Direct Reprogramming of Fibroblasts to Dendritic Cells for Immunotherapy

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Thesis submitted to Faculdade de Ciências e Tecnologias da Universidade de Coimbra for the degree of Master in Biochemistry

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July of 2016

“A True sign of intelligence is not knowledge but imagination”

Albert Einstein

ABSTRACT

The maintenance of cellular identity relies on stable and complex gene regulatory networks. However, several studies have shown that cell fate can be reversed or modified by defined sets of lineage specific Transcription Factors (TFs). The process of direct cellular reprogramming holds promise for the generation of autologous cells for regenerative medicine. In the immunotherapy field, the use of immune modulatory cells enables the manipulation of patients 'own immune system to target cancer cells. In this context we aim to apply direct cellular reprogramming for the generation of Dendritic Cells (DCs) as ideal antigen-presenting cells to kick-start adaptive immune responses.

Here, TF-mediated direct reprogramming approach was established to generate DCs from fibroblasts. First, we employ a combination of literature mining and computational analysis to identify candidate TFs to induce DC fate *in vitro*. Candidate TFs were selectively expressed in DC populations in both mice and humans and their disruption caused abnormal adaptive immune phenotypes in mice. This analysis generated 19 candidate TFs with key developmental roles in the DC lineage. We have expressed a set of these TFs using a reprogramming proven Doxycycline-inducible lentivirus in mouse embryonic fibroblasts (MEFs). Employing transgenic MEFs harbouring the DC-specific reporter Clec9a-Cre X R26-stop-Tomato, a minimal combination of 4 TFs was identified. This set of 4 TFs activated the DC-specific reporter and generated tdTomato+ cells. TdTomato+ cells acquired DC-like morphology with increased size and complexity. Moreover, a percentage of tdTomato+ cells expressed Major Histocompatibility Complex (MHC) Class II at the cell surface, a critical molecule for antigen presenting function. Finally, overexpression of the 4TFs in Human Dermal Fibroblasts generated cells with DC-like morphology. These morphological changes emerged with similar timing and efficiency in mouse and Human, supporting species conservation of transcriptional regulators underlying DC commitment.

Collectively, DC-like cells were generated via a TF-mediated direct reprogramming approach. The results presented in this study highlight the potential of direct reprogramming to a better understanding of transcriptional events underlying lineage specification and to generate immune modulatory cells for immunotherapy.

ACKNOWLEDGEMENTS

It is a pleasure to acknowledge those who made this thesis possible. First, I would like to give a grateful thank you to my supervisor, Dr. Carlos Filipe Pereira, for providing me a year full of new and refreshing experiences. It was a great pleasure to work with him. His expertise, understanding and generous guidance made it possible for me to work in a stimulating and challenging project with great interest for me. I will keep in mind his advice: “You can have fun, but not to much fun”! Likewise, I’m sincerely grateful for the opportunity to participate in the COHiTEC program and to be a part of the Blood Reprogramming Technologies (BRT) Team. I consider myself an entrepreneurship enthusiast and this was a truly remarkable experience. I hope one day many lives can benefit from BRT products!

I am deeply grateful to Cristiana Pires (Cris) for the vital support during this long year. Her extraordinary personality and attitude were a model for me and will continue to influence my actions throughout life. Cris, you are a great person and I hope I can continue working with the great mind you are.

Once again I would like to thank Filipe and Cris for the incredible friendly environment that allowed very constructive scientific and entrepreneurial discussions. You have great things to look forward in the future, just remember to not to be afraid of greatness, while some are born great and others achieve greatness, you have greatness thrust upon you.

I would like to thank all people who have passed through the Cell Reprogramming and Developmental Hematopoiesis lab for the help and cooperation during my thesis. Those were amazing moments spent in and outside the lab. In particular, I would like to thank to Claudia Raquel and Alexandra Gabriela for being great lab partners and for providing amazing social moments. In addition, I would like to thank the Polish girls (Monika and Julia) for the enthusiastic “coffee conversations”, Luis for the very constructive car discussions on the way between Coimbra and Cantanhede, and the “Tissue Engineering” people for the company and talks during lunch at the canteen.

I am indebted to my colleagues from my own town (Leiria) for being such great friends. I know we do not see each other very often, but I think good friends are like stars, you don't always see them but you know they are always there! I would like to thank to “Costeletas de Frango” for the great patient and support. You guys are great friends that I will never forget. In particular I would like to give a special thanks to Mumia, for being my housemate and for making this year bearable, and to Selas and Miguel for being great friends and an inspiration in Fado.

I would like to show my gratitude to all my friends from FAN-Farra Académica de Coimbra and from Grupo de Fados Da Tuna Universitária de Coimbra for providing good relaxing moments filled with music and companionship.

Finalmente gostaria de agradecer aos meus pais Ana Paula e Fernando Manuel por me ensinarem a ser a pessoa que sou hoje, aos meus avós paternos Manuel e Maria por estarem sempre presentes e por me indicarem os caminhos para a humildade, aos meus avós maternos Zé e Ermelinda por me ajudarem sempre que precisava, aos meus tios José e Cila pelo apoio incondicional e, em

especial, ao meu Irmão João por ser acima de tudo um grande amigo e parceiro que espero manter para toda a vida.

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ABBREVIATIONS

AML	Acute myeloid leukaemia
AP	Alkaline phosphatase
APC	Antigen-presenting cell
BM	Bone marrow
cDC	Conventional DC
cDNA	Complementary DNA
CDP	Common DC progenitors
CDS	Coding sequence
CLP	Common lymphoid progenitor
CMP	Common Myeloid progenitors
CSF-1	Colony stimulating factor 1
CSF-2	Colony stimulating factor 2
CTL	Cytotoxic T lymphocyte
CTLA4	Cytotoxic T lymphocyte-associated antigen 4
DC	Dendritic cell
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
Dox	Doxycyclin
E13.5	Embryonic day 13.5
ESC	Embryonic stem cell
FBS	Fetal bovine serum
FDA	Food and Drug Administration
Flt3L	Flt3 ligand
FSC	Forward scatter
GEO	Gene expression omnibus
GM-CSF	Granulocyte-Macrophage colony stimulating factor
GRN	Gene regulatory network
HDF	Human dermal fibroblasts
HSC	Hematopoietic stem cell
HSCT	Hematopoietic stem cell transplantation
IL	Interleukin
iPSC	Induced Pluripotent Stem cell
KD	Knock-down
KO	Knockout
LC	Langerhans cells
M-CSF	Macrophage colony stimulating factor
M2rtTA	Reverse tetracycline-controllable transactivator
MDP	Monocyte/DC precursors
MEF	Mouse embryonic fibroblast
MHC	Major histocompatibility complex
moDC	Monocyte-derived DC
MPP	Multipotent progenitor
OD	Optical density

PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
pDC	Plasmacytoid DC
RNA	Ribonucleic acid
SCNT	Somatic-cell nuclear transfer
SM	Small molecules
SSC	Side scatter
TAA	Tumor-associated antigens
TetO	Tetracycline operator minimal promoter
TF	Transcription factor
TGF-β	Transforming growth factor beta (TGF-β
TLR	Toll-like receptor
TNFα	Tumor necrosis factor α
WHO	World health organization

CHAPTER I

1. INTRODUCTION

1.1. REPROGRAMMING: HISTORICAL BACKGROUND

Shinya Yamanaka and John Gurdon were awarded with the 2012 Nobel Prize in Physiology or Medicine for the discovery that mature cells could be reprogrammed to pluripotency [1, 2]. This incredible breakthrough has changed our understanding of how cells and organisms develop, bringing new opportunities for regenerative medicine. Skin cells derived from diseased patients could be reprogrammed to a pluripotent state, examined and compared to healthy cells, and used to better understand disease mechanisms. However, it was not long ago that scientific community believed cell fate was permanent and irreversible.

Back to the late 19's, a genetic theory described by August Weismann hypothesized that inheritance could only be mediated by germ cells and that commitment to specific lineages would be accomplished by deletion or permanent inactivation of specific genetic codes [3]. Briggs and King later confirmed this assumption in 1952 by publishing a study regarding the transfer of embryonic cell nuclei in different developmental stages into enucleated *Rana pipiens* frog eggs [4]. In this experiment, they realized that when nuclei were taken from embryos in the blastula stage, the frogs experienced normal development. However, when nuclei were taken from later embryonic stages, there were major developmental abnormalities. These results lead to the conclusion that as cells differentiate, they suffer permanent changes to the nucleus.

The genetic theory known as "The Weismann barrier" was considered one of the first steps towards a better understanding of embryonic development, resulting in the development of a new model by Conrad Waddington, in which embryonic development is described as a ball rolling down ("differentiation") a mountain throughout distinct valleys (Figure 1) [5]. In this so-called "Waddington's landscape model", lineage commitment was thought to be permanent. However, a decade of change was coming with the first report regarding cellular reprogramming through somatic cell nuclear transfer (SCNT) in frogs [2]. This experiment performed by Dr. John Gurdon proved that, after all, lineage commitment could not be as permanent as was thought. In this experiment, a nucleus of a somatic cell belonging to intestinal epithelia was transferred to an enucleated egg and, after several divisions, an embryo was generated with the same genetic background as the somatic cell donor. Gurdon was considered a pioneer in developmental biology research by demonstrating that a somatic nucleus has the necessary genetic content to generate all cell lineages and that could be "reprogrammed" to an embryonic/pluripotent state by defined experimental conditions.

In the early 80s a new and world-changing cell line was developed. Known as Embryonic stem cells (ESCs), these embryo-derived self-renewable cell lines

were capable of generating all cell types in the adult mouse organism, a feature known as pluripotency [6, 7]. Eventually, the late 20th century was characterized by several reports regarding the successful generation of cloned mammals, i.e. sheep and mice, by SCNT [8, 9].

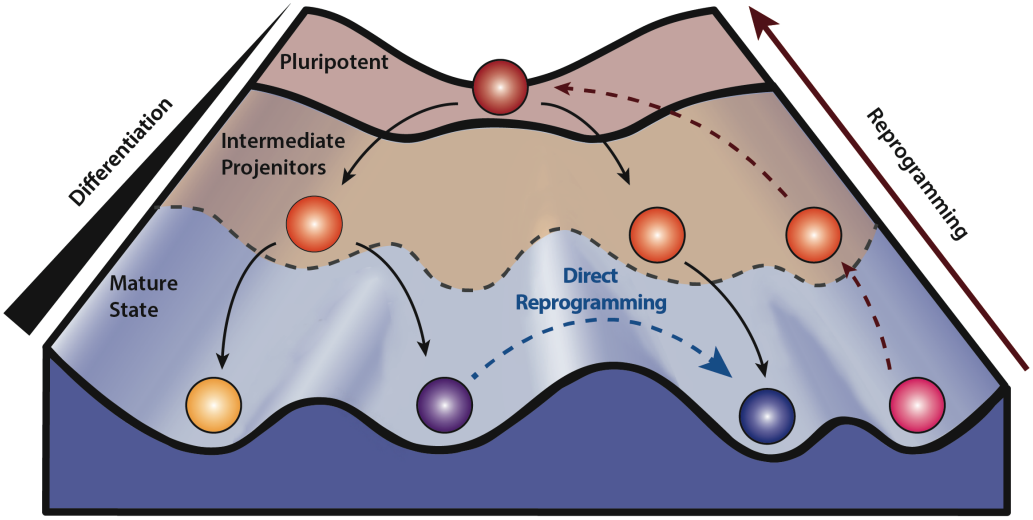


Figure 1. A Waddington perspective on cell fate changes. During normal development pluripotent stem cells (PSCs) (red) can pass through a complex differentiation process via intermediate progenitors (Orange), which results in the commitment to specific somatic cells (mature cells). Direct reprogramming (blue dashed arrow) takes advantage from the use of lineage specific factors to force a lineage-committed cell (violet) to change cell fate without passing through an intermediate pluripotent state. During "reprogramming", the overexpression of 4 TFs (Oct3/4, Sox2, Klf4, and c-Myc) is able to revert normal development, allowing mature cells (pink) to gain pluripotent features.

Simultaneously, parallel studies have shown that the gene expression profile of one cell could be changed through cellular fusion with a distinct cell type, resulting in the generation of a heterokaryon. Helen Blau documented this process for the first time in 1983, showing the activation of muscle-specific genes in human amniocytes after being fused with mouse muscle cells [10]. Later in the same year, Nabuo Takagi reported the reactivation of the entire inactive X chromosome in female somatic cells after fusion with murine embryonal carcinoma cells [11]. Only in the beginning of the 21st century, scientists reported the expression of pluripotency-associated genes in mouse somatic cells after being fused with pluripotent cells such as ESCs, suggesting that pluripotent cells are able to somehow reprogram somatic cells into a pluripotent state [12]. At this stage the scientific community was interested in deciphering the key "reprogramming factors" able to reconstruct the chromatin landscape by erasing the key genetic features that sustain somatic cell identity. Remarkably, the first evidence supporting the existence of such "reprogramming factors" appeared earlier in the 80s with studies showing the direct fate conversion of one cell to another by introducing a single transcription factor. An interesting set of experiments has busted the discovery of myoblast determination protein 1 (MYOD1) as a key muscle cell fate determinant [13, 14]. The ectopic expression of this protein was able to convert mouse fibroblasts to myoblasts that expressed

several myoblast marker genes [15]. Later, other subsequent studies showed that Transcription-factor mediated reprogramming could be achieved between mature cells from the same lineage. Holger Kulessa and colleagues have successfully reprogrammed myeloblasts into megakaryocyte and erythrocyte precursors by ectopic expression of erythroid transcription factor GATA-binding protein 1 (GATA1) [16]. Nico Heins and colleagues reprogrammed Glial cells into neuronal cells via overexpression of the PAX6 transcription factor [17]. Moreover, B cells could be converted into Macrophages by the ectopic expression of CCAAT/enhancer-binding protein-α (CEBPα) or CEBPβ [18]. These studies, among others, have provided strong evidences that lineage-specific transcription factors (TFs) may act as master regulators of cell identity and thus, may be utilized to induce cell fate transition.

As a result of these seminal works another light shined on the reprogramming field when Takahashi and Yamanaka successfully generated induced pluripotent stem cells (iPSCs) from somatic cells. This iPSCs reassembled functional ESCs with the ability to self renew and to generate cells from any germ layer[1]. In this exceptional work they have identified 4 TFs (Oct3/4, Sox2, Klf4, and c-Myc, or OSKM) able to permanently redirect the phenotype of fully mature fibroblasts to iPSCs. This experiment established the idea that TF-mediated direct cell fate transition could be accomplished between developmentally distinct cell lineages. This would rely on the ability of reprogrammed factors to overwhelm the pre-existent genetic and epigenetic codes, which requires high expression levels of the reprogramming factors, able to enforce the desired chromatin state. Beside that, another key aspect to consider in TF-mediated direct reprogramming is the stoichiometry when using a combination of several factors [19]. For instance, the mechanistic analysis of iPSC reprogramming have proved that factor stoichiometry not only influence the reprogramming process but also the quality of iPSCs [20].

The combinatorial overexpression approach established for generating iPSCs opened the way for the generation of multiple mouse and human cell types through direct reprogramming methodologies.

1.1.1. Direct Reprogramming

The rapid progression of the direct reprogramming field is a reflex of the increasing interest on finding alternative strategies for obtaining functional cells for regenerative medicine. Fundamentally, direct reprogramming can be described as the direct conversion of one cell type to another without passing through an intermediate pluripotent state [21]. This can be accomplished by the enforced expression of tissue-specific TFs. Cell fate is controlled by a complex gene regulatory network that actively maintain cellular identity. Nature has progressed to ensure the stability of patterns of gene regulation that are responsible for keeping each cellular identity. To induce cellular reprogramming, TFs must be able to activate genes that are typically repressed in the original cell [22]. These developmentally repressed genes are normally present in sites with

difficult chromatin accessibility. In that context, TF with increased reprogramming capacity have been shown to engage their target genes in close, nuclease-resistant chromatin prior to gene expression. These “pioneer TFs” have a primary role in cell reprogramming since they establish the competence for cell fate transition by modulating the chromatin structure. Wernig and colleges demonstrated the generation of induced glutaminergic neurons (iN) by overexpression of 3 TFs, i.e. Ascl1, Brn2 and Myt1l in mouse embryonic fibroblasts (MEFs) [23]. Generated iNs expressed several neuronal proteins, were capable of generating action potentials and functional synapses. Beside the generation of glutaminergic neurons, dopaminergic and motor neurons were also generated by direct reprogramming via overexpression of 2 other sets of TFs ([24], [25]). The 3 sets included the TF Ascl1. Follow-up experiments revealed the function of Ascl1 as a pioneer TFs during the early stage reprogramming of iNs. In fact, Ascl1 act first by establishing competence for the neuronal lineage, while the remaining ones act promoting neuronal type specification.

In the hematopoietic system Xie and colleges have reprogrammed B cells into Macrophages via overexpression of C/EBPα or C/EBPβ. The reprogrammed macrophages showed prototypical features of macrophages in terms of morphology and phagocytic capacity [18]. Combining the action of Pu.1 to C/ebpα converted fibroblasts into macrophage-like cells [26]. The reprogrammed cells displayed macrophage-like functions such as the ability to phagocytize small particles. Ieda and colleges have successfully reprogrammed postnatal cardiac or dermal fibroblasts directly into cardiomyocyte-like cells with the 3 TFs Gata4, Mef2c and Tbx5. These cells were capable of contracting spontaneously, had a cardiac-like phenotype and a gene expression profile similar to naturally occurring cardiomyocytes [27]. Zhao and colleges have directly reprogrammed fibroblasts into sweat gland-like cells through overexpression of 2 TFs, i.e. NF-kB and Lef-1, in fibroblasts. After transplantation, these cells were able to resemble fully reconstructed sweet glands [28]. Yamamoto K. and colleges have successfully converted Fibroblasts into functional osteoblasts by the utilization of osteoblasts-specific TFs, i.e. Runx2, Osterix, Oct4 and L-myc. The reprogrammed cells had similar gene expression profiles to the naturally occurring ones and were able to contribute to bone repair after transplantation into immunodeficient mice at artificial bone defect lesion sites [29].

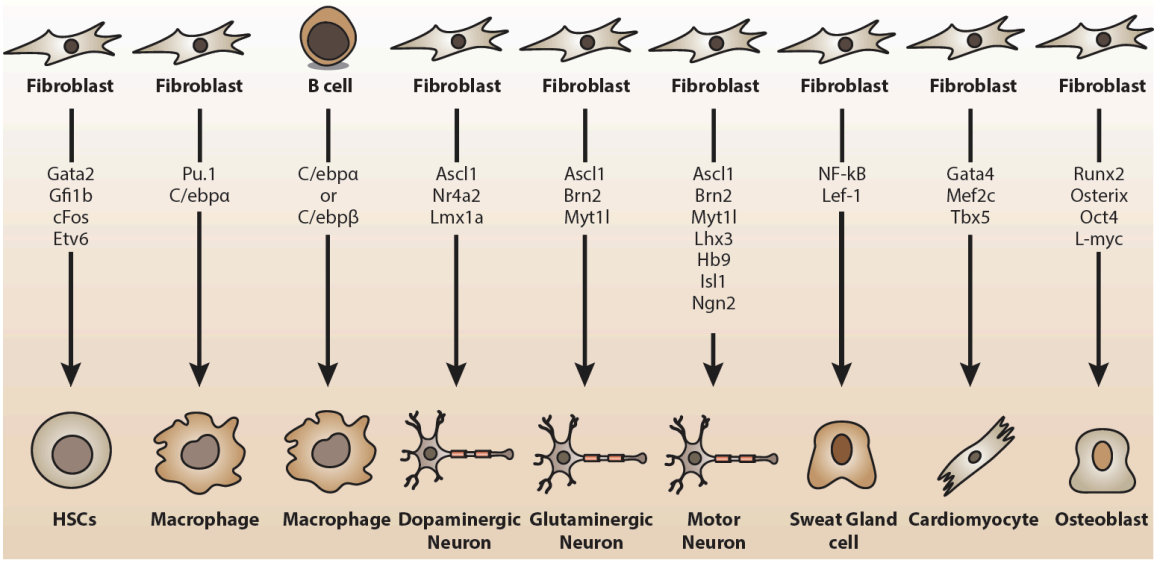


Figure 2. Examples of direct Reprogramming using a variety of transcription factors and input cells. TF-mediated direct reprogramming has been used for the generation of several cell types, including HSCs, Macrophages, neurons, Sweat gland cells, cardiomyocytes and osteoblasts.

This process has also been utilized for the generation of Hematopoietic Stem Cells (HSCs) and hematopoietic progenitor cells from various cell types such as fibroblasts and endothelial cells [30]. Pereira et al. have demonstrated the direct reprogramming of MEFs into hematopoietic progenitors with four TFs: Gata2, Gfi1, cFos and Etv6. These induced a dynamic, multi-stage hemogenic process that progresses through an endothelial-like intermediate [31]. This endothelial-to-hematopoietic transition was thought to occur *in vivo* in embryo and placenta. However, the progenitors responsible for the generation of HSCs *in vivo* were still to define. In that sense, Pereira took advantage of his *in vitro* reprogramming experiments to identify the *in vivo* phenotype for these hemogeneic precursors [32]). This was the first time a reprogramming experiment was shown to inform *in vivo* developmental processes, turning the system upside down, since developmental biology is usually the one informing reprogramming experiments.

Beyond lineage-specific TF overexpression, much attention has been given for the identification of alternative factors able to induce lineage reprogramming. In accordance to recent findings, epigenetic regulators, microRNAs and small molecules could be used for this end [21].

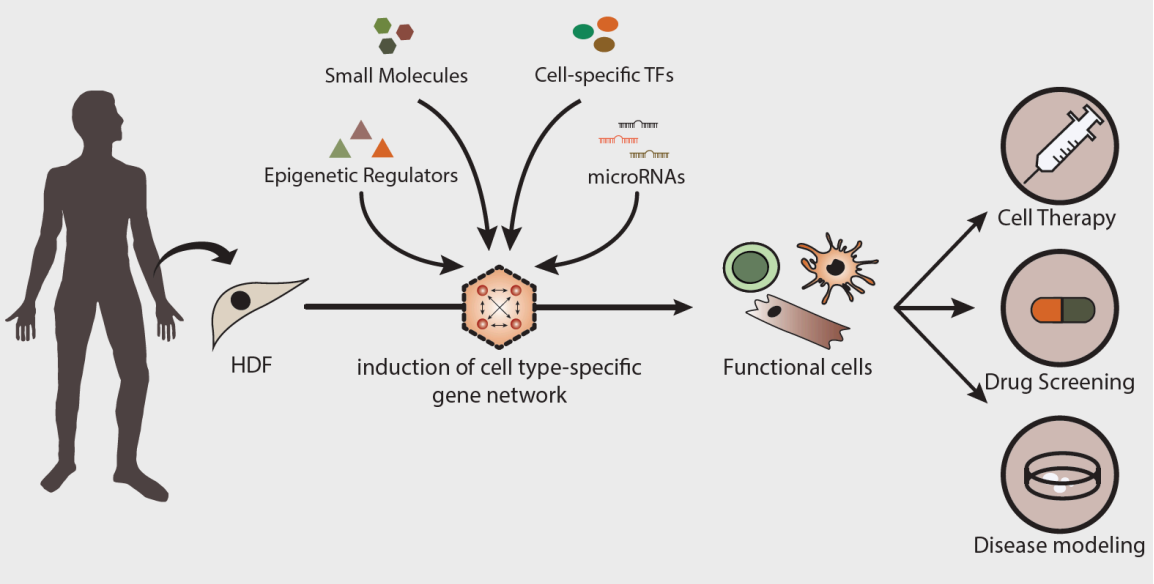


Figure 3. Alternative strategies applied in direct reprogramming aiming to generate functional cells for clinical applications. Distinct reprogramming factors can be used for directing cell fate towards distinct cell lineages. These factors, including small molecules, cell-specific TFs, epigenetic regulators and microRNAs, are able to induce a cell type-specific gene network, enabling the generation of functional cells with potential for cell therapy, drug screening and disease modelling.

Direct reprogramming requires the transition between different epigenetic states, which requires TFs that act, directly or indirectly, with epigenetic regulators for the proper remodelling of the chromatin landscape. Considering that, it is reasonable to hypothesize that chromatin remodelers may play a role in the process of cell fate transition. Takeuchi and Bruneau have reported that non-cardiac mesoderm could be transdifferentiated into cardiomyocytes through the ectopic expression of GATA4, Tbx5 and Baf60c, a cardiac-specific sub-unit of BAF chromatin remodelling complex [33]. During this process, Baf60c enabled the binding of Gata4 to cardiac genes, facilitating the reprogramming process. Furthermore, the relevance of epigenetic regulators for lineage conversion experiments has been emphasized by the emergence of studies showing that lineage conversion can be accomplished by the manipulation of epigenetic regulators alone. For instance, the deficiency of Dnmt1, a DNA methyltransferase, in beta-pancreatic cells was enough to induce cell fate transition to alpha-cells [34]. In this case, Dnmt1 deficiency was enabled the reactivation of Arx, a core gene implicated in the maintenance of alpha-cell identity [34]. Nevertheless, reprogramming by lineage-specific or general epigenetic regulators is still doubtful since their mechanism of action and specificity remains unclear. This issue is particularly relevant when considering non-specific epigenetic regulators and their putative role on promoting the activation of core regulatory networks, including TFs that are restricted to specific cell types. In that sense, the reprogramming potential of epigenetic regulators may rely on the interaction with other factors, such as TFs, that are critical for their function as cell fate changers [21].

Recently, microRNAs have been reported as potential candidates for driving cell fate changes. For instance, the overexpression of the neuronal-specific miRNAs miR-9/9* and miR-124 in human fibroblasts was able to induce

neuron-like cells expressing the neuronal marker MAP2 [35]. Still, overexpression of TFs was absolutely required for inducing functional human neuronal cell formation. In other study, cardiac lineage conversion could also be accomplished both *in vivo* and *in vitro* via an enriched miRNA cocktail alone, composed by miR-1, miR-133, miR-208 and miR-499 [36]. Later, a distinct study regarding the induction of functional induced neurons (iN) in mice has clarified a putative role for miRNAs in lineage commitment. In that experiment, Xue and colleagues have successfully generated iNs by inhibiting the miRNA regulator PTB, which is responsible for blocking miRNA-mediated activity of REST complex [37]. This allows the expression of several miRNA-regulated neuronal genes responsible for inducing the neuronal cell fate. Nevertheless, it seems that miRNA-induced lineage conversion is less efficient than TF-mediated reprogramming. The overexpression of miRNAs alone is able to induce the expression of several neuronal markers in human non-neuronal cells with numerous functional deficits [35]. However, TF-mediated reprogramming is able to promote a relatively complete induction of functional human neuronal cells. Although several studies have provided thorough insight into miRNA-mediated reprogramming, their mechanisms of action are still doubtful. The question here is how can they promote the expression of lineage-specific master genes if they regulate their targets by repression? For instance, the overexpression of a specific miRNA could inhibit the expression of master genes normally expressed in the original cells. This would create a disruptive unbalance between master regulators resulting in atypical lineage transitions. Other possible explanation would be the ability of miRNAs to repress the expression of specific epigenetic regulators, thus facilitating the expression of core target genes [21]. Although these possibilities need experimental confirmation, studies aiming to uncover miRNA molecular interactions during lineage conversion could be helpful for a better understanding of the mechanisms that are intrinsic to lineage reprogramming.

Several reprogramming strategies require genetic manipulation of the original cell. This raises health concerns regarding their use for therapeutic purposes in humans and, consequently, hinders their translation to the clinics. In that sense, a promising alternative is the use of small molecules (SMs) for inducing cell fate transition. SMs have several advantages over the overexpression of exogenous factors: they can be cell permeable, easily synthesized, preserved and standardized [38]. Economically, they are more cost-effective, which makes them an attractive alternative for the pharmaceutical industry. ,iPSCs can be generated using a combination of small molecules [39] as well as neuronal progenitor cells [40]. However, the identification of a SM cocktail able to efficiently replace the use of exogenous factors is still challenging. The transition between distinct cell fates relies on a complex interplay between several factors, i.e. TFs, epigenetic regulators and specific signalling pathways. Studies regarding natural examples of dedifferentiation or transdifferentiation have been providing true insight on key epigenetic mechanisms and signalling pathways strictly required for specific cell fate transitions [41]. In that sense, the same could be theoretically achieved through chemical reprogramming by using small molecules. Since TF-mediated reprogramming requires the activation of a core gene regulatory network for the target cell type, SM-mediated activation of factors belonging to this restrict group of genes could perhaps replace the use of TFs. However, the mechanisms used

by small molecules to promote this activation are barely understood and requires further refinement [21].

Functional cells derived from lineage reprogramming may have huge potential for distinct biomedical applications. These include disease modelling, drug development and cell therapy. In fact, reprogramming strategies have been applied in distinct biomedical fields not only to better understand disease progression but also to reach a potential curative treatment.

As previously mentioned, the generation of macrophages has already been accomplished via direct reprogramming. However, macrophages are characterized by a poor antigen-presenting capacity, which precludes their use for the induction of immunity in a clinical context. In contrast, Dendritic cells (DCs) are the professional antigen presenting cells (APCs) in the organism and their therapeutic potential have already been address in a diversity of studies and clinical trials. Therefore, there's momentum for the application of autologous directly reprogrammed DCs for controlling immunity and eliciting adaptive immune responses. These efforts will contribute to a deep insight on DC development, especially on the identification of the minimal TF network for DC specification, and also on the potential of programmed DCs for clinical applications.

1.2. DENDRITIC CELLS (DCS)

DCs are professional APCs located throughout the body that can be originated either from DC precursors or monocytes [42]. DCs are able to provide a crucial link between the external environment and the adaptive immune system through their ability to capture, process and present antigens to T cells, targeting them to different types of immune responses or to tolerance [43]. Firstly, DCs have to capture tumour antigens and process them through major histocompatibility complex (MHC) class I and MHC class II. Following their activation, DCs are able to migrate towards the local draining lymph nodes priming multiple B cell and T cell responses, a key feature of adaptative immunity [44]. The early protective efficacy is primarily conferred by the induction of antigen-specific antibodies produced by B lymphocytes. Beside that, the long-term protection against specific antigens requires the persistence of specific antibodies and the generation of immunological memory that could provide a rapid and efficient response after subsequent antigen exposure [45]. DCs, as professional APCs, have the ability to cross-present antigens, meaning that, in addiction to its classical ability to present exogenous antigens on MHC class II and endogenous antigens on MHC class I [46], they are also able to present exogenous antigens on MHC class I, a critical step for the generation of Cytotoxic T Lymphocyte responses (CTL) [47, 48].

This effective protection relies on a variety of immune responses associated with a very complex and diverse DC network. This network is essential for protecting the organism against invading pathogens and is only possible due to epigenetic changes associated with the normal cell development.

These epigenetic changes occur dynamically in a sequence of events during differentiation promoting different cell-type-specific chromatin landscapes and, consequently, differences in the DC transcriptional network. These processes provide distinct gene expression profiles and, consequently, the commitment of precursor cells to distinct DC subsets [43]. Although this immune lineage shares a considerable number of common functional and morphological features, multiple DC subsets have been identified with distinct immune functions in both mice and humans [46]. The existence of this genetically and functionally heterogeneous group was firstly noticed by the presence of different cell-surface molecule expression profiles. The mouse DC lineage can be divided in 4 major subsets: Conventional DCs (cDCs), Monocyte-derived DCs (moDCs), Plasmacytoid DCs (pDCs) and Langerhans cells (LC) (**Figure 2**).

