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Single cell resolution of Plasma Cell fate programming in health and disease

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Abstract

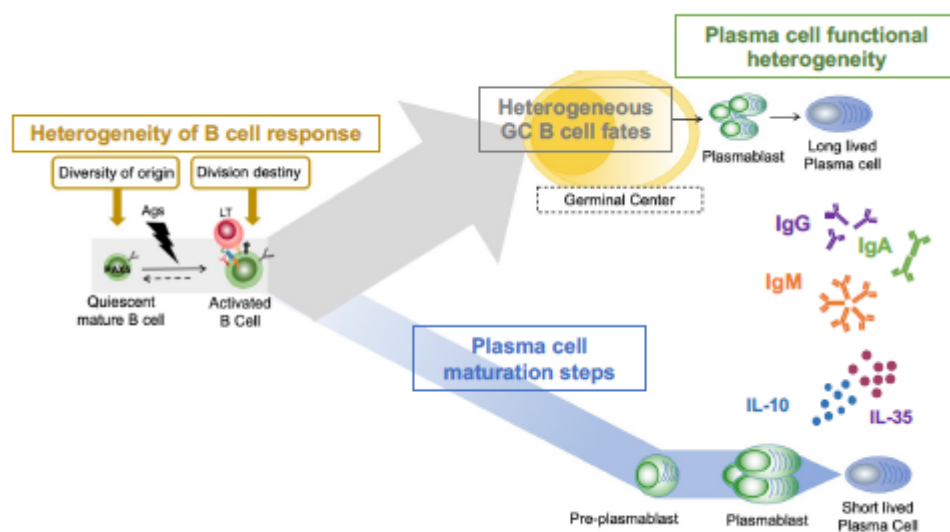
Long considered a homogeneous population dedicated to antibody secretion, plasma cell phenotypic and functional heterogeneity is increasingly recognised. Plasma cells were first segregated based on their maturation level, but the complexity of this subset might well be underestimated by this simple

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dichotomy. Indeed, in the last decade new functions have been attributed to plasma cells including but not limited to cytokine secretion. However, a proper characterization of plasma cell heterogeneity has remained elusive partly due to technical issues and cellular features that are specific to this cell type. Cell intrinsic and cell extrinsic signals could be at the origin of this heterogeneity. Recent advances in technologies like single cell RNA-seq, ATAC-seq or ChIP-seq on low cell numbers helped to elucidate the fate decision in other cell lineages and similar approaches could be implemented to evaluate the heterogeneous fate of activated B cells in health and disease. Here, we summarized published work shedding some lights on the stimuli and genetic program shaping B cell terminal differentiation at the single cell level in mice and men. We also discuss the fate and heterogeneity of plasma cells during immune responses, vaccination and in the frame of human plasma cell disorders.

Plasma cells form a heterogeneous population characterized by distinct maturation steps, phenotypes, and functions. Single cell analyses were at the origin of important new insights into B cell fate diversification after activation and may contribute to a better understanding of plasma cell heterogeneity.



Introduction

Following stimulation, B cells can terminally differentiate into antibody (Ab) secreting cells known as plasma cells (PCs) that are key for rapid and prolonged protection against infection. This phenomenon relies on the integration of different type of stimuli by B cells and is traditionally split into Thymus (T)-dependent and T-independent activation depending on the requirement for T cell help. This dichotomy originates from experimental immunization models while in real life both responses often co-exist. Indeed, most infections can induce both types of B cell activation through the integration of signalling triggered by the B cell receptor (BCR), Toll-like receptors (TLRs), activator and inhibitor

co-receptors (e.g. CD19, CD22, FcγRIIb) and by T-cell help (e.g. through CD40-CD40L interaction or through cytokine signalling). Additionally, B cell activation can be separated spatiotemporally into the extrafollicular response and the germinal centre (GC) response [1]. The extrafollicular response is a rapid reaction that can be both T-dependent and T-independent and that leads to the generation of a first wave of protective PCs secreting low affinity antibodies. In contrast, specialized microanatomical structures called GCs form through provision of growth and differentiation signals from a specialized subset of helper CD4⁺ T cells named follicular helper T (Tfh) cells [2]. GCs form upon infection in secondary lymphoid organs - including lymph nodes, spleen, Peyer's patches- and produce long-lived PCs and memory B cells involved in the long-term protective immunity [3]. This T cell-dependent response can be divided into two spatiotemporal events known as the pre-GC and the GC phase, the first one happening at the border of the B cell follicle, hence being coined extrafollicular, and the second one within the GC, a specialised structure described below.

Within the lymph nodes, naive B cells migrate towards concentrated B cell zones known as follicles in search for the antigen they are specific for either presented by subcapsular macrophages, follicular dendritic cells or soluble [4],[5]. Antigen-experienced B cells then relocate to the inter-follicular zone where they have to engage with their cognate antigen-specific pre-GC subset of Tfh (pre-Tfh) cell within a maximal two-days window after antigen priming [6],[7]. During this confined B cell-T cell interaction that last tens of minutes [8], B cells receive help in the form of various signals such as the membrane-bound CD40L, the ligand for the co-stimulatory receptor CD40, which is constitutively expressed on B cells, and secreted cytokines, that enable complete B cell activation, survival, proliferation [9] as well as immunoglobulin (Ig) class-switch recombination [10]. Moreover, the integration of these antigen and T cell-derived signals during the pre-GC phase guides activated B cells towards one of three fates [11]. A select number of B cells differentiate into PCs in the extrafollicular zone, generating the first wave of short-lived Ab secreting cells that provide the majority of early protective Abs. A separate subset differentiates into mainly unswitched memory B cells independently of the GC reaction. The remaining B cell subset is committed to the GC pathway. These GC-destined B cells relocate to the follicle centre and undergo expansive proliferation resulting in the formation of a mature GC. Within the GC, B cells capture antigen from follicular dendritic cells and then compete for Tfh help in order to undergo several rounds of somatic hypermutation (SHM) to improve BCR affinity. Only high-affinity GC B cells are positively selected for differentiation into either memory B cells or long-lived PCs whilst unfavourable GC B cells undergo apoptosis [12],[13]. Following their generation most PCs will wane away after 3 to 5 days. However, a fraction of them migrates to the bone marrow (BM) where they terminate their differentiation, become quiescent and persist in dedicated survival niches for long-period of time extending over several years and perhaps a lifetime for some of them [14]-[19]. Hence, the PC population encompasses long-lived cells and newly minted ones, that can express all Ig isotypes and that were likely generated from distinct types

