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The role of the C5a/C5aR1 axis activation on pulmonary dendritic cell function in allergic asthma

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Summary

Asthma is an inflammatory disease of the airways with an increasing prevalence in western countries. Thus, there is a vital need to better understand the mechanisms that control the development of the disease. Many immune cells are involved in the initiation of the disease. Here, the focus will be on dendritic cells due to their key role on the development of maladaptive Th2/Th17 responses. In steady state, the lung harbors $CD103^+$ and $CD11b^+$ conventional DCs (cDCs) as well as plasmacytoid DCs (pDC). In addition to cDCs, $CD11b^+$ $MHC\text{-}II^+$ monocytes reside in the lung and can differentiate into monocyte-derived DCs (mo-DCs) under inflammatory conditions. Both $CD11b^+$ cDC populations contribute to Th2 and/or Th17 development, whereas $CD103^+$ cDCs and pDCs promote tolerance. Recently, the transcription factors IRF4 and STAT5 have been identified as key components of a genetic program in $CD11b^+$ cDCs that drive Th2. STAT5 is downstream of TSLP, a cytokine of the IL-2 family that up-regulates co-stimulatory molecules and chemokines in DCs to help Th2 differentiation. IRF4 in pulmonary DCs is activated in response to TLR pathways stimulation and drives IL-10 and IL-33 production, which specifically promote Th2 differentiation. Several allergens including house dust mite (HDM) activate the complement system and generate the anaphylatoxins (ATs) C3a and C5a. These ATs exert their biologic function through their cognate G-protein coupled receptors (GPCRs) C3a receptor (C3aR), C5a receptor 1 (C5aR1) and C5a receptor 2 (C5aR2). Deficiency of the C3aR has been shown to attenuate the development of Th2/Th17 immune responses, whereas C5aR1 targeting during allergen sensitization enhanced Th2/Th17 immunity in several models of experimental asthma. Importantly, AT receptors are expressed on $CD11b^+$ cDCs and mo-DCs and regulate the production of IL-10, IL-12 and IL-23. At this point, it is unclear, how the ATs regulate the programming of DCs into a phenotype that drives Th2 immune responses in allergic asthma.

To shed light on the mechanism through which the ATs control the programming of DCs and more precisely the $CD11b^+$cDCs, I sensitized WT Balb/c mice intratracheally with a mixture of HDM and ovalbumin (OVA). Twenty-four hours later, I isolated the lungs and FACS-purified the $CD11b^+$cDCs based on the expression of C5aR1. I found that the majority of the $CD11b^+$ cDCs expressed C5aR1 under steady state conditions, whereas the frequency of $C5aR1^-CD11b^+$ cDCs increased after HDM/OVA *in vivo* stimulation. I performed phenotypic, functional and transcriptional characterizations of the $C5aR1^+$ and $C5aR1^-$cDCs, to better understand, how signaling through C5aR1 controls the programming of $CD11b^+$cDCs.

One of my main findings was that the C5a/C5aR1 axis controls $CD4^+$ T cell proliferation. While $C5aR1^-$ cDCs induced strong T cell proliferation, $C5aR1^+$ cDCs were poor inducers of T cell proliferation. In search for mechanisms, I observed that the lower potency of $C5aR1^+$ to drive T cell proliferation was associated with lower MHC-II and CD40 expression. As outlined above, TSLP is an important inducer of cDC maturation. Of note, I observed that TSLPR expression was much higher in $C5aR1^-$ than in $C5aR1^+$ cDCs after HDM/OVA sensitization. Co-culture experiments uncovered that OVA-tg $CD4^+$ T cells interacted more frequently with $C5aR1^-$ than with $C5aR1^+$ cDCs. Both pulmonary cDC populations induced strong Th2 differentiation and some Th17 differentiation. Mechanistically, I identified autocrine C5 production and cleavage into C5a in cDCs as the main source for C5aR1 activation. Importantly, *in vitro* OVA pulsing was a strong inducer of C5 production. C5a generation occurred in $C5aR1^-$ and $C5aR1^+$ cDCS. However, it was higher in $C5aR1^-$ cDCs than in $C5aR1^+$ cDCs. After T cell addition, the C5a generation was markedly enhanced in both $CD11b^+$ cDC populations. Interestingly, RNAseq analysis revealed 94 genes that were 2-10-fold higher expressed in $C5aR1^+$ than in $C5aR1^-$ cDCs affecting complement activation, cell differentiation and gene regulation

among others. The importance of C5a/C5aR1 axis activation for the regulation of T cell proliferation was verified *ex vivo* through specific targeting of C5aR1 in co-cultures of C5aR1$^+$ cDCs with OVA-tg CD4$^+$ T cells. Here, I found that such targeting markedly enhanced T cell proliferation. Further, I was able to show that C5aR1 is controlling T cell proliferation through CD40. Importantly, CD40/CD40L interaction-controlled T cell proliferation only when MHC-II expression was low, i.e. in C5aR1$^+$ cDCs or when the availability of the OVA peptide in the system was limited. In C5aR1$^-$ cDCs, in which MHC-II is strongly expressed, *in vitro* blockade of CD40-CD40L interactions did not affect the ability of C5aR1$^-$cDCs to induce strong CD4$^+$ T cell proliferation. The RNAseq analysis revealed almost 40 genes which were differentially expressed in each condition, but unfortunately, the function of most of these genes is currently unknown and thus, no information could be extracted at this time point. Finally, my findings suggest that C5aR1 is not controlling Ag uptake, processing and presentation as a mechanism to control T cell proliferation via the autocrine C5/C5a/C5aR1 loop.

Thus, from the available data, I propose a model, in which activation of C5aR1 in CD11b$^+$ cDCs through autocrine production of C5a drives a pathway that downregulates CD40 molecule expression. This downregulation of CD40 together with the reduced MHC-II expression in C5aR1$^+$ cDCs impairs the DC-CD4$^+$ T cell interaction at the level of synapse formation resulting in low allergen-specific Th cell proliferation by pulmonary C5aR1$^+$CD11b$^+$ cDCs. Suppression of C5aR1 signaling in CD11b$^+$ cDCs releases the break by upregulation of CD40, resulting in strong allergen-driven Th cell proliferation and allergic asthma development.

Zusammenfassung

Das Asthma bronchiale ist eine entzündliche Atemwegserkrankung mit steigender Prävalenz in westlichen Industrieländern. Daher ist ein besseres Verständnis der Pathomechanismen, welche zur Entstehung dieser Erkrankung beitragen, dringend erforderlich. Eine Vielzahl an Immunzellen tragen zur Initiierung der Erkrankung bei. In dieser Arbeit wurde der Fokus auf die Dendritischen Zellen (DCs) gelegt, aufgrund ihrer Schlüsselrolle in der Entwicklung der maladaptiven Th2/Th17 Immunantwort. Die Lunge enthält im homöostatischen Zustand $CD103^+$ und $CD11b^+$ konventionelle Dendritische Zellen (cDCs) sowie plasmazytoide Dendritische Zellen (pDCs). Darüber hinaus kommen $CD11b^+$ $MHC\text{-}II^+$ Monozyten in der Lunge vor, welche während einer Entzündungsreaktion zu Dendritischen Zellen monozytärer Herkunft (mo-DCs) differenzieren können. Beide Populationen der $CD11b^+$ DCs tragen zur Entwicklung der Th2 und/oder Th17 Antwort bei, wohingegen $CD103^+$ cDCs und pDCs tolerogen wirken. Erst kürzlich konnten die Transkriptionsfaktoren IRF4 und STAT5 als Schlüsselkomponenten des für die Th2-Immunantwort erforderlichen genetischen Programms in $CD11b^+$ DCs identifiziert werden. STAT5 ist dabei Teil einer Signalkaskade, welche durch die Bindung von TLSP initiiert wird. Dieses ist ein Zytokin der IL-2 Familie, welches in DCs die Expression von kostimulatorischen Molekülen und Chemokinen stimuliert, die für eine suffiziente Differenzierung zu Th2 Zellen erforderlich sind. Eine Vielzahl an Allergenen, darunter auch Hausstaubmilben (HDM), induzieren die Aktivierung des Komplementsystems und führen zur Generierung der Anaphylatoxine (ATs) C3a und C5a. Diese ATs wirken durch Bindung an ihre jeweiligen G-Protein gekoppelten Rezeptoren (GPCRs) - C3a Rezeptor (C3aR), C5aR1 und C5aR2. Es konnte gezeigt werden, dass eine C3aR-Defizienz die Entwicklung einer Th2/Th17 Immunantwort abmildert, wohingegen die Blockade des C5aR1 währeden der Sensitisierung des Allergens ebenjene in verschiedenen experimentellen Asthma-Modellen verstärkt. Alle AT-Rezeptoren werden von $CD11b^+$ cDCs und mo-DCs exprimiert und regulieren in diesen die Produktion von IL-10, IL-12 und IL-23. Hierbei ist jedoch unklar, wie die ATs die Programmierung von DCs in einen Th2-induzierenden Phänptypen im Kontext des allergischen Asthma bronchiale regulieren.

Um nachzuvollziehen, wie ATs die Programmierung von DCs bzw. genauer $CD11b^+$ cDCs kontrollieren, habe ich WT BALBb/c Mäuse intratracheal mit einer Mischung aus HDM und Ovalbumin (OVA) immunisiert. 24 Stunden später isolierte ich die Lunge und reinigte die $CD11b^+$ cDCs hinsichtlich C5aR1-exprimierenden und nicht-exprimierenden Zellen mittels FACS auf. Ich konnte nachweisen, dass die Mehrheit der $CD11b^+$ cDCs den C5aR1 unter physiologischen Bedingungen exprimieren, wogegen die Frequenz der $C5aR1^-CD11b^+$ cDCs nach HDM/OVA in vivo Stimulation anstieg. Im Anschluss daran führte ich phänotypische, funktionelle und transkriptionelle Charakterisierunngen der $C5aR1^+cDCs^-$ und $C5aR1^-$ cDCs-Populationen durch um besser zu verstehen wie die durch C5aR1 Aktivierung induzierten Signalwege die Programmierung *der* $CD11b^+$ cDCs kontrollieren.

Eines der Hauptergebnisse meiner Studie ist, dass die C5a/C5aR1 Achse die $CD4^+$ T Zell Proliferation kontrolliert. Während $C5aR1^-$ cDCs eine starke T-Zell-Proliferation induzierten, waren $C5aR1^+$ cDCs nur schwache Induktoren der T Zell Proliferationr. Auf der Suche nach Mechanismen bemerkte ich, dass die geringere Potenz von $C5aR1^+$ cDCs eine T-Zell-Proliferation zu induzieren mit einer geringeren MHC-II und CD40 Expression dieser Zellen einherging. Wie bereits eingehend beschrieben, ist TLSP ein wichtiger Induktor der cDC-Entwicklung. Interessanterweise konnte ich feststellen, dass die TSLPR-Expression in $C5aR1^-$ cDCs nach HDM/OVA Sensitisierung deutlich höher war als in $C5aR1^+$ cDCs. Ko-Kultur-Experimente zeigten, dass OVA-transgene $CD4^+$ T Zellen deutlich besser mit $C5aR1^-$ als mit $C5aR1^+$ cDCs interagierten. Beide pulmonale cDC-Populationen induzierten eine robuste T_H2- und gewisse T_H17-Differenzierung. Ich

konnte eine autokrine C5 Produktion und Spaltung in C5a durch cDCs als Hauptmechanismus der C5aR1-Aktivierung identifizieren. *In vitro* Stimulation mit OVA induzierte eine starke C5 Produktion. C5a Generierung wurde dabei sowohl in C5aR1$^+$ als auch C5aR1$^-$ cDCs beobachtet. Interessanterweise jedoch produzierten C5aR1$^-$ cDCs mehr C5a als C5aR1$^+$. Nach Hinzugabe von T Zellen verstärkte sich die Generierung von C5a in beiden CD11b$^+$ cDC-Populationen. Interessanterweise ergab die RNAseq-Analyse 94 Gene, die in C5aR1 2-10-fach höher exprimiert wurden als in C5aR1-cDCs, die unter anderem die Komplementaktivierung, Zelldifferenzierung und Genregulation beeinflussen. Die Relevanz der Aktivierung der C5a/C5aR1 Achse für die T Zell Proliferation wurde ex vivo durch spezifische C5aR1-Blockade in Ko-Kulturen von C5aR1$^+$ cDCs mit OVA-transgenen CD4$^+$ T Zellen verifiziert. Dabei stellte ich fest, dass die Blockade von C5aR1 in diesem Versuchsaufbau die T Zell Proliferation signifikant verstärkte. Darüber hinaus konnte ich zeigen, dass der C5aR1 die T Zell Proliferation über die Expression von CD40 reguliert. Wichtig dabei ist festzustellen, dass die CD40/CD40L-Achse die T Zell Proliferation nur kontrolliert, wenn MHC-II in geringem Ausmaß exprimiert wird, was zum Beispiel in C5aR1$^+$ cDCs oder bei limitierter Verfügbarkeit von OVA in der Probe der Fall ist. In C5aR1$^-$ cDCs, welche eine starke MHC-II-Expression zeigen, beeinträchtigte die in-vitro Blockade der CD40/CD40L-Achse die Fähigkeit zur Induktion einer T-Zell-Proliferation nicht. Zusammenfassend zeigen meine Ergebnisse, dass der C5aR1, aktiviert über die autokrine C5/C5a/C5aR1-Feedback-Schleife, die Fähigkeit zur Induktion einer T-Zell-Proliferation mechanistisch weder über die Antigen-Aufnahme, noch die Prozessierung oder Präsentation kontrolliert. Die RNAseq-Analyse ergab fast 40 Gene, die bei jeder Erkrankung unterschiedlich exprimiert wurden. Leider ist die Funktion der meisten dieser Gene derzeit nicht bekannt, sodass zu diesem Zeitpunkt keine weiteren Rückschlüsse bzgl. der Rolle dieser Gene für die cDC gezogen werden konnten.

Die vorhandenen Daten legen somit ein Modell nahe, in welchem die Aktivierung des C5aR1 in CD11b$^+$ cDCs durch autokrine Produktion von C5a einen Signalweg initiiert, welcher die CD40 Expression hemmt. Diese Reduktion der CD40 Expression zusammen mit der geringeren MHC-II-Expression der C5aR1$^+$ cDCs erschwert die DC-CD4$^+$ T-Zell-Interaktion auf der Ebene der Bildung der immunologischen Synapse. Dies resultiert in einer geringen Proliferation von Allergen-spezifischen Th Zellen. Die Inhibition des C5aR1-Signalweges in CD11b$^+$ cDCs enthemmt diesen Prozess durch Hochregulation der CD40-Expression, was zu einer starken Proliferation von Allergen-spezifischen Th-Zellen und der Entwicklung von allergischem Asthma bronchiale führt.

1. Introduction

1.1 The immune system

The earliest known reference to immunity was during the plague of Athens in 430 BC when Thucydides noted that people who had recovered from a previous bout of the disease could nurse the sick without contracting the illness a second time(Littman, 2009). However, it is Louis Pasteur, who is considered the father of immunology and one of the first who tried to deal with that field more scientifically and thoroughly. His pioneering work at the end of the 19th century about the germ theory of disease as well as the hope that all infectious diseases could be prevented by prophylactic vaccination awarded him immunology's paternity. However, it should be noted that Pasteur was working on the dawn of the appreciation of the microbial world, at a time when the notion of such a thing as an immune system did not exist, certainly not as we know it today, more than 130 years later (Smith et al., 2012).

The immune system consists of many different cell types, organs, proteins, and tissues. It is spread throughout the body and it is an integral part of it as it ensures its functional integrity. To achieve that, the immune system needs to be able to recognize any possible threats by using special sensors and eradicate them. But what do the sensors recognize as a threat? The threats can be both endogenous and exogenous. The immune system is equipped with sensors, like pattern recognition receptors (such as Toll-like-receptors) which can detect both types of threats. The receptors for both endogenous and exogenous threats may have evolved simultaneously as the integrity of the body is not only at risk upon infectious threats but also upon injury or cell death(Matzinger, 2002). In any of these cases, the immune system needs to deal with the threat either by clearing the dead cells and replacing them with new on the event of injury or trying to eliminate an infection(Köhl, 2006).

Most immune cells arise from precursors in the bone marrow (BM) and develop into mature cells through a series of changes that can occur in different parts of the body. Each of these cells has a function, and it takes the collaboration of several different cell types to elicit a proper immune response. There are eleven main cell types, which can further divide into sub-categories. These main cell types are B and T lymphocytes, natural killer (NK) cells, macrophages, monocytes, dendritic cells (DCs), innate lymphoid cells (ILCs), eosinophils, neutrophils, basophils, and mast cells(Murphy & Weaver, 2013).

The immune system starts to develop four to five months after conception, and this takes place in the primary lymphoid organs, which are the BM and the thymus. Many of the immune cells do not only originate from the BM but develop and mature there as well. Once the immune cells mature, their task is to leave the primary lymphoid organs and patrol the peripheral tissues. Some of these cells reside within tissues while others circulate in the bloodstream or the lymphatic system. The lymphatic system is a highly specialized system of vessels that drains extracellular fluid and is found everywhere throughout the body(Murphy & Weaver, 2013).

When immune cells migrate through the lymphatic system, they end up in secondary lymphoid organs, like the lymph nodes or the spleen. These organs are considered as centralized processing centers, in which information about invading pathogens is communicated to different cells that have different functions, and jointly they mount a protective response against a pathogen.

In case of a threat an immune response will be elicited making use of cellular and humoral immunity (is mediated by macromolecules found in extracellular fluids). Depending on the complexity of the organism which is exposed to the threat the immune system can consist only of innate immunity, which is found on

both primitive and complex organisms or of both innate and adaptive immunity. The latter is found in more evolutionary advanced organisms. One of the main differences between innate and adaptive immunity is that the former makes use of molecules that are encoded in the inherited genome as fully functional molecules. The sensors of the innate immunity detect and recognize groups of potential pathogens and/or internal signals of injury or abnormality in need of immune attention. In case of the adaptive immunity the involved molecules, are not pre-encoded in the inherited genome as complete molecules but are somatically created later. The somatic development of clonally diverse lymphocytes, each of which has a unique antigen recognition receptor that can be used to trigger its activation(Rimer et al., 2014). Strikingly, despite their differences both types of immunity, can develop immunological memory when the threat is exogenous. Immunological memory is the ability of the immune system to quickly and specifically recognize an Ag that the body has already been exposed to in the past and initiate an immune response at a shorter period. We notice the development of memory only in case of long-lived cells of the immune system like brain-resident macrophages (microglia) (Wendeln et al., 2018)or plasma cells(Khodadadi et al., 2019). Both subsystems of the immune system and the immunological memory will be analyzed in detail in the following sections.

1.1.1 The Innate immune system

The innate immune system is the older part of the immune system. The Russian zoologist, Elie Metchnikoff due to his pioneering research in immunology and more precisely, his discovery of phagocytes (macrophages) in 1882 is considered the father of innate immunity. For this great discovery, he won the Nobel prize in 1908(Kaufmann, 2008).

The innate immune system is found in primitive organisms and in plants and insects and has four principal components. (i) It provides a physical and chemical barrier to infectious agents, and it is primarily responsible for detecting the presence of invaders and abnormality, (ii) It consists of macrophages, DCs, monocytes, NK cells, ILCs, granulocytes (eosinophils, neutrophils, and basophils) and mast cells. However, the innate immune system is complicated and multifactorial, as it consists of (iii) humoral factors, including the complement system and (iv) cytokines and chemokines. Its complexity is needed because the innate immune system not only senses danger signals but builds the first line of defense. In cases of infection or injury, inflammation is initiated by inflammatory mediators released by the injured or resident immune cells, and/or the recruited circulating immune cells, which prevent the spread of the infection and at the same time promote the clearance of pathogens and wound healing(Medzhitov, 2002)(Murphy & Weaver, 2013)(Matzinger, 2002).

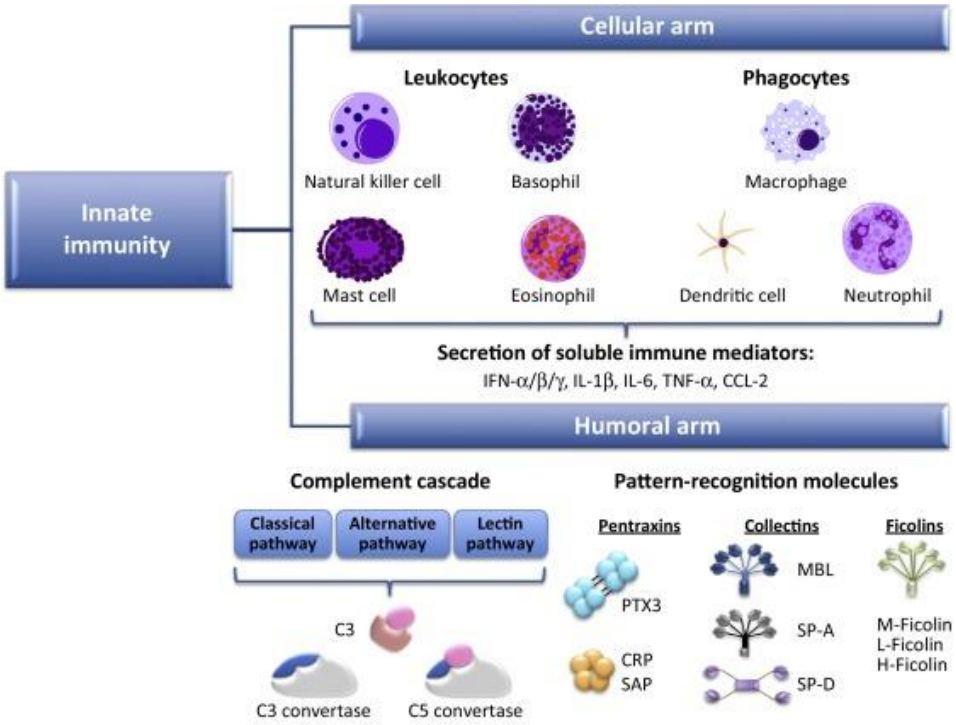


Figure 1:The two arms of innate immune system are the cellular and humoral compartments. The cellular arm of innate immunity consists of immune cells, like leukocytes and phagocytes, as well as immune mediators secreted by these cells. The humoral arm consists of the complement cascade and pattern-recognition molecules (PRMs). Efficient interaction between the two arms of innate immunity is vital to ensure a proper immune response to stimuli and facilitate adaptive immune response(Foo et al, 2015).

More specifically, acute inflammation starts through cells that reside in the tissues. These cells express receptors, known as pattern recognition receptors (PRRs), which recognize structures conserved among microbial species, known as pathogen-associated molecular patterns (PAMPs). PRRs also recognize endogenous molecules released from damaged cells, termed damage-associated molecular patterns (DAMPs). Currently, there are four different families of PRRs. These families include transmembrane or intracellular proteins such as Toll-like receptors (TLRs), C-type lectin receptors (CLRs), as well as cytoplasmic proteins like nucleotide oligomerization domain (NOD)-like receptors (NLRs) and retinoic acid-inducible (RIG)-I-like receptors (RLRs)(Takeuchi & Akira, 2010). These receptors expressed on professional immune cells such as DCs, macrophages and nonprofessional immune cells sense pathogens and different environmental stimuli. Further many of them also transduce strong maturation signals. Their contribution to inducing maturity is enigmatic considering that PRR ligands are not exclusive to pathogenic infection. Ligands are expressed by host commensals and can even be of host origin. It is still not entirely clear how DCs sense these ligands during steady state and yet maintain immune homeostasis(Takeuchi & Akira, 2010)(Medzhitov, 2002). These two opposing features make it challenging but also fascinating to delineate the mechanism of how innate immune specific cell types or cytokines achieve their different tasks(Iwasaki & Medzhitov, 2015).

The innate immunity is known as less specific and as the first line of defense given that in case of vertebrates the adaptive immune response will follow to give a more specific response. However, many recent reports support that innate immunity is much more robust than previously thought. There are

organisms which exclusively rely on it and are capable to deal with a great variety of pathogens and even develop immunological memory. In certain insect species, infection with non-lethal doses of pathogenic bacteria, or priming, confers a protective effect upon subsequent challenge with the same and/or different pathogen. These findings point out the ability of insects to exhibit a form of immune specificity. Further research has suggested that priming of the insect immune system is specific to the insect species and the type of pathogen. The protective effect varies in specificity from providing protection against a wide range of pathogens or specifically against the pathogen to which the insect was initially exposed. In addition, lifelong persistence of immune protection in insects can be accompanied with highly specific recognition of the priming agent(Zipfel et al., 2017). One of these organisms, which was also used to extensively study the innate immunity is the fruit fly, *Drosophila melanogaster*. How does *D. melanogaster* achieve a specific immune response against a viral infection accompanied by immunological memory? Upon viral infection, *D. melanogaster* makes use of haemocytes, circulating blood cells that phagocytose, produce antimicrobial peptides and take up double stranded RNA (dsRNA) to make functional small interfering RNAs (siRNAs). *In vitro* data show that cell lines of haemocytes produce circular copies of viral-derived DNA (vDNA) after RNA virus infection through a reverse-transcriptase-dependent mechanism(Goic et al., 2013). West et al showed that haemocytes produce circular vDNAs to serve as a template for the amplification of secondary vsRNAs *in vivo*, and vDNA production is dependent upon Ago2. vsRNAs are then packaged into exosome-like vesicles (ELVs) and are released from haemocytes to spread protective vsRNAs to distal sites. These are then processed into siRNAs and loaded into functional RNA-induced silencing complexes (RISCs). Notably, West et al and colleagues also showed that these exosomes can confer passive immunity. Exosomes purified from Sindbis-virus-infected flies prevented viral replication when injected into naive animals. This protection was both pathogen specific and long lasting. So, in some organisms the lack of the adaptive immune system does not prevent them from efficiently protecting themselves from pathogens by using alternative immune responses(West & Silverman, 2017)(Flemming, 2017).

1.2 Complement system

A very powerful compartment of innate immunity is the complement system. It was discovered in 1890 by Jules Bordet and in his view, its primary function was to boost the opsonization and killing of pathogens by antibodies (Abs). Therefore, its activity was to “complement” Ab’s tasks and this is how it received its name(Nesargikar & Chavez, 2012). But complement is more than that. It is a sophisticated network of soluble and membrane-bound proteins. More specifically, it consists of more than 30 plasma proteins, which are mainly produced by the liver and are usually circulating in serum and interstitial fluids. Most of these proteins are found in the precursor form, but they are proteolytically cleaved in response to the recognition of molecular components of microorganisms. It is noteworthy that the complement system becomes sequentially activated in an enzyme cascade – the activation of one protein enzymatically cleaves and activates the next protein in the cascade. These proteolytic cascades finally generate the effector complement components that favor the removal or destruction of the pathogen. There is canonical complement activation (classical, lectin and alternative pathways) and non-canonical (intracellular complement activation). I will firstly talk about the canonical complement activation. Complement can be activated systemically in the blood via three main routes: the classical pathway, which is Ab-triggered and was the first to be discovered, the second discovered pathway was the alternative one, which can be initiated by spontaneous hydrolysis and activation of the complement component C3, which can then bind directly to microbial surfaces. The third and last pathway to be discovered was the

lectin pathway, which recognizes and binds carbohydrates found on the surface of pathogens(Murphy & Weaver, 2013)(Nesargikar & Chavez, 2012)(Reis et al., 2019)(Noris & Remuzzi, 2013).

It is compelling that all these three pathways converge at the most essential and critical step in complement activation. When any of the pathways interact with the surface of a pathogen, then the C3 convertase is generated. Dependent on the complement pathway, the C3 convertase varies, but in any case, it is a multi-subunit protein with protease activity that cleaves the complement component 3 (C3). The C3 convertase covalently binds to the pathogen's surface, where it cleaves the C3 and generates large amounts of C3b, the primary effector molecule of the complement system and at a lower extent C3a, a peptide which induces inflammation. The cleavage of the C3 is a critical step in complement activation, as this step leads either directly or indirectly to many effector activities of the complement system. Once C3b is generated, it has a dual role. It can covalently bind to the microbial surface and act as an opsonin, allowing phagocytes, which have complement receptors to take-up and destroy the C3b-coated pathogen(Koski et al., 1983). However, it can also bind to C3 convertases generated from either the classical or lectin pathway, to form another multi-subunit protease, the C5 convertase. The C5 convertase cleaves the complement component C5 into the highly inflammatory peptide C5a and the C5b fragment. C5b is considered the initiator of the late events of the complement activation, during which a couple of additional complement proteins interact with C5b and form the membrane-attack complex (MAC) on the surface of the microbe, which forms a hole on the microbe's surface that results in cell lysis(Tegla et al., 2011)(Morgan, 1989)(Wallis et al., 2010)(Phillips et al., 2009)(Lu et al., 1990)(Bexborn et al., 2008)(Murphy & Weaver, 2013)(Harboe & Mollnes, 2008)(Rawal et al., 2008).

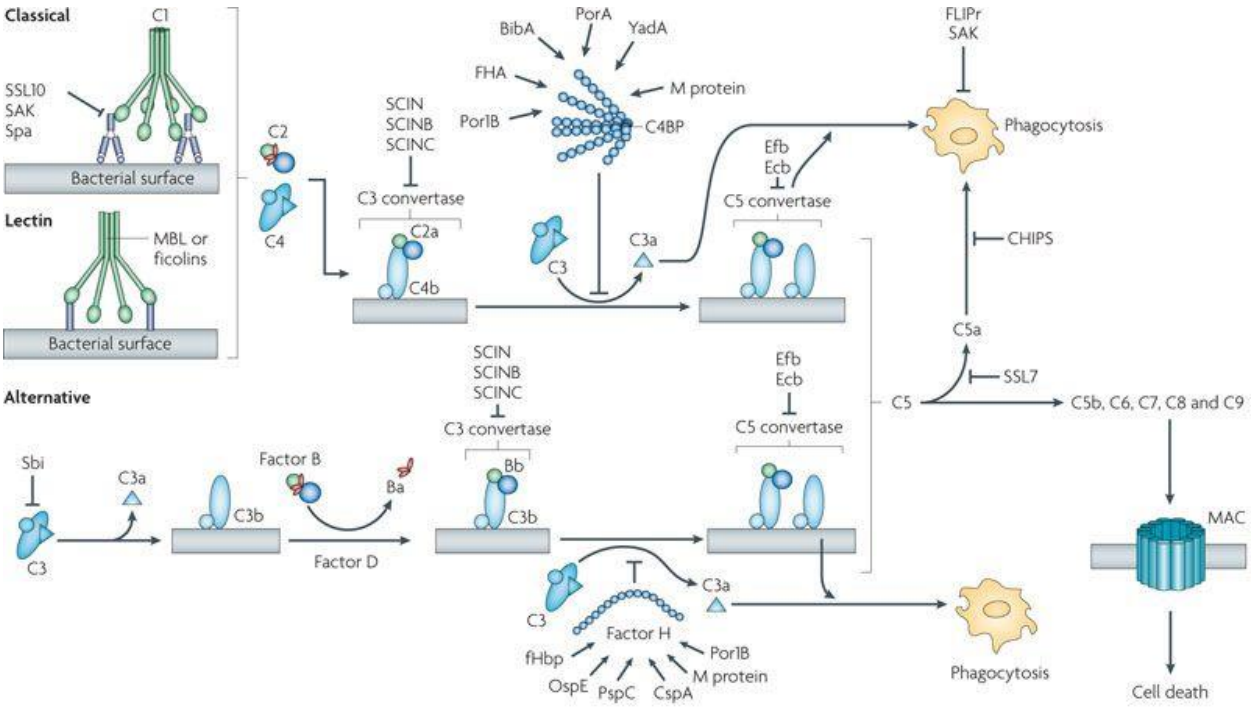


Figure 2: The complement cascade. The classical pathway, the lectin pathway and the alternative pathway. Through the formation of C3 convertases (C4bC2a for the classical and lectin pathways, and C3bBb for the alternative

pathway), these pathways culminate in the generation of the opsonin C3b and the anaphylatoxin C3a. Subsequent C5 convertase formation (C4bC2aC3b for the classical and lectin pathways, and C3bBbC3b for the alternative pathway) leads to C5b and anaphylatoxin C5a generation, with C5b initiating the formation of the MAC and its insertion into target membrane(Serruto et al, 2010).

1.2.1 The classical pathway

The classical pathway even though it is considered as part of the innate immune system, it is known to play a decisive role in both innate and adaptive immunity. Its first and most crucial component is the protein C1q, which acts as a link between the adaptive humoral response and the complement system by binding to Abs bound to antigens (Ags). However, this is not the only way that C1q can trigger complement activation. This protein can also directly bind to the surface of the pathogen and induce the activation of the complement system in the absence of an Ab. However, to be more precise, C1q does not act on its own but rather as a complex of the C1 proteins. The C1 complex consists of C1q and two more molecules, the zymogens C1r and C1s, which are bound to C1q. C1q has six globular heads, which are all kept together with a collagen-like tail, which is surrounded by the $(C1r: C1s)_2$ proteins. When more than one heads, of the C1q molecule bind to the pathogen's surface, then this causes a conformational change in the $(C1r: C1s)_2$ complex, which results to activation of an autocatalytic enzymatic activity in C1r; the active form of C1r then cleaves its associated C1s to generate an active serine protease(Gaboriaud et al., 2004)(Bally et al., 2009). Upon C1s activation, the C1s protease acts on the two components of the classical pathway, and it cleaves the protein C4, to generate the C4a and C4b. The C4b binds covalently to the surface of the pathogen(Gregory et al, 2003). The covalently attached C4b then binds one molecule of C2, making it susceptible, in turn, to cleavage by C1s, which results in the generation of C2b and C2a (it is also an active serine protease). The complex of C4b with the active serine protease C2a remains on the surface of the pathogen as the C3 convertase of the classical pathway. Its most important activity is to cleave large numbers of C3 molecules to produce C3b molecules that coat the pathogen surface. At the same time, the other cleavage product, C3a, initiates a local inflammatory response(Murphy & Weaver, 2013).

1.2.2 The lectin pathway

In case of the lectin pathway there is no need of Abs for its activation. The lectin pathway makes use of PRMs, known as mannan-binding lectin (MBL), ficolins (ficolin 1, 2 and 3) or collectins-10/-11, which recognize carbohydrate ligands on microbial surfaces and activate the complement system. The PRMs of the lectin pathway are soluble molecules, which consist of a collagen-like domain and a carbohydrate-binding domain. The ligand specificities of the PRMs of the lectin pathway include the carbohydrates like mannose, *N-acetylglucosamine* and b-glucan. The recognition structure of each of these molecules, defines two categories: the C-type lectins (MBL, collectin-10 and -11) and fibrinogen-like proteins (ficolin-1, ficolin-2 and ficolin-3). The effector functions of the PRMs are mediated through the serine proteases MASP-1, MASP-2 and MASP-3. In the past, MASP-2 was considered to be auto-activated and the only active serine protease in the lectin pathway. However, more recent data revealed that MASP-1 activates MASP-2 and is necessary for the activation of the lectin pathway. Once the PRMs form a complex with MASP-1 or MASP-2, they catalyze the cleavage of C4 and C2, as described in the previous paragraph. This finding highlighted the resemblance between the classical and the lectin pathway as MASP-1 and MASP-2 closely resemble C1r and C1s in their homology and way of action. Regarding the role of MASP-3, not much is known as it is the least studied serine protease of the lectin pathway. Some recent studies showed that MASP-1 activates MASP-3, which then cleaves pro factor D to factor D and thus has a role on the activation of the alternative pathway. Hence, the lectin pathway, induces complement activation in a way

similar to the classical pathway, forming a C3 convertase from C2a and C4b(Feinberg et al., 2003)(Gregory et al., 2004)(Garred et al., 2016)(Rosbjerg et al, 2017).

1.2.3 The alternative pathway

As mentioned above, the alternative pathway was the second one to be discovered, and this is how it took its name. In contrast to the classical and lectin pathway, the activation of the alternative pathways does not require neither Abs nor specific structures on the microorganisms. It rather gets activated due to spontaneous hydrolysis (tickover) of the thioester bond in C3 to form C3(H2O). The protein C3 is abundant in plasma and tickover results in a steady low-level production of C3(H2O). The C3(H2O) can then bind to factor B, which is cleaved by factor D, producing a short-lived C3(H2O)Bb convertase, which can cleave C3 into C3a and C3b. Much of this C3b is inactivated due to hydrolysis, but the rest binds through its thioester bond to the pathogen's surface and binds factor B, resulting to the formation of the C3 convertase and inducing C3b production. Usually, the C3 convertase of the alternative pathway is very short lived, and this pathway may be favored by Factor P (properdin), which can bind to the convertase and stabilize it. However, this is not the only function of properdin. It is known that it can also directly bind to microbial surfaces and thus it is believed that it can also act as a PRR. Because of its dual role, both as a stabilizer of the C3 convertase of the alternative pathway and as a PRR, properdin could direct the activity of the alternative pathway to pathogen surfaces. It is known that the alternative pathway also acts as an amplification loop for both the classical and lectin pathways and the presence of ligand bound pentraxins seems to mediate interaction between all three pathways(Bexborn et al., 2008)(Murphy & Weaver, 2013)(Harboe & Mollnes, 2008)(Rosbjerg et al., 2017).

1.2.4 Regulation of the complement system

It is evident that all these pathways result in inflammatory and destructive effects and considering that they also include some amplification steps (alternative pathway) could be of danger even for the host and thus should be strictly regulated. The immune system has taken care of that. Firstly, the activated complement proteins are immediately inactivated unless they bind to the surface of the pathogen on which their activation started. Secondly, there are many inhibitory proteins at several points of the pathway, that prevent the activation of complement on the surfaces of the healthy host cells, thereby protecting them from accidental damage. These inhibitory proteins do not only act in a protecting way for healthy host cells but inactivate complement once the clearance of pathogens is complete. The two main regulation mechanisms are: decay-acceleration activity (DAA) which increases the rate of dissociation of (C4b2a and C3bBb) C3 convertases, and factor I cofactor activity (CA), which results in the factor I-mediated cleavage of covalently bound C3b and C4b into inactive fragments incapable of reforming the C3 convertases(Nesargikar & Chavez, 2012). The pathways are regulated by both membrane-bound and fluid phase complement regulators that keep the complement system in check. There are several regulators of the complement system, and I will discriminate them as plasma and membrane bound. I will firstly talk about the plasma regulators. **C1 inhibitor** is one of them and is known to irreversibly bind to and inactivate C1r and C1s of the classical pathway and MASP-1 and MASP-2 of the lectin pathway, thus inhibiting the initiating steps of these activation pathways. Two additional plasma regulators, which have a key role on the inhibition of the alternative pathway are the **complement factor H** (CFH) and **complement factor I** (CFI). If one of them is missing, or fully dysfunctional, the activation of the alternative pathway in plasma is vigorous and leads to secondary complement deficiency via overconsumption of C3 and other complement components. CFI is a plasma serine protease that is able to permanently inactivate C3b to iC3b by proteolysis but needs a cofactor. iC3b then is cleaved into further fragments (C3dg and

C3c) by CFI, again with the need for cofactor proteins. CFH is one such cofactor. CFH controls the alternative pathway activation by competing with complement factor B in binding to C3b, acting as a cofactor for CFI in the C3b cleavage, decreasing the stability of the C3bBb convertase complex and accelerating the dissociation to C3b and Bb (decay accelerating activity). Regarding the CFI also has a regulatory role in the classical and lectin pathways. More specifically, it cleaves the C4b component of the C3 convertase C4bC2a in the presence of the plasma cofactor C4b-binding protein. Of interest, C4b-binding (C4-bp) protein can also favor C3b inactivation, although to a lesser degree. The plasma regulator C4bp regulates the inhibition of the classical and lectin pathways. It achieves that through binding to C4b and accelerates the decay of the C3 convertase. It can also act as a cofactor for the cleavage of C4b by factor I(Nesargikar & Chavez, 2012)(Noris & Remuzzi, 2013).

The focus will now be on the membrane-bound complement regulators. This category includes the membrane cofactor protein (**MCP/CD46**)(Hakulinen et al., 2004), the decay-accelerating factor (**DAF/CD55**)(Brodbeck et al., 2000), and the **CR1** (**CD35**)(Ahearn & Fearon, 1989). MCP acts as a cofactor for C3b and C4b cleavage by CFI, but it only protects those cells on which it is expressed. The DAF accelerates the dissociation of the C3bBb AP C3 convertase similarly to CFH in case of the alternative pathway, but it decreases the stability of the C3 convertase of the classic and lectin pathways, C4bC2a, by accelerating its dissociation to C4b and C2a. CR1 on circulating cells mainly acts as an immune adherence receptor to facilitate the removal of C3b/C4b-opsonized immune complexes and pathogens from the circulation, but it also exerts complement inhibitory activities. CR1 has cofactor activity for CFI-mediated cleavage of C3b to iC3b, and thereafter to C3c and C3dg, and of C4b(Zipfel & Skerka, 2009)(Ferreira & Pangburn, 2007)(Nesargikar & Chavez, 2012)(Noris & Remuzzi, 2013).

Table 1: Regulators of complement system

	Target molecules	Classical Pathway	Lectin Pathway	Alternative Pathway
DAF	C4b2b, C3bBb	+	+	-
Factor H	C3b	-	-	+
Factor I	C3b, C4b	+	+	+
CD46	C3b, C4b	+	+	+
C1-inhibitor	C1r, C1s, MASP-2	+	+	-
C4-bp	C4b	+	+	-
CR1	C3b, C4b	+	+	+

1.2.5 The anaphylatoxins and their role in health and disease

Two complement fragments, which are generated upon complement activation are the small peptides C3a and C5a, which are also known as ATs. These two peptides are generated as a result of the proteolytic cleavage of the proteins C3 and C5 respectively. They are exerting their biologic functions through activating specific receptors, to produce local inflammatory signals(Klos et al., 2009)(Klos et al, 2013)(Coulthard & Woodruff, 2015). C5a is known as the most potent inflammatory molecule among all activated complement fragments. Once activated, the ATs can mediate a great variety of effector functions(Nordahl et al., 2004). Both ATs can cause smooth muscle contraction and an increase in vascular permeability. Depending on the cell type that is activated, they can induce a variety of immune reactions. For instance, in macrophages(Murakami et al, 1993), neutrophils(Elsner et al, 1994b), and eosinophils(Elsner et al, 1994a) C3a and C5a can trigger oxidative burst and thus regulate the generation of reactive oxygen species. On top of that, the ATs also mediate the release of histamine form

basophils(Kretzschmar et al., 1993) and mast cells(el-Lati et al, 1994). In case of the eosinophils, C3a and C5a control the production of eosinophil cationic protein, their adhesion to endothelial cells as well as their migration(Takafuji et al, 1996)(DiScipio et al, 1999). C3a further promotes serotonin release from guinea pig platelets(Fukuoka & Hugli, 1988) and modulates synthesis of IL-6 and TNF-α from B cells and monocytes(Fischer & Hugli, 1997)(Fischer et al, 1999). C5a is known as a powerful chemoattractant for macrophages(Aksamit et al., 1981), neutrophils(Ehrengruber et al, 1994), activated B(Ottonello et al., 1999), and T cells(Nataf et al, 1999), basophils(Lett-Brown & Leonard, 1977) and mast cells. However, the ATs do not only have pro-inflammatory properties but they also mediate tissue regeneration(Mastellos et al, 2001)(Strey et al., 2003), tissue fibrosis(Strey et al., 2003)(Hillebrandt et al., 2005)(Addis-Lieser et al, 2005) as well as brain development(Bénard et al., 2004).

As mentioned on the previous section, the complement system needs tight regulation. Since the ATs are two potent complement fragments with pro-inflammatory activity, the immune system developed mechanisms to constrain their action through AT degradation. This is achieved through the action of carboxypeptidases, which closely control the two ATs and are capable of cleaving a C-terminal arginine residue, resulting in the generation of C3adesArg and C5adesArg(Bokisch & Müller-Eberhard, 1970)(Matthews et al, 2004). The C3adesArg does not have any receptor-mediated inflammatory function, but the C5adesArg retains 1-10% of its pro-inflammatory activity(Reis et al., 2012)(Sayah et al., 2003).

1.2.6 The anaphylatoxin receptors

As mentioned in the previous paragraph, the ATs exert their functions through specific receptors, known as anaphylatoxin's or complement's receptors. There are three known anaphylatoxin's receptors (ATRs), which are part of a superfamily of GPCRs, known as C3aR, C5aR1 and C5aR2. They share high-sequence homology and are closely related to other chemotactic receptors. Regardless of their similarity, the AT receptors differ in ligand specificity, signal transduction capacity, and function.

The **C3aR** exclusively binds C3a and recognizes neither its degradation product C3adesArg nor C5a(Crass et al., 1996)(Ames et al., 1996). Upon binding of C3a to C3aR, intracellular signal transduction is initiated via heterotrimeric G proteins. More specifically, depending on the cell population, signaling through C3aR can be mediated either by pertussis toxin sensitive or insensitive G proteins(Norgauer et al., 1993)(DiScipio et al., 1999). C3aR is expressed by cells of the myeloid origin like neutrophils, basophils, eosinophils, mast cells, monocytes, macrophages, DCs and microglia(Glovsky et al, 1979)(Daffern et al, 1995)(A Klos et al., 1992)(Zwirner et al., 1998)(Gutzmer et al., 2004). Quell et al also confirmed the expression of C3aR in the murine myeloid cells. More precisely, it was shown that the eosinophils express the receptor intracellularly, the mucosal DCs are also positive for its expression, the neutrophils express C3aR only upon activation and the expression of C3aR was also detected in the $SiglecF^-$ macrophages(Quell et al., 2017). However, the expression of C3aR is also found in non-myeloid cells like astrocytes in the inflamed brain(Ischenko et al., 2002), endothelial cells(Monsinjon et al., 2003), epithelial cells, smooth muscle cells, submucosal and parenchymal vessels of the lungs of patients with asthma(Fregonese et al., 2005). Some studies report the expression of C3aR on activated human $CD4^+$ T cells but not under steady state conditions(Martin et al., 1997), while in mice no C3aR expression was detected neither on naïve nor activated $CD4^+$ T cells(Quell et al., 2017).

Concerning **C5aR2**, it binds C5a but has a 20-fold higher affinity for the C5a-desArg(Cain & Monk, 2001)(Shoji Okinaga et al., 2003). The C5aR2 is uncoupled from G-proteins due to its lack of certain

intracellular motifs like DRY and NPXXY and thus it was suggested that its function is to act as a decoy-receptor, which only regulates C5aR1 and binds to excessive C5a(Okinaga et al., 2003). More precisely, according to Scola et al, the way that C5aR2 acts as an anti-inflammatory decoy receptor is by binding to C5a and this binding leads to ligand degradation and thus prevents the binding of C5a to C5aR1 that could cause inflammation(Scola et al, 2009). However, many recent studies question the view that C5aR2 is a decoy receptor and report that C5aR2 has either a pro- or anti-inflammatory role (Li et al, 2013)(H. Gao et al., 2005)(Croker et al., 2014). More specifically, it was shown that in neutrophils, C5aR2 deficiency results in an altered cytokine profile upon stimulation with C5a. The data showed that in this experimental setting, C5aR2 controlled the C5a-induced upregulation of IL-6, TNF-α, and CR3(H. Gao et al., 2005). Moreover, mice deficient for C5aR2 showed improved survival in the cecal ligation puncture model of sepsis. This finding is in agreement with the observation that upon C5aR2 deficiency reduced inflammatory cell infiltration and HMGB-1 production from macrophages and neutrophils is noticed(Rittirsch et al., 2008). C5aR2 is known to be expressed by neutrophils, eosinophils, in mucosa-associated DCs, in NK cells, in B lymphocytes, in case of macrophages tissue-specific differences were noticed and no C5aR2 expression was detected in T lymphocytes(Karsten et al., 2017).

The **C5aR1** is the best characterized receptor of the three. C5aR1 is a membrane glycoprotein that binds both C5a and its degradation product C5adesArg but with different affinities. C5aR1 binds to C5adesArg with 10-100 fold lower affinity in comparison to the C5a (Boulay et al, 1991)(Gerard & Gerard, 1991)(Reis et al., 2012). It is encoded in two exons by the C5AR1 gene on chromosome 19 in humans and chromosome 7 in mice, arising a seven transmembrane domain protein. C5aR1 undergoes several post-translational modifications. The receptor gets glycosylated at the N-terminus, which does not affect neither the ligand binding nor the receptor expression(Pease & Barker, 1993). Additionally, the N-terminus can get sulfated, which has an effect on ligand binding(Farzan et al., 2001)(Ippel et al., 2009)(Scola et al., 2007). The interaction between C5a and C5aR1 includes a dual binding motif, a recognition site for the disulfide-linked core of C5a, which is located in the N-terminal extra-cellular domain of the receptor and a second site, which involves Asp191/Glu199 and Arg206 of the second transmembrane loop of C5aR1, in binding Lys68 and the terminal carboxylate group of C5a, respectively(Monk et al, 2007). Once C5a binds to C5aR1, then the carboxy-terminus is heavily phosphorylated.

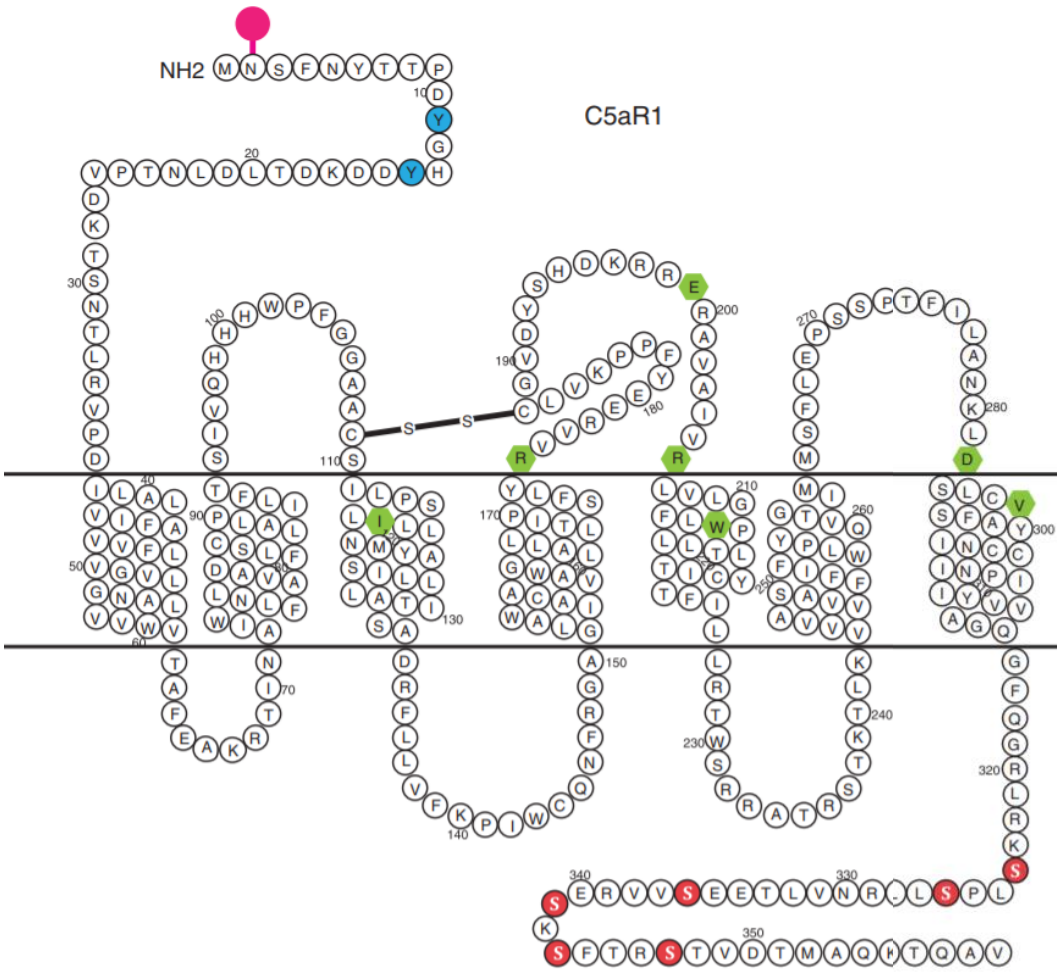


Figure 3: Sequence and domain structure of C5aR1. The pink symbol denotes the site for glycosylation and phosphorylation at the intra-cellular domains of the receptors, when the tyrosine sulfation sites at the extra-cellular domains are denoted by blue circles. The green circles denote the ligand-binding sites located at the extra-cellular domains (Verschoor et al, 2017).

