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C5aR1 functions in cellular networking of ILC2 in health and disease

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Table of Contents

Summary ... **1**

Zusammenfassung ... **4**

1 Introduction ... **8**

1.1 The immune system ... **8**

1.1.1 Adaptive Immune System ... 9
1.1.2 Innate Immune System ... 12
1.1.2.1 Cellular compartment of the innate immune system ... 13

1.2 The Complement System ... **30**

1.2.1 Non-Canonical activation of C3 and C5 ... 31
1.2.2 Role of Anaphylatoxins ... 34

1.3 Asthma ... **36**

1.3.1Cellular aspects of allergic Asthma ... 36

1.4 Lung Development ... **40**

1.5 AIMS ... **41**

2 . Material, Equipment, and Methods ... **42**

2.1Materials ... **42**

2.1.1 Chemicals ... 42
2.1.2 Antibodies for flow cytometry, immunofluorescence staining ... 45
2.1.3 Compounds used for flow cytometry ... 49
2.1.4 Consumables ... 50
2.1.5 Kits ... 52
2.1.6 Buffers and Solutions ... 52
2.1.7 Mouse strains ... 54
2.1.8 Equipment and Software ... 55

2.2 Methods ... **58**

2.2.1 Animals ... 58
2.2.2 Intratracheal application of substances ... 58
2.2.3 Models of experimental allergic asthma ... 59
2.2.4 Dysbiosis Models for lung development ... 59
2.2.5 Cell harvesting preparation from different organs ... 60
2.2.6 Analysis and purification of cell populations by flow cytometry and fluorescence-activated cell sorting ... 62
2.2.7 In vitro Developmental models using a co-culture system ... 65
2.2.8 Measurement of Airway Hyperresponsiveness ... 69
2.2.9 ELISA-based assays ... 69
2.2.10 Histology ... 70
2.2.11 Statistical Analysis ... 73

3 Results ... **74**

3.1 C5aR1 regulates the function of ILC2s ... **74**

3.2 Neither ILC2 nor ILC2p express C5aR1 at steady state ... **76**

3.3 C5a/C5aR1 axis enhances the ILC2 development through its function in BM-M-DC ... **77**

3.4 C5a/C5aR1 signaling axis in BM-M-DCs promotes proliferation and differentiation of ILC2 . **80**

3.5 C5a/C5aR1 axis regulates the ILC regulatory genes in a time-dependent manner 83
3.6 C5aR1 expression in BM-M-DCs impacts ILC2 STATs phosphorylation status via IL-23 84
3.7 BM-M-DCs regulate the phosphorylation of the STAT pathway in a time-dependent manner 88
3.8 C5a supports the pulmonary ILCs development through its function in MHC II cells............ 89
3.9 C5a supports the pulmonary myeloid cells-triggered ILCs development 91
3.10 C5a favors the survival of ILC2 and maturation of ILC2 93
3.11 C5a /C5aR1 axis on pulmonary myeloid cell independent from STAT4 phosphorylation 94
3.12 C5a/ C5aR1 signaling in DC regulates TSLP-R expression 94
3.13 C5a/C5aR1 signaling axis drives development of ILC2 upon crosstalk 95
3.14 C5a/C5aR1 signaling triggers IL-5 production by ILC2 upon AM crosstalk 97
3.15 Tonic C5/C5a production by AMs participates in ILC2 development 99
3.16 Tonic C5/C5s sustains AM driven IL-5 induction in ILC2 99
3.17 AM-derived soluble factors are required for ILC2 development..................................... 101
3.18 DC required cell-to-cell contact to regulate ILC2 104
3.19 C5aR1 might play role in airway function and cell recruitment during sensitization 104
3.20 C5aR1 might have an impact on the lung development.. 106
3.21 Early life exposure has a nonrecoverable effect on the small intestine 107
3.22 Early life exposure to ABX changes immune cell composition in the airway.................... 110
3.23 Early-life dysbiosis has a strong impact on lung inflammation and may associate with the accumulation of inflammatory Eosinophils 111
3.24 Eosinophilic subpopulations recruitment depends on IL-5... 114
3.25 ILC2s are not only potent source for IL-5 during pulmonary development 115
3.26 Early-life dysbiosis has an impact neither on the ILC2 progenitors nor ILC2s in bone marrow and spleen 117
3.27 Gut dysbiosis alters neutrophils accumulation in the lung... 119
3.28 Gut dysbiosis alters effector T cells in the spleen 120
3.28 Dysbiosis exacerbates baseline airway function.. 121
4 Discussion... 122
4.1 Expression of the C5aR1 in lymphoid cells in general and ILCs in particular 123
4.2 C5a/C5aR1 signaling in ILC2 in Allergic Asthma 124
4.3 Cellular network of ILC2.. 125
4.4 Phosphorylation of STATs on ILC regulation 126
4.5 C5a triggered phosphorylation of the STAT4 activated with IL-23.................................... 127
4.6 Classical ILC plasticity paradigm 129
4.7 C5aR1 signaling axis during ILC2-Macrophage crosstalk 129
4.8 C5a/C5aR1 axis regulates the ILC2 DC crosstalk 131

4.9 Immune cells in postnatal lung development 132
4.10 Gut -Lung axis during development 132
4.11 Conclusion and Future perspective 133
5 Abbreviations and symbols 137
List of Tables 143
List of Figures 144
List of Supplementary Figures 145
6 References 146
7 Supplementary Figures 165
8 Acknowledgement 168
CURRICULUM VITAE 170
CONFERENCE CONTRIBUTIONS 172
POSTER PRESENTATIONS 172
ORAL PRESENTATIONS 172
LIST OF PUBLICATIONS 174

Summary

Worldwide more than 300 million people are affected by asthma, a heterogeneous disease characterized by chronic airway inflammation. Allergic asthma is the most common asthma phenotype and is characterized by an inappropriate Type 2 immune response to common airborne allergens, including Eosinophilia, airway hyperresponsiveness (AHR), airway obstruction and inflammation, intermittent airflow, and mucus production. Although the past decades have witnessed progress in unraveling the cellular and molecular mechanism of the immune regulation of asthma, details are still unknown. Less than 10 years ago, a new set of innate immune cells was identified, the Innate Lymphoid cells (ILCs). The Innate Lymphoid cells type 2 (ILC2) are thought to play a key role during allergen sensitization by secretion of Th2 cytokines. In addition, soluble factors generated upon complement system activation or via allergen-driven proteolytic cleavage, the anaphylatoxins C3a and C5a, have been recognized as potent regulators of both the development and severity of the disease via their binding to their cognate receptors C3aR, C5aR1, and C5aR2. Interestingly, while C3a/C3aR and dendritic cells have been recognized as important regulators of ILC2 functions in allergic asthma animal models and patients suffering from allergic rhinitis, the role of C5a in an ILC2 development and functions remains poorly understood.

Starting with an evaluation of the impact of C5aR1 on ILC2 mediated function using intracellular staining for IL-5 on ILC2 in *C5ar1*$^{-/-}$ and WT mice exposed intratracheally (i.t.) once to house dust mite extracts (HDM), I observed a reduced expression of IL-5 in *C5ar1*$^{-/-}$ ILC2, compared to WT control, suggesting that C5aR1 regulates the ILC2 function. In line, former data from our group showed that the recruitment of inflammatory Eosinophils (iEOS) was dampened in the *C5ar1*$^{-/-}$ context upon a single HDM exposure. To track the C5aR1 expression, I used novel GFP-*C5ar1*$^{\text{flox/flox}}$ reporter knock-in mouse, antibody (C5aR1/CD88 clone 20/70) staining, and mRNA analysis. I could not detect an expression of C5aR1 either in ILC2 from different compartments such as the lung, spleen, bone marrow (BM), and Peyer's Patches or in their progenitors (ILC2p) population in the lung and the BM. These observations suggested that C5a does not signal directly in ILC2s, but rather functions through an intermediate cell.

Then, I established an *in-vitro* co-culture to investigate the impact of the C5a-induced cell signaling on myeloid cells in the development and function of ILC2. Using bone marrow (BM)-derived ILCp and macrophage/dendritic cells (BM-M-DCS) to investigate the impact of the C5a-induced cell signaling on myeloid cells in the development and functions of ILC2. Firstly, I observed that, in vitro, bone marrow (BM)-derived macrophage/dendritic cells (BM-M-DC)

promoted ILC2 development. Interestingly, in the presence of C5a, the differentiation of ILC2 was improved, while C5a had no direct effect on ILCp. Mechanistically, C5a-activated BM-M-DCs strengthened the expression of the transcription factor GATA3 hence favoring the differentiation of ILC2 after 24h of co-culture, and KLRG-1, thus increasing their maturation. As ILC development is driven by STAT proteins, I evaluate their phosphorylation status by flow cytometry. Intriguingly, I observed that C5a triggered BM-M-DCs favor the phosphorylation of STAT4 in ILC2 rather than STAT3 or STAT6. Mechanistically, STAT4 phosphorylation depends on the C5a-driven secretion of IL-23 by BM-M-DCs. As STAT4 is commonly associated with the regulation of the master transcription factor of the ILC1, Tbet, I followed the differentiation of ILCs for a longer period. Early on, at 24h of co-culture, the majority of the cells were ILC2 and ILC3. However, the number of ILC3 decreased gradually. While the ILC2 phenotype of cells persisted up to 48h after co-culture started, it shifted to ILC1 after 72 hours. However, this effect was independent of C5a. Nonetheless, these data suggested that BM-M-DCs play a role in plasticity between ILCs.

As it is now well accepted that BM-derived cells differ significantly from their pulmonary counterparts, I investigated the C5aR1 function on ILC2s using primary sorted pulmonary MHC class II (MHC-II) expressing cells, encompassing macrophages, DCs, and some alveolar epithelial cells. In line with the BM-derived cells experiments, MHC-II expressing cells drove ILC2 formation and the effect was strengthen in presence of C5a. To delineate the nature of the MHC-II$^+$ cells involved in this C5a-driven regulation, I sorted alveolar macrophages (AMs), dendritic cells, and interstitial macrophages (IMs) and repeated the co-cultures. Interestingly, although interstitial macrophages do not take a part in ILC2 development, the C5a/C5aR1 signaling axis on pulmonary DCs and alveolar macrophages was required for the ILC2 development and their function, as C5a triggered alveolar macrophages and DCs promoted the production of IL-5. Mechanistically, C5a triggered the secretion of IL-1α by the AMs and then the differentiation/functionality, while DCs participated in the ILC2 differentiation via cell-cell contacts. Collectively, my data give a first insight into C5a/C5aR1 function on AMs/DC-driven ILC2 development and functions thus opening some new therapeutic approaches to limit the recruitment of inflammatory Eosinophil (iEOS) during sensitization to allergens.

Interestingly, iEOS, not only accumulate in the lung after allergen exposure but is also found during early postnatal lungs. Therefore, I investigated the C5aR1 function on iEOS recruitment during post-natal lung development. My data showed that the accumulation of iEOS, at PND14 the reported peak of iEOS influx in the postnatal lung, significantly increase in *C5ar1*$^{-/-}$ mice compared to WT. In addition to C5aR1, numerous pieces of evidence outlined the existence of

windows during pregnancy and early in life during which environmental factors, including maternal microbiota, have a major role in shaping neonates' immunity. In agreement, the idea that early-life antibiotic (ABX) exposure may be associated with a higher risk of developing asthma later in life has been proposed. To investigate short- and long-term effects of ABX exposure on neonate pulmonary development and functions, mothers were exposed to three courses of widely used ABX (streptomycin vancomycin and ampicillin) or sucralose alone (Suc) as control from 10 days from postnatal day P10-20 via drinking water. Then, the cellular composition of the lungs was determined at different time points by flow cytometry and structural changes in lung offspring were appreciated by histological examination of lung sections. Interestingly, while Suc lungs showed an increase in iEOS infiltration at P14, I observed a significant increase upon ABX exposure, while the number of neutrophils, that peaked at the same time in controls, were lower upon ABX compared to Suc. Intriguingly, such a significant accumulation of iEOS was dependent on IL-5 production, but not necessarily from the innate lymphoid cells type 2 suggesting that ILC2 may play a more limited role in the iEOS influx during the postnatal lung development in comparison to sensitization. Additionally, Periodic Acid Shiff staining of sections showed that at P14 mucus secretion and alveolar damage were higher in neonates from ABX-exposed mothers compared to the control. In the long run, at P28, airway functions measurement by forced oscillatory technic upon methacholine trigger showed, in the ABX exposed group, an increase of sensitivity in the small and the total airway compared to Suc controls. Interestingly, this group was also characterized by marked and significant pulmonary neutrophilia. Finally, I also observed, using measures of intestine length between P14-28 and H&E scoring at P56, that maternal dysbiosis during the early life of offspring causes recoverable and unrecoverable damages to the gastrointestinal tract in the long run. Altogether my data suggest that indirect early life exposure to antibiotic exposure may facilitate the development of allergic asthma through impacts on cellular recruitment of cells in the lung and structural damage to the gastrointestinal tract.

Overall, the different studies I performed identified C5a/C5aR1 as an important signaling axis for the development and functions of pulmonary innate lymphoid cells, with a consequence in the recruitment of inflammatory Eosinophils in the lung upon allergen exposure. In contrast, during post-natal lung development, C5a/C5aR1 may have an important role in limiting the recruitment of iEOS into the lung, a phenomenon that I also observed in neonates whose mothers have been exposed to antibiotics. Thus C5a/C5aR1 signaling axis may display contrasting functions during life favoring the development of allergic diseases.

Zusammenfassung

Weltweit sind mehr als 300 Millionen Menschen von Asthma betroffen, einer heterogenen Krankheit, die durch eine chronische Entzündung der Atemwege gekennzeichnet ist. Allergisches Asthma ist der häufigste Asthma-Phänotyp und zeichnet sich durch eine unangemessene Typ-2-Immunreaktion auf häufige Allergene in der Luft aus, einschließlich Eosinophilie, Hyperreagibilität der Atemwege (AHR), Obstruktion und Entzündung der Atemwege, intermittierender Luftstrom und Schleimproduktion.

Obwohl in den letzten Jahrzehnten Fortschritte bei der Entschlüsselung der zellulären und molekularen Mechanismen der Immunregulation von Asthma erzielt wurden, sind die Einzelheiten noch immer unbekannt. Vor weniger als 10 Jahren wurde eine neue Gruppe von angeborenen Immunzellen identifiziert, die angeborenen lymphatischen Zellen (ILCs). Es wird angenommen, dass die angeborenen lymphoiden Zellen vom Typ 2 (ILC2) durch die Sekretion von Th2-Zytokinen eine Schlüsselrolle bei der Allergensensibilisierung spielen. Darüber hinaus sind lösliche Faktoren, die bei der Aktivierung des Komplementsystems oder durch allergenbedingte proteolytische Spaltung entstehen, die Anaphylatoxine C3a und C5a, über ihre Bindung an die entsprechenden Rezeptoren C3aR, C5aR1 und C5aR2 als wirksame Regulatoren sowohl der Entwicklung als auch des Schweregrads der Erkrankung erkannt worden. Während C3a/C3aR und dendritische Zellen als wichtige Regulatoren der ILC2-Funktionen in Tiermodellen für allergisches Asthma und bei Patienten mit allergischer Rhinitis anerkannt sind, ist die Rolle von C5a bei der Entwicklung und den Funktionen von ILC2 noch wenig bekannt. Ausgehend von einer Bewertung des Einflusses von C5aR1 auf die durch ILC2 vermittelte Funktion mittels intrazellulärer Färbung von IL-5 auf ILC2 in C5ar1-/- und WT-Mäusen, die einmalig intratracheal (i.t.) Hausstaubmilbenextrakten (HDM) ausgesetzt wurden, beobachtete ich eine verringerte Expression von IL-5 in C5ar1-/- ILC2 im Vergleich zur WT-Kontrolle, was darauf hindeutet, dass C5aR1 die ILC2-Funktion reguliert. Im Einklang damit zeigten frühere Daten unserer Gruppe, dass die Rekrutierung von entzündlichen Eosinophilen (iEOS) im C5ar1-/--Kontext nach einer einmaligen HDM-Exposition gedämpft war. Mit Hilfe von GFP-C5ar1flox/flox-Reporter-Knock-in-Mäusen, Antikörperfärbung (C5aR1/CD88-Klon 20/70) und mRNA-Analyse konnte ich weder in ILC2 aus verschiedenen Kompartimenten wie Lunge, Milz, Knochenmark (BM) und Peyer's Patches noch in deren Vorläuferpopulationen (ILC2p) in Lunge und BM eine Expression von C5aR1

nachweisen. Diese Beobachtungen deuten darauf hin, dass C5a nicht direkt in ILC2s Signale übermittelt, sondern über eine Zwischenzelle wirkt.
Anschließend habe ich eine In-vitro-Co-Kultur angelegt, um die Auswirkungen der C5a-induzierten Zellsignalisierung auf myeloische Zellen auf die Entwicklung und Funktion von ILC2 zu untersuchen. Mit aus dem Knochenmark (BM) stammenden ILCp und Makrophagen/dendritischen Zellen (BM-M-DCS) wurden die Auswirkungen der C5a-induzierten Zellsignalisierung auf myeloische Zellen auf die Entwicklung und Funktion von ILC2 untersucht. Zunächst stellte ich fest, dass *in vitro* aus dem Knochenmark (BM) stammende Makrophagen/dendritische Zellen (BM-M-DC) die Entwicklung von ILC2 fördern. Interessanterweise wurde in Gegenwart von C5a die Differenzierung von ILC2 verbessert, während C5a keine direkte Wirkung auf ILCp selbst hatte. Mechanistisch gesehen verstärkten C5a-aktivierte BM-M-DCs die Expression des Transkriptionsfaktors GATA3, wodurch die Differenzierung von ILC2 nach 24 Stunden Co-Kultur begünstigt wurde, und von KLRG-1, wodurch deren Reifung gefördert wurde. Da die Entwicklung der ILCs durch STAT-Proteine gesteuert wird, habe ich ihren Phosphorylierungsstatus mittels Durchflusszytometrie untersucht. Erstaunlicherweise konnte ich feststellen, dass C5a-ausgelöste BM-M-DCs die Phosphorylierung von STAT4 in ILC2 gegenüber STAT3 oder STAT6 begünstigen. Mechanistisch gesehen hängt die STAT4-Phosphorylierung von der C5a-getriebenen Sekretion von IL-23 durch BM-M-DCs ab. Da STAT4 in der Regel mit der Regulierung des Haupttranskriptionsfaktors von ILC1, T--bet, in Verbindung gebracht wird, habe ich die Differenzierung der ILCs über einen längeren Zeitraum verfolgt. Zu Beginn, d. h. nach 24 Stunden Co-Kultur, bestand die Mehrheit der Zellen aus ILC2 und ILC3, doch die Zahl der ILC3 nahm allmählich ab. Während der ILC2-Phänotyp der Zellen bis zu 48 Stunden nach Beginn der Co-Kultur bestehen blieb, verschob er sich nach 72 Stunden zu ILC1. Dieser Effekt war jedoch unabhängig von C5a. Nichtsdestotrotz deuten diese Daten darauf hin, dass BM-M-DCs eine Rolle bei der Plastizität zwischen ILCs spielen.
Da inzwischen allgemein anerkannt ist, dass sich Zellen aus dem Knochenmark deutlich von ihren pulmonalen Pendants unterscheiden, untersuchte ich die C5aR1-Funktion auf ILC2 unter Verwendung primärer sortierter pulmonaler MHC-Klasse-II-Zellen (MHC-II), die Makrophagen, DCs und einige Alveolarepithelzellen umfassen. In Übereinstimmung mit den Experimenten mit aus dem Knochenmark stammenden Zellen trieben MHC-II-exprimierende Zellen die Bildung von ILC2 an, und der Effekt wurde in Gegenwart von C5a verstärkt. Um die Art der MHC-II^{+}-Zellen zu bestimmen, die an dieser C5a-gesteuerten Regulierung beteiligt sind, habe ich Alveolarmakrophagen (AMs), dendritische Zellen und

interstitielle Makrophagen (IMs) sortiert und die Co-Kulturen wiederholt. Obwohl intestinale Makrophagen keine Rolle bei der Entwicklung von ILC2 spielen, war interessanterweise die C5a/C5aR1-Signalachse auf pulmonalen DCs und alveolaren Makrophagen für die Entwicklung von ILC2 und deren Funktion erforderlich, da C5a Stimulation alveolare Makrophagen und DCs aktivierte und diese die Produktion von IL-5 förderten. Mechanistisch gesehen löste C5a die Sekretion von IL-1 durch die AMs und dann die Differenzierung/Funktionalität aus, während DCs über Zell-Zell-Kontakte an der Differenzierung der ILC2 beteiligt waren.

Insgesamt geben meine Daten einen ersten Einblick in die Funktion von C5a/C5aR1 auf die AMs/DC-gesteuerte ILC2-Entwicklung und -Funktionen und eröffnen damit einige neue therapeutische Ansätze zur Begrenzung der Rekrutierung von iEOS während der Sensibilisierung auf Allergene.

Interessanterweise reichern sich iEOS nicht nur in der Lunge nach Allergenexposition an, sondern sind auch in der frühen postnatalen Lunge zu finden. Daher untersuchte ich die Funktion von C5aR1 bei der iEOS-Rekrutierung während der postnatalen Lungenentwicklung. Meine Daten zeigten, dass die Anhäufung von iEOS, bei PND14, dem gemeldeten Zeitpunkt des iEOS-Einstroms in die postnatale Lunge, bei C5ar1-/--Mäusen im Vergleich zu WT signifikant zunimmt. Neben C5aR1 gibt es zahlreiche Belege für die Existenz von Zeitfenstern während der Schwangerschaft und in der frühen Lebensphase, in denen Umweltfaktoren, einschließlich der mütterlichen Mikrobiota, eine wichtige Rolle bei der Gestaltung der Immunität der Neugeborenen spielen. In Übereinstimmung damit wurde vorgeschlagen, dass eine frühzeitige Antibiotikaexposition (ABX) mit einem höheren Risiko für die Entwicklung von Asthma im späteren Leben verbunden sein könnte. Um die kurz- und langfristigen Auswirkungen einer ABX-Exposition auf die Lungenentwicklung und -funktionen von Neugeborenen zu untersuchen, wurden die Mütter 10 Tage lang ab dem postnatalen Tag P10-20 über das Trinkwasser drei weit verbreiteten ABX (Streptomycin, Vancomycin und Ampicillin) oder Sucralose allein (Suc) als Kontrolle ausgesetzt. Anschließend wurde die zelluläre Zusammensetzung der Lungen zu verschiedenen Zeitpunkten mittels Durchflusszytometrie bestimmt, und strukturelle Veränderungen in den Lungen der Nachkommen wurden durch histologische Untersuchung von Lungenschnitten beurteilt. Interessanterweise zeigte sich in den Suc-Lungen eine Zunahme der iEOS-Infiltration bei P14, während die Zahl der Neutrophilen, die bei den Kontrollen zum gleichen Zeitpunkt ihren Höhepunkt erreichte, bei ABX im Vergleich zu Suc geringer war. Interessanterweise war eine solch signifikante Anhäufung von iEOS von der IL-5-

Produktion abhängig, aber nicht notwendigerweise von den angeborenen lymphoiden Zellen des Typs 2, was darauf hindeutet, dass ILC2 eine geringere Rolle beim iEOS-Einstrom während der postnatalen Lungenentwicklung im Vergleich zur Sensibilisierung spielen könnte. Darüber hinaus zeigte die PAS-Färbung (*Periodic acid-Shiff stain*) von Schnitten, dass bei P14 die Schleimsekretion und die alveoläre Schädigung bei Neugeborenen von ABX-exponierten Müttern höher waren als bei der Kontrollgruppe. Langfristig, bei P28, zeigte die Messung der Atemwegsfunktionen durch forcierte oszillatorische Technik bei Methacholin-Auslösung in der ABX-exponierten Gruppe eine Erhöhung der Empfindlichkeit in den kleinen und den gesamten Atemwegen im Vergleich zu den Suc-Kontrollen. Interessanterweise war diese Gruppe auch durch eine deutliche und signifikante pulmonale Neutrophilie gekennzeichnet. Schließlich beobachtete ich anhand der Messung der Darmlänge zwischen P14-28 und der H&E-Bewertung bei P56, dass die mütterliche Dysbiose während des frühen Lebens der Nachkommen wiederherstellbare und langfristig nicht wiederherstellbare Schäden im Magen-Darm-Trakt verursacht. Insgesamt deuten meine Daten darauf hin, dass eine indirekte frühkindliche Antibiotikaexposition die Entwicklung von allergischem Asthma durch Auswirkungen auf die zelluläre Rekrutierung von Zellen in der Lunge und strukturelle Schäden im Gastrointestinaltrakt begünstigen kann. Insgesamt ergaben die verschiedenen von mir durchgeführten Studien, dass C5a/C5aR1 eine wichtige Signalachse für die Entwicklung und die Funktionen der angeborenen lymphatischen Zellen in der Lunge darstellt, was sich in der Rekrutierung von entzündlichen Eosinophilen in der Lunge bei Allergenexposition niederschlägt. Im Gegensatz dazu könnte C5a/C5aR1 während der postnatalen Lungenentwicklung eine wichtige Rolle bei der Begrenzung der Rekrutierung von iEOS in die Lunge spielen, ein Phänomen, das ich auch bei Neugeborenen beobachtet habe, deren Mütter Antibiotika ausgesetzt waren. Somit kann die C5a/C5aR1-Signalachse im Laufe des Lebens gegensätzliche Funktionen aufweisen, die die Entwicklung von allergischen Erkrankungen begünstigen.

1 Introduction

1.1 The immune system

The word “immunity” originates from the Latin “*immunis*” meaning “exempt” of something, in our context exempt of infection. The first known literature example of the concept of exemption of infection after primoinfection dates back to 430 BC when Thucydides reported that people who had recovered from the Athens plague could nurse the infected patients without contracting the disease a second time [1].

The immune system consists of many layers of defense including physical barriers, lymphoid organs, humoral factors, and cellular components. The central function of the immune system is to eliminate threats through its ability to discriminate between antigens on its own cell “self” and foreign “non-self” materials. After recognition of both endogenous and exogenous threats via sensor molecules, the immune system builds up an effector response by recruiting various immune cells to the inflamed tissue [2]. Further, immune defense is mainly divided into three layers according to the time and duration of the response, cell types, and specificity: i) anatomic and physiological barriers, ii) innate immune system, and iii) adaptive immune system [3, 4].

The first line of defense includes physical and chemical barriers that are always ready to protect the body against invading microorganisms and to clear any remaining danger that may affect the host’s barrier. Physical barriers encompass skin and mucosal lining of the respiratory and gastrointestinal mucosal surface, sweat and saliva glands and their protective chemical secretions, low stomach pH, and bacteriolytic lysozyme coming from tears, saliva, and milk that prevent the pathogen from entering the body [5]. Then, the innate and adaptive immunity orchestrate the cells and humoral response which tightly work together to recognize and clear any threat that succeeds in crossing the physical barriers.

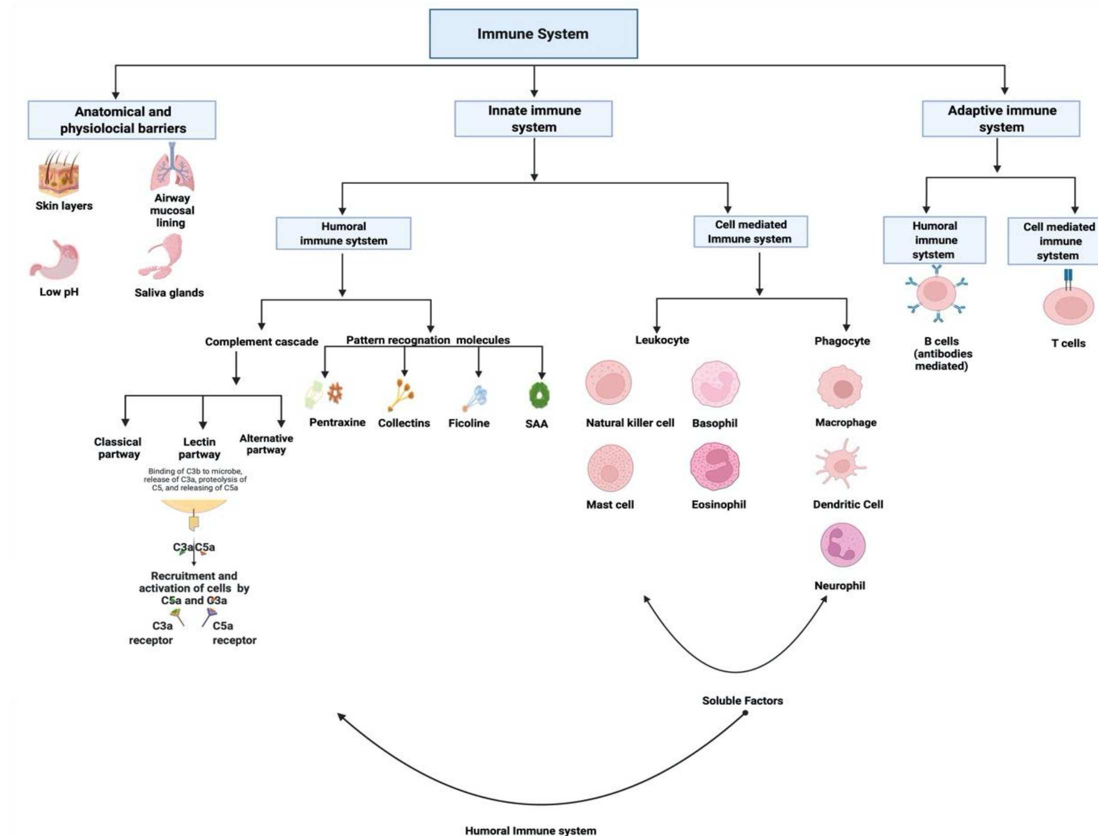

Figure 1. 1The 3 arms of the immune system. The first arm consists of anatomical barriers consisting of skin, mucosal surfaces, and physiological barriers including temperature, pH, oxygen tension, and various soluble chemical factors. The two other arms are humoral and cellular mediated. While the cellular-mediated immune system consists of the immune cell such as leukocytes and phagocytes, the humoral arm of the innate immune system consists of the complement cascade, pattern recognition molecules (PRMs), and soluble effector molecules. Alternatively, the immune system can also be divided into innate and adaptive immune responses that are fundamentally different in their mechanisms of action, although a synergy between them is essential for a fully effective immune response [6].

1.1.1 Adaptive Immune System

The discovery of the adaptive immune system was made by Paul Ehrlich, who is considered a father of the humoral adaptive immune system, with his theory on the side-chain of antibody formation and general principles of the way of antibodies for inducing neutralization and bacterial lysis [7]. The first evidence of an adaptive immune system was described in jawless vertebrates [8]. The jawless vertebrates develop lymphocyte-like cells that morphologically

resemble the T and B cells and it is narrated how antigenic challenges have a significant impact on their humoral and cellular immune response [9, 10].
In comparison to the innate immune response, the adaptive immune response, also known as the acquired response, is more specific but unlike the innate immune response is not immediate. However, while adaptive immunity does not respond quickly, it provides long-lasting memory defense. The adaptive immune response is generally divided into two main groups: i) the humoral response and ii) the cellular response which is carried out by highly mobile and specific T and B lymphocytes, and antigen-specific receptors [4].

1.1.1.1 B cells

B cells originate from the bone marrow [11, 12] and participate primarily in the humoral adaptive immune response by their ability to produce and secrete antibodies, known as immunoglobulins (Igs). These circulate in the bloodstream and spread through other body fluids, where they can bind specifically to target antigen antigens [13, 14]. B cells amplify their responses by recruiting innate effector mechanisms to effector response against invading microbes [6].
In addition to their role as antibody-producing cells, B cells have multiple effector functions such as neutralization and destruction of the antigen, phagocytosis, and complement activation [15]. Moreover, B cells also play a role as antigen-presenting cells (APC) and help maintain tolerance via the secretion of cytokines [16]. Besides the secretion of immunoglobulins, B cells express B cell receptors (BCR) on the surfaces which play an important role in specific antigen recognition. Antigen binding to BCR induces its internalization and increases the expression of peptide-major histocompatibility cell (MHC-II) complex on the cell membrane leading to the presentation of antigen-driven peptides to T cells [17].

1.1.1.2 T cells

T cells originate from a lymphoid progenitor in the thymus. Naïve T cells are quiescent and largely metabolically inactive till receiving the signal from peptide-driven antigen loaded on MHC-II at the surface of APC [18]. Upon recognition of antigen-derived peptides via MHC complex by T cell receptor (TCR) on their surface, T cells become activated, proliferate, and differentiate into effector T cells. Thus, mature T cells carry out various functions, including cell-mediated immunity, switching on the bactericidal function of macrophages, and enhancing the differentiation of the B and T cell cells to fully mature cells [19].

T cells are divided into two different subsets: $CD4^+$ and $CD8^+$ T cells. While $CD4^+$ T cell recognized antigen displayed on MHC class II, $CD8^+$ T recognized MHC class I loaded molecule. Then, $CD4^+$ T cells may differentiate into Th (T helper)1, Th2, Th9, Th17, Th22, Treg (regulatory T cells), and Tfh (follicular helper T cells) according to the type of the immune response taking place and cytokines necessary in the environment. All Th subsets undergo the production of different cytokines which mainly have effector, survival, or regulator functions and thus can be functionally distinguished. Thus, Th1 cells produce IFN-γ to maintain an immune response against extracellular parasites. Upon allergen exposure, Th2 cells produce Interleukin (IL)-4, IL-5, and IL-13, while Th17 cells produce mainly IL-17A, IL-17, and IL-22 for orchestrating immune response against extracellular bacteria and fungi [20]. Tfh, also known as follicular B helper T cells [21] plays a critical role in triggering germinal center (GC) B cells into antibody-secreting plasma and memory B cells [22]. On the other hand, Treg plays an immune suppressive role to maintain homeostasis and self-tolerance [23].
In contrast, naïve $CD8^+$ T cells differentiate into the cytotoxic T cell, a subset crucial for the lysis of target cells and tumor cells.
Finally, minor populations of the T cells (< 5%) of all T cell populations express the γδ TCR, and act as a part of the first line of defense against microbial invaders in the skin and mucosal surfaces [24], recognizing certain peptides and nonpeptides antigens, independent of the MHC class I or MHC class II molecules in δT cells [25].

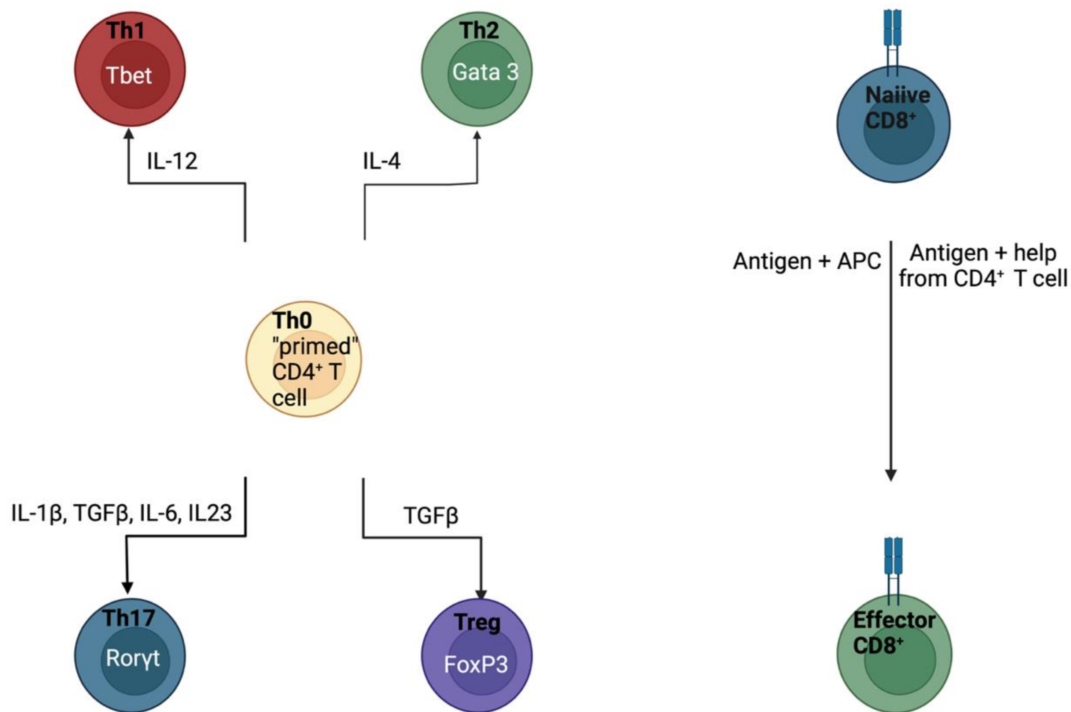

Figure 1. 2 Overview of T cell differentiation. While naïve CD4$^+$ T cells differentiate into T helper (Th)1, Th2, and Th17 cells as well as regulatory (Treg) T cells, naïve CD8$^+$ T cells differentiate into effector CD8$^+$ T cells. Mechanistically, Th1 differentiation depends on transcription factor Tbet and is included by IL-12; Th2 differentiation is dependent on GATA3 transcription factor and is induced by IL-4; Th17 differentiation depends on transcription factor RORγt and is induced by IL-1, TGF-β, IL-6, and IL-23. Finally, Treg differentiation depends on the transcription factor Foxp3 and required TGF-β [20].

1.1.2 Innate Immune System

In 1908, Russian scientist Ilya I. Mechnikov was awarded the Nobel Prize for his discovery of phagocytes and phagocytosis which is considered the starting point of understanding the innate immunity [26].

Innate immunity, described as “nonspecific” immunity, provides a spontaneous response to invading pathogens and dangers [27]. Typically, the first step of the innate immune response is the recognition of endogenous and exogenous danger and their elimination while sparing the host tissue [28]. In the late 1980s, Charles Janeway proposed the theory in which recognition

molecules named pattern recognition receptors (PRRs) will ensure recognition with low specificity [29]. Nonspecific recognition relies on a series of conserved germline-encoded receptors [28]. These include the Toll-like receptors (TLRs), Nucleotide oligomerization domain NOD-like receptors (NODs), C-type lectin receptors, a retinoic acid-inducible gene I (RIG-I receptors), and AIM-like receptors, which all can recognize specific conserved microbial structures known as a pathogen-associated molecular patterns (PAMPs) [30]. These microbial structures or "patterns" are composed of bacterial membrane components, lipopolysaccharides (LPS), unmethylated microbial DNA, and viral origin RNA [3, 31, 32]. In contrast to PAMPs, damage-associated molecular patterns (DAMPs) are molecules originating from damaged host cells that serve as a warning signal for the organisms [3]. They encompass nuclear high mobility group box 1 protein (HMGB-1), heat shock proteins, uric acid, and other endogenous alarmins released during cell lysis or tissue damage such as IL-33 [3, 33, 34]. DAMPs initiate the immune response via interacting with pattern recognition receptors [35], promoting sterile inflammation, which is very important for tissue healing but also activating numerous immune cells [35]. These activated immune cells release pro-inflammatory cytokine which further leads to the recruitment of cells to inflamed tissue and initiates the adaptive immune response [35].

The innate immune system consists of two arms. Firstly, a humoral part includes complement proteins, antimicrobial peptides, LPS binding protein (LBP), peptides, C-reactive protein, other pentraxins, and collectins [36]. Secondly, a cellular component provided by hematopoietic cells, including professional phagocytes (monocytes, neutrophils, and macrophages), basophils, Eosinophils, natural killer cells, and dendritic cells (DCs), Innate lymphoid cells (ILCs) and nonhematopoietic cells such as epithelial cells of the skin, airways, gastrointestinal tract [3, 27, 28].

1.1.2.1 Cellular compartment of the innate immune system

1.1.2.1.1 Epithelial cells

While epithelial cells (EC)s lining skin, airways and gastrointestinal tracts represent the very first line of defense [37] acting as a physical barrier, they also regulate type-2 mediated immune response upon allergen exposure or helminth infections via production of a variety of cytokines including IL-1α, IL-33, IL-25, GM-CSF, and TSLP, and inflammatory mediators such as uric acid, and ATP [38]. ECs use pattern recognition receptors to recognize immune insults, get activated, and released chemokines and cytokines that activate the immune cells [39].

1.1.2.1.2 The macrophage

The macrophage is well known to be a mobile effector cell that plays a crucial role in homeostasis and immune surveillance of tissues with its capacity to engulf and digest pathogens [40]. Macrophages arise as tissue-resident cells from embryonic origin but also as monocyte-derived cells from the blood [40]. They are widely spread in the body, and according to their origin, tissue of residence, and functions, are named differently such as alveolar macrophage in the lungs, Kupffer cells in the liver, osteoclast in the bones, and microglia in the brain [41]. As such, macrophages show variations in their functions, depending on the local environment [41]. For instance, microglia play an important role in neuronal well-being and responding to stress-triggered inflammation, while osteoclast degrades bone to normal remodeling and bone resorption [42, 43]. Consequently, due to their widespread distribution in tissues and their heterogeneity, macrophages play an important role in many diseases such as depression, Alzheimer's disease, Parkinson´s disease, diabetes endotoxemia, vascular diseases such as viral infections as well as tumor progression [43-46].

Originally, macrophages have been divided into two functional subtypes according to their activation state, environment, and functions: the so-called classically (M1) and alternatively activated (M2) macrophages [47]. While it originates from an *in-vitro* and well-defined set-up and therefore is an oversimplification, such an M1/M2 paradigm is a useful concept to better understand the macrophage biology [48] and has been widely used. However, it can be misleading when studying macrophage functions *in-vivo*, where both M1 and M2 macrophages co-exist. Nonetheless, macrophages become M1 while activated by microbial components such as lipopolysaccharide or interferon-gamma (IFN γ) and Toll-like receptor (TLR) ligands [49]. Such M1 macrophages release pro-inflammatory cytokines such as IL-12, IL-6, IL-23 tumor necrosis factor (TNF)-α, and IL-1-β which have strong microbicidal and tumoricidal activities [50]. In addition, M1 macrophages express co-stimulatory molecules such as CD80, and CD86 and membrane glycoproteins such as CD32 (FcgammaRII), and CD16 (FcgammaRIII) helping them to act as a bridge between the innate and adaptive immune system. However, they are generally poor antigen-presenting cells [51]. In addition, in response to inflammatory stimuli such as LPS, M1 macrophages produce nitric oxide (NO), reactive oxygen species (ROS), and reactive nitrogen species (NOS) to induce apoptosis necrosis and autophagy [52]. Finally, M1 macrophages secrete Th1 recruiting chemokines such as CXCL9, CXCL10, and CXCL11 [53]. In contrast to M1 macrophages, M2 Macrophages arise upon IL-

4 and IL-13 exposure via phosphorylation of STAT 6 [54]. They secrete anti-inflammatory cytokines such as IL-10, Tissue Growth Factor (TGF)-β and Galectin -1 (Gal-1) to suppress the inflammation, thus, contributing to tissue remodeling and hemostasis [49, 55]. Interestingly, Th-2-associated cytokines such as IL-33 could drive the M2 polarization leading to the secretion of CCL17, and CCL24 which triggers the recruitment of the Eosinophils to inflamed tissue [53].

Finally, macrophages are known to be highly plastic, with a polarization that is not fixed. Microenvironmental signals and tissue-specific genes extrinsic factors, epigenetic changes, and immune regulatory cells regulate the switch from the M1 to the M2 subtype or vice versa [56, 57].

1.1.2.1.2.1 Lung Macrophages

Macrophages are the most abundant innate immune cells in the lungs and display various functions such as maintaining homeostasis and immune surveillance, protecting the lung against the invader pathogen, clearance of microorganisms and repairing the tissue following injury [48]. At least two distinct macrophage populations are present in the steady-state lungs: the alveolar macrophages (AMs) and the interstitial macrophages (IMs) according to their location in the lung [58] and the expression of surface proteins [59].

The term alveolar macrophage coins cells residing in the airway of the alveoli, as well as cells attached to the alveolar epithelium [60], whereas macrophages residing in the interstitium are called interstitial macrophages. Furthermore, AMs are larger in size and present in a higher abundance than IMs [61]. Although both AMs and IMs share some common expressions of *bona fide* macrophage markers like CD64 and MerTK, they display marked differences in surface markers expression. For instance, while IM expresses low CD11c and Siglec-F and high level of CD11b, AMs express in contrast high CD11c and Siglec-F [58]. Besides their surface markers, transcription factors participating in their differentiation differ in AMs and IMs, with PPAR-γ, and Banc-2 being essential for AMs differentiation [62], and PU.1 being involved in the differentiation of both IMs and AMs [58].

On the one hand, AMs, the most abundant pulmonary immune cells that play a role in orchestrating the immune response, show an extensive ability to engulf foreign material and pathogens, although they are less efficient in antigen presentation than DCs due to their low expression of MHC-II [63]. Importantly, AMs play a dual role in lung function depending on the alveolar microenvironment, as they play an anti-inflammatory role under homeostatic

conditions, At the same time, they display pro-inflammatory functions upon inflammatory condition [64]. In general, AMs are the first cells to encounter dust, environmental allergens, bacterial, fungal, and viral pathogens and show immune suppressive function and induce efferocytosis [65] to maintain tolerance in the tissue. In such context, activation of the AMs leads to the secretion of anti-inflammatory molecules such as TGF-β and prostaglandin E2 (PGE2) [66]. In addition, AMs induce regulator cells (Treg) expansion by secretion of the TGF β [67]. In contrast, upon inflammatory conditions such as upon sensing pathogens in the airways or upon epithelial damage, AMs can be activated [68], thus orchestrating the recruitment of other immune cells to alveolar spaces [63]. Such activated AMs secrete various cytokines (e.g., IL-1β, TNFα, IL-6, and IL-8), chemokines [e.g., CXC-chemokine ligand 9 (CXCL9), CXCL10, CXCL1, CC-chemokine ligand 5 (CCL5), MIP-1α, MCP-1 and MCP-2] and NO [45, 58, 60, 63]. In addition, under low oxygen conditions, extracellular vesicles (EV) originating from epithelial cells (EC)s activate the pro-inflammatory functions of AMs, leading to the production of oxygen metabolites such as reactive oxygen species (ROS) [69]. Finally, recent studies emphasize the relationship between neutrophil extracellular traps (NETs) and AMs activation in the acute lung injury model [70, 71], as NETs formation triggers the pro-inflammatory function of AMs which results in massive tissue damage and necroptosis in a patient with severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) [71, 72].

On the other hand, IMs are phagocytic cells that act as a second line of defense against invading microorganisms and play immune regulatory functions with their capacity to produce IL-10 in response to microbial products [58]. Furthermore, it has been demonstrated that IMs could function as antigen-presenting cells via the expression of MHC-II at their surface [73]. Importantly, IM impairs both Th2-mediated Eosinophilic and Th17-mediated neutrophilic airway inflammation via the production of anti-inflammatory cytokines and the ability to decrease mucus formation [73]. In line with its protective role in airway inflammation, it has been shown that in response to low oxygen levels, the number of IMs in the lung increases, leading to an increase in pulmonary expression of anti-inflammatory genes [73].

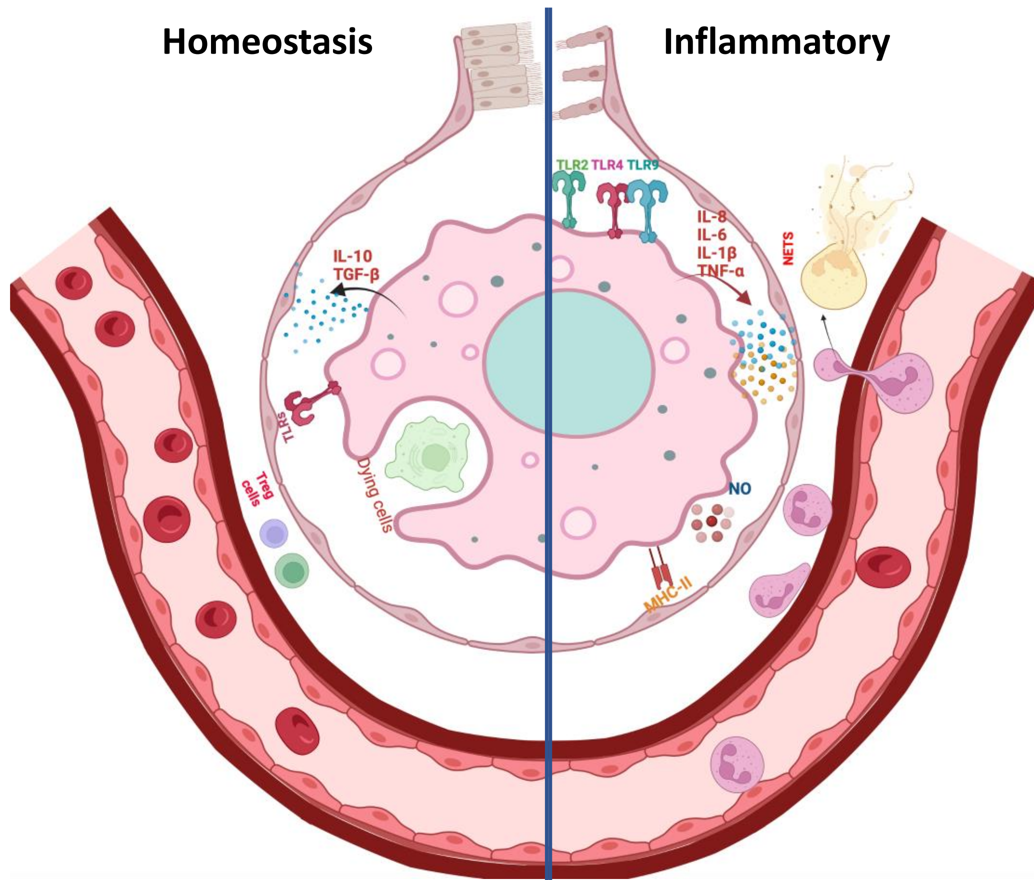

Figure 1. 3 Dual function of alveolar macrophages. Under homEOStatic/anti-inflammatory conditions, AMs secrete anti-inflammatory mediators, such as IL-10 and TGF-β. Upon inflammatory/pathological condition, AMs activate and produce inflammatory mediators, such as NO^-, IL-1β, IFNs, IL-6, IL-8, and TNFs. Inflammatory mediators amplify and perpetuate the inflammation, damaging lung alveoli and epithelium [72].

1.1.2.1.3 Dendritic cells

Dendritic cells (DCs) are known to be the most potent antigen-presenting cells (APC) together with B cells and thus act as a bridge between innate and adaptive immunity [74]. The initiation and orchestration of the immune responses are regulated by DCs, at mucosal surfaces, and in lymphoid organs after their recruitment from the blood [75-77]. Although DCs are innate immune cells with their way of recognition and response, they regulate the adaptive immune response by providing essential signals for activating a desired immune response.

1.1.2.1.3.1 Main functions of DCs

The main functions of DCs are capturing, processing, and presenting antigens to adaptive immune cells. Dynamically, DCs capture antigens in peripheral sites, and they migrate to T-dependent areas in lymphoid organs where they present antigen fragments loaded on MHC-II to naïve T lymphocytes [78, 79]. Then, depending on the context, they induce the T cell activation, proliferation, and differentiation into different T-helper cell (Th) subsets [80]. DCs are divided into two functional stages "immature" and "mature" DC. Firstly, immature DCs take up the antigen using their receptors (endocytosis receptors, phagocytosis receptors, and C-type lectin receptors) by phagocytosis and micropinocytosis [76]. However, upon disequilibrium of tissue homeostasis triggering activation by DAMPs, PAMPs, or inflammatory stimuli such as cytokines [74, 80], DCs undergo a developmental program named DC maturation [74]. That maturation induced by CD40/CD40L signal is characterized by the upregulation of co-stimulatory CD80/86 molecule as well as the high-capacity antigen-presenting and T cell priming [81, 82]. Matured DCs then process antigens as the next step since T cells lack the ability to recognize the whole antigen. DCs express several different receptors to initiate endocytosis and phagocytosis of antigens and pathogens [74]. The most abundant antigens receptors on DCs are C-type lectin family members and specifically in DCs, they include DEC205 and mannose receptor (MR) [74].