of B cells through distinct cellular processes. Despite these evident sources of heterogeneity, PCs have long been considered a homogeneous population with unique function to secrete large quantities of Abs. This view was challenged a few years ago with the observation that PCs were important cytokine producers [20],[21]. Interestingly, distinct PCs seem to produce different cytokines hence emphasizing the existence of PC subsets. A precise characterization of these subsets as well as the cellular and molecular signals involved in their generation are an active area of research because they represent potential therapeutic targets to manipulate the B cell response in order to optimize vaccine strategy but also to intervene in autoimmune and inflammatory diseases. These questions have been studied at the population level [11]. However, bulk analysis shows limitations in the context of heterogeneous B cell responses. Conventional single-cell approaches such as serial dilution or flow cytometry analysis can overcome these problems. Powerful single cell sequencing techniques represent a step forward in the characterization of B cell fate diversification after their activation.

Since the first transcriptome analysis at the single cell level in 2009 [22], a number of technological developments [23],[24] have enabled the growth of data collected at the single cell level at the origin of important new insights into cell identity, state and functions. More recently, single-cell ATAC-seq [25] and ChIP-seq [26] technologies have also been developed to study chromatin heterogeneity in term of structure via histone modification and chromatin accessibility to trans-factors regulating gene expression patterns. Simultaneous analysis of two or more omics data such as transcriptome, DNA methylation and chromatin accessibility can now be performed in the same cell [27].

Here, we summarized published work shedding some lights on the stimuli and genetic programs driving B cell terminal differentiation at the single cell level, as well as on the fate and heterogeneity of PCs.

At the origin of PC heterogeneity: Regulation of B cell activation and PC differentiation

Heterogeneity of individual B cell response to antigen stimulation

To understand how a B cell changes during differentiation towards PC fate, it is important to measure the sequence of decisions it makes. However, this complex and holistic process is still poorly understood for several reasons. B cell response to stimuli is heterogeneous and asynchronous. As a result, average of gene expression in population studies mask the specific phenotype of intermediate stages of PC differentiation. Another challenge in investigating this process is the scarcity of the activated B cells in transient states towards the PC fate. These have limited the progress made in the understanding of the gene circuitry governing early cell fate decision and PC commitment. The use of single cell methods has already proven their effectiveness in overcoming these problems.

The PC pool is composed of cells with diverse origins. Indeed, the propensity of a B cell to differentiate into PCs can be heavily influenced by the cell of origin in secondary lymphoid organs: naive, memory or marginal zone B cell, and by spatiotemporal interactions and signals that a B cell receives from its microenvironment in extrafollicular zone and GCs. It is therefore unsurprising that heterogeneous cell fate can be observed to arise from both *in vivo* and *in vitro* models that mimic T-dependent or T-independent responses. Recent advances made to better understand the origins of this heterogeneity have resulted in the generation of several mechanistic models thought to be responsible for terminal differentiation, including cell intrinsic, cell-extrinsic, stochastic or deterministic regulation [28]. The question of the relative contribution of each process in the many different activation scenarios, for example *in vitro* or *in vivo*, is unresolved, however, the end result must be consistent with the transcriptional programming required for the formation of Ab secreting cells [29]. Indeed, the PC differentiation process initiated upon antigen-specific priming is associated with a marked alteration in B cell morphology, gene expression profile and BCR affinity compared to their naive B cell precursor that provide them with specialized effector functions. Ab secretion is the hallmark of PCs, but other functions will be detailed below.

The ability of single naive B cells to give rise to multiple effector cell types was recently demonstrated *in vivo*. Terminal B cell fate tracing was studied by means of ingenious single cell transfer experiments in mouse models. A limiting dilution approach was used to track the primary response of individual antigen specific naive B cells transferred into recipient mice immunised with allophycocyanin [30]. The populations that differentiated from individual naive B cells were shown to be highly heterogeneous in terms of overall size and composition. However, more interestingly observed, was that single naive B cells were able to give rise to populations containing multiple effector cell types, namely short-lived PCs, GC-independent memory B cells, GC B cells or undifferentiated activated precursors (Fig. 1). Whereas some clonal populations contained only one effector cell type, many populations contained two or three and a few contained all four. Furthermore, the populations containing all four effector cell subsets exceeded their expected size, which appeared to correlate with the founder naive B cells demonstrating greater resistance to cell death. Consistent with previous reports, increasing clonal BCR affinity to antigen by the means of BCR transgenic MD4 B cells[31], resulted in an increased tendency to differentiate into short-lived PCs indicating that intrinsic heterogeneity linked to BCR shape the PC fate destiny. Within the GC, the clonal expansion of GC B cells is accompanied by BCR diversification through SHM which introduces point mutations into the variable sequence of Ig genes, a process known as affinity maturation. Memory B cell lineage was nicely demonstrated to be coupled with low-affinity BCR in an unbiased fate tracing approach [32]. This confirms that BCR affinity is one of the important determinants in the cell fate decision both in the extrafollicular pathway and GC reaction.

Division destiny

For long time, it has been observed that B cells undergo PC differentiation only after completing multiple cell divisions [33],[34]. Division destiny, determined as the generation at which cells cease to divide, is an inherited property that has the potential to internally program the fate of B cell progeny [35],[36]. Exploration of PC differentiation at the single cell level and by the means of defined cell culture systems has been powerful to define the relationship between cell proliferation and PC commitment. By using direct imaging of CpG-stimulated primary mouse B cells, it was observed that although the number of divisions each cell can undertake is variable, the descendants of each founder cell divide the same number of times. Furthermore, founder cell size could also pre-determine the division destiny of B cell progeny, as large cells present at the first division produced offspring that underwent multiple divisions before ceasing [37]. This suggests that division destiny may be a consequence of the stimulation experienced by the first cell that sets into place mechanisms capable of controlling the extent of division and ultimately B cell fate. More recently, Scharer et al. set up an adoptive transfer model of Cell trace-labelled splenic B cells to follow single cell destiny of the progeny following T-independent immunisations with LPS or NP-Ficoll [38]. In agreement with the results obtained in the context of *ex-vivo* stimulated cells, at least 8 cell divisions were required to trigger CD138⁺ PCs secreting Abs. More importantly, single cell-RNAseq (sc-RNAseq) studies performed in these experimental models revealed that an early cell fate decision towards PC differentiation occurs early (before the fifth division) in dividing B cells, confirming a key aspect of *in vitro* differentiation models. In the context of human PC differentiation, *ex-vivo* models provide also a highly accessible system to study at the single cell level the temporal dynamics of signalling pathways regulating transcription factors in conjunction with cell proliferation and differentiation. Such an approach using single-cell qRT-PCR revealed the heterogeneity of human B cell responses to minimal extrinsic factors such as the T-cell derived IL-2 signal driving PC differentiation [39]. In line with what was observed in mice, imprinting towards the PC program happened during the initial stages of B cell activation and proliferation.

