INDUCTION AND MAINTENANCE OF LONG-TERM IMMUNOLOGICAL MEMORY FOLLOWING INFECTION OR VACCINATION

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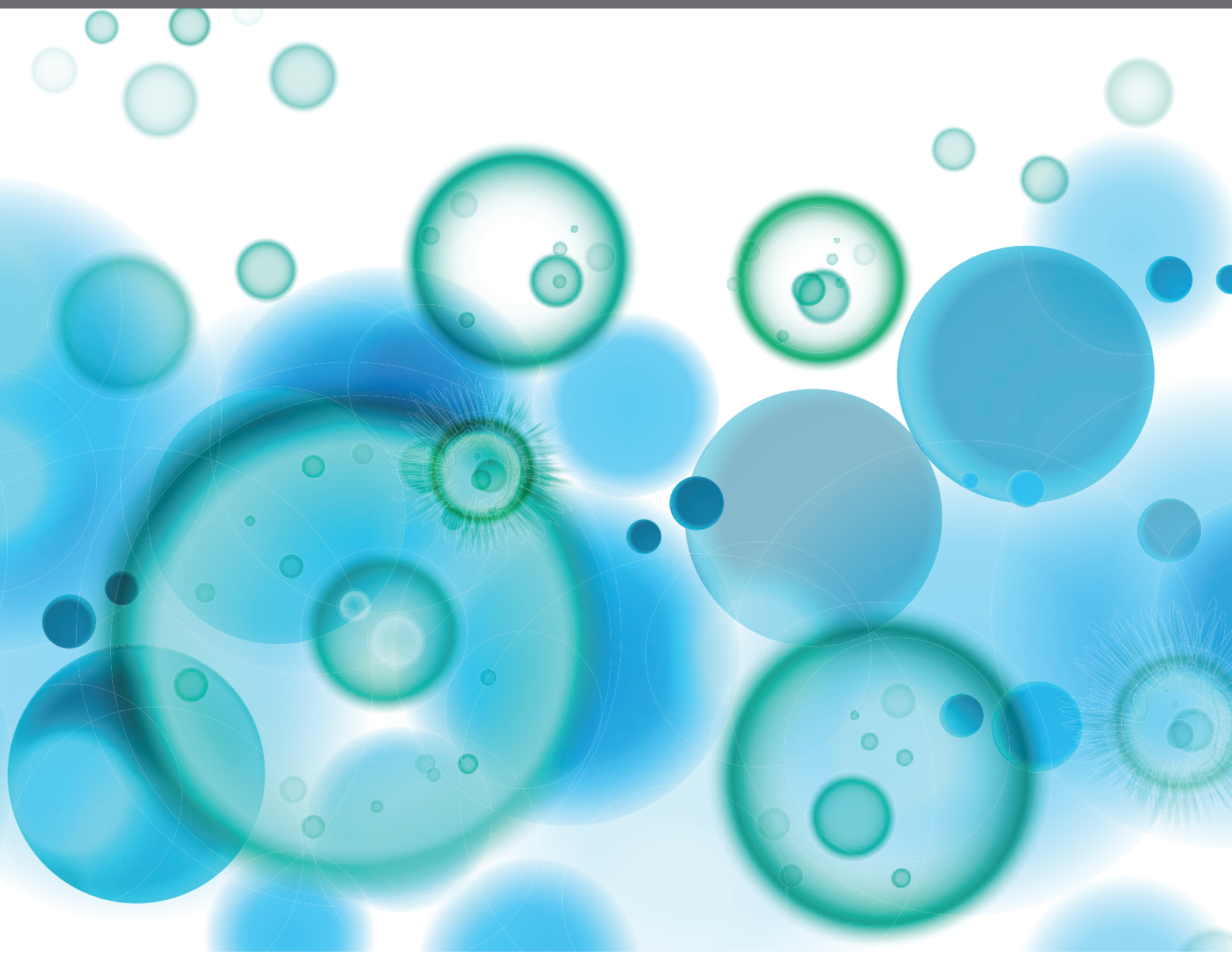

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INDUCTION AND MAINTENANCE OF LONG-TERM IMMUNOLOGICAL MEMORY FOLLOWING INFECTION OR VACCINATION

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EDITORIAL
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Editorial: Induction and Maintenance of Long-Term Immunological Memory Following Infection or Vaccination

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Editorial on the Research Topic

Induction and Maintenance of Long-Term Immunological Memory Following Infection or Vaccination

The term immunological memory is attributed to the phenomenon of qualitatively and quantitatively improved and/or enhanced antigen/epitope-specific recognition by various cells of the adaptive immune system with an ensuing improved and/or enhanced effector function. Because of the terminology used to describe this phenomenon, efforts have been focused on how to fit this phenomenon into various hypotheses, which all have to involve some form of remembrance of the previous interaction with a specific antigen/epitope. However, the way linguistics, language and words influence our thought process is an important area which was beyond the scope of this special issue.

How the adaptive leukocytes remember previous encounters has been mainly attributed to internal switching mechanisms involving prolonged cellular longevity, or interaction with antigens or fragments/epitopes thereof presented by select antigen presenting cells, such as follicular dendritic cells or long-term memory B cells. However, much remains to be elucidated regarding the mechanisms that underlie induction and maintenance of immunological memory through intrinsic or extrinsic pathways following infection or vaccination. In addition, there is sufficient evidence to strongly suggest important differences in induction and maintenance of memory in mucosal inductive and effector sites compared to systemic sites. In addition, the rules that dictate the fate of memory TH, CTL, and B cells appear to be differentiated divergently.

In this special issue, we had important contributions in most aspects of B and T cell memory of both mucosal and systemic origins. The study of the quality of antibodies produced by memory B cells as defined by their higher avidity against specific antigenic epitopes is of paramount importance. In this regard, Krueger et al. reported the existence of a recently described plasma cell population that originated from memory B cells, lived in bone marrow, and secondary lymphoid organs, rapidly produced higher avidity antibodies than primary plasma cells, but was short-lived. The same main authors reported another important discovery (Krueger et al.) that involved the essential role of TLR signaling, and hence innate responses, in the induction of the aforementioned memory B cell derived secondary plasma cells. The interplay of innate and adaptive responses in the maintenance of memory B cells has been studied and debated for some time. In 2006, Nemazee's team reported that MyD88-deficient mice were still able to respond normally to LPS-derived, as well as non-TLR-related, but inflammasome-inducing, vaccine adjuvants, such as Alum, hence suggesting that TLR signaling was not a pre-requisite for B cell responses using such adjuvants (1).

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Currently, vaccine schedules in humans, and even in animal models, are generally based on what schedule induces high enough acute responses, and it is not known which vaccination schedule induces the highest acute and memory responses. Therefore, the article by Mantile et al. was of high consequence as it identified a "consolidation phase" in the induction of epitope-specific memory B cells, as a window of time during which certain disrupting stimuli could hamper the generation of memory. This phenomenon, if confirmed by others, could have significant consequences on the vaccination schedules.

The issue of memory induction following vaccination is linked to vaccine adjuvants and delivery systems, and their nature as replicating, metabolically alive but non-replicating, or non-living and non-replicating (such as TLR agonists, Alum, etc.) The commonly unwritten rule is that vaccine adjuvants and delivery systems must induce pro-inflammatory innate responses in order to induce acute and memory responses, although this view has recently been challenged by the introduction of a vaccine adjuvant that induced strong adaptive B and T cell responses in the absence of strong innate immune responses (2, 3).

Whether memory cells reside locally in tissues, particularly in mucosal tissues which are the main portal of pathogen and antigen entry, or are derived from other peripheral sites, has been another topic of paramount significance in memory maintenance. Two original papers in this issue addressed this very problem elegantly. In an influenza A model of infection, Suarez-Ramirez et al., showed the significance of the mucosal homing receptor CD103 ($\alpha_E\beta7$) expression that can define tissue resident memory and primary CD8+ CTL in lungs and respiratory draining lymph nodes (LN), the mediastinal LN. Moreover, the check point inhibitor PD-1 and TGFβ also played differential roles on the primary/naïve and resident antigen-experienced CTL. As for the role of resident CD4+ memory TH cells, in a *Helicobacter pylori* model of infection, Liu et al. demonstrated the importance of local subserous vs. traditional remote vaccine administration for induction and maintenance of memory tissue resident CD4+ TH cells in the stomach, and these vaccine induced T_{RM} CD4+ cells played a main role in protection against *H. pylori* infection upon challenge. Because CD4+ TH cells, which recognize more diverse epitopes on influenza viruses than B cells, play a central role in B cell memory responses, Nelson and Sant devoted their paper on how CD4+ T cell imprinting and editing impacts overall memory B cell responses in humans against influenza virus infections.

Much of what we have inferred about the induction and maintenance of immunological memory in humans has been from animal and mostly murine studies. Therefore, the review by Palm and Henry was valuable for shedding light on the differences and similarities between B cell memory induction and maintenance following vaccination and infection in humans and mice. Influenza infections in humans and how to prevent them through induction of memory B cell responses that can be neutralize multiple seasonal or pandemic strains, was the focal point of the review by Auladell et al. with co-authorship by Dr. Peter Doherty, a Nobel prize winner, who has recently focused also on influenza vaccines. In this review, the role of B and follicular T cells in memory induction was examined. Special attention was also placed on the potential of inducing memory CTL responses against conserved regions of influenza viruses in the context of influenza infections and vaccinations, while bearing in mind that differences in HLA types may hamper such efforts in vaccine development. In their Perspective Article, Takamura and Kohlmeier highlighted the differences in T_{RM} vs. circulating lung CD8+ T cells, in the context of central and T_{EM} cells, and delineated special niches within lungs that mainly contain T_{RM}. Understanding how and which exogenous cytokines influence memory cells is highly valuable and in this context Kalia and Sarkar pointed out that IL-2 plays a key role in maintaining effector and memory CD8+ T cells, by triggering metabolic and transcriptional alterations in such cells.

Overall, this issue addressed many central questions, but many more remain unanswered on the path to understand and employ various memory stages and processes in vaccine development and vaccination schedules. Methods of tagging epitope-specific cells and tracking them throughout the evolution of acute vs. memory responses from first contact with the epitope until death with regards to their transcriptional signature at various stages, and their interactions with the antigen/pathogen as well as with other cells and cellular products could shed much needed light on the enigma of long-term immunological memory. Specifically, tagging epitope-specific IgM+ B cells throughout their lifetime, and to determine how some acquire many mutations, without switching to downstream isotypes such as IgG, IgA, or IgE, leading to secreted IgM with higher affinities, and whether and when they traffic to the bone marrow, may be a good start on this long path. Such studies may identify hitherto unknown ways of considering the concept of immunological memory (4).

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and has approved it for publication.

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MINI REVIEW
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Regulation of Effector and Memory CD8 T Cell Differentiation by IL-2—A Balancing Act

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Interleukin-2 (IL-2) regulates key aspects of CD8 T cell biology–signaling through distinct pathways IL-2 triggers critical metabolic and transcriptional changes that lead to a spectrum of physiological outcomes such as cell survival, proliferation, and effector differentiation. In addition to driving effector differentiation, IL-2 signals are also critical for formation of long-lived CD8 T cell memory. This review discusses a model of rheostatic control of CD8 T cell effector and memory differentiation by IL-2, wherein the timing, duration, dose, and source of IL-2 signals are considered in fine-tuning the balance of key transcriptional regulators of cell fate.

Keywords: IL-2, CD8 T cell memory, terminal effectors, autocrine, transcription factors, metabolism

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INTRODUCTION

Interleukin-2 (IL-2)–the first cytokine to be identified and characterized more than three decades ago—has emerged as a pleiotropic player in a variety of seemingly paradoxical immune functions. Originally discovered for its immunoenhancing role of promoting T cell expansion during mitogenic stimulation, IL-2 is also implicated in activation-induced cell death (AICD). Likewise, IL-2 promotes a variety of effector (cytotoxic CD8, T_{H1}) T cell responses, yet is indispensable for the development, maintenance and function of regulatory T cells (T_{reg})—the very cells that serve to suppress effector T cell responses. Further adding to the intrigue, even amongst the effector subsets, IL-2 promotes CD8, $T_{H1,}$ and T_{H2} effector responses, but suppresses inflammatory T_{H17} responses, and also inhibits the differentiation of follicular helper T (T_{FH}) cells required for B cell germinal center reactions in secondary lymphoid organs. Collectively, these findings support the thesis that IL-2 critically regulates the balance of immunostimulatory and immunosuppressive forces during immune responses to foreign antigens as well as self-antigens during homeostasis. While our understanding of the molecular, transcriptional, and metabolic regulation of CD4 T cell differentiation into T_{H1}, T_{H2}, T_{H17}, $T_{FH,}$ and T_{reg} subsets by IL-2 is abundant [see previous reviews (1–5)], the IL-2-dependent gene regulatory networks that drive effector and memory CD8 T cell differentiation remain to be fully defined. In this review we will focus on IL-2 regulation of CD8 T cell responses; alongside a summary of current literature in the context of CD4 and CD8 T cells, we will also discuss how this niche area is poised for significant advances owing to newer tools such as conditional ablation of IL-2 production and signaling in key subsets of immune cells in the physiologically relevant setting of immunocompetent hosts.

BALANCING PRIMARY AND SECONDARY CD8 T CELL IMMUNITY

CD8 T Cell Responses to Acute Infections

A typical CD8 T cell response to primary infection with acute viral or intracellular bacterial pathogens is characterized by three distinct phases—expansion, contraction, and memory. Upon stimulation with cognate antigen in conjunction with costimulatory and inflammatory ligands, naïve cells undergo massive clonal expansion (up to 50,000-fold) and concomitant effector differentiation to generate large numbers of cytotoxic T lymphocytes (CTL), which serve to control the pathogen by migrating to peripheral sites of infection and elaborating cytotoxicity against infected target cells and producing effector cytokines such as IFN-γ and TNF-α (6–11). It is now well-established that the effector CTL pool broadly contains two distinct subsets—(1) short-lived effector cells (SLECs), which are fated to rapidly die after pathogen clearance, and (2) memory precursor effector cells (MPECs) (12–16), which are imprinted with antigen-independent survival capabilities for mediating long-term protection against secondary challenge (17–19). Thus, supporting the concept of memory programming, or imprinting of cardinal memory properties during primary expansion (20–23), several studies have now demonstrated that the balance of MPECs and SLECs can be altered by manipulating the duration of antigen, IL-2 and other inflammatory cytokine signals (14–16, 24). In fact, as discussed later, the heterogeneity of the memory CD8 T cell pool is likely programmed by differential signals accrued during the primary expansion phase. IL-2 signals (paracrine or autocrine) in particular exert crucial roles in effector and memory differentiation and function.

Regulation of Effector CD8 T Cell Responses by IL-2

Optimal T cell activation with cognate peptide-MHC-I and costimulatory ligands result in IL-2 production and induction of IL-2Rα (CD25) expression, which along with IL-2Rβ (CD122, also used for IL-15 signaling), and IL-2Rγ (CD132, also referred to as common γ-chain as it is shared by other cytokines of the γ-chain family such as IL-4, 7, 9, 21) (5), forms the high affinity heterotrimeric receptor for robust IL-2 signal transduction and clonal expansion and effector differentiation (2). Much of the early work on IL-2 regulation of T cell responses relied on reductionist *in vitro* studies where amount and duration of TCR and IL-2 stimulation can be tightly controlled. These studies established a critical role for IL-2 as a T cell growth factor in driving cell cycle progression and expansion of CD8 T cells following TCR stimulation (25). Similar conclusions were reached following *in vivo* administration of IL-2, which engendered enhanced effector and memory pools of antigen-specific CD8 T cells (26–29). While these studies demonstrate that CD8 T cell differentiation events are amenable to manipulation by IL-2, physiological relevance of IL-2 in shaping a developing CD8 T cell response was uncovered following the development of *Il2* germline-deleted mice. Studies in IL-2 knockout mice are confounded by Treg deficiency and associated spontaneous lymphoproliferative disease (30, 31). Hence, irreconcilably disparate outcomes of reduced or unaltered expansion and effector differentiation were reported in the context of infections and peptide immunization in IL-2 knockout mice (32–35). Nonetheless, bypassing pleiotropic immune effects in straight IL-2 and IL-2Rα (CD25) knockout mice, subsequent studies engaged the strategy of adoptively transferring IL-2- or IL-2Rα-deficient TCR transgenic CD8 T cells into wild-type recipients. In these studies, enumeration of antigen-specific CD8 T cells in an otherwise wild-type milieu using congenic differences without the need for restimulation, clearly established a requirement for IL-2 signals in driving optimal primary expansion of antigen-specific CD8 T cells in secondary lymphoid as well as non-lymphoid tissues (36, 37). IL-2 promotes effector differentiation through STAT-5-mediated Blimp-1-dependent induction of effector molecules (16, 38–42). In this regard, proinflammatory cytokine signals such as IL-12, IFN-γ, and type-1 interferons (IFN-α/β)—commonly referred to as signal 3 for their role in promoting optimal clonal expansion of effector CD8 T cells—are believed to complement IL-2, possibly non-redundantly (43, 44). Such collaboration, particularly between IL-12 and IL-2 has been recently shown to be important for optimal expression of transcription factors T-bet and Blimp-1, which synergize to drive a terminal effector differentiation program in CD8 T cells (45).

Regulation of Memory CD8 T Cell Responses by IL-2

In addition to promoting CD8 T cell expansion and effector differentiation, IL-2 signals are also necessary for memory responses. IL-2Rα upregulation early after TCR stimulation is critical for formation of memory cells with robust secondary expansion capability (46, 47). Subsequent correlations of the duration of IL-2Rα expression with final memory outcome in a physiologically relevant setting—where the natural course of CD8 T cell response was not disturbed—revealed that rapid downregulation of IL-2Rα is equally important for memory development (16). Fate-tracking analyses showed that following an initial burst of IL-2 signals through IL-2Rα, curtailed expression of IL-2Rα and diminished IL-2 signaling is associated with memory fate, whereas prolonged expression of IL-2Rα and stronger IL-2 signaling drives terminal effector differentiation (16). Stronger IL-2 stimulation (100 U/ml) during *in vitro* priming also drives terminal differentiation compared to weaker signals (10 U/ml) (41). Similar findings have been reported in the DC-peptide immunization models as well as during murine infection with Lymphocytic choriomeningitis virus (LCMV), Listeria monocytogenes (LM), Vaccinia virus (VV), and Vesicular stomatitis virus (VSV) (16, 48). Moreover, constitutive activation of STAT-5 (key signal transducer of common γ-chain cytokines) also causes terminal differentiation (49). Consistent with the pro-proliferative role of IL-2, terminally differentiated effector CD8 T cells (SLECs) that express IL-2Rα for longer duration during an acute infection expand more than their memory-fated counterparts (MPECs) that downregulate the expression of IL-2Rα earlier (15, 16, 50–52). Together, these findings support the notion that metered IL-2 signals are required for optimal

protective immunity and present a model of rheostatic control of CD8 T cell fates by IL-2 during acute infections.

All memory cells that survive after clearance of a primary infection are not created equal. Protective CD8 T cell immunity, as we understand it today, consists of collaborative defense against secondary challenge through concerted actions by a complex mixture of memory cells with distinct phenotypes, location, migratory properties, polyfunctionality, antigen-independent longevity, and potential for mounting rapid and robust clonal expansion and effector functions upon secondary challenge (44). As is expected from a spectrum of effector CTLs—that develop in response to varying doses and durations of antigen perceived in a variety of immune contexts, such as dose and duration of cytokines (e.g., IL-2, IFN-I, IL-12, IL-21, TGFβ, etc.), costimulatory signals, CD4 T cell interactions—a veritable spectrum of memory cells exist in a host after antigen clearance. At the risk of oversimplifying the CD8 T cell memory complexity, one can arguably categorize memory cells broadly into two major subsets—lymphoid or central memory (T_{CM}), and non-lymphoid memory, which is further distinguished into tissue-resident memory (T_{RM}), and migratory memory. Defined by their location, central memory cells largely recirculate through secondary lymphoid organs; tissue-resident memory (T_{RM}) cells—true to their name—set up permanent residence at front-lines of pathogen exposure; whereas migratory memory cells comprise a heterogeneous population that is capable of recirculation to peripheral tissues, and may be further distinguished by intravascular staining methodology into the CX3CR1^{hi} effector memory subset (T_{EM}) which does not enter extravascular space, and the less differentiated CX3CR1^{int} memory subset capable of migration into extravascular spaces (53, 54). T_{RM} cells serve effectively as the first line of defense against infections by virtue of their key properties of location at barrier sites and rapid elaboration of effector functions (cytotoxicity against infected target cells and effector cytokine production). Consistent with their ability to recirculate through peripheral tissues, T_{EM} cells retain higher expression of effector molecules, and are believed to aid T_{RM} cells in protecting against secondary challenge along with the extravascular migratory memory cells. In contrast, T_{CM} cells largely downregulate their effector program after antigen clearance, but are capable of rapid upregulation of the effector program upon antigenic rechallenge, also have superior polyfunctionality (ability to coproduce multiple cytokines such as IL-2, IFN-γ, and TNF-α), and expand more vigorously to aid the T_{RM} and migratory cells during secondary challenge.

Developmentally, fate-tracking experiments show that effector CD8 T cells that rapidly downregulate IL-2Rα largely give rise to central memory and effector memory cells. In comparison, effector CTLs, with prolonged IL-2Rα expression, largely give rise to terminal effector and effector memory fates; and curtailed stimulation of these cells by adoptive transfer into infection-controlled recipients (removal of antigen, IL-2, and all other infection-related signals) results in less terminal differentiation, as evidenced by increased proportions of effector memory cells compared to short-lived effector cells. These observations are consistent with a role for increasing IL-2 in driving effector CD8 T cells progressively toward terminal differentiation. It is believed that T_{RM} cells arise from relatively less differentiated memory precursors, which first seed the peripheral sites such as skin and small intestines (55, 56). *In situ,* the precursors receive microenvironment-specific developmental cues that drive the expression of unique chemokine receptors, integrins, and transcription factors for T_{RM} cell tissue residency and local protection (56–61). Within the tissue, the transforming growth factor β (TGF-β) exerts a critical role in directing the T_{RM} differentiation program in concert with other tissue-specific signals (55, 56, 62, 63). While CD8 T_{RM} cells capable of IL-2 production have been recently reported in skin and liver (64, 65), and IL-2 signals have been shown to be important for maintenance of allergic T_{H2}-type cells in the lungs (66), murine studies directed at understanding whether early IL-2 signals are necessary for T_{RM} seeding of tissue sites, whether prolonged IL-2 signals compromise T_{RM} cells, and how TGF-β signals and other tissue-specific factors work in conjunction with IL-2 signals (synergistically or antagonistically) to drive the differentiation, maintenance, and recall function of T_{RM} cells within the local sites remains to be fully explored. Likewise, whether similar rules of progressive terminal differentiation with increasing IL-2 signals are also active in situations of chronic antigen stimulation—as occurs during persistent viral infections and cancers—remains to be defined.

AUTOCRINE AND PARACRINE PROGRAMMING OF T CELL FATES

During thymic development, T cell-derived IL-2 is critical for development of Treg cells. (67). During homeostasis, IL-2 is largely produced by CD25^{int} and CD25^{lo} CD4 T cells (activated by self-peptide and foreign peptide MHC-II complexes on DCs) (68), the regulatory T_{R1} subset in peyer's patches that also produces IL-10 and IFN-γ (69), and to some extent by NK, NKT, and CD8 T cells [evaluated by mRNA (68)]. Recent studies, involving IL-2 ablation in defined immune cells, have shown that T cell-derived IL-2 is critical for maintaining numbers and regulatory function of Treg cells in most secondary lymphoid organs, with the exception of mesenteric lymph nodes where DC-derived IL-2 was also observed to be important (67). During an immune response, activated CD4 T cells produce copious amounts of IL-2 (2), with other IL-2 producers being CD8 T cells (70), DCs (71), NKT cells (72), and mast cells (73). There is evidence that IL-2 may be transpresented by CD25 expressing DCs (74) to deliver high affinity IL-2 signals to CD8 T cells that lack CD25 expression and only express the intermediate affinity β/γ IL-2 receptor heterodimer—analogously to IL-15 transpresentation—thus, suggesting that IL-2 may be delivered in a context-specific manner *in vivo* depending on the nature and activation status of antigen-presenting DCs.

With highest levels in secondary lymphoid organs, IL-2 is believed to act in an autocrine or paracrine manner to support effector and memory CD8 T cell differentiation. We have previously shown that memory-fated effector CD8 T cells selectively retain the ability for robust IL-2 production in

response to antigenic stimulation compared to their terminally differentiated effector counterparts (8, 15, 16, 44). Likewise, polyfunctionality—the capacity for potent IL-2 production along with other effector cytokines such as IFN-γ and TNF-α in response to antigenic restimulation –is a hallmark property of lymphoid central memory CD8 T cells. Querying the functional relevance of autocrine IL-2 production by memory-fated CD8 T cells, studies involving ablation of *Il2* in a fraction of antigen-specific CD8 T cells during attenuated LM immunization (75) as well as acute LCMV infection (unpublished observations), demonstrate that the IL-2 needed for development of robust memory CD8 T cells capable of optimal secondary expansion is largely autochthonous. Since CD4 T cells are the major producers of IL-2, it was long presumed that IL-2 serves as the mode of CD4 help for development of protective memory CD8 T cells capable of robust secondary expansion. Thus, largely dismissing CD4 T cell-derived paracrine IL-2 as a mode of help, it is now proposed that CD4 T cells license DCs through the CD40-CD40L axis to induce memory-fated CD8 T cells to produce IL-2 (75). Autocrine IL-2 production through CD27 signals has also been shown to sustain survival of antigen-specific CD8 T cells in virus-infected non-lymphoid tissues (76, 77).

We further employed novel conditional IL-2 gene-deleted mice (78) to investigate whether autocrine IL-2 signals are specifically required during the programming phase of primary responses, or during secondary expansion (unpublished observations). Ablation of *Il2* in memory CD8 T cell immediately prior to rechallenge did not result in compromised secondary expansion, but ablation prior to primary infection resulted in defective recall responses. These data suggest that autocrine IL-2 signals during primary CD8 T cell expansion are required to institute a program of optimal secondary expansion. In the context of CD4 help to CD8 T cells, these instructive autocrine IL-2 signals are believed to in part promote the expression of a transcriptional corepressor, Nab2 for blocking TRAIL-mediated apoptosis during secondary expansion (79, 80). Defects in protective CD8 T cell immunity associated with IL-2Rα ablation are rescued by a strong bolus of exogenous IL-2 during primary expansion (47), further supporting the idea that IL-2 exerts an early instructive role. Whether secondary expansion defect associated with lack of autochthonous IL-2 maybe similarly rescued by excessive paracrine IL-2 signals remains unknown. Alternatively, it is possible that there are fundamental differences (quantitative and/or qualitative) between autocrine and paracrine IL-2 signals. In the case of CD4 T cells, autocrine IL-2 production in response to cognate antigen and CD70 signals during late stages of influenza A virus infection has been shown to be critical for upregulation of IL-7Rα (CD127) and survival into memory phase (81). More recently, T_{FH} and T_{H1} fates have been linked to autocrine and paracrine IL-2 signals, respectively, with different gene expression programs being triggered for lineage determination in IL-2-producing and non-producing CD4 T cells (82). While CD8 T cells that receive differential strength or duration of IL-2 signals have expectedly unique gene expression programs, it remains to be defined how autocrine and paracrine IL-2 signals impact CD8 T cell gene regulation and metabolism.

FINE-TUNING THE REGULATORS OF T CELL FATES

Transcriptional and Metabolic Regulation of CD8 T Cell Differentiation

IL-2 couples T cell expansion and effector differentiation through induction of multiple downstream signaling cascades. Expression of pro-differentiation transcription factors, Blimp-1 (16, 38, 40–42) and Id-2 (83), is largely mediated through STAT-5 activation in response to IL-2 stimulation (2) (**Figure 1**). Reciprocal suppression by IL-2 of transcriptional factors that promote T cell memory such as Bcl-6 (41, 84–88) (which also represses Blimp-1 expression) is believed to further fix the terminal effector differentiation program (45). IL-2 is believed to regulate the expression of Bcl-6 through activation of Akt, which serves to control the activity of Foxo family transcription factors (89), Activation of Akt also alters the expression of proteins involved in CD8 T cell trafficking such as CD62L, CCR7, and S1P1, so as to promote their migration to peripheral sites of infection and inflammation (90–92). In addition to activation of STAT-5 and Akt, which largely promote effector differentiation, IL-2 links effector differentiation with clonal expansion through activation of MAPK signaling and T cell activation, cell cycle progression and survival programs (89). Sustained expression of cMYC through IL-2 drives proliferation by upregulating cyclins and anti-apoptotic molecule B-cell lymphoma 2 (Bcl-2), and by downregulating p21 (93, 94). In addition to cell cycle regulators, Myc also controls key metabolic aspects of T cell activation and proliferation (95). Myc promotes glycolysis and glutaminolysis through upregulation of key enzymatic and transporter proteins (96, 97). In this regard, mTOR also serves as a primary hub to integrate environmental cues from growth factors such as nutrients and IL-2 to promote glycolysis (94, 98, 99), oxidative phosphorylation and anabolic processes such as protein, lipid, and nucleotide biosynthesis necessary to sustain proliferation (96, 97). How effector and memory CD8 T cell fates are defined *in vivo* through differential metabolic programming by varying IL-2 strength or duration remains to be elucidated.

A Model for Rheostatic Control of T Cell Fates by IL-2

With diverse outcomes of memory and terminal effector differentiation in CD8 T cells that receive short/weak as opposed to prolonged/strong IL-2 signals (as described earlier), it remains to be determined whether rheostatic control of transcriptional and metabolic regulation occurs. It is plausible that curtailed or weak IL-2 signals drive lower levels of STAT-5, Akt and mTOR activity, thus resulting in lesser proliferation, effector differentiation and trafficking to peripheral sites of infection. In contrast, strong and prolonged IL-2 signals may drive stronger STAT-5, Akt, mTOR and MAPK activity, thus leading to augmented proliferation, effector differentiation and migration to peripheral sites of infection, where the microenvironmental niches further reinforce the terminal differentiation programs through induction of receptors for inflammatory cytokines such as IL-12 (100), and inhibition of IL-7 (101) receptor levels.

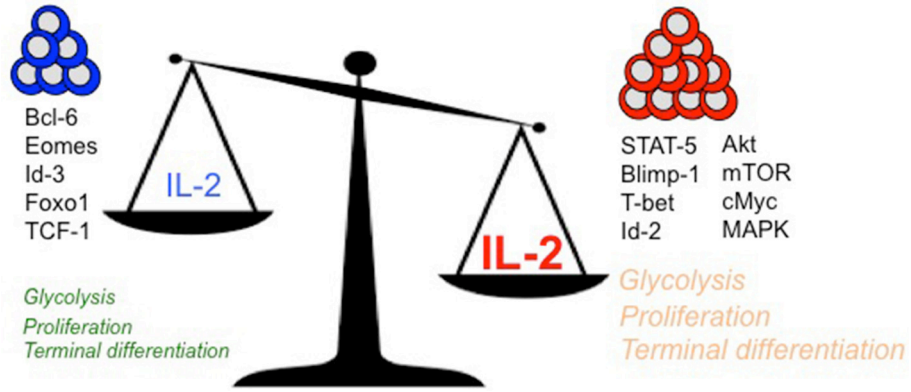

FIGURE 1 | Regulation of key intracellular signaling, transcriptional, and metabolic mediators of terminal effector vs. long-lived polyfunctional memory CD8 T cell fates are presented in the context of differential levels of IL-2 signals.

Notably, a role for Tregs has been implicated in regulating the amount of IL-2 signals to memory-fated CD8 T cells by acting as IL-2 sinks (102) during CD8 T cell expansion. During later stages in the absence of antigen (when IL-2 is limiting) also, Tregs continue to curtail T cell stimulation and proliferation to maintain memory CD8 T cell quiescence through CTLA-4 (103) and IL-10 (104) inhibitory mechanisms and possibly through IL-2 restriction (105). IL-2 is also bound to the extracellular matrix through heparan sulfate moieties (106) to presumably increase local concentrations, thus supporting the notion that strong and prolonged IL-2 signals can be achieved *in vivo*. Effectually, quantal differences in IL-2 signals may lead to differences in signaling thresholds that ultimately result in terminal effector gene expression patterns driven by Blimp-1, T-bet, Id-2, and cMyc, or in memory lineage gene expression patterns characterized by augmented Bcl-6, Eomesodermin and Id-3. Indeed, analogous rheostatic control of CD4 T cell fates by differential levels of IL-2 signaling has been reported in the balance of T_{H1} and T_{FH} fate determination (107, 108) through reciprocal regulation of T-bet and Bcl-6 by mTORC1-dependent control of the glycolysis gene expression program (109).

CONCLUDING REMARKS

Tightly coupled to antigen and costimulation, IL-2 signals follow close suit in T cell activation. In addition to driving expansion and effector differentiation, IL-2 regulates long-term memory outcome as well. Hence, it has been proposed as vaccine adjuvant (110) to augment the size of the memory pool. However, given its rheostatic regulation of terminal effector and memory fates (**Figure 1**), careful investigation into the dose and duration of IL-2 in a context specific manner is warranted to fine-tune the balance of terminal effector and memory lineages. Hence, based on the clinical need, timely and curtailed IL-2 signals might be exploited to augment memory outcome during vaccination. Alternatively—owing to its ability to induce proliferation and effector differentiation–strong and sustained IL-2 signals might be employed for immunotherapeutic interventions against cancers and chronic infections that rely on activation of a large pool of antigen-specific CD8 T cells. In this quest, IL-2 has gained particular recognition in treating melanomas and renal cell carcinomas (111) by augmenting the tumor-reactive CD8 T cell pool. In the case of gene-modified T cell immunotherapies also—for e.g., when patient T cells are bioengineered to express chimeric antigen receptors or TCRs directed against select tumor antigens–IL-2 is critical for expansion of CAR T cells to sufficient numbers for therapeutic benefit (112). Even in the case of PD-1 checkpoint blockade immunotherapy, IL-2 supplementation has offered combinatorial success with PD-1 blockade in boosting quantitative and functional aspects of exhausted CD8 T cells for enhanced viral control (113). Needless to say, the pleiotropic effects of IL-2 have posed significant hurdles such as off-target side effects of IL-2 administration—e.g., vascular leak syndrome due to activation of endothelial cells, or induction of immune regulation by Tregs. To minimize side effects, novel IL-2 muteins and immune complexes have been developed to selectively target IL-2 to either effector or regulatory T cells (5, 111, 114–116). By enhancing IL-2 binding to the β/γ heterodimer typically expressed on effector CD8 T cells, and thus directing IL-2 away from Tregs—which typically express high levels of IL-2Rα–these immune complexes and muteins provide a means to avoid concomitant induction of Treg suppression observed in case of rIL-2 administration that is counteractive to the desired outcome of effector differentiation. We envisage that detailed molecular dissection of the signal transduction and transcriptional networks downstream of IL-2 signaling *vis a vis* biological outcomes in individual immune cell-types will guide innovative immunomodulatory strategies designed for distinct clinical mandates. Along this concept, manipulations of the Bcl-6-Blimp-1 and CD27-CD70 axes are being considered with the goal of uncoupling effector differentiation effects of IL-2 from expansion effects (89). Beyond the binary terminal effector or memory outcomes conceived thus far, it is enticing to speculate whether rheostatic regulation of MPEC and SLEC differentiation states by controlling IL-2 signals might be exploited to balance the immediate therapeutic benefits and long-term protective outcomes during adoptive T cell therapy and therapeutic cancer vaccines.

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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Identification of a Consolidation Phase in Immunological Memory

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Long lasting antibody responses and immunological memory are the desired outcomes of vaccination. In general, multiple vaccine doses result in enhanced immune responses, a notable exception being booster-induced hyporesponsiveness, which has been observed with polysaccharide and glycoconjugate vaccines. In this study, we analyzed the effect of early booster doses of multimeric protein vaccine (1-11)E2 on recall memory to B epitope 1-11 of β-amyloid. Mice immunized with a single dose of (1-11)E2 stochastically display, when immunized with a recall dose 9 months later, either memory, i.e., an enhanced response to epitope 1-11, or hyporesponsiveness, i.e., a reduced response. Memory is the most common outcome, achieved by 80% of mice. We observed that a booster dose of vaccine (1-11)E2 at day 15 significantly reduced the ratio between the magnitude of the secondary and primary response, causing an increase of hyporesponsive mice. This booster-dependent disruption of recall memory only occurred in a limited time window: a booster dose at day 21 had no significant effect on the ratio between the secondary and primary response magnitude. Thus, this study identifies a consolidation phase in immunological memory, that is a time window during which the formation of memory is vulnerable, and a disrupting stimulus reduces the probability that memory is achieved.

Keywords: vaccine, boost, antibody, primary response, secondary response

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INTRODUCTION

Vaccination affords immunity from diseases by inducing immunological memory and long-lived antibody responses (1, 2). The identification of switches that regulate immunity is central to efforts of rational vaccine design (3, 4).

Immunological memory, i.e., the ability to mount an enhanced response to an antigen that has been previously encountered, is a system-level property of the immune system, that arises from an increase in the frequency of antigen-specific B and T lymphocytes as well as from the differentiation of antigen-experienced lymphocytes into qualitatively different cell populations, namely memory cells, which display faster response to antigen re-exposure and the ability to self-renew (5–7). The half-life of the antibody titer, which is a critical issue in vaccine development as it is linked to the duration of protection, displays considerable variation among different vaccines currently in use. In humans, a longitudinal study of the antibody titer to common viral and vaccine antigens found that antibody responses against tetanus and diphtheria antigens waned more quickly, with estimated half-lives of 11 and 19 years, respectively, whereas antiviral antibody responses were remarkably stable, with estimated half-lives ranging from 50 years for varicella-zoster virus to more than 200 years for other viruses such as measles and mumps (8). The antibody titer in the circulation reflects

the size of the antibody-secreting cells (ASC) pool, that includes different populations of ASC, that differ in their proliferative potential, life-span, and that are prominent in different temporal phases of the immune response, namely plasmablasts, short-lived plasma cells, and long-lived plasma cells (9). Of these, long-lived plasma cells ensure the long-term persistence of antibodies (10). Thus, the duration of antibody responses is related to the number and longevity of long-lived plasma cells. Survival vs. death of plasma cells is one of the key decisions that guide antibody production; understanding the control system of this decision, not only is potentially valuable for vaccine development, but also for treating disorders of antibody production in autoimmunity, allergy, and immunodeficiency (11).

The control system governing the quality and quantity of circulating antibody, far from being a single binary switch, comprises a series of decision points where B cells integrate many inputs influencing their fate (11); a crucial role is played by the Germinal Center (GC) reaction, a highly complex process involving a cascade of several distinct, timed events that are topographically segregated (12). The role of asymmetric cell division and stochastic events in this coordinated process of cellular differentiation and selection is still unresolved. Measures of the time to develop into a plasmablast, and to divide or die for thousands of cells suggested that each fate is pursued autonomously and stochastically and that the allocation of a proportion of B cell to each fate is a phenomenon of stochastic competition (13).

In this study, we set out to investigate the effect of the time delay between the first and the second dose of vaccine on the antibody titer trajectory during the primary and secondary response. Antibody titer/time curves reflect the contribution of antibody secreting cells that reside in different organs, namely the lymph nodes, the spleen, and the bone marrow, which become prominent in different time windows. While it is not feasible to analyze over time the development of different ASC populations in a single individual, serum can be sampled multiple times; thus, we took the approach of analyzing a single experimental parameter, namely the IgG antibody titer against a specific B epitope, in 50 genetically identical, age, and sex-matched mice over 1 year post vaccination. We monitored the primary response for 9 months, and then we administered a recall dose and monitored the secondary response for 3 months, sampling sera at 11 timepoints that we had previously identified as sufficient to capture the shape of the titer/time curve. We utilized as a model vaccine (1-11)E2, a multimeric protein designed to induce an antibody response against the β-amyloid peptide, a peptide involved in the pathogenesis of Alzheimer's Disease (14, 15). (1-11)E2 is an icosahedral protein nanoparticle, displaying 60 copies of peptide 1-11 of β-amyloid, at the N-terminus of self-assembling protein domain E2 (16). A single injection of (1-11)E2 induces recall memory to the displayed β-amyloid epitope in the majority of immunized subjects (16), making this multimeric protein a suitable antigen for the investigation of recall memory.

Mice immunized with a single dose of (1-11)E2 stochastically display, when immunized with a recall dose 9 months later, either memory, i.e., an enhanced response to epitope 1-11, or hyporesponsiveness, i.e., a reduced response. Memory is the most common outcome, achieved by 80% of mice.

When a booster dose of vaccine (1-11)E2 was administered at day 15, we observed a significant reduction of the ratio between the magnitude of the secondary and primary response, resulting in an increase of hyporesponsive mice. This booster-dependent disruption of recall memory only occurred in a limited time window: a booster dose at day 21 had no effect on the ratio between the secondary and primary response magnitude.

Hyporesponsiveness, defined as a lower antibody (Ab) level after the second immunization than after the first, has been observed after vaccination with polysaccharide or glycoconjugate vaccines (17). We report here, for the first time in our knowledge, that hyporesponsiveness also occurs in the case of a multimeric protein antigen and can be induced by a booster dose administered in a specific time window.

Thus, this study identifies a consolidation phase in immunological memory, that is a time window during which the formation of memory is vulnerable, and a disrupting stimulus reduces the probability that memory is achieved.

RESULTS

A Booster Dose Given 15 Days After Priming Impairs Immunological Memory to a B Cell Epitope

In this study, we set out to investigate the effect of the timing of a booster dose on immunological memory to a B cell epitope. Our model epitope is Aβ(1-11), consisting of the 11 amino acid N-terminal immunodominant B epitope of β-amyloid. Immunization against Aβ(1-11) was performed with antigen (1-11)E2, a recombinant protein comprising epitope 1-11 of β-amyloid and the E2 domain of the pyruvate dehydrogenase of *Bacillus stearothermophilus,* that self-assembles into a multimeric structure that includes 60 monomers (14).

For this study, we define memory as the ability to display an enhanced response to an antigen that has been previously encountered. In particular, in this study, the feature of the immune response that we analyze is the IgG antibody titer.

We monitored for 1 year post-immunization the IgG antibody titer against Aβ(1-11), in 50 mice undergoing a primary and a secondary response. The experimental setup and the definition of primary and secondary response are schematized in **Figure 1**. Mice were randomly allocated to four immunization schedules. The control group received only a single dose (SD), while treatment groups D7B, D15B, and D21B also received a booster dose, respectively, at day 7, 15, or 21 after the first dose. All mice received a recall dose 9 months after the first dose and were then monitored for 3 more months (**Figure 1**).

In order to establish whether the different treatment groups had developed immunological memory to Aβ(1-11), defined as the ability to display an enhanced recall response, we compared, within each group, the magnitude of the peak of the primary and secondary antibody response to Aβ(1-11) (**Figure 2A**). While the mice that received a single dose of vaccine and the group that received a booster dose at day 21 displayed a significantly

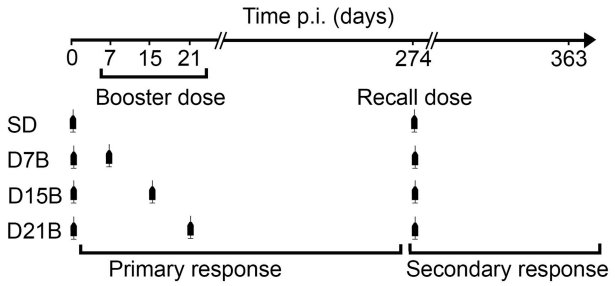

FIGURE 1 | Experimental design. 50 BalbC mice were randomly allocated to four immunization schedules. The control group received only a single dose (SD) of vaccine (1-11)E2, while treatment groups D7B, D15B, and D21B also received a booster dose, respectively, at day 7, 15, or 21 after the first dose. All mice received a recall dose 9 months after the first dose and were then monitored for 3 more months. The IgG antibody titer against Aβ(1-11) was monitored for 1 year post-immunization. The definition of primary and secondary response is shown: the primary response is defined as the response initiated by the first dose of vaccine (days 0–274 post immunization), the secondary response is defined as the response initiated by the recall dose (days 274–363 post immunization).

enhanced peak response to the recall dose, we observed no statistically significant difference in magnitude between the peaks of the primary and secondary total IgG response in the groups that received the booster dose at day 7 or 15. Thus, a booster dose administered within 15 days of the first dose abrogated immunological memory, defined as the ability to display an enhanced recall response.

Indeed, the effect of the booster dose given at day 7 on recall memory appeared less severe than the effect of the booster dose given at day 15. As shown in **Figure 2A**, both in the D7B and in the D15B group there is no statistically significant difference between the secondary and primary response peak, however the geometric mean titer of the secondary response displays a trend toward higher values in the D7B group (**Figure 2A**).

Among mice of the same treatment group, we observed a broad spread of the anti-Aβ(1-11) IgG titers at the peak of the secondary response (**Figure 2B**). In order to analyze the diversity of the fate of the immune response between individuals, we classified individual titer/time trajectories with respect to the ratio between the peak of the secondary response and the peak of the primary response, so as to be able to recognize "immunological memory," defined as an enhanced secondary response, at the level of the individual.

The ratio between the peak of the secondary response and the peak of the primary response ranged from 0.1 to over 100 (**Figure 2C**). We defined "memory" a secondary response 2-fold higher than the primary response, that is a ratio of the antibody titer of the secondary peak to the primary peak above 2, "equal response" a ratio comprised between 2 and 0.5, and "hyporesponsiveness" a ratio lower than 0.5.

All treatment groups included some mice that had developed memory to Aβ(1-11), albeit at different frequencies (**Figures 2C,D**). The ratio between the secondary and the primary peak was significantly lower in the D15B group, compared to the SD group and the D21B group (**Figure 2C**). The number of mice that displayed a memory response to Aβ(1-11) was minimal in the D15B group (**Figure 2D**).

In the analysis of the ratio between the secondary peak and the primary peak in individual mice (**Figure 2C**), while the D15B group is statistically different from the SD group ($p = 0.008$), the difference between the D7B group and the SD group is not statistically significant. In the classification of individual recall responses shown in **Figure 2D**, the D7B group appears intermediate between the SD group and the D15B; in the D7B group the percentages of mice displaying memory was lower than in the single dose group, but higher than in the D15B group (**Figure 2D**), whereas conversely in the D7B group the percentage of mice displaying hyporesponsiveness was higher than in the SD group but lower that in the D15B group.

In summary, only when the booster dose is given at day 15 there is a statistically significant reduction in the ratio between the magnitude of the secondary and primary response.

Hyporesponsiveness Is Unrelated to the Primary Response and to Antibody Titer at Recall

The antibody titers from day 0 to 274, shown in **Figure 3**, demonstrate that, differently from the recall response, the primary response was not reduced in the mice that received booster doses, compared to the mice that received only a single dose (**Figure 3**).

Moreover, we asked whether the ability to exhibit an enhanced response to the recall dose was related to the antibody titer at the time of recall.

We observed no significant difference in the anti-Aβ(1-11) antibody titer at the time of recall between mice that displayed a memory response to Aβ(1-11) and mice that did not (**Figure 4A**). Mice immunized with (1-11)E2 develop an antibody response both to the Aβ(1-11) peptide and to the scaffold protein domain E2. The antibody titer against E2 at the time of recall also did not differ between mice with and without memory to Aβ(1-11) (**Figure 4B**). Thus, the different fates in individual responses to the recall dose were not related to differences in the titer of circulating antibodies against the immunizing antigen at the time of recall.

Recall Memory to the E2 Carrier Protein Is Impaired by a Day 15 Booster

We asked if the booster-related effects on recall memory were limited to the Aβ(1-11) B cell epitope or extended to other B epitopes of the immunizing antigen (1-11)E2. Thus, we analyzed the IgG antibody titer trajectories against the carrier moiety E2. In accordance to what we observed in the response to the β-amyloid epitope (1-11), also in the response to the E2 protein the ratio between the peak of the secondary and primary response is significantly reduced ($p = 0.02$) in the group that received a booster dose at day 15, compared to the single dose group (**Figure 5**).

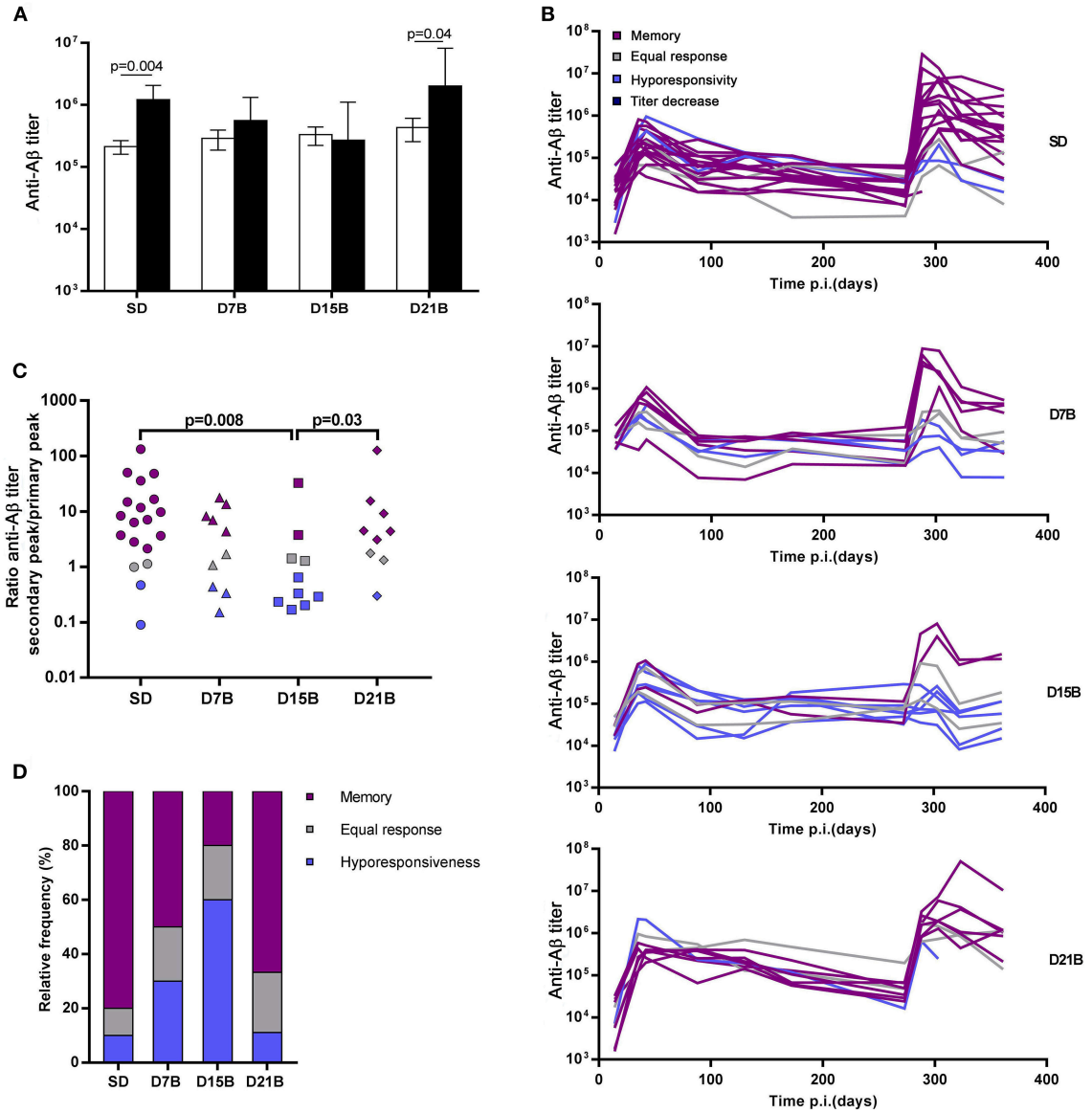

FIGURE 2 | Effect of booster doses on recall memory. **(A)** The histograms show the geometric mean titer of IgG against Aβ(1-11) at the peak of the primary response (open bars) and secondary response (black bars). Error bars represent the standard error of the mean (s.e.m.). Significant *P*-values calculated with the Wilcoxon rank sum test are shown. **(B)** The line graph shows the time course of the IgG titer against Aβ(1-11) in individual mice. Trajectories are color-coded based on the classification of response patterns as in **(B,C)**. **(C)** The dot plot shows the ratio between the peak titer of IgG against Aβ(1-11) in the secondary response and the primary response in individual mice. Each symbol represents one mouse. Significant *P*-values calculated with the Wilcoxon rank sum test are shown. **(D)** The histogram shows the relative frequencies of 3 patterns of response to recall, defined based on the ratio of the peak of the secondary response to the peak of the primary response as memory (ratio $>$ 2, violet), equal response (0.5 $\leq$ ratio $\leq$ 2, grey) hyporesponsiveness (ratio $<$ 0.5, blue).

Both in the single dose group and in the group that received the day 15 booster, the ratio between the secondary and primary response peak is highly correlated between the response to Aβ(1-11) and the response to E2 (Pearson correlation coefficient is 0.94 in the single dose group, and 0.99 in the D15B group).

This results demonstrate that the day 15 booster dose impaired recall memory not only to the Aβ(1-11) epitope, but also to other B epitopes of the E2 carrier.

DISCUSSION

The most notable finding in this study is that a booster dose of the multimeric protein antigen (1-11)E2, injected 15 days after the primary immunization, impaired the antibody response to a recall dose, administered 9 months later. In particular, the analysis of the trajectories of the antibody titer against B epitope Aβ(1-11) in individual mice revealed that a booster dose at day 15 resulted in fewer mice being subsequently able to exhibit

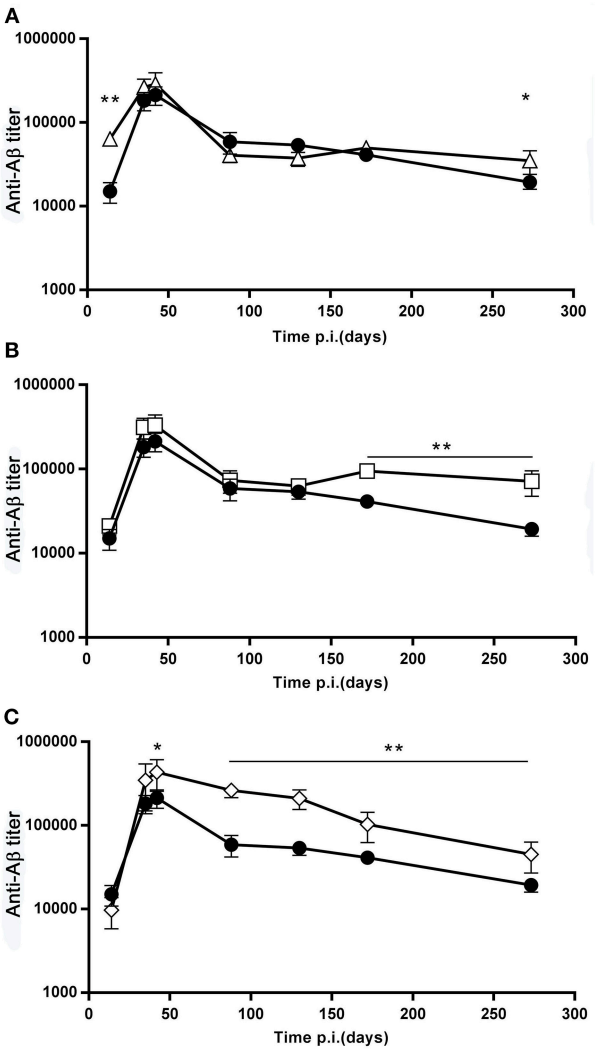

FIGURE 3 | Effect of booster doses on the primary response. **(A–C)** The graphs report the time course of the anti β-amyloid antibody titer, GMT ± SEM, of the SD group (full circles, $N = 20$), overlayed to the time course of the D7B group (open triangles, $N = 10$, **(A)**, the D15B group (open squares, $N = 10$, **(B)**, and the D21B group (open diamonds, $N = 10$, **(C)**. $^{*}p \leq 0.05$, $^{**}p \leq 0.02$.

an anamnestic response to the recall dose, and in some mice displaying hyporesponsiveness. While in the single dose group only 4/20 mice failed to mount an enhanced secondary response, in the day 15-boost group this happened in 8/10 mice. On the other hand, a booster dose given at day 21 after the primary immunization did not affect the fold ratio between the secondary and primary response.