C5aR1 is found on the majority of the myeloid cells like neutrophils, macrophages, eosinophils and DCs(Karsten et al., 2015)(Monk et al., 2007). More precisely, regarding the expression of C5aR1 by DCs, by using a GFP reporter mouse, Karsten et al demonstrated that the C5aR1 is expressed by the $CD11b^{+}$cDCs and mo-DCs but not by the $CD103^{+}$cDCs(Karsten et al., 2015). The expression of C5aR1 by T and B cells is under discussion as Karsten et al showed that there is no expression when Strainic et al could detect C5aR1 expression in T cells(Lalli et al., 2008)(Strainic et al., 2008)(Karsten et al., 2015).

Binding of C5a to C5aR1 in granulocytes and monocytes results in cytoskeletal remodeling and up-regulation of adhesion molecules/complement receptors (CR1, CR3/4), chemotaxis, apoptosis, granule release, neutrophil extra-cellular trap formation and synthesis of reactive oxygen metabolites(Klos et al., 2009)(Kemper & Köhl, 2013)(Kordowski et al., 2018)(Karsten et al., 2012)(Perianayagam et al., 2002)(DiScipio et al., 1999)(Aksamit et al., 1981). Activation of C5aR1 in DCs, controls the expression costimulatory molecules as well as the production of IL-12 family cytokines and transforming growth factor beta (TGF-β) and IL-6, thereby controlling the activation and differentiation of T cells(Hawlisch et al., 2005)(Schmudde et al., 2013)(Engelke et al., 2014)(Sheen et al., 2017).

When C5a binds to C5aR1 induces a complex signaling cascade, which results in the activation of kinases, guanosine triphosphate (GTP)-binding/regulatory proteins, transcription factors and other signaling enzymes or structural proteins. Signaling through C5aR1 can be mediated either by pertussis toxin sensitive (Giα2 and Giα3)(Skokowa et al., 2005) or insensitive (Gαs and Gα16) G proteins(Monk & Partridge, 1993). When C5a binds to C5aR1 results in calcium flux both from intracellular stores but also from the extracellular medium(Braun et al, 2003). Upon activation, the β-arrestins 1 and 2 bind to C5aR1, targeting it for receptor internalization via clathrin coated pits. The β-arrestins are important players on the regulation of the GPCR signal transduction and their binding depends on phosphorylation of the C-terminus of the receptor by G-protein coupled receptor kinases (GRKs). But GRKs do not only act as kinases but they have additional functions, as they interact with other components of intracellular signaling like Akt, MAPK/ERK kinase and PIEK-γ. It was shown that upon C5aR1 activation, there is downstream, activation of several components of signaling pathways like PI3K-γ kinase(Perianayagam et al, 2002)(la Sala et al, 2005), phospholipase C β2(Jiang et al., 1996), phospholipase D(Mullmann et al, 1990) and Raf-1/B-Raf mediated activation of MEK-1(Buhl et al, 1994).

1.2.7 Interaction of complement with other parts of innate immunity

Interestingly, inflammatory conditions often lead to simultaneous initiation of the complement pathway and activation of PRRs like TLRs, NLRs, NOD-, LRR- and pyrin domain-containing 3 (NLRP3) inflammasomes and CLRs on immune cells(Köhl, 2006). The engagement of complement receptors to their activation fragments triggers the recruitment of cytoplasmic adaptor molecules, enabling crosstalk with other signaling pathways(Klos et al., 2013)(Hajishengallis & Lambris, 2016).

One of best studied synergisms is the one between the complement receptors C3aR and C5aR1 with the TLRs. Studies showed that this synergism upregulates the expression of co-stimulatory molecules on APCs and induces the production of pro-inflammatory cytokines(Hawlisch et al., 2005)(Hajishengallis & Lambris, 2016)(Bosmann et al., 2012). An interesting observation is that C5a differentially modulates TLR4-induced responses in monocytes and macrophages, as it causes an enhanced production of inflammatory cytokines in monocytes when in macrophages it downregulates these responses(Seow et al., 2013). However, the crosstalk between C5aR1 and TLR4 is not confined on APCs but studies showed that in a sepsis model, this crosstalk controls the production of IFN-γ and TNF in NK and NKT cells(Fusakio et al., 2011). Additionally, in a rodent model of polymicrobial sepsis, it was shown that the activation of TLR2, TLR3 and TLR4 promotes the synthesis of factor B by macrophages and cardiac cells, resulting in activation of the alternative pathway(Zou et al., 2013).

Several studies showed that complement is involved in crosstalk with other cell receptors that modulate immune responses besides TLRs. More precisely, Huang et al showed that complement-mediated signaling promotes dectin 1-mediated phagocytosis by DCs and activation of NLRP3 inflammasomes(Huang et al., 2012). But C5aR1 also interacts with other members of the GPCRs family. An *in vitro* study, in which macrophages were infected with laboratory strains of HIV, showed that the CCR5-mediated infection of macrophages with HIV is C5aR1 dependent, as according to Fernandez et al the C5aR1 acts as an enhancer of CCR5-mediated HIV entry into macrophages(Fernandez et al., 2016).

All these findings suggest that complement through its cooperation with other parts of innate immunity shapes the type and magnitude of an immune response. Even though the majority of the data comes from animal models and thus the physiological relevance of these kind of crosstalk in humans needs further exploration, the current dogma is that complement both promotes the elimination of microbial intruders

and on top of that it also contributes to the repair and maintenance of tissue homeostasis and immune tolerance.

1.2.8 Non canonical pathways of complement

1.2.8.1 Local complement activation in tissues

So far, I focused on the canonical complement activation but as I mentioned above, there is non-canonical complement activation as well, which happens without initiation of the entire complement cascade and occurs independent of the convertase formation. The non-canonical pathway relies on proteases, which regulate the cleavage of C3 and C5 into C3a/b and C5a/b. Interestingly, non-canonical complement activation is rapid and requires less energy waste. It has been shown that almost every cell type can regulate the production of complement proteins(Morgan & Gasque, 1997). The kallikrein-related peptidase 14 (KLK14), which is found in tissues and biological fluids, is very effective at the C3 cleavage(Oikonomopoulou et al., 2013). Also, thrombin can efficiently generate biologically active C5a, thereby linking the coagulation with the complement system(Huber-Lang et al., 2006). Moreover, some phagocytic cells, like alveolar macrophages or Kupffer cells can produce and cleave C5 and are thus able to generate C5a independently of a C5 convertase(Huber-Lang et al., 2002a)(Morgan & Gasque, 1997). Cathepsin L is another protease, which has the capacity to activate C3 in the lysosomes of resting human T cells(Liszewski et al., 2013)(Kolev et al., 2014). In the context of allergic asthma, there is evidence that Dermatophagoides farinae-derived protease (Der p) 1, a protease found in HDM cleaves C3 and C5 into C3a and C5a, respectively(Maruo et al., 1997).

1.2.8.2 Intracellular complement activation and its functions

In the past when people were referring to either systemic or local complement activation, it was considered that it takes place in the extracellular space. Many complementologists started to doubt the notion that complement activation was confined to extracellular space and supported the concept that it can also happen intracellularly(Liszewski et al., 2013)(Arbore et al., 2016). In the beginning, people were suspicious and judgmental about that hypothesis, but nowadays, it is widely accepted not only that the complement system can get activated intracellularly but also that intracellularly activated complement has other non-immune related functions. So, the Kemper laboratory found big intracellular stores of C3 in resting human $CD4^+$ T cells and intracellular expression of C3aR and cathepsin L. They showed that cathepsin L can continuously cleave the intracellular C3 into C3a and C3b in resting T cells. The binding of intracellularly generated C3a to the intracellular C3aR maintains T cell survival via induction of low-level mTOR(Liszewski et al., 2013). Once these T cells get activated, the whole intracellular complement system instantly translocate to the cell surface, where C3a and C3b ligate the C3aR and CD46 receptors respectively and signal in autocrine way leading to IFN-γ production and Th1 responses(Liszewski et al., 2013). This finding is of outmost importance as it shows that while binding of C3a and CD46 on their surface receptors regulates Th1 induction, the intracellular C3 activation is needed for T cell survival. T cells in which C3aR expression is blocked do not survive. This study from Liszewski et al, nicely showed that the location of complement activation and the engagement to the receptor (intra VS extracellular) is critical for the functional outcome.

Interestingly, it was published that T cells are equipped with an intracellular C5 system as well, which is vital for normal T cell activation(Arbore et al., 2016). Similar to what was observed for C3, Arbore et al showed that human T cells also possess intracellular stores of C5 which can be cleaved into C5a by an unknown protease. When T cells get activated the intracellularly generated C5a binds to intracellular

C5aR1 which leads to elevated ROS production and subsequent activation of NACHT, LRR and PYD domains-containing proteins 3 (NLRP3) inflammasome. This finding was in agreement with the observations of Samstad et al, who showed that C5aR1 drives inflammasome activation in myeloid cells(Samstad et al., 2014). Arbore et al also detected intracellular expression of C5aR2, and signaling of either the C5a or C5a-desArg through C5aR2 results in a negative regulation of the C5aR1-driven NLRP3 inflammasome activity(Arbore et al., 2016).

Based on the findings that human T cells and myeloid cells are equipped with a great variety of complement components, receptors and regulators, some complementologists tried to compare the inflammasome with the intracellular complement system and thus the term "complosome" emerged. The data so far suggest that complosome has a regulatory role on the cell metabolic machinery. Strikingly, complosome is not confined on the immune cells and some recent studies reported its role in non-immune cells as well. Satyama et al associated intracellular C3 activation in intestinal mucosal cells with local ischemia/reperfusion injury(Satyam et al., 2017), when Jung et al suggested that complosome controls neuronal fitness as they showed that mesenchymal stem cells induced down-regulation of intracellular C3 expression in neurons to promote their survival under hypoxic conditions(Jung et al., 2016). Of note, the comlosome also interacts with intracellular pathogens and depending on the pathogen and cell type, this interaction can either promote pathogen clearance but in some cases, it can result in pathogen survival(Abdul-Aziz et al., 2016)(Chen et al., 2014)(Appledorn et al., 2008). The complex interactions between complosome and intracellular pathogens need further investigation on a bigger variety of cell types.

1.3 Dendritic cells (DCs)

Another member of innate immunity with a vital role in bridging innate and adaptive immunity are the DCs. These cells were discovered in the late 1970s by Ralph Steinman and Zanvil Cohn. Back then, the notion that DCs have a unique role in the immune system received much skepticism, and it took many years before their role was widely accepted(Steinman & Cohn, 1974). Finally, almost forty years after the initial discovery, Steinman's work was highly appreciated, and he won the Nobel prize in 2011. It was finally accepted that DCs could mount an adaptive immune response towards pathogens.

However, Steinman was not the first one to study DCs. In 1868, the pathologist Paul Langerhans observed a cell type in the skin, today known as Langerhans Cells (LCs). These cells had a stellate morphology, resembling the nerve cells and he mistakenly identified the LCs as nerve cells. Once the presence of DCs in lymphoid organs, and their unique role in the immune system was recognized, it was noticed that DCs share many immunogenic properties with LCs, which made scientists think that there is a DC family with more than one categories (Schuler et al., 1985).

This hypothesis was only the beginning in the studies followed, which revealed key features of DCs. More precisely, the studies revealed that in most nonlymphoid tissues, a cell type with similar phenotype with the so-called DCs was found. Upon Ag encounter, DCs take up Ag and traffic to the secondary lymphoid organs through the lymphatics. There, they would reside in the T cell zone and present a peptide fragment of the Ag to naïve T cells. Findings from these studies, support the concept of DCs acting as the sentinels of the immune system, having as their primary goal to guard the tissues and capture Ags in order to educate the T cells in response to peripheral cues. However, the way DCs act is not only to activate T cells but also tolerize them towards self-Ags, preventing autoimmune reactions (Banchereau & Steinman, 1998).

As already mentioned, DCs have a stellate morphology and extend dendrites at specific developmental stages. These dendrites give the cells their name, as in Greek denro or δένδρο means tree. In general, the separation and discrimination of DCs from other cells and specifically from other immune cells like macrophages and monocytes is difficult as they do not express exclusive DC markers. Therefore, a combination of expressed and absent surface markers has been used to identify DCs. More specifically, this combination includes the strong expression of MHC-II and the integrin CD11C and the absence of various lineage markers such as CD3 (T cell), CD19 (B cell), CD49b (natural killer cell), SiglecF (eosinophils) and Ly6G (granulocytes). At certain developmental stages, DCs also express costimulatory molecules including CD80 (B7.1), and CD86 (B7.2), CD40 and OX40L which are upregulated upon DC activation. To identify DC subtypes, additional markers are used(Plantinga et al., 2013) and in many cases transcription factors are also used for defining them (it will be discussed in detail later).

However, DCs are not only characterized by their morphology and the expression of several markers but also by their specific functions. DCs are widely known as APCs because an important function is to present antigens and induce a primary immune response in resting naïve T lymphocytes. Their dendritic extensions help them to form close contact with multiple T cells simultaneously. However, DCs need to go through a specific process before being able to successfully prime T cell responses. This process consists of four steps including i) Ag uptake, ii) Ag processing, iii) Ag loading on the groove of the MHC molecules and iv) Ag presentation and DC maturation(Mellman & Steinman, 2001).

1.3.1 Antigen uptake by dendritic cells

DCs capture the Ag at peripheral sites, and then they migrate to secondary lymphoid organs, where they encounter naïve T cells and activate them. The Ag can be taken up by three different mechanisms: phagocytosis, pinocytosis, and receptor-mediated endocytosis. When **phagocytosis** takes place, large particulates (cells or bacteria) are recognized by membrane receptors, which induce the formation of large endocytic vesicles, known as phagosomes. The formation of phagosomes requires the reorganization of the actin cytoskeleton, which is necessary for molding the plasma membrane around the phagocytosed particles. The basic principles apply of **pinocytosis** (macro- and micropinocytosis) as well. In the case of **macropinocytosis**, reorganization of the actin cytoskeleton is required, but in this process, the vesicle is formed around a large volume of extracellular fluid(Sallusto et al, 2002). To ensure a continuous and stable uptake of extracellular fluid, immature DCs make use of several members of the aquaporin family(Engel et al, 2000), and membrane channels that facilitate the elimination of excess water across the endosomal membranes(Engel et al., 2000). Engulfment of large portions of extracellular volume followed by the release of excess water results in a highly efficient concentration of solutes in the endosomal compartments of immature DCs(J. P. Lim & Gleeson, 2011). Another mechanism of pinocytosis is **micropinocytosis**. In this case, there is no requirement for actin polymerization. The vesicle formation takes place via the cytosolic protein clathrin, which is used for the formation of clathrin-coated-pits that surround the engulfed extracellular medium. Lastly, regarding the **receptor-mediated endocytosis**, DCs express a variety of receptors, which can be used for Ag uptake. These receptors include Fc receptors(Den Haan & Bevan, 2002)(Ravetch & Bolland, 2001)(Sallusto et al, 2002), PRRs like members of the C-type lectin family such as the mannose receptor(Sallusto et al., 2002), DEC-205(Mahnke et al., 2000), DC-SIGN(Van Kooyk & Geijtenbeek, 2003) and the TLR family(Blander & Medzhitov, 2006). Notably, the expression pattern of many of these antigen receptors varies among DC subsets, suggesting that the DC populations may be specialized at presenting antigens derived from different sets of pathogens(Caminschi et al., 2001)(Linehan et al, 1999)(Mommaas et al., 1999)(Valladeau et al., 2000)(Vremec & Shortman,

1997). Further not all these receptors act the same way. More specifically, it was shown that some of them deliver activatory whereas others deliver inhibitory signals, suggesting that they may have a dual role as both antigen receptors and modulators of the immune response(Den Haan & Bevan, 2002)(Van Kooyk & Geijtenbeek, 2003)(Chang et al., 2002)(Introna et al., 2003)(Blum et al, 2013).

1.3.2 Antigen processing by dendritic cells

Once DCs capture the Ag, then the second step is its processing, as T cells do not recognize whole Ags. As stated above, DCs use two different systems for Ag processing depending on the location of the Ag inside the cell. The first of the two is called the **endocytic pathway** and the other one the **cytosolic pathway**. The first is known to deal with exogenous components whereas the second deals with endogenous ones. However, what does endogenous and exogenous mean? With the term endogenous, we refer to components that are synthesized by the DCs themselves, exogenous means that these components were taken up by endocytosis. It should be mentioned here, that most cytosolic proteins are endogenous. However, under the conditions that they are exogenous, a mechanism known as cross-presentation is being used for the Ag presentation(Joffre et al, 2012). On the other hand, it is also misleading to assume that all the contents of the endosomes are exogenous, when in fact under steady-state conditions these contents are predominantly endogenous, comprising membrane proteins that are delivered to lysosomal compartments at the end of their life span, or standard components of the endocytic route such as proteases or ATPases (in autophagy)(Blum et al., 2013). In the following sections I will outline main differences between the two systems.

The **endocytic pathway** includes tubulovesicular structures and numerous proteases, known as cathepsins with variable substrate specificity and pH requirements(Mcgrath, 1999). The endocytic pathway consists of three major parts. The first one are the **early endosomes** (EEs), whose limiting membrane and lumen have a similar composition to the plasma membrane and the extracellular medium respectively. The second one are the **late endosomes** (LEs), which accommodate proteases that can only be found in the endocytic pathway and are more acidic in comparison to the EE. The third and last part are the **lysosomes**, which are the final station of the pathway and are highly acidic and proteolytic. Once DCs endocytose Ag, it predominantly moves along the EE-LE-Lysosome axis, ending up in the lysosomes. The endosomal proteases are then delivered to the endocytic route directly from the Golgi and are primarily retained in endocytic compartments(Chapman et al, 1997). Even though we still do not know how the decision making is done in each case, concerning the transmembrane proteins, it has been shown that they carry this information on their cytoplasmic region. It is this encoded information that determines how the protein will enter the endocytic route, whether it will leave and how(Kirchhausen et al, 1997). This information is probably being recognized by cytosolic proteins, which based on that, regulate protein sorting. It is still not well understood how this sorting mechanism interprets the information from the cytoplasmic region or in general how the mechanism works(Gruenberg, 2001)(Blum et al., 2013)(McCormick et al, 2005).

In cases of endogenous proteins, the second major Ag processing route, the **cytosolic system** is in action. Under these conditions, the proteins become ubiquitinated and headed to the proteasome for degradation. The proteasome is a multimeric complex located in the cytosol and consists of several proteolytic and regulatory subunits. In proteasomes, the proteins get fragmented into peptides. The proteasome digestion products usually require N-terminal trimming by cytosolic peptidases, and the produced peptides are ready to be transported to the endoplasmic reticulum (ER)(Rock et al, 2004)(Kessler et al., 2003). Once the peptides are generated, they are transferred to the rough ER for loading on the groove (Rock et al., 2002). The transfer of the peptides to the ER is needed as the MHC-I

molecules are synthesized there and are never exposed to the cytosol. Once the freshly synthesized MHC-I molecules enter the membranes of the ER, they bind to calnexin, a chaperone protein, which keeps the MHC-I molecule in a partially folded state. However, it seems that not all peptide-MHC-I complexes make use of the cytosolic pathway. It has been shown that in some cases when exogenous Ags are presented on MHC-I, the processing does not necessarily require transport to the cytosol and can thus be loaded in the endocytic pathway. This happens as reports are suggesting that endocytic compartments appear to "leak" part of their content or containing specific channels or translocators that permit egress of proteins or peptides to the cytosol utilizing size-limited channels or leak pathways(Kleijmeer et al., 2001)(Ackerman & Cresswell, 2004)(Bachmann et al., 1995)(Blum et al., 2013).

1.3.3 Antigen loading on MHC molecules

Where and how does peptide binding occur? Once again, depending on the Ag presentation system, MHC-I or MHC-II, the process is different. Regarding the loading of peptide to MHC-I, this happens in the endoplasmic reticulum as described in the previous paragraph, but things are a bit more complicated when it comes to MHC-II. There is the notion that as Ags get degraded gradually along the entire EE–LE–lysosomal track, MHC-II molecules become receptive to antigenic binding peptides, or polypeptide precursors, at all the stations of the endosomal pathway and the peptide-MHC-II complexes can exit from all compartments and move to the plasma membrane (Castellino and Germain, 1995; Driessen et al., 1999; Villadangos et al., 2000), (Driessen et al., 1999). This ability enables MHC-II molecules to sample peptides from the entire endocytic route, including peptides that may only be present in EEs because they do not survive the harsher conditions of LEs or lysosomes, and also peptides that require thorough degradation of their polypeptide precursors late in the endocytic route (Sercarz and Maverakis, 2003; Villadangos, 2001; Watts, 1997; Wolf and Ploegh, 1995)(Blum et al., 2013)(Roche & Furuta, 2015). Freshly synthesized MHC-II αβ dimers are transported to the endosomal compartments, where the peptides are kept. Unfortunately, the peptide cavity is highly undiscriminating, and there is the risk that it can promptly associate with other polypeptide chains in the ER(Busch et al., 1996), an event that would hinder the αβ dimers from acquiring the right peptides in the endosome. Therefore MHC-II molecules evolved a safety mechanism to prevent an improper loading of their groove and stabilize the conformation of the αβ dimer. The MHC-II molecules are synthesized as inactive pro-forms, in which peptide-binding cleft is occupied by the chaperone invariant chain (Ii). The Ii has a sorting motif in its cytoplasmic portion, which keeps the αβ-Ii complexes out of the secretory pathway but into the EE/LE stations of the endocytic pathway(Bakke & Dobberstein, 1990). However, it should be stated here, that up until now, we do not know the exact endocytic compartment in which Ii is cleaved, and the MHC-II encounters a peptide. Once the MHC II–Ii complexes reach their destination, the endocytic compartments, then the αβ dimers do not need the Ii anymore and eliminate it to regain their capacity to bind antigenic peptides. Once the MHC II–CLIP complex formation has taken place, the CLIP peptide must be substituted with antigenic peptides. This reaction involves the chaperone H-2M (HLA-DM in humans) which interact transiently with MHC II–CLIP, destabilizing the complex and facilitating the release of CLIP. This makes space for the antigenic peptides with the correct combination of amino acids to associate with the now vacant peptide-binding groove(Engelhard, 1994)(Vyas et al, 2008). However, in the endocytic compartments, we can also find a molecule known as H-2O (HLA-DO in humans) which acts as a negative regulator of H-2M, by binding to it and inhibiting both the H-2M, catalyzing the release of the CLIP and the binding of other peptides to the cleft of MHC-II. Under inflammatory conditions, the expression of H-2M is increased but this is not the case of H-2O, and this is how the H-2M can overcome the inhibitory effects of the H-2O. Once the formation of the MHC-II-peptide complex is complete, then they can be sorted from the endocytic route

toward the plasma membrane in transport vesicles. Notably, H-2M does not only have a role in removing CLIP, but it also acts as a peptide editor, as it promotes the exchange of low-affinity peptides for high-affinity ones(Katz & Sant2, 1994)(Kropshofer et al., 1996)(Blum et al., 2013)(Busch et al., 2005)(Roche & Furuta, 2015).

1.3.4 Antigen presentation and dendritic cell maturation

So once the peptide is loaded on the cavity of MHC molecules and transferred on the cell membrane, it is ready to be presented to T cells. Before DCs are ready to present the peptide to T cells, they have to go through a process, known as maturation. Upon maturation, there is a structural reorganization on the DCs as well as phenotypic and functional changes(Mellman & Steinman, 2001). Under steady state conditions, immature DCs express low levels of surface MHC and co-stimulatory molecules. In addition to that, a large amount of MHC-II is found accumulated within the cell's lysosomal compartments, together with internalized Ags, which were recently endocytosed(Inaba et al., 2000)(Trombetta et al, 2003a). The process of maturation commences shortly after the DCs receive adequate stimulus from their microenvironment. More specifically, a source of the maturation stimuli is the PRRs, whose role is not only to capture a pathogen but also to provide DCs with activatory signals. At the same time, under inflammatory conditions, DCs also receive signals from the epithelial cells, which release a variety of cytokines, known as alarmins that ligate many receptors found on the DCs and have an activatory effect on them as well. In addition to that, *in vivo*, DCs interact with other cell types like NK cells and others, and this contributes to their maturation as well(Roche & Furuta, 2015)(Vyas et al, 2008).

During the maturation process, the Ag uptake function of the DCs is gradually decreasing. Secondly, the MHC molecules escape from the lysosomal compartments to the cell surface, and they are “fixed” there, as they do not cycle anymore between the plasma membrane and the EEs. This is how upregulation of surface MHC-II is achieved upon maturation. However, this process needs to stop when the levels of MHC-II reach a certain level, and DCs achieve that by reducing the rate of MHC-II synthesis. Therefore, the expression levels of MHC-II are regulated by coordinated regulation of the MHC-II rate synthesis but also the rate of the peptide-MHCII internalization and degradation(Cella et al., 1997)(Rescigno et al., 1998)(Wilson et al., 2004). It is important to mention here, that the synthesis of the MHC-I follows a different pattern upon maturation, as it increases. This is consistent with the sustained turnover of surface MHC-I observed in mature DCs. Thirdly there is an upregulation of the co-stimulatory molecules expression and all together result in extension of the dendrites(Inaba et al., 2000)(Trombetta et al, 2003b)(Sallusto & Lanzavecchia, 2002)(West et al., 2004)(Chow et al., 2002)(Jensen, 2007).

Once the DCs mature, they are qualified to present peptides to T cells. For a successful Ag presentation, three signals are required. The first of these signals is the interaction between the peptide-MHC and the TCR. There is also an interaction between either the CD8 or CD4 molecule, depending on the T cell type, and a non-peptide binding region on the MHC molecule. The second signal is the interaction of CD40, OX40L, CD80, and CD86 on DC side with CD40L, OX40, and CD28 on the T cell side respectively. The third and last signal are cytokines released from the DCs which activate T cells and contribute to mounting a specific response(Wilson & Villadangos, 2005)(Mellman & Steinman, 2001).

1.3.5 Dendritic cell subtypes

DCs can be distinguished from other immune cell types by their morphology, phenotype, and functions. Importantly, DCs are not a homogeneous population. Nowadays, the developments in computational methods allow a more robust analysis of flow cytometry and CyTOF data. Making use of objective

algorithms to define cellular clusters, automated analyses increases the reproducibility of flow analysis by circumventing manual gating and its subjectivity.

Moreover, automated analyses assess the expression of all markers at once and are not influenced by the order in which cells are gated, as in manual sequential pairwise comparisons. Finally, these techniques simplify the visualization of the multidimensional datasets, which is particularly important when analyzing such data with more than 30 markers. However, application of these techniques for the study of DCs has remained limited. Nevertheless, with the help of this approach, we are now able to subdivide DCs more reliably (Guilliams et al., 2016) into three main subsets: conventional type 1 DCs (cDC1s), conventional type 2 DC (cDC2s), and pDCs.

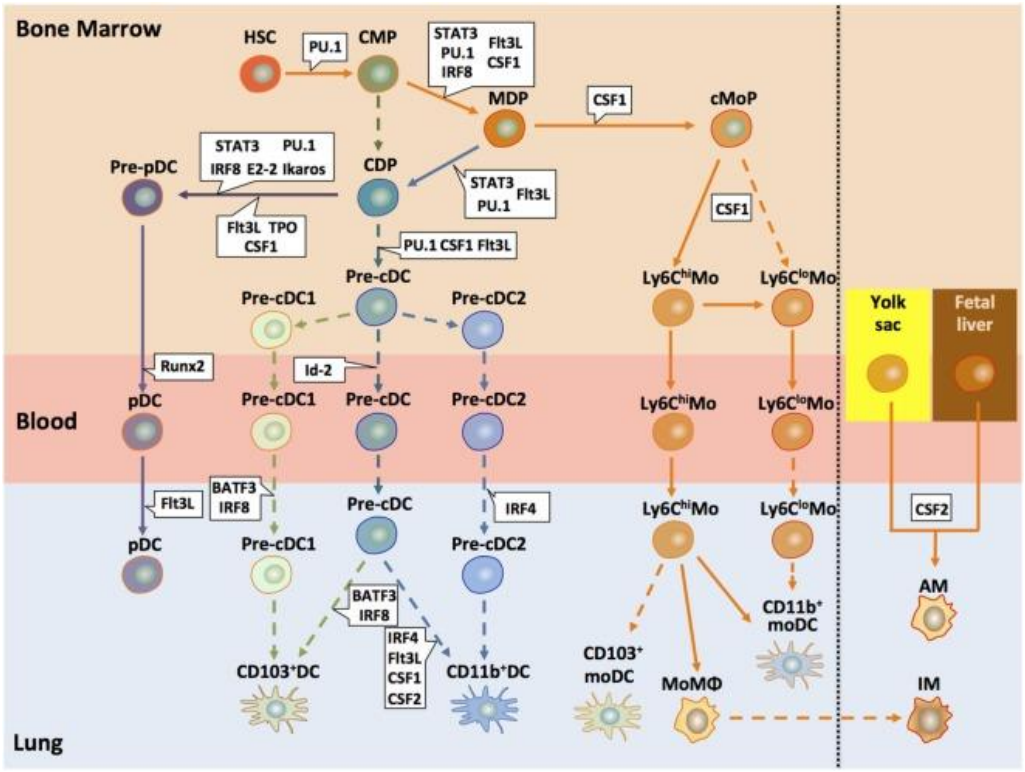


Figure 4: Differentiation of DCs and macrophages in mice. Summary of the current understanding of the differentiation of murine pulmonary DC and macrophage subsets suggests early lineage commitment of cDCs in the BM and differentiation of monocytes into different population with DC, macrophage, or suppressive functions(Hoffmann et al., 2016).

Plasmacytoid DCs (pDCs) morphologically resemble plasma cells. In response to a viral stimulus, they produce large amounts of interferon (IFN)-α. It is unclear if pDCs have an important role in Ag presentation because of their poor Ag presentation skills, and of their capacity to stimulate naïve T cells in comparison to other DC types (Krug et al., 2003). When stimulated, pDCs, acquire an immunogenic phenotype and prime T cells against viral Ag. Phenotypically these cells can be identified by the expression of B220, mPDCA-1, and Gr-1. (Colonna et al, 2004).

To distinguish pDCs from DCs identified by R. Steinman, the latter were renamed classical or conventional DCs (cDCs) and remain so today.

Classical Dendritic Cells (cDCs) refer to all DCs besides pDCs. cDCs are derived from hematopoietic stem cells and populate most lymphoid and non-lymphoid tissues. As mentioned above, they can be further subdivided into two functionally distinct lineages: the CD8α$^+$ (CD103$^+$) cDC1 lineage, and the CD11b$^+$ cDC2 lineage. cDCs originate from BM and go through several differentiation steps before they differentiate towards cDC1 and cDC2. DC-committed progenitor cells include the common DC progenitors (CDPs) and pre-DCs, which exit the BM and seed peripheral tissues before differentiating locally into mature cDCs. Where and when commitment to the cDC1 or cDC2 lineage occurs remains widely unknown.

cDC1 expressing CD8α (in lymphoid organs) and the integrin αE CD103 (in non-lymphoid organs) act like cells from the same lineage, based on their functional skills and transcription factor requirements. During the first steps of the differentiation in the BM, cDC1 are highly dependent on FLT3L for their differentiation (Liu et al, 2009) and in case of the non-lymphoid tissues there is also strong dependence on GM-CSFR for homeostasis of CD103$^+$cDCs *in vivo*(Greter et al., 2012). During development, cDC1 mainly depend on the transcription factors interferon regulatory factor 8 (IRF8), basic leucine zipper transcription factor ATF-like 3 (BATF3) and inhibitor of DNA binding 2 (ID2) (Ginhoux et al., 2009). All cDC1 express the surface markers chemokine receptor XCR1 and the C-type lectin receptor DNGR-1/CLEC9A. The former is the receptor for the XCR1 ligand chemokine (C motif) ligand 1 (XCL1), which is known to have a role in fostering an efficient interaction between cDC1 and CD8$^+$ T cells, whereas the latter acts as receptor for necrotic material, and is pivotal for the capture and routing of antigen into the MHC-I cross-presentation pathway for stimulation of CD8$^+$ T cells (Huysamen et al., 2008) (Dorner et al., 2009). Notably, cDC1 are the only DCs which are equipped with TLR3, that recognizes viral dsRNA and therefore cDC1 cells, are considered the primary sensors of viral infection.

cDC2/CD11b$^+$cDCs are the second family of cDCs. Cells of this family co-express CD4 and CD11b in the spleen and CD24 and CD11b in non-lymphoid organs. Up until now, it remains unclear whether the cDC2 cells found in lymphoid and non-lymphoid organs are a homogeneous population or divergent branches of the same ontogenic tree. By using additional markers, we can identify many cDC2 subsets in both lymphoid and non-lymphoid organs, which are usually characterized by different functional specializations. They mainly depend on the transcription factors IRF4, zbtb46 and stat5 and they selectively express the integrin CD11b(Tamoutounour et al., 2013) (Plantinga et al., 2013).

While some transcription factors for the development of the cDC1 cells have been identified that are required by both sub-categories (CD8$^+$ and CD103$^+$ cDCs), things are slightly different in case of the cDC2. The peculiarity with cDC2 is that these cells display tissue-specific transcription factor dependencies. For instance, splenic CD11b$^+$cDCs have a requirement for the neurogenic locus notch homolog protein 2 (Notch2)(Lewis et al., 2011), the V-rel reticuloendotheliosis viral oncogene homolog B (Relb) (Wu et al, 1998) and the lymphotoxin β receptor (Ltβr)(Kabashima et al., 2005), while in case of the intestinal CD11b$^+$CD103$^+$ there is the additional requirement for IRF4. On the other hand, the pulmonary CD11b$^+$cDCs are highly dependent on the STAT5 and IRF4 for their development (Bell et al., 2013) (Lugt et al., 2014). IRF4 regulates multiple functions of CD11b$^+$cDCs with the most profound being their development, migration and Ag presentation. It has been shown that IRF4 regulates DC migration through CCR7 and Ag presentation by its effect on MHC-II(Bajana et al, 2012) (Lugt et al., 2014). In case of the pulmonary CD11b$^+$cDCs, STAT5 has an essential role in DC maturation, as STAT5 deficient DCs failed to

upregulate co-stimulatory molecules, produce chemokines, and promote Th2 differentiation(Bell et al., 2013).

Of note, cDCs from both lineages, are not only genuine parts of the innate immunity as they bridge innate and adaptive immunity and initiate T cell activation.

1.4 The adaptive immune system

As already mentioned, vertebrates do not only rely on innate immunity for securing their integrity but make use of adaptive immunity, which is the second arm of the immune system. Adaptive immune system was discovered at the same time as the innate immune system. The discovery was made by Paul Ehrlich, whose research work revealed that, when people are immunized with foreign proteins like a protein from an animal or even bacterial toxins, they generate protective Abs that can be found in the blood(Kaufmann, 2008).

The adaptive or acquired immune system, is a subsystem of the overall immune system, which consists of highly specialized cells, the B and T lymphocytes. There are two big categories of T lymphocytes, the $CD4^+$ T cells, also known as T helper cells and the $CD8^+$ T cells, also known as cytotoxic T cells. It mediates humoral antibody responses and cell-mediated responses, which are carried out by B and T cells respectively. Both lymphocytes, carry lymphocyte antigen receptors. In case of the B cells, known as the B-cell receptor (BCR), it is in the form of immunoglobulins and can be either found as transmembrane receptors or secreted Abs. T cells express T-cell receptors (TCRs) as transmembrane receptors. These lymphocyte receptors are how T and B cells sense the presence of antigens in their environment. Responsible for the antigen recognition is the so-called variable region (V region) of the lymphocyte receptor. V region is characterized by variation in the amino acid sequence, which is a prerequisite for achieving high antigen specificity. In each lymphocyte receptor, the V region is bound to the constant region (C region), which provides effector or signaling function. Each of these lymphocytes carries many copies of an antigen receptor with a unique antigen-binding site. As mentioned above, the lymphocytes are the most abundant cells of the immune system. To be more precise, each individual carries billions of lymphocytes, allowing these cells to match with enormous variety of antigens. To generate such a diverse repertoire of lymphocyte receptors and a high specificity to defend against infection, an extraordinary mechanism has been developed to cope with that task. Considering the great variety of lymphocyte antigen receptors found in an individual, it is becoming clear that each receptor-chain cannot be encoded in full in the genome, as this would require more genes for antigen receptors than the number of genes in the entire genome(Iwasaki & Medzhitov, 2015)(Murphy & Weaver, 2013)(Von Behring & Kitasato, 2015)(LeBien & Tedder, 2008)(Kondo, 2010).

To overcome this obstacle, a mechanism has been developed which is known as gene rearrangement. The way it works is that the V region of the lymphocyte antigen receptors are encoded in several pieces. These pieces are assembled in the developing lymphocyte by DNA recombination to form a complete V-region sequence. The mechanism of gene rearrangement is common in T and B lymphocytes, but in the case of B cells, the immunoglobulins go through subsequent modifications to achieve greater effectivity of the Ab response. One of these modifications is known as somatic hypermutation, which introduces point mutation into the V region of activated B cells, resulting in a stronger binding to the antigen, a phenomenon known as affinity maturation, which is achieved as the immune response progresses(Murphy & Weaver, 2013).

But the modifications are not constrained on the V region, at least in case of Abs. The main task of the C region of a TCR is to support the V region and anchor the molecule in the membrane, so no modifications are required. Regarding immunoglobulins, the situation is slightly different. As mentioned above these molecules can be found in two versions, either as transmembrane receptors or as secreted Abs. In case of the latter, the C region can be of great importance for the diverse effector functions of the Ab. Abs are made in several different classes and they achieve that through a second modification which focuses on the C region of the immunoglobulins and is known as Ab class switching. Class switching enables Abs with the same antigen specificity to gain a different functional property(Von Behring & Kitasato, 2015)(Murphy & Weaver, 2013)(LeBien & Tedder, 2008).

However, in order to generate these specific immune responses, lymphocytes have first to recognize the antigen and become activated. Both B and T cells are activated in secondary lymphoid organs. T cells are only able to recognize antigens which are displayed on the surface of APCs, like DCs. More specifically, T cells are not able to recognize the whole Ag, but rather small peptide fragments derived from the pathogen's proteins(Sallusto & Lanzavecchia, 2002)(Rothoeft et al., 2006). The peptide fragments are displayed on the surface of cells by being loaded on a family of specific glycoproteins, which are known as MHC molecules and are encoded by a large cluster of genes(Wälchli et al., 2014). As mentioned above, there are two different classes of MHC molecules, known as MHC-I and MHC-II(Mellman & Steinman, 2001). The MHC-I is loaded with peptides which are derived from the cytosol and the MHC-I-peptide-complex is being recognized by the $CD8^+$ T cells. With a few exceptions, like erythrocytes, all cells are capable of presenting antigens via MHC I molecules. The MHC-II-peptide complexes are recognized by the $CD4^+$ T cells. The $CD4^+$ and $CD8^+$ T cells have distinct and very different functions, and protect the host from infection with different types of pathogens(Murphy & Weaver, 2013)(Mellman & Steinman, 2001)(Wilson & Villadangos, 2005).

However, TCR activation by MHC-loaded is not sufficient for T cell activation. They also require a second signal, the co-stimulatory signal, which is provided by the interaction of co-stimulatory molecules expressed on the membrane of APC and the T cell. The co-stimulatory molecules on the surface of APCs include CD40, OX40L, CD80, and CD86 and they interact with CD40L, OX40, or CD28 on the T cell side(Rothoeft et al., 2006).

Regarding B lymphocytes, these cells not only recognize whole Ags derived from pathogens but also act as APCs. Once a BCR binds an Ag, the Ag is taken up into the B cell through BCR, degraded and presented to T cells as peptide pieces in complex with MHC-II molecules on the cell membrane. T helper (T_H) cells, bind these MHC-II-peptide complexes through their TCR. Following TCR-MHC-II-peptide binding, T cells express the surface protein CD40L as well as cytokines. CD40L serves as a necessary co-stimulatory factor for B cell activation by binding the B cell surface receptor CD40, which promotes B cell proliferation, immunoglobulin class switching, and somatic hypermutation as well as T cell growth and differentiation. After B cells receive these signals, they are considered activated(LeBien & Tedder, 2008).

An individual can encounter a particular pathogen more than once in his life. Interestingly, the immune response that will be initiated on the second or other subsequent encounters with the same pathogen will be much faster and more specific in comparison to the first one. This happens due to the development of immunological memory upon the initial Ag encounter. In more detail, immunological memory is a unique property of the immune system as it can “store” information about a stimulus and can mount an

effective response once the stimulus is encountered again. The so called secondary immune response is stronger and faster in comparison to the primary immune response. It takes a smaller stimulus to trigger a secondary response and it lasts for many years after the first exposure(Murphy & Weaver, 2013)(Khodadadi et al., 2019).

Till recently, the immunological memory was closely connected with adaptive immunity and was considered to be its unique characteristic. The focus was set on memory B and T lymphocytes. Normally, once the primary immune response disappears, the effector cells, which were involved in the immune response are eliminated. However, a small number of them, like the T and B cells, together with the Abs remain in the body and consist the immunological memory. The cellular compartment of the immunological memory is found in a resting state and at the subsequent encounter with the same Ag, these cells have the capacity to rapidly respond and eliminate the Ag. The memory cells are known to have a long life and last up to several decades in the body(Khodadadi et al., 2019)(Murphy & Weaver, 2013)(Ratajczak et al., 2018).

However, the B and T lymphocytes are not the only long-lived cells in the body. Many innate immune cells, like alveolar macrophages, microglia and astrocytes have a long life. These data together with the concept of innate immune memory or trained immunity in plants and invertebrates was the trigger to start the discussions about innate immune memory in vertebrates and change the current view that it is simply an immediate mediator of host resistance and inflammation. Similar to what is known about adaptive memory, the features of innate memory would involve a priming event whereby after an initial exposure, the innate immune cells would be changed so that they could elicit a more robust and fast response in case of a subsequent encounter with the same or heterologous stimuli(Wendeln et al., 2018)(Netea et al., 2015)(Zipfel et al., 2017)(Saeed et al., 2015).

As mentioned above, the first knowledge about innate immune memory comes from plants and invertebrates. Data show that in plants, epigenetic alterations lead to the priming of genes encoding host defense molecules to respond upon re-exposure. For instance, in a study in which they used macrophages, they could confirm the theme of epigenetic modification during innate immune responses in vertebrates, describing the crosstalk between interferon-g (IFN-g) and IL-4 in the stimulation of macrophages. Using a genomic approach, Piccolo et al showed that a large fraction of the transcriptional and epigenomic changes induced by stimulation with IFN-g are reduced or suppressed by IL-4. These inhibitory effects of IL-4 *in vitro* are retained after the cytokine is washed out and cells are then treated with IFN-γ. Macrophages can therefore carry an ‘epigenomic memory’ of prior exposure to IL-4 (as an indicator of parasitic infection), such that there will be a more limited response to IFN-γ during a bacterial infection(Piccolo et al., 2017).

According to Kate Fitzgerald the chromatin modifications that accompany trained immunity are not the exclusive way of developing innate immune memory. She suggested that there is another possible mechanism, and this is long-term regulation of long non-coding RNA (lncRNA). Numerous lncRNAs are transcriptionally induced upon signaling via sensors of the innate immune system. Since lncRNAs can mediate both the activation of various classes of immunological genes and their repression, the pattern and timing of the induction of lncRNA could profoundly affect the type of immune responses to secondary stimulations(Netea et al., 2015).

So, the current understanding is that innate immune memory has several defining characteristics when compared to classical immunological memory. Firstly, it involves a set of cells (myeloid cells, NK cells, ILCs)

and germline encoded recognition and effector molecules like PRRs, which are different from those involved in classical immunological memory. Secondly, the better responsiveness to a secondary stimulus orchestrated by trained immunity is not specific for a particular pathogen and it is mediated through signals impinging on transcription factors and epigenetic reprogramming, when the classical immunological memory depends on gene rearrangement and proliferation of antigen-specific lymphocyte clones. The changes in innate immune memory cells are broadly defined as sustained changes in transcription programs through epigenetic rewiring, leading to changes in cell physiology that do not involve permanent genetic changes such as mutations and recombination. Finally, trained immunity relies on an altered functional state of innate immune cells that persists for weeks to months, rather than years, after the elimination of the initial stimulus(Saeed et al., 2015)(Netea et al., 2015). Thus, it is clear that there is also innate immune memory, but it is fundamentally different from the classical immunological memory.

1.5 Allergic asthma

The majority of the diseases that are observed in vertebrates and more precisely mammals make use of both innate and adaptive immunity. One of them is asthma, which is a chronic inflammatory disease of the airways with high associated morbidity, which was first described in Ancient Egypt.(Teeter & Manniche, 2003) The word “asthma” originates from the Greek άσθμα, which means "panting." The rates of asthma started to increase since the 1960s significantly and in 1990, 183 million people had asthma worldwide(Anandan et al, 2010). The last decades the prevalence of asthma dramatically increased. In 2015 the number of people who suffered from asthma, went up to 358 million when the same year 397.100 people died because of asthma especially in developing countries (GBD 2015 Disease and Injury Incidence and Prevalence Collaborators, 2016).

Asthmatic patients suffer from airway hyperreactivity (AHR), and mucus overproduction is resulting in recurrent episodes of chest tightness, breathlessness, wheezing, and coughing. Nowadays, it is widely accepted that asthma develops as a result of a maladaptive Th2/Th17 immune response towards innocuous aeroallergens in genetically susceptible individuals(Erle & Sheppard, 2014)(Tjota & Sperling, 2014)(Köhl et al., 2006)(Schmudde et al., 2013)(Plantinga et al., 2013)(Lambrecht & Hammad, 2003). There is no cure for asthma. The available medication can only alleviate the symptoms. The therapeutics usually target the effector phase of the disease trying to either reduce the inflammatory processes that drive the clinical symptoms or decrease the resistance in the airways and increase the airflow in the lung.

However, which are the factors that cause asthma, and is the reason that allergic diseases have dramatically increased during the last decades in western countries? Different factors contribute to the increased number of allergies. Some of these factors are included in the concept of the "Hygiene hypothesis," which was first introduced by Dr. David Starchan. According to him, the rise in allergic disease is related to changes in lifestyle and environmental exposure, including rapid improvements in sanitation, an increase in Cesarean-section and bottle versus breastfeeding, decrease of outdoor activities and altered diet, western diet with serious effects on the human microbiome, affecting immunotolerance and finally increasing the risk of allergic diseases(Okada et al, 2010)(Bloomfield et al., 2016). Also, a genetic predisposition is “required” for the development of asthma(Mukherjee & Zhang, 2011).

Two types of asthma cause allergic and the non-allergic (intrinsic) asthma. Most of the children and about 50% of the adults suffer from allergic asthma. The pathology is generated by allergen specific-allergic immunoglobulin E (IgE) antibodies, the frequently positive skin-prick test to the (lipo)proteins of common

inhaled or ingested allergens such as HDM, animal dander, fungal spores and plant or tree pollen. In children the onset of the disease is often characterized by eczema in the first year of life. At later times, the children develop allergic rhinitis and that also progresses to allergic asthma.

Nonallergic asthma usually develops later in life and is characterized by neither IgE reactivity to allergens in the serum nor any apparent involvement of the adaptive immune system such as Th2 cells. However, this fundamental discrimination of asthma types is an oversimplification, as in each of these two phenotypes, individuals with different and distinct pathophysiology are found. The different pathophysiologies detected in each phenotype, are called asthma endotypes(Akdis et al., 2011). These endotypes differ in terms of genetic susceptibility, risk factors, the age of onset, clinical presentation, prognosis and response to standard and new therapies. Asthma is therefore increasingly seen as a syndrome rather than as a single disease(Woodruff et al., 2009)(Wu et al., 2014).

1.5.1 Immune mechanisms underlying the development of allergic asthma

Allergic asthma is an inflammatory airway disease, which is developed in response to harmless aeroallergens. It is defined by an integrated response in the conducting airways of the lung. As a multicellular disease it involves atypical responses of many different pulmonary cell types. Many of these cell types, interact and collaborate to induce mucus overproduction, bronchial hyperactivity, airway wall remodeling, and airway narrowing. Together, it results in clinical symptoms including shortness of breath, wheezing and chest tightness(Wills-Karp, 1999)(Erle & Sheppard, 2014).

Does the past decade the following pathophysiological concept has evolved? Once an individual is exposed to harmless aeroallergens (i.e., HDM) the integrity of the epithelial barrier is disrupted. Responsible for the disruption are proteases found in the allergens, which cleave the tight junctions between the epithelial cells. Both the disruption of the epithelial barrier, as well as the ligation of the PRRs expressed by the epithelial cells causes the release of highly inflammatory cytokines. These cytokines include IL-33, TSLP, IL-25, and GM-CSF, which are also known as alarmins because they act as an alarm to alert the immune system for the invasion of intruders. However, epithelial cells also release endogenous “danger signals” such as uric acid, ATP and high mobility group box 1 (HMGB1)(Kool et al., 2012)(Willart et al., 2012)(Wills-Karp et al., 1999)(Wills-Karp et al., 1998). These alarmins activate DCs, ILC2s, and basophils(Van Dyken et al., 2016)(Lambrecht & Hammad, 2014). Once ILC2s and basophils get activated, they start to secrete more inflammatory cytokines resulting in the recruitment of additional immune cells to the site of infection. In the case of DCs, the disruption of the epithelial barrier makes it easier for them to take up Ags. Their concomitant activation by signals from epithelial cells (DCs express on their surface receptors for the alarmins but also receptors for the inflammatory mediators like uric acid and HMCB1) induces their activation/maturation resulting in their migration to the regional draining lymph nodes (dLNs), where naïve $CD4^+$ T cells are located. Once they interact with $CD4^+$ T cells and activate them, the T cells migrate to the lung and start secreting the type 2/17 cytokines (IL-4, IL-5, IL-13, and IL-17)(Williams et al., 2013)(Plantinga et al., 2013)(Schmudde et al., 2013). In the case of IL-5 and IL-13, it should be noted that are not exclusively produced by the $CD4^+$ T cells but by the ILC2s as well. Of note, ILC2s get activated very early upon allergen exposure and produce IL-5 and IL-13. IL-13 production by ILC2s during the early events of allergic asthma is critical for the migration of cDCs to the dLNs, where they induce the activation of Th cells. Thus, the data show that ILC2s have a vital role in generation of Th2 immune responses(Halim et al., 2014). There is a report stating that ILC2 cells were found to represent more than half of the cells producing Th2 cytokines in the lungs of mice subjected to OVA- and HDM-induced asthma(Wolterink et al., 2012)(Van Dyken et al., 2016). In both cases, IL-5 induces airway eosinophilia by acting on BM

progenitors and stimulating their development. Eosinophils are recruited to the lungs through the production of eotactic chemokines like eotaxins 1, 2 and 3 (CCL11, CCL24, and CCL26, respectively). Eosinophil-derived products such as eosinophil peroxidase cause bronchial hyperresponsiveness (BHR) directly and activate adaptive immunity through effects on DCs(Coyle et al, 1995)(Chu et al., 2014). Eosinophils are also capable of presenting Ag to already activated T cells(Wiese, unpublished data)(Hoogsteden et al, 2003). Eosinophils further contribute to airway remodeling by causing the damage of pulmonary structural cells. They do that by releasing extracellular DNA traps, as neutrophils do, which contain eosinophilic granules. Considering that the eosinophilic granules sustain the capacity for ligand-induced secretion, the DNA trap formation could result in increased concentrations of eosinophilic toxins like eosinophil-derived neurotoxin, cationic proteins (eosinophil peroxidase) and major essential protein, which can damage structural cells of lungs(Dworski et al, 2011)(Yousefi et al, 2012).

