Generation and Utilization of In Vitro Models of Dendritic Cells for the Study of Dendritic Cell Biology

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Thèse de doctorat ès sciences de la vie (PhD)

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par

Matteo PIGNI

Master de l'Université de Milan

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Prof. David Vernez, Président
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Résumé large public

Génération et Utilisation de Modèles *In Vitro* de Cellules Dendritiques pour l'Étude de la Biologie des Cellules Dendritiques.

Matteo PIGNI, Département de Biochimie (DB), CLE, Quartier UNIL-Épalinges.

Le système immunitaire est composé de deux branches principales : le système immunitaire inné et le système immunitaire adaptatif. Le système immunitaire inné fournit une réponse rapide, non spécifique de l'agent pathogène et est considéré comme la première ligne de défense contre les infections. A l'opposé, le système immunitaire adaptatif développe des réponses ciblées se perfectionnant durant une période d'induction plus longue pour combattre des menaces spécifiques, et donne lieu à une mémoire immunitaire à long terme spécifique de l'agent pathogène.

Les cellules dendritiques (CDs, en anglais *dendritic cells,* DC) sont des cellules immunitaires innées qui assurent leur fonction à la frontière entre l'immunité innée et l'immunité adaptative. En effet, les CDs surveillent constamment l'organisme en vue de déceler la présence éventuelle d'éléments pathogènes. Lorsqu'une menace potentielle est détectée, les CDs s'activent, alertent le système immunitaire adaptatif et initient le développement de la réponse immunitaire adaptative la plus appropriée.

En raison de ces fonctions uniques, la compréhension des CDs a des répercussions importantes dans de nombreux domaines comme par exemple la création de nouveaux vaccins, le développement de nouvelles stratégies pour le traitement de cancers par immunothérapie et la compréhension des mécanismes impliqués dans les maladies auto-immunes qui apparaissent lorsque le système immunitaire attaque de manière erronée l'organisme. Cependant, la rareté des CDs *in vivo*, leur fragilité extrême et leur sensibilité aux conditions de culture ont fortement ralenti la recherche sur les CDs.

Pour cette raison, notre groupe s'est intéressé à la conception d'une méthode permettant d'obtenir un nombre suffisant de CDs pour l'expérimentation. Ce but a été atteint lorsque nous avons réussi à générer une lignée de CDs fonctionnelles stables, appelée MutuDC1, à partir de souris transgéniques portant un oncogène dont l'expression est ciblée dans les CDs. Les cellules MutuDC1 peuvent être facilement maintenues en culture et peuvent fournir un nombre pratiquement illimité de CDs.

In vivo, les CDs sont très hétérogènes et peuvent être classifiées en plusieurs sous-groupes caractérisés par des fonctions différentes. Par conséquent, afin de mieux représenter cette diversité qui est observée *in vivo*, nous avons décidé de modifier notre méthode pour générer différentes lignées de MutuDC qui correspondent aux différents sous-groupes des CDs.

La première partie de cette thèse décrit les modifications introduites dans la méthode de génération de MutuDC et comment celles-ci nous ont permis de générer une nouvelle lignée de CDs, appelée $CD4^-$ MutuDC2, qui diffère de la lignée cellulaire MutuDC1 sur plusieurs aspects, y compris la fonction.

Dans la seconde partie de cette thèse, nous montrons un exemple du potentiel de notre lignée MutuDC pour étudier la biologie des CDs. Pour ce projet, nous nous sommes concentrés sur les mécanismes qui permettent aux CDs de détecter des pathogènes et de s'activer en leur présence. Nos expériences ont permis d'élaborer une nouvelle stratégie pour identifier des régulateurs moléculaires non décrits dans la littérature qui interviennent dans la détection du pathogène et l'activation des CDs.

En conclusion, le travail présenté dans cette thèse vise à fournir des outils pertinents afin de mieux comprendre la biologie des CDs, avec une attention particulière sur les mécanismes sous-jacents à la détection des pathogènes et à l'activation des CDs.

Summary

Generation and Utilization of *In Vitro* Models of Dendritic Cells for the Study of Dendritic Cell Biology.

Matteo PIGNI, Department of Biochemistry (DB), CLE, Quarter UNIL-Épalinges.

Toll-like receptors (TLRs) are a well-known family of innate receptors that can recognize a great variety of pathogen-derived molecules. During an infection, several TLR ligands can be detected simultaneously. It has been observed that specific combinations of TLR ligands can activate the innate immune cells cooperatively in a mechanism known as synergy. Synergistic activation of innate immune cells induces cytokine and chemokine production in a greater than additive manner and contributes to the development of the most appropriate immune response for every different pathogen. Synergy can be further amplified by specific host derived signals like IFNγ in a process defined super-synergy.

Dendritic cells (DCs) are a heterogeneous group of innate immune cells and are considered to be the most efficient antigen presenting cells. Many aspects of DC biology have been explained in great detail. However, the mechanisms that regulate synergistic and super-synergistic activation of DCs remain elusive.

One of the biggest challenges in the study of DCs is the difficulty to obtain sufficient numbers of cells for experimentation. In the past years, our group has developed a strategy to generate functionally stable DC lines, called MutuDCs, from the spleen of Mushi1 mice, a strain of SV40 large T oncogene (SV40LgT)-transgenic mice that spontaneously develop splenic DC tumors. Meticulous characterization of these cell lines has shown their equivalence to the splenic conventional DC type 1 (cDC1) subset.

For the project presented in this thesis, we introduced the SV40LgT transgene in a $Batf3^{-/-}$ genetic background. We exploited the selective absence of cDC1s that characterizes $Batf3^{-/-}$ mice to generate a new MutuDC line that we showed to be phenotypically and functionally analogous to the $CD4^{-}$ splenic conventional DC type 2 (cDC2) subset and that, therefore, we named $CD4^{-}$ MutuDC2 line.

In this thesis, we provide a description of the approach that we used to generate our MutuDC1 and $CD4^{-}$ MutuDC2 cell lines, with special attention on the phenotypic and functional characterization of the latter. Furthermore, we present an example of use of our DC model cell lines to facilitate the study of DCs. In particular, we describe the strategy that we have designed to investigate the molecular mechanisms of DC (super-)synergistic activation. Our results demonstrated that flow cytometric analysis of the expression of IL-12 and CCR7 allows to distinguish non-synergistic, synergistic and super-synergistic activation-states in MutuDC1s treated with CpG ODN, poly(I:C), and IFNγ either alone or in their binary or ternary combinations. This observation allowed to develop a sorting-based strategy to screen a pooled CRISPR/Cas9 genomic knockout library (more than 20000 genes) with the potential to reveal new regulators of (super-)synergistic activation of DCs.

Overall, the work presented in this thesis intends to provide valuable tools to better understand DC biology with a particular focus on the mechanisms underlying the (super-)synergistic activation of DCs.

Résumé

Génération et Utilisation de Modèles *In Vitro* de Cellules Dendritiques pour l'Étude de la Biologie des Cellules Dendritiques.

Matteo PIGNI, Département de Biochimie (DB), CLE, Quartier UNIL-Épalinges.

Les récepteurs de type Toll (en anglais *Toll-like receptors,* TLRs) appartiennent à une famille bien connue de récepteurs innés qui peuvent reconnaître une grande variété de molécules dérivées de pathogènes. Lors d'une infection, différents ligands de TLR peuvent être détectés simultanément. Il a été observé que des combinaisons spécifiques de ligands de TLR peuvent activer coopérativement les cellules immunitaires innées par un mécanisme appelé synergie. L'activation synergique de ces cellules immunitaires innées induit la production de cytokines et chimiokines de manière plus qu'additive et contribue au développement de la réponse immunitaire la plus appropriée pour chaque pathogène. La synergie peut être amplifiée par des signaux spécifiques dérivant de l'hôte, comme par exemple l'IFNγ, par un processus appelé super-synergie.

Les cellules dendritiques (CDs) constituent un groupe hétérogène de cellules immunitaires innées et sont considérées comme les cellules présentatrices d'antigène les plus efficaces. De nombreux aspects de la biologie des CDs ont été décrits en détail. Cependant, les mécanismes qui régulent l'activation synergique et super-synergique des CDs restent peu connus.

Un des plus grands défis de l'étude des CDs est la difficulté d'obtenir un nombre suffisant de cellules pour l'expérimentation. Au cours des dernières années, notre groupe a développé une stratégie pour générer des lignées de CDs fonctionnelles stables, appelées MutuDCs, provenant de la rate de souris Mushi1, une lignée de souris transgéniques pour l'oncogène grand T du SV40 (en anglais *SV40 large T oncogene,* SV40LgT) qui développent spontanément des tumeurs des CDs spléniques. La caractérisation précise de ces lignées cellulaires a montré leur équivalence avec un sous-groupe de CDs : les CDs conventionnelles de type 1 (en anglais *conventional DC type 1,* cDC1) de la rate.

Dans le cadre du projet présenté dans cette thèse, nous avons introduit le transgène SV40LgT dans un fond génétique Batf3$^{-/-}$. Nous avons exploité l'absence sélective des cDC1s qui caractérise les souris Batf3$^{-/-}$, pour générer une nouvelle lignée de MutuDCs analogue phénotypiquement et fonctionnellement au sous-groupe CD4^{-} cDC2 de la rate. Par conséquent, nous avons dénommée cette nouvelle lignée cellulaire CD4^{-} MutuDC2.

Dans cette thèse, nous décrivons l'approche que nous avons utilisée pour générer nos lignées cellulaires MutuDC1 et CD4^{-} MutuDC2, avec une attention particulière à la caractérisation phénotypique et fonctionnelle de cette dernière. De plus, nous présentons un exemple d'utilisation de nos lignées cellulaires pour faciliter l'étude des CDs. En particulier, nous décrivons la stratégie que nous avons établie pour étudier les mécanismes moléculaires de l'activation (super-)synergique des CDs. Nos résultats ont démontré que l'analyse de cytométrie en flux pour l'expression d'IL-12 et de CCR7 permet de distinguer les différents états d'activation non-synergiques, synergiques et super-synergiques des MutuDC1s traités avec CpG ODN, poly(I:C) et IFNγ seuls ou en combinaison binaire ou ternaire. Cette observation a permis de développer une stratégie fondée sur le tri cellulaire par cytométrie en flux pour le criblage d'une banque de CRISPR/Cas9 knockout (plus que 20000 gènes) qui pourrait révéler de nouveaux régulateurs de l'activation (super-)synergique des CDs.

En résumé, le travail présenté dans cette thèse a pour intention d'apporter des outils pertinents permettant de mieux comprendre la biologie des CDs avec une attention particulière sur les mécanismes sous-jacents à l'activation (super-)synergique des CDs.

Index

1. Introduction

1.1. Overview of the innate and the adaptive immune responses

The immune system is a complex network of cells and molecular factors that interact coordinately to defend the body from disease and disease-causing agents like pathogenic organisms and detrimental exogenous or endogenous molecules to which we are constantly exposed. The activity of the immune system needs to be finely regulated to allow the recognition of pathological signals and the development of the most appropriate response to each threat while maintaining the ability to discriminate self from non-self and the specificity to preserve the integrity of healthy cells and tissues.

The development of an immune response is a multilayered mechanism that depends on the cross-talk between two broad humoral and cellular subsystems, known as innate immune system and adaptive immune system, both of which comprise several different cell types with distinct function and distribution in the body. The main cell types that constitute the innate immune system are granulocytes (i.e. mast cells, neutrophils, basophils and eosinophils), innate lymphoid cells (ILCs), natural killer (NK) cells, monocytes, macrophages and dendritic cells (DCs), while the adaptive immune system comprises T cells and B cells.

Innate immunity is commonly considered as the first line of defense against infections because it is constantly and promptly available to respond to invading pathogens. The mechanisms involved in the innate response have a rather general specificity. Indeed, they depend on a group of innate receptors that mediate pathogen sensing through the recognition of numerous molecular structures that are broadly common among different pathogens. For long time, it was believed that the innate immune system did not possess the potential to give rise to immunological memory. However, in

the last decade, mounting evidence has demonstrated this notion to be inaccurate, paving the way to a new concept of innate immune memory named trained immunity. The mechanisms that mediate trained immunity are far from the classic concept of immunological memory which is characteristic of the adaptive immune system. Nevertheless, it is now known that numerous pathogen- or environmental-derived stimuli have the potential to induce metabolic and epigenetic changes that cause a transcriptional-program switch both in progenitor and terminally differentiated innate immune cells. These events prompt the innate immune system to induce a stronger and more efficient response to subsequent infections, with an effect that can be protracted for months or even years [1–3].

In contrast to the innate immune system, the adaptive immune system has a much higher target specificity which develops over a longer response-time on a case-by-case basis against each pathogen that escapes clearance by the innate immunity. This specificity is achieved thanks to surface receptors that are refined to allow every adaptive immune cell to recognize one unique epitope of a single antigen. The process of activation of antigen-specific adaptive immune cells involves a molecular mechanism known as antigen presentation in which specialized groups of immune cells expose pathogen-derived epitopes in a molecular configuration that T cells can recognize through their epitope-specific surface receptors. After recognition of their specific antigen, activated adaptive immune cells expand clonally and induce a multitude of mechanisms that allow them to kill infected cells, to prevent pathogen dissemination to healthy tissues and to help the innate immune system to clear the infection. As mentioned, adaptive immunity generates long-term immunological memory in the form of pathogen-specific cells which are able to start a faster and stronger adaptive response in case of subsequent encounters with the same pathogen.

1.1.1. The main cell types of the innate immune system

Mast cells (reviewed in [4]) are spread over the whole body but reside mainly at its contact points with the environment like the gastrointestinal tract, the skin and the respiratory tract. The cytoplasm of mast cells contains a large quantity of granules filled with pro-inflammatory mediators, cytokines, chemokines, antibacterial products and proteases. When mast cells come in contact with pathogens or potentially harmful molecules, they become activated and release the content of their granules through the so-called degranulation. The mediators that are released during degranulation induce a vast array of effects including recruitment and/or activation of other immune cells like neutrophils, macrophages, NK cells and DCs, increase of vascular permeability, vasodilation and remodeling of connective tissues. Additionally, they induce mechanisms intended to facilitate the physical elimination of the menace like increased fluid secretion and mucus production, smooth muscle contraction and increased peristalsis, resulting in coughing, vomiting and diarrhea.

Neutrophils (reviewed in [5–8]) are a group of relatively short-lived phagocytic cells (from ancient Greek: *phagein* = eating, *kytos* = cell. The mechanisms of phagocytosis are discussed in paragraph *1.3.1. Mechanisms of antigen capture, processing and presentation by DCs*) that circulate in the blood stream and are recruited to the tissues during inflammation. Similarly to mast cells, neutrophils contain large quantities of cytoplasmic granules in which inflammatory mediators, regulatory cytokines and antimicrobial products are stored. Pathogens are phagocytosed by neutrophils and killed through the release of cytotoxic and antimicrobial molecules from the granules into the phagocytic vesicles. Alternatively, neutrophils can remove pathogens from their surrounding environment either by releasing extracellularly the content of the granules or by forming extracellular DNA traps known as neutrophil extracellular traps (NETs) that ensnare the microorganisms and immobilize them to facilitate their killing by the neutrophils themselves or by other innate immune cells.

Eosinophils and *basophils* (reviewed in [5,9–11]) are granulocytes whose main described function is related to the elimination of pathogens like parasites that are excessively big to be phagocytosed by neutrophils. However, the content of their granules is not limited to antimicrobial and cytotoxic molecules but comprises several inflammatory mediators that have a central role in the recruitment and the regulation of other innate and adaptive immune cells. They share some mechanisms with neutrophils, like the ability to form extracellular DNA traps.

ILCs (reviewed in [12,13]) are the most recently described group of innate immune cells. ILCs lack the antigen specific receptors that characterize adaptive immune cells and are mostly devoid of innate receptors for direct detection of pathogens. Therefore, their function relies on their ability to sense and respond to cytokines, inflammatory mediators and other signaling molecules produced by innate immune or non-hematopoietic cells. ILCs have a very important role in the control and clearing of several classes of infecting pathogens thanks to their early production of numerous cytokines that typically characterize the more tardive T cell adaptive response. However, their spectrum of functions is much broader and include regulation of metabolism and of tissue regeneration and homeostasis, control of the symbiotic microbiota, mediation of the cross-talk between immune cells and non-hematopoietic cells and induction of secondary-lymphoid-organ development in the fetus.

NK cells (more recently classified as a subset of ILCs) (reviewed in [14,15]) are innate immune cells characterized by a tightly regulated cytotoxic function. Several activating and inhibitory receptors are present on the membrane of NK cells and allow them to interact with other cells of the body distinguishing self from non-self and identifying aberrant or damaged cells, like tumor cells, and cells infected by some classes of intracellular pathogens. The interaction with non-self or abnormal cells alters the balance between activating and inhibitory signals and causes the activation of NK cells that kill the target cell through the release of cytotoxic proteins or by direct induction of apoptosis.

Upon activation, NK cells produce important inflammatory mediators and cytokines that regulate the function of macrophages, DCs and T cells.

Monocytes (reviewed in [16,17]) are phagocytes that circulate in the blood and, during inflammation, are recruited to the inflamed tissues where they differentiate into subsets of macrophages or DCs and help to clear the infection, to present antigens and to regulate inflammation.

Macrophages (reviewed in [18–22]) are a heterogeneous group of phagocytic cells characterized by a broad variety of functional specificities and tissue localizations. In spite of their heterogeneity, however, macrophages have many well-known general characteristics and functional properties. As their name suggests (from ancient Greek: *makrós* = large, *phagos* = eater), macrophages are specialized in the engulfment and intracellular killing of pathogens. Additionally, they phagocytose and remove dead cells, debris and detrimental molecules of various origin. When activated, macrophages have a fundamental role in the production of cytokines which contribute to recruit and activate innate immune cells like DCs and neutrophils but also to promote or modulate inflammation. Macrophages are also known to be one of the three groups of APCs (APCs) (the other two being DCs and B cells).

DCs (reviewed [23]) are phagocytic cells characterized, in most cases, by a typical branched shape (from ancient Greek: *dendron* = tree). Their abundance in the body is rather limited, and yet they are found in almost every tissue and organ. DCs are considered the most efficient and specialized APCs. Upon encounter with pathogens, DCs internalize antigens and process them for presentation to T cells. At the same time, they induce the expression of specific surface molecules that provide the T cells with fundamental activating signals, and they start producing cytokines that contribute to the recruitment of other immune cells and to the orchestration of the adaptive immune response.

1.1.2. The main cell types of the adaptive immune system

As mentioned above, *T cells* (reviewed in [24–28]) do not recognize pathogens directly but react to antigens presented on the surface of APCs like DCs. The recognition of antigens happens with very high specificity and is mediated by the T cell receptor (TCR). Different TCRs possess distinct antigen-specificity and every T cell is characterized by a single type of TCR. A healthy immunocompetent organism possesses an enormous repertoire of TCRs and, in the presence of an invading pathogen, only the T cells whose TCR recognizes antigens derived from that specific pathogen are activated to become effector cells and to expand numerically. Effector T cells can be divided into different classes on the basis of their mechanism of action. Cytotoxic T cells (or cytotoxic T lymphocytes, CTL) are $CD8^+$ and can recognize and kill cells infected with intracellular pathogens. Helper T cells (Th) are $CD4^+$ and are divided into different subclasses with distinct capacity to regulate the activity of other innate or adaptive immune cells, in particular macrophages and B cells. Regulatory T cells (Treg) are $CD4^+$ and are activated through the same mechanisms described for other classes of effector T cells with the difference that their TCR specificity is directed toward self-antigens. This counterintuitive and apparently dangerous specificity is justified by the fact that the function of the Tregs is not related to the elimination of pathogens but rather to the maintenance of the mechanisms of self-recognition and self-tolerance.

Similarly to T cells, also *B cells* (reviewed in [29,30]) have a membrane receptor, the B cell receptor (BCR), that allows them to recognize antigens, but in contrast to T cells, B cells can directly detect antigens. Different BCRs have distinct antigen specificity, and every B cell possesses just one type of BCR. The complex of all the B cells present in a healthy immunocompetent organism represents its full repertoire of BCR specificities. B cells are the third group of antigen-presenting cells. Even if their capacity to internalize and present antigens is not limited to the specific ligand of their BCR, antigen internalization is incomparably more efficient when it is BCR-mediated, making it extremely more

likely for the B cells to present peptides derived from BCR-recognized antigens rather than from non-specific antigens. Once an antigenic epitope is recognized by a BCR, the antigen is internalized, together with the receptor, and processed for presentation. Antigen-derived peptides become then available on the surface of the B cell for recognition by T cells with the same antigen specificity as the B cell. Recognition of the antigen-derived peptides by activated Th cells, leads to B-cell activation, which induces the B cells to proliferate and triggers them to switch their isotype under the influence of cytokines in order to strengthen their specificity toward precise classes of pathogen, to mutate the variable regions of their BCRs in order to increase the affinity for their target epitope, and to produce soluble secreted forms of their BCRs, the antibodies. In some instances, the recognition of certain antigens in the presence of additional pathogen-derived signals can induce B cell activation and antibody production even in the absence of Th cells. The antibodies produced by activated B cells have several functions among which inhibiting the activity of pathogenic and detrimental molecules or helping the identification and clearance of pathogens by the innate immune cells.

1.2. Pathogen sensing by the innate immune cells

The broad molecular-recognition capacity of the innate immune system relies on a large set of germline-encoded receptors able to bind a great variety of molecular structures that are conserved among pathogens or commonly produced by tissue damage and pathological processes. These molecular structures are known as pathogen-associated molecular patterns (PAMPs), when derived from invading microorganisms, and damage- or danger-associated molecular patterns (DAMPs), when produced by tissue damage. Therefore, the numerous receptors that detect their presence are collectively known as pattern recognition receptors (PRRs) [31]. Detection of PAMPs or DAMPs by PRRs can trigger several signaling cascades that result in the innate immune cell activation and

in the induction of defense mechanisms like phagocytosis and pro-inflammatory cytokine production [31].

1.2.1. Innate immune receptor families

Most PRRs can be classified into six separate families on the basis of their molecular structure and signaling properties: nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs), C-type lectin-like receptors (CLRs), retinoic acid-inducible gene-I (RIG-I)-like receptors (RLRs), absent in melanoma 2 (AIM2)-like receptors (ALRs), oligoadenylate synthase (OAS)-like receptors (OLRs) and toll-like receptors (TLRs) [31,32].

NOD-like receptors - NLRs (reviewed in [33–35])

NLRs are a family of cytosolic receptors involved in the response to a broad variety of intracellular pathogens, host-derived DAMPs and environment-derived detrimental molecules.

The NLR family comprises a large number of members that share a relatively common structural organization [33] **(Figure 1)**. The N-terminal domain mediates downstream signaling and can belong to four different classes: acidic transactivation domain, baculoviral inhibitor of apoptosis repeat (BIR) domain, caspase activation and recruitment domain (CARD) or pyrin domain [36]. Four sub-families of NLRs are defined according to their N-terminal domain, namely NLRA (acidic transactivation), NLRB (BIR), NLRC (CARD) and NLRP (pyrin) [36] **(Figure 1)**. The central part of the NLRs typically contains a NOD domain, also called NACHT (NAIP, CIITA, HET-T and TP-1). This domain has dNTPase activity and is involved in NLR oligomerization [37]. The C-terminal region is a leucine-rich repeat which is involved in the recognition and binding of NLR ligands [33].

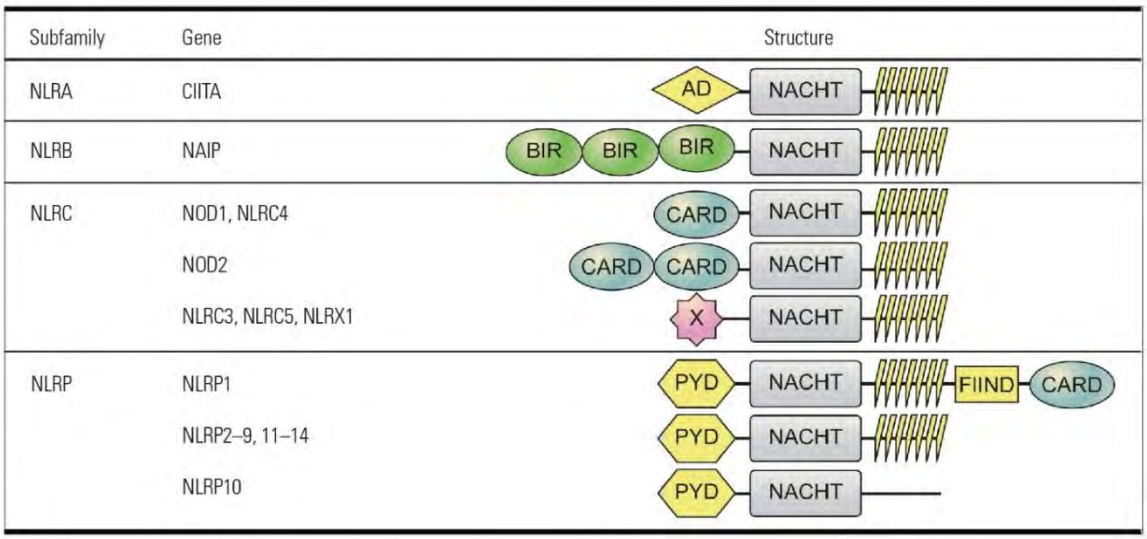

Figure 1. Classification and structure of NLRs. AD = acidic transactivation domain; BIR = baculovirus inhibitor of apoptosis repeat; CARD = caspase activation and recruitment domain; FIIND = function to find domain; NACHT = NAIP, CIITA, HET-T and TP-1; PYD = pyrin domain; X = unidentified; Yellow bars = leucine rich repeat. From [33].

Many different ligands are known to be recognized by and to activate NLRs and comprise peptidoglycan, flagellin, bacterial type III secretion system, anthrax lethal factor, cholesterol crystals, uric acid, alum, asbestos and silica. Upon activation, NLRs activate numerous downstream signaling mechanisms that can result in regulation of inflammation through the NF-κB and MAPK pathways, assembly and activation of inflammasomes (discussed below) **(Figure 2)** or transcription regulation, depending on the nature of the activating ligand as well as on the class of NLR involved in its recognition [33].

Two NLRC family members, NLRC1 and NLRC2, also known as NOD1 and NOD2, recognize components of bacterial cell wall and peptidoglycan [38,39]. In particular, NOD1 is activated by γ-D-glutamyl-meso-diaminopimelic acid (iE-DAP) from Gram-negative bacteria, while NOD2 recognizes muramyl dipeptide (MuDP) from Gram-positive and Gram-negative bacteria. Upon

ligand recognition, both the receptors induce the NF-κB and MAPK pathways by recruiting and activating the serine/threonine kinase RIP2 [40].

One of the most fascinating properties of several members of the NLR family is their ability to induce the assembly of a molecular complex known as inflammasome **(Figure 2)**. In general and simplistic terms, the inflammasome is a heteromeric protein scaffold in which an activated pyrin domain-containing member of the NLR family recruits the apoptosis-associated speck-like protein containing CARD (ASC) through a pyrin-pyrin domain interaction [35]. This complex recruits pro-caspase-1 through the CARD domain of ASC with consequent autocleavage-induced activation of caspase-1 [35]. Active caspase-1 mediates the activation, among several substrates, of IL-1β and IL-18 by cleavage of their inactive forms, pro-IL-1β and pro-IL-18 [41]. Additionally, it can induce a form of proinflammatory cell death called pyroptosis [41].

Numerous members of the NLR family can form an inflammasome upon activation. The best described are NLRP1, NLRP3 and NLRC4, but also NLRP6, NLRP7 and NLRP12 have this potential [35,42].

Several elements in the reality of pathogen sensing add much complexity to the regulation of inflammasome formation and activation. For example, the NLRP1 inflammasome might involve NOD2 in the recognition of the NLRP1 ligand MuDP [43,44]. Moreover, NLRP1 inflammasome does not strictly depend on ASC since NLRP1 is the only described NLRP to possess also a CARD domain that can directly recruit caspase-1 [45]. Another example is represented by NLRP3 which responds to a very vast range of pathogens and molecules among which viruses, bacteria, fungi, protozoa, uric acid, alum, asbestos and silica [46]. This broad specificity has been suggested to depend on the presence of co-receptors or upstream activators of NLRP3 rather than on direct recognition of these ligands by NLRP3 itself. Moreover, NLRP3 has been shown to induce the activation of caspase-1

through the formation of a caspase-11-dependent non-canonical inflammasome in response to Gram-negative bacteria [47–49]. Also the NLRC4 inflammasome shows a certain degree of complexity. NLRC4 is known to respond to flagellin and type III bacterial secretion system [50–52], but other members of the NLR family like NAIPs, which belong to the NLRB sub-family, could function as co-receptor for ligand recognition [53,54]. Moreover, NLRC4 lacks a pyrin domain and can form ASC-independent inflammasomes through direct recruitment of caspase-1. Nonetheless, NLRC4 can also participate in the formation of ASC-dependent inflammasomes. The ASC-dependent and ASC-independent NLRC4 inflammasomes have been found to favor respectively pro-inflammatory cytokine production and pyroptosis [34,55].

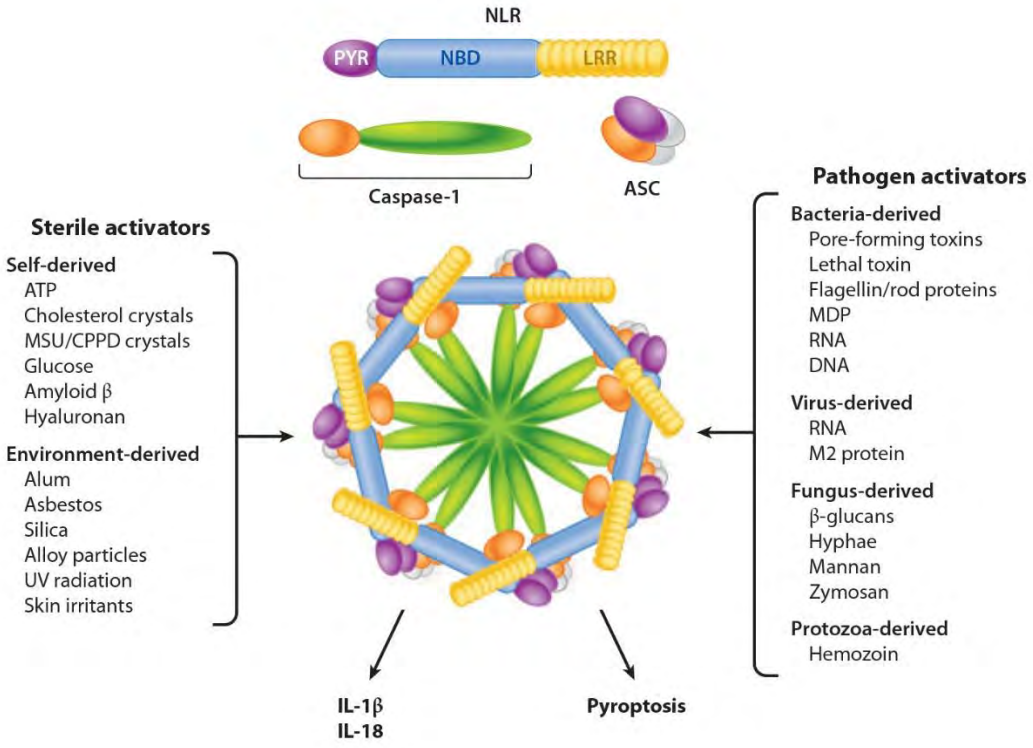

Figure 2. General structure of the NLRP inflammasome and examples of inflammasome activators. Inflammasome activation is induced by sterile activators as well as by host- and environment-derived molecules, including PAMPs from bacteria, viruses, fungi, and protozoa. The NLRP inflammasome is comprised of NLRPs, ASC, and caspase-1 assembled in a penta- or heptamer structure. Activation of the inflammasome leads to maturation and secretion of IL-1β and IL-18 and to inflammatory cell death by pyroptosis. ASC = apoptosis-associated speck-like protein containing a caspase recruitment domain;

CPPD = calcium pyrophosphate dihydrate; LRR = leucine-rich repeat; MDP = muramyl dipeptide; MSU = monosodium urate; NDB = nucleotide-binding domain (NACHT domain); PYR = pyrin domain. Adapted from [41].

The fourth sub-family of NLRs, NLRA comprises, so far, just one member also known as major histocompatibility complex (MHC) class II transactivator (CIITA) [36]. CIITA participates in a heteromeric protein complex that is formed on the MHC class II (MHC-II) promoter and regulates the expression of the MHC-II gene [56,57]. Similarly to CIITA, NLRC5 is another example of transactivating NLR that regulates MHC class I (MHC-I) expression by participating in a protein complex on the MHC-I promoter [58].

C-type lectin-like receptors - CLRs (reviewed in [59–62])

CLRs are a large family of soluble or transmembrane receptors that have been classified into 17 groups on the basis of their structure [63]. CLRs recognize carbohydrates like mannose, fucose and glucans through a Ca^{2+}-dependent or a Ca^{2+}-independent C-type lectin-like domain (CTLD) [60,64]. They are expressed by several groups of innate immune cells including DCs, macrophages, neutrophils and monocytes and allow the recognition of numerous types of pathogens comprising, in particular, fungi but also viruses, helminths and mycobacteria [59]. Some CLRs exert their function by enhancing or suppressing the signaling initiated by different PRRs [59], while other CLRs activate independently a direct signaling that results in the regulation of several effector functions like phagocytosis, respiratory burst, and production of the cytokines IL-23, IL-6, IL-1β and type I interferons (IFNs) [61,65,66]. An additional distinction can be made between CLRs that can signal directly through a cytoplasmic signaling domain and others that miss this domain and therefore need to recruit adaptor proteins [59,60].

Dectin-1 is one of the best characterized CLRs and is considered the prototypical example of CLR that signals directly through a cytoplasmic domain. Dectin-1 is activated by particulate but not soluble ligands through the recognition of β-glucans which are one of the main components of fungal cell-wall [67–69]. Ligand recognition by Dectin-1 triggers the activation of the spleen tyrosine kinase (SYK) through the cytoplasmic immunoreceptor tyrosine-based activation motif (ITAM) of Dectin-1 [70]. This, in turn, mediates the canonical and non-canonical nuclear factor (NF)-κB activation through the CARD9-Bcl10-Malt1 complex (CARD-containing protein 9 – B cell leukemia/lymphoma 10 – mucosa associated lymphoid tissue lymphoma translocation protein 1) [71,72]. Other downstream signaling targets of Dectin-1 activation include different mitogen-activated protein kinases (MAPKs), nuclear factor of activated T cells (NFAT), interferon regulatory factor (IRF) 1 and IRF5 [73–81]. Alternatively, in human DCs, Dectin-1 can induce a SYK-independent signaling that is mediated by an ITAM-mediated activation of v-raf-1 murine leukemia viral oncogene homolog 1 (Raf-1), resulting in the increased activation of the canonical NF-κB and the repression of the non-canonical NF-κB pathway [73]. Additionally, the SYK-dependent Dectin-1 signaling pathway has been reported to activate the canonical NLRP3-inflammasome, through the induction of reactive oxygen species production [82], and the non-canonical Malt-1-Caspase-8-ASC inflammasome [83–85].

Unlike Dectin-1, that signals directly through its ITAM-containing cytoplasmic domain, other CLRs like Dectin-2 need to recruit ITAM-containing adaptor proteins like the Fc receptor gamma chain (FcRγ) [86,87]. FcRγ activates a SYK-dependent signaling that induces the activation of the canonical NF-κB pathway, NFAT and MAPKs similarly to Dectin-1.

Examples of suppressive CLRs are the DC immunoreceptor (DCIR) and the myeloid inhibitory C-type lectin-like receptor (MICL) which have a cytoplasmic signaling domain containing an

immunoreceptor tyrosine-based inhibition motif (ITIM) which recruits phosphatases that inhibit cellular activation [64,88,89].

Other CLRs like Clec9A and CD205 can recognize DAMPs deriving from apoptotic or necrotic cells [90–92].

RIG-I-like receptors - RLRs (reviewed in [93,94])

RLRs are a small family of cytosolic sensors of foreign RNA from RNA viruses. The RLR family comprises three members: RIG-I, melanoma differentiation-associated gene 5 (MDA5) and laboratory of genetics and physiology 2 (LGP2) [95].

RIG-I and MDA5 have similar structural conformation **(Figure 3)**. Their N-terminal region contains a tandem CARD domain with signaling function, followed, in the central portion of the proteins, by a DExD/H box helicase domain with ATPase activity [95] and by a C-terminal domain involved in ligand binding and specificity [96].

In spite of this structural analogy, RIG-I and MDA5 have different ligand specificity. RIG-I recognizes the ends of short 5' tri-phosphorylated double strand (ds) RNA derived from viral genomes or from viral replication intermediates, but also single strand (ss) RNA with both secondary structure and tri-phosphorylation at the 5' end [97–102]. By contrast, MDA5 has a binding preference toward high molecular weight dsRNA [102–105].

Upon ligand recognition both RIG-I and MDA5 induce the activation of NF-kB, MAPKs and IRFs through a signaling cascade mediated by the CARD-containing adaptor mitochondrial antiviral signaling protein (MAVS). Therefore, ligand recognition by the RLRs triggers the induction of an antiviral state determined by the expression of type I IFNs, pro-inflammatory cytokines and IFN-stimulated genes (ISGs) [95,106–111].

LGP2 shares with the other two members of its receptor family a DExD/H box helicase domain with ATPase activity [95] **(Figure 3)**. It possesses a RIG-I-like RNA-binding C-terminal domain **(Figure 3)** that recognizes dsRNA blunt ends independently of the presence of a tri-phosphorylation [96,112–114]. However, the lack of an N-terminal signaling tandem CARD domain [95] **(Figure 3)** makes LGP2 unable of independent downstream signaling. By contrast, LGP2 is thought to have a functional role in the regulation of RIG-I and MDA5 activity [112–116].

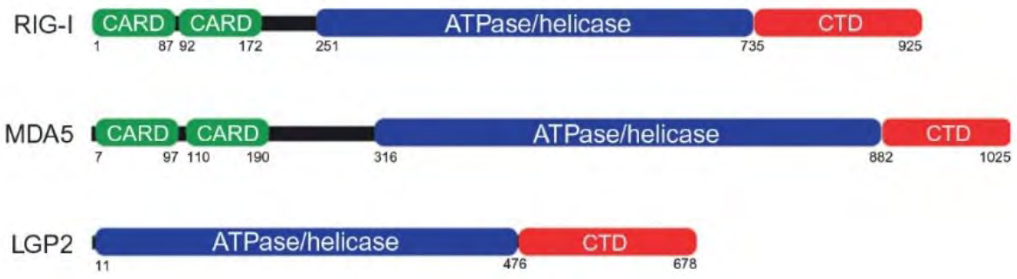

Figure 3. Structure of RLRs. CARD = caspase activation and recruitment domain; CTD = C-terminal domain. Adapted from [94].

AIM2-like receptors - ALRs (reviewed in [117])

ALRs are a family of proteins characterized by an N-terminal pyrin domain and one or multiple hematopoietic interferon-inducible nuclear antigen with 200 amino acid repeats (HIN) domains **(Figure 4)**. Because of these distinctive structural features, they are also indicated as PYHIN family [118,119]. So far, only two ALRs have shown PRR activity: AIM2 and the IFNγ-inducible protein 16 (IFI16) (considered the human ortholog of murine IFI204 [120] **(Figure 4)**) [121–123]. AIM2 and IFI16 are structurally different since AIM2 possesses a single C-terminal HIN domain, while IFI16 contains two of them [124] **(Figure 4)**.

AIM2 localizes in the cytosol and is activated by direct sequence-independent binding of cytoplasmic dsDNA from viral origin. Upon ligand recognition, multiple AIM2 molecules oligomerize on the

dsDNA and recruit multiple ASC molecules which, in turn, recruit caspase-1 to form an active AIM2-dependent inflammasome with consequent production of IL-1β and IL-18 [85,122,125,126].

In contrast to AIM2, IFI16 has predominantly a nuclear localization [127–129]. However, it functions as a sensor of foreign dsDNA both in the cytoplasm and in the nucleus [127,130,131]. IFI16 recognizes dsDNA in a sequence-independent but length-dependent way [132]. The cooperative oligomerization of multiple IFI16 molecules on a long stretch of naked dsDNA triggers the activation of the downstream signaling cascade [132]. Activated IFI16 recruits the stimulator of IFN genes (STING) which upon activation induces the production of type I IFNs and pro-inflammatory cytokines through activation of IRF3 and NF-κB [123,131,133–136]. Additionally, IFI16 has been shown to be able to interact with ASC and pro-caspase-1 to form a functional inflammasome [137].

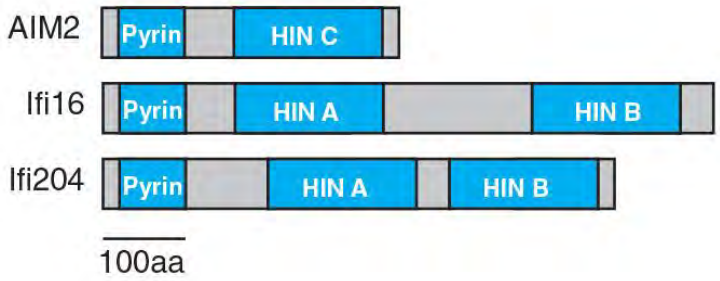

Figure 4. Structure of AIM2, IFI16 and IFI204. Three ALRs have been described to function as PRRs, namely AIM2, IFI16 and the murine paralog of this latter, IFI204. These three proteins belong to the PYHIN family. PYHIN proteins consist of a pyrin domain and at least one HIN domain. HIN domains can be divided into three subtypes, HIN A, HIN B and HIN C, on the basis of their conserved amino acid content. HIN = hematopoietic interferon-inducible nuclear antigen with 200 amino acid repeats. Adapted from [124].

OAS-like receptors - OLRs (reviewed in [32])

OLRs are a class of cytosolic nucleic acid sensors that in human comprises the 2'-5'-oligoadenilate synthase proteins (OAS) OAS1, OAS2, OAS3 and OASL and includes the structurally related cyclic-GMP-AMP (cGAMP) synthase (cGAS) [138,139].

OAS1, OAS2 and OAS3 are activated by dsRNAs and, upon recognition of their ligands, they catalyze the synthesis of 2'-5' oligoadenylates [139]. These products are able, in turn, to activate RNase L that degrades RNA and consequently inhibits protein synthesis [140,141]. OASL is the only member of this family to lack the oligoadenylate synthase enzymatic activity and, rather than directly inducing downstream-effector activation, it enhances the activation of the RLR RIG-I through its ubiquitin-like domain [142].

The recently identified DNA receptor cGAS is structurally related to the OAS proteins [139]. This enzyme binds free DNA in the cytosol and uses it as a substrate to produce cGAMP [143]. The downstream signaling cascade is mediated by STING that upon recognition of cGAMP induces the activation of IRF3 and NF-κB [134,135,144].

Independently of its role as adaptor, STING has been identified as a receptor of bacterial cyclic dinucleotides (CDNs) which are commonly produced by bacteria as second messengers [145,146]. Similarly to cGAMP, binding of CDNs activates STING and triggers its downstream signaling [146].

Toll-like receptors - TLRs (reviewed in [147,148])

TLRs are one of the most studied and best characterized families of PRRs. In mouse, there are 12 expressed TLRs, namely TLR1-9 and TLR11-13 [149–151], that are structurally similar to each other but differ in terms of cellular localization, binding specificity and downstream signaling.

All the TLRs are integral membrane proteins composed of a horseshoe-shaped leucine-rich ectodomain, which interacts with the TLR ligand, and a cytoplasmic tail that, upon TLR activation, initiates the downstream signaling cascade through its Toll/Interleukin-1 Receptor (TIR) domain [149–152] **(Figure 5)**.

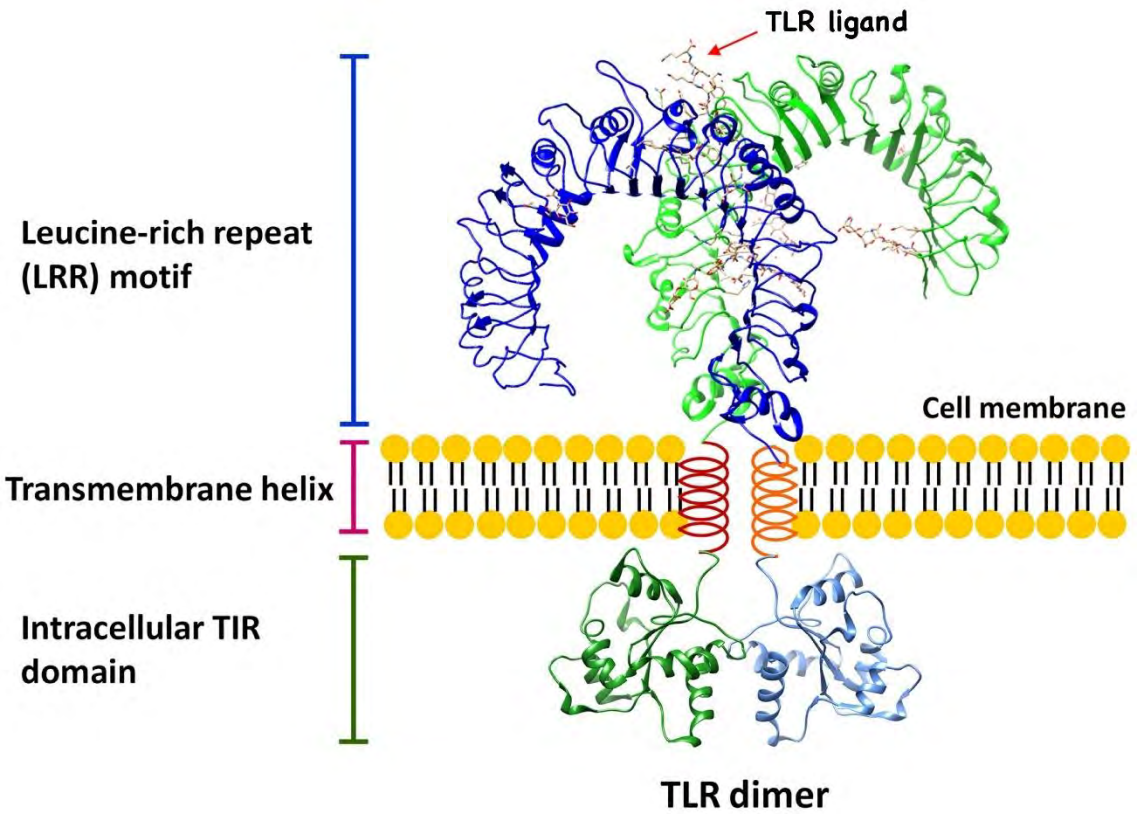

Figure 5. General structure of a TLR dimer. The LRR structure is based on the model of TLR1-TLR2 heterodimer (Protein Data Bank, PDB, ID: 2z7x) interacting with 6 tri-acylated lipopeptides, Pam3CSK4. The TIR domain homology model is based on TLR2 TIR structure (PDB ID: 1fyw). LRR = leucine-rich repeat; TIR = Toll/Interleukin-1 Receptor. Adapted from [153].

A first broad classification of TLRs can be made on the basis of their intracellular or extracellular localization. TLR1, TLR2, TLR4, TLR5 and TLR6 are expressed on the cell surface, while TLR3, TLR7, TLR8, TLR9, TLR11, TLR12 and TLR13 localize in the endosome [149,150].

TLRs interact with their ligands as homo- or heterodimers. In particular TLR2 dimerizes with TLR1 or TLR6, while all the other TLRs form mainly homodimers [149–152]. Each TLR dimer has different binding specificity. Within the group of surface TLRs, TLR1/2 and TLR2/6 heterodimers recognize a wide spectrum of cell wall components like triacylated or diacylated lipoproteins respectively, peptidoglycan, lipoteichoic acid, lipoarabinomannan and zymosan, TLR4 binds to lipopolysaccharide (LPS), which is present in the outer cell membrane of Gram-negative bacteria, and TLR5 recognizes flagellin, the main component of bacterial flagella [149–152]. Endosomal TLR signaling is mainly triggered by nucleic acids, with TLR3 sensing double strand RNAs, TLR7 and TLR8 recognizing single strand RNAs and TLR9 binding to unmethylated-CpG motif-containing DNAs of bacterial or viral origin [149–152]. TLR11, TLR12 and TLR13 are a very specialized subgroup of endosomal TLRs expressed in mouse but not functional (TLR11) or completely absent (TLR12 and 13) in human [154]. TLR11 and TLR12 mediate the recognition of *Toxoplasma gondii* profilin and can bind to their ligand as homo- or heterodimers [154]. TLR13 has been recently determined to specifically sense the 23S ribosomal RNA of the bacterial ribosome [155,156].

