Deciphering myeloid (progenitor) cell function and communication in (tumor) tissues

Lei, X.

Chapter 1

General Introduction and thesis outline

Myeloid cells that develop from hematopoietic precursors during a process called myelopoiesis, include granulocytes, monocytes, macrophages (MΦ), osteoclasts and dendritic cells (DC). Myeloid cells, in particular DC, play a crucial role in bridging innate immunity with adaptive immunity[1,2]. Emerging evidence indicates that beyond direct interaction with tumor cells or tumor stroma[3,4], the interactionn of myeloid cells with adaptive immune cells regulates cancer progression. Therefore, understanding how these cells and their precursors are regulated both locally and systemically is of great importance in the field of cancer immunology and immunotherapy.

Myelopoiesis and DC ontogeny

To maintain homeostasis of the mature blood system, strict regulation is required in processes controlling self-renewal, lineage specification, and differentiation of their precursors in the bone marrow (BM)[5,6]. Over the last decades, a classical model of cell differentiation was established for myelopoiesis at steady state in the adult individual. Following this model, the various myeloid lineage cells follow a hierarchical scheme starting with hematopoietic stem cells (HSC), followed by more dedicated multipotent progenitor (MPP), common myelopoietic progenitor (CMP)[7] and granulocyte/monocyte/macrophage progenitor (GMP) which is also defined as granulocyte/monocyte/osteoclast/DC progenitor (GMODP)[8], then unipotent progenitors such as the common monocyte progenitor (cMoP)[9] and common DC progenitor (CDP)[10]. The development of myeloid lineages is linked to distinct transcriptional factors (TF)[6,11], such as PU.1, and the CEBP and IRF families[2,6,12]. Notably, a continuum model has been proposed in recent years due to advances in single-cell (sc) technologies (Fig. 1). This model describes a progressive loss of multilineage differentiation potential of hematopoietic precursors at various developmental stages, suggesting that commitment to a restricted set of lineages can occur as early as the multipotent progenitor (MPP) stage, and continuously throughout hematopoiesis[13–15].

The DC, that is originally defined by its distinct morphology[16], is one of the cell types arising from myelopoiesis. DC subset identity is defined by ontogeny, combined with a certain level of tissue imprinting[11]. The traditional view is that three major DC subsets develop under homeostatic conditions: plasmacytoid (p)DC, classical DC type 2 (cDC2) and cDC1. The development of each of these DC lineages is controlled by distinct TF[6,11], such as *ZEB2* and *IRF8* for pDC, *IRF4* and *KLF4* for cDC2, and *BATF3* and *IRF8* for cDC1 (Fig. 1). These DC lineages are distinct from monocyte-derived DC

(moDC), that are derived from monocytes under inflammatory conditions[2,17,18]. The four main DC lineages can also be discerned by their key surface markers in combination with shared major histocompatibility class (MHC)-II expression. pDC express CD123, CD303, and CD304. cDC2 express CD1c and SIRPα. cDC1 express CD141, XCR1, and CLEC9A. moDC express CD14, CD206 and CD1a[19]. Recent studies have revealed remarkable homogeneity of cDC1 as a subset present in all healthy and diseased tissues analyzed, whereas cDC2 are found to exhibit heterogeneity[11,18,20]. Single cell (sc)RNA-sequencing (seq) analysis[17,18] identified a $CD1c^+$ DC population expressing monocyte-related genes (i.e., *FCN1, CD14*, and *CD163*) alongside genes associated with classical cDC2 (i.e., *CD1c, FCER1A*, and *CLEC10A*). Cytlak *et al*.[20] have further demonstrated that the cDC2 subset can be discerned into $CD1c^+CD163^-$ cDC2A and $CD1c^+CD163^+$ cDC2B subsets that develop along distinct hematopoietic trajectories. cDC2A arise from $IRF8^{high}CD123^+$ GMP together with pDC and cDC1, whereas cDC2B and monocytes derive from $IRF8^{low}CD33^+$ GMP (Fig. 1).

