ADVANCES IN PLASMA CELLS IN HEALTH AND DISEASE

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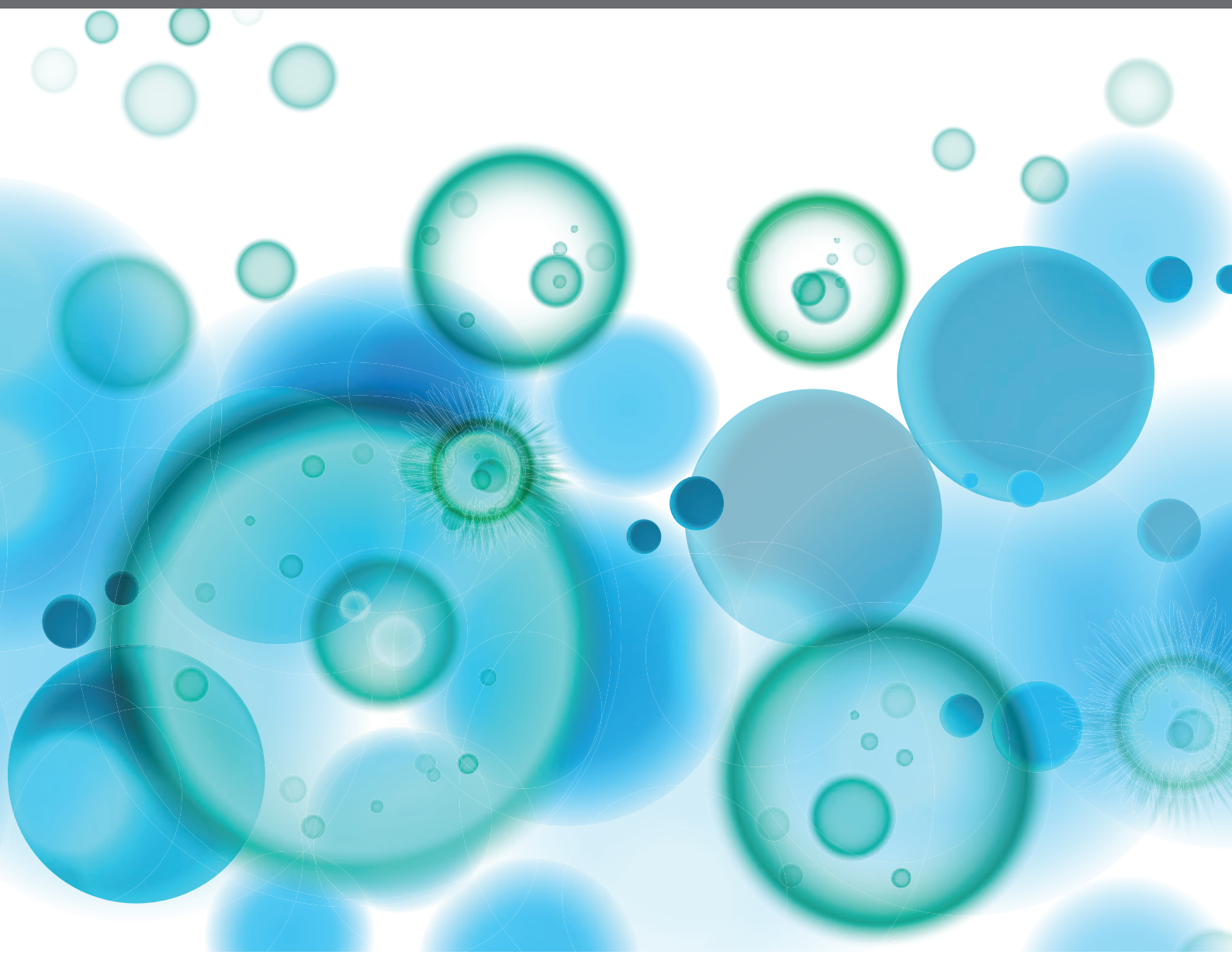

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ADVANCES IN PLASMA CELLS IN HEALTH AND DISEASE

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EDITORIAL
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Editorial: Advances in Plasma Cells in Health and Disease

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Keywords: niche, bone marrow, allergy, serological memory, plasma cell, multiple myeloma, immunoglobulin, antibody

Editorial on the Research Topic

Advances in Plasma Cells in Health and Disease

The plasma cell (PC) is a relatively fledging cell type in biomedical science because its functional recognition dates back only to the mid 20th century (1, 2). PCs are terminally differentiated immune effectors that develop from B lymphocytes following infection and vaccination, with the highly specialized function to manufacture and secrete antibodies, the effector molecules of humoral immunity. Circulating antibodies can persist in the absence of antigen and provide protection for a lifetime, affording serological memory. Due to their rapid disappearance in the circulation, PCs have long been thought to be short-lived and to maintain serum antibodies by continuous differentiation from B cells. It has recently become clear that PCs may survive and secrete protective antibodies for decades after antigen encounter (3–6).

The nature of the stimulus that triggers PC generation from short-lived antibody secreting cells (ASC) and the molecular programs underlying such transformation are elusive. Long-lived PCs (LLPCs) reside in dedicated niches, mainly located in the bone marrow (BM). Different cell types and signals combine to shape PC survival niches (5, 7). In humans, the phenotype of LLPCs is currently defined as CD19^{neg}CD138^{+}CD38^{hi}, but phenotypic, biological and functional heterogeneity is likely to exist within this population (8). Far from being quiescent, LLPCs display unrivaled immunoglobulin (Ig) secretion that requires unique organelle organization and adaptive proteostatic and metabolic features that excite great curiosity and represent formidable research challenges (9–11). The longevity of PCs, from their generation throughout their maintenance, relies on multiple factors, whose identification and mechanistic details are critical to advance our understanding of adaptive immunity, as well as the pathophysiology of PC-mediated autoimmune and neoplastic disorders (12). This collection highlights fundamental mechanisms of PC longevity in health and disease, namely, multiple myeloma and allergy, as well as novel PC functions and immunophenotypes in nonhuman primates.

Five review articles within this series discuss the extrinsic and intrinsic determinants of PC maintenance with specific focus and from different perspectives, offering a comprehensive and integrated view of LLPCs. Slifka and Amanna discuss the mechanisms underlying how the structural biology of multivalent antigens can induce durable protective immunity by LLPCs compared with monovalent antigens, an issue whose relevance for vaccine design has become tangible with the SARS-CoV-2 pandemic. Lindquist et al. review the dynamic nature of PC niches,

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the current knowledge on their molecular and cellular composition, and how its changes may influence PC function, with a specific focus on metabolism and new technologies to gauge it over time *in vivo*. Lightman et al. analyze the extrinsic and intrinsic factors of PC longevity within their niche, including continuous niche-generated signals unique to LLPC survival, metabolic fitness, specific bioenergetic cues, and cellular components of the LLPC niche itself. Khodadadi et al. depict a historical perspective on the recent discovery and characterization of PCs. In view of the incessant capacity of LLPCs to secrete antibodies, independently of antigen presence, T cell help or supply from precursors, they propose to call them *memory* PCs. They too discuss the determinants of PC longevity, namely, extracellular components — cellular compartments and soluble and membrane-bound molecular elements — and intracellular factors, related to differentiation and stress-adaptive pathways, metabolism, autophagy, and survival. Since PCs acquire longevity in inflamed tissues, they also review the inflammatory cellular and molecular mechanisms thought to support PC survival. Finally, Nguyen et al. review the molecular, functional and immunophenotypic features that hallmark the transition of human short-lived ASCs to LLPCs and the known cell-autonomous and nonautonomous factors required, with a specific perspective on the adaptive significance of the changes imparted to early minted ASCs as they mature into late BM LLPCs. Of technological relevance, they propose a minimal set of extrinsic conditions, combining secreted factors from BM stromal cells, APRIL and low oxygen tension as an experimental human BM mimic able to maintain human ASCs in culture for weeks for molecular *ex vivo* studies.

Not all PCs are protective. Aberrant PC generation and maintenance can result in pathogenic PCs in human disease, like PC dyscrasias, where transformed PCs gain enhanced proliferation and survival. The prototypical PC cancer is multiple myeloma, an age-onset malignancy characterized by the clonal expansion of PCs at multiple foci in the BM, typically resulting in lytic bone lesions, hypercalcemia, renal failure, anemia, and infections (13). Myeloma cells are the malignant counterpart of BM resident LLPCs; however, the exact cell of origin of this cancer remains unknown. Myeloma cells usurp the BM niche-specified pro-survival signals intended for LLPCs. Targeting such multi-cellular environmental niches holds great therapeutic potential against myeloma, but a comprehensive and translatable knowledge of the underlying circuits warrants more investigation (14). Barwick et al. discuss the cellular and genetic origin of multiple myeloma, reconstructing the milestone discoveries on clonal gammopathies and their interconnectedness with the advancing knowledge of PCs, with in-depth focus of the myeloma-driving genetic and epigenetic alterations in the context of PC differentiation and biology.

IgE PCs arise for protection against parasites, but can mediate allergic diseases. Ramadani et al. deployed a human *ex vivo* tonsil B cell culture system to investigate transcriptional profiles of IgE-expressing PCs and identified putative specific gene expression trajectories and regulatory networks.

Two additional original articles complete this series. PCs have functions beyond Ab secretion (15). Using mouse models, Meng et al. gauged the contribution of PCs to IL-10 provision in the BM. Besides confirming PCs as the chief source of IL-10, they tested its function and demonstrated a key role of IL-10 in driving myeloid lineage differentiation, an effect that appeared to increase with age. Lastly, Zhang et al. defined the surface immunophenotypic markers that identify antibody-secreting plasmablasts in the nonhuman primates, Chinese rhesus macaques. Of biotechnological and therapeutic relevance, this work may help isolate ASCs for efficient mAb cloning and evaluate antibody responses to vaccination or infection in these human-relevant animal species.

In summary, we present a unique collection of review and original articles dissecting the known mechanisms of PCs in health and disease to raise essential questions that still remain at large in the generation and maintenance of LLPCs.

AUTHOR CONTRIBUTIONS

All authors have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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ORIGINAL RESEARCH
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Transcriptional Analysis of the Human IgE-Expressing Plasma Cell Differentiation Pathway

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IgE is secreted by plasma cells (PCs) and is central to allergic disease. Using an *ex vivo* tonsil B cell culture system, which mimics the Th2 responses *in vivo*, we have recently characterized the development pathway of human IgE-expressing PCs. In this system, as in mice, we reported the predisposition of IgE-expressing B cells to differentiate into PCs. To gain a comprehensive understanding of the molecular events involved in the differentiation of human IgE^+ B cells into PCs we have used the Illumina HumanHT-12 v4 Expression BeadChip array to analyse the gene expression profile of *ex vivo* generated human IgE^+ B cells at various stages of their differentiation into PCs. We also compared the transcription profiles of IgE^+ and $IgG1^+$ cells to discover isotype-specific patterns. Comparisons of IgE^+ and $IgG1^+$ cell transcriptional profiles revealed molecular signatures specific for IgE^+ cells, which diverge from their $IgG1^+$ cell counterparts upon differentiation into PCs. At the germinal center (GC) stage of development, unlike in some mouse studies of IgE biology, we observed similar rates of apoptosis and no significant differences in the expression of apoptosis-associated genes between the IgE^+ and $IgG1^+$ B cells. We identified a gene interaction network associated with early growth response 1 (*EGR1*) that, together with the up-regulated IRF4, may account for the predisposition of IgE^+ B cells to differentiate into PCs. However, despite their swifter rates of PC differentiation, the transcription profile of IgE^+ PCs is more closely related to IgE^+ and $IgG1^+$ plasmablasts (PBs) than to $IgG1^+$ PCs, suggesting that the terminal differentiation of IgE^+ cells is impeded. We also show that IgE^+ PCs have increased levels of apoptosis suggesting that the IgE^+ PCs generated in our *in vitro* tonsil B cell cultures, as in mice, are short-lived. We identified gene regulatory networks as well as cell cycle and apoptosis signatures that may explain the diverging PC differentiation programme of these cells. Overall, our study provides a detailed analysis of the transcriptional pathways underlying the differentiation of human IgE-expressing B cells and points to molecular signatures that regulate IgE^+ PC differentiation and function.

Keywords: human IgE^+ B cells, IgE^+ plasma cell differentiation, gene expression, transcriptomics, apoptosis, cell cycling, allergic disease

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INTRODUCTION

IgE plays a central role in the pathogenesis of allergic disease (1, 2). Although IgE is the least abundant antibody in the circulation, its binding to the high affinity IgE receptor (FcεRI) on mast cells and basophils is critical for the manifestation of immediate hypersensitivity to allergens and allergic inflammation (1, 2). IgE is secreted by PCs, which represent the terminal stage of B cell differentiation, after immunoglobulin class switching to IgE in precursor B cells (3).

Important advances in understanding the regulation of IgE production have been made over the last decade. The predisposition of IgE-switched cells to develop toward the PC rather than the memory cell lineage is seen in both mouse and human systems (4–10). However, this could not be attributed to differences in the expression levels of the PC differentiation master regulator, Blimp-1 (7, 9). Studies by IgE and IgG1 domain swapping in mouse B cells show that membrane IgE (mIgE) signaling promotes antigen-independent PC differentiation of IgE^+ B cells (5, 10). The CH2-CH3 extracellular domains and the cytoplasmic tail contribute to this activity, but the key component was the extracellular membrane-proximal domain (EMPD) (5, 10).

The effect of mIgE signaling in PC differentiation has been suggested to involve IRF4 (5, 10), a transcription factor that regulates PC differentiation (11). However, we lack a more comprehensive knowledge of other molecular pathways that likely contribute to this process, especially in humans. Unlike in mouse, two isoforms of mIgE exist in humans, a short form (mIgEs), equivalent to the mouse mIgE, and a long form ($mIgE_L$) containing an EMPD that is 52 amino acids longer (12, 13). Expression of the $mIgE_L$ by the human IgE^+ B cells may also influence PC differentiation.

Using an *ex vivo* tonsil B cell culture system, stimulated with IL-4 and anti-CD40 *in vitro* to generate IgE^+ cells, we have recently characterized the developmental pathway of human IgE^+ and $IgG1^+$ PCs (7). In this system, we demonstrated that there are three discrete stages of IgE^+ PC development pathway, which we characterized phenotypically as IgE^+ GC-like B cells ($IgE^{lo}CD27^-CD138^-Bcl6^{hi}Pax5^{hi}Blimp1^{lo}$), IgE^+ PC-like "PBs" ($IgE^{hi}CD27^{++}CD138^-Bcl6^{lo}Pax5^{lo}Blimp1^{hi}$), and IgE^+ PCs ($IgE^{hi}CD27^{++}CD138^+Bcl6^{lo}Pax5^{lo}Blimp1^{hi}$) (7). A similar $IgG1^+$ PC development pathway was also observed. The IgE^+ cells displayed cell cycle and proliferation rates greater than their $IgG1^+$ cell counterparts, and interestingly we also observed that the differentiation of IgE^+ B cells into PCs is accompanied by the modulation of $mIgE_L$ and $mIgE_S$ surface expression (7). Here, to better understand the differentiation process of human IgE^+ B cells into PCs and to identify key regulators of this process, we have used the Illumina HumanHT-12 v4 Expression BeadChip array to define and compare the transcriptomes of *ex vivo* generated IgE^+ and $IgG1^+$ B cells at various stages of their differentiation into PCs.

Abbreviations: AID, Activation-induced cytidine deaminase; EMPD, Extra-membrane proximal domain; FDR, False discovery rates; GC, Germinal Center; GO, Gene ontology; GRN, Gene regulatory network; IPA, Ingenuity Pathway Analysis; $mIgE_L$, Long form of membrane IgE; $mIgE_S$, Short form of membrane IgE; PB, Plasmablast; PC, Plasma cell; SOM, Self-organizing map; WGCNA, Weighted gene co-expression network analysis.

METHODS

Cell Cultures

B cells were isolated from the dissected tonsil tissue on a density gradient (GE Healthcare) followed by incubation with aminoethyl isothiouronium bromide-treated sheep red blood cells to rosette T cells (TCS Biosciences). B cells were >95% $CD19^+$ as determined by flow cytometric (FACS) analysis. Purified tonsil B cells were induced to undergo class switching to IgE as previously (14). Briefly, 0.5×10^6 freshly purified tonsil B cells were stimulated with IL-4 (200 IU/ml; R&D Europe Systems Ltd.) and anti-CD40 antibody (0.5 μg/ml; G28.5; American Type Culture Collection). After day 7 the population of $IgG1^+$ and IgE^+-switched cells gradually increased to a maximum at 10 days when the cells were harvested for study.

FACS Sorting of IgE^+ and $IgG1^+$ Cells

Cultured cells were stained with a live/dead fixable stain dye (Life Technologies Ltd.) and anti-CD138 APC (Miltenyi Biotech) followed by fixation with 2% paraformaldehyde. Following washing with RNAsecure (Life Technologies Ltd.) treated PBS, supplemented with 100 U/mL of RNase inhibitor (Bioline Reagents Ltd.) and 5 mM DL-dithiothreitol (Sigma-Aldrich Ltd.), cells were permeabilized with 1% molecular grade triton ×100 (Sigma-Aldrich Ltd.) containing 250 U/mL of RiboSafe RNase inhibitor and 5 mM DL-dithiothreitol and intracellularly stained with anti-IgE FITC (Vector Laboratories) and anti-IgG1 PE (Miltenyi Biotech) for 45 min on ice. The $IgE^{lo}CD138^-$, $IgE^{hi}CD138^-$, and $IgE^{hi}CD138^+$cells and their respective IgG1 counterparts were FACS sorted into melting buffer (Invitrogen) containing 1,600 U/mL RiboSafe RNase inhibitors and 10 mM DL-dithiothreitol and used for total RNA extraction (see below).

RNA Isolation

Total RNA was isolated using a previously described protocol (7) for the PureLink FFPE total RNA isolation kit (Invitrogen). Briefly, cells were sorted into the melting buffer containing 1600 U/mL RNase inhibitor (Bioline) and 10 mM DTT (Sigma-Aldrich Ltd.) and stored at −80°C before proceeding to the proteinase K treatment for 15 min at 60°C. Subsequently the manufacturers instructions were followed, including the optional DNase digestion. The RNA was further cleaned using the RNeasy Mini Kit RNA Cleanup protocol (Qiagen). RNA concentrations were measured using the NanoDrop 2000 (Thermo Scientific) and RNA integrity assessed using the 2100 Bioanalyser instrument (Agilent Technologies, Inc.).

Illumina BeadChips Array

cDNA was synthesized and amplified from 40 ng RNA using the Ovation Pico WTA system V2 (NuGEN) and purified using the MiniElute Reaction Cleanup Kit (Qiagen). Yield and purity were measured using the 2100 Bioanalyser instrument and the RNA 6000 Nano kit (Agilent). Four microgram of amplified cDNA was biotin labeled with Encore Biotin Module (NuGen),

purified, concentrated and hybridized onto Illumina HumanHT-12 v4 Expression BeadChip array and scanned using the Illumina iScan platform. The data was then subjected to QC analysis and normalization using Illumina's Genome Studio Suite v1.0.

Microarray and Gene Network Analysis

Assessment of differential gene expression and statistical analysis was performed in Partek Genomics Suite 6.6. Unless otherwise stated 2 way ANNOVA analysis (comparing donor identity and cell phenotype) was undertaken to detect differential expression and the resultant gene lists were obtained by filtering results by FDR < 0.05 and $p < 0.05$ with fold changes >1.5. The PANTHER classification system (15) was used for the gene ontology (GO) analysis of the up-regulated and down-regulated genes. Unsupervised hierarchal clustering was undertaken by K-means clustering of standardized gene intensity values, normalized so that the mean is 0 and the standard deviation is 1 (z-score). Finally, gene regulatory networks were investigated using Ingenuity Pathway analysis (IPA) (Qiagen Bioinformatics) to identify known downstream targets of transcription factors (based on Ingenuity knowledge database of mammalian interactions) or using Weighted Gene Co-expression Network Analysis (WGCNA) analysis (16) to identify modules of highly correlated genes. We related these modules to external sample traits using the eigengene network methodology (17).

The array data has been deposited in NCBI's Gene Expression Omnibus (18) and are accessible through GEO Series accession number GSE99948.

RT-PCR

RT-PCR was performed using TaqMan MGB gene expression assays and TaqMan Universal PCR Master Mix on a Viia7 real-time PCR machine (Applied Biosystems). Gene expression was normalized to an endogenous reference gene 18s rRNA (Hs99999901_s1, Applied Biosystems). Off-the-shelf gene specific qPCR assays were purchased from applied biosystems utilizing Taqman MGB chemistry. All gene specific assays were multiplexed with the 18s endogenous control assay and run in triplicate. SDS software was used to determine relative quantification of the target cDNA according to the $2^{-(\Delta\Delta ct)}$ method.

FACS Analysis

To validate some of the differentially expressed genes we fixed, permeabilized, and stained cells as previously described (7). The antibodies used were as follows; anti-IL4R APC (R&D), anti-CD27 FITC (Biolegend), anti-CD38 PE-CY7 (Biolegend), anti-CD20 FITC (Biolegend), anti-IRF4 alexa 647 (Invitrogen), anti-IRF8 APC (Biolegend), anti-BLIMP1 APC (R&D), and anti-active Caspase 3 alexa 647 (BD Biosciences). To determine the rates of apoptosis the IL-4 and anti-CD40 cultured cells were harvested and the dead cells removed using the Easysep dead cell removal kit (Stemcell). The cells were then recultured for 24 h with IL-4 and anti-CD40, followed by staining for Annexin V (eBioscience) and live/dead fixable violet dead stain kit (Life Technologies). Data was collected on a BD FACSCanto (BD Biosciences) and events were analyzed using FlowJo software version 10.4.2 (Tree Star).

RESULTS

Transcriptional Profile of GC and PC Associated Genes Along the Differentiation Pathway of IgE$^+$ and IgG1$^+$ Cells

In order to determine the transcriptional profile of IgE$^+$ and IgG1$^+$ PCs, and their prescursors, after 10 days of culture with IL-4 and anti-CD40, tonsil B cells were sorted by flow cytometry into IgE$^+$ and IgG1$^+$ GC-like B cells, PC-like PBs and PCs (**Figure 1A**). Total RNA from the purified cells was isolated reverse transcribed, amplified and biotin labeled prior to transcriptional profiling using the Illumina HumanHT-12 v4 Expression BeadChip array.

To confirm and extend our phenotypic characterization of the IgE$^+$ and IgG1$^+$ PCs, and their prescursors, we compared the transcriptional profile of known regulators and markers of B cell differentiation into PCs (19–25) (**Figure 1B**). Genes previously associated with GC reactions were highly expressed in both IgE$^+$ and IgG1$^+$ GC B cells compared to IgE$^+$ and IgG1$^+$ PBs and PCs (e.g., *IL-4R* >3-fold, *STAT6* >2-fold, *AICDA* >4-fold, *BCL6* >3-fold). In contrast, genes associated with PC differentiation and functions were highly expressed in both PBs and PCs compared to IgE$^+$ and IgG1$^+$ GC B cells (e.g., *IRF4* >3.5-fold, *PRDM1* >4-fold, *XBP1* >4-fold). The differential expression of some of the genes was also confirmed at the protein level by flow cytometry (**Figure 1C**). Overall, the data shows that our previously characterized cell populations displayed a uniform profile with respect to these GC- and PC-associated markers, consistent with the designated phenotype of the populations.

Distinct Gene Expression Patterns at Different Stages of B Cell Differentiation Into PCs

To determine the gene expression changes during the differentiation of GC B cells into PCs, irrespective of Ig isotype, we performed a 2 way ANOVA, based on donor identity and cell phenotype, yielding 726 annotated genes that were differentially expressed by >1.5-fold ($P < 0.05$, and FDR < 0.05) between any of the cell types. To identify genes with distinct expression profiles across the three cell types we generated self-organizing maps (SOMs) and identified 6 different patterns of gene expression associated with either negative or positive regulation as cells differentiated into PCs (**Figure 2A** and **Supplementary Table 1**).

GO analysis of the clustered genes revealed that cluster 1, identifying genes which peaked at the PB stage, contained genes that were associated with *type I interferon signaling pathway* (GO:0060337, fold enrichment = 27.74), such as *IRF4*, required for PC differentiation (11), and *IRE1-mediated unfolded protein responses* (GO:0036498, fold enrichment = 21.85), which activates *XBP1* (26). Cluster 2 genes, which peaked at the PC stage, are involved in *co-translational protein*

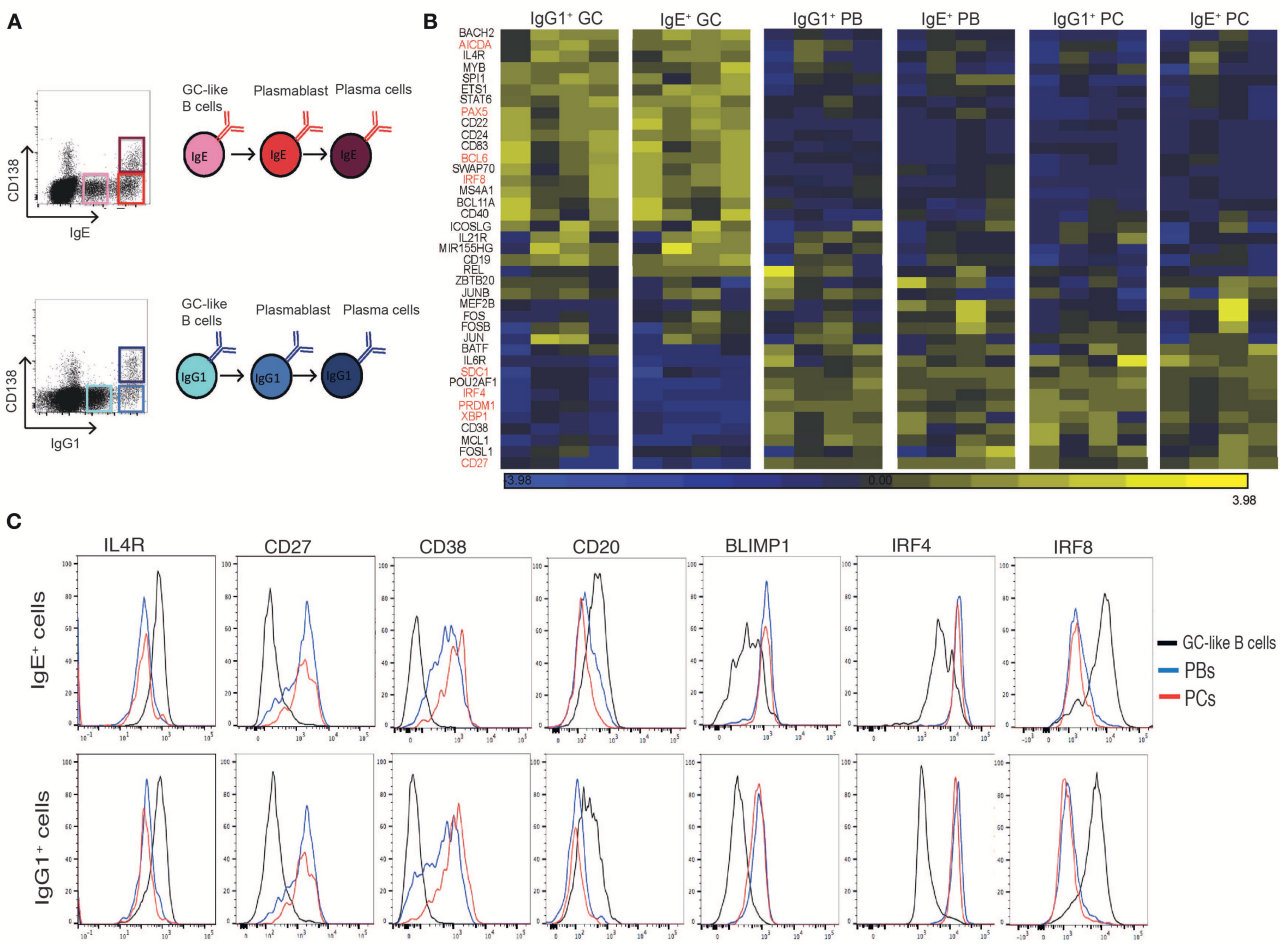

FIGURE 1 | Expression profile of GC and PC associated genes in sorted IgE$^+$ and IgG1$^+$ cell populations. **(A)** IL-4 and anti-CD40 stimulated tonsil B cells were harvested on day 10 of the culture and surface stained for CD138, intracellular IgE and IgG1 and FACS sorted into GC B cells (IgE^{lo} CD138$^-$ and IgG1^{lo} CD138$^-$), PBs (IgE^{hi} CD138$^-$ and IgG1^{hi} CD138$^-$), and PCs (IgE^{hi} CD138$^+$ and IgG1^{hi} CD138$^+$). **(B)** Heatmap of GC and PC associated genes in each of the sorted IgE$^+$ and IgG1$^+$ cell populations. Each column represents the gene expression profiles of the different phenotypic cell populations sorted from four different tonsil B cell cultures. **(C)** Flow cytometric validation of seven differentially expressed genes in IgE$^+$ and IgG1$^+$ cell populations. Data are representative of 6 experiments.

targeting to membranes (GO:0006613, fold enrichment = 13.75), *endoplasmic reticulum to cytosol transport* (GO:1903513, fold enrichment = 59.58), and *endoplasmic reticulum unfolded protein responses* (GO:0030968, fold enrichment = 25.53). Examples include *PRDM1*, the well-known regulator of PC differentiation (27), and *XBP1*, which plays a key role in protein folding, secretion and degradation (28). Expression of genes within cluster 3 also peaked at the PC stage. These genes were involved mainly in *protein N-linked glycosylation via asparagine* (GO:0018279, fold enrichment = 26.99) and *ER-associated ubiquitin-dependent protein catabolic process* (GO:0030433, fold enrichment = 15.64).

In contrast to clusters 1–3, genes within clusters 4–6 were down-regulated as B cells differentiated into PCs. Consistent with the phenotype of cells, these clusters contained genes previously shown to play an important role in establishing, maintaining or mediating GC reactions (19, 20, 24), including *IL4R* (cluster 4), *AICDA*, *FAS*, *IRF8* (cluster 5), *BCL6,* and *CIITA* (cluster 6). The main biological processes enriched within cluster 4 are the *cellular response to cytokine* (GO:0034097, fold enrichment = 4.59) and the *regulation of immune responses* (GO:0050776, fold enrichment = 4.02). Genes within cluster 5, primarily restricted to GC cells, were associated with various aspects of cell division, including *DNA unwinding involved in DNA replication* (GO:0006268, fold enrichment = 88.3), *cell cycle phase transition* (GO:0044770, fold enrichment = 7.78) and *DNA replication* (GO:0006260, fold enrichment = 11.88). Genes within cluster 6, repressed particularly in PB cells, are associated with *mitotic cell cycle phase transition* (GO:0044772, fold enrichment = 6.34) and *lymphocyte activation* (GO:0046649, fold enrichment = 4.93).

Since these clusters contain genes with highly correlated expression profiles, we also investigated whether they were known to be regulated by common transcription factors. GO analysis of transcription factor binding sites (TFBS) revealed that all 6 clusters were enriched for certain transcription

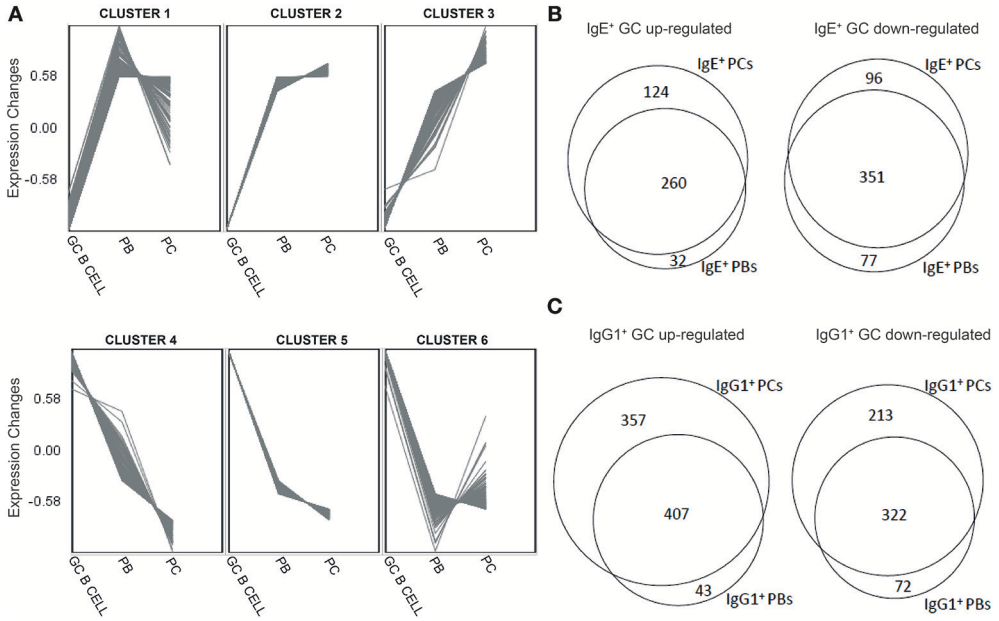

FIGURE 2 | Distinct gene expression patterns and identification of genes unique different stages of B cell differentiation into PCs. **(A)** Clustering of genes differentially expressed along the differentiation pathway of B cells into PCs regardless of the Ig isoform was undertaken by the production of unsupervised Self-organizing Maps (SOM). **(B)** Venn diagrams showing overlaps and differences between genes that were significantly ($p < 0.05$) up-regulated or down-regulated by >1.5-fold in IgE^+ cells along their differentiation pathway into PCs. **(C)** Venn diagrams showing overlaps and differences between genes that were significantly ($p < 0.05$) up-regulated or down-regulated by >1.5-fold in $IgG1^+$ cells along their differentiation pathway into PCs.

factor binding sites (TFBS) (**Table 1**), either specifically enriched in certain clusters (e.g., ETS2 and NFAT in cluster 1; PAX4 in cluster 3; NFY and FOXO4 in cluster 4; E12, PU1, and E2F in cluster 6) or in more than one cluster (e.g., SP1 and LEF1).

Next, to highlight the transcriptional changes during the PC differentiation of IgE^+ and $IgG1^+$ cells, we constructed a series of Venn analysis diagrams using genes differentially expressed (>1.5-fold change with a $P < 0.05$, FDR < 0.05) along their differentiation pathway into PCs (**Figures 2B,C**). The comparison showed that both IgE^+ PBs and IgE^+ PCs shared a core of differentially up-regulated (351) and down-regulated (260) genes compared to IgE^+ GC B cells, but also genes that distinguished IgE^+ PCs (96 up-regulated and 124 down-regulated) from PBs (77 up-regulated and 32 down-regulated) (**Figure 2B** and **Supplementary Table 2**). By comparison, while $IgG1^+$ PBs and $IgG1^+$ PCs also shared a core of differentially up-regulated (322) and down-regulated (407) genes compared to $IgG1^+$ GC B cells, the number of differentially expressed genes unique to $IgG1^+$ PCs (213 up-regulated and 357 down-regulated) more than doubled in comparison to that of IgE^+ PCs whereas those of $IgG1^+$ PBs were almost unchanged (72 up-regulated and 43 down-regulated) (**Figure 2C** and **Supplementary Table 2**). The GO analysis of these genes show that the main biological processes enriched with genes that are either up-regulated or down-regulated in IgE^+ and $IgG1^+$ GC B cells, compared to their more differentiated cell populations, are consistent with their phenotype (**Supplementary Table 2**).

The Transcriptional Profiles of IgE^+ and $IgG1^+$ Cells Diverge as PC Differentiation Proceeds

We have previously shown that IgE^+ and $IgG1^+$ cells display different biological properties with regards to their differentiation potential (7). Upon examining the expression levels of IRF4, which has been reported to be involved in the PC differentiation of mouse IgE^+ GC B cells (10), we observed a significantly higher expression of this transcription factor in IgE^+ cells at the GC stage compared to their $IgG1^+$ cell counterparts (**Figure 3A**). To better understand the molecular pathways underlying these biological differences we carried out a 2-way ANOVA analysis comparing the genes unique to each IgE^+ and $IgG1^+$ cell differentiation stage. As illustrated by the Venn analysis diagrams, IgE^+ GC B cells share a similar pattern of gene expression with the $IgG1^+$ GC B cells (1,532 similarly expressed genes), with only 7 up-regulated and 25 down-regulated genes in IgE^+ GC B cells (**Figure 3B** and **Supplementary Table 3**). At the PB stage of differentiation, IgE^+ cells had 940 unchanged, 26 down-regulated, and 35 up-regulated genes compared to $IgG1^+$ cells. However, at the PC stage, IgE^+ and IgG^+ cells diverge in their transcriptional profiles and display a more distinctly different profile with 1125 unchanged, 164 down-regulated and 255 upregulated genes in IgE^+ PCs compared to $IgG1^+$ PCs (**Figure 3B** and **Supplementary Table 3**).

To emphasize these diverging transcriptional profiles we subjected genes, the expression of which differed by >1.5-fold across any cell type, to hierarchal clustering (**Figure 3C**).

TABLE 1 | Summary of temporal clusters.

Cluster	Notable genes	Top GO biological process (fold enrichment >10, $p <$ E-05)	TFBS >10 genes
1	MCL1, IRF4	Type I interferon signaling pathway, endoplasmic reticulum unfolded protein response, cellular response to unfolded protein	ETS2 (11 genes $p < 0.0012$), AP4 (12 genes $p < 0.0019$), SP1 (17 genes $p < 0.0036$), NFAT (12 genes $p < 0.0088$)
2	CD27, PRDM2, IRF1, XBP1	Protein exit from endoplasmic reticulum	SP1 (14 genes $p < 0.0026$), LEF1 (13 genes $p < 0.0039$)
3	CD38, CD79A	Protein N-linked glycosylation via asparagine	SP1 (25 genes $p <$ 2.3e-5), LEF1 (21 genes $p < 0.00026$), MYC (11 genes $p < 0.0006$), PAX4 (11 genes $p < 0.0038$)
4	BCL11A, CD19, IL4R	*NS*	MAZ (17 genes $p < 0.0001$), NFY (12 genes $p < 0.00012$), AP4 (12 genes $p < 0.0005$), FOXO4 (13 genes $p < 0.0011$), SP1 (15 genes $p < 0.0027$)
5	AICDA, CCL17, CCL22, FAS, IRF8, MYB	DNA replication	SP1 (17 genes $p < 0.00026$), MAZ (11 genes $p < 0.0118$), LEF1 (12 genes $p < 0.0152$) E12 (11 genes $p < 0.0153$)
6	BATF3, BCL6, CD79B, CD83, SPIB	Mitosis	E2F (11 genes $p <$ 7.3e-9), SP1 (33 genes $p <$ 7.2e-9), ETS (19 genes $p <$ 2.2e07), LEF1 (28 genes $p <$ 1.4e-6), E12 (26 genes $p <$ 1.5e-6), MYC (15 genes $p <$ 1.3e-5), PU1 (12 genes $p <$ 1.2e-5)

The top GO biological processes with the lowest P-value and a fold enrichment threshold of >10 are shown. TFBS significant at 5% threshold and known to regulate >10 genes are shown. For a list of genes related to each of the clusters see ***Supplementary Table 1****. (ns, Not significant).*

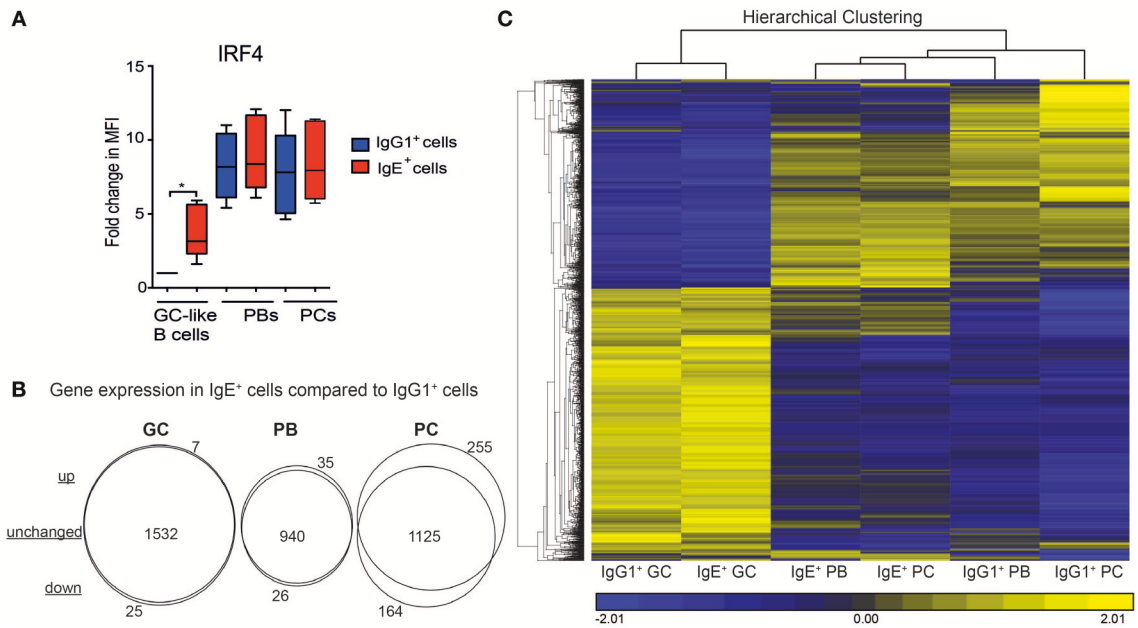

FIGURE 3 | The relationship between the IgE^+ and $IgG1^+$ cells along their differentiation pathway. **(A)** Expression levels of IRF4 in IgE^+ and $IgG1^+$ cells as determine by flow cytometry. Data show the fold change in median fluorescence intensity (MFI) of anti-IRF4 stained cells relative to $IgG1^+$ GC-like B cells ($n = 6$). Statistical analysis was performed using the One-Way ANOVA, Dunnett's test (*$P < 0.05$). **(B)** Visualization of gene expression differences between IgE^+ and $IgG1^+$ cells along their PC differentiation pathway. Genes differentially expressed (>1.5-fold, $p <$ 0.05) at each IgE^+ and $IgG1^+$ cell differentiation stage underwent a 2-way ANNOVA analysis. The number of genes that were significantly ($p < 0.05$) up-regulated or down-regulated by >1.5-fold in IgE^+ cells compared to $IgG1^+$ cells at GC, PB, and PC are highlighted by the Venn diagrams. **(C)**, Unsupervised K-means hierarchical clustering of all genes differentially expressed in IgE^+ and $IgG1^+$ cells along their differentiation pathway. Each column represents the mean gene expression profile from all four donors of the specified phenotypic group.

Clustering confirmed that IgE^+ and IgG^+ GC cells were most similar. However, while $IgG1^+$ PCs have a very distinct transcriptional profile, IgE^+ PCs are more closely related to IgE^+ and $IgG1^+$ PBs. This observation was especially surprising, considering that we and others have previously shown that IgE^+ cells are more prone to differentiation than $IgG1^+$ cells (4, 7, 9).

To explore the origins of IgE^+ and $IgG1^+$ cell differences, we undertook a gene regulatory network (GRN) analysis using the curated knowledge database in IPA, as well as a data-driven approach using WGCNA (16). IPA analysis on the differentially expressed genes between IgE^+ and $IgG1^+$ GC-like B cells identified a gene interaction network associated

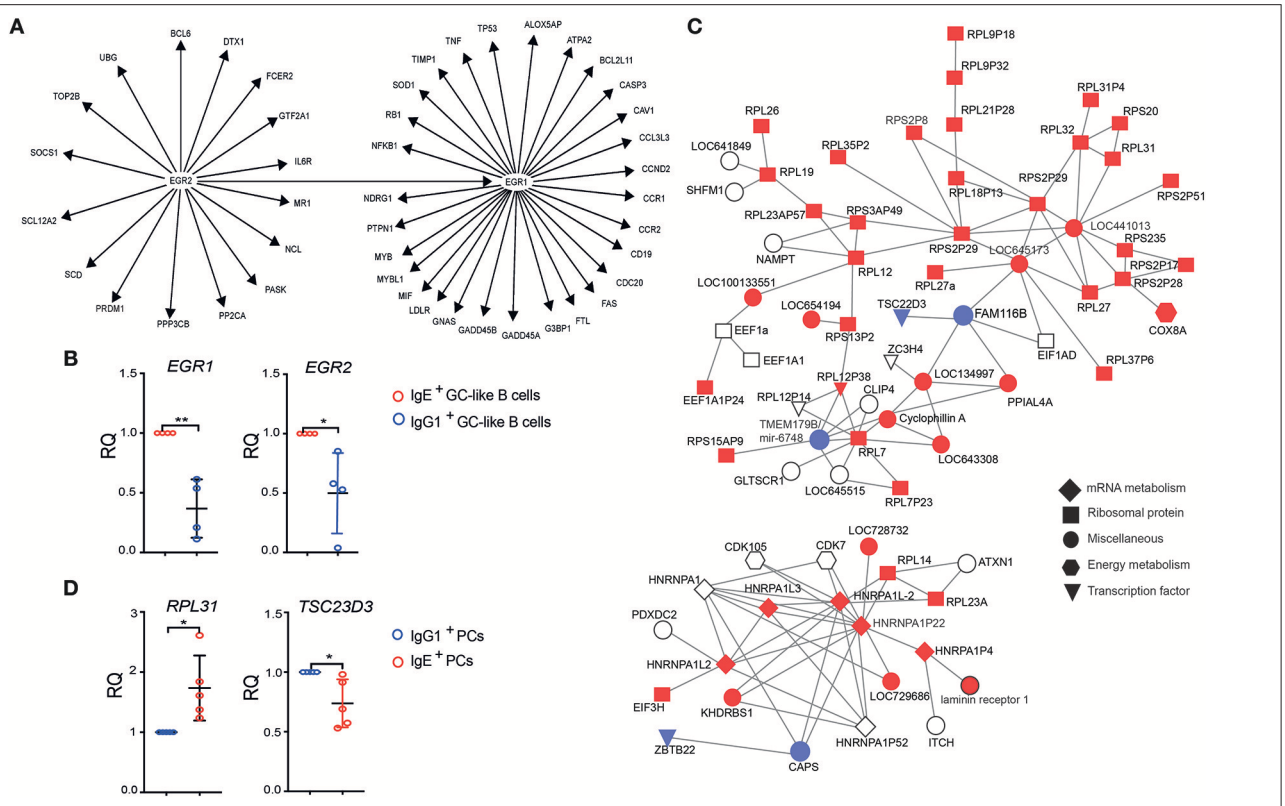

FIGURE 4 | Identification of gene interaction and co-expression networks associated with IgE$^+$ PC differentiation. **(A)** IPA was performed on genes that were differentially expressed between IgE$^+$ and IgG1$^+$ cells (>1.5-fold and $P < 0.05$). The gene network was identified based on the literature contained in the IPA knowledge database. Target genes of the EGR1 and EGR2, shown in the figure, were found to be differentially expressed by more than 1.5-fold ($P < 0.05$) either in IgE$^+$ and IgG1$^+$ GC-like B cells compared to PBs or PCs or in IgE$^+$ cells compared to IgG1$^+$ cells along their PC differentiation pathway. **(B)** RT-PCR validation of *EGR1* and *EGR2* expression in IgE$^+$ and IgG1$^+$ GC-like B cells. Data represent the mean +/– SD of the relative quantification (RQ). Statistical analysis was performed using the *t*-test with Welch's correction (*$P < 0.05$, **$P < 0.01$). **(C)** Identification of a module of highly correlated genes, by WGCNA analysis encoding a large number of ribosomal proteins, that is enriched in IgE$^+$ PCs. In total this network contains 547 genes, however, to improve network visibility only those with a weight above 0.075 are shown. This *de-novo* co-expression network was negatively correlated with the IgG1$^+$ PCs (correlation coefficient −0.65, $p = 0.003$). The genes up-regulated (red) or down-regulated (blue) by more than 1.3-fold in IgE$^+$ PCs compared to IgG1$^+$ PCs, whereas genes with <1.3-fold difference are shown as uncolored. The shape of each node reflects the biological function of each gene, as determined by GO analysis. More detailed information about the top candidate genes displayed in the network can be found in the **Supplementary Table 4**. **(D)** RT-PCR validation of RPL31, which is up-regulated, and TSC23D3 that is down-regulated in IgE$^+$ and IgG1$^+$ PCs. Data represent the mean +/– SD of the relative quantification (RQ). Statistical analysis was performed using the unpaired *t*-test with Welch's correction (*$P < 0.05$, **$P < 0.01$).

with the inducible zinc finger transcription factors, *EGR1* and *EGR2* (**Figure 4A**). The RT-PCR analysis confirmed the up-regulated *EGR1* and *EGR2* expression in IgE$^+$ GC-like B cells (**Figure 4B**). These transcription factors are known regulators of a number of genes and include those that are down-regulated (*CASP3*, *MYB*, *LDLR*, *GNAS*, *FTL*, *CCR2*, *CCND2*, and *NDRG1*) or up-regulated (*CAV1*, *FAS*, *CD19*, *G3BP1*, *LOX5AP*, *NFKB1*, *MYBL1*, *TNF*, *TP53*, *SOD1*) in IgE$^+$ and IgG1$^+$ GC B cells compared to their more differentiated cell counterparts. This network also contained genes upregulated (*NCL*, *FCER2*, *CDC20*, *CCL3L3*, and *CCR1*) or down-regulated (*PTPN1*, *GADD45A*, *GADD45B*, *TIMP1*, *NDRG1*, and *RB1*) in IgE$^+$ PCs compared to IgG1$^+$ PCs (**Figure 4A**).

In addition, WGCNA identified a co-expression network, which is enriched in IgE$^+$ PCs ($p = 0.003$), containing a large number of ribosomal components and the differentially expressed transcriptional regulator *TCS22D3* and guanine exchange factor (*FAM116B*) (**Figures 4C,D** and **Supplementary Table 4**).

Overall these data suggest that the IgE$^+$ and IgG1$^+$ cells adopt an increasingly different gene expression profile as they differentiate into PCs. The data also provide molecular signatures that may account for some of the differences seen in the later stages of IgE$^+$ and IgG1$^+$ cell differentiation.

Proliferative and Apoptotic Associated Genes Differentially Expressed in IgE$^+$ and IgG1$^+$ Cells

According to the GO analysis, among the most enriched biological processes associated with genes over-expressed in IgE$^+$ PCs, compared to IgG1$^+$ PCs, were *translation*

initiation (GO:0006413, fold enrichment = 12.74), *mitotic cell cycle phase transition* (GO:0044772, fold enrichment = 6.09) and *mitotic cellular division* (GO:0007067, fold enrichment = 4.67), suggesting that IgE^+ PCs are still cycling (**Supplementary Table 3**). These observations are consistent with our previously reported data (7), which show that the proliferative and cycling capacity of IgE^+ PB and PCs is greater than that of their $IgG1^+$ cell counterparts.

There are several differentially expressed genes that correlate with the enhanced proliferation of IgE^+ cells relative to their $IgG1^+$ cell counterparts (**Figure 5A**). Among these genes, *RB1*, an important regulator of the G1 checkpoint (29), and G*ADD45A*, a regulator of the G2-M checkpoint (30, 31), are upregulated in $IgG1^+$ PBs and PCs, but not in IgE^+ PBs and PCs, when compared to GC B cells (**Figure 5A**). Other negative regulators of the cell cycle progression up-regulated in $IgG1^+$ PBs and PCs include *CDKN2B*, *HUS1,* and *E4F1*. Conversely, we observe that IgE^+ PBs and PCs, unlike their $IgG1^+$ cell counterparts, up-regulate the expression of a number of genes associated with positive regulation of the cell cycle e.g., *CDC25B*, *MYC*, *CSK1B*, *FOXM1*, *CDCA3*, *AURKB*, *PLK4*, *CDC20*, *E2F2* (**Figure 5A**).

Contrary to recent reports suggesting that IgE^+ GC B cells undergo increased apoptosis compared to $IgG1^+$ GC B cells (5, 6), the expression of apoptosis-associated genes in $IgG1^+$ and IgE^+ GC B cells is similar (**Figure 5B**). The exceptions are the pro-apoptotic regulators *BNIP3*, *BNIP3L,* and *HKR*, which are increased in $IgG1^+$ GC cells, and *DAPK2*, increased in IgE^+ GC cells. However, Annexin V and dead cell staining of the cells after 24 h of culture, reveals that IgE^+ and $IgG1^+$ GC B cells have similar rates of apoptosis (**Figure 6A**). This is also supported by their similar levels of activated caspase-3 at day 10 of the culture with IL-4 and anti-CD40 (**Figure 6B**), suggesting that unlike in the mouse system these cells undergo apoptosis at a similar rate. In contrast, despite increased levels of *TNFRSF13B* (TACI) and *TNFRSF17* (BCMA)*, two* important contributors of PC survival (32, 33), in IgE^+ PBs and PCs (**Supplementary Table 3**), their rates of apoptosis and their expression levels of active caspase-3 are increased compared to their $IgG1^+$ cell counterparts (**Figures 6A,B**). We find that the expression of a number of apoptosis-associated genes was either up-regulated (e.g., *BNIP2*, *CASP*3, *FADD,* and MAP3K5) or down-regulated (e.g., *DAPK2*, *BNIP3*, *BNIP3L*, *BCL2L1,* and *CASP1*) in both IgE^+ PBs and PCs compared to their $IgG1^+$ cell counterparts (**Figure 5B**). In addition, *BAG1*, *TP53INP1,* and *TP73* were down-regulated and *BCL2L11*, *CASP10,* and *TNFRSF25* were up-regulated only in IgE^+ PCs (**Figure 5B**). The differential expression of *BCL2L1* and *BCL2L11,* which encode two well-characterized regulators of apoptosis, Bcl-xL, and Bim, respectively, in IgE^+ and $IgG1^+$ PCs was also confirmed by RT-PCR (**Figure 6C**).

Overall the data suggest that the apoptotic potential of IgE^+ cells increases as they differentiate into PCs and that IgE^+ PCs may be inhibited from exiting the cell cycle, a process that is required for the completion of the PC differentiation program (21, 22, 34).

DISCUSSION

A notable feature of IgE^+ B cell development is the predisposition of IgE^+ GC B cells to differentiate into PCs (6, 7, 9). In this study, we sought to obtain a better understanding of the IgE^+ PC differentiation process by analyzing gene expression in human B cells at discrete stages of PC differentiation. We also compared IgE^+ and $IgG1^+$ B cells to discover isotype-specific patterns.

We identified distinct gene expression patterns at different stages of B cell differentiation into PCs and found that at each stage both IgE^+ and $IgG1^+$ cells have distinct molecular signatures with well-characterized genes of B cell function and differentiation as well as other genes of unknown function. The analysis of genes recognized as critical for either the GC reaction or PC differentiation and function confirmed the phenotype of our previously characterized IgE^+ and $IgG1^+$ cells (7).

A previous study reported that the vast majority of mouse IgE^+ GC B cells undergo apoptosis, owing to low mIgE expression and the resulting weak BCR signaling (6). Thus, the canonical B cell differentiation programme is not observed. It was proposed that IgE BCR directly promotes the apoptosis of IgE^+ B cells (5, 35). However, the evidence for this is conflicting, and our results are more consistent with another study in the mouse, which also demonstrated similar rates of apoptosis in IgE^+ and $IgG1^+$ GC B cells (10). Shedding further light on this matter, recent work has revealed that the expression of the ε heavy chain itself on GC B cells leads to PC differentiation uncoupled from antigen activity (5, 10). This antigen-independent PC differentiation mediated by the IgE BCR involved IRF4. The increased levels of IRF4 expression in our *in vitro* generated IgE^+ GC-like B cells may also account, in part, for the accelerated PC differentiation of human IgE^+ B cells. Using the curated knowledge database in IPA, we have identified two other transcriptional regulators, EGR1 and EGR2, that may contribute to this process. EGR1 has been reported to regulate PC differentiation of B cells (36) and EGR2 to be associated with T cell differentiation (37, 38). In future studies it would be interesting to determine the mechanisms by which the different expression levels of these transcription factors affect the differentiation rates of IgE^+ and $IgG1^+$ B cells.

A novel finding of our study is that as IgE^+ and $IgG1^+$ B cells differentiate into PCs their transcriptional profiles diverge, with IgE^+ and $IgG1^+$ PCs showing the greatest difference. Consistent with our previously reported results (7), we observed that a number of genes involved in the regulation of the cell cycle are differentially expressed in IgE^+ cells. For example, the protein product of *RB1*, which is repressed in IgE^+ PBs and PCs, can block the S-phase entry and growth by binding to the E2F1 transcription factors and inhibiting its activity (29). Similarly, *GADD45A*, which can arrest the cell cycle at the G2-M checkpoint by suppressing the CDC2/Cyclin B kinase activity (30, 31), is also down-regulated in IgE^+ PBs and PCs. In contrast, *CDC25B* and *MYC*, two positive regulators of the cell cycle and proliferation (39, 40), are both expressed at elevated levels in the IgE^+ cells.

In addition, using a WGCNA approach, we identified a large number of ribosomal proteins enriched in IgE^+ PCs.

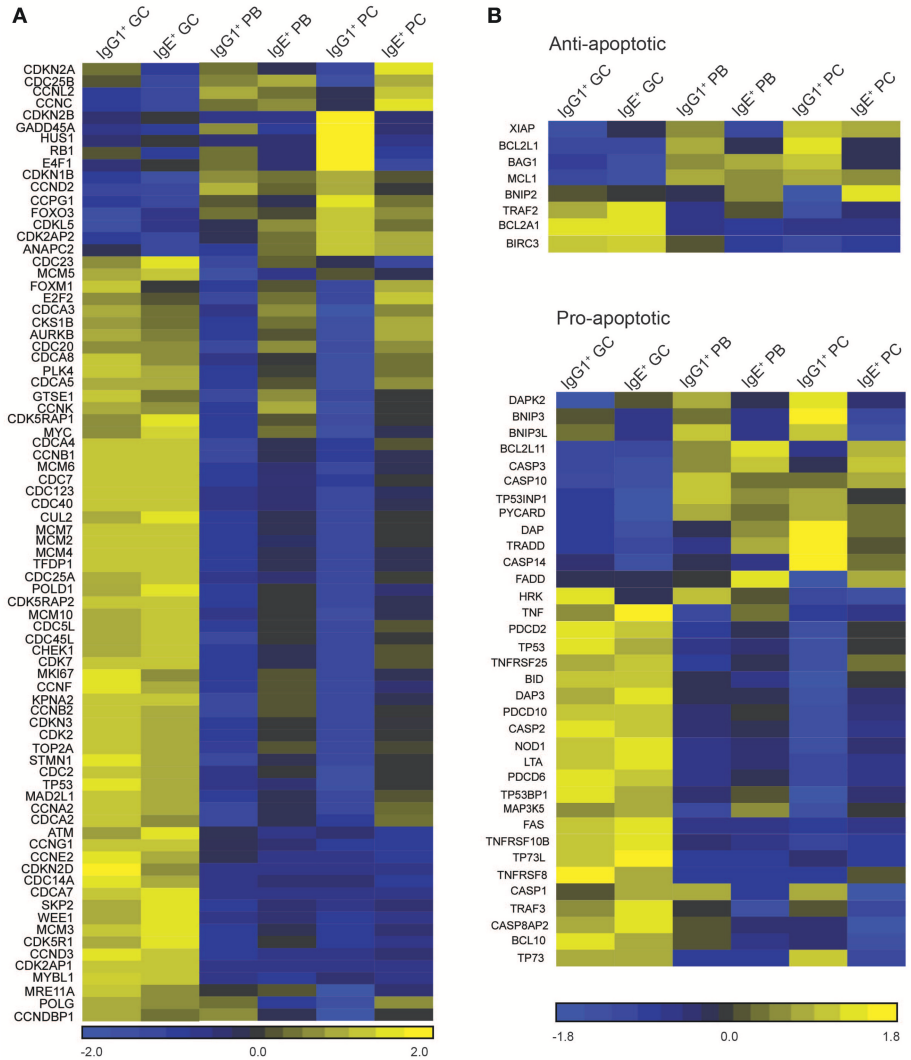

FIGURE 5 | Cell cycle/proliferation-associated genes differentially expressed in IgE$^+$ and IgG1$^+$ cells. **(A)** Heatmap of cell cycle/proliferation-associated and **(B)** pro- and anti-apoptotic genes differentially expressed along the PC differentiation pathway of both IgE$^+$ and IgG1$^+$ cells, and differentially expressed in IgE$^+$ cells compared to IgG1$^+$ cells. Each column in the heat maps shown represents the mean gene expression profile from all four donors of the specified phenotypic group.

It is known that the rate of translation is finely tuned to match cell proliferation (41, 42), and therefore increased ribosomal protein expression in the IgE$^+$ PCs, compared to IgG1$^+$ PCs, may be a consequence (or driver) of increased proliferation in these cells. Together these differences (and their downstream effects) may account for the maintenance of proliferative capacity as the IgE$^+$ B cells differentiate into PCs.

Intriguingly, the analysis of the gene expression data revealed that the transcriptional profile of IgE$^+$ PCs was more closely related to that of IgE$^+$ and IgG1$^+$ PBs than to IgG1$^+$ PCs. It might be that the failure of IgE$^+$ PCs to fully exit the cell cycle hinders their completion of the PC differentiation programme. Additionally, discrepancies between the IgE$^+$ and IgG1$^+$ PC transcriptional profiles might also be due to the up-regulation of the human mIgE$_S$ (7) on becoming PCs, which distinguishes these cells from non-IgE$^+$ PCs that down-regulate their mIg receptors as they become more dedicated to antibody secretion.

