Plasma cell output from germinal centers is regulated by signals from Tfh and stromal cells

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Germinal centers (GCs) are the sites where B cells undergo affinity maturation. The regulation of cellular output from the GC is not well understood. Here, we show that from the earliest stages of the GC response, plasmablasts emerge at the GC-T zone interface (GTI). We define two main factors that regulate this process: Tfh-derived IL-21, which supports production of plasmablasts from the GC, and TNFSF13 (APRIL), which is produced by a population of podoplanin+ CD157high fibroblastic reticular cells located in the GTI that are also rich in message for IL-6 and chemokines CXCL12, CCL19, and CCL21. Plasmablasts in the GTI express the APRIL receptor TNFRSF13B (TACI), and blocking TACI interactions specifically reduces the numbers of plasmablasts appearing in the GTI. Plasma cells generated in the GTI may provide an early source of affinity-matured antibodies that may neutralize pathogens or provide feedback regulating GC B cell selection.

Introduction

A hallmark of antibody responses to T-dependent antigens is the increase in affinity of antigen-specific antibodies in circulation. Antibody affinity maturation takes place in B cells differentiating in germinal centers (GCs; MacLennan, 1994; Victora and Nussenzweig, 2012). Before the initiation of GCs, some B cells rapidly mature into extrafollicular plasma cells (PCs) that generate an early low-affinity germline-derived antibody (MacLennan et al., 2003). Increases in antibody affinity are easily detectable after secondary immunization (Eisen and Siskind, 1964), but also noticeable during the primary response (Takahashi et al., 1998; Kang et al., 2015). Mutated PCs were found as early as 10 d after primary immunization (Jacob and Kelsoe, 1992; Smith et al., 1997), which is only a few days after the onset of mutational activity in primary GCs (Weiss et al., 1992; Jacob et al., 1993; McHeyzer-Williams et al., 1993). In carrier-primed responses, when T cell help is available immediately, extrafollicular and follicular B cell differentiation happens more rapidly, and mutated PCs are found in the splenic red pulp as early as 2 d after GC formation (Sze et al., 2000). Affinity-increased antibody can appear in blood at the same time (Zhang et al., 2013). Considering mutated GC-derived PCs compete with the initially formed extrafollicular PCs (Sze et al., 2000), this increase in circulating antibody is remarkably fast.

A recent study demonstrated that GCs mature, going through stages of preferential output of memory B cell or long-lived PCs homing to the bone marrow (Weisel et al., 2016). The antibody is not only important for pathogen defense, but it also has a role in regulating B cell selection in the GC by modulating antigen accessibility, shielding antigens from access by lower-affinity B cells (Zhang et al., 2013). For this antibody feedback to happen efficiently, it is critical that GCs produce affinity-matured PC output generating a higher-affinity antibody from an early stage. A recent study showed that the high-affinity antigen interaction of GC B cells triggers PC differentiation, whereas additional undefined signals from T follicular helper (Tfh) cells are necessary to fully induce PC differentiation (Kräutler et al., 2017). In the current study, we set out to test when and where PCs generated from GCs appear locally. We show that this starts from a very early stage of GC development. During the earliest stages of GC differentiation, PCs leave the GC by entering the T zone from the GC dark zone. Defining timing and location of PC output enabled us to identify factors that regulate the appearance of affinity-matured PCs from the GC. We show a role for IL-21, a B cell differentiation factor produced by Tfh cells that is also involved in extrafollicular PC differentiation (Linterman et al., 2010; Zotos et al., 2010; McGuire et al., 2015). We further demonstrate that the GC-T zone interface (GTI) contains a new T zone stromal cell subset producing APRIL, which can support differentiation of PCs in the GTI.
Results
Lymphocyte activation and the appearance of GC-derived plasmablasts
The timing and location of plasmablasts emerging in the spleen were tested by immunizing naive mice with sheep red blood cells (SRBCs). i.v. injection of SRBCs induces a synchronized onset of primary T and B lymphocyte activation, leading to extrafollicular plasmablast differentiation and formation of GCs. To follow plasmablast appearance, spleen sections were labeled for the transcription factor IRF4. IRF4 is expressed at low levels in activated B and T cells (Matsuyama et al., 1995; Klein et al., 2006; Sciammas et al., 2006), but is strongly induced as B cells initiate PC differentiation (unpublished data; Klein et al., 2006; Sciammas et al., 2006).