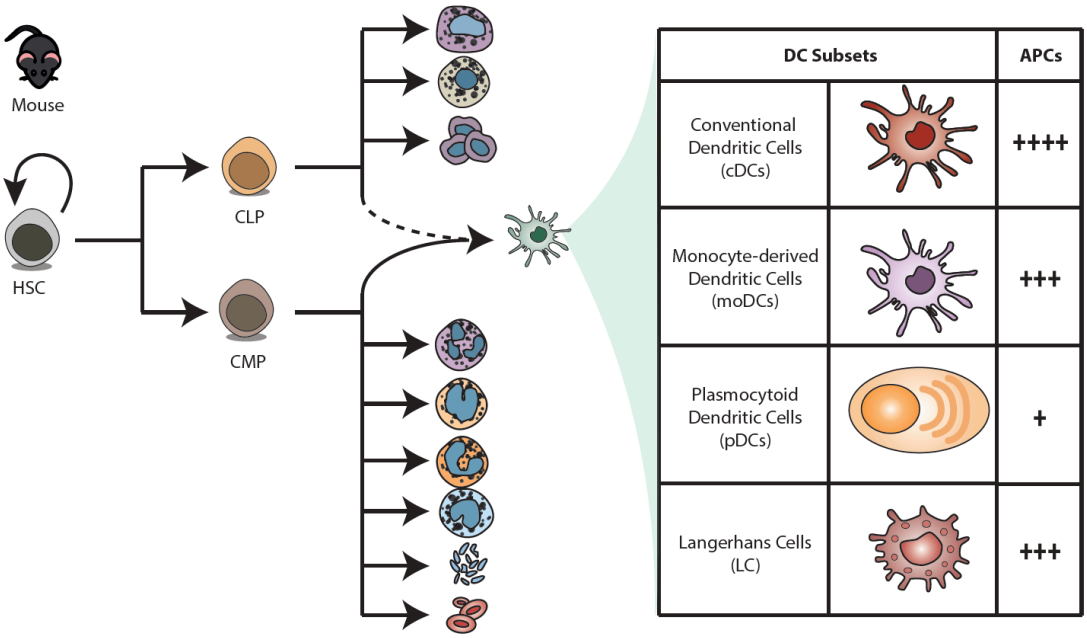


Figure 4. Schematic overview of the different DC subsets in mice. Dendritic cell development starts at the Hematopoietic stem cell (HSC) level, from which two progenitor populations emerge: Common lymphoid progenitors (CLPs) and Common Myeloid progenitors (CMPs). Together, both progenitor populations are able to generate all blood cells, including DCs that derive mainly from CMPs. The lymphoid origin of some DCs is under debate (10.1038/nri1127). In mice, the DC compartment can be divided in 4 subsets: Conventional DCs (cDCs), Monocyte-derived DCs (moDCs), Plasmacytoid DCs (pDCS) and Langerhans Cells (LCs). The best antigen presenting cells (APCs) are found in the cDC subset.

cDCs, mostly found in steady state, are specialized in antigen processing and presentation and, therefore, are able to induce immunity to any foreign invading-antigens as well as enforce tolerance to self-antigens [49]. This exceptional capacity is related to some key-aspects including its critical distribution in non-lymphoid tissues and in the spleen marginal zone, where they constantly interact with tissue and blood antigens; its superior antigen processing and presentation machinery [50, 51]; its ability to migrate loaded with tissue antigens to T cell zone of lymphatic nodes in both steady state or inflamed state [52] and, lastly, cDCs' superior ability to prime T cell responses [49]. Dependently

on their tissue localization and migratory pathways, this subpopulation can be separated in two distinct classes: migratory DCs, which are able to migrate from peripheral tissues, where they are found has early precursors specialized in antigen sampling, to the regional lymph nodes [53]; and lymphoid-resident DCs, which are majorly found in lymphoid organs, an ideal place to sense antigens or pathogens that are circulating in the bloodstream, and arise from precursor cells found on lymphoid tissues [54]. Additionally, the lymphoid-resident DC subset can be further subdivided in two distinct sub-populations: cDC1 and cDC2 [55]. The cDC1 sub-population comprises cells specialized in antigen cross-presentation, polarizing the Th1 subset of T helper cells. Th1 polarization is associated with the secretion of interferon (IFN)-γ and tumour necrosis factor (TNF)-β, which allows a particularly effective protection against tumoural cells and intracellular infections [56]. cDC2 sub-population is specialized in antigen presentation to CD4+ T cells, polarizing Th2 and Th17 subsets of helper T cells. Th2 polarization is associated with the production of majorly IL-4 and IL-5 signals, which are critical immune mediators associated with protection against intestinal parasites and contributing to the majority of antibody production. Th17 polarization is required for protection against specific bacteria, being also involved in the development or maintenance of autoimmune diseases. Taken together, cDC1 cells are usually defined as critically involved on acting against tumoural cells and intracellular pathogens while cDC2 subset is critically involved on acting against extracellular pathogens [57].

moDCs are derived from circulating blood monocytes that, under inflammatory conditions, are rapidly mobilized and differentiated into cells with prototypical features of DCs [58, 59]. Furthermore, moDCs have the capacity to present antigens to lymphocytes, constituting a reservoir of APC that may play an emergency-backup tool in cases of acute inflammation [46]. These cells are commonly utilized as precursor cells for the induction of DCs *in vitro* [60].

pDC are quiescent cells specialized in antiviral activity by producing large amounts of type one interferons (IFNs) [42]. This specific type of DC is characterized by a poor antigen-presenting capacity and by their still doubtful contribution to immune responses [61].

Lastly, LCs are normally present in the skin and, like migratory DCs, are mobilized to lymph nodes in order to present antigens [62].

Similar to murine DCs, human DCs can be divided into pDCs, cDCs and LCs, all comprising both resident and migratory DCs [63] (Figure 4). Resident DCs are found in lymphoid organs during their life cycle, while migratory DCs are found in non-lymphoid and peripheral tissues, being able to migrate through the lymph in order to reach the lymph nodes. Two main subsets of resident cDCs can be found in blood as well as in spleen, tonsil and lymph nodes: BDCA1/CD1c+ DCs and Clec9A+BDCA3/CD141+ DCs. Analysis of gene signature and phenotypic characteristics have suggested the homology of Clec9A+BDCA3/CD141+ DCs to murine cDC1s and BDCA1/CD1c+ DCs to cDC2s. However, while in the mouse Irf8 seems to be important for the cDC1 subset, in the human system it seems to be critical for both cDC1 and cDC2 lineages [64]. Tissue DC subsets are poorly characterized, except for the skin were 4 subsets of DC can be found: epidermal LCs, dermal CD1a+ DCs, dermal

CD14+ DCs and a minor population of dermal Clec9A+CD141+ DCs ([65], [66]. All these skin DC subsets are able to migrate towards the lymphatic vessels in order to reach skin-draining lymph nodes for presenting processed antigens. Tissue and blood Clec9A+CD141+ DCs are considered to belong to the same lineage as well as tissue and blood CD1c+ [63]. Regarding Human pDCs, they are characterized by an efficient production of interferon α (IFNα), which support their putative role on promoting resistance to viral infections ([67], [68]). Moreover, in contrast to murine pDCs, human pDCs have been shown to cross-present antigens efficiently ([69], [70], [71]). Ultimately, Human inflammatory DCs resembling murine moDCs in terms of phenotype and origin have also been identified, being potentially involved in the induction and maintenance of Th17 cellular responses. ([72]).

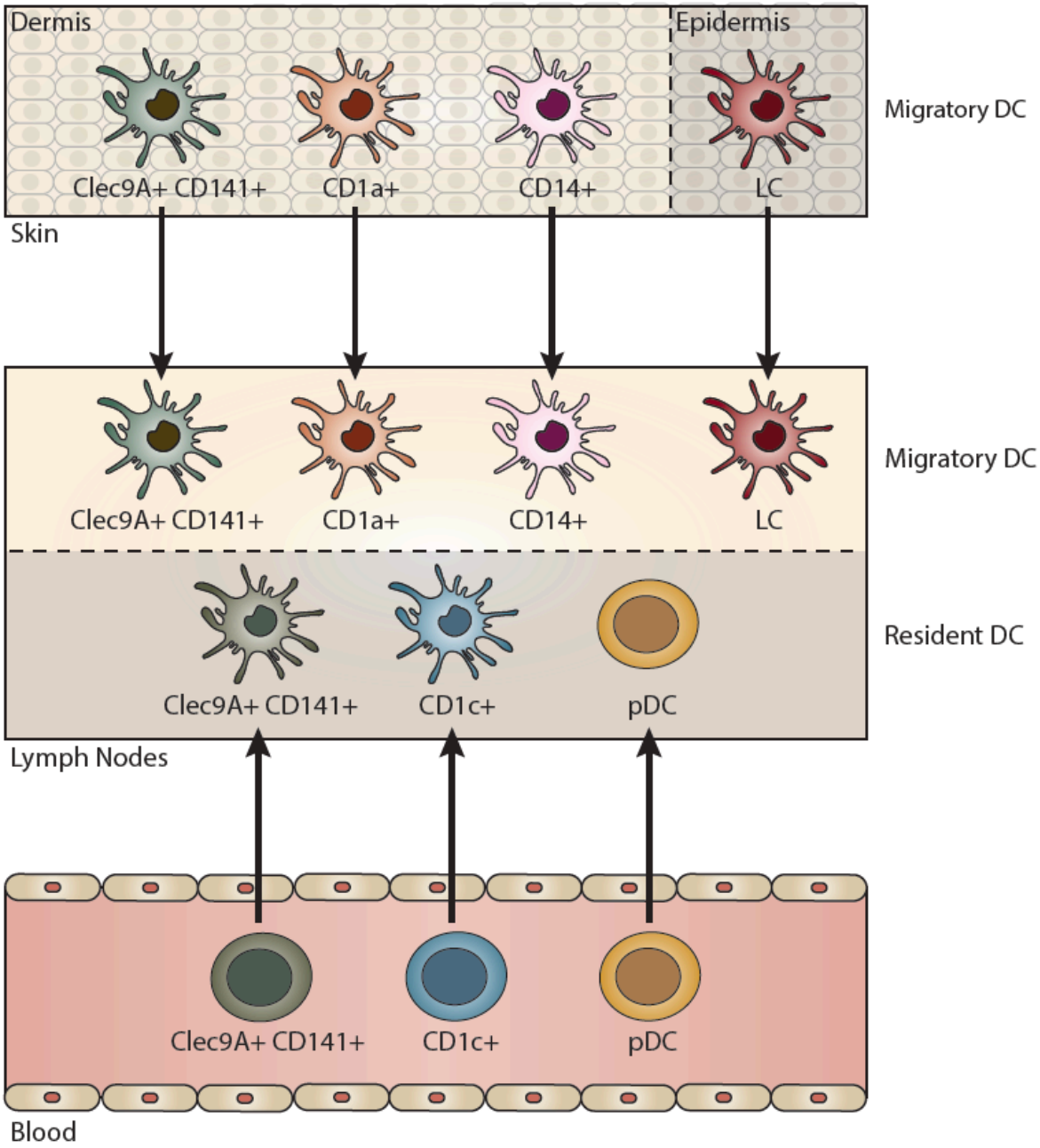


Figure 5. Schematic overview of the Human DC compartment. Skin DCs are the best-studied tissue DC. Migratory DCs comprise dermal Clec9a+ CD141+, CD1a+ and CD14+ DCs and epidermal Langerhans cells (LC). Migratory DCs are able to migrate to the draining lymph nodes. Resident DCs are typically present in the lymphoid organs, comprising pDCs, CD1c+ and Clec9a+CD141+ DCs. While blood CD1c+ and Clec9a+CD141+ DCs are thought to be a precursor form of resident CD1c+ and Clec9a+CD141+ DCs, blood pDC are though to be terminally differentiated while circulating in the blood.

1.2.1. DC DEVELOPMENT

DC development is not a synchronized linear event but is instead characterized by the continuous emergence of dendritic precursor cells in which commitment is acquired through several stages of maturation [49] (Figure 5). This differentiation process starts in the Bone Marrow (BM), where a population of HSCs is able to generate Multipotent Progenitors (MPPs) before giving rise to Common Myeloid progenitors (CMPs). These CMPs can then give rise to Monocyte/DC precursors (MDP) with potential to generate not only DCs but also monocytes. These cells differentiate to Common DC progenitors (CDP), cells with pDC and cDC potential. Following this non-synchronized maturation process, CDPs can originate pre-DCs, a sub-population of DC precursors characterized by a high degree of heterogeneity [73]. Recent findings have suggested that polarization to specific subsets of Conventional DCs occur still in the BM [55]. The pre-DC population can be divided in 2 distinct sub-populations regarding the phenotypic profile i.e, pre-cDC1 and pre-cDC2. These phenotypically distinct sub-populations were already primed to either the cDC1 or the cDC2 lineages and were the more mature DC-precursor population found at the BM level. These cells can then suffer migration to peripheral tissues where they complete their maturation process in accordance to peripheral cues. Beside the fact that DC-commitment to specific mature DC subsets still occurs in the BM, cDC1-commitment relies on important transcriptional events associated with the maintenance of the Irf8 auto-activation loop, which is Batf3-dependent [74].

As a result of the increasing interest on unrevealing the complexity of DC network efforts have been expanded for decoding the key developmental features of this cell lineage in order to explain the dynamics and functional proprieties of each DC sub-set. The regulation of DC development relies on distinct transcriptional players and on the presence of distinct cytokines.

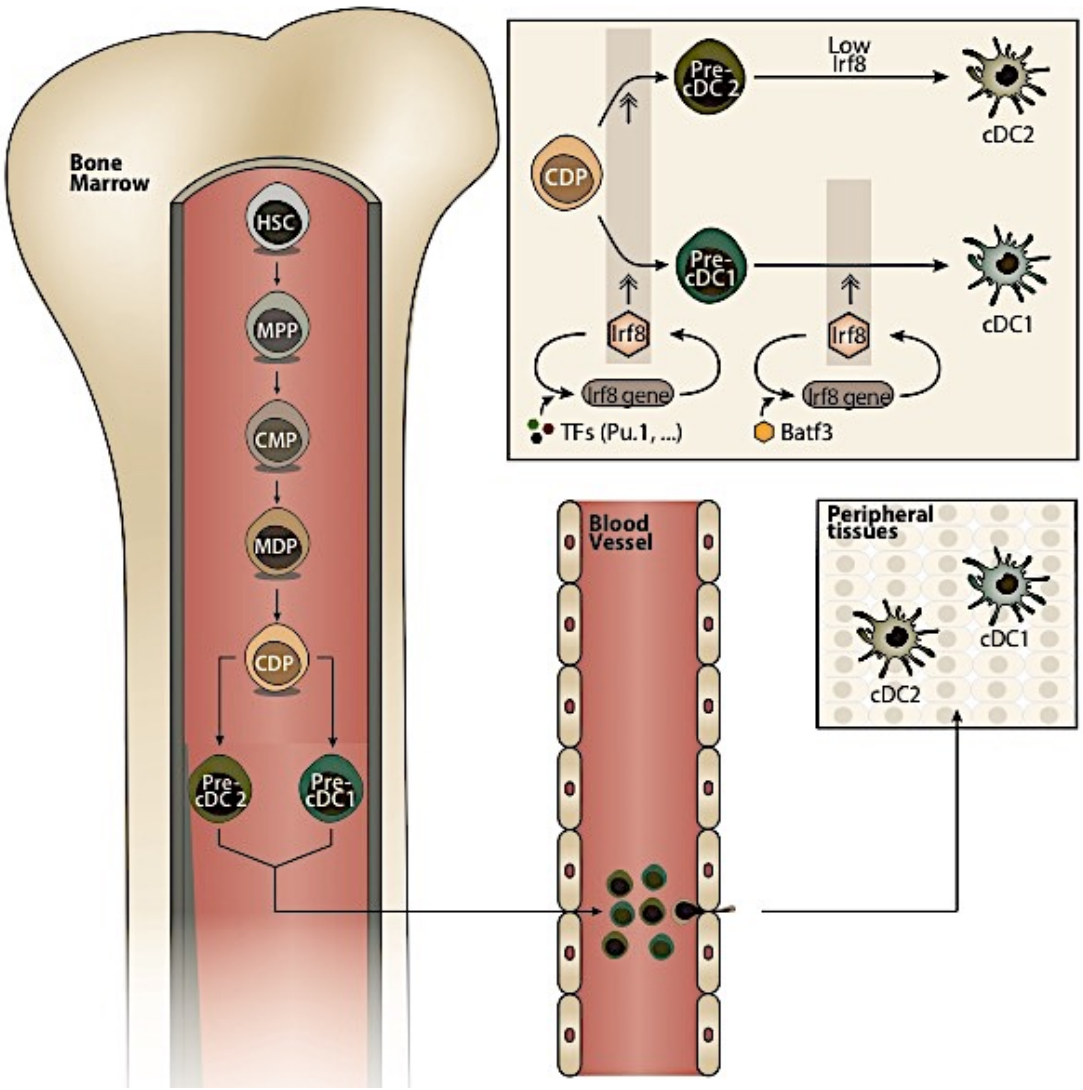


Figure 6. Dendritic cell development and specification of the cDC1 and cDC2 subsets. DC development starts in the bone marrow, with a population of Hematopoietic Stem Cells (HSCs). Progressively, HSCs give rise to Multipotent Progenitors (MPPs), Common Myeloid Progenitors (CMPs) and Common Dendritic cell Progenitors (CMP). The commitment to the conventional DC1 (cDC1) or conventional DC2 (cDC2) subsets occurs still in the bone marrow through pre-cDC1 and pre-cDC2 populations. After commitment, Pre-DCs migrate through the blood vessels in order to reach peripheral tissues, where they complete maturation, generating mature cDCs (cDC1 or cDC2). Commitment to the cDC1 lineage relies on the presence of Batf3, which promotes an auto-activation loop of the Irf8 gene enabling the persistence of Irf8 during cDC1 development.

1.2.2. Cytokines and their role in Dendritic Cell lineage commitment and differentiation

Currently it is well known that DC commitment to specific lineages and its subsequent differentiation is largely driven by extracellular signals, especially by distinct hematopoietic cytokines. According to Merad et al. 6 key cytokines are involved in this process, including: Flt3 ligand (Flt3l), Colony Stimulating Factor 1 (CSF-1), CSF-2, Lymphotoxin β, Transforming growth factor beta (TGF-β1) and Interleukin 4 (IL-4) [75].

Flt3L is a key regulator of DC commitment in hematopoiesis. Its specific receptor, FLT3, is expressed in circulating pre-cDC and pDC and in all tissue cDCs excluding LCs. Some studies suggest that FLT3/FLT3l pathway is associated with the regulation of peripheral DC's proliferation, indicating that the presence of this extrinsic signal provides the maintenance of homeostatic DC numbers [76];

CSF-1, also known as macrophage colony stimulating factor (M-CSF), works as a hematopoietic regulator that manage the survival, proliferation, and differentiation of macrophages [77].

CSF-2 (GM-CSF), also known as Granulocyte-Macrophage colony stimulating factor (GM-CSF), is involved in the regulation of myeloid lineage progression [78] by promoting the differentiation of hematopoietic progenitors and monocytes into cells that resemble mouse splenic cDCs [79]. GM-CSF seems to be a critical regulator of cDC survival in lymphoid tissues [80] and a key cytokine for generating DC-based vaccines for clinical use [81]. Notably, this cytokine seems to be implicated in the final stages of cDC maturation and therefore may be involved in the capacity to cross-present antigens [82];

Lymphotoxin β has been associated with the homeostasis and expansion of splenic DCs, suggesting their importance for maintaining these cells locally in the steady state [83].

TGF-β1 is normally associated with LC differentiation in both mice and humans [84, 85]. The transcription factor Runx3 regulates cDC responses to TGF- β1 and thus it is required for LC development [86];

Lastly, IL-4, a pivotal Th2 cytokine, is involved in the suppression of murine bone-marrow-derived cDCs/splenic DCs responses to type I interferon [87]. In fact, this cytokine suppresses cDC responses to the presence of Toll-like receptor 7 (TLR7) and TLR9 ligands inhibiting the expression of classic pro-inflammatory cytokines such as IL-12, Tumor necrosis factor α (TNFα) and IL-6. IL-4 also inhibits the increase in MHC class I expression, a very important IFN-induced response to stimulate antiviral adaptive immune responses. Furthermore, the suppressive effects on IFN response may play a role on HIV spreading in DCs, a natural reservoir for the retrovirus, since this cytokine increases the permissiveness of DCs to viral infection [88]. Nevertheless, it is usually utilized for the *in vitro* generation of mouse bone morrow-derived cDCs because DCs growth in GM-CSF supplemented with IL-4 are more potent stimulators of mixed lymphocyte reactions and more efficient in antigen presentation than cells growth in a medium containing GM-CSF alone [89].

The selection of a specific cocktail of cytokines is crucial to establish

specific *in vitro*-derived DC subtypes [90]. According to Belz et al, two *in vitro* models are utilized for investigating DC development and behaviour. The first one uses FLT3l to promote signalling through the FLT3 pathway aiming the generation of steady-state DC subsets. When BM precursors are cultured with Flt3L, pDCs and multiple lymphoid tissue-resident cDCs are generated [91-93]. A second one aiming the generation of monocyte-derived DCs, in which bone marrow precursors are cultured in a medium supplemented with GM-CSF and IL-4, giving rise to moDCs, the dominant inflammatory DC subset [94].

Collectively, several culture mediums supplemented with GM-CSF, IL-4, Flt3L, among others, have been utilized for the generation of DCs *in vitro* with distinct functional proprieties.

1.2.3. Dendritic Cells: Clinical Applications

The awareness that DCs are fundamental regulators of immune responses, in association with the development of techniques to obtain large numbers of DCs *in vitro* from isolated monocytes have boosted research on DC-based vaccination strategies [95]. The first clinical study was published in 1996 [96] and since then a considerable number of other clinical trials have been performed with promising results, specially in the cancer field. In fact, DC vaccination has proved to be efficient in inducing immunity. However, this efficacy relies on several DC factors, particularly the expression pattern and biological proprieties of particular receptors and on activation/maturation status of DCs [97].

Initially, these studies were based on the utilization of immature DCs. However, the investigators rapidly realized that these cells were inducing tolerance instead of immunity [95]. This fact resulted in a paradigm-switch to utilize mature DCs. DCs are normally pulsed with peptides, loaded with proteins or transfected with RNA encoding specific antigens, in addition to other alternative approaches that have also been used with encouraging results such as the generation of DC-tumour hybrids [98], the utilization of DC-derived exosomes [99] or even the combination of DC-based vaccination with other therapies such as chemotherapy [100, 101]. These important cells have been addressed in several studies with the objective of finding a rational design for novel DC vaccination techniques.

The methods for DC-generation *in vitro* have a major impact on vaccine's efficiency and their heterogeneity is associated with the observed largely variable clinical outcomes [60]. Indeed, the *ex vivo* DC manipulation can have 2 distinct genetic sources: autologous cells, which are removed from the own patient; and allogeneic cells, which are removed from healthy donors. Allogenic cells are normally associated with a higher probability of rejection, which makes the use of autologous cells a more promising strategy.

Most DC-based vaccines currently explored in clinical trials utilize mature antigen-loaded autologous DCs in order to promote antigen-specific T- and B-cell responses. DCs can be generated from distinct cell types such as monocytes, $CD34^+$ precursor cells, induced pluripotent stem cells (IPSC) [102] and embryonic stem cells (ESCs) [102, 103]. In most of the studies regarding induction of DCs *in vitro*, monocytes or CD34+ precursors obtained by cytapheresis directly from

patient's blood were used. This is a limiting factor because monocytes are associated with the production of low-efficient DCs while CD34+ hematopoietic progenitors and natural occurring DCs are found in limited number in peripheral blood mononuclear cells (PBMCs). On the other hand the utilization of IPSCs or ESCs, beside requiring expensive and technically challenging methods, may not be able to generate fully functional DCs due to the current inability to generate HSCs and definitive hematopoiesis from these cells. Moreover, DCs derived from cancer-bearing patients are frequently dysfunctional [60]. Therefore, there's still no ideal method for the *in vitro* generation of DCs. New insights are required to better understand transcriptional programming and, eventually, to translate this experimental data for the generation of patient-specific DCs. These efforts will contribute towards the development of more efficient vaccines for immunotherapy purposes with a wide range of applications.

1.3. Introduction to Cancer Immunotherapy

Manipulation of the patient's own immune system to treat cancer is an extremely promising strategy that could counteract the poor expectations on cancer prevalence for the next decades. Cancer is the second leading cause of death being responsible for approximately 13% of all deaths worldwide [104]. According to a recent study published in the British Journal of Cancer, 1 in 2 men and women will be diagnosed with cancer at some point of their lifetime [105]. Based on Cancer Research Institute, each year 14.1 million people are diagnosed with cancer worldwide. From those, nearly 8.2 million will die, which means that one person dies every 4 seconds due to cancer. Beside that, cancer deaths are expected to increase 2-fold from 2013 to 2030. These numbers are increasing not only due to the growth and aging of the population but also due to the increasing prevalence of already well-established risk factors such as smoking, obesity and overweight, sedentarism, viral infection, health disparities, among others [106]. Immunotherapy is an emerging field which as been extensively tested in the past years. In fact, the immune system, when manipulated properly, is able to attack cancer cells powerfully and systematically throughout the body. This immune-mediated attack is characterized by its extraordinary specificity, which is directly translated to fewer side effects, targeting specific antigens on cancer cells [44]. Moreover, it is also associated with the development of immunological memory, another key-feature of immunotherapy, providing a durable and persistent cancer protection [107].

The biology of cancer development can be described as a complex path with distinct crossings and obstacles that are responsible, alongside with individual genetic and immune features, for each patient-specific pathway on cancer progression [108]. This complexity makes each cancer unique, making it difficult to identify the fundamental abnormalities that support cancer homeostasis. However, a sequence of fundamental events generally associated with the emergence and progression of cancer can be projected [109]. First of all, a primary feature of cancer is the *tumour clonality*, which supports the idea that cancers are derived from single-cells that begin to proliferate atypically [110]. This characteristic was discovered on the basis of X chromosome inactivation

(XCI) process, which is a female mechanism to equalize the dosage of X-encoded genes [111]. Classically, one member of the X chromosome pair is inactivated, by chromatin compaction, during female embryonic development or upon differentiation of female ESCs. This inactivation process occurs randomly, which means that one X chromosome is inactivated in some cells while the other X chromosome is inactivated in other cells, resulting in the generation of tissues composed by a mixture of cells with distinct inactive X chromosomes [112]. However, tumour tissues are usually composed by cells with the same pattern of X inactivation, meaning that all those cells were derived from a single cell. However, the single-cell origin of the tumour do not suggest that the original progenitor cell had all the characteristics of a cancer cell [109]. Moreover, the development of cancer is a multi-step process that progressively becomes malignant through the gradual acquisition of genetic alterations [113, 114]. This developmental feature is supported by the fact that cancer incidence increases dramatically with age, which suggests that most cancers develop due to the accumulation of numerous abnormalities over time [115].

At the cellular level, cancer progression is defined as a multistep process implicating not only mutations but also a selection process in which cells with a progressively increasing ability to proliferate, survive, invade and promote metastasis are naturally conserved and selected. Therefore, we may highlight several key-steps underlying this process [109]. Firstly, *Tumour initiation* is thought to be the consequence of a genetic alteration that leads to a proliferative disorder at the single-cell level. This uncontrollable proliferation leads to the generation of cells resembling the progenitor cell status – *Tumour Progression*. While this process occurs, additional mutations arise within cells of the population. Our immune system is constantly sensing our tissues and thus it is able to identify cells with abnormal characteristics. However, some of these additional mutations confer a selective advantage to cells such as increased growth capacity and additional immune privilege status [116]. Consequently, such improved fitness will result in the prevalence of these cells within the tumour population- *clonal selection [110]*. This selection process continues to occur throughout tumour development, becoming continuously more rapid growing and with increasing malignancy.

Douglas Hanahan and Robert A. Weinberg have defined 6 hallmarks, 2 emerging hallmarks and 2 enabling characteristics typically associated with cancerous cells (**Figure 7**) [117].

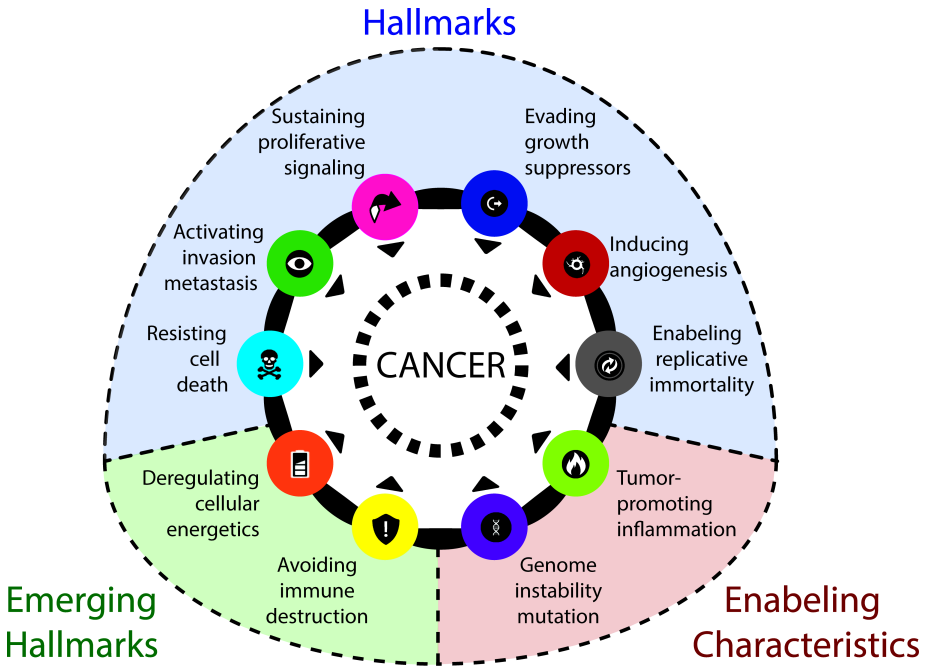


Figure 7. Cancer Hallmarks, emerging hallmarks and enabling characteristics.

The relevance of this cellular characteristics are truly important for cancer biology since their inhibition is able to harm tumour progression and thus, has some therapeutic effect. As a consequence, these hallmarks have provided a useful theoretical background for researchers to direct their own investigations on cancer biology, trying to identify possible therapeutic targets and alternative therapies for treating cancer patients. In fact, the past 2 decades have been characterized by a paradigm-switch from the utilization of non-specific cytotoxic agents to the use of selective and targeted therapeutics [118]. The first step towards cancer treatment was based on the use of chemical compounds – *chemotherapy*, able to kill highly-proliferative cells [119]. Despite their considerable toxicity and recurrent acquired resistance, chemotherapy is still the main pillar supporting cancer treatment. Recently, new advances on cancer progression and physiology have boosted the research on alternative treatment options such as targeted therapies and immunotherapy [120]. Targeted therapies have been shown to have impressive antitumoural responses on specific molecularly defined groups of cancer patients. However, since these therapies are directed to specific targets, they are frequently associated with regression followed by the emergence of drug-resistant variants [121]. These facts make therapies short-lived with a reduced overall clinical benefit.