Finally, DCs play an essential role in "cross-presentation", a concept referring to APCs not directly infected by a pathogen but are surrounded by infected cells that initiate the cytotoxic immune response via DC presentation of extracellular antigens to MHC-I molecules to CD8 T cells [83, 84]. Then activated naïve CD8 T cells by acquiring nominal antigen from other cell processes known as "cross-priming" which is essential for immune defense against viruses and tumors [85].

1.1.2.1.3.2 DC lineage and subsets

DCs are heterogenous populations that comprise different subsets, named according to their localization in the tissue, the expression of a surface marker, and their functions. In general, DCs divided into two distinct populations: plasmacytoid (p)DCs and conventional (c)DCs. All DCs derived from hematopoietic stem cells through common macrophage/dendritic cells progenitor (MDP), which give rise to monocyte/macrophage linage or common dendritic cell progenitor (CDP) [76, 86-88]. CDP further differentiates into pDC and preDC progenitors [88].

While pDCs complete their development in bone marrow, preDC is recruited to the vascular system to complete its maturation and differentiation into conventional DC subsets, such as CD8 α^{+} / $CD103^{+}$ DC, also known as a cDC1 or $CD11b^{+}$ DC, also known as cDC2 [88, 89].
The pDC is bone marrow-derived DCs that are first defined in the human blood [90, 91]. In addition, pDCs can reside in an area where inflammation, infection, and cancer occur. Interestingly, pDCs produce a massive amount of type 1 interferons (IFN α/β) and pro-inflammatory cytokines [92], thus controlling the viral infection [93]. In addition to their ability to regulate viral infection, pDCs could play both immunogenic and tolerogenic functions depending on their microenvironment and type of exposure, such as cancer pathogenesis [93].
cDC1 are composed of tissue organ resident $CD8\alpha^{+}$ DCs in lymphoid organs representing approximately 20% of all DCs and their equivalents in non-lymphoid organs tissue-resistance $CD103^{+}$ DC. cDC1 development is highly dependent on tyrosine kinase receptor FMS-like tyrosine kinase 3 ligand (Flt3L) and inhibition of the DNA protein-binding protein 2 (ID2), transcription of interferon regulatory factor 8 (IRF8), and basic leucine zipper transcription factor ATF-like 3 (BATF3) [86]. cDC1 is essential in the maintenance of central and peripheral tolerance due to the ability to produce a high amount of TGF β through self-reactive T cells at a steady state [94]. In addition, cDC1 is activated under the inflammatory condition to produce a massive amount of inflammatory cytokines such as IL-12p70 and IFN γ [89]. Furthermore, cDC1 does not activate $CD4^{+}$ T cells but plays a crucial role in the cross-presentation via MHC class I.
In contrast, cDC2 development is controlled by interferon regulatory 4 (IRF4), and unlike cDC1, mouse cDC2 is more efficient at MHC-II presentation and a $CD4^{+}$ T cell. Also, cDC2 is known to co-express $CD8^{-}$ cDC2 represents approximately 80% percent of all DC in the lymphoid organs in the mouse [89]. While in non-lymphoid organs cDC2 always express CD11b, in the lung can be either $CD103^{+}$ or $CD103^{-}$ and in the intestine, they may also express CD24 [89]. Furthermore, cDC2 can produce a massive amount of pro-inflammatory cytokines containing TNF-α, and IL-6 in response to bacterial infection. Recently, it has been shown that cDC2 may secrete type 1 IFN in PVM-specific TCR transgenic mice [95].

1.1.2.1.3.3 Pulmonary Dendritic

DCs play a vital role in the pathogenesis of various lung diseases. DCs reside in the respiratory tract where they continuously uptake antigens from airways and alveolar surfaces and migrate to the lymph nodes where they present processed peptides to T cells [96].

In lungs, DCs encompass 4 populations that circulate in response to lung infection or injury; The two conventional DC populations cDC1 ($XCR1^+$, $CD103^+$, $D64^-$, $CD11b^-$), and cDC2 ($XCR1^-$, $CD103^-$, $CD64^-$, $CD11b^+$) are supplemented by monocyte-derived (mo)DCs ($XCR1^-$, $CD103^-$, $CD64^+$, $CD11b^+$) and pDCs ($CD11c^+$, $GR1^+$, $B220^+$, PDCA-1^+) [97, 98]. In addition to their specific marker's expression, pulmonary DC populations are distributed differently. While the cDC1 population resides in the respiratory epithelium and is functionally active in the airway lumen, cDC2 mainly resides beneath the airway [99, 100]. In murine lungs, cDC1 expresses tight junction proteins claudin-1, claudin-7, and zonula-2 which form tight junctions with airway epithelial cells [100]. Formation of tight junction help DC to uptake antigen within the airway lumen without epithelial interruption and following antigen uptake, cDC1 migrates the T cell zone to draining mediastinal after viral infection. On the other hand, cDC2s are major producers of proinflammatory chemokines, which are responsible for recruiting inflammatory cells [101] and are essential for the induction of Th2 cell differentiation upon allergen exposure [102]. Furthermore, while under steady-state conditions, the number of the moDC in the lung is low, after exposure to antigen, pathogen, or inflammatory cytokines, monocytes rapidly recruited into the lung differentiate into the moDC and participate in the Th2 response [100]. Finally, evidence from the clinical and murine study suggested that pDC are associated with lung inflammation in response to viral infections such as severe Covid-19 infection [103], although they are also associated with atopic dermatitis, allergic asthma, and allergic rhinitis [104-106].

1.1.2.1.4 Eosinophils

Early investigations demonstrated that an association existed between increased levels of the Eosinophils (EOS) in peripheral blood and helminth infections, hence showing that they were essential to the Type 2 immune response [107]. However, EOS is known to be multifunctional granulocyte involved in many inflammatory diseases, including viral infection and allergic disease [108, 109]. Although Eosinophils normally account for only 1-3 percent of the peripheral leukocyte, the number of peripheral and tissue Eosinophils significantly increases the infection and allergy [110]. Interestingly EOS are pleiotropic cells, acting as terminal effector cells, eliminating microbial, pathogenic, and parasitic threats through the release of their granule proteins [58], as well as antigen-presenting cells (APC). Indeed, many reports showed that EOS express MHC-II and co- stimulation molecule CD80 and CD86 [58, 111, 112], migrate toward the mLN, and present the processed antigens to $CD4^+$ T cells, promoting their proliferation differentiation [113, 114], although it has been suggested that, they are only

able to induce the proliferation of effector but not naïve T cell [115]. In addition, EOS can regulate T cell polarization via the synthesis of an enzyme called indoleamine 2,3- dioxygenase (IDO), an enzyme involved in the oxidative metabolism of tryptophan, which regulates the Th1 and Th2 imbalance [113].

EOS granules possess specific secondary granules in their cytoplasm. These granules contain unique cationic toxic proteins which are stainable with acid aniline dye and Congo red [113, 116]. Eosinophil granules released various substances, including major basic protein (MBP) and EOS peroxidase (EPO), in response to helminth infection. Once at the site of injury, Eosinophils produce various oxygen species including superoxide, singlet oxygen, and hydrogen peroxide which catalyze the production of a highly toxic antimicrobial agent called hypohalous acid [110]. In addition, they can release collateral oxidative protein (ECP) which has cytotoxic and ribonuclease activity. In addition, ECP can induce T cell response and Ig synthesis by B cells and mucus and glycosaminoglycan production [113]. The last product of the Eosinophil granular enzyme is an Eosinophile-derived neurotoxin (EDN), which is a ribonuclease, and displays antiviral activity. Furthermore, mounting evidence suggests that Eosinophils undergo a distinct form of extracellular lytic cell death called EETosis by the formation of DNA- based Eosinophil extracellular trap (EET) under inflammatory condition [117]. Cytolytic EETosis considered to be an innate immune function against invaders. However, it could initiate a long-lasting adhesive luminal surface resulting in barrier dysfunction along with bacterial aggregation and biofilm formation which trigger reinfection and even chronicity of the disease [118].

In addition to its role in innate immunity and modulation of the immune-inflammatory system and tissue repair and remodeling, several Eosinophilic conditions in human patients are associated with systemic and tissue Eosinophilia in which Eosinophils are considered the main driver of the pathogenesis [119]. Current clinical results provide conclusive evidence harmful role of Eosinophils such as specific organ damage in localized infiltrative Eosinophilic entities; cardiac fibroelastosis, pulmonary fibrosis, esophageal stricture, obstructive intestinal masses and bronchospasm, and multiple organ damage in systemic Eosinophilic conditions such as vasculitis's, malignancies, HES complex [119]. Yet, a recent report suggests that EOS could also play role in female reproductive health, tumor surveillance, and adipose tissue remodeling [119]. Indeed, EOS could exhibit beneficial, harmful, and uncertain roles in health and disease.

1.1.2.1.4.1 Eosinophil subsets

Eosinophils develop in the bone marrow, where they can differentiate from hematopoietic stem cells into hybrid precursors with the properties of basophils and Eosinophils then mature as Eosinophils [110].

Under baseline conditions, Eosinophils reside in the thymus, mammary gland, uterus, and lamina propria of the gastrointestinal tract where they regulate the various biological functions [110, 113, 120, 121] such as maintaining Immunoglobulin (Ig) A production, enhancing the Peyer's patch formation and mucus production [122]. Recently, Eosinophils with ring shape nuclei were reported in a patient with skin lesions and patients with Crohn's disease [123]. In contrast, Eosinophils are recruited at the site of inflammation in inflammatory conditions. Endothelium involves in the trafficking of the Eosinophils into the inflammatory site by regulating the rolling, adhesion, and diapedesis. Depending on the inflammation site, Eosinophils traverse endothelium into the tissue. This process is regulated by the complex network, including cytokines (e.g., Interleukin (IL)-4, IL-5, and IL-13), chemokines (e.g., RANTES and eotaxin), other molecules (e.g., acidic mammalian chitinase) and lipid mediators (e.g., Platelet-activating factor and leukotriene C4) [113]. Recent studies have demonstrated the essential function of IL-5 and eotaxin in the Eosinophil trafficking [124]. IL-5 is a key player in the growth, differentiation activation, and survival of the Eosinophils and supply signal which induces expansion and mobilization of the Eosinophil to the lung [113]. Further, pieces of evidence show that eotaxin, CC chemokine, is a small protein responsible for Eosinophil recruitment to the lung by stimulating seven-transmembrane GPCR on leukocyte surface receptor called CCR3 (CC chemokine receptor) in response to allergen exposure [113, 124-127].

Recently, a small population of pulmonary resident EOS, which averaged 1.5 % of total $CD45^{+}$ hematopoietic cells, have been identified in murine naïve lung but not in the bronchoalveolar fluids [128]. Unlike the rEOS which express CD11c, lung resident Eosinophils exhibited $CD11c^{-}$ phenotype. Moreover, rEOS display a unique morphological ring shape nuclei in steady-state and following the development of house dust-mite-induced airway allergy [128]. The rEOS reside in the parenchyma and express the cell surfaces CD62L and CD125, intermediate levels of Siglec-F, and low levels of CD101 [108].

However, under allergic inflammatory conditions, EOS coexist with inflammatory Eosinophils (iEOS) which possess a unique shape with the segmented nucleus. iEOS is exclusively found

in peribranchial areas and express low levels of CD62L, intermediate levels of CD125 and CD11c, and high level of Siglec-F and CD101. [108, 129].

1.1.2.1.4.2 Eosinophils in lung diseases

Eosinophils are involved in the pathogenesis of several lung diseases, including asthma, acute and chronic Eosinophilic pneumonia, allergic fungal airway disease hypersensitivity pneumonitis, Eosinophilic granulomatosis with polyangiitis, Churg Strauss syndrome, DRESS syndrome, hyper Eosinophilic syndrome [130].

1.1.2.1.5 Neutrophils

Neutrophils are known as simple foot soldiers of innate immunity with "weapons" playing critical roles in acute inflammation [131]. They are polymorphonuclear leukocytes that are considered short-lived cells, with a half-life in the circulation in mice of about 1 h whereas 6-8 h in humans. However, during inflammation, the lifespan of the neutrophils significantly increases [132].

Neutrophils develop in bone marrow (BM), where they can differentiate from hematopoietic stem cells into myeloid precursors and then differentiate into segmented mature neutrophils [133]. They form a heterogenic population involved in phagocytosis, degranulation, and Neutrophil extracellular trap (NET) formation against a large range of pathogens including bacteria, fungi, and protozoa. NETs trap es the host`s defense by neutralizing and killing the pathogens [134]. Of note, in addition to its beneficial function on host defense, excessive NETosis could damage the epithelium in response to pulmonary infection contributing to acute lung damage [134].

In addition to their antimicrobial function, neutrophils play role in immune regulation via a broad array of cytokines and effector molecules of the humoral arm of the innate immune system. In inflammatory conditions, they recruit the site of inflammation by chemotactic molecules including cytokines, bacterial peptides, or complement components [135]. Once they are recruited into tissues, neutrophils engage in crosstalk with other immune cells, platelets, and endothelial cells and this orchestrates the adaptive immune response [136]. Neutrophils have also been shown to migrate to the lymph nodes, where they can interact with DCs to modulate the antigen presentation [136].

1.1.2.1.5.1 Neutrophils in lung diseases

Neutrophils are the most abundant circulating leukocyte which is implicated in a wide range of lung diseases, including chronic obstructive pulmonary disease (COPD), severe asthma pulmonary fibrosis, acute respiratory distress syndrome, and Respiratory syncytial virus (RSV) [137-140].

1.1.2.1.6 Innate Lymphoid Cells

Innate lymphoid cells (ILCs) are innate counterparts of T lymphocytes, which lack recognition activating gene (RAG)-dependent rearranged adaptive antigen receptors [141]. ILCs are a distinct arm of the innate immune system that participate in tissue homeostasis, metabolism, regeneration, growth, infectious diseases, chronic inflammation, and the cancer [141-143]. ILCs are controlled by multiple endogenous mammalian cell-derived factors such as neuropeptide hormones, eicosanoids, cytokines, and other alarmins [144]. In addition, ILCs are considered tissue-resident cells that commonly reside at barrier interfaces, while only a few of them could be found in the bloodstream [145]. The prototypical ILC populations are natural killer cells (NK), which were discovered in 1975, and the lymphoid tissue inducer (LTi) which were discovered in 1997. While Natural killer (NK) cells fulfill the function of $CD8^+$ cytotoxic T cells that play an important role in immediate response against viruses and tumor cells, LTi is crucial for the formation of lymph nodes during embryogenesis. ILC development depends on common cytokine receptors γ and inhibitors of DNA binding 2 (ID2) [146]. Only recently, has evidence highlighted the existence of several distinct non-cytotoxic ILC populations. ILCs consist of the ILC1s, ILC2s, and ILC3s that display similarities with CD4 T helper cell (Th)1, Th2, and Th17 cells respectively [141].

1.1.2.1.6.1 Identification and Nomenclature

ILCs were first identified very recently, in 2013 [147]. Since then, a clear nomenclature has been established and led to a division of ILCs into groups 1, 2, and 3 each of them containing the subgroup [143]. All groups exhibit a lymphoid morphology, lack cell surface molecules that characterized other immune cells (hence are described as lineage marker-negative Lin^-), and express the IL-7 receptor subunit-α (IL-7Rα / CD127) [143]. Further, subgroups within each group share similar patterns, such as cytokines profile, but functions exert variation. Group 1 ILCs, comprising NK cells and ILC1s, depend on the T-box transcription factor Tbet and produce interferon-gamma (IFN-γ) [148]. Group 2 is a single group, ILC2s, depending on

GATA3 and RORα , and produces IL-5 and IL-13 [149]. Finally, Group 3 ILCs encompass 3 subgroups of natural cytotoxicity receptor (NCR)⁻ cells, NCR⁺ cells, and LTi, all depending on transcription factor RORγt and producing IL-17 and/or IL-22. Group 3 of ILCs mediate early immune response to extracellular microbes such as fungus and bacteria [150-155].

A, cutting edge report highlighted that in fact, GATA3 is critical for the development of all CD127 ⁺ ILC lineage, including ILC1, ILC2, ILC3, LTi, and CD127 expressing NK cells other than intraepithelial lymphocytes (IELs) which are known to express both NK.1 and Nkp46 [156, 157]. Interestingly, the de-activation of GATA 3, negatively regulates Th1-associated genes including *Stat4* [157].

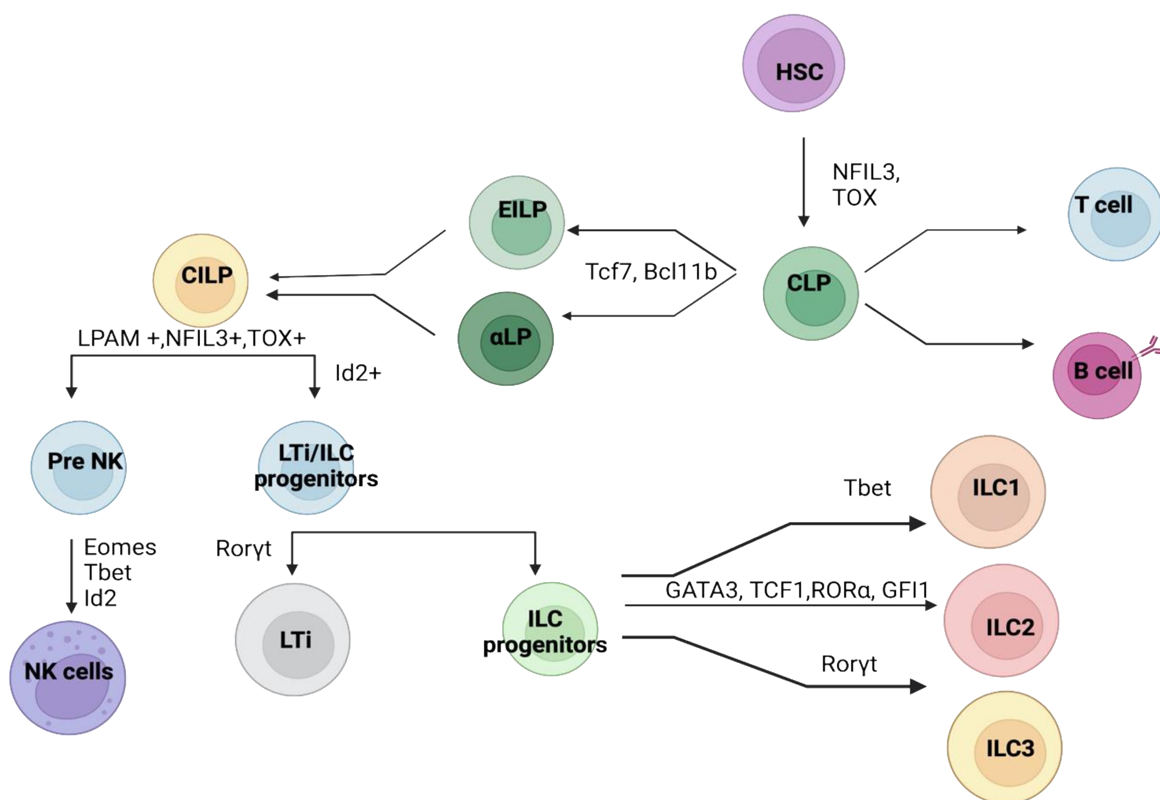

Figure 1. 4 Deciphering the family tree of ILCs. ILCs are derived from hematopoietic stem cells (HSC) which give rise to common lymphoid progenitor (CLP). CLPs differentiate into T and B cells or give rise to Common Innate lymphoid progenitor (CILP) from Early Innate lymphoid progenitor (LP) and alpha LP. Further, CILPs differentiate into pre-NK and LTi / Innate Lymphoid Cell (ILC) progenitors. While pre-NK cells differentiate into NK cells, Lti / ILC progenitors differentiate into Lti orILC progenitors, giving rise to ILC1, ILC2, and ILC3 [158].

1.1.2.1.6.2 ILC development "ILC-poiesis"

In humans, ILCs are derived from hematopoietic stem cells (HSCs) and differentiate into hematopoietic lymphoid-primed multipotent progenitors (LMPPs) [158]. LMPPs are characterized by their high levels of surface expression of cKit, Sca-1 Flt3 but not CD127 [158, 159]. These cells undergo rounds of differentiation during lymphopoiesis, differentiating into common lymphoid progenitors (CLP) which are identified as Lin^{-} Kit^{lo} $Sca\text{-}1^{lo}$ $Flt3^{+}CD127^{hi}$ [145]. CLPs have only lymphoid potential, they could differentiate T, B, and ILC progenitor [160]. Finally, ILC progenitors later differentiate into ILC1, ILC2, or ILC3 [161].

In contrast, ILC differentiation occurs in mice in the fetal liver and after birth in the spleen, bone marrow, and peripheral tissues. Like all lymphocytes, ILCs arise from common lymphoid progenitors (CLP) that give rise to differentiated common innate lymphoid progenitors (CILPs). This is achieved either via an Early innate lymphoid progenitor (EILP) [162], or a αLymphoid Progenitor (LP) characterized as $Lin^{-}\alpha4\beta7^{+}CD127^{+}FLT3^{-}$, that shows an increased expression of NFIL3 and ID2 [163] [164]. Such CILPs can further differentiate into NK cell precursors (NKP) or into common helper innate lymphoid progenitors (CHILP) that express GATA3 and PLZF [145]. While NKP cells differentiate into NK cells, CHILP gives rise to differentiate Lymphoid tissue inducer progenitors (LTiPs) and Innate lymphoid tissue progenitors (ILCs). Finally, LTiPs differentiate into LTis, while ILCPs differentiate into ILC1, ILC2, and ILC3 under the control of the transcription factors. [164].

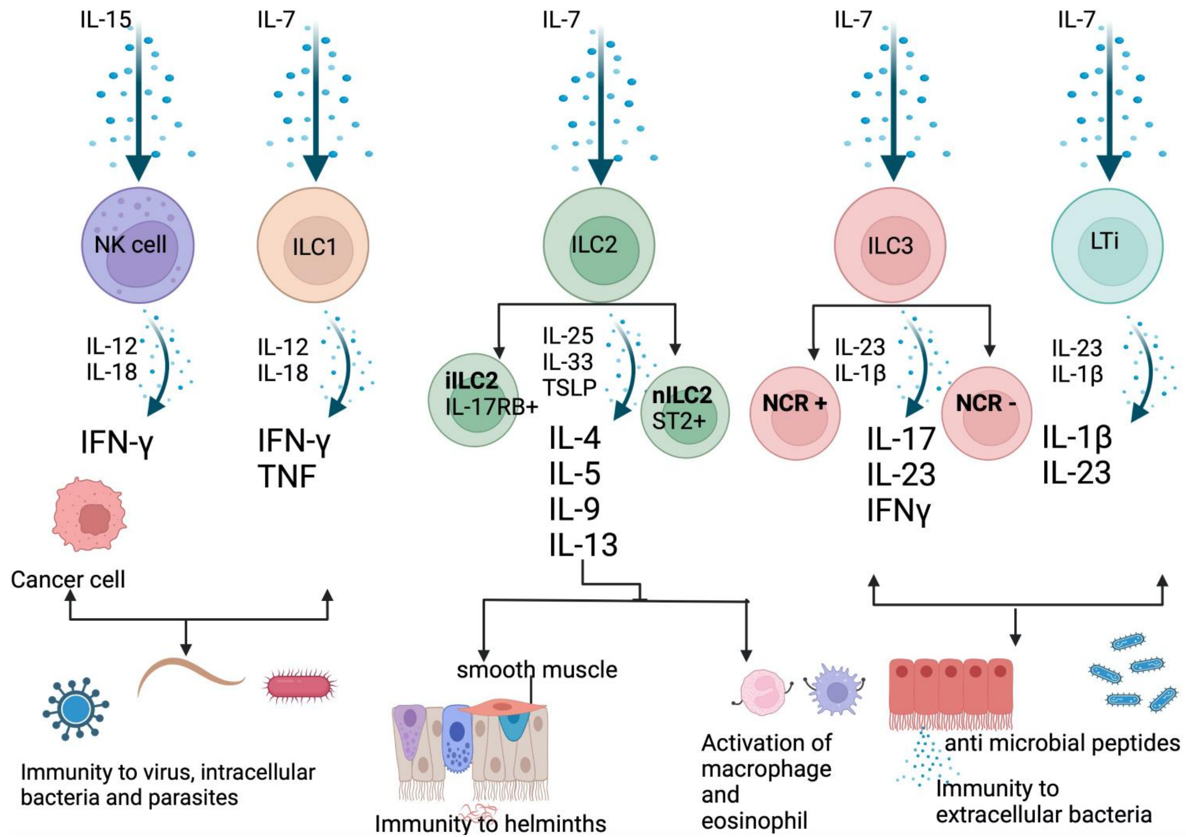

Figure 1. 5 ILC differentiation and functions. ILCs divide into 3 main groups. Group 1 ILCs encompass 2 subgroups, the NK cells, and the ILC1. Group 2 ILCs consist of nILC2 and iILC2. Group 3 ILCs are divided into 3 subgroups; NCR-, NCR+ ILC3, and LTi [141]. While IL-15 is required for NK cells, IL-7 is required for the survival of ILC1, ILC2, ILC3, and LTi. Further, IL-18 activates the NK cells [141], which releases IFN-γ. Similarly, IL-12 and IL-18 also activate ILC1, leading to IFN-γ and TNF-a secretion in response to intracellular bacteria, viruses, and parasite infections. Furthermore, TSLP, IL-25, and IL-33 activate ILC2, which plays a critical role in the orchestration of the immune response and triggers smooth muscle contraction by releasing Type 2 cytokines including IL-4 IL-5, IL-9, and IL-13 [165]. Being involved in Type 2 immune response, ILC2 plays a critical role in immunity against helminths. Finally, IL-23 and IL-1β activate ILC3s and LTi in response to anti-microbial peptides and extracellular bacteria. While ILC3s secrete IL-17, IL-23 and IFN-γ, LTi secretes only IL-17, IL-23 [165].

1.1.2.1.6.2.1 Group 1 ILCs

Group 1 ILCs comprise the natural killer (NK) cells and ILC1 per se. On the one hand, their location in the body allows the distinction between the two, since ILC1s reside in tissues, while NK cells circulate in the bloodstream [165]. Furthermore, while the NK cells and ILC1 development are controlled by the transcription factors Tbet, GATA 3, and Nfil3, ILC1 does not require the expression of the Eomersidermin (Eomes) transcription factor, which is

necessary for the maturation of NK cells. Functionally, although both NK cells and ILC1 cells produce IFN- γ, and TNF-a, their immunological functions are different [148]. Indeed, NK cells have potent cytolytic functions since they strongly express perforin [156] and participate in the clearance of intracellular microorganisms, including bacteria and parasites [148, 150, 156, 163, 166, 167]. In contrast, ILC1s play a role in the regulation of tissue inflammation, pathogenesis, homeostasis, metabolism, and host defense [163]. Further, it has been highlighted that in addition to its regulatory function ILC1s invoke an antiviral effect by producing IFN- γ in the oral mucosa [168, 169].
While NK cells are the most important antiviral and anticancer effector cells [146, 150, 170, 171], they participate in the development of potent adaptive responses as well as neurodegenerative diseases, acute vascular and traumatic damage as well as mental disorders [163, 167, 172]. Nevertheless, it has been reported that NK cells can acquire immunological memory similar to T and B cells [173, 174].

1.1.2.1.6.2.2 Group 2 ILCs

Group 2 innate lymphoid cells (ILC2s), discovered in 2016, have emerged as crucial players in type 2 immune responses and orchestration of cell recruitment, particularly at an early stage of worm expulsion or allergic inflammation [175]. They derive from CLPs through PLZF$^+$ common ILC precursors upon activation of transcription factors GATA3 and RORα as the main developmental drivers together with Bcl1b, TCF-1, and Gfi [142, 157, 175-181]. In addition, IL-7 is required for the ILC2 development and survival by maintaining their number in the tissue [182]. Functionally, ILC2s are activated indirectly when allergen and other cues trigger damage to the mucosal epithelium, inducing the release of immunological and biological alarmins by epithelial and immune cells [176]. These alarmins include IL-25 (also known as IL-17E), IL-33, thymic stromal lymphopoietin (TSLP), lipid mediators like eicosanoids (LTD_4, PGD_2), neuropeptides, and hormones like neuromeric U (NMU) or vasoactive intestinal protein (VIP) [183]. This activation of ILC2s by alarmins leads to the production of type 2 cytokines, including IL-5, IL-4, IL-9, IL-6, and IL-13 [176], thus the idea that ILC2 acts as an innate counterpart to Th2 lymphocytes. In addition, ILC2s produce amphiregulin that promotes various homEOStatic immunologic and pathologic processes, including helminth clearance, epithelial repair after injury, and beiging of white adipose tissue [37, 184-187]. Because of their ability to secrete Type 2 cytokines, ILC2s are key players in initiating Eosinophilia, goblet cell hyperplasia, mucus secretion, remodeling of mucosal tissue,

and IgE production [176]. In agreement, clinical studies outlined the importance of the ILC2s in asthma, allergic rhinitis, chronic rhinosinusitis, atopic dermatitis, Eosinophilic esophagitis, and food allergy [183]. Of note, ILC2s have both beneficial and detrimental roles. For instance; while ILC2s play a protective role against *Helicobacter pylori* by promoting IgA antibody production, they suppress beneficial type 1 immune response against *Cryptococcus neoformans* infection [188, 189].

ILC2s form a heterogenous Population of cells

ILC2 can be divided into two distinct populations; homeostatic or natural ILC2s (nILC2s cells) and inflammatory ILC2s (iILC2) [190]. While nILC2 reside naturally in tissues and respond to IL-33 [191], iILC2 cells are not present in the peripheral tissues at a steady state but appear in the lung, mLN, spleen, and liver, bone marrow, and blood after intraperitoneal administration of IL-25 or *N. brasiliensis* infections [192]. In line, iILC2 only respond to IL-25 as they lack the IL-33 Receptor (IL-33R, ST2), although they change their phenotype and become $ST2^{+}$ nILC2-like cells after IL-2 or helminth infections, including cells in transition from iILC2 to nILC2 [190]. While iILC2 are characterized as $ST2^{-}IL17RB^{+}$, nILC2 phenotype shows differentiation according to their location; for instance, $ST2^{+}$ nILC2 residing in the lung express low level of IL-17RB while in the adipose tissues, they do not express IL-17RB [190, 191]. Finally, recent evidence indicates that a portion of the lung iILC2 derived from resting ILC2 from intestinal lamina propria suggests ILC2s are able to migrate from one tissue to another one [193].

1.1.2.1.6.2.3 Group 3 ILCs

Group 3 ILCs are known for their capacity to produce the cytokines IL-17A, IL-17F, and/or IL-22 in response to signals from myeloid cells such as IL-1 β and IL-23 [194]. Similar to Th17 cells, their development, and functions depend on the transcription factor retinoid–related orphan receptor (RORγt) [154]; also similar to Group 2 ILCs, their development depends on IL-7Rα. Group 3 ILCs play a central role in the formation of secondary lymphoid organs during embryogenesis, barrier tissue homeostasis, regulation of host commensal mutualism, epithelial turnover, and tissue restoration in response to inflammation and infection, orchestration of adaptive immunity [145, 155, 195].

Group 3 ILCs are subdivided into at least 3 subgroups of NCR- and NCR+ ILC3 and LTi. In general, group 3 ILCs are characterized by the surface expression of c-kit (CD117) and Neuropilin-1 [196]. However, LTi, localized in the lymphoid tissue such as fetal mesenteric

lymph nodes that play a role in lymph nodes, crypto patches, and lymphoid follicles [197], express chemokine receptor CCR-6 and variable CD4 expression [198]. In contrast, natural-cytotoxicity receptor-positive ILC3 (NCR^+ ILC3) is defined by the expression of the NK cell-associated receptor (NKp46) and CD49a and localized in the lamina propria [195]. ILC3s act as innate immune sentinels of the gastrointestinal tract and maintain intestinal barrier function through the receptor GPR43 [195].

Furthermore, while ILC2s are commonly associated with allergic asthma, ILC3s appear to contribute to the pathogenesis of non-allergic asthma by sensing the environmental changes caused by smoking that correlate with disease severity [199]. In line, it has been shown that ILC3-associated genes were increased in nasal brushings if adult-onset severe asthmatic patients suggesting group 3 ILCs are associated with IL-17- driven neutrophil and Eosinophil responses in asthma [200]. Although ILC3s are only a minor immune cell population in the lung, they play a critical role in infectious and non-infections pulmonary diseases [150, 155, 157, 196, 201]. In line, it has been shown that lung function positively correlated with NCR^+ ILC3 frequency and a healthy lung microbiome diversity [201].

1.2 The Complement System

The complement system was first discovered in the late 19th century by Jules Bordet and Paul Elrich as a very powerful heat-labile serum circulating component of innate immunity [26]. Its primary function is to sustain microbial defense through coating pathogens with antibodies, the process of opsonization via activated complement proteins, and the lysis of target cells via the formation of a Membrane attack Complex (MAC). The whole ensures the rapid identification, labeling, and removal of microbial threats. Furthermore, the complement induces a potent inflammatory response through pro-inflammatory molecules and the recruitment of effector cells via the generation of anaphylatoxins (ATs) [7, 202]. A second time, complement ensures the bridging of innate and adaptive immunity through its functions in innate cells such as DCs. Although still controversial in mice, complement-derived ATs also play a crucial role in regulating T and B cell function and activation [203-205].

The complement system encompasses 50 soluble and membrane-bound proteins mainly produced by the liver and found in circulating serum, lymph, and intestinal fluids [206, 207]. Proteins are present in plasma in non-activated form [208], activation occurring via a series of proteolytic reactions, which can be initiated via sensing of exogenous or endogenous threats by C1q, mannan-binding lectin, or ficolins through three major pathways i) the classical

pathway (CP) (through complement protein C1); ii) the lectin pathway (LP); or iii) the alternative pathway (AP) [206, 207] Mechanistically, all pathways consist in series of sequential enzyme-substrate interactions and ultimately converge into the formation of a complement C3 convertase which cleaves C3, generating the opsonin C3b and the AT C3a [209]. These C3b and C3 convertase subsequently create a loop to enhance opsonization via C3b [36, 209, 210]. In the second step, C3b participates in the formation of a C5 convertase, cleaving C5 and generating the AT C5a and C5b. Then, the release of C5b results in further complement cascade activation and the assembly of membrane attack complex (MAC) that contains not only C5b but also other complement components, including C6, C7, C8, and C9, regulatory proteins clustering, and/or vitronectin [206, 207]. MAC is not a single molecular species but rather a family of water-soluble proteins to integral membrane proteins[211] The main function of the MAC is the rapid elimination of foreign cells, particularly gram-negative bacteria, and protozoan pathogens [211].

The classical pathway is initiated upon the binding of the C1 complex, consisting of C1q, C1r, and C1s proteins to antibodies or directly to the target's cell surface and ends with the lysis of the cell [212]. C1 complex acts as a link between the humoral response and complement system and has six globular heads from collagen tails surrounded by C1r and C1s proteins [212]. Activation of the C1 complex results in the cleavage of C4 into C4a and C4b to further generate the C3 convertase [212, 213]. In contrast, in the lectin pathway, antibody binding is not required for its activation [214], as it is activated when mannan-binding lectin (MBL), ficolin, or collectins recognized carbohydrate ligands on the surface of the microbial surface [215]. Upon lectin pathway activation, the cleavage of C4 and C2 is initiated, forming the C3 convertase [216]. Finally, unlike the classical and lectin pathway, the alternative partway is activated by spontaneous hydrolysis of the C3 to C3(H_2O) [217]. Upon hydrolysis, the factor B site is exposed resulting in the formation of the C3 convertase [218].

1.2.1 Non-Canonical activation of C3 and C5

For a long, it has been thought that C3 and C5 are activated by only liver-derived serum canonically circulating convertases. However, recent observations identified a non-canonical activation mechanism that regulates cleavage and activation of complement factors not only by serum-derived convertase but also by allergen-associated proteases and immune cells [219]. While the formerly involved cleavage by proteases [220], the latter non-canonical way to activate the complement is referred to as compose [221]. The first evidence of complosome

was identified in human $CD4^+$ T cells [221, 222]. was the identification of low expressed levels of C3 and C5 in the Endoplasmic reticulum (ER), endosome, and lysosome stores [221, 222]. While C3 is continuously cleaved by cathepsin L (CTSL) to generate C3b and C3, tonic C3a generation is important for T cell survival via activation of mammalian target rapamycin (mTOR), C3b, through an autocrine engagement of CD46. Various metabolic events, including expression of glucose, amino acid, and nutrition influx, glycolysis and oxidative phosphorylation (OXPHOS), and last but not least enhancement generation of intracellular C5 pools are regulated by CD46 [221, 222]. Further, intracellularly produced C5a stimulates the mitochondrial C5a that triggers ROS production and nucleotide-binding oligomerization domain-leucine-rich repeat-, and pyrin domain-containing protein 3 (NLRP3) inflammasome activation [223, 224].

Canonical and non-canonical activation led to the formation of C3a and C5a. Identified in 1970, C3a and C5a have been purified and characterized. These small peptides are called anaphylatoxins due to their ability to cause anaphylaxis when produced in large amount [225]. ATs are amino-terminal cationic fragments that are generated by multiple upstream pathways of the complement cascade as well as being generated by direct or indirect activation of immune cells (e.g., Neutrophil, macrophage) or platelets and coagulant cascade proteins. Human anaphylatoxins are 74-77 amino acids highly cationic proteins made up of four α helices in disulfide bonds. This unique structure imparts stability to the anaphylatoxins [226]. In normal conditions, CP and LP are activated by froing material under the tight control of complement regulators [210, 227]. However, under certain conditions, including tissue ischemia and reperfusion, it can be activated by not only froing material but also self-material, which leads to autologous damage [228]. In order to protect host cells from unspecific complement activation, various complement regulatory proteins take place [228]. These cell surface and fluid-phase proteins tightly control the complement system when the complement is hyperactivated.

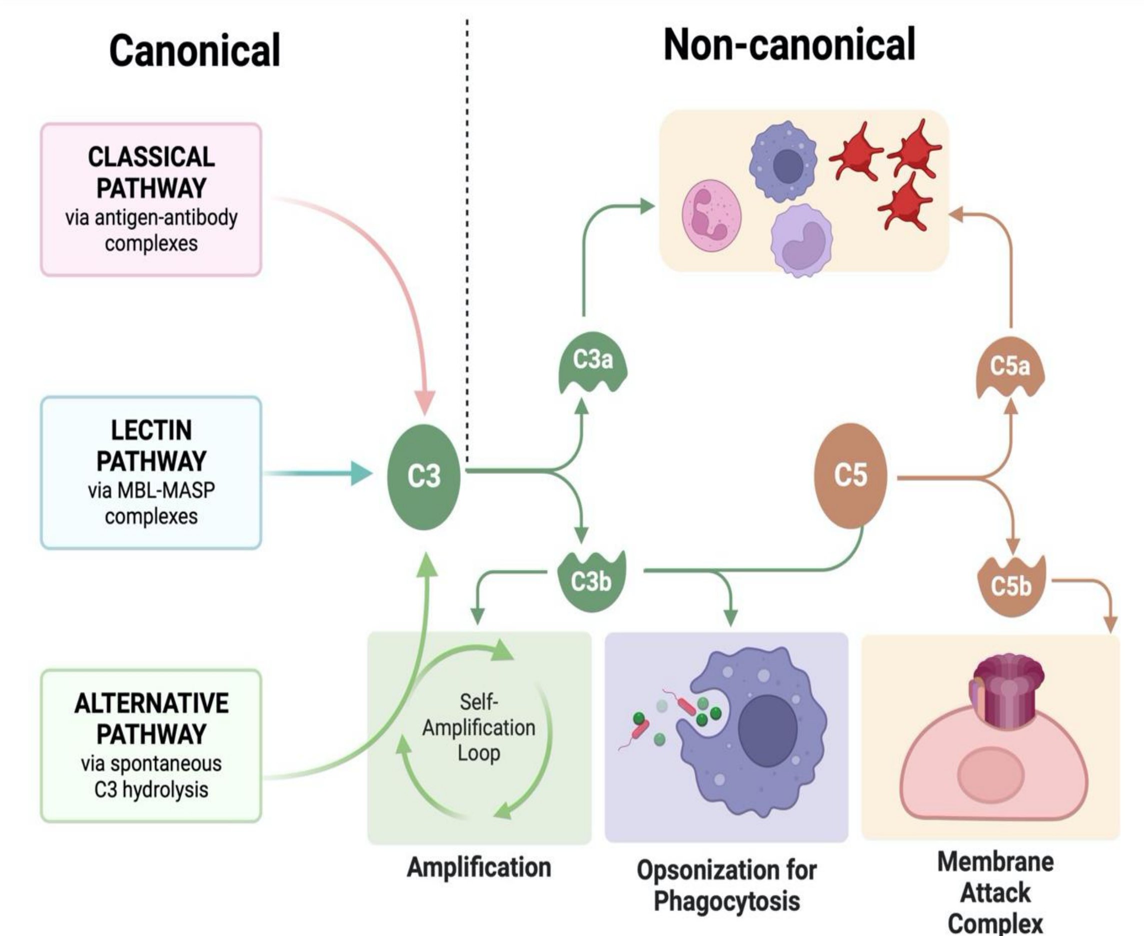

Figure 1. 6 Complement cascade and key canonical and non-canonical functions. The classical pathway is initiated upon recognition of antigen-antibody complexes, the lectin pathway via MBL-MASP complexes, and the alternative partway activates via spontaneous C3 hydrolysis. All 3 complement pathways after activation trigger the formation of C3 convertases. Then C3 is cleaved into C3a and C3b. In the specific case of the alternative pathway, C3b or C3b (H_2O) also initiates a positive feedback amplification loop. Subsequent to C3 activation, C3b participates in the formation of a C5 convertase that cleaves C5 into C5a and C5b. Later on, surface-bound C5b together with additional complement factors form by insertion of the Membrane Attack Complex (MAC). Furthermore, C3b is essential for opsonization and takes a role in the self-amplification loop, while C3a and C5a, collectively named Anaphylatoxins, bind to their cognate receptors: C3a receptor (C3aR), C5aR1, and C5aR2. Alternative to that canonical activation processes, recent studies identified intracellular immune cell proteases, as well as allergen-associated proteases as potent non-canonical pathways leading to the activation of C3 and C5 [223].

1.2.2 Role of Anaphylatoxins

Anaphylatoxins have been identified to play a diverse role in the health and disease [225]. Both C5a and C3a are inflammatory fragments playing role in the pathogenesis of inflammatory and immune-mediated diseases including tumor growth, sepsis, and autoimmune diseases [225]. In addition, ATs are chemotactic for inflammatory cells and trigger ROS release by inflammatory cells [229]. Furthermore, evidence shows that ATs trigger smooth muscle constriction and increase vascular permeability [230]. Of note, although both Ats are thought to be pro-inflammatory fragments, C5a is the most potent inflammatory fragment among all activated fragments [202, 226, 231].

As mentioned above, the complement system is tightly regulated by complement regulators [232-234]. In addition, the excessive pro-inflammatory activity of ATs is counteracted by the degradation of ATs through their desarginisation in their aminoterminal end, resulting in the formation of C3adesArg and C5adesArg [235]. Notably, whereas C3adesArg has no receptor-mediated function, C5adesArg retains reduced pro-inflammatory functions [236]. Anaphylatoxins show their multiple effector and regulatory functions via binding their conjugate receptor known as anaphylatoxin´s receptor (ATRs) which are members of G-protein-coupled receptors [232]. Of note, while C3a binds to C3aR, C5a and C5adesArg, bind to C5aR1 or C5aR2 [232].

1.2.2.1 C5a receptors

AT receptors belong to the largest member of the transmembrane (TM) receptors. There are two known receptors for C5a; C5aR1 (cluster of differentiation molecule 88, CD88) and C5aR2 (C5a-like receptor C5L2; G protein uncoupled receptor 77, GPR77) [233]. Although human C5aR2 shares a 37 % amino acid sequence identity with C5aR1, pieces of evidence show that there is a key difference between the receptors in an affinity [237] and most notably the in altered G protein binding [237].

C5aR1 is a membrane glycoprotein encoded in two exons by the *C5ar1* gene on chromosome 19 in humans and chromosome 7 in mice that result in a 7-TM domain protein [238]. C5aR1 is a G protein-coupled receptor (GPCR) coupled to Gαi and Gα16. Furthermore, it is a 42kDa protein that undergoes various post-translational modifications including an N terminus glycosylation, followed by a sulfatation which regulates the binding of its ligand [239]. Indeed, C5aR1 binds both C5a and its degradation product C5adesArg, although with different binding affinities. It has been reported that C5a has 10 -100 times more affinity to C5aR1 than

C5adesArg [239, 240]. Of note, upon binding C terminus is heavily phosphorylated, resulting in a ligand-induced internalization [240]. C5aR1 expression is found in many cells of myeloid origin including, neutrophils Eosinophils, macrophages, and dendritic cells [233]. Although C5aR1 is well characterized in the myeloid cells, several studies show the function of C5aR1 in T cells [241, 242]. It has been shown that activation of C5aR1 has a strong impact on the proliferation and differentiation of the T cells [243]. Similarly, the genetic absence or pharmacological targeting of C5aR1 on DCs limits the Th1 response and increases the frequency of Treg cells [244]. In addition to immune cells, expression of C5aR1 in non-immune cells, including epithelial endothelial and smooth muscle cells and neurons has been shown in mice [240, 245]. It is noteworthy that many of study show that strong C5aR1 is associated with asthma [246, 247]. For long, it was thought that C5aR1 exerts only pro-inflammatory functions. However, recent evidence proved that C5aR promotes either pro- or anti-inflammatory properties depending on the stage of asthma for instance during the sensitization [234].

On the other hand, other C5a/ C5adesArg receptor C5aR2 is uncoupled from G protein due to alteration in DRY and NPXXY motif, which was considered to mediate negative regulator C5aR1[237]. However, recent evidence showed that the binding of C5a to C5aR2 promotes pro- or anti-inflammatory response [237]. Of note, C5aR2 is able to regulate C5ar1 activation of MAPK-dependent signaling partway via its ability to bind β-arrestins [219, 248]. It has been reported that C5a/C5adesArg to its receptors, C5aR1 or C5aR2, initiates phosphorylation which results in association with β- arrestin [248, 249]. In addition, C5a-mediated cellular response is controlled by the ratio between the expression of C5aR1 and 2. For instance, while C5ar2/β-arrestin association results in ERK1/2 phosphorylation, C5aR2/β-arrestin causes the inhibition of the ERK1/2 phosphorylation [249]. Interestingly, evidence highlighted that the C5a response from bone marrow-derived dendritic cells, which only expressed C5aR1, is different from monocyte-derived DCs expressing both C5aR1 and C5aR2 due to activation of different downstream molecules [248, 250].

At the cellular level, both ATRs are demonstrated by reporter mice in different cells mainly from the myeloid origin [232, 233, 248, 251]. More specifically, in addition to myeloid cells, C5aR1 is expressed in cells NK, NKT cells, and T cells, as well as epithelial smooth muscle and neural cells [242, 251]. In comparison to C5arR1, C5aR2 is expressed lower level in the cells [248]. Studies using reporter mice show C5aR2 expression, mainly on neutrophils, Eosinophils, and dendritic cells [252].

1.3 Asthma

The word “Asthma” originates from the Greek meaning shortness of breath [253], It is a common chronic disorder affecting more than 300 million people worldwide [254]. Its prevalence increases, especially among children. Recent reports indicate that about 10 % of children and 5% of adults are affected by asthma in industrialized countries [254]. Asthma is a heterogeneous disease characterized by upper respiratory symptoms, including wheezing, shortness of breath, chest tightness, reversible airway obstruction, airway narrowing, airway wall thickening, and coughing resulting from complex interactions between genetic and environmental interactions [255]. Symptoms range from mild to severe, and even life-threatening [255].

Asthma can be divided into two clinical phenotypes: allergic asthma (extrinsic, atopic) and non-allergic asthma (intrinsic, non-atopic) [256]. Allergic asthma is the most easily recognized asthma phenotype and is associated with Eosinophilic airway inflammation and genetic susceptibility to produce immunoglobulin (Ig) E [256-258]. In addition, allergic asthma often starts early in life [253]. On a molecular and cellular level, atopic asthma is characterized by the development of a maladaptive Th2 inflammatory disease triggered by exposure to common airborne allergens, such as house dust (HDM) feces, fungal spores, and pollens [259]. In contrast, non-allergic asthma is not associated with either allergy or IgE level and often develops later in the lifetime triggered by various non-immunological phenomenons [260]. Non-atopic asthma is more often seen in males than females and is associated with chronic rhinosinusitis with nasal polyps and aspirin hypersensitivity [253, 261].

1.3.1Cellular aspects of allergic Asthma

1.3.1.1Sensitization to allergens

The initial and primary immune response to an inhaled allergen is initiated by the activation of the airway epithelium [262]. Placed at the interface between the internal and external environment, the airway epithelium provides the first line of defense via PRRs [262]. Such allergen exposure triggers disturbance in the integrity of the epithelial barrier through allergen-derived protease activity acting on tight junctions and/or protease-activated receptor (PAR2), resulting in dysregulation of permeability and integrity [263]. Furthermore, physical damage and epithelial lesions result in the secretion of alarmins, including TSLP, IL-33, and IL-25

which activate epithelial cells as well as immune cells [262]. Further, activated epithelium releases various molecules such as granulocyte-macrophage colony-stimulating factor (GM-CSF), CC chemokine ligand (CCL)-17, CCL-20, CXC chemokine ligand (CXCL)-2, IL-8, RANTES and danger molecules including uric acid, adenosine triphosphate, an high mobility group box1 [264]. Such events orchestrate innate immune functions in the lung via the activation, maturation, and recruitment of innate lymphoid cells type 2 (ILC2), dendritic cells, macrophages, and mast cells in the airway [37, 183, 265, 266]. Then, ILC2s produce Type2 cytokines, including IL-4, IL-5, and IL-13, further promoting the Th2 cell polarization [176]. These further triggers exacerbation of the allergic response via secretion of IgE by activated B and mast cells [180, 267].

1.3.1.2 In established asthma

The effector phase of asthma occurs upon subsequent encounters with previously exposed allergens during the sensitization [268]. It is the result of a response of IgE-loaded mast cells and basophils to allergen binding that triggers their degranulation and the release of mediators including histamine, protoplasts, leukotrienes, as well as lipid-derived mediators such as PGD, LTB_4, and LTC_4 [269]. These mediators cause a hypersensitivity reaction resulting in mucus oversecretion associated with airflow obstruction and remodeling of airway smooth muscle cells [38, 270, 271]. Further, histamine activates the other cells such as neutrophils and alveolar macrophages and promotes type 2 inflammation [108].