On the other hand, IL-13 acts on epithelial cells and drives goblet cell metaplasia followed by excessive mucus production. On bronchial smooth muscle cells it induces bronchial hyperactivity(Wills-Karp et al., 1998). The cytokine IL-4 like IL-13 acts on epithelial cells to induce goblet cell metaplasia. On B cells, it causes Ab class switching to IgE, which characterizes allergic asthma. For a long time, asthma had been defined by airway eosinophilia, when efforts made to better characterize the asthma endotypes, it was revealed that some patients show a neutrophil-dominant disease without Th2 cytokine response. The neutrophilia is often noticed in patients with late onset of the disease, who usually suffer from severe forms of asthma and less reversible airway obstruction(McKinley et al., 2008)(Manni et al., 2014). In some severe forms of allergic asthma, it was shown that IL-17A had an active role contributing in airway remodeling by promoting fibroblast proliferation (Bellini et al., 2011) and/or counterbalancing the anti-inflammatory role of T regulatory cells (Tregs)(Zhao et al., 2013)(Gour & Wills-Karp, 2015).

However, it seems that IL-17A has more than one role in allergic asthma development. In addition to the two roles, it was also shown that contributes to the recruitment of neutrophils in the lung, by stimulating bone marrow stromal cells to secrete G-CSF, which favors granulopoiesis(Schwarzenberger et al., 2000). Interestingly, both in mice and humans, IL-17 can also contribute to direct contraction of bronchial smooth muscle cells and therefore cause BHR in the absence of neutrophilic inflammation(Kudo et al., 2012). Of note $CD4^+$ T cells are not the exclusive producers of IL-17. $TCR\gamma\delta^+$ T cells, invariant natural killer T cells (NKT), and ILC3 cells are a source of copious IL-17, and the relative contribution to IL-17 production might differ in the various asthma endotypes. Lastly, regarding Th17 cells, it should be noted that the cytokine production by Th17 cells is resistant to inhibition by steroids(McKinley et al., 2008)(Wang & Wills-Karp, 2011).

Strikingly, studies are linking other Th cell subsets to the pathogenesis of the disease, and one of them is the Th9 cells producing IL-9, which is known to regulate the accumulation of mast cells in the airways, along with mucous cell metaplasia and BHR. However, it is still unclear, whether IL-9 is made by a dedicated Th9 cell subset or is also produced by Th2 cells(Elyaman et al., 2012).

Finally, in the lungs of asthmatic patients, we can also find Tregs. The exact role of Tregs in patients suffering from allergies is not entirely clear. In cases of severe asthma, the number of Tregs found in the blood and sputum is lower and these cells have lower suppressive potencies in comparison to Tregs from healthy individuals(Mamessier et al., 2008). Furthermore, the percentage of Tregs is lower in the BAL of pediatric patients with asthma in comparison to healthy children(Hartl et al., 2007). Clearly Tregs are functionally compromised in people with asthma, but this impairment is related only to their ability to

control Th2 responses. Tregs from the blood of HDM-sensitized children are able to suppress the production of Th1 and Th17 cytokines but not of Th2(Joller et al., 2014). This finding could support the notion that Tregs in asthmatic patients promotes the Th2 response instead of controlling it. In the mouse, a similar Treg cell population unable to suppress Th2 responses has been reported(Joller et al., 2014).

The activation of all these different cell types happens gradually, and it is known that depending on the phase of asthma (sensitization and effector phase), the cells and the inflammatory cytokines may have subtle functional differences.

1.5.2 The role of pulmonary dendritic cells in allergic asthma

DCs have a vital role in the development of allergic asthma. They are one of the first pulmonary cell types, which senses the allergen and gets activated to stimulate adaptive immune responses. In the past, it was believed that pulmonary DCs are a homogeneous population, as they were described as $CD11C^+MHCII^+$ (Sertl et al., 1986). Nowadays, it is known that there are at least four DC subtypes and that there is a division of labor among them. These pulmonary DC subsets include $CD11b^+$ and $CD103^+$ cDCs, pDCs and under inflammatory conditions monocyte-derived cDCs (mo-DCs)(Plantinga et al., 2013). The discrimination of the DC subtypes requires a big panel of markers as well as a strict gating strategy to accurately define them. In most studies, markers like CD11c, CD11b, MHC-II, SiglecF, CD103, and CD64 were used to distinguish the DC subsets on pre-gated lineage negative cells, although there are some studies, that make use of additional markers. The lack of an established and unanimously accepted panel of markers to define pulmonary DC and the differences in the isolation procedures (that resulted in liberation of DCs in different quantities) lead to inconsistencies between different studies, numbers and functions(Plantinga et al., 2013)(Misharin et al, 2013).

1.5.2.1 Subpopulations of pulmonary dendritic cells

1.5.2.1.1 Pulmonary pDCs

Under naïve conditions, a small number of pDCs is found in the lungs. Upon allergen exposure, pulmonary pDCs increase(Lewkowich et al, 2008), although they do not have an active role in the development of the maladaptive Th2/Th17 response as they have compromised Ag presentation ability in comparison to $CD11b^+$cDCs(Plantinga et al., 2013). In the context of allergic asthma, pDCs are mainly considered tolerogenic for two reasons. They induce Treg differentiation(Watkins et al., 2005)(de Heer et al., 2004) and act in trans by regulating the functions of cDCs during the crosstalk with naive T cells(Lewkowich et al., 2008)(Köhl et al., 2006) in a mechanism that involves the regulation of B7 molecule expression(Zhang et al., 2009).

1.5.2.1.2 Pulmonary $CD103^+$ cDCs

Since the cDC1/$CD103^+$ cDCs were discussed in more detail in a previous section, here I will focus on their role in the lung under allergic asthma conditions. The $CD103^+$cDCs are equipped with the tight junction proteins Claudin-1, Claudin-7, and ZO-2, which allow them to form tight junctions with epithelial cells and thus sample the airway lumen without barrier damage(Gaskin et al., 2006). Still, they prove to be less potent in Ag uptake in comparison to the $CD11b^+$cDCs in an HDM-induced asthma model(Plantinga et al., 2013). Their contribution to the Th2/Th17 skewing, which is typical for allergic asthma, is debatable(Plantinga et al., 2013)(Nakano et al., 2012)(Furuhashi et al., 2012). More precisely, Nakano et al. demonstrated that the $CD103^+$cDCs are capable of nicely taking up the Ag, migrating to the lymph nodes and eliciting a Th2 response(Nakano et al., 2012), when Furuhashi et showed that $CD103^+$cDCs induce a Th1 immune response(Furuhashi et al., 2012). Plantinga et al., did not confirm any of these

findings. In their hands the CD103$^+$cDCs mainly have a regulatory role(Plantinga et al., 2013). A possible reason for the different findings could be the nature of the allergen used in each study (HDM, OVA, and cockroach) or the administered amount of allergen. Especially regarding the used amount of the allergen, Plantinga et al showed that when they treated the mice with 10µg of HDM, then only the CD11b$^+$cDCs were able to take up the Ag and migrate to the dLNs. CD103$^+$cDCs were able to take up the Ag and migrate to the dLNs only when 100µg of HDM were administered to the mice(Plantinga et al., 2013). Thus, depending on the used amount of the allergen and its nature, different DC subpopulations might be involved in the initiation of the disease and lead to a different outcome.

1.5.2.1.3 Pulmonary monocyte-derived DCs (mo-DCs)

Under steady state conditions, the number of mo-DCs in the lungs is low(Schlitzer et al., 2013)(Robays et al., 2007). It is very challenging to adequately discriminate mo-DCs from the CD11b$^+$cDCs. CD64 and FcεR were revealed as mo-DC markers(Plantinga et al., 2013). Several groups use Ly6C as a marker but with poor results, as Ly6C is a marker that is downregulated once the mo-DCs home the lungs. As expected, after HDM exposure, the numbers of mo-DCs in the lungs increase(Plantinga et al., 2013). Even though they take up the Ag, their capacity to activate T cells is rather poor upon low allergen dose(Plantinga et al., 2013). Their primary role in the development of allergic asthma is the recruitment of eosinophils and monocytes(Robays et al., 2007). Mo-DCs produce cytokines and chemokines, including CCL24, CCL2, CCL4, CCL7, CCL9 and CCL12 that are important for activating and recruiting eosinophils and monocytes in response to allergen challenge(Plantinga et al., 2013)(Robays et al., 2007).

1.5.3 Role and location of pulmonary CD11b$^+$cDCs in allergic asthma

Along with CD103$^+$cDCs, CD11b$^+$cDCs are the dominant DC population in the lung. To be more precise, the cells that fall into that subset are CD11b$^+$CD64$^-$cDCs, and mo-DCs are excluded from this category as they are a category on their own. CD11b$^+$CD64$^-$cDCs take up the allergen *in vivo* and *in vitro*(Furuhashi et al., 2012) and migrate to the regional lymph nodes, shortly after allergen exposure(Plantinga et al., 2013). They do that by up-regulating C-chemokine receptor-7 (CCR7) expression, a G-protein coupled receptor. CCR7 is upregulated on activated DCs and is involved in the migration of DCs to the lymph nodes. CCR7 recognizes CCL19 and CCL21, which are produced in the lymph nodes and increase the expression of MHC-II and co-stimulatory molecules on DCs(Clatworthy et al., 2014). Due to their advanced Ag uptake and migratory skills, CD11b$^+$CD64$^-$cDCs are considered to be vital for eliciting the Th2/Th17 immune response(Plantinga et al., 2013)(Furuhashi et al., 2012). The epithelial barrier is critical on the education of CD4$^+$ T cells by CD11b$^+$CD64$^-$cDCs. Upon allergen exposure, the epithelial cells release danger signals and inflammatory cytokines which activate DCs and also license them to promote Th2/Th17 responses. The TSLP-STA5 axis promotes CD11b$^+$CD64$^-$cDCs activation through upregulation of co-stimulatory molecules and CCR7 and favors a Th2 response(Kitajima & Ziegler, 2013) (Bell et al., 2013). Another transcription factor with critical Th2 regulatory effects on CD11b$^+$CD64$^-$cDCs is IRF4. Williams et al. showed that IRF4 modulates IL-10 and IL-33 cytokine production to specifically promote Th2 differentiation and inflammation(Williams et al., 2013). Further, IRF4 can also license pulmonary CD11b$^+$CD64$^-$cDCs to prime a Th17 response(Schlitzer et al., 2013). On top of that, IRF4 enhances on CD11b$^+$CD64$^-$cDCs with outstanding Ag presentation abilities by enforcing the Ag processing and presentation abilities of these cells(Lugt et al., 2014).

Only a few studies dealt with the location of the CD11b$^+$CD64$^-$cDCs in the lungs. Most studies focused on functional discriminations of the cells and did not focus on their spatial distribution. CD11b$^+$CD64$^-$cDCs in

the lungs have been found in the lung parenchyma, i.e. the main conducting fraction(Holt et al., 2005), alveolar region(Cleret et al., 2006) and around the airways up to a distance of 200μm(Thornton et al., 2012). Thus, $CD11b^{+}CD64^{-}$cDCs seems to be broadly distributed within the lung.

1.6 The role of the C5aR1 in allergic asthma

Since the present thesis focused on the role of C5aR1 in the early events of the allergic asthma development, I will give a short overview of current knowledge about the role of C5aR1 both in the sensitization and effector phase of the disease.

The complement system is present from the early onset of allergic asthma to the effector phase. It also has a role on resolution of the disease. Reports show that complement is already activated under naïve conditions, but upon allergen exposure, it gets significantly increased(Krug et al, 2001)(Marc et al., 2004). The anaphylatoxin C5a, can be produced either by the canonical or non-canonical complement activation. Data is showing that the allergens' proteases could *in vitro* produce the ATs in serum in a dose and time-dependent manner(Maruo et al, 1997). Besides, ATs can also be produced by cellular mechanisms. For instance, alveolar macrophages can cleave C5 into C5a by a serine protease(Lang et al., 2002). There are more serine proteases in pulmonary immune cells with cleavage capacity of C5, like the granzyme B(Perl et al, 2012), found in neutrophils and lymphocytes as well as the aspartic protease Cathepsin D(Lang et al., 2012). C5a has distinct roles in the pathogenesis and the pathology of asthma, depending upon the conditions under which it is generated, the cell-types that become activated and the stage of the disease.

1.6.1 Sensitization phase

Many recent reports provide evidence that ATs have a regulatory role in the development and the magnitude of adaptive immune responses. There are many data showing that the C5a/C5aR1 signaling axis controls allergic asthma at the DC/T cell interface. More precisely, the data so far indicate that signaling through C5aR1 has a protective role against the development of a Th2 immune response during sensitization phase. More specifically, data from several groups showed that $C5^{-/-}$ mice exhibit an increased susceptibility to development of AHR and pulmonary inflammation(Drouin et al, 2006)(McKinley et al, 2006). *In vivo* targeting of C5aR1 during sensitization phase in both an OVA- and HDM-induced experimental allergic asthma model resulted in increased levels of Th2 cytokines and an overall increased airway inflammation(Köhl et al., 2006). Mechanistically, signaling through C5aR1 controls the ratio between cDCs and tolerogenic pDCs. Additionally, Zhang et al showed that the C5a/C5aR1 signaling axis prevents the development of maladaptive Th2 immunity in allergic asthma by regulating the accumulation of pulmonary pDCs expressing costimulatory molecules B7-H1 (PD-L1) and B7-DC (PD-L2), which can modulate the function of cDCs as well as regulatory T cells(Zhang et al., 2009). On top of that, inhibition of C5aR1 signaling correlates with enhanced release of the Th2 cell homing chemokines CCL17 and CCL22. The current understanding is that signaling through C5aR1 during allergen sensitization is vital for inducing tolerance against innocuous allergens. Events that prevent signaling through C5aR1 during the onset of the disease result in increased activation of cDCs, which will induce robust activation of naïve Th cells(Köhl et al., 2006).

1.6.2 Effector phase

The role of C5aR1 in the effector phase of allergic asthma is the opposite of what was observed in the sensitization phase. More specifically, when C5aR1 was *in vivo* blocked during the effector phase of experimental asthma, this resulted in a significant suppression of the disease-associated allergic phenotype in response to airway challenges with HDM, OVA or A. fumigates extract, accompanied by

lower numbers of eosinophils and lymphocytes in the BAL and lower AHR(Baelder et al., 2005)(Peng et al., 2005)(Köhl et al., 2006). Additionally, no changes on the expression levels of CCL17 and CCL22 were noticed under these conditions(Köhl et al., 2006). Mechanistically, signaling through C5aR1 may promote the production of Th2 cytokines through the recruitment and activation of eosinophils, basophils, neutrophils and mast cells. All the observations suggested that in an established allergic asthma environment, C5a has proallergic properties and the targeting of C5aR1 under these conditions leads to reduced airway inflammation and AHR.

Taken together, the data show that C5aR1 has a dual role in the development of the disease. But since in knock-out models and upon pharmacological targeting, signaling via the C5aR1 is either systemically or pulmonary blocked, it remains to be investigated at which cellular level the C5aR1-mediated regulation of disease development occurs.

1.7 The aim of the project

As discussed in the previous section, previous work both from my laboratory and other groups showed that C5aR1 regulates the development of maladaptive Th2 response at the DC/T cell interface(Köhl et al., 2006)(Drouin et al., 2006)(McKinley et al., 2006). However, the gating strategy used in the past could not properly discriminate all the pulmonary DC subsets and therefore it is of outmost importance to elucidate the mechanism by which C5a/C5aR1 signaling axis controls the functions of the DC subsets. Due to the pivotal role of the $CD11b^{+}CD64^{-}$cDCs in eliciting a Th2/Th17 response as well as the fact that $CD11b^{+}CD64^{-}$ cDCs express the complement receptor C5aR1(Karsten et al., 2015) it is believed that C5aR1 controls the development of a maladaptive Th2/Th17 response through these cells, but the molecular mechanisms underlying this C5aR1-mediated regulation of pulmonary $CD11b^{+}CD64^{-}$cDCs functions are still unclear. And even today all these evidences are still a hypothesis and incompletely understood. Therefore, the aim of thesis was to delineate the role of C5a/C5aR1 signaling in $CD11b^{+}CD64^{-}$cDC for the activation of $CD4^{+}$T cells in the sensitization phase of allergic asthma.

I defined the following aims:

1. **Delineate the functional and phenotypic differences between the $CD11b^{+}C5aR1^{+}$cDC and $CD11b^{+}C5aR1^{-}$cDC.**
    - Do the $CD11b^{+}C5aR1^{+}$cDC and $CD11b^{+}C5aR1^{-}$cDC have the same potency of activating OVA-transgenic-$CD4^{+}$ T cells?
    - Do the $CD11b^{+}C5aR1^{+}$cDC and $CD11b^{+}C5aR1^{-}$cDC have the same frequency of interactions with the OVA-transgenic-$CD4^{+}$ T cells?
    - Do the $CD11b^{+}C5aR1^{+}$cDCs and $CD11b^{+}C5aR1^{-}$cDCs sense the inflammatory signals released by the ECs upon HDM treatment the same way?
    - Do the $CD11b^{+}C5aR1^{+}$cDC and $CD11b^{+}C5aR1^{-}$cDC mature in a similar way upon HDM treatment?
    - Do the $CD11b^{+}C5aR1^{+}$cDC and $CD11b^{+}C5aR1^{-}$cDCs have the same potency to migrate to dLNs?
2. **Assess whether the $CD11b^{+}C5aR1^{+}$cDC and $CD11b^{+}C5aR1^{-}$cDC belong to the same cDC lineage or if they are different cell types.**
    - Is the transcriptional programming of $CD11b^{+}C5aR1^{+}$cDC and $CD11b^{+}C5aR1^{-}$cDC the same?

3. **Determine which is the source of C5a in CD11b$^+$C5aR1$^+$cDC - CD4$^+$ T cell and CD11b$^+$C5aR1$^-$cDC - CD4$^+$ T cell co-culture systems in response to allergen pulsing and T cell interaction.**
    - When do CD11b$^+$C5aR1$^+$cDC and CD11b$^+$C5aR1$^-$cDC start to make C5a upon *ex vivo* OVA pulsing?
    - Do CD4$^+$ T cells make any C5a?
    - Which is the trigger for the C5a production?
4. **Delineate the role of C5aR1 on the maturation profile of CD11b$^+$cDCs and their ability to induce CD4$^+$ T cell proliferation.**
    - Does C5aR1 control the maturation of the CD11b$^+$C5aR1$^+$cDCs?
    - Does C5aR1 regulate the ability of the CD11b$^+$C5aR1$^+$cDC to induce CD4$^+$ T cell proliferation?
5. **Define the activation pathways by which C5aR1 controls the tolerogenic potential of CD11b$^+$cDCs via MHC-II/CD40.**
    - Are there any difference in the transcriptional programming of C5aR1$^+$cDCs which were targeted with the C5aR1-specific mAb 20/70 in comparison to the C5aR1$^+$cDCs which were treated with the isotype control?
    - Which is the role of C5aR1 at the immunological synapse formation when MHC-II levels are low?

2. Material, Equipment and Methods

2.1 Materials

2.1.1 Chemicals

Table 2: Used Chemicals.

Substance	Manufacturer
Aluminum potassium sulfate ($KAl(SO_4)_2$)	Merck, KGaA, Darmstadt
Ammonium chloride (NH_4Cl)	Sigma-Aldrich Chemie GmbH, Steinheim
Aqua ad injectabilia	B. Braun Melsungen AG, Melsungen
BD FACS Flow Sheath Fluid	BD Biosciences Europe, Erembodegem, Belgium
β-Mercaptoethanol	Sigma-Aldrich Chemie GmbH, Steinheim
Bovine Serumalbumin (BSA)	Sigma-Aldrich Chemie GmbH, Steinheim
Carboxyfluoresceine-Succinimidylester (CFSE)	Life technologies Corporation, Carlsbad, USA
Chloral hydrate ($C_2H_3Cl_3O_2$)	Merck, KGaA, Darmstadt
Citric acid, crystalline	Merck, KGaA, Darmstadt
Compensation beads (anti rat/hamster)	BD Biosciences Europe, Erembodegem, Belgium
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich Chemie GmbH, Steinheim
DNase I from bovine pancreas	Sigma-Aldrich Chemie GmbH, Steinheim
DQ-OVALBUMIN (D-12053)	Invitrogen, Molecular Probes, Eugene
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
Fetal calf serum (FCS) (Lot A04304-0413)	PAA Laboratories GmbH, Pasching, Östereich
Formaldehyde solution, 37%	Sigma-Aldrich Chemie GmbH, Steinheim
Glacial acetic acid	Merck, KGaA, Darmstadt
CSF-2 (GM-CSF), recombinant murine	Peprotech Corporation, Rocky Hill, USA
House dust mite extract (lot 262538)	Greerlabs Laboratories Inc., Lenoir, 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
Ovalbumin, grade III	Sigma-Aldrich Chemie GmbH, Steinheim
Phosphate Buffered Saline (PBS)	PAA Laboratories GmbH, Pasching, Östereich
Penicillin-Streptomycin, 100x Liquid	Life technologies Corporation, Carlsbad, USA
Phloxine	Chroma Technology Corporation, Bellow Falls, USA
Potassium bicarbonate ($KHCO_3$)	Sigma-Aldrich Chemie GmbH, Steinheim
Rompun vet.	Bayer AG, Lervekusen
RPMI 1640	Life technologies Corporation, Carlsbad, USA
Sodium chloride	Sigma-Aldrich Chemie GmbH, Steinheim
Sodium dihydrogen phosphate (Na_2H2PO_4)	Sigma-Aldrich Chemie GmbH, Steinheim
Sodium hydrogen phosphate (Na_2HPO_4)	Sigma-Aldrich Chemie GmbH, Steinheim
Sodium iodate ($NaIO_3$)	Merck, KGaA, Darmstadt

Sodium pyruvate	Life technologies Corporation, Carlsbad, USA
Trypan blue	Life technologies Corporation, Carlsbad, USA
Tween 20	Sigma-Aldrich Chemie GmbH, Steinheim
Xylazine	Sigma-Aldrich Chemie GmbH, Steinheim

2.1.2 Antibodies and flow cytometry reagents

Table 3: Antibodies and reagents for flow cytometry

Antibody	Clone	Label	Manufacturer	Isotype	Conc. mg/ml	Dilution
anti-mouse CD16/32	93	-	eBioscience, Vienna, Austria	-	0.1	1:100
anti-mouse CD19	ID3	eF450	eBioscience, Vienna, Austria	Rat IgG2a	0.2	1:300
anti-mouse CD3e	145-2C11	eF450	eBioscience, Vienna, Austria	armenian Hamster IgG	0.2	1:300
anti-mouse CD49b	DX5	eF450	eBioscience, Vienna, Austria	Rat IgM	0.2	1:300
anti-mouse Ly6G	1A8	V450	BD Biosciences Belgium	Rat IgG2a	0.2	1:300
anti-mouse SiglecF	E50-2440	BV421	BD Biosciences Belgium	Rat IgG2a	0.2	1:300
anti-mouse CD11c	N418	APC	eBioscience, Vienna, Austria	armenian Hamster IgG	0.2	1:800
anti-mouse CD11c	HL3	PE-CF^{TM}594	BD Biosciences Belgium	armenian Hamster IgG	0.2	1:800
anti-mouse CD11b	M1/70	APC	eBioscience, Vienna, Austria	Rat IgG2b	0.08	1:800
anti-mouse CD103	2E7	PerCP-Cy5.5	BioLegend, London, UK	armenian Hamster IgG	0.2	1:800
anti-mouse CD64	X54-5/7.1	PE	BioLegend, London, UK	Mouse IgG1	0.2	1:800
anti-mouse CD88	20/70	PE-Cy7	BioLegend, London, UK	Rat IgG2b	0.2	1:300
anti-mouse CD24	M1/69	PE	BioLegend, London, UK	Rat IgG2b	0.2	1:800
anti-mouse CD301	LOM-14	PE	BioLegend, London, UK	Rat IgG2b	0.2	1:400
anti-mouse MHCII	M5/114.15.2	FITC	BioLegend, London, UK	Rat IgG2b	0.5	1:1500
anti-mouse MHCII	M5/114.15.2	APC-eF780	eBioscience, Vienna, Austria	Rat IgG2b	0.2	1:1500
anti-mouse MHCII	M5/114.15.2	FITC	Biolegend, London, UK	Rat IgG2b	0.5	1:1500
anti-mouse	IC10	APC	eBioscience,	Rat IgG2a	0.2	1:800

CD40			Vienna, Austria			
anti-mouse CD80	16-10A1	APC	eBioscience, Vienna, Austria	armenian Hamster IgG	0.2	1:800
anti-mouse CD86	GL-1	APC	BioLegend, London, UK	Rat IgG2a	0.2	1:800
anti-mouse OX40L	RM134L	APC	BioLegend, London, UK	Rat IgG2b	0.2	1:800
anti-mouse TSLPR	22H9	Biotin	BioLegend, London, UK	Rat IgG2a	0.2	1:400
anti-mouse CD127	A7R34	PE-Cy5	eBioscience, Vienna, Austria	Rat IgG2a	0.2	1:200
anti-mouse IL17RB	9B10	PE	eBioscience, Vienna, Austria	Rat IgG2a	0.2	1:400
anti-mouse CD197	4B12	APC	BioLegend, London, UK	Rat IgG2a	0.2	1:400
anti-mouse IL17A	TC11-18H 10	APC	Milteny Biotec GmbH, Bergisch Gladbach	Rat IgG1	0.2	1:400
anti-mouse antiFOXP3	FJK-16S	APC	eBioscience, Vienna, Austria	Rat IgG2a	0.2	1:200
anti-mouse IFN-γ	XMG1.2	APC	eBioscience, Vienna, Austria	Rat IgG1	0.2	1:200
anti-mouse IL13R	eBio13A	PE	eBioscience, Vienna, Austria	Rat IgG1	0.2	1:200
anti-mouse C5a	I52-1486	-	BD Biosciences, Erembodegem, Belgium	Rat IgG1	0.5	1:50
anti-mouse ST2	RMST2-2	APC	eBioscience, Vienna, Austria	IgG2a	0.2	1:400
anti-mouse CD4	RM4-5	PE-Cy7	eBioscience, Vienna, Austria	Rat IgG2a	0.2	1:400
Fixable Viability Dye	-	APC-eF780	eBioscience, Vienna, Austria	-	-	1:1500
Fixable Viability Dye		eF450	eBioscience, Vienna, Austria	-	100 tests	1:1500
anti-mouse SiglecF	E50-2440	PE	BD Biosciences Belgium	Rat IgG2a	0.2	1:400
InVivoMAb anti-mouse CD40L (CD154)	MR-1	-	BioXCell, NY, USA	Armenian Hamster IgG	6.69	Working concentration 5µg/ml
InVivoMAb anti-mouse MHCII (I-A/I-E)	M5/114	-	BioXCell, NY, USA	Rat IgG2b	7.96	Working concentration 10µg/ml & 1µg/ml

APC= Allophycocyanin, BV= Brilliant Violet, Cy= Cyanin dye, eF= eFlour, FITC= Fluoresceinisothiocyanat, PE= Phycoerythrin, PerCP= Peridinin chlorophyll protein, V= Violet

2.1.3 Consumables

Table 4: Consumables

Material	Manufacturer
BD Microtainer tube	BD Biosciences Europe, Erembodegem, Belgium
Cell strainer 40 µm	BD Biosciences Europe, Erembodegem, Belgium
ELISA-reservoir 25 ml	VWR International GmbH, Darmstadt
Filtertip 10 µl, 100 µl, 1000 µl	Sarstedt AD & Co., Nümbrecht
Microscope slide	Gerhard Menzel GmbH, Braunschweig
MACS Separation LS Column	Milteny Biotec GmbH, Bergisch Gladbach
Tracheal cannula for mouse (OD 1.2 mm, L 15mm)	Hugo Sachs, March-Hugstetten
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
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
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
Tube 5 ml	Sarstedt AD & Co., Nümbrecht
Tube 15 ml, 50 ml	Sarstedt AD & Co., Nümbrecht
Weighing dish	Greiner Bio-One GmbH, Frickenhausen

2.1.4 Kits

Table 5: Kits

Kit	Manufacturer
CD4 T cell isolation kit II	Milteny Biotec GmbH, Bergisch Gladbach
Mouse IL13 DuoSet	R&D Systems, Wiesbaden
Mouse IL17A DuoSet	R&D Systems, Wiesbaden
Mouse IFN-γ Duoset	R&D Systems, Wiesbaden
Agilent RNA 6000 Pico Kit	Agilent Technologies, USA
PKH26 Red Fluorescent Cell Linker Mini Kit for General cell membrane labeling	Sigma-Aldrich
RNeasy Micro Kit	Qiagen

2.1.5 Buffers and solutions

Table 6: Buffers and solutions

Buffer/Solution	Substance
10x anesthetic (BALB/c)	2% Rompun 50 mg/ml Ketavet
1x anesthetic (BALB/c) **(1:10 dilution of 10x anesthetic BALB/c in PBS)**	PBS 0.2% Rompun 5 mg/ml Ketavet
Block buffer	anti-mouse CD16/32 antibody 1:100 dilution in flow buffer
Digestion medium (lung cell isolation)	RPMI 1640 0.25 mg/ml Liberase TL and 0.5mg/ml DNaseI
MACS buffer	MACS BSA stock solution 1:20 dilution in PBS
Labeling buffer	5ml pre-warmed PBS 0.5 µl of 10mM CFSE
Formaldehyde solution	900 ml A. dest 7.8 g Na_2HPO_4 1.87 g NaH_2PO_4 100 ml Formaldehyde stock solution (37%)
Saponin buffer	0.2% saponin 20% FCS PBS
RLT buffer	Part of the RNeasy Micro Kit, Qiagen
RDD	Part of the RNeasy Micro Kit, Qiagen
RW1	Part of the RNeasy Micro Kit, Qiagen
Complete medium	RPMI 1640 10% FBS, heat inactivated 100 Units/ml Penicillin 100 µg/ml Streptomycin 2mM L-Glutamine
Pure medium (lung cell isolation)	RPMI 1640 without additives
Red blood cell lysis buffer (RBC)	A. dest 155 mM NH_4Cl 10 mM $KHCO_3$ 0.1 mM EDTA pH 7.2 sterile
Wash medium (lung cell isolation)	RPMI 1640 10% FBS, heat-inactivated 100 Units/ml Penicillin 100 µg/ml Streptomycin 2 mM L-Glutamine 0.5 mg/ml DNase I
FOXP3 transcription factor staining buffer set	eBioscience,Vienna, Austria

Components: **Fixation/Permeabilization concentrate** **Fixation/Permeabilization diluent** **Permeabilization buffer (PERM) (10X)**	

2.1.6 Mice

Table 7: Mouse strains

Name	Strain	Official name (symbol)	Breeder
Wildtype (WT)	BALB/c		Charles River, Breeding Laboratories, Sulzfeld, Germany
DO11.10RAG2$^{-/-}$	BALB/c	Tg(DO11.10)10Dlo	Internal breeding

2.2 Equipment and software

2.2.1 Equipment

Table 8: Equipment

Equipment	Manufacturer
BD FACS Aria™ III	Beckton Dickinson GmbH, Heidelberg
Biological Safety Cabinets	Nuaire Inc., Plymouth, USA
Centrifuge 5424	Eppendorf AG, Hamburg
Centrifuge 5424R	Eppendorf AG, Hamburg
Centrifuge 5810R	Eppendorf AG, Hamburg
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
Flow cytometer BD LSR II	Beckton Dickinson GmbH, Heidelberg
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 Magnetic Cell Separator	Milteny Biotec GmbH, Bergisch Gladbach
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
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
Agilent Bioanalyzer 2100	Agilent Technologies, USA

2.2.2 Software

Table 9: Software

Software	Company
BD FACSDiva 7.0	BD Biosciences, San Jose, USA
FlowJo_V10	FlowJo, LLD, Ashland, USA
Imaris 9.2.1	BITPLANE, an Oxford Instruments Company
GraphPad Prism 7.0	Graph Pad Software Inc., LaJolla, USA
Leica Application Suite 2.0.0	Leica Microsystems GmbH, Heerbrugg, Swiss
Microsoft Excel 365 Business	Microsoft Corporation, Redmond, USA
Microsoft Powerpoint 365 Business	Microsoft Corporation, Redmond, USA
Microsoft Word 365 Business	Microsoft Corporation, Redmond, USA

2.3 Methods

2.3.1 Mice

The DO11.10RAG2$^{-/-}$ mice on the BALB/c background were bred and kept in the specific-pathogen free (SPF) facility of the university of Lübeck (Gemeinsame Tierhaltung (GTH)) according to institutional and national guidelines. WT BALB/c mice were purchased from Charles River laboratories and used at 8-12 weeks of age. The studies were reviewed and approved by the Animal Care and Use Committee from the Schleswig Holstein state authorities - Ministerium für Landwirtschaft, Energiewende und ländliche Räume, Kiel, Germany (39 (75-6/16) Köhl and 39_2017-0301_Laumonnier). Mice were sacrificed by an overdose of anesthetic (200µl 76mg/ml Ketamin, 4.8mg/ml Xylazin), followed by cervical dislocation, if not stated otherwise.

2.3.2 Model of combined house dust mite (HDM)/ovalbumin (OVA)-mediated allergen sensitization

To induce allergen sensitization, WT BALB/c mice were anesthetized with 6.9mg/kg bodyweight Xylazin (Rompun, Pfizer) and 114.5 mg/kg bodyweight Ketamin (Ketavet, Bayer). The deeply narcotized mice were

fixed on a tripod by their incisors and an elastic band. The tongue was carefully pulled out and a total volume of 50µl of PBS with the dissolved HDM/OVA allergens was administered to the throat. More precisely, the mice were sensitized once by an intratracheal (i.t.) application of 100µg house dust mite (HDM) extract and 40µg ovalbumin (OVA) in 50µl PBS. 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 backflow of the administered substance. The animals were kept under red light to prevent them from cooling. Twenty-four hours after the one step HDM/OVA sensitization, the mice were euthanized, and the lungs were harvested for further analysis (Figure 5).

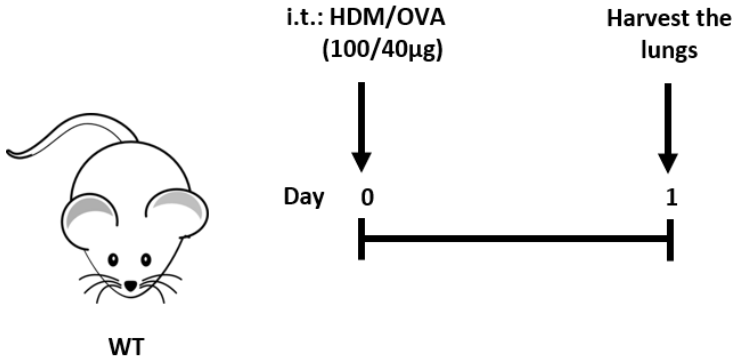


Figure 5: Model of HDM/OVA mediated allergen sensitization. WT BALB/c mice received HDM/OVA (100/40µg) i.t. dissolved in 50 ul PBS. Twenty-four hrs later the mice were euthanized, and the lungs were harvested for further analysis.

2.3.3 Organ removal and preparation of lung cell suspension

Before lung isolation, bronchioles were lavaged with 1 ml ice-cold PBS. For broncho-alveolar lavage (BAL), the rib cage was opened, the trachea was exposed to form a curve and was opened by a half incision on the upper third ring. The lavage of the bronchi was done by insertion of a 20-gauge catheter. The next step was the lung isolation. All the lobes were harvested from the thorax cavity and placed in a 40µm cell strainer in a 6-well plate with 5ml pure RPMI 1640 which was kept on ice. The lung tissue was chopped with scissors to small pieces and was digested for 45 min at 37°C on a shaker in the presence of freshly reconstituted 0.5mg/ml DNase and 0.25mg/ml Liberase TL. Subsequently, the lung tissue-containing cell strainer was transferred to a 50 ml Falcon tube. The single cell suspension was prepared by mechanical disruption of the lungs with the help of a 5ml syringe stamp and additional 10ml complete medium in the presence of 0.5mg/ml DNase. The smoothing step was repeated twice with a subsequent step of washing with 5 ml of complete medium (+DNA-I). The, the cell suspension was centrifuged for 10min at 350 x g at 4°C. The supernatant was discarded, and the pellet was re-suspended in 3ml RBCL-buffer for 3min at RT to lyse the erythrocytes. The lysis was stopped by adding 30 mls of PBS and the cell suspension was centrifuged for 5min, at 400 x g at 4°C. An amount of 20µl of the cell suspension was mixed with 20µl of trypan blue in an eppendorf tube and counted in a Neubauer counting chamber. Only living cells were counted and total numbers of cells were calculated using the following formula:

$$total\ cell\ count = \frac{counted\ cells}{number\ of\ counted\ big\ squares} \times dilution\ factor\ \times 10^4\ \times V(ml)$$

Once the cell number was determined, the single cell suspension was used either for flow-cytometry analysis, or fluorescence-activated cell sorting (FACS).

2.3.4 Fluorescence staining, flow cytometry analysis and fluorescence activated cell sorting

2.3.4.1 Surface fluorescence staining

The lung cell-suspension was adjusted to a density of $1x10^7$cells/ml, and 100µl per $1x10^6$ cells were used for each staining. The cells were centrifuged using the Centrifuge 5424R for 30s at max. speed at 4°C and resuspended in 100µl FC-block, for 20 min at 4°C to saturate unspecific binding sites. The cells were then centrifuged for 30s at max. speed at 4°C to remove the unbound antibodies from the FC-block and were resuspended in 100µl of the surface antigen staining with conjugated CD antibodies (20min incubation at 4°C), which was prepared in flow buffer, using the antibodies depicted in Table 2 at the given concentrations. The cells were washed with 1ml PBS to wash out unbound Abs and resuspended in 300µl flow buffer for analysis on a BD LSRII flow cytometer. The threshold was set on FSC at 35,000 to exclude small particles and cell debris.

2.3.4.2 Intra-cellular fluorescence staining

For the intra-cellular staining of the cytokines IFN-γ, IL-13, IL-17 and the transcription factor Foxp3 the Foxp3/Transcription Factor Staining Buffer Set was used, whereas for the complement products C5 and C5a, a formaldehyde-based fixation and saponin based permeabilization approach was used. In both cases, surface and live/dead staining were done before the permeabilization step.

2.3.4.2.1 Foxp3/Transcription Factor Staining Buffer Set

After the surface expression staining as described in 2.3.4.1 of the $CD4^+$ T cells, the Foxp3/Transcription Factor Staining Buffer Set was used and adapted to manufacturer's instructions for the staining of the aforementioned cytokines. The cells were fixed in 100µl fixation-buffer for 1 hour at 4°C. Then 100µl Fc-block in permeabilization buffer (PERM) was added and incubated for 20 min at 4°C. After centrifugation (using the Centrifuge 5424R) for 30sec at max speed at 4°C, the cells were incubated for 15 min at 4°C in the antibody master mix for the intracellular proteins prepared in PERM. Afterwards, the cells were washed twice in PERM and resuspended in 300µl flow buffer for the analysis on a BD LSR II.

2.3.4.2.2. Saponin-based permeabilization approach

For the intracellular staining of C5 or C5a, either freshly FACS-sorted $CD11b^+C5aR1^+$cDCs and $CD11b^+C5aR1^-$cDCs, or cells which were seeded in a 96-well U-bottom plate in the presence or absence of $CD4^+$ T cells, were used. In all cases, the cells were transferred into eppendorf tubes and centrifuged (using the Centrifuge 5424R) at maximum speed for 30sec at 4°C Then the cells were fixed for 30min at 4°C in 100µl formaldehyde (1.5%) centrifuged for 30s at max speed at 4°C and then resuspended in a saponin

(0.2%) buffer containing 20% FCS for 30 min at RT. After centrifugation, the cells were incubated with the respective primary antibody in 100µl dissolved in saponin-buffer for 20 min at 4°C. Cells were then centrifuged and incubated with the secondary antibody against rat IgG1 in 100µl saponin-buffer for 20min at 4°C. Afterwards, the cells were washed twice in saponin-buffer and resuspended in 300µl flow buffer for the analysis on a BD LSR II flow cytometer.

2.3.4.3 Determining cell populations and protein expression by flow cytometry

Flow cytometry is a laser-based technology, in which both the physical and the molecular characteristics of cells can be measured with a high flexibility. Single cells, passing the laser beam, can be distinguished by at least three factors. The first one is their size (forward scatter (FSC)), the second one is their granularity (side scatter (SSC)), and the last one is the expression of proteins, which were stained with fluorescently-labeled monoclonal antibodies. More specifically, the use of different antibody-combinations allows to determine the frequency of a certain cell population in a cell suspension. Therefore, by using an antibody-combination, I was able to determine the frequency of a certain dendritic cell population within the whole lung cells. On top of that, flow cytometry allows the analysis of functional cell properties such as cell proliferation. For the proliferation assay, a slightly different staining protocol was used. The cells of interest were labeled with Carboxyfluorescein succinimidyl ester (CFSE), which allows a successful proliferation analysis by dye dilution. In most stainings, the antibody panels that are used are complex, and the antibodies, tagged with different fluorochromes reveal different emission spectra, which partly overlap. This spectral overlap was corrected by compensation, which was done manually within the compensation matrix of the analysis-software FlowJo_V10 (FlowJo, LLD). Cells were analyzed on a BD LSR II analyzer or a BD FACS ARIA^{TM} III cell sorter with a flow-velocity of approximately 1,000cells/s. About 100,000 cells, if possible, were measured for further analysis with FlowJo_V10.

2.3.4.4 Purification of pulmonary dendritic cells by fluorescence activated cell sorting

For Fluorescence Activated Cell Sorting (FACS), a high number of cells was needed. Thus, to sort CD11b^{+} cDC cells subsets, lung tissue isolates from several mice had to be pooled. The cells were resuspended in MACS-buffer at a density of 2x10^{7}cells/ml. Shortly before sorting, the cells were filtered through a 40µm cell strainer to maintain a single-cell suspension. The sorting of the different DC subsets was based on the principle of flow-cytometry. It is important is to exclude any doublets found in the cell suspension, which could give a false-positive signal as well as dead cells and cell debris. The purification of DC subpopulations was done using a BD FACS ARIA^{TM} III cell sorter, equipped with a 100µm nozzle. The cells were sorted into 200µl complete medium supplemented with 20ng/ml GM-CSF.

2.4 Dendritic cell/ T cell co-culture

The co-culture system comprised either CD11b^{+}C5aR1^{+}cDCs and CD4^{+} transgenic (tg) T cells or CD11b^{+}C5aR1^{-}cDCs and CD4^{+} T cells.

2.4.1 Isolation and sorting of CD11b^{+}C5aR1^{+}cDCs and CD11b^{+}C5aR1^{-}cDCs

Mice were sensitized with a mixture of HDM/OVA as described in 2.3.2 section. The lungs were harvested, and the single cell suspension was prepared as described in section 2.3.3. The lung suspension was blocked and stained as described in 2.3.4. The cells were then sorted on a BD ARIA III^{TM}, using an established gating

strategy (Figure 6). Briefly, the first steps were to gate out doublets, dead cells, eosinophils, alveolar macrophages, neutrophils, T-, B- and NK cells. In the next step, I gated on DCs, which were $CD11c^+$ and $MHCII^{high}$. Then, by using CD103 and CD11b, three subsets of cDCs could be differentiated in the lung. At this step, the gate was set on cells which were $CD103^-CD11b^+$. Since it is known that in the lungs there are also monocyte derived DCs (mo-DCs), which are positive for CD11b, at this step it was important to gate out any mo-DCs. Thus, I used CD64 as a typical mo-DC marker and set the gate on $CD11b^+CD64^-$ cells. The last gate was set based on the expression of C5aR1 in the $CD103^-CD11b^+CD64^-$cDC subset. Using this strategy, I was able to differentiate two populations, which were sorted, i.e. $CD103^-CD11b^+CD64^-$ $C5aR1^+$cDCs (75%) and $CD103^-CD11b^+CD64^-C5aR1^-$cDCs (25%). The sort purity was 76%.

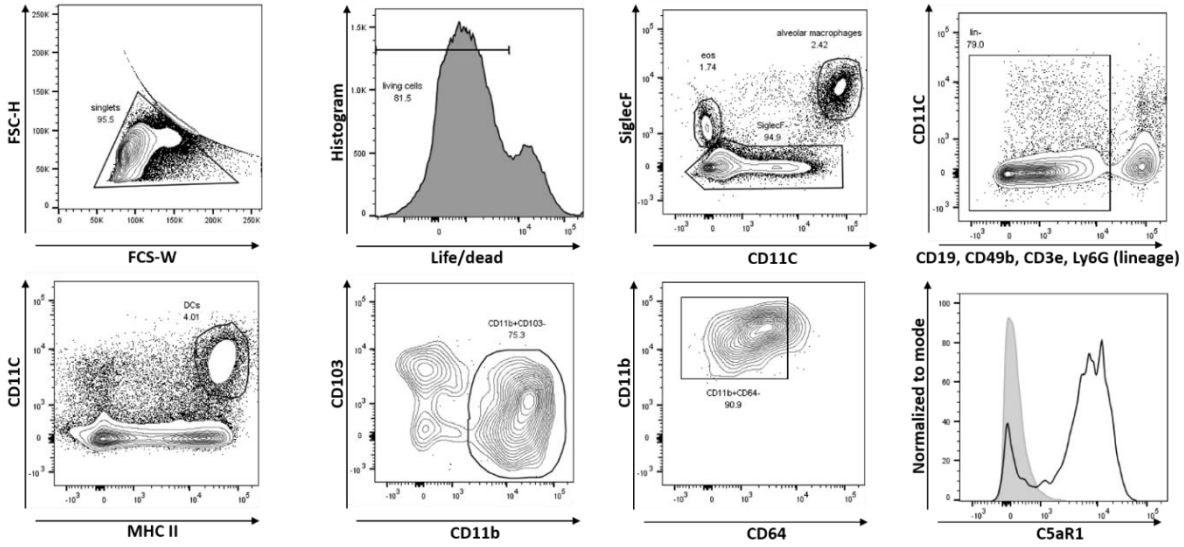


Figure 6: Gating strategy to purify pulmonary $CD11b^+C5aR1^+$cDCs and $CD11b^+C5aR1^-$cDCs. The two cDC cell populations were identified after sequential gating for: (i) single cells using forward scatter height vs forward scatter width, (ii) live/dead-stain negative cells, (iii) $SigleF^{neg}$ and $CD11C^{pos}$ cells, (iv) lineage negative cells, (v) $CD11C^+$ and $MHCII^{high}$ cells. At this step, three subpopulations of DCs were identified using the CD103 and CD11b markers; the gate was set on cells which were $CD103^-CD11b^+$, (vi) within the $CD11b^+$cDC fraction, $CD64^+$ mo-DCs can be found. Thus, the gate was set to $CD64^-CD11b^+$ cells. (vii) Using C5aR1 as a marker, I discriminated two populations, one negative for the expression C5aR1 (25%) and one which was positive (75%). So, the two populations that were sorted were: $SiglecF^-lin^-CD11c^+MHCII^+CD103^-CD11b^+CD64^-C5aR1^+$cDCs and $SiglecF^-lin^-CD11c^+MHCII^+CD103^-CD11b^+CD64^-$ $C5aR1^-$cDCs.

2.4.2 Isolation and CFSE-labelling of OVA-transgenic $CD4^+$ T cells

Splenic OVA-transgenic $CD4^+$ T cells were isolated from DO11.10 $RAG2^{-/-}$ mice. For this purpose, mice were sacrificed by CO_2 and cervical dislocation. The spleen was removed and placed in ice-cold PBS. The single cell suspension was prepared by mechanical disruption of the spleen, with the help of a 5ml syringe stamp and additional 10ml of PBS in the presence of 0.5mg/ml DNase. The spleen cells were then centrifuged for 10min at 350 x g at RT. The negative isolation of $CD4^+$ T cells was done with the $CD4^+$ T cell isolation kit from Miltenyi according to manufacturer's instructions. Briefly, the cell pellet was re-suspended in 400µl of MACS buffer and then incubated for 5min at 4°C in 100µl of biotin-antibody cocktail. The biotin antibody cocktail consists of biotin-conjugated antibodies against CD8a, CD11b, CD11c, CD19, CD45R (B220), CD49b (DX5), CD105, Anti-MHC-class II, Ter-119 and TCRγ/δ as primary labeling reagent. The cell suspension was then washed with 200 µl of MACS buffer and incubated for 10min at 4°C in 200µl anti-

biotin microbeads. The cells were then washed with 10mls MACS buffer and centrifuged for 5min at 400 x g at RT. The pellet was re-suspended in MACS buffer followed by magnetic cell separation using the MACS cell separation device. The cells were counted as described in section 2.3.3. Some of them were stained with an anti-CD4 antibody (in PE Cy7) to check for the purity of the isolated cell population with flow cytometry. Purity was always > 90%. For the CFSE labeling of the $CD4^+$ T cells, the CellTrace CFSE cell proliferation kit was used and the labeling was done following the manufacturer's instructions. Briefly, after the magnetic separation, the cells were centrifuged for 5min at 400 x g at RT and the cell pellet was re-suspended in 500µl of the labeling buffer (5ml of pre-warmed PBS +0.5µl of 10mM CFSE). The cells were incubated in the labeling buffer for 10min at 37°C in the dark. The reaction was stopped by adding 1ml ice-cold PBS and incubating the cells for 1min on ice. The cells were then centrifuged, and the pellet was re-suspended in complete medium. The labeling success was determined by flow cytometry. A bright, single-peak staining, with fluorescence intensity at 10^5 was considered as proper labeling. After successful CFSE labeling the $CD4^+$ T cells were added to a 96-well plate in which the $CD11b^+$cDCs were plated the day before.

2.4.3 Co-culture of $CD11b^+$ cDCs with CFSE-labeled OVA-transgenic $CD4^+$ T cells

The FACS-purified $CD11b^+C5aR1^+$cDCs and $CD11b^+C5aR1^+$cDCs were seeded in a 96-well U-bottom plate to ensure that cDCs and T cells were in close contact to each other, which is an important factor for DC survival. The cells were cultured in complete medium supplemented with 20ng/ml GM-CSF, which acts as a growth factor during DC differentiation. The cells were pulsed overnight with 10µM OVA (grade III) and in some cases with other blocking antibodies (Table 9). CFSE-labeled OVA-tg $CD4^+$ T cells were added the next day at a ratio of 1 : 2.5 [($CD11b^+$cDCs) : ($CD4^+$ T cells) = (1 : 2.5)]. After 4 days, the cell suspension was transferred to a 1.5ml eppendorf tube and centrifuged for 10min at 350 x g at 4°C. The supernatant was taken and frozen at -20°C for further analysis. The cells were stained with a fixable viability dye (APC-eF780) and CD4 following the protocol described in 2.3.4.1. The life/dead ratio and the CFSE-dilution of the OVA-tg T cells was analyzed on a BD LSRII flow cytometer.