Beside the advances on targeted therapies, immunotherapy has proven to be clinically relevant on treating oncogenic mechanisms [122]. Ipilimumab and Sipuleucel-T are two examples of well-successful immunotherapy-based treatments specially highlighted in extending cancer patients survival. Ipilimumab consists in an antibody directed against the specific cytotoxic T lymphocyte-associated antigen 4 (CTLA4), approved by the US Food and Drug Administration (FDA) as a first-line or second-line therapy for patients with advanced melanoma [123, 124]. These antibodies are able to block the inhibitory signal for activated T cells, busting the T cell-mediated attack and enhancing

tumour destruction. Sipuleucel-T is an active cellular immunotherapy approved by the US FDA to specifically treat castration-resistant prostate cancer patients, consisting in the utilization of autologous PBMCs, including antigen-presenting cells (APCs), such as DCs, activated ex vivo with a recombinant fusion protein consisting of a prostate cancer antigen fused with GM-CSF [125]. The clinical outcome of Ipilimumab in cancer treatment has illustrated how immunotherapy could achieve long-lasting responses through the generation of antigen-specific immunological memory. However, while considerable advances have been made in this field, the clinical outcome of the distinct cancer immunotherapies has been inconsistent, which may be associated with the potent immunosuppressive effects of well-established tumours and, in the case of DC-based vaccines, the variable efficiency of APCs generated *in vitro* [126, 127]. The analysis of both targeted agents and immunotherapy suggests a complementary and synergistic mechanism of action on cancer treatment, suggesting their use as a combinatorial therapy [120]. While targeted agents would induce a rapid tumour regression and a decrease on tumour-associated immunodepressive milieu, they would support the immune-mediated attack by limiting the protective tumour-microenvironment and promoting a more potent cytotoxicity. Moreover, targeted therapies can also be utilized do attenuate specific immune populations involved in immunosuppression, i.e. myeloid-derived suppressor cells (MDSCs) and forkhead box P3 (FOXP3)+ regulatory T (TReg) cells [128, 129]; to enhance tumour antigen presentation by DCs [130, 131] and to sensitize tumour cells for the immune-mediated attack [132, 133]. Further studies are needed in order to decipher the complex interplay between targeted agents and immunotherapy, providing a window for the specification of parameters such as the timing, dosage and sequence of administration - critical steps towards the integration of such agents in the context of immunotherapy.

Moreover, the generation of a clinically relevant anti-tumoural response may require the successful intervention in several immune processes through the rational design of synergetic combinations armed with agents targeting distinct steps on cancer progression.

1.4. RATIONAL DESIGN FOR THE GENERATION OF A POTENT ANTI-TUMOURAL VACCINE

The generation of a potent anti-tumoural vaccine relies on several aspects not only intrinsically related with DC function but also with the vaccination strategy itself, which requires the selection of specific antigens and loading procedures; maturation cocktails; routes of administration and cellular doses (**Figure 8**).

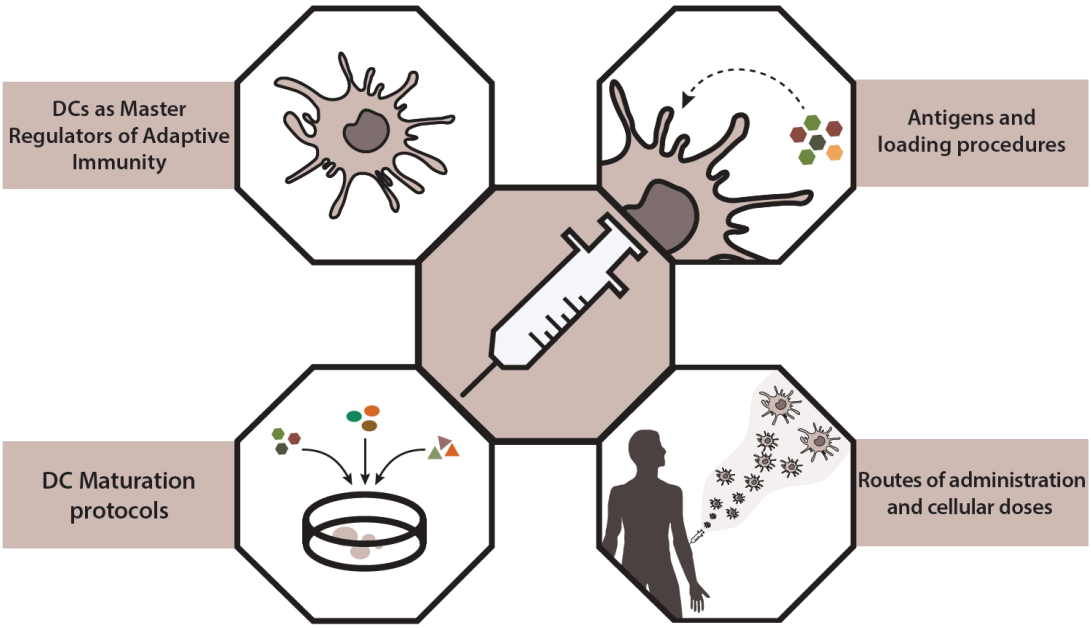


Figure 8. Rational Design for the generation of a potent anti-tumoural vaccine. Distinct aspects have to be considered during the design of a DC-based vaccine, i.e. type of DC, type of antigens and loading procedures, DC maturation protocols and routes of administration and cellular doses.

1.4.1. DENDRITIC CELLS AS MASTER REGULATORS OF ADAPTATIVE IMMUNITY

DCs are critical immune sentinels specialized in antigen capture, processing and presentation, being able to connect innate immunity and adaptative immunity. Therefore, they are ideal cells to kick-start adaptive immune responses. Mellman and colleagues have defined three key-steps required for mounting an effective antitumoural response [134]. The first one is the capture of tumour antigens, followed by processing and presentation, by DCs. The second one requires the presence of a suitable cocktail of activation and/or maturation signals that would allow DCs to differentiate, migrate to the lymph nodes, and present processed antigens to naïve T cells and B cells in an efficient way. The next step is associated with the expansion of enough T cells and B cells, to effectively produce a strong anti-tumoural response, and sufficient memory cells

to provide the desired long-term protection. Distinct methods have been utilized for the *in vitro* generation of DCs in a clinical context. These differ in terms of DC source, maturation cocktail, nature and procedure for antigen loading and route of administration [60]. In fact, the large-scale generation of stable DC able to induce antigen-specific Th1 responses has been a major goal in medicine with limited success, which suggests the need to develop new methods for the *in vitro* generation of DCs not only in enough number but also with an increased ability to uptake, process and present antigen to lymphocytes, promoting an efficient and specific immune-mediated response.

1.4.2. SELECTION OF ANTIGENS AND LOADING PROCEDURES

The correct selection of tumour-associated antigens and loading methods for the development of efficient DC-based vaccines has been considered critical. DCs have been pulsed with isolated or recombinant tumour antigens [135]; transfected with tumour messenger mRNAs [136]; transduced with antigen-coding genes [137] or even loaded with tumour cell lysates [138] or apoptotic tumour cells [139]. All these methods have been successfully utilized for the generation of an antigen-specific DC-mediated immune response, however with distinct efficiencies. Utilization of cell lysates or apoptotic bodies is advantageous since it encompasses multiple tumour-associated antigens (TAA). However, the utilization of short peptides is associated with its direct loading onto MHC molecules on the cell surface, while proteins and tumour lysates require internalization and processing. Nevertheless, the use of defined peptides is confined to the reduced number of known cancer antigens. Beside that, some of those antigens are restricted to specific HLA molecules, requiring the identification of patient's specific haplotype for its rational selection. Currently, 3 sets of antigens can be defined [140]: Tumour-specific antigens, oftenly associated with mutated proteins exclusively expressed on tumour cells; TAAs, which are endogenous antigens expressed on both tumour and normal cells usually deregulated in their expression levels or localization; and, the recently discovered class of antigens, the oncoantigens, expressed in the cell surface. This last category of tumour antigens is thought to be a suitable target for immunotherapies not only because of their relevant role in driving tumorigenesis but also because of their easily accessible location, which makes them ideal targets to elicit specific immune responses. Beside these promising oncoantigens, other types of antigens have been considered good therapeutic targets, especially in cancer prevention. For instance, cancer-testis (CT) antigens are a class of encouraging TAA candidates as novel targets for cancer prevention since they are overexpressed in a variety of distinct tumours, while they have a minimal expression in normal cells [141]. In fact, two CT antigens – *MAGE-A3* and *NY-ESO-1* - have been tested in clinical trials for their clinical efficacy in combination with adjuvants, being also in the top ten antigens in the Project for the Prioritization of Cancer Antigens [142].

1.4.3. Dendritic Cell Maturation protocols

Even though DC-based vaccines have proven to be efficient on promoting immunity, depending on the DC maturation status, these cells may target different types of immune responses. Immature DCs are normally characterized by its poor ability to migrate from the injection sites to the lymph nodes, inducing IL-10-producing antigen-specific regulatory T cells (Tregs) [143], causing tolerance. Beside the DC maturation status, cytokine profile is also a parameter of great importance. For instance, the expression of IL-12p70 and CCR7 has shown to be directly proportional to vaccine efficiency by promoting CTL responses and DC migration to lymph nodes, respectively [97]. Although several maturation protocols have been tested in the past decades, we still don't have an unanimously adopted cocktail for promoting the desired DC maturation status. In general, the more promising already-established maturation protocols utilize proinflammatory cytokines, i.e. CD40 ligand, in combination with TLR agonists [144].

According to literature, distinct culture media supplemented with GM-CSF, IL-4 and Flt3L have been utilized for the generation of DCs *in vitro* with distinct functional proprieties [49]. In order to increase DC immunogenicity, these cells are usually administrated in combination with adjuvants. For instance, clinical trials utilizing Human DC-based vaccination have addressed this issue. Between the most commonly utilized adjuvants, we can highlight GM-CSF, IL-2, IFN-α, and TLR agonists [145]. While GM-CSF promotes a stronger recruitment and maturation of DC, IFN-α enhances antigen cross-presentation, providing a maximum cytotoxicity. Other relevant point to consider in DC maturation protocols is the exposure-time to the stimuli required for the desired maturation. Normally, DCs are momentarily exposed to maturation/danger signals in peripheral tissues, taking 2-4 hours to reach the lymph nodes. However, most currently utilized protocols specify an exposure time of 24-48 hours, which may be contributing for the exhaustion of DCs, especially when considering the production of cytokines favouring the cytotoxic T lymphocyte activity (CTL) [146].

1.4.4. Route of administration and cellular doses

Since the efficacy of DC-based vaccines relies on the migration of enough DCs to the lymph nodes, the route of administration, frequency of vaccination and the number of cell injected are also relevant aspects to consider. In the past years distinct routes of administration have been tested, being all of them able to elicit specific antitumoural immune responses, although with distinct characteristics [60, 147]. For instance, DCs injected intravenously (i.v.) shows preferential accumulation in the liver and spleen, being detected also in lymph nodes. When intradermally (i.d) or subcutaneously (s.c.) injected, DCs remain in the injection site or are cleared by phagocytes, whereas less then 5 % of DCs are able to reach the LN. Considering that less than 3% of cells injected via i.d. or s.c. reach the LNs [148], another possibility would be to administrate the vaccine directly inside the lymph nodes, which requires the ultrasonic guidance of the needle by a radiologist [149]. This approach seemed to be promising because we

would be maximizing the number of DCs reaching the LN. However, the efficiency of this administration on promoting antitumoural T-cell responses was comparable or even inferior to the intradermal administration [150]. There's a tendency now to combine distinct routes of administration in order to obtain a vigorous response. Considering the cellular doses, the minimal number of DC capable of inducing immunity has not been defined yet. There's high variability between clinical trials regarding the dose of DCs utilized for therapeutic purposes, ranging between 1 dose of 0.3×10^6 cells to 3 doses of 200×10^6 cells [60]. While some studies have positively correlated the number of administrated DCs with the vaccine efficacy, other studies have shown that the administration of reduced numbers of DCs would be beneficial for the LN migration, enhancing anti-tumoural cytotoxicity. These data may suggest that the multiple i.d. injections with reduced numbers of DCs would be beneficial for cancer therapy.

1.5. DC-BASED VACCINE FOR ACUTE MYELOID LEUKAEMIA (AML)

Acute myeloid leukaemia (AML) can be described as an aggressive, clonal myeloid neoplasm characterized by an impaired myeloid maturation leading to the accumulation of myeloblasts in BM and blood [151]. In adult AML, intensive chemotherapy is able to promote total cytomorphological remission in almost 80% of patients [152]. However, the majority of those suffer a subsequent recurrence of the disease, especially in patients over 60 years-old and in specific cytogenic and molecular risk groups [153]. In this context, post-remission therapy is crucial to eliminate minimal residual disease (MRD) and, consequently, to achieve complete remission [154]. The most potent antileukemic effect has been achieved by allogeneic hematopoietic stem cell transplantation (HSCT), showing the lowest rate of relapse and a relevant overall survival in specific age groups [155]. However, this may not be suitable for certain patient groups due to donor availability, age or patient's comorbidity. Beside that, HSCT is associated with high rate of morbidity and mortality [156, 157]. Consequently, targeted therapies may be considered suitable alternative strategies to eliminate MRD in AML-patients not eligible for HSCT. Immunotherapy is able to induce a strong and specific immune response against defined cancers has boosted research in the field of tumour immunology. Consequently distinct cytotoxic T-lymphocyte (CTL) responses have been detected against leukaemia-associated antigens, i.e. Wilm's tumour protein 1 (WT1), human telomerase reverse transcriptase (hTERT), preferentially expresses antigen in melanoma (PRAME), proteinase 3, between others [152].

The awareness that DCs could be attractive potential candidates for tumour and anti-leukemic vaccination strategies has highlighted the potential use of DC-based vaccines for the induction of effective anti-tumoural responses.

Since monocytes or CD34+ precursors are usually used to generate DCs *in vitro* and these cells may be compromised in patients diagnosed with AML, there's momentum for the application of direct reprogramming for the generation of patient specific APCs aiming the post-remission therapy in AML.

1.6. AIMS OF THIS STUDY

Innate and adaptive immune systems act side by side as a self-defense mechanism against foreign threats. This complex interplay relies on the ability of dendritic cells DCs to detect non-self antigens and to induce a subsequent antigen-specific immune response. This unique properties of DCs as professional APCs have boosted research on DC-vaccine strategies for cancer therapy. In the context of AML, post remission therapy appears to be a good target for DC-based vaccines aiming at the elimination of minimal residual disease. Clinical trials are on going utilizing DC-mediated immunotherapy but have met limited success due to insufficient numbers and APC efficiency of *in vitro* monocyte-derived DCs. An alternative approach is required for the generation of autologous APCs.

Direct reprogramming allows the conversion of one cell to another via transcription factor expression. The direct reprogramming field has been mainly focused on the induction of distinct cell types such as hepatic, β-cells, neuronal cells and hematopoietic progenitors for regenerative medicine. There is momentum to generate autologous directly reprogrammed DCs for the control of immune responses and inducing immunity. The overall aim of this study is to generate professional APCs from fibroblasts via direct reprogramming. We will use combinatorial overexpression to define the minimal combination of TFs that is able to induce an efficient conversion of fibroblasts to DCs for AML immunotherapy.

This study aims the identification of a combination of TFs that specify the DC lineage. Initially, candidate selection will be performed by literature mining and bioinformatic analyses. Subsequently, selected TFs will be cloned in a reprogramming proven Doxycycline-inducible lentiviral system. This approach will allow the inducible overexpression of DC-specific TFs in mouse and Human fibroblasts and the identification of a minimal combination of TFs to induce efficient activation of a DC-specific reporter. Finally, expression of molecules involved in antigen presentation will be assessed as well as whether reprogrammed cells are stable upon exogenous transcription factor removal.

Collectively this study will contribute to a better understanding of DC specification by determining the minimal transcriptional network required for the establishment of the DC lineage and antigen-presenting capacity in an unrelated cell-type. In the future reprogrammed DCs may represent an alternative method for the *in vitro* generation of efficient autologous APCs for clinical use, in particular, for the generation of a personalized immunotherapy targeting the elimination of minimal residual disease in AML patients.

CHAPTER II

2. METHODS

2.1. BIOINFORMATIC ANALYSIS

Several bioinformatic tools were applied under the scope of this study. The screening of TFs with a potentially relevant role for the DC lineage was performed using BioGPSmatch (Pereira and Papatsenko, unpublished). This software allowed the identification of several potential TFs for both mouse and human systems. The expression level of all given genes was individually confirmed in *biogps.org* website, an extensible gene annotation portal that allows the user to verify the expression profile of single genes in distinct cell types or tissues, in order to avoid the selection of non-specific TFs. From this analysis, only TFs which expression was, at least, 3-fold higher than the overall median were considered. The mouse and human expression profile of selected TFs was extracted from GeneAtlas MOE430 and GeneAtlas U133A datasets (BioGPS.org), respectively. Expression data for the candidate TFs in bone-marrow derived macrophages and DCs, as well as for DC progenitor populations (MDPs, CDPs and pre-DCs), was obtained from Gene Expression Omnibus (GEO) under the accession numbers GSE62361 [158] and GSE66565 [74], respectively.

Expression data was analysed by cluster 3.0 (log-transformed data; centered genes; centered, clustered and normalyzed cell types) and displayed by Treeview.

Specificity of the Clec9a gene for the DC-lineage was assessed in Immunological Genome Project (www.immgen.org) ([159].). Single-cell data regarding the expression of Clec9a in DC progenitors, as well as regarding the expression of DCi1, DCi6, DCi5 and DCi4, was obtained from GEO under the accession number GSE60783 [55], and displayed in Prism 6.

Gene ontologies for the selected 19 TFs were acquired from Enrichr (Ma'ayan Laboratory - Computational Systems Biology lab, New York) while gene Phenotypes were acquired from Network2canvas, MGI – Mammalian Phenotype – Top 4 (Ma'ayan Laboratory - Computational Systems Biology lab, New York).

2.2. LENTIVIRAL-BASED INDUCIBLE EXPRESSION SYSTEM

The expression system used is a reprogramming proven Doxycycline (Dox)-inducible lentiviral vector containing the mOrange coding sequence (CDS) flanked by EcoRI restriction sites [31] (**Figure 1**). The mOrange CDS was replaced by individual candidate TF CDSs.

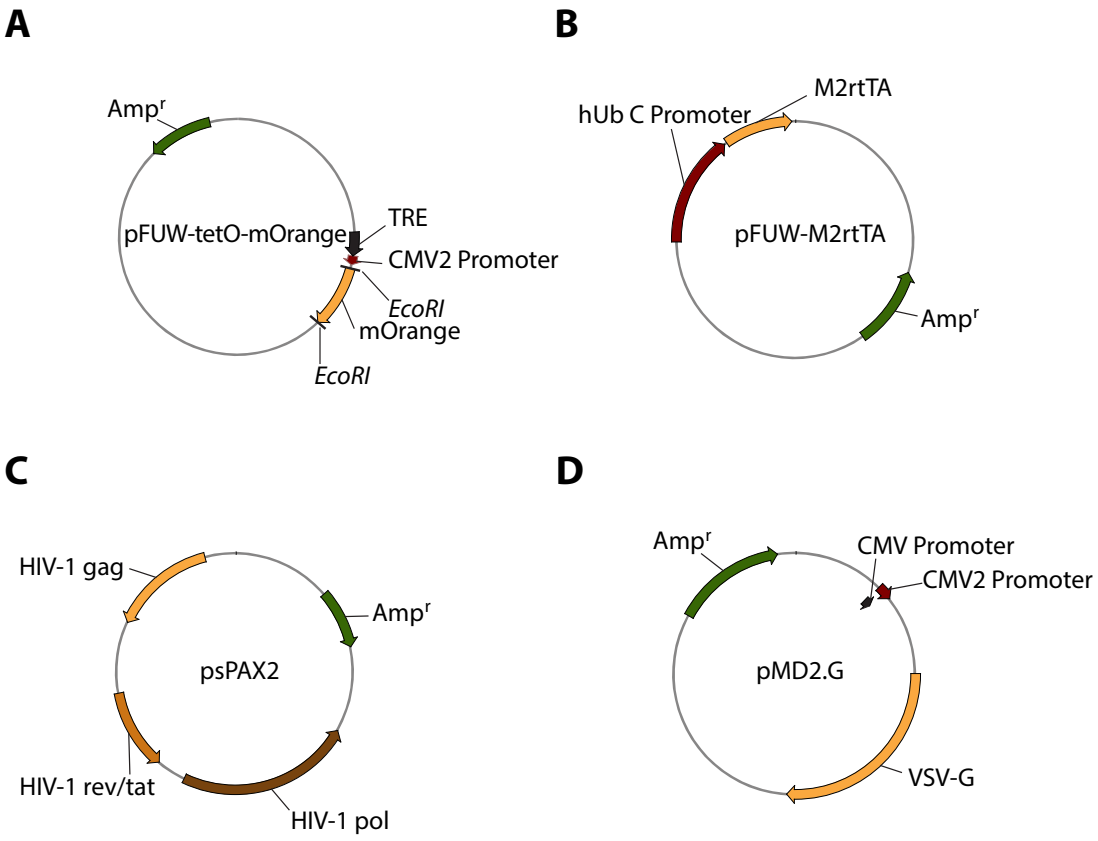


Figure 9. Lentiviral-based gene expression system used in this study. (A) pFUW-tetO plasmid was used for inducing the overexpression of TFs. mOrange is cloned downstream a tetracycline operator (Tetracycline Response Element, TRE) and a minimal CMV promoter (CMV2). mOrange is flaked by EcoRI restriction sites and was substituted by individual TFs. **(B)** pFUW-M2rtTA is a lentiviral vector containing the reverse tetracycline transactivator (M2rtTA) under the control of a constitutively active human ubiquitin C promoter (hUb C Promoter). (**C**) psPAX2 is a packaging plasmid that encodes the viral proteins gag, pol, rev and tat. (**D**) pMD2.G is an envelope vector encoding the envelope protein VSV-G. AMP^r, Ampicillin resistance gene.

2.3. POLYMERASE CHAIN REACTION (PCR)- BASED CLONING OF CANDIDATE TFS

The coding sequences (CDS) of each TF were inserted individually into the pFUW-tetO vector through a sub-cloning process for generating individual pFUW-tetO-TF plasmids [31] (**Figure 2**, exemplified for the TF Irf4).

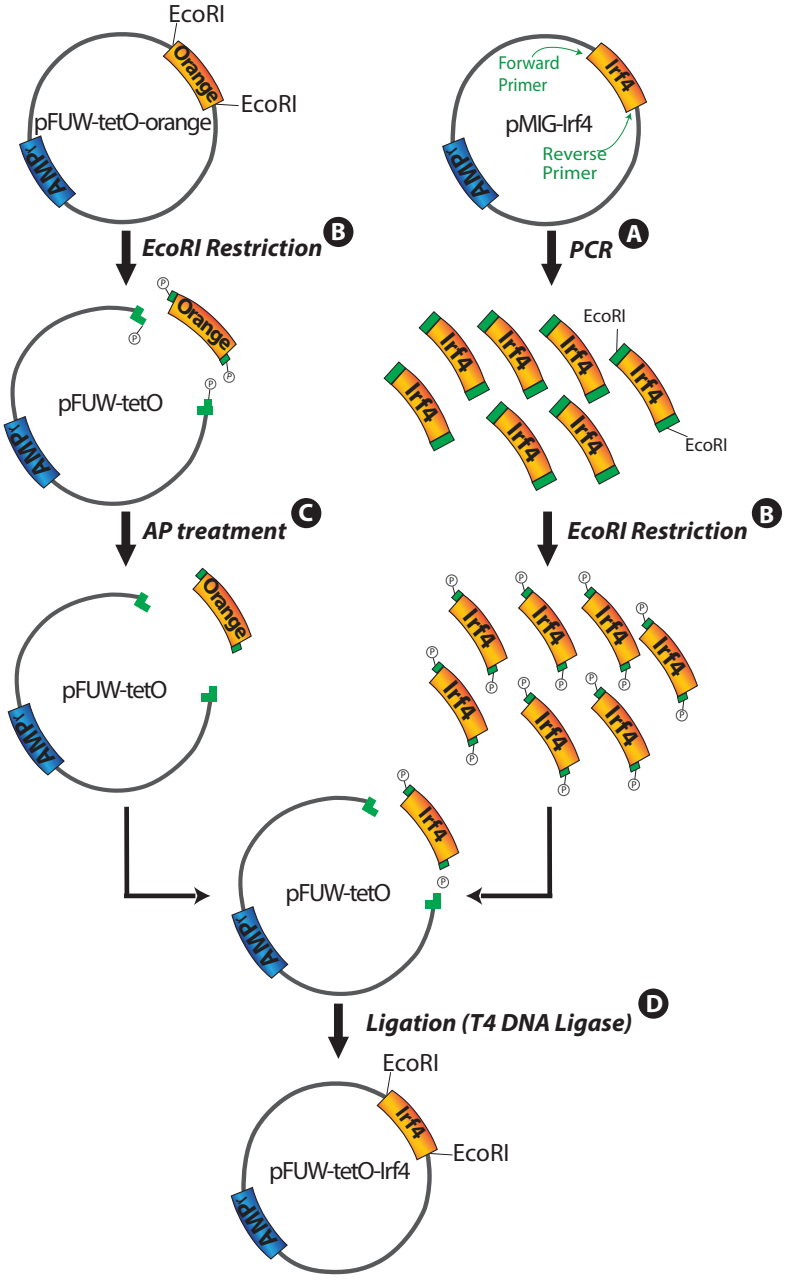


Figure 10. Strategy for cloning candidate TFs coding sequences in the pFUW-tetO plasmid. Cloning of Irf4 coding sequence in the pFUW-tetO plasmid is shown as an example of the PCR-based cloning strategy. **(A)** Irf4 coding sequence was amplified by PCR with cloning primers, allowing the insertion of EcoRI restriction sites at the beginning and end of the coding sequence. **(B)** Both PCR product and pFUW-tetO-mOrange original vector were submitted to an enzymatic restriction with EcoRI, enabling the generation of cohesive ends. **(C)** pFUW-tetO backbone was dephosphorylated in order to prevent re-circularization. **(D)** Irf4 coding sequence is inserted into the dephosphorylated pFUW-tetO backbone by enzymatic ligation.

Single TF CDSs were bought from 3 distinct providers (Openbiosystems; OriGene Technologies; Addgene) as part of their complementary DNA (cDNA) libraries or plasmid collections. Sequencing primers were designed (**Table 1**) and TF CDS sequences were analysed by Sanger sequencing (Sanger Sequencing Service, GATC Biotech). Sequencing results were aligned with the consensus CDS (NCBI) for the individual gene with vector NTI software.

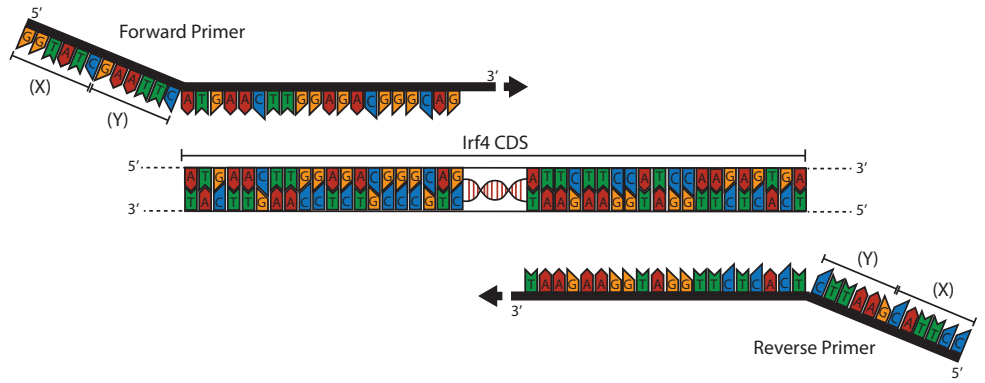
Table 1. Primers used for sanger sequencing. Primers for each original plasmid were designed for verifying TF CDS sequence by sanger sequencing.

Plasmids	Primer	Sequence
pFUW-tetO-DCi1 pFUW-tetO-DCI2 pFUW-tetO-DCI3	*pFUW-seq*	5'-TCCACGCTGTTTTGA-3'
pCMV6-DCI4 pCMV6-DCi9 pCMV6-DCI10 pCMV6-DCi15	*VP1.5*	5'-GGACTTTCCAAAATGTCG-3'
pSPORT-DCi5 pSPORT-DCi7 pSPORT-DCi8 pSPORT-DCi11 pSPORT-DCi14 pSPORT-DCi12 pSPORT-DCi17	*SP6*	5'-ATTTAGGTGACACTATAG-3'
pMIG-DCi6	*pMIG-seq*	5'-CACCCTAAGCCTCCGCCTCCTCT-3'
pMX-Hu-DCi13	*pMXs-seq*	5'-GACGGCATCGCAGCTTGGATACA-3'

Forward and reverse primers were designed (**Figure 4A**) including the first 20 nucleotides of the CDS or its reverse complementary sequence, respectively. Restriction sites were inserted upstream for either *EcoRI* (5'-GAATTC-3') or *MfeI* (5'-CAATTG-3') restriction enzymes. Extra nucleotides for maximizing restriction enzyme cutting efficiency were also inserted upstream restriction sites in both forward (5'-GGTATC-3') and reverse primers (5'-CCTTAC-3'). PCR amplifications using a proof-reading polymerase (Thermo Scientific Phusion Flash High-Fidelity PCR Master Mix) were performed in 20 μl reactions with the following cycling program: 1 initial saturation cycle (98°C, 10 seconds); 30 cycles of denaturation (98°C, 1 second), annealing (58°C, 5 seconds) and extension (72°C, 30 seconds); 1 final extension at 72°C for 1 minute). PCR-products were separated by gel electrophoresis in a 1% Agarose gel (Calbiochem OmniPur Grade Agarose), fallowed by image acquisition under U.V. light to confirm correct band size (**Figure 4B**). PCR-product bands were purified (GenElute Gel Extraction Kit,

Sigma) and submitted to enzymatic restriction. Inserts with *EcoRI* restriction sites present in the CDS sequence were restricted with *MfeI* (New England Biolabs), which is able to generate cohesive ends compatible with the ones generated by EcoRI restriction. Total purified PCR product was restricted at 37°C during 2 hours and 30 minutes, purified by gel electrophoresis, extracted from gel and DNA concentration was quantified by spectrophotometry (Nanodrop 1000, Thermo Fisher scientific).