Modulation and adaption of myelopoiesis is an integral part of an optimized immune response toward pathogens or sterile insults. During infection/inflammation, steady-state myelopoiesis undergoes a transition to emergency myelopoiesis by launching a unique hematopoietic response program that is aimed at greatly increasing myeloid cell output to meet the heightened demand[21,22]. This transition can be driven by pattern-recognition receptors (PRR) expressed by hematopoietic and non-hematopoietic cells in the bone marrow (BM) niches and affected tissues. These stress sensors induce the production of proinflammatory cytokines[21–23] and chemokines[24–26]. Myeloid progenitor cells also alter their expression of cytokine[27] and chemokine[26,28,29] receptors. Collectively, these effects lead to increased myeloid cell production in the BM, and the migration of both myeloid progenitors and differentiated myeloid cells into the affected tissues. Evidence also suggests enhanced proliferation and differentiation of myeloid progenitor cells *in situ*[26,30]. Of note, despite the obvious beneficial effects of inflammation-induced activation of the hematopoietic system, increasing evidence also points to detrimental effects of chronic inflammatory stress[31–33], such as cancer[34] on the functions of hematopoietic progenitor cells. Apart from external stress, somatic driver mutations can also alter the functions of myeloid progenitor cells. This can lead to the clonal dominance of a subset of myeloid progenitors and their progenies, a process referred to as clonal myelopoiesis. Clonal myelopoiesis has been linked to a proinflammatory phenotype of hematopoietic cells, immune dysfunction and myeloid neoplasia[35,36]. Although several studies demonstrated the involvement of NLRP3 inflammasome[37] and inflammatory cytokines[36], the

mechanistic connections between clonal myelopoiesis and functional dysregulation of myeloid progenitor cells remain active subjects of investigation.

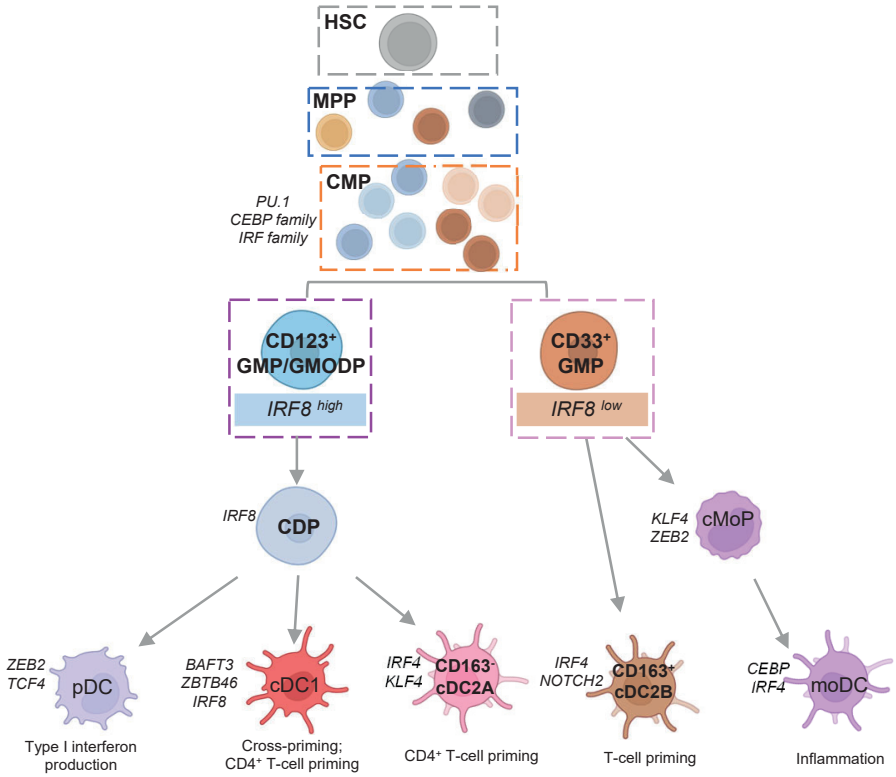

Fig. 1. Modified model of myelopoiesis and DC ontogeny. Illustration depicting continuum model of myelopoiesis in the adult that proposes lineage precommitment as early as the MPP stage and heterogeneity in downstream progenitor cells. Shown are ontogeny, key transcription factors and functions of pDC, cDC1, CD163$^-$ cDC2A, CD163$^+$ cDC2B and moDC. Abbreviations: HSC, hematopoietic stem cell; MPP, multipotent progenitor; CMP, common myelopoietic progenitor; GMP, granulocyte/monocyte/macrophage progenitor; GMODP, granulocyte/monocyte/osteoclast/DC progenitor; CDP, common DC progenitor; cMoP, common monocyte precursor; moDC, monocyte-derived DC.