Mechanistically, such hyperreactivity to previously encountered allergens result from the allergen-dependent epithelial barrier disruption initiated upon sensitization. Indeed, the activated ILC2 and type 2 inflammation encompassing IL-4, IL-5, and IL-13 release, participates in the IL-4-triggered IgE class switching and the expansion of vascular cell adhesion molecule (VCAM-1), the IL-13-induced mucus production and airway remodeling via goblet cell hyperplasia and smooth muscle cell hyperplasia [176]. In addition, Th2/ILC2-driven IL-5 favors the recruitment of Eosinophils [272]. Airway mucosal Eosinophilia, one of the hallmarks of allergic asthma, supports the increase in smooth muscle reactivity which is associated with the development of airway hyperreactivity (AHR) through the release of granule-proteins such as MBP and EPX [113], while Eosinophilic cysteinyl-leukotrienes increase vascular permeability, mucus production, and trigger the release of histamine from mast cells [115]. Further, EOS-derived TGF-β plays a role in the development of airway

remodeling which occurs as an airway response to injury [273]. In addition, TGF-β is known to induce apoptosis in airway epithelial cells and lead to the expansion of the goblet cells [273]. EOS are able to release type 2 cytokines, including IL-4, IL-6, IL-9, and IL-13, which induce type 2 immune responses [115].

In addition, DCs are indispensable during the effector phase of allergic response. Allergen exposure induces inflammation and recruitment of CD11b^{+} cDCs and monocyte-derived (mo)DCs which upregulate CCR7 and migrate to the mediastinal lymph node (mLN) to initiate the T proliferation [274]. The proliferation of the T lymphocytes induces the production of Th2 cytokines and triggers classical hallmarks of asthma, including Eosinophilia, airway remodeling, and mucus production [274].

1.3.1.3 Factors associated with increased or decreased risk of asthma in children

Although the development and persistence of asthma are driven by the gene-environment interaction [275], recent evidence outlined the existence of "windows of opportunity" during pregnancy and early in life, in which both biological and sociological factors have a role in shaping neonate's immunity to the outer world [276-278]. This holds true for asthma, as several studies support those environmental factors, including allergens (both inhaled and ingested), pollutants (e.g., tobacco smoke, NO_2, SO_2), psychosocial factors, microbes, microbial metabolites originating from the maternal microbiota, influence the asthma development [275]. It has been reported that the nutrition of the mother and baby is one of the important factors that influence asthma development [275]. Although there is no solid evidence showing that ingestion of any specific food during pregnancy increases the risk of asthma, a couple of studies from pre-birth cohort reported that maternal intake of food commonly considered allergic was associated with a decrease in allergy and asthma in offspring [279, 280]. Furthermore, despite conflicting evidence, some studies suggest that breastfeeding decreases wheezing in early life [275]. In addition to nutrition, dietary supplements for mothers and/or babies, including vitamin D supplementation, fish oil, long-chain polyunsaturated fatty acids, and probiotics are known to influence asthma development [253, 254, 281-285].

Finally, evidence suggests that human interaction with microbiota may be beneficial in preventing asthma. This theory is called as "hygiene hypothesis" also known as the "microflora hypothesis" or "biodiversity hypothesis" [286, 287]. In agreement, studies have outlined a lower risk of asthma among children raised on farms, compared to non-farmers [288, 289]. In addition, it has been shown that children whose has contact with lipopolysaccharide endotoxin

have a reduced risk of asthma [290]. In line, exposure of the infant to the mother's vaginal microflora through vaginal delivery may be beneficial for reducing asthma development in comparison to children born by cesarean section [291].

In addition, a growing understanding of antibiotics on health outcome and microbiome increase the new concern. Fecal microbiome analysis on a group of term and preterm infants whose mothers received intrapartum antibiotics showed a higher percentage of *Enterobacteriaceae* and lower *Lactobacillaceae* [292]. Not surprisingly, antibiotic use during pregnancy and with toddlers has been associated with the development of asthma in later life, which may be related to the microbiota [276, 277, 293-296].

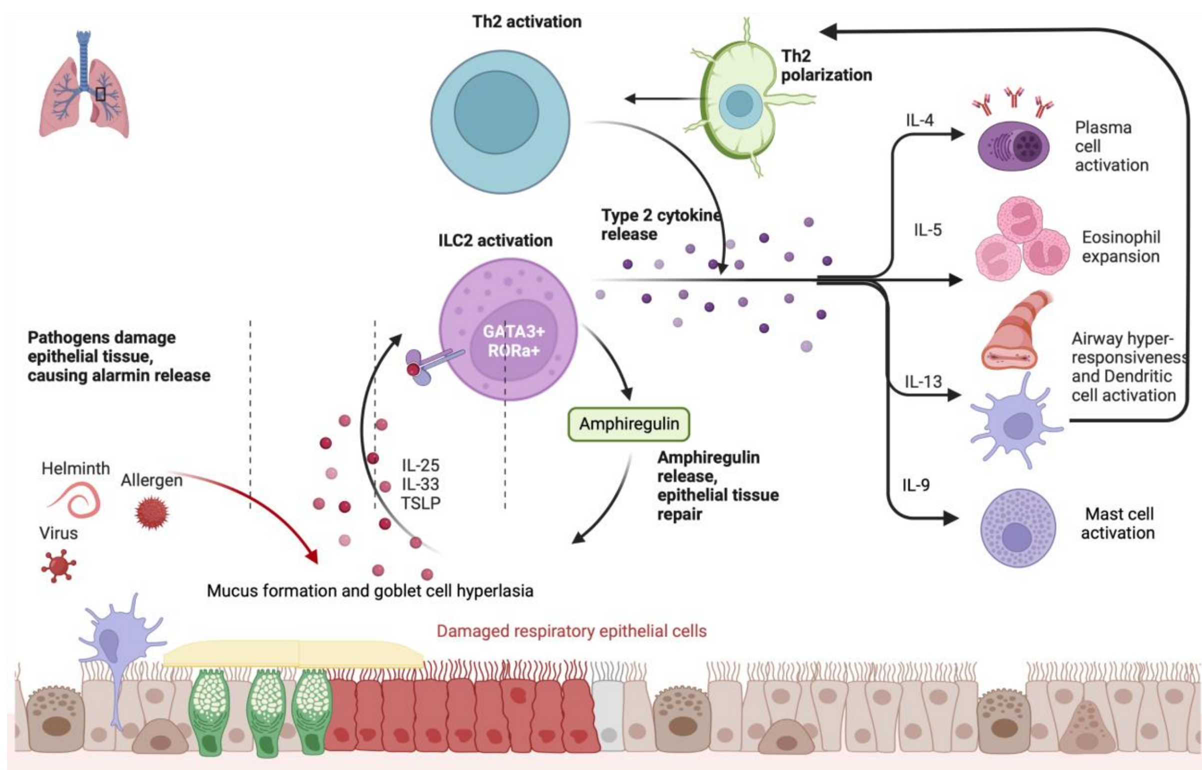

Figure 1. 7 Social networking upon allergen exposure. Upon allergen exposure, pathogens sensing, and damage sensing apparatus trigger the secretion of alarmins such as IL-25, IL-33, and Thymic stromal lymphopoietin (TSLP). Alarmins activate the ILC2s during sensitization that in turn helps dendritic cells which take up the allergen. Later, the task of the ILC2 is taken over by the Th2 cells. In addition, ILC2 together with Th2 cells regulates type 2 inflammation and takes part in epithelial tissue repair with amphiregulin secretion. IL-9 is essential for mast cell activation. IL-13 is critical for mucus hyperplasia and thus airway hyperresponsiveness. IL-5 is responsible for Eosinophil recruitment to inflamed tissue. IL-4 regulates the plasma cell activation and class switch [176].

1.4 Lung Development

The lung is primarily responsible for exchanging oxygen in the external environment with carbon dioxide to accommodate the needs of aerobic cellular respiration [297]. Lung development begins early in human gestation (around day 25) and embryonic day (E)9 in the mouse [298]. In humans and mice, the lung arises from two germ layers; the gut endoderm and the splanchnic mesoderm [299]. While gut epithelium originates from lung endoderm, the splanchnic mesoderm gives rise to the lung mesenchyme [299]. Lung development is divided into four stages; Embryonic or Pseudoglandular period, Canalicular period, Saccular period, and Alveolar period [300]. While each stage has characteristic features, there is some overlap between the beginning and end of each of these stages [300].

The embryonic period begins 24 to 26 days after fertilization and is complete by 7 weeks of gestation. Organ development-organogenesis occurs during this period. This period is characterized by the process of morphogenesis which regulates the lung bud formation and initial branching of presumptive airways [301]. During this period, epithelial cell progenitors differentiate into basal, neuroendocrine, ciliated, and secretory cells [299]. In addition, the mesodermal lung compartment differentiates into smooth muscle cells, lymphatic cells, Endothelial cells, Nerve cells, and Chondrocytes.

The canalicular stage (Human: week 17-26; mouse: Embryonic day (E)1 6.5- E17.5), is marked by the early development of pulmonary parenchyma, the subdivision of the respiratory bronchioles, and the multiplication of capillaries [300]. During this period, epithelial cells progenitors, also known as alveolar bipotential progenitors, are differentiated into type 1 and type 2 alveolar epithelial cells (AEC I and AEC II) [299].

The saccular stage (Human: week 26-40; mouse: E17.5-Postnatal day (P)5), is characterized by the formation of alveolar sacs and new arteries and vascular and respiratory tissue expansion [299]. In these stages, mesenchyme gets thinner to maintain the gas exchange [302].

In the uterus, gas exchange is performed by the placenta and as the baby is delivered, the lungs are used to breathe. While the lung of the newborn human at 40 weeks is functional**,** remodeling still occurs within the parenchyma and capillary network; this stage is named the alveolar stage (Human: week 36 to 8 years; mouse P5-P30) [300]. In this stage, the alveolar surface increase through subdivision of the alveolar sacs into mature alveoli [300]. This process is named the

alveolarization [300, 303]. Notably, double-layered capillaries become thinner and form single capillary networks during alveolarization to provide a more efficient gas exchange [300].

1.5 AIMS

The prevalence of allergic diseases, such as allergic asthma has continued to rise in developing nations in recent decades. The main form of allergic asthma is characterized by strong inflammatory Eosinophil which is driven by the secretion of IL-5 from ILC2s during sensitization. Further, the soluble factors anaphylatoxins C3a and C5a have been recognized as potent regulators of both the development and severity of the disease via their binding to their cognate receptors C3aR, C5aR1, and C5aR2. C3aR signaling in DCs has been recognized as an important regulator of ILC2 function in the allergic rhinitis [304-306], but the closely related ATR, C5aR1, in the ILC2 development remains elusive. Additionally, iEOS, not only accumulate in the lung after allergen exposure but it can be also found during early postnatal lungs. The main purpose of this thesis was to establish the functions of the anaphylatoxin receptor, C5aR1 on ILC2s in health and pulmonary disease.

1. To determine if the impact of C5aR1 deficiency on ILC2 functions is direct, through its expression in ILC2 or ILC2 precursors (ILC2Ps)
2. To determine whether the impact of C5aR1 deficiency on ILC2 functions is indirect, through complex networking between AM, DC, and ILC2.
3. To determine, if the recruitment of iEOS in the postnatal developing lung is altered by ATR deficiency
4. To determine, if maternal Antibiotic exposure during the early life of offspring is a risk factor for asthma development.

2 . Material, Equipment, and Methods

2.1Materials

2.1.1 Chemicals

Table 2. 1: Used chemical

Substance	**Manufacturer**
Acetyl-β-Methyl-Choline (Methacholine)	Sigma-Aldrich Chemie GmbH, Steinheim
Agorose, low melting point	Bio-Rad Labotories GmbH, Munich
Aqua ad injectabilia	B. Braun Melsungen AG, Melsungen
Ampicillin	USP Grade, Gold biotecnology Inc., St Louis, USA
BD FACS Flow Sheath Fluid	BD Biosciences Europe, Erembodegem, Belgium
	BD Biosciences Europe, Erembodegem, Belgium
Brilliant Stain buffer	eBioscience ™ Brefeldin A Solution,
Brefeldin A, 1000x	Thermo Fisher Scientific, Waltham, USA
Bovine serum albumin (BSA)	Sigma-Aldrich Chemie GmbH, Steinheim
C5a, human,recombinant	Hycult,Biotech, Uden,Netherland
Compensation beads (anti rat/hamster)	BD Biosciences Europe, Erembodegem, Belgium
Chloroform	Thermo Fisher Scientific, Waltham, USA
Cytoseal	Thermo Fisher Scientific, Waltham, USA
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich Chemie GmbH, Steinheim
DNase I from bovine pancreas	Sigma-Aldrich Chemie GmbH, Steinheim
Dulbecco`s Phosphate Buffered Saline	Life Technologies Corporation, Carlsbad, USA
Esmeron® (Rocuronium bromide)	Organon, Oss, Niederlande
Ethanol, absolute	J. T. Baker, Deventer, Netherlands
Ethanol, 70% denaturated	Carl Roth GmbH & Co. KG, Karlsruhe
Ethanol, 96% denaturated	Carl Roth GmbH & Co. KG, Karlsruhe
Ethylenediaminetetraacetic acid (EDTA)	Sigma-Aldrich Chemie GmbH, Steinheim
Gentamicin	USP Grade, Gold biotecnology Inc., St. Louis,
Glucose	USA
Glycerol	D-(+) Glucose, Sigma-Aldrich Inc., St. Louise,
	USA

	Glycerol for Flouresence microscopy, Merck KGaA, Darmstadt, Germany
Fetal calf serum (FCS) (Lot A04304-0413)	PAA Laboratories GmbH, Pasching, Östereich
Formaldehyde solution, 37%	Sigma-Aldrich Chemie GmbH, Steinheim
Fluoroshield™ histology mounting medium	Sigma-Aldrich Chemie GmbH, Steinheim
HEPES 1M	Chroma Technology Corporation, Carlsbad, USA
House dust mite extract (lot 262538)	Greerlabs Laboratories Inc., Lenoir, USA
Ionomycin	Sigma-Aldrich Chemie GmbH, St. Louis, USA
Isopropanol	Otto Fishar GmbH & Ko. KG, Saarbrücken
Ketamine hydrochloride	Sigma-Aldrich Chemie GmbH, Steinheim
L-glutamine (200 mM concentrate)	Life technologies Corporation, Carlsbad, USA
Liberase™ TL Research Grade	Roche Diagnostics International AG, Rotkreuz, Risch, Schweiz
MACS® BSA stock solution	Milteny Biotec GmbH, Bergisch Gladbach
MEM Non-Essential Amino Acids Solution (100X)	Thermo Fisher Scientific, Walham, USA
Monensin, 1000x	eBioscience ™ Monensin Solution, Thermo Fisher Scientific, Waltham, USA
Mowiol	Sigma-Aldrich Chemie GmbH, St. Louis, USA
Normal donkey serum control	Jackson Immunoresearch Laboratories, West Grove,USA
Normal mouse serum	Invitrogen, Carlsbad,USA
Dulbecco’s Phosphate Buffered Saline (PBS)	-Life technologies Corporation, Carlsbad, USA
Penicillin-Streptomycin, L-glutamin 100x Liquid	Life technologies Corporation, Carlsbad, USA
PMA	Phorbol 12-myristate 13-acetate, Sigma-Aldrich Chemie GmbH, St. Louis, USA
Potassium bicarbonate ($KHCO_3$)	Sigma-Aldrich Chemie GmbH, Steinheim
RPMI 1640	Life technologies Corporation, Carlsbad, USA
Sucrose	Carl Roth GmbH & Co. KG, Karlsruhe
Sucralose	Sigma-Aldrich Chemie GmbH, St. Louis, USA
Sodium chloride	Sigma-Aldrich Chemie GmbH, Steinheim
Sodium chloride, 0.9%	Berlin-Chemimie AG, Berlin

Sodium pyruvate	Life technologies Corporation, Carlsbad, USA
Sodium pentobarbitial	Provided by veterinary service, CCHMC, Cincinnati USA
Vancomycin	USP Grade, Gold biotecnology, St Louis, USA
Trypan blue	Life tecnologies Corporation, Carlsbad, USA
Tris	Carl Roth GmbH & Co. KG, Karlsruhe
Tween 20	Sigma-Aldrich Chemie GmbH, Steinheim
Water, sterile, deinoized	Ampuwa® Plastipur®, Fresenius Kabi GmbH, Bad Hamburg, Germany
Xylazine	Sigma-Aldrich Chemie GmbH, Steinheim
Recombinant mouse IL-7	Biolegend, UK
Recombinant mouse IL-2	PeprotechCorporation,Rocky Hill,USA
Mouse carrier free IL-33	eBioscience, Austuria
Murine GMC-SF	PeprotechCorporation,Rocky Hill,USA

2.1.2 Antibodies for flow cytometry, immunofluorescence staining

Table 2. 2: Antibodies used for flow cytometry, immunofluorescence staining

APC = Allophycocyanin, AF = Alexa Fluor, BV = Brilliant Violet, Cy = Cyanin dye, eF = eFlour, FC = flow cytometry, FITC = Fluorescein isothiocyanate, HRP = Horseradish peroxidase, IHC = Immunohistochemistry, PE = Phycoerythrin, PerCP = Peridinin chlorophyll protein, V = Violet, WB = Western blot

Antibody	Clone	Label	Manufacturer	Isotype	Conc. mg/ml	Appli-cation	Dilution
anti-mouse CD16/32	93	–	eBioscience, Vienna, Austria	–	0.1	FC	1:100
anti-mouse B220	RA3-6B2	PerCP-Cy5.5	Biolegend, London, UK	Rat IgG2a	0.2	FC	1:300
anti-mouse CD3e	145-2C11	PerCP-Cy5.5	eBioscience, Vienna, Austria	armenian Hamster IgG	0.2	FC	1:300
anti-mouse CD3	17A2	e450	eBioscience, Vienna, Austria	Rat IgG2a	0.5	FC	1:400
anti-mouse CD3	17A2	e450	eBioscience, Vienna, Austria	Rat IgG2a	0.5	IHC	1:100
anti -mouse CD44	IM7	PEAlexa 610	eBioscience, Vienna, Austuria	Rat IgG2b	0.2	FC	1:200
Anti-mouse CD62L	MEL-14	APC	eBioscience, Vienna, Austuria	Rat IgG2a	0.5	FC	1:300
anti-mouse CD49b	DX5	APC-Cy7	Biolegend, London, UK	Rat IgG2a	0.2	FC	1:300

anti-mouse Ly6G	1A8	PerCP-Cy5.5	BD Biosciences, Belgium	Rat IgG2a	0.2	FC	1:300
anti-mouse Ly6G	1A8	PE	BD Biosciences, Belgium	Rat IgG2a	0.2	FC	1:400
anti-mouse Ly6C	1A8	APC-Cy7	BD Biosciences, Belgium	Rat IgG2a	0.2	FC	1:400
anti-mouse SiglecF	E50-2440	BV421	BD Biosciences, Belgium	Rat IgG2a	0.2	FC	1:300
anti-mouse CD11c	N418	APC	eBioscience, Vienna, Austria	armenian Hamster IgG	0.2	FC, IHC	1:800
anti-mouse CD11c	N418	BV-711	Biolegend, London, UK	armenian Hamster IgG	0.2	FC, IHC	1:200
anti-mouse CD11c	N418	APC-Cy7	Biolegend, London, UK	armenian Hamster IgG	0.2	FC	1:300
anti-mouse CD11b	M1/70	PerCP-Cy5.5	BioLegend, London, UK	Rat IgG2b	0.08	FC	1:300
anti-mouse CD11b	M1/70	PE-Cy7	BioLegend, London, UK	Rat IgG2b	0.2	FC,IHC	1:100
anti-mouse CD25	PC6.1	BV421	BioLegend, London, UK	Rat IgG1	0.2	FC	1:200
anti-mouse CD25	PC6.1	PE	BioLegend, London, UK	Rat IgG1	0.2	FC	1:200
anti-mouse CD127	A7R34	PE-Cy7	eBioscience, Vienna, Austria	Rat IgG2a	0.2	FC	1:200
anti-mouse CD127	A7R34	APC	eBioscience, Vienna, Austria	Rat IgG2a	0.2	FC	1:200
anti-mouse CD64	X54-5/7.1	BV711	BioLegend, London, UK	Rat IgG1	0.5	FC	1:800
anti-mouse CD88	20/70	APC	BioLegend, London, UK	Rat IgG2b	0.2	FC	1:800

anti-mouse CD88	20/70	PE-Cy7	BioLegend, London, UK	Rat IgG2b	0.2	FC	1:200
anti-mouse CD101	Moushi	PE	eBioscience, Vienna, Austria	Rat IgG2a	0.25 µg/test	FC	1:6ß00
anti-mouse MHC-II	M5/114.15.2	FITC	BioLegend, London, UK	Rat IgG2b	0.5	FC	1:1500
anti-mouse MHC-II	M5/114.15.2	PE-Cy7	eBioscience, Vienna, Austria	Rat IgG2b	0.2	FC, IHC	1:1500
anti-mouse CD4	RM4-5	PE-Cy7	eBioscience, Vienna, Austria	Rat IgG2a	0.2	FC	1:400
anti-mouse CD4	RM4-5	V450	eBioscience, Vienna, Austria	Rat IgG2a	0.2	IHC	1:100
anti-mouse TSLP-R	22H9	PE	BioLegend, London, UK	Rat IgG2a	0.2	FC	1:200
anti-mouse F4/80	BM8	BV510	BioLegend, London, UK	Rat IgG2a	0.2	FC	1:400
anti-mouse KLRG-1	2F1	BV-711	eBioscience, Vienna, Austria	Syrian hamster IgG	0.2	FC	1:400
Anti-mouse/Human GATA3	TWAJ	PE	eBioscience, Vienna, Austria	Rat IgGb	5 µL (0.00375 µg)/test	FC, IHC	1:200
anti-mouse GATA3	HG3-31	AF-647	Santa Cruz, Dallas, USA	mOUSE IgG1	0.2	IHC	1:200
anti-mouse T1/ST2 (IL33R)	DJ8	PE	BD bioproducts, London UK	Rat IgG1	0.2	FC	1:800
anti-mouse LPAM	DATK32	PE	BioLegend, London, UK	Rat IgG2a	0.2	FC	1:200

anti-mouse LPAM	DATK3 2	APC	BioLegend, London, UK	Rat IgG2a	0.2	FC	1:200
anti-mouse BCL-2	BCL/10 C4	BV421	BioLegend, London, UK	Rat IgG2a	0.2	FC	1:200
anti-mouse Tbet	4B10	Alexa Flour 4888	BioLegend, London, UK	Rat IgG2a	0.2	FC	1:200
anti-mouse Rorγt	AFKJS-9	APC	eBioscience, Vienna, Austria	Rat IgG2a	0.2	FC	1:200
anti STAT3 (pY705)	p-49/P-STAT3	Alexa Flour 4888	BD Biosciences Belgium	Mouse IgG2a	50 test/ml	FC	1:300
Anti STAT4 (pY693)	p-A1513E 25T	APC	BD Biosciences Belgium	Rat IgG2a	50 test/ml	FC	1:400
anti STAT5 (pY694)	p-47	PEcy7	BD Biosciences Belgium	Mouse IgG1	50 test/ml	FC	1:600
anti STAT6, (y641)	p-CHI2S4 N	PE	eBioscience, Vienna, Austria	Mouse IgG1	0,125	FC	1:400
IL-5	TRFK-5	BV-421	eBioscience, Vienna, Austria	Rat IgG1a	0.2	FC	1:400
anti-mouse CD125	T21	BV-711	BD Biosciences Belgium	Rat Wistar IgG1	0.2	FC	1:400
anti-mouse CD19	ID3/CD 19	PerCP-Cy5.5	eBioscience, Vienna, Austria	Rat IgG2a	0.2	FC	1:300

anti-mouse CD27	LG.3A10	PerCP-Cy5.5	BioLegend, London, UK	Armenian hamster IgG	0.2	FC	1:300
anti-mouse CD5	53-7.3	PerCP-Cy5.5	BioLegend, London, UK	Rat IgG2a	0.2	FC	1:300
anti-mouse TCR-β	H57-597	PerCP-Cy5.5	BioLegend, London, UK	Armenian Hamster IgG	0.2	FC	1:300
anti-mouse CD45	30-F11	eflour 506	eBioscience, Vienna, Austria	Rat IgG2b	0,25	FC	1:300
anti-mouse CD90.2	53-2.1	BV605	BioLegend, London, UK	Rat IgG2a	0.2	FC	1:200
anti-mouse CD90.2	53-2.1	PE	BioLegend, London, UK	Rat IgG2a	0.2	FC	1:200
anti-mouse Ter-119/ Erithroid Cell antibody	Ter119	PerCP-Cy5.5	BioLegend, London, UK	Rat IgGb	0.2	FC	1:300
anti-mouse Ly-6A/E	D7	BV510	BioLegend, London, UK	Rat IgG2a	0.2	FC	1:300
anti-mouse Ki-67	SolA15	FITC	eBioscience, Vienna, Austria	Rat IgG2a	0.25 µg/test	FC	1:400

2.1.3 Compounds used for flow cytometry

Table 2. 3: Compounds used for flow cytometry, immunofluorescence staining.

AF = Alexa Fluor, DAPI=4′,6-diamidino-2-phenylindole, eF = eFluor, FC = flow cytometry, IHC = Immunohistochemistry.

Compound	Clone	Label	Manufacturer	Isotype	Conc. mg/ml	Application	Dilution
Fixable Viability Dye	–	eF780	eBioscience, Vienna, Austria	-	-	FC	1:1500
DAPI		-	Invitrogen, Paisley, UK		5.0	IHC	1:500

2.1.4 Consumables

Table 2. 4: Consumables.

Material	Manufacturer
Cell strainer 40 µm	BD Biosciences Europe, Erembodegem, Belgium
Cell strainer 100 µm	BD Biosciences Europe, Erembodegem, Belgium
Cannula 20 G	Vasofix® Safety, B.Braun AG, Melsungen, Germany
Cover glasses	Mezel Cover Glasses, 2x60mm, thickness I, VWR International GmbH, Darmstadt
Insulin syringes, 1mL	Becton Dickson Inc., Radnor, USA
Liquid Blocker pen	Liguid blocker PAP pen , Daido Sangyo Ltd., Tokyo, Japan
ELISA-reservoir 25 ml	VWR International GmbH, Darmstadt
FACS tube 5ml	Sarsted AD&Co., Nümbrecht
Filtertip 10 µl, 100 µl, 1000 µl	Sarstedt AD & Co., Nümbrecht
gentleMACS™ tube	Milteny Biotec GmbH, Bergisch Gladbach
LowRetention tube	
Microscope slide	Gerhard Menzel GmbH, Braunschweig
Micro tube 0.5 ml; 1.5 ml; 2 ml	Sarstedt AD & Co., Nümbrecht
Needle 26G	BD Biosciences Europe, Erembodegem, Belgium
Nitrile Powder-Free Examination Gloves	Ansell Healthcare GmbH, Munich

Parafilm	Parafilm Laboratory Sealing Film Bemis Inc., Neenah, USA
Petri dish 60x15 mm	Greiner Bio-One GmbH, Frickenhausen
Pipette tip 10 µl, 100 µl, 1000 µl	Sarstedt AD & Co., Nümbrecht
Pipette with tip 5 ml, 10 ml, 25 ml	Greiner Bio-One GmbH, Frickenhausen
Plate 6 well	Sarstedt Inc., Newton, USA
Plate 96 well (U bottom)	Greiner Bio-One GmbH, Frickenhausen
Pur-Zellin swab	Greiner Bio-One GmbH, Frickenhausen
Serological pipettes, 5ml, 10 ml , 25 ml	Sarstedt AG, Nümbrecht, Germany
Spatula	VWR International GmbH, Darmstadt
Single-use syringe 1 ml	B. Braun Melsungen AG, Melsungen
Syringe 5 ml, 10 ml	BD Biosciences Europe, Erembodegem, Belgium
Scalpel	Disponsable Sabre Surgical Scalpels, Swann-Monton Ltd., Sheffielf, UK
Tracheal cannula for mouse (OD 1.2 mm, L 15mm)	Hugo Sachs, March-Hugstetten
Tubes 5 ml, 15 ml, 50 ml	Sarstedt AD & Co., Nümbrecht
Tube stripe 0.2 ml	Sarstedt AD & Co., Nümbrecht
Weighing dish	Greiner Bio-One GmbH, Frickenhausen

2.1.5 Kits

Table 2. 5: Used Kits

Kit	**Manufactures**
Foxp3 intracellular staining kit	eBioscience, Vienna, Austria
Fixation /Permeabilization solution	Becton Dickinson Inc., Franklin Lakes, USA

2.1.6 Buffers and Solutions

Table 2. 6 : Buffers and solutions

Buffer /Solution	**Substance**
ACK Lysing buffer	Ammonium-Chloride-potassium Lysing buffer
	Lonza AG, Basel, Switzerland
Anesthetic 10x (BALB/c)	2% Xylazin
	50 mg/ml Ketamin
Anesthetic 1x (BALB/c)	PBS
	10% 10X anesthetic
Agarose Low Melt from Bio-Rad® 3%	0,3g Agarose Low Melt BioRad
	10 ml HEPES
BD brilliant violet staining buffer	Brilliant violet staining buffer
	1:10 dilution in MACS buffer
Complete Medium	RPMI 1640
	10% FCS, heat inactivated
	100 Unit/ml Penicillin
	100 µg/ml Streptomycin
	2mM L-Glutamine
Complete cell culture media for	RPMI 1640
BM- drived DCs	100 U/ml penicillin
	100 µg/ml Streptomycin
	10% heat-inactivated fetal calf serum (FCS)
	2 mM L-glutamine
	55 µm 2-mercaptoethanol

Cell culture media for Pulmonary or BM-derived ILC progenitor	RPMI 1640 medium
	100 U/ml penicillin
	100 μg/ml Streptomycin
	10% heat-inactivated fetal calf serum (FCS)
	2 mM L-glutamine
	%10 non-essential amino acids
	1mM sodium pyruvate
DNAse 1 reconstition	Sodium chloride, 0.9%
	100 mg/ML DNase 1
ELISA	
Fc block buffer	Anti- mouse CD16/32 antibody
	1:100 dilution in flow buffer
Ringer solution	5.6 mM KCl
	136.4 mM NaCl
	1 mM $MgCl_2$ x 6 H2O
	2.2 mM $CaCl_2$ x 2 H2O
	11mM Glucose
	10 mM HEPES
	pH 7,4 10N NaOH
HDM reconstitution	Ampuwa®(Aqua and injectabilia)
	10 mg/ml protein
HDM (i.t. application)	PBS
IMDM	Iscove`s Modified Dulbecco`s Medium
	Thermo FisherScientific, Waltham, USA
MACS buffer (Flow buffer)	PBS
	0.5% MACS® BSA stock solution
Sorting buffer	PBS
	1% MACS® BSA stock solution
PFA solution	PBS
	PFA (0.5% v/v) used for bone tissue fixation
PFA solution	PBS
	PFA (4% v/v) used for cell fixation
Red Blood Cell Lysis Buffer	dH_2O
	155 mM NH_4CI

	10 mM $KHCO_3$
	0.1 mM EDTA
	pH 7.2
	Sterile
Re-stimulation Medium	RPMI 1640
	10% FCS, heat activated
	100 Unit/ml Penicillin
	100 µg/ml Streptomycin
	2mM L-Glutamine
	55 µm 2-mercaptoethanol
5x Stimulation mix	PMA 250 ng/ml
	Iomycin 2500 ng/ml
	Brefeldin 5x
	Monensin 5x
	IMDM
Wash medium (lung cell isolation)	RPMI 1640
	10% FCS, heat inactivated
	100 Unit/ml Penicillin
	100 µg/ml Streptomycin
	2mM L-Glutamine
	0.5 mg/ml DNase 1
Wash buffer for (IHC)	100 ml 10X TBS 900 ml dH_2O 1 ml Tween-20 and mix
Blocking solution (IHC)	5 ml 1X TBST 250 µl normal mouse serum or donkey serum
20% EDTA	EDTA disodium salt,200g
	dH_2O 950 ml
	pH was adjusted to 7.4 with NaOH

2.1.7 Mouse strains

Table 2. 7 : Specification of used mouse strains

Name	Strain	Official name (symbol)	Breeder
C5ar1$^{-/-}$	BALB/c	C5ar1^{tm1Cge}	Internal breeding, ISEF, AG Köhl, Lübeck, Germany
Wildtype (WT)	BALB/c	BALB/cAnNCrl	Charles River, Breeding Laboratories,Sulzfeld, Germany
Wildtype (WT)	BALB/c	BALB/cAnNCrj	Internal breeding, ISEF, AG Köhl, Lübeck, Germany
Wildtype (WT)	C57BL/6	C57BL/6J	Jackson, USA
Wildtype (WT)	C57BL/6	C57BL/6JRj	Internal breeding, ISEF, AG Köhl, Lübeck, Germany
Wildtype (WT)	C57BL/6	C57BL/6J	Internal breeding, CCHMC, Lewkowich Lab, Lübeck, Germany
GFP-C5ar1$^{fl/fl}$	BALB/c	C5aR1TMJko	Internal breeding, ISEF, AG Köhl, Lübeck, Germany
C5-Lam (C5 deficient mouse, Hc0 allele from DBA/2J)	C57BL/6	B10.D2/oSnJ; C5-d	Internal breeding, AG Nording, Lübeck, Germany
LysMcre-GFP-C5ar1$^{fl/fl}$	C57BL/6	B6.C5aR1tm1JKo x B6.129P2-*Lys2t$^{m1(cre)Ifo}$*/J	Internal breeding, ISEF, AG Köhl, Lübeck, Germany
B6-LysMcre	C57BL/6	B6.129P2-*Lys2$^{tm1(cre)Ifo}$*/J	Internal breeding, ISEF, AG Köhl, Lübeck, Germany

2.1.8 Equipment and Software

2.1.8.1 Equipment

Table 2. 8 : Used equipment

Equipment	Manufacturer
BD^{TM} LSR II cell analyzer	Beckton Dickinson GmbH, Heidelberg
BD LSRFortessa™ cell analyzer	Beckton Dickinson GmbH, USA

Biological Safety Cabinets	Nuaire Inc., Plymouth, USA
Cell Sorter BD FACSAria™ III	Beckton Dickinson GmbH, Heidelberg
Cell Sorter BD FACSAria™ II	Beckton Dickinson GmbH, USA
Confocal laser scanning microscope LSM710	Carl Zeiss,Oberkochen
Centrifuge 5424	Eppendorf AG, Hamburg
Centrifuge 5424R	Eppendorf AG, Hamburg
Centrifuge 5810R	Eppendorf AG, Hamburg
Chemical hood	Waldner Laboreinrichtungen GmbH & Co KG, Wangen
Cytospin centrifuge Cellspin I	Tharmac GmbH, Walsolms
Dissecting scissors	WPI Deutschland GmbH, Berlin
ELISA-Reader Fluostar Omega 0415	BMG Labtech GmbH, Ortenberg
ELISA-Washer Nunc-Immuno™ Wash 12	Thermo Fisher Scientific Inc., Waltham, USA
FlexiVent	SCIREQ Scientific Respiratory Equipment Inc., Montreal, Canada
Forceps	WPI Deutschland GmbH, Berlin
Fridge, 4 °C and -20 °C combined	Liebherr-International Deutschland GmbH, Biberach an der Riß
Hot-air cabinet	Memmert, Schwabach
Incubator	Heraeus, Hanau
IR Direct Heat CO_2 Incubator	Nuaire Inc., Plymouth, USA
MACS multicolur flow cytometry	Miltenyi Biotec, Bergisch Gladbach Germany
Microscope Fluovert FS	Leica Mikrosysteme Vertrieb GmbH, Wetzlar, Germany
Microscope Leica DM IL LED	Leica Mikrosysteme Vertrieb GmbH, Wetzlar, Germany
Microscope camera Leica EC3	Leica Mikrosysteme Vertrieb GmbH, Wetzlar, Germany
MoFlo Legacy Cell Sorter	

Multichannel pipette Biohit M300	Sartorius Biohit Liquid Handling Oy, Helsinki, Finnland
Neubauer counting chamber, improved	VWR International GmbH, Darmstadt
pH-Meter Seven Easy PH S20-K	Mettler Toledo, Schwerzenbach, Schweiz
Pipetboy	Integra Biosciences AG, Zizers, Schweiz
Pipette (0,1-2,5 µl; 0,5-10 µl; 10-100 µl; 20-200 µl; 100-1000 µl)	Eppendorf AG, Hamburg
Precision balance LC6200S	Sartorius AG, Göttingen
Pure water system Nanopure Diamond D11931	Thermo Fisher Scientific GmbH, Bremen
Shaker Polymax 1040	Heidolph Instruments GmbH & Co. KG, Schwabach
Steam sterilizer E14 Hydromat	WEBECO Hygiene in Medizin und Labor GmbH & Co. KG, Selmsdorf
Suction system Vacusafe 158310	Integra Biosciences GmbH, Fernwald
Table centrifuge	Carl Roth GmbH & Co. KG, Karlsruhe
Ultra-low temperature freezer, -80 °C	SANYO Electrics Co., Japan
VacuGene Pump	Pharmacia, Belgium
Vortex-Genie 2	Scientific Industries Inc., New York, USA

2.1.8.2 Software

Table 2. 9: Used software for data collection and analysis.

Programm	**Firma**
BD FACSDiva 7.0	BD Biosciences, San Jose, USA
EndNote X7.8	Clarivate Analytics, Philadelphia, USA
FlexiVent Software 5.3	SCIREQ Scientific Respiratory Equipment Inc., Montreal, Canada
FlowJo 10	FlowJo, LLD, Ashland, USA
GraphPad Prism 8.0	Graph Pad Software Inc., LaJolla, USA
Imaris 9.2.1	Bitplane, Concord, USA
Microsoft Excel 2016	Microsoft Corporation, Redmond, USA
Microsoft Powerpoint 2016	Microsoft Corporation, Redmond, USA
Microsoft Word 2015	Microsoft Corporation, Redmond, USA

Image J	Wayne Rasband, USA
Imaris 9.2.1	Bitplane, Concord, USA
Matlab	Mathematical Computing Software, Simulink, California, USA
Legendplex software	BioLegend, London, UK

2.2 Methods

2.2.1 Animals

Wild-type (WT) mice were either purchased from Charles River Laboratories (BALB/cAnNCrl) or came from internal breeding (Balb/cAnNRj, WT C57BL/6JRj) in our specific-pathogen-free (SPF) animal facility from the University of Lübeck. In addition,
C5ar1$^{-/-}$, GFP-*C5ar1*$^{fl/fl}$, LysMcre-GFP-*C5ar1*$^{fl/fl}$, and C5 lam mice were also bred and kept in our specific-pathogen-free (SPF) animal facility from the University of Lübeck and the University of Cincinnati. Animals were used for organ removal and experimental allergic asthma studies. All studies were reviewed and according to protocols approved by the local authorities of the Animal Care and Use Committee from Schleswig-Holstein state authorities (Ministerium für Landwirtschaft, Energiewende, Umwelt und ländliche Räume, Kiel, Germany) (V312-7224.122-39 (37-2/13), V242-30278/2016 (50-4/16), V242-389/2016 (75-6/16), V242-23572/2018 (44-5/18) respectively). Mice were sacrificed by an overdose of 10x anesthetic (75-100µl 76mg/ml Ketamine, 4.8mg/ml Xylazine), followed by cervical dislocation if not stated otherwise.

2.2.2 Intratracheal application of substances

Mice were anesthetized with 6.9mg/kg bodyweight Xylazine and 114.5 mg/kg bodyweight Ketamine by intraperitoneal (i.p.) injection. Deeply narcotized mice were fixed on an immunization board by their incisors and an elastic band. The tongue was carefully pulled out and a total volume of 50 µl of the respective substance was applied to the throat. By pulling out the tongue and closing the nose at the same time, the inhalation of the fluid into the airways was forced. After the treatment, mice were transferred back into their cages and placed on bedding material to avoid any backflow of the administered substance. During narcosis, animals were kept (at a distance of 30-40 cm) under red light to prevent them from cooling.

2.2.3 Models of experimental allergic asthma

2.2.3.1 Induction of HDM-mediated sensitization

For the sensitization phase of experimental allergic asthma [303], WT and *C5ar1*$^{-/-}$ mice were sensitized by an intratracheal (i.t.) application of 100 µg house dust mite (HDM) extract in 50 µl PBS once on day 0 (Figure 2.1A) or twice on day 0 and 7 (Figure 2.1B). Control mice were treated the same way with 50 µl PBS. Mice were euthanized and organs were harvested 24-72 hours after the last immunization for further analysis.

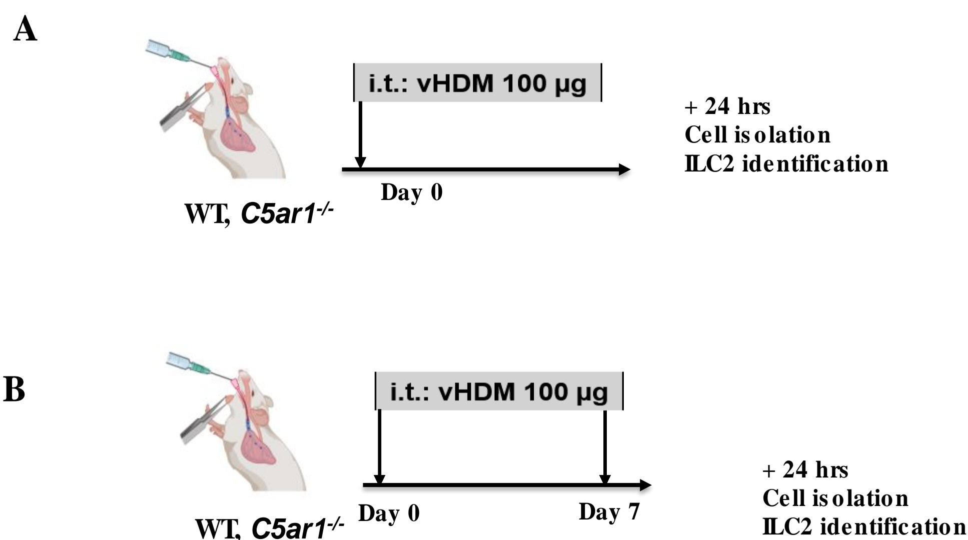

Figure 2. 1 Experimental models of house dust mite (HDM)-sensitization in Balb/c mice. Mice were sensitized by an intratracheal (i.t.) application of 100 µg house dust mite (HDM) extract in 50 µl PBS. (A) Once on day 0 or (B)Twice on days 0 and 7.

2.2.4 Dysbiosis Models for lung development

To investigate the effects of dysbiosis on neonate pulmonary development and functions, mothers were exposed to Sucralose (0.5mg/ml) or three courses of widely used ABX sucralose plus antibiotics (ABX; 1mg/ml each of vancomycin hydrochloride, ampicillin sodium, gentamicin sulfate; Goldbio, St. Louis, MO) via drinking water. Water bottle contents were replaced with freshly made supplemented water approximately every 4-6 days. Sucralose was supplied to counteract the bitterness of ABX, thus control animals received sucralose-supplemented water. ABX cocktail was provided from postnatal day 10 (PN10) to PN20 (Figure 2.2). Starting from post-natal day 20 mice were exposed to regular water. Different post-natal

day (P)-10, P14, P16, P18, P20, P24, P28, P28, P35 and P56 organs were collected for flow cytometric analysis 2.2.6.1 and immunohistochemistry 2.2.10.

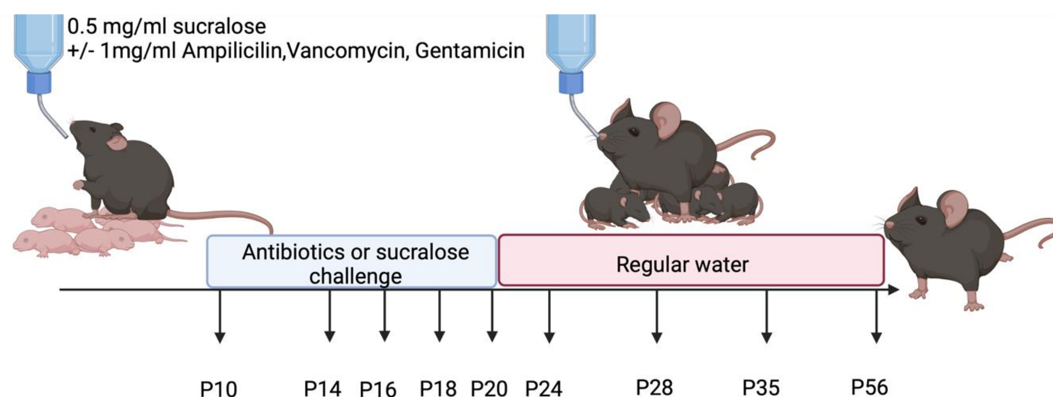

Figure 2. 2 Dysbiosis model. For dysbiosis, mothers were exposed to three courses of widely used ABX (streptomycin vancomycin and ampicillin and Suc) or sucralose alone (Suc) as a control from 10 days from postnatal day PND10-20 via drinking water. Starting from post-natal day 20 mice were exposed to regular water.

2.2.5 Cell harvesting preparation from different organs

2.2.5.1 Bronchoalveolar lavage

To obtain the bronchoalveolar lavage (BAL) the murine thorax was opened, and the trachea was freed of surrounded muscles and opened at the upper third part by half-incision. A catheter (Vasofix® Safety, 18 G, 1.33X45mm) was inserted and lung lobes were flushed once with 1 ml ice-cold PBS for adult mice or mice at P14 300 ml with (Vasofix® Safety, 20 G). BAL fluid was collected in a 1.5 ml microtube, centrifuged (10 min, 250 x g, 4°C) and erythrocytes lysed with 100 µl red blood cell lysis buffer (RBCL buffer) or 100 µl Ammonium-Chloride-potassium Lysing buffer (ACK), (2 min, room temperature (RT). By adding 900 µl PBS cells were washed and the cell number was determined (see section 2.2.5.5). Cells were used for subsequent tSNE flow cytometric analysis (see 2.2.6.1).

2.2.5.2 Lung cell isolation

For a collection of lung tissue, a 6-well plate was equipped with 40 µm cell strainers, pre-filled with 5 ml RPMI 1640, and kept on ice. After performing bronchoalveolar lavage (see 2.2.5.1) for removing cells in the alveolar space, either all lung lobes or only the right lobes (in case of histology sampling) were collected into the cell strainer and then gently chopped by using dissection scissors. The lung tissue was digested for 45 min at 37 °C in presence of 0.25 mg/ml Liberase TL (Roche) and 0.5 mg/ml DNase I (Sigma) under gentle shaking. After incubation, the lung tissue-containing cell strainer was transferred to a 50 ml tube, and lung cells were excavated by mechanical disruption with a plunger of a 5 ml syringe. The cell strainer was washed with 5 ml digestion medium and additionally with 10 ml wash medium (2.6). The single cell suspension was centrifuged (4 °C, 350 x g, 10 min), the supernatant discarded and erythrocytes lysed with 3 ml of Red Blood Cell Lysis buffer (3 min, RT). The reaction was terminated by the addition of PBS to a final volume of 30 ml and the cell number was determined (see 2.2.5.5). Subsequently, the cells were used for flow cytometric analysis (see sections 2.2.6.1), and fluorescence-activated cell sorting (FACS) (see 2.2.6.1).

2.2.5.3 Bone marrow isolation

For bone marrow isolation, femurs and tibias were removed from mice and placed into a sterile cell culture dish. Using forceps and small scissors, muscle, and fibrous tissues were cut away from the bone, and the bone was wiped with 70% ethanol to remove any excess muscle fibers. Muscle-freed femurs and tibias were placed in the cold phosphate-buffered saline (PBS) (without $Mg2^+$ and $Ca2^+$). To isolate cells from mouse femora and tibia, bones were flushed with 3 mL PBS (without $Mg2^+$ and $Ca2^+$) supplemented with 1% fetal calf serum. Flushing by done using a 21G needle attached to a 10 mL syringe through a 40 µM cell strainer. The cells were collected to 50 mL and the cell strainer was washed with an additional 8 mL PBS. The cell suspension was centrifuged (5 min, 350xg 4°C) and the supernatant was discarded. Bone marrow cells from naive mice were subjected to 3 mL red blood cell lysis (RBCL) buffer for 3 min at RT. The reaction was stopped by the addition of a large volume of PBS. The isolated bone is used for BM-derived DC or ILC2 culture (2.3.7.1-2.2.7.3) or flow cytometric analysis (2.2.6.1).

2.2.5.4 Spleen cell isolation

For a collection of spleens, a 6-well plate was equipped with 40 µm cell strainers, pre-filled with phosphate-buffered saline supplemented with 10% fetal calf serum, and kept on ice. Spleens were removed from mice and placed into cell strainers. Spleens-containing cell strainer was transferred to a 50 mL tube homogenized with a plunger of a 5 ml syringe into a single-cell suspension using additional 5 mL phosphate-buffered saline supplemented with 10% fetal calf serum. The single cell suspension was centrifuged (4 °C, 350 x g, 10 min), the supernatant discarded and erythrocytes lysed with 1 mL ammonium chloride-Tris (ACT) buffer and incubated on ice for 10 min. The reaction was stopped by the addition of a large volume of PBS, isolated cells were used for flow cytometric analysis (2.2.6.1).

2.2.5.5 Determination of the cell number

To count the cell concentration, a hemocytometer was used. To distinguish dead cells from living cells, an aliquot from cell suspension was mixed with a 1:1 ratio trypan blue. From the mixture, 10 µl of the sample was loaded. The loaded hemocytometer is then placed on the microscope stage of a transmitted light microscope. The number of viable cells was calculated according to the following formula:

Total cells/ml = (Total cells counted x Dilution factor x 10,000 cells/ml)/ Number of squares counted

2.2.6 Analysis and purification of cell populations by flow cytometry and fluorescence-activated cell sorting

2.2.6.1 Principals of flow cytometry

Flow cytometry is a laser-based measurement technology that rapidly analyzes single cells or particles as they flow past single or multiple lasers and has the ability to collect information from millions of cells in seconds [307]. Flow cytometry contains three main components: fluid, optics, and electronics. The fluid system transport particles to the laser beam. The optics systems consist of the sample stream and optical filters for better detection with the photomultiplier tubes (PMTs). The electronic system converts the detected light signals to electronic signals that can be further processed by computer. Flow cytometry uses the laser as

a light source to produce a scattered and fluorescent light signal that is read by detectors. Light scattering occurs when particles deflect incident laser light depending on the physical properties of particles such as their size and internal complexity. Various factor affects the scattering for instance cell`s membrane, nucleus, and any granular material inside the cells. While forward-scattered light also known as FSC detects cell-surface area or size, side-scattered light (SSC) detects cell granularity or internal complexity.

In addition to FSC and SSC detection, flow cytometry detects fluorescent-labeled cells. Usually, flow analysis starts with fluorescent labeling in a single-cell suspension. A fluorescent-labeled cell absorbs light energy over a range of wavelengths that is specific to the cell. This absorption of light cause electron to be increased to a higher energy level. The excited electron quickly decays and emits excess light. The emitted light is directed to optics, where it is split into defined wavelengths and channeled by several filters and mirrors within the flow cytometry. Moreover, the filtered fluorescent light is delivered to the detection system which allows each PMT can detect the fluorescent at a specific wavelength, and voltage pulses are recorded by electronics. For the data analysis, Flow-Jo 10 was used.

In addition to traditional 2D flow analysis, new technologies allow the user to do 3D analysis with tSNE. T-Distributed Stochastic Neighbor Embedding (tSNE) is an algorithm for performing dimensionality reduction, allowing visualization of complex multi-dimensional data in fewer dimensions while still maintaining the structure of the data.

2.2.6.2 Principals of cell sorters

A cell sorter is a specific type of flow cytometry that can purify and collect samples. Users can gate the population of interest in the cell sorters and collect it into a collection vessel. To separate, the cells oscillate the sample stream of liquid at high frequencies by these it generates drops. The drops are given either positive or negative for desired parameters through a metal reflection plate. According to its charge, it is directed to the collection vessel.

