Monocytes and their doppelgängers: an immunological crossroads

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Recent advances have uncovered monocyte-like "doppelgänger" populations that challenge traditional classifications. These findings reveal cell subsets with overlapping features yet distinct origins, such as GMP-derived and MDP-derived cells. This complexity raises questions about the true nature of monocyte identity and their roles in health and disease.

In Fyodor Dostoevsky's "*The Double: A Petersburg Poem*," the protagonist, Yakov Golyadkin, is portrayed as an antisocial, paranoid individual who encounters his doppelgänger - an exact physical replica of himself but with a radically different personality. Initially, their actions appear to complement each other, but over the course of several days, the doppelgänger seamlessly integrates into and ultimately appropriates Golyadkin's life. This unsettling development raises the question of whether Golyadkin and his double are truly separate entities, or if the doppelgänger is merely a manifestation of Golyadkin's own psychological breakdown. This motif of identity confusion extends beyond literature into the scientific realm. Scientists are rapidly identifying and naming new cell populations by utilising single cell technologies and fate mapping models, which regularly create identity confusion. However, it is crucial to thoroughly understand both the origin and function of the newly discovered cell subsets, and to carefully evaluate their nomenclature to determine whether their actions go beyond their ontogeny. This issue has recently emerged in the field of monocyte research with the identification of monocyte-like 'doppelgänger' populations that exhibit phenotypical traits of classical monocytes but seem to vary in their origin, function or migration behaviour. This raises the questions: Are all these populations actually monocytes, do they really represent distinct subsets, and what should be considered a monocyte in the first place?

Monocytes form the circulating component of the mononuclear phagocyte system (MPS), which also includes resident macrophages and FLT3L-dependent, *Zbtb46* expressing conventional dendritic cells (cDCs). A distinguishing feature of monocytes is their ability to rapidly migrate into inflamed tissues in large quantities, where they act as a versatile "emergency squad". This dynamic response allows monocytes to adopt either a pro-inflammatory or regulatory phenotype, influenced in part by environmental and spatial cues, to help return the tissue back to a healthy physiological state (*1*). Compared to cDCs, the development and survival of monocyte-derived cells depend on signalling through CSF1R.

Monocyte development has attracted considerable attention recently. Monocytes were considered to arise from Monocyte/DC progenitors (MDP) - identified among the Granulocyte and Macrophage Progenitor (GMP) population - which have lost their potential to generate granulocytes. MDP subsequently differentiate into unipotent

Common DC progenitors (CDP) or Common Monocyte Progenitors (cMoP), of which the latter gives rise to classical monocytes (*1*). These classical monocytes, defined as Ly6C^{Hi} MHCII^{-} monocytes, enter the bloodstream and develop into non-classical Ly6C^{Low} monocytes (*2-4*) or alternatively leave the circulation destined to become monocyte-derived tissue macrophages. However, this model, with MDPs as the exclusive monocytic precursor population and a homogenous classical monocyte population at its centre, was recently challenged by the discovery of several 'doppelgänger' cell populations within the classical monocyte gate and revealed a dual origin of these cells.

Monocyte heterogeneity and doppelgängers

More than a decade ago, the Randolph laboratory first described classical monocyte heterogeneity by identifying Ly6C^{+} MHCII-expressing cells in the blood (*5*). These cells were incorporated in the Immunological Genome Project, and, in contrast to conventional Ly6C^{Hi} monocytes, MHCII-expressing cells were characterised by *Cd209a* and *Cd74* expression (immgen.org). Unbiased single cell sequencing (scRNA-seq) of blood monocytes (CD11b^{+} CD115^{+}) reinforced the notion of monocyte heterogeneity. Beside classical and non-classical monocytes that were connected by an intermediate cluster of cells, the presence of a fourth *Cd209a*$^{+}$ population was evident (*4*) (**Table 1**).

Although scRNA-seq has advanced our understanding of monocyte heterogeneity, it does not address ontogeny. A landmark study revealed that monocytes may in fact develop through two distinct pathways, the GMP and MDP routes, leading to a heterogeneous pool of cells that resemble classical monocytes from different origins (*6, 7*). The GMP-derived cells exhibited a gene expression pattern reminiscent of their granulocyte precursors (including typical neutrophilic genes such as *Elane*, *Prtn3* and *Ctsg*) and were named neutro-like monocytes. In contrast, MDP-derived cells express MHCII-related genes were termed DC-like monocytes (*6*).