DC functions and their roles in tumor immunity

DC are professional antigen-presenting cells (APC) and function as the sentinels of the immune system. Concomitant to antigen sampling, DC can sense various entities such as microbes, infected- and damaged/dead cells via PRR such as Toll-like receptors, which leads to their activation[38]. The key function of DC is the regulation of antigen-specific T-cell responses. DC can present exogenous antigens via MHC-I to CD8$^+$ T-cells in a

process termed cross-presentation, and via MHC-II to $CD4^+$ T-cells. Apart from triggering the T-cell receptor (TCR) by presenting its ligand, peptide/MHC complex, DC can also express co-stimulatory molecules including CD80, CD86 and CD70, and produce cytokines including IL-12 and IL-15 to regulate T-cell activation, proliferation and differentiation[39,40]. Additionally, DC also express co-inhibitory molecules such as PD-L1 and PD-L2[41] to control the T-cell response and to balance between tolerance and immunity. Thus, DC serve not only as central coordinators of the immune responses against threats, but also as regulators of immune homeostasis at steady-state. Notably, DC that exhibit a "semi-mature" state characterized by low expression of MHC-II and co-stimulatory molecules[42,43], decreased expression of proinflammatory cytokines, along with tolerogenic attributes[44,45], are designated as tolerogenic, as opposed to immunogenic DC.

Each DC subset also possesses its unique functional properties. Although pDC are major sources of type I interferon (IFN-I) especially upon viral infection, they are generally considered to be suppressive in the tumor micro-environment (TME) Tumor-infiltrated pDC have impaired IFN-I production[46] and can induce regulatory T-cell (Treg) activation via ICOS-ligand(L)[47] or IDO[48]. Both cDC2 and cDC1 can be subdivided into migratory and lymph node (LN) resident subsets. Evidence suggests that resident cDC can receive antigens from the migratory population[49]. cDC2 can induce polarization of naïve $CD4^+$ T-cells into T helper (Th)1, Th2 or Th17 cells upon exposure to extracellular pathogens[2,50]. Recent studies have also indicated that cDC2 can present soluble antigens to $CD8^+$ T-cells in the lymph nodes (LN)[51,52], as well as provide co-stimulation in the TME to support further effector differentiation of stem-like T-cells that have been (semi)primed in tumor draining lymph nodes (tdLN)[53]. The role of cDC2 in cancer immunology is less established, partly due to their high degree of heterogeneity[54]. cDC1 are specialized in cross-presenting cell-associated antigens and relaying $CD4^+$ T-cell help to $CD8^+$ T-cells[51,52,55]. The cDC1 subset is required for $CD8^+$ T-cell mediated spontaneous tumor regression, and enhances survival benefits in various preclinical models employing adoptive T-cell transfer or immune checkpoint blockade (ICB) therapy[56–58]. The importance of cDC1 for anti-tumor immunity in part reflects their ability to acquire antigens from tumor cells and transport these tumor antigens to tdLN to initiate *de novo* T-cell responses[49,59]. Moreover, emerging evidence suggests that cDC1 regulate anti-tumor immunity directly within the TME, by *in situ* tumor antigen presentation[60–62], as well as local provision of chemokines[61,63], cytokines[64] and co-stimulatory signals[53,65]. Notably, multiple transcriptome-based immune-cell profiling studies of the TME have recently revealed a tumor-infiltrating "mature" DC state, which

is present exclusively in tumor tissues[66–68]. Both tumor-infiltrating cDC1 and cDC2 are shown to acquire this conserved "mature" DC program characterized by the expression of immune stimulatory-, regulatory, migratory and antigen processing genes[52,54,66–68]. Importantly, these "mature" DC are enriched within "immune" niches and associated with improved prognosis[52,62,68–70]. Finally, the exact functions of moDC *in vivo* is not yet fully elucidated, but they are not critical for T cell priming (ref). However, due to their ease of generation in large-scale *in vitro*, moDC have been utilized in numerous anti-cancer DC vaccine trials[71,72].

