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Reprogramming cancer cells to antigen-presenting cells

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UNIVERSIDADE D COIMBRA

DOCTORAL DISSERTATION

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Cell reprogramming has highlighted the cellular plasticity of somatic cells, while direct lineage conversion promoted the identification of the transcription factor combinations that gatekeep the cell identity for various cell types. Additionally, cell reprogramming products have opened new avenues for regenerative medicine and repair. Cancer cells were shown to be amenable to cell reprogramming strategies; however, previous efforts to reprogram cancer cells aimed at decreasing tumorigenic drive. In this thesis, I leveraged direct cell reprogramming to enhance tumor immunogenicity and overcome major immune evasion mechanisms. In **Study I**, I participated in the identification of the transcription factors that impose a cDC1-lineage in unrelated cell types, including mouse and human fibroblasts, within 9 days of reprogramming. Overexpression of PU.1, IRF8, and BATF3 led to cDC1-like morphology and induced the expression of hematopoietic marker CD45 and professional APC marker MHC class II (MHC-II). Additionally, combining the expression of PU.1, IRF8, and BATF3 in a polycistronic cassette improved reprogramming efficiency and demonstrated that higher levels of PU.1 are required to initiate reprogramming. In **Study II**, single-cell RNA and chromatin immunoprecipitation (ChIP)-sequencing informed the cooperation between the cDC1-specific factors to silence the fibroblast program and kickstart the dendritic cell state as early as day 3 of reprogramming. In **Studies III** and **IV**, I demonstrated that dendritic cell reprogramming endowed mouse and human cancer cells with professional APCs machinery and function, including the secretion of cytokines (interleukin-12) and chemokines (CXCL10) with important roles in antitumor immune responses. Moreover, tumor-APCs responded to inflammatory stimuli, engulfed dead cells and other exogenous antigens, and primed naïve CD4+ and CD8+ T cells. Importantly, I have also shown that cDC1 reprogramming enhances tumor cells' immunogenicity by increasing MHC-I molecules and, consequently, the presentation of tumor antigens at the cell surface, leading to higher cytotoxic T cell-mediated cell death *in vitro*. I also showed that primary cancer cells and cancer-associated fibroblasts (CAFs) are amenable to cDC1 reprogramming. Finally, intratumoral infusion of tumor-APCs *in vivo* synergized with immune checkpoint inhibitors to delay tumor growth, resulting in increased mice survival.

The results presented here show that cDC1 reprogramming enhances antitumor immunity by combining cDC1's antigen processing and presenting abilities with the endogenous generation of tumor antigens. This thesis lays the groundwork for generating novel immunotherapies based on endowed APC function through direct reprogramming.

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To Filipe Dias

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List of Articles

Articles included in this thesis.

Direct reprogramming of fibroblasts into antigen-presenting dendritic cells.

F. F. Rosa*, C. F. Pires*, I. Kurochkin, A. G. Ferreira, A. M. Gomes, L. G. Palma, K. Shaiv, L. Solanas, C. Azenha, D. Papatsenko, O. Schulz, C. R. e Sousa, C.-F. Pereira, *equal contribution. Science Immunology 3, eaau4292 (2018). DOI: 10.1126/sciimmunol.aau4292

Study II

Single-cell RNA-seq informs efficient reprogramming of human somatic cells to cross-presenting dendritic cells.

F.F. Rosa, C. F. Pires, I. Kurochkin, E. Halitzki, T. Zahan, N. Arh, O. Zimmermannova, A. G. Ferreira, H. Li, S. Karlsson, S. Scheding, C.-F. Pereira. Science Immunology 7, eabg5539 (2022). DOI: 10.1126/sciimmunol.abg5539

Study III

Restoring tumor immunogenicity with dendritic cell reprogramming.

O. Zimmermannova*, A. G. Ferreira*, E. Ascic#, M. V. Santiago#, I. Kurochkin#, M. Hansen, O. Met, I. Caiado, I. E. Shapiro, J. Michaux, M. Humbert, D. Soto-Cabrera, H. Benonisson, R. Silvério-Alves, D. Gomez-Jimenez, C. Bernardo, M. Bauden, R. Andersson, M. Höglund, K. Miharada, Y. Nakamura, S. Hugues, L. Greiff, M. Lindstedt, F. F. Rosa, C. F. Pires, M. Bassani-Sternberg, I. M. Svane, C.-F. Pereira. *equal contribution, #equal contribution. Science Immunology 8, eadd4817 (2023). DOI: 10.1126/sciimmunol.add4817

Study IV

Reprogramming mouse and human cancer cells to antigen-presenting cells.

A. G. Ferreira, O. Zimmermannova, I. Kurochkin, E. Ascic, F. Akerström, C.-F. Pereira. (*accepted, in press, 2023)* Bio-protocol.

ADDITIONAL ARTICLES NOT INCLUDED IN THIS THESIS

Cell fate reprogramming in the era of cancer immunotherapy.

O. Zimmermannova, I. Caiado, A. G. Ferreira, C.-F. Pereira. Frontiers of Immunology 12 (2021). DOI: 10.3389/fimmu.2021.714822

Gata2 mitotic bookmarking is required for definitive hematopoiesis.

R. Silvério-Alves, I. Kurochkin, A. Rydström, C. V. Echegaray, J. Hayder, M. Nichols, C. Rode, L. Thelaus, A. Y. Lindgrem, A. G. Ferreira, R. Brandão, J. Larsson, M. F. T. R. de Brujin, J. Martin-Gonzalez, C.-F. Pereira. Nature Communications 14, 4645 (2023). DOI: 10.1038/s41467-023-40391-x

ABBREVIATIONS

ACN	Acetonitrile
ACT	Adoptive T cell-based therapy
AGC	Automatic gain control
AML	Acute myeloid leukemia
APC	Antigen-presenting cell
B-ALL	B- acute lymphocytic leukemia
BCR	B cell receptor
BL	B cell lymphoma
BM	Bone-marrow
BM-DC	Bone-marrow derived dendritic cell
CAFs	Cancer-associated fibroblasts
CAR	Chimeric antigen receptor
cDC	Conventional dendritic cell
CDP	Common or conventional dendritic cell progenitor
CEA	Carcinoembryonic antigen
CFSE	Carboxyfluorescein succinimidyl ester
ChIP-seq	Chromatin immunoprecipitation sequencing
CLP	Common lymphoid progenitor
CLR	C-type lectin receptors
CMP	Common myeloid progenitor
CMV	Cytomegalovirus
CRISPR	Clustered regularly interspaced short palindromic repeats
CTL	Cytotoxic T lymphocyte
CTV	CellTrace Violet
DAMPs	Damage-associated molecular pattern molecules
DDA	Data dependent analysis
DIA	Data independent analysis
Dox	Doxycycline
ESCs	Embryonic stem cells
FA	Formic acid
FACS	Fluorescent-activated cell sorting
FDA	Food and Drug Administration
FDR	False discovery rate
FMO	Fluorescence minus one
GI	Gastrointestinal

GM-CSF	Granulocyte-macrophage colony-stimulating factor
GSEA	Gene set enrichment analysis
h	hours
HDFs	Human dermal fibroblasts
HEFs	Human embryonic fibroblasts
hiDC	Human induced dendritic cell
HLA	Human leucocyte antigen
HSCs	Hematopoietic stem cell
HSPCs	Hematopoietic stem and progenitor cell
HSV-1	Herpes Simplex Virus-1
ICI	Immune checkpoint inhibitors
iDC	Induced dendritic cell
IFN	Interferon
IL	Interleukin
iPSCs	Induced pluripotent stem cells
L	liter
LPS	Lipopolysaccharide
M1/M2	Macrophage type 1 or macrophage type 2
MACS	Magnetic-activated cell sorting
M-CSF	Macrophage colony-stimulating factor
MEFs	Mouse embryonic fibroblasts
MEP	Megakaryocytes-erythroid progenitor
MFI	Median fluorescent intensity
MHC	Major histocompatibility complex
mg	Milligram
µg	Microgram
mL	Milliliter
min	Minutes
moDC	Monocyte-derived dendritic cell
ms	milliseconds
MS	Mass spectrometry
MSC	Mesenchymal stromal or stem cell
MuLV	Murine leukemia virus
ng	Nanogram
NSCLC	Non-small cell lung cancer
OSKM	OCT4, SOX2, KLF4, MYC
OVA	Ovalbumin
PAMPs	Pathogen-associated molecular pattern molecules
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline solution
PCA	Principal component analysis
PDAC	Pancreatic ductal adenocarcinoma
pDC	Plasmacytoid dendritic cell

PIB	PU.1, IRF8, and BATF3
Poly(I:C)	Polyinosinic:polycytidylic acid
PRRs	Pattern recognition receptors
RNA-seq	RNA-sequencing
s	Seconds
SCC	Squamous cell carcinoma
SFFV	Spleen focus-forming virus
STING	Stimulator of interferon genes
TAAs	Tumor-associated antigens
TAMs	Tumor-associated macrophages
tdLN	Tumor-draining lymph nodes
tdT	tdTomato
TCGA	The Cancer Genome Atlas
TCR	T cell receptor
TFA	Trifluoroacetic acid
Th1/Th2	T cell helper type 1 or T cell helper type 2
TIL	Tumor-infiltrating lymphocyte
TLR	Toll-like receptor
TMB	Tumor mutational burden
TME	Tumor microenvironment
TNF	Tumor necrosis factor
Treg	Regulatory T cell
VEGF	Vascular endothelial growth factor

ABSTRACT

For the past two decades, immunotherapy revolutionized cancer treatment. However, responses vary significantly among eligible patients and some cancer types are not yet open to immunotherapy. Several mechanisms contribute to immunotherapy resistance, including loss of antigen presentation machinery and immunosuppression. Conventional dendritic cells type 1 (cDC1) are a rare population of professional antigen-presenting cells (APCs) that specialize in recognizing, processing, and cross-presenting antigens to cytotoxic CD8+ T cells and orchestrating complex immune responses. During carcinogenesis, the role of cDC1 is to capture tumor-associated antigens (TAAs) and stimulate effector immune cells to build an immune response against cancer. In cancer patients, cDC1s are dysfunctional or excluded from the tumor microenvironment (TME). Furthermore, cancer cells downregulate key components of antigen presentation pathway, including major histocompatibility complex class I (MHC-I), allowing them to evade immune surveillance. Therefore, there is a need for strategies that counteract cancers' mechanisms of immune evasion.

Cell reprogramming has highlighted the cellular plasticity of somatic cells, while direct lineage conversion promoted the identification of the transcription factor combinations that gatekeep the cell identity for various cell types. Additionally, cell reprogramming products have opened new avenues for regenerative medicine and repair. Cancer cells were shown to be amenable to cell reprogramming strategies; however, previous efforts to reprogram cancer cells aimed at decreasing tumorigenic drive. In this thesis, I leveraged direct cell reprogramming to enhance tumor immunogenicity and overcome major immune evasion mechanisms. In **Study I**, I participated in the identification of the transcription factors that impose a cDC1-lineage in unrelated cell types, including mouse and human fibroblasts, under 9 days of reprogramming. Overexpression of PU.1, IRF8, and BATF3 led to cDC1-like morphology and induced the expression of hematopoietic marker CD45 and professional APC marker MHC class II (MHC-II). Additionally, combining the expression of PU.1, IRF8, and BATF3 in a polycistronic cassette improved reprogramming efficiency and demonstrated that higher levels of PU.1 are required to initiate reprogramming. In **Study II**, single-cell RNA and chromatin immunoprecipitation (ChIP)-sequencing informed the cooperation between the cDC1-specific factors to silence the fibroblast program and kickstart the dendritic cell state as early as day 3 of reprogramming. In **Studies III** and **IV**, I demonstrated that dendritic cell reprogramming endowed mouse and human cancer cells with

professional APCs machinery and function, including the secretion of cytokines (interleukin-12) and chemokines (CXCL10) with important roles in antitumor immune responses. Moreover, tumor-APCs responded to inflammatory stimuli, engulfed dead cells and other exogenous antigens, and primed naïve CD4+ and CD8+ T cells. Importantly, I have also shown that cDC1 reprogramming enhances tumor cells' immunogenicity by increasing MHC-I molecules and, consequently, the presentation of tumor antigens at the cell surface, leading to higher cytotoxic T cell-mediated cell death in vitro. I also showed that primary cancer cells and cancer-associated fibroblasts (CAFs) are amenable to cDC1 reprogramming. Finally, intratumoral infusion of tumor-APCs in vivo synergized with immune checkpoint inhibitors to delay tumor growth, resulting in increased mice survival.

The results presented here show that cDC1 reprogramming enhances antitumor immunity by combining cDC1's antigen processing and presenting abilities with the endogenous generation of tumor antigens. This thesis lays the groundwork for generating novel immunotherapies based on endowed APC function through direct reprogramming.

POPULAR SUMMARY IN ENGLISH

The immune system is a security system responsible for finding and dealing with daily threats, such as microbes and cancer, thus maintaining the human body's health. Over the last 20 years, doctors have been using immunotherapy that improves our immune system's capacity to recognize and fight cancer. However, immunotherapy does not always work for certain cancers, as cancer cells find ways to hide from this security system and avoid being eliminated. Within our immune system, a unique and rare group of cells works as elite sentinels. They are called conventional dendritic cells type 1 (cDC1), and they can find cancer cells and alert other cells that will attack cancer directly. However, in many cancer patients, these sentinels do not work properly and sometimes cannot get close enough to the cancer cells to do their job. Cancer cells also avoid being attacked by the defense team arranged by cDC1 by hiding specific proteins, called MHC-I, from the cell surface, impairing their recognition as intruders and limiting immunotherapy success. Therefore, there is a need for solutions that target cancer cells directly and force them to reveal themselves to the immune system. Direct cellular reprogramming, where a cell can be converted to adopt another cell's function or job, provides an exciting strategy to convince cancer cells to act as cDC1. This can be accomplished by introducing special proteins that overwrite the initial cancer cell program and masquerade cancer cells as cDC1.

In this thesis, I helped identify the special proteins called transcription factors, PU.1, IRF8, and BATF3, responsible for masquerading a cancer cell into a cDC1-like cell. Within 9 days of reprogramming, cancer cells start looking and acting like cDC1. By assessing individual cells, I also showed that reprogrammed cancer cells rapidly lose their cancerous potential and fully adopt cDC1 skills as early as day 3 of reprogramming. These cancer cells turned sentinels responded to inflammatory stimuli to start producing alarm signals, called cytokines and chemokines, that are important to organize the attacking team of cells that will later recognize and fight cancer directly. Reprogrammed cancer cells are apt at eating dead cells and showing pieces of their original cancerous program. Like a trojan horse, reprogrammed cancer cells can act from within the tumors and expose the intruders to other immune cells, making them more visible. Finally, I showed that injecting these detective cancer cells into live mice could boost the immune system. Combined reprogrammed cancer with other immunotherapy drugs significantly slowed cancer growth and increased mice survival. The results I presented in this thesis open new

avenues for generating immunotherapies based on direct cell reprogramming strategies.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Immunförsvaret är ett säkerhetssystem som ansvarar för att hitta och hantera dagliga hot, som mikrober och cancer, och därigenom upprätthålla den mänskliga kroppens hälsa. Under de senaste 20 åren har läkare använt en teknik som kallas immunoterapi som förbättrar vårt immunsystems förmåga att känna igen och bekämpa cancer. Men för vissa cancerformer fungerar immunoterapi inte alltid eftersom cancerceller hittar sätt att gömma sig från detta säkerhetssystem och undvika att bli eliminerade. Inom vårt immunsystem finns det en unik och sällsynt grupp av celler som fungerar som elitvakter. De kallas konventionella dendritiska celler av typ 1 (cDC1) och de kan hitta cancerceller och larma andra celler som direkt kommer att attackera cancern. Men hos många cancerpatienter fungerar dessa vakter inte korrekt och kan ibland inte ens komma tillräckligt nära cancercellerna för att göra sitt jobb. Dessutom undviker cancerceller också att bli attackerade av försvarsteamet som organiserats av cDC1 genom att dölja specifika proteiner, kallade MHC-I, från cellens yta, vilket hindrar att de identifieras som inkräktare och begränsar framgången med immunoterapi. Därför finns ett behov av lösningar som direkt riktar sig mot cancerceller och tvingar dem att visa sig för immunsystemet. Direkt cellulär omprogrammering, där en cell kan omvandlas för att anta en annan cells funktion eller arbetsuppgift, kan vara en strategi för att få cancerceller att agera som cDC1. Genom att introducera speciella proteiner kan man ersätta det ursprungliga cancercellprogrammet och maskera cancercellerna som cDC1.

I denna avhandling bidrog jag till att identifiera de speciella proteiner som kallas transkriptionsfaktorer, PU.1, IRF8 och BATF3, som är ansvariga för att maskera en cancercell till en cDC1-liknande cell. Efter endast 9 dagar av omprogrammering börjar cancerceller se ut och bete sig som cDC1. Genom att studera enskilda celler, visade jag dessutom att omprogrammerade cancerceller snabbt förlorar sin cancerösa potential och helt antar cDC1-färdigheter redan på dag 3 av omprogrammeringen. Dessa omprogrammerade cancerceller reagerade på inflammatoriska stimuli och började producera larmsignaler, så kallade cytokiner och kemokiner, som är viktiga för att organisera det angripande teamet av celler som senare kommer att känna igen och direkt bekämpa cancern. Omprogrammerade cancerceller är skickliga på att äta döda celler och visa delar av sitt ursprungliga cancerösa program. Likt en trojansk häst kan omprogrammerade cancerceller verka inifrån tumörerna och utsätta omkringliggande tumörceller för andra immunceller,

vilket gör dem mer synliga. Slutligen visade jag att genom att injicera dessa detektivcancerceller i levande möss kunde de stärka immunsystemet. Kombinationen av omprogrammerad cancer med andra immunoterapiläkemedel bromsade cancerutvecklingen och ökade mössens överlevnad betydligt.

De resultat jag presenterar i denna avhandling öppnar nya vägar för framställning av immunoterapier baserade på direkta cellomprogrammeringsstrategier.

RESUMO POPULAR EM PORTUGUÊS

O sistema imunitário é um sistema de segurança responsável por encontrar e lidar com ameaças diárias, tal como micróbios e células do cancro, e, assim, manter a saúde do organismo humano. Nos últimos 20 anos, os médicos começaram a explorar imunoterapia como forma de melhorar a capacidade do sistema imunitário para detetar e combater o cancro. No entanto, para certos tipos de cancro, a imunoterapia nem sempre é eficaz, pois as células cancerígenas adotam mecanismos para se esconder deste sistema de segurança e evitar ser eliminadas. Dentro do nosso sistema imunitário existe um grupo de células único e raro que atua como sentinelas, que são designadas células dendríticas convencionais do tipo 1 (cDC1). Estas células patrulham o corpo humano à procura de intrusos, incluindo células do cancro, e, quando os encontram, conseguem capturá-los e apresentá-los a outras células que, consequentemente, irão formar uma resposta imunitária contra a ameaça. Contudo, em doentes de cancro, estas células dendríticas não funcionam corretamente, nem conseguem interagir diretamente com as células do cancro para fazerem o seu trabalho. Adicionalmente, as células do cancro conseguem também esconder a sua identidade do resto das células do sistema imunitário e evitar ser atacadas ao não expressar proteínas específicas, chamadas de MHC-I, à sua superfície. Estes mecanismos impedem que as células de cancro sejam reconhecidas e limitam a eficácia da imunoterapia. Assim, há uma necessidade de encontrar alternativas terapêuticas que obriguem as células de cancro a revelar a sua identidade às células efetoras do sistema imunitário. A reprogramação celular direta surge como uma estratégia interessante para converter, ou reprogramar, a identidade de uma célula de cancro e obrigá-la a adotar a função de uma célula dendrítica. Isto é possível através da introdução de proteínas especiais, conhecidos como fatores de transcrição, que alteram a identidade da célula original mudando a expressão genética para a da célula desejada, neste caso, o de uma célula dendrítica.

Neste trabalho, eu ajudei a identificar estes fatores de transcrição que codificam a identidade de uma célula dendrítica, conhecidos pelos seus símbolos PU.1, IRF8, e BATF3. Durante 9 dias de reprogramação, as células cancerígenas adotam a forma estrelada da célula dendrítica e começam a expressar marcadores específicos desta célula. Além disto, eu mostrei que as células do cancro perdem muito rapidamente o potencial tumorigénico e adquirem as funções das células dendríticas, logo a partir do terceiro dia de reprogramação. As células do cancro reprogramadas respondem a estímulos inflamatórios para produzir sinais de alarme, conhecidos por citocinas e quimiocinas, que são importantes para organizar uma resposta imunitária eficaz

contra o cancro. As células reprogramadas são ainda capazes de fagocitar células mortas e mostrar marcadores da sua identidade original. Tal como o "cavalo de Troia", após a reprogramação, as células do cancro conseguem infiltrar-se no tumor e expor a sua identidade às células efetoras que conseguem assim atacar tanto as células reprogramadas como as outras células cancerígenas. Finalmente, eu mostrei que ao injetar estas células reprogramadas em ratinhos com cancro da pele, elas podem incentivar o sistema imunitário a atacar o cancro. A combinação entre células reprogramadas com medicamentos usados atualmente em imunoterapia diminui significativamente o crescimento de tumores nos ratinhos e aumentou a sua sobrevivência. Os resultados que aqui apresentei abrem novas oportunidades para o desenvolvimento de novas imunoterapias baseadas em reprogramação celular.

INTRODUCTION

Cancer is a complex, multi-factorial disease marked by a series of genetic and epigenetic alterations that affect oncogenic drivers and silence tumor suppressor genes, leading to unrestrained growth, resistance to apoptotic signals, and the creation of a supportive tumor microenvironment (TME) that invades and colonizes healthy tissue (1).

Despite the first discoveries interconnecting the immune system and cancer control being first published in the 19th century (2), it took over a century for the emergence of immuno-oncology as a field of research. Today, it is well established that the immune system plays a role in different stages of cancer development: from its inception to its evolution and now, potentiated by immunotherapies, to its demise.

In this section, I will explore the intricate relationship between cancer and the immune system, how immunotherapy revolutionized cancer treatments, and how it contributed to our understanding of cancer development. Moreover, I will explain the importance of antigen presentation and the crucial role of dendritic cells in cancer therapy as the foundation for immunotherapy success. I will then introduce the concept of cell fate reprogramming and how it can be utilized to generate novel approaches for immunotherapy.

THE RISE OF IMMUNO-ONCOLOGY

The immune system is equipped to deal with foreign agents and plays an important role in identifying and eradicating damaged cells to restore homeostasis. During carcinogenesis, somatic cells acquire mutations that endow them with improved fitness and survival, allowing them to grow unchecked. However, rampant growth is often accompanied by additional mutations and chromosomic instability. These alterations can lead to the accumulation of misfolded or aberrant proteins, which, in turn, can be easily identified by the immune system as foreign agents and swiftly eliminated.

The complex relationship between cancer and the immune system started in the 19th century, with Rudolph Virchow hypothesizing that tumors resulted from a “severe irritation” of the tissue in 1863 (2). It would take half a century until researchers started postulating that the human body had mechanisms to keep tumor

growth in check, thus given birth to the concept of “immune surveillance” (3, 4). Indeed, it is current knowledge that the lack of a functional adaptive immune system predisposes mammalian hosts to a higher probability of developing cancer (5, 6). However, it was also clear that those mechanisms could fail and lead to cancer development.

As the immune surveillance hypothesis was being developed, the sole proof of immune cells' ability to detect and remove tumor-associated antigens (TAAs) was from the groundbreaking studies of Gross in 1943, Foley in 1953, and Klein in 1960. The first two studies reported that the inoculation of primary methylcholanthrene-induced sarcomas resulted in the rejection of tumors in different mice if the lesions were small enough, suggesting an inherent capacity to induce an immune response against cancer (7, 8). At the same time, Klein and colleagues were on the verge of reporting that mice, after exposure to a primary tumor, generated lymph node cells against cancer that could reject and confer resistance to a second establishment of the same tumor type (9). Paradoxically, these immune cells were incompetent at controlling or eliminating the original tumor, as it developed in the first place.

Later, research surrounding the discovery of the first TAA in melanoma patients, MAGE-1, cemented the evidence that the human body could identify cancer cells as foreign and generate tumor-specific CD8+ T cells (10). However, Bruggen’s studies, together with Klein’s, also exposed the glaring inability of the immune system to avoid and eliminate tumors, suggesting that the tumors promoted an immune-privileged microenvironment that secluded antitumor-specific T cells from acting.

Targeting the immune system against cancer.

Simultaneously with Virchow’s hypothesis formulation, William Bradley Coley, and other researchers before him observed tumor regression in patients affected by a concurrent streptococcal skin infection, which seemed to stimulate the immune system to eliminate both diseases in the host. These observations allowed Coley to develop Coley’s toxins and deliberately treat over 1000 sarcoma patients with bacterial vaccines during his career (2, 11), in one of the first recorded attempts at harnessing the immune system against cancer. However, by Coley’s observations, this treatment was only effective in a small fraction of patients suffering from sarcomas (11). The development of chemotherapy and radiotherapy and the discovery of hormone therapy for hormone-sensitive cancer dictated that Coley’s approach would be replaced by more reliable techniques (2). Despite this setback, a remnant of the bacterial era of immunotherapy is still practiced today. The tuberculosis vaccine has been used to treat early-stage bladder cancer since 1976 with promising results, with its mechanisms of action well cataloged (2). From Coley’s early steps in immunotherapy, the field of immuno-oncology has since matured, supported by the first seminal works on T cell biology (2).

A T cell centric view of immunotherapy.

Adoptive T Cell Therapies

T cells have always been at the center stage of cancer immunotherapy, with some relevant exceptions occurring in the past decade. First discovered in the 1960s, T cells are an important cellular component of the immune system. T lymphocytes can be distinguished from other blood cells by the expression of the T cell receptor (TCR), whose role is to recognize pathogenic antigens – small peptides that are processed intracellularly - bound to the major histocompatibility complex (MHC) molecules at the surface of a cell (12). The T cell population can be divided into two well-defined lineages characterized by the expression of CD4 or CD8. CD4+ T cells recognize antigens bound to MHC class II (MHC-II). They manipulate immune responses by producing cytokines and chemokines that activate or inhibit other immune effector cells. On the other hand, T cells expressing the CD8 co-receptor detect antigens presented in the MHC-I context and directly attack infected or damaged cells. Their cytotoxic activity is crucial to detect and eliminate cancer cells.

The infiltration of CD8+ T cells in tumors has been known for almost half a century and has helped predict cancer outcomes; the higher the lymphocytic infiltration, the better the prognosis (13-15). Complementally, Rosenberg *et al.* utilized *ex vivo* expanded tumor-infiltrating lymphocytes (TILs) to cure tumor-bearing mice. This approach would be later applied in melanoma metastatic patients (16-19) (**Figure 1**), with 20% of patients showing durable responses and 95% of them reporting ongoing responses past the 3-year mark (19). The discovery and development of *ex vivo* TILs would pave the way for adoptive T cell-based therapies (ACT). Currently, there are clinical trials to test the efficacy in several tumor types – head and neck, cervical, ovarian, and lung cancer.

Another prominent example of ACT is the development of chimeric antigen receptor (CAR)-T cells (**Figure 1**). Autologous T cells collected from patients are engineered to express modified (chimeric) TCRs that bind to specific antigens expressed by cancer cells (20). Several clinical trials attested their safety and efficacy in treating lymphoma, acute lymphocytic leukemia, and, recently, multiple myeloma (20-22). CAR-T cell therapies had their breakthrough in 2017 after approval by the Food and Drug Administration (FDA), and until 2023, 6 different treatments have been authorized to be used in the clinic. Moreover, there have been efforts to turn this therapeutic modality beyond blood cancers (21). CAR-T cell therapies are currently being adapted to several types of solid cancer, including ovarian and renal cancer, glioma, and hepatoma (23). However, solid tumors impose important barriers interfering with CAR-T cell efficacy, including tumor antigen heterogeneity in quality and quantity, tumor infiltration, and an immunosuppressive environment (23). Engineering CAR-T to target better solid cancers and overcome the challenges imposed by these will be a decisive direction for the future of CAR-T in immunotherapy (23-26).

Immune checkpoint inhibitors

The molecular studies surrounding T cell biology and the TCR signaling pathway elucidated key regulatory mechanisms crucial for competent T cell function. Notably, researchers found that correct T cell activation required a second signal, termed co-stimulation, through activation of CD28, a T cell receptor belonging to the immunoglobulin superfamily – a group of evolutionarily related proteins that share similarities with antibodies (12, 27, 28).

Conversely, T cells also possess receptors that negatively affect their function. A series of studies from the 1980s to the early 2000s revealed the existence of two members of the immunoglobulin superfamily – CTLA-4 and PD-1 (29-32). Contrary to the steady-state expression of CD28, T cells express a low basal level of CTLA-4, and stronger expression levels are induced after T cells have been activated (12). Due to their similarity, CD28 and CTLA-4 share the same ligands of the B7 family, CD80 and CD86 (33, 34), expressed by antigen-presenting cells (APCs). CTLA-4 has an opposite function to CD28, whereas the first actively inhibits T cell activation and proliferation by stopping CD28 from binding to its ligands (33, 35-37). CTLA-4 expression in T cells, especially in regulatory T cells (Tregs), is critical in the induction of immunological tolerance. Indeed, CTLA-4 insufficiency in both humans and rodents causes severe generalized multi-organ lymphocytic infiltration and autoimmunity due to a lack of CD28 control (38-41).

Like CTLA-4, PD-1 is highly expressed in T cells upon TCR activation. This checkpoint inhibitor binds to its ligands, also from the B7 gene family, PD-L1 and PD-L2, present in APCs (31) and cancer cells (42-44). The PD-1 axis controls T cells by inducing T cell exhaustion, a dysfunctional state where T cells stop proliferating and performing their effector functions (45-47). However, unlike CTLA-4, disturbing the PD-1 axis leads to the late development of a heterogeneous group of autoimmune-related diseases affecting peripherical tissues (48-51).

The first report that blocking CTLA-4 with antibodies reverted its negative effect in T cells immediately followed by James P. Allison's discovery that infusion of anti-CTLA antibodies in live mice mediated tumor rejection (52, 53) fundamentally changed the course of immuno-oncology (**Figure 1**). Generalized recognition of harnessing the immune system against cancer resulted in the nomination of cancer immunotherapy for "Breakthrough of the Year" (54) by Science in 2013 and the 2018 Nobel Prize in Physiology or Medicine for James P. Allison and Tasuko Honjo.

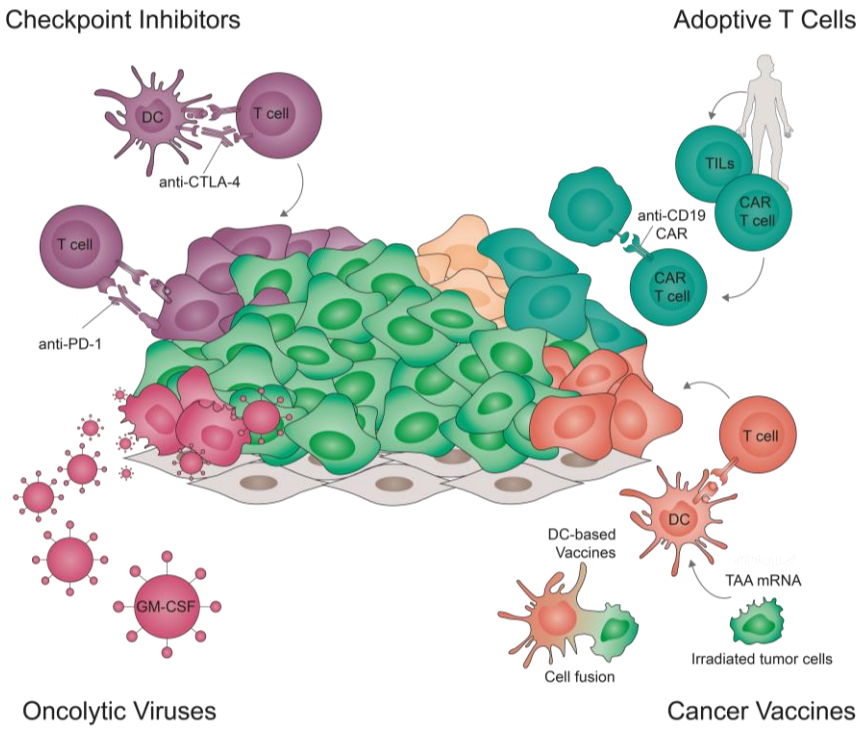

Figure 1 | Current strategies for cancer immunotherapy. Schematic representation of current immunotherapies being developed or in clinical practice. Checkpoint inhibitors (top left) are based on antibodies targeting negative regulators of T cell activation. These encompass receptors of the immunoglobulin superfamily CTLA-4 and PD-1 expressed by T cells. Adoptive T cell therapies (ACT) (top right) include engineered autologous T cells to express chimeric antigen receptor (CAR), mostly against hematological malignancies, as illustrated by the anti-CD19 CAR-T cells. *Ex vivo* expanded tumor-infiltrating lymphocytes (TILs) are currently in clinical trials for head and neck cancer, cervical carcinoma, ovarian carcinoma, and non-small cell lung carcinoma. Oncolytic viruses (bottom left) exploit cancer cells' vulnerability to viral infections. This immunotherapy is often accompanied by the introduction of immunomodulatory molecules such as GM-CSF to increase therapeutic potential. Finally, cancer vaccines (bottom right) take advantage of dendritic cells' (DC) capacity for antigen presentation. Cancer vaccines can be administrated as mRNA-encoding tumor-associated antigens (TAAs), irradiated tumor cells, or cell fusion products between tumor cells and APCs. Adapted from Zimmermannova, Caiado (55).

The successful preclinical studies demonstrating tumor regression and prolonged immunity in various cancers after immune checkpoint blockade rapidly led to clinical trials proving their efficacy in human patients (2, 12, 55). Ipilimumab, an antibody targeting CTLA-4, was the first immune checkpoint blockade therapy approved by the FDA in 2011 to treat unresectable, high-grade melanoma (56). Anti-CTLA-4 treatment in melanoma patients has increased short and long-term survival, with 22% of patients reporting more than three years survival rate (57, 58).