A



B

TFs	Primer Forward	Primer Reverse	Restriction enzyme	Product size (bp)
DCi1	GGTATCGAATTCatgttacaggcgtgcaaaat	CCTTACGAATTCgaaaggtagggtgtccagga	EcoRI	819
DCi2	GGTATCGAATTCatgccgcgctcattcctggt	CCTTACGAATTCtcatttgagtccatgctgag	EcoRI	1470
DCi3	GGTATCGAATTCatggatgctgatgagggtca	CCTTACGAATTCttagctcatgtggaagcggt	EcoRI	1548
DCi4	GGTATCGAATTCatgtcgcaagggcccccgc	CCTTACGAATTCtcatcgtggtagacagctgg	EcoRI	357
DCi5	GGTATCGAATTCatgtgtgaccggaacggcgg	CCTTACGAATTCttagacggtgatctgttgat	EcoRI	1275
DCi6	GGTATCGAATTCatgaacttggagacgggcag	CCTTACGAATTCtcactcttggatggaagaat	EcoRI	1353
DCi7	GGTATCCAATTGatgccggtggaacggatgcg	CCTTACCAATTGttaacagctcttgacacggg	Mfel	1050
DCi8	GGTATCGAATTCatggctcagtggaaccagct	CCTTACGAATTCtcacatgggggaggtagcac	EcoRI	2313
DCi9	GGTATCCAATTGatgaacaaccgaaaggaaga	CCTTACCAATTGtcagtgactccagggtctga	Mfel	1302
DCi10	GGTATCGAATTCatgccctgcgtccaagccca	CCTTACGAATTCttagaaaggtagggtgtcca	EcoRI	1878
DCi11	GGTATCCAATTGatggcctccccggctgacag	CCTTACCAATTGtcagcaggctttggggagct	Mfel	2124
DCi12	GGTATCGAATTCatgcatcaccaacagcgaat	CCTTACGAATTCtcacatctgtcccatgtgat	EcoRI	2013
DCi13	GGTATCGAATTCatggactacgactcgtacca	CCTTACGAATTCttagtagccagtgaggtatg	EcoRI	1095
DCi14	GGTATCCAATTGatgcagctgagaaaaatgca	CCTTACCAATTGttacctggagtccgaagccg	Mfel	1389
DCi15	GGTATCGAATTCatgcttgctctggaggctgc	CCTTACGAATTCtcagacatgccgggaggctg	EcoRI	804
DCi16	GGTATCGAATTCatgaatttcccactgggga	CCTTACGAATTCtcaaagtttctcagaactta	EcoRI	576
DCi17	GGTATCGAATTCatgccgagtcgccgcgctgc	CCTTACGAATTCctacgtggcttcaggccctg	EcoRI	1668
DCi18	GGTATCGAATTCatggcatcgaacagcatctt	CCTTACGAATTCtcagtagggccgccacacgg	EcoRI	1290
DCi19	GGTATCCAATTGatgaaagccttcagtccggt	CCTTACCAATTGttagccacagagtactttgc	Mfel	405

Figure 11. Primer design for PCR-based cloning strategy. (A) Scheme exemplifying the strategic design of primers for the TF Irf4. Each primer is composed by a sequence for improving restriction enzyme binding (X), a restriction site (Y) and 20 nucleotides from the CDS or reverse complementary sequence. **(B)** Table showing amplification primers for each TF with the corresponding restriction enzyme and product size.

The pFUW-tetO backbone, obtained through EcoRI restriction of 1 μg of pFUW-tetO-mOrange, was treated with alkaline phosphatase (AP, from calf intestine, Roche) at 37°C during 15 minutes, promoting dephosphorylation of 5´ and 3´ ends, a critical step to prevent plasmid re-ligation, and separated from mOrange by gel electrophoresis. The linear and dephosphorylated pFUW-tetO

plasmid was subsequently extracted from agarose gel, DNA concentration was quantified by spectrophotometry and purified DNA was kept at -20°C until further use. The ligation reaction (T4 DNA ligase, New England Biolabs) for each TF was performed with approximately 30 ng of pFUW-tetO backbone, in 10 μl reactions with a 1:3 molar ratio of pFUW-tetO during 1 hour at room temperature. A negative control including water instead of insert DNA was used (ligation without insert) to compare the number of colonies to the background levels. The ligation product was immediately used for bacterial transformation or was kept at -20°C for further use.

2.4. INDUCTION OF CHEMICALLY COMPETENT BACTERIA

Two distinct protocols were used for the induction of competent *Escherichia coli* (*E. coli*). As initial stock we have used chemically competent E. coli (NEB DH5-alpha Competent *E. coli* (High Efficiency)).

The first protocol was based on Chung et al. [160, 161]. A 5 ml overnight culture of *E.coli* was growth in LB Broth media (Vegetable LB Broth (Lennox), Pronadisa) at 37° C with shaking (180 rpm). In the following morning, the 5 ml culture was diluted in 50 ml LB Broth in a 200 ml conical flask (1:100 ratio) and grown at 37° C to an Optical Density at a wavelength of 600nm (OD600) of 0.5 to 0.6. The 50 ml culture was split into two 50 ml falcon tubes and incubated on ice for 10 minutes. All subsequent steps were carried out at 4° C. Both falcons were centrifuged at 3000g for 10 minutes and each pellet was resuspended in 5.5 ml of chilled TSS buffer [10% Polyethylene glycol 8000 (VWR, AMRESCO); 10 mM Mgcl2 (Ambion, Nalgene); 5% Dimethyl sulfoxide (Fisher BioReagents)]. Bacteria were aliquoted in chilled microcentrifuge tubes (200 μl/tube) and kept at -80° C until further use.

The second protocol was based on Inoue et al. [162]. A single *E.coli* colony was picked from a plate previously incubated for 16 hours at 37°C, transferred into 25 ml LB Broth in a 500 ml Erlenmeyer flask and incubated for 7 hours at 37°C with shaking (250 rpm). After 7 hours, the 25 ml culture was used to inoculate three 1 L Flasks, each one containing 250 ml of LB Broth. One flask received 10 ml of starter culture while the other two received 4ml or 2ml. The three flasks were incubated overnight at 20°C with shaking (120 rpm). In the following morning, the OD600 of each overnight culture was measured and continuously monitored every 30 min until the OD of one culture has reached 0.55. This culture was transferred to an ice bath for 10 minutes while the other two cultures were discarded. After a 10 minute incubation on ice, cells were harvested by centrifugation at 2500 g for 10 min at 4°C. The supernatant was discarded and remaining drops were carefully removed with a vacuum aspirator. The cell pellet was carefully resuspended in 80 ml of ice-cold Inoue

transformation buffer (ITB) [163] [55 mM MnCl2*4H2O (Alfa Aesar); 15 mM CaCl2*2H2O (Emsure, Merck Millipore); 250 mM Kcl (VWR chemicals); 10 nM PIPES pH 6.7 (Alfa Aesar); filtered through a 0.45-μm filter (Corning)] and harvested again by centrifugation at 2500 g for 10 min at 4° C. The medium was discarded and cells were carefully resuspended in 20 ml of ice-cold ITB. Then, 1.5 ml of DMSO was added to the cell suspension, gently mixed and incubated on ice for 10 min. Aliquots of 500ul were dispensed in chilled microcentrifuge tubes, snap-frozen through immersion in a liquid nitrogen bath and placed at -80 ° C until further use. To access the transformation efficiency of each batch of competent bacteria, the number of colonies per μg of plasmid DNA was calculated.

2.5. TRANSFORMING COMPETENT BACTERIA WITH PLASMID DNA

To transform competent bacteria, a tube of competent *E.coli* was removed from the -80°C freezer and thawed on ice. Ligation products or 10 μl of 10 to 100 ng of plasmid DNA were used for bacterial transformation. 50 μl of ice-cold competent bacteria were mixed with the DNA solution and incubated on ice for 30 minutes. After the incubation on ice, a heat-shock was applied by rapidly transferring the DNA-bacteria mixture to a 42°C water bath. After the 1 minute, the mixture was immediately put on ice for 5 minutes. Then, 500 μl of LB Broth were added to the mixture and incubated on a water bath at 37°C for 30 minutes, allowing the bacteria to express the ampicillin resistance gene encoded by the plasmid. After 30 min, the bacterial suspension was spread in LB agar plates (Vegetable LB Agar (Lennox), Pronadisa) supplemented with 100 µg/mL ampicillin (G Biosciences) using glass beads. Plates were kept at room temperature until the liquid was completely absorbed. Beads were removed, plates were inverted and incubated at 37°C for 16 hours. Two controls were included in each transformation: a positive control, 80 ng of pFUW-tetO-mOrange plasmid, and a negative control, ligation without the insert.

2.6. SCREENING FOR POSITIVE COLONIES BY COLONY PCR OR ENZYMATIC RESTRICTION

After transforming competent bacteria, the screening for positive colonies was performed by PCR amplification (NZYTaq Master Mix, NZYtech) using insert-specific primers (Figure 4B) or by enzymatic restriction. For each LB agar plate >10 colonies were assessed for the presence of the insert. Positive (original plasmid containing the insert) and negative (pFUW-tetO-mOrange) controls were included. The amplification product size was accessed by gel electrophoresis fallowed by analysis under U.V. light. Colonies with appropriate band size were

sent to the Sanger sequencing service (GATC Biotech, Germany). Alternatively, individual bacterial clones were picked and minicultures were grown overnight. Next day, plasmid DNA was extracted (NZYMiniprep kit, NZYTech) and restricted with EcoRI enzyme at 37°C during 2 hours and 30 minutes. Restricted plasmids were analyzed after gel electrophoresis under U.V. light. Colonies with 2 bands, one corresponding to the pFUW-tetO backbone and another corresponding to the gene CDS size, were sent for sequencing.

2.7. CELLS AND CELL CULTURE CONDITIONS

Distinct cellular systems were used under the scope of this work, either 293T cell line (ATCC), a highly transfectable derivative of human embryonic kidney 293 cells, primary cultures of C57BL/6 MEFs, Clec9a-Cre X R26-stop-tdTomato MEFs (collaboration with Professor Caetano Reis e Sousa, The Francis Crick Institute, London, UK)) and adult Human dermal fibroblasts (HDFa, ScienCell). All tissue culture reagents were from Thermo Fisher Scientific unless stated otherwise. 293T cells and MEFs were maintained in growth medium [Dulbecco's modified eagle medium (DMEM) supplemented with 10% (v/v) foetal bovine serum (FBS) (Gibco), 2mM L-Glutamine and antibiotics (10 μg/ml Penicillin and Streptomycin)], grown until confluence, dissociated with TrypLE Express and frozen for further use in 10% dimethyl sulfoxide (DMSO), 90% FBS. All cells were maintained at 37° C and 5% (v/v) CO2.

2.8. TRANSFECTION: VIRAL PRODUCTION IN 293T CELL LINE

A 2nd-generation lentiviral system was utilized for the generation of viral particles, consisting in 3 distinct plasmids: a lentiviral transfer plasmid; a packaging plasmid (psPAX2.G) encoding viral proteins, i.e. Gag, Pol, Rev and Tat genes; and an envelope vector (pMD2) encoding the envelope protein VSV-G that infect both mouse and human cells (Figure 5). As lentiviral transfer plasmid we have used the pFUW-tetO plasmid [155], which drives the gene of interest under the control of a doxycycline-responsive promoter and a minimal CMV promoter, or the pFUW-M2rtTA, a previously described lentiviral vector containing the reverse tetracycline transactivator M2rtTA under the control of a constitutively active human ubiquitin C promoter [164]. In this system, the M2rtTA protein is responsible for bridging doxycycline to the doxycycline-inducible promoter. Cells were transfected at 30-50% confluence in 10 cm plates. For each 293T plate a mixture of 10 μg of transfer plasmid, 10 μg of psPAX2.G and 5 μg of pMD2 was prepared in a 15 ml conical tube and adjusted to a final volume of 500 μL with autoclaved water. Then, 62.5 μl of 2M CaCl2 (Merck Millipore, Massachusetts, EUA) were added to the mixture and, subsequently, using a Pasteur pipette and a pipet aid, bubbles were continuously released into the DNA

mixture while carefully adding 500 μL of 2x BES-buffered saline (Sigma) drop-wise. This step is critical for generating DNA complexes that are required for cellular transfection. The resulting solution was incubated at room temperature (RT) for 15 min. Meanwhile, media from 293T cells seeded the day before (passaged1:6) was aspirated and replaced by 10 ml of fresh growth medium without antibiotics. The DNA mixture was then evenly distributed drop wise in the 293T cell plate, fallowed by overnight incubation at 37°C. After 24h incubation, expression of fluorescent protein mOrange was confirmed by fluorescence microscopy. 293T culture media was aspirated, replaced by 4 ml of fresh growth medium and incubated at 32°C, 5% (v/v) CO2. Supernatants were collected at 36 h, 48 h and 60 h post-transfection and stored at 4°C. Viral-containing supernatants were filtered through a 0.45 μm low protein binding filter (Corning) and combined with filtered pFUW-M2rtTA virus at a 1:1 ratio. Virus were kept at 4°C and used fresh for direct reprogramming experiments (Figure 5).

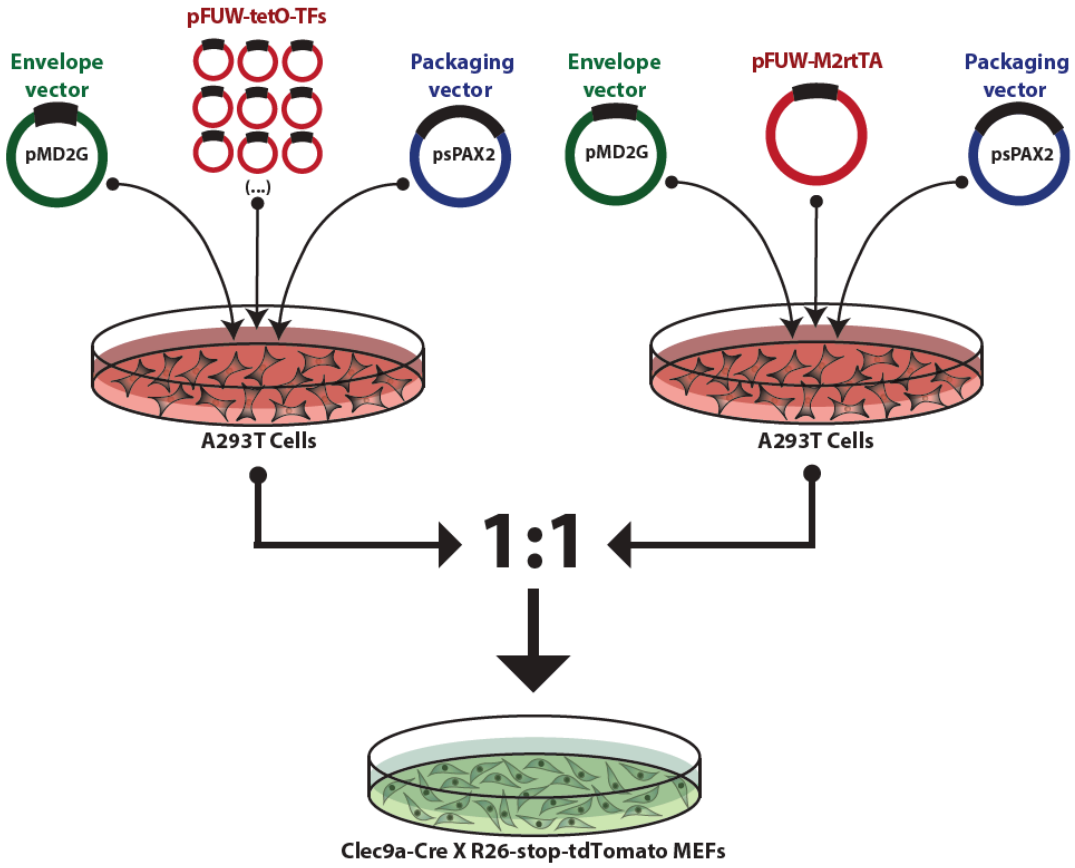


Figure 12. A second-generation lentiviral system was used for screening DC fate inducing TFs in cultured MEFs. A293T cell line was transfected with a mixture of viral plasmid and packaging constructs expressing the viral packaging functions and the VSV-G protein. Viral supernatants were collected after 36, 48 and 60 hours, filtered and used for MEF transduction (50% pFUW-tetO-TF viral cocktail, 50% pFUW-M2rtTA viral suspension).

2.9. LENTIVIRAL TRANSDUCTION OF MEFS AND HDFS

Fibroblasts were plated in gelatin-coated (Sigma) 6-well plates at a density of $0.5x10^5$ MEFs or $0.2x10^5$ HDFs per well. Subsequently, 2 transduction rounds were performed in subsequent days in the presence of 8 μg/ml of polybrene. This cationic polymer increases gene transfer efficiency via neutralization of the negative electrostatic repulsion between the cell surface and the viral particles, increasing virus absorption [165]. Culture media was replaced for 8 hours with normal growth medium 12 hours after the first transduction. To induce reprogramming, culture media was replaced 12h after the second transduction by growth medium supplemented with 1 μg/ml of doxycycline (Sigma-Aldrich) (day 0). To induce macrophage reprogramming [26] in MEFs culture media was supplemented with 10 ng/ml of Macrophage-Colony Stimulating Factor (M-CSF). During the experiment, culture media was replaced every 2 or 3 days with fresh media and cells were analyzed by fluorescence microscopy and flow Cytometry at defined time-points.

2.10. FLOW CYTOMETRY ANALYSIS

To analyze Clec9a-driven expression of tdTomato by flow cytometry, transduced MEFs were dissociated with TrypLE Express, resuspended in 200 μL PBS 5% FBS and kept at 4ºC prior analysis in a BD Accuri C6 Flow Cytometer (BD Biosciences). Sample acquisition was performed with the standard configuration 3-blue-1-red (533/30 filter in FL1; 585/40 in FL2, 670 LP in FL3 and 675/25 in FL4). tdTomato fluorescence was analyzed in the FL2 channel.

For the analysis of the CD45 or MHCII cell surface marker expression, dissociated cells were incubated with APC-Cy7 rat anti-mouse CD45 (Clone 30-F11, BD Biosciences) antibody or Alexa Fluor 647 rat anti-mouse I-A/I-E (Clone M5/114.15.2, BD Pharmingen) diluted in PBS 5% FBS at 4ºC for 30 minutes with rat serum (1/100, GeneTex) to block unspecific binding. Finally, MEFs were washed with PBS 5% FBS, resuspended in 100 μL of PBS 5% FBS and analyzed in a BD Accuri C6 Flow cytometer using the FlowJo software. CD45 APC-Cy7 and I-A/I-E Alexa Fluor 647 fluorescence were analyzed in FL4 channel. Flow cytometry results were analyzed using the FlowJo software (FLOWJO, LLC, version 7.6).

2.11. FLUORESCENCE ACTIVATED CELL SORTING (FACS)

Clec9a reporter MEFs were isolated from E13.5 embryos and expanded until confluence. Prior sorting, MEFs were incubated at 4ºC for 30 minutes with APC-Cy7 CD45 antibody diluted in PBS 5% FBS. Subsequently, MEFs were washed with PBS 5% FBS, resuspended in PBS 5% FBS and tdTomato- CD45- MEFs were purified in BD FACSAria III (BD Biosciences).

For assessing the stability of induced cells, tdTomato+ cells at day 15 were purified using BD FACSAria III and cultured in the absence or presence of doxycycline. FACS data was processed in FlowJo software.

2.12. IMAGE ACQUISITION AND ANALYSIS

Bright field and fluorescent images of cells in culture were acquired using an inverted microscope (Zeiss AxioVert 200M). Images were further processed using Adobe Photoshop CS6 and Adobe Illustrator CS6.

CHAPTER II

3. RESULTS

3.1. IDENTIFICATION OF DC-INDUCING TRANSCRIPTION FACTORS IN MOUSE AND HUMAN SYSTEMS

Somatic cells can be directly reprogrammed to other mature cell types by the overexpression of a small number of lineage restricted TFs. Inducible lentiviral systems can be used to promote the overexpression of lineage-specific TF and so, to induce lineage conversion [31]. Four complementary approaches were used to identify candidate TFs for the induction of the DC fate: (i) literature analysis to reveal DC transcriptional regulators, (ii) ChIP-seq dataset analysis to uncover how TFs influence DC commitment and development, (iii) genome-wide transcriptional profiling to identify genes with high expression levels in mature DCs and DC precursors relative to other blood cell types and tissues, and (iv) Single cell transcriptome analysis to exclude misleading averages of whole population cell analysis (**Figure 13**).

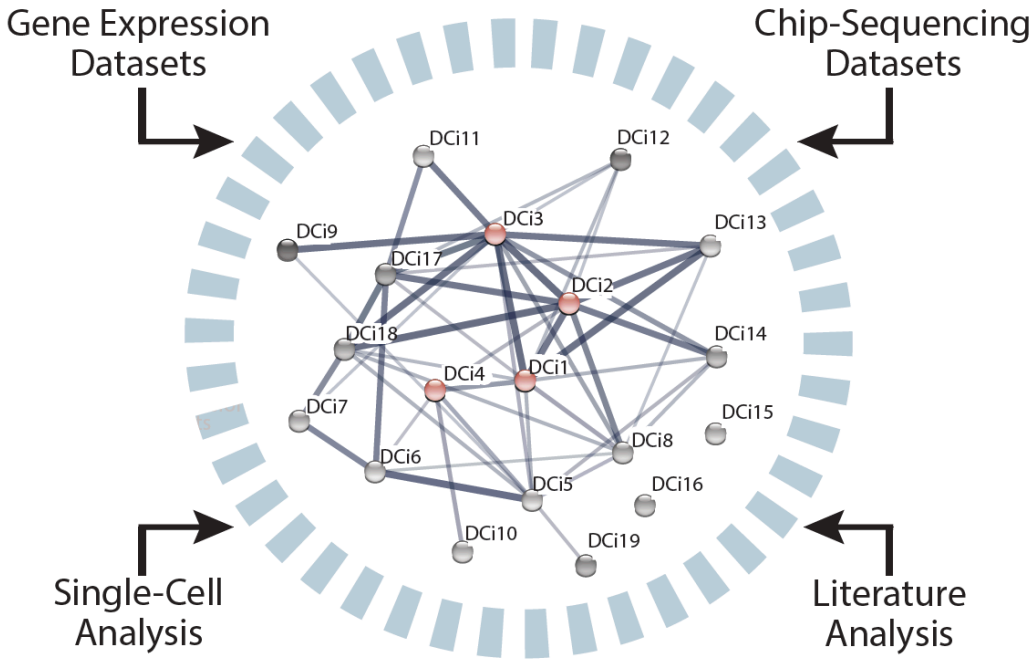


Figure 13**. Distinct approaches for the selection of DC-inducing TFs.** Candidate TFs (circles) to induce dendritic cells (DCs) were selected by the analysis of ChIP-Sequencing datasets, mouse and human gene expression profiles, single-cell data and literature. The figure shows the predicted interactions between 17 out of 19 selected TFs with a minimum interaction score of 0.4 (medium confidence). Analysis based on STRING- known and predicted protein-protein interactions, network line thickness indicates the strength of the data support. Red circles indicate a putative minimal TF network for inducing the DC fate.

To identify TFs restricted to the DC lineage an analysis was performed using a recently written program (BioGPSmatch; Pereira and Papatsenko, unpublished). This analysis gives more significance to gene expression restriction across distinct tissue hierarchies rather than only one system (for example within the hematopoietic system). This degree of cell-type specificity is an important feature of reprogramming factors [166]. This computational analysis revealed 26 genes for mouse CD8α+ cDCs (***Supplementary table 1***), 69 genes for mouse CD8 α - cDCs (***Supplementary table 2***), 72 genes for mouse plasmacytoid DCs (***Supplementary table 3***) and 137 genes for Human BDCA4+ DCs (***Supplementary table 4***). Reassuringly, this unbiased analysis based of cell-type specificity identified 6 TFs (DCi4, Smyd1, DCi8, Med13, DCi13 and Tsc22d1) that were previously implicated in the specification and/or function of pDCs, CD8a+ DCs and CD8a- DCs. The expression levels of all candidate genes were individually confirmed in *biogps.org* website, in order to avoid the selection of non-specific TFs. From this analysis only TFs which expression was, at least, 3-fold higher in DCs than the overall median were considered.

Despite the current efforts on unrevealing the transcriptional network of the DC lineage [167], we still do not fully understand how this transcriptional program is set during development both temporally and spatially. This knowledge would allow the precise regulation of the DC compartment and a better understanding of developmental processes naturally associated with the DC lineage. However, the analysis of previous studies addressing the transcriptional entities involved in the commitment and differentiation of DCs it provides literature-based support for the selected candidate transcriptional regulators. In that sense, loss-of-function studies in mouse models are particularly informative since they provide clues for the potential role of TFs during DC commitment to distinct subsets. The TF DCi1 seems to be intrinsically required for the generation of all DC lineages since *DCi1*-deficient mouse shows impaired DC development [168-170]. In addition, ablation of *DCi2* in hematopoietic progenitors makes them unable to develop into DCs, developing into macrophages instead [171]. This suggests that DCi2 may possibly be a critical cell fate switch between DCs and Macrophages. Deficiency of DCi8, DCi11 and DCi6 also impaired the specification of cDCs. DCi8-deficient hematopoietic cells were unable to respond to Flt3-ligand stimuli and failed to generate mature cDCs, resulting in impaired cDC specification [172, 173]. Ablation of *DCi11* in mice resulted in a selective reduction in CD4+ cDCs and CD8 α + cDCs [174, 175]. *DCi6*-KO mice showed a decrease in the total number of CD11c-high cDCs and CD4+ DCs, lacking specifically myeloid-derived DCs [176, 177]. In fact, the commitment of cDC precursors to the CD4+ DC lineage seems to rely not only in DCi6 but also in DCi7, DCi18 and DCi17 since their absence critically impairs CD4+ DC specification [178] [86]. [179-182]. Still in the cDC compartment, CD8 α + DC specification seems to rely on the TFs DCi14, DCi5, DCi19 and DCi4. While the ablation of *DCi14* and *DCi5* in mice induces the selective loss of CD8 α + DCs in lymphoid tissues [183-186], the absence of

DCi19 and DCi4 impairs the number of CD8α + DCs in both spleen and non-lymphoid tissues [74, 187-191]. Murine *DCi12*, *DCi16* and *DCi3* ablation impairs the development and maintenance of pDCs [192-197]. In fact, it has been hypothesized that DCi3 could control pDC differentiation by silencing a large array of genes. Finally, the ablation of *DCi9* results in the generation of cDCs with impaired phenotype, ablation of *DCi13* and *DCi10* profoundly impact DC function. For instance, deletion of *DCi10* considerably decreases activation-induced apoptosis and *DCi13*-KO results in the generation of DCs showing a remarkably reduced BrdU uptake and reduced T-cell priming activity during infection.

After combining unbiased bioinformatic analysis with literature mining from loss-of-function studies, we identified a pool of 19 candidate TFs ranked as strong candidates to induce DC lineage (**Table 2**).

Table 2. Deletion of candidate TFs impairs DC development, function and specification. Literature analysis of loss-of-function studies showing selected TF phenotypes within DC cell lineage.

TFs	Gene Bank ID	Phenotype	References
DCi4	NM_030060.2	Impaired CD8α+ DC specification;	Hildner K. et al, Science 2008 Edelson BT. Et al, J. Exp. Med. 2010 Martínez-López M. et al, Eur. J. Immunol. 2015
DCi15	NM_019866.1	Impaired plasmocytoid DC specification;	Schotte R. et al, J. Exp. Med. 2004
DCi5	NM_001301811.1	Impaired plasmocytoid and CD8α+ DC specification	Schiavoni G. et al, J. Exp. Med. 2002 Tsujimure H. et al, J Immunol. 2003 Tamure T. et al, J Immunol. 2005
DCi1	NM_001025597.2	Impaired DC specification	Carotta S. et al, Immunity 2010 Anderson K. et al, J Immunol. 2000 Guerriero A. et al, Blood 2000
DCi6	NM_013674.1	Impaired conventional DC specification	Tamure T. et al, J Immunol. 2005 Suzuki S. et al, Proc. Natl Acad. Sci. USA 2004
DCi8	NM_01030931.1	Impaired conventional DC specification	Laouar Y. et al, Immunity 2003
DCi12	XM_006525762.2	Impaired plasmocytoid DC specification;	Cisse B. et al, Cell 2008 Ghosh H. S. et al, Immunity 2010
DCi3	NM_001220765.2	Impaired plasmocytoid DC specification;	Wu L. et al, Immunity 1997 Allman D. et al, Blood 2006
DCi19	NM_010496.3	Impaired CD8α+ and CD103+ DC specification;	Hacker C. et al, Nature Immunol 2003 Ginhoux F. et al, J. Exp. Med. 2009
DCi16	NM_016707.3	Impaired plasmocytoid DC specification;	Wu X. et al, PLoS ONE 2013 Ippolito GC. et al, Proc. Natl Acad. Sci. USA 2014
DCi17	NM_001290457.1	Impaired CD8α$^-$ DC specification;	Wu L. et al, Immunity 1998 Clark GJ. et al, Immunology 1999
DCi9	NM_027656.2	Impaired conventional DC phenotype;	Satpathy AT. et al, J. Exp. Med. 2012
DCi18	NM_019732.2	Impaired CD11b(+)Esam(hi) DC specification;	Dicken J. et al, PLoS ONE 2013
DCi2	NM_001127216.1	Impaired conventional and plasmocytoid DC specification;	Rathinam C. et al, Immunity 2005
DCi7	NM_008391.4	Impaired plasmocytoid and CD4$^+$DC specification;	Ichikawa E. et al, Proc. Natl Acad. Sci. USA 2004
DCi14	NM_017373.3	Impaired CD8α+ DCs specification;	Kashiwada M. et al, Blood 2011
DCi16	NM_009744.3	Impaired conventional DC specification	Ohtsuka H. et al, J Immunol 2011 Zhang T. et al, PLoS ONE 201
DCi13	NM_001033081.2	Impaired DC function	Wumesh K. et al, Nature 2014
DCi10	NM_006981.3	Impaired DC function;	Wang T. et al, Blood 2009

Gene ontology enrichment analysis of this gene set of 19 TFs highlighted their fundamental role on leucocyte and DC differentiation and activation (p-value: 25^{-13}). By analysing the Mouse Genome Informatics mutant phenotype database, genetic disruption of this gene set is associated with mouse abnormal immune phenotypes, in particular abnormal adaptive immunity and abnormal antigen presentation (p-value: 25^{-13}), fundamental features of the DC compartment (**Figure 14**).

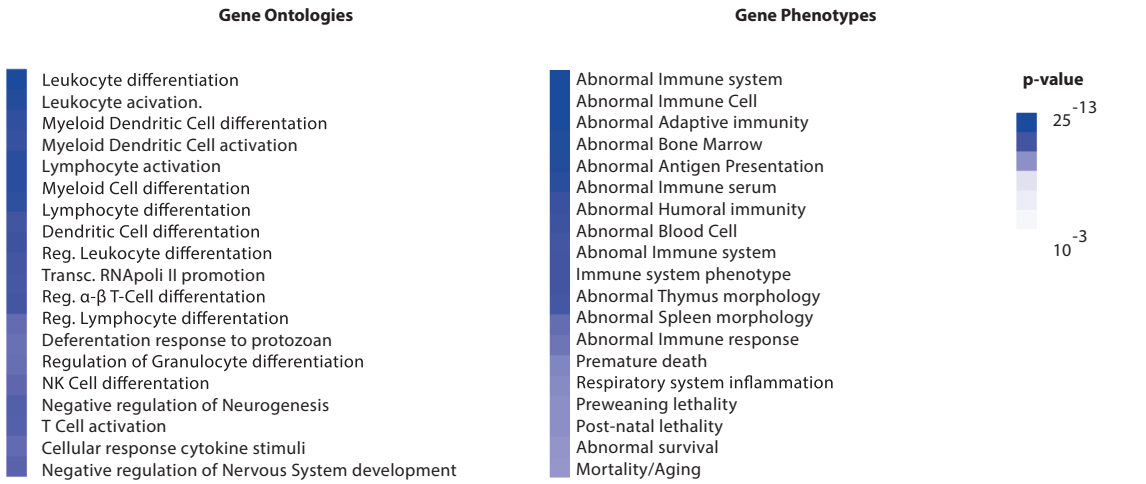


Figure 14 Biological processes and mammalian phenotypes for the selected pool of TFs. Bioinformatic analysis showing that selected TFs are highly relevant for biological processes related with DC development and function, i.e. Myeloid Dendritic cell differentiation and activation (left panel) and that their ablation induces immune phenotypes in mice, i.e. abnormal immune system and abnormal adaptive immunity. Gene ontologies were acquired from Enrichr (Ma'ayan Laboratory - Computational Systems Biology lab, New York) while gene Phenotypes were acquired from Network2canvas, MGI – Mammalian Phenotype – Top 4 (Ma'ayan Laboratory - Computational Systems Biology lab, New York). More relevant gene ontologies and gene phenotypes are represented, blue means high relevance while white means low relevance. Gene ontologies and gene phenotypes are represented, blue means high relevance while white means low relevance, left column showing respective p-values.