As seen in the mouse (10), the increased rates of apoptosis suggests that the IgE$^+$ PCs generated in our tonsil B cell cultures may be short-lived PCs, which could account for some of the transcriptional differences between IgE$^+$ and IgG1$^+$ PCs. Similarly, a recent study, published during the review of our manuscript, reaffirmed the immature transcriptional program and relatively poor survival capacity of differentiated IgE$^+$ cells isolated from the blood of peanut allergic patients (43). In support of this, our data show that IgE$^+$ PCs down-regulate *BCL2L1* (Bcl-xL), which prevents apoptosis during the PC differentiation by sequestring Bim (44), a pro-apotoic

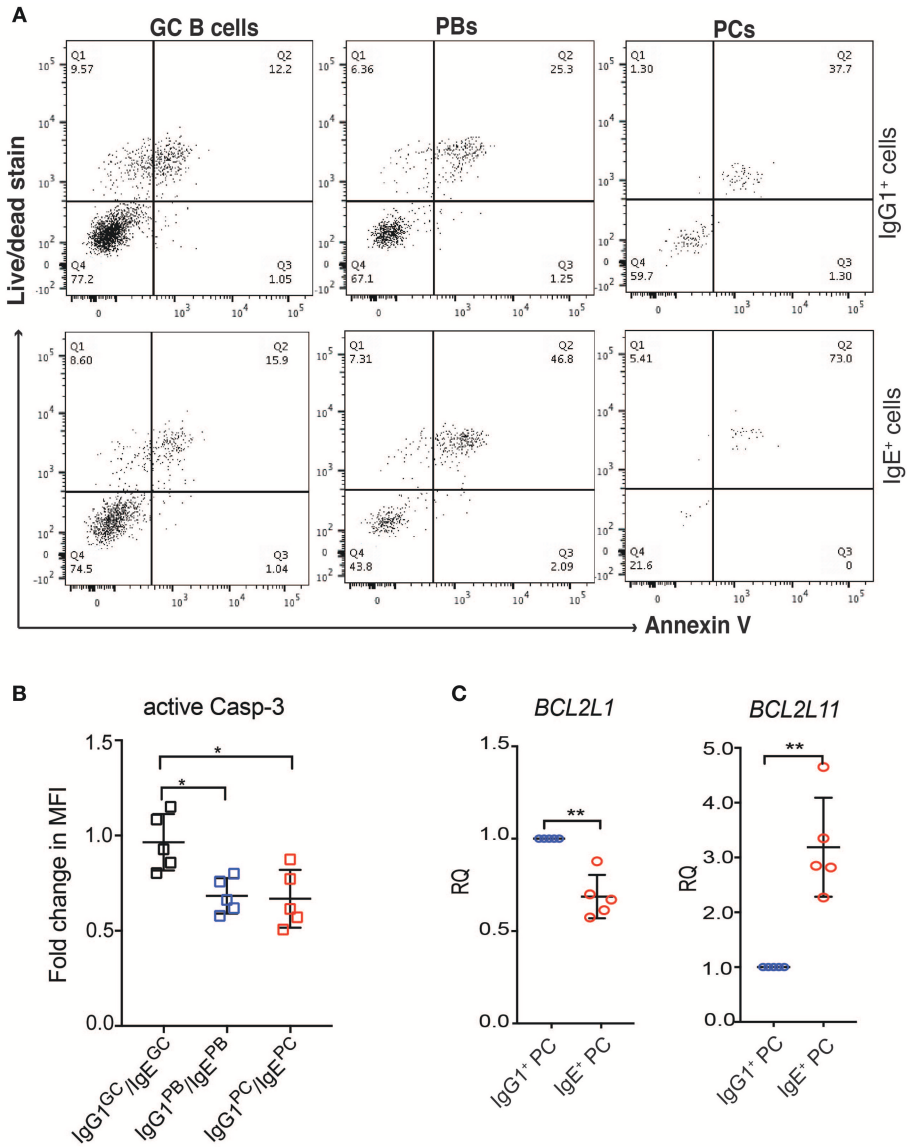

FIGURE 6 | IgE$^+$ PCs have increased rates of apoptosis compared to IgG1$^+$ PCs. **(A)** After 24 h of reculture with IL-4 and anti-CD40, the IgE$^+$ and IgG1$^+$ cells were stained with annexin V and a live/dead fixable dye. The lower left quadrant within each dot plot (negative for Annexin V and live/dead stain) corresponds to the viable cells and the data shown are representative of three different experiments. **(B)** On day 10 of the culture, the activity of Caspase 3 was determined by staining with anti-active Caspase 3 antibody. The data show the fold change in MFI of active Caspase 3 within each IgG1$^+$ cell population made relative to their respective IgE$^+$ cell counterparts. Statistical analysis was performed using the one way ANNOVA test with Bonferroni correction (*$P < 0.05$). **(C)** RT-PCR validation of *BCL2L11* and *BCL2L1* expression in IgE$^+$ and IgG1$^+$ PCs. Data represent the mean +/– SD of the relative quantification (RQ). Statistical analysis was performed using the unpaired *t*-test with Welch's correction (*$P < 0.05$, **$P < 0.01$).

protein encoded by *BCL2L11* (45, 46), which is up-regulated in IgE$^+$ PCs. Other pro-apoptotic associated genes that are up-regulated in IgE$^+$ PCs, and which could account for their higher rates of apoptosis, include *FADD* (fas associated death domain) and *MAP3K5* (47–49). However, despite their increased rates of apoptosis, IgE$^+$ PCs were expressing significantely higher levels of *TNFRSF13B* and *TNFRSF17*, which encode two very important regulators of PC survival, the transmembrane activator and CAML interactor (TACI) and the B cell maturation antigen (BCMA), respectively (32, 33, 50, 51). The differential expressions of pro- and anti-apoptotic associated genes suggests that IgE$^+$ and IgG1$^+$ PCs may have different survival requirements, possibly related to the microenvironment in which they reside (52, 53). This is highlighted by the serum IgE titres and the IgE-mediated responses after immunosuppressive treatments that do not affect the long-lived PCs (54–57), demonstrating the presence of long-lived IgE$^+$ PCs. Further work is needed to test the predicted effects

of the cell cycling and apoptosis-associated genes on IgE^+ PC differentiation and survival.

In summary, we have defined the molecular signature of the human IgE^+ and $IgG1^+$ cell differentiation into PCs. We show that the transcriptional profile of IgE^+ and $IgG1^+$ cells diverges as these cells differentiate into PCs. At the GC stage of development, we observe similar rates of apoptosis between IgE^+ and $IgG1^+$ cells. However, IgE^+ B cells have increased levels of IRF4 and EGR1 which may predispose these cells into PC differentiation. Significantly, IgE^+ PCs have an immature gene expression profile that is more related to IgE^+ and $IgG1^+$ PBs than to $IgG1^+$ PCs. They continue cycling and exhibit increased rates of apoptosis. Overall, our data furthers our understanding of the molecular events involved in the regulation of PC differentiation of IgE^+ B cells and the longevity of the generated IgE^+ PCs.

DATA AVAILABILITY

The datasets generated for this study can be found in NCBI Gene Expression Omnibus, GSE99948.

ETHICS STATEMENT

Tonsils were obtained from children undergoing routine tonsillectomies as a result of tonsillitis. Full written informed consent was given by parents or legal guardians of the donors. The study was conducted at and in accordance with the recommendations of King's College London and Guy's and St Thomas's NHS Foundation Trust and the protocol was approved by the London Bridge Research Ethics Committee (REC number 08/H0804/94).

AUTHOR CONTRIBUTIONS

FR and DF designed and performed experiments, analyzed data, and wrote the paper. HB performed experiments and analyzed data. HG designed experiments, analyzed data, and wrote the paper. All authors reviewed the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.00402/full#supplementary-material

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The Maintenance of Memory Plasma Cells

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It is now well accepted that plasma cells can become long-lived (memory) plasma cells and secrete antibodies for months, years or a lifetime. However, the mechanisms involved in this process of humoral memory, which is crucial for both protective immunity and autoimmunity, still are not fully understood. This article will address a number of open questions. For example: Is longevity of plasma cells due to their intrinsic competence, extrinsic factors, or a combination of both? Which internal signals are involved in this process? What factors provide external support? What survival factors play a part in inflammation and autoreactive disease? Internal and external factors that contribute to the maintenance of memory long-lived plasma cells will be discussed. The aim is to provide useful additional information about the maintenance of protective and autoreactive memory plasma cells that will help researchers design effective vaccines for the induction of life-long protection against infectious diseases and to efficiently target pathogenic memory plasma cells.

Keywords: plasma cells, memory plasma cells, long-lived plasma cells, maintenance, survival, bone marrow, inflammation, autoreactivity

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HISTORICAL ASPECTS OF MEMORY PLASMA CELLS

The term "plasma cell" (PC) was introduced by the anatomist Wilhelm Waldeyer in 1875, but it is doubtful whether he was actually referring to the same cells now known as antibody-secreting plasma cells. In 1895, Marshalko described oval cells with a strong basophilic cytoplasm and an eccentric nucleus containing coarse heterochromatin, which indeed corresponds to the current morphological definition of plasma cell (1). Its role as an antibody-secreting cell (ASC) was first demonstrated by Astrid Fagraeus in 1947 (2). Max Cooper and Robert Good identified lymphocytes (later termed as B lymphocytes) in the bursa of Fabricius of chickens, which is equivalent to the human bone marrow as the precursor of plasma cells (3). The phenotype and isotype of plasma cells can differ based on the type of activated B cells (naïve or memory, lymph nodes or spleen, and B1 or B2) and stimuli (T-independent or T-dependent antigens) (4). Bone marrow (BM) plasma cells are the main source of circulating antibodies (5–8). Plasma cells are specialized to secrete large amounts of antibodies (about 10^3 per second) (9, 10). For many years, the general opinion was that plasma cells are short-lived since they can only survive a few days under *in vitro* conditions Therefore, it was postulated that plasma cells are replenished via the constant activation of memory B lymphocytes (3, 11).

In 1997, Andreas Radbruch's group showed that antigen-specific plasma cells generated in ovalbumin (OVA)-immunized mice were maintained in the bone marrow for up to 120 days without proliferation (12, 13). At about the same time, Slifka et al., using an entirely different technical approach, demonstrated that plasma cells can persist in murine bone marrow for more than 1 year, even if their precursors were blocked (6). Recently, Hammarlund et al. observed the survival of antigen-specific plasma cells induced by vaccination in the bone marrow of rhesus macaques, a species with a lifespan similar to humans, for more than one decade in spite of sustained memory B cell depletion (14).

Plasma cells can be generally divided into two distinct categories based on their lifespan: (a) short-lived plasma cells/plasmablasts (proliferating cells with a life span of 3–5 days) and (b) long-lived plasma cells (non-proliferating cells with a life span of several months to lifetime). The term- antibody secreting cells (ASCs) refers to both short-lived and long-lived plasma cells. It is not fully understood whether long-lived plasma cells represent the final differentiation stage of short-lived plasma cells, or whether short- and long-lived plasma cells belong to completely separate plasma cell populations (15). While long-lived plasma cells are mainly formed during germinal center reaction secreting high-affinity class switched antibodies located in BM, short-lived plasma cells are mainly formed in extra-follicular sites of secondary lymphoid organs expressing low-affinity IgM antibodies (16, 17). The competence to become a long-lived plasma cell is distinct from the basic ability to become a plasma cell (18). It is presumed that not all plasma cells are long-lived *per se*. In our opinion, long-lived plasma cells fulfill the criteria of memory cells as they continuously secrete the antibodies independently of their precursor cells (B cells), T cell help and antigen presence. Therefore, we suggest to use the term "memory plasma cell."

We have proposed that, in order to become a memory cell, short-lived plasmablasts need a special environment: the so-called plasma cell survival niche (19, 20). This survival niche is composed of cellular components and soluble factors derived from these cells. If migratory plasmablasts reach the survival niche and receive survival factors there, they will become memory plasma cells; otherwise, they will remain short-lived and die. The bone marrow is the primary niche for memory plasma cells. Of note, plasma cells can survive for decades in the hypoxic bone marrow, and it has been shown that hypoxic condition enhances the survival of human plasma cells *in vitro* (21). Therefore, the hypoxic environment could be one of the factors that contribute to the long-term survival of memory cells. The number of plasma cell survival niches in a given organ is limited. This, in turn, limits the number of memory plasma cells per organism (22). A recently introduced mathematical model provides a possibility to quantify the niche-related dynamics of plasma cells (23). However, the long half-life of plasma cells is a new area of exploration. Most of our current knowledge about memory plasma cells is from mouse models. However, we should also consider some differences between human and mice (24). There are many questions to be answered, for example, whether the internal trigger for transformation into memory plasma cells is the intrinsic program of plasma cells, or if it is related to external signals from the plasma cell survival niche.

EXTRINSIC SURVIVAL FACTORS (SIGNALS)

Extracellular factors can be divided into two general categories: cellular compartments and molecular compartments.

Cellular Compartments

Cellular compartments of plasma cell survival niches are composed of stromal cells (key players) and originated hematopoietic cells (accessory cells).

Stromal Cells

Stromal cells are a complex network of various subpopulations, including fibroblasts, endothelial cells, fat cells, and reticular cells, almost all of which are bone marrow stromal cells of mesenchymal origin (25). They provide signals by secreting growth factors or by making direct cell-cell contacts needed for hematopoiesis (including the progression of B-lymphoid lineage cells) or for the survival of memory plasma cells (26, 27). *In vitro* studies show that co-culture of plasma cells with stromal cells significantly increases the life span of plasma cells (27). Reticular stromal cells, a minor subpopulation of stromal cells, express CXC-chemokine ligand 12 (CXCL12, a ligand of CXCR4 expressed on plasma cells) and are scattered throughout the bone marrow (28). It has been shown that high numbers of plasma cells are in contact with these CXCL12-expressing cells in CXCL12/GFP reporter mice (28). Furthermore, *in vivo* intravital microcopy studies have demonstrated that direct contacts form between plasma cells and reticular stromal cells, that reticular stromal cells form a static component of the plasma cell survival niche, and that about 80% of plasma cells directly contact reticular stromal cells in a non-random fashion (29). However, a recent study has shown that cell-cell contact is not necessary for the survival of human bone marrow plasma cells *in vitro* (21).

Fibroblasts form part of the survival niches for memory plasma cells in the bone marrow by producing IL-6 and CXCL12 (30, 31). Other evidence shows that fibroblasts from the lymph nodes (LN) and spleens of mice and humans can also promote plasma cell survival *in vitro* (27, 32). A new subset of fibroblastic reticular cells (FRCs) that form dense meshworks in the medullary cords of lymph nodes, where many plasma cells reside, has been recently identified. Medullary FRCs have also been described as major local producers of plasma cell survival factors IL-6, BAFF, CXCL12 and APRIL. "*In vitro*, medullary FRCs alone or in combination with macrophages promote plasma cell survival while other LN cell types do not have this property" (33).

Another hypothesis of how stromal cells and plasma cells communicate involves the release of extracellular vesicles from bone marrow-derived mesenchymal stromal cells (MSCs). This novel mechanism of cell-cell communication over short and long distances supports the concept of *ex vivo* survival of human antibody secreting cells (34).

Hematopoietic Niche Components

Hematopoietic niche components (HNC) such as megakaryocytes (35), basophils (36, 37), dendritic cells and monocytes/macrophages (38), myeloid progenitors (39), neutrophils (40), and eosinophils (41) act as accessory cells of the plasma cell niche. Hematopoietic cells are associated with memory plasma cells in bone marrow and support their survival mainly by secreting the survival factors APRIL (a proliferation-inducing ligand) and IL-6 (see below). Plasma cells express BCMA (B- cell maturation antigen) and IL-6R (IL-6 receptor), receptors for APRIL and IL-6, respectively. Among hematopoietic components, eosinophils are the best characterized cells as source of APRIL and IL-6 (42, 43). In accordance with the importance of eosinophils in maintaining memory plasma cells, animal studies have shown that the number of plasma cells is significantly reduced in the bone marrow and gastrointestinal tract of eosinophil-deficient ΔdblGATA1 mice (41, 43, 44). However, two more recent, independent studies suggest that eosinophils are not essential for plasma cell survival in the bone marrow (45, 46). This discrepancy might be due to the effects of different environmental factors, especially microbiota. Microbiota can play a role during early life and may thus influence the generation of plasma cells and total immunoglobulin concentrations in adult animals. A new study indicates that microbiota-specific IgA-producing gut plasma cells generated during infancy live for many decades (47). This suggests that signals from the microbiota can impact on plasma cell pools. However, eosinophils are not the only APRIL source that supports the survival of plasma cells. APRIL can be produced by a variety of other bone marrow cells (35, 48).

Considering the short life span of cells of hematopoietic origin compared to that of long-lived memory plasma cells, the maintenance of static memory plasma cells does not depend on a single cellular source of survival factors. The multicomponent plasma cell survival niche model suggests that different dynamic hematopoietic cells can compensate for the loss of a particular cell type (48). Regulatory T cells (Tregs)—the other type of cells of hematopoietic origin—also play a supportive role in the maintenance of memory plasma cells. It has been reported that the loss of T regulatory cells correlates with the reduction of memory plasma cell populations in the bone marrow. Although the mechanism for this remains unclear, the close association of Treg cells and plasma cells suggests that communication between these populations takes place through cell-cell contact or soluble factors (49). In the bone marrow Treg cells express high levels of Treg effector molecules CTLA-4, and that deletion of CTLA-4 results in elevated plasma cell numbers. These findings indicate a possible regulatory effect of CTLA-4 expression on Tregs, a population which acts on the plasma cell pool in the bone marrow (49). However, it is also known that plasma cell survival depends on constitutive signals through CD80/CD86, which is presented by CD11c cells under T regulatory cell control (49, 50).

Molecular Niche Components

Molecular niche components (MNC) include soluble factors and membrane-bound factors.

Soluble Factors

Soluble factors that contribute to plasma cell survival are cytokines and chemokines. A wide variety of cytokines, including IL-6 and members of the tumor necrosis factor (TNF) superfamily (APRIL, BAFF, and TNF-alpha), play an important role. IL-6 binds to the IL-6 receptor expressed on plasma cells, enhances their survival and maintains antibody titers *in vitro* (51). However, plasma cell survival is not significantly decreased in IL-6-null mice (51). In human, IL-6 is mandatory for *in vitro* generation and survival of memory plasma cells in combination with either APRIL or stromal cell-soluble factors (52). APRIL (a proliferation-inducing ligand) and BAFF (B-cell activating factor) are also important factors for memory plasma cell maintenance (53). Structurally, APRIL and BAFF are very similar cytokines that belong to the TNF superfamily. Both can bind with high affinity to B-cell maturation antigen (BCMA) and to transmembrane activator and calcium-modulator and cyclophilin ligand interactor (TACI), which is expressed by B cells at various stages of maturation; however, only BAFF can react with the BAFF receptor (BAFF-R) (54). Plasma cells express BCMA and TACI, but only low levels of BAFF-R (55). APRIL, which competes with BAFF for receptor binding sites, is expressed by eosinophils (41), megakaryocytes (35) and myeloid-derived cells, including monocytes, macrophages, and dendritic cells (41, 54). APRIL and BAFF bind to BCMA (their shared receptor) and promote plasma cell survival by inducing the anti-apoptotic molecule Mcl-1 of the Bcl-2 family (see below) (56). Neutralization of BAFF and APRIL with TACI-Ig depletes plasma cells in the bone marrow, whereas the presence of either BAFF or APRIL alone is sufficient to sustain the plasma cell population (53, 57). Unlike IL-6 deficiency, BCMA deficiency has a great impact on the loss of memory plasma cells (57). TNF-alpha has also been shown to support the survival of human plasma cells *in vitro* (30, 51). Taken together, these data suggest that the TNF superfamily and IL-6 are essential for the long-term maintenance of plasma cells in the bone marrow.

Chemokines and their receptors are crucial for the control of lymphocyte trafficking. CXCL12 (also known as SDF1) and its receptor CXCR4 are important for the migration of plasmablasts to the bone marrow for final differentiation into plasma cells, and for the maintenance of effective humoral immunity (17, 58). CXCL-12 has two main effects on plasmablasts and memory plasma cells. First, it acts as a chemokine and guides the plasmablasts from secondary organs to the bone marrow (59) and, second, it acts as a survival factor for plasma cells (as was shown *in vitro*) (51, 60). CXCL12-expressing stromal cells guide plasmablasts (expressing CXCR4) toward unique environments rich in anti-apoptotic survival factors in the bone marrow for their survival (61). In humans, the migration of plasmablasts requires glucose oxidation, which is controlled by CXCL12/CXCR4-mediated activation of the protein kinase AKT (62). CXCL12 itself also promotes plasma cell survival in murine bone marrow *in vitro* and *in vivo* (17, 27). Hence, the chemokine CXCL12 promotes the entry of CXCR4-expressing plasma cells into the bone marrow and the long-term survival of plasma cells (42).

Membrane Bound Factors: Memory Plasma Cell Surface Markers and Adhesion Molecules

Memory Plasma Cell Surface Markers

The phenotype of plasma cells might provide useful information about how external stimuli trigger intrinsic signals and play a role in the maintenance of memory plasma cells. The expression of CD138, TACI, BCMA, Sca-1, Ly6C, Ly6K, CD28, SLAMF7, and CD98 is a hallmark of mouse plasma cells. The human genome lacks direct homologs of murine Ly6A, Ly6C1/2, Ly6K (63). In mice, short-lived plasma cells can be distinguished from long-lived memory plasma cells by high expression of B220 (a relatively B-cell-specific isoform of CD45) and MHCII (63, 64). Human plasma cells are characterized by the co-expression of CD138 and CD38, which allows for the identification of plasma cells in the bone marrow or in cell suspensions from tissues by flow cytometry. These terminally differentiated B cells lose the ability to express CD19 and CD20 (B cell marker) on the cell surface while retaining cell surface expression of CD27. However, there is a diverse range of phenotypic markers of plasma cells (65).

CD138 (syndecan-1, Sdc-1) is a member of the syndecan family of four structurally related cell surface heparan sulfate proteoglycans (HPSGs) (66). Among non-hematopoietic cells, CD138 expression is high on epithelial cells and lower on a variety of other cell types, including endothelial cells and fibroblasts (67). Plasma cells at higher level (68) and pre-B cells at lower level (69) are the only hematopoietic cells that express CD138. High expression of CD138 on plasma cells is a hallmark of their identification, which is upregulated during differentiation from plasmablasts into plasma cells (69). CD138 is involved in many cellular functions, including cell-cell adhesion and cell-matrix adhesion (70). *In-vitro* plasma cells adhere to type I collagen of the bone marrow stromal matrix via CD138 (synecan-1) (71). Some investigators doubt that CD138 is important for plasma cell function under normal conditions (72), but recent evidence shows that CD138 plays a direct cell-intrinsic role in plasma cell survival *in vivo* (73). These authors suggest that CD138 plays a major role in protecting plasma cells from premature apoptosis by using its heparin side chains to substantially increase IL-6 and APRIL presentation to their receptors on plasma cells, leading to increased cytokine signaling and higher expression of the pro-survival proteins Bcl-2 and Mcl-1. Surface expression of CD138 on plasma cells does not impair its early differentiation or proliferation, but rather promotes or correlates with the survival of mature plasma cells.

CD38 is a type II transmembrane glycoprotein. Its extracellular domain acts as an enzyme that converts nicotinamide adenine dinucleotide (NAD^+)/$NADP^+$ into cADPR, ADP-ribose and NAADP, all of which are intracellular calcium-mobilizing agents (74). CD38 is expressed on most thymocytes, some activated peripheral blood T cells and B cells, plasma cells, and dendritic cells. Memory plasma cells express high levels of CD38 compared to their precursors (75, 76). Apparently, this CD38 molecule is distinct from CD38 on other cells because a lamprey monoclonal antibody that recognizes a unique epitope of the CD38 ectoenzyme specifically reacts with plasmablasts and plasma cells in healthy individuals and in most human multiple myelomas (77). CD38 molecules on the plasma membrane are in close contact with the BCR complex and with molecules regulating homing (CXCR4 and CD49d) (78).

CD19, a co-receptor of the BCR complex, is one of the earliest and most specific markers of B-lineage cells (79). Plasma cells in human bone marrow express CD19 in a heterogenic manner. The majority of plasma cells express CD19, but a minor group of plasma cells is $CD19^{neg}$. There is now increasing evidence that memory plasma cells among the $CD19^{neg}$ plasma cell population are enriched in human bone marrow (80–82). A recent study using a new staining protocol of plasma cells in mice, could also detect IgG-secreting cells with $CD19^{low}$ $B220^{low}$ $CD138^{high}$ Blimp-1^{high} in bone marrow which are most likely memory plasma cells (76). Lack of CD19 expression may be considered as a candidate marker for memory plasma cells maintaining long-term memory, but its mechanism is unknown (83). Compared to $CD19^{pos}$ plasma cells, $CD19^{neg}$ bone marrow plasma cells have a prosurvival mature phenotype: low expression of CD95 and high expression of Bcl2 and less proliferating Ki67 cells. This is a sign of long-term stability of this subset in human bone marrow (84, 85). It has been recently demonstrated that $CD19^{neg}$ $CD45^{neg}$ plasma cells persist for at least two decades in the human small intestine (82). This study also has shown that $CD19^{neg}$ plasma cells isolated from the small intestine of elderly subjects contain rotavirus-specific clones. These findings support the lifetime selection and maintenance of protective plasma cells in the human intestine (82). Therefore, $CD19^{neg}$ plasma cells are not restricted to plasma cells in the bone marrow, but can also be detected in the gut. Of note, it has been demonstrated that CD19 loss can occur in a subset of plasmablasts at an early stage of the immune response and, thus, is not strictly dependent on plasma cell aging (79).

Adhesion Molecules

A variety of different adhesion molecules such as very late antigen 4 (VLA-4, integrin $\alpha4\beta1$), lymphocyte function-associated antigen 1 (LFA-1, integrin $\alpha_L\beta2$), endothelial-cell selectin (E-selectin) ligand, platelet selectin (P-selectin) ligand, CD11a, CD18, CD44, and CD93 are expressed on plasma cells (20). VLA-4 and LFA-1 have a high impact on the survival of plasma cells. VLA-4 binds to VCAM-1 on stromal cells, and extracellular matrix components fibronectin and osteopontin present in the bone marrow. LFA-1 binds to three different molecules of the immunoglobulin superfamily: ICAM-1, ICAM-2, ICAM-3. Both VLA-4 and LFA-1 probably act by fixating the plasma cells in their niches. Their importance for bone marrow plasma cell survival has been demonstrated by co-blockade of LFA-1 and VLA-4 adhesion molecules *in vivo,* which resulted in a transient 75% reduction of bone marrow plasma cells in wild-type mice (86). However, the administration of integrin-blocking antibodies do not lead to strong plasma cell depletion in lupus prone mice (87). CD93 has also been suggested to promote plasma cell survival by functioning as an adhesion molecule (88) CD44 binds to hyaluronic acid, a protein of the extracellular matrix (89). CD44 itself is a surface marker of mesenchymal stem cells (90) and is involved in cell–cell and cell–extracellular matrix adhesion. Bone marrow plasma cells express high levels of CD44, which prolongs

the survival of human plasma cells *in vitro* (30, 51). It has been reported that cell contact between plasma cells and stromal cells via a CD44 variant isoform induces IL-6 production by stromal cells (91).

Several other cell-surface proteins like CD28 (92) and CD37 promote various aspects of plasma cell longevity (93). CD28 expressed on bone marrow plasma cells has been shown to be essential for plasma cell longevity (50, 92), and CD28-CD80/86 interaction modulates short-lived and memory plasma cell function (94). CD28 expression triggers an intrinsic survival signal, possibly through activation of the NF-κB pathway and by upregulation of BCMA. This protection is specific to bone marrow memory plasma cells and, at least in malignant plasma cells, is mediated by CD28's engagement of CD80/86 on myeloid cells and subsequent IL-6 secretion (95). CD37, a tetraspanin protein, is essential for the clustering of VLA4 molecules on B cells necessary for activation of the Akt survival pathway. CD37-defient mice have reduced numbers of IgG secreting plasma cells compared to wild-type mice (93).

In summary, multiple molecules expressed on plasma cells contribute to plasma cell survival. The above-mentioned examples show that progress is being made, but many questions remain unanswered. Thus, more data is needed for a better understanding of the processes controlling plasma cell homing and longevity (96).

INTRACELLULAR FACTORS AND MECHANISMS

Many intracellular factors and mechanisms are related to memory plasma cell programming. Together, they build up a complex network that controls various biological functions of memory plasma cells, including their differentiation, maintenance, and death as well as antibody synthesis and secretion. These intracellular factors and mechanisms have complicated functions and influence each other. This might explain why some studies have yielded conflicting findings and conclusions. The real pathways, especially how these intracellular factors and mechanisms communicate with the extracellular factors, are not comprehensively understood. Here, we focus on some recently described factors and mechanisms that are considered to correlate with the survival and maintenance of memory plasma cells.

Differentiation-Related Factors

The differentiation of activated B cells into plasma cells requires coordinated expression changes in hundreds of genes. Interferon regulatory factor 4 (IRF4), B lymphocyte-induced maturation protein 1 (Blimp-1), and X-box-binding protein 1 (XBP-1) are the three most important transcription factors guiding the plasma cell development program (17, 97). IRF4 is required for class switch recombination, germinal center (GC) B cell formation, and plasma cell differentiation (98–100). IRF4 functions are dose-dependent. Low levels of IRF4 or even transient induction of IRF4 is sufficient to induce GC B-cell formation, while high concentrations of IRF4 promote the generation of plasma cells and antagonize the GC fate by repressing *Bcl6* and by activating both Blimp1 and Zbtb20 (zinc finger and BTB domain-containing protein 20) (17, 100, 101). It has been shown that plasma cells residing in murine bone marrow disappeared immediately after conditional inactivation of *Irf4*, and that the effect can last for the whole observation time period of several weeks (102). Therefore, in addition to the defects in GC B-cell formation (100) and plasma cell differentiation caused by the loss of IRF4, the available results indicate that IRF4 plays an essential part in memory plasma cell survival, potentially by regulating some key survival molecules, such as myeloid cell leukemia 1 (Mcl-1) (102).

Blimp-1 is a transcriptional "master regulator" that is necessary for plasma cell differentiation (103, 104). During the B cell to plasma cell transition, 648 genes are upregulated and 424 are downregulated. Blimp-1 activates 38% (245) of these upregulated genes and represses 41% (105) of the downregulated ones (106). It directly regulates several transcription factors and important gene programs to facilitate the post-mitotic state of mature plasma cells (17, 107, 108). Within the B cell lineage, Blimp-1 is exclusively expressed in plasma cells, and its expression is higher in mature memory plasma cells than in short-lived plasma cells (plasmablasts) (109). By using a conditionally Blimp-1 deficient mouse model, it has been shown that Blimp-1 is required for the maintenance of memory plasma cells in the bone marrow and for the long-term maintenance of antigen-specific immunoglobulin in serum. In this mouse model, the number of memory plasma cells in the bone marrow decreases 4-fold, resulting in a drop in antigen-specific IgG1 levels in serum 3 to 4 weeks after inactivation of *Prdm1*, which encodes Blimp-1 (110). However, by using a GFP reporter mouse model to track plasma cells at higher resolution, another study more recently has demonstrated that plasma cell numbers in the bone marrow and spleen remain stable for many weeks in spite of a lack of Blimp-1, although the Blimp-1 deficient plasma cells lost their ability to secrete antibodies (102). Similar results are obtained after transferring B cells from these mice into B- and T-cell-deficient $Rag1^{-/-}$ mice after conditional Blimp-1 inactivation. Furthermore, this study suggests that Blimp-1 is essential for the establishment of the full plasma cell transcriptome but that once it has been established, plasma cell identity is maintained independently of Blimp-1 (102).

Endoplasmic Reticulum Stress-Related Factors

Memory plasma cells continuously secrete antibodies which allow the immune system to maintain a stable humoral immunological memory over long periods (8). To maintain stable levels in serum, one plasma cell secrets about 10^3 antibodies per second, approximately 2 ng per day (111, 112). To maintain this large-scale and stable antibody synthesis and secretory capacity, plasma cells require a specialized machinery, and metabolic activity. The endoplasmic reticulum (ER) is the major organelle for the synthesis and folding of secreted and transmembrane proteins. Plasma cells have continuous ER stress. When protein-folding requirements exceed the processing

capacity of the ER, the accumulated misfolded and unfolded proteins trigger the unfolded protein response (UPR), resulting in the adjustment of protein synthesis and the enhancement of ER folding capacity as well as increased degradation of misfolded proteins and enhanced ER biogenesis (113, 114). However, when these attempts fail and ER stress is unabated, UPR signaling typically switches to a pro-apoptotic mode known as the terminal UPR (115). The pro-apoptotic factor Chop (C/EBP homologous protein) is a characteristic marker for terminal UPR-induced apoptosis.

There are three regulatory arms of the UPR: PERK (protein kinase RNA activated (PKR)-like ER kinase), ATF6α (activating transcription factor 6α), and IRE1 (inositol-requiring enzyme-1) (113). Although mature B cells express high levels of PERK and ATF6α, physiologically, both PERK and ATF6α are dispensable for plasma cell differentiation, immunoglobulin secretion and survival (116, 117). Blimp-1 is intimately involved in the UPR. It directly regulates *Atf6* and 38% of the downstream genes of the UPR (102). As a component of the IRE1 branch, XBP-1 is an important transcription factor associated to UPR, which induces the transcription of a wide variety of ER-resident molecular chaperones and protein-folding enzymes that work together to increase ER size and function (118). Additionally, the induction of *Xbp1*, which is downstream of Blimp-1, is required for this marked ER expansion and increased protein synthesis (119). XBP-1 is required for the generation of plasma cells. In XBP-1 deficient mice, immunoglobulin levels are low and plasma cells are notably absent (120). However, later studies suggest that XBP-1 is required more specifically for immunoglobulin production (121–123). Conditional inactivation of *Xbp1* has no effect on the size of the plasma cell population, while XBP-1 deficiency in bone marrow plasma cells results in a global decrease in immunoglobulin transcripts and protein expression which correlates with reduced immunoglobulin secretion (102), but does not have a direct effect on the maintenance of memory plasma cells. Based on the current evidence, it seems that the three main arms of UPR do not directly influence the survival and longevity of memory plasma cell.

The inducible nitric oxide synthase (iNOS), which can be induced by XBP-1 (124), is associated with various mammalian physiology functions, including ER stress. iNOS has been found to modulate components of the UPR. Several mRNA levels related to ER stress are significantly lower in iNOS-deficient plasma cells (125). Both iNOS deficiency and iNOS inhibitor treatments cause plasma cells to have shorter life spans *in vitro* and *in vivo*. Bone marrow memory plasma cell numbers are significantly lowered in iNOS-deficient mice and wild-type mice treated with an iNOS inhibitor, and this decrease is accompanied by a significant decrease in the levels of antigen-specific antibodies. The effect of iNOS on the ER suggests that it has an effect on plasma cell survival. The finding that iNOS is also required for plasma cell responses to IL-6 and APRIL suggests an additional contribution of iNOS to the maintenance of memory plasma cells (125, 126).

Another system linked to ER stress is the ubiquitin-proteasome system, which is responsible for the degradation of not needed and misfolded proteins inside the cell. Bortezomib can inhibit the proteasome function and induce the efficient depletion of plasma cells, including memory plasma cells, in lupus mice (127). After bortezomib treatment, mRNA levels of Chop, a characteristic marker for the terminal UPR, increase 40-fold in splenic plasma cells and 20-fold in bone marrow plasma cells, resulting in the induction of terminal UPR and cell death. Another mechanism contributing to bortezomib-induced cell death is the inhibition of anti-apoptotic transcription factor NF-kB activity (127).

Autophagy

As misfolded proteins accumulate in the ER, autophagy functions as a crucial adaptive "self-eating" process by which autophagosomes envelop and degrade cellular components, and thus ameliorate ER stress. Similarly to the unfolded protein response, autophagy can result in either cell survival or cell death (114). Autophagy is a catabolic process related to lysosomal activity. There are three major types of autophagy: macroautophagy, chaperone-mediated autophagy (CMA), and microautophagy. *Microautophagy* involves direct invagination of the lysosomal membrane. C*haperone-mediated autophagy* involves the direct translocation of proteins into lysosomes. *Macroautophagy* leads to the integration of cytoplasmic material into vesicles that ultimately fuse with lysosomes; it is central to lymphocyte homeostasis, which is under the control of autophagy-related gene (ATG) products (128). Using mice in which *Atg5* is conditionally deleted in B lymphocytes, antibody responses are significantly diminished during antigen-specific immunization, parasitic infection, and mucosal inflammation (129). Moreover, *Atg5*-deficient B cells retain the ability to produce immunoglobulin and undergo class-switch recombination, but are impaired in their ability to terminally differentiate into plasma cells and, therefore, are unable to mount an effective antibody response, since the total plasma cell numbers in spleen and mesenteric lymph nodes are significantly low after immunization (129). However, another study with *Atg5*-deficient mouse model has shown that the antigen-specific plasma cell number in spleen is similar to the control group 14 days after immunization (130). Memory plasma cells from the bone marrow have higher autophagic activity than B cells. *Atg5*-deficient plasma cells have a larger ER and more ER stress signaling, which leads to higher expression of Blimp-1 and immunoglobulins, and to increased antibody secretion (130). The enhanced immunoglobulin synthesis is associated with more death of mutant plasma cells. The immunized *Atg5*-deficient mice have normal GC responses, but a 90% reduction of antigen-specific bone marrow memory plasma cells, demonstrating that Atg5 is specifically required for the maintenance of bone marrow memory plasma cells (130). Similarly, the *in vitro* study has shown that a lack of autophagy causes a substantial increase in the death of murine plasma cells and that 1 year after immunization, Atg5-deficient mice have remarkably fewer antigen-specific memory plasma cells in the bone marrow than wild-type mice (131). Another study using a murine autophagy-deficient autoimmune model has revealed that a decrease in memory plasma cells in the bone marrow is accompanied by a decrease in serum anti-dsDNA IgG

antibody levels (132). These findings confirm that autophagy is important for the maintenance of memory plasma cells. So far, the contribution of other autophagy factors is not known.

Metabolism

As they require the secretion of large quantities of glycosylated antibodies, which consumes 90% of their glucose utilization, plasma cells have high metabolic and energy pressure. Human and murine memory plasma cells can robustly engage pyruvate-dependent respiration and take up more glucose, which is essential for the generation of pyruvate. Targeting mitochondrial pyruvate carriers Mpc1 and Mpc2 *in vitro* reduces the survival of memory plasma cells significantly (133). The conditional *Mpc2*-deficient mice result in a significant loss of bone marrow memory plasma cells and a corresponding reduction of antigen-specific antibody titers in serum. These findings suggest that glucose uptake and mitochondrial pyruvate import promote the long-term persistence of memory plasma cells (133, 134). Interestingly, glucose can stabilize the expression of Mcl-1 (135, 136), which is essential for the survival of memory plasma cells (see below). Other nutrients besides glucose also contribute to plasma cell functions. Amino acids are the basis for antibody synthesis. Expression of CD98, a common subunit of many amino acid transporters and thus a marker of amino acid availability, is controlled by the transcription factor Blimp-1 and is very highly expressed in plasma cells, especially memory plasma cells (64, 102, 117). CD98 deficiency leads to severe antibody defects, and autophagy contributes to the metabolism of amino acids as cellular components are recycled, whereby the autophagy activity is higher in memory plasma cells than in plasmablasts (117). The metabolism of short-chain fatty acids (SCFAs) produced by gut microbiota are involved in plasma cell differentiation and promote antigen-specific host antibody responses (137).

Ectonucleotide pyrophosphatase/phosphodiesterase 1 (ENPP1), first identified as a membrane alloantigen, is involved in ATP-derived energy production. ENPP1 expression gradually increases during B cell differentiation to plasma cells, and bone marrow plasma cells show higher ENPP1 expression than their splenic counterparts in both mice and humans. Furthermore, bone marrow memory plasma cells express about 2-fold more ENPP1 than plasmablasts (138). ENPP1 deficiency does not affect GC formation or plasmablast migration. However, plasma cells residing in the bone marrow of $Enpp1^{-/-}$ mice take up less glucose and the frequency of antigen-specific memory plasma cells is significantly lower in the bone marrow than wild-type controls (138). ENPP1-deficient plasma cells have an impaired glycolysis pathway, which leads to reduced levels of energy production. Considering the 2-fold higher ENPP1 expression in bone marrow memory plasma cells, it suggests that ENPP1 allows bone marrow memory plasma cells to consume more glucose in order to better fuel higher antibody production levels and longer survival times (138).

Anti-apoptotic Factors

The NF-κB family of transcription factors governs the expression of multiple genes involved in cell survival, proliferation and effector functions. The primary contribution of NF-κB to lymphocytes is to assure cell survival. The anti-apoptotic functions of NF-κB are crucial for lymphocytes, even after they become mature (139). As NF-κB is involved in activities such as proliferation, activation, and GC formation during B cell differentiation (140–142), it influences the differentiation of plasma cells. Several factors like BAFF-BAFF-R axis and CD40-CD40L axis have been shown to activate the NF-κB pathway and mediate B and plasma cell differentiation. Adhesion molecules such as ICAM-1 and VCAM-1 are also regulated by NF-κB signaling (143). These factors are involved in the construction of survival niches for memory plasma cells, probably indirectly, through the NF-κB pathway.

Ras-related in brain 7 (Rab7) inhibition and knock-out studies provide further evidence of the role of the NF-κB pathway in the maintenance of memory plasma cells. Rab7 is a small GTPase that plays a B cell–intrinsic role in antibody response and promotes class-switch recombination by mediating NF-κB activation (144). One study has showed that Rab7 activity inhibition or Rab7 gene knockout results in reduced numbers of plasma cells, including memory plasma cells, and that it consequently suppresses IgG anti-dsDNA autoantibody responses, prevents the development of disease symptoms, and extends the lifespan of lupus mice (145); Rab7 also decreases the expression of several genes associated with memory plasma cells survival, including *Cxcr4, Irf4, Mcl1,* and *Atg5,* but not *Prdm1* and *Xbp1*. Interestingly, the apoptosis of cultured $CD19^{-}CD138^{hi}$ plasma cells induced by Rab7 inhibition can be prevented by enforced NF-κB activation (145). In another study, the treatment of lupus mice with resveratrol, a small polyphenol anti-inflammatory agent, enhances the expression of FcγRIIB on B cells and plasma cells, resulting in a marked depletion of plasma cells in the spleen and bone marrow, thereby decreasing serum autoantibody titers and ameliorating lupus nephritis; the authors have concluded that this upregulation of FcγRIIB is NF-κB dependent (146).

Bcl-2, Bcl-xL, and Mcl-1 are anti-apoptotic members of the Bcl-2 family expressed on plasma cells (56, 147). Various studies have shown that Bcl-2 and Bcl-xL are involved in plasma cell differentiation (148, 149), but the presence of both is not crucial for the survival of existing plasma cells (56, 150). Mcl-1 expression, regulated by the BCMA, is higher in bone marrow plasma cells than in plasma cells residing in other lymphoid organs (56). BCMA is a receptor for APRIL, which is an important survival factor for memory plasma cells, as described above. BCMA is an essential factor for the survival of memory plasma cells in the bone marrow. $BCMA^{-/-}$ mice have a 20% decrease in plasma cells in the bone marrow compared to their wild-type counterparts (57). The enzyme γ-secretase directly cleaves BCMA and releases soluble BCMA, which acts as a decoy that neutralizes APRIL. The inhibition of γ-secretase *in vivo* enhances BCMA surface expression in plasma cells and increases their number in the bone marrow (151). Another study confirms the importance of the APRIL-BCMA axis in plasma cell survival in the bone marrow and indicates that this process requires the transcriptional induction of Mcl1 (56). After deletion of Mcl1, the percentage and absolute numbers of total plasma cells and antigen-specific plasma cells are significantly lower than in wild-type mice, which highlights the important

role of this APRIL/BCMA/Mcl-1 signaling pathway for the long-term maintenance of memory plasma cells. The study from a mouse plasma cell line has shown that Blimp-1 can positively regulate the expression of the BCMA gene (152). Although the induction of BCMA is part of a transcriptional program during plasma cell differentiation, however, investigations in Blimp-1 deficient GFP reporter mice have indicated that the BCMA-mediated plasma cell survival pathway is independent of Blimp-1 (56). So far, this APRIL/BCMA/Mcl-1 pathway appears to be the best-characterized survival pathway of memory plasma cells (96).

MicroRNAs

MicroRNAs (miRNAs) are small, non-coding RNA molecules (containing about 20–22 nucleotides) that post-transcriptionally regulate gene expression in plants and metazoans. Until now, about 2,500 human and 1,900 mouse miRNAs which are functionally involved in most physiological cellular processes, including proliferation, development, and differentiation, have been identified (153–155). Many miRNAs are involved in B cell and plasma cell biology, for example, miR-30, miR-217, miR-28, miR-150, miR-155, miR-361, miR-125b, miR-181b, miR-21, miR-24-3p, miR-148a, and miR-17-92, etc. (156). MicroRNA-150 is specifically expressed in mature lymphocytes; it directly targets the transcription factor c-Myb (157), which is required for newly generated plasma cells migrating toward CXCL12 and therefore regulates the establishment of the memory plasma cell pool (158). MicroRNA-155 is required for the B-cell response to antigens. In miR-155-deficient mice, the number of GC B cells is reduced. B cells lacking miR-155 show a reduced GC response and failed secretion of class-switched, high-affinity IgG1 antibodies (159, 160). MicroRNA-125b regulates GC B-cell responses by targeting transcription factors IRF-4 and Blimp-1, and thereby inhibiting plasma cell differentiation (161). MicroRNA-24–3p has been identified as a direct mediator of human plasma cell survival, which is upregulated by IL-6 and CXCL12. Under induced ER stress, the upregulation of miR-24-3p expression by IL-6 can rescue plasma cells from apoptosis through the mitogen-activated protein kinase pathway (162). MicroRNA-148a can protect immature B cells from apoptosis and regulate B cell tolerance; it is upregulated in lymphocytes from lupus patients and lupus mice and accelerates the development of autoimmunity (163). It has been shown that miR-148a is upregulated in activated naive murine B cells, that it is the most abundant miRNA in memory plasma cells in both humans and mice, and that it promotes plasmablast differentiation and survival *in vitro* (164). A significant decrease in serum antibody levels and plasma cell numbers has been observed in conditional B-cell-specific miR-148a knockout mice with and without immunization, and in tamoxifen-inducible miR-148a-deficient mouse, the number of bone marrow memory plasma cells is significantly reduced, suggesting that miR-148a controls the differentiation of B cells into plasmablasts and the survival of memory plasma cells (165).

Other Factors

Zbtb20 is a broad complex, tramtrack, bric-à-brac, and zinc finger (BTB-ZF) protein expressed in GC B cells; it is upregulated during plasma cell differentiation and is highly expressed in memory plasma cells in an IRF4-dependent manner. Zbtb20 conditional knockout mice are characterized by a blunted antibody response and a significant loss of plasma cells in the bone marrow; these findings indicate that Zbtb20 is essential for the maintenance of memory plasma cells in the bone marrow and for the persistence of antigen-specific immunoglobulin levels in serum (101). In this study the expressions of *Bcma* and *Mcl1* in plasma cells are similar in knockout and wild-type mice (as determined by quantitative RT-PCR), whereas another study in Zbtb20-deficient mice demonstrates the reduced expression of *Mcl1* in bone marrow plasma cells (as analyzed by single-cell quantitative RT-PCR), suggesting that Zbtb20 may be required for the maximal expression of Mcl-1 (166). Interestingly, the requirement for Zbtb20 appears to be dependent on the type of adjuvant used. After alum-adjuvanted immunization, antigen-specific memory plasma cells fail to accumulate in the bone marrow, leading to a progressive loss of antibody production, whereas adjuvants that activate TLR2 and TLR4 restore long-term antibody production by inducing compensatory survival pathways in the plasma cells of Zbtb20-deficient mice (166).

Another regulator expressed in plasma cells is the tyrosine kinase Lyn, a negative regulator for many signaling pathways. Lyn attenuates signal transducer and activator of transcription 3 (STAT3) signaling, which can mediate the upregulation of Blimp-1 during plasma cell differentiation (167), of STAT3 responses to IL-6, and of the IL-6/JAK/STAT3 pathway, therefore supporting plasma cell survival and immunoglobulin secretion (168). A study in Lyn-deficient mice has showed that, in the absence of Lyn, memory plasma cells accumulate and have improved survival, and that the expression of CXCR4 on plasma cells is enhanced. Furthermore, cultured Lyn-deficient plasma cells show better *in vitro* survival with IL-6 but not with APRIL, indicating that Lyn regulates the survival of memory plasma cells through the IL-6/STAT3 pathway (169).

The lifestyle of memory plasma cell is complex. It involves many intracellular factors and mechanisms, all of which influence the maintenance of memory plasma cells more or less. Apparently, communication between the different factors and mechanisms is essential for establishing a survival network for memory plasma cells (**Table 1**; **Figure 1**). However, the signaling pathways, especially the patterns of connecting extracellular factors, are still bewildering and need to be further investigated.

MAINTENANCE OF MEMORY PLASMA CELLS IN INFLAMED TISSUES

The bone marrow is the primary survival niche for memory plasma cells. In addition, secondary lymphoid organs like the spleen provide a limited number of plasma cell survival niches. However, in the presence of chronic inflammation, memory plasma cells can also be detected in inflamed tissues (20, 170, 171), such as kidneys (105, 172–176), central nervous system (177–179), lungs (180, 181), nose (182), lymph nodes (183), salivary glands (184, 185), joints (186, 187), and tonsils (188, 189). The infiltration of local plasma cells appears to be associated with

TABLE 1 | Extrinsic and intrinsic factors contributing to the maintenance of memory plasma cells.

External factors		Internal factors and mechanisms
Cellular compartments	**Molecular compartments**	
➢ Stromal Cells (mesenchymal origin) • CXCL12+ cells ➢ Hematopoietic-origin cells • Megakaryocytes • Basophils • Dendritic cells • Monocytes/ Macrophages • Neutrophils • Eosinophils • Regulatory T cells	➢ Soluble Factors • Cytokines ○ TNF-superfamily (APRIL, BAFF, TNF-a) ○ IL-6 • Chemokines ○ CXCL-12 ➢ Membrane-bound Factors • Plasma cell (PCs) surface markers ○ CD138 ○ CD38 ○ CD19 • Adhesion molecules expressed on PCs ○ VLA-4 ○ LFA-1 ○ CD93 ○ CD44 ○ CD28 ○ CD37	➢ Differentiation related Factors • IRF4 • BLIMP-1 • XBP-1 ➢ Endoplasmic reticulum (ER) stress related factors and mechanism • UPR • iNOS • Ubiquiton-proteasome response ➢ Autophagy and metabolism • Anti-apoptosis factors • NF-kB family • Bcl-2 Family ➢ Others • Zbtb20 • Tyrosine Kinase Lyn • MicroRNA

the severity of the inflammatory disease. Little is known about how the memory plasma cells are maintained in the inflamed tissues and what kinds of survival niches support them. In this section, we will review recent findings regarding the presence of memory plasma cells in inflamed tissues.

CXCL12 Axis

The CXCL12/CXCR4 axis supports the maintenance of memory plasma cells in the bone marrow, as described above. In lupus prone mice (NZB/W), CXCL12 expression is higher than in healthy mice. Various studies have shown that CXCL12 levels are elevated in the kidneys of older mice with nephritis (174, 190, 191), and that neutralization of CXCL12 with a monoclonal antibody at an early age can prevent the development of proteinuria and prolong the survival (190). Treatment with AMD3100, a CXCR4 blocker, significantly decreases the number of memory plasma cells in the kidneys of NZB/W mice (175). Similar results have been achieved by inhibiting CXCR4 with the antagonist CTCE-9908 in another murine lupus model (191). About 60% of plasma cells in inflamed NZB/W kidneys are in contact with $CD11b^{+}$ macrophage-like cells (173), which are the prime source of CXCL12 (192). Using a collagen-induced arthritis model, it has been observed that CXCL12 expression is increased in the inflamed joints, and that treatment with the CXCR4 blocker AMD3100 provides clinical benefits (193). In rheumatoid arthritis (RA), the expression of CXCL12 in synovial tissues is increased (194, 195), and CXCL12 seems to be predominantly expressed by endothelial venules and synoviocytes in the synovial tissues (196–199). These findings resemble the pictures of the CXCL12-expressing stromal cells organizing the survival niches for memory plasma cells in the bone marrow (170).

In a murine model of induced experimental autoimmune encephalomyelitis (EAE), an upregulation of CXCL12 has been detected in the inflamed spinal cord where memory plasma cells are localized (179). Similar co-localization of memory plasma cells with CXCL12-expressing epithelial and infiltrating mononuclear cells has been detected in the salivary glands of patients with primary Sjögren's syndrome (184). However, in a hepatitis virus-induced central nervous system inflammation mouse model, virus-specific IgM and IgG antibodies are detected in the spinal cord, and plasma cells are predominantly found in demyelinated lesions and adjacent white matter (178). While, mRNA levels of CXCL12 do not always exceed baseline levels throughout this inflammatory condition (178).

APRIL/BAFF Axis

APRIL and BAFF expression levels are higher in the inflamed kidneys of NZB/W mice (174). In human lupus nephritis, elevated mRNA levels of APRIL and BAFF have been detected in renal biopsies from patients refractory to immunosuppressive therapies (200). In RA, BCMA and APRIL expression levels are higher in synovial fluid (187, 201), and the APRIL levels closely correlate with the local plasma cell counts. The main source of APRIL is infiltrating neutrophils and CD68+ macrophages, and CD138+ plasma cells are found to be in tight contact with CD68+ macrophages in the zones of high concentration of secreted APRIL (202). Furthermore, APRIL and BAFF released by microglia and astrocytes in the spinal cord are present in the same area in which memory plasma cells are found. Notably, the memory plasma cells are strongly positive for both APRIL and BAFF, which suggests that a self-sufficiency mode may exist under inflammatory and autoimmune disease conditions (179). Moreover, the mRNA levels of BCMA, TACI, APRIL and BAFF in the spinal cord are increased during inflammation, indicating that these factors may support the survival of local plasma cells (178).

In inflamed nasal tissues from patients with granulomatosis with polyangiitis (GPA), plasma cells are found in close proximity to APRIL secreted by macrophages, giant cells, and epithelial cells. (182). In allergen-challenged mice, BAFF levels are significantly increased in lung and bronchoalveolar lavage fluid (203), and the number of eosinophils is increased and associated with infection and allergic diseases. Although eosinophils have been shown to support the maintenance of memory plasma cells in the bone marrow and of IgA+ plasma cells in the intestine (44), the number of IgA+ plasma cells is not reduced in the lungs of eosinophil-deficient mice (204).

Others Factors

Under inflammatory conditions, plasma cells are detectable in the inflamed joints of patients with RA and osteoarthritis (186, 187, 205, 206). Many factors like ICAM1, VCAM1, VLA-4, and IL-6 are increased in inflamed synovial tissues (207–209), which may build up the microenvironment needed for the maintenance of

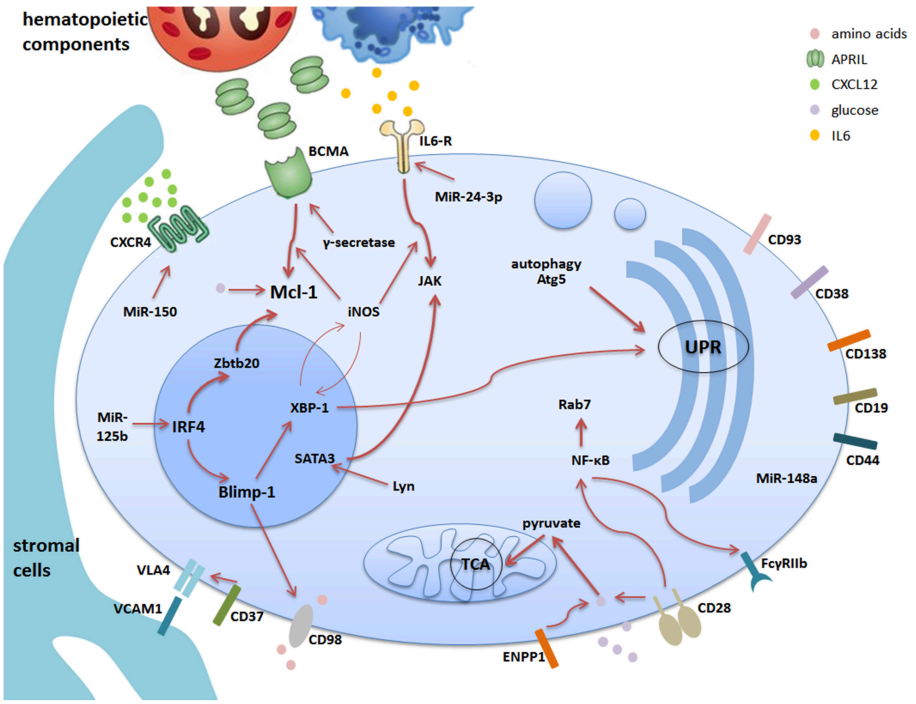

FIGURE 1 | The survival network for memory plasma cells in the bone marrow. Bone marrow is the major site of memory plasma cells. The stromal cells together with hematopoietic cells construct the microenvironment supporting the survival of memory plasma cells via cell-contact or secretion of soluble factors. Different plasma cell surface molecules are communicating with intracellular factors via different pathways regulated by internal factors and mechanisms. This cartoon summarizes the current understanding of the complex network consisting of stromal and hematopoietic cells, soluble factors, receptors and signaling pathways. The thicker arrows indicate pathways, which are key players in the maintenance of memory plasma cells.

local plasma cells. Other types of cells may also contribute to the establishment of survival niches in inflamed joints. In RA, nurse-like cells produce enhanced levels of many cytokines (e.g., IL-6) and promote B cell survival and differentiation. Interestingly, transmission electron microscopy analysis of RA synovial tissues has shown numerous plasma cells are surrounded by synovial long slender cytoplasmic dendritic cells with spines or finger-like protrusions, which cell membrane appears to be fused or very tightly attached to the cell membrane of the paired plasma cell (210). Immunofluorescence staining studies also show that both CD14+ dendritic cells and CD138+ plasma cells in synovial tissue reside in close proximity (211, 212).

In an adapted multiple sclerosis murine model of EAE, VCAM-1 is upregulated; memory plasma cells are localized in areas of increased VCAM-1 expression (179). Further research has shown that, in the inflamed salivary glands of patients with Sjögren's syndrome, Ki67 negative memory plasma cells are tightly juxtaposed to the ductal and acinar epithelia, which highly express IL-6 (184).

Plasma cells are also detectable in lung tissues during allergic airway inflammation. Nerve growth factor (NGF) and neurotrophin-3, mainly secreted by local T cells and macrophages, appear to support the survival of these plasma cells, which express neurotrophin receptors due to upregulation of the anti-apoptotic protein Bcl2. One study has showed that inhibition of neurotrophin receptors significantly reduces local plasma cell numbers and serum antibody levels, and that overexpression of NGF results in higher plasma cell counts in the perialveolar area. These data suggest that NGF might be essential for the local survival for plasma cells (213). In patients with chronic bronchitis and obstructive pulmonary disease, plasma cells are particularly abundant in the subepithelium and interstitium between submucosal gland acini, where they are co-localized with IL-4-positive cells. The latter cells are identified as CD68+ monocytes/macrophages and CD20+ B cells and, interestingly, over 60% of the plasma cells themselves express IL-4 in these inflamed tissues (214).

So far, investigators who have tried to explain the survival mechanisms for memory plasma cells under inflammatory conditions based their studies on the assumption that the memory plasma cell environment in inflamed tissues is like that in the bone marrow, but if this is actually so in reality is still unclear. Current findings suggest that the maintenance of plasma cells in inflamed tissues is supported by inflammatory cells (**Table 2**). In addition, plasma cells themselves are able to secrete their own survival factors such as APRIL, BAFF, and IL-6. Transcriptome comparisons of bone marrow memory plasma cells and splenic plasmablasts revealed more than 900 differentially expressed transcripts between these two types of plasma cells (117). Taking into account the different behavior of memory plasma cells in inflamed tissue, there must be differences between the memory plasma cells in bone marrow

TABLE 2 | Contributors to the survival of memory plasma cells in inflamed tissues.

Inflammation	Factors	local sources
Kidney	CXCL12	Macrophages
	APRIL, BAFF	Not clearly defined
Joint	CXCL12	Endothelial cells, synoviocytes
	APRIL	Infiltrating neutrophils, macrophages
	IL-6	Nurse-like cells
	Cell-cell contact	Dendritic cells
	ICAM1, VCAM1, VLA-4	Not clearly defined
CNS	APRIL, BAFF	Microglia and astrocytes, plasma cells
	CXCL12, VCAM-1	Not clearly defined
Salivary gland	CXCL12	Epithelial infiltrating mononuclear cells
	IL-6	Ductal and acinar epithelia
Nose	APRIL	Macrophages, giant cells and epithelial cells
Lung	NGF, neurotrophin-3	Local T cells, macrophages
	IL-4	Monocytes/macrophages, B cells, plasma cells
	BAFF	Not clearly defined

and those in inflamed tissues, healthy memory plasma cells, and their autoreactive counterparts. A study comparing the transcriptomes of these plasma cells would help to understand the survival mechanisms of (autoreactive) memory plasma cells under inflammatory conditions.

CONCLUSION

Due to their longevity, memory plasma cells residing in the bone marrow are crucial for maintaining humoral immunity independently of memory B cells, T cell help, and antigen presentation. A stable immune memory provides long-term protection against pathogens. The survival of memory plasma cells is supported by a physiological survival niche established by different cells and extra- and intra-cellular factors and mechanisms. Upsetting the niche environment will disturb the survival of memory plasma cells and, ultimately, humoral immune memory. More information on the maintenance of memory plasma cells may improve vaccination strategies.

However, if immune tolerance fails, autoreactive plasma cells can be generated and become memory plasma cells surviving in bone marrow and chronically inflamed tissues where they contribute to autoimmune pathology. Under these disease conditions, memory plasma cells are considered to be therapeutic targets (170, 172, 215). Compared to plasmablasts, memory plasma cells are resistant to most conventional therapies, including conventional immunosuppression and B cell depletion as such. This makes it clinically challenging to target memory plasma cells (87, 171, 216, 217). At present, only a few approaches are able to target memory plasma cells efficiently. Since these methods do not distinguish between memory plasma cells secreting protective and pathogenic antibodies, there is a need to develop strategies that selectively targets the pathogenic cells without affecting the protective humoral memory. Therefore, a better understanding of the lifestyle of memory plasma cells will give us clues for developing new approaches to target these cells. Although many advances have been made in research on the survival and maintenance of memory plasma cells, as we have described in this review, the path to truth is still covered with a veil that needs to be lifted in further investigations in the future.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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REVIEW
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In the Right Place, at the Right Time: Spatiotemporal Conditions Determining Plasma Cell Survival and Function

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Plasma cells (PCs), the B lineage cells responsible for producing and secreting antibodies (Abs), are critical cellular components of the humoral immune system. While most of the antibody-secreting cells in the body have a rather short lifetime of a few days, some of them can become long-lived and persist in the body over the entire life span of an individual. The majority of these long-lived plasma cells secretes protective antibodies against pathogens, and are thereby crucial for the humoral component of immunological memory. The generation of these protective antibody-secreting cells can be triggered by an exposure to pathogens, and also by vaccination. Although the majority of plasma cells are protective, sometimes long-lived plasma cells produce autoreactive antibodies, which contribute to the pathogenesis and perpetuation of chronic autoimmune diseases, including lupus erythematosus, rheumatoid arthritis, or multiple sclerosis. In order to promote the formation of protective antibody-secreting cells and to target pathogenic plasma cells, it is crucial to understand the signals which promote their longevity and allow them to exert their function. In recent years, it has become clear that plasma cells depend on extrinsic factors for their survival, leading to the concept that certain tissue microenvironments promote plasma cell retention and longevity. However, these niches are not static structures, but also have dynamic features with respect to their cellular composition. Here, we review what is known about the molecular and cellular composition of the niches, and discuss the impact of dynamic changes within these microenvironments on plasma cell function. As plasma cell metabolism is tightly linked to their function, we present new tools, which will allow us to analyze metabolic parameters in the plasma cell niches *in vivo* over time.

Keywords: plasma cells, survival, bone marrow, inflammation, intravital 2P microscopy, tissue niches, metabolism

ANTIBODY SECRETING CELLS—THE CELLULAR BASIS OF HUMORAL IMMUNITY

Humoral immunity is mediated by antibodies, produced by cells highly specialized to synthesize and secrete large quantities of proteins (1). Antibody-secreting cells are traditionally divided into plasmablasts and plasma cells, on the basis of their capacity to further proliferate; as the name suggests, plasmablasts retain the capacity to proliferate, while plasma cells are considered to be post-mitotic. While most of the antibody-secreting cells in the body have a rather short lifetime of a few days, some of them can become long-lived and persist in the body over the entire life span of an individual, as described 20 years ago (2, 3), thereby providing the cellular basis of long-term antibody responses.

