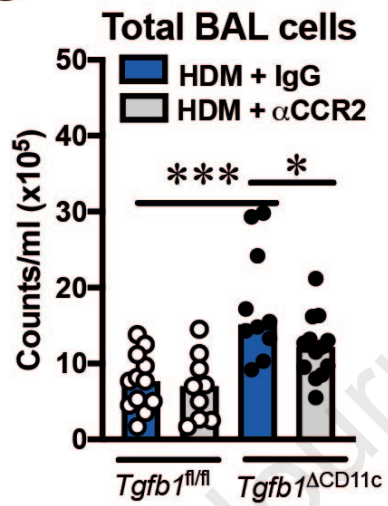


Airway
epithelial cell







A**C****D**