TFs normally cooperate by promoting the activation or repression of specific target genes. By interacting with each other, these proteins enable the generation of gene regulatory networks (GRNs) particularly associated with each cell type. Since reprogramming TFs are involved in the regulation of fundamental processes such as cellular specification, these GRNs are assumed to be highly conserved between species [198]. For the induction of pluripotency or direct reprogramming the combinations of TFs are conserved between mice and Human [19]. The same was observed with the combination of TFs that efficiently induce hemogenic program in mouse and human fibroblasts [31]. To access the specificity of candidate TFs for the DC lineage in both mice and human systems, expression data for the 19 candidate TFs in distinct cell types or tissues was extracted from BioGPS.org. Importantly, it was observed that the majority of the 19 TFs are highly enriched in both mouse and human DCs **(Figure 15)** when compared to other tissues or cell types.

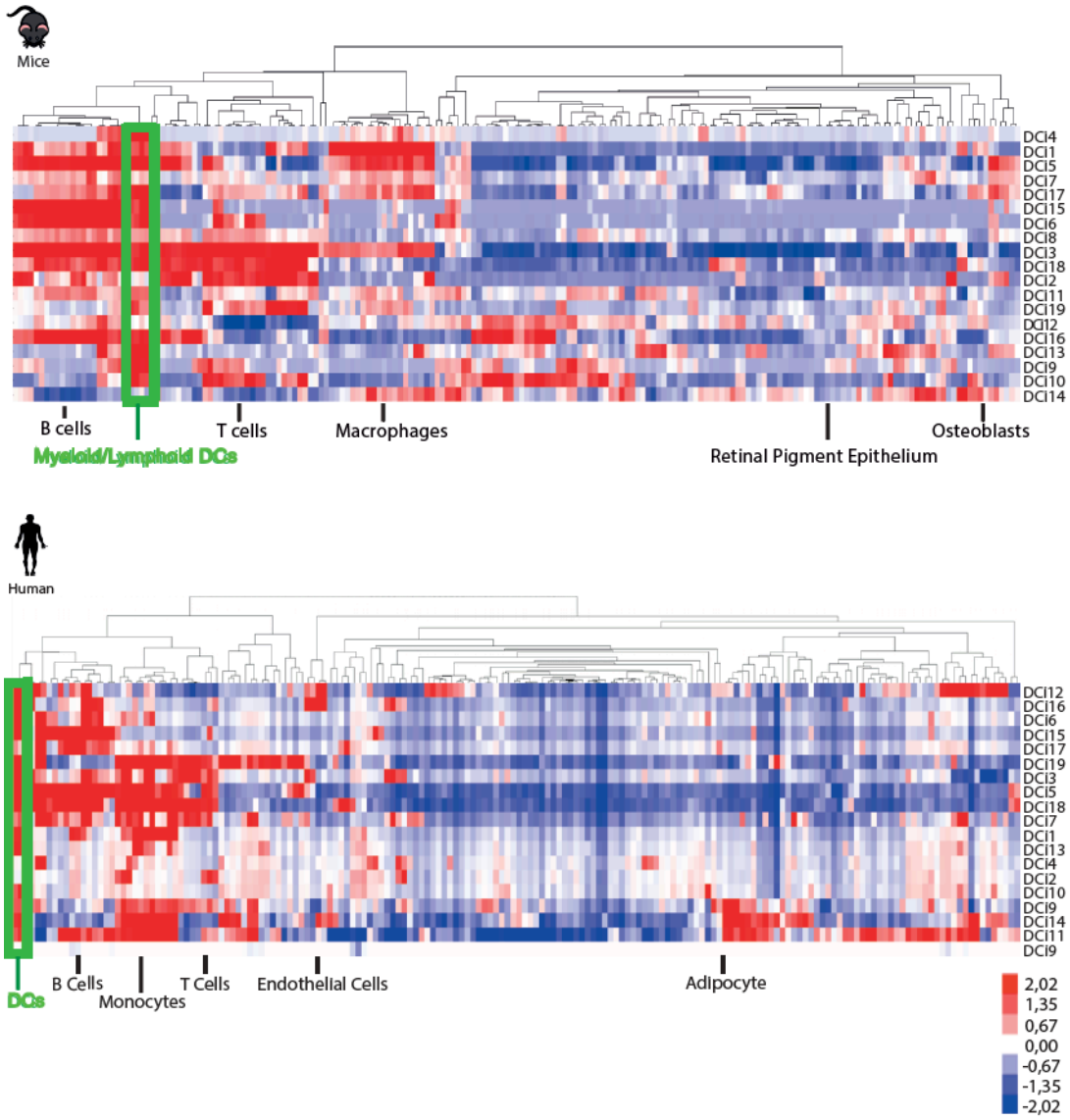


Figure 15. Expression of 19 candidate Transcription factors is enriched in the DC population in both mouse and human. Heat maps showing gene expression profile of the selected 19 TFs across multiple mouse and human tissues and cell types (Upper panel: mouse GeneAtlas MOE430; Lower panel: human GeneAtlas U133A). The majority of the 19 TFs are enriched in DC populations (green box, left) when compared to other tissues in both mice and humans. Gene expression data was obtained from BioGPSmatch, analysed by cluster 3.0 (log-transformed data; centred genes; centred, clustered and normalized according to cell types) and displayed by Treeview. Red indicates increased expression, whereas blue indicates decreased expression over the mean.

Macrophages and DCs share common cellular features and arise from a common myeloid progenitor (MDP). Under steady-state conditions they express approximately 4000 genes, 96% of which are mutual [199]. However, they differ considerably in terms of functionality, for instance, DCs have a superior antigen presenting capacity [200]. Gene expression data for DC and Macrophage populations derived from GM-CSF-stimulated bone marrow cultures was extracted in order to access the specificity of selected TFs regarding both cell types (**Figure 16, Left panel**) [158]. The TFs DCi4, DCi15, DCi5, DCi17, DCi9

and DCi10 were specifically confined to the DC populations while the TFs DCi6, DCi8, DCi19, DCi16, DCi18, DCi14, DCi11 and DCi13 were considerably enriched in DCs in comparison to macrophages. In contrast, the TFs DCi1 and DCi3 seemed to be more expressed in macrophages than in DCs. Overall, the selected 19 TFs are enriched in DCs suggesting that they may be able to impose a DC-specific GRN in fibroblasts that is distinct from macrophages.

Regarding the distribution of transcriptional regulators during the development of the DC lineage, core TFs may have distinct temporal and spatial contribution for the ontogeny of DC lineage and can act as cell-fate mediators for specific subsets. Considering the phenotypic and functional heterogeneity of the cDC lineage, it was hypothesized that while several TFs may be critical for the development of the DC lineage as a whole, other TFs may play a more restrict role in specifying particular subsets inside the cDC lineage, i.e. cDC1 or cDC2. To access this hypothesis, stage-specific gene expression profile for the 19 candidate TFs was analysed in populations of sorted CDPs, pre-cDC1s, pre-cDC2s, cDC1s and cDC2s **(Figure 16, right panel)**. The gene expression data was obtained under the accession number GSE66565 [74]. Several TFs seem to be more expressed in one cDC subset than the other. For instance, DCi4, DCi5, DCi9 and DCi13 TFs are apparently more expressed in cDC1s while the expression of DCi6 and DCi16 is enriched within the cDC2 subset. Considering the interplay between distinct TFs, it is important to emphasize the stage-dependent expression as a requirement for the specification of each DC subset. In fact, DCi5 seems to be considerably expressed in CDPs however, cDC1-commitment seems to rely on important transcriptional events associated with the maintenance of DCi5 expression. Actually, DCi5 expression is maintained by an auto-activation loop, which is dependent on DCi4-DCi5 interaction at the protein level [74]. Considering that, the cDC2-specification seems to rely on the absence of DCi4. When DCi4 is not expressed, the DCi5 auto-activation does not occur. Consequently, cDC2 commitment seems to rely on the replacement of DCi5 by DCi6 [74].

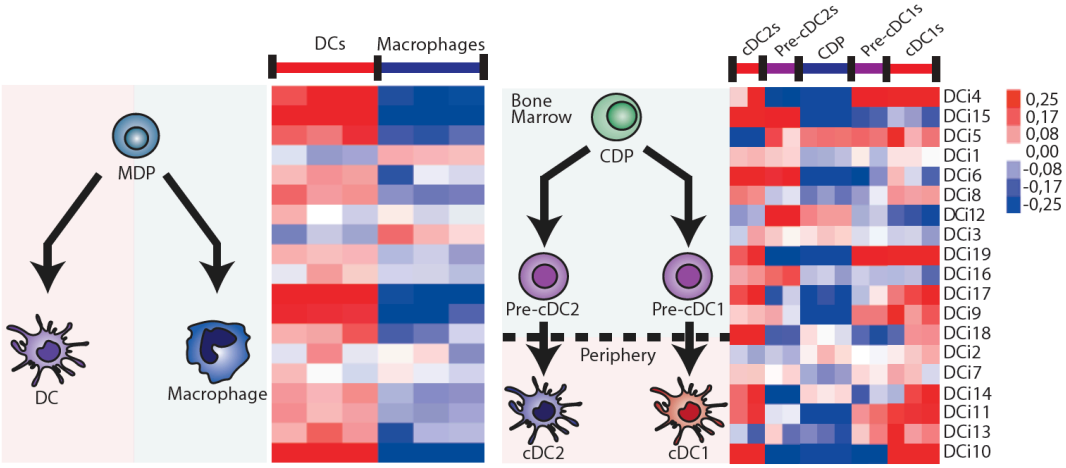


Figure 16. The 19 candidate TFs are confined to the DC lineage and their expression level is correlated with DC commitment and differentiation. Heat map showing increased gene expression of the 19 candidate TFs in mouse DCs when compared to macrophages derived from bone marrow cultures. (Left panel, GSE62361). Heat map showing gene expression of the 19 candidate TFs in common DC progenitors (CDP), pre-conventional DC (pre-DCs) and conventional DCs (cDCs). The 19 candidate TFs increasily expressed during DC differentiation (Right panel, GSE66565). Gene expression data was obtained from Gene expression omnibus, analysed by cluster 3.0 (log-transformed data; centered genes; centered, clustered and normalyzed according to cell types) and displayed by Treeview. Red indicates increased expression, whereas blue indicates decreased expression over the mean.

In summary, this combined analysis allowed the identification of a list with 19 TFs that are a) restricted to mouse and human DCs b) functionally important to DCs c) enriched in DCs when compared to macrophages and d) expressed during DC lineage specification. This analysis supports the potential of these 19 TFs or more restricted combinations for the direct reprogramming of fibroblasts to DC-like cells.

3.2. PCR-BASED CLONING OF DC-INDUCING TRANSCRIPTION FACTORS

The induction of desired cell-types by direct reprogramming has been accomplished by doxycycline-inducible lentiviral transduction of specific TFs in MEF or HDF cultures. For instance, Pereira et al. have accomplished the direct reprogramming of MEFs to hemogenic cells by the overexpression of 4 TFs (Gata2, DCi2b, cFos and Etv6) using inducible lentiviral vectors [31]. For this purpose, coding sequences (CDS) of each TF were subcloned individually into the *EcoRI* sites of the pFUW-tetO backbone. In this system, expression of TFs is controlled by a tetracycline operator minimal promoter (tetO). In the presence of Doxycycline, cells were co-transduced with a constitutive lentivirus encoding the tetracycline controllable transactivator (M2rtTA) and pFUW-tetO-TF expressing corresponding TFs. In order to

establish this reprogramming proven Dox-inducible lentiviral system for DC direct reprogramming, CDSs for the selected DC-inducing TFs were acquired (**Table 3**). The TFs DCi1, DCi3 and DCi2 were already cloned in the pFUW-tetO backbone while the other templates were acquired from distinct cDNA libraries in different vector backbones. The CDSs for DCi16, DCi18 and DCi19 were not available at the time and so will not be considered for the subsequent experiments. Before sub-cloning the 16 TFs into the inducible lentiviral vector, the integrity of the CDS was confirmed by alignment of the of the sequence obtained by Sanger sequencing with the reference consensus CDS from NCBI of the sequence obtained by Sanger sequencing with the reference consensus CDS from NCBI.

Table 3. 16 out of 19 TF Coding-sequences were acquired. Table showing the original vector, species, source, NCBI reference and sequencing primer for each TF CDS. TFs in dark grey represent templates that are not available.

TF	Original vector	Specie	Original Source	NCBI Reference	Sequencing primer
DCi1	pFUW-tetO-DCi1	Mouse	Lab	NM_001025597.2	pFUW-seq
DCi2	pFUW-tetO-DCi2	Human	Lab	NM_001127216.1	pFUW-seq
DCi3	pFUW-tetO-DCi3	Human	Lab	NM_001220765.2	pFUW-seq
DCi4	pCMV6-DCi4	Mouse	Origene	NM_030060.2	VP1.5
DCi5	pSPORT-DCi5	Mouse	Openbiosystems	NM_001301811.1	SP6
DCi6	pMIG-DCi6	Mouse	Addgene	NM_013674.1	pMIG-seq
DCi7	pSPORT-DCi7	Mouse	Openbiosystems	NM_008391.4	SP6
DCi8	pSPORT-DCi8	Mouse	Openbiosystems	NM_001030931.1	SP6
DCi9	pCMV6-DCi9	Mouse	Origene	NM_027656.2	VP1.5
DCi10	pCMV6-DCi10	Human	Origene	NM_006981.3	VP1.5
DCi11	pSPORT-DCi11	Mouse	Openbiosystems	NM_009744.3	SP6
DCi12	pMXs-Hu-DCi12	Human	Addgene	NM_001033081.2	pMXs-seq
DCi13	pSPORT-DCi13	Mouse	Openbiosystems	NM_017373.3	SP6
DCi14	pCMV6-DCi14	Mouse	Origene	NM_019866.1	VP1.5
DCi15	pSPORT-DCi15	Mouse	Openbiosystems	XM_006525762.2	SP6
DCi16	pSPORT-DCi16	Mouse	Openbiosystems	NM_001290457.1	SP6
DCi17	----------	----------	----------	----------	----------
DCi18	----------	----------	----------	----------	----------
DCi19	----------	----------	----------	----------	----------

PCR-based cloning allows the amplification of specific CDS, adding at the same time restriction sites to both 3' and 5' ends so that they can be easily cloned in the pFUW-tetO backbone. For that purpose, selected TFs were amplified by PCR from acquired plasmids using a high fidelity Taq polymerase to minimize amplification errors and purified by gel electrophoresis in order to confirm expected product size for each insert. For instance, the amplification of DCi4 (357 bp), DCi5 (1275 bp), DCi6 (1353 bp), DCi8 (2313 bp), DCi9 (1302 pb) and DCI13 (1095 bp) resulted in single bands with the expected size (**Figure 17, A**). PCR products were then extracted from gel and used for enzymatic restriction.

The vector plasmid pFUW-TetO-mOrange was also prepared by restriction with *EcoRI*, followed by dephosphorylation in order to minimize re-ligation without insert. Restricted vector and inserts were then subject to a ligation reaction. To control the efficiency of the ligation reaction, restricted pFUW-TetO backbone and mOrange inserts were used as depicted in (**Figure 17, B**). After ligation, resulting products (in triplicate) were purified by electrophoresis (**Figure 17, C**). Circular pFUW-tetO-mOrange plasmid was included as a positive control. A negative control was also included consisting in the ligation cocktail without the ligase enzyme. As expected 2 bands corresponding to the non-ligated pFUW-TetO backbone and m-Orange were observed. Importantly, on the ligation products, it was observed a band corresponding to the expected ligation product pFUW-TetO-mOrange. Another band was also observed that probably corresponds to the re-ligated empty vector.

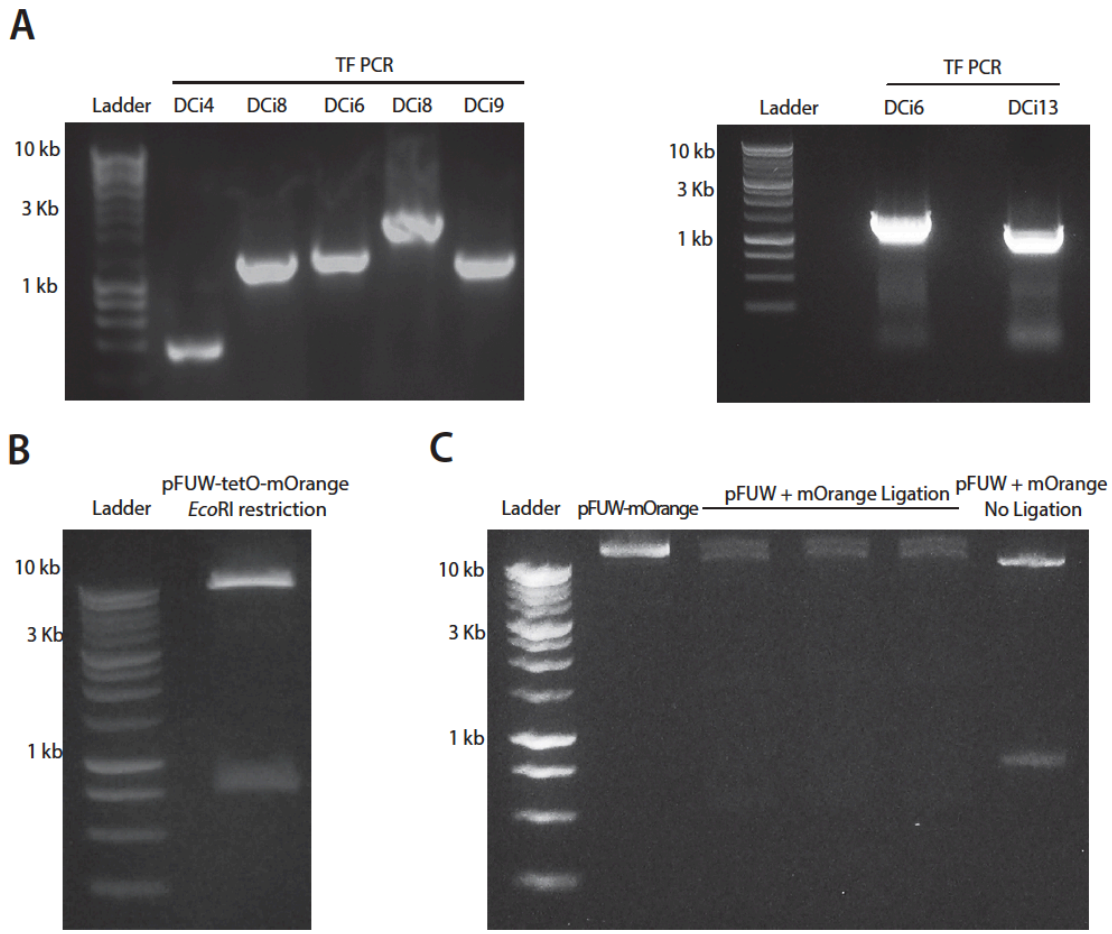


Figure 17. Analysis by gel electrophoresis for PCR-based cloning strategy. A. DNA bands derived from PCR amplification of DCi4, DCi5, DCi6, DCi8, DCi9 and DCI13 TFs. **B.** DNA bands derived from *EcoRI* restriction of the pFUW-tetO-mOrange plasmid, showing mOrange (711 bp) and pFUW-tetO (9132 bp) **C.** Controls for the ligation reaction: circular pFUW-tetO-mOrange vector (pFUW-mOrange), ligation reaction of the pFUW-tetO backbone with the mOrange CDS (pFUW + mOrange Ligation; 3 lanes) and pFUW-tetO backbone with mOrange CDS without Ligase enzyme (No ligation).

After verifying the efficacy of ligation enzymatic reactions, competent bacteria were needed for amplifying the desired cloning product. Some bacteria are able to take up foreign DNA from the environment by horizontal gene transfer. The process of gene transfer by transformation requires the presence of DNA in the environment and bacteria capable of up-taking free extracellular genetic material [201]. Genetic modifications to create bacterial strains that could be efficiently transformed, maintaining plasmid DNA without rearrangement have been made. This natural process can be usually induced through chemical or electrical stimuli, generating the commonly known “competent bacteria”.

Chemically competent E.coli cells were generated for this project. Two distinct protocols were tested and evaluated for the ability to generate more efficient competent cells: a protocol based in Chung et al. [160, 161] and a

protocol based in Inoue et al. [162]. Bacteria generated from both protocols, as well as commercially available competent cells, were transformed in parallel with the same amount of DNA, plated in LB agar plates supplemented with ampicillin and cultured overnight at 37ºC (**Figure 18**). The efficiency of each batch was accessed by colony count after bacterial transformation and expressed as number of colony forming units per µg of DNA. As expected, the commercially available competent bacteria were very efficient, having an efficiency of almost 35700 CFUs *per* µg of plasmid DNA. As regards to the induced competent bacteria, the ones generated by the first protocol (Chung et al.) had different efficiencies, for instance, when compared to the commercially available competent bacteria (**Figure 18, A)**, the CB1 and the CB2 batches generated 3-fold less CFUs (12000 CFUs) (**Figure 18, B)** and 12-fold less CFUs (3090 CFUs) per μg of plasmid DNA (**Figure 18, C)**, respectively. For the generation of CB1 batch, TSS buffer was freshly prepared while, for the CB2 batch, TSS buffer prepared the week before was used. This may explain the differences in efficiency of both batches. The alternative protocol, based on Inoue et al., generated less efficient competent bacteria, giving rise to 1432 CFUs per μg of DNA (**Figure 18, D)**. The CB1 batch was used for subsequent experiments as the more efficient induced competent cells in the laboratory.

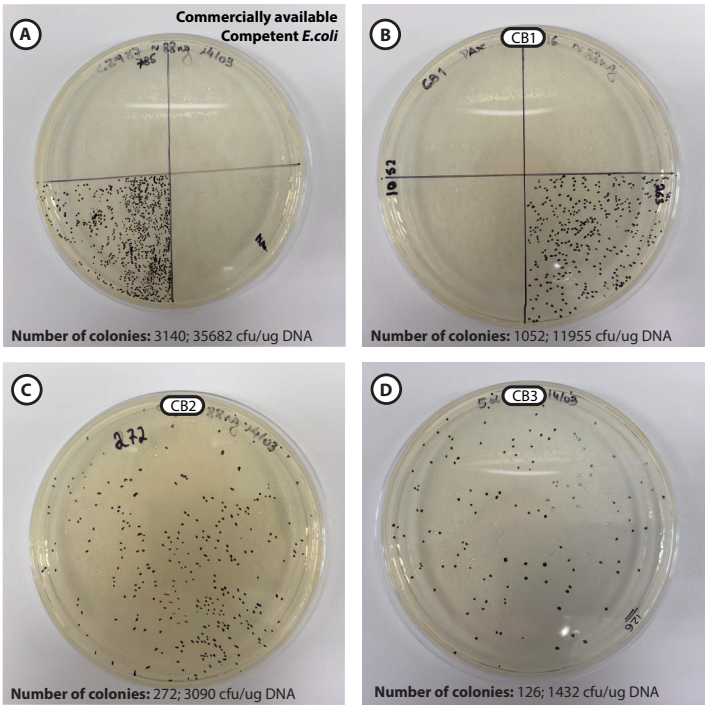


Figure 18. Testing the efficiency of competent *E.coli*. Two distinct methods were used for inducing chemically competent bacteria. Competence was evaluated by transforming bacteria with approximately 88ng of plasmid DNA (psPAX2). **A.** Commercially available NEB® 5-alpha Competent E. coli (High Efficiency): **B,C**. Competent bacteria generated with a protocol based on Chung et al. **D.** Competent bacteria generated with a protocol based on the Inoue method (Inoue et al., Gene 1990).

Efficient competent bacteria were transformed with the products derived from ligation reactions of each individual TF and the pFUW-tetO backbone (**Figure 19, A**). After overnight culture, 2 distinct approaches were used to identify clones that have successfully acquired the desired ligation product: PCR-based (colony PCR) or restriction-based screen. For each ligation, at least 10 colonies *per* plate were analysed by PCR using insert-specific primers (**Figure 19, B**). The original plasmid with the specific insert was added as a positive control. A negative control consisting in a colony picked up from the transformation of pFUW-tetO plasmid without insert was also included. Bacterial clones with appropriate inserts, were picked, expanded and plasmid verified by Sanger sequencing (**Figure 20**). Alternatively, colonies were grown in overnight cultures for DNA purification and a restriction digestion with *Eco*RI enzyme was performed (**Figure 19, C**). Colonies with 2 clear bands, one corresponding to the pFUW-tetO backbone and another corresponding to the expected insert size, were verified by Sanger sequencing (**Figure 19**).

9 TFs from the candidate 19 TFs were successfully cloned in the pFUW-tetO plasmid. The remaining TFs were not cloned due to amplification failure or unsuccessful ligation or identification of a positive clone. Further optimization of the PCR and ligation conditions is required for completing the cloning of these genes. Subsequent experiments were performed with 9 TFs.

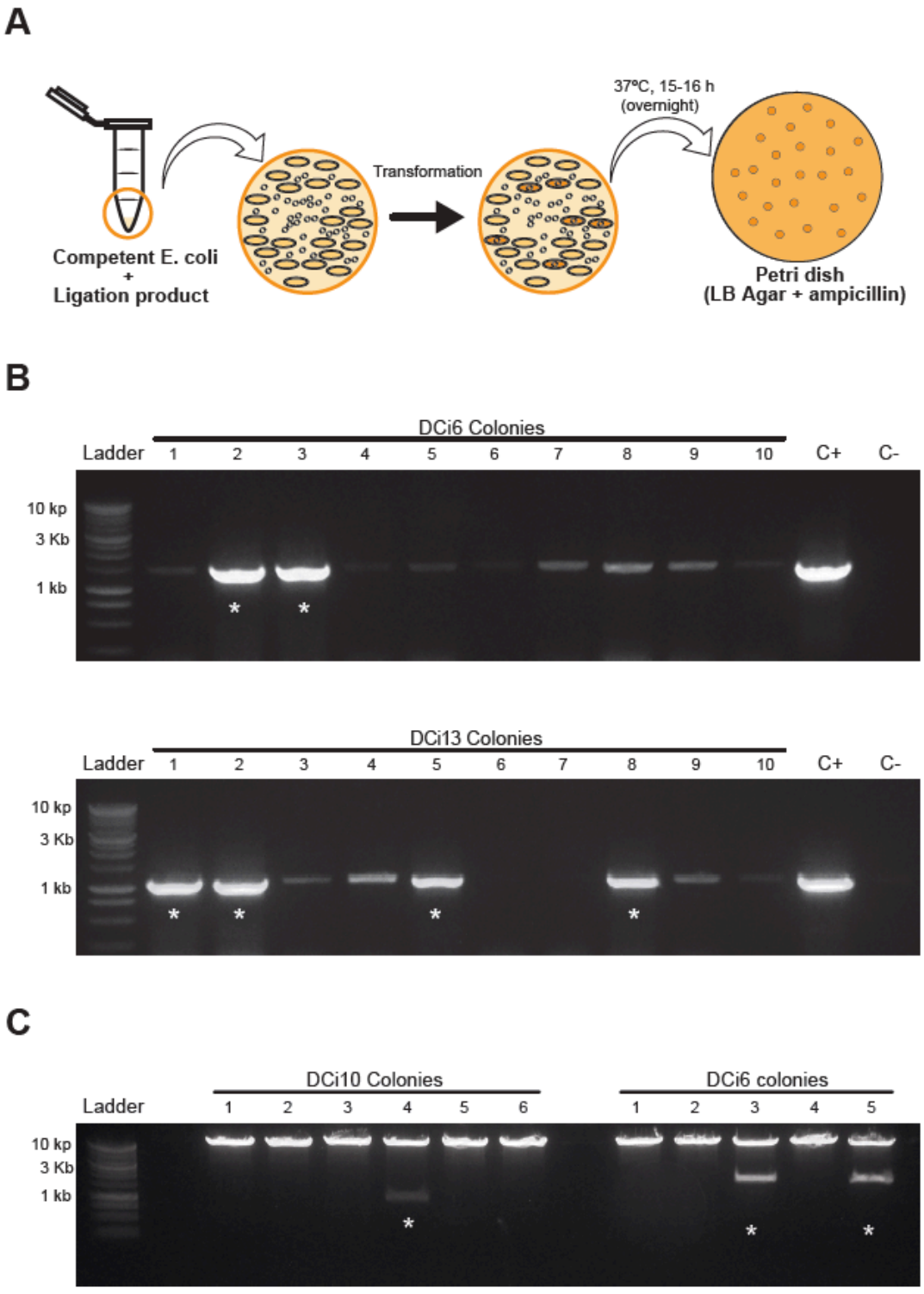


Figure 19. Screening of bacterial clones by colony PCR and *EcoRI* restriction. A. Scheme representing *E. coli* transformation with a ligation product, in which only a portion of bacteria captures efficiently the plasmid (Orange clones) which encodes for a Ampicillin resistance gene. Transformed bacteria survive in the LB Agar plate supplemented with Ampicillin after an overnight incubation at 37°C. **B.** The screening of resistant clones was performed either by colony PCR (upper and middle panels) or by *EcoRI* restriction (bottom panel). Colonies marked with an asterisk were considered positives and were sent for sequencing confirmation.