PLASMA CELL GENERATION, MIGRATION, AND SURVIVAL UNDER PROTECTIVE/PHYSIOLOGIC CONDITIONS

Plasma cell differentiation from activated B cells begins in secondary lymphoid organs. During the early phases of an immune response, after the initial B cell activation has taken place but before the formation of germinal centers, a first wave of antigen-specific plasma blasts is generated. These cells are generally considered short-lived (4) and relocate apart from the B cell zones, in the splenic red pulp (RP) (5), or the medullary cords (MC) of lymph nodes (6), respectively. These areas contain $Gr1^{int}CD11b^{hi}F4/80^{+}$ macrophages which are a source of APRIL, a potent survival factor for plasma cells. Under conditions where no germinal centers are formed, e.g., in immune responses to T cell-independent antigens or when germinal center formation is experimentally blocked, B cells differentiate into extrafollicular plasma blasts, which later can become long-lived plasma cells (7), in agreement with the concept that extrinsic factors determine the capacity of plasma cells to survive (5).

On their way from the B cell zone to the medullary cords, migratory plasma blasts pass by a microenvironment rich in the growth factor IL-6, stemming from perivascular CD11c+ cells in the T cell zone. Directed migration from the B cell follicles to the medullary cords (8) is guided by a gradient of CXCL12, produced by medullary fibroblastic reticular cells (9). These specialized stromal cells are also a local source of plasma cell survival factors IL-6, BAFF, and APRIL, and synergize with the myeloid cells in promoting plasma cell survival at this location. Upon settling in the medullary cords, they become mature plasma cells, and cease their migration (10) and proliferation (6).

During later phases of primary as well as recall T-dependent immune responses, plasma blasts are generated during germinal center reactions. Germinal centers develop in B cell follicles in the course of T-dependent responses, and represent unique microanatomical structures, where B cells proliferate and encounter antigen deposited on the surface of follicular dendritic cells in the germinal center light zone (11). After B cell receptor-mediated antigen uptake, they present the antigen to T cells, and in turn receive T cell help. Successful antigen presentation to T cells induces further B cell proliferation and somatic hypermutation of their immunoglobulin (Ig) variable genes in the dark zone, followed by the selection of B cells with higher affinity B cell receptors (12). Multiple rounds of B cell proliferation and selection result in the affinity maturation of the B cell immune response, and in the output of memory B cells and antibody secreting cells. Plasma blast differentiation is initiated by antigenic stimulation in the light zone, and their further maturation occurs after they have localized to the dark zone (13). A recent study has identified the germinal center-T zone interface (GTI) as an important site for plasma cell maturation during the early maturation phases of the GC response, using a model of primary immunization (14). The GTI shares characteristics with the RP/MC microenvironment as it is rich in IL-6, APRIL and CXCL12, produced by stromal cells in this region. In addition, IL-21 from Tfh cells, known to promote plasma blast differentiation, is abundant in these areas. Plasma blasts leave the GTI migrating in the direction of the medullary cords/red pulp areas (14). Whether these migrating cells feed directly into the short-lived pool or leave the SLOs to become long-lived in the bone marrow is currently unclear. Using pulse-chase experiments, Weisel et al. (15) found that the majority of long-term resident plasma cells in the bone marrow were actually derived from late GC phases. However, antigen-specific plasma cells accumulate as early as day 3 in the bone marrow (16, 17). This may be explained by the possibility that plasma cells of a higher affinity can gradually replace the earlier generated low-affinity ones in the bone marrow, but this remains to be formally proven. Further, the mechanism by which plasma cells could compete on the basis of affinity remains unclear, as plasma cells lack membrane expression of their antigen receptor, and antigen is not retained in the bone marrow to a similar extent as on follicular dendritic cells. Nevertheless, a bulk/first wave of plasma blasts leaves the SLO and migrates to the bone marrow in the time frame of a week after immunization in mice (16, 17), and a peak of antigen-specific plasma blasts can be detected in the blood of humans at day 6 and 7 after immunization (18), indicating a similar time course in humans.

There are distinct molecular requirements for cellular entry or exit into tissue: the receptor for sphingosine-1-phosphate S1PR1

Abbreviations: Abs, Antibodies; APRIL, A proliferation-inducing ligand; ASC, Antibody-secreting cell; BAFF, B-cell activating factor; BCMA, B cell membrane antigen; CNS, Central nervous system; CSF, Cerebrospinal fluid; CXCL, Chemokine X ligand; CXCR, Chemokine X receptor; EAE, Experimenal autoimmune encephalitis; ER, Endoplasmic reticulum; FCCP, Carbonyl cyanide-p-trifluoromethoxyphenylhydrazone; FLIM, Fluorescence lifetime imaging microscopy; GAPDH, gyceraldehyde-3-phosphate-dehydrogenase; GC, germinal center; GRIN, Gradient refractive index; GTI, Germinal center-T cell zone interface; HSC, hematopoietic stem cell; HSPC, hematopoietic stem and progenitor cell; LDH, lactate dehydrogenase; LIMB, Longitudinal imaging of the bone marrow; MC, medullary cords; MOG, myelin oligodendrocyte glycoprotein; MS, multiple sclerosis; mTOR, mammalian target of rapamycin; NADH, Nicotinamide adenine dinucleotide; NADPH, Nicotinamide adenine dinucleotide phosphate; NAD(P)H, either NADH or NADPH; NMDAR, N-methyl-d-aspartate receptor; NZB/W F1, New Zealand black × New Zealand white, F1 generation; PC, Plasma cell; PDH, Pyruvate dehydrogenase; RNA, Ribonucleic acid; RP, red pulp; SLE, systemic lupus erythematosus; SLO, Secondary lymphoid organ; VCAM, Vascular cell adhesion molecule.

(19), whose expression on plasma blasts is modulated by mir-17-92 (20), and integrin receptor beta 2 (21) are required on plasma blasts to leave lymph nodes, while the chemokine receptor CXCR4 (16, 17) is required for bone marrow homing. Plasma blasts also transiently respond to CXCR3-ligands, however, at what stage of plasma blast migration these chemokines play a role is not known (17).

The CXCR4 ligand CXCL12 is produced in high amounts by bone marrow stromal cells (22). Notably, plasma blasts deficient in the transcription factor c-Myb exhibit a deficiency in migration toward CXCL12 gradients *in vitro* and mislocalize to the T cell zone in the spleen, indicating that they are not able to reach the red pulp (23). Thus, CXCR4 seems to not only control access to "exit points" for extravasation from secondary lymphoid organs, but migration to specific domains within lymphoid tissues. The nature of these egress sites has not yet been defined in detail. Plasma blasts in the red pulp occur in clusters, which indicates that these sites are present within the sinusoidal vessel structures of this compartment. Shp1 deficient plasma blasts are able to migrate to the red pulp, but do not form clusters and are impaired in their bone marrow homing capability due to an enhanced binding to integrin α4β1 to its ligand VCAM-1, which results in an impaired capacity to migrate (24). Integrin α4β1 (VLA-4) has been implied in multiple aspects of plasma cell biology, and seemingly contradictory results may be explained by its different functions in varying microenvironments. For example, integrin β1 activation by the cochaperone Mzb1 has been shown to contribute to the relocation of plasma blasts (25), however, this seems to mainly affect their entry into the bone marrow, not their egress from SLOs. CXCL12 has also been shown to activate α4β1 (26), and VCAM-1 mediated stimulation of α4β1 impacts on the survival of plasma cells (27). This particular function seems to depend on CD37, which regulates the membrane distribution of α4β1, thereby enabling signaling via the Akt survival pathway (28).

MICROENVIRONMENTS OF PLASMA CELL NICHES IN THE BONE MARROW

It has long been known that plasma cells accumulate in the bone marrow (29). Long-lived plasma cells were first described in this organ (2, 3), and as it is the primary locus of humoral memory, the bone marrow microenvironment has been the most intensively studied plasma cell niche.

The entry points and routes which plasma cells use to enter the bone marrow from the blood are not completely identified yet, but they are likely similar to the ones used by hematopoietic stem and progenitor cells (HSPCs). Bone marrow vasculature comprises small arterioles, which regulate the blood flow into the parenchyma. These vessels progressively increase their diameter and connect to a network of sinusoids, which are characterized by large lumina (30, 31). The fenestrated endothelia and the discontinuous structure of their underlying basement membrane (32), in combination with low blood flow velocities make this vascular compartment the preferred entry site for cells, as has been shown for HSPCs (33).

Plasma cell survival crucially depends on a combination of extrinsic signals, among them adhesion molecules (27). After crossing the endothelium, plasma blasts migrate to specialized microenvironments ("niches") in the bone marrow parenchyma. Their migration is directed by stromal-derived factor 1α (CXCL12). Upon arrival at its niche, a motile plasma blast loses its responsiveness to chemokines (17) and docks onto stromal cells (34, 35). The newly arrived plasma blasts then becomes sessile, and remains constantly in close contact with the stromal cell (36). This contact seems to be based on α4β1 (VLA-4) and αLβ2 (LFA-1) on plasma cells interacting with their respective ligands on stromal cells, as only the combined blockade of both adhesion molecules by antibodies has been shown to effectively deplete plasma cells from the bone marrow (37). The stromal cells on which plasma cells colocalize have been shown to be VCAM-1$^+$ (34), however, a recent study provided evidence that fibronectin, another ligand of α4β1 integrin, also mediates plasma cell survival (38). Less is known about which of the ligands for αLβ2 (of which there are 6: ICAM1-5 and JAM-A) are of relevance for plasma cells in their niches, and fibronectin also binds CD44, which has been shown to mediate plasma cell survival *in vitro* (27). It is currently not known whether the stromal cells, which support plasma cell niches constitute a functionally specialized subset within the bone marrow stroma. Although most reticular bone marrow stromal cells express CXCL12, there is evidence for considerable heterogeneity among the stromal compartment (39), and it will be important to analyze the relationship of hematopoietic cell types to these various stromal subsets *in situ* using multiplexed microscopy (40). The term "stromal cell" is known to encompass a wide range of cell types—in the lymph node, for example, single-cell RNA sequencing was used to define 9 different stromal cell populations (41). Four of these were previously known (follicular dendritic cells, marginal reticular cells, perivascular cells, and T-zone reticular cells), while the rest had not been previously appreciated or defined. Given the wide range of cell types and processes that occur within the bone marrow, it is unlikely that bone marrow stromal cells will be less heterogenous than lymph node stromal cells.

The niches provide plasma cells not only with signals for their adhesion, but also directly promote PC survival. BCMA on plasma cells is a crucial mediator of survival signals (42) via its ligands APRIL and BAFF (43). BCMA signaling regulates the expression of the anti-apoptotic protein Mcl-1 in bone marrow plasma cells (44). Multiple different cellular sources of APRIL have been reported in the bone marrow. Eosinophils have been shown to produce APRIL and also IL-6, another survival factor for plasma cells, and support plasma cell survival *in vitro* (45). The same group also showed that bone marrow plasma cell numbers are reduced in eosinophil-deficient or –depleted mice to about half the numbers present in controls. In contrast, two recent studies by other labs (46, 47) found that eosinophils were actually dispensable for plasma cell survival in the bone marrow. These discrepancies could be in part explained by environmental factors, for example differences in the microbiome of animals, as the two latter studies used littermate controls, which Chu et al. did not. In addition, these findings indicate that more than one cell type may contribute to BCMA-dependent plasma

cell survival. Indeed, APRIL and IL-6 have also been shown to be produced by megakaryocytes in the bone marrow (48). Plasma cell numbers are reduced in megakaryocyte-deficient c-mpl mice, whereas raising megakaryocyte numbers pharmacologically is accompanied by an increase in plasma cell numbers, suggesting that megakaryocyte-derived APRIL and IL-6 are physiologically relevant to bone marrow plasma cells.

Although most studies have focused on APRIL, the other ligand of BCMA, BAFF, likely also plays a role in plasma cell survival, as a reduction in bone marrow plasma cells is only observed in APRIL$^{-/-}$ mice, if BAFF is blocked as well (43). The redundancy of the hematopoietic cell types contributing to plasma cell niches is further underlined by reports of several other cell types, which have been reported to support their survival via different mechanisms: Ly6C^{hi} monocytes have been shown to produce APRIL and co-localize with bone marrow plasma cells in mice (49), and myeloid precursors are a major source of APRIL in humans (50). In mice, basophils have been shown to support plasma cell survival by producing IL-4 and IL-6 *in vitro* and *in vivo* (51). Dendritic cells have also been shown to make functional contacts with bone marrow plasma cells: CD80/86 on the DC surface interacts with CD28 on bone marrow plasma cells, which promotes their survival by activation of the Vav and NF-κB pathways (52). Consequently, CD28$^{-/-}$ mice have a reduction in bone marrow plasma cells (53). By intravital microscopy, bone marrow plasma cells have been shown to form contacts with CD11c^{+} cells as well as T regulatory cells (54), but the mechanisms by which these two cell types regulate niches are not fully elucidated. Deletion of CTLA-4 in the Tregs resulted in an increase in plasma cell numbers, which may indicate that they are able to modulate the function of the niche dendritic cells. On the other hand, depletion of Tregs induced by systemic infection caused a reduction of the bone marrow plasma cell population, indicative of their role in plasma cell maintenance.

Due to its function in hematopoiesis, with blood cells constantly being generated and leaving the tissue, the bone marrow is a very dynamic tissue (55), with far more cells being generated and leaving than cells remaining resident and resting in this organ. On the order of a hundred million cells per gram of bone marrow are produced per day. Other hematopoietic cells which contribute to the niches may be only transient niche inhabitants, as has been shown for eosinophils (36), and the transient or migratory cells may not need to physically contact plasma cells. For example, APRIL can be produced in a soluble form by cells that are not in contact with plasma cells, and a local accumulation of APRIL in the niches could be achieved by its retention on the tissue via proteoglycans (56) or by directly binding to syndecan-1 (CD138) on the surface of plasma cells (57). Later in this review, we will address some interesting new findings about the dynamics of the bone marrow, and how this relates to its cellular and structural components. A summary of signals that have been described to promote plasma cell survival in the bone marrow is shown in **Figure 1**.

LIFETIME AND NICHES OF PLASMA CELLS GENERATED IN INTESTINAL IMMUNE RESPONSES

In contrast to their counterparts in the bone marrow, antibody-secreting cells in the intestine, the largest compartment of these cells in the body, were for a long time considered to be short-lived. Intestinal B cells migrate from the sites of their activation, Peyer's patches or mesenteric lymph nodes, through the blood to the lamina propria underlining the intestinal epithelium, where they secrete their antibodies, mostly IgA, at the frontline of defense. Their migration is guided by the chemokine receptors CCR9 and CCR10 (58). The high rate of proliferation among intestinal antibody-secreting cells led to the conclusion that these IgA+ cells have an average life span of less than a week in mice (59). However, studies in rats demonstrated the presence of a small, non-proliferative population of antibody-secreting cells (60). In mice, EdU pulse-chase experiments showed that long-lived IgA^{+} plasma cells are indeed generated in mucosal immune responses after oral immunization (61), although the short-lived plasma cells outnumber their long lived counterparts in the lamina propria, reflecting the continuous antigenic stimulation occurring at the mucosal interface. Plasma cells generated in intestinal immune responses also contribute to the long-lived plasma cell pool in the bone marrow.

Both intestinal and bone marrow long-lived plasma cells persist without antigenic stimulation (62, 63). The molecular structure of the survival niches in the gut is quite similar to the ones in the bone marrow, although the cells providing this structure are tissue-specific and thus quite different: the intestinal epithelium appears to be the main source of APRIL in mice (61) as well as humans (64). Eosinophils (45) and neutrophils (56) in the intestine also produce APRIL.

Human plasma cells in cultures of intestinal biopsies have been shown to survive without proliferation and secrete antibodies over several weeks, in a BCMA and IL-6 dependent manner (65). By using the analysis of cellular turnover in transplanted intestines and by calculating the age of individual cells using carbon-14 in DNA, the same lab showed that CD19^{-} plasma cells showed little to no turnover and persisted for decades, while CD19^{+} plasma cells were dynamically exchanged (66). Plasma cell populations differing in their CD19 expression have also been described in the bone marrow (67), with the CD19^{-} subset containing a static population providing long-term immunity, whereas the CD19^{+} subset appears to have more dynamic features of cells responding to a new antigenic challenge (68). The finding of heterogeneity among long-lived plasma cell populations is intriguing, and it will be interesting to investigate whether they differ in terms of requirements regarding their microenvironment, or whether they can occupy the same niche. Similarly, it will be important to investigate whether the recently described IL-10 producing subset of natural regulatory plasma cells (69) occupies a specific niche.

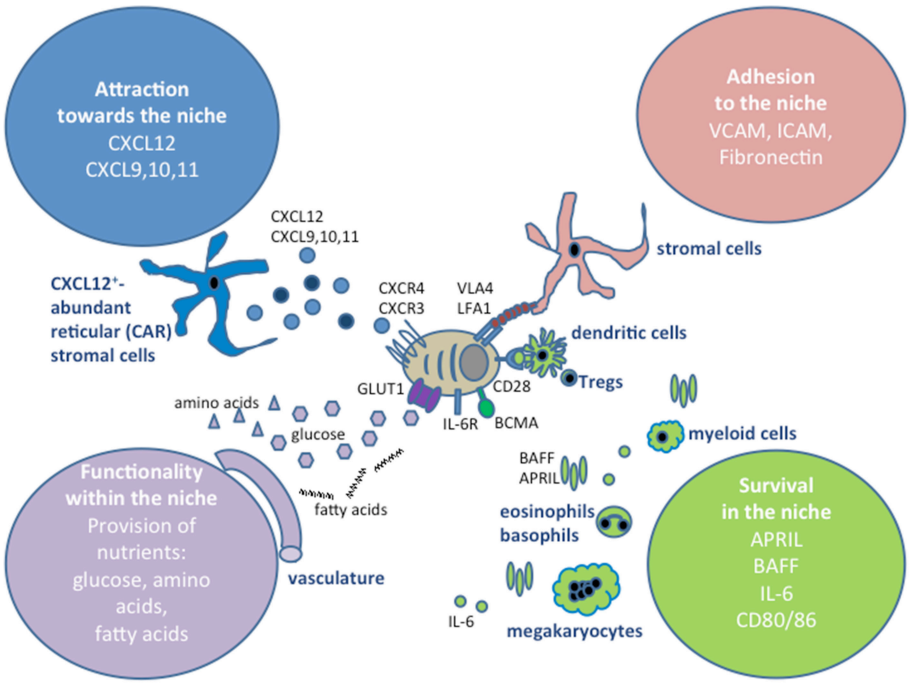

FIGURE 1 | Extrinsic factors defining plasma cell niches. The extrinsic signals can be grouped into four categories, depending on whether they affect the migration of plasma cells into the niches, retain plasma cells at those sites, promote their survival or impact on their functionality, for example by providing nutrients. In this graph, the cell types that have been shown to contribute to the niches in the bone marrow are shown, however, the cells types involved in securing these four categories vary between the tissues where plasma cells are localized, as discussed in the text.

PLASMA CELLS IN INFLAMMATORY NICHES

Bone marrow and the intestine represent physiological locations for long lived plasma cells, however, plasma cells can also accumulate in chronically inflamed tissues. In autoimmune diseases like systemic lupus erythematosus (SLE), immune thrombocytopenia, autoimmune hemolytic anemia, and myasthenia gravis, plasma cells secreting autoantibodies are critical contributors to pathogenesis (70). Plasma cells have been shown to localize in inflamed kidneys of lupus-diseased NZB/W F1 mice (71) as well as in SLE patients (72, 73). The majority of the kidney plasma cells in the mouse model are long-lived (74), and they contribute to the local production of renal autoantibodies (75). Anti-dsDNA and –nucleosome autoantibodies have been shown to be present in glomerular deposits, a hallmark of nephritis (76, 77), although plasma cells secreting other specificities are also found in inflamed NZB/W F1 kidneys (71).

The long-lived autoreactive plasma cells pose a problem for therapy of these diseases, as they have been shown to be refractory to conventional immunosuppressive therapies (78) and downregulate B cell markers commonly used for therapy with B cell-depleting biologicals, such as CD20 (79). In fact, in immune thrombocytopenia patients treated with anti-CD20 (Rituximab), long-lived plasma cells are found to persist in the spleen (80), whereas they are absent in spleens of non-treated patients. Similar reports have also been observed in warm autoimmune hemolytic anemia patients (81). Plasma cell persistence in the B cell-depleted patients is accompanied by elevated splenic BAFF levels, which may indicate that the decreased local BAFF consumption (81, 82) permits *de novo* generation of niches for long-lived plasma cells in the spleen. Conversely, although the proteasome inhibitor bortezomib was shown to effectively deplete both short- and long-lived plasma cells in NZB/W F1 mice (83), as normal cellular turnover leads to continuous antigenic exposure to nucleosomes and other self-antigens, long-lived plasma cells are continuously generated in SLE-prone mice (84) and patients (85), and sustained depletion can only be achieved by combining bortezomib and B cell depletion (86). Together, this demonstrates that an effective therapy must take into account the interdependence of the various B cell differentiation stages and their requirements for cytokines.

Autoantibody-mediated diseases and the option to treat them using plasma cell depletion have gained increasing attention in the field of neurology in recent years. The central nervous system (CNS) represents a special segregated niche for plasma cells as well as for antibodies, as the blood-brain barrier separates the CNS tissue and cerebrospinal fluid from systemic blood flow in healthy individuals. Anti-N-methyl-D-aspartate receptor (NMDAR)-encephalitis is a recently described autoantibody-mediated immune disease. Patients present with heterogeneous symptoms, ranging from seizures and dyskinesia to frank psychosis, and antibodies against the NR1-subunit

of the NMDAR have been shown to be pathogenic (87). Bortezomib reduced antibody titers and improved the clinical outcome in NMDAR patients resistant to other therapies (88). This suggests plasma cell targeting is an option also for the treatment of neuroinflammatory diseases such as neuromyelitis optica (NMO), which is clearly associated with autoantibodies specific for the water channel protein aquaporin 4 (AQP4), as well as with antibodies against myelin oligodendrocyte protein (MOG) in a subset of patients (89). Oligoclonal bands, indicative of intracerebral antibody production, have been regarded as hallmarks in the diagnosis of multiple sclerosis (MS) for decades (90), and B cell-depleting therapies are now utilized successfully to treat MS at least as effectively as conventional therapies [reviewed in (91)]. Axopathic and demyelinating antibodies are present in a subset of patients with MS, but there seems to be a high degree of mechanistic heterogeneity in this disease (92) and plasma cells may also have a role in ameliorating neuroinflammation by providing anti-inflammatory cytokines (93). In experimental autoimmune encephalomyelitis, a mouse model for MS, plasma cells accumulate in the CNS during peak disease and persist in the chronic state (94). Although the actual lifetime of plasma cells in CNS tissue from MS patients has not yet been determined, the fact that oligoclonal bands in the CSF recognize the same epitopes over time (90), together with the finding that $CD138^{+}$ cells in brain tissue from MS patients are non-dividing (94), suggests that plasma cells persist in chronically inflamed human CNS. In EAE, the finding that tissue-specific, resident CNS cells such as astrocytes contribute to the formation of these niches supports the idea that various cell types in many different tissues can support the retention of plasma cells and

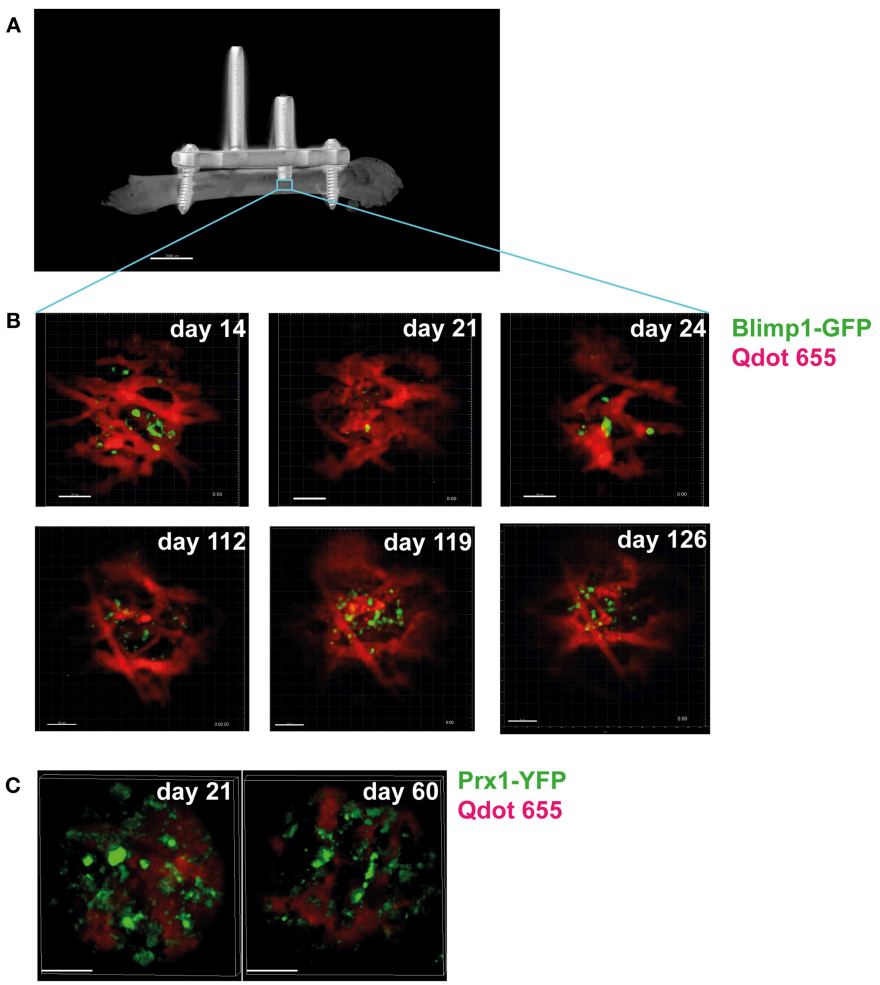

FIGURE 2 | Longitudinal intravital imaging of plasma cells in bone marrow niches. **(A)** Micro-CT image of a mouse femur with an implanted device enabling longitudinal intravital microscopy of the bone marrow (LIMB). This device consists of a titanium-alloy based internal fixator used to fix an imaging port (arrow), which holds a gradient-refractory index (GRIN) lens onto the femur of live mice (55). The lens can be installed to reach deep into the bone marrow, thereby allowing imaging of tissue areas previously inaccessible by intravital microscopy (blue square), such as plasma cell niches, and allows imaging of the same area over months. **(B)** Longitudinal imaging of plasma cell niches reveals dramatic changes in the vascular structure (red) surrounding the plasma cells (green) over time. Time points (days) after implantation of the device are indicated. Scale bar 50 μm. **(C)** Changes are also present in the stromal compartment, as revealed by imaging of reporter mice expressing yellow fluorescent protein in bone marrow stromal cells [Prx-Cre (108) x R26R-eYFP (109)]. Stromal cells are shown in green, vessels in red. Scale bar 50 μm.

transform into plasma cell niches, and that chronic inflammation can induce this process, even in tissues that are void of peripheral immune cells in healthy individuals.

Taken together, strategies for the future include a more selective targeting of plasma cells within the inflammatory niches, but this requires a more detailed knowledge of the cell types forming the niches in various organs under a range of healthy and pathologic conditions. For example, the niches for long-lived plasma cells in inflamed lupus kidneys are not well characterized. A recent publication reported the presence of APRIL-producing macrophages promoting plasma cell accumulation in renal lesions of patients with IgG4-related disease (95), but whether these macrophages are also abundant in kidneys from SLE patients remains to be investigated. The adhesion and survival signals required by plasma cells for persistence are provided by the niche, and are independent of the tissue of residence. As chronic inflammation can promote niche formation in widely varying tissues, the mechanisms by which the various cell types present in these tissue niches provide these signals and thereby affect plasma cell function may also have commonalities.

This review is focused on plasma cells, however, their malignant counterparts, multiple myeloma cells, were actually the first cells that could be efficiently targeted and eliminated, by therapeutically exploiting their high levels of protein production. using proteasome inhibitors. Bortezomib has been clinically used to treat patients with multiple myeloma for over a decade. In 2008, Voll et al. for the first time used Bortezomib to successfully target plasma cells in humoral autoimmunity, using a mouse model of SLE (83). Since then, it has been successfully used to treat human SLE (85, 96) and therapy-refractory anti-NMDA receptor encephalitis (88). More specific proteasome inhibitors such as carfilzomib and ixazomib have been developed, to reduce side effects. As plasma cells no longer express most of the surface molecules characteristic of the B cell lineage, they cannot be targeted by antibodies or cell-based therapies directed against B cells, for example CD20 or CD19 (79, 97). However, cell therapies targeting alternative antigens such as BCMA have demonstrated pre-clinical success and are in clinical trials (98, 99).

The bone marrow stromal microenvironment has been shown to support myeloma cell survival, immune evasion and expansion via contact-dependent mechanisms as well as the secretion of soluble mediators, by inducing the expression of transcription factors such as c-Myc, JunB, and c-Maf, hypo-methylation of histone H3 in promoter regions of anti-apoptotic genes such as *IGF-1* and *Bcl2*, and by modulating miRNA expression. Therefore, future therapeutic strategies should also include targeting the interaction between malignant plasma cells and their microenvironment [reviewed in (100)].

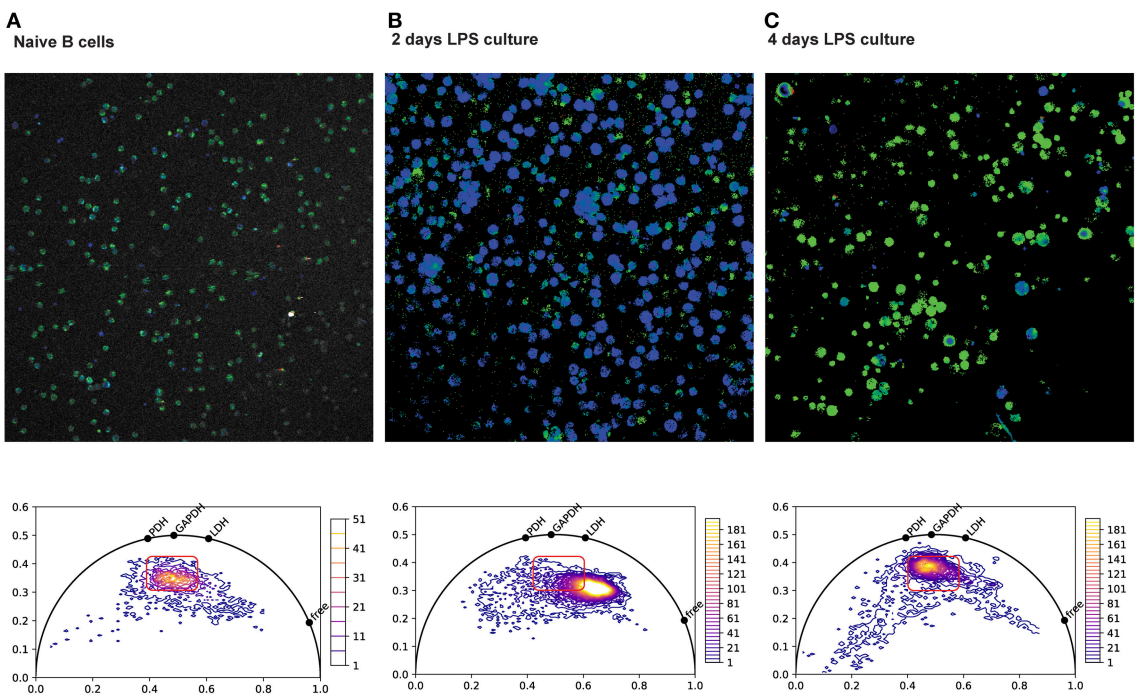

FIGURE 3 | NAD(P)H fluorescence lifetime imaging (FLIM) of splenic plasma cells. Top row: Color-coded NAD(P)H fluorescence lifetime images of splenic B cells isolated by negative immuno-magnetic selection, imaged directly (**A**, naive), or after two **(B)** or four **(C)** days of culture in LPS. Bottom row: Corresponding phasor plots of NAD(P)H fluorescence lifetimes from the images in the top row. Phasor plots are generated from the discrete Fourier transform of the fluorescence decay at each pixel in the image. Points on the semicircle correspond to mono-exponential decay, and points within the semicircle correspond to the intensity-weighted vector sum in of their components. Shorter decay times are along the right of the semicircle, and longer decay times are to the left (132). Hence, pure free NAD(P)H with a fluorescence lifetime of ∼400 ps appears further to the right on the phasor semicircle than NADH bound to lactate dehydrogenase (LDH) at 1,600 ps, NADH bound to gyceraldehyde-3-phosphate-dehydrogenase (GAPDH) at 2,050 ps and NADH bound to pyruvate dehydrogenase (PDH) at 2,470 ps. After 2 days of LPS culture, the rapidly proliferating cells show a marked shift toward shorter NAD(P)H lifetimes, consistent with more free NAD(P)H and a more glycolytic metabolism dominated by LDH.

IMPACT OF THE MICROENVIRONMENT ON PLASMA CELL METABOLISM AND FUNCTION

Lymphocytes' activation states are tightly coupled to their metabolism. Resting cells have relatively low metabolic and synthetic requirements, and typically use oxidative metabolism (101, 102). Upon activation, lymphocytes increase their metabolic and synthetic capacity in preparation for proliferation. Plasma cells, due to their high biosynthetic demands, require an adequate supply of glucose and amino acids. Recent studies examining the metabolism of plasma cells have shown that although plasma cells take up large amounts of glucose, this is predominantly used for antibody glycosylation, not for metabolism (103). Both short- and long-lived plasma cells had similar basal oxidative respiration *in vitro*, but long-lived bone marrow plasma cells had a markedly higher oxidative respiratory capacity, as measured by oxygen consumption after addition of the ionophore FCCP (103). *In vivo*, disruption of the mitochondrial pyruvate transporter Mpc2 led to shortened lifespan and loss of long-lived plasma cells, and decreased antigen-specific antibody titers. Although bone marrow plasma cells used long-chain fatty acids for basal respiration, there were no evident differences between the mitochondrial size or potential in short- or long-lived plasma cells. However, despite their basal use of oxidative respiration, long-lived bone marrow plasma cells took up more glucose than short-lived splenic plasma cells, irrespective of proliferative status (103).

Although the bone marrow is heavily perfused, a significant fraction of the parenchyma is markedly hypoxic. Technical measurements of oxygen tension within tissue are difficult, as invasive measurements with a microelectrode invariably compromise the tissue integrity. Most of the data on tissue oxygen levels is therefore indirect, using expression of hypoxia-induced proteins, or binding of the exogenous hypoxia label pimonidazole, which covalently binds to cellular macromolecules at oxygen tension <10 mmHg, and is detectable by immunostaining (104). An optical method using the phosphorescence lifetime of a platinum porphyrin-coumarin-343 nanoparticle was used to visualize the oxygen tension within calvarial bone marrow (105), which unexpectedly found that the endosteal region is less hypoxic than the perisinusoidal regions, due to small penetrating arterioles. This was somewhat controversial, as it had previously been reported that the endosteum was more hypoxic than the perisinusoidal regions, due to the relatively small diameter of the endosteal arteries (106, 107). This same report used the subset of perivascular Nestin$^+$ cells that define the quiescent HSC niche to demonstrate that the pO2 was higher in the Nestin$^+$ vessels near the bone surface than in the larger-diameter, Nestin$^-$ vessels deeper in the bone marrow cavity. This suggests that HSC quiescence is not regulated by hypoxia *per se*, and raises the possibility that the relative lack of cell division in the Nestin$^+$ niches may lead to a comparatively low usage and demand for O_2, and thereby contribute to the relative hyperoxia of the Nestin$^+$ niche.

It should be noted that these measurements were only conducted over a limited time frame, due to technical limitations of the cranial bone marrow imaging window; it was therefore not possible to image the same region over a prolonged interval to examine the degree to which the oxygen tension in individual regions varies over time.

Recently, a method developed by our group (55) that permits longitudinal imaging of bone marrow over the time course of several months has demonstrated that the structure of the blood vessels in the bone marrow dynamically changes, on a time scale of days (small vessels) to weeks (larger vessels). This is also evident in vessels surrounding bone marrow plasma cells (**Figure 2**). This strongly suggests that the variability in the provision of nutrients and O_2 does not only depend on spatial conditions, but also changes over time. It is currently unclear how long-lived plasma cells deal with these changes, since they have a high biosynthetic demand, given that they can secrete >1,000 antibody molecules per second (110). To permit this massive amount of protein synthesis and secretion, plasma cells dramatically up-regulate the unfolded protein response (111, 112). Autophagy is one component of this, which is required for the survival of long-lived plasma cells in the bone marrow (113). Cre-mediated deletion of the autophagy factor Atg5t in B cells led to increased ER size and ER stress signaling, which led to higher BLIMP-1 expression and increased antibody secretion (114). However, this higher-level antibody synthesis caused a pronounced decrease in viability, leading to lower antibody responses and markedly lower numbers of long-lived bone marrow plasma cells, with the plasma cells in the bone marrow all having escaped Atg5 deletion (114).

This up-regulation of the unfolded protein response is intrinsic to the plasma cell fate, and is controlled by the master plasma cell transcription factor Blimp1 (115). Interestingly, high-level antibody secretion is not necessary for plasma cell survival, as deletion of the unfolded protein response-related transcription factor Xbp1 specifically in B-lineage cells led to markedly reduced antibody synthesis, but no appreciable differences in plasma cell proliferation, differentiation, or cell number in both resting and immunized mice (116). This is consistent with the observation that the rate of antibody secretion is not uniform across all plasma cells (117). It may suggest that plasma cell antibody synthesis should not be expected to be constant over time, but to vary widely in all plasma cells, and to be influenced by the perfusion and oxygenation status of the plasma cell niche. The finding that inhibition of the cellular nutrient sensor mammalian target of rapamycin complex 1 (mTORC1) blocks antibody synthesis without affecting long-lived plasma cell survival is very interesting in this context (118). Of note, mTOR-inhibition not only prevents the development of lupus nephritis (119), it also effectively attenuates established disease in NZB/W F1 mice (120).

TOWARD UNDERSTANDING THE LINK BETWEEN PLASMA CELL METABOLISM AND FUNCTION *IN VIVO*: NEW TOOLS ON THE HORIZON

Taken together, there is a strong demand for methodologies that allow monitoring of plasma cell metabolism over time *in vivo*. In general, metabolically active cells have less free NAD(P)H and more enzyme-bound NAD(P)H. It has been known since the 1960s that the fluorescence of the endogenous cofactor NAD(P)H can be used to measure the metabolic activity of cells (121). The fluorescence lifetime of NAD(P)H gives information about the relative amounts of free and protein-bound NAD(P)H, and reflects the enzymes to which NAD(P)H is bound (122, 123). Actively proliferating cells have been shown to have more free NAD(P)H, in addition to a longer fluorescence lifetime (124, 125). However, malignant cell lines have shorter NAD(P)H fluorescence lifetimes than relatively benign, more slowly-proliferating cell lines. As the fluorescence lifetime for free NAD(P)H is markedly shorter than for enzyme-bound NAD(P)H, this indicates that there is not a simple 1:1 correspondence between cell proliferation and shorter NAD(P)H half-lives. Instead, it will depend on the specific NAD(P)H-binding enzymes in the cells of interest: on their NAD(P)H fluorescence lifetimes, and the extent to which they are bound.

We and others have recently developed methods to "fingerprint" specific NAD(P)H –binding enzymes, using the lifetime of NAD(P)H fluorescence (126–131), and are applying these methods to visualize the metabolic status of single cells in tissue. Our data demonstrates that B cell blasts have a markedly shorter NAD(P)H half-life than do naive splenic B cells (**Figure 3**) (133). As cell proliferation slows down, the NAD(P)H lifetime lengthens again, to values similar to those of naive B cells. This is consistent with increased levels of free NAD(P)H in rapidly proliferating cells, as previously reported (124, 125), in addition to a shift from oxidative to glycolytic metabolism. Bone marrow plasma cells, sorted and analyzed *in vitro* (data no shown), had a fluorescence lifetime similar to that of naive B cells, consistent with a largely oxidative metabolism. Although plasma cells take up large amounts of glucose, as it is used for antibody glycosylation (103), it has no bearing on the cellular metabolic status.

Despite being a promising approach for deciphering the metabolic activity of cells *in vivo*, in tissue context, NAD(P)H fluorescence lifetime imaging (FLIM) has been used only in the last few years for this purpose, with most of the studies investigating single time points (127–129, 134). As of this writing, there have been no NAD(P)H-FLIM studies concerned with the metabolism of long-lived plasma cells in the bone marrow. The lack of *in vivo* NAD(P)H-FLIM studies is due to technical limitations in acquiring and analyzing images of fluorescence lifetimes that have only recently been overcome. For example, frequency-domain FLIM technologies are prone to numerical artifacts when analyzing complex exponential fluorescence decays (135). Further, they are based on field-detection using cameras, which makes their use in highly scattering media such as tissue very difficult. Only a few years ago, time-domain FLIM technologies, of which time-correlated single-photon counting is the best suited for deep-tissue *in vivo* studies (126), relied on analyzing less than 5% of the emitted photons. This necessarily required extremely high photon counts, incompatible with deep-tissue or dynamic imaging. First the parallelization of detection and photon counting and the use of hybrid photomultiplier detection in FLIM led very recently to the successful acquisition of time-lapse FLIM movies (126, 130). These time-lapse FLIM experiments make us confident that NAD(P)H-FLIM combined with longitudinal bone-marrow imaging will open new insights into the enzymatic and metabolic lifestyle of long-lived plasma cells. There are significant technical challenges in measuring NAD(P)H fluorescence lifetimes *in vivo*, in particular the relatively low intrinsic fluorescence of NAD(P)H, and the high density of cells. However, despite these challenges, *in vivo* NAD(P)H FLIM remains the most promising method of imaging the metabolic status of single cells, and provides an unparalleled view into the metabolic and functional dynamics of cells in their native tissue context-especially the plasma cell niche.

CONCLUDING REMARKS

Recent progress in the fields of immunometabolism and single-cell analysis and has led to an improved understanding of plasma cell biology. Due to the importance of the microenvironment in promoting plasma cell function and survival, functional analyses of plasma cells in the tissue context will be key in order to fully understand their lifestyle. This will be critical in developing better ways to target pathologic plasma cells in autoimmunity, malignant plasma cells in multiple myeloma, and also to enhance antibody secretion to improve the efficacy of vaccination.

AUTHOR CONTRIBUTIONS

RL generated NAD(P)H-FLIM data. RN and AH generated longitudinal intravital imaging data of the plasma cell niches. All authors reviewed literature and contributed to writing of the manuscript.

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Role of Multivalency and Antigenic Threshold in Generating Protective Antibody Responses

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Vaccines play a vital role in protecting our communities against infectious disease. Unfortunately, some vaccines provide only partial protection or in some cases vaccine-mediated immunity may wane rapidly, resulting in either increased susceptibility to that disease or a requirement for more booster vaccinations in order to maintain immunity above a protective level. The durability of antibody responses after infection or vaccination appears to be intrinsically determined by the structural biology of the antigen, with multivalent protein antigens often providing more long-lived immunity than monovalent antigens. This forms the basis for the Imprinted Lifespan model describing the differential survival of long-lived plasma cell populations. There are, however, exceptions to this rule with examples of highly attenuated live virus vaccines that are rapidly cleared and elicit only short-lived immunity despite the expression of multivalent surface epitopes. These exceptions have led to the concept that multivalency alone may not reliably determine the duration of protective humoral immune responses unless a minimum number of long-lived plasma cells are generated by reaching an appropriate antigenic threshold of B cell stimulation. Examples of long-term and in some cases, potentially lifelong antibody responses following immunization against human papilloma virus (HPV), Japanese encephalitis virus (JEV), Hepatitis B virus (HBV), and Hepatitis A virus (HAV) provide several lessons in understanding durable serological memory in human subjects. Moreover, studies involving influenza vaccination provide the unique opportunity to compare the durability of hemagglutinin (HA)-specific antibody titers mounted in response to antigenically repetitive whole virus (i.e., multivalent HA), or detergent-disrupted "split" virus, in comparison to the long-term immune responses induced by natural influenza infection. Here, we discuss the underlying mechanisms that may be associated with the induction of protective immunity by long-lived plasma cells and their importance in future vaccine design.

Keywords: vaccines, antibody, immunological memory, VLP, HPV, JEV, HAV

INTRODUCTION

During an antigenic insult triggered by vaccination or infection, a series of immunological events unfold including the activation, proliferation, differentiation, and coordination of antigen-specific B cells and T cells that, if successful, will develop into an appropriate immune response that provides protective immunity upon re-encounter with the pathogen of interest. Pre-existing antibodies maintained in the circulation or along mucosal surfaces represent the first line of defense against reinfection and a better understanding of the parameters associated with inducing long-term antibody responses is key to the development of better, more effective vaccines.

ROLE OF PLASMA CELLS AND MEMORY B CELLS IN MAINTAINING LONG-TERM ANTIBODY RESPONSES

Humoral immunity is built upon two main antigen-specific B cell populations; memory B cells (MBC) and plasma cells (PC). Although detailed discussion of MBC subsets and differentiation pathways are beyond the scope of this review, the reader is referred to several excellent reviews on these topics (1–3). In a broad sense, MBC and PC populations are often polar opposites in terms of basic phenotype and function. Class-switched MBC do not secrete antibodies *per se* but instead maintain expression of membrane-bound immunoglobulin that assists in their immunosurveillance capacity and increases their ability to bind and present antigen to $CD4^+$ T cells through MHC Class II (4, 5). Upon activation, MBC are able to proliferate and differentiate into antibody-secreting plasmablasts/PC as well as generating more MBC. In contrast, long-lived PC are defined as terminally-differentiated non-dividing cells that no longer express membrane-bound immunoglobulins due to constitutive secretion of antibody molecules (6, 7). Fully differentiated PC also down-regulate MHC Class II expression (6, 7), resulting in a reduced ability to present peptide epitopes to $CD4^+$ T cells. Early immunological studies indicated that PC (and most likely plasmablasts) had a relatively short lifespan, on the order of a few days to a few weeks (8–11). This finding was in stark contrast to the long-lived serum antibody responses that are commonly found after infection or vaccination and this led to several MBC-dependent theories for maintaining long-term antibody production, mostly involving reactivation and proliferation of MBC in order to repopulate short-lived plasma cell numbers. These theories have been described previously in detail (12–15) and questions regarding the need for MBC to replenish long-lived PC populations have been largely resolved based on analysis of PC survival after B cell depletion studies performed in mice (6, 16–18), humans (19–26), and non-human primates (NHP) (27). In addition, a recent case study using high-throughput sequence analysis of bone marrow-resident PCs from human subjects identified the persistence of multiple bone marrow PC clonotypes over a 6.5-year period, further supporting the concept of intrinsic PC longevity (28). Mice are a well-characterized model for studying humoral immune responses, but since mice live only 1–3 years, it is difficult to determine the true long-term survival of PC in the absence of MBC. Clinical studies in humans provide insight into the long-term durability of antibody responses after B cell depletion, but since $CD20^+$ B cell depletion by rituximab may not fully deplete B cells from all lymphoid tissues (29–34), one could argue that a small proportion of residual MBC may still contribute to the maintenance of the PC pool. B cell depletion experiments performed in NHP provides another useful approach by combining the attributes of a versatile animal model with the ability to measure long-term antibody responses that can be followed for many years similar to those achieved in clinical studies. In longitudinal experiments spanning ∼10 years of study, we monitored antiviral antibody responses to adenovirus, rhesus cytomegalovirus (RhCMV), and a measles-like paramyxovirus in addition to direct vaccine-mediated antibody responses to tetanus and three pertussis antigens including pertussis toxin, filamentous hemagglutinin (FHA), and pertactin (27). To determine the role of MBC in maintaining serum antibody levels, a group of animals had their peripheral MBC removed by anti-CD20 depletion in addition to surgical removal of the spleen and draining lymph nodes to eliminate the possibility of residual MBC populations or persisting antigen in the form of immune complexes on follicular dendritic cells (FDCs) residing in these lymphoid sites. Serum antibody responses to the aforementioned virus and vaccine antigens were monitored for several years and antibody titers in the MBC-depleted animals remained as durable as those observed in the non-depleted control animals. This indicates that MBC are not required to maintain antigen-specific PC populations and long-term antibody production. As a secondary approach to identifying long-lived PC, BrdU (bromodeoxyuridine) incorporation studies were performed at the time of vaccination and non-dividing $BrdU^+$ PC could be detected by histology for up to 10 years after immunization/BrdU administration. Together, these independent approaches provide further support for the theory that non-dividing PC can persist for many years and continue to maintain serum antibody levels in the absence of an intact MBC pool.

It is important to note that although MBC are not required for directly maintaining PC numbers and serum antibody titers, they still play an important role in host defense (3). In cases in which pre-existing antibody responses are too low to block infection, MBC may traffic to sites of infection as well as contribute to rapid anamnestic antibody responses. Another interesting attribute of MBC is that they have been found to develop a broader range of specificities than those observed in the primary PC pool and have the capacity to provide protection against variants of the original pathogen that escape neutralization by immunodominant serum antibodies (35). For some viruses such as hepatitis B virus (HBV), MBC may contribute directly to protection since cases have been reported of vaccinated individuals who are at least transiently infected with HBV and seroconvert to non-structural HBV antigens but do not develop into a carrier state or show clinical manifestations of disease (36, 37). This indicates that for pathogens that are slow at inducing a disease state, there may be enough time for anamnestic MBC-mediated immune responses to participate in clinically relevant immune defense.

MULTIVALENT WHOLE-VIRUS OR VIRUS-LIKE PARTICLE (VLP) VACCINES INDUCE LONG-TERM IMMUNITY

One commonly held misconception regarding immunological memory is the idea that live-attenuated vaccines elicit life-long immunity after a single immunization whereas non-replicating protein vaccines elicit only short-lived immunity (38). This assertion most likely comes from prior clinical observations in which natural infection in childhood often leads to lifelong immunity but when vaccines against those same pathogens are developed using inactivated or live-attenuated strains of the wild-type pathogen, in many cases a booster vaccination is required in order to sustain host immunity above a protective threshold (38). Here, we provide four examples of multivalent VLP or whole-virus vaccines that elicit long-term, potentially life-long immunity after completion of the recommended vaccination series.

Human papilloma virus (HPV) is a non-enveloped DNA virus that is 55 nm in diameter that causes cervical cancer as well as a number of other genital and oropharyngeal cancers. Globally, cervical cancer is the third leading cause of cancer among women with an estimated 530,000 new cases and 275,000 annual deaths (39). There are currently three HPV vaccines on the market; Cervarix (HPV16/18), Gardasil (HPV6/11/16/18), and Gardasil-9 (HPV6/11/16/18/31/33/45/52/58). Each vaccine is formulated with alum except that Cervarix contains AS04, a combination of alum and the toll-like receptor 4 (TLR4) agonist, monophosphoryl lipid A (MPL). Each vaccine is composed of VLP comprising an array of 360 copies of the L1 major capsid protein arranged in a densely packed pentameric array (40). HPV vaccination was initially designed as a 3-dose vaccination series. However, in a Phase III trial of Cervarix in Costa Rica, researchers were surprised to find that 4-year vaccine efficacy against persistent HPV16/18 infection was similarly high among women who received 1, 2, or 3 doses of vaccine (41). Further analysis of serum antibody titers provided a unique opportunity to determine the kinetics of antibody decay in relation to different booster vaccination regimens (**Figure 1A**) (42). Following the 3-dose regimen, high titers of anti-HPV16 (and anti-HPV18) were maintained for the 4-year span of observation. Similar results showing a well-maintained plateau of antibody production have been observed in prior studies with clinical observations of stable protection against disease and stable antibody titers for up to 9 years after vaccination (39). Likewise, long-term antibody responses have also been observed after Gardasil-9 vaccination, indicating that even when 9 closely related VLP antigens are combined into one vaccine, long-term immunity to each of the individual virus serotypes is maintained (44). We calculated that after an initial antibody decay rate of $T_{1/2}$ = 14 months from years 1 to 2 after a 3-dose regimen in the Costa Rican study, the antibody half-life from years 2 to 4 post-vaccination slows to ~$T_{1/2}$ = 3.5 years (**Figure 1A**). Following a 2-dose schedule (either 0/1 month or 0/6 month regimen), the initial antibody decay rates appeared to be similar to the 3-dose series ($T_{1/2}$ = 11.6 and 14 months for the 0/1 and 0/6 schedules, respectively) and between years 2 and 4, the antibody $T_{1/2}$ = 2.9–6.7 years. Although the 0/6 vaccination group achieved higher peak antibody titers compared to the 0/1 group, by 4 years post-vaccination the antibody titers appeared similar. These results may be unique to HPV since booster immunization studies with other vaccines indicate that longer time intervals between booster vaccinations often results not only in higher peak titers, but

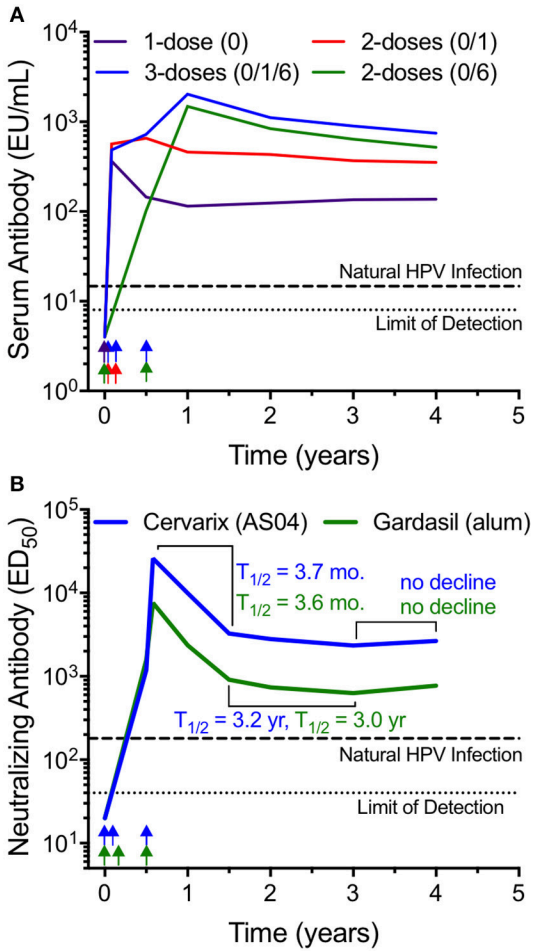


FIGURE 1 | Long-term antibody maintenance after vaccination with HPV VLP vaccines with or without Toll-like receptor 4 (TLR4) agonists. **(A)** Serum antibody responses to HPV16 were monitored by ELISA for up to 4 years following various vaccination schedules (1 dose; 2 doses at 0 and 1 month; 2 doses at 0 and 6 months; 3 doses at 0, 1, and 6 months) with AS04-adjuvanted, VLP-based Cervarix® HPV vaccine. The graph was prepared using data provided in **Table 1** from Safaeian et al. (42). Longitudinal analysis was limited to subjects with sera available at each time point, with group sizes ranging from 52 to 140. **(B)** Neutralizing antibody responses against HPV16 were assessed longitudinally following a three-dose vaccination schedule with either Cervarix (AS04 adjuvant), or Gardasil® (alum-only adjuvant). The graph was prepared by combining the HPV16-specific neutralizing antibody titers from 3 age groups (18–26, 27–35, and 36–45 years of age) using the geometric mean of the data presented in **Figure 2** from Einstein et al. (43). This dataset was comprised of 136 subjects that received Cervarix and 139 subjects that received Gardasil. For both panels, antibody levels after natural HPV infection are shown as a dashed line and the limit of detection is shown as a dotted line. Approximate vaccination time points are indicated by arrows. EU; ELISA Units, ED_{50}; 50% effective dose.

also appears to increase levels of antibodies during the early plateau phase of the vaccine-mediated immune response (37, 45). Importantly, the peak and plateau levels of anti-HPV antibodies are similar following the 2-dose and 3-dose schedules and based on the information provided to the ACIP (Advisory Committee on Immunization Practices) showing that a) the 3-dose schedule elicits long-term protection and b) a 2-dose schedule elicits similar antibody titers to a 3-dose schedule, in 2016 the ACIP changed their HPV vaccine recommendations from a 3-dose to a 2-dose schedule for girls and boys ages 9 through 14 years (46).

Interestingly, following a single HPV vaccination the initial antibody decay rate from 1 to 12 months was $T_{1/2} = 6.7$ months but showed no appreciable decline from 2 to 4 years post-vaccination. The single dose vaccination schedule is noteworthy because although antibody titers plateaued at levels that were about 5-fold lower than that achieved by the 2-dose or 3-dose series, these antibody titers are still maintained above the levels observed after natural infection and it shows that a booster vaccination (or the accompanying re-activation of MBC during a boosted immune response) is not required in order to generate long-lived PC that can maintain protective steady-state antibody production. This also indicates that HPV vaccination may be one of the few vaccines capable of inducing prolonged, potentially life-long immunity after a single vaccination. However, this may pertain only to protection against vaccine-targeted serotypes HPV16 and HPV18 since further analysis indicates that a single dose of vaccine was suboptimal compared to the 3-dose series at inducing cross-reactive antibody responses to other clinically relevant HPV serotypes including HPV31 and HPV45 (47). This suggests that similar to other vaccines (38), even the impressive immunological memory induced by HPV vaccination might be further improved by administering at least one booster vaccination.

The role of TLR agonists in generating durable antibody responses is a question of considerable interest and much has been learned by studies involving HPV vaccination. Unlike inactivated whole virus particles that may contain viral RNA with the potential to trigger TLR7 activation, HPV VLP do not contain viral RNA yet still induce long-lived, potentially lifelong antibody responses (**Figure 1**). During clinical development of Cervarix, HPV16/HPV18 VLP vaccine antigen was prepared either with AS04 (i.e., alum plus MPL) or with alum alone to assess the role of TLR4 engagement by MPL on vaccine-induced antibody responses during HPV vaccination (48). Peak anti-HPV titers occurred at 1 month after the third dose of vaccine and were ~2- to 3-fold higher among the subjects immunized with the MPL-containing AS04 formulation compared to vaccination with HPV containing only the alum adjuvant regardless of whether HPV-specific ELISA or pseudo-neutralizing assays were employed (48). However, by 3.5 years after the last administered dose of vaccine, there was only a 1.5-fold (HPV16) to 2.1-fold (HPV18) difference in virus-specific antibody titers, indicating that although TLR4 stimulation may increase peak antibody levels, the difference in the magnitude of antibody responses becomes relatively minor within the first few years after vaccination.

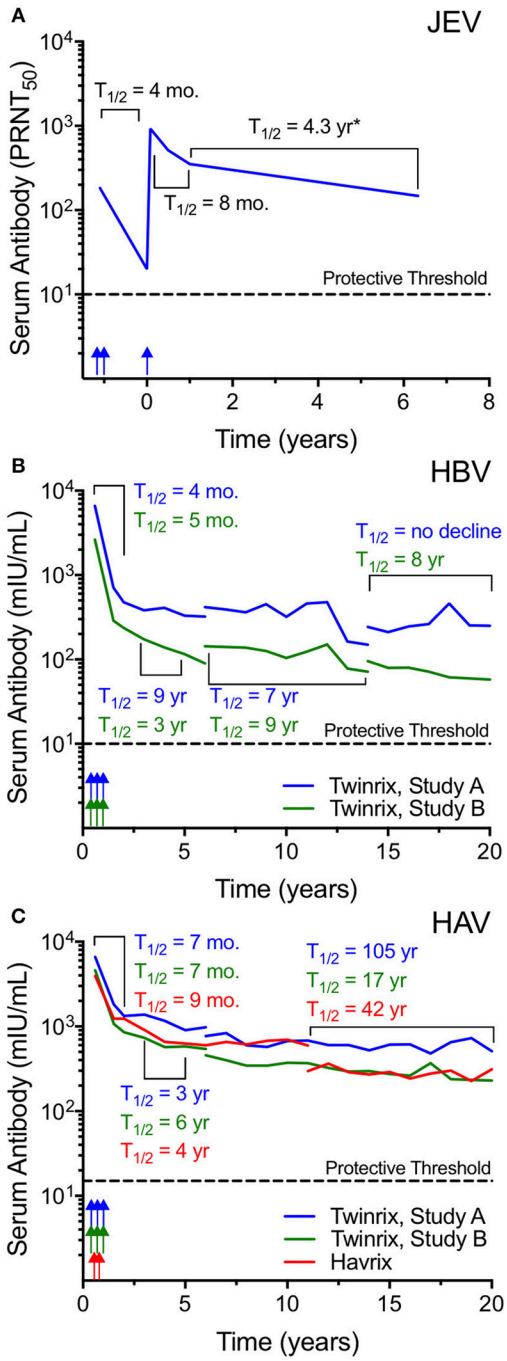


FIGURE 2 | Inactivated whole-virus vaccines induce long-lived antibody responses. **(A)** The alum-adjuvanted, inactivated whole-virus Ixiario® JEV vaccine was used to immunize 67 subjects with a two-dose primary series spaced 28 days apart followed by a third dose administered at 15 months after the conclusion of the primary series. The figure was constructed using the primary data provided in Table 2 of Paulke-Korinek et al. (53). Serum samples were assessed for JEV-specific neutralizing activity (plaque-reduction neutralizing titer 50%; $PRNT_{50}$) for up to 6 years post-vaccination. The putative protective threshold of $PRNT_{50} \geq 10$ is shown as a dashed line. Vaccination time points are indicated by arrows. **(B,C)** Vaccine-induced serum antibody responses were monitored for up to 20 years in subjects receiving either a combined hepatitis B virus (HBV) and hepatitis A virus (HAV) vaccine

(Continued)

FIGURE 2 | (Twinrix®), or an HAV-only vaccine (Havrix®) (54, 55). HBV surface antigen (HBsAg) forms pleomorphic, irregular-shaped VLP whereas HAV consists of purified, inactivated whole-virus and both vaccines are formulated with alum. The Twinrix vaccine **(B,C)** was administered as a three-dose series (0, 1, and 6 months) and included 2 cohorts (Study A and Study B). Subject attrition resulted in fewer subjects included at later time points with 18/107 subjects in Study A and 22/116 subjects in Study B completing visits up to 20 years post-vaccination. The Havrix vaccine (**C**) was administered as a two-dose series at 0 and 6 months (55) with 34/76 subjects completing the 20 year study. Color-coded arrows indicate the approximate vaccination schedule. At various points, assay kits for HBV and HAV antibody measurements changed, and these changes are indicated by line breaks. Data for geometric mean antibody titers were obtained from clinical study reports available on-line (https://gsk-clinicalstudyregister.com/) and antibody half-lives were calculated for the select periods as indicated. The putative protective threshold for HBV and HAV are indicated with dashed lines. mIU/mL; milli-international units per milliliter. *JEV-specific antibody half-life may underestimate the durability of antibody responses after vaccination due to inclusion of the 1-year time point, a period in which antibody responses have not plateaued and are still declining rapidly.

Another longitudinal study comparing Cervarix (AS04) to Gardasil (alum-only) provides further insight into the role of TLR stimulation for inducing long-lived antibody responses (**Figure 1B**) (43). Following each 3-dose vaccination series, anti-HPV antibody titers peak at 7 months (i.e., 1 month after the final vaccination) and decay with similar initial half-life estimates of $T_{1/2} = 3.7$ and 3.6 months for Cervarix and Gardasil, respectively. From 1.5 to 3 years post-primary vaccination (i.e., 1–2.5 years post-final vaccination), antiviral antibodies decay with slower, albeit similar kinetics with half-life estimates of $T_{1/2} = 3.2$ and 3.0 years, respectively. By 3–4 years post-primary vaccination, anti-HPV antibody titers have plateaued, with no further evidence of decline following administration of either vaccine formulation. Together, these studies demonstrate that a standard alum formulation provides durable antiviral immunity and addition of a TLR4 agonist appears to play little to no role in the overall durability of long-lived vaccine-mediated antibody responses.