Table 10: Different *in vitro* conditions used for the co-culture between $C5aR1^+$cDCs – $CD4^+$ T cells and $C5aR1^-$cDCs – $CD4^+$ T cells

Condition A

$CD11b^+C5aR1^-$cDCs	**$CD11b^+C5aR1^+$cDCs**	**$CD11b^+C5aR1^+$cDCs**
GM-CSF	**GM-CSF**	**GM-CSF**
OVA	**OVA**	**OVA**
	Anti-C5aR mAb (20/70)	**Isotype control**

Condition B

$CD11b^+C5aR1^-$cDCs	**$CD11b^+C5aR1^+$cDCs**	**$CD11b^+C5aR1^+$cDCs**
GM-CSF	**GM-CSF**	**GM-CSF**
$OVA^{323-339}$	**$OVA^{323-339}$**	**$OVA^{323-339}$**
	Anti-C5aR mAb (20/70)	**Isotype control**

Condition C

$CD11b^+C5aR1^-$ cDCs	$CD11b^+C5aR1^+$ cDCs	$CD11b^+C5aR1^+$ cDCs	$CD11b^+C5aR1^+$ cDCs	$CD11b^+C5aR1^+$ cDCs	$CD11b^+C5aR1^+$ cDCs
GM-CSF	GM-CSF	GM-CSF	GM-CSF	GM-CSF	GM-CSF
OVA	OVA	OVA	OVA	OVA	OVA
		Anti-C5aR mAb (20/70)	Anti-C5aR mAb (20/70)	Isotype control	Isotype control
	antiCD40L		antiCD40L		antiCD40L

Condition D

$CD11b^+C5aR1^-$cDCs	$CD11b^+C5aR1^-$cDCs	$CD11b^+C5aR1^-$cDCs
GM-CSF	GM-CSF	GM-CSF
OVA	OVA	OVA
Anti-MHCII mAb	Anti-MHCII mAb	Isotype control
Isotype control	antiCD40L	antiCD40L

Condition E

$CD11b^+C5aR1^-$ cDCs	$CD11b^+C5aR1^-$ cDCs	$CD11b^+C5aR1^-$ cDCs	$CD11b^+C5aR1^-$ cDCs	$CD11b^+C5aR1^-$ cDCs
GM-CSF	GM-CSF	GM-CSF	GM-CSF	GM-CSF
$OVA^{323-339}$ (5µg/ml)	$OVA^{323-339}$ (500ng/ml)	$OVA^{323-339}$ (50ng/ml)	$OVA^{323-339}$ (5ng/ml)	$OVA^{323-339}$ (5ng/ml)
				antiCD40L

Condition F

$CD11b^+C5aR1^+$ cDCs	$CD11b^+C5aR1^+$ cDCs	$CD11b^+C5aR1^+$ cDCs	$CD11b^+C5aR1^+$ cDCs	$CD11b^+C5aR1^+$ cDCs
GM-CSF	GM-CSF	GM-CSF	GM-CSF	GM-CSF
$OVA^{323-339}$ (5µg/ml)	$OVA^{323-339}$ (500ng/ml)	$OVA^{323-339}$ (50ng/ml)	$OVA^{323-339}$ (5ng/ml)	$OVA^{323-339}$ (5ng/ml)
				Anti-C5aR1 mAb (20/70)

Table 11: Concentration of the $OVA^{323-339}$ peptide and the neutralizing Abs used in section 2.4.2

	Working concentration	Working concentration	Working concentration	Working concentration
Anti-C5aR1	5µg/ml	-	-	-
Anti-CD40L	5µg/ml	-	-	-
Anti-MHCII	100pg/ml	10pg/ml	-	-
$OVA^{323-339}$	5µg/ml	500ng/ml	50ng/ml	5ng/ml

2.4.3 ELISA

The production of IL-13, IL-17 and IFN-γ in T-cell co-cultures was determined with DuoSet ELISA-kits (R&D systems). The detection limit for IL-17 was 15.6pg/ml, for IFN-γ 31.3pg/ml and for IL-13 62.5pg/ml. The cytokine concentrations were determined following the manufacturer´s instructions using half of the recommended amount of volume (instead of using 100µl, I used 50µl). Briefly, the plate was coated with 50µl of the specific capture antibody overnight at RT. Then the capture antibody was decanted, and the plate was washed 3 times with washing buffer. Afterwards, the plate was blocked with 150µl PBS/BSA (1%) for 1 hour and washed again 3 times with washing buffer. Then the standards and samples (50µl) were added in duplicates to the plate and incubated for 2 hours at RT. The plate was washed again 3 times with washing buffer and 50µl of the respective biotinylated detection antibody were added for another 2 hours at RT. After another wash, 50µl of streptavidin-horse-radish-peroxidase were added and incubated for 20min at RT in the dark. After washing, 50µl of substrate were added for 20min at RT in the dark. The reaction was stopped with 25µl 1M H_2SO_4, and the ELISA-plate was analyzed on a FluoStar Omega 0415 reader (BMG Labtech).

2.5 Laser scanning microscopy to assess dendritic cell T cell interactions

To determine the interaction between $CD11b^+$ cDCs and T cells, the FACS-purified sensitized $CD11b^+C5aR1^+$cDCs and $CD11b^+C5aR1^-$cDCs were seeded in a 96-well U-bottom plate in complete medium supplemented with 20ng/ml GM-CSF and pulsed with 10µM OVA. Eighteen hours later, the cells were collected into an Eppendorf tube and labeled with the red fluorescent dye PKH26 (membrane labeling) according to manufacturer's instructions. Briefly, the collected cells were washed with 1ml pre-warmed serum-free medium and centrifuged for 5min at 400 x g. The supernatant was aspirated. Then 100µl of diluent C (is an aqueous solution designed to maintain cell viability, while maximizing dye solubility and staining efficiency during the labeling step) were added to 1 x 10^5 cells. The diluent was mixed with the cells by gently pipetting up and down. Immediately prior to staining, in a separate Eppendorf, the dye solution was prepared by adding 0.4µl of PKH26 ethanolic solution dye to 100µl Diluent C. The dye was quickly pipetted into the Eppendorf tube, which already contained the cells. Then, the cells were mixed with the dye by gently pipetting the cell solution up and down. Afterwards, the cells were incubated with the dye for 5 min at RT in the dark with periodic mixing. The staining was stopped by adding 1ml of pre-warmed complete medium. The sample was centrifuged at 400 x g for 10min at RT. The supernatant was discarded, the cells were washed in 1ml pre-warmed complete medium and centrifuged again at 400 x g for 10 min at RT. The supernatant was aspirated, and the pellet was resuspended in 50µl of pre-warmed complete medium. The OVA tg $CD4^+$ T cells were negatively isolated by MACS and labeled with CFSE as described in section 2.4.1. CFSE-labeled-$CD4^+$ T cells (1 x 10^5) were resuspended in 50µl of pre-warmed

complete medium and transferred together with the PKH26-labeled cDCs in an Eppendorf tube. One end of the incubation chamber was unscrewed, and the mixture of DC-T cells was added by continuously pipetting to avoid bubbles. Afterwards, 700µl of pre-warmed complete medium was slowly added to both ends of the chamber to avoid perturbation of the cells. The cells were incubated for 30min at 37°C and subsequently transferred to the FV1000 confocal microscope to assess the DC-T cell interactions. The DC-T cell interactions were recorded for 5h by taking one picture per minute.

2.6 Antigen uptake and processing assay

The antigen uptake and processing assay was performed using either CD11b$^+$C5aR1$^+$cDCs from naïve WT mice or WT mice sensitized one time with HDM/OVA as described in section 2.3.2. In both cases, FACS-purified CD11b$^+$C5aR1$^+$cDCs were seeded in a 96-well U-bottom plate in complete medium supplemented with 20ng/ml GM-CSF. Fifty percent of these cells were treated with mAb 20/70, which specifically blocks the interaction of C5a with C5aR1. Eighteen hours after the *in vitro* targeting of C5aR1, the cells were pulsed with 10µg/ml FITC-OVA for 60min (Ag uptake assay) and 10µg/ml DQ-OVA for 120 and 240min (Ag uptake/processing assay) at 37°C or 4°C as a control (Table 10). Uptake and processing were detected in the FITC channel on a BD LSRII flow cytometer.

Table 12: Ag uptake & processing *in vitro* conditions

Condition A

CD11b$^+$C5aR1$^-$cDCs	**CD11b$^+$C5aR1$^+$cDCs**	**CD11b$^+$C5aR1$^+$cDCs**
GM-CSF	**GM-CSF**	**GM-CSF**
	Anti-C5aR mAb (20/70)	**Isotype control**
FITC-OVA (60min)	**FITC-OVA (60min)**	**FITC-OVA (60min)**

Condition B

CD11b$^+$C5aR1$^-$cDCs	**CD11b$^+$C5aR1$^+$cDCs**	**CD11b$^+$C5aR1$^+$cDCs**
GM-CSF	**GM-CSF**	**GM-CSF**
	Anti-C5aR mAb (20/70)	**Isotype control**
DQ-OVA (120min)	**DQ-OVA (120min)**	**DQ-OVA (120min)**

Condition C

CD11b$^+$C5aR1$^-$cDCs	**CD11b$^+$C5aR1$^+$cDCs**	**CD11b$^+$C5aR1$^+$cDCs**
GM-CSF	**GM-CSF**	**GM-CSF**
	Anti-C5aR mAb (20/70)	**Isotype control**
DQ-OVA (240min)	**DQ-OVA (240min)**	**DQ-OVA (240min)**

2.7 RNA sequencing

I determined the transcriptional profile of C5aR1^{+}CD11b^{+}cDC and C5aR1^{-}CD11b+ subpopulations in response to one-time HDM/OVA immunization. Further, I assessed the impact of C5aR1 targeting on the transcriptional activity of C5aR1+CD11b+ cDCs after one-time HDM/OVA immunization. For this purpose, I performed two RNA sequencing experiments. For the first experiment, FACS-purified CD11b^{+}C5aR1^{+}cDCs and CD11b^{+}C5aR1^{-}cDCs from HDM/OVA sensitized mice were used (Figure 7). Immediately after sorting, the cells were centrifuged for 5min at 500 x g at 4°C, the supernatant was aspirated, and the pellet was snap frozen in liquid nitrogen. For the second experiment, the impact of C5aR1 targeting was tested. For this purpose, the FACS-purified CD11b^{+}C5aR1^{+}cDCs from HDM/OVA sensitized mice were seeded into 96-well plates as described in section 2.5.1, Table 9, condition A. Half of these cells were treated with the neutralizing mAb 20/70 targeting C5aR1; the other half of the cells was treated with the appropriate isotype control. Eighteeen hours later, the cells were collected, pelleted, the supernatant was aspirated, and the pellet was frozen at -80°C (Figure 8). In both experiments the same RNA extraction protocol was used.

2.7.1 RNA extraction

For the RNA extraction, the pellets were thawed and the Qiagen microRNA easy kit was used. The extraction was done according to the manufacturer's instructions. Briefly, the samples were thawed, and the pellet was immediately resuspended in 75µl of RLT buffer (contains a high concentration of guanidine isothiocycanate, which supports the binding of RNA to the silica membrane) (+β-mercaptoethanol) and vortexed for 30s to disrupt the plasma membrane. Seventy-five µl of 70% ethanol were added to the Eppendorf tube and the total volume of 150µl was transferred into a column that was provided with the kit. The columns were centrifuged for 15s at 10.000 x g at RT. The fluid was discarded and 350µl of RW1 (contains a guanidine salt, as well as ethanol, and is used as a stringent washing buffer that efficiently removes biomolecules such as carbohydrates, proteins and fatty acids, that are non-specifically bound to the silica membrane) (provided with the kit) were added to the samples, which were then centrifuged again using the same conditions as before. Again, the fluid was discarded and 10µl of DNase + 70µl of RDD (provides efficient on-column digestion of DNA and ensures that the RNA remains bound to the column) buffer was added to each sample. The entire volume was pipetted on the membrane and the samples were incubated for 15min at RT. Then, 350µl of RW1 (provided with the kit) were added and the samples were centrifuged for 15s at 10.000 x g at RT. The fluid was discarded, and the column was placed into a new 2ml collection tube. Five hundred microliters of RPE (its main function is to remove traces of salts, which are still on the column due to buffers used earlier in the protocol) (provided with the kit) were added and the samples were centrifuged for 15s at 10.000 x g at RT. The fluid was discarded and 500µl of 80% ethanol were added and the samples were centrifuged for 2min at 10.000 x g at RT. The fluid was discarded, and the column was transferred into a new 2ml collection tube. The samples were centrifuged with lid left open for 5min at 20.000 x g at RT. The fluid was discarded, and the column was transferred into a 1.5ml collection tube. The bound RNA was eluted with 14µl RNAse free water. The samples were incubated for 10min at RT and then centrifuged for 1min at 20.000 x g at RT. Finally, the RNA quality of each sample was tested with the Agilent Bioanalyzer. All samples were of high quality (on the following section I will clarify what is considered high quality and how this evaluated). They were shipped to the

DNA Core facility of Cincinnati Children's Medical Center, Ohio, USA, where the RNAseq was performed.

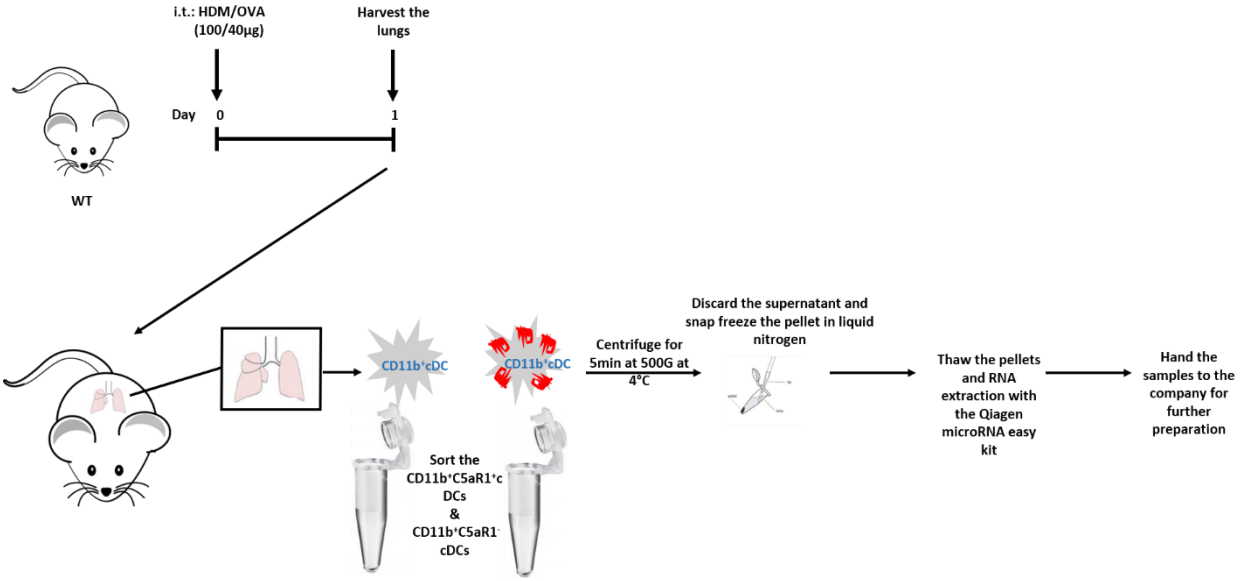


Figure 7: Flow chart for RNAseq sample preparation of CD11b^{+}C5aR1^{+} and CD11b^{+}C5aR1^{-} cDCs. WT mice were sensitized intra-tracheally with a mixture of HDM/OVA (100/40µg) and 24h later the mice were euthanized, and the lungs were harvested. After enzymatic lung digestion, the CD11b^{+}C5aR1^{+}cDCs and CD11b^{+}C5aR1^{-}cDCs were FACS-purified. The cells were centrifuged for 5min at 500 x g at 4°C, the supernatant was aspirated, and the pellet was snap frozen in liquid nitrogen. For the RNA extraction, the Qiagen microRNA easy kit was used, the RNA quality was checked with the Agilent Bioanalyzer and the samples were subsequently shipped to the DNA core of Cincinnati Children's in Ohio, USA for RNA sequencing.

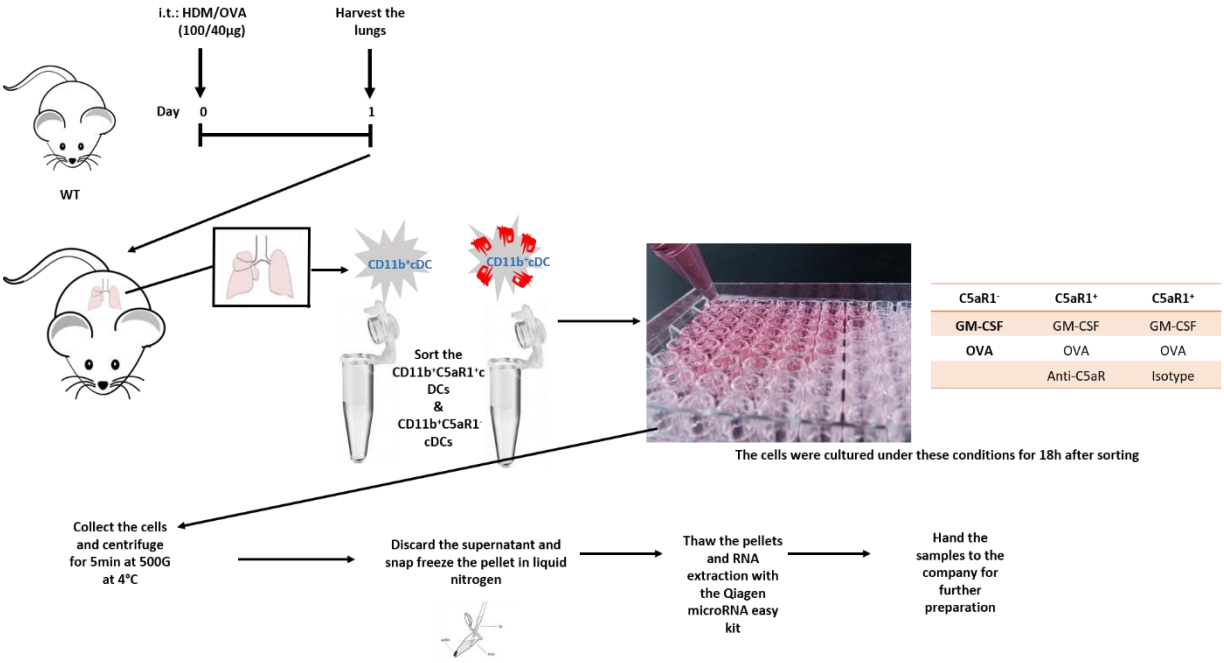


Figure 8: Flow chart for RNAseq sample preparation of CD11b$^+$C5aR1$^+$ cDCs in the presence or absence of C5aR1 targeting. WT mice were sensitized intra-tracheally with a mixture of HDM/OVA (100/40µg) and 24h later the mice were euthanized, and the lungs were harvested. The CD11b$^+$C5aR1$^+$cDCs and CD11b$^+$C5aR1$^-$cDCs were FACS-purified. The cells were seeded in 96-well plates in complete medium (+20ng/ml GM-CSF), pulsed with OVA and the C5aR1$^+$cDCs, were splitted into two wells. One half of these cells were treated with antiC5aR mAb (20/70) and the second half with the appropriate isotype control. Eighteen hours later, the cells were collected, centrifuged for 5min at 500 x g at 4°C, the supernatant was aspirated, and the pellet was snap frozen in liquid nitrogen. Before proceeding with RNA sequencing, the T cell proliferation was evaluated to check if the in vitro blocking of C5aR1 was efficient. The CD11b$^+$C5aR1$^-$cDCs were not used for the sequencing but only as control for evaluating the T cell proliferation. For the RNA extraction the Qiagen microRNA easy kit was used, the RNA quality was checked with the Agilent Bioanalyzer and the samples were subsequently shipped to the DNA core of Cincinnati Children's in Ohio, USA for RNA sequencing.

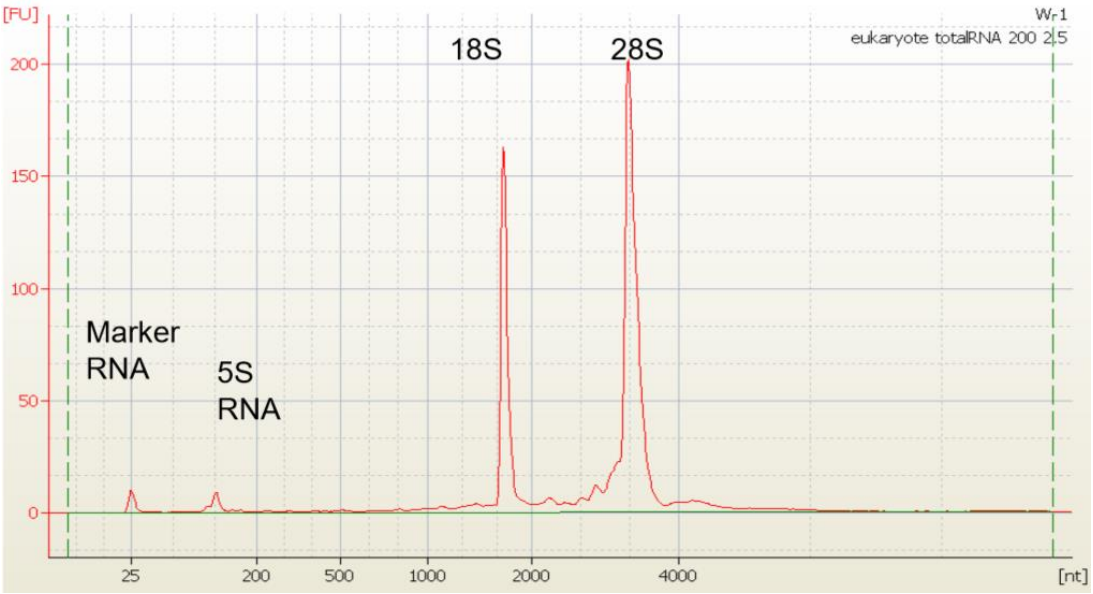
2.7.2 RNA 6000 Pico assay

2.7.2.1 Principles of Nucleic Acid Analysis on a Chip

The electrophoretic assay is based on traditional gel electrophoresis principles for the analysis of RNA. The chip has sample wells, gel wells and a well for a ladder. Micro-channels are fabricated in glass to create interconnected networks among these wells. During chip preparation, the micro-channels are filled with a polymer gel and fluorescence dye. Once the wells and channels are filled, the chip becomes an integrated electrical circuit. The 16-pin electrodes of the cartridge are arranged so that they fit into the wells of the chip. Each electrode is connected to an independent power supply. The charged biomolecules are electrophoretically separated by size. Dye molecules intercalate into RNA strands. These complexes are detected by laser-induced fluorescence. Data are translated into gel-like images (bands) and electropherograms (peaks). With the help of a ladder that contains components of known sizes, a standard curve of migration time versus fragments size is plotted. From the migration times measured for each fragment in the sample, the size is calculated. One marker runs with each of the samples bracketing the overall sizing range. The "lower" and "upper" markers are internal standards used to align the ladder

data with data from the sample wells. Quantitation is done with the help of the ladder area. The area under the ladder is compared with the sum of the sample peak areas. The area under the “lower” marker is not taken into consideration. For total RNA assays, the ribosomal ratio is determined, giving an indication on the integrity of the RNA sample. Additionally, the RNA integrity number (RIN) can be utilized to estimate the integrity of total RNA samples based on the entire electrophoretic trace of the RNA sample, including the presence or absence of degradation products (Figure 9).

A



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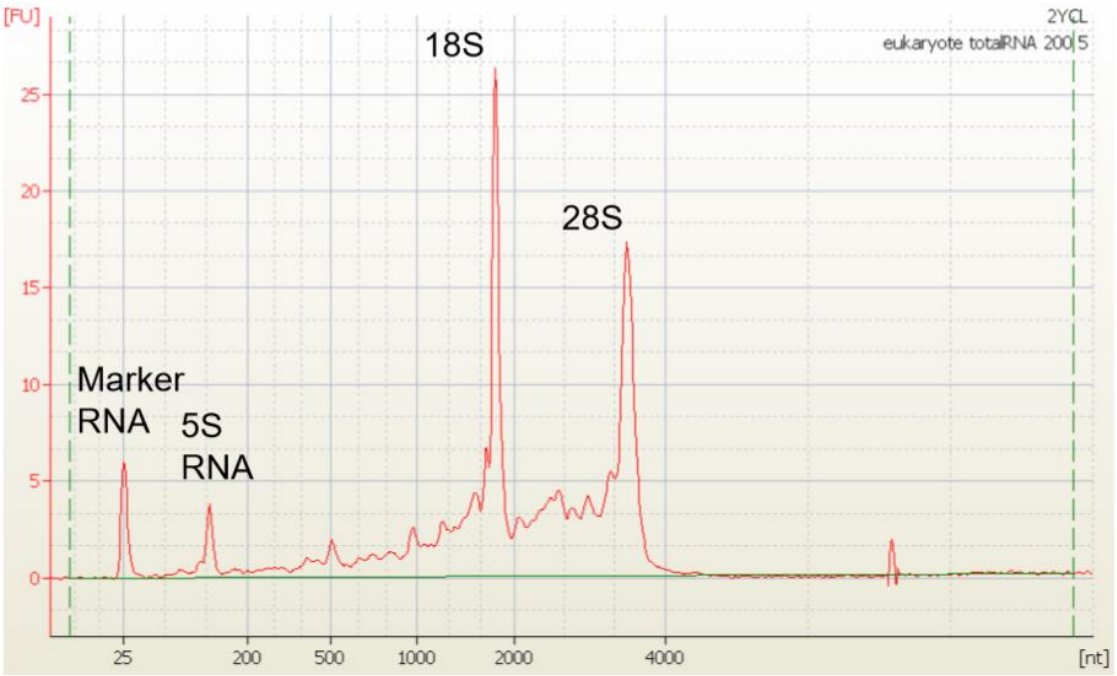

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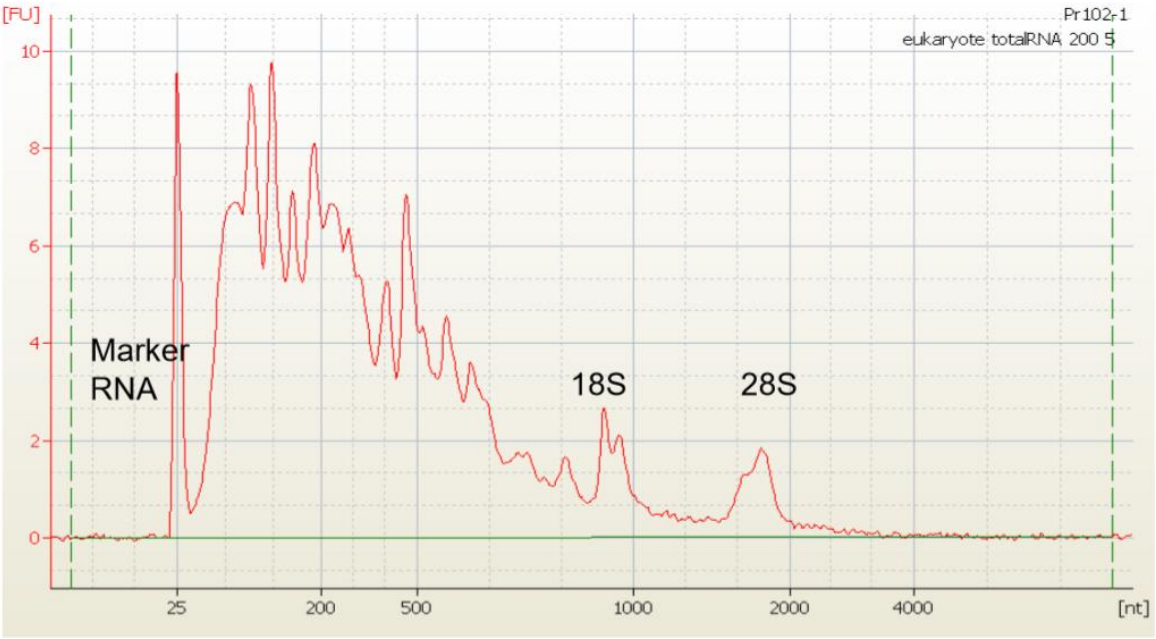


Figure 9: RNA quality assessment using the Agilent Bioanalyzer 2100. The RNA integrity of a sample is calculated using an algorithm known as RNA integrity number (RIN). The analyzer assigns a number according to how much signal is detected between the 5S and 18S band, between the 18S and 28S bands, and after the 28S band. A RIN number of 10 is perfect score. (A) RNA peaks of a successful sample run with RIN=10, detecting one marker peak and two ribosomal peaks. (B) The RIN=7.3 as several small peaks are detected before the 18s peak, between the 18s and 28s and one peak after the 28s peak. A small of that quality is not recommended for RNA sequencing. (C) In this sample the RNA is highly degraded and the RIN=2.3.

2.7.3 RNA sequencing

Next Generation polyA stranded RNA-sequencing was performed by the CCHMC DNA Sequencing and Genotyping Core using Illumina TruSeq kits and Illumina HiSeq 2000 sequencing system following manufacturer's instructions and resulting in ~20 million paired end reads per sample. Briefly, mRNA was fragmented using the Fragment, Prime, Finish Mix. The synthesis of the first strand of cDNA followed by reverse transcribing the cleaved RNA fragments that were primed with random hexamers into first strand cDNA using reverse transcriptase and random primers. The next step was the synthesis of the second strand of cDNA. This process removes the RNA template and synthesizes a replacement strand, incorporating dUTP in place of dTTP to generate ds cDNA. Then the adenylation of the 3' ends follows. A single 'A' nucleotide is added to the 3' ends of the blunt fragments to prevent them from ligating to one another during the adapter ligation reaction. A corresponding single 'T' nucleotide on the 3' end of the adapter provides a complementary overhang for ligating the adapter to the fragment. The next step is the ligation of the adapters. This process ligates multiple indexing adapters to the ends of the double stranded cDNA, preparing them for hybridization onto a flow cell. Then the PCR amplification step follows. Quality control analysis on each sample library and quantification of the DNA library templates is performed. The last step of the protocol includes the normalization and pooling of the libraries.

2.7.4 Analysis of RNA sequencing

For the analysis of the data we collaborated with the System Biology for Inflammatory Disease department of the University of Lübeck. Professor Busch used a combination of different tools to perform a multidimensional analysis of the data. More precisely, a pseudoalignment was performed using Kallisto and the principal component analysis (PCA) shows how similar or dissimilar the samples from each independent experiment were. Next and in order to identify the differentially regulated genes among the different groups, sleuth was used. Heatmaps were made, which showed the 40 most upregulated genes in each group of cells. The expression values are given as transcripts per million (TPM). A volcano plot was used to depict differentially regulated genes with p-value < 0.05. This plot shows the difference in expression along the x axis and the significance of differential expression along the y axis.

2.8 Statistics

Statistical analysis was performed, using GraphPad Prism version 7 (GraphPad Software, Inc.). Normal distribution of data was tested using the Kolmogorov-Smirnov and D'Agostino-Pearson tests, some after log transformation. When groups were normally distributed, statistical differences between two groups were analyzed by unpaired t test. If more than two groups were evaluated, the groups were first analyzed by an analysis of variance (one-way ANOVA), and in case of significance, followed by a Tukey´s test. P-values < 0.05 were considered as statistically significant. * represents $p < 0.05$; ** represents $p < 0.01$; *** represents $p < 0.001$ and **** represents $p < 0.0001$.

3. Results

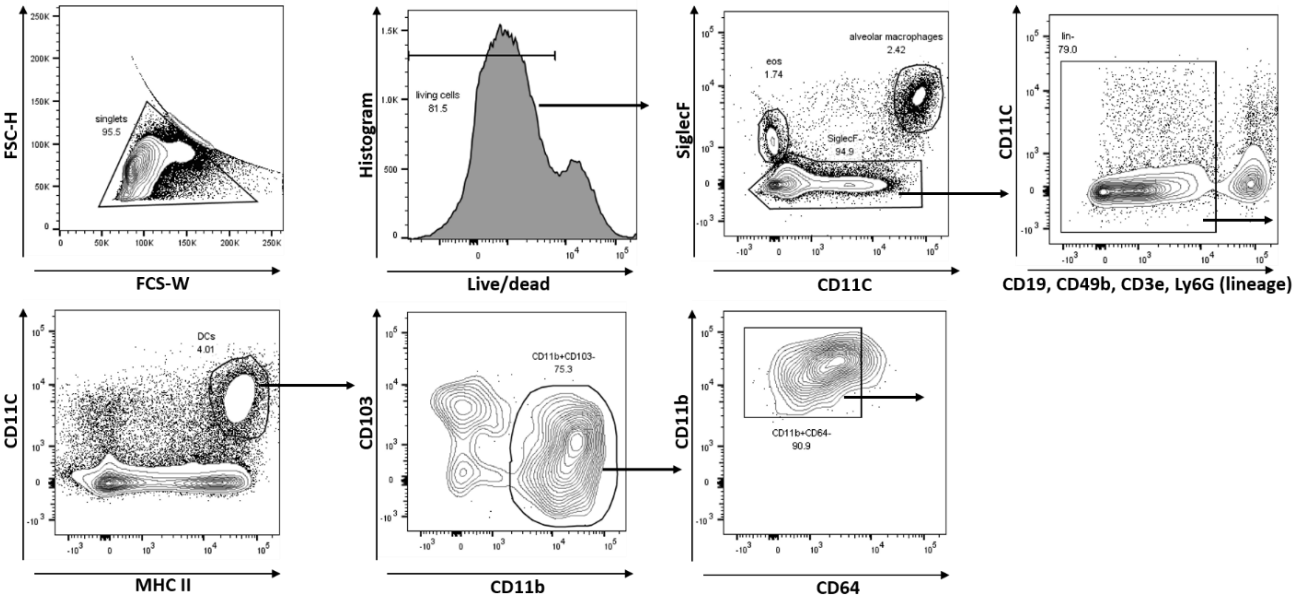
3.1 Identification and functional characterization of C5aR1$^+$ and C5aR1$^-$CD11b$^+$ conventional dendritic cell (CD11b$^+$cDCs) subsets in HDM/OVA-sensitized BALB/c mice

Under steady state conditions, the lung harbors CD103$^+$, CD11b$^+$ conventional DCs (cDCs) and plasmacytoid DCs (pDCs). In addition to cDCs, CD11b$^+$CD64$^+$ monocytes, residing in the lung can acquire a DC phenotype under inflammatory conditions and present antigens to T cells. After HDM treatment, the pulmonary epithelial cells release inflammatory cytokines like TSLP, GM-CSF, IL-33 and IL-25 (Lambrecht & Hammad, 2014), (Plantinga et al., 2013). However, HDM treatment also activates the complement system and generates the ATs C3a and C5a (Köhl et al. J Clin Invest 2006). These ATs exert their biologic functions through their cognate G-Protein Coupled Receptors (GPCR), C3aR, C5aR1 and C5aR2. Importantly, C5aR1 is expressed on CD11b$^+$ cDCs (Karsten et al. J. Immunol. 2015). Previously, the Köhl laboratory has shown that targeting or genetic deletion of C5aR1 resulted in an increased allergic phenotype characterized by strong airway hyperresponsiveness, mucus production and airway inflammation that was associated with strong maladaptive Th2 immune responses (Köhl et al. J Clin Invest 2006). This increased allergic phenotype was associated with a strong increase in CD11b$^+$cDCs in the lung of C5aR1-deficient or C5aR1-targeted mice. At this point, it was unclear, how C5aR1 regulates the programming of CD11b$^+$cDCs into a phenotype that activates CD4$^+$ T cells and drives Th2 immune responses in allergic asthma. Therefore, I aimed to delineate the role of C5a/C5aR1 signaling in CD11b$^+$cDC for the activation of CD4$^+$ T cells in the sensitization phase of allergic asthma. More precisely, my aims were to i) functionally and phenotypically characterize the CD11b$^+$cDCs based on the expression of C5aR1 and determine their C5/C5a production, ii) determine the mechanisms underlying C5aR1 driven regulation of pulmonary CD11b$^+$cDCs.

3.1.1 Pulmonary D11b$^+$cDCs can be phenotypcially differentiated into C5aR1$^+$ and C5aR1$^-$ subsets

To identify pulmonary CD11b$^+$cDCs in wildtype (WT) BALB/c mice I used the following gating strategy: first, macrophages and eosinophils were excluded as SiglecF$^+$ cells. Within the SiglecF$^-$ fraction, lineage$^+$ cells comprising B cells (CD19), T cells (CD3e), NK cells (CD49b) and neutrophils (Ly6G) were excluded. Among the lineage$^-$ cells, DCs were identified as CD11C$^+$MHC-II^{high} cells. These DCs were further subdivided into CD103$^+$CD11b$^-$ cDCs and CD103$^-$CD11b$^+$ cells comprising CD11b$^+$CD64$^-$cDCs and CD11b$^+$CD64$^+$ mo-DCs. Additionally, a population which was CD11b$^-$CD103$^-$ was identified. Based on this gating strategy I sorted SiglecF$^-$CD19$^-$CD3e$^-$CD49b$^-$Ly6G$^-$CD11C$^+$MHCII^{high}CD103$^-$CD11b$^+$CD64$^-$DCs by FACS (Figure 10A). Using C5aR1 as an additional marker, I found that CD11b$^+$cDCs are a heterogeneous population, comprising C5aR1$^+$ (85%) and C5aR1$^-$ (15%) subpopulations under steady-state conditions (Figure 10B). Importantly, 24h after 1-step HDM/OVA intratracheal (i.t.) immunization the majority of the CD11b$^+$cDCs was still positive for C5aR1 (75%), but the frequency of C5aR1$^-$cDCs increased (25%) (Figure 10B and 10C).

A


FSC-H
singlets
95.5
FCS-W
Histogram
living cells
81.5
Live/dead
SiglecF
alveolar macrophages
2.42
eos
1.74
SiglecF+
94.9
CD11C
CD11C
lin-
79.0
CD19, CD49b, CD3e, Ly6G (lineage)
CD11C
DCs
4.01
MHC II
CD103
CD11b+CD103-
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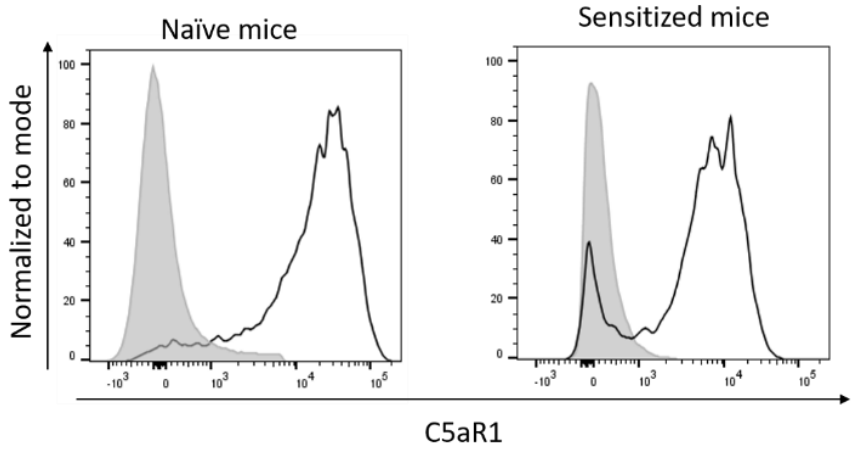
CD11b+CD64-
90.9
CD64

B


Naïve mice
Sensitized mice
Normalized to mode
C5aR1

C

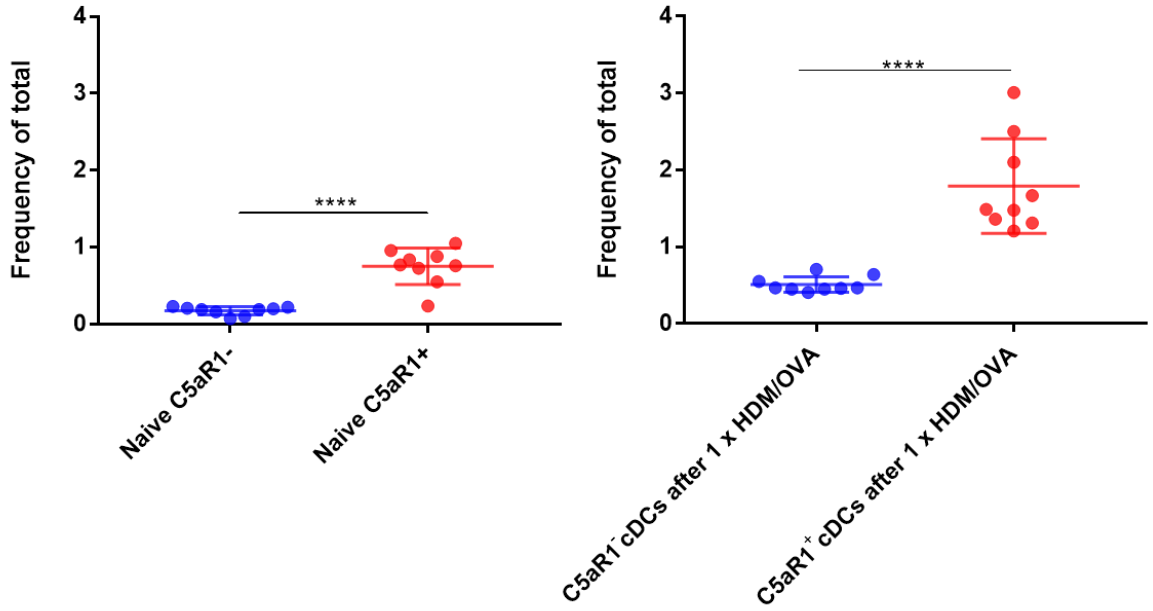


Figure 10: Pulmonary CD11b$^+$cDCs are a heterogeneous population based on the expression of C5aR1. (A) Gating strategy to identify pulmonary CD11b$^+$cDCs in WT naïve and sensitized BALB/c mice. The CD11b$^+$cDCs are: SiglecF$^-$ lin$^-$CD11C$^+$MHCII^{high}CD103$^-$CD11b$^+$CD64$^-$. (B) Histogram showing the expression of C5aR1 in pulmonary CD11b$^+$cDCs from naive mice and animals sensitized 1 x i.t. with a combination of HDM and OVA. The majority of naïve CD11b$^+$cDCs is C5aR1$^+$ (85%), and only a minor fraction of the population is C5aR1$^-$ (15%). Twenty-four hrs after 1 step HDM/OVA (100µg/40µg) i.t. sensitization, the majority of the CD11b$^+$cDCs is still C5aR1$^+$ (75%), but the group of C5aR1$^-$ increased to 25%. The histograms are representative of data from 10 mice. The HDM/OVA immunization and the isolation of the lungs 24 hrs later, applies also to the subsequent figures. (C) Frequency of pulmonary CD11b$^+$C5aR1$^-$ and CD11b$^+$C5aR1$^+$cDCs in naïve (left panel) and sensitized (right panel) mice immunized 1x i.t. with HDM/OVA. Shown are the frequencies of the two subsets within total lung cells; n= 10, data were analyzed by unpaired t-test. ****p<0.0001

More precisely, under naïve conditions, the frequency of C5aR1$^+$cDCs was 0.80% and for C5aR1$^-$cDCs 0.1% of total lung cells. After 1 step HDM/OVA sensitization, the frequency of the C5aR1$^+$ subset increased 3-fold to 2.5%; for the C5aR1$^-$ it increased 6-fold and reached almost 0.6%. These data show that pulmonary CD11b$^+$cDCs are not a homogeneous population but can be differentiated into two subsets, i.e. CD11b$^+$C5aR1$^+$cDCs and CD11b$^+$C5aR1$^-$cDCs.

3.1.2 C5aR1$^+$ and C5aR1$^-$cDCs exert different potencies to drive OVA transgenic (tg) CD4$^+$ T cell proliferation

After the characterization of CD11b$^+$cDCs as a heterogeneous population, I wondered whether the phenotypic differences are associated with functional differences. To address this question, I performed an assay that evaluates the potency of the two CD11b$^+$cDC sub-populations to drive OVA-specific CD4$^+$ T cell proliferation. More precisely, 24h after 1 step HDM/OVA immunization, the C5aR1$^+$ and C5aR1$^-$cDCs were FACS-purified, *in vitro* pulsed with OVA and 18h later co-cultured with CFSE-labeled-OVA-tg-CD4$^+$ T cells from DO11.10 RAG2$^{-/-}$ mice. Four days later, the proliferation was evaluated. The *in vitro* induction of CD4$^+$ T cell proliferation was visualized by the dilution of CFSE, an intracellular dye, emitting within the FITC channel.

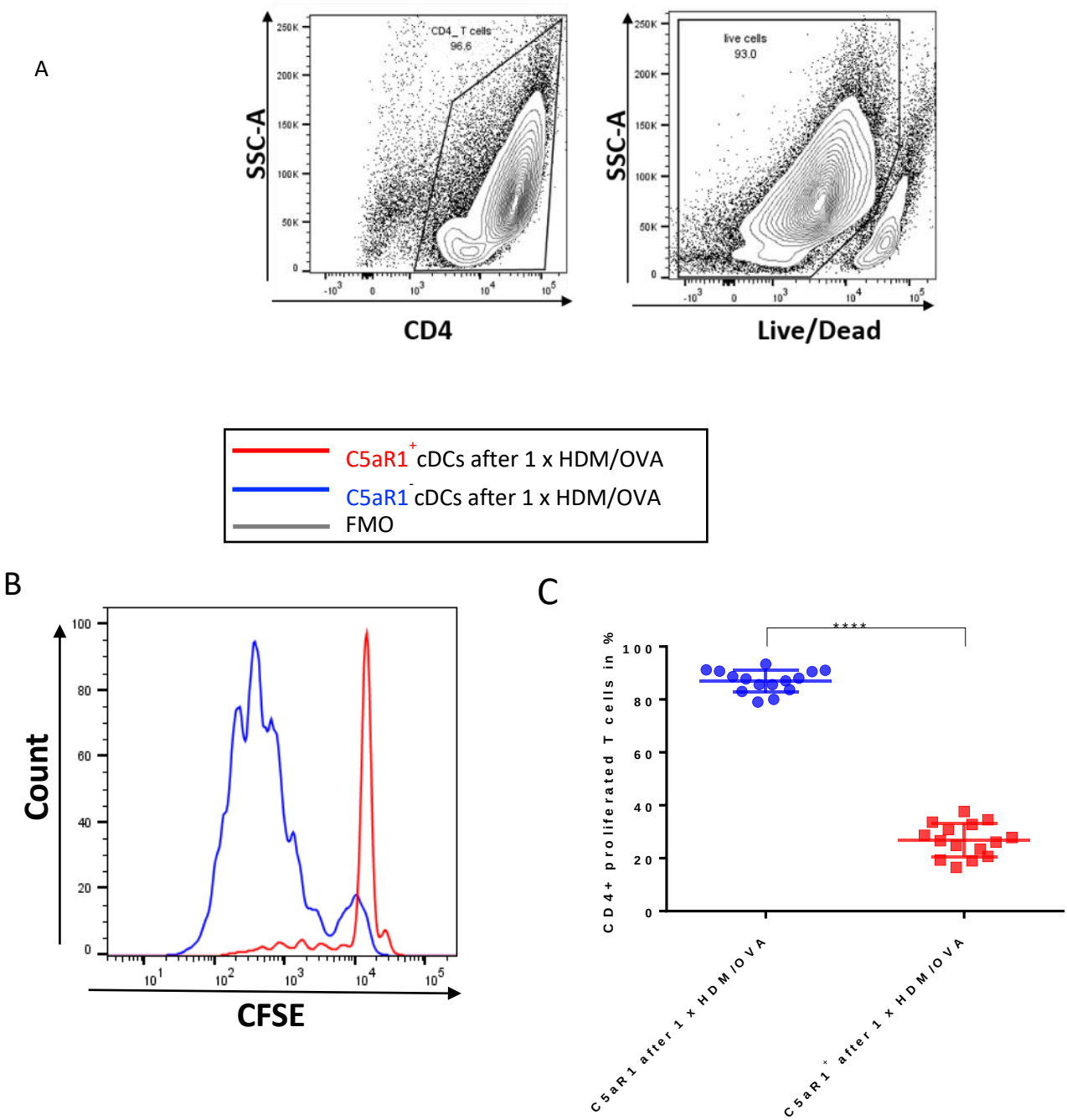


Figure 11: Proliferation of OVA-transgenic CD4⁺ T cells in response to co-culture with OVA-pulsed C5aR1⁺ and C5aR1⁻ cDCs. WT mice were treated once with a mixture of HDM/OVA (100 µg/40 µg) i.t. C5aR1⁺ and C5aR1⁻ CD11b⁺ cDCs from WT mice were purified from digested lung tissue by FACS 24h after the treatment. For four days, both cell populations were co-cultured with CFSE-labeled OVA transgenic CD4⁺ T cells in the presence of OVA (10 µM) and GM-CSF (20 ng/ml). (A) Dot-plots showing the gating strategy to identify living untouched OVA-tg CD4⁺ T cells purified by MACS. (B) Histogram showing the CFSE signal in OVA tg CD4⁺ T cells after four days co-culture with C5aR1⁺ or C5aR1⁻ cDCs. (C) Frequency of proliferated OVA tg CD4⁺ T cells, n = 15 per group. Differences between group were evaluated by unpaired t-test. ****p<0.0001

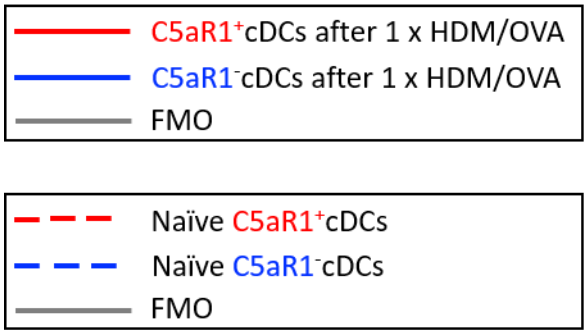
To evaluate the T cell survival, the cells were stained with the fixable viability dye eF450. After four days, the percentage of living T cells was typically between 90-93 % in T cell co-cultures with both C5aR1⁺ and

C5aR1$^-$cDCs, demonstrating that both CD11b$^+$cDC populations promote T cell survival. Even though both populations could promote T cell survival equally well, this was not the case for the T cell proliferation. More specifically, C5aR1$^+$cDCs had a much lower potency of priming OVA-specific T cell responses in comparison to the C5aR1$^-$cDCs. Clearly, the data showed that the two CD11b$^+$cDC subsets found in the lungs of sensitized mice after 1 step HDM/OVA immunization were functionally different.