Anti-PD-1 antibodies, nivolumab, and pembrolizumab were first approved for non-small cell lung carcinoma (NSCLC), metastatic melanoma, and bladder cancer (12, 59, 60). Since then, antibodies targeting CTLA-4 and the PD-1 axis have been implemented in the clinic alone or in combination to treat various cancers with varied degrees of success (12, 55, 61). PD-1 blockade has seen greater success in

carcinogenic-derived cancers such as melanoma (57, 59, 61, 62), lung (59, 63-65), head and neck (66, 67), urothelial (68-71), gastric (72) and liver carcinomas (73). Coincidentally, these tumors have also been shown to have higher mutational burdens. In the past 5 years, anti-PD-1 has also been used to treat Hodgkin's lymphoma and triple-negative breast cancer with improved survival in PD-L1-positive patients (74-77).

The impressive accomplishment of immune checkpoint inhibitors (ICI) relies on their mechanism of action. By blockading T cell negative regulators, anti-CTLA-4 and anti-PD-1 effectively reactivate the immune system against cancer cells. Pre-clinical studies and post-clinical trial analysis have provided evidence that antitumor immunity confers long-lasting immunological memory benefits, which can potentially protect the host against relapse (53, 58, 61, 62, 78-80).

Exploiting alternative immunotherapies against cancer.

Oncolytic viruses

Oncolytic viruses (**Figure 1**) are a promising therapeutic approach that uses native or modified viruses that replicate selectively in cancer cells. The ability of viruses to infect and kill cancer cells has been known for almost a century. The pursuit of oncolytic viruses as immunotherapy only gained significant interest in the last 20 years (81). This was made possible by various studies that revealed the ability of viruses like Influenza and Herpes Simplex type I (HSV-1) to reduce tumor growth (82, 83). A clinical trial performed on melanoma patients also demonstrated good clinical outcomes after using an oncolytic virus encoding GM-CSF, despite the gap in knowledge regarding oncolytic viruses' mechanisms of action (84).

Oncolytic viruses selectively infect, replicate, and lead the tumor cells to immunogenic cell death. This process is thought to trigger the release of type I interferon (IFN), danger-associated molecular patterns (DAMPs), pattern-associated molecular patterns (PAMPs), and TAAs, including neoantigens which can be recognized by several effector immune cells from the innate and adaptive immune system (81).

Several contenders for oncolytic therapy are based on viral backbones such as HSV-1 (81). Genetic modifications can help reduce pathogenicity and enhance tropism selectivity for cancer cells. Additionally, oncolytic viruses can be manipulated to include transgenes to boost immune responses, such as TAAs, co-stimulatory molecules, and cytokines like granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin (IL) -2, IL-12, or tumor necrosis factor (TNF)-α, which can be incorporated into their genome.

Currently, four oncolytic viruses are approved for cancer treatment (85). Talimogene laherparepvec (T-VEC) is used to treat unresectable, recurrent melanoma. T-VEC uses a weakened HSV-1 backbone to infect cancer cells and encodes GM-CSF, which is necessary for the recruitment and maturation of APCs.

Multiple clinical trials have used T-VEC as a monotherapy or combined with other therapies, confirming safety and efficacy (85). The reported overall response rate percentages are between 20% and 50% across the different clinical trials, showing that T-VEC benefits patients with early metastatic disease (85). Finally, other cancer indications currently in focus for oncolytic virus therapy include head and neck squamous carcinoma, pancreatic cancer, glioma, NSCLC, and bladder carcinoma (86-92).

Cancer Vaccines

Vaccination against cancer has been studied for several decades with mixed results. Cancer vaccines are designed to stimulate the immune system by unleashing the antigen-presenting capacity of professional APCs, such as dendritic cells, leading to polyclonal T cell-specific responses (**Figure 1**). Traditionally, cancer vaccines were based on administering antigen-encoding vectors directly into the body or utilizing cell-based vaccines. In the latter approach, APCs are first loaded with the patient's tumor-specific antigens by various methods.

For the longest time, cancer vaccination strategies have exploited professional APCs as vehicles for TAAs delivery and trigger antitumor immunity. Spiluleucel-T is the only cancer vaccine in this category approved by the FDA to treat prostate cancer patients. In this therapy, autologous peripheral blood mononuclear cells (PBMCs), which include APCs, are activated with prostatic acid phosphatase fused with GM-CSF (93). In a study from 2014, Spiluleucel-T increased activated T cell infiltration with TME in treated patients (94). Another method of combining various cancer antigens with professional antigen presentation is fusing cancer and dendritic cells. This approach generated antigen-specific T cells against acute myeloid leukemia (AML) in patients, extending remission for 71% of patients enrolled in the trial (95).

Pulsing autologous dendritic cells or monocyte-derived dendritic cells (moDCs) with TAAs has also been extensively studied (96). The most used approach involves the use of moDCs. Despite requiring extensive *in vitro* manipulation, moDCs manufacture and properties have been, by far, the most well characterized. To obtain moDCs, monocytes are isolated from PBMCs, which are then matured and activated outside the body before being infused into patients. Both autologous and allogeneic approaches are possible in this context, with results showing safety and immunogenicity (97, 98). The mechanisms of action of moDCs seemingly favor the induction of CD8+ memory T cells and have lower antigen presentation capacity, which might contribute to underwhelming clinical outcomes, with only 5% to 15% of patients responding positively (99, 100). Interesting alternatives to moDCs rely on purified subsets of dendritic cells such as plasmacytoid dendritic cells (pDCs) or conventional type 2 dendritic cells (cDC2). A phase I clinical trial met feasibility, tolerance, and safety objectives imposed on assessing allogeneic pDCs vaccination in metastatic melanoma patients (101). In one patient, anti-melanocytes specific T cells were found in resected metastasis and circulating in the blood after the

vaccination protocol. Importantly, the patients did not generate anti-allogenic responses, increasing the probability of these cells being used in the future as "off-the-shelf" vaccines (101). Another small study where patients were vaccinated with *ex vivo* stimulated cDC2 demonstrated that vaccination with dendritic cells is well tolerated and promoted progression-free survival in some patients (102).

Other cellular-based strategies include lethally irradiated cancer cells to drive immunity. GVAX, for example, couples engineered cancer cells targeting pancreatic ductal adenocarcinoma (PDAC) to express GM-CSF (103-106) (**Figure 1**). Although GVAX therapy, either alone or in combination with other approaches, was initially considered safe, it was later found to be less effective than chemotherapy (107). Additionally, the properties of GM-CSF have been reported to be both pro-tumorigenic and antitumorigenic, hindering its broad adoption in the clinic (108).

The most recent efforts in the cancer vaccination field have especially focused on developing RNA-based vaccines encoding neoantigens or TAAs (109). Most cancer vaccines target shared tumor antigens – antigens commonly found in different patients. These include "self" antigens that are present in normal tissues but are dysregulated and overexpressed in certain cancers, such as melanoma-associated antigens, or "non-self" antigens, including viral E6 and E7 proteins of high-risk human papillomavirus linked to the etiology of cervical and head and neck cancers (110, 111). In patients with unresectable melanoma, administering an RNA vaccine against four TAAs mostly expressed in melanoma triggered an immune response in more than 75% of the patients who participated in the study (112). Moreover, patients mounted effective polyclonal CD4+ and CD8+ T cells against at least one of the antigens present in the vaccine, and the T cell pool was maintained for more than a year.

On the other hand, personalized cancer vaccines based on patients' neoantigens have reemerged in the spotlight with two independent works. At the end of 2022, Moderna and Merck announced the results of a phase IIb trial that assessed the efficacy of their RNA-based vaccine coding 34 melanoma neoantigens based on the patients' mutational signature combined with anti-PD1 (113, 114). The report stated a reduction of recurrence risk or death by 44% compared with ICI alone, which granted the approach the designation of breakthrough therapy by the FDA at the beginning of 2023. In May of the same year, Rojas *et al.* described the results of a phase I trial evaluating a neoantigen vaccine developed using resected PDAC tumors (115). The researchers observed that half of the patients receiving the RNA vaccine elicited substantial *de novo* antigen-specific T cell responses targeting more than one antigen encoded by the vaccine (115). In glioblastoma patients, another phase I/Ib trial showed that neoantigen vaccination promoted antigen-specific T cell infiltration within the TME (116). It is worth noting that only a small percentage of the antigens used in these approaches are immunogenic, and it is common for patients to report immune responses against one or two antigens among the more than 20 peptides used in tandem in these vaccines.

Cancer vaccines based on the direct delivery of TAAs have been proven safe and efficient in promoting antitumor immunity, especially when combined with ICI (109). Recent technologies like whole-exome sequencing, mass spectrometry-based immunopeptidomics, single-cell RNA sequencing, and antigen prediction algorithms have advanced the development of personalized cancer vaccines by providing more precise methods for identifying neoantigens.

CURRENT CHALLENGES AND LIMITATIONS OF CANCER IMMUNOTHERAPY

The implementation of immunotherapy had a slow start initially, but over the past decade, it has become a fundamental pillar for the treatment of advanced malignancies, besides conventional therapies (117). Clinical trials evaluating various immunotherapy strategies have consistently reported that patients who respond to immunotherapy strategies have a longer overall survival rate and a lower chance of tumor recurrence. This is attributed to the creation of immunological memory, which provides a long-lasting effect. Nevertheless, all immunotherapy modalities face their challenges and limitations.

For example, T cell-based therapies must overcome manufacturing limitations before being universally and consistently employed. TIL isolation requires highly infiltrated tumors and large resected samples, limiting this treatment to only a fraction of patients (118). Although employing “off-the-shelf” allogeneic CAR-T cells, if successful, in the future may partially offset the high cost associated with this strategy, other issues regarding toxicity are difficult to predict. CAR-T-based therapies require extremely specific cancer antigens with low expression in normal tissues, lest they provoke irrevocable damage to the host’s body (119). CAR-T cell toxicity can range from cross-reaction with other proteins not expressed in tumor cells, allergy-like symptoms, neurological toxicity, and cytokine release syndrome, which leads to multi-organ failure and death (118, 119).

ICI's promise of unprecedented survival rates in previously untreatable patients revitalized the hope for universal cancer treatments. However, anti-CTLA-4 and anti-PD1 have been shown to cause unexpected side effects targeting all organs (120). As observed in the previously described knockout mice (39, 40, 50), blockade of CTLA-4 and PD-1/PD-L1 axis can lead to autoimmune-related consequences affecting the gastrointestinal tract, skin, joints, pancreas, lungs, heart, and brain, among others (120).

Aside from immune-related adverse side effects, all immunotherapy strategies described until now have also been confronted with 60% to 80% of patients who do not benefit from the treatment (19, 58, 118). Only a small percentage of eligible patients benefit from immunotherapy, and responses can vary greatly. Notably, the probability of experiencing a relapse is never zero, indicating that patients can

acquire resistance to immunotherapy (117). Moreover, patients with common cancers such as breast, prostate, pancreas, and colon cancers are more prone to resist immunotherapy than for other malignancies (118, 121-123). Therefore, resistance to immunotherapy is not uncommon and imposes a daunting challenge to be surpassed.

Common mechanisms for immunotherapy resistance can result from the intrinsic properties of tumor cells and extrinsic mechanisms (118). In the following chapter, I will explore some common mechanisms that lead to immunotherapy failure.

Hot and Cold Tumors: Defining Immunotherapy Response

The observations that lymphocytic infiltration correlated with better prognosis in cancer patients are still prevalent today (2, 13, 15, 124). A recent review by Jérôme Galon, the pioneering immunologist behind the Immunoscore – a tool that implements T cell infiltration when grading tumor severity –, has collected data from nearly 300 studies involving approximately 70000 patients diagnosed with various common cancers (124). Their systematic approach demonstrated that the immune compartment within the TME has higher predictive value across the tumors studied over other tumor characteristics such as mutational burden, microsatellite instability, and carcinogen origin (124). His work summarizes years of evidence that the presence of immune effectors such as CD8+ T cells, CD4+ T helper cells type 1 (Th1), B cells, NK cells, macrophages type 1 (M1), and dendritic cells within the TME correlated with better prognostic outcomes compared to known immunosuppressive populations like macrophages type 2 (M2), Treg and CD4+ T cell helper type 2 (Th2).

The lack of CD8+ T cell infiltration within the TME is generally associated with poor responses to ICI and other immunotherapies. Tumors that have low infiltration are commonly referred to as "cold tumors" by the Immunoscore. Various factors underlying the generation of immune deserts within the TME manifest differently among cancers and patients with the same cancer type. The immunosuppressive environment promoted by tumor-associated macrophages (TAMs), usually polarized toward pro-tumorigenic M2, cancer-associated fibroblasts (CAFs), Tregs, as well as dysregulation of the cytokines and chemokines network within the TME are well-described factors underlying antitumor immune effectors exclusion, exhaustion, or malfunction (125). However, this thesis will focus on mechanisms revolving around antigen presentation, which is critical for immunotherapy success.

Antigen presentation: the cornerstone of the cancer immunity cycle.

The cancer immunity cycle (**Figure 2**), first described by Daniel Chen and Ira Mellman a decade ago, divides the antitumor immune response into cyclic steps that can self-propagate and, in theory, amplify T cell responses (126). Cellular instability

in cancer cells leads to the production of TAAs and abnormal proteins that can be released into the TME, which professional APCs can take. Professional APCs upholster the newly processed antigens on their surface, complexed with MHC-I and MHC-II molecules. After migrating to the nearby lymph nodes, APCs prime and activate T cells into cytotoxic T lymphocytes (CTLs) and, through chemokine and cytokine secretion, promote T cell trafficking into the TME. Upon infiltration, CTLs recognize the antigens presented by tumor cells on their surface and effectively kill the target tumor cells. The antitumor immune response is a complex process that can fail at multiple points. In cancer patients, the chain of events required for effective antitumor immune responses is often disrupted, including the first steps involving antigen presentation.

Kickstarting immunity – the role of professional antigen-presenting cells.

Professional APCs are immune sentinels that scavenge the host's body for threats to homeostasis. These cells stand at the border between innate and adaptive immune systems, for they are one of the first barriers against microbial infections and can orchestrate complex immune responses involving T and B lymphocytes. While B cells can perform antigen presentation by receptor-mediated endocytosis, macrophages and dendritic cells scavenge their surroundings to phagocyte possible antigens.

Macrophages are a heterogeneous population of phagocytic cells originating from the maturation of circulating monocytes. Importantly, tissue-resident macrophages assume different names according to their respective location, but all share a common progenitor and function (**Figure 3**). Macrophages are important in tissue surveillance, infection, wound healing, and muscle repair (127). Under specific conditions, macrophages can actively contribute to neurodegeneration and atherosclerosis. Due to their high cellular plasticity and environmental adaptability, their ambiguous role in immunity extends to macrophages' dual role in cancer immunity. In cancer, pro-inflammatory polarization of macrophages towards the M1 phenotype contributes to tumor clearance by cytokine production, generation of reactive oxygen species, and antigen presentation from engulfed dead cells. Contrastingly, the TME can force macrophage polarization towards an anti-inflammatory program M2, which strongly correlates with poor prognosis in cancer patients (128). TAMs promote tumor proliferation, metastasis, angiogenesis, and immunosuppression but can be manipulated to repolarize into M1 macrophages (129, 130).

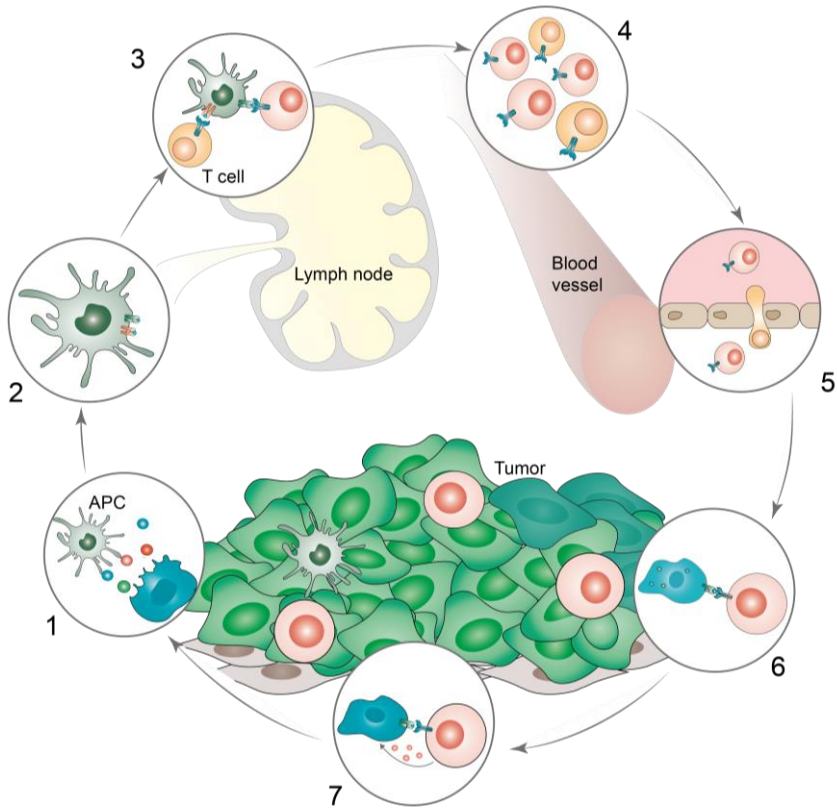

Figure 2 | The cancer-immunity cycle. Visual representation of the cancer-immunity cycle as described by Chen *et al.* (2013). The cyclic nature of antitumor immune responses starts with antigen release by cancer cells (step 1), followed by uptake and processing by professional APCs (step 2). APCs migrate to peripheric lymph nodes, prime T cells (step 3), and exert chemoattractant functions to promote immune effector migration towards the tumor (step 4). Finally, T cells infiltrate the tumors (step 5), where they can recognize cells expressing the cognate antigens (step 6) and unleash their cytotoxic activity on tumor cells (step 7). The cycle self-propagates with more antigens being released and captured by other APCs. Adapted from Chen and Mellman (126).

Dendritic cells as professional APCs – ontogeny, diversity, and function

Dendritic cells are professional APC that play a key role in immune responses. In the past, macrophages and dendritic cells were confused as part of the same population of cells necessary for priming T cells *in vitro* (131). However, Steinman and Cohn were the first to identify a distinct stellate cell with protrusions resembling dendrites (from the Greek origin “dendron,” meaning “tree”) that contained fewer lysosomes than macrophages (132). With advancements in molecular biology, transcriptomic analysis, and lineage tracing, we now know that dendritic cells represent an independent cell lineage separate from macrophages despite sharing phagocytic activity and phenotypic markers (133).

Myeloid-derived dendritic cells are designated conventional dendritic cells and can be divided into cDC1 and cDC2 (133). Like all leucocytes, dendritic cells are the product of a multistep process that continuously generates dendritic cell precursors from hematopoietic cells (HSCs) in the bone marrow (BM) (**Figure 3**). HSCs, through multiple division and differentiation processes, lead to lymphoid-myeloid-primed progenitors, which in turn branch into common lymphoid progenitors (CLPs) (134), common myeloid progenitors (CMPs) (135) and megakaryocytes-erythroid progenitors (MEPs) (136). These precursors are set apart by differential expression of specific transcription factors determining their lineage potential. For example, GATA1 expression in MEPs is crucial for the normal development of megakaryocytes and erythrocytes (137). On the other hand, GATA3, TCF7 (also known as TCF1), and BCL11B, in combination with Notch ligands, lock CLP fate towards T cell generation in the thymus (138). Despite the role of PU.1 (encoded by the gene *Spi1*) in CLP lineage commitment (139), PU.1 is a master regulator of almost all myeloid genes necessary for the generation of monocytes, macrophages, and dendritic cell progenitors (140-145). Moreover, the abrogation of PU.1 in adult HSCs results in a drastic reduction of the cDC compartment (146).

CMPs undergo further differentiation steps to generate granulocyte progenitors (basophils, eosinophils, neutrophils, and mast cells), monocyte progenitors (Langerhans cells, macrophages, and moDCs), and lastly, common, or conventional dendritic cell precursors (CDP) (**Figure 3**) (147-149). Surprisingly, the path through which cDC subsets emerge from CDPs has been only elucidated in the past decade. This was only possible by generating genetic models that uncovered the expression of C-type lectin receptor (CLR) CLEC9A (also known as DNGR1) and ZBTB46 in cDC1 precursors (150-152). While still in the BM, CDPs differentiate in uncommitted pre-DCs, pre-cDC1, and pre-cDC2, which migrate to lymphoid and nonlymphoid tissues where they receive external cues and fulfill their terminal differentiation potential into cDC1 and cDC2 (153, 154).

The distinction between cDC1 and cDC2 depends on the transcription factors required for their specification, their molecular expression patterns, and some of their functional properties (**Figure 3**). First, pre-cDC1 commitment is established in the CDP pool by IRF8, BATF3, ID2, and NFIL3 expression (149, 154). Early in cDC1 commitment, CDP bias towards the first subset requires high expression levels of IRF8 (155). This is controlled by the activity of the +41-kb enhancer portion at the *Irf8* gene locus (156, 157). During the CDP transition to pre-cDC1, NFIL3 induces high levels of ID2, leading to ZEB2 downregulation, a repressor of cDC1 development. Later in cDC1 specification, expression of IRF8 becomes dependent on BATF3 (154, 157, 158). Knockout models for *Nfil3*, *Id2*, *Batf3*, and *Irf8* show different levels of cDC1 impairment. Loss of ID2 and NFIL3 results in cDC1 deficiency (159, 160). Conditional deletion of *Irf8* before the pre-DC stage leads to reduced cDC1 cell numbers, skewing CDP differentiation into cDC2 (161). *Batf3* knockout does not impair the emergence of pre-cDC1 cells but induces

selective loss and dysfunction of the mature cDC1 compartment (154, 158). This, together with IRF8's dependence on BATF3 at later stages of differentiation, suggests that the interplay between IRF8 and BATF3 is key for full cDC1 specification and maintenance.

Phenotypically, cDC1 and cDC2 cells express the pan-hematopoietic marker CD45, CD135, CD11c, and MHC-I and MHC-II while lacking markers for T, B, NK, and erythrocyte lineage markers (133). Migrating cDCs express co-stimulatory molecules CD80, CD86, and chemokine receptor CCR7 (162). Contrastingly to cDC2, which is known to express CD4, splenic cDC1 cells are identified by the expression of CD8α, with an exception for circulating cDC1, which expresses E-cadherin-binding integrin αE (CD103) or CD24 instead. More importantly, all cDC1 express XCR1, a chemokine receptor, and CLRs CD205, and CD207, in addition to the already mentioned CLEC9A (133). cDC1 cells also express higher amounts of FLT3, which binds to FLT3L, the responsible factor for generating all cDC subsets (163, 164). Interestingly, generating bone marrow-derived dendritic cells (BM-DCs) using FLT3L to complement GM-CSF cultures produces a higher proportion of functional cDC1 (165).

Although cDC2 cells are more prevalent among conventional dendritic cells, we have limited knowledge about their biology. The population of cDC2 is considerably more diverse, and their heterogeneity starts during their differentiation. IRF8 homologs, including IRF4 and IRF2, together with TRAF6, regulate cDC2 development (133). Although the loss of IRF4 affects cDC2 function, it is not strictly required for cDC2 specification (166, 167). Over the years, different subgroups of cDC2 have been segregated through different dependence on the Notch signaling pathway or different requirements for KLF4 expression (166-168). Expression of T-bet and RORγt also identified two subgroups of cDC2 in the spleen (169). Recently, a cDC2-committed progenitor has been identified that expresses higher levels of cDC2-associated genes at the CDP level (170) (**Figure 3**). However, there is still a need for a deeper understanding of the molecular mechanisms driving cDC2-specification.

Mirroring the hazy cDC2 development, there is little uniformity in their phenotypical markers. Parallel to CD8 expression in cDC1, only a fraction of cDC2 express CD4, but most express high levels of SIRPα and CD11b (133, 171). Interestingly, cDC2 are known for lacking cDC1, including CD8 and XCR1 or CLEC9A receptors, but different subgroups within the cDC2 population express different markers (133, 150, 162, 169).

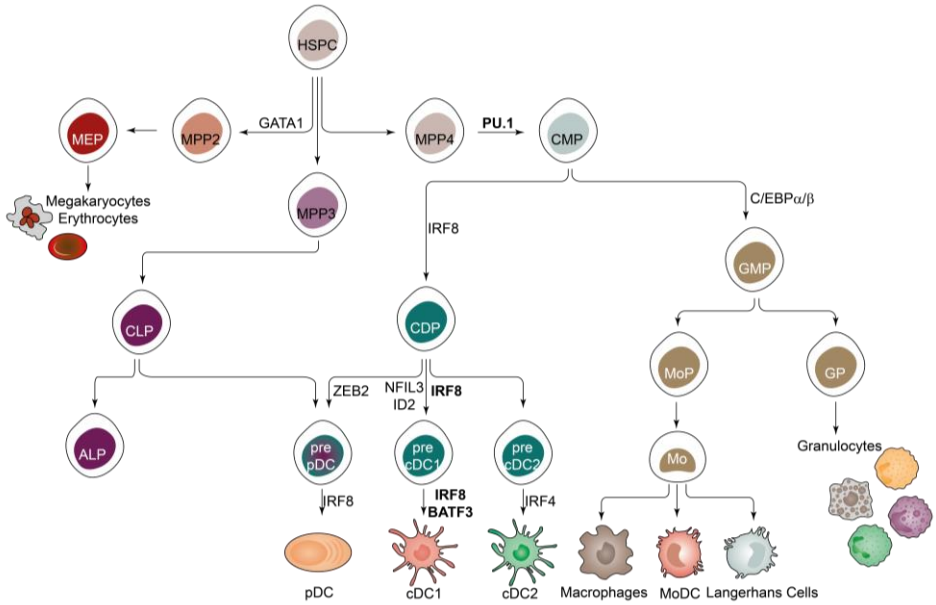

Figure 3 | Development of myeloid dendritic cells. Hematopoiesis gives rise to all circulating and tissue-resident blood cells, including dendritic cells. Hematopoietic stem and progenitor cells (HSPC) divide and originate multipotent progenitors (MPPs) that can generate the different progenitors that will differentiate into the megakaryocyte-erythroid lineage (MEP), common lymphoid lineage (CLP) and common myeloid lineage progenitors (CMP) depending on the environmental cues and transcription factors expressed at specific stages of development. MEPs will differentiate in mature megakaryocytes and erythrocytes, while CLP gives rise to all T, B, and NK cell lymphoid lineages. CMP will branch into common dendritic cell progenitors (CDP) and granulocyte-macrophage progenitors (GMP). Conventional dendritic cells type 1 and 2 (cDC1 and cDC2) arise from CDP committed to either lineage. The cDC1 subset is characterized by lineage-defining transcription factors BATF3 and IRF8, while NFIL3 and ID2 repress ZEB2 and block CDP maturation into plasmacytoid dendritic cells (pDC). Conversely, cDC2 is the most heterogenous population, with some researchers proposing a division within this subset. Nonetheless, cDC2 cells also arise from pre-committed CDP and express high levels of IRF4. The origin of pDCs has yet to be completely deciphered, as there is also evidence that some CLPs can generate pDC. ALP – all-lymphoid progenitors; GP – granulocyte progenitors; MoP – monocyte progenitors; Mo – monocytes; moDC – monocyte-derived dendritic cells. Adapted from Anderson, Dutertre (172).

cDC populations are highly specialized APCs capable of migrating to peripheric lymph nodes and prime *de novo* adaptive immune responses (133). Dendritic cells possess a vast arsenal of molecules fine-tuned for antigen sensing and capture and migration to peripheric lymphoid tissues where they can interact with T, B, and innate immune effectors such as NK cells. First, dendritic cells express diverse pattern recognition receptors (PRRs) that recognize PAMPs. Toll-like receptors (TLRs) and CLRs sense extracellular or internalized microbial antigens. PRRs also include cytosolic DNA sensors, which recognize cytoplasmic infection by viruses and certain bacteria. Self-antigens exposed by damaged cells, or DAMPs can be recognized by PRRs and initiate dendritic cell activation programs (133). Expression of PRRs varies across the different dendritic cell subsets. For example, human and mouse cDC1 express higher levels of TLR3 responsible for detecting double-stranded RNA and facilitating CD8+ T cell priming (173). CLEC9A binds

to filamentous actin, which is only exposed in damaged cells (151, 174). Signaling through CLEC9A allows cDC1 to sense cell debris and stimulate CTL responses (175).

Upon PRR stimulation and inflammatory stimuli, dendritic cells express more MHC molecules necessary for antigen presentation along with co-stimulatory molecules and chemokine receptors (133). The latter is critical for cDC to follow chemokine gradients, driving them from the periphery to draining lymph nodes, where they will interact with other immune cells (133). Lymph-node resident dendritic cells can also sense and capture free antigens traveling through the lymphatic system (176, 177). After migration to lymph nodes, dendritic cells prepare to transmit the antigenic information to other immune cells, including other dendritic cells (133). To stimulate CD4 and CD8 T cells, dendritic cells express the antigen-presenting molecules and co-stimulatory machinery along with IL-12 (178, 179). Functional specialization of dendritic cells is simultaneously dependent on their specific transcriptional program. Despite dendritic cells' inherent capacity to compensate each other in case of failure of one subset (133), cDC1 are undoubtedly excellent antigen presenters. cDC1 are endowed with machinery that allows them to present antigens through MHC-I, a cross-presentation process, to stimulate CD8+ T cells (133, 180, 181). Murine and human cDC1 produce vast amounts of IL-12 resulting from high levels of IRF8 in this subset (179, 182, 183). Moreover, cDC1 can also prime CD4+ T cells (179, 181, 184, 185).

Human dendritic cells have been found to follow similar ontogeny processes and express the same phenotypical identifiers as their murine counterparts (133, 186). Research on the heterogeneity of human dendritic cells has been largely driven by single-cell gene expression analysis. Despite species-specific characteristics, XCR1 and CLEC9A remain canonical markers of human cDC1 in addition to CD141 (133). Moreover, human cDC1 differentiation also depends on BATF3 and IRF8 (187, 188). On the other hand, human cDC2 cells are more heterogeneous and lack a uniform classification. Efforts to classify human DCs are still ongoing, with more relevant examples set by Nir Hacohen and Alexander Rudensky's respective groups. Villani et al. (2017) distinguished six subsets among human dendritic cells using single-cell RNA sequencing. By their classification, DC1 corresponds to cDC1, cDC2 is divided into DC2 and DC3 populations, and DC6 refers to pDC (189). Furthermore, Brown *et al.* discovered two subsets of cDC2, designated by cDC2A and cDC2B, separated by expression of T-bet and RORγt and CLEC10A in mice and humans (169).

There is another specific dendritic cell population known as pDCs (**Figure 3**), characterized by their plasma-like shape and ability to produce high amounts of type I IFN in response to viruses (147, 190). Early research suggested that pDCs descended from CDP (147, 191, 192), but some studies argued that pDC ontogeny was more closely related to lymphopoiesis (147, 169, 189, 193-195). On the other hand, it has also been discovered that pDCs share their origin with cDC1 through a common CX3CR1+ progenitor (196). This new understanding of pDC ontogeny

aligns with previous research demonstrating the interplay between ID2 and ZEB2 in cDC1 and pDC development (157, 197). Much like their origin, pDCs inherent antigen presentation is under debate. The first studies reporting pDCs' ability to prime T cells might have suffered from contaminants from other cDC (193-195), which reflects the artificial culture conditions hindering a thorough understanding of their function. More recently, there are some studies addressing whether pDCs can function as professional APCs (198).

Through their outstanding antigen sensing and presentation capacity, dendritic cells are fundamental pillars in fighting against pathogenic threats and maintaining homeostasis. Moreover, dendritic cell function extends further than T cell stimulation. Dendritic cells also interact with B cells and are powerful orchestrators of the innate immune system (133). For these reasons, it is unsurprising that dendritic cells play an important role in the cancer immunity cycle.

The Role of cDC1 in Cancer

It is important to recognize that moDCs, cDC2, and pDCs also play a role in antitumor immunity, especially due to their biological abundance, relatively easy manipulation, and use in cancer vaccine approaches (55, 133, 180, 199-201). However, this section will summarize the literature surrounding cDC1 antitumor immunity. The cDC1 subset has been implicated in antitumor immunity for over a decade, especially due to its flagship cross-presentation ability (202). In recent years, other facets of cDC1 biology have emerged, positioning this population of professional APCs as a central player in antitumor responses.

Mouse models lacking BATF3-dependent cDC1 fail to reject highly immunogenic fibrosarcomas (158). Proteomic and transcriptional analysis revealed the unique myeloid landscape in the TME (203). cDC1 was one of the myeloid populations found within the TME along with TAMs M1 and M2, albeit at lower proportions than the latter. Interestingly, regressing tumors had a more substantive presence of cDC1 within their TME, and tumoral cDC1 was apt at cross-presenting antigens to infiltrating CTLs (203). More recently, the application of imaging-based deep learning to identify the spatial organization of cDC1 interaction with CD8+ T cells within the TME revealed that cDC1-CD8+ T cells clusters appear at higher frequencies in highly immunogenic tumors in niches that promote CD8+ T cell activation (204). Furthermore, therapeutic vaccination with splenic cDC1 loaded with TAAs was sufficient to elicit tumor-specific CD8+ and CD4+ responses *in vivo (205)*.

Various independent lines of research in mouse and humans have demonstrated that cDC1 presence within the tumor is required for successful immunotherapy (202, 206-209). Administration, expansion, and activation of cDC1 at the tumor site synergize with ICI and improve immunotherapy response in several models (206, 210-213). In turn, ICI can promote the accumulation of cDC1 in the TME and enhance dendritic cell function (214-216). Checkpoint blockade can promote T cell release of IFN-γ. Intratumoral dendritic cells respond to IFN-γ by producing IL-12,

which supports CTL function (215). In alignment with these findings, detecting dendritic cell gene signatures within the TME and higher ratios of intratumoral cDC1 compared to other myeloid subsets is correlated with better prognosis and response to ICI (203, 209, 217).

