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CICLO XXVIII

PROFILING THE MOLECULAR MECHANISMS DRIVING THE FATE OF HUMAN B CELLS IN RESPONSE TO VACCINATION

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1. Abbreviations

Ab: antibody.

Ag: antigen.

APC: antigen presenting cell.

ASC: antibody secreting cell.

BCR: B cell receptor.

Cq: quantification cycle.

DC: dendritic cell.

GC: germinal center.

HCL: hierarchical clustering.

Ig: immunoglobulin.

LDA: linear discriminant analysis.

MBC: memory B cell.

PB: plasmablast.

PBMC: peripheral blood mononuclear cell.

PC: plasmacell.

PCA: principal component analysis.

PCR: polimerase chain reaction.

qPCR: quantitative PCR.

RT-qPCR: reverse transcription quantitative PCR.

Tfh: follicular helper T cell.

VH: heavy chain immunoglobulin variable region.

VL: light chain immunoglobulin variable region.

2. SUMMARY

Antigen (Ag) encounter activates B cells to proliferate and mature through the formation of germinal centers. Here somatic hypermutation of the variable regions and Immunolgobulin (Ig) isotype switching lead the high affinity Ag-specific clones to two possible differentiation outcomes: antibody (Ab) secreting plasmablasts (PB) or quiescent memory B cells (MBC). The molecular mechanism that drives the fate of a human B cell to differentiate into PB or MBC is poorly understood. Recent studies have provided new insights into the transcriptional program responsible for B cell maturation in mice or human bulk populations. The limited availability of samples and the difficulties in isolating Ag-specific MBCs from peripheral blood make this analysis particularly challenging in humans. We collected samples from human donors that received the seasonal influenza vaccine; those were processed and sorted immediately after the bleed at two different time points: day 8 and day 22 post vaccination, namely the peaks of PBs and MBCs response respectively. The blood samples were used to collect PBs, Ag-specific MBCs and naive B cells (NAIVE) by flow cytometry sorting, exploiting classical surface markers strategies. A new protocol was set up to allow qPCR analysis of multiple genes from sorted single human B cells. This protocol was first used in a pilot study on cells sorted from a first vaccinee, to perform gene expression profiling of 21 relevant genes that allowed us to discriminate the three different B cell populations. Then we up-scaled and optimized the protocol taking advantage of the 96.96 Fluidigm Dynamic Array technology, which enables to perform RT-qPCR for 96 single cells against 96 target genes in one single reaction. This new high-throughput approach was then applied to 240 single cells belonging to Ag-specific MBCs, PBs and NAIVE B cells (80 each) of a second vaccinee, to perform gene expression profiling of 96 genes involved in several pathways of B cell differentiation. By performing unsupervised hierarchical clustering on all the cells, we observed that NAIVE, PBs and MBCs clustered separately and it was possible to identify signatures of gene expression characterizing the three populations. Linear Discriminant Analysis, a dimensionality-reduction analysis, shows that PBs are particularly different from MBCs and NAIVE, that instead share more similarities. By performing statistical analysis we identified the significant differentially expressed genes, which include genes involved in known B cell expression networks and, interestingly, also novel observations (*FOXP1, POU2AF1, IRF2*). We then compared the gene expression profile of Ag-specific MBCs with MBCs isolated from a healthy donor, to investigate possible differences in the expression patterns of recently activated MBCs and steady-state MBCs. With this analysis we identified 16 genes with a significant

differential expression level, denoting a more active profile for the recently activated MBCs isolated from the vaccinee. To further investigate the heterogeneity of Ag-specific MBCs we also recovered immunoglobulin VH sequences from the same cells by sequencing the specific PCR products. Correlation studies showed only weak association between B cell receptor (BCR) maturation (in terms of VH mutation rate) and gene expression data. Conversely, significant association was found between the expression of two genes and the Ig isotype. In particular *RORα* is associated with IgA, while *TBX21* with IgG, in accordance to previous studies performed on mouse bulk B cell populations. The genes identified with this study could be further investigated as they represent potential markers of B cell response to human vaccination.

3. RIASSUNTO

Nell'ambito del processo di attivazione dovuto all'interazione con l'antigene (Ag), le cellule B proliferano e iniziano un processo di maturazione terminale attraverso la formazione dei centri germinativi (GC). All'interno dei GC, a seguito dell'ipermutazione somatica delle regioni variabili del recettore delle cellule B (BCR) ed il cambiamento di isotipo delle immunoglobuline, i cloni che hanno raggiunto alta affinità per l'Ag possono andare incontro a due possibili destini: differenziamento in plasmablasti (PB) che secernono anticorpi (Ab) o in cellule B della memoria quiescenti (MBC). Il meccanismo molecolare che determina il destino delle cellule B umane durante il differenziamento tardivo in PB o MBC è poco conosciuto. Studi recenti hanno rivelato nuovi aspetti del programma trascrizionale responsabile della maturazione di cellule B in topo o in popolazioni di cellule umane, ma la disponibilità limitata di campioni e la difficoltà nell'isolamento di MBC Ag-specifiche da sangue periferico hanno reso l'analisi di questi tipi cellulari particolarmente complicata. Per questo sono stati raccolti campioni di sangue da donatori sottoposti a vaccinazione stagionale contro l'influenza. Questi campioni sono stati processati immediatamente dopo il prelievo, effettuato in corrispondenza di due particolari momenti: 8 e 22 giorni dopo la vaccinazione, rispettivamente picchi della risposta mediata da PB e da MBC . I campioni di sangue periferico sono stati usati per l'isolamento di PB, NAIVE e MBC Ag-specifiche sfruttando marcatori di superficie. Per l'analisi del profilo di espressione genica è stato ottimizzato un metodo che permette di effettuare qPCR di numerosi geni in cellule B umane isolate come singola cellula. Tale approccio è stato usato inizialmente per uno studio pilota dell'espressione di 21 geni di interesse su cellule isolate da un primo soggetto, permettendoci di discriminare cellule appartenenti alle tre diverse popolazioni. Successivamente questo protocollo è stato ottimizzato sfruttando la tecnologia del 96.96 Dynamic Array prodotto da Fluidigm, sistema che permette di effettuare RT-qPCR su 96 singole cellule per 96 geni in una singola reazione. Con questo metodo ad alta resa abbiamo analizzato 240 singole cellule appartenenti alle popolazioni di MBC Ag-specifiche, PB e NAIVE (80 cellule ciascuna) di un secondo soggetto, permettendoci di analizzare il profilo di espressione di 96 geni coinvolti nelle vie di differenziamento delle cellule B. Attraverso un'analisi statistica di raggruppamento gerarchico dei dati di espressione appartenenti a tutti i campioni processati, abbiamo riunito sotto tre gruppi diversi per espressione genica le cellule appartenenti alle tre diverse popolazioni e abbiamo identificato i geni che le caratterizzano. La Linear Discriminant Analysis, una tecnica di riduzione dimensionale supervisionata, sottolinea come i PB siano

particolarmente differenti da MBC Ag-specifiche e NAIVE, che invece condividono un profilo più simile. Sfruttando diversi metodi di analisi statistica, sono stati identificati i geni significativamente espressi in maniera diversa tra le tre popolazioni. Così facendo sono stati individuati sia geni il cui ruolo nella maturazione delle cellule B è noto, sia geni conosciuti principalmente per la loro funzione in altri processi o altre fasi dello sviluppo di queste cellule (*FOXP1, POU2AF1, IRF2*). Inoltre abbiamo confrontato i profili di espressione delle MBC Ag-specifiche con MBC isolate da un donatore sano non vaccinato, per identificare possibili differenze nei profili di espressione di MBC recentemente attivate e MBC circolanti, lontane dall'attivazione Ag-specifica. Tale analisi ha identificato 16 geni espressi differentemente in maniera significativa, evidenziando un profilo di espressione che denota uno stato di attivazione per le MBC recentemente contattate dall'Ag. Per studiare ulteriormente l'eterogeneità delle MBC Ag-specifiche, tramite PCR abbiamo amplificato e sequenziato le regioni variabili delle catene pesanti (VH) delle immunoglobuline espresse dalle stesse cellule, ma gli studi di correlazione mostrano solo deboli associazioni tra maturazione del BCR (in termini di tasso di mutazione delle VH) e dati di espressione genica. Al contrario, è stata individuata associazione significativa tra la selezione dell'isotipo del BCR e l'espressione di due geni, in particolare l'espressione di *RORα* è associata alla classe IgA, mentre *TBX21* all'IgG, in accordo con studi precedenti effettuati in popolazioni di cellule B murine. In conclusione, i geni identificati da questo studio come discriminanti delle MBC recentemente attivate dall'Ag potrebbero essere ulteriormente studiati in qualità di potenziali marker della risposta B alla vaccinazione in uomo.

4. INTRODUCTION

Efficacy of vaccination relies heavily on the production of long lasting protection. There are several important differences between primary and secondary antibody responses, concerning both quantity and quality of the antibodies produced (Chart 1).

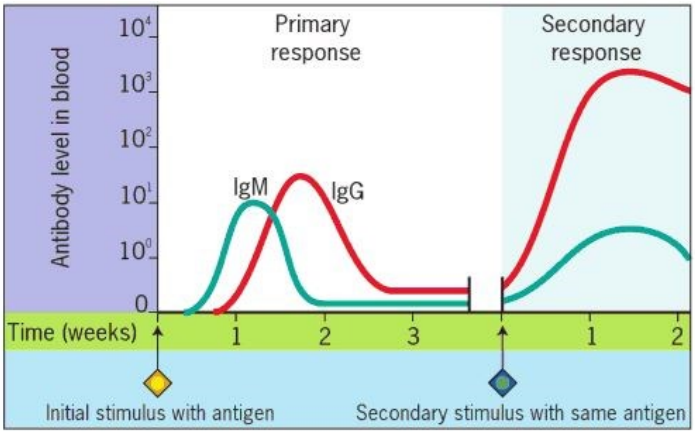


Chart 1: *Primary and secondary antibody responses*[161].

On secondary responses the lag phase is reduced and the antibody titers are typically higher. Moreover, the first contact with an antigen leads to an initial production of IgM and a lagged IgG production, whereas when the antigen is re-introduced the response is generally IgG dominated and characterized by higher affinity antibodies. Thus, between primary and recall responses, isotype switch and affinity maturation take place. These differences are a consequence of the different B cell subset kinetics of response to the antigen (Chart 2).

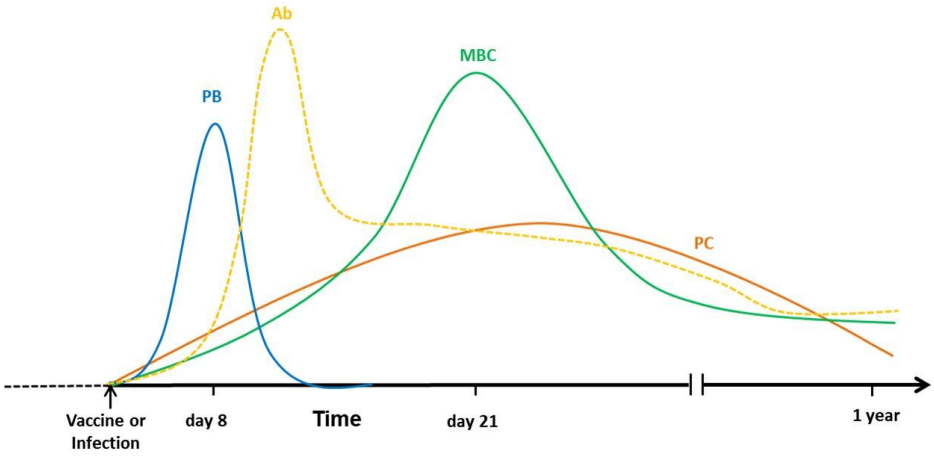


Chart 2: *Immune response kinetics of different B cell populations.*

Briefly, naïve B cells in peripheral lymphoid organs are activated to proliferate and differentiate into antibody-secreting plasma cells (peaking 8 days after vaccination) and memory B cells (peaking 21-30 days after vaccination). Some plasma cells may migrate to and survive in the bone marrow for long periods. In secondary responses, memory B

cells are quickly activated to differentiate in plasmablast and produce larger amounts of antibodies, often with more heavy-chain class switching and affinity maturation. Therefore the activity of a small number of surviving memory B cells generated during the primary immune response is believed to be responsible of this improved responsiveness. Dissecting the peculiar proprieties characterizing the memory compartment is then fundamental to understand the basis of humoral memory and hence to improve vaccine development.

4.1. Early B cell development

B lymphocytes are essential antibody-producing cells of the immune system. They develop from hematopoietic stem cells (HSCs) that originate from bone marrow [1]. The pluripotent HSC with its extensive self-renewal potential regenerates all blood cell types throughout life by differentiating to progenitor cells with gradually restricted developmental potential. HSCs first differentiate into multipotent progenitor (MPP) cells, then they commit to either the lymphoid or erythroid-myeloid lineages, resulting in the formation of the common lymphoid (CLP) cells [1]. From here, their development into B cells occurs in several stages, each marked by various gene expression patterns and immunoglobulin H chain and L chain gene loci arrangements, the latter due to B cells undergoing V(D)J recombination as they develop [2]. B cells undergo two types of selection while developing in the bone marrow to ensure proper development. Positive selection occurs through antigen-independent signaling involving both the pre-BCR and the BCR. If these receptors do not bind to their ligand, B cells do not receive the proper signals and cease to develop [3,4]. Negative selection occurs through the binding of self-antigen with the BCR: if the BCR can bind strongly to self-antigen, then the B cell undergoes clonal deletion, receptor editing or anergy [4]. This negative selection process leads to a state of central tolerance, to avoid self-antigen binding by mature B cells present in the bone marrow [2]. To complete development, immature B cells migrate from the bone marrow to the spleen and pass through the T1 and T2 transitional stages [5]. While migrating to the spleen and after spleen entry, they are considered T1 B cells [6]. Within the spleen, T1 B cells turn into T2 B cells and then differentiate into either follicular (FO) B cells or marginal zone (MZ) B cells depending on signals received through the BCR and other receptors [7]. After differentiation, they are considered mature B cells, or naive B cells and they start to circulate between secondary lymphoid organs (lymph nodes) through the peripheral system.

4.2. Germinal center dynamics and late B cell differentiation

In order for our bodies to mount a successful humoral immune response, B cells must first encounter, and then be activated by, their cognate antigens. Following activation, a coordinated cascade of events involving several different cell types drives antigen-engaged B cells to diversify the antibody they produce in order to increase affinity for the antigen and alter its effector function to produce the best possible response to said antigen[8]. During this process, the B cell differentiates into either an antibody secreting cell (plasmablast (PB) or PCs) or a long lived MBC. This sequence of events takes place within a specialized immunological environment termed the germinal center (GC).

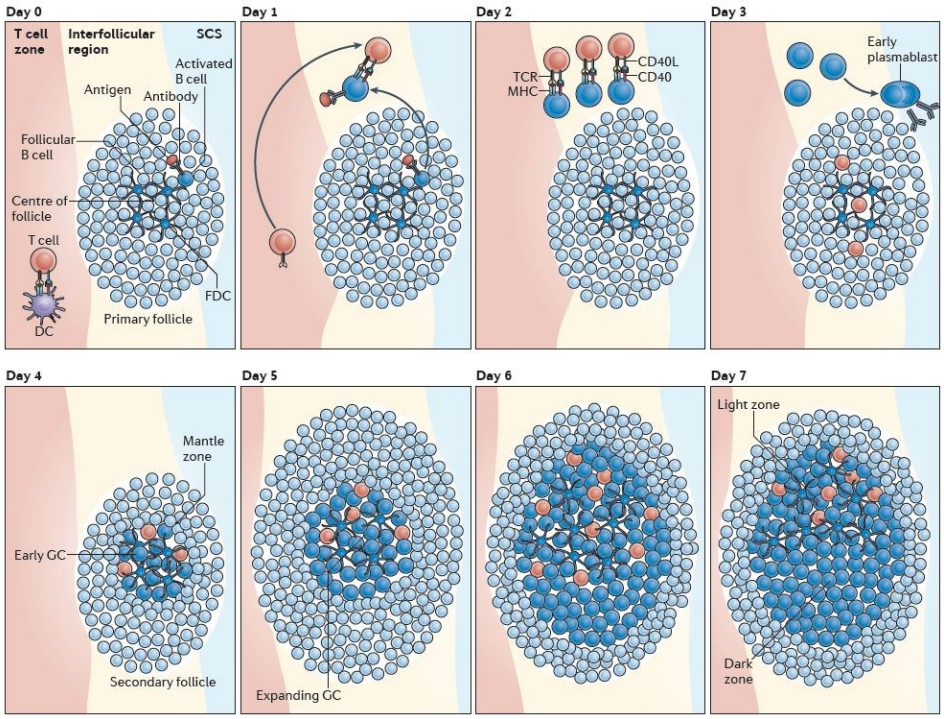


Chart 3: *B cell activation by the antigen* [8].