It is possible to speculate that in our experiment the booster dose interfered with a different stage of the GC reaction, depending on its precise timing. Pre-existing GC can be populated by new B cell clones following a booster immunization (18, 19). It has been suggested that B cells that acquire antigen can enter GCs at all stages of the response, and that antigen is one of the main limiting factors (18). The GC response undergoes a temporal switch in its output; memory B cells and long-lived plasma cells are produced at separate points in time (20). In particular, unswitched memory B cells are generated early in the response, followed by switched memory B cells, and finally by a delayed appearance of isotype-switched bone marrow long-lived plasma cells (20). We never observed, in prime-boosted mice, a reduced primary response compared to single dose mice, indicating that booster doses did not inhibit ASC development, as shown in **Figure 3**.

Neutralizing serum immunoglobulin can inhibit the secondary response and have differential effects on B cell populations that mediate early and late memory (21). In our experiment the antibody titers at the time of the recall dose were in the same range in mice that then demonstrated an enhanced secondary response (memory) and in those that did not; therefore we can rule out that circulating antibodies inhibited the secondary response.

From our experiment, it is not possible to establish whether an impaired development of memory cells or a dominant inhibitory mechanism caused the observed hyporesponsiveness.

Several studies have reported that booster doses of polysaccharide vaccines can induce unresponsiveness. Unconjugated meningococcal polysaccharide vaccination induces antibody hyporesponsiveness, that impairs antibody responses to subsequent injections of meningococcal polysaccharide (MPS) or meningococcal conjugate vaccines. Administering MPS as a probe to assess conjugate vaccine-induced immunologic memory also can extinguish subsequent memory anticapsular antibody responses, whereas conjugate vaccination regenerates memory B cells (22). A mechanism that has been proposed for the hyporesponsiveness caused by polysaccharide antigens is that the polysaccharide, a T independent antigen, may stimulate the existing pool of memory B cells to differentiate into plasma cells and secrete antibody without replenishment of the memory B cell pool (22). A study on the effect of 1, 2, or 3 boosters of pneumococcal polysaccharide with 16 day intervals, in mice primed with a pneumococcal conjugate concluded that booster-induced hyporesponsiveness is caused by abrogation of conjugate-induced GC reaction and depletion of polysaccharide-specific Antibody-secreting cells, resulting in no homing of new specific long-lived plasma cells to the bone marrow (23). At difference with our study, the pneumococcal polysaccharide booster reduced the antibody titer of boosted mice, compared to the PBS control; instead, we did not observe a titer reduction. A difference in the study design is the age of the mice at the time of priming. The study on the effect of pneumococcal polysaccharide was performed on neonatal, 7 days old mice, whereas our study was performed on adult, 8 weeks old mice.

For human vaccines currently in use, the minimum interval to next dose recommended by the Advisory Committee on Immunization Practices is between 4 weeks and 5 years. The day 15 boost has been widely utilized to vaccinate mice against β-amyloid with β-amyloid 1–42 (24) recombinant bacteriophages (25) and recombinant proteins (14). Agent-based simulations of the response to our model vaccine predicted that a booster

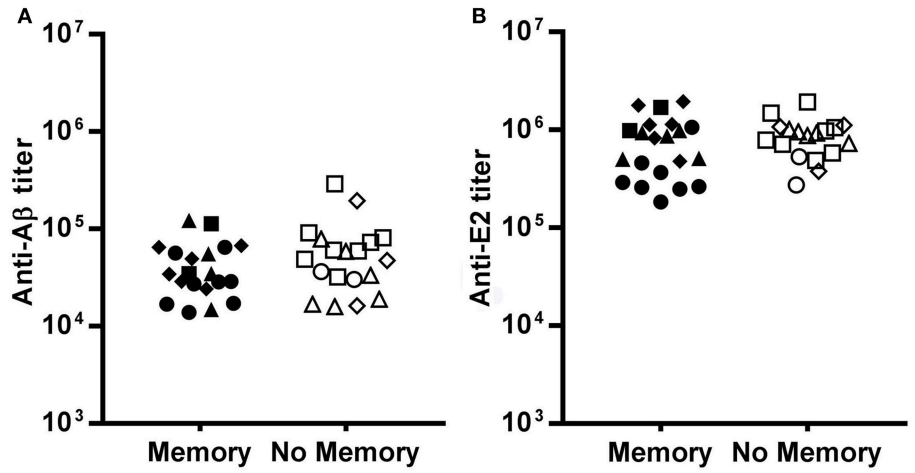

FIGURE 4 | Pre-existing serum titers at recall. The dot plots show the IgG titer against Aβ **(A)** and E2 **(B)** at day 273, the day before the recall dose, in mice that displayed memory or no memory against Aβ. Each dot represents a mouse of the SD group (circles), D7B group (triangles), D15B group (squares), D21B group (diamonds). There is no statistically significant difference between memory and no memory mice as regards the antibody titer against Aβ and E2.

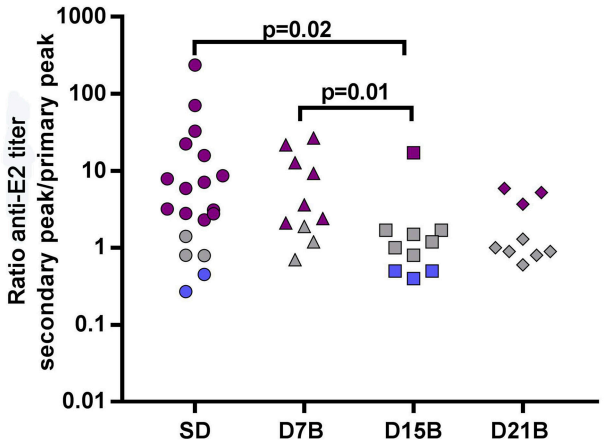

FIGURE 5 | Effect of booster doses on recall memory to E2. The dot plot shows the ratio between the peak titer of IgG against E2 in the secondary response and the primary response in individual mice. Each symbol represents one mouse. The same color code of **Figure 2** is used: violet (memory), grey (equal response), blue (hyporesponsiveness). Significant *P*-values calculated with the Wilcoxon rank sum test are shown.

dose would be inefficient if given earlier that a few months after the first dose (26), however, the study did not investigate booster-induced unresponsiveness to recall.

The results of this study show that there is a consolidation phase in immunological memory to the Aβ(1-11) epitope; there is a time window, after immunization with the vaccine (1-11)E2, during which the fate of the secondary response to the Aβ(1-11) epitope is vulnerable, and a disrupting stimulus reduces the probability that memory is achieved.

Interestingly, the results we obtained analyzing the antibody response to the β-amyloid epitope and the carrier epitopes were similar, in that a booster injection at day 15 caused a reduced probability of a subsequent enhanced secondary response to both the β-amyloid and the E2 carrier protein. In our classification of responses as memory, equal response and hyporesponsiveness, some mice fall into a different as regards the response to (1-11) and the response to E2. A possible explanation for this discordance lies in the fact that the E2 response reflects the cumulative behavior of more cells, and therefore more often falls into the intermediate pattern, i.e., "equal response." In fact, E2 is a larger antigen than Aβ(1-11), comprising 257 amino acids vs. 11 amino acids, and the response to E2 reaches a titer 7 times higher than the response to Aβ(1-11), indicating that more clones are involved in the response to E2 than in the response to Aβ(1-11).

A word of caution is needed regarding the generalization of the kinetics that we observed, as it is possible that different types of antigen, adjuvants, or injection routes, and different dose may be associated with differences in the kinetics of the response.

This study paves the way to investigating early correlates of immunological memory development, by analyzing the molecular and cellular effects of memory-disrupting stimuli.

MATERIALS AND METHODS

Mice

All experiments were performed on female BalbC mice. Mice were purchased from Charles River Laboratory, Italy. The first dose of vaccine was injected when the mice were 8 weeks old.

Model Vaccine

The vaccine (1-11)E2 is a multimeric protein. The monomer, that self-assembles into a 60-mer complex, consists of a fusion protein that includes the first 11 N-terminal residues of the β-amyloid peptide, DAEFRHDSGYE, and a bacterial protein domain, from the E2 subunit of the Acyl-transferase of *Bacillus stearothermophilus* (14, 15). The (1-11)E2 protein was produced in E. coli and purified and stored as previously described (14, 15).

Each vaccine dose consisted of 130 μg of (1-11)E2 protein (carrying 6 μg of the β-amyloid epitope 1-11) mixed with 100 μl of Freund's adjuvant, in a final volume of 200 μl. Complete Freund's adjuvant was used in the first injection, and incomplete Freund's adjuvant was used in subsequent shots. The vaccine was injected intraperitoneally.

Immunization and Bleeding Schedules

We have monitored, for a total of 12 months, the time course of the antibody response in 50 individual BalbC mice, undergoing 4 different dosing schedules. All dosing schedules included a first dose given when the mice were 2 months old, and a recall dose given 9 months after the first dose. Twenty mice only received these 2 doses, while other groups, of 10 mice each, also received a booster dose, respectively, 1, 2, or 3 weeks after the first dose.

Blood was collected from the tip of the tail, with heparinized microhematocrit capillaries, at the following time points after the first dose: day 14, 35, 42, 88, 130, 172, 273, 288, 302, 323, and 361. Blood was left at room temperature for 30 min, then centrifugated at 6,000 rpm for 30 min. The serum was divided into aliquots and stored at −80°C.

Antibody Titer Measures

The antibody titer was measured by ELISA assays, performed as previously described (15).

Each serum was tested against synthetic peptide 1-11 of β-amyloid. Synthetic peptide 23–29 of β-amyloid was used as a negative control. The titer of serum was defined as the dilution yielding an absorbance value equal to 2-fold the background value obtained against the negative control.

Statistical Analysis

The Wilcoxon rank sum test was performed to determine the statistical significance of observed differences.

ETHICS STATEMENT

This study was carried out in accordance with European Union Laws and guidelines (European Directive 2010/63/EU) and in accordance with the authorization 161/2015-PR released by the Italian Ministry of Health.

AUTHOR CONTRIBUTIONS

AP contributed to the conception and design of the study and wrote the manuscript. FM and AC performed the experiments. FM, AC, PDB and AP contributed to the data analysis, interpretation, and manuscript revision, and read and approved the submitted version.

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RNA and Toll-Like Receptor 7 License the Generation of Superior Secondary Plasma Cells at Multiple Levels in a B Cell Intrinsic Fashion

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Secondary plasma cells (PCs) originate from memory B cells and produce increased levels of antibodies with higher affinity compared to PCs generated during primary responses. Here we demonstrate that virus-like particles (VLPs) only induce secondary PCs in the presence of toll-like receptor (TLR) 7 and if they are loaded with RNA. Furthermore, adoptive transfer experiments demonstrate that RNA and TLR7 signaling are required for secondary PC generation, both at the level of memory B cell as well as PC differentiation. TLR7-signaling occurred in a B cell intrinsic manner as TLR7-deficient B cells in an otherwise TLR7-competent environment failed to differentiate into secondary PCs. Therefore, RNA inside VLPs is essential for the generation of memory B cells, which are competent to differentiate to secondary PCs and for the differentiation of secondary PCs themselves. While we have not tested all other TLR or non-TLR adjuvants with our VLPs, these data have obvious implications for vaccine design, as RNA packaged into VLPs is a simple way to enhance induction of memory B cells capable of generating secondary PCs.

Keywords: memory B cells, secondary plasma cells, virus-like particles, toll-like receptor 7, anti-viral immunity, adaptive immunity

INTRODUCTION

Antibodies are the critical effector molecules induced by prophylactic vaccination and are responsible for anti-viral and anti-bacterial protection. PCs are the principle cell type producing antibodies. A number of different antibody forming cells (AFCs) have been described. At an early stage of the primary immune response, short-lived AFCs derived from marginal zone or follicular B cells are found in extra-follicular foci in secondary lymphoid structures (1). A second wave of PCs is generated by the germinal center (GC) reaction of which some are also short-lived. However, a subset of GC derived PCs is long-lived and resides in secondary lymphoid organs as well as bone marrow (BM) for months and even years (2–4). It has been known for decades that memory B cells can differentiate to PCs after secondary antigen encounter (5, 6). We have recently described the particular phenotype of these PCs, which we coined secondary PCs, as they derive from memory B cells during secondary responses, in a VLP immunization model (7). In contrast to PCs induced

during primary responses, they produce increased levels of high affinity antibodies. Unexpectedly, secondary PCs are short-lived and disappear a few days after their induction (Krueger et al., under review[1]).

The Th cell dependence of PC induction varies with the type of B cell progenitor. B1 cells, which provide only 1% of splenic B cells and are usually found in the peritoneal and pleural cavity, are a major source of natural antibodies produced in a Th cell independent manner (8). Early extra-follicular PCs, which often produce IgM, may be induced in the absence of T cell help in many cases, in particular if Th cell independent antigens are used for immunization (9). In contrast, GC-derived PCs are often isotype-switched and their generation requires T cell help and CD40L (10, 11). As opposed to GC-derived primary PCs, secondary PCs derived from memory B cells can be induced in the absence of CD40L (12). Hence, secondary PCs provide an early wave of antibodies in a relatively Th cell independent fashion during secondary responses, in a way similar to the short-lived extra-follicular PCs induced during primary responses.

Most antibody responses are driven by follicular Th cells (13, 14). However, presence of TLR-ligands, such as RNA, may overcome the requirement of follicular Th cells and other Th cells may take over (15–21).

There is a large number of adjuvants that are able to induce strong and long-lived B cell and antibody responses (22). Even though TLR-ligands are potent enhancers of B cell responses (23, 24), there is not an absolute requirement for the presence of TLR-ligands in order to induce protective B cell responses. Nevertheless, TLR-ligands play an important role for the generation of antibody responses during natural infections and many natural or artificial TLR-ligands are in development for adjuvants formulation (25–28) often in combination with classical adjuvants such as Alum (29). Monophosphoryl lipid A (MPL), a synthetic TLR4-ligand, is part of marketed vaccines since decades (30–32) and CpGs, a synthetic ligand for TLR9, have recently been approved for use in combination with hepatitis B vaccine (33). Furthermore, natural TLR-ligands are components of many widely used vaccines; in particular RNA, which is part of almost all live and inactivated viral and bacterial vaccines (34, 35). Single stranded RNA (ssRNA) is recognized by TLR7/8 in the endosome and RNA-sensing molecules in the cytosol and enhances antibody responses in many ways. B cells recognize RNA associated with the antigen via TLR7/8 and respond with increased production of IgG and in particular with a shift to the IgG2a subclass (17, 36, 37), enhanced B cell proliferation and increased BCR hypermutation (38, 39). This mechanism is dependent on TLR7-signaling in B cells and independent of RNA sensing in DCs (17). Similar B cell intrinsic pathways have been described for TLR9 (36, 37) which drives antibody responses in an IRF5-dependent way (40) and promotes B cell survival (28). The IgA subclass is particularly interesting with respect to TLR7-signaling, as systemic IgA responses need TLR signaling in B cells, while mucosal IgA responses need TLR signaling in DCs (41, 42). In addition, it has recently been shown that IgG responses against gram-negative bacteria require RNA-sensing in DCs followed by activation of TRIF and further downstream the inflammasome pathway (35, 43). The requirement for B cell intrinsic TLR signaling varies with time and is more important early than late during the GC response (34, 39), a finding that is consistent with the fact that the early GC response is more important than the late response to drive long-lived antibody responses (44). Furthermore, recent work indicated a temporal switch in GC reactions, where memory B cells are shown to emerge early during the response, whereas long lived PCs are a late output of the GC (20).

We have previously shown that immunization with VLPs derived from the RNA bacteriophage Qβ elicits strong and sustained IgG antibody responses with a prominent role for packaged *E.coli* RNA in driving class switch to IgG2a and IgA antibodies (42, 45–47). During recall responses, MBCs rapidly and quantitatively differentiate into secondary PCs (7). Here we show that RNA and TLR7-signaling in B cells synergize for the regulation of the secondary PC response. Absence of RNA or TLR7-signaling resulted in complete failure to generate memory B cells competent of forming secondary PCs. Moreover, stimulation of memory B cells generated in the presence of RNA, also failed to result in secondary PC induction in the absence of TLR7-signaling during recall. Hence, generation of secondary PCs is regulated by RNA and TLR7-signaling at multiple levels.

MATERIALS AND METHODS

Study Design

The goal of this study was to further characterize secondary PCs, which were generated by MBCs after Ag challenge. To achieve this, adoptive transfers in allotypic mice (Ly5.1/Ly5.2, IgHa/IgHb, TLR7 KO/WT, and TLR7 KO BM chimeras/WT BM chimeras) were performed. This enabled us to study primary and secondary immune responses in the same animal. All mice were kept according to cantonal veterinary guidelines at the central animal facility (Department of Biomedical Research) of the University of Bern and controlled laboratory experiments were performed in accordance with ethical principles and guidelines of the Cantonal Veterinary Office Bern, Switzerland. Animals were randomly assigned to the different groups. MBCs were generated by VLP immunization of mice. The control naïve mice remained untreated. At the same time, B cells were isolated from memory and naive mice and transferred into recipients. Upon immunization with VLPs, serum samples, spleens, and BM were collected and subjected to ELISA, ELISPOT, and FCM analysis. The investigators who performed the experiments, assessed, analyzed, and quantified the results were not blinded and aware of which group a sample was taken from. Individual groups consisted of four mice. All experiments were performed in at least two independent biological replicates. For the ELISA and ELISPOT in **Figures 1D,E** and day 6 FCM experiment only one replicate was performed. Data were collected at previously determined time points. All data were included in the analysis.

[1]Krueger CC, Thoms F, Keller E, Vogel M, Bachmann MF. Virus-specific secondary plasma cells produce elevated levels of high-affinity antibodies but are short lived. *Front Immunol.* (under review).

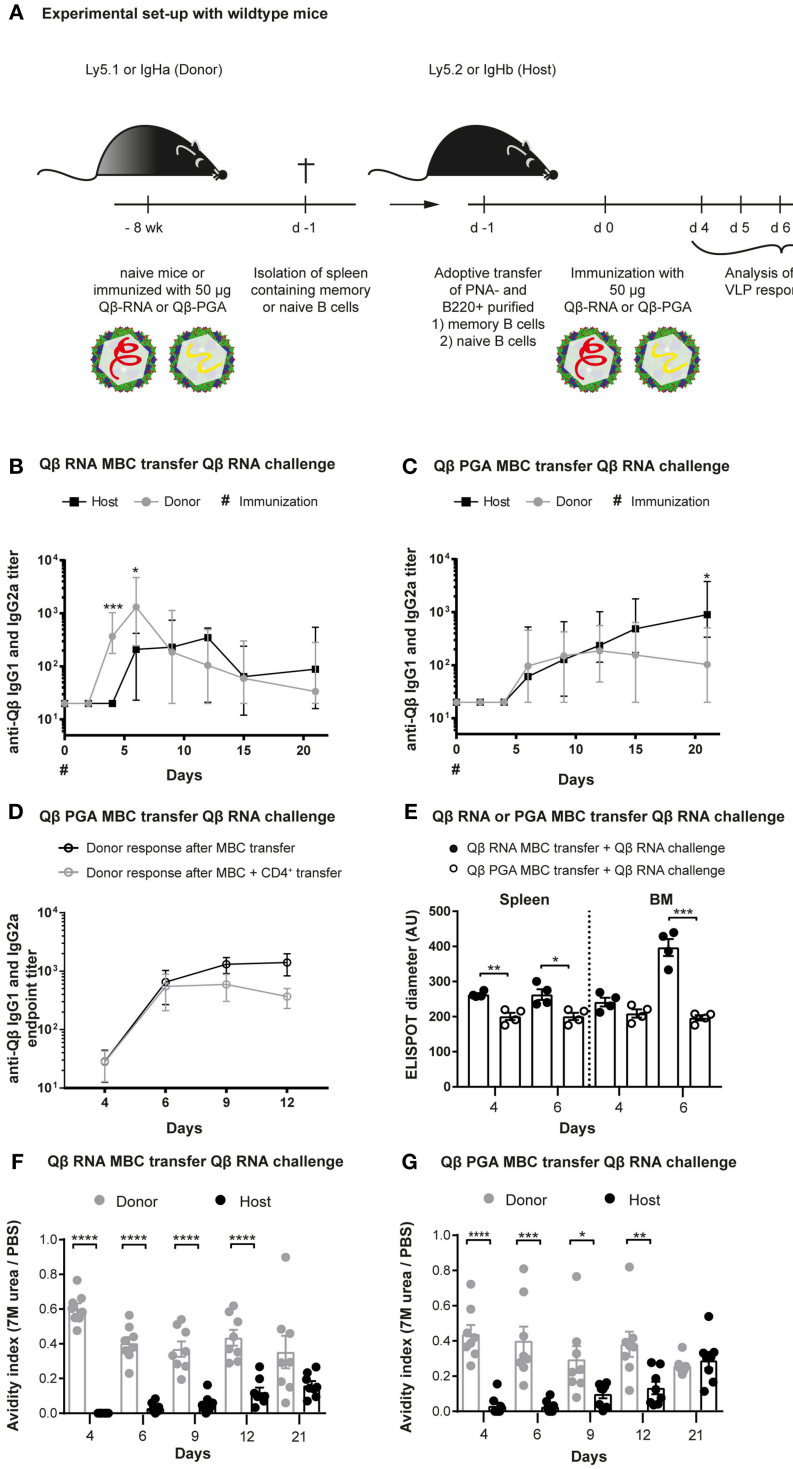

FIGURE 1 | Memory B cells generated in presence of bacterial RNA generate secondary PCs after challenge with VLPs containing RNA. **(A)** Congenic mice (Ly5.1 or IgHa) were immunized with 50 µg Qβ VLPs containing RNA **(B,E,F)** or polyglutamic acid (PGA) **(C–E,G)** i.v. Eight weeks after immunization spleens of immunized and naïve mice were isolated and PNA^{-} $B220^{+}$ **(B,C,E–G)** and $CD4^{+}$ **(D)** MACS purified cells were transferred into host mice (Ly5.2 or IgHb). Recipient mice were immunized with 50 µg Qβ-RNA or Qβ-PGA i.v. 1 day after the transfer. Spleens, bone marrow, and serum were taken at several time points after challenge. **(B,C)** The anti-Qβ IgG1 and IgG2a antibody titers in the serum were determined by ELISA. Ha and Hb allotype specific detection antibodies were used to discriminate between donor (IgHa, gray circles) and host (IgHb, black squares) responses. **(D)** The endpoint titer of anti-Qβ IgG1 and IgG2a antibodies in the serum was determined by ELISA. Donor-derived responses after memory B cell (black open circles) or memory B cell and memory $CD4^{+}$ T cell (gray open circles) transfer were detected using

(Continued)

FIGURE 1 | Ha allotype specific detection antibodies. **(E)** Quantification of the spot diameter in ELISPOT assays after transfer of memory B cells induced with 50 μg Qβ-RNA (black circles) or Qβ-PGA (open circles) and challenge with 50 μg Qβ-RNA. A modified ELISA was performed to determine the avidity index of the sera after transfer of memory B cells generated in presence **(F)** or absence **(G)** of bacterial RNA. Mean with SEM. *P*-values were obtained using an unpaired *t*-test. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$. $n = 4$ mice per group. Data representative of 2 independent experiments, except for D and E, where only one experiment was performed.

Mice

C57BL/6JRccHsd wildtype mice were purchased from Envigo (Horst, The Netherlands). The IgHa [B6.Cg-Gpi1<a> Thy1<a> Igh<a> (Stock No. 001317)] mouse strain was purchased from the Jackson Laboratory (USA). We thank Prof. Annette Oxenius for the kind donation of the Ly5.1 (B6.SJL-Ptprc<a> Pepc<b>/BoyJ) mouse strain, Prof. Dr. Pål Johansen for the kind donation of the TLR7 KO (B6.129P2-Tlr7tm1Aki) mouse strain and Prof. Andrew Macpherson for the kind donation of the JH KO (B6.129P2-Igh-Jtm1Cgn/J) mouse strain.

Generation of BM Chimeras

C57BL/6JRccHsd wildtype mice were lethally irradiated by the application of 1,300 cGy as a split dose of 2 × 650 cGy with a 4 h interval, using a Gammacell 40 (GC40) research irradiator (Best Theratronics). Irradiated mice were reconstituted with 25 × 10^7 donor bone marrow cells, consisting of 80% JH KO and 20% TLR7 KO or 20% C57BL/6JRccHsd WT cells, respectively, i.v. Antibiotics [Baytril (1.25 ml/l) and Bactrim Nopil (5 ml/l)] were supplied in the drinking water for 2 weeks. After 6 weeks, reconstitution of the BM chimeras was analyzed by staining of B cell (B220) and T cell (CD3) markers using FCM. BM chimeras were immunized with 50 μg Qβ VLPs formulated in 150 μl phosphate buffer intravenously 10 weeks after irradiation.

Antigen

The bacteriophage derived Qβ virus-like particles (VLPs) self-assemble and enclose bacterial RNA during their production in *E. coli*. The purification process is described elsewhere (48). VLPs without RNA were generated by disassembling the particles in presence of DTT in acidic conditions. This results in dimer formation, which were purified by size exclusion chromatography. Afterwards, the dimers were reassembled with polyglutamic acid (PGA) (17). VLPs containing B type CpGs (1668) were prepared as described previously (49, 50). Briefly, RNA inside the VLPs was digested using RNAse A (1.2 mg/ml for 3 mg/ml VLPs) for 3 h at 37°C. RNA digestion was confirmed using a 1% agarose gel stained with peqGreen dye. VLPs were repackaged by adding 1.125 μg CpG oligonucleotides to 20 μg RNAse digested VLPs for 3 h at 37 °C and repackaging was confirmed on a 1% agarose gel.

Immunization

To induce primary immune responses and generate memory B cells against the VLPs, mice were immunized intravenously (i.v.) with 50 μg Qβ-RNA or Qβ-PGA. To challenge adoptively transferred MBC or naive cells, recipient mice were immunized with 50 μg of either Qβ-RNA, Qβ-PGA or Qβ-CpG i.v. For administration the VLPs were formulated in 150 μl phosphate buffer.

Adoptive Transfer

MBCs were generated by immunization of congenic donor mice (Ly5.1, IgHa, TLR7 KO, WT, or BM chimeras). At least 8 weeks after immunization donor mice were sacrificed and spleens isolated in RPMI media containing 2% FCS and antibiotics. A single cell suspension of the spleens was prepared and red blood cells were lysed using ACK buffer (0.15 M ammonium chloride, 0.01 M potassium hydrogen carbonate, pH 7.2–7.4). The splenocytes were PNA^- and $B220^+$ MACS purified. For PNA negative purification splenocytes were labeled using PNA-biotin (Vector Labs, B-1075) and PNA^+ cells were depleted by Strepravidin MicroBeads (Milteny Biotec, 130-048-101) according to the manufacturer's protocol. Positive selection using B220 and CD4 MicroBeads (Milteny Biotec, 130-049-501, 130-117-043) was performed according to the manufacturer's protocol.

Purified cells from 1/3 of a donor spleen (Ly5.1, IgHa, TLR7 KO, WT, or BM chimeras ∼1–3 × 10^6 cells of which ∼0.05–0.1% are specific for the antigen) were adoptively transferred i.v. into congenic host mice (Ly5.1, Ly5.2, or IgHb). Control mice received PNA^- and $B220^+$ purified splenocytes from naïve congenic mice. One day after memory B cell transfer host mice were challenged with 50 μg of either Qβ-RNA, Qβ-PGA or Qβ-CpG i.v. formulated in 150 μl phosphate buffer.

ELISPOT

Spleens from mice after adoptive transfer were isolated and a single cell suspension was prepared. To collect BM cells, tibia and femur were flushed with RPMI media containing 2% FCS and antibiotics. After red blood cell lysis with ACK buffer, cell numbers of splenocytes and BM cells were determined using the Cellometer mini (Nexcelom, USA). 5 × 10^5 cells were seeded per well on MAIPS ELISPOT plates (Millipore, MAIPS4510) previously coated with 10 μg/ml Qβ VLPs overnight at 4°C and blocked with 2% BSA in PBS for at least 2 h. After performing a 2-fold dilution series, cells were incubated for 5 h at 37°C and 5% CO_2. Subsequently cells were washed off and bound specific antibodies produced by PCs were detected using a goat anti-mouse IgG antibody (EY laboratories, AT-2306-2) followed by a donkey anti-goat alkaline phosphatase secondary antibody (Jackson Immunoresearch, 705-055-147). Spots were visualized by the AP Conjugate Substrate Kit (BioRad, 1706432) and counted using an EliSpot Reader (AID, Germany).

ELISA

Serum samples were obtained from blood collected at the indicated time points during experiments using Microtainer tubes (BD, 365967). Corning half area 96 well plates were coated with 1 μg/ml Qβ VLPs overnight at 4°C. Sera were 1:10 pre-diluted and 1:4 further serial diluted to analyse a total of 7 dilutions per sample. Qβ-specific antibodies were detected using

mouse anti-mouse IgG for both allotypes. IgHa-specific (biotin ms anti-ms IgG1[a] (10.9), biotin ms anti-ms IgG2a[a] (8.3) from BD) and IgHb-specific (biotin ms anti-ms IgG1[b] (B68-2), biotin ms anti-ms IgG2a[b] (5.7) from BD) antibodies were detected using horseradish peroxidase (HRP) labeled streptavidin (Jackson ImmunoResearch, 016-030-084).

Total Qβ-specific antibodies were detected using goat anti-mouse IgG-HRP (Jackson ImmunoResearch, 115-035-071).

The absorbance readings of the tetramethylbenzidine (TMB) color reaction at 450 nm for the serum samples were interpreted as OD50 or endpoint antibody titers. The OD50 antibody titers are defined as the reciprocal of the dilution that reaches half of the maximal optical density (ODmax). The endpoint antibody titers are defined as the reciprocal of the last dilution above the threshold, which is set above the background level.

Avidity ELISA

Serum samples were obtained from blood collected at the indicated time points during experiments using Microtainer tubes (BD, 365967). Corning half area 96 well plates were coated with 1 μg/ml Qβ VLPs overnight at 4°C. Sera of the different time points were applied with a 1:10 pre-dilution and 1:4 further serial diluted. After 1 h incubation, the sera were washed off and the plates washed 3 times 5 min either with 7 M urea in PBST (PBS containing 0.05% Tween20) or PBST only. Qβ specific antibodies were detected using mouse anti-mouse IgG for both allotypes. IgHa-specific (biotin ms anti-ms IgG1[a] (10.9), biotin ms anti-ms IgG2a[a] (8.3) from BD) and IgHb-specific (biotin ms anti-ms IgG1[b] (B68-2), biotin ms anti-ms IgG2a[b] (5.7) from BD) antibodies were detected using horseradish peroxidase (HRP) labeled streptavidin (Jackson ImmunoResearch, 016-030-084). Total Qβ-specific antibodies were detected using goat anti-mouse IgG-HRP (Jackson ImmunoResearch, 115-035-071). The absorbance readings of the tetramethylbenzidine (TMB) color reaction at 450 nm served as basis for avidity index calculation. The avidity index (AI) was calculated by AI_x = OD (dilution x) + urea/OD (dilution x)–urea.

Flow Cytometry (FCM)

For FCM staining spleens of mice after adoptive transfer were isolated in RPMI supplemented with 2% FCS and antibiotics and a single cell suspension was prepared. Red blood cells were lysed using ACK buffer prior to staining. Fc receptors were blocked using an anti-CD16/32 antibody (2.4G2, BD). To discriminate Qβ-specific plasma cells (PCs) from Qβ-specific activated and CS B cells, surface immunoglobulins (Ig) of specific cells were blocked using unlabelled Qβ VLPs. PCs were further stained with and characterized as IgM (polyclonal, Jackson ImmunoResearch), IgD (11-26c (11-26), eBioscience), CD4 (H129.19, BD), CD8 (53-6.7, BD), GR1 (RB6-8C5, BD), CD11b (M1/70, BD), CD11c (HL3, BD) negative (all antibodies labeled with PE), and B220-PE-Cy7 (RA3-6B2, BD) low. To detect Qβ specific PCs by intracellular staining of specific Ig, splenocytes were permeabilized using FACS lysing solution (BD, 349202) containing 0.04% Tween20 and stained with Alexa Flour 488 labeled Qβ VLPs. The congenic marker Ly5.1 (antibody labeled with APC, A20, eBioscience) identified all transfer derived B cells.

Qβ VLPs were labeled with the Alexa Flour 488 protein labeling kit (Thermo Fisher Scientific, A10235) according to the manufacturer's instructions.

Statistics

Statistical analysis was performed using GraphPad Prism Version 7.01 (GraphPad Software, USA). Statistically significant differences between two groups were calculated using unpaired *t*-tests. Statistical significance was defined as $p < 0.05$.

RESULTS

RNA Drives the Generation of Memory B Cells Competent of Forming Secondary PCs

We have previously demonstrated that vaccination with Qβ VLPs containing bacterial RNA leads to the formation of long-lasting humoral memory. Upon immunization, isotype-switched memory B cells as well as PCs are generated in a Th cell-dependent manner (7, 12, 44, 45). During secondary responses, VLP specific memory B cells do not re-enter GCs but differentiate to short-lived secondary PCs independent of T cell help (7, 12). The hallmark of secondary PCs is increased production of high affinity antibodies early after activation (Krueger et al., under review). To further study the mechanism of secondary PC generation, adoptive transfers of memory B cells using congenic mice were performed. Briefly, memory B cells were generated by immunizing wildtype (WT) donor mice with Qβ VLPs containing either bacterial RNA (Qβ-RNA) or polyglutamic acid (Qβ-PGA), a negatively charged polymer serving as surrogate for RNA to enable VLP-assembly, which, however, does not stimulate TLRs (**Figure 1A**). Purified memory B cells of immunized or naïve donor mice were transferred into congenic recipient mice expressing a different IgH- or Ly5- allotype. We did not co-transfer memory $CD4^+$ T cells after Qβ-RNA priming, as we have previously observed that their presence has no influence on VLP specific memory B cell responses (7, 12). Upon cell transfer and challenge with Qβ-RNA the specific VLP antibody response of transferred memory B cells and host B cells was assessed within recipient mice (**Figures 1B,C**). As observed before, when memory B cells were induced with Qβ-RNA and challenged with Qβ-RNA, the on- and offset as well as the magnitude of the antibody response derived from memory B cells was significantly faster and higher compared to the host's primary antibody response (**Figure 1B**). Memory B cell derived IgG titers raised within 4 days and peaked early at day 6 post-immunization. In contrast, the host's primary response became detectable on day 6 and peaked at day 12 after immunization. However, if memory B cells were generated with Qβ-PGA instead of Qβ containing RNA, memory B cell derived antibody responses were not increased but rather similar to the host's antibody response after challenge with Qβ-RNA (**Figure 1C**; **Figure S1A**). Therefore, the memory antibody response resembled the one of the primary response if VLPs deprived of RNA were used for memory B cell generation indicating that RNA inside the VLPs

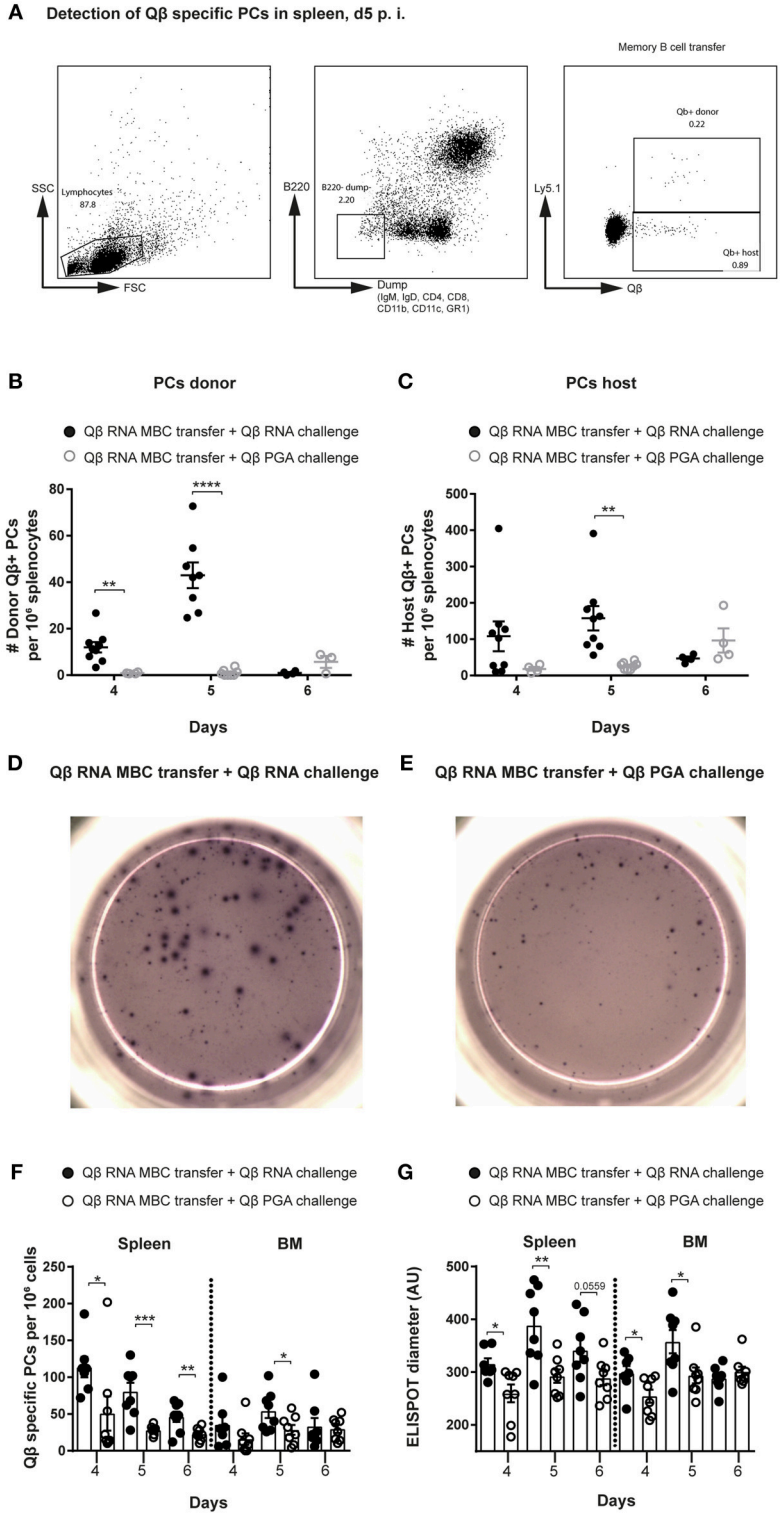

FIGURE 2 | Presence of bacterial RNA during challenge of memory B cells is essential to generate secondary PCs. Memory B cells were generated by immunizing Ly5.1 mice (donor) with 50 μg Qβ-RNA. One day after transfer of MACS purified memory B cells, allotypic Ly5.2 hosts were challenged with 50 μg Qβ-RNA or 50 μg Qβ-PGA, respectively. **(A)** Representative FCM plots for the gating strategy to identify Qβ-specific PCs in the spleen 5 days after transfer and challenge. B220^{low} cells not expressing IgM, IgD, CD4, CD8, CD11b, CD11c, or GR1 were analyzed for their intracellular binding of labeled Qβ VLPs. The congenic Ly5 marker was used to discriminate transfer from host derived PCs. **(B,C)** FCM analysis of the specific PC compartment at day 4, 5, and 6 after transfer of memory B cells induced with Qβ-RNA and challenged with Qβ-RNA (black circles) or Qβ-PGA (gray circles). Number of Qβ-specific donor **(B)** or host **(C)** derived PCs within the B220^{low}, IgM, IgD,

(Continued)

FIGURE 2 | CD4, CD8, CD11b, CD11c, and GR1 negative compartment, binding Qβ intracellularly after membrane permeabilisation. Representative images of ELISPOTs of the spleen at day 5 after Qβ-RNA memory B cell transfer and Qβ-RNA **(D)** or Qβ-PGA **(E)** challenge. **(F)** Numbers of Qβ-specific PCs in spleen and BM 4, 5, and 6 days after memory B cell transfer and challenge with either Qβ-RNA (black circles) or Qβ-PGA (open circles) were determined by ELISPOT. **(G)** Quantification of the spot diameter in ELISPOT assays after memory B cell transfer and challenge with either Qβ-RNA (black circles) or Qβ-PGA (open circles). Mean with SEM. *P*-values were obtained using an unpaired *t*-test. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$. $n = 4$ mice per group. Data representative of 2 independent experiments, except for day 6 of B and C, where only one experiment was performed.

was crucial for enhanced antibody responses during secondary Ag challenges. A similar donor response was seen when Qβ-PGA memory B cells were transferred in presence or absence of memory $CD4^+$ T cells (**Figure 1D**) and challenged with Qβ-RNA. Consequently, like in Qβ-RNA secondary responses, co-transfer of memory $CD4^+$ T cells had no influence on the VLP specific memory B cell response. Of note, memory B cells generated with VLPs containing RNA also failed to generate increased IgG levels if the boost was performed with Qβ devoid of RNA (**Figure S2A**). Thus, enhanced IgG responses were only detectable when memory B cells were generated and boosted with Qβ-RNA (**Figures 1B,C**; **Figures S1A**, **S2A-C**). The increased IgG response produced by memory B cells generated in presence of RNA could also be verified in ELISPOT assays of spleen and BM, where the spot diameter correlates with the amount of antibodies produced by one PC (**Figure 1E**).

We further investigated the antibody response by performing avidity ELISAs (**Figures 1F,G**). The avidity index was determined by a modified ELISA where washes with 7 M urea were performed, which dissociates low avidity antibodies but high avidity antibodies remain bound. The avidity index of secondary response antibodies after Qβ-RNA induced memory B cell transfer and Qβ-RNA re-stimulation was high as of day 4 and stayed significantly higher compared to the primary response antibodies until day 21 (**Figure 1F**). A similar, but less pronounced, observation of the avidity increase was made, when Qβ-PGA induced memory B cells were transferred and re-stimulated with Qβ-RNA (**Figure 1G**). In strong contrast, primary response antibodies reach comparable avidity only after 21 days in the absence of memory B cell transfer (**Figure S1B**). These data indicate that RNA is required to generate memory B cells capable of generating secondary PCs but affinity maturation occurred to a large degree in the absence of RNA.

Presence of Bacterial RNA During Challenge of Memory B Cells Is Essential to Generate Secondary PCs

After induction of memory B cells with Qβ-RNA in Ly5 allotypic wildtype mice, MACS purified memory B cells were adoptively transferred into allotypic recipients, which were challenged with Qβ-RNA or Qβ-PGA 1 day after transfer (**Figure 1A**). Antigen-specific PCs were identified as $B220^{low}$, IgM, IgD, CD4, CD8, CD11b, CD11c, GR1 negative and by intracellular Qβ binding after membrane permeabilisation using flow cytometry (FCM). Donor derived cells ($Ly5.1^+$) were discriminated from host derived cells ($Ly5.2^+$) using the Ly5 marker (**Figure 2A**). Donor, hence memory B cell derived Qβ-specific PCs, were significantly increased on day 4 and 5 after challenge with Qβ-RNA compared to Qβ-PGA (**Figure 2B**). This difference was less prominent looking at host derived antigen specific PCs generated early during the primary response (**Figure 2C**).

The increased PC response generated by transferred memory B cells boosted with Qβ-RNA in comparison to Qβ-PGA correlated with PC numbers detectable in spleen and BM analyzed by ELISPOT (**Figures 2D–F**). In addition, secondary PCs generated in presence of Qβ-RNA were capable to produce more antibodies shown by the increased spot diameter observed in ELISPOT analysis (**Figures 2D,E,G**). The peak of the PC number and spot diameter in spleen and BM after transfer of Qβ-RNA-primed memory B cells followed by challenge with Qβ-RNA was around day 4 and 5, thereafter PC numbers rapidly declined, demonstrating the short-lived nature of the secondary PCs. In contrast, boosting with Qβ-PGA resulted in slower but more sustained responses (**Figure 2B**, day 6). The early PC population detectable in spleen and BM after naïve cell transfer in control experiments generating a primary response was smaller and produced less antibodies (**Figures S1C,D**).

Thus, bacterial RNA as a TLR7 ligand is not only important in the generation of memory B cells but also during their differentiation to secondary PCs with increased ability to secrete high-affinity antibodies.

RNA Induced TLR7 Signaling Must Be Present for Induction of Memory B Cells Competent to Differentiate Into Secondary PCs

To assess whether TLR7 was involved in the generation of memory B cells competent to differentiate into secondary PCs, adoptive transfer experiments using TLR7 KO mice were performed. Memory B cells were induced in TLR7 KO or WT mice by Qβ-RNA vaccination. MACS purified memory B cells were then transferred into Ly5-allotypic WT mice and challenged with Qβ-RNA, Qβ-PGA, or Qβ-CpG (**Figure 3A**). As observed with Qβ-PGA (**Figures 1C,G**), the total anti-Qβ IgG titer and avidity was significantly lower at early time-points when TLR7 deficient memory B cells were transferred (**Figures 3B,C**). Therefore, the humoral immune responses observed in the absence of TLR7 correlated well with the data observed in the absence of RNA, indicating that memory B cells competent to differentiate to secondary PCs fail to differentiate in the absence of TLR7 signaling.

PC numbers and spot sizes in spleen and BM obtained from ELISPOT assays were consistent with the antibody responses and corroborated the findings using RNA-free VLPs (**Figures 3D,E**). Host mice that received WT memory B cells exhibited significantly increased PC frequencies in the spleen

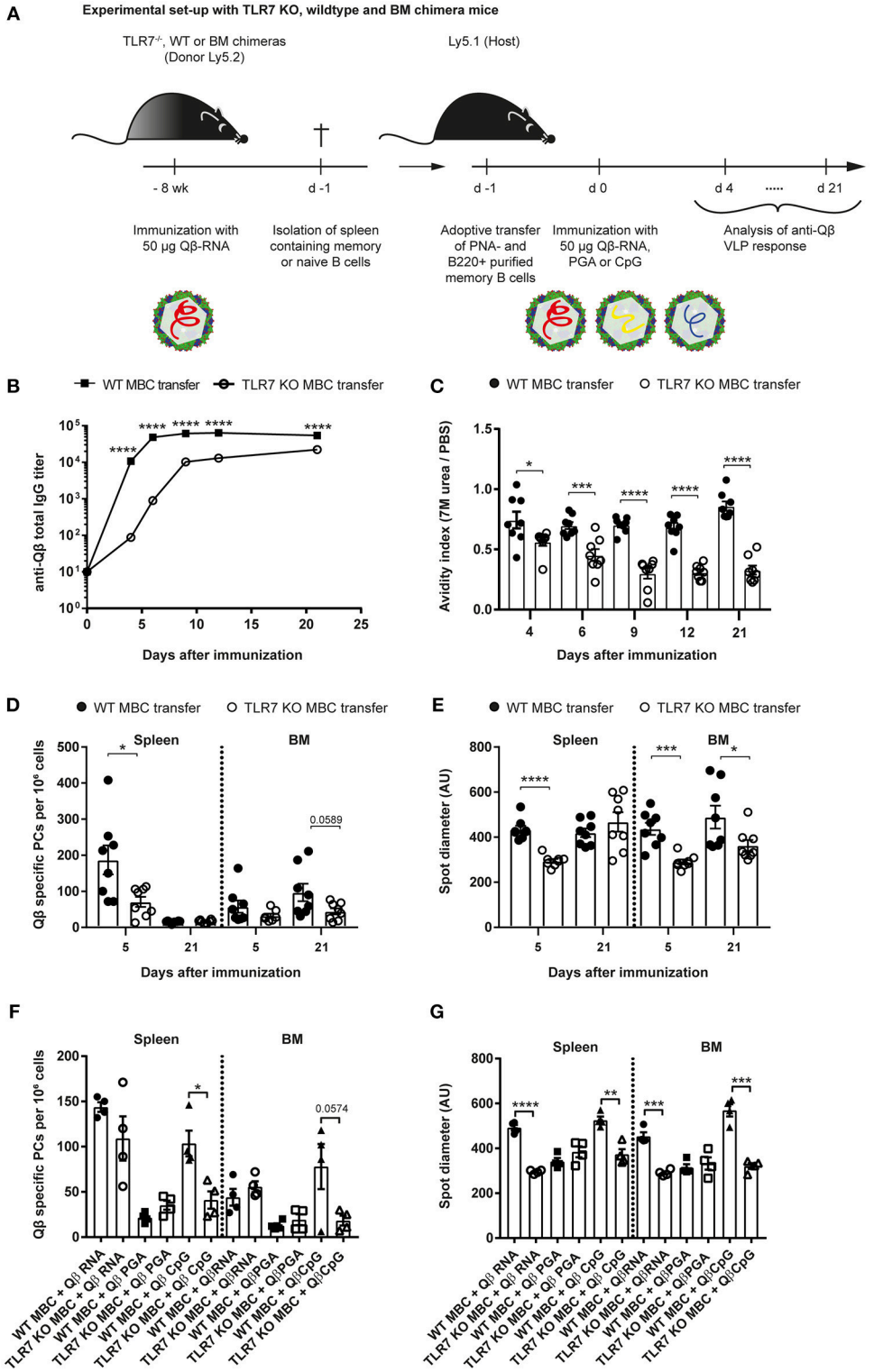

FIGURE 3 | TLR7 must be present during memory B cell induction to generate secondary PCs after challenge with VLPs. **(A)** Memory B cells were induced in TLR7 KO, WT, or BM chimeras by immunization with 50 µg Qβ-RNA i.v. Eight weeks after immunization spleens were isolated and PNA^{-} B220^{+} MACS purified cells were transferred into host mice (Ly5.1). One day after the transfer, recipient mice were challenged with 50 µg Qβ-RNA **(B–E)** or Qβ-RNA, Qβ-PGA, and Qβ-CpG **(F,G)** i.v. Spleens, bone marrow, and serum were taken at several time points after challenge. **(B)** Qβ-specific total IgG titers after WT (black squares) or TLR7 KO (open circles) memory B cell transfer were determined by ELISA. **(C)** The avidity index after WT (black circles) or TLR7 KO (open circles) memory B cell transfer was determined by a

(Continued)

FIGURE 3 | modified ELISA. **(D,E)** ELISPOT assays were used to determine the number **(D)** of Qβ specific PCs and the spot diameter **(E)** produced by these in spleen and BM on days 5 and 21 after WT (black circles) or TLR7 KO (open circles) memory B cell transfer. **(F,G)** ELISPOT assays were used to determine the number **(F)** of Qβ specific PCs and the spot diameter **(G)** in the spleen and BM on day 5 after WT (black shapes) or TLR7 KO (open shapes) memory B cell transfer and challenge with Qβ-RNA, Qβ-PGA, or Qβ-CpG, respectively. Mean with SEM. *P*-values were obtained using an unpaired *t*-test. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$. $n = 4$ mice per group. Data representative of 2 **(B–E)** or 1 **(F,G)** independent experiments.

5 days after VLP challenge (**Figure 3D**). This difference was less prominent in the BM. The spot diameter was significantly increased in spleen and BM after WT memory B cell transfer compared to TLR7 KO memory B cell transfer early during the response. This difference was maintained in the BM but gone in the spleen by day 21, likely because most secondary PCs had died by then (**Figure 3E**). In conclusion, as increased antibody production and spot size are a hallmark of secondary PCs, these cells were only generated when WT memory B cells were transferred, which could receive RNA-mediated TLR7 signals during their generation and re-stimulation.

To assess whether TLR7 KO memory B cells were competent to differentiate to secondary PCs, adoptive transfers of WT and TLR7 KO memory B cells and challenge with Qβ-RNA, Qβ-PGA, or Qβ-CpG were performed. Qβ-CpG contained B-type CpG oligodeoxynucleotides, which trigger myeloid differentiation primary response 88 (MyD88) signaling in B cells via TLR9. The memory B cell response toward the three challenge antigens was determined by ELISPOT at day 5 after challenge. TLR7 KO memory B cells exhibited a reduced capacity to generate secondary PCs in response to Qβ-RNA and Qβ-CpG challenge, shown by decreased spot number (**Figure 3F**) and spot size (**Figure 3G**) in the spleen and BM. The spot number and spot sizes generated were comparable to the ones of WT and TLR7 KO memory B cells challenged in the absence of any TLR ligand (Qβ-PGA) (**Figures 3F,G**), which failed to differentiate to secondary PCs (**Figure 2**). WT memory B cells on the contrary differentiated to secondary PCs after reactivation with Qβ-RNA and Qβ-CpG (**Figures 3F,G**), indicating that TLR9 stimulation can compensate for TLR7 when memory B cells were induced in presence of TLR7 ligands. Consequently, TLR7 signaling is indispensable during memory B cell priming for imprinting the ability of secondary PC generation after antigen challenge, as MyD88 signaling induced by TLR9 stimulation is not able to compensate for the defect seen after TLR7 KO memory B cell transfer. Moreover, presence of TLR7 or TLR9 signaling is sufficient for secondary PC formation after challenge of memory B cells generated in the presence of TLR7 signaling.

B Cell Intrinsic TLR7 Signaling Is Needed to Generate Memory B Cells Capable of Differentiating to Secondary PCs

To test whether TLR7 signaling was intrinsically required in B cells for secondary PC generation, mixed BM chimeras of JH knockout (KO) with WT or TLR7 KO BM were generated. TLR7 KO BM chimeras exclusively lack TLR7 in B cells, whereas WT BM chimera B cells were sufficient for TLR7 in all cells. Two months after reconstitution, both BM chimeras were immunized with Qβ-RNA (**Figure 4A**). MACS purified memory B cells were transferred into recipient WT mice and challenged with Qβ-RNA (**Figures 3A**, **4A**). From day 4 after challenge the anti-Qβ total IgG titer as well as the affinity was significantly lower when memory B cells from TLR7 deficient BM chimeras were transferred (**Figures 4B,C**). The difference was particularly pronounced before day 9 after challenge, representing the time span when secondary PCs were dominating the response, providing the early wave of antibodies. As observed before, the differences became smaller at later time-points, as the host response developed (**Figures 4B,C**).

PC numbers and spot sizes examined in spleen and BM correlated well with the antibody responses (**Figures 4B–E**). After transfer of TLR7 KO BM chimera memory B cells, PC numbers in the spleen were significantly lower compared to WT BM chimera memory B cell transfer at day 5 after challenge. This difference was absent 21 days after challenge, again indicating that secondary PCs are short-lived (**Figure 4D**). Moreover, spot diameters, which correlate with the amount of antibodies produced by PCs, were smaller in spleen and BM at day 5 after challenge, when TLR7-deficient BM chimera memory B cells were transferred compared to WT BM chimera memory B cells (**Figure 4E**). Taken together, the data presented here clearly demonstrate that RNA and TLR7 signaling are required in a B cell intrinsic fashion for the generation of memory B cells and their differentiation to secondary PCs, which are capable to produce vast amounts of high affinity antibodies early during secondary responses.

DISCUSSION

B cell responses are controlled and regulated at multiple levels. As a key step, B cell specificity is cross-checked by available cognate T cell help and the presence of innate stimuli indicative of an infection. This is exemplified by the primary B cell response against viruses where specificity alone is driving the initial response by efficient cross-linking of BCRs by the repetitive viral surface (51). This results in a Th cell independent IgM response, which is, however, short-lived. Only the presence of cognate T cell help results in a GC response and isotype-switching. In this way, the immune system validates the BCR-signals by the presence of Th cells specific for the same antigen, which indicates that the recognized antigen is non-self. Presence of PAMPs, in particular TLR-ligands, is a second checkpoint, which implies that the antigen is not only non-self but most likely an infectious agent. This results in augmented antibody and Th cell responses. Here we demonstrate that TLR-signals, in particular TLR7, are also key in secondary B cell responses as they cause the differentiation of a subset of memory B cells capable to rapidly differentiate into secondary PCs upon re-exposure to the same antigen plus TLR7-ligand.