3.1.3 Sensitized C5aR1$^+$cDCs express significantly lower levels of MHC-II and the co-stimulatory molecule CD40 in comparison to C5aR1$^-$cDCs

As outlined in detail in the introduction (chapter 1.3.4), the activation of CD4$^+$ T cells requires three signals, which are typically provided by professional APCs such as DCs. More specifically, these signals are: i) the interaction between the TCR on the T cells and the peptide-MHCII complex on the DCs, ii) the co-stimulatory molecules (e.g. CD40, CD80, CD86, and OX40L) on the DCs and the (CD40L, CD28, and OX40) on the T cells and iii) polarizing cytokines released by DCs or other innate immune cells. The significantly reduced potency of the C5aR1$^+$cDCs to efficiently drive CD4$^+$ T cell proliferation suggested that the formation of immunologic synapse is disturbed at the level of MHC-II/TCR and/or costimulatory molecule.

To address this question, I decided to first characterize the two CD11b$^+$cDC subsets phenotypically. I determined the expression of MHC-II and the co-stimulatory molecules CD40, CD80, CD86, OX40L in C5aR1$^+$ and C5aR1$^-$ cDCs, both under steady-state conditions and 24h after sensitization to HDM/OVA (Figure 12). The same number of events (10.000 events) was recorded for each subpopulation to avoid any bias.



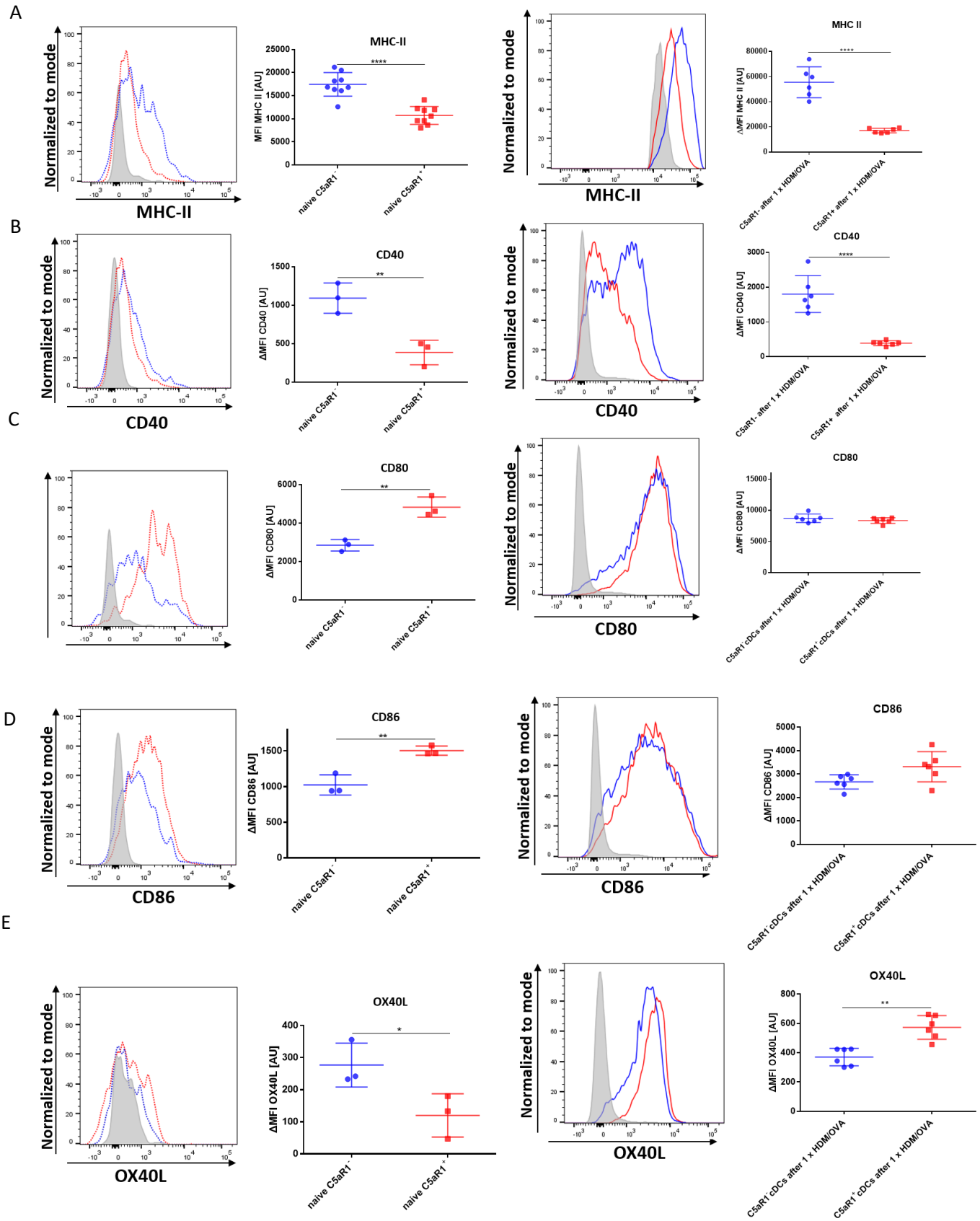

A
Normalized to mode
MHC-II
MFI MHC II [AU]
naive C5aR1-
naive C5aR1+
****
ΔMFI MHC II [AU]
C5aR1- after 1 x HDM/OVA
C5aR1+ after 1 x HDM/OVA
B
CD40
ΔMFI CD40 [AU]
**
C
CD80
ΔMFI CD80 [AU]
C5aR1- cDCs after 1 x HDM/OVA
C5aR1+ cDCs after 1 x HDM/OVA
D
CD86
ΔMFI CD86 [AU]
E
OX40L
ΔMFI OX40L [AU]
*

Figure 12: Association of C5aR1 expression on CD11b$^+$ cDCs with MHC-II (A), CD40 (B), CD80 (C), CD86 (D) and OX40L (E). The cDCs were either from naïve WT mice (left panel) or from WT mice treated once with HDM/OVA (100 µg/40 µg) (sensitized mice) i.t (right panels). Twenty-four hrs after the in vivo treatment, the expression of MHCII and other co-stimulatory molecules (CD40, CD80, CD86, and OX40L) was evaluated by measuring 10.000 events for each of the two subpopulations. The graphs show the ΔMFI of the expression of the examined molecules by the two subsets as mean ± SEM, n = 6 per group; differences between groups were evaluated by unpaired t-test, * $p < 0.05$; **$p< 0.01$; **** $p <0.0001$.

I found that C5aR1$^-$cDCs and C5aR1$^+$ cDCs from naïve mice both expressed MHC and co-stimulatory molecules. Upon 1 x HDM/OVA immunization, MHC-II expression increased in both cell populations (Figure 12A). Both CD11b$^+$cDC subsets also expressed CD40, however, upon 1 x HDM/OVA immunization, the expression of CD40 increased only in the C5aR1$^-$ group, whereas its expression levels remained almost unchanged in case of C5aR1$^+$cDCs (Figure 12B). I also observed that both C5aR1$^-$ and C5aR1$^+$cDCs from naïve mice expressed CD80 and they both upregulated the expression upon 1 x HDM/OVA immunization (Figure 12C). The expression of CD86 followed a similar pattern as CD80, as both populations expressed that molecule under steady state conditions and upregulated its expression upon allergen contact (Figure 12D). Lastly, regarding the expression of OX40L, I observed that C5aR1$^-$cDCs and C5aR1$^+$ cDCs from naïve mice both expressed that molecule, but the exposure to HDM/OVA only affected the C5aR1$^+$cDCs, which increased the expression of OX40L, whereas no changes were observed for the C5aR1$^-$cDCs (Figure 12E).

Next, I compared the expression of MHC-II and co-stimulatory molecules in C5aR1$^+$ and C5aR1$^-$ cDCs from naïve WT mice. Here, I found that MHC-II expression in C5aR1$^-$ cDCs was significantly higher than that in C5aR1$^+$ cDCs. Similarly, CD40 and OX40L expression was higher in C5aR1$^-$ cDCs than in C5aR1$^+$ cDCs. In contrast, CD80 and CD86 expression was significantly higher in C5aR1$^+$ than in C5aR1$^-$ cDCs (Figure 12 A-E, left panels).

Finally; I assessed MHC-II and costimulatory molecule expression in C5aR1$^+$ and C5aR1$^-$ cDCs after 1 x HDM/OVA immunization. Similar to what I had observed under steady state conditions, MHC-II and CD40 expression was significantly higher in C5aR1$^-$ as compared to C5aR1$^+$ cDCs. CD80 and CD86 expression in both cDC populations was similar, whereas OX40L expression was higher in C5aR1$^+$ cDCs than in C5aR1$^-$ cDCs (Figure 12 A-E, right panels).
Based on these findings, I hypothesized that the impaired potency of C5aR1$^+$ cDCs to drive T cell proliferation results from the decreased MHC-II and CD40 ligand expression.

3.1.4 OVA-tg CD4$^+$ T cells interact more frequently with the C5aR1$^-$cDCs than with C5aR1$^+$cDCs

As outlined above, strong CD4$^+$ T cell proliferation requires strong immunological synapse formation between cDCs and T cells. To directly assess synapse formation between the two pulmonary CD11b$^+$ cDCs subsets and OVA-tg CD4$^+$ T cells, I labeled both cell populations and monitored their interactions for 300 minutes by confocal microscopy (Figure 13).

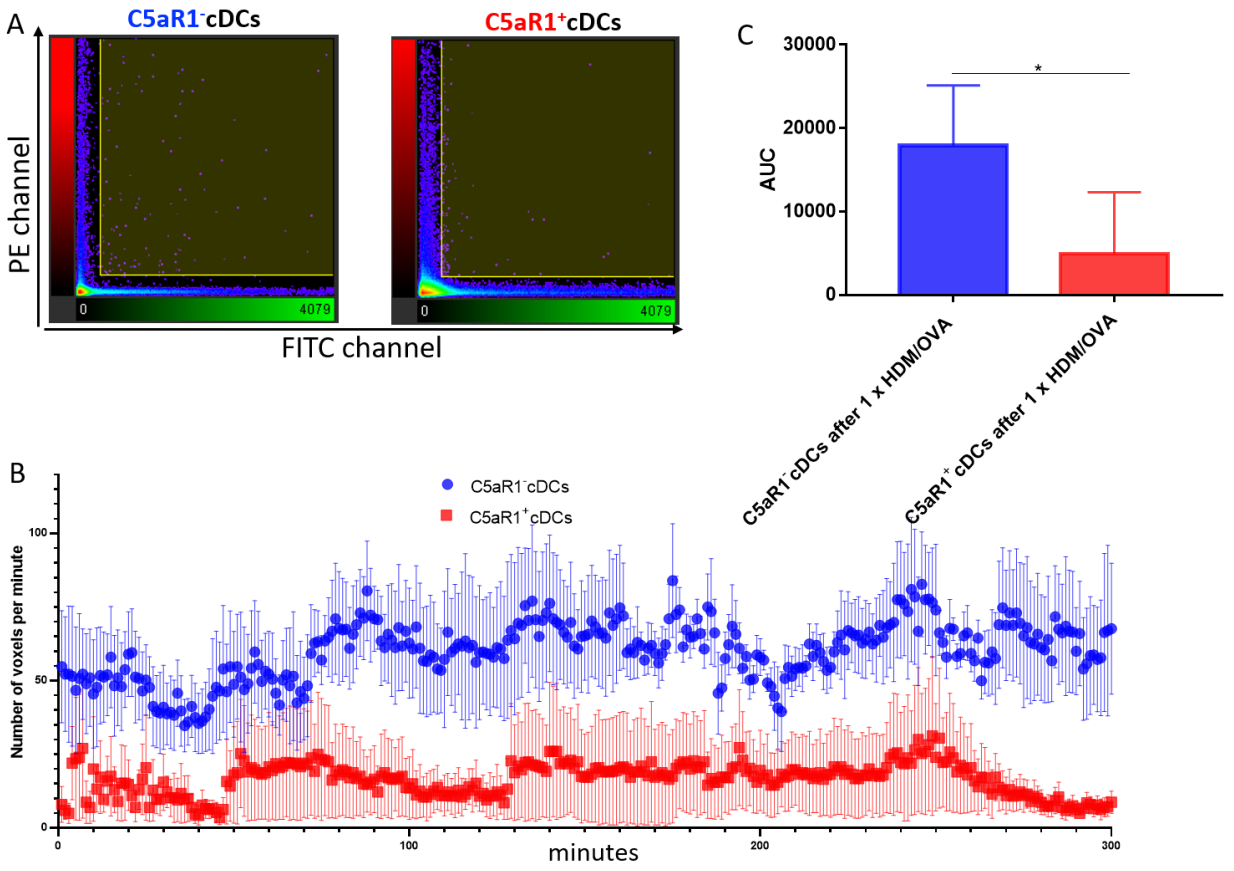


Figure 13: Kinetic of the interaction between OVA-tg CD4⁺ T cells, C5aR1⁻ and C5aR1⁺. WT mice were treated once with a mixture of HDM/OVA (100 µg/40 µg) i.t. C5aR1⁺ and C5aR1⁻CD11b⁺cDCs from WT mice were purified from lung tissue by FACS 24h after sensitization. The cDCs cultured in the presence of OVA Ag (10µM) and GM-CSF (20 ng/ml) overnight. The following day, they were labeled with PKH26, a red fluorescent dye, emitting in PE for membrane labeling and transferred in a channel slide together with CFSE-labeled OVA transgenic CD4⁺ T cells. Using an FV1000 confocal microscope, the interactions between DCs and T cells were monitored for 300 minutes, taking one picture per minute. To visualize the interactions between the two cell populations, the Imaris™ coloc tool was used. It operates on two channels (PE and FITC) simultaneously and measures the degree of overlap between the two channels. (A) Intensity histogram of the PE/FITC channels, which reflects the distribution of voxel (in computer-based modeling, a voxel is a unit of graphic information that defines a point in three-dimensional space) pair intensities occurring in the two selected channels. The range of intensity pairs considered as colocalized can be defined on the histogram as channel thresholds, marked with the two yellow lines. (B) Number of voxels per minute per sample recorded for 300 min using either C5aR1⁻ cDCs (blue) or C5aR1⁺ cDCs (red), n=4 per group. (C) Quantitative evaluation of the curves shown in (B). The area und the curve (AUC) was determined for evaluation; n=4 per group. Differences between groups were assessed by unpaired t-test, *<0.05.

I observed more interactions between OVA-tg CD4⁺ T cells and C5aR1⁻ than with C5aR1⁺ cDCs during the 300 minutes observation period (Figure 13 A-C). In particular, the number of interactions between T cells and C5aR1⁺ cDCs during the first 2h was very low, strongly suggesting that the low MHC-II expression in C5aR1⁺ cDCs prevented optimal antigen presentation via MHC-II and sustained interaction via CD40. Also, at the end of the observation period (260-300 min), a marked reduction of CD4⁺ T cell interaction with C5aR1⁺ cDCs occurred, whereas the high level of interaction was unchanged between C5aR1⁻ cDCs and

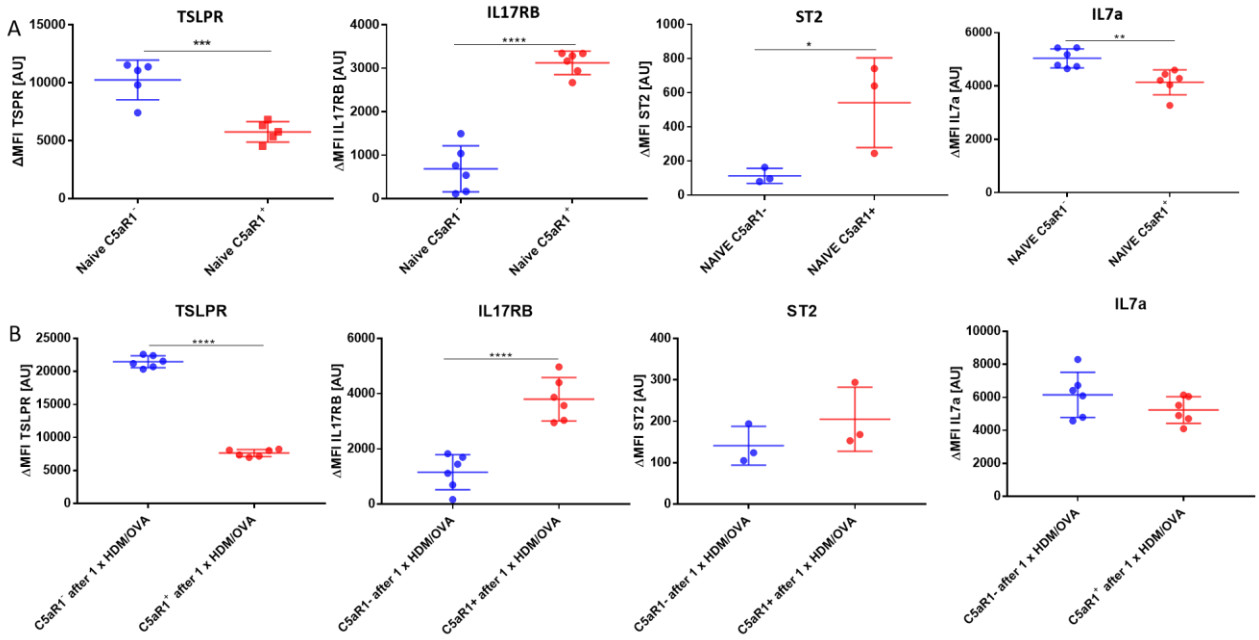
OVA-tg CD4$^+$ T cells. The data indicate that OVA-tg CD4$^+$ T cells only transiently engage with C5aR1$^+$ cDCs and that this engagement is less stable than that with C5aR1$^-$ cDCs.

3.1.5 C5aR1$^+$cDCs express significantly higher levels of IL17RB but significantly lower levels of TSLPR in comparison to the C5aR1$^-$cDCs

The distinct expression levels of MHC-II, CD40, CD80, CD86 and OX40L in C5aR1$^+$ and C5aR1$^-$ cDCs in response to allergen sensitization may suggest that these two CD11b$^+$cDC subpopulations are not able to sense the signals from their environment in the same way and due to that, they showed a different maturation profile.

Work from several groups during the past few years revealed an essential role of ECs in cDC maturation. It was shown that the ECs do not only act as a passive barrier for allergen uptake but recognize allergens through the expression of pattern recognition receptors and mount an innate immune response. Upon allergen recognition such as HDM/OVA exposure, ECs release cytokines/alarmins, such as IL-1, IL-25, IL-33, TSLP, and GM-CSF, and endogenous danger signals, such as high-mobility group box 1, uric acid, and ATP, that activate the pulmonary DC network (Lambrecht & Hammad, 2014). Therefore, the next step to further characterize the two CD11b$^+$cDC fragments was to examine the expression of specific alarmin receptors and compare their ability to sense the inflammatory signals released by the epithelium.

To assess whether the observed differences in the maturation profile between the sensitized C5aR1$^-$ and C5aR1$^+$cDCs could result from their different ability to sense their micro-environment, I determined the expression of ST2, the receptor of IL-33, TSLPR, the receptor for TSLP, IL17RB, the receptor for IL-17B and IL17E (IL-25), and the IL-7 receptor α-chain (IL-7a), the receptor for TSLP and IL-7 in C5aR1$^+$ and C5aR1$^-$ DCs from naïve and HDM/OVA-sensitized mice (Figure 14).



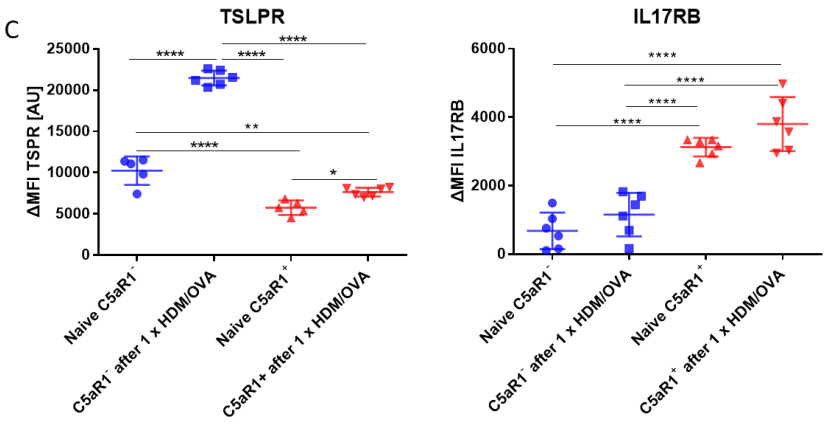


Figure 14: Association of alarmin receptor and C5aR1 expression in CD11b$^+$cDCs from naïve and HDM/OVA-sensitized WT mice. (A) Alarmin receptor expression in C5aR1$^-$ or C5aR1$^+$ cDCs from naïve WT mice. (B) Alarmin receptor expression in C5aR1$^-$ or C5aR1$^+$ cDCs from WT mice treated once with HDM/OVA (100 µg/40 µg) i.t. (C) Comparison of the TSLPR (left) and IL17RB (right) expression between naïve and sensitized C5aR1$^-$cDCs as well as between naïve and sensitized C5aR1$^+$cDCs. The graphs show the ΔMFI of the expression of the examined molecules by the two subsets as mean ± SEM, n = 3-6 per group. Differences between groups in (A) and (B) were compared by unpaired t-test; * indicates significant differences between C5aR1$^-$ and C5aR1$^+$ cDCs; *p<0.05, **p<0.01, *** p < 0.001 and ****p<0.0001. Values shown in C are the mean ± SEM, n=6 per group and the data were analyzed using an ANOVA test, followed by Tukey's post-hoc test; * indicates significant differences between naïve and sensitized C5aR1$^-$cDCs as well as between naïve and sensitized C5aR1$^+$cDCs; **p<0.01 and ****p<0.0001.

Under naïve conditions, the C5aR1$^+$cDCs expressed higher levels of ST2 in comparison to the C5aR1$^-$cDCs. However, upon 1 step HDM/OVA the expression pattern of that alarmin receptor slightly changed. The C5aR1$^+$cDCs decreased its expression, whereas the expression of ST2 remained unchanged for the C5aR1$^-$ group, which resulted in similar expression levels between these two groups upon allergen contact (Figure 14A-C). In case of IL-7a, I observed that naïve C5aR1$^-$cDCs expressed significantly higher levels of this receptor in comparison to C5aR1$^+$cDCs. Interestingly, upon HDM/OVA exposure, both populations slightly increased the expression of IL-7a, although, the C5aR1$^+$cDCs to a higher extent. Eventually, both cDC populations expressed similar levels of IL-7a (Figure 14A-C). Regarding the expression of IL-17RB, I noticed that already under naïve conditions the C5aR1$^+$cDCs expressed significantly higher levels in comparison to the C5aR1$^-$cDCs. However, I found no changes in the expression pattern of that receptor upon HDM/OVA sensitization (Figure 14A-C). Concerning the expression of TSLPR, the C5aR1$^-$cDCs expressed significantly higher levels in comparison to the C5aR1$^+$cDCs both under naive and inflammatory conditions. More precisely, this was the only molecule, which was significantly upregulated in both CD11b$^+$cDC subsets after 1 step HDM/OVA sensitization. Importantly, although both populations upregulated the TSLPR, the upregulation in C5aR1$^-$ cDCs was much stronger than that observed in C5aR1$^+$ cDCs (Figure 14A-C).

3.1.6 C5aR1$^+$ and C5aR1$^-$cDC drive mixed Th2/Th17 immune responses

Next, I aimed to delineate the differentiation program initiated in naïve OVA tg CD4$^+$ T cells upon co-culture with either C5aR1$^-$ or C5aR1$^+$cDCs. The CD11b$^+$ DC populations contribute to Th2 and/or Th17 development (Plantinga et al., 2013). More precisely, 24h after 1 step HDM/OVA immunization, the C5aR1$^+$, and C5aR1$^-$cDCs were FACS-purified, *in vitro* pulsed with OVA and 18h later were co-cultured with

CFSE-labeled-OVA-tg-CD4$^+$ T cells from DO11.10 RAG2^{-}/$^{-}$ mice. Four days later, the T cell differentiation was evaluated by: (i) ELISA, i.e. measuring the concentrations of cytokines in the supernatants of C5aR1$^-$ and C5aR1$^+$/T cell co-culture); and (ii) intracellular cytokine staining. More precisely, on day 4 of the co-culture, I stimulated the cells with ionomycin and phorbol 12-myristate13-acetate (PMA) and stained the T cells for the intracellular expression of the cytokines IL-13 and IL-17A) (Figure 15). The cytokine IL-13 is associated with a Th-2 response, IL-17 is typical for a Th-17 response, FOXP3 for a T regulatory response and IFN-γ for a Th-1 response.

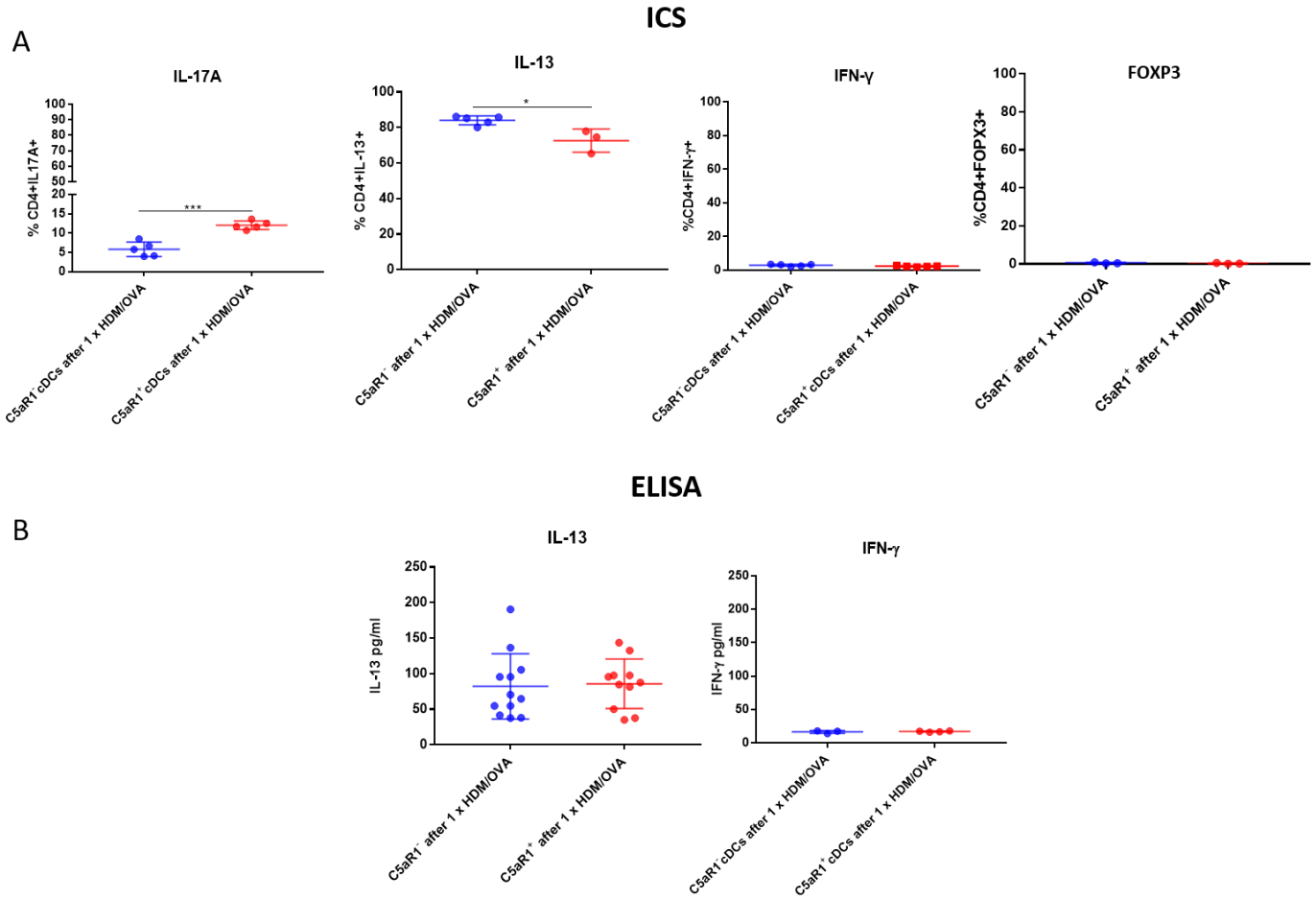


Figure 15: Sensitized C5aR1$^-$ and C5aR1$^+$cDCs drive a mixed Th2/Th17 response. WT mice were treated once with HDM/OVA (100 µg/40 µg) i.t. Twenty-four hrs later, pulmonary C5aR1$^+$ and C5aR1$^-$ CD11b$^+$cDCs from WT mice were FACS-purified. For four days, they were co-cultured with CFSE-labeled OVA transgenic CD4$^+$ T cells in the presence of OVA (10 µM) and GM-CSF (20 ng/ml). On day 4, the differentiation of T cells was evaluated by intra-cellular cytokine staining (A) and ELISA (B). Shown is the percentage of proliferated CD4$^+$ T cells expressing IL-13, IL-17A, IFN-γ and FOXP3 (A only) as the mean ± SEM, n = 3-5 per group for the ICS and n=6-12 for ELISA. Differences between groups were assessed by unpaired t-test; *indicates significant differences between T cell co-cultures of C5aR1$^-$ vs. C5aR1$^+$ cDCs; * $p < 0.05$, ***$p<0.001$ (N.D.=not detected).

C5aR1$^-$ and C5aR1$^+$ cDCs induced the differentiation of IL-17-producing Th17 cells (Fig. 15). However, the C5aR1$^-$cDC – T cell co-cultures resulted in a lower frequency of IL-17A$^+$ producing T cells (5 ± 3%) in comparison to the C5aR1$^+$cDC – T cell co-cultures (10± 5 %; Figure 15A). When I checked IL-17A production

by ELISA, I noticed that in case of the C5aR1$^-$cDC – T cell co-cultures, IL-17 production was below the detection limit of the assay, whereas in the C5aR1$^+$cDC – T cell co-cultures , I measured 50 ± 45pg/ml IL-17A (Fig 15B).

In addition to IL-17A, C5aR1$^+$ and C5aR1$^-$ / T cell co-cultures resulted in the differentiation of IL-13 producing Th2 cells. The frequency of IL-13-producing Th2 cells was between 70 ± 5% and 80 ± 5% in co-cultures of C5aR1$^+$ and C5aR1$^-$ with T cells, respectively (Fig. 15A). Thus, C5aR1$^+$ and C5aR1$^-$ cDCs drove a dominant Th2 response, which was somewhat more pronounced with C5aR1$^-$ cDCs. When I checked the IL-13 production by ELISA, I noticed that the IL-13 protein production from the T cells of both groups was identical (Fig. 15B).

As expected, both CD11b$^+$cDC subpopulations did at best drive a very low frequency of IFN-γ-producing or FOXP3-expressing T cells. Also, INF-γ production by ELISA was minor (Fig. 15).

As I have shown in section 3.1.2, C5aR1$^-$cDCs induced robust CD4$^+$ T cell proliferation and the frequency of activated T cells in that co-culture system was significantly higher in comparison to the C5aR1$^+$cDC – T cell co-cultures. Surprisingly, I noticed that in the C5aR1$^+$ group of cells, even though the number of activated T cells was lower in comparison to the C5aR1$^-$cells, the secreted amount of IL-13 and IL-17A was either the same or even higher. This finding indicates that each T cell in the C5aR1$^+$ group produced markedly higher amounts of both IL-13 and IL-17A in comparison to the C5aR1$^-$cDCs. Taken together, the data demonstrate that the C5aR1$^-$ cDCs were more potent to drive T cell proliferation than the C5aR1$^+$ cDCs, but their ability to induce Th2 and Th17 cytokine production in individual T cells was much lower than that of their C5aR1$^+$ counterparts.

3.1.7 CD11b$^+$C5aR1$^+$cDCs express lower levels of CCR7 in comparison to CD11b$^+$C5aR1$^-$cDCs

It is known that once the CD11b$^+$cDCs take up the Ag and get activated, they migrate to the draining lymph nodes (dLNs), where they encounter naïve CD4$^+$ T cells and activate them (Clatworthy et al., 2014). Since my data showed that the C5aR1$^-$cDCs were the ones, which efficiently elicited T cell proliferation, I wanted to know whether they had a better potency to migrate to dLNs in comparison to C5aR1$^+$cDCs. To do that, I checked for the expression of CCR7, which is upregulated on activated DCs and critical for the migration of DCs to the dLNs(Clatworthy et al., 2014). CCR7 recognizes CCL19 and CCL21, which are produced in the lymph nodes and increase the expression of MHC-II and co-stimulatory molecules on DCs(Clatworthy et al., 2014). The expression of CCR7 was tested 24h after a 1 step HDM/OVA sensitization (Figure 16).

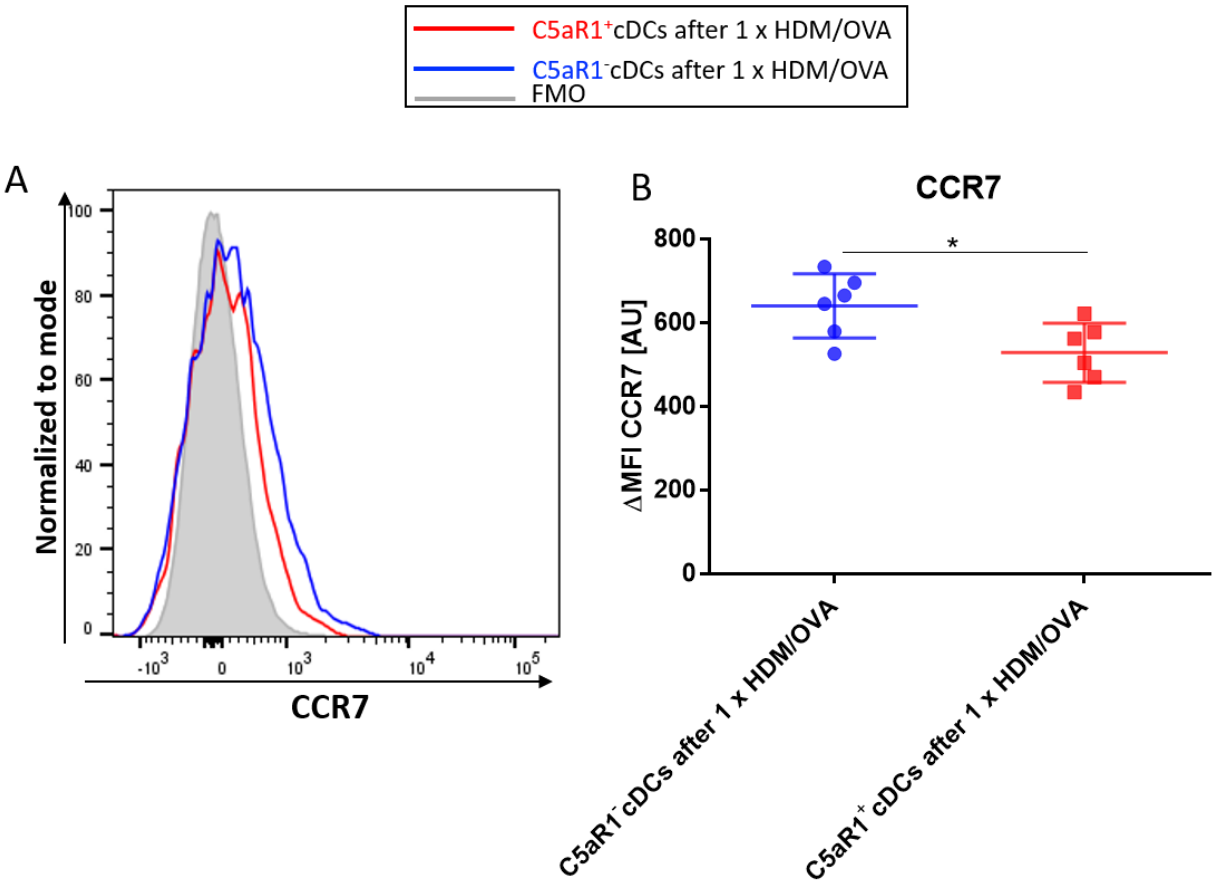
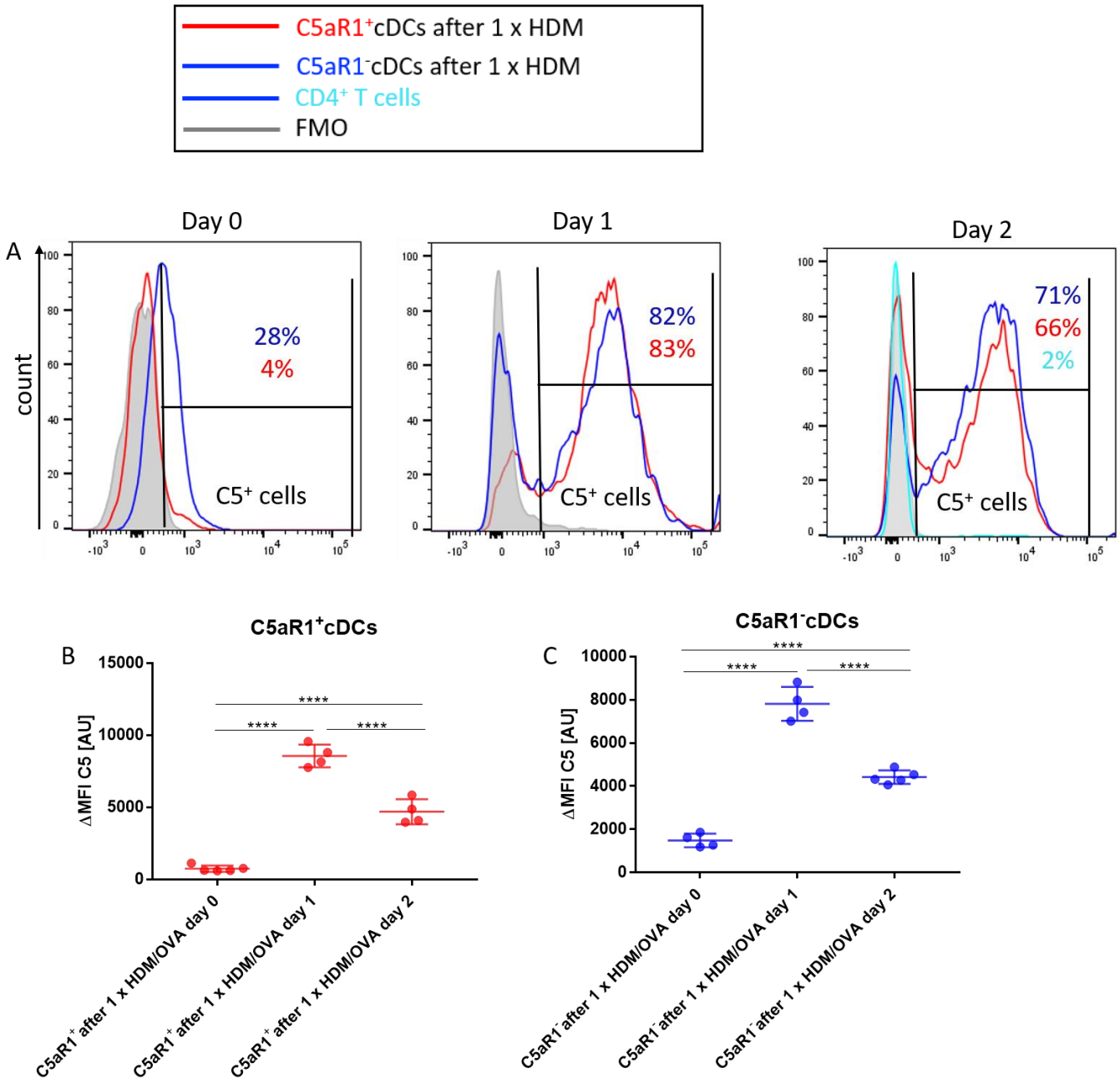


Figure 16: Association of C5aR1 expression on CD11b⁺ cDCs and CCR7 expression in CD11b⁺ cDCs subsets. The expression of CCR7 was evaluated by flow cytometry. Altogether 10.000 events were measured for each sub-population. (A) Histogram showing CCR7 expression in C5aR1⁺ and C5aR1⁻ cDC subsets. The figure is representative of one experiment. (B) Quantitative assessment of CCR7 expression in C5aR1⁻ and C5aR1⁺ cDCs. Shown is the ΔMFI for CCR7 within the two subsets. Data shown are the mean ± SEM, n = 6/group, * $p < 0.05$. Differences between groups were determined by unpaired t-test.

The data show that the C5aR1⁻cDCs expressed significantly higher levels of CCR7 in comparison to the C5aR1⁺cDCs. Given that CCR7 is critical for migration into the dLNs these findings may suggest that C5aR1⁻ cDCs migrate more efficiently to the dLNs where they may interact more efficiently with T cells due to their higher expression levels of MHC-II and CD40.

3.1.8 Impact of *ex vivo* OVA-pulsing and T cell co-culture on C5 production and C5a generation from sensitized C5aR1⁺ and C5aR1⁻ cDCs

My findings that C5aR1⁺ and C5aR1⁻ cDCs show a distinct potency to drive T cell proliferation and differentiation *in vitro* in co-cultures with CD4⁺ T cells, suggest that C5aR1 is activated during the co-culture to promote the observed effects. However, at this point, it was unclear, which cells might produce C5 and cleave it into C5a during the cDC/T cell co-culture. To determine the source of C5, I analyzed whether C5aR1⁻ and C5aR1⁺cDCs were making C5 on day 0, i.e. when cells were sorted after 1 step HDM/OVA sensitization, on day 1, when DCs were *in vitro* pulsed with OVA, but before the T cells were added to the system and on day 2, i.e. when DCs and T cells had interacted for 24h hours (Figure 17).


C5aR1⁺cDCs after 1 x HDM
C5aR1⁻cDCs after 1 x HDM
CD4⁺ T cells
FMO
A
Day 0
Day 1
Day 2
count
28%
4%
82%
83%
71%
66%
2%
C5⁺ cells
B
C5aR1⁺cDCs
ΔMFI C5 [AU]
C5aR1⁺ after 1 x HDM/OVA day 0
C5aR1⁺ after 1 x HDM/OVA day 1
C5aR1⁺ after 1 x HDM/OVA day 2
C
C5aR1⁻cDCs
C5aR1⁻ after 1 x HDM/OVA day 0
C5aR1⁻ after 1 x HDM/OVA day 1
C5aR1⁻ after 1 x HDM/OVA day 2
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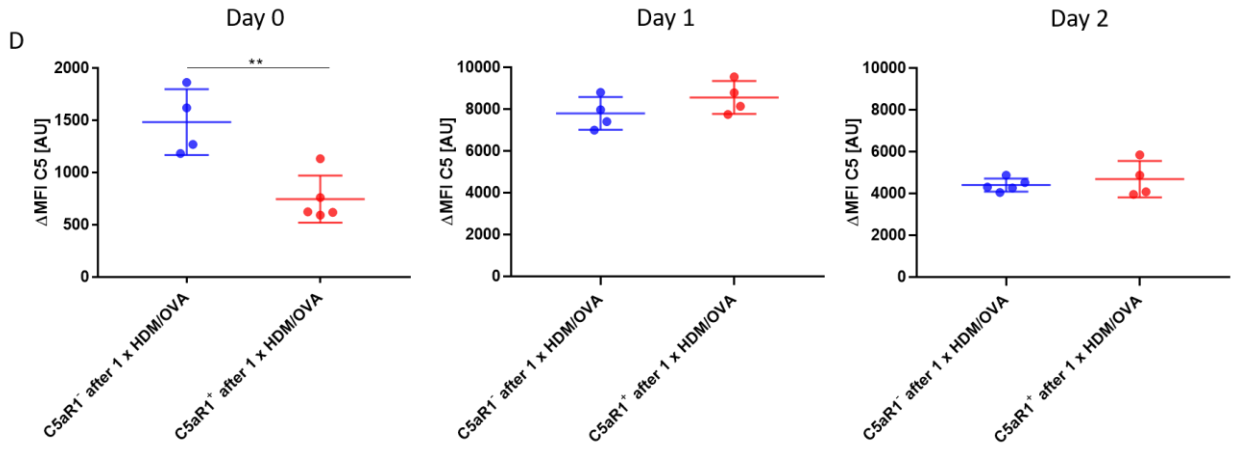
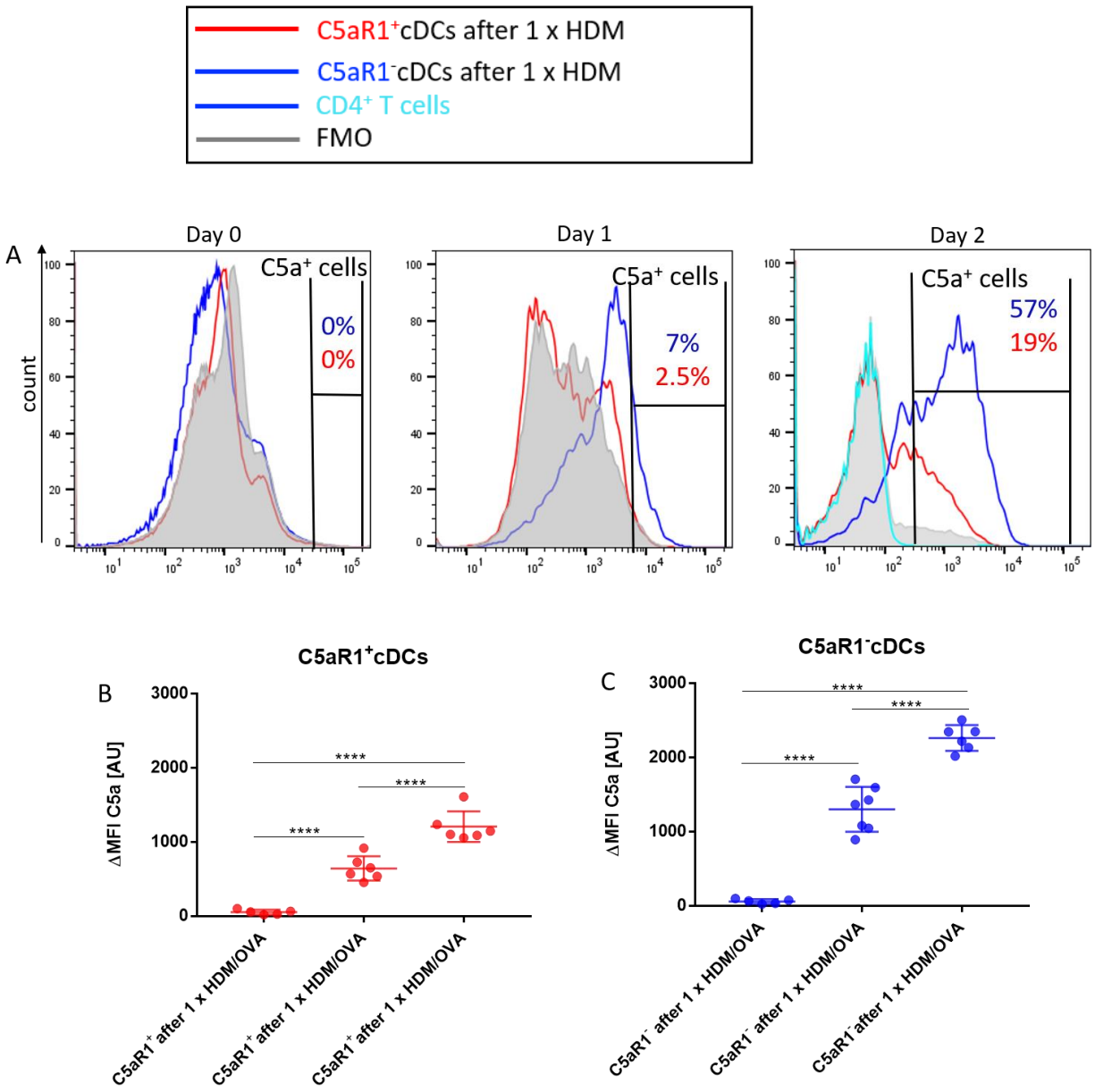


Figure 17: Impact of allergen-pulsing and T cell co-culture on C5 production from pulmonary C5aR1$^-$ and C5aR1$^+$ cDCs. (A) Histograms showing C5 generation in C5aR1$^+$ and C5aR1$^-$ cDC subsets directly after FACS purification on day 0, after OVA-pulsing on day 1 and after addition of OVA-tg CD4$^+$ T cells on day 2. (B) Quantitative evaluation of C5 production in C5aR1$^+$cDCs. (C) Quantitative evaluation of C5 production in C5aR1$^-$cDCs on day 0, 1 and 2. (D) Comparison of C5 production in C5aR1$^-$ and C5aR1$^+$ cDCs on days 0, 1, and 2. The figures in B, C and D show the ΔMFI of the C5 expression. Data shown are the mean ± SEM, n=4 per group; in (B) and (C) data were analyzed by ANOVA test, followed by Tukey's post-hoc test; *indicates significant differences from day 0 to day2; ** $p < 0.01$ and **** $p < 0.0001$. (D) Data were analyzed by unpaired t test; *indicates significant differences between C5aR1$^-$ and C5aR1$^+$ cDCs; ** $p < 0.01$.

On day 0, around 4 % of C5aR1$^+$ (Figure 17A, B) and 28 % of C5aR1$^-$ cDCs (Figure 17A-D) homogenously produced C5, directly after sorting. After OVA-pulsing on day 1, the C5 production in C5aR1$^+$ and C5aR1$^-$ cDCs markedly increased. In contrast to day 0, 83 % of C5aR1$^+$ and 82 % of C5aR1$^-$ cDCs produced C5. After addition of T cells on day 2, the C5 production significantly decreased in both the C5aR1$^+$ and C5aR1$^-$ cDCs and only 66% of C5aR1$^+$ cDCs and 71% of C5aR1$^-$ cDCs still expressed C5. To determine the individual C5 expression in C5aR1$^+$ and C5aR1$^-$ cDCs on days 0,1 and 2, I compared the ΔMFI. As shown in Figure 17D, C5 production was significantly higher in C5aR1$^-$ cDCs than in C5aR1$^+$ cDCs on day 0. In contrast, C5 production increased to the same level in C5aR1$^+$ and C5aR1$^-$ cDCs at day 1 and the expression levels were also similar on day 2. Importantly, I found no C5 production in the T cells (Figure 17A)

These finding suggest that OVA-pulsing is an important inducer of C5 production in C5aR1$^+$ and C5aR1$^-$ cDCs. The fact that I observed C5 production in only a subfraction of cDCs may suggest that these cDCs have efficiently taken up the antigen. Further, DC/T cell interaction reduced the potency of pulmonary CD11b$^+$ cDCs to produce C5.

Next, I aimed at studying the impact of OVA-pulsing and DC/T cell interaction on intracellular C5a generation from DC-produced C5. On day 0, none of the two CD11b$^+$cDC subsets produced C5a (Figure 18A-D). Only after *in vitro* pulsing with OVA, the cDCs started to make C5a. In C5aR1$^+$ cDCs, 2.5% of cells produced C5a, whereas in C5aR1$^-$ cDCs, 7% were C5a producers. Strikingly, the C5a production significantly increased after the addition of T cells on day 2 (Figure 18B and 18C). After the addition of T cells 19% of C5aR1$^+$ and 57% of C5aR1$^-$ cDCs produced C5a. Interestingly, C5aR1$^-$cDCs were making more C5a in comparison to the C5aR1$^+$cDCs on day 1 after OVA pulsing and on day 2 after addition of the T cell. In accordance with my findings that T cells make no C5, I also found no production of C5a (Figure 18A, D).