GCs are not permanent structures, but rather arise transiently within the lymphoid tissue in response to a T cell-dependent antigen (Chart 3). Lymph nodes are composed of multiple lobules surrounded by lymph-filled sinuses enclosed by a capsule [9]. Naive B and T cells from the circulation cycle continually through the lymph node, residing within distinct areas of the lobule where they can interact with antigen-presenting cells (APCs) to survey the antigenic environment. The outer layer (cortex) contains follicles of naive B cells and follicular dendritic cells (FDCs). These follicles are separated from the T cell zone (paracortex), which contains naive T cells and dendritic cells (DCs), by the interfollicular zone [10]. Circulating naive B cells bear the chemokine receptor CXCR5

and so are attracted to the lymphoid follicles by the chemokine CXCL13, which is expressed by resident FDCs and marginal reticular cells [11]. Similarly, circulating naive T cells expressing the chemokine receptor CCR7 are recruited to the T cell zone by fibroblastic reticular cell expression of the CCR7 ligands, CCL19 and CCL21 [12]. Once in the follicles, naive B cells interact with antigen via their B cell receptor (BCR). BCR binding to cognate antigen activates the B cell, triggering internalization of the BCR and presentation of antigen on the cell surface in the context of major histocompatibility complex (MHC) class II molecules [13].BCR engagement also up-regulates expression of the chemokine receptor CCR7, which promotes B cell migration to the periphery of the T cell zone where its ligands are expressed abundantly. During this phase of activation, the B cells continue to maintain expression of CXCR5; the balance between CXCL13 expression in the follicles and CCL19/21 expression in the T cell zone positions B cells at the border of the T cell zone [14]. Meanwhile, naive T cells in the T cell zone encounter their cognate antigen, here presented by DCs, initiating commitment towards a T follicular helper (Tfh) cell phenotype [15]. Tfh cell commitment is accompanied by CCR7 down-regulation and CXCR5 upregulation, promoting Tfh cell migration to the T/B cell boundary where they can support B cell expansion [16,17]. Two days after antigen encounter, activated B cells find their cognate Tfh cells and form long-lived interactions that result in full B cell activation and proliferation [18,19]. At this time, a subset of activated B cells moves away from the extrafollicular sites and differentiate into short-lived PB. These cells secrete IgM, providing immediate protection to the individual, but with low specificity [20]. After 3 days, the activated Tfh and B cells migrate into the center of the follicle, where the B cells start to proliferate rapidly. By this time the B cells begin to express the master regulator B cell lymphoma 6 protein (BCL6), which drives the acquisition of the GC B cell phenotype [21]. The rapid proliferation of activated GC B cells within a network of FDCs pushes aside the resident follicular B cells to form the early GC over days 5–6. By day 7 the rapid proliferation of GC B cells, coupled with the continued influx of activated GC cells, results in the polarization of the fully formed GC into two distinct microenvironments called dark and light zones (Chart 4). In the dark zone, densely packed GC B cells, referred to as centroblasts, divide rapidly and undergo SHM. Centroblasts are retained in the dark zone by their expression of the chemokine receptor CXCR4, the ligand of which, CXCL12, is expressed abundantly by dark zone stromal cells [22]. Down-regulation of CXCR4 and up-regulation of CD83 and CD86 allow the GC B cells to migrate from the dark zone into the light zone, a less densely packed

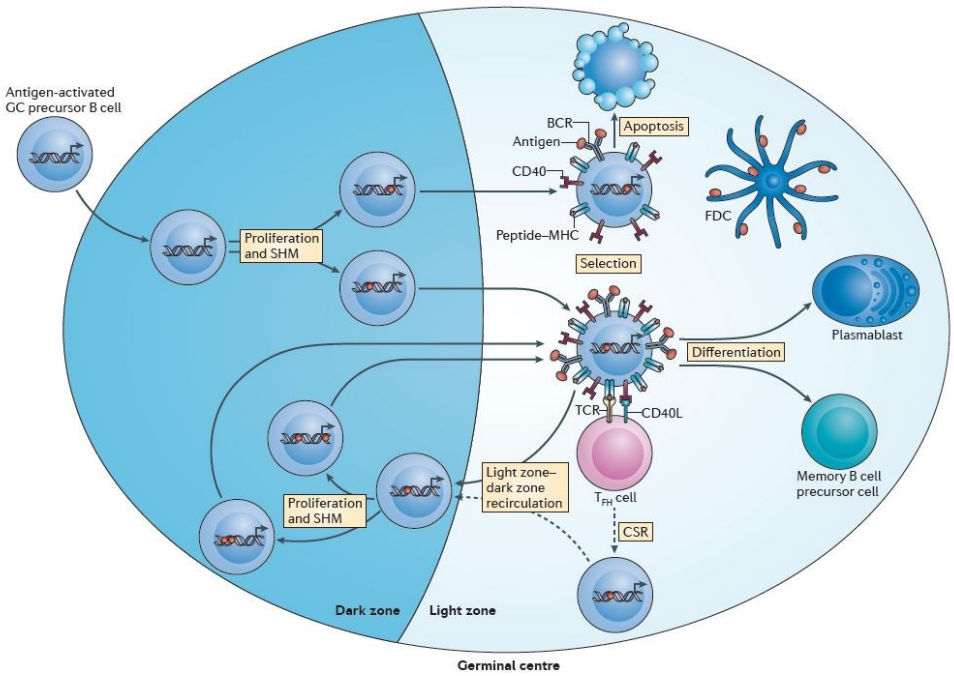


Chart 4: *Germinal center dynamics and late B cell differentiation* [8].

compartment populated with Tfh cells, macrophages and FDCs [23]. In the light zone, B cells undergo a process of selection where B cells producing higher-affinity antibodies compete for available antigen [24] and/or T cell help [25], thus receiving survival signals via BCR binding. Selection promotes centrocyte re-entry into the dark zone for further rounds of mutation and selection [26]. Conversely, lower-affinity B cells receive no survival cues and undergo apoptosis [27]. In parallel, CSR drives apoptosis of undesirable B cell clones through deletion of the Ig heavy chain in a process called locus suicide recombination. This prevents BCR expression and thus eliminates the survival signals the BCR transmits, inducing apoptosis [28]. Having survived selection in the light zone, GC B cells can do one of three things: they can re-enter the dark zone for additional rounds of proliferation and somatic hypermutation (SHM) [29]. Alternatively, GC B cells can leave the GC and differentiate into PB (precursors of antibody-secreting PCs) or they can differentiate to form long-lived MBC to enable a rapid response upon re-encountering the same antigen [30,31]. Co-ordination of all the events described above is controlled by a number of master regulator transcription factors.

Among these factors, some are responsible of maintaining B cell identity. Paired box protein 5 (PAX5) is the master regulator of B cell identity and is expressed throughout B cell development [32], from pro-B cells [33] to mature GC B cells [34]. PAX5 binds directly to thousands of DNA sites in B cells and functions by both activating and repressing gene expression [32]. During early B cell development PAX5 is required for

the initial commitment of lymphoid progenitors to the B cell fate and VDJ recombination of the Ig locus [33,35,36]. In mature B cells it regulates the expression of genes critical to B cell identity, including components of the B cell receptor (Ig heavy chain and CD79A), CD19, CD21, B lymphocyte kinase (BLK), interferon regulatory factor IRF4 and IRF8 [32]. In addition, PAX5 further reinforces B cell identity by repressing the expression of lineage inappropriate genes, including Fms-like tyrosine kinase 3 (FLT3), CCR2 and CD28, which are expressed in PCs following PAX5 down-regulation, and macrophage colony-stimulating factor (M-CSF) receptor, NOTCH1, RAMP1, LMO2 and CCL3, which are expressed in common lymphoid progenitors and myeloid cells [37]. As PAX5 promotes and maintains the expression of the B cell transcriptional program, its down-regulation is required for differentiation into committed Ig-secreting PCs [38]. Remarkably, PAX5 directly represses the expression of one of the master regulators of the PC program, XBP1, and its down-regulation is required for Ig secretion [39,40].

As mentioned earlier, BCL6 is essential for GC formation [41] and is considered the master regulator of the GC, where it controls gene expression programs in both GC B cells and in Tfh cells [21,42] . Within these cells BCL6 functions predominantly as a transcriptional repressor, directly suppressing multiple genes involved in the DNA damage-sensing pathway, including TP53, ATR and CHEK1 and regulators of the cell cycle, p21, p53 [43,44]; this establishes a transcriptional program that allows both the rapid proliferation of cells and the tolerance of DNA damage essential to SHM. In addition, BCL6 controls the migration of B cells into the follicle. One of the critical functions of BCL6 appears to be the repression of PC differentiation, in this case mediated by repression of BLIMP1 [45]. Although BCL6 functions as a transcriptional repressor, it also induces AID expression indirectly in GC B cells [41]. Similarly, repression of SPI1, IRF8 and MYB is also relieved, all regulators of the GC transcriptional program.

Two transcription factors, B lymphocyte-induced maturation protein 1 (BLIMP1, also known as PR domain zinc finger protein 1, PRDM1) and X-box-binding protein 1 (XBP1) are essential for orchestrating PC differentiation [30]. BLIMP1 is a transcriptional repressor that, within the B cell lineage, is expressed exclusively in antibody-secreting cells; at lower levels in PBs and higher levels in mature PCs [46,47]. During PC commitment, BLIMP1 represses the expression of the B cell-specific regulators PAX5, BCL6, ID3 and Spi-B transcription factor (Spi-1/PU.1-related) (SPIB) [48], thus allowing expression of XBP1. However, although XBP1 appears to act downstream of BLIMP1 in

the regulatory network [49], BLIMP1 is necessary, but not sufficient for XBP1 expression [38]. Furthermore, BLIMP1 is not required for initiation of the PC differentiation program as pre-plasmablasts form in the absence of BLIMP1 [50]. XBP1 acts downstream of BLIMP1 and is a key regulator of PC development, but it is not absolutely required for the formation of antibody-secreting cells [51]. Rather, XBP1 appears to act predominantly to set up the cells to allow for the secretion of vast quantities of Ig [52], inducing endoplasmic reticulum remodelling, activation of mechanistic target of rapamycin (mTOR) [53] and autophagic pathways [54] and the induction of the unfolded protein response [52]. Although much is known regarding the interconnections that exist between the regulatory networks of these B cell lineage master regulators, questions remain as to exactly what initiates each pathway. In addition, controversy still surrounds the issue of PC longevity. Short-lived PCs play a critical role in the immune response and undergo a 'traditional' differentiation program, exiting the cell cycle, undergoing terminal differentiation followed by rapid cell death. However, while it is clear that a long-lived PC population is maintained in the bone marrow [55], it is still not clear how this population is maintained. Recent data suggest that active autophagy might account for the longevity of these cells [56], protecting these cells from apoptosis, possibly in combination with some degree of ongoing homeostatic proliferation [57].

IRF4 is a member of the IRF (interferon regulatory factor) superfamily of transcription factors that shows relatively weak DNA binding on its own. Therefore, in order to exert its diverse functions it binds DNA co-operatively with a host of other transcription factors, including IRF8, PU.1 and Spi-B [58,59]. IRF4 plays an essential role in isotype-switching, with IRF4-deficient mice failing to induce AID expression and undergo CSR when stimulated in vitro [60,61]. IRF4 may regulate AID expression through co-operative binding with BATF, a transcription factor essential for AID expression [62]. IRF4 is induced rapidly upon BCR ligation [63,64] and is reported to be required for BCL6-induction and entry into the GC reaction. However, it is not required for maintenance of the GC [60]. In addition to establishing the GC reaction, IRF4-deficient mice also fail to make mature PCs [60,61] and this defect is a result of failure to induce BLIMP1 expression [61]. However, it was also suggested that the failure to induce PC differentiation is independent of BLIMP1 expression and instead is due to a loss in XBP1 expression [60]. The ability of IRF4 to initiate two distinct cell fate transitions, GC B cell and PC differentiation, originates from its differing expression levels at these times. IRF4 is expressed at low levels in naive B cells but is up-regulated during PC differentiation

[65]. It is thought that the strength of the BCR signaling, as determined by the affinity of the BCR for antigen, determines the level of IRF4 induction. This, in turn, determines whether the GC B cell program or the PC differentiation program is initiated: initially, low concentrations of IRF4 activate AID and BCL6 expression. As the GC reaction continues Ig affinity increases, leading to increased BCR signaling and elevated IRF4 expression, favoring BLIMP1 expression [63,66], BCL6 repression [67] and extinguishment of the GC program. These divergent functions of IRF4 are mediated through its ability to associate with different binding motifs. At lower concentrations, IRF4 co-operates with PU.1 and BATF, facilitating binding to ETS-IRF or AP-1-IRF composite motifs and coordinating the GC program. At high concentrations, resulting from hypermutation-driven high-affinity BCR- antigen recognition, IRF4 favors binding to interferon sequence response elements (ISREs), shifting the cells' expression profile towards the PC program [63].

IRF8 is another member of the IRF transcription factor superfamily, but unlike IRF4 is expressed abundantly in centroblasts [68] and down-regulated in centrocytes [69]. IRF8 was proposed initially to regulate BCL6 and AID positively; IRF8 over-expression in human B cells increased the abundance of BCL6 and AID transcripts, while siRNA-mediated knock-down of IRF8 in a murine GC-derived B cell line had the opposite effect [68]. However, more recently, IRF8-deficient mice have been shown to display only minor reductions in AID and BCL6 expression and have a normal antibody response [70]. While the phenotype of IRF8-deficient B cells is relatively minor, knock-out of both IRF8 and its common binding partner PU.1 result in heightened PC differentiation and class-switch recombination [65]. This mouse model showed that IRF8:PU.1 are together able to help maintain the B cell program by promoting expression of PAX5 and BCL6 and concurrently repressing BLIMP1.

Another critical component of the humoral immune response is cell death, which allows autoimmunity prevention, drives affinity maturation and terminates the response once the challenge has been met. Conversely, inhibition of apoptosis is essential for immunological memory. Apoptosis induced by the loss of environmental signals such as growth factor withdrawal or loss of BCR signaling is initiated by pro-apoptotic members of the BCL2 family of proteins (including BIM, BAD, BIK and BAX), while it is prevented by the anti-apoptotic BCL2 factors (BCL2, BCLXL and myeloid cell leukaemia 1 (MCL1)). Thus, a B cell's apoptotic potential is determined by the balance between pro-apoptotic and anti-apoptotic signaling. Accordingly, B cells undergoing affinity maturation in the GC

show low expression of a number of anti-apoptotic factors, such as BCL2, while express pro-apoptotic factors such as FAS and BAX abundantly [71]. As such, GC B cells appear to be destined to apoptosis unless rescued by BCR signaling. More recently, MCL1 has been identified as the main anti-apoptotic regulator of GC B cells and MBCs [72]. Due to the requirement for DNA recombination, mutation and rapid proliferation, B cells are prone to lymphoma development at various stages of B cell ontogeny. Of these, a number are derived from the GC stage, including follicular lymphoma, diffuse large B cell lymphoma, Hodgkin's lymphoma and Burkitt's lymphoma. In many of these cases either translocation of the BCL2 gene or up-regulation of one of the anti-apoptotic BCL2 family members can be demonstrated and probably plays a role in the transformation process [73].

It has become increasingly apparent that the different B cell expression programs are controlled by a highly coordinated regulatory network. Within this network, multiple points of positive and negative feedback ensure the mutually antagonistic expression of the master regulators, augmented by an increasing number of secondary factors that reinforce these networks and contribute towards sensing the progress of the GC reaction. Initially, the B cell-specific expression pattern is established by PAX5, which not only regulates the expression of proteins critical to B cell function but also drives the expression of IRF4 (at a low level), IRF8 and BACH2. Together, these factors inhibit the expression of the master regulators of PC differentiation, BLIMP1 and XBP1; PAX5 directly represses XBP1, while IRF8, in combination with PU.1, both maintains PAX5 and inhibits BLIMP1. BLIMP1 is also suppressed actively by BACH2. Following activation of the B cell via BCR engagement, BCL6 is activated by IRF4/PU.1. BCL6 controls not only the establishment of the GC fate, initiating the diversification pathways and rapid proliferation of the B cells, but also further represses BLIMP1. Although much has been elucidated as to how these pathways repress B cell differentiation into PCs, it is less clear how the path is set towards favoring terminal differentiation to PCs, essential for the final success of the GC reaction. As SHM produces Igs of ever-increasing affinity, BCR signal strength increases, in turn increasing IRF4 expression. Increased IRF4 expression then starts to activate BLIMP1, which in turn represses BCL6 and PAX5. Once BLIMP1 accumulates, it represses multiple genes responsible for maintaining B cell identity, including BCL6. This, in turn, allows the expression of genes responsible for PC identity, driven in part by IRF4. Finally, suppression of PAX5 relieves repression of XBP1, allowing establishment of the full secretory program. Although critical, the network described

above appears not to be the whole story. The rapid proliferation of B cells is a necessary part of the GC response, but it now seems likely that this process also plays an active role in determining cell fate. It has been known for many years that a cell's potential to undergo CSR is determined (at least in part) by the number of divisions it has undergone [74,75]. Later, it was shown that a B cell's potential to undergo differentiation into an antibody-secreting cell was also dependent upon division number [74]. Together, these data suggest that B cells possess some form of division counting mechanism that changes an individual cell potential to undergo cell division, apoptosis and differentiation [30]. Further, recent studies suggest that individual naive B cells may have a restricted potential with regard to the number and type of effector cells (PC, MBCs and GC cells) into which their progeny can differentiate [76]. Clonal populations that resisted apoptosis and divided more times were more likely to give rise to multiple effector subsets. In addition, clones bearing higher-affinity antigen receptors underwent higher levels of cell division and generated a greater ratio of PCs to MBCs than clones bearing lower-affinity receptors [76]. Thus a combination of BCR signaling, cell division and apoptosis appears to determine the response of an individual B cell following antigen encounter. Much is now known about the molecular network regulating the GC response and PC differentiation, both of which are controlled largely by the expression of a small number of master regulators. However, for what concerns MBC, no deterministic transcription factor has been found so far.

4.3. Generation of memory B cells

In T cell-dependent B cell responses, accumulating evidence shows that antigen-activated proliferating B cells begin to follow one of three fates by differentiating into extrafollicular short-lived PCs, GC-independent memory B cells or GC-dependent memory B cells [77].

Affinity-dependent B cell selection occurs at the B cell–T cell border as a result of T cell help, which could affect B cell fate decisions [78]. Among the various signals provided by T cells, the CD40 signal alone can induce activated B cells to differentiate down the memory pathway but not into GC cells [79]. In addition to the CD40 signal, cytokine signaling is probably required for germinal center B cell differentiation. Indeed, interleukin-21 (IL-21) was shown to upregulate the expression of B cell lymphoma 6 (BCL-6) in B cells, which is a crucial transcription factor for germinal center formation and maintenance [80,81]. Hence the formation of durable Tfh cell–B cell conjugates to provide adequate T cell help could enable B cells to differentiate into GC B cells.

However, if the duration of conjugate formation is fairly short, B cells are more likely to join the GC-independent MBC pool. Given that class switching but not somatic hypermutation occurs during this early period, BCR specificities of the GC-independent MBCs are likely to reflect those of the initial responding B cells. Therefore, the GC-independent MBCs may enable the host to maintain a broad range of antigen-specific B cells possibly providing protection against pathogens that bear related but distinct antigens and epitopes.

As reported above, for what concerns GC-dependent MBC the precise mechanism of formation is still unclear. One hypothesis is that there is a master regulator of transcription that directs the cells towards a memory B cell fate, but so far no single deterministic transcription factor for MBCs has been elucidated. An alternative idea is that MBCs differentiate stochastically from GC B cells and that a survival advantage is sufficient for MBC differentiation [82].

It was previously assumed that MBCs are only formed during T cell-dependent immune responses and therefore that conventional B2 cells are the exclusive participants in MBC generation. However, recent data show that B1 cells can also generate MBCs during T cell-independent immune responses [83,84]. B1 cells are the most abundant B cells in the peritoneal cavity but they are also present at a low but detectable frequency in the spleen [85]. Although T cell-independent MBCs can be generated, it seems that their recall response is quantitative, rather than qualitative. Thus it is unclear whether T cell-independent MBCs have an intrinsic advantage compared with their naive B cell counterparts to respond more rapidly and more robustly to the antigen.

4.4. Heterogeneity of memory B cells

During the primary immune response, several types of MBCs are generated, suggesting the idea that these have distinct functions [86]. Two decades ago, it was hypothesized that there are two distinct types of MBCs (IgM^+ and IgG^+ cells) which are activated and function in a distinct manner during reinfection [87]. Two groups have recently addressed this question and they have reached a similar conclusion that upon antigen re-challenge, IgG^+ MBCs preferentially differentiate into PB, whereas IgM^+ MBCs proliferate more and enter the GC reaction [88,89]. However, it seems that there is functional heterogeneity even within the IgM^+ or IgG^+ MBC pools, and it cannot be excluded that IgG^+ MBCs can re-enter germinal centers or that IgM^+ MBCs might produce a PB response. A more recent study has proposed that other markers (CD80 and programmed cell death 1 ligand 2 (PDL2)) are more functionally relevant to MBC subsets;

$CD80^-$ $PDL2^-$ MBCs enter the GC reaction, whereas $CD80^+$ $PDL2^+$ MBCs promptly differentiate into PBs upon restimulation [90]. The above-mentioned studies mainly used MBCs expressing IgG1 or expressing mixtures of IgG1, IgG2a and IgG2b. However, a recent study shows the need to functionally characterize each isotype of MBC [91]. Transcription factors that are induced in B cells by cytokines are important for regulating subsequent B cell behaviour in the primary response; for example, interferon-γ (IFNγ)-induced T-bet (also known as TBX21) expression is known to be important for IgG2a class switching. Interestingly, such transcription factors are also important for the survival of immunoglobulin class-specific MBC [91]. Expression of T-bet or retinoic acid receptor-related orphan receptor-α (RORα) in IgG2a+ or IgA+ MBCs, respectively, is higher than in naive B cells, and these transcription factors are crucial for memory cell survival, probably by controlling the transcription of genes that encode cell-surface BCR components [91]. As each subclass of immunoglobulin has unique biological activities as a result of its Fc portion, targeting particular transcription factors for developing antibody isotype- skewing vaccines could be an important strategy for immunotherapeutic applications. In summary, these recent studies of MBCs expressing IgM, IgG2a and IgA have shown that the origin, the function and the longevity of MBCs could differ between cells expressing different antibody isotypes. Therefore, questions arise about how such heterogeneity is induced and whether different types of MBCs are coordinately activated upon secondary infection.