(Tumor-reactive) T-cell (cross)priming and differentiation

The priming of naive T-cells is initiated by a primary recognition of specific peptide/MHC complexes in lymphoid tissues. This process consists of a series of biophysical, biochemical, metabolic and epigenetic changes that lead to clonal expansion of activated T cells and their differentiation into effector cells and/or long-lived memory cells. According to the paradigm, antigen-specific T-cell priming in the LN occurs in three distinct phases[73,74]. In phase I, T-cells that recognize antigen engage in brief contacts with DC, resulting in the upregulated expression of adhesion molecule CD69. In phase II, these T-cells decrease their motility and form synapses with DC where antigen, co-stimulation and cytokine signals are provided. In phase III, T-cells restore their motility and begin to proliferate and differentiate.

Antigen cross-presentation, a process by which exogenous antigens are taken up by DC and presented on MHC-I[75], is the foundation of successful $CD8^+$ T-cell cross-priming. TCR signals and environmental cues such as co-stimulatory signals and cytokines influence $CD8^+$ T-cell activation and differentiation. Apart from effector and memory differentiation, a distinct trajectory termed the "exhaustion" pathway[76,77] is also recognized in cancer and chronic infection. It is characterized by progressive loss of cytotoxic effector function and acquisition of inhibitory receptors such as PD-1 and TIM-3, driven by persistent antigen exposure. Notably, a stem-like memory stage of $CD8^+$ T-cell differentiation characterized by expression of TCF-1 and intermediate levels of PD-1 has recently been discovered both in both the tdLN and the TME[78–80]. The stem-like cell state is associated with proliferative capacity, self-renewal and long-term persistence, and is considered as the starting point of the "exhaustion" trajectory[76,77,81,82]. Importantly, increasing evidence[78,83] supports the association between stem-like $CD8^+$ T-cells and efficacy of ICB, positioning them as promising targets for cancer immunotherapies.

As for $CD8^+$ T-cells, clonal expansion and T helper (Th) differentiation of $CD4^+$ T cells rely on the strength and duration of peptide/MHC-II–TCR interactions, co-stimulation[84], and cytokine milieu[85]. In addition, other environmental factors, such as metabolic changes[86] and microbiome[87], may alter the states of $CD4^+$ Th cell subsets and their effector functions. Although the concept of "exhaustion" has been described for $CD4^+$ T-cells in the classical mouse model of chronic infection[88], the differentiation trajectory leading to this state and its clinical implication have only recently gained attention in cancer research[81,89,90]. Tumor-reactive PD-$1^+$$CXCL13^+$$CD4^+$ T-cells are heterogenous and are recently characterized by an expression profile previously assigned to either proliferating cells (i.e., *THYM, TOP2A*), $CD4^+$ T follicular helper (Tfh) cells (i.e., *TCF7*, *BCL6, CXCR5*), or "exhausted" cells (i.e., *HAVCR2, LAG3*)[90,91]. Notably, Tfh cells exhibit a transcriptional profile closely resembling that of stem-like $CD8^+$ T-cells[92], and a gradual transition process may exist from classical *IL21*$^+$ Tfh to *IFNG*$^+$ Tfh/Th1[90]. Consequently, it is likely that there is a continuum in the differentiation spectrum of $CD4^+$ T-cells in these states, mirroring the differentiation spectrum of their $CD8^+$ T-cell counterparts.