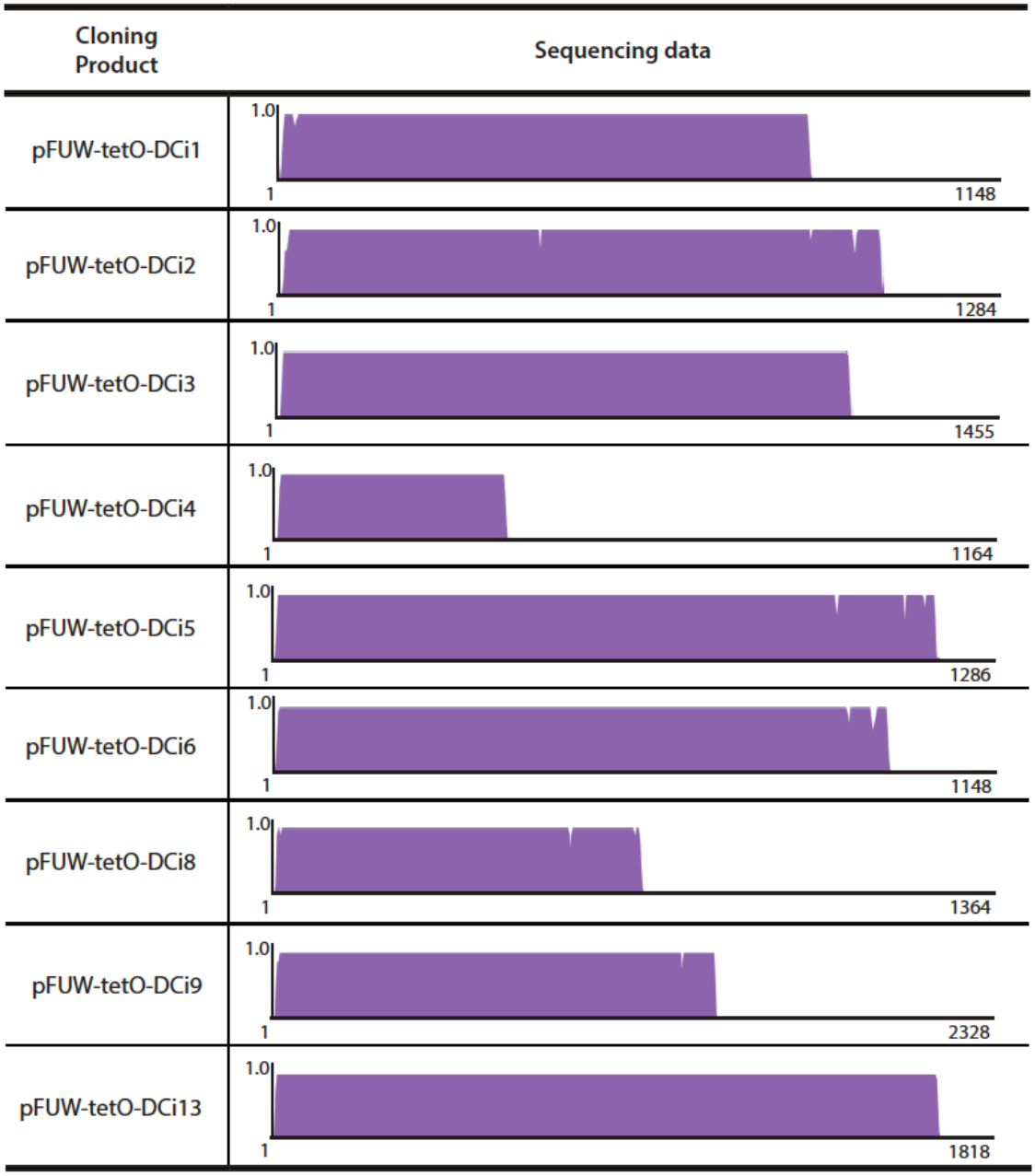


Figure 20. Sequence verification of cloned products in the pFUW-tetO vector. Sanger sequencing results were aligned with the corresponding CDS in Vector NTI software. Corresponding CDS were derived from Gene Database, NCBI. Alignment quality is displayed as purple graphics. Each gap was individually confirmed to verify sequencing errors.

3.3. DIRECT REPROGRAMMING OF MEFS INTO DC-LIKE CELLS

The success of TF-mediated direct reprogramming experiments relies on the ability to force high expression levels of transcription factors that specify the desired cell fate. The identified transcriptional network involved in DC commitment and differentiation will be tested to promote the direct conversion of fibroblasts to DCs. TF-mediated reprogramming has been accomplished using inducible lentiviral systems [31]. The present study aims to generate DC-like cells through a direct reprogramming approach.

3.4. OPTIMIZATION OF THE DOXYCYCLINE (DOX)-INDUCIBLE LENTIVIRAL SYSTEM

Second-generation inducible lentiviral plasmids encoding for the candidate DC-inducing TFs were generated (pFUW-TetO-TF). For producing lentiviral particles, combinations of pFUW-TetO-TFs, packaging (pPAX2) and envelop (pMD2.G) plasmids were used.

Because expression of multiple TFs is a requirement in direct reprogramming experiments, production of lentiviral particles and transduction of fibroblasts were optimized in order to increase the probability of co-transducing fibroblasts with the selected pool of TFs. 2 distinct DNA quantities (10µg and 20µg) of control plasmid pFUW-tetO-mOrange were used for 293T transfection (**Figure 21, A**). 24 hours after transfection, mOrange expression was detected by fluorescence microscopy in 293T cell cultures. When double amounts of transfer plasmid DNA (as well as packaging and envelope plasmids) were used, the number of mOrange-positive cells increased (**Figure 21, A**). As expected, mOrange expression is not detected in 293T cultures transfected with pFUW-M2rtTA.

Freshly isolated lentiviral supernatants were then used to transduce C57BL/6 MEFs. In this inducible system, expression of the fluorescent protein only occurs in MEFs co-transduced with FUW-TetO-mOrange and FUW-M2rtTA viral particles. Increasing volumes of both types of lentiviral particles (250 to 2000 μL of each) were tested. Dox was added to culture media and mOrange expression in transduced MEFs was observed by fluorescence microscopy 2 days after (**Figure 21, B**). mOrange-positive (mOrange+) cells were quantified by flow cytometry to find the condition that allows an efficient transduction (**Figure 21, C**). As expected, the percentage of mOrange+ cells increased along the defined volume range, reaching a maximum value of approximately 70%. Treatment with 2000 μl of each viral preparation induced a similar percentage of mOrange+ cells with both initial DNA transfection quantities. Therefore 10 μg of plasmid DNA, 10 μg of pPAX2 and 5ug of pMD2.G were selected for 293T transfections. Furthermore, 1000 μL of

FUW-TetO-TF and FUW-M2rtTA viral particles were selected for subsequent MEF transductions.

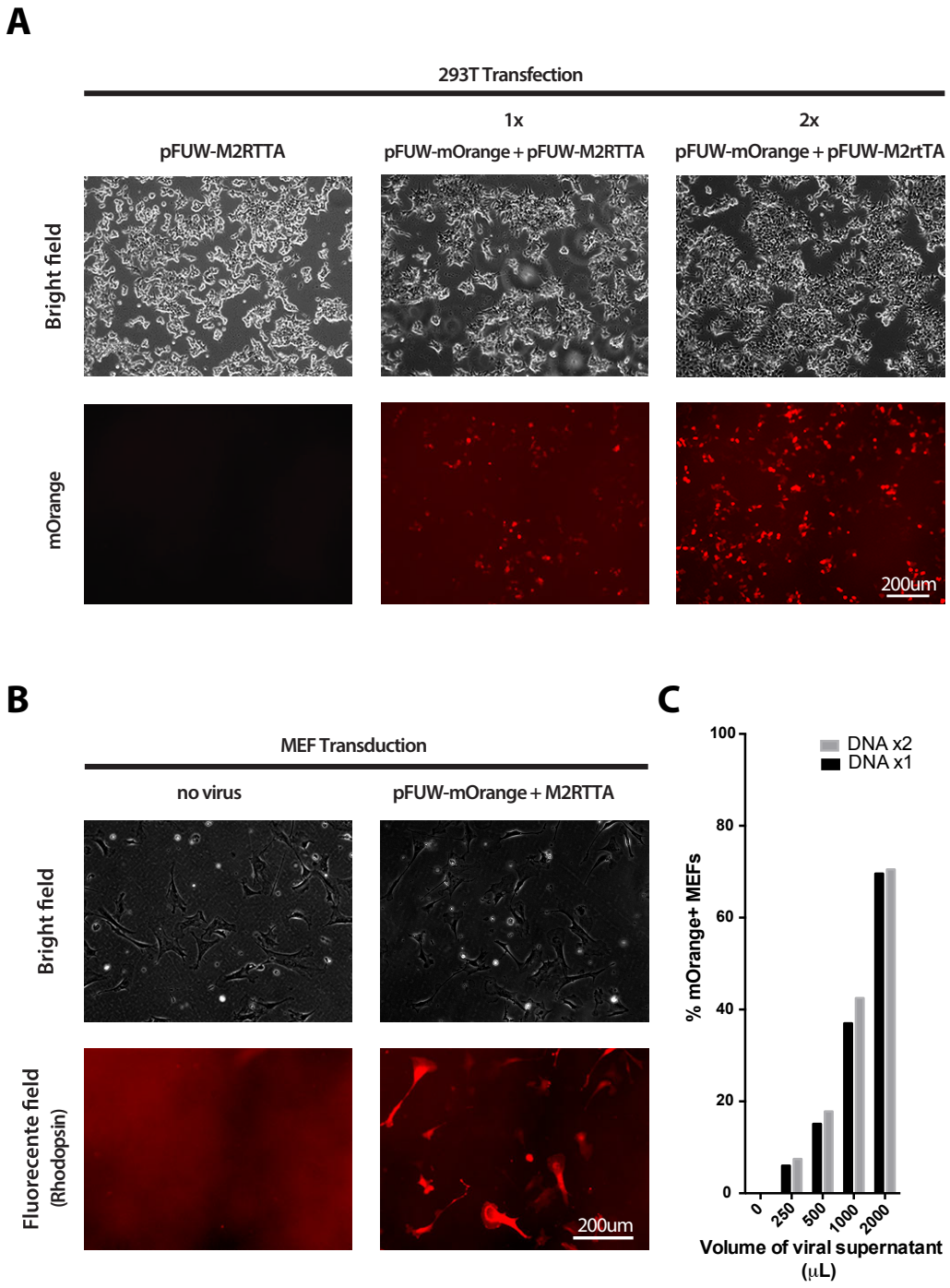


Figure 21. Optimization of 293T transfection and MEF transduction. A. mOrange Expression after 293T cells transduction with pFUW-tetO-mOrange plasmid, pPAX2 and pMD2.G (1x: 10ug of transfer plasmid, 10ug of pPAX2 and 5ug of pMD2.G; 2x: 20ug of transfer plasmid, 20ug of pPAX2 and 10ug of pMD2). **B.** C57BL/6 MEFs express mOrange after transduction with lentiviral particles encoding pFUW-tetO-mOrange and pFUW-M2rtTA. mOrange expression was accessed by fluorescence microscopy (rhodopsin filter) 5 days after Dox supplementation. **C.** Percentage of cells expressing mOrange was assessed by flow cytometry 5 days after Dox supplementation. Variations of DNA quantity and viral supernatant volume were considered.

3.5. IDENTIFICATION OF A REPORTER SYSTEM FOR THE DC LINEAGE

A key requirement for a successful direct reprogramming strategy is the selection of a good reporter system restrictively expressed in the cell lineage one wants to generate. DCs and macrophages are mononuclear phagocytes with some overlap in terms of morphological, phonotypical and functional features [199]. This fact makes the identification of DCs problematic. However, a recent study has identified a gene that allows the genetic tracing of the cDCs lineage based on their ontogenetic progeny from committed precursors [202]. C-type lectin domain family 9, member a (Clec9a) gene, encodes for DNGR-1, an endocytic receptor of the C-type lectin superfamily that is selectively expressed at high levels in CD8α cDCs and at low levels in pDCs. DNGR-1 is able to detect performed intracellular ligands exposed upon loss of membrane integrity after primary or secondary necrosis. Expression of Clec9a enables the identification of CDPs and its subsequent progeny (pre-DCs and mature DCs) (**Figure 22, A**)

To confirm Clec9a DC-specificity, expression of Clec9a was investigated within the hematopoietic system and within DC progenitors. In the hematopoietic system, Clec9a is highly expressed in splenic CD8+ DCs while slightly less expressed in both CD8+ DCs in thymus and splenic pDCs (**Figure 22, B**). Regarding DC progenitors, Clec9a expression is detected in CDPs and pre-DCs while in MDPs is absent (**Figure 22, C**). This data supported Clec9a as a marker for the genetic tracing of CDPs in BM and their progeny in lymphoid tissues. Considering the aim of this study, a reporter system based on Clec9a expression would be ideal for screening for DC cell fate induction. In this context, MEFs from genetically modified mice harbouring a Clec9a reporter system were isolated (Clec9a-Cre X R26-stop-Tomato, unpublished).

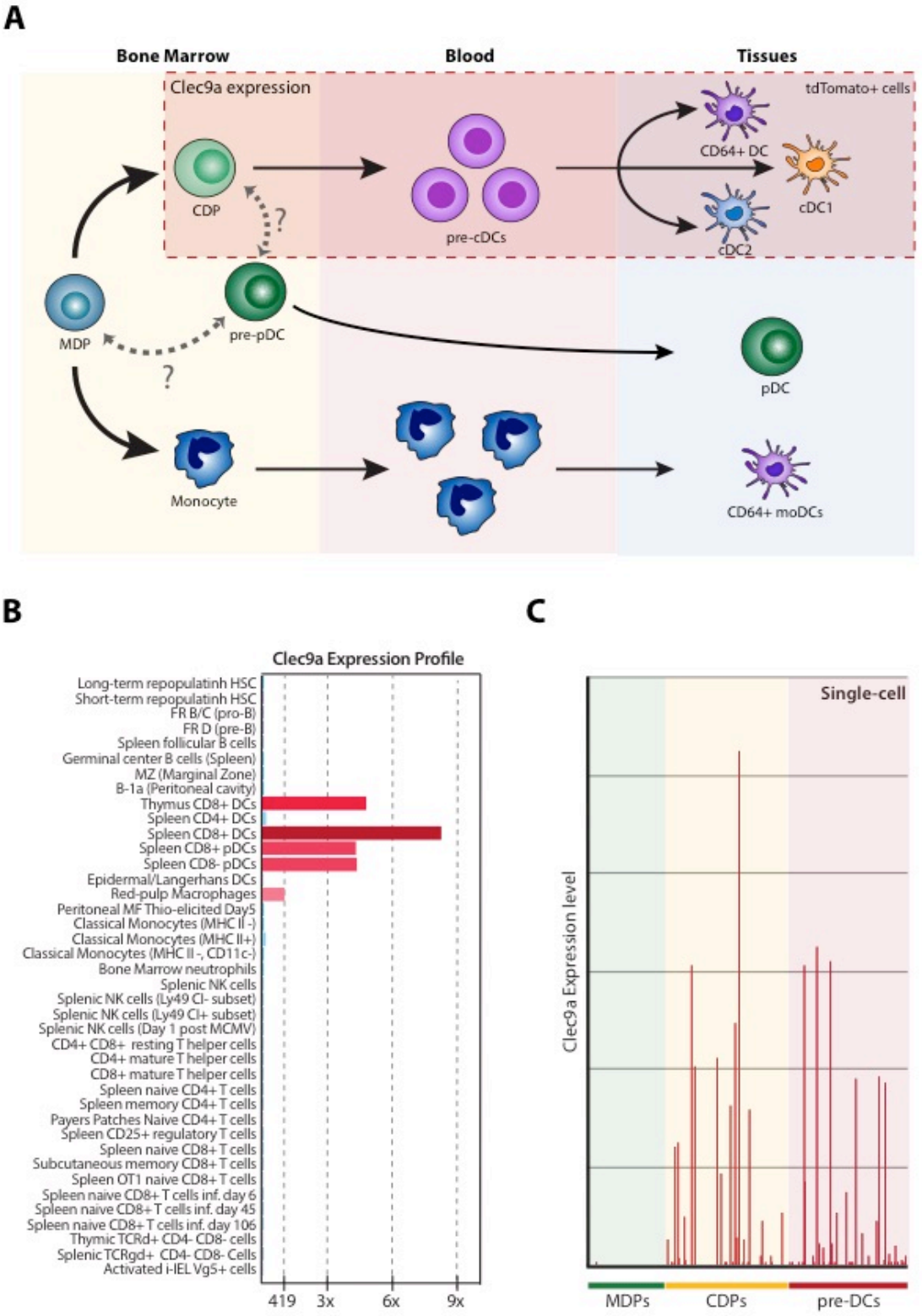


Figure 22. Expression of Clec9a is restricted to the DC-lineage. (**A**) Clec9a-Cre X R26-stop-Tomato double transgenic mouse enables identification of cDC and their committed precursors (CDP and pre-DCs), but not other leukocytes, due to the exclusivity of Clec9a expression. **(B)** Expression profile of Clec9a in different hematopoietic cell lineages obtained from data available in Immunological Genome Project (www.immgen.org), showing Clec9a expression is specifically restricted to DCs. (**C**) Gene expression of Clec9a gene at single cell level (GSE60783). DC-committed precursors, such as CDPs and pre-DCs, express Clec9a, contrarily to Monocyte DC Progenitors (MDPs).

3.6. SCREENING OF CANDIDATE DC-INDUCING TFS USING CLEC9A REPORTER MEFS

Before proceeding with the direct reprogramming experiments it was important to assure that direct reprogramming events were derived from the reprogramming strategy itself and not from the expansion of pre-existing hematopoietic cells. For that purpose it was important to exclude any cellular contamination with hematopoietic and rare tdTomato+ cells in the initial MEFs (**Figure 23, A**). Residual CD45+ and tdTomato+ cells were removed by cell sorting prior to transduction with DC-inducing TFs (**Figure 23, B**). MEFs used for the following experiments were tdTomato$^-$ CD45$^-$ with 99.8% purity (**Figure 23, C**).

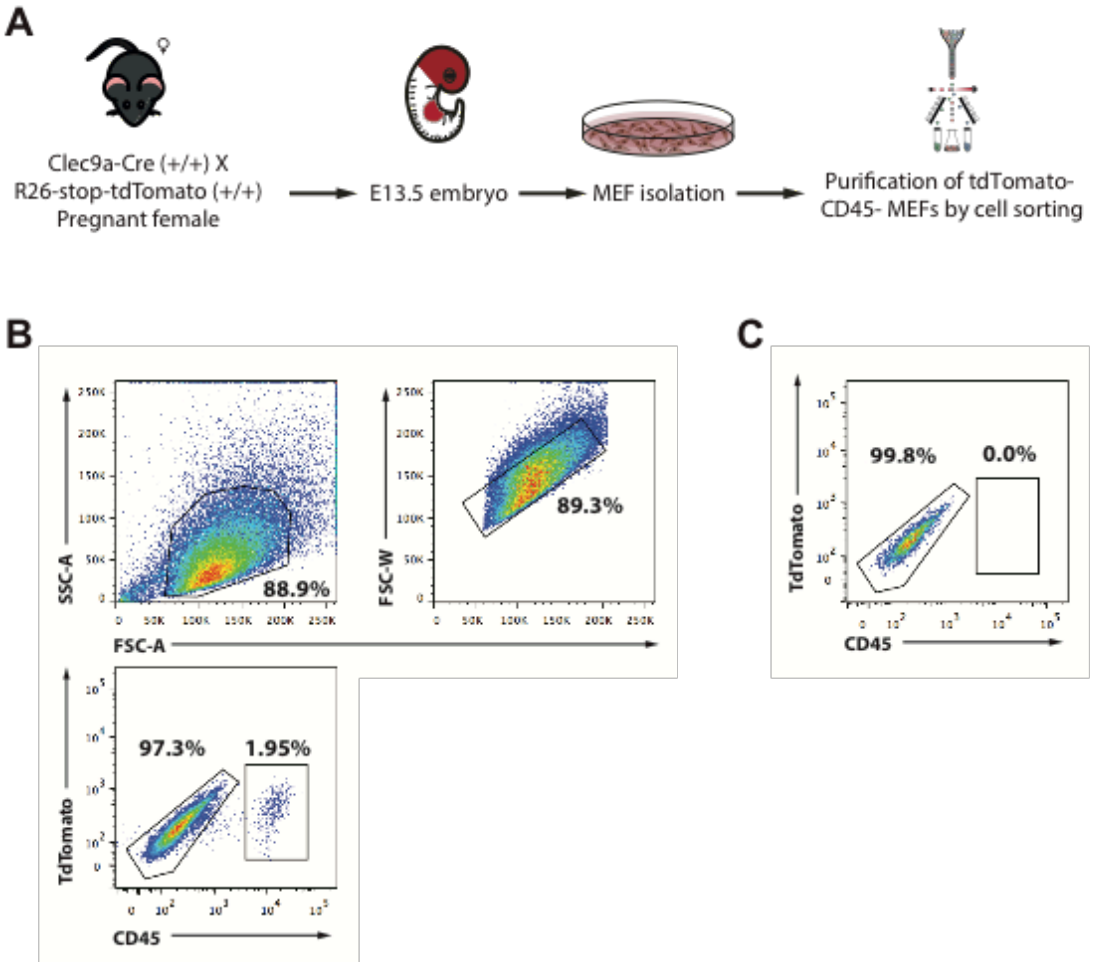


Figure 23. Strategy to obtain Clec9a reporter MEFs to screen candidate TFs. (A) Clec9a reporter mouse pregnant females were used to isolate MEF at embryonic day E13.5. After removal of the head, fetal liver and all internal organs, MEFs were cultured until confluency. MEFs were sorted to remove residual CD45+ and TdTomato+ cells that could represent cells with hematopoietic potential. (B) Gating strategy to remove residual CD45+ and TdTomato+ cells. Double negative MEFs, around 97% of the population, were sorted. (C) Purity confirmation of the sorted population.

The experimental strategy for the direct reprogramming of MEFs to DC-like cells is outlined in **Figure 24**. In Clec9a-Cre X R26-stop-Tomato mice, Cre expression leads to Cre-mediated excision of a stop codon flanked with loxP sites. This stop codon is surrounded upstream by a constitutive promoter – Rosa26, and downstream by a tandem version of the red fluorescent protein dTomato – tdTomato (**Figure 24, A**). After Cre expression driven by the Clec9a promoter and stop codon excision, tdTomato is constitutively expressed. The reporter is expected to be activated after commitment to the cDC lineage in CDPs, pre-DCs and mature cDCs. The access to this reporter mice enabled the development of a system in which the induction of DC-like cells from MEFs can be analysed quantitatively by reporter-based flow cytometry.

For the reprogramming experiments, different conditions were tested, such as M2rtTA viral particles alone or co-transduced with DCi1 and Cebp/a, pool of all the available 9 TFs or a more restricted pool of 4 TFs **(Figure 24, A).** M2rtTA alone was included as a negative control since we do not expect the activation of the reporter with this condition. The Pu.1 and Cepb/a condition was included as a stringent control for inducing macrophage cell fate as described by Feng et al. Because macrophages do not express Clec9a, we do not expect to have tdTomato+ cells in these cultures. Notwithstanding testing all available candidate DC-inducing TFs, a restricted pool of 4TFs (DCi1, DCi6, DCi5 and DCi4) were also included as the strongest candidates for their described key role on DC development, particularly for cDCs.

After transduction, MEFs were analysed for the presence of tdTomato+ cells by fluorescence microscopy at days 2, 5, 8, 12 and 15 after dox supplementation and by flow cytometry at days 5, 8, 12 and 15 (**Figure 24, B**).

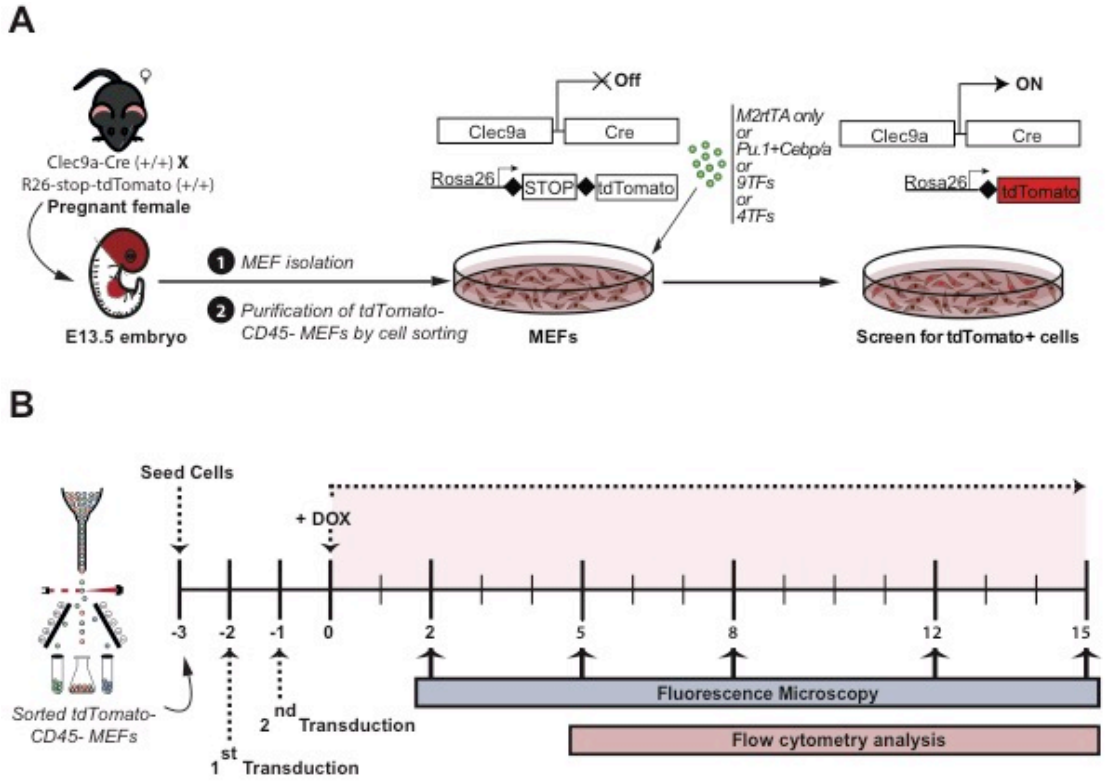


Figure 24. Strategy for screening candidate TFs able to reprogram MEFs to DC-like cells. A) Clec9a reporter mouse pregnant females were used to isolate MEF at embryonic day E13.5. After removal of the head, fetal liver and all internal organs, MEFs were cultured until confluency**. B)** Proposed experimental timeline for screening tdTomato+ MEFs. Purified MEFs are seeded the day before the first transduction. After Doxycycline supplementation at day 0, tdTomato+ cells were analysed by Fluorescence microscopy (days 2, 5, 8, 12, 15) and by flow cytometry (days 5, 8, 12, 15).

3.7. DCI1, DCI6, DCI5 AND DCI4 ARE SUFFICIENT AND NECESSARY FOR CLEC9A-REPORTER ACTIVATION IN MEFS

Two days after the addition of Dox tdTomato+ cells were observed in both MEF cultures transfected with 9 TFs and 4 TFs, suggesting the ability of both sets of 9 and 4 TFs to activate the Clec9a promoter (**Figure 25**). On the other hand, tdTomato+ cells were not found in cultures transduced with Pu.1 and Cebp/α.

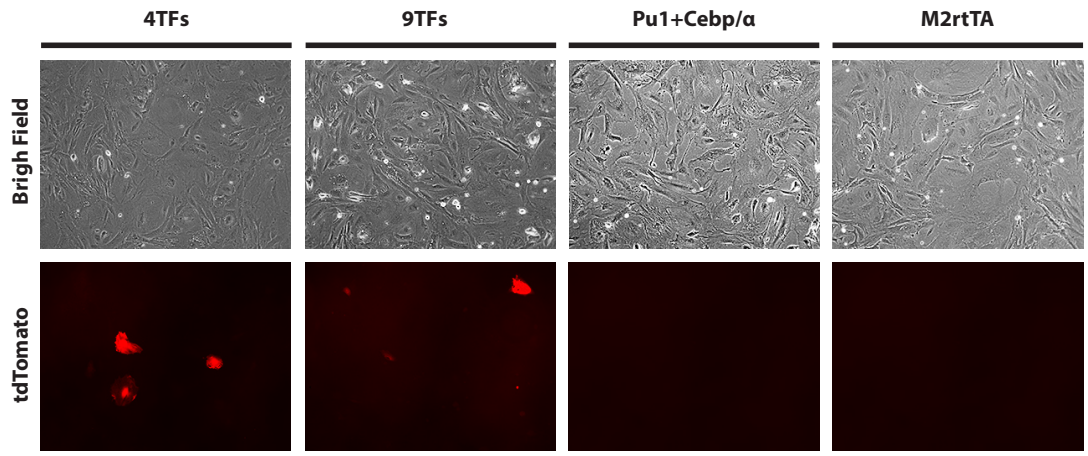


Figure 25. tdTomato+ cells start to be identified at day 2 after doxycycline supplementation. MEF cultures were transduced with 4 TFs (DCi1, DCi6, DCi5 and DCi4), 9 TFs , DCi1+Cebp/α or M2rtTA. MEF cells were analysed by fluorescent microscopy 2 days after the addition of Dox.

To assess the efficiency of the direct reprogramming process, tdTomato+ MEFs were analysed by flow cytometry at distinct time-points. At day 5, 2.28% of MEFs transduced with 4 TFs were tdTomato+ while with all 9 TFs 4-fold less tdTomato+ cells were generated (0.59%) (**Figure 26, A and B**). In contrast, tdTomato expression was not detected in MEFs transduced with M2rtTA or DCi1+Cebp/α. These results suggest that DCi1, DCi6, DCi5 and DCi4 are sufficient for efficient activation of the reporter. The percentage of tdTomato+ cells increases up to 3.44% in the 4TF treatment at day 8, from which remains constant. Regarding the condition with 9 TFs, percentage of tdTomato+ cells increases up to 0.59% at day 5, which continues to increase slowly up to 0.81% at day 15. tdTomato expression was not detected at any time-point in both M2rtTA and DCi1+Cebp/α transductions. The induction of CD45-positive cells was confirmed in MEF cultures transduced with DCi1+Cebp/a at day 10 (5.54%) (**Figure 7, C**).

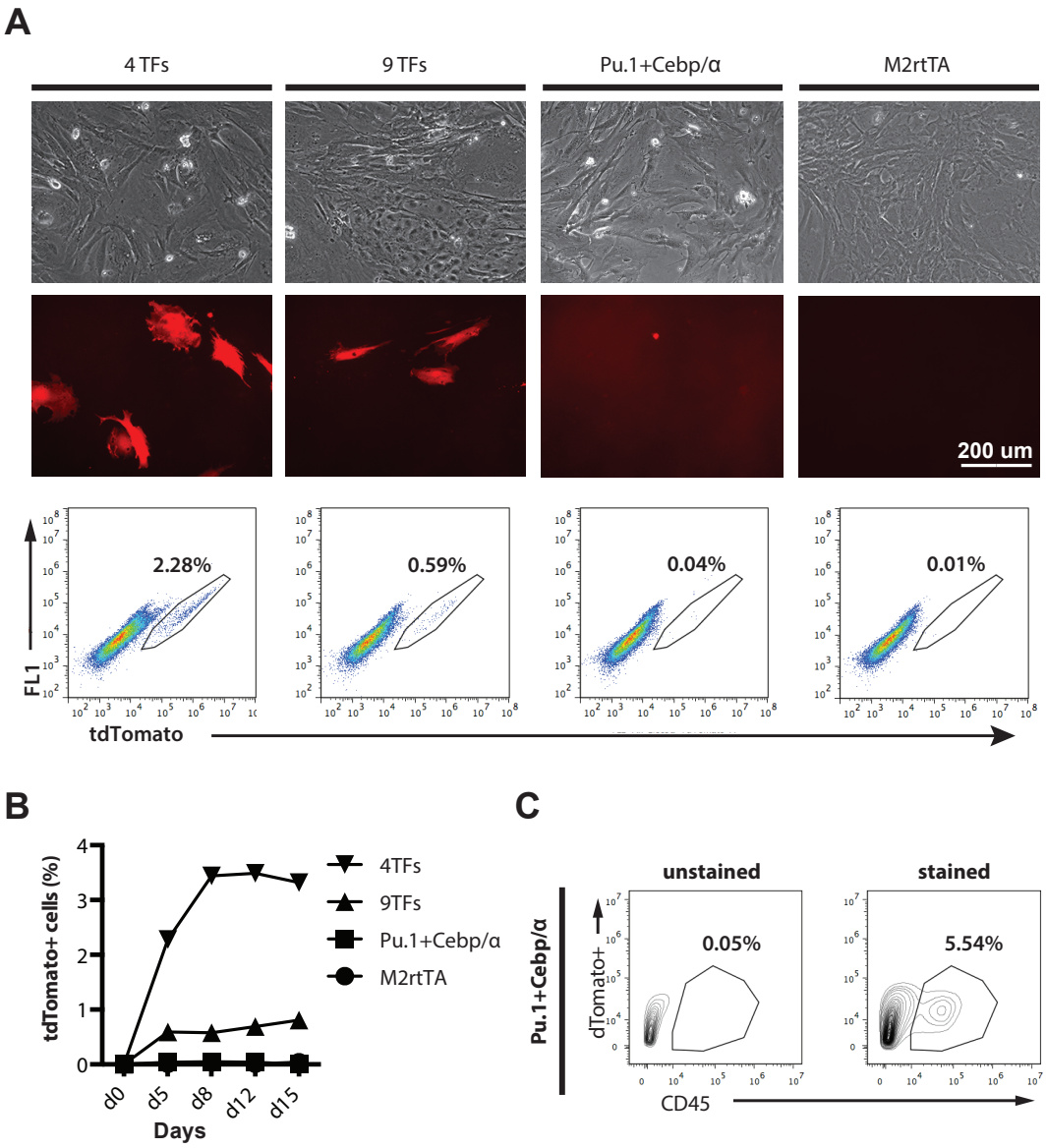


Figure 26. Combination of DCi1, DCi6, DCi5 and DCi4 induces efficient activation of the DC-specific reporter. MEF cultures were transduced with 4 TFs (DCi1, DCi6, DCi5 and DCi4), 9 TFs , DCi1+Cebp/α or M2rtTA. **A.** MEF cells were analysed by fluorescent microscopy and flow cytometry 5 days after the addition of Dox. **B.** MEF cells were analysed by flow cytometry at days 5, 8, 12 and 15 after the addition of Dox. **C.** Surface expression of CD45 was assessed by flow cytometry in MEF cells transduced with DCi1+ Cebp/ α at day 10 after addition of dox.