Binding of a TLR to its ligand induces a signaling cascade **(Figure 6)** which culminates in the expression or the up-regulation of a wide variety of effectors comprising pro-inflammatory cytokines and chemokines. Ligand-induced dimerization of the TLR ectodomains leads to the recruitment of adaptor proteins to the cytoplasmic tails of the receptors through a TIR-TIR interaction. Four adaptor proteins have been described: myeloid differentiation primary response gene 88 (MyD88), MyD88 adaptor-like (MAL) (also called TIR-domain containing adaptor protein (TIRAP)), TIR-domain-containing adaptor-inducing interferon-β (TRIF) and TRIF-related adaptor molecule (TRAM). Every TLR signals through one or more specific adaptor proteins among which MyD88 and TRIF are the most important mediators of TLR signaling. On this basis, TLRs are classified as MyD88- or TRIF-dependent. In particular, all the TLRs except TLR3 signal through MyD88

(together with MAL in the case of TLR1/2 and TLR2/6 heterodimers). On the contrary, TLR3 signals exclusively through the TRIF-dependent pathway. TLR4 is the only TLR that can signal trough both MyD88 and TRIF in association with MAL or TRAM, respectively [149–152]. Triggering of the MyD88-dependent pathway induces a signaling cascade which leads to phosphorylation and activation of the IκB kinase (IKK) complex, which comprises the subunits IKKα, IKKβ and IKKγ, and of mitogen-activated protein kinases (MAPKs) such as ERK1/2, c-Jun terminal kinase (JNK) and p38. Activated IKK phosphorylates the NF-κB inhibitor IκB with consequent release and nuclear translocation of NF-κB. Moreover, activated MAPKs phosphorylate and activate the AP-1 transcription factor family. Overall, the activation of AP-1 and NF-κB leads to the transcription of pro-inflammatory cytokines like IL-6, TNFα, IL-1β and IL-12. Additionally, in a pathway that is specific just for one subset of DCs, the plasmacytoid DCs (discussed in paragraph *1.3.3. Developmental and functional heterogeneity of DC subsets*), IRF7 can be recruited directly through activation of the MyD88-dependent pathway and phosphorylated by IKKα. Phosphorylated IRF7 moves to the nucleus and activates the transcription of IFNα and IFNβ [147,149,150,152].The TRIF-dependent pathway, on the other hand, is mainly involved in the phosphorylation of IRF3, which consequently activates transcription of type I IFNs [149,150,152]. Nevertheless, TRIF can also drive the activation of IKK, with consequent translocation to the nucleus and activation of NF-κB [152].

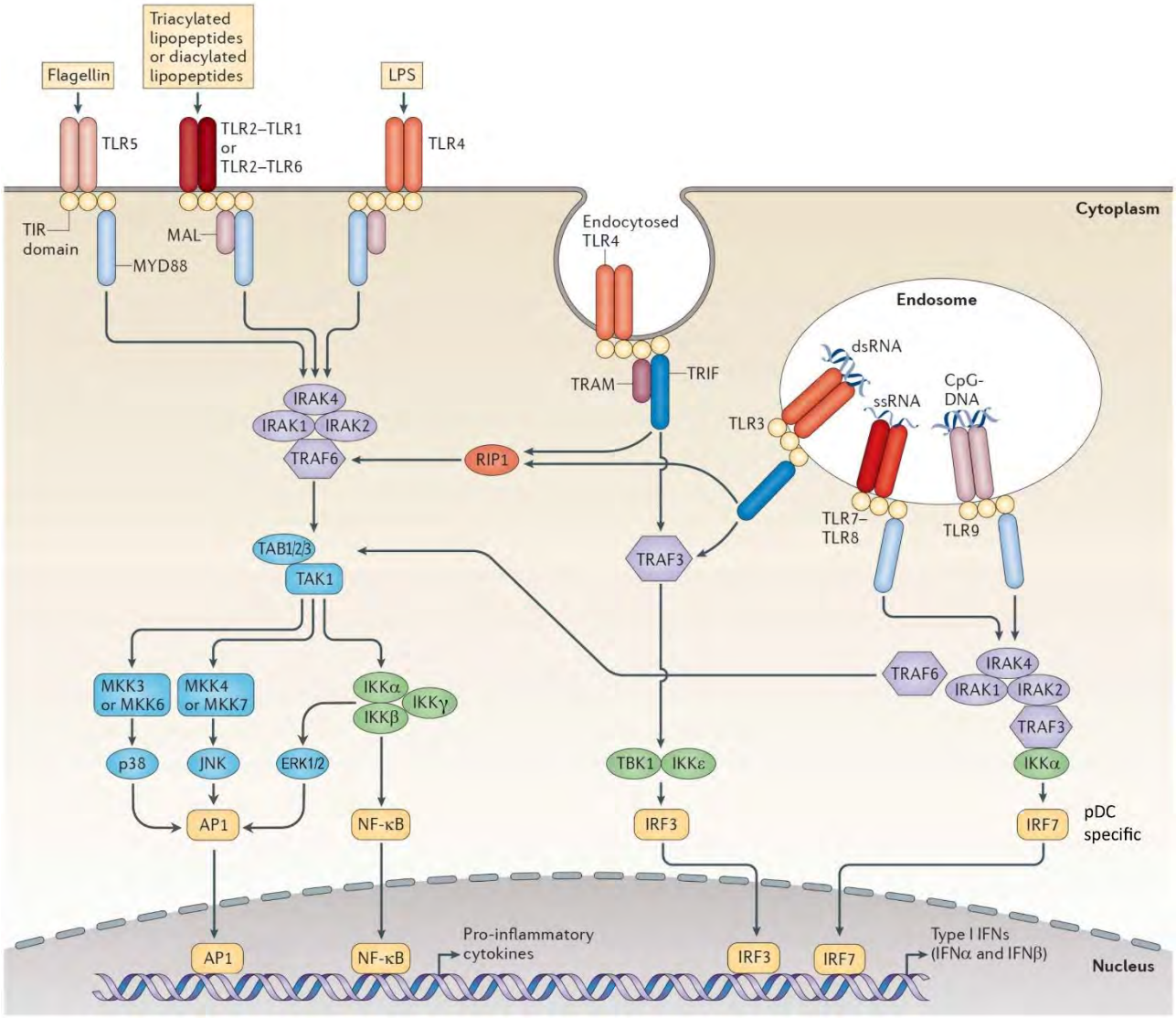

Figure 6. TLR localization and signaling pathways in mouse and human. TLR5, TLR4, and the heterodimers of TLR2–TLR1 or TLR2–TLR6 localize on the cell surface, whereas TLR3, TLR7–TLR8, and TLR9 localize in the endosomes where they sense microbial and host-derived nucleic acids. TLR4 localizes at both the plasma membrane and the endosomes. TLR signaling is initiated by ligand induced dimerization of receptors. Following this, the TIR domains of TLRs engage the TIR domain-containing adaptor proteins MyD88 and MAL or TRIF and TRAM. TLR4 moves from the plasma membrane to the endosomes in order to switch signaling from MyD88 to TRIF. Engagement of the signaling adaptor molecules stimulates downstream signaling pathways that lead to the activation of the MAPKs JNK, p38 and ERK1/2 and to the activation of transcription factors. Two families of transcription factors that are activated downstream of TLR signaling are NF-κB and IRFs, but other transcription factors, such as AP-1, are also important. A major consequence of TLR signaling is the induction of pro-inflammatory cytokines, and in the case of the endosomal TLRs, the induction of type I IFN. AP-1 = activator protein 1; dsRNA = double stranded RNA; ERK = extracellular signal-regulated kinase; IKK = inhibitor of NF-κB

kinase; IRAK = IL-1R-associated kinase; IRF = interferon regulatory factor; JNK = JUN N-terminal kinase; LPS = lipopolysaccharide; MAL = MYD88-adaptor-like protein; MKK = MAP kinase kinase; MyD88 = myeloid differentiation primary-response protein 88; NF-κB = nuclear factor-κB; RIP1 = receptor-interacting protein 1; rRNA = ribosomal RNA; ssRNA = single-stranded RNA; TAB = TAK1-binding protein; TAK = TGFβ-activated kinase; TBK1 = TANK-binding kinase 1; TIR= Toll–IL-1-resistence; TRAF = TNF receptor-associated factor; TRAM = TRIF-related adaptor molecule; TRIF = TIR domain-containing adaptor protein inducing IFNβ. Adapted from [151] on the basis of KEGG Toll-like receptor signaling pathway – Mus musculus (mouse) - 04620 8/28/17 © Kanehisa Laboratories.

1.2.2. Signaling cross-talk between simultaneously engaged PRRs

As mentioned, the innate immune system has more limited pathogen specificity than the adaptive immune system. Nevertheless, the innate immune cells are able to discriminate separate classes of pathogens and, on this basis, to control the induction of the most appropriate kind of response for each of them. Since distinct microorganisms can share some structural characteristics but, at the same time, differ in their infection mechanism, a first level of control is implicit in the recognition of the same PAMPs at different cellular locations. For example, flagellin can be recognized by TLR5 in the extracellular environment and by NLRC4 in the cytoplasm, with the activation of separate signaling pathways and the consequent induction of different effector mechanisms. However, in the context of an infection by single or multiple pathogens, numerous PAMPs are concurrently present and exposed, making it very likely for different PRRs to be simultaneously or sequentially activated by their ligands. Even if the downstream signaling pathways described for many PRRs have been characterized individually, the study of concomitant activation of several PRRs has highlighted that these pathways do not behave just as isolated signaling units but instead they integrate with each other at various levels. This molecular cross-talk between different PRR-induced signaling pathways promotes qualitatively and quantitatively [157–165] distinct effector mechanisms that are selectively activated by the specific combinations of detected ligands rather than by the individual

ligands. Moreover, different immune cells can respond differently to the same combination of PRR ligands, adding a further level of complexity to the regulation of PRR activation and innate immune responses [166–171].

The numerous examples of PRR-signaling integration that have been described can be divided into three general classes on the basis of the mode of interaction.

The first of these classes includes a type of interaction that can be defined as dependent or consequential, namely all that cases in which the possibility to activate one PRR signaling pathway strictly depends on the prior activation of a distinct PRR. One example of this mode of integration is represented by the NLRP3 inflammasome whose function in mouse macrophages depends on the activation of the NF-kB signaling pathway through other PRRs, like a TLR [35,172,173] or Dectin-1 [82], to induce the expression of NLRP3 itself and of the inactive pro-forms of IL-1β and IL-18 that are among the final targets of cleavage by caspase-1.

The second class of signal integration mechanisms can be defined inhibitory since it includes the cases in which the induction and activation of downstream effectors mediated by one PRR is partially or completely blocked by the activation of a second PRR. Several works have shown such kind of interactions. For example, RIG-I can inhibit TLR-mediated IL-12 production through activation of IRF3 that binds on IL-12 promoter and blocks transcription of this gene [174]. NLRX1 has been shown to attenuate TLR-induced NF-kB activation by physically interacting with TRAF6 and IKK [175,176], and to reduce RIG-I signaling by directly interacting with MAVS [176]. Similarly, NLRP6 negatively regulates TLR-induced MAPK and NF-kB activation by influencing the phosphorylation state of IkB and ERK, and NLRC3 reduces NF-kB activation by affecting the ubiquitination and activation of TRAF6 [177]. Also, NLRC3 inhibits STING-mediated downstream signaling by blocking

STING trafficking upon activation [178]. Finally, the autocrine effect of TLR2-induced IL-10 production has been shown to block TLR3- or TLR4-mediated IL-12 production [179].

The third class of PRR signal integration mechanisms can be named synergistic PRR activation and comprises all the cases in which the simultaneous activation of different PRRs induces a quantitatively greater-than-additive response if compared with the response of the single receptors. Also in this case, several examples are known. NOD1 and NOD2 synergize with TLR3, TLR4 and TLR9 for cytokine production [180–182]. Simultaneous activation of Dectin-1 and TLR2 induces synergistic production of TNF by increasing IkB degradation and, therefore, by enhancing NF-kB activation [166,183]. TLR9 is synergistic with STING for the production of IL-12 and type I IFNs [159]. However, one of the best-known examples of synergistic PRR activation is the positive interaction between TLR-dependent signaling pathways that induces synergistic production of pro-inflammatory cytokines [157,160–162,165,170,184,185]. Not every possible TLR-ligand combination shows this cooperative effect. Indeed, several studies have demonstrated the requirement of a cross-talk between the MyD88-dependent (all TLRs except TLR3) and the TRIF-dependent (TLR3 and TLR4) signaling pathways to trigger a synergistic response to TLR ligands [157,161,169,184,186,187]. Interestingly, not only the composition of the ligand combination, but also the temporal sequence of encounter with the single ligands play a non-negligible role in the synergistic activation [157,162,169,186]. A similar effect can be observed after treatment with combinations of single TLR ligands with other host-derived signals like IFNγ or CD40L [157,188,189]. Treatment with a combination of synergistic TLR ligands and host-derived supplementary signals can sustain and further increase the synergistic effect, in a way which has been referred to as super-activation or super-synergy [157,186].

Even though, as discussed, numerous examples of PRR cooperative activation are known, the molecular details of these signal-integration processes are in most cases still elusive. Further studies

are needed to precisely describe the molecular aspects of PRR-signal integration. However, in many cases, these mechanisms appear to be mediated by autocrine cytokine effects [179], by differential post-translational modification of PRR-signaling mediators [165] and by regulation of effector-gene transcription through cooperation of differentially activated transcription factors [190].

1.3. Overview of DC biology

By virtue of their functions, DCs have a central role in linking innate and adaptive immunity (reviewed in [23]). DC research is very active because of the clear implications that the role of DCs has in several fields like the study of the mechanisms of autoimmune diseases, the creation of new vaccines and the development of new approaches for the treatment of infectious diseases and for cancer immunotherapy. This thesis describes the development of a new DC line and provides examples of the use of *in vitro* models of DCs for the study of DC biology. Therefore, the next paragraphs will focus on the molecular and biological details of DC ontogeny, phenotype and function.

1.3.1. Mechanisms of antigen capture, processing and presentation by DCs

DC are considered the most specialized APCs due to their unmatched ability to capture antigens from a broad variety of sources and to process them for presentation to $CD8^+$ or $CD4^+$ T cells.

In order to be presented to T cells, antigens are processed through a partial degradation followed by loading of the resulting products into molecular complexes with proteins of the MHC family, which are essential for antigen recognition by the TCR [191]. Protein-derived antigens are presented by means of two structurally related protein complexes, namely MHC-I and MHC-II, whose loading with antigenic peptides represents the result of three distinct antigen processing/presentation pathways known as MHC-I presentation, MHC-II presentation and MHC-I cross-presentation [191]. Typically, peptides derived from proteins of intracellular origin are loaded on MHC-I molecules

which selectively mediate antigen recognition by the TCR of $CD8^+$ T cells [191]. Therefore, this presentation pathway concerns all the self-proteins expressed by the DC, but also all the foreign antigenic proteins that are synthesized in the cytoplasm of intracellular-pathogen-infected cells as a part of the life/infection cycle of the invading organism [191]. By contrast, the classic antigen presentation pathway for extracellular antigens is mediated by MHC-II which interacts specifically with $CD4^+$ T cells [192]. A third possibility of antigen processing and presentation is represented by the so-called MHC-I cross-presentation which is an alternative pathway that mediates loading of extracellular antigens on the MHC-I complex [193].

MHC-I presentation **(Figure 7)**

In the classic MHC-I presentation pathway, cytosolic peptides derived mainly from incomplete or misfolded proteins, also known as defective ribosomal products (DRiPs), are degraded by the activity of the proteasome [194]. The proteasome-cleaved protein fragments are then translocated to the endoplasmic reticulum (ER) through the ATP-dependent transporter associated with antigen processing (TAP) [195]. Here, the peptides can undergo further processing through the activity of the ER aminopeptidase associated with antigen processing (ERAAP) which trims the protein fragments to satisfy the peptide-length requirements of 8-10 amino acids needed for loading on MHC-I molecules [196,197]. In the ER, TAP recruits newly synthesized MHC-I molecules and several chaperones and enzymes to form a protein complex known as peptide loading complex (PLC) which facilitates peptide loading on MHC-I and stabilizes, until peptide binding, the otherwise unstable unloaded MHC-I [191]. Within this molecular context, MHC-I loading takes place through a quality control-guided mechanism that is regulated by the glucosylation state of the N-terminal extremity of MHC-I [191]. Indeed, the glucosylated unloaded MHC-I has high affinity for the PLC.

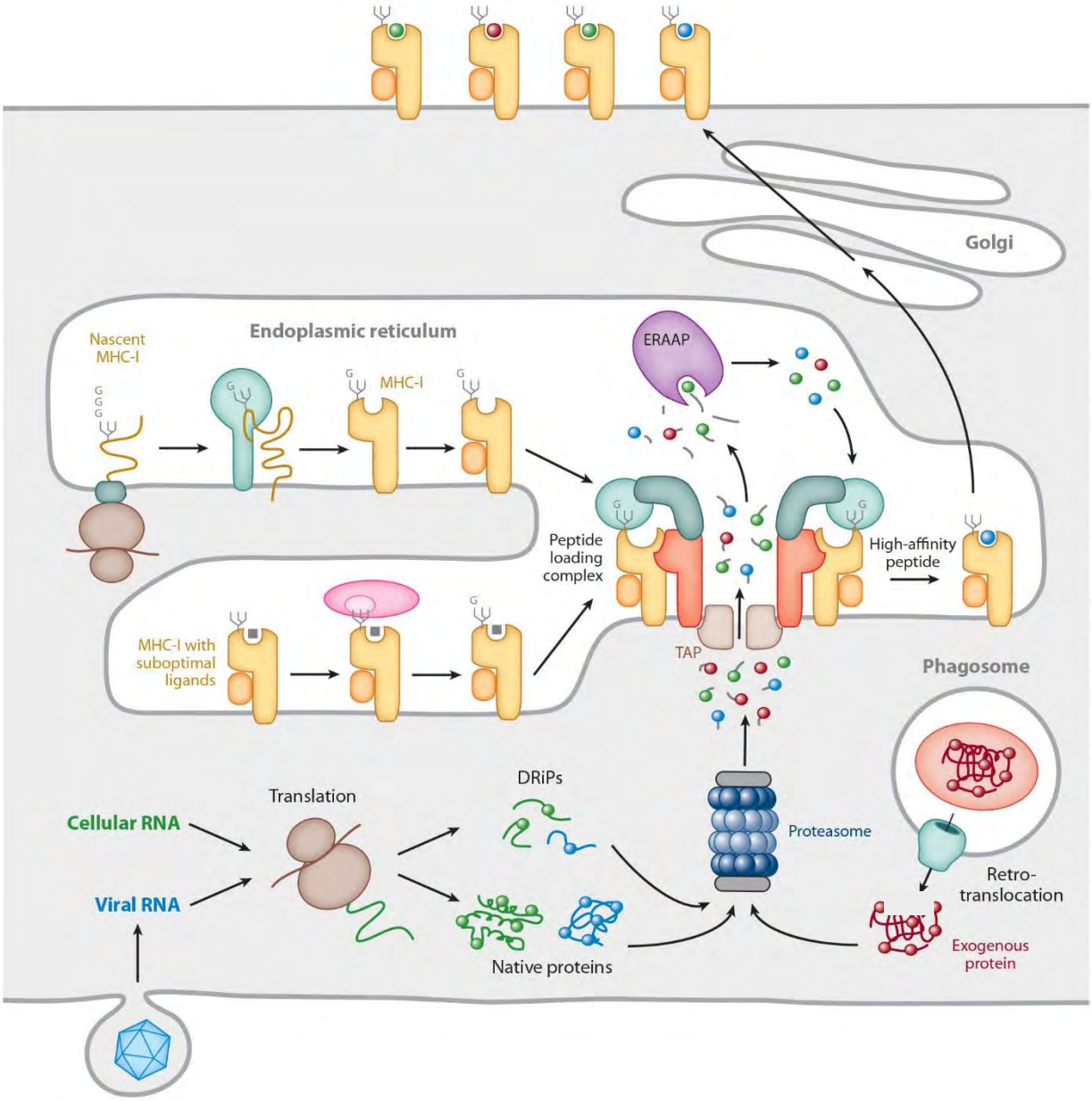

Figure 7. Processing and loading of antigenic peptides on MHC-I for MHC-I presentation or for the cytosolic pathway of MHC-I cross-presentation. A single N-linked terminal glucose residue (G) permits the interaction of MHC-I with chaperones at several stages during its folding and assembly. The empty MHC-I, which is inherently unstable, is then recruited in the peptide loading complex (PLC). The association of MHC-I with the PLC both stabilizes the empty MHC-I molecule and maintains the binding groove in a conformation that favors high-affinity peptide loading. During this step, the N-glucosylation is removed by a specific glucosidase. MHC-I molecules with suboptimal peptides are reglucosylated allowing reentry of the MHC-I into the PLC and exchange for high-affinity peptides. Peptides translocated into the ER by the transporter associated with antigen processing (TAP) originate primarily from the proteasomal degradation of endogenous proteins or DRiPs. These proteins may arise from the translation of either self or foreign (i.e., viral) RNA or, in the case of cross-presentation,

derive from translocation of endosomal or phagosomal proteins into the cytosol. Many of the peptides that are delivered into the ER are longer than the 8–10 residues preferred by MHC-I molecules and undergo trimming by the ER aminopeptidase known as ERAAP. MHC-I:peptide complexes are released and then transit to the cell surface for recognition by CD8+ T cells. Adapted from [191].

Upon peptide binding, the glucose residue on MHC-I becomes accessible to a glucosidase that removes it. These events cause a reduction of MHC-I affinity for the PLC. Low affinity peptides can be lost because of weak interaction with MHC-I or due to recognition of the low-affinity-peptide:MHC-I complex as faulty. In both cases the peptide leaves the MHC-I binding-groove and MHC-I is re-glucosylated. The absence of a peptide in the binding groove and the re-glucosylation restore the affinity of MHC-I for the PLC leading to a new cycle of peptide loading. High affinity peptide:MHC-I complexes are transported to the cell membrane and exposed on the surface for presentation to $CD8^+$ T cells.

Mechanisms of antigen internalization **(Figure 8)**

While the surface exposure of MHC-I complexes with cytoplasmic-antigen-derived peptides is a process shared by virtually any nucleated cell in the body, one of the most characterizing features of APCs is their ability to internalize, or endocytose (from ancient Greek: *éndon* = within, *kytos* = cell), antigens from the extracellular environment and to direct them into a specialized pathway whose final goal is their presentation to $CD4^+$ T cells through a peptide:MHC-II protein complex [192]. Several mechanisms allow APCs to internalize antigens from the extracellular environment, but the predominant ones are receptor-mediated endocytosis, phagocytosis and macropinocytosis [192].

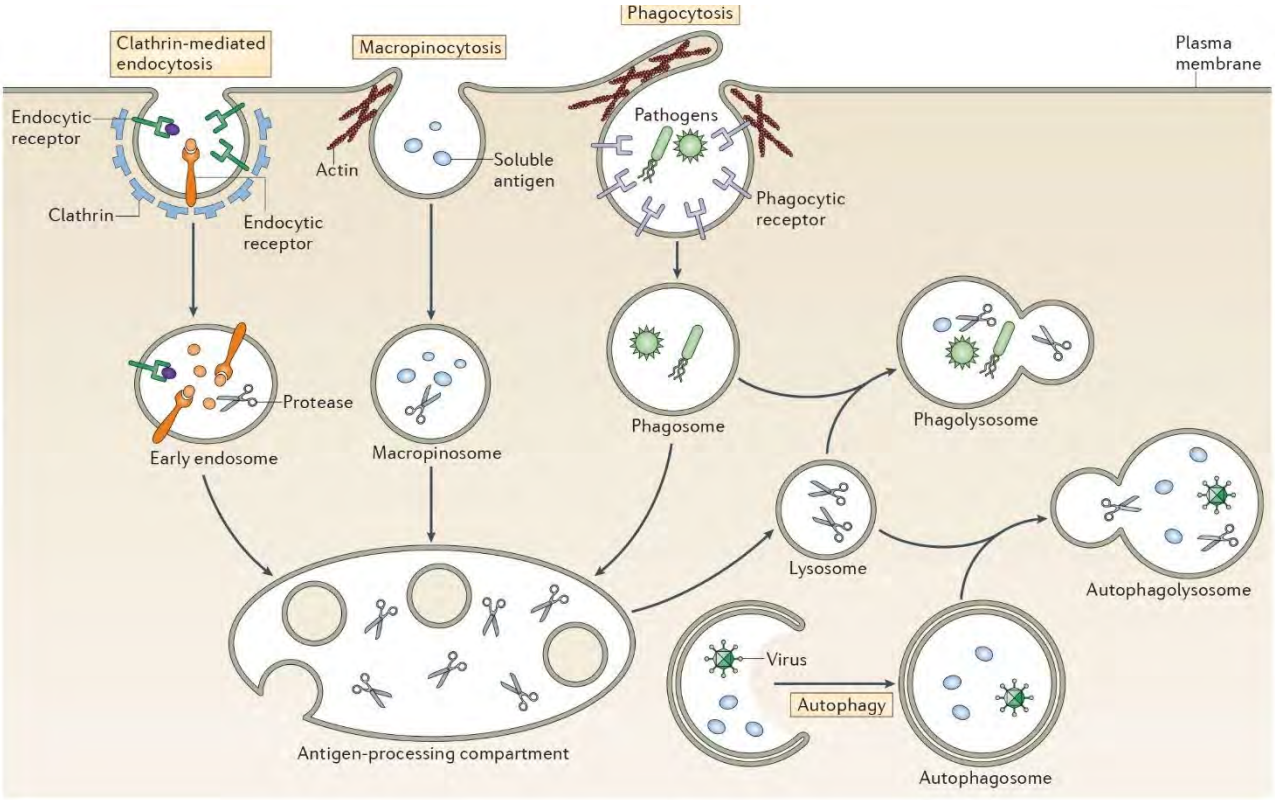

Figure 8. Pathways of antigen internalization in APCs. Antigens can enter the endocytic pathway of antigen-presenting cells (APCs) through several distinct mechanisms. Receptor-mediated endocytosis via clathrin-coated vesicles requires antigen binding to endocytic receptors on APCs which results in their internalization into early endosomes. Macropinocytosis is an actin-dependent process that leads to the uptake of soluble material into the cell in a macropinosome. In each of these processes, the internalized early endosomes eventually fuse with multivesicular late endosomal–lysosomal antigen-processing compartments. It is in these compartments that internalized-antigen proteolysis and peptide:MHC-II-complex formation take place. Phagocytosis is an endocytic process in which free or receptor-bound particles enter the cells through membrane-derived phagosomes that are formed by actin-dependent membrane reorganization. Phagosomes are not particularly rich in proteases or MHC-II and, after fusion with lysosomes or potentially with MHC-II-containing late endosomal–lysosomal compartments, the resulting phagolysosomes generate peptide:MHC-II complexes. Autophagy is a process by which membranes, often derived from the endoplasmic reticulum (ER), envelop cytosolic antigens to form an autophagosome. Upon autophagosome fusion with lysosomal compartments, the resulting autophagolysosome generates peptide:MHC-II complexes. Adapted from [192].

Numerous surface receptors in APCs can induce the activation of receptor-mediated endocytosis. Examples are the Fcγ receptors, which recognize opsonized antigenic structures, the two C-type

lectin family members CD205, which detect ligands respectively from necrotic or apoptotic cells, and mannose receptor, which binds specific glycan residues on glycoproteins [192]. Ligand-bound receptors are recruited and clustered in specific areas of the cell membrane which are coated with clathrin on the cytoplasmic side. Here, multiple molecules of clathrin interact with each other to form a concave three-dimensional scaffold that bends the cell membrane toward the cytoplasm. The extremities of the intruding bubble are eventually fused together to release a clathrin coated vesicle within the cytoplasm. At this point, the clathrin coating dissociates from the vesicle and releases it for trafficking in the cytoplasm [198]. All the primary endosomal vesicles fuse with an organelle called the early endosome from which the endocytosed material can be recycled to the cell membrane or directed to degradation. The degradative branch of the process of endocytosis and vesicle trafficking entails a sequence of passages during which the luminal pH of the vesicles is progressively reduced and simultaneously the recruitment and the activation of proteolytic enzymes are induced. The early endosome matures to become a late endosome which has a lower pH, thanks to the activity of ATP-dependent protonic pumps, and contains proteases that are activated in an acidic environment. Moreover, the late endosome is characterized by a more complex structure than the early endosome since its lumen contains numerous intra luminal vesicles (ILVs) that are constantly formed from its outer membrane. For this reason, the late endosome represents a form of multivesicular body (MVB). The last event of endosome maturation is accomplished when the late endosome is fused to the lysosome in which the pH is lower than in the late endosome and several additional proteases are present and active. Fusion of the late endosome with the lysosome leads to the final degradation of the endocytosed material [199,200].

Phagocytosis is a clathrin-independent endocytic mechanism that can be mediated by surface receptors and that allows phagocytic cells to internalize large particulate extracellular materials like bacteria, viruses and components of necrotic or apoptotic cells. The internalized material is held

within a vesicle named phagosome that undergoes a process of maturation and eventually fuses with lysosomes to form the phagolysosome in which the low pH and the lysosomal proteases can degrade the vesicular content [201,202].

Macropinocytosis is a nonspecific actin-dependent endocytic mechanism that mediates the internalization of large quantities of soluble and particulate extracellular material into vesicles called pinosomes that are formed by ruffling and extrusion of the plasma membrane. Similarly to endosomes and phagosomes, also pinosomes mature and fuse to lysosomes leading to the degradation of their content. Macropinocytosis is a process of primary importance in APCs and especially in DCs where it is constitutively active allowing them to constantly sample their surrounding microenvironment [203,204].

MHC-II presentation **(Figure 9)**

As discussed, in APCs, the extracellular materials internalized through endocytic mechanisms can be processed and loaded on MHC-II molecules for presentation to $CD4^+$ T cells.

Peptide generation and loading on MHC-II takes place within the so-called MHC-II compartment, also indicated as MIIC, which is an MVB with characteristics of late endosomes [192]. MHC-II is synthesized in the ER with the help of the chaperone CD74, also known as invariant chain (Ii) [205]. Ii is a transmembrane protein that occupies the peptide binding groove of MHC-II. By doing so Ii stabilizes the complex since MHC-II, similarly to MHC-I, is rather unstable when unloaded [206]. The cytoplasmic domain of Ii contains a signal sequence that targets MHC-II on the cell surface or directly to the MIIC [207–209]. The Ii:MHC-II complex expressed on the cell surface is rapidly internalized through clathrin-dependent endocytosis and then it is directed to the endocytic compartment [210]. The acidic conditions in the endocytic vesicles promote a partial degradation of Ii that leaves a peptide of approximately 20 amino acids, called class II associated Ii peptide (CLIP), in the binding

groove of MHC-II [191,192]. In the MIIC, the internalized extracellular proteins are cleaved by lysosomal-proteases [211] into peptides of about 18-20 amino acids, which is the optimal length for peptide binding to MHC-II [212–214]. The chaperone H2-DM, which is able to remove low affinity peptides from MHC-II, promotes the dissociation of CLIP from MHC-II and helps loading MHC-II with the antigenic peptides of endocytic origin [215,216]. Eventually, the peptide:MHC-II complexes are targeted to the cell membrane for presentation to $CD4^+$ T cells. From here, peptide:MHC-II complexes can also be recycled through the endocytic compartment and therefore MHC-II molecules can undergo multiple cycles of peptide-loading and transport on the surface for antigen presentation [192].

Even if the most part of the peptides presented through MHC-II are of extracellular origin, a considerable fraction of peptide:MHC-II complexes contains peptides of intracellular origin [217]. Presentation of intracellular or self-antigens on MHC-II molecules can be achieved through phagocytosis of components derived from apoptotic or necrotic cells [191,192]. However, one of the main routes of intracellular-antigen acquisition for MHC-II presentation is represented by the mechanism of autophagy [217,218] **(Figure 8)**. The endocytic process of autophagy strongly resembles the mechanism of phagocytosis. However, during autophagy, intracellular components, including soluble fractions, protein aggregates and even entire organelles, are captured within vesicles known as autophagosomes that originate from the ER. Similarly to other endocytic pathways, autophagosomes eventually fuse with lysosomes to form the autolysosomes where the vesicular content is degraded and recycled or directed to the MHC-II presentation pathway [217,218].

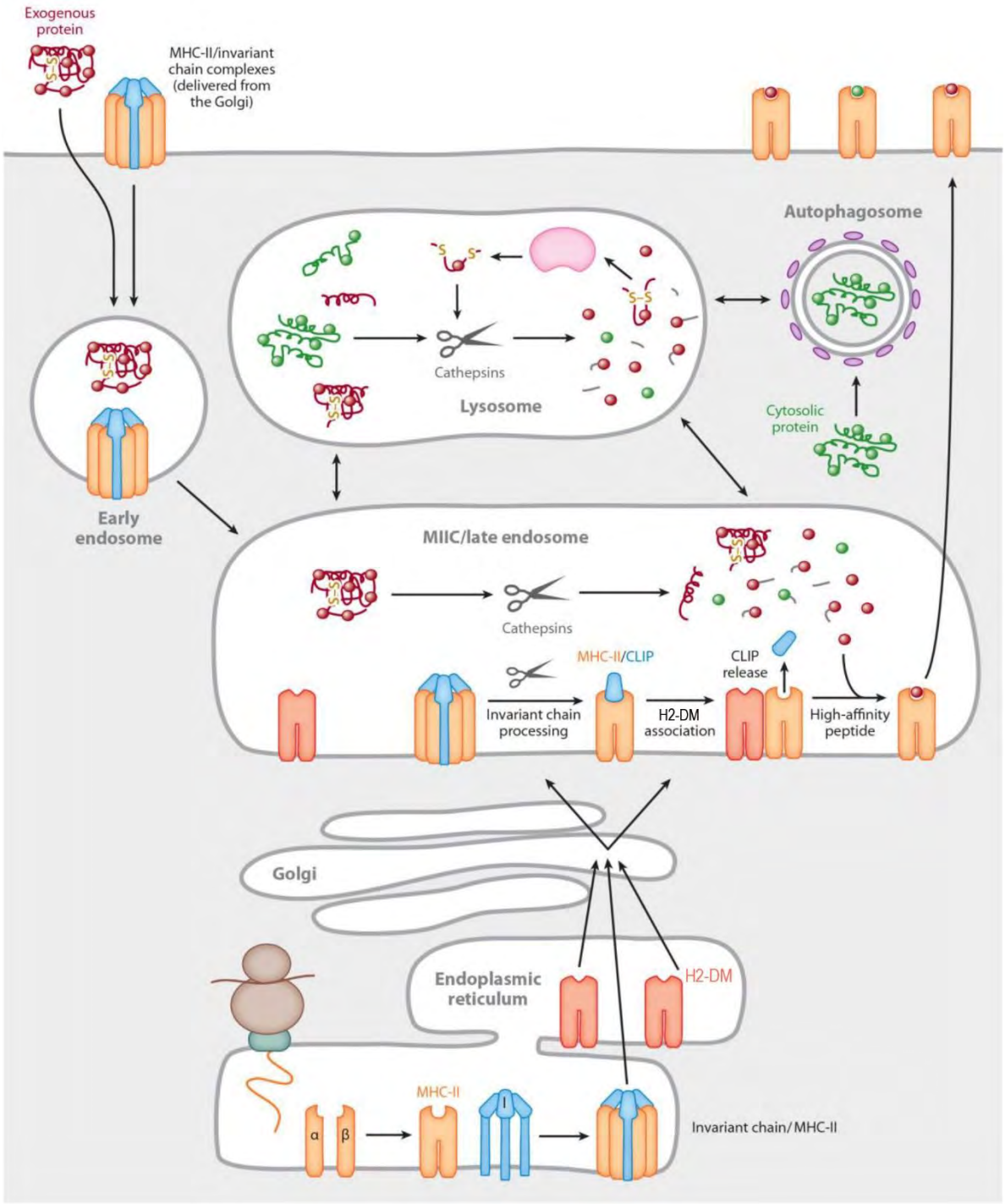

Figure 9. Processing and loading of internalized antigens on MHC-II for antigen presentation to CD4⁺ T cells. MHC-II associates with invariant chain (Ii) trimers to form nonamers. These complexes transit to mature endosomes either via the trans-Golgi network (TGN) or by recycling from the cell surface. Within endosomes, Ii is sequentially proteolyzed to yield the residual Ii fragment, class II-associated invariant chain peptide (CLIP). Displacement of CLIP from the ligand groove of MHC-II is mediated by the MHC-II-related chaperone H2-DM. Antigens delivered to late endosomes by phagocytosis, pinocytosis, endocytosis and autophagy are processed by cathepsins and oxidoreductases. Acquisition

of high-affinity peptides by MHC-II is facilitated by H2-DM. The MHC-II:peptide complexes are subsequently transported to the cell surface for recognition by CD4+ T cells. Adapted from [191].

MHC-I cross-presentation

The third main mechanism of protein-derived antigen presentation is the MHC-I cross-presentation pathway that allows loading of extracellular antigens on MHC-I molecules for presentation to $CD8^+$ T cells [193].

Antigens internalized through the receptor-mediated endocytic pathway, the phagocytic pathway or the macropinocytic pathway can all be efficiently cross-presented [193,219]. However, it is thought that milder conditions in the endocytic vesicles in terms of pH and proteolytic potential can favor cross-presentation over MHC-II presentation [191,193,220–222]. Therefore, an additional level of regulation during antigen internalization might play a role in the decision between MHC-I cross-presentation and MHC-II presentation.

Two mechanisms of antigen processing have been described for cross-presentation, namely the cytosolic pathway **(Figure 7)** and the vacuolar pathway **(Figure 10)** [193].

In the cytosolic pathway, the internalized proteins are transported from the endocytic vesicles to the cytoplasm to be degraded by the proteasome. Even though the exact mechanism that mediates this transport is still object of debate, some hypotheses have been proposed. One possibility is represented by the ER-associated degradation (ERAD) which is a pathway typically active in the ER to dispose of misfolded proteins [223]. During this process, misfolded proteins are transported to the cytoplasm with the help of chaperones and, once there, they are directed to the proteasome through ubiquitination [224]. Therefore, considered the documented presence of ER proteins in the endocytic compartment, it is likely that an ERAD-like mechanism could mediate the process of

cytoplasmic transfer of internalized antigens for cross-presentation [225–228]. However, other observations point to the existence of parallel mechanisms of cytosolic transport of endocytic proteins. For example, it is known that administration of exogenous cytochrome *c* can mediate apoptotic protease activating factor-1 (Apaf1)-dependent apoptosis of cross-presenting cells [229]. This indicates that cytochrome *c* is transported into the cytoplasm in a native biologically active state which is incompatible with an ERAD-like mechanism. Indeed, other studies have demonstrated that a process of endocytic vesicle disruption probably participates in the transport of internalized proteins to the cytoplasm by destabilizing the vesicular membrane and therefore promoting the release of the vesicular content [230]. Independently of the transport mechanism, once in the cytoplasm, the internalized antigens are degraded by the proteasome. It was believed that, after proteasomal degradation, the antigenic peptides were directed to the classic MHC-I loading pathway. However, subsequent studies have shown that a mechanism of retro-transfer of the peptides to the endocytic compartment might as well be involved in the process of cross-presentation. Indeed, as mentioned, ER proteins are present in the endocytic vesicles allowing therefore the formation of the TAP-dependent MHC-I PLC, and hence the loading of antigenic peptides on MHC-I, within the endocytic compartment [231].

In contrast to the cytosolic pathway, the vacuolar pathway of antigen cross-presentation does not involve transport of antigens to the cytoplasm nor does it depend on the participation of the proteasome. In this pathway, extracellular antigens are degraded through acidification and proteolytic activity of the endocytic vesicle and are loaded on MHC-I molecules directly within the very same vesicle [232–235].

Both in the case of direct vesicular degradation of internalized proteins or TAP-dependent retro-transport of peptides into the endocytic compartment, MHC-I molecules must be transported to the vesicular lumen to receive the processed antigenic peptides. One possibility of MHC-I-transport

mechanism consists in the direct trafficking of MHC-I-containing vesicles from the ER to the endocytic compartment through a yet unidentified pathway. An intriguing hypothesis proposes a role of Ii in this process [236,237]. However, this mechanism has not been conclusively proven. In parallel to the direct transport from the ER, it is known that MHC-I molecules are constantly recycled to the endocytic compartment from the cell membrane [238,239]. Once in the endocytic compartment, MHC-I molecules are loaded with antigenic peptides generated through one of the mechanisms discussed above and finally the peptide:MHC-I complexes are transported to the cell membrane for presentation to $CD8^+$ T cells.

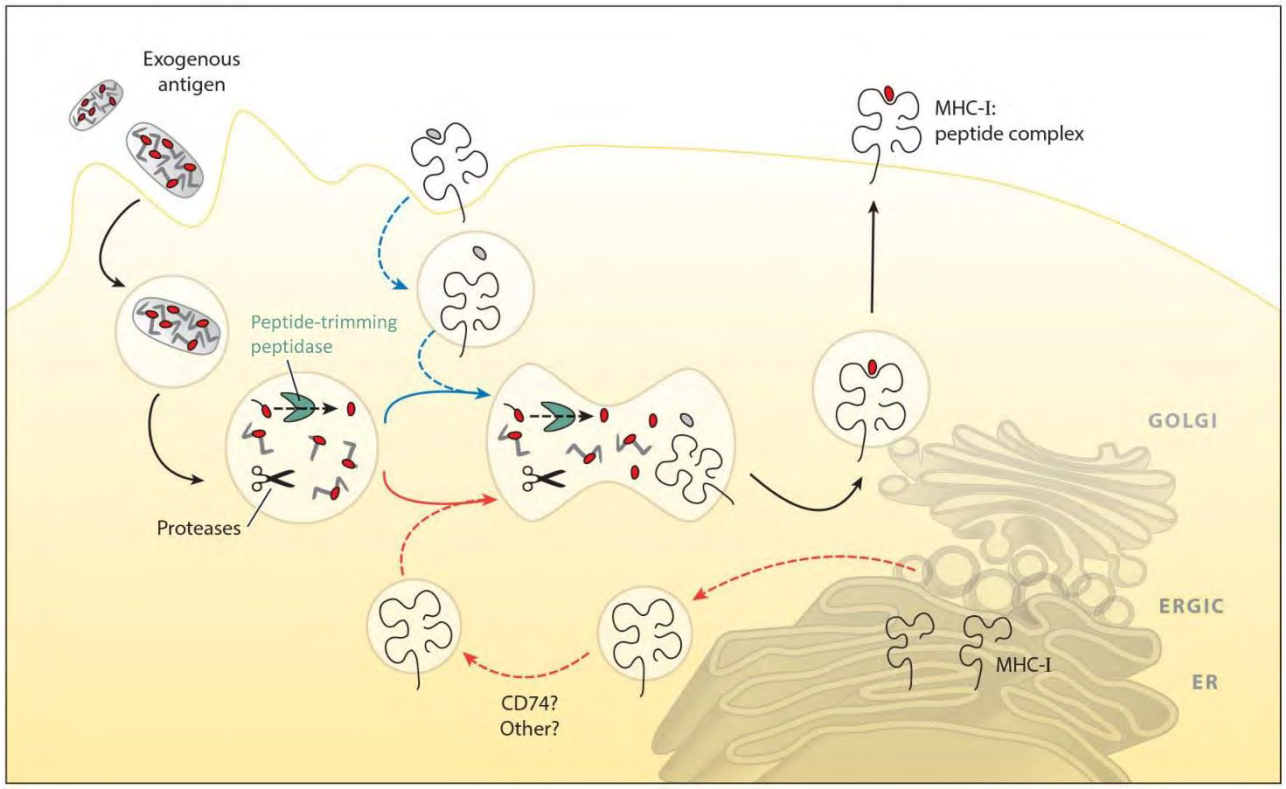

Figure 10. The vacuolar pathway of MHC-I cross presentation. Exogenous antigens are internalized via phagocytosis, pinocytosis, or receptor-mediated endocytosis. The antigens are cleaved by proteases within the endocytic compartment (primarily by cathepsins) and can be further trimmed by specific peptidases. MHC-I molecules are recruited either from the plasma membrane (blue lines) or from the ER (red lines). Peptides are loaded onto MHC-I in the endosome, and the complexes are then presented at the plasma membrane. Dashed lines indicate steps in the pathway that are not fully

understood. ER = endoplasmic reticulum, ERGIC = ER-Golgi intermediate compartment. Adapted from [193].

1.3.2. Phenotypic and functional maturation of DCs upon activation

After the discovery of the central role of DCs in the activation of T cells, much evidence has been collected showing that, upon the occurrence of specific conditions like the presence of an invading pathogen, DCs can undergo a maturation process that entails several phenotypic/functional modifications aimed to fully actualize their potential as APCs. In this process, DCs gradually progress from a fully immature state, in which they are particularly efficient at sampling their microenvironment through internalization mechanisms, to a fully mature state in which they become strongly immunogenic and therefore able to induce the activation of T cells [240].

T cell activation is known to rely on three signals that are often indicated as signal 1, signal 2 and signal 3 [241]. Signal 1 is received by a T cell when its TCR recognizes its specific antigen in a peptide:MHC complex on the surface of an APC. Signal 2 is delivered by the interaction of a broad group of receptors/ligands that are found on the surface of T cells and APCs. The ligand/receptor pairs can be described as a very complex network of co-signaling molecules that, according to the kind of activating signal encountered by the APC, provide the interacting T cell with co-inhibitory or co-stimulatory signals that regulate T cell proliferation and activation. The balance of co-stimulatory and co-inhibitory signals contributes to determine the final fate of the T cell and therefore to regulate the development of the most appropriate T cell response [242]. In the context of priming and activation of naïve T cells, the predominant co-stimulatory molecules expressed by the APC are CD80 and CD86 which bind their co-stimulatory receptor CD28 on the surface of the T cell [242]. Signal 3 is represented by the fine balance of all the pro- and/or anti-inflammatory signals received by the T cell through the cytokines that are present in its surrounding environment [25,243,244].

Activated immune cells produce cytokines, which can signal directly to the T cell, and chemokines, that can recruit other immune cells which in turn produce more cytokines and chemokines. The combination of cytokines that T cells sense in their surrounding environment influences profoundly their activation and determines the development of the distinct effector T cell subsets [25,243,244].

In the immature state, DCs constantly capture self and foreign antigens through the internalization mechanisms discussed above. These antigens are then processed and loaded on MHC molecules for presentation to T cells. In the absence of an activating signal, DCs continuously replenish their supply of MHC-I and MHC-II by expressing new MHC molecules or by recycling the already existing ones from the cell surface. Therefore, immature DCs express very high levels of MHC-I and MHC-II, even if MHC-II expression is localized mainly within the intracellular compartment rather than on the cell surface [192]. The encounter of DCs with PAMPs or DAMPs, derived for example from an infection or an injury, can induce DC activation through engagement of PRRs. Upon activation, the antigen internalization capacity of DCs strongly increases for a limited time after which it decreases considerably [240,245–249]. More recent studies, however, have shown that the endocytic capacity of DCs is not completely abrogated after activation, since, even when fully mature, DCs still maintain their ability to capture antigens through different endocytic mechanisms [248,250,251]. MHC-II molecule expression is also rapidly increased upon DC activation and subsequently inhibited in the fully mature state of DCs [245]. Simultaneously, antigen degradation is enhanced and the peptide:MHC-II complexes are transported more efficiently to the cell membrane where they are stabilized [192]. Overall, these events that take place during DC maturation clearly favor the capacity of DCs to provide a robust signal 1 while diminishing their propensity to capture and present new antigens that they could meet between the moment of their activation and the encounter with an antigen-specific T cell.

DC activation induces upregulation of several other surface markers that have a functional implication in the process of T cell priming. One example is the C-C motif chemokine receptor 7 (CCR7) that is one of the main factors involved in the migration of DCs from the tissues to the associated draining lymph nodes [252]. This process of migration is of particular importance because it allows DCs to transport antigens to the T cell zones of secondary lymphoid organs where the interaction with T cells and the induction of an appropriate adaptive response actually take place.

Signal 2, needed for the regulation of T cell activation, is provided by co-stimulatory molecules like CD80 and CD86 that are also upregulated by DCs upon activation [242]. Another important co-stimulatory molecule upregulated by DCs following activation is CD40. The interaction of CD40 with its ligand, CD40L, which is expressed mainly but not exclusively on activated $CD4^+$ T cells, contributes to the delivery of signal 2 to the T cell, but simultaneously promotes and sustains DC maturation by inducing upregulation of CD80, CD86 and MHC-II and by increasing the production of proinflammatory cytokines through the NF-κB and MAPK pathways [253]. Additionally, CD40-CD40L interaction is considered one of the main mechanisms through which $CD4^+$ Th cells can promote and stimulate the capacity of DCs to activate $CD8^+$ T cells [253].