SRBCs induced rapid extrafollicular plasmablast differentiation from day 3 to 5 after immunization (Fig. 1 A). Similar to responses to other antigens (Jacob and Kelsoe, 1992; Toellner et al., 1996, 1998), these appeared in the bridging channels connecting the T zone with the red pulp (Fig. 1 A), but peaked by day 5 (Fig. 1 B). T cell activation, indicated by the significant increase of Il4 mRNA (Fig. 1 C) and appearance of IRF4int T cells (Fig. 1 A), occurred by day 2 after immunization. A rise in germline IgG1 transcripts suggests that cognate T–B interaction must have happened at the same time (Fig. 1 D).

GCs appeared in a significant amount from day 4 after immunization (Fig. 1 A). Throughout the GC response, IRF4high cells are found at the GTI, suggesting that output of PCs from the GC takes place in this area (Fig. 1 A). Although antibody-forming cells located close to the GC in T zones of lymph nodes have been noticed before (Mohr et al., 2009; Meyer-Hermann et al., 2012), it was surprising to find that these are prevalent among the earliest stages of GC development and peak within 24 h after the emergence of GCs (day 5; Fig. 1 F). Less frequently, IRF4high cells were observed in the periphery of the GC light zone (Fig. 1 A), although this occurs more often at later stages (Angelin-Duclos et al., 2000).

The early onset of PC differentiation at the GTI is not only seen in primary SRBC responses, as challenge of carrier-purified mice with soluble chicken gamma globulin (CGG) coupled to 4-hydroxynitrophenyl (NP) induced even larger numbers of IRF4high PCs at the GTI (Fig. 1, G and H). Again, relative to the size of the GC, appearance of these cells was maximal within 2 d of the appearance of GCs (Fig. 1 H; Toellner et al., 1996). Occasionally, IRF4int B cells could be observed in the GC light zones (Fig. 1 G, open arrowheads).

In summary, these observations are consistent with recent data that GC B cells are selected in the GC light zone through interactions with antibody and Tfh cells (Kräutler et al., 2017), then migrate toward the GC dark zone and GTI, further up-regulating IRF4 expression. At the GTI, they exit the GC as plasmablasts (Meyer-Hermann et al., 2012).

Characterization of IRF4-expressing cells on the GTI
To confirm that the IRF4high cells emerging at the GTI are plasmablasts and to test whether these cells still express GC related markers that could be exploited for isolation and characterization, expression of a large range of antigens associated with GC and PC phenotype was tested by immunohistology at the peak of the response, 5 d after SRBC immunization. This shows IRF4high cells appear in a narrow area rich in CD4 T cells, bordering the IgD+ GC (Fig. 2 A). IRF4high cells in the GTI were found along a narrow strand of IgD+ B cells, identifying this area as the border between the GC-containing follicle and the T zone (Fig. 2 A). IRF4high cells in this area were in close contact with CD4 T cells. Lower numbers of IRF4-expressing cells were found in the light zone of the GC, often in contact with CD4+ Tfh cells (Fig. 2 A). Ki-67 staining shows that IRF4high cells in the GTI were still in cell cycle (Fig. 2 B). Furthermore, they accumulated BrdU injected i.p. 2 h before tissue analysis (unpublished data). This identifies them as blasts. Many, but not all, were IgG switched. They expressed Ig as strongly as extrafollicular plasmablasts in the red pulp (Fig. 2 C). Most cells lacked B220 (Fig. 2 D) and expressed Blimp1 (Fig. 2 E) and CD138 (Fig. 2 F), which again is consistent with these cells being plasmablasts. Interestingly, IRF4high cells found in the outer light zone of the GC expressed lower levels of Blimp1 and lower levels of CD138 (Fig. 2, E and F). Because IRF4 is upstream of Blimp1 and differentiation to PCs (Sciammas et al., 2006), this indicates that the IRF4high cells in the outer light zone are an earlier maturation stage than the Blimp1high and CD138high plasmablast in the GTI. Plasmablasts in the GTI were not associated with markers expressed in the GC, including peanut agglutinin binding (Fig. 2 G), BCL6 (Fig. 2 H), or CD21 (not depicted). IRF4high cells strongly expressed CXCR4 (Fig. 2 I), and some of them expressed CCR7 (not depicted), which may aid their migration to the GTI (Bannard et al., 2013).