Single cell resolution of transcriptional dynamics - heterogenous GC B cell fates and PC trajectories

B cell response is under complex cascades of timed events highly regulated and coordinated at the molecular levels. Recent studies pointed out the intricate relationship between chromatin remodelling, epigenetic modifications and gene regulatory networks shaping the PC fate [40],[41]. However, the initial commitment toward PC differentiation remains elusive. Multiple forms of evidence exist in support of an interconnected network of critical transcription factors rather than the existence of a unique master regulator that would direct B cell fate toward PC. Indeed, the transition

of a naive B cell precursor to PC is governed by a well-characterized gene regulatory network of transcription factors whose molecular requirement has been primarily determined by gene-deficient mouse models. It involves on one hand transcription factors promoting B cell fate maintenance including PAX5, BACH2, PU.1, IRF8 and BCL6 that are silenced in PCs, and on the other hand PC transcription factors such as BLIMP1, IRF4 and XBP1 regulating the PC transcriptional program [29],[42]. The role of each of these key transcription factors during terminal B cell differentiation has been well defined as they coordinate a program shared by all mature B cell subsets to ensure PC formation (Fig. 1). However, it is unclear how the B cell transcription program interprets and integrates external signals through transcriptional, post-transcriptional and/or epigenetic mechanisms. The amount of multi-omic data collected over terminal B cell differentiation has been growing thanks to high throughput sequencing technology. Classical time-series transcriptome analyses from various systems modelling PC differentiation in human [43]-[46] and in the mouse system [47] revealed a differentiation continuum of gene expression changes. However, to date, only few genes important in initiating and controlling the transitions and progression of naive B cell to early PC precursors have been described. Furthermore, the hierarchical relationship between transcription factors remains unclear. Sc-RNAseq profiling is expected to provide the real dynamic of their expression thanks to the pseudo-temporally ordering of single cells along the differentiation path. Such an approach has inferred the temporal dynamics of PC generation spanning from mouse follicular naive B cells, transferred into mice, through to PCs, following LPS immunisation [38]. Importantly, it revealed an early bifurcation point separating activated B cells between a PC differentiation path associated with MYC activation and OXPHOS (Fig. 1), and a non-differentiation path associated with an inflammatory response. In agreement with previous data showing a major role for BACH2 in B cell fate decision [39],[48],[49], the level of BACH2 activity was found differentially expressed at this molecular switch. Furthermore, B cells deficient for IRF4 failed to engage towards this differentiation path, identifying IRF4 as a major actor of this early commitment towards PC fate. This is in agreement with the mutually antagonistic relationship between IRF4 and IRF8 that was proposed previously to regulate the initial bifurcation in activated B cell fate [50].

In the context of cellular heterogeneity of GC B cells, characterizing the key steps and targets that initiate and control the transition and progression of the activated B cell towards memory or PC destiny was problematic. Classical models of the GC have recognized the existence of two GC B cell differentiation states known as centroblasts and centrocytes which are localized in two histological compartments of the GC, the dark zone (DZ) and the light zone (LZ), respectively (Fig. 2). The phenotypic markers CXCR4, CD83 and CD86 have been used to discriminate these populations using flow cytometry in human and mouse [51]-[53]. Then, GC B cells have been shown to transit back and

forth between DZ and LZ (a dynamic reviewed in [54]). However, rather than two different stages of differentiation, single cell transcriptomic profiling and BCR analysis revealed the huge heterogeneity of GC B cells that moved forward the canonical concept toward a more dynamic vision of cell fate determination coupled with BCR diversity. Indeed, several recent studies have started to analyse single-cell gene expression carried out in human[55]-[58], coupled in some cases with surface phenotype and BCR sequencing. They revealed a continuum of cells between DZ and LZ, as well as cluster of cells corresponding to intermediate states of differentiation towards memory B cells and PCs. Memory B cell precursors are clustered by their expression profiles of *CCR6*, *BANK1*, *KLF2* [56],[59] while PC committed cells expressed *PRDMI* and *XBPI* [58]. The expression of the chemokine receptor CCR6 has localized pre-memory B cells in the LZ [60]. The choice of PC fate, whether it is initiated in LZ or DZ, is unresolved. Tfh and stromal cells within the GC primed PC precursors [61]-[63]. However, PC differentiation may emerge from DZ as BLIMP1⁺ (encoded by *Prdm1*, the master PC transcription factor repressing the B cell fate and regulating PC functions) was found to be expressed at low levels by a subset of proliferating DZ cells in Blimp1 reporter mouse models [64]. With the aim to better understand timing and hierarchy of PC transcription factor expression, a single cell GC B cell feeder assay developed by Kuraoka et al. [65] was used to follow the proliferative capacity and the differentiation destiny of GC B cells expressing low levels of Blimp1 [64]. Interestingly, their results suggested that cells at an early time point of Blimp1 expression may be competent to differentiate but may not be totally committed to PC fate as they were capable to reverse Blimp1 expression *in vitro*. Altogether, those studies characterized transcriptional plasticity at the origin of B cell diversity. Whether it accounts for PC heterogeneity is still not known.