The activation of DCs through PRR stimulation can induce them to express numerous cytokines and chemokines comprising type I IFNs, IL-6 and IL-12 [254]. The Th1-inducing cytokine IL-12, in particular, is produced in large quantities by some subsets of DCs [23,255] (discussed in paragraph *1.3.3.Developmental, phenotypic and functional heterogeneity of DC subsets*). Through direct production of cytokines or through chemokine-mediated recruitment of other cytokine-producing immune cells, DCs can profoundly influence the signal 3 sensed by activated T cells and therefore shape the development of the most appropriate T cell subsets [254].

1.3.3. Developmental, phenotypic and functional heterogeneity of DC subsets

All the biological aspects described so far have been intentionally discussed from a perspective that simplistically excludes any subclassification of DCs and rather considers the DC compartment as a functionally homogeneous immunological entity. In fact, DCs are very heterogeneous and can be divided in several subclasses characterized by distinct, even if often dynamically overlapping, developmental pathways, phenotypes and functional specificities [23,256,257].

For many years in the history of DC research, the classification of DCs has been guided by the analysis of the expression of surface markers that, to a certain degree, are DC-subset specific [23,255]. However, the deepening of our understanding of DC biology has revealed that DCs and, more in general, mononuclear phagocytes constitute an extremely plastic system where the distinct cell subsets cooperate through complementary and/or overlapping modes of action [23,258]. Therefore, if on one hand the classification systems based on functional or phenotypic considerations have allowed to define numerous DC subsets and to reveal their fundamental characteristics, on the other hand they have contributed to generate some confusion between analogous subsets in different tissues and organisms, but also between separate mononuclear phagocyte lineages with overlapping phenotypic and/or functional properties. Therefore, the recent introduction of a new classification system based on ontogeny has substantially simplified the process of categorization of mononuclear phagocytes [259]. Through this method, DCs can be easily recognized as an independent lineage with different precursors than monocytes and macrophages.

The analysis of DC developmental pathways allows to make a first distinction between two main groups of DCs, namely conventional DCs (cDCs) and plasmacytoid DCs (pDCs). The cDC subset shows additional heterogeneity and can be further divided into type 1 cDCs (cDC1s) and type 2 cDCs (cDC2s) [259]. This first classification level based on ontogeny lays the foundations for a second level

of classification that, on the basis of functional and phenotypic characteristics, divides cDC1s and cDC2s in the numerous subsets found in different tissues and organs [257].

According to their location in the body, all the different DC subsets can be divided into two groups, namely lymphoid tissue resident DCs, which conclude their development and spend their entire life within secondary lymphoid organs, and non-lymphoid tissue DCs which constantly sample the tissues and, especially upon activation, migrate loaded with antigens through the lymphatics toward the draining lymph nodes for antigen presentation [256].

At the dawn of the study of DCs, DC development from specifically committed precursors had not been described yet. On the contrary, it was believed that DCs derived directly form activated monocytes [259–261]. Even if this concept has been extensively revised, it is true that, in conditions of inflammation, monocytes can be recruited from the blood and differentiate, within the inflamed tissue, into an additional subset of DCs known as monocyte derived DCs (moDCs) [262].

Finally, Langerhans cells (LCs) have been considered for years as the prototypic example of DCs [263]. Their classification has been more recently revised thanks to the new ontogeny-based categorization system [259]. However, for historical reasons and by virtue of their singular biological identity, LCs have been included and briefly described in this section dedicated to the different DC subsets.

Early stages of DC development

DCs originate in the bone marrow (BM) mainly from common myeloid progenitors (CMPs) **(Figure 11)** derived from hematopoietic stem cells [264–266].

Factors of primary importance throughout the differentiation of DCs are the surface receptor FMS-like tyrosine kinase 3 (FLT3) and the transcription factor PU.1 [267,268]. Indeed, PU.1 is required for

the expression of FLT3 [269]. The growth factor FLT3 ligand (FLT3L), which is both necessary and sufficient to drive DC differentiation [267,270], binds to FLT3, and activates a signaling cascade that, through the signal transducer and activator of transcription 3 (STAT3), induces the expression of PU.1 [271] that in turn favors the myeloid commitment [272] and upregulates the levels of FLT3 [271,273].

The CMPs give rise to the macrophage and DC progenitors (MDPs) [274] **(Figure 11)** which can further differentiate either into the common monocyte progenitors (cMoPs) [275] **(Figure 11)** or into the common DC progenitors (CDPs) [276,277] **(Figure 11)**.

The commitment of MDPs to the DC lineage and, therefore, the differentiation to CDPs involve the activity of IRF8 [278] whose upregulation at the MDP stage is mediated by the transcription factor PU.1 [279].

CDPs represent the last common differentiation stage in DC development before the commitment of the progenitors to the different DC subsets. Indeed, CDPs can differentiate into cDC-committed or pDC-committed progenitors known respectively as pre-cDCs and pre-pDCs [276,277,281].

Pre-cDCs can be further divided into pre-cDC1s and pre-cDC2s [282,283] **(Figure 11)** and are characterized by the expression of transcription factors which are not present in pre-pDCs and pDCs, like Zbtb46 [284,285], which is maintained also in the differentiated cDCs, and Id2 [286], that is one of the determinants of cDC1 commitment [284–286].

Pre-pDCs complete their development into pDCs in the BM from where they migrate, through the blood circulation, to secondary lymphoid organs and peripheral tissues [287]. By contrast, pre-cDCs leave the BM at the stage of cDC1- or cDC2-committed progenitors and, through the blood stream, they reach secondary lymphoid organs and peripheral tissues [288,289] where they conclude their development into the several cDC subsets described *in vivo* [257].

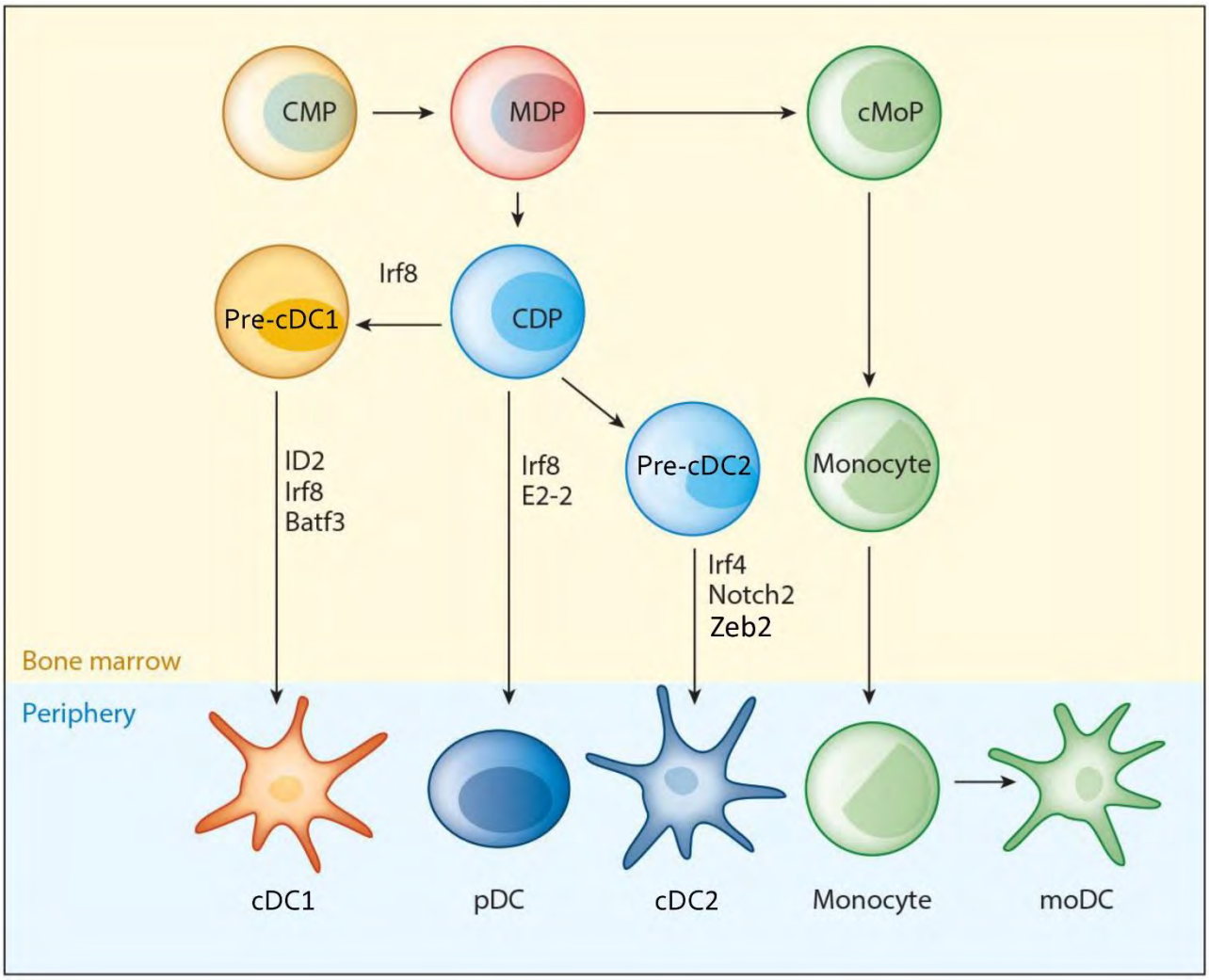

Figure 11. Stages and transcription factors for DC development. A scheme showing myeloid lineage development from the CMP, indicating transcription factors required for particular transitions between stages. cDC = conventional DC; CDP = common DC progenitor; cMoP = committed monocyte progenitor; CMP = common myeloid progenitor; DC = dendritic cell; MDP = macrophage-DC precursor. Adapted from [280].

Type 1 conventional DCs – cDC1s

As explained, cDC1s derive from pre-cDC1 progenitors, that are characterized by high or intermediate levels of CD24 [288]. cDC1 development depends on the transcription factors IRF8, Id2 and Batf3, and the knockout of any of them causes severe defects in the cDC1 compartment [257,282,290–292]. In particular, Batf3$^{-/-}$ mice lack almost completely cDC1s at the steady state but

show normal development of all the other DC subsets [290]. In the differentiation of cDC1s, fundamental functions are carried out by Id2, which antagonizes the transcription factor E2-2 (one of the main drivers of pDC development) [293], and by IRF8, whose autoactivation allows to maintain the high levels of IRF8 that are required for the commitment of the DC progenitors to the cDC1 subset [282]. Batf3, acts late in the process of cDC1 development, at the pre-cDC1 stage, and is required to maintain high levels of IRF8 in the late stages of cDC1 differentiation [282].

Terminally differentiated cDC1s express MHC-II and CD11c at high levels and are positive for CD24, XCR1 and the C-type lectins CD205 and Clec9A [23,294–299], which are linked to the cross-presentation of self-antigens derived from apoptotic and necrotic cells [221,300]. In the lymphoid organs, cDC1s are characterized by expression of the α subunit of CD8 which exclusively distinguishes them from other DC subsets [301]. On the contrary, in non-lymphoid tissues, cDC1s do not express CD8α but are mostly characterized by the expression of CD103, in spite of the existence of several $CD103^-$ cDC1 subsets in tissues like the skin and the intestine [257]. Several studies have also reported that numerous subsets of cDC1s, especially in lung, oral mucosa and nose, can express langerin (CD207), a marker that was originally thought to be exclusive for LCs [257].

CDC1s are the only DCs to express TLR3 [302] and, upon activation, they are among the main producers of the Th1-inducing IL-12p70 [303–305] whose expression involves the cDC1-characterizing transcription factor IRF8 [306]. CDC1s are also considered the most efficient cross-presenting DCs [222,290,307,308] thanks to a precise regulation of antigen degradation in the endocytic compartment, an effective phagosome-to-cytosol transport and a gene expression profile oriented to MHC-I presentation [23,222]. All these elements show a preferential role of cDC1s in the response to intracellular pathogens, in the activation of cytotoxic $CD8^+$ T cells and in the induction of a Th1 phenotype in $CD4^+$ T cells.

Type 2 conventional DCs – cDC2s

Differently from cDC1s, cDC2s derive from $CD24^{int/-}$ cDC2-committed progenitors [288]. One of the most important transcription factors for cDC2 development and function is IRF4 [309–311], even if other transcription factors like IRF2 and RelB are involved in their differentiation [280,312]. IRF8 expression is downregulated during the transition from the pre-cDC2 stage to the terminally differentiated cDC2s and concomitantly IRF4 levels gradually increase [282].

The marker CD172a (SIRPα) is expressed specifically by cDC2s and allows to distinguish them from cDC1s [294,313,314]. In most cases, the integrin CD11b is expressed by cDC2s. However, $CD11b^{lo/-}$ cDC2s are found, for example, in dermis [257,294,315]. In contrast to cDC1s, cDC2s are negative for XCR1 [294,313]. In the mouse, splenic cDC2s can be divided into two subsets on the basis of their expression of CD4 and ESAM. The $ESAM^+$ cDC2s coincide almost completely with the $CD4^+$ cells, therefore the two cDC2 populations can be recognized as $CD4^+ESAM^+$ and $CD4^-ESAM^-$, respectively [255,315]. In non-lymphoid tissues, a multitude of surface markers are differentially expressed on several subsets of $CD11b^+CD172a^+$ cDC2s. For example, in the intestine and the nose, different cDC2 subsets can be distinguished through the analysis of CD103 expression, and in the lung, the nose, the skin and the intestine some subsets are positive for CD24, often but not necessarily, in association with CD103 expression [257].

From a functional point of view, little is known about the differences and the specificities of each cDC2 subset. From a generic perspective, cDC2s express a wider TLR pattern than the cDC1s but lack expression of TLR3 [302]. Upon activation, they can produce large amounts of cytokines like IL-6 and IL-23 but, in contrast to cDC1s, show limited or negligible production of IL-12p70 whose expression is inhibited by IRF4 [316]. The function of cDC2s in the context of T cell activation is believed to be mostly associated with the development of Th2 and Th17 responses [257,280,309,317–320]. In

contrast to cDC1s, cDC2s are known to be very inefficient at antigen cross-presentation [222,307,308,321]. Indeed, their gene expression profile is markedly oriented toward MHC-II presentation [322] also thanks to the role of IRF4 in the expression of MHC-II presentation-related genes [323]. Several transcription factors, like Notch2, Klf4 and Zeb2, have been associated with the differentiation of specific cDC2 subsets and have been shown to influence differentially the development of Th2 and Th17 responses [257,280]. Therefore, it has been speculated that distinct cDC2 subsets, developmentally dependent on different transcription factors, might induce preferentially either Th17 or Th2 type responses [257,280]. However, it has not been possible to identify markers to clearly distinguish this putative Th2- and Th17-inducing cDC2 subsets, and a consistent model has not been clearly demonstrated yet.

Plasmacytoid DCs – pDCs

PDCs, in opposition to cDCs, can develop even in the absence of IRF8 during the early and late stages of their ontogeny. However, IRF8 is expressed at high levels in pDCs and is extremely important for their correct functionality [291]. As mentioned, the mutually exclusive expression of E2-2 or Id2 defines the commitment of CDPs toward the pDC or the cDC lineage, respectively [293,324]. E2-2 is considered one of the main determinants of pDC development and function [293,325] and it is involved in the maintenance of high levels of IRF8 [293].

Terminally differentiated pDCs are $B220^{+}$ $Ly\text{-}6C^{+}$ and are characterized by the selective expression of PDCA-1 and Siglec-H [326–330]. In contrast to other DCs, pDCs express intermediate or low levels of MHC-II and CD11c [326–328].

In functional terms, pDCs are regarded as one of the main innate effectors against viral infections [286]. Indeed, pDCs express high levels of TLR7 and TLR9 which, upon recognition of their ligands, signal through the MyD88-mediated pathway that activates IRF7, whose constitutive expression in

pDCs is regulated by E2-2 [293,325]. Activation of IRF7 induces a fast and massive production of type I IFNs [286]. The cytokine production capacity of pDCs following PAMP recognition, however, is not limited to type I IFNs, but includes also other pro-inflammatory cytokines like IL-6 and IL-12 whose expression is induced by the activation of the NF-κB pathway [286,331]. The role of pDCs in antigen presentation and T cell activation is strongly debated. It has been shown that, upon activation, pDCs upregulate MHC-II and co-stimulatory molecules and that they are able to process antigens and to present and cross-present them through peptide:MHC complexes both *in vitro* and *in vivo* [332]. However, pDCs are much less efficient than cDCs at antigen presentation and, at least in some models of infection, their role in T cell activation appears to be very limited [333–335].

Monocyte-derived DCs – moDCs

During inflammation, monocytes derived from the cMoPs can differentiate into a monocyte-derived subset of cells with DC characteristics known as monocyte-derived DCs (moDCs) or inflammatory DCs [262,336]. Among different monocyte subpopulations, moDCs are thought to derive from Ly-6C$^+$ monocytes [337–340] in a macrophage colony-stimulating factor receptor (M-CSFR)-dependent way [341].

After their differentiation, moDCs upregulate several DC markers like Zbtb46, CD11c and MHC-II [342,343]. They express high levels of co-stimulatory molecules and can migrate to the lymph nodes in a CCR7-dependent manner [343]. Their phenotype is similar to that observed in cDC2s because of the expression of markers like CD172a and CD11b [336]. Therefore, despite a different ontogeny, the two subsets are difficult to distinguish just on the basis of their phenotypic characteristics. However, few markers that appear to be specifically expressed by moDCs have been identified and comprise CD64 (FcγRI) and FcεRI [294,336]. Moreover, CD26 is expressed exclusively by cDCs but does not appear to be expressed by cells of monocytic origin [257,294].

From the functional point of view, moDCs have been implicated in the response to several infectious and non-infectious diseases. One of their first discovered characteristics was their strong ability to produce high quantities of TNF-α and of the inducible nitric oxide synthase (iNOS) that initially earned them the name of Tip (TNF-α/iNOS-producing) DCs [344]. Several works have demonstrated *in vitro* and *ex vivo* the capacity of moDCs to activate $CD4^+$ or $CD8^+$ T cells through both direct antigen presentation and cross-presentation [256,343]. *In vivo*, moDCs have been shown to have an important role in several infection models and to be able to prime T cells, even if with lower efficiency than cDCs. MoDCs are thought to largely contribute to the development of Th1 responses. However, they have also been implicated in the development of Th2 and Th17 responses [256,258].

Langerhans cells – LC

As mentioned, LCs have been considered for many years as a prototypical example of DCs. Recently, this idea has been extensively revised after the discovery that LCs do not share the same progenitors as DCs nor do they depend on the DC-defining growth factor FLT3L [259,263]. LCs originate during fetal development in the yolk sack and in the liver from where they migrate to seed the skin [345,346]. After birth, the LC precursors undergo numerical expansion, acquire a dendritic morphology and upregulate markers like MHC-II and Langerin [347,348]. Terminally differentiated LCs constitute a population of self-renewing cells that localize in the epidermis [349]. Therefore, despite having some DC-like phenotypic characteristics, LCs appear to be more closely related to macrophages on the basis of their ontogeny [259].

Like DCs, LCs are able to sample their surrounding environment and internalize exogenous antigens. One characteristic feature of LCs is their ability to extend their dendrites between the keratinocytes to sample the surface of the skin and mediate the development of humoral responses against

possibly harmful agents found in this context, in a process called preentive immunity [263]. Activated LCs can produce pro-inflammatory cytokines through the activation of the NF-κB pathway, and upon activation they upregulate co-stimulatory molecules and the chemokine receptor CCR7 which mediates their migration to draining lymph nodes [350–353]. LCs possess the capacity to present and cross-present antigens and to induce T cell activation even if less efficiently than cDCs [351,354–357]. They have been shown to participate in the development of Th17 and Th2 responses and, on the contrary, to have a limited role in Th1 development [351,352,357,358].

1.3.4. Murine model systems in the study of DCs

One of the most limiting aspects in DC research is the difficulty to obtain sufficient quantities of viable and non-activated cells for experimentation [295,359]. Indeed, DCs are rather scarce *in vivo*, and *ex vivo* they are very sensitive to prolonged culturing and easily undergo spontaneous activation and cell death [360,361]. For these reasons, in the past years, considerable effort has been dedicated to simplifying the access to sufficient quantities of DCs *in vivo*, *ex vivo* and *in vitro* [362].

One of the first and most diffused procedures to generate large quantities of DCs *in vitro* is based on the supplementation of bone marrow or peripheral blood cultures with the granulocyte-macrophage colony-stimulating factor (GM-CSF), frequently in the presence of IL-4 [363–366]. However, this method produces differentiated cells that mainly resemble inflammatory moDCs rather than steady state or lymphoid-organ resident DCs [367]. Another example of growth factor-dependent DC differentiation *in vitro* employs the main DC-inducing growth factor, FLT3L [267,270]. Cultivation of bone marrow in the presence of FLT3L generates mixed cell populations composed of different DC subsets that can be related phenotypically and functionally to splenic DCs, despite lacking the expression of the subset-distinctive markers CD8α and CD4 [296,368,369]. Remarkably, analogs of splenic cDC1s, cDC2s and pDCs are all generated under these culture conditions, even

though the relative abundance of the three subsets deviates from the numerical distribution of their splenic counterparts, highlighting a preferential development of cDC1 and pDC equivalents [296]. Thus, in spite of a very close resemblance of these cells to steady state lymphoid-organ resident DCs, further purification steps are required to isolate individual subsets. Moreover, the process required to generate these DC equivalents is relatively time consuming and is subject to the limitations that derive from the sensitivity of DCs to long term culturing.

Injection or overexpression of the same growth factors in mice can promote the expansion of several DC subsets *in vivo*. In particular, daily administration of FLT3L has been shown to enormously increase the numbers of murine DCs in multiple organs and tissues [370–373]. Interesting alternatives, that allow to promote a similar expansion while overcoming the disadvantages related to the daily handling and treatment of mice, are the injection of tumors secreting FLT3L [374] and the generation of transgenic murine lines that overexpress FLT3L either constitutively [375] or in an inducible way [376]. Analysis of splenic DCs from FLT3L-transgenic mice has shown a considerable numerical increase of all the main DC subsets, even if the effect appears to be more pronounced for pDCs and, to a lesser extent, for cDC1s [375]. Most importantly, the FLT3L-induced expansion does not affect phenotype and functionality of DCs [371,372,375,376]. Therefore, these approaches represent relevant tools that can be exploited as effective sources of DCs both for *in vitro* and for *in vivo* experimentation. Analogously, some strategies have been developed to induce DC expansion *in vivo* through administration of GM-CSF to mice. Surprisingly, daily injection of GM-CSF in mice was found to have a negligible effect on DC abundance [371], probably because of a very short half-life of the exogenous GM-CSF *in vivo*. Indeed, the use of a polyethylene glycol-modified GM-CSF, that has increased half-life, induced a substantial expansion of $CD11c^+$ cells [377]. Interestingly, this effect was restricted to $CD11b^+$ DCs but did not affect $CD11b^-$ cells [377]. Other similar approaches were based on the use of GM-CSF-secreting tumors as a constant source of GM-CSF *in vivo*

[378,379]. Also in these cases, a significant increase of DC numbers could be observed. However, regardless of the adopted strategy, the characterization of the GM-CSF-induced cells showed very often morphological, phenotypic and functional features that were compatible with their belonging to the moDC subset. Moreover, in the case of approaches based on GM-CSF-secreting tumors, the characteristics of the expanded DCs might have been partially related to the presence or the development of the tumors.

The generation of homogeneous immortalized DC lines is a valuable alternative and allows to overcome some of the technical difficulties that remain associated to these methods, like the sensitivity and the functional instability of *ex vivo* generated DCs, as well as the requirement for long differentiation protocols and the possible need for laborious cell purification steps. In the past years several DC lines have been generated and used to study multiple aspects of DC biology. However, some features of many of these cell lines might constitute a constraint in their use for specific applications. For example, in several cases, phenotypic and functional characteristics of distinct *in vivo* DC subsets coexist, indicating a possible limitation for their employment in the study of DC-subset-restricted mechanisms. Additionally, some of these DC lines need special culture conditions, like constant presence of growth factors or incubation at specific permissive temperature, which might represent a complication in their maintenance and could hinder their use for specific applications. Also, in some cases, the phenotypic and functional stability of these cells in long-term culture is not known. This represents an important additional factor to keep into consideration when working with DC lines whose passage number has not been tracked or in the case of cell lines which, after many years since their derivation, have reached very high passage numbers.

Our group has developed a strategy to generate stable immortalized DC lines from murine spleen. Similarly to many other approaches, our method is based on the use of the simian virus 40 large T oncogene (SV40LgT) to induce tumorigenic transformation and immortalization of DCs. However,

while most strategies entail immortalization of isolated DCs *in vitro* through lentiviral transduction of the cells with SV40LgT, our approach is rather uncommon because it exploits SV40LgT-induced tumorigenic transformation of splenic DCs *in vivo*. Indeed, we have generated several transgenic mouse lines, called Mushi mice, that carry a construct comprised of SV40LgT and of an internal ribosome entry site (IRES)-linked enhanced green fluorescent protein (EGFP) reporter sequence under transcriptional control of the 5.7 kb CD11c proximal promoter. The expression of the transgene, which is almost exclusively observed in DCs [380,381], causes DC tumorigenic transformation that leads to the development of multisystem histiocytosis (from which the name Mushi) in the mice [381]. We observed that, depending on the copy number of the transgenic construct, different Mushi mouse lines developed histiocytosis at different ages and with different severity [381]. For this reason, we selected the Mushi1 line in which the transgene levels were sufficient to cause the tumorigenic transformation of DCs without altering their phenotypic and functional properties. Mushi1 mice develop histiocytosis at an age of around 4 months and, once sick, they show enlarged spleen, low hematocrit and mesenteric lymphadenopathy [381]. From the spleen of diseased Mushi1 mice, we generated several DC lines that we called MutuDCs (murine tumor DCs) [382,383]. The phenotypic and functional characterization of the MutuDC lines derived from Mushi1 mice showed that these cells share all the main features with splenic cDC1s (spl-cDC1s) [382], therefore we refer to them as MutuDC1s.

2. Aims

This thesis describes and discusses the results of two separate projects. The first one had the goal to generate new subsets of MutuDC lines, while the second one aimed to characterize the mechanisms of synergistic and super-synergistic activation of DCs. Therefore, each of the following sections, including this one, is divided into two separate thematic blocks titled respectively *Generation of new MutuDC lines* and *Study of the molecular mechanisms of synergistic and super-synergistic DC activation*.

2.1. Generation of new MutuDC lines

As mentioned above, all the MutuDC lines derived from Mushi1 mice belong to the spl-cDC1 subset. Over the years, MutuDC1s have proven to be a very reliable and powerful model for the study of cDC1s. However, model cell lines representative of other DC subsets were still missing. Therefore, our first aim was to derive new DC lines with characteristics of other DC subsets than the spl-cDC1s. To this end, we planned to generate a new strain of Mushi1 mice with a severe defect in cDC1 development. Through a derivation method analogous to the one described for MutuDC1s [382,383], we aimed to derive new Mutu cell lines and, once established them, to characterize them phenotypically and functionally.

2.2. Study of the molecular mechanisms of synergistic and super-synergistic DC activation

In parallel, we exploited our previously derived MutuDC1 line to investigate TLR signaling and the synergistic and super-synergistic integration of TLR and host-derived signaling in spl-cDC1s. In particular, since little is known about the molecular mechanisms that underlie these effects, the second aim of our work was the identification of genes that specifically and directly regulate

synergistic/super-synergistic activation of spl-cDC1s. To address this question, we chose an unbiased approach based on the use of a lentiviral CRISPR/Cas9 pooled library targeting several thousands of mouse genes. In the preliminary stage of this project, our purpose was to identify one or more synergistically and/or super-synergistically expressed genes that could possibly be used as markers to clearly discriminate, by flow cytometric analysis, between non-synergistic, synergistic and super-synergistic activation of non-transduced MutuDC1s. Once established one or more suitable activation-state-defining markers to use as an experimental readout, we aimed to lentivirally transduce MutuDC1s with the CRISPR/Cas9 pooled library and, by doing so, to generate a pooled population of single knockout cells to use in a loss of function-based flow cytometric screening by positive and/or negative selection (see paragraph *3.2.1.CRISPR/Cas9-library screening approach* for a detailed description of the screening strategy). Our final goal was the identification of genes whose knockout causes dysregulation of (super-)synergistic activation of MutuDC1s.

3. Materials and Methods

3.1. Generation of new MutuDC lines

The majority of the experimental procedures and reagents used for this project are explained in detail in the section **MATERIALS AND METHODS** of paragraph *4.1.1. Establishment and characterization of a functionally competent type 2 conventional dendritic cell line*. The additional procedures and materials that were used in this project are described in the following paragraphs.

3.1.1. Long-term storage of cells in liquid nitrogen

Cells were resuspended at a density of 1-3x10^6 cells/mL in ice-cold freezing medium prepared as follows: 40% complete DC-culture medium (IMDM+GlutaMAX™ Supplement (31980, GIBCO), 10 mM HEPES (15630, GIBCO), 0.075% $NaHCO_3$ (from 7.5% NaHCO3 stock solution, 25080, GIBCO), 50 µM b-mercaptoethanol (31350, GIBCO), 8% heat inactivated FCS (tested for toxicity toward DC cultures), 50 U/mL penicillin, 50 µg/mL streptomycin (15070, GIBCO)), 50% heat inactivated FCS (tested for toxicity toward DC cultures), 10% dimethyl sulfoxide (DMSO) (AppliChem Panreac A1584,0100). The cell suspensions were divided in 1 mL aliquots into cryotubes (SARSTEDT CryoPure Tube, ref. 72.380) and frozen at -80 °C using freezing containers (Nalgene Mr. Frosty, ref. C1562-1EA) containing isopropanol pre-cooled at 4 °C. After freezing, the cells were transferred in liquid nitrogen for long term storage.

3.1.2. T cell activation assay in the presence of exogenous IL-12p70, IL-4 and anti-CD28

The experiments were carried out as described in the section **MATERIALS AND METHODS** of paragraph *4.1.1. Establishment and characterization of a functionally competent type 2 conventional dendritic cell line*. Recombinant mouse IL-12p70 (eBioscience, ref. 14-8121) or mouse IL-4

(eBioscience, 14-8041, discontinued) were diluted in complete DC-culture medium and added to the co-cultures at the final concentrations of 5 ng/mL and 30 ng/mL, respectively.

The anti-CD28 antibody was purified through a protein A/protein G-conjugated sepharose column from the supernatant of a culture of an anti-CD28-secreting hybridoma (clone 37.51). The purified antibody was used at the final concentration of 2,5 µg/mL.

3.2. Study of the molecular mechanisms of synergistic and super-synergistic DC activation

3.2.1. CRISPR/Cas9-library screening approach

The experimental approach to identify genes whose knockout causes a dysregulation of DC synergistic and/or super-synergistic activation was designed as follows:

- Establishment of one or more DC activation markers that are synergistically and super-synergistically regulated in MutuDC1s. These markers must allow to unequivocally distinguish between non-synergistic, synergistic and super-synergistic activation of DCs in a flow cytometric analysis. Once identified, the markers are used as a readout during the screening process
- Lentiviral transduction of MutuDC1s with the CRISPR/Cas9 library. Cell transduction is carried out at a low multiplicity of infection (MOI) of 0,3 to obtain a pooled population of single knockout cells, thus cells which carry, at most, only one CRISPR/Cas9 construct targeting a single gene
- Selection of transduced cells with puromycin to exclude non-transduced cells
- Activation of the transduced MutuDC1s with a synergistic or a super-synergistic combination of signals

Screening after activation with a synergistic signal combination

- Three cell populations are identified on the basis of their activation state and sorted: unresponsive (non-activated), non-synergistically activated and synergistically activated
- Genomic DNA is extracted from the cells in each population and the single guide RNAs (sgRNAs) are sequenced to identify the corresponding knockout genes
- The frequency of every sgRNA in each of the above-mentioned populations is compared with the original composition of the library. The candidate regulators of synergistic activation are either under-represented or over-represented in the distinct sorted populations

Screening after activation with a super-synergistic signal combination

- Three cell populations are identified on the basis of their activation state and sorted: non-activated/non-synergistically activated, synergistically activated and super-synergistically activated
- Genomic DNA is extracted from the cells in each population and the sgRNAs are sequenced to identify the corresponding knockout genes
- The frequency of every sgRNA in each of the above-mentioned populations is compared with the original composition of the library. The candidate regulators of synergistic and/or super-synergistic activation are either under-represented or over-represented in the distinct sorted populations

3.2.2. Genome CRISPR knock-out (GeCKO) v2 pooled library

The genome CRISPR knock-out (GeCKO) v2 library is a pooled collection of sgRNAs targeting more than 20000 mouse genes and more than 1000 mouse miRNAs. Each gene or miRNA is targeted by 6 different sgRNAs. The whole collection of sgRNA is divided into two half-libraries, namely GeCKO A and GeCKO B, each containing 3 sgRNAs per gene or miRNA. Paragraph *8.2.1. Genome-scale CRISPR*

Knock-Out (GeCKO) v2.0 pooled libraries includes a more exhaustive description of the two half libraries and the detailed protocol designed and used to amplify them with no loss of representation. For the work presented in this report, the one-vector lentiviral GeCKO system was used. The backbone vector of all the GeCKO-library constructs was the lentiCRISPR v2 plasmid **(Figure 12)**.

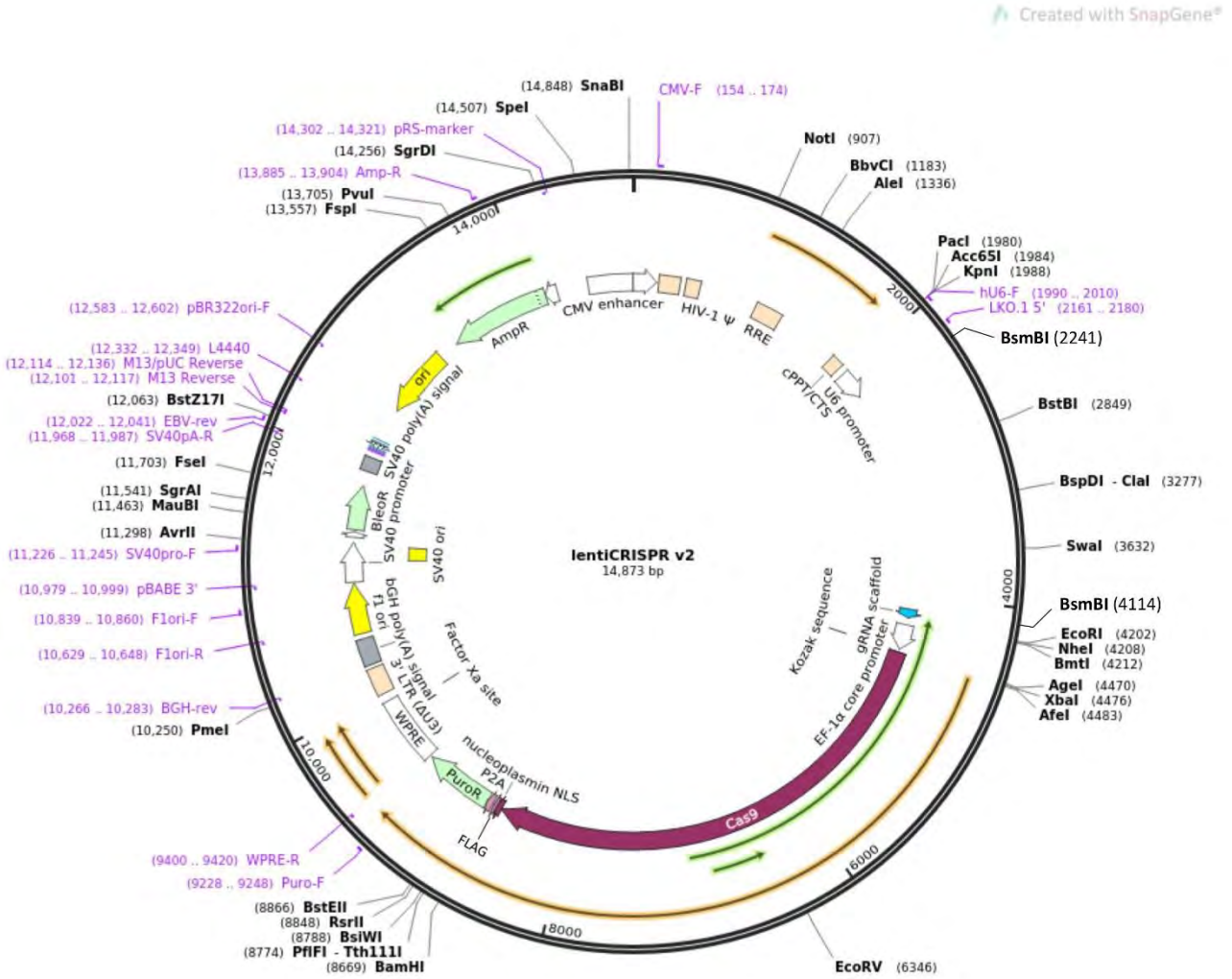

Figure 12. Map of plasmid lentiCRISPR v2. Addgene reference number 52961. Originally published in [384].

GeCKO A and B libraries were kindly provided by Professor Fabio Martinon (University of Lausanne, Lausanne, Switzerland). For library amplification, electrocompetent bacteria (Endura™ ElectroCompetent Cells, Lucigen, ref. 60242) were used. Transformed bacteria were grown at 37 °C.

3.2.3. Design and cloning of lentiCRISPR v2 constructs targeting individual genes

Paragraph *8.2.2 LentiCRISPR v2 and lentiGuide-Puro: lentiviral CRISPR/Cas9 and single guide RNA* provides a detailed description of the lentiCRISPR v2 one-vector system. The above-mentioned paragraph also includes the protocol that was used to clone specific sgRNAs in the lentiCRISPR v2 plasmid. For guide sequence design, the online CRISPR design tool at http://crispr.mit.edu was used. For target guide-sequence cloning the following materials were used: lentiCRISPR v2 plasmid **(Figure 12)** (Addgene, ref. 52961), BsmBI (NEW ENGLAND BioLabs, ref. R0580), custom designed oligonucleotides (Invitrogen), T4 Polynucleotide Kinase (PNK) (NEW ENGLAND BioLabs, ref. M0201), T4 DNA Ligase Reaction Buffer (NEW ENGLAND BioLabs, ref. B0202), T4 DNA Ligase (NEW ENGLAND BioLabs, ref. M0202). Plasmid DNA was amplified by using homemade One Shot™ Sbl3™ Chemically Competent *E. coli* cells (propagated from Thermo Fisher SCIENTIFIC, ref. C7373-03) were used. Midipreparations of plasmid DNA (PureYield™ Plasmid Midiprep System, Promega, ref. A2492) were used to sequence the amplified plasmids. Maxipreparations of plasmid DNA (PureYield™ Plasmid Maxiprep System, Promega, A2393) were prepared and used for transfections.

3.2.4. lentiCRISPR v2 lentiviral particle production

To produce lentiviral particles carrying lentiCRISPR v2 constructs (either single-gene targeted constructs or one of the GeCKO half-libraries) the following envelope and packaging plasmids were used: psPAX2 (**Figure 13)** (Addgene, ref. 12260) and pCMV-VSV-G **(Figure 14)** (Addgene, 8454).

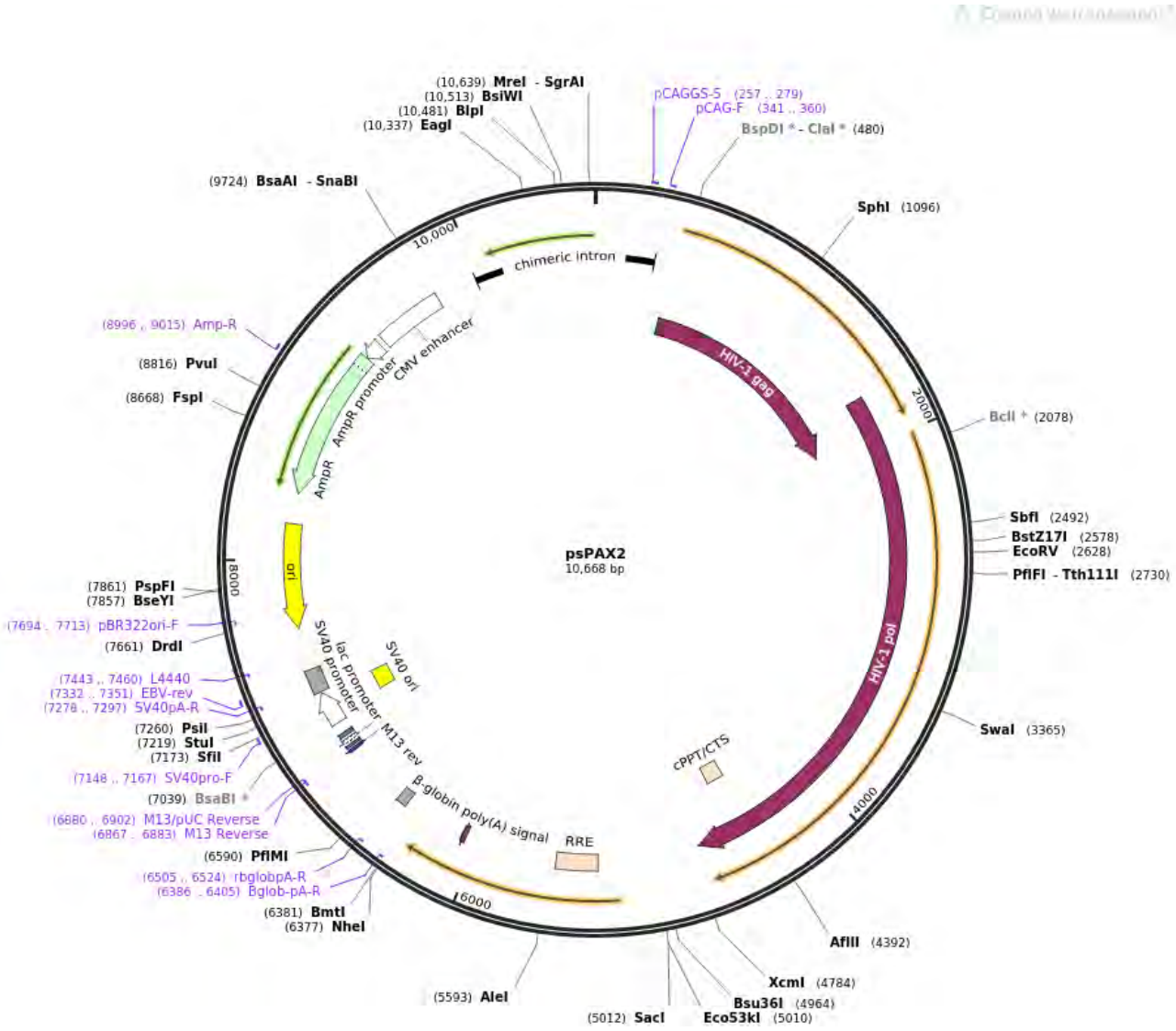

Figure 13. Map of plasmid psPAX2. Addgene reference number 12260. Trono lab packaging and envelope plasmids (unpublished).

A transfection mix was prepared as follows: 40 µg lentiCRISPR v2 construct, 30 µg psPAX, 20 µg pCMV-VSV-G in 3 mL of serum-free DMEM (DMEM, high glucose, GlutaMAX™ Supplement, pyruvate, Thermo Fisher SCIENTIFIC, ref. 31966-021). A quantity of polyethylenimine (PEI) between 90 and 135 µg was added to the mix (1-1,5 µg PEI/µg DNA).

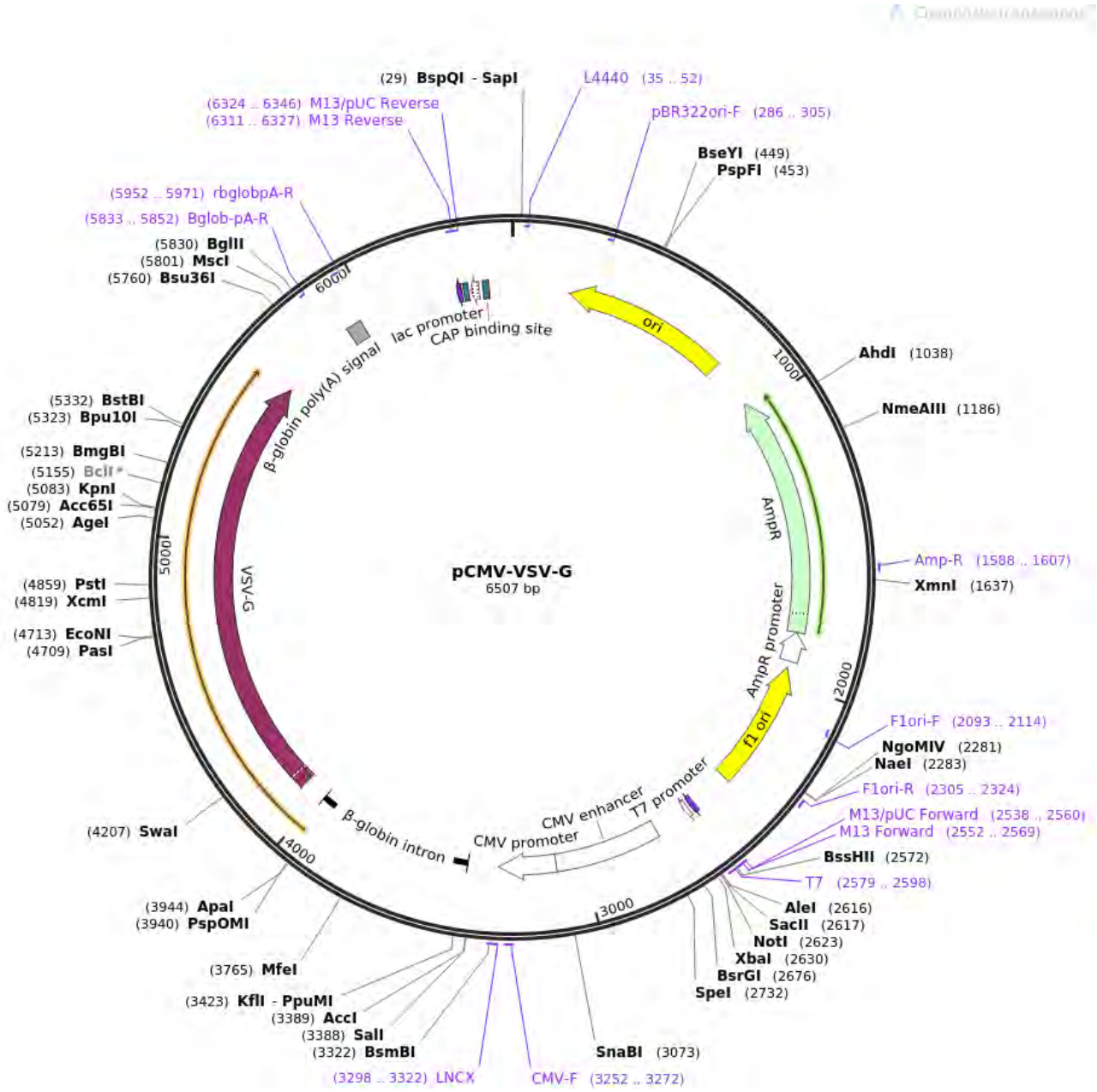

Figure 14. Map of plasmid pCMV-VSV-G. Addgene reference number 8454. Originally published in [385].

After vortexing, the transfection mix was incubated at room temperature for 15 minutes and subsequently diluted with 12 mL of complete HEK-cell culture medium (DMEM, high glucose, GlutaMAX™ Supplement, pyruvate + 10% heat inactivated FCS (tested for toxicity toward DC cultures)). A confluent T150 flask of HEK293T cells was incubated with the 15 mL of transfection mix

overnight (15 hours). At the end of the incubation, the supernatant was removed and replaced with 15 mL of viral-collection medium (Opti-MEM™ I Reduced Serum Medium, GlutaMAX™ Supplement (Thermo Fisher SCIENTIFIC, ref. 51985-026) + 1% heat inactivated FCS (tested for toxicity toward DC cultures)). After 24 hours, the supernatant was harvested and replaced with 15 mL of fresh viral-collection medium. The collected viral-particle-containing supernatant was filtered through a 0.22 µm syringe filter unit and stored at 4 °C. The second fraction of viral-collection medium was harvested after 24 hours and filtered as described above. The viral particles were then precipitated for storage at high concentration. To do so, the two viral-particle-containing fractions were pooled and mixed with 5,7 mL of sterile PBS, 1,3 mL of sterile 5 mM NaCl and 8 mL of sterile PEG 8000 50%. This viral suspension was mixed on a rotating wheel overnight at 4 °C and subsequently centrifuged at 4000 rpm, 4 °C, 1 hour. The supernatant was removed, and the pellet was resuspended in 200 µl of PBS. The viral particles were aliquoted and stored at -80 °C or used immediately to transduce MutuDC1 cells.