4.5. Peculiar characteristics of memory B cells

Key functional features of MBCs are their longevity and their rapid and robust responses to antigen re-exposure, which are the basis of vaccine success. Haematopoiesis is a well-known example of a biological system with long-term functions. In this system, the long-term maintenance of homeostasis depends on the co-existence of somatic stem cells and more committed progenitor cells [92]. The stem cells ensure the efficient replacement of more committed cells, but at the same time maintain themselves through a process of self-renewal. The more committed progenitor cells can be quickly differentiated into more mature cells following exogenous stimulation. It was postulated that such a stem cell-based mechanism might be similarly used by the humoral memory system, which requires bi-functionality to efficiently make effector cells upon re-encountering pathogens and simultaneously continue to maintain the responsive memory state. As IgG^+ MBCs seem to have a greater propensity to differentiate towards PCs than IgM^+ MBCs do, it could be suggested that the IgM^+ MBC compartment contains

more stem cell-like cells, whereas class-switched MBCs, such as IgG^+ MBCs, correspond to committed progenitor cells. This proposal requires further study but would be similar to the situation for memory $CD8^+$ T cells, for which substantial evidence of a stem cell-based model has recently been provided [93].

To determine which types of cells and molecules are required for MBC survival, previous studies have used IgG^+ MBCs as a target. Those can persist in the absence of T cells or input from precursor cells, but experiments in mice have suggested that there is a requirement for FDCs for the maintenance of IgG^+ MBCs [94]. In these mice, the primary IgG response was unaffected, but the secondary antibody response was significantly decreased. Notably, the impaired memory response corresponded with the reduced frequency of antigen-specific MBCs. Inducible deletion of phospholipase Cγ2 (PLCγ2) after the generation of $IgG1^+$ MBCs substantially decreased the size of the memory compartment, which suggests a requirement for BCR signaling for $IgG1^+$ MBC survival [95]. In terms of a requirement for antigen, genetic studies showed that cognate antigen was not necessarily required after the generation of IgG^+ MBCs, which implicates a tonic-like BCR signal in the maintenance of IgG^+ MBCs [96]. As a result, factors that participate in expression of the BCR components (class-specific immunoglobulin heavy and light chains, Igα and Igβ) and tonic BCR signaling molecules could be potential determinants of memory B cell survival. The differential persistence of IgM^+ and IgG^+ MBCs was recently shown; Ag-specific IgM^+ MBCs persisted for 500 days after priming, whereas the number of IgG^+ MBCs declined by many fold during this time period [89]. This could be explained by differences in the self-renewal activity of IgM^+ and IgG^+ MBCs (as discussed above) and/or by the existence of differential B cell survival mechanisms. Consistent with the existence of differential B cell survival mechanisms, blocking the receptors for B cell-activating factor (BAFF; also known as TNFSF13B) and a proliferation-inducing ligand (APRIL; also known as TNFSF13) did not affect the survival of IgG^+ MBCs *in vivo* but had a marked effect on naive IgM^+ B cells [97]. Therefore, the differential usage of BAFF and/or APRIL might be one cause of differential survival between IgM^+ and IgG^+ MBCs in mice, although this requires further clarification and may not apply to human B cells. However, in humans, vaccinations and infections are known to elicit stable populations of IgG+ MBCs [98]. Thus, it would be interesting to test the possibility of heterogeneity between IgG1+ MBCs in terms of their self-renewal and their survival ability. In T cell-dependent primary B cell responses, it is well known that the production of high-affinity class-switched antibodies requires Tfh cells and FDCs. Thus, it is worth

considering both B cell-intrinsic and B cell-extrinsic mechanisms to account for the robust responsiveness of the memory compartment. MBCs rapidly differentiate into PBs that produce class-switched antibodies that are capable of clearing the infection far more quickly than naive B cells. To explain the rapid response of $IgG1^+$ MBCs compared with IgM^+ naive B cells, two non-mutually exclusive models have been traditionally assumed. In the first one, the unique IgG1 cytoplasmic domain structure of 28 highly conserved amino acid residues (compared with the IgM cytoplasmic tail, which consists of three amino acids) is thought to be the primary factor accounting for differences in responsiveness, while in the second model, other changes such as alterations in transcription factor levels that take place during priming are thought to explain the differences. In support of the first model, several *in vitro* biochemical studies have shown differential signaling activity of IgM and IgG1 BCRs. To assess the contribution of the two models, a mouse IgG+ 'naive' B cell line was recently established by nuclear transfer from an $IgG1^+$ MBC, thus enabling for the first time a direct comparative analysis of naive-type $IgG1^+$ B cells and antigen-experienced memory-type $IgG1^+$ B cells. Antigen-experienced, but not naive, IgG1+ B cells rapidly differentiated into PCs, which indicates that stimulation history (a BCR-extrinsic factor) is important in determining the response [99]. Furthermore, the transcription factor BTB and CNC homologue 2 (BACH2), which is known to repress differentiation towards PCs, was expressed at a lower level in $IgG1^+$ MBCs than in $IgG1^+$ naive B cells, thus favoring the differentiation of $IgG1^+$ MBCs to PCs over germinal center entry. Due to data showing that before the induction of BLIMP1 expression (and so PC differentiation) there are several intermediate states between activated B cells and PCs, we propose that $IgG1^+$ MBCs could be into such an intermediate state by the downregulation of BACH2 [50]. Given that the BACH2 level of IgM^+ MBCs was more similar to that of naive B cells, IgM+ MBCs are probably also more similar to naive B cells in terms of their differentiation state and their ability to enter the germinal center pathway. These data shows the importance of stimulation history for the robust responsiveness of IgG^+ MBCs, but it does not exclude a role for the IgG1 cytoplasmic domain.

4.6. B cell receptor formation and maturation

B cells recognize and respond to foreign antigens through specialized polymorphic membrane receptors: the B cell receptor (BCR). Diversification of the antibody repertoire is essential for the normal operation of the human adaptive immune system. Three molecular mechanisms contribute to the diversity of the immune repertoire of B

cells: V(D)J recombination, class-switch recombination (CSR) and somatic hypermutation (SHM). These three mechanisms involve DNA damage, modification and the cellular DNA-repair machinery. The chromosomal organization of the genes that encode for the BCR is highly conserved between the receptor-chain loci, as well as between species (Chart 5).

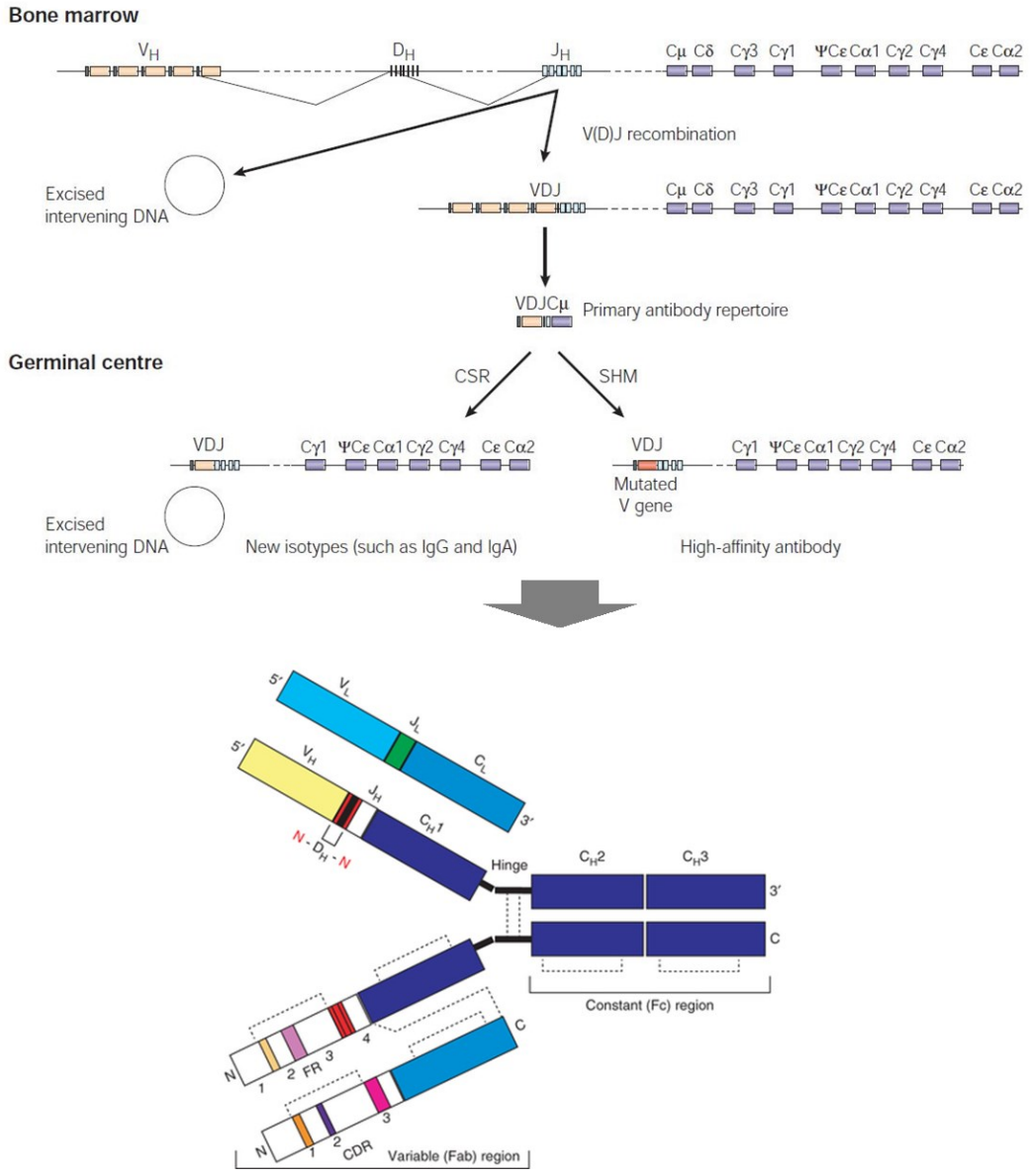


Chart 5: *Chromosomal organization and recombination of the human immunoglobulin heavy chain locus and schematic structure of immunoglobulins* [108,162].

The variable antigen-recognition domains of these receptors are encoded by many scattered gene segments of three types (variable (V), diversity (D) and joining (J)) which are somatically rearranged, in appropriate cell lineages, before their expression [100]. So, V(D)J recombination generates the diversity of B-cell primary immune repertoires [101–

103]. During the initial phase of V(D)J recombination, the lymphoid-specific recombinase-activating gene 1 (RAG1)/RAG2 factors, together with ubiquitous DNA architectural proteins (high mobility group, HMG, proteins), recognize and bind to recombination signal sequences (RSSs) that flank all variable (V) and joining (J) segments and introduce a DNA double-strand break at the border of the RSS. On the chromosome, coding ends are left as hairpin-sealed structures, whereas signal ends, which are excised from the chromosome, are blunt and 5' phosphorylated. The subsequent steps are taken care of by the DNA-repair machinery of the non-homologous end-joining (NHEJ) apparatus[104]. The DNA-double-strand break is first identified by the DNA-dependent protein kinase (DNA-PK) complex (formed by the Ku70–Ku80 heterodimer and the DNA-PK catalytic subunit, DNA-PKcs). Before re-ligation, the hairpins at the coding ends are first opened, presumably by the Artemis–DNA-PKcs complex. The XRCC4–DNA-ligase IV complex carries out the ligation step. The terminal deoxynucleotidyl transferase (TDT) further increases the diversity of the coding joint by adding non-templated nucleotides (N).

In the case of B cells, two additional mechanisms, which are triggered after antigen recognition, further optimize the antibody response [105]. Class-switch recombination (CSR) allows a previously rearranged IgH variable domain to be expressed in association with a different constant (C) region, leading to the production of different isotypes (IgG, IgA or IgE) (which mediate antigen elimination by different routes) without changing antibody specificity. The variable domains of immunoglobulins can also increase their affinity for antigen through the accumulation of somatic hypermutations (SHMs) within

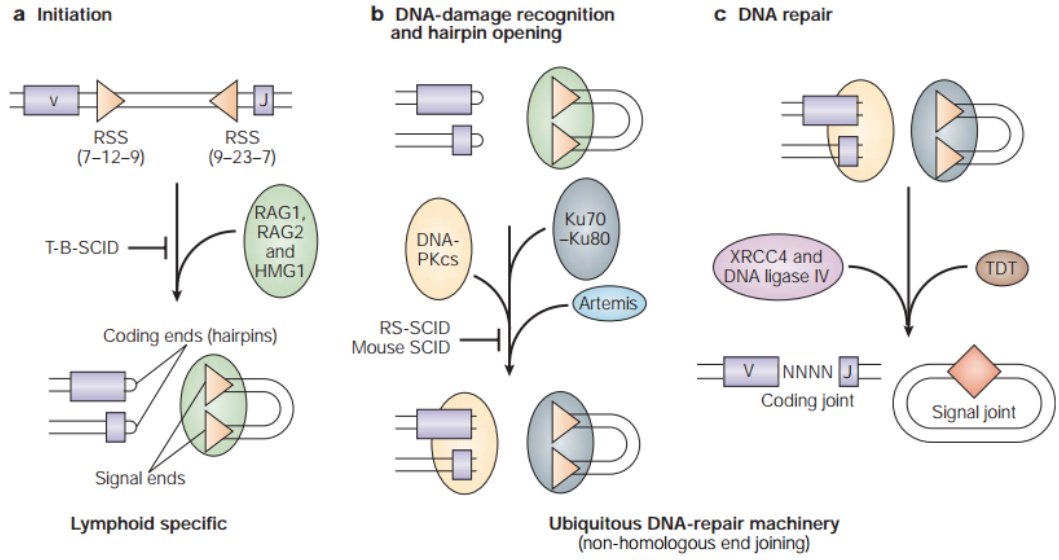


Chart 6: *Molecular mechanisms involved in VDJ recombination* [162] .

the V gene segment. These two B-cell specific antibody-maturation processes take place after antigenic stimulation, in the germinal centers of peripheral lymphoid organs, whereas V(D)J recombination occurs in the bone marrow (Chart 6). CSR involves recombination between two different switch (S) regions that are located upstream from each C region of IgH, with deletion of the intervening DNA. Replacement of the Cμ region by a C region of another class of immunoglobulin (Cγ1–4, Cα1–2 or Cε) results in the production of different isotypes (IgG1–4, IgA1–2 or IgE) with the same V region, and therefore, the same specificity and affinity for the antigen. The nature of the produced isotype determines its activity (half-life, ability to bind Fc receptors or to activate complement) and the location to which it is delivered (such as IgA in the mucosa) [106].

SHM introduces mutations in the V region and its flanking regions with high frequency. These mutations, which are essentially missense mutations and more rarely deletions or insertions, occur in the complementarity-determining regions (CDRs) and target specifically the Arg-Gly-Tyr-Trp motifs. Normally, SHM is eventually followed by the positive selection of B cells that express a BCR with high affinity for antigen, whereas B cells that express a BCR with low affinity are deleted by apoptosis or recirculating in the GC to undergo further rounds of modification. This selection process occurs in close contact with follicular dendritic cells [107].

The rapidly emerging technology of B cell receptor BCR sequencing enables determination of the antibody repertoire [108]. BCR repertoire analysis can enhance our understanding of the effect of pathogen exposure and immune status on antibody repertoire, and facilitate identification of new vaccine targets. For example, BCR sequencing of circulating B cells in various human populations showed that both age and chronic viral infection altered the B cell repertoire [109]. Also, immunoglobulin sequencing of B cells isolated from recently immunized individuals identified vaccine-specific BCR sequences [110,111].

4.7. Gene expression studies and combination with BCR repertoire analysis

Gene expression profiling studies are traditionally performed using whole-transcriptome microarrays or RNA sequencing. For the past decade, microarrays capable of simultaneously measuring the expression of large numbers of genes in specific cell populations have been considered the gold standard for transcriptomic analyses. More recently, next-generation sequencing approaches that allow for rapid genome-wide sequencing have gained popularity.

Typically, transcriptomic studies are performed on whole blood or isolated cell populations, and differentially expressed genes are compared during the course of infection or vaccination to highlight key mechanisms involved in protection [112–115]. Among those, Querec *et al* studied the immune mechanisms driving protection for the yellow fever vaccine YF-17D and identified transcriptomic signatures in PBMC from vaccinated individuals that could predict the magnitude of the $CD8^+$ T cell immune response [113]. Another study showed that the immunogenicity of the inactivated trivalent seasonal influenza vaccine could be predicted by a gene signature in PBMCs [112]. These examples all utilized redundant transcriptomic analysis, where the full transcriptome was analyzed. This approach remains expensive, requires a further validation step for genes of interest (traditionally RT-qPCR), and requires a relatively large amount of starting material, which is problematic for rare cell populations. Also, for some applications, a targeted panel of genes rather than the complete transcriptome is sufficient to address a given question. Hence, there has been a growing interest in the development and application of high-throughput multiplex gene expression systems, such as the Fluidigm systems, which focus on a specific panel of target genes. Recent technological advances in the field of transcriptomics, such as those described above, can also be applied to single cell gene profiling [116] . Gene expression studies at the single cell level have thus far highlighted the fact that individual cells from an apparently homogenous population (such as effector or memory cells) can display high heterogeneity at the mRNA level [117]. For example, using the multiplex high-throughput RT-qPCR Fluidigm system for single cell gene expression profiling, Arsenio *et al* revealed new insights into the fate of CD8+ T cells effector and memory subsets during bacterial infection that were masked when the analysis was performed on pooled cells [118].

Considering what has been described so far in this introduction, it is clear that each step of B cell maturation is the result of a complex interplay between transcriptional regulation and BCR signaling. Thus it is becoming increasingly important to characterize B cells at both levels simultaneously, in order to get the most information possible from each sample, especially for rare human B cell populations. This is possible by combining gene expression studies with Ig repertoire analysis. As reported above, recent technology advancement allows performing this kind of analysis even at single cell level. Combination of gene expression and Ig repertoire analysis at single cell level could be used to further investigate MBC heterogeneity. Indeed Ig repertoire studies allow for

the identification of Ig clonal expansion elicited by vaccination. Different MBC clonal families being at different maturation stages (in terms of accumulated BCR modifications and affinity for the antigen) could be characterized by a peculiar gene expression signature. The identification of such signature may become a biomarker for mature MBC subsets and be used to follow them during a vaccination response. A first attempt of gene expression analysis/BCR sequencing combination was performed by Weinstein *et al*, where single Ag-specific and Ag-nonspecific mouse B cells were used for gene expression profiling and BCR sequencing, finding correlations between the two[119]. A broader study is the one by McHeyzer-Williams *et al*, where they performed a deep and accurate analysis of murine GC dynamics during recall responses at single cell level[120].
Most of the studies investigating gene expression profiling and B cell function were initially performed in murine models and generally there is good correspondence with humans. However this is not always the case, and human lymphocyte biology cannot always be easily extrapolated from animals studies [121]. Gaining this kind of information in humans is fundamental to better understand humoral B cell immunity, but it is also crucial knowledge for next-generation vaccine design. Knowing what determines the formation of particular MBC responses (different Ig isotype and thus different effector functions) could drive the definition of new adjuvants strategies that help in eliciting the appropriate immune response to the pathogen of interest or to address specific age-related response impairments. For instance, this is the case of Tbet and Rorα that, as mentioned earlier, were indicated as responsible of specific Ig class expression in MBC, being potential targets for isotype-skewing vaccines. Combination of gene expression and Ig repertoire analysis could be used to further investigate MBC heterogeneity. Ig repertoire studies allow identification of clonal expansion elicited by vaccination. Moreover the identification of biomarkers characterizing specific B cell populations could be used to identify such populations when assessing the vaccine efficacy in clinical trials. In addition this could lead to the detection of new B cell subsets with possible vaccine efficacy predictive potential.