C5aR1+cDCs after 1 x HDM
C5aR1-cDCs after 1 x HDM
CD4+ T cells
FMO
A
Day 0
Day 1
Day 2
C5a+ cells
0%
0%
7%
2.5%
57%
19%
count
B
C5aR1+cDCs
ΔMFI C5a [AU]
C5aR1+ after 1 x HDM/OVA
C
C5aR1-cDCs
C5aR1- after 1 x HDM/OVA
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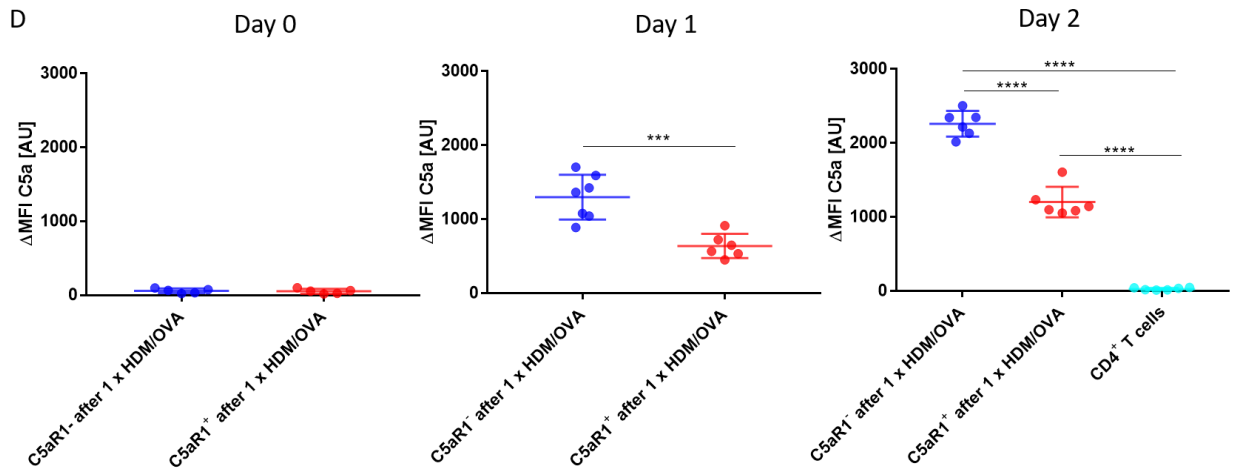


Figure 18: Impact of allergen -pulsing and T cell co-culture on C5a production from sensitized C5aR1$^-$ and C5aR1$^+$cDCs. (A) Histograms showing C5a generation in C5aR1$^+$ and C5aR1$^-$ cDC subsets directly after FACS purification on day 0, after OVA-pulsing on day 1 and after addition of OVA-tg CD4$^+$ T cells on day 2. (B,C) Quantitative evaluation of C5a production in C5aR1$^+$cDCs (B) and C5aR1$^-$ cCDs (C) on days 0, 1 and 2. (D) Comparison of C5a production in C5aR1$^-$ and C5aR1$^+$ cDCs on days 0, 1, and 2. The figures in B, C and D show the ΔMFI of the C5a expression. Data shown are the mean ± SEM, n=6 per group. in (B) and (C) data were analyzed by ANOVA test, followed by Tukey's post-hoc test; *indicates significant differences from day0 to day2; **** $p < 0.0001$. (D) Data were analyzed by unpaired t test; *indicates significant differences between C5aR1$^-$ and C5aR1$^+$ cDCs; *** $p < 0.001$ and **** $p < 0.0001$.

To directly assess the impact of T cells on C5a production from CD11b$^+$cDCs, I compared the C5a generation on day 2 in the presence or absence of CD4$^+$ T cells. In case of the C5aR1$^-$cDCs, the levels of C5a production were similar between the two groups, indicating that the trigger for the C5a generation was the allergen and the interaction of the cDCs with the CD4$^+$ T cells had no influence. However, in case of C5aR1$^+$cDCs, the CD4$^+$ T cells played a role in the production of C5. More precisely, I found that in both CD11b$^+$cDC subsets, the trigger was the allergen, but in case of the C5aR1$^+$cDCs, the interaction of the DCs with the CD4$^+$ T cells resulted in lower generation of C5a, suggesting that the CD4$^+$ T cells could act as limiting factor for the production of C5a (Figure 10). These findings suggest that T cells differentially impact on the potency of the two different CD11b$^+$ subsets to generate C5a from C5.

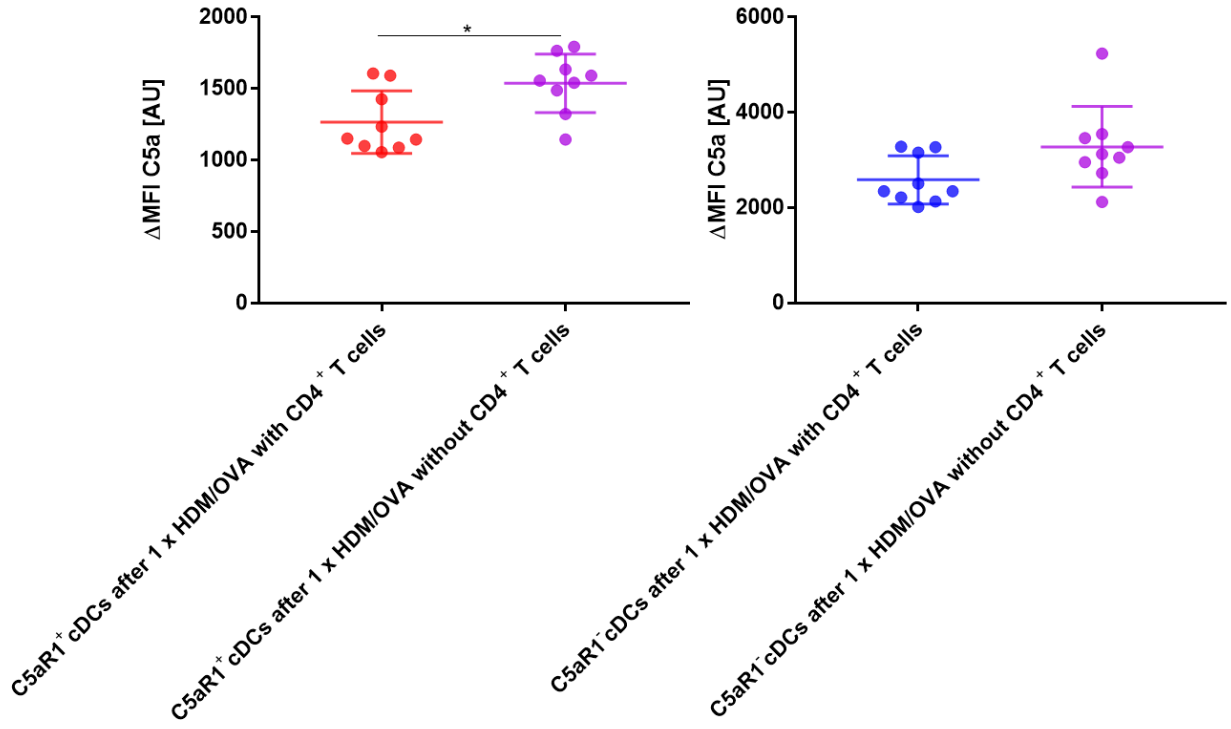


Figure 19: Impact of T cells on OVA-driven C5a production in sensitized C5aR1⁻ and C5aR1⁺cDCs. WT mice were treated once with HDM/OVA (100 µg/40 µg) i.t. C5aR1⁺ and C5aR1⁻cDCs from WT mice were purified from lung tissue by FACS 24h after the treatment. On day 2 post sorting, the intracellular expression of C5a was evaluated in OVA-pulsed cDCs that were co-cultured system with or without OVA-tg CD4⁺ T cells. The figure shows the ΔMFI of the C5a expression by the two cDCs subsets. Data shown are the mean ± SEM. n = 9, and were analyzed by unpaired t test; *indicates significant differences between C5aR1⁻ and C5aR1⁺ cDCs; *p < 0.05

3.1.9 Bulk RNA sequencing of C5aR1⁺ and C5aR1⁻cDC after 1 step HDM/OVA immunization revealed distinct transcriptional activity in the two CD11b⁺cDC subsets

As outlined above, C5aR1⁻ and C5aR1⁺cDCs exert phenotypical differences including distinct MHC-II, costimulatory molecule and alarmin receptor expression that are associated with a distinct potency of the two CD11b⁺ cDC subsets to drive T cell proliferation and differentiation. To delineate the activation pattern of sensitized C5aR1⁺ and C5aR1⁻ cDCs in more detail, I performed bulk RNA sequencing (RNAseq) of FACS-purified CD11b⁺C5aR1⁻ and CD11b⁺C5aR1⁺cDC, 24h after one step HDM/OVA sensitization (Figure 20). In order to perform RNAseq, a minimum of 300ng of RNA yield is required. Due to the low number of pulmonary C5aR1⁻ and C5aR1⁺ cDCs that can be sorted from a single mouse, ten mice had to be pooled to meet the requirements. In case of the C5aR1⁺cDCs, this resulted in a sufficient RNA yield (700ng), but in case of the C5aR1⁻cDCs, the yield was around 200ng. Even though, the DNA core at CCHMC, which performed the RNA sequencing recommends to use 300ng of RNA, it is also possible to work with lower amounts (150-200ng). Thus, for the initial library preparation, I used half of the amount of each sample (350 and 100 ng). Unfortunately, the first approach to prepare the cDNA libraries failed. So, in the second approach, the DNA core from CCHMC, decided to pool all the C5aR1⁻ samples, in order to reach the 300ng of RNA yield and to ensure successful library preparation and sequencing. Regarding the C5aR1⁺cDCs,

there was no need for pooling, since the RNA yield of one sample was sufficient. Thus, instead of acquiring data from three independent experiments, I had to use pooled RNA from the three independent experiments. Both for the C5aR1$^+$ and C5aR1$^-$ cDCs, a paired end sequencing was performed, and the samples received almost 20 x10^6 reads.

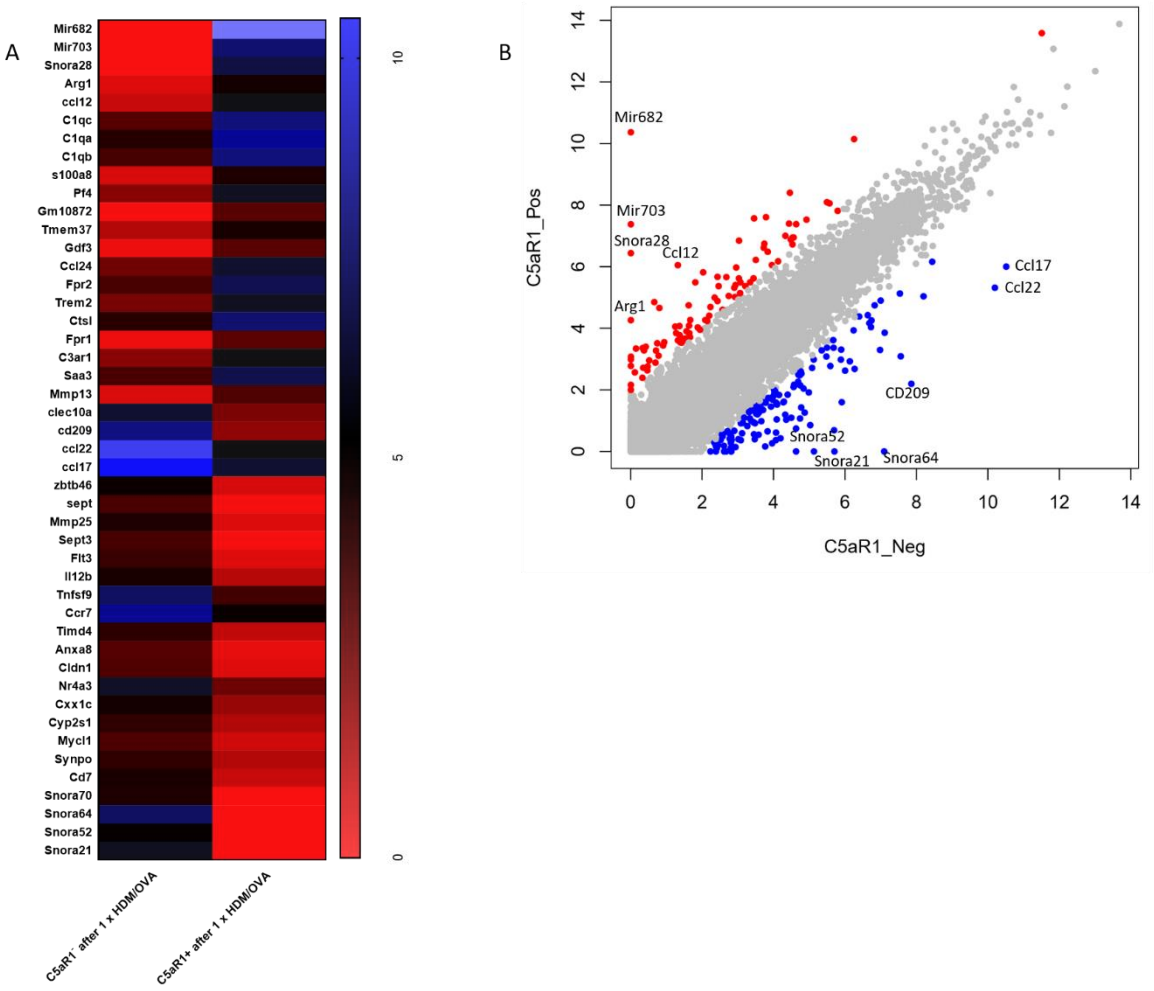


Figure 20: RNAseq analysis of sensitized C5aR1$^+$ and C5aR1$^-$cDCs. WT mice were treated once with HDM/OVA (100 µg/40 µg) i.t. C5aR1$^+$ and C5aR1$^-$cDCs from WT mice were FACS-sorted 24h after the sensitization, and the samples were prepared for RNA sequencing. (A) Expression of 40 genes (rows) with the most significant difference in expression in C5aR1$^+$ and C5aR1$^-$cDCs (columns). (B) Scatter plot for genes with a >4-fold change (blue= high in C5aR1$^-$ cDCs; red=4-fold high in C5aR1$^+$cDCs, grey= genes with a 2-fold or lower fold change between the two groups of cells).

The analysis revealed that the two CD11b$^+$cDC subsets did not cluster together and by setting the cut off to 4 fold, there were almost 100 genes from each population that were highly expressed in one of the two populations whereas their expression was much lower or in some cases even zero in the corresponding population. More precisely, genes with a 4-fold or higher change within the C5aR1$^+$ group of cells comprised Arg1, Mir682, Mir703, Snora28, Ccl12, C1qc, C1qb and s100a8. The genes Snora70,64,52 and 21, zbtb46, ccl17, Ccl22, cd209, Cd7 and clec10a showed a stronger expression in the C5aR1$^-$cDCs, with a 4-fold or higher change in comparison to their expression levels on the C5aR1$^+$cDCs.

3.1.10 CD301, CD24 and CD209 discriminate different subsets of CD11b$^+$cDCs

In a next step, I started to verify some of the RNA profiling results. I focused on cell surface molecules that I could easily use to further phenotype the CD11b$^+$ cDCs. I assessed the expression of CD209 and CLEC10A in the two cDC populations together with CD24 (Figure 21).

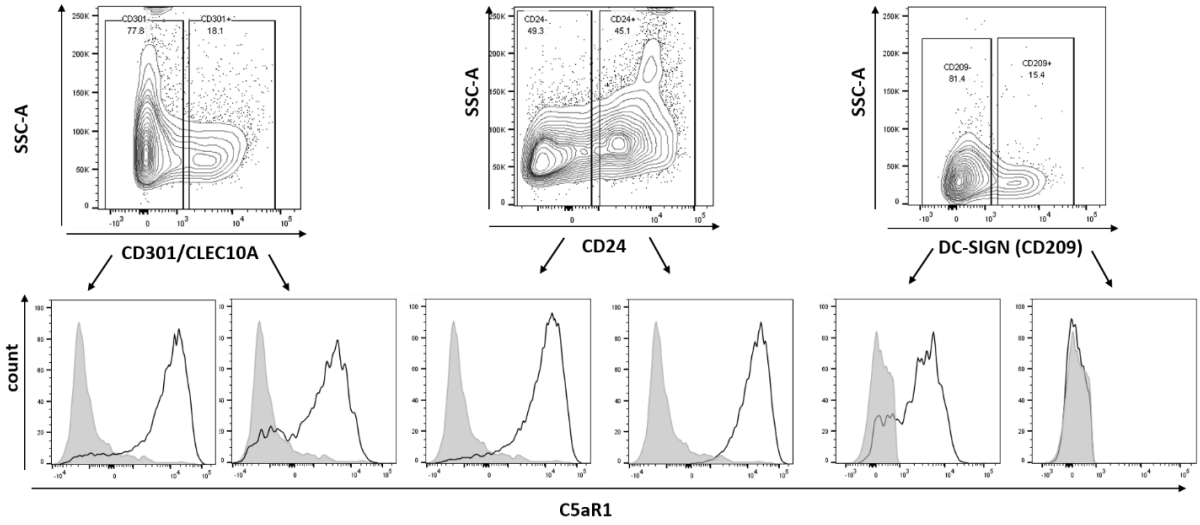


Figure 21: CD301, CD24 and CD209 reveal different subsets of CD11b$^+$cDCs. WT mice were treated once with HDM/OVA (100 µg/40 µg) i.t. 24h after the treatment, the expression of CD301, CD24, and CD209 was evaluated by measuring 10.000 events for each of the two sub-populations. The dot plots show the expression of CD301, CD24, and CD209 by CD11b$^+$CD64$^-$cDCs. The histograms show C5aR1 expression in the different CD11b$^+$cDCs.

I could confirm that CD209hi CD11b$^+$cDCs did not express C5aR1. However, in the CLEC10A$^+$CD11b$^+$cDCS only a subpopulation was C5aR1$^-$, whereas the majority expressed C5aR1. Similarly, CD24 was expressed on C5aR1$^-$ and C5aR1$^+$cDCs. Thus, the flow cytometric data confirmed some results obtained from RNAseq. Clearly, they demonstrate that the CD11$^+$CD64$^-$DCs are heterogeneous.

3.2 Direct evaluation of the role of C5aR1 on sensitized CD11b$^+$C5aR1$^+$cDCs

3.2.1 *In vitro* targeting of C5aR1 partially restored the potency of C5aR1$^+$cDCs to induce CD4$^+$ T cell proliferation

The different sets of experiments I performed so far (phenotypic characterization, functional studies, and RNA sequencing), all indicated that the C5aR1$^-$ and C5aR1$^+$cDCs are two cDC subsets with distinct functions. However, at this time point, it was still not clear, whether these differences between the two CD11b$^+$cDC subsets were controlled by signaling through C5aR1 or not. To directly assess the role of C5aR1 on CD11b$^+$cDCs, I decided to focus on C5aR1$^+$cDCs and block C5aR1 using a neutralizing Ab against the receptor *in vitro* (Köhl et al. J Clin Invest 2006). In more detail, 24h after one step HDM/OVA immunization, the C5aR1$^+$ cDCs were FACS-purified and *in vitro* pulsed with OVA. Half of the cells were treated with a neutralizing Ab against C5aR1, and the rest of the cells were treated with the appropriate isotype control. Eighteen hours later, both groups of C5aR1$^+$cDCs were co-cultured with CFSE-labeled-CD4$^+$ T cells from OVA-tg DO11.10 RAG2$^{-/-}$ mice. Four days later, T cell proliferation was evaluated.

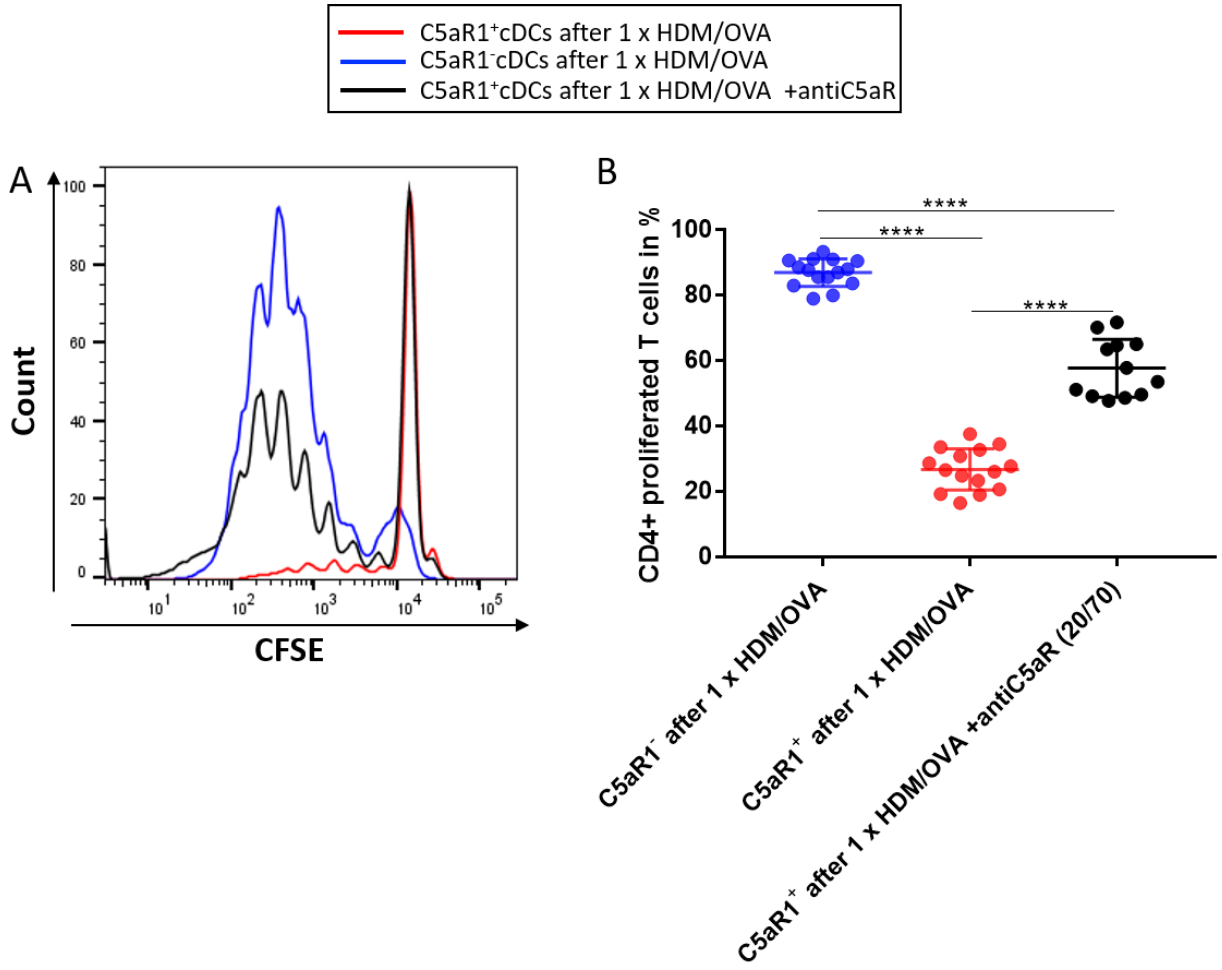


Figure 22: Impact of *in vitro* targeting of C5aR1 in C5aR1$^+$ cDCs on CD4$^+$ T cell proliferation. WT mice were treated once with a mixture of HDM/OVA (100 µg/40 µg) i.t. C5aR1$^+$ and C5aR1$^-$ CD11b$^+$ cDCs from WT mice were purified from lung tissue by FACS 24h after the treatment. Half of the C5aR1$^+$cDCs were treated with 5µg/ml of a neutralizing Ab against C5aR1 (20/70) and the other half with the appropriate isotype control. For four days, all the cells were co-cultured with CFSE-labeled OVA transgenic CD4$^+$ T cells in the presence of OVA (10 µM) and GM-CSF (20 ng/ml). After four days in co-culture, the T cell proliferation was determined by evaluating the intensity of the CFSE signal by flow cytometry. (A) Histogram showing the reduction of the CFSE signal in proliferated T cells stimulated with C5aR1$^+$ cDCs in the presence or absence of anti-C5aR1 mAb 20/70. (B) Quantification of T cell proliferation in the different treatment groups. Values shown are the mean ± SEM; n=12. Data were analyzed by ANOVA, followed by Tukey's posthoc test; *indicates significant differences between T cell co-cultures with C5aR1$^-$ or C5aR1$^+$cDCs or C5aR1$^+$cDCs +anti-C5aR1; ****p< 0.0001.

Interestingly, *in vitro* blocking of C5aR1 significantly restored the potency of C5aR1$^+$cDCs to prime CD4$^+$ T cell responses. More precisely, isotype control Ab treated C5aR1$^+$cDCs resulted in activation of 30 ± 10% of the CD4$^+$ T cells. In contrast, upon *in vitro* C5aR1 blockade, the frequency of activated CD4$^+$ T cells increased to 60 ± 10% showing that signaling through C5aR1 partially controls the ability of pulmonary CD11b$^+$cDCs to activate T cells.

3.2.2 C5aR1 targeting in C5aR1$^+$cDCs has no impact on allergen uptake

When I saw the effect of the *in vitro* blocking of C5aR1 on the ability of C5aR1$^+$cDCs to drive T cell proliferation, I considered that the signaling through C5aR1 might suppress one of the basic DC functions

like Ag uptake and processing. To test this hypothesis, I FACS-purified naïve C5aR1$^+$cDCs and treated them with either FITC-OVA or DQ-OVA in the presence or absence of the neutralizing C5aR1 mAb 20/70 (Figure 19). When DCs digest DQ-OVA, the dye emits at 515nm in the FITC-channel. Once the DQ-OVA is digested and the fragments accumulate in the cell's organelles in high concentrations, the fluorophores can form excimers, which can be visualized using a red light-sensitive extended pass filter (PE-Texas Red channel).

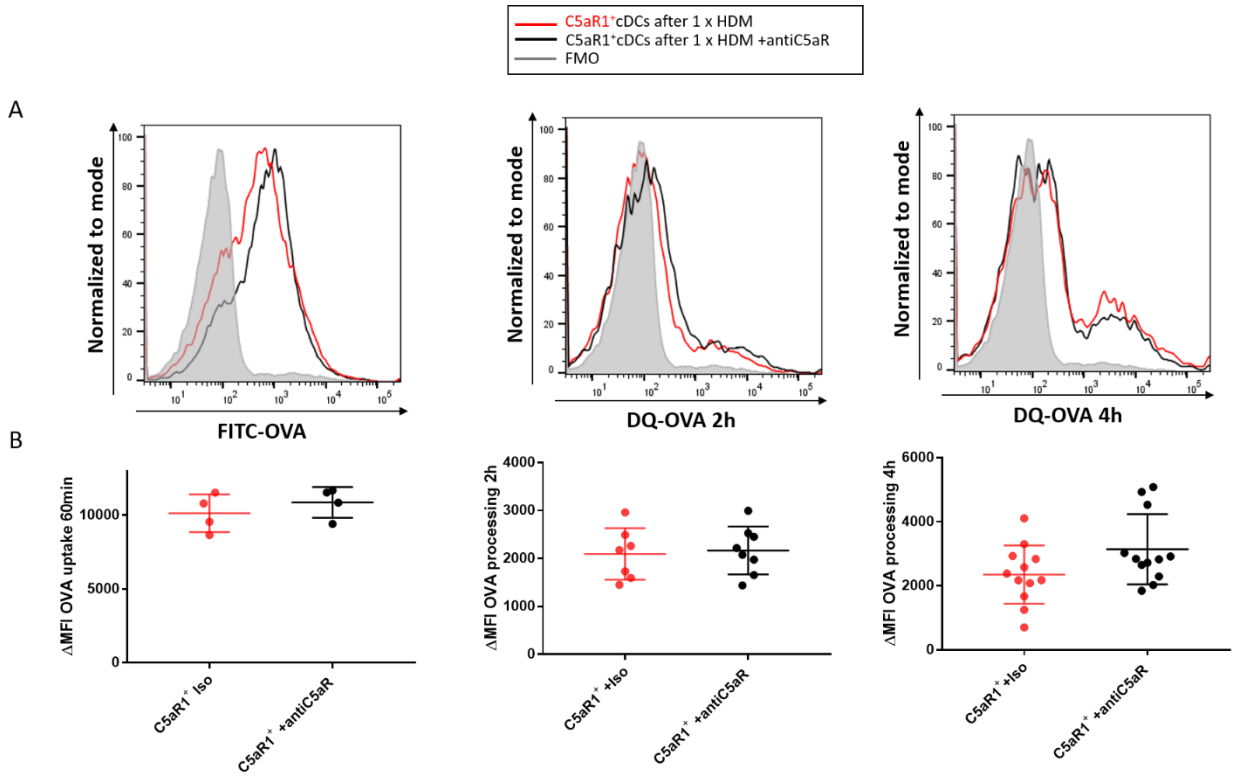


Figure 23: C5aR1 targeting in C5aR1$^+$ cDCs has no impact on allergen uptake. The C5aR1$^+$cDCs from naïve mice were FACS-sorted and put in culture. Half of the C5aR1$^+$cDCs were treated overnight with 5µg/ml of a neutralizing Ab against C5aR1 (20/70) and the other half with the appropriate isotype control. The following day, the cells were pulsed either with FITC-OVA (1h) to assess Ag uptake or DQ-OVA (2 and 4h) for Ag-uptake/processing. (A) Histogram showing the ΔMFI of the OVA-uptake using FITC-OVA or DQ-OVA. (B) Quantification of the FITC-OVA or DQ-OVA by the two different CD11b+ cDC subsets. Shown is the mean ± SEM, n = 4 per group for 1h, n = 8 for 2h and n = 12 for 4h. Data were analyzed by unpaired t-test; *indicates significant differences between C5aR1$^+$ + isotype ctrl or C5aR1$^+$ + antiC5aR1 mAb 20/70.

I found no differences in Ag uptake after 1, 2 or 4h between the anti-C5aR1-treated-C5aR1$^+$cDCs and isotype-Ab-treated-C5aR1$^+$cDCs, suggesting that the different potencies of C5aR1-targeted cDCs to drive T cell proliferation did not result from different potencies in Ag uptake during the first 4h after Ag pulsing.

3.2.3 *In vitro* blockade of C5aR1 results in significant upregulation of CD40 but has no effect on MHC-II expression

Since C5aR1 targeting had no impact on allergen uptake and processing, I focused next on the expression levels of MHC-II and the co-stimulatory molecule CD40 on the C5aR1$^+$cDCs. My previous experiments (Fig. 12), showed that the C5aR1$^+$cDCs expressed low levels of CD40 and MHC-II, two molecules which are known to be critical players in the activation of T cells. Thus, I assessed MHC-II and CD40 expression 18h after *in vitro* targeting of C5aR1 and before I added the T cells to the system (Figure 24).

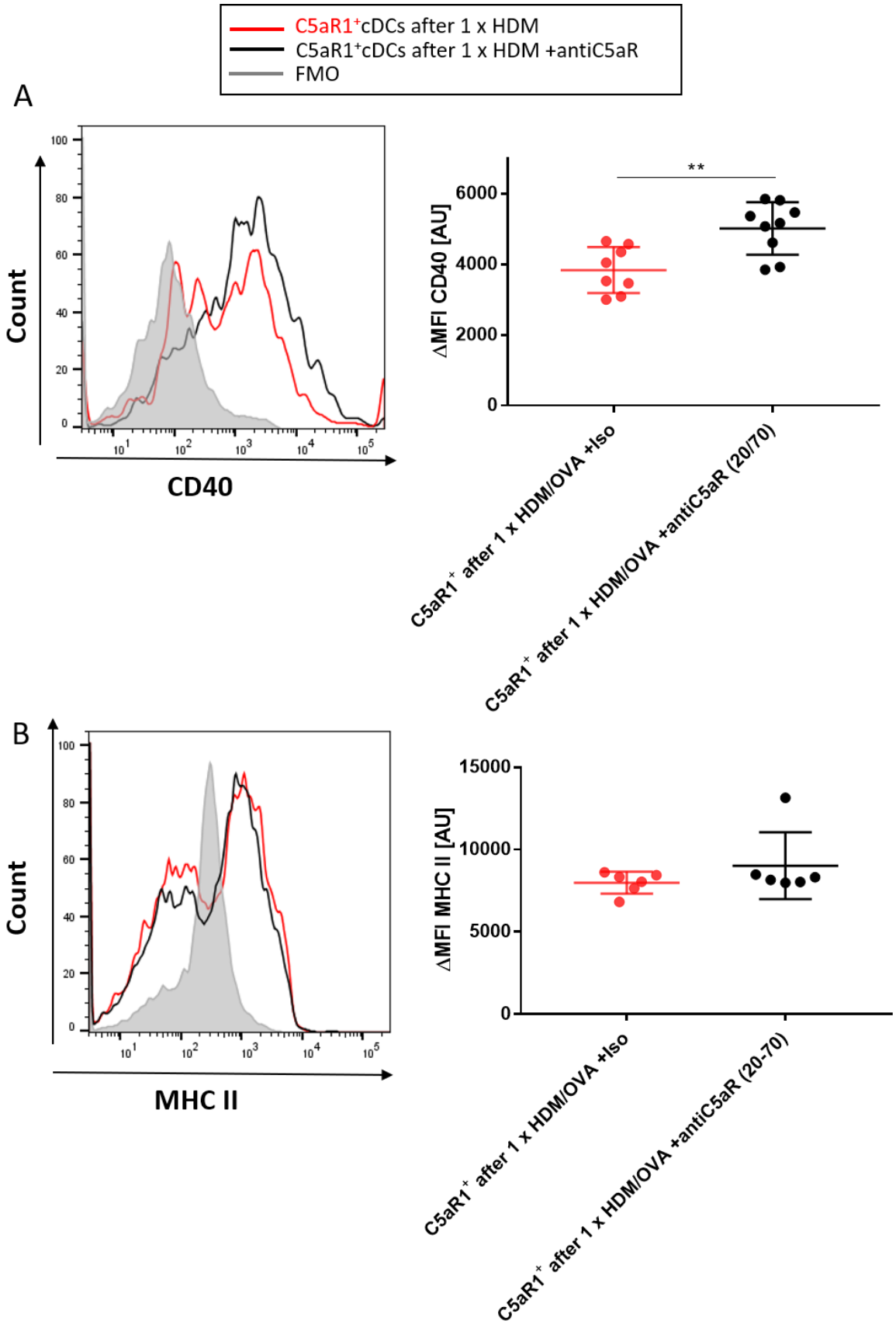
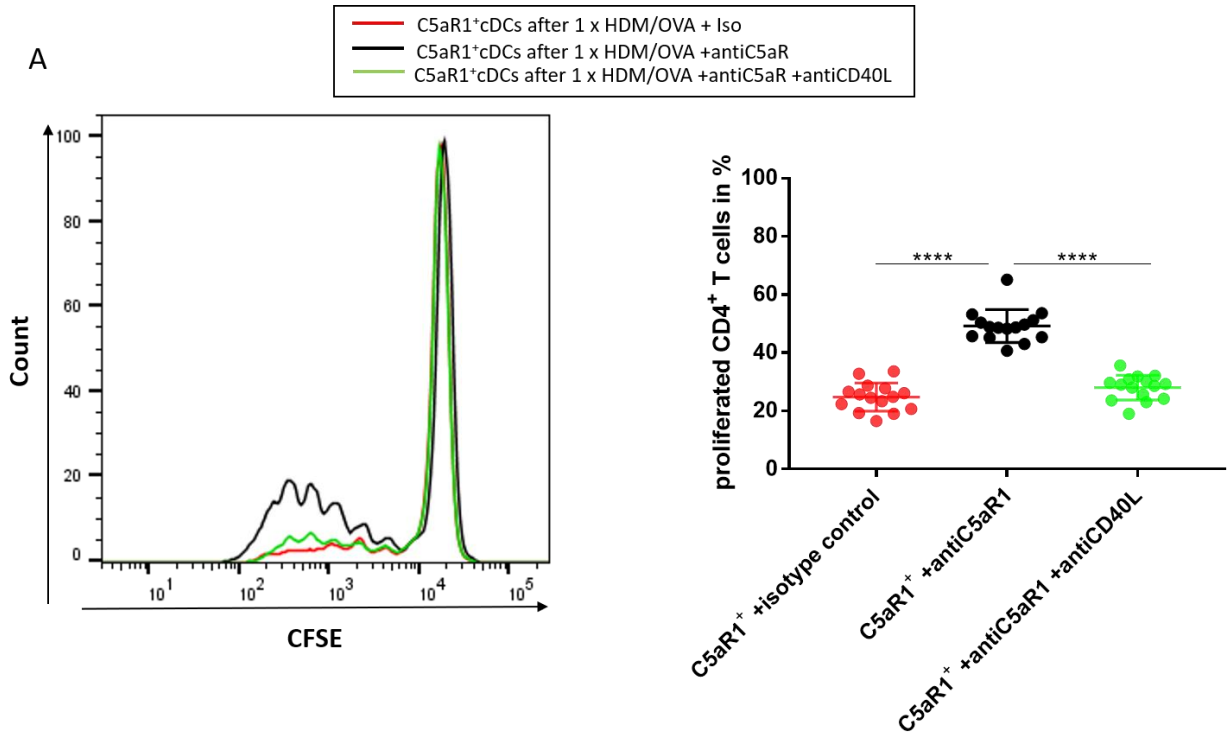

C5aR1+cDCs after 1 x HDM
C5aR1+cDCs after 1 x HDM +antiC5aR
FMO
A
Count
CD40
ΔMFI CD40 [AU]
**
C5aR1+ after 1 x HDM/OVA +Iso
C5aR1+ after 1 x HDM/OVA +antiC5aR (20/70)
B
Count
MHC II
ΔMFI MHC II [AU]
C5aR1+ after 1 x HDM/OVA +Iso
C5aR1+ after 1 x HDM/OVA +antiC5aR (20-70)

Figure 24: Impact of C5aR1 targeting on MHC-II and CD40 expression in C5aR1$^+$ cDCs. WT mice were treated once with a mixture of HDM/OVA (100 µg/40 µg) i.t. C5aR1$^+$CD11b$^+$cDCs from WT mice were purified from lung tissue by FACS 24h after the treatment. Half of the C5aR1$^+$cDCs were treated with 5µg/ml of a neutralizing Ab against C5aR1 (20/70) and the other half with the appropriate isotype control. 18h after the in vitro blocking of C5aR1, the expression of CD40 and MHCII was evaluated. (A) Histogram showing CD40 expression (left) and quantitative assessment in C5aR1$^+$ + isotype ctrl Ab and C5aR1$^+$ + antiC5aR1 mAb (right). (B) Histogram showing MHC-II expression (left) and quantitative assessment in C5aR1$^+$ isotype ctrl Ab and C5aR1$^+$ + antiC5aR1 mAb (right). The figures show the ΔMFI of the expression of the examined molecules by the two subsets as mean ± SEM, n = 6 per group. Data were analyzed with unpaired t-test; * indicates significance between the two treatment groups, ** p < 0.001.

I observed a significant up-regulation of CD40 whereas the expression of MHC-II was unchanged 18h after *in vitro* blocking of C5aR1. These data suggest that activation of C5a/C5aR1 axis suppresses the expression of CD40 and that this regulation may impact on the activation of CD4$^+$ T cells.

3.2.4 Simultaneous *in vitro* blockade of CD40-CD40L interactions and C5aR1 reverses the increased potency of C5aR1$^+$cDCs to induce CD4$^+$ T cell proliferation in response to C5aR1 blockade alone

In light of the regulatory effect of C5AR1 targeting on CD40 expression, I decided to further assess the role of CD40 by *in vitro* blocking the CD40-CD40L interactions using a neutralizing Ab against CD40L. I expected that this approach will define the importance of CD40 in the activation of CD4$^+$ T cells in C5aR1$^-$ and C5aR1$^+$cDCs (Figure 25).



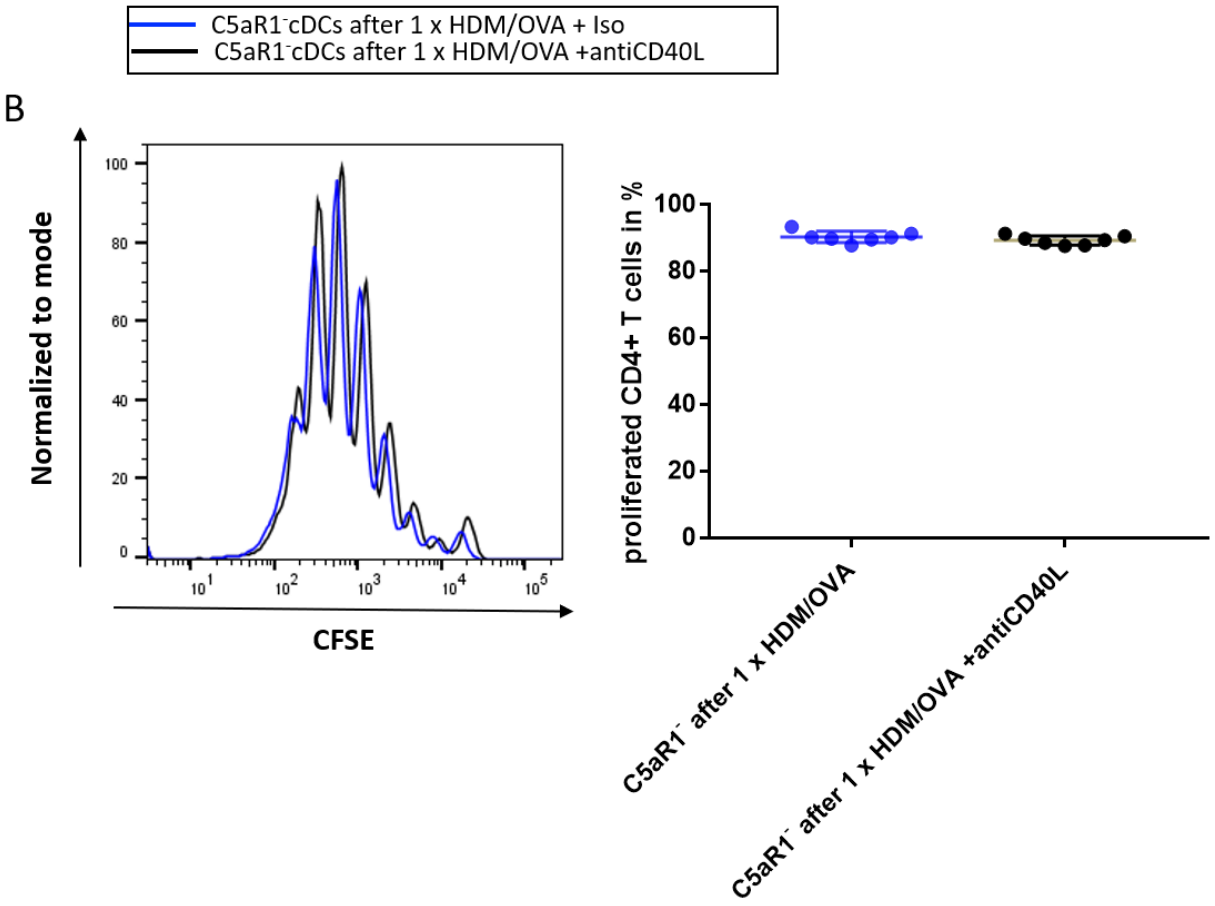
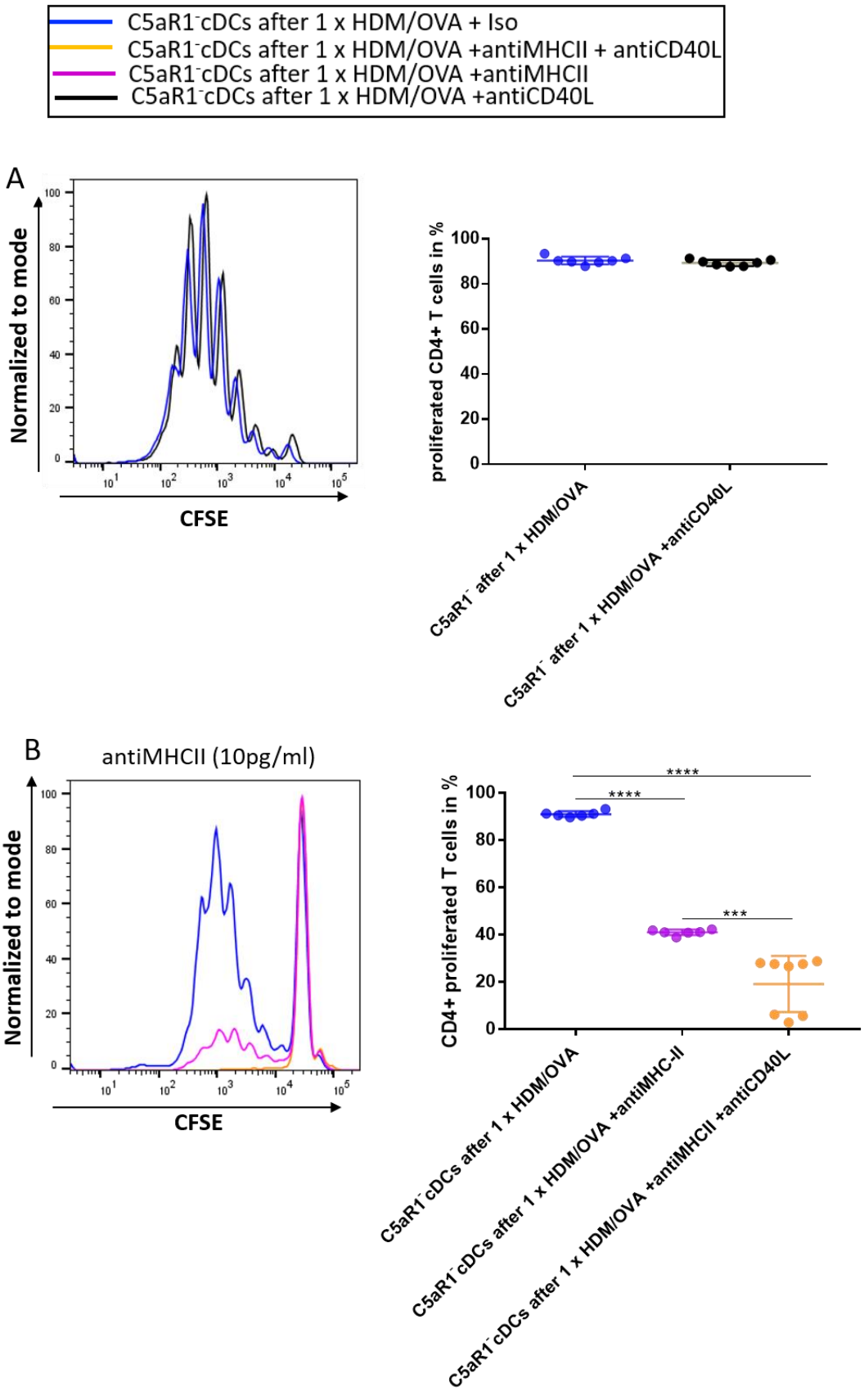


Figure 25: Impact of *in vitro* CD40-CD40L blockade on the potency of C5aR1$^+$cDCs to induce CD4$^+$ T cell proliferation in response to C5aR1 blockade alone. WT mice were treated once with a mixture of HDM/OVA (100 µg/40 µg) i.t. C5aR1$^+$ and C5aR1$^-$ CD11b$^+$cDCs from WT mice were purified from lung tissue by FACS 24h after sensitization. (A) Histogram (left) showing the impact of C5aR1 targeting vs. C5aR1 and CD40/CD40L targeting on T cell proliferation. The graph on the right side shows the quantitative evaluation of the different treatment strategies. Values shown are the mean ± SEM; n= 15 per group. Data were compared by ANOVA followed by Tukey's posthoc test. *indicates significant differences between T cell co-cultures with C5aR1$^+$ or C5aR1$^+$cDCs + anti-C5aR or C5aR1$^+$cDCs + anti-C5aR1 + antiCD40L; ****p< 0.0001. (B) Histogram (left) showing the impact of CD40/CD40L targeting on T cell proliferation in C5aR1$^-$ cells. The graph on the right side shows the quantitative evaluation of CD40/CD40L blockade. Values shown are the mean ± SEM; n= 7 per group. Data were analyzed by unpaired t-test; *indicates significant differences between T cell co-cultures with C5aR1$^-$ or C5aR1$^-$cDCs +antiCD40L; *p< 0.05.

As shown above, C5aR1 targeting partially restored the potency of C5aR1$^+$ cDCs to drive T cell proliferation (Fig. 22). When C5aR1 and CD40L were simultaneously targeted *in vitro*, I observed a decreased potency of the C5aR1$^+$cDCs to induce CD4$^+$ T cell proliferation (Figure 25). In fact, the T cell proliferation was as low the one observed in untreated C5aR1$^+$cDCs (Figure 11). These findings suggest that CD40-CD40L interaction in C5aR1$^+$cDCs is critical for T cell proliferation. In contrast, targeting of the CD40-CD40L axis in C5aR1$^-$cDCs did not affect T cell proliferation suggesting that only under conditions of low MHC-II expression the CD40-CD40L targeting controls T cell proliferation (Fig. 25).

3.2.5 CD40-CD40L interaction controls the activation of $CD4^+$ T cells under low MHC-II expression

To further test the hypothesis that in $C5aR1^-$cDCs the strong expression of MHC-II dominated the activation of T cells and compensated for the loss of CD40 (as shown in Figure 25), I setup an experiment, in which I targeted MHC-II used a neutralizing Ab. More precisely, I used different concentration of the neutralizing MHC-II Ab to determine whether I could recapitulate the effect that under conditions of low MHC-II signaling CD40 (as shown in $C5aR1^+$ cDCs in Fig. 25) would affect DC-driven T cell proliferation.