In cancer, cDC1 cells' antitumor activities combine the cross-presentation of TAAs inside the TME and tumor-draining lymph nodes (tdLN) with the recruitment of other immune effectors and CTL activity support (202). Cross-presentation of TAAs depends on the correct function of vesicular trafficking of antigens. Loss of WDFY4, a regulator of intracellular vesicular trafficking, or overexpression of YTHDF1, a post-transcriptional RNA modifier implicated in cancer progression, abolishes presentation of tumor antigens by cDC1 to CD8+ T cells and allows tumor progression (218, 219). Recognition of intracellular DNA originating from necrotic cancer cells by stimulator of interferon genes (STING) is also necessary for optimal T cell activation by cDC1 (220). CCR7-dependent migration of cDC1 to tdLN is crucial for antitumor immunity as it promotes direct antigen presentation in a highly stimulatory environment (221). Importantly, intratumoral cDC1s secrete CXCL9 and CXCL10, which promotes T cell infiltration within the TME and supports the reactivation of CTLs (202, 207, 208, 215, 222, 223). Tumor rejection mediated by cDC1 is mediated by type I IFN signaling (224, 225). Finally, cDC1 establishes a bidirectional relationship with innate immune effectors such as NK cells to promote antitumor immunity. NK cells secrete CCL5 and XCL1, which attract cDC1 to the tumor milieu (226, 227). In turn, cDC1 produces a pro-inflammatory cytokine cocktail and chemokines that recruit, promote, and maintain NK-mediated anticancer cytotoxicity in the TME (228). Altogether, these data reveal that cDC1 plays a crucial role in antitumor immunity by influencing the TME with soluble factors and attracting and enabling effective adaptive and innate immune responses.

cDC1 dysfunction in tumors

Despite cDC1's important role in cancer immunity, the TME imposes severe obstructions to their normal function (**Figure 4**). Recruitment of cDC1 is impaired in melanomas with active β-catenin as they reduce CCL4 expression (208). The viability of NK cells is also affected in the TME, which, in turn, reduces cDC1 infiltration and viability (226). IL-6, produced by cancer and immune cells, undermines cDCs and moDCs differentiation (180). Vascular endothelial growth factor (VEGF) was found to repress FLT3L activity *in vivo*, blocking cDC1 differentiation (229). VEGF is also produced in large amounts by tumor cells, thus constituting yet another mechanism through which TME might be hostile to cDC1 presence (229). The TME also inhibits cDC1 activation and reduces antigen presentation. The expression of TIM3 by cancer cells can hinder intracellular DNA recognition by PRRs (230). Activation of TLR2 by tumor-derived proteoglycans induces chronic expression of IL-6 and anti-inflammatory cytokine IL-10 and upregulation of their receptors, contributing to direct immunosuppression of cDC1 within the TME (231). TAMs are another source for IL-10 and stop IL-12

production by cDC1 (232). Tumor-infiltrating cDC1s are also prone to endoplasmic reticulum stress due to the harsh conditions within the TME. This, in turn, results in less antigen presentation capacity and reduced T cell activation (180, 233). Oxidized lipid metabolites derived from the TME accumulate in cDC1 by binding to components of MHC-I translocation to the cell surface, thus preventing effective antigen presentation (234). Recently, it has also been shown that cDC1 circulating in pancreatic mouse models and patients decreases during carcinogenesis, promoted by increased apoptosis and maturation impairments (235). Collectively, these data prove that, even though cDC1s have the potential to orchestrate an efficient antitumor immune response, the harsh conditions inside the TME undermine optimal function.

Tumor immunogenicity and antigen presentation by cancer cells.

Complementing professional APCs' role in tumor immunity, tumor cells are active participants in tumor recognition, or lack thereof, by the immune system. Tumor mutational burden (TMB), expression of neo-antigens and TAAs, and antigen presentation machinery contribute to tumors' intrinsic ability to stimulate or avoid immune responses, also known as tumor immunogenicity (236, 237).

Chromosomic instability leads to the accumulation of genetic defects by cancer cells. Consequently, high mutational rates can generate aberrant proteins that can be degraded in small peptides and presented on cancer cells' surface in MHC-I molecules. Additionally, unique peptides originating from dysregulated RNA splicing, post-translational modifications, and viral proteins constitute new sources of "non-self-antigens" in cancer cells that can trigger immune responses. In immuno-oncology, these peptides are called neo-antigens (125). Neoantigens can originate from single-nucleotide variants, insertion and deletion mutations, gene fusions, non-coding regions, alternative RNA splicing, and protein variants (238). In recent years, there has been growing evidence that higher mutational burdens correlate with better immunotherapy response.

Exome sequencing of NSCLC biopsies from patients treated with anti-PD1 showed that a high frequency of mutations strongly correlated with clinical response to ICI (239). Prior to this study, whole-exome sequencing of 64 melanoma patients uncovered a specific neoantigen landscape that, when present, was associated with stronger responses to CTLA-4 inhibition (240). In alignment with these findings, pan-cancer stratification studies based on TMB and ICI response generated predictive models to identify patients who benefit the most from ICI (239-241).

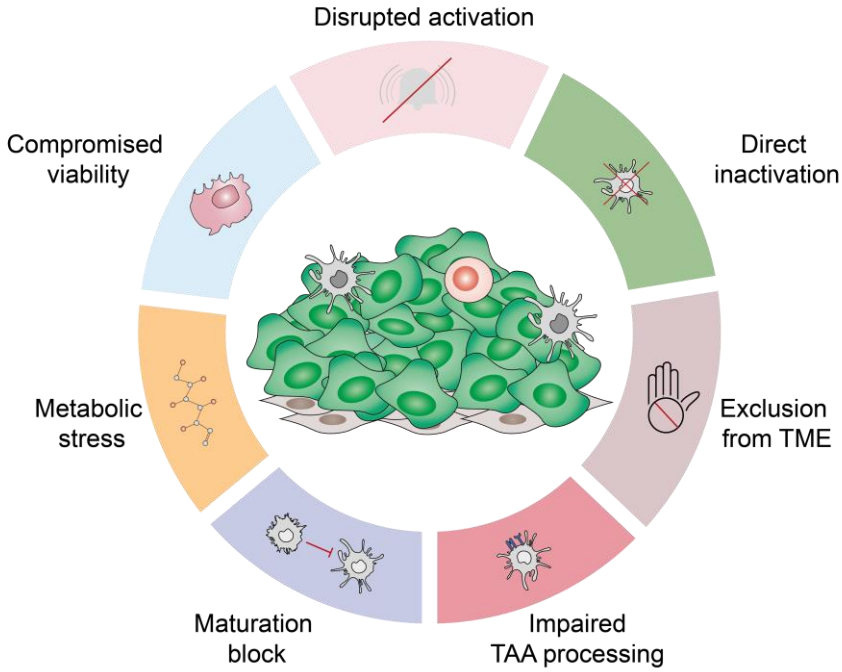

Figure 4 | Mechanisms of cDC1 dysregulation in tumors. The tumor microenvironment (TME) suppresses cDC1 function by targeting fundamental features of their biology. Tumor cells release metabolites that directly and indirectly exclude cDC1 from the TME, induce metabolic stress, and impair tumor-associated antigens (TAAs) processing. Tumor cells and other components of the TME, like macrophages, can directly inactivate cDC1 function and compromise cDC1 viability. Adapted from Wculek, Cueto (180).

Systematic reviews on the predictive value of TMB for carcinogen-driven carcinomas such as NSCLC and melanomas have concluded that patients with higher TMB respond more effectively to ICI (242, 243). Notably, cancer cells can also share "self-antigens" with healthy tissue by overexpressing too much of certain proteins, lineage-specific differentiation markers, or cancer germline antigens (238). However, these non-mutated tumor antigens might be presented during T lymphocyte development to induce "self-tolerance". The naturally occurring TCRs for these antigens might have low affinity for them, meaning that reactive T cells will be less effective (244).

T cells targeting neoantigens can be found circulating in the peripheral blood of patients and expand upon anti-PD1 treatment (245). Next-generation sequencing revealed several shared neoantigens among multiple myeloma patients and the presence of neoantigen-specific T cells that could be leveraged against cancer (246). Another study found shared neoantigens in colorectal, stomach, and endometrial adenocarcinomas with high chromosomic instability and corresponding neoantigen-specific CD8+ T cells in healthy donors and cancer patients (247). In a mouse model for squamous cell carcinoma (SCC), researchers identified polyclonal neoantigen-specific CD4+ T cells that promoted CD8+ T cell-mediated immune responses and

limited tumor growth (248). These suggest that neoantigens are prone to generate an immunogenic response and one of the reasons underlying the positive correlation between TMB and efficient ICI responses.

At the same time, immunologically visible tumors can also undergo immunoediting. This process puts selective pressure on cancer cells by eliminating those that express antigens on their surface and promoting the proliferation of cancer cells that acquire mechanisms to avoid the immune system (**Figure 5**). For example, a recent genetic screen using clustered regularly interspaced short palindromic repeats (CRISPR) to study mechanisms of immune evasion in murine syngeneic models for breast cancer unveiled the inactivation of certain tumor suppressor genes only in the presence of an adaptive immune system (249). Whereas *in vitro*, cancer cells that lose CUL3 stop proliferating, transplanted cancer cells lacking this protein into immunocompetent mice are positively selected and contribute to tumor growth. This can be explained by CUL3 forming complexes that promote PD-L1 degradation (250). Thus, loss of CUL3 helps cancer escape the immune system by stabilizing PD-L1 expression.

Another mechanism cancer cells evolve to avoid the immune system is the loss of antigen presentation machinery on their surface (**Figure 5**) (237). All somatic cells express MHC-I, which serves as a diagnostic tool for CD8+ T cells to assess cell health. On the other end of the spectrum, complete abrogation of MHC-I expression in cells triggers NK cells' cytotoxic activities (251).

During protein degradation, the resulting peptides can be loaded on MHC-I complexes and showcase their origin to CD8+ T cells. Aberrant proteins or viral products can be recognized as foreign, and the diseased cell is targeted for T cell-mediated cell death. In humans, MHC-I is encoded by human leucocyte antigen (HLA) class I. In melanoma and NSCLC patients, loss of heterozygosity for HLA-I genes and mutations on beta-2-microglobulin (B2M), a protein necessary for MHC-I expression on the cell surface, are related to acquired resistance to cancer immunotherapy, and constitute barriers to effective ICI (252, 253). In many of the most frequent human cancers, low expression, or complete loss of HLA molecules or B2M, have been reported with implications for ICI response (236, 237).

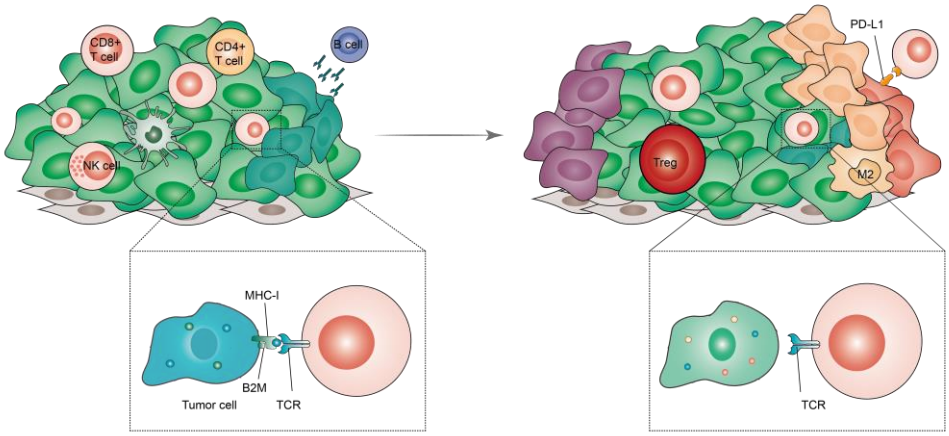

Figure 5 | Tumor landscape editing by the immune system. The adaptive immune system eliminates tumor cells that express neoantigens. Consequentially, tumor cells undergo selective pressure to exploit mechanisms that confer advantages over the immune system. Expression of PD-L1 to inhibit T cell function and loss of antigen presentation machinery are only two of the mechanisms depicted that cancer cells adopt to escape immune detection.

A recent study quantifying the loss of HLA-I molecules in 9000 tumors from the Cancer Genome Atlas (TCGA), in addition to two cohorts of pancreatic cancer and metastatic melanoma patients, identified the pervasiveness of its occurrence. All cancers from TCGA exhibited low levels of mRNA encoding for HLA-I molecules ranging from 6% to 86% (254). Loss of MHC-I molecules can occur through different pathways, resulting from genetic lesions or epigenetic deregulation. While the former is irreversible, epigenetic deregulation can be easily reverted. Somatic lesions, which involve the complete loss of all alleles encoding MHC-I or B2M, essentially abolish all MHC-I expression, lead to ICI resistance and can be found in up to 40% of patients, depending on the cancer (254, 255). On the other hand, one of the most common direct MHC-I alterations found in cancers is the partial loss of HLA alleles (254, 256, 257). This ensures that cancer cells can simultaneously escape CD8+ T cell recognition by low expression levels of MHC-I and avoid NK cell detection (251, 256, 257). Partial loss of HLA alleles substantially diminishes the neoantigen repertoire that can be presented and safeguards residual HLA presentation that can repress NK cells' main triggers (251, 256).

Furthermore, disturbances in genes involved upstream of the MHC-I pathway limit its expression on the cell surface, impacting tumor antigen presentation. For example, NLRC5, the master transcription factor regulating key proteins of the MHC-I pathway, including B2M, MHC-I, antigen processing proteins such as immunoproteasomes, and antigen transporters like TAP1, is often downregulated in cancers (258). Mutations and epigenetic modifications rendering NLRC5 ineffective have been described in cancer, resulting in MHC-I depletion (258-260). Loss of IRF1 and IRF2 results in downregulation of MHC-I and upregulation PD-L1 (261, 262). Proteasomes and immunoproteasomes degrade intracellular proteins into small peptides, which generate the antigens that will bind to MHC-I. Higher

expression of immunoproteasome subunits PSMB9, PSMB10, and PSMB8 was associated with better prognosis in NSCLC patients, whereas decreased levels of immunoproteasome expression accompanied epithelial to mesenchymal transition with worse outcomes (263). Defects in the expression of TAP1 and TAP2 have been known to exist in breast, cervical, colorectal, lung, and renal cell carcinomas (264-269). Therefore, all intervenient parts of the MHC-I pathway can be subjected to mutation or allelic loss, transcriptional dysregulation, epigenetic silencing, as well as post-transcriptional and translational modifications, which result in loss of MHC-I on cancer cells' surface with varying degrees of restoration potential (237, 270).

A study from 2019 attempted to calculate tumor immunogenicity score as a function of the TMB and antigen presentation machinery score. The latter is calculated from the total mRNA expression of antigen-presenting machinery genes present in tumor samples, which includes immunoproteasome subunits, TAP1, TAP2, B2M, and the three allelic forms of HLA-I among other genes belonging to the MHC-I pathway (236). According to this study, the status of antigen-presentation machinery expression heavily influences ICI response. For example, moderately mutated tumors with low antigen presentation machinery scores tend to have poor ICI responses. Such is the case for prostate and breast cancers, whereas tumors with higher expression levels of antigen-presentation machinery genes tend to respond much more effectively to ICI treatment. Moreover, this and other studies have noticed that tumor immunogenicity can be manipulated, antigen presentation in cancer cells can be restored, and antitumor immune responses reinstated if the defects in antigen-presentation pathways originate from reversible lesions (236, 237, 270).

Other mechanisms that affect tumor immunogenicity have been described. Increased tumor heterogeneity, for example, can also contribute to poor immunogenicity. The number of tumor clones affects immune infiltration. Surprisingly, the higher number of clones in melanoma mouse models and human samples is correlated with poorer survival. Independently of the TMB, a lower number of cancer clones means that most mutations occurred in very few initial clones; in contrast, a high level of diversity implies an even and diverse dispersion of neoantigen presence across all the different clones that might have occurred at different points of carcinogenesis. Therefore, the presence of neoantigens is diluted across several clones and, as such, increasing the difficulty for the immune system to eliminate all the tumorigenic clones (271).

Despite MHC-II expression being mostly restricted to professional APCs, tumor cells may also start expressing these molecules under certain circumstances, such as IFN-γ stimulation. MHC-II restricted neoantigens have been identified in mouse models, and the role of CD4+ T cells in driving antitumor immunity has been highlighted in recent years (248, 272). The presence of MHC-II molecules in tumor cells has been correlated with improved survival, greater infiltration of lymphocytes, and enhanced tumor immunity (273). Interestingly, cancer evolution involves the generation of mutations that are less likely to be presented by MHC-II

molecules, resulting in lower tumor immunogenicity (274). This demonstrated the importance of MHC-I for effective anticancer immunity.

In summary, carcinogenesis offers many opportunities for cancer cells to be recognized by the immune system by generating neoantigens. However, in addition to other immunosuppressive mechanisms, tumorigenesis is often accompanied by perturbations on antigen presentation pathways, leading to poor tumor immunogenicity and hindering effective ICI response.

Strategies to enhance tumor immunogenicity.

Loss of tumor immunogenicity is a common evasion mechanism found across several types of cancers. For several years, this has raised the question of whether these mechanisms can be reversed, and tumor immunogenicity restored. Cancer vaccines can potentially increase the professional antigen presentation of tumor-derived antigens. However, moDCs-based vaccination has encountered some limitations (55, 275). While pre-clinical studies have demonstrated that cDC1-derived vaccination effectively halts tumor progression and synergizes with ICI (203), this approach is also faced with constraints. cDC1 is the rarest population of dendritic cells circulating in the blood and within TME (133, 202). Autologous cDC1 from cancer patients can be dysregulated, and the TME can still suppress their function (180, 233, 235). Whether their delivery is done by dendritic cell vaccines or through nucleic acid vehicles, neoantigen vaccines are also an attractive opportunity to improve tumor immunogenicity (238). However, identifying personalized neoantigen peptides can become costly and time-consuming, has limited accuracy, and is insufficient for tumors with low TMB (238). An “off-the-shelf” approach exploiting commonly found neoantigens or TAAs can offset the research and validation costs involved in *de novo* identification of neoantigens. Still, it can also be met with acquired resistance mechanisms such as loss of neoantigen expression, induction of unwanted toxicity when antigens are shared with healthy tissue or poor antigenic immunogenicity (125, 238).

On the other hand, direct manipulation of tumor intrinsic immunogenicity has been explored in diverse settings. In cancer cells whose loss of MHC-I expression stems from allelic loss or inactivating mutations in genes involved in MHC-I expression, traffic, and processing, gene replacement therapy or gene editing would be required to substitute the lost genes. Recovering HLA-I and B2M expression by ectopic expression of these genes was demonstrated before the turn of the century (276). Garrido and colleagues have shown that delivery of B2M in an adenovirus package recovered HLA-I expression in HLA-I-negative cancer cells (276). Intratumoral injection of adenovirus encoding B2M sequence in human xenografts models also restored HLA-I expression and increased antigen-specific T cell recognition (276, 277). However, approaches like the one described here would require that almost all cancer cells, including metastasis, be efficiently transduced and repaired, lest MHC-I negative clones escape immune effectors again (270). It is

also possible to convert cancer cells into surrogate APCs by delivery of MHC-II-inducing factors. High endogenous expression of MHC-II induced by ectopic expression of *Ciita*, the transcription factor regulating the expression of genes involved in the MHC-II pathway, drives tumor rejection in murine cancer models (278). Transfected cancer cells could process and present antigens into MHC-II molecules, triggering IFN-γ producing CD4+ T cells (278).

For tumors where the MHC-I pathway is only restricted by downregulatory mechanisms, recovery of tumor immunogenicity is, in theory, easier to achieve. For example, treatment with type I and II IFN has been well-known to stimulate MHC-I and MHC-II expression (279-281). A small phase Ib/II clinical trial assessed the antitumor efficacy of the combination of anti-PD1 blockade and pegylated-IFNα2b in stage IV melanoma patients. Results showed that combining the two treatments elicited one complete response and several partial responses with relative safety (282). However, IFN-based treatments for cancer have had limited success (283). Type I IFN have been shown to have a dual role in cancer. Whereas acute exposure to IFN has cytotoxic properties and can potentiate antitumor immunity, chronic exposure instigates pro-survival mechanisms and hinders cancer therapy (283). Furthermore, chronic antigen exposure without co-stimulatory molecules and inflammatory cytokines results in T cell anergy (284).

Although clinical trials have not always yielded favorable outcomes for IFN treatment, the molecular pathways affected by type I and II IFN play a critical role in the success of immunotherapy. Various CRISPR screenings have uncovered several genes necessary for intrinsic tumor immunogenicity (285-287). Consistent with reported deficiencies in antigen presentation machinery in cancer cells, loss-of-function of genes encoding antigen presentation machinery and proteasomes induced the most resistance to CTL-mediated destruction in mutated cancer cells (285). Interestingly, despite not including a guide RNA for IFN-γ pathway genes, the authors discovered that APLNR, a G-protein-coupled receptor that is found mutated across several tumors, was among the genes responsible for effective antitumor responses by CD8+ T cells. APLNR directly interacts with JAK1 to potentiate IFN-γ responses (285). Notably, other screens that aimed at finding targets that sensitize cancer cells to immunotherapy also identified IFN-related modulators. Loss of PTPN2 and SETDB1 leads to tumor rejection, increased antigen presentation to CD8+ T cells and subsequent activation, and increased sensitivity to ICI in mouse models (286, 287). While the first is a negative IFN-γ regulator by dephosphorylation of JAK1 and STAT1, the second is a histone methyltransferase whose targets include a cluster of IFN genes. Likewise, STING agonists also induce potent antitumor immune responses through activation of the IFN pathway and increased MHC-I expression (288-290). These data strongly suggest that IFN signaling is a solid target for direct and indirect manipulation that can unlock tumor immunogenicity.

Epigenetic modulators targeting DNA methyltransferases and histone deacetylases have been shown to induce MHC-I expression in cancer cells (291,

292). Importantly, there are already several FDA-approved drugs for cancer treatment that target DNA methylation and histone deacetylation, even though the exact mechanism of action remains to be elucidated (270). For example, azacytidine, used for treating myelodysplastic syndromes and AML, induced PD-1 and IFN-γ signaling in cancer cells. In a limited trial, combining azacytidine with anti-PD-1 improved overall survival in responders among AML patients (293). Despite being approved for blood cancers, these drugs have achieved poor results in solid tumors, especially when used as single agents (291). Combination with ICI has been effective in pre-clinical models and small clinical studies (292-296). However, cytotoxicity, broad targets, and unknown mechanisms of action limit the potential adoption of epigenetic modifiers in the clinic (270).

Although there are many different methods to target immunogenicity, there is still a need for a comprehensive therapy that combines epigenetic and transcriptional regulation of antigen presentation machinery. This therapy should trigger IFN signaling pathways and potentiate antitumor immunity. To bridge this gap, we must explore alternative methods for immunotherapy that go beyond the common toolbox currently used for the therapies described here.

CELL FATE REPROGRAMMING

Conrad Waddington described cell fate as a ball rolling down a hill, a one-way irreversible path that embryonic stem cells (ESCs) take to ultimately differentiate into the diverse tissues of an adult organism (297). The pioneer work of John Gurdon started to challenge this concept after the somatic cell nuclear transfer of terminally differentiated cells into enucleated eggs (298, 299). These experiments revealed that the nucleus of a cell contained the important genetic information for the development of a full organism. Moreover, a pluripotent state could be achieved when the nucleus of a cell, independently of its status, was placed in the right cytoplasmic context. The cloning of Dolly, the sheep, in 1997 was the stepping stone cementing that somatic adult cells could be reversed to a pluripotent stem cell state (300). In the early 21st century, Shinya Yamanaka and colleagues identified the combination of transcription factors mediating somatic cell conversion to pluripotent stem cells (301-303). Today, the transcription factors are known as the OSKM factors, standing for OCT4, SOX2, KLF4, and MYC, and the process is called cellular reprogramming, where cell fate conversion is accompanied by transcriptional and epigenetic changes that allow an adult cell to become an induced pluripotent stem cell (iPSC) (304). These seminal works would grant Shinya Yamanaka and John Gurdon the 2012 Nobel Prize in Physiology or Medicine.

The ability to alter cell identity using just a few transcription factors has presented exciting prospects for exploring the basic principles of cell identity. It also offered a novel method for producing uncommon cellular subtypes for drug testing and disease modeling, creating new possibilities for regenerative medicine and cell replacement therapy (304). Since the discovery of the OSKM combination, iPSCs have been successfully generated from keratinocytes, melanocytes, neurons, hepatocytes, and peripheral blood cells besides fibroblasts (301-303, 305-310).

Like ESCs, iPSCs retain pluripotency, as they can generate all germ-layer cells when given the appropriate stimuli. The generation of donor-specific iPSCs surpasses ethical issues regarding the use and manipulation of ESCs while providing a valuable source of cells that can be differentiated into the desired cell for regenerative medicine. Moreover, iPSCs enable *in vitro* engineering for necessary genetic corrections or enhancements before their intended application (55, 311). iPSCs can also unveil cellular and molecular pathogenesis underlying human disease and provide a tool for high-throughput drug screening tests.

On the other hand, iPSCs lines can have diverse backgrounds, which impact their differentiation capacity, leading to heterogenous populations of desired differentiated cells and contaminating teratoma-inducing pluripotent progenitors (301, 312). For this reason, the clinical use of iPSC continues to face challenges. New protocols are currently in development to eliminate iPSC contaminants from ex vivo differentiation. Particularly, in a primate model for Parkinson's Disease, researchers isolated iPSCs-derived dopaminergic progenitors by the expression of CORIN (313). In contrast, antibodies targeting iPSCs' marker CD30, and

antimitotic agents eliminate pluripotent stem cells from iPSCs-derived cardiomyocytes, allowing safer clinical trials using iPSCs (314).

Direct lineage reprogramming

Identifying the transcriptional network regulating pluripotency also allowed other researchers to ask whether different cell fates could be coded under such restrictive, minimal networks of transcription factors. Before the OSKM findings, various studies demonstrated that cell fate was plastic and that there were transcription factors capable of overwriting the original lineage commitment of differentiated cells. Such was the case for the overexpression of MyoD activating a muscle program in different cell lines and exogenous expression of GATA1 in myelomonocytic cell lines, transdifferentiating them into eosinophils, erythroblasts and thrombocytic cells (315-317). The latter was also a prime example of GATA1 showcasing its role as a master regulator of hematopoietic differentiation. Overexpression of PAX6 and C/EBPα/β in glial and B cells, respectively, could override the original genetic program and induce neuronal and macrophage-like cell fates (318, 319).

The concept of direct reprogramming, or transdifferentiation, was thus born and reflected "the process of inducing a desired cell fate, by converting somatic cells from one lineage to another bypassing intermediate pluripotent or multipotent states" (304). Direct reprogramming strategies offer multiple advantages over iPSCs-derived products as they are faster to obtain, easier to manufacture, and more suited for clinical applications as they do not carry the same oncogenic risk promoted by pluripotent stem cells (55). Since those first studies reporting the lineage conversion of muscle-like cells and macrophages, many cell types have been now generated through direct reprogramming. While current technology allows cell lineage conversions mediated by many methodologies, such as small molecules, micro-RNAs, and epigenetic modifiers, understanding the transcriptional network behind cell identity is the foundation of direct reprogramming (304, 320).

Transcription factors as instructors of cell fate

A combinatorial approach of 14 transcription factors necessary for heart development identified GATA4, MEF2C and TBX5 induced cardiomyocyte-like fate in cardiac fibroblasts. *In vivo* transdifferentiation improved myocardial function after infarction (321, 322). Inducing neuronal phenotypes has also been extensively pursued by different groups. The combination of ASCL1, BRN2 and MYTL1 reprograms mouse embryonic fibroblasts (MEFs) and adult tail fibroblasts into functional, generic neuronal cells that can form functional synapses (323). Spinal motor neurons were obtained using the previous three transcription factor in addition to four more factors (324). Caiazzo and others have successfully identified the transcriptions factors to reprogram fibroblasts into dopaminergic neurons,

GABAergic neurons, and astrocytes (325-327). Dopaminergic neurons can be rapidly generated by overexpression of MASH1, NURR1, and LMX1A in mouse and human fibroblasts (325). Staggered FOXG1 and SOX2 expression, followed by ASCL1, DLX5, and LHX6 induced GABAergic neurons (326). In turn, NF1A, NFIB, and SOX9 gatekeep astrocytes' cellular identity (327). Fibroblasts can be converted into functional induced hepatocytes by overexpression of different combinations including FOXA3 and HNF1A (328, 329). OCT4, RUNX2, MYC and OSX could reprogram human fibroblasts into osteoblasts that promoted bone repair *in vivo* (330, 331). Besides cardiomyocytes, neurons and hepatocytes, the combinations of master transcription factors that impose the fates of Sertoli cells, adipocytes, chondrocytes, and insulin-producing β-cells were identified in the past two decades (332-335). Altogether, these studies are compounding evidence that, under careful overexpression of the right lineage-instructing transcription factors, cell commitment can be rewritten across cell types and germ-layers (304). Importantly, identifying lineage-specific factors opens new avenues for regenerative medicine and tissue repair with paramount implications in diseases affecting the myocardial muscle, neurodegenerative diseases, diabetes, and liver disease.

Reprogramming hematopoiesis and immune cell fates.

Both cell reprogramming and direct reprogramming strategies opened novel avenues to generate a wide range of blood cells (**Figure 6**). iPSCs can be differentiated into hematopoietic stem and progenitor cells (HSPCs) through a stepwise process requiring the formation of embryoid bodies and the development of the mesoderm layer. Most protocols also require specialized media to support HSPCs, cytokine cocktails that further support the desired cell type and, in some cases, specialized feeder-layers that can provide external signaling to promote cell growth and differentiation (55, 336). On the other hand, direct reprogramming approaches have elucidated the range of transcription factors necessary for converting somatic cells into HSPCs. Overexpression of GATA2, GF1B, FOS, and ETV6 induces a time-dependent, multistep process that leads to the generation of hemogenic endothelium that ultimately gives rise to HSPCs, resembling embryonic hematopoiesis (337-339). The combination of ERG, GATA2, LMO2, RUNX1c, and SCL generated hemogenic endothelium with the potential to differentiate into granulocytes, macrophages, erythrocytes, and megakaryocytes (340). These examples illustrate how cell reprogramming and direct lineage conversion can be used to simulate development and generate abundant sources for blood derivatives.

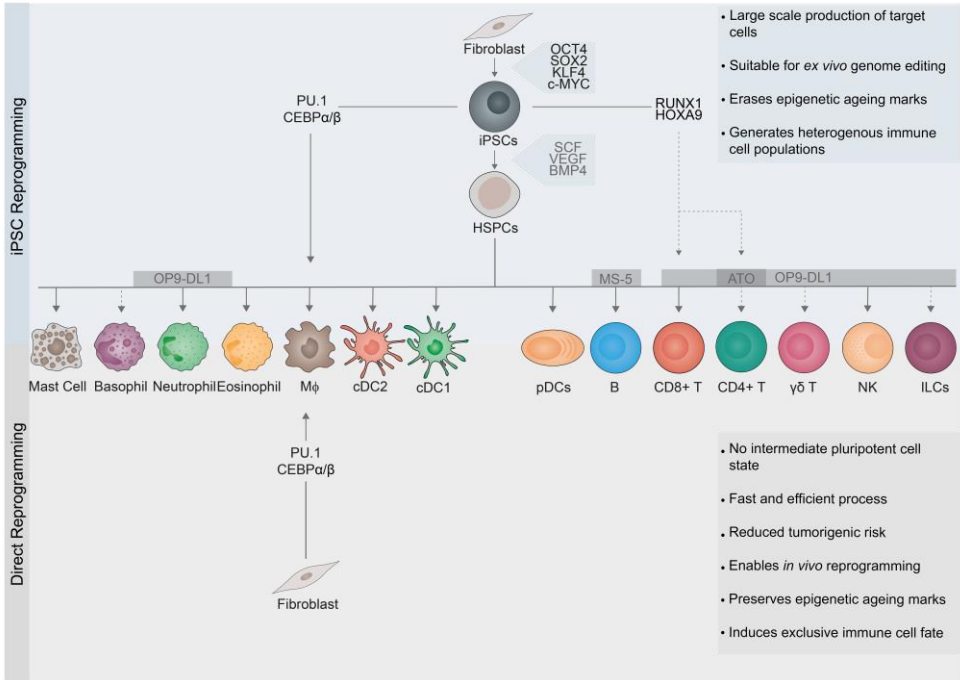

Figure 6 | Cell reprogramming of immune cell fates. Overexpression of OCT4, SOX2, KLF4, and c-MYC generates induced pluripotent stem cells (iPSCs), which can be differentiated into hematopoietic stem and progenitor cells (HSPCs) by cytokine cocktails, including stem cell factor (SCF), vascular endothelial growth factor (VEGF), and bone morphogenetic protein 4 (BMP4). Differentiating HSPCs into different lineages involves complex protocols using cytokine combinations and feeder layers like MS-5, OP9-DL1, and artificial thymic organoids (ATO) to support lymphopoiesis. iPSCs constitute a source for large-scale production of immune cells that can be genetically engineered to express desired phenotypes, such as expression of CAR. Reprogramming rewrites the original epigenetic and transcriptional programs and removes aging footprints. However, obtaining immune cells from iPSCs is challenging (dashed lines) and generates heterogeneous populations. Direct reprogramming bypasses intermediate pluripotent cell states with reduced tumorigenic potential. It is also a faster, more efficient, and more faithful process that can be done *in vivo.* The direct reprogramming of fibroblasts to macrophages induces an exclusive macrophage (Mϕ) phenotype. Transcription factors can also be used in forward reprogramming to accelerate iPSCs differentiation. cDC - conventional dendritic cells; pDCs – plasmacytoid dendritic cells; NK – natural killer; ILCs – innate lymphoid cells. Adapted from Zimmermannova, Caiado (55).

Reprogramming immunity.

All cell identities reflect complex transcriptional, epigenetic, and functional unique signatures. Advances in next-generation sequencing helped distinguish and better characterize immune cells, identify novel cell types, and uncover the gene networks instructing cell fate. While being extensively studied in regenerative medicine, cell reprogramming can be harnessed to manipulate anti-cancer immunity. In particular, iPSCs were used to produce cancer vaccines. More importantly, cell reprogramming offers an opportunity to generate rare immune cells, including T, NK, and dendritic cells at a clinically relevant scale.

Cancer vaccines using iPSCs.

Human embryonic stem cells and iPSCs share a potential oncogenic genetic program and can induce immune responses (341-344). Prophylactic immunization with an iPSC-vaccine recently conferred protection against multiple cancer types in different mouse strains (342). The authors demonstrated that iPSCs may share the expression of known and unknown TAAs since vaccinated mice could both reject breast cancer and limit the growth of teratomas. Furthermore, the antitumor effect was attributed to IFN-γ producing T cells, and vaccination increased intratumoral infiltration of B and T cells at an early cancer stage. Altogether, iPSCs-derived vaccines can be an interesting, patient-specific approach to elicit responses against a large spectrum of tumor antigens. However, more studies need to be done to address whether patient-derived iPSCs can express tumor and patient-specific TAAs, or just the ones shared with teratomas.