3.2.5. Transduction of MutuDC1s with lentiCRISPR v2 constructs

MutuDC1 cells were seeded at a density of 10^4 cells/cm^2. Lentiviral-particle dilutions were prepared in complete DC-culture medium supplemented with polybrene at a concentration of 7 µg/mL. MutuDC1 cells were transduced overnight (15 – 17 hours) with 150 µL/cm^2 of lentiviral-particle suspension. After transduction, the lentiviral-particle-containing supernatant was removed and replaced with fresh DC-culture medium. Before selection with puromycin, the cells were let recover for 48 hours, replacing the culture medium every 24 hours. To select transduced cells, a selection medium was prepared by diluting puromycin in complete DC-culture medium at the final concentration of 0,5 µg/mL. The cells were incubated with the selection medium for 3 days.

3.2.6. Cell viability assessment and functional titration of lentiviral particles

To assess lentiviral functional titers MutuDC1s were seeded in a 96-well plate at a density of $3x10^4$ cells/well. Serial two-fold lentiviral dilutions were prepared in complete DC-culture medium supplemented with 7 μg/mL polybrene and used to transduce MutuDC1s in sextuplicate (11 dilutions of lentiviral particles in two sets of triplicates), including a no-virus control sextuplicate. The number of cells in every well of the first set of triplicates was estimated 48 hours post-transduction through a cell viability assay (AlamarBlue™ Cell Viability Reagent, Thermo Fisher SCIENTIFIC, ref. DAL1100) by interpolation with a standard curve of MutuDC1 cell densities. The second set of triplicates was selected with 0,5 μg/mL of puromycin for 3 days. A cell viability assay was carried out, as described above, to determine the number of cells that survived the selection. The percentage of transduced cells was calculated for every lentiviral dilution as the ratio between the number of cells that survived the selection for every lentiviral dilution (averages of each triplicate) and the number of cells that were measured 48 hours post-transduction (averages of each triplicate). The MOI was calculated for every lentiviral dilution as follows assuming Poisson's distribution of infection probability: $MOI = -\log_e(1-P)$, where P is the percentage of infected cells at a certain lentiviral dilution, that is the percentage of puromycin resistant cells.

3.2.7. PCR of sgRNA sequences from lentiCRISPR v2 constructs integrated in genomic DNA

Genomic DNA was extracted from transduced or non-transduced MutuDC1s by incubation of cell pellets at 95 °C in 50 mM NaOH for 30 minutes under agitation, followed by addition of 8% of 1 M Tris-HCl pH 8. For the PCR, the following primers were used: v2Adaptor F (5' – AATGGACTATCATATGCTTACCGTAACTTGAAAGTATTTCG – 3') and v2Adaptor R (5' – TCTACTATTCTTTCCCCTGCACTGTTGTGGGCGATGTGCGCTCTG – 3'). The following program was used:

1st PCR program

1. 5 min 95 °C
2. 30 sec 95 °C
3. 30 sec 55,5 °C
4. 30 sec 72 °C repeat from 2. 29x
5. 5 min 72 °C

Taq DNA polymerase from *Thermus aquaticus* (SIGMA-ALDRICH, ref. D6677)

3.2.8. Activation of MutuDC1s

MutuDC1s were stimulated with CpG or poly(I:C) as described in the section **MATERIALS AND METHODS**, subsection **TLR Stimulation and Cytokine Detection**, of paragraph *4.1.1. Establishment and characterization of a functionally competent type 2 conventional dendritic cell line*. IFNγ (ThermoFisher SCIENTIFIC, ref. BMS326) was used at a final concentration of 100 U/mL.

3.2.9. Cell staining and flow cytometry

Extracellular staining: MutuDC1s were incubated for 30 minutes on ice with a staining mix composed of the appropriate antibody diluted in a 1:2 solution of FACS buffer (3% FCS, 5 mM EDTA in PBS) and supernatant from hybridoma 2.4G2 (Fc receptor blocking). In the case of staining with anti-CCR7 antibody, the cells were incubated with the staining mix at 37 °C, according to manufacturer's indications. When a biotin-conjugated antibody was used, the cells were first incubated with the antibody as described above and, after extensive washing with FACS buffer, they were incubated for 30 minutes on ice with a mix composed of Brilliant Violet 421™-conjugated streptavidin (BioLegend, ref. 405225) diluted in FACS buffer. The cells were analyzed immediately after staining or fixed in 1% paraformaldehyde (PFA) at room temperature for 10 min, stored at 4 °C and analyzed

within 3 days after the staining. Intracellular staining: after the desired extracellular staining, MutuDC1s were fixed as described above. Fixed cells were incubated for 30 minutes at room temperature with a mix composed of the appropriate antibody diluted in a 1:2 solution of saponin buffer (3% FCS, 0,5% saponin in PBS) and supernatant from hybridoma 2.4G2. The cells were analyzed immediately after staining or stored at 4 °C and analyzed within 3 days after the staining. Alternatively, for intranuclear staining with an anti-FLAG monoclonal antibody the eBioscience™ Foxp3/Transcription Factor Staining Buffer Set (Thermo Fisher SCIENTIFIC, ref. 00-5523-00) was used according to manufacturer's instructions.

Flow cytometric data were acquired with BD LSR-II or BD LSRFortessa cytometers (BD Biosciences) and analyzed with FlowJo (version 10.0.8r1, Tree Star, Inc.).

The monoclonal antibodies that were used were specific for: CD62E (clone REA369, Biotin, Miltenyi Biotec), CD194 (CCR4) (clone 2G12, Brilliant Violet 421™, BioLegend), CD197 (CCR7) (clone 4B12, APC, Biotin, eBioscience), CD200 (OX2) (clone OX-90, APC, BioLegend), CTLA-4 (clone 1B8, PE, eBioscience).

3.2.10. Protein extraction and Western blotting

The composition of the main solutions used for protein SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) and Western blotting is listed below.

- Laemmli buffer (sample buffer) 4x: 250 mM Tris-HCl pH 6,8, 40% Glycerol, 8% SDS, 100 mM dithiothreitol (DTT), 0,002% bromophenol blue.
- Running gel: Acrylamide/Bis-acrylamide (ratio 37,5:1) at the desired percentage, 375 mM Tris-HCl pH 8,8, 0,1% sodium dodecyl sulfate (SDS), 0,1% ammonium persulfate (APS), 0,1% Tetramethyl ethylenediamine (TEMED).

- Stacking gel: Acrylamide/Bis-acrylamide (ratio 37,5:1) 4%, 125 mM Tris-HCL pH 6,8, 0,1% SDS, 0,1% APS, 0,1% TEMED.
- Transfer buffer: 25 mM Tris base, 19,2 mM Glycine, 0.03% SDS, 20% Ethanol.
- Tris-Buffered Saline Tween (TBST): 150 mM NaCl, 50 mM Tris-HCl pH 7,5, Tween20 0,1%.
- Tris-Glycine buffer: 25 mM Tris base, 19,2 mM Glycine, 0,1% SDS.

Total-protein extraction was carried out as follows: MutuDC1s were lysed with radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris-HCl pH 8, 150 mM NaCl, 1% Triton x-100, 0,5% sodium deoxycholate, 0,1% SDS, protease inhibitor (SIGMA-ALDRICH, ref. 11836153001)) for 30 minutes on ice, vortexing every 10 min. The samples were centrifuged at 17000 x g, 15 min, 4 °C and the supernatants (protein extracts) were used immediately or stored at -80 °C for future testing.

Total-protein samples were mixed with Laemmli buffer, incubated at 95 °C for 10 minutes and centrifuged at 17000 x g for 5 minutes. The samples were loaded on a polyacrylamide gel and the proteins were separated through SDS-PAGE. The proteins were transferred on a 0,45 µm nitrocellulose membrane for 2 hours at 350 mA, 4 °C in pre-cooled transfer buffer. A Ponceau S solution was used to reveal the proteins on the nitrocellulose membrane and to visually quantify the homogeneity of loading. All the washing steps were carried out in TBST. The membrane was blocked with 5% skimmed milk in TBST for 1 hour at room temperature. The membrane was incubated overnight with the appropriate primary antibody diluted in 5% milk-TBST at 4 °C. After extensive washing the membrane was incubated for 2 hours at room temperature with the appropriate HRP-conjugated secondary antibody diluted in 5% milk-TBST. Antibody binding was revealed with the SuperSignal™ West Pico Chemiluminescent Substrate (Thermo Fisher SCIENTIFIC, ref. 34080) and detected through exposition of an X-ray film.

4. Results

4.1. Generation of new MutuDC lines

4.1.1. Establishment and characterization of a functionally competent type 2 conventional dendritic cell line

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Summary

The exclusive generation of cDC1-like MutuDCs (MutuDC1s) from Mushi1 mice and the lack of cDC2-like DC model lines, induced us to design a new strategy to derive a cDC2-like MutuDC line. We generated a new mouse strain in which the SV40LgT was introduced in a Batf3$^{-/-}$ genetic background where cDC1 development is severely impaired. The new Batf3$^{-/-}$ Mushi1 mouse strain developed histiocytosis but with different characteristics if compared with Mushi1 mice in terms of disease onset and involvement of secondary lymphoid organs. The cell line derivation process carried out from the spleen of diseased Batf3$^{-/-}$ Mushi1 mice allowed to obtain several immortalized cell lines. Among them, one was chosen for the phenotypic and functional characterization that is described in this article. Our results clearly showed that this newly derived MutuDCs have phenotypic and functional characteristics that distinguish them from cDC1s, from MutuDC1s and from moDCs. By contrast, their features are consistent with their belonging to the CD4^{-} subset of splenic cDC2s (spl-cDC2s) [386].

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Establishment and Characterization of a Functionally Competent Type 2 Conventional Dendritic Cell Line

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Dendritic cells (DCs) are the most potent antigen presenting cells and possess an incomparable ability to activate and instruct T cells, which makes them one of the cornerstones in the regulation of the cross-talk between innate and adaptive immunity. Therefore, a deep understanding of DC biology lays the foundations to describe and to harness the mechanisms that regulate the development of the adaptive response, with clear implications in a vast array of fields such as the study of autoimmune diseases and the development of new vaccines. However, the great difficulty to obtain large quantities of viable non-activated DCs for experimentation have considerably hindered the progress of DC research. Several strategies have been proposed to overcome these limitations by promoting an increase of DC abundance *in vivo*, by inducing DC development from DC progenitors *in vitro* and by generating stable DC lines. In the past years, we have described a method to derive immortalized stable DC lines, named MutuDCs, from the spleens of Mushi1 mice, a transgenic mouse strain that express the simian virus 40 Large T-oncogene in the DCs. The comparison of these DC lines with the vast variety of DC subsets described *in vivo* has shown that all the MutuDC lines that we have generated so far have phenotypic and functional features of type 1 conventional DCs (cDC1s). With the purpose of deriving DC lines with characteristics of type 2 conventional DCs (cDC2s), we bred a new Batf3$^{-/-}$ Mushi1 murine line in which the development of the cDC1 subset is severely defective. The new MutuDC line that we generated from Batf3$^{-/-}$ Mushi1 mice was phenotypically and functionally characterized in this work. Our results demonstrated that all the tested characteristics of this new cell line, including the expression of subset-determining transcription factors, the profile of cytokine production and the ability to present antigens, are comparable with the features of splenic CD4^{-} cDC2s. Therefore, we concluded that our new cell line, that we named CD4^{-} MutuDC2 line, represents a valuable model for the CD4^{-} cDC2 subset.

Keywords: dendritic cell, cell line, conventional DC subset, cDC1, cDC2, cell culture, spleen, mouse

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INTRODUCTION

Dendritic cells (DCs) are a heterogeneous lineage of innate immune cells with a unique and essential role in the initiation and orchestration of the adaptive immune response (1). Upon encounter with pathogens, DCs become activated and undergo a series of functional modifications that include induction of cytokine and chemokine production, regulation of surface marker expression and increase of antigen presentation efficiency (2).

Pathogen sensing by DCs is achieved by means of a broad group of surface receptors called pattern recognition receptors (PRRs). PRRs recognize specific molecular motifs, collectively known as microbe associated molecular patterns (MAMPs), which are conserved among pathogenic and non-pathogenic microorganisms (3, 4). One of the most studied groups of PRRs is the toll like receptor (TLR) family which in mouse comprises twelve members, namely TLR1-9 and TLR11-13 (4). To carry out their function, TLRs form binary protein complexes that can be either homodimeric or heterodimeric, as in the case of TLR1/2 and TLR2/6, (5, 6), and that recognize MAMPs with distinct ligand specificity. The recognition of a MAMP by its specific TLR dimer triggers a signaling cascade that culminates in the activation of DCs with consequent increase of surface markers, like MHC-II and co-stimulatory molecules, and regulation of several effector genes, including pro-inflammatory cytokines and chemokines (7, 8).

DCs constantly endocytose self and foreign antigens, but it is especially following activation and maturation that they become highly efficient at forming peptide-MHC complexes for antigen presentation to T cells (9). Endogenous self-antigens or intracellular pathogen-derived antigens are presented to $CD8^+$ T cells through direct MHC-I presentation. Instead, exogenous antigens, endocytosed from the extracellular environment, can be presented either to $CD4^+$ T cells, by means of peptide-MHC-II complexes, or to $CD8^+$ T cells via the alternative MHC-I pathway known as cross-presentation (9–11). Together with antigen presentation through peptide-MHC complexes, the upregulation of co-stimulatory molecules and the induction of cytokine production that follow DC activation provide the three canonical signals required for T cell activation (2).

As previously mentioned, DCs are very heterogeneous and can be classified into several distinct subsets. At the steady state, DCs are roughly divided into non-lymphoid tissue DCs, which during inflammation circulate loaded with antigens from the peripheral tissues to the draining lymph nodes through the lymphatics (12, 13), and lymphoid tissue-resident DCs, which differentiate and dwell in the lymphoid organs (14, 15). Additionally, under inflammatory conditions, blood circulating monocytes can differentiate into a subset of DCs known as monocyte-derived/inflammatory DCs (moDCs) (12, 16–20). The steady-state DC population is composed of two main subsets: plasmacytoid DCs (pDCs), which are known to be major producers of IFNα during antiviral response (21, 22), and conventional DCs (cDCs) (23). CDCs are subdivided into type 1 cDCs (cDC1s) and type 2 cDCs (cDC2s) on the basis of their ontogeny (24). In particular, the cDC1 subset includes all the cDCs whose development depends on the basic leucine zipper ATF-like transcription factor 3 (Batf3) (25, 26) and on the interferon regulatory factor 8 (IRF8) (25, 27, 28), while the cDC2 subset comprises all the cDCs that are independent of these transcription factors and that, by contrast, develop in an interferon regulatory factor 4 (IRF4)-dependent manner (29–31). Notably, this ontogeny-based classification system can be extended to cDCs across separate organs and species (32), overcoming the confusion generated by the phenotypic variability of analogous cDC subsets in different tissues. The different DC subsets express a wide variety of subset- and tissue-specific surface markers (1, 32) among which CD8α, CD11b, and CD4 have been traditionally used in mouse to discriminate the splenic resident cDC1s (spl-cDC1s) and cDC2s (spl-cDC2s) (33).

This varied assortment of DC subsets reflects a diversified array of functional specificities in terms of pathogen sensing, cytokine production and antigen presentation (34, 35). For instance, cDC1s are characterized by high levels of TLR3 and TLR9 but display selective lack of TLR7, while cDC2s have a wider TLR profile but show limited or absent TLR3 expression (36). Upon activation, cDC1s produce considerable quantities of IL-12, while cDC2s are known to be poor producers of this cytokine (33, 37, 38). Additionally, cDC1s are specialized in MHC-I-mediated cross-presentation of extracellular antigens, and hence they are more addressed toward cytotoxic T lymphocyte priming, while cDC2s are mainly oriented to MHC-II-mediated presentation and helper T cell activation (39–41).

In the past years, our group has developed a new method to generate immortalized DC lines from the spleen of a murine model of multisystem histiocytosis named Mushi1 (multisystem histiocytosis line 1) (42). Mushi1 mice carry a transgenic construct that contains the simian virus 40 Large T-oncogene (SV40LgT) and an IRES-linked EGFP reporter under the control of the 5.7 kb CD11c proximal promoter that restricts the transgene expression almost exclusively to DCs (42, 43). Between 3 and 5 months of age, Mushi1 mice develop splenic tumors caused by tumorigenic transformation of spl-cDC1s (42). From the tumors, stable cell lines named MutuDCs (murine tumor DCs) can be derived (44). These cells can be easily cultured through standard procedures without additional growth factors and are stable in long term culture for a minimum of 40 passages. Meticulous analysis of MutuDCs has clearly demonstrated that they share with spl-cDC1s all the main phenotypic and functional characteristics including the cross-presentation ability, and hence we refer to them as MutuDC1s (44).

In this work, we describe the derivation of the new $CD4^-$ MutuDC2 line from splenic tumors of $Batf3^{-/-}$ Mushi1 mice and we illustrate how the selective absence of cDC1s in this genetic background (26) allowed to obtain a cell line whose features are different from spl-cDC1s. We also show and discuss the characterization of these cells demonstrating that their phenotype and function are consistent with their belonging to the $CD4^-$ spl-cDC2 subset.

MATERIALS AND METHODS

Mice

C57BL/6JOlaHsd mice were purchased from Harlan Laboratories. Mushi1 (42), OT-I, and OT-II transgenic mice were maintained and bred in our own facility. $Batf3^{-/-}$ mice in a C57BL/6J background (26) were provided by Prof. Kenneth M. Murphy (Washington University School of Medicine in St. Louis, St. Louis, MO). All the animals were housed and bred under specific pathogen free conditions and used at an age of at least 8 weeks. Genomic DNA from pups of $Batf3^{-/-}$ x Mushi1 litters was extracted by incubation of ear samples at 95°C in 600 μL of 50 mM NaOH for 30 min followed by

addition of 50 μL of 1 M Tris-HCl pH 8. The genomic DNA was used for genotypic screening by PCR using the following primers: pCD11c (5′-GGCAGCTGTCTCCAAGTTGCTCAG-3′) and RβGR1 (5′-GGGTCCATGGTGATACAAGGG-3′) (42). All animal experiments were performed after approval by the cantonal veterinary office (Service de la consommation et des affaires vétérinaires, Département du territoire et de l'environnement, Permission no. VD2490.1).

Culture Conditions and Generation of MutuDC Lines

Cells were kept in culture at 37°C in a humidified incubator with 5% CO_2. Complete culture medium was composed as follows: IMDM+GlutaMAX™ Supplement (31980, GIBCO), 10 mM HEPES (15630, GIBCO), 0.075% $NaHCO_3$ (from 7.5% $NaHCO_3$ stock solution, 25080, GIBCO), 50 μM β-mercaptoethanol (31350, GIBCO), 8% heat inactivated FCS (tested for toxicity toward DC cultures), 50 U/mL penicillin, 50 μg/mL streptomycin (15070, GIBCO). Cells were harvested by treatment with a non-enzymatic cell dissociation buffer (5 mM EDTA, 20 mM HEPES in PBS). The derivation of the new $CD4^-$ MutuDC2s was carried out as previously described for other MutuDCs (44, 45). Spleens from diseased $Batf3^{-/-}$ Mushi1 were cut with a scalpel and filtered through a 40 μm cell strainer to obtain single cell suspensions. The splenocytes were seeded in serial two-fold dilution in a 24-well plate at a starting density $\geq 10^7$ cells/well. After 8–16 h, non-adherent cells were removed by washing the wells. The adherent cells were maintained in 24-well plates for 5–10 passages during which the wells were frequently washed to remove non-adherent/dead cells or debris, and culture medium was changed periodically. In the earliest passages the cells were always kept at high density and split at a maximum 1:2 dilution. When the cells became able to tolerate 1:6 splitting, the cultures were progressively expanded. The MutuDCs chosen for the characterization described in this work have been numbered 20956A (clone A from mouse HAO-20956).

Light Microscopy

Pictures of cells were obtained by photographing the cultures directly with the EVOS™ FL Color Imaging System (AMEFC4300, ThermoFisher SCIENTIFIC).

Splenocyte Isolation, Staining, and Antibodies for Flow Cytometry

Spleens were cut into small pieces with a scalpel and incubated for 20 min at 25°C in a freshly prepared collagenase D/DNase I solution composed as follows: RPMI 1640+GlutaMAX™ Supplement (61870, GIBCO), 2% FCS, 1 mg/mL collagenase D (11088866001, ROCHE), 40 μg/mL DNase I (10104159001, ROCHE). After the digestion, the spleens were filtered through a 40 μm cell strainer to obtain single cell suspensions. All the washing steps and acquisitions were carried out in FACS buffer (3% Fetal Bovine Serum, 5 mM EDTA in PBS). For the staining, the cells were incubated for 30 min on ice with a staining mix composed of the appropriate antibody combinations diluted in a 1:2 solution of FACS buffer and supernatant from hybridoma 2.4G2. The cells were analyzed immediately after staining or fixed in 1% paraformaldehyde at room temperature for 10 min, stored at 4°C and analyzed within 3 days after the staining. For intracellular staining the eBioscience™ Foxp3 / Transcription Factor Staining Buffer Set (00-5523-00, ThermoFisher SCIENTIFIC) was used according to manufacturer's instructions. Flow cytometric data were acquired with BD LSR-II or BD LSRFortessa cytometers (BD Biosciences) and analyzed with FlowJo (version 10.0.8r1, Tree Star, Inc.). The fluorochrome-conjugated monoclonal antibodies that were used were specific for: B220 (CD45R) (clone RA3-6B2, Alexa Fluor 700, eFluor 450, PE, PE-Cy7, eBioscience), CD4 (clone RM4-5, APC, Pacific Blue, PE-Cy7, BioLegend, or APC, eFluor 450, PE-Cy7, PerCP-Cy5.5, eBioscience), CD8α (clone 53-6.7, PE-Cy7, BD Biosciences, or APC, APC-eFluor 780, eFluor 450, PE-Cy7, PerCP-Cy5.5, eBioscience), CD11b (clone M1/70, APC, PE, BioLegend, or APC, APC-eFluor 780, eBioscience), CD11c (clone N418, APC, PacificBlue, PE-Cy7, BioLegend, or eFluor 450, PE, PE-Cy7 eBioscience), CD24 (clone M1/69, APC, BD Biosciences, or eFluor 450, eBioscience), CD40 (clone 1C10, APC, PE, eBioscience), CD80 (clone 16-10A1, Brilliant Violet 421, BioLegend, or APC, eBioscience), CD86 (clone GL1, Alexa Fluor 700, BioLegend, or APC, eBioscience), CD64 (FcγRI) (clone X54-5/7.1, PE, BioLegend), CD172a (clone P84, APC, PE, BD Biosciences), CD205 (clone NLDC-145, APC, BioLegend, or clone 205yekta, PerCP-eFluor 710, eBioscience), CD206 (MMR) (clone C068C2, PE, BioLegend), CLEC9A (CD370) (clone 7H11, APC, PE, BioLegend, or clone 42D2, PE, eBioscience), F4/80 (clone BM8, APC, BioLegend, or eFluor 450, eBioscience), FcεRIα (clone MAR-1, PE, BioLegend), FLT3 (CD135) (clone A2F10, PE, eBioscience), Gr-1 (clone RB6-8C5, APC, BioLegend, or PE, eBioscience), IFNγ (clone XMG1.2, PE, BD Biosciences, or PE, BioLegend, or PE, PE-Cy7, eBioscience), IRF4 (clone 3E4, PE-Cy7, eBioscience), IRF8 (clone V3GYWCH, APC, eBioscience), MHC-II (clone M5/114.15.2, PerCP, BioLegend, or Alexa Fluor 700, PE, eBioscience), PDCA-1 (CD317) (clone eBio129c, PE, eBioscience), TLR5 (clone ACT5, Alexa Fluor 647, BioLegend).

TLR Stimulation and Cytokine Detection

$CD4^-$ MutuDC2s and MutuDC1s were seeded at a density of 2.5×10^5 cells/cm^2 in 96-well or 48-well plates and incubated for 24 h with 450 μL/cm^2 of the following TLR agonists diluted in complete medium: Pam3CSK4 (150 ng/mL, tlrl-pms, InvivoGen), poly(I:C) (8.5 μg/mL, tlrl-pic, InvivoGen), LPS from *E. coli* (100 ng/mL, tlrl-peklps, InvivoGen), ultrapure flagellin from *B. subtilis* (100 ng/mL, tlrl-pbsfla, InvivoGen), FSL-1 (100 ng/mL, tlrl-fsl, InvivoGen), Gardiquimod™ (1 μg/mL, tlrl-gdqs, InvivoGen), CpG ODN 1826 (1 μM, TriLink BIOTECHNOLOGIES). In all the experiments each condition was plated in technical triplicate. The supernatants were analyzed by ELISA for the presence of IL-6, IL-10, IL-12/IL-23 p40, IL-12p70, and MCP-1(CCL2) using the following kits according to manufacturer's instructions: Mouse IL-6 ELISA Set (555240, BD Biosciences) or Mouse IL-6 ELISA Ready-SET-Go! (88-7064, eBioscience), Mouse IL-10 ELISA Set (555252, BD Biosciences) or Mouse IL-10 (Interleukin-10) ELISA Ready-SET-Go! (88-7104, eBioscience), Mouse IL-12 (p40) ELISA Set

(555165, BD Biosciences), Mouse IL-12 (p70) ELISA Set (555256, BD Biosciences), Mouse CCL2 (MCP-1) ELISA Ready-SET-Go! (88-7391, eBioscience).

RNA Extraction, cDNA Synthesis, and RT-qPCR

Total RNA from CD4$^-$ MutuDC2s and MutuDC1s was extracted with the RNeasy Plus Mini Kit (74134, QIAGEN) according to manufacturer's instructions and stored in RNA secure (AM7005, Thermo Fisher SCIENTIFIC). The synthesis of cDNA was carried out using random nonamers and the M-MLV reverse transcriptase kit (M1701, Promega) or the SuperScript™ Reverse Transcriptase kit (18064014, Thermo Fisher SCIENTIFIC) according to manufacturer's instructions, with the addition of RiboLock RNase Inhibitor (EO0381, Thermo Fisher SCIENTIFIC). DNA/RNA hybrids were removed with RNase H (70054Y, Thermo Fisher SCIENTIFIC). cDNAs were purified using the QIAquick PCR Purification Kit (28104, QIAGEN). RNA and cDNA yields were quantified by Nanodrop spectrophotometry (Thermo Fisher SCIENTIFIC). RT-qPCR was carried out using KAPA SYBR® FAST qPCR kit for LightCycler®480 (KK4611, SIGMA-ALDRICH) on a LightCycler®480 (384-well plate, 5 μL reaction) from Roche Diagnostics. The following primers were used at the final concentration of 500 nM: TLR3 FW (5′-GCGTTGCGAAGTG AAGAA-3′), TLR3 REV (5′-TCGAGCTGGGTGAGATTT-3′), TLR5 FW (5′-CCTCATCTCACTGCATACC-3′), TLR5 REV (5′-TATTACCAACACGGGGCT-3′), ACTB FW (5′-CTGAACCC TAAGGCCAACCGTG-3′), ACTB REV (5′-GGCATACAGGG ACAGCACAGCC-3′). Every sample was analyzed in technical triplicates.

T Cell Activation Assays

Ovalbumin-specific CD8$^+$ and CD4$^+$ T cells were isolated from spleens and lymph nodes (brachial, inguinal and mesenteric) of OT-I and OT-II mice, respectively, and purified using the following MACS or EasySep™ kits: CD4$^+$ T Cell Isolation Kit, mouse (130-104-454, Miltenyi Biotec), CD8a$^+$ T Cell Isolation Kit, mouse (130-104-07, Miltenyi Biotec), EasySep™ Mouse CD4+ T Cell Isolation Kit (19852, STEMCELL™ TECHNOLOGIES), EasySep™ Mouse CD8+ T Cell Isolation Kit (19853, STEMCELL™ TECHNOLOGIES). The T cell isolation kits were used following manufacturer's protocols except for the buffers that were prepared as follows: MACS buffer (0.5% FCS, 2 mM EDTA in PBS), EasySep buffer (2% FCS, 1 mM EDTA in PBS). A fraction of the purified T cells was stained with fluorochrome-conjugated monoclonal antibodies specific for TCR β chain (clone H57-597, Brilliant Violet 510, BioLegend) and for either CD4 (clone RM4-5, APC, BioLegend or eBioscience) or CD8α (clone 53-6.7, APC, eBioscience) and analyzed by flow cytometry to assess T cell purity. The purified T cells were stained with the cell proliferation dye eFluor™ 670 (65-0840, eBioscience) or eFluor™ 450 (65-0842, eBioscience). CD4$^-$ MutuDC2s and MutuDC1s were plated in 96-well round bottom plates at a density of 10^4 cells/well and incubated for 6–8 h with the ovalbumin-derived peptides $OVA_{332-339}$ (Protein and Peptide Chemistry Facility, UNIL) or $OVA_{257-264}$ (Protein and Peptide Chemistry Facility, UNIL) or with the full-length ovalbumin (vac-pova, InvivoGen) in the presence of CpG ODN 1826 (1 μM, TriLink BIOTECHNOLOGIES). At the end of the incubation, the supernatants were removed and the wells were washed gently with fresh complete medium. The proliferation dye-labeled T cells were plated with the MutuDCs at a density of 10^5 cells/well. After 3 days (CD8$^+$ T cells) or 4 days (CD4$^+$ T cells) of co-culture, the supernatant was removed from each well and the cells were restimulated for 6 h with phorbol 12-myristate 13-acetate (PMA) (10 ng/mL, P8139, SIGMA-ALDRICH), ionomycin (500 ng/mL, I0634, SIGMA-ALDRICH) in the presence of brefeldin A (00-4506-51, eBioscience). The T cells were stained with fluorochrome-conjugated monoclonal antibodies and analyzed by flow cytometry for proliferation and IFNγ production.

Statistical Analyses

All the statistical analyses were carried out using GraphPad Prism 7.04.

RESULTS

Generation of Batf3$^{-/-}$ Mushi1 Mice and Derivation of CD4$^-$ MutuDC2s

In the Mushi1 mouse, the development of splenic tumors is invariably caused by spl-cDC1 transformation (42). In this study, we aimed to design a strategy to favor the transformation of spl-cDC2s over spl-cDC1s with the purpose of generating new spl-cDC2-like MutuDC lines. To do this, we crossed Mushi1 mice with Batf3$^{-/-}$ mice (**Figure 1A**) to introduce the CD11c:SV40LgT transgenic construct in the Batf3$^{-/-}$ genetic background where all the cDC1s are selectively absent (26, 46).

Batf3$^{-/-}$ Muhi1 mice started showing signs of histiocytosis between 6 and 8 months of age (**Figure 1B**), with a delay of around 3 months if compared with Mushi1 mice (42). Similarly to sick Mushi1 mice, diseased Batf3$^{-/-}$ Mushi1 mice developed splenic tumors characterized by splenomegaly (**Figure 1C**). Additionally, in Batf3$^{-/-}$ Mushi1 mice the progression of the disease was occasionally associated with peripheral lymphadenopathy (**Figure 1D**) that instead was never observed in Muhi1 mice.

Cell line derivation from Batf3$^{-/-}$ Mushi1 splenic tumors was carried out as previously described (44, 45) and is schematized in **Figure 2A**. This procedure allowed to generate numerous immortalized cell lines from several mice. After a first exploratory phenotypic analysis, the most promising cell line, that in this work is named CD4$^-$ MutuDC2 line, was chosen for further phenotypic and functional characterization. At the steady state, the stable CD4$^-$ MutuDC2s have round morphology with few or absent dendritic processes (**Figure 2B**). They are adherent and have a slight tendency to cluster (**Figure 2B**). In conditions of prolonged culture, the CD4$^-$ MutuDC2s can be split at densities as low as 1.5×10^4 cells/cm^2 and maintain their phenotype and function for at least 40 passages. With the increment of passage number, moderate increase of clustering tendency and slight reduction of adherence can be observed.

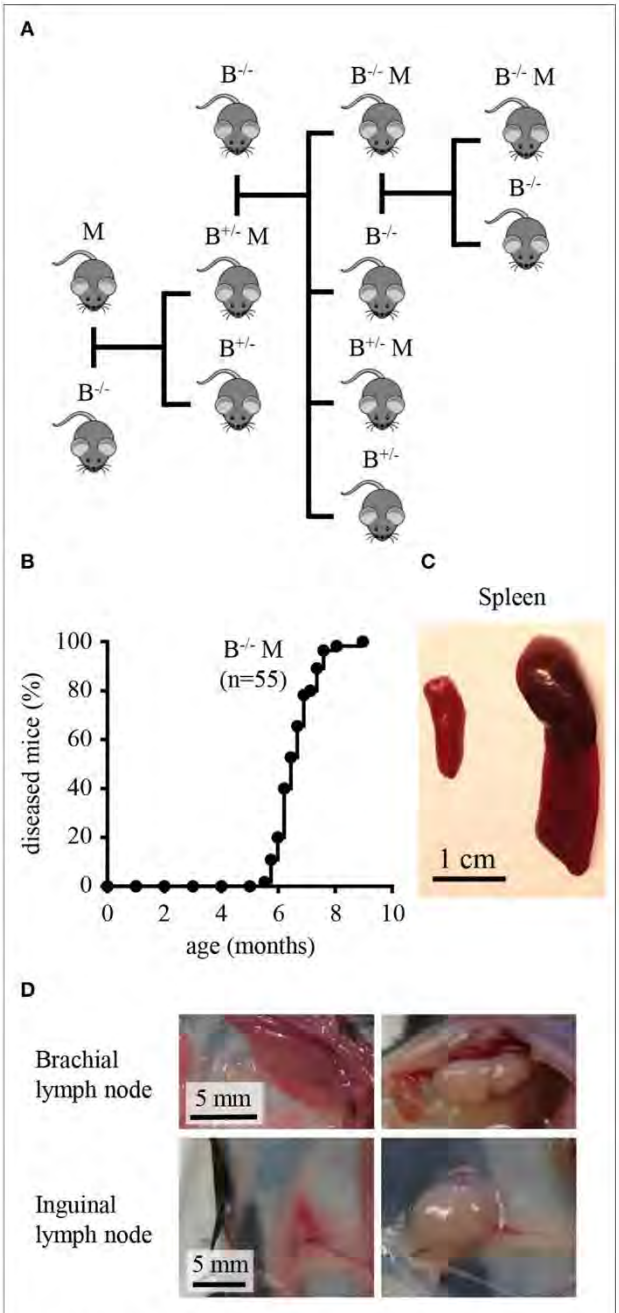

FIGURE 1 | Batf3$^{-/-}$ Mushi1 mice develop splenic tumors with occasional peripheral lymphadenopathy. **(A)** Breeding strategy to generate the new Batf3$^{-/-}$ Mushi1 mouse strain. Mushi1 mice are heterozygous for a transgenic construct which contains the SV40 Large T-oncogene under the control of the murine CD11c promoter. Batf3$^{-/-}$ mice (B$^{-/-}$) were crossed with Mushi1 mice (M). Batf3$^{+/-}$ Mushi1 mice (B$^{+/-}$ M) from the offspring were then backcrossed with B$^{-/-}$ mice. Starting from the second generation, the strain was maintained by breeding B$^{-/-}$ M with B$^{-/-}$ mice. **(B)** Age-dependent cumulative percentage of diseased Batf3$^{-/-}$ Mushi1 mice. **(C,D)** Compared with healthy Batf3$^{-/-}$ Mushi1 controls (left), diseased mice (right) show **(C)** spleen enlargement **(D)** and occasionally develop peripheral lymphadenopathy.

When analyzed by flow cytometry, the CD4$^-$ MutuDC2s were found to be homogeneous (**Figures 2C,D**) and to express the SV40LgT-associated reporter EGFP even if at lower levels than the previously derived MutuDC1s (**Figure 2D**). This observation indicates lower expression of SV40LgT in the CD4$^-$ MutuDC2s than in the MutuDC1s. In Mushi mice, higher levels of SV40LgT correspond to earlier onset of histiocytosis suggesting a dose-dependency of SV40LgT (42). Therefore, the lower expression of SV40LgT observed in the CD4$^-$ MutuDC2s might explain the delayed development of splenic tumors in Batf3$^{-/-}$ Mushi1 mice.

CD4$^-$ MutuDC2s Are Phenotypically Similar to CD4$^-$ spl-cDC2s

To assess the phenotypic resemblance of the CD4$^-$ MutuDC2s to spl-cDCs, we analyzed their expression of numerous extracellular and intracellular markers in comparison to fresh spl-cDCs. To do this, splenocytes from C57BL/6 mice were isolated, stained with fluorochrome-conjugated monoclonal antibodies and compared by flow cytometry with equally stained CD4$^-$ MutuDC2s (**Figures 3A–E**). Spl-cDCs were defined as CD11c^{hi}MHC-II^{hi} cells, and within this population, spl-cDC1s and spl-DC2s were distinguished on the basis of either CD8α or CD11b expression (**Figure 3A**). We observed that, similarly to fresh spl-cDCs, the CD4$^-$ MutuDC2s are CD11c^{hi}MHC-II^{hi}F4/80$^{lo/-}$B220$^-$Gr-1$^-$PDCA-1$^-$ (**Figure 3B**). At the steady state, the expression of the co-stimulatory molecules CD80 and CD86 was found to be higher than in spl-cDCs in which the levels of these markers were low but detectable (**Figure 3B**). By contrast, CD40 expression, that was low in spl-cDCs, was not detectable in the CD4$^-$ MutuDC2s (**Figure 3B**).

As mentioned, IRF8 and IRF4 have a primary role in the development of cDC1s and cDC2s, respectively (24). Fully differentiated cDC1s and cDC2s maintain a divergent expression of these two transcription factors. Indeed, spl-cDC1s express high levels of IRF8 and display low IRF4 expression, while, by contrast, both the CD4$^+$ and the CD4$^-$ spl-cDC2s have low levels of IRF8 and higher expression of IRF4 (31, 42), even though IRF4 deficiency affects more severely the development of the former subset (31). In order to correctly determine the belonging of the CD4$^-$ MutuDC2s to either the spl-cDC1 or the spl-cDC2 subset, we analyzed them for the expression of IRF4 and IRF8. Our results showed that the CD4$^-$ MutuDC2s express high levels of IRF4 and low levels of IRF8, with a profile that mirrors almost perfectly the pattern observed in spl-cDC2s as opposed to spl-cDC1s (**Figure 3C**). Consequently, we concluded that the CD4$^-$ MutuDC2s belong to the spl-cDC2 subset.

To further prove the phenotypic similarity of the CD4$^-$ MutuDC2s to spl-cDC2s we analyzed them for the expression of several spl-cDC subset-characterizing markers in comparison with fresh spl-cDC1s and spl-cDC2s. The spl-cDC2-specific markers CD11b and CD172a (47–50) appeared to be strongly expressed in the CD4$^-$ MutuDC2s at an even higher level than in spl-cDC2s, while CD4 expression was found to be absent comparably to the CD4$^-$ subpopulation of spl-cDC2 (**Figure 3D**). Analysis of the spl-cDC1-specific markers CD8α,

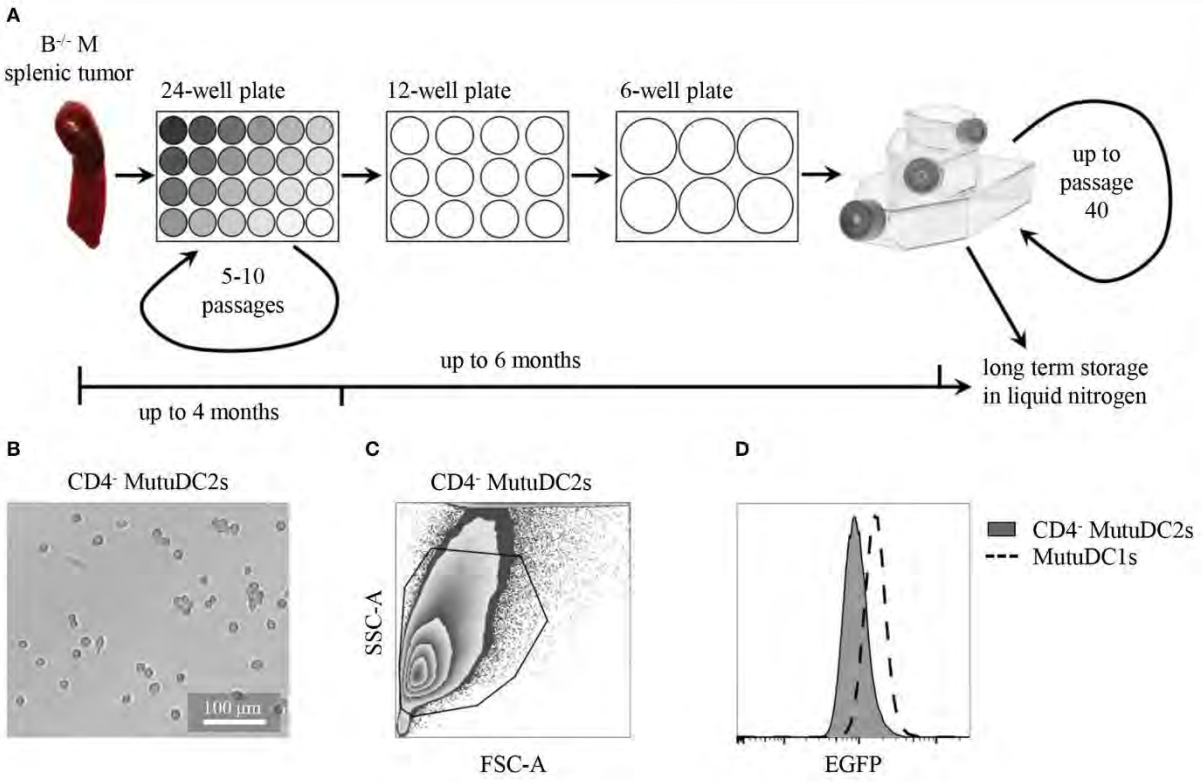

FIGURE 2 | CD4$^-$ MutuDC2s are derived from Batf3$^{-/-}$ Mushi1 splenic tumors. **(A)** Schematic representation of CD4$^-$ MutuDC2-derivation procedure. Splenocytes from Batf3$^{-/-}$ Mushi1 (B$^{-/-}$ M) splenic tumors were isolated and seeded in serial two-fold dilutions in 24-well plates. The adherent cells were maintained in 24-well plates for 5–10 passages until they became accustomed to culture conditions. The cultures were progressively expanded into plates and flasks with larger growth surfaces and frozen or used for experiments. **(B)** Light microscopy image of CD4$^-$ MutuDC2s. **(C)** Flow cytometric analysis and gating of CD4$^-$ MutuDC2s. **(D)** Flow cytometric comparison of expression of the SV40LgT-associated reporter EGFP in CD4$^-$ MutuDC2s and in MutuDC1s.

CLEC9A, CD205, and CD24 (40, 51–53) revealed that the CD4$^-$ MutuDC2s are CD8α$^-$ and CLEC9A$^-$ but express CD205 and CD24 at higher levels than the spl-cDC2s (**Figure 3D** and **Supplementary Figure 2**). However, consistently with a spl-cDC2 phenotype, the expression of these two markers is lower in the CD4$^-$ MutuDC2s than in the MutuDC1s (**Figure 3F**).

Since some characteristics of the CD4$^-$ MutuDC2s are consistent with the hypothesis of their belonging to the moDC subset, we analyzed them for the expression of moDC- or cDC-distinguishing markers to exclude the possibility of a monocytic origin. The two markers FcεRIα and CD64, which are considered very specific for moDCs (20), are respectively negative and low in the CD4$^-$ MutuDC2s, with expression profiles that are very close to the ones observed in spl-cDCs (**Figure 3E**). CD206, known as macrophage mannose receptor (MMR), is also considered a moDC-characterizing marker even if its expression in cDC subsets is debated (20, 54–57). Analysis of CD206 expression in the CD4$^-$ MutuDC2s showed that they express CD206 at low levels (**Figure 3E**) in line with a small cell population that is present within the cDC gate (**Figures 3A,E**). One of the main inducers of DC development is the the FMS-like tyrosine kinase 3 ligand (FLT3L) (58, 59). Therefore, its receptor FLT3 is one of the most characteristic markers of DCs. Accordingly, when analyzed by flow cytometry, spl-cDCs were found to be FLT3$^+$ (**Figure 3E**). Surprisingly, the analysis of surface levels of FLT3 on the CD4$^-$ MutuDC2s showed no detectable expression of this marker (**Figures 3E,G**). However, intracellular analysis of its expression in the CD4$^-$ MutuDC2s revealed high intracellular levels of FLT3 (**Figure 3G**).