5. OBJECTIVE

A successful vaccine-induced humoral immune response relies on long lasting protective antibodies with appropriate isotype and high affinity for the immunizing antigen. To achieve this, antigen-activated B cells enter a process of BCR maturation and isotype switch selection that results in the production of short-lived antibody-secreting plasmablasts and long-term survival memory B cells. These outcomes are achieved within transient structures called germinal centers, residing in the follicles of secondary lymphoid organs. The molecular mechanism driving the fate of a human B cell to differentiate into a plasmablast or a memory B cell is poorly understood and many questions about memory B cells development remain unanswered, especially in humans. The goal of this study is to further characterize the molecular dynamics of late human B cell differentiation in response to vaccination, with a particular focus on memory B cells. We want to address this questions performing gene expression profiling at single cell level, thus investigating true population heterogeneity. Besides, comparing gene expression patterns induced by vaccination with the profile of steady-state circulating populations, we aim at identifying signatures of recent antigen stimulation. Additionally, combining gene expression analysis with B cell receptor sequence analysis, we explore possible correlations between expression signatures and BCR maturation, in order to identify mature subpopulations of memory B cells. Ultimately this work aims to identify putative biomarkers of efficacious B cell responses induced by vaccination.

6. MATERIALS AND METHODS

6.1. Human cells

Two anonymous healthy donors participating to the seasonal Influenza vaccination campaign were selected for the study. Blood and plasma samples were collected at day 8 and day 21 after one dose of undisclosed influenza vaccine upon approval of the informed consent. An additional sample from an anonymous healthy blood bank donor was collected after written informed consent was provided and ethical approval granted. All peripheral blood mononuclear cells (PBMCs) samples were isolated right after the bleed and diluted 1:2 in HBSS. PBMCs were isolated by conventional centrifugation over a Ficoll gradient and resuspended in PBS.

6.2. Antigen labelling

H1-California (Protein Sciences) and HSA (Sigma-Aldrich) were chemically labeled with Alexa Fluor 647 succinimidyl ester (Molecular Probes, Invitrogen) following the manufacturer's instructions. Each protein antigen was incubated with the dye at a molar ratio of 1:10 for 1 hour at room temperature and then loaded into a Zeba desalting spin column (Thermo Scientific) to remove the unbound dye. The degree of labeling was determined by measuring the absorbance of conjugated protein at the relevant wavelength for each fluorochrome by spectrophotometry. Protein concentrations were calculated with the Bradford Protein Assay (Giotto Biotech).

6.3. Flow cytometry analysis and sorting

Fresh PBMCs were divided in tubes containing approximately $7x10^6$ cells. First they were stained with 100µl of 1:500 Live/Dead Aqua (Invitrogen) for 20min in the dark and washed with PBS. Then 50 µl of PBS containing 20% rabbit serum were added for further 20 min at 4°C to saturate Fc receptors. After washing with PBS, PBMCs were stained with 50µl of a pre-titrated monoclonal antibodies mix diluted in PBS-1%FBS for 1h at 4°C in the dark. PBMCs from day8 after vaccination were stained with anti-CD19 APC (Clone SJ25C1, Becton Dickinson, Franklin Lakes, NJ, US), anti CD20 PrCPCy5.5 (Clone L27, Becton Dickinson, Franklin Lakes, NJ, US), anti CD27-PE (Clone L128, Becton Dickinson, Franklin Lakes, NJ, US), anti CD38-A700 (Clone HIT2, ExBio, Prague, CZ), anti IgG-V450 (Clone G18-145, Becton Dickinson, Franklin Lakes, NJ, US) and anti IgM-FITC (Clone G20-127, Becton Dickinson, Franklin Lakes, NJ, US), to identify Plasmablasts (PB) and Naïve B cells (NAIVE). PBMCs from day22 after vaccination were stained with CD20 PrCPCy5.5 (Clone L27, Becton Dickinson, Franklin Lakes, NJ, US), anti CD27-PE (Clone L128, Becton Dickinson, Franklin Lakes, NJ, US), anti IgG-V450 (Clone G18-145, Becton Dickinson,

Franklin Lakes, NJ, US), anti IgD-A700 (Clone IA6-2, Becton Dickinson, Franklin Lakes, NJ, US) and Ag-Alexa647, to identify antigen specific Memory B cells (Ag+MBC). After washing with 1,5 ml of PBS-1% FBS, cells were resuspended in 500µl of PBS-EDTA 5mM and stored on ice before sorting at BD FACSAria™. PB (CD19+/CD20dim/CD27++/CD38++), Ag-spec MBC (CD20+/CD27+/Ag+) and NAIVE (CD19+/CD27-) populations were sorted as single cells in 96 well plates containing 5µl of lysis buffer, consisting of 1mg/ml Ultrapure BSA (Ambion) and 1U/well Ribolock (Thermo) diluted in PCR grade water (Life Sciences). Lysates plates were quickly put on dry ice and then stored at -80°C.

6.4. cDNA synthesis and pre-amplification

Plates of lysates were thawed on ice and immediately used to perform reverse transcription through the SuperscriptIII Reverse Transcriptase Kit (Life technologies). 5µl of lysates present in each well were mixed with non-specific primers (0.25µl of 100µM oligodT and 0.25µl of 100µM random hexamers, QIAGEN), 0.5µl of 10mM dNTPs (Life Technologies) and 1µl of PCR grade water (Life Technologies). The plate was then incubated at 65°C for 5 min. A mixture of 2µl of 5X RT buffer, 0.5µl of DTT, 2.5U of SuperscriptIII and 0.5U of RNaseOUT (Life Technologies) was added to each well, after the plate had been at least 1 min on ice. This final mix was put in the thermocycler and incubated 5 min at 25°C, 60 min at 50°C, 15 min at 55°C, 15 min at 70°C and then put on ice again. To verify if the quality of the material was suitable for further steps, a test qPCR was performed mixing 1µl of cDNA with Taqman Universal Master Mix II (Life Technologies), 0.5µl of 20X *B2M* Taqman Assay (Life Technologies) and 3,5µl of PCR grade water. The qPCR plate was put in the qPCR machine (Lightcycler480II) and incubated 2 min at 50°C and 10min at 95°C to allow activation of the enzyme and then cycled for 40 cycles denaturing 15s at 95°C and annealing/extending 1min at 60°C. If an acceptable amount of wells resulted positive then the cDNA was pre-amplified to increase the amount of specific cDNA, using all gene-specific primers in a short multiplex amplification reaction. In Subject A (SbjA), 5µl of cDNA were mixed with 12.5µl of 2X Preamplification mastermix (Life Technologies), 1.3µl of 0.860µM previously prepared Taqman assay mix (containing all target genes assays), 1.3µl of 0.86µM VH-κ-λ forward primer mix, 1.3µl of 4.5µM CH-κ-λ reverse primer mix and 3.6µl of PCR grade water. 5µl of the pre-amplified product was then diluted 1:8 in PCR grade water for gene expression analysis, while the remainder was used undiluted for repertoire analysis. In Subject B (SbjB) and the healthy donor the pre-amplification protocol was slightly

different due to the different number of genes: 5µl of cDNA were mixed with 12.5µl of 2X Preamplification mastermix (Life Technologies), 7µl of 0.16µM previously prepared Taqman assay mix (containing all target genes assays), 0.25µl of 4.5µM VH-κ-λ forward primer mix and 0.25µl of 4.5µM CH-κ-λ reverse primer mix. The plate underwent the following PCR program: 10 min at 95°C, 18 cycles of 15s at 95°C and 4 min at 57°C. 1µl of the pre-amplified product was then diluted 1:8 in PCR grade water for gene expression analysis, while the remainder was used undiluted for repertoire analysis. The sequences of primers and the Taqman Assay IDs are shown in Table 1-2.

6.5. Single cell qPCR

Gene expression data in SbjA was obtained performing one qPCR amplification per target gene separately for each plate of single cells. The qPCR reaction mix for each well is prepared combining 1µl of diluted pre-amplified cDNA with 5µl of 2X Taqman Universal Master Mix II (Life Technologies), 0.5µl of 20X Taqman assay, and 3.5µl of PCR grade water in qPCR specific 96 well plates. The plate was put in the qPCR machine (Lightcycler480II, Roche) and incubated 2 min at 50°C and 10min at 95°C to allow activation of the enzyme and then cycled for 40 cycles denaturing 15s at 95°C and annealing/extending 1 min at 60°C. Raw data were collected using the Lightcycler 480 II software and analysed as reported below.

Gene expression data in SbjB and the healthy donor was obtained using the Biomark™ HD system (Fluidigm). The sample mix was prepared combining 2.7µl of diluted pre-amplified cDNA with 0.30µl of 20X Sample Loading Reagent (Fluidigm) and 3µl of Taqman Universal Master Mix II (Life Technologies). The assay mix was prepared mixing 3µl of each of the 96 20X Taqman Assays with 3µl of 2X Assay Loading Reagent (Fluidigm). Samples and assays were loaded on the 96.96 Dynamic Array™ IFC after priming, and then run on the Biomark™ HD qPCR machine. ROX has been used as passive reference. Expression data has been retrieved using the Biomark "Data Collection" software and Biomark "Real Time PCR Analysis" software using Linear Derivative baseline correction and "Auto Detectors" Cq threshold method. Further analysis methods are reported below. The Taqman assay ID are shown in Table 1.

6.6. Single cell Ig PCR and sequencing

The undiluted pre-amplified cDNA was used to amplify the immunoglobulins VH regions with the Q5 High-Fidelity DNA polymerase (New England BioLabs). 4µl of product were mixed with 5µl of 5X Reaction Buffer, 5µl of 5X GC Enhancer, 0.5µl of 10mM dNTPs (Life

Technologies), 1.25µl of 10µM VH forward primer mix, 1.25µl of 10µM CH reverse primer mix and 7.75µl of PCR grade water. The PCR program used was as follows: 30s at 98°C, 5 cycles of 10s at 98°C, 1min at 57°C and 1min at 72°C and 45 cycles of 10s at 98°C, 1min at 60°C and 1min at 72°C, and 7min at 72°C. The PCR products were visualized on a 2% agarose gel stained with GelRed (Biotium) to check for the presence of 350-450bp VH products. The PCR products were purified with Agencourt Ampure beads (Beckman Coulter) and finally sequenced with the ABI 3730xl 96 capillary DNA analyzer (Applied Biosystems). Two or more sequencing reactions were performed for each PCR product by using the same forward and reverse primer mixes as the Ig PCR (or single primers when needed). The sequences of primers and the Taqman Assay IDs are shown in Table 1-2.

6.7. Quantification of antibodies in human plasma

These experiments were performed using the Gyrolab® system, a technology that performs miniaturized immunoassay in a high-throughput manner allowing measuring the antigen-antibody bindings. The fluorescence intensity signal of each data-point is automatically provided by the instrument through the Gyrolab® evaluator software and it is proportional to the quantity of antigen specific antibodies present in the plasma sample. For total Ab quantification, plasma samples (diluted 1:2 during PBMC isolation) were diluted 1:250 (total 1:500), 1:500 (total 1:1000) and 1:1000 (total 1:2000) in RexxipH™ Buffer. For Ag-specific Ab quantification, plasma samples were diluted 1:25 (total 1:50) in RexxipH™, except for H3N2 IgA that were diluted 1:12,5 (total 1:25). They were run using the quantification method to define the concentration of total or Ag-specific IgG-M-A Ab present in the plasma samples. For capturing we used: Goat Anti-Human IgG-biotin #109-065-003 Jackson, Goat anti-Human IgM-biotin #109-065-043 Jackson, Goat anti-Human IgA-biotin #109-065-011 Jackson. Seasonal Flu Ag 2013/14 (H1N1California-biotin 248ug/mL, H3N2Texas-biotin 286ug/mL and B Massachusetts-biotin 250ug/mL). The concentration in the assay was 100ug/mL. For detection we used anti-Human IgG A-647 (Fc Specific Jackson), anti-Human IgM A-647 (Fc Specific Jackson) and anti-Human IgA A-647 (Fc Specific Jackson). The concentration in the assay was 25nM.

6.8. Data analysis

Sorting was performed on BD FACSAria™ and data exported with FACSDiva™ software, while further analysis were performed using the FlowJo© (Tree Star), software.

For SbjA, pre-processing of raw data was performed using the LightCycler Software (Roche). Then, further pre-processing steps were carried out using GenEx (multiD), while hierarchical clustering (HCL) heatmaps and Principal Component Analysis (PCA) were generated using MultiExperiment Viewer (MeV, http://www.tm4.org) . The cycling threshold (Cq) values from individual cells were transformed into relative mRNA abundance by subtraction of the Cq value from a baseline of 30, followed by conversion to linear scale by expressing each expression value in relation to an arbitrary reference level, that in our case was the most expressed sample for each particular assay. Then data was converted to $\log_2$ scale to perform further statistical analysis. HCL was carried out using Euclidean Metrics.

For SbjB and the healthy donor, pre-processing of single cell gene expression raw data was performed directly on the Biomark™ HD computer, using the Real Time PCR Analysis software (Fluidigm) to check the quality of all amplification curves. The Cq threshold method used was set to "Auto Detectors", which calculates independently a threshold for each detector (Taqman assay) on a chip. We performed baseline correction with the default Linear Derivative method. Data were exported and used for descriptive statistic, which were carried out with R (R Foundation for Statistical Computing, Vienna, Austria; http://www.R-project.org/). HCL heatmaps and Violin plots were generated using the SINGuLAR R package (D. Wang and G. Sun (2014). fluidigmSC: Fluidigm SINGuLAR Analysis Toolset. R package version 3.5.2.). Statistical tests were performed either in R, Graphpad Prism® or Mev. The cycling threshold (Cq) values from individual cells were transformed into relative mRNA abundance by subtraction of the Cq value from a baseline of 30 and expressed in log base 2. HCL heamaps were created using Euclidean Metric for sample clustering while Pearson metric was used to cluster genes.

In all cases single cells that didn't express *B2M* and that presented low levels of *18S* were removed from the analysis. Comparisons between more than two populations were performed with the one-way ANOVA test, Bonferroni adjusted. Further comparisons between 2 populations were performed on the ANOVA significant genes with Tukey Kramer test.

VH sequence chromatograms were analyzed with Sequencher® (Gene Codes), while alignments with germline sequences were performed as in ref [122]. Correlation studies to combine gene expression data and sequence data were performed using GraphPad Prism®.

7. Results

The purpose of my study is to investigate the late stages of B cell differentiation by comparing the different gene expression profiles induced by the antigen (Ag) encounter in human memory (MBC), plasmablast (PB) and naive (NAIVE) B cells. B cells, specifically resting populations like MBC and NAÏVE, are particularly delicate and contain low amounts of mRNA available for analysis. Also, we want to investigate gene expression at a single cell level to appreciate the true variability within the populations and not just a mean value of expression as in microarray-based strategies. Therefore we choose an approach that guarantees the highest sensitivity, widest dynamic range and the least sample manipulation steps possible: single cell RT-qPCR. We performed several tests to check the possibility to apply this approach on available clinical trial samples, condition that implies a freezing step of peripheral blood mononuclear cells (PBMCs) before cell staining and sorting by flow cytometry. These tests show that at single cell level it is not possible to obtain comparable results from previously frozen samples, even when additionally fixed trying to better preserve mRNA [123]. Therefore we collected fresh blood samples from people recruited for the annual Flu vaccination campaign (2013/2014). To avoid any bias coming from the freezing/thawing procedure, those samples have been processed and sorted immediately after the bleed at two different time points, Day 8 and Day 22 post vaccination, collecting PB, NAIVE and Ag-specific MBC (Fig. 1A).

7.1. The single-cell RT-qPCR approach successfully identifies Ag^+MBC, PB and NAIVE B cells isolated from human samples by gene expression profile analysis in a pilot study

The single-cell qPCR protocol was first applied on cells isolated from vaccinee Subject A (SbjA), as reported in the Methods section. Single cells were sorted from freshly separated PBMCs using classical surface markers to isolate B cells belonging to Ag^+MBC, PB and NAIVE B cell populations, as indicated in Fig. 1B. We analyzed the expression of a restricted number of genes, selected because of their involvement in B cell differentiation (Table 1, star-marked genes), using a classical qPCR approach. Unsupervised hierarchical clustering (HCL) of gene expression data shows that cells belonging to different populations can be distinguished by analyzing the gene expression profile (Fig. 2A). Principal Component Analysis (PCA) shows that while PBs are well segregated, Ag^+MBC and NAIVE B cells are more similar populations (Fig 2B). Dissecting the gene scores on PC1 we identify *IRF4*, *IRF8*, *PRDM1* (BLIMP1), *XBP1* and *CD19* as the major genes that are differently expressed between PBs and the other two populations.

In particular *IRF4*, *PRDM1* (BLIMP1) and *XBP1* are expressed at higher levels in PBs. On the other hand, by looking at the gene scores on PC2 we identify the expression of *IRF4*, *MCL1* and *CD19* as responsible of the difference between MBCs and NAIVE B cells. Focusing on the Ag^+MBC population alone, HCL identifies 4 clusters of cells (Fig. 2C), but not all of them are visible in the PCA (Fig. 2D). Specifically, cluster 2 is scattered over the other groups and no longer noticeable in PCA, whereas cluster 4 separates into two additional groups (Fig. 2C-D, orange square, circle and arrows). The significant genes involved in the diversity of the MBC groups are *IRF4* and *CD19* (ANOVA $p<0.05$). We also recovered the immunoglobulin heavy chain variable region (Ig VH regions) sequences expressed by each single cell and analyzed the sequences, as previously reported, to identify clonotypes [122]. Comparing the distribution of clonotypes between NAIVE B cells, PBs and Ag^+MBCs, we observe clonal expansion in the memory population (Fig. 3). In summary, with this preliminary study we successfully applied the combined single-cell qPCR protocol to human B cells, including particularly challenging populations like Ag^+MBC. We were also able to identify the genes involved in population discrimination and the expressed VH sequences, offering the means to perform gene expression–Ig correlation studies.