C5aR1-cDCs after 1 x HDM/OVA + Iso
C5aR1-cDCs after 1 x HDM/OVA +antiMHCII + antiCD40L
C5aR1-cDCs after 1 x HDM/OVA +antiMHCII
C5aR1-cDCs after 1 x HDM/OVA +antiCD40L
A
Normalized to mode
CFSE
proliferated CD4+ T cells in %
C5aR1- after 1 x HDM/OVA
C5aR1- after 1 x HDM/OVA +antiCD40L
B
antiMHCII (10pg/ml)
Normalized to mode
CFSE
CD4+ proliferated T cells in %
****
****
***
C5aR1-cDCs after 1 x HDM/OVA
C5aR1-cDCs after 1 x HDM/OVA +antiMHC-II
C5aR1-cDCs after 1 x HDM/OVA +antiMHCII +antiCD40L

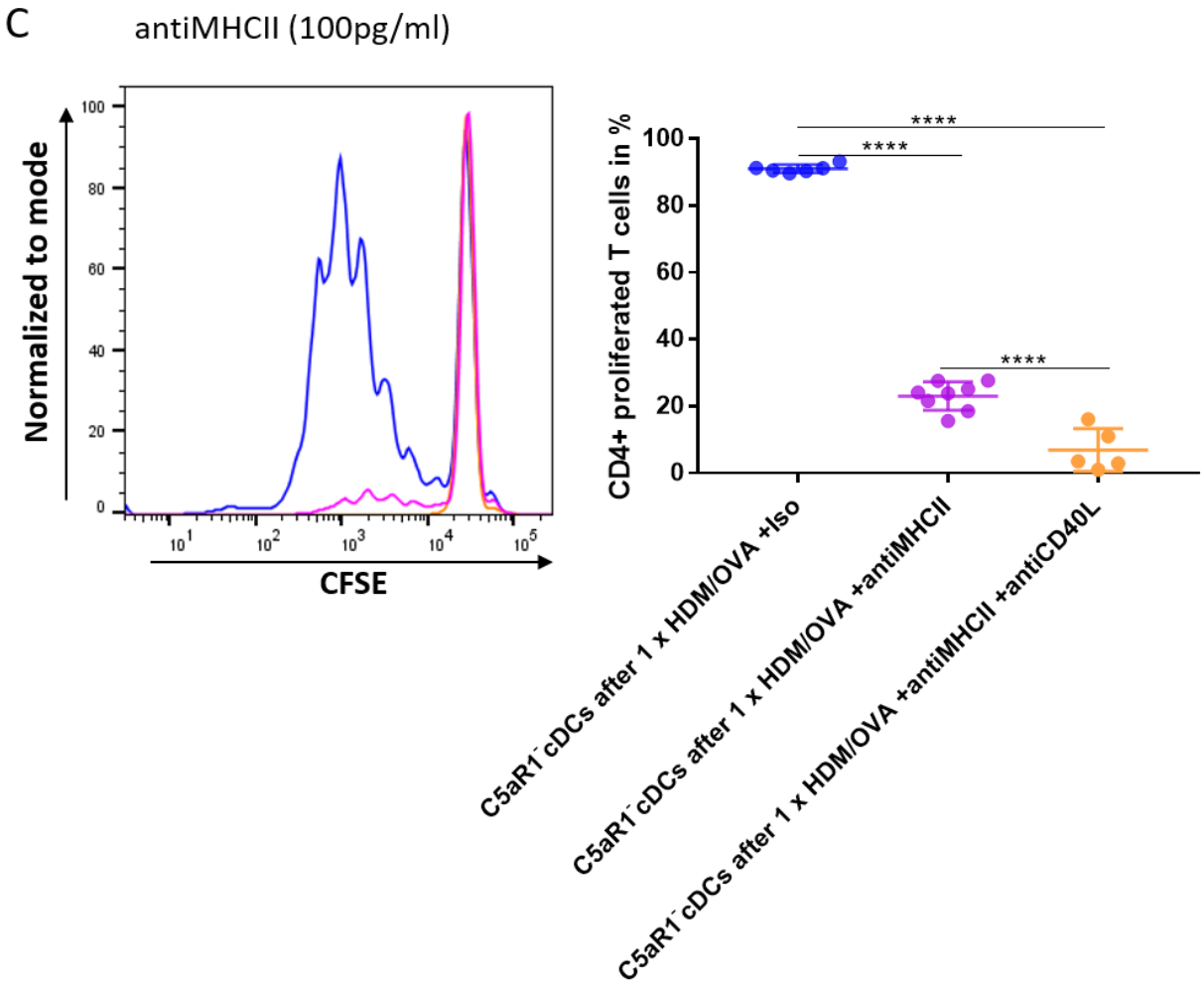


Figure 26: The effect of MHC-II targeting on C5aR1⁻ cDC -driven T cell proliferation in response to CD40L neutralization. WT mice were treated once with a mixture of HDM/OVA (100 µg/40 µg) i.t. C5aR1⁻ cDCs from WT mice were purified from lung tissue by FACS 24h after allergen sensitization. The cells were cultured under six different conditions. Some of the C5aR1⁻cDCs were treated with a neutralizing Ab against MHCII (100pg/ml or 10pg/ml), some with the Ab against MHCII (100pg/ml or 10pg/ml) and with a neutralizing Ab against CD40L (5µg/ml), some with the neutralizing Ab against CD40L (5µg/ml) and the rest with the appropriate isotype control. The cells were co-cultured with CFSE-labeled OVA transgenic CD4⁺ T cells for four days in the presence of OVA (10 µM) and GM-CSF (20 ng/ml). After four days in co-culture, the T cell proliferation was determined by evaluating the intensity of the CFSE signal by flow cytometry. (A) Histogram (left) showing the impact of CD40/CD40L targeting on T cell proliferation in C5aR1⁻ cells. The graph on the right side shows the quantitative evaluation of CD40/CD40L blockade. Values shown are the mean ± SEM; n= 7 per group. Data were analyzed by unpaired t-test; *indicates significant differences between T cell co-cultures with C5aR1⁻ or C5aR1⁻cDCs +antiCD40L; *p< 0.05. (B) Histogram (left) showing the impact of MHC-II (10pg/ml) targeting on T cell proliferation in C5aR1⁻ cells or C5aR1⁻ cells in which there was a simultaneous CD40/CD40L blockade. The graph on the right side shows the quantitative evaluation of MHC-II blockade. (C) Histogram (left) showing the impact of MHC-II (100pg/ml) targeting on T cell proliferation in C5aR1⁻ cells or C5aR1⁻ cells in which there was a simultaneous CD40/CD40L blockade. The graph on the right side shows the quantitative evaluation of MHC-II blockade. Values shown are the mean ± SEM; n= 5-8 per group. Data were analyzed

by ANOVA test, followed by Tukey post-hoc test; *indicates significant differences between T cell co-cultures with C5aR1$^-$ or C5aR1$^-$cDCs +anti-MHC-II or C5aR1$^-$cDCs +anti-MHC-II +anti-CD40L; ***p< 0.001 and ****p<0.0001.

In a first set of experiment, I targeted CD40L in C5aR1$^-$ cDCs. As shown before (Fig. 11) C5aR1$^-$ cDCs were strong inducers of T cell activation driving proliferation in around 90% of the T cells. Targeting of CD40L had no effect (Fig. 25). Next, C5aR1$^-$cDCs were treated with a neutralizing Ab against MHCII at a concentration of 10pg/ml. This regimen reduced the potency of C5aR1$^-$ cDCs to drive T cell proliferation to 40%. Interestingly, CD40L targeting further decreased T cell proliferation to 20% (Fig. 26B). When I increased the concentration of the neutralizing MHC-II Ab to 100 pg/ml, T cell proliferation decreased further to 20% (Fig. 26C), Anti-CD40L treatment almost completely abolished T cell proliferation in C5aR1$^-$ cDCs. These findings strongly support the view that under conditions of low MHC-II expression as observed in C5aR1$^+$ cDCs CD40 signaling is critical for T cell proliferation.

3.2.6 CD40-CD40L interaction control the activation of CD4$^+$ T cells when MHC-II peptide loading is limited

At this point, my results suggested that the C5a/C5aR1 axis controlled the CD4$^+$ T cell proliferation through CD40 when the number of MHC-II molecules is limited. However, the experimental setup with OVA as model allergen was not able to assess whether a limited amount of allergen within the peptide grove of MHC-II will also sensitize to CD40/CD40L. To address that question, I sensitized WT mice exclusively with HDM *in vivo*. After 24h, I FACS-purified the C5aR1$^+$ and C5aR1$^-$cDCs and pulsed them *in vitro* with OVA$^{323-339}$peptide, which specifically binds to the TCR of OVA-tg DO11.10 RAG2$^{-/-}$ mice. I used 4 different 10-fold dilutions of the peptide starting with 5ug/ml and compared the impact of decreased peptide MHC-II loading on the potency of C5aR1$^-$ and C5aR1$^+$cDCs to drive proliferation of OVA-tg CD4$^+$ T cells (Figure 27).

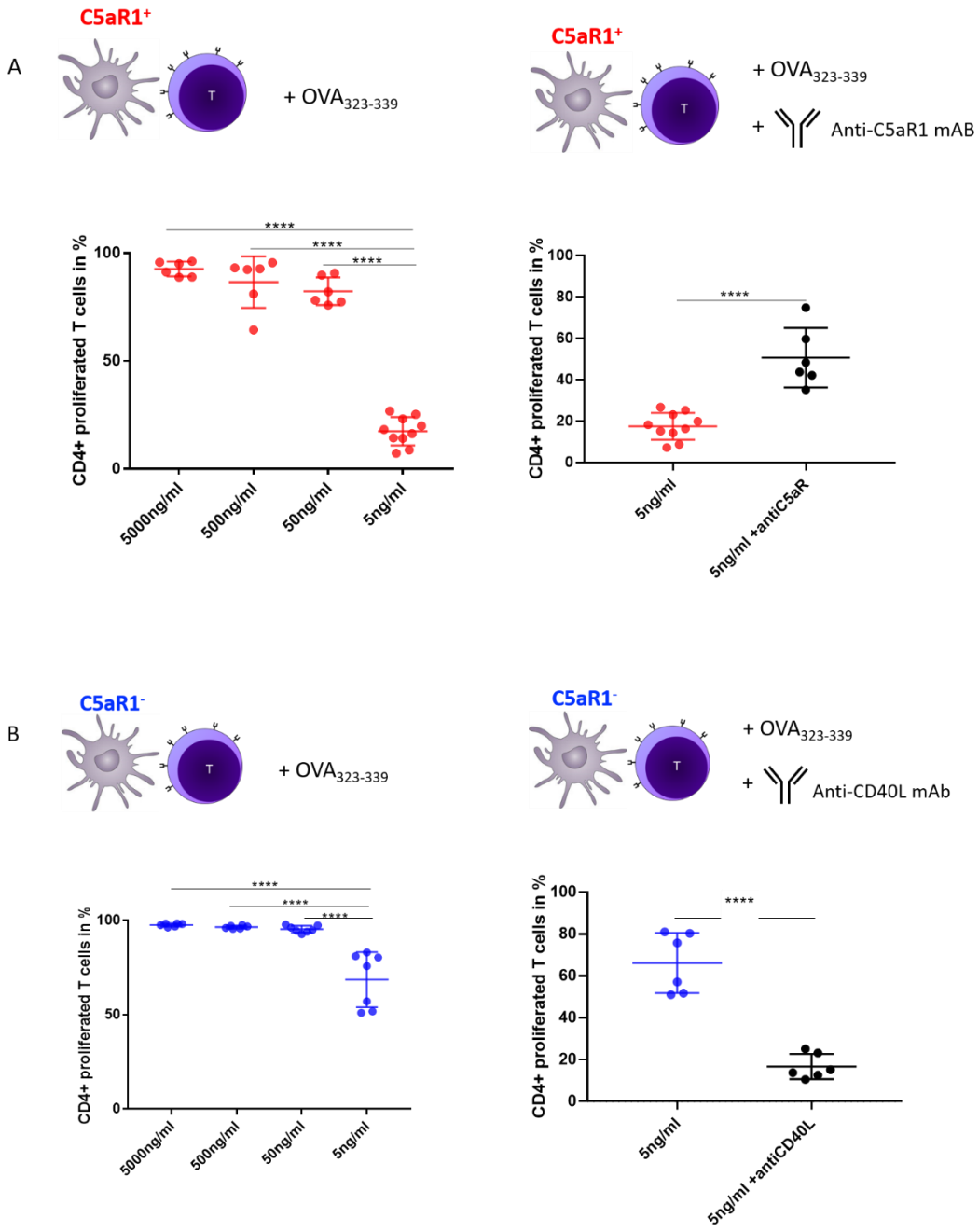


Figure 27: Impact of low OVA$^{323-339}$ peptide concentrations on the proliferation of OVA-tg CD4$^+$ T cells stimulated with C5aR1$^+$ (A) or C5aR1$^-$ cDCs (B). WT mice were treated once with HDM (100 µg) i.t. C5aR1$^+$ (A) or C5aR1$^-$(B) cDCs from WT mice were purified from lung tissue by FACS 24h after the treatment. The cDCs were co-cultured with CFSE-labeled OVA transgenic CD4$^+$ T cells in the presence of OVA$^{323-339}$ peptide (5000ng/ml, 500ng/ml, 50ng/ml and 5ng/ml) and GM-CSF (20 ng/ml). (A) Frequency of proliferated T cells in response to OVA peptide pulsing of C5aR1$^+$cDCs in the absence (left) or presence of a neutralizing C5aR1-specific mAb (20/70) (B) Frequency of proliferated T cells in response to OVA peptide pulsing of C5aR1$^-$cDCs in the absence (left) or presence of a neutralizing CD40L-specific mAb; values shown are the mean ± SEM; n= 6-10 per group. (A+B, left graphs) data were analyzed by ANOVA , followed by Tukey post-hoc test; *indicates significant differences between T cell co-cultures with C5aR1$^-$ (5000ng/ml VS 500ng/ml VS 50ng/ml VS 5ng/ml) or C5aR1$^+$cDCs (5000ng/ml VS 500ng/ml VS 50ng/ml VS 5ng/ml); ****p< 0.0001. (A+B, right graphs) data were analyzed with unpaired t test; *indicates significant

differences between T cell co-cultures with C5aR1$^-$ and C5aR1$^-$ +antiCD40L or C5aR1$^+$ and C5aR1$^+$ +antiC5aR1; ****p<0.0001.

When I pulsed both subsets with high OVA$^{323-339}$ concentrations (5000ng/ml and 500ng/ml), I noticed that C5aR1$^-$ and C5aR1$^+$cDCs induced robust T cell proliferation without any significant differences (Figure 27A, B). However, when the availability of the OVA$^{323-339}$ in the system was reduced (50ng/ml and 5ng/ml), I observed a gradual reduction in the percentage of proliferated CD4$^+$ T cells, which were co-cultured with the C5aR1$^+$cDCs (Figure 27A). The effect was most profound, when I used the lowest concentration. At this OVA peptide concentration (5 ng/ml) C5aR1$^+$ cDCs induced proliferation in about 20% of T cells, which is similar to what I had observed with C5aR1$^+$ cDCs from mice, immunized with HDM/OVA and pulsed with OVA *in vitro* (Figure 11). In case of C5aR1$^-$ cDCs, only the lowest tested concentration of OVA peptide (5 ng/ml) decreased T cell proliferation to 80%, which was similar to what I had observed when C5aR1$^+$ cDCs were loaded with 50ng/ml of OVA peptide (Figure 27 A vs B).
It has been shown (Rothoeft et al., 2006) that the activation of CD4$^+$ T cells requires both peptide presentation but also co-stimulation. My findings (sections 3.2.1 + 3.2.3 + 3.2.4) showing that the potency of C5aR1$^+$cDCs to efficiently drive T cell proliferation was enhanced through CD40 upregulation after C5aR1 targeting, which highlighted the vital role of the CD40-CD40L interactions for the activation of T cells. Thus, the obvious next step to corroborate these findings was to determine if: (i) anti-C5aR1-treatment of C5aR1$^+$cDCs pulsed with 5ng/ml of OVA$^{323-339}$ peptide would enhance T cell proliferation; and (ii) *in vitro* anti-CD40L-targeting of C5aR1$^-$cDCs pulsed with 5ng/ml of the OVA$^{323-339}$ would decrease T cell proliferation. As shown in Figures 27 A, B, C5aR1$^+$ cDCs clearly induced stronger T cell proliferation whereas C5aR1$^-$ cDCs markedly lost most of their potency to drive T cell proliferation.

My findings clearly support the view that low allergen peptide loading increases the importance of CD40/CD40L interaction as a critical factor for T cell proliferation and that C5a/C5aR1 axis activation in C5aR1$^+$ cDCs tips the balance in T cell activation through the control of CD40.

3.2.7 Impact of C5aR1 targeting on the transcriptional activity of C5aR1$^+$ cDCs to better understand the pathways by which C5aR1 activation controls the maturation and the activation of C5aR1$^+$ cDCs in response to HDM/OVA sensitization.

For this purpose, I decided to perform RNA sequencing of FACS-purified CD11b$^+$C5aR1$^+$cDCs, which had been pulsed with OVA and treated for 18h *in vitro* either with the neutralizing Ab against C5aR1 or with the appropriate isotype control.
I collected samples from two independent experiments, in which C5aR1$^+$ cDCs were treated with either the C5aR1-neutralizing mAb 20/70 or the appropriate isotype control antibody. These experiments were performed at CCHMC and the samples were sequenced at the DNA core of the University of Cincinnati (UC). The DNA core at the UC can work with lower amount of RNA in comparison to the DNA core from CCHMC. However, duo to the low number of C5aR1$^+$cDCs that can be isolated from one mouse, I had to pool 6 mice to reach the 100ngr of RNA yield that was required. The samples were sequenced as single end. The analysis was performed by Hauke Busch (Lübeck Institute of Experimental Dermatology; Systems Biology laboratory).

The samples were firstly analyzed by performing a pseudoalignment using Kallisto. The principal component analysis (PCA) showed that the treatment groups did not cluster together. The control experiment HS2, in which the cells were treated with the isotype control was very distinct from the others.

This lack of group clustering indicates that more experiments are required to compare the RNA profile of the two groups (Figure 28A).

In order to increase the number of the RNAseq experiments and better understand the impact of C5aR1 targeting on the transcriptional programming of C5aR1$^+$cDCs, I repeated the experiment, but this time the experiments were performed in Lübeck and the samples were sequenced by the DNA core of CCHMC. In more detail, I collected samples from three independent experiments. The experimental design was the same between the two experiments. Unfortunately, the RNA yield in five out of six samples was low. More precisely, in two of the samples, the RNA yield was in range that no libraries could be generated. This happened in two of the three isotype treated C5aR1$^+$ cDCs. Thus, only one sample from that group and three from the anti-C5aR1-treated-C5aR1$^+$cDC group were sequenced as paired ends. The analysis was performed once again by Hauke Busch.

The analysis of the results showed that the RNA expression pattern of the three anti-C5aR1-treated samples was very heterogenous and that the samples did not cluster together (Figure 28B). A possible explanation for the detected heterogeneity is that the samples did not have the same number of reads. As mentioned above, only one of the samples (KA5) had a high RNA yield and thus, resulted in a higher number of reads in comparison to the rest of the samples (Figure 28C). This effect is apparent in the PCA, with KA5 separating from the other samples (Figure 28C). Regarding the isotype-treated group of C5aR1$^+$cDCs (KA4), the PCA shows that it separates from the rest, however, since there are no replicates of that condition, the perception from that experiment can only be minor (Figure 28B).

On top of that the PCA compares the two RNAseq experiments (Figure 28B). The one performed exclusively in Cincinnati (samples HS1, HS2, HS3 and HS4) and the one which was partially performed in Lübeck (sample preparation) and in Cincinnati (RNA sequencing) (samples (KA1, KA3, KA4 and KA5). The PCA shows that there is a strong separation between these two experiments, indicating a strong batch effect, which might be due to the different library preparation, the different number of reads and/or the fact that the mice were kept in different animal facilities and different batches of FCS were used for the cell culture (Figure 28 B, C).

Since the data between the two experiments are not directly comparable, we did not perform any further comparisons and focused on the last experiment, which was performed in Lübeck.

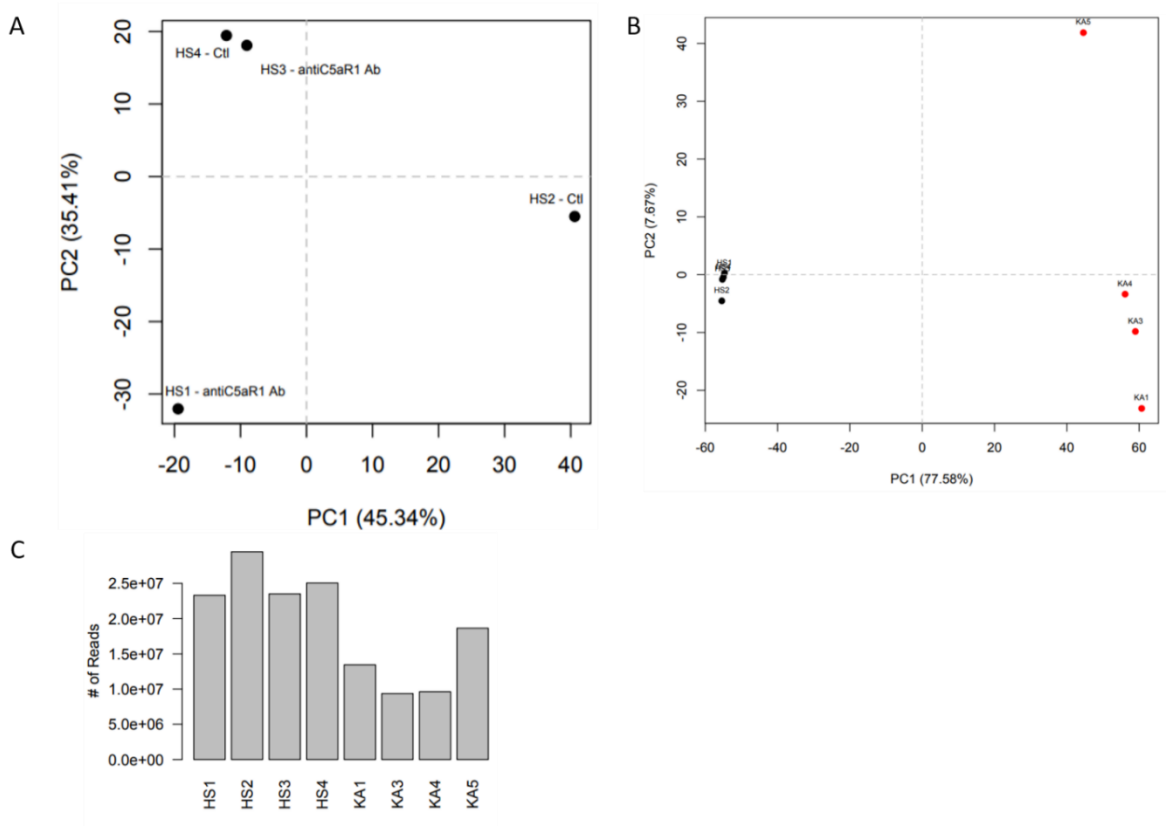
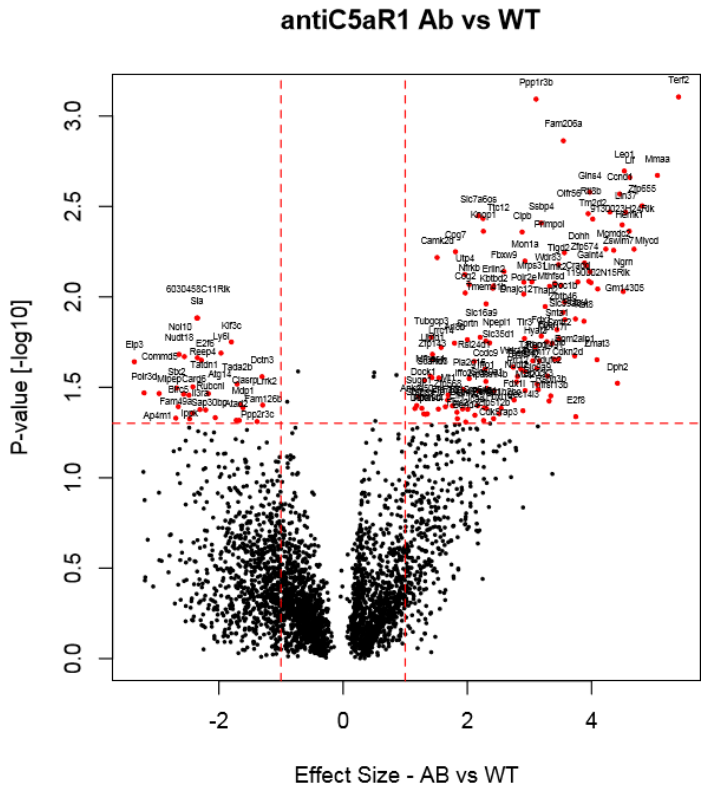


Figure 28: Quality assessment of the RNAseq data. (A) The principal component analysis shows similarities between samples HS1 (anti-C5aR1 treated mAb), HS2 (isotype-Ab treated), HS3 (anti-C5aR1 treated mAb) and HS4 (isotype-Ab treated). (B) The principal component analysis shows similarities between samples KA1 (anti-C5aR1 treated mAb), KA3 (anti-C5aR1 treated mAb), KA4 (isotype-Ab treated) and KA5 (anti-C5aR1 treated mAb). It also shows how dissimilar the two RNAseq experiments were. (C) Number of reads determined in samples HS1-4 and KA1,3,4 and 5.

As mentioned above, even though the RNA expression pattern of the three anti-C5aR1-treated samples was very heterogenous, we detected 40 genes which were found to be upregulated in all three samples when they were compared to the isotype treated control (KA4) (Figure 29B). The most strongly expressed genes were Tm2d2, Camk2d, Slc7a6os, Ppp1r3b, Ttc12, Fbxw9, Limk2 and Nfrkb. The expression levels of the aforementioned genes were 4 to 6-fold higher in the anti-C5aR1-treated samples in comparison to the isotype-treated control, in which the expression of some of the genes was zero.

We also identified the 40 genes which were strongly expressed in the isotype-treated sample (KA4) but not in the anti-C5aR1-treated samples (Figure 29C). These genes were mt-Nd3, Chchd1, Rps19bp1, Gm7334, Zfp385a, Zfp706, Sdr39u1, Auh and Gm8369. The expression levels of the aforementioned genes were 2 to 4-fold higher in the isotype-treated control in comparison to the anti-C5aR1-treated samples.

A


antiC5aR1 Ab vs WT
P-value [-log10]
0.0
0.5
1.0
1.5
2.0
2.5
3.0
-2
0
2
4
Effect Size - AB vs WT

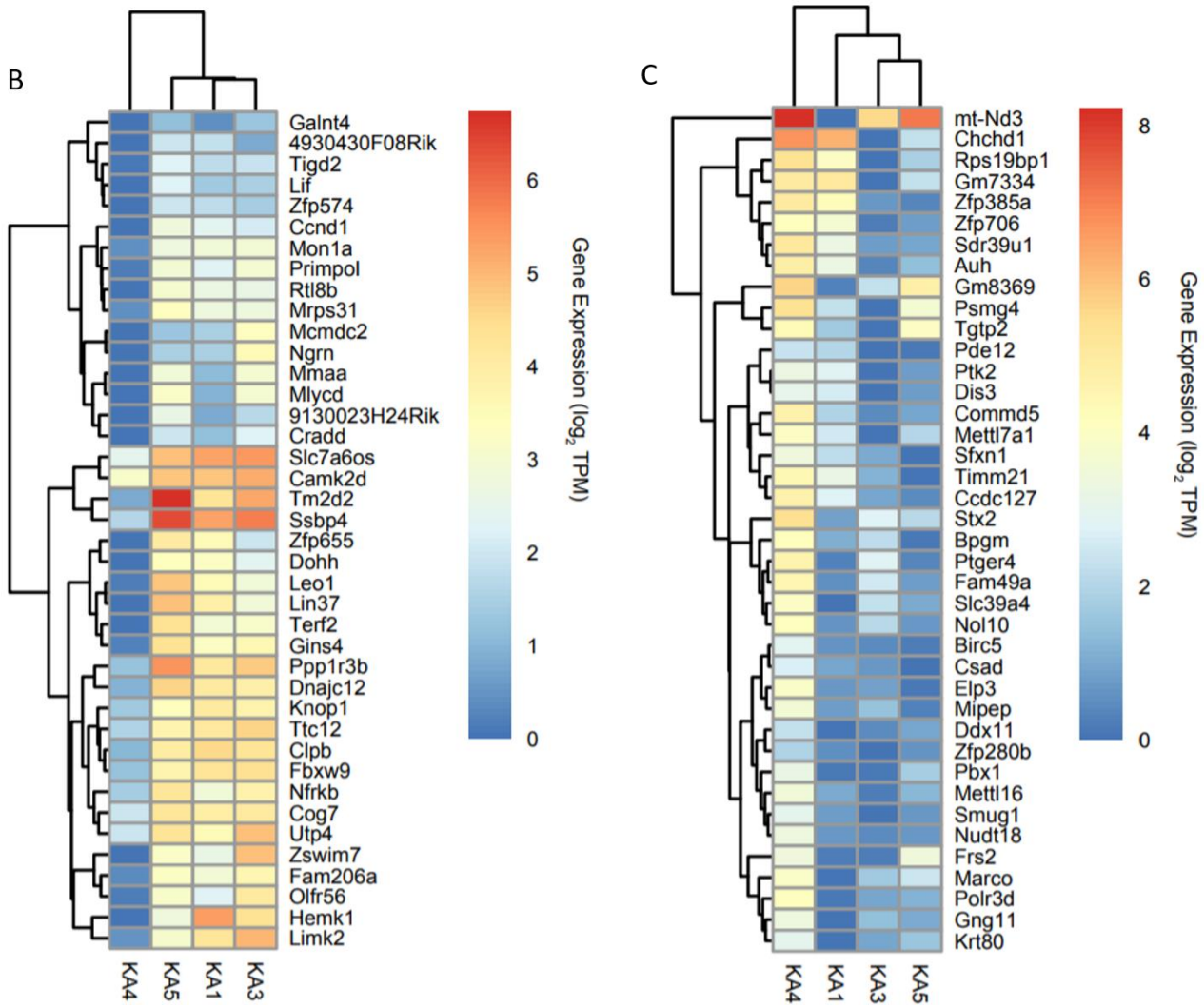


Figure 29: RNAseq analysis of antiC5aR1-treated-C5aR1^{+} and isotype-treated-C5aR1^{+}cDCs. WT mice were treated once with HDM/OVA (100 µg/40 µg) i.t. C5aR1^{+} cDCs from WT mice were FACS-sorted 24h after the sensitization. The cells were put in culture in the presence of OVA (10 µM) and GM-CSF (20 ng/ml). Half of the C5aR1^{+}cDCs were treated with 5µg/ml of a neutralizing Ab against C5aR1 mAb (20/70) (samples KA3, KA3 and KA5) and the other half with the appropriate isotype control (KA4). 18h later the samples were collected and were prepared for RNA sequencing. (A) Volcano plot for genes with a >2-fold change. (B)Expression of 40 genes (rows) which were strongly upregulated in the anti-C5aR1 treated samples in comparison to the isotype treated group of cells (columns). (C) Expression of 40 genes (rows) which were strongly upregulated in the isotype treated group of cells in comparison to the anti-C5aR1 treated samples (columns).

Interestingly, by comparing the RNAseq experiment which was described in section 3.1.9 with this one (samples KA1, KA3, KA4 and KA5) we could not find any matches. More precisely, there was no match between the genes which were found to be upregulated in the KA1, KA3 and KA5 samples with the genes which were got upregulated in the C5aR1^{-}cDCs (section 3.1.9, Figure 20A). The situation was similar when the KA4 sample was compared with the C5aR1^{+}cDCs. No matches were detected between these two samples (section 3.1.9, Figure20A).

4. Discussion

Allergic asthma is an inflammatory disease of the airways with associated morbidity, which is increasing in developed countries(Anandan et al., 2010). Several immune and non-immune cells are involved in the development of the disease(Wills-Karp, 1999)(Erle & Sheppard, 2014). Under steady state conditions, the lung harbors CD103$^+$ and CD11b$^+$ cDCs as well as pDCs. In addition to cDCs, CD11b$^+$ MHC-II$^+$ monocytes reside in the lung and differentiate into mo-DCs under inflammatory conditions. Both CD11b$^+$ cDC populations contribute to a mixture of Th2/Th17 development, whereas CD103$^+$ cDCs and pDCs promote tolerance(Watkins et al., 2005)(de Heer et al., 2004)(Zhang et al., 2009)(Furuhashi et al., 2012)(Plantinga et al., 2013)(Bell et al., 2013). In the present thesis, I showed that C5a/C5aR1 signaling axis controls CD4$^+$ T cell proliferation. Further, I found that C5a is made by both CD11b$^+$cDC subsets upon *ex vivo* OVA pulsing and acts in an autocrine way. Signaling through C5aR1 on CD11b$^+$cDCs resulted in minor T cell proliferation, which was significantly restored upon *in vitro* blocking of C5aR1. The *in vitro* targeting of C5aR1 was accompanied by upregulation of CD40, suggesting that C5aR1 is controlling the proliferation through CD40. However, my findings suggest that CD40 is necessary for T cell activation only when the levels of MHC-II are low or when the availability of MHC-II peptide loading is limited. In C5aR1$^-$ cDCs, which strongly express MHC-II, the *in vitro* blockade of CD40-CD40L interactions did not affect the ability of C5aR1$^-$cDCs to induce strong CD4$^+$ T cell proliferation and the situation only changed when I *in vitro* blocked the CD40-CD40L interactions. Finally, regarding the basic DC functions, like Ag uptake, processing and presentation, my current data suggest that they are C5aR1 independent.

4.1 Pulmonary CD11b$^+$ cDCs are a heterogeneous population based on the expression of C5aR1

Several studies exist, which investigated the role of C5aR1 in allergic asthma. Some of them focused on the sensitization phase and/or effector phase of the disease. Based on the hypothesis that C5aR1 controls the development of allergic asthma at the DC/T cell interphase, several reports focused on the role of C5a/C5aR1 axis activation during allergic sensitization. Previously, it has been shown that C5aR1 activation has a dual role regarding the development of allergic asthma. During the sensitization phase it protects from whereas in the effector phase it drives the development of allergic asthma. However, the mechanisms underlying this complex role are incompletely understood (Köhl et al., 2006). The Köhl lab already began to uncover the mechanism through which C5aR1 exerts a protective role during the sensitization phase. The current understanding is that C5aR1 controls maladaptive Th2/Th17 development by suppressing the frequency of pulmonary CD11b$^+$cDCs and increasing the frequency of pDCs(Zhang et al., 2009). It is still not known if the protective role of C5aR1 results from a direct regulatory effect on CD11b$^+$cDCs during primary allergen sensitization or only through the control of the frequencies between CD11b$^+$cDCs and pDCs. In addition to the modulation of the distinct cDC subsets, the functions of pDCs are altered by pulmonary C5aR1 blockade. After HDM exposure, pDCs express less of the co-stimulatory molecules PD-L1 and PD-L2, both of which regulate Th2 cytokine production from CD4$^+$ T cells (Schmudde et al., 2013)(Köhl et al., 2006)(Zhang et al., 2009).

Until recently, due to the lack of a reliable gating strategy to properly identify all cDC subsets, it was impossible to reveal the exact mechanism through which the C5a/C5aR1 signaling axis exerts its protective role during HDM sensitization. A few years ago, Plantinga et established a flow cytometry-based gating strategy, which allowed the identification of at least four different subsets of pulmonary DCs. It is noteworthy that this elegant study also showed that there is a division of labor among the various lung DCs(Plantinga et al., 2013). In the context of allergic asthma, pDCs are tolerogenic as, they induce Treg differentiation(Watkins et al., 2005)(de Heer et al., 2004) and act in trans by regulating the functions of cDCs during the crosstalk with naive T cells(Lewkowich et al., 2008)(Köhl et al., 2006) in a mechanism that

involves the regulation of B7 molecule expression(Zhang et al., 2009). According to Plantinga et al, CD103$^+$cDCs also have a tolerogenic role, while data both from Lambrecht and Vermaelen group, show that the primary role of mo-DCs under allergic asthma conditions is to produce cytokines and chemokines, like CCL24, CCL2, CCL4, CCL7, CCL9 and CCL12, which are important for activating and recruiting eosinophils and monocytes in response to allergen challenge(Plantinga et al., 2013)(Robays et al., 2007). CD11b$^+$CD64$^-$cDCs are identified as the main migratory subset, which drives a Th2/Th17 cell mediated immunity in the lymph nodes(Plantinga et al., 2013). I took advantage of this gating strategy and identified four cDC subpopulations in the lung. Using CD103 and CD11b as markers, I identified three cDC subsets; i.e. CD103$^+$CD11b$^-$cDCs, CD103$^-$CD11b$^-$cDCs and CD103$^-$CD11b$^+$cDCs. However, as already mentioned CD103$^-$CD11b$^+$cDCs are not a homogeneous population as they include CD11b$^+$cDCs and mo-DCs. To discriminate these two subsets, I used CD64 as a marker, which is typically expressed by mo-DCs. Thus, I further subdivided the CD103$^-$CD11b$^+$ cDCs into CD11b$^+$CD64$^-$ cDCs and CD11b$^+$CD64$^+$ mo-DCs. Different studies confirm the differentiation of the different pulmonary DC subsets although the absolute cell numbers vary as different identification strategies and different cell isolation protocols have been used (Plantinga et al., 2013)(Nakano et al., 2012)(Hoffmann et al., 2016).

According to Plantinga et al., the CD11b$^+$CD64$^-$cDCs were the main players for the development of allergic asthma, as independently of the Ag dose, they were the dominant cell type that took up antigen and migrated to the draining lymph nodes to activate naïve CD4$^+$ T cells. Using a GFP C5aR1 knock-in mouse, Karsten et al. showed that the pulmonary CD11b$^+$ cDCs express C5aR1(Karsten et al., 2015). Altogether, these data made me hypothesize that the protective role of C5aR1 during HDM sensitization(Köhl et al., 2006) was due to direct signaling of C5aR1 on the CD11b$^+$ cDCs. To test this hypothesis, I firstly checked the expression of C5aR1 by pulmonary CD11b$^+$cDCs, both under steady-state conditions and after one-time HDM/OVA sensitization. Strikingly, I noticed that the CD11b$^+$CD64$^-$cDC population is heterogeneous. Under steady-state conditions, the majority of the CD11b$^+$CD64$^-$cDCs (85%) were C5aR1$^+$, and only a minor fraction of the cells was C5aR1$^-$ (15%). This distribution slightly changed in the sensitized CD11b$^+$CD64$^-$ cDCs. Here, the frequency of the C5aR1$^+$cDCs decreased to 75% and the frequency of C5aR1$^-$cDCs increased to 25%. My data clearly showed that the discrimination of cDCs from other cells of the mononuclear phagocyte system is challenging due to the shared expression of several surface markers.

The mechanisms driving the increased frequency of the C5aR1$^-$cDCs upon HDM/OVA sensitization is still unclear. I consider the following scenarios. First, a massive proliferation of the naïve C5aR1$^-$cDCs after HDM exposure may occur. However, in this case, an extensive proliferation of C5aR1$^-$cDCs would be required, which is an unlike event as DCs are not known to heavily proliferate(Steinman & Nussenzweig, 1980). Under inflammatory conditions, DC can be mobilized and migrate into the inflamed tissue. Based on this notion, the second scenario is that the increased frequency of C5aR1$^-$cDCs might be due to cell recruitment to the lungs upon HDM/OVA sensitization through CCR2(Plantinga et al., 2013). This possibility could be evaluated by either using CCR2$^{-/-}$ mice or *in vivo* blocking CCR2. Fanny Ender, a former PhD student in the Köhl lab, performed that experiment. What she did was to use a selective CCR2 antagonist to block the interaction of CCL2 with its cognate receptor CCR2 prior to the exposure of mice to HDM. Upon *in vivo* CCR2 blockade she observed a minor decrease in the number of CD103$^-$CD11b$^+$cDCs in the lung, which did not reach statistical significance. However, this experiment cannot exclude the possibility that the second scenario holds true, as Fanny Ender did not specifically check if the ratio of C5aR1$^-$ versus C5aR1$^+$ CD11b$^+$cDCs was affected by the CCR2 blockade. She only commented on the cell numbers of the CD103$^-$CD11b$^+$cDCs. On top of that, the n number (n=3) was very low and considering the

variation among the cell numbers of the three used mice, it is hard to draw any conclusions about the role of CCR2 in the recruitment of C5aR1$^-$cDCs to the lungs. The third and last scenario deals with the possibility that naïve C5aR1$^+$cDCs downregulate the expression of the C5aR1 under inflammatory conditions. The investigation of this hypothesis is more demanding. Ideally, a C5aR1 fate-mapping mouse would be needed, which would allow to track the expression or silencing of C5aR1 by the CD11b$^+$cDCs during the development of the disease. Unfortunately, for the present thesis, the use of such a mouse was not feasible, but it would be a handy tool for future experiments.

4.2 The CD11b$^+$C5aR1$^+$ cDCs have a significantly lower potency to prime CD4$^+$ T cell responses in comparison to the CD11b$^+$C5aR1$^-$ cDCs

My finding that CD11b$^+$cDCs expressed C5aR1 was in line with observations from Karsten et al.(Karsten et al., 2015) and my hypothesis. The identification of a second CD11b$^+$cDC subpopulation which was C5aR1$^-$ was somewhat unexpected, especially because Plantinga et al. considered CD11b$^+$CD64$^-$cDCs as a homogeneous population(Plantinga et al., 2013). I next decided to functionally characterize these two subsets. First, I evaluated the potential of the two subsets to induced proliferation of naïve CD4$^+$ T cells. My data showed that the C5aR1$^+$cDCs had a very low potency at triggering CD4$^+$ T cell proliferation in comparison to the C5aR1$^-$cDCs, which were very potent. Plantinga et al. claimed that the CD11b$^+$cDCs, treated as one population, was the most potent pulmonary cDC population at inducing efficient T cell proliferation(Plantinga et al., 2013). It was surprising to see that the efficiency at priming T cell responses which was attributed from Plantinga et al. to the CD11b$^+$CD64$^-$cDCs was mainly driven by the 25% of the whole CD11b$^+$ cDC population.

4.3 The C5aR1$^+$cDCs are less mature in comparison to the C5aR1$^-$cDCs and this is accompanied by a lower number of interactions between the C5aR1$^+$cDCs and CD4$^+$ T cells

Once I observed this substantial difference between the two CD11b$^+$cDC subsets to activate T cells, I decided to setup experiments aimed to delineate the mechanisms underlying these functional differences. For successful T cell proliferation, T cells interact with cDCs in a highly dynamic environment in secondary lymphatic tissues such as lymph nodes, where they require to achieve a level of TCR stimulation sufficient to drive their activation. The signals that lead to T-cell activation are generated at the level of the immunological synapse, a specialized area of contact between T cells and APCs. At the synapse, the TCRs are sequentially triggered by peptide–MHC complexes, a process that allows the signal to be sustained for as long as the synapse is in place. Synapses are stable in the absence of disturbing influences, but they can be disrupted by cell division, by the death of APCs or by external influences, such as collagen or chemokines. T cells continuously search for antigen and can rapidly shift from one APC to another offering a higher level of stimulation. While the duration of TCR stimulation depends on the synapse, the intensity of the signal that T cells receive is dependent both on the level of peptide–MHC complexes and the level of costimulatory molecules that amplify the signaling process(Rothoeft et al., 2006)(Boisvert et al., 2014)(Grakoui et al., 2017)(Wetzel et al., 2014). Thus, considering the great importance of the immunological synapse for an efficient T cell proliferation, I speculated that the compromised ability of the C5aR1$^+$cDCs to efficiently activate CD4$^+$ T cells would be due to decreased expression levels of MHC-II and/or co-stimulatory molecules. Indeed, my data showed that the C5aR1$^+$cDCs expressed significantly lower levels of both MHC-II and CD40. The lower expression levels of these two molecules could explain the lower potency of the C5aR1$^+$cDCs to drive efficient CD4$^+$ T cell proliferation. It is well appreciated that the maturation status of DCs is vital to ensure strong T cell interactions and it has a critical impact on the initiation of the response. Benvenuti et al. showed that

immature DCs fail to activate T cells and only when the DCs are pulsed with very high concentrations of peptide, they managed to partially activate T cells (Benvenuti et al., 2014). Clearly, C5aR1$^+$cDCs were not completely immature as they upregulated the expression levels of MHC-II and CD40 24h after HDM sensitization, however, not to the extent that resulted in strong immunological synapse formation. My data rather suggest that they were in a partially mature status, which allowed some T cell proliferation in contrast to the immature DCs used in the study by Benvenuti et al. (Benvenuti et al., 2014). To further strengthen the data, I decided to track the interactions between C5aR1$^+$cDCs – CD4$^+$ T cells and C5aR1$^-$ cDCs – CD4$^+$ T cells *in vitro*. For the purpose, the DCs were labeled with PKH26, a red fluorescent dye and the T cells with CFSE. I tracked the interactions of the cells for 5h using a confocal microscope taking one picture per minute. From the first minutes of interactions between the C5aR1$^+$cDCs – CD4$^+$ T cells and the C5aR1$^-$cDCs – CD4$^+$ T cells, I noticed a higher number of interactions for the C5aR1$^-$cDCs, which was more profound after the first 2h of the co-culture. I did not expect any differences in the frequency of interactions between the C5aR1$^-$ and C5aR1$^+$cDCs during the first 2h of the co-culture. As mentioned, once naïve CD4$^+$ T cells are co-cultured with DCs, they start to explore their environment and make loose contacts with DCs to see whether they find an MHC-II molecule loaded with the appropriate peptide. Thus, I expected to see no differences at this stage of DC/T cell interaction. However, Benvenuti et al. demonstrated during the first hour of DC - T cell interaction that the adhesions of T cells to immature DCs was significantly lower as compared to mature DCs. Clearly, the C5aR1$^+$cDCs are less mature than the C5aR1$^-$cDCs. Benvenuti et al. pulsed the immature DCs with a very high amount of a peptide, which could trigger T cell proliferation and compared this treatment with unpulsed DCs. Looking at the percentage of T cells establishing contacts with mature DCs, they found that 50% or 65% of the T cells established long contacts with mature DCs in the absence and the presence of peptide. For immature DCs, the frequency decreased to 10 or 20% in the absence or presence of peptide. These data demonstrate that the maturation status and the peptide loading determine the interaction of T cells with DCs. Another finding that may explain why I observed a lower number of interactions between the C5aR1$^+$cDCs – CD4$^+$ T cells in comparison to the C5aR1$^-$cDCs – CD4$^+$ T cells was the short duration of the interaction in case of the former group of cells. More precisely, Benvenuti et al. observed that immature DCs are mainly forming short contacts in the range of 10-100s. Considering that I took one picture every 60s, it is highly likely that I missed some of the interactions between the C5aR1$^+$cDCs – CD4$^+$ T cells, which did last less than 60s(Benvenuti et al., 2014).

4.4 High levels of TSLPR expression in C5aR1$^-$cDCs in comparison to C5aR1$^+$cDCs may account for their stronger maturation status

The next set of experiments was designed to shed light on the mechanisms underlying the low maturation status of the C5aR1$^+$cDCs. As already mentioned, ECs release several inflammatory cytokines, so-called alarmins, upon HDM sensitization, which can activate pulmonary DCs (Zhou et al., 2005)(Roan et al., 2012). Thus, I explored the possibility that the C5aR1$^+$cDCs lacked or showed reduced expression levels of alarmins receptors and consequently, were less sensitive to maturation signals provided by HDM-activated ECs. I found that among the different tested receptors, TSLPR was significantly upregulated in both CD11b$^+$cDC subsets upon HDM sensitization. However, already under naïve conditions the C5aR1$^-$ cDCs expressed significantly higher levels of TSLPR in comparison to the C5aR1$^+$cDCs. Even though both subpopulations, upregulated TSLPR, the C5aR1$^-$cDCs did that to a greater extent, and its expression was even higher under inflammatory conditions. This finding was of utmost importance considering the pivotal role of the TSLP/TSLPR signaling on the induction of maturation to CD11b$^+$cDCs. Bell et al., previously showed that TSLPR through its downstream target, the transcription factor STAT5 controls a genetic

program in CD11b$^+$ cDCs that drives CD4$^+$ T cell activation and Th2 differentiation(Bell et al., 2013). In DCs, it up-regulates co-stimulatory molecules and chemokines that help T cell activation and Th2 differentiation. The vital role of TSLPR on induction of DC maturation was shown in a study performed by Zhou et al. They showed that bone marrow-derived dendritic cells (BM-DCs) upregulated the expression levels of MHC-II and CD40 when cultured in the presence of TSLP(Zhou et al., 2005). Taken together, my findings suggest that C5aR1$^+$ cDCs are less sensitive to HDM-driven upregulation of TLSPR in response to alarmin release from EC. At this point, it remains to be explored whether C5aR1 activation during HDM sensitization *in vivo* directly suppresses TSLPR upregulation in an autocrine fashion or whether C5aR1 activation acts in trans on other lung cells that control TSLPR expression. Whatever the mechanism might be, my findings clearly provide a rationale for the decreased maturation status of C5aR1$^+$CD11b$^+$ cDCs.

4.5 C5aR1$^+$ and C5aR1$^-$ CD11b$^+$ cDCs drive a mixed Th2/Th17 response

TSLPR controls T cell differentiation and favors Th2 differentiation(Kitajima & Ziegler, 2013)(Bell et al., 2013). When I determined the nature of Th response elicited by C5aR1$^+$ and C5aR1$^-$cDCs, I noticed that they drove a mixed Th2/Th17 response. This finding was in line with what others found before, when assessing the nature of the Th response elicited by pulmonary DCs (Plantinga et al., 2013) or BM-DCs(Schmudde et al., 2013). TSLP has been shown to induced Th2 commitment as shown by Kitajima et al. (Kitajima & Ziegler, 2013). Skin TSLPR$^-$/$^-$ CD11b$^+$cDCs failed to activate T cells and mount a Th2 response. They also showed that TSLP upregulated CCR7 and promoted their migration to the lymph nodes(Kitajima & Ziegler, 2013). According to Bajana et al., CCR7 is not exclusively regulated by TSLP but by IRF4 as well(Bajana et al., 2012). They showed that upon induction of skin inflammation, CD11b$^+$ dermal DCs in IRF4$^-$/$^-$ mice did not express the chemokine receptor CCR7 and failed to migrate to cutaneous lymph nodes(Bajana et al., 2012). Loss of the transcription factor STAT5 in DCs resulted in their inability to respond to TSLP. Th2 responses in mice with DC-specific loss of STAT5 resembled those seen in mice deficient in the receptor for TSLP. Bell et al. showed that the TSLP-STAT5 axis in DCs is a critical component for the promotion of type 2 immunity at barrier surfaces(Bell et al., 2013). Another transcription factor with a vital role in the licensing of CD4$^+$ T cells is IRF4 (according to the first RNAseq experiment, section 3.1.9, the expression of irf4 is 2-fold higher in the C5aR1$^-$cDCs in comparison to the C5aR1$^+$cDCs, see supplementary). Even though it was known that IRF4 favors the Th2 response(Williams et al., 2013)(Gao et al., 2013), Schlitzer et al. showed that IRF4 also controls the production of IL-23 and therefore the induction of Th17 responses in mice and humans. They could confirm the need of IRF4 for Th17 responses both under steady-state conditions and in an Aspergillus fumigatus model (Schlitzer et al., 2013). Besides these transcription factors, there is also strong evidence that complement and more precisely C5aR1 has a regulatory role on the elicitation of a mixed Th2/17 response upon allergic inflammation. In a very elegant study, Schmudde et al. showed that OVA-pulsed BM-DCs from C5aR1$^-$/$^-$ mice produced similar levels of Th2 cytokines as BM-DCs from WT mice. This finding is in agreement with my data, as I noticed that both C5aR1$^-$ and C5aR1$^+$ cDCs produced similar levels of IL-13. By contrast, Schmudde et al. showed that IL-17A production was significantly reduced in C5aR$^-$/$^-$ BM-DC co-cultures. My data confirmed this observation, as the percentages of CD4$^+$ T cells that were co-cultured with C5aR1$^-$cDCs produced significantly lower levels of IL-17A in comparison to the CD4$^+$ T cells co-cultured with the C5aR1$^+$cDCs(Schmudde et al., 2013).