Reprogramming into T and B lymphocytes.

Due to its high complexity, lymphopoiesis is tremendously difficult to recreate *ex vivo* as it spans multiple organs, environmental cues, and complex transcriptional and epigenetic regulation. In immunotherapy, particularly for ACT, having an unlimited supply of immune cells would solve the issues caused by the low availability of patients' T, NK, or dendritic cells. To date, there have been no successful attempts at direct reprogramming T or B lymphocytes from unrelated somatic cells.

On the other hand, the differentiation of T and B cells from iPSCs has benefited from extensive research and protocol optimization in the past decades (**Figure 6**). The natural CD8+ and CD4+ T cell development can be recapitulated through iPSCs differentiation in time-consuming conditions involving artificial thymic organoids (ATO), and complex cytokine cocktails (345, 346). T cells can also be used as a source for iPSCs, which also improves the outcome of re-differentiation. By using antigen-specific T cells to generate iPSCs, the parental TCR is locked in its original rearrangement even after redifferentiation to T cells again, which ensures the maintenance of antigen-specificity and overcomes a major limitation for *ex vivo* lymphopoiesis protocols (347, 348). The exhausted T cell pool found in cancer patients can then be rejuvenated from T cell-derived iPSCs that maintain their original TAA specificity (347, 349).

Although *in vitro* direct reprogramming has not yielded fruitful conversions to T cell fate, transient expression of HOXB5 in B cell progenitors at the pro-pre-B stage, followed by transplantation into mice, blocks B cell commitment and converts these cells into early T cell progenitors, which then mature to fully functional T lymphocytes (350). Furthermore, functional T cells can be generated *in vivo* via forward reprogramming, which utilizes transcription factors to facilitate the differentiation of iPSCs. Guo et al. overexpressed RUNX1 and HOXA9 during the early endothelial-to-hematopoietic differentiation process from iPSCs. The

resulting progenitors were then formatted to express thymus-homing molecules that would migrate to the thymus and fully mature in induced T cells with a varied TCR repertoire that populate the blood of immunodeficient mice (351). Importantly, genetic engineering of the original iPSCs to express tumor antigen-specific TCR or CAR can endow newly differentiated T cells with desired antitumor properties.

In striking contrast with T cells, B cell development does not need thymic stimulation. However, generating B cells from iPSCs is equally challenging and lengthy. iPSCs can differentiate in B cell precursors expressing CD45, CD19, and CD10. Although these precursors induced the first steps of B cell receptor (BCR) rearrangement, indicating the adoption of an early pre-B commitment, they failed to acquire a mature B cell phenotype (352). Later, Carpenter and colleagues reported a more successful endeavor by adapting culture conditions to use MS-5 as the supporting stroma (353). The arising B cells had fully rearranged BCR genes and expressed immunoglobulin M at the cell surface.

Reprogramming into NK cells.

In recent years, NK cells have received wide attention from the research community as a promising new immunotherapy for cancer (251). As mentioned before, NK cells' mechanism of action is to kill cells that do not express MHC-I on their surface, as these molecules repress NK stimulation and prevent inadequate activation against the "self". Although the conditions found in the TME do not support NK cell antitumor properties, *ex vivo* activation, expansion, and genetic modifications have been shown to contribute positively to tumor control. Several clinical trials are assessing the efficacy of NK cell-based therapies against AML, lymphoma, multiple myeloma, pancreatic cancer, and other solid tumors (251). However, NK cell-based immunotherapy faces many challenges, including high manufacturing costs, potent adverse immune reactions, and high variability in the efficacy of generating NK cells from different donors. Despite not being constricted to HLA haplotypes, allogeneic NK cells are obtained inefficiently from donor blood, yielding highly impure NK and monocyte mixtures (354). iPSCs can be leveraged to overcome most of the hurdles NK cell-based therapies face by providing an unlimited source for standardized, ready-to-use NK cells with known haplotypes.

Differentiation of NK cells from iPSCs requires a two-step protocol in which, first, hematopoietic progenitors are generated, followed by the addition of a cytokine cocktail supporting NK maturation (**Figure 6**) (355). Additionally, iPSCs-derived NK can also be engineered to express CAR, which confers NK cells with antigen specificity and avoids the PD1/PD-L1 suppressive axis CAR-Ts are subjected in solid tumors (251). Importantly, iPSC-derived NK cells with or without CAR engineered in their genome constitute the first immunotherapy resulting from a cell reprogramming product to reach clinical trials for blood malignancies and solid tumors (251). For example, preliminary results for a small clinical trial assessing an iPSC-derived NK cell immunotherapy were recently reported.

Combination of iPSC-NK cells with anti-CD20 enhanced lymphoma cell killing and showed complete responses in three patients (356).

Although NK cell development, phenotype, and transcriptional profile have been well documented (357), the minimal gene network regulating NK cell lineage identity is yet to be completely deciphered. Direct reprogramming protocols for generating induced NK cells have yet to be published. However, conditional deletion of BCL11B in thymocytes and mature T cells inhibited T cell development and lineage maintenance and promoted NK cell transdifferentiation instead (358).

Reprogramming into granulocytes.

Granulocytes exert an important role in innate immunity as the first line of defense against pathogens. Although they do not act directly against cancer cells, granulocytes could substantially help to recover immune competence in cancer patients undergoing chemotherapy or bone marrow transplantation who are highly susceptible to opportunistic bacterial and fungal infections. Like other differentiation processes from iPSCs, most protocols generating granulocytes involve the induction of hematopoietic progenitors, followed by terminal differentiation with cytokines (**Figure 6**). This combination generally yields eosinophil-rich populations. Upon co-culture with OP-DL1, neutrophils can also be differentiated from iPSCs (359-361). Like other immune cells, the lineage-specific transcription factors that can impose a granulocytic cell identity have not been identified. Therefore, a strategy for direct reprogramming into granulocytes does not exist yet, and a large-scale manufacturing could rely solely on iPSC differentiation.

Reprogramming into professional antigen-presenting cells.

The rapid generation of autologous and allogenic professional APCs could propel further advancement of current cancer vaccination approaches. Several protocols for differentiating iPSCs into macrophages and conventional dendritic cells (**Figure 6**). Macrophages can be obtained from iPSCs by introducing IL-3 and M-CSF in the culture conditions (359, 362). However, iPSCs-derived macrophages also show low-polarization profiles, expressing a mixed phenotype between M1 and M2 programs and simultaneously secreting pro- and anti-inflammatory cytokines (363). Nonetheless, macrophage differentiation can be utilized for immunotherapy. For example, in a study by Senju *et al.*, genetic modification of iPSC to express a membrane-bound form of a single chain antibody targeting CD20 allowed the generation of macrophages that specifically engulfed malignant cells in a mouse model for B cell acute lymphoblastic leukemia (B-ALL) (364). Others have studied the introduction of CAR to drive antitumor immunity based on antigen-specific phagocytosis (365, 366).

Interestingly, macrophages were the first immune cells for which the lineage instructing transcription factors were revealed by successfully converting B cells

(319). The combined expression of C/EBPα, C/EBPβ, and PU.1 suppressed the B cell lineage commitment transcription factor PAX5, downregulated CD19 expression, and triggered a myeloid program, leading to MAC-1 expression among other markers. Four years after the first publication, Thomas Graf and colleagues published the direct reprogramming of fibroblastic cells using C/EBPα and PU.1 to instruct myeloid lineage, upregulate hematopoietic and macrophage-specific genes, and endow unrelated cells with phagocytic capacity in less than 8 days of culture (367). Recently, the same transcription factors were employed in the forward reprogramming approach. Overexpression of PU.1 and C/EBPα in iPSCs to generate macrophages that exhibit microglia characteristics when co-cultured with induced neurons (368).

Due to their importance in antitumor immunity and their rarity in peripheral blood, dendritic cells are an excellent target population for large-scale production by reprogramming approaches. However, protocols differentiating dendritic cells from iPSCs have produced heterogeneous populations with immature phenotypes, tolerogenic properties, and poor antigen presentation. iPSCs-derived dendritic cells result from multistep processes involving different cytokine cocktails that can favor one subset over the others. For example, the co-culture of iPSCs-derived CD43+ progenitors in OP9 feeder layers in the presence of FLT3L, SCF, and GM-CSF potentiates the generation of pDCs. At the same time, adding IL-4 to the cytokine cocktail enriches cDC populations, which can respond to inflammatory stimuli (369). Choi *et al.* reported a system to generate hematopoietic progenitors with an elevated myeloid potential to differentiate into neutrophils, eosinophils, macrophages, and dendritic cells (370). Sachamitr *et al.* approach utilized different adherence surfaces, growth factors, and cytokines supplements, ultimately resulting in CD141+ dendritic cells resembling circulating cDC1 populations (371). These iPSCs-derived CD141+ cDC1 were endowed with co-stimulatory phenotype upon TLR activation, antigen presentation capacity, and priming of naïve T cells. On the other hand, these cells could not secrete IL-12 in large amounts and displayed tolerogenic properties resembling tissue-resident dendritic cells. Furthermore, dendritic cells resulting from iPSCs differentiation protocols showcase arrested development in a fetal-like phenotype, with many characteristic markers of adult dendritic cells being expressed at low levels and stunted antigen presentation capacity (364, 371). To circumvent these drawbacks, iPSCs can be obtained from cell reprogramming of peripheral cDC1 and differentiated back to a cDC1 phenotype, improving the rejuvenated dendritic cells' properties (372).

Furthermore, the original iPSCs can be modified to express TAAs such as carcinoembryonic antigen (CEA), thus conferring any differentiated dendritic cell capable of expressing and presenting these antigens. Indeed, this was tested in a subcutaneous mouse model for colon cancer expressing CEA. Immunization of mice with iPSCs-CEA-derived dendritic cells resulted in activation of CEA-specific CD8+ T cells, which exerted cytotoxic activity against MC38 colorectal carcinoma cells expressing CEA. Additionally, immunized mice demonstrated a higher

capacity to control tumor growth, and tumor tissues had significant infiltration of CD8+ T cells compared to non-vaccinated mice and other controls while not suffering from adverse events caused by the vaccination (373).

The generation of dendritic cells through direct reprogramming would elucidate the lineage instructing transcription factors to impose a dendritic cell phenotype and pave the way for developing the next generation of dendritic cell-based vaccines. Furthermore, identifying the gene networks regulating other immune cell identities will provide an invaluable source for rare populations crucial for cancer immunotherapy.

Reprogramming cancer cells into pluripotency.

In the context of cancer treatment, cell reprogramming strategies might not be limited to the generation of immune cells to advance cancer immunotherapy. Inducing pluripotency and transdifferentiation protocols can also be leveraged to revert the oncogenic fate and restart benign programs in cancer cells. Carcinogenesis involves transcriptional and epigenetic changes that share roots with the necessary steps required for reprogramming (374). Chromatin remodeling, histone modification, DNA methylation, and overexpression and repression of distinct transcription factors are mirrored in malignant transformation and cellular reprogramming. The parallels between both processes highlight the potential for cancer cells to share the same cellular plasticity that somatic cells enjoy and be amenable to reprogramming (55). Fusion between fibroblasts and mouse cancer cells attenuated malignancy as hybrids failed to induce tumors in mice 91% of the time (375). The nuclear transfer of melanoma cancer cells into oocytes generated embryonic stem cells capable of generating teratomas. Moreover, these embryonic stem cells could generate chimeric mice, albeit with a higher predisposition for cancer (376). These experiments suggested that nuclei of cancer cells can be reprogrammed to pluripotency and differentiate in multiple benign states depending on their cellular context. Therefore, transcription factor-based lineage conversion can provide opportunities to reverse tumorigenic potential after terminal differentiation (377) and, on a basic research level, contribute to our knowledge of cancer heterogeneity, cancer stem cells, and oncogenic drivers underlying different malignancies as cancer-derived iPSCs clones retain their mutational signature (378).

In the last fifteen years, several groups reported the cell reprogramming of various cancers to pluripotency (**Figure 7**). Overexpression of the complete OSKM combination or smaller sets induced pluripotency in melanoma (379, 380), blood-related malignancies (378, 381-383), gastrointestinal cancer cells (384), pancreatic cancers (385), sarcomas (377), bladder and breast carcinomas (386, 387) and glioblastomas (388, 389). Despite the general low efficiency observed across studies (390), differentiation of cancer-derived iPSCs into benign phenotypes was possible even in the presence of constitutive oncogenic drivers. For example, iPSCs originating from BRAF-mutated melanomas could be differentiated in fibroblastic

and neuronal cells, whereas myeloblast-derived iPSCs harboring BCR-ABL fusion protein could give rise to engraftable hematopoietic cells (379, 382). These studies and the strong epigenetic nature of cancer have highlighted how the tumorigenic program can be rewritten into pluripotency and further shaped into multiple unrelated terminally differentiated phenotypes. However, in certain cases, differentiation towards the original identity can reinstate the original tumorigenic potential. Teratoma differentiation of pancreatic cancer-derived iPSCs resembled the development of pancreatic intraepithelial neoplasia and progressed toward invasive human pancreatic cancer (385). Hematopoietic progenitors from AML-iPSCs demonstrated enhanced proliferative potential and specialization bias towards myeloid lineage followed by reactivation of the aberrant transcriptional and epigenetic AML-specific signatures (383).

Despite the common observation that reprogramming cancer cells towards pluripotency attenuates tumorigenicity upon redifferentiation, the gene network and subsequent transcriptional and metabolic changes triggered by iPSCs reprogramming have been implicated in carcinogenesis. Moreover, incomplete reprogramming and pluripotent cells contamination hinders the full adoption of the method without unequivocal dissipation of safety, specificity, and efficiency concerns (390-392). In contrast, direct reprogramming strategies may overcome cell reprogramming main limitations while modifying somatic cell identities and disrupting oncogenic drive (55).

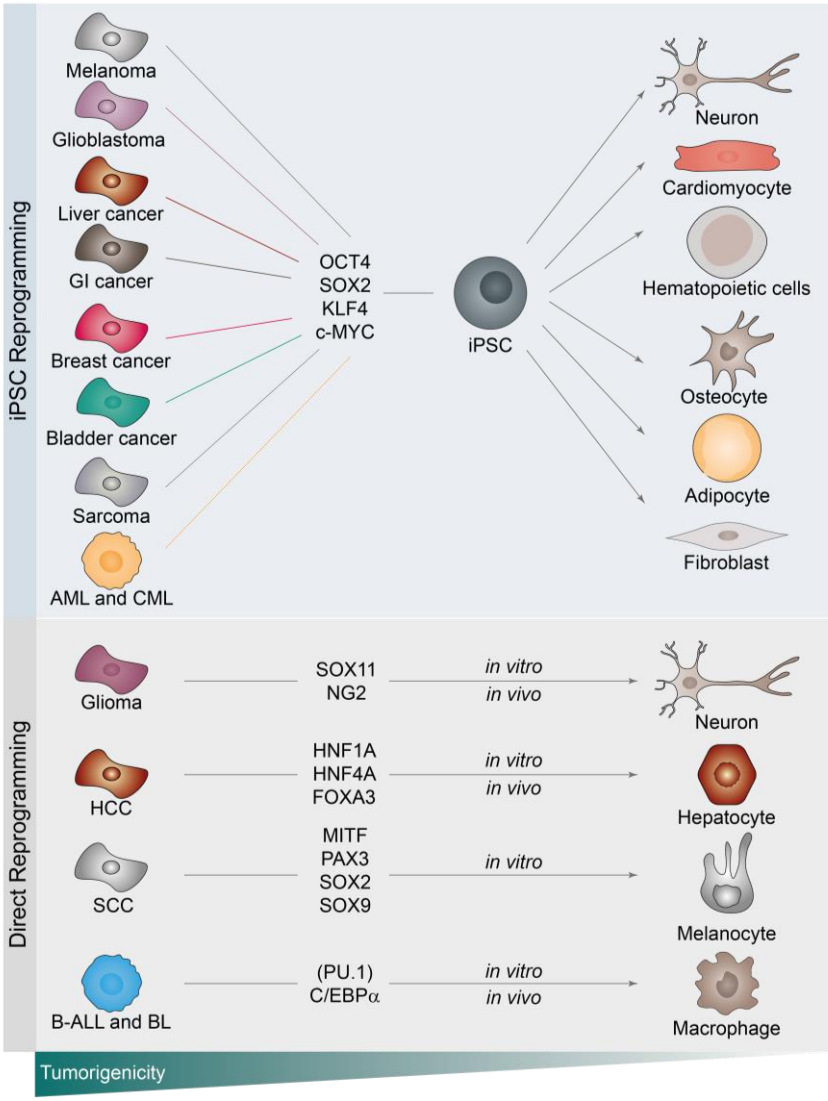

Figure 7 | Cancer cell reprogramming strategies. Several cancer cells from melanoma, glioblastoma, sarcoma, liver, gastrointestinal, breast, and bladder cancer, as well as blood malignancies, have been successfully reprogrammed towards induced pluripotent stem cells (iPSCs) by overexpression of OCT4, SOX2, KLF4, and c-MYC. Differentiation of cancer-derived iPSCs to their original programs or different cell fates generally leads to loss of tumorigenicity in the final cell identity. Direct reprogramming of cancer cells is also possible by overexpressing lineage-defining factor that can epigenetically and transcriptionally rewrite the oncogenic program and reinstate the original benign function of the cell that initiated the cancer (glioma to neuron, hepatocellular carcinoma (HCC) to hepatocyte or squamous cell carcinoma (SCC) to melanocyte). Moreover, lineage-specific factors can also alter cell fate in cancer cells, endowing them with a benign identity that can also have functional properties for immunotherapy (reprogramming towards macrophage). B-ALL – B cell acute lymphocytic leukemia; BL – B cell lymphoma; GI – gastrointestinal. Adapted from Zimmermannova, Caiado (55).

Instructing benignity in cancer cells through direct cell reprogramming.

Direct reprogramming through lineage-instructive transcription factors was also shown to impose a terminal cell identity in cancer cells while installing a benign cell program (**Figure 7**). Human glioma cells can be forced to undergo differentiation to neuron-like cells and enter post-mitotic arrest by overexpression of NGN2 and SOX11. Importantly, transplantation of reprogrammed cancer cells into the striatum of immunodeficient mice failed to generate tumors, suggesting loss of tumorigenic potential. Intratumoral delivery of lentiviral particles encoding NGN2/SOX11 reduced tumor burden and extended survival of treated mice (393). A SCC cell line was successfully transdifferentiated into melanocytes through ectopic expression of MITF, PAX3, SOX2, and SOX9. This process also resulted in loss of tumorigenic drive in the original cancer cells, with mitotic arrest, and failure to produce viable tumors in mice (394). HNF1A, HNF4A, and FOXA3 imposed the unique hepatocyte phenotype in liver cancer cell lines, endowing reprogrammed cancer cells with hepatocyte-like morphology and function. Cancer-derived hepatocytes acquired typical features of mature hepatocytes and metabolized drugs. Cell- and patient-derived xenografts of liver cancer could be reprogrammed towards functional hepatocytes *in vivo* while transplanted cancer-derived hepatocytes engrafted diseased livers without signs of tumorigenesis (395).

While these studies have shown successful transitions towards benignity exploiting direct cell reprogramming strategies, it is also important to note that not all cell identity conversions will result in loss of tumorigenic potential. This has been observed during the transdifferentiation of pro-B leukemic cells towards T cell lineage. The resulting cells resembled early T precursors, although their real identity could not be assigned to any naturally occurring developmental stage. Importantly, these T-like cells retained the original malignant phenotype and progressed as T lineage leukemia (396).

Inducing immune cells.

Inducing certain cell types, like neurons from glioblastomas, would require that all tumor cells convert to the wanted cell fate, integrate into the damaged tissue, survive, and acquire long-term functionality. These requirements set an unattainable standard for cancer cell reprogramming. In the case of immune cell identities, long-term engraftment and function might not be limiting factors. For example, professional APCs must only live long enough to present the newly acquired antigens or transfer them to other APCs to mount a robust immune response that can be maintained in memory T and B cells and plasma cells (133). Moreover, the loss of antigen presentation machinery is one of the mechanisms underlying immune evasion, as stressed throughout the first part of this introduction. Thus, restoration

of antigen presentation and, in turn, reinstalment of tumor immunogenicity holds immense potential to rebuild antitumor immune responses.

Until now, the macrophage was the only immune cell with lineage-instructing factors deciphered and shown to impose a defined cell identity in unrelated somatic cells through direct reprogramming. The combination of PU.1 and C/EBPα transdifferentiated a wide range of B cell lymphoma (BL) and leukemia cells into macrophage-like cells (**Figure 7**). The results showed downregulation of CD19 and increased MAC-1 expression mirroring transcriptomic changes that led to the acquisition of a phagocytic phenotype and loss of tumorigenicity (397). In the same vein, McClellan *et al*. successfully reported the direct reprogramming of primary human B-ALL cells into macrophage cells using the same combination of transcription factors, which reduced their leukemogenicity (398). Once the macrophage identity was established after 7 days of reprogramming, reprogrammed cancer cells no longer depended on exogenous factors as they had already activated the essential macrophage regulatory network to preserve cell identity (397).

However, all these studies' main objective was to instruct benignity independently of the target cell fate. Additionally, macrophages have a dual role in cancer, and the stability of tumor-derived macrophages can become compromised within the TME. As such, there is still a gap in the cell reprogramming field to bridge the induction of benignity and functional reprogramming in cancer cells. The identification of other professional APCs lineage-restrictive networks that can induce antigen presentation, potentiate IFN signaling pathways, and boost antitumor responses remains unexplored. To this end, cDC1 harbors the ideal phenotype for professional APCs, combining antigen presentation and cross-presentation capacity and cytokine secretion that make them excellent for T cell activation. In addition, the well-characterized phenotype of dendritic cells like cDC1 can be leveraged to profile reprogrammed cells upon identifying the gene network regulating their lineage identity. As such, I hypothesize that identifying the lineage instructing factors for cDC1 identity will pave the way for novel immunotherapies based on direct reprogramming strategies.

AIMS

1. Identify the minimal transcription factor combination able to enforce a cDC1-like identity in somatic cells (Study I).
2. Employ single-cell transcriptomic analysis to understand the dendritic cell reprogramming process and improve reprogramming efficiency (Study II).
3. Explore dendritic cell reprogramming factors to induce antigen presentation in mouse and human cancer cells (Studies III and IV).
4. Restore antitumor immunity with cDC1 reprogramming strategies (Studies III and IV).

SUMMARY OF RESULTS

Antigen presentation is a keystone mechanism in the cancer immunity cycle, as failure to recognize cancer antigens can result in uncontrolled tumor growth. Tumors with low expression of antigen presentation molecules correlate with inadequate immune infiltration and unfavorable immunotherapy outcomes.

In the past decade, researchers have shown that somatic cells can be reprogrammed to other cell identities by overexpressing lineage-restricted transcription factors. While direct reprogramming strategies have been employed in cell therapy and regenerative medicine, I envision its application to manipulate immune responses, especially against cancer.

In the first two studies (**Study I and II**), I participated in the identification and characterization of the minimal combination of transcription factors that converts a somatic cell into an induced dendritic cell (iDC). We have shown that this process is conserved between mouse and human fibroblasts and uncovered the crucial role of PU.1 while establishing a cDC1 program in unrelated cell types.

In **Studies III and IV**, I hypothesized that cDC1-reprogramming could be used to manipulate the immune system in the context of cancer. I showed in-depth characterization of PIB-reprogrammed murine and human cancer cells, which I called tumor-APCs. I have also demonstrated that dendritic cell-reprogramming factors overwrite the original tumorigenic transcriptional profile in favor of a gene signature commonly shared between cDC1, iDC, and tumor-APC.

Finally, I demonstrated that reprogrammed cancer cells can elicit anti-tumor immunity *in vivo*, potentially delaying tumor growth. This chapter summarizes and highlights the key findings from each study, laying the groundwork for this dissertation.

DIRECT REPROGRAMMING OF SOMATIC CELLS TO INDUCED DENDRITIC CELLS (STUDY I AND II)

Identification of PU.1, IRF8, and BATF3 as cDC1-lineage instructors.

Engineering different cell fates in somatic cells requires strong transcription factor candidates for the desired cell lineage. Transcription factors highly expressed in the desired cell fate and loss, or gain-of-function studies provide insights into the importance of each transcription factor during cell specification and development, offering invaluable information regarding lineage-instructing factors.

To identify such candidates for cDC1 lineage, we employed two different strategies. First, gene expression profiles of dendritic cell populations in humans and mice were compared to a broad range of other cell types and tissues, generating an unbiased list of the most enriched transcription factors restricted to dendritic cell-fate. After obtaining a list of potential reprogramming factors, we verified their expression within the dendritic cell compartment compared to other hematopoietic cells for reference. To complement this information, literature mining was used to determine the role of potential candidates in dendritic cell specification and development by looking at loss-of-function studies in mouse models. These two strategies allowed the identification of eighteen potential transcription factors that could comprise the minimal gene network necessary to instruct cDC1-lineage.

To assess whether these candidates could assert a cDC1-fate in unrelated cell types, a mouse reporter model that informs the appearance of dendritic cell-related cells was required. CLEC9A is a necrosis-sensing receptor whose expression demarks commitment towards the dendritic cell lineage (150, 151). In this model, the CLEC9A drives the expression of tdTomato through a Cre-based system, permanently labelling cells where CLEC9A has been activated (**Figure 8A**). MEFs from this mouse model were transduced with lentiviral particles encoding the eighteen transcription factors that were identified before, alone or in combinations (**Figure 8A**). Minus one experiments narrowed down the combination of transcription factors to induce a dendritic cell-fate in unrelated somatic cells to be PU.1 together with IRF8, and BATF3 (**Figure 8B**). The ectopic expression of the three-factor cocktail triggered CLEC9A expression (**Figure 8B**), and removing any transcription factor from this pool abolished tdTomato (**Figure 8C).** Thus, PU.1, IRF8, and BATF3 emerged as the minimal necessary and sufficient transcription factor network to instruct dendritic cell-lineage. Moreover, reporter activation is sustained until 15 days of reprogramming (**Figure 8D**).

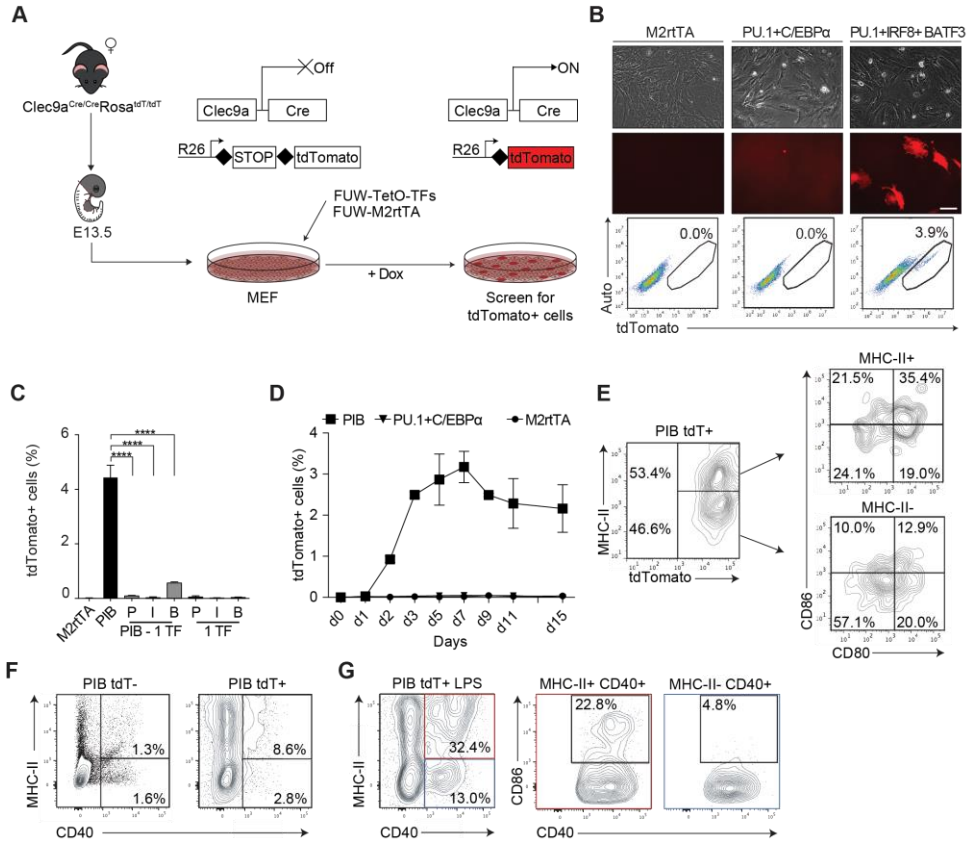

Figure 8 | Identification of PU.1, IRF8, and BATF3 (PIB) as instructors of cDC1 phenotype in mouse fibroblasts. (A) Schematic representation of the strategy employed to screen for cDC1-inducing factors. Clec9a-tdTomato mouse embryonic fibroblasts (MEFs) collected at embryonic day 13.5 were transduced with lentiviral particles encoding for transcription factors (TF) under a tetracycline-inducible system (TetO). Transduced MEFs were cultured in the presence of doxycycline (Dox) and screened for tdTomato expression based on Clec9a activation. **(B)** MEFs transduced with reverse tetracycline transactivator (M2rtTA), macrophage-inducing factors PU.1 and CEBPα or PIB were analyzed by fluorescence microscopy and flow cytometry for tdTomato detection 5 days post-Dox induction. Scale bar: 200 μm. **(C)** Quantification of the percentage of tdTomato+ cells 5 days post-Dox induction by flow cytometry. MEFs were transduced with a three-factor pool (PIB), a two-factor pool after each factor was removed from the original (PIB – 1TF), or individual factors (1 TF). (n=3) **(D)** Flow cytometric analysis of tdTomato expression kinetics over 15 days of continuous Dox supplementation. **(E)** Co-expression of tdTomato and MHC-II was assessed through flow cytometry at day 5. Co-expression of co-stimulatory molecules CD80 and CD86 were also evaluated within MHC-II+ and MHC-II- populations. **(F)** Co-expression of CD40 and MHC-II in tdTomato- (tdT-) or tdTomato+ (tdT+) in PIB-transduced MEFs at day 7 post-Dox supplementation. **(G)** PIB-transduced MEFs were stimulated with lipopolysaccharide (LPS) overnight before flow cytometry acquisition to assess the co-expression of MHC-II, CD40, and CD86. Mean±SD is represented. p-value: ****p<0.0001.

Inducing an APC immunophenotype through ectopic expression of PIB.

Professional APCs engage T cells through peptides complexed with MHC molecules that bind to specific TCR that recognizes the peptide, constituting the first signal for naïve T cell activation (28). However, co-stimulatory molecules are necessary to make sure the T cell is appropriately engaged in antigen recognition. Lack of this secondary signal can result in anergic T cells (27). The presence of CD80 and CD86 ensure a quick T cell activation but also provide a safety net for response inhibition, as both ligands bind CTLA-4, necessary for T cell downregulation. Moreover, CD40 is another essential co-stimulatory molecule that modulates T-cell priming and differentiation (28).

Approximately half of the tdTomato+ population express MHC-II, within which three-quarters express CD80, CD86, or both (**Figure 8E**). We also observed that reprogrammed MEFs upregulated CD40 (**Figure 8F**) and further increased its expression in response to stimulation with lipopolysaccharide (LPS) from bacterial origin (**Figure 8G**). Overexpression of PU.1, IRF8, and BATF3 in an unrelated cell type, such as MEFs, activates the dendritic cell-lineage restricted marker, CLEC9A, and triggers the expression of essential molecules for antigen presentation, such as MHC-II and co-stimulatory molecules.

Overexpression of PIB endows somatic cells with dendritic cell-abilities.

Professional APCs scavenge the host's organism for pathogens and foreign antigens to present them to T cells (133, 175). cDC1 are especially apt at screening the environment for free-roaming proteins and necrotic bodies (150, 151, 175). tdTomato+ cells acquire competency at sensing and uptaking death cells and ovalbumin (OVA) (**Figure 9A, B**).

I have discussed the importance of proper antigen presentation through MHC-TCR engagement and co-stimulatory signaling to ensure T cell priming and differentiation (28). Importantly, cytokine secretion by APCs is the third pillar of T cell conditioning, which directs differentiation toward the appropriate immune response depending on the type of pathogen. As mentioned before, cDC1 are excellent antigen cross-presenters, and the cytokines produced after antigen encounters provide optimal CD8+ T cell cytotoxic responses and direct CD4+ T cells towards a Th1 phenotype via both type 1 IFN and IL-12 secretion (179, 188). We detected pro-inflammatory cytokines in supernatants collected from cultured tdTomato+ cells, including the intracellular presence of IL12p40 after TLR stimulation (**Figure 9C, D**).

After confirming antigen uptake and cytokine production, we assessed whether tdTomato+ cells could prime and sustain naïve T cell activation. Standard assays for antigen presentation take advantage of mice whose populations of T cells express

only one TCR. This TCR is specifically targeted to an antigen, and T cells can only be primed after APCs exhibit this antigen in the correct MHC molecule. OT-I and OT-II strains have CD8+ and CD4+ T cells that only recognize OVA 257-264 (SIINFEKL) or OVA 323-339, respectively. After feeding tdTomato+ cells with processed peptides from OVA 323-339 or total protein, we observed CD4+ T cell activation and expansion by the presence of CD44 and carboxyfluorescein succinimidyl ester (CFSE) dilution (**Figure 9E, F**). These data indicate that reprogrammed cells can uptake exogenous proteins, process and load peptides into MHC-II, exhibit them at the cell surface, and prime naïve CD4+ T cells.

Another feature that is fundamental to characterize cDC1 is antigen cross-presentation (158). We evaluated antigen cross-presentation by pulsing reprogrammed tdTomato+ cells with OVA before co-culturing these cells with OT-I CD8+ T cells. After 3 days of co-culture, we could observe CD8+ T expansion, as indicated by CellTrace Violet (CTV) dilution and CD44+ expression, indicating that tdTomato+ cells could prime naïve T cells through cross-presentation (**Figure 9G, H**).