CD4$^-$ MutuDC2s Are Weakly Activated by poly(I:C) or Flagellin and Express Low Levels of TLR3 but Have High Expression of TLR5

The existence of several distinct DC subsets reflects a great variety of differential functional specificities, including fundamental aspects related to pathogen sensing and cytokine production (34, 35). Spl-cDC1s are known to be the only spl-DC subset to express TLR3 (36) and represent one of the primary producers of IL-12p70 during inflammation. By contrast, spl-cDC2s lack TLR3 expression and are weak producers of IL-12p70 (33, 36–38), but express IL-6, IL-23, and MCP-1(CCL2) (42, 60, 61). To assess the TLR expression profile of the CD4$^-$ MutuDC2s and their ability

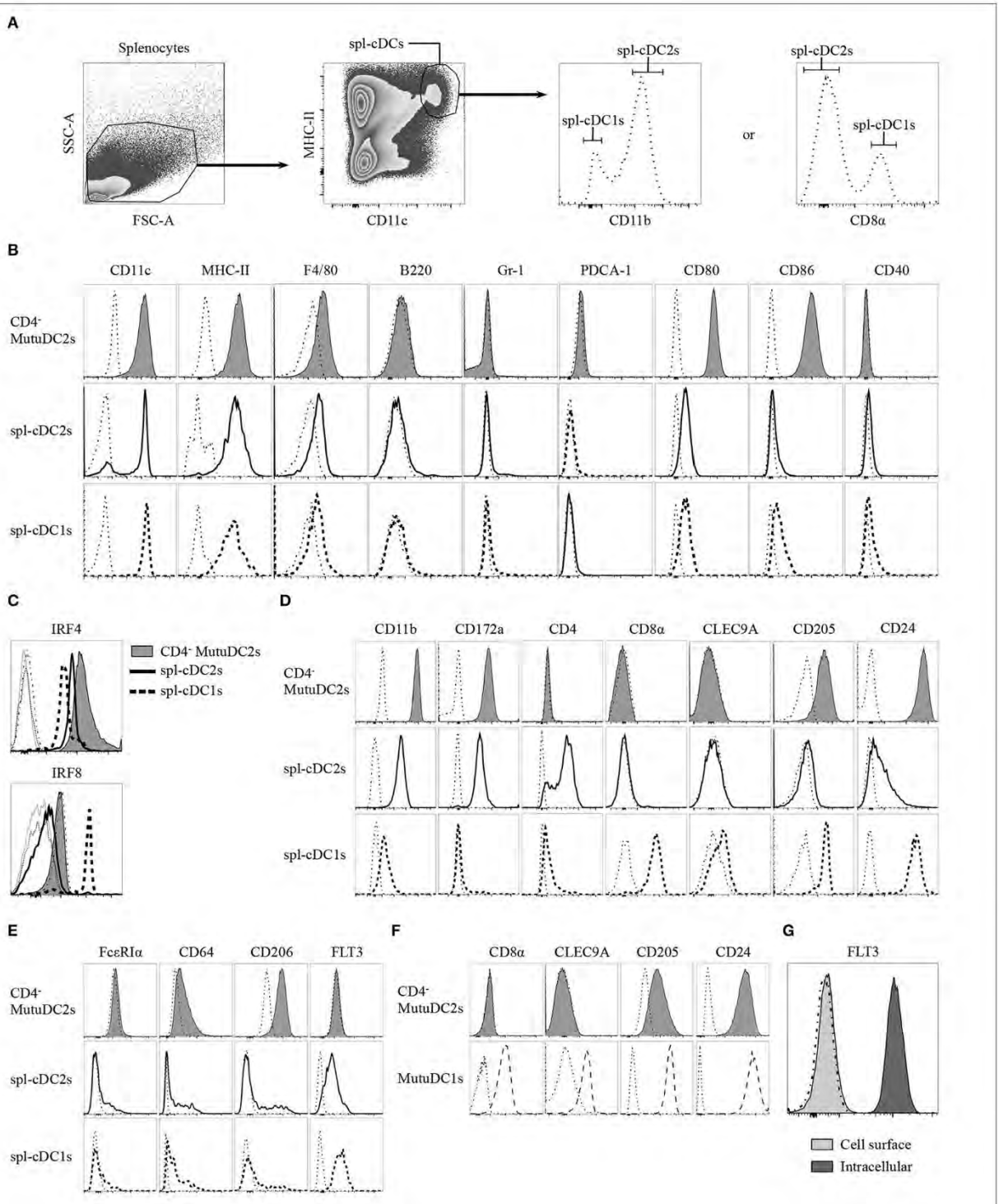

FIGURE 3 | CD4⁻ MutuDC2s share the surface and intracellular marker expression profile with CD4⁻ spl-cDC2s. **(A–E)** Splenocytes from C57BL/6 mice were isolated by digestion of spleens with collagenase D followed by filtration through a 40 μm cell strainer. CD4⁻ MutuDC2s, MutuDC1s and splenocytes were stained with different antibody cocktails that always contained anti-MHC-II and anti-CD11c antibodies to distinguish spl-cDCs. Antibodies specific for either CD8α or CD11b were included in every staining cocktail to identify spl-cDC1s and spl-DC2s. The cells were analyzed by flow cytometry. **(A)** Gating strategy to discriminate spl-cDCs and their subsets. The dash-dotted lines show the fluorescence-minus-one (FMO) controls acquired for each marker. **(B)** CD4⁻ MutuDC2s and spl-cDC subsets were

(Continued)

FIGURE 3 | compared for the expression of the indicated surface markers. The gating strategies applied to analyze CD11c and MHC-II expression in the different spl-cDC subsets are detailed in **Supplementary Figure 1**. The dash-dotted lines show the FMO controls acquired for each marker. **(C)** CD4$^-$ MutuDC2s and spl-cDCs were compared for the expression of the spl-cDC subset-specific transcription factors IRF4 and IRF8. FMO controls: CD4$^-$ MutuDC2s (dash-dotted line), spl-DC1s (dotted line), spl-DC2s (thin solid line). **(D)** CD4$^-$ MutuDC2s and spl-cDC subsets were compared for the expression of the indicated spl-cDC subset-specific surface markers. The dash-dotted lines show the FMO controls acquired for each marker. **(E)** CD4$^-$ MutuDC2s and spl-cDC subsets were compared for the expression of the indicated moDC or spl-cDC characterizing surface markers. The dash-dotted lines show the FMO controls acquired for each marker. **(F)** CD4$^-$ MutuDC2s and MutuDC1s were compared for the expression of the indicated spl-cDC1-specific surface markers. The dash-dotted lines show the FMO controls acquired for each marker. **(G)** Intracellular analysis of FLT3 expression in CD4$^-$ MutuDCs. The dash-dotted line shows the background fluorescence of unstained cells. All the results are representative of two to six independent experiments.

to respond to TLR stimulation, we treated them with different TLR ligands and measured their production of IL-6, IL-12/IL-23 p40, IL-12p70, and MCP-1(CCL2) (**Figure 4A** and data not shown). Additionally, considering the ability of some DC subsets to produce the anti-inflammatory cytokine IL-10 in response to TLR stimulation (62), we measured also IL-10 levels in the supernatants of stimulated CD4$^-$ MutuDC2s. The majority of the tested TLR ligands induced robust production of IL-6 and MCP-1(CCL2). With a similar ligand-dependent pattern, modest IL-12/IL-23 p40 secretion was observed, especially following treatment with the TLR4 ligand LPS to which the MutuDC1s respond weakly (44). However, after stimulation of the CD4$^-$ MutuDC2s with the TLR3 ligand poly(I:C) or the TLR5 ligand flagellin, we measured only low or undetectable production of IL-6, IL-12/IL-23 p40, and MCP-1(CCL2) (**Figure 4A**). These findings indicated a limited capacity of the cells to respond to TLR3 and TLR5 stimulation. In addition, none of the tested TLR ligands induced production of IL-12p70 and IL-10 at detectable levels. By contrast, in the same conditions the MutuDC1s responded mainly to TLR3 and TLR9 ligands by producing IL-12/IL-23 p40, IL-12p70, IL-6, and IL-10, while they failed to secrete detectable levels of MCP-1(CCL2) (data not shown).

We reasoned that a low expression of TLR3 and TLR5 could explain the weak responsiveness of the CD4$^-$ MutuDC2s to poly(I:C) and flagellin. To test this possibility, we measured by RT-qPCR the levels of TLR3 and TLR5 mRNAs in the CD4$^-$ MutuDC2s in comparison to the MutuDC1s which are known to have high expression of TLR3 (44) and low levels of TLR5 (unpublished RNA-seq data-sets). In line with our hypothesis, TLR3 expression was found to be around eight-fold lower in the CD4$^-$ MutuDC2s than in the MutuDC1s (**Figure 4B**). Surprisingly, the CD4$^-$ MutuDC2s showed five- to six-fold higher expression of TLR5 than the MutuDC1s (**Figure 4B**). Flow cytometric comparison of the CD4$^-$ MutuDC2s with fresh spl-cDCs further confirmed this observation by showing that TLR5 levels are higher in the CD4$^-$ MutuDC2s than in spl-cDC2s (**Figure 4C**).

CD4$^-$ MutuDC2s Are Capable of MHC-I-Mediated and MHC-II-Mediated Priming of CD8$^+$ and CD4$^+$ T Cells but Do Not Cross-Present Antigens Through MHC-I

Upon activation, DCs undergo a process of maturation which entails several morphological and functional modifications which contribute to the efficient priming of T cells, including upregulation of co-stimulatory molecules, increase of surface MHC and development of dendrites (2, 63). To test the ability of our cell line to undergo such maturation, we activated the CD4$^-$ MutuDC2s with CpG ODN and analyzed them by flow cytometry. The activated CD4$^-$ MutuDC2s increased their adherence and showed modified morphology characterized by more tapered shape, enlarged size and presence of granules in the cytoplasm (data not shown). The levels of MHC-II, CD80 and CD86, which are already high in resting CD4$^-$ MutuDC2s (**Figure 3B**), were increased after stimulation with CpG ODN (**Figure 5A**). Also CD40, which is not expressed by CD4$^-$ MutuDC2s in the resting state (**Figure 3B**), was upregulated under these conditions (**Figure 5A**).

To test the ability of the CD4$^-$ MutuDC2s to activate T cells, we pulsed them, in the presence of CpG ODN, with increasing concentrations of the ovalbumin (OVA)-derived peptides $OVA_{257-264}$ or $OVA_{323-339}$, which are respectively restricted to MHC-I and MHC-II. OVA-specific CD8$^+$ or CD4$^+$ T cells were isolated respectively from OT-I and OT-II mice, stained with a proliferation dye and co-cultured with OVA peptide-pulsed CD4$^-$ MutuDC2s. The percentage of activated T cells, defined as proliferating IFNγ^+ cells, was measured by flow cytometry (**Figure 5B**). This analysis showed that, in the presence of CD4$^-$ MutuDC2s pulsed with increasing concentrations of MHC-I-restricted or MHC-II-restricted OVA peptides, the percentage of activated CD8$^+$ or, respectively, CD4$^+$ T cells increased accordingly (**Figure 5C** left, and **Supplementary Figure 3**). These results demonstrate that the CD4$^-$ MutuDC2s have retained the ability to induce MHC-I-mediated and MHC-II-mediated T cell activation.

In an analogous experimental setup, we pulsed the CD4$^-$ MutuDC2s with increasing concentrations of the full-length OVA (OVA_{FL}). When co-cultured with OVA-specific CD4$^+$ T cells, the OVA_{FL}-pulsed CD4$^-$ MutuDC2s induced T cell activation in an antigen concentration-dependent manner (**Figure 5C** bottom right and **Supplementary Figure 3**). Thus, this result not only confirmed that the CD4$^-$ MutuDC2s have retained the capacity to activate T cells, but also showed that they are capable of endocytosing and processing extracellular antigens for presentation on MHC-II. By contrast, when we pulsed the CD4$^-$ MutuDC2s with increasing concentrations of OVA_{FL} and co-cultured them with OVA-specific CD8$^+$ T cells, we did not observe T cell activation. Instead, in the same conditions the MutuDC1s were able to induce CD8$^+$ T cells to proliferate and produce IFNγ (**Figure 5C** top right

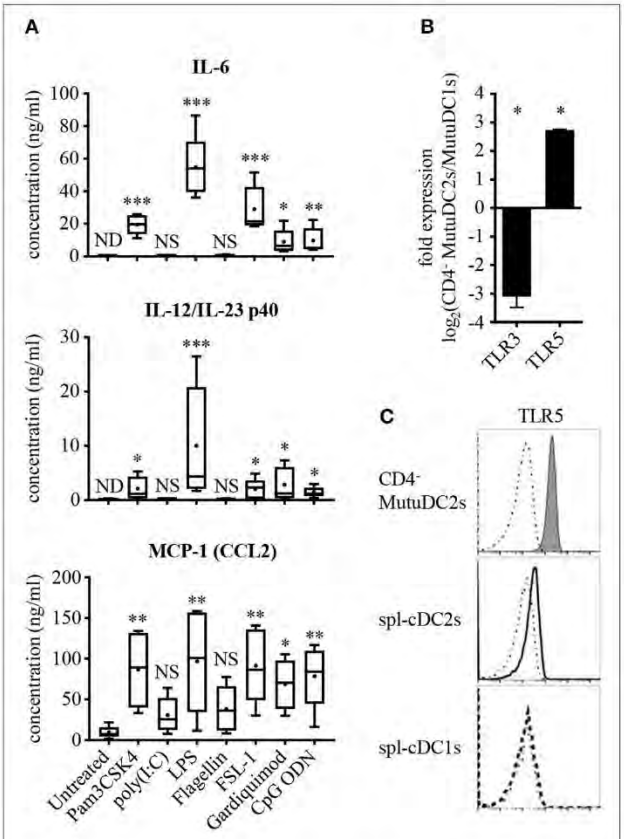

FIGURE 4 | CD4$^-$ MutuDC2s respond weakly to TLR3 and TLR5 ligands and have low TLR3 expression but high levels of TLR5. **(A)** CD4$^-$ MutuDC2s were stimulated with specific TLR ligands: TLR1/2 ligand Pam3CSK4, TLR3 ligand poly(I:C), TLR4 ligand LPS, TLR5 ligand flagellin, TLR2/6 ligand FSL-1, TLR7 ligand Gardiquimod, TLR9 ligand CpG ODN. After 24 h, the supernatants were collected and analyzed by ELISA to determine the concentration of IL-6, IL-12/IL-23 p40, and MCP-1(CCL2). The graphs show the results of five independent experiments represented as box-and-whiskers plots: mean (+), median (horizontal line), interquartile range (box), min/max values (whiskers). To assess significance, every tested condition was compared with the untreated control by Kruskal-Wallis testing followed by uncorrected Dunn's testing (*$p <$ 0.05, **$p < 0.01$, ***$p < 0.001$, NS, not significant; ND, not detectable). For the statistical analysis, all the measures below the lower detection limit of the assay were replaced with the value of the detection limit. **(B)** Expression of TLR3 and TLR5 was measured by RT-qPCR in CD4$^-$ MutuDC2s compared with MutuDC1s. Fold expression in CD4$^-$ MutuDC2s relative to MutuDC1s was calculated with the $2^{-\Delta\Delta Ct}$ method using β-actin expression as a reference for normalization. Data are presented as mean and SD of the $\log_2$ fold expression values from two independent experiments. The results were analyzed by two-tailed unpaired *t*-testing (*$p < 0.05$). **(C)** Flow cytometric analysis of TLR5 expression in CD4$^-$ MutuDC2s compared with spl-cDC1s and spl-cDC2s. The isolation of splenocytes and the staining were carried out as described in **Figure 3**. The dash-dotted lines show the FMO controls not stained with anti-TLR5 antibody. The results are representative of two independent experiments.

and **Supplementary Figure 3**). These results showed that the CD4$^-$ MutuDC2s are not capable of antigen cross-presentation in contrast to the MutuDC1s which are known to share this functional feature with their splenic counterpart (44).

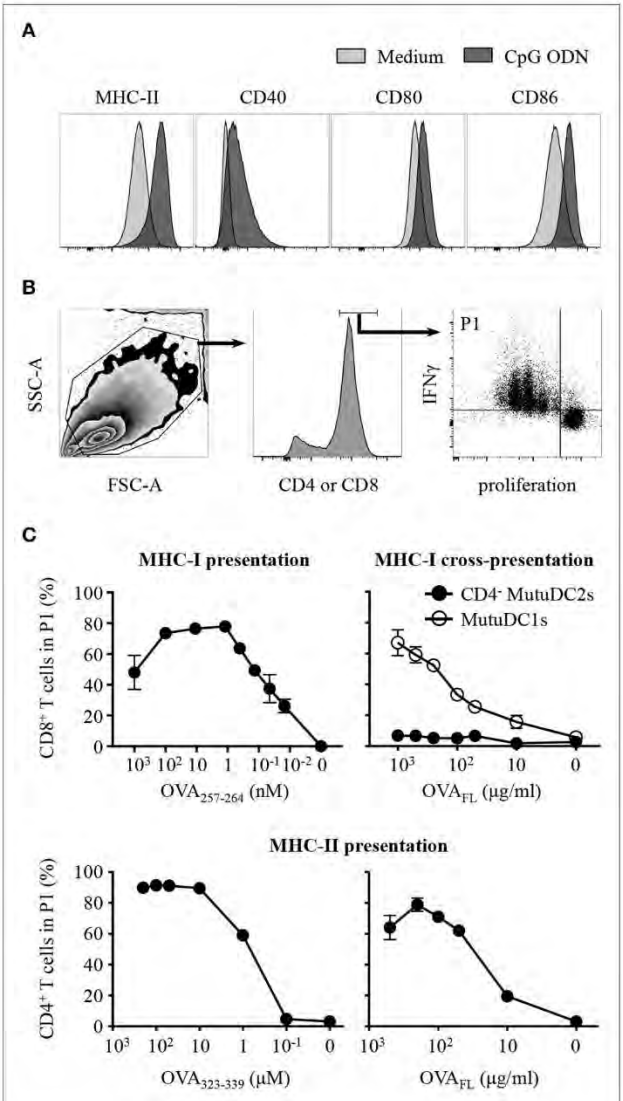

FIGURE 5 | CD4$^-$ MutuDC2s upregulate co-stimulatory molecules upon activation and display MHC-I-mediated and MHC-II-mediated activation of T cells but fail to cross-present antigens through MHC-I. **(A)** CD4$^-$ MutuDC2s were incubated with medium or with CpG ODN. After 20 h they were stained with fluorescent-labeled antibodies specific for MHC-II, CD80, CD86, or CD40 and analyzed by flow cytometry. The graphs relative to untreated CD4$^-$ MutuDC2s are the same shown in **Figure 3B**. **(B,C)** CD4$^-$ MutuDC2s or MutuDC1s were pulsed, in the presence of CpG ODN, with full length ovalbumin (OVA_{FL}) or with the MHC-I-restricted peptide $OVA_{257-264}$ or with the MHC-II-restricted peptide $OVA_{323-339}$ at the indicated concentrations. OVA-specific CD8$^+$ or CD4$^+$ T cells were isolated respectively from OT-I and OT-II mice and labeled with a proliferation dye. In the MHC-I T cell activation assay (**C**, top left), the CD8$^+$ T cells were co-cultured for 3 days with $OVA_{257-264}$-pulsed CD4$^-$ MutuDC2s. In the MHC-I antigen cross-presentation assay (**C**, top right), the CD8$^+$ T cells were co-cultured for 3 days with either OVA_{FL}-pulsed CD4$^-$ MutuDC2s or OVA_{FL}-pulsed MutuDC1s. In the MHC-II T cell activation assay (**C**, bottom), the CD4$^+$ T cells were co-cultured for 4 days with either $OVA_{323-339}$-pulsed or OVA_{FL}-pulsed CD4$^-$ MutuDC2s. At the end of the co-cultures, the T cells were restimulated with PMA and ionomycin in the presence of brefeldin A and prepared for flow

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FIGURE 5 | cytometric analysis by staining them with fluorescent-labeled antibodies specific for IFNγ and either CD4 or CD8α. **(B)** Gating strategy. The gate P1 contains the activated T cells defined as proliferating IFNγ$^+$ cells. **(C)** The percentage of T cells in the gate P1 measured in each tested condition was plotted against the respective antigen concentration. The results are presented as mean and SD of technical triplicates and are representative of two to three independent experiments.

DISCUSSION

DCs are well known for their scarcity *in vivo* that limits tremendously their accessibility for experimentation (64, 65). In addition, isolated DCs are very sensitive to long term culture and under these conditions they easily undergo functional modification, spontaneous activation and cell death (66, 67). For these reasons, significant efforts have been made to create suitable and reliable models for the study of DC biology. Several procedures have been proposed to simplify the access to sufficient amounts of viable and non-activated DCs for experimentation by differentiating DC progenitors *in vitro* and by expanding the number of DCs *in vivo* (68). However, despite their indisputable value, these methods still display disadvantages including the need for additional steps of cell isolation and purification, as well as the sensitivity, the functional instability and the limited lifespan in long term culture of the cells that they generate. Therefore, a widely pursued alternative to the *in vitro*-differentiation and *in vivo*-expansion strategies is represented by stable DC lines. Several approaches have allowed to generate stable DC lines (68) which in most cases have demonstrated to be very good models to study single aspects of DC biology (69–74). However, specific functional features of different DC subsets are often found concomitantly in several DC lines, raising doubts about their ability to maintain and fully represent the DC-subset functional specificities observed *in vivo* (75–83). Moreover, the need for special culture conditions, like growth at low permissive temperature (77, 83) or constant supplementation with growth factors (78, 84, 85), possibly represents an additional technical complication for the maintenance of some DC lines in long-term culture. Additionally, a relevant and yet often neglected caution is required when the capacity of the DC lines to maintain fully unaltered their phenotypic and functional properties over the passages, and especially at high passage number, has not been extensively ascertained.

Among the different examples of oncogene-based approaches to immortalize DC lines, several strategies are based on *ex vivo* transduction or transfection of DCs with the SV40LgT (77, 83, 86). Our approach is different because it is based on the generation of SV40LgT-transgenic mice and on the transgene-induced transformation of DCs *in vivo* with consequent development of DC tumors (42). Our group generated several SV40LgT-transgenic murine lines where the transgene expression was restricted to DCs (42). Among them we selected the one, the Mushi1 line, in which the transgene expression was sufficient to induce transformation of DCs and development of DC tumors, but not high enough to cause functional modifications of the cells. Finally, from the DC tumors we were able to derive the stable immortalized MutuDC lines (44). In parallel, we generated with a similar strategy several KO and transgenic MutuDC lines by crossbreeding the parental KO or transgenic murine lines with Mushi1 mice (44, 45). This crossbreeding approach can be applied to introduce virtually any kind of mutation in the cell lines or to derive them from any different genetic background. Thus, this strategy confers great versatility to our method and represents one of its main advantages. Additionally, the recent development and diffusion of genome-editing methods has enormously increased the possibilities to modify our cell lines *in vitro* (44, 45).

All the MutuDC lines generated so far from Mushi1 mice derive from spl-cDC1s (44), and therefore they are named MutuDC1s. To obtain new MutuDC lines with phenotype and functional properties of spl-cDC2s, we applied the crossbreeding strategy to Batf3$^{-/-}$ mice which are devoid of spl-cDC1s (26, 46). As expected, the Batf3$^{-/-}$ Mushi1 mice developed splenic tumors, even if with a delayed onset of the disease if compared with Mushi1 mice (42). Given the concentration-dependency of the SV40LgT (42), this difference can be explained by our previous observation that in Mushi1 mice the levels of the transgene expression are lower in spl-cDC2s than in spl-cDC1s (42). Consequently, the oncogenic transformation is likely to occur earlier in spl-cDC1s than in spl-cDC2s, possibly explaining why all the MutuDCs generated from Mushi1 mice derive from spl-cDC1s.

From the splenic tumors of Batf3$^{-/-}$ Mushi1 mice, we derived the new CD4$^-$ MutuDC2 line whose characterization is described in this work. The expression of the SV40LgT was measured in the CD4$^-$ MutuDC2s through the analysis of its associated EGFP reporter. We found that the CD4$^-$ MutuDC2s express lower levels of the transgene than the previously derived MutuDC1s, reflecting the difference observed between spl-cDC2s and spl-cDC1s in Mushi1 mice.

To assess the belonging of our CD4$^-$ MutuDC2s to one of the spl-DC subsets, we analyzed their surface and intracellular marker expression profile and compared them with freshly isolated spl-DCs. The CD4$^-$ MutuDC2s display high levels of CD11c and MHC-II with lack of the pDC-specific markers B220, Gr-1 and PDCA-1. Additionally, in their resting state they are CD80^{hi} and CD86^{hi}. These observations demonstrated that the CD4$^-$ MutuDC2s have a relatively mature spl-cDC phenotype. Analysis of expression of the subset-determining transcription factors IRF4 and IRF8 showed that, comparably to spl-cDC2s, the CD4$^-$ MutuDC2s express high IRF4 and low IRF8. Therefore, we concluded that they belong to the spl-cDC2 subset. Additional surface marker analysis further corroborated this conclusion by showing that the CD4$^-$ MutuDC2s strongly express the spl-cDC2-distinctive markers CD11b and CD172a and have low levels of CD4, comparably to the CD4$^-$ spl-cDC2 subset. The markers CD8α, CLEC9A, CD205, and CD24 are known to be specifically expressed by spl-cDC1s (40, 51–53). In our CD4$^-$ MutuDC2s, CD8α and CLEC9A are not expressed, but the levels of CD205 and CD24 are higher than in fresh CD4$^-$ spl-cDC2s. However, the comparison between the CD4$^-$

MutuDC2s and the MutuDC1s shows that the expression of CD205 and CD24 is still lower in the former, in agreement with the differences observed *in vivo* between spl-cDC2s and spl-cDC1s. It should be mentioned that the levels of CD205 are also higher in the MutuDC1s if compared with freshly isolated spl-cDC1s (44). This increase of CD205 expression both in the $CD4^-$ MutuDC2s and in the MutuDC1s reminds of what is observed in overnight cultures of *ex vivo* spl-cDCs in the presence of GM-CSF, where CD205 expression is upregulated both in spl-cDC2s and in spl-cDC1s but remains higher in the latter (87). Concerning CD24 expression in the $CD4^-$ MutuDC2s, we cannot fully explain the upregulation of this marker. However, it has been shown that $CD24^{int}$ and, to a lesser extent, $CD24^{hi}$ DC precursors maintain the potential to generate spl-cDC2s (18). Moreover, several examples of $CD11b^+CD24^+$ cDC2s, which share at least partially developmental origin, phenotype and functional characteristics with spl-cDC2s (23, 29, 32, 35), can be found among the mucosal cDC populations in lung (29, 88), small intestine (32), and nose (89).

Many of the phenotypic characteristics that we described are consistent with a moDC phenotype. However, in Mushi1 mice, SV40LgT expression is driven by the 5.7 kb CD11c proximal promoter which restricts the transgene expression to DCs (43). Indeed, the SV40LgT-associated reporter, the IRES-linked EGFP, is not detectable in $CD11c^{lo}$ macrophages and monocytes, demonstrating no expression of the transgene in these cell types (unpublished results). This makes it highly unlikely that the cell line here described is monocyte-derived. Consistently, the analysis of the moDC-specific markers Ly-6C (Gr-1), FcεRIα, and CD64 (20), which are negative or low in the $CD4^-$ MutuDCs, highlights this conclusion. In further agreement with the results discussed above, the $CD4^-$ MutuDCs express high levels of the DC-defining marker FLT3, in spite of an unusual intracellular localization. When we generate DC lines, they become able to grow at low densities only after several passages. We think that this is due to the fact that, during the derivation process, we select for cells that can secrete growth factors that favor DC growth. Consistently, at low densities the cells preferentially grow in conditioned medium. A likely growth factor is FLT3L whose binding to its receptor FLT3 has been shown to induce the dimerization and internalization of FLT3 (90). Therefore, the presence of FLT3L in the culture medium would explain the intracellular localization of FLT3 that we observe in the $CD4^-$ MutuDC2s.

Distinct profiles of TLR and cytokine expression represent an additional characterizing difference between splenic DC subsets. For instance, spl-cDC1s are known to express mainly TLR3 and TLR9 (36) and to be major producers of IL-12p70 (37, 38). On the contrary, they do not produce the chemokine MCP-1(CCL2) (42). The picture is reversed in spl-cDC2s which express a broader array of TLRs, with the well described exception of TLR3 (36), and produce MCP-1(CCL2), IL-6, and IL-23 but only limitedly IL-12p70 (37, 38, 60, 61). Consistently, after stimulation with the ligands of TLR1/2, TLR4, TLR2/6, TLR7, and TLR9, our $CD4^-$ MutuDC2s responded by producing IL-6, MCP-1(CCL2), and IL-12/IL-23 p40 but did not express detectable levels of IL-12p70. Additionally, in line with the limited cytokine production observed after stimulation with the TLR3 ligand poly(I:C), transcription analysis showed very low levels of TLR3 mRNA. We also observed that the $CD4^-$ MutuDC2s exhibit a generally weak responsiveness to treatment with the TLR5 ligand flagellin, in apparent contrast with their relatively high expression of TLR5.

Several studies in the past have analyzed TLR5 expression in different DC subsets. For example, intestinal lamina propria $CD11b^+$ cDC2s show high levels of TLR5 and rely considerably on this receptor for detection of pathogens, maturation and induction of cytokine production (70, 91, 92). By contrast, our knowledge is less precise regarding spl-cDCs. Indeed, while some studies have shown that TLR5 is expressed by spl-cDCs, in particular by spl-cDC2s (36, 93), in other cases TLR5 was found to be very low or absent (70, 91, 92, 94). Interestingly, just one study could show *in vitro* a connection between TLR5 expression and direct flagellin-induced maturation of spl-cDCs (93), while in other reports this effect was not observed (94, 95). Additionally, even in the former case, flagellin always induced a very limited or absent production of cytokines like IL-12 and IL-6. These observations are consistent with a differential tissue- and DC subset-specific role of TLR5. In agreement with this hypothesis, TLR5 was shown to function also as an endocytic receptor that mediates the uptake of flagellin promoting MHC-II presentation of flagellin epitopes to $CD4^+$ T cells (96). Further investigation of this model showed that the $CD4^-$ spl-cDC2s are the main subset among the spl-cDCs to carry out this TLR5-mediated flagellin processing pathway (97). Our results show very low, and yet detectable, levels of TLR5 in spl-cDC2s as opposed to spl-cDC1s that are $TLR5^-$ (**Figure 4C**). Instead, TLR5 expression is high in the $CD4^-$ MutuDC2 line. However, the upregulation of TLR5 displayed by the $CD4^-$ MutuDC2s, together with their low responsiveness to flagellin, appears to integrate perfectly in the context of the functional specialization of the $CD4^-$ spl-cDC2s, rather than representing a divergence of the $CD4^-$ MutuDC2 line from its splenic counterpart.

In addition to the induction of cytokine production, the encounter of DCs with a pathogen initiates a process of maturation that causes several functional modifications including upregulation of the co-stimulatory molecules CD40, CD80, and CD86, increase of the surface levels of MHC molecules and reduction of the antigen-uptake capacity of DCs, accompanied by an increase of their ability to process antigenic peptides and load them into MHC complexes for presentation to T cells (2, 7–9). All these elements are essential to ensure the efficient priming of T cells and to initiate an appropriate adaptive response. Upon treatment with CpG ODN, the $CD4^-$ MutuDC2s upregulate CD40, CD80, and CD86 and increase their surface levels of MHC-II. When pulsed, in the presence of CpG ODN, with an ovalbumin-derived MHC-II-restricted peptide or with the full-length ovalbumin, they are able to take up the antigen and present it to antigen-specific $CD4^+$ T cells. We speculate that the need of a rather high antigen

concentration in our presentation assays could be linked to the relatively mature phenotype that the CD4$^-$ MutuDC2s show at the steady state, which might imply a reduced endocytic capacity in favor of a higher antigen processing and presentation efficiency. The CD4$^-$ MutuDC2s also proved to be very efficient in the direct MHC-I-mediated activation of CD8$^+$ T cells. Therefore, their inability to cross-present antigens to CD8$^+$ T cells, even at the highest concentrations, mirrors precisely the functional distinction that is observed *in vivo* between the cross-presenting spl-cDC1s and the non-cross-presenting spl-cDC2s and further distinguishes the CD4$^-$ MutuDC2s from moDCs (19, 56, 98).

In conclusion, we have exploited the versatility of our SV40LgT-based derivation method to generate the new CD4$^-$ MutuDC2 line which has striking phenotypic and functional resemblance to the CD4$^-$ spl-cDC2 subset. This cell line has already proven to be a reliable model for the study of cDC2s. Indeed, in a recent report from our group, resistance to collagen induced arthritis (CIA) was partially recovered by the adoptive transfer of CD4$^-$ MutuDC2s in a CIA-susceptible CD11b$^{-/-}$ mouse model, highlighting a potential tolerogenic role of cDC2s in the modulation of autoimmune responses (99). Furthermore, the CD4$^-$ MutuDC2s have been successfully transduced with a CRISPR-Cas9 editing system to generate a new CD11b$^{-/-}$ CD4$^-$ MutuDC2 line (unpublished data). As we have shown, the new CD4$^-$ MutuDC2s are simple to culture and can be expanded at will, providing a virtually unlimited source of cells for experimentation. They can be easily manipulated with standard experimental techniques and display great stability of their phenotypic and functional properties in long term culture as long as their passage number is maintained below 40-50. For these reasons the CD4$^-$ MutuDC2s represent a new valuable tool for the study of DC biology.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.

AUTHOR CONTRIBUTIONS

MP designed and performed experiments and data analysis, wrote the article and revised the reviewed drafts. DA generated the Batf3$^{-/-}$ Muhi1 mouse line and participated in the cell line derivation. MS helped with the experimental design and performed preliminary experiments. HA-O conceived the project, acquired the founding, supervised experimental design and data analysis and reviewed the article drafts. All the authors reviewed the submitted version of the article.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2018.01912/full#supplementary-material

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Supplementary Material

Establishment and Characterization of a Functionally Competent Type 2 Conventional Dendritic Cell Line

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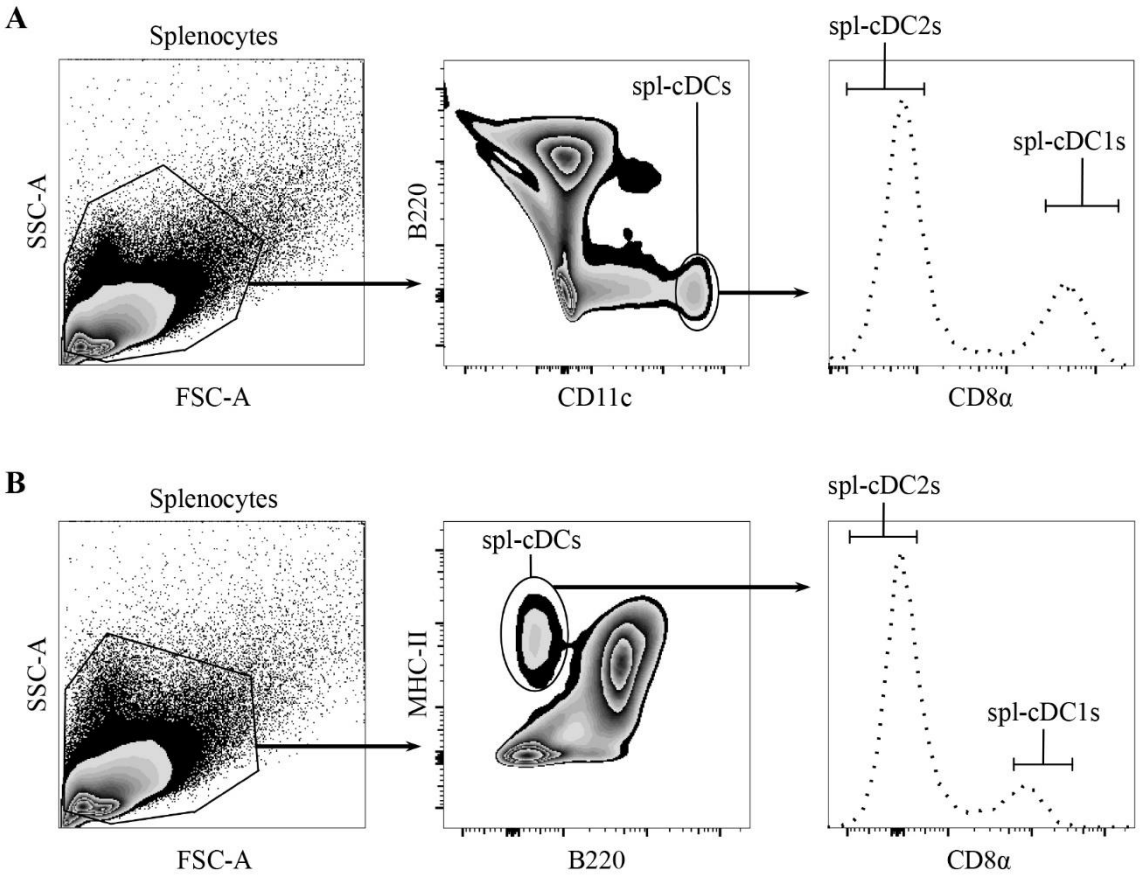
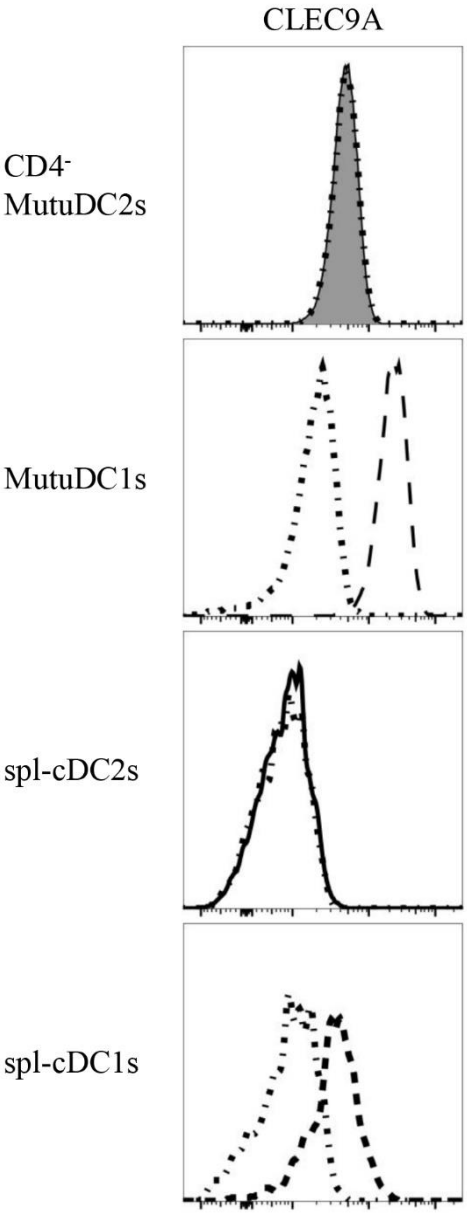
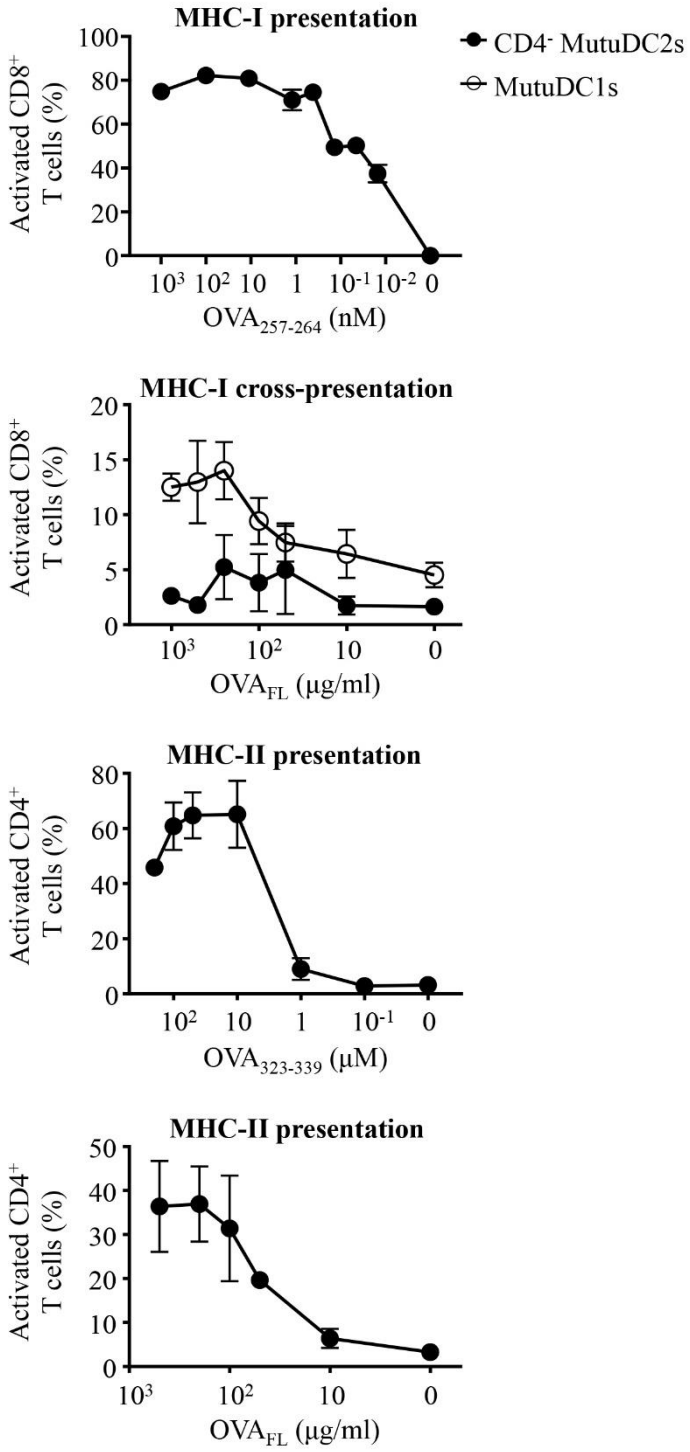


Supplementary Figure 1. Gating strategy for the analysis of CD11c and MHC-II expression in spl-cDC subsets. Splenocytes from C57BL/6 mice were isolated by digestion of spleens with collagenase D followed by filtration through a 40 µm cell strainer. The cells were analyzed by flow cytometry after staining with fluorescent-conjugated antibodies specific for B220, MHC-II, CD11c and CD8α. Spl-cDCs were identified through the analysis of either (**A**) B220 and CD11c expression or (**B**) MHC-II and B220 expression. (**A,B**) In both cases, the spl-cDC subsets were distinguished on the basis of CD8α expression.



Supplementary Figure 2. CD4$^-$ MutuDC2s do not express CLEC9A. Splenocytes were isolated as described in **Figure S1**. CD4$^-$ MutuDC2s, MutuDC1s and splenocytes were analyzed by flow cytometry after staining with fluorescent-conjugated antibodies specific for MHC-II, CD11c, CD8α and CLEC9A. The spl-cDC subsets were identified through the analysis of MHC-II, CD11c and CD8α expression as reported in **Figure 3**. The dash-dotted lines show the fluorescence-minus-one controls not stained with anti-CLEC9A antibody. The results are representative of two independent experiments.



Supplementary Figure 3. CD4$^-$ MutuDC2s activate T cells through MHC-I and MHC-II but do not cross-present peptides through MHC-I. Biological replicate of the experiment illustrated in **Figure 5C**. The results are presented as mean and SD of technical triplicates and are representative of two to three independent experiments.

4.1.2. Preliminary characterization of additional cell lines obtained from Batf3$^{-/-}$ Mushi1 spleens

The derivation process that we have previously presented [382,383,386] is not always successful. To be able to generate new cell lines, numerous repetitions of the procedure might be needed either for multiple wells of serially diluted cells isolated from a single spleen or even for splenocytes isolated from different spleens. Therefore, at the beginning of a derivation, the splenocytes isolated from tumoral Batf3$^{-/-}$ Mushi1 spleens were seeded in serial two-fold dilutions in one separate plate for each spleen. Each well of every plate was considered and cultivated independently from any other well in the plate. This precaution was necessary to avoid any possible cross-contamination with cell populations of undesired features that, we observed, occur and quickly prevail in some of the wells. This also implied that every well in which the culture was able to tolerate the first 3-5 passages was subjected to a preliminary phenotypic evaluation to determine the potential of the cells as candidate DC-subset model cell lines.

During the derivation of the CD4^{-} MutuDC2s, we repeated the derivation procedure several times with multiple spleens. Even though the most part of the wells that were seeded at the beginning of the procedure did not yield stable or viable cell lines, we were able to obtain numerous stable cultures that we classified with a serial number (ID number of the mouse of origin followed by one or two alphabet letters) before their characterization. After a preliminary phenotypic characterization, we unexpectedly observed that the majority of these cell lines expressed CD11c at low levels and MHC-II at lower levels than the MutuDC1s. We selected one representative cell line, the 17005B Mutu cells **(Figure 15)**, and further characterized it.

Flow cytometric analysis showed that the 17005B Mutu cells expressed EGFP homogeneously and at lower levels than the MutuDC1s **(Figure 15B)**. They were negative for Gr-1, B220 and PDCA-1,

which excluded their belonging to the pDC subset **(Figure 15C)**, and did not express the spl-cDC1 characterizing markers CD8α and Clec9A **(Figure 15D)**. The two other cDC1-characterizing markers that we analyzed, CD205 and CD24, were expressed, but at lower levels than in MutuDC1s **(Figure 15D)**. By contrast, the cDC2-specific markers CD11b and CD172a were expressed at high levels by the 17005B Mutu cells but not by the MutuDC1s **(Figure 15E)**. CD4 expression was measured and found to be absent in the 17005B Mutu cells **(Figure 15E)**.

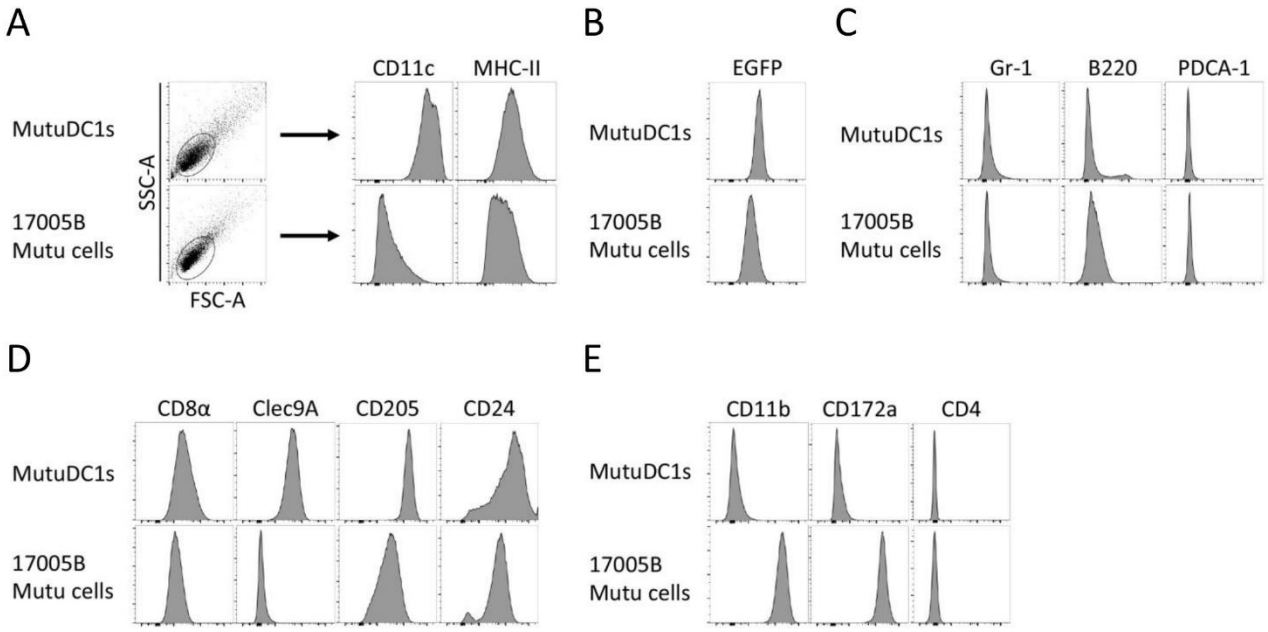


Figure 15. 17005B Mutu cells have a spl-cDC2-like phenotype but express low levels of CD11c and MHC-II. MutuDC1s and 17005B Mutu cells were stained with fluorescent-labeled antibodies specific for the indicated surface markers and compared by flow cytometry. Data are representative of a minimum of 3 independent experiments. **(A)** MutuDC1s and 17005B Mutu cells were compared for the expression of the indicated cDC characterizing surface markers. **(B)** Expression of the SV40LgT transgene in MutuDC1s and 17005B Mutu cells was assessed through analysis of the levels of its associated reporter EGFP. **(C)** MutuDC1s and 17005B Mutu cells were compared for the expression of the indicated pDC-characterizing markers. **(D)** MutuDC1s and 17005B Mutu cells were compared for the expression of the indicated spl-cDC1-characterizing markers. **(E)** MutuDC1s and 17005B Mutu cells were compared for the expression of the indicated spl-cDC2-characterizing markers.

We reasoned that, in spite of the low expression of CD11c and MHC-II, the phenotype of the 17005B Mutu cells was consistent with their belonging to the $CD4^-$ spl-cDC2 subset.

Our hypothesis was further corroborated by the analysis of expression of IRF4 and IRF8 that showed high levels of IRF4 and low levels of IRF8 **(Figure 16)**.

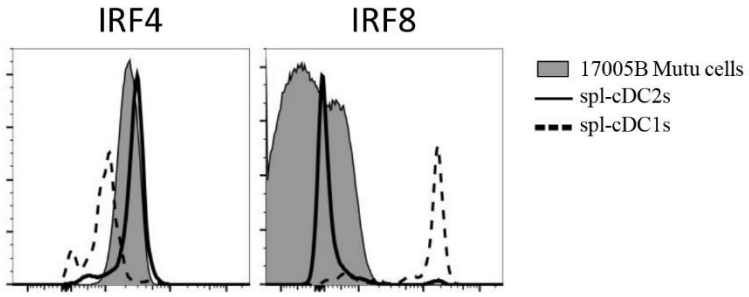


Figure 16. 17005B Mutu cells express high levels of IRF4 and low levels of IRF8 similarly to spl-cDC2s. Splenocytes from C57BL/6 mice were isolated by digestion of spleens with collagenase D followed by filtration through a 40 µm cell strainer. 17005B Mutu cells and splenocytes were stained with an antibody cocktail that contained anti-MHC-II and anti-CD11c antibodies to distinguish spl-cDCs. An anti-CD8α antibody was included in the staining cocktail to identify spl-cDC1s and spl-DC2s. For the details of the gating strategy refer to "**FIGURE 3 |** $CD4^-$ MutuDC2s share the surface and intracellular marker expression profile with $CD4^-$ spl-cDC2s." contained in paragraph *4.4.1. Establishment and characterization of a functionally competent type 2 conventional dendritic cell line*. Stained cells were analyzed by flow cytometry for the expression of the cDC subset-characterizing transcription factors IRF4 and IRF8.

As described for the $CD4^-$ MutuDC2s, we tested the TLR expression profile of the 17005B Mutu cells by activating them with different TLR ligands and subsequently by measuring their cytokine and chemokine production. The 17005B Mutu cells responded to the majority of the tested TLR ligands by producing IL-6, MCP-1 (CCL2) and IL-12p40 **(Figure 17)**. By contrast, none of the tested conditions induced detectable production of IL-12p70 and IL-10 (data not shown). Except for a weak induction of MCP-1 secretion, the TLR3 and TLR5 ligands, poly(I:C) and flagellin, respectively, were never able to induce detectable production of any of the tested cytokines **(Figure 17)**.