Distinct layers of plasma cell heterogeneity

Maturation

The PC compartment is highly heterogeneous and can be subdivided according to maturation state, Ig isotype expression, and life span (Fig. 3). PC differentiation from activated B cells is a continuous process that involves the pre-plasmablast (pre-PB), the plasmablast (PB) and the mature PC stage. The principal phenotypic markers for these different stages of maturation are described below and summarized in Table 1. PB precursors, so-called pre-PBs, have been identified in various studies. Arce et al. demonstrated a CD38^{neg/low} IgG-secreting precursor PC population in human tonsils [66]. The existence of CD38^{neg/low} Ig-secreting cells was corroborated by *in vitro* studies that identified two PB populations upon CD40-ligand withdrawal: a proliferating, CD38⁺ subset and a non-proliferating CD38⁻ subset, which might be the precursors of long-lived and short-lived PCs, respectively [67].

Along this line, *in vitro* differentiation of memory B cells to PCs involves a transitional stage of CD27^{high}/CD38⁻ cells that secrete high titers of Ig [68]. Moreover, CD38⁻/CD138⁻/CD20^{neg/low}/CD27^{low} Ig-secreting pre-PBs preceded the generation of CD38^{high}/CD27^{high} PBs *in vitro*. These pre-PBs were CD138-negative, secreted lower amounts of Ig compared to PBs/PCs and were detectable in human tonsils and lymph nodes, but not in the blood and BM [69]. Whether all PC subsets can become long lived is still a matter of debate with PC longevity potentially depending on both cell intrinsic and cell extrinsic features. Noteworthy, PCs can also originate from the B1 compartment, a specialized B cell subclass localized mainly in the pleural and peritoneal cavities, capable of self-renewal and a major source of natural IgM [70]. Transfer of adult peritoneal B1 cells into B1 cell-deficient neonate mice reconstituted B1 PCs, Blimp1-dependent as well as Blimp1-independent IgM- and IgG3-secreting B1 cells [70],[71].

CD138 (Syndecan-1) in combination with B220 has been frequently used to identify Ab-secreting murine PBs/PCs. As B220 is gradually downregulated during PC differentiation, murine PBs are characterized as CD138⁺/B220⁺, whereas mature PCs are CD138⁺/B220^{low/neg}. However, the CD138⁺/B220^{low/neg} fraction is heterogeneous and contains non-PCs [72],[73]. Therefore, additional markers for delineation of the PC pool are required. Newly generated PBs in contrast to mature PCs are proliferating and can be identified by Ki-67 staining. In addition, differentiation of PBs to PCs is accompanied by a gradual increase of Blimp1 expression with a peak in mature PCs [74]. Thus, many studies on murine PCs have been conducted in the Blimp1-GFP reporter mouse model [75]. However, Blimp1 expression is not restricted to PCs and can be detected in T cell subsets [76]. Recently, a combined staining with the Transmembrane activator and CAML interactor (TACI) and CD138 has been established to reliably identify the PB/PC population by flow cytometry [72]. Furthermore, the combination of TACI and CD138 with B220 and CD19 allows the reliable identification of three distinct murine PB/PC subsets: the TACI⁺/CD138⁺/B220⁺/CD19⁺ PB population (P1), the TACI⁺/CD138⁺/B220^{low/neg}/CD19⁺ early PC population (P2) and the non-proliferating TACI⁺/CD138⁺/B220^{low/neg}/CD19^{low/neg} mature P3 PC subset. Blimp1 expression gradually increases from P1 to P3. The P3 BM PC fraction includes IgG PCs and therefore, presumably contains long-lived PCs [72].

Similar to murine PCs, CD138 is highly abundant on the surface of human PCs [77]. However, in contrast to mouse PCs, human PCs are characterized by high CD27 and high CD38 expression. By using a combined CD19, CD38 and CD138 staining, four different PC subsets were identified in human BM [78]. The CD19⁻/CD38^{high}/CD138⁺ population included non-cycling, long-lived IgG PCs specific for viral antigens 40 years after infection. Along this line, Mei and colleagues found an enrichment of IgG⁺ PCs in the CD19⁻ human BM PC fraction [79]. Moreover, by using the carbon-14 dating method, Landsverk and colleagues demonstrated that CD19⁻ IgA⁺ PCs in the lamina propria of

the human small intestine can be further subdivided in CD45⁺ and CD45⁻ subsets that harbour long-lived PCs with average life spans of 11 and 22 years, respectively [80]. Importantly, infant BM was deficient for CD19⁻ PCs, but already contained CD19⁺ PBs/PCs. CD19⁺ BM PBs/PCs might, therefore, differentiate to CD19⁻ PCs during childhood or adolescence [79]. Hence, downregulation of CD19 expression might be a predictive but not yet clearly established marker for discriminating long-lived PCs from short-lived PCs. Longevity, however, is not restricted to IgG⁺ PCs, because IgA⁺ PCs with a life span of several years up to decades can be detected in the human small intestine as well [80].

Importantly, loss of CD19 expression might not always correlate with the transition of early PBs to late long-lived PCs. For example, the emergence of vaccine-specific CD19⁺, CD19^{low} and CD19⁻ PCs was demonstrated during acute immune responses following influenza vaccination. Furthermore, CD19⁻ PBs were generated by either T cell-dependent or T cell-independent stimulation in parallel to CD19⁺ PBs *in vitro* [81]. Upon T cell-dependent immunization *in vivo*, newly formed IgM-secreting PBs and longer-lived IgG-secreting PCs emerged in parallel in the BM for months, revealing high dynamics and the presence of a heterogeneous PC pool that co-exists in the BM: short-lived B220⁺ cells, B220⁻ cells with an intermediate half-life and a rare long-lived B220⁻ population [82]. Of note, CD19⁻ PCs that secrete natural IgM and IgG3 can also originate from the B1 cell compartment [70]. Besides the downregulation of CD19, various other markers have been suggested to be predictive for PC longevity, such as CD93 and BCMA. CD93-deficient mice showed normal initial humoral immune responses. The maintenance of antibody secretion and BM PC numbers, however, were perturbed, indicating that CD93 contributes to longevity of BM PCs [83]. Similar findings were reported for mice with a deletion of the *tnfrsf17* gene (encoding for BCMA) [84].