7.2. High-resolution characterization of B cell populations by gene expression profiling analysis in human peripheral blood

Having established the reliability of the single cell qPCR approach, we upscaled the technique to a more high-throughput setting by using the Fluidigm 96.96 Dynamic Array technology for quantitative real time PCR analysis, allowing for simultaneous measurement of 96 genes in 96 individual cells isolated from fresh PBMCs of a second vaccinee, Subject B (SbjB) (Fig. 1C). We selected a total of 96 target genes involved in several pathways related to B cell differentiation (Table 1) and analyzed their expression over 70 NAIVE B cells, 65 Ag^+MBCs and 75 PBs without applying any normalization to housekeeping genes. By analyzing the gene expression patterns we find the cells clustering by B cell phenotype (Fig. 4A). We then applied a supervised method for dimensionality reduction to enhance group separation: Linear Discriminant Analysis (LDA). In this way we can graphically display the expression data of all populations and it's possible to appreciate the similarity of Ag^+MBCs and NAIVE in comparison to PBs (Fig. 4B). We identified 61 significant differentially expressed genes among the three populations as a result of the ANOVA test and represented the expression distributions for each of these genes using violin plots (Fig. 4C). By comparing the 3 populations, we

observe that few genes are detected almost exclusively in PBs: *PRDM1* (BLIMP1)*, XBP1, TNFRSF17* (BCMA)*, IL2r, RORα, KI-67* and *CD138*, but while the *PRDM1* (BLIMP1) and *XBP1* are expressed at high levels and throughout the whole population, the last ones are represented only by a small fraction of cells. Another group of genes shows a higher number of positive cells in PBs than in the other populations (*ENTPD1* (CD39*), IGBP1, CD81*, *KLF2, IRF4, IRF2*). Also, the high expression of *PRDM1* (BLIMP1), *XBP1* and *IRF4* together with downregulation of *IRF8*, *SPIB* and *PAX5* confirms the classical signature of PBs differentiation [30]. PBs also share similar expression patterns with MBCs for genes coding for signaling cytokines *(AKT1-2, MTOR*) and genes involved in activation and survival (*CD86, TNFRSF13B* (TACI)), responsiveness (*SATB1, KLF2, BACH2*), maturation (*ZBTB32*)*,* migration (*GNAI1*) and antibody secretion (*ATF6)*. On the other hand, PBs and NAIVE are less alike: among the few shared genes, a small number shows a matching expression distribution (*CD80, STAT5, TBX21* (Tbet)*, PIK3CA*), while the majority of themis not expressed in comparison with Ag^+MBC (*GPR183* (EBI2)*, HIF1α, IL10RA*). It is also noteworthy how *POU2AF1*, a gene with an important role in GC formation and maintenance, is expressed at high levels also at later stages in both PBs and MBCs, and to a lesser extent in NAÏVE B cells. In the same way *FOXP1*, whose role is described mainly in early B cell development and GCs, seems to be expressed by all MBCs and NAIVE cells, but very poorly by PBs. In summary PBs, though sharing a little similarity with the other populations, remains the most distinct and well defined group, while MBCs and NAIVE are more interconnected.

7.3. Gene expression activation signatures of Ag-specific memory B cells in comparison to naive B cells

To further investigate the differences between Ag^+MBCs and NAÏVE B cells, we repeated the same analysis focusing on these two populations. By looking at the LDA we see that the two populations are separated, but partially overlapping (Fig. 5A). In fact the direct Ag^+MBC-NAIVE comparison resulted in 32 significant differentially expressed genes out of the 61 previously identified (Fig. 5B). Most of the genes are expressed at higher levels in Ag^+MBCs indicating greater transcriptional activity in these cells. In particular, among the genes showing exclusive or higher expression frequency in Ag^+MBCs, some are involved in activation (*CD80, CD86*), proliferation (*ZBTB32, IL10RA*), survival (*MCL1, TNFRSF13C* (BAFFR)*, TNFRSF13B* (TACI)), migration (*GPR183* (EBI2)*, GNAI2*) and maturation (*MTOR, TBX21* (Tbet)*, STAT1, TNFRSF13B* (TACI)*, TNFRSF13C* (BAFFR)). Conversely, the genes that have higher expression frequency in NAIVE are mainly

involved in quiescence and cell cycle regulation (*SATB1, ZBTB16, BACH2*), but we also find a higher expression of *IL4R* and *CCR7*. Interestingly *GPR183* (EBI2)*, CCR7* and *MTOR* are known for their involvement during early B cell activation and class switch recombination, while their role during the late stage of differentiation has not been described yet. Moreover *SATB1* codes for a chromatin organizer that promotes quiescence in hematopoietic stem cells and its role in mature B cells has not been investigated yet. While the functional relevance of these genes remains to be confirmed, these data provides further support to explore the overall functional properties of these cell types. It also further exacerbates the responsive profile of Ag^+MBC in comparison to NAIVE B cells.

7.4. Investigating the heterogeneity of the antigen-specific memory B cell population

There is growing awareness that B cell memory is constituted by multiple layers and is more heterogeneous than once thought [90]. To investigate the possibility of Ag^+MBC subpopulations, we used the HCL clustering tree (Fig. 6A). Setting the least restrictive threshold possible (0.51) on the clustering dendrogram, we could separate Ag^+MBCs in two groups, as highlighted in the PCA graph (Fig. 6B), but unfortunately we didn't identify any significant differentially expressed gene (T test, $p>0.05$)

7.5. Effect of antigen activation on circulating memory B cells

To confirm previous data obtained on SbjA and SbjB, we isolated single PBs and MBCs from a healthy donor following the same procedure as before (Fig. 1D). Applying HCL and LDA to the healthy donor gene expression data, we distinguished the two populations and confirmed the classical gene expression patterns previously observed in the vaccinee (Fig. 7A-B). The Ag^+MBC population was sorted at the peak of memory B cells response towards one of the specific vaccine Ags, having been recently in contact with the Ag. In order to investigate if the Ag encounter triggers a specific gene expression signature, we compared the gene expression profile of Ag^+MBCs and steady-state circulating MBCs. Therefore we looked for gene expression differences between Ag^+MBCs (vaccinee) and steady-state MBCs (healthy donor), identifying 16 significant genes involved in several functions (Fig. 7C-D). Ag^+MBCs show higher expression frequencies of genes involved in activation (*CD80, CD86*), GC dynamics (*BCL6, SPIB, IRF8*), survival (*MCL1*), protein secretion (*ATF6*) and BCR signaling (*CD22*), while a lower expression of genes involved in quiescence (*KLF9*), Ab secretion (*IL10RA*), hypoxia

response (*HIF1α*) and GC homing (C*XCR5*). *TBX21* (Tbet) and *RORα,* that are suggested to be involved in immunoglobulin isotype selection, will be further examined below. In general these data denoted a more active profile for the recently activated Ag^+MBCs isolated from the vaccinee.

7.6. Gene expression signatures of B cell maturation

The main and most known humoral effector function of B cells is conveyed by the BCR they bear and eventually secrete. The maturation of B cells is strictly related to the affinity of their BCR and its affinity maturation is obtained modifying the BCR sequence through SHM and CSR in the germinal centers [105]. Starting from this premise, we hypothesized a possible link between BCR maturation and gene expression clustering. Therefore, to investigate this hypothesis, we recovered the BCR VH sequences of the same cells used for gene expression analysis. First, we performed a clonotype analysis to check the sequence clonal dynamics during the vaccinee immune response. Indeed in the vaccinee we observe clonal expansion in both PB and Ag^+MBC in comparison to the NAIVE population, which instead shows absence of clonotypes like the healthy donor populations (FIG. 8A). Second, we performed a correlation study on the vaccinee sequences to look for associations between the BCR maturation, in terms of BCR mutation rate, and gene expression. Unfortunately we were able to identify only few and weak correlations (Fig. 8C). Lastly, we focused on the isotype carried by the BCRs. Going over the class distribution across the three populations, we notice a peculiar clustering of the Ig isotypes (Fig. 8B). In particular we find a striking high percentage of circulating IgA in PBs. Looking at FACS data (Fig. 1C) this condition is confirmed, but Ag specific ELISA performed on the vaccinee serum shows very low Ag specific IgA at Day8, suggesting the presence of unspecific PB in the population we isolated (Supplementary Fig. 1). This segregation of Ig isotypes prevented the identification of class specific gene expression signatures using an unsupervised method. Hence we focused on two transcription factors whose association with Ig isotype selection have been suggested in recent literature[91]: *RORα* and *TBX21* (Tbet). Pooling the vaccinee sequences together and re-grouping them by Ig class we identify significant association between *RORα* expression and IgA, while *TBX21* is associated to IgG (Fig. 8D). In conclusion this dataset allowed us to detect Ig clonal expansion in response to vaccination and to associate *TBX21* and *RORα* to the expression of a particular Ig isotype in humans, but we weren't able to identify strong connections between the BCR maturation and the expression of any of the selected 96 genes.

8. Discussion

The major goal of this work was to better characterize the transcriptional networks of B cell populations at different stages of terminal differentiation in humans, with a particular interest in the discrimination between memory B cells (MBC) and plasmablasts (PB). Once assessed the specific profile of each population, we examined in depth the peculiarity of memory B cells recently activated by the encounter with the antigen through the comparison with steady-state circulating memory B cells. To get this information we chose to analyze samples from a single vaccination schedule, so that we could work in the context of the same immune response and isolate cells responding to the same antigen. The limited availability of samples and the difficulties in isolating Ag-specific MBCs from peripheral blood make this analysis particularly challenging in humans. In fact antigen-specific cells account for a small amount of the total lymphocytes within the blood, so that the presence of molecular changes in antigen-specific cells and biologically relevant signatures could be masked by experimental noise from non-specific counterparts. This limitation has been addressed by adapting a method that enables RT-qPCR from different B cell populations (Ag^+MBC, PB, NAIVE) at single cell level. With the recent advances in the field of systems biology, this approach offers a time-effective way to perform high resolution and high sensitivity gene expression profiling of a high but selected number of target genes. It represents an alternative to transcriptomic analysis, without requiring further steps of validation or large amounts of starting material, so it's particularly indicated for rare and delicate cell populations like antigen specific B cells.

In principles, following activation, the B cell diversifies the antibody it produces increasing its affinity for the antigen and altering its effector function, thus tailoring the response to the faced immunological challenge. The B cell then differentiates into either a specialized antibody-secreting cell (PB) to face the antigen or a long-lived MBC, so that a more rapid (and specific) response can be mounted upon re-encountering the same antigen. This sequence of events takes place within specialized immunological environments called germinal centers (GC). Successful completion of the GC reaction therefore relies upon careful regulation and co-ordination of B cell movement, division, apoptosis, differentiation, DNA repair and recombination. This is achieved through the activation (and repression) of multiple transcriptional programs that interact in a series of complex regulatory networks. Although much as been discovered regarding the coordination of the GC response, a number of fundamental questions remain unanswered, especially about the signals driving activated B cells towards terminal

differentiation into PB or MBC. Much is known about the molecular dynamics regulating GC response and PB differentiation [120], however so far no deterministic transcription factor for MBC has been found. We addressed this question by selecting a set of probes to profile the expression of genes known or suggested to be involved in various aspects of B cell differentiation, with a particular focus on genes with a putative differential expression in MBC subsets. Then we interrogated single B cells isolated from human vaccinees and belonging to three different B cell populations (Ag^+MBC, PB and NAIVE) for their gene expression profile. By doing that, we first confirmed the classical network of master regulators driving PB differentiation, which involves upregulation of *PRDM1* (BLIMP1), *XBP1*, *TNFRSF17* (BCMA) and *IRF4* and downregulation of *IRF8, SPIB* and *PAX5* [30,63,124]. We also confirm the importance of *MCL1,* that has been proposed as the main anti-apoptotic factor in mature B cells [27]. However, in contrast with what postulated by Vikstrom *et al*, in our dataset *MCL1* is highly expressed in both circulating Ag^+MBC and PB, while *BCL2*, another key anti-apoptotic factor, is expressed at low frequencies and with no significant difference between populations. Thus, *MCL1* expression seems to be important not only for tissue lymphocytes, but also for the maintenance of peripheral B cells. The expression of these genes, except for *TNFRSF17* (BCMA) that was not included in the first pilot experiment, is the same for both SbjA and SbjB and the expression patterns are similar in both experiments.

We also made some interesting observations about genes which are better known for their roles in other stages of B cell differentiation. One of these genes is *FOXP1,* which in B cells is mainly known for its role during early pro/pre-B cell development [125]. Moreover it is reported to be involved in coordinating transitions between cell proliferation and differentiation in many biological contexts [126]. Recently it was suggested that FOXP1 has a role also at a more mature stage of B cell development: in human tonsillar B cell subpopulations FOXP1 shows the opposite expression pattern to BCL6 and shares a part of its target genes. Therefore these proteins may have antagonistic roles. In fact it was showed that *FOXP1* needs to be downregulated after naive B cells activation to ensure appropriate GC formation and transit [126]. We show that in humans *FOXP1* is expressed at high levels in Ag^+MBCs and NAIVE, while it is mostly not expressed in PBs. This suggests that FOXP1 could have a potential role in cell fate decision during GC reaction, in particular in favoring MBC versus PB differentiation. One of the target genes of the repressor activity of *FOXP1* is *POU2AF1*. This gene is known to be involved in GC reaction, specifically in promoting proliferation and class

switch recombination in response to low levels of *IRF4*. We observed an exceptional high expression of *POU2AF1* in Ag^+MBC and PB. While its role in PB differentiation is already reported in literature [127], its role in MBC differentiation remains unidentified. Considering that *IRF4* is generally down regulated in MBC, *POU2AF1* activation may be under the control of different and unknown factors in this population. Another gene that we see expressed especially in PB, but also in a subset of MBC and NAIVE, is *IRF2*. This transcription factor is involved in innate immunity and has hardly been described in mature B cell differentiation transcriptional networks. Little is known about its interaction with other genes belonging to these pathways, except that it may compete with *PRDM1* (BLIMP1) for the binding of a set of target genes. BLIMP1 and IRF2 may interact in the context of plasmacytic differentiation, in which a role for BLIMP1 is well documented. IRF2 levels remain constant during B cell development [128], while BLIMP1 is induced during terminal differentiation to plasma cells. Thus, regulation of target genes that both proteins can bind could be achieved by changes in relative protein concentrations during induction of *PRDM1* (BLIMP1) and differences in binding affinities for specific sites [129,130].

The hallmarks of memory B cells include self-renewal, longevity and rapid response upon secondary Ag encounter [131–133]. In fact when we compared Ag^+MBCs to the NAIVE population we found signatures of higher expression for activation, proliferation, survival and maturation markers. Among these, *GPR183* (EBI2)*, GNAI2* and *MTOR* are particularly interesting because their role in MBC is not known yet. EBI2, together with CCR7 and CXCR4, plays an important role in positioning B cells in the follicle during activation. After activation, EBI2 needs to be downregulated by BCL6 in order to have affinity maturation in GC, otherwise the B cell will differentiate in PB without passing through GCs [134]. However, it is still unknown whether EBI2 plays a role downstream of GCs by regulating the generation, migration and/or function of the effectors of long-term humoral immunity. Another gene that we find particularly expressed by all Ag^+MBC is *GNAI2*. This gene codes for an alpha subunit of guanine nucleotide binding proteins and is a transducer in various transmembrane signaling systems. In B cells it is reported to be involved in B cell motility and specifically to regulate the entrance of murine B cells into peripheral lymph nodes and cause an increase in chemokine receptor signaling [135]. *GNAI2* role in MBC (but also in PB and NAIVE) could be connected to this function.

In mouse models, B cell-specific deficiencies in *MTOR* impair germinal center formation, decrease the production of IgG isotypes in response to immunization and lead to a

decrease in affinity maturation of antibodies in *vivo* [136]. Other studies also suggested that overall *MTOR* signaling controls the ability of B cells to divide and to differentiate [137–139]. We observed that a subset of Ag^+MBC (and PB), but not NAIVE, expresses *MTOR*. This probably contributes in giving Ag^+MBC a more active and responsive profile than NAIVE.

Our data shows that few genes are actually upregulated in NAIVE cells, specifically the ones involved in cell cycle regulation (*ZBTB16, BACH2*), which are believed to keep naive cells quiescent [99,131]. Interestingly we also see a higher frequency of expression of *SATB1* that, besides being known for its role in Th2 differentiation [140], is also involved in regulating hematopoietic stem cell maintenance versus lineage commitment [141]. Thus, in NAIVE B cells, *SATB1* could have a similar role in promoting their quiescence.

Lately it is becoming increasingly clear that the MBC compartment is all but homogenous [79,99,142–146]. It was initially postulated that the expression of different Ig isotypes (specifically IgM and IgG) defined functional MBC subsets [88,89,147]. However recent works in mouse models revealed that Ig isotypes are not necessarily the only markers involved in functional distinction of MBC subpopulations, but the expression of receptors like CD80 and PD-L2 could better define MBC activity in terms of ability to differentiate into antibody-secreting cells (more "memory-like") or enter GC (more "naïve-like") on secondary responses [90]. Moreover it was previously reported that also CD73 is expressed in MBC subsets and was associated with the maturity of BCR [142,148]. Considering the importance of elucidating the molecular mechanisms at the basis of MBC functions and their implications in vaccine design, we investigated if such heterogeneity could be appreciated also from our data. In our case this meant looking for subsets among switched MBCs, since the populations we isolated from the vaccinees are mainly constituted by $CD27^+IgG^+$MBCs (Fig. 1B-C). In SbjA, having a very small set of genes to analyze, we identified two genes (*CD19* and *IRF4*) which were significantly discriminating Ag^+MBCs in 4 subpopulations. Unfortunately, when we increased the number of genes to profile in SbjB, we couldn't define any significant subset based on gene expression data of our 96 selected gene-set; raising the number of target genes caused an increased variability of the expression data in SbjB and masked what we previously observed in SbjA. Notably, the extended gene-set used for SbjB includes the aforementioned *CD80*, *PDCD1LG2* (PDL2) and *CD73* genes: in fact we detect expression of *CD73* in small subsets of Ag^+MBCs and NAIVE, and *CD80* expression in an Ag^+MBC subset, while *PDCD1LG2* (PDL2) is not expressed at all in our cells. However their

expression patterns were not statistically relevant to identify subsets within the Ag^+MBC population we analyzed.

We also repeated the same analysis workflow for PBs and MBCs isolated from a healthy donor. PBs and MBCs discriminating genes followed the same patterns as in the vaccinees. Moreover this experimental set-up allowed us to investigate the vaccination-induced signatures specific of MBC by comparing the vaccinee profile to the steady-state profile of a healthy donor, in order to find out whether there was a transcriptional difference between recently activated and quiescent circulating MBCs. This comparison revealed a more active profile for Ag^+MBCs, characterized by signatures of recent GC transition. In fact we find higher expression of activation markers, few cells that are still expressing *BCL6* and a higher expression level of *SPIB* and *IRF8* which are involved in GC dynamics [21,41,124,149,150]. On the other hand, we were also able to highlight a signal of exit from the GC, conveyed by reduced frequencies of *CXCR5* expression, a chemokine receptor responsible for GC homing. Since MBCS do re-circulate between lymphoid compartments during an ongoing humoral response [151], this signal is not completely shut down, but the trend of the expression distribution is opposite to the one of the healthy donor. Another indicator of activation in terms of transcriptional activity is the higher expression of *ATF6*, a gene encoding for a sensor of ER stress [152,153]. ATF6 is involved in the unfolded protein response, a process which have been proposed to be predictive of good vaccine responses in humans [154] and in particular a robust biomarker of later emergence of protective antibody titers [112]. In fact, *ATF6* together with *XBP1* is expressed also in PBs at high levels. Activation signals in Ag^+MBCs match with lower expression of the quiescent factor *KLF9*, a cell-cycle regulatory gene which is described to be downregulated in human spleen-derived MBCs and one of the potential reasons of their increased responsivity in comparison to naive B cells upon secondary response [131]. Ag^+MBCs also express higher levels of *CD22*, which is a response regulator involved in inhibitory control of BCR signaling [155], mirroring recent activation of BCR by the Ag. CD22 also regulates time course of B cell response by functioning as crucial regulator of B cell division after Ag stimulation and its ablation results in rapid B cell differentiation and Ab production [156], in accordance with our data where PB show no expression of *CD22*. Human B cell differentiation is regulated by the actions of numerous cytokines, with IL-10 produced by T follicular helper cells (Tfh cells), being key factor in promoting proliferation, isotype switching, PC differentiation, and secretion of most Ig isotypes by not only naive B cells, but also memory B cells,

including both IgM^+ and isotype-switched subsets [157]. Both Ag^+MBCs and healthy donor MBCs express the receptor of IL-10, but our data indicates that Ag^+MBCs that have recently exited the GC show a subset of cells that do not express it. Also, we detected in both MBC populations the expression at high frequency of *HIF1α*, which is considered a master regulator of the hypoxia response and is also involved in the unfolded protein response [158]. As for *IL10RA*, a group of Ag^+MBCs do not express *HIF1α*, while in the healthy donor the whole population does.