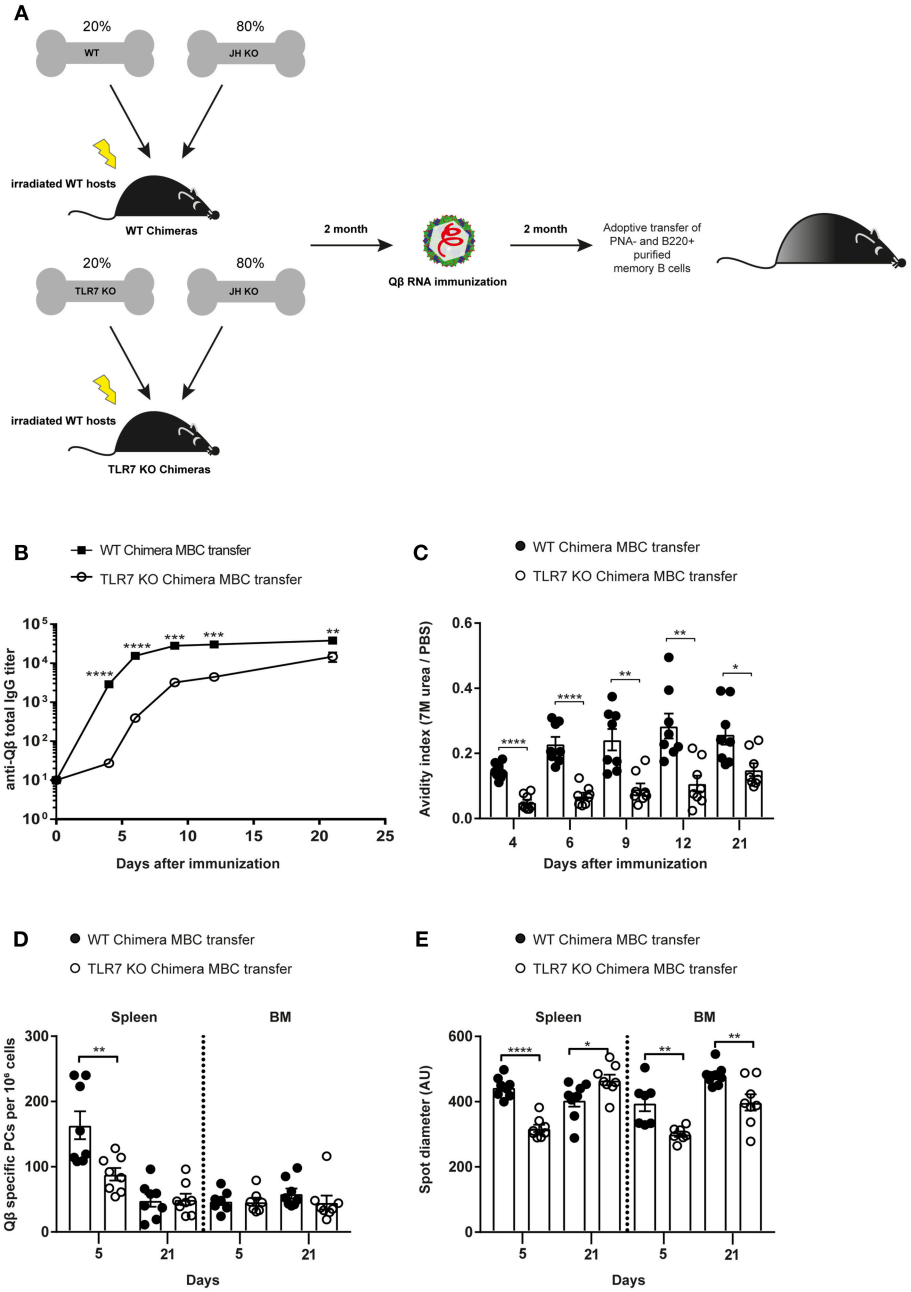

FIGURE 4 | B cell intrinsic TLR7 signaling is needed to generate memory B cells capable of producing secondary PCs. **(A)** Mixed BM chimeric mice with 20% WT or TLR7 KO and 80% JH KO BM were generated. Eight weeks after reconstitution the WT and TLR7 KO chimeras were immunized with 50 μg Qβ-RNA. Memory B cells from BM chimeric mice were transferred into congenic Ly5.1 recipients after 8 weeks. Recipient mice were challenged with 50 μg Qβ-RNA 24 h after the adoptive transfer. **(B)** The anti-Qβ total IgG titer in the serum after WT chimera (black squares) or TLR7 KO chimera (open circles) memory B cell transfer was determined by ELISA. **(C)** The avidity index of antibodies from sera generated after WT chimera (black circles) or TLR7 KO chimera (open circles) memory B cell transfer and challenge was calculated after performing a modified ELISA. ELSIPOT assays of splenocytes and BM cells were performed to determine PC number **(D)** and spot diameter **(E)** 5 and 21 days after WT chimera (black circles) or TLR7 KO chimera (open circles) transfer and challenge. Mean with SEM. *P*-values were obtained using an unpaired *t*-test. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$. $n = 4$ mice per group. Data representative of two independent experiments.

The adaptive immune system is confronted with the choice between speed and specificity. As clonal selection and, in the case of B cells, hypermutation need time to develope, high specificity comes at the cost of time. As many pathogens may be fatal within a week, highly specific antibody responses would be too late to provide protection (52). Broadly cross-reactive antibody responses, on the other hand, always carry the risk of non-desired recognition of self-antigens. The solution the immune system

found during primary responses is the early and rapid generation of poly-reactive IgM antibodies, which are especially potent at recognizing repetitive surfaces due to their deca-valence. To balance the potential of IgM antibodies to cross-react, these responses are, however, short-lived and eventually replaced by highly specific bivalent IgG antibodies, which are controlled by cognate T cell help and presence of TLR-ligands.

We demonstrate here that secondary antibody responses may follow a similar pattern. Pre-existing, TLR7- and Th cell experienced memory B cells differentiate rapidly to secondary PCs, which produce large amounts of high affinity IgG antibodies. This differentiation occurs without direct interaction with cognate Th cells (12) but needs again the presence of RNA or CpG oligodeoxynucleotides to engage TLR7/9 signaling to ensure presence of an infectious agent. However, since the pathogen may have evolved over time, these high affinity IgG antibodies may primarily recognize the original pathogen rather than the current version. For this reason, similar as the IgM antibodies generated during the primary response, this early wave of secondary IgG antibodies is replaced by a more specific second wave of antibodies derived from naïve B cells, which again will require presence of Th and TLR7-ligands for optimal specificity.

The present data further underscore the importance of RNA-sensing in B cells. We and others have previously shown that TLR signaling in B cells drives primary B cell responses and is, at least for viral particles, more important than TLR7 signaling in DCs (16–19). Here we extend these findings to secondary B cell responses and demonstrate that B cell-intrinsic TLR7-signaling is essential for imprinting the ability to differentiate to secondary PCs, as vaccination with VLPs deprived of RNA induces affinity matured memory B cells which lack the potential to generate secondary PCs. Moreover, this signaling pathway is also key for driving the differentiation of these secondary PCs from memory B cells. Hence, TLR7 signaling in B cells is essential for the shaping of both primary and secondary B cell responses. These data have obvious implications for vaccine design as the major vaccines based on virus-like particles, those against hepatitis B virus, human papilloma virus and malaria do not package RNA. Future vaccine platforms may therefore be based on VLPs incorporating RNA in order to allow formation of memory B cells capable of differentiating into secondary PCs to provide the first wave of rapidly produced protective antibodies during re-infection.

AUTHOR CONTRIBUTIONS

CK, EK, and FL performed all experiments. CK, FT, and MB designed all the experiments. MV interpreted results and contributed to the scientific discussion. CK, FT, and MB wrote the manuscript. All authors read and 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.2019.00736/full#supplementary-material

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Establishment and Maintenance of Conventional and Circulation-Driven Lung-Resident Memory CD8$^+$ T Cells Following Respiratory Virus Infections

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Antigen-specific CD8$^+$ tissue-resident memory T cells (T_{RM} cells) persist in the lung following resolution of a respiratory virus infection and provide first-line defense against reinfection. In contrast to other memory T cell populations, such as central memory T cells that circulate between lymph and blood, and effector memory T cells (T_{EM} cells) that circulate between blood and peripheral tissues, T_{RM} cells are best defined by their permanent residency in the tissues and their independence from circulatory T cell populations. Consistent with this, we recently demonstrated that CD8$^+$ T_{RM} cells primarily reside within specific niches in the lung (Repair-Associated Memory Depots; RAMD) that normally exclude CD8$^+$ T_{EM} cells. However, it has also been reported that circulating CD8$^+$ T_{EM} cells continuously convert into CD8$^+$ T_{RM} cells in the lung interstitium, helping to sustain T_{RM} numbers. The relative contributions of these two mechanisms of CD8$^+$ T_{RM} cells maintenance in the lung has been the source of vigorous debate. Here we propose a model in which the majority of CD8$^+$ T_{RM} cells are maintained within RAMD (conventional T_{RM}) whereas a small fraction of T_{RM} are derived from circulating CD8$^+$ T_{EM} cells and maintained in the interstitium. The numbers of both types of T_{RM} cells wane over time due to declines in both RAMD availability and the overall number of T_{EM} in the circulation. This model is consistent with most published reports and has important implications for the development of vaccines designed to elicit protective T cell memory in the lung.

Keywords: tissue-resident memory, CD8$^+$ T cells, memory T cell maintenance, lung, respiratory virus infections

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INTRODUCTION

Memory CD8$^+$ T cells in non-lymphoid tissues are optimally positioned to mediate rapid responses to invading pathogens. They comprise at least two distinct subpopulations: tissue-resident memory T cells (T_{RM} cells) and effector memory T cells (T_{EM} cells). T_{RM} cells are a non-circulating population that typically, but not exclusively, expresses a specific array of surface markers (e.g., CD69, CD103, and CD49a) and possess gene-expression profiles that are associated with tissue retention (1). In contrast, T_{EM} cells lack the expression of these molecules and continuously

circulate between blood and non-lymphoid tissues (2). The vast majority of memory $CD8^+$ T cells in most non-lymphoid tissues are T_{RM} and play the predominant role in protective immunity (3, 4). In contrast, memory $CD8^+$ T cells in the circulation have minimal, if any, impact on immediate local protection (3, 5). However, it is possible that the small numbers of $CD8^+$ T_{EM} cells that transit through the tissues at the time of reinfection may contribute to protection.

The lung appears to differ from other non-lymphoid tissues in that it harbors relatively large numbers of both tissue-circulating T_{EM} and T_{RM} cells in a number of distinct niches (3, 6). Furthermore, these memory $CD8^+$ T cell subpopulations alter their phenotypes and functions in response to environmental factors present in distinct compartments of the lung (7, 8). Thus, a complete understanding of the phenotypic and functional features of these memory T cell populations in each of these lung compartment has been hampered by the challenges of isolating pure populations for analysis. This has resulted in confusion in the field. In this perspective, we attempt to resolve these issues and outline a model that explains the generation and maintenance of diverse populations of memory $CD8^+$ T cells in the lung.

MEMORY $CD8^+$ T CELLS IN THE LUNG

The tissues that comprise the barrier surfaces of the body typically consist of an epithelial layer that overlays a stromal layer, such as the epidermis and dermis in the skin and the epithelium and lamina propria in the intestine. These tissues differ considerably and provide distinct anatomical and biological niches for the maintenance of memory $CD8^+$ T cells (9). Consistent with other barrier tissues, the lung airways (epithelium) and the lung interstitium (stroma) host phenotypically and functionally distinct memory $CD8^+$ T cell populations.

Memory $CD8^+$ T cells in the lung airways are localized primarily in the epithelial layers of the bronchiole and are readily isolated by bronchoalveolar lavage (BAL) (10–12). Since the lung airways are anatomically separated from blood vessels, there are few, if any, blood cell contaminants in BAL samples (unless the blood vessels are damaged by poor technique or infection). Consequently, it is possible to interpret the data on T cells isolated by BAL without using intravascular (i.v.) staining to distinguish contaminating cells from the blood (13). Such BAL data indicate that large numbers of antigen-specific memory $CD8^+$ T cells are present in the lung airways for several months following recovery from a respiratory virus infection (14, 15). These airway T cells do not return to the circulation or the lung interstitium under steady-state conditions (12), suggesting that they are T_{RM}. However, since they have relatively short lifespans (presumably due to cell-extrinsic factors, such as their biophysical removal by the barrier function of airway mucosa) (16), their maintenance depends on continual influx of memory cells from the interstitium (16, 17). This continual replenishment of memory pool does not fit with the definition of T_{RM} and, as such, is a unique feature memory $CD8^+$ T cells in the lung airways. Upon recruitment to the airways, the cells receive antigen-independent local environmental cues to acquire an activation phenotype (e.g., upregulation of CD69) and to completely downregulate the integrin LFA-1 (CD11a) (7, 16). As a result, memory $CD8^+$ T cells in the airways lose cell contact-mediated cytolytic activity (11). Nevertheless, these cells can confer antigen-specific protection by rapidly secreting interferon (IFN)-γ in the face of antigenic challenge (18, 19).

Memory $CD8^+$ T cells in the lung interstitium can be purified by enzymatic digestion of lung tissues after removal of the BAL. However, cells prepared this way are contaminated with small numbers of memory $CD8^+$ T cells that had been trapped in the airways, although a certain fraction of these cells (i.e., cells that are exposed in the airway environment more than 48 h) can be distinguished by their reduced expression of CD11a (16). Interstitial cells prepared by enzymatic digestion are also contaminated with blood derived T cells from the capillaries. Therefore, prior i.v. staining is necessary to discriminate cells in the interstitium from those in the pulmonary capillary bed (13). It is important to point out two things here. First, data regarding parenchymal cells that have been isolated without i.v. staining must be cautiously interpreted given the significant degree of blood cell contamination. For example, before researchers began discriminating cells in the lung tissue and the lung vasculature, lung interstitium had erroneously been considered to be a "permissive tissue" that was readily accessible to memory $CD8^+$ T cells in the circulation (20–22). However, a more detailed analysis has revealed that, as with other mucosal tissues, the migration of circulating memory $CD8^+$ T cells into the lung interstitium is minimal in uninfected lung interstitium (6, 23, 24). Second, because memory $CD8^+$ T cells in the lung interstitium (i.e., negative for i.v.-injected antibody) include both T_{RM} and small numbers of tissue-circulating T_{EM}, parabiosis approaches are necessary to distinguish these populations. Using these approaches, we and others have formally demonstrated that a large proportion of memory $CD8^+$ T cells in the lung interstitium are T_{RM} cells (**Figure 1**) (3, 6). It has also become evident that $CD8^+$ T_{RM} and T_{EM} cell populations are maintained in distinct compartments of the lung interstitium: the former is predominantly localized within the site of tissue repair and regeneration around the bronchiole (we termed these Repair-Associated Memory Depots: RAMD), while the latter are widely and sparsely distributed in unaffected areas of the interstitium (6). Unlike memory $CD8^+$ T cells in the airways, $CD8^+$ T_{RM} cells in the lung interstitium are a stable population (6). Hence, memory $CD8^+$ T cells in the lung interstitium comprise a mixture of stable (T_{RM}) and dynamic (T_{EM}) memory populations that are maintained independently.

The true phenotypes of memory T cells in the lung interstitium are best revealed through parabiosis studies in which a pair of influenza virus infected mice are surgically joined after memory has been established and rested until leucocytes in the blood of each mouse are equilibrated. Non-circulating host $CD8^+$ T cells in the lung predominantly, but not exclusively, express T_{RM} markers CD69, CD103, and CD49a that facilitate tissue-retention while partner-derived T_{EM} cells are mostly negative for these markers (**Figure 1**) (6). The small fraction of

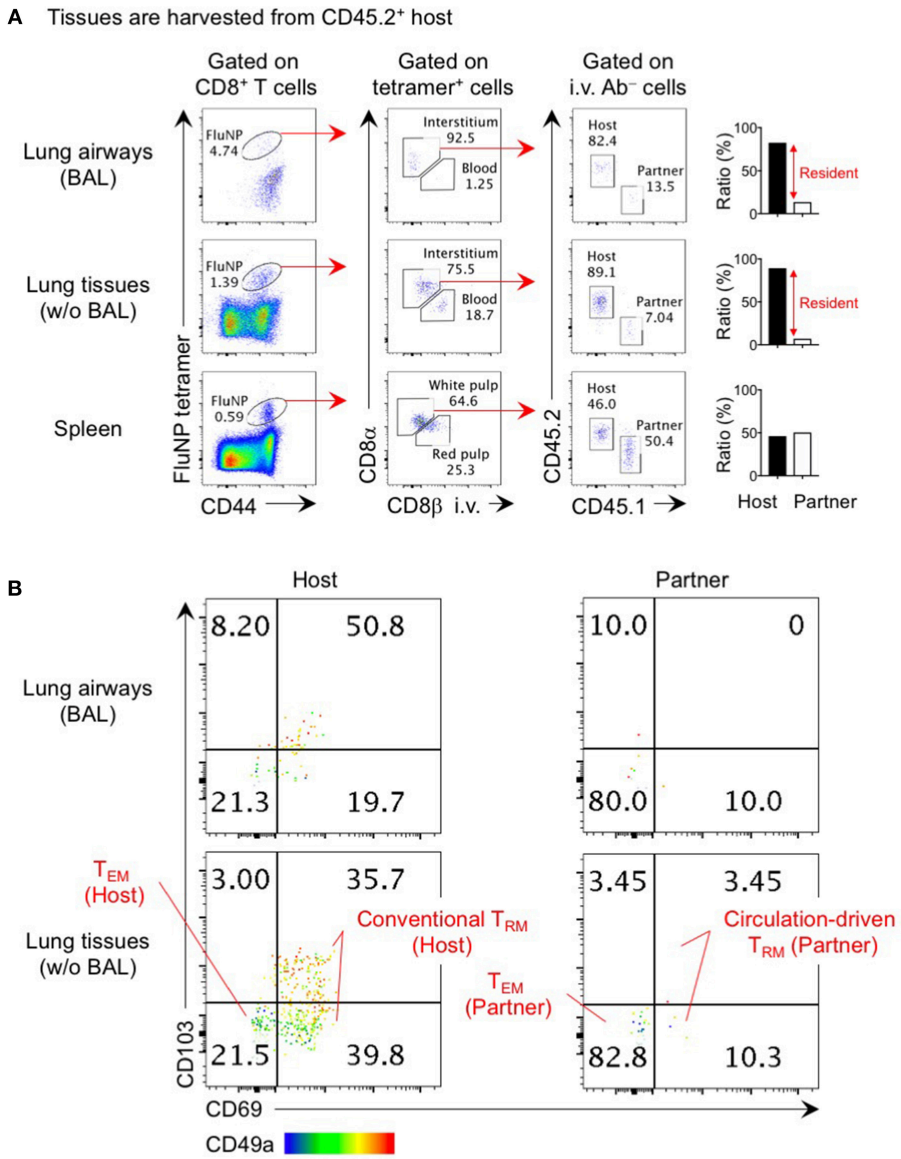

FIGURE 1 | Analysis of lung T_{RM} and T_{EM} by parabiosis experiment. Congenically distinct mice (CD45.1$^+$ and CD45.2$^+$) were infected i.n. with influenza virus x31 (300 EID_{50}) and subjected to parabiotic surgery 35 days later. Day 21 after the surgery, mice were injected i.v. with 1 μg anti-CD8β 3 min prior to tissue harvest. Cells in the lung airways were recovered by BAL. Lung tissues were digested by collagenase D, and enriched by centrifugation in 40/80% Percoll gradient. Cells were stained with influenza $NP_{366-374}/D^b$ tetramer and fluorescent-conjugated antibodies. Data shown are derived from a CD45.2$^+$ parabiont. Plots shown in **(A)** indicate the gating strategy of host- and partner-derived antigen-specific CD8$^+$ T cells in the spleen, lung interstitium and airways. Bar graphs show ratio of host and partner cells among i.v. antibody negative cells in individual mouse. Plots shown in **(B)** indicate the expression of CD69, CD103, and CD49a on host- and partner-derived $NP_{366-374}/D^b$ tetramer$^+$ CD8$^+$ T cells in the lung airways and interstitium. Host cells are the mixture of a large proportion of T_{RM} (CD69$^+$ CD49a$^+$ CD103$^+$ and CD69$^+$ CD49a$^+$ CD103$^-$) and a minor population of T_{EM} (CD69$^-$ CD49a$^-$ CD103$^-$). The former population may include a small number of circulation-driven T_{RM} converted from host T_{EM}. The data also show how circulation-driven T_{RM} cells are a relatively small population and are difficult to identify in individual animals.

host CD69$^-$ CD103$^-$ CD49a$^-$ cells likely represent the host-derived T_{EM} population. It is interesting that a sizable fraction of host CD69$^+$ CD49a$^+$ cells in both the lung interstitium and airways lack the expression of another T_{RM} marker, CD103 (**Figure 1**) (6, 25). The lack of CD103 on some T_{RM} is consistent with subpopulation of T_{RM} found in the intestinal lamina propria, brain, and liver (26–28). In this regard, i.n. infection of CD103 knockout mice with influenza virus resulted in partial, but not complete, loss of CD8$^+$ T_{RM} in these tissues (29). These data also indicate that the CD103 marker does not efficiently discriminate T_{RM} from T_{EM} in the lung. Given the diversity of memory CD8$^+$ T cell populations in the lung, it is critical to precisely identify each population to avoid misinterpretation and confusion.

GENERATION AND MAINTENANCE OF CANONICAL T_{RM} CELLS IN THE LUNG

Following initial priming in the draining lymph nodes (LN), effector $CD8^+$ T cells migrate to the inflamed tissues where they receive local instructive signals that promote their subsequent differentiation into T_{RM} (9, 30). Transforming growth factor-β1 (TGF-β) is a common factor in most non-lymphoid tissues that drives T cell expression of CD103 and thereby promotes integrin αEβ7-mediated adhesion to E-cadherin on epithelial cells. A variety of cells, such as macrophages and stromal cells in the interstitium, and epithelial cells, are known to produce the latent form of TGF-β in the lung during early phases of an influenza virus infection (25, 31). As with the intestine (32–34), $CD103^+$ dendritic cells (DC) in the lung interstitium may play a role in the local conversion of TGF-β into the active form through integrin αvβ8, and promote the establishment of $CD103^+$ $CD8^+$ T_{RM} cells in the lung (35). In the absence of TGF-β signaling, $CD8^+$ T_{RM} cells in the whole lung (i.e., a mixture of cells in the airway and interstitium) completely lack the expression of CD103 (35, 36), although the number of antigen-specific $CD8^+$ T cells in the whole lung is not affected (36). This suggests that the establishment of $CD103^-$ $CD8^+$ T_{RM} cells in the lung interstitium and airways is not dependent of TGF-β.

Since there is limited space for cells to inhabit in normal lung tissue, newly created anatomical niches are required for the establishment and long-term maintenance of $CD8^+$ T_{RM} cells in the lung (6, 9, 37). Upon respiratory virus infection, infection-induced cytolysis and disruption of infected cells by antigen-specific effector $CD8^+$ T cells both contribute to tissue damage. A broad spectrum of cells including immune cells as well as basal cells (e.g., distal airway stem cells) accumulate at sites of damage to mediate the repair process which can be virtually observed as cytokeratin-expressing cell aggregates (Krt-pods) (38), thereby providing special niches for the establishment of $CD8^+$ T_{RM} cells in the lung interstitium (6, 37). Thus, lung $CD8^+$ T_{RM} cells may be specifically committed to protect weak spots (tissues undergoing repair) in the lung against reinfection (24). The structural characteristics of these T_{RM} depots (RAMD) differ from inducible bronchus-associated lymphoid tissue (iBALT) as most $CD8^+$ T_{RM} cells in the RAMD do not form organized lymphoid structures (iBALT consists of $CD4^+$ T cell cluster that surround B cell follicles) (6). This is consistent with the fact that unlike $CD4^+$ T cells and B cells that act cooperatively, $CD8^+$ T_{RM} cells can act alone upon recall. Furthermore, our timed parabiosis approach (joining pairs of mice at various time points before and after infection) clearly demonstrated that $CD8^+$ T cells recruited to the lung later than the peak of T cell response in the lung (around day 10 post influenza virus infection) failed to from T_{RM} (6). This indicates that lung T_{RM} niches are occupied at the peak of tissue damage and are no longer available for latecomer $CD8^+$ T cells including T_{EM} cells. It is well known that $CD8^+$ T_{RM} cells in the lung display relatively shorter longevity relative to T_{RM} in other tissues as T_{RM} cell-mediated heterosubtypic immunity to influenza virus lost at 4–6 months post-infection (5, 8). The decline in the size of the RAMDs overtime as tissue repair proceeds would explain the limited longevity of lung $CD8^+$ T_{RM} cells as compared to $CD8^+$ T_{RM} cells in other non-lymphoid tissues (6, 37). Similarly, the elevated proapoptotic activities of $CD8^+$ T_{RM} cells in the whole lung can be attributed to the concomitant loss of environmental factors that potentially support the homeostasis of T_{RM} (8).

It has been established that concurrent $CD4^+$ T cell responses also contribute to the establishment of $CD8^+$ T_{RM} cells in the lung (39). In contrast to other mucosa (female reproductive tract) where $CD4^+$ T cells play an indirect role in promoting optimal positioning of $CD8^+$ T_{RM} cells by triggering the local production of inflammatory chemokines (40), $CD4^+$ T cell help in the lung confers prolonged survival and improved functionality of $CD8^+$ T cells by transcriptionally modulating the metabolism to maintain higher spare respiratory capacity (41), a hallmark of T cell memory (42). $CD4^+$ T cell-derived IFN-γ also acts directly on $CD8^+$ T cells to downregulate the expression of T-bet. This leads to memory $CD8^+$ T cell rescue from T-bet-mediated repression of CD103, thereby promoting T_{RM} formation (43). Given the differential distribution of $CD8^+$ and $CD4^+$ T_{RM} (RAMD and iBALT, respectively), it seems likely that the primary involvement of $CD4^+$ T cell help during $CD8^+$ T_{RM} formation is exerted during the acute phase of infection (41).

A recent study has shown that cell-intrinsic factors also contribute to the durability of T_{RM} in the lung. $CD8^+$ T_{RM} cells generated from memory $CD8^+$ T cells that had previously experienced multiple antigen encounters exhibit superior longevity compared to these generated from naïve $CD8^+$ T cells (44). Reciprocal adoptive transfer approaches using a mixture of memory and naïve T cell receptor (TCR) transgenic T cells revealed that T_{RM} cells derived from memory cells preferentially occupy lung T_{RM} niches compared to T_{RM} cells derived from naïve cells (44). This suggests that there may be increased frequencies of T_{RM} precursors ($KLRG1^{lo}$ effector cells) among memory-derived $CD8^+$ T cells, compared to naïve $CD8^+$ T cells following activation in the draining LN. It is also possible that memory-derived $CD8^+$ T cells may be capable of receiving additional instructive signals, such as 4-1BB signals for up-regulation of pro-survival factors, when cells are recruited to the RAMD and acquire resultant longevity (45).

Cognate antigen-driven local reactivation is also indispensable for the establishment of lung $CD8^+$ T_{RM} cells. The best example for this is the impact of route of infection/vaccination on the establishment of $CD8^+$ T_{RM} cells in the lung. Intranasal (i.n.) infection elicits robust populations of $CD8^+$ T_{RM} cells in the lung interstitium and airways, whereas non-pulmonary route of infection do not (5, 6, 19, 23, 24, 35, 46–49). In the case of the skin and genital tract, forced recruitment of circulating $CD8^+$ T cells to the mucosa using inflammatory stimuli or topical administration of chemokines is sufficient to establish local T_{RM}, an approach referred to as "prime and pull" (50, 51). However, we and others have shown that the exposure of $CD8^+$ T cells to the lung environment is insufficient to promote subsequent

differentiation of these into long-lived lung T_{RM} (6, 23, 35). Instead, local reactivation induced by pulmonary administration of trace amount of antigen during the process of "prime and pull" is necessary for converting circulating $CD8^+$ T cells into lung T_{RM} cells (6, 23). Thus, both cell-intrinsic and extrinsic factors are necessary for complete conversion of these cells to T_{RM}. First, pulmonary administration of antigen generates antigen-bearing target cells that are eliminated by antigen-specific $CD8^+$ T cells, leading to the creation of damage and repair-associated T_{RM} niches (6). Second, local reactivation provides cell-intrinsic effects such as prolonged expression of CD69 and CD49a necessary for retention (6, 23), and upregulation of interferon-induced transmembrane protein 3 (IFITM3) for survival (52). Furthermore, TCR signaling may protect T_{RM} cells from a damage/danger-associated molecular pattern (DAMP)-induced cell death (53). Interestingly, there is differential expression of CD103 on distinct epitope-specific $CD8^+$ T_{RM} cells in the lung, irrespective of their localization, suggesting that difference in the extent of antigen presentation or subset of antigen presenting cells (APC) involved may also influence lung T_{RM} biology (25).

While it is unclear which APC provide local antigen signaling, the delivery of antigen to pulmonary DC by antibody-targeted vaccination (conjugate of antigen and antibody specific for DC) significantly facilitates the establishment of $CD103^+$ $CD8^+$ T_{RM} cells in the lung (35). Furthermore, $CD103^+$ respiratory DC are known to continually carry residual antigen from the lung to the draining LN, suggesting that respiratory DC are the primary source of local antigen signaling (54). Given the unique ability of $CD103^+$ respiratory DC to provide strong stimulatory signals in the draining LN, thereby generating effector $CD8^+$ T cells that preferentially home back to the lung (55), local reactivation by respiratory DC may promote terminal effector maturation rather than memory differentiation (56–58). Thus, other APC subsets, such as pulmonary macrophages, that accumulate in the RAMD during the early phase of infection may be necessary to provide the optimal antigen signaling required for T_{RM} development (59, 60).

CONVERSION FROM T_{EM} TO T_{RM}: A MINOR PATHWAY OF T_{RM} DEVELOPMENT IN THE LUNG

Despite the inefficiency of the non-pulmonary route of infection/immunization in establishing lung $CD8^+$ T_{RM} cells, several studies have nevertheless reported the deposition of $CD8^+$ T_{RM} cells in the lung following systemic infections (3, 61–63). Such blood-borne T_{RM} are derived from effector cells that have undergone less differentiation (defined as null to intermediate expression of CX3CR1 and lack of KLRG1 expression and including exKLRG1 cells that have downregulated this molecule during the contraction phase) (64, 65). Adoptive transfer of splenic memory clearly revealed the emergence of a small fraction of $CD103^+$ $CD69^+$ $CD8^+$ T cells in the whole lung (8). The appearance of $CD8^+$ T cells exhibiting T_{RM} phenotypes was also evident in our parabiosis experiments (**Figure 1**) (6), indicating that some levels of T_{EM} to T_{RM} conversion occurs in the lung. These cells exhibited a T_{RM} gene-expression signature and their tissue-residency was also confirmed by parabiosis (3, 61). Since several cytokines, such as TGF-β, IL-33, and tumor necrosis factor (TNF)-α, are reported to drive T_{EM} to T_{RM} conversion (66), the formation of blood-borne $CD8^+$ T_{RM} cells in the lung likely depends on TNF, and its effect is prominent in previously infected lung tissues as compared to naive lung tissues (8). Because partner cells are also detected in the lung airways after parabiotic surgery (**Figure 1**) (6), circulating memory $CD8^+$ T cells can reach to this tissue at basal levels, and CXCR3 plays a role in this recruitment (67). Treatment with pertussis toxin (PTx), which inhibits G protein-coupled chemokine receptors, significantly reduced the number of whole lung $CD8^+$ T_{RM} cells (including the dynamic population in the airways), suggesting that not only migration from the lung interstitium to the airway, but also the entrance of circulating $CD8^+$ T_{EM} cells into the lung depends on chemokine signaling (8). Despite their relatively low numbers, blood-borne lung $CD8^+$ T_{RM} cells confer some extent of protection against respiratory virus challenge (61–63). It should be emphasized, however, that this protection is far inferior to that mediated by *bona fide* lung $CD8^+$ T_{RM} cells generated by intranasal infection/immunization (5, 19, 23, 24, 48, 49). It is well known that the phenotype and function of memory $CD8^+$ T cells in the circulation continues to change over time after infection, with central memory T cells (T_{CM} cells) emerging as the predominant subset (64, 68–70). This leads to reduced numbers of memory $CD8^+$ T_{EM} that can be recruited to the lung and the eventual loss of a dynamic population of memory $CD8^+$ T cells in the lung (8).

FUTURE PERSPECTIVE

In **Figure 2**, we suggest a model by which the diverse populations of memory $CD8^+$ T cells are generated and maintained in the distinct compartments of the lung. Although the ontogeny of lung T_{RM} and T_{EM} differs, some levels of conversion from T_{EM} to T_{RM} occurs within the lung interstitium and also following recruitment to the airways. Furthermore, although lung airway memory $CD8^+$ T cells are a non-circulating population, the maintenance of their numbers depends on the continual influx of new cells from the lung interstitium. Thus, precise discrimination of each population is critical for future studies to avoid confusion in the field (2). Based on the model, it is likely that the limited longevity of conventional lung $CD8^+$ T_{RM} cells and eventual loss of blood-borne lung $CD8^+$ T_{RM} cells both contribute the rapid decay of total $CD8^+$ T_{RM} cells in this tissue (**Figure 2**). In other words, such a short-lived nature of lung memory $CD8^+$ T cells may, in a sense, be programed to avoid unnecessary pathogenesis in this tissue (71). Hence, multiple combinations of strategies to extend the longevity of both T_{RM} and T_{EM} should be considered for the development of vaccines against respiratory infectious pathogens. Since additional tissue damage is required to create new T_{RM} niches, strategies that enable the effective establishment

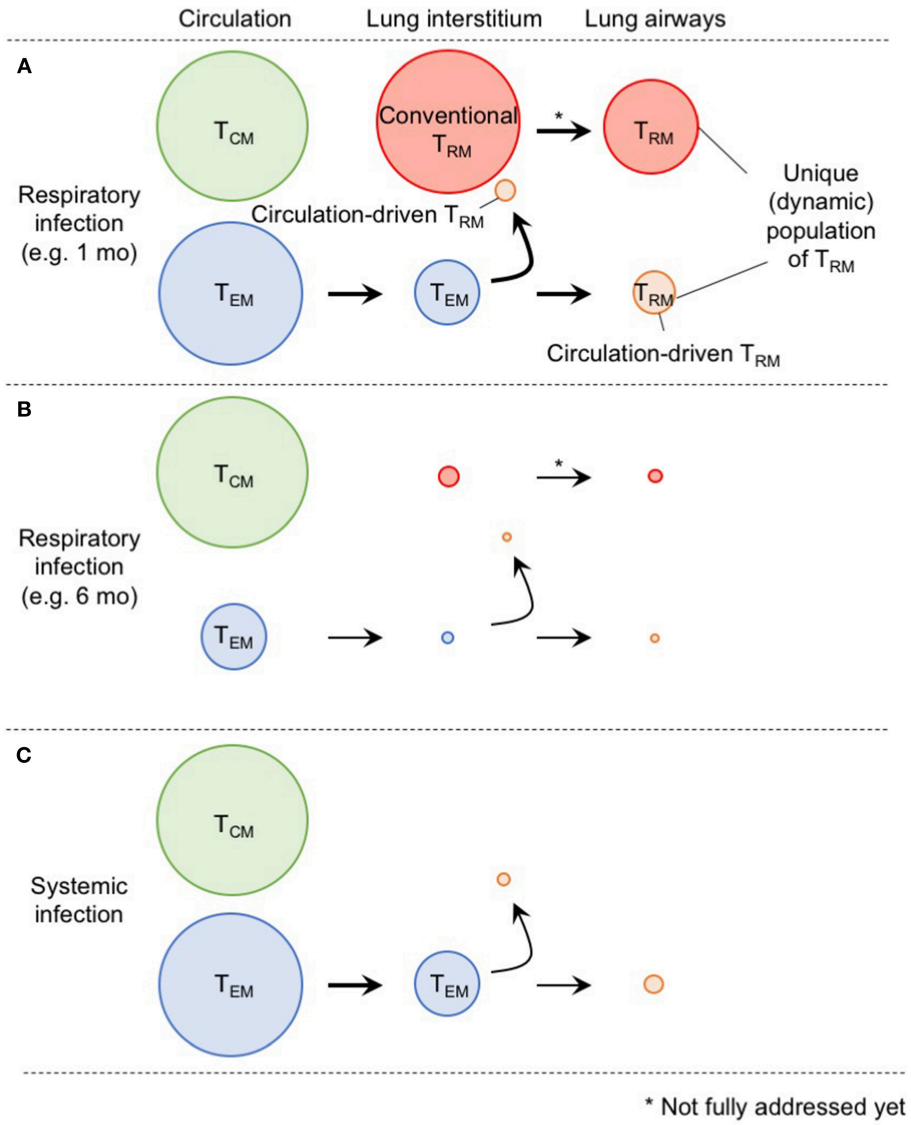

FIGURE 2 | A comprehensive picture of memory CD8$^+$ T cell populations in the lung. **(A)** Memory CD8$^+$ T cells in the lung interstitium comprise a major population of conventional T_{RM} and a smaller population of T_{EM}. Some of the latter also give rise to T_{RM} in response to TNF secreted in the conditioned lung that experience prior virus infection. Both host and partner cells in the interstitium are likely recruited to the lung airways and undergo phenotypic changes induced by environmental factors in this tissue. Although lung airway memory CD8$^+$ T cells represent non-circulating population, and thus, are recognized as T_{RM}, continual replacement is required for their maintenance. The size of the circles indicates the relative sizes of the respective populations in the lung. **(B)** As T_{EM} cells in the circulation decrease overtime after infection, input of T_{EM} to the lung interstitium and airways also decrease. Full recovery from the tissue damage, and resultant decrease of the size of RAMDs leads to reduction in the number of host CD8$^+$ T_{RM} cells in the lung interstitium and airways. Consequently, the animals lost CD8$^+$ T cell-mediated protective immunity in the lung. **(C)** Because of the lack of local antigen, *bona fide* CD8$^+$ T_{RM} cells are not generated in the lung interstitium and airways. Although some T_{EM} cells give rise to T_{RM} in the lung, the extent is less than infection-experienced lung.

of T_{RM} (including conversion from T_{EM} to T_{RM}) without the induction of undesirable pathogenesis should be considered in the future.

ETHICS STATEMENT

The studies utilizing laboratory animals were carried out in strict accordance with the Act on Welfare and Management of Animals of the government of Japan and the Regulations for the Care and Use of Laboratory Animals of Kinki University. The protocol for the present study was approved by the Institutional Animal Experimentation Committee of Kinki University Faculty of Medicine (Permit Number: KAME-26-025). All surgery was performed under anesthesia, and all efforts were made to minimize suffering.

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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PERSPECTIVE
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Imprinting and Editing of the Human CD4 T Cell Response to Influenza Virus

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Immunity to influenza is unique among pathogens, in that immune memory is established both via intermittent lung localized infections with highly variable influenza virus strains and by intramuscular vaccinations with inactivated protein-based vaccines. Studies in the past decades have suggested that the B cell responses to influenza infection and vaccination are highly biased by an individual's early history of influenza infection. This reactivity likely reflects both the competitive advantage that memory B cells have in an immune response and the relatively limited diversity of epitopes in influenza hemagglutinin that are recognized by B cells. In contrast, CD4 T cells recognize a wide array of epitopes, with specificities that are heavily influenced by the diversity of influenza antigens available, and a multiplicity of functions that are determined by both priming events and subsequent confrontations with antigens. Here, we consider the events that prime and remodel the influenza-specific CD4 T cell response in humans that have highly diverse immune histories and how the CD4 repertoire may be edited in terms of functional potential and viral epitope specificity. We discuss the consequences that imprinting and remodeling may have on the potential of different human hosts to rapidly respond with protective cellular immunity to infection. Finally, these issues are discussed in the context of future avenues of investigation and vaccine strategies.

Keywords: CD4 T cells, vaccine, human immunology, Influenza virus, imprinting

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OVERVIEW

Immunological memory to influenza is established by infection and vaccination. Epidemiological studies suggest that children in North America are typically infected with seasonal influenza at a rate of 5–15% each year, depending on age and history of vaccination (1–3). In the U.S., it is now recommended that all children at 6 months of age and older receive yearly vaccination (4). Currently licensed vaccines include either intranasal inoculation of cold adapted influenza vaccines (CAIV), such as Flumist®, or inactivated influenza vaccine (IIV), delivered via intramuscular injection. Typically, the first vaccinations are with IIV, delivered in infants as sequential vaccinations separated by 28 days between prime and boost. After 2 years of age, children can be administered CAIV intranasally, with the goal of boosting cellular and local immunity in the respiratory tract. Thus, by many different mechanisms, CD4 T cells specific for influenza viral antigens are established early in life. Worldwide, most adults have likely first encountered influenza by infection, because influenza vaccines were not widely used until the last two decades. In contrast, most young children in the US could have been exposed to influenza antigens first by vaccination.

The human host confronts influenza antigens in diverse forms and at somewhat unpredictable intervals through periodic infections and yearly vaccinations. How these different types of encounters with influenza virus and its antigens affect CD4 T cell memory and phenotype is critically important to understand, because this accumulated memory will influence all subsequent responses. Despite the importance of this issue, currently our knowledge is quite limited. The concept of "imprinting" of influenza immunity has garnered a great deal of interest recently but this has largely been in the context of the B cell response (5–8). Here we consider the potential impact of CD4 T cell imprinting and editing of the human CD4 T cell repertoire to influenza and the potential consequences this might have on protective immunity to infection.

CHARACTERISTICS OF THE CD4 T CELL RESPONSE TO INFECTION AND VACCINATION

Two aspects of the CD4 T cell response to infection are strikingly different from that of the B cell repertoire. First, the epitope specificity is tremendously diverse in human CD4 T cells, consisting of perhaps hundreds of different epitopes. This reactivity is determined in part by the multiple viral proteins targeted by CD4 T cells, stable binding of the antigenic peptide to MHC class II molecules (9–11) and by the precursor frequency of the CD4 T cells in the host to any given peptide (12). Even mice that express only one to two MHC class II molecules elicit CD4 T cells specific for 25–80 different peptide epitopes, distributed across both surface virion proteins such as hemagglutinin (HA) and neuramindase (NA), and internal virion proteins such as nucleoprotein (NP) and matrix protein (M1) (13–15). These antigen specificities have also been observed in humans (16–22). Due to expression of multiple HLA-class II isoforms and heterozygosity, humans can express as many as ten different class II molecules. As a result, they are likely to respond to a much wider array of peptide epitopes than do typical inbred mice. This complexity makes it extremely difficult to quantify reactivity to any particular influenza-derived peptide. Also complicating estimation of the diversity of the primary response of human CD4 T cells to infection are limitations in sampling tissues that are at the site of the response. Procedures are currently being developed to more broadly survey lymph nodes and the respiratory tract after infection (23–25). We believe that more efforts of this type are essential to understand the dynamic features of human immunity to influenza and long-term memory in the human host. However, at present, we can only estimate the breadth of human CD4 T cell immunity based on extrapolation of studies in animal models.

The second important distinction between human influenza-specific B cells and CD4 T cells is the functional complexity of the elicited response to infection. Accumulated studies to date have shown that the effector function and fate of CD4 T cells after priming by influenza infection are heterogeneous, and include follicular helper cells ("Tfh"), that remain in the lymph node for extended periods of time and facilitate B cell responses, prototypical Th1 cells that either enter recirculation or home to the lung to establish tissue resident memory, and cytotoxic CD4 T cells that are primarily detected in the respiratory tract [reviewed in (26)] (27, 28). Each of these subsets has distinct transcriptional profiles (29). The elements within the lung draining lymph node that control commitment to alternate fates of CD4 T cells are not well-understood. Differentiation decisions during CD4 T cell priming have been attributed to the local microenvironment, particularly cytokines (30, 31), but in the case of influenza infection, and dominant Th1 biased response, many other distinct functional subsets of CD4 T cells quickly emerge. Beyond the cytokine milieu, there are other parameters suggested to shape the CD4 T cell response to infection, including the impact of T cell receptor affinity (32, 33) and the epitope density that CD4 T cells encounter as they enter the antigen draining lymph node (34, 35).

In contrast to the diversity in specificity and functionality elicited by CD4 T cells in response to infection, vaccination with licensed vaccines is currently designed to elicit HA-specific neutralizing antibodies. Early vaccines were produced from isolated virions that were simply chemically inactivated prior to administration to humans (36). These early whole inactivated vaccines were highly immunogenic, likely due to the viral RNA content, and contained diverse influenza proteins (37). Since the 1960s, vaccine production has been progressively modified to be less reactogenic in order to increase compliance and safety, and to be more highly enriched for the HA protein, as our understanding of the role of neutralizing antibody in sterilizing protection from influenza has grown. Accordingly, the CD4 T cell responses to influenza vaccines have become focused in specificity and more limited in inflammatory response (38, 39). A recently licensed influenza vaccine now contains only pure HA proteins (Flublok® Quadrivalent) (40), with the relevant HA from each circulating strain isolated from transfected insect cells, thus further focusing the immune response to the HA proteins. Whether increasingly purified influenza vaccines endow the host with more or less protection from infection is not known at this time. This may ultimately limit the specificity of CD4 T cells to highly diverse HA proteins, diminishing cross protection against diverse influenza strains. Protein vaccines delivered in the absence of adjuvant to naïve individuals elicit CD4 T cells of limited functional complexity (41–44). Both of these features may limit the overall protective capacity induced by influenza vaccines.

IMPRINTING AND EDITING IN THE CD4 T CELL RESPONSE AMONG DIFFERENT AGE GROUPS AND INDIVIDUALS

By imprinting, we refer to the possibility that certain types of influenza confrontations, determined by age (e.g., the very young) or type (e.g., infection), permanently bias subsequent responses. Editing refers to the possibility that the CD4 T cell repertoire is remodeled with each subsequent encounter with influenza viruses and vaccines. Knowledge of these issues is essential in order to both predict and potentially design new

vaccines that most effectively poise the host for future immunity. Although imprinting in influenza immunity is most commonly discussed with regard to the B cell response, we propose here that imprinting may also have a dramatic impact on the specificity, phenotype and persistence of the CD4 T cell repertoire.

Unlike animal models of infection or vaccination that might experience primary and perhaps secondary immune responses, the human immune system is primed and boosted with influenza antigens numerous times over a lifetime. **Figure 1** illustrates the way these events may vary by the single parameter of age. The oldest individuals (>65 years of age) were likely exposed to influenza first through infection, and have had numerous subsequent exposures to distinct circulating H1N1, H2N2, H3N2, and influenza B viruses through infection [reviewed in (36, 47)]. Thus, based on periodic infections with different influenza viruses, we would expect that this oldest cohort of individuals would have accumulated a highly diverse CD4 T cell repertoire to distinct virus proteins. However, based on evolving vaccine recommendations, the immune repertoire of the over 65 cohort would have been perturbed by yearly vaccination for the past 1–2 decades [reviewed in (36, 48)]. Individuals in the 50–60 year old demographic may display the same pattern of early-life influenza virus exposure, but may not have received the yearly influenza vaccination suggested for older people. Conversely, children 15 years old and younger may have had their first confrontation with influenza through intramuscular vaccination with vaccines comprised of proteins from multiple virus strains, and enriched for HA. Whether and how frequently young children experience influenza infections is quite difficult to know with certainty, because many infections cause only mild disease, particularly among vaccinated individuals.

The simplest prediction of these scenarios is that older adults would have the largest epitope diversity of CD4 T cells, specific for many influenza virus proteins, with the most diverse functional potential, generated by each infection, while the youngest cohort might have a highly enriched HA-specific CD4 T cell repertoire generated largely by vaccination and perhaps boosted periodically by mild infections.

This simple model discussed above fails to account for several features of influenza immunity. First, in terms of the circulating repertoire of memory CD4 T cells that accumulates in humans, the potential requirement for periodic boosting to sustain CD4 T cell specificities is not clear. Also, it is not known if different functional subsets (e.g., Tfh vs. cytolytic cells) differ in this regard. Our own studies have shown that humans vaccinated with an H5N1 vaccine maintain some of the CD4 T cells specific for the unique H5-HA peptides for at least 5 years and that they can be recalled (49). This argues that if attrition does occur in humans, due to failure to boost, it is not complete within this time frame. Also, the impact of competition among CD4 T cell responses that likely occurs during complex immune responses, such as that induced by infection and vaccination is not yet well-understood, particularly during sequential, periodic confrontations (50, 51).

If intermittent boosting is required, some epitope specificities may become enriched for over time while others may decay. Current licensed inactivated vaccines typically contain some NP and M1, derived from the vaccine donor strain (52), which may be of sufficient quantity to boost pre-existing immunity generated by infection. Consequently, many humans may accumulate CD4 T cells specific for the most highly conserved epitopes within these internal virion proteins. The broad reactivity of these CD4 T cells could allow them to provide cross-reactive immunity against many influenza strains, particularly if their functional and lung homing potential induced by the original infection is maintained. Enrichment of these specificities over time with vaccination could be beneficial for the human host. If re-stimulation is required, then it is possible that unique epitopes in HA and NA proteins from viruses that are no longer in circulation disappear over time. Thus, the repertoire might be edited by "pruning" of some epitope specificities.

In support of the idea that adults may accumulate CD4 T cells specific for highly conserved HA-derived epitopes with age is a study showing that relative to younger subject, older adults display higher levels of highly conserved H1-reactive CD4 T cells, localized to epitopes mainly in the HA2 domain (53). In addition to the positive and negative effects of intermittent boosting of the CD4 T cells by conserved epitopes and losses due to attrition by neglect, it is also possible that there is loss of some potential epitope specificities due to the competitive advantage that memory cells have. Our laboratory has found that in sequential heterosubtypic infections in mice, CD4 T cells specific for NP epitopes that are conserved between the two viruses expand preferentially over new HA-derived epitope specificities present in the second virus (54), likely due to their higher abundance and greater sensitivity to antigen, both enhanced in memory T cells. Thus, editing of the CD4 T cell repertoire can depend on the sequence of viruses encountered. Also important to consider is that because of error prone polymerase in influenza virus, T cell epitopes in influenza proteins can accrue small mutations, leading to emergence of variants that may stimulate only a subset of the memory CD4 T cells. Documented evidence for this is more common with CD8 T cells because of the greater availability of MHC-peptide tetramers and well-defined short peptides of 8–10 amino acids, allowing easily deduced binding registers to MHC class I proteins. MHC class II molecules, in contrast, bind peptides of highly variable length (12–25 amino acids), due to a peptide binding pocket that is open at both ends and often have poorly delineated MHC binding registers. In animal models, well-defined variant peptides for CD4 T cells behave as altered peptide ligands, inducing modified functionality (55–59) or modified T cell receptor usage (60). An additional potential mechanism responsible for CD4 T cell repertoire editing, particularly after infection, are the possible negative effects of robust IFN-γ production on priming and expansion of new CD4 T cells. Human influenza-specific CD4 T cells in adults produce abundant IFN-γ (17, 18, 53, 61, 62) perhaps reflecting their original priming by infection. If these cells are recruited into the response to vaccination, elicitation of new CD4 T cell epitope specificities could be dampened via a complex network of suppression initiated by IFN-γ (10, 63, 64). It is known that T cell primed by infection can establish long-term memory in the respiratory tract (27, 65, 66), which

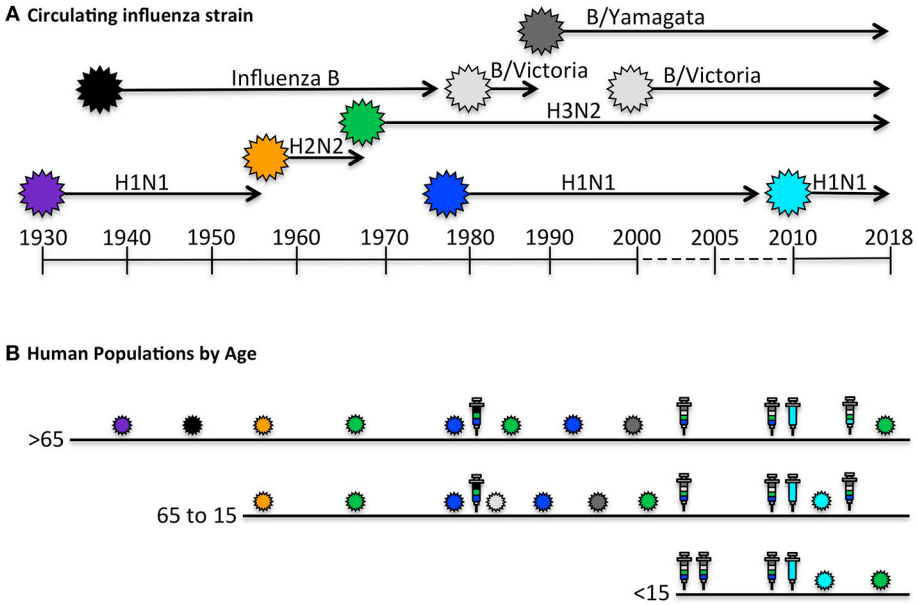

FIGURE 1 | Human exposure to influenza viral antigens. **(A)** Shown are the seasonal influenza strains that have circulating since the first H1N1 virus was isolated in 1933 (45–47). At times, there has been only a single strain documented to be circulating, such as H1N1 from 1933 to 1957, after which H2N2 was circulating for approximately a decade. Influenza B was identified in approximately 1940 and has been co-circulating since, in different lineages (Victoria and Yamagata). Influenza H3N2 reappeared in 1968 and H1N1 began to recirculate with H3N2 in 1977. The H1N1 "seasonal" virus was replaced in 2009 with the novel pandemic "swine origin" virus which has dominated with H3N2 and influenza B in the last decade. **(B)** The human immune system encounters influenza antigens intermittently through both infection and vaccination, depicted by the colored influenza virions indicated in **(A)**, and in syringes, respectively. Seasonal influenza vaccines, shown in multiple colors, contain HA derived from each circulating strain, while pandemic vaccine formulations contain a single HA. Persons over 65 years of age, indicated in B, have had decades of exposure to distinct H1N1, H2N2, H3N2, and Influenza B isolates via infection, but limited exposure to vaccination until later in life, when we expect they would have already accumulated a diverse CD4 T cell repertoire. Persons 15–65 years of age have likely encountered diverse viral strains via infection, and depending on age, have likely had intermittent vaccinations. In contrast to older age group, the youngest age cohort (<15 years old), may have had their first encounter with influenza derived antigens, especially HA, in the form of a prime-boost immunization. Thus, we predict that older adults would have a CD4 T cell repertoire with diverse antigen specificity and functional potential that was largely generated by infection, while younger individuals may have CD4 repertoire that is enriched in HA-specific cells and generated largely by vaccination and perhaps boosted periodically by mild infections. The specificity and function of the circulating memory populations in adults will depend on the factors discussed in the text.

endows them with the capacity for rapid protective responses to infection. It is possible that infection also seeds T cells in the periphery that preferentially return to the lung upon a secondary infection, based on their dominant Th1 phenotype and associated chemokine receptors (31) or priming via a lung draining antigen presenting cells after infection (67). Such infection-primed CD4 T cells may have priority for persistence as they were generated in the context of a robust inflammatory response and activation of many cells in the innate compartment.

ESSENTIAL STUDIES TO RESOLVE THE IMPACT OF CD4 T CELL IMPRINTING AND EDITING IN THE INFLUENZA SPECIFIC CD4 T CELL REPERTOIRE

Resolution of the mechanisms that might underlie imprinting and editing of the CD4 T cell response is exciting to consider. First, and probably most informative, are longitudinal cohort studies that track the evolving immune response to infection and vaccination from early childhood to adulthood, where immune confrontations could be precisely monitored and documented (68). The best design would encompass both unvaccinated subjects, who will likely be primed first by infection and perhaps sequentially with different virus strains, and vaccinated subjects, who may have their first encounter with inactivated vaccines. Also critical in identifying factors that control imprinting will be improvements in approaches that allow low abundance human CD4 T cells, specific for single or selected epitopes from vaccines or viruses, to be quantified and characterized in these longitudinal studies. With refinement of these approaches, the functional fate and persistence of elicited CD4 T cells can be evaluated. For example, use of selected HLA-peptide tetramers coupled with either single cell sequencing or multiparameter flow cytometry would be extremely valuable. Finally, because of the potential of heterologous immunity—immunity generated by unrelated pathogens—to play a role in T cell responses (69, 70), it would be valuable to begin to develop methods to identify the array of pathogens and vaccines that an individual has been exposed to that may have shaped their existing T cell repertoire, an option that is feasible with carefully monitored cohorts. If immunological imprinting is unique to early childhood infection, then it is possible that some vaccine-specific responses in adults are drawn from heterologous infections established in childhood and then boosted by vaccination. This CD4 T cell repertoire may be distinct in several ways. First, the responses

to vaccination might contribute to protection or lung pathology, depending on the effector phenotype elicited by the first infection (71, 72). Second, the cross- reactive response may have a narrowed breadth in TcR sequence, which might possess more limited efficacy and later cross-reactivity to variant influenza strains (70). With the help of advances in computational studies and data science, it may be possible to identify predictable events confronting the immune system that perturb and ultimately control the repertoire of CD4 T cells specific for influenza.