Collectively, this identifies IRF4high cells in the GTI as plasmablasts (Fig. 2 J). The cells may have been induced to differentiate in the GC light zone and arrive via migration along the GC outer zone (Liu et al., 1992). Expression of CXCR4 and CCR7 may guide their migration toward the GTI (Bannard et al., 2013; Rodda et al., 2015). These chemokine receptors also would have the potential to support further movement of plasmablasts toward splenic bridging channels (Hargraves et al., 2001). GCs in lymph nodes have a similar GTI that contains plasmablasts that appear to be migrating toward the medulla (Mohr et al., 2009).

In vivo migration of plasmablasts at the GTI
Several strategies were used to more directly show that plasmablasts emerging in the GTI are derived from adjacent GCs. A small number of observations could be made using intravital multiphoton microscopy. Mice received adoptive transfers of NP-specific B cells from B18i/k−/−/Prdm1GFP/Cdt1mKO2 mice. These mice express GFP under the control of the Prdm1 promoter (Kallies et al., 2004), and express a fast-folding version of monomeric Kusabira Orange (mKO2) during the G1 phase of the cell cycle (Sakaua-Sawano et al., 2008), indicating fate decision toward PC differentiation by GFP, whereas GCs can be identified by mKO2 expression. Plasmablast migration was followed 6 d after foot immunization with NP-CGG in complete Freund’s adjuvant. Observations showed the presence of Blimp1-GFP-expressing cells in the GC and in the zone between the GC and the medulla (Fig. 3 A). Blimp1-expressing cells were found in large numbers in the medulla and in smaller numbers in GCs and the space between the GC and the medulla.
Blimp1-expressing cells in the GC preferentially moved toward the GTI, with few traversing into the surrounding space. Similar to earlier observations (Fooksman et al., 2010), Blimp1-expressing cells between the GC and medulla were mobile, whereas cells in the medullary cords were more or less stationary (Fig. 3 B). Analysis of the movement of plasmablasts along the GC-medullary axis showed a slightly higher tendency of net migration toward the medulla (Fig. 3 C).
To test whether cells in the GTI are clonally related to cells in the GC, Confetti mice (Snippert et al., 2010) were induced to randomly express CFP, hrGFP, YFP, or dsRFP and immunized with SRBCs. After 5 d, the distribution of different colors in GCs and adjacent GTIs were observed by microscopy (Fig. 3D). Comparing the distribution of different colors (Fig. S1) by principal component analysis revealed a positive correlation between GCs and plasmablasts in the adjacent GTI (Fig. 3D).

To further test whether cells in the GTI originate from the GC, Aicda-CreERT2 mice (Dogan et al., 2009) were crossed onto mTmG mice (Muzumdar et al., 2007) permitting Aicda-expressing cells to express GFP after induction of Cre by tamoxifen. Mice were immunized with SRBCs, and Cre was induced 4 d after immunization. 5 and 6 d after immunization, GFP+ cells in the GC, GTI, and in extrafollicular foci in bridging channels were analyzed. This showed that 24 h after Cre induction, 9.0% of GC cells and 1.8% of GTI plasmablasts were GFP+, whereas PCs in bridging channels were GFP−. Another 24 h later, 19% of GC B cells were GFP+, and 8.3% of GTI plasmablasts and 1.1% of bridging channel PCs had become GFP+.

These observations are consistent with plasmablasts in the GTI being descendants of adjacent GCs in transit toward bridging channel or medullary PC niches (Sze et al., 2000; Mohr et al., 2009) with a 24 h delay between cells expressing Aicda in the GC and the first arrival of their descendants in the GTI. These timings correlate very well with earlier observations on germinal center dynamics (Victora et al., 2010).