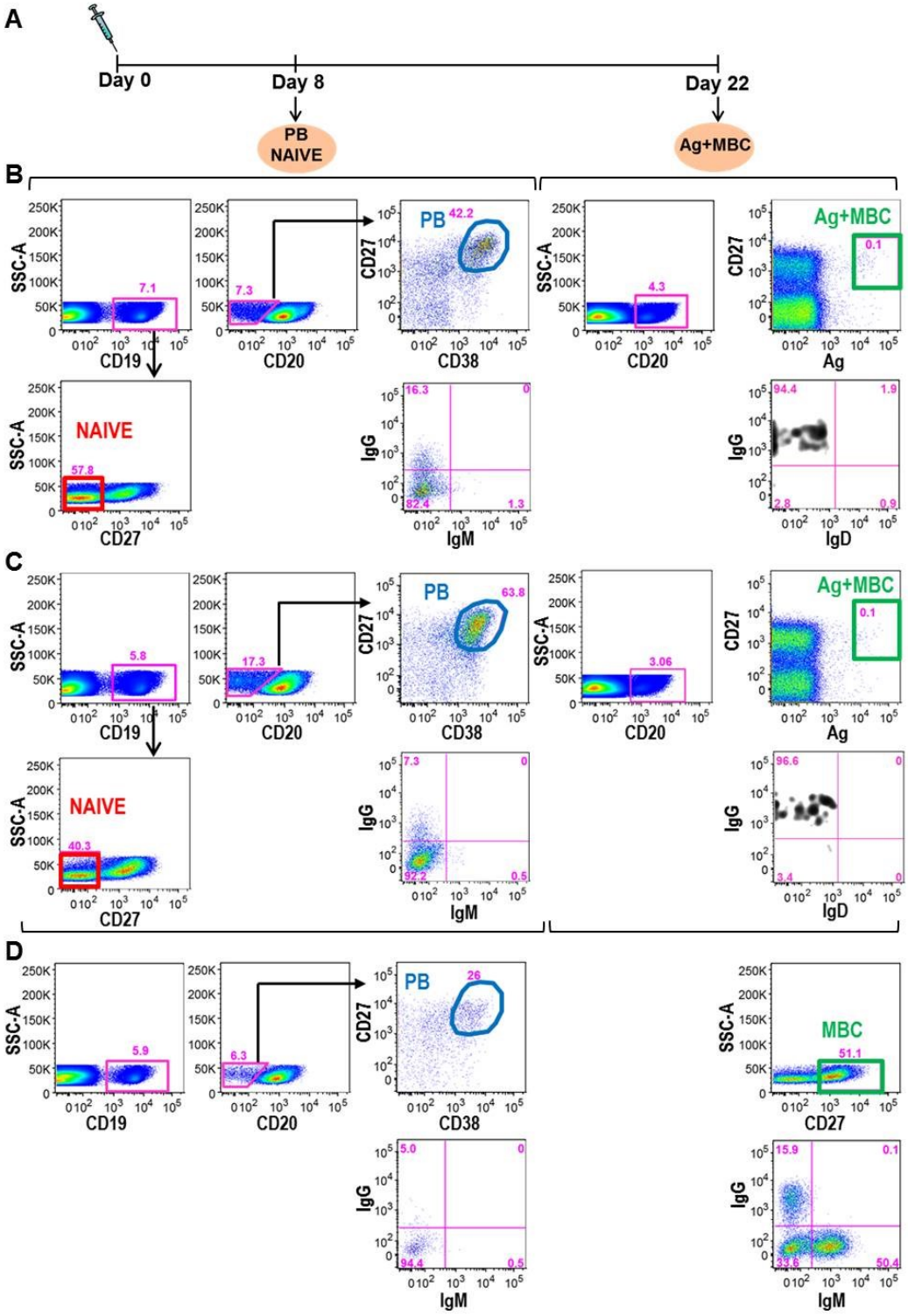
Performing a simultaneous VH sequence analysis for the same single cells, we identified clonal expansion in the vaccinee PB and Ag^+MBC populations. Similarly to the vaccinee NAIVE population, all healthy donor VH sequences belonged to single clonotypes instead. Considering that the injected influenza vaccine is unknown, we cannot directly compare our repertoire analysis with previous studies. Also, the sample size available for repertoire analysis is quite limited. Anyhow this wasn't the reason behind the Ig sequencing experiments. Our main interest was to look for potential connections between the BCR maturation and transcriptional signatures. Unfortunately our dataset didn't allow us to identify any particular correlation of gene expression data with BCR VH mutation rate (that we used as an indicator of BCR maturation), but we did detect an association with Ig isotype. Notably we identified an association of *TBX2*1 (Tbet) and *RORα* expression with IgG and IgA isotype respectively. In murine models it was revealed that B cell memory is organized in class-specific subsets, each with separate central transcriptional regulators [91]. Specifically, transcriptional regulators Tbet and RORα control divergent IgG2a and IgA memory B cell subsets respectively to coordinate separate functions within these B cell compartments. Tbet is used by many cell types in response to inflammatory stimuli, with focus on the clearance of intracellular pathogen [159]. Also, $IgG2a^+$ B cell memory relies selectively on a Tbet dependent program to establish and maintain subset integrity, according to Wang *et al* study. Similarly, IgA^+ B cell memory is specialized to protect the mucosal surfaces [160] and the selective use of transcriptional regulator RORα enhances this unique memory B cell function. Importantly, these unique developmental programs can be exploited for directed immunotherapeutic applications and future class-skewing vaccine formations.

Overall this type of analysis identified new putative actors in the late differentiation pathway of B cells and offered further support to transcriptional data provided by murine studies. Moreover we identified genes that characterize recently-activated MBC that could be used as indicators of efficacious B cell responses. Extending this approach

to a larger number of subjects and including different formulations of a vaccine of interest or different age ranges could be helpful in understanding the evolution of adaptive immune responses following vaccination in different contexts. Having access to bigger sample sizes could also allow performing more extensive Ig repertoire analysis, thus helping to uncover associations to particular gene expression patterns that could lead to the characterization of mature and high affinity B cell subsets. Lastly, this approach that combines phenotype, gene expression and Ig sequence data at single cell level, allowed us to highlight and exploit the variability of our samples to better describe the true heterogeneity of B cell populations, that wouldn't have been possible using microarray based approaches or other techniques that measure mean expression values of the whole population.

9. FIGURES AND TABLES

Figure 1


A
Day 0
Day 8
Day 22
PB
NAIVE
Ag+MBC
B
SSC-A
CD19
7.1
CD20
7.3
CD27
CD38
PB
42.2
CD20
4.3
CD27
Ag
Ag+MBC
0.1
NAIVE
57.8
CD27
IgG
IgM
16.3
0
82.4
1.3
IgG
IgD
94.4
1.9
2.8
0.9
C
SSC-A
CD19
5.8
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17.3
CD27
CD38
PB
63.8
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3.06
CD27
Ag
Ag+MBC
0.1
NAIVE
40.3
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IgG
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7.3
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IgG
IgD
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IgG
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15.9
0.1
33.6
50.4

Fig. 1 Isolation of B cell populations from human samples. (A)Vaccination schedule of SbjA and SbjB.The specific B cell populations have been isolated day 8 and day 22 post vaccination. (B)(C)Flow cytometry analysis of plasmablast (PB) ($CD19^{+}CD20^{dim}$ $CD27^{hi}CD38^{hi}$) and naive B cell (NAIVE) ($CD19^{+}CD27^{-}$) populations from day 8 and antigen-specific memory B cell (Ag^{+}MBC) ($CD20^{+}CD27^{+}Ag^{+}$) from day 22, isolated from SbjA and SbjB respectively. (D) Flow cytometry analysis of plasmablast (PB) ($CD19^{+}CD20^{dim}CD27^{hi}CD38^{hi}$) and memory B cell (MBC) ($CD19^{+}CD20^{+}CD27^{+}$) populations isolated from one single blood sample of a healthy donor.

Figure 2

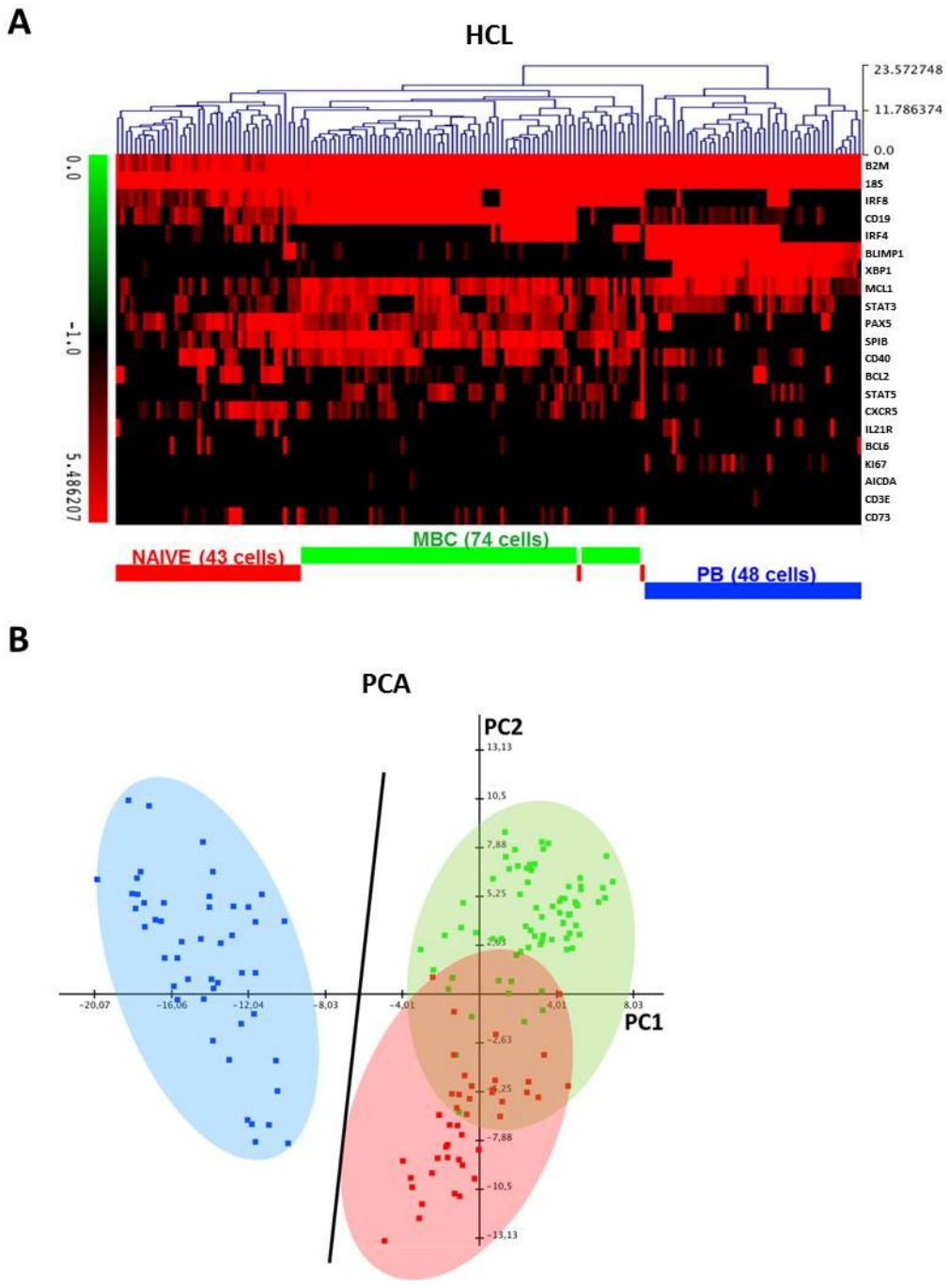
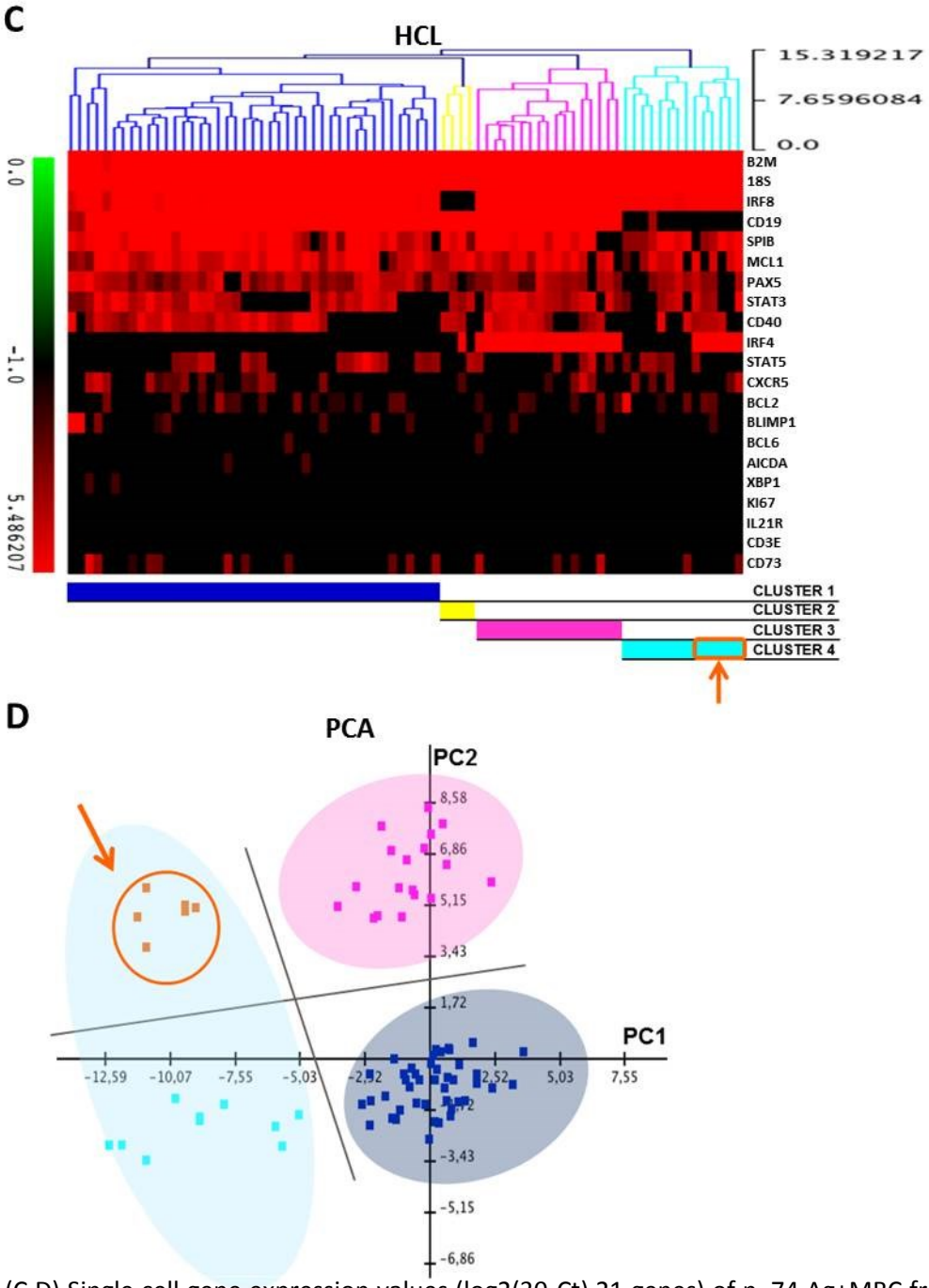


Fig. 2 Pilot study: gene expression analysis of B cell populations in SbjA. (A,B) Single cell gene expression values (log_2(30-Ct), 21 genes) of n=165 total B cells from SbjA at days 8 and day22 after vaccination were analyzed by unsupervised hierarchical clustering (A, heatmap with dendogram of single B cells in columns and genes in rows) and Principal Component Analysis (B, dot plot shows the position of every cell on the space defined by the first two principal components PC1 and PC2 with colored circles identifying population grouping).



(C,D) Single cell gene expression values (log2(30-Ct),21 genes) of n=74 Ag+MBC from SbjA at day22 after vaccination were used for unsupervised hierarchical clustering (C, heatmap with dendogram of single B cells in columns and genes in rows) and Principal Component Analysis (D, dot plot shows the position of every cell on the space defined by the first two principal components PC1 and PC2 with colored circles identifying clusters of Ag+MBC); clusters identified by hierarchical clustering are represented below the heatmap in different colors; arrows indicate a subgroup of cluster 4 identified through PCA. For clarity purposes, protein IDs instead of gene IDs are used.