2.2.6.3 Compensation in Flow Cytometry

The compensation is a method used by flow cytometric analysis, to correct for fluorescence spillover, i.e., removing the signal of any given fluorochrome from all detectors except the one devoted to measuring that dye. For the following study, I used two different strategies to create a compensation matrix. As a first one, I used the bead-based compensation method. In this method, 1µl of fluorophore-labeled antibody was added into every tube which contain one drop

of the positive bead and one drop of the negative anti-rat rat/anti-hamster compensation bead were single stained compensation beads and incubated for 5 min in dark. After incubation, 300 µl of flow buffer was added to each tube. Prepared compensation beads were used to design a compensation matrix that could automatically be calculated by Diva software of flow cytometry. To compensate GFP signal, I used a single staining method. A tube with an unstained fluorophore was used as a negative control. Cells were stained with each fluorophore separately and measured in the wavelength. The percentage of fluorescence attributed to overlapping emission was calculated and used to generate a matrix.
However, unfortunately, compensation calculation does not always work as it is expected, to correct spectral overlap, even better, manual compensation also used.

2.2.6.4 Fluorescence staining of surface markers

For fluorescence staining, single-cell suspension was prepared as described in 2.2.6. Single-cell suspension was adjusted to 0,1- $1x10^6$ and incubated in 100 µl flow buffer (PBS- 0,5% BSA) supplemented with 1:100 dilution of anti-mouse CD16/CD32 Fc-block on ice for 15 min to eliminate non-specific bindings to Fc receptors II and III. Unbound antibodies were removed by washing cells with a flow buffer. Subsequently, cells were centrifuged at 4°C, 30 seconds (s), maximum speed, and the supernatant was aspirated.
After removing unbound antibodies with Fc Block, a specific cell surface marker was used for the identification of the cells, antibodies are listed in Table 2.2 and used as specified dilution antibody mix prepared in flow buffer as diluent. Staining was performed after Fc block at 4°C for 20 min in dark. Subsequently, unbound antibodies were removed by washing the cells with 900 µl of PBS, and centrifugated at 4°C for 30 sec at max speed. The supernatant was aspirated. Cells were resuspended in a 300 µl flow buffer and analyzed with a BD™ LSR II, Fortesa I-III, or BD FACS Aria™ . For analysis of the cell population, 1-$2x10^5$ cells were recorded when possible. Thresholds were set at 25,000 in order to exclude small particles, cell debris, and erythrocytes.

2.2.6.5 Intracellular fluorescence staining

Intracellular fluorescence staining is a method to detect intracellular molecules including phosphorylated signaling proteins and cytokines. To stain intracellular molecules, the cells should be fixed in a suspension containing cross-linking agent (e.g., formaldehyde) to ensure the stability of the cell membrane. Subsequently, cells should be permeabilized in suspension

to allow antibodies to bind intracellular targets. In this study, Foxp3 intracellular staining kits were used for the detection of nuclear antigens such as transcription factors GATA3, RORγT, and Tbet and secreted cytokines according to manufacturer`s recommendations.

2.2.6.6 Cytokine detection with flow cytometry

Isolated cells (2.3.6) from WT, *C5ar1*$^{-/-}$ or *LysMcre-C5aR1*$^{fl/fl}$ mice seeded at a density of $2x10^6$ cells/200µl in re-stimulation medium (DMEM 10% FCS, Penicillin, streptomycin) in a 96-well U-bottom plate and then kept overnight at 4°C. The next day, the cells were stimulated with 500ng/ml Ionomycin and 50ng/ml phorbol 12-myristate 13-acetate (PMA) in the presence of brefeldin (1x) and monensin (1x) according to manufacturer’s instructions for 4 hours at 37°C, 5%CO_2. Then, cells were harvested, and levels of IL-5 expressed by ILC2 were determined by flow cytometry. Fixable viability dye was used for the identification of living cells. ILC2 were identified as viable, Lineage [CD11c, CD11b, CD3e, CD5, CD19, Ly6G, TCR β, CD49R, CD45b] negative, CD90.2$^+$, CD127$^+$, and CD25$^+$ cells. After fixation and permeabilization, IL-5 was stained intracellularly using the Foxp3/Transcription factor staining buffer according to the to manufacturer's instructions (eBioscience).

2.2.6.7 Intracellular fluorescence staining for phospho- STAT

To stain phospho-STAT3, 4, 5, and 6, cells were fixed with 4% paraformaldehyde at 4 °C for an hour and permeabilized with 90% ice-cold methanol at −20 °C overnight. Subsequently, other cell surface markers, p-STATs markers, and intracellular staining performed as are described in 2.3.7.4 and 2.3.7.5.

2.2.7 In vitro Developmental models using a co-culture system

2.2.7.1 *In-vitro* differentiation of BM-derived macrophage/dendritic cells

To generate BM-derived macrophage/ dendritic cells, a single-cell suspension was prepared as it is described in 2.2.5.3 from bone marrow. 10×10^6 bone marrow cells per well were cultured in tissue-culture-treated 6-well plates in 5 ml of complete medium (RPMI 1640 supplemented with glutamine, penicillin, streptomycin, 2-mercaptoethanol,10% heat-inactivated fetal calf serum, and GM-CSF (20 ng/ml, Peprotech). On day 2, the culture was supplemented with

additional GM-CSF (20 ng/ml, Peprotech). Half of the medium was removed on day 4 and centrifuge and at room temperature (380G, 8 min), the supernatant was aspirated. Cells were added back in a new medium supplemented with GM-CSF (2×, 40 ng/ml) and warmed at 37°C. On day 9, non-adherent cells and in the culture supernatant and loosely adherent cells harvested by gentle washing with PBS were pooled and used as the starting source of material for co-culture experiments.

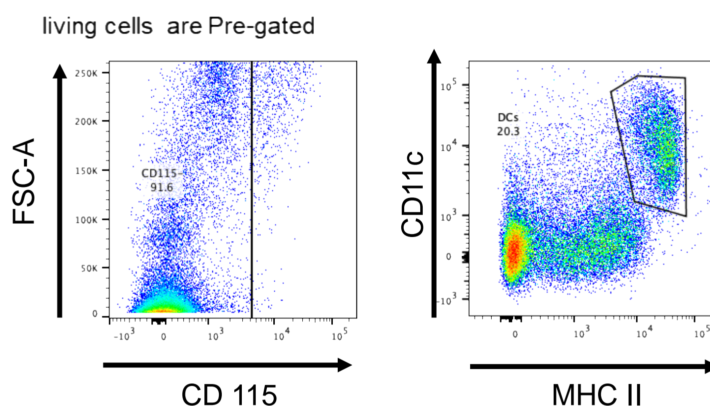

Figure 2. 3 Flow cytometric analysis after 9 days of BM-M-DC culture. Macrophages are identified as CD115+ (macrophage colony-stimulating factor receptor or CSF-1 receptor), and Dendritic cells are identified as CD115- CD11c+MHC-II^{+} population.

2.2.7.2 BM-derived ILC Culture

Bone marrow cells from mice were subjected to red blood cell lysis (2.2.5.3), counted (2.2.5.5), and stained with Fixable Viability Dye, CD45 and CD11c, CD11b, CD3e, CD5, CD19, Ly6G, CD27, TCR β , CD49R, CD45b, Ter119 at the cataloged dilution and cells were resuspended in 1% PBS-BSA solution. CD45^{+}, viable, Lineage [CD11c, CD11b, CD3e, CD5, CD19, Ly6G, CD D27, TCR β , CD49R, CD45b, CD27, Ter119] negative cells (ILC progenitor) were sorted in ILC media (containing PMI 1640 medium, 100 U/ml penicillin, 100 µg/ml Streptomycin, 10% heat-inactivated fetal calf serum (FCS), 2 mM L-glutamine, %10 non-essential amino acids 1mM sodium pyruvate) with IL-7 (20 ng/ml). Sorting was performed using a 70-micron nozzle, and cell sorting efficiency was 94-98 %. 1 × 10^{6} bone marrow cells per well were cultured in tissue-culture-treated 6-well plates in 3 ml of ILC medium with IL-7 (20 ng/ml) and IL-2 (20 ng/ml). On day 4, the culture was supplemented with additional IL-7 (20 ng/ml) and

IL-2 (20 ng/ml). On day 5, the culture was supplemented with an additional 2 ml of ILC medium with IL-7(20 ng/ml) and IL-2 (20 ng/ml), and IL-33 (20 ng/ml). On day 9, non-adherent cells and in the culture supernatant and loosely adherent cells harvested by gentle washing with PBS were pooled and used as the starting source of material for co-culture experiments.

2.2.7.3 *In vitro* developmental co-culture system

1× 10^5 bone marrow-derived ILC cells were cultured in tissue-culture-treated 96-well U bottom plates in 200 µl of ILC medium or with 1× 10^5 bone marrow-derived DCs cells in 200 µl of ILC medium. Later cells were supplemented with or without 20 mM C5a for 24h. Subsequently, cells were used for fluorescence staining.

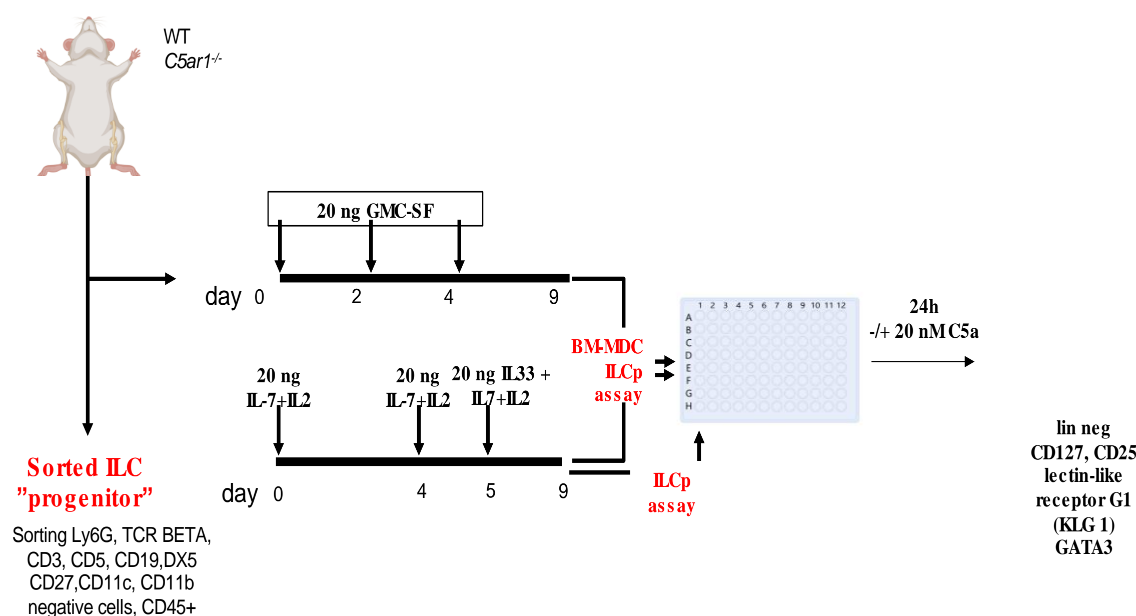

Figure 2. 4 In vitro developmental co-culture system. BM-derived ILCs were cultured with or without C5a for ILCp assay. For the developmental assay, BM-derived DC and ILCp ILCs were cultured in a 1:1 ratio with or without 20 nM C5a.

2.2.7.4 Pulmonary developmental assay

Lung cells were isolated as described in section 2.2.5.2. Isolated cells were stained with Fixable Viability Dye, CD45 and CD11c, CD11b, CD3e, CD5, CD19, CD27, Ly6G, TCR β , CD49R, CD45b, Ter119, and MHC-II at the cataloged dilution, and cells were resuspended in 1% PBS-BSA solution. $CD45^+$, viable, Lineage negative cells so-called ILCPs, and MHC-II positive cells were sorted separately in ILC media (containing PMI 1640 medium, 100 U/ml penicillin,

100 µg/ml Streptomycin, 10% heat-inactivated fetal calf serum (FCS), 2 mM L-glutamine, %10 non-essential amino acids 1mM sodium pyruvate) with IL-7 (20 ng/ml). Sorting was performed with a 70-micron nozzle and 94-98 % sorting efficiency. 1×10^5 sorted ILCs cells were cultured in tissue-culture-treated 96-well U bottom plates in 200 µl of ILC medium or with 1×10^5 MHC-II$^+$ cells in 200 µl of ILC medium. Later cells were supplemented with or without 20 mM C5a for 24h. Subsequently, cells were used for fluorescence staining.

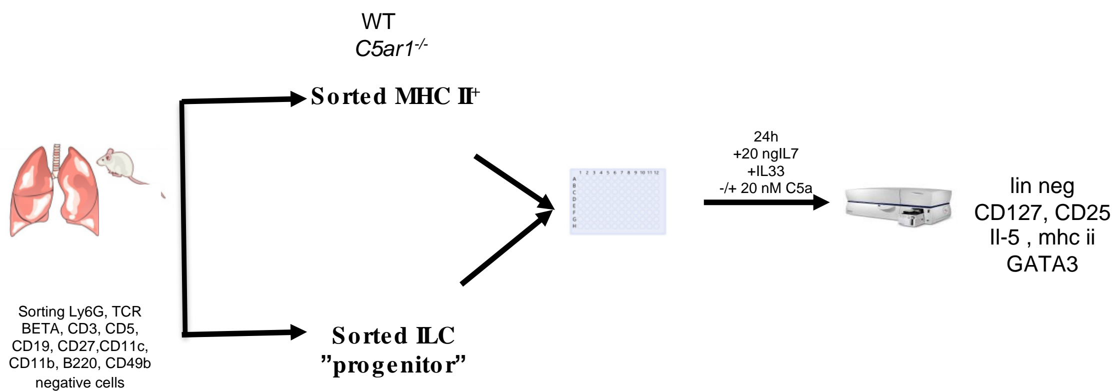

Figure 2. 5 Pulmonary developmental co-culture system. Pulmonary ILCs were cultured with or without C5a for ILCp assay. For the developmental assay, MHC-II$^+$ and ILCp were cultured in a 1:1 ratio with or without 20 nM C5a.

2.2.7.5 *In-vitro* co-culture system

Lung ILCp cells were sorted as described in 2.3.7.4. In addition, cells were stained with Siglec-F, CD11c, MHC-II, CD11b, and F4/80 at the cataloged dilution, and cells were resuspended in 1% PBS-BSA solution. Lung alveolar macrophages (Siglec-F$^+$, CD11c^{high}), DCs (MHC-II$^+$, CD11c$^+$), and interstitial macrophages (F4/80$^+$, CD11b$^+$) were sorted in a complete medium containing RPMI 1640 supplemented with glutamine, penicillin, streptomycin, 2-mercaptoethanol,10% heat-inactivated fetal calf serum. Sorting was performed with a 70-micron nozzle and 94-98 % sorting efficiency. For the developmental assay, sorted ILCs cells were cultured in tissue-culture-treated 96-well U bottom plates in 200 µl of ILC medium or with sorted AM, DC, and IM Cells in a 1:1 ratio for 24h and stimulated with and without 20nM C5a.

2.2.8 Measurement of Airway Hyperresponsiveness

AHR was measured 24H after allergen exposure. Mice were anesthetized by i.p. injection of 50 µl Ketavet/Rompun (76 mg/ml and 4.8 mg/ml respectively, Pfizer/Bayer). Muscle relaxation was achieved by injection of 50 µl of Esmeron (10 mg/ml, Organon). AHR was measured in anesthetized mice that were mechanically ventilated using a FlexiVent (SciReq) system as described by [308]. Aerosolized Acetyl-β-Methyl-Choline (methacholine) (0, 2.5, 5, 10, 25, and 50 mg/ml; Sigma-Aldrich) was generated by an ultrasonic nebulizer and delivered in-line through the inhalation port for 10 seconds. Airway resistance was measured two minutes later.

2.2.9 ELISA-based assays

ELISA (enzyme-linked immunosorbent assay) is a plate-based assay technique designed for detecting and quantifying soluble substances such as peptides, proteins, antibodies, and hormones. ELISAs are typically performed in 96-well polystyrene plates, which passively bind antibodies and proteins.
In principle, the plate is coated with a specific coating antibody overnight at RT. Then the capture antibody is decanted and covers all unsaturated surface-binding sites of the plate with blocking antibody and incubated. Following the standards and samples are added in duplicates to the plate and incubated. The plate is washed again 3 times with washing buffer and biotinylated detection antibodies are added. After 3 times washing 50 µl streptavidin-horse-radish-peroxidase was added and incubated for 20 min at RT in the dark. After washing 50 µl of the substrate was added for 20 min at RT in the dark. The reaction was stopped with 25 µl 1M H_2SO_4 and the ELISA plate was analyzed on the FluoStar Omega 0415.

2.2.9.1 ELISA

IL-1α concentrations in the supernatants from 24h co-culture or lung supernatant 24h after the second HDM exposure was measured by DuoSet ELISA kits (R&D Systems) according to the manufacturer's instructions. The minimum detectable dose of mouse IL-1α is less than 2.5 pg/ml.

2.2.9.2 Multiplex

The LEGENDplex is a bead-based immunoassay that utilizes the same basic principles of sandwich immunoassays, whereby a soluble analyte is captured between two antibodies. The LEGENDplex Mouse Inflammation Panel is used from BioLegend. This multiplex assay allows simultaneous quantification of 13 mouse cytokines, including IL-1α, IL-1β, IL-6, IL-10, IL-12p70, IL-17A, IL-23, IL-27, MCP-1, IFN-β, IFN-γ, TNF-α, and GM-CSF. The plate was analyzed on MACS multicolor flow cytometry.

2.2.10 Histology

2.2.10.1 Inflation fixation

The mouse was euthanized using sodium pentobarbital (100-150ul depending on the age and size of the mouse). Once the mouse stops breathing descending aorta was cut as a secondary kill to open the circulatory system. The rib cage was cut through to expose the heart and lungs. The system was flushed with 1x PBS by inserting a needle into the right ventricle to Blanche lungs until the lungs become whitened as they cleared. The tracheae were exposed by making a shallow cut in the skin at the throat and continuing to remove unwanted skin/tissue layers until the trachea is visible. A small incision was made in the trachea just below the thickest "ring", just big enough to insert the blunt tip and the suture around the trachea tightened off just below where the blunt needle was inserted, to make a tight seal and prevent formalin from leaking. Another suture was made throw around the trachea just below where the blunt needle tip ends. The formalin was let to flow into the lung via a catheter. The height of the formalin was kept at 25 cm above the mouse, to ensure gravitational pressure is sufficient to inflate the lungs but not excessive enough to damage lung structures for 5min.

The second suture was tied off throwing below the tip of the blunt needle. The blunt needle was removed. The inflated lungs were carefully removed from the chest cavity. Lungs were immersed in formalin and let sit overnight-24 hours. Formalin was decanted and replaced with 30% EtOH. On the rotational shaker, the lungs were gently agitated for 15 min. Later, replace 30% with 50% EtOH. Gently agitate for 15 min. Replace 50% with 70% EtOH. Gently agitate for 15 min. 70% EtOH rinses were repeated 2 more times Lungs were placed in tissue cassettes dorsal side down and closed cassette lid. Cassettes were labeled and placed in 70% EtOH and submitted to the Pathology core for cut and staining.

2.2.10.2 Periodic Acid-Schiff staining

A Periodic Acid-Schiff Stain is a staining method that detects polysaccharides and mucosubstances [309], such as glycogen, glycolipids, glycoproteins, and mucins. Free hydroxyl groups of saccharides are oxidized which generates violet complexes with the fuchsine sulfate of the Schiff reagent. Amount of the mucus can be calculated by violet color formation.

Lungs and intestines samples were thoroughly rinsed with 1x PBS, placed in embedding cassettes, and fixed in 4% (w/v) PFA fixative or ready-to-use formalin solution for 24 hours at RT. After washing with tap water for 2-4 hours, samples were dehydrated and paraffinized.

Microtome sections were generated from paraffin blocks precooled to -20°C with a thickness of 4-6 µm. Sections were first collected in a 20°C water bath and then transferred to a 45°C water bath for flat mounting on slides. Finally, mounted slides were dried at 37°C before proceeding with histochemical staining.

For deparaffinization and rehydration, slides were sequentially immersed in the following: three times xylene for 5-10 min each, two times 100% ethanol, 96% ethanol, 80% ethanol, and two times 70% ethanol for 3 min each followed by two times rinsing in ddH_2O. Subsequently, slides were incubated in ascetic acid for 3 min, followed by 1% periodic acid for 10 min. This was followed by Schiff's reagent for 15 min. Finally, samples were counterstained with Mayer's hemalum solution and mounted with Aquatex® solution. cell PAS-positive and PAS-negative airways were counted by light microscopy and the percentage of PAS-positive airways was calculated to quantify mucus production.

2.2.10.3 Hematoxylin and EOSin (H&E) staining

The lung and intestine tissues were stained with H&E to determine inflammatory cell accumulation. In theory, positively charged hematoxylin binds to negatively charged structures such as DNA. Then it appears to be blue, and that negatively charged EOSin counterstains other structure which appears shades of red. Before staining, the histological slices were deparaffinized by decreasing ethanol series rehydrated.

Table 2. 10: Protocol of the H&E staining. Lung and intestine tissue slices were re-hydrated, stained with Hemalaun and EOSin, and mounted with mounting medium.

Solution	Incubation time
100% ethanol	5 min
96% ethanol	5 min
90% ethanol	5 min
80% ethanol	5 min
70% ethanol	5 min
A. dest	5 min
Hemalaun	10 min
EOSin-ploxine solution	35 s
96% ethanol	20 sec
100% ethanol I	1 min
100% ethanol II	1 min
100% ethanol III	2 min
Xylol I	5 min
Xylol II	5min
Xylol III	5 min
Xylol IV	5 min
Mointing with Entellan®	

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2.2.10.4 Mouse lung section via vibratome

The mouse was euthanized using sodium pentobarbital (100-150ul depending on age and size of the mouse). To remove the lungs chest cavity was opened by cutting along the mediastinum up to the throat. Thorax and muscles around the trachea were removed to access the diaphragm. Vena cava was cut in the abdomen area. 3ml ice cold Ringer solution containing 5% was injected into the right ventricle using a small cannula. The heart was removed immediately after perfusion. The thread was prepared with a knot in the middle of the trachea. Agarose (3% LM agarose in ringer solution) filled cannula kept at 37°C for at least 1 hour. After incubation, it was slowly inserted into the trachea below the knot to fill the agarose into the lung. The knot was tightened while the cannula was out of the trachea. Lungs were removed and placed into the beaker with iced HEPES and kept at least for 5 min. Different lung lobes were separated and dried. Lung lobes were glued with Vet bond on the top of the metal plate. Metal molds were placed on the plate and filled with agarose. Later metal plate was kept on the ice at least for 5 min. 300µm sections were sliced by vibratome and collected in an ice-cold Ringer buffer. The lung sections were fixed with 1% PFA in PBS (15 min, RT, shaking) washed three times with

PBS (15 min, RT, shaking), and incubated 20% sucrose solution overnight at 4°C. 300µm sections were stored at -20°C in 20% sucrose solution.

2.2.10.5 Immunofluorescence staining

Lung sections were incubated with an IHC blocking buffer (20 min, RT). Antibody antibodies are listed in Table 2.2 and used as specified dilution antibody mix prepared in IHC blocking buffer incubated overnight at 4°C. Following incubation, the section was incubated with 5% normal serum of host species for 60 min RT. The sections were washed three times with PBS and embedded in mowiol media. Images were acquired with an LSM 710 confocal laser-scanning microscope (Carl Zeiss).

2.2.10.6 Decalcification of bone

For bone cross-section, bone was dissected and removed from soft tissue. Dissected bones were fixed with 0.5 % paraformaldehyde (PFA) and tissue was washed with distilled H_2O. The tissue was placed in 20% EDTA with shaking at 4°C. This may take one day to several weeks depending on the age of the animal and tissue size. Use 20X more volume of EDTA solution to saturate tissue. EDTA solution was changed twice a week. Decalcification is complete when a bone is soft and pliable. The tissue was rinsed with distilled H_2O, 3 times and kept in 70% ethanol (ethanol mixed with distilled H_2O).

2.2.11 Statistical Analysis

Statistical analysis was performed using GraphPad Prism (version 7.0a; GraphPad Software). The graphs are presented as scatter plots with bars showing the individual samples and the mean ± standard error of the mean (SEM). Statistical differences between the two groups were assessed by the Student's t-test. Comparisons involving multiple groups were first evaluated by performing an analysis of variance (ANOVA) followed by a performance of Tukey's multiple comparison test. A p-value < 0.05 was considered statistically significant (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$

3 Results

3.1 C5aR1 regulates the function of ILC2s

Anaphylatoxins (ATs), C3a and C5a, are cleavage products of complement factors generated by activation of the complement system or allergen-derived proteases. Both ATs are found in large quantities in the bronchoalveolar lavage fluid of allergic asthma patients and involve in the regulation of allergic diseases and severity. In particular, C5a exerts its multiple effector and regulatory functions via binding to its cognate receptors, C5aR1, and C5aR2. Interestingly, C5a has pleiotropic roles during the effector and sensitization phases of allergic asthma. During sensitization, allergen exposure also triggers tissue damage which results in the generation of the so-called alarmins from the disturbed epithelium. These molecules, Interleukin (IL)-25, IL-33, and TSLP, are potent activators of type II innate Lymphoid cells (ILC2). While C3aR has recently been shown to play a key role in ILC2 function during allergic asthma, I aimed at delineating the role played by C5a/C5aR1 signaling on ILC2 during sensitization.
In the murine model of single intra-tracheal (i.t.) house dust mite exposure, I observed, an increase in ILC2 numbers with HDM exposure (Figure 3.1A). However, I did not observe any changes in the numbers of the ILC2s between WT and *C5ar1*$^{-/-}$ with allergen exposure (Figure 3.1A). On the other hand, I observed, there were no changes by intracellular staining, and that the ILC2-derived IL-5 from *C5ar1*$^{-/-}$ mice exposed to HDM was markedly and significantly reduced compared to ILC2 from WT control (Figure 3.1B). These data suggested that C5aR1 regulates the function of ILC2 during the sensitization phase of allergic asthma.

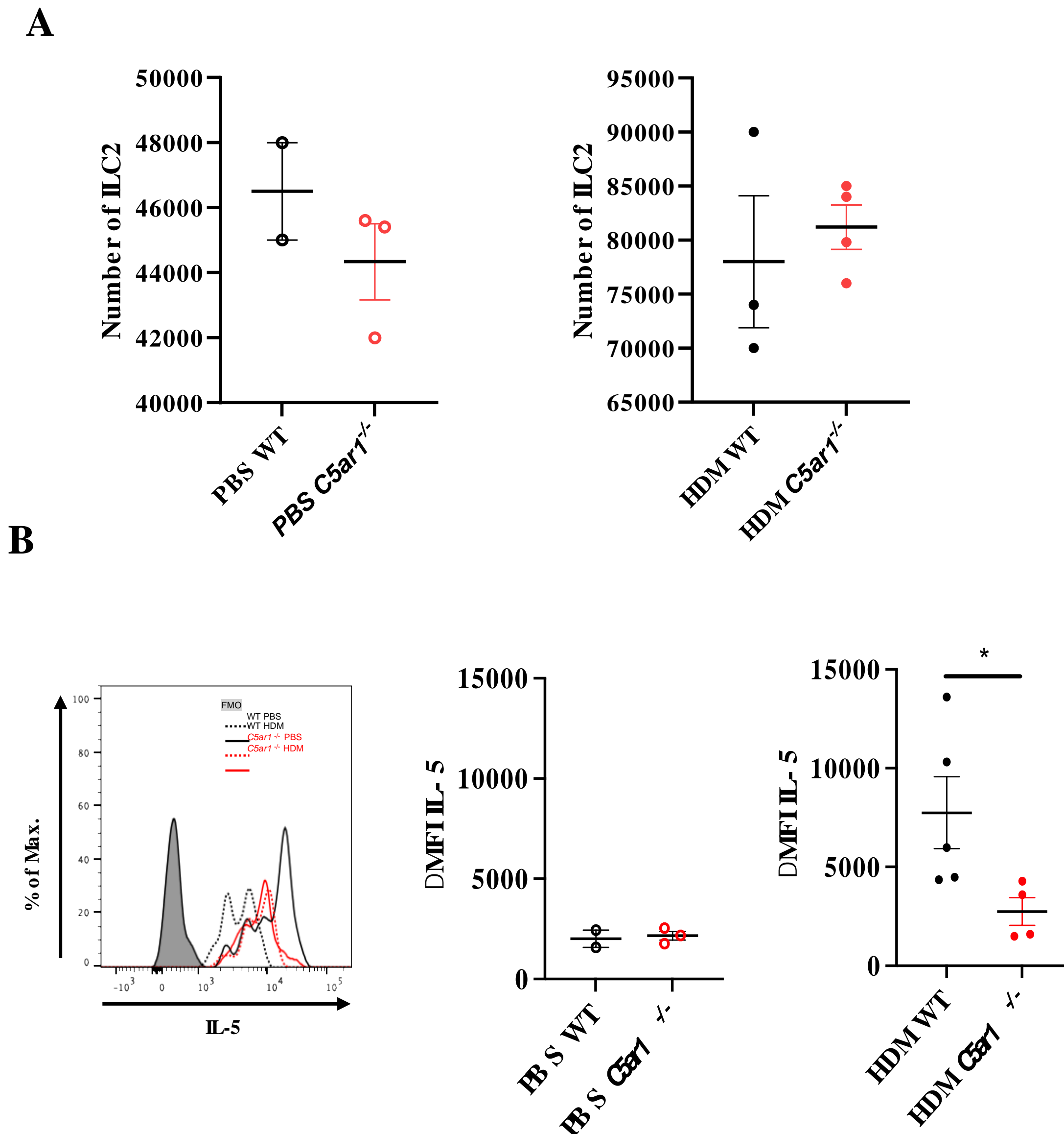


Figure 3. 1 C5a/ C5aR1 regulates the ILC2 function. ILC2 were identified as Lineage- [CD11c, CD11b, CD3e, CD5, CD19, Ly6G, CD27, TCR β , CD49R, CD45b, CD27, Ter119], CD90.2+, CD25+, CD127+. **(A)** Numbers of ILC2 in WT (black), or *C5ar1*$^{-/-}$ (red) mice were exposed i.t. to PBS or 100 µg HDM, and the lungs were harvested 24h later. **(B)** Intracellular expression of IL-5 in ILC2 cells upon one-time HDM exposure WT (black) or *C5ar1*$^{-/-}$ (red) mice were exposed i.t. to PBS (dotted line) or 100 µg HDM (solid line). Data are representative of n=2-5 animals, from 2 independent experiments. The graphs show the mean value IL-5 normalized to the FMO control (grey) ΔMFI ± SEM. Data were analyzed by unpaired t-test, * $p<0.05$.

3.2 Neither ILC2 nor ILC2p express C5aR1 at steady state

C5a is a proinflammatory molecule that is highly chemoattractant and activator for neutrophils, macrophages, and mast cells [306]. The C5a binds to C5aR1, a 45 kDa 7-TransMembrane protein with high affinity. Although C5aR1 expression has been reported in various tissues, organs (kidney, liver, lung, skin) and organ systems (the vascular system, central nervous system) connective and lymphoid tissues, its expression seems to be restricted to cells of myeloid origin including monocytes, macrophages, neutrophils, basophils, and Eosinophils [232]. In immune cells, the expression of C5aR1in a cell of lymphoid origin including NK cells, NKT cells, and T cells as well as non-immune cells such as epithelial cells, the smooth muscle cell is still a matter of debate [232, 242, 251]. Although, I observed that C5aR1 deficiency altered IL-5 secretion by ILC2, at this point it was unclear if C5aR1 was expressed by ILC2s and /or their progenitor, ILC2Ps. To delineate whether C5aR1 directly influences the function of ILC2s or the effect via its progenitor, I checked expression at a steady state on ILC2s / ILC2Ps. To track C5aR1 expression, I used GFP-C5aR1 reporter mice and a C5aR1 antibody staining. Data show that in both bone marrow and lung, neither ILC2 nor ILC2p express C5aR1 (Figure 3.2). This suggests that C5a does not signal directly in ILC2s, but rather functions through an intermediate cell.

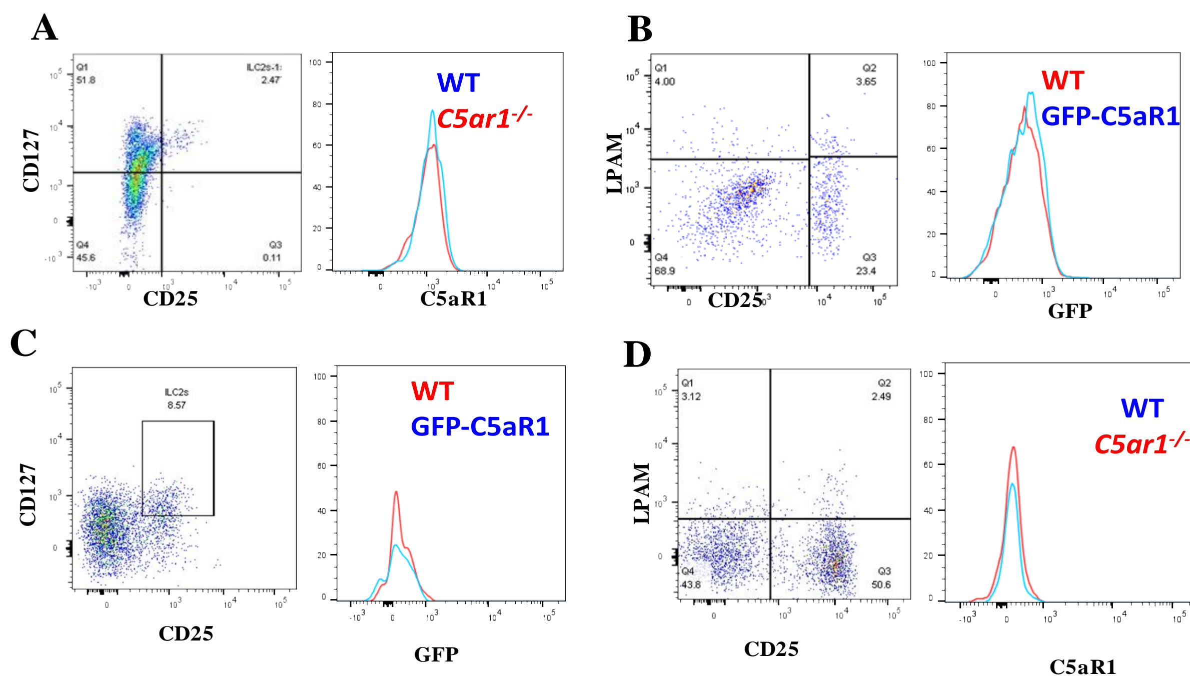


Figure 3. 2 ILC2 and its progenitor ILC2p do not express C5aR1. The rosa histograms show WT/FMO signals; the green lines correspond to signals in GFP-C5aR1 knock-in mice or signals of the

C5aR1 mAb (clone:20/70) staining. Flow cytometric analysis of GFP- or C5aR1 signal in ILC2 progenitors (ILCp) and ILC2 **(A)** BM ILC2, **(B)** Lung ILC2, **(C)** BM ILCp, **(D)** Lung ILCp. Neither ILCp (LPAM+ CD25+) nor ILC2 (CD127+CD25+) shows a positive signal for C5aR1 or a GFP reporter as shown by flow cytometry.

3.3 C5a/C5aR1 axis enhances the ILC2 development through its function in BM-M-DC

Since myeloid cells, in particular macrophages and dendritic cells (DCs) express high levels of C5aR1, and since bone marrow from cross-section contains a lot of GFP-C5aR1 positive cells (Figure 3.3A), I next examined if bone marrow-derived myeloid cells express C5a/C5aR1. To delineate this, I developed an *in vitro* assay, where bone marrow (BM)-derived ILCs were cultured alone or in the presence of BM-derived macrophage/dendritic (BM-M-DC) cells from WT and, GFP-C5aR1$^{fl/fl}$ animals in presence or absence of C5a. I observed there was no GFP signal in IL2 or any changes in C5aR1 expression in ILC2 with C5a stimulation. When I co-culture the cells with BM-M-DC, I observed a GFP signal in culture (Figure 3.3B). This suggests that BM-M-DCs express C5aR1.

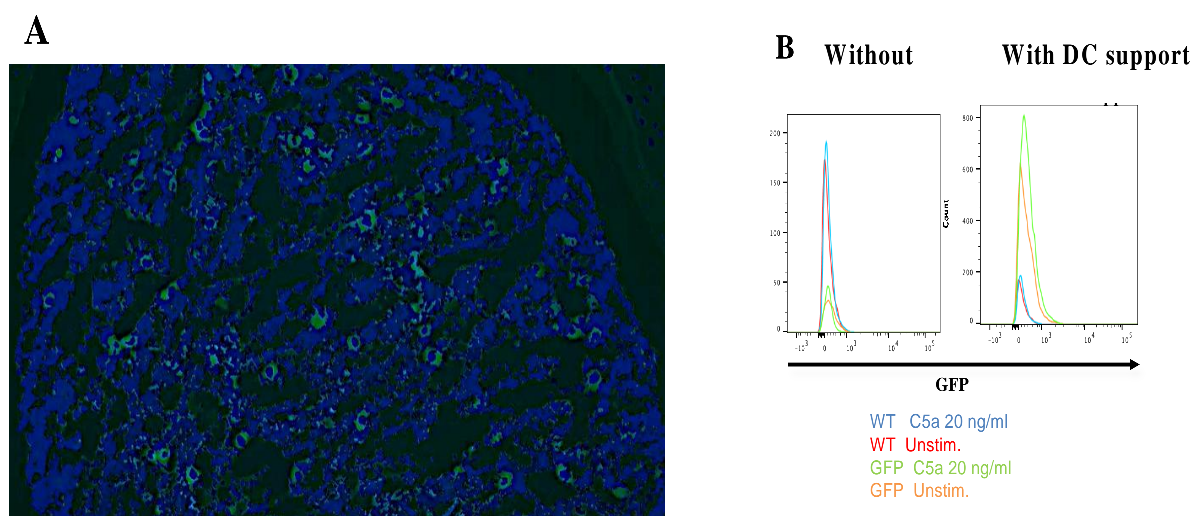
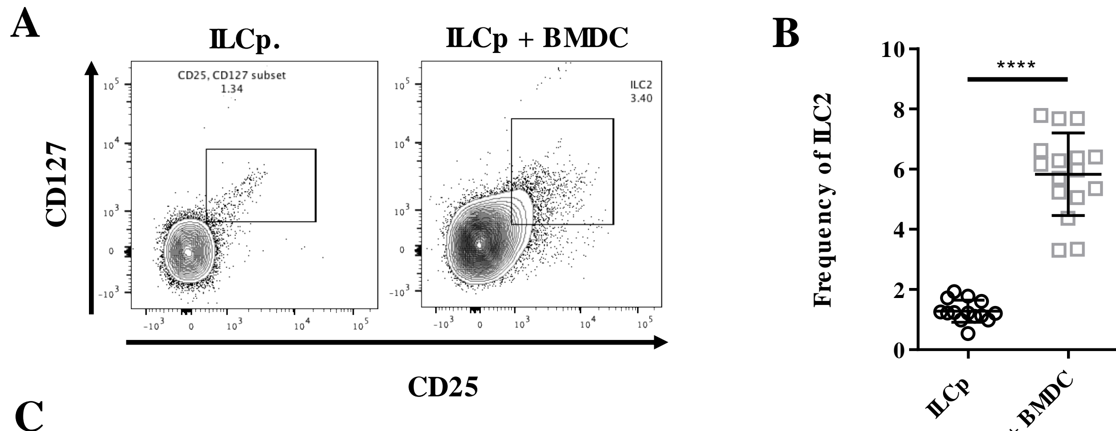


Figure 3. 3 Myeloid cells in bone marrow express C5aR1. (A) Precision Cut slice from the bone of GFP reporter mice (GFP signal is green) mouse stained with DAPI (blue) for labeling DNA at steady state. **(B)** Histogram representative GFP signal when ILCP cultured with BM-M-DC or without, WT used as a control for GFP signal, blue WT C5a stimulated, rosa WT unstimulated, Green GFP C5a stimulated, Orange GFP unstimulated.

In order to investigate the impact of C5a-induced cell signaling on myeloid cells in the development and function of the ILC2s, I used an *in-vitro* approach where I sorted ILC

progenitor [CD45$^+$, viable, Lineage$^-$ (CD11c, CD11b, CD3e, CD5, CD19, Ly6G, CD D27, TCR β , CD49R, CD45b, CD27, Ter119)] and, supplemented with IL-7 (20 ng), IL-2 (20 ng), and, IL-33 (20 ng) to generate bone marrow-derived ILCs. I cultured 1×10^5 bone marrow-derived ILC/ILCp cells with 1×10^5 bone marrow-derived DCs cells in WT and *C5ar1*$^{-/-}$. Following I stimulated the cells with or without 20 mM C5a for 24h and stained cells with surface markers expression of ILC2 was checked with flow cytometry. Interestingly, the frequency of ILC2 improved when cultured with BM-M-DC (Figure 3.4A-B) suggesting that BM-M-DC supports the development of ILC2. Further, while *in vitro* BM-derived ILC2s failed to develop in the presence or absence of C5a stimulation (Figure 3.4D), the development of ILC2 when cultured with BM-M-DCs was significantly improved upon C5a exposure (Figure 3.4E). To confirm whether C5a/C5aR1 signaling plays an important role in suggested ILC2 development indirectly through its function in BM-M-DC, I cultured BM-derived ILCp generated from WT animal with BM-M-DC from *C5ar1* deficient animal or the other way around (Figure 3.4F). In line, the absence of *C5ar1* in BM-M-DC impaired the development of ILC2 in comparison to WT-derived BM-M-DC, while *C5ar1* deficiency in ILCp does not change the development of the ILC2 (Figure 3.4F). Interestingly, the frequency of the ILC2 was also reduced when *C5ar1* deficient BM-M-DC were cultured with WT ILCs, independently of C5a (Figure 3.4E), suggesting that there might be an autonomous production of C5a in the BM-M-DC. Altogether, my findings clearly show that BM-M-DCs act as feeder cells for ILC2s and support their development in a C5a/C5aR1-dependent manner.


A
ILCp.
ILCp + BMDC
CD25, CD127 subset
1.34
ILC2
3.40
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CD25
B
Frequency of ILC2
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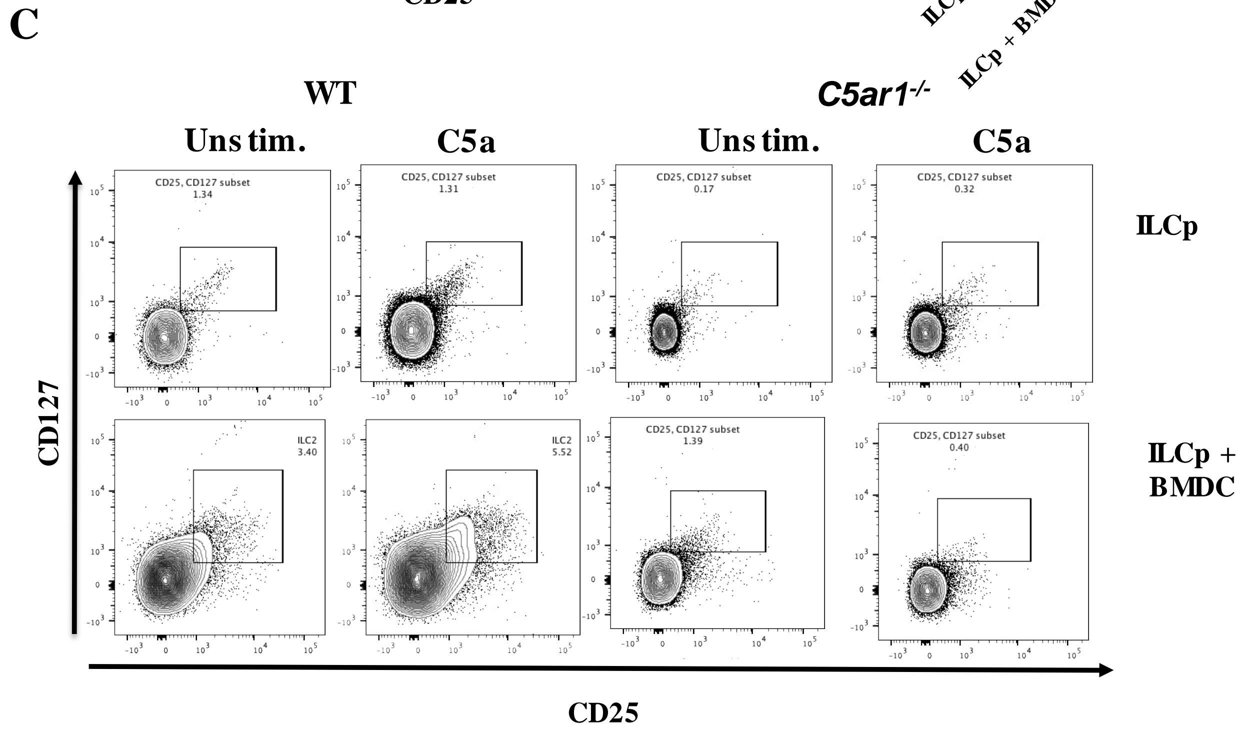
C
WT
C5ar1-/-
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CD25, CD127 subset
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0.17
CD25, CD127 subset
0.32
ILCp
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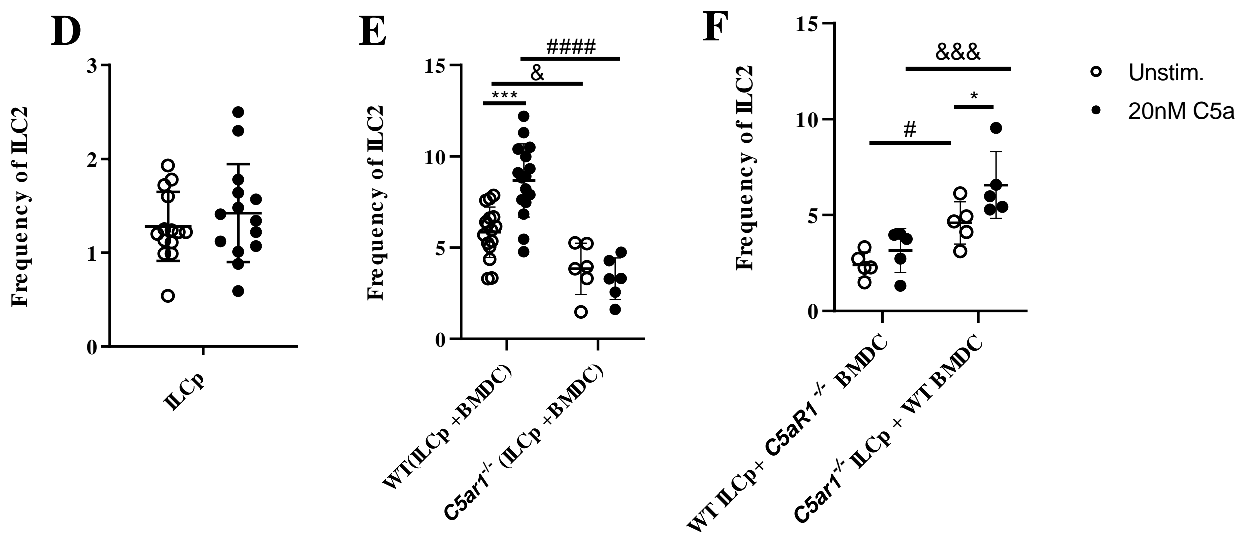
D
Frequency of ILC2
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WT(ILCp +BMDC)
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WT ILCp+ C5aR1 -/- BMDC
C5ar1-/- ILCp + WT BMDC
Unstim.
20nM C5a

Figure 3. 4 Bone marrow-derived macrophages/dendritic cells support ILC2 development. Frequency of ILC2 (Lineage^{-}, CD25^{+}, CD127^{+}) in Lineage^{-} [CD11c, CD11b, CD3e, CD5, CD19, Ly6G, CD27, TCR β , CD49R, CD45b, CD27, Ter119], population. Black-filled round representative of culture with C5a stimulation, empty round representative of without C5a stimulation. **(A)** Representative dot plots of BM-derived ILC2 from cultures BM-ILCp and BM-M-DC. **(B)** Percentage of (Lineage^{-}, CD25^{+}, CD127^{+}) ILC2s were assessed 24h after co-cultures BM-ILCp/M-DCs started. **(C)** Dot plots are representative of BM-derived ILC2 from cultures with BM-M-DC in the presence or absence of 20 nM C5a. **(D)** Percentage of BM-derived ILC2s cultured alone in the presence or absence of 20 nM C5a in WT mice. **(E)** Percentage of BM-derived ILC2s 24h after co-cultures started in presence of WT and *C5ar1*$^{-/-}$ BM-M-DCs with or without C5a (20 nM). **(F)** Percentage of ILC2 in co-culture from mixture phenotype. +/- SEM, n=5-19 cultures from 5 independent experiments. Statistical differences were assessed by unpaired T-test (Figures B and D) or by ANOVA (Figures E and F); *, &, # $p<0.05$; ***, &&& $p<0.001$ (for better discrimination; & and # were used as *).

3.4 C5a/C5aR1 signaling axis in BM-M-DCs promotes proliferation and differentiation of ILC2

To better understand the mechanisms by which C5a/C5aR1 in BM-M-DC populations control the development of ILC2 remained elusive. Since Ki67 is a nuclear protein associated with cellular proliferation, I measured the Ki67 expression in BM-derived ILC-M/DC co-culture with flow cytometry to see whether the increase in the percentage of the ILC2s, is due to its impact on proliferation. Data shows in absence of the C5aR1, fluorescence main intensity (MFI) of Ki67 in ILC2s was reduced compared to WT (Figure 3.5B), suggesting that C5a/C5aR1 signaling favors the ILC2 development by enhancing their proliferation.

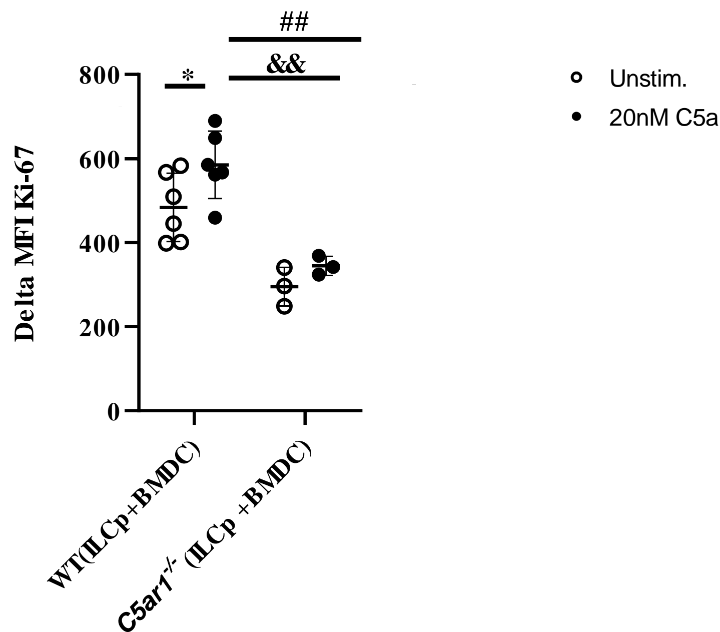


Figure 3. 5 Expression of Ki67 in ILC2. Black-filled round representative of culture with C5a stimulation, empty round representative of without C5a stimulation. Data show the mean fluorescence normalized to the FMO control +/- SEM, n=3-6 cultures from 2 independent experiments. Statistical differences were assessed by ANOVA, * $p<0.05$; &&, ## $p<0.01$.