THE POSSIBILITY OF ELIMINATING THE "ONE SIZE FITS ALL" VACCINE STRATEGY

Currently, licensed vaccines are largely designed via a single platform with a limited and focused goal. Inactivated vaccines introduce HA from each circulating virus strain via intramuscular injection with the goal of eliciting neutralizing antibodies to the circulating influenza strains. The intranasal platform of Flumist, designed to provide more local and cellular immunity in the respiratory tract (73), has had uneven performance and appears to be most effective in young children (74). There have also been many recent initiatives to design vaccines that provide broadly protective immunity (75–80). With our increasing appreciation of the complexity and complementary nature of protective immunity to influenza, and specifically the multitude of functions that CD4 T cells play (27, 51, 81), there is likely to be increased focus on development of vaccines that prime or replenish particular specificities and functionalities. For example, if early-life exposures to influenza do effectively imprint the specificity and function of CD4 T cells, vaccines that establish the most robust and diverse repertoire of T cells may be most critical for infants and young children. In this regard, it is interesting to consider the potential consequence of widespread influenza vaccination beginning in infants. If childhood exposure is uniquely capable of imprinting specificity and functionality the immune system, then these early exposures to influenza primarily through vaccination might prime a limited CD4 repertoire. This repertoire could be enriched in HA reactivity Additionally, these CD4 T cells primed at peripheral sites without innate activators may have less lung homing potential and polyfunctionality and may instead be enriched for IL-2 or other Th2 biased responses, which are more typical of neonates (82). Conversely if adults who have received primarily inactivated, HA enriched vaccines are deficient in broadly reactive CD4 T cells, and are lacking established tissue resident memory cells, they may benefit from vaccine platforms that boost local immunity in the respiratory tract reactive with highly conserved proteins such as NP and M1. Alternate vaccine strategies for different individuals will require more sensitive and accurate approaches to define the components of the influenza specific immune repertoire that are deficient in the human host.

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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Gastric Subserous Vaccination With *Helicobacter pylori* Vaccine: An Attempt to Establish Tissue-Resident CD4+ Memory T Cells and Induce Prolonged Protection

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Tissue-resident memory T (Trm) cells are enriched at the sites of previous infection and required for enhanced protective immunity. However, the emergence of Trm cells and their roles in providing protection are unclear in the field of *Helicobacter pylori* (*H. pylori*) vaccinology. Here, our results suggest that conventional vaccine strategies are unable to establish a measurable antigen (Ag)-specific memory cell pool in stomach; in comparison, gastric subserous injection of mice with micro-dose of Alum-based *H. pylori* vaccine can induce a pool of local CD4+ Trm cells. Regional recruitment of Ag-specific CD4+ T cells depends on the engagement of Ag and adjuvant-induced inflammation. Prior subcutaneous vaccination enhanced this recruitment. A stable pool of Ag-specific CD4+ T cells can be detected for 240 days. Two weeks of FTY720 administration in immune mice suggests that these cells do not experience the recirculation. Immunohistochemistry results show that close to the vaccination site, abundant CD4+T cells locate on epithelial niches, independent of lymphocyte cluster. Paradigmatically, Ag-specific CD4+ T cells with a phenotype of CD69+CD103- are preferential on lymphocytes isolated from epithelium. Upon *Helicobacter* infection, CD4+ Trm cells orchestrate a swift recall response with the recruitment of circulating antigen-specific Th1/Th17 cells to trigger a tissue-wide pathogen clearance. This study investigates the vaccine-induced gastric CD4+ Trm cells in a mice model, and highlights the need for designing a vaccine strategy against *H. pylori* by establishing the protective CD4+ Trm cells.

Keywords: tissue-resident memory T cells, CD4+ T cells, subunit vaccine, *Helicobacter pylori*, immunological memory

INTRODUCTION

In addition to effector memory T (Tem) cells and central memory T (Tcm) cells, tissue-resident memory T (Trm) cells are the third subset of memory T cells that reside in the non-lymphoid tissues without entering recirculation (1). Proximity to the entry points of pathogens and their state of differentiation ensure that Trm cells can rapidly react to local infection (2–5). A study

reveals that a subset of effector T cells within non-inflamed tissues manipulate the potential to differentiate into Trm cells after adaption to local survival cues (6). However, in most scenarios of immunization/infection, Trm cells emerged after the resolution of local inflammation (1, 7). Trm cell populations are well-characterized in terms of Trm cells derived from CD8+ T cells or generated in response to invasive pathogens, but are less well-understood in terms of Trm cells derived from CD4+ T cells or generated in response to non-invasive pathogenic bacteria (3).

Helicobacter pylori (*H. pylori*) is a highly successful pathogen that colonizes the stomach of humans (8). Development of vaccines is one of the desirable alternative strategies to eliminate the threat of *H. pylori*. Previous clinical trials have demonstrated that many attempts fail to provide sufficient protection against *H. pylori* in human (9, 10). Evidence obtained from mice suggests a strong ability of this bacterium to alter the detection of pattern recognition receptors (PRRs) and subvert host immune system by producing multiple virulence factors (11). When facing this pathogen, host immune system is unable to orchestrate a potent response to purge the infection. Most infected individuals develop asymptomatic chronic gastritis, which sustains over their lifetimes if no antibiotic intervention. It is commonly accepted the need for CD4+ T cells, rather than CD8+ T cells or antibody-mediated responses, in providing protection (12, 13). Multiple studies using conventional vaccine strategies show that vaccination reduces *H. pylori* colonization in mice (13–18). Yet, the emergence of gastric Trm cells in these studies remains enigmatic. Dependence solely on recalling circulating memory T cells induced by conventional vaccination may result in a delay and "miss the boat" for optimal protection. Establishing a CD4+ Trm pool in stomach by vaccination and exploring the generation, maintenance, and behavior of these cells are attractive. However, the first-line challenges are how to send these pathogen-specific CD4+ T cells into the tissue "battlefield" and make sure that a CD4+ Trm pool can be detected. To address these gaps in the field, by using intracellular cytokine staining, we assessed the magnitude of antigen (Ag)-specific CD4+ cells after various vaccinations and found a measurable pool of Ag-specific CD4+ Trm cells in mice that vaccinated with micro-dose of Alum-based *H. pylori* vaccine in gastric subserosa layer (GSL). The characteristics and mechanism of protection against *H. pylori* were further investigated in these cells. This study proposes a notion that investigators should take into account a subset of Trm cells when planning an *H. pylori* vaccine strategy.

MATERIALS AND METHODS

Vaccine Preparation

Purified CCF protein and GEM particles were prepared and stored according to previous protocols (19, 20). Briefly, the CCF protein was expressed by *Escherichia coli* Rosetta (DE3) cells with pET-28a-CCF. The protein was first purified by nickel affinity chromatography (GE Healthcare), followed by anion-exchange chromatography with DEAE Sepharose FF (Amersham Pharmacia Biotech AB, Sweden). The purity of CCF was confirmed by Coomassie blue staining. The GEM particles were prepared by *Lactococcus lactis* NZ9000 cells using a hot-acid water bath. Vaccine with Alum was prepared with an equal volume of CCF solution and Alum adjuvant. CpG ODN 1826 was obtained from Sangon Biotech Co., Led. (China, Shanghai) and dissolved in CCF solution before intranasal vaccination.

Animals and Immunizations

Eight-week-old female C57BL/6J mice were obtained from the Comparative Medicine Center of Yangzhou University and bred at the China Pharmaceutical University Animal Experimental Center. All animal experiments were approved by the Animal Ethical and Experimental Committee of China Pharmaceutical University. The immunizations were performed according to the timetables in the figures and the doses of antigen and adjuvants are indicated in the figure captions or special region of the figure.

Gastric Subserous Layer Vaccination

Mice were anesthetized with 15 mg/kg Xylazine and 100 mg/kg Ketamine, and placed on a body temperature heating pad. After shaving the right abdomen, a 1.5 cm incision was made above the stomach. After laparotomy, the stomach was localized, and 5 μl vaccine preparation (Volume, CCF solution: Alum = 1:1, containing ∼7.5 μg CCF) was injected into the gastric subserous layer of the greater curvature using a Hamilton syringe with a 33 G needle. Then, suturing with PGA absorbable sutures was performed using uninterrupted sutures for the peritoneum and interrupted sutures for the skin incision (Shanghai Pudong Jinhuan Medical Products Co., Ltd.).

Preparation of Single-Cell Suspensions From Gastric Tissue

Single-cell suspensions were prepared as a previous study with modifications (21). Briefly, the whole stomach was isolated, cut through the lesser curvature, and the contents were removed before being placed into 15 ml RPMI 1640 containing 10 mM HEPES, 10% FBS, 4 mM EDTA, and 0.5 mM dithiothreitol. Gastric epithelial lymphocytes were isolated by shaking at 250 rpm and 37°C for 30 min. Tissues were then minced and incubated with another 15 ml RPMI 1640 containing 10 mM HEPES, 10% FBS, 4 mM EDTA, and 0.5 mM dithiothreitol for 15 min to isolate the remaining lymphocytes. Supernatants were passed through a 70 μm cell strainer. After washing and centrifugation, cell pellets were resuspended in an appropriate medium for further analysis or culture.

Preparation of Single-Cell Suspensions From Lymphoid Organs and Blood

The spleen and mesenteric lymph nodes were isolated and gently pushed through a 70 μm cell strainer. After extensive washing, cells from the lymph nodes were collected. The cells from the spleen and blood were suspended in 7 ml erythrocyte lysis buffer (Biolegend) and washed twice with 10 ml PBS containing 5% FBS. Cells were collected for FACS analysis or stimulated *in vitro*.

Antigen-Specific CD4+ T Cell Analysis

Single-cell suspensions from the stomach were purified with 67/44% Percoll gradients. The cells at the interface were collected and washed with 7 ml RPMI 1640 containing 10% FBS. To detect

Ag-specific CD4+ T cells, purified single-cell suspensions from the stomach, MLN, spleen or blood were stimulated with 1×106 naïve, CFSE-labeled splenocytes that were preloaded with CCF in RPMI 1640 containing 10% FBS and 5 μg/ml BFA for 12 h. After collection, cells were stained for intracellular cytokines.

FACS Analysis

For IFN-γ and IL-17 intracellular cytokine staining, *in vitro* restimulated cells were first stained with anti-CD4 (GK1.5) and anti-CD90.2 (30-H12) antibodies, then fixed and permeabilized with Intracellular Staining Fixation/Permeabilization Wash Buffer (Biolegend, San Diego, CA) and stained intracellularly with anti-IFN-γ (XMG1.2) and anti-IL-17 (9B10) antibodies. For cell phenotype detection, single-cell suspensions were stained with the following antibodies: anti-CD3ε (145-2C11), anti-CD90.2 (30-H12), anti-CD45 (30-F11), anti-CD4 (GK1.5 or RM4-4), anti-CD11b (M1/70), anti-CD8α (53-6.7), anti-CD19 (6D5), anti-MHC class II (M5/114.15.2), anti-CD69 (H1.2F3), anti-CD25 (3C7), anti-CD44 (IM7), anti-CD103 (2E7), anti-TCRγδ (UC7-13D5), anti-Ly6C (HK1.4), anti-Gr-1 (RB6-8C5), anti-CD11c (N418), and anti-F4/80 (BM8) purchased from Biolegend or BD Pharmingen. Multiparameter analyses were performed on a BD FACS Aria II or a BD FACS Calibur flow cytometer.

Immunofluorescent Histology

For gastric histology, the longitudinal specimens were fixed with 4% paraformaldehyde, embedded in paraffin, and stained with hematoxylin and eosin (HE). For CD4 immunofluorescent staining, 20 or 10 μm frozen sections were cut and dried at room temperature. After blocking, these sections were stained with an Alexa Fluor® 488-anti-CD4 (GK1.5, Biolegend) antibody and/or purified anti-CD11b (M1/70, Biolegend) or anti-CD8α (53–6.7, Biolegend) antibody followed by goat anti-rat IgG2a/IgG2b Alexa Fluor® 488/594 antibody (Biolegend). The slides were washed and counterstained with DAPI to visualize cell nuclei, and images were acquired with a Panoramic 250 Flash III Scanner (3D Histech). The number of CD4+ cells in each section was counted in a 0.5 μm × 0.5 μm area with highest CD4+ cell signaling.

Quantitative RT–PCR

Gastric RNA extraction and reverse transcription were carried out as described previously (22). PCR amplification was performed with a conventional TaqMan method. TaqMan gene primers and probes were designed by Sangon Biotech Co., Led. (China, Shanghai) based on the following sequence numbers: CCL5, Mm01302427_m1; CXCL9, Mm00434946_m1; CXCL10, Mm00445235_m1; GADPH Mm99999915_g1.

FTY720 Treatment

For FTY720 treatment, 1 mg/kg FTY720 was injected i.p. daily to block circulating memory T cell egress from the lymphoid nodes according to the design of the experiments.

Neutralizing Antibody Experiments

Immune mice were i.p. injected with 100 μg anti-CD4 antibody (GK1.5, BioXcell), anti-RatIgG1, anti-IFN-γ (XMG1.2, BioXcell) and anti-IL-17A (17F3, BioXcell) antibody every 2 days to deplete CD4+ T cells, IFN-γ and IL-17A according to the design of experiments.

H. pylori Challenge

Helicobacter pylori SS1 was cultured as previously described (22). Sixty days after the last vaccination, the mice were challenged with 1×10^9 CFU *H. pylori* SS1 (determined by turbidimetry) by gavage in 200 μl of 0.2% sodium bicarbonate solution.

Quantitative Culture of *H. pylori*

Quantitative culture of *H. pylori* was performed as previously described (22). Briefly, half of the stomach was homogenized in 500 μl Brain Heart Infusion (BHI) broth and plated at a series of dilutions on BHI plates. The bacterial colonization was calculated at the whole organ level.

Statistics

GraphPad Prism 7.0 software was used for statistical analyses. The differences between the groups were assessed using the Kruskal–Wallis test or Mann–Whitney *U*-test. $P < 0.05$ was considered statistically significant.

RESULTS

Ag-specific CD4+ Effector T Cells Are Present in Stomach After Conventional Vaccinations, but Fail to Give Rise to a Formidable CD4+ Memory T Cell Pool

An outstanding question in the field of *H. pylori* vaccinology is whether conventional vaccinations can induce an Ag-specific CD4+ cell population in the stomach. Here, we used a recombinant *H. pylori* subunit vaccine, CCF, as a model Ag. CCF was constructed by multi-epitopes from *H. pylori* urease, and self-adjuvant regions from *Salmonella typhimurium* phase I flagellin FliC and cholera toxin B (20). To detect Ag-specific CD4+ T cells in stomach, we isolated total purified leukocytes from whole stomach of immune mice and co-cultured these cells with 1×10^6 Ag-preloaded, CFSE-labeled naive splenocytes for 12 h in the presence of Brefeldin A (BFA). Two crucial effector cytokines, IFN-γ and IL-17A, for anti-*H. pylori* immunity were used to identify Ag-specific T cells. In the preliminary data, we found that for conventional vaccinations, Ag-specific CD4+ cells that produced only IFN-γ or IL-17A were rare, but the combination of IFN-γ and IL-17A allowed for the detection of more Ag-specific CD4+ T cells in these groups (**Supplementary Figure 1**).

Previous studies suggest that specific vaccinations can evoke a transient state that which allows Teff cell migration into non-lymphoid tissues at effector stage (6, 23). To detect gastric Ag-specific CD4+ T cells after conventional vaccinations, we performed different vaccine administrations on the mice and compared the gastric Ag-specific CD4+ T cells at Day 7 and Day 30 (**Figures 1A,B**). Naïve mice were used as a negative control to exclude non-specific staining, and mice receiving gastric subserosa layer (GSL) vaccination were used as a positive control. Ag-specific CD4+ T cells could be observed on Day 7 in stomach of mice receiving subcutaneous (s.c.), intranasal

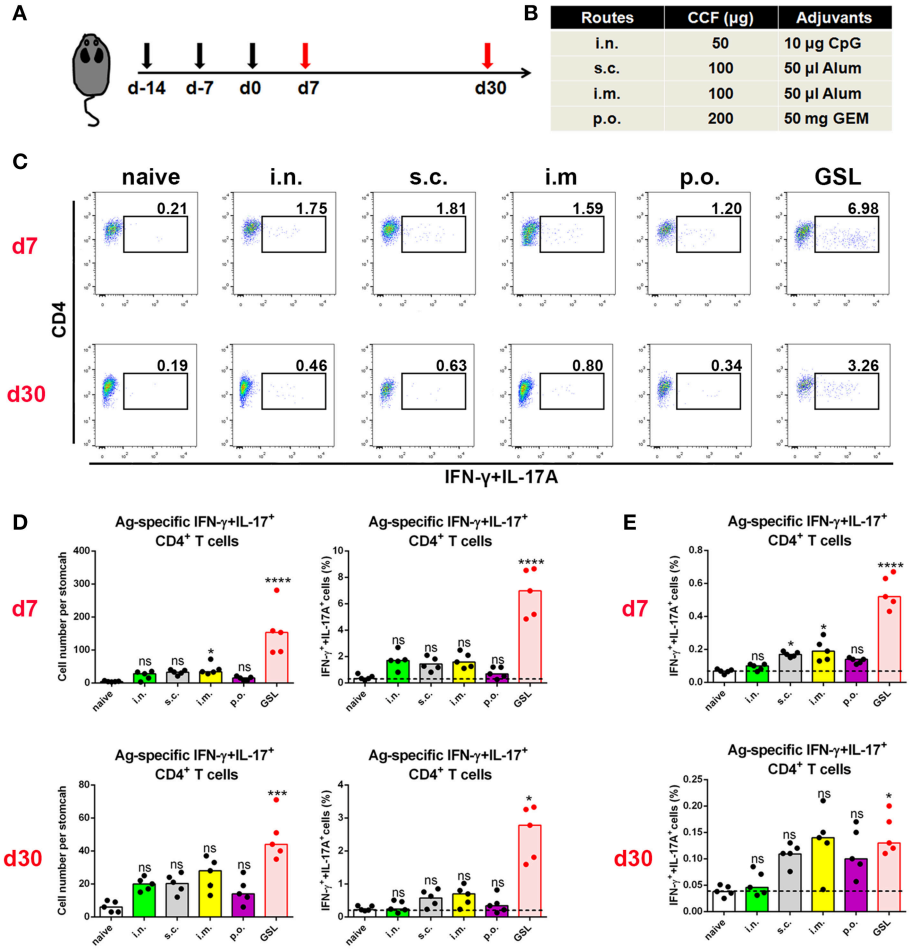

FIGURE 1 | Conventional vaccinations failed to induce a durable Ag-specific CD4+ memory T cell pool in the stomach. **(A)** C57BL/6J mice were immunized at Day-14, Day-7, and Day 0 with different vaccine strategies **(B)**. At Day 7 and Day 30 after the last vaccination, mice were sacrificed and the Ag-specific CD4+ T cells in stomach were measured by intracellular cytokine staining. Purified cells were restimulated with Ag-preloaded, CFSE-labeled, naïve splenocytes for 12 h in the presence of 5 μg/ml BFA. IFN-γ- and/or IL-17A-producing CD90.2+CD4+ cells were identified as Ag-specific CD4+ T cells. At Day 7 (top) and Day 30 (bottom) after the last vaccination, gastric Ag-specific CD4+ T cells from these immunized mice were analyzed **(C)**. Absolute number and frequencies of gastric Ag-specific CD4+ T cells among total CD4+ T cells at Day 7 (top) and Day 30 (bottom) were quantified **(D)**. The frequencies of Ag-specific CD4+ T cells from MLN among total CD4+ T cells at Day 7 (top) and Day 30 (bottom) were quantified **(E)**. In all graphs, dots represent individual data points and columns represent median values. $^{*}P < 0.05$, $^{***}P < 0.001$, $^{****}P < 0.0001$, *ns* = not significant. The Kruskal–Wallis test (vs. naïve) was used. Data were pooled from two individual experiments with $n = 5$ mice per group.

(i.n.), intramuscular (i.m.) and oral (p.o.) vaccinations, but the number was much lower than that in the GSL control mice (**Figures 1C,D**). To investigate whether these Ag-specific CD4+ T cells could form a gastric memory T cell pool, we compared the number of gastric Ag-specific CD4+ T cells among different groups on Day 30. Results revealed that few Ag-specific CD4+ T cells were detected in these groups except for GSL (**Figures 1C,D**). Moreover, the expansion of Ag-specific CD4+ T cells was observed on Day 7 in the MLN from mice receiving i.m. or s.c. administration (**Figure 1E**). These findings suggest that conventional vaccine strategies can drive some of Ag-specific CD4+ T cells presenting in stomach, but these cells fail to give rise to a formidable memory T cell pool.

Gastric Subserosa Layer Vaccination Recruits Abundant Ag-specific CD4+ T Cells Into Stomach

Development of an *in situ* vaccine strategy might be of utmost importance to establish a strong CD4+ Trm pool in stomach (24, 25). GSL injection has been used for local anesthesia and for the development of gastric ulcer or *in situ* tumor animal models. Given that orientation of visible blood vessels is from lesser curvature to greater curvature, we hypothesized that the non-vascular zone of greater curvature was a feasible region to establish a local Ag depot. To verify this, we formulated CCF with Alum adjuvant, performed the laparotomy to access stomach, and injected 5 μl vaccine into GSL (**Figures 2A,B**). Acute

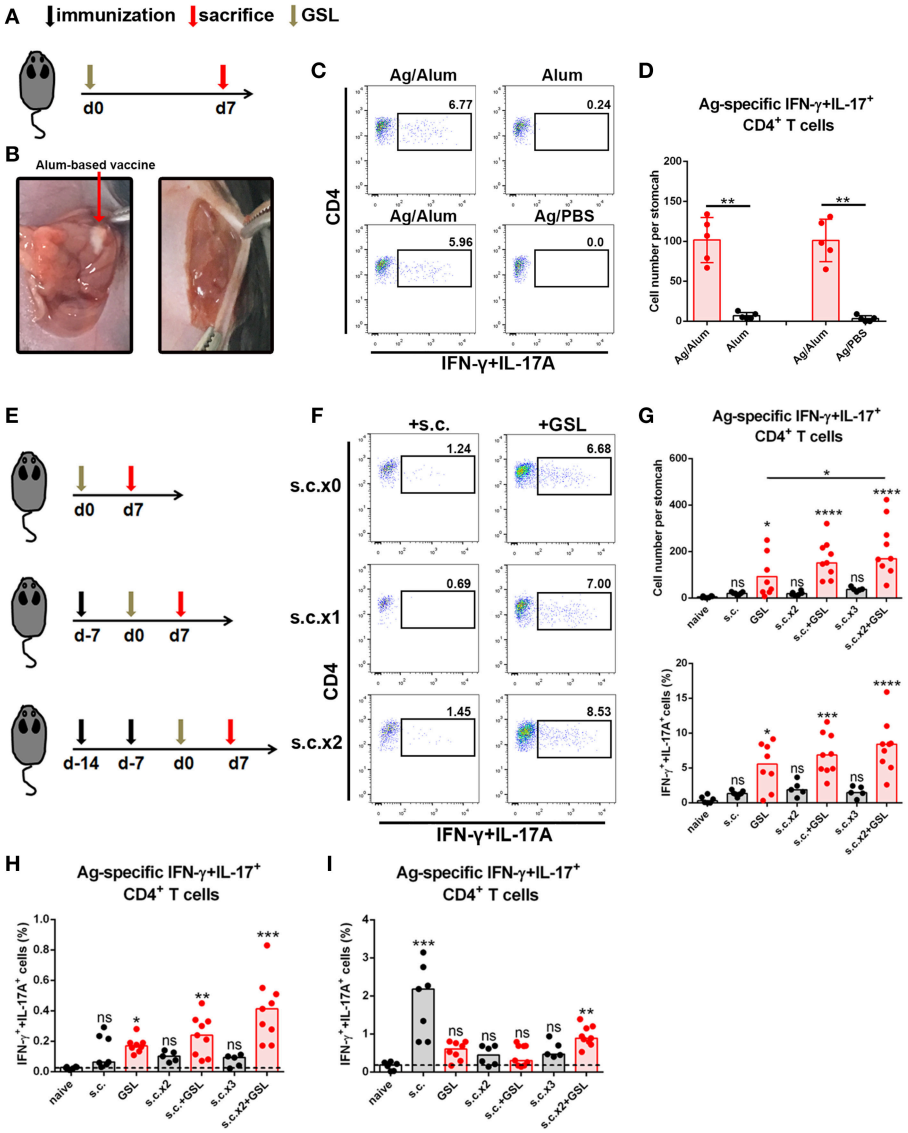

FIGURE 2 | A vaccine strategy triggered abundant Ag-specific CD4+ T cells infiltration of the stomach. **(A)** C57BL/6J mice were immunized at Day 0 by GSL and sacrificed on Day 7. **(B)** Details for GSL vaccination are as follows: an incision was made above the stomach and 5 μl vaccine formulation (containing ~7.5 μg Ag) was injected into the subserosa layer of the stomach. The incisions in peritoneum and skin were sutured. **(C)** Gastric Ag-specific CD4+ T cells were analyzed in mice that GSL vaccinated with Ag/Alum, Alum and Ag/PBS. **(D)** Absolute number of gastric Ag-specific CD4+ T cells at Day 7 were quantified **(E)** C57BL/6J mice were immunized with one of six different strategies. **(F)** Gastric Ag-specific CD4+ T cells in each group were analyzed as described before. Absolute number and frequencies of gastric Ag-specific CD4+ T cells among total CD4+ T cells at Day 7 were quantified **(G)**. The frequencies of Ag-specific CD4+ T cells from MLN **(H)** and spleen **(I)** among total CD4+ T cells were quantified. In all graphs, dots represent individual data points and columns represent median values. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$, *ns* = not significant. The Kruskal–Wallis test (vs. naïve) or Mann–Whitney U test (for two groups) was used. Data were pooled from six individual experiments with $n = 5$–9 mice per group.

inflammation, which was characterized by mucosal swelling, was observed in the vaccination site, and abundant Ag-specific CD4+ T cells could be detected 5–7 days later. To clarify the requirement for Ag-specific CD4+ cell recruitment in GSL vaccination, mice were injected with Ag/Alum, Alum alone, or Ag/PBS solution, respectively. Injection of Alum alone failed to recruit any Ag-specific CD4+ T cells, but induced regional tissue swelling (**Figures 2C,D**). In comparison, GSL injection of Ag/PBS solution did not recruit Ag-specific CD4+ T cells into stomach either, and no tissue alteration was observed in the injection site. We found that Ag/PBS solution could be absorbed within hours after injection, indicating that a sustained-release vehicle (i.e., Alum adjuvant) was necessary for Ag-specific T cell recruitment.

In consideration of the low dosage of vaccine used in GSL vaccination, we modified the vaccine strategy with an additional

step to create the following protocol: i) subcutaneous vaccination to induce a systemic Ag response, followed by ii) a surgical operation to inject a micro-dose of vaccine into GSL. To test this strategy for the induction of an Ag-specific T cell response, we performed six kinds of vaccination programs that included multiple rounds of subcutaneous vaccination and/or a GSL vaccination (**Figure 2E**). As expected, some of gastric Ag-specific CD4+ T cells were detected in mice receiving subcutaneous vaccination alone, but abundant these cells were observed in GSL-vaccinated mice. Performing two rounds of subcutaneous vaccination significantly boosted the number of Ag-specific CD4+ T cells recruited by GSL vaccination (**Figures 2F,G**). We also examined the percentages of Ag-specific CD4+ T cells in spleen and MLN. s.c. vaccination did not induce plenty of Ag-specific CD4+ T cells in MLN, whereas Ag-specific CD4+ T cells were abundant in mice receiving s.c. vaccination plus GSL vaccination (**Figure 2H**). In the different vaccination programs, the levels of splenic Ag-specific CD4+ T cells were variable (**Figure 2I**). We also tested whether GSL injection of mice with Alum or Ag/PBS solution after s.c. vaccination could induce plenty of Ag-specific CD4+ T cells into stomach. The results showed that without regional Ag exposure and adjuvant-induced inflammation, recruitment of Ag-specific CD4+ T cells was limited (**Supplementary Figure 2**). These results indicated that introduction of a systemic Ag-specific response enhanced the regional Ag-specific CD4+ T cell recruitment induced by GSL vaccination.

Taken together, these data indicate that the local Ag encounter, Ag vehicle phase and systemic immune response all contribute to the maximum recruitment of Ag-specific CD4+ T cells induced by GSL vaccination.

Ag-specific CD4+ T Cells Retain in Stomach Long-Term Without Recirculation

To investigate whether a durable Ag-specific CD4+ Trm pool was formed after s.c.x2 +GSL vaccination, we counted the number of Ag-specific CD4+ T cells in stomach over the following 8 months (**Figure 3A**). Age-matched naïve mice were served as negative controls to exclude the influence of age. In immune mice, the granulation tissue induced by the Alum adjuvant was stiffened at Day 30 and shrank in the following months (**Figure 3B**). No gland atrophy or metaplasia was observed in the mucosa near the vaccination site during Days 30–60 (**Supplementary Figure 3**). On the other hand, contraction of the infiltrating Ag-specific CD4+ T cell population was complete within 30 days and a stable Ag-specific CD4+ memory T population could be detected for at least 240 days (**Figures 3C,D**).

In the next step, to investigate whether these CD4+ memory T cells experienced recirculation via the lymphovascular system, we treated immune mice with FTY720 daily to inhibit lymphocyte egress from lymph nodes for 14 days (**Figure 4A**). Even though circulating CD4+ T cells were decreased by more than 100-fold in blood and Ag-specific CD4+ memory T cells vanished from blood, the number of Ag-specific CD4+ memory T cells was stable in stomach (**Figures 4B,C**), suggesting a characteristic of local retention. Moreover, the absolute number of Ag-specific CD4+ memory T cells was not altered in MLN, but significantly decreased in spleen after FTY720 administration (**Figures 4B,C**).

To verify whether these Ag-specific CD4+ Trm cells were sensitive to systemic CD4 antibody depletion, we i.p. injected immune mice with anti-CD4 antibody (**Figure 4D**). No CD4+ T cells could be detected in blood or spleen, whereas Ag-specific CD4+ cells in stomach remained numerically unchanged and measurable, although with lower CD4 expression (**Figures 4E,F**), implying their retention in a distinct anatomical location which was less affected by circulation. Collectively, these data reveal that infiltrating Ag-specific CD4+ Teff cells can form a long-lived Trm pool and most of these cells may be separated from circulation.

Distribution of These CD69+CD103-CD4+ Trm Cells Is Dependent on Epithelial Architecture of Stomach

We next examined the location of CD4+ T cells induced by GSL vaccination. Stomach was isolated and cut through lesser curvature. As shown in **Figure 5A**, vaccination region was sniped longitudinally and used for immunohistological staining of CD4+ T cells. Six groups with different vaccination programs were involved in this experiment. Mice were sacrificed on 7-day or 30-day post GSL vaccination. The results indicated that abundant CD4+ T cells could be observed in the gastric mucosa near the vaccination site at 7-day post GSL vaccination (**Figures 5B,D**). Infiltration of CD4+ T cells was restricted to the adjacent mucosa, as few CD4+ cells presented in the mucosa of non-vaccination site. Density of CD4+ T cells was not associated with Ag exposure and prime. Extremely low density of CD4+ T cells was observed in stomach of naïve and s.c. immunized mice in the same perspective. Additionally, only some CD4+ T cells appeared around the vaccination site, even though the surrounding region was enriched with immune cells (**Figure 5D**). At 30-day post GSL vaccination, density of CD4+ T cells on mucosa of vaccination site was decreased (**Figures 5C,E**). Notably, at this time point, most CD4+ T cells were located close to the epithelium, which was confirmed by the distinct epithelial architecture of stomach (**Figures 5E,F**). For instance, in the body mucosa close to the cardia equivalent, CD4+ Trm cells formed a chain; in the middle of the body mucosa, CD4+ Trm cells were evenly distributed in the epithelial region of gastric pit; and in the transition region, both two retention patterns could be observed. Moreover, 10 μm sections of gastric tissue indicated that these CD4+ T cells were in contacted with the epithelial cells (**Figure 5E**, bottom). Parallelly, detection of Ag-specific CD4+ T cells in the epithelium or lamina propria also suggested that at memory stage, most of Ag-specific CD4+ cells were located on epithelial regions (**Figures 5G,H**). These data demonstrate that CD4+ T cells recruited by GSL vaccination preferentially infiltrate the adjacent mucosa and survive in the special niches of the epithelium.

Previous studies suggest that CD4+ Trm cells are maintained in vagina, intestine and skin, and cluster with macrophages, dendritic cells (DCs) and CD8+ T cells (24, 26, 27). To examine whether other immune cells were responsible for CD4+ Trm

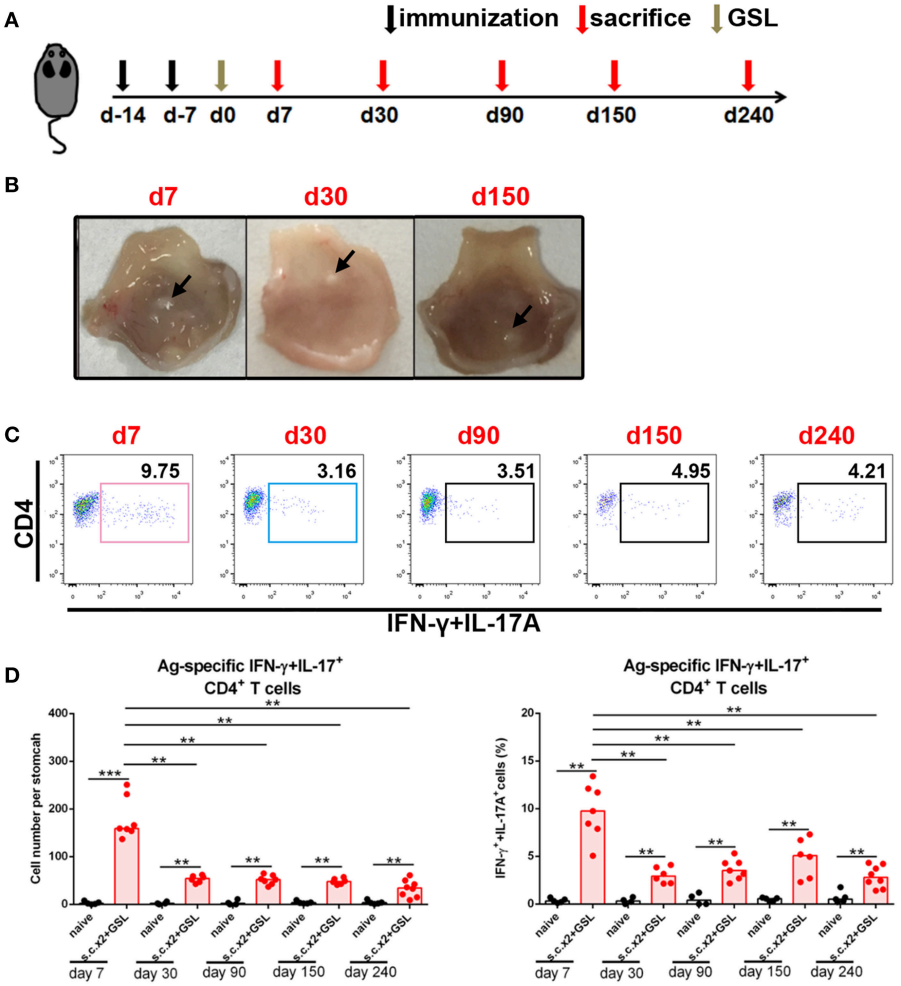

FIGURE 3 | Ag-specific CD4+ Teff cells contracted and sustained in stomach long-term. **(A)** C57BL/6J mice were s.c. immunized with Ag/Alum at Days-14 and-7, and treated with GSL vaccination at Day 0. **(B)** Mice were sacrificed on Day 7, Day 30, and Day 150, and their stomachs were unfolded. Arrows indicated granulation tissue in the vaccination sites. **(C)** Mice were sacrificed on the day indicated in the timetable (red arrows), and the gastric Ag-specific CD4+ T cells in each time point were analyzed as described previously. **(D)** Absolute number and frequencies of gastric Ag-specific CD4+ T cells among CD4+ T cells at each time points were quantified. In all graphs, dots represent individual data points and columns represent median values. $^{**}P < 0.01$, $^{***}P < 0.001$. Mann–Whitney U test was used to compare two groups. Data were pooled from six individual experiments with $n = 4$–8 mice per group.

cell residence in stomach, we analyzed the types of infiltrated immune cells at whole organ level on Day 7 and Day 30 (**Figure 6A**). All cell types except macrophages, i.e., neutrophils, inflammatory monocytes, mo-DCs, B cells, γδ T cells, CD4+ T cells and CD8+ T cells, were expanded on Day 7 (**Figure 6B**). However, flow cytometric analysis showed that most of the immune cells seceded from stomach before Day 30, suggesting that the increased gastric immune cell content induced by GSL vaccination was not sustainable at the whole organ level. To visualize the regional relationship between CD4+ T cells and innate immune cells, an immunolocalization assay was performed and the images indicated no direct relationship between the CD4+ and CD11b+ cells during the memory stage, as the CD11b+ cells preferentially enveloped the vaccination site (**Figure 6C**). Next, we measured several chemokines CCL5, CXCL9 and CXCL10 that are critical for CD4+ T cell recruitment (26). Compared with memory stage, CCL5 and CXCL10 levels were significantly increased in the vaccination site during effector stage (**Figure 6D**). Interestingly, at memory stage, CCL5, CXCL10, and TGF-β1 levels at the vaccination site were lower than those of naïve mice (**Figure 6E**). We reckoned that the granuloma structure might affect regional homeostasis. In total, these data demonstrate that a distinct migration and retention pattern of CD4+ T cells is induced by GSL vaccination.

Next, we isolated intraepithelial T cells from the vaccination site and analyzed their phenotypes at the memory stage. CD4+ T cells from the vaccination site expressed CD69 and CD44, but expressed little CD103 and no CD62L; on the contrary, CD8+ T cells in this region expressed CD103, CD69, and CD44 (**Figure 6F**). Parallelly, we measured the expression of

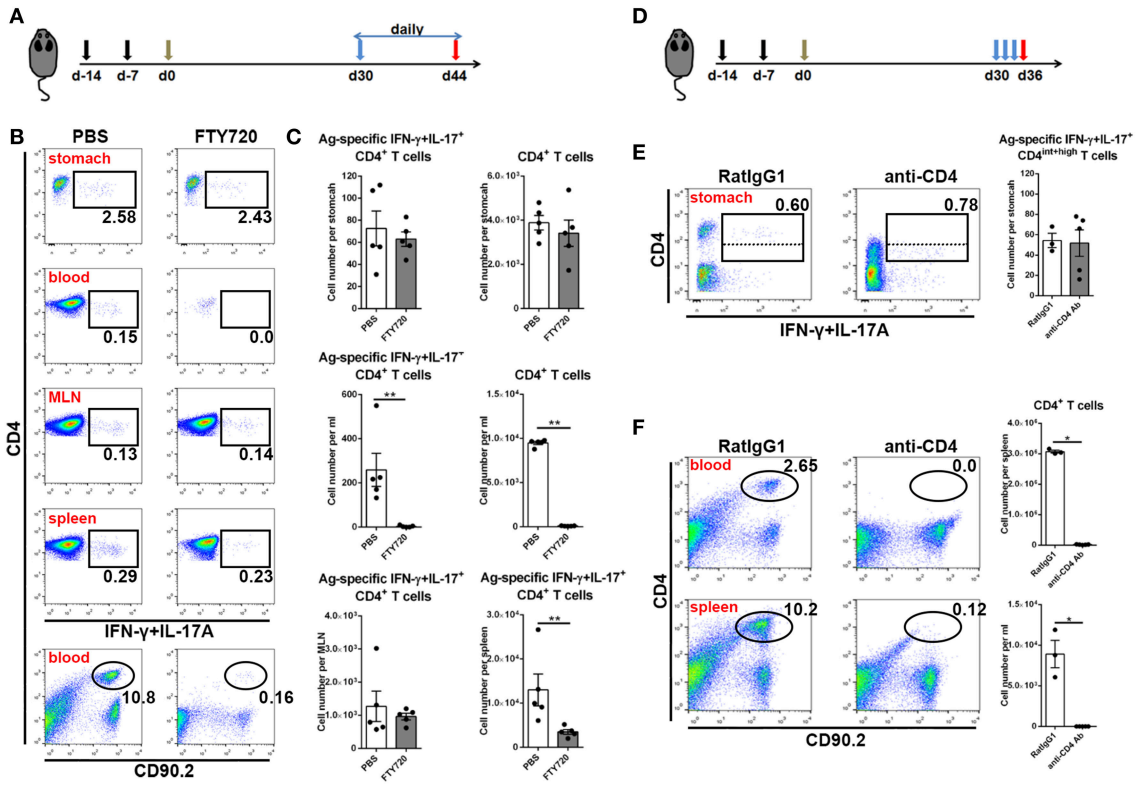

FIGURE 4 | Gastric Ag-specific CD4+ memory T cells induced by GSL vaccination experienced little recirculation and were located separately from the blood. **(A)** C57BL/6J mice were s.c. immunized with Ag/Alum at Days-14 and-7, and treated with GSL vaccination at Day 0. Thirty days later, the mice were i.p. injected with 1 mg/ml of FTY720 or PBS daily for 14 days. **(B)** Ag-specific CD4+ T cells among CD4+ T cells in the stomach, blood, MLN and spleen and CD4+ T cells among total leukocytes were analyzed. **(C)** Absolute number of Ag-specific CD4+ T cells in stomach, MLN, blood and spleen and CD4+ T cells in stomach and blood at the whole organ level were quantified. **(D)** 30-day post GSL vaccination, mice were i.p. injected with 100 μg anti-CD4 antibody or anti-RatIgG1 antibody at Days 30, 32 and 34. Mice were sacrificed on Day 36. **(E)** Ag-specific CD4+ T cells among total CD90.2+ cells in stomach were shown (left), and the absolute number of Ag-specific CD4+ T cells in stomach was quantified (right). **(F)** CD4+ T cells among total leukocytes in blood or spleen were shown (left), and the absolute number of CD4+ T cells in blood or spleen was quantified (right). In all graphs, dots represent individual data points and columns represent mean and SEM. $^{*}P < 0.05$, $^{**}P < 0.01$. Mann–Whitney U test was used to compare two groups. Data were pooled from two individual experiments with $n = 3$–5 mice per group.

CD69 and CD103 on Ag-specific CD4+ Trm cells. Almost all these cells displayed a CD69+CD103-phenotype (**Figure 6G**). These data indicate that GSL vaccination induces intraepithelial CD69+CD103- CD4+ Trm cells in stomach.

Vaccine Strategy Involving CD4+ Trm Cells Provides Rapid and Long-Term Protection Against *Helicobacter* Insult

Vaccination programs used in previous studies generally performed *H. pylori* challenge on ∼14 days after the last vaccination (28, 29). At this time point, Teff cells induced by conventional vaccinations might not completely attenuate in blood or stomach of immune mice. A previous clinical study indicated that the protective effects induced by oral vaccination continued to attenuate in the years that followed (30). Here, we tested the differences between conventional vaccinations and vaccine strategies involving GSL vaccination in terms of rapid and long-term protection. Mice were divided into nine groups and subjected to different immunization programs (**Figure 7A**). *H. pylori* challenge was performed 60 days later. At Day 63, colonization of *H. pylori* was determined by quantitative culture. Substantial reductions of bacterial colonization could be found in mice that received GSL vaccination, and s.c.x2+GSL vaccination provided the optimal protection at this time point (**Figure 7B**), indicating that these mice demonstrated rapid antimicrobial responses and prolonged protection. In comparison, most conventional vaccine strategies failed to reduce bacterial colonization and no reduction of *H. pylori* colonization was observed in mice receiving Alum alone by GSL vaccination. Furthermore, we investigated the limit of anti-microbial response involving CD4+ Trm cells (**Figure 7C**). Results showed that s.c.x2+GSL vaccination induced a drastic reduction of *H. pylori* load at day 0–14 and the bacterial load was sustained at a low degree during day 14–30 post challenge (**Figure 7D**). A numerical advantage of reduction was observed between s.c.x2+GSL vaccination and GSL vaccination. These results imply that s.c.x2+GSL vaccination induces rapid and long-term protection against *H. pylori*.

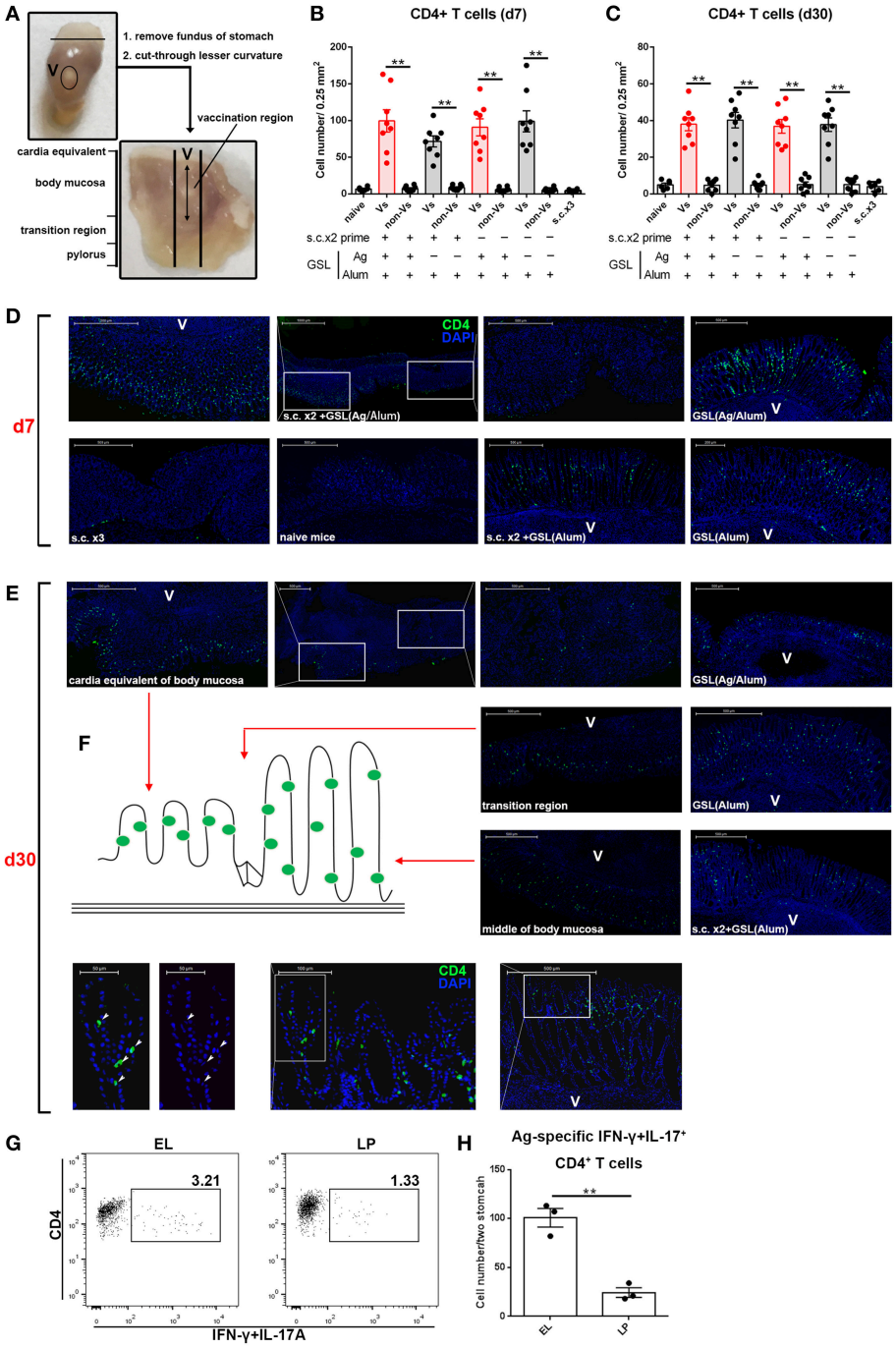

FIGURE 5 | GSL vaccination drove CD4+ T cell infiltration of the mucosa and retention within the epithelium. **(A)** Stomach was dissected as photo indicated and anatomical position was shown in the macroscopic perspective. Gastric tissue from mice that were treated with GSL vaccination was cut longitudinally and stained with Alexa Fluor® 488-anti-CD4 antibody. CD4+ T cells were quantified on immunofluorescent staining of 20 μm frozen sections from Day 7 **(B)** or Day 30 **(C)** post GSL vaccination. **(D,E)** Representative images of immunofluorescent staining (green, CD4; blue, DAPI; V, vaccination site). **(F)** Schematic plan of CD4+ cell location (left) and the distribution of CD4+ cell on gastric middle mucosa and transition region (right). The precise positions of CD4+ cells were determined by immunofluorescent staining of 10 μm frozen sections (green, CD4; blue, DAPI; V, vaccination site). $^{**}P < 0.01$. Mann–Whitney U test was used to compare two groups. Data were repeated 3–8 times. **(G)** EL or lamina propria (LP) lymphocytes were pooled from two immune mice at memory stage. Ag-specific CD4+ T cells were quantified **(H)**. Six mice ($n = 6$) were used in this experiment. $^{**}P < 0.01$, unpaired t-test.

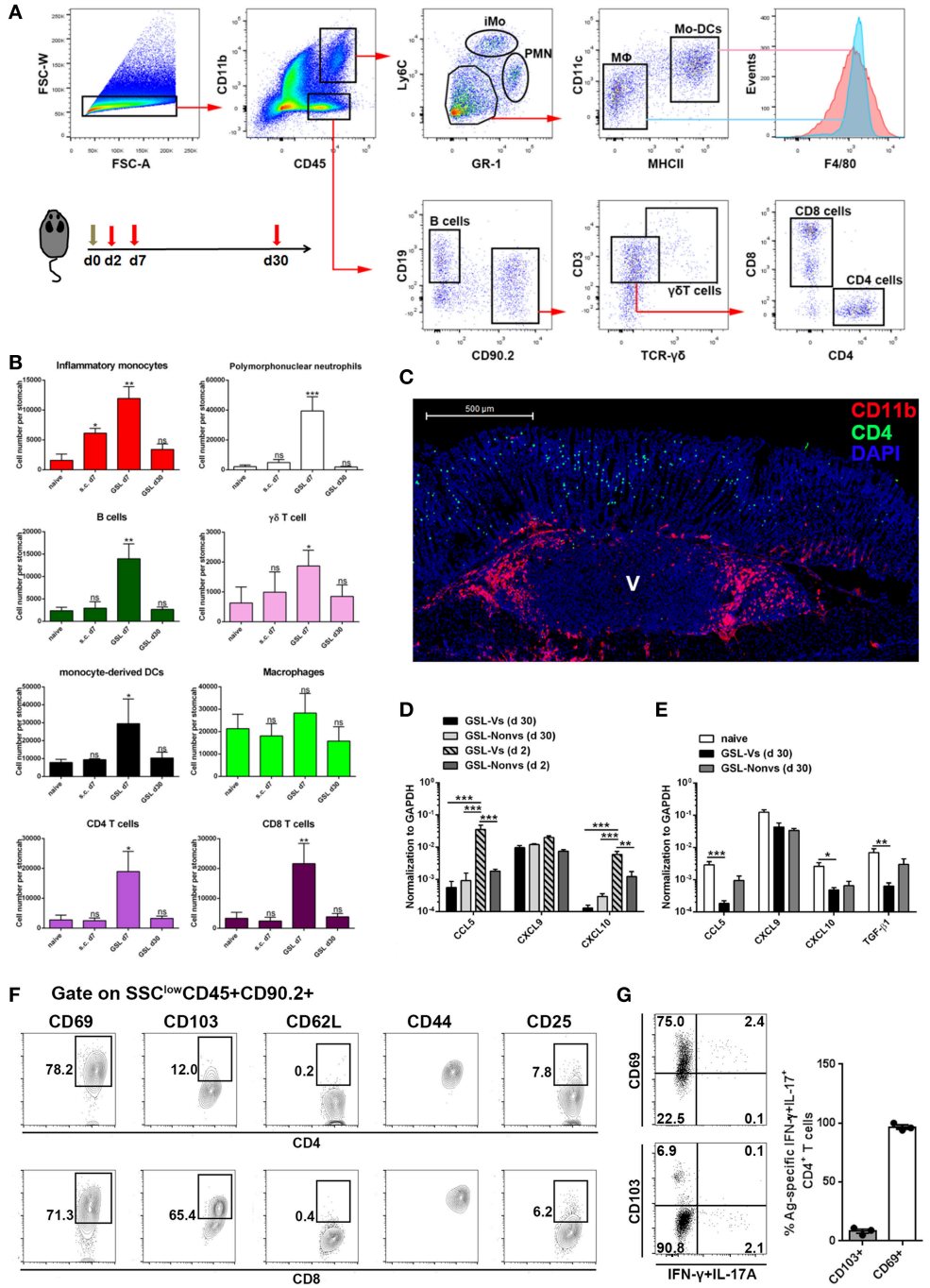

FIGURE 6 | The maintenance and phenotype of CD4+ Trm cells. **(A)** GSL vaccination was performed at Day 0 and mice were sacrificed on Day 7 and Day 30 to analyze immune cell infiltration using the indicated gated strategy. **(B)** Absolute number of innate and adaptive immune cells at the whole organ level was quantified. $^*P < 0.05$, $^{**}P < 0.01$. The Kruskal—Wallis test (vs. naïve) was used. Columns represent mean and SD. Data were pooled from two individual experiments with $n = 5$–6 mice per group. **(C)** Immunofluorescent staining of 20 μm frozen sections at Day 30 (green, CD4/CD11b; red, CD11b; blue, DAPI; V, vaccination site) post GSL vaccination. Data were repeated at least three times with similar results. **(D)** CCL5, CXCL9, and CXCL10 mRNA were measured at Day 2 and Day 30 by qRT-PCR. GSL-Vs: vaccination site; GSL-Non vs: non-vaccination site. **(E)** CCL5, CXCL9, CXCL10 and TGF-β1 mRNA were measured at Day 30 by qRT-PCR. **(F)** Phenotypes of intraepithelial CD4+ T cells and CD8+ T cells in the vaccination region. **(G)** Expression of CD69 and CD103 was analyzed on Ag-specific CD4+ Trm cells. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. Mann–Whitney U test was used to compare two groups. Data were pooled from two individual experiments with $n = 3$–5 mice per group. Columns represent mean and SEM.