4.6 Autocrine C5 and C5a generation in C5aR1$^+$ and C5aR1$^-$ cDCs but not in CD4$^+$ T cell after *ex vivo* exposure to OVA

The substantial difference in DC maturation between C5aR1$^+$ and C5aR1$^-$ cDCs, associated with distinct potencies to drive T cell proliferation and differentiation raised the question about the importance of autocrine or paracrine C5aR1 activation during DC/T cell interaction. Thus, I checked Intracellular expression of C5a and observed that both CD11b$^+$cDC subsets started making C5a upon *ex vivo* OVA-pulsing. Interestingly, the CD4$^+$ T cells could not make any C5a, demonstrating that C5a generated by the CD11b$^+$cDCs acted in an autocrine way. The C5a production by the CD11b$^+$cDCs was in line with findings from others who showed that BM-derived APCs produced C3a and C5a during the cognate APC/T cell interaction(Lalli et al., 2008)(Strainic et al., 2008). The main differences between my data and these two studies are that: first, I used primary cDCs from the lung; second, the C5a was DC- and not T cell-derived and third, the C5a produced by CD11b$^+$cDCs could activate only C5aR1 on DC, as I found no C5aR1 expression by CD4$^+$ T cells confirming the results obtained by the Köhl lab using a GFP-C5aR1 knock-in mouse (Karsten et al., 2015). More precisely, I showed that on day 0, i.e., when cells were sorted after 1 step HDM/OVA sensitization, no C5a expression occurred, suggesting that at this time point the *in vivo* source of C5a could be either the alveolar macrophages or epithelial cells(Huber-Lang et al., 2002)(Morgan & Gasque, 1997). As shown, the C5aR1$^-$cDCs expressed higher levels of C5a in comparison to the C5aR1$^+$cDCs. This difference could either result from higher levels of C5 expression in C5aR1$^-$cDCs or stronger expression of serine proteases which lead to more efficient cleavage of C5 into C5a in comparison to the C5aR1$^+$cDCs. Another explanation could be that in case of C5aR1$^+$cDCs some of the C5a was bound to C5aR1 thereby masking the epitope of the detection Ab. Indeed, on day 0, the C5aR1$^-$cDCs expressed significantly higher levels of C5 in comparison to the C5aR1$^+$cDCs, suggesting that stronger C5 expression accounts for the higher expression levels of C5a. However, this does not exclude the possibility that the C5aR1$^-$cDCs also express higher levels of serine proteases. I also assessed the potential impact CD4$^+$ T cells on C5a generation as we found increased C5 and C5a levels after DC/T cell co-culture. Thus, I determined the C5a expression levels on day 2, when DCs and T cells had interacted for 24h hours. In a second setup, I did not add any T cells to the system. Thus, I evaluated the C5a production when DCs were *in vitro* pulsed with OVA for two days. In the case of the C5aR1$^-$cDCs the presence or absence of the CD4$^+$ T cells in the co-culture system did not affect the C5a expression levels suggesting that the allergen and not the T cells are the main driver. However, the situation was slightly different regarding the C5aR1$^+$cDCs. Upon CD4$^+$ T cell addition, the levels of C5a decreased significantly. However, due to the high inter-assay variation of the C5a levels, the findings require additional confirmation in future experiments. It should also be stated here, that for all cell culture conditions the RPMI medium was supplemented with FCS. Even though there are reports for the presence of C5a in the serum, we can exclude the possibility that the C5a was from calf origin, firstly because the Ab which was used for the detection of C5a was mouse-specific. Secondly, on day 2 of co-culture both CD11b$^+$cDCs made more C5 and C5a than on day 1 suggesting *de novo* synthesis in the pulmonary cDCs.

4.7 C5aR1$^+$cDCs and C5aR1$^-$cDCs express a distinct pattern of genes

So far, many differences between the C5aR1$^-$ and C5aR1$^+$ cDCs had been noticed, both phenotypically and functionally. To go one step further, I decided to perform an RNAseq experiment and compare the transcription profile of the two cell populations. The RNAseq data confirmed our initial observations that these two CD11b$^+$cDC subsets are two distinct populations which do not cluster together. On the C5aR1$^-$ cDC subset, we detected high expression levels of Snora21, Snora52, Snora64, clec10A, cd209, ccl22,

ccl17, zbtb46 and Cd7. These genes were either not expressed at all by the C5aR1$^+$cDCs or their expression was considerably lower. I picked two genes, which were very highly expressed by the C5aR1$^-$cDCs, i.e. clec10A, and cd209 and checked whether they were also expressed at the protein level. Concerning the expression of CD209, a DC-specific C-type lectin, with a strong adhesion function, the cells which expressed CD209 were almost exclusively C5aR1$^-$, whereas the CD209$^-$ cells were C5aR1$^+$. This finding could explain the lower potency of the C5aR1$^+$cDCs to efficiently drive CD4$^+$ T cell proliferation. It is known that the initial interaction of T cells with DCs is Ag-independent and allows scanning of the peptide-MHC class II complex repertoire by the TCR. The abundance of appropriate MHC-peptide complexes is low to mediate significant adhesion by itself, and therefore adhesion molecules are essential for an efficient TCR engagement. One of these molecules is ICAM-3, which is recruited in the contact region of APC with T cells. Geijtenbeek et al. showed that CD209 is the counter-receptor for ICAM-3 and the CD209-ICAM-3 contact stabilizes intimate DC-T cell membrane contact transiently to enable efficient TCR engagement(Geijtenbeek et al., 2000). The situation was slightly different regarding the expression of CLEC10A at the protein level. CLEC10A is also a C-type lectin receptor that is known as macrophage galactose-type-C-type lectin, and in mice, it has two homologs CD301a (MGL1) and CD301b (MGL2), while in humans and rats there is only one homolog. CD301a is mainly expressed on a subset of macrophages and immature DCs and CD301b on cDCs. When I checked for its expression within the CD11b$^+$CD64$^-$cDCs, I found three subpopulations. Only the SSC^{high}CLEC10A$^-$ were exclusively C5aR1$^+$, while the other two subsets, SSC^{low}CLEC10A$^-$ and SSC^{low}CLEC10A$^+$ consisted of C5aR1$^+$ and C5aR1$^-$ cells. The inability to identify a population, which was CLEC10A$^+$C5aR1$^-$ (as suggested from the RNAseq data) might be due to the use of an Ab clone that recognizes both homologs and thus cannot discriminate cDCs from the macrophage/immature DC subsets. In future, this experiment should be repeated using an Ab specific for either CD301a or CD301b. Transient depletion of CD301b$^+$ DCs in response to subcutaneous injection of OVA along with papain or alum was associated with less effective accumulation and decreased expression of CD69 by polyclonal CD4$^+$ T cells in the lymph node. Further, the authors observed decreased IL-4 production by OVA-specific OT- II transgenic CD4$^+$ T cells and significantly impaired Th2 cell development upon infection with Nippostrongylus brasiliensis. These results identified CD301b$^+$ dermal DCs as the key mediators of Th2 immunity (Kumamoto et al., 2014). Currently, the exact impact of CD301b on pulmonary cDCs is unknown, but the data from the Kumamoto study along with my data suggest that it might regulate T cell activation in the lungs as well.

Importantly, the RNAseq data further confirmed the hypothesis that the pulmonary CD11b$^+$CD64$^-$cDCs are not a homogeneous population. I also demonstrated this by the distinct expression of the 4 different surface markers CD24, CD209, CD301, and C5aR1 in CD11b$^+$ cDCs. This finding highlighted the diversity of this cDC population suggesting that additional molecules may exist that define even more CD11b$^+$ cDC subpopulations. Even though the gating strategy established by Plantinga et al. was better as compared to what had been used in the past, it neglects the diversity of cDC subsets which may exert distinct functions regarding the initiation of maladaptive immunity(Plantinga et al., 2013). Many scientists in the DC field tried other ways to better discriminate cDCs from macrophages or monocytes. In a very elegant study, Satpathy et al. identified the transcription factor zbtb46 as an additional marker to properly discriminating cDCs from cells of the mononuclear phagocyte system. They checked for the expression of zbtb46 within the pulmonary cDCs using the gating strategy established by Plantinga et al. In our hands this gating strategy under steady state conditions reveals that the CD103$^-$CD11b$^+$cDCs are a heterogeneous population regarding the expression of CD64. However, by using CD64 as a marker, we ended up with almost 93-95% of the cells being CD11b$^+$CD64$^-$ and 5-7% being CD11b$^+$CD64$^+$cDCs. When

Satpathy et al. checked for the expression of zbzt46 within the pulmonary CD103$^-$CD11b$^+$cDCs under steady-state conditions, they noticed that only half of the cells were zbzt46$^+$, suggesting that 50% of the cells were not bona fide cDCs. Even though I did not find the same 50% ratio of cDCs versus non cDCs when I used C5aR1 as a maker, these findings indicate that CD64 is a poor marker to discriminate bona fide cDCs and non cDCs. My data further support the findings by Satpathy as the RNAseq data obtained with C5aR1$^-$ and C5aR1$^+$cDCs showed that only the former population of cells expressed high levels of zbtb46 while the latter did not express zbtb46 at all. Since Satpathy et al., showed that zbtb46 is an exclusive marker for cDCs, C5aR1$^+$cDCs may belong to the monocyte/macrophage lineage (Satpathy et al., 2012). This finding could explain their poor potency to activate naïve CD4$^+$ T cells. Neither macrophages nor monocytes are very potent in activating naïve T cells. The RNAseq experiment revealed two more genes, ccl17 and ccl22 whose expression was almost 3-fold higher in the C5aR1$^-$cDCs in comparison to the C5aR1$^+$cDCs. CC chemokine ligand 17 (CCL17), also known as thymus and activation-regulated chemokine (TARC), and CCL22, also known as macrophage-derived chemokine (MDC), both are ligands for CC chemokine receptor 4 (CCR4), preferentially expressed on Th2 polarized effector cells. Normally these chemokines are secreted by myeloid DCs but not pDCs, and they regulate the pulmonary homing of Th2 cells (Facchetti et al., 2002). The Köhl lab previously found high concentrations of CCL17 and CCL22 in CD11c$^+$ DC/CD4$^+$ lymphocyte co-cultures of anti-C5aR1–treated mice after initial and repeated allergen exposure and in C5aR1$^-$/$^-$ mice. The high CCL17/CCL22 concentrations resulted in the recruitment of high numbers of Th2 effector cells, which were responsible for the elevated levels of Th2 cytokines that were observed upon C5aR1 absence. The data from that study suggested that C5a regulated homing of Th2 cells through negative regulation of CCL17/CCL22 production from pulmonary myeloid DCs(Köhl et al., 2006). My findings are in agreement with these previous observations, as CCl17 and CCl22 were significantly higher expressed in C5aR1$^-$cDCs than in C5aR1$^+$cDCs, probably because of a negative feedback loop comprising C5a/C5aR1 activation in C5aR1$^+$ cDCs. Surprisingly, the RNAseq experiment pointed towards CD7, which was highly expressed by the C5aR1$^-$but not C5aR1$^+$ cDCs. CD7 is expressed by thymocytes or mature T lymphocytes. Thus, we did not expect to detect a strong expression of that molecule in a group of pulmonary CD11b$^+$cDCs(Subrahmanyam et al., 2003). However, a novel population of human cord blood cells which is CD45RA$^+$CD7$^+$ has been identified as progenitors of NK/DC cells. Thus, this finding could indicate that the C5aR1$^-$cDCs could serve as a progenitor population for the generation of mature DCs(Canque et al., 2000). In future studies the expression of CD7 at the protein level should be tested, to confirm the RNAseq data before further exploring the role of CD7 in pulmonary CD11b$^+$cDCs. Lastly, high expression levels of three small nucleolar RNAs (snora21, snora52 and snora64) were detected in the C5aR1$^-$cDC group of cells, while their expression was almost zero for the C5aR1$^+$cDCs. The small nucleolar RNAs represent a group of noncoding RNAs (snoRNAs) with diverse functions. The past ten years, research in the field of snoRNAs revealed several novel and unexpected functions for noncoding RNAs. Some snoRNAs play essential roles in the nucleolytic processing of rRNAs, but the majority of them function as guide RNAs in the post-transcriptional synthesis of 2'-O-methylated nucleotides and pseudo uridines in rRNAs, small nuclear RNAs (snRNAs) and probably other cellular RNAs, including even mRNAs(Kiss, 2002). There is data that several snoRNAs have a role in various cancers and different snoRNAs have a role in the initiation, proliferation, tumor growth, the cell cycle, apoptosis and metastasis. The dysregulated snoRNAs in certain cancers are projected to become novel biomarkers for diagnosis or the evaluation of therapeutic efficacy. Among these snoRNAs, upregulated expression in cancer is considered to have an oncogenic function, while downregulated expression may exert tumor suppressive effects(Baral et al., 2018). Unfortunately, so far, the biological significance of snoRNAs has

not been studied as extensively as in oncology. Thus, we still cannot predict if the expression of these three snoRNAs by the C5aR1$^-$cDCs could have an impact on their ability to promote T cell proliferation. However, what could be done in the future and would probably be informative, is to check for the expression of these three snoRNAs both under naïve conditions and at a later time point during the C5aR1$^-$ cDC – T cell co-culture, to firstly see if their expression changes over time and, if so, to also compare their expression levels at these time points with the C5aR1$^+$cDCs.

By focusing on the C5aR1$^+$cDCs, I detected high expression levels of Mir682, Mir703, Snora28, Arg1, ccl12, C1qa, C1qc, C1qb and s100a8. The expression levels of all nine genes were almost zero in the C5aR1$^-$cDCs with the exception of C1qa. This gene was moderately expressed by the C5aR1$^-$cDCs, but still its expression levels were 2-fold lower than its expression levels in the C5aR1$^+$cDCs. M2 macrophages typically express most of these markers (Arg1, C1qa, C1qc, C1qb and ccl12), which were found to be strongly expressed in the C5aR1$^+$ cDC group of cells. More precisely, macrophages are heterogeneous and undergo various phenotypic changes in response to microenvironmental stimuli. The M1 and M2 types are best known. Among other markers, type-I arginase (Arg-I), the complement component C1q, CCL12 or monocyte-chemoattractant-protein 5 (MCP-5) are associated with the M2 phenotype, and widely used as the markers for characterization of the two macrophage phenotypes(Munder et al., 1999)(Bronte & Zanovello, 2005)(Sarafi et al., 1997)(Castellano et al., 2008)(Mascarell et al., 2017)(Castellano et al., 2010)(Spivia et al., 2014). The complement component C1q which is known to act as a pattern recognition receptor is involved in apoptotic cell clearance and polarizes macrophages towards the M2 anti-inflammatory phenotype. During the apoptotic cell clearance, C1q dampens M1, and this may be at least partially responsible for the autoimmune phenotypes of both human and mice genetically deficient in C1q. However, C1q is not exclusively made neither by macrophages nor exerts its anti-inflammatory effects through these cells(Spivia et al., 2014)(Schlitzer et al., 2015). More precisely, while at an immature state, DCs produce C1q, which is functionally active in complement activation and can bind to apoptotic cells. However, upon DC maturation, C1q is immediately downregulated *in vitro*. Thus, immature DCs, cells with tolerogenic properties are a rich source of C1q both *in vivo* and *in vitro* but they downregulate C1q on maturation(Castellano et al., 2008). According to Mascarell et al., C1q has a regulatory role in an experimental allergic asthma OVA model, as treatment with C1q led to downregulation of AHR, eosinophilia, and ILC2 infiltrates in bronchoalveolar lavages, as well as allergen-specific Th2 cells in the lungs. In that model, C1q exerted its anti-inflammatory properties through pDCs(Mascarell et al., 2017). However, the C5aR1$^+$ cDCs examined in the present study are partially mature cells and thus, if these cells were cDCs, it is surprising that they highly express C1qa, C1qb, and C1qc of the C1q molecule. So, even though the expression of C1q and its regulatory roles have also been described in DCs, none of the observations apply to the present study. My RNAseq data indicate that the Ca5R1$^+$cDCs are not cDCs but macrophages. Of course, these findings are no proof that the C5aR1$^+$cDCs are indeed M2 macrophages and not immature DCs or myeloid derived suppressor cells (MDSCs). Clearly, several studies also report the production of Arg1 and C1q by immature DCs(Munder et al., 1999). Arg1 is an enzyme of the urea cycle and converts L-arginine into L-ornithine and urea. It is produced by MDSCs and M2 macrophages and is also used as a marker to identify the latter. However, Munder et al. showed that macrophages were not the exclusive producers of Arg1, but DCs can also make it upon culture with IL-4 and IL-10(Munder et al., 1999). It is very challenging to compare findings from different groups as they use of different markers or sometimes even the same markers, but some consider a marker typical for macrophages when others consider them specific markers for DCs. Due to the low numbers of tissue DCs, several studies have used BM-DCs. Many of these findings need to be interpreted with caution, as a seminar paper by Helft et al.

showed that the great majority (almost 75%) of the previously known BM-DCs are BM-macrophages (Helft et al., 2015). Therefore, additional evidence is needed before drawing conclusions regarding the identity of the C5aR1$^+$cDCs. Even though it is not entirely clear whether C5aR1$^+$CD11b$^+$ pulmonary cells are a subset of cDCs or M2 macrophages, I wanted to better understand the mechanisms underlying their low potency to drive T cell proliferation. Rodriguez et al. performed two studies showing how Arg1 expression could block T cell proliferation. It seems that the presence of Arg1 reduces the availability of L-Arg resulting in weak T cell proliferation. The absence of L-Arg (i) arrests stimulated T cells in G0-G1 phases, which is associated with an inability to upregulate the expression of cyclin D3 and cdk4 but not D1, cyclin D2 and cdk6(Rodriguez et al., 2007) and (ii) induces molecular changes including low expression of the CD3ζ chain(Correa et al., 2005). Lastly, monocyte chemoattractant protein 5 (MCP-5) or CCL12, which was highly expressed by the C5aR1$^+$cDCs but not by the C5aR1$^-$cDCs, is a product of activated macrophages and is a potent monocyte chemotactic factor that signals through CCR2(Sarafi et al., 1997). Clearly, all of the highly expressed genes on the C5aR1$^+$cDCs are markers of M2 macrophages. In future studies the morphology of the cells needs to be studied as well as the expression of additional macrophage markers like MerTK and F4/80. Also, phagocytosis assays may help to distinguish between cDCs and macrophages. I also noticed that the C5aR1$^+$cDCs express high levels of S100 calcium binding protein A8 (s100A8) while its expression was zero in C5aR1$^-$cDCs. S100A8 is an inflammatory mediator released by cells of the myeloid origin. These intracellular molecules are released to extracellular compartments in response to cell damage, infection, or inflammation, and function as proinflammatory danger signals. More precisely, there are several studies, which showed that MDSCs synthesize and secrete S100A8 and express receptors for S100A8. These cells are found under inflammatory conditions and they have been extensively studied using cancer models. It has been published that blocking of S100A8 or the S100A8 receptor RAGE on MDSCs from patients with gastric cancer abrogated T cell effector function(Wang et al., 2013). This finding further supports the hypothesis that the poor potency of C5aR1$^+$cDCs to induce T cell proliferation could be due to the fact that this group of cells are MDSCs. It would be interesting to block S100A8 and see if the suppressive effect could be reversed leading to stronger T cell proliferation. Lastly, I also detected high expression levels of two micro RNAs (miRNAs), Mir682 and Mir703. MiRNAs are noncoding RNAs that are critical RNA regulators and bind to the 3' untranslated region (UTR) of mRNA. Recently, miRNAs have been shown to regulate immune processes, however not much is known about that. A recent study showed that mir214 induces DC switching from tolerance to immunity by targeting β-catenin signaling (Gu et al., 2015). Unfortunately, there is no data about the role of mir703 and mir682. However, the fact that they are both exclusively expressed by the C5aR1$^+$ group of cells, in combination with the other genes which are highly expressed by the C5aR1$^+$cDCs, make me speculate that these two miRNAs could act in a way similar to mir214. Of course, additional studies are required to support this hypothesis, but the current data suggest this as a possible explanation.

Interestingly, the group of genes that was highly expressed by the C5aR1$^-$cDCs all suggest that this population belongs to the group of cDCs, which are known to efficiently activate CD4$^+$ T cells and induce a strong proliferation. On the other hand, the group of genes which were strongly expressed by the C5aR1$^+$cDCs, unanimously described a population of cells which acts as MDSCs or M2 macrophages. Even though, the data are interesting and promising, they have to be interpreted with caution before drawing any conclusions as I have only one replicate per condition and, therefore, this experiment needs to be repeated before drawing final conclusions.

4.8 Impact of C5aR1 i*n vitro* targeting on CD4⁺ T cell proliferation, antigen uptake as well as CD40 and MHC-II expression

So far, all the data showed that the two C5aR1⁺ and C5aR1⁻ cell populations were transcriptionally, phenotypically and functionally different. I discriminated them based on the expression of C5aR1, but, at this point, I still did not know, if C5aR1 activation is critical for the different functional behavior of C5aR1⁺ cDCs. To directly assess the role of C5aR1, I decided to focus only on the C5aR1⁺cDCs and *in vitro* block signaling through C5aR1 using a neutralizing Ab against C5aR1. The *in vitro* blockade significantly restored the potency of C5aR1⁺cDCs to drive CD4⁺ T cell proliferation in comparison to the isotype-treated C5aR1⁺cDCs. Once I noticed the increase in the percentage of the proliferated T cells, I hypothesized that signaling through C5aR1 may suppress Ag uptake and/or processing by C5aR1⁺cDCs. I delineated this possibility using naïve FACS-purified C5aR1⁺cDCs and *in vitro* pulsed them with either FITC-OVA or DQ-OVA, in the presence or absence of an anti-C5aR1 mAb. The results showed that both DC functions were C5aR1-independent as I did not observe any differences between the C5aR1-targeted-C5aR1⁺cDCs and the isotype-treated-C5aR1⁺cDCs. However, this experimental setup does not entirely prove that the Ag uptake/processing is C5aR1 independent because the used cells were naïve and not exposed to the inflammatory signals released from the ECs. As mentioned above, such inflammatory signals significantly contribute to the maturation of the DCs. Thus, in future experiments it will be critical to compare the C5aR1-targeted-C5aR1⁺cDCs with the isotype-treated-C5aR1⁺cDCs for their Ag uptake abilities after they allergen sensitization *in vivo*. There are at least two ways to do this. Firstly, I could repeat this experiment under the same conditions, but this time supplement the medium with a cocktail of inflammatory cytokines released by the ECs. However, because this approach is a bit challenging especially for finding the optimal concentration of each cytokine in order to recapitulate to the *in vivo* conditions, it might be more feasible to treat the mice once with HDM and 24h later to FACS-purify the cells. This treatment will ensure exposure of the cDCs to the inflammatory cytokines and still keep the cells “naïve” with regard to OVA Ag uptake.

Next, I decided to focus on the importance of the immunological synapse and check the impact of signaling through C5aR1 on synapse formation. At this point, I already knew that the C5aR1⁺cDCs express low levels of MHC-II and CD40, which could result in a compromised immunological synapse formation and therefore weak CD4⁺ T cell proliferation. Thus, I hypothesized that the improved potency of the C5aR1⁺cDCs to drive CD4⁺ T cell proliferation upon *in vitro* targeting of C5aR1 results from an upregulation of the expression levels of one of these two molecules. To test this hypothesis, I compared the expression levels of CD40 and MHC-II between the isotype-treated- C5aR1⁺cDCs and the anti- C5aR1-treated- C5aR1⁺cDCs 18h after *in vitro* blockade of C5aR1 and before the CD4⁺ T cells were added to the system. Interestingly, I noticed that the *in vitro* blocking of C5aR1 resulted in a significant upregulation of CD40, but the expression levels of MHC-II remained unchanged, at least at this time point. This finding suggested that the main effect of C5a/C5aR1 axis activation is the control of CD40 but not MHC-II expression. However, since I assessed only one time point, I cannot exclude regulation of MHC-II at a later or earlier time point. Clearly, kinetic experiments will help to clarify this issue.

4.9 The CD40-CD40L interactions control the CD4⁺ T cell proliferation when the levels of MHC-II are low

The upregulation of CD40 in C5aR1⁺ cDCs upon C5aR1-targeting was an interesting observation, considering the importance of that molecule for the formation of an immunological synapse. Thus, I decided to investigate its role in more detail. For this purpose, I used a neutralizing Ab against CD40L to

in vitro block the CD40-CD40L interactions and checked whether this treatment could alter the improved $CD4^+$ T cell proliferation observed upon *in vitro* blockade of C5aR1. It was intriguing to see that the hypothesis was correct as the simultaneous blockade of C5aR1 and the CD40-CD40L interactions decreased T cell proliferation. I also determined the contribution of the CD40-CD40L interaction for T cell proliferation on the $C5aR1^-$cDCs. However, in contrast to $C5aR1^+$ cDCs, the T cell proliferation remained unchanged upon *in vitro* blockade of CD40L. I hypothesized that the lacking impact on T cell proliferation is due to the high expression of MHC-II, which dominates the response and compensates for the loss of CD40. To test this hypothesis, I used a neutralizing Ab against MHC-II in two concentrations (10pg/ml and 100pg/ml). Indeed, using these two concentrations, I could detect a markedly decreased percentage of proliferated T cells, which was similar to that observed with the $C5aR1^+$cDCs. These data demonstrate that the limited the availability of MHC-II sets the threshold for CD40-driven regulation of T cell proliferation.

4.10 CD40-CD40L interactions control T cell proliferation when antigen peptide loading of MHC-II is limited

I performed additional experiments to verify the importance of CD40/CD40L interaction for T cell proliferation. For this purpose, I setup an experiment, in which I limited the amount of peptide loaded onto MHC-II. I pulsed $C5aR1^+$ and $C5aR1^-$ cDCs with the OVA peptide ($OVA^{323-339}$). I titrated the peptide from 5µg/ml to 5ng/ml. In both subsets of cDCs, I noticed a strong T cell proliferation when the cells were pulsed with 5µg/ml, 500ng/ml and 50ng/ml. However, the percentages of the proliferated T cells were significantly decreased when the cells were pulsed with the 5ng/ml. In case of the $C5aR1^+$cDCs, the proliferation dropped down to 20%, whereas for the $C5aR1^-$cDCs, the drop was mild and 80% of the T cells still proliferated. This difference between the two populations was expected as in case of the $C5aR1^-$cDCs, even though the peptide concentration was low, the expression of CD40 was high and could ensure better adhesion of T cells to DCs. Next, I pulsed $C5aR1^+$cDCs with 5ng/ml OVA peptide and at the same time *in vitro* block C5aR1. As shown in previous experiments, this *in vitro* blockade causes a CD40 upregulation and a stronger T cell proliferation. The expectation was that limited amount of peptide would result in a stronger need for co-stimulation to achieve a better T cell proliferation. My data confirmed this hypothesis and further highlighted the vital role of CD40 for a proper T cell proliferation which is controlled through C5aR1. The next step was to evaluate the need of CD40-CD40L interactions in case of the $C5aR1^-$cDCs. Once again, I pulsed the $C5aR1^-$cDCs with the lowest concentration of the OVA peptide (5ng/ml), which was accompanied by the *in vitro* blockade of CD40L. This treatment resulted in a dramatic decrease in $CD4^+$ T cell proliferation, which dropped to 20%. There are at least two explanations for this result. Firstly, in case of the Ag-pulsed cells, the amount of the loaded peptide on the groove of the MHC-II is probably higher than the one when the cells are pulsed only with the 5ng/ml. Unfortunately, I was not able to test this, because there is no Ab available that quantifies the amount of the $OVA^{323-339}$ peptide loaded on the BALB/c background. What I could do instead, is to *in vitro* block the CD40-CD40L interactions when the $C5aR1^-$cDCs are pulsed with the highest concentration of the peptide, which resulted in a strong T cell proliferation. However, even that approach would not prove the case, as Bertho et al., showed that a T cell could distinguish between peptide-loaded class II MHC complexes depending on their mode of peptide acquisition. Whereas T cell ligation of endosome-derived peptide/MHC-II allows endosomal tubulation, DCs loaded with exogenously added peptide induced no morphological transformation of endosomal compartments upon interaction with Ag-specific T cells(Bertho et al., 2014).

4.11 *In vitro* blocking of C5aR1 markedly changed the transcriptional program C5aR1$^+$cDCs

So far, my data showed that *in vitro* blocking of C5aR1 significantly restored the ability of C5aR1$^+$cDCs to activate CD4$^+$ T cells through CD40 upregulation. To elucidate differences in the activation pattern of C5aR1$^+$ cDCs in response to *in vitro* targeting of C5aR1 I decided to perform an RNA sequencing experiment. The data showed that many genes were differentially expressed in C5aR1-targeted cDCs. Within the anti-C5aR1-treated C5aR1$^+$cDCs, I detected high expression levels of Slc7a6os, Camk2d, Tm2d2, Ssbp4, Ppp1r3b, Dnajc12, Knop1, Ttc12, Fbxw9 and Nfrkb. These genes were strongly expressed in all anti-C5aR1-treated samples and their expression was either zero or very low in the isotype-treated C5aR1$^+$cDCs. After thorough screening of the literature, I did not find any genes whose expression has been reported in relation to specific DC functions. Several years after the sequencing of the mouse genome and according to the latest release of the Panther (Protein ANalysis THrough Evolutionary Relationships) Classification System, there is no information about the biological process of a big number of genes. I used both the PANTHER and the UniProt, another database which contains a large amount of information about the biological function of a protein, trying to see if any of these genes cluster together and thus we could at least identify pathways which are regulated through C5aR1. Unfortunately, by checking on both databases, I noticed that some of the genes fall in the group of genes with a so far unknown biological function. These genes are Tm2d2, Dnajc12, Knop1, Ttc12 and Fbxw9. Regarding, Camk2d, it known that it is a calcium/calmodulin-dependent protein kinase involved in the regulation of Ca^{2+} homeostasis, Ssbp4 is known to act as a positive regulator of transcription by RNA polymerase II, Ppp1r3b, acts as a glycogen targeting subunit for phosphatase PP1. It facilitates interaction of the PP1 with enzymes of the glycogen metabolism and regulates its activity. The current knowledge about Nfrkb, another gene which was among the most upregulated genes in the anti-C5aR1-treated C5aR1$^+$cDCs, is that it is a putative regulatory component of the chromatin remodeling INO80 complex, which is involved in transcriptional regulation, DNA replication and probably DNA repair. Lastly, not a lot is known about Slc7a6os as well, as the first study investigating its role in vertebrates was published in 2015 and used zebrafish as a model organism. Benini et al, published that Slc7a6os plays a critical role in the development of the central nervous system in zebrafish. Even though I found no relevant publication, according to the gene ontology resource, Slc7a6os has a role in the hematopoietic progenitor cell differentiation(Benini et al., 2015). This information could be of interest since it could imply, that the *in vitro* targeting of C5aR1, could affect the differentiation procedure and could result in a progenitor population for the generation of mature DCs.

The identification of the most strongly upregulated genes in the isotype-treated C5aR1$^+$cDCs was a bit more challenging, As shown in figure 29C, genes which were strongly expressed by the isotype-treated C5aR1$^+$cDCs were in some cases also strongly expressed at least by one of the antiC5aR1-treated samples. Eventually, I identified the following genes that were strongly upregulated in the isotype-treated but not the anti-C5aR1 Ab-treated group of cDCs: mt-Nd3, Chchd1, Rps19bp1, Commd5, Stx2 and Nol10. I used the same tools which were mentioned on the previous paragraph to get some information about the function of each gene. I noticed that not a lot is known about the role of any of these genes regarding DC biology. More precisely, Chchd1 is known to have a role in the mitochondrial protein translation, Commd5 is a known negative regulator of cell proliferation and also cell cycle G2/M phase transition, Stx2 is suggested to have a role in intracellular protein transport, mt-Nd3 is the core subunit of the mitochondrial membrane respiratory chain NADH dehydrogenase that probably belongs to the minimal assembly required for catalysis and lastly, Rps19bp1, is a direct regulator of SIRT1. Interestingly, a study published by the Verdin group showed that SIRT1 deacetylates RORγt and thus enhances Th17 cell generation(Lim

et al., 2015). As shown in section 3.1f, the C5aR1$^+$cDCs were able to drive a more profound Th17 response in comparison to the C5aR1$^-$cDCs, which could probably be mediated through Rps19bp1.

After identifying the genes which were most upregulated in each group of cells, I also compared them with the genes from the first RNAseq experiment (section 3.1i). Unfortunately, I found no matches. Especially in case of the C5aR1$^+$cDCs and the isotype-treated C5aR1$^+$cDCs, I was expecting to identify some commonly upregulated genes. However, this was not the case. Of course, these two populations are not directly comparable as the RNAseq was performed at different time points after FACS-purification, (C5aR1$^+$cDCs immediately after FACS purification and in case of isotype-treated C5aR1$^+$cDCs, 18h after the cells had been *in vitro* cultured in the presence of GM-CSF and OVA) and the cells were metabolically different. Also, when I compared the transcription profiles of C5aR1$^-$cDCs with that of the anti-C5aR1-treated C5aR1$^+$cDCs, I found no matches. I anticipated that the *in vitro* blockade of C5aR1 would induce a transcription profile similar to C5aR1$^-$cDCs as it results in a CD40 upregulation and better ability to prime T cell responses. However, I already had evidence that these two cell populations differ in their expression of MHC-II, which did not change after C5aR1 targeting. On top of that, as mentioned above, the two RNAseq experiments were performed at different time points. Therefore, it was not surprising that I did not detect any commonly upregulated genes.

In any case, it is obvious that this experiment needs to be repeated. Firstly, the different anti-C5aR1-treated samples markedly differed in the number of reads. In case of the isotype treated group of cells, I had no replicates, so it is not possible to extract reliable information. Even though this last experiment was also performed in Cincinnati using the same experimental design, it is difficult to draw any conclusion due to the different library preparations and the batch effects. Clearly, this experiment needs to be repeated with several replicates to get better and reliable insights into the transcription profile of C5aR1$^+$ cDCs in the presence or absence of C5aR1 targeting.

Summary/Working model

Taken together, my findings suggest that the C5a/C5aR1 axis defines two distinct pulmonary CD11b$^+$ cDCs that exert important roles in the maturation and activation process of such cDCs and their potency to drive naive T cell proliferation and differentiation. In this model (Figure 30), OVA allergen (1) pulsing of C5aR1$^+$cDCs results in C5a generation (2). Signaling through C5aR1 (3) keeps the expression of CD40 low (4) and together with the low MHC-II expression, it results in an impaired immunological synapse formation between C5aR1$^+$cDCs – CD4$^+$ T cells and resulting in poor activation of naive CD4$^+$ T cells (5).

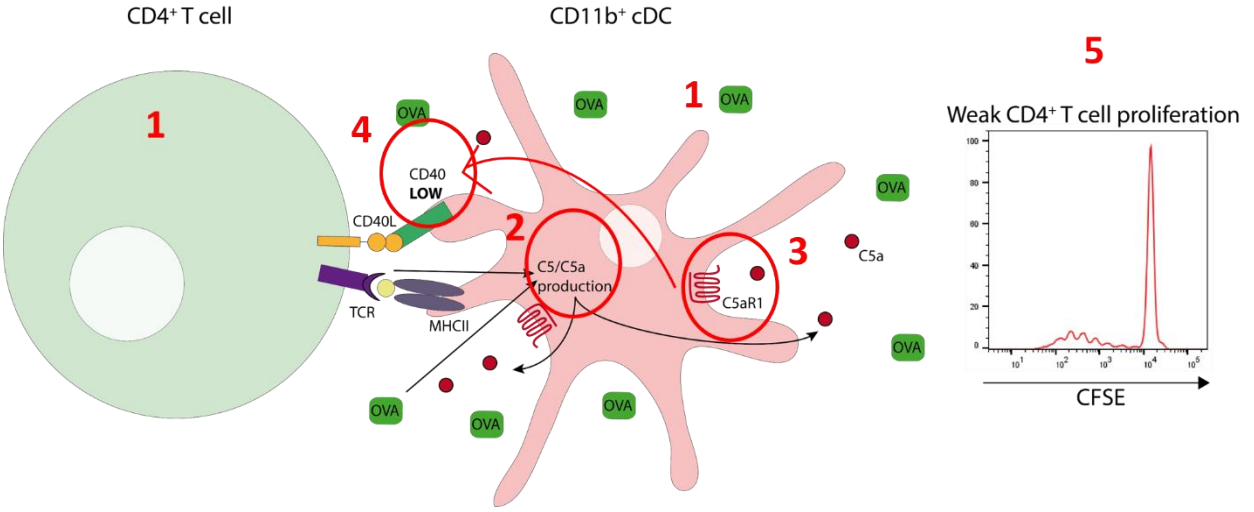


Figure 30: Model detailing the impact of C5a/C5aR1 axis activation in C5aR1⁺ cDCs on the activation of CD4⁺ T cells.

Under conditions, in which C5aR1 is downregulated or targeted, e.g. by a neutralizing C5aR1 mAb (1), the break is released (2) resulting in the upregulation of CD40 (3) thereby increasing the interaction between cDCs and T cells at the immunological synapse, eventually activating T cells through strong CD40/CD40L interactions, which lead to strong CD4⁺ T cell proliferation (4).

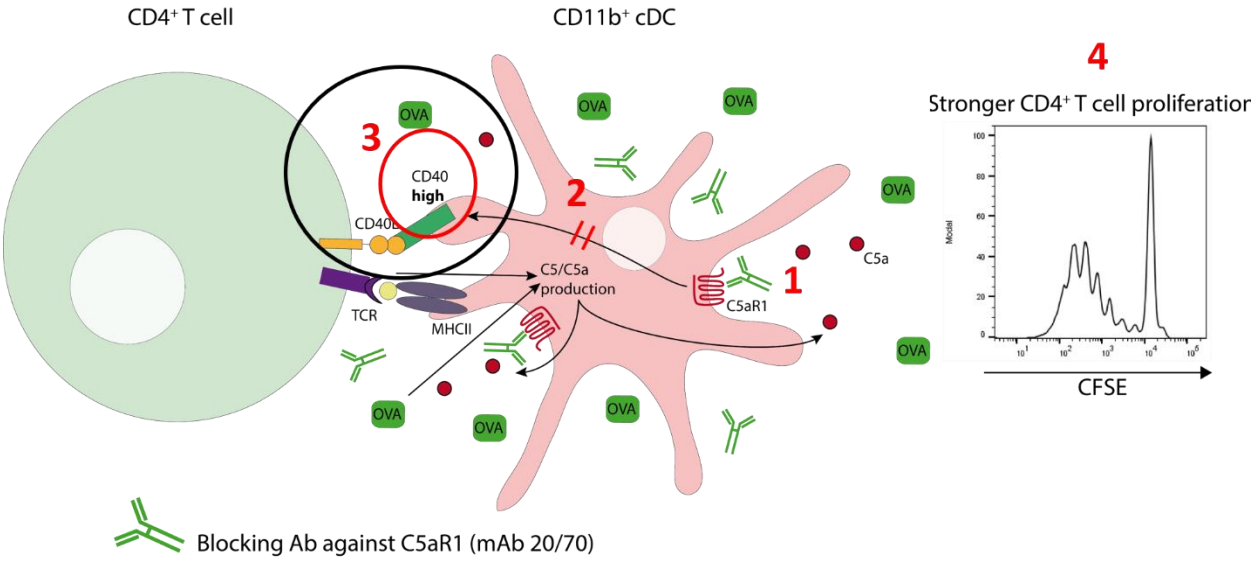


Figure 31: Model detailing the impact of C5a/C5aR1 axis targeting in C5aR1⁺ cDCs on the activation of naive CD4⁺ T cells.

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Supplementary

1st RNAseq experiment (see section 3.1.9)

	C5aR1_Neg	C5aR1_Pos	Neg-Pos
chr13:75782493-75782589 | Mir682	0	10.36616919	-10.366169
chr5:98904574-98904683 | Mir703	0	7.376846014	-7.376846
chr12:112779156-112779277 | Snora28	0	6.438851721	-6.4388517
chr7:16832091-16844889 | C5ar1	1.314261477	6.049994445	-4.735733
chr7:106628072-106628217 | Snord15b	0	4.261590989	-4.261591
chr10:24635012-24647276 | Arg1	0.656350835	4.849208662	-4.1928578
chr4:136445716-136448829 | C1qc	3.448726549	7.568001694	-4.1192751
chr4:136451830-136454759 | C1qa	4.451547427	8.398517118	-3.9469697
chr7:20281592-20284515 | Apoe	6.248109737	10.14192831	-3.8938186
chr3:90472992-90473956 | S100a8	0.798276184	4.658851269	-3.8605751
chr4:136436060-136442092 | C1qb	3.781013425	7.607197136	-3.8261837
chr11:81859213-81861025 | Ccl7	3.02722307	6.843355793	-3.8161327
chr7:20256927-20262213 | Apoc2	2.022630582	5.816392349	-3.7937618
chr11:81915346-81916901 | Ccl12	1.802867351	5.489481923	-3.6866146
chr5:91201460-91202409 | Pf4	2.424089007	5.668845283	-3.2447563
chr15:76097216-76100269 | Gm10872	0.142988441	3.340424117	-3.1974357
chr1:121963953-121970357 | Tmem37	1.618177563	4.744586305	-3.1264087
chr6:34253933-34267443 | Gm6644	0.00224077	3.073635173	-3.0713944
chr6:122555420-122560089 | Gdf3	0.332516612	3.358451122	-3.0259345
chr5:136045806-136048913 | Ccl24	2.949421007	5.972382487	-3.0229615

chr17:18024787-18030916 | Fpr2	3.736171894	6.745882689	-3.0097108
chr17:48485725-48491601 | Trem2	2.672323501	5.660577846	-2.9882543
chr6:47622224-47622398 | Rn4.5s	0	2.987675583	-2.9876756
chr14:69181443-69200129 | Adamdec1	0.418334615	3.39947601	-2.9811414
chr13:64464521-64471614 | Ctsl	4.426767933	7.397606186	-2.9708383
chr17:18013434-18020903 | Fpr1	0.367469479	3.278423438	-2.910954
chr6:122797157-122806175 | C3ar1	2.456753591	5.367063482	-2.9103099
chr7:53967367-53971046 | Saa3	3.717725769	6.614617096	-2.8968913
chr5:114243737-114250361 | Tmem119	1.239361378	4.053137409	-2.813776
chr1:89999831-90115573 | Ugt1a6b	0.000108714	2.777750038	-2.7776413
chr9:7272513-7283333 | Mmp13	0.739154233	3.511733681	-2.7725794
chr2:160189412-160192801 | Mafb	4.626754509	7.379568981	-2.7528145
chr1:172949056-172959892 | Fcgr4	3.497522953	6.218323958	-2.720801
chr6:142438768-142456463 | Ldhb	1.360437323	4.071599653	-2.7111623
chr8:40667053-40728032 | Msr1	4.324846598	7.00252251	-2.6776759
chr16:33829750-33949424 | Itgb5	3.830823203	6.482706329	-2.6518831
chr10:92951512-92979488 | Hal	2.345112464	4.99506386	-2.6499514
chr3:103539316-103541924 | Olfml3	0.932653099	3.546079531	-2.6134264
chr6:122527809-122552462 | Apobec1	4.916108758	7.526788727	-2.61068
chr11:81849078-81850954 | Ccl2	5.488219344	8.095882235	-2.6076629
chr9:21047360-21052887 | S1pr5	1.661147591	4.266254332	-2.6051067
chr17:48386895-48398213 | Trem3	3.023771649	5.618814209	-2.5950426
chr3:105950471-105970482 | Chi3l3	1.266516713	3.844897797	-2.5783811

chr8:118227152-118230794 | Maf	0.703115765	3.274172189	-2.5710564
chr2:36085945-36107789 | Ptgs1	0.904020049	3.438467189	-2.5344471
chr8:77617516-77624492 | Hmox1	5.56031449	8.056160802	-2.4958463
chr14:79688556-79701442 | Rgcc	2.928836459	5.405144868	-2.4763084
chr17:57498108-57622952 | Emr1	2.226866077	4.69133874	-2.4644727
chr11:84771255-84779556 | Car4	2.42370993	4.883620816	-2.4599109
chr1:132762353-132777367 | Faim3	1.627620845	4.08349422	-2.4558734
chr4:44544377-44723312 | Pax5	0.111722171	2.567387249	-2.4556651
chr13:20885987-21116121 | Aoah	0.513549362	2.954937014	-2.4413877
chr14:51710751-51725826 | Rnase4	2.894590794	5.316978383	-2.4223876
chr11:62945011-62973048 | Pmp22	3.069031869	5.489767697	-2.4207358
chr1:74421776-74432625 | Slc11a1	4.5252295	6.944155663	-2.4189262
chr5:139452928-139470907 | Pdgfa	1.367795983	3.774281039	-2.4064851
chr6:123212124-123225286 | Clec4d	4.47785215	6.876345041	-2.3984929
chr8:112099027-112103072 | Hp	4.553956383	6.948425639	-2.3944693
chr4:32646654-32658637 | BC024582	0.34668463	2.715559558	-2.3688749
chr3:10204342-10208576 | Fabp4	1.556046772	3.901417221	-2.3453704
chr1:195047834-195090239 | Hsd11b1	0.774386897	3.10787196	-2.3334851
chr2:117937657-117952869 | Thbs1	2.739102806	5.048611112	-2.3095083
chr11:94204608-94254290 | Abcc3	1.377079315	3.672097162	-2.2950178
chr10:75411056-75412391 | Vpreb3	0.469137873	2.759253857	-2.290116
chr11:109301391-109314673 | LOC100503496	1.317031945	3.606821212	-2.2897893

chr6:131169252-131197380 | Klra2	2.193017876	4.408147986	-2.2151301
chr1:195098353-195099382 | G0s2	1.634858087	3.840422949	-2.2055649
chr17:48403660-48414724 | Treml4	4.524427314	6.723531258	-2.1991039
chr5:144090986-144094383 | 0610040B10Rik	0.687313362	2.882195011	-2.1948816
chr11:69496078-69499056 | Tnfsf13	3.433814084	5.620976355	-2.1871623
chr7:4189355-4195065 | Lilra5	2.08409829	4.265706926	-2.1816086
chr19:11395528-11410636 | Ms4a7	0.461440549	2.630985508	-2.169545
chr13:94827750-94965364 | Lhfpl2	3.203489353	5.365996915	-2.1625076
chr6:47626562-47626736 | Rn4.5s	0	2.15897679	-2.1589768
chr6:124462638-124473458 | C1ra	1.507576476	3.664551105	-2.1569746
chr15:79921763-79949931 | Syngr1	1.871776637	4.027242536	-2.1554659
chr12:99440509-99497547 | Galc	1.386110117	3.539890832	-2.1537807
chr7:19470038-19475787 | Pglyrp1	3.33816338	5.491721645	-2.1535583
chr16:30065442-30067882 | Hes1	1.565168203	3.674630131	-2.1094619
chr13:89794914-89882117 | Vcan	1.419964896	3.5290713	-2.1091064
chr3:95538070-95543492 | Ecm1	3.945729421	6.053652246	-2.1079228
chr6:115311238-115440419 | Pparg	2.13857531	4.240924865	-2.1023496
chr11:82988687-83003718 | Slfn4	2.913023423	5.013672172	-2.1006487
chr9:58372106-58388747 | Cd276	1.53159356	3.62946154	-2.097868
chr7:3958674-4014806 | Lair1	3.057387884	5.142478791	-2.0850909
chr10:116714596-116719328 | Lyz2	11.50162325	13.58428138	-2.0826581
chr9:118515826-118810121 | Itga9	0.323214405	2.392784506	-2.0695701
chr18:53405315-53550479 | Snx24	1.656336189	3.711494907	-2.0551587

chr5:44210131-44234707 | Bst1	4.121197799	6.174472335	-2.0532745
chr4:19803984-19849713 | Atp6v0d2	2.565894457	4.600317335	-2.0344229
chr17:24300831-24306340 | Atp6v0c-ps2	5.788837071	7.813608057	-2.024771
chr6:145806762-145813940 | Bhlhe41	1.934682558	3.951298705	-2.0166161
chr2:144091717-144091938 | Snord17	0	2.00032457	-2.0003246
chr13:30841126-30858796 | Irf4	6.389449795	4.380639416	2.00881038

Abbreviations and Symbols

-/-	knock-out
°C	degree Celsius
%	percent
AHR	airway hyperresponsiveness
AT	anaphylatoxin
ATR	anaphylatoxin receptor
BAL	broncho alveolar lavage
BMDC	bone marrow dendritic cell
BSA	bovine serum albumin
C3aR	c3a receptor
C5aR1	c5a receptor 1
C5aR2	c5a receptor 2
CCR	CC-chemokine receptor
CD	cluster of differentiation
cDC	conventional dendritic cell
CFSE	carboxylfluorescein succinimidyl ester
CLIP	class II-associated invariant chain peptide
GM-CSF	granulocyte-macrophage colony stimulating factor
DC	dendritic cell
EC	epithelial cell
ER	endoplasmic reticulum
et al.	at alii
FACS	fluorescence activated cell sorting
FBS	fetal bovine serum
FITC	fluorescein isiothiocyanate
g	gram (unit)
h	hour

HDM	house dust mite
i.e.	in example
i.p.	intraperitoneal
i.t.	intratracheal
MFI	mean fluorescence intensity
mg	milligram
MHC	major histocompatibility complex
Min	minute
µl	micro liter
ml	milliliter
mo-DC	monocyte-derived dendritic cell
OVA	ovalbumin
PBS	phosphate buffered saline
PE	phycoerythrin
rpm	rounds per minute
RT	room temperature
WT	wild type

Conference contributions (oral presentations)

The C5a/C5a receptor 1 axis controls pulmonary tolerance at the level of pulmonary CD11b^{+}conventional dendritic cells

Konstantina Antoniou, Tillman Vollbrandt, Harinder Singh and Jörg Köhl

HOROS Symposium, Obergurgl 2018

The C5a/C5a receptor 1 axis controls pulmonary tolerance at the level of pulmonary CD11b^{+}conventional dendritic cells

Konstantina Antoniou, Tillman Vollbrandt, Harinder Singh and Jörg Köhl

Allergy meets infection symposium, Lübeck 2018

The C5a/C5a receptor 1 axis controls pulmonary tolerance at the level of pulmonary CD11b^{+}conventional dendritic cells

Konstantina Antoniou, Tillman Vollbrandt, Harinder Singh and Jörg Köhl

International Complement Workshop (XXVVII ICW), Santa Fe 2018

C5a receptor 1 activation controls Th cell proliferation by pulmonary CD11b^{+}cDC through antigen processing, MHC-II and CD40 expression

Konstantina Antoniou, Tillman Vollbrandt, Harinder Singh and Jörg Köhl

15th International Symposium on Dendritic Cells, Aachen 2018

The C5a/C5aR1 axis controls allergen-driven CD4^{+} T cell proliferation by CD11b^{+} pulmonary dendritic cells

Konstantina Antoniou, Tillman Vollbrandt, Harinder Singh and Jörg Köhl

Autumn Immunology Conference (AIC), Chicago 2017

The C5a/C5aR1 axis controls allergen-driven CD4^{+} T cell proliferation by CD11b^{+} pulmonary dendritic cells

Konstantina Antoniou, Tillman Vollbrandt, Harinder Singh and Jörg Köhl

Immunology Symposium, Deer Creek 2017

The role of complement in transcriptional Th2 programing of pulmonary dendritic cells in allergic

Konstantina Antoniou, Tillman Vollbrandt, Harinder Singh and Jörg Köhl

IRTG 1911 Retreat, Boltenhagen 2016

List of publications

1. Wiese AV, Ender F, Quell KM, **Antoniou K**, Vollbrandt T, König P, et al. (2017) The C5a/C5aR1 axis controls the development of experimental allergic asthma independent of LysM-expressing pulmonary immune cells. PLoS ONE 12(9): e0184956. https://doi.org/10.1371/journal.pone.0184956
2. Quell KM[1], Karsten CM[1], Kordowski A[1], Almeida LN[1], Briukhovetska D[1], Wiese AV[1], Sun J[1], Ender F[1], **Antoniou K[1]**, Schröder T[1], Schmudde I[2], Berger JL[2], König P[2], Vollbrandt T[3], Laumonnier Y[4], Köhl J[4,5]. J Immunol. Monitoring C3aR Expression Using a Floxed tdTomato-C3aR Reporter Knock-in Mouse. J Immunol July 15, 2017, 199 (2) 688-706; DOI: https://doi.org/10.4049/jimmunol.1700318

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