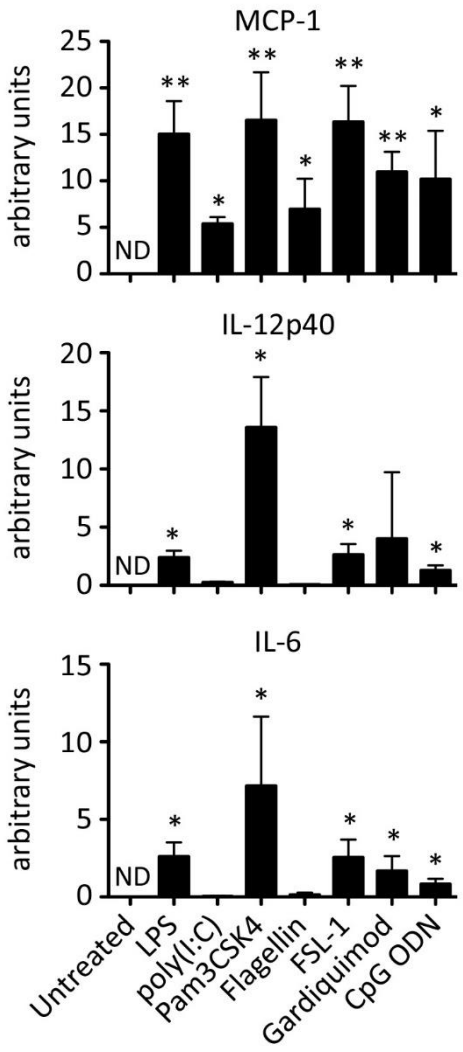


Figure 17. 17005B Mutu cells are activated weakly by TLR3 and TLR5 ligands. 17005B Mutu cells were activated with specific TLR ligands: TLR1/2 ligand Pam3CSK4, TLR3 ligand poly(I:C), TLR4 ligand LPS, TLR5 ligand flagellin, TLR2/6 ligand FSL-1, TLR7 ligand Gardiquimod, TLR9 ligand CpG ODN. After 24 hours of incubation the supernatants were collected and analyzed by ELISA to determine the concentration of MCP-1 (CCL2), IL-12p40 and IL-6. Independent experiments were compared as follows: the experiment that showed the highest absolute values of cytokine production was used as a reference. The ratio between the absolute values observed in every condition of the reference experiment and the respective values observed in each of the other experiments was calculated. For every experiment, the calculated ratios were averaged out to determine a correction factor. The absolute values of cytokine production of each experiment were multiplied by the respective correction factor. The distinct corrected cytokine-production levels were individually compared with untreated controls through Mann-Whitney U testing to assess statistical significance. For the statistical analysis, all the measures below the lower detection limit of the assay were replaced with the value of

the detection limit. *p < 0.05, **p < 0.01, ND = not detectable. Data are mean ± SD from 4 independent experiments.

To analyze the functional properties of the 17005B MutuDCs, we tested their ability to present ovalbumin (OVA) and OVA-derived peptides to OT-I and OT-II cells in an *in vitro* T cell activation assay. It is worth specifying that, in chronological terms, the derivation of the 17005B Mutu cells preceded the establishment of the $CD4^-$ MutuDC2s described above. This explains, at least partially, some of the choices that were made in the definition of the experimental conditions. Indeed, during this stage of MutuDC derivation, we erroneously assumed that all the different MutuDC lines would have presented antigens at similar peptide concentrations, possibility that was proven wrong during the subsequent characterization of the $CD4^-$ MutuDC2s. Therefore, the establishment of the experimental conditions was based on our experience with the previously derived MutuDC1s. In the conditions that we tested, the 17005B Mutu cells were able to induce strong activation of $CD8^+$ T cells when pulsed with the $OVA_{257-264}$ peptide either in the presence or the absence of CpG ODN or LPS **(Figure 18)**. On the opposite, and in contrast to MutuDC1s, they were not able to cross-present full-length OVA (OVA_{FL}) to $CD8^+$ T cells, as demonstrated by the low percentages of activated $CD8^+$ T cells measured in the co-cultures even after stimulation of the 17005B Mutu cells with CpG ODN or LPS **(Figure 18)**. Additionally, when pulsed with the MHC-II-restricted $OVA_{323-339}$ peptide, in the absence of TLR ligands, the 17005B Mutu cells were not able to induce $CD4^+$ T cell activation **(Figure 18)**. By contrast, in the presence of either CpG ODN or LPS they presented the $OVA_{323-339}$ peptide to $CD4^+$ T cells, even if much less efficiently that the MutuDC1s in the same conditions **(Figure 18)**.

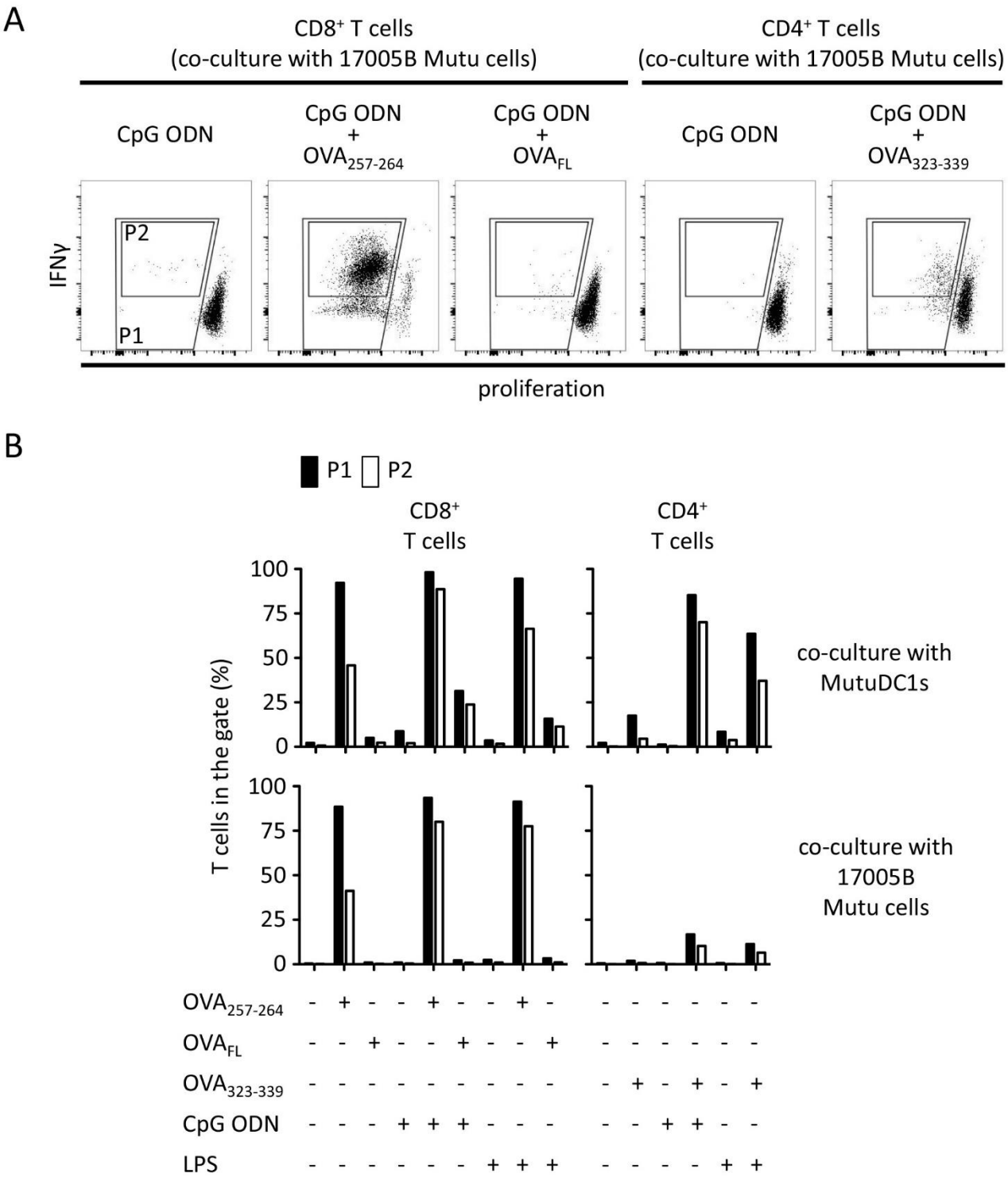


Figure 18. 17005B Mutu cells are able to induce MHC-I-mediated activation of CD8$^+$ T cells but induce limited MHC-II-mediated CD4$^+$ T cell activation and fail to cross-present antigens through MHC-I. 17005B Mutu cells or MutuDC1s cells were pulsed for 6 h with $OVA_{257-264}$ (1 µM), OVA (25 µg/mL) or $OVA_{323-339}$ (50 nM) in the presence or the absence of CpG ODN (1 µM) or LPS (100 ng/mL), and subsequently co-cultured with CD8$^+$ or CD4$^+$ T cells purified from OT-I and OT-II mice, respectively. The T cells had previously been stained with the eFluor® 670 proliferation dye and were plated in a 10:1

ratio with the Mutu cells. On the third day of co-culture, the cells were re-stimulated with PMA and ionomycin in the presence of Brefeldin A. The cells were stained extracellularly with fluorochrome-conjugated anti-CD4 and anti-CD8 antibodies and intracellularly with an anti-IFNγ fluorochrome-conjugated antibody. T cells were analyzed by flow cytometry for proliferation and IFNγ production. **(A)** Gating strategy for the flow cytometric analysis of T cells. Dot plots of representative experimental conditions are shown. Gate P1 includes all the proliferating T cells; gate P2 includes all the proliferating T cells that produce IFNγ. **(B)** Quantification of the gates P1 and P2 in the several tested conditions. The bars represent the fraction of cells in the relative gate calculated as percentage of the total number of T cells. Data are representative of 3 independent experiments.

To investigate the possible explanations of this low $CD4^+$ T cell-activation capacity, we stimulated 17005B MutuDCs or MutuDC1s with either CpG ODN or LPS for 7 hours and measured their levels of MHC-II, CD40, CD80 and CD86. We observed that, while the MutuDC1s were able to upregulate MHC-II and co-stimulatory molecules in these conditions, the 17005B MutuDCs showed low or undetectable upregulation of these markers **(Figure 19)**.

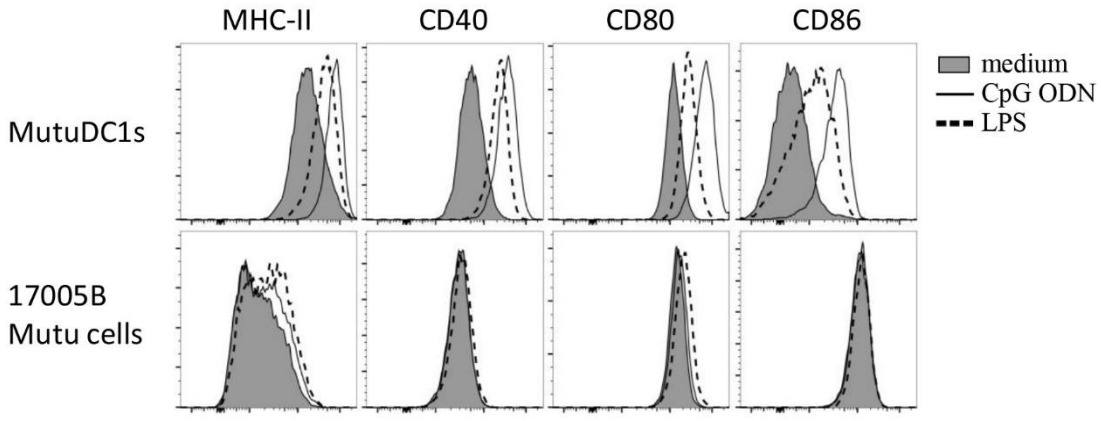


Figure 19. 17005B Mutu cells show weak or absent upregulation of MHC-II and co-stimulatory molecules upon activation with CpG ODN or LPS. MutuDC1s or 17005B Mutu cells were incubated with medium, CpG ODN or LPS. After 7 h they were stained with fluorescent-labeled antibodies specific for MHC-II, CD40, CD80 or CD86 and compared through flow cytometric analysis. Data are representative of two biological replicates.

Therefore, we hypothesized that the inefficient MHC-II antigen presentation that we had observed might depend on insufficient or lacking T cell co-stimulation. Additionally, we reasoned that the possible absence of cytokines involved in T cell differentiation like IL-12p70, whose production by the 17005B MutuDCs was never detected, and IL-4, which is not produced by cDCs [387], might contribute to our previous results. Therefore, we tested these hypotheses through a $CD4^+$ T cell activation assay in which we pulsed the 17005B cells with $OVA_{323-339}$ in the presence of CpG ODN and subsequently we added to the co-cultures IL-12p70, IL-4 and exogenous co-stimulation (anti-CD28 activating antibody) either alone or in binary combinations composed of anti-CD28 with IL-12p70 or IL-4. Our analysis showed no improvement in $CD4^+$ T cells-activation capacity of the 17005B Mutu cells in any of the tested conditions if compared with our previous experimental setup (data not shown). Therefore, we concluded that the inefficient MHC-II-mediated antigen presentation that we observed in the 17005B Mutu cells likely depended on their low expression of MHC-II.

As mentioned, most of the cell lines that we obtained from $Batf3^{-/-}$ Mushi1 spleens were characterized by high expression of CD11b and CD172a with low levels of MHC-II and CD11c. However, in the preliminary phenotypic characterization of our cell lines, we observed also other recurrent phenotypes in several wells.

For example, in the article included above (paragraph *4.1.1. Establishment and characterization of a functionally competent type 2 conventional dendritic cell line*), we have described the spl-cDC2-like phenotype of the $CD4^-$ MutuDC2s.

Additionally, we obtained several cell lines, named 21735A, 21733C and 20876B Mutu cells, characterized (except in the case of the 20876B Mutu cells) by intermediate levels of CD11c and MHC-II and by variable expression levels of B220 and PDCA-1 **(Figure 20)**. This observation induced us to hypothesize the possible belonging of these cell lines to the pDC subset. However, our

hypothesis was strongly contradicted by the high levels of CD11b expression displayed by these cell lines **(Figure 20)**. Therefore, concerning the belonging of these Mutu cell lines to a DC subset, further investigation would be needed to allow any conclusion.

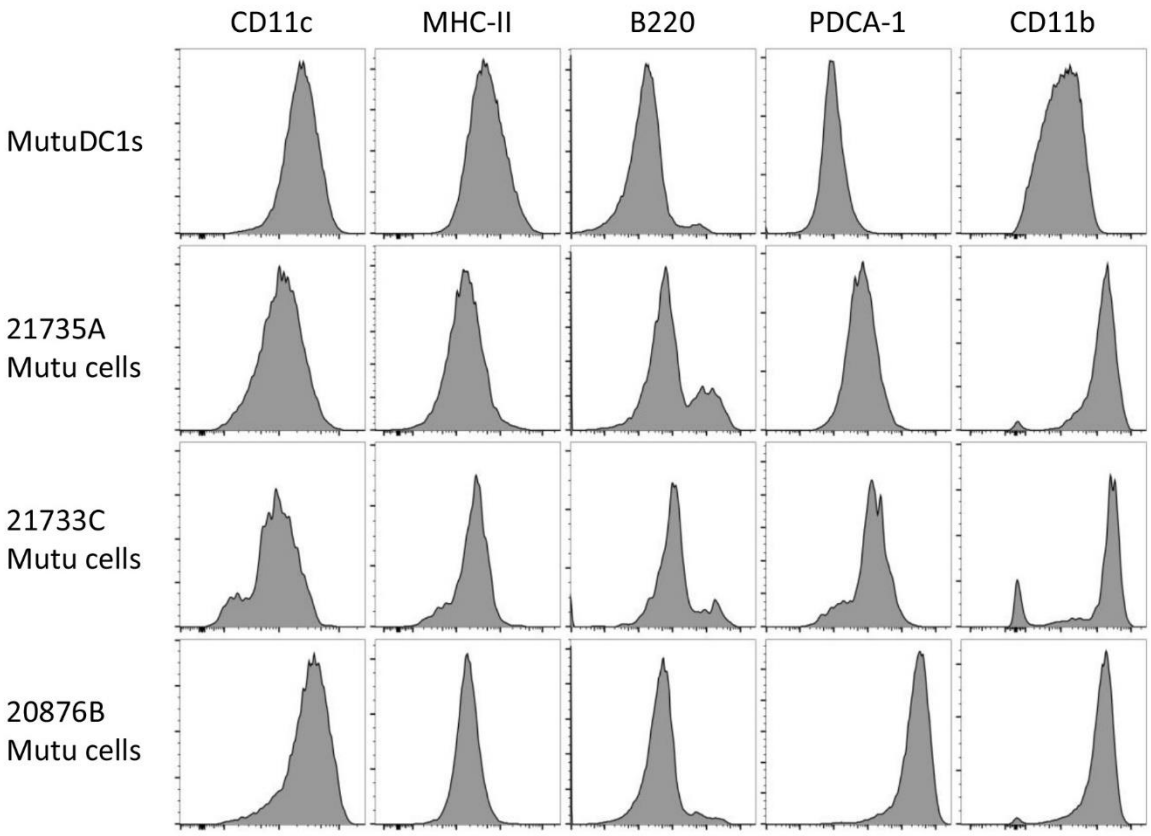


Figure 20. Several Mutu cell lines derived from Batf3$^{-/-}$ Mushi1 spleens show phenotypic characteristics of pDCs. MutuDC1s, 21735A Mutu cells, 21733C Mutu cells and 20876B Mutu cells were stained with fluorochrome-conjugated antibodies specific for CD11c, MHC-II, CD11b and for the pDC-characterizing markers B220 and PDCA-1. Flow cytometric analysis was carried out to compare 21735A Mutu cells, 21733C Mutu cells and 20876B Mutu cells with MutuDC1s for the expression of the indicated markers.

4.2. Study of the molecular mechanisms of synergistic and super-synergistic DC activation

4.2.1. Expression of IL-12 and CCR7 allows to distinguish between non-synergistic, synergistic and super-synergistic activation of MutuDC1s

The cell sorting-based approach that we want to apply to screen our pooled knockout library (see paragraph *3.2.1. CRISPR/Cas9-library screening approach*) intrinsically relies on a robust readout that permits to clearly separate non-synergistically, synergistically and super-synergistically activated cells out of the same population. For this reason, to define good sorting conditions for our screening, we analyzed the expression of several candidate marker genes.

If compared with other DC subsets, cDC1s are known to express the highest levels of IL-12 in response to TLR9-signaling activation (MyD88-dependent pathway) [304]. Moreover, they are the only cDCs that express TLR3 (TRIF-dependent signaling) [302], and upon activation with poly(I:C) (TLR3 ligand), they produce high amounts of IL-12 [304]. Our MutuDC1s are a model of cDC1s and, similarly to what is observed with freshly isolated cDC1s, their activation with poly(I:C), CpG ODN and IFNγ in binary or ternary combinations induces them to produce IL-12 at synergistic or super-synergistic levels, respectively [382]. Therefore, we considered the hypothesis that IL-12 expression after MutuDC1 activation with poly(I:C), CpG ODN and IFNγ either alone or in their binary or ternary combinations could represent a good readout for our screening.

To examine this possibility, we stimulated MutuDC1s for 24 hours with poly(I:C), CpG ODN, IFNγ and their binary or ternary combinations. The cells were stained with a fluorescent-labeled antibody specific for IL-12p40 and analyzed by flow cytometry **(Figure 21A)**. This analysis revealed that the IL-12 expression profile does not suffice to discriminate the different subpopulations that we aimed

to sort. Indeed, as already mentioned, our screening approach depends on the capacity to simultaneously distinguish non-synergistic, synergistic and super-synergistic activation but, in the tested conditions, the peaks that represent these three activation states are extensively overlapped and do not allow a clear separation **(Figure 21A)**.

Therefore, we decided to analyze the expression profile of other possible surface or intracellular markers, searching for genes with clear synergistic and/or super-synergistic regulation. We examined an mRNA-seq data set, and we identified five genes that showed the desired expression profile: CCR4, CCR7, CD62E, CD200 and CTLA4 (data not shown). To assess their validity as markers in our strategy, we stimulated the MutuDC1s with poly(I:C), CpG ODN, IFNγ and their binary or ternary combinations. After 24 hours of stimulation, we analyzed by flow cytometry the expression levels of the five candidate markers. Two of them, namely CD200 and CCR7, showed synergistic and super-synergistic expression **(Figure 21B,C)**.

CD200 was found to be bimodally expressed already by untreated MutuDC1s, with a $CD200^{-}$ and a $CD200^{lo}$ population **(Figure 21B)**. Cell activation with poly(I:C) and CpG ODN, either alone or in combination, induced modest upregulation of CD200 at slightly different degrees, and exclusively in the initially $CD200^{lo}$ cells **(Figure 21B)**. IFNγ alone induced negligible upregulation of CD200 **(Figure 21B)**. The ternary ligand combination and all the binary treatments involving IFNγ caused strong upregulation of CD200 in both the starting $CD200^{-}$ and $CD200^{lo}$ cells **(Figure 21B)**. This effect was especially evident with the combination of CpG ODN and IFNγ. The proportion of $CD200^{hi}$ cells was more increased after treatment with either the binary combination of CpG ODN and IFNγ or the ternary combination of ligands than in any other condition tested **(Figure 21B)**. However, the bimodal expression of CD200, even if observed in all of our experiments, was found to be extremely variable among several biological replicates. Moreover, the fact that CD200 expression remains bimodal after stimulation with the distinct ligands and ligand combinations adds further complexity

to the process of discrimination of the different activation states, since it generates additional overlaps between their corresponding peaks. Due to these reasons, we excluded CD200 as a possible marker for our screening strategy.

CCR7 showed a clear synergistic and super-synergistic expression after activation of MutuDC1s with binary or ternary combinations of poly(I:C), CpG ODN and IFNγ **(Figure 21C)**. However, as we observed in the case of IL-12 expression, the overlap between the peaks representing the different activation states was found to be too broad to allow a precise separation.

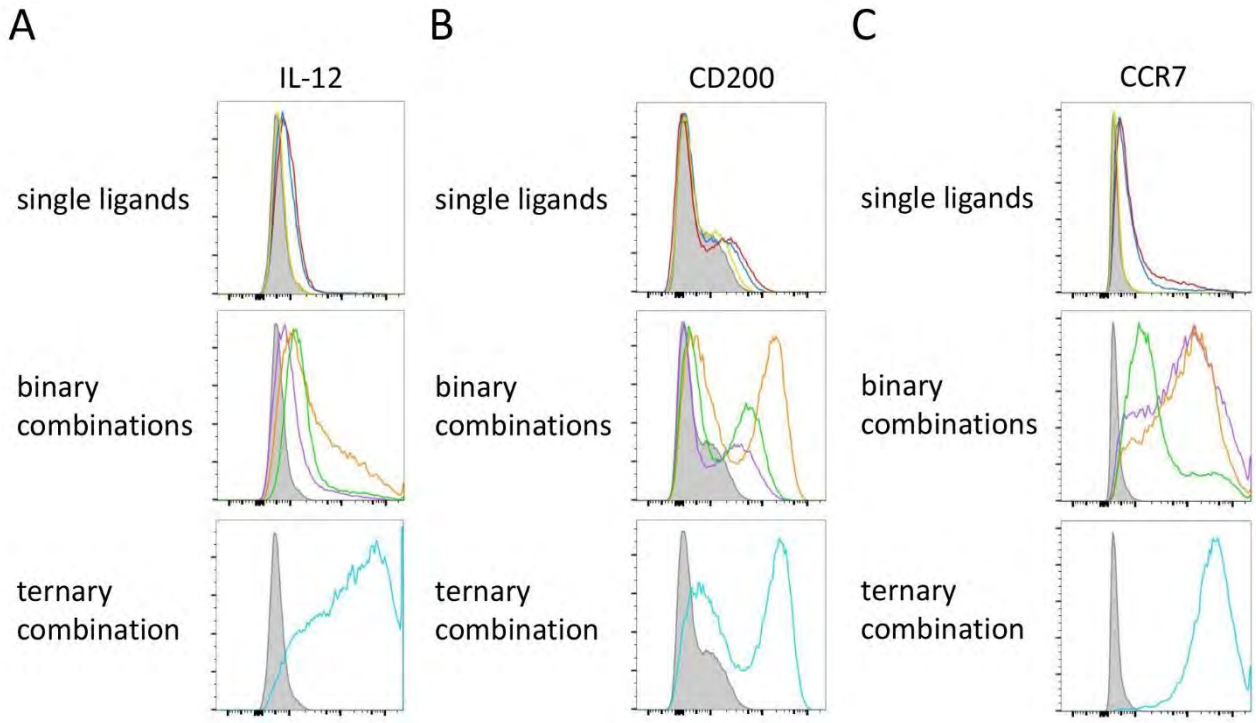


Figure 21. MutuDC1s upregulate IL-12 mainly after super-synergistic activation, while CD200 and CCR7 are increased upon synergistic activation and their levels are maintained in super-synergy. MutuDC1s were incubated for 24 hours with poly(I:C) (8.5 µg/mL), CpG ODN (1 µM), IFNγ (100 U/mL) alone or in binary and ternary combinations. **(A)** The cells were incubated for 6 h with Brefeldin A, stained intracellularly with an anti-IL-12p40 fluorochrome-conjugated antibody and analyzed by flow cytometry. **(B, C)** The cells were stained extracellularly with fluorescent-labeled antibodies specific for **(B)** CD200 or **(C)** CCR7 and analyzed by flow cytometry. From top (single ligands) to bottom (ternary combination of ligands), the peaks that correspond to the different ligands and ligand combinations are shown in distinct colors (single ligands: blue = poly(I:C), red = CpG ODN, yellow = IFNγ; binary

combinations of ligands: violet = poly(I:C) + CpG ODN, green = poly(I:C) + IFNγ, orange = CpG ODN + IFNγ; ternary combination of ligands: cyan = poly(I:C) + CpG ODN + IFNγ) and are compared with untreated cells (shadowed gray). Data are representative of 3 independent experiments.

Nevertheless, we reasoned that if on the one hand the analysis of individual markers does not suffice to discriminate precisely the non-synergistic, the synergistic and the super-synergistic activation states, on the other hand the simultaneous analysis of multiple markers might allow a better distinction. To test this hypothesis, we stimulated MutuDC1s with poly(I:C), CpG ODN, IFNγ and their binary or ternary combinations for 24 hours and analyzed them by flow cytometry for the expression of CCR7 and IL-12. This experiment showed that CCR7 expression was cooperatively upregulated mostly by binary combinations of ligands, with negligible or very limited effect on the levels of IL-12 **(Figure 22)**. By contrast, the ternary stimulation was able to super-synergistically induce IL-12 expression in addition to the synergistic upregulation of CCR7, with an exiguous or absent fraction of single positive or double negative cells **(Figure 22)**. Therefore, we concluded that the simultaneous analysis of CCR7 and IL-12 expression by activated MutuDC1s allows to distinguish, within the same population, the non-synergistically activated cells as $CCR7^{lo}$ $IL\text{-}12^{-}$, the synergistically activated cells as $CCR7^{hi}$ $IL\text{-}12^{-/lo}$ and the super-synergistically activated cells as $CCR7^{hi}$ $IL\text{-}12^{hi}$.

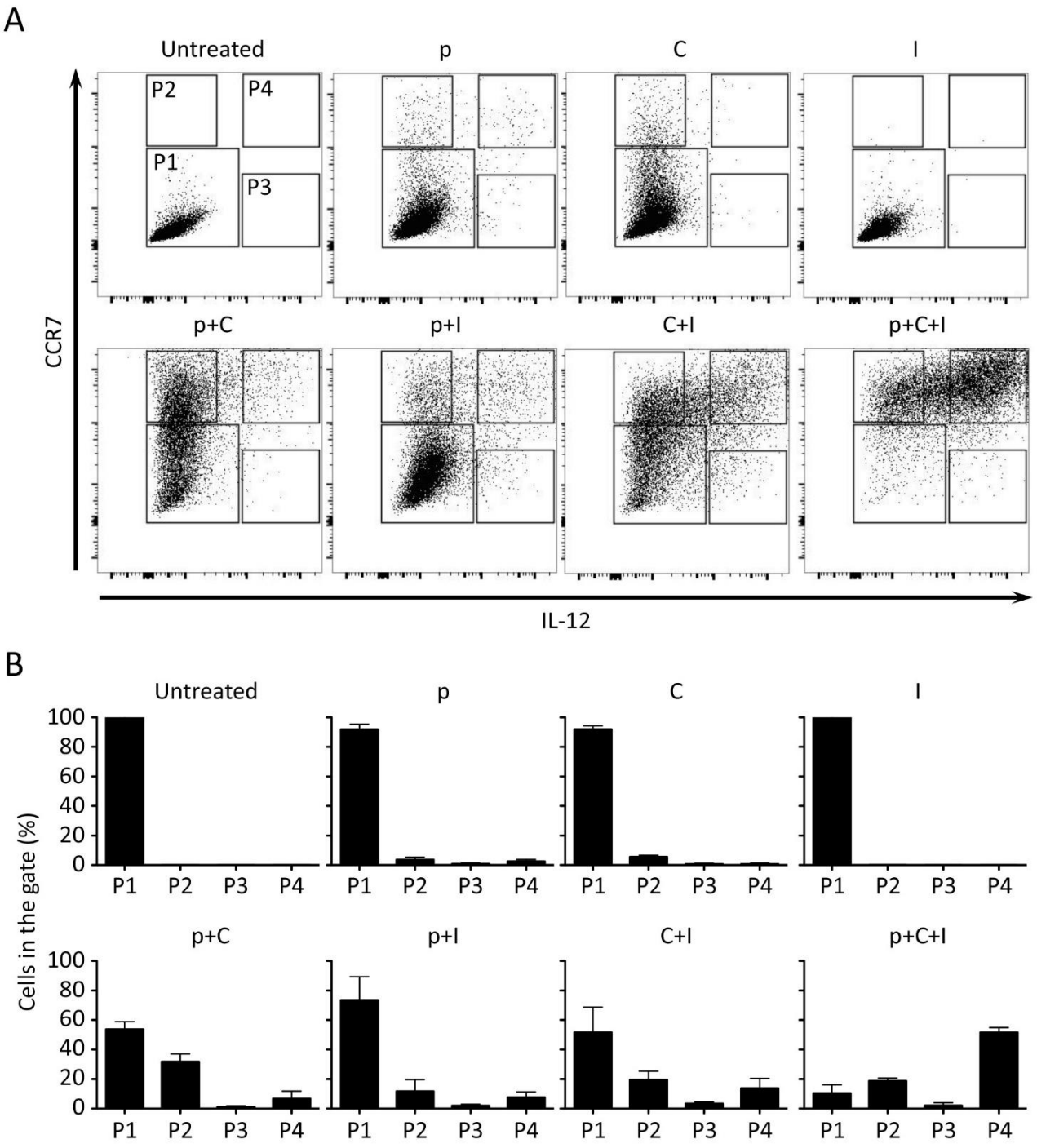


Figure 22. Simultaneous analysis of IL-12 and CCR7 expression allows to discriminate non-synergistic, synergistic and super-synergistic activation states of MutuDC1s. MutuDC1s were incubated for 24 hours with poly(I:C) (p) (8.5 µg/mL), CpG ODN (C) (1 µM) and IFNγ (I) (100 U/mL) either alone or in binary or ternary combinations. Brefeldin A (10 µg/mL) was added during the last 6 h of incubation. The cells were collected and stained extracellularly with a fluorochrome-conjugated anti-CCR7 and intracellularly with a fluorochrome-conjugated anti-IL-12p40 antibody. The expression of CCR7 and IL-12 was assessed by flow cytometry. **(A)** Dot plot representation of the results, and gating strategy.

Gate P1 includes the non-synergistically activated and/or the unresponsive cells; gate P2 and possibly P3 include mainly synergistically activated cells; gate P4 includes mainly super-synergistically activated cells. **(B)** Quantification of gates P1, P2, P3 and P4 in the tested conditions. The fraction of cells in each gate is indicated as percentage of the total number of cells. Data are mean ± SD from 3 independent experiments.

4.2.2. Lentivirally transduced MutuDC1s integrate the lentiCRISPR v2 constructs and express the selection marker but fail to express Cas9 and to undergo gene knockout

Once defined an appropriate readout for our screening approach, our work focused on the establishment of the best experimental conditions for the lentiviral infection and the selection of transduced MutuDC1s. For our screening, we decided to use the genome CRISPR knock-out (GeCKO) v2 pooled lentiviral library which is cloned in the backbone plasmid lentiCRISPR v2 (Figure 12) and is divided into two half-libraries called GeCKO A and GeCKO B (for a detailed description see paragraph *3.2.2. Genome CRISPR knock-out (GeCKO) v2 pooled libraries*).

In a first stage, we produced lentiviral particles carrying either the GeCKO A or the GeCKO B library, we assessed the capacity of the MutuDC1s to tolerate the lentiviral infection and we estimated the functional titer of the lentiviral particles. To do this, GeCKO A or GeCKO B lentiviral particles were used in serial dilution to transduce MutuDC1s **(Figure 23A)**. Measurements of cell viability carried out 48 hours after the infection showed that, even after incubation with the highest lentiviral concentrations, the viability of MutuDC1s was only slightly affected if compared with non-transduced cells **(Figure 23B left)**, indicating that most of the cells were able to tolerate the transduction. The viability values measured in each well were used to calculate an estimate of the total cell number post-transduction. To determine the functional titer of the lentiviral particles, we

exploited the puromycin-resistance selection marker contained in the lentiCRISPR v2 constructs. The transduced cells were selected for three days with puromycin and subsequently their viability post-selection was measured. Depending on the initial concentration of viral particles **(Figure 23A)**, different viability values were measured, indicating that different proportions of MutuDC1s survived the selection. Also in this case the viability values were used to estimate the absolute number of puromycin-resistant cells generated with each viral dilution. The percentage of transduced cells was calculated as the ratio between the number of puromycin-resistant cells and the total number of cells post-transduction **(Figure 23B right)**. From this value, the functional titer of the lentiviral particles was estimated.

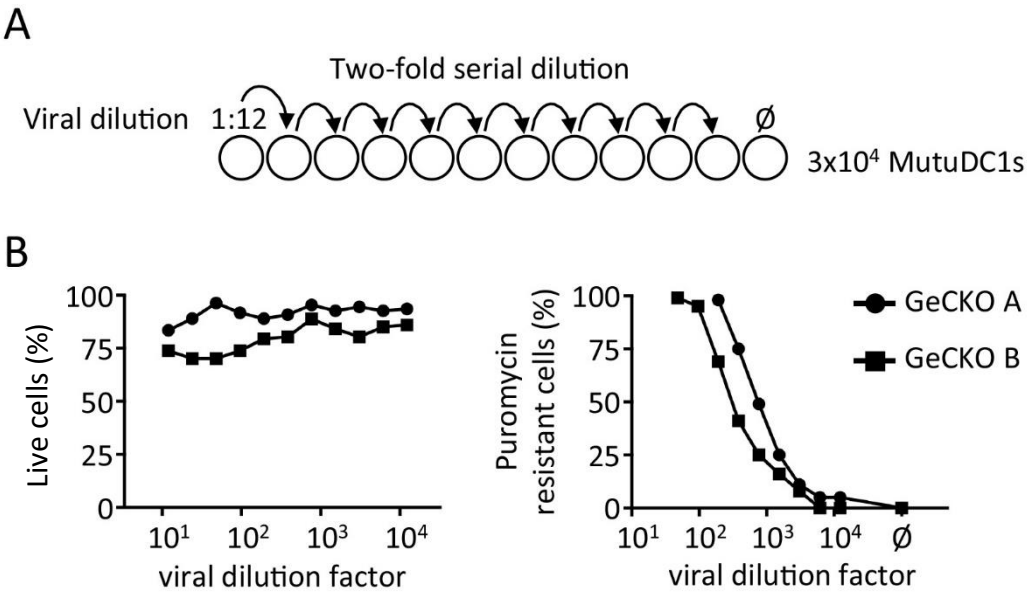


Figure 23. Transduction of MutuDC1s with different concentrations of lentiCRISPR v2 viral particles shows limited effect on cell viability and allows a consistent generation of puromycin resistant cells. Representative example of lentiCRISPR v2 viral particle titration. For each half-library (GeCKO A and GeCKO B), MutuDC1s were seeded in 6 rows of a 96-well plate at a density of $3x10^4$ cells/well. One aliquot of lentiviral particles was diluted 1:12 in polybrene-containing medium and subsequently a serial two-fold dilution of the lentiCRISPR v2 particles was prepared. All the wells located in the same column of the plate were incubated with the same concentration of lentiviral particles. After transduction and cell recovery, a standard curve of different MutuDC1 densities was seeded in an empty row and used as a reference in a cell viability assay to estimate the number of viable cells in

each well of the first 3 rows of the plate. The remaining 3 rows were selected with puromycin and, subsequently, a new viability assay was carried out to estimate the number of cells that survived the selection. **(A)** Schematic representation of viral dilution preparation and plating. The symbol Ø indicates the non-transduced controls. **(B left)** The number of cells that survived the transduction was estimated for every viral concentration and the percentage of surviving cells was calculated as ratio with the non-transduced control. **(B right)** The number of puromycin-resistant cells was estimated for every lentiviral concentration. Percentages were calculated as ratio of these values and the number of cells before selection. Data are average of technical triplicates. The symbol Ø indicates the non-transduced controls.

Considering that for our sorting approach we aimed to work with a pooled population of single knockout cells, for the following experiments a low MOI of 0,3 - 0,5 was chosen since, in these transduction conditions, the likelihood of a cell undergoing multiple infections, and therefore multiple knockouts, is very limited.

To assess Cas9 expression in MutuDC1s, we transduced them with the GeCKO A or B lentiviral particles at an MOI of 0,3 and selected them with puromycin (transduced cells were named: MutuDC1-GA or MutuDC1-GB). MutuDC1-GA and MutuDC1-GB cells were intranuclearly stained with a fluorochrome-conjugated anti-FLAG antibody, since in the GeCKO v2 constructs a FLAG tag is included at the C-terminal extremity of Cas9 **(Figure 12)**. Flow cytometric analysis of the transduced MutuDC1s showed no detectable expression of Cas9 **(Figure 24)**.

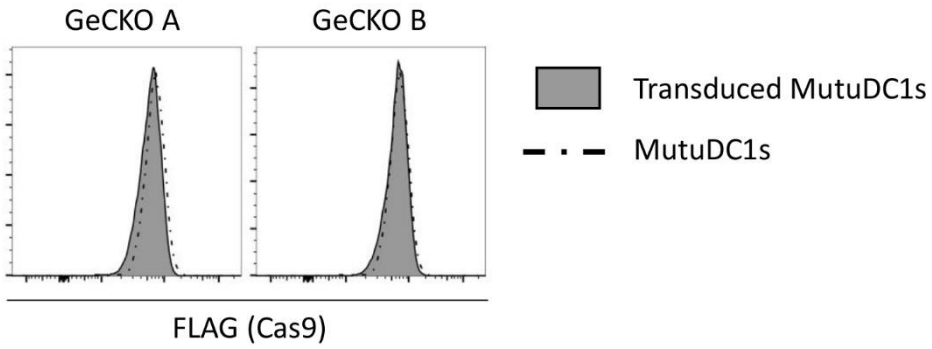


Figure 24. Cas9 expression is not detectable by flow cytometric analysis of MutuDC1-GA and MutuDC1-GB cells. MutuDC1-GA and MutuDC1-GB cells were intranuclearly stained with a fluorochrome-conjugated anti-FLAG antibody and compared by flow cytometric analysis with non-transduced MutuDC1s. Results are representative of 3 independent experiments.

To explain this result, we hypothesized that the FLAG tag could be rendered inaccessible to the antibody by the tertiary structure of Cas9. Therefore, we prepared a total-protein extract from the transduced MutuDC1s and tested the expression of Cas9 by Western blotting with an anti-FLAG antibody. Also in this case, we did not detect Cas9 expression (data not shown).

On the basis of these observations, we decided to retest the correct integration of the lentiCRISPR constructs in the MutuDC1-GA and MutuDC1-GB cells. To do this we collected a sample of cells form both the MutuDC1-GA and the MutuDC1-GB cultures and analyzed their genomic DNA through PCR by amplification of lentiviral constructs from the common sgRNA-flanking regions. This analysis showed a clear amplification in the genomic DNA samples from MutuDC1-GA and MutuDC1-GB cells but not from non-transduced MutuDC1s **(Figure 25)**, demonstrating the presence of detectable lentiCRISPR v2 constructs integrated in the genome of transduced cells.

Figure 25. MutuDC1-GA and MutuDC1-GB cells show integration of the GeCKO A and GeCKO B lentiCRISPR vectors in their genome. Genomic DNA was extracted from MutuDC1-GA, MutuDC1-GB and non-transduced MutuDC1 cells. A PCR was carried out using the v2Adaptor F and R primers and the 1st PCR program (see materials and methods). For each reaction an estimated quantity of 40 ng of genomic DNA was used. 1 ng of purified GeCKO A plasmid-DNA was used as a positive control.

In parallel, we performed a second cycle of puromycin selection on the MutuDC1-GA and MutuDC1-GB cultures. Under these conditions, both the cultures showed negligible mortality, demonstrating that the vast majority of MutuDC1-GA and MutuDC1-GB cells had correctly integrated the lentiCRISPR v2 constructs in their genome.

Considering that even very low levels of Cas9 should in theory suffice to generate a gene knockout, we aimed to test if lentiCRISPR v2-transduced MutuDC1s could successfully knockout target genes in spite of our inability to detect Cas9 expression. To this end, we designed two new sgRNAs targeting the gene coding for the ELAV-like protein 1 (ELAVL1, also known as human antigen R (HuR)), an RNA-binding protein that our group has found to be involved in the regulation of synergy through mRNA stabilization (Gupta et al., unpublished results). Two new lentiCRISPR v2 constructs, named 239E1 and 240E1 **(Figure 26)**, were generated by introducing the sequences of the *Elavl1*-targeting sgRNAs in the lentiCRISPR v2 plasmid.

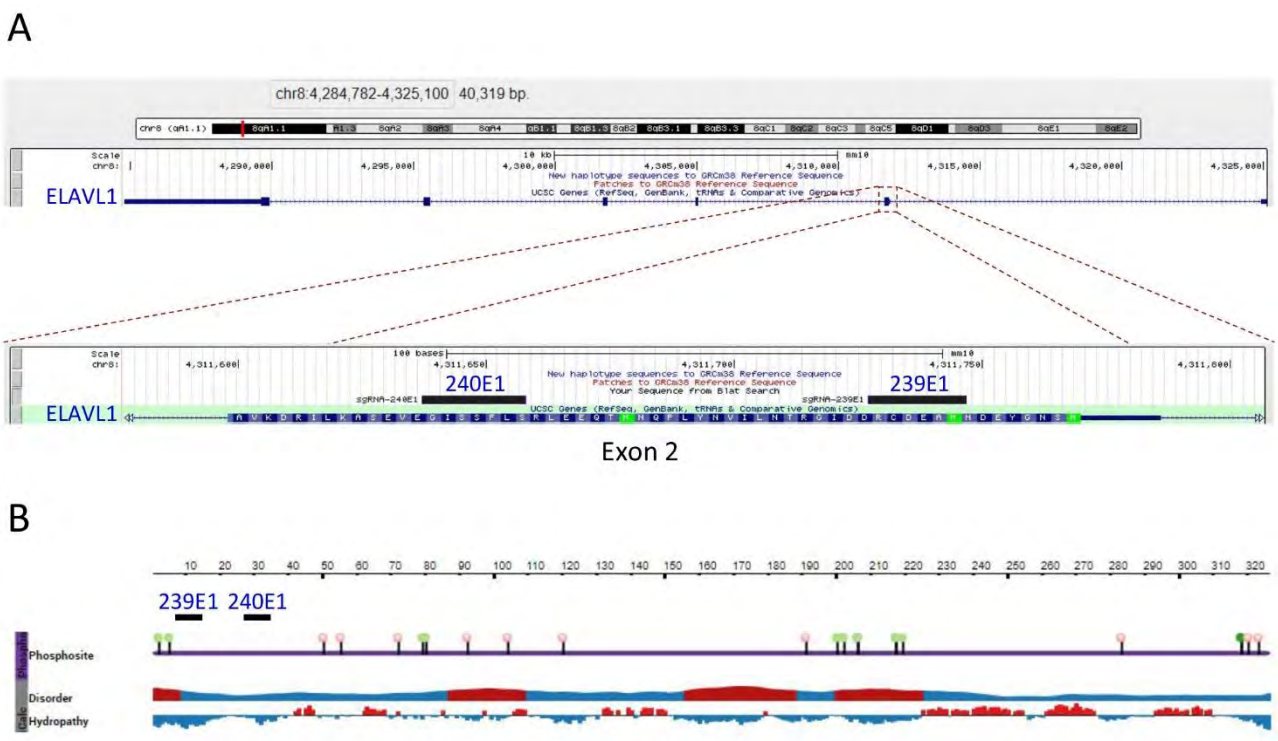


Figure 26. *Elavl1*-targeting lentiCRISPR v2 constructs. (A) Genomic map of the *Elavl1* locus. The coding sequence of *Elavl1* is located on the negative strand. The intron/exon structure is highlighted as follows: intron = thin blue line; exon = thick blue line; untranslated regions are shown as thinner areas within the exons. An enlarged representation of exon 2 is shown with indication of the position of the constructs 239E1 and 240E1 (black horizontal bars). Figures are adapted from the UCSC Genome Browser on Mouse Dec. 2011 (GRCm38/mm10) Assembly. **(B)** Schematic representation of ELAVL1 protein sequence with indication of annotated post-translational modification sites (Ubiquitylation = pink; Phosphorylation = green), predicted protein disorder (Ordered = blue; Disordered = red) and calculated hydropathy (Hydrophilic = blue; Hydrophobic = red). The protein regions whose coding sequences are targeted by the lentiCRISPR 239E1 and 240E1 are indicated by two black horizontal bars. Figure is adapted from the Protein Feature View of PDB entries mapped to a UniProtKB sequence, ELAV-like protein 1 - P70372 (ELAV1_MOUSE).

Lentiviral particles carrying the 239E1 or 240E1 constructs were produced and tested as described above for the GeCKO A and B lentiviral particles. MutuDC1s were transduced with the 239E1 or 240E1 lentiviral particles at an MOI of 0,3 and subsequently selected with puromycin. The selected cells were analyzed by intracellular flow cytometry for the expression of Cas9. Also in this case,

staining with a fluorescent-labeled anti-FLAG antibody did not show detectable levels of Cas9 **(Figure 27)**.

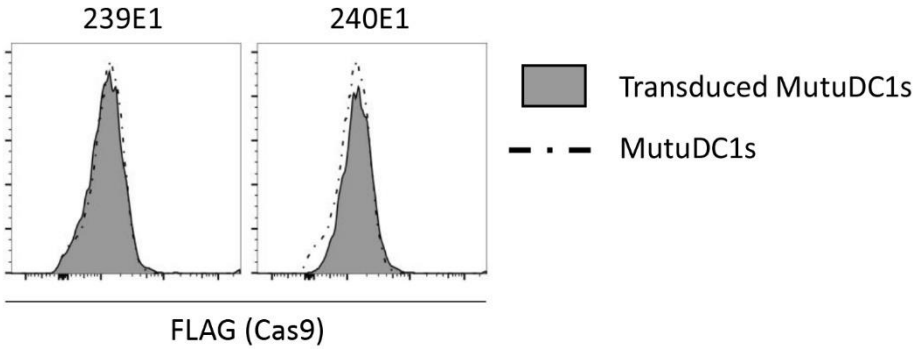


Figure 27. Cas9 expression is not detectable by flow cytometric analysis of MutuDC1-239E1 and MutuDC1-240E1 cells. MutuDC1-239E1 and MutuDC1-240E1 cells were intranuclearly stained with a fluorochrome-conjugated anti-FLAG antibody and compared by flow cytometric analysis with non-transduced MutuDC1s. Results are representative of 3 independent experiments.

Total protein extracts were prepared from MutuDC1-239E1 and MutuDC1-240E1 cells and analyzed by Western blotting with anti-FLAG and anti-ELAVL1 antibodies. This analysis confirmed our previous observation that the transduced cells did not express detectable levels of Cas9 (data not shown). Moreover, if compared with non-transduced MutuDC1s, the MutuDC1-239E1 and MutuDC1-240E1 cells did not show clear reduction of ELAVL1 expression **(Figure 28)**. Therefore, also in consideration of our previous results, we concluded that, in these experimental conditions, the MutuDC1s fail to express Cas9 and to undergo CRISPR/Cas9-mediated gene knockout.