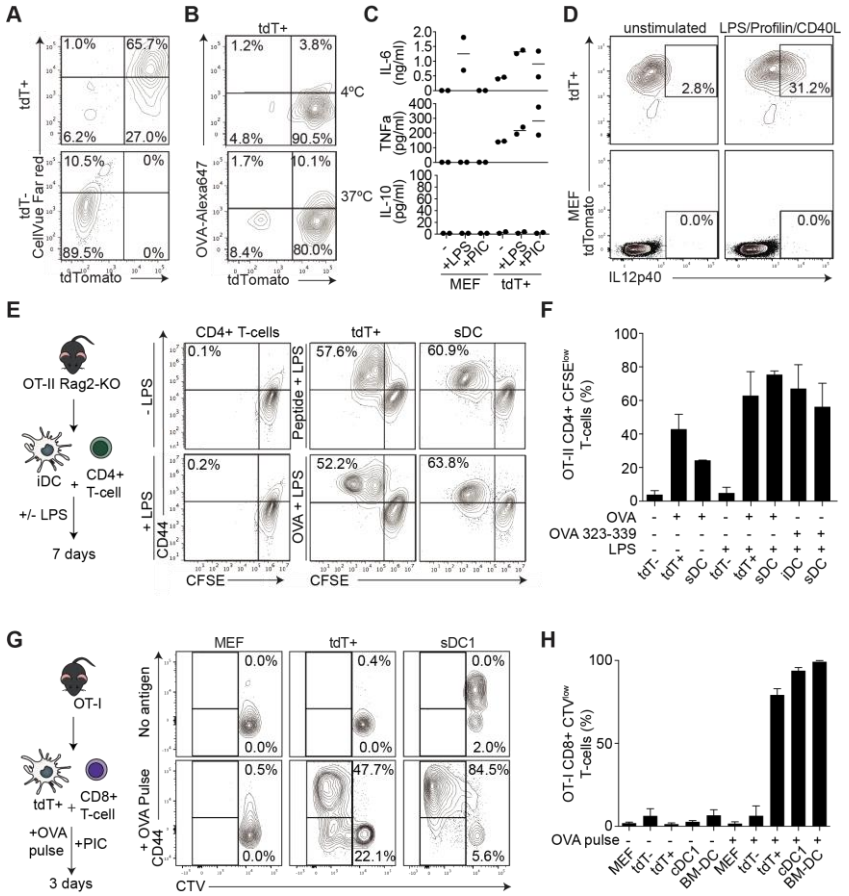

Figure 9 | Functional reprogramming of MEFs to APC. (A) tdTomato negative (tdT-) and positive (tdT+) cells were pulsed with dead cells labeled with CellVue-Far red. Uptake was detected by the presence of CellVue within the tdTomato- and tdTomato+ population by flow cytometry. **(B)** tdTomato+ cells were cultured in the presence of ovalbumin-AlexaFluor 648 (OVA-Alexa647), and uptake was measured by flow cytometry. **(C)** Cytokine secretion of IL-6, TNFα , and IL-10 by MEFs and tdTomato+ cells with and without LPS and polyinosinic:polycytidylic acid (Poly(I:C); PIC) stimulation**. (D)** Expression of IL12p40 was measured by flow cytometry in MEFs and tdTomato+ cell before and after stimulation with LPS/profilin/CD40L. **(E)** CD4+ T cells from OT-II Rag2-KO mice were labeled with CFSE and co-cultured in the presence of tdTomato+ or splenic dendritic cells (sDC). After 7 days of co-culture in the presence of ovalbumin (OVA) or processed peptide and LPS, CFSE dilution and CD44 expression were measured as a synonym of T cell expansion and activation. **(F)** Quantification of $CFSE^{low}$ population within OT-II CD4+ T cells after co-culture with tdTomato-, tdTomato+, or sDC with or without LPS stimulation. **(G)** and **(H)** OT-I CD8+ T cells were labeled with CellTrace Violet (CTV) and co-cultured with MEFs, tdTomato+, tdTomato-, splenic conventional dendritic cell type 1 (cDC1) or CD103+ bone-marrow-derived dendritic cells (BM-DC) for 3 days after the short pulse with ovalbumin (OVA). T cell activation was measured by flow cytometry and quantified as CTV dilution and CD44 expression. Mean±SD is represented.

Polycistronic vectors increase reprogramming efficiency and generate cDC1-like cells with high fidelity.

Reprogramming somatic cells towards iPSC was first reported to have low efficiencies (301, 302). Poor recovery yield could be attributed to the necessity of transducing somatic cells with multiple viral transcripts (399). Given the potential use of iPSCs in regenerative medicine, this strategy quickly raised concerns regarding the high risk of insertional mutagenesis, the potential to activate proto-oncogenes in progeny cells, and the reactivation of silent viral transcripts (399, 400). Therefore, there was an effort to develop better tools to deliver transcription factors more efficiently and safely (303, 309, 399-401). Polycistronic cassettes ensure that transduced cells get the complete combination of transcription factors in one plasmid resulting in higher reprogramming efficiencies (309, 400).

I hypothesized that the same strategy could be applied to cDC1 reprogramming. I generated the polycistronic cassette containing PU.1, IRF8, and BATF3 separated by self-cleaving 2A peptides (**Figure 10A**). Previously, it was reported that the order in which sequences were introduced and the order and type of 2A sequences used impacted expression levels and, subsequentially, reprogramming efficiencies (401, 402). I took advantage of the systematic comparison from Liu *et al.* (2017) to decide the final sequences to use in our constructs and the desired placement to introduce them. I finally developed two cassettes differing only by PU.1 and IRF8 position. Both cassettes have P2A and T2A sequences between factors, expressing BATF3 in the third position. When comparing the reprogramming efficiency, polycistronic vectors induced more cells expressing Clec9a-tdTomato than pooled individual factors (**Figure 10B**). The tricistronic cassette for PU.1, IRF8, and BATF3 had a higher yield in both tdTomato+ and MHC-II expression than the second construct or the individual factors (**Figure 10C**). We then confirmed that the position in the vector influenced protein expression levels for PU.1 and IRF8, with the first position in the vector correlating with higher expression levels (**Figure 10D**). These data indicated that high levels of PU.1 are crucial for successful cDC1 reprogramming and that polycistronic vectors ensure an efficient way of generating iDC.

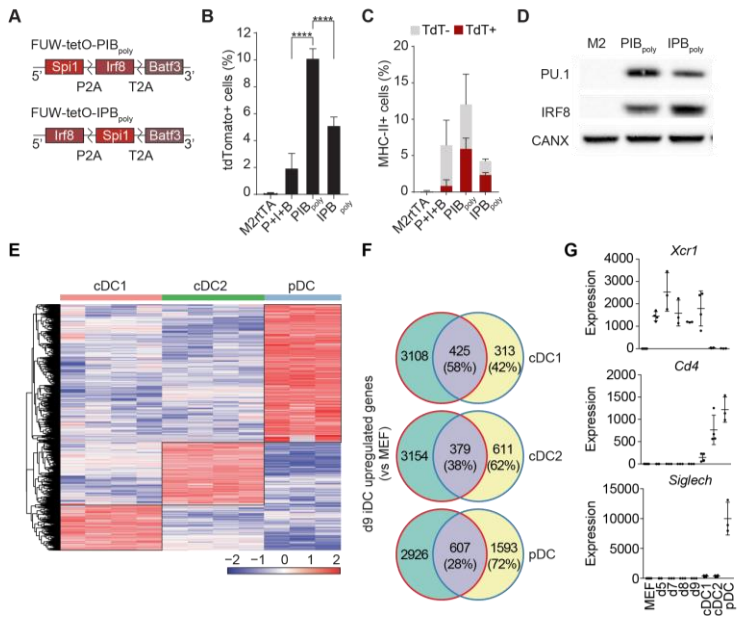

Figure 10 | Imposing a cDC1 fate through polycistronic vectors. (A) Sequences for *Spi1*, *Irf8*, and *Batf3* were inserted sequentially interspaced with 2A self-cleaving peptides in two different placement orders, PIBpoly and IPBpoly. **(B)** Comparison of reprogramming efficiency given by % of tdTomato+ (tdT+) cells after overexpression of individual factors or polycistronic vectors. **(C)** MHC-II expression in tdTomato- (tdT-) and tdTomato+ cells after reprogramming with individual factors, PIBpoly, or IPBpoly. **(D)** Protein expression for PU.1, IRF8, and calnexin (CANX) at day 5 after MEFs were transduced with M2rtTA (M2) or polycistronic vectors, PIBpoly and IPBpoly. **(E)** Heatmap for differentially expressed genes between splenic cDC1, cDC2, and pDC. **(F)** MEFs transduced with PIBpoly were purified by FACS for tdTomato+ MHC-II+ CD45+ and subjected to population mRNA-sequencing (mRNA-seq) at days 5, 7, 8, and 9 of the reprogramming process along with MEFs, splenic cDC1, cDC2, and pDC. Venn diagrams show the intersection between upregulated genes in induced dendritic cell (iDC), cDC1, cDC2, and pDC. **(G)** Gene expression for *Xcr1*, *Cd4*, and *Siglech* as examples for genes specific for cDC1, cDC2, and pDC, respectively. Mean±SD is represented. p-value: ****p<0.0001.

Finally, I compared the gene signatures from splenic cDC1, cDC2, and pDC with iDC to evaluate whether direct reprogramming could impose a faithful cDC1 identity in MEFs. Indeed, iDC shares a higher percentage of identity with cDC1 than with cDC2 and pDC (**Figure 10E, F**). This was also confirmed by RNA expression for genes that define cDC1, cDC2, and pDC populations, such as *XCR1*, *CD4*, and *Siglech* (**Figure 10G**).

In summary, for **Study I**, I have helped establishing the minimal transcription factor network that converts cell fate towards a cDC1-lineage, endowing unrelated cells with APC machinery and functional properties (403).

Constitutive promoters potentiate dendritic cell reprogramming with higher lineage fidelity.

As mentioned earlier, it has been shown that polycistronic cassettes overcome reprogramming obstacles and increase reprogramming efficiency. However, we also observed incomplete reprogrammed cells whose gene signature is closer to the original cell state (404). These findings are consistent with previous studies stating

that, during successful reprogramming, cells respond by downregulating the original program. Still, unsuccessful reprogramming generally results from regression toward their original state (405-407). Therefore, it was hypothesized that more robust expression levels of reprogramming factors could lead to higher efficiency and irreversible rewriting of cellular identity. To evaluate this hypothesis, the PU.1, IRF8 and BATF3 cassette was introduced into various lentiviral vectors with different constitutive promoters followed by assessment of their reprogramming efficiency of Clec9a-tdTomato MEFs and human embryonic fibroblasts (HEFs) (**Figure 11A-D**). Overexpression of PU.1, IRF8, and BATF3 under spleen focus-forming virus (SFFV) showed remarkable efficiency in reprogramming MEFs towards a cDC1 cell fate when compared to other constitutive promoters and the original dox-inducible vector (**Figure 11A, B**). This result agrees with prior studies where overexpression of the OSKM factors under the SFFV promoter increased iPSC reprogramming (408). In HEFs, we observed a strikingly higher percentage of a CD45+HLA-DR+ population in SFFV-PIB reprogrammed cells, approximately 21%, compared to the nearly null population acquired with tetO-PIB (**Figure 11C**). Pro-inflammatory cytokines further enhanced the maturation of reprogrammed HEFs (**Figure 11D**).

To dissect the extent of the reprogramming process between the different strategies, we used a predictive algorithm (scPred) to affiliate the gene signatures of the reprogrammed HEFs with the corresponding dendritic cell type (**Figure 11E**). ScPred can identify weighted gene signatures after being trained on a single-cell RNA-sequencing (RNA-seq) dataset where the identity of the cells is known. The query samples are then evaluated against the training dataset and affiliated to a determined population if the gene signatures match. Considering this, we used the dataset Villani *et al*. (2017) generated, where they identified new dendritic cell gene signatures and progenitors (189), to train the algorithm and compare it to HEFs-derived iDC.

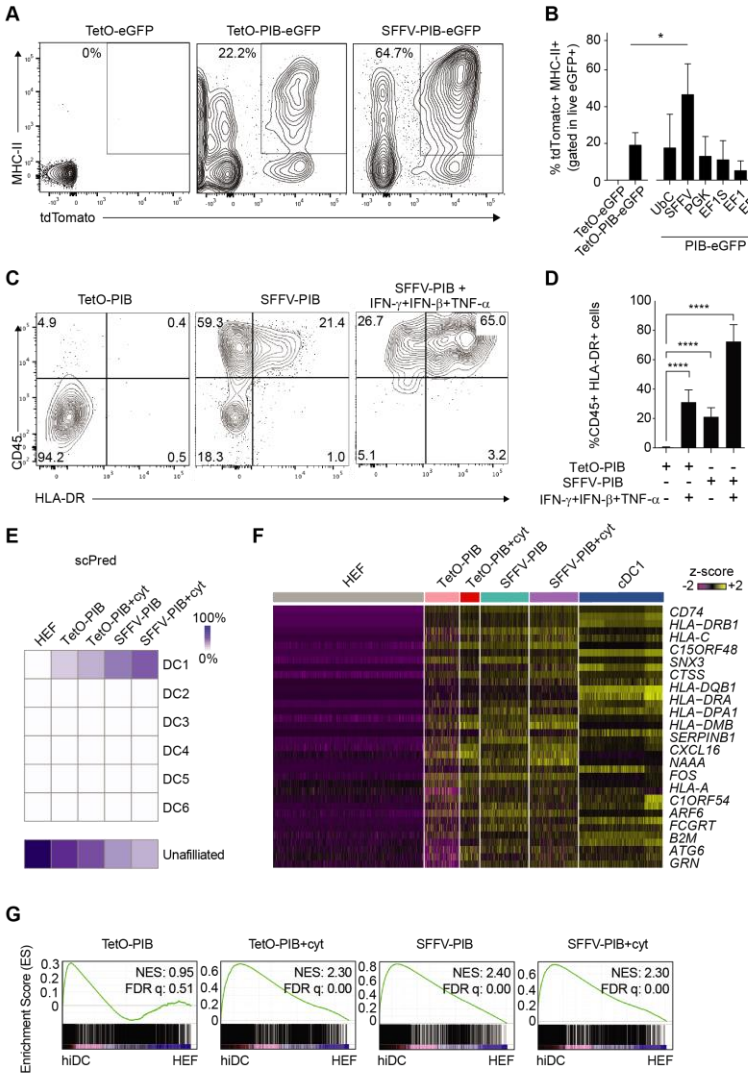

Figure 11 | Constitutive expression of PU.1, IRF8, and BATF3 (PIB) improves reprogramming fidelity towards cDC1-lineage. (A) Clec9a-tdTomato MEFs were transduced with polycistronic vectors coding for eGFP or PIB-eGFP and characterized by the expression of tdTomato and MHC-II by flow cytometry at day 9 of reprogramming. **(B)** Quantification of reprogrammed tdTomato+ MHC-II+ cells after Clec9a-tdTomato MEFs were transduced with PIB-eGFP under dox-inducible (TetO) or constitutive promoters (UbC, SFFV, PGK, EF1S, EF1, and EF1i). **(C)** HEFs were transduced with PIB under TetO and SFFV promoters in the presence or absence of pro-inflammatory cytokines, IFN-γ, IFN-β , and TNF-α. Reprogramming efficiency was measured at day 9 by expression of a hematopoietic marker, CD45, and professional APC marker, HLA-DR, by flow cytometry. **(D)** Quantification of CD45+ HLA-DR+ population in HEFs-derived hiDC after overexpression of PIB as mentioned in **C**. **(E)** HEFs-derived hiDC were sorted and profiled by single-cell RNA-Seq. Heatmap shows affiliation probability of hiDCs, generated under different conditions, towards different human dendritic cell subsets. **(F)** Heatmap shows the expression of commonly upregulated genes between hiDCs generated under different conditions and cDC1 gene signature. **(G)** Gene Set Enrichment Analysis (GSEA) was performed for genes implicated in successful cDC1 reprogramming. Normalized enrichment score (NES) and false discovery rate (FDR) are shown. Mean±SD is represented. p-value: *p<0.1, ****p<0.0001.

HEFs-derived hiDC from overexpression of PU.1, IRF8, and BATF3 under TetO and SFFV promoters in the presence or absence of pro-inflammatory cytokines, were sorted based on the expression of CD45+HLA-DR+ to be profiled by single-cell RNA-seq. SFFV-derived iDC affiliated with cDC1 gene signature with higher fidelity. This further validated that PU.1, IRF8, and BATF3 drive identity reconfiguration towards the cDC1 lineage in unrelated somatic cells. Cytokine signaling synergized with enforced expression of PU.1, IRF8, and BATF3 to increase overall reprogramming efficiency and frequency of the double positive population for CD45 and HLA-DR (**Figure 11D, E**). This was also evident by the presence of genes participating in IFN signaling and antigen presentation pathways, such as *HLA-A*, *HLA-DRA*, *CD74*, and *B2M* in hiDCs originated from the constitutive expression of PU.1, IRF8, and BATF3 with cytokines compared to hiDCs derived from inducible vectors (**Figure 11F**). Finally, we identified a set of genes characteristic of successful reprogramming (404). hiDCs originating from SFFV-driven expression of PU.1, IRF8, and BATF3 express more genes belonging to IFN, oncostatin M, IL-6, and TNF-α signaling pathways while lacking expression of cell cycle genes (**Figure 11G**).

These results showed that constitutive overexpression of cDC1-lineage specific factors, PU.1, IRF8, and BATF3, impacts reprogramming efficiency and yields better iDC due to higher commitment towards the cDC1 identity.

PIB-driven lineage conversion is independent of the original cell state.

One of the main advantages of direct reprogramming is the possibility of bypassing the pluripotent stem cell state. In practice, it is difficult to eliminate residual and partially reprogrammed pluripotent stem cells after directed differentiation of iPSCs. On the other hand, direct reprogramming protocols have higher efficiency than iPSCs protocols in general, with source material and specificity of the defined factors imposing small limitations. Good cell sources for direct reprogramming are readily available in the human body and require minimally invasive collection methods and handling. Thus, human dermal fibroblasts (HDFs) and mesenchymal stromal cells (MSC) emerge as potential candidates for generating high numbers of cDC1.

To support applicability, we reprogrammed primary HDFs and MSC from three healthy donors for each cell origin using the constitutive promoter that supported high-fidelity iDCs—reprogramming HDFs generated around 20-30% of CD45+ HLA-DR+ cells without cytokines (**Figure 12A**). The pro-inflammatory cytokine cocktail has enhanced reprogramming efficiency in synergetic cooperation with PU.1, IRF8, and BATF3 (**Figure 12B**). Additionally, hiDCs originated from HDFs express co-stimulatory molecules CD40 and CD80, mimicking the reprogramming trajectory studied in MEFs (**Figure 12 C**) (403).

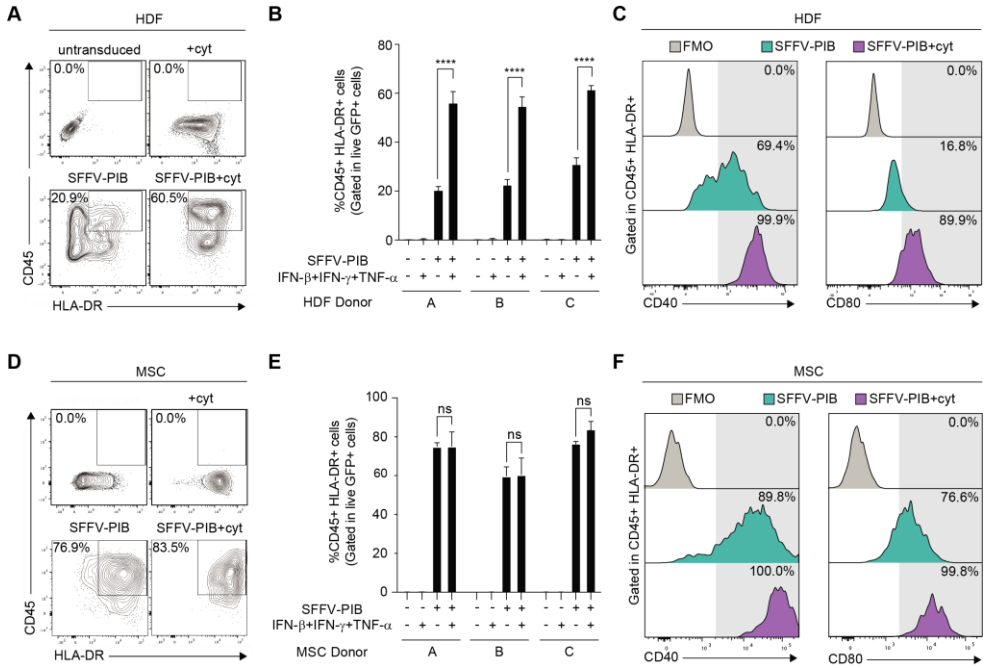

Figure 12 | Reprogramming towards cDC1 lineage is independent of original cell identity. (A) Human dermal fibroblasts (HDFs) were transduced with a constitutive vector encoding the PU.1, IRF8, and BATF3 (PIB) polycistronic cassette. Reprogramming efficiency was measured by the presence of CD45+ and HLA-DR+ populations after 9 days of reprogramming in the presence or absence of the pro-inflammatory cytokines IFN-γ, IFN-β and TNF-α (SFFV-PIB + cyt). **(B)** Quantification of CD45+ HLA-DR+ in hiDCs derived from HDFs from 3 different donors, A, B, and C, before and after reprogramming in the presence or absence of cytokines. **(C)** Expression of co-stimulatory molecules, CD40 and CD80, in reprogrammed HDFs after constitutive expression of PIB without cytokines (green) or with cytokines (purple). **(D)** Reprogramming efficiency of hiDCs derived from human mesenchymal stromal (MSC) cells was assessed by flow cytometry through the expression of CD45+ HLA-DR+ after 9 days of constitutive expression of PIB in the presence or absence of cytokines. **(E)** Quantification of CD45+ HLA-DR+ in hiDCs originated from MSC from 3 different donors, A, B, and C, before and after reprogramming in the presence or absence of cytokines. **(F)** Expression of co-stimulatory molecules, CD40 and CD80, in reprogrammed MSC in the same conditions as stated in **E**. Mean±SD is represented. p-value: ****p<0.0001, ns – not significant.

Furthermore, enforced expression of PU.1, IRF8, and BATF3 was sufficient to efficiently generate CD45+ HLA-DR+ populations in MSC-derived hiDCs. The use of cytokines did not impact the generation of CD45+ HLA-DR+ cells (**Figure 12D, E**), but we observed a stronger median fluorescent intensity (MFI) for co-stimulatory molecules CD40 and CD80 was observed (**Figure 12F**). This suggests that pro-inflammatory cytokines during reprogramming impact the maturation state of hiDCs originating from MSC. However, it also becomes clear that the impact of cytokine signaling in cDC1-lineage conversion can depend on the original cell type and that only some somatic cell states will benefit from it.

In the end, the constitutive expression of PU.1, IRF8, and BATF3 proved its ability to instate a highly committed cDC1 program in different cells, independently

of their species, or original cell state, highlighting the conserved network of factors and downstream elements constituting a cDC1 identity.

Interactions between PU.1, IRF8, and BATF3 in open chromatin determine cDC1 identity.

Reprogramming directly results from successful chromatin changes surpassing epigenetic barriers imposed during cell differentiation. The minimal transcriptional network of PU.1, IRF8, and BATF3 constitutes the backbone of cDC1 identity. Understanding how they cooperate to establish a new cell state will also deepen our knowledge regarding cDC1 development during hematopoiesis. As such, chromatin immunoprecipitation sequencing (ChIP-seq) was performed 2 days after forced expression of PU.1, IRF8, and BATF3 individually, or together **(Figure 13A**).

Individually, PU.1 was the factor that showed the most affinity to bind chromatin, followed by IRF8 and BATF3 (**Figure 13B**). When all three factors were expressed together, the chromatin sites where these factors bind increased more than 2-fold for PU.1, nearly 70-fold for IRF8, and 35-fold for BATF3. This suggests that PU.1 has an independent capacity to bind genomic DNA and facilitates cooperative binding of IRF8 and BATF3 between the three factors. We also saw an overlap of over five thousand peaks bound by all factors (**Figure 13C**) with special relevance in regions of the genome with PU.1-IRF and IKZF motifs (**Figure 13D, E**).

Integration of single-cell RNA-seq data with ChIP-seq data revealed how chromatin modulation can affect transcriptional rewiring of the original cell state in the early stages of reprogramming. It was observed that, together or individually, PU.1, IRF8, and BATF3 can target fibroblast-specific genes to repress them and activate cDC1-specific genes (**Figure 13F**).

Additionally, all transcription factors, individually or in cooperation, seem to have a higher affinity to open chromatin sites like promoters and enhancers (**Figure 13G**). Interestingly, PU.1 alone shows binding capacity to poised transcription start sites in heterochromatin regions, although it heavily favors open chromatin sites. These findings agree with previous studies showing PU.1 ability to redistribute partner transcription factors in lymphoid and myeloid cells depending on prior chromatin status (145).

Together, our results indicate that PU.1 interacts with chromatin first at euchromatin regions, especially in active transcription start sites, and cooperates with IRF8 and BATF3 to repress fibroblast genes and kickstart the cDC1-lineage signature (**Figure 13H**).

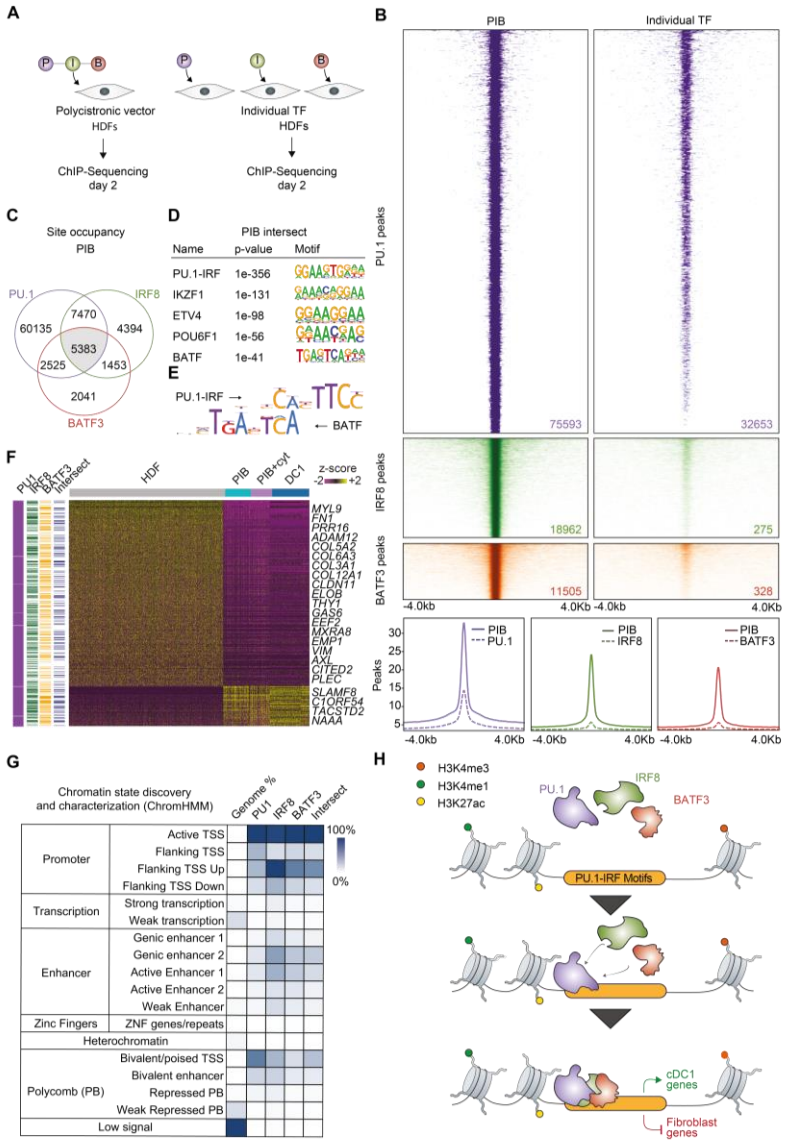

Figure 13 | cDC1 identity requires the interaction between PU.1 and open chromatin. (A) Schematic strategy for profiling chromatin binding sites of PU.1, IRF8, and BATF3 (PIB) in the early stages of reprogramming. Human dermal fibroblasts (HDFs) were transduced with full polycistronic cassette PIB or individual factors. **(B)** Heatmaps show the distribution of PU.1, IRF8, and BATF3 placement across the genome when expressed together (left) or individually (right). The mean peak signal intensity is shown at the bottom. **(C)** Venn diagram for overlapping peaks between PU.1, IRF8, and BATF3. **(D).** *De novo* prediction analysis of motifs occupied by all PIB factors in cooperation. **(E)** Motif comparison between enriched PU.1-IRF interactions and BATF motif. **F.** Heatmap shows differentially expressed genes between HDFs and human induced dendritic cells (hiDCs) at day nine and chromatin binding by individual transcription factors alone or in cooperation at day two of reprogramming. **(G)** Heatmap shows the percentage of occupancy of total PU.1, IRF8 and BATF3 together or individually in different chromatin regions. **(H)** Schematic model for the initiation of cDC1 reprogramming. TSS, transcription start site.

RESTORING ANTITUMOR IMMUNITY WITH DENDRITIC CELL REPROGRAMMING (STUDY III AND IV)

Mouse and human cancer cells can be reprogrammed to cDC1-like cells.

The results of the first two studies in this dissertation established the minimum network necessary to instruct a cDC1 fate in somatic cells, resulting in functional APCs. Loss of antigen presentation machinery is one of the mechanisms underlying immune surveillance failure and one of the most common cancer evasion mechanisms. In **Study III**, I hypothesized that antigen presentation could be imposed in cancer cells by direct reprogramming with PU.1, IRF8, and BATF3, which, in turn, could restore the cancer immunity cycle *in vivo* (**Figure 14A**).

First, I tested our hypothesis in poorly immunogenic mouse cell lines representing lung carcinoma (LLC) and melanoma (B16), which respond poorly to immunotherapy (286, 409, 410). The ectopic expression of PU.1, IRF8, and BATF3 resulted in the emergence of the CD45+ MHC-II+ population in transduced cancer cells (**Figure 14B-D**), which we have used as an indicator of successful reprogramming efficiency based on **Study I**. These markers also represent the acquisition of hematopoietic and professional antigen presentation signatures. Then, I evaluated CLEC9A expression within the CD45+MHC-II+ population of transduced cells. Reprogrammed LLC and B16 express CLEC9A (**Figure 14E**), indicating reprogrammed cancer cells acquire a cDC1-like immunophenotype. Moreover, we could reprogram a broad panel of solid and blood malignancies from human origin (**Figure 14F, G**) (411) with varying degrees of efficiency. The percentage for CD45+ HLA-DR+ cells ranged from 0.2±0.1 to 94.5±7.6%. The observed disparity of reprogramming efficiency across different cell lines coincided with previously reported difficulties in inducing pluripotency and neural cell fate in endodermal-derived cells (305, 411, 412). Human glioblastoma-derived tumor-APCs also expressed cDC1 markers such as CLEC9A, CD226, and CD11c and adopted cDC1-like morphological features such as a stellate shape and formation of dendrites (**Figure 14H, I**).

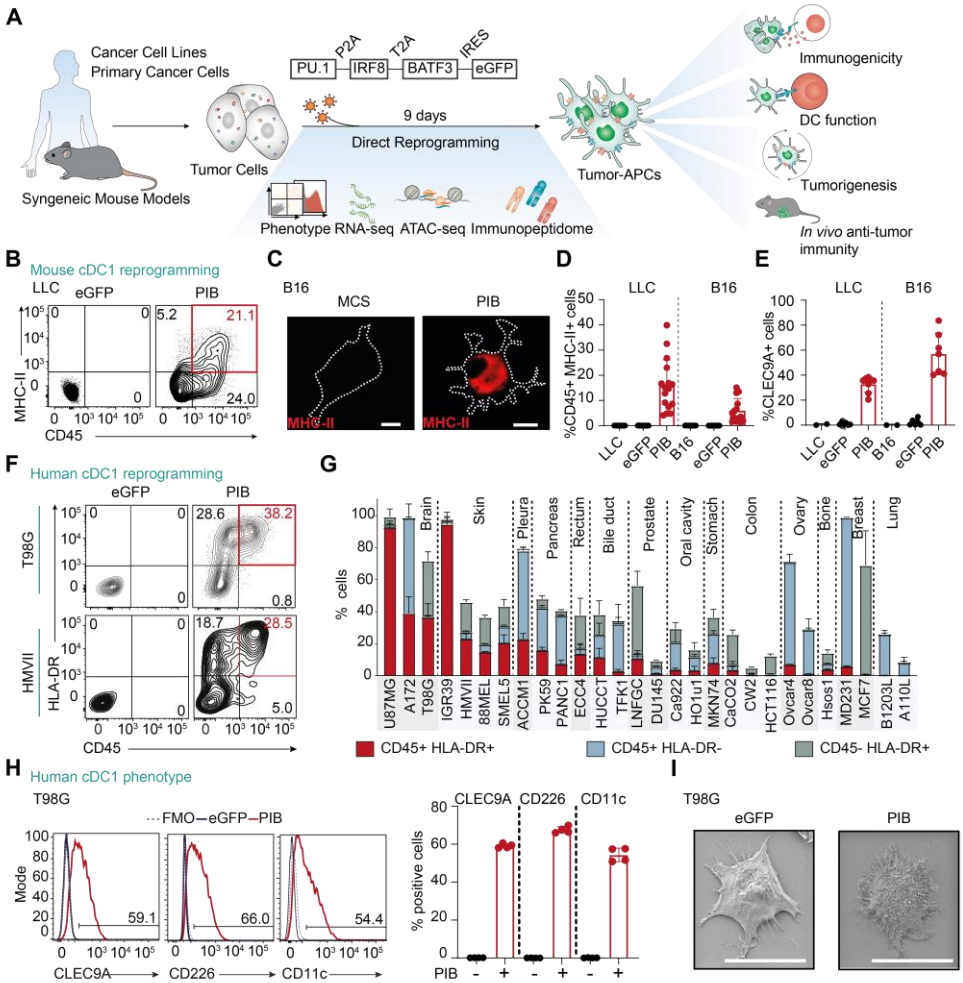

Figure 14 | PU.1, IRF8, and BATF3 (PIB) reprogramming imposes a cDC1 phenotype in cancer cells. (A) Schematic description of the direct cell reprogramming process to induce cDC1-like cells in cancer cells. Cancer cells of mouse and human origin were subjected to the polycistronic vector encoding for the three transcription factors, PIB. Reprogrammed tumor-antigen presenting cells (tumor-APCs) were evaluated *in vitro* and *in vivo*. **(B)** Flow cytometric analysis of murine lung carcinoma cell line (LLC) before (eGFP transduced) and after reprogramming (PIB transduced). Reprogrammed cells acquire CD45 and MHC-II on the cell surface. **(C)** Micrographs showing MHC-II expression in reprogrammed mouse melanoma cells (B16). Scale bars: 20 μm. **(D)** Reprogramming efficiency quantified by the percentage of the emerging double positive population for CD45+MHC-II+ in mouse cancer cells. **(E)** Quantification of CLEC9A+ cells gated in reprogrammed mouse cancer cells. **(F)** Reprogramming efficiency of human glioblastoma (T98G) and melanoma (HMVII) cell lines. **(G)** Quantification of reprogrammed (CD45+HLA-DR+) and partially reprogrammed cancer cells (CD45+HLA-DR- or CD45-HLA-DR+) in 28 human cancer cell lines. **(H)** Expression and quantification of cDC1 specific markers before and after reprogramming of glioblastoma cell line. Fluorescence minus one (FMO) is shown. **I.** Scanning electron microscopy of T98G before and after reprogramming depicting the acquisition of a dendritic cell-like shape. Scale bars: 20 μm. Mean±SD is represented.