Japanese encephalitis virus (JEV) is an enveloped flavivirus that is endemic in South-East Asia and the Western Pacific. The JEV vaccine, IXIARO, is FDA-approved for use in the U.S. for the purposes of protecting international travelers and military personnel who plan on entering JEV-endemic areas. The vaccine contains alum-adjuvanted formalin-inactivated whole virus that is ∼50 nm in diameter. Flaviviruses are secreted from infected cells as a mixture of mature and immature virus particles. In its immature form, flavivirus particles contain 60 heterotrimers of the envelope and premembrane protein arranged in an icosahedral configuration (49, 50) whereas in its mature form, the virus is comprised of 180 copies of the envelope protein in 90 antiparallel dimers arrayed in a herringbone fashion (51, 52). Following a primary JEV vaccination series of doses administered at 0 and 1 month, the geometric mean titers (GMT) reached 1:183 by 1 month after the second dose (**Figure 2A**) (53). Antibody titers declined rapidly ($T_{1/2} = 4$ months) during the first year after vaccination to a GMT of 1:20 and even though 96% of subjects were still seropositive at this time point, this represents a neutralizing titer that is only 2-fold above the protective threshold of 1:10. A third dose of vaccine resulted in much higher antibody titers that peaked at 1:927 by day 28 after the third dose and this elicited long-term immunity in which 96% of subjects remained seropositive (GMT = 1:148) when examined at 6 years after final vaccination (**Figure 2A**). The authors used mathematical modeling to estimate the duration of protection using a log linear function with a structural break at 6 months post-booster vaccination and predicted that protection would last 14 years. When we estimated antibody half-life based on this dataset there was approximately an 8 month half-life from the peak through the first year after the third dose and from 1 to 6 years after booster vaccination the average antibody $T_{1/2} = 4.3$ years. However, these estimates are likely to be an underestimation of the true durability of the antiviral immune response because they are based in part on the 1 year time point when antibody responses are still rapidly declining and based on the data in **Figures 1**, **2**, and other studies (14, 56–58), antibody decay rates typically do not reach their stable kinetic profiles until 3–4 years after vaccination or infection. This means that the >90% seroprotection rate observed at 6 years after booster vaccination may represent a plateau that could be maintained for much longer than the estimated 14-year time frame. Future studies will require obtaining samples during the plateau phase of the immune response between 3 and 10 years post-vaccination in order to accurately predict the persistence of immunity at later time points but it is possible that a 3-dose regimen could provide long-term and potentially lifelong protection against JEV.

Hepatitis B virus (HBV) is a major cause of human morbidity and mortality with an estimated 620,000 HBV-associated deaths occurring globally in 2000 and implementation of routine infant immunization is anticipated to reduce of HBV-associated fatalities by >80% (59). The native HBV particle is ∼42 nm in diameter whereas the HBV vaccine contains subviral particles comprised of hepatitis B surface antigen (HBsAg) that are ∼22 nm in size (60, 61). *In vivo*, the HBV HBsAg is produced in vast excess as a decoy molecule and it is pleomorphic with shapes that range from spherical or ovoid to rod-shaped, tubular or even filamentous (37). There are several recombinant HBV VLP vaccines on the market with at least 25 vaccines that have been pre-qualified by the World Health Organization (37). The Twinrix™ vaccine is a combination vaccine against HBV and hepatitis A virus (HAV) that is formulated with alum and the duration of vaccine-mediated antibody responses to Twinrix have been monitored for 20 years among two cohorts; Study A and Study B (**Figures 2B,C**) (54). Following a 3-dose schedule administered at 0, 1, and 6 months, anti-HBV antibody responses decline rapidly for the first 2 years after vaccination ($T_{1/2} = 4$–5 months) followed by more durable maintenance of antibody levels thereafter (**Figure 2B**). Over the 20 years of observation, there were several technical changes that resulted in breaks in the longitudinal studies when switching from one serological assay to another. From 3 to 5 years post-vaccination, antibody $T_{1/2} = 3$–9 years, from 6 to 14 years post-vaccination, HBV-specific antibody $T_{1/2} = 7$–9 years, and from 14 to 20 years after vaccination, anti-HBV titers continued to decline with $T_{1/2} = 8$ years to infinity

(i.e., no decline observed in Study A). The apparent increase in antibody levels near the end of Study A appears to be an aberration when compared to the earlier time points within this cohort as well as to the decay rates observed among Study B subjects. The increase in antibody from 15 to 20 years post-vaccination may be due to exposure event(s) or possibly due to the limitations associated with study subject attrition with fewer individuals included at these later time points [at Year 20 there were 28/150 subjects (18.6%) in Study A and 25/131 (19%) subjects remaining in Study B]. Mathematical modeling predicted that ≥50% of vaccinated subjects would maintain antiviral antibodies at ≥10 mIU/mL for 40 years after vaccination (54). The decline in antibody titers below this threshold in itself may not be of great concern because although it is recognized that serological titers of ≥10 mIU/mL protect against HBV infection, as long as subjects have received at least 3 doses of an HBV vaccine and developed an initial antibody response, they continue to be protected against chronic HBV infection and clinical disease (36, 37).

Hepatitis A virus (HAV) is endemic in many low- and middle-income countries with millions of people at risk and it is associated with ∼15,000 deaths annually (62). HAV is a non-enveloped icosahedral virus particle that is ∼27 nm in diameter (63–66) and comprised of 32 capsomeres (each capsomere containing 5 protomers with each protomer made of 3 proteins; VP1, VP2, and VP3). There are a number of inactivated HAV vaccines on the market including Twinrix, Havrix, Avaxim, Epaxal, Healive, and VAQTA. The durability of HAV vaccination (Havrix) following a 2-dose schedule (55) was compared to the HAV titers elicited after the 3-dose Twinrix vaccination series containing both HAV and HBV antigens (**Figure 2C**). Similar to HBV (**Figure 2B**), anti-HAV antibody titers declined rapidly during the first 2 years after vaccination with $T_{1/2}$ = 7–9 months. This was followed by a slower antibody decay rate of $T_{1/2}$ = 3–6 years from 3 to 5 years after vaccination. There was more variability at the later time points measured from 11 to 20 years post-vaccination with antibody $T_{1/2}$ = 105, 17, and 42 years for Twinrix Study A, Twinrix Study B, and Havrix, respectively. Similar to HBV, the increased variability in half-life estimates is likely due to the attrition of participating study subjects over time but nevertheless the data shows that antibody responses are durably maintained well-above the protective threshold. Moreover, this data indicates that there is no further benefit to a 3-dose schedule over a 2-dose schedule (Twinrix vs. Havrix) in terms of the induction of long-term and potentially life-long immunity against HAV. Mathematical modeling studies of anti-HAV antibody responses indicate that they are likely to persist for 25 years, 40 years or even longer (55, 67, 68) without requiring further booster vaccination in order to maintain long-lived PC numbers and protective serum antibody levels.

Review of the vaccine-induced antibody responses to the 4 representative viruses described in **Figures 1**, **2** show several similarities as well as some interesting differences. In terms of similarities, the early kinetics of antibody decay during the first 1–2 years after booster vaccination declined with a 4- to 14-month half-life for each of the vaccines. From 2 to 4 years post-booster vaccination (HPV), 3–5 years post-booster vaccination (HBV, HAV), or 1–6 years post-booster vaccination (JEV), antibody responses declined with approximately a 3- to 9-year half-life regardless of the vaccine antigen under study. This indicates that for alum-adjuvanted protein vaccines, there may be a relatively common set of early decay rate curves that could potentially be used to predict long-term antibody maintenance. In contrast, at >5 years post-vaccination, comparison of HBV and HAV titers revealed a different story. Although their first two stages of decay rate kinetics were similar, at later time points the HBV titers continued to decline with a 7–9-year half-life similar to that observed from samples taken 3–5 years post-vaccination whereas the HAV titers had stabilized with a 17-, 42-, or 105-year half-life (roughly, average half-life of 55 years). These differences are unlikely to be due to variability in vaccination procedures since the Twinrix studies included both HBV and HAV antigens within the same vaccine formulation. Another proposed reason for the more rapid antibody decay rate kinetics observed with HBV vaccination is that the HBV subviral particle/VLP is a relatively small particle (∼23 nm) compared to other VLP such as HPV. However, the intact HAV virus particle is similar in size (∼27 nm) and so it seems unlikely that particle size alone can explain the differences in long-term antibody maintenance between HAV and HBV obtained from the same vaccine (Twinrix). The 7–9-year half-life observed at late time points after HBV vaccination is reminiscent of other data with monovalent protein antigens such as tetanus and diphtheria, which have longitudinal half-lives of 11–19 years, respectively (58, 69). In contrast to the more rigid structure of the non-enveloped HAV particle, HBV HBsAg-based VLPs are unusual because they are about half of the size of the native infectious virus particle, contain small 1 nm pores that are thought to be channels for small molecules (70), and are pleomorphic with examples of different sized spherical, ovoid, or even filamentous forms observed *in vivo* (37, 70–72). During clinical vaccine production in yeast cells, more recent studies noted that by electron microscopy, HBV VLPs were 23.09 nm in diameter (range; 20–30 nm), but when the same vaccine material was measured by dynamic light scattering (DLS), the average particle size was 55.91 nm and the size distribution of HBV VLP ranged broadly from 20 to 180 nm (60). It is believed that larger particles were not observed because of a sterile filtration step during the manufacturing process that would have removed particles>200 nm in diameter. However, this indicates that in practice, the yeast-derived HBsAg/VLPs produced in clinical vaccine preparations may often be dimeric or constitute even larger agglomerates (confirmed by electron microscopy) and if these antigenic preparations are aggregates (60, 73) that have a disregular order, we speculate that multimeric crosslinking of the B cell receptor (BcR) on antigen-specific B cells could be suboptimal. This may explain why antiviral antibodies mounted against the HBsAg subviral particles have a half-life that is more similar to alum-adjuvanted non-repetitive proteins such as tetanus and diphtheria, rather than the more long-lived antibody responses induced by the highly repetitive structures on HAV particles within the same vaccine formulation (**Figure 2C**).

INFLUENZA: COMPARISON OF REPETITIVE VS. NON-REPETITIVE ANTIGEN

Despite more than 60 years of vaccine research and development, influenza remains a pressing public health concern, with the US experiencing >200,000 hospitalizations and over 20,000 deaths per year (74–76). The 2017–2018 season was particularly severe with ∼900,000 influenza-associated hospitalizations and 80,000 influenza-associated deaths in the US (77). This indicates that despite vaccination coverage in 2017–2018 of up to 47% of the US population (and ∼68% of children aged 6 months to 4 years) (77), more work is needed to find new and improved vaccines against this potentially life-threatening disease. Influenza A is responsible for the majority of human infections and while comprised of many different subtypes, it is H1N1, H2N2, and H3N2 that are the primary subtypes associated with pandemics and large seasonal outbreaks observed over the last 100 years (78, 79). Until recently, the durability of immune responses to influenza in the absence of reinfection were difficult to determine due to the potential for re-exposure to the same strains or similar antigenically-related "drifted" strains of the virus that caused the initial infection. However, a recent study in Finland describing the 2009 influenza A/H1N1 pandemic [A(H1N1)pdm09] has revealed evidence that subtype-specific immunity against influenza may indeed be lifelong. Using banked serum samples obtained in 2005 (i.e., prior to the pandemic of 2009), the study found that ≤3% of samples from subjects born between 1950 and 2005 had cross-reactive hemagglutinin inhibition (HAI) antibodies to A(H1N1)pdm09 (80). This indicated that cross-reactive H1N1 strains had not been circulating during this prolonged 55-year span of time. In contrast, 96% of subjects born between 1909 and 1919 had HAI titers of ≥1:10 against A(H1N1)pdm09 and this was likely due to maintenance of antiviral antibody titers for decades after recovery from the H1N1 1918 Spanish flu pandemic because of the structural and sequence similarities of the hemagglutinin (HA) surface antigen between the H1N1 1918 Spanish flu and the 2009 pandemic A(H1N1)pdm09 strain. Remarkably, ∼55% of the 1909–1919 cohort still retained HAI titers of ≥1:40, suggesting that these were potentially seroprotective levels of antiviral antibodies and this could explain multiple epidemiological reports of pre-existing antibodies and reduced disease among individuals over 65 years of age during the 2009 pandemic (80). These results are not unique to the H1 influenza antigen since similar results were observed 77 years after an H3-like influenza pandemic in 1889-91 that, "*...left a lifelong immunological imprint on ≥80% of those who were aged ≤21 years at the time*" (81). Together, these results indicate that natural infection can elicit lifelong subtype-specific immunity against influenza. Although annual variations within a subtype (antigenic drift) as well as changes between subtypes over time (antigenic shift) have made influenza vaccine development challenging (82), there is concern that current influenza vaccines induce only transient immunity that may not be sufficiently maintained for the duration of a single season even in the absence of demonstrable antigenic drift (83). What could be some of the reasons for the disparity between lifelong immunity induced by natural influenza infection vs. rapidly waning immunity after influenza vaccination?

Despite clear evidence that natural influenza can induce long-term immunity, recent studies have shown that the efficacy of current detergent-disrupted or "split" influenza vaccines may wane rapidly over the course of a single influenza season (84). One large study performed over the course of seven influenza seasons and involving 44,959 patients, found that the odds ratio (OR) for testing positive for any influenza increased by ∼16% for each additional 4-week period following vaccination (85). The authors of this study assumed a peak vaccine efficacy (VE) of 40% on October 1st, and using this assumption along with the measured decreases in VE over time, these results indicate that protection could decline to as little as ∼20% by the peak of an average influenza season (**Figure 3A**), leaving a substantial proportion of the population at risk. In a comparable report encompassing a nationwide influenza network involving five locations and four influenza seasons (2011/12–2014/15), US researchers demonstrated declining VE for influenza A (both H3N2 and H1N1pdm09) and influenza B, with rates in absolute VE dropping by ∼7–11% per month (88). Declining immunity to H3N2 was particularly pronounced, dropping to a VE point estimate of 0% by ∼5 months after vaccination. A similar multicenter trial performed among multiple European countries, spanning the 2010/11 through 2014/15 seasons, demonstrated peak VE of 50.6% for the H3N2 component at 38 days post-vaccination, which dropped to 0% VE by ∼3 months (89). As noted above, one explanation for intra-season waning of protective immunity could be due to antigenic drift resulting in immunological escape of circulating influenza strains as the season progresses. However, several studies suggest that mechanisms distinct from antigenic mismatch may be contributing to waning vaccine efficacy (90, 91). In one study performed in the UK during the 2011/12 influenza season, protective immunity declined from 53% VE at <3 months post-vaccination to only 12% VE at points ≥3 months post-vaccination (90). These differences in VE could not be attributed simply to antigenic drift because HA antigens for ≥78% of A/H3N2 strains isolated during early (Oct-Jan) or late (Feb-Apr) periods remained antigenically similar to the vaccine strain. This suggests that despite a nearly 5-fold drop in VE over the course of the season, the antigenic characteristics of circulating influenza strains remained relatively stable. In Spain during the 2011-2012 influenza season, VE efficacy waned rapidly over time with VE = 52%, 40%, and 22% at 3.5 months, 3.5–4 months, and >4 months, respectively after vaccination even though no changes in circulating virus strains were identified throughout the season (83). During the 2015/16 influenza season in Canada, early season (January-February) VE against A/H1N1pdm09 reached 62%, but dropped to a non-significant level of 19% during the March-April period of observation. This decline occurred even though only 1 of the 467 A/H1N1pdm09 viruses isolated during the season was considered antigenically distinct from the vaccine strain (91). Together, these independent studies demonstrate that regardless of antigenic drift, the

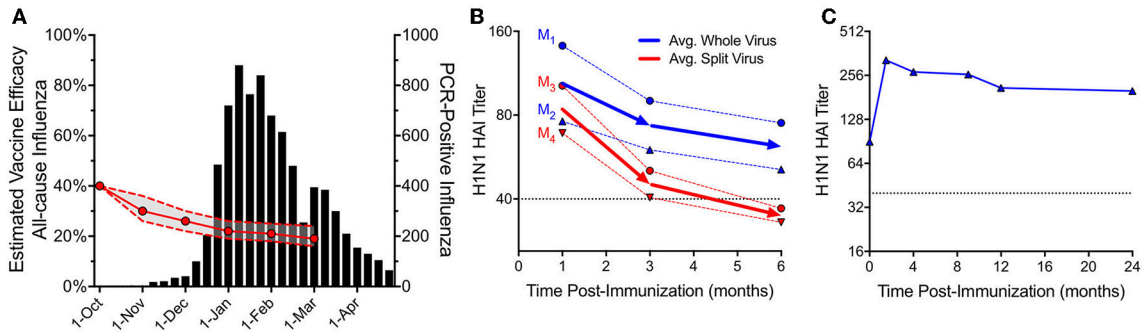


FIGURE 3 | Protective immunity after vaccination with repetitive (whole-virus) or non-repetitive (split-virus) influenza vaccines. Current inactivated influenza vaccines contain detergent-disrupted or "split" virus antigens and these formulations lose protective efficacy rapidly during the course of a single season. **(A)** A study of 7 influenza seasons involving 44,959 patients showed that the odds ratio (OR) for testing positive with influenza virus increased by ~16% for each 28-day period after vaccination. If vaccine efficacy (VE) was 40% starting on October 1st, we estimate that the subsequent decline in VE would result in only 20% VE during the peak of an average influenza season. The VE and associated 95% confidence intervals were inferred from the ORs in Table 2 (VE = [1÷OR] × 40%) of Ray et al. (85). Data for total PCR-positive influenza tests from 2010/11 to 2016/17 seasons were adapted with permission from **Figure 1** in Ray et al. (85). **(B)** In 1977, hemagglutination inhibition (HAI) responses to 2 commercial whole virus (WV) vaccines (dotted blue lines, M_1, manufacturer 1; M_2, manufacturer 2) and 2 split virus (SV) vaccines (dotted red lines, M_3, manufacturer 3; M_4, manufacturer 4) were monitored in parallel following a single immunization of adults (n = 46-56/group) (86). HAI serum titers were adapted with permission from **Figure 1** in Cate et al. (86) with the average of each group (WV or SV) shown as solid lines. **(C)** In 1953, a single dose of WV vaccine was administered to 45 young adults and HAI titers were monitored longitudinally for up to 2 years after vaccination (87). Antibody titers against the PR8 (A/H1N1) strain of influenza were adapted with permission from **Figure 1** in Salk et al. (87). For panels **(B)** and **(C)**, the putative protective HAI antibody level of 1:40 is shown as a dotted line.

failure of current detergent-split influenza vaccines to elicit durable protection is likely due to rapidly waning immunity after immunization.

Could changes in modern influenza vaccine manufacturing processes be playing a role in low overall immunogenicity and rapidly waning protective immunity? Although vaccine developers utilized inactivated whole virus (WV) influenza vaccines for several decades, concerns over reactogenicity led to the development of detergent-split virus (SV) vaccines (92). The first split vaccine was licensed in the US in 1968 to reduce reactogenicity observed with earlier WV formulations (93). Subsequently, a series of clinical trials comparing WV and SV formulations for the 1976 swine flu demonstrated higher reactogenicity of WV formulations in children and these results are credited with influencing later decisions to recommend using only SV-type influenza vaccines in this age group (92). At the time, industry leaders cautioned that preference from the medical community for SV formulations could lead to the eventual disappearance of WV vaccines (94). This was a concern because SV vaccines are poorly immunogenic compared to their WV counterparts. For example, children vaccinated with WV vaccines showed fourfold or greater rise in antibody responses in 69-100% of subjects whereas those vaccinated with SV formulations produced fourfold responses in 0-23% of the vaccine recipients (94, 95). Likewise, 65–93% of 3–5-year old children receiving WV formulations demonstrated HAI titers of ≥1:20 whereas 0–10% of children receiving SV preparations mounted HAI responses of ≥1:20 (94). The concerns over vaccine reactogenicity outweighed the merits of immunogenicity and Maurice Hilleman noted at the time that, "*The production of vaccines in this country is very closely geared to consideration of costs for preparation and to preferences in the medical community. Responsible manufacturers are sensitive to these demands and can and will produce whatever kind of vaccine is wanted*" (94). In addition to issues of poor seroconversion rates among young and naïve vaccine recipients, another potential concern with SV vaccine preparations involved the durability of vaccine-induce immune responses. Contemporary comparisons of vaccine-mediated antibody persistence between matched WV and SV influenza formulations are scarce. However, during the clinical testing of the 1976 A/H1N1 swine flu vaccine, groups of seronegative adults received one of four different commercially manufactured vaccines including two WV vaccines or two SV vaccines and antibody titers were monitored for up to 6 months after immunization (**Figure 3B**) (86). Titers declined rapidly from 1 to 3 months post-immunization with slower decay rate kinetics observed from 3 to 6 months post-vaccination. However, the authors noted a significant difference ($P < 0.05$) in the rate of antibody decline between the two vaccine types, with WV vaccines declining by 40% over a 5-month period, compared to an average decrease of nearly 60% for SV vaccines, leading to an improvement in serum HAI titers for the WV vaccines by the end of the observation period. These differences become important as antibody titers decline to near a putative protective threshold such as an HAI titer of 1:40. The observation that WV vaccines elicit antiviral immunity that was on average still maintained above 1:40 at 6 months post-vaccination whereas SV vaccines induced antibody responses that declined to <1:40 may explain the epidemiological findings of short-lived protective immunity in our current populations during this short period of time after vaccination (**Figure 3A**). In contrast to SV vaccines, it is possible that WV vaccines will induce more durable immunity and in a separate study in which HAI titers were monitored for

up to 2 years following WV influenza vaccination in pre-immune subjects (87), the HAI titers peaked at 6 weeks post-vaccination, declined during the first year after vaccination, but then remained relatively stable for at least 2 years above the protective threshold (**Figure 3C**). This work suggests that re-introduction of optimized, multivalent WV influenza vaccine formulations may provide a feasible approach to improving the durability of current seasonal influenza vaccine regimens if issues surrounding potential reactogenicity are addressed.

ROLE OF MULTIVALENCY AND ANTIGENIC THRESHOLD IN DETERMINING DURATION OF PROTECTIVE IMMUNITY

The impact that antigen structure (i.e. monomeric, multimeric, aggregated) can have on antigen processing, cellular immunity and subsequent B cell responses has been well described (96–99). Many of the concepts regarding the role of B cell epitope multivalency, antigenic thresholds, and the Imprinted Lifespan model for generating long-lived PC (**Figure 4**) have been previously described (14, 38). According to the Imprinted Lifespan Model, exposure to haptens or monovalent self antigens are most likely to elicit only short-term immunity due to the lack of B cell receptor crosslinking/activation and the absence of $CD4^+$ T cell help. Vaccines containing monomeric foreign protein antigens such as tetanus or diphtheria do not induce substantial B cell receptor crosslinking but due to peptide-specific $CD4^+$ T cell help, these antigens are immunogenic and induce protective immune responses that can last for many years. Vaccines containing multimeric foreign protein antigens would be expected to elicit the highest level of B cell receptor cross-linking that, in addition to cognate $CD4^+$ T cell help, will "imprint" the activated B cells to proliferate and differentiate into plasma cell populations that are more long-lived than those elicited by monovalent antigens and may in some circumstances result in life-long immunity.

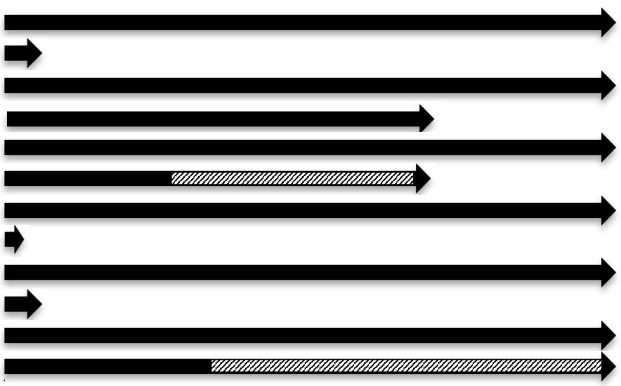
The concept that all viral infections are expected to elicit lifelong immunity stems mainly from observations of the durable immunity induced by natural infection. Live-attenuated vaccines have been developed to mimic the protective immune responses elicited by natural infection but without prolonged and/or high-titer infection causing the clinical manifestations of that particular disease. The steps taken to ensure attenuation and increased safety often comes at the cost of reduced immunogenicity due to lower antigen load and this associated reduction in antigenic threshold may result in short-lived or only partial protective immunity in the absence of at least one booster vaccination (**Table 1**). Although natural infection with dengue virus (DENV) may not provide complete protection against reinfection (100), it is generally believed to provide long-term immunity since the majority of DENV cases and the more severe disease is identified more often among children than adults, indicating that adults living in endemic communities are afforded serotype-specific protection presumably from prior childhood infection (101). Based on this premise, it was expected that a live-attenuated dengue virus vaccine would likewise induce durable protective immunity, but recent studies indicate that this was not the case. Following 3 doses of the live-attenuated Dengvaxia vaccine (comprised of a chimeric yellow fever virus expressing dengue serotype-specific envelope proteins), children 2–5 years of age in the ASIA (CYD14) longitudinal cohort showed ∼36% VE (Vaccine Efficacy=1–hazard ratio) during the first year after vaccination, but no vaccine-mediated protection remained by 3 years post-vaccination (102). Likewise, children among cohorts aged 6–8 years or 9–11 years of age showed 79% VE and 50% VE during the first year after vaccination but protection dropped to undetectable levels by 4 years and 3 years post-vaccination, respectively. Dengvaxia VE was not statistically significant during the first year after vaccination for two of the three age groups and it is notable that even trends toward vaccine-mediated protective immunity were subsequently lost within 3–4 years after a 3-dose vaccination series (102). Moreover, lack of vaccine efficacy and concerns with potential vaccine-associated exacerbation of disease among previously seronegative subjects (103) have resulted in the Dengvaxia vaccine indication being changed to include only dengue-seropositive individuals.

It is well-established that childhood measles infection induces lifelong immunity (104), with serum antibody responses that show little or no decay when measured longitudinally for over 20 years (58). In contrast, after achieving 95–99% seroconversion to measles after primary MMR (measles, mumps, rubella) vaccination (105), there is evidence suggesting that immunity may wane (106) and a 2-dose schedule is recommended in order to raise the antigenic threshold and induce antibody titers that plateau above the protective threshold. Likewise, when we examined the durability of measles-specific antibodies among a small group of MMR vaccine recipients, the antiviral antibody levels were stable but in three out of four subjects, the titers were maintained at or below the protective threshold of 1,000 ELISA Units/mL, equivalent to 0.2 IU/mL [(58); Supplemental Appendix] whereas the majority of antibody responses to natural measles infection were maintained at much higher levels, most likely due to the prolonged infection and exposure to higher antigenic load during natural infection vs. vaccination (38, 58). Similar to measles, mumps is another common childhood infection that typically results in life-long immunity. However, recent studies have indicated that there may be a greater waning of MMR vaccine-mediated immunity against mumps then that observed against measles and the ACIP currently recommends a 3rd dose of MMR vaccine be administered to persons previously vaccinated with 2 doses who are at increased risk for contracting mumps during an outbreak (107).

Although smallpox has been eradicated, studies involving different smallpox vaccines have been informative in understanding long-term antiviral immunity. Vaccinia virus strains used in standard scarification/transcutaneous smallpox vaccination have not been specifically attenuated for vaccine use and they elicit long-term antibody and T cell-mediated immunity similar to Variola virus itself (108). In contrast, the MVA (modified vaccinia Ankara) strain of vaccinia virus was heavily attenuated by serial *in vitro* passage in chicken embryo fibroblast cells, resulting in a virus that is essentially replication-deficient in humans. Unlike replicating strains of

TABLE 1 | Examples of antiviral immunity generated from wild-type viruses vs. live-attenuated vaccine strains.

Virus or vaccine target	Repetitive structure?	Reach antigenic threshold?*	Duration of Immunity: Years / Decades / Lifelong
Dengue	Yes	Yes	
Dengvaxia	Yes	No	
Measles	Yes	Yes	
Measles (MMR)	Yes	Partial	
Mumps	Yes	Yes	
Mumps (MMR)	Yes	Partial	
Vaccinia-Dryvax	Yes	Yes	
Vaccinia-MVA	Yes	No	
Varicella zoster virus	Yes	Yes	
VZV-Oka	Yes	Partial	
Yellow fever	Yes	Yes	
YFV-17D/17DD	Yes	Partial	



**Antigenic Threshold is defined as follows: Yes: indicates that the infection achieves a high level of antigenic stimulation that induces antigen-specific B cells to proliferate and differentiate into a sufficient number of PC to provide long-term antibody production above a protective threshold. Partial: a partial achievement of antigenic threshold is defined as a smaller degree of antigenic stimulation resulting in shorter duration of immunity that may decline below the protective threshold but can be improved by booster vaccination. No: If there is little or no achievement in reaching an antigenic threshold then this means that even after booster vaccination, antiviral immunity will decline to below protective levels in the majority of individuals in <1–4 years. Cross-hatched arrows indicate that immunity may be long-lived in a subpopulation of individuals. MMR, measles, mumps, rubella; MVA, modified vaccinia Ankara; VZV, varicella zoster virus.*

vaccinia virus that induce high seroconversion rates (>90%) and long-term immunity after a single vaccination [antibody $T_{1/2} = 92$ years (58)], immunization with 2 doses of MVA result in ∼71% seroconversion for neutralizing antibodies (≥1:10) at the peak of the immune response before declining to 28.8% seropositive status within 2 months after booster vaccination (109). Another study provided similar results; within 6 months after booster MVA vaccination, antibody titers waned to near baseline levels and seroconversion rates declined to 35–54%, depending on the formulation and immunization route (110).

Chickenpox is caused by varicella zoster virus (VZV) and similar to measles and mumps, it was considered to be a childhood disease because after recovery from primary infection, it was rare to get chickenpox twice. VZV is an α-herpesvirus that establishes latency within the infected host and therefore it was believed that this vaccine would require only one shot to elicit lifelong immunity. Instead, although high levels of protection are observed during the first 1–3 years after primary vaccination, protective immunity continuously wanes thereafter, resulting in a >30-fold increase in the risk of symptomatic breakthrough cases by 9 years after vaccination (111). In 2006, the ACIP recommended a second dose of varicella vaccine be administered to improve long-term vaccine-mediated immunity.

Yellow fever virus (YFV) causes an acute viral infection and similar to other acute viral infections (dengue, measles, mumps, vaccinia, etc.) recovery from natural YFV infection induces essentially lifelong immunity (112). Development of live-attenuated YFV vaccines are believed to also induce lifelong immunity and primary seroconversion rates of >95% have been noted in multiple clinical trials (113). However, despite initially high rates of seroconversion and long-term immunity among the majority of vaccinated subjects, it appears that 20–30% of YFV-immunized adults may lose immunity within the first 5–10 years after primary vaccination (113–115). Bearing in mind that initial seroconversion rates are substantially lower among children [69–85% primary seroconversion (116, 117)], there is concern that subsequent waning immunity might result in an even larger number of children/adolescents who become unprotected or seronegative if a booster dose is not administered. For these reasons, some YFV-endemic countries such as Brazil have continued to maintain booster vaccinations against yellow fever.

THE IMPRINTED LIFESPAN MODEL

In 2007, we published a longitudinal study in which we found a surprising difference in the durability of serum antibody responses mounted against monovalent protein antigens such as tetanus and diphtheria toxins vs. the persistence of antibody responses to multivalent antigens associated with measles, mumps, rubella, or vaccinia infection (58). Based on these observations, we proposed the Imprinted Lifespan Model of long-lived PC (14, 38), wherein PCs are imprinted with a predetermined lifespan based on the magnitude and quality of B cell signaling that occurs during the induction of an antigen-specific humoral immune response (**Figure 4**). This model is based primarily on a set of 4 core tenets. Tenet #1: Long-lived PC are defined as terminally differentiated non-dividing cells that continuously secrete antibodies without requiring further stimulation. Since most PC lose the expression of surface-bound immunoglobulins and down-regulate MHC Class II expression, they are less likely to sense their antigenic microenvironment and will have little or no direct antigen-specific interactions with $CD4^+$ T cells. Bearing this in mind, we propose that the intrinsic lifespan of these non-dividing cells must be determined during the induction of the humoral immune response when the responding B cells first encounter their cognate antigen. Tenet #2: The majority of long-lived PC reside in the bone

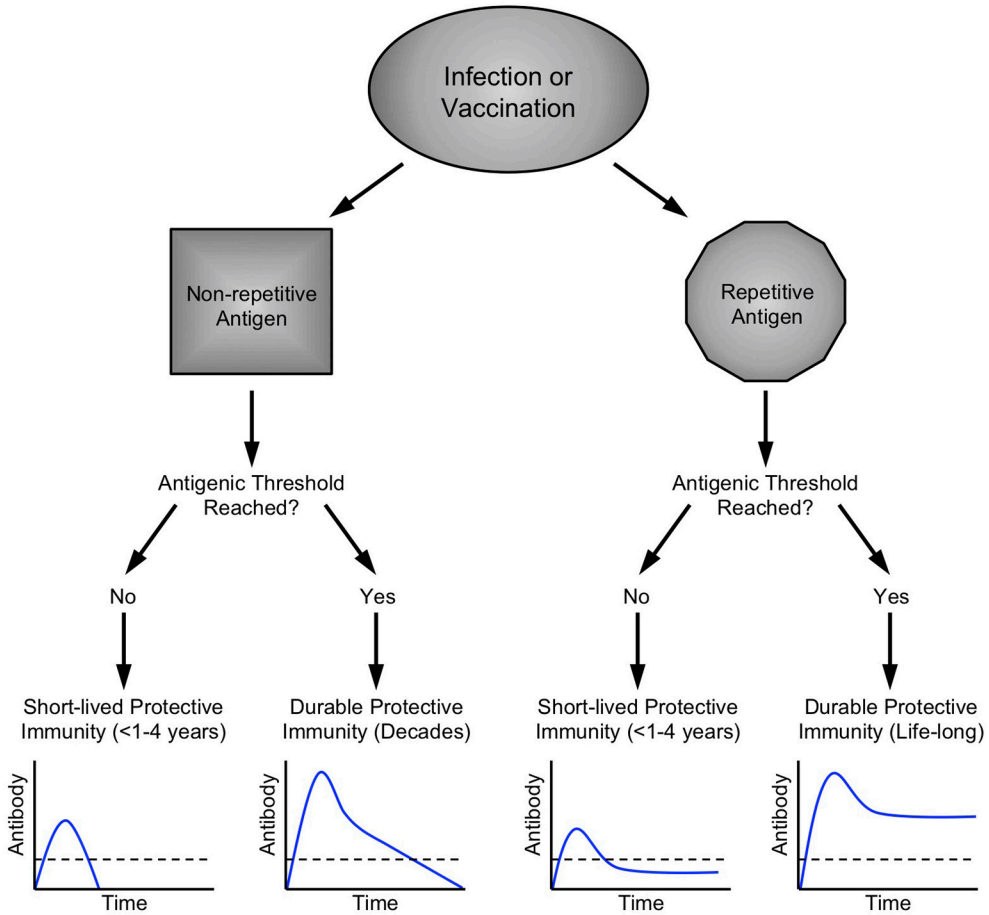


FIGURE 4 | Imprinted lifespan model of humoral immunity. Prior longitudinal studies have indicated that different vaccine and virus antigens elicit serum antibody responses of varying duration (58). Since long-lived PC are terminally-differentiated non-dividing cells that can survive for months or even years after the initial antigenic insult has been resolved (27), our hypothesis is that PC lifespan must be determined or "imprinted" at the time of B cell activation during the initial stages of an active immune response. Microbes such as viruses and bacteria have surface antigens that are highly repetitive and these structural attributes are likely to cause increased B cell activation by binding to multiple immunoglobulin molecules on the responsive B cell and trigger increased signal transduction in comparison to B cell activation induced by non-repetitive or monovalent antigens. B cell activation is further enhanced via T cell help during T-dependent immune responses to foreign antigens. In addition to the structural attributes of a particular antigen, another important parameter is antigenic load (118). If an infection is resolved too quickly or if a vaccine has low antigen content, is rapidly degraded, or contains no adjuvant, then there will be fewer germinal center reactions, fewer activated B cells and T cells, and fewer PC that enter the long-lived pool. Bearing this in mind, we postulate that a certain antigenic threshold must be met in order to elicit a sufficient number of PC to maintain antibody responses above a protective threshold (shown as a dashed line in each graph) and this set point will differ depending on the level of protection needed for each pathogen or toxin. Contemporary detergent-disrupted influenza vaccines provide an example of non-adjuvanted non-repetitive antigen that does not reach the antigenic threshold. Under these circumstances, transient, low levels of immunity rapidly wane to below a protective threshold, often before the end of single influenza season. In contrast, booster vaccination against other non-repetitive alum-formulated antigens such as tetanus or diphtheria is able to elicit high antibody titers that decline faster than that observed with highly repetitive viral antigens (58), but nevertheless provide long-term immunity that may last for decades. Among repetitive antigens, the duration of immunity is also likely to be influenced by antigenic threshold. For highly attenuated vaccine strains of viruses such as MVA or Dengvaxia (**Table 1**), viral antigen may be cleared too quickly, resulting in the induction of fewer long-lived PC due to insufficient antigen load with an end-result of long-term antibody production that resides near or below the protective threshold. On the other hand, natural infections with viruses such as measles, vaccinia, influenza, etc., (**Table 1**) or immunization with optimized and adjuvanted VLP or whole-virus vaccines (**Figures 1**, **2**) will potentially induce lifelong immunity by presenting repetitive epitopes while also achieving sufficient antigenic load that, together with appropriate T cell help, will result in long-term antibody production that is maintained at or above protective levels.

marrow and since there is only finite space within the bone marrow compartment for new PC, it is possible that resident PC are imprinted with a lifespan of varying duration based on the probability that the antigen represents a relevant and protective target epitope. Antibody responses to soluble self antigens or carbohydrates are normally avoided due to the monomeric nature of the soluble antigen and the lack of T cell help. Highly repetitive/multimeric carbohydrate antigens may be recognized as being from an invading microbe based on their multivalent structures and although they are capable of eliciting

T cell-independent antibody responses by virtue of their ability to activate B cells through crosslinking of the B cell receptor (BCR), these types of antibody responses are often short-lived unless the antigen is conjugated to a carrier protein in order to allow the secondary signals to be initiated through critical B cell:T cell interactions (14, 38, 119). Tenet #3: Foreign protein antigens capable of triggering the activation of B cells can be divided into two general categories; monovalent and multivalent. The surface structures on particulate antigens such as viruses and bacteria are often highly repetitive (i.e., multivalent) whereas internal proteins are more likely to be monomeric (e.g., non-structural proteins, viral or bacterial RNA/DNA polymerases, etc.), the induction of PC with a preferentially longer lifespan against highly repetitive antigens could provide a long-term advantage to the host by not crowding the bone marrow with PC to potentially less protective epitopes among internal microbial proteins. However, even monomeric foreign proteins can be dangerous to the host and therefore antibody responses to secreted virulence proteins or toxins (tetanus toxin, diphtheria toxin, pertussis toxin, etc.) is also needed, but since these proteins lack the repetitive nature of microbial surface antigens, the PC responses would be imprinted with an intermediate lifespan that may last for years or decades rather than being dedicated to nearly a lifetime of antibody production in the bone marrow. Tenet #4: In order to achieve long-term protective immunity, an antigenic threshold must be reached in order to generate sufficient B cell activation, proliferation, and differentiation into a minimum number of PC to maintain antibody production at levels above a protective threshold for that particular pathogen or toxin.

Exposure to a non-repetitive foreign antigen during infection or vaccination will trigger initial B cell activation and antibody production (**Figure 4**). If this is a primary exposure/vaccination then it is possible that the antigenic threshold is not reached and antibody responses will peak and then decline below protective levels shortly thereafter. Influenza vaccination with detergent-disrupted "split" virus falls into this category, with only short-lived immunity that may or may not last for a single influenza season (**Figure 3A**). However, if an appropriate adjuvant is included [e.g., alum (120, 121)] to create an antigen depot and/or if booster vaccination is performed, then the peak immune response is likely to be higher and after the early stages of rapid antibody decline (14) (**Figures 1**, **2**, and **3**), it is anticipated that antibody levels will decay at a slow rate that is potentially maintained for years above the protective threshold. Antibody responses to tetanus and diphtheria toxins fall into this category. After the initial rapid antibody decay that occurs for 1–3 years post-vaccination, anti-toxin antibody responses decline with an estimated half-life of 11–14 years (58, 69, 122) for tetanus and 19–27 years (58, 122) for diphtheria, respectively. Interestingly, diphtheria-specific antibody decay rates observed among people naturally exposed to endemic diphtheria are higher than those vaccinated after elimination of indigenous diphtheria but the antibody decay rate kinetics appear to be essentially the same (123). This suggests that the antibody decay rates for monomeric antigens will be similar regardless of whether the exposure comes from an infection or through vaccination. Although antitoxin antibody responses are likely to continue declining over time, their initial peak levels are sufficiently above the protective threshold to allow protective immunity to persist for many years. Notably, the World Health Organization does not recommend adult booster vaccination against tetanus or diphtheria once the initial 5-dose vaccination series has been completed (124, 125). Likewise, we have examined the incidence of tetanus and diphtheria among countries that do or do not routinely vaccinate adults and found that continued vaccination of the adult population may no longer be needed once the childhood vaccination series has been completed (A. Slifka, L. Gao, M. Slifka; manuscript submitted). This indicates that even monomeric antigens can elicit lifelong immunity if booster vaccination reaches a minimum antigenic load resulting in antibody titers that are sufficiently higher than what is needed to be maintained above a protective threshold.

Following infection or vaccination with highly repetitive antigens, protective immunity can be short-lived or long-lived depending on the antigenic threshold. Following infection/vaccination with highly attenuated live viruses such as MVA (109, 110) or Dengvaxia (102), antiviral immunity may crest above the protective threshold but decline to levels that reside below those needed for long-term immunity, presumably because the viral antigens are cleared too rapidly to elicit a large PC population that can survive and maintain antibodies above protective levels. In contrast, when highly repetitive antigens are expressed during natural infection such as measles, mumps, etc. (**Table 1**) or when immune responses are elicited through booster vaccination with live-attenuated viruses or immunization with highly repetitive whole-virus or VLP antigens formulated with an appropriate adjuvant (**Figures 1**, **2**), then long-term antibody responses can be generated that plateau above the protective threshold during the maintenance phase and result in potentially life-long immunity.

Understanding the underlying mechanisms for inducing lifelong antibody responses is key to rational vaccine design. Natural influenza infection results in lifelong subtype-specific antiviral immunity whereas current detergent-disrupted influenza vaccines often fail to protect against disease for even a single season (**Figure 3A**) even in the absence of appreciable antigenic drift (83, 91). The poor immunogenicity of detergent-split influenza vaccines has been demonstrated in head-to-head comparisons with whole-virus vaccines (**Figure 3B**) and with the contemporary lack of high vaccine efficacy resulting in large scale hospitalizations and nearly 80,000 deaths in the US during the 2017/2018 season (77), it is time that we re-evaluate the design of future vaccines with an emphasis on the structural integrity of vaccine antigens. It is possible that even small but fundamental changes in vaccine design could improve early antiviral immune responses (**Figure 3B**) as well as lead to more durable antibody responses that last beyond a single flu season (**Figure 3C**).

The role of germinal center reactions including interactions with different $CD4^+$ T cell subsets, adhesion molecules, cytokines, chemokines, metabolic pathways, and many other aspects of the immediate microenvironment encountered during

both initial B cell stimulation as well as PC homing and survival in the bone marrow microenvironment are all important areas that are worthy of further investigation. More studies are needed to better understand the dynamics of B cell activation during vaccination or infection and we believe that optimization of these parameters, especially with regard to antigenic threshold (to determine antibody magnitude) and antigen structure (to determine antibody persistence), will lead to improved vaccines in the future that elicit more durable immunity not only against influenza but for other major human pathogens as well.

AUTHOR CONTRIBUTIONS

MS and IA contributed to manuscript drafting and revision. Both authors read and approved the submitted version

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REVIEW
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Survival of Long-Lived Plasma Cells (LLPC): Piecing Together the Puzzle

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Durable humoral immunity is dependent upon the generation of antigen-specific antibody titers, produced by non-proliferating bone marrow resident long-lived plasma cells (LLPC). Longevity is the hallmark of LLPC, but why and how they survive and function for years after antigen exposure is only beginning to be understood. LLPC are not intrinsically long-lived; they require continuous signals from the LLPC niche to survive. Signals unique to LLPC survival (vs. PC survival in general) most notably include those that upregulate the anti-apoptotic factor Mcl-1 and activation of the CD28 receptor expressed on LLPC. Other potential factors include expression of BCMA, upregulation of the transcription factor ZBTB20, and upregulation of the enzyme ENPP1. Metabolic fitness is another key component of LLPC longevity, facilitating the diversion of glucose to generate pyruvate during times of stress to facilitate long term survival. A third major component of LLPC survival is the microenvironment/LLPC niche itself. Cellular partners such as stromal cells, dendritic cells, and T regulatory cells establish a niche for LLPC and drive survival signaling by expressing ligands such as CD80/CD86 for CD28 and producing soluble and stromal factors that contribute to LLPC longevity. These findings have led to the current paradigm wherein both intrinsic and extrinsic mechanisms are required for the survival of LLPC. Here we outline this diverse network of signals and highlight the mechanisms thought to regulate and promote the survival of LLPC. Understanding this network of signals has direct implications in increasing our basic understanding of plasma cell biology, but also in vaccine and therapeutic drug development to address the pathologies that can arise from this subset.

Keywords: long-lived plasma cells (LLPC), plasma cell survival, plasma cell niche, plasma cell function, humoral responses

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INTRODUCTION

Plasma cells (PC) represent an essential arm in humoral immunity as the main line of defense against infection and re-infection. As the primary producers of circulating immunoglobulin (Ig), these cells provide vital durable and protective immunity against a multitude of pathogens. Longitudinal analysis of antigen-specific antibody titers from vaccinated humans demonstrates that the predicted half-life of the measles titer is 3,014 years (1). This is a testament to the long-lived protection that PC can provide. Unlike other immune cell subsets such as T cells or B cells, the complexity of the varying PC subsets is only beginning to be understood. Plasma cell generation occurs primarily upon T cell-dependent differentiation of B lymphocytes to PC in germinal center reactions (2, 3). The current paradigm proposes that two general types of PC develop from these interactions: short-lived plasma cells (SLPC) and long-lived plasma cells (LLPC) (4, 5). The LLPC

subset characteristically thought to traffic to and reside in the bone marrow (BM), is the subset that provides long-term and sustained antibody production that is maintained for decades to the lifetime of an individual (6–8). The germinal center reactions, through somatic hypermutation and class-switch recombination, allow for the selection of high-affinity antibody producing PC, which is proposed to be the major precursors of LLPC (9, 10). However, there is relatively little understanding of the driving force behind why these LLPC can become long lived.

SPECIALIZED NICHES FOR LLPC

There has been considerable research into the biology of PC as a whole, from how they are generated and the key transcriptional programs involved, to their ability to traffic to various organs (11–15). Further studies have elucidated the cellular and molecular components of various organ-specific niches occupied by PC (16–18). However, there is relatively little understanding of what distinguishes the ability of LLPC to survive in contrast to SLPC. LLPC are not intrinsically long-lived, as their survival is critically dependent on the ability to access and use a fixed number of specific pro-survival niches in the BM, secondary lymphoid organs, mucosa, and sites of inflammation (5, 7, 19–24). The BM is traditionally thought to be the primary organ of LLPC residence. It provides a dynamic infrastructure amenable to the formation of a complex microenvironment and allows for the generation of cell-type specific niches more easily than other less plastic organs (5, 6).

More recent work has illuminated the fact that LLPC do not only reside within the BM. Of human PC, about 80% are located in gut-associated lymphoid tissue (GALT) and produce primarily IgA (25). This allows for tolerance to the commensal bacteria in our gut, while also providing protection against unfavorable microbial and dietary antigens. It was originally thought that continual activation of B cells within the mucosa supplied the pool of IgA-producing PC in the gut (26, 27). However, new studies have highlighted that PC in the gut can also persist for long periods of time. Antibodies specific to *Escherichia coli* were detected 112 days after exposure (28). Examination of intestinal biopsies kept in culture contained non-proliferating IgA-producing PC for >4 weeks (29). Another study showed that 9 months post-immunization with both an IgA-inducing antigen (cholera toxin) and a T-dependent antigen (Ova), antigen-specific PC could be detected in the Lamina Propria (LP) but also within the BM (21). This suggests that survival niches present in the gut could be similar to those in the BM and that mucosal PC can utilize these niches in the same way as BM PC, as well as contributing to the BM LLPC pool (21).

It is traditionally thought that most BM LLPC secrete IgG antibodies, and those that reside in mucosal sites such as the gut produce IgA antibodies, however, about 40% of BM PC also produce IgA (30). It has also been reported that there are long-lived low-affinity IgM producing PC within the BM that appear to occupy different niches (20). Furthermore, allergic sensitization with ovalbumin generates IgG, IgA, and IgE secreting PC that can be found in the BM for an extended period (31). Therefore, evidence suggests that the longevity of a PC is not primarily driven by its antibody isotype—but rather by the nature of the pathogen, the characteristics of initial B cell activation elicited by the pathogen, and the niche the resultant PC homes to. This broad framework suggests that the primary role of SLPC is in protection against frequent (and less severe) endemic infections that is sustained by recurrent and antigen-dependent B cell reactivation; whereas LLPC provide sustained protective immunity against infrequent (but more severe) epidemic infections in an antigen-independent fashion. It has also been proposed that early B cell/plasmablast activation signals determine whether PC enter and respond to survival signals in the PC niche (32–34). Because the space available to LLPC is finite, LLPC longevity requires both access to the survival niche and the ability to respond to the niche's unique pro-survival signals.

LLPC AND MEMORY B CELLS ARE NOT ONE AND THE SAME

It was originally proposed that sustained antibody responses resulted from constant replenishment of a SLPC pool by continuous memory B cell re-stimulation (35). However, observations that BM transplantation caused non-allergic individuals to acquire allergies through transfer of allergen-specific IgE production lent credence to the idea that these PC were long-lived, due to the absence of antigenic re-stimulation in these patients (36, 37). Further studies have shown *in vivo* that some PC subsets are indeed long-lived and that absence of antigen plus depletion of memory B cells through radiation did not abrogate the ability of this LLPC subset to continue to produce high-affinity antigen-specific antibodies (38, 39). Other studies have shown that prolonged therapeutic depletion of the total B cell pool did not affect antigen-specific BM PC numbers or antibody titers in vaccinated murine models (40), nor did it affect antibody titers against childhood vaccines in humans (41). In the human studies, vaccine-specific antibody titers were maintained following anti-CD20 monoclonal antibody treatment (which targets B cells but not PC), despite clear depletion of the memory B cell pool (41). Sustained B cell aplasia caused by CD19 CAR-T cells (which also target B cells but not PC) in adult and pediatric acute lymphocytic leukemia patients also had no effect on serum vaccine antigen-specific antibody titers nor PC numbers in either the BM or ileum and colon (42). A study examining PC dynamics from biopsies of transplanted duodenum found that 1-year post-transplantation, $CD38^+$ PC from the donor could still be identified. Further characterization identified $CD19^-$ and $CD19^+$ PC present in these biopsies, where the $CD19^-$ PC subset represented a stable population with a potentially long lifespan (24). Lastly, characterization of subsets of human PC demonstrated that $CD19^-$ PC in the BM were predominantly IgG secreting with a mature phenotype and remained settled in the BM after systemic vaccination (43). This demonstrates that LLPC are

indeed distinct from other B cell subsets, however the key factors that distinguish these cells are only recently beginning to be characterized.

A UNIQUE TRANSCRIPTIONAL PROFILE

During B cell to PC differentiation, many genes are downregulated including those involved in antigen presentation and BCR function. Concurrently, there is upregulation of the PC lineage defining transcription factor *PRDM1* (BLIMP-1) and genes involved in protein translation and the unfolded protein response (*XBP-1*) (11, 44). An avenue to distinguish this LLPC subset is through their transcriptional profile. A recent study of human PC has established a unique profile that distinguishes between early PC, circulating blood PC, and long-lived BM-resident CD138^{+} PC (14). The signature that distinguishes LLPC contains a significant number of genes that are downregulated and only a handful of genes that are upregulated. This latter group includes the anti-apoptotic genes *Mcl-1* and *ZNF667*, ER stress-associated genes including *EROILB* and *MANF*, the cation transport ATPase *ATP12A*, and *TFBS* and *SRF* that play roles in retention of hematopoietic progenitor cells in the BM. Many of these genes are associated with the central function of PC to produce significant amounts of protein in the form of immunoglobulin—although the fact that this function is not unique to LLPC suggests that there may be additional unappreciated biology of these genes in LLPC.

Identification of a transcriptional profile that is unique to LLPC faces a significant experimental obstacle—namely the ability to identify a pure population of LLPC to analyze. Anatomic location has traditionally been the way that SLPC and LLPC were distinguished from each other, due to the lack of distinct phenotypic markers distinguishing these subsets. However, as noted above, recent sophisticated phenotypic profiling has made it increasingly clear that LLPC are not the only PC present within the BM. Recent work looking at PC precursor populations has identified that several waves of trafficking can occur post-antigen exposure and PC differentiation—resulting in other PC aside from LLPC being in the BM (34). Furthermore, surface markers that were traditionally used to identify LLPC have been recently shown to variably stain different PC subsets—for example, analysis of PC in the BM compartment have shown that there is varying expression of surface markers such as CD38 and CD19 (43, 45). These phenotypic differences are clearly associated with functional differences, such as whether these PC are producing high-affinity or low-affinity antibodies (46).

Because of the recent increase in PC subset heterogeneity, analysis of the total BM PC population compared to PC from other compartments may not pinpoint the specific factors and interactions responsible for reinforcing and sustaining the transcriptional signature necessary for the LLPC subset. Thus, one approach in defining the key transcriptional networks unique to LLPC, is to first identify the upstream receptors and signal transduction pathways that uniquely support LLPC survival and longevity.

INTRINSIC SIGNALS FOR LLPC SURVIVAL AND FUNCTION

BCMA

We now know that there are intrinsic signals uniquely utilized by LLPC that regulate their long-term survival and function (**Table 1**). It is well-established that factors such as A Proliferation Inducing Ligand (APRIL), Interleukin-6 (IL-6), B cell Activating Factor (BAFF), CD44 and CXCL12 can support survival of both SLPC and LLPC in various organs (4, 12, 29, 47–49). One of the receptors for both BAFF and APRIL is B cell maturation antigen (BCMA), which is highly upregulated on PC (50). Genetic knockout of *Tnfrsf17* (which encodes BCMA) resulted in a significant loss of antibody secreting cells (ASCs) in the BM compared to wild-type (Wt) controls, 6–8 weeks post-immunization. However, germinal center responses and early antigen-specific serum IgM and IgG titers were normal, indicating loss of BCMA primarily affected the LLPC subset (50). Additional studies have demonstrated that murine memory B cell survival is not dependent on BCMA, and BCMA is only induced in human B cells committed to PC differentiation (47, 51). This suggests that reliance on BCMA for survival is specific to the PC compartment. Furthermore, studies in multiple myeloma (MM), a BM-resident Ig-class switched LLPC neoplasm, showed that BCMA significantly promoted MM survival and proliferation (52). Therapeutically targeting BCMA in MM has shown high selectivity against the MM and PC populations (53), supporting a PC-restricted role for the receptor. Activation of BCMA by BAFF increases expression of the anti-apoptotic molecule Mcl-1, pointing to the mechanistic basis by which the BAFF/BCMA axis promotes long-term survival (50). An increase in the gene expression for Mcl-1 in BM PC was observed in Wt mice compared to BM PC from BCMA$^{-/-}$ mice, where both Mcl-1 mRNA and protein expression were significantly lower in the knockouts (54). Furthermore, loss of Mcl-1 causes a significant decrease in antigen-specific titers 21 days post immunization, consistent with an effect on the LLPC subset (54). As with BCMA, Mcl-1 is also a critical factor for MM survival (55, 56), which is consistent with the broader concept that much of myeloma biology is in fact the biology of normal LLPC (57).

STAT3

The importance of IL-6 in PC function and survival has been extensively studied (49, 58). IL-10 and IL-21 have also been implicated in human PC maturation and survival (59–61). IL-21 is predominantly produced by T follicular helper (Tfh) cells (62), which are involved in many phases of the humoral immune response from B cell activation to maturation and differentiation of B memory cells into PC (63). A study looking at human tonsil, splenic, lymph node, and BM PC indicated that BM PC had very low expression of IL-21R and that IL-21 only enhanced Ig secretion from secondary lymphoid PC but not

TABLE 1 | Summary of intrinsic factors supporting LLPC survival.

Intrinsic Factors of LLPC survival	
BCMA	Upregulation of anti-apoptotic genes
STAT3	Responds to IL-6, IL-10, and IL-21 signaling in PC, initiating downstream survival signaling associated with these cytokines
Aiolos	Promotes high-affinity antibody producing PC.
CD93	Possible connection with BLIMP-1—regulating mature LLPC phenotype and production of high-affinity antibodies
CD28	Signaling through the Vav/Grb2 motif can induce NFκB signaling and BLIMP-1 expression. This receptor can engage ligands CD80/CD86 to promote back signaling through DC and upregulation of IL-6
Autophagy (Atg5)	Recycling mechanism—supplying metabolic substrates and elimination of misfolded protein
Metabolic Profile	LLPC uptake glucose for antibody glycosylation. They also utilize glycolysis and mitochondrial pyruvate import under non-optimal conditions
ENPP1	Enzyme that is a glucose homeostasis-regulator of glucose and the metabolic pathway in LLPC

BM PC (64). More recent work, however, has postulated that Tfh cells influence blood Ag-induced PC, which can contain precursors of the LLPC subset (65). Whether this effect is directly through IL-21 or the effect of IL-21 on B cell subsets and upregulation of receptors like IL-6R is uncertain. How these individual cytokines promote BM PC survival and function is still unclear; however, they all initiate STAT3 signaling (66–68). Activation of STAT3 plays an important role in the ability of PC to respond to these various cytokine signals (68). Impairment of this pathway inhibits cytokine-dependent increase in viability and IgG secretion. Furthermore, STAT3 activation appears to be a prerequisite for the ability of PC to respond to APRIL and BAFF. This suggests a critical role for STAT3 as a key downstream signaling node transducing stimuli in LLPC that reinforces their survival and longevity (68).

Aiolos

Other factors restricted to the lymphoid lineage have been studied for their possible role in supporting the LLPC subset. Aiolos, part of the Ikaros gene family of nuclear regulators that function in modulating chromatin structure, is one of these factors (69). Absence of this gene does not affect B cell development or the early SLPC response but does affect long-term PC survival (70). Immunization normally causes a first wave of low-affinity antibodies produced primarily by SLPC, and then a second wave of higher affinity antibodies from BM LLPC (10). Upon deletion of Aiolos, there is a significant decrease in the production of high-affinity antibodies in the BM 120 days after primary immunization with no effect on the low-affinity antibody response (71). Additional studies show that the deletion of this gene does not cause a homing defect. This suggests that Aiolos upregulation is required for high-affinity antigen-specific LLPC, unlike other factors like BLIMP-1, which are required for generation of all PC. The exact mechanism remains to be understood, but the ability of Aiolos to promote high-affinity antibodies is important for enhanced protective immunity long term.

CD93

Another starting point for identifying specific receptors involved in LLPC survival and longevity are those receptors that are highly upregulated on BM PC compared to mature B cells. Greenlee et al. observed that CD93, which has been largely characterized for its role in intercellular adhesion and clearance of apoptotic cells, is not present on mature B cells but is highly upregulated during PC differentiation (72). Upon immunization with either T-independent (TI) or T-dependent (TD) antigens, CD93 is further upregulated in BM PC, and the $CD93^{+}CD138^{+}$ PC have increased expression of BLIMP-1, XBP-1, IRF4, and produce higher-affinity antibodies—suggesting that this receptor identifies the LLPC subset (73). Consistent with this, loss of CD93 diminished the persistence of long-term antibody titers post immunization (73). Loss of Blimp-1 decreases CD93 expression only in PC, as CD93 is still present at low levels in Blimp-1-deficient immature B cells. This connection to Blimp-1, which is most highly expressed in LLPC, led the authors to hypothesize that CD93 is necessary to promote the mature phenotype of LLPC and production of high-affinity antibodies. This is further corroborated by a recent study looking at measles titers of vaccinated school children in which the most highly expressed genes in high responder groups were CD93, IL-6, and CXCL12-implicating CD93 involvement in the generation of long-lived antibody responses in humans (74).

CD28

Upon activation, B cells downregulate the B cell lineage-defining transcription factor Pax5 (75). Pax5 was found to directly inhibit *CD28* gene expression in B cells, and the downregulation of Pax5 leads to upregulation of CD28 as an immediate/early event in B→PC differentiation. In this study loss of CD28 impaired short term induction of antigen-specific antibody titers, although long-lived antibody responses were not assessed. Previous studies in CD28 global knockouts and signaling-deficient CD28 receptor knock-in mice had demonstrated significant defects in antibody responses (76–79), but this was attributed to defects in T cell help. Subsequent work from our lab has shown that CD28 has an essential PC-intrinsic role in the survival of LLPC and the maintenance of durable antigen-specific IgG antibody titers after vaccination (80). Compared to Wt mice, unvaccinated $CD28^{-/-}$ mice have comparable numbers of splenic PC but have significantly fewer BM PC. *In vitro* studies demonstrated that CD28 activation alone (without a "signal 1" that is necessary for T cells) enhances the survival of purified BM PC, but not splenic PC, in response to serum-starvation-induced death. *In vivo,* the loss of CD28 expression specifically in the B cell lineage (B cells do not express CD28, only PC) resulted in the inability to sustain antigen-specific antibody titers in these murine models (80). Work done by Njau et al. also looked at the effect of CD28 on PC. Initial studies with global $CD28^{-/-}$ mice showed a significant increase in IgM antigen-specific responses compared to Wt mice, but found a significantly lower antigen-specific IgG response compared to Wt controls 60 days post immunization,

which they attributed to a defect in T-cell help (81). However, in contrast to Rozanski et al. specific loss of CD28 in the B cell lineage resulted in a significant increase in antigen-specific antibody titers compared to Wt PC. The basis of the differences between these two studies is unclear, although the immunization strategies (both the antigen and in particular the adjuvants used) were different. Regardless both studies establish that CD28 is present on PC and has a role in affecting antibody responses.

Studies of signaling pathways emanating from the CD28 receptor in LLPC demonstrated that loss of downstream Vav/Grb2 mediated PLCγ1 signaling caused a loss of BM LLPC, while loss of downstream PI3K/AKT signaling had no effect (82). Interestingly, although murine SLPC also express CD28, direct activation of the receptor does not induce downstream activation of PLCγ1 and NFκB—indicating a signaling basis for why SLPC are not responsive to CD28 and suggesting that CD28 has different activation thresholds in SLPC vs. LLPC. Further downstream, CD28 activation augments BLIMP-1 expression, a master regulator of PC identity and antibody production (82, 83). Consistent with the role CD28 plays in normal LLPC, we have also found that activation of CD28 confers a major pro-survival/chemotherapy resistance signal in MM (84, 85).