PC differentiation and maturation stages were also investigated at the transcriptional level and the fine mapping of these processes have followed technical and experimental advances. The transition between activated B cells and bona-fide PBs has been particularly studied with early works characterizing the transcriptome of human CD20⁻CD38⁻ pre-PB by microarray-based analysis [69]. This was recently completed by RNAseq analyses comparing the transcriptome of these pre-PB with memory B cells and the more mature subsets of PB and PC generated *in vitro* [44]. In another model of human naive B cell differentiation into PBs, these final stages of differentiation have been characterized with ATAC sequencing and hydroxymethylation profiling [45]. More detailed analyses highlighted a role for Enhancer of Zeste Homolog 2 (EZH2), the catalytic subunit of Polycomb Repressive Complex 2 (PRC2), as a key regulator of this transitional stage by pausing PC differentiation and ensuring amplification of this cellular compartment by proliferation [85]. The role

of BLM, a DNA helicase involved in genome stability, was underscored in this subset [44]. Many other transcription factors and epigenetic enzymes demonstrate an interesting expression profile in this pre-PB subset and remain to be studied at the functional level. In line with this, ChIPseq analyses of the well-known IRF4 XBP1 and BLIMP1 PC transcription factors helped to elucidate their relative contribution to the gene regulatory network involved at the transition between the B cell fate and the PC fate [46]. Like in many fields, the advent of sc-RNAseq analyses has pushed forward the identification and characterization of PC maturation stages specially in the context of human immune response and disease [56],[86]-[90]. In particular, the very first step of PC differentiation were captured by Holmes et al. that described 6 clusters of PBs. Three of them were coined “GC light zone like cells” and could correspond to the very first steps of PC commitment within the GC. One cluster was similar to the previously mentioned pre-PB as they were enriched for cell cycle and proliferation transcriptional signatures. Finally, the two last clusters were expressing bona fide PB markers and display decreased expression of the proliferation transcriptional signature [56]. This discrimination between pre-PBs and PBs based on cell cycle signature was also reported by a sc-RNAseq study of dengue virus infected patients [87]. Further sc-RNAseq studies in mouse and human reported that short-lived and long-lived BM PCs also present distinct transcriptomic profiles with cell cycle, metabolic and ribosomal pathways discriminating both stages [40],[86],[91]. However, a bit disappointingly, no further insight into what regulates PC functional heterogeneity within the BM emerged from these studies. Whether this is due to technical limitations linked to the sequencing approach used requires further investigation. Interestingly, a study recently published dresses a more complete atlas of PC maturation stages from human secondary lymphoid organs, blood and BM, and described 13 transcriptional profiles covering this process. Furthermore, expression of CD39 was proposed as a marker to distinguish newly generated BM PCs from long-lived ones [90]. Of note, most of these studies concern human PC differentiation and equivalent work in mouse models are still scarce. Furthermore, these sc-RNAseq analyses did not establish a link between PC function, B cell of origin and maturation stage.

Function

What could be the relevance of having distinct PC subsets? A wealth of literature suggests that PC function might not be limited to Ab secretion. Actually, a very large part of cytokine secretion from the B cell lineage originates from PBs or PCs. The best documented case concerns secretion of IL-10. Indeed, at steady state but also after salmonella infection or in the course of experimental autoimmune encephalomyelitis (EAE), PCs were consistently reported as the main producers within the B cell lineage of this key cytokine in the mouse [20],[92]-[96] but also in humans [20]. PC-derived IL10 was shown to have immunosuppressive effects on dendritic cells, NK cells and T cells and to promote the

myeloid skewing of the haematopoiesis observed with age [20],[92],[97]. Furthermore, IL-10-producing PCs were observed in solid tumour microenvironment where they negatively affect anti-tumour T cell immunity [98],[99]. Interestingly, IL-10 production is also a feature of human and murine myeloma cells and can contribute to disease associated immunodeficiency via inhibition of neutrophils [100]. PC-mediated cytokine secretion is not limited to IL-10 as IL-35⁺ and GM-CSF⁺ PCs were also shown to be protective in the context of EAE [21] and microbial sepsis [101], respectively. Moreover, PCs may also contribute to antimicrobial defences via the production of IL-17 in response to *Trypanosoma cruzi* infection [102], of TNF- α and iNOS in the gut [103] and of Granzyme B in human tonsils [104]. Importantly, studies that have looked in parallel at distinct effector functions report that the PCs expressing IL-10 and IL-35 after EAE induction [21], expressing GM-CSF or IL-10 in the spleen [101] or expressing TNF- α and iNOS in the gut [103] are not the same ones, hence re-enforcing the notion of distinct PC subsets within the same immunological context. Altogether, these studies suggest that PCs are important and versatile modulators of their environment through the production of cytokines adapted to the challenges faced by the organism. Moreover, these functions of PCs might be detrimental in the case of solid tumours or during immune-ageing.

The phenotype of these distinct PC subsets is still unclear. Recently, LAG3 was proposed as a surface marker for IL-10-primed PCs [95]. No specific marker has been identified so far for the other cytokine producing PCs. Moreover, the origin of this functional heterogeneity is only partially understood and is likely to be multifactorial. As seen above, the B cell type, the stimulation received, and the tissue of origin might all be involved in PC function. For example, a B1a origin was proposed for GM-CSF⁺ and LAG3⁺ PCs [95],[101] despite these two cell subsets being distinct, while both follicular and MZ B cells stimulated with trypomastigotes can differentiate into IL-17⁺ PCs [102]. In terms of stimulation, TLR-dependent signalling seems necessary for rapidly promoting IL-10 secretion by PCs [20],[21],[92],[95],[105] and for inducing GM-CSF⁺ PCs [101] while TLR plus CD40/CD40L signalling is required for IL-35 production by PCs [21]. Although it was initially thought that PBs might be responsible for most cytokine production, it is now clearly established that this feature is also shared by mature PCs, hence suggesting that maturation is not the key to this functional heterogeneity [21],[95],[106]. In EAE where the protective role of IL-10-producing PCs is now well established, the tissular origin of these cells is also debated with draining lymph nodes, spleen and gut proposed as the non-exclusive sources of this cell type [20],[21],[96]. Finally, another characteristic of these cytokine-producing PC subsets is the isotype they express with most of them being of the IgM and IgA isotype as will be detailed below.