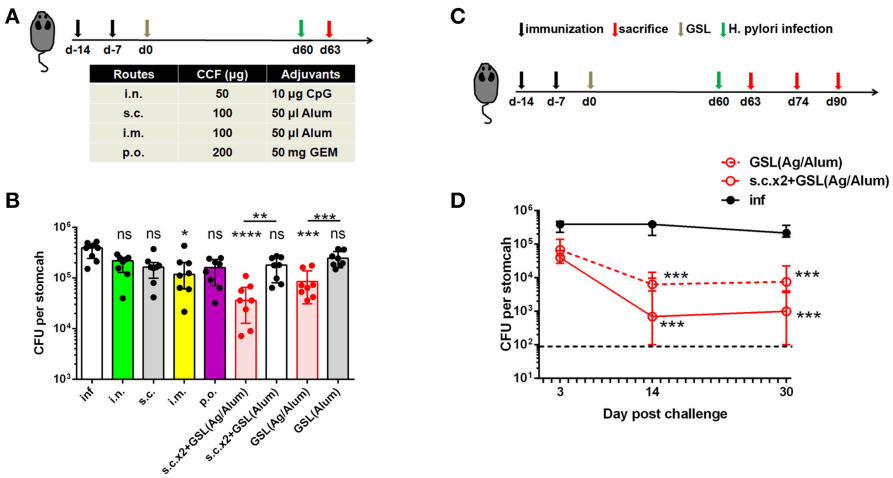

FIGURE 7 | s.cx2+GSL vaccination provided prolonged and rapid protection against *Helicobacter* insult. **(A)** C57BL/6J mice were immunized at Day-14, Day-7, and Day 0 with different vaccine formulation described in the table. *Helicobacter* challenge was performed on Day 60 and mice were sacrificed on Day 63 to test the protective effects. **(B)** *H. pylori* colonization was determined by the quantitative culture on Day 63. $^{*}P < 0.05$, $^{***}P < 0.001$, $^{****}P < 0.0001$, *ns*, $P > 0.05$. The Kruskal–Wallis test (vs. naïve) was used. Data were pooled from two individual experiments with $n = 8$ mice per group. **(C)** Mice experienced s.cx2+GSL vaccination were challenged at Day 60 and H. pylori colonization was determined on Day 63, Day 74, and Day 90 **(D)**. $^{**}P < 0.05$, $^{***}P < 0.01$. Mann–Whitney U test was used to compare two groups. In all graphs, columns represent median and interquartile. Data were pooled from two individual experiments with $n = 6$–8 mice per group.

Reactivated CD4+ Trm Cells Trigger a Rapid Systemic Th1/Th17 Cellular Response to Support Tissue-Wide Anti-microbial Response

Next, we investigated the immunological mechanism of protective response and the role of circulating lymphocytes in mice receiving s.c.x2+GSL vaccination. Administration of FTY720 to immune mice impaired the protective effects, and depletion of CD4+ T cells by an anti-CD4 antibody completely abrogated protection (**Figure 8A**), indicating the vital roles of circulating memory lymphocytes and CD4+ cells in protective response induced by GSL vaccination. Furthermore, IFN-γ and IL-17A depletion by neutralizing antibody partly suppressed the protective response in immune mice, highlighting the roles of IFN-γ and IL-17A in providing protection (**Figure 8C**). Analysis of Ag-specific immune response at day 3 post challenge suggested that a potent Ag-specific Th1/Th17 cell response in stomach and the expansion of Ag-specific Th17 cells in spleen already could be detected (**Supplementary Figure 4A**). Next, we compared the Ag-specific Th1/Th17 cell responses in immune mice treated with or without FTY720 in stomach, blood, spleen, and MLN at 7-day post challenge. FTY720 administration significantly dampened the expansion of Ag-specific Th1 and Th17 cells in stomach and blood (**Figures 8D–F**). In addition, after preventing lymphocyte egress from the lymph nodes with FTY720, the percentages of Ag-specific Th1 cells in spleen and MLN were increased by 3-fold; however, an increase in the percentage of Ag-specific Th17 cells was only observed in MLN (**Figures 8D–F**). Because of the powerful ability of Th17 cells to induce an anti-microbial response, reactivated MLN-settled Ag-specific memory Th17 cells might be a vital source of gastric Th17 cells that contribute to the recruitment of innate inflammatory cells and trigger tissue-wide protection. In fact, at 7-day post challenge, the mucosa near the vaccination site showed fewer inflammatory cell infiltration as compared with the distal mucosa (**Supplementary Figure 4B**), possibly suggesting a tissue-wide inflammatory cell infiltration was temporally delayed to the regional infiltration. These data highlight the alarming function of these intraepithelial CD4+ Trm cells, and indicate that regionally positioned CD4+ Trm cells can trigger tissue-wide *H. pylori* clearance through the recruitment of circulating Th1/Th17 cells.

DISCUSSION

Stomach is an inhospitable digestive organ that is inhabited by only ∼200 different species (8). The notorious one is *H. pylori*, which is a helical rod-shaped organism that is in contact with the gastric epithelium and influences the physiology of gastric stem cell pool by inducing chronic inflammation (31). Evidence indicates that as long as 50,000 years of co-evolution with human have conferred multiple capabilities (e.g., secretion of virulence factors and remodeling of autologous constituents) on *H. pylori* to adapt to the milieu of stomach and escape the host defensive mechanisms (32–35). Likewise, these capabilities lead to an undesirable outcome for *H. pylori* vaccines. Conventional vaccine strategies have been extensively tested in the field of *H. pylori* vaccinology (15, 16, 36–38), while no study identified an Ag-specific cellular response in stomach. Here, we demonstrated that by using intracellular cytokine staining, a small population of Ag-specific CD4+ T cells could be measured during effector stage rather than memory stage of these immunizations, suggesting conventional vaccine strategies were less effective to induce a measurable CD4+ Trm pool

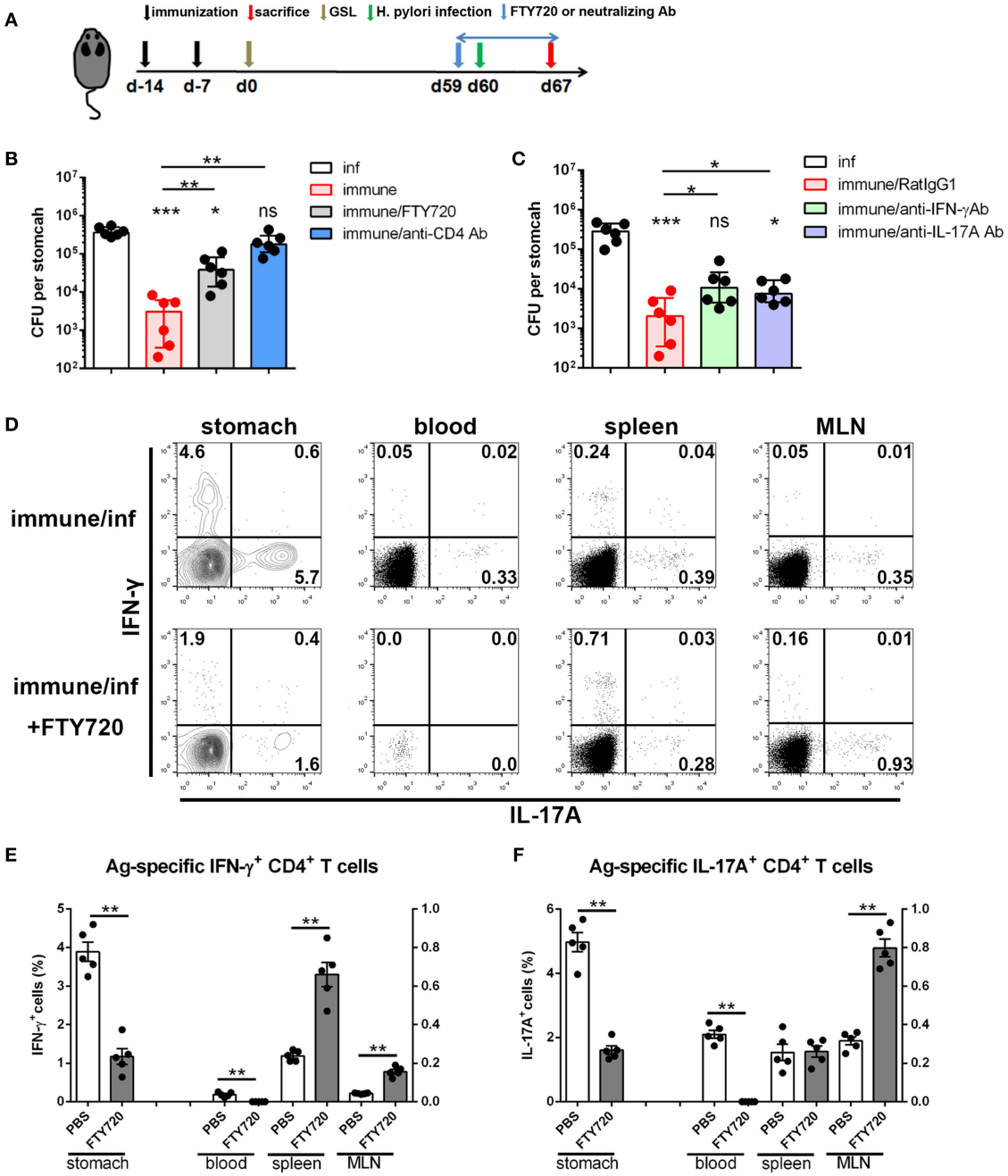

FIGURE 8 | The immunological mechanism of protective response in the mice experienced s.c.x2+GSL vaccination. **(A)** Mice experienced s.cx2+GSL vaccination. *H. pylori* challenge was performed on Day 60. FTY720 administration (1 mg/kg) was performed daily during Days 59–67. Neutralizing antibody (i.p.) injection was performed on Days 59, 61, 63, and 65. *H. pylori* colonization were determined on Day 67 after FTY720 and anti-CD4 antibody administration **(B)** or after anti-IFN-γ and anti-IL-17A antibodies administration **(C)**. $^{*}P < 0.05$, $^{***}P < 0.001$, *ns* = not significant. The Kruskal–Wallis test (vs. naïve) was used or The Mann–Whitney U test was used to compare two groups. Dots represent individual data points and columns represent median and interquartile. Data were pooled from two individual experiments with $n = 6$ mice per group. **(D)** Immune mice were administrated with 1 mg/kg FTY720 daily during Days 59–67 and sacrificed on Day 67 for Ag-specific CD4+ T cell analysis. The percentages of Ag-specific IFN-γ **(E)** or IL-17A **(F)** CD4+ T cells among total CD4+ T cells in stomach, blood, spleen, and MLN were quantified. $^{**}P < 0.01$, The Mann–Whitney U test was used to compare two groups. Dots represent individual data points and columns represent mean and SEM. Data were pooled from two individual experiments with $n = 5$ mice per group.

for further investigation. Technological barrier for identifying endogenous Ag-specific CD4+T cells in stomach is obvious. The inefficiency of lymphocyte isolation from stomach of mice has already been proved that 98.3% lymphocytes loss in stomach using flow cytometry for counting (2, 39). A need for purification to *ex vivo* culture and extensive steps of intracellular cytokine

staining may lead to the additional cell loss (40). Thus, we decided to establish a mice model with a measurable CD4+ Trm pool.

Past studies have revealed that almost always Trm cells form within the tissue after resolution of inflammation or infection (4, 41). Most *H. pylori* vaccines, including the only licensed *H. pylori* vaccine, are composed of non-infectious *H. pylori* Ags that are poorly immunogenic (30). In consideration of the importance of local inflammation, we employed GSL injection to deliver a micro-dose of Alum-based *H. pylori* vaccine into stomach and subcutaneous vaccination was performed before GSL vaccination to mount the infiltrating Ag-specific CD4+ T cells. This immunization strategy established a stratified immune memory involving the local long-lived CD4+ Trm cells and adjacent lymph node-settled memory T cells. Upon GSL vaccination, the regional dissemination of Ag-specific CD4+ T cells was dependent on local Ag recognition and adjuvant-induced inflammation, as either injection with Ag in PBS or delivering Alum adjuvant alone showed no effect on Ag-specific CD4+ T cell recruitment.

Our results also provided insights of CD4+ Trm cells induced by GSL vaccination in terms of migration properties, location/development, and cell surface phenotype. For migration properties, the magnitude of CD4+ Trm cells was stable after FTY720 administration, suggesting they were accord with the important identification parameter that undergoes little or no recirculation (1). For location/development, we found that in GSL immune mice, CD4+ Trm cells showed a distinct pattern of intraepithelial retention. Three phases of their development can be described as followed: in acute phase, CD4+ T cells infiltrated into the mucosa along with the elevated levels of CCL5 and CXCL10 and expansion of various innate immune cells; during the phase of inflammation resolution, more than 60% of CD4+T cells, including Ag-specific CD4+T cells, withdrew from stomach; in memory stage, CD4+Trm cells were distributed along with the architecture of gastric epithelium and keep stable in magnitude for long-term. An elegant study indicates that vaginal CD4+ Trm cells induced by an attenuated herpes simplex virus 2 sustain in a unique lymphocyte structure, named memory lymphocyte cluster, which is located in parenchyma tissue (26). Half of skin CD4+ T cells persist in peri-follicular clusters that accurately equilibrate with the blood lymphocytes during steady state, and infection can increase the immune cell content of these clusters (27). Our data showed that no lymphocyte clusters were observed in stomach of immune mice in memory stage. Residential pattern of the CD4+ Trm cells in our study was different from the prevailing view that after resolution of infection/inflammation, CD4+ Trm cells are preferentially localized within parenchymal tissues, while CD8+ Trm cells adhere to epithelial layers (1). Current knowledge about Trm cells is primarily obtained from invasive pathogens, which can disseminate into the host organ. Differences on types of vaccine, tissue architecture and inflammatory signaling may be responsible for the outcome of CD4+ Trm cell location/development (1, 25, 42, 43). In addition, the cell surface phenotypes of Ag-specific CD4+ Trm cells isolated from GSL immune mice were exclusively CD69+CD103-. A recent study in the context of *Candida albicans* infection finds that non-recirculating skin CD69+CD4+ Th17 cells are sufficient to trigger sterilizing immunity (44). Also, Ag-specific CD4+ Trm cells reported by N. Iijima and A. Iwasaki's study expressed CD69 but little CD103 (26).

Trm cells within peripheral tissues provide strong protection against pathogenic insult (26, 45, 46). Inducing a potent mucosal immune memory is favored as *H. pylori* restrictedly survive in epithelium of stomach with less invasiveness. During *H. pylori* insult, evenly distributed intraepithelial CD4+ Trm cells are optimally positioned to eliminate the window period and initiate a protective response immediately. GSL vaccination-induced CD4+ Trm cells are long-lived and pathogen-specific, therefore providing prolonged protection that was highly sensitive to *H. pylori* insult. As Th1 and Th17 immunity was highlighted in the anti-microbial response of *H. pylori* vaccine (28, 29), we reported that CD4+ Trm cells induced by GSL vaccination sustained in stomach for long-term and rapidly reactivated to recruit circulating Th1/Th17 cells to clear gastric *H. pylori*. Tissue-autonomous protection was found in the immune mice, but superior antimicrobial effects were dependent on the engagement of circulating lymphocytes (**Figure 8B**). It might be that protective CD4+ Trm cells induced by GSL vaccination were restricted to adjacent mucosa at low magnitudes and needed the help of circulating Th1/Th17 cells to trigger tissue-wide protection (41). This observation is consistent with previous study that small numbers of Trm cells trigger an antiviral state through amplifying innate or adaptive immune signals (47).

In the present study, we attempted to introduce a vaccine-induced CD4+ Trm pool in stomach and evaluated its protective efficacy. Alum-based vaccine was used for GSL vaccination, but we found an undesirable granuloma that affected regional homeostasis. Employing a biocompatible vehicle with the characteristics to spread in GSL is interested to improve the outcome of this model. Our further study will use the silk fibroin to replace Alum adjuvant to extend the CD4+ Trm cell distribution, increase their magnitudes, and prevent the form of granuloma. A more attractive but challenging question is how to design a feasible delivered system that targets stomach. The laparotomy used in mice is impractical in humans. Combining endoscopy technology with GSL vaccination is also less feasible for the translational application. Recently, an impressive study reports an ingestible self-orienting system for oral delivery of macromolecules that deliveries insulin through gastric mucosa (48). This delivered system is applicable for stomach-targeted vaccination after some adjustment.

Overall, our study developed a mice model with a strategic CD4+ Trm pool in stomach. CD4+ T cells induced by GSL vaccination preferentially infiltrated the adjacent mucosa, and then restrictively sustained in the epithelial region adjacent to the vaccination site. The underlying mechanism of local maintenance is currently unknown but may be associated with the metabolism of free fatty acids and TGF-β signals, similar to the maintenance requirements for intraepithelial

CD8+ Trm cells (49). Principally, our results indicate that pathogen-specific CD4+ Trm cells within the gastric epithelium can catch the best chance to sound the alarm, orchestrate the defense response, and provide prolonged protection. The notion that developing vaccine strategies involves a Trm population may shed new light on the development of *H. pylori* vaccines.

ETHICS STATEMENT

All animal experiments were approved by the Animal Ethical and Experimental Committee of China Pharmaceutical University.

AUTHOR CONTRIBUTIONS

WL and YX designed all experiments. WL performed surgical operation. ZZ, SL, CH, NX, and AH conducted the animals. WL, ZZ, and SL analyzed the data. ZZ prepared the reagents and the experiments. WL, YX, LZ, ES, and TX discussed the results and wrote the manuscript. All authors have reviewed this manuscript before submission.

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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.2019.01115/full#supplementary-material

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Immunity to Respiratory Infection Is Reinforced Through Early Proliferation of Lymphoid T_{RM} Cells and Prompt Arrival of Effector CD8 T Cells in the Lungs

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Cross-protection between serologically distinct strains of influenza A virus (IAV) is mediated by memory CD8 T cells that recognize epitopes from conserved viral proteins. Early viral control begins with activation of tissue-resident memory CD8 T cells (T_{RM}) cells at the site of viral replication. These CD8 T cells do not act in isolation, as protection against disseminated infection is reinforced by multiple waves of effector cells (T_{EFF}) that enter the lungs with different kinetics. To define how a protective CTL response evolves, we compared the functional properties of antiviral CD8 T cells in the respiratory tract and local lymphoid tissues. When analyzed 30 dpi, large numbers of antiviral CD8 T cells in the lungs and mediastinal lymph nodes (MLNs) expressed canonical markers of T_{RM} cells (CD69 and/or CD103). The check point inhibitor PD-1 was also highly expressed on NP-specific CD8 T cells in the lungs, while the ratios of CD8 T cells expressing CD69 and CD103 varied according to antigen specificity. We next used *in vitro* experiments to identify conditions that induce a canonical T_{RM} phenotype and found that that naïve and newly activated CD8 T cells maintain CD103 expression during culture with transforming growth factor-beta (TGFβ), while central memory CD8 T cells (T_{CM}) do not express CD103 under similar conditions. *In vivo* experiments showed that the distribution of antiviral CTLs in the MLN changed when immune mice were treated with reagents that block interactions with PD-L1. Importantly, the lymphoid T_{RM} cells were poised for early proliferation upon reinfection with a different strain of IAV and defenses in the lungs were augmented by a transient increase in numbers of T_{EFF} cells at the site of infection. As the interval between infections increased, lymphoid T_{RM} cells were replaced with T_{CM} cells which proliferated with delayed kinetics and contributed to an exaggerated inflammatory response in the lungs.

Keywords: immune regulation, respiratory infection, cytotoxic T cell, viral immunity, immunological "memory"

INTRODUCTION

The 1918 influenza pandemic caused more deaths in the period of a single year than any other emerging infectious disease (1). Intermittent infections with new strains of avian influenza A virus (IAV) raise concerns that another global pandemic could begin at any time (2, 3). Efforts to protect public health include regular vaccination with inactivated viral products. Because the antibodies do not bind viruses with modified surface proteins, these vaccines provide little protection against infection with new strains. Frequent vaccine failures emphasize the need for broadly protective vaccines that target multiple different serotypes (4). As models predict that dependence on a single vaccine could increase the severity of future pandemics (5), preparations for mass vaccination should encompass different methods of immunization. Since a local route of vaccine delivery is required to populate the lungs with antiviral memory CD8 T cells (6), vectored vaccines may be the best method for inducing broad immunity in the respiratory tract (7).

Cytotoxic T lymphocytes (CTLs) enhance immunity by destroying host cells that support viral replication (8–10). Responses to new strains of IAV develop slowly, while naïve CD8 T cells undergo clonal expansion in the local lymph nodes. Several days pass before effector CD8 T cells (T_{EFF}) enter the lungs and destroy host cells that support viral replication (8–10). During the delay, replicating virus spreads to the lower respiratory tract, where T cell-derived cytokines contribute to defuse alveolar damage (11, 12). We have previously shown that less damage occurs when viral dissemination is impeded by tissue-resident memory CD8 T cells (T_{RM}) in the airways (6). The role of T_{RM} cells in immunity was discovered after MHCI tetramers were used to quantify CTLs in the lungs during the recovery phase of infection. Investigators found that the numbers of memory CD8 T cells in the circulation did not change while protective immunity declined (13, 14). Importantly, some anti-viral CTLs expressed CD69 in the lungs and gradually disappeared as protective immunity declined. The presence of these activated CTLs prompted us to explore how long viral peptides were presented to CD8 T cells during the recovery phase of infection. We found that the mediastinal lymph node (MLN) contained residual viral peptides until at least 2 months after intranasal (i.n.) inoculation (15). Importantly, the remaining peptides induced abortive proliferative responses from naïve CD8 T cells, while T_{CM} cells show no signs of activation (16). After completing several rounds of cell-division, the responding cells displayed a partially-activated phenotype as indicated by increased CD44, CD11a, and CD69 expression.

We used parabiosis experiments to explore how antiviral memory CD8 T cells survey the lungs during recovery from infection (15, 17). These experiments showed that some CD8 T cells left the circulation during acute viral infection and remained lodged in the walls of the airways after infectious virus had been eliminated (15, 18). A majority of the resident cells displayed a canonical T_{RM} phenotype, exemplified by stable CD69 and/or CD103 expression. In addition, more than 80% of antiviral CD8 T cells in the MLNs were non-circulating (host-derived) cells that expressed CD69 and/or CD103 (15, 17), including some cells that expressed the immune checkpoint inhibitor programmed death-1 (PD-1). For parabiosis experiments, we used mice that were housed in specific pathogen free (SPF) facilities. As expected, very few CD8 T cells expressed CD69 or PD-1 before IAV infection. In contrast, both markers were widely expressed on CD8 T cells in lymph nodes recovered from human cadavers and out-bred mice that had been exposed to diverse environmental pathogens, indicating a response to microbial products (19, 20).

Although the contributions of mucosal T_{RM} cells to antiviral immunity are widely recognized, the functional properties of lymphoid T_{RM} cells remain poorly defined (21). Here 5-bromo-2′-deoxyuridine (BrdU) has been used to analyze the proliferative responses of antiviral memory CD8 T cells in the lungs and local lymphoid tissues after heterosubtypic challenge. By altering the length of time between primary and secondary IAV infection, we show that defenses in the lungs of immune mice are reinforced by early proliferation by T_{RM} cells in the lung-draining lymph nodes and prompt arrival of T_{EFF} cells at the site of viral replication.

Multiple receptors control access to the circulation, including CD69 and CD103 (αeβ7 integrin) which is expressed on some subsets of CD8 T cells during stimulation with TGFβ. Studies have shown formation of pulmonary T_{RM} cells requires local exposure to antigen and/or TGFβ (17, 22, 23), but we have limited knowledge of the signals that are involved in maintenance of these specialized cell populations. For the current study, we compared the surface antigens on T_{RM} cells that recognize three different viral epitopes and found that phenotypes of lymphoid T_{RM} cells varied according to antigen-specificity. T_{RM} cells that were specific for an epitope encoded in the nucleoprotein (NP) gene expressed surface markers that were consistent with a response to persisting viral peptides (i.e., CD69 and/or PD-1, without CD103). PD-1 was highly expressed on CD8 T cells in the lungs. In contrast, CTLs that were specific for an epitope encoded in the acid polymerase (PA) gene included larger percentages of cells that expressed CD103 in combination with CD69, while PD-1 was largely absent. A two-step culture system has been used to explore how circulating CD8 T cells respond to stimulation with TGFβ. Our data indicate that responses to this cytokine are influenced by the timing and quantity (or context) of antigen exposure in local tissues.

MATERIALS AND METHODS

Mice and Reagents

OTI mice (24) were bred and housed at UCONN Health, in accordance with institutional guidelines. C57BL/6 mice were purchased from Charles River. Frozen MHCI molecules ($NP_{366-374}/D^b$, $PA_{324-333}/D^b$) were supplied by the NIH tetramer facility (Emory University, Vaccine Center at Yerkes, Atlanta, GA) and ($OVA_{257-264}/K^b$) MBL International corporation. Tetramers were made at UCONN Health. Virus stocks were grown in fertilized chicken eggs (Charles River), titered and stored as described previously (15). Between 8 and 20 weeks after birth, anesthetized mice were infected intranasally with 2×10^3 PFU WSN-OVA_I (H1N1) (25). For secondary infections, mice received 5×10^3 PFU X31-OVA (H3N2) (26). Blocking antibodies to PD-L1 (B7H1) and isotype control

were purchased from Bio-Xcell (West Lebanon, NH.). Mice received 250 μg of blocking antibody (100 μl saline) given twice by i.p. injection. Mice were infected (i.v.) with 5,000 CFU recombinant *Listeria monocytogenes* expressing chicken ovalbumin (LM-OVA) (27). Experiments were performed in accordance with guidelines and protocols approved by the University of Connecticut Health Center Institutional Animal Care and Use Committee (IACUC).

Adoptive Cell Transfer and Sample Preparation for Flow Cytometry

Naïve CD8 T cells were isolated from spleens and pLN of CD45.1$^+$ OTI mice using Mojosort isolation kits (Biolegend). OTI cells were labeled with CFSE according to directions from the manufacturer (Molecular Probes). Mice received 3×10^5 OTI cells by I.V. injection. For flow analysis, chopped lung tissue was digested using 150 U/ml collagenase (Life Technologies, Rockville, MD, USA) in RPMI containing 1 mM $MgCl_2$, 1 mM $CaCl_2$, 5% FBS and incubated at 37°C for 90 min. Non-adherent cells were enriched on 44/67% Percoll gradients and spun at 400 *g* for 20 min. Washed lymphocytes were incubated with antibodies that block Fc-receptors for 15 min, then stained with anti-CD8 and MHCI tetramers for 1 h at room temperature. Antigen-experienced CTLs were identified using high CD44 and CD11a expression. NP-specific CTLs were phenotyped using Tetramer-PE, CD103-AF647, CD69-PerCP, and PD-1 FITC. OVA and PA-specific CTLs were phenotyped using Tetramer-APC, CD103-FITC, CD69-PerCP, and PD1-PE. BrdU was administered by i.p. injection 4 h before cell analysis. BrdU incorporation was measured by intracellular staining, according to instructions from the manufacturer (BD Biosciences). To visualize cells that were close to X or Y axis, scales on some contour plots were adjusted using the bi-exponential function in Flowjo® software.

Cell Culture

Naïve CD8 T cells were stimulated with plate-bound anti-CD3/CD28 and rIL-2 (20 U/ml) in 24 well plates. Cultures were supplemented with SB-431542 (10 μM) or vehicle (0.1% DMSO) (28). Other wells were exposed to exogenous TGFβ (10 ng/ml). Cells were suspended in RPMI containing FBS, L-glutamine, β-mercapthoethanol, sodium pyruvate, Hepes, and antibiotics. At 48 h, CTLs were transferred to new wells and stimulated with activated TGFβ (10 ng/ml) and rIL-2 for an additional 48 h (no antigen).

Confocal Microscopy

MLNs were fixed in 4% PFA/PBS for 60 min at 4°C and cut into thick sections (350 microns) using a vibratome. Sections were pre-incubated with antibodies to block Fc-receptors (15 min at 4°C) and stained with biotin-conjugated antibodies to CD11c (eBioscience) diluted in 2% FBS/PBS solution. After extensive washing, sections of fixed MLNs were stained overnight at 4°C with streptavidin-PE antibody (Life Technologies), Pacific blue-conjugated anti-CD31, Alexa Flour 488- conjugated anti-CD45.1 (BioLegend, San Diego, CA, USA), eF660-conjugated LYVE-1, and B cells were detected with V500-conjugated anti-B220 (BD Biosciences). After extensive washing, stained tissues were mounted on slides using Shandon Immu-Mount (Thermo Electron, Pittsburgh, PA, USA). Images were recorded using a Zeiss LSM880 confocal microscope with an inverted Axio Observer. Fluorescence was detected using: an argon laser for emissions at 458, 488, and 514 nm; a diode laser for emissions at 405 and 440 nm; a diode-pumped solid-state laser for emissions at 561 nm; and a HeNe laser for emissions at 633 nm. Images were analyzed using the colocalization function in Imaris suite software (Bitplane, South Windsor, CT, USA).

Histology

Lungs were fixed in 4% PFA/PBS at 4°C for 24–48 h. After washing, lungs were stored in 70% ethanol until processing. Hematoxylin and eosin (H&E) staining was performed by the Histology Core at the UCONN Health. Images were takes at 5X and 20X normal magnification.

Statistical Analysis

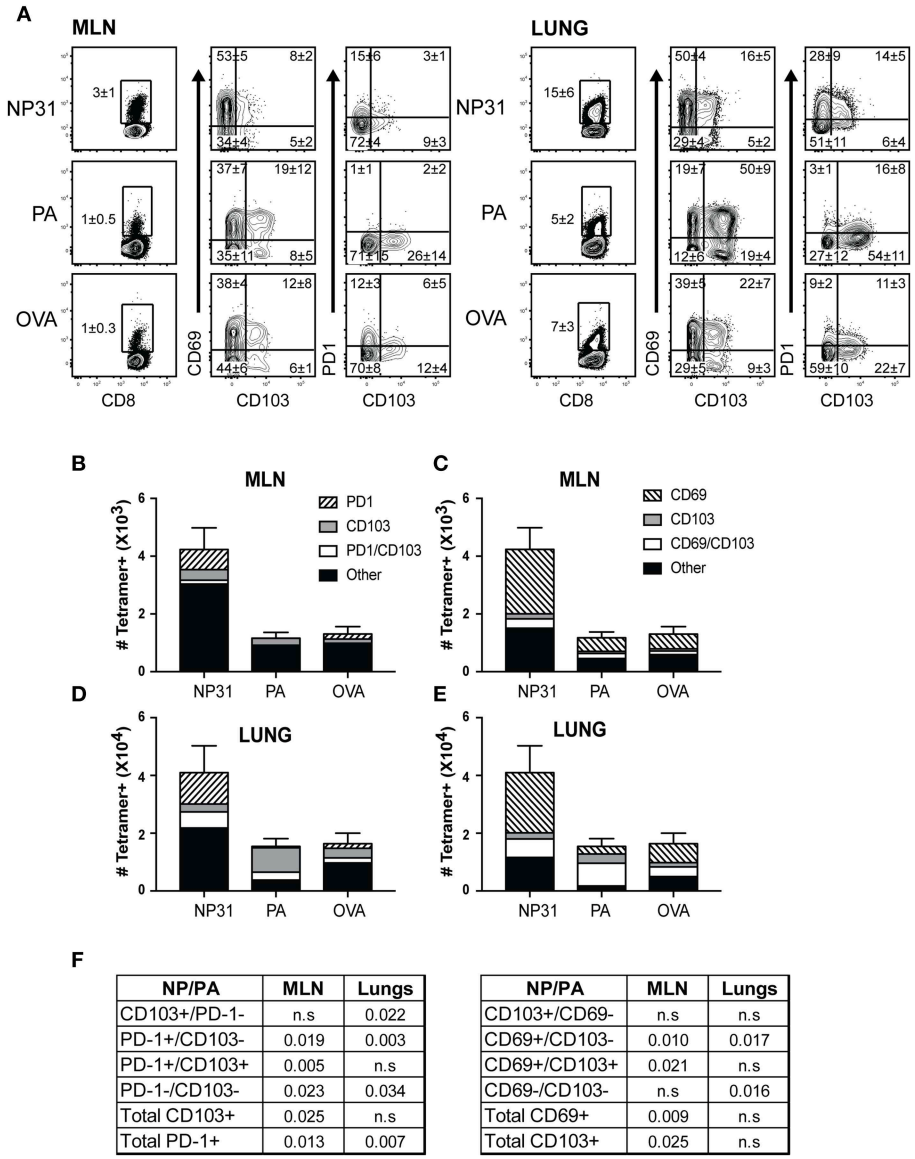
Statistical significance was determined using an unpaired two-tailed Student *t*-test. Horizontal lines indicate comparisons between samples, with *p* values from groups of 5/6 mice. NS, $P > 0.05$; $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$.

RESULTS

Lymphoid T_{RM} Cells Are Receptive to Signals That Promote Lodgment in Peripheral Tissues

To examine the phenotypes of antiviral CD8 T cells during recovery from IAV infection, C57BL/6 mice were infected with a recombinant virus (WSN-OVA_I) encoding the SIINFEKL peptide (25). Virus-specific CD8 T cells were analyzed 30 dpi, using MHCI tetramers containing peptides encoded by the nucleoprotein ($NP_{336-374}/D^b$), acid polymerase ($PA_{224-233}/D^b$), and ovalbumin genes ($OVA_{257-264}/K^b$). The lungs and MLNs both contained antiviral CD8 T cells that expressed canonical markers of T_{RM} cells (**Figure 1A**). NP-specific CD8 T cells were the dominant subset at both locations (**Figures 1B–E**). CD69 was expressed on large percentages of antiviral CD8 T cells identified with all three tetramers. In contrast, the percentages of virus-specific CD8 T cells that expressed CD103 and/or PD-1 varied according to antigen-specificity (**Figure 1A**). A majority of NP-specific CD8 T cells lacked CD103, while PD-1 was highly expressed in lungs. Conversely, only small percentages of PA-specific CTLs expressed PD-1 and CD103 was highly expressed in the lungs. The OVA-specific CTLs displayed an intermediate phenotype. Gates for analyses were set using non-CD8 T cells.

Tonic signaling from the TGFβ receptor reinforces expression of αeβ7 integrin (CD103) on T_{RM} cells (17, 29). We next investigated whether naïve and/or circulating memory CD8 T cells express CD103 *in vivo*. We previously found that T_{CM} cells develop with delayed kinetics after IAV infection, due to the influence of persisting viral peptides. To avoid this complication, C57BL/6 mice were infected with recombinant *L. monocytogenes* encoding the chicken ovalbumin gene (LM-OVA) (27). After 32 days, CD8 T cells were recovered from the spleens and analyzed for CD103 expression (**Figure 2A**). After gating CD62L$^+$ cells,



F

NP/PA	MLN	Lungs
CD103+/PD-1-	n.s	0.022
PD-1+/CD103-	0.019	0.003
PD-1+/CD103+	0.005	n.s
PD-1-/CD103-	0.023	0.034
Total CD103+	0.025	n.s
Total PD-1+	0.013	0.007

NP/PA	MLN	Lungs
CD103+/CD69-	n.s	n.s
CD69+/CD103-	0.010	0.017
CD69+/CD103+	0.021	n.s
CD69-/CD103-	n.s	0.016
Total CD69+	0.009	n.s
Total CD103+	0.025	n.s

FIGURE 1 | The phenotypes of T_{RM} cells vary according to antigen-specificity. Antiviral CTLs were recovered from the lungs and MLN 35 dpi with WSN-OVA_I. Antigen experienced CTLs were identified using high CD11a and CD44 expression. **(A)** Contour plots show antigen-experienced CTLs analyzed with MHCI tetramers. The tetramer$^+$ CTLs were analyzed for CD103, CD69, and PD-1 expression. Percentages of cells in each quadrant are means ± SD (n = 5/group). Gates were set using non-CD8 T cells. **(B–E)** Bar graphs show total numbers of tetramer$^+$ CTLs, plotted using means ± SD (n = 5/group). Shading shows **(B,D)** ratios of cells expressing CD103 and/or PD-1, **(C,E)** ratios of cells expressing CD103 and/or CD69. **(F)** The numbers of CTLs in each quadrant were compared for the NP and PA epitopes. P-values were calculated using Student's t-tests.

naïve and central memory CD8 T cells (T_{CM}) were distinguished using CD44 and CD11a (top row). The overlaid histograms show that CD103 expression on naïve CD8 T cells, but not T_{CM} cells. Similarly, CD103 was expressed on naïve CD8 T cells from uninfected mice and was absent when the TGFβ receptor was not expressed (bottom row).

We used a transfer model to create a homogeneous supply of OVA-specific T_{CM} cells for *in vitro* experiments. Naïve CD8 T cells were recovered from the peripheral lymph nodes of OTI mice which express a transgenic receptor that is specific for SIINFEKL peptide presented in the context of H-$2K^b$ ($OVA_{257-264}/K^b$) (24). Enriched OTI cells were transferred to C57BL/6 mice 48 h before infection (i.v.) with LM-OVA. After 3 months, T_{CM} cells ($CD62L^{high}/CD11a^{high}$) were sorted from the spleens using CD45.1 expression. A two-step culture system was used to compare the phenotypes of naïve and T_{CM} cells after stimulation with TGFβ (**Figure 2B**). To induce cell proliferation, purified OTI cells (naïve and T_{CM}) were stimulated with plate-bound anti-CD3/CD28 and recombinant IL-2 (20 U/ml). After 48 h, live cells were transferred to clean wells and cultured for

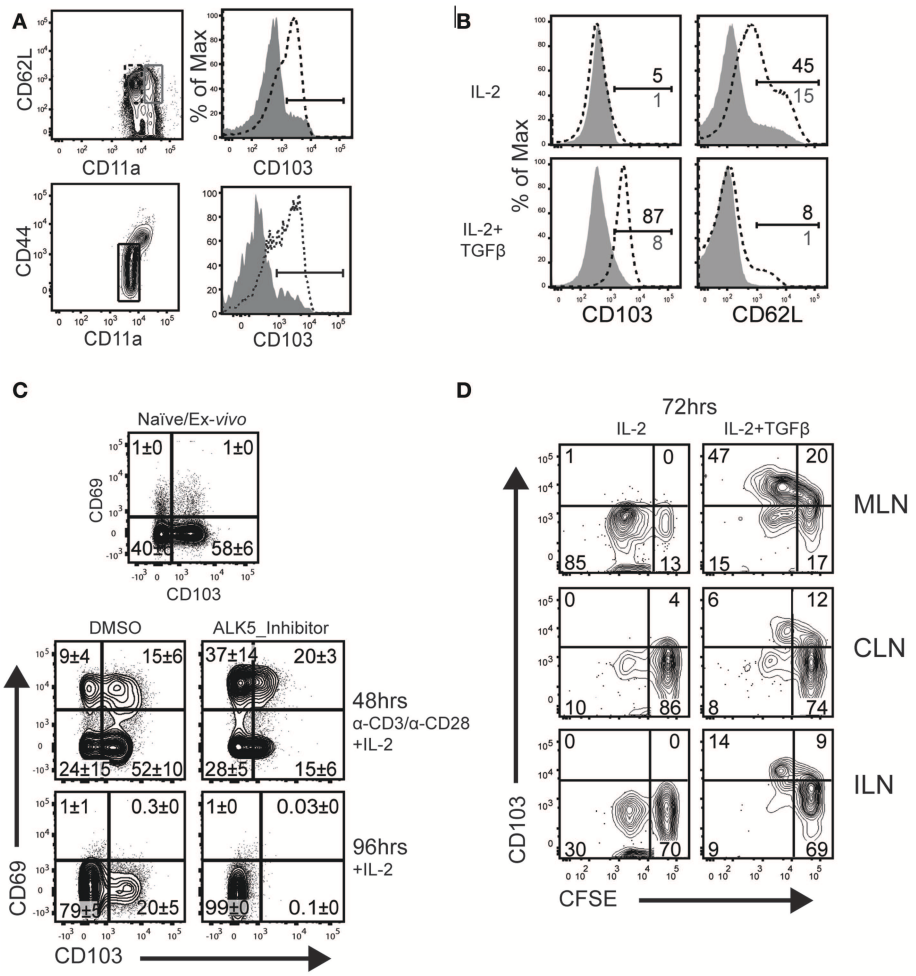


FIGURE 2 | T_{CM} do not upregulate CD103 during culture with TGFβ. **(A)** C57BL/6 mice were infected systemically (i.v.) with LM-OVA. CD62L-positive CD8 T cells in the spleen were analyzed 32 dpi. The contour plots indicate gates that were used for analyses. Histograms (top row) show CD103 expression on naïve CD8 T cells, while T_{CM} cells do not express CD103 (gray shading). Lower panels show CD103 expression naïve OTI cells from the spleens of uninfected mice (bottom row, dashed line). Naïve OTI cells do not express CD103 after ablation of the TGFβ receptor (bottom row, gray shading). **(B)** Naïve OTI cells were transferred to B6 mice 48 h before infection with LM-OVA. After 3 months, T_{CM} cells were sorted from the spleens using CD45.1 expression. Purified CD8 T cells (naïve and T_{CM}) were stimulated with plate-bound anti-CD3/CD28 and rIL-2 (20 U/ml) for 48 h. Live cells were transferred to new wells (no antigen) and cultured for 48 h with rIL-2 plus/minus TGFβ (10 ng/ml). **(C)** Naïve OTI cells were cultured with plate-bound anti-CD3/CD28 and rIL-2. In addition, some wells were supplemented with SB-431542 (10 μM) to avoid stimulation with serum-derived TGFβ. After 48 h, activated OTI cells were analyzed for CD103/CD69 expression (top panels). Additional cells were cultured for an additional 48 h with rIL-2 and no antigen stimulation (lower panels). **(D)** Naïve OTI cells were labeled with CFSE-dye and transferred to mice that were previously (30 d) infected with WSN-OVA_I. The MLNs were recovered 5 days after transfer and lymphocytes were cultured for 72 h with rIL-2 plus/minus TGFβ. Three experiments gave similar results.

an additional 48 h in fresh media, supplemented with rIL-2, plus/minus TGFβ (10 ng/ml). The naïve OTI cells down regulated CD103 during antigen stimulation and expression did not return during extended culture with rIL-2. Importantly, CD103 was re-expressed when the cultures were supplemented with TGFβ. Conversely, T_{CM} cells lacked CD103 expression when cultured under similar conditions.

Respiratory infection promotes formation of mucosal T_{RM} cells which express CD103 in combination with CD69. CD69 is expressed on CD8 T cells soon after antigen stimulation (or exposure to selected cytokines), and quickly disappears when the stimulus is removed (30). Here, cultured CTLs were used to identify conditions that induce the canonical T_{RM} phenotype of dual CD69 and CD103 expression (**Figure 2C**). Naïve OTI cells were stimulated with anti-CD3/CD28 and rIL-2 as previously described. Fetal bovine serum contains small quantities of TGFβ. To prevent stimulation with this cytokine, replicate wells were supplemented with an inhibitor (SB-431542) that prevents phosphorylation of TGFβ receptor I (ALK5) (31), or the vehicle (DMSO) control. The first samples were analyzed 48 h after antigen stimulation, when approximately 20% of OTI cells expressed CD69 in combination with CD103 (top row). The percentages of $CD69^+$ cells increased when the ALK5 inhibitor was present, while CD103 was partially down-regulated. Higher

percentages of $CD69^+$ CD8 T cells indicate that the inhibitor prevented suppression from TGFβ during early T cell activation (32). Other OTI cells were stimulated with antigen for 48 h and transferred to new wells containing fresh medium with rIL-2, for an additional 48 h without antigen (lower panels). Approximately 20% of OTI cells maintained CD103 expression during extended culture with rIL-2, while both markers (CD103 and CD69) were down regulated when the cultures contained the ALK5 inhibitor (SB-431542) (**Figure 2C**). These data confirmed CD103 expression on newly activated CD8 T cells is reinforced by stimulation with TGFβ.

We previously used transfer experiments to determine how long viral peptides were presented to CTLs in the tissues of IAV infected mice (15). To detect persisting OVA peptides, naïve OTI cells were labeled with CFSE-dye and transferred to mice that had previously been infected with WSN-OVA_I. The donor cells were analyzed 5 days after transfer, when reduced intensity of the CFSE-dye showed that some OTI cells proliferated *in vivo*. Here, a similar protocol was used to determine whether CD8 T cells remain receptive to TGFβ after completing several rounds of cell division in the MLN. C57BL/6 mice were infected with WSN-OVA_I and CFSE-labeled OTI cells ($CD11a^{low}/CD44^{low}$) were transferred 30 dpi. After 5 days, lymphocytes were recovered from the MLNs and cultured for 48 h with rIL-2 (20 U/ml), plus/minus TGFβ (10 ng/ml). Diluted CFSE-dye showed that some OTI cells divided in the MLN between 30 and 35 dpi and maintained CD103 expression during culture with TGFβ (**Figure 2D**). Together, these studies show that naïve CD8 T cells respond to "late" antigen stimulation in the MLN and remain receptive to environmental cues that encourage lodgment in peripheral tissues. Importantly, T_{CM} did not respond to late antigen presentation (16), or express CD103 when cultured with TGFβ.

The Distribution of Lymphoid T_{RM} Cells Changes During Therapeutic Blockade of PD-1L

PD-1 is a costimulatory molecule that delivers negative-signals to T cells during interactions with APCs. Newly activated CTLs transiently express PD-1 during antigen stimulation, while stable expression has been linked to chronic antigen exposure and suboptimal T_{EFF} function (33, 34). During chronic infections, many CTLs express PD-1 in combination with other inhibitory receptors and exhibit symptoms of exhaustion (33, 35). Sustained PD-1 expression is linked to broad functional changes including reduced motility of activated CTLs during interactions with APCs (35). Whether PD-1 plays a role in maintenance of lymphoid T_{RM} cells has not been explored.

Tetramer analyses showed that substantial numbers of antiviral CTLs maintained PD-1 expression during recovery from IAV infection (**Figure 1**). To explore whether PD-1 plays a role in maintenance of lymphoid T_{RM} cells, we examined the distribution of antiviral CTLs in the MLNs of IAV infected mice during treatment with antibodies that block interactions with PD-1 ligand (PD-L1) (36).To visualize antiviral CTLs in the MLN, naïve ($CD11a^{low}CD44^{low}$) OTI cells were transferred to B6 mice 48 h before infection with WSN-OVA_I. After 30 days, the recipient mice were treated twice with antibodies that block interactions with PD-L1, or an isotype control. MLNs were recovered 5 days after the first antibody treatment (35 dpi) and analyzed by scanning confocal microscopy (**Figures 3A–G**). OTI cells were widely distributed in the MLNs from both groups of animals. When control antibodies were used (**Figures 3A–D**) clusters of OTI cells (1 or 2 per MLN) were found in close proximity with high endothelial venules (HEVs) (**Figure 3B**), while additional OTI cells were adjacent to $LYVE^+$ vessels. Areas of direct cell contact are shown in white (**Figures 3D,G**). No clusters of OTI cells were visible after PD-L1 blockade (**Figures 3E–G**). The numbers of $CD45.1^+$ cells in the MLNs decreased during treatment with antibodies to PD-1L (**Figure 3H**). We found no difference in BrdU incorporation during the antibody treatments (data not shown). Taken together, these data indicate that small clusters of OTI cells dispersed during PD-L1 blockade, while some activated CTLs may have been released into the circulation. A single lobe from the lungs of each mouse was analyzed by H&E staining to evaluate changes in pathology during PD-L1 blockade (**Figure 4**). Infection-induced pathology was detected in the lungs of all animals and the areas of lymphocytic infiltration did not substantially change during treatment with antibodies that block PD-1L. Images were taken at 5X normal magnification and total areas of lymphocytic inflammation were measured using ImageJ software ($P = 0.7221$, $n = 6$/group).

T_{EFF} Cells Enter the Lungs With Variable Kinetics After Heterosubtypic Challenge

Heterosubtypic immunity disappears between 4 and 6 months after IAV infection (6, 37). To understand how lymphoid T_{RM} cells respond to secondary infection with a different strain of IAV, mice were primed with WSN-OVA_I (H1N1 serotype) and later challenged with X31-OVA (H3N2 serotype). Both viruses encode the SIINFEKL peptide, but express different surface proteins (25, 26). To explore how the functional properties of antiviral memory CD8 T cells evolve with time, we adjusted the interval between recurrent infections from 1 to 4 months. For optimal protection, secondary infections were administered between 30 and 35 dpi. For simplicity, this time interval is referred to as early recall (ER). To study the responses of antiviral memory CD8 T cells as immunity declined, additional mice were reinfected between 120 and 160 dpi. This interval is referred to as late recall (LR). On different days after secondary infection, groups of 5 mice received a single dose of BrdU and virus-specific CTLs were analyzed 4 h later (**Figure 5**). The contour plots show gated populations of antigen-experienced CD8 T cells (CD44-high, CD11a high) analyzed using tetramers specific for the NP, PA and OVA epitopes (**Figure 5A**). Unpaired student's *t*-tests were used to compare the rates of BrdU incorporation in the MLNs (3 dpi) during early and late recall. This experiment showed

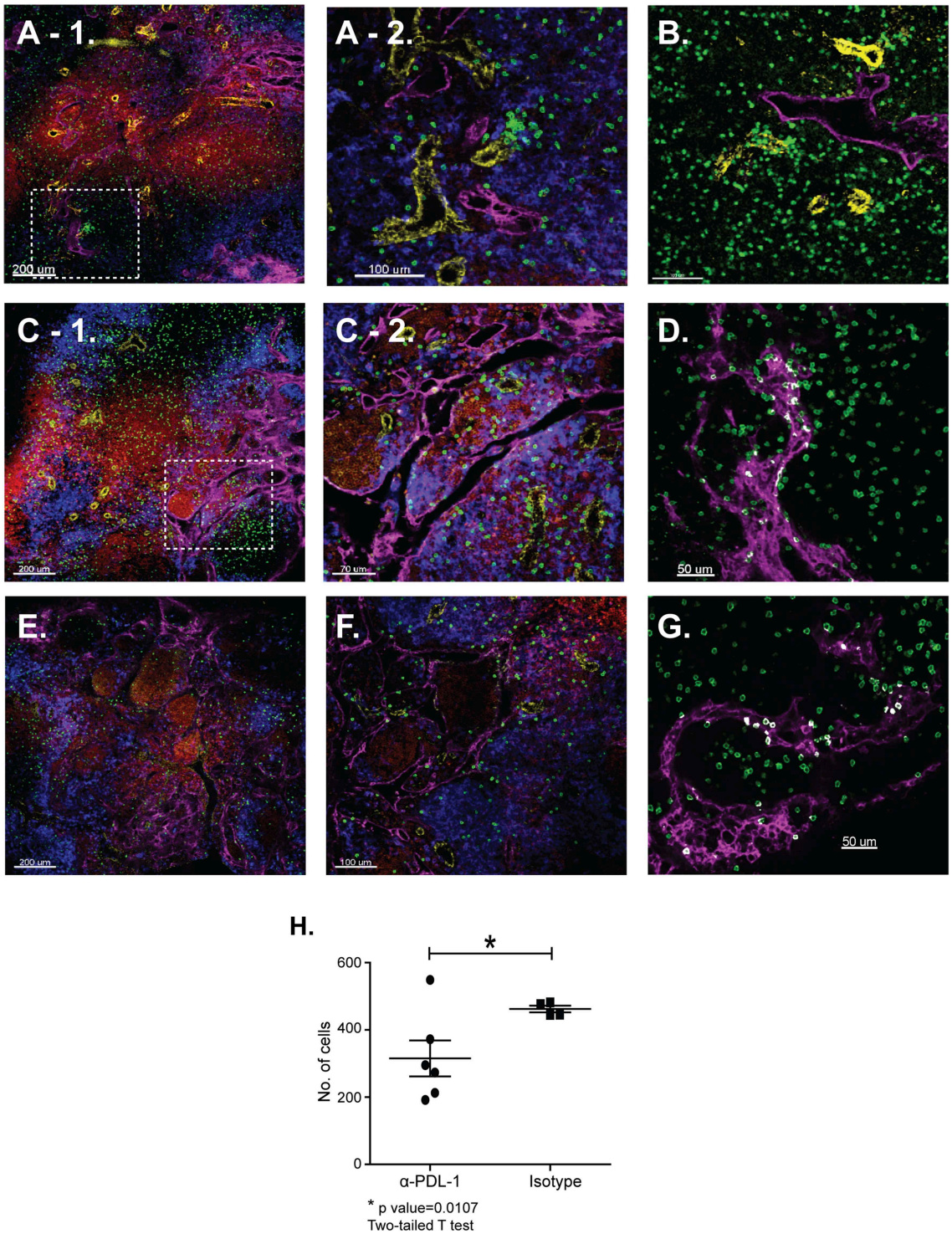


FIGURE 3 | The distribution of lymphoid T_{RM} cells changes during PD-1L blockade**.** Naïve OTI cells were transferred to B6 mice 48 h before infection with WSN-OVA_I. At 30 and 32 dpi, antibodies that block interactions with PD-L1 (or isotype control) were administered by IV injection (250 μg). After 35 days sections of fixed MLN were stained with antibodies that are specific for CD45.1 (green); CD31 (yellow); CD11c (blue); B220 (red), and LYVE-1 (magenta). Z-stacks were recorded at 10X and 20X normal magnification. **(A–D)** MLNs analyzed after treatment with control antibodies. **(E,F)** MLNs analyzed after treatment with antibodies that block interactions with PD-L1. The inset boxes (dashed lines) mark the locations of enlarged images shown in **(A-2,C-2)**. **(D,G)** The Imaris software colocalization function was used to detect contacts (white) between OTI cells (green) and $LYVE^+$ vessels (Magenta). **(H)** Numbers of OTI cells per 10 micron Z-stack (*$P = 0.0107$).

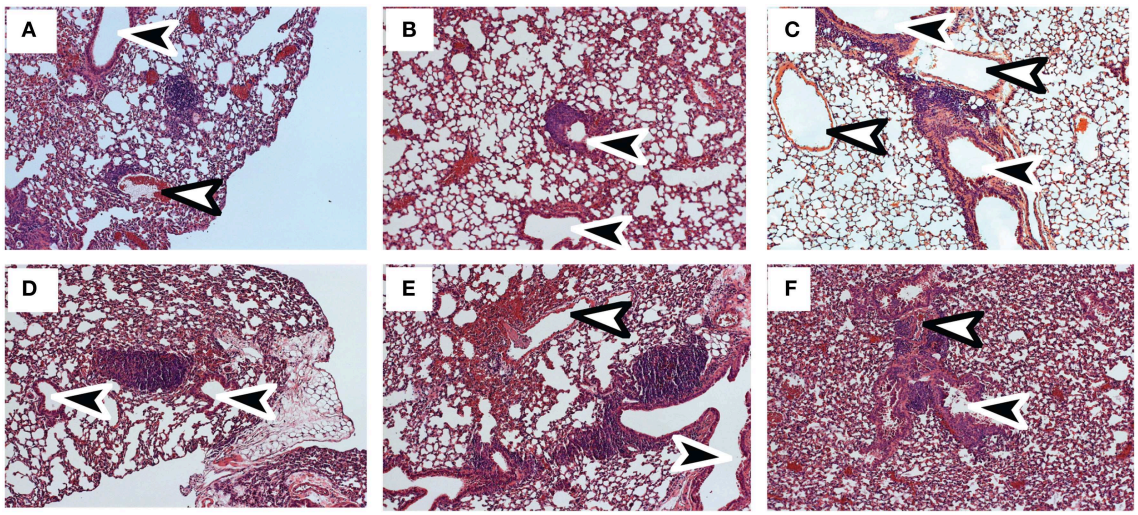


FIGURE 4 | Infection-induced pathology is not substantially altered by PD-L1 blockade. C57BL/6 mice were infected with WSN-OVA_I and treated twice with **(A–C)** antibodies to PD-L1 or **(D–F)** isotype control. Sections of fixed lung tissue were stained 35 dpi using hematoxylin and eosin. Images were recorded at 20X normal magnification. Arrows indicate Bronchioles (black) and blood vessels (white).

that anti-viral CTLs proliferated in MLNs with accelerated kinetics after early recall. The percentages of CD8 T cells that incorporated BrdU were significantly different for all three tetramers (NP, $P = 0.0126$; PA, $P = 0.0205$; and OVA, $P = 0.0002$). The bar graphs show total numbers of tetramer$^+$ cells in the lungs and MLNs, with shading to indicate cells that contained BrdU (**Figure 5B**). Robust BrdU incorporation was not detected in the MLN until 4 days after late recall and T_{EFF} cells accumulated in the lungs with delayed kinetics. Weak proliferation by PA-specific CTLs contributed to a change in epitope dominance after reinfection, as reported previously (38, 39).

Pathology in the lungs was evaluated 4 dpi, using H&E staining (**Figures 6A–D**). After early recall, the blood vessels were surrounded with pronounced mononuclear leukocytic infiltrates (**Figure 6A**), while the conducting airways were largely unobstructed (**Figure 6B**). Although perivascular and peribronchial infiltrates were less prominent after late recall (**Figure 6C**), the airways were heavily congested with mucus and mononuclear cells, including lung macrophages (**Figure 6D**). Taken together, these data show that lymphoid T_{RM} cells play an integral role in the response to reinfection and prompt dissemination of T_{EFF} cells to the lungs. As the interval between infections increased, lymphoid T_{RM} cells were replaced with T_{CM} cells which proliferated with delayed kinetics. We previously found that T_{CM} contributed to an exaggerated T_{EFF} response in the lungs as the infection progressed (6, 40), and does not prevent cellular obstruction in the airways.