$CD4^+$ T-cell "help"

Although cDC are specialized in antigen processing and (cross)presentation, they alone are sometimes not enough to prime efficient $CD8^+$ T-cell responses, such as during tumor progression. The concept of $CD4^+$ T-cell "help" in enhancing CTL responses dates back to the 1970s[93,94]. Initially, cytokine secretion such as IL-2 by $CD4^+$ T-cells[95] was considered as a major mechanism. Later, the role of DC in $CD4^+$ T-cell "help" (DC licensing) was discovered[96,97]. Only recently, the cDC1 was pinpointed as the DC type relaying $CD4^+$ T-cell "help" for the generation of CTL responses against cell-associated antigens in the context of cancer[51,52]. Currently, cognate DC licensing via the CD40-CD40 ligand (L) axis[51,97,98] and its downstream CD70-CD27 axis[99,100] are considered the primary mechanisms of "help", although there is some evidence indicating that non-cognate DC licensing can occur[101]. Subsequently, "helped" cDC1 increase the expression of co-stimulatory molecules, cytokines, and anti-apoptotic molecules (e.g., Bcl-xL)[52,102]. Thus optimized "helped/ licensed" cDC1 can instruct optimal clonal expansion, effector and memory differentiation of the $CD8^+$ T-cells[51,52,99]. Furthermore, given the evidence linking metabolic programming and epigenetic modifications to DC functions[103–105], it is reasonable to speculate that $CD4^+$ T-cell "help" may modify these processes in cDC1,

although direct evidence remains limited.

The timing and location of $CD4^+$ T-cell "help" have been long-standing areas of interest. T-cell priming is the resultant of sequential interactions, guided by chemokines, with different DC types in lymphoid tissues. A two-step priming model (Fig. 2A) has been established based on intravital imaging to outline this process[40]. Initially, $CD4^+$ and $CD8^+$ T-cells are activated independently and asynchronously by migratory cDC2 and cDC1 respectively[106,107]. In a subsequent priming step, $CD4^+$ T-cells deliver the "help" signal through interactions with the same LN-resident cDC1, which also prime $CD8^+$ T-cells via cross-dressing[108] or cross-presentation[106,109]. Notably, recent studies have underscored the significance of migratory cDC1 in early $CD4^+$ T-cell priming in the secondary lymphoid organs[51], particularly in response to cell-associated tumor antigens. Thus a modified model[110] (Fig. 2B) has been recently proposed, wherein either migratory or LN-resident cDC1 can serve as the platforms for $CD4^+$ T-cell "help" in secondary lymphoid organs. It is likely that $CD4^+$ T-cells are activated first, enabling cDC1 to transmit "help" signals to $CD8^+$ T-cells. So far there is insufficient evidence supporting the capacity of LN-resident cDC1 to initiate $CD4^+$ T-cell priming (Fig. 2C). Nevertheless, these recent findings do not exclude the possibility that cDC2 serve as APC for $CD4^+$ T-cells[111,112]. Interestingly, increasing evidence also suggest that cDC, PD-$1^+$$CXCL13^+$$CD4^+$Th cells and PD-$1^+$($TCF1^+$)$CD8^+$ T-cells form close interactions in specific tumor niches (e.g., tertiary lymphoid structures (TLS), stem-immunity hub)[62,69,70,113], and $CD8^+$ T-cell effector differentiation happens not only in the tdLN but also in the TME[53,62]. Thus, a scenario in which the transmission of "help" by cDC1 can also take place in certain tumor niches has also been proposed[52].

Despite the absence of MHC-II on many tumors, the requirement for $CD4^+$ T-cells in cancer immunotherapy is increasingly appreciated[91,114,115]. The ability of $CD4^+$ T-cells to interact with MHC-II^+ DC (DC licensing) is crucial for persistent anti-tumor $CD8^+$ T-cell responses both in adoptive cell transfer[116] and cancer vaccine[115] settings. Studies using single-cell transcriptomics have identified transcriptional signatures of tumor-specific $CD4^+$ T-cells in the TME, and revealed a positive correlation between the presence of tumor-specific $CD4^+$ T-cells and tumor-specific $CD8^+$ T-cells and DC activation in the TME, as well as with patient survival[81,91]. Therefore, successful targeting of tumor-specific $CD4^+$ T-cells may provide a multifaceted attack on MHC-II negative cancer by supporting the activation of DC, which in turn promote the priming and functions of a diverse $CD8^+$ T-cell response to multiple tumor antigens.