Activation of CD28 on MM directly transduces a pro-survival signal, and also signals back to dendritic cells (DC) through the CD28 ligands CD80 and CD86 expressed on these DC (84–86). This back signaling through CD80/CD86 upregulates both IL-6 and the immunosuppressive enzyme indoleamine 2,3-dioxygenase (IDO) expression/activity in the DC to promote further MM survival. Thus, CD28 both directly delivers a pro-survival signal to the myeloma cell and modulates the DC in the niche to further support MM survival. Conversely, *in vivo* blockade of the CD28-CD80/CD86 interaction with CTLA4-Ig (which is FDA approved for the treatment of rheumatoid arthritis) significantly enhanced the sensitivity to chemotherapy in a murine model of MM (84). Given the significant shared biology between LLPC and MM, this bidirectional signaling axis between CD28 and CD80/CD86 is now being investigated as previously unrecognized pro-survival factors for LLPC.

Autophagy

Because LLPC are continually generating and secreting high levels of Ig protein, they require unique metabolic pathways to deal with the protein load and ER stress. Induction of the Unfolded Protein Response (UPR) has been clearly identified as essential for B cells to differentiate to PC (87). A second mechanism implicated in the adaptation to this stress is autophagy. Autophagy's primary function is to sustain cellular metabolism when nutritional starvation occurs. In yeast, to counterbalance the stress caused by ER expansion, autophagic trimming is employed to remove excess ER (88). Within the immune compartment, mature T cells rely on autophagy-mediated turnover of mitochondria (mitophagy) to control intracellular production of reactive oxygen species (89, 90). Autophagy also appears to be critical for LLPC survival. Because antibody-producing PC are highly biosynthetic, it would be predicted that autophagy plays key roles in both processing/eliminating/recycling misfolded immunoglobulins as well as supporting the fuel and metabolic substrate requirements of the PC—in particular since the BM microenvironment may face highly variable nutrient availability due to changing levels of hematopoiesis during stress (91, 92). Analysis of the secretome from mesenchymal stromal cells (known to be part of the BM niche) identified two major survival factors of BM PC, fibronectin (FN-1) and the YWHAZ protein, which were shown to downregulate mTORC1 signaling in BM PC (17). Autophagy induction normally occurs after inhibition of mTOR (target of rapamycin), an evolutionarily-conserved protein kinase. mTOR is a key regulator in cell growth and response to the nutrient status and stress signals in a cell- and is known to negatively regulate autophagy (93). Thus, these factors in the niche could further influence the ability of LLPC to upregulate autophagy as a survival mechanism. Other studies have demonstrated that in mice knocked out for *Atg5*, which plays a central role in initiating autophagy, there is a significant defect in high-affinity LLPC post-immunization with a TD antigen (94). While all PC are generating Ig at high levels, it is possible that LLPC are more dependent on autophagy over their lifespan for both elimination of misfolded protein as well as a recycling mechanism to supply metabolic substrates, to reinforce the metabolic fitness of these cells during periods of nutrient deprivation.

Metabolism

Recent studies have begun to connect the metabolic energy production pathways used by LLPC to those used by memory T cells (which need to rapidly respond to activation to become highly biosynthetic and proliferate). CD28 co-stimulation in naïve T cells switches their fuel utilization to fatty acids and mitochondrial oxidation to sustain their metabolic demands, and this has been shown to be required for the generation of memory T cells (95, 96). CD28 co-stimulation also induces changes in mitochondria in memory T cells—elongated mitochondria with increased cristae structure are associated with enhanced (spare) respiratory capacity compared with naive T cells. Similarly, work on the metabolic phenotype of LLPC by Lam et al. has interestingly shown that both mouse and human LLPC have a greater maximal respiratory capacity than SLPC, and although they have increased ability to take up glucose (via Glut1 upregulation), they constitutively use long chain fatty acids as their primary fuel source—and glucose appears to be primarily used for antibody glycosylation (97, 98). However, under possibly non-optimal conditions they are also capable of using glycolysis and mitochondrial pyruvate import. The importance of this "alternative" metabolism program was demonstrated by the finding that loss of the ability to import pyruvate into the mitochondria significantly decreased LLPC frequency and abrogated sustained long-lived antibody response 22 weeks post vaccination (97). Similar work looking at IgA-secreting PC in the LP exhibited higher expression of glycolysis-related metabolites than naïve B cells in Peyer's Patches (PP) (99). It is proposed that the switch from IgM to IgA requires a metabolic shift and preferential use of the glycolytic pathway. This group also observed that splenic SLPC did not have very many glycolytic metabolites- indicating that both the BM and gut are unique microenvironments that

could contribute to this shift in the metabolic phenotype of LLPC.

ENPP1

Further investigation into the potentially unique metabolic characteristics of LLPC has led to the discovery of other previously unrecognized pathways. The transmembrane glycoprotein ectonucleotide pyrophosphatase/phosphodiesterase 1 (ENPP1) is highly expressed on PC, but its function has only recently been demonstrated. This enzyme was previously characterized for its role in bone formation, glucose homeostasis and downregulation of the insulin signaling pathway (100). ENPP1$^{-/-}$ mice were also found to have a significant reduction of BM LLPC 12 weeks post immunization with TD dependent antigen and *C. chabaudi* infection, but no effect was observed on splenic PC (101). ENPP1$^{-/-}$ PC take up less glucose than their Wt counterparts and exhibit an impairment in glycolysis, suggesting a role for ENPP1 as a regulator of glucose and the metabolic pathway found in LLPC (101).

Parallels Between Mice and Men

The relevance of some of the above factors in supporting LLPC survival were recently supported by characterization of human BM PC. These studies elegantly demonstrated the heterogeneity of BM PC in mice is also evident in humans, of which only a fraction appear to be the bonafide long-lived subset. This study defined 4 subsets of PC found in the BM: CD19^{+}CD38^{hi}CD138^{-} (subset A), CD19^{+}CD38^{hi}CD138^{+} (subset B), CD19^{-}CD38^{hi}CD138^{-} (subset C), and CD19^{-}CD38^{hi}CD138^{+} (subset D) (45). Subset D did not proliferate in comparison to the other three subsets and was CD20 negative, HLA-DR negative but CD28 positive (45). This subset had the highest number of IgG specific antibodies against tetanus, measles, mumps, and influenza. Interestingly, this subset also showed a distinctive RNA transcriptome signature for enhanced autophagy. This finding suggests that those components uncovered in murine studies are relevant to human PC and are fundamental to LLPC function and survival.

EXTRINSIC SIGNALS AND CONTRIBUTION OF THE NICHE

General PC Partners: Stromal Cells, Megakaryocytes, and Basophils

The PC-intrinsic signaling and downstream responses detailed above do not occur in a vacuum. LLPC are critically dependent upon signals they receive from the niche, as LLPC cultured alone *in vitro* rapidly die (102). Studies of the LLPC BM niche have identified several different cell types that can provide survival signals to the PC (**Figure 1**). The first cell type to be identified were reticular and mesenchymal stromal cells within the BM. They have high expression of CXCL12, the ligand for CXCR4 expressed on PC, which is responsible for their trafficking into the BM (18, 103). However, even though initial studies demonstrated these stromal cells supported PC survival *in vitro*, this survival was not sustained—and *in vivo*, they did not appear to have an essential role in supporting LLPC survival (48, 104). Furthermore, the PC-survival factors like APRIL, BAFF, and IL-6 are not secreted by these cells. Yet, it has recently been shown that BM mesenchymal stromal cells secrete fibronectin (FN-1) and other proteins (YWHAZ) that can sustain LLPC survival *in vitro,* in combination with hypoxia and factors like APRIL (17). FN-1 binds integrins, collagen and CD138 (highly expressed on LLPC), and is proposed to play an important role in tethering PC to the BM extracellular matrix. Studies in MM have also shown that integrin-mediated binding to fibronectin facilitates resistance to chemotherapy-mediated killing (105).

Other studies have identified megakaryocytes as part of the LLPC niche. Megakaryocytes can produce APRIL and IL-6, and mice that do not have the ability to form megakaryocytes have a reduced number of BM PC when compared to Wt mice (106). Furthermore, upon immunization with ovalbumin (Ova), it was observed that Ova-specific PC were in direct contact with megakaryocytes in the BM. However, functional studies of PC post-immunization in the absence of megakaryocytes did not show any effect in the late stage of antigen-specific antibody responses. Basophils are another cell type reported to support PC survival. In *in vitro* cultures of basophils and BM PC, the presence of basophils supported the survival of these PC and markedly increased the Ig production of these cells (107). However, functional studies of the effect of basophils on antigen-specific BM PC responses were only assessed *in vitro*, therefore it is still uncharacterized how these cells might affect BM LLPC in the *in vivo* setting. It was determined that the survival effects seen by basophils are most likely due to their ability to secrete IL-4 and IL-6. Thus, megakaryocytes, and basophils may play an important role in establishing the LLPC niche and possibly LLPC access, however, it remains unclear how they contribute to long term survival and maintenance of LLPC.

Eosinophils

Another cell subset thought to provide critical survival cytokines to PC, like IL-6, and APRIL, are eosinophils. It was reported that eosinophils co-localize with PC in both the BM and gut associated lymphoid tissue (GALT) and that eosinophil-deficient mouse have significantly fewer PC at steady state and post-exposure to antigen (108, 109). For IgA-secreting PC, eosinophils are necessary for promoting IgA production and maintaining immune homeostasis in the GALT (110). In a study of PC in the lamina propria one-third of the PC were found to be localized next to eosinophils (21). With respect to the BM niche, it was found that lack of eosinophils caused LLPC in the BM to undergo apoptosis. Upon reconstitution of eosinophils, the number of PC was transiently increased, suggesting a role for these cells in homing of PC to the BM as well as maintaining the PC population once there (108). Once activated an eosinophil can promote B cell differentiation into IgM secreting PC that is independent of the induction of germinal centers and generation of high-affinity IgG secreting

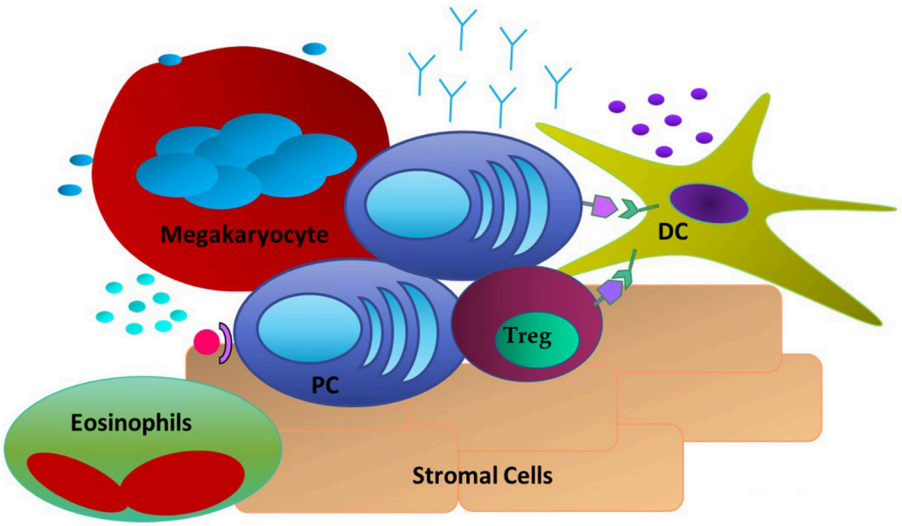

FIGURE 1 | Extrinsic Factors in the LLPC niche. PC are associating with DC through CD28: CD80/CD86 interactions and with Tregs. Stromal cells expressing CXCL12 act as a homing signal to $CXCR4^+$ PC. Megakaryocytes and Eosinophils are also present and have been shown to produce soluble factors such as APRIL and BAFF to promote PC survival.

PC (111). However, more recent studies have questioned whether eosinophils are a necessary component for LLPC survival and function, at least in murine models this has not been conclusive (112). Therefore, the current literature suggests that eosinophils may play a critical role in the generation and trafficking of PC, for both mucosal sites and the BM, but their contribution to the survival, maturity, and long term function of LLPC remains unsettled.

Dendritic Cells

Parallel studies in the niche have focused on cellular components that express the ligands that can engage receptors on LLPC that support survival and function. Our work has demonstrated a key role for CD80, and CD86, the natural ligands for CD28. An absence of these ligands in global knockout mice significantly diminished BM LLPC numbers and durable antigen-specific titers (80). The cells that have the highest expression of CD80 and CD86 are myeloid (conventional) DC. Other myeloid cells (monocytes, eosinophils) can also have significant expression. Our lab and others have demonstrated co-localization of DC with PC occurs within the BM. Additionally this supports LLPC survival *in vitro* (in a contact and CD28-dependent fashion) and *in vivo* (80, 113). This interaction has further significance as DC can produce significant amounts of the pro-PC survival cytokine IL-6, and that CD80/CD86 activation is one pathway that induces DC IL-6 production (86). As noted above, DC also significantly enhances the survival of MM in a CD28-CD80/CD86 dependent manner. This suggests a bidirectional signaling axis in the niche to support LLPC both directly and indirectly through modulation of cellular partners to produce essential survival factors.

T Regulatory Cells

Another significant partner recently identified within the LLPC BM niche are T regulatory (Tregs) cells in close physical association with $CD11c^+$ DC. High number of Tregs have been shown to co-localize with PC in the BM *in vivo*, and this is necessary to homeostatically support BM PC populations (113). Ablation of Tregs during infection caused a significant reduction in the number of BM PC. The phenotype of these BM Tregs was consistent with an activated subset with increased suppressive capabilities, and most closely matched the profile observed in injured muscle. Interestingly, unlike splenic Treg cells that have little CTLA-4 expression, these BM Tregs express CTLA-4—which is a member of the CD28 superfamily that also binds CD80 and CD86. In the absence of Treg expression of CTLA-4, there was a 3-fold increase in the number of BM PC. The authors proposed that $CTLA\text{-}4^+$ Tregs contribute to the maintenance of an immune-privileged niche for LLPC in part by buffering this niche against systemic inflammatory changes, as well as maintaining LLPC homeostasis by limiting the PC pool (113). However, what role CTLA-4 itself is playing within the PC niche is unclear. It is possible that through expression on Tregs it acts in a homeostatic way to limit the amount of PC able to traffic into and reside within the BM. However, it could also be binding to the CD80/CD86 receptors on niche DC, causing upregulation of cytokines like IL-6 to help support PC survival. Alternatively, Treg CTLA-4 may compete for and limit LLPC CD28 binding to these ligands on the DC, as part of homeostatic regulation of the LLPC population. It is interesting to speculate that this cellular triumvirate of LLPC, DC, and Tregs is modulated by dynamic molecular interactions between CD28, CD80/CD86, and CTLA-4, facilitating a stable niche that critically supports LLPC survival and continued function.

TABLE 2 | Importance of intrinsic factors in B cell and PC subsets.

Intrinsic factors	Cell type	Expression	Differentiation	Survival	References
BCMA	*Mature B cell*	Yes	Not necessary	Not necessary	(114)
	SLPC	Yes	Not necessary	No	(50)
	LLPC	Yes	Not necessary	Yes	(50)
	Mucosal LLPC	Yes	Unknown	Yes	(115)
STAT3	*Mature B cell*	Yes	Yes	Yes	(116)
	SLPC	Yes	Yes	Yes	(68)
	LLPC	Yes	Yes	Yes	(68)
	Mucosal LLPC	Unknown	Unknown	Unknown	
Aiolos	*Mature B cell*	Yes	Yes	Necessary for homeostasis	(70)
	SLPC	Yes	Not necesssary	Not necessary	(71)
	LLPC	Yes	Yes	Yes	(71)
	Mucosal LLPC	Unknown	Unknown	Unknown	
CD93	*Mature B cell*	No	Not necesssary	Not necessary	(117)
	SLPC	Yes	Yes	Not necessary	(73)
	LLPC	Yes	Yes	Yes	(73)
	Mucosal LLPC	Unknown	Unknown	Unknown	
CD28	*Mature B cell*	No	No	No	(75)
	SLPC	Yes	No	No	(80)
	LLPC	Yes	No	Yes	(80)
	Mucosal LLPC	Yes	No	No	(118)
Autophagy	*Mature B cell*	Yes	Not necesssary	Yes	(119)
	SLPC	Yes	Yes	Yes	(120, 121)
	LLPC	Yes	Yes	Yes	(94)
	Mucosal LLPC	Unknown	Unknown	Unknown	
Metabolic shift: glycolysis and mitochondrial alterations	*Mature B cell*	Yes	Yes	Yes	(122)
	SLPC	No	No	No	(97, 99)
	LLPC	Yes	Yes	Yes	(97)
	Mucosal LLPC	Yes	Yes	Yes	(99)
ENPP1	*Mature B cell*	Yes (terminally differentiated GC B cells)	No	Unknown	(101, 123)
	SLPC	Yes	No	No	(101)
	LLPC	Yes	No	Yes	(101)
	Mucosal LLPC	Unknown	Unknown	Unknown	

CONCLUDING REMARKS

This review has identified some of the components that form the networks that allow for survival and maintenance of LLPC. Long-term generation of protective antibodies by this subset is crucial for durable protective immunity. Understanding these key molecular and cellular components that support LLPC survival and longevity gives us tools for utilization of new vaccination strategies as well as therapeutic strategies against diseases that arise from this cell, such as MM and autoimmune syndromes.

It has become clear that there are distinct subsets of PC defined by anatomic location, phenotype, function, and longevity (**Table 2**). Evidence suggests that PC making high affinity neutralizing Ig (which would be particularly important in rapidly lethal pathogens that characteristically cause epidemics) are key members of the LLPC subset and are generated upon germinal center reactions and then traffic to the BM; where they have unique characteristics that allow them to unlock and occupy/utilize the LLPC niche. It is now clear that this is a significant oversimplification given the heterogeneity of the BM PC populations and niches present in other organs like the gut. Nonetheless, a growing body of evidence demonstrates that the "traditional" LLPC that produce lifelong protective antibody titers, requires both PC intrinsic and niche components to be long-lived. These interactions can then lead to downstream PC-intrinsic signaling that buttresses the ability of the LLPC subset to survive over decades in their microenvironment; which in the BM is hypoxic and crowded with rapidly proliferating cells (especially reactive BM responding to infection) competing for space and nutrients, and in mucosal sites where there is constant exposure to both helpful and harmful microbes. Thus, the key aspects that underlie the longevity of LLPC likely reinforce the mechanisms that allow for effective adaptation to these stresses over a lifetime.

AUTHOR CONTRIBUTIONS

SL primary author and drafted the bulk of the review, substantial contributions to conception and design. AU provided revisions to content of manuscript. KL contributed to conception and design, provided critical revisions and final approval, and provided funding.

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REVIEW
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Cell of Origin and Genetic Alterations in the Pathogenesis of Multiple Myeloma

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B cell activation and differentiation yields plasma cells with high affinity antibodies to a given antigen in a time-frame that allows for host protection. Although the end product is most commonly humoral immunity, the rapid proliferation and somatic mutation of the B cell receptor also results in oncogenic mutations that cause B cell malignancies including plasma cell neoplasms such as multiple myeloma. Myeloma is the second most common hematological malignancy and results in over 100,000 deaths per year worldwide. The genetic alterations that occur in the germinal center, however, are not sufficient to cause myeloma, but rather impart cell proliferation potential on plasma cells, which are normally non-dividing. This pre-malignant state, referred to as monoclonal gammopathy of undetermined significance or MGUS, provides the opportunity for further genetic and epigenetic alterations eventually resulting in a progressive disease that becomes symptomatic. In this review, we will provide a brief history of clonal gammopathies and detail how some of the key discoveries were interwoven with the study of plasma cells. We will also review the genetic and epigenetic alterations discovered over the past 25 years, how these are instrumental to myeloma pathogenesis, and what these events teach us about myeloma and plasma cell biology. These data will be placed in the context of normal B cell development and differentiation and we will discuss how understanding the biology of plasma cells can lead to more effective therapies targeting multiple myeloma.

Keywords: multiple myeloma, MGUS, plasma cell, B cell, genetics, epigenetics, IgH translocations, MYC

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A BRIEF HISTORY OF PLASMA CELLS AND MALIGNANCY

The study of malignancies that would ultimately be resolved to plasma cells was intertwined with, and necessary for the discovery of plasma cells and their function. Perhaps the first report of the plasma cell malignancy multiple myeloma described as "*mollities ossium*" by Samuel Solly in 1844 characterized two cases of patients who presented with symptoms including fatigue, bone pain, and multiple fractures (1). The author noted, that although rare, these were certainly not the first cases. Upon autopsy it was revealed that the bone marrow of both patients was replaced with a red substance filled with distinctive looking large cells [reviewed by Kyle and Rajkumar (2)]. The second patient noted that his urine stiffened his clothes, and a sample was sent for examination

by Dr. Henry Bence Jones who confirmed the semi-solid urine would liquefy upon heating but resume its viscous consistency upon cooling (3, 4). Dr. Bence Jones emphasized the importance of obtaining urine samples for diagnosis, a practice that continues today.

Contemporaneous observations in immunity would lay a foundation for understanding the cellular source of these neoplasms and the Bence Jones proteins. The seminal work of John Fewster and Edward Jenner in smallpox demonstrated acquired immunity, which Jenner would later use to successfully protect patients through inoculation with cowpox (5). This led to discoveries in 1890, where Emil von Behring and Kitasato Shibasaburo showed that the serum of animals immunized with sub-lethal doses of dipetheria and tetanus contained an antitoxin (6). This proved the existence of an adaptive humoral immune system. The following year Paul Ehrlich described this antitoxin component as an "antibody" and in his 1908 Nobel laureate speech predicted the existence of cells that recognize these toxins using a "toxin receptor" and amazingly anticipated that "the antitoxin is nothing else but discharged components of the cell, namely receptors discharged in excess" (7). Although the term "plasma cell" had already been coined (8), it would be more than 40 years before the cellular source of this immunity was discovered.

Several more cases of *mollities osseum* were reported and in 1873 J. von Rustizky coined the phrase multiple myeloma (9). In 1900 James H. Wright concluded that the cells prevalent in multiple myeloma "are essentially plasma cells, or immediate descendants of them" (10). However, this did not explain the presence of proteinurea or Bence Jones proteins. In 1947, plasma cell formation was correlated with antibody production implicating plasma cells as the cellular source of antibodies (11). Korngold and Lipari determined in 1956 that multiple myeloma patients often had "electrophoretically homogeneous" Bence Jones proteins (12), which would later be shown to be identical to protein in the serum of the same patients (13). These monoclonal proteins corresponded to one of the two immunoglobulin light chains that were named kappa and lambda after Korngold and Lipari. Later the delineation of T and B lymphocytes (14) [reviewed by Max Cooper (15)] would lead to the identification of B cells as the precursors to plasma cells.

Advances in electrophoresis and the invention of the immunoblot allowed for more routine testing of immunoglobulin proteins in the serum and urine. In 1961, Jan Waldenström described a monoclonal band in patients with hypergammaglobulinemia many of whom had multiple myeloma or macroglobulinemia, but other patients had no symptoms of malignancy (16). Importantly, Waldenström delineated monoclonal proteins as indicative of neoplasm or a pre-malignant disease (now known as monoclonal gammopathy of undetermined significance or **MGUS**). This was in contrast to polyclonal proteins that were indicative of an inflammatory response.

Today, the cellular and molecular etiology of multiple myeloma as well as the programming of normal B cell development and plasma cell differentiation have been elucidated to a great extent. Like their discoveries, we have learned much about multiple myeloma from studying the normal processes of plasma cell differentiation and *vice versa*. Despite the incredible progress made and knowledge gained, over 130,000 new cases of multiple myeloma occur every year worldwide (17), including over 30,000 cases in the US alone (18). It is now known that myeloma is a progressive disease preceded by an asymptomatic stage called MGUS (19, 20) often followed by an intermediate stage referred to as smoldering multiple myeloma (**SMM**), prior to symptomatic newly diagnosed multiple myeloma (**NDMM**), and finally relapsed and/or refractory multiple myeloma (**RRMM**). Despite the incredible progress made, it is still very difficult to identify MGUS patients who will progress from those whose condition will remain benign. This is a major problem as MGUS is present in 3% of the population over 50 years of age, and progresses to multiple myeloma at a rate of ∼1% per annum (21, 22). There is now a formidable arsenal of therapies for multiple myeloma, and thus far the most successful agents are targeted at plasma cell biology, which is largely retained by multiple myeloma (23). While most patients benefit from these treatments, ultimately and unfortunately, most still succumb to disease resulting in almost 100,000 deaths per year worldwide (17).

B CELL DEVELOPMENT, PLASMA CELL DIFFERENTIATION, AND MYELOMAGENESIS

B cell development, much like plasma cell neoplasms, progresses through a series of well-defined stages. Current data suggest that a distinguishing attribute of plasma cell malignancies is the differentiation state at which the transformation manifests. This defining characteristic can be exploited to better identify vulnerabilities of multiple myeloma through the study of non-malignant B cells and plasma cells (23). A comprehensive description of these processes has been provided for both B cell development (24, 25) and plasma cell differentiation (26–29), and is beyond the scope of this current review. However, a brief description of these processes is essential to understanding the mechanistic underpinnings and etiology of myelomagenesis.

Like all immune cells, B cells are derived from hematopoietic stem cells that primarily develop in the bone marrow (30, 31) or fetal liver (32). Hematopoietic stem cells can successively differentiate into multi-potent progenitors, common lymphoid progenitors, and eventually mature B cells through the stages pre-pro-B, pro-B, pre-B, immature B, and transitional B cells. In the mouse, this process requires the transcription factors including E2A (33), PU.1 (34), and PAX5 (35) as well as interleukin 7 (IL7) cytokine signaling (36). It is important to point out there are key differences in human B cell development (37, 38), which is not dependent upon IL7 (39). However, in both mice and humans the recombination activated genes, RAG1 and RAG2, physically recombine the variable (V), diversity (D), and joining (J) segments of the immunoglobulin genes (40, 41). Mechanistically, RAG proteins work by recognizing and excising recombination signal sequences, which are conserved heptamer and nonamer sequences separated by a spacer (42).

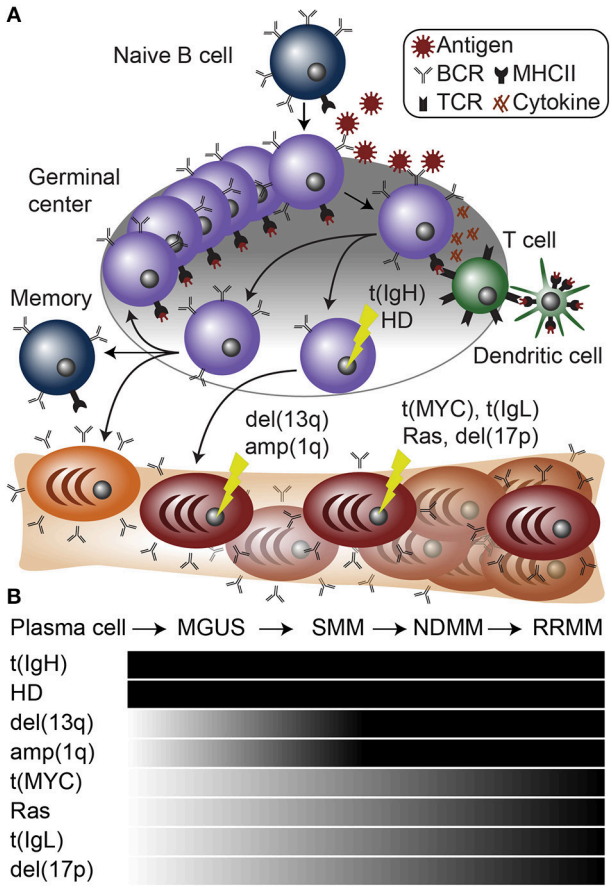

FIGURE 1 | Plasma cell differentiation and myelomagenesis. **(A)** Schematic of B cell differentiation, plasma cell development, and myelomagenesis. Lightning bolts represent genetic mutations common in myeloma. **(B)** Diagram of stages of myeloma progressing from a normal plasma cell to monoclonal gammopathy of undetermined significance (MGUS), smoldering multiple myeloma (SMM), newly diagnosed multiple myeloma (NDMM), and relapse/refractory multiple myeloma (RRMM). Below are common mutations in myeloma and the stages at which they appear.

This proceeds first at the immunoglobulin heavy chain (**IgH**) D → J segments (pre-pro-B), and then V → DJ segments (pro-B). If a productive (in-frame) IgH gene is recombined, it is then transcribed, translated, and expressed on the surface with a surrogate light chain (composed of VPREB and IGLL1), which triggers light chain recombination at the V → J segments (light chains contain no diversity segments) marking the pre-B stage. This occurs first at the kappa light chain and if no productive allele is made, then at the lambda light chain. Surface expression of the paired heavy and light chains—referred to as the B-cell receptor (**BCR**)—marks the immature B cells stage, after which B cells can transition from the bone-marrow into the periphery and secondary lymphoid tissues where they mature.

Mature naïve B cells are mitotically (43) and transcriptionally quiescent (44, 45), but surveil the environment for pathogens which are recognized by toll-like receptors (**TLR**) (46) and the BCR. B cell activation that occurs without cytokine help from T cells, referred to as T-cell independent activation, generally results in acute and shorter lived B cell and plasmablast responses. In contrast, antigens that invoke T cell-dependent (**TD**) cytokine stimulation induce a more complex B-cell activation that results in selection of B cells with higher-affinity B-cell receptors and longer lasting immunity. However, this process is prone to genomic errors that contribute to oncogenesis. Indeed, current data suggests that almost all of myeloma is initiated by mutations associated with TD responses. TD B-cell activation requires BCR-mediated endocytosis of protein antigens, which are subsequently degraded and ectopically presented by the major histocompatibility complex class II (**MHC-II**) (47). When an antigen peptide presented by MHC-II on a B cell is recognized by a cognate T cell receptor (**TCR**), this induces an immunological synapse and T cell stimulation. This causes T cell expression of CD40 ligand (CD40L) (48) that induces B cell CD40 signaling (49), as well as polar release of T cell cytokines IL4 (50, 51), IL21, and IL6 (52) resulting in potent B cell activation. In particular, IL6 not only induces B cell activation, but is a potent growth stimulant for plasma cells and myeloma (53). This stimulation induces rapid B cell proliferation, which forms a lymphoid structure called a germinal center [**Figure 1A**; reviewed in (54, 55)]. During the germinal center reaction, B cells continuously cycle through rounds of division and selection for high-affinity antibodies, which are made through two types of somatic alterations termed somatic hypermutation (**SHM**) (56) and class-switch recombination (**CSR**), both of which are mediated by the activation-induced cytidine deaminase (**AID**) (57). AID deaminates cytosines on single-stranded DNA resulting in mutations of the immunoglobulin heavy and light chains or SHM. SHM of the heavy and light chains has the potential to increase antibody-antigen affinity through mutation of the complementarity determining region. This results in more efficient antigen uptake and presentation, resulting in more T cell stimulation and selection of B cell clones with high-affinity antibodies to a given antigen. CSR occurs when IgH somatically recombines the constant region μ and its splice isoform δ with one of the alternative constant regions γ3, γ1, α1, γ2, γ4, ε, or α2. CSR occurs via AID-dependent recombination of switch regions located just 5' of each constant region resulting in recombination of a new IgH constant region (58–60). This process requires DSBs, and can result in aberrant recombination with other genomic regions causing translocations. Indeed, there is now substantial evidence that myeloma initiating alterations are a result of errors in CSR.

PRIMARY GENETIC EVENTS IN GAMMOPATHIES

A dichotomy of genetic aberrations accounts for the large majority, if not all of myeloma initiating events. First, approximately half of myeloma cases contain an aneuploidy of several odd numbered chromosomes including 3, 5, 7, 9, 11, 15, 19, and 21. This is referred to as hyperdiploidy (**HD**), and will be further discussed below. The second type of founding genetic event is almost mutually exclusive with hyperdiploid myeloma and involves translocations of the IgH locus (61) (**Figure 1B**).

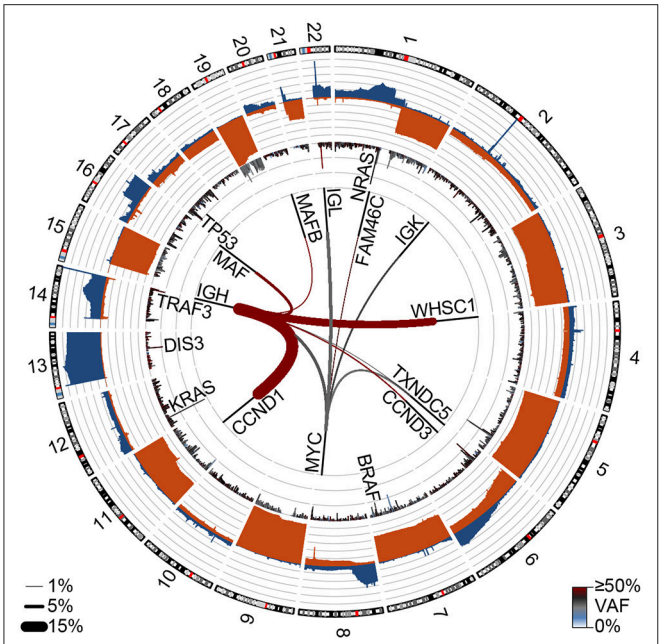

FIGURE 2 | Genetic events in newly diagnosed multiple myeloma (NDMM). Circos plot showing copy number losses (blue) and gains (orange) in the outer ban (gray lines indicate 10% of the population). Mutations are shown on the inner ban, where the frequency of non-synonymous mutations and the variant allele frequency (VAF) are shown for 500 kb regions. Translocations are shown on the inside where the frequency is denoted by line thickness (key bottom left) and color denotes the VAF. Data are from 850 NDMM patients part of the MMRF CoMMpass study (dbGaP phs000748.v7.p4).

IgH translocations juxtapose the IgH enhancers to one of a half dozen oncogenes including any of the three Cyclin D genes (CCND1-3), WHSC1 (also known as NSD2 or MMSET), MAF, or MAFB [reviewed in (62–64)] (**Figure 2**). When present, these translocations are clonal alterations (i.e., present in all tumor cells) in all stages of MGUS or myeloma and emanate from the IgH constant chain switch regions implicating them as errors in CSR that occurred during B cell activation in the germinal center (65, 66). Consistent with this, more than 90% of myelomas express class-switched IgH constant chains and almost all display SHM identifying them as post-germinal center cells.

Cyclin D Dysregulation

Cyclin D dysregulation is the most common type of IgH translocation, which involve t(11;14), t(12;14), and t(6;14) translocations that juxtapose the IgH enhancer(s) with CCND1 (15–20% of NDMM), CCND2 (∼1%), and CCND3 (1–4%), respectively (65, 67, 68). All three Cyclin D genes function by activating CDK4 and CDK6 that in turn phosphorylate and inactivate RB allowing for E2F activation and cell cycle progression (63). Although these translocations result in aberrant expression of their respective Cyclin D genes, overexpression of at least one Cyclin D gene appears to be an early and unifying event in plasma cell malignancies (69). For instance, IgH translocations to MAF or MAFB result in high levels of CCND2 (70); IgH-WHSC1 translocations result in moderate levels of CCND2, and hyperdiploid disease results in overexpression of CCND1 (located on chromosome 11) or expression of both CCND1 and CCND2 (69). Conversely, CCND3 expression is less frequent and seems to be primarily a result of t(6;14) (69). Although most Cyclin D translocations occur at the switch region breakpoints, a subset of t(11;14) CCND1 translocations originate from the V(D)J region, suggesting that they may be the result of errors in V(D)J recombination during B cell development (71). Earlier work indicated myeloma-specific idiotypes reacted with some pre-B cells from the same patient, suggesting some myeloma may have origins in B cell development (72). However, these early studies were limited to two cases and it is unclear if pre-B cells with a myeloma idiotype harbor the genetic mutations that result in malignancy. Furthermore, should these errors occur during B cell development, it is not clear what causes these to manifest in myeloma rather than mantle cell lymphoma, which routinely have t(11;14) translocations originating from the V(D)J region (73). Regardless, it is clear that these translocations result in aberrant CCND1 expression, which predisposes to malignancy.

IgH-WHSC1 or t(4;14)

IgH-WHSC1 or t(4;14) are the second most common translocation, occurring in ∼15% of NDMM, and in most cases results in the dual dysregulation of both WHSC1 and FGFR3 (74, 75). These are mostly reciprocal translocations that occur almost exclusively at the IgM switch region and split WHSC1 and FGFR3 on the telomeric side of chromosome 4p. This often results in IgH-WHSC1 fusion transcripts and/or loss of the 5′ exons of WHSC1 (76). Subsequently, the IgH Eμ enhancer drives expression of WHSC1 on the derivative of chromosome 4, while the IgH 3′ enhancers drive expression of FGFR3 on the derivative of chromosome 14 (62, 74). For some time it was unclear whether FGFR3 or WHSC1 was the definitive oncogenic factor, however, ∼25% of t(4;14) myelomas do not have the reciprocal FGFR3 translocation and lack FGFR3 expression (76, 77). This suggests that WHSC1 is the essential transforming element, although FGFR3 overexpression and activating mutations likely contribute to pathogenesis. It is also possible that FGFR3 expression is required for MGUS initiation but is subsequently lost in a subset of cases. WHSC1 is now known to be a histone 3 lysine 36 (H3K36) methyltransferase that catalyzes di-methylation of histone 3 lysine 36 (H3K36me2) (78, 79). Pervasive H3K36me2 in t(4;14) myeloma is associated with accessible chromatin and gene dysregulation (79). However, how WHSC1 results in myelomagenesis or CCND2 upregulation is not well understood and difficult to trace due to the genome-wide effects H3K36me2.

IgH-MAF and IgH-MAFB

IgH-MAF and IgH-MAFB translocations are the least common class of primary IgH translocation, and result from t(14;16) and t(14;20), respectively (80, 81). These are present in approximately 5–10% of NDMM cases, with MAF being more common than MAFB (66). MAF induces expression of CCND2 through a MAF binding motif in the CCND2 promoter, as well as Integrin B7 leading to increased adhesion to bone marrow stromal cells

(70). In addition to upregulating MAF, t(14;16) translocation breakpoints disrupt WWOX, a tumor suppressor gene in breast and prostate cancers, where it is also commonly deleted (82, 83). The contribution of WWOX to t(14;16) myeloma is still unclear as there is little to no evidence of biallelic inactivation and most research has focused on the oncogenic effects of MAF (62).

Hyperdiploidy

Hyperdiploidy is the other common type of initiating genetic event in plasma cell malignancies. Hyperdiploidy is almost mutually exclusive with IgH translocations, and hyperdiploid myeloma tend to have a better prognosis than IgH-translocated multiple myeloma (61, 66, 84). Unlike IgH translocations, it is very difficult to trace the oncogenic effects of hyperdiploidy to a causative element(s) due to the aneuploidy of numerous chromosomes. Compounding the difficulty of pinpointing the pathogenic elements in hyperdiploid myeloma, model systems for hyperdiploid myeloma are lacking. For instance, of the roughly 80 multiple myeloma cell lines, more than 90% have IgH translocations and there are no commonly used hyperdiploid cell lines (62). The good prognosis of hyperdiploid myeloma and lack of cell line models suggests that hyperdiploidy rarely results in extramedullary disease or plasma cell leukemia as most cell lines are derived from patients with disease that is independent of the bone marrow microenvironment (85). Hyperdiploidy is also hypothesized to occur during rapid germinal center proliferation that results in chromosome segregation errors. However, it is not clear if this is one catastrophic event or a series of sequential errors that must occur prior to a clonal outgrowth.

Both IgH translocations and hyperdiploid myeloma are found to be clonal at all stages of gammopathy, which is consistent with them being founding genetic events (**Figure 1B**). Despite these large genomic changes, almost all myeloma has multiple genetic events present upon diagnosis, suggesting that primary events initiate MGUS, but are not sufficient to cause symptomatic disease.

SECONDARY GENETIC EVENTS IN GAMMOPATHIES

In addition to the primary genetic events described above, presentation of myeloma is regularly accompanied by several other major chromosome abnormalities including deletion of chromosome 13q [del(13q)], amplification of chromosome 1q [amp(1q)], and deletion of chromosome 1p [del(1p)] (**Figure 1**). All three of these alterations involve regions tens of megabase in size and thus similar to hyperdiploidy, pinpointing the causative element(s) is difficult. However, contributing elements are speculated for all of these aberrations with varying degrees of supportive data.

Del(13q)

Del(13q) occurs in almost 50% of NDMM (86) and is found to be clonal, but is less frequent in MGUS where it is either sub-clonal or clonal (87). Del(13q) co-occurs with t(4;14) and t(14;16) myeloma and was once considered to be marker of poor prognosis, but this outcome appears to have been overcome by the use of proteasome inhibitors (88). Generally, the entire arm of 13q is lost, and contains several loci that may contribute to myeloma pathogenesis. Notably RB1, which prevents cell cycle progression by sequestering E2F transcription factors, is located on 13q14. However, 13q loss is primarily mono-allelic and rarely are there mutations or deletions that result in biallelic RB1 inactivation (64). In contrast to RB1, the exosome endoribonuclease DIS3 is mutated in ∼10% of NDMM and ∼75% of these mutations occur in del(13q) myeloma suggesting biallelic loss of DIS3 occurs in most DIS3 mutated myeloma (66, 89, 90). Finally, 13q14 is also deleted in Chronic Lymphocytic Leukemia (CLL), albeit in a more punctate fashion, allowing researchers to pinpoint the DLEU2/miR-15a/16-1 locus as a minimally deleted region. Deletion of this region causes a lymphoproliferative disease in mice (91). A similar analysis independently identified the same locus as a minimally deleted region in myeloma (92), but it remains to be determined whether DLEU2, miR-15a, or miR-16-1 have tumor suppressor function in myeloma.

Amp(1q)

Amp(1q) occurs in 40% of patients and is associated with worse prognosis (93, 94). The poor prognosis appears to be dose-dependent as patients with 4 or more copies of chromosome 1q do worse than those with three (95). These additional copies of 1q likely have a proportional effect on expression of 1q genes, as a gene signature of high-risk myeloma is composed of a large number of 1q genes (96, 97). Putative oncogenes may include CKS1B, which facilitates ubiquitinylation and degradation of the cyclin dependent kinase inhibitor CDKN1B ($p27^{KIP1}$) (98). Approximately two-thirds of amp(1q) coincide with del(13q), which is a significant co-occurrence between the two events (90, 99). If CKS1B and RB1 are the myeloma-inducing genetic alterations on amp(1q) and del(13q), respectively, questions remain as to why two alterations are needed in the same pathway in addition to overexpression of a Cyclin D gene. This might be explained by the somewhat rare nature of cell cycle progression in myeloma where <1% of cells are actively synthesizing DNA (64). Alternatively, it may be a polygenic effect or other genes may be responsible for the deleterious effects of these alterations. One such gene on chromosome 1q is MCL1, a BCL2-family anti-apoptotic protein that is induced during plasma cell differentiation and essential for plasma cell and myeloma cell survival (100–102). There are now MCL1 inhibitors in early phase clinical trials, and it will be important to understand if these are more effective against myeloma with amp(1q) that overexpresses MCL1 (103–105), as discussed further below.

Del(1p)

Del(1p) occurs in 20–25% of patients and often co-occurs with hypodiploidy (loss of chromosomes). Unlike hyperdiploidy, hypodiploidy is associated with worse prognosis (106, 107) as is del(1p) (94). The region lost on 1p often includes the cyclin dependent kinase inhibitor CDKN2C, and similar to amp(1q), two-thirds of del(1p) also coincides with del(13q) and mono-allelic loss of RB1. Another promising candidate myeloma suppressor gene located on chromosome 1p, is FAM46C, a non-canonical poly(A) polymerase (108, 109). Inactivating mutations in FAM46C result in a cell survival advantage whereas

overexpression causes an unfolded protein response and cell death (110). In addition to being lost in ∼25% of NDMM by del(1p), FAM46C is also mutated in ∼10% and translocated in ∼2.5% of NDMM, supporting its role as a *bona fide* tumor suppressor in multiple myeloma (66, 89, 90).

GENETIC EVENTS OF PROGRESSION IN MGUS AND MYELOMA

MYC Structural Variants

MYC structural variants are pervasive in B cell malignancies and myeloma is no exception. MYC structural variants are sometimes present in MGUS, present in ∼35% of NDMM, and even more common in RRMM and myeloma cell lines (66, 111). This suggests that MYC alterations promote disease progression. This is further supported by a mouse model of myeloma, in which AID-induced MYC expression only results in myelomagenesis in mouse strains prone to MGUS (112, 113). This suggests that MYC cannot initiate MGUS, but facilitates MGUS progression to myeloma. Consistent with this, IgH-MYC [t(8;14)] translocations are distinct from other IgH translocations in that they are found at sub-clonal levels in NDMM and have extragenic IgH breakpoints (66, 112). Such MYC alterations in myeloma are distinct from other B cell malignancies such as Burkitt lymphomas, where immunoglobulin-MYC translocations are a near universal primary event and IgH-MYC translocations have breakpoints in the IgH switch regions (114, 115). In myeloma, MYC structural variants are spread across at least two broad regions and serve to amplify or transpose large enhancers to drive MYC expression (66, 112, 116). Interestingly, almost all MYC translocations are also accompanied by copy number alterations, with most showing large duplicated sequences at both translocation breakpoints (66, 117). This appears to be a common phenomenon present at other secondary translocations in myeloma and other cancers, however, it is rare at myeloma primary translocations that originate from the CSR regions (66, 117). This key insight into the mechanistic basis of secondary and complex translocations could be explained by synthesis-dependent strand annealing of DSBs with long single-stranded overhangs. Indeed, AID deaminates cytosines on single stranded DNA and is known to initiate genomic instability at heavily transcribed regions of the genome (118), such as the intragenic regions of PVT1, where MYC translocations commonly occur, as well as at the immunoglobulin enhancers.

Immunoglobulin Light Chain Kappa (IgK) and Lambda (IgL)

Immunoglobulin light chain kappa (IgK) and lambda (IgL) enhancers are often co-opted in complex secondary translocations that drive oncogene expression. IgL translocations occur in ∼10% of MGUS and NDMM, but up to 20% of RRMM or myeloma cell lines, whereas IgK translocations are more rare, occurring in <5% of NDMM (64, 66). This is surprising given that two-thirds of human B cells and myeloma cells express IgK and only one-third express IgL. The higher prevalence of IgL translocations can be explained by B cell ontogeny, where IgK VJ rearrangement deletes the IgK enhancer if a productive IgK product is not made (119). Thus, without an enhancer the IgK region is inert if translocated, and consequently all IgK-translocated myelomas express IgK (66). Conversely, IgL-translocated myelomas are found at the normal ratio of two-thirds IgK expressing to one-third IgL expressing, which indicates that the IgL enhancer is constitutively active, and equally prone to translocation even in IgK-expressing myeloma (66). We recently showed that translocations of the IgL locus, but not the IgK locus, were prognostic of poor outcome (66). This was even true when restricting the analysis to the same translocated oncogene. For instance, approximately 40% of both IgK and IgL translocations occur to MYC, but only patients with IgL-MYC translocations have a poor prognosis, despite similar baseline levels of MYC expression from each translocation (66). This suggests that distinct enhancers are differentially susceptible to therapeutic perturbation and myeloma is not only a disease of oncogenes but also an "enhanceropathy."

Deletion of 17p [del(17p)] Including TP53

Deletion of 17p [del(17p)] including TP53 is also a marker of poor outcome as well as of genomic instability. Unlike several other prognostic markers TP53 status as a high-risk marker has not waned in the face of modern therapies that target plasma cell biology (107). Del(17p) is rare in MGUS, present in ∼10% of NDMM but present in the majority of plasma cell leukemias and associated with extramedullary disease (66, 107, 120, 121). TP53 mutations also occur in ∼5% of NDMM, but are primarily restricted to samples with del(17p), suggesting a step-wise progression where del(17p) predisposes to biallelic loss of TP53 by selection for cells with TP53 mutation. The co-occurrence of TP53 mutations with 17p loss results in exceedingly poor outcomes (95), and provides strong evidence that TP53 is the functional tumor suppressor inactivated by del(17p).

Aberrant NF-κB Signaling

Aberrant NF-κB signaling results from both inactivating mutations of genes that suppress NF-κB signaling (e.g., TRAF3) as well as aberrant upregulation of genes the promote NF-κB signaling (e.g., MAP3K14) (89). There is a broad mutational spectrum encompassing dozens of genes, mostly mutated at a low frequency, that converge on the non-canonical NF-κB pathway in ∼20% of NDMM (122, 123). Non-canonical NF-κB signaling provides a pro-survival signal and growth advantage to myeloma cells (122, 123), but it is possible that it occurs by a variety of mechanisms. For instance, NF-κB was discovered as a transcription factor that regulates kappa light chain expression (124), but is now known to also regulate IgH and human IgL expression (125). This suggests that NF-κB signaling may enhance expression of oncogenes translocated to immunoglobulin enhancers in myeloma.

This non-canonical NF-κB signaling in myeloma is in contrast to other B cell malignancies such as Waldenstrom's macroglobulinemia (lymphoplasmacytic lymphoma), where over 80% of cases harbor activating mutations in MYD88 that result in canonical NF-κB signaling (126, 127). This may impart a more "innate-like" B cell response and explain why Waldenstrom's macroglobulinemia is almost exclusively IgM expressing whereas IgM expressing myeloma is very rare. Indeed,

long-term follow-up of MGUS cases, 15% of which are of the IgM isotype, indicate that IgM MGUS patients progress almost exclusively to Waldenstrom's macroglobulinemia or non-Hodgkin's lymphoma whereas class-switched MGUS cases progress to multiple myeloma (128).

Ras Signaling

Ras signaling is a common alteration in myeloma but rare in MGUS (129). KRAS and NRAS are the two most commonly mutated genes in myeloma, each present in ∼20% of NDMM (66, 89, 95). Counterintuitively, 15% of KRAS-mutated patients also have NRAS mutations. Given the subclonal nature of most Ras mutations, it is conceivable that in cases where both KRAS and NRAS are mutated, these occur in distinct and non-overlapping clonal populations. Alternatively, it may suggest that not all Ras mutations uniformly activate MAPK signaling. Indeed, this has been recently confirmed by phosphoproteomics in myeloma cell lines (130). In contrast, FGFR3 mutations appear to be a more potent inducer of MAPK signaling and are mutually exclusive with NRAS and KRAS mutations (130, 131).

THE MOLECULAR PROGRAM OF MULTIPLE MYELOMA

As noted above, translocations and chromosomal aberrations serve to dysregulate oncogenes and tumor suppressor genes and given the broad array of mutations in myeloma, it is not surprising that these result in distinct gene expression subtypes. Over a decade ago Zhan et al. used cDNA microarrays to classify myeloma into 7 gene expression subtypes, which mostly reflected the founding genetic mutations (132). These expression subtypes include two CCND1 subtypes, CD-1 and CD-2, both driven by t(11;14) translocations, but CD-2 tended to express more B-cell like markers such as CD20; the HY subtype corresponded with genetic hyperdiploidy; the MF subtype corresponded with MAF and MAFB translocations; the MS corresponded with WHSC1 translocations (WHSC1 was commonly referred to as MMSET at the time); LB or low bone disease was not well defined by gene expression or discernable baseline genetics; PR represented a proliferative disease with poor outcome (132). These subtypes of myeloma are well conserved, as segregation of myeloma based on translocations and Cyclin D expression (TC subtype) resulted in groups with similar characteristics (69). Another independent study from Europe identified 10 groups based on gene expression, which corresponded with those from Zhan et al. but provided slightly more granularity (133). Furthermore, we recently found that gene expression subtypes from Zhan et al. were largely conserved in yet another independent data set using a newer technology (RNA-seq) (66). Thus, it appears the initiating genetic alterations of myeloma appear to imprint a gene expression program such that myeloma is really several diseases.

The gene expression subtypes described above correlate with primary genetic events in myeloma, but the impact of secondary and tertiary genetic mutations on gene expression are harder to discern. This may be due to the often sub-clonal nature of these alterations, which likely result in their effects on gene expression being diluted out by cells without the alteration when profiled *en masse*. Emerging single-cell technologies may eventually be able to address this difficult problem, which would ideally require simultaneous profiling of DNA and RNA. Early experiments of single-cell RNA-seq have provided intriguing data indicating the inter-sample heterogeneity of myeloma cells was segmented into distinct gene expression programs, whereas those from SMM, MGUS, and plasma cells from healthy donors where more homogenous (134). This suggests that sub-clonal genetic differences may underlie these variations in the gene expression program, and has important implications for myeloma cell plasticity and the ability of current therapies to effectively eradicate all clones.

PLASMA CELL DIFFERENTIATION AND EPIGENETIC DYSREGULATION IN MULTIPLE MYELOMA

Although the gene expression program appears to be driven by primary genetic alterations, there is clearly a cascade of molecular events that result from these abnormalities. Furthermore, genetic events alone cannot fully explain the gene expression program or phenotype. For instance, the most common translocation in myeloma, t(11;14), is also a defining characteristic of mantle cell lymphoma (73), and t(8;14) translocations that occur as secondary events in myeloma are present as primary events in the majority of Burkitt lymphomas (114, 115). Unlike myeloma, both these lymphomas are believed to originate from pre-germinal center B cell, suggesting that the combination of cancer genetics and cell differentiation state determine the cellular phenotype. Indeed, plasma cell differentiation involves dramatic changes in gene expression, epigenetic reprogramming, and cell morphology (45, 135–140). Thus, genetic alterations may manifest in different phenotypes given their timing in the context of the epigenetic landscape of the cell of origin. These epigenetic changes involve DNA methylation, which primarily occurs on cytosines in CpG dinucleotides (**Figure 3**). DNA methylation at promoters or enhancers usually functions to repress gene expression by occluding transcription factor binding (141), whereas intragenic DNA methylation corresponds with high levels of gene expression and serves to help prevent transcription from cryptic promoters (142). During germinal center formation and plasma cell differentiation, the histone 3 lysine 27 (H3K27) methyltransferase EZH2 represses plasma cell differentiation genes (e.g., PRDM1, IRF4) by depositing the repressive H3K27me3 histone modification, thereby prolonging the germinal center response (143, 144). The rapid cellular proliferation during the germinal center results in a genome-wide DNA hypomethylation, thereby facilitating activation of plasma cell enhancers, which have the ability to induce plasma cell differentiation (45, 137–139, 145). This explains why activating mutations in EZH2 give rise to germinal center B cell lymphomas (143). These and other epigenetic processes serve to activate plasma cell enhancers and super-enhancers, which are often co-opted to drive oncogene expression (112, 116, 146). Determining the unique trans-acting factors in plasma cell and myeloma cell enhancers may provide an effective way to therapeutically target multiple myeloma (147).

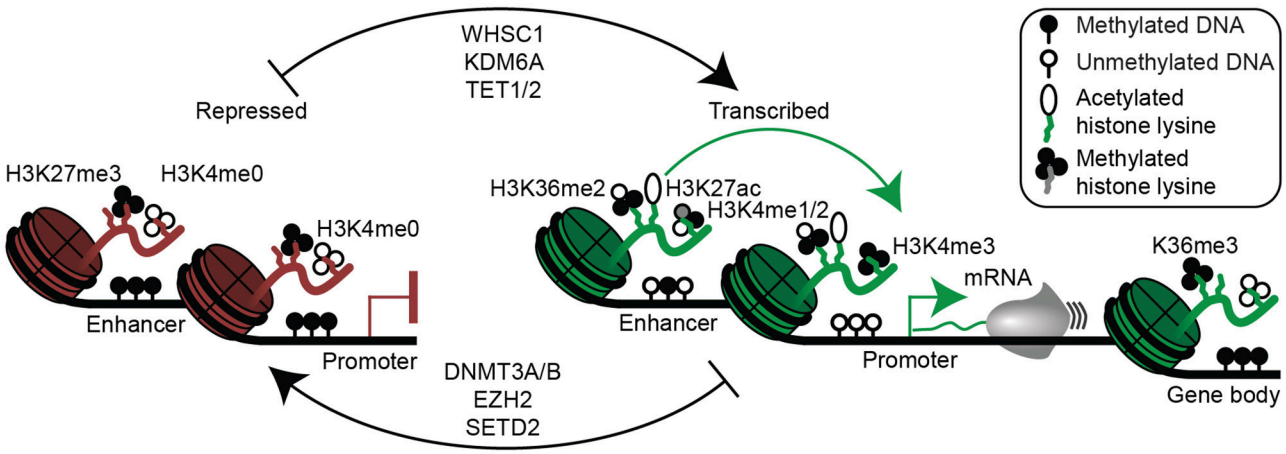

FIGURE 3 | Epigenetic mechanisms of gene regulation in plasma cells and multiple myeloma. Epigenetic modifications associated with gene repression are shown (left) and include histone 3 lysine 27 trimethylation (H3K27me3), lack of histone 3 lysine 4 methylation (H3K4me0), as well as enhancer and/or promoter DNA methylation. Epigenetic modifications permissive to gene transcription are shown (right) and include H3K36me2 (mediated in part by WHSC1), histone 3 lysine 27 acetylation (H3K27ac), H3K4me1/2 at enhancers, H3K4me3 at promoters, and absence of enhancer and promoter DNA methylation. Gene bodies (far right) of actively transcribed genes are often demarcated with H3K36me3 and DNA methylation. Certain enzymes that mediate activating (top) and repressive (bottom) epigenetic modifications relevant to multiple myeloma are shown (middle).

As noted above, one of the most common translocated genes in myeloma is the H3K36 methyltransferase, WHSC1, resulting in a genetic alteration with widespread epigenetic effects. This results in a global increase of H3K36me2 and increased chromatin accessibility as well as a commensurate inhibition of the repressive mark H3K27me3 (78, 79). How this may specifically promote oncogenesis, is still being determined.

In addition to aberrant WHSC1 expression due to t(4;14) translocations, there are a number of mutations in epigenetic enzymes that confer survival advantages to myeloma cells. These include mutations in the H3K27me3 demethylase KDM6A (also known as UTX), where loss of KDM6A function results in increased proliferation, adhesion, and clonogenicity of myeloma cells (148). Unlike the genome-wide effects seen as a result of WHSC1 overexpression, only focal changes on H3K27me3 were observed with KDM6A ablation (148).

Recently, mutations in the isocitrate dehydrogenases, IDH1 and IDH2, have been reported (90). Isocitrate dehydrogenases normally produce α-ketoglutarate, but when mutated result in the accumulation of D-2-hydroxyglutarate, which inhibits Jumonji-C histone demethylases and TET family methylcytosine hydroxylases that require α-ketoglutarate as a co-factor. A consequence of IDH mutations includes altered histone modifications and DNA hypermethylation (149). This may alter the function of transcription factors, such as MYC and MAX, which bind CpG containing E-box elements and are sensitive to DNA methylation state (146, 150). Indeed, we recently showed that loss of function mutations in MAX occur in ∼3% of myelomas and alter its binding affinity to methylated and hydroxymethylated E-box transcription factor binding sites (150).

Other modifiers of the DNA methylation pathway mutated in myeloma include TET2 (90), which oxidizes DNA methylation (151) allowing for its removal by base excision repair, as well as the *de novo* DNA methyltransferase DNMT3A (90), which catalyzes DNA methylation at unmethylated CpGs (152). We recently showed that conditional deletion of both *de novo* DNA methyltransferases in B cells results in a loss of DNA methylation at B cell enhancers as well as increased B cell activation and plasma cell differentiation in response to immunization (137). However, the functional impact of these enzymes in myeloma has yet to be elucidated.

In addition to the recent discoveries of mutations in enzymes that regulate DNA methylation, early observations in multiple myeloma showed promoter DNA hypermethylation and gene silencing of the cyclin-dependent kinases inhibitors CDKN2B (p15) and CDKN2A (p16), suggesting they were incapable of preventing cell cycle progression (153). SOCS1, a suppressor of cytokine signaling including the key myeloma cytokine IL6 (53), is also aberrantly silenced by DNA hypermethylation (154, 155). Despite these punctate hypermethylation events, recent genome-wide analyses have found myeloma is mostly characterized by widespread hypomethylation as compared to plasma cells from healthy individuals (156–159). This DNA hypomethylation appears to be progressive as it is more severe in NDMM and RRMM than in MGUS and SMM (156, 159). Indeed, as part of the Multiple Myeloma Research Foundation's CoMMpass project we are performing whole genome bisulfite sequencing on a large cohort of multiple myeloma and have found pervasive hypomethylation organized into megabase domains that are devoid of gene expression. In contrast, DNA methylation was retained in the gene bodies of highly expressed genes. Given the pre-clinical data showing that myeloma cells are sensitive to the DNA methylation inhibitors, such as 5-azacytidine and decitabine (160, 161), the selective sensitivity of multiple myeloma to demethylating agents has yet to be shown *in vivo*.

THERAPEUTIC VULNERABILITIES OF PLASMA CELLS

As our understanding of plasma cell and myeloma biology has improved, so too has our ability to treat myeloma effectively. Like most malignancies diagnosed in the mid twentieth century, myeloma was initially treated with cytotoxic chemotherapy that derived its benefit from attacking any rapidly dividing cell in the body. The alkylating agent melphalan was the first effective treatment for myeloma and in combination with the corticosteroid prednisone formed the backbone of myeloma therapy for 40 years (162, 163). The next major advance came in the 1980's with the introduction of high dose chemotherapy and autologous stem cell rescue, a procedure that is still routinely performed on the majority of eligible myeloma patients today (164, 165). However, it is the addition of novel plasma cell targeted therapy that has had the greatest impact on the improvement in overall outcomes for myeloma patient over the last two decades.

Proteasome Inhibitors

Proteasome inhibitors target the ability of both normal and malignant plasma cells to produce thousands of antibodies per second. In order to sustain such rapid levels of protein production, the cells rely heavily upon a number of quality control pathways for survival, and it is these pathways that have proven to be an Achilles heel for myeloma. In all cells, protein synthesis occasionally results in the production of misfolded and non-functional peptides that must be quickly disposed of to prevent their accumulation. These peptides are tagged with ubiquitin, which targets them for degradation by the proteasome system. Given the marked protein synthesis activity in myeloma cells, the amount of misfolded protein is similarly amplified, making myeloma even more dependent on the proteasome (166). Proteasome inhibitors block the degradation of misfolded proteins, allowing them to accumulate and ultimately induce cell death through the unfolded protein response (**Figure 4**). Although the proteasome plays an outsized role in myeloma cells by controlling the unfolded protein response, it has a number of other functions including regulation of signaling pathways, cell-cycle, and DNA repair. Proteasome inhibitors may therefore contribute to cell death through multiple mechanisms. There are currently three proteasome inhibitors approved for myeloma, bortezomib (167), carfilzomib (168), and ixazomib (169). These agents are often combined with dexamethasone, a corticosteroid with anti-lymphocyte activity, and an immunomodulatory drug, particularly during induction therapy (170), but also during maintenance (171) and relapse (172, 173).

Immunomodulatory Imide Drugs (IMiDs)

Immunomodulatory imide drugs (IMiDs) include thalidomide, the notorious anti-nausea medicine developed in Europe during the 1950s. Despite extensive testing in animals with no side-effects, thalidomide resulted in severe birth defects and in most cases death. However, discovery of the anti-angiogenic properties of thalidomide (174) led to clinical trials which showed it to be an effective agent in treating myeloma (175). This spurred the development of more potent and less toxic analogs of thalidomide, including lenalidomide (176, 177) and pomalidomide (178), now approved for the treatment of myeloma. Despite their efficacy, the mechanism by which IMiDs exert their effect was only recently discovered. IMiDs alter the target specificity of the CUL4A-DDB1-Cereblon E3 ubiquitin ligase (179), and in myeloma, this leads to the degradation of two key plasma cell transcription factors, Ikaros (IKZF1) and Aiolos (IKZF3) (180, 181) (**Figure 4**). Importantly, IMiDs bind Cereblon through an interaction at residue 391, which is not conserved in mice (182), explaining why thalidomide had no effect on animal studies originally conducted in the 1950s. IMiD-mediated degradation of IKZF1 and IKZF3 results in myeloma cell growth arrest as well as activation of T cells (180, 181), both of which may contribute to anti-myeloma effects of IMiDs. Through loss of IKZF1 and IKZF3, IMiDs also lead to down regulation of IRF4, another essential plasma cell transcription factor (183). IRF4 in turn regulates the expression of MYC (184), a potent oncogene in numerous lymphoid malignancies.