To understand the extent of cancer reprogramming to tumor-APCs, I profiled completely (CD45+MHC-II+) and partially (CD45+MHC-II- or CD45-MHC-II+) reprogrammed cancer cells **(Figure 15A**) (411) by population RNA-seq. I compared these populations with mock transduced cancer cells (d0), the previously reported iDC population, and naturally occurring cDC1.

The gene signature of reprogrammed cancer cells clustered closer to natural cDC1 (**Figure 15B**). Importantly, we found gene transcripts for cDC1-specific markers, such as *Clec9a* and *Xcr1*, in mouse and human cancer cell reprogramming systems (**Figure 15C**) (411). I found that overexpression of ectopic PU.1, IRF8, and BATF3 factors increased overall mRNA expression of these factors but also resulted in upregulation of endogenous *Irf8* and *Batf3* (**Figure 15D, E**), which indicates that PU.1, IRF8, and BATF3 impose a stable transcriptomic change in tumor-APCS and the maintenance of acquired cDC1 identity is not dependent on continuous expression of exogenous transcription factors.

The emergence of partially reprogrammed cancer cells, as indicated by the expression of only one of the reprogramming markers, was consistent with previously reported reprogramming processes (367, 392, 403, 404). While this generally means partial retention of the original program, partially reprogrammed cancer cells still show signs of transcriptome overhaul to express a tumor-APC gene signature, which was generated based on cDC1 genes commonly upregulated during reprogramming (**Figure 15F**). Importantly, this gene signature contains genes associated with antigen processing, presentation, and T cell priming, suggesting that tumor-APCs acquire competence to trigger immune responses and still showcase tumor antigens (**Figure 15G**).

Epigenetically, tumor-APCs have undergone modifications to open chromatin as early as day 3, corresponding to the emergence of complete and partially reprogrammed cells this early in the reprogramming process (411). PU.1 and IRF8 interactions at the chromatin level led to the initiation of cDC1 reprogramming from day 0 to day 3, as observed in **Study II** (404). Thus, the reprogramming process occurs in a stepwise manner, where the first upregulated genes are related to cDC1 lineage establishment (*ZNF366*). Interestingly, the endogenous expression of *IRF8* and *BATF3* is already established at day 5 of reprogramming. At the same time, genes related to cDC1 function (*CLEC9A*, *XCR1*) are recruited later, suggesting the acquisition of a mature tumor-APC program (411).

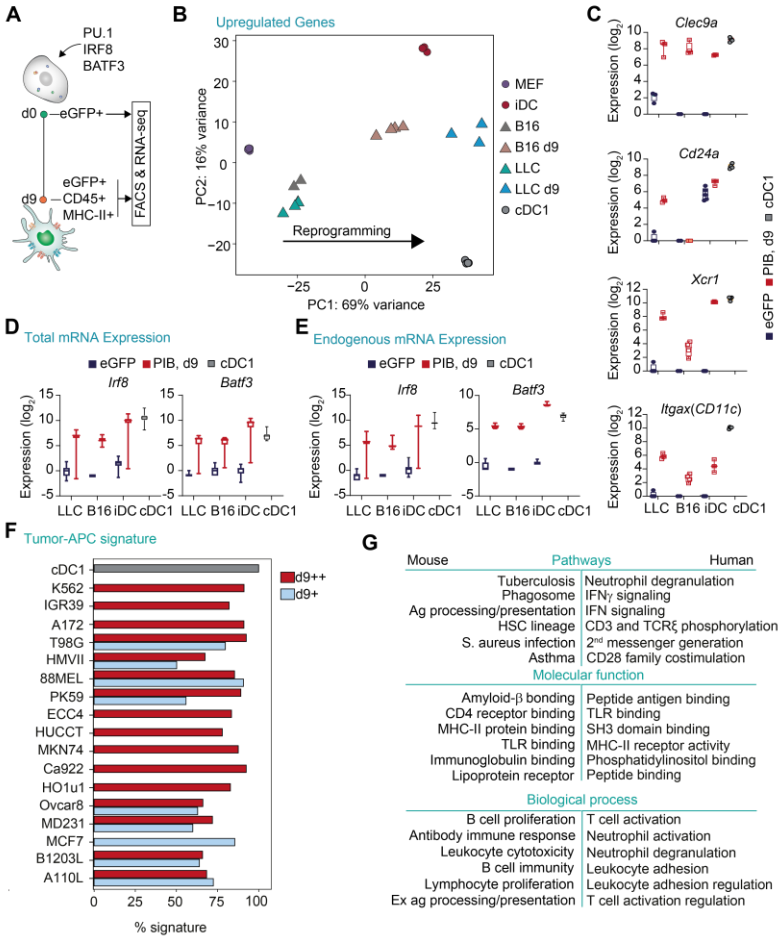

Figure 15 | PU.1, IRF8, and BATF3 (PIB) impose a cDC1-like gene signature in mouse and human cancer cells. (A) Cancer cells were reprogrammed with PIB for 9 days. Mouse tumor-APCs were sorted by fluorescence-activated cell sorting (FACS) at day 9 (d9) based on the triple expression of eGFP+CD45+MHC-II+ and assessed by RNA-sequencing (RNA-seq). Cancer cells transduced with eGFP-only coding vector were included as day 0 (d0) controls. Human tumor-APCs were purified based on their ability to generate complete reprogrammed tumor-APCs (eGFP+CD45+HLA-DR+, d9++) or partially reprogrammed tumor-APCs (eGFP+CD45+HLA-DR- or eGFP+CD45-HLA-DR+, D9+). **(B)** Principal component analysis (PCA) of tumor-APCs and control cancer cells. Data was integrated with previous RNA-seq datasets for mouse embryonic fibroblast (MEFs), induced dendritic cells (iDC), and splenic cDC1 (GSE103618). The arrow represents a reprogramming path. **(C)** mRNA expression levels of genes related to cDC1 signature, *Clec9a*, *Cd24a*, *Xcr1*, and *Itgax* (CD11c). **(D)** Total mRNA expression and **(E)** endogenous expression of *Irf8* and *Batf3*. Minimum, maximum, and mean are shown in the boxplots. **(F)** Percentage of reprogrammed (red) and partially reprogrammed (blue) human cancer cells that acquire a defined tumor-APC gene signature that is common to human peripheral blood cDC1 (grey). **(G)** Top 6 pathways and gene ontologies upregulated in mouse and human tumor-APCs.

Altogether, these data demonstrate that PIB-induced reprogramming is a universally conserved process that induces profound alterations in the epigenome and transcriptome of cancer cells, overwriting the original cancerous program with a cDC1-like signature. The nature of the changes implemented in tumor-APCs

suggests a mature and functional cDC1-like phenotype, thus opening opportunities to assess whether these alterations are reflected in enhanced tumor immunogenicity.

Tumor-APCs are more immunogenic and present tumor-associated antigens.

RNA-seq data uncovered a stepwise activation of antigen presentation machinery in mouse and human-derived tumor-APCs (411). Additionally, we found the upregulation of transcripts of MHC-I and MHC-II master regulators, *Nlrc5* and *Ciita*, respectively. These are crucial for maintaining MHC-I and MHC-II downstream genes active (237, 278, 410). On the other hand, we detected the upregulation of IFN-γ, STING, and TLR-induced maturation pathways in tumor-APCs, which have been correlated with increased antitumor immunity and CTL sensitivity (220, 285-287, 289, 411).

A combination of flow cytometric and fluorescence microscopy analyses detected increased expression of MHC-I and B2M at the cell surface in murine tumor-APCs (**Figure 16A-D**). To evaluate whether tumor-APCs could present TAAs, we employed mass-spectrometry (MS)-based immunopeptidomics on reprogrammed mouse melanoma cells, revealing that tumor-APCs could present a higher number of peptides predicted to bind to MHC-I compared to control or IFN-γ treated cancer cells (411). Moreover, melanoma-associated antigens such as TYR, TYRP1, TYRP2, and p30gag were all found among the list of peptides predicted to be shown by tumor-APCs even at higher levels than IFN-γ stimulated melanoma (**Figure 16E**), validating the hypothesis that tumor-APCs can efficiently present endogenous tumor antigens.

IFN-γ has been shown to increase MHC-I expression in cancer cells, and recent CRISPR-based screenings identified upstream regulators of the IFN pathway that affect tumor immunogenicity (285-287). Still, TCR engagement without co-stimulatory molecules ends in T cell anergy (284). Therefore, we assessed if tumor-APCs expressed costimulatory molecules CD40, CD80, and CD86 necessary for TCR engagement. Reprogramming by PU.1, IRF8, and BATF3 drove tumor-APCs to express costimulatory molecules in contrast with IFN-γ stimulation of control cancer cells (**Figure 16F, G**), indicating that tumor-APCs express all the antigen presentation machinery necessary to activate T cells.

Next, we addressed tumor-APCs' ability to present endogenous antigens. We reprogrammed OVA-expressing cell lines (B16-OVA and LLC-OVA) and co-cultured the tumor-APCs with OVA-specific OT-I CD8+ T cells as described previously. OVA-expressing tumor-APCs, but not control cells, acquired the ability to prime naïve T cells and trigger T cell activation and expansion ($CD44+CTV^{low}$) (**Figure 16H**).

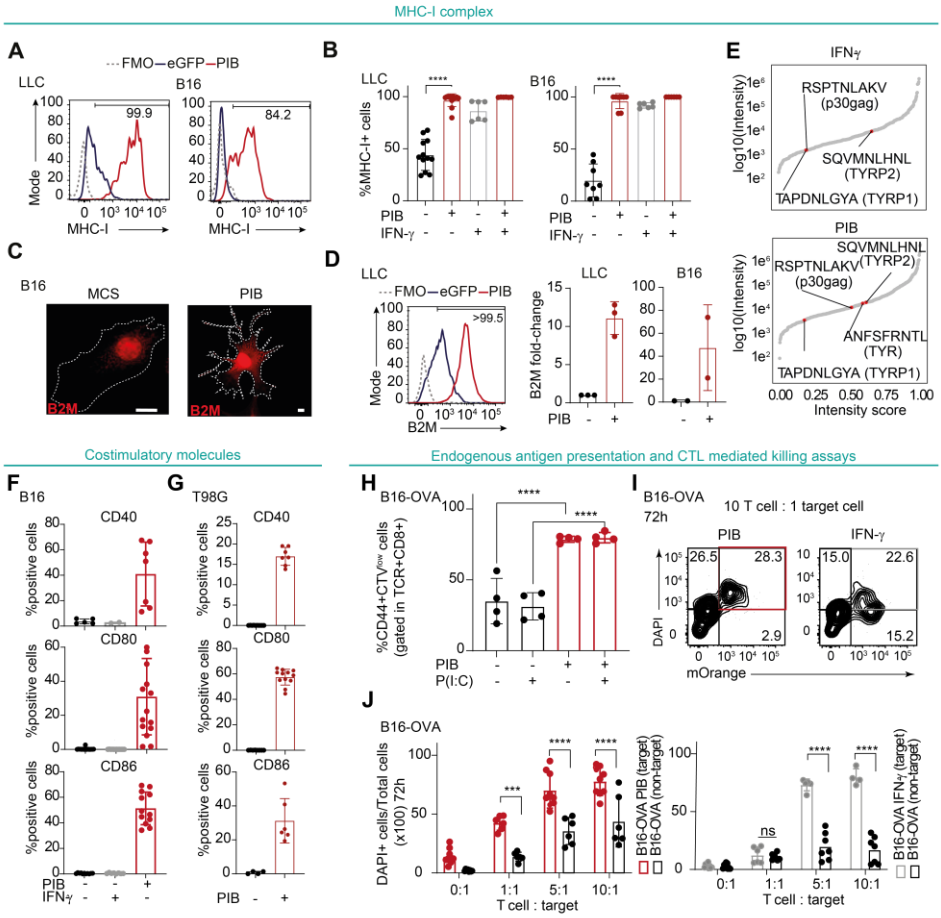

Figure 16 | Tumor-APCs become immunogenic. (A) Flow cytometric analysis of MHC-I expression at the cell surface of cancer cells (black) and tumor-APCs (red) derived from mouse lung carcinoma (LLC) and melanoma (B16). Fluorescence minus one (FMO) was included as control staining. **(B)** Quantification of the percentage of MHC-I+ cells gated in reprogrammed cancer cells at day 9 or eGFP-transduced cancer cells in the presence or absence of IFN-γ. **(C)** Micrographs depict beta-2-microglobulin (B2M) upregulation in mouse melanoma after reprogramming. **(D)** Representative flow cytometry plot and quantification of the percentage of B2M+ gated in reprogrammed cancer cells. **(E)** Ranking by the normalized intensity of predicted peptides that bind MHC-I obtained by immunopeptidomics analysis from IFN-γ treated mouse melanoma and tumor-APCs. Canonical melanoma tumor-associated antigens are highlighted. **(F)** Expression of CD40, CD80, and CD86 co-stimulatory molecules in eGFP-transduced, IFN-γ treated and reprogrammed B16 and **(G)** T98G-derived tumor-APCs. **(H)** B16-OVA derived tumor-APCs at day 3 of reprogramming were co-cultured with OT-I naïve CD8+ T cells. The plot represents the quantification of CD8+T cell proliferation assessed by CTV dilution and expression of CD44 (CD44+CTV^{low}). **(I)** Representative flow cytometry plot for T cell-mediated killing of B16-OVA target cells (mOrange+) reprogrammed or stimulated with IFN-γ measured 3 days (72h) after co-culture establishment. The percentage of target dead cells (mOrange+ DAPI+) is highlighted in red (PIB) and grey (IFN-γ). **(J)** Quantification of cell killing for target and non-target B16-OVA cells after co-culture with increasing ratios of OT-I T cells after 72h. p-value: ***p<0.001, ****p<0.0001, ns – not significant.

Importantly, PU.1, IRF8, and BATF3 endowed tumor-APCs with endogenous antigen presentation skills independently of TLR stimulation with Poly(I:C).

Then, I developed a flow cytometric assay to evaluate tumor-APCs susceptibility to CTL-mediated killing based on a non-radioactive assay to measure CTL activity *in vitro* (413). First, I genetically modified B16-OVA to express mOrange before PU.1, IRF8, and BATF3 reprogramming or IFN-γ stimulation (target, mOrange+) and mixed with unmodified B16-OVA (non-target, mOrange-). I co-cultured this cell mix for 3 days with increasing ratios of activated OT-I CD8+ T cells (**Figure 16I, J**). I observed increasing percentages of dead target cells (DAPI+mOrange+) compared to non-target cells along culture time and higher starting T cell numbers. Consistent with poor endogenous antigen presentation capacity (411), IFN-γ stimulated cells also demonstrated low susceptibility to CTL-mediated cytotoxicity, only achieving the same dead cell ratio with higher T cell starting numbers when compared to 1:1 T cell to target ratio needed for tumor-APC successful killing. Moreover, tumor-APCs needed less time to be targeted by CTLs, showing more cell death at early time points than control cells. Additionally, tumor-APCs seemed to promote a bystander effect on non-target cells at later time points and at a higher scale than IFN-γ stimulated cancer cells (414). We also validated these results against naturally occurring TAAs by co-culturing B16-derived tumor-APCs with T cells targeting gp100/pmel, demonstrating that tumor-APCs are more easily targeted for CTL-mediated killing than cancer cells. These data indicate that tumor-APCs show superior tumor antigen presentation, resulting in improved immune recognition and elimination by activated T cells.

Tumor-APCs are endowed with cDC1-like functional properties.

The TME disrupts cDC1's capacity for scavenging, phagocytosis, cytokine secretion to attract other immune players, and T cell activation with exogenous antigens (133). I have described how reprogramming cancer cells to tumor-APCs can enhance the intrinsic immunogenicity of cancer cells and promote T cell responses. However, conferring cDC1 functional properties to tumor-APCs would directly impact the TME and help restore the cancer immunity cycle. To this end, we conducted experiments that would allow us to evaluate cytokine secretion, phagocytosis, antigen processing, and, lastly, naïve T cell priming by tumor-APCs (**Figure 17A**). I first detected the necessary transcripts for cDC1 cytokines and chemokines, such as *Ifnb* and *Cxcl10* (207), in tumor-APCs samples, which indicates a primed, mature state (**Figure 17B**). Upon TLR stimulation, we confirmed that mouse and human tumor-APCs released pro-inflammatory cytokines IL12p70, IL-1β, IL-6, and chemoattractants like CXCL10, among others (411), which are required for T cell recruitment and activation within the TME (202, 207, 223) (**Figure 17C**).

Additionally, the expression of PU.1, IRF8, and BATF3 endowed reprogrammed cancer cells with phagocytic capacity as demonstrated by the engulfment of fluorescently-labeled OVA (**Figure 17D, E**). Furthermore, I assessed tumor-APCs'

ability to process engulfed antigens by detecting DQ-OVA cleavage through fluorescent emission. Reprogrammed cancer cells, but not control cells, could effectively cleave DQ-OVA (**Figure 17F)**, a process that could be abolished in the presence of proteasome inhibitors. This is consistent with increased immunoproteasome transcripts and intracellular protein found in tumor-APCs compared to control cancer cells (411).

To confirm that tumor-APCs can prime CD8+ T cells, I pulsed B16 and LLC-derived tumor-APCs with OVA peptides and whole protein and co-cultured them with naïve T cells (**Figure 17G, H**). Murine tumor-APCs promoted CD8+ T cell activation at similar levels achieved by BM-DCs. T cell priming was established in reprogrammed cancer cells as early as day 3, in agreement with early activation of antigen presentation signature in tumor-APCs. Moreover, human tumor-APCs could also engulf cytomegalovirus (CMV) peptides and efficiently activate CMV+CD8+T cells (**Figure 17I**). Although reprogrammed cells were competent to exert cDC1 functions already at basal conditions, TLR activation with poly(I:C) or LPS further potentiated cytokine secretion and cross-presentation ability by tumor-APCs.

Moreover, partially reprogrammed cancer cells have the same machinery to acquire professional cDC1 function and can perform dead cell phagocytosis (411).

To bridge the initial findings with clinical applicability, I sought to reprogram human primary cancer cells and CAFs. Ectopic expression of PU.1, IRF8, and BATF3 reprogrammed over 35 primary tumor samples to cDC1-like cells. More importantly, tumor-APCs obtained from primary melanoma cells were capable of T cell priming and being recognized by TILs, confirming our prior results in murine cancer cell lines (411).

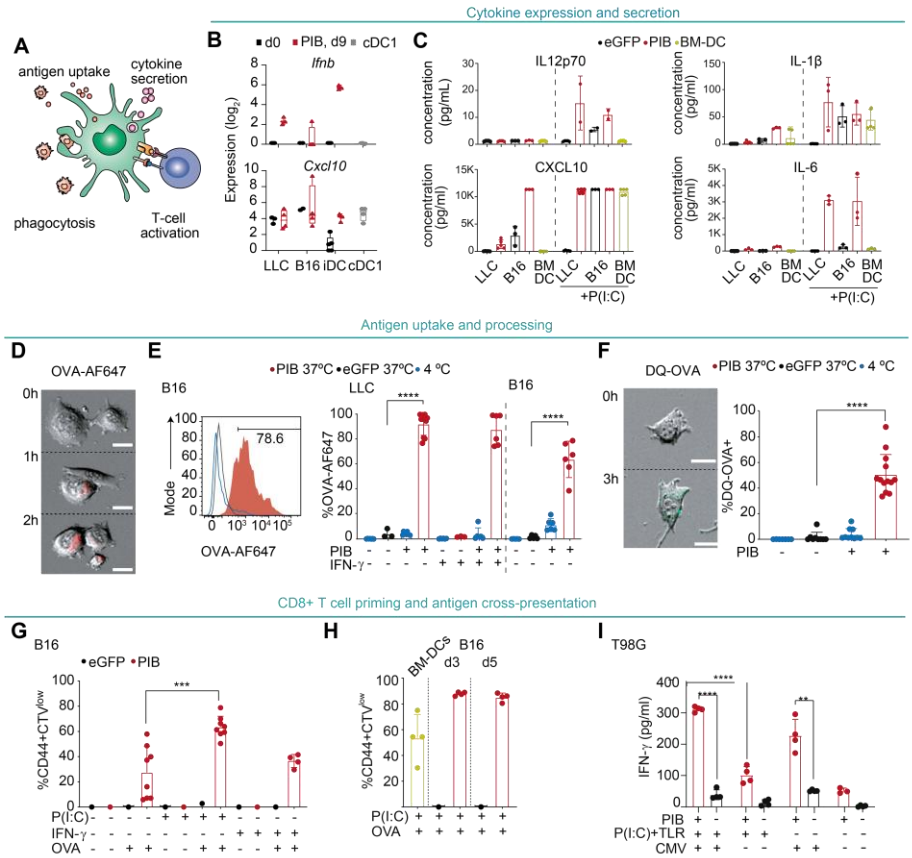

Figure 17 | PU.1, IRF8, and BATF3 (PIB) reprogramming confers cDC1 function to tumor-APCs. (A) Tumor-APCs were evaluated for their capacity to secrete cytokines, phagocytosis, antigen processing, and naïve T cell priming. **(B)** Mouse cancer cell lines (LLC and B16) were transduced with PIB and purified based on the expression of eGFP+CD45+MHC-II+ (d9). Cancer cells transduced with eGFP (d0), MEFs, iDC, and splenic cDC1 were used as reference controls. Box plots represent mRNA expression levels for cytokines related to cDC1 function, *Ifnb*, and *Cxcl10*. **(C)** Quantification of IL12p70, IL-1β, CXCL10, and IL-6 in supernatants collected from eGFP- (black) and purified PIB- (red) transduced mouse cancer cells with or without poly(I:C) (P(I:C)) stimulation. Bone-marrow-derived dendritic cells (BM-DCs) were used for controls (green). **(D)** Micrographs representing phagocytosis of fluorescently-labeled ovalbumin (OVA) (OVA-AlexaFluor647, OVA-AF647, red) by LLC. Scale bars: 25 µm. **(E)** Flow cytometric analysis of OVA-AF647 uptake by reprogrammed LLC and B16 in the presence or absence of IFN-γ. Incubation was done at 37º and 4ºC for reference. **(F)** Fluorescent microscopy analysis for DQ-OVA processing (left) and flow cytometric quantification of the percentage of DQ-OVA+ cells at 37º and 4º C by tumor-APCs and eGFP-transduced cancer cells (right). **(G)** Quantification of proliferative OT-I CD8+ T cells ($CD44+CTV^{low}$) percentage after co-culture with tumor-APCs pulsed with OVA. **(H)** Tumor-APCs purified at days 3 and 5 of reprogramming were pulsed with exogenous antigen and co-cultured with OT-I CD8+ T cells before flow cytometry to measure CD8+ proliferation. **(I)** T98G-derived tumor-APCs were pulsed with cytomegalovirus (CMV) peptide and co-cultured with CMV-specific T cells. The plot represents the quantification of IFN-γ in supernatants collected after co-culture of reprogrammed T98G (red) with CMV-specific CD8+ T cells. T98G cells transduced with eGFP were used as controls (black). Mean±SD is represented. p-value: *p<0.1, **p<0.01, ***p<0.001, ****p<0.0001.

Tumor-APCs have low tumorigenic potential and induce antitumor immunity *in vivo*.

Reprogramming cancer cells to pluripotency and direct reprogramming towards benignity have been demonstrated to decrease tumorigenicity of the resulting cell fate (55). Likewise, RNA-seq analysis showed gradual silencing of cell cycle genes along the cDC1 reprogramming process, suggesting mitotic arrest, while tumor suppressor genes *TP53*, *RB1*, and *CDKN1A* were activated. Additional assays demonstrated that complete and partially reprogrammed tumor-APCs lose proliferation capacity and tumorigenic potential *in vitro*. This suggests that the cDC1 program drives cancer cells to a stable, benign state independently of cell origin (411).

More importantly, these findings were validated *in vivo* by injecting tumor-APCs subcutaneously in NXG mice (**Figure 18A**). While mice injected with control transduced cancer cells slowly grew tumors and had to be sacrificed within 5 months, animals implanted with tumor-APCs survived tumor-free for the duration of the experiment (**Figure 18B**).

Finally, I addressed tumor-APCs' capacity to induce antitumor immune responses *in vivo*. As such, we established subcutaneous B16-OVA tumors in C57b/6 mice and reprogrammed B16 tumor-APCs before stimulating them with poly(I:C) followed by OVA protein pulse. Pulsed tumor-APCs were injected intratumorally at days 7, 10, and 13 (**Figure 18C**). Mice treated with tumor-APCs showed increased survival and delayed tumor growth delay compared to mice treated with PBS or transduced cancer cells with an empty vector (**Figure 18D**).

Moreover, I found a higher proportion of OVA and murine leukemia virus (MuLV) reactive T cells circulating in the peripheral blood of mice treated with tumor-APCs (**Figure 18E**). At the same time, there was an increase of pmel-specific T cells in tumor-draining lymph nodes (411). These results suggest that tumor-APCs elicited antigen-specific T cell-mediated responses, delaying tumor growth. Importantly, tumor-APCs treatment led to the infiltration of CD8+, CD4+ T cells, and NK cells within the TME (**Figure 18F**). TILs also presented a more activated immunophenotype by displaying CD44+PD-1+ and CD44PD-1- (**Figure 18G**), which indicates that administration of tumor-APCs alters the TME by attracting robust immune effectors and eliciting antitumor responses.

To exclude the contribution of naturally occurring cDC1 to the immune response mounted against cancer in wildtype mice, I validated the experiments in BATF3 knockout mice that lack the endogenous the cDC1 compartment. Tumor burden and decreased and overall survival greatly increased in mice treated with tumor-APCs in comparison with control mice (411).

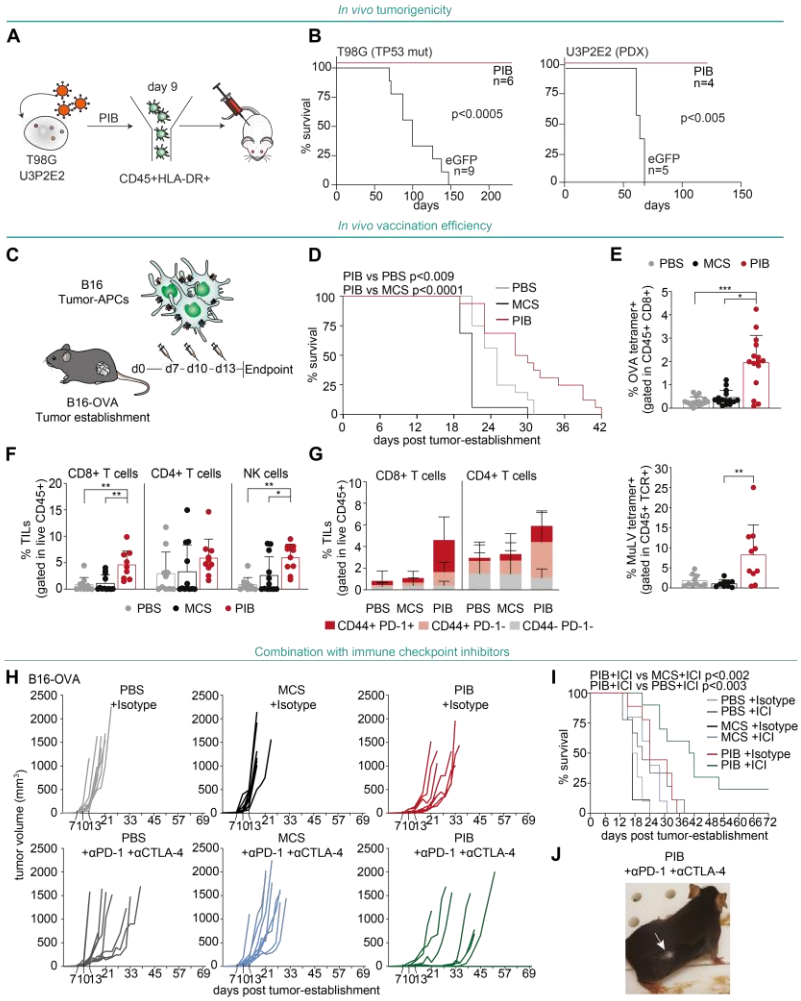

Figure 18 | Tumor-APCs lose tumorigenicity and promote antitumor immunity *in vivo*. (A) Tumor-APCs derived from glioblastoma and bladder carcinoma (U3P2E2) were purified by FACS on reprogramming day 9 and injected in NXG mice to evaluate tumorigenicity *in vivo*. **(B)** Survival curves of NXG mice transplanted with reprogrammed cancer cells (red) and eGFP-transduced cells (black). **(C)** Schematic overview of the experimental procedure to evaluate antitumor properties of tumor-APCs. B16-OVA tumors were injected intratumorally at day 7, 10, and 13 post-tumor establishment with B16-derived tumor-APCs pulsed with OVA protein and stimulated with poly(I:C). **(D)** Survival curve for mice injected with tumor-APCs (PIB, red). Phosphate buffered saline (PBS) solution (grey) and empty-vector-transduced B16 cells (black) were used as references. **(E)** Peripheral blood from injected mice was collected and assessed by flow cytometry. Plots represent the quantification of the percentage of Ova-specific (top) or MuLV-specific T cells within the CD45+CD8+ population circulating in the blood of treated mice at day 14 post-tumor establishment. **(F)** Quantification of tumor-infiltrating lymphocytes (TILs) and **(G)** Characterization of CD4+ and CD8+ TILs by the expression of CD44 and PD-1. **(H)** Mice with established tumors were treated with checkpoint inhibitors (ICI, anti-PD-1, anti-CTLA4) in combination with tumor-APCs. Plots represent tumor growth and **(I)** survival curves for mice treated with ICI alone or combined with tumor-APCs. Isotype controls (IgG2a and IgG2b) were used as ICI references. PBS and transduced cancer cells were used as controls. **J.** Photograph of a cured mouse with combination therapy depicts depigmentation (white arrow) on tumor regression site. Mean±SD is represented. Mean±SD is represented. p-value: *p<0.1, **p<0.01, ***p<0.001.

Additionally, tumor-APCs also confer a greater advantage compared to fibroblast-derived iDC. The first express endogenous TAAs while iDC need to incorporate exogenous antigens and process them before being able to present them. This phenomenon might contribute to tumor-APCs being responsible for mounting a more robust immune response against cancer than iDC (411).

Most immunotherapy regimens combine various approaches (12, 61), so I sought to understand whether tumor-APCs could improve ICI treatment in resistant melanoma like B16. Combining ICI with tumor-APCs treatment resulted in a more prominent extension of survival, a robust effect on tumor growth delay, and even complete regression in some mice (**Figure 18H-J**). These data show that tumor-APCs can synergize with ICI to revert cancer *in vivo*.

This study supports the hypothesis that tumor-APCs generated *in vitro* present endogenous antigens and acquire cDC1-like functions, potentiating antitumor immunity responses.

Tumor-APCs present antigens in the MHC-II context.

In **Study IV**, I further characterized tumor-APCs regarding their immunophenothypical markers. cDC1 and other APCs can express immune checkpoint molecules such as PD-1 and VISTA. The presence of these molecules ensures that the immune responses orchestrated by dendritic cells do not generate overreactions. Indeed, murine-derived tumor-APCs can start expressing some of these markers, PD-1, ICOS and TIM-3 as a result of the reprogramming process. However, these markers can also be found in naturally occurring cDC1 from both species (**Figure 19A**).

I also described the magnetically activated cell sorting (MACS)-based method we developed to purify tumor-APCs faster and more efficiently. MACS allows 4 to 6 times faster purifications than traditional, fluorescent-activated cell sorting (FACS), producing more viable cells after the process (415, 416). Whereas FACS requires flow cytometric cell sorters, MACS requires fewer instruments and is scalable without adding processing time. To enrich tumor-APCs, we use CD45+ and MHC-II+ markers as targets for magnetic labeling (**Figure 19B**). Flow cytometric analysis is used to assess the quality of the enrichment by comparing samples before sorting (pre-MACS) with positive (post-MACS(+)) and negative (post-MACS(-)) fractions regarding transduction (eGFP+) and either CD45, MHC-II, or both markers (**Figure 19C, D**). We could recover most transduced cells (post-MACS(+)) with MACS, while we could only detect around 33% of transduced cells in the negative fraction (**Figure 19E**). We consistently registered only around 6% of partially and completely reprogrammed cells in the negative fraction. In contrast, we recovered almost 73% of tumor-APCs in the positive fraction, a 10-fold increase compared to the unsorted culture (**Figure 19F**). Additional quality control of tumor-APCs can be done by evaluating MHC-I expression (**Figure 19G, H)**.