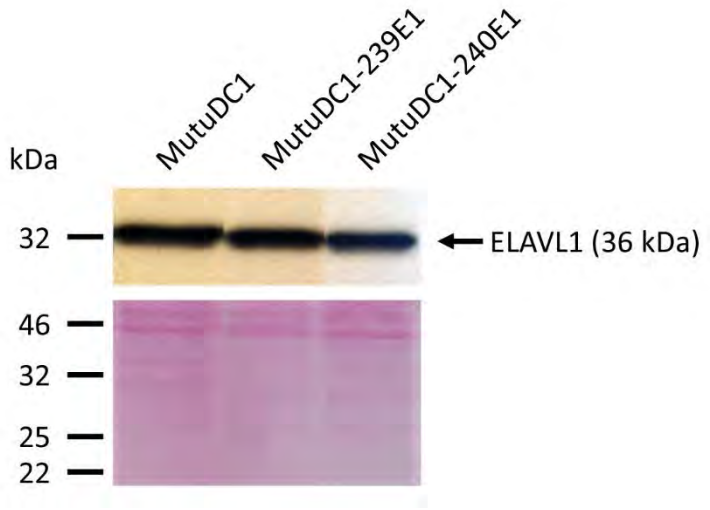


Figure 28. Expression of ELAVL1 in MutuDC1-239E1 and MutuDC1-240E1 is not reduced if compared with non-transduced MutuDC1s. Total protein extracts from MutuDC1-239E1, MutuDC1-240E1 or non-transduced MutuDC1 cells were prepared by lysing $2x10^6$ cells in 150 µl of RIPA buffer. ELAVL1 expression was analyzed by Western blotting (top) after SDS-PAGE of 5 µl of protein extracts on a 15% polyacrylamide gel. Ponceau S staining (bottom) was used to estimate the homogeneity of protein loading among the different samples.

5. Discussion

5.1. Generation of new MutuDC lines

The difficulties of studying DCs depend largely on their paucity *in vivo* and on their high sensitivity to *in vitro* conditions. Additionally, the current understanding of DC biology has likely just started to unveil the extreme complexity of the DC lineage, showing a multitude of DC subsets with unique functional and phenotypic features that further complicate the efforts toward a global understanding of this sophisticated cell network. These aspects contribute to explain the great need for suitable model systems to facilitate DC research, also in consideration of the central role of DCs in the regulation of mechanisms, such as self-tolerance and induction of adaptive immunity toward pathogens, that have clear implications, for example, in the pathogenesis of autoimmune diseases and in the creation of new vaccination strategies.

We have extensively shown and discussed the generation and characterization of two of the DC lines developed in our laboratory. As explained, our cell lines are not the first models available for the study of DCs *in vitro*, however, to our knowledge, they are one of the only examples, if not the first one, in which DC lines succeed in representing individual DC-subsets with their phenotypic and functional specificity.

In this thesis, we show in particular the generation and characterization of a cDC2-like DC line. These cells, named $CD4^-$ MutuDC2s, were extensively compared with freshly isolated cDC1s and cDC2s but also with our previously derived cDC1-like MutuDC1s. The characteristic cDC markers FLT3, CD11c, MHC-II, CD40, CD80 and CD86 are all expressed by the $CD4^-$ MutuDC2s at comparable or higher levels than in freshly isolated cDCs. By contrast, the $CD4^-$ MutuDC2s lack the expression of markers that might have suggested a monocytic origin or their belonging to the pDC subset. The expression

of several cDC2-specific markers, together with the lack of cDC1-associated phenotypic features, strongly corroborates the conclusion of their belonging to the cDC2 subset making them a *bona fide* model of cDC2s. This is further supported by their profile of expression of TLRs, cytokines and chemokines as well as by their capacity to process and present antigens to $CD4^+$ T cells through MHC-II but not to $CD8^+$ T cells through MHC-I cross-presentation.

The MutuDC1s and the $CD4^-$ MutuDC2s are just two examples of cell lines derived through our method. Indeed, this approach has already shown in several instances the potential to generate cell lines with characteristics of different DC subsets other than cDC1s and cDC2s.

For example, one of the prevailing phenotypes that we observed during the derivation of the $CD4^-$ MutuDC2s was characterized by low levels of CD11c and MHC-II. Among the numerous DC subsets described *in vivo*, one has been shown to be characterized by low levels of CD11c and to lack MHC-II expression. These DCs, named L-DCs, are considered to be the *in vivo* counterpart of the long-term culture (LTC) DCs that are generated *in vitro* from hematopoietic progenitors with the support of a monolayer of stromal cells but without the need of exogenous cytokines [388–390]. L-DCs have been partially characterized and shown to express CD11b but to lack the expression of CD172a. Moreover, in L-DCs, the surface markers CD80 and CD86 are expressed at relatively high levels but, similarly to MHC-II, they are not upregulated upon cell activation. Functional analysis of L-DCs has shown that they can cross-present antigens but have a limited capacity to activate $CD4^+$ T cells through MHC-II presentation. Our 17005B Mutu cells showed several characteristics similar to the L-DCs. Indeed, 17005B Mutu cells have low levels of CD11c and MHC-II, fail to upregulate MHC-II and co-stimulatory molecules upon activation and have limited capacity to activate $CD4^+$ T cells. By contrast, their expression of high CD172a levels and their inability to cross-present antigens might contradict the hypothesis of their belonging to the L-DC subset. Several aspects of L-DC phenotypic and functional specificity are still unexplored, therefore our analysis of TLR expression profile and

cytokine production in the 17005B Mutu cells does not allow to draw any better conclusion about their possible belonging to this DC subset. Additionally, the observation, that we made at a later time than the derivation of the 17005B Mutu cells, that distinct Mutu cell lines might need different concentrations of antigens for presentation to T cells suggests that the inefficient MHC-II presentation shown by the 17005B Mutu cells could depend on a mere technical inaccuracy that might have concealed their functional characteristics. In consideration of this possibility, it would be necessary to repeat the T cell activation assays with antigen-pulsed 17005B Mutu cells to assess if the functional features that we have described remain unvaried even at higher concentrations of antigenic peptides. Therefore, with regard to the unequivocal determination of the belonging of the 17005B Mutu cells to a specific DC subset, a thorough comparison of this cell line with freshly isolated DCs, in particular with L-DCs and/or LTC-DCs, would be needed. In a long-term perspective, should the results indicate that the 17005B Mutu cells do not belong to any of the DC subsets observed *in vivo*, it would be interesting to determine their developmental origin and assess the existence of an *in vivo* counterpart of this cell line both in healthy and tumoral spleens with a particular attention to the possible specific generation of these cells in the latter context.

As we have shown, during our cell-line derivations we have also obtained three stable Mutu cell lines, the 21735A, the 21733C and the 20876B Mutu cells, with potential characteristics of pDC lines. Indeed, they have intermediate levels of both CD11c and MHC-II and show expression of B220 and PDCA-1. However, their high levels of CD11b strongly contrast with the hypothesis of their belonging to the pDC subset. To investigate their possible belonging to this subset a more detailed phenotypic and functional characterization of 21735A, 21733C and 20876B Mutu cells would be needed. In particular, the analysis of E2-2 and IRF7 expression as well as the determination of their TLR expression profile, with special focus on TLR7 and TLR9, and the assessment of their capacity to

produce type I IFNs upon activation could provide strong indications in favor of or against our hypothesis.

5.2. Study of the molecular mechanisms of synergistic and super-synergistic DC activation

There is mounting evidence that integration of different pathogen- and host-derived signals is required to develop the most appropriate innate immune response. Numerous publications demonstrate that DCs can be synergistically activated by specific combinations of TLR ligands and that this effect can be increased to a super-synergistic level by the presence of host-derived signals like IFNγ or CD40L [157,160–162,165,170,184,185,188,189]. Even though the signaling pathways activated by the different TLRs after recognition of their ligands are well understood and have been extensively described, the mechanisms that regulate the synergistic activation and the super-synergistic integration of host-derived signals are still mostly uncharacterized.

In the context of synergistic activation by binary combinations of TLR ligands, the prevailing and most accepted model is that a cross-talk between the MyD88- and TRIF-dependent pathways is needed to induce synergy [157,161,169,184,186,187]. However, there are examples where cooperativity between MyD88-dependent TLRs was observed [391], and overall the mechanisms that allow the integration of the two pathways are still completely obscure. One of the proposed models suggests a type I IFN-mediated paracrine effect on synergy [185,186,392], but contrasting results have been obtained by different groups, since in many cases type I IFN receptor knockout, type I IFN blocking antibodies or exogenous addition of type I IFNs did not produce any significant effect on synergistic activation [157,161,169]. Other works indicate that synergy might depend on a sustained and prolonged activation of the signaling pathways downstream of TLRs [157,164]. In addition, there are many studies based on the use of knockout models or chemical inhibitors of

several members of the TLR-signaling pathways such as NF-κB, MAPKs, JNK, AP-1 and IRFs [161,163,170,185,391,393]. All these studies clearly showed the importance of single factors or branches of the TLR-signaling cascade for the synergistic effect and were even able to demonstrate a gene- and/or stimulus-specific role for some of them [187]. However, the use of inhibitors or knockout models mostly failed to describe the mechanisms that underlie a synergistic cross-talk between different TLRs. Indeed, synergistic response to binary combinations of TLR ligands most likely relies on the functional integrity of the signaling pathways induced by the individual TLRs. In other terms, after recognition of their ligands, both the synergistic TLRs activate specific signaling pathways that can partially or completely overlap with each other and/or culminate on the same effectors, and we might think that synergy lies on the direct or indirect integration of these individual signaling pathways at unique convergence points. In these terms, the disruption, upstream of the synergistic convergence points, of one of the single branches of the signaling cascades, using inhibitors or knockout models, would likely cause an indirect and non-specific loss of synergy with limited potential to highlight the mechanisms that specifically regulate signal integration. Moreover, we can hypothesize that multiple sequential regulatory events might control the contribution of an individual element of the TLR-signaling pathway to synergy by cumulatively enhancing its functional state. However, this mechanism would remain unnoticed if the primary function of the signaling factor itself in the signaling cascade were abolished through inhibition or knockout. In other terms, one possible interpretation of synergy is that the function of individual known TLR-activated effectors might be enhanced by the binary combination of synergistic TLR-ligands through mechanisms such as stabilization, sustained activation or upregulation of the signaling factor themselves. In this scenario, knockout or chemical inhibition of the individual downstream effectors would affect also the response to the single TLR-ligands and would therefore conceal the mechanisms of synergistic functional enhancement.

Regarding the synergistic cell activation by combined stimulation with a TLR ligand and IFNγ, most of the work has been carried out in macrophages while little has been investigated in DCs. In macrophages, treatment with IFNγ induces a pre-activation state that enhances cell sensitivity to subsequent stimulation with TLR ligands, with consequent synergistic increase of cytokine and chemokine production. This effect has been named priming, even if some studies have demonstrated that the positive effect of IFNγ on TLR signaling is maintained regardless of the temporal sequence of addition [188,394]. Several mechanisms have been suggested to explain this effect. Synergistic activation of macrophages by IFNγ/TLR-ligand combinations seems to be mainly mediated by IRF1, which represents a point of intersection of the TLR- and the IFNγ-signaling pathways, and by STAT1, which is the main regulator of the IFNγ signaling and can be activated by the paracrine effect of type I IFNs which, in turn, can be induced by the TLR-signaling cascade [188,394]. The proposed mechanisms include transcriptional regulation through cooperation of IFNγ- and TLR-induced transcription factors on the promoters of cytokine genes or through STAT1- and IRF1-mediated chromatin remodeling that favors binding of TLR-activated transcription factors to cytokine gene promoters and regulatory elements [394]. Other possible mechanisms imply direct cross-talk between the pathways through shared regulators, but also IFNγ-dependent inhibition of TLR-induced negative feedback loops [188,189]. However, nearly nothing is known about the integration of the IFNγ signaling with the synergistic response induced by binary combinations of TLR ligands. In DCs this effect has been observed [157,186] but not explored and there is no evidence that the same regulatory mechanisms demonstrated in macrophages are shared also by DCs.

One of the main limitations of the approaches used so far to study synergy and super-synergy is the fact that, by operating nearly exclusively on known members of the TLR- and IFNγ-signaling cascades, they do not keep into account the possible role of undescribed complementary pathways that specifically mediate the (super-)synergistic cross-talk and that directly regulate (super-)synergy.

Microarray and kinome analyses support this possibility by showing the existence of unique sets of genes and proteins which are regulated by combinations of TLR agonists, but not by treatment with the single ligands [164,165]. Moreover, previous work done in our laboratory further corroborates this idea by revealing that mRNA stability, controlled by mRNA-binding proteins, participates in the synergistic and super-synergistic activation of DCs (Gupta et al., unpublished results).

Therefore, to explore the possibility that synergy and super-synergy are regulated by undescribed complementary pathways, we have decided to use a high-throughput unbiased strategy. As explained, the approach that we propose consists in the flow cytometric screening of a pooled population of single knockout MutuDC1s generated through lentiviral transduction with a genome scale CRISPR/Cas9 pooled library.

To establish the screening strategy, we have identified CCR7 and IL-12 as markers that allow to distinguish different activation states of MutuDC1s. In particular, our data demonstrated that, upon synergistic activation, non-transduced MutuDC1s strongly upregulate CCR7 but express low levels of IL-12, while, upon super-synergistic activation, they maintain high expression of CCR7 and additionally induce abundant production of IL-12. This allows to distinguish a $CCR7^{lo/-}$ $IL\text{-}12^{-}$ population of unresponsive/non-synergistically activated cells, a $CCR7^{hi}$ $IL\text{-}12^{lo/-}$ population of synergistically activated cells and a $CCR7^{hi}$ $IL\text{-}12^{hi}$ population of super-synergistically activated cells.

In our screening strategy, the screening target is represented by any gene-knockout that causes a dysregulation of (super-)synergistic cell activation. The identification of these targets relies on the capacity to distinguish the knockout cells that show altered (super-)synergistic activation, namely any transduced cell in which the cell activation state does not correspond to the activating treatment received. As we have shown, the recognition of such candidate targets can be achieved by flow cytometric analysis of CCR7 and IL-12 expression. For example, after treatment of

transduced cells with a super-synergistic ligand combination, all the super-synergistically activated cells are expected to be in the CCR7^{hi} IL-12^{hi} gate while all the cells in which the gene knockout has caused a loss of super-synergistic activation are not expected to be found there. Therefore, if compared with the original composition of the library, the CRISPR constructs which target genes involved in the regulation of super-synergy are expected to be missing or less frequent in the CCR7^{hi} IL-12^{hi} gate and symmetrically to be enriched in the CCR7^{hi} IL-12$^{lo/-}$ gate and/or in the CCR7$^{lo/-}$ IL-12$^{lo/-}$ gate. Similar reasoning delineates the strategy to identify candidate regulators of cell synergistic activation with any binary combination of CpG ODN, poly(I:C) and IFNγ. Even in the alternative hypothesis of a negative regulation mechanism of (super-)synergy in which, for example, a stimulation threshold existed below which negative regulatory factors inhibited synergistic or super-synergistic activation, the approach that we propose would allow to identify possible (super-)synergy regulators. Indeed, knockout of these regulators would induce a state of hyperactivation of the cells after stimulation with the single ligands or with their binary combinations, and consequently we would expect the constructs that target these regulators to be enriched in the CCR7^{hi} and/or, depending on the initial stimulation conditions, in the CCR7^{hi} IL-12^{hi} gates.

These few examples show that this approach conveniently allows to carry out simultaneously screenings by positive selection (enriched construct frequency) and negative selection (decreased construct frequency). Moreover, by comparing the constructs of interest identified in the different sorted subpopulations, it allows to distinguish, at the same time, regulators of all the possible transitions from one activation state to the other. Our strategy has high potential to identify regulators of synergy and super-synergy as long as they carry out their function through pathways which are complementary to the canonical TLR- and IFNγ-signaling cascades. At the same time, one of the strengths of this method is that the identification of known elements of the TLR- and IFNγ-

signaling pathways through our screening would represent a strong internal control of the validity of the approach.

However, there is no reason to exclude the possibility that synergy and super-synergy depend on differential regulation of elements of the canonical TLR- and IFNγ-signaling pathways. This hypothesis includes several possible mechanisms. For example, as mentioned before, transcription factors downstream of the individual signaling cascades could cooperatively bind on promoters and regulatory elements of synergistically and super-synergistically transcribed genes or induce chromatin modification to favor transcription initiation. Different and cumulative levels of regulation of known elements of the individual signaling cascades might have a role, without involving additional complementary factors. In general, all the possible mechanisms that explain synergy and super-synergy through an enhancement of the function of known elements of the TLR- and IFNγ-signaling cascades would be impossible to highlight by using the approach that we propose. The same would be true if redundancies in the signaling pathways were able to compensate for the gene knockout.

Our results suggest another possible interpretation of synergy and super-synergy. Indeed, it is possible to notice that upon stimulation with binary or ternary combinations of ligands, not only is the marker expression level increased at the single cell level (i.e. higher fluorescence intensity), but also the number of activated cells is increased **(Figure 22)**. This indicates that synergy and super-synergy might depend, at least partially, on a population effect. In this scenario, the synergistic and super-synergistic levels of cytokines and of the relative mRNAs, measured respectively by ELISA and RT-qPCR, might partially depend on the synergistic or super-synergistic increase of the number of cells producing them in addition to the synergistically or super-synergistically enhanced production by each activated cell. Similarly to the mechanisms described above, also this interpretation of

synergy and super-synergy would imply the activity of positive and/or negative regulators that hence would be likely identified through our screening approach.

From a technical point of view, after the identification of CCR7 and IL-12 as screening markers, we have dedicated much effort to determining the most suitable transduction conditions. Indeed, for several reasons discussed below, this experimental step is essential for the success of the approach that we propose, and its establishment has proven to be particularly delicate in our model system.

First of all, we aimed to determine a precise functional titer of the lentiviral particles, because this parameter allows an accurate control over the MOI used during cell transduction and is the only way to ensure that every transduced cell ideally integrates only one CRISPR/Cas9 construct and therefore undergoes just a single gene-knockout. This is important because multiple knockouts in the same cell could increase the chances of false positive or false negative results.

In second instance, we focused on the knockout efficiency in transduced cells. Indeed, in the type of pooled strategy that we propose, this parameter must be maximized to increase the statistical power of the screening and to limit as effectively as possible the levels of background signal detected during the process of target-construct identification. The latter point can be explained by considering, for example, a screening by negative selection. In that case a low knockout efficiency would increase the occurrence of false negative events by increasing the frequency of actual screening target-constructs in the negative population, and therefore it would make much more difficult to recognize those constructs as actual targets. Moreover, to reach statistical significance, the screened population must be composed of a number of knockout cells that exceeds the complexity of the library by at least 100 folds. This means that, with the GeCKO A and B half-libraries, each of which targets roughly $2x10^4$ genes with 3 sgRNA constructs per gene, the final number of knockout cells needed for a single experiment would be of at least $6x10^6$. However, with a low

knockout efficiency, this number would inevitably increase and could easily reach levels sufficient to compromise the technical feasibility of the experiment.

When MutuDC1s were transduced with the GeCKO A and B libraries, they correctly integrated the CRISPR/Cas9 constructs in their genome and expressed the selection marker sequence contained therein. However, we were not able to show the expression of Cas9. Using the same vector (lentiCRISPR v2 plasmid) in which the GeCKO libraries are cloned, we created new CRISPR/Cas9 constructs to knockout the putative (super-)synergy regulator ELAVL1. Transduced MutuDC1s integrated the CRIPR/Cas9 constructs and expressed the selection marker but did not show Cas9 expression nor did they appear to have undergone ELAVL1 knockout. Similar results were obtained when other members of our group tried to induce the knockout of other genomic targets in MutuDC1s (data not shown). Additionally, we did similar observations in the past, when we tried to use an shRNA-based knockdown strategy in MutuDC1s (data not shown). Similarly, when lentiviral expression-constructs were used to transduce MutuDC1s, the levels of the exogenous proteins were often found to be low or non-detectable even if, in some instances, RT-qPCR analysis of transduced cells showed transcription of these coding sequences (data not shown). The lentiCRISPR v2 vector was used to generate additional constructs targeting the integrin CD11b. These constructs were successfully used to knockout CD11b in $CD4^-$ MutuDC2s. However, in the most successful case, the knockout efficiency was lower than 70% and subsequent repetitions of the same protocol produced poorly reproducible results (data not shown). Overall, these observations indicate a potential refractoriness of our cells to CRIPR/Cas9-knockout generation and, more in general, to lentiviral-based approaches. So far, we have not been able to determine if this pattern depends on procedural inaccuracies or on possible mechanisms that allow the MutuDCs to inhibit transcription or translation of genes introduced through lentiviral transduction. An alternative interpretation, at least in the case of the CRISPR/Cas9- or shRNA-based strategies, is that, by knocking out or down

specific genes, we introduce a selective pressure that can favor the growth of cells that manage to escape the knockout or the knockdown events. If this were true, we would probably not be able to identify knockout or knockdown events at a population level and we would need to clonally expand single transduced cells to isolate potential knockout or knockdown candidates. To explore this hypothesis, we are currently producing different lentiCRISPR v2 constructs targeting EGFP (which is expressed by MutuDC1s). EGFP knockout is not expected to introduce any selective pressure and can be easily monitored by flow cytometric analysis allowing an immediate estimation of the knockout efficiency on live cells. Once we will have determined the reasons for the low knockout efficiency and established the best experimental set up for the strategy that we propose, we will use these conditions to proceed with the CRISPR/Cas9-library screening and the identification of (super-)synergy regulators.

In conclusion, synergistic and super-synergistic activation are extremely complex mechanisms that undoubtedly rely on several levels of regulation and depend on the integration of numerous and articulate regulatory pathways. Nonetheless, we think that the approach that we propose, even if with clear limitations, has the potential to help clarifying some details of the molecular aspects of synergy and super-synergy. Moreover, we think that, once overcome the technical difficulties, this strategy can prove to be a very powerful approach that can likely be extended to numerous biological questions other than DC (super-)synergistic activation.

6. Abbreviations

Apaf1	apoptotic protease activating factor-1
APC	antigen presenting cell
BIR	baculoviral inhibitor of apoptosis repeat
Bcl10	B cell leukemia/lymphoma 10
BCR	B cell receptor
BM	bone marrow
CARD	caspase activation and recruitment domain
CCR7	C-C motif chemokine receptor 7
CD	cluster of differentiation
CD40L	CD40 ligand
cDC	conventional DC
cDC1	cDC type 1
cDC2	cDC type 2
CLIP	class II associated Ii peptide
CLR	C-type lectin-like receptor
CMP	common myeloid progenitor
CTLD	C-type lectin-like domain
DAMP	damage associated molecular patter
DC	dendritic cell
DCIR	DC immunoreceptor
DRiP	defective ribosomal products
EGFP	enhanced green fluorescent protein
ER	endoplasmic reticulum
ERAAP	ER aminopeptidase associated with antigen processing
FcRγ	Fc receptor gamma chain
FLT3	FMS-like tyrosine kinase 3
FLT3L	FLT3 ligand
GeCKO	genome CRISPR knock-out
GM-CSF	granulocyte and macrophage colony-stimulating factor
iE-DAP	γ-D-glutamyl-meso-diaminopimelic acid

IFI16	IFNγ-inducible protein 16
IFN	interferon
Ii	invariant chain
IkB	inhibitor of kB
IKK	IκB kinase
IL	interleukin
IRES	internal ribosome entry site
IRF	IFN regulatory factor
ISG	IFN-stimulated gene
ITAM	immunoreceptor tyrosine-based activation motif
ITIM	immunoreceptor tyrosine-based inhibition motif
LC	Langerhans cell
MAL	MyD88 adaptor-like
Malt-1	mucosa associated lymphoid tissue lymphoma translocation protein 1
MAPK	mitogen-activated protein kinase
MAVS	mitochondrial antiviral signaling protein
M-CSFR	macrophage colony-stimulating factor receptor
MDP	macrophage and dendritic cell progenitor
MHC	major histocompatibility complex
MHC-I	MHC class I
MHC-II	MHC class II
MICL	myeloid inhibitory C-type lectin-like receptor
MINCLE	macrophage inducible Ca^{2+}-dependent lectin
moDC	monocyte derived DC
MuDP	muramyl dipeptide
Mushi	multisystem histiocytosis
MutuDCs	murine tumor DCs
MyD88	myeloid differentiation primary response gene 88
NFAT	nuclear factor of activated T cells
NF-κB	nuclear factor-kB
NLR	nucleotide-binding oligomerization domain-like receptor
PAMP	pathogen associated molecular pattern

pDC	plasmacytoid DC
PLC	peptide loading complex
PRR	pattern recognition receptor
Raf-1	v-raf-1 murine leukemia viral oncogene homolog 1
SYK	spleen tyrosine kinase
Spl-cDC	splenic cDC
STAT3	signal transducer and activator of transcription 3
SV40LgT	simian virus 40 large T oncogene
TAP	transporter associated with antigen processing
TCR	T cell receptor
TipDC	TNF-α/iNOS-producing DC
TIR	Toll/Interleukin-1 Receptor
TLR	toll-like receptor
TRAF	TNF receptor associated factor
TRAM	TRIF-related adaptor molecule
TRIF	TIR-domain-containing adaptor-inducing interferon-β

7. References

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8. Appendix

8.1. Other publications

8.1.1. Derivation and utilization of functional CD8$^+$ dendritic cell lines

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Chapter 3

Derivation and Utilization of Functional CD8$^+$ Dendritic Cell Lines

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Abstract

It is notoriously difficult to obtain large quantities of non-activated dendritic cells ex vivo. For this reason, we produced and characterized a mouse model expressing the large T oncogene under the CD11c promoter (Mushi mice), in which CD8α$^+$ dendritic cells transform after 4 months. We derived a variety of stable cell lines from these primary lines. These cell lines reproducibly share with freshly isolated dendritic cells most surface markers, mRNA and protein expression, and all tested biological functions. Cell lines can be derived from various strains and knockout mice and can be easily transduced with lentiviruses. In this article, we describe the derivation, culture, and lentiviral transduction of these dendritic cell lines.

Key words Dendritic cell, Cell line, Tissue culture, Ex vivo derivation

1 Introduction

It is very difficult to obtain large numbers of the different subsets of dendritic cells (DCs) that can have unique or overlapping functions. Many DC subsets have been described in mouse. The major lymphoid tissue-resident DC subsets can be distinguished by their expression of CD8α (these DCs are major cross-presenter and are better for CD8 T cell priming), CD11b (these DCs are better for CD4 T cell priming), or CD45RA (these DCs are termed plasmacytoid DCs (pDC) and are the major type I interferon producers after viral infection) [1, 2]. In other tissues, several migratory subsets are found such as epidermal Langerhans cells, dermal CD103$^+$ DC (these are equivalent to the lymphoid tissue-resident CD8$^+$ DCs), and CD11b$^+$CD103$^-$ DC subsets. These migratory DCs migrate to secondary lymphoid organs upon microbial infection and inflammation, leading to their activation and differentiation, and present encountered antigens to T cells. A few millions of total DCs, including 100,000–400,000 DCs of the major subsets, are obtained per mouse spleen after sorting ex vivo [3]. An alternative is the derivation of bone-marrow-derived DCs with growth and

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differentiation factors such as GM-CSF, with or without IL-4 (yielding mostly CD11b$^+$ DC subsets, up to 100 million per mouse depending on the procedure, with 40% contaminating non-DCs, including macrophages), or Flt3 ligand (Flt3L) (yielding mainly CD8-like DCs which do not express CD8 but are closely related to this subset [4], but also CD11b$^+$ DC and pDC subsets, a few millions per mouse). However, further purification steps are required to obtain pure DC subsets using these isolation and in vitro differentiation procedures [4–6]. Isolated cells are often stressed or activated after the long isolation procedures, and, in addition, many cells die within 24–48 h of in vitro culture. The in vitro life span can be slightly extended by adding growth and differentiation factors or by using Bcl-2 overexpression or cells deficient for proapoptotic molecules [7].

Injection of Flt3L into mice, adoptive transfer of tumors secreting Flt3L, and more recently, generating transgenic mice with Flt3L overexpression allowed generation of different DC subsets in the spleen in about 30-fold higher numbers than from normal mice [8, 9].

Alternatively, several groups have established cell lines after retroviral transduction with oncogenes, transgenesis, or from primary cultures of splenocytes with growth factors [10–17]. All these lines have been cultured for many passages and have lost many of the initially described functions typical of DCs.

Despite the advances in method development, it is still difficult to obtain sufficient numbers of DCs to perform large-scale drug screenings, subcellular fractionations, proteomics, etc.

For these reasons, we generated transgenic mice, which spontaneously develop DC tumors, and derived stable cell lines with controlled low passage numbers [18–21]. These mice (called Mushi mice) are transgenic for the SV-40 large T oncogene under the CD11c promoter and generate spontaneous DC tumors. Cell lines derived from these tumors are surprisingly similar to freshly isolated DCs. They grow up to one-two million per mL with divisions every 1.5 days, require no additional growth factors, grow slightly adherent allowing the removal of dead cells before isolation, and share all tested functions with freshly isolated DCs such as protein and mRNA expression, activation-induced cytokine and chemokine secretion, co-stimulatory molecule expression (CD40, CD70, CD80, CD86), antigen (cross)-presentation, etc.

Mushi mice develop exclusively CD8$^+$ DC tumors spontaneously between 4 and 4.5 months of age. The reason for the preferential outgrowth of CD8$^+$ DC tumors is most likely due to the threefold higher expression of the large T oncogene in this subset compared to CD11b$^+$ DCs with the CD11c promoter fragment used to generate the transgenic mice [21]. Tumors first develop in spleens, but also infiltrate the liver, mesenteric lymph nodes, thymus, and bone marrow. From these tumors, DC lines (termed MuTu DC) can be derived. They have very similar functions to freshly isolated splenic

DCs with the advantage that they are not activated during the isolation procedure and keep being viable. In addition, the cell lines can be easily transduced with lentiviruses for overexpression or shRNA to knockdown gene expression [19, 20]. Knockout or transgenic DC lines can be obtained by crossing the Mushi mice with the corresponding knockout or transgenic mice (*see* Table 1). Here, we describe the derivation, culture, and lentiviral transduction of the MuTu DC lines. We recently were able to derive the first CD11b and pDC lines from BatF3 KO large T transgenic mice (*see* Table 1).

2 Materials

2.1 Derivation of $CD8^+$ DC Lines

1. Microcapillary tubes.
2. Critoseal Capillary Tube Sealant (Fisher Scientific).
3. Microhematocrit centrifuge with a hematocrit rotor.
4. Microhematocrit tube capillary reader (McCormick Scientific).
5. Scissors, forceps and scalpel.
6. Mesh or cell strainer and syringe plunger.
7. PBS.
8. Complete medium: IMDM-glutamax, 8–10% heat-inactivated fetal calf serum (FCS), 10 mM Hepes, 50 μM 2-mercaptoethanol (*see* **Note 1**), and, if required, 50 U/mL penicillin and 50 μg/mL streptomycin. Adjust with $NaHCO_3$ to 308 mOsm, if required (*see* **Note 2**). The medium is not supplemented with additional growth factors (*see* **Notes 3** and **4**). Prepare fresh medium and keep at 4 °C for a maximum of 2 weeks.
9. Culture vessels (*see* **Note 5**).
10. Humidified incubator set at 37 °C with 5% CO_2 (*see* **Note 6**).

2.2 Freezing

1. CryoTubes.
2. Containers for slow freezing filled with fresh ice-cold isopropanol. Precool at 4 °C for a minimum of 16 h, and replace the isopropanol after using the container five times.
3. Freezing medium: complete medium, 50% FCS, 10% DMSO. Keep at 4 °C.

2.3 Cell Culture

1. Complete medium. Pre-warm before use.
2. Cell dissociation buffer: PBS, 5 mM EDTA (non-enzymatic). Pre-warm at room temperature before use.
3. Culture vessels (*see* **Note 5**).

2.4 Lentiviral Transduction

1. 293T cells.
2. 293T culture medium: DMEM, 4.5% glucose, 10% FCS, 2 mM glutamine, penicillin/streptomycin. Prepare fresh medium every week.

Table 1
List of currently available MuTu DC lines

Cell line	DC type	Genotype (all but the Langerhans cell line are GFP$^+$ large T$^+$)	Strain background	Lentiviral transduction	References
MuTu1940	CD8	WT	C57BL/6	–	[19, 20]
MuTu BALB/c	CD8	WT	BALB/c	–	Not yet published
MuTu1 NOD	CD8	WT	NOD	–	Not yet published
MuTu IFNI R$^{-/-}$	CD8	Type I IFN receptor KO	C57BL/6	–	[20]
MuTu TLR3$^{-/-}$	CD8	TLR3 KO	C57BL/6	–	[20]
MuTu TLR9$^{-/-}$	CD8	TLR9 KO	C57BL/6	–	[20]
MuTu MAVS$^{-/-}$	CD8	MAVS/Cardiff KO	C57BL/6	–	Not yet published
MuTu NOX2$^{-/-}$	CD8	NADPH Oxidase 2 KO	C57BL/6	–	Not yet published
MuTu H-2 K$^{b-/-}$	CD8	H-2 K^b KO	C57BL/6	–	Not yet published
MuTu CD11b	CD11b	BatF3 KO	C57BL/6	–	Not yet published
MuTu pDC	pDC?	BatF3 KO	C57BL/6	–	Not yet published
MuTu LC	Langerhans cell?	Generated from Langerin promoter-large T transgenic mouse. Has human CD2 as reporter	C57BL/6	–	Not yet published
MuTu IFNβ, IFNI R$^{-/-}$	CD8	Interferon β secreting, type I IFNR KO	C57BL/6	+	[20]

MuTu IL-2	CD8	IL-2 producing	C57BL/6	+	Not yet published
MuTu IL-10	CD8	IL-10 producing	C57BL/6	+	Submitted
MuTu IL-12	CD8	IL-12 producing	C57BL/6	+	Not yet published
MuTu IL-15	CD8	IL-15 producing	C57BL/6	+	Not yet published
MuTu IL-35	CD8	IL-35 producing	C57BL/6	+	Submitted
MuTu TGFβ	CD8	TGFβ producing	C57BL/6	+	Submitted
MuTu Active TGFβ	CD8	Constitutively active TGFβ producing	C57BL/6	+	Submitted
MuTu Arginine	CD8	Arginase producing	C57BL/6	+	Not yet published
MuTu Indo	CD8	Indoleamine 2,3-dioxygenase producing	C57BL/6	+	Not yet published
MuTu CTLA-4	CD8	Surface CTLA-4 expressing	C57BL/6	+	Not yet published
MuTu PD2L	CD8	Surface PD2-L expressing	C57BL/6	+	Not yet published
MuTu luciferase	CD8	Luciferase expressing	C57BL/6	+	[18]

The MuTu1940 DC line is the best characterized, already distributed to many labs. From several KO mice, we have independently derived cell lines with similar phenotype and function. All the lentiviral-transduced cell lines are originally MuTu1940 cell lines at low passage numbers. They generally keep the expression of the transduced gene for at least ten passages, but it is recommended to test them from time to time. If they lose expression, we thaw another sample or lentivirally re-transduce a MuTu dendritic cell line

3. Plasmid psPAX2 (Addgene).
4. Plasmid pENV (Addgene).
5. Plasmid containing the lentivector of interest.
6. 0.5 M $CaCl_2$.
7. 2× HeBS (HEPES-buffered saline): dissolve 1.64 g NaCl, 1.19 g HEPES free acid, and 0.02 g Na_2HPO_4 in 80 mL of water. Adjust pH to 7.05 and filter sterilize. Keep at 4 °C.
8. 0.45 μm syringe filters (Millex-HV PVDF, Durapore).
9. 10 cm Petri dishes.
10. Puromycin.
11. Humidified incubator set at 37 °C with 5% CO_2.

3 Methods

3.1 Derivation of $CD8^+$ DC Lines from Tumor-Bearing Mushi Mice

1. When mice start looking pale, measure their hematocrit (*see* **Note 7**). Bleed the mice.
2. Collect the blood in a microcapillary.
3. Seal the microcapillaries with Critoseal Capillary Tube Sealant.
4. Spin the microcapillaries (15,000 × *g* for 3 min) in a microhematocrit centrifuge with a hematocrit rotor.
5. Determine the hematocrit by placing the tube in a microhematocrit tube capillary reader.
6. If the hematocrit is below 0.3 (*see* **Note 8**), sacrifice the mouse.
7. Remove the organs from which you want to isolate MuTu DC.
8. Dissociate the organs by pressing them through a fine mesh, or cell strainer, using a syringe plunger (*see* **Note 9**).
9. Wash the cells in PBS by centrifugating at 360 × *g* for 5–10 min.
10. Culture the cells in 6- or 24-well plates in at least ten replicates. Prepare twofold serial dilutions from 10^7 cells/mL to 10^6 cells/mL (*see* **Note 10**).
11. Replace the medium after overnight culture, discarding overgrown cultures. This step will remove many dead cells as the DC lines already start adhering.
12. Replace half the medium at least weekly until confluent adherent cultures are observed. This may take several weeks.
13. Once the medium starts changing color, split the cells very gently into culture vessels of the same size in two to three serial dilutions (*see* **Notes 11** and **12**).
14. Repeat **step 13** until the lower dilutions start growing (this may take several months) (*see* **Notes 13** and **14**).

15. Keep track of passage numbers (*see* **Note 15**).
16. Expand the culture by diluting the cells at 10^5 cells/mL in the largest possible volume. Go to **step 6** of Subheading 3.3 and/or.
17. Freeze many tubes of cells at early passage.

3.2 Freezing MuTu DC Lines

1. Place the freezing vials at 4 °C 1 h before use.
2. Dissociate the cells by incubating them for 10 min in cold cell dissociation buffer.
3. Centrifuge the cells at $360 \times g$ for 5 min.
4. Label the precooled freezing vials (*see* **Note 16**).
5. Gently dissociate the dry pellet.
6. Resuspend the cells at 3×10^6/mL in ice-cold freezing medium.
7. Transfer the cells to the vials (1 mL per vial). Close the tubes well.
8. Place the tubes quickly in the freezing container and place the container at −70 °C (*see* **Note 17**).
9. After a minimum of 2 days, transfer the tubes into liquid nitrogen for long-term storage.

3.3 Culture of MuTu DC Lines

1. Thaw the cells by warming up the tubes quickly until most ice crystals have melted and dilute dropwise with ice cold complete medium.
2. Centrifuge the cells at $360 \times g$ for 5 min.
3. Count the cells.
4. Seed the cells in culture vessels in twofold serial dilutions from 10^6 cells/mL to 10^5 cells/mL in complete medium.
5. Once the cells reach confluence ($1–2 \times 10^6$ cells/mL), you need to dilute them for expansion. Cells will be seeded into new culture vessels at 10^5/mL - 5×10^5/mL (*see* **Note 18**).
6. Remove the medium supernatant.
7. Dissociate the cells by adding pre-warmed PBS containing 5 mM EDTA. Just cover the culture vessel bottom.
8. Incubate for 5–10 min at room temperature.
9. Gently tap the culture vessels or pipette the cells up and down to detach them from the culture vessels.
10. Spin the cells at $360 \times g$ for 5 min.
11. Remove the supernatant.
12. Dissociate the dry pellet by gently tapping the tube.
13. Resuspend the dissociated pellet in the desired volume of complete medium.
14. Discard the culture when passage 40 is reached (*see* **Notes 19** and **20**).

3.4 Lentiviral Transduction

1. On day 1, seed 293T cells in a 10 cm Petri dish (two million cells in 8 mL of 293T culture medium per dish).
2. On day 2, refresh the medium with pre-warmed 293T culture medium. Allow transfection solutions to equilibrate at room temperature.
3. In a 15 mL Falcon tube, add to 250 µL of sterile distilled water 5 µg of psPAX2 plasmid, 15 µg of pENV plasmid, and 20 µg of the lentivector of interest.
4. Add 250 µL of 0.5 M $CaCl_2$. Mix well by vortexing.
5. Add the above 500 µL dropwise (one drop per second) to 50 µL of 2× HeBS by vortexing at full speed the 15 mL Falcon tube continuously.
6. Incubate for 20 min at room temperature, without the cap, under the hood.
7. Add the precipitate of DNA/calcium phosphate (total volume of 1 mL) to the 293T cells dropwise, slightly tilting the plates for mixing slowly.
8. Incubate the plates into the incubator for a maximum of 18 h.
9. On day 3, check on the microscope for the formation of a precipitate between the cells. Remove the 293T cells supernatant carefully (total volume of 9 mL) and refresh the medium with 9 mL of pre-warmed 293T culture medium.
10. Split the MuTu DC at 10^5 cells/mL in 6-well plates (25,000 cells/cm^2).
11. On day 4, filter the 293T cell supernatant containing viral particles using 0.45 µm filters.
12. The viral supernatants can be stored for a few days at 4 °C or frozen at −70 °C. However, viral titers may drop.
13. On day 5, remove the medium of the MuTu DC and replace it with lentivirus-containing medium (*see* **Note 21**).
14. 48 h after lentiviral infection, add puromycin to the MuTu DC medium (0.5 µg/mL).
15. Allow 3 days for efficient lentiviral transduction.

4 Notes

1. In our experience, the cells will not survive if 2-mercaptoethanol is omitted.
2. In some instances, when human osmolarity medium (290 mOsm) is used, cells may suffer. We then adjust osmolarity to 318 mOsm by adding 7.5 % sodium bicarbonate solution at 1/100 dilution to increase the osmolarity.

3. The MuTu DC lines require rich media. We recommend RPMI1640 or IMDM. The cells do not grow in DMEM.
4. The batch of fetal calf serum is important: about one in three serum batches quickly kill the cells in our experience. In general, survival of cells correlates with low LPS in the serum, but we do not know whether LPS or other contaminants are toxic for the lines. It is advised to test several batches for growth of the MuTu DC lines.
5. All types of culture vessels we tested work well: culture flasks of any size, Petri dishes, 96-, 48-, 24-, 6-well plates, etc. Non-tissue culture-coated plates or non-coated Petri dishes also work, but cells adhere less. However, as the cells grow adherent, roller bottles are not recommended.
6. Some groups grow DC in 10% CO_2. Check if your medium is at the right pH at this CO_2 concentration.
7. When MuTu tumors heavily infiltrate the bone marrow, hematopoiesis is disturbed and the mice become pale. We usually measure the hematocrit when they start becoming pale.
8. Usually, mice have a hematocrit between 0.4 and 0.5.
9. Alternatively, you can digest the organs with collagenase using the methods provided in Chapter 5 from this book.
10. In parallel to the primary culture, we often transfer tumor splenocytes into T cell-deficient Rag2 KO or CD3ε KO mice. They can also be transferred into CD8β T cell-depleted mice using 1 mg of H35-17.2 monoclonal antibody just before intravenous injection of one million tumor splenocytes [18]. Within a month, much more aggressive tumors grow in which DCs make up for more than 40% of splenocytes. This gives (1) a second chance to derive cell lines, and (2) it is generally easier to derive cell lines after adoptive transfer.
11. It can help to add 10% of conditioned medium from an established DC line.
12. If you plate multiple wells, ensure that you add the medium before distributing the cells and gently mix after distribution. Otherwise, the cells may adhere quickly at high local concentrations.
13. During this phase, fibroblasts may overgrow which will terminate the culture. To avoid this, we prepare many replicates and discard the ones in which fibroblasts overgrow.
14. It can take up to 6 months and up to ten passages to obtain easy growing functional DC lines.
15. It is important to keep track of the passage numbers to avoid selecting for lines that have lost some of their functions.
16. Use a pencil, not ink, to avoid removing the label if isopropanol spills over the tubes.

17. Keep the cells for as short as possible at 4°C before moving them to −70 °C.
18. Established cell lines are seeded above 10^5 cells/mL. Seeding at lower concentrations leads to a prolonged lag phase from which they usually slowly recover. If too diluted, the cells may die. Using 0.2 μm-filtered conditioned medium from parallel confluent DC line cultures helps to overcome this lag phase and even allows cloning. Let the cells grow to near confluence (not more than 1×10^6/mL), and split them before the medium is too consumed (slightly orange), otherwise they may lose functions quickly, as you will select for variants.
19. In general, established DC lines keep their functions for at least 40 passages if they are maintained properly. We do not let them overgrow and discard them if they do. They can remain functional much longer, but at late passages we often see decreased cytokine production upon stimulation and decreased cross-presentation capacity. It is worth testing the cells from time to time for cytokine production and cross-presentation, as these are the first functions they lose after prolonged culture.
20. We have observed that after adoptive transfer of aged cell lines with reduced functionality into Rag KO, CD3 KO, or CD8 T cell-depleted C57BL/6 mice (as they are rejected by a CD8 T cell response to the strong large T transgene [18]), they may regain their functions. Some of the cell lines increase their CD11b expression in culture. After such an adoptive transfer, they become $CD11b^{low}$ again.
21. Infection may be increased by adding 7 μg/mL Polybrene.

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8.1.2. CD11b regulates the Treg/Th17 balance in murine arthritis via IL-6

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FRONTLINE | Research Article

CD11b regulates the Treg/Th17 balance in murine arthritis via IL-6

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Th17 cells are often associated with autoimmunity and been shown to be increased in CD11b$^{-/-}$ mice. Here, we examined the role of CD11b in murine collagen-induced arthritis (CIA). C57BL/6 and CD11b$^{-/-}$ resistant mice were immunized with type II collagen. CD11b$^{-/-}$ mice developed arthritis with early onset, high incidence, and sustained severity compared with C57BL/6 mice. We observed a marked leukocyte infiltration, and histological examinations of the arthritic paws from CD11b$^{-/-}$ mice revealed that the cartilage was destroyed in association with strong lymphocytic infiltration. The CD11b deficiency led to enhanced Th17-cell differentiation. CD11b$^{-/-}$ dendritic cells (DCs) induced much stronger IL-6 production and hence Th17-cell differentiation than wild-type DCs. Treatment of CD11b$^{-/-}$ mice after establishment of the Treg/Th17 balance with an anti-IL-6 receptor mAb significantly suppressed the induction of Th17 cells and reduced arthritis severity. Finally, the severe phenotype of arthritis in CD11b$^{-/-}$ mice was rescued by adoptive transfer of CD11b^{+} DCs. Taken together, our results indicate that the resistance to CIA in C57BL/6 mice is regulated by CD11b via suppression of IL-6 production leading to reduced Th17-cell differentiation. Therefore, CD11b may represent a susceptibility factor for autoimmunity and could be a target for future therapy.

Keywords: Arthritis · Autoimmunity · CD11b integrin · Dendritic cells · Th17

See accompanying Commentary by Christoph Garbers and Stefan Rose-John

Additional supporting information may be found in the online version of this article at the publisher's web-site

Introduction

Collagen-induced arthritis (CIA) is the gold standard for rheumatoid arthritis (RA) model. It can be induced by immunizing susceptible mice with native type II collagen (CII) and complete Freund's adjuvant (CFA). Immunization is performed in the base of the tail [1]. The histopathological characteristics of this model are mononuclear cell infiltration of multiple joints, resulting in chronic inflammation, bone erosion, and progressive cartilage destruction. Susceptibility to CIA is defined by MHC genes. Indeed, only H-2^{q} or H-2^{r} MHC haplotypes make mice susceptible to the disease, therefore the C57BL/6 strain of mice (H-2^{b}) is resistant [2, 3].

Proinflammatory cytokines such as TNF-α, IL-1, and IL-6 mediate inflammation in CIA, although antibodies specific for

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CII are needed for complete development of the disease [4]. However, there is now growing evidence implicating the newly characterized IL-17-producing T-cell population (Th17) in the pathogenesis of CIA [5]. It has been shown that the inhibition or overexpression of IL-17 in the joints suppresses or worsens joint inflammation and damage, respectively [6]. Moreover, Hirota et al. have demonstrated that the induction of T-cell self-reactivity in SKG mice that causes autoimmune arthritis depends on the generation of Th17 cells [7]. SKG mice have a spontaneous mutation of the gene encoding an SH2 domain of ZAP-70.

The CD11b integrin (CD11b/CD18, Mac-1, or $\alpha_M\beta_2$) is a member of the beta 2 integrin family of adhesion receptors. Several studies have demonstrated that absence of CD11b/CD18 is implicated in the development of several inflammatory diseases [8, 9]. The mechanisms by which this integrin controls these diseases are not well known. Although, CD11b integrin is classically associated with inflammatory responses [10, 11], recent reports have revealed that this integrin also plays significant immunoregulatory roles [12–14]. Indeed, in a spontaneous model of systemic lupus erythematosus, CD11b deficient mice exhibit severe glomerulonephritis that associates with increased neutrophil infiltration in the kidneys [15]. Similarly, CD11b deficiency enhances inflammation in a murine model of tubulointerstitial nephritis [16].

The mechanisms by which CD11b controls these opposite effects are not well known. In order to explore the role of CD11b in CIA pathogenesis, it is needed to consider, on the one hand, its function in leukocyte recruitment and on the other hand, its capacity to promote immune unresponsiveness.

In our previous study of peripheral tolerance, we have reported that CD11b deficiency leads to increased production of IL-17 that interferes with the establishment of the oral tolerance [17]. In the present study, we sought to further dissect the impact of increased Th17 differentiation in CD11b deficient mice on arthritis development using CIA as a model of rheumatoid arthritis.