DISCUSSION

Coordinated changes in homing receptor expression control the distribution of pathogen-specific CTLs in peripheral and lymphoid tissues. Naïve CD8 T cells follow gradients of sphingosine-1-phosphate (S1P) during transit through blood and lymph (41). Transit through lymphoid tissues is inhibited when CD8 T cells upregulate CD69 during antigen stimulation and the receptor for S1P (S1PR1) is modulated from the cell surface (42). A similar mechanism is required for T_{RM} cells to settle in infected tissues, where CD69 expression can be induced by local exposure to antigen and/or inflammation (43). Although the mechanism(s) that reinforce CD69 expression on T_{RM} cells have not been identified, chronic exposure to TGFβ prevents re-expression of S1P1 through negative regulation of Krupple-like factor 2 (KLF2) (23, 44). TGFβ also promotes retention of T_{RM} cells at barrier surfaces by maintaining CD103 expression, which in turn mediates interactions with a structural protein (E-cadherin) expressed on epithelial cells (17, 29, 45). Local concentrations of TGFβ increase during tissue repair and encourage T_{RM} cells to accumulate near inflamed tissues (46). Consistently, images of the lungs taken 30 dpi with IAV showed that the airway epithelium was densely populated with T_{RM} cells that expressed CD103 (6). Kinetic studies have shown that pulmonary T_{RM} cells gradually disappear from the lungs as protective immunity declines, while some replenishment occurs as small numbers of CTLs arrive from other tissues (15, 47). The origin of CTLs that enter the lungs during the recovery phase of infection is unknown, but may include CTLs that are released from the MLN during a response to persisting viral peptides (40).

Here, a two-step culture system has been used to define conditions that induce newly activated CTLs to express a canonical T_{RM} phenotype. We found that naïve OTI cells expressed CD69 in combination with CD103 soon after antigen stimulation. CD103 was eventually down regulated in the presence of antigen and returned during subsequent culture with TGFβ. A similar pattern was observed when naïve CD8 T

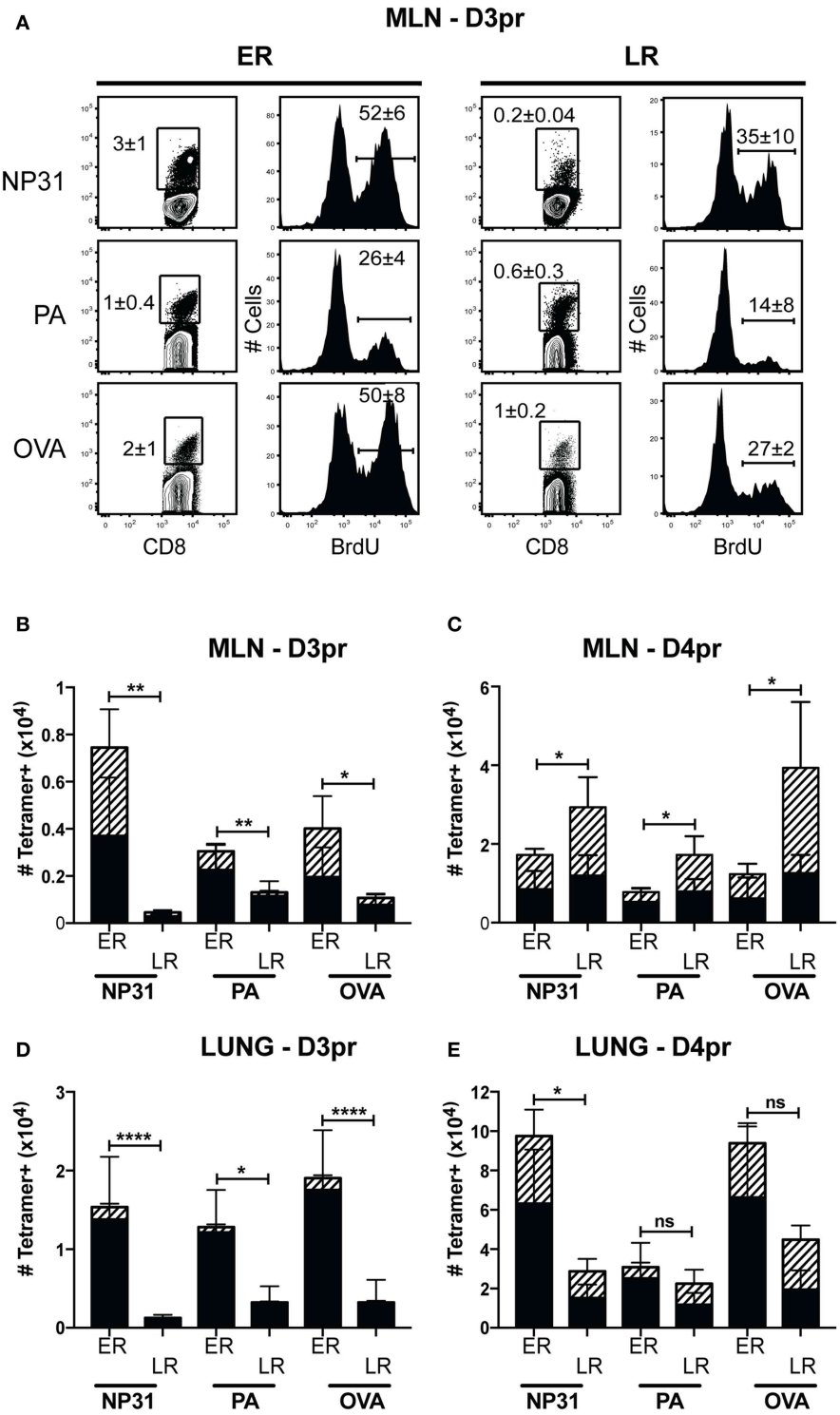


FIGURE 5 | Early proliferation by lymphoid T_{RM} cells corresponds with increased numbers of anti-viral CTLs in the lungs. C57Bl/6 mice were infected with WSN-OVA$_I$ and challenged with X31-OVA. Secondary infections were administered between 30 and 35 dpi (ER), or 120–160 dpi (LR). On the days indicated, each mouse received a single dose of BrdU (given by IP injection) and antiviral CTLs were analyzed 4 h later. **(A)** On day 3 post recall (D3pr), the MLNs were analyzed for antigen-experienced CTLs using high CD11a and CD44 expression. The contour plots show frequencies of tetramer$^+$ cells. Histograms show BrdU incorporation within the tetramer gates. Percentages are means ± SD ($n = 5$/group). **(B–E)** The bar graphs show total numbers of Tetramer$^+$ CTLs, including BrdU$^+$ cells (hatched shading). Bars are means ± SD ($n = 5$/group). The numbers of cells that incorporated BrdU after ER and LR were compared using unpaired *T*-tests. NS, $P > 0.05$; *$P < 0.05$; **$P < 0.01$; ****$P < 0.0001$.

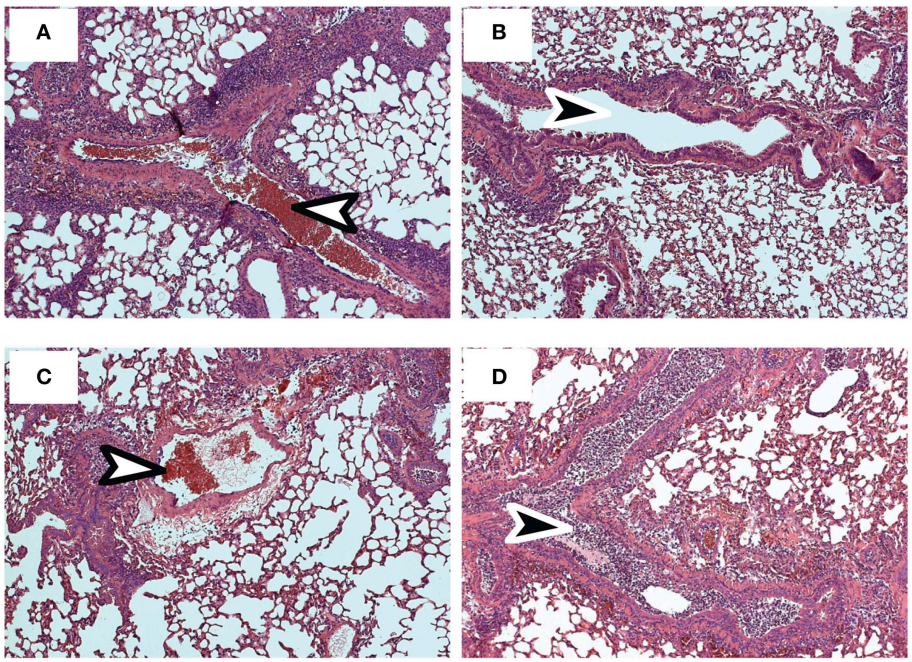


FIGURE 6 | Sections of fixed lung tissue were stained with H&E 4 dpi. Representative images from 5 mice are shown. **(A,B)** Lungs after early recall **(C,D)** Lungs after late recall. Arrows indicate Bronchioles (black) and blood vessels (white).

cells were exposed to antigen *in vivo*. A transfer model also showed that naïve OTI cells completed multiple rounds of cell division in the MLN during late antigen presentation. Importantly, the responding CD8 T cells expressed CD103 when cultured with TGFβ. We previously showed that T_{CM} cells do not respond to late antigen presentation in the MLN 30dpi (16). When analyzed *in vitro,* T_{CM} cells did not express CD103 during culture with TGFβ, either before or after antigen stimulation. Together, these experiments show that naïve CD8 T cells respond to suboptimal antigen stimulation in the MLN and are receptive to factors in the local environment that promote extended residence in peripheral tissues. Importantly, CTLs become resistant to TGFβ after committing to the T_{CM} lineage.

Data from other models show that T_{RM} cells display variable phenotypes during infection with different pathogens. One report showed that T_{CM} cells trafficked to the skin during vaccinia virus infection and converted to $CD69^+$ T_{RM} cells, without CD103 expression (48). Lymphoid T_{RM} cells displayed a similar phenotype during recovery from LCMV infection (19). To analyze CD8 T cells responses after IAV infection, we used mice that were bred in SPF facilities. Consequently, naïve CD8 T cells were the principal source of T_{RM} cells in this study. Tetramer analysis showed that the percentages of T_{RM} cells expressing CD103 varied according to antigen specificity (**Figure 1**). Whereas, large percentages of PA-specific T_{RM} cells expressed CD103, this marker was largely absent from NP-specific T_{RM} cells, while PD-1 was highly expressed in the lungs. It is important to note that the NP antigen is more abundant than PA during acute viral infection (49) and that NP peptides persist in the MLNs for approximately 2 months (15). Since CD8 T cells upregulate PD-1 and lose CD103 during antigen stimulation, many NP-specific T_{RM} cells displayed a phenotype ($PD\text{-}1^+CD69^+$CD103-negative) that was consistent with a response to persisting viral peptides (15). Some tumors contain intraepithelial lymphocytes (IEL) that express PD-1 in combination with CD103 (50). Some PA-specific T_{RM} cells expressed a similar phenotype in the lungs after IAV infection. Variations between these markers suggest that the phenotypes of T_{RM} cells are influenced by the timing and quantity or context of antigen exposure in local tissues.

We have found that T_{CM} cells respond to secondary IAV infection with delayed kinetics and contribute to a robust inflammation in the lungs as the infection progresses (6). The alveoli are surrounded by delicate membranes, which can be readily damaged by high concentrations of T cell-derived cytokines. Our data caution that some poorly designed vaccines may trigger a robust inflammatory response in the lungs during respiratory infection, by promoting formation of T_{CM} cells without T_{RM} in the local tissues (40, 51).

ETHICS STATEMENT

Experiments were performed in accordance with guidelines and protocols approved by the University of Connecticut Health Center Institutional Animal Care and Use Committee (IACUC).

AUTHOR CONTRIBUTIONS

JS-R and KC were responsible for all experimentation and data processing. The Manuscript was written and edited by LC and JS-R. Histology was done by the histology core (UCONN Health) and evaluated by SB. All authors participated in editing the manuscript.

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REVIEW
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Recalling the Future: Immunological Memory Toward Unpredictable Influenza Viruses

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Persistent and durable immunological memory forms the basis of any successful vaccination protocol. Generation of pre-existing memory B cell and T cell pools is thus the key for maintaining protective immunity to seasonal, pandemic and avian influenza viruses. Long-lived antibody secreting cells (ASCs) are responsible for maintaining antibody levels in peripheral blood. Generated with $CD4^+$ T help after naïve B cell precursors encounter their cognate antigen, the linked processes of differentiation (including Ig class switching) and proliferation also give rise to memory B cells, which then can change rapidly to ASC status after subsequent influenza encounters. Given that influenza viruses evolve rapidly as a consequence of antibody-driven mutational change (antigenic drift), the current influenza vaccines need to be reformulated frequently and annual vaccination is recommended. Without that process of regular renewal, they provide little protection against "drifted" (particularly H3N2) variants and are mainly ineffective when a novel pandemic (2009 A/H1N1 "swine" flu) strain suddenly emerges. Such limitation of antibody-mediated protection might be circumvented, at least in part, by adding a novel vaccine component that promotes cross-reactive $CD8^+$ T cells specific for conserved viral peptides, presented by widely distributed HLA types. Such "memory" cytotoxic T lymphocytes (CTLs) can rapidly be recalled to CTL effector status. Here, we review how B cells and follicular T cells are elicited following influenza vaccination and how they survive into a long-term memory. We describe how $CD8^+$ CTL memory is established following influenza virus infection, and how a robust CTL recall response can lead to more rapid virus elimination by destroying virus-infected cells, and recovery. Exploiting long-term, cross-reactive CTL against the continuously evolving and unpredictable influenza viruses provides a possible mechanism for preventing a disastrous pandemic comparable to the 1918-1919 H1N1 "Spanish flu," which killed more than 50 million people worldwide.

Keywords: T cells, B cells, influenza, immunological memory, vaccine

INTRODUCTION

Successful vaccination relies on the induction of long-term immunological memory. Exposure to an infectious virus elicits acute effector responses that mediate acute pathogen control, along with the generation and maintenance of T cell and B cell memory capable of protecting against re-exposure. At sufficient levels, neutralizing antibody (Ab) can prevent re-infection while, especially if such protection is partial, the rapid recall of memory $CD8^+$ cytotoxic T lymphocytes (CTLs) facilitates enhanced pathogen control. Seasonal influenza results from the emergence of an occasional, highly infectious variant selected as a consequence of Ab-driven mutational change in the viral envelope hemagglutinin (HA) and/or neuraminidase (NA) proteins. Pandemic influenza A viruses, on the other hand, arise from gene reassortment of two different influenza A virus (IAV) subtypes infecting the same cells. As a consequence, the influenza research and control community face the continuing challenge of producing new vaccines to control emerging threats.

Most of the existing products utilize inactivated virus, or isolated viral HA and NA proteins, that stimulate influenza strain-specific antibody immunity and B cell memory, but do not prime the much more cross-reactive $CD8^+$ CTL compartment. The challenge is thus to add a T cell-targeted vaccine component that promotes CTL memory for the rapid recall of anti-viral CTL effectors to the respiratory tract for early virus control and/or induce cross-protective B cells. In this review, we focus on the nature of optimal memory B cell and T cell generation and ask how we might use this knowledge to overcome the limitations of seasonal influenza vaccines by developing feasible strategies for both inducing and maintaining long-term, cross-reactive immunological memory.

The Burden of Seasonal Influenza

Seasonal influenza virus is a global health problem. In the United States, influenza virus infections causes 9.2–35.6 million cases of illness, 140,000–710,000 hospitalizations and 12,000–56,000 deaths per year (1). Globally, it is estimated that every year 290,000–650,000 respiratory deaths are due to seasonal influenza (2). The World Health Organisation (WHO) recommends annual influenza vaccination for people at high risk of developing severe disease, and for those in contact with high-risk individuals. Vulnerable groups include the elderly (>65 years), young children (6–59 months), Indigenous populations, patients with chronic medical conditions, pregnant women, and health-care workers (3). National health authorities in the countries with an advanced public health system recommend annual vaccination for everyone 6 months of age and above, both to protect individuals and to limit the spread of the virus through the community (4, 5).

Influenza Virus Evolution Poses a Challenge for Long-Term Humoral Immunity and Vaccine Effectiveness

Influenza viruses attach to host cells via HA binding to cell surface sialic acids (6, 7). Protective antibodies (Abs) block virus attachment by binding to the antigenic sites (8–11) proximate to the sialic acid receptor binding pocket on the HA head. Such Abs are the best correlate for influenza immunization and are measured using the hemagglutination inhibition (HAI) assay, which detects Abs blocking the capacity of the virus to agglutinate red blood cells by binding to sialic acids on their surface (12). The influenza virus RNA polymerase lacks proof-reading function, with the consequence that there is a constant emergence of mutants (affecting viral fitness and/or immune recognition) carrying substitutions that arise randomly across the genome. Antibody-mediated immune pressure drives the selection of viruses expressing variant HAs and NAs (13, 14) that, if their "fitness" is not unduly compromised, have the potential in nature to cause the process that has long been called antigenic drift (15, 16). Clearly, for a drifted strain to emerge as a clinical problem, its HA must be sufficiently changed to escape neutralization by pre-existing antibodies induced broadly in human populations by past infections and/or vaccinations. The reality that individuals who were once protected are now at risk from the new variant strain is the basis for frequently reformulating seasonal influenza vaccines (17). In contrast, through the process of antigenic shift, influenza viruses incorporate a completely new HA or NA (18), which adds a new virus into the epidemiological mix. When it comes to antibody-mediated selection, the A/H3N2 strains have consistently shown the greatest antigenic drift for the three types of influenza viruses that co-circulate globally and cause seasonal epidemics (A/H1N1, A/H3N2, and influenza B viruses) (16, 19). In general, more extensive epidemics (with increased morbidity and mortality) occur when a novel, seasonal A/H3N2 drifted strain emerges (16, 20, 21).

Multi-component influenza vaccines are designed to elicit serum antibodies against the HAs of one A/H1N1 strain, one A/H3N2 strain and one (or two) influenza B viruses (Yamagata or Victoria) (22). Increased antibody titres induced by vaccination decrease the risk for infection caused by any strains antigenically similar to those included in the vaccine (23, 24), although they confer limited or no protection against other types or subtypes (including drifted variants) of influenza (25). The global WHO network closely monitors the circulation of influenza viruses in humans and other species, including birds, across the northern and southern hemispheres, whereby information derived from the antigenic and genetic characterization of these strains, along with epidemiological data, is used to select the strains to be incorporated into an upcoming seasonal vaccine (26). This strategy can fail, at least in part, as vaccine preparation takes at least 6 months and the product may no longer match all 3 (or 4) circulating viruses by the time it is released (27). Moreover, pre-existing immunity in humans can be highly variable due to age and prior exposures via infection and/or vaccination (28–34). The level of pre-existing human immunity is considered but often difficult to interpret due to high heterogeneity. First-infection ferret antisera is used to identify and characterize new influenza strains, yet repeated exposures to A/H3N2 variants affect Ab quantity and quality, which makes vaccine-strain selection even more challenging (35). Both immunological responses to influenza viruses and influenza vaccine effectiveness are undoubtedly affected by the combination of antigenic drift and prior immunity. Influenza virus evolution has been widely

studied, yet it is still largely unknown how cross-reactive B cell memory impacts on Ab responses to new strains.

B Cell Memory and Imprinting Against Prior Strains

The idea that immunological memory could impact negatively on Ab responses to novel influenza strains first emerged in the early 1950s, when Francis and Davenport observed that the exposure to a new influenza strain induced higher titres of Abs against variants encountered in childhood than against the prevailing strain (36–39). They proposed the colorfully named concept of "original antigenic sin" (OAS), which states that Abs generated against the first antigen (Ag) encountered in childhood would be repeatedly and preferentially induced at every exposure, even if the epitope remained as a minor secondary antigen. This was considered to be sinful, i.e., detrimental for protection against following influenza infections, since the Abs induced poorly neutralized the most recent strain that had actually triggered them (40, 41). Molecular level analyses of B cell receptor usage have since confirmed that memory B cells elicited by a priming Ag can participate in the immune response toward a structurally related, boosting Ag (42, 43). While it is clear that somatic mutation of the immunoglobulin (Ig) variable (V) region takes place, the extent to which this leads to increased affinity for the priming vs. boosting variant remains controversial (42). These molecular analyses are consistent with more recent observations that Ab boosting is broad, and greatest against more similar viruses, differing somewhat from the OAS concept that centers on the initial antigen encountered (44, 45). Efforts to understand why prior vaccination enhances vaccine effectiveness in some influenza seasons, yet attenuates it in others, has led to further refinements to the OAS hypothesis, namely that imprinted B cell memory responses are not inevitably "sinful" i.e., ineffectual (31). Hensley et al. propose that Ab become focused on selected epitopes which are relatively conserved between successive strains due to a form of competitive dominance by memory B cells and that while this may result in high Ab titres and clinical benefit it may, alternatively, compromise protection if the epitope is altered in future strains. This hypothesis is based on molecular and serological analyses that document focused HI Ab responses in selected individuals (29, 30, 46–49).

At the cellular level, it is clear that memory B cells respond more rapidly than their naïve precursors. Hence, antibody responses may become focused on epitopes that were present in earlier strains because memory B cells specific for those epitopes become rapidly activated at the expense of naïve B cells, which need a higher threshold to respond (50, 51). Memory B cells that bear affinity matured antigen receptors may also be better able to compete with existing Abs for inducing antigen than naïve B cells (52). Several strategies have the potential to promote naïve B cell activation and broaden the Ig response. These include giving repeated vaccine doses (39), increasing the amount and concentration of antigen (53), and adding adjuvants (54). Another suggested mechanism that may promote the enhanced engagement of memory (vs. naïve) B cells is that T regulatory cells (T_{regs}) induced by the initial encounter reduce the amount of antigen presented on dendritic cells, thus diminishing the antigen availability for naïve B cells, promoting a memory B cell boost at the expense of naïve B precursors (55).

Current Strategies to Improve Seasonal Influenza Vaccine Effectiveness

Strategies to increase seasonal influenza vaccine effectiveness (VE), like high-dose or adjuvanted vaccines, are still under evaluation. Pooled analysis of multiple studies showed that high-dose vaccines significantly reduce the risk of laboratory-confirmed influenza cases in the elderly when the vaccine and the circulating strains are well-matched, but not when they are mismatched. The HAI geometric mean titres after vaccinating with the high-dose vaccine were significantly higher compared to the standard-dose vaccine for the H3 component. However, the proportion of participants with seroprotective HAI Ab levels (HAI titer $\geqq$ 1:40 or 1:32) was the same using both vaccines (56). Similarly, high-dose vaccines showed significant increases in VE with a reduction in mortality among the elderly by 36.4% in the 2012–2013 season, when H3N2 viruses were predominantly circulating (57). Nonetheless, seasonal VE on that season was only of 11% for that particular age group (58), indicating that a high-dose vaccine, despite increasing VE, did not induce an epidemiologically significant improve in overall H3N2 VE. Alternatively, the use of a standard-dose influenza vaccine with the MF59 adjuvant (Novartis) can reduce laboratory-confirmed influenza cases as well as hospitalizations due to influenza in the elderly (59) and seasonal trivalent vaccines formulated with this adjuvant are now available for those >65 years old (FluAd, Sequiris).

In addition to MF59, other adjuvants licensed for use with inactivated or sub-unit-based influenza vaccines include Alum-containing formulations ($AlPO_4$ or $Al[OH]_3$) and oil-in-water emulsions, AS03 (GSK) and AF03 (Sanofi Pasteur). The benefits of using these adjuvants to increase seroprotective antibody titres are widely reported in a number of clinical studies, including in individuals who are most susceptible to influenza-related illness. Compared to non-adjuvanted vaccine responses, formulation of mono- and multi-valent influenza vaccines with MF59 induces substantially higher HAI titres and seroconversion rates in children (60–63) with similar improvements observed in the young and elderly using AS03 (64). These formulations are generally well-tolerated and safe, however, incidences of narcolepsy associated with the use of an AS03-adjuvanted A/H1N1pdm2009 vaccine (Pandemrix) limits the use of this adjuvant in the young. Nevertheless, both MF59 and AS03 have been shown to accelerate the induction of vaccine-mediated responses as demonstrated by the use of adjuvanted vaccines in healthy adults (65, 66), children (67) and in the elderly (68), wherein a single vaccination dose is sufficient to induce seroprotective levels of antibody within as little as 3 weeks. In this regard, these adjuvants, along with AF03 or Alum, provide dose-sparing capabilities for mass vaccination of the wider population; similar levels of protection attained with unadjuvanted vaccines can be achieved with using substantially smaller amounts of HA antigen or less vaccination doses when formulated with adjuvant

(69–72). Several studies have also demonstrated the ability of MF59 to induce cross-reactive antibodies against non-vaccine matched strains in prime-boost regimens. Priming of subjects with a clade 0 H5N3 vaccine formulated with MF59 followed by a boost with a clade 1 H5N1 vaccine containing the same adjuvant results in high titres of cross-neutralizing antibody against H5N1 clade 0, 1 and 2 viruses (73–75). These results thus highlight the role that adjuvants can play in generating and broadening the cross-specificity of naïve and pre-existing B cell memory, the possible underlying mechanisms of which are discussed further in subsequent sections below.

Influenza vaccines designed to target Abs toward the conserved epitopes in the HA stem are also under intense study. While heterosubtypic protection with group 1 HA stem vaccines (i.e., H1 and H5 viruses) lacking the highly variable HA head has been demonstrated in animal models (76), studies on group 2 HA stem vaccines (i.e., H3 and H7 viruses) are more limited. Although promising results are observed when immunizing mice with conserved HA stem epitopes from the H3 subtype, by way of cross-clade neutralizing activity (77, 78), immunogenicity and protection are not maintained when using larger animal models like ferrets (78). Therefore, further studies are needed to develop a human B cell-based universal influenza vaccine, with consideration into the potential for influenza viruses to escape from HA-stem targeted Abs (79).

Dissecting the B Cell Response

Activation of naïve B cells can elicit short-lived ASCs (also called plasmablasts), long-lived antibody-secreting plasma cells (LLPCs), and memory B cells. The fate of B cells is considered to be highly orchestrated, depending on the mode of stimulation, the affinity of their B cell receptors (BCR, or surface Ig) for antigen and their location (80–82). In the periphery, within secondary lymphoid organs (SLO), naïve B cells are activated by BCR/Ag binding and, depending on whether T cell help is provided, they will continue the response in a T cell-dependent (TD) or T cell-independent (TI) manner. B cell memory resulting from a TI response expresses and produces IgM capable of engaging at broadly low affinity with antigens via multivalent BCR engagement, plus toll-like receptor (TLR), and/or complement engagement (83). In TD responses, B and T cell interaction occurs when antigen is captured through the BCR of specific naïve B cells and presented via cell-surface MHC-II glycoproteins to $CD4^+$ helper T cells specific for peptides from the same antigen (84, 85). All B cells activated in this manner either move into lymph node follicles and generate germinal centers (GCs) or differentiate into extrafollicular plasmablasts (86, 87). Through this array of processes, different classes of memory B cells are generated, which can be distinguished by their passage through the GC, location and Ig isotype (81).

In the GC, B cells undergo intense proliferation and broaden their BCR diversity through somatic hypermutation, a process whereby point mutations, insertions, and deletions are introduced within Ig V gene hotspots to generate a broad array of B cell clones with a broad spectrum of affinities for the immunizing Ag (88). This process results in the generation of memory B cells with high-affinity surface Igs and surface $Ig^{+/-}$ plasma cells that maintain serum immunoglobulin levels against the foreign invader. The GC is also the site where a large proportion of BCR-defined clones undergo class switch recombination (CSR), exchanging the Ig isotypes originally expressed (IgM and IgD) for IgG, IgA, or IgE (88–90). The sequential generation of long-lived memory B cells in the GC starts from unswitched memory B cells, followed by class-switched memory B cells and, finally, by LLPC that travel to the bone marrow and other sites (91). The later each B cell population appears, the higher its affinity for Ag (92). Hence, B cells with lower affinity BCRs have a greater propensity to enter, and persist in, the memory pool. Intriguingly, such memory-directed B cells show enhanced Bach2 transcription factor expression when compared to their counterparts with higher BCR affinity, and Bach2 expression inversely correlates with the strength of the B-T follicular helper (Tfh) cell interaction. This suggests that B cells with lower affinity receive weaker T cell help and express higher levels of Bach2, which is clearly a key factor in memory B cell fate determination (92). In addition, expression levels of Blimp-1, the key regulator of plasma cell differentiation and CSR, are regulated by Bach2. Higher Bach2 levels decrease Blimp-1, promoting B cell differentiation toward an unswitched memory fate. The aryl hydrocarbon receptor (AhR), a ligand-induced nuclear transcription factor, is highly induced in B cells upon BCR engagement. AhR promotes Bach2 expression, which in turn suppresses Blimp-1 and therefore the B-Tfh cell interaction becomes weaker and B cell CSR and differentiation into plasma cells are suppressed (93), indicating that it may be a potential target in promoting the generation of low-affinity IgM^+ B cell memory upon vaccination. This is of particular relevance for the design of the next generation influenza vaccines since, as discussed below, as there is an increasing body of evidence suggesting that low-affinity IgM^+ memory B cells capable of identifying a broad range of epitopes should be targeted by influenza vaccination.

Heterogenous Memory B Cell Phenotypes Have Different Roles in Secondary Responses

The various modes of TD and TI B cell activation generate memory B cells with varying isotypes and affinities (summarized in **Figure 1**), some bearing highly mutated Igs generated via the GC reaction and others maintaining germline, less specific and more cross-reactive Abs (52, 81, 94). While it is generally accepted that memory B cells show an enhanced capacity for terminal differentiation into ASC, regardless of phenotype and affinity, there is less consensus regarding their propensity to (re)-enter GC reactions. Contrary to early thinking, it is now generally accepted that both IgG^+ and IgM^+ memory B cells can re-enter GC reactions, albeit they are more predisposed to differentiate into ASC during recall responses (52, 95–97). Similarly, whether or not GCs form during recall responses together with the character of the memory B cell subsets that participate may depend on the type and amount of antigen, inflammatory signals and the availability and quality of cognate Tfh cells (98). There is evidence that unswitched memory B cells bearing germline BCRs have a greater propensity to enter the GC reaction (99). In particular, IgM^+ cells with the least mutated V genes were

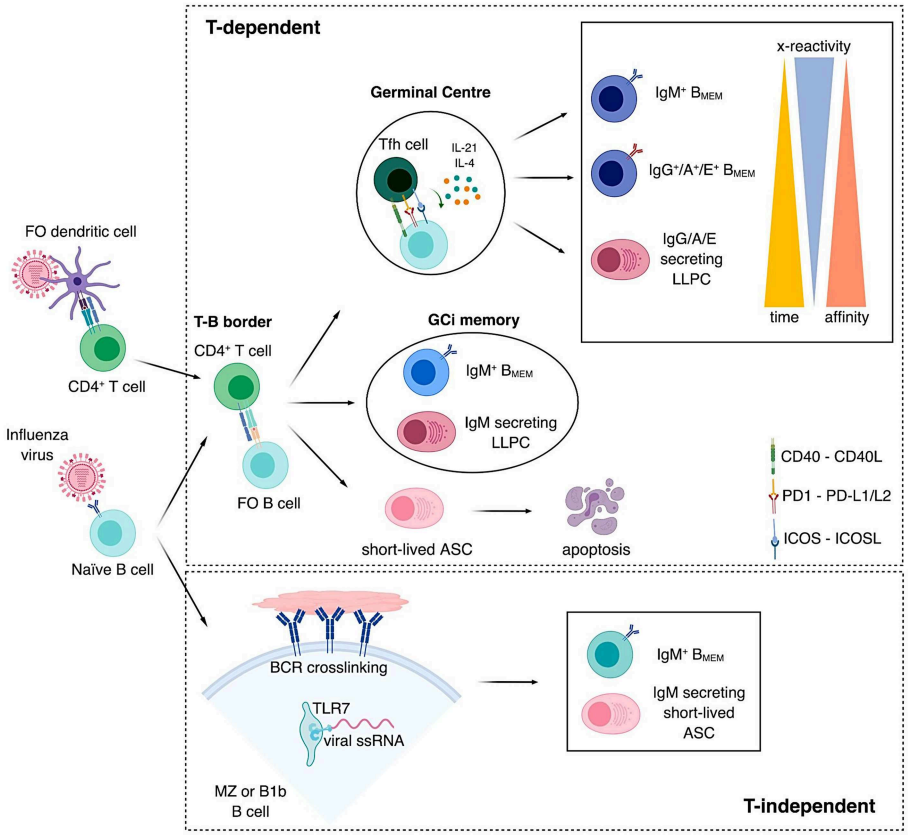

FIGURE 1 | Pathways to B cell memory. Naïve B cells become activated by direct recognition of antigens expressed on the surface of the pathogen. Top panel: Follicular (FO) naïve B cells become activated within the lymph node through a T cell-dependent pathway. $CD4^+$ T cells become activated by recognizing viral peptides processed by FO dendritic cells and presented on their surface by MHC-II molecules. After becoming activated, both $CD4^+$ T cells and B cells, travel to the T-B border in the lymph node, where they interact. Three outcomes can follow this interaction. (i) A germinal center (GC) is formed, $CD4^+$ T cells polarize into T follicular helper (Tfh) cells and FO B cells differentiate into GC B cells. In the GC, B cells undergo rapid proliferation and somatic hypermutation of the Ig V regions in their B cell receptors (BCR), due to their interaction with Tfh cells through CD40-CD40L, PD1-PD-L1/L2, ICOS-ICOSL among others and the secretion of cytokines such as IL-4 and IL-21, affinity maturation takes place and those B cells that increase affinity toward their Ag are selected. Some of these B cells will also class-switch. These interactions result in the generation of IgM^+ memory B cells (B_{MEM}), $IgG^+/A^+/E^+$ B_{MEM} or IgG/A/E secreting long-lived plasma cells (LLPC) in this order in time. The later these cells are generated, the higher affinity and lesser cross-reactivity they have toward the antigen or antigen variants, respectively. (ii) Not all B cells enter the GC after interacting with their cognate activated $CD4^+$ T cells in the T-B border, IgM^+ B_{MEM} and IgM secreting LLPCs are also generated outside of the GC, in a GC-independent (GCi) manner. (iii) Short-lived antibody secreting cells (ASC) are generated early after activation to generate a rapid response against the pathogen. These short-lived ASC will undergo apoptosis and do not contribute to the generation of B cell memory. Bottom panel: Some protein antigens provide highly repetitive antigenic structures, which induce strong BCR crosslinking. Viral single-stranded RNA (ssRNA) together with other danger signals also activate toll-like receptors such as TLR7. These strong signals are enough to activate B cells in a T cell-independent (TI) manner and generate short-lived IgM secreting ASC and IgM^+ B_{MEM}. B1b and marginal zone (MZ) B cells are activated in a TI manner and provide a faster response against the pathogen.

more prominent within GCs during the recall response to a variant viral protein antigen rather than to the original inducing antigen when sequentially immunizing mice with variant Dengue envelope proteins with 63% amino acid identity (100). However, when using HAs from more closely related influenza viruses, with ~82% sequence identity, the GC response was dominated by highly mutated memory B cells, which led to a worsened antibody response as compared to the primary encounters, even in the presence of an adjuvant (101). In the elderly, a poor adaptive capacity of B cells toward the drifted influenza epitopes has also been demonstrated. This resulted in the expansion of B cell memory targeting mostly conserved but less potent epitopes (102). In contrast, memory B cell expansion after H3N2 infection reflected imprinting toward strains encountered early in life but also adaptation to the infecting virus (103). These studies suggest that a certain degree of an antigenic difference is needed to induce a protective secondary antibody response by stimulating broadly cross-reactive low-affinity IgM^+ memory B cells. High-dose and adjuvanted vaccines may improve VE when influenza vaccines strains are antigenically-different. The propensity for IgM^+ memory B cells to dominate recall GC responses may be further determined by pre-existing antibodies that may outcompete the BCRs from low affinity naïve and IgM^+ memory B cells, but not high affinity IgG^+ memory B cells, for antigen (52, 96, 104).

In consideration of the potential for influenza Ab responses to become focused on epitopes present in successive vaccine strains to the detriment of recognizing future variants, it seems

appropriate to think in terms of future vaccines that maintain plasticity and heterogeneity within the B cell response. For example, vaccination strategies that recall IgM^+ memory B cells with less-mutated BCR repertoires, while also inducing naïve populations together with cognate Tfh cell memory to facilitate memory GC formation (98), may tend to skew the overall response toward the generation of more cross-reactive Abs against variant epitopes.

T Cell-Independent B Cell Responses Against Influenza

In contrast to TD Ags, which are generally proteins that cannot induce cross-linking of multiple BCRs, TI Ags are generally multivalent polysaccharides or other molecules that contain a repetitive array of antigenic epitopes that have that BCR-polymerization propensity. This paradigm is, however, challenged by the finding that high doses of a monomeric protein Ag can also elicit an exclusive TI B cell response (105, 106). In mouse experiments, both TD and TI B cells give rise to short-lived plasma cells and memory B cells (107–109) and contribute not only to resolving primary influenza virus infection, but also to more effective control of virus replication and symptoms after secondary challenge (110). The recall capacity of TI memory B cells is largely a result of Ag driven clonal expansion, however, like other memory B cells, TI memory B cells are able to respond more readily to Ag than their naïve counterparts.

The capacity of inactivated whole (vs. split) virion vaccines to induce superior influenza virus-specific antibody responses (111–113) may in part be due to the greater induction of TI B cell responses (114). Notably, when TI B cell responses were induced Ab affinity and neutralizing activity was enhanced. The ability of inactivated whole virions to induce TI B cell responses is linked to the presence of single-stranded RNAs that activate B cells via a TLR7-dependent mechanism (114), hence TLR7/8 agonists should be considered as potential adjuvants for seasonal influenza vaccines.

Importance of Location for Influenza-Specific Memory B Cells

Unlike LLPCs, memory B cells persist as tissue-resident or circulating among the SLO (115). Memory B cells resulting from a local infection also localize in the affected organs. This occurs following influenza virus infection when influenza-specific memory B cells can be found, not only in lymphoid organs, but also in the lungs. Moreover, memory B cells are also differentially distributed among the lymphoid tissues, indicating that trafficking is influenced by local tissue factors (116, 117). After influenza re-exposure, lung-resident memory B cells differentiate into plasmablasts, providing IgG and IgA *in situ* that quickly neutralizes the virus (117, 118). In general, IgA^+ memory B cells seem to localize preferentially to the blood and to tissue sites of pathology, while IgG^+ memory B cells are broadly distributed among tissues that may, or may not, be directly involved in the disease process (116, 117). B cell memory and secreted IgA located in the lungs are essential to provide a quick and effective response against influenza viruses upon exposure, yet current influenza vaccines fail to strongly boost IgA responses (119). Antigen reaching the mucosa of the lung is required to potentially induce stronger IgA responses and for the generation of lung-resident memory B cells, which establish early after infection. The varied location of memory B cells according to their isotype, together with the fact that different environments drive B cell class-switching to a specific isotype, are of particular interest for vaccine design, particularly where (as in influenza) mucosal surfaces are the primary site of infection.

T Follicular Helper Cell Memory: Recent Advances in Influenza Vaccination

When the GC contracts, the GC Tfh cells exit and develop into resting memory Tfh cells with a less polarized Tfh phenotype (120–125). Tfh cells with a resting memory phenotype both recirculate in blood and can be found in BM, spleen, and lymph nodes (126–128). Circulating Tfh (cTfh) cells are the most accessible subset in humans. Of increasing research interest, cTfh cells are heterogeneous and can be classified into different subsets based on surface marker expression. Resting cTfh cells express CCR7, which differentiates them from their GC counterparts. When cTfh cells become stimulated, they downregulate CCR7 to traffic to the GC (129). Three different subsets of cTfh cells can be distinguished according to the surface expression of the chemokine receptors CXCR3 and CCR6, which are involved in inflammatory-homing and epithelial and mucosal site-homing, respectively (130, 131). The Tfh1 cells are $CXCR3^+$ $CCR6^-$, express the T-bet transcription factor and secrete the Th1 cytokine IFNγ. Conversely, the $CXCR3^-CCR6^-$ Tfh2 set expresses the transcription factor GATA3 and produces the Th2 cytokines IL-4, IL-5, and IL-13. Then the Tfh17 cells $CXCR3^-CCR6^+$ cells express the transcription factor RORγT and secrete the Th17 cytokines IL-17A and IL-22 (132).

An overall consensus on the functional implications of the different Tfh subsets regarding B cell help is yet to emerge. While the Tfh1 cells are thought not to be efficient B cell helpers, the opposite is true for the Tfh2 and Tfh17 populations (132, 133). However, human studies on the cTfh response following influenza vaccination demonstrate an increase of circulating, activated cTfh1 cells peaking on day 7 after vaccination that positively correlates with the generation of protective Ab responses and the presence of ASCs in blood (115, 134). In the context of influenza immunization, when culturing human cTfh1 cells isolated at day 7 after priming with either naïve or memory B cells, the cTfh1 cells stimulate memory B cell differentiation into plasmablasts, while naïve B cells remain resting. Yet, naïve B cells cultured with Tfh2 and Tfh17 cells can differentiate into plasmablasts (134). Because Tfh cells are essential to induce a proper B cell response and we speculate that naïve B cells are not being sufficiently stimulated due to epitope masking by pre-existing Abs and memory B cells, it could be possible that mainly Tfh1 cells are stimulated after influenza vaccination at the expense of Tfh2 and Tfh17.

Anti-viral $CD8^+$ T Cell Responses

Seasonal influenza vaccines are designed to elicit an Ab response. However, the natural influenza virus infection additionally elicits cellular immunity ($CD8^+$ T cells, $CD4^+$ T cells, MAIT cells,

NK cells) to eliminate the infection. Because influenza viruses are under constant selective pressure, the long-term protective value of any vaccine that targets a specific HA and/or NA will inevitably be compromised with time, immune $CD8^+$ T cells are critical for recovery and provide some protection against severe influenza disease, including that resulting from infection with a previously unencountered avian strain. This likely reflects that influenza-specific $CD8^+$ T cells tend to recognize HLA-bound peptides derived from more conserved, internal virus proteins. The question is whether vaccines that promote such $CD8^+$ T cell memory can, when combined with the classical products that induce virus-specific Ig response, provide better protection against, in particular, a newly invasive pandemic strain. An overview comparison between B and T cell responses after influenza virus drift and shift and how they complement each other is shown in **Table 1**.

Adaptive T cell immunity is mediated primarily by T cells, expressing the CD4 or CD8 co-receptors, respectively. During influenza virus infection, viral proteins are degraded by the proteasome and processed into smaller peptide fragments. These fragments are bound to MHC molecules and carried to the cell surface for presentation. These peptide/MHC complexes (pMHC) are recognized by clonally expressed TCRs on $CD4^+$ or $CD8^+$ T cells, leading to their activation and recruitment into the virus-specific immune response. The $CD8^+$ cytotoxic T lymphocytes act as sentinels, recognizing and killing virus-infected targets, an essential step for virus clearance. Following activation, $CD8^+$ T cells also secrete anti-viral cytokines (especially IL-2, IFN-γ, and TNF-α) which further recruit innate and adaptive immune cells into sites of influenza virus-induced pathology and induce anti-viral responses in infected cells (141, 142). When it comes to CTL killing, the secretion of perforin, granzymes and FAS ligand can all be involved in the process of inducing the apoptosis of virus-infected cells (143, 144). Additionally, the expression of TRAIL on CTLs can lead to the elimination of influenza virus infected cells, with a resultant decrease in mortality (145).

T Cell Fate: to Die or Become Memory

Formation of memory $CD8^+$ T cells is essential for the protection against re-encountered pathogens. Our understanding of key factors determining the fate of $CD8^+$ T cells during influenza is still limited but crucial for the development of a $CD8^+$ T cell activating vaccine. During differentiation from naïve to effector, to memory status, $CD8^+$ T cells transiently express cell surface molecules that are considered to be predictive of cellular fate and function. Surface expression of IL-7R and KLGR1 on effector $CD8^+$ T cells can, at least in some situations, differentiate between $CD8^+$ T cells designated as memory precursor effector cells and short-lived effector cells (146). Compared to the IL-$7R^{lo}KLGR1^{hi}$ set, $CD8^+$ T cells expressing high levels of IL-7R and low levels of KLGR1 are 10-fold more likely to survive (147) in mice infected with lymphocytic choriomeningitis virus (LCMV). However, it should be noted that these profiles may not be exclusive, as $KLRG1^+$ $CD8^+$ T cells are detectable after LCMV infection is cleared (148), and the survival value associated with the IL-$7R^{hi}KLGR1^{lo}$ set for LCMV is less obvious for influenza virus infection (149). Additionally, the discovery of other early markers of memory formation during *Listeria monocytogenes* and vesicular stomatitis virus infection, including expression of ID3 transcription factor (150) and IL-2Rα cytokine receptor, showed that $CD8^+$ T cell memory generation is certainly multi-factorial (151, 152). Identifying markers of successful memory formation is crucial for evaluation of novel influenza vaccine responses and should be considered in future influenza vaccine studies. More recently, high-throughput sequencing is facilitating the emergence of a broader picture for $CD8^+$ T cell differentiation. Single-cell RNAseq of $CD8^+$ T cells at the acute phase of LCMV infection indicates that there may be two distinct populations of antigen-induced $CD8^+$ T cells that share genes either with "terminal effector" or "memory" cells (153). Compared to naïve $CD8^+$ T cells, the "terminal effector-like" set can be shown to have upregulated more than 900 different genes, while the "memory-like" cells only upregulated 27 genes (153). This suggests that the differentiation of "terminal effector" $CD8^+$ T cells mandates the upregulation of hundreds of genes involved in both clonal expansion and the mediation of a spectrum of effector functions, while the establishment of $CD8^+$ T cell memory requires only the involvement of a few key genes to maintain lymphocyte quiescence. Although the exact factors mediating distinct $CD8^+$ T cell fates during early division following viral infection are still in the process of elucidation, experiments with TCR-transgenic mouse models indicate that TCR signaling strength (154), as reflected in IL-2R, IFN-γR, and mTOR levels during mitosis and asymmetrical division (155–157) is key to the generation of anti-viral $CD8^+$ T cell memory. This is an exciting area of research that should, as it unfolds, give a much better understanding of both the molecular basis of CTL memory formation, and provide key measurement parameters that will allow us to skew early vaccine responses so that they provide optimal memory that gives long-lasting protection when recalled by further pathogen challenge.

Importance of Generating Long-Term T Cell Memory

As mentioned above, memory $CD8^+$ T cells are important for eliciting long-term, broadly cross-reactive immunity to influenza viruses, and are thought to mediate the protective function mainly via the killing of virus-infected targets (158). Virus-specific $CD8^+$ effector T cells also produce proinflammatory cytokines, and the breadth of cytokine production (termed polyfunctionality) often correlates with efficient protection against pathogens, including influenza viruses (159). Polyfunctional memory $CD8^+$ T cells (producing IFN-γ, TNF, IL-2, and MIP-1β) (160) are thought to operate via augmented cytolytic activity via dual IFN-γ/TNF expression (161), IL-2-mediated enhancement of $CD8^+$ T cell memory function (162) and increased IFN-γ secretion on a per cell basis (163). One example of the protective capacity of these polyfunctional memory $CD8^+$ T cells is the induction of long-lasting memory $CD8^+$ T cells against variola (smallpox) virus induced by the Vaccinia vaccine Ankara (164). When $CD8^+$ T cells were primed with influenza virus nucleoprotein (NP) expressed by either a recombinant Vaccinia virus or in *Listeria monocytogenes*, the more polyfunctional NP-specific $CD8^+$ T

TABLE 1 | The clinical outcome and the B and T cell memory responses after exposure to influenza viruses are summarized below.

	Influenza antigenic site change			
		Antigenic drift *Genetic changes in Ag sites alter Ab binding*		**Antigenic shift**
	None	**Minimal**	**Major**	**Exchange of surface Glycoproteins**
Clinical outcome		Little to no symptoms	Unpredictable (135)	Dependent of $CD8^+$ T cell response • Limited by HLA alleles (136, 197) • Prior exposure to influenza (137–139) T cell memory pool and quality of T cell response (137–139) ⇒ Severe to fatal outcome with prolonged hospitalizations (137)
B cell response	Robust memory B cell response and protective Ab production (135)	Dominated by memory B cells against preserved antigenic sites, yielding a protective but focused Ab response that may not protect against future drift.	Cross-reactive memory B cells produce an early unadapted Ab response to limit virus replication and symptoms, and enter GC reactions to generate updated memory and PCs If enough Ag available, naïve B cells react and generate updated B cell memory	Very limited (if any) protection by memory B cells (31, 140) Response driven by naïve B cells
$CD8^+$ T cell response		Cross-reactive Not responsive if B cells neutralize the virus		Cross-reactive but not neutralizing immunity Host-specific differences

cells were generated following Vaccinia virus exposure. Mice vaccinated with the Vaccinia virus showed also a greater level of protection against a normally lethal IAV challenge compared to *the Listeria monocytogenes* vaccine group counterparts (165). This indicates, that not only the quantity of memory $CD8^+$ T cells is critical for the protection but also their quality. Insights into key factors inducing these polyfunctional $CD8^+$ T cells could improve a T cell-based vaccine therefore vastly.

Memory $CD8^+$ T cells can be divided conceptually into central and effector T cell memory sets, based on their expression profiles for the CD62L and CCR7 surface proteins (166) that are known to affect cell localization and function (167). The $CD62L^{hi}CCR7^{hi}$ "central memory" $CD8^+$ T cells (T_{CM}) can be found in the spleen, blood and lymph nodes, and display superior functions compared to their $CD62L^{lo}CCR7^{lo}$ effector memory $CD8^+$ T cell (T_{EM}) counterparts, mainly in terms of their proliferative capacity and IL-2 production profiles (168). In addition, a highly specialized population of tissue-resident (T_{RM}) memory $CD8^+$ T cells expressing $CD103^+CD69^+$ can persist in sites of pathology subsequent to virus clearance (169). Following the secondary challenge, $CD103^+CD69^+$ T_{RM} set is able to expand and secrete cytokines, including IFN-γ and TNF, as well as generate more polyfunctional progeny (69% of cells capable of secreting three cytokines), when compared to $CD103^-CD69^+$ (21%) and $CD103^-CD69^-$ (16%) parent subsets (160, 169). In the context of influenza, persistence of influenza-specific $CD8^+$ T_{RMs} correlates strongly with protection when mice are challenged with a serologically distinct IAV that shares common internal proteins (170). The T_{RM} population develops from precursors lacking KLRG1 (171, 172) and further studies on T cell receptor (TCR) repertoires suggest that they arise from the same naïve pool as T_{CM} set (173). T_{RM} generation is largely regulated by a series of transcription factors (174), such as Runx3 which is crucial for T_{RM} establishment across a range of tissues (175), and Bach2 which is recognized to restrain the terminal differentiation of effector T cells and help with formation of long-term memory T cells (176). The differentiated T_{RM} phenotype is associated with changes in key transcription factors, including downregulation of Kruppel-like factor 2 (KLF2), TCF1 (177), T-bet, and Eomes (178, 179) as well as upregulation of Hobit, Blimp1 (177) and AhR (180), Nur77 (181), and Notch (182), required for the maintenance of T_{RMs}. While the previously named transcription factors are universal hallmarks of T_{RM} formation, T_{RM} heterogeneity among cells generated at different tissue sites suggest that microenvironmental cues are important for site-specific T_{RM} differentiation. Indeed, generation of the lung T_{RM} set is influenced by transforming growth factor β (TGF-β) along with the presence of IFN-γ-secreting $CD4^+$ T cells following influenza virus infection (183, 184). While the generation of influenza-specific T_{RMs} has recently been shown to be vital for robust protection, unlike T_{RMs} generated within the skin or gut (185–187), lung-resident T_{RMs} do not offer long-term

protection, rather they require a constant supply of circulating T_{EM}s cells to replenish the niche over time (188) (summarized in **Figure 2**). In humans, influenza-specific lung-resident T_{RM} cells show a high degree of TCR-sharing with other influenza-specific lung T_{EM} cells, suggesting that both memory cell subsets originate from the same precursors (160). Our understanding of the protective role of memory $CD8^+$ T cells in influenza virus infection also comes from experiments with a C57BL/6 mouse model lacking antibodies, where increased numbers of influenza-specific memory $CD8^+$ T cells and T_{RM} cells led to markedly reduced influenza-induced morbidity (189). Similarly, primary vaccination with a single-cycle, non-replicative H3N2 IAV induced $CD8^+$ T cells capable of protecting against a heterologous (H1N1) lethal challenge (190), an effect that was diminished for mice that had been depleted of $CD8^+$ T cells after vaccination. These studies highlight the potential of long-term memory $CD8^+$ T cells protecting against severe influenza virus infections. A potential that is not harnessed in the current vaccine strategy.

$CD8^+$ T Cells Recognize Highly Conserved Influenza Epitopes

$CD8^+$ T cells can confer broad cross-protection across different seasonal, pandemic and avian influenza IAV strains due to their ability to recognize relatively conserved viral peptides derived from internal influenza components (NP, M1 and PB1, PB2). The best defined human $CD8^+$ T cell influenza epitope is the immunodominant $M1_{58-66}$ peptide bound to the HLA-A*02:01 molecule (191–193). This peptide is highly conserved within different influenza A subtypes spanning 100+ years (136), including the 1918 and 2009 pandemic H1N1 strains as well as highly pathogenic H5N1 avian viruses (194). Analysis of immunogenic peptide profiles for the avian H7N9 influenza virus established that it shared six universal $CD8^+$ T cell epitopes conserved at ~100% prevalence in human influenza A viruses circulating since the catastrophic Spanish 1918 influenza. These universal human influenza-specific $CD8^+$ T cells epitopes include HLA-A*02:01/$M1_{58-66}$, HLA-A*03:01/$NP_{265-273}$, HLA-B*08:01/$NP_{225-233}$, HLA-B*18:01/$NP_{219-226}$, HLA-B*27:05/$NP_{383-391}$ (although mutants were found in some H3N2 strains) and HLA-B*57:01/$NP_{199-207}$ (136). The population coverage by the universal HLAs varies greatly across ethnicities. Fifty-six percent of Caucasians displaying at least one universal HLA, while such coverage reached only 16% in the Alaskan and Australian Indigenous populations (136), highlighting the vulnerability of Indigenous populations toward newly-emerged influenza viruses. Additionally, our recent studies found broadly cross-reactive $CD8^+$ T cell responses directed toward the HLA-B37-restricted NP_{338} epitope across IAVs (195), and excitingly, for the HLA-A*02:01-restricted PB1-derived epitope across influenza A, B and C viruses (196). The latter introduces a new paradigm

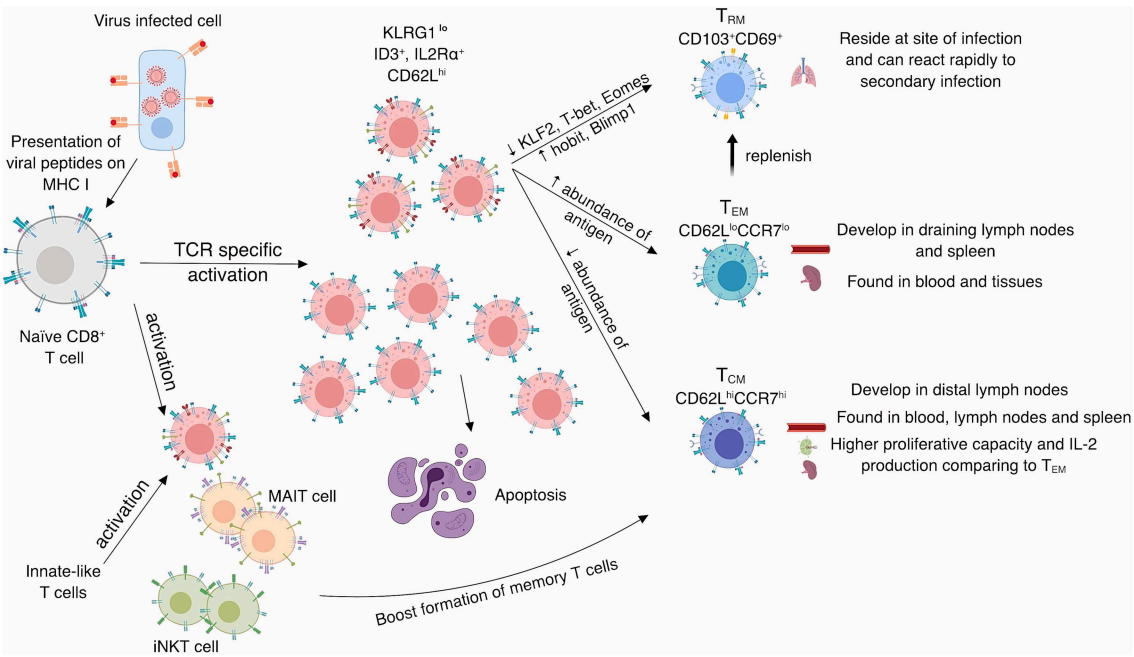

FIGURE 2 | $CD8^+$ T cell memory formation. Naïve $CD8^+$ T cells become activated by recognition of viral peptides presented in the context of MHC-I molecules on the surface of virally-infected APCs. Activated $CD8^+$ T cells divide and differentiate into effector $CD8^+$ T cells, which kill virus-infected cells and secrete cytokines to induce an anti-viral milieu. After viral clearance, mainly $KLRG1^{lo}$, $ID3^+$, $IL2R\alpha^+$, and $CD62L^{hi}$ $CD8^+$ T cells develop into $CD8^+$ memory T cells, while the remaining ~90–95% of $CD8^+$ T cells undergo apoptosis. Memory formation can be augmented by innate-like T cells (iNKT and MAIT cells). Memory $CD8^+$ T cells are divided based on surface marker expression, known to impact their localization. While T_{CM} and T_{EM} can be found in blood and tissues, T_{RM} reside at the site of infection where they can rapidly respond towards a secondary infection. T_{CM} can be also found in lymph nodes and display higher proliferative capacity and IL-2 production compared to their T_{EM} counterparts.

whereby $CD8^+$ T cells can potentially confer a measure of previously unrecognized cross-reactivity across all human influenza A, B and C viruses, a key finding for the design of universal vaccines.