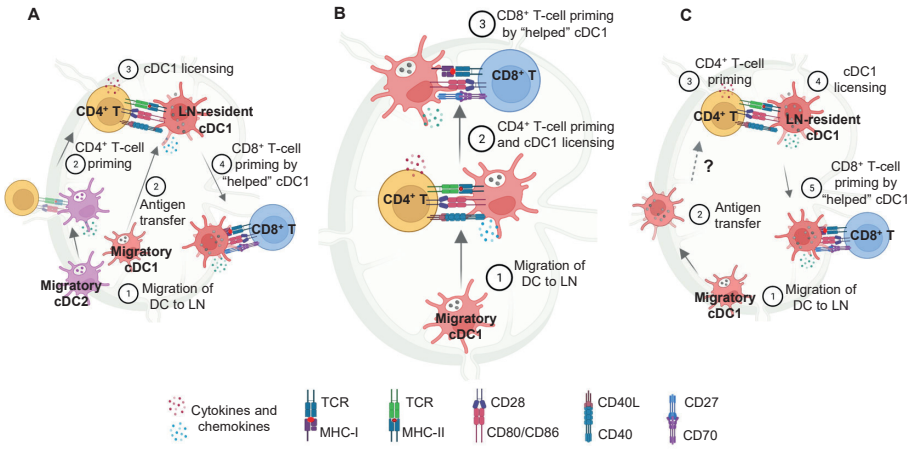

Fig. 2. Model of CD4$^+$ T-cell licensing of cDC1 to induce CTL responses. (A) Migratory cDC2 can activate CD4$^+$ T-cells. Migratory cDC1 can transfer antigens to LN-resident cDC1. Subsequently, CD4$^+$ T-cells deliver the "help" signal through interactions with the same LN-resident cDC1 to (cross)prime efficient CTL responses. (B) Migratory cDC1 have the capacity to activate CD4$^+$ T-cells. Subsequently, "help" signals from CD4$^+$ T-cells are transmitted via the same DC to (cross)prime efficient cytotoxic CD8$^+$ T-lymphocyte (CTL) responses. (C) Migratory cDC1 can transfer antigens to LN-resident cDC1. It is possible that LN-resident cDC1 can also prime CD4$^+$ T-cells, although evidence is insufficient (dotted line with question mark). After CD4$^+$ T-cell activation, "help" signals are transmitted via the same DC to (cross)prime efficient CTL responses.

Description of the chapters in this thesis

The role of DC in CD4$^+$ T-cell "help" for anti-tumor CTL responses was discovered in 1990s. Since then, ample mouse studies have been conducted aiming to discover the mechanism of the "help" signals. In **chapter 2**, I delve into the molecular mechanisms of CD4$^+$ T-cell "help" as a critical signal that can successfully activate human cDC1 and describe the clinical significance of cDC1 licensing in human cancers.

So far, the mechanism of CD4$^+$ T-cell "help" to cDC1 is largely attributed to CD40/CD40L axis based on mouse studies. However, some studies argue that other mechanisms[51,117,118] may also be involved. In **chapter 3**, I explore the role of type I interferon (IFN-I) signaling as part of cDC1 licensing machinery, and identify tumor-infiltrating Ki67$^+$CXCL13$^+$CD4$^+$ T-cells as IFN-I producers in the context of help delivery to cDC1 in the tumor.

With the intention to employ the knowledge of DC biology in cancer treatments, in **chapter 4,** I summarize the findings made by us and others regarding tumor-infiltrating DC states and immune checkpoints targeting DC for antibody-based modulation in cancer.

As a continuation of chapter 4, in **chapter 5**, I explore the potential use of human cDC1-like cells generated *in vitro* from blood-derived myeloid progenitors to facilitate the development of adoptive T-cell transfer therapies.

As an integral component of my exploration into myeloid cell biology, the focus of chapters 6 and 7 is shifted to myeloid progenitors and myelopoiesis. In **chapter 6**, I investigate how myeloid progenitors respond to local inflammation within the context of toll-like receptor (TLR)5 stimulation and delineate the clinical implication of such response. In **chapter 7**, I describe our effort to identify the driver mutation-carrying myeloid progenitors among both high- and low-risk patients with Langerhans cell histiocytosis, leveraging our ability in generating a substantial number of progeny from myeloid progenitor cells *in vitro*.

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