We found that genetic inactivation of CD11b confers the disease-resistant C57BL/6 mouse strain susceptibility to arthritis. The development of autoimmune arthritis in CD11b deficient mice is highly dependent on Th17 cells secreting IL-17, a proinflammatory cytokine capable of recruiting and activating neutrophils and other inflammatory cells. In addition, we showed that the development of arthritis in CD11b-deficient mice can be inhibited by adoptive transfer of CD11b-expressing dendritic cells (DCs). Most importantly, we demonstrated that neutralization of IL-6 signaling activity using an IL-6R-specific blocking antibody can rescue CD11b-deficient mice from disease development. Moreover, this mAb treatment was effective also after induction of the Th17-cell response 21 days after the primary immunization. Thus, this study suggests that CD11b integrin on DCs is required to suppress the emergence of autoimmune diseases. It also suggests that the activity of CD11b/CD18 could determine sensitivity and disease severity in the development of rheumatoid arthritis.

Results

CIA of high severity and incidence in CD11b$^{-/-}$ mice

To evaluate the requirement of CD11b integrin for the development of CIA, the standard protocol for CIA induction was applied in C57BL/6 background. CD11b$^{-/-}$ and control WT mice were immunized at the base of the tail on day 0 with CII/CFA and then boosted with CII/IFA on day 21. The onset and progression of arthritis was assessed using a clinical score, based on redness, swelling of joints, and the footpad thickness. CD11b$^{-/-}$ mice developed severe joint inflammation evidenced by marked swelling and erythema of the hind paws and forepaws (Fig. 1A). Mice were assessed for 50 days after the second immunization. Arthritis scores were only observed in CD11b$^{-/-}$ mice with a peak of disease from day 28 to day 50 (Fig. 1B). Sixty five percent of CD11b$^{-/-}$ mice developed arthritis with a mean onset on Day 25 after initial immunization (Fig. 1C). Interestingly, around 20% of the CD11b$^{-/-}$ mice had developed arthritis before the second immunization of CII-CFA.

In addition, histological analysis was used to confirm the clinical assessments. H&E and S/FG staining revealed that immunized WT mice had no significant inflammatory cell infiltration, nor cartilage and bone damage resulting in healthy joints (Fig. 1D). In contrast, the arthritis lesions in the CD11b$^{-/-}$ mice exhibited typical histological features as previously described in arthritic mice, namely, synovitis, cartilage destruction, architectural changes of joint, and a massive lymphocytic infiltration (Fig. 1E).

Collagen II specific antibodies are key factors in the pathogenesis of CIA [18]. To better characterize the humoral immune mechanisms underlying CIA development by CD11b$^{-/-}$ mice, we measured anti-collagen antibody titers of total IgG, IgG1, and IgG2a in the serum of CD11b$^{-/-}$ and wild-type (WT) immunized mice on Day 40. At this time point, we found that CD11b$^{-/-}$ mice exhibited significantly higher levels of collagen-specific IgG1 Abs in comparison to WT mice indicating a predominant Th2 immune response. On the other hand, the levels of anti-collagen IgG2a and total IgG Abs were not different in both groups of mice (Fig. 1F).

Taken together, these results indicate that CD11b has a role in controlling arthritis severity in C57BL/6 mice.

Increased frequency of Th17 cells and reduced Treg cells in CD11b$^{-/-}$ mice with CIA

We characterized the adaptive immune system at the steady state and no significant difference between CD11b$^{-/-}$ and WT mice could be observed (Supporting Information Fig. 1). However, when we studied T helper cell polarization twenty days after the second CII immunization, in vitro, PMA/ionomycin treatment of inguinal lymphocytes for 6h revealed significantly higher percentages of IL-17-producing CD4$^+$ T cells in immunized CD11b$^{-/-}$ mice compared to WT mice (Fig. 2A). We also examined IL-17 production in the T-cell culture and found that the amount of IL-17 in the culture supernatants from PMA/ionomycin stimulated

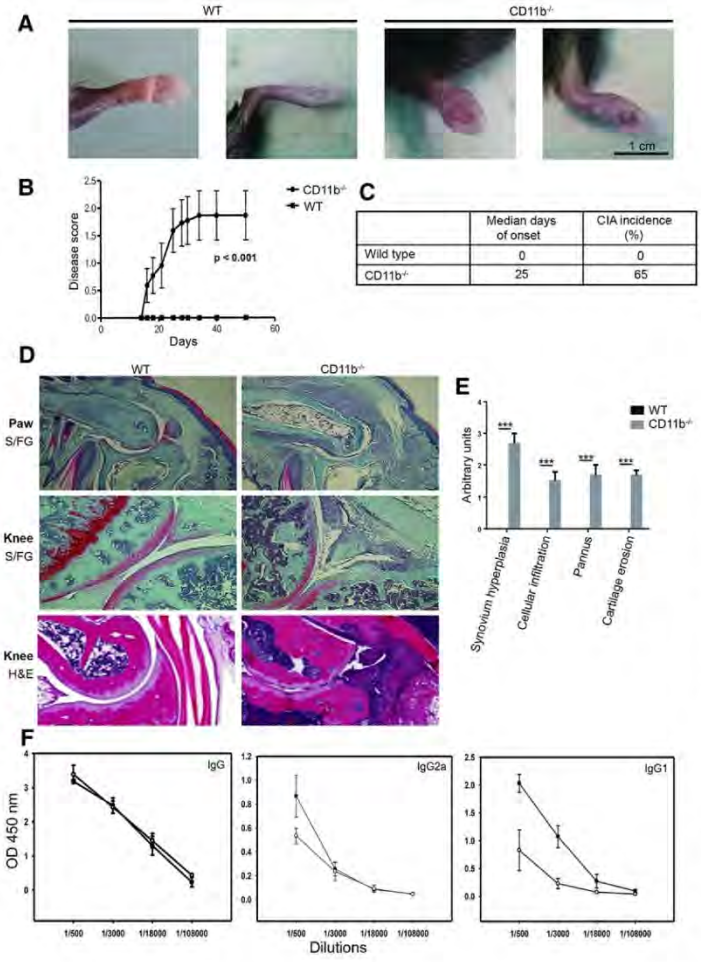

Figure 1. CIA incidence and severity are increased in CD11b$^{-/-}$ mice. Mice immunized with CII on day 0 and 21 were monitored two times per week for the development of arthritis. (A) Representative photographs from hind limb and paws on day 30 showing severe arthritis in CD11b$^{-/-}$ mice. Images are representative of five independent experiments. (B and C) Clinical scores and incidence of arthritis in WT and CD11b$^{-/-}$ mice. Results shown are pooled from three independent experiments ($n = 20$ mice/group). (D) Histological examination of the joints from WT and CD11b$^{-/-}$ mice on day 40. Paraffin-embedded hind paw and knee sections were stained with H&E or S/FG. Original magnification ×100 for H&E and S/FG. Images are representative of three independent experiments. (E) Quantification of synovium hyperplasia, pannus, cellular infiltration and cartilage erosion in the joints of CIA mice. Data are shown as mean ± SEM ($n = 8$ mice/group) and are representative of three independent experiments. (F) CD11b$^{-/-}$ mice had higher anti-collagen, IgG1 levels than WT mice. Total IgG as well as Serum titers of anti-mouse CII IgG1 and IgG2a were measured at day 40. Data are means ± SEM. Results shown are pooled from two separate experiments ($n = 8$ mice/group). $^{*}p<0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; Mann–Whitney U tests.

inguinal lymph node cells of immunized CD11b$^{-/-}$ mice was significantly higher than that of WT mice (15-fold) (Fig. 2B). To further examine the importance of IL-17 production in CIA induction, we determined whether IL-17 was produced in the joint cell lysates. We found higher levels of IL-17 in the joints cell lysates of CD11b$^{-/-}$ mice compared to those of WT mice (Fig. 2C). According to these results, CII immunization in CD11b$^{-/-}$ mice tends to polarize CD4^{+} T cells toward a Th17 response. This supports the concept that CIA is a Th17-mediated disease.

IL-10-producing B cells can modulate autoimmune diseases by affecting the frequency and equilibrium of both Treg and Th17 cells [19]. In order to examine the role of IL-10 in our model, we stimulated splenocytes from diseased mice for 6 h with LPS and analyzed IL-10 production by flow cytometry. We observed that CD11b$^{-/-}$ mice had a significant reduction of IL-10-producing B cells compared with the WT mice, suggesting that B cells with regulatory activity were also reduced in CD11b$^{-/-}$ mice (Fig. 2D).

We next studied FoxP3^{+} Treg cell development in CIA sick mice. We found that immunized CD11b$^{-/-}$ mice developed fewer numbers of FoxP3^{+} Treg cells in their draining lymph nodes than their equivalent in WT mice (Fig. 2E).

Reduced CIA severity in CD11b$^{-/-}$ mice after blocking IL-6 activity

IL-6 is a potent inducer of Th17 differentiation and probably has an important role in the pathogenesis of RA [20, 21]. To explore the role of IL-6 in CIA, serum IL-6 levels were measured and correlated with disease severity. We found that sick CD11b$^{-/-}$ mice had significantly higher serum IL-6 levels than those of WT controls (Fig. 3A). Supernatant from magnetically enriched DCs or freshly isolated peritoneal macrophages indicates that both types of cells could be responsible for this increase (Supporting Information Fig. 2B and C). We next tested the action of a blocking antibody against

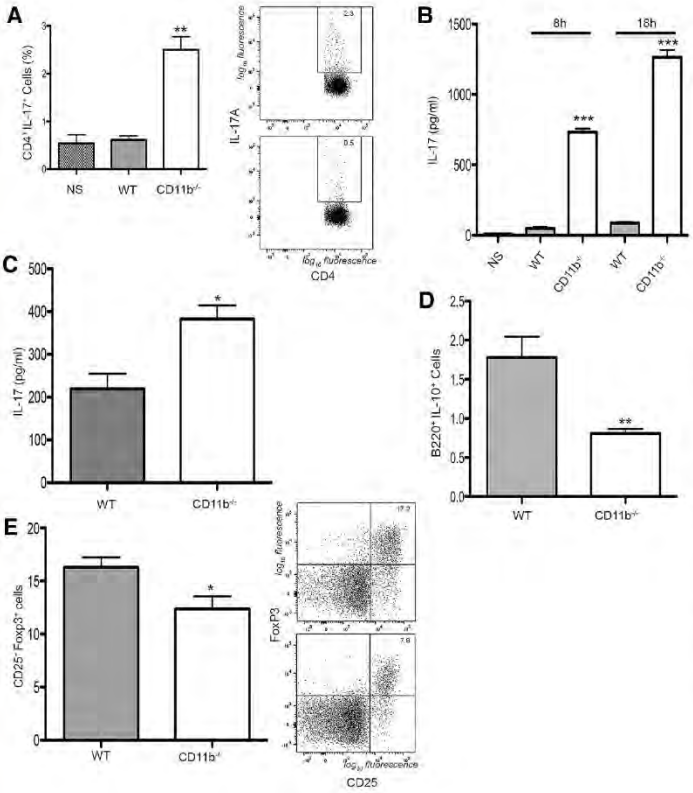

Figure 2. Increased frequency of Th17 cells and reduced Treg cells in CD11b$^{-/-}$ mice with CIA
(A) IL-17 production in CD4 T cell populations of ILN from immunized WT and CD11b$^{-/-}$ mice on day 20 after second immunization. IL-17 production was determined in inguinal lymph node single cell suspensions by intracellular staining after ex vivo stimulation with PMA/ionomycin in the presence of brefeldin A for 6 h. (B) IL-17 in culture supernatants of PMA/ionmycin stimulated inguinal lymph node cells. (C) Assessment by ELISA of IL-17 levels in the joint cell lysates of CIA mice. (D) IL-10 production in B220$^+$ cells in inguinal lymph node from immunized WT and CD11b$^{-/-}$ mice on day 30 after immunization. IL-10 production was examined by intracellular staining after *ex vivo* stimulation with LPS in the presence of brefeldin A. (E) Percentages of CD4$^+$CD25$^+$ Foxp3$^+$ T cells in inguinal lymph nodes from collagen-immunized WT and CD11b$^{-/-}$ mice on day 30 after immunization. (A–E) Data are shown as mean ± SEM (n = 6 mice/group) and are pooled from two independent experiments. $^*p<0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; Mann–Whitney U tests.

IL-6R, an anti-IL-6R mAb (15A7), in vitro, on Th17 differentiation. As shown in Fig. 3B, the addition of IL-6R neutralizing mAbs to the co-culture of isolated DCs from both WT and CD11b$^{-/-}$ mice and isolated WT PMA/ionomycin activated CD4$^+$ T cells potently inhibited Th17 differentiation in the presence of TGFβ.

To evaluate the action of anti-IL-6R mAb (15A7) on disease severity in vivo, both 15A7 and IgG isotype-matched control antibodies were administrated intraperitoneally into CD11b$^{-/-}$ mice at weekly intervals starting from the time of the second immunization. The 15A7 treatment showed a significant decrease of disease severity in comparison to IgG isotype-matched control IgG (Fig. 3C). Twenty days after the second CII immunization, inguinal CD4$^+$ T cells were analyzed by flow cytometry in order to assess cytokines patterns. As shown in Fig. 3D, a significant reduction in Th17 cells was observed in mice treated with 15A7 compared to isotype-matched control IgG treated mice. Intriguingly, the percentage of Treg cells became comparable to WT mice after 15A7 treatment (Fig. 3E).

Adoptive transfer of CD11b$^+$ DCs reduces the severity of arthritis in CD11b$^{-/-}$ mice

Previous studies demonstrated the potential of CD11b$^+$ DCs to ameliorate CIA [22–26]. We therefore, examined the potential of a newly produced CD11b$^+$ DC line to exert an immune regulatory effect in vivo. The advantage of using clonal CD11b$^+$ DC lines is to have pure cells, thus excluding the potential effect of contaminating cells. As reported in Fig. 4A, CD11b$^{-/-}$ mice given CD11b$^+$ DCs showed significantly reduced disease severity compared with the untreated mice. It has been reported that the ligation of CD11b on DCs inhibits IL-6 production induced by TLR stimuli [27]. We therefore cross-linked CD11b on the DC line using M1/70 mAb and found that CD11b ligation suppressed CpG-induced IL-6 production (Fig. 4B).

Discussion

We show here that genetic deletion of the CD11b integrin confers H-2^b mice with susceptibility to collagen-induced arthritis. Despite the standard protocol for CIA induction, no WT mice developed arthritis. Even increasing mycobacterial load and collagen concentrations did not result in arthritis in C57BL/6 mice. This confirms that H-2^b mice are resistant to the disease as previously described in various articles [3]. In addition, we showed that the protective Treg/Th17 balance is inverted in CD11b$^{-/-}$ mice after CII immunization. Most importantly, we demonstrated a major protective function of an anti-IL-6R mAb on CIA development in CD11b deficient mice when applied late after immunization (day 20).

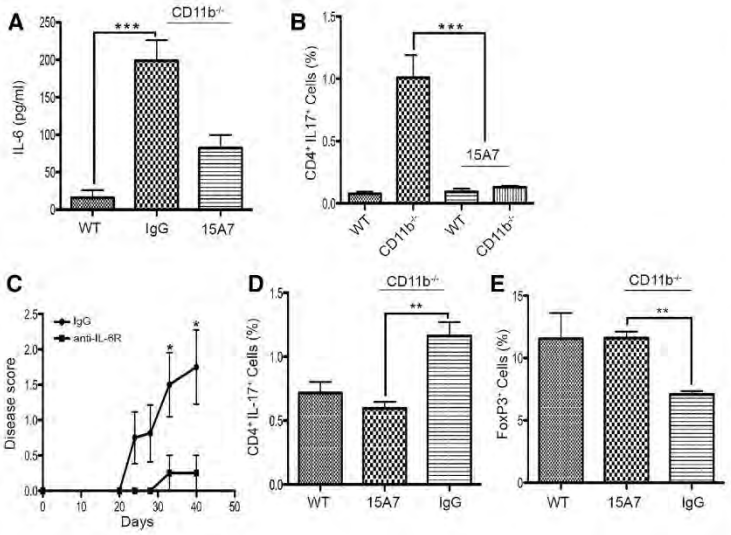

Figure 3. Effect of anti-IL-6R treatment on arthritis and Th17 differentiation. (A) Levels of IL-6 in serum samples from CIA mice were quantified by ELISA. Data are means ± SEM (n = 5 mice/group) and are representative of two independent experiments. (B and C) DCs isolated from untreated WT and CD11b$^{-/-}$ mice were cultured with CD4$^+$ T cells, stimulated with anti-CD3 and TGF-β in the presence or absence of anti-IL-6R mAb 15A7. After 3 days, CD4$^+$ T cells were stained for IL-17-producing Th17 (B) and Foxp3$^+$ Treg cells (C). (B and C) The data are expressed as means ± SEM (n = 8–9) and are representative of three independent experiments. (D) Clinical scores of arthritis in WT and Cd11b$^{-/-}$ mice treated with anti-IL-6R or isotype-matched control mAbs. (E) Intracellular staining of IL-17 and FoxP3 in CD4$^+$ T cells isolated from inguinal lymph nodes of mice treated with anti-IL-6R or isotype-matched control mAbs on day 21, analyzed on day 40. (D and E) Data are shown as mean ± SEM (n = 9 mice/group) and are pooled from two independent experiments. $^*p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$; Mann–Whitney U tests.

Our findings correlate with the key role of IL-17 CIA physiopathology reported by previous research [6, 28]. Murphy et al also showed that a C57BL/6 resistant mice with a IL-12-deficiency become susceptible to CIA and hold higher amounts of IL-17–producing T cells [29].

IL-6 is a pleiotropic cytokine. Its signaling pathway can be triggered by two forms of the same receptor: membrane IL-6 receptor (IL-6R) on target cells or soluble IL-6R in the serum. After interacting with IL-6, soluble IL-6R can form a complex together with membrane gp130. This pathway in opposition to the classical pathway, is called IL-6, trans-signaling [30]. The decreased IL-6 levels upon anti-IL-6R treatment could be explained by the positive feedback loop between IL-6 and IL-17 [31]. Indeed, IL-6 induces IL-17, which will in turn induce IL-6 production. In our case, as IL-6 signaling is inhibited by the receptor-neutralizing antibody, the loop is interrupted and IL-6 levels are decreased.

We did not observe differences in soluble IL-6 receptor quantities in WT or CD11b$^{-/-}$ mice but observed a parallel increase in immunized WT and CD11b$^{-/-}$ mice.

In addition to its many roles, IL-6 is involved in the effector phase of autoimmune arthritis Clinical trials has shown that blocking IL-6 pathway with an anti–IL-6R antibody (tocilizumab) treatment is very efficient in established RA in human patients [32, 33]. Nevertheless, it remains unclear how the production of IL-6 is controlled at molecular and cellular levels.

Besides its key role in cell migration and adhesion, increasing evidence supports the notion that CD11b is also involved in the suppression of proinflammatory cytokines and in tolerance maintenance. Behrens et al. have shown that following CD11b ligation, DCs are less immunogenic [27]. In addition, it has been reported that CD11b deficiency resulted in increased inflammatory cytokine production by DCs both in vitro and in vivo upon TLR activation. Han et al. have demonstrated that after TLR stimulation, CD11b is activated and promotes MyD88 and TRIF degradation through recruitment of Cbl-b and syk in macrophages. This results in inhibition of TLR-mediated responses [13]. A similar pathway could possibly be found in DCs.

When mice were treated on day 21 after collagen immunization with anti-IL-6R mAb treatment, we observed that CD11b$^{-/-}$ mice developed less severe arthritis than the control mice. Anti-IL-6R treatment inhibited the induction of Th17 cells confirming the already described role of the IL-6 cytokine in regulating Th17 development [20]. Interestingly, anti-IL6R mAb treatment at a time point where the Treg/Th17 balance is already established was able to prevent disease development. In this context, TGF-β, a key factor for Treg cell differentiation and of high relevance for tolerance induction, could be another important molecule implicated in our model. However, no significant difference was observed in the serum of our mice, although a slight decrease of seric TGFβ in immunized CD11b$^{-/-}$ mice was noted (Supporting Information Fig. 2A). Further studies should be performed to investigate its role.

In the present report, we demonstrated that arthritic CD11b$^{-/-}$ mice had significantly higher levels of seric collagen-specific IgG1 than WT mice. Whereas development of CIA can be caused by collagen-specific IgG2a [18], others suggested that other isotypes might be of importance in the induction of arthritis. In support to our findings, Maccioni et al showed that IgG1 was the dominant isotype in the K/BxN mouse model [34]. In accordance with the role of Th17 induced IgG1 class switch, Hickman-Brecks et al. have reported that they are able to induce IgG1 dominant responses in the Th17 polarized KRN7T-bet$^{-/-}$ adoptive transfer model of arthritis [35].

Previous studies have shown an effecient treatment of established murine CIA by adoptive transfer of in vitro modified DCs

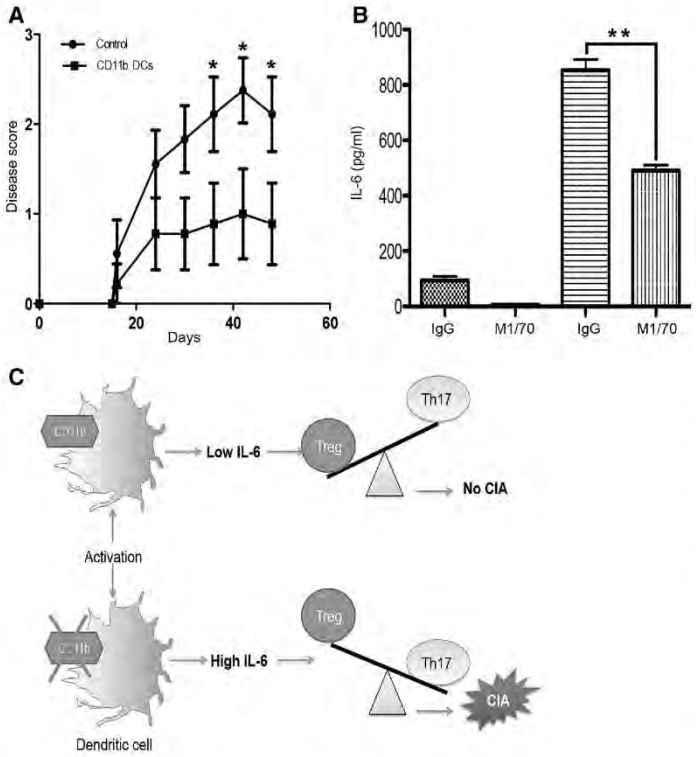

Figure 4. Adoptive transfer of CD11b$^+$ DCs ameliorates arthritis severity in CD11b$^{-/-}$ mice. (A) Clinical scores in CIA mice after CD11b$^+$ DC transfer. CD11b$^+$ DCs were used for adoptive transfer into CD11b$^{-/-}$ mice 1 day before the second immunization. The severity of arthritis was compared between control and DC transferred CD11b$^{-/-}$ mice. Data shown are means ± SEM (n = 16 mice/group) and are pooled from two separate experiments. (B) Crosslinking of CD11b on DCs with M1/70 in vitro decreases levels of the IL-6 cytokine. Twenty minutes after ligation, CD11b DCs were stimulated with CpG. IL-6 was quantitated by ELISA after overnight culture. Data are mean ± SEM and are representative of three independent experiments. There were three biological replicates per experiment. (A and B) $^*p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$; Mann–Whitney U tests. (C) Model showing the mechanisms whereby CD11b controls CIA by suppressing IL-6 production. Deficiency of CD11b causes an increase in IL-6 production by DCs leading to an altered balance in Treg/Th17 differentiation, which causes arthritis.

[23, 25, 26]. In agreement with these observations, our results demonstrated that our newly developed CD11b$^+$ DC line is able to inhibit the development of arthritis. However, further studies are required to better understand how these CD11b DCs induce their immunosuppressive function.

Altogether, the results point toward a role of CD11b to prevent autoimmunity. As previously mentioned, CD11b alteration or deficiency has been shown to promote SLE and other inflammatory diseases [8, 36]. Autoimmune diseases represent an important health issue in Western countries and optimal treatments remain difficult to find. It is crucial to find new targets for therapies. CD11b seems to be a promising target for treatment. Adoptive transfer of tolerogenic CD11b$^+$ cells could be considered as it is performed with Treg cells [37]. Gene therapy with CD11b induction is another possible alternative.

To conclude, this work identified a new connection between CD11b, IL-6, and arthritis. The results extend the crucial role of CD11b in the regulation of the immune system from previous studies. The finding that resistant H-2^b mice become susceptible to CIA after CD11b loss opens the possibility for a parallel role in human autoimmune susceptible patients. Therefore, therapeutic strategies targeting CD11b and its anti-inflammatory functions would potentially be novel therapies to treat patients suffering from autoimmune diseases.

Materials and methods

Mice

WT (WT) and CD11b$^{-/-}$ mouse strains were in the C57BL/6Jbackground, used at age of 8–13 weeks and sex-matched. WT mice were purchased from Harlan. CD11b$^{-/-}$ micewere provided by Prof. Britta Engelhardt (Theodor Kocher Institute, University of Bern). All in vivo experimental protocols were approved by the Swiss Federal and Cantonal Veterinary authorities.

Antibodies and reagents

APC–anti–mouse IL-17 (cloneTC11-18H10.1) was purchased from BioLegend. PerCP–anti-CD4 (cloneL3T4) was obtained from BD Biosciences. PE–anti–mouse IL-10 (cloneJES5-16E3) and PE-Foxp3 (clone FJK-16s) were purchased from eBioscience. M. tuberculosis (H37Ra) was purchased from Difco Laboratories. Chicken collagen II (CII) and CFA were obtained from sigma. Horseradish peroxidase-conjugated goat anti-mouse IgG or isotype-specific anti-IgG1 or anti-IgG2a were obtained from Invitrogen. An optimal dose (1 mg/mouse) of IL-6R mAb (clone 15A7, Bio-X-Cell) or isotype-matched control IgG was injected

intraperitoneally on days 21, 28, and 35 after induction of CIA.

Collagen-induced arthritis

Native chicken type II collagen (Sigma) was dissolved in 10 mM acetic acid overnight at 2 mg/mL. 100 μg collagen emulsified in Complete Freund's Adjuvant (CFA, Sigma) containing 5 mg/mL mycobacterium tuberculosis was injected intradermally at the base of tail. Twenty one days after the first injection a booster injection of 100 μg of native chicken collagen was done in Incomplete Freund's Adjuvant intradermally (IFA) at the base of the tail. All immunization reagents were purchased from DIFCO (Basel, Switzerland).

Clinical and histological assessment of arthritis

The mice were assessed two times per week for redness and swelling of limbs and a clinical score from 1 to 4 ((i) slight edema and erythema limited to ankle; (ii) slight edema and erythema from the ankle to the tarsal bone; (iii) moderate edema and erythema the ankle to the tarsal bone; (iv): edema and erythema from the ankle to the entire leg) was given for each limb for up to 50 days. An overall mean score was calculated by dividing the sum of the four scores per mouse by four (average severity per paw). At the end of the experiment, the paws of the mice were taken, fixed, decalcified, and embedded with paraffin. Joint sections (5 μm) were stained with Safranin-O-fast green (S/FG) or hematoxylin and eosin (H&E) and evaluated for histological alterations including synovial hyperplasia, pannus formation, cellular infiltration and cartilage erosion. Histological sections were graded independently by two observers unaware of mouse genotype using an established scoring system (from 0: no histological sign to 3: most severe).

ELISA for detection of antibodies specific to collagen II and cytokines

Peripheral blood was isolated frommice by cardiac puncture. Collagen-specific Abs were captured from serum using 10 μg/mL CII coated wells and were detected with horseradish peroxidase-conjugated goat anti-mousewhole IgG (Sigma) or isotype-specific IgG1, clone 2H12B4 and IgG2a, clone 1F10C2 (Chondrex) at serial dilutions. The plates were developed using TMB as substrate. The color development was monitored by measurement at 450 nm in a microplate reader (model 450, Bio-Rad, Richmond, CA). For IL-17 detection in knee joints, they were homogenized on ice with a tissue grinder. The lysate was centrifuged twice at 4000 × *g* for 5 min at 4°C and the supernatant were assessed for the detection of IL-17 using specific ELISA kit (eBioscience).

Intracellular cytokine staining

Intracellular staining was performed on single cell suspensions from the draining LNs and restimulated with PMA, ionomycinand brefeldinin complete media for 5 h at 37°C. The splenocytes were stained with different combinations of cell surface antibodies. After intracellular IL-17 and Foxp3 staining, cells were analyzed in a LSRII flow cytometer (BD) and data examined using FlowJo software.

Splenic DC subset isolation and culture

Spleens were digested for 20 min at 37°C with collagenase D (1 mg/mL) and DNAse I (40 μg/mL) in RPMI-1640 complemented with 2.5% FCS for optimal DC isolation. $CD11c^+$ DCs were isolated using anti-CD11c antibody-coupled magnetic micro-beads (Miltenyi Biotech). Purified $CD4^+$ T cells were co-cultured with DCs and anti-CD3 Abs (145.2C.11, homemade) in the presence or absence of anti-IL-6R antibody or control IgG (20 μg/mL). After 4 days, $CD4^+$ T cells expressing intracellular IL-17 and Foxp3 were quantitated by flow cytometry.

DC and macrophage isolation for cytokine analysis

DCs were isolated as described above and cultured for 15 h with presence or absence of LPS (200 ng/mL) or CpG (1 μmol/L). Macrophages were obtained by lavage of the peritoneal cavity with ice cold PBS. Cells were plated in RPMI-1640 complemented with 10% FCS. After 2-h incubation at 37°C, non-adherent cells were removed by PBS wash and medium replacement. Supernatant was collected and used for ELISA and LEGENDplex™ detection of IL-6, TNFα, IL-1, soluble IL-6R.

PAI-1/luciferase assay for TGF-β

Transfected mink lung epithelial cells (TMLCs) were cultured in DMEM supplemented with 10% FCS, penicillin, streptomycine, L-glutamine and G418 (200 μg/mL). TMLC were plated at 1.6×10^4 cells per well in a 96-well plate. 100 μL of mouse serum was directly added to adherent cells after removing culture medium, and incubated overnight at 37°C 5% CO2. After incubation, 100 μL Steady-Glo reagent (Steady-Glo assay system, Promega) was added. Luminescence was measured at a luminescence reader after 10 min.

Treatments in vitro

Stimulation of CD11b-expressing DCs was performed using CpG (2 mM, TriLink) for 18 h. Cross-linking CD11b antibody (clone M1/70, homemade) was used at 10 μg/mL. Supernatants were collected and then IL-6 levels were measured by ELISA.

Adoptive transfer of CD11b$^+$ DCs

We recently generated a new fully functional CD11b DC line on the C57BL/6 background (article in preparation). CD11b$^+$ DCs (2×10^6 cells per mouse) were injected intraperitoneally (I.P.) 20 days after the first immunization. To avoid rejection of CD11b$^+$ DCs via an anti-large T-cell response [38], the CD8β$^+$ T cells were depleted with two injections of 250 μg of anti-CD8β (clone H35) antibody i.p. (one 1 day before the DC transfer and the other 3 days later).

Statistical analysis

Data represent mean ± SEM. Statistical analyses and calculation of p values were performed using Prism5 (GraphPad Software). Statistical analyses were achieved through the nonparametric Mann–Whitney test. Significant differences are considered for $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$.

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Conflict of interest: The authors declare no financial or commercial conflict of interest.

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Abbreviations: **WT**: Wild-type · **CIA**: Collagen-Induced Arthritis · **S/FG**: Safranin-O-fast green · **H&E**: Hematoxylin · **CFA**: Complete Freund's Adjuvant · **IFA**: Incomplete Freund's Adjuvant intradermally · **CII**: Native chicken type II collagen · **RA**: Rheumatoid Arthritis · **Th17**: IL-17-producing T cellpopulation · **Treg**: Regulatory T cells

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8.2. Supplementary materials and methods

8.2.1. Genome-scale CRISPR Knock-Out (GeCKO) v2.0 pooled libraries

Related references: [384,395]

GeCKO Genome-scale CRISPR Knock-Out | LENTIVIRAL CRISPR TOOLBOX

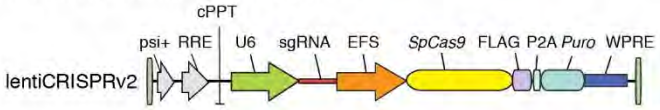
MIT ZhangLab

Genome-scale CRISPR Knock-Out (GeCKO) v2.0 pooled libraries

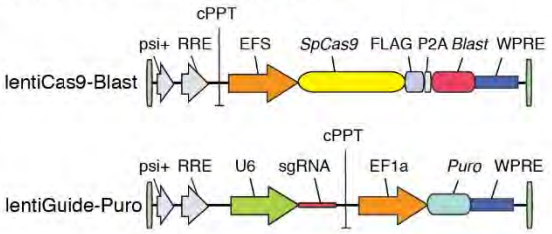
CRISPR (**C**lustered **R**egularly **I**nterspaced **S**hort **P**alindromic **R**epeats) is a microbial nuclease system containing a combination of CRISPR-associated (Cas) genes as well as non-coding RNA elements capable of programming the specificity of the CRISPR-mediated nucleic acid cleavage. Using lentivirus, we delivered the type II CRISPR nuclease system to facilitate genome editing in mammalian cells in a pooled library targeting early consecutive exons (Shalem*, Sanjana*, *et al.*, Science 2014). **Here we describe how to amplify GeCKO v2.0 DNA plasmids to have sufficient quantity to produce lentivirus, while maintaining full library representation**.

Library description: The GeCKO v2 libraries consist of specific sgRNA sequences for gene knock-out in either the human or mouse genome. Each species-specific library is delivered as two half-libraries (A and B). When used together, the A and B libraries contain 6 sgRNAs per gene (3 sgRNAs in each library). We recommend screening the entire library (A and B) when possible but if adequate representation cannot be obtained with the entire library, screening can be performed with one half-library. Since more cells are needed in the screen as number of constructs in the library increases, this design has the flexibility of screening with just the A library (3 sgRNAs per gene) at a smaller scale or screening the full library (6 sgRNAs per gene). Both A and B libraries contain 1000 control sgRNAs designed not to target in the genome. The A library also targets miRNAs (4 sgRNAs per miRNA). Each library is available in a 1 vector (lentiCRISPRv2) or 2 vector (lentiCas9-Blast and lentiGuide-Puro) format. The 2 vector format with the library in lentiGuide-Puro has the advantage of higher titer for the library virus but requires cells to already contain Cas9 (usually genomically integrated using lentiCas9-Blast). See p. 3 for full library specs.

One vector lentiviral GeCKO system



Two vector lentiviral GeCKO system



Lentiviral production: Since these vectors enable lentiviral delivery of both Cas9 and sgRNA for targeted gene knock out, it is important to perform these experiments in a lab with the appropriate biosafety level and controls, which can vary between different institutions. Before starting any lentiviral work, please ensure compliance with your Environmental Health and Safety office and government / organization / university. Briefly, to make lentivirus, lentiCRISPR (with sgRNA cloned) must be co-transfected into HEK293(F)T cells with the packaging plasmids pVSVg (AddGene 8454) and psPAX2 (AddGene 12260). As a positive control for viral production, we use a CMV-EGFP lentiviral transfer plasmid (eg. AddGene 19319).

Representation: Each GeCKO library is a pool of many different vectors mixed together. The libraries contain 122,417 (human) or 130,209 (mouse) unique sgRNAs. To ensure no loss of representation, it is important to amplify the library using the protocol given on the next page. **The GeCKO library should not be transformed using chemically competent cells or amplified in liquid cultures. Please read over the entire protocol before starting library amplification.**

Citation: Please reference the following publications for the use of this material.

Improved lentiviral vectors and genome-wide libraries for genetic screening in human and mouse cells using the CRISPR nuclease Cas9. Sanjana NE*, Shalem O*, Zhang F *submitted*.

Genome-scale CRISPR-Cas9 knockout screening in human cells. Shalem O*, Sanjana NE*, Hartenian E, Shi X, Scott DA, Mikkelsen T, Heckl D, Ebert BL, Root DE, Doench JG, Zhang F (2014). *Science*, 343, 83-7. DOI: 10.1126/science.1247005

Online resources: Please see *http://www.genome-engineering.org/gecko/* for GeCKO library sequences, vector maps, protocols, discussion forums and technology updates.

GeCKO v2.0 library specifications

	GeCKO v2 human library	GeCKO v2 mouse library
Species	human	mouse
Number of genes targeted	19,052	20,661
Targeting constructs per gene	6 per gene (3 in Library A, 3 in Library B)	6 per gene (3 in Library A, 3 in Library B)
Number of miRNA targeted	1864	1175
Targeting constructs per miRNA	4 per miRNA	4 per miRNA
Control (non-targeting) sgRNAs	1000	1000
Total sgRNA constructs	122,417 (65,386 in Library A, 58,031 in Library B)	130,209 (67,405 in Library A, 62,804 in Library B)
Viral plasmid vector	Single and dual vector: lentiCRISPR v2 and lentiGuide-Puro	Single and dual vector: lentiCRISPR v2 and lentiGuide-Puro

All library sequences for screen readout are available for download at *http://www.genome-engineering.org/gecko/*

GeCKO pooled electroporation, plating, determination of transformation efficiency and maxi prep

Follow these instructions for each library (A and B). Amplify and prep half-libraries A and B **separately** as follows:

1. Dilute the GeCKO library to 50 ng/uL in water or TE (if not already diluted).

2. Electroporate the library
   a. Add 2 uL of 50 ng/uL GeCKO library to 25 uL of electrocompetent cells with an efficiency of ≥10^9 cfu/ug. We have had success with many electrocompetent cells, including NEB DH5a cells, Invitrogen DH5a cells and Lucigen Endura. In our hands, the Lucigen Endura cells (cat # 60242) have yielded the highest efficiency with the GeCKO library.
   b. Electroporate using the manufacturer's suggested parameters/protocol.
   c. Recover in 975 uL recovery media (i.e. media provided with cells) and transfer to a loosely capped tube with an additional 1 mL of recovery media.
   d. Repeat for a total of 4 electroporations and rotate at 250 rpm for 1 hour at 37 C

3. Plate a dilution to calculate transformation efficiency
   *Note the library plasmids have **ampicillin resistance** – prepare all plates accordingly.*
   a. Pool all 8 mL of electroporated cells. Mix well.
   b. Remove 10 uL and add to 1 mL of recovery media, mix well, and plate 20uL onto a pre-warmed 10cm petri dish (ampicillin). This is a 40,000-fold dilution of the full transformation and will enable you to estimate transformation efficiency to ensure that full library representation is preserved.

4. Plate the transformations
   Follow Step a) if your lab has 24.5 cm^2 bioassay plates for large-scale bacteria culture; otherwise follow Step b), which substitutes 20 standard (10 cm round) petri dishes.
   a. Plate 4mL of transformation on each of 2 pre-warmed 24.5 cm^2 bioassay plates (ampicillin) using a spreader. Spread the liquid culture until it is largely absorbed into the agar and won't drip when turned upside down.
   b. Alternatively, spread 400 uL of transformation mix per petri onto 20 pre-warmed petri dishes (ampicillin).

5. Grow all plates inverted for **14 hours** at **32 C**. Growth at this lower temperature reduces recombination between the lentiviral long-terminal repeats. (Growth at 37 C is also acceptable if 32 C is not possible.)

6. Calculate transformation efficiency
   a. Count the number of colonies on the dilution plate.
   b. Multiple this number of colonies by 40,000 for the total number of colonies on all plates.
   c. Proceed if the total number of colonies is at least 6 x 10^7. This efficiency is equivalent to 50X colonies per construct in the GeCKO library.

7. Harvest colonies
   a. Pipette 10 mL of LB onto each 24.5 cm^2 bioassay plate (or, 500 uL per 10 cm petri dish)
   b. Scrape the colonies off with a cell spreader/scraper.
   c. Pipette off the liquid plus scraped colonies into a tube and repeat the procedure a second time on the same plate with additional 5-10 mL. *Note: Weigh this tube prior to adding any liquid to it.*

8. Weigh the bacterial pellet to determine the proper number of maxiprep columns to use
   a. Spin down all liquid to pellet the bacteria and then discard the supernatant.
   b. Weigh the bacterial pellet and subtract the weight of the tube

9. Maxi-prep for downstream virus production and future amplification
   a. Using a maxi scale plasmid prep, each column can handle approximately 0.45 g of bacterial pellet.
   b. Perform a sufficient number of maxi preps so as to not overload a column.

10. Proceed to transient transfection of HEK293(F)T cells with maxi-prepped GeCKO lentiCRISPR library and appropriate packaging plasmids. lentiCRISPRv2 and lentiGuide-Puro backbone vectors are compatible with both 2nd and 3rd generation lentiviral packaging plasmids (see www.addgene.org/lentiviral/packaging)
    a. If producing virus from the full library, transfect cells with both A and B half-library plasmids.

8.2.2. LentiCRISPRv2 and lentiGuide-Puro: lentiviral CRISPR/Cas9 and single guide RNA

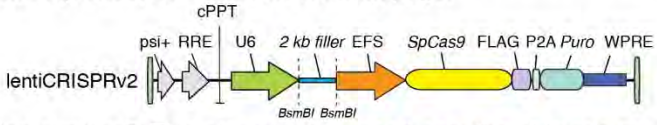
Related references: [384,395]

GeCKO Genome-scale CRISPR Knock-Out | LENTIVIRAL CRISPR TOOLBOX | MIT ZhangLab

LentiCRISPRv2 and lentiGuide-Puro: lentiviral CRISPR/Cas9 and single guide RNA

CRISPR (**C**lustered **R**egularly **I**nterspaced **S**hort **P**alindromic **R**epeats) is a microbial nuclease system involved in defense against invading phages and plasmids. CRISPR loci in microbial hosts contain a combination of CRISPR-associated (Cas) genes as well as non-coding RNA elements capable of programming the specificity of the CRISPR-mediated nucleic acid cleavage. Lentiviral CRISPR/Cas can infect a broad variety of mammalian cells by co-expressing a mammalian codon-optimized Cas9 nuclease along with a single guide RNA (sgRNA) to facilitate genome editing (Shalem*, Sanjana*, *et al.*, Science 2014). Protocols for cloning into the lentiviral transfer plasmid and general considerations for producing lentivirus are described below. **Separate protocols are available for amplifying the genome-scale CRISPR knock-out (GeCKO) libraries. This protocol is for creating individual lentiviral CRISPR plasmids targeting a single genomic locus.**

lentiCRISPRv2 (one vector system): This plasmid contains two expression cassettes, hSpCas9 and the chimeric guide RNA. The vector can be digested using *BsmB*I, and a pair of annealed oligos can be cloned into the single guide RNA scaffold. The oligos are designed based on the target site sequence (20bp) and needs to be flanked on the 3' end by a 3bp NGG PAM sequence, as shown on the next page.



lentiGuide-Puro (two vector system): This plasmid expressed only the chimeric guide RNA. It does *not* contain Cas9. Please use lentiCas9-Blast (a separate lentiviral construct that delivers hSpCas9 and blasticidin resistance) to first integrate Cas9 into your cell line. The lentiGuide-Puro vector can be digested using *BsmB*I, and a pair of annealed oligos can be cloned into the single guide RNA scaffold. The oligos are designed based on the target site sequence (20bp) and needs to be flanked on the 3' end by a 3bp NGG PAM sequence, as shown on the next page.

Which vector to use: lentiCRISPRv2 is identical to the original lentiCRISPRv1 but produces nearly 10X higher titer virus. lentiGuide-Puro produces >100X higher titer virus over lentiCRISPRv1 and should be used in cell lines where Cas9 has already been integrated in (e.g. using the separate lentiCas9-Blast lentivirus). For applications where Cas9 cannot first be introduced (e.g. primary cells), lentiCRISPRv2 is recommended. After transduction, use puromycin to select for cells with lentiCRISPRv2 or lentiGuide-Puro.

Lentiviral production: Before starting any lentiviral work, please ensure compliance with your Environmental Health and Safety office and government/organization/university. Briefly, to make lentivirus, a transfer plasmid (e.g. lentiCRISPRv2 or lentiGuide-Puro) must be co-transfected into HEK293(F)T cells with the packaging plasmids pVSVg (AddGene 8454) and psPAX2 (AddGene 12260). As a positive control for viral production, we often use a CMV-EGFP lentiviral transfer plasmid (eg. AddGene 19319).

Target design notes and online resources: For application of Cas9 for site-specific genome editing in eukaryotic cells and organisms, we have computationally identified suitable target sites for the *S. pyogenes* Cas9 and calculated most likely off-targets within the genome. Please visit *http://www.genome-engineering.org* to access these Cas9 target design tools. Complete plasmid sequences, protocols, a discussion forum and additional information can be found at the Zhang Lab GeCKO website: *http://www.genome-engineering.org/gecko/* .

Citation: Please reference the following publications for the use of this material.

Improved lentiviral vectors and genome-wide libraries for CRISPR screening. Sanjana NE*, Shalem O*, Zhang F. *Nature Methods* (2014).

Genome-scale CRISPR-Cas9 knockout screening in human cells. Shalem O*, Sanjana NE*, Hartenian E, Shi X, Scott DA, Mikkelsen T, Heckl D, Ebert BL, Root DE, Doench JG, Zhang F (2014). *Science*, 343, 83-7. DOI: 10.1126/science.1247005

Target Guide Sequence Cloning Protocol

In order to clone the target sequence into the lentiCRISPRv2 or lentiGuide-Puro backbone, synthesize two oligos of the following form. **All plasmids have the same overhangs** after *BsmBI* digestion and the same oligos can be used for cloning into lentiCRISPRv2, lentiGuide-Puro or lentiCRISPRv1.

```
Oligo 1 ---->  5' - CACCGNNNNNNNNNNNNNNNNNNN      - 3'
               3' -      CNNNNNNNNNNNNNNNNNNNNCAAA - 5' <---- Oligo 2
```

Example oligo design: Note that the NGG PAM is **not** included in the designed oligos.

```
Genomic   5' -...GACCACAGTCTGATCAGTTTTCCTTGGGCTGCAA...- 3'
Sequence  3' -...CTGGTGTCAGACTAGTCAAAAGGAACCCGACGTT...- 5'
                                          NGG PAM
Oligo 1 ---->  5' - CACCGCAGTCTGATCAGTTTTCCTT      - 3'
               3' -      CGTCAGACTAGTCAAAAGGAACAAA - 5' <---- Oligo 2
```

Oligonucleotide ordering tips: Standard de-salted oligos (usually the most inexpensive synthesis) are sufficient for cloning. If not already resuspended, dilute each oligo to 100 μM in sterile water or TE.

* * * * *

Lentiviral vector digestion, oligo annealing and cloning into digested vector:

1. Digest and dephosphorylate 5ug of the lentiviral CRISPR plasmid with *BsmB*I for 30 min at 37C:

5 ug	lentiCRISPRv2 **or** lentiGuide-Puro
3 ul	FastDigest *BsmB*I (Fermentas)
3 ul	FastAP (Fermentas)
6 ul	10X FastDigest Buffer
0.6 ul	100 mM DTT (freshly prepared)
X ul	ddH_2O
60 ul	total

2. Gel purify digested plasmid using QIAquick Gel Extraction Kit and elute in EB.

   If *BsmBI* digested, a ~2kb filler piece should be present on the gel. **Only gel purify the larger band**. Leave the 2kb band.

3. Phosphorylate and anneal each pair of oligos:

1 ul	Oligo 1 (100 μM)
1 ul	Oligo 2 (100 μM)
1 ul	10X T4 Ligation Buffer (NEB)
6.5 ul	ddH_2O
0.5 ul	T4 PNK (NEB M0201S)
10 ul	total

   Please use the T4 **Ligation** Buffer since the buffer supplied with the T4 PNK enzyme does not include ATP (or supplement to 1mM ATP).

Put the phosphorylation/annealing reaction in a thermocycler using the following parameters:

37°C	30 min
95°C	5 min and then ramp down to 25°C at 5°C/min

4. Dilute annealed oligos from **Step 3** at a 1:200 dilution into sterile water or EB.

5. Set up ligation reaction and incubate at room temperature for 10 min:

X ul	*BsmB*I digested plasmid from **Step 2** (50ng)
1 ul	diluted oligo duplex from **Step 4**
5 ul	2X Quick Ligase Buffer (NEB)
X ul	ddH_2O
10 ul	subtotal
1 ul	Quick Ligase (NEB M2200S)
11 ul	total

Also perform a negative control ligation (vector-only with water in place of oligos) and transformation.

6. Transformation into **Stbl3 bacteria**.
Lentiviral transfer plasmids contain Long-Terminal Repeats (LTRs) and must be transformed into recombination-deficient bacteria. We use homemade Stbl3 (propagated from Invitrogen C7373-03) and get excellent plasmid yields. Although other RecA- strains may work, we have found the most consistent transformations and yields using Stbl3.

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