Influenza-induced morbidity and mortality can correlate with the expression of certain HLAs, including HLA-A*24:02, A*68:01 or B*39:01 alleles, as shown during the 2009 H1N1 pandemic (197). Analysis of peptide scores demonstrated that HLA-A*24:02 is more likely to bind variable (rather than conserved) viral regions (197). Similarly, we have previously shown that some HLA alleles, including HLA-A*24:02 and A*68:01, are less able to elicit robust immune responses toward the highly conserved NP and M1 peptides (136). Both HLA-A*24:02 and A*68:01, in particular, are found at higher frequencies for Indigenous populations world-wide (136, 197), which may explain the disproportionate impact of pandemic influenza viruses on Indigenous peoples during both (otherwise mild) 2009 pH1N1 pandemic and 1918–1919 (H1N1) Spanish "flu catastrophe" (198–202).

Thus, given the broad potential for cross-protective capacity mediated by $CD8^+$ T cells, along with more recent evidence that this effect may indeed be operating in nature to protect people, this aspect of immunity is of considerable interest in terms of developing improved influenza vaccines. However, it is important to note that designing peptide-based T cell vaccines that only cover the major HLA types would clearly be disadvantageous for Indigenous populations globally (203). Further research on $CD8^+$ T cell epitopes found in high risk populations is therefore of highest importance to protect people of highest vulnerability.

$CD8^+$ T Cells Can Confer Broad Cross-Protection for Heterologous IAV Strains

In the context of newly emerging influenza virus infections in people, correlative studies suggest that established $CD8^+$ T cell memory confers cross-reactive immunity against severe influenza disease, as observed during the 2009 pandemic H1N1 (pH1N1) outbreak (139, 204). The high (∼70%) conservation of $CD8^+$ and $CD4^+$ T cell epitopes contributing to pre-existing memory may have been a significant factor in the generally mild outcomes of the 2009 H1N1 pandemic (138). Sridhar et al. showed that individuals with higher numbers of $CD8^+$ T cells recognizing conserved influenza epitopes fared better following natural infection with the 2009 H1N1 virus (139). The importance of $CD8^+$ T cell-mediated immunity was further highlighted in 2013 following the emergence of the novel avian H7N9 strain (205, 206), which killed ∼40% of the infected patients. In H7N9-infected individuals, rapid recovery from hospitalization was associated with the presence of significantly more IFN-γ-secreting $CD8^+$ T cells when compared to the situation for those who died (207) and recovered (206).

Development of CTL-Based Vaccines

Lessons Learned From the Yellow Fever Vaccine

While the initial experience of IAV infection generally occurs in the first 6 years of life (208), our understanding of both the primary IAV-specific $CD8^+$ CTL response and the transition to influenza-specific T cell memory is very limited for humans. Though one paper by Mbawuike et al. reported on primary infection in infants as early as 6 to 13 months of age (209), studies of such influenza exposures in infants are rare, and have not been performed using contemporary approaches for the analysis of T cell-mediated immunity. The closest we have for humans of any age when it comes to the formation of memory $CD8^+$ T cells following first virus encounter is for the live-attenuated 17D yellow fever (YF) vaccine. As might be expected from a plethora of mouse experiments, recent YF vaccination studies showed that deuterium-labeled, epitope-specific $CD8^+$ CTLs expanded initially following vaccination, before undergoing a contraction phase characteristic of $CD8^+$ T cell memory. These vaccine-induced YF-specific memory $CD8^+$ T cells persisted in the blood for at least 2 years after YF vaccination, with an average deuterium half-life decay rate of 493 days (210). A similar YF vaccination study in mice demonstrated that, after initial contraction, the long-lived $CD8^+$ T cell memory pool remained consistent in size (211), indicating a potential advantage of a $CD8^+$ T cell that would need fewer revaccinations compared to the annual recommendation necessary for the seasonal influenza vaccine. Unfortunately for influenza vaccination, the current IIV used in humans does not induce any $CD8^+$ T cell responses that can be targeted for such a longevity analysis (115).

Vaccination Approaches to Induce Memory $CD8^+$ T Cells

Different influenza vaccination approaches are currently being investigated in order to induce long-lasting cross-protective immunity. The only licensed vaccines capable of inducing $CD8^+$ T cell immunity, such as the YF vaccine, use live-attenuated pathogens. These are not recommended for influenza "high-risk" groups such as pregnant women, immunosuppressed individuals and the elderly. Therefore, new vaccination strategies need to be developed if we are to protect such vulnerable people. Vogt et al. showed that changing the route of vaccine administration of a quadrivalent inactivated influenza vaccine from intramuscular (i.m.) to transcutaneous induces the expansion of vaccine component-reactive $CD8^+$ T cells. Interestingly, the vaccine was also able to induce $M1_{58-66}$-specific responses in a HLA-A*02:01-positive donor, although this was only observed in one individual (212). Another approach currently in development is the *Flu-v* $CD8^+$ T cell-activating vaccine (213) containing four 21–35 amino acid-long peptides from internal influenza proteins, which can potentially bind to multiple HLA allelic forms, including the highly prominent HLA-A*02:01. This approach was protective for HLA-A2 transgenic mice and was also capable of inducing IFN-γ-expressing $CD8^+$ T cells across all the participants ($n = 15$) in a phase 1b vaccine trial (213, 214). The *Flu-v* product showed that the vaccine reduces both the viral titer and the symptom score after H3N2 virus challenge in humans (215). However, due to the unknown HLA-restriction of the immunogenic epitopes, the HLA coverage of this vaccine is still to be determined. To circumvent the need for prior knowledge of HLA-restricted epitopes to be included in a universal T cell-based vaccine, particularly for less common HLA allelic variants, full-length influenza proteins have been expressed in Vaccinia virus

Ankara vaccine vectors. Berthoud et al. showed that a viral vector encoding for the two internal proteins NP and M1 could induce some $CD8^+$ T cell responses (216).

Overall, development of an effective, long-lasting, cross-reactive influenza vaccine relies on an individuals' capacity to generate polyfunctional lung-resident $CD8^+$ T cells. However, difficulties in identifying cross-reactive epitopes caused a bottleneck in the development of a universal influenza vaccine. Due to the propensity of IAV to trigger severe outbreaks with pandemic potential, murine models have thus far been developed to test the effectiveness of IAV vaccines based on conserved internal proteins (217–219). While mice immunized with these vaccines can elicit protective $CD8^+$ T cell responses, the molecular mechanisms which govern formation of protective memory responses still require further validation in mice, and ultimately in humans.

Innate and Bystander T Cell Activation During Influenza Virus Infection

In addition to the activation and proliferation of $CD8^+$ T cells in a peptide-MHC dependent manner, T cells can also become activated via antigen-independent mechanisms, resulting in proliferation of polyclonal T cells (220). In an influenza mouse model, adoptive transfer of TCR-transgenic OT-I $CD8^+$ T cells, which recognizes the ovalbumin peptide, into influenza-infected mice, showed that these OT-I cells can non-specifically expand in the lungs of influenza-infected mice. This suggests that $CD8^+$ T cells can become activated independently of their TCRs during primary influenza virus infection (221). Similarly, highly activated $CD38^+HLA\text{-}DR^+$ $CD8^+$ T cells, numerically greatly exceeding influenza-specific $CD8^+$ T cell pools, were found in patients hospitalized with severe H7N9 disease (137), suggesting bystander activation of at least some $CD38^+HLA\text{-}DR^+$ $CD8^+$ T cells. Despite the evidence that bystander $CD8^+$ T cell activation occurs during influenza virus infection, the importance of these cells in terms of viral clearance and the induction of long-term memory is poorly understood. To date, the most solid evidence for the role of bystander activation has been observed in innate-like T cells. These cells, unlike conventional $CD8^+$ T cells, recognize non-peptide antigens presented by orthologous MHC I-like molecules. They rapidly secrete cytokines following activation and can mediate some level of protection before adaptive immunity is sufficiently advanced (222). Recently, we demonstrated that mucosal-associated invariant T (MAIT) cells become activated during IAV infections in humans and mice (223, 224). These MAIT cells recognize riboflavin-derivative antigens produced by microbial pathogens (225), but can be variously activated by IL-12/IL-18 (224), IL-15, or type I interferons (226). Using a murine model, we showed that MAIT cells rapidly accumulate and become activated in the infected lung and contribute to protection against IAV infection (223). Similarly, invariant Natural Killer T (iNKT) cells, which recognize lipid antigens presented by CD1d, can protect against murine IAV (227–229). In addition, iNKT cells induced by inactivated influenza A virus vaccination in conjunction with alpha-galactosylceramide, an iNKT cell antigen, can boost influenza-specific memory $CD8^+$ T cells and protective immunity in mice (230). The exact contribution of innate T cells vs. conventional $CD8^+$ T cell-mediated immunity against influenza viruses is a subject of further investigation. These new insights help to understand the wider range of vaccine responses thus offering us opportunities to generate better strategies to fight against influenza.

CONCLUDING REMARKS

Although current seasonal influenza vaccines can promote the induction of highly specific, long-term memory B cells that produce antibodies against the viral HA1 domain, these antibodies are generally unable to combat newly emerging influenza viruses, including novel pandemic stains and antibody-selected "seasonal" variants that have accumulated mutations in those epitopes surrounding the receptor binding pocket. Generation of high-affinity neutralizing Abs against conserved surface epitopes remains a constant challenge to provide long-lasting and cross-protective B cell memory, and as such, more work is needed to better understand B cell responses against natural infection vs. vaccination, in order to design better B cell- or antibody-based universal vaccines. On the other hand, an influenza vaccine capable of stimulating $CD8^+$ T cell responses would generate long-term T cell memory against conserved epitopes without the need for annual vaccination. In addition, a role for innate-like T cells in influenza protection is increasingly emerging, which could potentially be important both for the development of novel therapeutics and for boosting (or maintaining) long-term memory. As a consequence, substantial efforts are being made globally to exploit both innate and adaptive immune components for the development of novel influenza vaccines that induce long-lasting B cell/antibody and/or cross-reactive T cell immune memory populations.

AUTHOR CONTRIBUTIONS

MA and AF wrote the sections on influenza and humoral immunity. LH and XJ wrote the sections on cellular immunity. BC wrote the section on vaccine adjuvants. TN, KK, and PD wrote and modified the original manuscript, and the revised versions.

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REVIEW
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Remembrance of Things Past: Long-Term B Cell Memory After Infection and Vaccination

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The success of vaccines is dependent on the generation and maintenance of immunological memory. The immune system can remember previously encountered pathogens, and memory B and T cells are critical in secondary responses to infection. Studies in mice have helped to understand how different memory B cell populations are generated following antigen exposure and how affinity for the antigen is determinant to B cell fate. Additionally, such studies were fundamental in defining memory B cell niches and how B cells respond following subsequent exposure with the same antigen. On the other hand, human studies are essential to the development of better, newer vaccines but sometimes limited by the difficulty to access primary and secondary lymphoid organs. However, work using human influenza and HIV virus infection and/or immunization in particular has significantly advanced today's understanding of memory B cells. This review will focus on the generation, function, and longevity of B-cell mediated immunological memory (memory B cells and plasma cells) in response to infection and vaccination both in mice and in humans.

Keywords: B cell memory, vaccination, mouse vs. human, influenza virus, infection

INTRODUCTION

One of the hallmarks of our immune system is the ability to "remember" past exposure to pathogens. Such exposure can be from infection or vaccination, and by remembering we are, ideally, fully protected from infection upon future encounter with the same pathogen (1). Although humoral immunological memory is mediated in part by serum antibodies secreted by long-lived plasma cells (LLPCs), these cells are usually not described as memory B cells. Instead, memory B cells are defined as long-lived and quiescent cells that are poised to quickly respond to antigen upon recall (2–5).

Both memory B cells and antibody-secreting cells (ASCs) are the product of antigen activation and, most often, interaction with cognate T helper cells. They can be IgM^+ or immunoglobulin class-switched, and display germline or affinity-matured antigen receptors (B cell receptors; BCRs) (2, 6–8). Although generation of memory B cells does require ligation of CD40 (9), an early burst of both memory B cells and ASCs can form independently of GCs, as well as in T-cell independent responses (10–16). However, T-cell independent memory responses are beyond the scope of this review and will therefore not be thoroughly discussed here.

The terminal differentiation of B cells into ASCs is governed by a gene-regulatory network and modified by environmental stimuli as reviewed in Nutt et al. (17). ASCs can be divided into short-lived ASCs, including short-lived plasma cells and plasmablasts, and LLPCs. Plasmablasts are considered a transient population and can be either precursors of plasma cells (short- and/or long-lived; mainly in mice) or terminally differentiated effector cells activated during ongoing immune responses (mainly in humans) (18–23). In mice, within 2–4 d after infection, plasmablasts are found in extra-follicular zones and differentiate into plasma cells that secrete large quantities of antibodies. This early humoral response of lower affinity usually lasts a few days (24). In contrast, activation and differentiation of B cells within GCs allow the generation of plasma cells of high affinity that will then migrate to the bone marrow, where they can survive for decades and provide long-term humoral protection (25). Such LLPCs are key to maintaining long-term humoral immunity after infection or vaccination. They persist in the absence of antigen for decades after the original exposure (26). Although they exist in multiple lymphoid organs, the bone marrow is the home of the majority of plasma cells in mice (27, 28).

Most of what we know about the generation of plasma cells and memory B cells comes from mechanistic studies in mice. Because of massive differences between mice and humans in terms of life span and cell populations/phenotypes, the biology of mouse and human B cells differs. It is therefore important to also look toward *in vivo* lessons we have learned from humans.

LESSONS FROM MOUSE STUDIES

The Plasma Cell vs. Memory B Cell Fate Decision

Following antigen activation with a T-dependent antigen, naïve B cells will interact with cognate T cells at the border between the B- and T-cell zones in the secondary lymphoid organs (**Figure 1a**). Here, the activated B cells will proliferate and make their first fate decision: whether to differentiate into extrafollicular ASCs or germinal center (GC)-independent memory B cells, or to move deeper into the follicle to form a GC (**Figure 1b**). A similar choice must then later be made in the light zone (LZ) of the GC, further discussed below. Although the molecular mechanisms for this decision have been extensively studied they have still not been completely elucidated, especially for memory B cell generation. Several studies have addressed the possibility of a "master transcription factor" for memory B cell differentiation, similar to Bcl-6 for GC B cells and IRF-4/Blimp-1 for plasma cells (29, 30). Although Bach2, or specifically high expression of Bach2, in LZ GC B cells has been pointed out as a factor promoting differentiation to memory B cells, a transcription factor unique to memory B cells is yet to be found (31–37). As recently reviewed, ZBTB32, KLF2, ABF-1, and STAT5 have been associated with memory B cell generation, but further studies are needed to understand their role (38).

Affinity

There is general consensus in the field that initial affinity for the antigen influences which differentiation pathway will be chosen by an antigen-activated B cell. Newly activated B cells with a relatively high affinity for the antigen will differentiate into short-lived extra-follicular ASCs (39). This ensures that the first burst of secreted antibody has enough affinity for the antigen to opsonize it and form immune complexes that will be directly cleared by phagocytosis, activate complement, and/or be presented on follicular dendritic cells (FDCs), thereby driving affinity maturation in the GC (30, 40). Conversely, antigen-activated B cells of lower affinity typically develop into GC-independent memory B cells. These are most often unmutated and unswitched (IgM+), although class-switched GC-independent memory B cells have been described (13). The GC-independent memory B cells provide a means of retaining adaptability potential within the memory B cell pool, and these cells can either be recruited later in the same response or recalled upon secondary encounter with the antigen.

GC Responses

The third fate choice for antigen-activated B cells is to upregulate Bcl-6 and move deeper into the follicle and start a GC reaction [excellently reviewed in Victora and Nussenzweig (30), Mesin et al. (40)]. Briefly, the GC B cells will go through multiple rounds of division in the dark zone (DZ) of the GC, each time introducing mutations in their antigen receptor (B cell receptor; BCR). This process of somatic hypermutation (SHM) leads to affinity maturation and ensures that B cells will specialize their binding to a particular antigen. The mutated B cells will then move to the LZ, where the new BCR will be tested against the antigen presented on FDCs. The B cells that manage to form a BCR with high enough affinity will receive survival signals and either return to the DZ to go through another round of division and SHM, or exit the GC as a plasma cell or a memory B cell.

Similarly to extrafollicular fate decisions, BCR affinity to the antigen seems to play a role also in the GC (37, 41, 42). High-affinity B cells can bind and endocytose more antigen, and consequently present more antigen-derived peptides on class II MHC (MHCII). This higher density of peptide:MHCII on high-affinity B cells gives them an advantage in competing for access to T-follicular helper (Tfh) cells (43–45). In addition, each interaction with a Tfh cell is prolonged and intensified due to a feed-forward loop depending on peptide:MHCII density and CD40:CD40L ligation (45). This enhanced CD40:CD40L interaction causes down-regulation of Bcl6 and turning on of IRF-4 in the GC B cells, allowing them to differentiate into $Bcl6^{lo}CD69^{hi}$ plasma cell precursors before exiting the GC as plasma cells (46) (**Figure 1c**). In addition, IL-21 secreted from Tfh cells is required for plasma cell differentiation (47), further demonstrating the importance of long and strong B:T interactions for this fate decision. A fraction of the plasma cells leaving the GC will home to the bone marrow, where their survival depends on a number of factors in the plasma cell niche (**Figure 1d**). This will be further discussed below.

Memory B cells, on the other hand, are generated from low-affinity GC B cells in the LZ and will eventually enter the

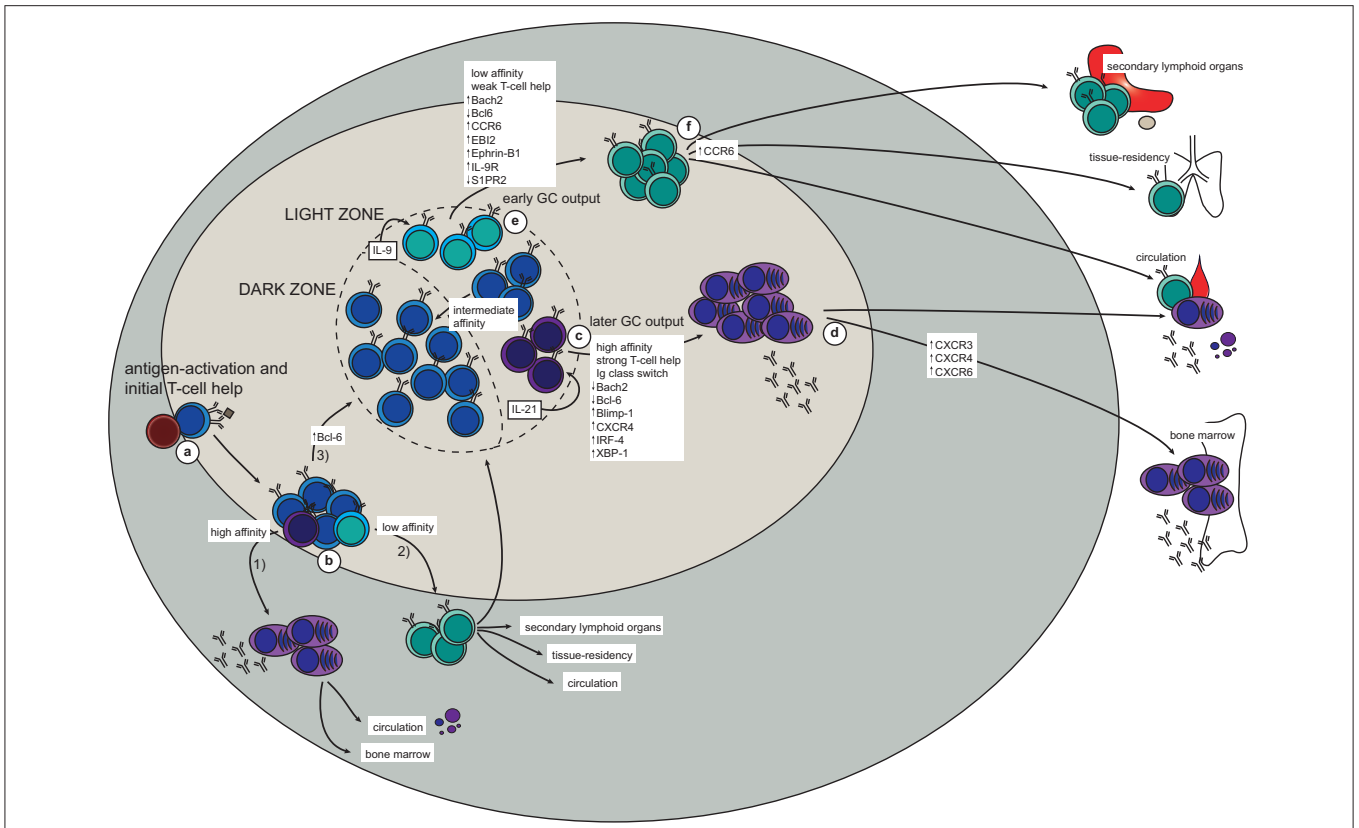

FIGURE 1 | The generation of memory B cells and plasma cells in a T-dependent response (based on mouse studies). **(a)** Antigen-activation brings B- and T cells in contact at the T-B border in secondary lymphoid organs. **(b)** After initial proliferation in the outer follicle, the B cells make their first fate choice: (1) differentiation into extrafollicular (mostly short-lived) plasma cells (higher affinity), (2) differentiation into very early memory B cells (lower affinity), or (3) up-regulation of Bcl-6 and formation of a germinal center (GC). **(c,d)** In the GC, a similar selection process takes place in the light zone (LZ). Here, high-affinity LZ GC B cells receive strong T-cell help and consequently down-regulate Bach2 and Bcl-6 while turning on the plasma cell transcriptional program (Blimp-1, IRF-4, XBP-1; including up-regulation of CXCR4) **(c)**. The plasma cell precursors will then either enter the circulation as short-lived antibody-secreting cells, or they will upregulate CXCR3, CXCR4, and CXCR5 to allow migration to the bone marrow plasma cell niche **(d)**. Here survival factors produced by stromal cells and other adjacent cells (including eosinophils and macrophages) promote their differentiation into long-lived plasma cells, which continue to secrete antibodies for the duration of the lifetime of the host. **(e,f)** Due to the weaker T-cell help received by low-affinity LZ GC B cells, these will not be instructed to turn on either the plasma cell or the GC B cell transcriptional program. Instead, up-regulation of Bach2, CCR6, EBI2, Ephrin-B1, and IL-9R, together with down-regulation of Bcl-6 and S1PR2, promote differentiation to memory B cells **(e)**. To maximize the likelihood of secondary antigen encounter memory B cells will then position themselves strategically in secondary lymphoid organs, become tissue-resident at the site of infection, or patrol as recirculating cells **(f)**.

circulation as patrolling cells or take up residence in lymphoid or target organs (**Figures 1e,f**). The observation that memory B cells consistently are of lower affinity and have fewer mutations than plasma cells indicate that the former are generated before affinity maturation has allowed for the production of high-affinity BCRs. Indeed, an extensive study shows that memory B cells are formed early in the response whereas LLPCs are a later product (15). This temporal discrepancy also fits well with the Bach2 dynamics in memory B cells. Bach2 is required for memory B cell differentiation and only early GC B cells express Bach2, with the expression starting to decline from day 10 (37). Moreover, these experiments show that T cell help, in the form of CD40:CD40L interaction, represses Bach2-expression in GC B cells in a dose-dependent manner. Thus, B cells with higher affinity typically have a lower expression of Bach2 and are therefore predisposed to choose re-entry to the DZ or commitment to the plasma cell transcriptional program. Conversely, relatively weak T cell help, as would be the case for lower-affinity B cells, maintains a relatively high Bach2-expression in LZ B cells, thus favoring a memory B cell fate (37). It is not clear how Bach2 determines memory B cell fate, but it is believed to act as a suppressor of transcription, particularly of *Prdm1* (encoding Blimp-1) and of pro-apoptotic factors such as Bim and Puma (37, 48–51). Thus, it seems likely that lack of strong signaling, and consequently lack of instructions to start the plasma cell or GC B cell transcriptional program forces activated B cells into memory fate. Interestingly, memory B cells and naïve B cells, which are both quiescent with persisting differentiation potential, have similar transcriptional profiles, with the important exception of memory B cells seemingly being hardwired for quick responses (31, 33, 34, 36, 52).

Selection of B cells with a relatively low affinity into the memory compartment early in the response thus ensures that a certain poly-reactivity is maintained within the memory B cell

pool. Indeed, preservation of germline, or close to germline, encoded BCRs in memory B cells provides the memory B cell pool with clones that are able to respond quickly while still maintaining a higher degree of flexibility in terms of antigen binding. This flexibility would be lost should only memory B cells with high-affinity mutated BCRs persist in the memory pool. This idea can be illustrated by the observation that around 10% of memory B cells recognize variant antigen better than wild type protein, thus allowing for breadth of protection in a way that LLPCs do not (53). Conversely, by choosing only the highest-affinity GC B cells for plasma cell fate, the quality of the secreted antibodies is ensured to be very high.

Immunoglobulin Isotype

Another proposed determinant factor of plasma cell vs. memory B cell differentiation is immunoglobulin isotype. B cells that have switched to IgG, IgE, or IgA are more prone to differentiate to plasma cells than memory B cells (54–58). Interestingly, a recent study showed that even when B cells are forced to switch to IgG1 independently of AID, thus uncoupling the effects of SHM and class-switch recombination (CSR), the switched GC B cells were predominantly differentiating into plasma cells (58). Moreover, transcriptional analysis of IgM^+ and $IgG1^+$ GC B cells in the LZ revealed altered signaling through Nur77 in the switched B cells, associated with increased expression of chemokines associated with exit from the GC into the plasma cell compartment (58). Together, these studies indicate that intrinsic properties of a non-IgM BCR, probably in their signaling capacity, influences the plasma cell vs. memory B cell fate decision.

Marking Memory B Cell Precursors

Studies aiming at defining memory B cell precursors in the GC have found differential expression of several markers on subsets of GC B cells in the LZ. One such marker is the chemokine receptor CCR6, which has been shown to be dispensable for the initial generation but required for correct positioning of memory B cells as well as for optimal recall responses (59, 60). These $CCR6^+$ GC B cells are generally of lower affinity, and have a phenotype closely resembling that of memory B cells (e.g., up-regulated EBI2 and S1PR1, and down-regulated S1PR2) (60). A recent study describes a population of Ephrin-$B1^{high}S1RP2^{low}$ GC B cells as memory precursor cells in the LZ, positioned close to the edge of the GC (61). In addition, a study focused on plasma cell precursors in the GC LZ proposes that a fraction of GC B cells in the LZ presenting as $Bcl6^{low}CD69^{low}$ are memory B cell precursors (46).

Finally, IL-9R is expressed on memory B cells as well as on a subset of LZ GC B cells concluded to be memory B cell precursors (62, 63). In addition to Bach2-requirement, optimal memory B cell generation also needs Tfh-derived IL-9 (63), and signaling through IL-9R on memory B cells is required for their recall response (64). Taken together, memory B cell precursors may be found in the GC LZ and present as $CCR6^+S1PR2^{low}$Ephrin-$B1^{high}Bcl6^{low}CD69^{low}IL\text{-}9R^+$. However, further studies are needed to fully elucidate whether this phenotype really corresponds to a committed memory B cell precursor.

The Memory B-Cell Niche and Recall Responses

Upon re-exposure to an antigen the memory recall response will be faster, stronger, and more specific than a naïve response. Protective memory depends first on circulating antibodies secreted by LLPCs (**Figure 2a**). When these are not sufficient for immediate pathogen neutralization and elimination, memory B cells are recalled. It is therefore of vital functional importance that memory B cells are stationed at strategic sites where they can maximize their chance of encountering antigen (**Figure 2b**). The spleen, including the marginal zone, is a major reservoir for memory B cells in both mice and humans (14, 65–67), as is the subcapsular sinus (SCS) of lymph nodes (68). Both the splenic marginal zone and the lymph node SCS are abundant with CD169+ macrophages, which are specialized in presenting unprocessed antigen to B cells (69, 70). It has been demonstrated that both naïve and memory B cells interact with CD169+ macrophages in the SCS, and that upon antigen recall the memory B cells quickly form SCS proliferative foci (**Figure 2c**), or form new GCs (68). This was also seen in human lymph nodes. Interestingly, the largest output from the SCS proliferative foci is short-lived plasma cells (ASCs), whereas the new GC is a site for further affinity maturation and CSR with very stringent quality controls that limit plasma cell differentiation (42). Importantly, both the SCS proliferative foci and the GC also foster memory B cells that may participate in another recall response or be recruited later in the same response. In addition to the spleen and lymph nodes, memory B cells are found in the bone marrow, Peyers' patches, gingiva, mucosal epithelium of tonsils, the lamina propria of the gastro-intestinal tract, and in the circulation (67, 71–76). It has not been convincingly demonstrated that the bone marrow, or any other tissue (apart from the spleen and the lymph nodes) contains functional memory B cells or if these memory B cells simply recirculate from the blood to the tissues. These are all anatomical sites where antigen may breach the barriers or be carried to via the circulation, and the memory B cells located here act as sentinels should pre-existing antibodies not provide adequate protection.

Importantly, memory B cells can also seed sites of infection, where they are maintained as tissue-resident memory B cells (77–79). Here they are quickly activated after pathogen invasion without the need for antigen transportation to draining lymph nodes, thus shortening the time for plasma cell differentiation and antibody production on secondary exposure. Interestingly, in the case of influenza virus infection, broadly reactive memory B cells are enriched in the lung-resident pool, thus conferring quick and cross-reactive protection at the site of infection (80).

Upon re-exposure to antigen, memory B cells can quickly proliferate and differentiate into plasma cells. Alternatively, they will re-enter GCs for another round of affinity maturation and CSR. This decision depends on BCR affinity and isotype in addition to differential expression of CD80 and PD-L2 (**Figures 2d–f**). These surface markers denote functionally different memory B cells independent of immunoglobulin isotype (2, 4, 7, 8, 65). Importantly, the heterogeneity of the memory

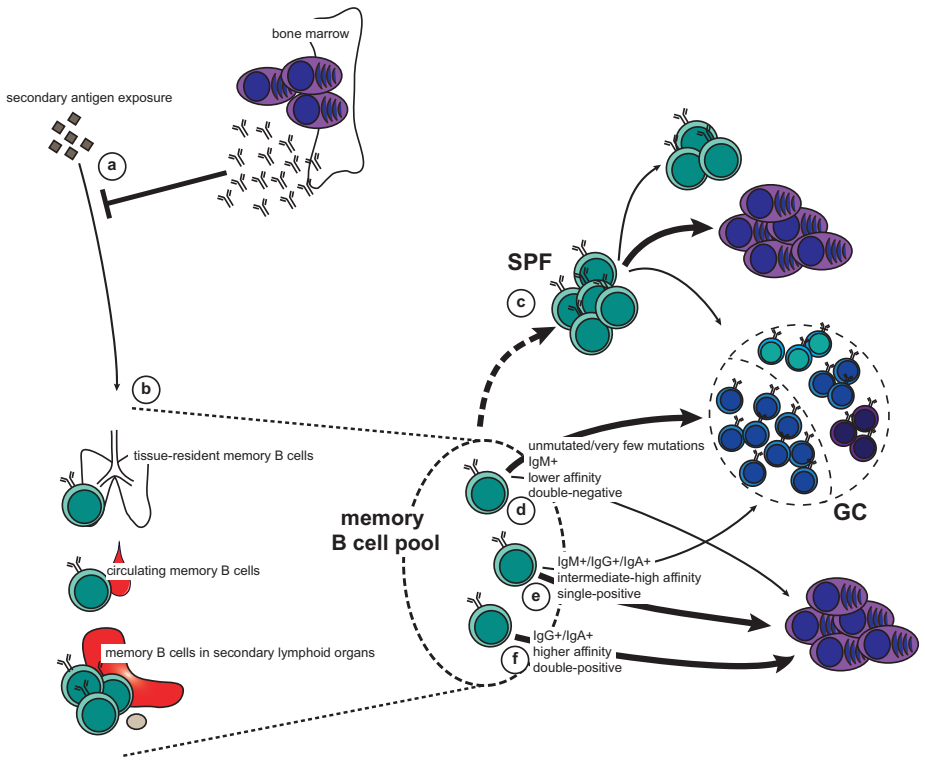

FIGURE 2 | The memory recall response to secondary antigen exposure. **(a,b)** Pre-existing antibodies secreted by long-lived plasma cells (LLPCs) constitute the first line of defense **(a)**. If this is not sufficient for immediate neutralization and elimination of the antigen, memory B cells will be engaged. This can happen either directly in the affected tissue (tissue-resident memory and circulating memory B cells), or when antigen is carried to secondary lymphoid organs **(b)**. **(c)** Activated B cells in lymph nodes can form subcapsular sinus proliferative foci (SPF) upon antigen-dependent re-activation. Although it is unclear which memory subset constitute the SPF, it is known that the main output is plasmablasts, but that this is also the fostering site for new memory B cells as well as cells entering GCs. **(d–f)** Depending on their phenotype, different fate decisions will be made by the reactivated memory B cells: new germinal centers (GCs) are typically formed by IgM^+, usually unmutated, $CD80^-PD\text{-}L2^-$ (double-negative) memory B cells of lower affinity **(d)**. In addition, both IgM^+ and switched memory B cells that express either CD80 or PD-L2 (single-positive) have retained the capacity to seed GCs **(e)**. However, the bulk of these cells, together with some of the IgM^+ double-negative memory B cells, will differentiate directly into plasmablasts **(c,d)**. Finally, switched, high-affinity memory B cells that are double positive for CD80 and PD-L2 exclusively form new plasmablasts **(f)**.

B cell compartment allows for a functional breadth of memory recall responses.

Unswitched (i.e., IgM^+) memory B cells are often derived from GC-independent or very early GC responses. They frequently do not express CD80 and/or PD-L2, and carry few, if any, mutations (7, 65). The IgM^+ memory B cell pool thus keeps a breadth of reactivity similar to that of naïve B cells but with the advantage of being able to rapidly respond to antigen (31, 33, 34, 36, 52). This breadth is particularly important for mounting rapid recall responses to variant antigens, such as influenza virus. On the other hand, recalled IgG+ memory B cells tend to rapidly differentiate into plasma cells without re-entering a GC (4). This is comparable to the fate chosen by switched B cells in the primary GC response (54–58). However, these observations may not be exclusively dependent on immunoglobulin isotype. Indeed, when further dissecting the memory B cell compartment, it becomes apparent that $CD80^-PD\text{-}L2^-$ IgM^+ memory B cells preferentially enter GCs upon recall, whereas those expressing CD80 and/or PD-L2 typically generate rapid IgM^+ and IgG^+ plasma cell responses (4, 7, 8, 74, 81). Similarly, IgG^+ memory B cells single-positive for CD80 or PD-L2 can differentiate to ASCs while retaining the capacity to seed GCs, whereas double-positive IgG^+ memory B cells only generate ASCs (8). These findings are further supported by studies demonstrating that IgG^+ and IgA^+ memory cells can engage in new GC reactions (5, 75).

LESSONS FROM HUMAN STUDIES

Human Plasmablasts

In humans, most studies consider plasmablasts as blood short-lived ASCs generated in acute B cell responses to infection or vaccination that transiently contribute to the serum antibody. In a secondary systemic immune response to a protein antigen such as tetanus toxoid or an inactivated influenza virus vaccine, antigen-specific IgG-secreting plasmablasts with somatically mutated VH gene rearrangements are generated from memory B cells (20, 82). It is also the case following influenza, Ebola, or Dengue virus infection (22, 83–85). It remains an open debate whether human plasmablasts are precursors of and how many

do become LLPCs. Evidence suggests that once the infection is cleared, the majority of ASCs undergo apoptosis, while a small proportion may go on to further differentiate into LLPCs (86). The heterogeneity seen in human ASCs from tonsil, blood, and bone marrow reveals stages of increasing maturity, and local profiles of adhesion molecule expression suggest a multi-step model for plasma cell differentiation (82, 87). In human blood when plasmablasts appear between days 6 and 8 after vaccination, they are migratory and attracted by CXCL12 and could migrate to tissues, such as the bone marrow (88, 89).

Plasmablasts have also been described as a "steady state" population where the majority express IgA. They express CCR10 and the adhesion molecule β_7 integrin and they are attracted by CXCL12 suggesting that they come from mucosal immune reactions and can return to mucosal tissue. Approximately 40% of LLPCs in human bone marrow are IgA^+, non-migratory, and express β_7 integrin and CCR10, suggesting a substantial contribution of mucosal plasma cells to bone marrow resident LLPCs (90). After tetanus vaccination, $IgG^+CD62L^+\beta_7$ integrin$^-$ dividing, vaccine-specific, and migratory plasmablasts appear in the blood, as do non-dividing, non-migratory, $CD62L^-$ plasma cells of different specificities (90).

A recent study identified survival factors from the bone marrow niche that favors maturation of human blood ASCs to LLPCs *in vitro* (91). IL-6 and two members of the tumor necrosis factor (TNF) superfamily: BAFF (B-cell activating factor of the TNF family; also known as BLyS in humans) and APRIL (a proliferation-inducing ligand) are known to be important survival signals (92), as well as is CXCL12 (93). Additional factors secreted by the bone marrow niche such as fibronectin and YWHAZ are important for LLPC maturation (91).

LLPCs

Migration to and From the Bone Marrow

Human LLPCs freshly isolated from the bone marrow have high expression of the chemokine receptors CXCR4 and CXCR6 and responsiveness in *in vitro* migration assays to the chemokines CXCL12 and CXCL16. The chemokine CCL28 has also been shown to attract human bone marrow plasma cells *in vitro* (94). Two interesting populations have been observed in the blood of tetanus toxoid immunized individuals: a population of migratory plasmablasts expressing CXCR3 and CXCR4, and a population resembling mature plasma cells of the bone marrow. These findings suggest that these cells are likely to be resident LLPCs mobilized from their survival niches in the bone marrow, in competition with newly generated plasmablasts (88).

In the Bone Marrow

Mesenchymal stromal cells (MSC) in the human bone marrow microenvironment provide factors that support LLPC survival (95–97). Cytokines of the TNF superfamily (BAFF, APRIL and TNF-α), IL-6 family, CD80/CD86, CD44 binding to hyaluronic acid, and VLA-4 binding to VCAM-1/fibronectin promote survival of plasma cells. CXCL12 promotes entry of cells to the bone marrow as well as plasma cell survival (86). BAFF seems to be important for human plasmablast differentiation whereas APRIL is the key to long-term survival in the bone marrow (98). An interesting study demonstrated that extracellular vesicles from bone marrow-derived MSCs support *ex vivo* survival of human ASCs (99).

In humans, the bone marrow contains both $CD19^+$ and $CD19^-$ LLPCs (26). The majority of $CD19^-$ LLPCs are actually found in the bone marrow, compared to the blood, spleen and tonsils. Interestingly, $CD19^-$ LLPCs are enriched in IgG^+ cells and carry fewer VH mutations compared to $CD19^+$ LLPCs. Only $CD19^-$ LLPCs resist to mobilization into the blood following immunization, and are resistant to depletion by Rituximab. In addition, $CD19^-$ LLPCs were not found in the bone marrow of 5–7 months old infants while $CD19^+$ LLPCs were present. This study suggests a multi-layer model of LLPCs in the human bone marrow with $CD19^+$ LLPCs being a dynamic component and $CD19^-$ a more static component permitting both adaptation and stability of humoral protection (100). A more recent study of the same populations but this time in response to influenza virus vaccination suggests that newly generated ASCs can acquire a mature plasma cell phenotype that is accompanied by loss of CD19 expression at an early stage of differentiation, and that aging is not an obligate requirement for a $CD19^-$ state to be established (101). Finally both $CD19^+$ and $CD19^-$ vaccinia-specific LLPCs were detected in the BM more than 35 years after the eradication of smallpox, suggesting that the LLPC pool may be maintained by a process in which vaccinia-specific B cells differentiate into LLPCs in the BM (26).

Outside of the Bone Marrow

Compared to the bone marrow niche, fibroblasts from the lymph nodes and the spleen have been poorly characterized in both mice and humans. A few studies have shown that stromal cells in the spleen and lymph nodes might promote plasma cell survival *in vitro* (102, 103). Recently, a new subset of fibroblasts (FRCs for fibroblastic reticular cells) in the lymph nodes have been described both in mice and humans as the main cell type in contact with plasma cells to guide them in their migration (104).

Mucosal Plasma Cells

Plasma cells are very abundant in mucosal tissues. They are located both in the connective tissue (lamina propria) and in lymphoid organs such as the tonsils in the oral cavity and Peyer's patches in the gut. The majority of these plasma cells secrete IgA antibodies, and humans also have a substantial IgM^+ plasma cell population in the mucosa (105). B cells in the respiratory tract and IgA responses in the gastrointestinal tract in have been nicely reviewed in Kato et al. (106) and Bunker and Bendelac (107), respectively, and are both beyond the scope of this review.

Human Memory B Cells

A great variety of B cell subsets have been identified in the tonsil, spleen, and peripheral blood and represent different stages of development of a naive B cell into a memory B cell. In the human tonsil, at least five distinct subpopulations of mature human B cells (Bm1–Bm5) have

been identified. Concisely, naive B cells belong to the Bm1 and Bm2 subpopulations whereas fully differentiated memory B cells belong to the Bm5 subset (108–110). Interestingly IgG transcripts in the tonsil had accumulated twice as many mutations as the IgM transcripts suggesting that reentry of selected B cells in the GC to generate higher affinity BCRs is a possibility (109).

As we previously stated, memory B cells are mainly generated in the GCs in secondary lymphoid organs. After leaving the GCs, memory B cells either join the recirculating pool of lymphocytes, or home to antigen draining sites. Memory B cell niches outside of the blood have been described and memory B cells have been found in the bone marrow, the tonsil and the spleen (111). Additionally a population of tissue based memory B cells expressing Fc receptor-like 4 (FCRL4) instead of CD27 has been described (112, 113). In the blood and bone marrow, human memory B cells can be divided in three main populations: $CD19^+CD27^+IgM^+IgD^+$ (similar to marginal zone (MZ) B cells), $CD19^+CD27^+IgM^+IgD^-$ (IgM-ONLY) and class-switched $CD19^+CD27^+IgM^-$ (IgG^+ or IgA^+) (114, 115). An in-depth flow cytometry analysis of human bone marrow and blood samples showed that compared to the blood, the bone marrow was enriched in both MZ and switched B-cells (116). In the spleen, two main phenotypically distinct B cell populations exist and localize to separate areas of the lymphoid tissue. Mantle zone B cells ($IgD^{high}IgM^+CD21^+CD23^+$) are unmutated and believed to be naive B cells, whereas MZ B cells are $IgD^+IgM^{high}CD21^{high}CD23^{\pm}$ and exhibit somatic mutations (117–119). It has been demonstrated that CD148, as well as CD27, are markers for memory B cells present in the human spleen (120). More recently, a population of IgG^+ memory B cells residing in the MZ of the spleen have been found and examined. IL-21 and BAFF have been demonstrated to be important for the differentiation of these IgG^+ splenic memory B cells into plasma cells (121).

$CD19^+CD27^+IgM^+IgD^+$ (Also Called Human MZ B Cells)

The spleen is an important organ in the defense against encapsulated bacteria. A population of "IgM memory B cells" controlling *Streptococcus pneumoniae* is observed in the spleen (122). Additionally, the human peripheral B-cell compartment displays a large $CD19^+CD27^+IgM^+IgD^+$ memory B cell population, resembling the splenic MZ B cells. In fact, by CDR3 spectratyping and gene-expression profiling, it has been demonstrated that $CD19^+CD27^+IgM^+IgD^+$ memory B cells are circulating splenic MZ B cells. These memory B cells have a mutated BCR, provide a pre-diversified immune repertoire and are involved in T-independent responses (123). They can develop in the absence of a spleen, but splenectomy in older individuals dramatically reduces the number of blood MZ B cells (122, 124). Finally, when compared to switched memory B cells in children <2 year of age, $CD27^+IgM^+IgD^+$ memory B cells in the spleen and blood do not display any signs of antigen-driven activation and expansion despite the many antigenic challenges experienced during childhood, suggesting a developmental diversification outside of T-dependent and T-independent responses (125).

$CD19^+CD27^+IgM^+IgD^-$ (IgM-Only) and Class-Switched $CD19^+CD27^+IgM$

By tracking tetanus toxoid-specific memory B cells ($CD3^-CD19^+CD20^+CD27^+$) at steady state, it has been showed that the spleen is the largest reservoir of memory B cells followed by the tonsil. Bone marrow and blood memory B cells express surface IgG and IgA at similar frequencies, while the tonsil contained more IgA memory B cells compared to other locations. IgG^+ memory B cells were enriched in the spleen and the tonsil compared to the bone marrow and the blood and IgM^+IgD^+ memory B cells were reduced in the tonsil compared to other locations. Interestingly, the absence of spleen and tonsils does not affect secondary responses to tetanus, suggesting an organ independent maintenance and reactivation for human memory B cells (111). Memory B cells that reside in lymphoid organs and recirculate after re-exposure to antigen are phenotypically the same and do not represent different stages of maturity. Additionally, it has been demonstrated that the human spleen is a major reservoir of long-lived vaccinia-specific memory B cells (66). Indeed, anti-smallpox IgG^+ memory B cells were specifically enriched in the spleen, confirming that the spleen is a major reservoir for long-lived memory B cells.

Finally, high-throughput VH sequencing on paired blood and spleen samples revealed that IgM sequences from clones shared between the MZ and the memory IgG/IgA (switched) compartments displayed a mutation and repertoire profile of IgM-only and not of MZ B cells. Thus the "IgM-only" subset appears as the only subset showing precursor–product relationships with $CD27^+$ switched memory B cells, indicating that they represent GC-derived IgM memory B cells and that IgM-only and MZ B cells constitute two distinct entities (126).

Human IgG and IgA Responses Induced by Infection and Vaccination

The route by which an antigen enters the body (systemic vs. mucosal) and the nature of the antigen are factors that direct the immune response class-switching patterns. Protein antigens usually trigger B cells receiving T-cell help while polysaccharide antigens induce CSR in the absence of T-cell help. Moreover, BAFF and APRIL have been shown to stimulate CSR to IgG and IgA in human B cells (127). Polysaccharide B cell responses to vaccination in humans have been reviewed in Mitchell et al. (23), while the kinetics of ASC responses to infection have been reviewed in Carter et al. (128).

IgG

Antibody responses to soluble protein antigens and membrane proteins primarily induce IgG1, but are accompanied with lower levels of the other subclasses. Viral infections in general lead to IgG antibodies of the IgG1 and IgG3 subclasses (129). On the other hand, antibody responses to bacterial capsular polysaccharide antigens is almost only restricted to IgG2 (130). IgG4 antibodies are often formed following repeated or long-term exposure to antigen in a non-infectious setting (131).

IgA

Homeostatic IgA responses employ a polyreactive repertoire to bind to a broad subset of microbiota species and tend to be of low affinity. In contrast, mucosal pathogens and vaccines elicit high-affinity, T-cell dependent antibody responses (107, 132). Mucosal IgA responses through a T-cell dependent reaction that place in mucosal lymphoid follicles, such as intestinal Peyers' patches and mesenteric lymph nodes (together called MALT for Mucosa-Associated Lymphoid Tissues) (132). Human IgA subtypes show distinct anatomical expression patterns, with monomeric IgA1 dominating in the serum and dimeric IgA2 in the gut (133).

Very few studies in humans have compared the induction of IgA and IgG secreting cells following various routes of immunization. An early study compared oral, intranasal and systemic influenza virus vaccines in healthy adults. Both systemic and intranasal immunizations induced mainly IgG^+ influenza-specific B cells in the blood after vaccination while the oral route induced IgA^+ influenza-specific B cells in the blood. Additionally, oral and intranasal administration of antigen-induced IgA influenza-specific antibodies in external secretions (134). These results were confirmed later on by multiple studies reporting a bursting population of IgG^+ antigen-reactive plasmablasts in the blood after secondary tetanus toxoid vaccination (88), influenza virus vaccination or infection (20, 83, 135), as well as acute dengue virus infection (22). In addition, immunization of African green monkeys with a live-attenuated H5N1 influenza vaccine resulted in more serum IgG neutralizing antibodies than IgA (136).

A study employing Ad26/Env (HIV) vaccination in rhesus macaques demonstrated highly coordinated IgG and IgA responses in both peripheral blood and mucosal compartments (137). It remains unclear to this day how related IgG and IgA plasmablasts/plasma cells are and what the relationship between mucosal and systemic antibody responses looks like. While a study suggested that mucosal and systemic humoral immune responses are regulated independently of each other based on the observation that systemic vaccination does not seem to impact peripheral IgA^+ plasmablast numbers (90, 138), another study revealed that in celiac disease patients, the same antigen-reactive B cell clones that give rise to gut plasma cells also contribute to the serum IgG and IgA antibody pool. However, serum IgA antibodies had a molecular composition (IgA1 vs. IgA2 and J chain level) distinct from that of IgA antibodies secreted in the gut, suggesting the involvement of different plasma cell populations (139). Finally, analysis of long-term transcriptional profile between blood IgG and IgA influenza-reactive plasmablasts as well as influenza-negative IgA plasmablasts did not reveal any specialization based on isotype. These data suggest that IgG and IgA vaccine–positive plasmablasts are largely similar, whereas IgA vaccine–negative plasmablasts appear to be transcriptionally distinct from antigen-induced peripheral blood plasmablasts (140).

Lessons From HIV

Significant efforts in the HIV field are focusing on the design of vaccines that would induce the generation of broadly neutralizing antibodies (bNAbs). Understanding the immunology behind the development of antibody potency and breadth following immunization is crucial in this context, not only to the HIV community (141). The success of most vaccines relies on the generation of antibodies to provide protection against subsequent infection. As discussed earlier in this review, Tfh cells are critical for the production of high-affinity B cell clones in the GC and thus the generation of long term memory, i.e., memory B cells and LLPCs (142).

The feasibility of assessing GCs and Tfh responses from human lymph nodes has been limited, as GC B cells do not circulate in the blood, and lymph nodes are rarely sampled (143). Recently, fine needle aspirates of the draining lymph nodes were used to longitudinally sample GC B cells and GC Tfh cells in non-human primates. The lymph node fine needle aspiration technique has proven effective in terms of how many cells were recovered from the biopsy as well as in not disrupting the ongoing GC. The authors found that neutralizing antibodies in non-human primates correlate with GC B cell magnitude and Tfh help quality (144). They also found that GCs peak weeks after the initial immunization. This means that a classic immunization (one injection of antigen) is not optimal for "feeding" the peak GC response. Proteins that are not of extreme stability can be degraded, exposing epitopes that would normally be hidden or non-existent on a more native protein conformation. Slow immunogen release could improve the availability of intact antigen and epitopes of interest for the duration of the GC response (145).

Germline-targeting strategies aim to activate B cell precursors with potential interest for bNAbs generation, so that they will enter the GC, be selected and affinity matured and will generate memory B cells. Studying HIV-reactive B cell lineages to infer unmutated ancestral BCRs that represent what a naïve B cell would express is the key to a B-cell lineage vaccine strategy (146). A vaccination protocol based on B-cell lineage differs from classic protocols in the fact that they may prime with one immunogen and boost with another or with a sequence of several different immunogens (147–150).

It has been recently demonstrated that only immunogens above a certain affinity and in multimeric form are capable of inducing GCs dominated by B cells from a bNAb precursor starting with low precursor frequency (151). These B cells successfully competed in GCs, underwent somatic hypermutation and differentiated into memory B cells. Overall this study demonstrates that germline-targeting immunogens can overcome affinity, avidity, and inter-clonal GC competition challenges with high-affinity multimeric designs.

Lessons From Influenza

Plasmablasts have been extensively studied in humans, especially in the context of influenza vaccination and infection. Little is known about B cells that become activated but do not differentiate into plasmablasts. A subset of antigen-reactive B cells called ABCs for "Activated B Cells" has been described and was found to be transcriptionally distinct from the ASC population and committed to the memory lineage (152). ABCs and ASCs share hemagglutinin (HA)-reactive clones following influenza vaccination. Our laboratory also described a post-GC

population of B cells that phenotypically resemble memory B cells but that have low expression of CD21 (classical memory B cells are $CD19^{+}CD27^{+}CD21^{high}$). We demonstrated that the $CD21^{low}$ population was comprised of recent GC graduates that were refractory to GC reentry and seemed to be predisposed to differentiation into long-lived plasma cells (153). Although clonally related to memory B cells and plasmablasts, $CD21^{low}$ B cells form distinct clades within phylogenetic trees based on the accumulation of variable gene mutations. Another study demonstrated that HA-reactive $CD21^{low}$ B cells are enriched in the blood compared to the tissues while there was an enrichment of $CD27^{+}CD21^{high}HA^{+}$ B cells in all tissues. Both $CD21^{+}$ and $CD21^{low}$ populations were not maintained in the peripheral blood at 1 year post-vaccination (154).

Additionally, it is of great interest to understand how different vaccine compositions will affect the generation of memory B cells and LLPCs. Seasonal influenza vaccines exist as live-attenuated influenza virus (LAIV), which more closely resembles natural immunity after infection, or as inactivated vaccines. LAIV have been used mostly in children but do not induce strong systemic antibody responses in adults (155). The same was true for two different avian pandemic LAIV vaccines (H5N1, H7N9), although these vaccines elicited a long-term immune memory that was revealed after administration of a matched inactivated vaccine (156–158). To understand how LAIV vaccines can prime such a memory response, a detailed analysis of B cell responses in systemic and local lymphoid tissues in a non-human primate model was performed (136). Interestingly, the authors found that the LAIV vaccine induced robust GCs in the mediastinal (lung-draining) lymph node and that both HA-reactive plasmablasts and memory B cells were found in the mediastinal lymph nodes after immunization.

Finally, it is believed that adjuvants can modulate humoral responses and retain antigen at the site of injection. Most studies have been done with alum and it remains unknown how other adjuvants (such as AS03 and MF59) act on GCs and antigen release (159). In the context of influenza vaccines, adjuvanted vaccines administered in patients with impaired immune responses, such as infants and the elderly, were shown to be beneficial (160–162). Additionally a study showed that the adjuvant AS03 induced an increased activation of naïve B cells and an increased adaptability of recalled memory B cells, improving immunogenicity (163).