Expression of mRNA coding for IL21 in the GC and APRIL in the GTI
To test signals regulating plasmablast differentiation from the GC, sections of spleens taken from carrier CGG–primed mice...
Figure 3. Dynamics and motility of PCs of different localization during immune response. NP-specific B cells from B18i/k−/−/Blimp1GFP/Cdt1mKO2 mice were transferred into C57BL6 recipients followed by foot immunization with 10 µg NP-CGG in CFA 24 h later. Popliteal lymph nodes were analyzed 6 d later. (A) A 57-µm maximum intensity Z-projection from two-photon microscopy image stacks of a GC and medulla (M) in a live popliteal lymph node. NP-specific transferred B cells and plasmablasts in proliferation are mKO2+ and Blimp1-expressing plasmablasts, and PCs are GFP+. SHG, second harmonic generation. Bar, 100 µm. Dashed lines indicate the outline of the GC and medulla. Tracks of GFP+ cells in GC, area between GC, and medulla and within medullary cords are shown. Track starts are indicated with light green arrowheads. (B) Persistence of tracks (track displacement length/track length) of GFP+ or mKO2+ cells in different areas. Horizontal bar indicates median. (C) Displacement of cells moving in up (toward the GC) or down (toward the medulla) direction. Displacement normalized to track observation period. Data merged from three independent experiments (n = 3). Each symbol corresponds to one cell. Two-sided Mann-Whitney test. (D) Spleen section from Rosa26CreERT2 mouse stained with IRF4. Colored arrows indicate the color of fluorescent protein expressed by IRF4+ GTI plasmablasts. Representative pictures are from two independent experiments (n = 10). Correlation of first principle component of CFP, GFP, YFP, and RFP expression patterns between GC and corresponding GTI (right). Dashed lines indicate the outline of the GC and B cell follicles. Spearman rank correlation, r = 0.719. (E) AID-CreERT2 × ROSA26mT/m mouse spleen section taken 48 h after tamoxifen induction on day 4. Stained for IRF4 and IgD. White arrowheads indicate GFP+ IRF4high cells. (F) Quantification of AID-GFP+ in each group at 24 h and 48 h after tamoxifen induction. Data merged from three independent experiments (two to three mice each time). Nonpaired two-tailed Student’s t test. ***, P = 0.0002; ****, P < 0.0001.
5 d after challenge with NP-CGG were separated into the B cell follicle, GC, GTI, extrafollicular PC foci, and T zone using laser microdissection (Zhang et al., 2016). Real time PCR (RT-PCR) for expression of Cd3e, Cd19, Pax5, and several other GC- and PC-associated genes demonstrate that the technique distinguishes these specific splenic microenvironments (Fig. S2). Despite the GTI being quite narrow, which may lead to contamination from neighboring areas, there was 20× lower Bcl6 and Aicda mRNA detected compared with neighboring GCs (Fig. S2).

Detection of chemokines and receptors organizing follicular migration showed, as expected, Cxcl12 being highly expressed in the GTI and extrafollicular PC areas (Fig. 4 A). The CXC12 receptor CXCR4, being involved in GC light zone–dark zone translocation, was strongest expressed in GC cells. Although CXCR4 protein was strongly expressed on plasmablasts in the GTI (Fig. 2 I), mRNA expression was not higher in the GTI than in follicular areas, which may reflect posttranscriptional regulation (Al-Souhibani et al., 2014) or dilution of signal by contaminating non-PCs.

Expression of cytokines supporting B cell differentiation (Il4, Il6, Il10, Il21, Tnfsf13 [April], and Tnfsf13b [Baff]) was tested. The expression of most cytokines was below detection level, except Il21, Baff, and April mRNA. Il21 mRNA was expressed at more than 100× higher levels within microdissected GCs than in other areas (Fig. 4 B). Mean expression of its receptor was also strongest within the GC. Baff mRNA was present throughout all B cell areas (Fig. 4 G). Interestingly, April mRNA, which is expressed in B cell follicles, was absent from GCs but present in the GTI at similar levels to follicles. Tnfsf13c (Baffr) mRNA was strongly expressed in follicles and GCs, but found at 10× lower levels in the GTI. However, mRNA coding for Tnfrsf13b (Taci), a receptor that binds both BAFF and APRIL, although absent from the GC, was reexpressed in the GTI. Extrafollicular PC foci expressed even higher levels of Taci mRNA. Tnfrsf17 (Bcma) mRNA, another receptor binding BAFF and APRIL, was detected reliably in extrafollicular PC foci, but there was no significant expression in the GTI. Collectively, these data indicate that B cells developing in the GC receive various differentiation signals, including IL-21 (Shulman et al., 2014). Furthermore, the expression of APRIL, specifically expressed in the GTI, and TACI suggests roles in mediating differentiation and survival in the GTI (Mackay and Schneider, 2008).