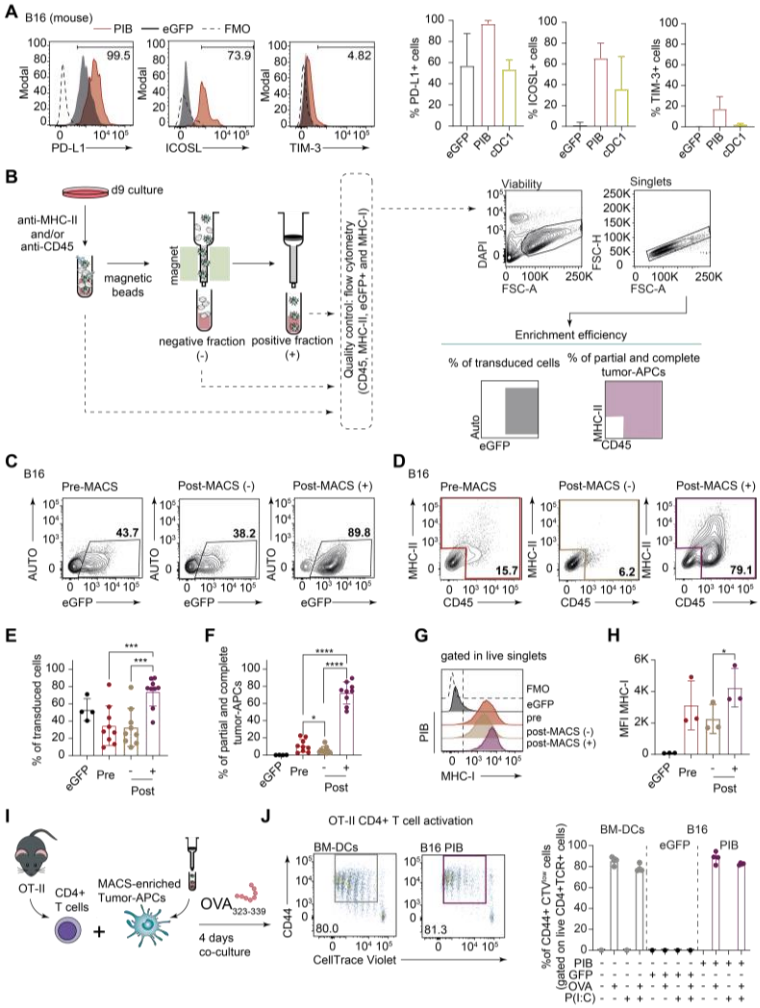

Figure 19 | Enriched tumor-APCs can present antigens in the MHC-II context. (A) Tumor-APCs derived from murine melanoma cells were assessed by flow cytometric analysis for the expression of PD-L1, ICOSL and TIM-3. Expression was compared to eGFP-transduced cells and splenic cDC1. **(B)** Illustration depicting a MACS-based protocol to enrich mouse melanoma-derived tumor-APCs based on the expression of CD45 and MHC-II. Tumor-APCs are labelled with magnetic beads before being recovered through a strong magnetic field. The post-MACS-positive fraction (+) contains purified tumor-APCs collected after removing the column from the magnetic field. Quality control is assessed by flow cytometric analysis. Gating strategy is shown. **(C)** Flow cytometric analysis for eGFP expression and **(D)** representative plots depicting a successful MACS procedure. Enrichment efficiency is compared to pre-MACS and post-MACS negative fractions. **(E)** Quantification of eGFP+ cells represents the total percentage of transduced cells at different protocol steps. **(F)** Quantification of the percentage of partially and completely reprogrammed tumor-APCs pre- and post-MACS purification. eGFP-transduced cancer cells were measured as a reference control. **(G)** Flow cytometric analysis of MHC-I expression in pre- and post-MACS fractions. **(H)** Median fluorescent intensity (MFI) for MHC-I expressed by cancer cells and tumor-APCs after purification. **(I)** MACS-purified tumor-APCs are pulsed with OVA (323-339) peptides before co-culture with naïve OT-II CD4+ T cells. **(J)** T cell proliferation is measured by flow cytometric analysis of CD44 expression (CD44+) and CellTrace Violet (CTV) dilution (CTV^{low}). Mean±SD is shown. p-value: *p<0.1*** p-value < 0.001; **** p-value < 0.0001.

More importantly, this purification method yields approximately $2x10^6$ total cells per $1x10^6$ cells seeded before PIB-driven reprogramming, ensuring a pure population that can be used for *in vitro* and *in vivo* studies.

Finally, I showed that tumor-APCs could present exogenous antigens in the MHC-II context. We pulsed reprogrammed cancer cells with OVA peptides (323-339), presented in MHC-II complexes to CD4+ T cells, before co-culturing them with naïve OT-II CD4+ T cells. Similarly to CD8+ T cell priming, mouse melanoma-derived tumor-APCs trigger CD4+ T cell expansion and activation as demonstrated by CTV dilution and CD44+ expression within CD4+TCR+ T cells (**Figure 19I, J**).

General Discussion and Future Perspectives

In the past decades, three fields developed parallelly and constitute the pillars of the work I presented during this dissertation. Firstly, immunotherapy revolutionized cancer treatment with incredible success in treating highly metastatic, progressive cancer diseases previously considered untreatable. Secondly, the discovery of dendritic cells and their role in orchestrating innate and adaptive immune responses incited various studies characterizing the different dendritic cell subsets and their implication in cancer. The third pillar of this thesis, cellular reprogramming, has opened a multifaceted toolbox that spans tissue replacement and repair in regenerative medicine to potential cancer treatment by promoting benign cell identities and halting tumorigenic drive. My thesis work focused on exploring the applicability of direct cellular reprogramming for cancer immunotherapy.

Despite the many immunotherapy successes, the spectrum of malignancies treatable with immunotherapy is limited, mainly due to intrinsic cancer heterogeneity, immunosuppressive milieu, lack of suitable tumor antigens, or even lower TMB. Furthermore, low tumor immunogenicity and lack of functional cDC1 were identified as the barriers behind the pitfalls of immunotherapy. More importantly, they prove that antigen presentation is key to efficient antitumor immune responses. Cancer cells evolve through genetic mutations and immune selection to avoid the immune system by downregulating important genes of antigen presentation pathway. While there have been efforts to manipulate tumor immunogenicity, systemic toxicity and off-target side effects have, for now, hindered the broad application of drugs that have been shown to increase antigen presentation in cancer cells. Conversely, cDC1 has also stood as a key player in antitumor immunity. Their presence in the TME facilitates ICI success, and cancer vaccines based on this subset have met favorable preclinical outcomes. Moreover, the discovery of cDC1 role in cancer has cemented that antigen presentation is fundamental for the cancer immunity cycle and that the loss of antigen presentation machinery, either by suppression of the cDC1 compartment or by downregulation of these proteins by cancer cells, results in poor immune responses against cancer. Dendritic cell-based vaccines have also faced manufacturing challenges and variable clinical outcomes, which have hindered a wider adoption for cancer treatment.

This dissertation aimed at intertwining cellular reprogramming with cDC1 biology to overcome common immunotherapy pitfalls and introduce a novel strategy for developing anti-cancer therapies. To this end, I first participated in the identification of the minimal combination of transcription factors that could impose a cDC1-like cell fate in somatic cells. **Studies I** and **II** employed single-cell RNA sequencing data to unravel the mechanisms behind cDC1 reprogramming. ChIP-seq data helped dissect the interactions between PU.1, IRF8, and BATF3 in the early settlement of a cDC1 identity. Additionally, the development of polycistronic vectors encoding PU.1, IRF8, and BATF3 was crucial to first establish PU.1 as the necessary transcription factor to kickstart the reprogramming process by binding open chromatin regions and recruiting the other two factors to repress fibroblastic identity and enforce a cDC1 program. More importantly, combining the polycistronic vectors with constitutive promoters increased the frequency of completely reprogrammed cDC1-like cells. Aside from implementing a cDC1-like program, reprogrammed somatic cells with PU.1, IRF8, and BATF3 express cDC1-specific markers and are endowed with functional antigen presentation machinery. These findings lead me to hypothesize whether the cDC1 reprogramming strategy could be exploited to induce antigen presentation in cancer cells and restore tumor immunogenicity. In **Studies III** and **IV**, I have demonstrated that forced expression of cDC1 lineage-specific factors indeed forces transcriptional and epigenetic changes in cancer cells originating from the human and the mouse species (**Figure 20**). Reprogrammed cancer cells expressed cDC1-specific markers and acquired antigen presentation machinery, enhancing endogenous antigen presentation. Furthermore, tumor-APCs could present exogenous antigens and priming naïve CD4+ and CD8+ T cells. Finally, we demonstrated that tumor-APC vaccination increases T and NK infiltration in the TME and increases the circulation of tumor antigen-specific T cells, leading to a delay in tumor growth. More importantly, tumor-APCs synergize with ICI to improve mice survival (**Figure 20**).

The cellular identity of cDC1 is imposed in cancerous and non-cancerous cells by the combined expression of PU.1, IRF8, and BATF3. PU.1 is implicated in all myeloid lineages (140-146). Adult HSCs require PU.1 to differentiate into cDC and pDC compartments, and, importantly, PU.1 regulates FLT3 expression, which is necessary during cDC1 development (146). Abrogation of IRF8 in different stages of cDC1 development has demonstrated that this transcription factor drives cDC1 lineage commitment and maintenance (161). BATF3, in turn, was discovered to be a key factor in cDC1 maturation and function (158). The connection between the three factors during cDC1 development has been well documented. PU.1 reshapes chromatin around the IRF8 gene locus and kickstarts expression of the latter in dendritic cell progenitors (417), while BATF3 promotes self-sustaining activation of IRF8 at later stages of cDC1 cell lineage specification (154). These results align with the observations published in **Studies I** and **II**.

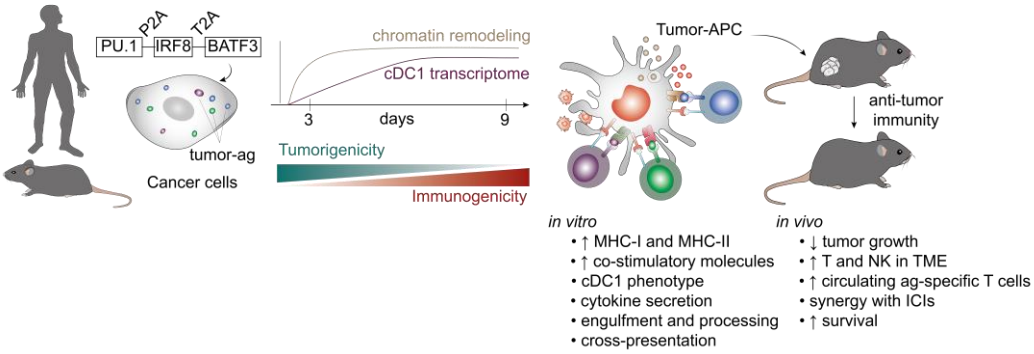

Figure 20 | Restoring antitumor immunity through cDC1 reprogramming strategies. Mouse and human cancer cells were reprogrammed into tumor-APCs through overexpression of PU.1, IRF8, and BATF3. The reprogramming process is fast, with rapid chromatin remodeling around day 3, followed by a stepwise adoption of a conventional dendritic cell type I (cDC1) transcriptome until day 9. Tumor-APCs lose tumorigenicity and upregulate antigen presentation machinery that enhances intrinsic tumor immunogenicity. Additionally, tumor-APCs acquire cDC1 functional features as PU.1, IRF8, and BATF3 endow them with exogenous antigen engulfment and presentation to CD4+ and CD8+ T cells. Finally, intratumoral injection of tumor-APCs delays tumor growth, promotes T and NK cell infiltration in the tumor microenvironment (TME), and synergizes with immune checkpoint inhibitors (ICI) to prolong survival. Ag – antigen.

The three transcription factors cooperate to impose a cDC1 identity in somatic cells, and missing one or two factors from this combination abrogates the establishment of a successful reprogramming path. Furthermore, the increased reprogramming efficiency observed with polycistronic vectors demonstrated that cDC1 lineage commitment results from regulated stoichiometry between the three factors, in accordance with previous studies.

Single-cell RNA-seq analysis of iDC outlined the trajectory of somatic cells undergoing cDC1 lineage conversion. While the reprogramming process is asynchronous, successful reprogramming was correlated with the activation of endogenous *Spi1*, *Irf8*, and *Batf3*. Moreover, cDC1 reprogramming triggers an inflammatory signaling pathway associated with a more faithful lineage conversion. Pro-inflammatory pathways have been shown to prime somatic cells for efficient reprogramming processes. The TLR3 agonist, Poly(I:C), enhances chromatin plasticity by regulating epigenetic modifiers that aid the reprogramming factors to trigger pluripotency in somatic cells (418). During direct cell reprogramming towards cardiomyocytes, loss of innate immunity genes, including TLR3, impacts reprogramming efficiency by altering DNA methylation status (407). This aligns with my results, showing that the addition of pro-inflammatory cytokines, IFNs, and TNF-α synergized with PU.1, IRF8, and BATF3 increasing reprogramming efficiency (**Study II**). On the other hand, MSCs were the exception to the effect of cytokines in cDC1 reprogramming. This suggests that MSCs' inherent plasticity confers them an advantage for optimal reprogramming towards the cDC1 fate or that MSCs are less responsive to inflammatory stimuli.

In stark contrast with the gene signatures associated with successful reprogramming processes, failure to downregulate the original cell program led to reprogramming resistance and even cell fate reversal (405-408). While cDC1 reprogramming does not transit through intermediate or progenitor states to achieve a mature phenotype, reprogramming efficiency can potentially limit their application. In **Studies I** and **II**, delivery optimization with polycistronic constructions and constitutive promoters increased the yield of iDCs. These results align with previously published data showing that high expression levels of ASCL1 are necessary for neuronal reprogramming and MEF2C, GATA4, and TBX5 for cardiomyocyte generation (405, 419). Moreover, iPSC reprogramming also benefits from the constitutive expression of the OSKM factors driven by the SFFV promoter, indicating that overcoming a certain expression threshold might be a requirement for efficient cell conversion processes (408). Interestingly, I observed that cDC1 reprogramming is a stepwise process through which cells transit through partially reprogrammed populations before reaching complete cDC1-like features. This was not unexpected due to previous reports on partially reprogrammed cells arising from different reprogramming processes. Further optimizations to my strategy will make it possible to improve reprogramming efficiency. The data generated for **Studies I** and **II** could be explored further to assess what genes and epigenetic modifiers contribute to successful reprogramming. This could also improve other reprogramming systems by boosting reprogramming efficiency and facilitating clinical translation of reprogramming-based approaches.

Study II uncovered the early events underlying cDC1 reprogramming through ChIP-seq analysis. PU.1, IRF8, and BATF3 interact with each other to induce cDC1 identity in human fibroblasts. Chromatin remodeling is a necessary step for efficient lineage conversions, and it is often the result of the engagement of lineage-restrictive factors to activate and repress certain genes. Whether lineage-instructive factors can bind closed chromatin and act as pioneer factors or cooperate to repress the original program and induce the final cell fate genes has been the research subject for the past decade (420, 421). Recently, PU.1 was described as a non-classical pioneer transcription factor whose capacity to bind and remodel chromatin depends on the cell state (145). Despite being unable to bind closed chromatin, forced expression of PU.1 in cells that naturally lack PU.1 expression leads to extensive chromatin remodeling and kickstarts the expression of myeloid genes by interacting and distributing other transcription factors (145). Accordingly, during dendritic cell reprogramming, PU.1 preferentially occupies promoter and enhancer sites in open chromatin. IRF8 binding sites in cDC1-specific genes are enriched with motifs targeted by transcription factors belonging to ETS and AP-1 families, whose members include PU.1 and BATF3, respectively (422). Moreover, our data revealed that BATF3 binds chromatin effectively only in the presence of PU.1 and IRF8. This contrasts with the pioneer function of BATF in CD4+ T cells, where BATF alone binds closed chromatin, recruits CTCF, and cooperates with ETS1 to initiate transcription (423). Together with my observations, these data suggest that

PU.1 recruits IRF8 and BATF3 to repress fibroblastic genes and activate a cDC1 program. In the future, it would be interesting to study whether cooperation between the three cDC1-lineage instructors binds to regulatory regions within the *Irf8* during a reprogramming time-course, including *loci* that have been shown to demark cDC1 commitment and lineage maintenance (154-157).

We demonstrated that overexpression of PU.1, IRF8, and BATF3 endows murine and hiDC with functional features of natural cDC1, including responsiveness to TLR stimuli and secretion of the pro-inflammatory cytokine IL-12. Secretion of IL-12 by dendritic cells enhances antitumor immunity by promoting CTL activity, including NK, and inducing Th1 polarization (215, 424). Furthermore, reprogrammed dendritic cells actively engulf exogenous antigens and activate antigen-specific cytotoxic CD8+ T cells. Combined with the induction of antigen presentation machinery in iDCs, these data allowed me to hypothesize that dendritic cell reprogramming strategies could be employed to induce professional antigen presentation in cancer cells to counteract common immune evasion mechanisms.

In **Studies III** and **IV**, I demonstrated that cDC1 reprogramming is a conserved process across mouse and human cancer cell lines, independently of their germ layer of origin. Importantly, patient-derived cancer cells and CAFs were successfully reprogrammed into tumor-APCs, suggesting that cDC1-reprogramming can be employed in various cancer cell types and primary samples. Like somatic cells undergoing cDC1 reprogramming, cancer cells follow a stepwise reprogramming process that relies on fast chromatin remodeling that initiates the expression of cDC1 lineage genes already at day 3. Subsequently, the activation of endogenous expression of PU.1, IRF8, and BATF3 could help maintain cDC1-like identity in tumor-APCs.

Unlike most reprogramming strategies manipulating tumors, cDC1 converts cancer cells into immunologically enhanced cells that can secrete cytokines, process antigens, and trigger antigen-specific immune responses (55). Previously, leukemic cells were reprogrammed into macrophage-like cells with benign phenotype (397, 398). Only recently, this strategy was used to generate a cancer vaccination approach, leveraging cancer-derived macrophages as a vehicle for tumor antigens (425). Similarly to the reprogramming process I described here, murine syngeneic leukemic and solid cancers were amenable to reprogramming toward macrophage-like cells after inducible expression of PU.1 and CEBP/α for 7 days (425). Reprogrammed cancer cells *in vivo* could activate antigen-specific CD4+ and CD8+ T cells to elicit durable antitumor immunity (425). Together with my recent publications, this study further suggests that reprogramming cancer cells towards antigen presentation represent a new modality for antitumor immunity. Nonetheless, interactions between macrophages and tumor cells have been shown to promote metastatic behavior, and their presence within the TME is largely pro-tumorigenic (130). On the other hand, cDC1 are prime examples of professional APCs that excel in CD8+ T cell stimulation and orchestrating antitumor immune responses (210). In **Study III**, I showed that tumor-APCs engage naïve CD8+ T cells by presenting

endogenous antigens and cross-presenting exogenous antigens triggering T-cell stimulation. Moreover, this enhanced antigen presentation capacity enables T cell recognition *in vitro* and elicits antitumor immunity *in vivo*. Functional cDC1 cells are crucial for antitumor response, and their absence within the TME correlates with resistance to immunotherapy (215). Moreover, the cDC1 compartment is often functionally impaired in cancer patients compared to healthy counterparts (235). Although cDC1-based vaccines had promising preclinical studies, current methods fail to generate them at a relevant scale for clinical use (109). Therefore, cDC1 reprogramming provides a new tool to increase and enhance this compartment by promoting the presentation of TAAs.

Indeed, I showed that PU.1, IRF8, and BATF3 increase tumor immunogenicity by eliciting antigen presentation pathways (MHC-I, MHC-II, co-stimulatory molecules, and immunoproteasome) and activating the IFN signaling pathway. In general, successful immune surveillance of tumors and good response to immunotherapy largely rely on the sufficient immune recognition of tumor cells. Notably, cancers that retain antigen presentation signatures are associated with better patient prognosis and response to ICI (237). Previous studies demonstrated that tumor immunogenicity can be enhanced by activating the IFN pathway and induction of co-stimulatory molecules or enforced cytokine expression (105, 426). Direct and indirect modulation of IFN signaling through gene knockout or STING activation have shown their importance during tumor cell recognition and CTL-mediated cancer cell killing (285-287, 290). Increasing MHC-I molecules in melanoma cells lacking IFN overrode intrinsic resistance to ICI and ACT (410). Oncolytic viruses that couple immunogenic cell death with GM-CSF can stimulate immune responses *in situ* (105). However, the clinical application of these strategies faced major limitations spanning from systemic toxicity to eliciting protumorigenic effects and the inability to counteract common resistance mechanisms (118, 270). Reprogramming cancer cells to tumor-APCs activated IFN pathways, STING, and enabled functional antigen presentation. Importantly, immunopeptidomics analysis revealed that tumor-APCs have increased presence of endogenous TAAs at their surface compared to non-reprogrammed or IFN-γ treated cancer cells. The improved immunogenicity of tumor-APCs is translated to T cell priming and recognition *in vitro* and *in vivo*. Besides T cell priming, tumor-APCs modulate anti-cancer immunity at multiple levels. Tumor-APCs secrete pro-inflammatory cytokines, including IL12, chemokine receptors like XCR1, and secrete chemokines, such as CXCL10, which is an important element for the recruitment of TILs (202, 207). Consequently, intratumoral injection of tumor-APCs recruited T and NK cells to the TME, synergizing with ICI to delay tumor growth and increase survival in treated mice. These data suggest that tumor-APCs can exert cDC1-like function within the TME and restore antitumor immunity. In the future, it will be interesting to dissect further the mechanisms underlying antitumor immunity mediated by tumor-APCs and whether lacking IFN pathways or chemokine receptors will impact their function.

While aiming to eradicate tumorigenicity through direct reprogramming strategies might face potential hurdles due to partial retention of parental identity and low transduction efficacy (55, 320), cDC1 reprogramming offers a strategy to rewrite cell identity that is sufficient to elicit antitumor immunity and halt tumorigenicity for most cancer cells. Partially reprogrammed cancer cells can operate like cDC1 as they are endowed with antigen engulfment and processing capabilities. More importantly, partially reprogrammed tumor-APCs, like completely reprogrammed cancer cells, have arrested cell cycle and maintain the expression of tumor antigens necessary to elicit immune responses. This suggests that incomplete reprogramming could still support clonal expansion of tumor antigen-specific T cells while not contributing to tumor burden. It has been recently reported that it is possible to reverse aging landmarks and restore the original function of neurons without losing cell identity or generating pluripotent stem cells through partial cell reprogramming (427). This data, combined with the loss of mitotic capacity, shows data cDC1 reprogramming strategies can still be leveraged against the heterogeneity of cancer cells to induce tumor-specific responses despite variable reprogramming efficiency across different tumors. Nonetheless, refining the strategy to increase target lineage fidelity or ensuring the inactivation of residual tumor cells will be crucial for their clinical application.

In the future, I envision the use of cDC1 reprogramming as a strategy to restore antitumor immunogenicity *in situ*. As a result of years of developing safer gene delivery methods based on non-integrative viral vectors and non-viral delivery systems, *in vivo* reprogramming has become a reality (428-432). Moreover, reprogramming directly inside the target organism takes advantage of internal cell sources, limits possible immune rejection, and has lower risks for tumor formation and increased reprogramming efficiency and fidelity (433). In an *in vivo* setting, reprogramming cells can receive other environmental cues that are difficult to recapitulate *in vitro*. Cellular reprogramming with OSKM factors *in vivo* has been achieved before, with cells undergoing the reprogramming process demonstrating higher plasticity and capacity to generate embryo-like structures, including extraembryonic tissues, in contrast to their laboratory counterparts (434). This demonstrates that the microenvironment where reprogramming occurs has a profound effect on plasticity and can influence reprogramming outcomes. Notably, senescence significantly reduces reprogramming efficiency *in vitro*. However, reprogramming *in vivo* triggers senescence and pro-inflammatory signaling cues that promote higher cellular plasticity (435). While pluripotency factors can be used to revert aging hallmarks *in vivo* (436), the risk of oncogenic drive will hinder their potential application outside of *ex vivo* production of the desired target cells (434). As such, employing direct reprogramming strategies is more promising for therapeutic endeavors. Direct reprogramming of resident murine cardiac fibroblasts using GATA4, MEF2C, and TBX5 induced a myocardial program in these cells that integrated with the surrounding cardiomyocytes and attenuated infarct size (437). Delivery of SOX2 into an injured spinal cord, coupled with silencing of the p53

pathway, induced the reprogramming of glial cells into a diverse population of long-lived neurons (438). Others have reprogrammed acinar pancreatic cells into insulin-producing β-cells and converted myofibroblasts into functional hepatocytes that could alleviate liver fibrosis (439, 440). More importantly, *in vivo* cancer reprogramming was already demonstrated by converting hepatocellular carcinoma cells into hepatocytes (395). Despite the many examples and potential applications in regenerative medicine, it was only recently that direct reprogramming was leveraged for immunotherapy (411, 425). Although I did not explore non-integrative viral vectors or other nonviral delivery mechanisms for reprogramming, employing these alternatives may turn the cDC1 reprogramming process into a safer and scalable strategy for immunotherapy. In the future, it will also be interesting to compare macrophage reprogramming with cDC1 reprogramming *in vivo* and their potential for harnessing enhanced presentation of tumor antigens to trigger antitumor immune responses. Independently of the target cell, both cDC1 and macrophage reprogramming have demonstrated that it is possible to reprogram cancer cells into tumor-APCs and overcome immune evasion mechanisms.

Through the course of 4 studies published during my Ph.D. studies, I demonstrated that PU.1, IRF8, and BATF3 impose a cDC1-lineage identity in unrelated cell types from human and mouse origin. More importantly, the same factor combination enhances tumor immunogenicity by activating IFN, STING, and antigen presentation pathways in cancer cell lines and primary cancer tissue and promotes *in vivo* antitumor responses driven by cDC1-like functions. Moreover, reprogramming cancer cells into tumor-APCs can be accomplished by a single gene therapy strategy targeting many cancer types and intrinsic cancer heterogeneity. Lastly, cDC1 reprogramming *in situ* will combine the benefits of oncolytic virus, dendritic cell-based vaccines, and loss of tumorigenicity wrapped in one package. Despite the possible need for matching vector serotypes towards specific cancer types, PU.1, IRF8, and BATF3 can be used universally to convert tumor cells into tumor-APCs.

These data provided a proof-of-principle for engineering functional immune cell fates and their future utilization for cancer immunotherapy. In the future, cDC1 reprogramming can be translated into an “off-the-shelf” therapy, enforcing the presentation of patient-specific cancer antigens, and reflecting intrinsic tumor heterogeneity. Finally, the data I generated supports the development of alternative methods for *in vitro* tumor antigen-specific TIL expansion and the creation of novel platforms for the identification of tumor neoantigens based on enhanced antigen presentation by tumor-APCs.

MATERIALS AND METHODS

This section describes the main methods used in this dissertation. An expanded version of this section can be found in each published study's method section appended to this thesis.

Mice

Animal care and experimental procedures were performed in accordance with Swedish and Portuguese guidelines and regulations after approval from their respective local ethical committees. C57BL/6j females aged 6 to 10 weeks were acquired from Scanbur and Charles River. C57BL/6-Tg(TcraTcrb)1100Mjb/J (OT-I, The Jackson Laboratory) and B6.Cg-tg(TcraTcrb)425Cbn/J (OT-II, The Jackson Laboratory) mice were bred in-house. NOD-Prkdc^{scid}-IL2rg^{Tm1}/Rj (NXG) females aged 6 to 7 weeks were purchased from Janvier. Clec9a$^{Cre/Cre}$ (150) animals were crossed with Rosa26-stop$^{flox\text{-}tdTomato}$ reporter mice to generated double homozygous Clec9a$^{Cre/Cre}$ Rosa$^{tdTomato/tdTomato}$ (Clec9a-tdTomato). OTII-transgenic/Rag2 knockout (KO) (OT-II/Rag2KO) used in **Study I** were provided by L. Graça. All mice were kept under a fixed 12-hour light/dark cycle, under controlled temperature (23 ± 2 ºC), with free access to food and water.

MEFs isolation and culture

MEFs were isolated from embryos of Clec9a-tdTomato, or C57BL/6 mice were isolated as previously described (150, 339). A single-cell suspension was obtained and plated in 0.1% gelatin-coated 10-cm tissue culture dishes in growth media. Cells were grown for 2 to 3 days until full confluency, dissociated with TrypLE Express, and frozen in fetal bovine serum (FBS) and 10% dimethyl sulfoxide (Sigma). MEFs used for screening and in the following experiments were sorted for tdTomato- and CD45- with a purity of >99% and expanded up to four passages.

BM and spleen isolation.

Total BM cells were harvested from tibias and femurs by crushing with pestle and mortar. Freshly isolated spleens were homogenized using the frosted ends of two sterile slides or pushed against a nylon mesh. Cells were harvested in phosphate-buffered saline (PBS) supplemented with 2% FBS and filtered through a 70-µm cell

strainer (BD Biosciences). Red blood cells were lysed with BD Pharm Lyse (BD Biosciences) for 8 min at room temperature, protected from lysis. Lysis was stopped by the addition of volumes of PBS with 2% FBS.

Generation of BM-DCs.

Total BM cells were plated in petri dishes ($5x10^6$ cells per plate) in RPMI complete media supplemented with Flt3L (200 ng/mL), and GM-CSF (5 ng/mL) as previously described to generate cDC1-like CD103+ BM-DCs (165).

Cell culture.

LLC-OVA cells were generated by stable expression of truncated cytoplasmic OVA with pHAGE-cOVA-IRES-Puro. B16-mOrange and B16-OVA-mOrange were generated by transduction with SFFV-mOrange, and mOrange+ cells were purified by FACS. Both cell lines were expanded from single cell–sorted clones and used in experiments from passage 3 to 10 after transduction. Human embryonic kidney (HEK) 293T cells, MEFs, HEFs (passages 3 to 8), HDFs (passages 3 to 8), mouse cancer cell lines and human cancer cell lines were maintained in growth medium [Dulbecco's modified Eagle's medium supplemented with 10% (v/v) FBS, 2 mM l- glutamine, and antibiotics (penicillin and streptomycin, 10 μg/mL)]. Alternatively, some cells were cultured using RPMI 1640, Ham's Nutrient F10 Mixture (F10), and Ham's NutrientF12 Mixture (F12) growth supplemented as indicated before. B16-OVA complete growth medium culture was supplemented with geneticin (0.4 mg mL−1),whereas LLC-OVA was supplemented with puromycin (1×10^{-3} mg mL^{-1}). Primary CD8+ OT-I T cells, CD4+ T cells, and CD103+ BM-DCs were cultured in RPMI 1640 supplemented with 10% FBS, 1% penicillin-streptomycin, 1% GlutaMAX, 1% sodium pyruvate, and 2-mercaptoethanol (50 mmol dm^{-3}). Cell dissociation from tissue culture plates as done with TrypLE Express for 5 to 8 min at 37°C. All cells were cultured in a humid environment at 37°C and 5% CO_2. When mentioned, IFN-γ (1×10^{-9}mg mL^{-1}) (PeproTech), Poly(I:C) (10×10^{-3}mg mL−1) (InvivoGen), and LPS (1×10^{-4}mg mL^{-1}) (Enzo) were added 24 hours before analysis.

Molecular cloning and lentiviral production.

Coding regions of each candidate transcription factor were individually cloned into the pFUW-TetO vector where expression is under the control of the tetracycline operator and a minimal CMV promoter (FUW-TetO-TF) (339, 441). For the polycistronic vectors, coding sequences for mouse and human genes encoding PU.1, IRF8 and BATF3 were cloned together in the pFUW-TetO plasmid interspaced with 2A self-cleaving peptides (402). The first two coding sequences lacked the stop codon. A lentiviral vector containing the reverse tetracycline transactivator M2rtTA under the control of a constitutively active human ubiquitin C promoter (FUW-

M2rtTA) was used for co-transductions with pFUW-TetO constructs (339, 442). For constitutive overexpression, the polycistronic cassette was subcloned into lentiviral vectors with constitutive promoters: pFUW-UbC, pRRL.PPT-SFFV, pRRL.PPT-PGK, pRRL.PPT-EF1S, pHAGE2-EF1, and pWPXL-EF1i (gift from D. Trono; Addgene, plasmid # 12257) (187, 417, 443). To induce reprogramming in cancer cells, we used the lentiviral vectorpRRL.PPT.sf.PIB.i2eGFP expressing human or mouse transcription factors in polycistronic constructions. Empty pRRL.PPT.sf.MCS.i2eGFP served as a mock control throughout reprogramming experiments. For defined experiments, lentiviral vectors lacking IRES2-eGFP were used. HEK 293T cells were transfected with a mixture of transfer plasmid and packaging constructs expressing the viral packaging functions and the envelope VSV-G protein. The day before transfection,$7x10^6$ cells were seeded in a 15-cm dish to achieve approximately 80% confluence after 1 day. The next day, cells were transfected as follows: 10 μg of lentiviral vector, 7.5 μg of psPAX2.G lentiviral packaging vector, and 2.5μg of pMD2 envelope vector were combined with 60 μl of polyethylenimine (1 mg/mL) (PEI; linear 25 kDa, Polysciences) in 2 mL of OptiMEM (Thermo Fisher Scientific) and incubated for 15 min at room temperature. The PEI-OptiMEM mixture was added dropwise to HEK 293T cells and, 12 h later, replaced with fresh medium. Viral supernatants were collected 48, 60, and 72 h after transfection, filtered through a 0.45-μm cellulose acetate filter (low protein binding), concentrated with Amicon centrifugal filters (Millipore) or with the Lenti-X Concentrator (Takara) and stored at -80 ºC. Lentiviral titers were estimated with a Lenti-X qRT-PCR titration kit.

Viral transduction and reprogramming.

Clec9a-tdTomato or C57BL/6 MEFs, HEFs, and HDFs were seeded at a density of $4x10^4$ cells per well whereas MSCs were seeded at a $5x10^4$ cells per well on 0.1% gelatin-coated six-well plates. On the following day, cells were incubated overnight with a ratio of 1:1 FUW-TetO-TFs/FUW-M2rtTA, SFFV-eGFP or SFFV-PIB-eGFP lentiviral particles in media supplemented with polybrene (8 μg/mL). When testing combinations of transcription factors, equal multiplicities of infection of each individual viral particle were applied. Cells were transduced twice in consecutive days in the presence of polybrene (8 μg/mL), and media replaced in between transductions. After the second transduction with inducible vectors, growth media were supplemented with Dox (1 μg/mL), and this was considered day 0. Alternatively, cancer cells were seeded at 10^6 cells per 6-well plate and were transduced once 12 h after plating with constitutive vectors. Media were changed every 2 to 3 days for the duration of the cultures. For leukemic cell lines, polybrene was combined with spinfection (800g, 60 min, room temperature). When indicated, culture conditions included IFN-γ ($1x10^{-9}$ mg/mL), Poly(I:C) (25 μg/mL), LPS (100 ng/mL; Sigma), 2-mercaptoethanol ($1×10^4$ μM), l-glutamine (2 μmol/mL), GM-CSF (10 ng/mL), IL-4 (20 ng/mL), and Flt3L (100 ng/mL). Cytokines were added

at day 2 of reprogramming and kept for the duration of the process until analysis. LLC-OVA and B16-OVA were kept in antibiotic selection throughout reprogramming. Reprogramming efficiency was defined as the percentage of CD45+MHC-II/HLA-DR+ cells gated on live, transduced eGFP+ cells.