Further, it is well appreciated that ILC2-specific functions require the expression of the transcription factor GATA3. Therefore, I evaluated the expression of ILC2s' GATA3 in ILC-Macrophage/DC co-cultures. In line with the previous observation regarding the nullity impact of C5a in the development of ILC2, C5a did not change BM- ILC2s' GATA3 expression when ILCPs cultured alone in both WT and *C5ar1* deficient BM-M-DCs (Figure 3.6), confirming that C5aR1 nor C5aR2 (another known receptor for C5a) directly impact on the ILC2 terminal development. In contrast, BM-M-DCs increase the GATA3 expression in ILC2 for both WT and *C5ar1* deficient mice. Furthermore, supplementation with C5a increased the GATA3 expression in co-cultures with WT BM-M-DCs, but not in the *C5ar1* deficient BM-M-DCs (Figure 3.6A-B).

In addition to transcription factors implicated in the development, fully matured ILC2 are characterized in mice by the expression of Killer cell lectin-like receptor subfamily G member-1 (KLRG-1)[310]. Therefore, I evaluated the expression of KLRG on ILC2 cells and showed that while C5a stimulation increased KLRG-1 expression in ILC2, there were no changes in *C5ar1* cells (Figure 3.6C-D), supporting that C5a/C5aR1 signaling axis in BM-M-DCs

participates to the terminal development and maturation of ILC2s via the regulation of GATA3, and KLRG1 expression.

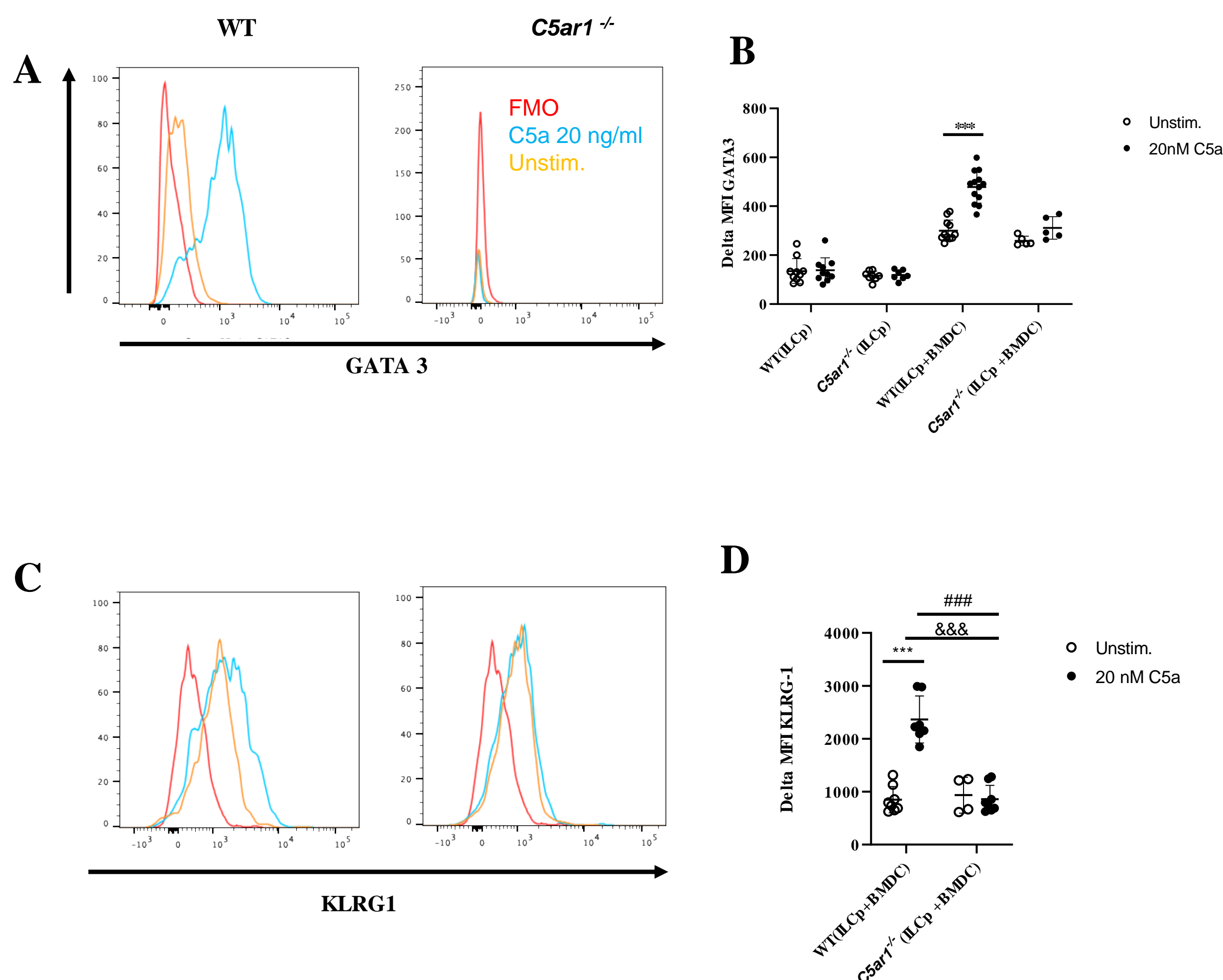


Figure 3. 6 BM-M-DCs allow the development of ILC2 from ILC progenitors in a C5aR1-dependent manner via the activation of GATA3 and KLRG1. Black-filled round representative of culture with C5a stimulation, empty round representative of without C5a stimulation. Expression in ILC2 from co-cultures in presence of C5a of the intracellularly stained transcription factor GATA3 and the surface lectin-like receptor KLRG1 **(A)** g*ata3* expression in co-culture assessed 24h after the co-culture start, histograms are representative of n=5-19 cultures from 5 Independent experiments **(B)** ΔMFI of GATA3 in BM-derived ILC2 in co-culture. Data show the mean fluorescence of GATA3, normalized to the FMO control (ΔMFI), n= 5-19 cultures from 5 Independent experiments Statistic descriptions of the test and significance. FMO, C5a stimulated and unstimulated are highlighted in rosa, green and yellow respectively**.** **(C)** KLRG1 expression in co-culture assed after 24h later, histograms are representative of n=4-7 from 3 Independent experiments. **(D)** Data show the mean fluorescence of KLRG-1, in BM-derived ILC2 and co-

culture normalized to the FMO control (ΔMFI), n=4-7 from 3 Independent experiments. KLRG-1 in BM-derived ILC2 and co-culture. Statistical differences were assessed by ANOVA, ***, &&&, ### $p<0.001$.

3.5 C5a/C5aR1 axis regulates the ILC regulatory genes in a time-dependent manner

Using BM-derived cells, I observed a significant development of ILC2 in presence of both BM-derived macrophage/dendritic cells (BM-M-DCs) and C5a. As mentioned in the introduction, ILC2s are highly plastic cells, although it is poorly understood as well as whether C5a/C5aR1 signaling could regulate the overall ILCs' fate and or plasticity. To understand the role of C5a/C5aR1 signaling on plasticity, using the same BM-derived- co-culture model, I evaluated by flow cytometry GATA3, T-bet, and RORγt expression in lin negative cells, as indicators for ILC1, ILC2, and ILC3, at different time points (24, 48, and 72h) in presence or absence of C5a. In agreement with the previous observation, 24 h after co-culture started in presence of C5a, ILC2 ($lineage^-$, $GATA3^+$) cell frequency (in $lineage^-$ cells) was the highest. Later, the impact of C5a disappeared and the frequency of ILC2 (in $lineage^-$ cells) reached its maximum level at 48h independently from C5a (Figure 3.7B), while at 72h the frequency of ILC2 dropped. Interestingly, the frequency (in $lineage^-$ cells) of the ILC3 ($lineage^-$, RORγt) gradually dropped over the whole time (Figure 3.7C). In contrast, inversely to the ILC2, the frequency of ILC1 increased over time with the same kinetic, reaching a maximum of 72h after the start, independently from C5a (Figure 3.7A). These data suggest that BM-M-DC regulates the ILCs' plasticity, C5a/C5aR1axis seems to be dispensable for the plasticity.

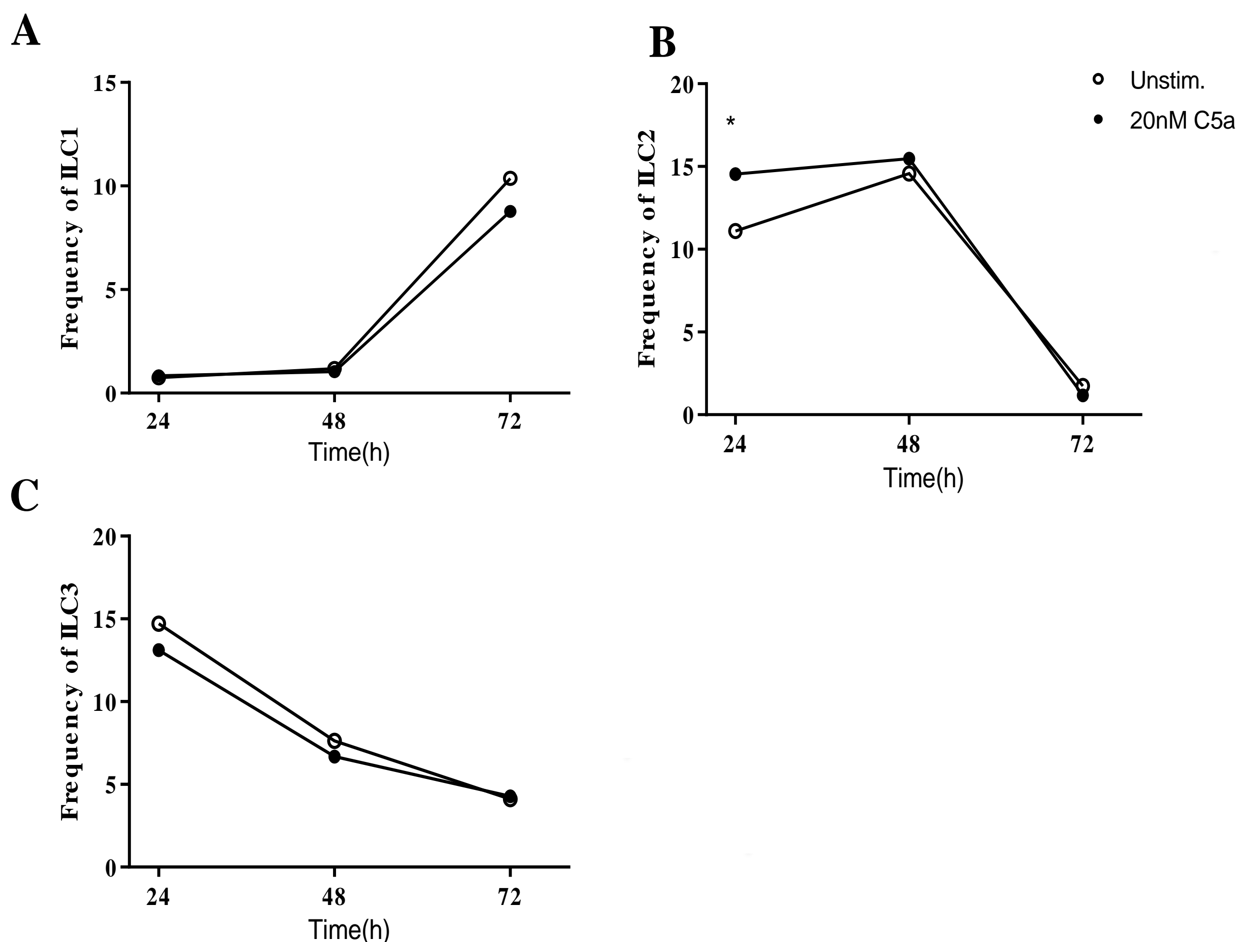


Figure 3. 7 C5a/C5aR1 axis regulates the developments Frequency of ILC1, ILC2, and ILC3 in Lineage$^-$ [CD11c, CD11b, CD3e, CD5, CD19, Ly6G, CD27, TCR β , CD49R, CD45b, CD27, Ter119], (**A**) ILC1 frequency (% of Lineage$^-$ cells), an intracellularly stained transcription factor of lineage negative T-bet positive, ILC1 from co-cultures in the presence-absence of the C5a in co-culture assessed after 24, 48 and 72h later. (**B**) ILC2 frequency (% of Lineage$^-$ cells), intracellularly stained transcription factor of lineage negative GATA3 positive, ILC2, from co-cultures in the presence-absence of the C5a in co-culture assed after 24,48 and 72h later. (**C**) ILC3 frequency (% of Lineage$^-$ cells), intracellularly stained transcription factor of lineage negative RORγ*t* positive, ILC3, from co-cultures in the presence-absence of the C5a in co-culture assessed 24, 48, and 72h later. +/- SEM, n=4 cultures from 2 independent experiments. Statistical differences were assessed by ANOVA, * p<0.05.

3.6 C5aR1 expression in BM-M-DCs impacts ILC2 STATs phosphorylation status via IL-23

Cytokines activating the Janus kinases (JAKs) and members of the signal transducer and activation of transcription (STAT) family are crucial for the development and function of innate lymphoid cells [311]. I was wondering, could C5a/C5ar1 regulates the ILC2 development via

STATs phosphorylation in ILC2s. In order to address this, I assessed the phosphorylation of STAT3, 4, 5, and 6 in ILC2s by flow cytometry. While, there was no clear activation of STAT 3, 4, or STAT6 in absence of C5a, cells deriving from co-cultures pulsed with C5a showed a significant increase in STAT4 phosphorylation 24 h after the start of culture (Figure 3.8), suggesting C5a/C5aR1 signaling in BM-M-DC might take a role ILC2 development via STAT4 activation.

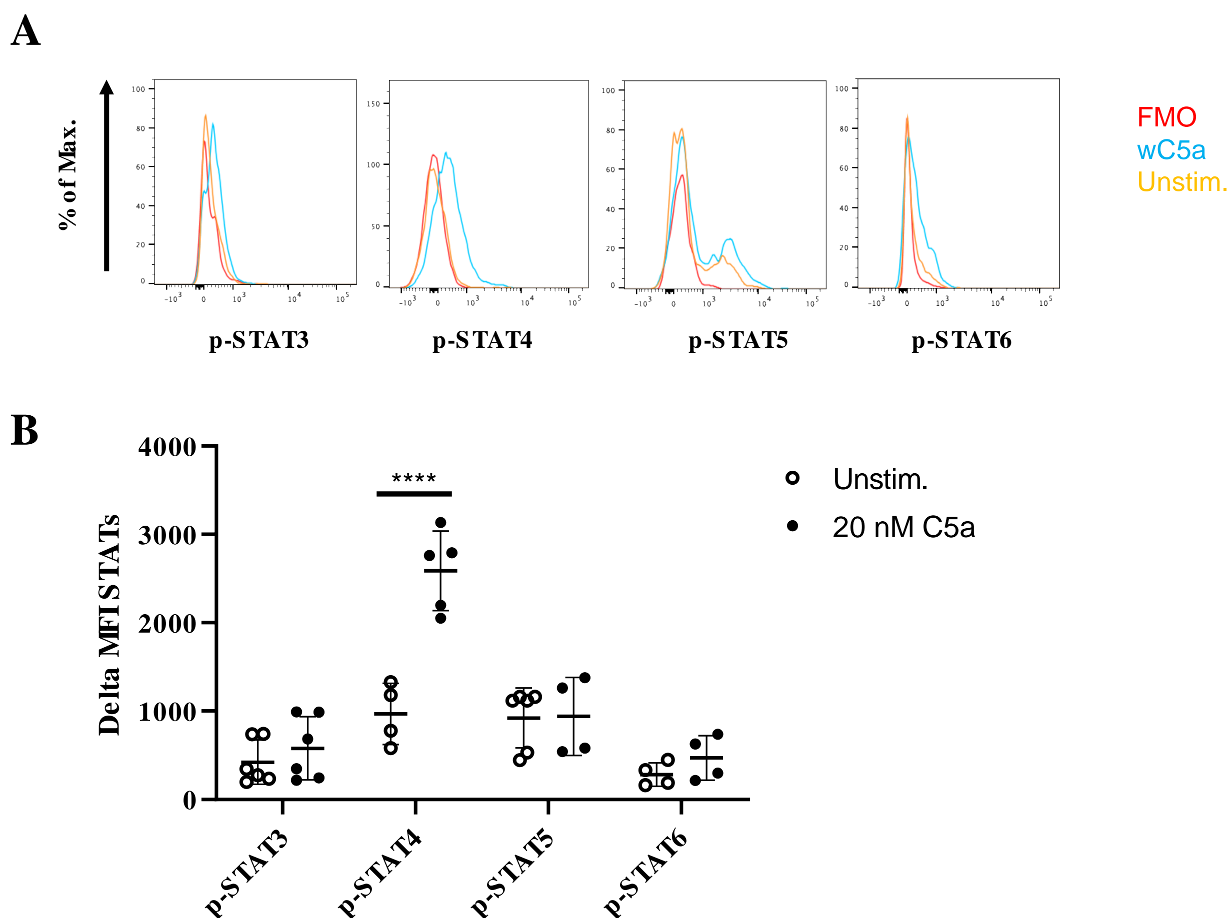


Figure 3. 8 C5aR1 signaling axis regulates STAT4 phosphorylation in ILC2. Phosphorylation of STATs in ILC2 from co-cultures in the presence or absence of C5a. **(A)** Intracellularly stained p-STAT3, p-STAT4, p-STAT5, and p-STAT6 in BM-ILC2 obtained by co-culture with BM-M-DCs were assessed 24h after start by flow cytometry. Histograms are representative of n=4-6 cultures from 3 independent experiments. C5a stimulated and unstimulated are highlighted in rosa, green, and yellow respectively. **(B)** Scatter plots showing the mean fluorescence normalized to the FMO control (ΔMFI) of p-STAT3, p-STAT4, p-STAT5, and p-STAT6 in co-culture +/- SEM, n=4-6 cultures from 3 independent experiments. The graphs show the mean value ΔMFI ± SEM normalized to the FMO control in rosa. Statistical differences were assessed by ANOVA, *** $p<0.001$.

Mechanistically, STAT4 is phosphorylated after binding of a variety of cytokines, including IL-12, IL-23, type 1 interferon (IFN), and IL-27, to their receptors at the membrane, the dimerization of p-STAT4 and its translocation from cytoplasm to the nucleus to regulate gene expression [312]. In order to understand how BM-M-DC activates the STAT phosphorylation in ILC2, I used a multiplex assay which allows me to look at various cytokines at the same time. I measured the presence of cytokines including IL-1α, Tumor Necrosis Factor (TNF)α, IL-1β, IFNγ , IL-23, IL-12p70, IL-10, Monocyte chemoattractant protein 1 (MCP-1), IL-6, IL-27, IFN-β, and, IL-17A, in supernatants from *in vitro* co-cultures. I detected measurable secretion of IL1-α, TNFα, IL-1β, IL-23, IL-10 and MCP-1. Interestingly, C5a stimulation increased IL-23 release from cultured cells (Figure 3.8.1B) and reduced but not completely suppressed the IL-10 secretion (Figure 3.8.1E), a known anti-inflammatory cytokine. In line, although C5a stimulation affects the IL-1-α, pro-inflammatory cytokines, and secretion, it did not reach statistical significance.

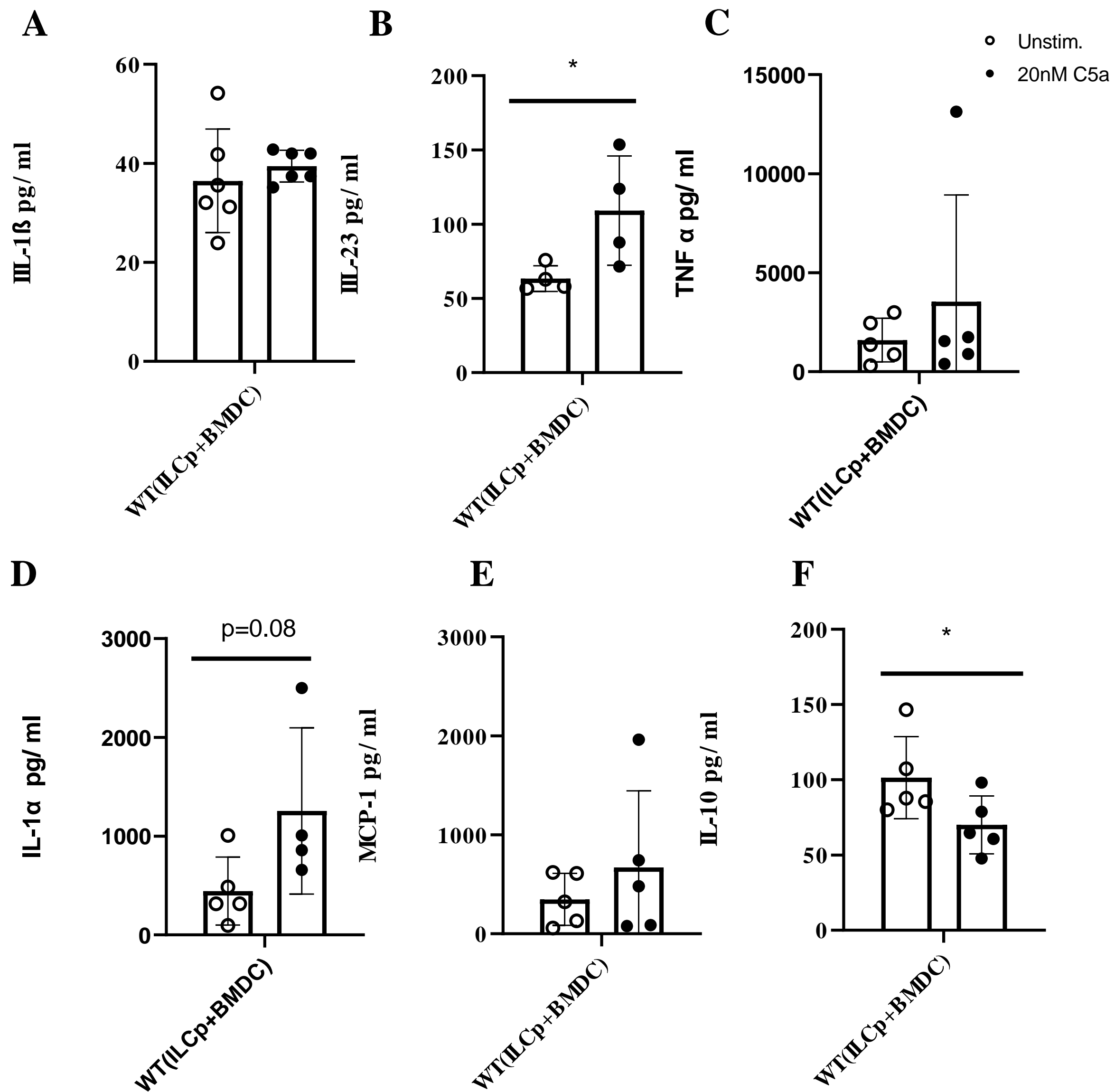


Figure 3.8.1 C5a/C5aR1 signaling drives the secretion of cytokines that are involved in the activation of STA T4. Cytokines level were assessed by multiplex assay 24 h later of co-culture in the presence or absence of C5a in pg/ml. Data show detectable levels of IL-1β **(A)**, IL-23 **(B)**, TNFα **(C)**, IL-1α **(D)**, MCP-1 **(E)**, and IL-10 **(F).** Data shown are mean +/- SEM, n=4-5 from 4-5 independent co-culture, and were analyzed using an unpaired t-test, * $p<0.05$.

3.7 BM-M-DCs regulate the phosphorylation of the STAT pathway in a time-dependent manner

Evidence suggested that BM-M-DCs regulate the ILC differentiation via changing of TFs. In addition, I observed that C5a/C5aR1 might regulate the STAT4 phosphorylation status via IL-23 secretion. Yet, it was unknown, whether C5a regulates STAT phosphorylation in long term. Therefore, I assessed the phosphorylation status of STAT4, 5, and 6 over time with flow cytometry up to 72h. Similar to the previous observation, after 24h C5a stimulation increased the phosphorylation of STAT4, after that phosphorylation gradually dropped irrespective of C5a stimulation (Figure 3.9A), possibly if C5a is used up in the first 24h. In contrast, the phosphorylation of STAT5, a bit higher at 24h was equal starting at 48h, (Figure 3.9B) suggesting that C5a, with the same dynamic favor the dephosphorylation of STAT5. Interestingly, the same phenomenon could be observed for STAT 6, which was actively dephosphorylated in presence of C5a at 48 h (Figure 3.9C). My data suggested that, while C5a is necessary to activate STAT4 in ILC2, it limits the phosphorylation of STAT5 and STAT 6, thus participating in the plasticity of ILCs.

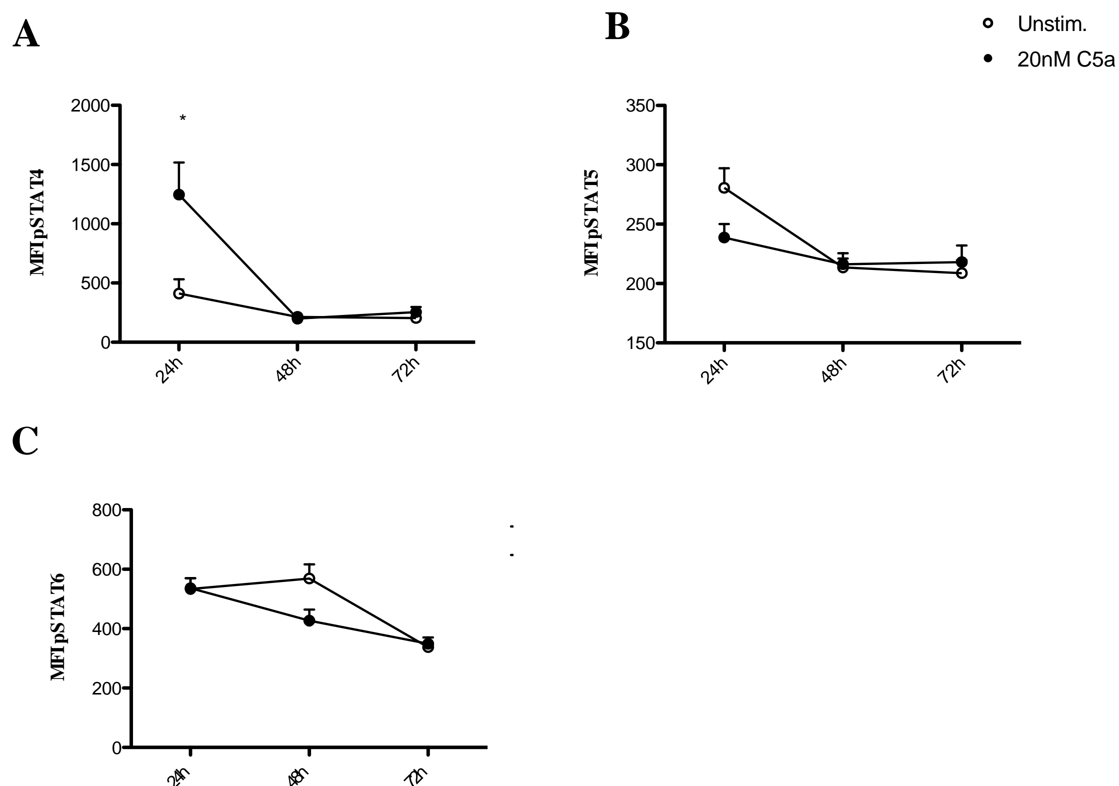


Figure 3. 9 BM-M-DC regulates the phosphorylation of STATs in ILC2s over time. Scatter plots showing the mean fluorescence (MFI) in ILC2 from co-cultures in the presence or absence of C5a of the

assessed after 24, 48, and 72h later, normalized to the FMO control of **(A)** STAT4 **(B)** STAT5 and **(C)** STAT6. Data are mean values +/- SEM, n=4 cultures from an experiment. Statistical differences were assessed by ANOVA, * $p<0.05$.

3.8 C5a supports the pulmonary ILCs development through its function in MHC-II cells

Although evidence shows that *in vitro* derived myeloid cells have an impact on the development of the ILC2, it is unclear how to translate the knowledge from the BM-derived model to the lung microenvironment since BM-derived cells are different from pulmonary cells. To delineate if crosstalk between the myeloid and innate lymphoid cells exists in pulmonary cells, I generated cultures using sorted pulmonary MHC-II $^+$ cells, as well as lung ILCPs [CD45$^+$, viable, Lineage$^-$ (CD11c, CD11b, CD3e, CD5, CD19, Ly6G, CD27, TCR β , CD49R, CD45b, CD27, Ter119)], in the presence or absence of C5a. I assessed the MHC-II driven C5a/C5aR1 axis impact on ILC2 using flow cytometry. My data showed that C5a stimulation on ILCp did not change the percentage of ILC2 in WT and *C5ar1*$^{-/-}$. In addition, while sorted pulmonary MHC-II$^+$ cells either from WT or *C5ar1*$^{-/-}$ had no impact on the frequency of ILC2, C5a stimulation increased the frequency of ILC2 (in Lineage$^-$ cells) cells when ILCp were cultured together with MHC-II$^+$ cells from WT origin but not from *C5ar1*$^{-/-}$ mice (Figure 3.10A). These data suggested that C5a/C5aR1 signaling in pulmonary MHC-II$^+$ cells support the development of ILC2 cells.

Then, I checked the GATA3 expression in pulmonary ILC2 from co-cultured with MHC-II$^+$ cells by intracellular staining. I observed solely in WT, that C5a/C5aR1 signaling axis enhanced the GATA3 expression ILC2 (Figure 3.10B). In addition, the expression of KLRG-1 in ILC2 increased when it is stimulated with C5a. In both cases, no induction could be observed in absence of C5aR1 (Figure 3.10A-B), confirming BM-derived data (Figure 3.6D). While this evidence suggested that C5a-triggered MHC-II$^+$ cells support the development of ILC2 via GATA3 expression, it was not yet clear whether C5a- triggered MHC-II$^+$ cells also play role in the function of ILC2s. In allergic asthma, ILC2-driven Eosinophil recruitment, controlled by IL5 secretion, to inflamed tissues is one of the most important features. Data shows that in response to C5a, MHC-II$^+$ cells increase IL- 5 secretion in ILC2 (Figure 3.10C). In addition, I evaluated STAT4 phosphorylation status by flow cytometry. Data shows that similar to BM-derived macrophages/Dendritic cells, MHC-II$^+$ cells are activated phosphorylation of STAT4 upon C5a stimulation (Figure 3.10E).

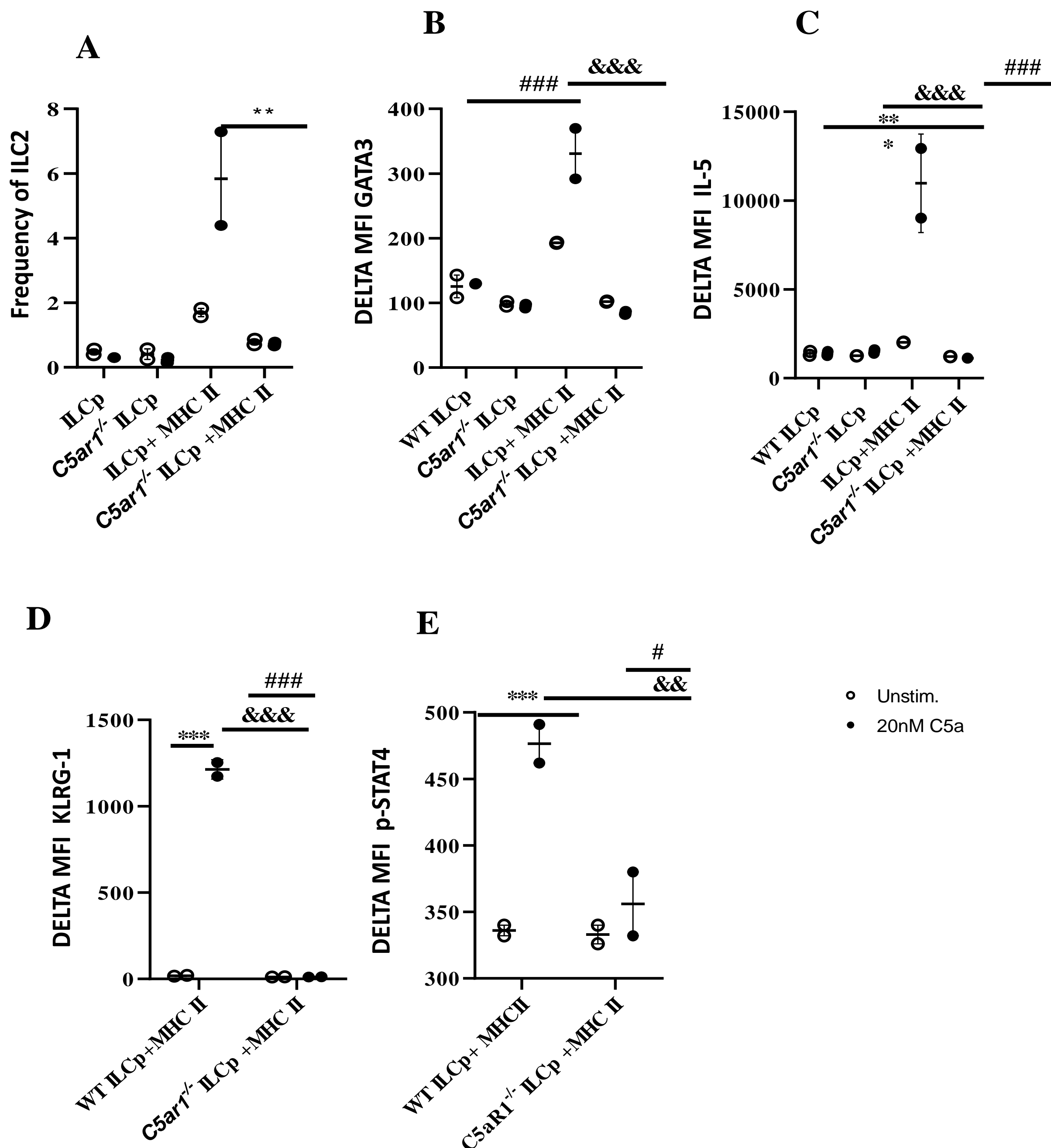


Figure 3. 10 Pulmonary MHC-II cells support ILC2 development. Percentage of ILC2s in Lineage⁻ [CD11c, CD11b, CD3e, CD5, CD19, Ly6G, CD27, TCR β , CD49R, CD45b, CD27, Ter119] cells. **(A)** The percentage of ILC2s (Lineage⁻, CD127⁺, CD25⁺), were assessed 24h after co-cultures started with sorted pulmonary MHC-II⁺ cells in the presence or absence of C5a using cells from WT or *C5ar1*$^{-/-}$ mice. **(B)** GATA3 expression in ILC2 generated by co-culture 24h later, data shows the mean fluorescence (MFI) normalized to the FMO control**. (C)** KLRG-1 expression in pulmonary ILC2 and co-culture, data show the mean fluorescence (MFI) normalized to the FMO control. **(D)** Intracellular expression of IL-5 in ILC2 cells

absence of C5a in WT mice. Data show the mean fluorescence (MFI) normalized to the FMO control. **(E)** p-STAT4 expression in pulmonary ILC2 and co-culture, data show the mean fluorescence (MFI) normalized to the FMO control. Data are mean values ± SEM, n=2 from one experiment, statistical differences were assessed using Kruskall-Walllis, ** p<0.01; ***, ###, &&& p<0.001.

3.9 C5a supports the pulmonary myeloid cells-triggered ILCs development

Evidence *in-vitro* primary pulmonary data shows C5a triggered MHC-II$^+$ cells supports the development of the ILC2. However, MHC-II is expressed by non-immune cells such as; epithelial cell [313] and many immune cells including Interstitial macrophages [59], Alveolar macrophages [314], and Dendritic cells [85]. Thus, it is required to understand more specifically which myeloid cells trigger the ILC2 development through C5a/C5aR1. To address the missing piece in the puzzle, I sorted different pulmonary myeloid cells, according to their surface expression markers, including (Siglec-F$^+$, CD11c$^+$) Alveolar Macrophages (AM)s, (CD11c$^+$, MHC-II$^+$) Dendritic cells (DC)s, and (F4/80$^+$, CD11b$^+$) Interstitial macrophages (IM)s, and culture for 24h in the presence or absence of C5a with sorted ILCPs [CD45$^+$, viable, Lineage$^-$ (CD11c, CD11b, CD3e, CD5, CD19, Ly6G, CD D27, TCR β , CD49R, CD45b, CD27, Ter119)]. In line with previous observations on BM-derived macrophage/DC and pulmonary MHC-II$^+$ cells, C5a stimulation of ILCp cells did not favor the formation of ILC2s (Figure 3.11C). Interestingly, none of the cells from myeloid origin participate in the ILC2 development without C5a stimulation (Figure 3.11A). In contrast, C5a-activated AMs and DC supported the development of ILC2s (Figure 3.11B-D-E), while IM had no impact on ILC2 development with or without C5a (Figure 3.11B-D-E).

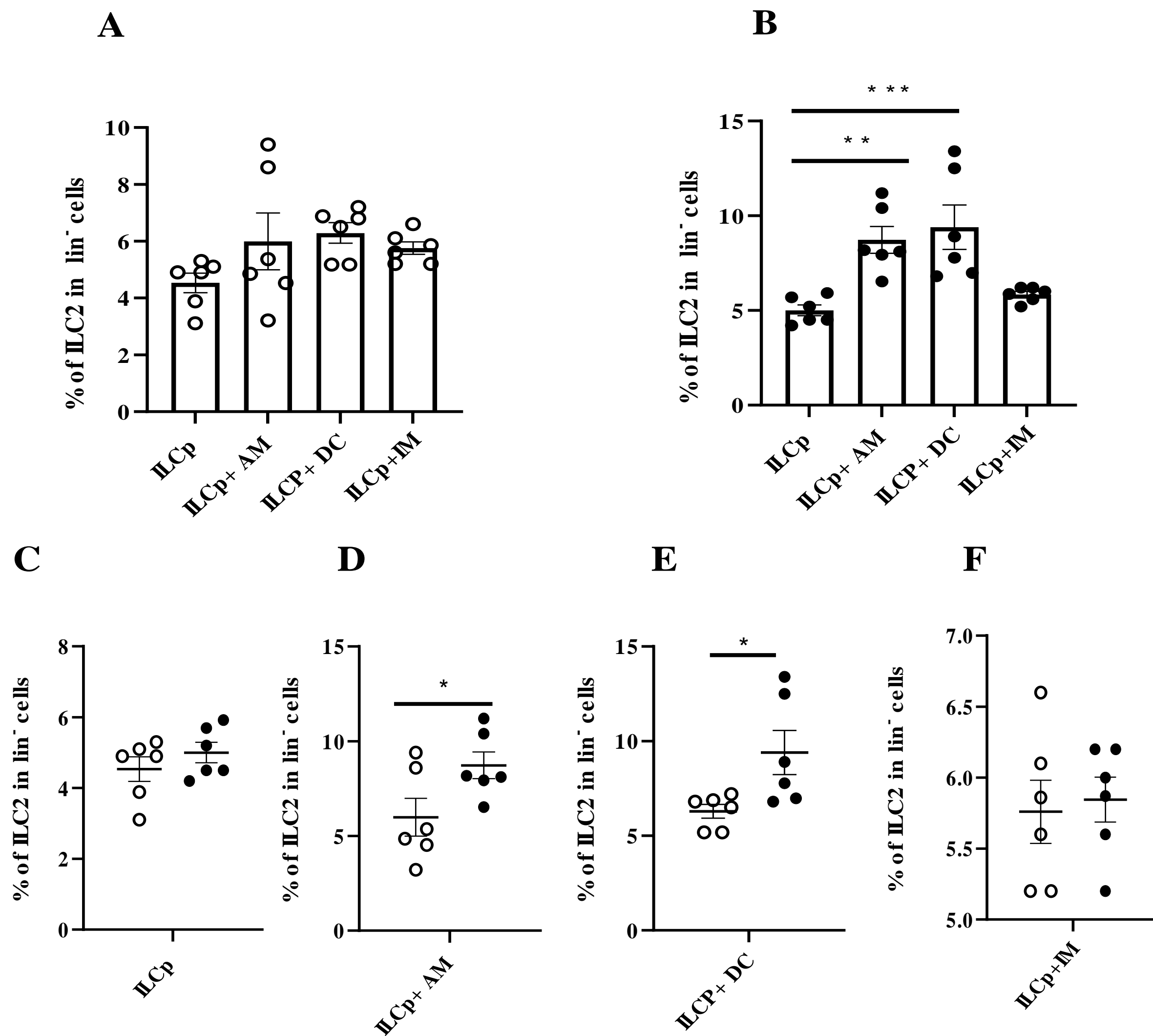


Figure 3. 11 Pulmonary APCs support ILC2 development. Percentage of ILC2s in Lineage$^-$ [CD11c, CD11b, CD3e, CD5, CD19, Ly6G, CD27, TCR β , CD49R, CD45b, CD27, Ter119], Scatter plots show mean value +/- SEM, n=5-6 from 3 independent experiments. Black-filled round representative of culture with C5a stimulation, empty round representative of without C5a stimulation. **(A)** Percentage of ILC2s (Lineage$^-$, CD127$^+$, CD25$^+$), were assessed 24h after co-cultures started in absence of C5a in WT mice. Statistical differences were assessed by ANOVA **(B)** Percentage of ILC2s (Lineage$^-$, CD127$^+$, CD25$^+$),were assessed 24h after co-cultures started in presence of C5a in WT mice. Statistical differences were assessed by ANOVA ** $p<0.01$, *** $p<0.001$.**(C)** Percentage of ILC2s (Lineage$^-$, CD127$^+$, CD25$^+$), were assessed 24h after in the presence or absence of C5a. Statistical difference differences were assessed by unpaired t-test. **(D)** Percentage of ILC2s (Lineage$^-$, CD127$^+$, CD25$^+$), were assessed 24h after AM-ILCp co-cultures started in culture in the presence or absence of C5a. Statistical difference differences were assessed by unpaired t-test * $p<0.05$**.** **(E)** Percentage of ILC2s (Lineage$^-$, CD127$^+$, CD25$^+$), were assessed 24h after DC-ILCp co-cultures started in culture in the presence or absence of C5a. Statistical difference differences were assessed by unpaired t-test * $p<0.05$. **(F)** Percentage of ILC2s (Lineage$^-$, CD127$^+$, CD25$^+$), were assessed

24h after IM-ILCp co-cultures started in culture in the presence or absence of C5a Statistical difference differences were assessed by unpaired t-test.

3.10 C5a favors the survival of ILC2 and maturation of ILC2

As C5a-triggered AMs and DCs increased the frequency of ILC2, I was wondering if C5a via AMs and DCs, supported the survival of ILC2. In order to address this, I measured the expression of the anti-apoptotic marker, Bcl2 by flow cytometry. Interestingly, C5a stimulation boosted the survival of ILC2s in ex vivo culture both in co-cultures with DCs or AMs, although the latter showed a much stronger effect (Figure 3.12A). KLRG-1 is commonly associated with maturation. In addition to its function in maturation, it has been shown that KLRG-1 expression involves in the survival of $CD8^+$ T cells via controlling of the Bcl-2 expression in the LCMV infection model [315]. I was wondering if KLRG-1 expression alters by C5a stimulation via its impact on AMs and DCs. Flow cytometric analysis shows that KLRG-1 expression in ILC2s increased by C5a stimulation in AM-ILCps culture (Figure 3.12B). Suggesting that AMs and DCs follow a different strategy to crosstalk with ILC2s.

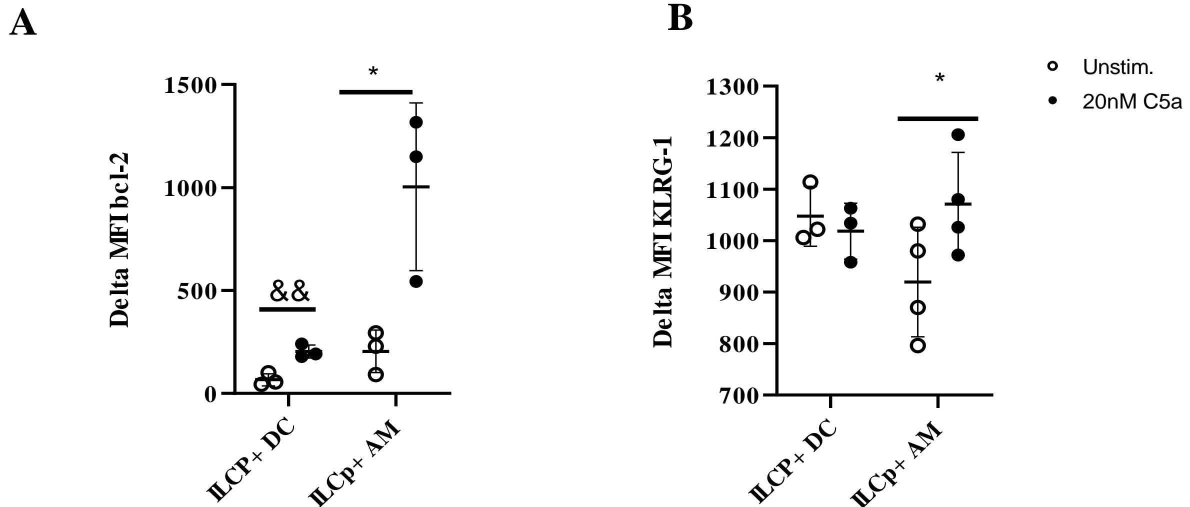


Figure 3. 12 Pulmonary APCs support ILC2 survival. (A) Bcl-2 expression in co-culture, data show the mean fluorescence normalized to the FMO control +/- SEM in ILC2s assessed, 24h after co-cultures started in the presence or absence of C5a. n=3 from 2 independent experiments. Data were analyzed by ANOVA, * p<0.05,** p<0.01. **(B)** KLRG-1 expression in co-culture, data show the mean fluorescence (MFI) normalized to the FMO control ILC2s assessed 24h after co-cultures started in ex vivo culture in the presence or absence of C5a. n=3-4 from 2 independent experiments, Data were analyzed by ANOVA, * p<0.05.

3.11 C5a /C5aR1 axis on pulmonary myeloid cell independent from STAT4 phosphorylation

Since, one of the main pieces of evidence I observed using BM-derived cells, was the observation that C5a/CaR1 on BM-M-DCs activates the phosphorylation of STAT4 in ILC2. Therefore, I stimulated AMs or DCs and evaluated the status of STAT4 in ILC2 in co-culture in the presence or absence of C5a. However, neither in the absence nor in presence of C5a, AMs or DCs triggered the phosphorylation level of STAT4 in ILC2s (Figure 3.13).

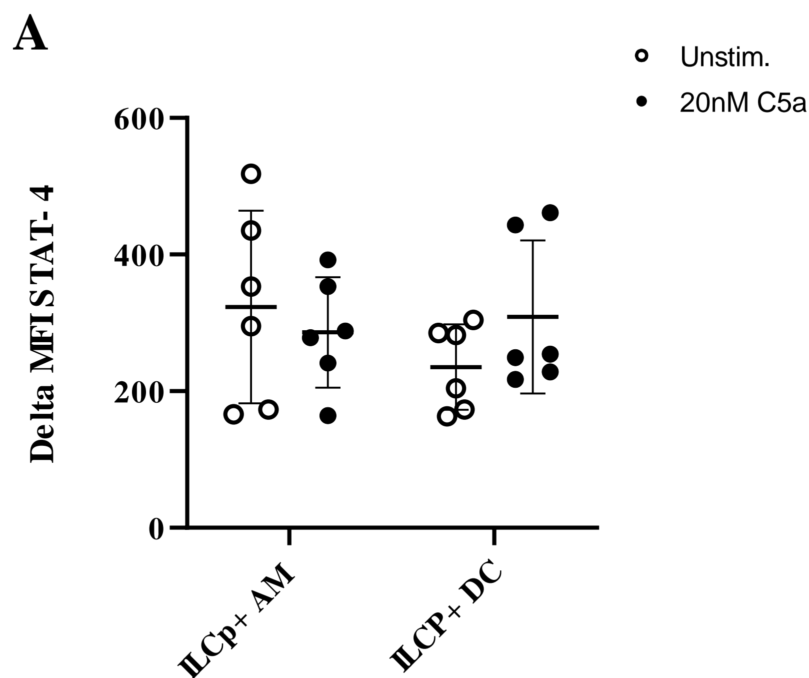


Figure 3. 13 C5a/C5ar1 in AM and pulmonary DC, did not change the STAT4 phosphorylation status in ILC2. Black-filled round representative of culture with C5a stimulation, empty round representative of without C5a stimulation. p-STAT 4 expression in co-culture, data show the mean fluorescence (MFI) normalized to the FMO control ILC2s assessed 24h after co-cultures started in presence or absence of C5a. Data were analyzed by ANOVA.

3.12 C5a/ C5aR1 signaling in DC regulates TSLP-R expression

Allergen exposure results in the secretion of alarmins including IL-33 and TSLP which activates the ILC2 via ST2 and TSLP-R, respectively [180]. Of note, pulmonary inflammatory

ILC2 known to express ST2 on its surface and TSLP were often associated with severe asthma. I was wondering if C5a/C5aR1 signaling has an impact on ST2 or TSLP-R on ILC2s. In the co-culture system, I observed that there was no increase in ST2 expression in ILC2 upon C5a (Figure 3.14A), while C5a/C5aR1 signaling in DCs negatively regulate TSLP-R expression (Figure 3.14B).

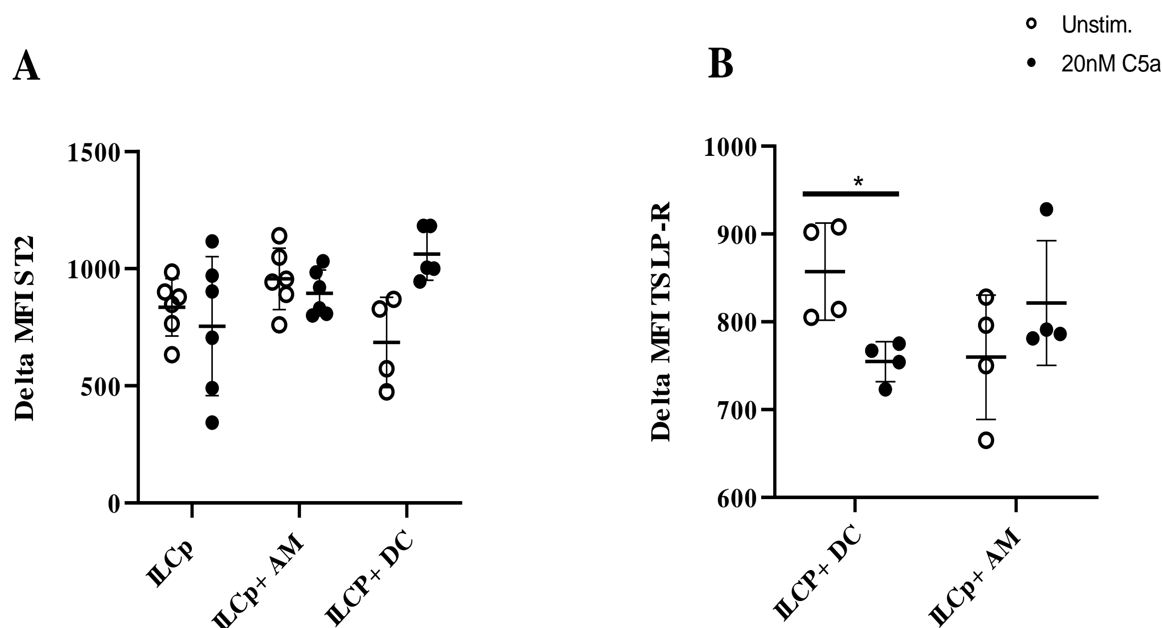


Figure 3. 14 C5a/C5aR1 signaling in DCs negatively regulates TSLP-R expression in ILC2. (A) Surface expression of ST2 in ILC2 in co-culture, data show the mean fluorescence normalized to the FMO control (Delta FMO). Black-filled round representative of culture with C5a stimulation, empty round representative of without C5a stimulation. **(B)** Surface expression of TSLP-R in ILC2 from co-culture, data show the mean fluorescence normalized to the FMO control (Delta FMO). +/- SEM, n=4-6, from 2-3 independent experiments were analyzed by ANOVA, * $p<0.05$.