A role for Tfh signals for the differentiation of early GC-derived plasmablasts.

T cell–derived signals have been suggested to have a role for PC differentiation from the GC (Kräutler et al., 2017). IL-21, which is produced by Tfh cells, has been shown to regulate GC development (Linterman et al., 2010; Zotos et al., 2010). IL-21 not only regulates GCs, but also affects early extrafollicular PC appearance independent of GCs (Zotos et al., 2010; Lee et al., 2011). This makes IL-21 a potential regulator of GC-derived plasmablast differentiation.

Il21 and Il21r expression was tested from in vivo–activated T and B cells. To provide larger numbers of antigen–specific B cells, NP-binding B220+ cells from quasimonoclonal (QM) enhanced YFP (eYFP) mice were transferred into wild type hosts 1 d before immunization with SRBCs coupled with NP. During the first 3 d after immunization, activated B and T cells were isolated as eYFP+ B cells and CD62Llow CD4 T cells. During the GC phase, B220+ Fas+ eYFP+ GC B cells and CD4+ PD1+ CXCR5+ Tfh cells were sorted. Despite the role of IL-21 in extrafollicular plasmablast differentiation, activated extrafollicular T helper cells during the first 3 d of the response expressed Il21 mRNA just above detection level (Fig. 5 A). Tfh cells, isolated from day 4, expressed 100× more Il21 mRNA than activated Th cells from the extrafollicular phase of the response, with the strongest expression within the first 2 d of the GC response (Fig. 5 A). Il21r expression was significantly induced during the extrafollicular phase of B cell activation and increased further once B cells formed GCs (Fig. 5 B). GC B cells isolated at day 5 expressed on average three to four times more IL21r mRNA than nonactivated B cells or Tfh cells (Fig. 5 C). Flow cytometry showed increased levels of IL-21R on activated B cells and GC B cells, with slightly higher expression in light zone B cells (Fig. 5 D).

To test the effect of Il21 deficiency on PC differentiation from the GC, plasmablast numbers in the GTI were quantified 7 d after immunization of Il21/Il21r-deficient mice with NP coupled to keyhole limpet hemocyanin (NP-KLH). As shown earlier (Zotos et al., 2010), GCs on average were 50% smaller in the absence of IL-21 (Fig. 5 E); the number of GTI plasmablasts, however, was reduced disproportionally more in the absence of IL-21, at 63% reduction in GTI plasmablasts per GC volume (Fig. 5 E). This corresponds to an 83% reduction of total GTI plasmablasts per spleen. Similar results were seen in BALB/c mice deficient in IL-21 receptor (IL21R) after primary immunization with NP-CGG in alum, with a 47% reduction in GC-derived plasmablasts (unpublished data). This points toward IL-21 being a major factor regulating the induction of GC-derived plasmablast differentiation.

To confirm that the emergence of GC-derived plasmablasts is dependent on Tfh–B cell interactions, mice were treated for 24 h with a blocking anti–CD40L antibody. This interval was chosen based on the known kinetics of GC cell recirculation (Victora et al., 2010) and output (Fig. 3 F). CD40L blocking or control antibody was given i.v. to mice at 4 d after primary SRBC immunization. As expected (Foy et al., 1996), this short blockade led to increased GC B cell apoptosis, but no significant loss of GC volume (Fig. 5 F). Plasmablast output at the GTI was reduced to 70% of control (Fig. 5 F). Importantly, quantification of total CD19+ PCs by flow cytometry did not show a reduction within 24 h (Fig. 5 F), indicating that GTI plasmablasts are a population derived from recent Tfh interactions and, therefore, are sensitive to Tfh signal blockade.