MYC Aberrant Expression

MYC aberrant expression occurs in the majority of myeloma cases through amplification, translocation, or transcriptional dysregulation (66, 112). Many MYC translocations result in its juxtaposition to immunoglobulin enhancers where the BET bromodomain protein BRD4 is highly associated. As a result, BRD4 inhibitors and degraders are currently being investigated as a method of downregulating MYC expression and inhibiting myeloma cell proliferation (185–187). IMiDs may also target MYC expression through inhibition of IKZF1- and/or IKZF3-regulated enhancers translocated to MYC (66) (**Figure 4**).

Immune-Based Therapies

Monoclonal antibodies against cell surface antigens highly expressed on malignant cells have been an important part of cancer therapy since the introduction of rituximab two decades ago. Like other cells of the immune system, plasma cells express cell surface markers that distinguish them from other cells, many of which continue to be expressed on myeloma. The transmembrane glycoprotein CD38 and the immunoreceptor SLAMF7 are the targets of the two monoclonal antibodies currently approved for the treatment of multiple myeloma, daratumamab (188, 189) and eloztuzumab (190), respectively. Daratumumab is capable of inducing complement dependent cytotoxicity, antibody dependent cellular cytotoxicity (ADCC) by NK cells, and antibody dependent cell phagocytosis (ADCP) by macrophages (191, 192), while elotuzumab acts primarily through ADCC and enhancement of anti-myeloma NK cell activity by crosslinking SLAMF7 on the two cell types (193–195) (**Figure 4**). Development of biologics that target plasma cells has been limited by the number of plasma cell specific markers, and thus a number of other potential targets on myeloma cells are being studied, including GPRC5D (196) and sulfated HLA-I epitopes (197). BCMA is an important cell survival receptor on plasma cells and is the target of the

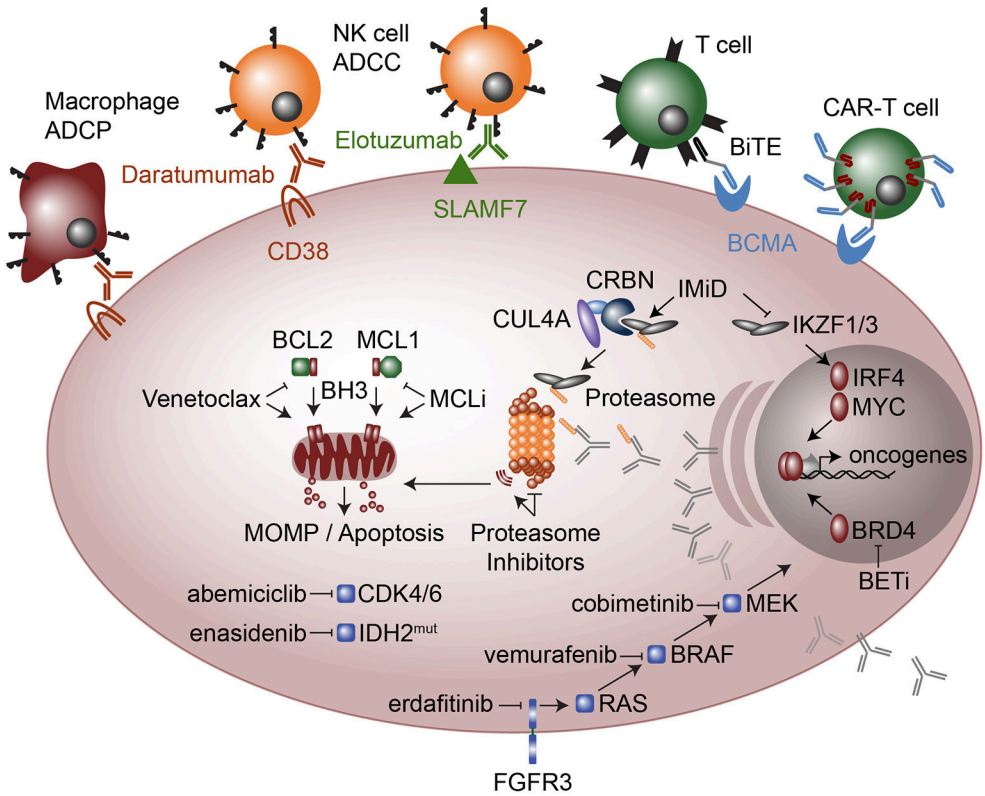

FIGURE 4 | Therapeutic modalities in multiple myeloma. Cellular targeted therapies (top) include chimeric antigen receptor T-cells (CAR-T cells) that target B cell maturation antigen (BCMA) and Bispecific T cell engagers (BiTE), which are two conjugated antibodies, one that recognizes the CD3 receptor on T cells while the other antibody recognizes BCMA. Monoclonal antibodies elotuzumab and daratumumab target SLAMF7 and CD38, respectively and result in myeloma cell killing by Natural Killer (NK) cell mediated antibody-dependent cellular cytotoxicity (ADCC) and in the case of daratumumab also by Macrophage antibody-dependent cellular phagocytosis (ADCP). Molecular modalities include immunomodulatory imide drugs (IMiD; top right) that bind Ikaros (IKZF1) and Aiolos (IKZF3) to Cereblon (CRBN) as part of an E3 ubiquitin ligase complex, which subsequently ubiquitinates IKZF1 and IKZF3 marking them for proteasomal degradation. Proteasome inhibitors (center) result in proteotoxic stress and the unfolded protein response, which plasma cells are particularly sensitive due to their high levels of antibody production. Anti-apoptosis inhibitors (middle left) include MCL1 inhibitors (MCLi) and BCL2 inhibitors such as venetoclax which induce mitochondrial outer membrane permeabilization (MOMP) and apoptosis. Therapeutics targeted at intracellular signaling include the cyclin dependent kinase 4 and 6 (CDK4/6) inhibitor abemiciclib and the mutant IDH2 inhibitor enasidenib. FGFR3 which is highly expressed in most t(4;14) myeloma and sometimes has activating mutations, is targeted with erdafitinib. FGFR3 feeds into RAS / MEK / MAPK signaling, which is targeted with drugs against BRAF (vemurafenib) and MEK (cobimetinib). Finally, a new class of drugs that target transcriptional activators such as bromodomain and extra-terminal (BET) inhibitors the block or degrade BRD4 are being used to target the enhancer machinery present at large enhancers that are often translocated in myeloma such as those found at the immunoglobulin loci.

first generation of myeloma directed chimeric antigen receptor (CAR)-T cells and bi-specific T cell engaging (BiTE) antibodies, which are conjugated antibodies binding both myeloma cells and T cells. A neutralizing antibody against the BCMA ligand APRIL is also being developed, as are monoclonal antibodies that deliver cytotoxic drugs more specifically to the antigen expressing cell, so called antibody drug conjugates (ADCs). ADCs targeting the plasma cell markers CD138, CD74, and CD48 are currently undergoing clinical trials.

Targeting Tumor Specific Biology

Targeting tumor specific biology has been successfully used to treat CML and a number of solid tumors with common driver mutations, but given the degree of genetic heterogeneity in myeloma, this has been less successful than plasma cell directed therapy. Nonetheless, treatment guided by specific oncogenic events in an individual patient's tumor remains an active area of investigation. As described above, alterations in the Ras-MAPK pathway occur in approximately 40% of patients. Although no direct Ras inhibitors exist, treatment with inhibitors of downstream kinases such as BRAF (vemurafenib) and MEK (trametinib, cobimetinib) have been reported in a small number of cases (198–201). Cobimetinib for Ras and Raf mutated patients is also being incorporated into a larger precision medicine trial known as MyDrug (202). Additional arms of this study include inhibitors of IDH2 (enasidenib), FGFR3 (erdafitinib), and CDK (abemiciclib) (**Figure 4**).

BCL2 Family Inhibitors

BCL2 family inhibitors represent a new class of drugs that may have applications in a broad range of malignancies. Pro- and anti-apoptotic members of the BCL2 family exist in a delicate state of

balance that regulates the survival of both normal and malignant cells (203, 204). The anti-apoptotic proteins BCL2, BCL2L1 (also known as BCL-xL), and MCL1 bind to and sequester pro-apoptotic proteins BIM, BAX, and BAK, preventing them from activating the apoptotic pathway. As normal cells transform into malignant cells they become even more dependent on the anti-apoptotic proteins for survival, leaving them sensitive to inhibitors of the BCL2 family and providing a potential therapeutic window (205–207). Venetoclax, navitoclax (ABT-263), AZD5991, AMG176, and S63845 induce tumor apoptosis by disrupting the function of BCL2, BCL2L1, and/or MCL1 (103, 104, 208). Venetoclax, a BCL2 specific inhibitor, has been approved for the treatment of chronic lymphocytic leukemia (CLL), which originates from BCL2-dependent B cells (209, 210). In contrast, plasma cells upregulate and become dependent upon MCL1, reducing their dependence upon BCL2 (100, 211). As a consequence, myeloma is primarily dependent on MCL1 and inhibitors of MCL1 have shown promising pre-clinical activity (103, 104). Surprisingly, a subset of myeloma characterized by the t(11;14) translocation is co-dependent on BCL2 and responds to BCL2 inhibition with venetoclax (212–218). Dexamethasone further enhances sensitivity to venetoclax by increasing expression of BIM and its binding to BCL2 (219). The biological basis for the BCL2 co-dependence in t(11;14) myeloma remains a mystery, however gene expression profiling of myeloma patient samples did reveal that t(11;14) were composed of two gene expression groups, CD1 and CD2, where CD2 had increased expression of B cell markers such as CD20, PAX5, and VPREB3, suggesting a possible connection with B cells and BCL2 dependence. The bone marrow microenvironment may also play a role in maintaining plasma cell MCL1 dependence through stromal cell mediated secretion of the plasma cell survival cytokine IL6 (220, 221).

SUMMARY

Throughout the history of multiple myeloma, we have learned a great deal about normal plasma cells from studying the malignant form and *vice versa*. While tremendous progress in the treatment of myeloma has been made over the past 25 years, due in a large part to therapies targeting plasma cell biology, myeloma remains an incurable disease. This necessitates not only the continued study of plasma cell and myeloma biology, but also the germinal center B cell origins of the disease. Clues of these origins are provided by epidemiological correlations. For instance, patients with Gaucher's disease accumulate lysolipids due to a deficiency in glucocerebrosidase, and are more prone to monoclonal gammopathies (222). This was recently leveraged to identify lysolipids as an antigen driving the gammopathy (223). Similarly, it is realized that MGUS incidence increases with obesity and has a higher prevalence in African Americans and in males (64, 223, 224). It will be important to sort out the genetic vs. environmental factors in each of these cases in hopes of minimizing risk of MGUS development. Likewise, it will be very important to identify factors that influence progression of MGUS to myeloma. Clinical trials testing therapeutic intervention to minimize risk of disease progression in SMM are already underway. However, given that 3% of adults over the age of 50 have MGUS (64), less toxic approaches at early stages of clonal gammopathy are needed to minimize chances of disease. Here, even interventions with small effects may have a large impact on cumulative disease burden. It would also be very valuable to accurately identify cases of MGUS that will develop into myeloma. Given the likelihood that myeloma may never be completely eliminated by preventative approaches, better models of disease will be needed to effectively develop the next generation of therapies. For instance, although we have learned a lot from patient derived cell lines models, these do not provide tractable comparisons of different genetic alterations without confounding background genetics. Although CRISPR has revolutionized gene editing, it has yet to be co-opted to induce myeloma translocations and it is unclear if this is possible. However, it is encouraging that other genetic approaches have been able to induce such translocations in murine B cells (225). Finally, given the dependence of most myeloma on the microenvironment, better *in vivo* models will also be needed. Significant efforts have yielded a mouse model of myeloma driven by AID-dependent MYC expression (113), and a humanized mouse capable of sustaining the human immune system including myeloma (226). These systems will need to be further exploited and expanded to better understand how the different genetic subtypes of myeloma respond to therapy and to delineate microenvironmental interactions and dependencies that can be leveraged to better treat multiple myeloma.

DATA AVAILABILITY

Figure 2 was generated by analysis from 850 newly diagnosed patients from the MMRF CoMMpass study (dbGAP phs000748.v7.p4).

AUTHOR CONTRIBUTIONS

BB and VG wrote the manuscript. LB and PV provided editorial input.

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Bone Marrow Plasma Cells Modulate Local Myeloid-Lineage Differentiation via IL-10

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Bone marrow plasma cells have been reported to represent a major source of IL-10; however, the impact of plasma cell derived IL-10 in that tissue remains poorly understood. We confirm in this study that even in the absence of acute immune reactions, mature plasma cells represent the dominant IL-10+ cell population in the bone marrow, and identify myeloid-lineage cells as a main local target for plasma cell derived IL-10. Using Vert-X IL-10 transcriptional reporter mice, we found that more than 50% of all IL-10+ cells in bone marrow were CD138+ plasma cells, while other IL-10+ B lineage cells were nearly absent in this organ. Accordingly, IL-10 was found in the supernatants of short-term cultures of FACS-sorted bone marrow plasma cells, confirming IL-10 secretion from these cells. IL-10+ bone marrow plasma cells showed a B220−/CD19−/MHCII low phenotype suggesting that these cells represent a mature differentiation stage. Approximately 5% of bone marrow leucocytes expressed the IL-10 receptor (IL-10R), most of them being CD115+/Ly6C+/CD11c− monocytes. Compared to littermate controls, young B lineage specific IL-10 KO mice showed increased numbers of CD115+ cells but normal populations of other myeloid cell types in bone marrow. However, at 7 months of age B lineage specific IL-10 KO mice exhibited increased populations of CD115+ myeloid and CD11c+ dendritic cells (DCs), and showed reduced F4/80 expression in this tissue; hence, indicating that bone marrow plasma cells modulate the differentiation of local myeloid lineage cells via IL-10, and that this effect increases with age. The effects of B cell/plasma cell derived IL-10 on the differentiation of CD115+, CD11c+, and F4/80+ myeloid cells were confirmed in co-culture experiments. Together, these data support the idea that IL-10 production is not limited to early plasma cell stages in peripheral tissues but is also an important feature of mature plasma cells in the bone marrow. Moreover, we provide evidence that already under homeostatic conditions in the absence of acute immune reactions, bone marrow plasma cells represent a non-redundant source for IL-10 that modulates local myeloid lineage differentiation. This is particularly relevant in older individuals.

Keywords: B cells, plasma cells, IL-10, monocytes, aging

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INTRODUCTION

Though most plasma cells are formed in peripheral tissues, the number of plasma cells in the bone marrow steadily increases with age (1). Bone marrow plasma cells are the major source for the production of memory antibodies (2, 3). These cells are preferentially but not exclusively generated during T-dependent immune reactions within germinal centers (4, 5). Compared to plasma cells from other tissues, they exhibit an altered immunophenotype and reduced susceptibility to therapeutic intervention (6–8).

Bone marrow is one of the major primary lymphoid organs after birth, where hematopoietic stem and precursor cells continuously give rise to new lymphoid and myeloid lineage cells (9, 10). A subpopulation of bone marrow myeloid cells expresses CD115, the macrophage colony-stimulating factor (M-CSF) receptor. This population consists of monocytes and "common progenitors of conventional and plasmacytoid dendritic cells" (11), which can further differentiate into macrophages, DCs and osteoclasts (12). CD115+ monocytes/common myeloid progenitors and their progeny exhibit important functions for the maintenance of hematopoietic stem and progenitor cells in the bone marrow, innate and adaptive immunity, wound healing and bone homeostasis. Thereby, these cells are crucial for the outcome of a variety of infectious and inflammatory diseases, such as tuberculosis and atherosclerosis, among others (11, 13–18). Plasmacytoid DCs resemble other bone marrow derived myeloid lineage cells which have a profound capacity to produce inflammatory type 1 interferon, a cytokine of crucial importance for immune protection against viral infection and relevant for the pathogenesis of autoimmune diseases (19).

Factors controlling the expansion and differentiation of bone marrow monocyte/macrophages include M-CSF and the "granulocyte-macrophage colony stimulating factor" (GM-CSF), among others (20–22). Interleukin (IL)-10 is a cytokine with pleiotropic functions (23, 24), which has been reported to promote the maturation of human monocytes into macrophages *in vitro*, while inhibiting their differentiation to DCs (25). Similarly, this cytokine was shown to control monocyte differentiation to macrophages during peritoneal infection in mice (26). Moreover, IL-10 has been reported to restrict the growth of monocyte-derived DCs by the inhibition of cytoprotective autophagy leading to increased apoptosis (27). Several studies indicate that age-related changes in IL-10 production of various cell populations contribute to the age-related chronic progressive increase in the proinflammatory status, sometimes referred to as "inflammaging," and changes of immune responses in older individuals (28–32).

B cell differentiation into CD138+ plasmablasts *in vitro* is accompanied by the up-regulation of IL-10 production (33). Accordingly, CD138+ plasmablasts/plasma cells represent the major population of IL-10+ cells in the spleen, as demonstrated by using IL-10 transcriptional reporter Vert-X mice (33). Some two decades ago, studies by Simon Fillatreau and David Gray identified B lineage cells as an important source of anti-inflammatory IL-10 in experimental autoimmune encephalomyelitis (34). More recent studies have now revealed that the relevant IL-10+ B lineage cells in this model actually represent CD138+ plasmablasts (35, 36). These plasmablasts were induced during experimental autoimmune encephalomyelitis (EAE) inflammation independent of germinal centers and were selectively found in the draining lymph nodes (36). The same authors demonstrated that these IL-10+ plasmablasts inhibit the activation of pathogenic T cells and thereby control EAE inflammation via modulation of dendritic cell functions. Upon treatment with rituximab, a reagent that selectively depletes B cells and plasmablasts, some multiple sclerosis patients developed increased disease severity, and this effect might be explained by a protective role of B cells/plasmablasts in these patients (37).

As shown by our group, the formation of IL-10+ plasma cells in the spleen can be stimulated by induction of a strong T-dependent reaction when mice are injected with goat-anti mouse IgD. These plasma cells efficiently suppressed the C5a-mediated neutrophil migration and inhibited autoimmune skin inflammation in a model of Epidermolysis bullosa acquisita (38). Furthermore, we found that bone marrow resident murine MOPC315.BM myeloma plasma cells produce IL-10 that mediates increased susceptibility to bacterial infection (38). In aged apolipoprotein E-deficient mice, a model for atherosclerosis, IL-10+ B lineage cells, many of them exhibiting an CD138+ plasma cell phenotype, have been also found within artery tertiary lymphoid organs, i.e., atherosclerosis-associated lymphoid aggregates surrounding the affected arteries (39). During Salmonella infection a novel "regulatory" CD138+ plasma cell population was found that is characterized by the expression of the inhibitory receptor LAG-3+, which following Toll-like receptor stimulation rapidly produces IL-10 (40).

Collectively, these data indicate that following acute immune stimulation, plasmablasts/plasma cells represent an important source of the anti-inflammatory cytokine IL-10, that can dampen autoimmune and infection driven inflammation but can also increase susceptibility to infection. IL-10+/IgM+ bone marrow plasma cells have been shown to be a major local source of IL-10 which may support the formation of immunization induced class-switched plasma cells (41).

In this study, we have confirmed that plasma cells are the dominant source of IL-10 within the bone marrow and have shown that CD115+/Ly6C+ monocytes are a main local target of this cytokine. Furthermore, our data provide evidence that under homeostatic conditions, plasma cell IL-10 is required for normal formation of bone marrow monocytes and DCs in older mice.

RESULTS

Plasma Cells Are the Dominant Source of IL-10 in Bone Marrow and CD115+ Myeloid Cells Represent a Major Target

Data from Il10Venus IL-10 reporter mice indicate that bone marrow plasma cells represent a major local source of IL-10 (41). In this study, we analyzed the expression of IL-10 in the bone marrow plasma cell compartment under non-inflammatory

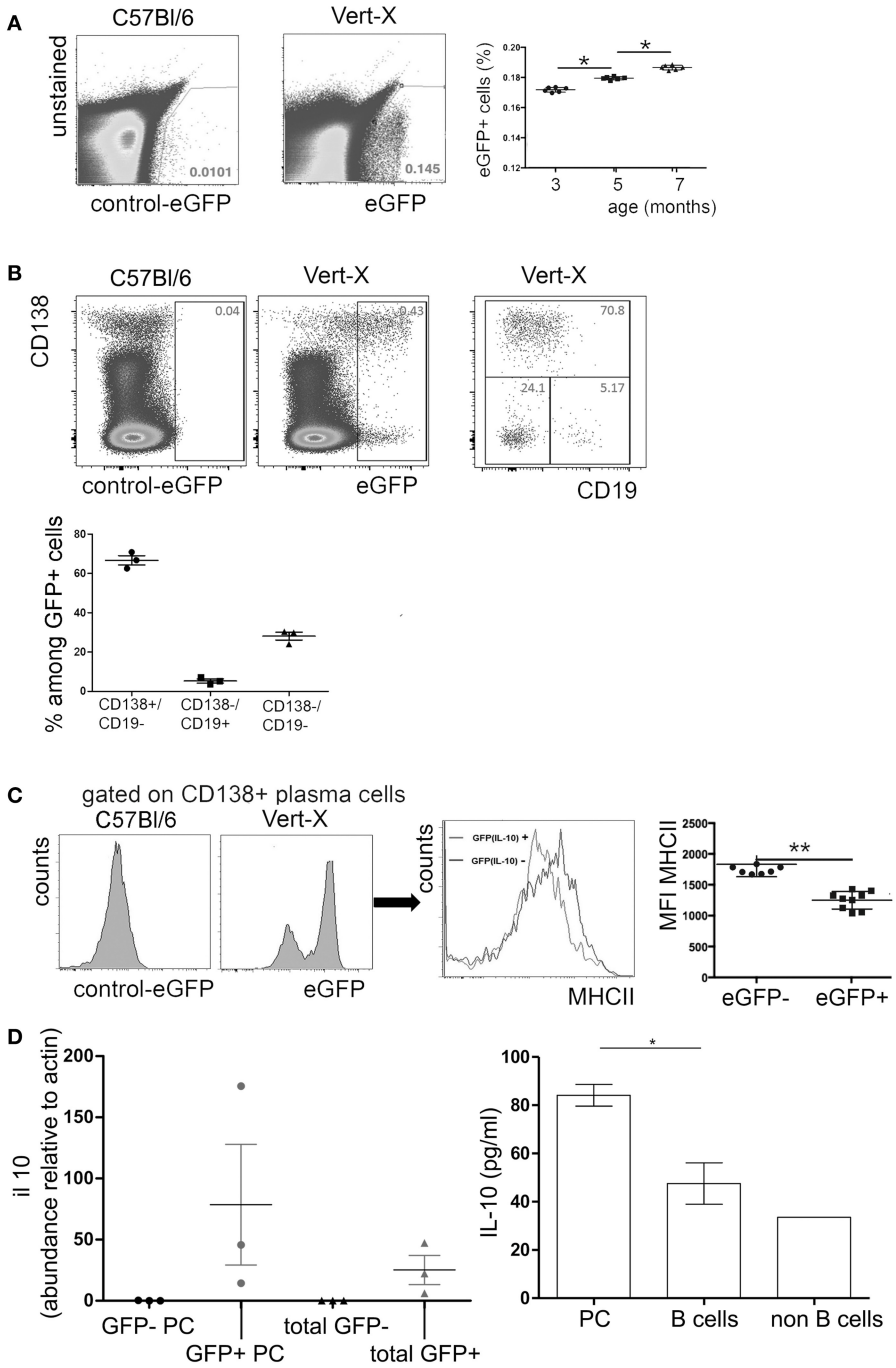

FIGURE 1 | IL-10 expression in murine bone marrow. Single cell suspensions were prepared from femurs and tibia of naïve Vert-X IL-10 transcriptional reporter mice between 3 and 7 months of age. Cells were counterstained for cell type specific markers and analyzed by flow cytometry. **(A)** Representative FACS plots of eGFP (IL-10) expression in Vert-X mice and C57BL/6 controls (left) and frequencies of eGFP (IL-10)+ cells among total bone marrow leucocytes (right). Pooled data from two independent experiments are shown ($n = 3$ for each experiment). **(B)** Gated eGFP (IL-10)+ cells were analyzed for CD19 and CD138 expression (upper row). Frequencies of CD19+/CD138− B cells, CD19−/CD138+ plasma cells and CD19−/CD138− non-B lineage cells among eGFP (IL-10)+ cells (lower plot). Data represents results obtained from one of two independent experiments ($n = 3$) **(C)** Comparison of MHCII expression between eGFP (IL-10)+/CD138+ plasma cells and eGFP (IL-10)−/CD138+ plasma cells. Representative FACS plots and the mean fluorescence intensity (MFI) of MHCII are shown for GFP- and GFP- plasma cells, as indicated. Statistics (Mann-Whitney test). Each dot represents data from a single mouse. Pooled data from three independent experiments are shown ($n = 3$ for each experiment). **(D)** Left: GFP+ plasma cells (GFP+ PC), GFP- plasma cells (GFP- PC), GFP+ non plasma cells (total GFP+), GFP- non plasma cells (total GFP-) from femurs and tibia of Vert-X mice were sorted by FACS and IL-10 expression was determined by real time PCR. Relative il10 mRNA levels compared to actin are shown. Right: Bone marrow plasma cells (PC), B cells, and non B cells from C57/Bl/6 mice were FACS-sorted and cultured in complete RPMI medium plus 20 ng/ml IL-6 for 20 h, when the supernatants were collected and the IL-10 levels were analyzed by ELISA. Data represents results obtained from one experiment ($n = 3$). $^{*}P < 0.05$, $^{**}P < 0.01$.

steady-state conditions in IL-10 transcriptional reporter (Vert-X) mice. These mice express enhanced green fluorescent protein (eGFP) under the control of the IL-10 promoter. Previous studies showed that eGFP expression of individual cells corresponds well with expression of IL-10 protein in Vert-X mice (33, 38). Under steady-state conditions, ∼0.1 to 0.2% of total bone marrow cells were eGFP+ and these frequencies increased with age (**Figure 1A**). Approximately 60% of the eGFP+ population exhibited a mature CD138+/CD19− plasma cell phenotype (**Figure 1B**). CD19+/CD138− B lineage cells represented only a very minor fraction of ∼5% of the of eGFP+ population, while CD19−/CD138− non-B lineage cells made up for ∼30% of eGFP+ cells. eGFP+/CD138+ plasma cells showed reduced expression of MHCII compared to eGFP−/CD138+ plasma cells (**Figure 1C**) and were mostly B220 negative (data not shown). These results are in accordance with previous findings (5, 41), suggesting that IL-10+ bone marrow plasma cells represent a highly mature differentiation stage.

In accordance with previous findings (33, 38), eGFP expression did correlate well with IL-10 mRNA expression in sorted cells and IL-10 could be also detected in supernatants of sorted bone marrow plasma cells after short-term culture (**Figure 1D**), indicating that eGFP expression indeed reflects production of IL-10 protein.

In order to identify potential local target cells of IL-10, the expression of the IL-10R was investigated. Approximately 4 to 7% of total bone marrow leucocytes expressed IL-10R and the narrow majority of them were CD115+ myeloid lineage cells (**Figure 2A**), i.e., monocytes and common myeloid progenitors (11). In fact, all CD115+ cells in the bone marrow showed IL-10 receptor expression, as measured by flow cytometry (**Figure 2B**). Quantification of IL-10RA mRNA by real time PCR of FACS sorted bone marrow subpopulations revealed that CD115+/Ly6C−/CD11c+ DCs and CD115+/Ly6C+/CD11c−monocytes express high levels of mRNA for the IL-10 receptor in comparison to CD115−/Ly6C−/CD11b+/Ly6G+ neutrophils and CD115−/CD11c−/F4/80+ macrophages (**Figure 2C**).

Together, these data confirm that bone marrow plasma cells represent the dominant source for IL-10 during steady state conditions and indicate that CD115+/Ly6C+/CD11c−monocytes are a potential local target for this cytokine.

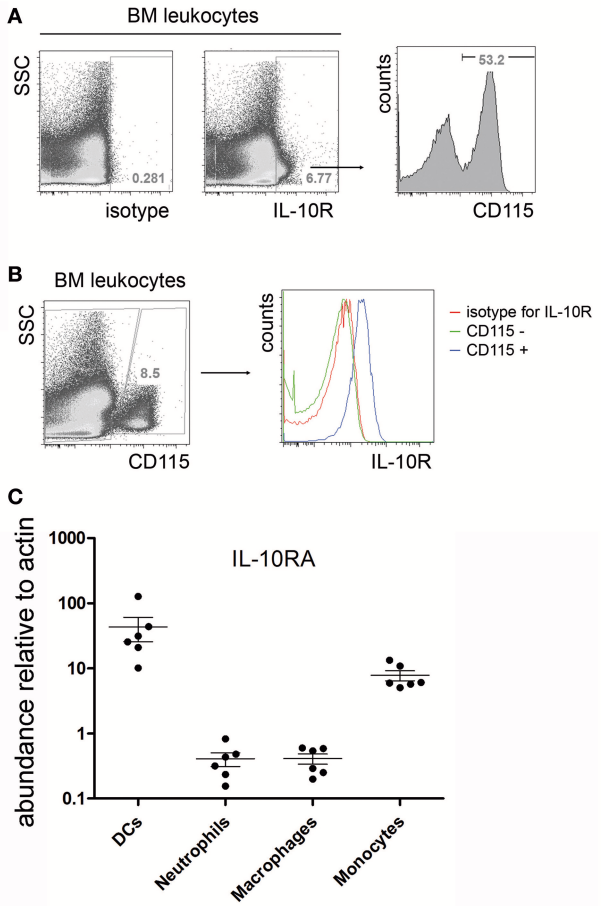

FIGURE 2 | IL-10R expression in the bone marrow. Single cell suspensions from femurs and tibia of C57BL/6 mice were stained for IL-10R and CD115. **(A)** Representative flow cytometric staining for IL-10R compared to an isotype control (left). IL-10R+ cells were electronically gated and analyzed for the expression of CD115 (right). **(B)** IL-10R expression on gated CD115+ bone marrow monocytes. CD3+, B220+, and Gr-1+ cells were excluded from the analysis by electronic gating. Data represents results obtained from one of two independent experiments ($n = 7$). **(C)** Bone marrow dendritic cells (CD115+ Ly6C− CD11c+), neutrophils (CD115− Ly6C− CD11b+ Ly6G+), macrophages (CD115− CD11c− F4/80+), and monocytes (CD115+ Ly6C+ CD11c−) were sorted by FACS and IL-10RA expression was determined by real time PCR. Relative il10ra mRNA levels compared to actin are shown. Pooled data from two independent experiments are shown ($n = 6$).

IL-10 From Activated B Cells/Plasma Cells Modulates the Formation of Multiple Myeloid Cell Types *in vitro*

IL-10 derived from plasmablasts in lymph nodes has been shown to alter the maturation of DCs (36). IL-10 is also known to modulate the differentiation of monocytes to macrophages in human cell culture experiments and during peritoneal infection in mice (25, 26). Moreover, IL-10 down-modulates monocyte/macrophage differentiation into osteoclasts (42, 43).

In order to investigate if plasma cells could modulate the differentiation of CD115+ monocytes from bone marrow via IL-10, CD115+ cells were isolated from primary bone marrow cultures by removal of non-adherent cells. Purity of CD115+ cells was >95%. The adherent cells showed a CD115+/CD11b+ F4/80−, Ly6G−, and CD11c− phenotype of monocytes/common myeloid progenitor cells (**Figure 3A**). These cells were cultured for another 2 days with or without addition of recombinant IL-10 and subsequently analyzed for the expression of CD115+ and F4/80+. Addition of recombinant IL-10 lead to a reduction of the CD115+ populations and an expansion of the F4/80+ populations (**Figure 3B**).

Next, recombinant IL-10 was replaced by IL-10+ B cells/plasma cells. Purified monocytes/common myeloid progenitor cells were supplemented with purified B cells cultured

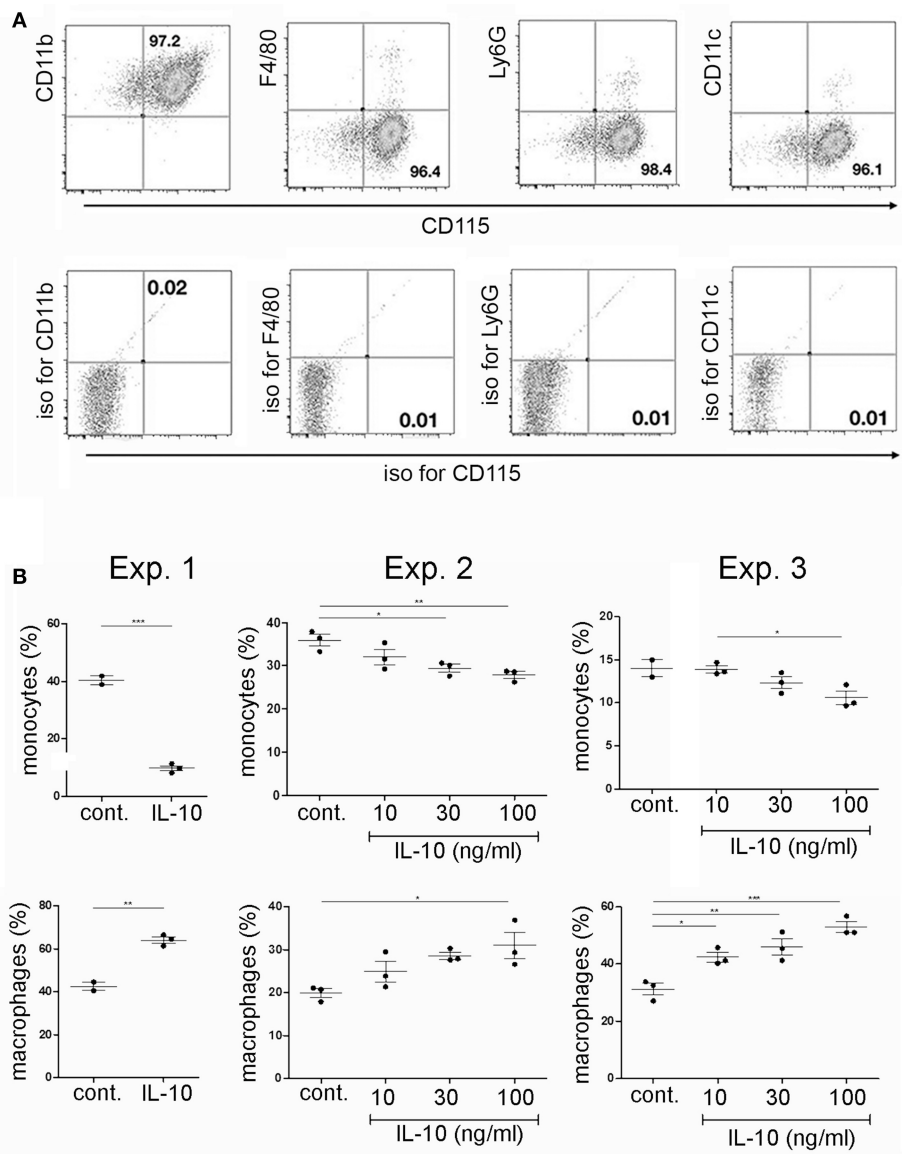

FIGURE 3 | Effects of recombinant IL-10 on monocyte/macrophage differentiation *in vitro.* Primary bone marrow cells were stimulated with M-CSF. After two days of culture, monocytes/common myeloid progenitors were isolated by removal of non-adherent cells. **(A)** Purified adherent cells were analyzed by flow cytometry for the expression of various myeloid markers. **(B)** Purified adherent cells were cultured for another 2 days in the presence of M-CSF and RANKL, with or without addition of various concentrations ofrecombinant IL-10 and subsequently analyzed for the presence of CD115+ and F4/80+ cells. Data from three independent experiments are shown separately, as indicated. Statistics: one-way ANOVA. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$.

for 4 days with LPS, containing substantial numbers of CD138+ plasma cells/plasmablasts. Approximately 50% of the *in vitro* generated CD138+ plasma cells/plasmablasts in these cultures produced IL-10, as indicated in cultures from IL-10 reporter (Vert-X) mice (**Figure 4**).

Comparable to what was observed after addition of recombinant IL-10, co-cultured activated B cells/plasma cells lead to a reduction of ∼20% in the frequencies and absolute numbers of CD115+ cells, but to a 4-fold expansion of F4/80+ macrophage populations. Addition of blocking anti-IL-10R antibodies reversed these effects (**Figure 5A**). F4/80+ cells generated in these cultures showed a CD115+ phenotype. The impact of activated B cells/plasma cells on the formation of CD11c+ DC was less clear. The frequencies of CD11c+ cells increased about 2-fold in cultures where activated B cells/plasma cells were added. Blockade of IL-10 did not reverse this effect (**Figure 5B**).

These data suggest that activated B cells/plasma cells can promote the formation of CD11c+ cells in an IL-10 independent manner, at least *in vitro*. Though it is known that IL-10 inhibits cytoprotective mechanisms in monocyte derived DC leading to increased apoptosis (27), the mechanism of how activated

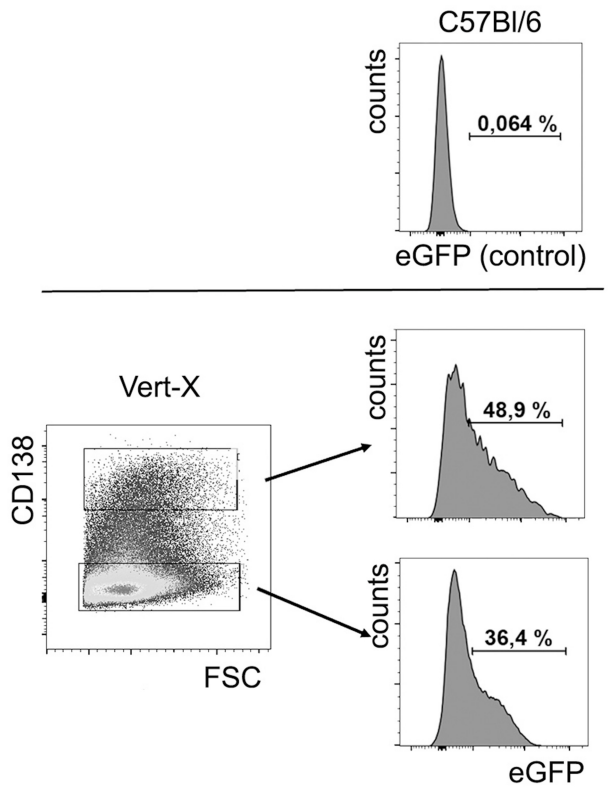

FIGURE 4 | *In vitro* generated plasma cells produce IL-10. B cells were isolated from spleens of IL-10 reporter Vert-X mice and C57BL/6 controls and activated by LPS. After 4 days in culture, IL-10 (eGFP) expression was analyzed in CD138+ plasma cells and CD138- cells. Upper panel: cells from C57BL/6 were used as a negative control for eGFP expression. Lower panel: eGFP expression in CD138+ plasma cells and in CD138− cells, as indicated. Representative FACS data from one of three independent experiments are shown ($n = 3$).

B cells/plasma cells promote the generation of DC remains to be elucidated.

At day 5, the formation of polynucleated "tartrate-resistant acid phosphatase" positive osteoclast-like cells could be observed in the culture by microscope. Addition of activated B cells/plasma cells reduced the formation of osteoclasts up to 8-fold. This effect increased with the numbers of activated B cells/plasma cells added and was dependent on IL-10, as indicated by the following treatment with blocking anti-IL-10R antibodies (**Figure 6**).

These data demonstrate that activated B cells/plasma cells can modulate the expansion and further differentiation of primary bone marrow CD115+ monocytes/common myeloid progenitors into macrophages, DCs and osteoclast-like cells via IL-10 *in vitro*.

Bone Marrow Plasma Cell Derived IL-10 Modulates Myeloid Lineage Cells *in vivo* in an Age-Dependent Manner

In order to investigate if B lineage IL-10 could be relevant for the differentiation of myeloid lineage cells *in vivo*, the populations of CD115+, CD11c+, and F4/80+ cells in bone marrow were analyzed in B cell–specific IL-10 knockout mice (CD19 Cre/IL-10 flox/flox) and compared to their littermate controls. Given the fact that CD138+ plasma cells represent the dominant source for IL-10 in this organ while other B lineage cells contribute only minimal to the local production of this cytokine, we expect that these mice represent a suitable model to study the effect of plasma cells derived IL-10 on the local phenotype of bone marrow myeloid cells. Bone marrow plasma cell populations are known to increase with age (1). In our experiments, between the ages of 3 to 7 months the numbers of bone marrow plasma cells increased ∼4-fold (**Figure S1**). At 7 months of age, B cell–specific IL-10 knockout mice exhibited about 20% increased frequencies and numbers of CD115+ bone marrow myeloid cells, while the expression of the macrophage marker F4/80 was reduced compared to their littermate controls (**Figures 7A,B**). Though we could not identify a distinguished population of F4/80+ cells, F4/80 expression might be an indicator for the presence of a specialized endosteal macrophage subtype in bone marrow, termed osteomacs (44).

In accordance with the expected IL-10 mediated suppression of dendritic cell formation observed *in vitro*, aged B cell–specific IL-10 knockout mice showed increased frequencies and numbers of CD11c+ dendritic cells in the bone marrow (**Figure 8**). Younger B cell–specific IL-10 knockout mice of 10 to 14 weeks of age, did not show differences in F4/80+ or CD11c+ cell populations compared to age matched wild type mice (**Figure S2**). Together, these data suggest that plasma cell derived IL-10 exhibits a non-redundant effect on local myeloid differentiation, and that this effect increases with age.

In order to investigate possible changes of the architecture of bones, the skeletal structures of femurs of 5 months old B cell–specific IL-10 knockout mice and littermate controls were analyzed by micro-computed tomography (micro-CT). No differences were observed between the volume and structure of bones of the two mouse lines (**Figures 9A,B**), suggesting that plasma cell derived IL-10 does not play a non-redundant function in bone homeostasis. However, this result could not rule out the possibility that plasma cell derived IL-10 becomes a relevant modulator of osteoclastogenesis and bone constitution at an even later age, and/or that IL-10 from non-plasma cells exhibits redundant functions.

The changes in CD11c+, F4/80+, and CD115+ myeloid populations in the bone marrow of aged B cell–specific IL-10 knockout mice correspond well with the effects of IL-10 from B cell/plasma cells observed in our co-culture experiments. The finding that ∼90% of IL-10+ B lineage cells in bone marrow were CD138+ plasma cells implies that plasma cell derived IL-10 modulates local myeloid differentiation in the bone marrow.

In conclusion, IL-10 from bone marrow plasma cells affects CD115+ myeloid cells and DC and macrophage differentiation in an age-dependent manner, while it may have no non-redundant impact on bone homeostasis.

DISCUSSION

The findings presented in this paper indicate that bone marrow plasma cells provide an important local source for IL-10 that is relevant for normal formation and differentiation of

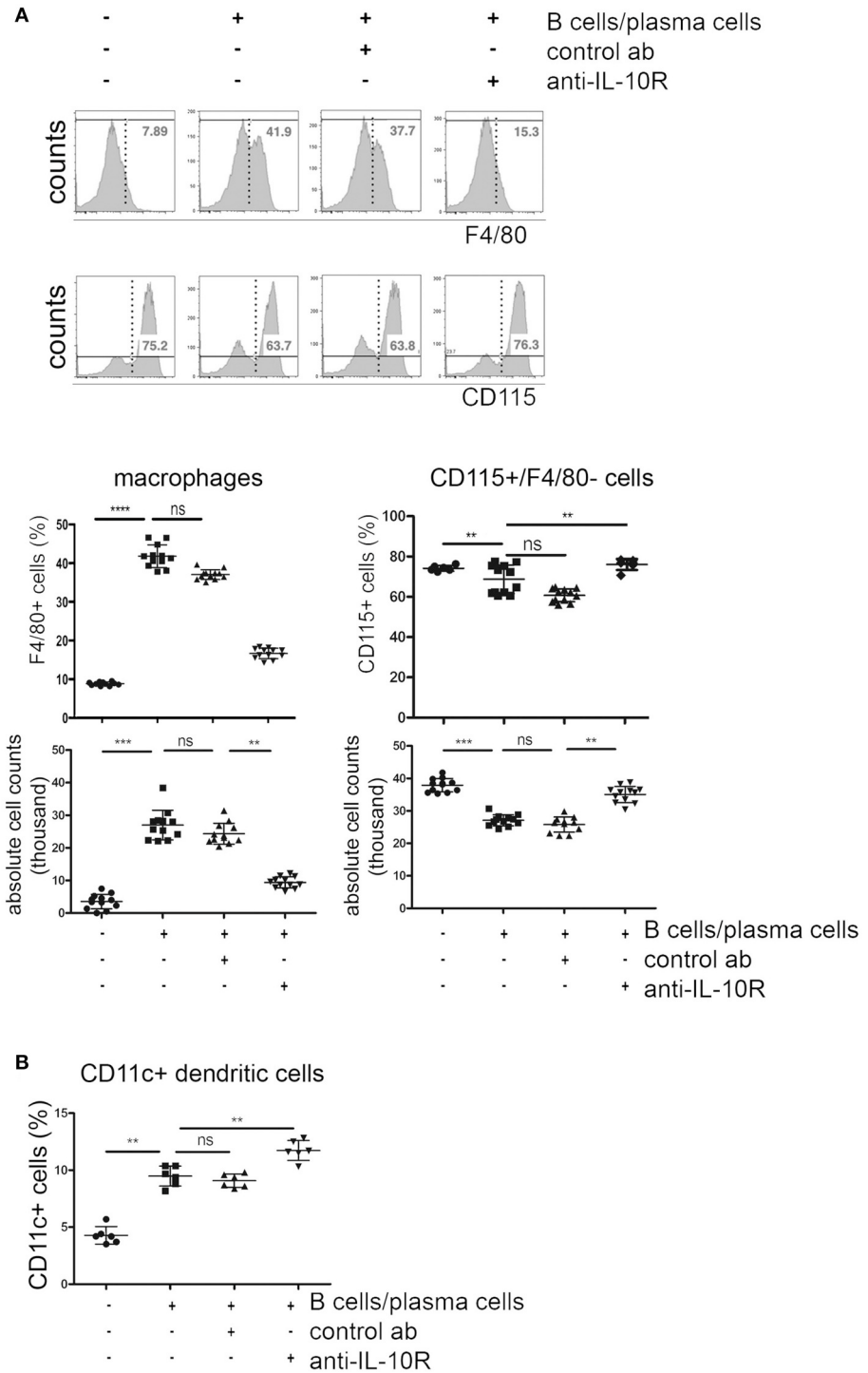

FIGURE 5 | Effects of IL-10+ B cells/plasma cells on myeloid differentiation *in vitro*. Primary bone marrow monocytes were stimulated with M-CSF and RANKL and cultured with or without addition of *in vitro* activated B cells/plasma cells, IL-10R blocking antibodies and control antibodies, as indicated. Three days later, CD115+ monocytes, F4/80+ macrophages, and CD11c+ cells were quantified by flow cytometry. **(A)** Representative FACS plots (upper panel) and statistical analysis (lower panels) of frequencies and absolute numbers of F4/80+ and CD115+/F4/80− cells are shown. Pooled data from four independent experiments are shown ($n = 3$ per experiment). **(B)** Frequencies of CD11c+ cells. Each dot represents the result from one well. Pooled data from two independent experiments are shown ($n = 3$ per experiment). Statistics: one-way ANOVA. $^{**}P < 0.01$, $^{***}P < 0.001$ and $^{****}P < 0.001$.

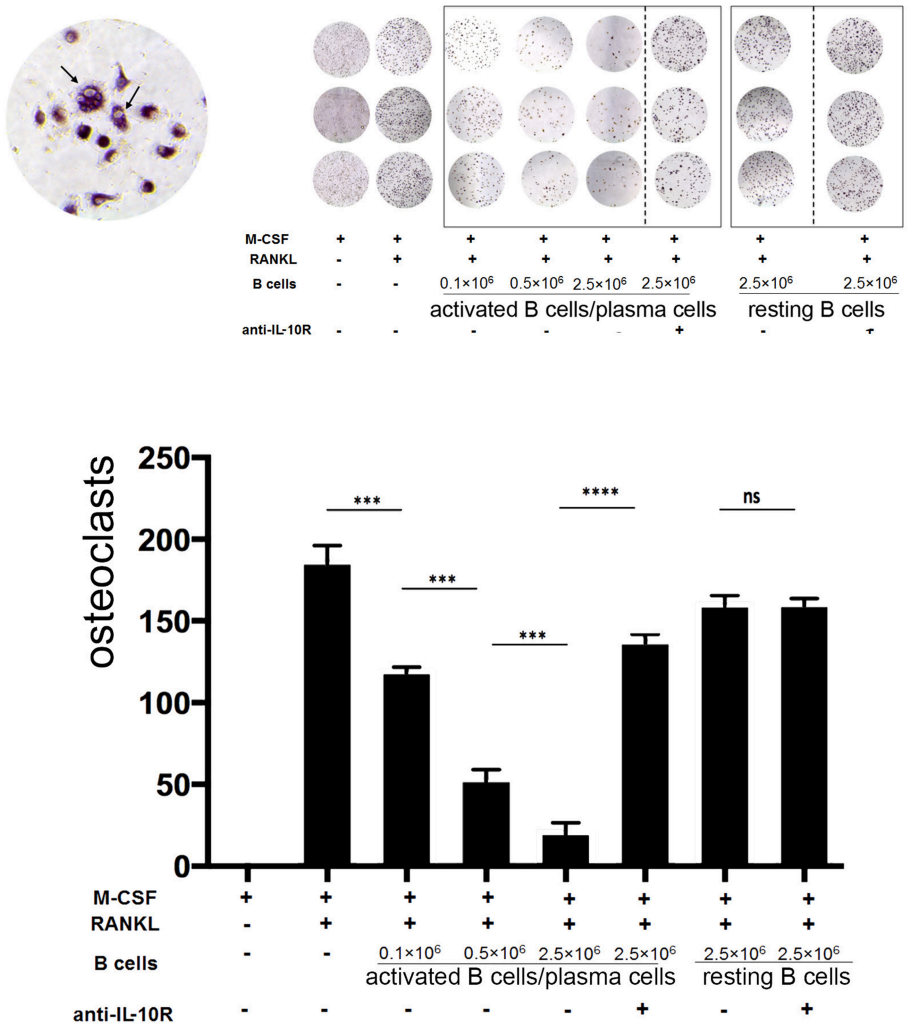

FIGURE 6 | Activated B cells/plasma cells modulate osteoclast differentiation *in vitro* via IL-10. Primary bone marrow monocytes were stimulated with M-CSF and RANKL and cultured with or without addition of *in vitro* activated B cells/plasma cells, naïve B cells, IL-10R blocking antibodies and control antibodies, as indicated. Upper panel: At day 5 to 7, the formation of polynucleated "tartrate-resistant acid phosphatase" positive osteoclast-like cells was observed by microscope. Lower panel: statistical analysis (example: day 7). Statistics: Mann-Whitney test. Data represents results obtained from one of five independent experiments ($n = 3$). ***$P < 0.001$ and ****$P < 0.0001$.

myeloid populations in older mice already under homeostatic conditions. Previous reports on IL-10+ CD138+ plasmablasts and plasma cells in LN and spleen indicate that these cells are formed in the course of autoimmune diseases or infections and exhibit the capacity to limit the inflammatory reaction accompanying these conditions (36, 38, 40). In contrast, bone marrow IL-10+ plasma cells are already present and relevant under steady state conditions. Though a population of "natural regulatory plasma cells" that exhibit the capacity to rapidly up-regulate IL-10 expression has been detected in spleens of naïve mice, these cells require additional signals provided via a Toll-like receptor (TLR)-driven mechanism to up-regulate IL-10 production (40). In contrast, isolated bone marrow plasma cells described in this study secreted IL-10 into the culture supernatant without TLR-stimulation, suggesting that these cells constitutively produce IL-10. Moreover, in IL-10 transcriptional reporter mice, these plasma cells were identified by eGFP expression, again suggesting that they already produce IL-10 in naïve mice. Therefore, we assume that IL-10+ bone marrow plasma cells are not related to IL-10+ plasmablasts/plasma cells induced in peripheral lymphoid tissues in response to external or auto-inflammatory stimulation. Hence, IL-10 is produced by a specialized subset of "natural regulatory plasma cells" relevant during conditions of infection (40), but is also produced by a considerable proportion of mature bone marrow plasma cells that seem to modulate myeloid cells under steady state. Our data are in line with previous reports showing that IL-10 is a potent modulator of monocytes/macrophage differentiation and osteoclast formation. In particular, this cytokine has been shown to inhibit the differentiation of GM-CSF stimulated

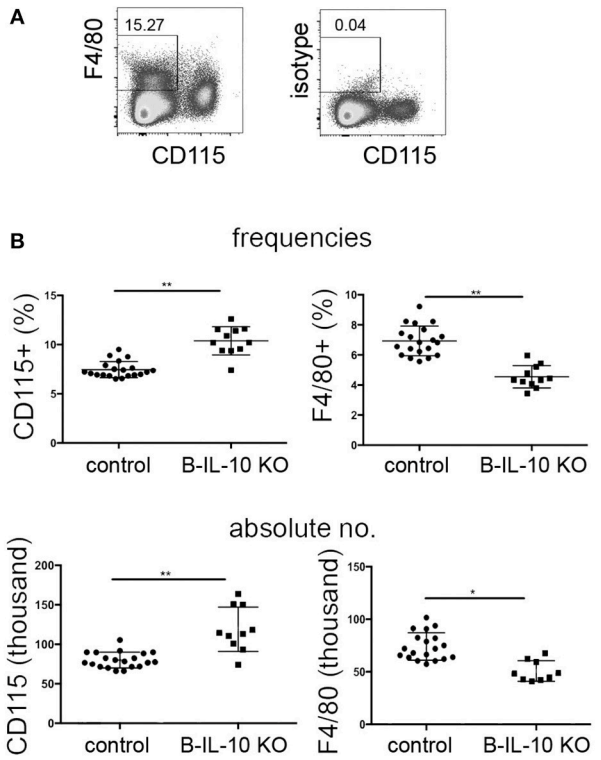

FIGURE 7 | Myeloid differentiation is altered in the bone marrow of B lineage deficient IL-10 KO mice. Single cell suspensions from femurs and tibia of 7 months old B cell specific IL-10 KO mice and their littermate controls were stained for cell lineage markers and analyzed by flow cytometry. **(A)** Representative staining for CD115 and F4/80 are shown. **(B)** Frequencies (upper panel) and absolute numbers (lower panel) of CD115+ and F4/80+ cells in IL-10 KO mice and controls, as indicated. Representative data from one of two experiments are shown. Each dot represents data from a single mouse. Statistics: *t*-test. $^{*}P < 0.05$ and $^{**}P < 0.01$.

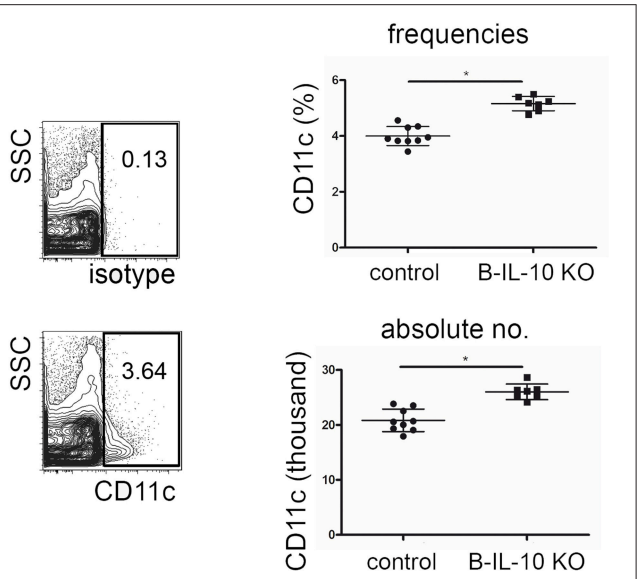

FIGURE 8 | Dendritic cell populations are expanded in the bone marrow of old B lineage deficient IL-10 KO mice. Single cell suspensions from femurs and tibia of 7 months old B cell specific IL-10 KO mice and their littermate controls were stained for cell lineage markers and analyzed by flow cytometry. Representative CD11c staining and isotype control (left). Frequencies (upper panel) and absolute numbers (lower panel) of CD11+ cells in IL-10 KO mice and controls, as indicated. Pooled data from two experiments are shown. Each dot represents data from a single mouse. Statistics: *t*-test. $^{*}P < 0.05$.

human monocytes into CD1a+ and MHC II high DCs, but instead promoted the formation of macrophages (25). Our results show that IL-10 exhibits similar effects on murine myeloid cell cultures and that bone marrow of aged B lineage specific IL-10 KO mice contains larger monocyte populations but smaller macrophage populations. In these mice, plasma cells represent the dominant source of IL-10 within B lineage cells. Together, these data indicate that bone marrow plasma cell derived IL-10 modulates the formation of monocytes and macrophages during hematopoiesis.

In mice infected with cecal bacteria, IL-10 has been shown to be essential for the differentiation of monocytes into a particular population of MHC II(lo) macrophages that efficiently can phagocytose apoptotic cells (26). In accordance with this observation, another study has shown that IL-10 constrains inflammation-induced macrophage phagocytosis of healthy self-cells (45). Recent findings also showed that the anti-inflammatory effects of IL-10 on macrophages are mediated by a metabolic reprograming of those cells and by eliminating their dysfunctional mitochondria (46). In murine sepsis, IL-10 has been reported to suppress the expression of IL-27 by activated F4/80+CD11b+ macrophages in an STAT3-dependent pathway (47). In accordance with a central role of IL-10 mediated signals on macrophage differentiation, mice exhibiting macrophage-specific IL-10R deficiency have been demonstrated to show impaired conditioning of monocyte-derived macrophages resulting in spontaneous development of severe colitis (48). Moreover, IL-10 seems also to play a critical role in regulating the switch of muscle macrophages from an M1 to an M2 phenotype in injured muscles *in vivo* (49). Though the importance of IL-10 mediated signals for monocyte/macrophage differentiation is well documented, most studies neither have identified the relevant cellular sources nor have addressed the question of whether the effect of IL-10 is age-dependent.

The effects of bone marrow plasma cell derived IL-10 on myeloid lineage cells observed in the present study apparently increase with age. This finding is in line with an overwhelming amount of literature showing that aging is accompanied by several changes of the immune system leading to increased vulnerability of older individuals to infectious diseases and reduced response to vaccination (31, 50–53). Among the factors associated with age-related changes of immune functions is IL-10. Our data suggest that the accumulation of IL-10+ plasma cells within the bone marrow contributes to the aging of the immune system and the related immune dysfunctions often observed in older individuals.

In aged mice, mononuclear myeloid cells have been reported to suppress the production of innate inflammatory

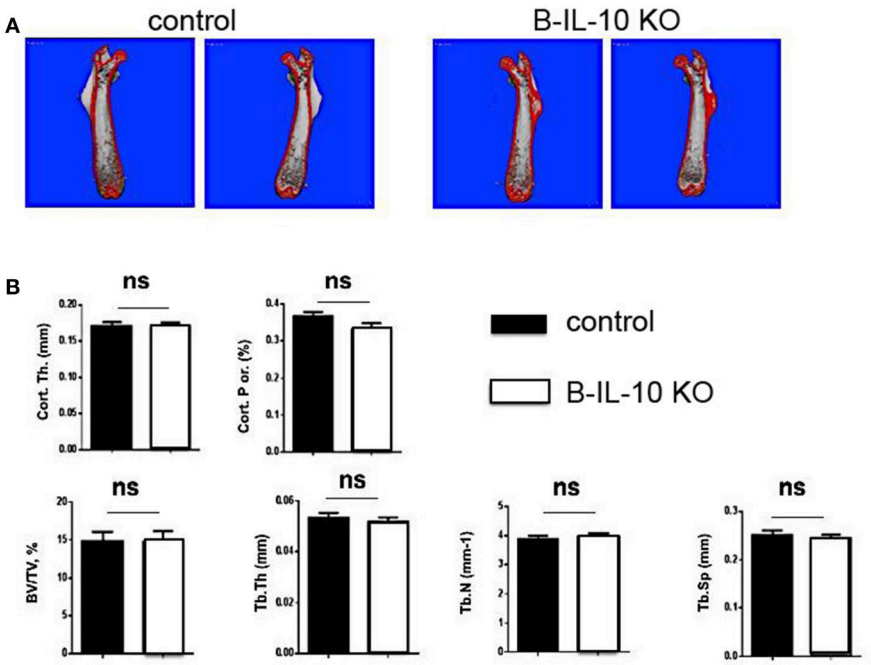

FIGURE 9 | B cell deficient IL-10 KO mice exhibit normal bone formation. Femurs were dissected from B cell specific IL-10 deficient mice and littermate controls and the bone thickness and bone volume density (BV/TV) was analyzed. **(A)** Imaging of the femurs by μCT. **(B)** Statistical analysis of BV/TV. B-IL-10 KO = B cell specific IL-10 deficient mice; control = littermate controls. Statistics: *t*-test ($n = 10$ and 19 for B cell specific IL-10 deficient mice and littermate controls, respectively).

cytokines (29). Impaired proliferation of aged human peripheral blood mononuclear cells was found to be related with increased IL-10 production (31). Moreover, human T cells exhibit age-related changes in their capacity to produce IL-10 following re-stimulation (32). IL-10 production has been reported to be oppositely affected during aging in different rat strains (30), hence indicating the existence of a genetic component influencing age-related changes in IL-10 functions. The impact of bone marrow plasma cell derived IL-10 on myeloid cells described in this study needs to be elucidated regarding the development of clinically relevant age-related immunodeficiency.

In addition to their crucial role for immune protection, monocytes, macrophages and dendritic cells play multiple roles in other physiological and pathological processes, such as wound healing, autoimmune inflammation and atherosclerosis (54–58). Further studies are required to investigate the impact of plasma cell derived IL-10 on myeloid lineage differentiation in the bone marrow, and its contribution to the age-related changes observed on these processes.

METHODS

Mice

8-12 week-old C57BL/6 mice were purchased from Charles River Laboratories (Sulzfeld, Germany). IL-10 reporter (Vert-X) mice were provided by Prof. Christopher L. Karp (University of Cincinnati College of Medicine, Cincinnati, Ohio, US), and CD19 Cre and IL-10 flox/flox mice were provided by Dr. Axel Roers, Dresden, Germany. Mice were kept and experiments were performed at the animal facilities of the University of Luebeck. All of the procedures performed for research purposes were approved by the governmental administration of the state of Schleswig-Holstein, Germany.