CONCLUSION

The generation of memory B cells and long-lived plasma cells is crucial to the long-term effectiveness of vaccines. Understanding how to induce these different populations and modulate their effects both in animal models and human is essential to the design of better vaccines. Thus, the design of new immunogens, how to release them, as well as the mechanisms of actions of various adjuvants are the future of vaccines protecting against challenging or emerging infectious diseases.

AUTHOR CONTRIBUTIONS

A-KP and CH contributed ideas and wrote the review. A-KP designed the figures.

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Virus-Specific Secondary Plasma Cells Produce Elevated Levels of High-Avidity Antibodies but Are Functionally Short Lived

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Most vaccines aim at inducing durable antibody responses and are designed to elicit strong B cell activation and plasma cell (PC) formation. Here we report characteristics of a recently described secondary PC population that rapidly originates from memory B cells (MBCs) upon challenge with virus-like particles (VLPs). Upon secondary antigen challenge, all VLP-specific MBCs proliferated and terminally differentiated to secondary PCs or died, as they could not undergo multiple rounds of re-stimulation. Secondary PCs lived in bone marrow and secondary lymphoid organs and exhibited increased production of antibodies with much higher avidity compared to primary PCs, supplying a swift wave of high avidity antibodies early after antigen recall. Unexpectedly, however, secondary PCs were functionally short-lived and most of them could not be retrieved in lymphoid organs and ceased to produce antibodies. Nevertheless, secondary PCs are an early source of high avidity antibodies and induction of long-lived MBCs with the capacity to rapidly differentiate to secondary PCs may therefore be an underestimated possibility to induce durable protection by vaccination.

Keywords: adaptive immunity, anti-viral immunity, memory B cells, secondary plasma cells, virus-like particles

INTRODUCTION

B cells differentiate to antibody secreting plasma cells (PCs) upon activation by their cognate antigen (Ag) within and outside of B cell follicles. At an early stage of the primary immune response, antibody–forming cells (AFCs) derived from follicular or marginal zone (MZ) B cells are rather short-lived and survive for a few days only (1). Meanwhile, follicular B cells form GCs where MBCs and long-lived PCs are generated in a mostly T cell dependent fashion (2–5).

Activated B-lymphocytes are driven to the PC pathway by up-regulation of the transcription factors B lymphocyte maturation protein 1 (Blimp-1), Interferon regulating protein 4 (IRF 4), and X-box-binding protein 1 (XBP 1) (6–8). Differentiation of activated B cells into AFCs needs a harmonized change in the gene expression of these cells. Shi et al. delineated the transcriptional profile during this differentiation process (9). PCs are terminally differentiated and arrested in the G1 phase of the cell cycle being incapable of further growth or proliferation (10, 11). To be able to secrete large amounts of antibodies, PCs are committed to their protein synthesizing machinery

and undergo major structural adaptations by increasing the size of the endoplasmic reticulum and Golgi apparatus (12). To cope with these changing conditions PCs induce the unfolded protein response as well as autophagy (13–15). These stress-regulating processes are necessary for survival as PCs can secrete the tremendous amount of up to 10'000 antibodies per second (16). Sizeable amounts of antibodies that are rapidly available are required to neutralize microorganisms and prevent infection. Antibodies furthermore play a key role in immunity and promote the crosstalk between the innate and adaptive immune system. Besides classical neutralization of toxins and pathogens, they are able to opsonize microbes and infected cells for phagocytosis, enabling their elimination, and promote antigen presentation thereby regulating inflammation (17).

PCs are found in secondary lymphoid organs and the bone marrow (BM) where they can survive for days, months, or even years. There is an ongoing debate whether long-term antibody responses are a result of persisting antigen leading to re-stimulation and differentiation of memory B cells to PCs or whether they are derived from intrinsically long-lived PCs. Several studies are in favor of the first hypothesis that persistent antigen or infection and polyclonal memory B cell activation is required (18–22). Nevertheless, evidence is growing that PCs can persist in the absence of continuous stimulation (23–25). It was shown that PCs require cell-intrinsic and extrinsic survival signals such as cytokines and adhesion molecules from nursery cells like monocytes, eosinophils, and megakaryocytes for long-term survival in BM niches (26–29). Once they reach the BM and successfully compete for a niche, PCs have a lifespan varying from a few months to years and even decades during which they constantly secrete antibodies (30, 31). In contrast to PCs, which do not express surface Ig, MBCs respond to secondary Ag encounter. They exhibit the intrinsic ability to respond with a proliferative burst faster compared to naïve B cells (32) and were found to seed new GCs and/or differentiate into PCs (33–37). Antibody responses generated during secondary responses are usually of higher affinity for the cognate Ag compared to those of a primary response.

We have previously shown that immunization with VLPs derived from the RNA bacteriophage Qβ elicit strong and sustained IgG antibody responses by activation of MZ and follicular B cells with the latter forming GCs (38–40). MBCs and PCs were rapidly generated and detectable as early as 3 days and up to several months after immunization in spleen and BM (41, 42). Here we show, that MBCs generated against Qβ proliferated during Ag recall experiments but exclusively differentiated into secondary PCs and failed to respond to multiple rounds of Ag stimulation. Secondary PCs exhibited the unique ability to produce 30 times more antibodies of increased affinity compared to primary PCs. The secondary PCs were found in spleen as well as in BM early on day 4 but almost completely disappeared by day 6 after Ag re-encounter from both organs. In addition, antibodies produced by secondary PCs were cleared from the system within weeks indicating that secondary PCs are functionally short-lived. Inducing MBCs that differentiate into secondary PCs by vaccination could represent a novel pathway for efficient and rapid control of infectious diseases by the induction of an early wave of high affinity antibodies.

MATERIALS AND METHODS

Study Design

The goal of this study was to further characterize secondary PCs, which were generated by MBCs after Ag challenge. To achieve this, adoptive transfers in allotypic mice (Ly5.1/Ly5.2 and IgHa/IgHb) were performed. This enabled us to study primary and secondary immune responses in the same animal. All mice were kept according to Cantonal Veterinary guidelines at the central animal facility (Department for BioMedical Research) of the University of Bern and controlled laboratory experiments were performed in accordance with ethical principles and guidelines of the Cantonal Veterinary Office Bern, Switzerland. Animals were randomly assigned to the different groups. MBCs were generated by VLP immunization of mice. The control naïve mice remained untreated. At the same time, B cells were isolated from memory and naive mice and transferred into recipients. Upon immunization with VLPs, serum samples, spleens, and BM were collected and subjected to ELISA, ELISPOT, and FCM analysis. The investigators who performed the experiments, assessed, analyzed, and quantified the results were not blinded and aware of which group a sample was taken from. Individual groups consisted of 4–5 mice. All experiments were performed in at least 2 independent biological replicates, apart from intracellular FCM analysis of PCs at day 6 after challenge. Data were collected at previously determined time points. All data were included in the analysis.

Mice

C57BL/6JRccHsd wildtype mice were purchased from Envigo (Horst, The Netherlands). The IgHa (B6.Cg-Gpi1<a> Thy1<a> Igh<a> (Stock No. 001317)) mouse strain was purchased from the Jackson Laboratory (USA). We thank Prof. Annette Oxenius for the kind donation of the Ly5.1 (B6.SJL-Ptprc<a> Pepc<b>/BoyJ) mouse strain.

Antigen

The bacteriophage derived Qβ virus-like particles (VLPs) self-assemble and enclose bacterial RNA during their production in *E. coli*. Due to their particulate and repetitive structure, the VLPs are highly immunogenic. The purification process is described elsewhere (43).

Immunization

To induce primary immune responses and generate MBC against the VLPs, mice were immunized intravenously (i.v.) with 50 μg Qβ VLPs. To challenge adoptively transferred MBC or naive cells, recipient mice were immunized with 50 μg Qβ VLPs i.v. For intravenous administration the VLPs were formulated in 150 μl sterile PBS.

Adoptive Transfer

MBCs were generated by immunization of congenic donor mice (Ly5.1 or IgHa). At least 8 weeks after immunization

donor mice were sacrificed and spleens isolated in RPMI media containing 2% FCS and antibiotics. A single cell suspension of the spleens was prepared and red blood cells were lysed using ACK buffer (0.15 M ammonium chloride, 0.01 M potassium hydrogen carbonate, pH 7.2–7.4). The splenocytes were PNA^- and $B220^+$ MACS purified. For PNA negative purification splenocytes were labeled using PNA-biotin (Vector Labs, B-1075) and PNA^+ cells were depleted by Strepravidin MicroBeads (Milteny Biotec, 130-048-101) according to the manufacturer's protocol. Positive selection using B220 MicroBeads (Milteny Biotec, 130-049-501) was performed according to the manufacturer's protocol.

Purified cells from 1/3 of a donor spleen (Ly5.1 or IgHa; ∼1–3×10^6 cells) were adoptively transferred i.v. into congenic host mice (Ly5.2 or IgHb). Control mice received PNA^- and $B220^+$ purified splenocytes from naïve congenic mice. One day after MBC transfer host mice were challenged with 50 μg Qβ VLPs i.v.

Flow Cytometry (FCM)

For FCM staining tissues (spleen, BM, kidney, lymph nodes (LN), liver, lung) of mice after adoptive transfer were isolated in RPMI supplemented with 2% FCS and antibiotics and single cell suspensions were prepared. Blood was collected in phosphate buffer containing heparin (1–2 units/ml). Red blood cells were lysed using ACK buffer prior to staining. Fc receptors were blocked using an anti-CD16/32 antibody. Qβ specific class switched (CS) B cells were identified as IgM, IgD, CD4, CD8, GR1, CD11b, CD11c negative (all antibodies labeled with phycoerythrin (PE)), and positive for B220 labeled with PE-Cy7 and Qβ VLP labeled with Alexa Flour 488. To discriminate Qβ specific PCs from Qβ specific activated and CS B cells, surface immunoglobulins (Ig) of specific cells were blocked using unlabeled Qβ VLPs. PCs were further stained with and characterized as IgM, IgD, CD4, CD8, GR1, CD11b, CD11c negative (all antibodies labeled with PE) and B220-PE-Cy7 low. To detect Qβ specific PCs by intracellular staining of specific Ig, splenocytes were permeabilized using FACS lysing solution (BD, 349202) containing 0.04% Tween20 and stained with Alexa Flour 488 labeled Qβ VLPs. The congenic marker Ly5.1 (antibody labeled with APC or PerCP-Cy5.5) identified all transfer derived B cells. Dead cells were stained by the addition of propidium iodide solution (PI, Sigma, 10 μg/ml) directly before acquisition. For detection of dead cells after fixation and permeabilisation, the Fixable Viability Dye eFluor 520 (eBioscience, 65-0867-14) was used according to the manufacturer's instructions.

Qβ VLPs were labeled with the Alexa Flour 488 protein labeling kit (Thermo Fisher Scientific, A10235) or Alexa Flour 647 NHS Ester (Thermo Fisher Scientific, A20006) according to the manufacturer's instructions.

Data acquisition was performed on a FACS Canto (BD) and analyzed using FlowJo V10.1 (Flowjo, LLC, USA). All antibodies were purchased from BD Biosciences and Biolegend.

ELISPOT

Spleens from mice after adoptive transfer were isolated and a single cell suspension was prepared. To collect BM cells, tibia and femur were flushed with RPMI media containing 2% FCS and antibiotics. After red blood cell lysis with ACK buffer, cell numbers of splenocytes and BM cells were determined using the Cellometer mini (Nexcelom, USA). 5×10^5 cells were seeded per well on MAIPS Elispot plates (Millipore, MAIPS4510) previously coated with 10 μg/ml Qβ VLPs overnight at 4°C and blocked with 2% BSA in PBS for at least 2 h. After performing a 2-fold dilution series cells were incubated for 5 h at 37°C and 5% CO_2. Subsequently cells were washed off and bound specific antibodies produced by PCs were detected using a goat anti-mouse IgG antibody (EY laboratories, AT-2306-2) followed by a donkey anti-goat alkaline phosphatase secondary antibody (Jackson Immunoresearch, 705-055-147). Spots were visualized by the AP Conjugate Substrate Kit (BioRad, 1706432) and counted using an EliSpot Reader (AID, Germany). The spot size was quantified with the EliSpot 7.0 iSpot software of the EliSpot Reader as the average surface area of the spot.

CFSE Proliferation

To analyse the proliferation of transferred cells, the donor cells were labeled with CFSE (Biolegend, Cat No. 423801) after MACS purification and before transfer into congenic hosts, according to the manufacturer's protocol. FCM staining was carried out similarly as described above. In this case, Qβ specific CS B cells were detected with VLPs labeled with Alexa Flour 647.

Splenocyte Cell Culture

Spleens from mice that had received memory or naïve B cells were isolated 5 and 6 days after VLP challenge. A single cell suspension of splenocytes was prepared. After red blood cell lysis with ACK buffer, cell numbers of splenocytes were determined using the Cellometer mini (Nexcelom, USA). 10×10^6 cells were seeded in 1 ml RPMI media containing 10% FCS and antibiotics per well in 24 well plates (Falcon Multiwell, Corning). The cells were incubated for 72 h at 37°C and 5% CO_2. Cell supernatants were harvested and the antibody content determined by ELISA.

ELISA

Serum samples were obtained from blood collected at the indicated time points during experiments using Microtainer tubes (BD, 365967). Corning half area 96 well-plates were coated with 50 μl of 1 μg/ml Qβ VLPs overnight at 4°C. Sera were 1:10 pre-diluted and 1:4 further serial diluted to analyse a total of 7 dilutions per sample. Qβ specific antibodies were detected using mouse anti-mouse IgG for both allotypes. IgHa-specific (biotin ms anti-ms IgG1[a] (10.9), biotin ms anti-ms IgG2a[a] (8.3) from BD) and IgHb-specific (biotin ms anti-ms IgG1[b] (B68-2), biotin ms anti-ms IgG2a[b] (5.7) from BD) antibodies were detected using horseradish peroxidase (HRP) labeled streptavidin (Dako).

Cell supernatants were used undiluted and a 1:2 serial dilution was performed. An anti-Qβ monoclonal antibody (purified from hybridoma cells) was used as a standard to quantify specific antibodies in the supernatants. Qβ specific antibodies were detected using goat anti-mouse IgG-HRP (Jackson ImmunoResearch, 115-035-071).

The absorbance readings of the tetramethylbenzidine (TMB) color reaction at 450 nm for the serum samples were interpreted as OD50 antibody titers. The OD50 antibody titers are defined

as the reciprocal of the dilution that reaches half of the OD max. The anti-Qβ monoclonal antibody standard curve was used to calculate antibody concentrations in the cell supernatants.

Avidity ELISA

Serum samples were obtained from blood collected at the indicated time points during experiments using Microtainer tubes (BD, 365967). Corning half area 96 well-plates were coated with 50 µl of 1 µg/ml Qβ VLPs overnight at 4°C. Sera of the different time points were applied with a 1:20 pre-dilution and 1:4 further serial diluted. After 1 h incubation, the sera were washed off and the plates washed 3 times 5 min either with 7M urea in PBST (PBS containing 0.05% tween 20) or PBST only. Qβ specific antibodies were detected using mouse anti-mouse IgG for both allotypes. IgHa-specific (biotin ms anti-ms IgG1[a] (10.9), biotin ms anti-ms IgG2a[a] (8.3) from BD) and IgHb-specific (biotin ms anti-ms IgG1[b] (B68-2), biotin ms anti-ms IgG2a[b] (5.7) from BD) antibodies were detected using horseradish peroxidase (HRP) labeled streptavidin (Dako). The absorbance readings of the tetramethylbenzidine (TMB) color reaction at 450 nm served as basis for avidity index calculation. The avidity index (AI) was calculated by AI_x = OD (dilution x) + urea / OD (dilution x)–urea.

Antibodies/Reagents

Antibody/reagent	Clone	Company	Conjugate	Detection	Catalog number
goat anti-ms IgG	polyclonal	EY laboratories		donkey anti-goat alkaline phosphatase (AP)	AT-2306-2
donkey anti-goat AP	polyclonal	Jackson ImmunoResearch	AP		705-055-147
anti-ms IgG1[a]	10.9	BD	biotin	Streptavidin HRP (Dako)	553500
anti-ms IgG2a[a]	8.3	BD	biotin	Streptavidin HRP (Dako)	553533
anti-ms IgG1[b]	B68-2	BD	biotin	Streptavidin HRP (Dako)	553502
anti-ms IgG2a[b]	5.7	BD	biotin	Streptavidin HRP (Dako)	553504
goat anti-ms IgG	polyclonal	Jackson ImmunoResearch	HRP		115-035-071
anti-ms CD16/32	2.4G2	BD			553142
anti-ms IgM	polyclonal	Jackson ImmunoResearch	PE		115-116-075
anti-ms IgD	11-26c (11-26)	eBioscience	PE		12-5993-83
anti-ms CD8a	53-6.7	BD	PE		553032
anti-ms CD4	H129.19	BD	PE		553653
anti-ms CD11b	M1/70	BD	PE		553311
anti-ms CD11c	HL3	BD	PE		553802
anti-ms GR1	RB6-8C5	BD	PE		553128
anti-ms B220	RA3-6B2	BD	PE-Cy7		552772
anti-ms CD45.1	A20	eBioscience	APC		17-0453-82
anti-ms CD45.1	A20	Biolegend	PerCP/Cy5.5		110727
anti-ms CD38	90	Biolegend	PerCP/Cy5.5		102722
Anti-ms IgG	Polyclonal	eBioscience	Biotin	Streptavidin APC/Cy7	13-4013-85
Peanut Agglutinin (PNA)		Vector Laboratories	Biotin	Streptavidin APC/Cy7	B-1075
Streptavidin HRP		Dako	HRP		P0397
Streptavidin APC/Cy7		BD	APC/Cy7		554063
Fixable Viability Dye eFluor 520		eBioscience	eFluor 520		65-0867-14

Statistics

Statistical analysis was performed using GraphPad Prism Version 7.01 (GraphPad Software, USA). Statistically significant differences between two groups were calculated using unpaired *t*-tests. Statistically significant differences between more than 2 groups were determined using a one-way ANOVA followed by Tukey's or Sidak's multiple comparisons test. Statistical significance was defined as $p < 0.05$. The best fitting line was calculated by linear regression.

RESULTS

Memory B Cell Derived Secondary PCs Produce Antibodies of Higher Avidity

We have previously shown that MBCs are generated against Qβ VLPs in a T cell-dependent manner (35, 38, 39, 44, 45). During secondary responses, these MBCs do neither extensively proliferate nor join GC reactions (35). T cell help, however, is essential for low-level MBC proliferation but dispensable for differentiation to secondary PCs during secondary immune responses (44). To reveal insights in the mechanism and kinetics of secondary PC formation from MBCs after antigenic re-stimulation, adoptive transfer experiments using congenic mice were performed (**Figure 1A**). To this end, MBCs were generated by immunizing donor mice (Ly5.1 or IgHa) with 50 µg Qβ VLPs. Eight weeks post immunization, splenocytes from donor mice were isolated and PNA^- and $B220^+$ MBCs were purified

by MACS, excluding transfer of GC B cells. Splenocytes from naïve mice were subjected to the same treatment and served as controls. We have previously shown that the presence of memory T follicular helper cells does not influence the MBC response (35, 44). Therefore, purified MBCs were transferred alone. Donor-derived (Ly5.1$^+$) MBCs were shown to preferentially home to secondary lymphoid organs, namely lymph nodes (LN), and spleen (**Figure S1A**) and the majority of Qβ-specific donor MBCs were found in the spleen (**Figure S1B**).

To analyse the humoral immune response after memory or naïve B cell transfer and Qβ VLP challenge, immunoglobulin heavy chain allotype mice were used as shown in **Figure 1A**. MBCs were induced in donor mice (IgHa) and adoptively transferred into recipient mice (IgHb). The recipient mice were challenged with Qβ VLPs 1 day after the transfer and splenocytes, BM as well as serum were collected at the indicated time points to determine CS B cells (outlined in **Figure 1B**), PCs (outlined in **Figure 1C**) as well as anti-Qβ antibody titers (**Figure 2**). The donor derived secondary response was discriminated from the host's primary response using allotype specific detection antibodies for IgG1 and IgG2a in ELISA, as these are the main isotypes induced by Qβ immunization (46) (**Figure 2**). Donor derived antibodies after MBC transfer started to rise from day 4 after challenge, peaked around day 6 and then declined until day 20 (**Figure 2A**). In contrast, host antibody titers only started rising from day 6 and peaked at day 12. The peak titer of the host antibodies was lower than from the donor, indicating that MBC-derived secondary PCs dominated the early response. In addition, the relatively rapid decline of the donor-derived antibody titer is a clear indicator that the functional response of secondary PCs is unexpectedly short-lived.

Whether donor-derived MCBs could undergo a tertiary response was assessed next. To this end, recipient mice were challenged a second time with VLPs on day 61. Surprisingly, only the host-derived but not the donor-derived antibody response could be boosted, demonstrating that MBCs cannot participate twice in a humoral response after challenge with VLPs (**Figure 2A**). This suggests that essentially all MBCs generated against VLPs instantly differentiated to secondary PCs after re-stimulation without supplying a new MBC population. As expected, transferred naïve donor cells did not respond to the VLP challenge, as they also did not engage in the primary response (**Figure 2B**). Of note, host antibody levels were elevated after naïve B cell transfer compared to the host response in presence of MBCs (**Figures 2A,B**). This indicates that the presence of MBC derived secondary PCs suppresses the hosts humoral response after VLP challenge, confirming earlier observations (35).

In order to analyze the antibody avidity of the secondary antibody response, a modified ELISA was performed. For this purpose, low avidity antibodies were dissociated by treatment with 7 M urea. Only high avidity antibodies remain bound under these conditions (47, 48). Comparing the OD values of urea vs. PBS treated sera, an avidity index was calculated. The primary response antibodies of the host started to increase in avidity between day 6 and 9 after immunization (**Figures 2C,D**). The avidity increase proceeded until day 21. In marked contrast, avidity of antibodies derived from secondary PCs was high as of day 4 after challenge and did not further increase (**Figure 2C**). Thus, secondary PCs are not only superior in antibody production but also in antibody avidity.

MBCs Do Not Extensively Proliferate Before Differentiating to Secondary PCs Upon Cognate Antigen Challenge

To be able to study proliferation of MBCs before differentiation to PCs, purified B cell populations were labeled with CFSE prior to adoptive transfer. One day after transferring the MACS purified and CFSE labeled B cells, congenic recipient mice (Ly5.2) were challenged with 50 μg Qβ VLPs. Flow cytometric analysis of the Qβ specific CS B cells (**Figure 1B**) showed that all MBCs had proliferated as essentially no CFSE$^+$ Ly5.1$^+$ Qβ-specific cells could be observed (**Figure 3A**, right histogram). Nevertheless, there was a robust number of CFSE$^+$, Ly5.1$^+$, B220$^+$ cells, which were not specific for Qβ, demonstrating survival of labeled cells upon adoptive transfer (**Figure 3A**, left histogram). The proliferation seen in this subset could be attributed to bystander proliferation or plasma blasts generated after proliferation and differentiation of MBCs, which had already downregulated surface BCR expression but are still B220$^+$. Thus, essentially all MBCs proliferated but this proliferation was not extensive and of short duration, as few MBCs accumulated but rather rapidly differentiated into secondary PCs (see below).

Transferred MBCs Are Detectable in the Specific B Cell Compartment Only at Early Time Points Upon VLP Challenge

In order to follow the MBC response upon transfer and challenge, the specific CS B cells were analyzed in the spleen by flow cytometry (FCM). VLP-specific CS B cells of donor (**Figure 3B**) and host (**Figure 3C**) origin were visualized as defined in **Figure 1B** and viable (**Figures S2A,B**). An increased number of Qβ specific donor derived cells was found, when MBCs were transferred compared to naïve B cell transfer on day 4 and 5 after VLP challenge (**Figure 3B**). This difference was more pronounced on day 5 but was already absent on day 6 post immunization. The host response in the CS B cell compartment was comparable between memory and naïve B cell transfer on day 4 and 5 after challenge (**Figure 3C**). However, the host B cell response seemed to be slightly impaired at day 6 when MBCs were present suggesting that MBCs suppress the response of the naïve host B cells (**Figure 3C**). This was consistent with the reduced host antibody titer in the presence of MBCs observed above (**Figures 2A,B**).

Secondary PCs Are Rapidly Induced but Are Functionally Short-Lived

To characterize the secondary PC population ELISPOT assays of spleen and BM were performed. As suggested by the antibody responses, secondary PCs occurred very promptly and reached high numbers 4 days after Ag challenge but the population rapidly contracted within the next 2 days (**Figure 4A**). Similar observations were made in the BM (**Figure 4B**). Besides

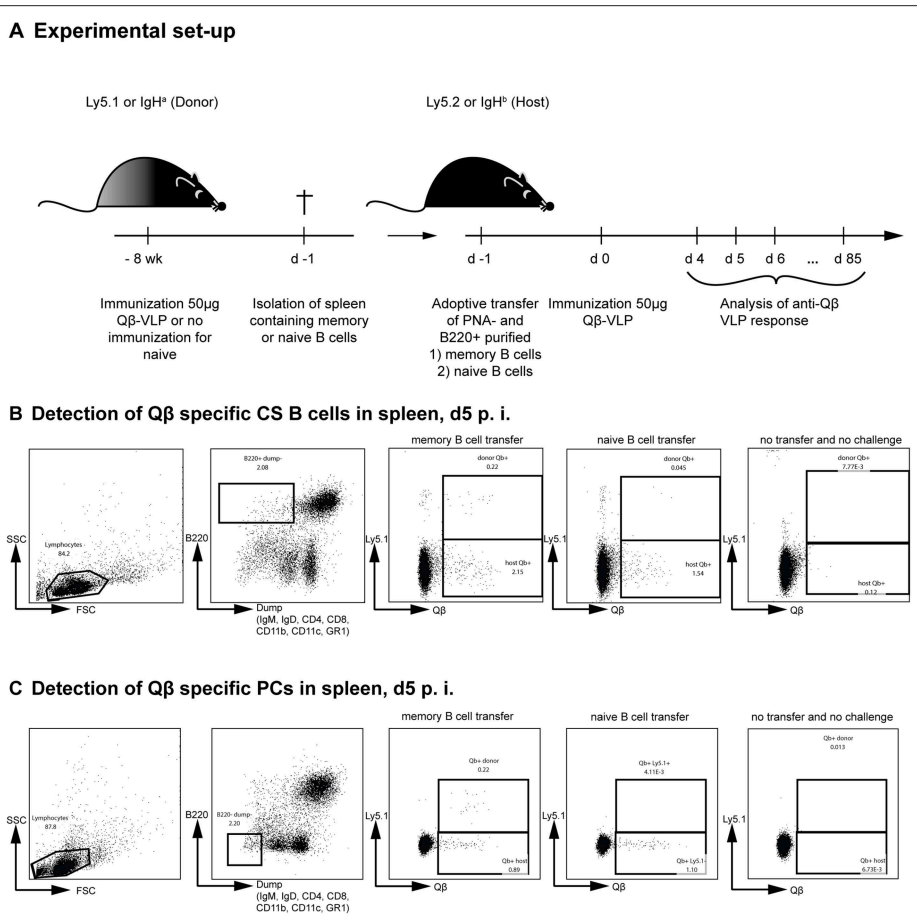

FIGURE 1 | Adoptive transfer of Qβ VLP specific or naïve B cells and flow cytometric analysis of Qβ specific CS B and plasma cells in the spleen. **(A)** Congenic mice (Ly5.1 or IgHa) were immunized with 50 µg Qβ VLPs i.v. Eight weeks after immunization spleens of immunized and naïve mice were isolated and PNA$^-$ B220$^+$ MACS purified cells were transferred into host mice (Ly5.2 or IgHb). Recipient mice were immunized with 50 µg Qβ VLPs i.v. 1 day after the transfer. Spleens, bone marrow, and serum were taken at several time points after challenge. **(B)** Representative FCM plots for the gating strategy to identify Qβ specific CS B cells in the spleen 5 days after immunization. B220$^+$ cells not expressing IgM, IgD, CD4, CD8, CD11b, CD11c, or GR1 were analyzed for their binding of labeled Qβ VLPs. The congenic Ly5 marker was used to discriminate transfer from host derived CS B cells. **(C)** Representative FCM plots for the gating strategy to identify Qβ specific PCs in the spleen 5 days after immunization. B220^{low} cells not expressing IgM, IgD, CD4, CD8, CD11b, CD11c, or GR1 were analyzed for their intracellular binding of labeled Qβ VLPs. The congenic Ly5 marker was used to discriminate transfer from host derived PCs cells.

ELISPOT analysis, PCs were enumerated by FCM, where the same pattern emerged. Secondary PCs occurred rapidly and peaked between days 4 and 5 but were largely absent by day 6 (**Figure 4C**). Thus, secondary PCs are induced within a few days but appear to be short-lived. As observed above for the CS B cells, the PC compartment of the host is similar on days 4 and 5, whereas it is slightly decreased on day 6 when MBCs were transferred (**Figure 4D**).

Transfer Derived Secondary PCs Show Enhanced Capacity to Produce Antibodies in Spleen and BM

As previously described, one hallmark for secondary PCs is their enhanced capacity to produce antibodies after cognate antigen challenge (35). An indicator for enhanced antibody production during MBC responses was the spot size in ELISPOT assays, as it is correlating with the amount of antibodies produced by one PC. Representative images of ELISPOTs from splenocytes after memory or naïve B cell transfer and challenge with VLPs are shown (**Figure 5A**). Every spot on the plate represents one Qβ specific PC and the spot diameter correlates the amount of antibody that is produced by one PC. Spot diameters of specific PC populations in spleen and BM were analyzed 4–6 days after adoptive transfer of memory or naïve B cells that were challenged with VLPs. The spot diameter from spleen and BM was always greater when MBCs were transferred. The most significant difference however was observed on day 5 after Qβ VLP challenge, representing the peak of the secondary PC response (**Figures 5B,C**). This observation was confirmed by FCM on day 5 after MBC transfer and challenge, as the mean fluorescent intensity (MFI) of intracellular anti-IgG binding was increased in donor-derived compared to host PCs (**Figure 5D**). As the MFI of intracellular Qβ binding correlated with the amount of

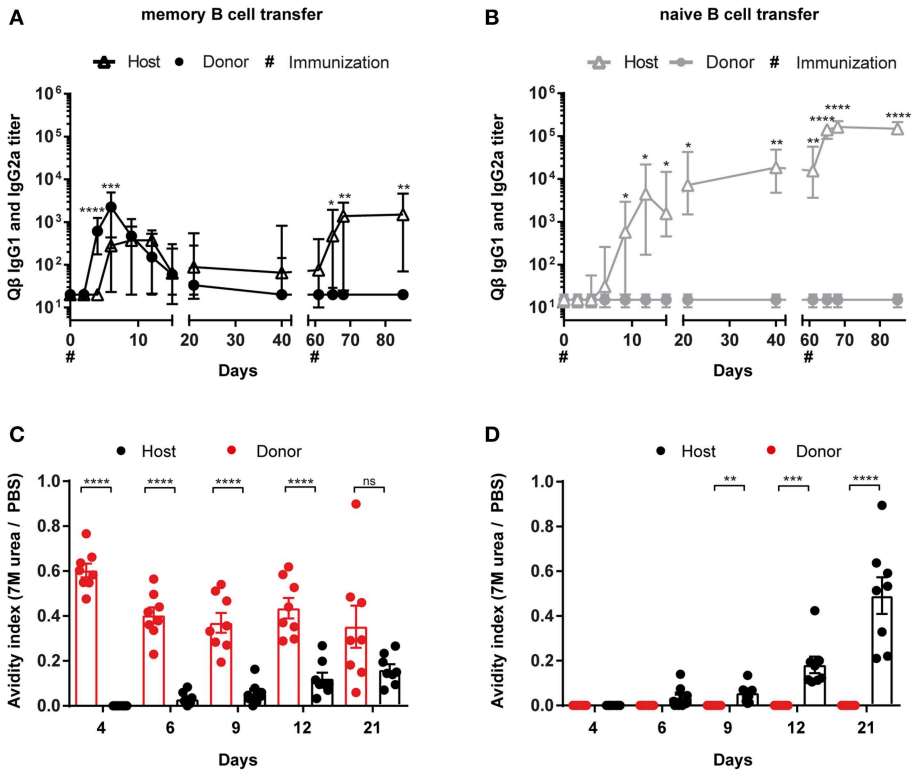

FIGURE 2 | Memory B cell derived secondary PCs produce antibodies of higher avidity. **(A)** MBC responses were initiated by vaccinating IgHa mice with 50 μg Qβ VLPs. After 8 weeks, PNA^{-} and $B220^{+}$ MACS purified B cells from memory **(A)** or naïve **(B)** donor mice were transferred into congenic recipients (IgHb). Recipient mice were challenged with 50 μg Qβ VLPs 24 h and 61 days after the transfer. The anti-Qβ IgG1 and IgG2a antibody titers in the serum were determined by ELISA on days 0, 2, 4, 6, 9, 12, 15, 21, 42, 61, 65, 68, and 85. Using Ha and Hb allotype specific detection antibodies, donor (IgHa), and host (IgHb) responses were discriminated. To determine the avidity index of IgGs in the sera after memory **(C)** or naïve **(D)** B cell transfer and VLP challenge, a modified ELISA was performed. Mean with SEM. *P* values were calculated using an unpaired *t* test. $n = 4$ mice per group. Data representative of 2 independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

intracellular anti-IgG binding it can serve as a surrogate for the amount of antibody present inside PCs (**Figure 5D**). The MFI of intracellular Qβ binding was significantly increased 4 and 5 days after challenge with Qβ when MBCs were transferred (**Figure 5E**). Taken together the results of the spot size and intracellular staining of spleen and BM-derived PCs indicated that secondary PCs produced increased amounts of antibodies.

Both spot size and intracellular staining with Qβ-VLPs may not linearly correlate with antibody production. To estimate the amount of antibodies produced by secondary vs. primary PCs more directly, splenocytes were collected and cultured from mice 5 and 6 days after adoptive transfer and VLP challenge. Whole splenocytes were seeded into 24 well-plates for 72 h and frequencies of specific PCs were quantified by FCM at the beginning of the culture. The amount of anti-Qβ antibody in cell culture supernatants of splenocytes harvested 5 and 6 days after challenge was ∼30 fold increased when MBCs were transferred, again demonstrating that secondary PCs produced elevated antibody levels (**Figure 5F**). The total amount of anti-Qβ antibody decreased from day 5 to day 6 after memory transfer (**Figure 5F**) further demonstrating their short lived nature. Nevertheless, the amount of specific antibody per PC stayed the same (**Figure 5G**). After naïve transfer, on the contrary, the amount of antibody per PC increased over time, as the primary response evolved (**Figure 5G**). This massively increased protein production by secondary PCs illustrates the stress these PCs may be exposed to, probably resulting in the short live span.

DISCUSSION

Long-lived PCs are crucial for sustained immune protection through secretion of specific antibodies (24). However, PCs do not always become long-lived during infection or vaccination because most of the PCs die early during the immune response. In fact, during primary immunogenicity studies using VLPs, we observed that the PC population in spleen emerged on day 4, peaked at day 7 and subsequently declined rapidly, followed by a phase of more stable PC frequencies (35). Hence, most PCs formed initially against Qβ are short-lived (41). This short lifespan may be a result of the irrevocable cell cycle arrest which PCs usually enter and therefore cannot maintain a cellular pool by means of proliferation. In contrast, the state of irreversible cell cycle quiescence must be controlled by mechanisms to enable long-term PC survival. Moreover, the ephemerality of the early

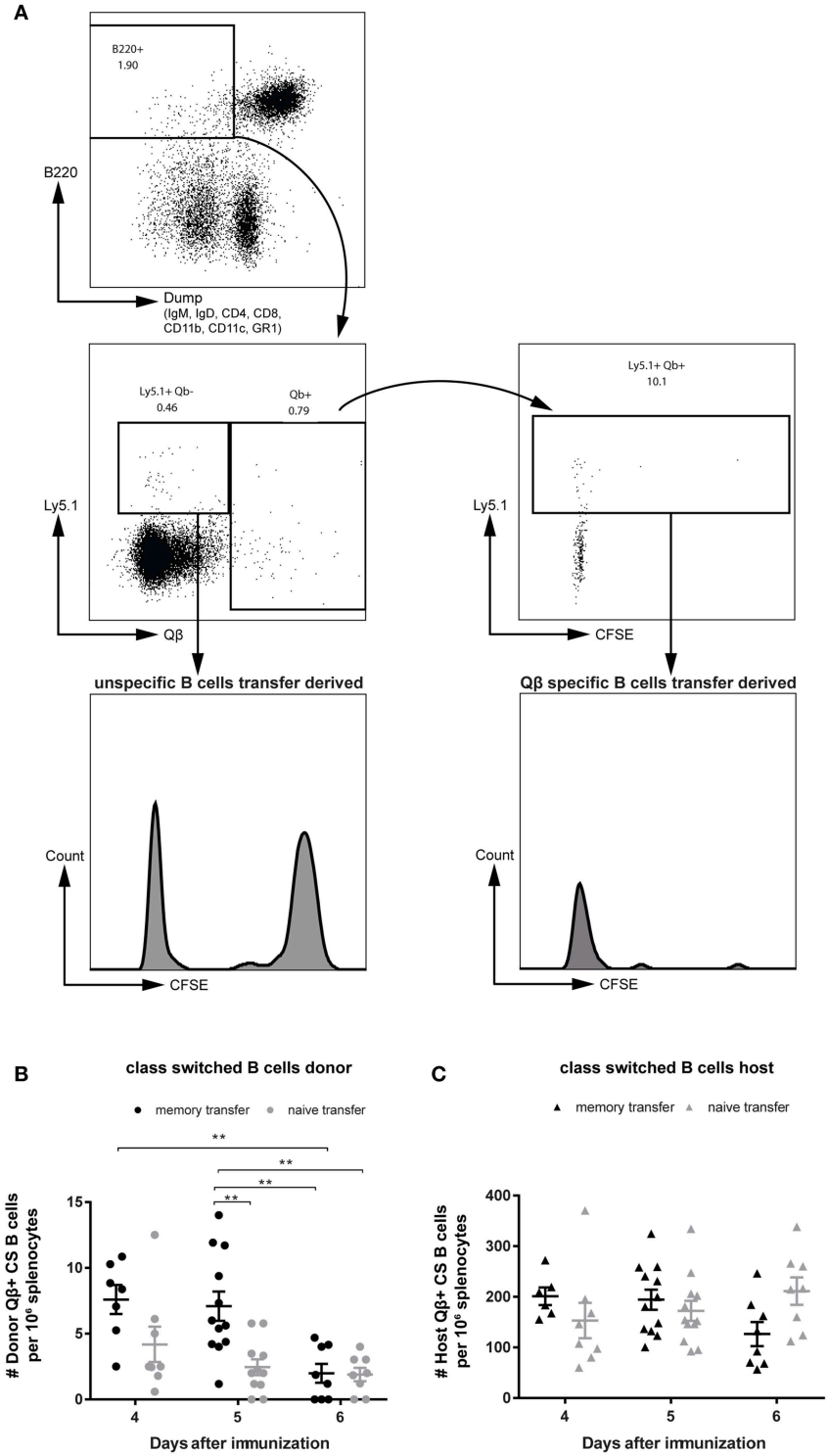

FIGURE 3 | MBCs do not extensively proliferate before differentiating to secondary PCs upon cognate antigen challenge. PNA^{-}, B220^{+} MACS purified cells from Qβ immune (8 weeks post immunization) or naïve Ly5.1 mice were labeled with CFSE and transferred into congenic hosts. Recipient mice were challenged 24 h later with 50 μg Qβ VLPs i.v. **(A)** Representative FCM plot to identify Qβ specific CS B cells in the spleen 5 days after challenge. B220^{+} cells negative for IgM, IgD, CD4, CD8, CD1b, CD11c, and GR1 were analyzed for their Qβ binding. CFSE dilution was examined to prove proliferation of donor derived cells (Ly5.1^{+}). **(B,C)** Number of Qβ specific CS B cells on day 4, 5, and 6 after challenge identified by FCM as B220^{+}, negative for IgM, IgD, CD4, CD8, CD11b, CD11c, GR1, and binding Qβ. Qβ specific donor **(B)** derived cells were distinguished from host derived cells **(C)** using the Ly5 congenic marker. Mean with SEM. *P* values were obtained using a one-way ANOVA followed by Tukey's multiple comparisons test. $^{**}p < 0.01$. $n = 4$ mice per group. Data representative of at least 2 independent experiments.

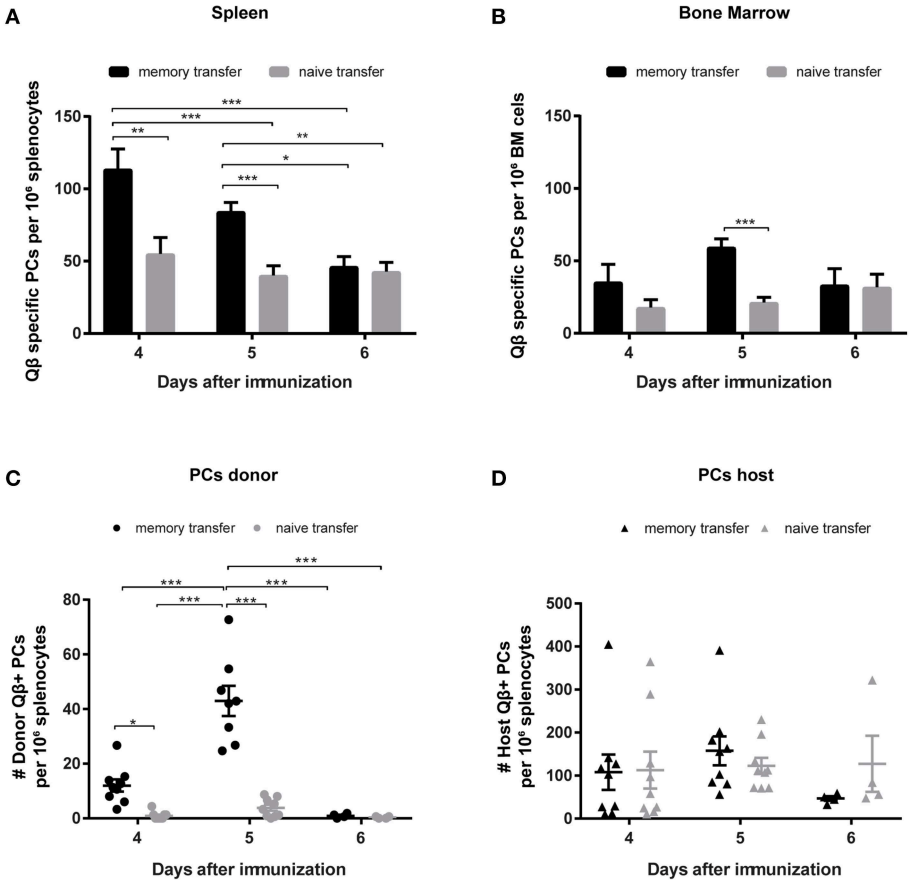

FIGURE 4 | Transfer of memory B cells leads to an increased number of PCs, which are rapidly induced but short-lived. Congenic Ly5.1 mice were immunized with Qβ VLPs to generate MBCs. Eight weeks after the immunization PNA^{-}, $B220^{+}$ MACS purified cells from memory or naïve mice were transferred into congenic hosts. One day after the transfer, recipient mice were challenged with Qβ VLPs and the anti-Qβ PC response in spleen and BM was elucidated by ELISPOT and FCM. Number of Qβ specific PCs in spleen **(A)** and BM **(B)** on day 4, 5, and 6 after challenge determined by ELISPOT. FCM analysis of Qβ specific PCs within the $B220^{low}$, IgM, IgD, CD4, CD8, CD11b, CD11c, and GR1 negative compartment, by intracellular Qβ binding after membrane permeabilisation. Qβ specific donor derived PCs **(C)** were distinguished from host derived PCs **(D)** using the Ly5 congenic marker. Mean with SEM. *P* values were obtained using a one-way ANOVA followed by Tukey's multiple comparisons test. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. $n = 4$ mice per group. Data representative of at least 1–2 independent experiments.

PCs could also be due to ER stress caused by the massive antibody production. Cell intrinsic constraints like unfolded protein response (UPR) and autophagy can rescue PCs from cell death but the cells additionally require sufficient nutrients, external survival signals and a survival niche (30, 49–52). Long-lived PCs are found in both the spleen and BM. However, the numbers of niches is finite, thus restricting the number of PCs with access to them (1). In fact, most VLP specific PCs reaching the BM do not survive as the number of PCs rapidly declines initially also in the BM (45). The constant competition for space and survival signals of PCs within the BM may provide an opportunity to manipulate PC survival for long-term antibody production upon vaccination as well as for therapies of malignant PC diseases (53). Moreover, CD28 has been shown to be expressed by human and mouse PCs (54, 55). Engagement of CD28 with CD80/CD86 derived from cellular partners in the PC niche was demonstrated to be important for BM long-lived PC survival, half-life and sustained antibody responses. Downstream signaling of CD28 induces BLIMP1 upregulation and is therefore involved in regulating PC differentiation and maintenance (55, 56). Furthermore, CD28 was shown to regulate glycolysis in long-lived PCs providing glycolytic end products for oxidative energy production and biosynthesis (57). Additionally CD28 regulated mitochondrial metabolism and respiration which favored survival of long-lived PCs (58, 59). In contrast to long-lived PCs, CD28 exhibited a higher activation threshold in short-lived PCs and therefore had no positive impact on their survival (55). Together with limited access to CD80/CD86 molecules derived from BM PC niche cells, the increased threshold of CD28 activation could be reasons for the short-lived nature of the secondary PCs. We are currently assessing a potential role of CD28 in the lifespan of primary and secondary PCs.

A population of MBCs is maintained after the decline of immune responses and may be activated upon re-infection to rapidly differentiate into PCs, which secrete antibodies. However, it has also been reported that MBCs can re-enter GCs and interact with T follicular helper cells shaping the immune response and generating a new pool of MBCs. In analogy to memory T

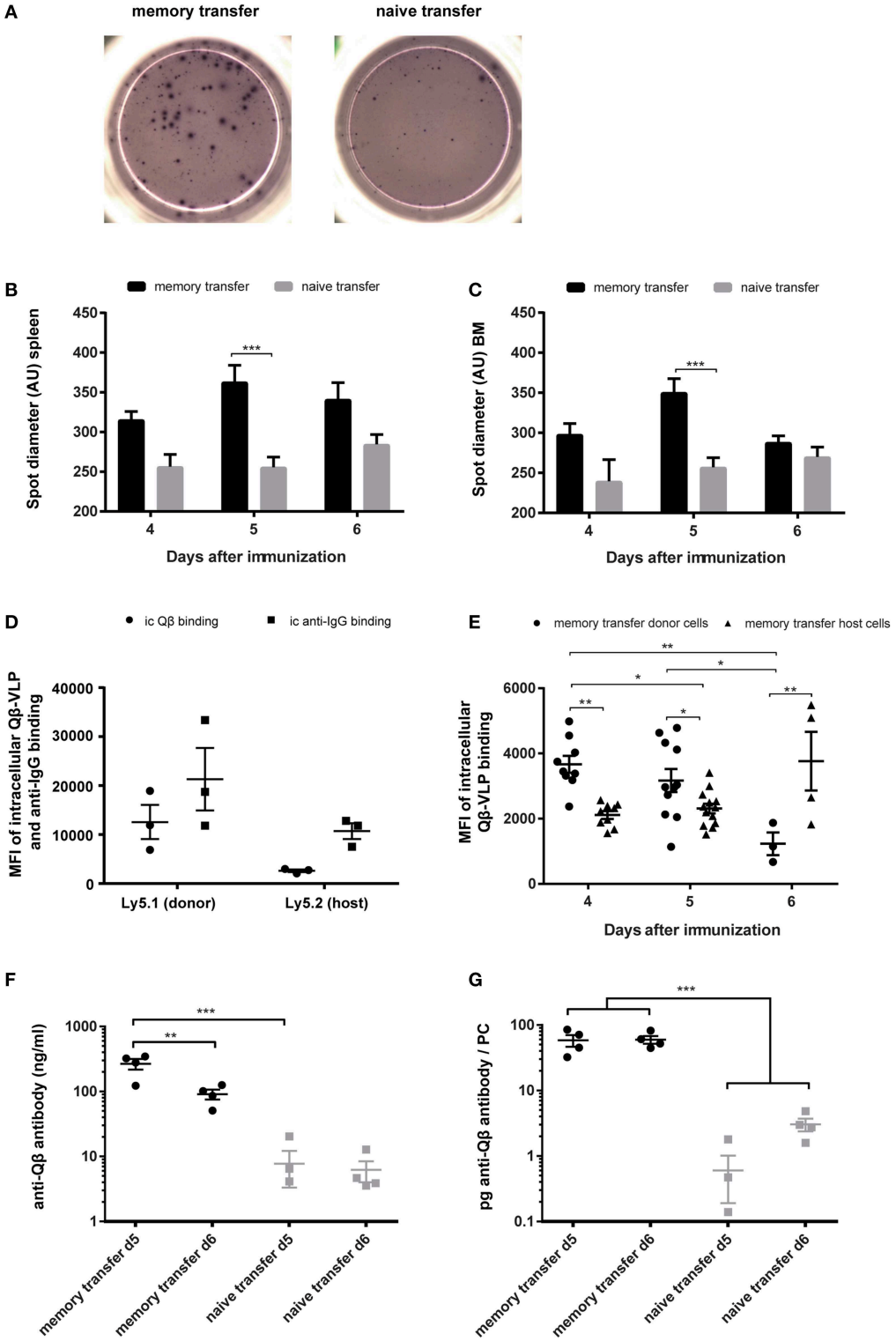

FIGURE 5 | Transfer derived secondary PCs show enhanced capacity to produce antibodies in spleen and BM. PNA^{-}, B220^{+} MACS purified cells from Qβ immune (8 weeks post immunization) or naïve Ly5.1 mice were transferred into congenic hosts. One day after the transfer, recipient mice were challenged with 50 μg Qβ VLPs i.v. Splenocytes and BM cells were analyzed by ELISPOT and FCM on day 4, 5, and 6 after challenge. **(A)** Representative images of anti-Qβ ELISPOTs on day 5 after challenge in the spleen. Quantification of spot diameter in spleen **(B)** and BM **(C)** on day 4, 5, and 6 after challenge. **(D)** Quantification of mean intracellular Qβ and

(Continued)

FIGURE 5 | anti-IgG binding of donor and host derived PCs on day 5 after memory transfer and challenge. **(E)** Quantification of mean intracellular Qβ-VLP binding of donor and host derived PCs after memory transfer. **(F,G)** Splenocytes were isolated 5 and 6 days after challenge and cultured for 3 days *in vitro*. Secreted antibodies in the cell supernatant were determined by ELISA. **(F)** Amount of anti-Qβ antibody secreted during the 72 h splenocyte cell culture. **(G)** Amount of specific antibodies produced per PC 5 and 6 days after memory or naïve cell transfer and challenge. Mean **(B,C,F,G)** or geometric mean **(D,E)** with SEM. *P* values were obtained using a one-way ANOVA followed by Tukey's multiple comparisons test. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. $n = 4$ mice per group **(A–C,E–G)**, $n = 3$ mice per group **(D)**. Data representative of 1–2 independent experiments.

cells, these two different effector functions of MBCs define two distinct cellular compartments: the effector MBCs differentiating into PCs for rapid antibody production and the central MBCs playing a role in re-initiating the GC response and maintaining the MBC pool (60). The secondary PCs described herein are derived from effector MBCs that were generated by a single round of immunization using Qβ-VLP. B cell intrinsic toll-like receptor (TLR) 7 stimulation was shown to be essential for MBC generation that were capable of differentiating to secondary PCs (61). Intriguingly, VLP specific MBCs only responded a single time to Ag re-stimulation, namely by terminal differentiation into short-lived PCs. These secondary PCs were $B220^{-}$ and no longer carried their Ig on the surface and therefore could not be further stimulated with the Ag. Baptista et al. studied PC differentiation in response to innate stimuli in the absence of antigen and observed that TLR9 signaling by CpG failed to differentiate follicular B cells into PCs whereas TLR4 stimulation by lipopolysaccharide (LPS) induced antibody production, PC surface markers such as CD138 and canonical transcription factors like IRF4, BLIMP1 or XBP1 (62). Furthermore, ligation of BCR and TLR7 was shown to drive PC differentiation (63, 64). Therefore, the downregulation of BCR and B220 expression and increased antibody production seen in response to MBC re-stimulation with VLPs containing bacterial RNA are clear signs of PC differentiation. Nevertheless, we never found a homogenous $CD138^{+}$ cell population using VLPs for vaccination. Consequently, further work needs to be done to determine the expression of classical PC transcription factors and surface markers to characterize the phenotype of secondary PCs in more detail.

The avidity of the antibodies secreted by secondary PCs was very high at early time points after immunization, a level which antibodies generated during a primary response only reached by day 20 upon VLP immunization. This finding is consistent with the notion that secondary PCs derive from MBCs which have undergone avidity maturation in a GC reaction (45). Thus, secondary PCs provide the host with a rapid wave of high-avidity antibodies. The great amount of VLP-specific IgG, which is present early during the recall response, is most likely responsible for the suppression of the host response. Link et al. demonstrated that VLPs complexed to specific IgGs were taken up by macrophages in the subcapsular sinus and did not efficiently reach B cell follicles and follicular dendritic cells, leading to antigen deprival for naïve B cell activation (65).

Surprisingly, secondary PCs did not have a long functional lifespan, neither in spleen nor in BM. In fact, most of them disappeared from lymphoid organs within 6 days after Ag re-stimulation. The dominant pool of long-lived PCs induced in the presence of MBC was derived from primary B cells. The early death of secondary PCs is probably a consequence of the enhanced antibody production, which increases cellular stress levels. Access to niches seems less important, as numbers of primary and secondary PCs are similar at day 6 after challenge, and yet only primary PCs become long-lived. The increased antibody production in secondary PCs more likely accounts for the short lifespan due to increased ER stress as well as accelerated demand for nutrients. The fact that secondary PCs produce at least 30 times more antibody than primary PCs underscores this point. It has been shown that PCs are able to adapt their metabolism according to the changing environment, but secondary PCs may be induced too quickly to produce very large amounts of antibodies, to be able to adapt their metabolism sufficiently. In addition, they may not live long enough to actually find a niche allowing their long-term survival. In this respect, secondary PCs behave more like innate cells, which usually respond very rapidly but are short-lived as well. In terms of surface marker and transcription factor expression, secondary PCs most likely do not differ extensively from other PC populations. However, there are substantial functional differences.

The fact that during viral infection all MBCs differentiate into functionally short-lived secondary PCs has interesting biological implications as it keeps the antibody repertoire flexible and adaptable to the changing world of pathogens, as e.g., influenza viruses. Secondary PCs produce an early wave of high avidity antibodies specific for the strain of pathogen previously encountered. Under this early protective antibody umbrella, naïve B cells are activated, initiate a novel GC reaction and generate high avidity PCs and MBCs for the current version of the pathogen. This mechanism ensures that the antibody repertoire is not frozen to the specificity for a single version of a pathogen but remains adaptable to their evolution. In this respect, the here presented mechanism ensures that original antigenic sin does not limit the dynamics and broadness of the antibody repertoire too extensively (25, 66, 67).

In summary, we demonstrate here that upon challenge with viral particles MBCs differentiate rapidly into secondary PCs, providing the host with an early wave of high avidity antibodies. Thus, induction of effector MBCs, which can provide rapid and effective protection by differentiating into secondary PCs, may be a promising alternative that should be considered in vaccine development.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of the Bundesamt für Lebensmittelsicherheit und Veterinärwesen (BLV) and the guidelines of the Cantonal Veterinary Office Bern, Switzerland. The protocol was approved by the Cantonal Veterinary Office Bern, Switzerland.

AUTHOR CONTRIBUTIONS

CK and EK performed all experiments. CK, FT, and MB designed all the experiments and wrote the manuscript. MV interpreted results and contributed to the scientific discussion. All authors read and 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.2019.01831/full#supplementary-material

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