3.13 C5a/C5aR1 signaling axis drives development of ILC2 upon crosstalk

Evidence shows that C5a-triggered myeloid cells, AMs, and DCs, favor ILC2 development. Yet, if the C5a/C5aR1 signaling axis plays a role directly on AMs and DCs or indirectly via other cells acting on AMs and DCs is ill-defined. I was wondering about the role of C5aR1 signaling in LysM-expressing cells, on the development of ILC2. Here, I determined directly the role of C5a-induced signaling in DCs and AMs on pulmonary ILC2 development using LysMCre-C5aR1conditional KO mice in which C5aR1 is specifically deleted in LysMCre-expressing cells, neutrophils, macrophages, and moDC [316]. Similarly, sorted ILCP [$CD45^{+}$,

viable, Lineage$^-$ (CD11c, CD11b, CD3e, CD5, CD19, Ly6G, CD D27, TCR β , CD49R, CD45b, CD27, Ter119)] was cultured with sorted AMs (Siglec-F$^+$, CD11c$^+$), or sorted DCs (CD11c$^+$, MHC-II$^+$), or alone as a control. Using such cells, I observed that, similar to previous data, in GFP-C5aR1^{flflf} control mice, C5a/C5aR1 signaling supported the ILC2 development (Figure 3.15). Further, although significantly reduced using conditionally deficient AMs, C5a in LysM-Cre-C5aR1 AMs persisted to provide support for the ILC2 compared to no C5a (Figure 3.15A). Suggesting, that C5a/C5aR2 might play a role. In contrast, C5a-triggered DCs, in GFP-C5aR1$^{fl/fl}$ control mice, supported ILC2s development (Figure 3.15B). while C5a didn't drive the ILC2 development when DCs specifically deleted C5aR1, suggesting that only the C5a/C5aR1 signaling axis was involved in the development of ILC2 upon DC crosstalk.

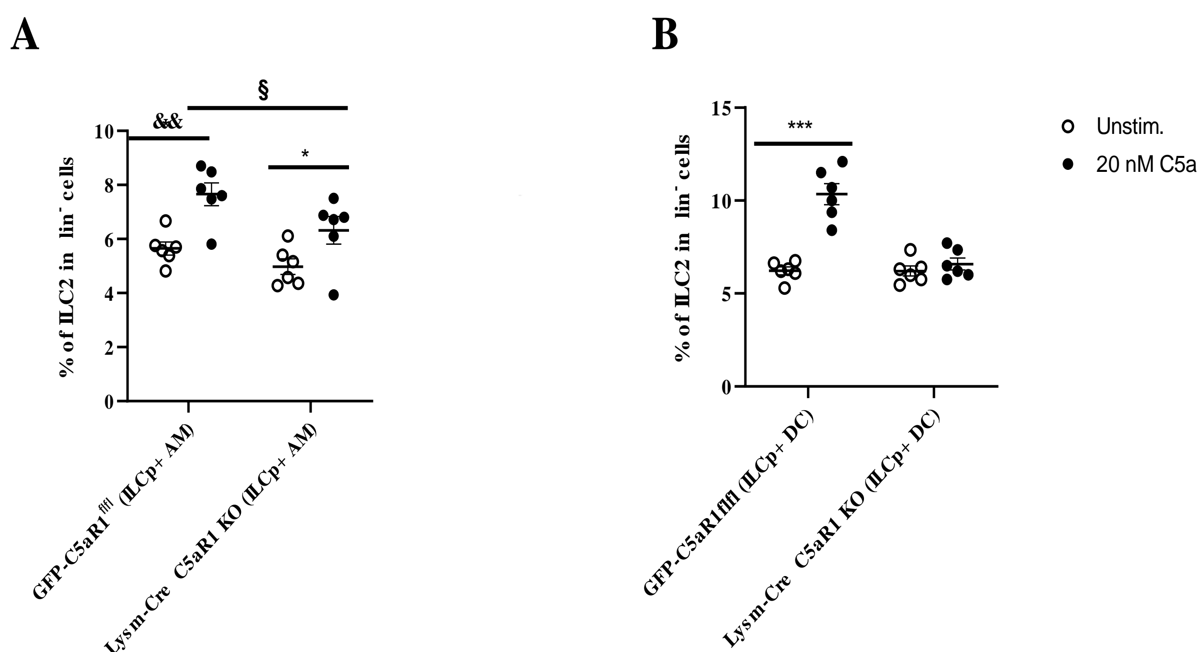


Figure 3. 15 While C5a/C5aR1 signaling axis drives the development of ILC2 upon DC crosstalk, C5a/C5aR2 might drives AM-ILC2 crosstalk. percentage of the ILC2s in lineage-negative cells, GFP-C5aR1^{flffl} and LysM-Cre C5aR1 KO are used to delineate the function of C5a/C5aR1 signaling on ILC2 development. **(A)** The percentage of Lineage negative CD127+ CD25+ ILC2s assessed 24h after AM-ILCp co-cultures started in presence or absence of C5a. **(B)** The percentage of Lineage negative CD127+ CD25+ ILC2s assessed 24h after DC-ILCp co-cultures started in the presence or absence of C5a. n=6, from 3 independent experiments. Data were analyzed by ANOVA, *,§ $p<0.05$, && $p<0.01$, *** $p<0.001$.

3.14 C5a/C5aR1 signaling triggers IL-5 production by ILC2 upon AM crosstalk

As ILC2s orchestrates the recruitment of immune cell to inflamed tissues via type 2 cytokines in general and IL-5 in particular, I aimed to appreciate the importance of C5a/ C5aR1 signaling axis, in the intracellular IL-5 expression in ILC2 upon sorted ILCp [$CD45^{+}$, viable, $Lineage^{-}$ (CD11c, CD11b, CD3e, CD5, CD19, Ly6G, CD D27, TCR β , CD49R, CD45b, CD27, Ter119)] co-cultures with sorted (Siglec-F^{+}, $CD11c^{+}$) Alveolar Macrophages (AM)s, ($CD11c^{+}$, MHC-II^{+}) Dendritic cells (DC)s ($CD11c^{+}$, MHC-II^{+}), from LysM-cre C5aR1 and GFP-C5aR1 reporter mice as a control. Interestingly, C5a stimulation DC was critical for ILC2 development, it is fruitless for ILC2 function in terms of IL-5 secretion (Figure 3.16B). On the other hand in AMs, in absence of the C5a stimulation in GFP reporter mice and the presence or absence of conditional KO, IL5 production was at a similar level. However, it increased with C5a stimulation in the control group (3.16C), this suggests that C5a/C5aR1 signaling in AMs regulates ILC2 function (Figure 3.16C).

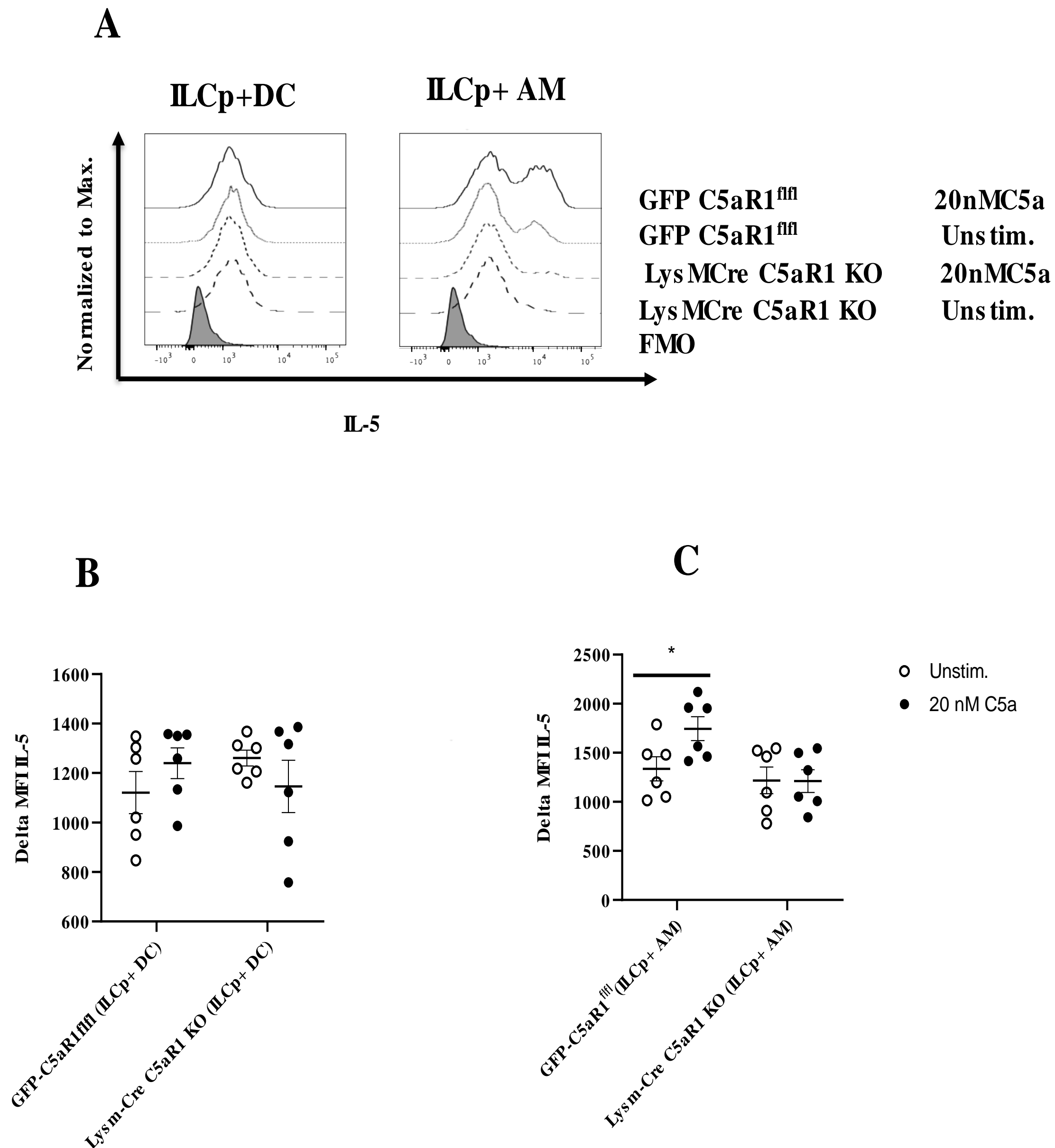


Figure 3. 16 AM regulates ILC2 function through C5a/C5aR1 signaling. Black-filled round representative of culture with C5a stimulation, empty round representative of without C5a stimulation. **(A)** Representative histograms showing the intracellular expression of IL-5 in ILC2 after cultures with DCs or AMs sorted from GFP C5aR1$^{fl/fl}$ or LysM-cre-C5aR1 mice. Graphs show FMO (grey), LysM-cre-C5aR1 unstimulated (dashed) or stimulated (dotted), and GFP-C5aR1 unstimulated (grey line) or stimulated (dark line). **(B)** Intracellular stained IL-5 in ILC2 from DC-ILCp co-culture. Data show the mean fluorescence of IL-5 normalized to the FMO control (ΔMFI) assessed 24h after co-cultures started in the presence or absence of C5a. The graph shows the mean ΔMFI value of IL-5. ± SEM n=6, from 2 independent experiments. **(C)** Intracellular stained IL-5 expression in AM-ILCp co-culture. The graph shows the mean ΔMFI value of IL-5 ± SEM. n=6, from 2 independent experiments. Data were analyzed by ANOVA, * $p<0.05$.

3.15 Tonic C5/C5a production by AMs participates in ILC2 development

Recently, it has been shown that not only C5a could be generated exogenously, but that it could also be produced endogenously [205, 246]. While in the previous sections, I reported that C5a stimulation activates myeloid cells that regulate ILC2 development through it is receptors, yet, it is not fully appreciated whether tonic C5/C5a production from myeloid origin cells has an impact on ILC2 development. To clarify this, I used C5 deficient mice that produced, due to a mutation introducing a STOP codon [317], a short form of C5 that can't be cleaved and thus form C5a, and WT mice as a control. When co-cultured ILCp with AMs (Siglec-F^{+}, CD11c^{+}), the ILC2 development is significantly reduced when using C5-deficient AM in comparison to WT mice (Figure 3.17). In contrast, no changes were observed in ILC2 development while using DCs (CD11c^{+}, MHC-II^{+}) (Figure 3.17), suggesting that a tonic C5a/C5a production by AMs participates in ILC2 development, while DC must be supplemented with C5a to drive ILC2 development.

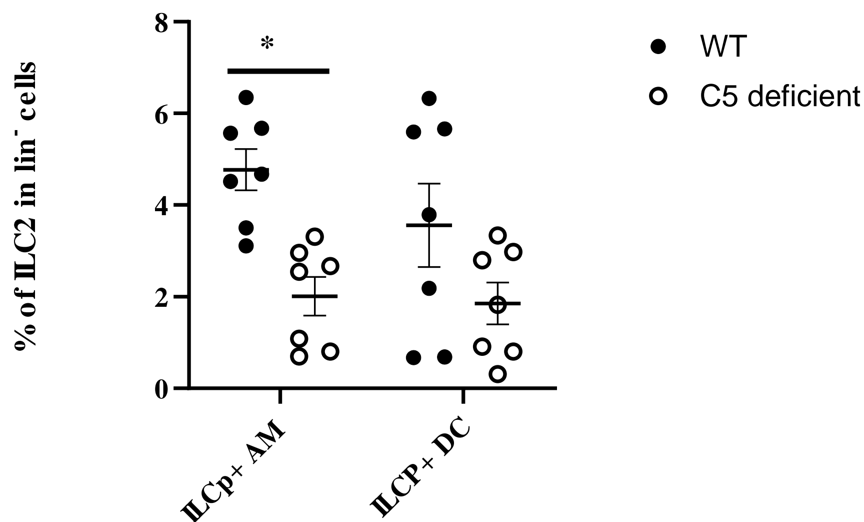
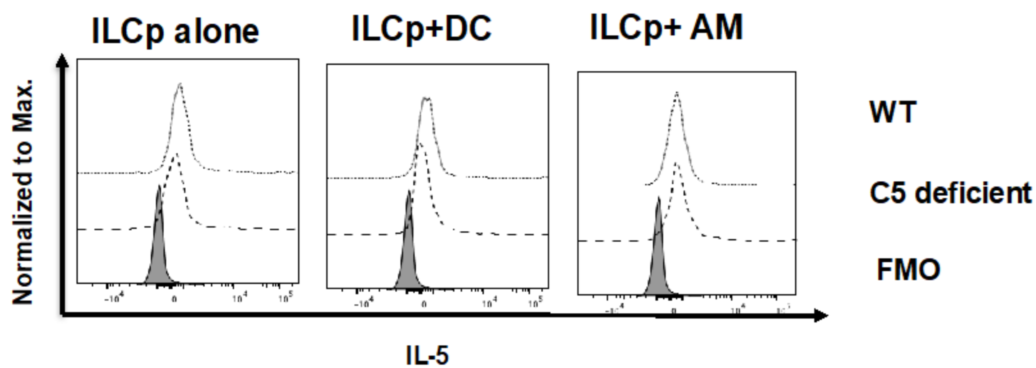


Figure 3. 17 Tonic C5/C5a production by AMs participates in the ILC2 development. Percentage of the CD127+ CD25+, ILC2s in lineage negative cells assessed after 24h after, WT and C5 deficient mice are used to delineate the function of tonic C5/C5a production on ILC2 development. ± SEM n=5-6, from 2 independent experiments. Data were analyzed by ANOVA, * $p<0.05$.

3.16 Tonic C5/C5s sustains AM driven IL-5 induction in ILC2

Although my data suggested that tonic C5/C5a production supports the development of ILC2, I was wondering, if, in addition to its role in development, tonic C5/C5a from AM and /or DC participates in the function of ILC2. To answer this, I measured the intracellular IL- 5

expression from innate lymphoid cell type 2 by flow cytometry. Similar to my previous results, while AMs, without the addition of C5a, were able to increase IL-5 in ILC2, it was impaired significantly in C5-deficient AMs (Figure 3.18B). In contrast, DCs did not sustain IL-5 production from ILC2 in WT mice, and interestingly C5 deficiency decreased IL-5 production.



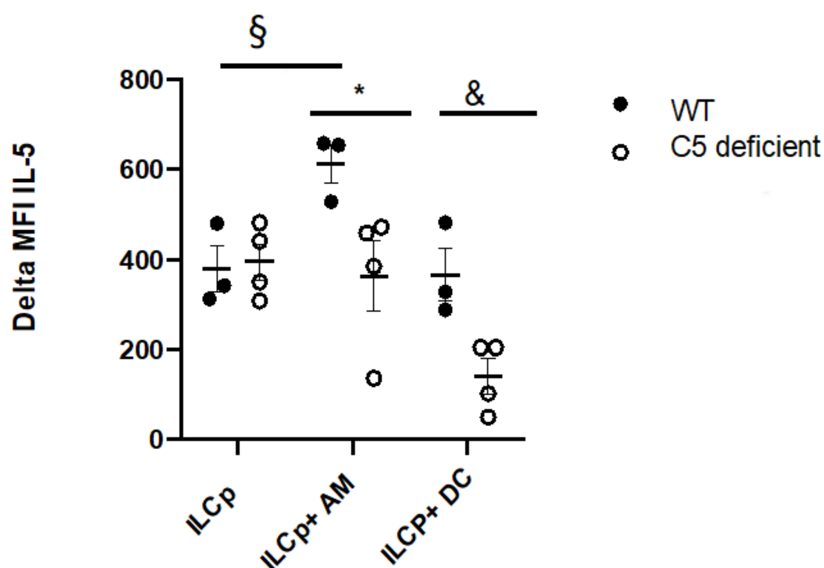


Figure 3. 18 Tonic C5/C5a sustains AMs- driven IL-5 induction in ILC2. (A) Histograms are representative intracellular expression of IL-5 in ILC2 from WT or C5 deficient mice assessed 24h after co-cultures started. **(B)** Intracellular stained IL-5 expression in ILC2 when ILCp culture with DC, AM- ILCp, data show the mean fluorescence (MFI) normalized to the FMO control ILC2s assessed after 24h after co-cultures started. ± SEM n=3-4, from 2 independent experiments. Data were analyzed by ANOVA, *,§,& $p<0.05$.

3.17 AM-derived soluble factors are required for ILC2 development

Although my data supported the idea that both AM and DC maintain ILC2 development through the activation of their anaphylatoxin receptors via endogenous and exogenous C5a, how mechanistically the crosstalk between myeloid cells and ILCp occurs remained elusive. Firstly, a soluble factor, such as cytokines, may be released by myeloid cells upon C5a and might drive ILC2 development. To address this question, I sorted AMs (Siglec-F^{+}, CD11c^{+}), ILCp, and DC populations (CD11c^{+}, MHC-II^{+}), and stimulated them for 24 h with C5a. The next day, I spun down cells and kept the supernatant of each cell type to use as conditioned media. Then, I freshly sorted ILCp again but this time instead of C5a stimulation, I stimulated the cells with the conditioned supernatants. Data show that only AM-derived supernatant, but not supernatants coming from stimulated ILCp or DCs, improved the ILC2 development (Figure 3.19A-B). Furthermore, I measured ILC2-derived IL-5 by flow cytometry and observed that AM-derived soluble factors regulated the functions of ILC2 (Figure 3.19C-D).

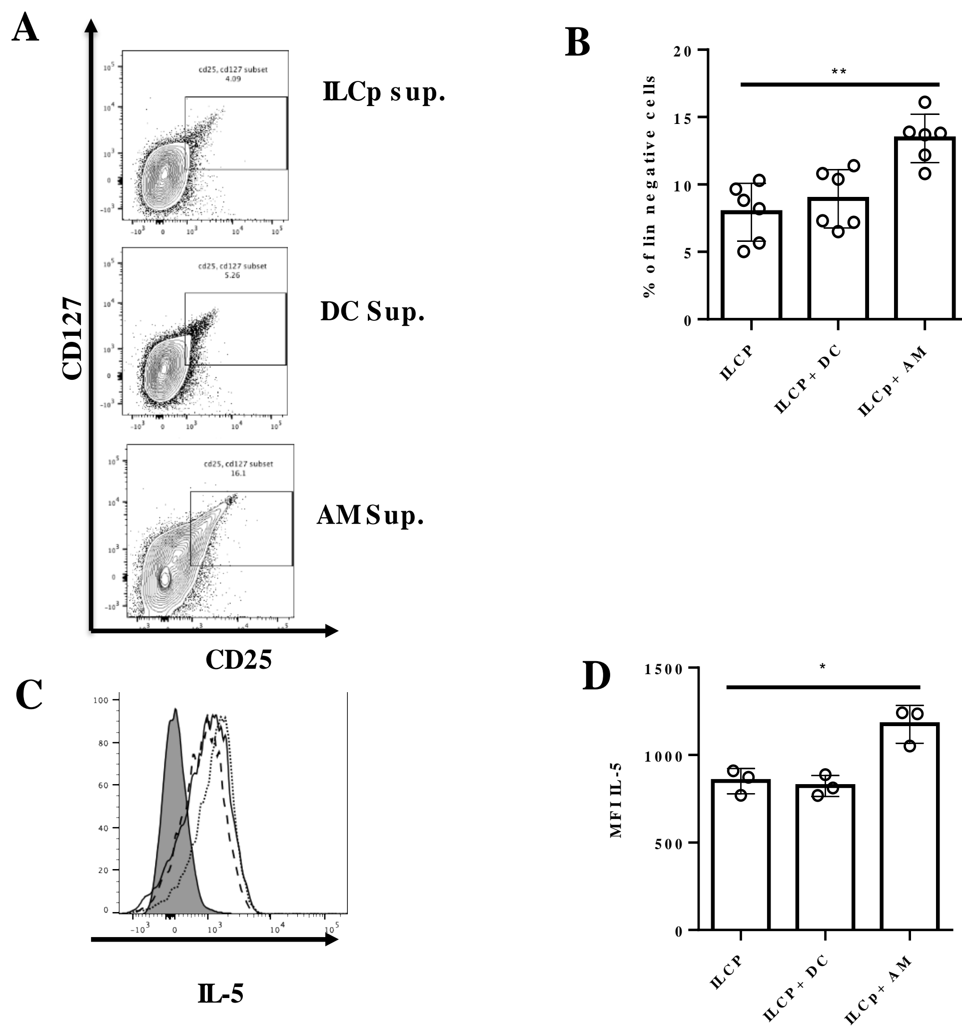


Figure 3. 19 Soluble factors released by AM are required for ILC2. **(A)** Histograms are representative of ILC2 from stimulated supernatant of AM, ILCp, and DC. **(B)** Frequency of ILC2, 24h after stimulated with ILCp, DC, and AM-derived supernatant respectively. **(C)** Histograms are representative of intracellular IL-5 staining from stimulated supernatant of AM, ILCp, and DC. FMO (Grey), AM (dotted line), or DC (solid line). **(D)** Intracellular stained IL-5 expression in ILC2 when it is cultured together with stimulated supernatant of DC, AM- ILCp, data show the mean fluorescence (MFI) normalized to the FMO control ILC2s assessed after 24h after co-cultures started. n=3-6, Data were analyzed by ANOVA, * $p<0.05$, ** $p<0.01$.

To identify the AM-derived soluble factor involved in the development and/or functions of ILC2, I performed a multiplex assay from the supernatant derived from AM. Although many cytokines were under detection level, only IL-1 α secretion was increased by C5a stimulation. Since ELISA is a more reliable method for cytokine detection, I performed ELISA to measure IL-1 α in stimulated and unstimulated supernatant from co-cultured (AM-ILCp). In line with the multiplex, ELISA measurement revealed that C5a stimulation increased the IL-1 α production from AMs, suggesting that AM-derived IL-1 α regulates ILC2 via C5a (Figure 18.1A).

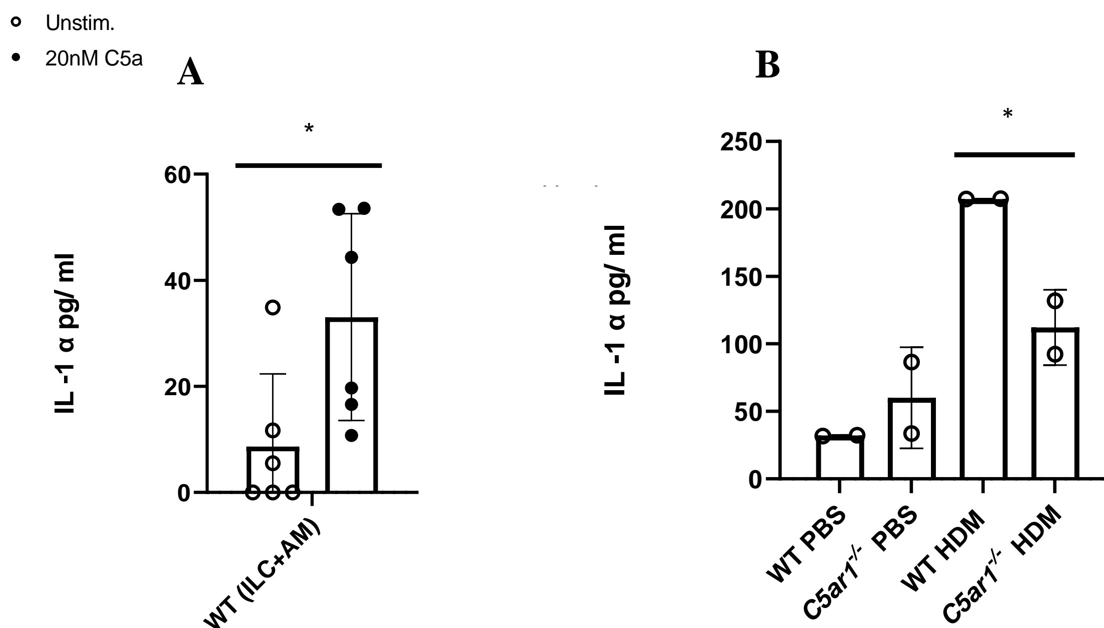


Figure 3. 19. 1 AMs derived IL-1 α regulates the ILC2 via C5a. **(A)** IL-1 α level was assessed with ELISA assay 24 h later of co-culture in the presence or absence of C5a in pg/ml. **(B)** IL-1 α level assessed with ELISA assay 24h after i.t. 2xHDM /PBS treatment. Data were analyzed by unpaired t-test, * $p<0.05$.

Till here, with numerous evidence, I showed that C5a/C5aR1 signaling axis functions in the cellular crosstalk between AMs/DCs and ILC precursors. However, I was wondering whether these basic cellular mechanisms could be translated to asthmatic conditions. It is known that during sensitization to an allergen, ILC2s are getting close contact with Alveolar macrophages [318]. I sensitized the mice by treating them i.t. twice with house dust mite (HDM) extract, a common allergen to humans, or PBS as a control. After 24 h, supernatant from digested lungs was collected and the level of IL-1α was measured. Then, ELISA was used to measure IL-1a. In line with the in vitro data, data shows that sensitized lungs show an IL-1α signature compared to the PBS control (Figure 3.19.1B). In contrast, *C5ar* deficient mice produced less IL-1α in comparison to WT-sensitized mice.

Since my previous observations using BM-derived cells show that C5a-activated Macrophage/Dendritic cells impact ILC2 phosphorylation of STAT 4 status via IL-23, I was wondering if this holds true for cytokines which induce the phosphorylation of STAT4 in pulmonary exposed mice. Sorted ILCp and AMs from 2x HDM i.t. treated mice were cultured in the presence or absence of C5a and the cytokines level in the supernatant of co-culture was measured by multiplex assay. I observed that IL-12p70, IL-23, and IL-27 levels were indeed increased upon C5a stimulation in co-cultures (Figure 3.19.2A-B-C).

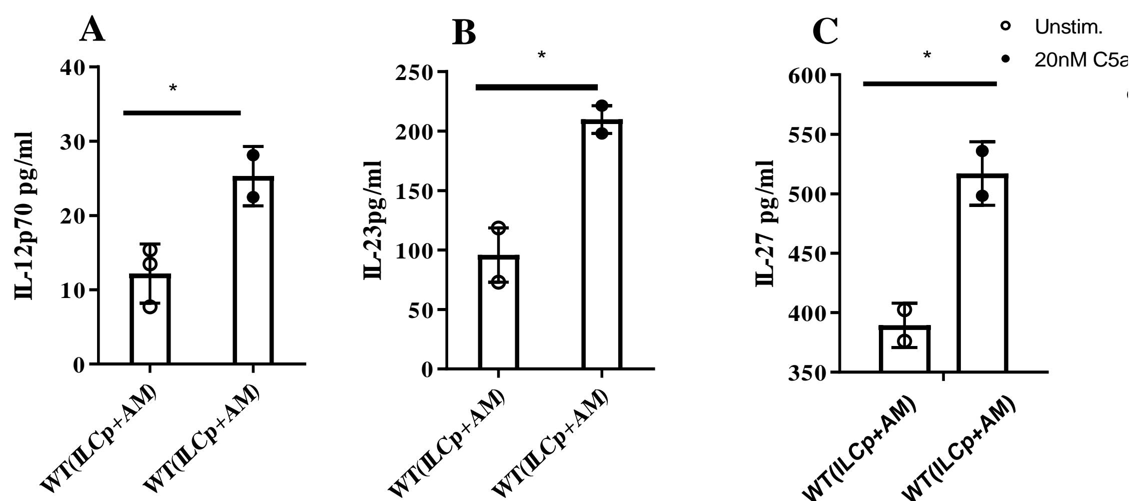


Figure 3. 19. 2 C5a/C5aR1 signaling drives the production and secretion of cytokines during sensitization. Cytokines level assessed in the supernatant of i.t. 2xHDM mice with multiplex assay after 24 h later of co-culture in the presence or absence of C5a in pg/ml **(A)** Level of IL-12p70 in the supernatant

of co-culture (**B**) Level of IL-23 in the supernatant of co-culture. (**C**) Level of IL-27, in the supernatant of co-culture. ± SEM n=2,3 Data were analyzed by unpaired t-test, * $p<0.05$.

3.18 DC required cell-to-cell contact to regulate ILC2

Although C5a-triggered DCs support the ILC2 development, soluble factors derived from DCs, in contrast to AMs, did not improve the ILC2 development. In order to understand the strategy used by DCs to crosstalk with ILC2, I treated the mice i.t. 2x with HDM and visualized the cell composition by confocal microscopy (Figure 3.20). Interestingly there were no ILC2s in the alveolar space, but AMs. In contrast, in the tissue CD11+DCs localized in close contact with $CD3^{-}$GATA3+ILC2s, suggesting that while AMs-derived cytokines regulate the ILC2 via C5a, DCs may require cell-to-cell contact.

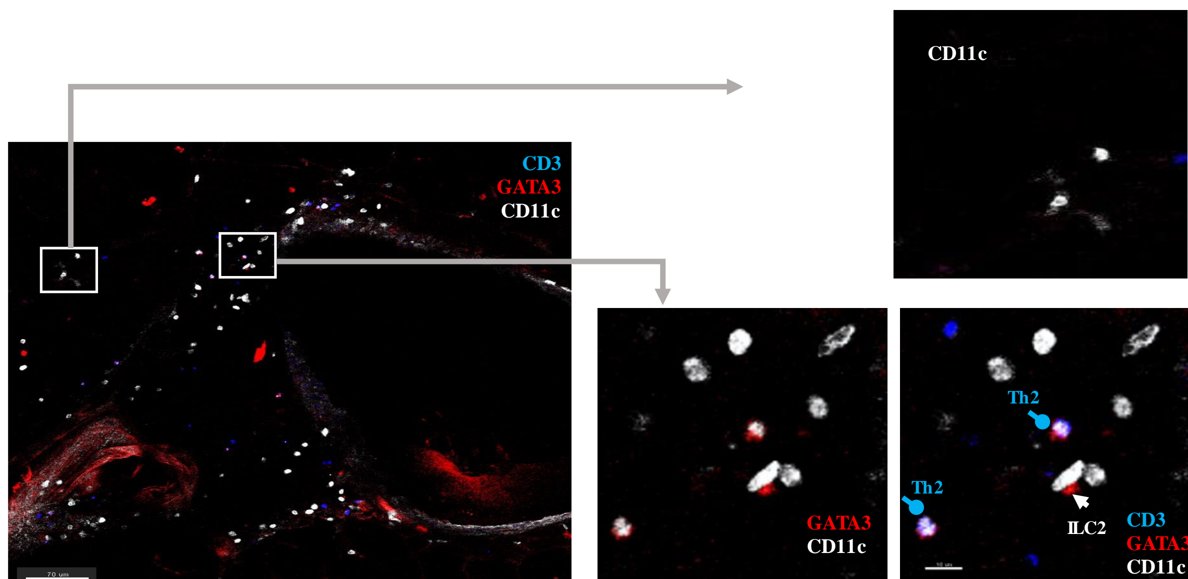


Figure 3. 20 DCs trigger ILC2 function and development through different mechanisms. Precision Cut slice from lungs of mouse exposed 2x to HDM stained with CD3 (T cells), GATA3 (Th2, ILC2 cells), and CD11c+ AMs and DCs. The distinction between AMs and DCs was based on their localization in the alveolar space or the tissue and their shape.

3.19 C5aR1 might play role in airway function and cell recruitment during sensitization

In order to have an idea of whether C5a/C5aR1 signaling on the cellular composition of the lung and airway functions, I treat the mice with i.t 2xHDM or PBS. Upon HDM exposure, resident and inflammatory Eosinophil were recruited into the lung (Figure 3.21A-B). However,

surprisingly the recruitment was not necessarily regulated by C5aR1, as the recruitment was similar in *C5ar1*$^{-/-}$ mice to the WT (Figure 3.21B). It is worth mentioning that, in the very first experiment (3.1) data suggests that C5aR1 plays a critical role in the Eosinophile recruitment after 1x allergen exposure data. Here using 2 allergen exposure, I could not observe any changes in Eosinophil recruitment. This might be because 2 allergen exposure is no longer sensitization, the dual role of the C5aR1 in allergic diseases has been shown [234]. Furthermore, in absence of the C5aR1, the airway resistance in response to methacholine tended to decrease (Figure3.21C).

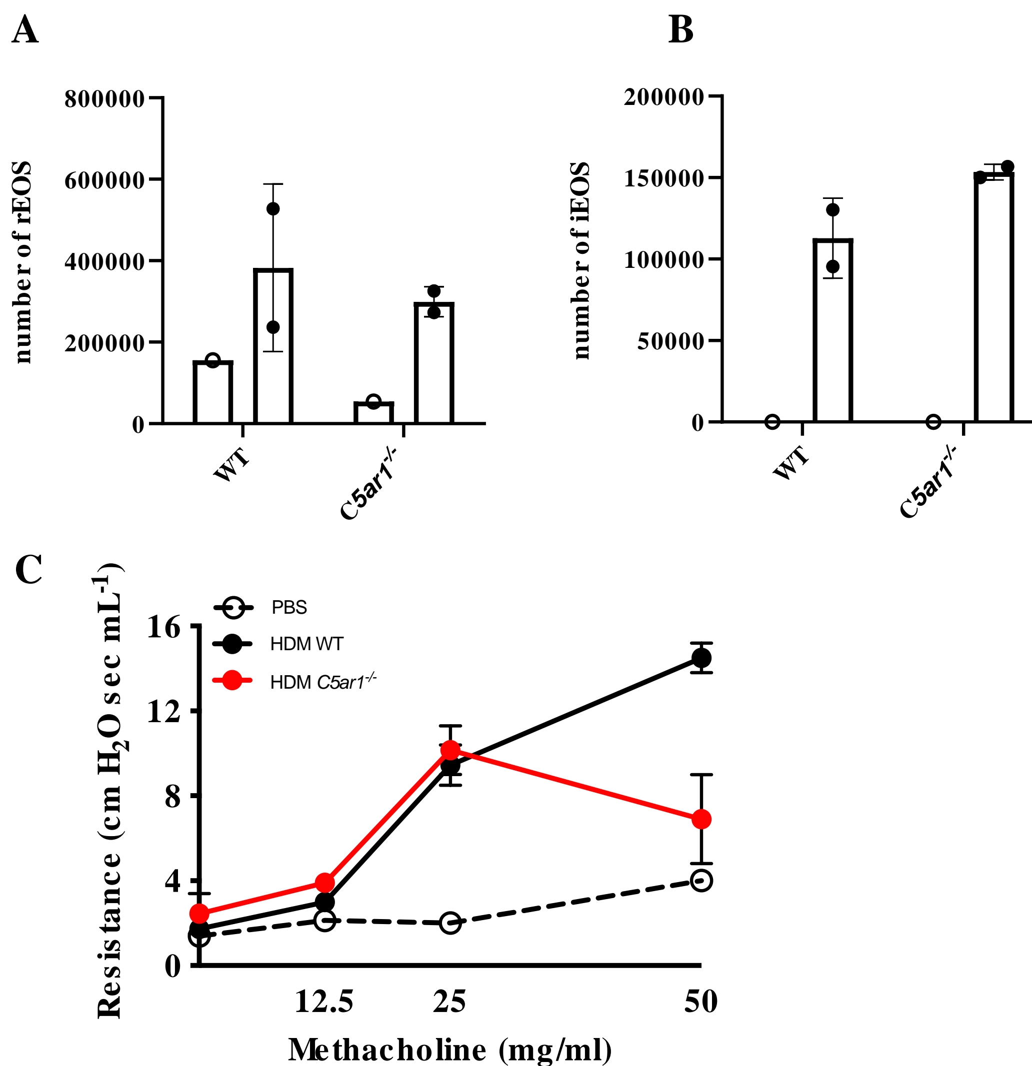


Figure 3. 21 C5a/ C5aR1 signaling axis is important for ILC2 accumulation in the lung. Mouse exposed i.t 2x to HDM or PBS as a control, 24h after from last challenge single staining performed (A) Data shows the number of SiglecF^{+}CD125^{int}CD101^{-}CD11^{-} resident Eosinophils (rEOS). Data are mean values ± SEM, n=2 from one experiment, statistical differences were assessed using Kruskall-Walllis**. (B)** Data shows the number of SiglecF^{+}CD125^{int}CD101^{+}CD11^{+} inflammatory Eosinophils (iEOS). Data are mean values ±

SEM, n=2 from one experiment, statistical differences were assessed using Kruskall-Walllis**.** **(C)** Data shows airway function measurement by forced oscillatory technic upon methacholine. n=1-2.

3.20 C5aR1 might have an impact on the lung development

Altogether my data support the idea that the C5a/C5aR1 signaling axis regulates the development and severity of allergic asthma via it is function in myeloid cells and the subsequent activation of further ILC2. As a consequence, ILC2-released type 2 cytokines including IL-5 promote Eosinophil infiltration to the inflamed lung. Besides such pathophysiologic conditions, Eosinophils together with ILC2s, mast cells, and basophils are infiltrating the perinatal lung during the phase of bulk/primary septation which is critical for alveolarization. This period ranges from the post-natal day (P) 3 to P18 in mice. However, there is almost no information about C5aR1 function during this early lung Eosinophilia. Recently it has been shown by many investigators, that Eosinophilic infiltration reaches its maximum level in mice at day P14. In particular, I observed that inflammatory Eosinophils (iEOS) take place not only in allergen exposure but also in lung development.

Then, using *C5ar1*$^{-/-}$ (KO), *C5ar1*$^{+/-}$ (heterozygote), and Wild type (WT) mice, I evaluated the number of various immune cells which play role in asthma progression including Eosinophils (EOS), alveolar macrophages (AM) and neutrophil. Interestingly, C5aR1 was dispensable for the infiltration of AMs, neutrophils, and EOS (Figure 3.22E-F). In contrast in absence of C5aR1, the recruitment of iEOS increased (Figure 3.22G-H). Although the reason for such increased infiltration remained elusive, these data suggested for the first time that C5aR1 might play role in iEOS infiltration during lung development.

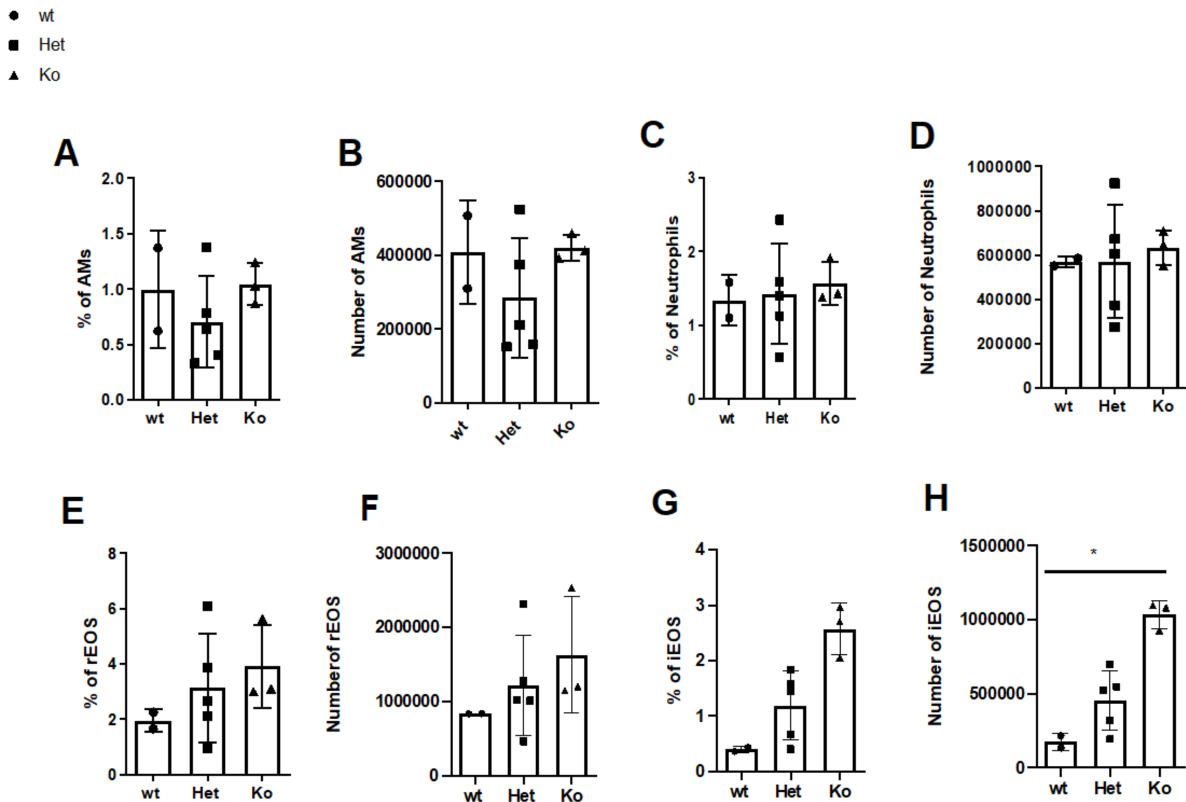


Figure 3. 22 C5aR1 might be important for Eosinophils. (A) Frequency of Alveolar macrophages (AM) in *C5ar1*$^{-/-}$ (KO), *C5ar*$^{+/-}$ (heterozygote), and Wild type (WT) at p14. **(B)** Numbers of AM in *C5ar1*$^{-/-}$, *C5ar*$^{+/-}$, and WT at p14. **(C)** Frequency of neutrophils in *C5ar1*$^{-/-}$, *C5ar*$^{+/-}$, and WT at p14. **(D)** Numbers of neutrophil in *C5ar1*$^{-/-}$, *C5ar1*$^{+/-}$ and WT at p14. (E) Frequency of resident Eosinophil (rEOS) in *C5ar1*$^{-/-}$, *C5ar*$^{+/-}$, and WT at p14. (F) Number of n rEOS in *C5ar1*$^{-/-}$, *C5ar1*$^{+/-}$, and WT at p14. **(H)** Frequency of inflammatory Eosinophil (iEOS) in *C5ar1*$^{-/-}$, *C5ar1*$^{+/-}$, and WT at p14. **(I)** Numbers of iEOS in *C5ar1*$^{-/-}$, *C5ar*$^{+/-}$, and Wild type WT at p14 ± SEM n=2-4 from one experiment, statistical differences were assessed using ANOVA, * $p<0.05$.

3.21 Early life exposure has a nonrecoverable effect on the small intestine

Not only genetic factors, such as *C5ar1* could influence early lung development, as recent evidence suggests the existence of “windows of opportunity” during pregnancy and early in life during which environmental factors. including microbial metabolites originating from the maternal microbiota, have a major role in shaping neonates' immunity to the outer world. Of note, commensals can influence immune cell behavior which causes immune cell populations in the juvenile lung to also influence later life immune characteristics. Thus, the idea that early-life antibiotic (ABX) exposure may be associated with a higher risk of developing asthma later in life has been proposed but remains debatable. To investigate short- and long-term effects of ABX exposure on neonate pulmonary development and functions, mothers were exposed to

three courses of widely used ABX (streptomycin vancomycin and ampicillin and Suc) or sucralose alone (Suc) as a control from 10 days from postnatal day P10 till 20 via drinking water. Firstly, I investigated the effects of early life ABX exposure in the development of the main mucosal surface that could affect by microbial changes, namely the intestine. I measured the length of the intestine from the different days starting from P14-till P56. Interestingly, at P14, after 4 days of ABX exposure and when most immune cells accumulate in the lung [319], I observed a major decrease in the length of the intestine of the offspring, which suggests an ongoing intestinal inflammation (Figure 3.23C). However, the length of intestines from early life ABX or Suc came similar after p18. In addition to the short-term effect, I evaluated how early-life dysbiosis could affect the long-term development of the intestine. Therefore, I collected the small intestines of the different groups of mice at P56 and sent them to the pathology core for cutting and staining with Hematoxylin and EOSin (H&E). The histological score is obtained according to crypt architecture, muscle thickness, lymphocytic infiltration, and goblet cell depletion. Although the length of intestines from the different groups soon after become similar, the histological score shows that early life exposure has a non-recoverable effect on small intestines (Figure 3.23B).

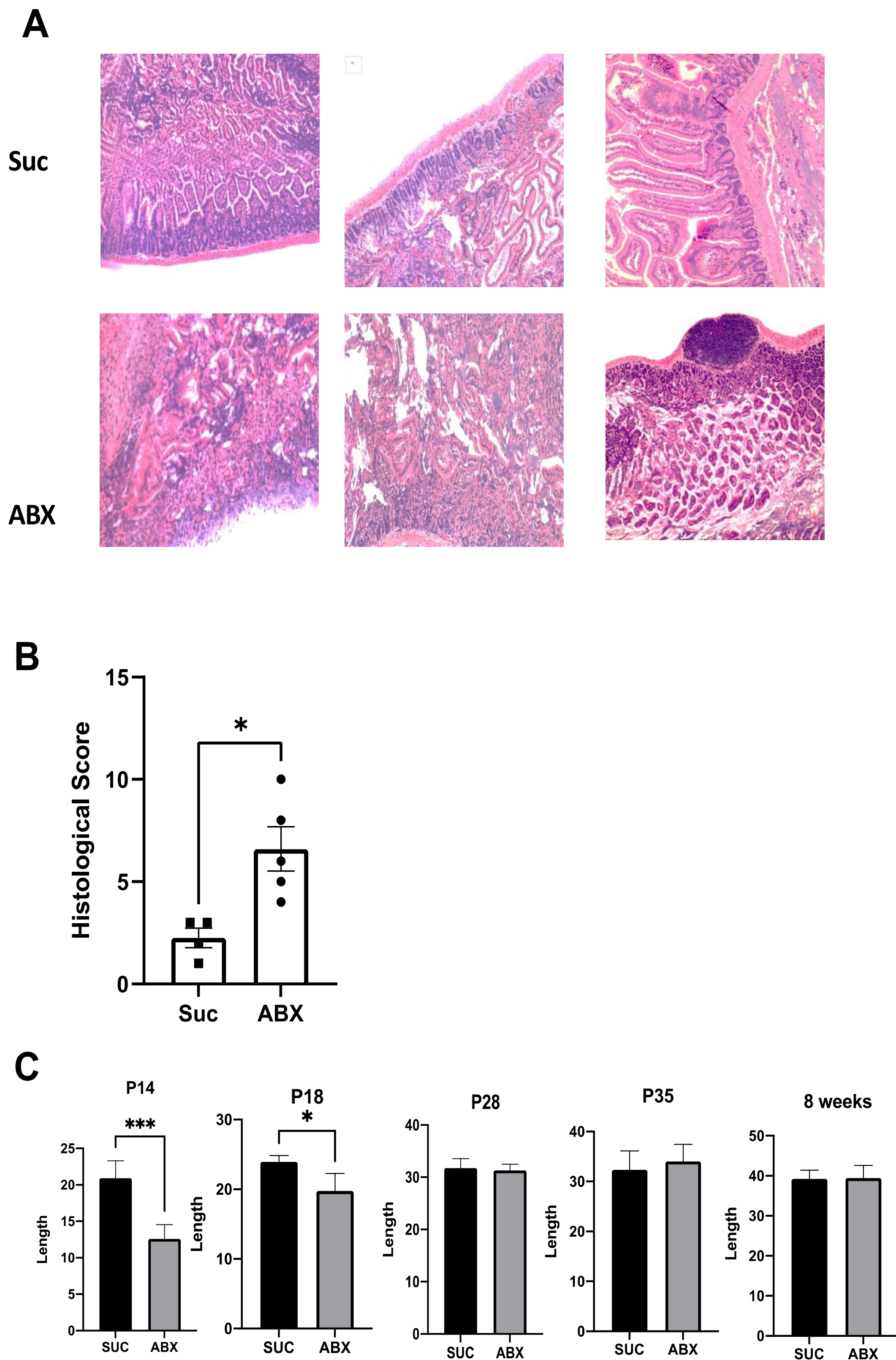


Figure 3. 23 Early life ABX exposure shows a nonrecoverable effect on the small intestine. (A) Intestines from offspring those exposed to Suc or ABX in their early life were fixed, cut, and stained with Hematoxylin and EOSin (H&E). **(B)** Histological scores were estimated with consideration of the following criteria: Crypt architecture, muscle thickness, lymphocytic infiltration, goblet cell depletion at P56 +/- SEM, n=4-5, Statistical differences were assessed by a Student's t-test. * $p<0.05$, ** $p<0.01$, *** $p<0.001$. **(C)**

Length of intestine at postnatal day 14 (P14), P18, P28, P35, P56 +/- SEM, n=16-20, from 3 independent experiment. Statistical differences were assessed by a student ‘s t-test. * $p<0.05$, *** $p<0.001$.

3.22 Early life exposure to ABX changes immune cell composition in the airway

Overall, my data shows that maternal antibiotic exposure during the early life of offspring promotes a higher basal airway hypersensitivity and a pro-inflammatory status, thus increasing the risk of developing severe asthma. I was wondering if early life exposure to ABX, has an impact on the cell composition in the airway. Using t-distributed stochastic neighbor embedding (t-SNE) technique, I visualized high-dimensional data giving each data point a location in a three-dimensional map in the bronchoalveolar fluid of offspring (at P14) of mothers exposed to sucralose or ABX. Data clearly shows that early-life dysbiosis, affect the immune cells in BALF, as well as different immune cell marker expression in particular Siglec-F, CD64, and MHC-II, which are seemingly most affected by dysbiosis (Figure 3.24). More details can be found in supplementary figure 3.