To pinpoint the origins of these cells, a fate mapping system was employed to track and trace the development of these cells. The GMP fate mapping mouse (*Ms4a3*^{Cre}) surprisingly revealed that the majority of monocytes (~95%) originated from GMP, while only a small fraction of *Cd209a*-expressing cells (~3-5%) remained unlabelled, indicating their MDP origin (*8*). Owing to their distinct DC signature, including labelling in homozygous *Zbtb46*$^{Gfp/Gfp}$ mice and antigen presenting abilities, MDP-derived cells were classified as blood pro-DC3, which give rise to tissue DC3 (*8*). These cells are equivalent the human DC3 counterpart, which also shares several overlapping characteristics with both monocytes and cDC, while lacking the monocyte markers CD88 and CD89 (*9-11*). These data challenged the notion that MDPs are precursors of monocytes and established a new paradigm in which classical monocytes actually originate from GMPs. Based on these results, the neutro-like monocytes reported by Yáñez *et al*. (*6*) or R1 cells by Menezes *et al*. (*12*) may correspond to classical monocytes, whereas DC-like monocytes (*6*), R2 cells (*12*), CD135^{+} monocytes (*13*) and *Cd209*$^{+}$ cells (*4*) appear to resemble some features of pro-DC3 (**Table 1**).

An additional piece of this ontological puzzle was recently added by findings obtained with a double reporter mouse system (the Ms4a3^{Cre} GMP fate mapper crossed with mononuclear phagocyte reporter *Cx3cr1*^{Gfp} mice). In this model, it was anticipated that all monocytes would exhibit both fluorescent protein markers, but instead, two Ly6C^{Hi}

monocyte subsets were detected within the bone marrow and peripheral circulation: a double labelled CD319^{-} GMP-derived monocyte subset (~90% of the classical monocyte gate) and a smaller fraction of Ly6C^{+} CD319^{+} MDP-derived cells characterised by only GFP expression (~10%). These MDP-derived cells expressed certain DC3 genes (*Cd209a*, *Tmem176a* and *Cd74*) but lacked the expression of the key DC markers FLT3, ZBTB46 and CD11c (*14*) (**Table. 1**). Consequently, it was proposed that CD319^{+} MDP-derived cells do not belong to the DC lineage, but rather represent a classical monocyte subset (*14*). These data are consistent with the observations that R2 cells were not labelled in heterozygous *Zbtb46*$^{Gfp/+}$ mice and developed independently of FLT3L despite expressing CD135 (*12*), which justifies their inclusion in the monocyte lineage.

The outcomes of these studies parallel Dostoevsky's doppelgänger motif: Do MDP-derived pro-DC3 and CD319^{+} cells constitute the same population or two distinct entities that share a partially overlapping transcriptional program (**Fig. 1**)? This is not merely a matter of semantics but could have significant implications for the physiological and pathological functions of these cells. If MDP-derived cells are equivalent to pro-DC3, they should have the ability to present antigens and migrate to lymph nodes. Indeed, DC3 cells are capable of presenting antigens and play an essential role in Th17 polarisation, whereas monocyte-derived cells performed poorly in these assays (*8, 9, 11*). Therefore, it remains to be determined whether CD319^{+} MDP-derived cells are capable of and involved in antigen presentation. On the other hand, if MDP-derived CD319^{+} cells belong to the monocyte family, they should have the potential to infiltrate tissues and develop CSF1R-dependent into macrophages. In fact, both GMP- and MDP-derived populations were shown to infiltrate the lungs and gut and differentiate into tissue-resident cells following transfer experiments (*14*). Curiously, only MDP-derived cells demonstrated the ability to colonise the dura mater after experimental elimination of endogenous macrophages from this particular niche (*14*). Another recent study followed this line of enquiry and examined whether ontologically distinct GMP- or MDP-derived subsets have different capabilities to repopulate the skin epidermal Langerhans Cell (LC) pool following graft versus host disease (GvHD). Given that LC display characteristics of DC, such as migration to draining lymph nodes during pathological processes, it can be inferred that LC's DC-like characteristics are possibly a legacy of their origin. Accordingly, classical GMP-derived monocytes may not represent the precursors of monocyte-derived LC during GvHD but rather their MHCII-expressing MDP-derived doppelgänger subset with DC attributes. Surprisingly, both GMP- and MDP-derived cells accumulated in the skin during GvHD and subsequently gave rise to LC (*editor please add new science immunology reference*).

Although tissue residency is a defining feature of macrophages, DCs also function as homeostatic tissue-resident immune cells. Initial findings indicate that DC3 can be found in various tissues under steady state conditions (*15*), challenging the idea that tissue residency is unique to macrophages. However, unlike long-lived self-renewing tissue macrophages (*3, 16*), cDCs are short-lived and require continuous replenishment from the bone marrow (*17*). Examining the retention time of cells derived from GMPs or MDPs within tissues could offer valuable insight into their cellular identity. However, current studies have only examined GMP- or MDP-derived cells in tissues up to 12 days after transfer (*14*) or, in the context of LC replacement during GvHD, it remains to be determined whether GMP- or MDP-derived LCs

dominate the niche over time due to differences in longevity and proliferation (*editor please add new science immunology reference*). Additional evidence is required to determine if tissue infiltrating GMP- or MDP-derived cells depend either on FLT3 (ligand FLT3L) or CSF1R (ligands CSF1 or IL-34) for their development and express ZBTB46 after differentiation, which could act as a crucial criterion for lineage determination.