Figure 3

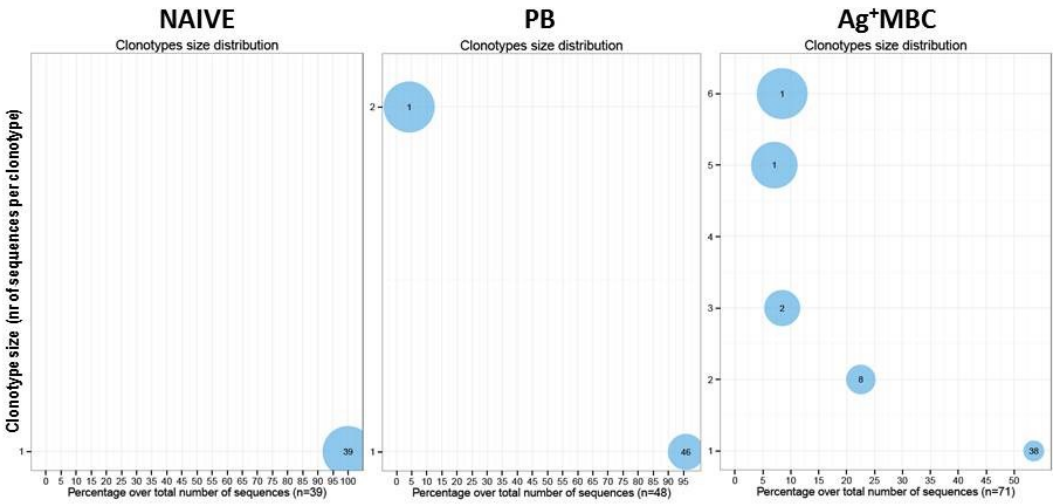
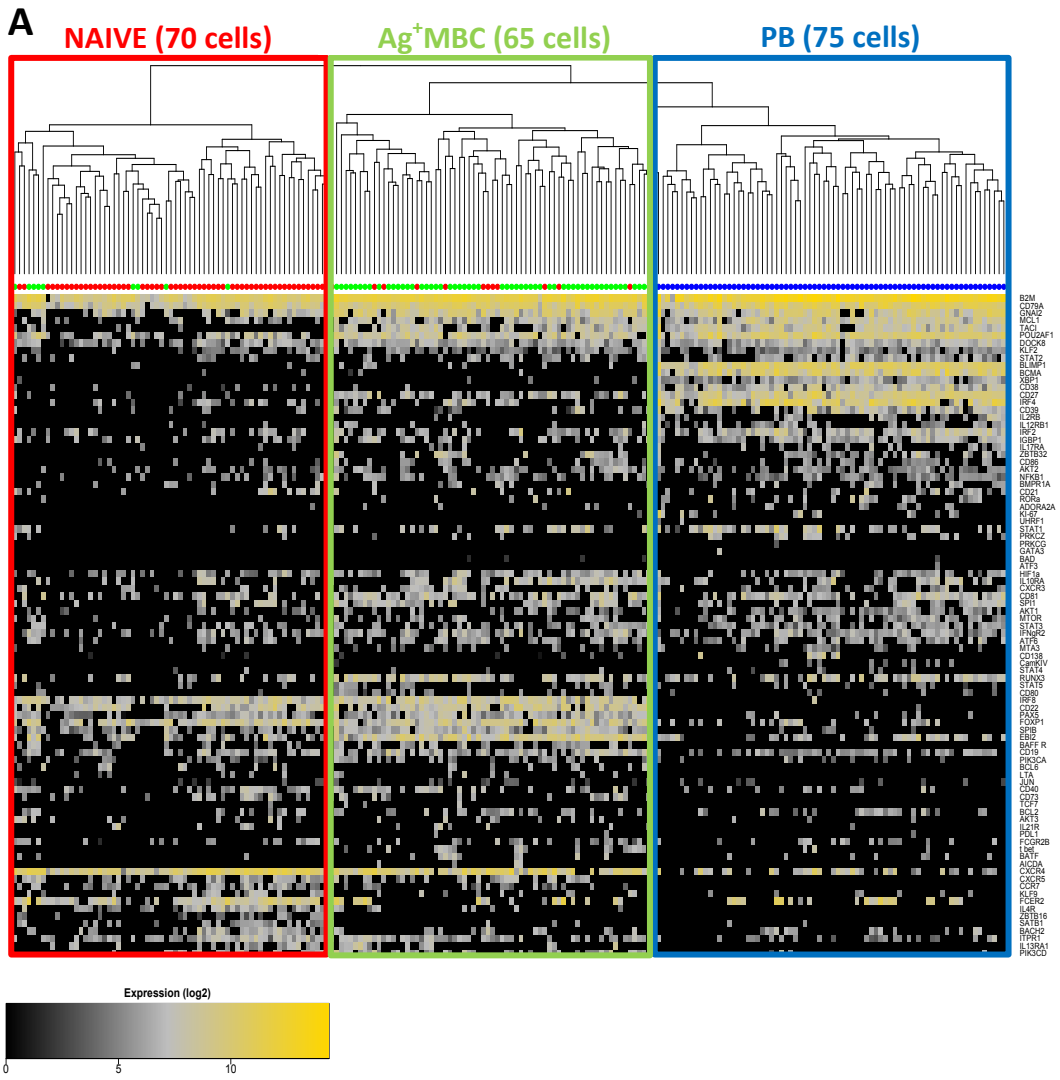


Fig. 3 Pilot study: Ig repertoire analysis of B cell populations in SbjA. Ig clonotype analysis performed on NAIVE, PB and Ag^{+}MBC with circles indicating clonotype size and numbers inside circles indicating the number of clonotypes of that particular size.

Figure 4


A
NAIVE (70 cells)
Ag+MBC (65 cells)
PB (75 cells)
Expression (log2)
0
5
10
B
LDA
LD2
LD1
Ag+MBC
NAIVE
PB

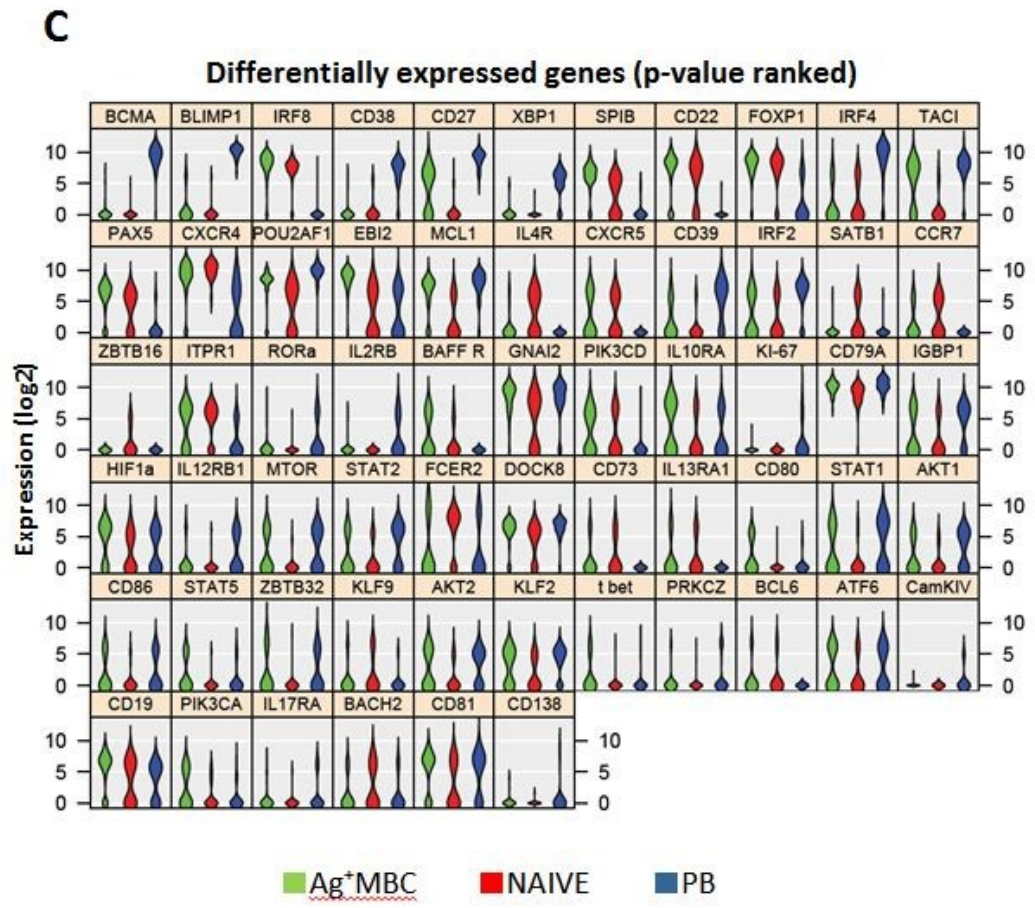


Fig. 4 Gene expression analysis of all SbjB B cell populations. (A) Single cell gene expression values (log_2(30-Ct), 96 genes) of n=210 total B cells from SbjB at days 8 and day22 after vaccination were used for unsupervised hierarchical clustering (heatmap with dendogram of single B cells in columns and genes in rows) and (B) Linear Discriminant Analysis (dot plot shows the position of every cell on the space defined by the first two linear discriminant components LD1 and LD2). (C) Violin plots depicting expression distribution of significant differentially expressed genes (ANOVA $p<0.05$) resulting from Ag^+MBC, PB and NAIVE population comparison, ranked by p-value from top-left to bottom-right. For clarity purposes, protein IDs instead of gene IDs are used.

Figure 5

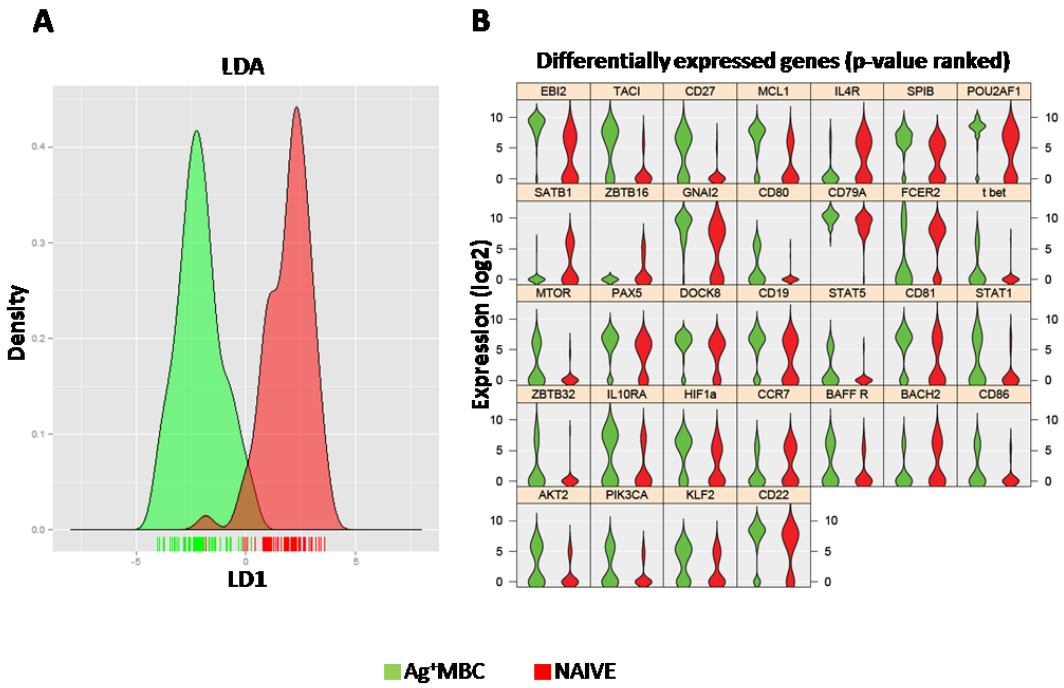


Fig. 5 comparison of Ag$^+$MBC-NAIVE gene expression profiles in SbjB. (A) Linear Discriminant Analysis of n=65 Ag$^+$MBC and n=70 NAIVE single cell gene expression values (log_2(30-Ct), 96 genes) (histogram shows the distribution of cells on the space defined by the first linear discriminant component LD1). (B) Violin plots depicting expression distribution of significant differentially expressed genes (Tukey test, $p<0.05$) resulting from Ag$^+$MBC versus NAIVE comparison, ranked by p-value from top-left to bottom-right. For clarity purposes, protein IDs instead of gene IDs are used.

Figure 6

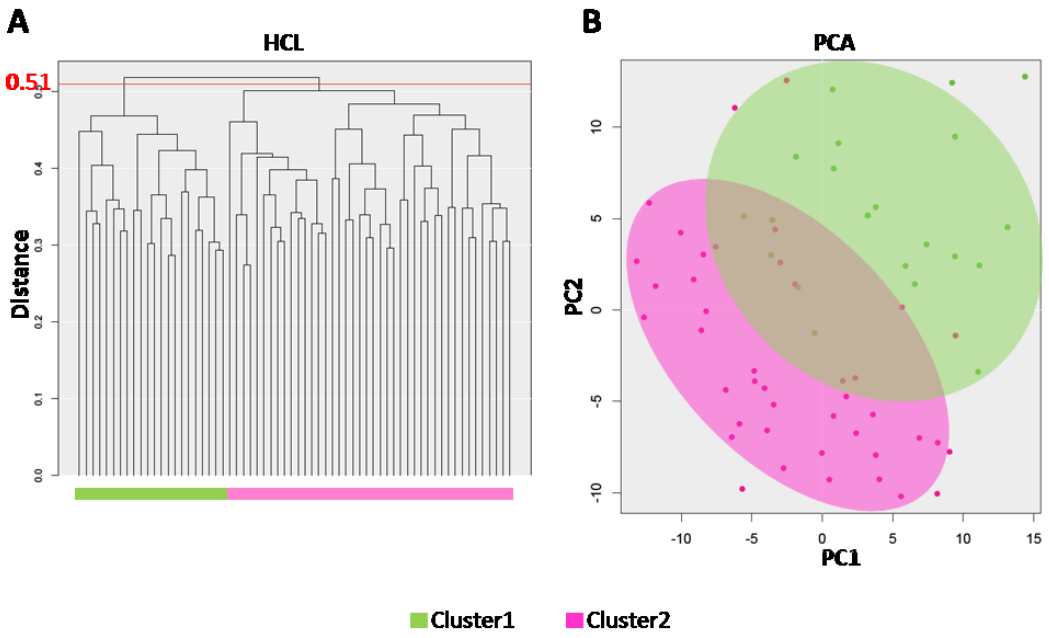
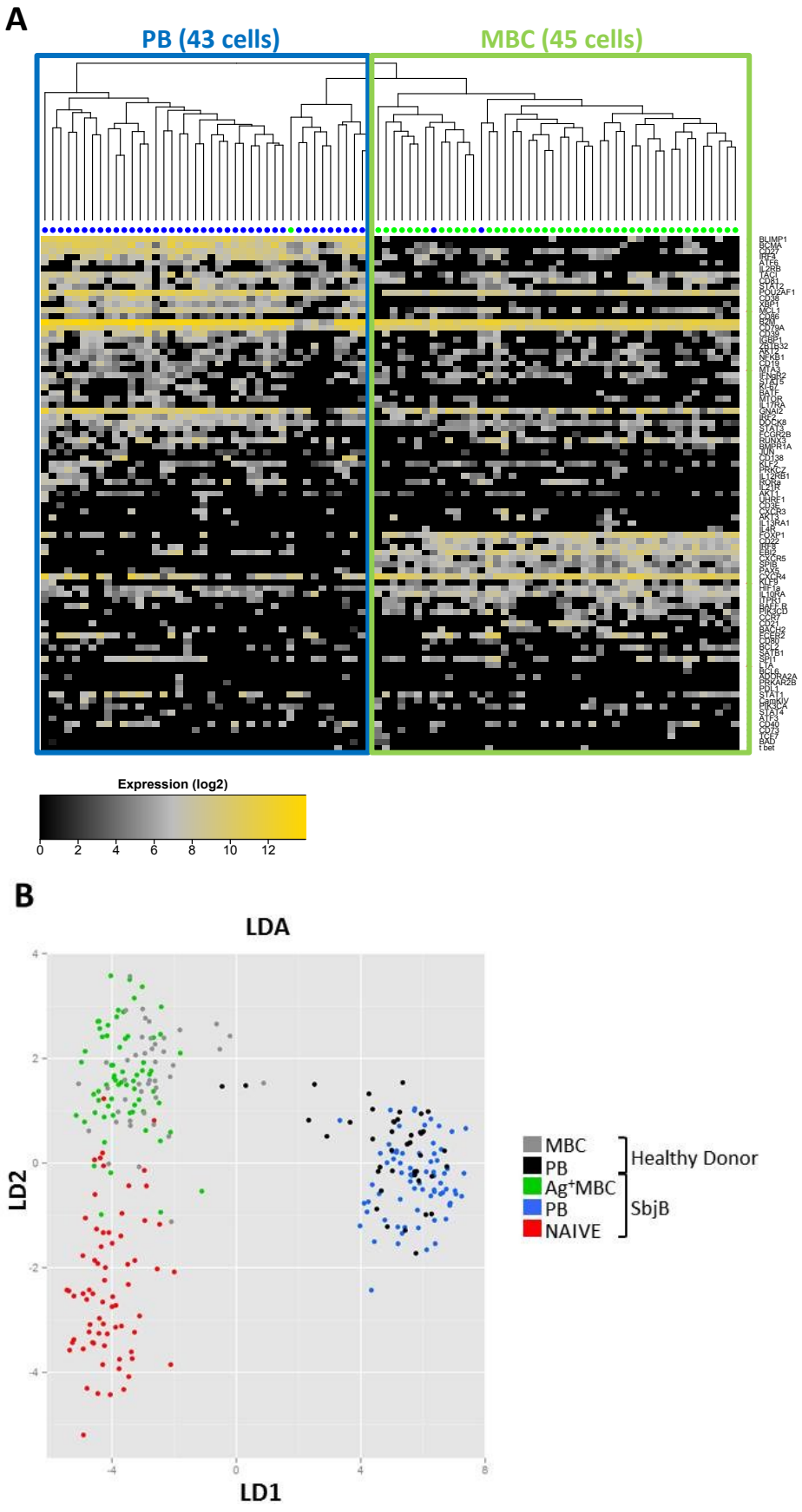


Fig. 6 Subpopulations identification in Ag^{+}MBC of SbjB. (A) Ag^{+}MBC clustering dendogram where the red line indicates the threshold distance (0.51) set to partition the population in two subsets based on gene expression patterns. (B) Principal Component Analysis showing the distribution and intersection of the identified clusters of Ag^{+}MBC.

Figure 7


A
PB (43 cells)
MBC (45 cells)
Expression (log2)
0
2
4
6
8
10
12
B
LDA
LD2
LD1
MBC
PB
Ag+MBC
PB
NAIVE
Healthy Donor
SbjB

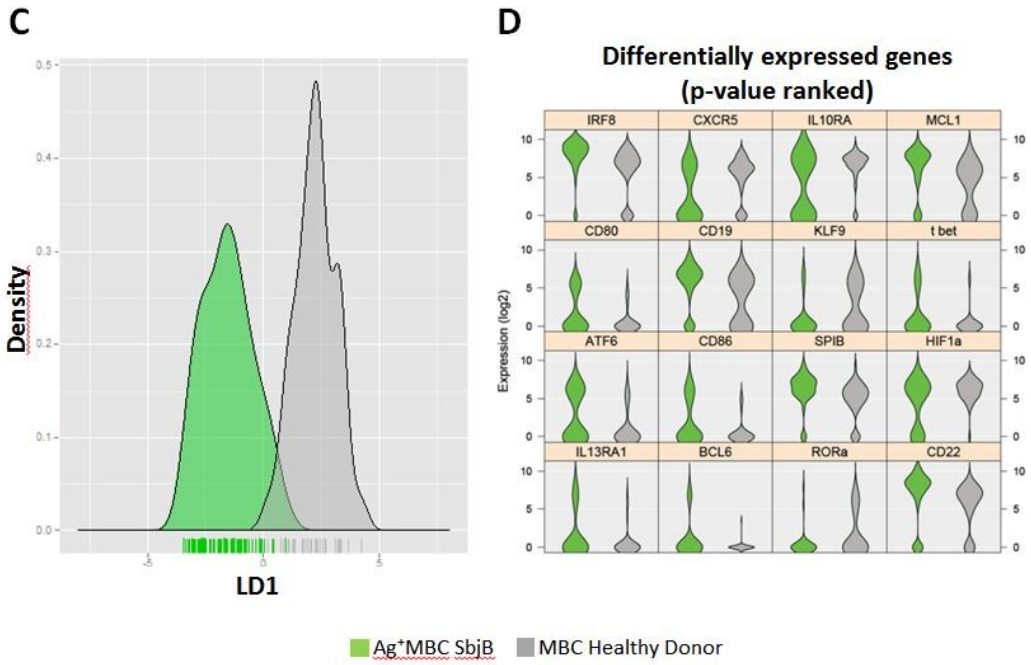
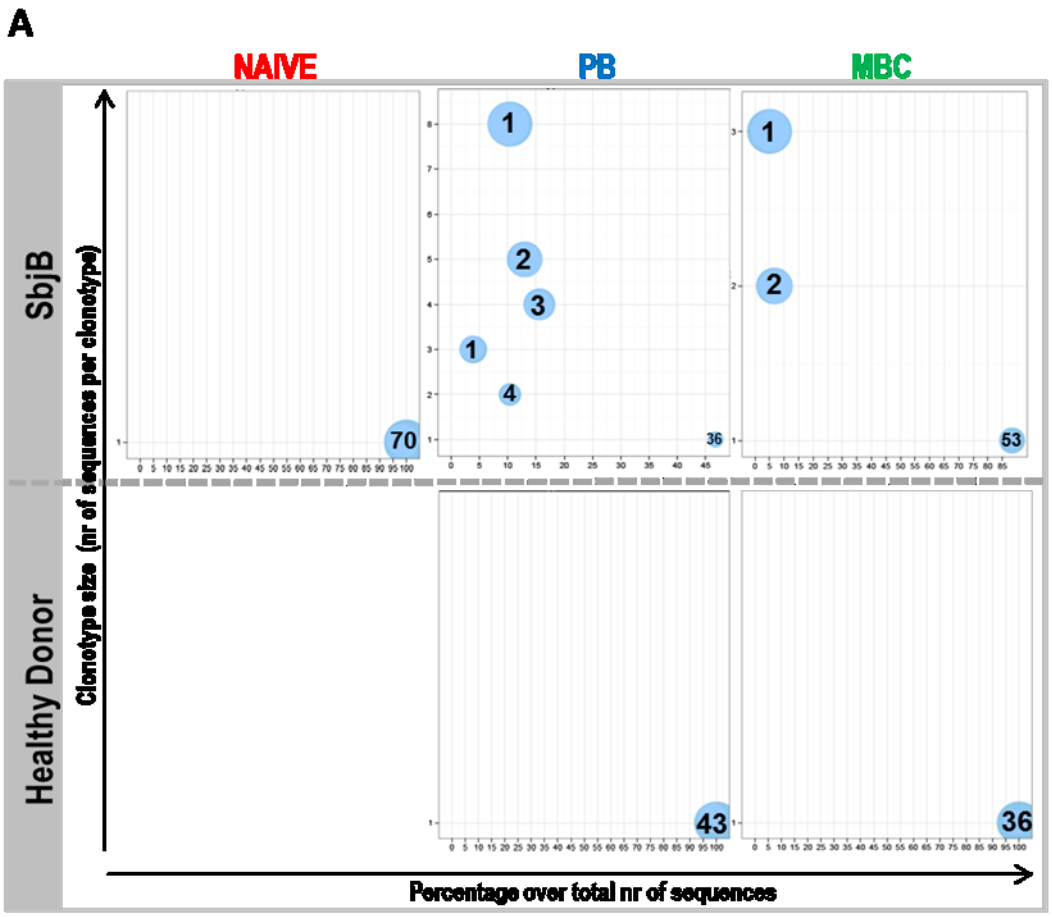