3.8. TDTOMATO+ CELLS UNDERGO PROFOUND MORPHOLOGICAL CHANGES DURING REPROGRAMMING

After transducing with DC-inducing TFs, emerging morphological features, i.e. increased cell size and cytoplasmic projections, can be assessed by fluorescence microscopy [203]. After day 5, morphology of cells expressing tdTomato was characterized by fluorescence microscopy. The morphological changes and the expression of tdTomato appear to be correlated. While some tdTomato+ cells showed numerous and thin dendrites dispersed in many directions from the spherical cell body **(Figure 27, C)**, others showed extended and branched cytoplasmatic projections derived from the cell body **(Figure 27, B, D, H and L)**. Others showed a flattened morphology characterized by a vast cytoplasm with a prominent nucleus and a small nucleus versus cytoplasm ratio (**Figure 27, A, E, F, I and J)**, showing in some cases quite evidenced ruffles at the surface **(Figure 27, F)**. In some cases, several fluorescent vesicles with an intense fluorescent signal were found inside the cytosol (**Figure 27, M and J**). In fact, these vesicles seemed to be widely dispersed throughout the cytoplasm. Several other morphology variants were continuously found throughout the experimental timeline. No obvious direct correlation was found between the experimental time course and the emergence of morphological features described behind, meaning that the morphological heterogeneity was constant from day 5 to day 15.

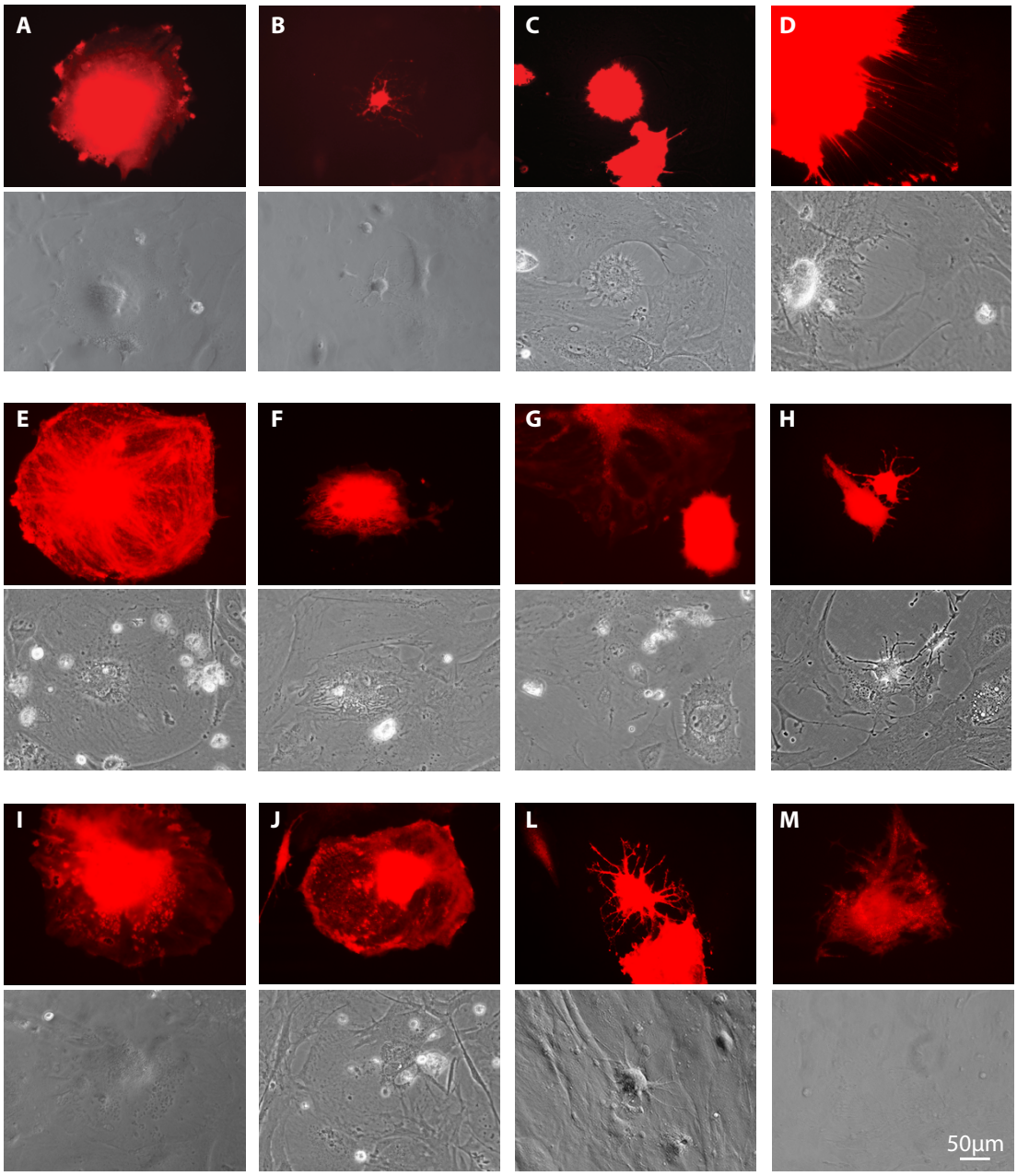


Figure 27. tdTomato+ cells show heterogenous DC-like morphology. Fluorescent microscopy photos were taken to capture the heterogeinity of tdTomato+ induced cells. Highlighted are increased size and clear cytoplasmatic projections.

In order to evaluate cell size and complexity/granularity, tdTomato+ cells were analysed by flow cytometry for Forward Scatter (FSC) and the Side Scatter (SSC) (**Figure 28**). While the FSC parameter gives the predicted cell size by measuring the laser beam that passes around the cells, the SSC parameter gives the intracellular complexity by measuring the amount of the laser bean that bounces-off from particles inside the cell. The expression of the selected 4 TFs resulted in an increased size of the live population 12 days after dox addition. Increased complexity of the cells was observed from day 5 to day 12. The increased size and complexity was particularly marked in tdTomato+ cell population 5 and 12 days after dox addition consistent with the morphologies observed by fluorescent microscopy.

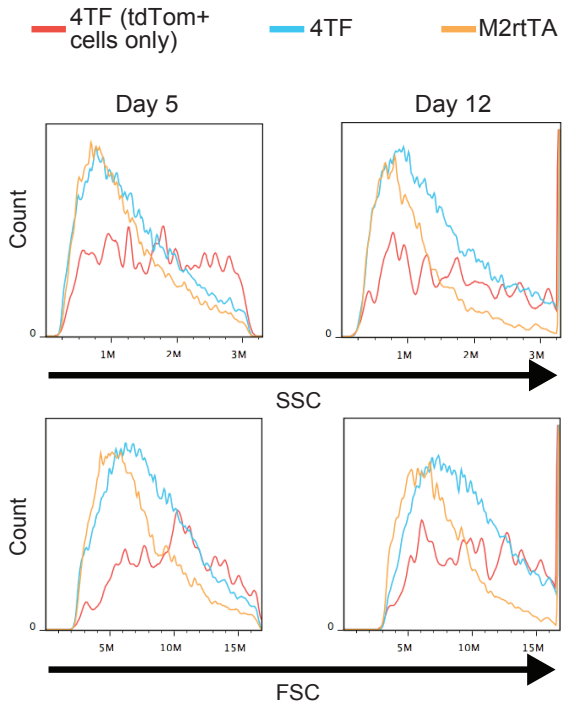


Figure 28. Size and complexity of induced Clec9a-tdTomato+ cells. Flow cytometry histograms showing size (FSC) and complexity (SSC) of transduced MEFs. MEF cultures were transduced with 4TFs and compared with cells transduced with M2rtTA alone. tdTom+ (red line) represent a gated population of tdtomato+ cells of MEFs transduced with DCi1, DCi6, DCi5 and DCi4 (4 TFs).

The aim of this study is to generate DC able to efficiently initiate an adaptive immune response by presenting antigens to lymphocytes. A key feature for the establishment of APCs relies on the capacity to not only process pathogenic or “self” proteins into small peptides but also on the capacity to express MHC-II molecules able to carry antigenic peptides to the cell surface [204]. In that sense, surface expression of MHC-II was accessed by flow cytometry after incubation with anti-MHC-II antibodies (**Figure 29**). Approximately 27% of tdTomato+ cells expressed MHC-II at the cell surface

at day 8. This result suggests that induced tdTomato+ cells may function as APCs as they express MHC-II molecules at the surface.

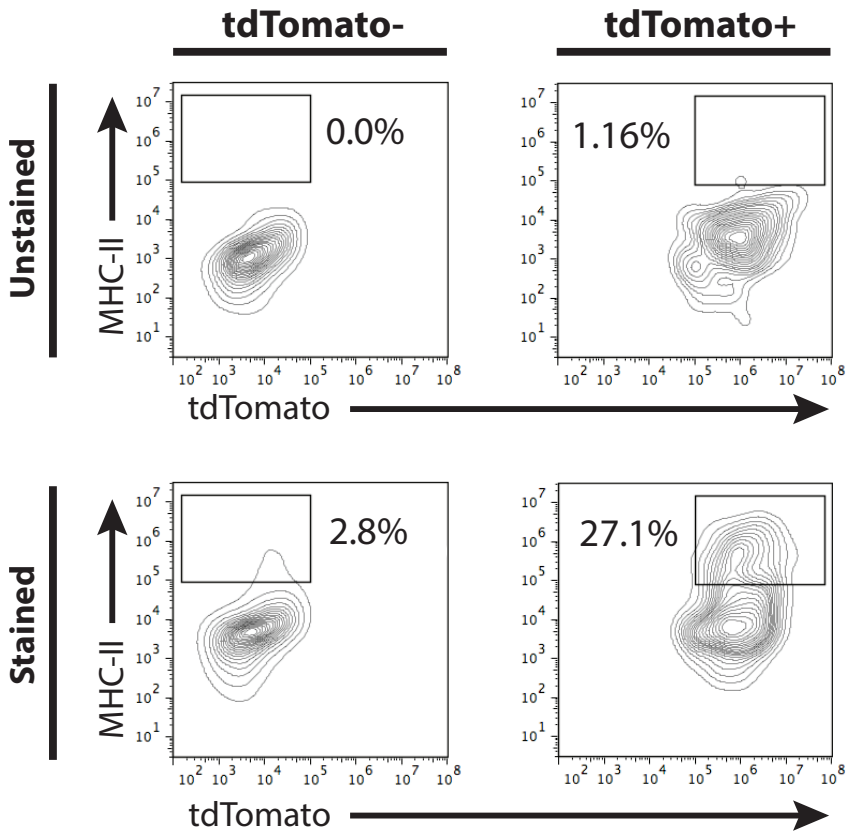


Figure 29. Induced Clec9-TdTomato+ cells express MHC-II at the cell surface. Flow cytometry analysis of MHC-II expression in TdTomato-negative (left) and TdTomato-positive (right) populations 8 days after transduction with 4 TFs.

3.9. THE FOUR TFS DCI1, DCI6, DCI5 AND DCI4 ARE REQUIRED FOR THE EFFICIENT REPORTER ACTIVATION

Two distinct pools of TFs were previously tested for their potential to induce DC-like cells. Since the pool of 4TFs was able to induce 4-fold higher activation of the reporter when compared to the pool of 10 TFs, the next logic step was to test the relevance of each one of the 4 TFs for the activation of the reporter. Each one of the 4 TFs was individually excluded from the pool in order to assess their importance for conversion. MEFs were transduced with 4TFs-DCi1, 4TFs-DCi6, 4TFs-DCi5 and 4TFs-DCi4 combinations as well as with each one of the 4 TFs individually (**Figure 30**). Percentage of cells expressing tdTomato was assessed by flow cytometry at day 8. Remarkably, the removal of each one of the TFs impaired the induction of tdTomato+ cells.

The exclusion of DCi1 or DCi5 generated 4-fold less tdTomato+ cells while the exclusion of DCi4 generated 7-fold less tdTomato+ cells. The exclusion of DCi6 was the most impactful, generating 20-fold less tdTomato+ cells. Conversely, the individual transduction of DCi1, DCi5 and DCi4 did not induce tdtomato+ cells. Transduction with DCi6 alone generated 11-fold less tdtomato+ cells when compared to the 4 TF pool. These results support the selection of the pool of 4 TFs as a minimal set of TFs able to robustly induce Clec9a-reporter activation and DC cell fate in fibroblasts.

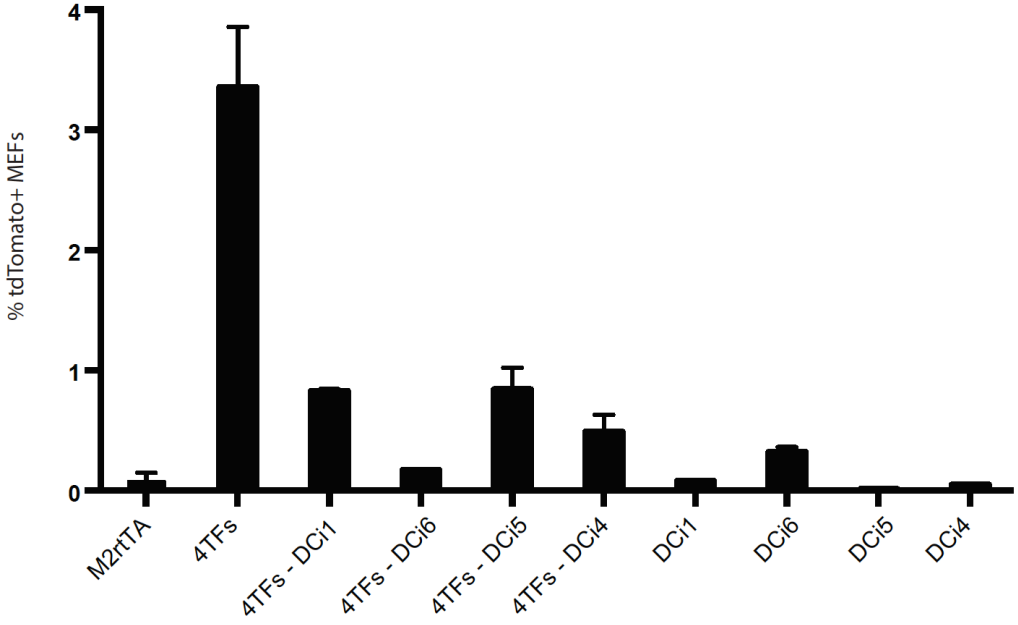


Figure 30. Elimination of each TF from the 4 TF pool dramatically impact reporter activation. Quantification of TdTomato+ cells after removal of individual TFs from the pool of 4TFs or expression of the individual TFs alone.

In fact, the relevance of DCi1, DCi6, DCi5 and DCi4 for the DC lineage is supported by several reports described in the literature mining and available gene expression data. For instance, the myeloid master regulator DCi1 is expressed in all cDCs and its inactivation within hematopoietic progenitors or CDPs ablates DC differentiation [169]. DCi1 has been implicated in transcriptional mechanisms responsible for high-order chromatin structure remodelling at the DCi5 gene, promoting the commitment to the DC fate [205]. In fact, DCi1 fails to drive DC differentiation in the absence of DCi5. DCi5 and DCi6 are two TFs implicated as DC fate promoters to the cDC subsets, i.e. cDC1 and cDC2 respectively [74]. cDC1 commitment has been associated with increased expression of DCi5, which expression relies on the DCi5 auto-activation loop maintained by DCi4. For assessing the stage specific expression of each TF, expression of the 4 TFs in MDPs, CDPs, and pre-DCs was analysed at a single cell level (**Figure 31**). During DC differentiation, DCi1 seems to be expressed at similar levels in the three stages. DCi6

expression seems to be restricted to a small population of pre-DCs. DCI5 expression suffers a considerable increase at the CDP stage while DCi4 seems to be activated in the transition between CDPs and pre-DCs.

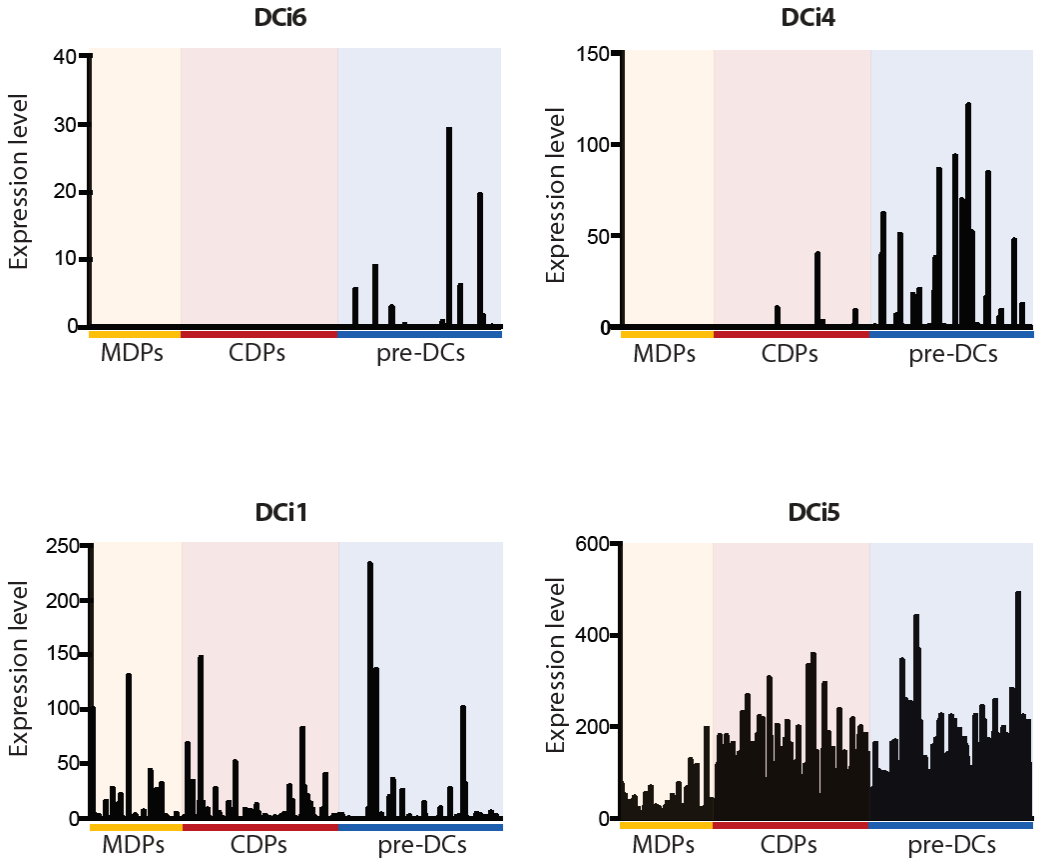


Figure 31. Expression profiles of DCi1, DCi6, DCi5 and DCi4 at the single cell level. Gene expression of *DCi1*, *DCi6*, *DCi5* and *DCi4* genes at single cell level (GSE60783) in DC progenitors (MDPs, CDPs, and pre-DCs). Expression level is expressed in reads per kilobase of exon model per million mapped reads (RPKM) values.

3.10. MORPHOLOGY OF TDTOMATO+ CELLS REMAIN STABLE AFTER REMOVAL OF EXOGENOUS TF EXPRESSION

At day 15 after Dox supplementation, tdTomato+ cells transduced with the 4 TFs were sorted and cultured in the presence or absence of Dox. The morphology and total number of cells was evaluated at day 22, 29 and 36 (**Figure 32, A, B and C)**. After day 15 the morphology of tdTomato+ cells remained similar in both conditions (-dox,+dox) **(Figure 32, B)**. The number of tdTomato+ cells counted by fluorescence microscopy decreased with time in both conditions. Therefore Dox removal does not impact the morphology and number of tdTomato+ cells. These results suggest that the induced DC cell fate is stably imposed by the 4 TFs.

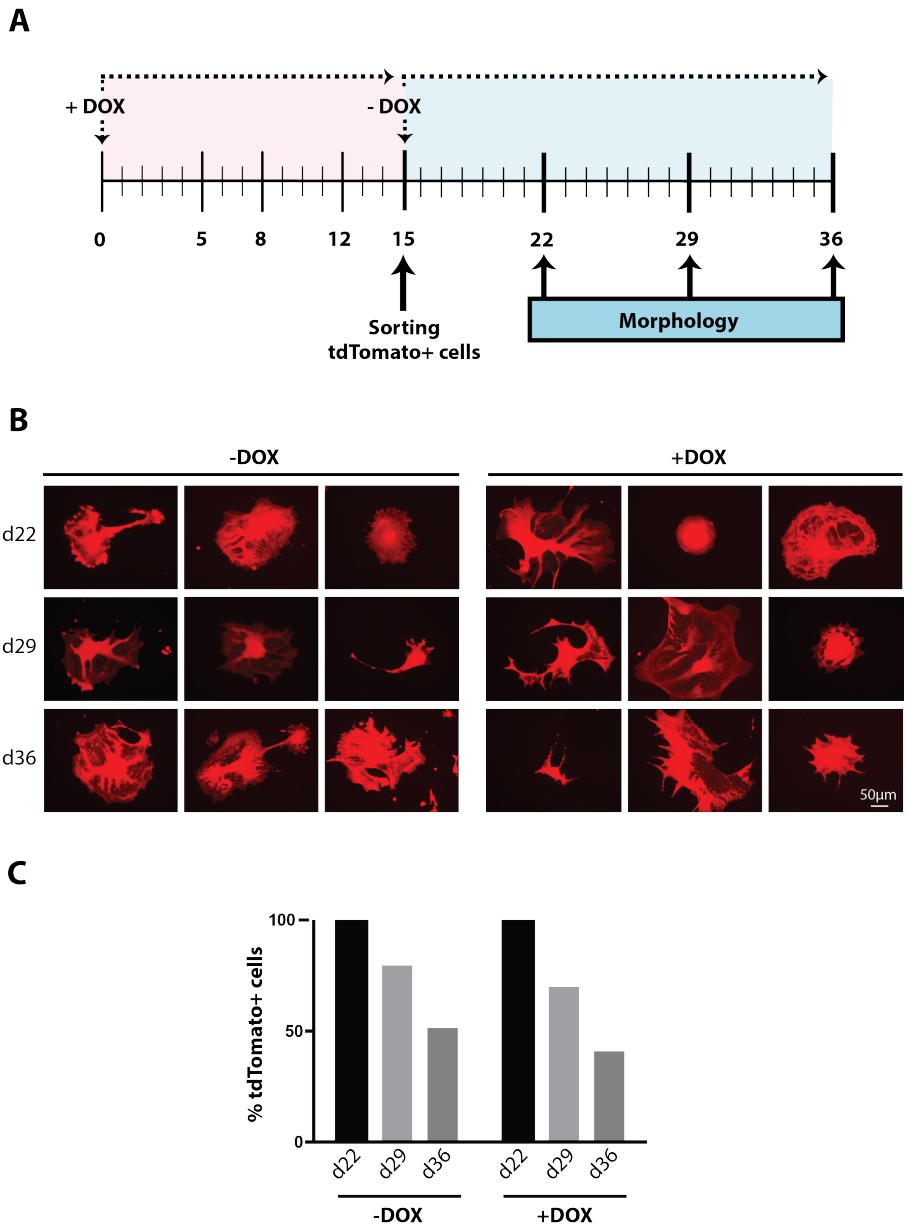


Figure 32. Stability of reprogrammed DCs after removal of exogenous TFs. (**A**) Experimental outline. At day 15 after transduction, TdTomato+ cells were sorted and cultured with or without Dox that leads to the downregulation of the exogenously expressed 4TFs (**B**) Fluorescent microscopy images showing that TdTomato+ cells retain DC-like morphology when cultured in the absence of Dox until day 36. (**C**) Quantification of tdTomato+ cell number after Dox removal to access cell viability. Cultures with Dox were maintained and included as control.

3.11. DCi1, DCi6, DCi5 AND DCi4 ARE SUFFICIENT TO INDUCE MORPHOLOGICAL CHANGES IN HUMAN FIBROBLASTS

Since MEF transduction with the 4 TFs induced an efficient activation of the reporter and TFs involved in fundamental processes are highly conserved between species [198], we recapitulated the experiments in Human Dermal Fribroblasts (HDFs).

For the reprogramming experiment in HDFs, transduction with 4 TFs and 9 TFs, as well as M2rtTA only, were used to induce morphological changes after enforced expression of TFs **(Figure 33, A)**. Remarkable changes in cellular morphology were observed 8 days after Dox supplementation. HDFs transfected with M2rtTA showed typical fibroblastic morphology characterized by bipolar appearance and elongated shapes, while HDF cultures transfected with 4TFs showed not only cells with typical fibroblastic morphology but also flattened cells very distinct from surrounding HDFs (**Figure 33, B**). These morphologically distinct cells had considerably irregular structure and were very heterogeneous. Some showed thin cytoplasmic extensions dispersed in many directions from a small cell body. While some cells were delimited by a considerably smooth borderline, others were delimited by an irregular boundary characterized by small, and some times branched, cytoplasmic extensions spread around the cell surface. Morphological changes started to be evident at day 5, which correlates with the timing of Clec9a-reporter activation in MEFs. This experiment shows that the same pool of TFs is able to efficiently induce the activation of the Clec9a reporter in MEFs is also able to induce marked morphological changes in cultures HDFs, suggesting that transcriptional regulators involved in cDC commitment may be conserved in both mice and humans.

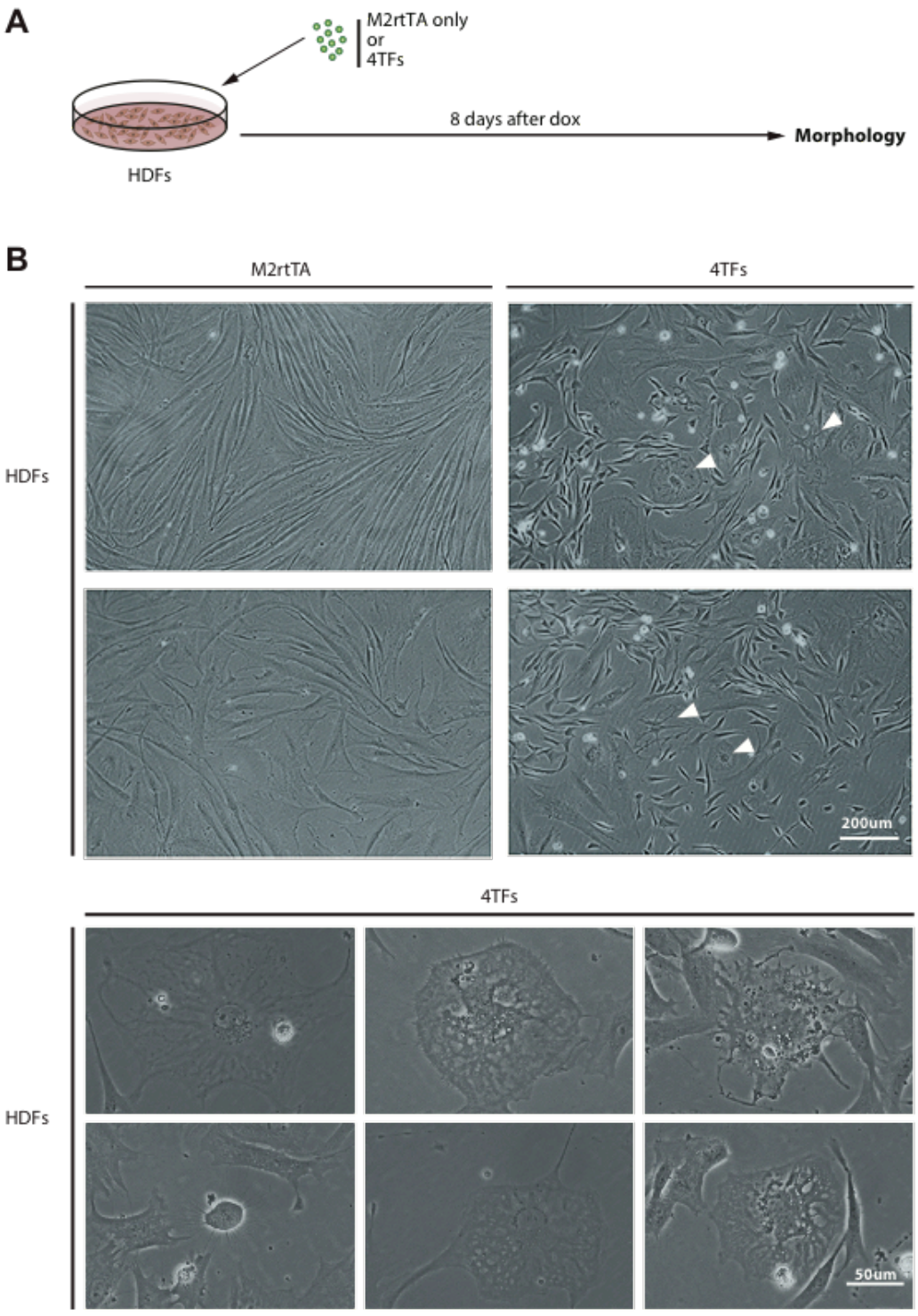


Figure 33. Selected pool of 4TFs induces DC-like morphology in human fibroblasts. (**A**) HDFs fibroblasts were transduced with FUW-M2rtTA lentiviral particles alone or in conjunction with pool of 4TFs, and cultured in the presence of Dox. Morphologic alterations were monitored during 8 days. (**B**) HDFs co-transduced with M2rtTA and 4TFs display distinctive morphologies when compared with HDFs transduced with M2rtTA alone (top panels). White arrowheads mark cells with typical DC-like morphology (top right), also displayed in higher magnification (bottom panels).