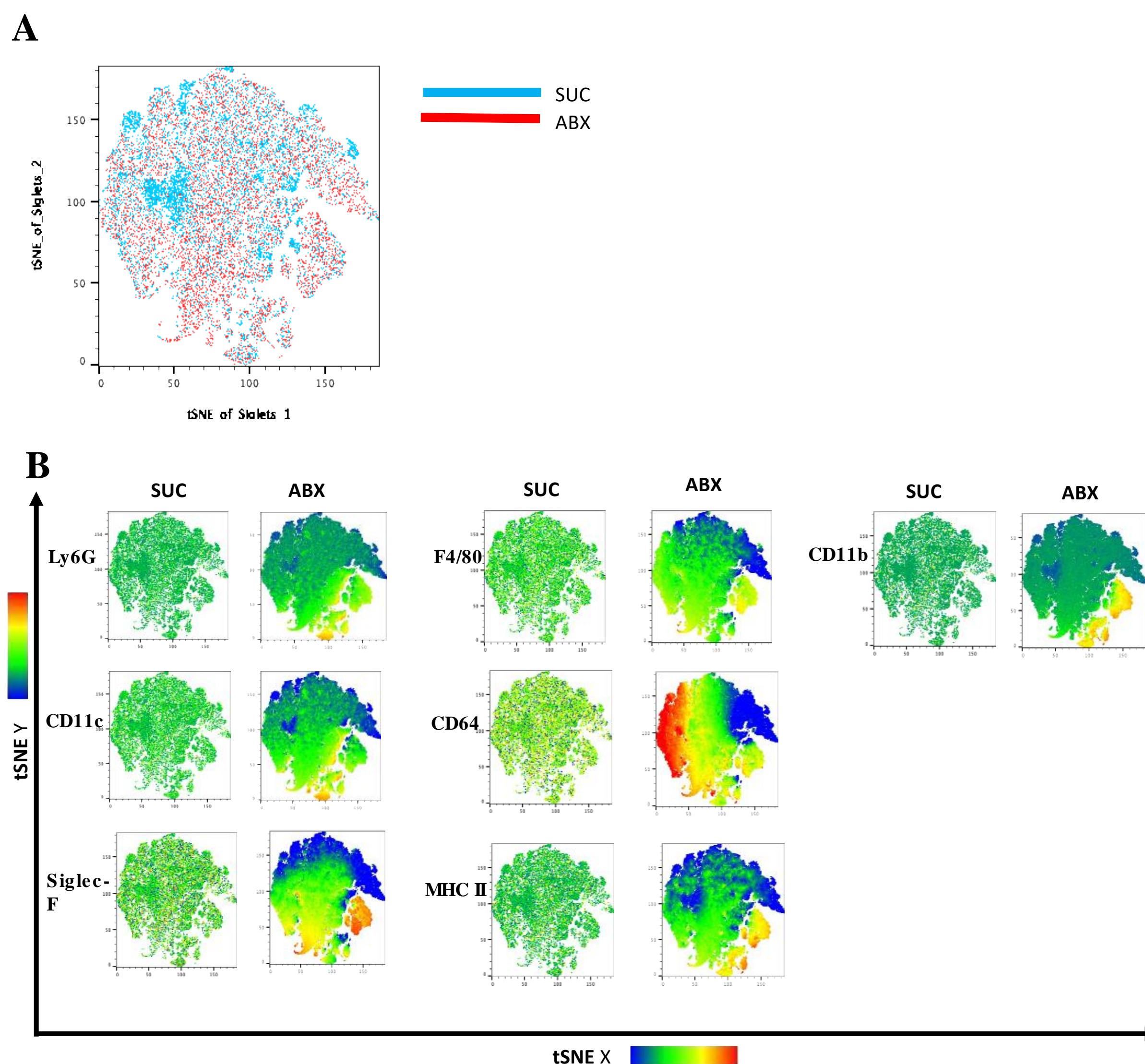


Figure 3. 24 Early life exposure to ABX altered expression of cell surface markers. Data shows tSNE analysis in BALF at P14, a Mean of 5000 cells selected for analysis. **(A)** Main population differences, blue representative sucralose exposed population, red representative ABX exposed mice. **(B)** A heat map is representative marker expression in two different groups, blue representative of the least expression, and red representing the highest expression n=8.

3.23 Early-life dysbiosis has a strong impact on lung inflammation and may associate with the accumulation of inflammatory Eosinophils

For long Eosinophils were considered a homogenous population, however, recently, the existence of other Eosinophil populations has been reported as so-called inflammatory Eosinophils. Only during the allergen exposure, inflammatory Eosinophils (iEOS) are seen in the BALF, and lung [129], [128], and Wiese, Duhn, and Korkmaz et at., in revision. These iEOS can be distinguished from regulatory Eosinophils by their surface expression of CD11c

and CD101. Interestingly, recent studies show that iEOS are also recruited during lung development. Taking into account the impact of ABX in the development of the intestine, I wanted to assess the impact of ABX on early lung cell recruitment. Then, mothers were exposed the ABX (streptomycin vancomycin and ampicillin and Suc) or sucralose alone (Suc) as control from 10 days from postnatal day P10-20 via drinking water, and the cellular composition of the lung was determined at different time points by flow cytometry. Interestingly, and in contrast to rEOS (Figure 3.25A), I observed a significant increase in iEOS recruitment at P14 upon ABX compared to sucralose (Figure 3.25B). Afterward, the number of iEOS gradually decreased after a certain time they disappeared with the same dynamic in ABX and sucralose-exposed groups (Figure 3.25C).

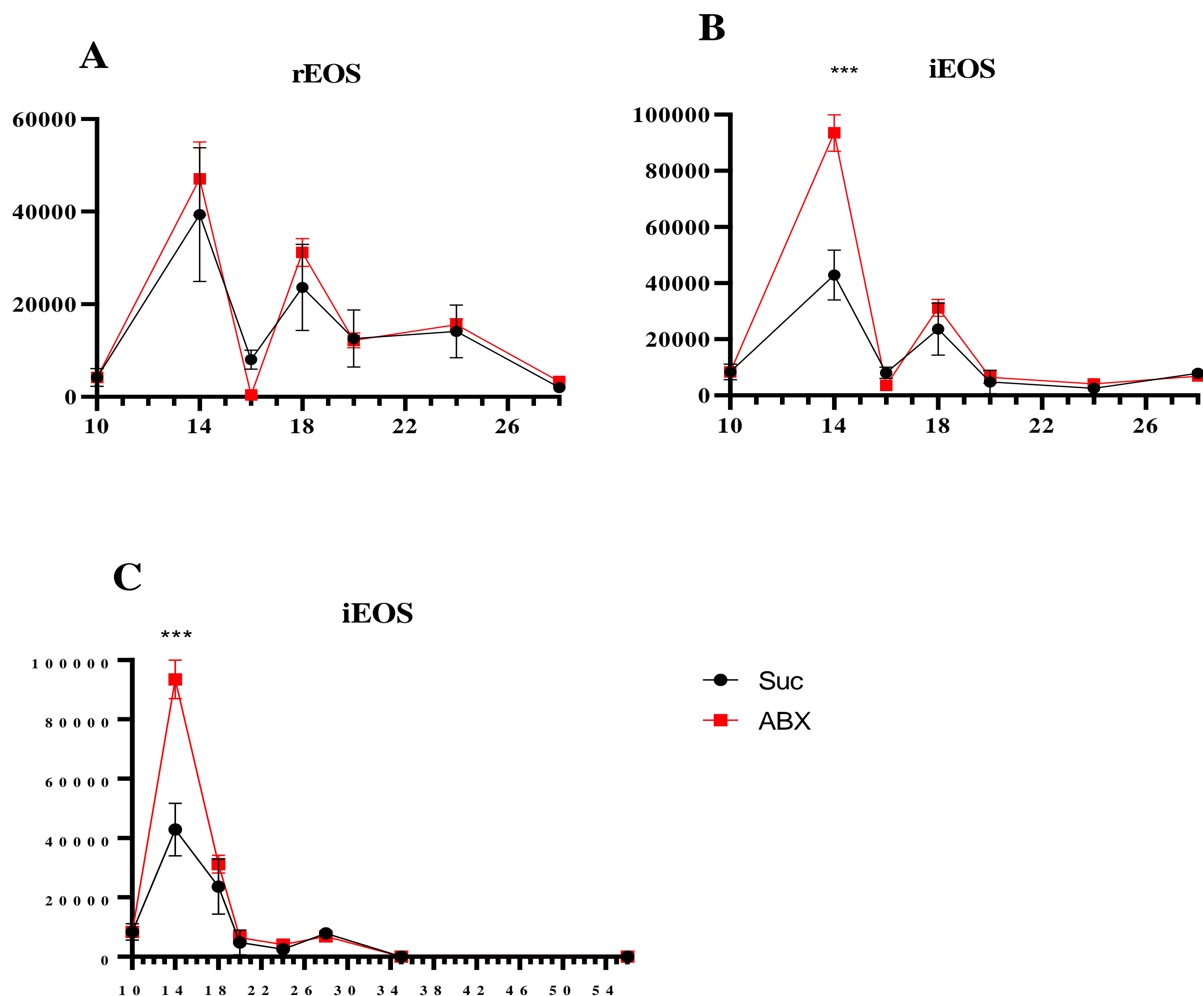


Figure 3. 25 Waves of the Eosinophils are transiently infiltrating the perinatal lung. Single-cell isolation was performed at post-natal day (P)-10, 14, 16, 18, 20, 22, 24, and 28 **(A)** Data shows the number of SiglecF$^+$, CD125^{int}, CD101$^-$, CD11$^-$ resident Eosinophils (rEOS) +/- SEM, for each day n=10-30 animals from 1-3 independent experiments**. (B)** Data shows the number of SiglecF$^+$ CD125^{int}, CD101$^+$, CD11$^+$, and inflammatory Eosinophils (iEOS). They peaked in developing lungs at P14 upon ABX. **(C)** Numbers of

inflammatory iEOS in the lung including +/- SEM, for each day, n=10-30 animals from 1-3 independent experiments. Statistical differences were assessed by two-way ANOVA *** p<0.001

Then, I examined if the accumulation of Eosinophils at P14 causes a structural change in the lung. Therefore, I performed the histological examination in offspring after PAS (Figure 3.25.1A) and H&E staining (Figure 3.25.1B). Analysis showed that at P14, mucus secretion, and alveolar damage were significantly higher in neonates from ABX-exposed mothers compared to sucralose controls.

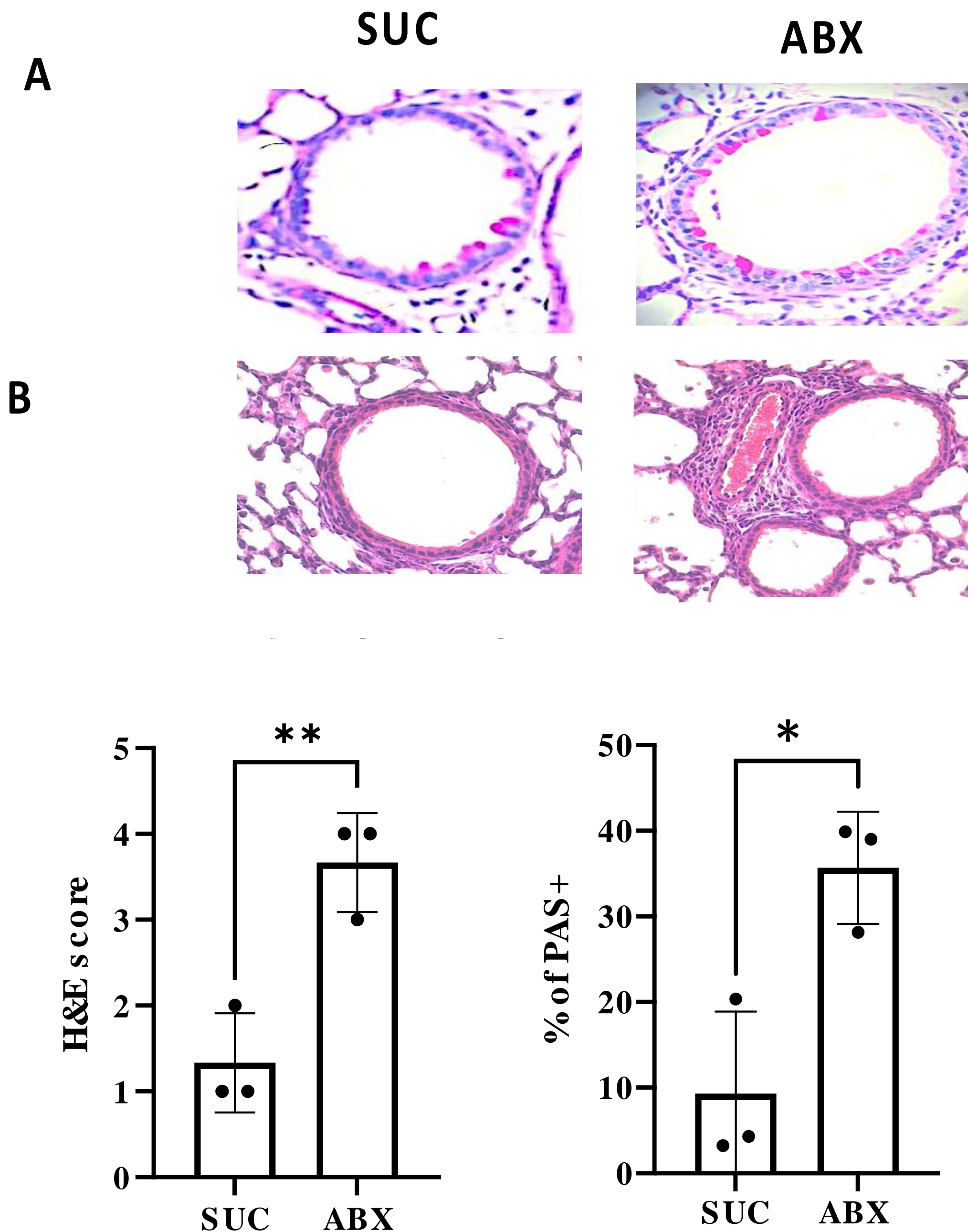


Figure 3. 25. 1 Mucus production and pulmonary cellular infiltration are enhanced upon ABX exposure at P14. Inflation fixation was performed, and slides were stained with **(A)** Periodic Acid-Schiff

Stain or **(B)** Hematoxylin and EOSin (H&E). +/- SEM, n=4-5 from 2 independent experiments, statistical differences were assessed by a Student‘s t-test. * p<0.05, ** p<0.01.

3.24 Eosinophilic subpopulations recruitment depends on IL-5

Additionally, I was wondering, whether the infiltration of Eosinophils upon ABX, in particular iEOS at P14, is regulated by IL-5 as in allergic inflammation. To clarify this, I inhibited the IL-5 secretion in of spring via neutralization of IL-5, using intraperitoneally (i.e.) injection of anti-IL-5 antibody or PBS as a control. Inhibition of IL-5 reduced the number of rEOS in comparison to PBS in the sucralose and ABX groups group (Figure 3.26A). In addition to rEOS, iEOS number reduced in absence of IL-5 (Figure 3.26B) in both early life ABX exposed mice and the control group. Furthermore, although data did reach the statistic seemingly IL-5 has an impact on dysbiosis-driven iEOS accumulation (Figure 3.26B).

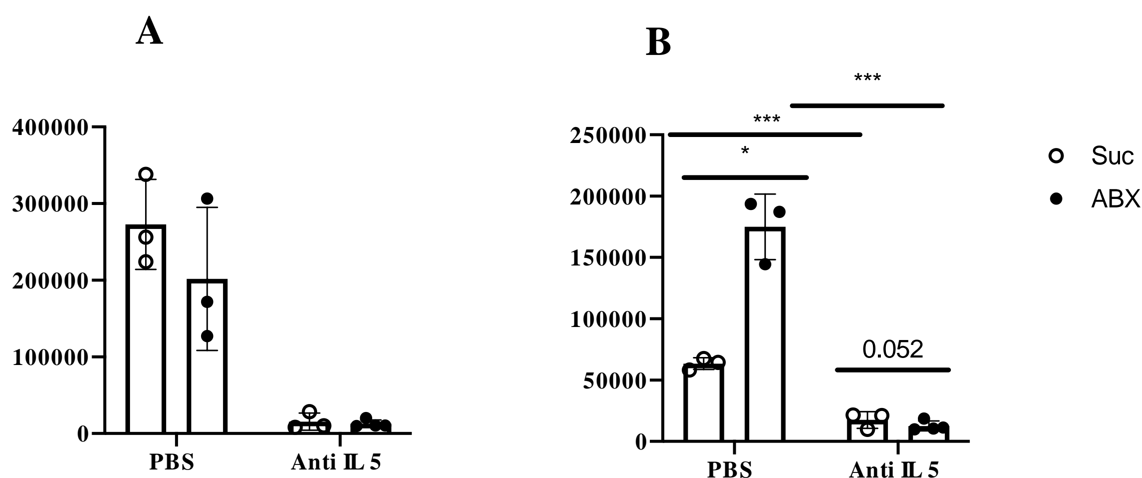


Figure 3. 26 Eosinophilic subpopulation's pulmonary recruitment depends on IL-5. (A) Numbers of the rEOS, in mice receiving PBS or anti-IL-5 (8 µg/ml). Lung cells were isolated and anti-IL-5 treatment vanished the number of rEOS in both ABX and Suc-exposed neonates. **(B)** Numbers of the iEOS. +/- SEM, n=3-4 Statistical differences were assessed by ANOVA, * p<0.05, *** p<0.001.

Eosinophils are usually stored in compartments where they could be mobilized upon IL-5 activation from bone marrow. I evaluated the impact of the inhibition of IL-5 on EOS in the bone marrow at P14. Interestingly, the BM, in the control group showed a high frequency of iEOS, while the rEOS are less frequent (Figure 3.26.1A-B). Upon ABX exposure, the rEOS frequency increased. Then upon IL-5 neutralization, while the percentage of iEOS does not change Suc-exposed the high ABX-triggered frequency of rEOS decreased significantly in the bone marrow (Figure 3.26.1A).

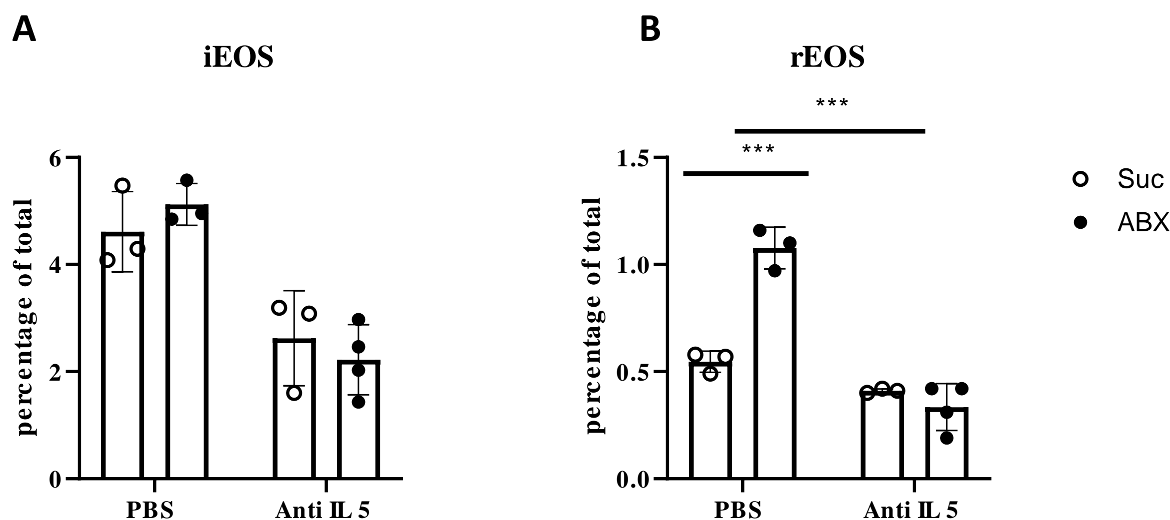


Figure 3.26. 1 BM-derived Eosinophile depends on IL-5. (A) Percentage of the rEOS, in mice receiving PBS or anti-IL-5 (8 µg/ml). BM cells were isolated and anti-IL-5 treatment vanished the number of rEOS in both ABX and Suc-exposed neonates. **(B)** Percentage of the iEOS. +/- SEM, n=3-4 Statistical differences were assessed by ANOVA, * p<0.001** p<0.01

3.25 ILC2s are not only potent source for IL-5 during pulmonary development

As mentioned, ILC2s play a critical role in allergic diseases via their ability to orchestrate cell mobilization through type 2 cytokines. In particular, ILC2-derived IL-5 regulates the infiltration of iEOS to inflamed tissue. By similarity, I was wondering if ILC2-derived IL-5 would also regulate the iEOS recruitment to the lung upon dysbiosis. Firstly, I evaluated the accumulation of ILC2s in the lungs between P10 till P21 by flow cytometry. While ILC2 reached the first peak at P14, they reached their maximum at P24 (Figure 3.27A), However, my data showed that early-life dysbiosis had no impact on ILC2 accumulation in the lung (Figure 3.27A). Furthermore, I evaluated the amount of intracellular IL-5 in ILC2 in control and ABX-exposed groups and showed that IL-5 expression increased in ILC2 with the same dynamic, at P14 and P24 (Figure 3.27A). However, dysbiosis showed no effect on IL-5 production (Figure 3.27B). Altogether, these data suggest that the peak of accumulation of iEOS is uncoupled from the ILC2 presence or functions

As other sources of IL-5 encompass Th2 cells. that reached the maximum number during adulthood in the lung, I nonetheless evaluated IL-5 secretion from non-ILC2 cells, including

Th2 cells, identified as Lin$^+$ cells. At P14, dysbiosis failed at increasing the IL-5 secretion compared to sucralose control (Figure 3.27C).

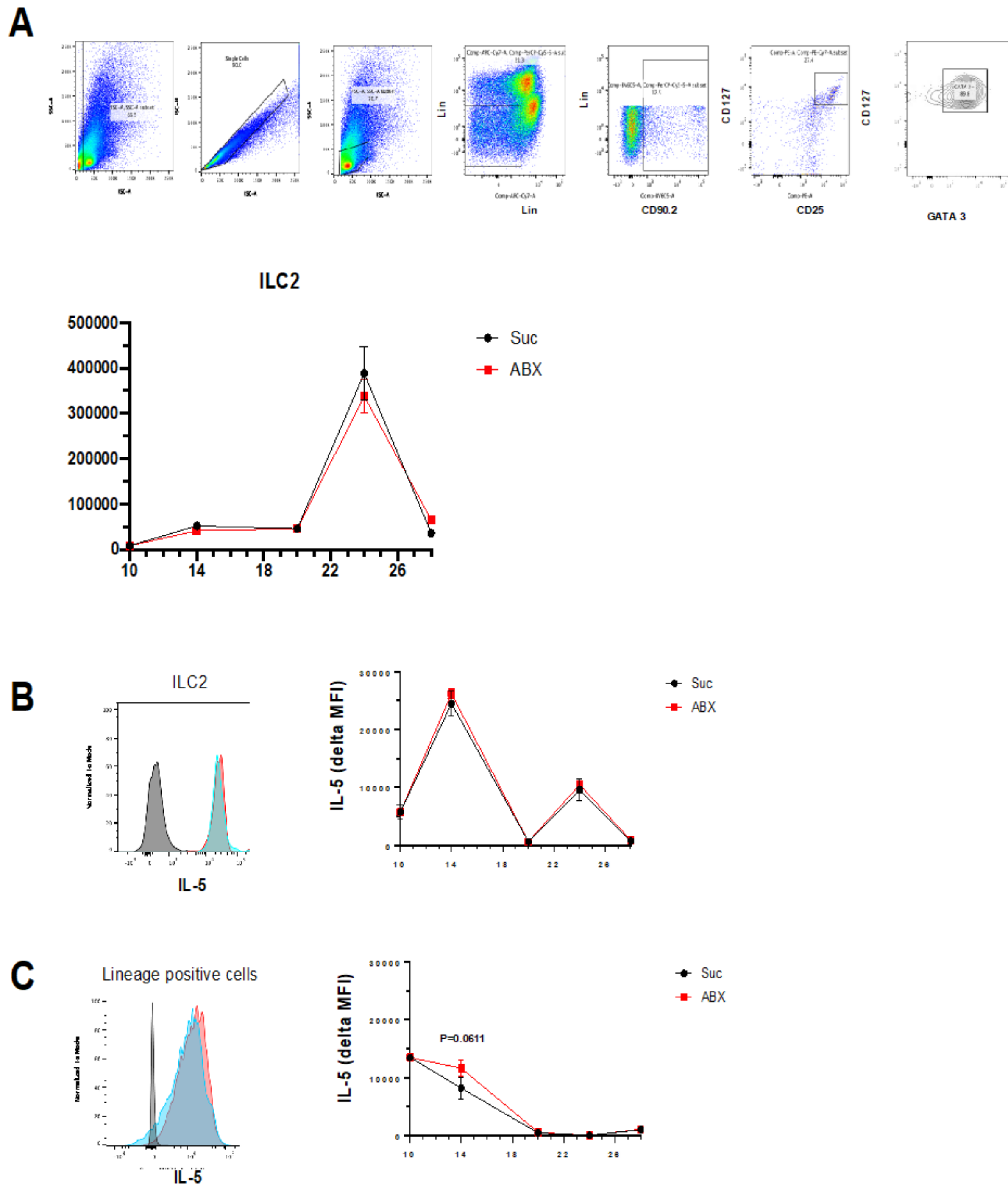


Figure 3. 27 ILC2 is only not a potent source for IL-5 during pulmonary development. Single-cell isolation was performed on post-natal days (P) 10, 14, 20, 24, and 28. **(A)** Graph representative of ILC2 gating strategy, Lin negative, CD90.2$^+$, CD127$^+$, CD25$^{+,}$ and GATA3 data shows numbers of the ILC2 in the lung. **(B)** Histograms are representative data showing the mean fluorescence (MFI) normalized to the FMO control grey, early life exposure to ABX and Suc highlighted in rosa, green, and respectively. The

intracellularly stain IL-5 mean fluorescence in ILC2 cells Lin^-, $CD90.2^+$, $CD127^+$, CD25+, $GATA3^+$. **(C)** Histograms are representative data showing the mean fluorescence (MFI) normalized to the FMO control grey, early life exposure to ABX and Suc highlighted in rosa, green, and respectively. The intracellular staining of IL-5 mean fluorescence intensity in non-ILC2 cell Lin^+ intracellularly stain IL-5 mean fluorescence in Lin^+. +/- SEM, n=10-30 from 1-3 independent experiments. Statistical differences were assessed by ANOVA.

3.26 Early-life dysbiosis has an impact neither on the ILC2 progenitors nor ILC2s in the bone marrow and spleen

To exclude that ILC2s are not involved in lung development upon dysbiosis at another level, I evaluated if early-life ABX exposure would alter ILC2 progenitors. Since it has recently been shown that ILC2s derive not only from bone marrow progenitors but also from spleen [165, 320], I evaluated ILC2 and ILC2p numbers in these two compartments. Interestingly, early life exposure to ABX, does not change the bone marrow-derived ILC2s and their progenitors ILC2Ps (Figure 3.28A-B). Although in comparison to lung and bone marrow-derived ILC2 and ILC2Ps, numbers of splenic ILC2s and ILC2Ps were lower (Figure 3.28E), I was still able to identify ILC2s and ILC2Ps in the postnatal spleen (3.28C-D). In addition, similar to bone marrow, splenic ILC2 or ILC2Ps do not seem to be affected by early-life dysbiosis.

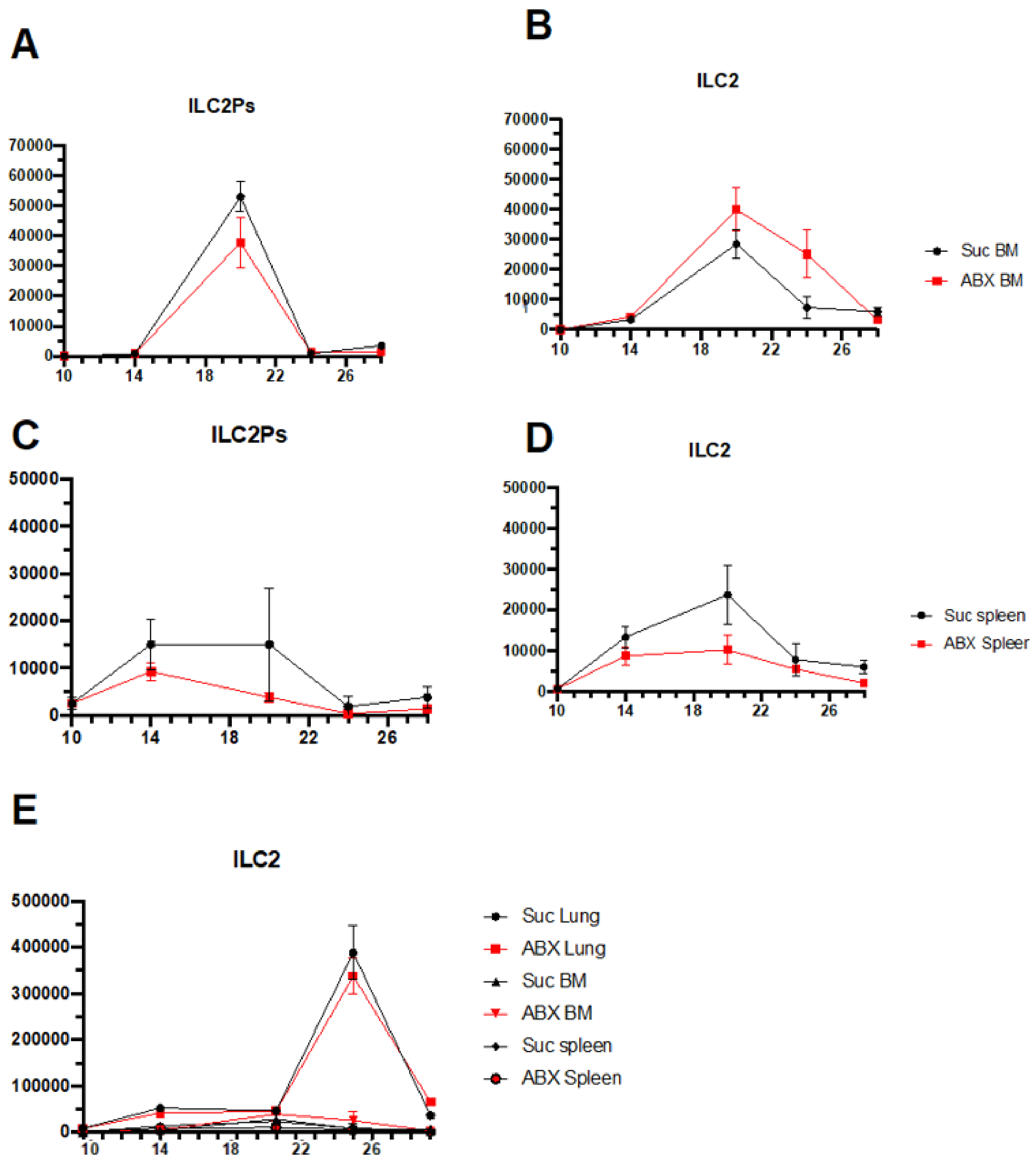


Figure 3. 28 Early life dysbiosis has no impact on splenic and BM-derived ILC2s and ILC2Ps. Single-cell isolation was performed on the post-natal day (P) 10,14, 20,24, and 28, ILC2s are identified with marker expression as Lin^-, $CD127^+$, $LPAM^+$, $CD135^-$, $KLRG\text{-}1^+$, and CD25+. ILC2Ps are identified with marker expressions as Lin^-, $CD127^+$, $LPAM^+$, $CD135^-$, $KLRG\text{-}1^-$, and $CD25^+$. (A) Number of ILC2Ps in the bone marrow. (B) Number of ILC2s in the bone marrow. (C) Number of ILC2Ps in spleen. (D) Number of ILC2s in spleen. (E) Numbers of ILC2Ps and ILC2s in spleen, lung, and bone marrow. $^+$. +/- SEM, n=10-30 from 1-3 independent experiments. Statistical differences were assessed by ANOVA, ** p<0.01, *** p<0.001.

3.27 Gut dysbiosis alters neutrophils accumulation in the lung

Finally, next to the EOS and ILC2, the alveolar macrophages are prominent cells that have been implicated in the development and progression of asthma. Furthermore, the presence of neutrophils has been associated with a severe form of asthma. Neutrophil accumulation in the lung results in airway colonization, bronchoconstriction, incomplete airflow obstruction, and AHR [321]. I was wondering about early life dysbiosis effect on the AM or Neutrophil accumulation. Intriguingly, early-life dysbiosis caused a delayed pulmonary accumulation of AMs (Figure 3.29A), with a peak at P24, compared to sucralose controls which peaked at P14 (Figure 3.29A). Similarly, while the number of neutrophils, that peaked at P14 in controls, was lower upon ABX compared to Suc (Figure 3.29B), they reached a higher and more significant number, in the long run, at P56, in the ABX exposed group (Figure 3.29C).

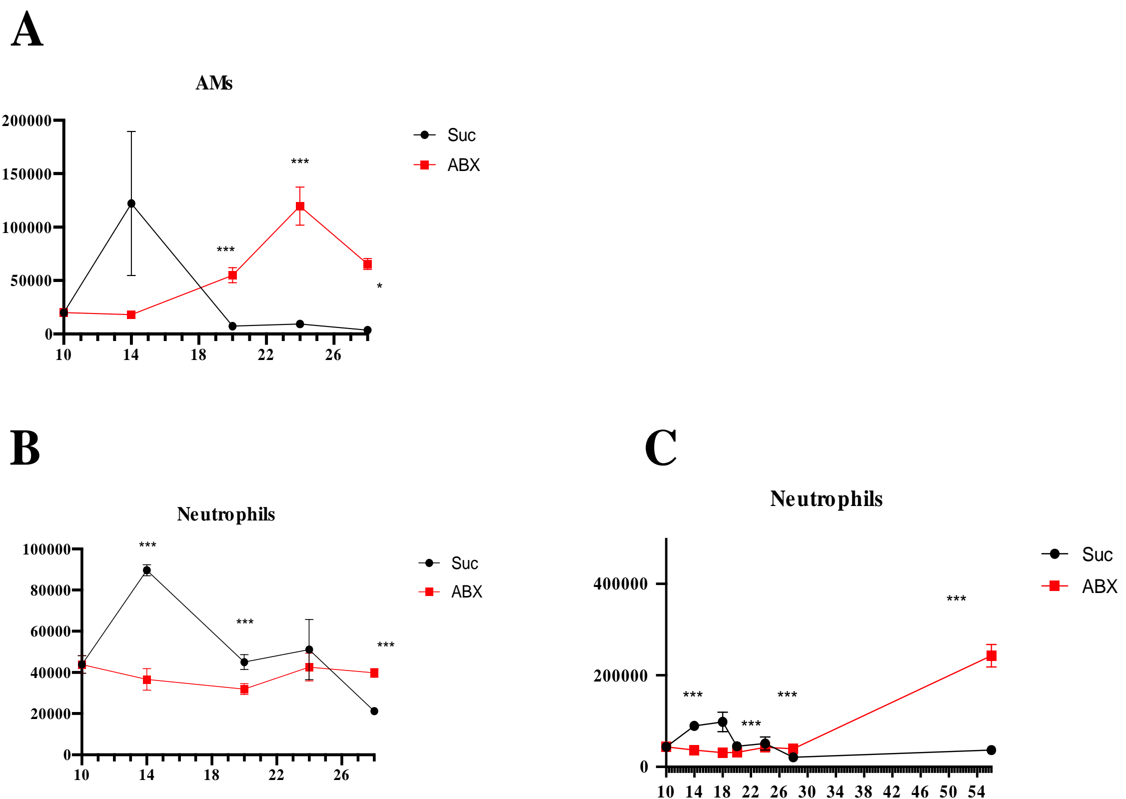


Figure 3. 29 Early life dysbiosis alters the accumulation of AM and Neutrophils in the lung (A) Neutrophil, Siglec-F$^-$, CD125$^+$, numbers at P10, P14, P20, P24, 28. **(B)** Neutrophil, Siglec-F$^-$, CD125$^+$, numbers at P10, P14, P18, P20, P24, P28, P56. **(C)** Alveolar macrophages, Siglec-F$^+$CD125^{int}, CD101^{high}

numbers at P10, P14, P20, P24, P28. +/- SEM, n=10-30 from 1-3 independent experiments. Statistical differences were assessed by ANOVA, ** p<0.01, *** p<0.001.

3.28 Gut dysbiosis alters effector T cells in the spleen

Finally, since T cells are important the airway inflammation and play a crucial role in asthma worsening, I wondered, whether dysbiosis also has an impact systemically on T cell populations. To clarify this, I examined the distribution of effector and memory T cells in the spleen by flow cytometry in ABX and sucralose groups. Although, there were no changes in central memory T cells (CMt) (CD3+, CD4+ CD62L+, CD44+) (Figure 3.30). Interestingly, the frequency of CD44+CD62L- (EMt) cell also known as effector memory T cells in young mice (P28) decreased, suggesting our model is not only affect the lung but also cause systemic dysfunction.

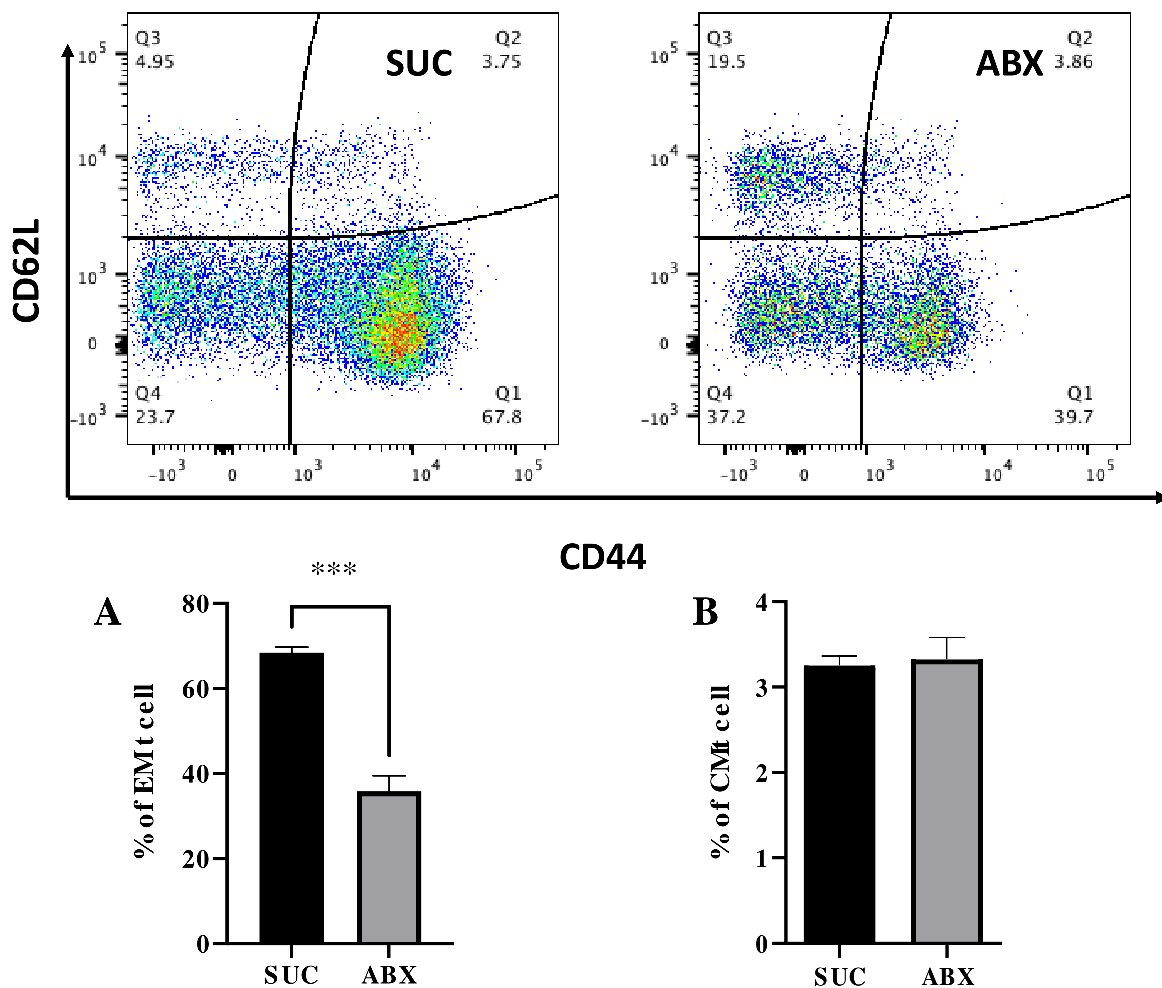


Figure 3. 30 Early life dysbiosis alters effector T cells. (A) Percentage of the effector-memory T cells (EMt) in CD3+, CD4+ cells. The Effector memory T (cells were identified as CD3^{+}, CD4+, CD62L^{-}, and

CD44⁺, and **(B)** Percentage of the central-memory T cells (CMt) in CD3+, CD4+ cells. Central memory T cells (CMt) were identified as (CD3+, CD4+ CD62L+, and CD44+) in 4 weeks (P28) old mice that were exposed to ABX from P10 to P20. The figure is representative of the gating strategy in ABS vs Suc when CD3 CD4 double-positive cells are pre-gated. SEM, n=6 from 3 independent experiments. Statistical differences were assessed by student unpaired T test *** $p<0.001$.

3.28 Dysbiosis exacerbates baseline airway function

While my data showed that early-life dysbiosis affects the immune cell composition and lung function which could be associated with allergic asthma, I was wondering if early-life dysbiosis could change the baseline of airway function in long run. Airway function measurement by forced oscillatory technic upon dose-response of methacholine trigger showed, in the ABX exposed group, increased sensitivity in the total (Figure A) and small (Figure B) airways compared to the Sucralose controls. In contrast, no difference could be measured in large (Figure C) airways, small airway provides more resistance than large airways.

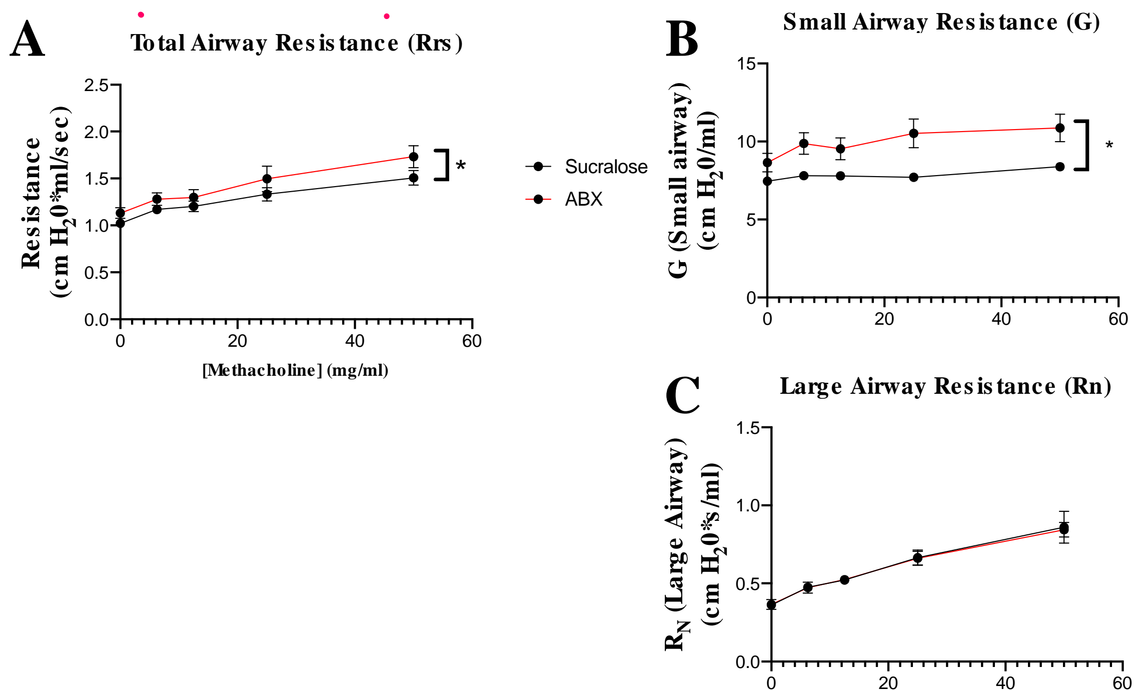


Figure 3. 31 Dysbiosis exacerbates baseline airway function. Data shows airway function measurement by forced oscillatory technic upon methacholine at P28. **(A)** Data show total airway resistance to methacholine. **(B)** Data shows small airway resistance to methacholine. **(C)** Data shows a large airway resistance measurement to methacholine. +/- SEM, n=8, Statistical differences were assessed by ANOVA, * $p<0.5$.

4 Discussion

Asthma is a common chronic disorder of the airway, which prevalence and severity increased dramatically in recent decades [254, 255, 283]. Next to a non-atopic form of asthma, the allergic form of the disease exhibits cardinal features such as airway remodeling, excessive mucus production, smooth muscle cell construction, airway hyperresponsiveness to a variety of specific and non-specific stimuli, chest tightness, pulmonary Eosinophilia, and elevated concentration of serum immunoglobulin E (IgE). Although extensively studied in the last decades, all the mechanisms leading to pulmonary allergic asthma have not been solved yet. The main form of allergic asthma is characterized by strong Eosinophilia, which during sensitization and established asthma is driven by IL-5 secretion by Type 2 inflammatory cells. While Th2 cells are involved in type 2 immune response during the course of the established disease, Innate lymphoid cell type 2 (ILC2s) play a key role in the establishment of the disease. ILC2s are one of the most critical players in the regulation of asthma via type 2 cytokines during sensitization [176]. In addition to ILC2s, various studies have reported the regulatory function of the complement system, in particular the anaphylatoxins (AT) C3a and C5a and their cognate receptors (ATRs) C3aR, C5aR1, and C5aR2, in asthma development and severity [232]. However, while most of the studies regarding the ATRs in allergic asthma focused on myeloid cells and their interactions with T cells, few took into consideration other immune cells, such as ILCs. Recently, C3aR has been shown to play a role in ILC2 functions during allergic asthma [305]. In contrast, the functions of C5aR1, the main receptor for C5a which has been well appreciated as an important regulator of asthma via functions in dendritic cells haven't been explored.

In this study, I investigated the role of C5aR1 in ILC2 cells using C5aR1 reporter knock-in mouse, *C5ar1*$^{-/-}$, C5 deficient, and LysM-cre C5ar1$^{-/-}$ mouse strain. In addition, I aimed to study new insight into molecular mechanisms in ILC2-myeloid cell crosstalk in the pulmonary context. Last but not least, the data in this study provide various important insight into how early-life dysbiosis shape the neonate´s immunity to outer work.

4.1 Expression of the C5aR1 in lymphoid cells in general and ILCs in particular

The complement system is a crucial mechanism of immune defense, and many immune cells can bind complement cascade side products and initiate cellular mechanisms [322]. While at the cellular level, C5aR1 is mainly expressed by myeloid cells [322], the role it plays in direct activation of the cell of lymphoid origin, in particular B and T cells [242, 323{Dunkelberger, 2012 #342]] remains controversial.

Although the expression and role of the C5aR1 on T cells have been a major concern in understanding the role of ATR in the development of allergic asthma, its function if the Th2-mirror cell of the innate compartment, the innate lymphoid cells [324], has not been fully explored. Interestingly, my data showed that, in the absence of C5aR1, HDM-triggered IL-5 secretion by ILC2 decrease during sensitization. Such defect in IL-5 translated into reduced recruitment of inflammatory Eosinophils, supporting the idea that C5aR1 is critical for ILC2 functions. Although my data suggested such an important role of C5aR1 in ILC2, I could not detect C5aR1 in pulmonary ILC2. ILC2s arise from its progenitor, ILC2P, in the bone marrow [147]. Similar to ILC2, I could not detect C5aR1 expression in ILC2Ps.

The putative role and expression of C5aR1 in ILCs is still a matter of debate. Qing et al. show that NK cells, a form of Group 1 innate lymphoid cells, are regulated by complement receptors via conventional DCs [323]. Furthermore, C5aR expression was reported in NK and NKT cells upon *E.coli-induced* sepsis, and C5a/C5aR signaling regulates the function of NK and NKT cells [325]. In addition to its function in Th1 cell responses, C5a has been shown to have a role in Th2-mediated diseases such as allergic asthma [326]. In contrast, ILC2s, which are innate counterparts of Th2 cells, rely on GATA binding protein 3 for their development and function [142]. However, ILC2s lack antigen-specific receptors but are still able to respond to TSLP, IL33, and IL-25 [180]. Innate lymphoid cells play a critical role in immune regulation and tolerance. The AT receptor C3aR expression on the ILC2s is controversial [305, 327], as the latter study, using a floxed tdTomato-C3aR reported knock-in mouse showed no C3aR expression on ILC1, ILC2, and ILC3 in the lung but in intestinal ILC3 [327]. Recently, it has been shown that in HDM-exposed mice a significant level of C3a is present in the BAL fluid [305]. In addition, in absence of the C3aR, AHR to methacholine was lower in comparison to WT and along with the number of Eosinophils and IL-5 and IL-13 expression at mRNA level which suggests that C3a/ C3aR signaling is required for the development of allergen-induced type 2 responses in the lung [305]. The number of revealing publications about C5aR1

expression on ILC2s is limited, and there is no clear evidence about the expression of C5aR1 by ILC2. Although Gour et al. reported expression of C5aR1, a closer look at the data shows that they observed C5aR1 expression, at a level 1,5 times greater than in T effector cell in a microarray technique in gut lamina propria [305]. In contrast, a recent study revealed that in an OVA-driven allergic asthma model, not only the expression of C5aR1 in pulmonary ILC2 could not be confirmed in a floxed green fluorescent protein (GFP)-C5aR1 knock-in mouse either at steady state or upon allergic inflammation [247]. In line, the inhibition of complement factor 5 reduces the type 2 responses without affecting the group of ILC2 in an effector phase of HDM-induced asthma, studies using GFP-C5aR1 reporter mice, as an alternative technique to antibody staining, failed to detect C5aR1 expression in lymphoid cells [242, 251]. Interestingly, no C5aR1 signaling was observed with using reporter mice on the resting or activated T cells [233, 242, 247, 251]. In contrast, my data confirm that C5a is important in lymphoid cell function. Indeed, besides my observation of IL-5 production in ILC2, C5aR1 plays a role in T cell trafficking and responses [251, 328, 329]. More specifically, various pieces of evidence show that complement molecules can be activated intracellularly and play a role in $CD4^+$ T cell survival, proliferation, differentiation into Th1, Th2, and Th17 effector, and function of the T cells [203, 205, 329-331]. A study dealing with *Pseudomonas aeruginosa* infection exerts that, in the absence of the C5aR has reduced the ability to clear the infection [332]. Another study shows that blocking the C5a receptor with a C5aR peptide antagonist inhibits the flu-specific $CD8^+$ T cells. [332]. In addition, the C5a/C5aR1 signaling axis regulates the TLR-4 mediated response through activation of PI3K and ERK1/2 [330].

4.2 C5a/C5aR1 signaling in ILC2 in Allergic Asthma

Although asthma is a common chronic inflammatory characterized by Th2-mediated immune responses, compiling evidence shows that anaphylatoxins play a critical role in regulating asthma diseases and severity [285, 305, 316, 329, 331, 333].