To test the role of IL-21 on plasmablast output more directly, blocking soluble IL-21 receptor was injected, and tissue was analyzed with similar timing as in the CD40L blocking experiment. This led to effects very similar to blockade of CD40 signaling with no significant effect on GC (Fig. 5 G) or extrafollicular PC numbers (not depicted), but effects on B cell apoptosis and plasmablast output through the GTI (Fig. 5 G). Lastly, the effect of IL-21 injection 4 d after SRBC immunization was tested 24 h later. This led to a significant increase of GC-associated plasmablasts without changing GC size (Fig. 5 H). Collectively, these results demonstrate that T cell–B cell interaction and IL-21 are major factors regulating plasmablasts emerging from GCs at the GTI.
The GTI contains a stromal cell niche that can produce APRIL
Stromal cells have been shown to play a role in directing GC cell migration (Bannard et al., 2013) and to support lymphocyte survival and differentiation (Cremasco et al., 2014; Fasnacht et al., 2014; Zehentmeier et al., 2014). Plasmablasts emerging at the GTI were in intimate contact with podoplanin (PDPN)-positive fibroblastic reticular cells (FRCs; Fig. 6 A). FRCs in the GTI differed from FRCs in the center of the T zone with higher expression of CD157 (Fig. 6 B), usually associated with follicular stroma (Cyster et al., 2000). FRCs within the T zone expressed lower levels of CD157, and this decreased toward the central arteriole (Fig. 6 C). Splenic GTI stroma typically was oriented parallel to the follicle–T zone border, with some extensions penetrating into the GC (Fig. 6 B). PDPN+ CD157+ cells did not occur in other local niches of PC development. Instead, other cells associated with PC survival were observed in these sites: splenic bridging channels (Fig. 6 D) contained CD11chigh dendritic cells (García de Vinuesa et al., 1999), while in the red pulp (Fig. 6 E), F4/80+ myeloid cells (Mohr et al., 2009) were found. These cell types were less abundant in the GTI (Fig. 6 F). CD157high PDPN+ stroma was also found in the GTI of lymph nodes (Fig. 6, G and H).

The microdissection experiments showed that the GTI is rich in April mRNA. To test whether the stromal cells in the GTI are sources of TACI ligands, cells were flow sorted from popliteal...
Figure 5. A role of IL-21 for the differentiation of early GC-derived plasmablasts. (A) Ii21 mRNA expression in naive CD4+ T cells (Nai. T), CD4+ CD62L-low activated T cells (Act. T), CD4+ CXCR5− PD1− non-Tfh cells, and CD4+ CXCR5+ PD1+ Tfh cells at different times after immunization of recipients of eYFP-NP-specific B cells with NP-SRBCs i.v. **, P = 0.0073. (B) Ii21r mRNA expression at different times after immunization in B220high eYFP- naive (Nai. B) or eYFP-activated B cells (Act. B cells) or B220high Fashigh eYFP− GC B cells. *, P = 0.028. (C) Ii21r mRNA 5 d after NP-CGG immunization of carrier-primed recipients of eYFP-NP-specific B cells in B220high eYFP- naive B cells, B220high Fashigh eYFP− GC B cells, CD4+ PD1− non-Tfh cells, or CD4+ PD1+ Tfh cells. ***, P = 0.0079. Two-tailed Mann-Whitney test. (D) IL21R expressed on CD86high CXCR4low light zone (LZ) and CD86low CXCR4high dark zone (DZ) B cells measured by FACS at day 5 after SRBC. Each diamond represents one animal. (E) GC size and IRF4+ plasmablasts in the GTI per GC area assessed from spleen sections of IL21/IL21Rko and wild type (C57BL6) mice 7 d after i.p. injection with NP-KLH in alum. **, P = 0.004. Data merged from two independent experiments. (F) Effect of 24 h MR1 treatment 4 d after immunization, showing caspase 3+ apoptotic GC cells, GC size, GTI plasmablasts per GC area, and total GTI plasmablasts per spleen section, quantified from immunohistochemically stained spleen sections. Total number of CD138+ PCs per spleen measured by flow cytometry. Tissues taken 5 d after immunization with SRBCs i.v. and 24 h after i.v. injection of anti-CD40L or control antibody. **, P = 0.006; ***, P = 0.0002; ns, not significant. (G) GC size, GTI plasmablasts per GC area, and Caspase 3+ apoptotic GC cells quantified from spleen sections. Tissues taken 5 d after SRBC immunization and 24 h after i.v. injection of IL21R-Fc or control protein. **, P = 0.006. (H) GC size and IRF4hi GTI plasmablasts per GC area assessed 5 d after SRBC and 24 h after injection of IL-21 i.v. **, P = 0.0023. Each symbol represents one animal. Data merged from two independent experiments (n = 10). Two-tailed unpaired Student’s t test.
lymph nodes 8 d after primary immunization with NP-CGG in alum and Bordetella pertussis. CD45+, EpCAM+, CD31, and PDPN+ stroma was separated first from CD31+ lymphatic endothelial cells (LECs) and blood endothelial cells (BECS; Link et al., 2007) and then into MadCAM+ CD157− medullary FRCs (MeFRCs) and MadCAM+ CD157− T zone reticular cells (TRCs). This TRC fraction should contain the CD157high GTI-associated stroma (Fig. 7 A). Furthermore, lymphocytes, CD11c+ dendritic cells (DCs), and CD11b+ macrophages were sorted. As expected (Luther et al., 2000), TRC expressed the highest amounts of Ccl19 and Ccl21 mRNA (Fig. 7 A). They also expressed high amounts of Cxcl12 (Fig. 7 A), which may attract CXCR4+ plasmablasts emerging from theGC to make contact. TRCs contained at least 9× more Baff mRNA than most other stromal populations (Fig. 7 B). Their April mRNA levels were only matched by MeFRC and macrophages (Mohr et al., 2009). This shows that stroma in the GTI may well be able to specifically support plasmablast differentiation through TACI signals. Further, TRCs produced high levels of Il6 mRNA, which is a cytokine that can collaborate with IL-21, inducing PC differentiation (Dienz et al., 2009).