Antibodies

Anti-mouse antibodies used in flow cytometry staining analysis: anti-CD11b (clone M1/70.15.11, in house production); anti-CD19 (clone 1D3, BioLegend, Fell, Germany); anti-CD138 (clone 281-2, BioLegend); anti-CD210 (IL-10 receptor, clone 1B1.3, in house production); F4/80 (clone BM8, in house production): CD115 (clone AFS98, BioLegend); anti-B220 (clone RA3.B2, in house production); anti-GR1 (clone RB6-8C5, in house production); anti-Ly6G (clone 1A8, BioLegend); anti-CD4 (clone GK1.5, eBioscience, Frankfurt, Germany); Ly-6C (clone HK1.4, BioLegend), CD11c (clone N418, Biolegend).

Flow Cytometry

Single cell suspension of spleens and bone marrow (femurs and tibia) were prepared and filtered through a 70 μm cell strainer (BD Falcon). The primary cells were resuspended (10^7 cells/ml) in PBS containing 0.5%. BSA 0.5%. *In vitro* generated CD115+ cells were generated as described below. To harvest them, culture supernatants were removed and cells were incubated with 2 mM EDTA for 10 min, and then harvested with cell scrapers.

Fc receptors were blocked with anti-CD16/CD32 for 15 min (5 μg/ml in PBS/BSA, clone 2.4G2, in house production). Subsequently, cells were washed with ice-cold PBS/0.5%BSA,

and incubated with fluorescent labeled antibodies for 10 min on ice. After washing twice, cells were re-suspended in PBS/0.5% BSA/2 mM EDTA, and analyzed in an LSRII flow cytometer (BD Biosciences). The resulting data were analyzed using the FlowJo software.

Myeloid Cell Cultures

In order to generate sufficient numbers of CD115+ cells, primary bone marrow were cultured (250×10^3 cell / well) in 48-well plates for 2 days in RPMI1640 (Gibco) medium containing 30 ng/ml M-CSF (R&D). After 2 days, non-adherent cells were removed and the purity of CD115+ cells was determined by flow cytometry.

In order to stimulate the expansion and further differentiation of these CD115+ cells into DCs or macrophages, at day 0 the culture was supplemented with 30 ng/ml M-CSF and 50 ng/ml RANKL (R&D) in RPMI1640 (Gibco). After 2 days of culture alone, or together with recombinant IL-10 or activated B cells/plasma cells, cells were harvested and analyzed by flow cytometry.

To generate osteoclast-like cells, cultures of CD115+ cells were prolonged for up to seven days. These cultures were supplemented with 30 ng/ml M-CSF and 50 ng/ml RANKL at days 0 and 3 in RPMI1640 (Gibco). In order to quantify osteoclast like cells, Tartrate-resistant acid phosphatase (TRAP) staining was performed with a commercial kit (Sigma-Aldrich). Briefly, cells were washed twice with cold PBS to remove non-adherent cells, and then fixed with 250 µl 4% PFA for 3 min. After washing twice with PBS, 250 µl TRAP solution was added and incubated for 15 min. Samples were washed again two times and mounted with glycerin/PBS (1:1). Purple cells with ≥3 nuclei were quantified as osteoclasts by microscope.

B Cell and Plasma Cell Isolation and Culture

For the co-culture experiments, B cells were isolated from spleen using a MACS B cell isolation kit (Miltenyi Biotech, Berdgisch Gladbach, Germany). The isolated B cells were incubated in complete RPMI medium with 10 µg/ml LPS (Sigma-Aldrich) in 48 well plates. For the measurement of IL-10 in the supernatant of cultures, CD138+ plasma cells, CD138− CD19+ B cells and CD138− CD19− non B cells from bone marrow were sorted by FACS using a MoFlo Legacy (Beckman Coulter) and cultured in 98 well plates (20 thousand/cells well) in complete RPMI medium plus 20 ng/ml IL-6. This cytokine was added because it improved the survival of isolated plasma cells, which otherwise die very quickly.

IL-10 ELISA

IL-10 in the supernatant of the murine B cell/plasma cell cultures was measured using a mouse IL-10 ELISA kit (Biolegend) according to the instructions of the manufacturer.

RNA Isolation and Real Time PCR

Bone marrow plasma cells (CD138+), non-plasma cells (CD138−), dendritic cells (CD115+ Ly6C− CD11c+), neutrophils (CD115− Ly6C− CD11b+ Ly6G+), macrophages (CD115− CD11c− F4/80+) and monocytes (CD115+ Ly6C+ CD11c−) were sorted using a BD FACS ARIA III. RNA was isolated using Trizol reagent according to the manufacturer's instructions (Zymo Research). Reverse transcription reaction of total RNA was performed using a QuantiNova Reverse Transcription Kit (Qiagen), including the procedure for removal of contaminating genomic DNA, according to manufacturer's instructions. Quantitative PCR was done using QuantiNova SYBR Green PCR Kit (Qiagen) iQSyber green (Biorad) on a CFX96 real-time PCR system (Biorad) using the specific following primers (Metabion): IL-10 forward 5′-GCGCTGTCATCGATTTCTCC-3′ and reverse 5′-GGCCTTGTAGACACCTTGGTC-3′; IL-10RA forward 5′- GAGCCTAGAATTCATTGCATACG-3′ and reverse 5′-GTACTGTTTGAGGGCCACTT-3′; actin forward 5′-GCACCACACCTTCTACAATGAG-3′ and reverse 5′-AAATAGCACAGCCTGGATAGCAAC-3′ (used as internal control for all samples). Real-time RT-PCR data were analyzed using CFX Manager Software 3.1 (Bio-Rad).

µCT Scanning

µCT analysis of the fixed femur was performed using a µCT40 desktop cone-beam microCT (Scanco Medical, Switzerland) with a voxel size of 10 µm. Thereby, trabecular bone was evaluated in the distal metaphysis in a volume situated 2,100 µm to 600 µm proximal of the distal growth plate. Cortical bone was evaluated in a volume of 1,000 µm length situated in the middle of the diaphysis.

Statistics

Statistical calculations were performed using GraphPad Prism (GraphPad Software, La Jolla, USA). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. Statistical tests are indicated in the individual figure legends.

ETHICS STATEMENT

The animal experiments conducted in this study were done in strict accordance with the German regulations of the Society for Laboratory Animal Science (GVSOLAS), and the European Health Law of the Federation of Laboratory Animal Science Associations (FELASA). All animal experiments were approved by the respective local Committee on the Ethics of Animal Experiments of the state Schleswig-Holstein (Ministerium für Landwirtschaft, Umwelt und ländliche Räume des Landes Schleswig Holstein).

AUTHOR CONTRIBUTIONS

LM, LA, A-KC, TL, JL, CL, KH, UK, and DW performed experiments. LM, LA, J-PD, and RM designed the experiments and wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.01183/full#supplementary-material

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REVIEW
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Factors Affecting Early Antibody Secreting Cell Maturation Into Long-Lived Plasma Cells

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Antibody secreting cells (ASCs) are terminally differentiated cells of the humoral immune response and must adapt morphologically, transcriptionally, and metabolically to maintain high-rates of antibody (Ab) secretion. ASCs differentiate from activated B cells in lymph nodes and transiently circulate in the blood. Most of the circulating ASCs undergo apoptosis, but a small fraction of early ASCs migrate to the bone marrow (BM) and eventually mature into long-lived plasma cells (LLPCs). LLPC survival is controlled both intrinsically and extrinsically. Their differentiation and maintenance programs are governed by many intrinsic mechanisms involving anti-apoptosis, autophagy, and metabolism. The extrinsic factors involved in LLPC generation include BM stromal cells, cytokines, and chemokines, such as APRIL, IL-6, and CXCL12. In humans, the BM $CD19^{-}CD38^{hi}CD138^{+}$ ASC subset is the main repository of LLPCs, and our recent development of an *in vitro* BM mimic provides essential tools to study environmental cues that support LLPC survival and the critical molecular mechanisms of maturation from early minted blood ASCs to LLPCs. In this review, we summarize the evidence of LLPC generation and maintenance and provide novel paradigms of LLPC maturation.

Keywords: immunoglobulin, B cell, antibody-secreting cell, long-lived plasma cell, differentiation, maturation, maintenance

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INTRODUCTION

A key aspect of the adaptive immune response is the rapid production of high-affinity antibodies (Abs). This antibody production is the function of an antibody secreting cell (ASC), which arises from naïve (or memory) B cells as they encounter antigen, activate, proliferate, and differentiate. In draining lymph nodes, the B cell to ASC differentiation can occur either in the germinal center (GC) reactions (which has implications for ASC longevity; see below) or outside GC, as a part of an extrafollicular response (1–3) (**Figure 1A**). Differentiated ASCs subsequently egress out of lymph nodes and circulate in the blood (**Figure 1B**). Despite their critical function, ASCs are rare and comprise of no more than ∼0.01–1% of the total cellularity in the circulation and lymphoid tissues.

In the blood and secondary lymph organs (SLOs), the ASC presence is transient (days to a few weeks) during primary and secondary immune responses (4–7). After vaccination or during infection, ASCs appear in circulation for days and quickly disappear from the blood while serum Ab titers remain (6, 8–10). The bimodal characteristics of serum Ab decay after infection or long-lived vaccines suggests that the large initial burst of ASCs is responsible for the peak of serum Ab titers and the secondary decay is due to ASCs with longer half-lives (11).

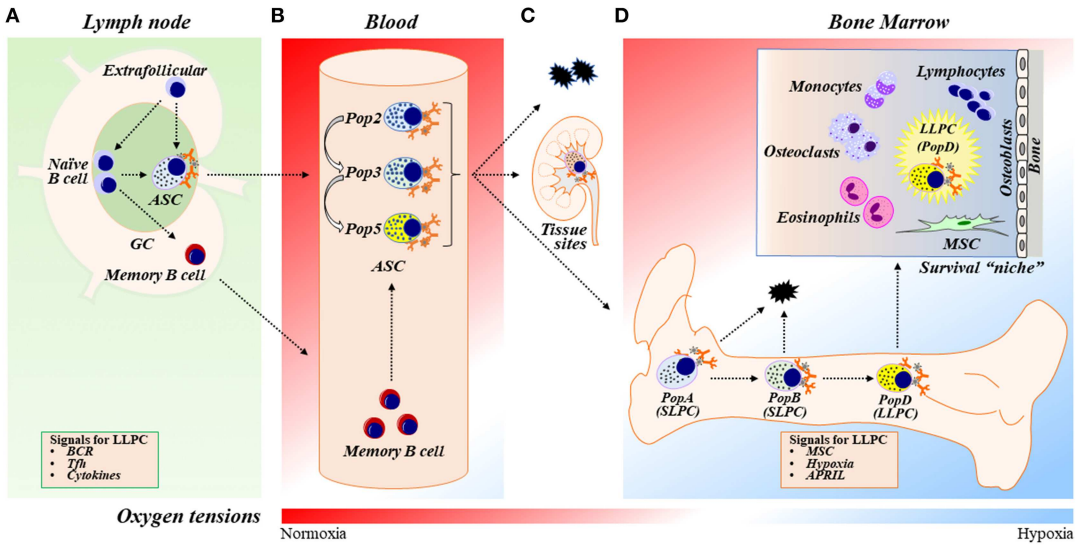

FIGURE 1 | ASC differentiation, maturation, and LLPC generation and maintenance. **(A)** In response to vaccination or infection, naïve B cells proliferate and differentiate into ASCs within germinal center (GC) reactions in secondary lymphoid organs (SLOs) such as lymph nodes (LNs) or outside of GCs in extrafollicular responses. ASCs then egress out of SLOs and circulate in the blood transiently. Naïve B cells can also differentiate into memory B cells, circulate systemically, and further differentiate into ASCs upon activation. The two-step model of LLPC generation consists of the GC reaction and the BM maturation process [see also **(D)**]. In the GC reaction, B cells may receive signals such as BCR stimulation, Tfh (or other Th cell) interactions, and cytokines (such as IL-21 and IFNγ) in the GC that are important during ASC differentiation to ultimately become a LLPC. **(B)** Human blood ASCs are heterogeneous and can be classified into distinct populations (Pops), including Pop2, Pop3, and Pop5, based on their surface protein expression (see also **Table 1**). **(C)** Most blood ASCs apoptose. However, a fraction migrates to inflammatory or other tissue "niches" (e.g., mucosa and bone marrow) and become tissue-specific ASCs. **(D)** A small number of blood ASCs emigrate to the BM, a physiologically hypoxic environment. Human BM ASCs can be identified based on surface protein expression as PopA (SLPCs), PopB (SLPCs), and PopD (LLPCs), respectively, in the BM (**Table 1**). LLPCs acquire longevity and are maintained within the dedicated BM survival "niche." ASC survival factors include BM MSCs (and/or their secreted factors), cytokines (i.e., APRIL), and hypoxic conditions. BM, bone marrow; GC, germinal center; ASC, antibody-secreting cells; SLPC, short-lived plasma cells; LLPC, long-lived plasma cells; BCR, B-cell receptor; Tfh, T follicular helper cells; MSC, mesenchymal stromal/stem cells.

In the circulation, the majority of circulating ASCs undergo apoptosis; yet a specialized fraction further matures into long-lived plasma cells (LLPCs) as they migrate to BM or other tissues (12) (**Figures 1C,D**). LLPCs are quiescent, terminally-differentiated, non-dividing cells that survive after the antigen vanishes and is responsible for protection (4, 13–15). It is thought that LLPCs constitutively produce specific Abs for years or even decades after an initial infection or immunization (12), with evidence that they can persist independently of memory B cells (11, 15–17). Hence, generating LLPCs is the main objective of an ideal vaccine.

Many studies show that ASCs derived from GC reactions, rather than extrafollicular responses, have the potential for LLPC formation and survival (12, 18–21). After B cell receptor (BCR) cross-linking and costimulation, a number of cytokines (particularly IL-21 and IFNγ), through help from T follicular helper cells (Tfh), and affinity maturation have significant intrinsic influence on an ASC generated through GC responses to become a LLPC (20–28). Most recently, IFNγ through B cell intrinsic T-bet expression is required for LLPC generation (29). Thus, the GC appears to be important for the formation of LLPCs, yet the overall molecular mechanisms and programs that govern these processes are largely unexplored.

Some investigators show that LLPCs can be generated independently of B cell maturation (i.e., in the absence of GC formation) (30–32). However, these studies showed persistence of ASCs for only 3–4 months and it is unclear if they are indeed long-lived. Nonetheless, whether a memory B cell generated initially through a GC response and re-stimulated extrafollicularly has similar intrinsic LLPC potential is not entirely known. Thus, it is still debated if some ASCs derived extrafollicularly from memory vs. naïve B cell origins may have long-lived survival potential.

In this review, we focus on the molecular and cellular processes involved in the adaptation of ASCs after exiting the GC into the medullary cords and efferent lymphatics and upon entering the blood, and eventually the BM niche (**Figure 1D**). Throughout the review, we will refer to "differentiation" as the process in which a B cell becomes an ASC (through GC or non-GC reactions), and "maturation" as the process in which an early minted ASC develops or transforms into a LLPC. The term "maintenance" will be used for survival of the ASCs. On this basis, we will discuss the role of BM "microniches" in both the maturation and the maintenance of BM LLPCs.

TRANSFORMATION OF B CELLS TO ASCS AND THEN TO LLPCS

The prime directive of ASCs is to synthesize and secrete Abs that provide protection against microorganisms. ASCs produce large amounts of Abs, typically at a rate of between ∼10 and

1,000 (and can be up to ∼5,000–10,000) Ab molecules per cell per second, which corresponds to ∼0.2–22 pg/cell/day (33–38). To perform this heroic feat, ASCs undergo fundamental changes in morphology and homeostasis during terminal differentiation, which include altering cellular structure, surface protein expression profile, metabolism, and other cellular and molecular programs. In this section, we discuss these changes by contrasting the recent findings of human blood and BM ASCs.

Cellular Structure

To accommodate the production of copious amounts of proteins, ASCs expand their cytoplasm, endoplasmic reticulum (ER), and Golgi apparatus during differentiation and maturation, enabling increased capacity for Ab synthesis and secretion (39) (**Figure 2**). Early ASCs lose their proliferative capacity and reduce the nucleus size by condensing their chromatin. The decrease in nuclear area and the repositioning of the nucleus closer to the cell membrane enable ASCs to accommodate additional cellular machinery necessary for protein production. Overall, these morphological changes are orchestrated to result in assembling an Ab factory.

Blood ASC Phenotypes

As ASCs differentiate and mature, they alter their surface protein expression profile. These proteins can be used as the phenotypic markers for identifying subsets within the ASC population. Notably, there is no universally accepted or "standardized" phenotype to identify ASCs—in humans or in mice (which make it difficult to compare across studies). Nevertheless, human ASCs in general lose their surface immunoglobulins and can typically be identified based on the expression of one or more of the following cell surface markers: CD19, CD27, CD38, CD138, and BCMA (40–46) (**Figure 2**). In mice, blood ASCs typically reduce or lose the expression of B220, MHC-II, and BCR. Additional markers such as Sca-1, TACI, and CD98, have also recently been used for identifying ASCs in mice—on the basis of CD138 expression (43, 45–49); however, it is unclear how this combination translates to humans or other animal models.

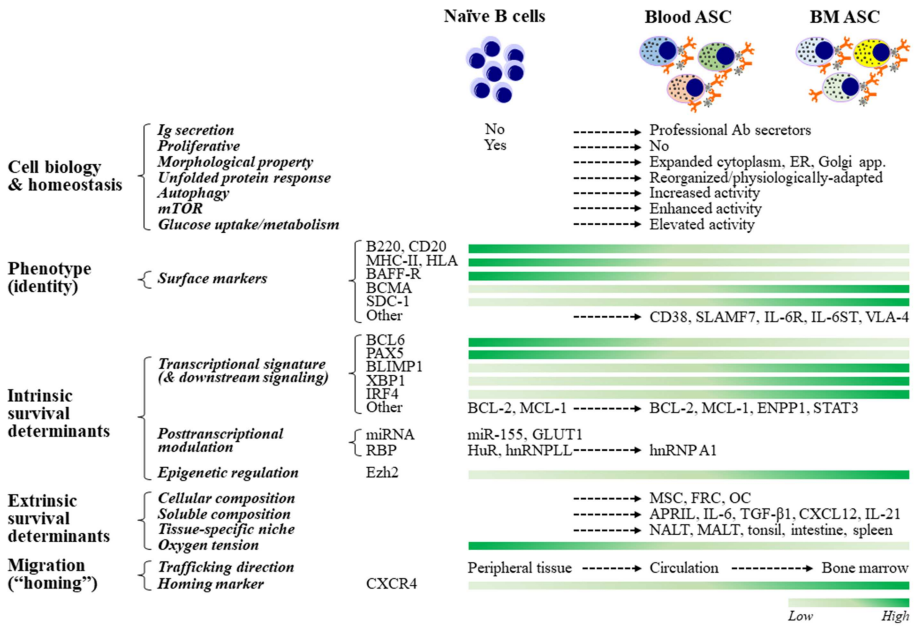

FIGURE 2 | ASC cell biology, fate determination, and resultant phenotypes. ASCs adapt their cellular biology, machinery, and morphology to maintain homeostasis while producing copious amounts of antibodies. For example, ASCs must upregulate the unfolded protein response (UPR) to inhibit apoptotic pathways that would be engaged due to increased Ig synthesis. Other pathways that act in concert with the UPR to support ASC homeostasis are autophagy, mTOR, and glucose metabolism. Although no phenotype-based "universal identity" of blood and bone marrow ASCs presently exists, ASCs are typically identified by increased expression of CD38, CD138, and BCMA, and decreased expression of B220, MHCII, and BCR. The commitment to the ASC fate of naïve B cells is regulated transcriptionally, post-transcriptionally, and epigenetically. Maintenance of B cell programs is mainly controlled by Bcl6 and Pax5, and promotion of B cell differentiation and ASC commitment are essentially governed by Blimp1 (encoded by *Prdm1*), Xbp1, and Irf4. The role of post-transcriptional (through certain miRNAs and RBPs) and epigenetic (such as via *Ezh2*) regulation is increasingly being recognized. The lifespan of ASCs ranges from a few days to several decades and is thought to be chiefly determined extrinsically, which is largely dependent on their residency. Accordingly, LLPCs reside in a specialized, dedicated BM compartment ("survival niche") that is physiologically hypoxic and is formed and maintained by both cellular (such as MSCs, FRCs, and OCs) and soluble (such as APRIL, IL-6, TGF-β1, and CXCL12) components. Other tissue-specific niches capable of maintaining ASCs have also been described. ASC migration (or "homing") from peripheral tissues or SLOs toward survival niches is primarily directed by binding of CXCR4 to its ligand, MSC-secreted chemokine CXCL12 (SDF1α). Certain miRNAs and RBPs include miR-155, which is involved in cytokine production, and HuR and hnRNPLL, which modulate IgH mRNA turnover and translation. ASC, antibody-secreting cells; LLPC, long-lived plasma cells; ER, endoplasmic reticulum; SLO, secondary lymphoid organs; MSC, mesenchymal stromal cells; FRC, fibroblastic reticular cells; OC, osteoclasts; NALT, nasal-associated lymphoid tissues; MALT, mucosa-associated lymphoid tissues.

In humans, blood ASCs are typically identified as $CD19^{int}CD27^{hi}CD38^{hi}$ (gated on $CD3^{-}CD14^{-}IgD^{-}$ cells) (41). However, our recent studies along with others have demonstrated that human blood ASCs are heterogeneous and can be further subgrouped on the basis of the additional expression of CD19 and CD138 (40, 50) (**Figures 1B**, **2**, **Table 1**). Of the five different ASC subsets we identified, three were $CD19^{+}$ whereas two were $CD19^{-}$ subsets. Among them, populations 2 (pop 2; $CD19^{+}IgD^{-}CD27^{+}CD38^{hi}CD138^{-}$) and 3 (pop 3; $CD19^{+}IgD^{-}CD27^{+}CD38^{hi}CD138^{+}$) are the most abundant, and population 5 (pop 5; $CD19^{-}IgD^{-}CD27^{+}CD38^{hi}CD138^{+}$) resembles LLPCs by surface markers (**Table 1**). The other ASC subsets contained a mixed population of B cells and other cell types and thus, the study focused on pops 2, 3 ($CD19^{+}$), and 5 ($CD19^{-}$). Based on RNAseq analysis, pops 2 and 3 are very similar transcriptionally whereas pop 5 is quite distinct (**Figure 1B**). More interestingly, all pops 2, 3, and 5 have similar potential for survival in an *in vitro* BM mimetic system, suggesting the importance of extrinsic factors in their survival.

Blood ASC Migration

The exit of ASCs from SLOs into the blood is guided by the expression of their S1P receptor 1 (S1P1) (51, 52). Subsequently, in concert with changes in surface markers, blood ASCs modulate the expression of chemokine receptors that direct them to local tissues (**Figure 1C**). For example, CXCR3 directs ASCs to inflamed tissues, CCR9 guides them to the small intestine, CCR10 directs the cells to a variety of tissues, including the colon, lung, trachea, or mammary glands (53), and, importantly, CXCR4 leads them to the BM rich in CXCL12 (54–56). It is also thought that ASCs are retained in the BM by CXCR4 because they fail to accumulate in the BM microenvironment in $CXCR4^{-/-}$ mice (54).

It still remains unclear how the expression of chemokine receptors on ASCs is regulated, although transcription factor c-Myb may be involved (57). In the absence of c-Myb, no ASCs are detected in the BM. Whether this phenomenon is due to inability of c-Myb deficient ASCs to migrate along a CXCL12 gradient or if c-Myb is involved in ASC survival will need further elucidation. Nonetheless, the migratory capability of ASCs appears to be decreased as they mature (55, 58), along with upregulation of Blimp-1 (59–61). This phenomenon may serve as another means of retaining ASCs in the BM or other tissues. Clearly, migration to specific sites rich in survival factors enhances ASC survival; but whether fundamental intrinsic differences are imprinted during ASC differentiation or whether maintenance is solely dependent on homing markers alone is not well-established. Thus, the nature (intrinsic) or nurture (extrinsic) is a critical debate for LLPC generation. To further its complexity, ASC maturation to a LLPC may actually require a combination of both.

TABLE 1 | Phenotype of blood and BM ASC subsets*.

ASC subsets	Blood	Pop2	Pop3	Pop5
	BM	PopA (SLPC)	PopB (SLPC)	PopD (LLPC)
FACS markers	CD19	+		neg
	CD138	neg	+	
	CD38	++		

BM, bone marrow; ASC, antibody-secreting cell; SLPC & LLPC, short- & long-lived plasma cell; neg, negative.
Color represents similar surface markers.

BM LLPC Phenotype

As previously mentioned, LLPCs are non-dividing, long-lasting terminally differentiated ASCs. In mice and humans, most LLPCs reside in the BM (12, 62, 63) (**Figure 1D**). These specialized ASCs persist and are thought to autonomously and continuously produce Abs for decades or beyond, independently of antigen stimulation (13–15). BM ASCs are also thought to be unresponsive to further antigenic stimulation (64). However, unlike IgG ASCs, a recent report identified a population of IgM BCR expressing ASCs that may sense antigen (65). In mice, ASCs in the spleen and the BM are identified based on the expression of B220, CD138, and Sca-1 subsets, with implications of $B220^{-}$ ASCs as mature subsets (46–48). Human BM ASCs are heterogeneous, with few overlapping markers with mice, and can be grouped on the basis of expression of CD19, CD27, CD38, and CD138 (66) (**Figure 1D**, **Table 1**). To identify the human BM LLPC subset, we interrogated each compartment for microbial specificity against viral infection (measles and mumps) that occurred decades ago and found that only the $CD19^{-}CD38^{hi}CD138^{+}$ subset contained these long-lived viral specificities. We assigned the $CD19^{+}$ BM populations as short-lived plasma cells (SLPCs), which include pops A and B that did not comprise of the long-lived specificities (66) (**Table 1**). Others have alluded to these results but did not show long-lived antigen specificities (67). Thus, it appears that the ASC fraction that downregulates CD19 expression and escapes apoptotic cell death eventually becomes LLPCs.

INTRINSIC REGULATION OF ASC DIFFERENTIATION AND MAINTENANCE

The B cell to ASC differentiation involves an extensive reorganization of transcriptional networks (68, 69) (**Figure 2**). In order for ASCs to synthesize and secrete large amounts of Abs, these regulatory networks massively upregulate transcription of the heavy and light chain mRNA transcripts (up to 100–1,000-folds). ASCs have unique transcriptomes where >70% transcripts encode the IgH and IgL chains for Ab synthesis (49). The differentiation of ASCs is regulated at the transcriptional, post-translational, and epigenetic levels. In this section, we discuss the intrinsic cellular programs—including signaling pathways and metabolic alterations as well as transcriptional, post-transcriptional, and epigenetic networks—involved in regulation of ASC differentiation, maturation, and maintenance.

Transcriptional Regulatory Networks

Aspects of ASC biology, metabolism, homeostasis, migration, and survival are controlled by their distinct gene expression

programs (or transcriptional regulatory networks) (49, 70, 71). These regulatory programs promote features to produce large quantities of Abs and to shed B cell characteristics. They function through two groups of antagonistic transcription factors, in which acquisition of plasma cell-specific transcription results in termination of B cell-specific transcriptional program (39, 72–74).

Transcription factors maintaining the B cell program are mainly BCL6 and PAX5 (75), although Bach2 and IRF8 are also important (39, 69). BCL6 is highly expressed in GC B cells and promotes cell proliferation and survival. Transcription factors facilitating ASC differentiation include Blimp1 (encoded by *Prdm1*) (76), XBP1, and IRF4 (39, 60, 61, 69, 77–79). These ASC-"specific" transcription factors are uniquely upregulated in plasma cell transcriptome (49, 70), with Blimp1 and XBP1 required for Ab secretion whereas IRF4 is likely essential for survival (61, 80–82). Importantly, Blimp1 and XBP1 play a multifunctional role in both instructing differentiation and maintaining homeostasis. Blimp1 inactivation downregulates the unfolded protein response (UPR), thus negatively affecting the cell secretory capabilities (60, 61). Also, Blimp1 positively regulates mTORC1 activity (61) as loss of Raptor negatively affects ASC maturation and Ab secretion (but not survival) (48). Finally, XBP1 has been shown to be an important regulator of the UPR (83).

In addition to these two reciprocally-regulated groups of transcription factors, a number of other factors have been found differently expressed by ASCs in distinct compartments (i.e., SLOs, blood, and BM). Examples include anti-apoptotic factors, such as Mcl-1, Bcl-2, BclxL, Bim, and BCLw, which are increased, and pro-apoptotic factors, such as Bax and BID, which are decreased, to promote ASC maintenance (84–87). Importantly, expression of Mcl-1 appears to be required for ASC survival in the BM and is mediated by BCMA, a TNF receptor (85), potentially under the control of IRF4 (71) or Zbtb20 (61, 88–90).

Signaling and Metabolism

During differentiation, ASCs adapt and utilize various signaling pathways to accommodate the need for high-rate Ab production and maintenance of homeostasis (**Figure 2**). Thus, activation of the UPR, the cellular response to ER stress due to accumulation of misfolded (damaged) proteins, is essential for increased Ab secretion during ASC differentiation from B cells (61, 81, 91, 92). In most cells, upregulation of the UPR indicates the inability of the cells to recycle proteins via ubiquitination or signify the hijacking of this pathway (i.e., by virus). In these situations, the UPR typically results in apoptosis as a means to eliminate a virally infected or dysfunctional cell. In the case of ASCs, the UPR is upregulated due to stress from the massive protein secretion in order to maintain the cell's survival since this imbalance could quickly lead to apoptosis. This pathway strongly involves XBP1 through ATF6 and IRE-1 during differentiation (61, 91, 92). Nevertheless, little is known about the regulation of this pathway in human ASCs after differentiation or how it may be modulated to ensure the maintenance of LLPCs. Thus, identifying the molecular mechanisms that regulate this pathway could lead to modulating LLPC formation and maintenance.

Antibody secreting cells (ASCs) must also adapt their metabolism to produce Abs during circadian nutrient fluctuations while maintaining cellular homeostasis. As terminally-differentiated cells, ASCs do not actively remodel their cytoplasm through cell division but rely on autophagy to recycle their protein aggregates and organelles in order to optimize energy for survival. Multiple autophagy genes, such as *Atg5*, *Atg9*, and *Atg13,* are upregulated in ASCs and likely play a role as LLPCs mature (49, 66, 93). This increased autophagic activity enables LLPCs to regulate the production of Abs when nutrients are limited (93). Interestingly, hypoxia is a known inducer of autophagocytic pathways, and we recently showed that hypoxic conditions enhance the survival of human ASCs *in vitro* (94). Whether autophagy programs are upregulated prior to BM localization or in response to the hypoxic BM microenvironment is still not clear.

Another important signaling pathway for ASCs is the mTOR. The mTOR kinase is a major regulator of many cellular processes, including survival and proliferation. mTOR signaling, mainly through the mTORC1 signaling complex (mTORC1), regulates the biosynthesis of cellular macromolecules, including proteins, nucleic acids, and fatty acids, as well as glycolysis and organelle biosynthesis (95, 96). Blimp1 positively regulates mTOR signaling as B cells differentiate into an ASC (48, 61, 97). However, in humans, as ASCs mature into BM LLPCs, mTORC1 activity is downregulated (94) and autophagy is increased (66). Additionally, the survival of human BM LLPCs, unlike that of BM SLPCs or early minted blood ASCs, is resistant to mTOR inhibitors, illustrating downregulation of mTOR pathways for LLPC maturation (94). Similar findings were corroborated in mouse studies (48).

As ASCs evolve into LLPCs, major metabolic changes occur. In addition to differences in mTOR signaling that functionally distinguishes LLPCs from SLPCs, a sundry of metabolic mechanisms differs between these two cell types. LLPCs have higher glucose uptake than SLPCs, and utilize glucose for Ab glycosylation (98). Moreover, LLPCs have increased expression of the glucose transporter, GLUT1 (98, 99). Recently, the ATP-degrading enzyme ENPP1, a regulator of glucose metabolism, was shown to be required for the development and survival of LLPCs in mice (100). In both mice and human, higher maximal respiratory capacity was shown in LLPCs compared to SLPCs (98), suggesting differences in respiratory capacity that may be linked to survival advantages. Nonetheless, more studies are warranted to understand whether versatility of energy utilization may determine survival advantages in unique nutrient-deprived environments.

Post-transcriptional Modulation

Post-transcriptional regulation of mRNA expression is a major mechanism to rewire the transcriptome and proteome. This type of regulation defines the fate of mRNAs, including immunoglobulin transcripts, through multiple steps of mRNA processing, including alternative splicing of pre-mRNAs, mRNA stability and turnover, 3' UTR regulation, and translational control (101–103). The two key players in post-transcriptional regulation of protein expression are non-coding RNAs (mainly

miRNAs) and associated RNA-binding proteins (RBPs) (101, 103, 104). In committed ASCs, post-transcriptional regulation is employed in several processes and can play a role in the magnitude of immunoglobulin expression (**Figure 2**). The processing of *IgH* mRNA is modulated post-transcriptionally (which requires Blimp-1) (60, 61). Expression of the glucose transporter GLUT1 is also regulated at the post-transcriptional level (98, 99). However, whether post-transcriptional regulation of mRNA and non-coding RNAs plays a role in LLPC maturation needs further evaluation.

RNA-binding proteins (RBPs) are involved in regulating the B cell programs and thus, can also influence ASC differentiation (101, 102). An example is HuR, an RBP splicing regulator ubiquitously found in the nucleus and capable of nucleo-cytoplasmic shuttling (105). Depletion of HuR results in unbalanced mitochondrial metabolism and impaired B cell proliferation and differentiation, which has a negative impact on ASC differentiation (106). Another major RBP involved in ASC differentiation is the splicing factor hnRNPLL, a member of the hnRNP (heterogeneous nuclear ribonucleoprotein) family. hnRNPLL is specifically induced in ASCs and, through regulating mRNA alternative splicing and stability, facilitates B cell differentiation and ASC Ab secretion (107). Also, hnRNPLL and the transcription elongation factor ELL2 (elongation factor, RNA polymerase II, 2), a regulator of pre-mRNA processing in plasma cells, modulate the ratio of secreted vs. membrane-encoding IgH transcripts. Most interesting, ELL2 was responsible for differentially processed transcripts such as BCMA (108).

Our recent integrated transcriptomic and proteomic analysis distinguishing early minted ASCs and BM LLPCs identified a novel RBP, hnRNP A1, as a potential post-transcriptional modulator (94). Blocking hnRNP A1 led to reduced survival of both human blood ASCs and BM LLPCs (109). Thus, unlike HuR or hnRNPLL, which are involved in regulating the differentiation of B cells to ASCs, hnRNP A1 may be unique and important in the regulation of the maturation of early blood ASCs and BM LLPCs. Altogether, these data highlight the important role of post-transcriptional regulation in the differentiation as well as maturation of ASCs, which can also influence the survival of LLPCs.

Epigenetic Regulation

Ezh2, a histone methyltransferase that catalyzes trimethylation of histone H3 (H3K27me3), has been shown to epigenetically regulate gene expression during ASC differentiation. *Ezh2* expression is upregulated during ASC differentiation, and *Ezh2*-deficient ASCs exert reduced levels of the UPR, glucose metabolism, and mitochondrial respiration, resulting in decreased Ab secretion (110, 111) (**Figure 2**). Thus, *Ezh2* plays a role in ASC differentiation by promoting metabolic changes that are important for Ab secretion. Interestingly, this enzyme can interact directly with Blimp1 (60) and represses the B cell program (110). It will be important to understand if *Ezh2* expression is modified as ASCs mature into a LLPC.

EXTRINSIC FACTORS IN ASC MATURATION AND MAINTENANCE

The notion that some ASC subsets exist for only a few days whereas others live for decades is thought to be chiefly determined extrinsically, i.e., largely dependent on where ASCs reside. In both mice and humans, most LLPCs reside in a specialized BM microenvironment, known as the survival niche (12, 62, 63) (**Figure 1D**). Removal of ASCs from this specialized residence leads to rapid cell death. Thus, these niches enable ASC maintenance and longevity—via intracellular crosstalk or direct ASC interactions with neighboring cells and/or through soluble factors produced locally by neighboring cells (12, 112, 113). It is believed that these niches support only a limited ASC number due to their physical confines (112). Thus, the BM environment must be stable to protect their resident cells yet dynamic enough to adapt to new arrivals.

Although LLPCs are highly enriched in the BM (63), recent studies have described several tissue-specific niches (TSNs) that afford LLPC survival advantages. LLPCs can also be found, albeit at low frequencies, in other tissues throughout the body, such as the nasal-associated lymphoid tissues (NALT) (114), human tonsillar lymphoid tissues (115), human mucosa or mucosa-associated lymphoid tissues (MALT) (116, 117), human intestine (118–120), spleen (67, 121), and human salivary gland microenvironment (in primary Sjögren's syndrome) (122) (**Figure 2**). In mice, the spleen appears to provide extrinsic signals to determine the cell survival (71, 123). Thus, outside the BM, TSNs may also be capable of maintaining LLPCs, but may be unique for specific isotypes or specificities (114–116, 118, 120, 122).

BM Niches: Cells Involved in ASC Maintenance

The BM niche is the primary "home" for LLPCs. This ASC niche represents a highly-complex microenvironment which includes multiple cell types such as mesenchymal stromal/stem cells (MSCs) (113, 124), eosinophils (125, 126), megakaryocytes (127), basophils (128), monocytes (129), macrophages (130), dendritic cells (131), T cells (132), osteoclasts (133), and fat cells (134) (**Figure 1D**). Notably, the BM is physiologically hypoxic—with oxygen tensions range ∼1–6%, depending upon the vicinity of blood vessels (135–138). Despite the rich cellular BM composition, it still remains a mystery whether LLPCs preferentially home to the BM or pro-actively adapt upon arrival to the extreme hypoxic BM environment.

MSCs (and Derivatives)

Mesenchymal stromal/stem cells (MSCs) are the principal organizer of the BM microniche involved in supporting LLPC survival. Evidence shows that this support is mediated through cell-cell contacts and/or soluble signals (62, 94–145). BM ASCs are in direct contact with MSCs (126, 146). This interaction is thought to be mediated mainly via ASC adhesion receptors, VLA-4 and LFA-1 (140, 145, 147, 148) and their natural ligands, VCAM-1 and ICAM-1, respectively, on MSCs, and are thought to

anchor them to the extracellular matrix (ECM) (149). MSCs also assist ASC survival through the secretion of soluble molecules. For example, MSCs secrete CXCL12 that interacts with CXCR4 on ASCs (54, 55, 62, 150), which direct them to close proximity of MSCs. In addition to chemokines, MSCs secrete IL-6 that is critical for ASC differentiation and survival (62). Moreover, interaction of VLA-4 and fibronectin was shown to be essential for MSC-mediated survival of ASCs (148). MSCs also secrete TGF-β1 and TGF-β2, which are involved in B cell homeostasis and IgA responses in mice (139, 151–154). Finally, MSCs produce ECM-modifying molecules, such as heparan sulfate (HS) (130, 155, 156), which is an unconventional "receptor" that induces APRIL oligomerization and may trigger BCMA-mediated survival signaling of ASCs (157, 158).

The interaction between MSCs and ASCs via MSC-produced CXCL12 and ASC membrane-bound CXCR4 is well-established (159). However, it was unclear whether cell-cell contact between MSCs and ASCs was essential (126, 139, 144, 159) or dispensable (94, 150). Possible explanations for this discrepancy may be due to distinct MSC subsets for each study. Our recent work confirms that human BM-derived MSCs provided sufficient soluble factors for ASC survival without the need for cell-cell contact (94). While the persistence of BM ASCs is supported in general by MSCs, a unique subset of $CXCL12^+$ MSCs are obligatory to form and maintain the survival niche (126, 146). Alternatively, the requirements for the survival of ASCs as they mature into LLPCs may vary, supporting a model that different MSC subsets are needed for distinct ASC populations (specific to blood or BM).

Recently, a unique subset of lymph node stromal cells found in the medullary cords provided a major source of ASC survival factors, including CXCL12, IL-6, APRIL, and BAFF (160). Distinct from T zone fibroblastic reticular cells (FRCs), the medullary FRC subset (MedRCs) together with macrophages promoted ASC survival by soluble signals similar to our observations with BM stromal cells. Additionally, FRCs at the GC-T cell zone interface may also have similar characteristics (160, 161). In summary, particular stromal cells in specific locations (i.e., lymph nodes, BM, or other tissue sites) may ultimately orchestrate ASC survival.

Extracellular vesicles (EVs) from BM MSCs provide additional evidence that MSC-ASC cell-cell contacts are non-essential (162). Both MSCs and ASCs are rare cell types in the BM microniche and EVs may provide survival factors over greater distances compared to the local paracrine factors which are limited by proximity. MSC-derived EVs have been shown to be involved in multiple myeloma (163), but our group recently showed that human BM MSC-derived EVs can support healthy ASC survival *in vitro* (162). Although the exact mechanism was not determined, EVs probably mediate the delivery and transfer of diverse biologically-active molecules over greater distances.

Eosinophils and Osteoclasts

In addition to MSCs, ASCs are also surrounded by other cell types in the BM microenvironment. Among them, eosinophils (125) and osteoclasts (OCs) (133) (**Figure 1D**) have been shown to promote ASC survival. Eosinophils have high-turnover, are located in the ASC vicinity, and produce survival factors, including IL-6 and APRIL (and BAFF) (125, 126). Although Chu et al. showed that eosinophils are needed for ASC survival (125), other investigators found that they are not essential (164–166). This discrepancy may be attributed to redundancy of ASC survival factors among a variety of accessory cells. Monocyte-derived OCs, which are able to support myeloma cell growth (167), also appeared to support the survival of ASCs *in vitro* (133). Interestingly, OCs produce BAFF and APRIL (168), but their ASC survival benefits may actually be independent of either APRIL or BAFF (133).

Other Accessory Cell Types

Other accessory cell types that are capable of promoting ASC maintenance include megakaryocytes (125, 127), basophils (125, 128), dendritic cells (131), Tfh (161, 169), and Treg (125, 170). Again, some of these cell types in BM niche may have redundant functions or secrete overlapping factors with other BM cell types and thus, may play overlapping roles for ASC survival. For example, IL-6 is paramount for ASC survival and is produced by both MSCs and eosinophils. Similarly, APRIL, another important ASC survival factor, can be secreted by neutrophils, eosinophils, megakaryocytes, osteoclasts, as well as BM MSCs (125, 127, 171). At this point, redundant functions from many BM cell types facilitate ASC persistence; however, the BM MSCs appear to be essential beyond IL-6 production for ASC survival (39, 94, 125, 126, 172). The precise soluble factors and molecular mechanisms are not known, but specific clues suggest the MSC surrounding ECM.

BM Niches: Signals and Factors Implicated in ASC Maturation and Maintenance

In addition to via cell-cell interactions, BM niches also support the process of ASC maturation by soluble components that ultimately alter the cell phenotype into a LLPC.

APRIL/BAFF:BCMA and APRIL:SDC-1 Signaling

The APRIL/BAFF:BCMA signaling axis is a well-characterized pathway in ASC survival (161, 173, 174). APRIL or BAFF, two members of the TNF family of ligands, promotes ASC survival and longevity *in vitro* (125, 175, 176), in mice (123, 129, 177), and *ex vivo* (in humans) (94). These signals are primarily triggered and delivered through the interactions of BAFF/APRIL with one of their shared receptors, BCMA (176). BCMA is predominantly expressed on terminally differentiated B cells with upregulation on LLPCs (178) (**Figure 2**). BCMA is required for maintenance of ASCs residing within BM niches, which likely acts through APRIL (or BAFF) in combination with IL-6 (176) or $CD4^+$ T cells (123). Moreover, APRIL also promotes IgA PC survival in human mucosa (116). Molecularly, APRIL mediates cell survival primarily by activation of Akt, Erk1/2, JNK, and NF-κB signaling pathways, which leads to upregulation of antiapoptotic genes. APRIL:BCMA has shown to sustain expression of Mcl-1, an important Bcl2-family antiapoptotic factor that is expressed in ASCs (85, 176).

Syndecan-1 (SDC-1), otherwise known as CD138, a heparan sulfate proteoglycan (HSPG), has been shown to be a pro-survival factor for ASCs (179) (**Figure 2**). While BAFF predominately

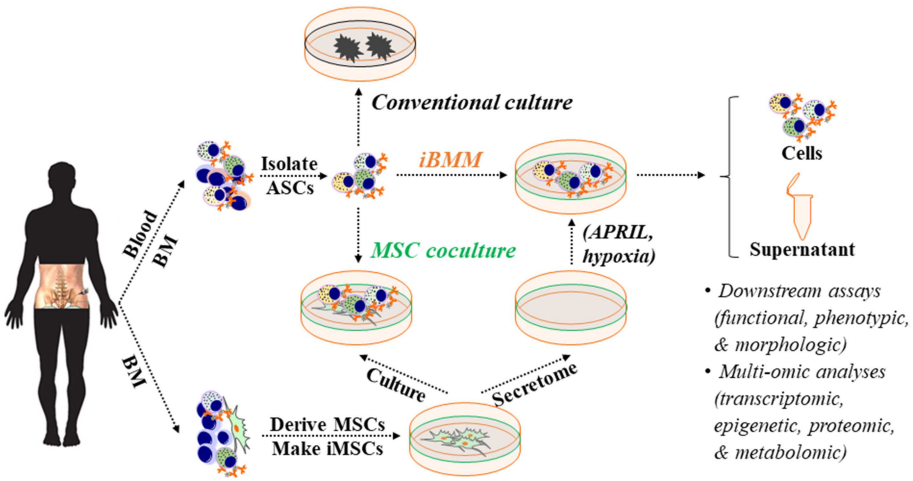

FIGURE 3 | An *in vitro* BM microniche mimic (iBMM) for studying human ASCs *ex vivo*. Primary MSCs are derived from human autologous or allogeneic BM samples, expanded, and irradiated. Populations or single blood or BM ASCs are isolated and co-cultured with BM-derived iMSCs or in the cell-free secretome that is harvested from BM-derived MSCs. Typically, blood or BM ASCs quickly die off *ex vivo* or in conventional culture but survive for weeks under these conditions. The cellular materials and supernatants obtained from the *ex vivo* ASC cultures can be collected for downstream assays and multi-omic analyses for studying human ASC biology. BM, bone marrow; ASC, antibody-secreting cells; MSC, mesenchymal stromal/stem cells; iMSC, irradiated MSC; APRIL, a proliferation-inducing ligand.

binds BAFF receptor (BAFF-R; which is expressed on immature B cells), APRIL can bind through HSPGs (129, 180, 181) and thereby through SDC-1, APRIL oligomerizes and triggers BCMA mediating ASC survival. Alternatively, binding to APRIL may enable syndecans to deliver survival signals through intracellular tails (182). In humans, SDC-1 expression had been used as a LLPC surrogate (3, 42, 180); however, our group recently showed that SDC-1 can be found in early blood ASCs even though SDC-1^{neg} ASCs could eventually upregulate SDC-1 in our *in vitro* BM mimetic cultures (40). Not surprisingly, both SDC-1^{+} and SDC-1^{neg} ASCs survived similarly, concluding that upregulation of SDC-1 plays a role in ASC maintenance.

Although there was some debate whether BAFF and APRIL equally support ASC survival (177), BAFF provides no survival advantage in our *in vitro* BM mimetic cultures (**Figure 3**). Only APRIL enhanced *ex vivo* survival of human early minted blood ASCs (94). Also, BAFF and APRIL do not appear to be involved in the pro-survival support to ASCs of OCs, which is reported to be fully cell-cell dependent (133). To our surprise, APRIL provided no additional enhancement over the BM MSC secretome for BM LLPCs (183) (**Figure 4**). Together, these data suggest that APRIL may only be needed transiently in the early blood ASC phase, permanently altering the phenotype into the LLPC maturation program.

IL-6:IL-6R and IL-6:IL-6ST Signaling

IL-6 signaling has been known to play a critical role in ASC differentiation and long-term survival (**Figure 2**). IL-6 is important for the differentiation of B cells into ASCs and acts as a pro-survival factor during ASC migration (112). Inhibiting IL-6 signaling leads to a profound reduction of human ASCs (94, 150). Early minted blood ASC subsets have a relatively high expression of the IL-6R (~32–77%), as shown in our recent study (40). Thus, during the acute phase of an infection when B cells differentiate to ASCs, when there is an abundance of free IL-6, ASCs signal through IL-6 through the cell surface IL-6 receptor (IL-6R). However, after resolution of the infection during steady state, free IL-6 levels decrease dramatically with most of it bound to the soluble IL-6 receptor (sIL-6R). The fact that, unlike blood ASCs, BM ASC have low expression of the surface IL-6R (66), might have implicated distinct mechanisms for IL-6 signaling in early minted ASCs and LLPCs.

Fortunately, IL-6 can act through two distinct signaling pathways: classic and trans-signaling. In the classic pathway, IL-6 first binds to the membrane-bound IL-6R (also known as gp80 or CD126); this interaction leads to dimerization and activation of the signal transducing protein gp130 (also known as IL-6ST or CD130), a transmembrane protein important for signal transduction following cytokine engagement through activation of the Jak-1/STAT and Akt/MAPK/ERK-1/2 signal transduction pathways, to eventually upregulate STAT3 (184–186). In the absence of IL-6R, IL-6 has no binding affinity for IL-6ST; therefore, only cells that express IL-6R can be stimulated by IL-6 (187). Thus, the effect of IL-6 through classic signaling is rather limited because it is restricted to only cell types that express the IL-6R such as early minted blood ASC (40, 188, 189). The trans-signaling activates the IL-6/STAT3 through extracellular IL-6 initially binding to its soluble IL-6R (sIL-6R), then this IL-6/sIL-6R complex subsequently binds to IL-6ST (190). Through trans-signaling, IL-6 (and the sIL-6R) stimulates cells that lack the membrane-bound IL-6R. Interestingly, IL-6ST is virtually expressed on all cells in the body (191), including BM LLPCs. It will be interesting to corroborate evidence from the transcriptomes of early minted ASCs and BM LLPCs for IL-6ST expression and investigate whether IL-6 trans-signaling plays a role in LLPC survival.

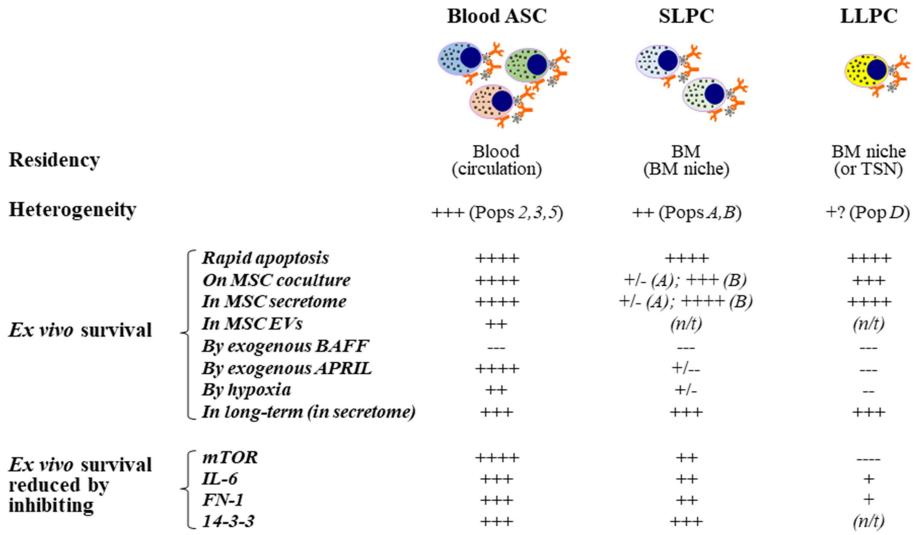

FIGURE 4 | Extrinsic cues for human *ex vivo* ASC maintenance: Findings from using the *in vitro* human BM mimic. Blood ASCs circulate transiently while short- and long-lived PCs reside in tissue-specific niches like the BM. Both circulating ASC and local PC populations are heterogeneous in humans and can be classified into distinct subsets based on their expression of CD19, CD38, and CD138. These populations are named Pop2, Pop3, or Pop5 in blood and PopA, PopB, or PopD in the BM. Typically, each of these ASC populations undergo rapid apoptosis *ex vivo*; however, they can survive for several weeks to months when cultured on BM MSCs, cultured in the cell-free MSC secretome, or cultured in BM MSC-derived extracellular vesicles, albeit at a lower potency. Using this approach, we have evaluated the effect of molecules on *ex vivo* human ASC survival by adding exogenous molecules, altering the environment where the cells are cultured, and by inhibiting pathways. APRIL and hypoxia offer survival advantages to human blood ASCs but appear to have little or no effects on human BM SLPCs and LLPCs. In contrast, BAFF does not increase survival of ASCs *ex vivo* even though it is essential for B cell survival. Interestingly, inhibition of mTOR has also elucidated a dependence on this pathway for human blood ASCs but less reliance on this pathway for BM LLPCs. Other pathways have been evaluated similarly as indicated. ASC, antibody-secreting cells; PC, plasma cells; SLPC, short-lived PCs; LLPC, long-lived PCs; MSC, mesenchymal stromal/stem cells; BM, bone marrow; SLO, secondary lymphoid organs; TSN, tissue-specific niches; ex-, exogenous; EVs, extracellular vesicles; N/T, not tested or not available; long-term survival, >8 weeks.

CXCL12:CXCR4 Signaling

As aforementioned, CXCR4 (CD184) and its ligand CXCL12 (SDF-1) play an essential role in directing blood ASCs to BM (42, 54, 55). Whether CXCL12 can directly also act as an ASC survival factor *in vivo* (192), or merely functions as a retention factor to maintain ASCs in survival niches is not known (193). One study showed that CXCL12:CXCR4 signaling promotes ASC survival *in vitro* (62). Nonetheless, further studies are needed to discriminate survival vs. homing mechanisms of CXCL12.

Other Soluble and Membrane-Bound Survival Factors

Additional survival factors reported to date include TGF-β1 (as mentioned previously) (139, 153, 154), IGF-1, TNF-a, HGF, VEGF, IL-21 (125, 130, 159, 161, 173), and fibronectin-1 (FN-1) and YWHAZ (14-3-3 zeta/delta) (94, 148) (**Figure 2**). Among some of the cell surface molecules involved in ASC survival are CD28 (131, 194, 195), CD93 (196), CD44 (62), CD37 (197), FcgRIIb (198), LFA-1, CD49d (VLA-4), CD49e (VLA-5) (130, 142, 148), CD73 (199), CD13 (YWHAZ receptor) (200, 201), and CD123 (IL-3R) (202). Although some of the factors, such as CD28 (131, 195) and CD93 (196), have been shown to be important for the Ab secretion and survival of BM ASCs, the other factors will probably need further evaluation to understand if they are essential or redundant factors for healthy LLPC maturation and maintenance.

NOVEL BM MIMETIC SUPPORTS ASC SURVIVAL AND MATURATION *EX VIVO*

Mouse and human ASCs undergo rapid apoptosis *ex vivo* (62, 94, 142, 162). Therefore, studies to understand human ASC survival *in vitro* have not been possible. To overcome this issue, we developed a novel culture system that mimics the human BM microniche to support ASC survival *ex vivo* to interrogate mechanisms of LLPC maturation (94). To establish this system, we derived and expanded primary human BM MSCs, which are spindle-shaped, plastic-adherent, and phenotyped as $CD90^{+}CD73^{+}CD45^{-}CD19^{-}$ (94) (**Figure 3**). We initially utilized irradiated BM MSCs as feeders for *in vitro* cultures of ASCs but quickly found their pro-survival support was entirely driven through paracrine effects, thus defining cell-free BM-derived MSC secretome (94). In contrast, we did not find prolonged ASC survival from cell types found in the blood (94).

We also found that the BM MSC secretome alone was not sufficient for long-lived ASC survival and exogenous APRIL and BM hypoxic conditions provided enhanced longevity (**Figures 3**, **4**). We refer to the BM MSC secretome, exogenous APRIL, and hypoxic conditions as the human cell-free *in vitro* BM mimetic (iBMM). Previous systems supported human ASCs with co-cultures of supportive cells for a few days or from *in vitro* generated ASCs (139, 175, 203). Our unique system

maintains freshly isolated human ASCs *ex vivo* for 8–12 weeks and beyond (94).

The development of iBMM has been critical for studying human ASCs *in vitro*. It provides powerful tools to follow the human ASC maturation process in the BM microniche to characterize the survival factors, signaling pathways, or metabolic programs important for LLPC generation. Moreover, by coupling recent advances in single cell molecular analysis, epigenetics, proteomics, and metabolomics together with the *in vitro* iBMM, we will be able to dissect the mechanisms of LLPC generation and provide novel insights to their survival and modulation of Ig secretion.

Additionally, the iBMM or cell-free ASC survival system provides a rapid novel system for monoclonal Ab discovery. Currently, monoclonal antibody discovery is limited by cost and time, limiting the ability to identify rare clones. With this system, a priori selection of rare but targeted ASC clones can be identified prior to monoclonal Ab generation. For example, Abs for only neutralizing epitopes can be selected for monoclonal Ab generation, thereby decreasing cost and time.

The ability to support human blood and BM ASC survival *ex vivo* for months may also provide novel assays to measure vaccine durability. Most vaccine candidates go through extensive clinical testing to assess immunogenicity, safety, and efficacy, and at this time, assessment of vaccine longevity requires the tincture of time. By using the BM mimetic, antigen specificity and neutralization capacity (i.e., protectiveness), of the Ab secreted from circulating ASCs can easily be assessed prior to serum level. Additionally, durability of the vaccines may also be assessed *in vitro* using the iBMM.

In summary, the generation of LLPCs is complex and involves both intrinsic and extrinsic factors. B cell differentiation to an ASC is immensely intricate involving GC and non-GC reactions together with upregulating the ASC programs. Although ASC differentiation is necessary it is not sufficient for LLPC generation, which involves BM survival factors to maintain ASC Ab secretion as well as other extrinsic elements in the microniche that appear to fundamentally transform early minted ASCs into a LLPC phenotypes. The novel iBMM culture system provides tools for studying LLPC generation and maintenance. Coupling with the recent advances in transcriptomics, at both a high-throughput scale and single-cell resolution, this system has the potential to answer questions about the intrinsic and extrinsic factors that regulate differentiation, maturation, and longevity of human LLPCs. Despite the progress in understanding the extrinsic cues that support ASC survival and Ab secretion, more studies are needed to characterize the complex maturation and maintenance of LLPCs.

AUTHOR CONTRIBUTIONS

DN, CJ, and FL wrote the manuscript. IS provided important editorial input.

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Phenotypic Characterization of Chinese Rhesus Macaque Plasmablasts for Cloning Antigen-Specific Monoclonal Antibodies

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Rhesus macaques (*Macaca mulatta*) are used as a human-relevant animal species for the evaluation of vaccines and as a source for cloning monoclonal antibodies (mAbs) that are highly similar to human-derived antibodies. Although antibody-secreting plasmablasts in humans are well-defined and can be easily isolated for mAb cloning, it remains unclear whether the same phenotypic markers could be applied for isolating antibody-secreting plasmablasts from Chinese rhesus macaques. In this study, we evaluated a series of cell surface and intracellular markers and identified the phenotypic markers of plasmablasts in Chinese rhesus macaques as $CD3^-CD14^-CD56^-CD19^-CD27^-CD20^{-/low}CD80^+HLA\text{-}DR^+CD95^+$. After influenza virus vaccination, the plasmablasts in peripheral blood mononuclear cells (PBMCs) increased transiently, peaked at day 4–7 after booster vaccination and returned to nearly undetectable levels by day 14. Antigen-specific enzyme-linked immunosorbent spot (ELISPOT) assays confirmed that the majority of the plasmablasts could produce influenza virus-specific antibodies. These plasmablasts showed transcriptional characteristics similar to those of human plasmablasts. Using single-cell PCR for immunoglobulin heavy and light chains, most mAbs cloned from the $CD3^-CD14^-CD56^-CD19^-CD27^-CD20^{-/low}CD80^+HLA\text{-}DR^+CD95^+$ plasmablasts after vaccination exhibited specific binding to influenza virus. This study defined the phenotypic markers for isolating antibody-secreting plasmablasts from Chinese rhesus macaques, which has implications for efficient cloning of mAbs and for the evaluation of plasmablast response after vaccination or infection in Chinese rhesus macaques.

Keywords: plasmablast, B cell, Chinese rhesus macaques, monoclonal antibodies, influenza virus, vaccination

INTRODUCTION

Non-human primates (NHPs) are widely used as a model for the evaluation of human vaccines and for cloning monoclonal antibodies (mAbs) against influenza virus, HIV, Ebola virus, and other pathogens (1–6). Antibody-secreting plasmablasts are a good source for cloning antigen-specific mAbs after vaccination or infection with a pathogen (7–10). In humans, plasmablasts have been well-characterized and defined as $CD3^{-}CD19^{+}CD20^{-/low}CD27^{hi}CD38^{hi}$ cells (7, 11). In humans, plasmablasts peak around 7 days after influenza virus vaccination (7, 12), and influenza virus-specific antibody-secreting cells account for up to 6% of all B cells (7). However, the same phenotypic markers may not be useful for identifying rhesus macaque (*Macaca mulatta*) plasmablasts. Previous studies investigating how to identify rhesus macaque plasmablasts and their kinetics after vaccination have mostly been focused on Indian rhesus macaques (8, 9). Moreover, the phenotypic markers used to characterize antibody-secreting cells in rhesus macaques have been inconsistent among different laboratories. In Indian rhesus macaques, one paper reported that the plasmablasts were $CD3^{-}CD16^{-}CD20^{-/low}$HLA-$DR^{+}CD14^{-}CD11c^{-}CD123^{-}CD80^{+}$ cells, and the magnitude of the simian immunodeficiency virus (SIV) gp140-specific plasmablast response following booster vaccination was significantly higher at day 4 than at day 7 (8). Another paper suggested that the Indian rhesus macaque plasmablasts were $CD3^{-}CD19^{low/+}CD20^{-/low}sIgG^{-}CD38^{+}CD27^{-/+}$ cells, and the plasmablasts peaked at day 7 after vaccination with live attenuated dengue viruses (9). It is important to note that in these two reports, a different set of cell surface markers were used. The research on the phenotypic markers of plasmablasts from Chinese rhesus macaques is even more lacking. One study focused on antibody-secreting plasma cells in bone marrow from Chinese rhesus macaques (13), but there have been no further studies on plasmablasts in the peripheral blood. In recent years, Chinese rhesus macaques have been increasingly used in biomedical research, possibly due to the restricted supply of Indian rhesus macaques, as India banned the exportation of rhesus macaques, while the exportation of Chinese rhesus macaques has increased in recent decades (14, 15).

The divergence between Indian and Chinese rhesus macaques has been estimated to have occurred about 162,000 years ago (16). A study reported that an HIV vaccine-induced specific T cell responses and lymphoproliferative responses in Chinese rhesus macaques were significantly weaker than those in Indian rhesus macaques, but antibody responses were stronger in Chinese rhesus macaques (17). Therefore, the immune response to an antigen may not be identical between Indian and Chinese rhesus macaques. At present, the phenotypic markers and kinetic responses of plasmablasts after vaccination in Chinese rhesus macaques remain unclear. Therefore, there is a need to define suitable phenotypic markers for identification and isolation of plasmablasts of Chinese rhesus macaques, which should greatly facilitate vaccine and antibody research using Chinese rhesus macaques.

In this study, we characterized the phenotypic markers of plasmablasts from the peripheral blood of Chinese rhesus macaques. Using influenza virus as a model antigen, we also demonstrated that antigen-specific mAbs could be efficiently cloned using single-cell PCR from flow cytometry sorted single plasmablasts after vaccination.