My data provide the first insight into the role of C5a/C5aR1 in ILC2 function during the first 24 h after initial allergen exposure, the so-called sensitization phase. I could show that ILC2 produced IL-5 in a C5aR1-dependent manner, which correlates with increased Eosinophilia. IL-5 is a well-known mobilizer of eosinophils and thus is critical for the Eosinophilic response [334]. Interestingly, C5a is chemotactic for immune cells playing a critical role in the recruitment of the granulocytes into the airway [226], is associated with the release of histamine from basophil and mast cells and secretion of Eosinophilic cationic protein [226], and such

mechanisms could be at play here. Intriguingly, a recent study showed that during the effector phase of asthma, C5 is dispensable for the ILC2 function [285]. However, current knowledge in ILC2 biology indicates that ILC2 plays a major role in sensitization rather than the effector phase in which Th2 cells are taken over the functions of ILC2 the in Allergic asthma [176, 183].

Intriguingly, in contrast to my study, Köhl et al. reported that during sensitization to HDM, C5a exerts a protective role in the allergic asthma development [331]. However, in contrast to my study, that study pharmacologically targeted the lung-specific C5aR1, whereas I used constitutively C5aR1-deleted mice. Furthermore, Köhl et al. either use OVA or 4 x HDM models of allergy[335], whereas I only used 1x HDM. In 2007, it has been shown that AHR differs within the OVA group and HDM group [336].

It is important to note that I measured AHR 24 h after the second allergen exposure, and we could observe a decrease in the susceptibility to methacholine the absence of the C5aR1. On the other hand, Köhl et al. measured 72 h after the fourth challenge. These findings might indicate that time after the last exposure might play a role in the response [337].

4.3 Cellular network of ILC2

Numerous studies have reviewed that ILC2 responses can be regulated by various factors, e.g. hormones, cytokines, lipid mediators, neuropeptides, lipid mediators, as well as the alarmins derived from epithelial cells [180, 267, 338]. My data showed that a direct action of the C5a anaphylatoxin was not involved in the development of ILC2, in contrast to the C3a axis shown to be associated with the functions and development of ILC2 via the expression of C3aR upon allergen-driven inflammation [305]. My data and older report from our group showed that C5aR1 was absent of both steady states or activated ILC2 independent of the model used [247]. Rather, I observed an unsuspected role of C5aR1 in macrophages and DCs in vitro using BM-derived and primary pulmonary cells, beyond its classical function in the DC/T cell crosstalk [246] that sustained the crosstalk between myeloid cells and ILC2. I could identify DC-ILC2 and AM-ILC2 contacts, enforced by C5a/C5aR1 axis, via cell-cell contact and soluble factors, respectively.

It is now well accepted that in addition to structural cells, including epithelial cells, and smooth muscle fibroblasts, ILC2s are able to interact with cells belonging to the adaptive and innate immune system by cell-cell contact or communication by soluble factor [318, 339]. ILC2s are tissue residence cells with a limited capacity to recognize microbial signals[340]. In contrast, myeloid cells are highly conserved sensory machinery to recognize danger signals, pathogens,

foreign antigens, and invading microorganisms that act as a superior sensor of the microbial signals to ILC homeostasis and regulate the function [341]. In addition to receiving soluble factors from other cell types, recent studies show that ILC2s can orchestrate the other cell type to maintain cellular responses by releasing type 2 cytokines [342]. It has been shown that activated ILC2-derived IL-13 does not induce the migration of DCs into draining lymphoid but also promotes CCL17 expression which is crucial for controlling type 2 inflammation[343]. Furthermore, to transduce cytokine signaling, ILC2s express many cytokines receptors including IL-2Ra, IL-4Ra, IL-9R, IL-17Rb, IL-R11 and TSLPR, DR3, IL-1R2, and IL-12Rb1[344]. In the presented study, ILC2s are identified with their surface marker, including lineage negative, CD 25(IL-2Ra), and CD127 (IL-7R) double-positive cells.
In line, IL-33, known to be an alarmin of ILC2s, is produced by many immune cells, including macrophages, and dendritic cells might regulate the development of ILC2 [345]. These might explain why BM-M- DC supports the ILC2 development, although cultures of BM-M-DCs pulsed with HDM did not allow the detection of IL-33[202].

4.4 Phosphorylation of STATs on ILC regulation

It is well known that cytokines activating the Janus kinases (JAKs) and members of the signal transducer and activator of transcription (STAT) pathways are involved in the terminal development of the Innate lymphoid cells [346]. Although molecular mechanisms initiated by C5a in BM-M-DC on the ILC2 phosphorylation were very elusive, I showed that it results in phosphorylation of STAT4, but not STAT3, 5, and 6, suggesting that C5a might be involved in STAT phosphorylation of lymphoid cells like T cells and ILC2s. Recently, the role of the STAT phosphorylation on the mDC-ILC2 crosstalk has been shown [304], and a couple of studies indicate that C5a activates the ERK pathway and could also activate STAT pathways [347-350].
Intriguingly, STAT6 has been shown to regulate the IL-4 production from ILC2s upon lung inflammation [351]. In general, and unlike the T cells, ILC2 development is independent of Rag genes, but their development tightly relies on transcription factors, including Eomesodermin (EOMES), T box expressed in T cells (T-BET), GATA-3, and RORγt [352]. Transcription factors are signal-dependent and are able to detect changes in the microenvironment to initiate a host immune response. While STAT 6 is commonly associated with GATA3 expression and group 2 innate lymphoid cell function, STAT4 is associated with Tbet expression and group 1 innate lymphoid cells [353, 354]. Further, It has been shown that

the development of the ILCS and NK cells, while mainly regulated by the JAK-STAT5 signaling axis [152, 355] can also be regulated by STAT4 [356, 357]. Interestingly, phosphorylation of the STAT 4 significantly increased within 24h upon C5a stimulation. It has been shown that C5a generation causes phosphorylation of STAT 4 which is associated with early inflammation in diabetic wounds [358]. Similarly, the role of the complement system in STAT phosphorylation has been reported in the atherosclerosis [210]. Although there is no study dealing with C5a functions on STAT4 phosphorylation in ILC2s, it has been shown that C5a peptides restore STAT4 phosphorylation in $C5^{-/-}$ T cell [204]. Interestingly, unlike the Th1/Th2 paradigm, Yagi et al. show that STAT 4 is important maintenance of the GATA3 expression [157], which might explain why I observed an increase in STAT4 phosphorylation in ILC2 within the first 24 h. Mechanistically STAT is localized in the cytoplasm, it is phosphorylated after cytokines bind to the membrane receptors and only then dimerized while translocating to the nucleus to regulate the gene expression [312]. As it takes much time to initiate the gene expression after STAT4 phosphorylation occurs, it might explain why I observed a maximum level of Tbet expression at 72 hours.

4.5 C5a triggered phosphorylation of STAT4 activated viaIL-23

Mechanistically, C5a-driven stimulation of BM-M-DC participates in the STAT4 phosphorylation in ILC2. However, the absence of C5aR1 in ILC2 suggests an indirect C5a effect. Furthermore, although C5a has already been associated with various cell signaling pathways, such as (PI3K) ([359], p38 mitogen-activated protein kinase (p38MAPK), and extracellular signal-regulated kinase (ERK)[360], there is seldom evidence that links between C5aR1 and STAT signaling [210]. In addition, the kinetic activation I observed, with a maximum of STAT4 phosphorylation reached 24h after stimulation, suggested that C5a may drive the secretion of cytokines involved in STAT4 signaling. Interestingly, I detected an increased concentration of IL-23 in the supernatant of BM-M-DCs stimulated with C5a. This was similar to data showing that C5aR1 signaling participates in the IL-23 production from HDM pulsed BM-M-DCs [306]. IL-23 is heterodimeric cytokines p 40 and p19 that binds IL-23 receptor and IL-12Rβ1 [361], is produced by macrophage and dendritic cells [362], and plays a crucial role in host defense and pathogenesis of autoimmune diseases [363, 364]. Further, IL-23 is known to activate the transcription of STAT4 [285]. IL-23 also plays an important role in allergic asthma through the regulation of TSLP and IL-33 expression by the epithelial cell, which are both involved in the airway hyperresponsiveness [365, 366]. In line,

intranasal application of anti-IL-23 antibody reduced the allergic reaction and significantly decreased the ILC2 number [367].

Nonetheless, the role of C5a on IL-23 expression remains controversial. While the IL-23 production from TLR-4 stimulated macrophages are dose-dependent on C5a via activation of PI3K-Akt and MEK1/2-ERK1/2 pathways [350], and C5aR1 is important for the IL-23 secretion by BM-M-DCs [306], another study shows that C5a negatively regulates TLR-4-induced IL-23 expression [330]. Conversely, this suggests that C5a might either trigger a response differently according to stimulation, as C5a inhibits the IL-23 release upon TLR-4 stimulation, while HDM stimulation triggers its secretion through C5aR1, or that the differences in responses originated from functional discrepancies between BM-M-DCs and other macrophages populations that have been used in various studies. In agreement, such functional variability has been highlighted in the expression pattern of C3aR in various macrophage populations [327]. Therefore, my data suggest that even BM-M-DCs and peritoneal macrophages behave differently in response to C5a. Supporting this, transcriptomic analysis has indeed shown that BM-derived macrophages are different from pulmonary macrophages [368].

Because IL-23 can induce several DNA bindings complexes, including STAT3-STAT4 heterodimer and STAT4-STAT4 homodimer, it is not only involved in Th1 polarization but also plays role in the Th17 expansion [361, 369]. Interestingly, during the first 24 h of co-culture, I observed a high percentage of ILC3 cells, the innate counterpart of Th17 cells. Such dual functions of IL-23 on ILC2s have been identified, and this regulatory function of IL-23 has been identified using AMP-activated protein kinase (AMPK) deficiency in macrophages and cDC in hookworm infection [370]. Further, intranasal anti-IL-23 administration with a low dose of HDM leads to the activation of both ILC2 and ILC3 [371], while anti-IL-23 administration during the sensitization suppresses the ILC2 in cigarette smoke-promoted asthma [367]. In contrast, a study shows that STAT1-deficient mice significantly increase their expression of IL-23, which further promotes the differentiation of the ILC2 to the ILC3 [151]. In line with my finding, an *in vitro* study shows that ILC2s expansion upon IL-23 stimulation requires the upregulation of the transcription factor RORc level without any changes of *gata3* at mRNA level [149].

4.6 Classical ILC plasticity paradigm

Although it seems independent of C5a, my data also showed that BM-M-DC act as a modulator of ILC plasticity, as between 48 and 72h after the start of co-cultures, ILCs switched from an ILC2 to an ILC1 phenotype. It has been recently recognized that ILC2s consist of several subpopulations that undergo certain plasticity depending on their microenvironments [372]. Although ILC2s normally produce type 2 cytokines and amphiregulin, they can be re-programmed into IFN-γ-producing ILC1-like cells under inflammatory conditions [373]. This shift from ILC2 to ILC1 depends on the cytokines produced by macrophages and dendritic cells, including IL-1, IL-12, and IL-27 [374]. Interestingly, Engelke et al. showed that HDM stimulation of BM-M-DC increases the release of IL-1 and IL12p40 [306], which are known to be trans differentiation cytokine of ILCs [372]. Notably, ILC1-like ILC2s are still able to produce IL-13 [375]. Further, in certain conditionsILC2s are also able to produce IL-10[376] and IL-17 [377, 378]. Similarly, in vitro studies show that ILC2s produce IL-10 in the presence of IL-4 and retinoic acid, or IL-17, when stimulated with IL-1β, IL-6, IL-23, and TGF-β, becoming the so called-called ILC3-like ILC2 [141]. In addition, IL-17 production from ILC2 has been reported during *Candida* infection associated with inflammatory ILC2 expressing a high level of KLRG-1 [191].

4.7 C5aR1 signaling axis during ILC2-Macrophage crosstalk

The data in this study provide important insights on ILC2 development, on the one hand, that BM-M-DCs support the terminal development of ILC2s, and on the other hand, that the C5a/C5aR1 signaling axis plays an important role in these mechanisms via the secretion of IL-23 and the phosphorylation of STAT4. I could also extend this observation to pulmonary MHC-II+. Distinguishing pulmonary macrophage subpopulations from DC allowed me to refine the functions of these cells and C5a/C5aR1 in the development of ILC2.

Macrophages are highly conserved sensory machinery that recognize danger signals, pathogens, foreign antigens, and invading microorganisms to protect host immunity [379]. Unlike the macrophages, ILCs are mostly tissue residents with limited capacity to recognize microbial signals for that reason is critical to have a superior signal from macrophages to control ILC function and homeostasis [153]. Interestingly, Macrophage-ILC2 interaction has been shown to be bidirectional, as while IL33-elicited ILC2s promote the M2 macrophages upon infection with *N. brasiliensis* [380], macrophages are also able to secrete IL-33 which is

the main alarmins of the ILC2s and regulate the ILC2 derived IL-13 secretion [381, 382]. In addition, I could show that IL-1α, a major proinflammatory cytokine derived from macrophages inducing the expression of other cells [383, 384], was released by AMs upon C5a. In the context of papain activation, IL-1α regulates the ILC2s [385]. However, while intercellular C5aR1 activation regulates the IL-1β production [386], C5aR1 function on the IL-1α signaling is unknown. However, since inflammasome activation also leads to the release of IL-1a[387], C5a/C5aR1 may also participate in such events. Interestingly, it has been recently shown that stimulator of IFN gene (STING)-mediated type I interferon response in alveolar macrophages inhibits the ILC2-driven type 2 inflammation upon allergic asthma [388]. Further, in PPARγ deficient macrophages, inflammasome-activated IL-1α expression, and secretion are dampened by IFN-α [389]. Interestingly, C5a suppresses the STING activation [390], hence may favor the development and function of ILC2, a function that will be lost upon allergic inflammatory conditions, as C5aR1 is downregulated in macrophages upon allergic inflammation [247]

Interestingly, macrophages are the earliest immune cells arising in the fetal lung, where they appear at E10 days of pregnancy and expand during the postnatal development of the lung, increasing during the alveolarization [391]. Similarly, ILC2s appear during pregnancy and reach their maximum during the bulk primary septation stage of the murine lung development [392]. Of note, and as resident alveolar macrophages, ILC2s are tissue-resident cells that colonized the lungs during the postnatal lung development [392] with only a few being replaced by newly generated ILC2. These suggest that ILC2-macrophage interaction could occur very early during the saccular and alveolar stages of lung development [393].

Interestingly, unlike the BM-M-DC and the pulmonary MHC-II$^+$ cells, sorted AMs co-cultures did not change the STAT4 phosphorylation status of the ILC2s. However, after 2x HDM exposure, I could measure IL-23, IL-12p40, and IL-27 cytokines in co-culture supernatants. It has been shown that IL-1α acts as a cofactor for the IL-12 secretion [394]. This suggests that, per se, secretion of IL-23, IL-12p40, and IL-27 from pulmonary cells upon C5a activation, synergistically amplified IL-1α, which down-regulates the STAT4 quicker than BM-M-DCs and pulmonary MHC-II cells. Alternatively, as MHC-II$^+$ cells encompass more than simply AMs, but also IMs [395], type II alveolar epithelial cells (AECII) [313], and DCs, it could be that synergistic action of different cells is required for STAT4 phosphorylation. Of note, while IMs are positive for C5aR1 [251], no evidence support supports a positive expression of C5aR1 in AECII [251].

IL-12 is known as transdifferentiating cytokines of ILC2, which induce plasticity from ILC2 to ILC1 [180]. Although C5a negatively regulates the TLR-4-induced IL-12 synthesis in the peritoneal macrophages [330], it has been observed that blocking C5a in human monocytes as well as C5a deficiency on the macrophage in murine impair the IL-12 production [396, 397].

4.8 C5a/C5aR1 axis regulates the ILC2 DC crosstalk

My data showed that DC-ILC2 crosstalk is important for the ILC2 expansion, survival, and/or development, as the frequency of the ILC2s as well as Bcl-2 was further increased upon C5a activation. Furthermore, in contrast to AMs, my data support the idea that a physical interaction between ILC2 and DCs is required for their crosstalk. It has been shown that when HDM pulsed *C5ar1$^{-/-}$* BMDCs transfer into the WT mice, they failed to drive the pulmonary allergy [306]. Although, Engelke et al. did not directly evaluate the BMDC drive C5a/ C5aR1 signaling axis function on ILC2. Recently, it has been shown that ILC2s express the inducible costimulatory ICOS molecule and its ligand ICOS-L [79], important signaling implicated in the survival of ILC2s via the control of Bcl-2 expression [79, 338]. Interestingly, ICOS-L expression on the dendritic cells has been detected in respiratory tract inflammation. [398], the inhibition of C5aR1 decreases ICOS-L (B7-RP1) on mDCs upon HDM exposure [286], suggesting a possible role of ICOS - ICOS-L in the crosstalk ILC2 and DCs in the C5a-driven ILC2 survival Besides, it has been shown that C5a/C5aR1 signaling axis regulates the conventional dendritic cells and T cells interactions via regulation of CD80 and CD86 expression in C5aR1^{+} cDC2, 24h after HDM/OVA treatment [324], while CD40 followed an opposite regulation. Interestingly, CD80 / CD86 activation in ILC2 has not been reported. Instead, these stimulatory molecules were shown to be expressed in ILC2 and regulate the crosstalk with T cells in the helminth infection [399]. Since CD40L has been reported to be expressed in human ILC2, [400], one could also speculate that C5a/C5aR1 interfere with any functions related to the activation of ILC2 via CD40/CD40L.

4.9 Immune cells in postnatal lung development

From our first breath, lungs are exposed to various microorganisms such as bacteria and viruses, and neonatal immune cells protect from most infections at an early stage [401]. My data confirm that innate immune cells, such as ILC2, Eosinophils, macrophages, and neutrophils, are transiently homing to the lungs during their postnatal development. Lung development is a complex process regulated by mesenchymal and epithelial cells [402]. During the first two weeks after birth, the lung undergoes dramatic changes including alveolar volume and septal thickness until P28 [402]. Several type 2 immune cells like mast cells, Eosinophils, ILC2s, basophils, and macrophages are present in low numbers at birth but increase during the alveolarization and thus could associate with an increase in IL-33 level [391, 392, 402]. Intriguingly, although IL-5 was required, my data showed that Eosinophil recruitment was uncoupled from ILC2s and T cells I failed to detect IL-5 changes in both. In adult mice, ILC2 and Th2 cells are the main sources of IL-5, which regulates the recruitment of inflammatory Eosinophils [129, 265, 272, 342, 403, 404], but it has been shown that mast cells, basophile, γδT cells, NK and NKT cells, and non-hematopoietic cells including epithelial cells can also produce IL-5 [405]. In addition, maternal serum and breast milk are potent sources of IL-5 [406].

4.10 Gut -Lung axis during development

Pregnancy and early life represent a window in which microbial metabolites originating from the maternal microbiota have a major role in shaping neonates' immunity. The data in this study provide several important insights into the impact of early-life dysbiosis on asthma development. My data show that early life exposure to antibiotics is sufficient to induce long-term damage to the intestine and exacerbates lung pathology through the alteration of innate immune cell recruitment during postnatal lung development. So far, it is unclear how the administration of ABX to the mother turn into the dysbiotic condition in the pups. Additionally, there is little evidence of the transmission for antibiotics through the maternal milk [406]. Nonetheless, maternal dysbiosis increases the accumulation of inflammatory Eosinophils in the lung at P14. In contrast, in early-life dysbiosis models, ABX exposure led to reduced neutrophilia during the development but was followed by a marked accumulation in the long term. Interestingly, this was increasing the airway responsiveness baseline in pups later in the age [278] . Notably, commensals can also influence immune cell behavior, which impacts later

life immune characteristics in particular NK cells, macrophages, and T cells [407]. Various studies have shown that fluid particles and microorganisms exposed to the nasal cavity of the mice can be found shortly after in the gastrointestinal tract suggesting the existence of a gut-lung axis [408]. In addition, upon antibiotic-depleted microbiota CD4+ T cells induce a response to the allergen [409]. As early life antibiotic exposure dramatically alters the microbiome in the long term [277, 295], it is associated with weight gain, metabolic imbalance, and susceptibility to diseases such as Inflammatory bowel diseases (IBD), allergies, and asthma [276, 293, 294, 410]. Furthermore, early-life dysbiosis and disruption of microbial maturation have been linked with increased asthma risk and severity, while antibiotics-driven intestinal dysbiosis exacerbates lung pathology [277]. However, the immunological mechanisms underpinning this connection are poorly understood [282, 296]. Metha et al. showed early-life dysbiosis of IL-17, IL-13, and IFNγ secretion, as well as an increase in the γδ T cells associated with severe asthma [411, 412]. In line, children developing asthma may have lower gut microbial diversity in comparison to healthy children in the first year of life suggesting that commensals are playing a critical role [284]. Finally, although early-life dysbiosis triggered increased inflammatory Eosinophil recruitment, it gives its place later to CD125 $^{+}$neutrophils, while IL-5 receptor alpha (CD125) expression on neutrophils has been shown in human and murine [413]. Interestingly it might be a reflection of possible plasticity between Eosinophils, as it has been reported that microenvironmental adaptation initiates a plastic switch between neutrophils and Eosinophils [414]. Interestingly, not only neutrophils are sensitive to IL-5 signaling via CD125, but in addition, typical neutrophil marker Ly6G expression has also been identified at the surface of the Eosinophils [415].

4.11 Conclusion and Future perspective

Innate lymphoid cells type 2 (ILC2), play key roles during allergen sensitization by IL-5 secretion. Summarizing my data, using an *in vitro* developmental assay, I observed that bone marrow (BM)-derived macrophage/dendritic cells (BM-M-DC) promoted ILC2 development in a C5aR1-dependent manner. Further, stimulation of BM-M-DC with C5a increased the differentiation of ILC2, while C5a had no direct effect on ILC2. Mechanistically, C5a-activated BM-M-DC strengthened the expression of GATA3 hence favoring the differentiation of ILC2 after 24h of co-culture. Intriguingly, we observed that C5a-activated BM-M-DCs concomitantly secreted interleukin (IL)-23 secretion and that STAT4, a downstream target of IL-23R, was phosphorylated in ILC2. As STAT4 has been described as a transcription factor

important for the development of ILC1 as well, I followed the differentiation of ILC for a longer period. Interestingly, I observed that the ILC2 phenotype persisted up to 48h after co-culture started, but shifted to ILC1 after 72h. Overall, my data show that C5a contributes to the development of the ILC2 through a unique indirect pathway via myeloid cell activation. Further our data also suggest that C5a may play a role in the plasticity between ILCs (Figure 4.1).

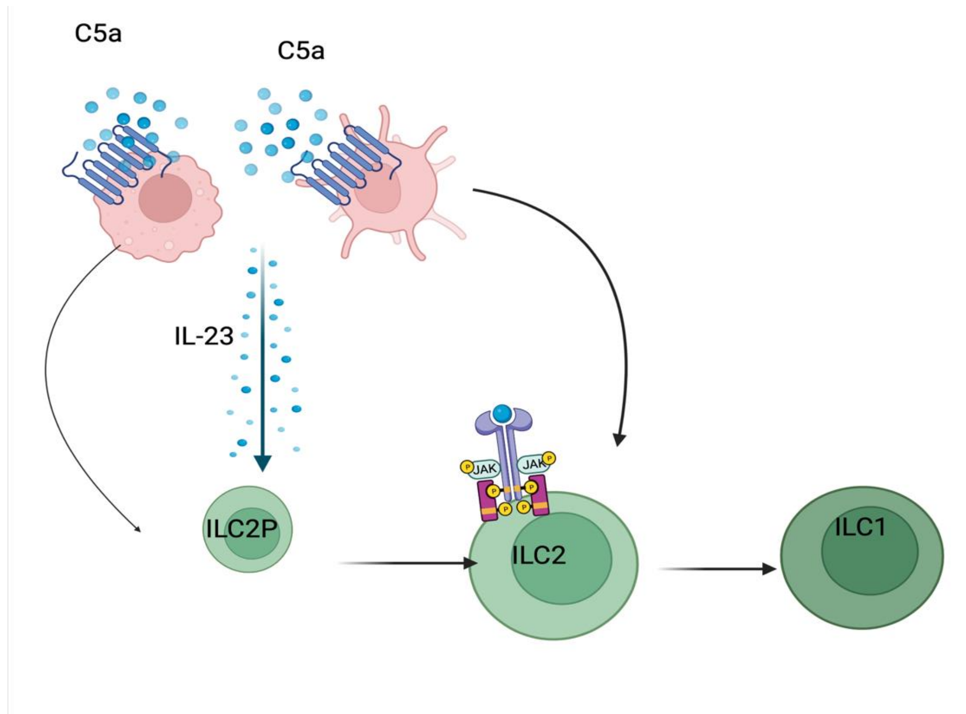


Figure 4. 1 Model for in-vitro experiments.

Further, observation suggests that both AM and DCs actively communicate with ILC2 to support its development and in this communication C5a/C5aR1 signaling axis plays a key role. While AMs tonically produce C5a, DCs must be supplemented with C5a to provide a support for ILC2. Furthermore, while AMs derive ILC2 via soluble factors for instance IL-1α, DCs required cell-to-cell contact (Figure 3.33).

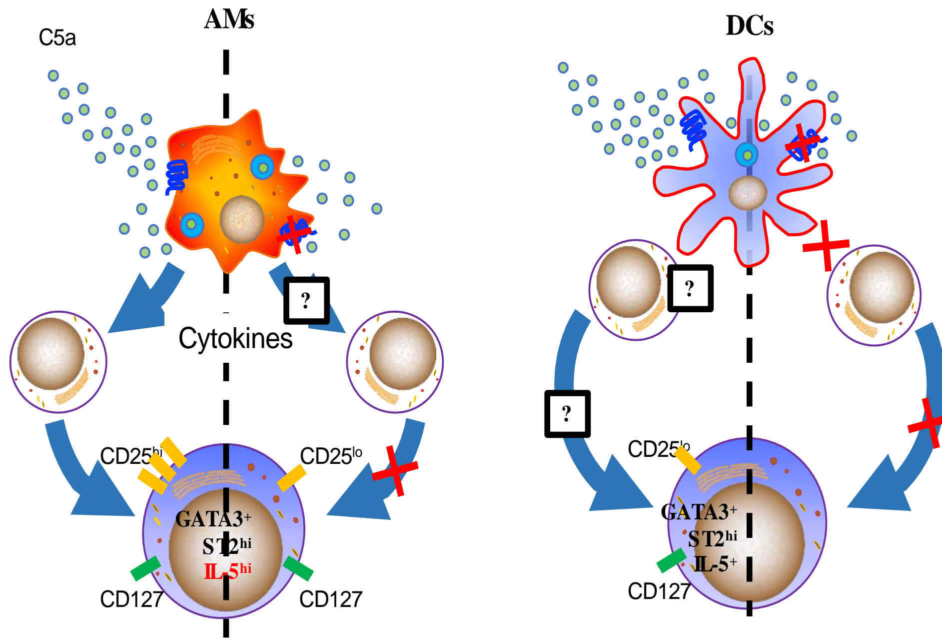


Figure 4. 2 Conclusion of AMs/DCs-ILC2 crosstalk.

Moreover, I could demonstrate that the C5aR1 axis is not only important for ILC2 development but also for its function on IL-5 production which is the main driver of inflammatory Eosinophil recruitment via its function in myeloid cells (Figure 4.3). This suggests that C5a signalling axes may be a potential pharmacological target to regulate ILC2 development in allergic asthma.

Another biological condition where inflammatory Eosinophils take place is development. However, ILC2-triggered Eosinophil recruitment failed in the dysbiosis model. On the other hand, accumulation of iEOS, mucus secretion, and alveolar damage was higher at P14. In the long run, airway function measurement by forced oscillary technic upon methacholine trigger showed, in the ABX exposed group, an increase of sensitivity in the small and the total airway compared to Suc controls. Overall, my data shows that maternal antibiotic exposure during the early life of offspring promotes a higher basal airway hypersensitivity and a pro-inflammatory status, thus increasing the risk of developing severe asthma.

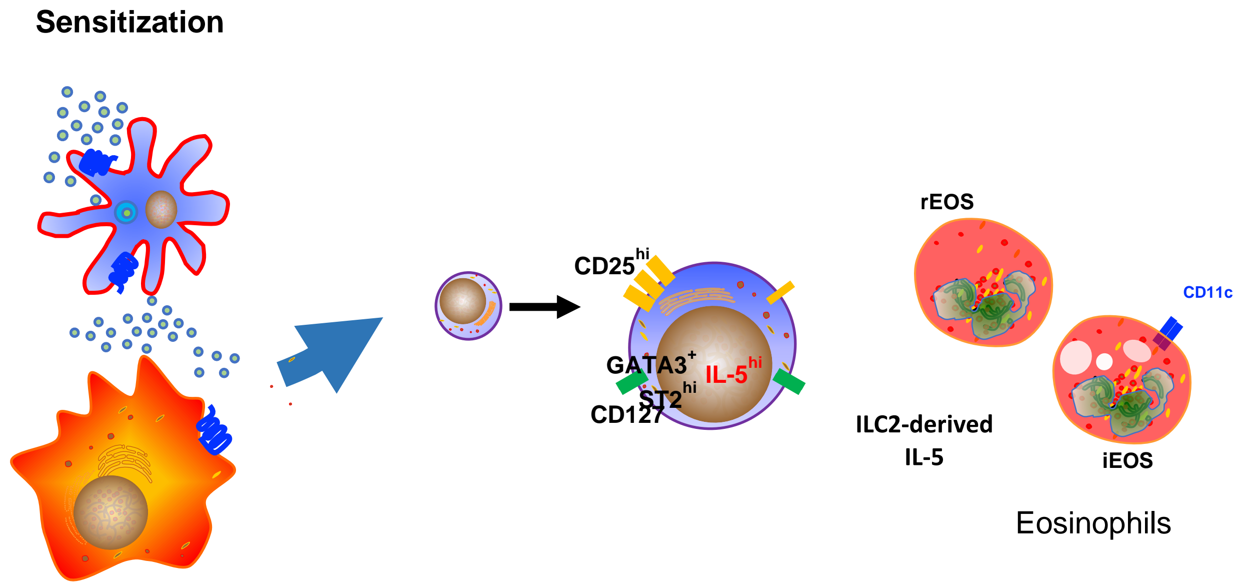


Figure 4. 3 Visual conclusion of sensitization of asthma. During sensitization, C5a/C5aR1 signaling axis driven from antigen-presenting cells, including macrophages and dendritic cells, support ILC2 development and function.

5 Abbreviations and symbols

-/-	Knock out
°C	Degree celsius
%	Percent
*, &, #	Indication of p-value In casse of comperison of more than two groups indicates significant difference between the grups * p<0.05,** p<0.01, *** p<0.001 & p<0.05,&& p<0.01, &&& p<0.001 # p<0.05,## p<0.01, ### p<0.001
AHR	Airway hyperresponsiveness
™	trademark
AT	Anaphylatoxin
ATR	Anahylatoxin receptor
AM	Alveolar macrophage
ABX	Antibiotic
AP	alternative pathway
APC	antigen-presenting cells
BAL	Bronchoalveolar lavage
BM-M-DC	Bone marrow derived macrophage-dendritic cells
BCR	B cell receptor
BCL-2	B cell lymphoma 2
BM	Bone marrow
BSA	Bovine serum albumin
C3aR	C3a receptor
C5aR1	C5a receptor 1
C5aR2	C5A receptor 2
CCL	CC-chemokine ligand
CCR	C-C chemokine receptor

CD	Cluster of differentiation
cDC	Conventional dendritic cell
CDP	Dendritic cell progenitor
CLP	Common Lymphoid progenitor
CMP	Common myeloid progenitor
CILP	Common innate lymphoid progenitor
CHILP	Common helper innate lymphoid progenitors
CO_2	Carbon dioxide
COPD	chronic obstructive pulmonary disease
CP	Classical pathway
CR	Complement receptor
CRP	C reactive protein
CTSL	Cathepsin L
CXCL	Chemokine C-X-C motif ligand
CXCR	Chemokine C-X-C motif ligand RECEPTOE
DAF	Decay-accelerating factor
DAMP	Damage-associated molecular pattern
DAPI	Diamidino-phenylindole
DC	Dendritic cell
DMSO	Dimethl sulfoxide
dH_2O	Deionized water
E	Embryonic day
EC	Epithelial cell
EDTA	Ethlenediaminetetraacetic acid
EDN	Eosinophile-derived neurotoxin
EET	Eosinophil extracellular trap
eF	EFluor
EILP	Early innate lymphoid progenitor
EV	extracellular vesicles
ERK	Extracellular signal-regulated kinase
elisa	Enzyme-linked immunosorbent assay
et al.	Et alii
EOS	Eosinophil

FACS	Flourescence activated cell sorting
FBS	Fetal bovine serum
FCS	Fetal calf serum
FcεR1	Fc epsilon receptor 1
FITC	Fluorescein isothiocyanate
FoxP3	Forkhead box P3
FCS	Forward scatter
g	Gram (unit)
g	Gravity(in centrifugation steps)
G	Small airway resistance
GATA3	GATA binding protein
GFP	Green fluorescent protein
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GRK	G protein-coupled receptor kinase
h	Hour
HDM	House dust mite
H&E	Hematoxylin-EOSin
HSC	Hematopoietic stem cell
iEOS	Inflammatory Eosinophil
IFN	interferon
Ig	Immunoglobulin
IM	Interstitial macrophage
IL	Interleukin
IHC	immunohistochemistry
ILC	Innate lymphoid cell
ILCP	Innate lymphoid cell progenitor
ILC1	Innate lymphoid cell type 1
ILC2	Innate lymphoid cell type 2
ILC2P	Innate lymphoid cell type 2 progenitor
ILC3	Innate lymphoid cell type 3
IEL	intraepithelial lymphocytes
i.p.	intraperitoneal

i.t	Intratracheal
HSC	Hematopoietic stem cells
HMGP-1	nuclear high mobility group box 1 protein
JAK	Januskinase
kg	Kilogram
LPS	Lipopolysaccharide
Ly6G	Lymphocyte antigen 6 complex locus G6D
KO	Knock out
KLRG-1	Killer cell lectin like receptor G1
LCMV	Lymphocytic choriomeningitis virus
LTi	Lymphoid tissue inducer
LTiP	Lymphoid tissue inducer progenitor
LBP	LPS binding protein
LP	Lectin pathway
mAb	Murine antibody
MAC	Membrane attack complex
MAPK	Mitogen activated protein kinase
MBL	Mannose-binding lectin
MFI	Mean fluorecence Intensity
μg	Microgram
μl	Microliter
mg	milligram
MHC	Major histocompatibility complex
Min	Minute
MR	Mannose receptor
Mo-DC	Monocyte-derived dendritic cell
NOD	Nucleotide-binding oligomerization domain
Muc	Mucin
mLN	Mediastinal lymph node
μM	Micromolar
NCR	Natural-cytotoxicity receptor
NET	Neutrophil extracellular trap
NMU	Neuromeric U

NO	Nitric oxide
NOS	Reactive nitrogen species
NK	Natural Killer
OVA	Ovalbumin
OXPHOS	Oxidative phosphorylation
PAS	Periodic acid schiff
PAMP	Pathogen-associated molecular patterns
PBS	Phoshate buffered saline
PE	Phycoerthrin
PECy	PE-cyanine
PGE-2	prostaglandin E2
Per-CP-Cy	Peridinin chlorophyl protein-cyanine
PMA	Phorbol 12-myristate 13-acetate
PVM	Pneumonia virus of mice
RBC	Red blood cell lysis
rEOS	Resident Eosinophil
RT	Room temperature
Rpm	Rounds per minute
Rn	Large airway resistance
RIG-I	retinoic acid-inducible gene I
Rrs	Airway resistance (total)
ROS	Reactive oxygen species
RORγT	Retinoic acid-related orphan receptor gamma T
RSV	Respiratory syncytial virus
s	Second(s)
SEM	Standard Error the mean
Siglec	Sialic acid-binding immunoglobulin-type lectin
SSC	Side scattered light
SCFA	Short chain fatty acid
STAT	Signal transducer and activator of transcription
ST2	soluble interleukin 1 receptor-like 1
Suc	Succarose
SARS-Co2	

	Severe acute respiratory syndrome coronavirus 2
Th	T helper cell
TM	Trademark
TM	Transmembrane
TCR	T cell receptor
tdTomato	Tandem-dye Tomato
TF	Transcription factor
Tfh	T folicular helper
Treg	Regulatory T
TLR	Toll like receptor
TNF	Tumor necrosis factor
TSLP	Thymic stromal lymphoeitin
3D	3 dimentional
TGF-β	Transforming growth factor-β
Vs.	Versus
VIP	Vasoactive intestinal protein
WT	Wild type

List of Tables

Table 2. 1: Used chemical 42
Table 2. 2: Antibodies used for flow cytometry, immunofluorescence staining 45
Table 2. 3: Compounds used for flow cytometry, immunofluorescence staining. 49
Table 2. 4: Consumables. 50
Table 2. 5: Used Kits 52
Table 2. 6 : Buffers and solutions 52
Table 2. 7 : Specification of used mouse strains 54
Table 2. 8 : Used equipment 55
Table 2. 9: Used software for data collection and analysis. 57
Table 2. 10 : Protocol of the H&E staining. 72

List of Figures

Chapter 1

Figure 1. 1The 3 arms of the immune system.... 9
Figure 1. 2 Overview of T cell differentiation. 12
Figure 1. 3 Dual function of alveolar macrophages.. 17
Figure 1. 4 Deciphering the family tree of ILCs.. 25
Figure 1. 5 ILC differentiation and functions.. 27
Figure 1. 6 Complement cascade and key canonical and non-canonical functions.). 33
Figure 1. 7 Social networking upon allergen exposure.. 39

Chapter 2

Figure 2. 1 Experimental models of house dust mite (HDM)-sensitization in Balb/c mice.. 59
Figure 2. 2 Dysbiosis model.. 60
Figure 2. 3 Flow cytometric analysis after 9 days of BM-M-DC culture. 66
Figure 2. 4 In vitro developmental co-culture system. 67
Figure 2. 5 Pulmonary developmental co-culture system.. 68

Chapter 3

Figure 3. 1 C5a/ C5aR1 regulates the ILC2 function.. 75
Figure 3. 2 ILC2 and its progenitor ILC2p do not express C5aR1. 76
Figure 3. 3 Myeloid cells in bone marrow express C5aR1. 77
Figure 3. 4 Bone marrow-derived macrophages/dendritic cells support ILC2 development.). 80
Figure 3. 5 Expression of Ki67 in ILC2. 81
Figure 3. 6 BM-M-DCs allow the development of ILC2 from ILC progenitors in a C5aR1-dependent manner via the activation of GATA3 and KLRG1.. 82
Figure 3. 7 C5a/C5aR1 axis regulates the developments Frequency of ILC1, ILC2, and ILC3 in total cell 84
Figure 3. 8 C5aR1 signaling axis regulates STAT4 phosphorylation in ILC2. 85
Figure 3.8.1 C5a/C5aR1 signaling drives the secretion of cytokines that are involved in the activation of STAT4 ...89
Figure 3. 9 BM-M-DC regulates the phosphorylation of STATs in ILC2s over time. 88
Figure 3. 10 Pulmonary MHC II cells support ILC2 development. 90
Figure 3. 11 Pulmonary APCs support ILC2 development. 92
Figure 3. 12 Pulmonary APCs support ILC2 survival. 93
Figure 3. 13 C5a/C5ar1 in AM and pulmonary DC, did not change the STAT4 phosphorylation status in ILC2.... 94
Figure 3. 14 C5a/C5aR1 signaling in DCs negatively regulates TSLP-R expression in ILC2. 95
Figure 3. 15 While C5a/C5aR1 signaling axis drives the development of ILC2 upon DC crosstalk, C5a/C5aR2 might drives AM-ILC2 crosstalk.. 96
Figure 3. 16 AM regulates ILC2 function through C5a/C5aR1 signaling. 98
Figure 3. 17 Tonic C5/C5a production by AMs participates in the ILC2 development.. 99
Figure 3. 18 Tonic C5/C5a sustains AMs- driven IL-5 induction in ILC2. 100
Figure 3. 19 Soluble factors released by AM are required for ILC2. 102
Figure 3.19.1 AMs derived IL-1α regulates the ILC2 via C5a...106
Figure 3.19.2 C5a/C5aR1 signaling drives the production and secretion of cytokines during sensitization...108
Figure 3. 20 DCs trigger ILC2 function and development through different mechanism.. 104
Figure 3. 21 C5a/ C5aR1 signaling axis is important for ILC2 accumulation in the lung. 105
Figure 3. 22 C5aR1 might be important for Eosinophils. 107
Figure 3. 23 Early life ABX exposure shows a nonrecoverable effect on the small intestine. 109
Figure 3. 24 Early life exposure to ABX altered expression of cell surface markers. 111
Figure 3. 25 Waves of the Eosinophils are transiently infiltrating the perinatal lung. 112
Figure 3.25.1 Mucus production and pulmonary cellular infiltration are enhanced upon ABX exposure at P14 ...119
Figure 3. 26 Eosinophilic subpopulation's pulmonary recruitment depends on IL-5. 114
Figure 3.26.1 BM-derived Eosinophile depends on IL-5 ...,...121

Figure 3. 27 ILC2 is only not a potent source for IL-5 during pulmonary development.. 116
Figure 3. 28 Early life dysbiosis has no impact on splenic and BM-derived ILC2s and ILC2Ps............................. 118
Figure 3. 29 Early life dysbiosis alters the accumulation of AM and Neutrophils in the lung. 119
Figure 3. 30 Early life dysbiosis alters effector T cells.. 120
Figure 3. 31 Dysbiosis exacerbates baseline airway function.. 121

Chapter 4
Figure 4. 1 Model for in-vitro experiments. 134
Figure 4. 2 Conclusion of AMs/DCs-ILC2 crosstalk. 135
Figure 4. 3 Visual conclution of sensitization of asthma. 136

List of Supplementary Figures

S 1 Gating strategy of Innate lymphoid cell type 2. 165
S 2 Surface staining of ILC2 after 9 days culture.. 166
S 3 Gating strategy of Eosinophils SUC exposed mice vs. ABX exposed mice at P14.. 166
S 4 tSNE anaysis from ABX vs. SUC exposed mice at P14 in the BALF. 167

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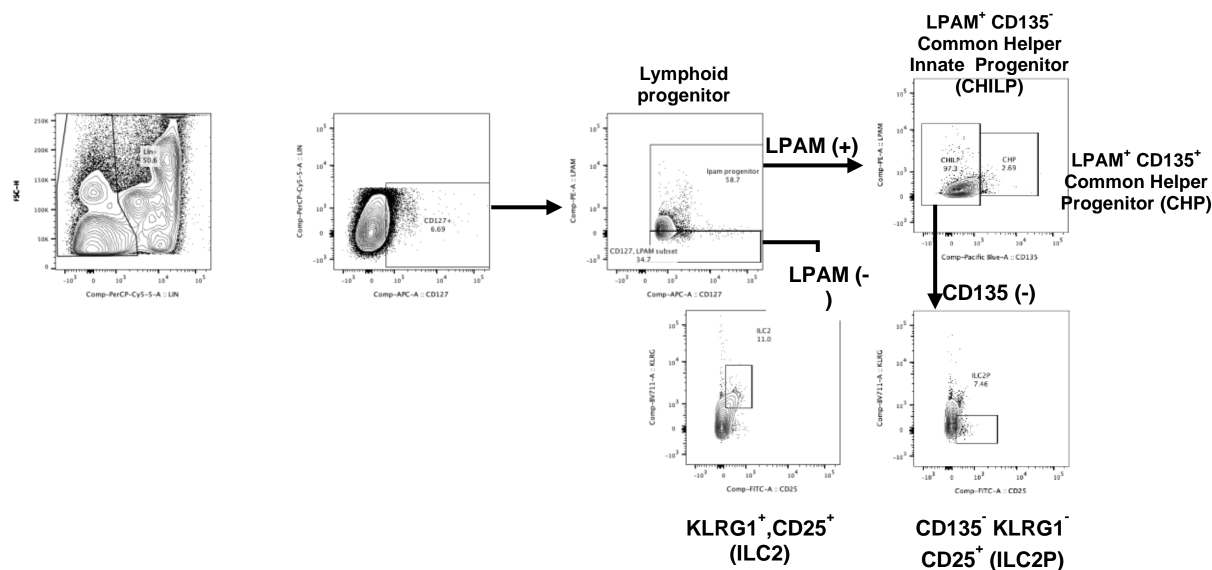
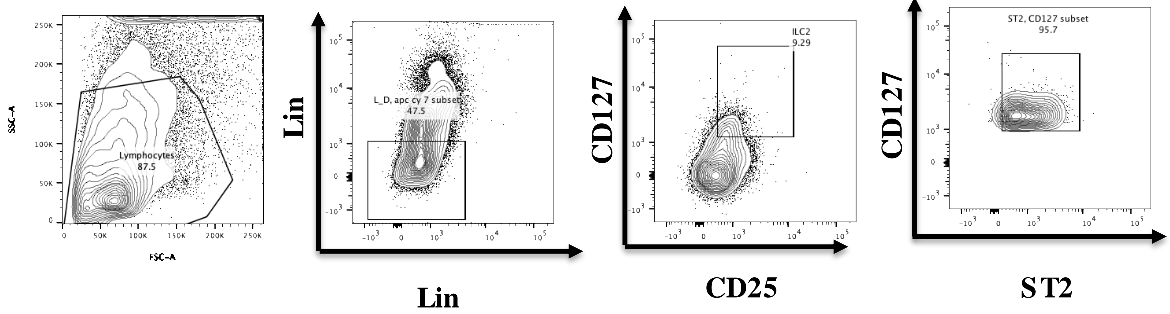
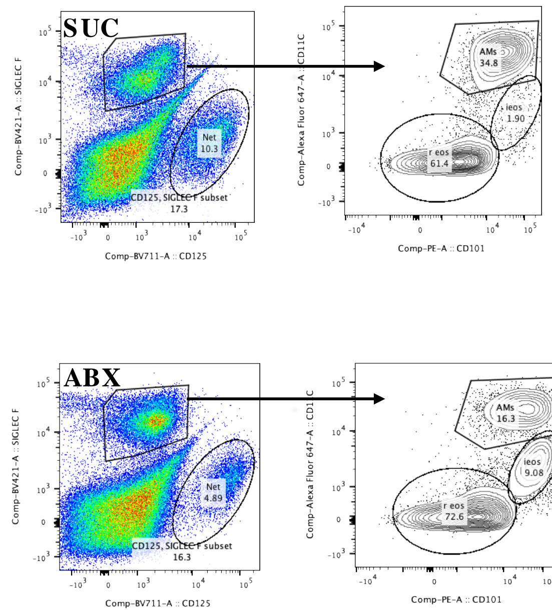
7 Supplementary Figures



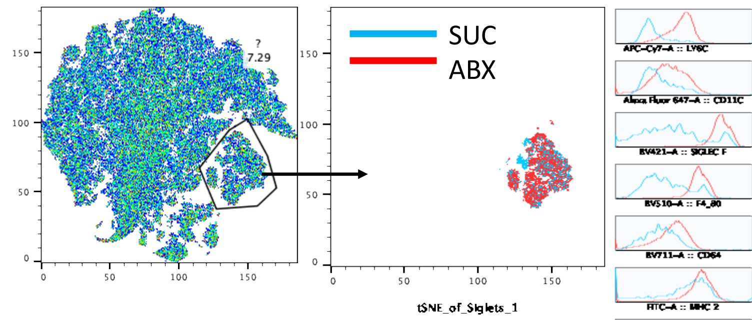
S 1 Gating strategy of Innate lymphoid cell type 2. Common Helper Progenitor, Common Helper Innate Lymphoid progenitor, Innate lymphoid cell type 2 progenitors. ILC2s are identified as Lin-CD127+LPAM-KLRG-1+CD25+, CHPs are identified as Lin-CD127+LPAM+CD135+, CHILPs are identified as Lin-CD127+LPAM+CD135-, ILC2Ps are identified as Lin-CD127+LPAM+CD135-KLRG-1-CD25+ in the bone marrow. Lineage marker are CD3e, TCRβ, CD19, B220, Ly6G, CD11c, CD11b, CD27, CD5.

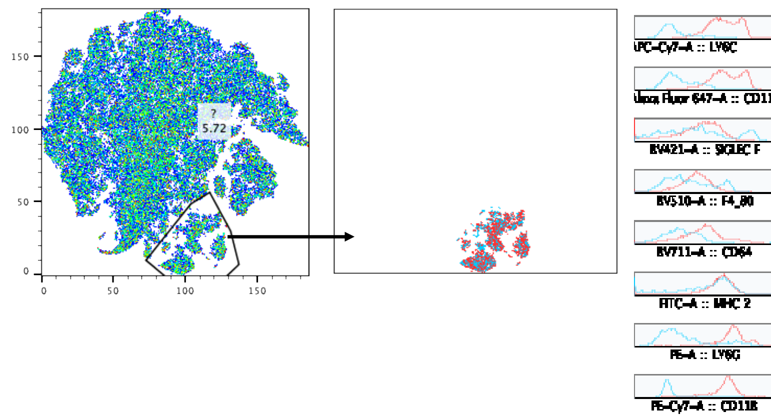


S 2 Surface staining of ILC2 after 9 days culture. Lineage negative population including viability dye. CD25 and CD127 double-positive cells express ST2.



S 3 Gating strategy of Eosinophils SUC exposed mice vs. ABX exposed mice at P14. iEOS are defined by Siglec-F^{+},CD125^{Int},CD11c^{+}, CD101$^{int/+}$, rEOS are defined by Siglec-F^{+},CD125^{Int},CD11c^{-} .





S 4 tSNE analysis from ABX vs. SUC exposed mice at P14 in the BALF. Different population analysis.

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In vitro pulmonary Eosinophil and T cell co-culture technique

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(Davos, Switzerland)

- 2022 WIRM 7th World Immune regulation Meeting, Short- and long-term effects of post-natal dysbiosis in pulmonary development and function (Davos, Switzerland)
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- 2021 WIRM 5th World Immune Regulation Meeting C5aR1 participates in the development of the Innate lymphoid cells via DC functions (Virtual)
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LIST OF PUBLICATIONS

- Anna V. Wiese, Jannis Duhn, **Rabia Ülkü Korkmaz**, Katharina M. Quell, Ibrahim Osman, Fanny Ender, Torsten Schröder, Ian Lewkowich, Simon Hogan, Markus Huber-Lang, Franziska Hoffman, Peter König, Jörg Köhl, Yves Laumonnier **C5aR1 activation controls inflammatory Eosinophil recruitment and functions in experimental allergic asthma (In review)**
- Christopher C. Udoye, Christina N. Rau, Sarah M. Freye, Larissa N. Almeida, Sarah Vera-Cruz, Kai Othmer, **Rabia Ü. Korkmaz**; Ann-Katrin Clauder, Timo Lindemann, Markus Niebuhr, Kathrin Kalies, Andreas Recke, Hauke Busch, Anke Fähn- rich, Fred D. Finkelman, Rudolf A. Manz, **BCR-binding-properties influence the nature of the IgE response and relative IgG1 and IgE production, Mucosal Im- munol. 2022 Sep 16.**
- Quell, K.M.; Dutta, K.; **Korkmaz, Ü.R.**; Nogueira de Almeida, L.; Vollbrandt, T.; König, P.; Lewkowich, I.; Deepe, G.S., Jr.; Vershoor, A.; Köhl, J.; Laumonnier, Y.,**GM-CSF and IL-33 Orchestrate Polynucleation and Polyploidy of Resident Murine Alveolar Macrophages in a Murine Model of Allergic Asthma. Int. J. Mol. Sci. 2020, 21, 7487.**
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