Ig isotype

PC differentiation is associated with a transition from the production of membrane Ig to secreted Ig and as such the dogma was that PCs do not express surface BCR. Over the years, however, evidence accumulated to suggest that this was not always the case, and that even fully differentiated PCs may still express some membrane Ig [101],[107],[108]. It was finally established that, both in mice and in humans, IgA⁺ and IgM⁺ PCs still express a functional BCR at their surface while IgG⁺ PCs do not [106],[109],[110]. The complete consequences of this paradigm-shifting finding are still to uncover. First works reported that BCR crosslinking on IgA⁺ PCs may promote survival [109] while on IgM⁺ PCs it may induce a transcriptional switch towards chemokine/cytokine secretion including IL-10 [106]. Although more work is required on this topic, these pioneering studies suggest that IgA⁺ and IgM⁺ PCs can respond to an antigen specific trigger through their surface BCR while IgG⁺ PCs cannot, hence denoting an extra layer of heterogeneity among PCs based on their isotype. In line with these observations, most of the cytokine-secreting PCs described were of the IgM or IgA isotype. IL-35 and GM-CSF were reported to be mainly produced by IgM⁺ PCs [21],[101]. TNF- α and iNOS production in the gut seems restricted to IgA⁺ PCs [103]. For IL-10, IgM⁺ PCs were initially reported as the main producing source [21],[94],[95],[106]. More recently, gut-derived IgA⁺ PCs were also suggested as an important source of immunosuppressive IL-10 in EAE [96]. Moreover, in the tumour environment, while IgG⁺ PCs are thought to be associated with good prognostic, IgA⁺ PCs were reported to be “pro-tumour” notably through the secretion of IL-10 [98],[99]. Whether these isotype-specific cytokine productions are induced by BCR-crosslinking on the PCs or whether they are imprinted during B cell activation is still to fully determine.

On top of cytokine production, the isotype they express may also contribute to other features of PCs. A recent paper suggests that IgG⁺ PCs secrete more Ig than IgM⁺ PCs and that this increased ER stress needs to be compensated by ER- or mitochondrial-derived ROS production [111]. Moreover, multi-parametric comparative analysis of IgA⁺, IgM⁺ and IgG⁺ PCs by CyTOF or RNAseq suggest that based on their isotype PCs clustered separately with IgG⁺ and IgM⁺ PCs being the most distinct [112],[113]. Interestingly, bulk RNAseq analysis of PCs based on their isotype also suggested that they might express distinct homing and adhesion molecules[113]. This might be due to distinct tissues of origin or could translate the requirement for distinct survival niches, something already proposed for BM IgM⁺ PCs [108]. Further work will be required to assess if this is indeed the case *in vivo*.

Single cell approaches to better understand tumour cell identity and plasticity

Terminal B cell differentiation involves a diversity of transcriptional and epigenetic modifications that can be hijacked by oncogenic events leading to the emergence of several forms of lymphoproliferative disorders, each specific entity corresponding to a certain state of B cell maturation towards PC

differentiation [114]. Most human B cell lymphomas derive from GC B cells that are therefore considered as cell of origin (COO). Indeed, the GC is a site of intense B cell proliferation and SHM that can favour the emergence of lymphomas [115]. Bulk analyses led to the widely accepted notion that tumour cells are locked at a transitional state on their way to exiting the GC as either PC or memory B cells. However, a new concept of tumour plasticity emerged from recent findings revealed by transcriptional and phenotypic profiling at single-cell level, to explain tumour B cell heterogeneity and drug resistance [55],[56],[116]. Single-cell RNA profiling of GC B cells underlined a much higher heterogeneity than previously recognized allowing for tumour cell identity to be redefined in these complex GC-derived lymphomas [55],[56]. In follicular lymphoma, the most frequent indolent non-Hodgkin lymphoma (NHL), sc-RNAseq revealed that tumour cells are not blocked at a particular transitional stage, in contrast they keep some plasticity for diverse fate and functions including capability to express transcriptional profiles belonging to the more differentiated PC subset [55] (reviewed in [117]). Whether this transcriptional diversity accounts for cellular microenvironment, environmental cues, and/or genetic heterogeneity as suspected by Andor et al. remains to be investigated in more detail [118]. Another recent study combining sc-RNAseq and flow cytometry explored the intratumor heterogeneity in term of transcriptome, genetic and drug-response of a small number of NHL patients looking at both malignant and non-malignant cells to defined the sources of cellular heterogeneity [116]. This study highlighted the power of single cell technologies to resolve each malignant subpopulation within a tumour, as well as the corresponding biomarkers and cognate drugs that might be of diagnostic value and enable successful personalized therapy.

In diffuse large B cell lymphoma (DLBCL), a clinically aggressive and heterogenous group of diseases, phenotypic profiling of malignant B cells by mass cytometry informed on both intertumoral and intratumoral heterogeneity and provided some evidence for genotype/phenotype relationship [119]. Importantly, single cell profiling of GC B cell helped to better classify subgroups of patients related to newly described single cell COO associated with different clinical outcomes [56]. While previous COO classification for DLBCL was based on two subsets: the GC B cell-like (GCB) DLBCL thought to originate from LZ B cells and the activated B cell-like (ABC) DLBCL which appear to originate from cells committed to PB differentiation [120], the newly described transitory GC B cell fates challenged this classification and even reversed the concept for some ABC-DLBCL that appear to be committed towards a memory fate at odds with PB commitment [56]. In line with this, a new experimental model supported the emerging concept that memory B cells could be the COO for some ABC-DLBCL entities [121].

A caricatural example of intra-tumour heterogeneity is Waldenström Macroglobulinemia (WM), a particularly heterogeneous post-GC NHL with BM involvement. WM pathology is heterogeneous at the level of clinical presentation and course [122]. The most striking aspect is the intra-tumour cell

fate heterogeneity, ranging from mature B cells to PCs, suggesting an ongoing PC differentiation from clonal B cells believed to be memory like cells with PC differentiation ability [123]. In line with this, recent flow cytometry analysis revealed a continuum of the PC marker CD138 expression between the malignant B cell subset and the more differentiated subset infiltrating the BM [124]. Part of these differentiated WM tumour cells, the plasmacytoid lymphocytes, co-expressed the pan-B-cell markers CD19 and CD20. Lack of heavy-chain class switching is also a hallmark of WM B cells. Hence, a disrupted B-cell fate transition into PC is believed but the mechanism behind the characteristic WM phenotype remains unknown. In the context of this pathology, a combination of relevant pre-clinical mouse models and single cell omics studies have a huge potential to better characterize the cellular complexity, cell identity and dynamic of malignant cells that should provide important cues on the way in which WM B cells differentiate, how they respond to immune stimuli and on the transcription factor network that governs the peculiar lymphoplasmacytic differentiation arrest.