MATERIALS AND METHODS

Vaccination of Chinese Rhesus Macaques and Human Volunteers

The Chinese rhesus macaques were housed at the experimental animal center of Guangzhou Institutes of Biomedicine and Health (GIBH), Chinese Academy of Sciences. The animals were cared for in conformance with the guidelines of the US National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals and the policies and procedures of GIBH. The protocol was approved by our Institutional Animal Care and Use Committee. The eight Chinese rhesus macaques used in this study (no. R080066, R063585, R080040, R080039, R061217, R052419, R071510, and R081210) were 3–5 kg and 6–9 years old. They were immunized via intramuscular injection of 15 μg of inactivated recombinant viruses with one of these vaccines: (1) 2015–16 trivalent seasonal influenza vaccine (A/H1N1/California/2009, A/H3N2/Switzerland/2013, B/Phuket/2013), (2) A/H5N6/Guangzhou/2014, and (3) A/H7N9/Anhui/2013 in A/Puerto Rico/8/34 (PR8) background in 1 ml. Five healthy volunteers received the 2015–2016 trivalent seasonal influenza vaccine via intramuscular injection (Aleph Biomedical Co. Ltd., Dalian, China) after signed informed consent was obtained.

Isolation of Peripheral Blood Mononuclear Cells (PBMCs)

Fresh blood samples from the Chinese rhesus macaques or humans were collected in Plasma Preparation Tube (PPT) tubes containing K_2EDTA (BD Biosciences) at different time points after vaccination. PBMCs were isolated with OptiPrep lymphocyte separation solution (Axis Shield Poc As, Oslo, Norway) by following the manufacturer's instructions. Briefly, the tubes were centrifuged at 1,000 g for 30 min at room temperature, with slow acceleration and deceleration, and PBMCs were collected. The remaining red blood cells among the PBMCs were lysed by incubation with ammonium-chloride-potassium (ACK) lysis buffer for 5 min at 4°C and the PMBCs were then washed twice with Roswell Park Memorial Institute (RPMI)-1640. For enzyme-linked immunosorbent spot (ELISPOT) assay, the cells were suspended in R10 medium (RPMI-1640, 0.05 mM 2-mercaptoethanol, 1 mM sodium pyruvate, 2 mM L-glutamate, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid [HEPES], and 10% fetal bovine serum) supplemented with penicillin/streptomycin (1×). For cell staining, the cells were suspended in 2% fetal bovine serum (FBS) in phosphate-buffered saline (PBS).

Antigen-Specific or IgG-Secreting Cell ELISPOT Assays

B cell ELISPOT assays were performed as previously described (18), with minor modifications. Briefly, MultiScreen 96-well filter plates (Merck Millipore, Darmstadt, Germany) were coated with 10 μg/ml inactivated influenza virus or anti-human IgG Fc antibody (Sigma) overnight at 4°C for enumeration of influenza virus-specific antibody-secreting cells or total IgG-secreting cells, respectively. The wells were washed with PBS and blocked with R10 medium for 2 h at 37°C. Whole PBMCs or PBMC-derived fluorescence-activated cell sorting (FACS)-sorted cells (described in the section below) were plated and incubated overnight in a 5% CO_2 incubator at 37°C. The plates were washed with PBS-Tween 20 (PBST), followed by incubation with biotinylated anti-human IgG and horseradish peroxidase (HRP)-conjugated streptavidin (BD Pharmigen). Spots were developed using 3-amino-9-ethylcarbazole (AEC) substrate (BD Pharmigen). To stop the reaction, the plates were washed with water. Spots of antibody-secreting cells were counted using an ELISPOT reader (Bioreader 4000, BIOSYS, Germany). The number of spots is reported as the number of antigen-specific cells or IgG antibody-secreting cells per million cells.

Analytical Flow Cytometry and Cell Sorting

For analytical flow cytometry, PBMCs were surface stained with fluorescein-labeled or biotin-labeled antibodies (Panel 1: CD3-Pacific Blue, CD14-phycoerythrin [PE], CD56-PE, CD19-PE-Cyanine7 [Cy7], CD20-PE-CF594, CD27-peridinin chlorophyll protein [PerCP]-Cy5.5, CD80-allophycocyanin [APC]-Cy7, human leukocyte antigen DR isotype [HLA-DR]-APC, CD95-fluorescein isothiocyanate [FITC], and IgG-biotin; Panel 2: CD3-PE, CD14-PE, CD56-PE, CD19-Pacific Blue, CD20-PE-CF594, CD27-PerCP-Cy5.5, CD80-Alexa Fluor 700, HLA-DR-APC, and CD95-FITC) for 30 min in the dark at 4°C (**Supplementary Table 1**). This was followed by the addition of V500-coupled streptavidin for Panel 1. The PBMCs were then washed with 2% FBS in PBS and resuspended in 2% FBS in PBS. In some experiments, intracellular staining was conducted after surface staining. The cells were permeabilized and underwent intracellular staining using a Foxp3/transcription factor staining buffer set (eBioscience). The stained cells were run on a BD LSRFortessa cell analyzer (BD Biosciences). All data were analyzed using FlowJo software (Tree Star, Ashland, OR, USA).

For sorting the macaque plasmablasts, fresh isolated PBMCs were surface stained with a cocktail of fluorescein-labeled antibodies (CD3-Pacific Blue, CD14-PE, CD56-PE, CD19-PE-Cy7, CD20-PE-CF594, CD27-PE, CD27-PerCP-Cy5.5, CD80-APC-Cy7, HLA-DR-APC, and CD95-FITC) in the dark at 4°C (**Supplementary Table 1**). These cells were sorted as bulk cells for subsequent functional ELISPOT assays (described in the section above). To identify plasmablasts from Chinese rhesus macaques, a sequential flow cytometry gating strategy was developed to enrich for plasmablasts: (1) extended lymphocyte gate that included plasmablasts was made on the forward scatter-area (FSC-A)/side scatter-area (SSC-A); (2) singlet lymphocytes were identified using the SSC-A/side scatter-width (SSC-W) and FSC-A/forward scatter-width (FSC-W); (3) a lineage-negative gate ($CD3^-CD14^-CD56^-$) to exclude granulocytes, $CD3^+$ T cells, $CD14^+$ monocytes, and $CD56^+$ NK cells.

Quantitative Real-Time (qRT)-PCR for Assessing the Expression of IgG and Transcription Factors

Total RNA was extracted from the sorted cells using an extraction kit (Axygen, NY, USA) and reverse transcribed into cDNA using an iScript RT kit (Bio-Rad, Hercules, CA, USA). The cDNA then served as templates for qRT-PCR and were amplified using a Bio-Rad CFX96 Real-time PCR Detection System (Bio-Rad) with QuantiFast SYBR Green PCR Master Mix (Qiagen, Hilden, Germany). Cycle threshold [C(t)] values and melting curves were analyzed with CFX Manager 3.1 (Bio-Rad). The expression of IgG and the following 11 transcription factors was assessed: Ki67, paired box 5 (Pax-5), B-cell CLL/lymphoma 6 (Bcl-6), basic leucine zipper transcription factor 2 (BACH2), zinc finger and BTB domain-containing 20 (ZBTB20), interferon regulatory factor 4 (IRF-4), X-box-binding protein 1 (XBP-1), B lymphocyte-induced maturation protein 1 (Blimp-1), T-box transcription factor (T-bet), myeloid cell leukemia 1 (MCL1), POU class 2-associating factor 1 (POU2AF1/OBF1). The expression levels were determined by comparison with the levels of beta-actin (**Supplementary Table 2**). The data are reported as the mean values of at least three experiments.

Single-Cell PCR for Cloning mAbs

Reverse transcription and single-cell IgG cloning were carried out as previously described (19), with a small modification. Briefly, single plasmablasts were sorted directly into 96-well PCR plates containing 20 μl lysis buffer. The lysis buffer was composed of 40 U RNaseOUT, 6.25 mM dithiothreitol (DTT), 5 μl 5 × First-Strand buffer (250 mM Tris-HCl, pH 8.3, 375 mM KCl and 15 mM $MgCl_2$), and 0.0625 μl Igepal (Sigma). Next, 150 ng random hexamers (Takara), 0.4 mM dNTPs (Fermentas), and 50 U Superscript III (Invitrogen) was added, with a total volume of 5 μl. cDNA synthesis was performed for 50 min at 50°C, 5 min at 85°C, and cooling at 4°C. The cDNA was stored at −80°C. Antibody genes were amplified from the cDNA by nested PCR, using previously described IgG heavy and light chain-specific primers (20), with some modifications (**Supplementary Tables 3**, **4**). Briefly, all PCR reactions were performed in 96-well plates in a volume of 25 μl per well, which contained 2.5 μl cDNA, 2.5 μl 10 × PCR buffer (Qiagen), 0.5 μl 10 mM dNTP (Fermentas), 1 U HotStar Taq Plus (Qiagen), and 0.5 μM of each primer mixture. The PCR program was initiated by incubation at 94°C for 5 min, followed by 50 cycles at 94°C, for 30 s, 55°C (1st) or 60°C (2nd), for 30 s, and 70°C for 1 min, and a final elongation step at 70°C for 7 min before cooling to 4°C. The 2nd round PCR products were evaluated on 2% agarose gels, purified using QIAquick spin columns (Qiagen) and sequenced using 2nd round PCR reverse primers.

Cloning was carried out using an In-Fusion HD Cloning kit (cat. no. 638909; Clontech). Briefly, 50 ng purified PCR products

were mixed with 2 ml In-Fusion HD enzyme premix and 100 ng linearized vector. Water was added to create a total volume of 10 ml. The reaction mixture was incubated for 15 min at 50°C and then placed on ice. About 4 ml of the product was used to transform TOP10 Chemically Competent *E. coli* (TransGen Biotech, Beijing, China). Five colonies for each single cell PCR were picked for sequencing confirmation. All five sequences were identical. This has been rectified in Material and Methods.

Antibody Sequence Analysis and Expression

Regarding the single sorted plasmablasts, to identify the V(D)J family gene usage and their complementarity-determining region 3 (CDR3) lengths, V domain sequences were analyzed for the immunoglobulin (Ig) heavy chain variable gene cluster (IGHV), Ig kappa light chain variable gene cluster (IGKV), and Ig lambda light chain variable gene cluster (IGLV) using IMGT/V-QUEST (http://www.imgt.org) and the international ImMunoGeneTics information system. Heavy- and light-chain plasmids were co-transfected into 293T cells for transient expression. The supernatants were harvested at 3–5 days after transfection. Antibodies were purified with Protein A beads (cat. no. CA-PRI-0100; Repligen) according to the manufacturer's instructions.

Antibody-Binding Enzyme-Linked Immunosorbent Assay (ELISA)

The plasmablast-derived mAbs were evaluated using ELISA. First, 1 μg/ml inactivated influenza virus (H3N2/Switzerland/2013) was coated onto 96-well plates at 4°C overnight. Serial dilutions of the supernatants of the 293T cells or purified antibodies in PBS were incubated in the wells for 2 h. The plates were washed and HRP-conjugated goat anti-human IgG was added. The reaction was developed with 3,3′,5,5′-tetramethylbenzidine (TMB) substrate according to the manufacturer's instructions (Merck Millipore). Optical density (OD) at 450 nm was measured. The minimum mAb concentration indicating antigen-specific binding was defined as an OD value ≥2-fold the OD value of the negative control.

FACS Titration of mAb Binding to Influenza Virus Infected Cells

FACS titration of mAbs binding to influenza virus infected cells were performed as previously described with minor modifications (21). Briefly, Madin-Darby canine kidney (MDCK) cells grown in 6-well plate were inoculated with influenza virus H3N2/Switzerland/2013 for 2 h and then incubated in DMEM culture medium containing 0.3% bovine serum albumin (BSA) and N-tosyl-L-phenylalanyl chloromethyl ketone (TPCK)-trypsin (1 μg/ml, Sigma) at 37°C for 24 h. Infected cells were collected and permeabilized using a fixation/permeabilization solution kit (BD). Cells were intracellular stained by primary antibody (puritified plasmablast-derived mAbs) at different concentration and fluorescein-labeled secondary antibody (IgG-APC-H7). The stained cells were run on a flow cell analyzer.

Data Analysis

Flow cytometric data were analyzed using FlowJo v10 software (Tree Star, Inc., Ashland, OR, USA). Statistical analyses and the construction of graphs were conducted using GraphPrism 5.01 (GraphPad Software Inc., La Jolla, CA, USA). Two-tailed *p*-values were calculated, and differences were considered significant when $p < 0.05$.

RESULTS

The Cell Surface Markers for Human Plasmablasts Could Not Be Used for Identifying Chinese Rhesus Macaque Plasmablasts

In humans, the cell surface markers for identifying plasmablasts have been established as $CD3^-CD19^+CD20^{-/low}CD27^{hi}CD38^{hi}$ (7, 11). Based on these cell surface markers, we observed an increase in influenza virus-specific plasmablasts after vaccination with a seasonal influenza virus vaccine in human volunteers. $CD3^-CD19^+CD20^{-/low}CD27^{hi}CD38^{hi}$ cells peaked at approximately 7 days and decreased by 14 days after vaccination (**Figure 1**). The secretion of influenza virus-specific antibodies by these cells was confirmed by B cell ELISPOT against influenza viruses. We first assessed the cross-reactivity of a variety of anti-human antibodies to ensure that they recognize the same proteins from Chinese rhesus macaques (**Supplementary Table 1**).

When the same cell surface markers were used for sorting cells from PBMCs obtained from Chinese rhesus macaques vaccinated with influenza viruses, few $CD3^-CD19^+CD20^{-/low}CD27^{hi}CD38^{hi}$ cells were observed to secrete influenza virus-specific antibodies (**Figure 1**). Therefore, the cell surface markers for identifying human plasmablasts are not useful for identifying plasmablasts from Chinese rhesus macaques. It is thus necessary to define appropriate markers for identifying plasmablasts from Chinese rhesus macaques.

Plasmablasts Induced After Vaccination Were Primarily CD19 Negative

To identify plasmablasts from Chinese rhesus macaques, we vaccinated Chinese rhesus macaques intramuscularly with influenza viruses. PBMCs were collected at 0, 4, 7, 14, and 28 days after vaccination and analyzed by FACS using a series of antibodies against cell surface markers. It is known that human plasmablasts generated after vaccination are positive for the proliferation marker Ki67 (7). Therefore, we verified the expression of the intracellular marker Ki67 in the antibody-secreting plasmablasts (**Supplementary Figure 1A**). Previous reports on plasmablasts from Indian rhesus macaques were controversial, as one paper reported that plasmablasts producing antigen-specific antibodies are $CD19^-$ (8), whereas another paper reported that antigen-specific antibody-secreting cells are $CD19^+$ (9). To clarify whether CD19 is expressed on antibody-secreting plasmablasts from Chinese rhesus macaques, we designed a flow cytometry strategy involving a singlet lymphocyte gate (which was extended to include B cells and plasmablasts)

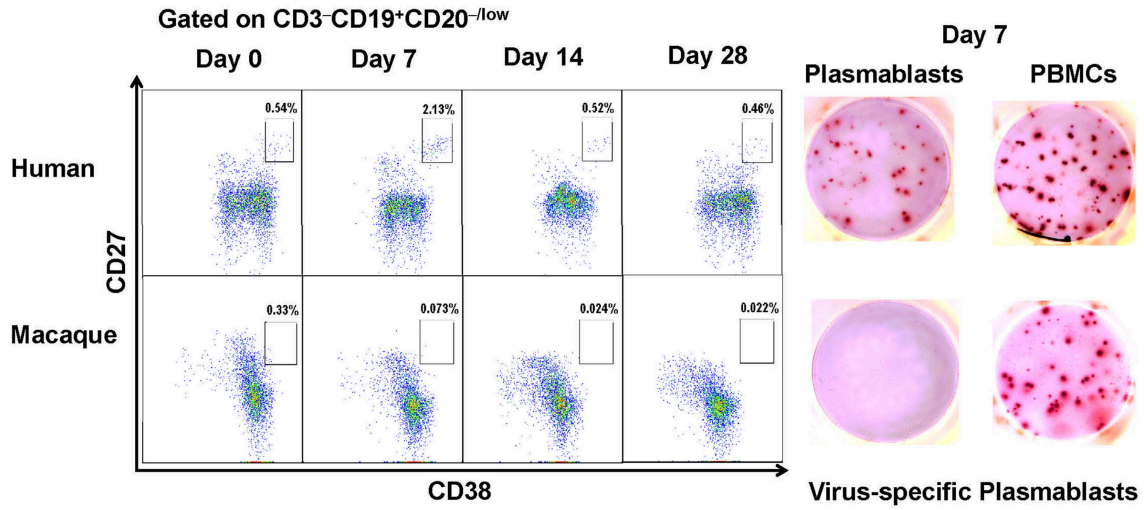

FIGURE 1 | Antibody-secreting plasmablasts from Chinese rhesus macaques are phenotypically distinct from human antibody-secreting plasmablasts. Plasmablasts in PBMCs were investigated using flow cytometry. The frequencies of plasmablasts, using human plasmablast gate ($CD3^-CD19^+CD20^{-/low}CD27^{hi}CD38^{hi}$), are shown for a representative human donor and a Chinese rhesus macaque at 0, 7, 14, 28 days after vaccination. Representative ELISPOT results showing reactivity to influenza viruses H1N1/California/2009 and H3N2/Switzerland/2013 at day 7 after vaccination. Each well contained 400 $CD3^-CD19^+CD20^{-/low}CD27^{hi}CD38^{hi}$ cells or 2×10^5 PBMCs ($n = 4$). The experiment has repeated a minimum of three times.

and a lineage-negative gate ($CD3^-CD14^-CD56^-$) to exclude granulocytes, $CD3^+$ T cells, $CD14^+$ monocytes, and $CD56^+$ NK cells (**Figure 2A**). After the remaining cells were stained to detect the proliferation marker Ki67, we found that >90% of the $Ki67^+$ cells were $CD19^-$ (**Figure 2B**). Thus, compared with $CD19^+$ cells, $CD19^-$ cells had a higher level of $Ki67^+$ (**Figure 2C**), suggesting that these $CD19^-$ cells were recently proliferating cells and are more likely to be the antibody-secreting cells.

To confirm the findings from the Ki67 analysis, $CD19^-$, and $CD19^+$ cells from PBMCs obtained at 7 days after vaccination were sorted from among the cells in the lineage-negative gate ($CD3^-CD14^-CD56^-$) and tested for antibody secretion using an antigen-specific ELISPOT assay for influenza viruses. The result showed that >85% of influenza virus-specific antibody-secreting cells were in the $CD19^-$ cell population, whereas <15% of them were in the $CD19^+$ cell population (**Figures 2D,E**). Thus, most of the antigen-specific antibody-secreting cells were $CD19^-$ (**Figures 2D,E**). To further confirm this phenotype, PBMCs were sorted from the $CD3^-CD14^-CD56^-$ cells based on positive or negative CD19 expression, and the expression of IgG mRNA in $CD19^+$ and $CD19^-$ cells was determined by qRT-PCR. Compared to the $CD19^+$ cells, the $CD19^-$ cells had a higher level of IgG expression (**Figure 2F**). This result demonstrated that, after vaccination, the antigen-specific plasmablasts from Chinese rhesus macaques are phenotypically distinct from their human counterparts, and they are primarily $CD19^-$ cells (**Figures 2G–I**).

Plasmablasts Induced After Vaccination Were Primarily CD27 Negative

It has been reported that CD27 is a marker expressed by human memory B cells and is also highly upregulated in human plasmablasts (7, 22). There was a discrepancy between two previous studies regarding CD27 expression in antibody-secreting plasmablasts from Indian rhesus macaques. One study showed that antibody-secreting plasmablasts were $CD27^-$ (8); another study reported that antibody-secreting cells could be either $CD27^+$ or $CD27^-$ cells (9). Therefore, we analyzed the expression of the proliferation marker $Ki67^+$ in $CD27^+$ and $CD27^-$ cells in the lineage-negative gate ($CD3^-CD14^-CD56^-CD19^-$) cell population. Although there was no significant difference in $Ki67^+$ cells between the $CD27^+$ and $CD27^-$ cells (**Figures 3A-3**, **4**), the mean fluorescence intensity (MFI) of $Ki67^+$ was higher among the $CD27^-$ cells than the $CD27^+$ cells (**Figure 3B**). This observation suggested that most $CD27^-$ cells were recently proliferating cells; these cells are more likely to be antibody-secreting plasmablasts.

We evaluated the secretion of antigen-specific antibodies from $CD27^-$ and $CD27^+$ cells that were sorted from among the cells in the lineage-negative gate ($CD3^-CD14^-CD56^-$). The antigen-specific ELISPOT assay revealed that >90% of the influenza virus-specific antibody secretion was from $CD27^-$ cells, whereas <10% was from $CD27^+$ cells (**Figures 3C,D**). Therefore, most antigen-specific antibody-secreting cells were in the $CD27^-$ cell population and not in the $CD27^+$ cell population. To further confirm this observation, the expression of IgG mRNA in the $CD27^+$ and $CD27^-$ cells was determined by qRT-PCR. Compared to the $CD27^+$ cells, the $CD27^-$ cells had a higher level of IgG expression (**Figure 3E**). This demonstrated that after vaccination, the antigen-specific antibody-secreting plasmablasts from Chinese rhesus macaques were primarily $CD27^-$, and they are phenotypically distinct from their human counterparts (**Figures 3F–H**).

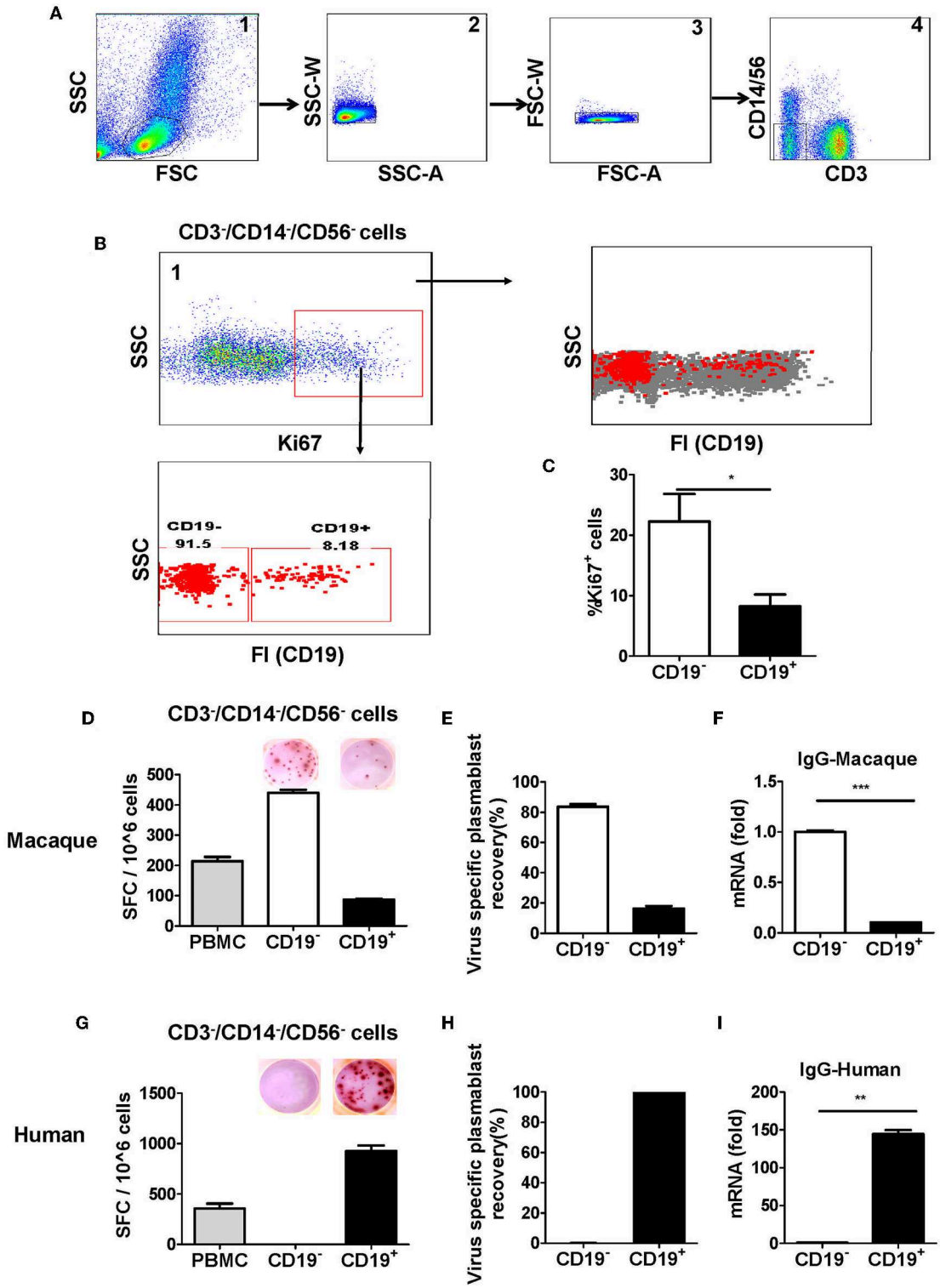

FIGURE 2 | Antibody-secreting plasmablasts from Chinese rhesus macaques are primarily Ki67^{+}CD19^{-} cells. A gating strategy was designed to exclude lineage cells (CD3^{+} T cells, CD14^{+} monocytes, and CD56^{+} NK cells) and the remaining cells were analyzed for intracellular Ki67. **(A)** Representative flow cytometry analysis showing the following sequential gating strategy to enrich possible plasmablasts: (1) extended lymphocyte gate that included plasmablasts; (2, 3) singlets; (4) CD3^{-}CD14^{-}CD56^{-} cells. **(B)** Representative flow cytometry analysis showing CD19 expression on the surface of intracellular Ki67^{+} cells. **(C)** Flow cytometry analysis showing the percentage of intracellular Ki67^{+} cells among CD19^{-} and CD19^{+} cells ($n = 4$, Mean ± SEM). PBMCs from rhesus macaques **(D,F)** ($n = 4$) and

(Continued)

FIGURE 2 | human volunteers **(G,I)** ($n = 3$) were sorted from among the $CD3^-CD14^-CD56^-$ cells based on positive or negative CD19 expression. They were subsequently subjected to influenza A virus-specific ELISPOT assay and qRT-PCR to assess the mRNA expression levels of IgG. Data points represent the plasmablast numbers observed per million PBMCs or other cell subsets. Values represent the mean percentage of total Ig-secreting plasmablasts recovered from each cell population in Chinese rhesus macaques (Mean ± SEM) **(E)** ($n = 4$) and human volunteers **(H)** ($n = 3$). The experiment has repeated a minimum of three times. FSC, forward scatter; SSC, side scatter; FI, fluorescence intensity; SFC, spot-forming cells. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

Plasmablasts Induced After Vaccination Were $CD3^-CD14^-CD56^-CD19^-CD27^-CD20^{-/low}CD80^+HLA\text{-}DR^+CD95^+$

To further characterize plasmablasts from Chinese rhesus macaques, we sorted cells with positive and negative expression of several human plasmablast markers, including HLA-DR, CD80, CD20, and CD95 from $CD3^-CD14^-CD56^-$ cells. The sorted cells were tested by ELISPOT for the secretion of influenza virus-specific antibodies. The expression of IgG mRNA in the cells was determined by qRT-PCR. Most of the antibody secretion activities were detected from $HLA\text{-}DR^+$, $CD80^+$, $CD20^{-/low}$, or $CD95^+$ cells (**Figures 4A,D,G,J**). These subsets expressed higher levels of IgG mRNA (**Figures 4B,E,H,K**). These results demonstrated that plasmablasts from Chinese rhesus macaques are $HLA\text{-}DR^+/CD80^+/CD20^{-low}/CD95^+$ cells, which is similar to human plasmablasts (**Supplementary Figure 1B**, **Figures 4C,F,I,L**).

Taking the results together, plasmablasts from Chinese rhesus macaques can be defined as $CD3^-CD14^-CD56^-CD19^-CD27^-CD20^{-/low}CD80^+HLA\text{-}DR^+CD95^+$ cells. Next, we isolated these cells, as well as $CD3^+$ T and $CD20^+$ B cells, for comparative characterization. The $CD3^-CD14^-CD56^-CD19^-CD27^-CD20^{-/low}CD80^+HLA\text{-}DR^+CD95^+$ cells from Chinese rhesus macaques were $Ki67^+$ and intracellular IgG^+ (**Figure 5A**), which is similar to human plasmablasts (**Figure 5B**). It is known that the fate of B cells is controlled by the expression of a network of transcription factors, including Blimp-1, IRF-4, and the spliced isoform of XBP-1 (23). These transcription factors drive the differentiation of B cells into plasmablasts and induce the expression of the molecular machinery required for antibody secretion. As assessed by intracellular staining and flow cytometry, we observed that IRF-4, XBP-1, and Blimp-1 expression levels were highest in plasmablasts relative to B and T cells (**Figure 5A**). Pax-5 is a transcription factor that helps B cells to maintain their identity (23). In both macaques and humans, Pax-5 expression was lower in plasmablasts than in B cells (**Figure 5B**). Using qRT-PCR, we also measured the mRNA transcription level of 11 transcription factors and IgG in $CD3^-CD14^-CD56^-CD19^-CD27^-CD20^{-/low}CD80^+HLA\text{-}DR^+CD95^+$ cells and $CD20^+$ B cells. Plasmablasts from Chinese rhesus macaques expressed higher levels of IRF-4, XBP-1, Blimp-1, Ki67, T-bet, MCL1, POU2AF1/OBF1, and IgG (**Figure 5C**) than $CD20^+$ B cells. In contrast, the expression levels of Pax-5, Bcl-6, BACH2, and ZBTB20 were lower in plasmablasts than in $CD20^+$ B cells (**Figure 5C**). We also observed a similar mRNA transcriptional profile in human plasmablasts using the following gating strategy: $CD3^-CD19^+CD20^{-/low}CD27^{hi}CD38^{hi}$ (**Figure 5D**).

Based on these findings, we sorted plasmablasts based on the $CD3^-CD14^-CD56^-CD19^-CD27^-CD20^{-/low}CD80^+HLA\text{-}DR^+CD95^+$ phenotype from among the PBMCs collected at different time points after the Chinese rhesus macaques received a booster vaccination of influenza virus. We found that the magnitude of influenza virus-specific plasmablast responses was significantly higher at day 4 than at day 7 after the booster vaccination. Up to 60% of the IgG-secreting plasmablasts were influenza-specific at day 4 (**Figures 6A–C**). Therefore, we concluded that HLA-DR, CD80, and CD95 expression combined with a lineage-negative gate ($CD3^-CD14^-CD56^-CD19^-CD27^-CD20^{-/low}$) can be used to isolate plasmablasts from Chinese rhesus macaques.

Antigen-Specific mAbs Could Be Cloned From $CD3^-CD14^-CD56^-CD19^-CD27^-CD20^{-/low}CD80^+HLA\text{-}DR^+CD95^+$ Plasmablasts

One important application of plasmablast identification is the use of single-cell PCR to clone heavy and light chain-paired mAbs from single plasmablasts. To demonstrate this utility, at day 4 after booster influenza virus vaccination, we sorted $CD3^-CD14^-CD56^-CD19^-CD27^-CD20^{-/low}CD80^+HLA\text{-}DR^+CD95^+$ plasmablasts into one 96-well plate, with one cell per well. The single cells were subjected to nested PCR with Ig heavy and light chain specific primers, as described previously (20) (**Supplementary Tables 3**, **4**). Overall, either Ig heavy or light chain amplicons could be obtained from 86 out of the 96 isolated plasmablasts, confirming that these cells expressed antibodies. We sequenced 17 mAbs with both Ig heavy and light chains successfully amplified from the same cell (**Supplementary Table 5**). An expression plasmid for each heavy and light chain pair was constructed and transfected into 293T cells to express full-length IgG1 antibodies, as previously described (2). After transfection, antibodies secreted into the culture medium were used for ELISA using influenza virus (H3N2/Switzerland/2013) as the antigen. Eleven out of the 17 mAbs (64.7%) showed positive reactivity to influenza virus H3N2/Switzerland/2013 (**Figure 7**), confirming that cells sorted based on the $CD3^-CD14^-CD56^-CD19^-CD27^-CD20^{-/low}CD80^+HLA\text{-}DR^+CD95^+$ phenotype are indeed antibody-secreting plasmablasts. We also confirmed the binding of some mAbs, including mAbs H7, B9, and B10. A previously published human broad-spectrum anti-influenza virus mAb MEDI8852 was used for comparison (24). These mAbs were expressed in 293 cells and were purified using protein A agarose. mAb H7 exhibited higher affinity with saturation of binding achieved at low IgG concentrations. mAbs B9 and B10 showed comparable binding to H3N2 infected

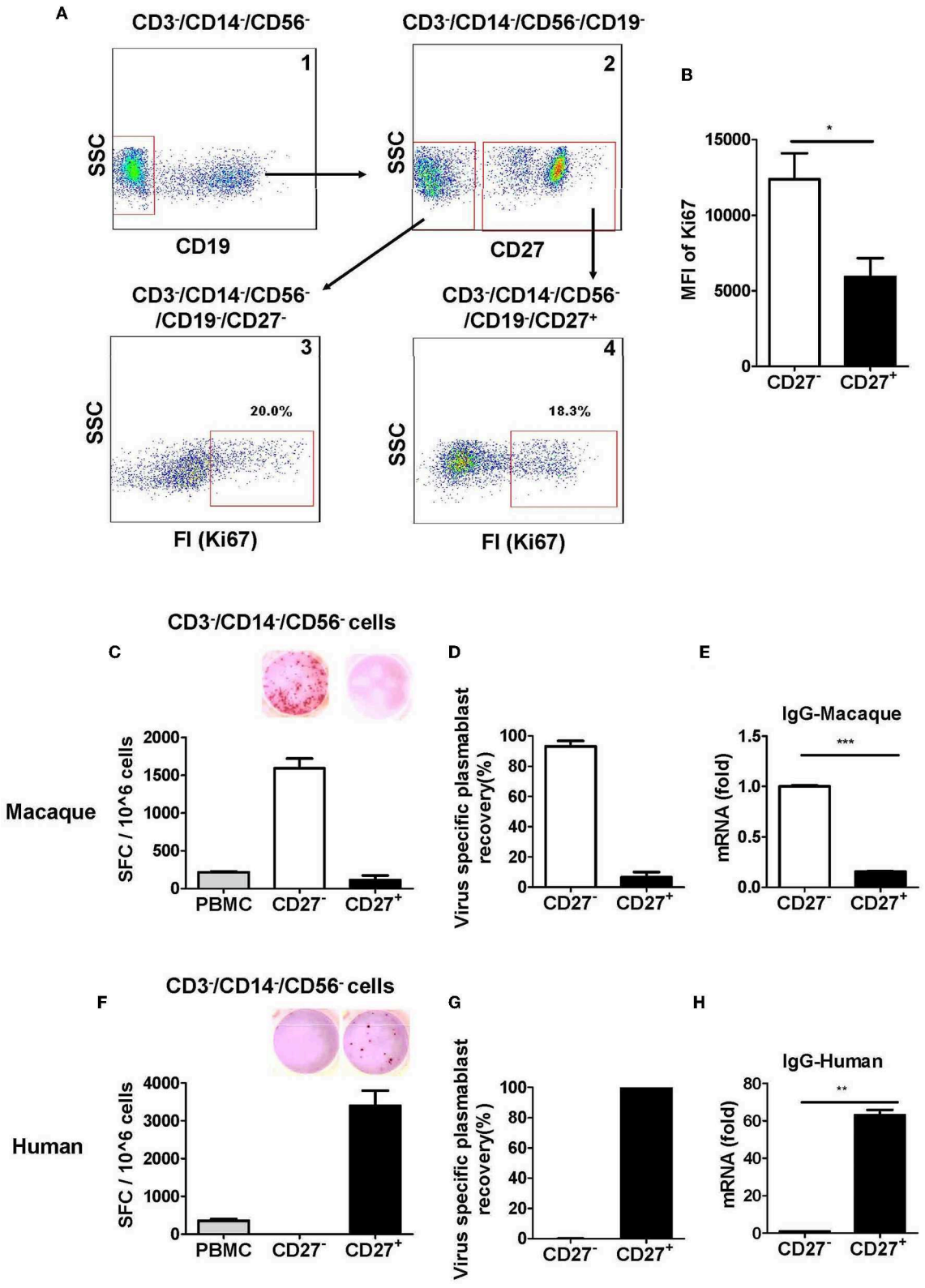

FIGURE 3 | Antibody-secreting plasmablasts from Chinese rhesus macaques are primarily Ki67$^+$CD19$^-$CD27$^-$ cells. **(A)** Flow cytometry analysis showing intracellular expression of Ki67$^+$ in CD27$^-$ or CD27$^+$ cells. **(B)** Mean fluorescence intensity (MFI) of Ki67 expression in CD3$^-$CD14$^-$CD56$^-$CD19$^-$CD27$^-$ and CD3$^-$CD14$^-$CD56$^-$CD19$^-$CD27$^+$ cells subsets. PBMCs from rhesus macaques **(C,E)** ($n = 4$) and human volunteers **(F,H)** ($n = 3$) were sorted from among the

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FIGURE 3 | $CD3^-CD14^-CD56^-$ cells based on positive or negative CD27 expression. They were subsequently subjected to influenza A virus-specific ELISPOT assay and qRT-PCR to assess the mRNA expression levels of IgG. Data points represent the plasmablast numbers observed per million PBMCs or other cell subsets. Values represent the mean percentage of total Ig-secreting plasmablasts recovered from each cell population (Mean ± SEM). **(D)** ($n = 4$) and human volunteers **(G)** ($n = 3$). The experiment has repeated a minimum of three times. FI, fluorescence intensity; FSC, forward scatter; SSC, side scatter; SFC, spot-forming cells. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

cells as mAb MEDI8852 (**Supplementary Figure 2**). We also estimated the affinity constants (Ka) of these mAbs from FACS data. When the binding rate of H3N2 infected cells to antibody increased to 50%, the reciprocal of corresponding antibody concentration is the Ka of that antibody, as shown in **Table 1**.

DISCUSSION

NHPs, especially rhesus macaques, are regarded as the most human-relevant animal models for evaluating experimental vaccines, pathogen infections and immune responses (1, 3, 6, 25–27). Compared to other animal species (apart from chimpanzees), the immunoglobulin gene of rhesus macaques shares the highest degree of homology with that of humans (28, 29). Rhesus macaques have been exploited as a valuable source for cloning therapeutic mAbs with a high degree of similarity to human antibodies (2, 4, 5, 8, 9). Although transgenic mice carrying human immunoglobulin genes have been developed and used to generate humanized antibodies, most of these mice are commercially protected and maybe not accessible. We previously reported that mAbs obtained from Chinese rhesus macaques could achieve >97% homology with human mAbs (2). Antibody-secreting plasmablasts have been used as a source to clone antigen-specific mAbs from humans and rhesus macaques (7–10). However, little information is available about the phenotypic characterization and kinetics of plasmablasts from Chinese rhesus macaques after infection or vaccination. In this study, we used influenza virus as a model antigen for defining the phenotypic markers of plasmablasts from Chinese rhesus macaques and for verifying the cloning of antigen-specific mAbs.

It has been well-accepted that human plasmablasts can be defined as $CD3^-CD19^+CD20^{-/low}CD27^{hi}CD38^{hi}$ cells. However, these cell surface markers cannot simply be applied to identify plasmablasts from Chinese rhesus macaques (**Figure 1**). Although human and rhesus macaques genome are 95.3% identical (26), some proteins may have variations in amino acid sequences. Therefore, an anti-human mAb that recognizes a particular epitope on a human protein may not find the same epitope on macaque cells. We thus firstly assessed the cross-reactivity of a variety of anti-human antibodies to ensure that they also recognize the same proteins in Chinese rhesus macaques (**Supplementary Table 1**). Finally, we defined the phenotype of antibody-secreting plasmablasts from Chinese rhesus macaques as $CD3^-CD14^-CD56^-CD19^-CD27^-CD20^{-/low}CD80^+$HLA-$DR^+CD95^+$.

We observed that HLA-DR is expressed on Chinese macaque plasmablasts, as previously described for their human and Indian macaque counterparts (7, 8, 13). CD80, also known as B7.1, provides costimulatory signals to T cells and is a typical marker of B cell activation. CD80 is upregulated on activated B cells, and this marker has been described as a regulator of germinal center development as well as antibody-secreting cells in mice (30), and it is expressed on human and Chinese and Indian macaque plasmablasts. CD95, also known as death receptor Fas, has been identified as a key regulator of activation-induced B-cell death. CD95 is highly expressed on activated B cells (31). We found that CD95 was expressed on both human and Chinese macaque plasmablasts. The most striking differences between humans and Chinese rhesus macaques were observed for CD19 and CD27 in our study. While human plasmablasts expressed high levels of CD19 and CD27, the macaque plasmablasts were low or negative for both markers (**Supplementary Table 6**).

In this study, the expression pattern of a series of transcription factors between human and macaque human plasmablasts were compared to further confirm the identity of Chinese rhesus macaque plasmablasts isolated based on the markers we proposed. Among these transcription factors, Blimp-1 is a key factor that can drive the maturation of B cells into antibody-secreting cells (32, 33). Recently, POU2AF1/OBF1 was found to act upstream of Blimp-1 (34). Moreover, the maintenance of MCL1 expression in antibody-secreting cells is crucial for their survival, and the survival pathway is independent of the Blimp-1-dependent component of antibody-secreting cell differentiation (35). POU2AF1/OBF1 and MCL1 were found to be highly expressed on vaccination-induced plasmablasts from Chinese rhesus macaques. These two factors may play important roles in triggering plasmablast differentiation and supporting cell survival.

Importantly, plasmablasts isolated from Chinese rhesus macaques were confirmed functionally. Sorted plasmablasts were tested using antigen-specific ELISPOT assays to detect the secretion of antibodies that react to influenza virus. In this study, we focused on IgG-producing plasmablasts. Most human plasmablasts have been shown to be IgG-secreting cells, but there are minor quantities of IgA- and IgM-secreting cells at 7 days after vaccination with influenza virus (7). A significant number but not all cells were positive for IgG production (**Figures 6A,B**). Some cells may not produce antibodies due to damages during isolation, which could account for 30–50% of FACS-sorted cells (8). Among the plasmablasts that produced a detectable amount of IgG, most were antigen-specific (**Figures 6A–C**). Therefore, antigen-specific mAbs can be cloned directly at a high success rate from freshly sorted plasmablasts. On the other hand, plasmablasts can be cultured

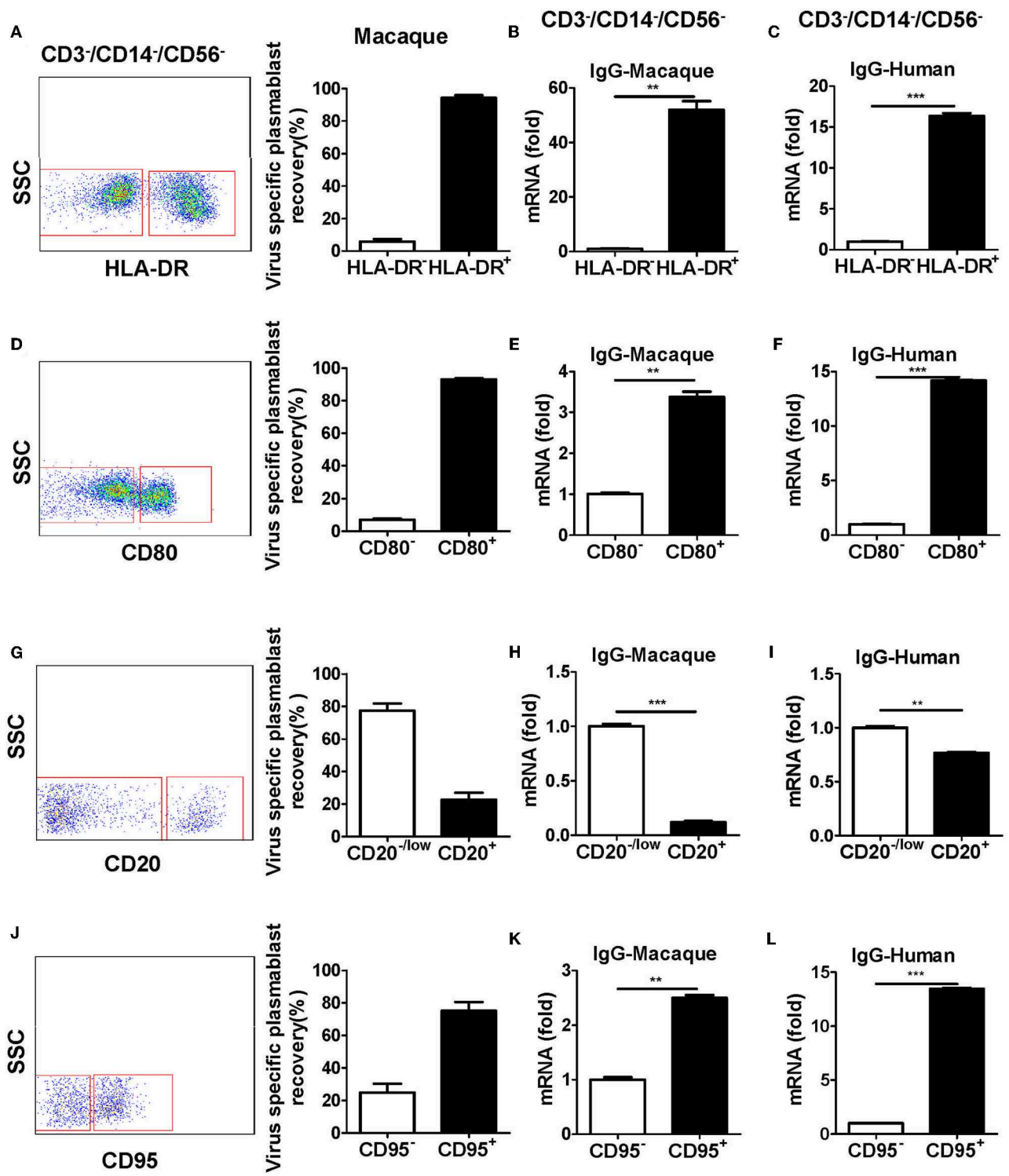

FIGURE 4 | Antibody-secreting plasmablasts from Chinese rhesus macaques are $CD3^-CD14^-CD56^-CD19^-CD27^-CD20^{-/low}CD80^+HLA\text{-}DR^+CD95^+$ cells. PBMCs from rhesus macaques ($n = 4$) were sorted from among the $CD3^-CD14^-CD56^-CD19^-$ subset cells based on the positive or negative expression of HLA-DR **(A)**, CD80 **(D)**, CD20 **(G)**, or CD95 **(J)** and subsequently subjected to influenza virus-specific ELISPOT assay. Values represent the mean percentage of total Ig-secreting plasmablasts recovered from each cell population (Mean ± SEM). PBMCs from Chinese rhesus macaques ($n = 4$) and human volunteers ($n = 3$) were sorted from among the $CD3^-CD14^-CD56^-$ cells based on the positive or negative expression of HLA-DR **(B,C)**, CD80 **(E,F)**, CD20 **(H,I)**, or CD95 **(K,L)** and qRT-PCR was used to assess the mRNA expression levels of IgG. The experiment has repeated a minimum of three times. $^{**}P < 0.01$; $^{***}P < 0.001$.

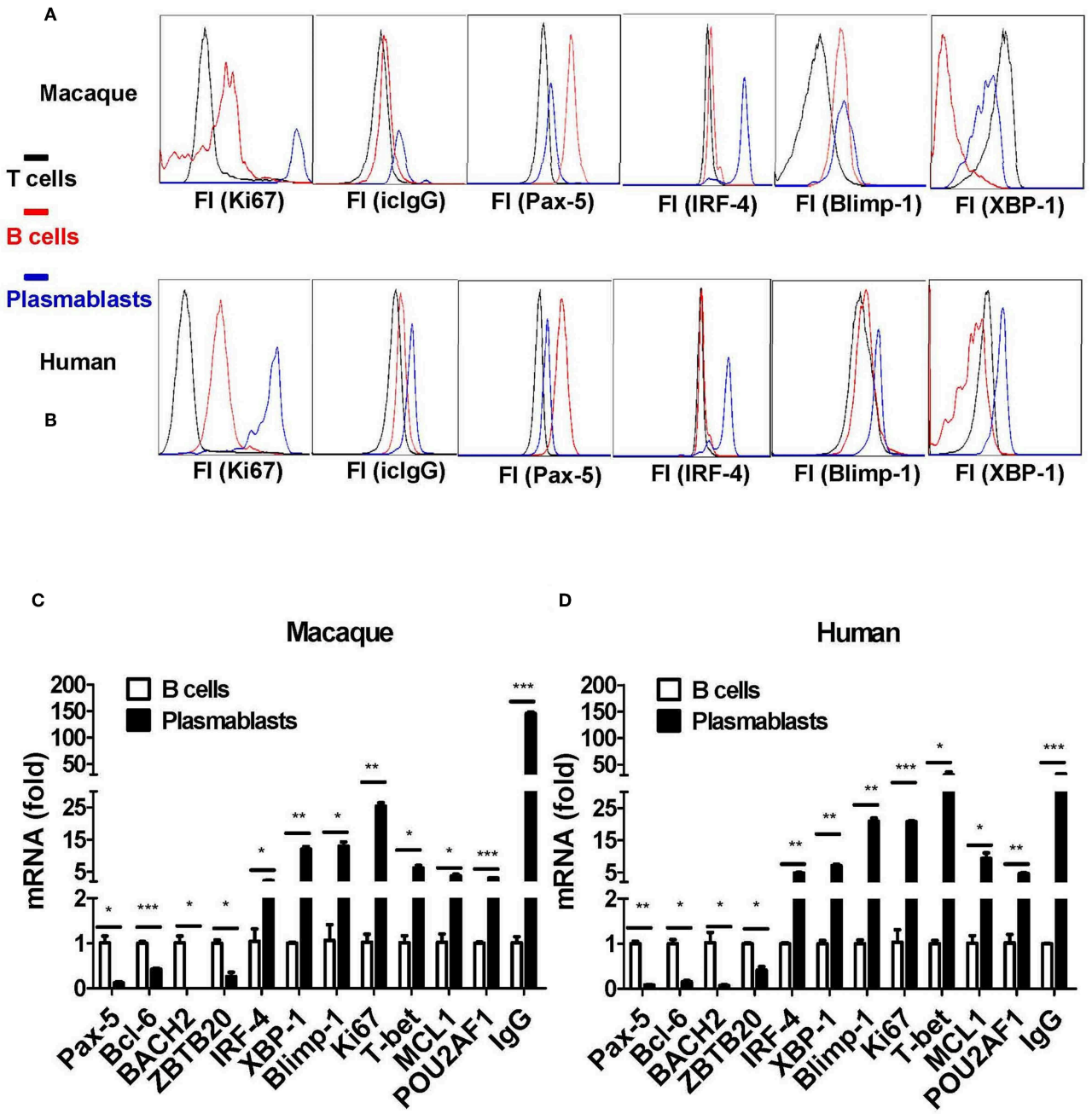

FIGURE 5 | Transcriptional characterization of antibody-secreting plasmablasts from Chinese rhesus macaques. **(A)** Intracellular protein expression of Ki67, IgG, Pax-5, IRF-4, Blimp-1, and XBP-1 by plasmablasts compared with T ($CD3^+$) or B ($CD20^+$) cells from Chinese rhesus macaques ($n = 4$). **(B)** Intracellular protein expression of Ki67, IgG, Pax-5, IRF-4, Blimp-1, and XBP-1 by plasmablasts compared with T ($CD3^+$) or B ($CD20^+$) cells from human volunteers ($n = 3$). **(C)** Macaque ($n = 4$) plasmablasts and B cells ($CD20^+$) were sorted at day 7 after booster vaccination. The mRNA levels of Pax-5, Bcl-6, BACH2, ZBTB20, IRF-4, XBP-1, Blimp-1, Ki67, T-bet, MCL1, POU2AF1/OBF1, and IgG were detected by qRT-PCR. **(D)** Human ($n = 3$) plasmablasts and B cells ($CD20^+$) were sorted at day 7 after booster vaccination. The mRNA levels of Pax-5, Bcl-6, BACH2, ZBTB20, IRF-4, XBP-1, Blimp-1, Ki67, T-bet, MCL1, POU2AF1/OBF1, and IgG were detected by qRT-PCR. The Chinese rhesus macaque plasmablast gating strategy was as follows: $CD3^-CD14^-CD56^-CD19^-CD27^-CD20^{-/low}$ $CD80^+HLA\text{-}DR^+CD95^+$. The human plasmablast gating strategy was as follows: $CD3^-CD19^+CD20^{-/low}$ $CD27^{hi}CD38^{hi}$. The experiment has repeated a minimum of three times. Each bar represents Mean ± SEM. Fl, fluorescence intensity; icIgG, intracellular IgG. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

for 1–2 days followed by antigen-specific ELISA to identify single plasmablast that produces antigen-specific antibodies. However, single-cell culture of plasmablasts is a delicate process which demands to maintain high cell viability and antibody secretion. More antigen proteins may be consumed to perform ELISA screen.

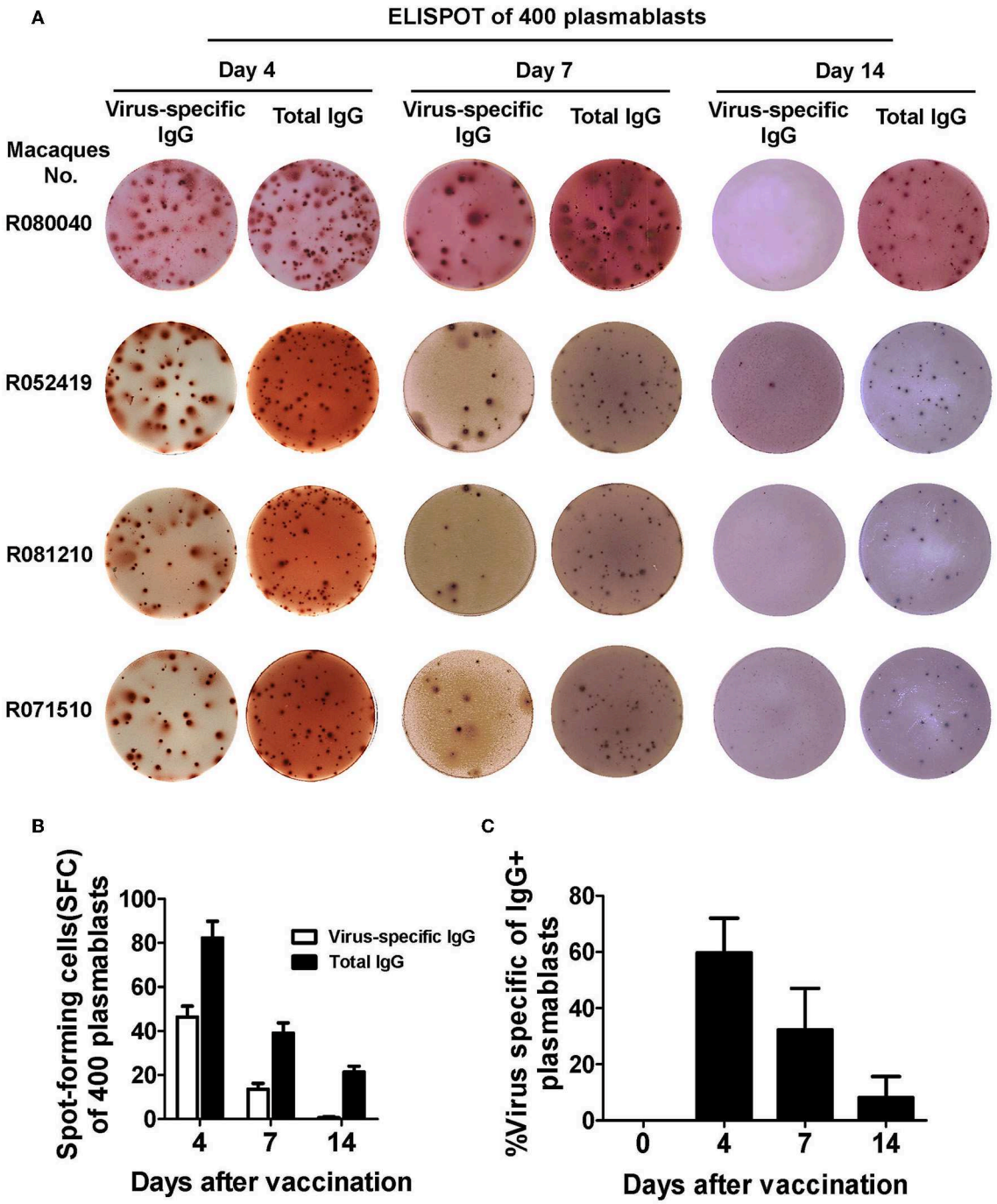

FIGURE 6 | Functional confirmation of antibody-secreting plasmablasts from Chinese rhesus macaques by ELISPOT assay. **(A)** 400 sorted Chinese rhesus macaque plasmablasts were plated with inactivated influenza virus or anti-human IgG. Representative examples of the ELISPOT assay results for macaque plasmablasts at day 4, 7, and 14 after vaccination (macaque no. R080040, R052419, R081210 and R071510). **(B)** Spot-forming cells (SFC) secreting virus-specific or total IgG among 400 sorted Chinese rhesus macaque plasmablasts at day 4, 7, and 14 after vaccination ($n = 4$, Mean ± SEM). **(C)** Percentage of influenza virus-specific plasmablasts among IgG-secreting plasmablasts at indicated time points after booster vaccination. The Chinese rhesus macaque plasmablast gating strategy was as follows: $CD3^-CD14^-CD56^-CD19^-CD27^-CD20^{-/low}CD80^+HLA\text{-}DR^+CD95^+$ ($n = 6$, Mean ± SEM). The experiment has repeated a minimum of three times.

In this study, we demonstrated that among the 17 mAbs cloned from sorted plasmablasts, 11 mAbs were specific for influenza virus. Another approach to clone antigen-specific mAbs is to use fluorescence-labeled antigens as probes to isolate antigen-specific memory B cells (4, 36, 37). However, when the binding epitopes of an antigen are not defined or available,

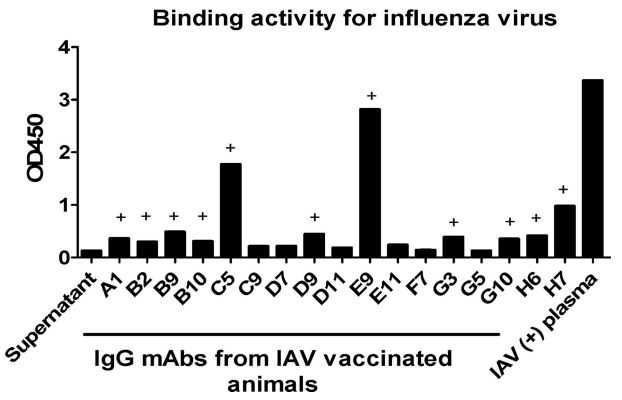

FIGURE 7 | Plasmablast-derived mAbs isolated from Chinese macaques display specific reactivity to influenza viruses. Single plasmablasts from Chinese rhesus macaques were sorted into a 96-well plate at day 4 after vaccination and were used to generate cDNA. Subsequently, the cDNAs were used as templates in nested PCRs with IgG heavy and light chain-specific primers. The paired IgG heavy and light chain sequences were cloned into antibody expression vectors and expressed in 293T cells after transfection. The mAbs produced were tested by ELISA. Inactivated influenza virus was coated onto a 96-well plate at a concentration of 1 μg/ml. At 72 h post transfection, 100 μl IgG expression supernatant was used as the primary antibody. HRP-conjugated anti-human antibody was used as the detection antibody. Influenza A virus (IAV)-positive plasma was used as a positive control. The OD450 values of the blank control were subtracted from those of the other wells. (+) The minimum mAb concentration indicating antigen-specific binding was defined as an OD value ≥2-fold the OD value of the negative control.

cloning mAbs directly from plasmablasts after vaccination would have a significant advantage. This allows characterization of B cell responses and cloning of mAbs based on plasmablasts from peripheral blood after vaccination or infection (7, 10, 11). The magnitude of the plasmablast response has been shown to correlate directly with neutralizing antibody titers and the potential to clear the infection. In humans, up to 70% of plasmablasts are antigen-specific at the peak of their response at 7 days after booster vaccination. Therefore, antigen-specific plasmablasts are excellent sources for cloning antigen-specific mAbs (7, 12). Earlier studies in Indian rhesus macaque showed that antibody-secreting cells peaks at either 4 and 7 days after the booster vaccination, but these two studies used a different set of markers (8). In our study, we found that up to 60% of IgG-secreting plasmablasts from Chinese rhesus macaques were antigen-specific at day 4, the magnitude of influenza virus-specific plasmablast responses was higher at day 4 than at day 7 after the booster vaccination (**Figures 6A–C**). It is possible that the peak of plasmablast response may vary due to antigen or adjuvant used, as well as the route of vaccination.

Influenza virus-specific memory B cells can be rapidly reactivated to yield plasmablasts after booster vaccination. Kinetic analysis of plasmablast responses is important for the design and appropriate timing of sample collection in vaccine and antibody studies. In humans, the number of plasmablasts in the peripheral blood peaks at day 7 after booster vaccination with influenza virus (7, 12). The rapid accumulation of plasmablasts suggests that these cells are the result of rapid clonal expansion. Somatic hypermutations in the immunoglobulin genes lead to the generation of high-affinity antibodies. Antigen-specific plasmablasts accumulate more somatic mutations than other B cell populations in humans (7). In this study, we found that plasmablasts exhibited a robust response at day 4 after booster vaccination in Chinese rhesus macaques, and this response occurred earlier than in humans after booster vaccination, also with influenza virus. Therefore, Chinese rhesus macaque plasmablasts may have a faster clonal expansion after vaccination. In the future, it would be desirable to analyze antibody repertoires at different days after vaccination to understand more details in antibody clonal expansion and somatic hypermutation.

TABLE 1 | Affinity constant (Ka) measurements of mAbs by FACS.

mAbs	Ka (L/Mol)
MEDI8852	2.02×10^8
H7	2.68×10^8
B9	1.31×10^9
B10	6.97×10^8

In summary, we defined the phenotypic markers for isolating antibody-secreting plasmablasts from Chinese rhesus macaques. Using this phenotype definition, single plasmablasts could be sorted using flow cytometry. mAbs with paired Ig heavy and light chain gene sequences could be cloned into expression plasmids using single-cell PCR for further analysis. This study should facilitate the evaluation of vaccination-induced plasmablast response and the efficient cloning of antigen-specific mAbs from Chinese rhesus macaque plasmablasts.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of Human Research Ethics Review Committee of Guangzhou Institute of Biomedicine and Health with written informed consent from all subjects. All subjects gave written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by the Human Research Ethics Review Committee of Guangzhou Institute of Biomedicine and Health. This study was carried out in accordance with the recommendations the guidelines of the NIH Guide for the Care and Use of Laboratory Animals and the policies and procedures of Guangzhou Institute of Biomedicine and Health and the protocol was approved by the Institutional Animal Care and Use Committee of Guangzhou Institute of Biomedicine and Health.

AUTHOR CONTRIBUTIONS

LC and PL conceived and designed the experiments. PL, FZ, LW, JLi, JLu, YF, YY, PH, WF, and RL performed the experiments. PL, FZ, ZZ, and XN analyzed the data. WP, CL, and HY contributed reagents and materials. PL, FZ, YT, XN, and LC wrote the paper. All authors commented on the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.02426/full#supplementary-material

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