Flow cytometric analysis and FACS

For screening of candidate factors, transduced Clec9a-tdTomato MEFs were dissociated with TrypLE Express. For the analysis of surface marker expression, dissociated mouse and human cells were incubated with adequate antibodies diluted in PBS with 2% FBS at 4°C for 30 min in the presence of rat or mouse serum (1/100, GeneTex) to block unspecific binding. To exclude dead cells, 7-Aminoactinomycin D (7AAD, Thermo Fisher Scientific) or 4´,6-diamidino-2-phenylindole (DAPI) were added shortly before analysis or sorting. Alternatively, fixable viability dye eFluor520 or LIVE/DEAD Near IR Fixable Stain was used to determine live cells. Cells were washed and resuspended in PBS with 2% FBS and analyzed in Accuri C6, FACSAria III, LSR Fortessa or LSR Symphony A1 (BD Biosciences). Flow cytometry data were analyzed using FlowJo software. Unless stated otherwise, all flow cytometry analyses were performed in single live cell gates.

To purify C57BL/6 and Clec9a-tdTomato MEFs, cells were incubated cells at 4°C for 30 min with anti-CD45 antibody followed by sorting. For functional experiments, dendritic cells were first enriched using Pan-DC Enrichment beads (Miltenyi). For isolation of MEFs-derived reprogrammed dendritic cells, cells were dissociated using TrypLE Express and resuspended in PBS with 2% FBS, and tdTomato- or tdTomato+ cells were purified. When mentioned, cells were incubated with rat anti-mouse I-A/I-E (MHC-II) and CD45 antibodies in the presence of rat serum for 30 min at 4°C. Human dendritic cells were enriched from PBMCs by negative selection using magnetic-activated cell sorting (MACS) using the Pan-DC Enrichment Kit (Miltenyi Biotec) according to the manufacturer's protocol. HLA-DR+CD11C+CD141+ cDC1s, HLA-DR+CD11C+CD141−CD1C+ cDC2s, and HLA-DR+CD11C−CD123+ pDCs were sorted and used for single-cell RNA-seq profiling. To purify CD45+, CD45+HLA-DR−, CD45+HLA-DR+, and CD45+HLA- DR+CD226+hiDC1s, cells were dissociated using TrypLE Express; resuspended in PBS containing 2% FBS; incubated at 4°C for 30 min with anti-CD45, anti-HLA-DR, and anti-CD226 antibodies in the presence of mouse serum; and purified in FACSAria III. For isolation of human primary MSCs, lineage-depleted BM mononuclear cells were incubated in blocking buffer [PBS without Ca2/Mg2, human normal immuno-globulin (3.3 mg/mL) (Octapharma), and 1% FBS], followed by antibody staining. CD45−CD271+ MSCs were sorted and used for reprogramming experiments. All purifications using flow cytometry were performed in a FACSAriaIII machine (BD Biosciences).

Magnetic-activated cell sorting (MACS)

Purification of reprogrammed cancer cells for functional assays was performed either at day 5 or day 9 post-transduction. Cells were dissociated and resuspended in staining FACS buffer supplemented with 2% penicillin-streptomycin. Briefly, 10^7 cells were incubated with 2 μL rat serum for 15 min, followed by 5 min incubation with 60 ng rat anti-mouse MHC-II and CD45 coupled with biotin. Cells were washed twice before incubation with anti-biotin magnetic beads (Miltenyi Biotec) for 15 min. All incubations were performed on ice. Labeled cells were purified in LS MACS columns (Miltenyi) according to manufacturer's recommendations. Purity of enriched populations was assessed by washing and consequently staining with fluorescent-conjugated antibodies for CD45, MHC-II and MHC-I.

Fluorescence microscopy and immunofluorescence

Clec9a-driven tdTomato in MEFs was visualized directly on six-well plates under an inverted microscope (Zeiss AxioVert 200 M), and images were processed with AxioVision and Adobe Photoshop software. DAPI (1 μg/mL; Sigma) and phalloidin–Alexa Fluor 488 (50 μg/mL; Sigma) were used to stain nuclei and F-actin, respectively. Reprogrammed B16 cells were MACS-enriched and fixed with 4% paraformaldehyde for 20 min and permeabilized with 0.4% Triton X-100. Blocking for 30 min with 2.5% bovine serum albumin and 10% goat serum (Abcam), was followed by overnight incubation at 4 ºC with anti-mouse MHC-II and anti-mouse B2M. Cells were then washed and incubated with secondary antibodies coupled with fluorochromes (Invitrogen) for 1 h 30 min. Cells were washed and imaged on CellDiscoverer 7 microscope (Zeiss), at 20x magnification. Data acquisition, image analysis and export were performed with the ZEN 2.5 blue software (Zeiss).

Live Cell Imaging

$5x10^3$ MACS-purified PIB-transduced LLC cells expressing either CD45 or MHC-II were seeded per well on a black 96-well μ-Plate (Ibidi) and incubated in the presence of $10x10^{-3}$ mg/mL AlexaFluor-647-labeled OVA (Thermo Fisher) or 0.1 mg/mL DQ-OVA (Thermo Fisher), followed by continuous recording for up to 4 hours. All live cell imaging was performed using the CellDiscoverer 7 microscope (Zeiss). Acquisition, image analysis and export were performed with ZEN 2.5 blue edition software (Zeiss).

Scanning electron microscopy

T98G-derived tumor-APCs and control eGFP+ cells were FACS-purified 9 days after transduction with PU.1, IRF8, and BATF3 or eGFP vectors respectively, plated in 0.1% gelatin-coated coverslips, cultured overnight, and prepared as described previously (403). Analysis was performed with Jeol JSM-7800F FEG-SEM.

Antigen uptake and processing analysis

Reprogrammed cells were resuspended at $1x10^6$ cells/mL (LLC, MACS-purified) or at $0.5x10^6$ cells/mL (T98G) of growth medium, followed by addition of $10x10^3$ mg/mL OVA-AF647 or 0.1 mg/mL of DQ-OVA and incubated at 37 ºC for 30-60 min. Fluorescence microscopy required up to 2 h of incubation for imaging OVA uptake and up to 4 h of incubation for visualization of OVA processing. Alternatively, the experiment was conducted on ice to inhibit active antigen uptake and processing. Changes in fluorescence referring to the internalization of labeled ovalbumin were analyzed by flow cytometry.

Dead cell phagocytosis

HEK 293T cells were exposed to ultraviolet irradiation (50 J/m2) to induce cell death and labeled with the CellVue Claret Far Red Fluorescent Cell Linker Kit (Sigma). Purified tdTomato+ and tdTomato- populations at day 10 and PIBpoly-transduced HEFs at day 9 were incubated with far red–labeled dead cells overnight, washed with PBS with 2% FBS, and analyzed in FACSAria III. DAPI staining was used to exclude floating or membrane-adherent dead cells. Dead cell incorporation was quantified in live tdTomato- and tdTomato+ cells or HLA-DR+ and HLA-DR− cells using far red staining. Alternatively, B16 or LLC cells were harvested, washed, and exposed to UV light (45 min/3x10 min with 24 h intervals. Dead cells were fluorescently labeled with CellVue Claret Far Red Fluorescent Cell Linker Kit (Sigma). Reprogrammed human eGFP+CD45+HLA-DR+ or mouse eGFP+CD45+MHC-II+ and control eGFP+ T98G/LLC cells were FACS-sorted on day 9 of reprogramming were incubated with dead cells for 4 to 8 h before dead cell phagocytosis was evaluated by flow cytometric analysis.

Cytokine expression and secretion analysis

Levels of IL-6, IL-10, and TNF-α were assessed in supernatants of purified tdTomato+ cells at day 9 or in control MEFs 10 days after doxycycline. Day 9 reprogrammed cells (T98G, FACS-purified 10 000/well eGFP+CD45+HLA-DR+ or eGFP+CD45-HLA-DR+ or bulk primary melanoma cells, 20 000/well) were seeded into flat bottom 96-well plates. When indicated, cells were stimulated overnight with LPS (100 mg/mL), Poly(I:C) (25 μg/mL), and R848 (5 μg/mL) (Invivogen). Subsequently, 50 μl of culture supernatants were harvested and processed with LEGENDplex Human Inflammation Panel 1 (Biolegend) according to manufacturer's instructions. eGFP transduced cells were used as controls. Human dendritic cells (separated by negative selection using pan-DC enrichment kit (Miltenyi Biotec) from healthy peripheral blood) and moDCs served as reference. Alternatively, mouse CD103+ BM-DCs, non-transduced, eGFP transduced, and reprogrammed CD45+MHC-II+ MACS-enriched B16 and LLC cells were seeded at a density of $6.5x10^4$ cells/well in 96-well plates and incubated overnight in the presence or absence of Poly(I:C). Supernatants were collected 10 h post-seeding and

analyzed with LegendPlex Mouse Anti-Virus Response Panel (13-plex) or CBA Mouse Inflammation kit (BD Biosciences) according to manufacturer's protocol. Triplicates were performed per condition. Analysis was performed on Accuri C6, FACSCanto, or LSR Fortessa (BD Biosciences) and data were analyzed using FCAP (BD Biosciences) or LEGENDplex (Bio-Legend) softwares. For IL12p40, PIB-transduced Clec9a- tdTomato MEFs at day 9 were incubated overnight in the presence of LPS (100 ng/mL), profilin (100 ng/mL) (Sigma), and CD40L (1 μg/mL; BioLegend). On the following day, Golgiplug (1 μl/mL; BD Biosciences) was added and incubated at 37 °C for 4 hours. Cells were then harvested and stained intracellularly for IL-12p40 using Cytofix/Cytoperm (BD Biosciences).

Western Blot

C57BL/6 MEFs transduced with polycistronic vectors were harvested 48 hours after the addition of Dox. Cells were resuspended in radio-immunoprecipitation assay buffer (Thermo Scientific) for 20 min, and protein extracts were diluted 1:2 in Laemmli buffer (Bio-Rad) with 5% 2-ME (Sigma) and boiled at 98 °C for 8 min. Samples were run in NuPAGE 4 to 12% bis-tris (Invitrogen) SDS-PAGE gels using XCell Sure Lock (Invitrogen) and MOPS SDS running buffer (Invitrogen). Transfer was done using iBlot (Thermo Scientific) dry system for 7 min. Membranes were incubated overnight with unconjugated primary antibodies against PU.1, IRF8, or calnexin and with donkey anti-rabbit horseradish peroxidase–conjugated secondary antibody diluted at 1:4000. Membranes were incubated with ECL prime (Amersham) for 5 min, and data were acquired in ChemiDoc (Bio-Rad).

Population RNA-seq library preparation and sequencing

For population RNA-seq, total RNA from 3000 to 5000 FACS sorted iDCs, mouse and human cancer cells lines and respective tumor-APCs, HEFs and hiDCs. was extracted with TRIzol reagent (Thermo Fisher Scientific). cDNA was generated with the SMARTSeq v4 Ultra-low input RNA kit (Takara) and amplified with 8 PCR cycles and purified with AMPure XP beads (Beckman). cDNA was analyzed using the Agilent High sensitivity DNA kit on an Agilent Bioanalyzer 2100 (Agilent). Library preparation was performed using the Nextera XT DNA library preparation kit (Illumina) according to the manufacturer's protocol. Following tagmentation of cDNA, forward and reverse indexes were added by 12 cycles of PCR. The libraries were normalized with beads, pooled, and sequenced on an Illumina NextSeq 500 machine (75-bp paired-end).

RNA-seq analysis

Paired-end reads were mapped to the human or murine genomes (Ensembl, release 93) using STAR v2.5.3a (444) with default settings except sjdbOverhang 74 --quantMode GeneCounts. Resulting gene counts were further processed with R package DESeq2 (445) and normalized using RLE method. DESeq2 package and

used for performing differential expression analysis based on Wald test. We defined upregulated genes by a fold change (FC) > 0 and Benjamini Hochberg (BH) corrected p-value < 0.05 and downregulated genes by FC < 0 and BH-corrected p-value < 0.05. PCA was performed using plotPCA function from DESeq2 package. Normalized counts were log2 transformed and visualized using ggplot2 R package (https://ggplot2.tidyverse.org/) or GraphPad Prism software.

Dendritic cell-specific gene list was defined as differential expression behavior between cDC1 and cDC2, cDC1 and pDC, cDC2 and pDC (adjusted P-value < 0.1 and log2foldChange > 0.5) and excluding genes that were over expressed in MEFs compared to cDC1, cDC2 and pDC (log2foldChange > 0.5). To find the relationship between all samples we calculated Pearson correlation and clustered the samples using method Ward.d2. The resulting clustering was reordered according to first principal component and visualized using pheatmap package. Also, principal component analysis was performed to explore relationships among day 5, 7, 8 and 9 iDC samples.

Gene set enrichment analysis

GSEA between all possible conditions and states of HEFs-derived iDCs were performed against C7: immunologic signatures from the Molecular Signatures Database. Alternatively, GSEA was performed using successful and unsuccessful cDC1 reprogramming gene signatures. The analysis was performed using normalized UMI counts using default parameters, P = 1 for calculation of enrichment statistic, normalized enrichment scores, and rank by calculating difference of means scaled by the SD.

For the human cancer cell dataset, functional enrichment analysis was performed for differentially expressed genes for each cancer cell line and tumor-APC signature using clusterProfiler (BH-adjusted P < 0.1) (446) and top 6 categories were selected. For the mouse cancer dataset, gene list enrichment analyses were generated with KEGG pathways, gene ontologies, biological processes and molecular functions were obtained through EnrichR (447). Input gene set consisted of commonly upregulated genes in all murine reprogramming processes (foldchange >9, 27 genes). Results for each database were ordered based on log10(p-value) and the top 6 pathways, processes and functions plotted accordingly.

Tumor-APC signature

The tumor-APC signature was defined as commonly upregulated genes between CD45+HLA-DR+ cells at day 9 and eGFP transduced cells (day 0), in at least 75% of human cancer lines. To order cell lines by reprogramming efficiency, we calculated for each gene the average difference between day 9 and day 0 and normalized it to the difference between cDC1 and day 0 for individual cancer cell lines. The median for each cancer cell line was used as reprogramming efficiency.

Antigen presentation signature

Gene lists for antigen presentation gene, IFN-γ and STING pathways were curated based on human or murine KEGG pathways list for antigen presentation, cytosolic-DNA sensing pathway, and literature review. The expression value of those genes was z-transformed and subjected to MinMax function (values higher than defined Max value were set to Max value, and lower than Min value were set to Min value). Gene lists were clustered using complete linkage method and visualized using pheatmap R package (https://cran.r-project.org/web/packages/pheatmap/index.html). To estimate activation of antigen presentation signature, we used a similar procedure as described for tumor-APC signature gene list.

Single-cell RNA sequencing

HEFs, hiDCs at d3, d6, and d9 (CD45+HLA-DR− and CD45+HLA-DR+); cDC1s; cDC2s; and pDCs from peripheral blood (from three individual donors) were FACS sorted for single-cell RNA-seq. Purified cells were loaded on a 10X Chromium (10X Genomics) according to the manufacturer's protocol. hiDC1s at d9 reprogrammed in the presence and absence of cytokines from HEFs and HDFs and CD45+HLA- DR+CD226+ hiDC1s were also profiled. Alternatively, single-cell RNA-seq was performed in T98G cells transduced with PIB-eGFP or with eGFP lentiviral particles (day 0). Nine days after reprogramming, 5000 to 10,000 transduced eGFP+ cells expressing at least one of the reprogramming markers,CD45 or HLA-DR, were FACS-purified and resuspended in PBS containing 0.04% bovine serum albumin (BSA). Day 0 controls were processed similarly. Cells were loaded on a 10x Chromium (10x Genomics) without multiplexing. Single-cell RNA libraries were prepared using the Chromium Single Cell 3+ v2 Reagent Kit (10x Genomics). Indexed sequencing libraries were quantified with a High Sensitivity DNA analysis kit (Agilent) and Agilent Bioanalyzer. Indexed libraries were pooled in an equimolar ratio and sequenced with an Illumina NextSeq 500 machine. Coverage was between 40,000 and 200,000 reads per single cell.

Dendritic cell subset classification

We used scPred (189) classification algorithm and two publicly available dendritic cell single-cell expression datasets (188, 223) for subset affiliation. To train the classifier using the scPred method (implemented as R library), we used the default parameters for get FeatureSpace and trainModel, as defined in tool vignette. To predict the assignment of dendritic cells isolated from PBMCs to publicly availably dendritic cell subsets, we used the scPredict function with default parameters. Alternatively, training of classifiers with available dendritic cell data was performed using 7,000 variable genes. Next, to adjust for cancer line expression background, we normalized cells by gene expression levels in non-reprogrammed

cells. We then classified normalized expression levels from the reprogramming dataset. We used a probability threshold of 0.99 to classify cells into classes. For classification of iDCs, we used the scPredict function with a threshold of 0.99 separately for each donor and then combined the number of cells affiliated to each subset.

Chromatin immunoprecipitation sequencing.

HDFs were transduced with TetO-PIB or vectors encoding individual factors (TetO-PU.1, TetO-IRF8, or TetO-BATF3) and M2rtTA. Transduced HDFs were expanded, and ChIP was performed 48 h after the addition of Dox using antibodies for human PU.1, IRF8, and BATF3.

ChIP-seq analysis and data visualization

ChIP-seq analysis was performed on the raw FASTQ files. FASTQ files were mapped to the human hg38 genome using the Bowtie 2 program, allowing for 2-bp mismatches. Mapped output files were processed through MACS v2.1.0 analysis software to determine peaks. Peak annotation was performed using ChIPseeker R library. For genome tracks, bigwig files were created from bam files with deepTools (https://deeptools.readthedocs.io/en/develop/) and explored using the UCSC Genome Browser. For chromatin state fold enrichment analysis, enrichment scores for genomic features, such as PU.1, IRF8, and BATF3 ChIP-seq peaks and histone marks, were calculated using the ChromHMM Overlap Enrichment (http://compbio.mit.edu/ChromHMM/). ChromHMM segmentation, containing 18 different chromatin states, was down-loaded from the Roadmap website (www.roadmapepigenomics.org/tools) and used for analysis. Enrichment scores were calculated as the ratio between the observed and the expected overlap for each feature and chromatin state based on their sizes and the size of the human genome. For *de novo* motif discovery, findMotifsGenome.pl procedure from HOMER was used on PU.1, IRF8, and BATF3 separately. HOMER was run using default parameters and input sequences comprising ±100 bp from the center of the top 2500 peaks. Co-bound regions by PIB were found using the findOverlapsOfPeaks function in the ChIPpeakAnno R library (www.biomedcentral.com/1471-2105/11/237). Co-bound regions were used for *de novo* motif discovery using HOMER. To evaluate the similarity of the two sets based on the intersections, we calculated the Jaccard statistic using MACRO-APE (https://opera.autosome.ru/macroape/compare). To produce the heatmaps and profile plots, we used deepTools in the reference-point mode where each feature (such as peaks of a transcription factor or histone marks) was aligned at PU.1, IRF8, or BATF3 summits and tiled the flanking up- and downstream regions within ±4 kb.

Purification of MHC-I

Anti–MHC-I monoclonal antibody (28-8-6S) was purified from the supernatant of HB-51 hybridoma cells (ATCC) using Protein A–Sepharose 4B beads (Invitrogen). Antibodies were cross-linked to Protein A–Sepharose 4B beads at a concentration of 5 mg of antibodies per 1 mL volume of beads. For this purpose, the antibodies were incubated with the Protein A–Sepharose 4B beads for 1 h at room temperature. Chemical cross-linking was performed by the addition of dimethyl pimelimidate dihydrochloride (Sigma-Aldrich) in 0.2 M sodium borate buffer pH 9 (Sigma-Aldrich) at a final concentration of 20 mM for 30 min. The reaction was quenched by incubation with 0.2 M ethanolamine pH 8 (Sigma-Aldrich) for 2 h. Cross-linked antibodies were kept at 4°C until use. To improve sensitivity, two groups of samples were processed: 3 biological replicates of $2x10^8$/each B16 cells intended for data dependent acquisition (DDA) measurement to generate a spectral library generation, and 3 biological replicates of samples of 10^6 sorted B16 cells that were either GFP transduced, reprogrammed, or IFN-γ treated, and control cells, for data independent acquisition (DIA) measurement. B16 cells were lysed in phosphate buffered saline containing 0.50% sodium deoxycholate (Sigma-Aldrich), 0.2 mM iodoacetamide (Sigma-Aldrich), 1 mM EDTA, 1:200 Protease Inhibitor Cocktail (Sigma-Aldrich), 1 mM phenylmethylsulfonylfluoride (Roche), and 1% octyl-β-D glucopyranoside (Sigma-Aldrich) at 4°C for 1 h. Samples of $2x10^8$ cells were lysed in 4 mL, samples of $1x10^6$ cells in 1 mL lysis buffer. Lysates were cleared by centrifugation with a table-top centrifuge (Eppendorf Centrifuge) at 4°C at 20,000 g for 50 min. MHC-I molecules were purified by incubating the cleared lysates with HIB antibodies cross-linked to Protein A–Sepharose 4B beads in affinity columns for 3h at 4°C. 300 µL of beads were used for samples of $2x10^8$ and 200 µL for samples of 10^6 cells. The affinity columns were then washed as follows: 2 column volumes of 150 mM NaCl in 20 mM Tris-HCl pH 8, 2 column volumes of 400 mM NaCl in 20 mM Tris-HCl pH 8, and again 2 column volumes of 150 mM sodium chloride in 20 mM Tris-HCl pH 8. Finally, the beads were washed in 1 column volume of 20 mM Tris-HCl pH 8. MHC complexes and the bound peptides were eluted at room temperature by adding twice 500 µL of 1% trifluoroacetic acid (TFA). Sep-Pak tC18 96-well plates (Waters), preconditioned with 1 mL of 80% acetonitrile (ACN) in 0.1% TFA and then with 2 mL of 0.1% TFA, were used for the purification and concentration of MHC-I peptides. Elutions containing MHC-I molecules and peptides were loaded in the Sep-Pak tC18 96-well plates and the C18 wells were then washed with 2 mL of 0.1% TFA. The MHC-I peptides were eluted twice with 250 µL of 28% ACN in 0.1% TFA. MHC-I peptides containing elutions were transferred into Eppendorf tubes. Recovered peptides were dried using vacuum centrifugation (Thermo Fisher Scientific) and stored at −20°C.

Mass spectrometry acquisition of immunopeptidome data.

Prior to MS analysis, MHC-I peptide samples were resuspended in 8µL of 2% ACN and 0.1% formic acid (FA), and 10% iRT suspension (Biognosys AG). Then, two technical replicates of 3 µL per biological samples were loaded on the column for each measurement by LC-MS/MS. The LC-MS system consists of an Easy-nLC 1200 (Thermo Fisher Scientific) coupled online to Q Exactive HF and or HF-X mass spectrometer (Thermo Fisher Scientific). Peptides were separated on a 450-mm homemade column of 75-µm inner diameter packed with ReproSil Pur C18–AQ 1.9-µm resin (Dr. Maisch GmbH). For DDA the analytical separation was performed for a period of 130 min using a gradient of H_2O/FA 99.9%/0.1% (solvent A) and CH_3CN/FA 80%/0.1% (solvent B). The gradient was run in sequential, linear steps: 0 to 110 min (2%-25% B), 110 to 114 min (25%-35% B), 114 to 115 min (35%-100% B), and 115 to 130 min (constant at 100% B) at a flow rate of 250 nL/minute. The mass spectrometer was operated as follows: full-scan MS spectra were acquired from m/z = 300–1,650 at a resolution of 60,000 (m/z = 200) with a maximum injection time of 80 ms. The auto gain control (AGC) target value was set to 3×10^6 ions. MS/MS spectra were acquired at a resolution of 30 000 (m/z = 200) using a top 20 method with an isolation window of 1.2 m/z, and a collision energy of 27 (HCD). For all MS/MS scans ions were accumulated to an AGC target value of $2x10^5$ with a maximum injection time of 120 ms. Precursors with a charge of 4 or more were excluded from fragmentation. Dynamic exclusion was set to 20s. For DIA the analytical separation was performed for a period of 65 min using a gradient of H_2O/FA 99.9%/0.1% (solvent A) and CH_3CN/FA 80%/0.1% (solvent B). The gradient was run in sequential, linear steps: 0 to 52 min (2%-25% B), 52 to 54 min (25%-35% B), 54 to 55 min (35%-100% B), and 55 to 65 min (constant at 100% B) at a flow rate of 250 nL/minute. The DIA method consisted of one full-scan MS spectra, acquired from m/z = 300–1,650 (Resolution = 60 000, ion accumulation time = 60 ms), and 22 MS/MS scans. For each MS/MS scan the automatic gain control (AGC) target value was set to 3×10^6 ion, resolution was set at 30 000 and a stepped normalized collision energy (25.5, 27, 30) approach was used. Maximum ion accumulation was set to auto, fixed first mass to 200 m/z. The overlap between consecutive MS/MS scans was 1 m/z.

MS data processing.

All DDA files were used to generate a library with a database search using fragpipe v17.1, including MSFragger v3.5. The FASTA file contained all reviewed mouse uniprot entries, as well as the manually added uniprot sequences P11269, P03386, Q3UFS3, and Q2TA50. The search space comprised unspecifically digested peptides with a length of 8 to 14 amino acids. Variable modifications included methionine oxidation and N-terminal acetylation and no fixed modifications were applied. Maximal precursor and fragment ion deviation was set to 20 ppm. Peptide, ion, and peptide spectrum match FDRs were set to 0.01, no

protein FDR was applied. DIA files were searched with the library described above using SpectroNaut 16 with a precursor q-value cut-off of 0.01.

MS data analysis

Peptide binding affinities were predicted with NetMHCpan (448) and MixMHC (449). Peptides were considered as binders when they received a rank percentage of 2% or less by either of the two tools for at least one allele. The intensity of each peptide and the number of identifications per biological replicate were averaged for each replicate. MHC-I peptides predicted as binders were ranked by intensity (highest intensity = rank 1). The ranks were then normalized by dividing each rank by the total amount of binders per sample and subsequently transformed ($f(x) = x - 1$), yielding in an intensity score that ranged from 0 to 1, where 0 indicates the lowest intensity in a sample and 1 indicates the highest intensity in a sample. Data analyses were done with Julia and R, visualizations were created with R.

T cell killing assays

CD8+ T cells from spleen of OT-I mice were enriched using a mouse CD8+ T cell isolation kit (Miltenyi Biotec) according to manufacturer's protocol. 6-well untreated plates were coated with anti-CD3 and anti-CD28 at $2x10^{-3}$ mg mL^{-1} for 2 h at 37 ºC and washed 3x before seeding $1x10^6$ T cells per mL in complete growth media (RPMI) supplemented with murine IL-2 (Peprotech, 100 U mL^{-1}) and IL-12p70 (Peprotech, $2.5x10^{-3}$ mg/mL). After 24 h of activation, T cells were re-seeded at $1x10^6$ cells per mL in complete RPMI supplemented with murine IL-2 for 48 h on new untreated plates to allow T cell expansion. MACS-sorted reprogrammed mOrange+ B16-OVA cells or IFN-γ treated cells were seeded with non-fluorescent B16-OVA (mOrange-) in equal numbers, 24 hours before co-culture with T cells. Expanded T cells were added in ratios of 0:1, 1:1, 5:1, 10:1 T cell to target cell. B16 cells not expressing OVA were used to assess assay specificity. For flow cytometric analysis, cells were resuspended and stained for viability (DAPI) and anti-CD3 and measured at indicated time points post co-culture with T cells**.**

Naïve CD8+ T cell isolation, T cell priming and cross-presentation assays.

CD8+ T cells from spleen of OT-I mice were enriched using a naïve mouse CD8+ T cell Isolation kit (Miltenyi). Enriched CD8+ T cells were labeled with 5 μM CTV (Thermo Fisher) at room temperature for 20 min, washed, and counted. FACS-sorted tdTomato+ and tdTomato- cells at day 8, MEFs, freshly isolated splenic cDC1 cells (CD11c+ MHC-II+CD8+), and CD103+ BM-DCs were incubated at 37 °C with OVA protein (100 μg/mL). Alternatively, MACS-sorted reprogrammed tumor cells, non-reprogrammed cancer cells, eGFP-transduced cancer cells, and CD103+BM-DCs were incubated at 37°C with OVA peptide (SIINFEKL, T cell priming assays) or protein (cross-presentation assays). OVA-

expressing cancer cells were not incubated with exogenous OVA. Cells were incubated overnight with Poly(I:C) or IFN-γ, where indicated. After extensive washing, 20^4 MEFs-derived APCs or $5x10^3$ tumor-APCs were incubated with 10^5 CTV-labeled OT-I CD8+ T cells in 96-well round- bottom tissue culture plates with Poly(I:C) (25 μg/mL). After 3 days of coculture, T cells were collected, stained, and analyzed in BD LSRFortessa. T cell proliferation (dilution of CTV staining) and activation (CD44 expression) were determined by gating live, single, TCRβ+ CD8+ T cells. The threshold for data plotting was fixed at 1000 events within live cell gating.

Naïve CD4+ T cell isolation and antigen presentation.

CD4+ T cell isolation and antigen-presenting assays CD4+ T cells from spleen of OT-II/Rag2KO mice were enriched using the Dynabeads Untouched Mouse CD4 Cells Kit (BD Biosciences). Enriched CD4+ T cells (purity, ≥85%) were labeled with 5 μM CFSE at room temperature for 10 min, washed, and counted. $2x10^4$ PIB-transduced MEFs or $2x10^4$ splenic CD11c+ MHC-II+ cells were incubated with $2x10^4$ CFSE-labeled OT-II CD4+ T cells in 96-well round-bottom tissue culture plates with OVA protein (10 μg/mL) or the OVA323-339 peptide (10 μg/mL) in the presence or absence of LPS (100 ng/mL). After 7 days of coculture, T cells were collected and stained for CD44. T cell proliferation (dilution of CFSE staining) and activation (CD44 expression) were analyzed in Accuri C6.

Alternatively, naïve CD4+ T cells were recovered from freshly isolated spleens from OT-II mice using Naïve CD4+ T cell isolation kit (Miltenyi). Isolated T cells were labeled with CTV as previously described for naïve CD8+ T cells and following manufacturer's protocol. $1x10^4$ MACS-purified tumor-APCs and CD103+ BM-DCs were plated in round-bottom 96-well plate and incubated with OVA323-339 overnight. APCs were stimulated with Poly(I:C), where indicated. CTV-labelled naïve CD4+ T cells (10^5 cells) were co-cultured with APCs for 4 days before flow cytometric analysis. T cell proliferation was measured as described for CD8+ T cells using CD44+ and CTV dilution as markers for T cell expansion and activation gated in live, single, TCRβ+, CD4+ T cells.

Tumor establishment and treatment.

To establish tumors, $2×10^5$ B16OVA were injected subcutaneously into the right flanks of recipient mice. For B16-OVA tumor challenges, 6- to 10-week-old age-matched C57BL/6 females were used. Tumor volumes were monitored with a digital caliper and calculated using the formula V=L×W×H× 1/2. Survival was determined by predefined endpoints (tumor size >1500 mm3, tumor ulceration, and signs of animal suffering). For all treatments with tumor-APCs, cancer cells were transduced with SFFV-PIB and purified by MACS with anti-CD45 and anti–MHC-II antibodies at day 5. Twenty-four hours before injection, tumor-APCs, or control cells were stimulated with Poly(I:C). For B16-OVA tumor experiments, B16-

derived tumor-APCs were additionally pulsed with OVA protein and washed extensively. For combinatorial treatment with ICIs, mice received 200 μg of anti–PD-1 (Bio X Cell, clone 29F.1A12) and 200 μg of anti–CTLA-4 (Bio X Cell, clone 9H10) or rat immunoglobulin G2a (IgG2a) (Bio X Cell, clone 2A3) and IgG2b (Bio X Cell, cloneLTF-2) isotype control antibodies intraperitoneally at days 7, 10, and 13. Animals were randomized after tumor inoculation before further treatments.

T cell isolation from PBMCs and tetramer staining

14 days after B16-OVA tumor establishment and 7 days after the first intra-tumoral tumor-APC injection, peripheral blood was collected from the tail vein into heparin tubes to prevent clotting. Erythrocytes were lysed using BD Pharm Lyse according to the manufacturer's protocol and cells labeled with H-2Kb OVA (SIINFEKL) or H-2Kb MuLV p15E (KSPWFTTL) tetramers along with anti-CD45, anti-CD8 or anti-TCR antibodies.

Immunophenotyping of tumors, tumor-draining and non-draining lymph nodes

On day 18, tumors were excised and chopped into pieces of 2 mm diameter. Tumor tissue was further mechanically and enzymatically processed for 1 h at 37°C using a digestion mixture of 1 mg/mL Collagenase D (Sigma-Aldrich), 10 μg/mL DNase I (Sigma-Aldrich) and 5 mM $MgCl_2$ in PBS under rotation. The resulting cell suspension was passed through a 70μm filter and divided equally for flow cytometric analysis using separate antibody staining panels. The lymphoid panel included antibodies for CD45, TCRβ, CD8α, CD4, CD44, PD-1 and NK1.1. The myeloid panel included antibodies for CD45, CD11c, MHC-II, CD64, Ly6C, CD103, CD11b, Ly6G. Populations were defined according to: CD8+ T cells (CD45+TCRb+CD8+), CD4+ T cells (CD45+TCRb+CD4+), NK cells (CD45+NK1.1+), neutrophils (CD45+MHC-II-CD11b+Ly6G+), monocytes (CD45+MHC-II-CD11b+Ly6c+), macrophages (CD45+MHC-II+CD11c+Ly6c-CD64+) and dendritic cells (CD45+MHC-II+CD11c+CD64-Ly6c-CD11b+).

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GABRIELA FERREIRA was born in 1993 in Braga, Portugal. Her goal to become a scientist led her to study Biomedical Sciences, followed by a master's degree in Molecular Genetics. During her studies, she found her passion for immunology, genetic engineering, and cancer biology. In late 2017 she was accepted to the Ph.D. programme in Experimental Biology and Biomedicine and soon after that, enrolled in a joint degree between Lund University, Sweden, and Universidade de Coimbra, Portugal. The work in this dissertation focused on exploring direct reprogramming strategies to increase tumor immunogenicity and develop new strategies for immunotherapy.

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