Another characteristic of classical monocytes is their capacity to develop into non-classical monocytes. It is therefore reasonable to ask whether both GMP-derived monocytes and MDP-derived cells possess the ability to convert into non-classical monocytes. Non-classical monocytes may be viewed as terminally differentiated macrophages residing in the blood (*3*). These monocytes are less likely to migrate into tissues compared to other monocyte subsets. Instead, they predominantly remain in the bloodstream, where they continuously monitor the vasculature by crawling along endothelial cells. This surveillance helps to maintain the integrity of blood vessels during homeostasis (*18*). Recent findings indicate that the survival of non-classical monocytes relies on their interaction with the vascular endothelium, which is facilitated by the CX_3CL1-CX_3CR1 axis and LFA-1. This interaction permits non-classical monocytes to bind to CSF1 tethered to the endothelium, which is a crucial factor for their survival (*19*). Concerning their origin, transfer and fate-mapping experiments have shown that non-classical monocytes arise from classical monocytes in rodents and humans (*2-4, 20*). scRNA-seq profiling has revealed that non-classical monocytes are connected to GMP-derived classical monocytes through an intermediate stage, but they do not appear to be related to *Cd209a*-expressing cells, which are likely to be derived from MDPs (*4*). However, when $Ly6C^{Hi}$ GMP- or MDP-derived cells are transferred into the bloodstream of recipient mice, both cell types gradually lose their Ly6C expression over time and phenotypically resemble non-classical monocytes (*14*). Alternatively, if the injected MDP-derived cells are pro-DC3, it is possible that they lose Ly6C expression during their development into $Ly6C^{-}$ DC3 after transfer (*8*). Whether these transferred cells exhibit a transcriptomic signature similar to that of non-classical monocytes remains to be shown.

The role of monocyte heterogeneity during pathology

The existence of various classical monocyte-like populations suggests that each subset may fulfil a unique function that collectively contribute to a coordinated immune response. Supporting this idea, studies have demonstrated that bacterial LPS injection results in an increase in GMP-derived monocytes, whereas CpG injection, mimicking viral infection, leads to an increase in MDP-derived cells (*6, 14*). $iNOS^{+}$ macrophages are a characteristic feature of *Listeria monocytogenes* infection and originate from circulating classical monocytes (*21*). Given the heterogeneity among classical monocytes, it is plausible that only a specific subset can acquire certain specialised phenotypes. Indeed, *in vitro* and *in vivo* studies have confirmed that R1 or GMP-derived classical monocytes are uniquely specialised to produce $iNOS^{+}$ macrophages following exposure to *Listeria monocytogenes* (*12*).

Significant progress has been made in the context of solid tumours and inflammation, in which a conserved molecular "state" of cDC has recently been identified. These cells are termed "mature DCs enriched in immunoregulatory molecules" or mregDC (*22*) and characterised by the expression of *LAMP3*, *PDCD1LG2* and *CCR7*, which are associated with regulatory, immunogenic and migratory gene programs. It appears that both cDC1 and cDC2 subsets can acquire an mregDC state upon interacting with

or internalising cell-associated antigens (*22, 23*). Therefore, it is plausible that pro-DC3 may also differentiate into mregDC when exposed to the tumour environment. Additionally, R2 or MDP-derived cells can up-regulate *PDCD1LG2* in a PU.1- and CSF2-dependent manner (*12*). Nonetheless, the specific contribution of this subset to cancer development requires further investigation.

Conclusion

The discovery of several monocyte doppelgänger populations has significant implications for laboratory research. Relying solely on traditional methods that use a limited set of markers to identify classical monocytes does not do justice to the potential heterogeneity and doppelgänger subsets masked as classical monocytes. For instance, relying on such methods could unintentionally include pro-DC3 cells, which could distort antigen-dependent T-cell stimulation experiments. Moreover, classical monocyte-derived cells have been associated with a variety of diseases, including cardiovascular conditions, autoimmunity, cancer, and infections. It is crucial to determine whether the reported functions of classical monocytes in these pathological conditions can be attributed to classical monocytes or whether they are influenced by the presence of a specific monocyte subset and doppelgänger populations. This will require a reassessment of the role of each monocyte population in distinct disease contexts.

At the crossroads of monocyte research, it is essential to provide a clear and comprehensive definition of these cells that extends beyond their cytokine dependency, surface marker expression and origin. This definition must also encompass functional aspects such as lymph node homing and antigen presentation. As Dostoevsky's Golyadkin remarks to his physician Rutenspitz: '…*till a more convenient moment, when everything will be discovered and the mask falls off certain faces, and something comes to light' (24)* (**Fig. 1**).

Figure 1. The multiple faces of classical monocytes. The identification of monocyte-like 'doppelgänger' populations that all express Ly6C and CD115 (CSFR1).

Table 1. Ly6C$^+$ cell subsets

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