Fig. 7 Vaccinee (SbjB) versus healthy donor comparison of MBC populations. (A) Single cell gene expression values (log_2(30-Ct), 96 genes) of n=88 total B cells from the healthy donor were used for unsupervised hierarchical clustering (heatmap with dendogram of single B cells in columns and genes in rows). (B) Linear Discriminant Analysis showing that MBC (grey) and PB (black) from healthy donor overlap with the respective vaccinee populations (green Ag^+MBC, blue PB). (C) Linear Discriminant Analysis of SbjB Ag^+MBC and healthy donor MBC single cell gene expression values (log_2(30-Ct), 96 genes) (histogram shows the distribution of cells on the space defined by the first linear discriminant component LD1). (D) Violin plots depicting expression distribution of significant differentially expressed genes (Tukey test, $p<0.05$) resulting from SbjB Ag^+MBC versus healthy donor MBC comparison, ranked by p-value from top-left to bottom-right. For clarity purposes, protein ID instead of gene ID is used.

Figure 8


A
NAIVE
PB
MBC
SbjB
Healthy Donor
Clonotype size (nr of sequences per clonotype)
Percentage over total nr of sequences
70
36
53
43
36
B
Ag+MBC
PB
NAIVE
77%
4%
19%
18%
78%
4%
3%
7%
90%
0%
50%
100%
IgG
IgA
IgM

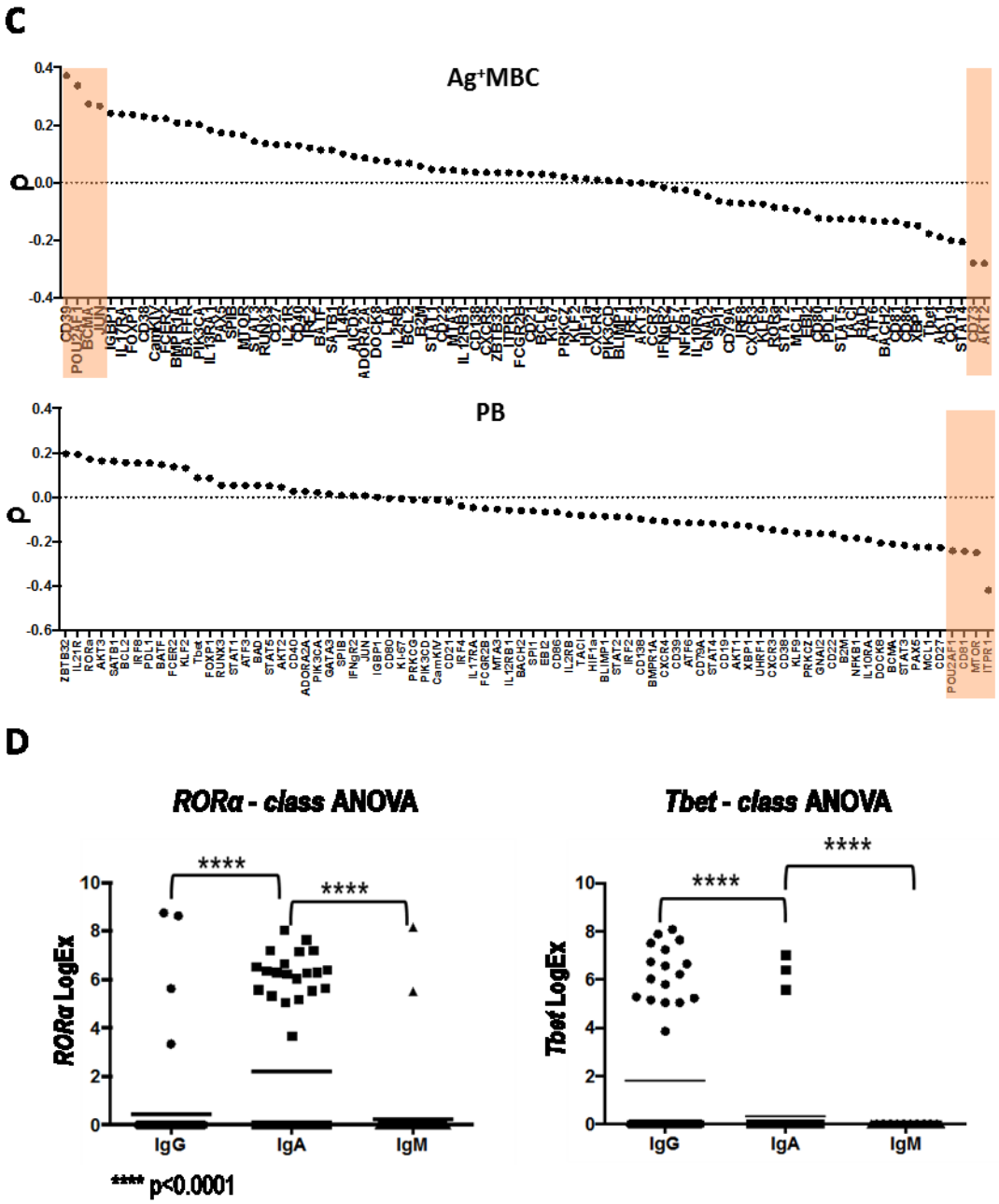
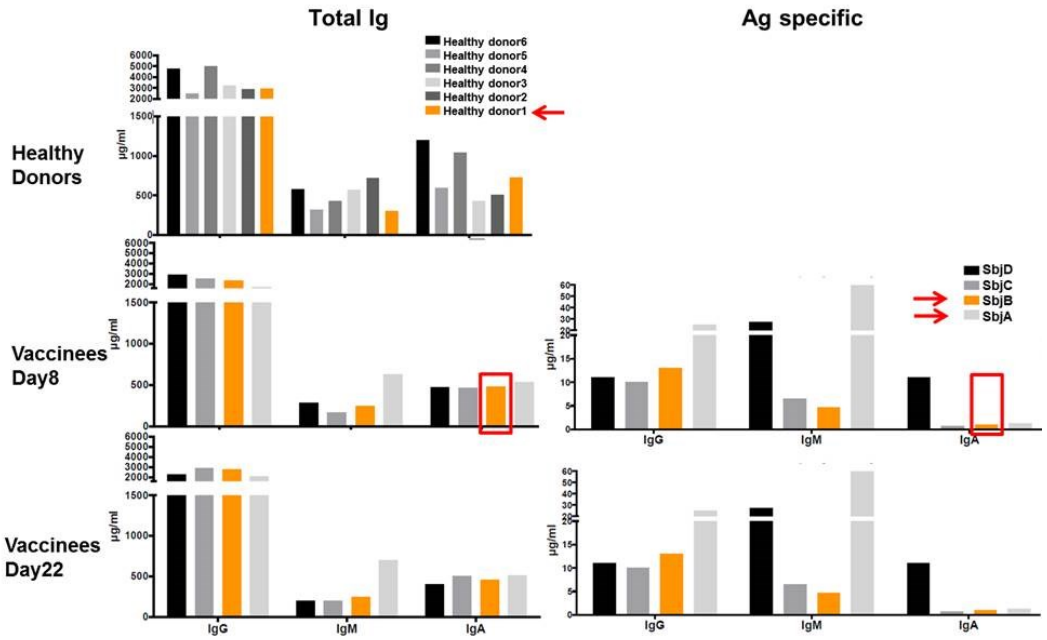


Fig. 8 Ig Repertoire analysis and correlations with gene expression data. (A) Ig clonotype analysis performed on SbjB and healthy donor populations, with circles indicating clonotype size and numbers inside circles indicating the number of clonotypes of that particular size. (B) Ig isotype distribution over Ag^{+}MBC, PB and NAIVE populations in SbjB. (C) Spearmann correlation study of VH full gene mutation rate and gene expression on SbjB Ag^{+}MBC (top) and PB (bottom); dots represent ρ values for each gene, orange color identifies significant correlations ($p<0.05$). (D) Association of *Rorα* (left) and *Tbet* (right) expression with Ig isotype (ANOVA, ****$p<0.0001$). For clarity purposes, protein IDs instead of gene IDs are used.

Supplementary Figure 1



Supplementary Fig. 1 Quantification of total and Ag-specific antibodies in vaccinees and healthy donors plasma. Histograms reporting total Ig (left) and Ag-specific (right) titers in plasma of 6 healthy donor (top row) and 4 vaccinees at Day8 (middle row) and Day22 (bottom row) after vaccination for IgG, IgM and IgA isotypes. Red arrows indicate subjects discussed in this work; red squares indicate Ag-specific titers of SbjB. These results were obtained performing high-throughput ELISA on the Gyrolab Workstation.

Table 1 (continues on next page)

Gene ID	Protein ID	Taqman ID	Group ID
CD80	CD80	Hs00175478_m1	Activation
CD86	CD86	Hs01567026_m1	
PDCD1	PD1	Hs01550088_m1	
CD274	PDL1	Hs01125301_m1	
PDCD1LG2	PDL2	Hs01057777_m1	
BAD	BAD	Hs00188930_m1	Apoptosis
*BCL2**	BCL2	Hs00608023_m1	
*MCL1**	MCL1	Hs01050896_m1	
ADORA2A	ADORA2A	Hs00169123_m1	ATP
ENTPD1	CD39	Hs00969559_m1	
*NT5E**	CD73	Hs00159686_m1	
CD22	CD22	Hs00233533_m1	BCR
CD79A	CD79A	Hs00998119_m1	
CD81	CD81	Hs00174717_m1	
DOCK8	DOCK8	Hs00405736_m1	
FCER2	FCER2	Hs01077046_g1	
FCGR2B	FCGR2B	Hs01634996_s1	
IGBP1	IGBP1	Hs00426831_mH	
PIK3CA	PIK3CA	Hs00907957_m1	
PIK3CD	PIK3CD	Hs00908667_g1	
PRKAR2B	PRKAR2B	Hs00176966_m1	
PRKCG	PRKCG	Hs00997697_g1	
PRKCZ	PRKCZ	Hs00177051_m1	
AKT1	AKT1	Hs00178289_m1	
AKT2	AKT2	Hs01086102_m1	
AKT3	AKT3	Hs00178533_m1	
MTOR	MTOR	Hs00234508_m1	
CAMK4	CamKIV	Hs01009421_m1	Calcium
ITPR1	ITPR1	Hs00181881_m1	
*MKI67**	KI67	Hs01032443_m1	Cell cycle
KLF2	KLF2	Hs00360439_g1	
KLF4	KLF4	Hs00358836_m1	
KLF9	KLF9	Hs00230918_m1	
UHRF1	UHRF1	Hs01086727_m1	
ZBTB16	ZBTB16	Hs00957433_m1	
*18S**	18S	Hs99999901_s1	Control
*B2M**	B2M	Hs00984230_m1	
TNFRSF13C	BAFFR	Hs00606874_g1	Cytokine receptor
TNFRSF17	BCMA	Hs03045080_m1	
BMPR1A	BMPR1A	Hs01034913_g1	
IFNGR2	IFNgR2	Hs00194264_m1	
IL10RA	IL10RA	Hs00155485_m1	
IL12RB1	IL12RB1	Hs00538167_m1	
IL13RA1	IL13RA1	Hs00609817_m1	
IL17RA	IL17RA	Hs01064648_m1	
*IL21R**	IL21R	Hs00222310_m1	
IL2RB	IL2RB	Hs01081697_m1	
IL4R	IL4R	Hs00965056_m1	
TNFRSF13B	TACI	Hs00963364_m1	

Table 1 (continues from previous page)

Gene ID	Protein ID	Taqman ID	Group ID
CXCR3	CXCR3	Hs01847760_s1	Homing
CXCR4	CXCR4	Hs00607978_s1	
*CXCR5**	CXCR5	Hs00173527_m1	
*AICDA**	AICDA	Hs00757808_m1	Hypermutation
CCR7	CCR7	Hs01013469_m1	Migration
GPR183	EBI2	Hs00270639_s1	
GNAI2	GNAI2	Hs01064686_m1	
LTA	LTA	Hs04188773_g1	
HIF1A	HIF1a	Hs00153153_m1	Oxidative stress
SDC1	CD138	Hs00896423_m1	Phenotype
*CD19**	CD19	Hs00174333_m1	
CR2	CD21	Hs00153398_m1	
CD27	CD27	Hs00386811_m1	
CD38	CD38	Hs01120071_m1	
*CD3E**	CD3E	Hs01062241_m1	
*CD40**	CD40	Hs01002913_g1	
ATF3	ATF3	Hs00231069_m1	Transcriptional factor
ATF6	ATF6	Hs00232586_m1	
BACH2	BACH2	Hs00222364_m1	
*BCL6**	BCL6	Hs00153368_m1	
*PRDM1**	BLIMP1	Hs00153357_m1	
FOXP1	FOXP1	Hs00908900_m1	
GATA3	GATA3	Hs00231122_m1	
IRF2	IRF2	Hs01082881_g1	
*IRF4**	IRF4	Hs01056533_m1	
*IRF8**	IRF8	Hs00175238_m1	
JUN	JUN	Hs99999141_s1	
MEF2B	MEF2B	Hs04188747_m1	
MTA3	MTA3	Hs00383033_m1	
NFKB1	NFKB1	Hs00765730_m1	
*PAX5**	PAX5	Hs00172003_m1	
POU2AF1	POU2AF1	Hs01573371_m1	
RORA	RORa	Hs00536545_m1	
RUNX3	RUNX3	Hs00231709_m1	
SATB1	SATB1	Hs00161515_m1	
SPI1	SPI1	Hs02786711_m1	
*SPIB**	SPIB	Hs00162150_m1	
STAT1	STAT1	Hs01013996_m1	
STAT2	STAT2	Hs01013123_m1	
*STAT3**	STAT3	Hs00374280_m1	
STAT4	STAT4	Hs01028017_m1	
*STAT5A**	STAT5	Hs00234181_m1	
TBX21	Tbet	Hs00203436_m1	
TCF7	TCF7	Hs00175273_m1	
*XBP1**	XBP1	Hs00231936_m1	
ZBTB32	ZBTB32	Hs00998475_g1	
BATF	BATF	Hs00232390_m1	

Table 1: List of IDs, Taqman Assay codes and categories of the genes (and respective proteins) analyzed in this study.

Table 2

Primer ID	Sequence
Preamp VH1 FW	CACTCCCAGGTGCAGCTGGTGCAG
Preamp VH2 FW	TGGGTCTTRTCCCAGGTCACCTTG
Preamp VH3 FW	AAGGTGTCCAGTGTSAGGTGCAG
Preamp VH4 FW	GTCCTGTCCCAGGTGCAGCTGCAG
Preamp VH5 FW	GAGTCTGTTCCGAGGTGCAGCTGG
Preamp IgG FW	GTGCCAGGGGGAAGACCGATG
Preamp IgA FW	GCMGAGGCTCAGCGGGAAGAC
Preamp IgM RV	GAGACGAGGGGGAAAAGGGTTG
Preamp Vk1 FW	CAGGTGCCAGATGTGHCATCCAG
Preamp Vk2 FW	CTGGATCCAGTGSGGATATTGTGATG
Preamp Vk3 FW	CCCAGATACCACCGGAGAAATTGTG
Preamp Vk4 FW	CTCTGGTGCCTACGGGGACATCGTG
Preamp Vk5 FW	CTGATACCAGGGCAGAAACGACAC
Preamp Ck RV	GAACACTCTCCCCTGTTGAAGCTCTTTG
Preamp VL1 FW	GGTCCTGGGCCCAGTCTGTGCTG
Preamp VL2 FW	GGTCCTGGGCCCAGTCTGCCCTG
Preamp VL3 FW	TCTGTGRCCTCCTATGAGCTGAC
Preamp VL4FW	CTCTCGCAGCCTGTGCTGACTCA
Preamp VL5 FW	GTTCTTGGGCCAATTTTATGCTG
Preamp VL6 FW	GGTCCAATTCTCAGGCTGTGGTG
Preamp VL7 FW	GAGTGGATTCTCAGACTGTGGTG
Preamp VL8 FW	GTCAGTGGTCCAGGCAGGGCTGAC
Preamp CL RV	GTGCTCCCTTCATGCGTGACC
Ig PCR VH1 FW	CCAGGTGCAGCTGGTGCAGTCTG
Ig PCR VH2 FW	CCAGGTCACCTTGAAGGAGTCTGGTC
Ig PCR VH3 FW	TGAGGTGCAGCTGGTGGAGTCTGGGGGAG
Ig PCR VH4 FW	CCAGGTGCAGCTGCAGGAGTCGGG
Ig PCR VH5 FW	CCAGGTGCAGCTGCAGCAGTGGGG
Ig PCR IgG RV	CGATGGCCCCTTGGTGGARGCTG
Ig PCR IgA RV	ACCTTGGGGCTGGTCGGGGATG
Ig PCR IgM RV	GTTGGGGCGGATGCACTCCCTG

Table 2: Immunoglobulin-specific primers used for PCR amplification. Primers specific for the V region of all Ig chains were introduced in the pre-amplification reaction mix ('Preamp' ID prefix). VH specific primers were used in the Ig PCR and sequencing procedure ('Ig PCR' ID prefix).

10. ACKNOWLEDGMENTS

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