CHAPTER III

4. DISCUSSION

In 1973, Ralph M. Steinman identified a cell type responsible for orchestrating the complexity of immune responses – DCs. For this discovery, Steinman was awarded with the 2011 Nobel Prize in Physiology or Medicine. The role of DCs is now well established as pivotal regulators of innate and adaptive immune responses [206]. These ”immune sentinels” have been intensively studied as potential therapeutic option for treating a wide variety of health problems, ranging from infectious and inflammatory diseases to cancer However, the clinical outcome of DC-based therapies has been inconsistent, which may be associated with the decreased efficiency of *in vitro* generated DCs. In that context, new methods are required for generating efficient APCs for clinical application Although the generation of DCs from iPSCs have been suggested, the clinical use of somatic cells derived from iPSCs have substantial concerns regarding the length of time required for the process, the decreased efficiency and safety issues linked to the possibility of teratoma formation. In that sense, direct reprogramming appeared as a promising alternative to bypass these limitations by avoiding the pluripotent state and its disadvantages. In this context, the present study aimed to generate DCs via TF-mediated direct lineage reprogramming.

Since direct reprogramming is generally achieved by overexpressing lineage-instructive TFs, 19 candidate TF with key developmental roles in the DC lineage were identified. The expression of the 19 candidate TFs is restricted to DC populations in both mice and humans and their disruption in mice impairs DC development and function, creating abnormal immune phenotypes. Selected TFs are increasingly expressed during DC development and, importantly, are restricted to DCs when compared to macrophages. Even though macrophages and DCs belong to developmentally close lineages, macrophages are characterized by a poor antigen-presenting ability, precluding their use for the induction of immunity. Additionally, their generation by direct reprogramming has already been accomplished by overexpression of DCi1 and Cebp/α [26]. From the initial 19 candidates, 9 TFs were successfully cloned into inducible lentiviral vectors and assessed for their capacity to induce the DC-fate in cultured MEFs and HDFs. MEFs collected from Clec9a-Cre X R26-stop-tomato mice do not express the fluorescent protein tdTomato. Lentiviral-induced overexpression of 9 TFs in MEFs induced the activation the DC-specific reporter in MEFs generating approximately 0.6% of tdTomato+ cells at day 8. Since the self-reinforcing nature of gene regulatory networks underlying specific cell fates is expected to rely on a small number of key TFs. A more restricted pool of TFs was selected for assessing their capacity to induce the activation of the DC reporter. Remarkably, the combination of DCi1, DCi6, DCi5 and DCi4 generated 4-fold more of tdTomato+ cells (3.4%) at day 8. The Clec9a-

tdtomato reporter was not detected when M2rtTA only or the macrophage-inducing TFs DCi1 and Cebp/α were overexpressed in MEFs, supporting the specificity of the reporter system to the DC lineage.

During reprogramming, fibroblast cells undergo striking morphological changes characterized by increased cell size and complexity. Both were expected since DCs are large in size and develop abundant intracellular vesicles due to the high level of endocytic activity [207]. The population of induced tdtomato-positive cells was highly heterogeneous. While some tdTomato+ cells showed a mature DC-like morphology characterized by the existence of extended cytoplasmatic projections, others showed an immature-like morphology characterized by a smoother membrane surface [208]. Since in vitro cultured Bone Marrow-derived DCs were described to adopt a mature morphology only after stimulation with lipopolysaccharide (LPS), poly I:C or thymic stromal lymphopoietin (TSLP), the emergence of tdTomato+ cells with dendrites is already remarkable. Expression of both DCi6 and DCi5 is correlated with the expression of surface molecules typically associated with a mature DC phenotype, i.e. CD80, CD86 and MHC II [209]. Thus, emergence of cells with prototypical features of mature DCs may be due to the forced overexpression of Irfr4 and DCi5. On the other hand, certain tdTomato+ cells showed a considerably big cell size with a vast cytoplasm and a prominent nucleus. A better phenotypic characterization of induced DC-like cells at the single cell level by flow cytometry or gene expression analysis will be required to explain this levels of heterogeneity. In addition, the manipulation of culture condition to provide maturation stimulus, such as supplementation with LPS will be tested.

We have identified a minimal TF-network able to establish the DC specification since the removal of each one of the 4 TFs dramatically impact the number of tdTomato+ cells generated during reprogramming. Individual overexpression of the TFs DCi1, DCi6, DCi5 or DCi4 was not sufficient to impose this cell identity conversion. This suggests a cooperative and synergistic effect between these transcription factors for establishment of the DC-like fate. Moreover, the induced tdTomato+ cells at day 15 seem to stably acquire the new cell identity as they are independent on the doxycycline-induced overexpression of the 4 TFs. The stability of the reprogrammed cell fate is a key parameter to address complete cellular reprogramming. Whether TF-downregulation would negatively or positively impact the function of induced DCs need to be further investigated.

Importantly, approximately 27% of tdTomato+ cells expressed MHC-II at the cell surface, a molecule that is highly expressed in professional APCs. These surface molecules are responsible for presenting processed peptides to lymphocytes in order to orchestrate an antigen-specific immunological response. Since the aim of this project is to generate professional APCs, the surface expression of this molecule is of great relevance, suggesting the

ability of the 4 TFs not only to activate the DC-specific reporter but also promotes the expression of MHC-II molecules. In fact, the expression of MHC-II is regulated by several TFs. The master regulator cofactor class II transactivator (C2ta) controls MHC-II expression in mice and DCi1 positively regulates the expression of C2ta in cDC. Therefore DCi1 has a pivotal role in promoting the expression of MHC-II molecules [210]. Furthermore, DCi6 and DCi5 might be good candidates for determining the function of DCi1 in stimulating MHC-II expression. In fact, DCi1 functions not only as a monomeric TF but also as a dimeric complex with either DCi6 or DCi5 [211]. These dimeric forms may differentially impact the expression level of MHC complexes in DCs and, consequently, the capacity to process and present antigens. For instance, DCi5-DCi1 dimer has been proposed to act as a key player in promoting C2ta transcription [212]. Since DCi4 maintains the autoactivation loop of DCi5 required for the commitment to the cDC1 subset, it is reasonable to speculate that DCi4 expression may also impact indirectly the expression of MHC molecules. Moreover, DCi6 has been reported to stimulate preferentially the expression of genes encoding core components of the MHC class II pathway required for efficient generation of peptide-MHC class II complexes [209].

Similarly, lentiviral-mediated overexpression of the same 4 TFs in Human cells (HDFs) is able to profoundly impact cellular morphology. In fact, 8 days after transduction, some HDFs suffered a profound morphologic shift, transiting from an elongated bipolar morphology to a flattened shape with features of the human DC compartment. These findings suggest that commitment to the DC lineage may rely on basal transcriptional events that are conserved in both mouse and human.

Collectively, the overexpression of DCi1, DCi6, DCi5 and DCi4 may be sufficient to orchestrate a gene regulatory network capable of directing the DC fate. The cooperative mechanism of the 4TFs underlying the establishment of a DC gene regulatory network is proposed in **Figure 34**. Similarly to the role of Ascl1 in establishing the competence to the neuronal lineage in iNs, DCi1 seems to work as a pioneer TF during Myeloid development in both mice and humans [213-215]. In fact, the specification to distinct myeloid and lymphoid lineages may rely on additional lineage-specific TFs that, in combination with DCi1, are able to specify the GRN to specify each hematopoietic cellular identity. The direct reprogramming of fibroblasts to macrophage-like cells relies on the overexpression of DCi1 in combination to Cebp/α [26]. While DCi1 seems to confer accessibility to target enhancers responsible for establishing the competence for the myeloid lineage, Cebp/α appears to establish the commitment to the macrophage cell fate [22]. Indeed Cebp/a by itself is sufficient to convert a hematopoietic cell-type (B-cell) into macrophages [18]. In the DC compartment, DCi1 triggers DCi5 chromatin remodelling and transcription in a concentration-dependent manner.

Accordingly, this remodelling may rely on the binding of DCi1 to the -50 kb DCi5 enhancer. This induces a chromatin loop able to promote the DCi5 autoactivation and, consequently, the MDP-to-CDP transition [205] (specification). The DCi5 autoactivation loop seems to drive the full maturation process from CDPs to cDC1s. However, while the same transcriptomic event enables the commitment to the pre-cDC1 lineage, the DCi5 autoactivation loop becomes DCi4-dependent during commitment to the cDC1 lineage. DCi6 is expressed at this stage, where pre-cDCs can either start expressing DCi4 and become committed to the cDC1 lineage by expressing high levels of DCi5; or they lose the potential to express DCi5 and become committed to the cDC2 lineage which requires DCi6 [74]. The removal of DCi6 from the 4 TF pool had a big impact in reporter activation. The identification of the precise DCi6 transcriptional events underlying DC commitment and clec9a activation may need to be re-evaluated in light of our results. Moreover, as previously mentioned, DCi6 stimulates the expression of genes encoding the machinery required for antigen presentation via MHC-II. This might be important for the expression of MHC-II observed in clec9a-tdTomato+ cells.

The reported combination of 4 TFs is sufficient to activate the DC-specific reporter but will the tdTomato+ cells be able to process antigens, migrate to the lymph nodes and initiate an antigen-specific immunological response? This is the *one million dollar question* that will have to be addressed in a near future. By now, it is important to establish the pillars that eventually will sustain this technology. Considering that only 9 of 19 TFs identified as potential candidates for inducing the DC fate *in vitro* were successfully cloned and tested, it is important to access the potential of each TF to induce APCs. Since the combination of Pu1, DCi6, DCi5 and DCi4 was able to induce efficient reporter activation, the effect of adding each one of the remaining 15 TFs will be assessed to maximize reprogramming efficiency. In fact, this screening will rely not only on the activation of the DC-specific reporter, but also on a secondary screening in which the expression of additional surface molecules that correlate with APC function (MHC-II and CD80, CD486 and CD40 co-stimulatory molecules). This analysis will be critical for the appropriate selection of the minimal TF network since some TFs may increase the APC ability without affecting the reporter activation. Once the minimal TF network is established, tdTomato+ cells will be characterized in terms of phenotype, global gene expression profile and functionality. While the phenotypic and gene expression analysis will give an idea of the DC subset tdTomato+ cells belong, the functional characterization will reveal if these cells are capable of functioning as an APC.

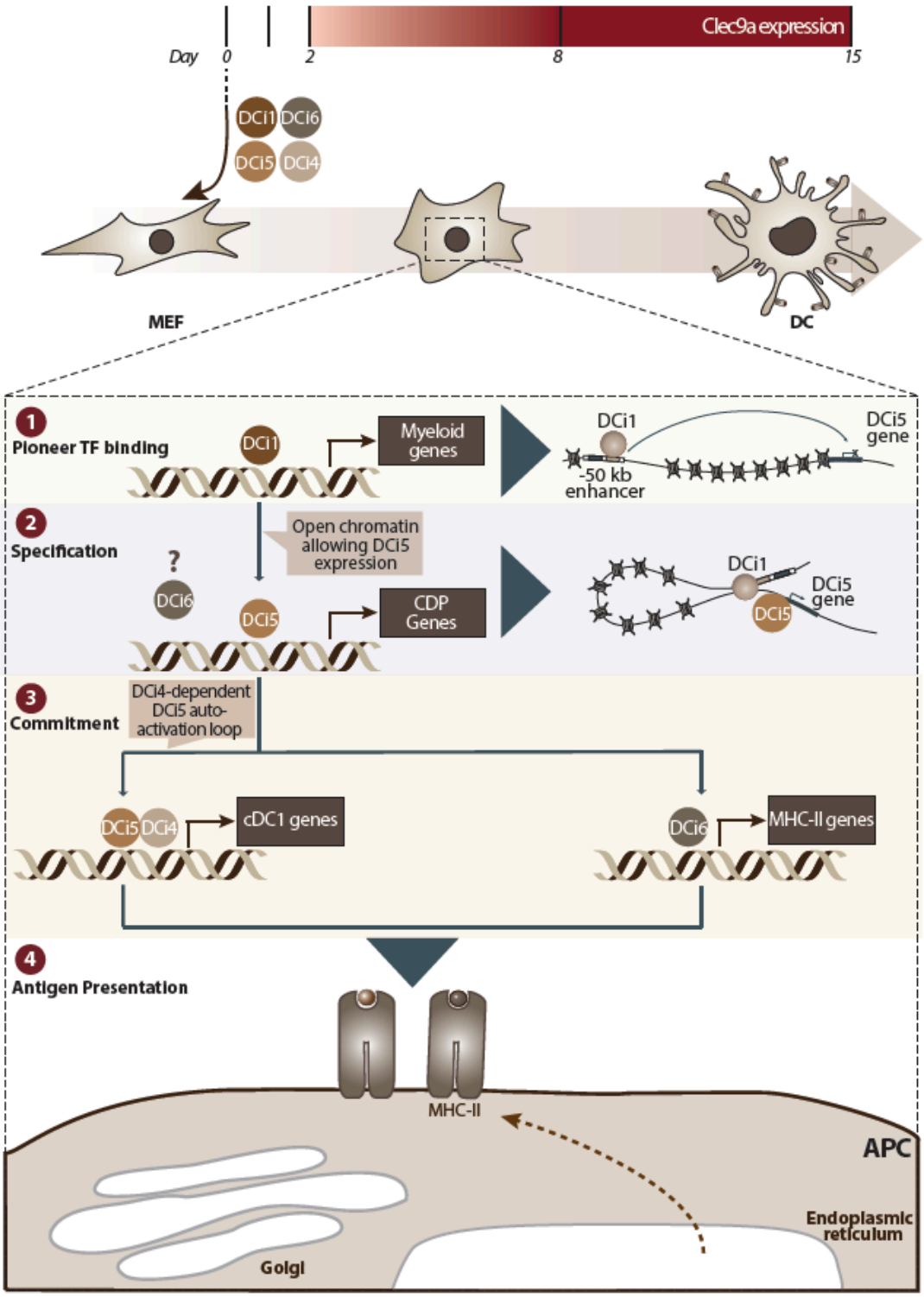


Figure 34. A model for the role of DCi1, DCi6, DCi5 and DCi4 in the direct reprogramming to Dendritic Cells. During reprogramming to Dendritic Cell (DC) fate may rely on DCi1 as a pioneer Transcription Factor (TF) able to induce high-order chromatin structure remodeling at the *DCi5* gene (*Pioneer TF binding*). The continuous expression of DCi5 may drive specification to the cDC lineage (*specification*). DCi5 expression become DCi4-dependent at the transition between pre-cDC1s and cDC1s (*commitment*). The generation of professional Antigen Presenting Cells (APCs) relies on subsequent transcriptional events. While DCi4 and DCi5 cooperate for maintaining high levels of DCi5 and, consequently, promoting the expression of cDC1 genes, DCi6 enables the generation of the required machinery for antigen presentation through Major Histocompatibility Complex Class II (MHC-II) molecules (*Antigen Presentation*). DCi6 may play an important role in this process. CDP: Common Dendritic Cell Progenitors; MEF: Mouse Embryonic Fibroblast.

The direct reprogramming of fibroblasts to DCs seems to be an alternative and attractive approach to obtain autologous DCs for therapy purposes. In fact, the focus is gradually moving towards the use of the patients' own immune system to treat cancer. In 2010, the FDA approval of Sipuleucel-T (PROVENGE, Dendreon Corporation) as an active cellular immunotherapy for treating advanced castration-resistant prostate cancer set up the therapeutic interest of DC-based therapies. Despite this DC-based vaccine hasn't been able to meet the high clinical expectations, the global DC-based therapy outlook is encouraging. In fact, several DC-based therapies in phase III clinical trial are likely to move forward. These include Eltrapuldencel-T, DCVax-L, AGS-003 and DCVAC/PCa [216]. Interestingly, DC and Chimeric Antigen Receptor T cell Therapies market is expected to become a multi-billion dollar market over the coming years. Indeed, these therapies have cumulatively raised over $500 million in venture funding rounds and its market is expected to witness an annual growth rate of approximately 30% between 2014 and 2024. DC-based therapies have emerged as an extremely promising field of immunotherapy. Several drivers are directly associated with this trend. DCs are able to generate a tumour-antigen specific immunological response and, by being autologous, the chances of being rejected by patients' body are significantly low. Additionally, therapy with DC-based vaccines is associated with reduced side effects as compared to conventional therapies and is associated with the generation of immunological memory. However, there are several current limitations. The manufacturing process for autologous DCs is considerably complex and patient-dependent, requiring the isolation of monocytes or CD34+ hematopoietic progenitors by cytapheresis and the subsequent culture in specialized GMP cleanrooms for obtaining DC in appropriate clinical conditions. This complex procedure results in considerably high cost of production. Moreover, it is directly correlated with other drawbacks, namely the generation of low-efficient APC derived from monocytes and the difficulty on collecting a minimal amount of CD34+ hematopoietic progenitors for preparing the vaccine. In addition, these precursor cells are commonly compromised in cancer-bearing patients, precluding their use for the induction of immunity. In contrast, fibroblasts are not affected by aggressive blood malignancies and are easily expanded *in vitro*.

The discovery of TFs capable of inducing the DC cell fate *in vitro* opened new doors for alternative, perhaps creative, ways of treating cancer. The species conservation of the minimal transcription network identified suggests the possibility that the DC reprogramming may work from other cell-types in addition to fibroblasts, as it is case in the induction of neurons or the induction of pluripotency. One interesting possibility arising from this study is the direct reprogramming of cancer cells into DCs capable of presenting their own cancer antigens to lymphocytes. This is a possibility that we will certainly address in the future.

The utilization of DCs for immunotherapy purposes has been studied not only for cancer treatment but also within other backgrounds. In fact, DCs could also be utilized for cancer prevention, treatment of infectious diseases or even transplantation. In the past 20 years prophylactic vaccines have been utilized as an immunopreventive therapy able to prevent carcinomas induced by viruses, i.e. Hepatitis B virus (HBV) and Human Papillomavirus (HPV) [140]. Despite the huge success of prophylactic vaccines on preventing viral-induced cancers, >80% of human cancers are induced by host DNA mutations rather than viral infection [217]. Therefore, there's an opportunity for the generation of an autologous DC-based vaccine that could efficiently prevent the development of non-viral cancers. This could be accomplished by the generation and manipulation of reprogrammed DCs, promoting the ideal DC maturation status able to provide an extensive immunological memory against specific tumor antigens.

Regarding infectious diseases, prior studies have proved the ability of DC to generate a protective immune response against distinct pathogens, i.e. protozoan, bacteria and virus [44]. One promising example is the Human immunodeficiency virus [87] [218]. This virus has evolved to evade our immune system by distinct mechanisms and is nowadays considered one of the bigger issues of worldwide health. Presently, the treatment is basically a combined antiretroviral therapy (cART), which refers to the utilization of drug combinations in order to control HIV infection [219]. However, cART is not able to eradicate the infection on HIV patients, therefore requiring the therapy throughout life. Considering these facts, new efforts are required for the development of viable therapeutic strategies against HIV infection. DCs, as critical APCs, play an important role on HIV dissemination by naturally allowing its transmission to CD4+ T cells through the virulogical synapse [220]. This initial contact between DCs and virus as been shown to result in distinct outcomes, either a strong immune response by T lymphocytes or a facilitated dissemination among CD4+ T cells, leading to a chronic HIV-infection characterized by the progressive loss of functional CD4+ T cells [221]. Moreover, other functional deficiencies, i.e. HIV-associated impaired antigen-presentation [222], are making HIV therapy an extremely challenging demand. One can hypothesise that the proper manipulation of autologous reprogrammed DCs, in association with the careful identification of adjuvants in order to manipulate and redirect the outcome of DC-HIV interaction, would provide a suitable clinical outcome in HIV patients, paving the road to the next generation of HIV therapies.

The manipulation of immune system in order to promote immune-tolerance of allografts also represents another possible application of reprogrammed DCs. In fact, the signaling pathways involved in the induction of tolerogenic DCs (tol-DCs) have been discussed by several authors [223-225]. The generation of engineered tol-DCs with immature phenotype and an anti-inflammatory cytokine profile has been accomplished by the genetic

manipulation of DCs or by the utilization of cytokines, immunosuppressive agents and other molecules capable of inducing tol-DCs. There is much optimist regarding the clinical utilization of tol-DCs in promoting survival and quality of life of transplanted recipients. However, there are some critical obstacles that remains to be elucidated, specially regarding the stability of the immature phenotype of tol-DCs, that remain to be clarified before proceeding with its translation to clinics [226].

Taking together, I have defined a strategy for converting fibroblasts to DC-like cells, involving lentiviral-based overexpression of the TFs DCi1, DCi6, DCi5 and DCi4. The proposed future experiments aim to optimize the minimal TF network required for the generation of APCs from fibroblasts, the characterization of the reprogrammed cells' identity and their capacity to induce an immunologic response in a clinical context. The findings reported in this study highlight the importance of direct reprogramming strategies not only for developing alternative methods of obtaining functional cells for clinical use, but also for providing deeper insights regarding the transcriptional control of each cell lineage.

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6. SUPPLEMENTARY DATA

Supplementary table 1. Relevant genes for the mouse CD8a+ cDC Lineage obtained from BioGPSmatch scored by p-value.

CD8a+ DCs		
Name	Score	P-value
Aire	10,99	4,74E-04
DCi4	10,27	7,11E-04
DCi8	7,02	9,47E-04
Insm1	6,4	1,18E-03
Smyd1	5,34	1,42E-03
DCi13	4,53	1,66E-03
Tsc22d1	4,23	1,89E-03
Sh3pxd2b	4,2	2,13E-03
Zfa	4,13	2,37E-03
Notch4	4,04	2,61E-03
Zfp111	3,96	3,08E-03
Ciita	3,71	3,32E-03
Med13	3,58	3,79E-03
Asb2	3,54	4,03E-03
Arid4a	3,53	4,26E-03
Etv3	3,42	4,50E-03
Hr	3,39	4,74E-03
Mtpn	3,34	4,97E-03
Zic2	3,27	5,21E-03
Tbx21	3,23	5,45E-03
Tox2	3,22	5,68E-03
Cbfa2t3	3,18	5,92E-03
DCi19	3,11	6,16E-03

Supplementary table 2. Relevant genes for the mouse CD8a- cDC Lineage obtained from BioGPSmatch and scored by p-value.

CD8a- DCs		
Name	Score	P-value
Zglp1	10,99	4,74E-04
DCi4	9,52	7,11E-04
Nkx1-2	8,66	9,47E-04
Supt7l	7,36	1,18E-03
Zfp248	7,15	1,42E-03
Zfp334	7,04	1,66E-03
Nhlh1	6,6	1,89E-03
Dbx2	6,5	2,13E-03
Emx1	6,47	2,37E-03
Wasl	5,73	2,61E-03
Spic	5,36	2,84E-03
Ascl3	5,09	3,08E-03
Rfx2	4,95	3,32E-03
Ikzf4	4,81	3,55E-03
Med13	4,75	3,79E-03
Zeb1	4,56	4,03E-03
Zfp398	4,5	4,26E-03
Cnot2	4,48	4,50E-03
Ankrd55	4,4	4,74E-03
Smyd1	4,34	4,97E-03
Cbfa2t3	4,22	5,21E-03
Asb2	4,19	5,45E-03
Ciita	4,16	5,92E-03
Foxh1	4,02	6,16E-03
Tsc22d1	3,99	6,40E-03
Mtpn	3,98	6,63E-03
Sox10	3,92	6,87E-03
Tbx21	3,87	7,34E-03
Klf17	3,87	7,34E-03
Tmpo	3,8	7,58E-03
DCi8	3,77	7,82E-03
Hoxb7	3,71	8,29E-03
Zfp358	3,67	8,53E-03
Pou5f1	3,62	9,00E-03
Insm1	3,62	9,00E-03
DCi13	3,61	9,24E-03
Med17	3,54	9,47E-03
Kcnip3	3,51	9,71E-03
Lmx1b	3,46	9,95E-03
Cphx	3,44	1,02E-02
Snai3	3,42	1,04E-02

Aebp2	3,37	1,09E-02
Pias4	3,32	1,11E-02
Pax8	3,31	1,16E-02
DCi6	3,31	1,16E-02
Zfp81	3,25	1,23E-02
Tbx22	3,25	1,23E-02
Aire	3,25	1,23E-02
Pkmyt1	3,23	1,28E-02
Foxd4	3,23	1,28E-02
Zbtb32	3,22	1,33E-02
Hira	3,22	1,33E-02
Polr2e	3,21	1,35E-02
Zfp626	3,19	1,40E-02
Nfatc4	3,19	1,40E-02
Gmcl1	3,17	1,42E-02
Msx2	3,15	1,44E-02
DCi17	3,09	1,47E-02
Etv6	3,08	1,49E-02
Ikbkb	3,07	1,52E-02
Kank1	3,06	1,54E-02
Nr6a1	3,05	1,61E-02
Etv3	3,05	1,61E-02
Ankrd5	3,05	1,61E-02
Rxrb	3,04	1,63E-02
Lcorl	3,03	1,66E-02

Supplementary table 3. Relevant genes for the mouse B220+ pDC Lineage obtained from BioGPSmatch and scored by p-value.

B220+ pDCs		
Name	Score	Pvalue
Gcm2	35,46	4,74E-04
Runx2	22,34	7,11E-04
Smyd1	13,38	9,47E-04
Cnot2	7,47	1,42E-03
Hand1	7,29	1,66E-03
Hnf4a	6,28	1,89E-03
DCi10	5,77	2,13E-03
Foxb1	5,68	2,37E-03
DCi8	5,59	2,61E-03
Nr6a1	5,44	2,84E-03
Nr1i2	5,21	3,08E-03
Nfya	5,2	3,55E-03
Nfe2l3	5,2	3,55E-03
Arhgap17	5,17	3,79E-03
Tlx2	4,86	4,26E-03
Cbx7	4,86	4,26E-03
Fank1	4,81	4,74E-03
DCi16	4,81	4,74E-03
Zbtb7a	4,8	4,97E-03
Uncx	4,77	5,21E-03
Med13	4,73	5,45E-03
Eomes	4,6	6,16E-03
Med17	4,41	6,63E-03
Ralgapa1	4,4	6,87E-03
Notch4	4,37	7,34E-03
Foxh1	4,35	7,58E-03
Trnp1	4,28	7,82E-03
Sik1	4,25	8,05E-03
Mtdh	4,09	8,29E-03
DCi4	4,07	8,53E-03
Pax8	4	8,76E-03
Tbx21	3,97	9,24E-03
DCi13	3,97	9,24E-03
Glyctk	3,95	9,47E-03
Ank2	3,89	9,71E-03
Phf7	3,88	9,95E-03
Mga	3,86	1,02E-02
Fev	3,84	1,04E-02
Rai1	3,83	1,07E-02
Tsc22d1	3,72	1,09E-02
Cphx	3,61	1,14E-02
Ercc8	3,59	1,16E-02

Myst3	3,48	1,18E-02
Anks3	3,42	1,21E-02
Zfp248	3,38	1,23E-02
Bhlha15	3,36	1,26E-02
Zdhhc13	3,35	1,28E-02
Trip4	3,34	1,30E-02
DCi15	3,32	1,33E-02
Obox3	3,31	1,35E-02
Atf7ip	3,3	1,37E-02
DCi12	3,29	1,40E-02
DCi7bp2	3,28	1,44E-02
Atf5	3,28	1,44E-02
Prrx1	3,27	1,49E-02
Arid3b	3,27	1,49E-02
Papolb	3,25	1,54E-02
Lcorl	3,25	1,54E-02
Gmeb2	3,24	1,56E-02
Zscan2	3,16	1,61E-02
Zfp113	3,14	1,63E-02
Mycn	3,12	1,66E-02
Hif1a	3,11	1,68E-02
Wasl	3,09	1,71E-02

Supplementary table 4. Relevant genes for the human BDCA4+ DC Lineage obtained from BioGPSmatch and scored by p-value.

Human BDCA4+ DCs

Name	Score	Pvalue
DCI13	35,67	9,80E-04
PLXNC1	21,82	1,47E-03
DCI6	20,81	1,96E-03
SOX4	16,97	2,45E-03
STAT2	16,61	2,94E-03
POU4F1	14,61	3,43E-03
EXOC2	14,47	3,92E-03
KLF6	13,97	4,41E-03
ZFYVE26	13,06	4,90E-03
TGIF2	13	5,39E-03
ETV6	12,9	5,88E-03
TFCP2	11,92	6,37E-03
DCI12	11,87	6,86E-03
DCI10	11,12	7,35E-03
ZC3H11A	10,69	8,33E-03
CUX2	9,77	8,82E-03
MEF2C	9,21	9,31E-03
KIAA0415	9,1	9,80E-03
SMARCC1	8,44	1,03E-02
NFX1	8,38	1,08E-02
RC3H2	7,88	1,13E-02
DCI16	7,78	1,18E-02
HIST1H1D	7,65	1,23E-02
TFEC	7,4	1,27E-02
TRIT1	7,21	1,37E-02
TBX19	7,2	1,42E-02
YY1	6,88	1,52E-02
CUL4B	6,88	1,52E-02
RIOK2	6,68	1,57E-02
EGR2	6,59	1,62E-02
ZNF134	6,41	1,72E-02
MAX	6,41	1,72E-02
JUN	6,34	1,81E-02
GABPA	6,34	1,81E-02
ZNF45	6,17	1,86E-02
NCOA3	5,96	1,96E-02
TOX4	5,94	2,01E-02
SPI1	5,92	2,06E-02
IGHM	5,9	2,11E-02
DCI15	5,66	2,30E-02
IRF7	5,58	2,35E-02

NFAT5	5,45	2,40E-02
STAT1	5,33	2,50E-02
AKAP8	5,28	2,55E-02
HHEX	5,23	2,60E-02
RERE	5,16	2,65E-02
NCOA1	5,05	2,70E-02
NR3C1	5,03	2,75E-02
ZNF335	4,99	2,79E-02
ZNF589	4,81	2,84E-02
ZNF281	4,78	2,89E-02
ZFP161	4,71	2,94E-02
MXD4	4,7	2,99E-02
IRF5	4,62	3,04E-02
CBFB	4,59	3,09E-02
PBX2	4,56	3,19E-02
HLA-DQB1	4,56	3,19E-02
ZNF124	4,46	3,33E-02
PPP1R10	4,46	3,33E-02
ZNF22	4,41	3,38E-02
TFAM	4,37	3,43E-02
MEF2A	4,35	3,48E-02
ZZZ3	4,32	3,58E-02
ZNF394	4,3	3,68E-02
DACH1	4,28	3,73E-02
RELA	4,26	3,77E-02
DCI5	4,12	3,97E-02
KLF4	4,06	4,02E-02
SP110	4,04	4,07E-02
KLF13	4,02	4,12E-02
ZEB1	3,99	4,26E-02
UBE2K	3,98	4,31E-02
ZGPAT	3,97	4,36E-02
NFKB1	3,92	4,51E-02
MLL	3,87	4,56E-02
NFATC1	3,84	4,61E-02
SMAD3	3,78	4,71E-02
STAT6	3,64	4,80E-02
ZNF136	3,62	4,95E-02
ZXDC	3,56	5,05E-02
PLEK	3,5	5,10E-02
TRMT1	3,49	5,15E-02
NFKB2	3,47	5,20E-02
E2F5	3,39	5,29E-02
NFATC3	3,38	5,34E-02
CREB1	3,35	5,49E-02
WHSC1	3,33	5,54E-02
ZBTB43	3,32	5,59E-02

BRPF1	3,31	5,69E-02
RNF113A	3,3	5,74E-02
REL	3,29	5,83E-02
POU2F2	3,28	5,88E-02
PBX3	3,26	6,03E-02
FOSB	3,25	6,08E-02
ZNF263	3,18	6,27E-02
RNASE2	3,12	6,37E-02
ZNF419	3,11	6,42E-02
ZNF592	3,1	6,52E-02
NOC4L	3,1	6,52E-02
ZNF266	3,06	6,62E-02
MYB	3,05	6,67E-02
DPF2	3,04	6,72E-02
TCF7	3,03	6,76E-02