Single cell approaches have also been successfully used to classify and characterize PC disorders including Monoclonal gammopathy of undetermined significance (MGUS), amyloid light chain (AL) amyloidosis and multiple myeloma (MM). Compared to normal BM PCs, and reminiscent to GC B cell derived lymphomas, MGUS, MM and AL cells display a high degree of interindividual heterogeneity [86],[90],[125]. Moreover, intra-tumour heterogeneity was prevalent and only partially linked to genomic aberrations, hence suggesting some trans-regulation of the transcriptional profile of these malignant cells [86],[125]. In parallel, such an approach has allowed to infer the potential COO of MM and AL as blood/BM CD39⁺ PCs and secondary lymphoid organs PCs, respectively [90] and has highlighted transcriptional changes associated with myeloma extramedullary manifestations, including altered metabolic profile and autocrine production of CXCL12 [125],[126]. Last but not least, there is hope for this approach to improve patient's care by optimizing treatments and understanding potential resistance. For example, the use of sc-RNAseq analyses allowed the detection of rare circulating malignant cells in MM patients after treatment and remission and revealed that their transcriptional profile is similar to the one before treatment [86]. The application and relevance of this technique in the daily clinical practice remain to fully establish.

Conclusions

As discussed above, PCs are much more than one subset of Ab secreting cells. Studying the complex mechanisms that control generation of PC subsets is of key importance not only to optimize protective immune responses but also to understand PC disorders and the failure to complete cell fate transition, the major causes of lymphomas. This may have therapeutic implications, therefore, requiring further clarification.

The evident heterogeneity in B cell responses and the average of gene expression on bulk populations have masked important trends in B cell fate decisions and PC fates that might have led to incomplete conclusions. Transcriptome wide approaches, such as sc-RNAseq, represent a new system of biological approach that can reconstruct cell lineage with an unbiased vision. In recent years the application of single cell profiling to the study of heterogenous B cell responses has contributed to better understand the dynamics of signal integration and gene expression that drive the transition of B cells to PCs. It is also now evident that tumour B cells and their normal activated B cell or PC counterparts are much more heterogenous than what was thought. Thus, at the single cell level, we now have the potential to better understand the temporal dynamics of signal integration and gene expression that may trigger distinct PC subsets. However, single cell analysis of PCs may be subject to technical limitations. Indeed, it has been difficult to identify PC subsets through sc-RNAseq and few studies have managed to link functional heterogeneity and specific phenotype. Important questions remain regarding the impact of tissue localization on PC heterogeneity and reversely whether the different PC subsets necessitate distinct environmental cues and transcriptional programming to ensure their functions.

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Conflict of interest:

The authors declare no financial or commercial conflict of interest.

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Figure legends:

Figure 1. Plasma cell programming via the extrafollicular pathway.

Schematic model of B cell response heterogeneity and cellular dynamic of mature B cell following antigen (Ag) priming. Single cell analyses have uncovered the capacity of a single naive B cell to generate multiple effector cell types: short-lived plasma cells (PCs), germinal centre (GC)-independent memory B cells or GC B cells. Heterogeneity of this response results from the origin of B cells being stimulated, the affinity and class of the BCR, and external cues such as cytokines and cognate interactions with T cells (LT). Extrafollicular PC differentiation proceeds along a defined course visualized by the blue path. Single cell analyses provided the possibility to characterize the precise stage where activated B cells gain the capacity to follow the PC differentiation path. A partial list of molecular regulators and temporal aspects are shown in boxes. Upregulation of IRF4 is key to the subsequent orchestration of the PC program, preceding BLIMP1 induction and the orchestration of the final differentiation that is coupled with epigenetic reprogramming. Multiple rounds of division are shown that are essential for progression towards the PC fate.

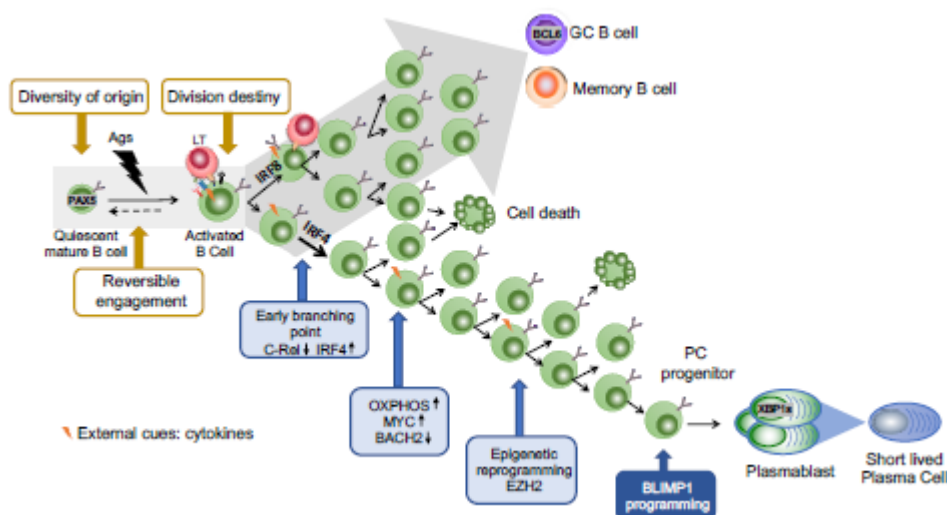


Figure 2. Activated B cell diversity in germinal centre.

Schematic model of the germinal centre (GC) response dynamic in the context of T cell help. Single-cell BCR sequencing revealed that class switch recombination (CSR) occurs mainly in human activated B cells prior to GC entry. Then, GC B cell migrates back and forth between the dark zone (DZ) and the light zone (LZ). In DZ, GC B cells expand and undergo somatic hypermutation (SHM) of their immunoglobulin genes. In LZ, GC B cells are selected based on their affinity through cellular interactions with follicular dendritic cell (FDC) and T_{FH} or undergo apoptosis. In contrast to bulk analyses that have defined the centroblast (CB) and centrocyte (CC) differentiation states, single-cell RNAseq analyses have provided an unbiased approach to identify multiple transitory cell types between LZ and DZ which fill out a continuum of gene-expression profiles. Intermediate states of GC B cell differentiation towards memory B cells (pre-memory GC B cell subset expressing CCR6, BANK1, KLF2) and plasma cells (the pre-plasmablasts (PB) expressing the PC transcription factors PRDM1 and XBP1) were also previously hidden by averaging effects in bulk analyses. BM: Bone marrow.