To enrich reticular cells in the GTI (GTIRCs), separating them from inner T zone TRCs, TRCs were subdivided according toCD157 expression into CD157int TRCs and CD157high cells (Fig. S3 A). This confirmed the high expression of Ccl19, Ccl21, and Cxcl12 mRNA in GTIRCs, distinguishing them from MeFRC (Fig. 7 C). Although there was no difference in Baff mRNA expression between CD157int TRC and GTIRC (Fig. 7 D), April and Il6 were more abundantly expressed in the GTIRC-enriched population, comparable to expression levels found in medullary stroma (Fig. 7 D), known to support PC development (Mohr et al., 2009). Flow cytometry also showed that the GTIRC fraction expressed higher levels of ICAM-1, VCAM-1, and CD44 (Fig. 7 E). Experiments designed to test their function in vitro failed, because, different from CD157int FRC, the CD157high fraction did not survive in isolation (Fig. 7 F and Fig. S3, B and C). These data indicate that stroma in the GTI is different from central T zone TRCs and has the potential to interact with GC-derived plasmablasts via adhesion molecules, chemokines, and cytokines.

APRIL produced by stroma in the GTI supports plasmablast differentiation.

The data indicate that APRIL produced in the GTI supports plasmablast output. Immunohistology of spleen sections taken 5 d after SRBC immunization confirm the pattern of TACI expression seen by microdissection: TACI is expressed at low levels in follicular B cells, absent on GC B cells, and is strongly expressed on plasmablasts emerging in the GTI, as well as on extrafollicular PCs in the bridging channels and the red pulp (Fig. 8 A). To test whether TACI signaling regulates emergence of plasmablasts from GCs, mice were injected with TACI-Fc fusion protein 4 d after primary immunization with SRBCs. TACI-Fc is a soluble decoy for BAFF and APRIL, and hence may abrogate ligand-mediated signaling through all BAFF and/or APRIL receptors (Bosseen et al., 2008). Although within 24 h this did not lead to significant effects on GC size, there was a 30% reduction of GTI plasmablast numbers (Fig. 6 B). Parallel experiments in mice undergoing carrier-primed responses to NP-CGG that were treated with TACI-Ig for 24 or 48 h before analysis on day 5 showed that GTI plasmablast numbers were reduced by 42 and 62%, respectively (Fig. 6 C), whereas there was no reduction in GC size (not depicted). TACI signals are essential for PC survival, which should lead to loss of PCs in red pulp and bridging channels. This, however, was only obvious after 48 h TACI-Ig blockade (Fig. 8 C). To test whether APRIL is the TACI ligand supporting emergence of PCs in the GTI, the experiment was repeated using anti-APRIL blocking antibody, which again led to a significant reduction of plasmablasts in the GTI within 24 h (Fig. 8 D), supporting the conclusion that GTIRC-produced APRIL supports plasmablast differentiation in the GTI.