Figure 2

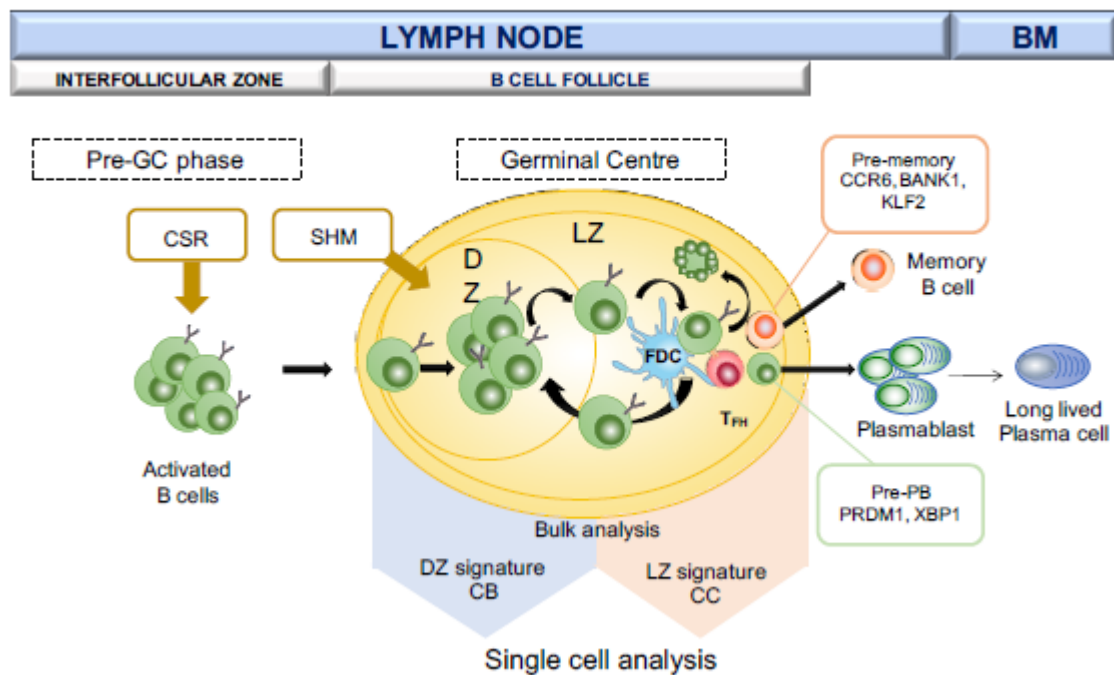


Figure 3: Determinants of plasma cell heterogeneity

Distinct layers of heterogeneity were reported for plasma cells (PCs): They can be separated based on their maturation stage from the pre-plasmablast (pre-PB) to the PC stage. PCs were also segregated between short-lived and long-lived PCs. PCs may also differ based on the isotype they express with IgA^+ and IgM^+ PCs expressing a surface BCR contrary to IgG^+ PCs. Moreover, IgM^+ PCs mainly secrete pentameric antibodies (Abs), IgA^+ PCs secrete dimeric or monomeric Abs while IgG^+ PCs only secrete monomeric Abs. Cytokine secretion by PCs of distinct isotype is also displayed. The respective contribution of the original stimulus, of the BCR isotype and of the environment to the generation and activity of these subsets is still to determine.

Figure 3

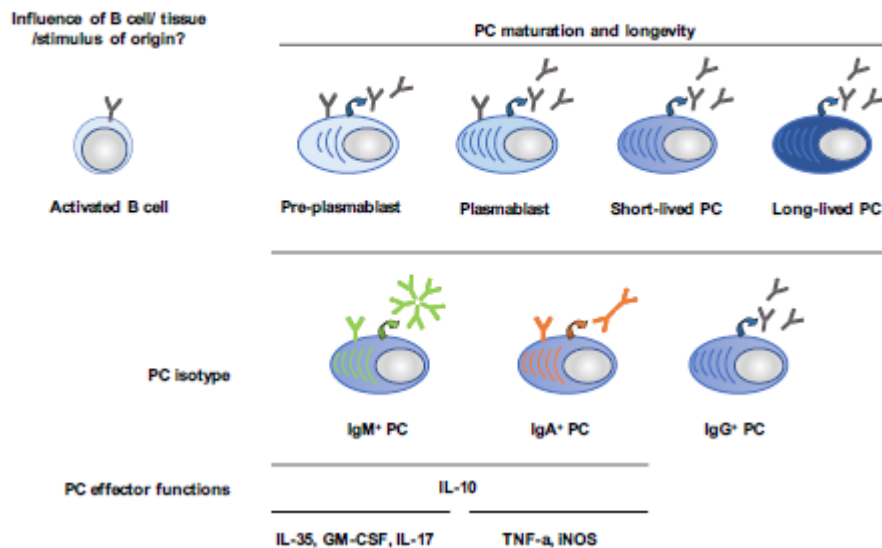


Table 1 : Surface markers for human and mouse plasma cell maturation stages :

	Pre-plasmablasts	Plasmablasts	Plasma cells	Long-lived plasma cells
<u>Human</u> :	CD38 ^{neg/low}	CD38 ^{high}	CD38 ^{high}	CD38 ^{high}
	CD27 ^{low}	CD27 ^{high}	CD27 ^{high}	CD27 ^{high}
	CD138 ⁻	CD138 ⁻	CD138 ⁺	CD138 ⁺
	CD20 ⁺	CD20 ⁺	CD20 ⁻	CD20 ⁻
	CD19 ⁺	CD19 ⁺	CD19 ⁺	CD19 ⁻
	CD45 ⁺	CD45 ⁺	CD45 ⁺	CD45 ⁻
<u>Mouse</u> :	TACI ⁺	TACI ⁺	TACI ⁺	TACI ⁺
	CD138 ⁺	CD138 ⁺	CD138 ⁺	CD138 ⁺
	CD19 ⁺	CD19 ⁺	CD19 ⁺	CD19 ⁻
	B220 ⁺	B220 ⁺	B220 ⁻	B220 ⁻