Discussion

We show here that plasmablasts emerge locally from the earliest stages of the GC response into a specialized microenvironment, which is the border between GC dark zone and T zone, or GTI. From here they may migrate to other local survival niches such as the splenic extrafollicular foci or the lymph node medulla or to the bone marrow. We show that the process of plasmablast differentiation starts very early during GC development and that IL-21 and TACI ligands regulate this process. While IL-21 is well known to be produced by Tfh cells, APRIL is produced by a new CD157high PDPN+ stromal population located in the GTI. These GTIRCs are in close contact with plasmablasts emerging at this site. GTI PCs also produce mRNA coding for Cxcl12, Baff, and IL-6, which may attract and then support differentiating plasmablasts.

During their several weeks of existence, GCs mature. Not only does the affinity of GC B cells and antibody increase over time, GCs also seem to vary their main types of output cells: a recent study shows preferential output of affinity-matured long-lived PCs that persist in bone marrow at late stages of the GC response, whereas younger GCs preferentially produce memory B cells (Weisel et al., 2016). We have not tested whether PCs seen in the GTI during the earliest stages of the GC reaction home to bone marrow or not. The findings by Weisel et al. (2016) suggest plasmablasts in the GTI may differentiate only locally and do not migrate to bone marrow, or it is also possible that GC-derived PCs first replace PCs in local niches and only at later stages start to appear in the bone marrow. Recent studies have shown that PC differentiation in the GC is induced in higher-affinity B cells (Kräutler et al., 2017), whereas memory B cell output is delayed and of lower quality (Goenka et al., 2014b; Suan et al., 2017). The higher-affinity antibody produced at an early stage of the GC response may not only provide rapid defense against pathogens, but also function by regulating the GC itself. Antibodies reentering GCs can restrict accessibility of the antigen on follicular dendritic cells and raise the GC B cell selection threshold through antibody feedback (Zhang et al., 2013). It is not known whether this antibody reenters GCs by active transport of diffusion. Antibody-producing cells located in the GTI are placed ideally to provide such antibodies locally.

A strong drive toward plasmablast differentiation at the earliest stages of the GC response may reflect the easy access to antigen held on follicular dendritic cells, which at this stage is still complexed by low-affinity antibody derived from the
Figure 6. IRF4$^{hi}$ PCs in close contact with PDNP$^{+}$ CD157$^{hi}$ reticular cells in the GTI of spleen and lymph node. (A) Triple immunofluorescence of spleen sections 5 d after SRBC immunization stained for IgD on follicular B cells, IRF4 in plasmablasts, and PDNP on T zone stroma. (B) Adjacent section stained for IgD, PDNP, and CD157 on follicular stroma and GTI. (C) Semiquantitative estimate of CD157 staining intensity along a line from the CD157$^{hi}$ follicle to the center of the T zone. Ratio: CD157 staining intensity divided by PDNP staining intensity. (D) CD11c and PDNP staining to illustrate different stroma associated with IRF4$^{hi}$ PCs in the GTI and red pulp bridging channels. (E) F4/80 and IRF4$^{hi}$ PCs in red pulp. Bar, 50 µm. (F) Quantification of IRF4$^{hi}$ cells in close contact with PDNP$^{+}$ CD157$^{hi}$ reticular cells, CD11c$^{+}$ cells, and F4/80$^{+}$ cells in different areas. Each symbol represents one spleen section. Two-tailed unpaired Student’s t test. **, P = 0.0034. (G) Large numbers of IRF4$^{hi}$ cells present in the GTI of a popliteal lymph node 8 d after subcutaneous foot immunization with NP-CGG. Bar, 200 µm. Close-up showing IRF4$^{hi}$ cells in contact with PDNP$^{+}$ reticular cells in the GTI (arrowheads). (H) Adjacent section stained for PDNP and CD157 shows reticular cells in the GTI coexpressing PDNP and high levels of CD157 (arrowheads). Bar, 50 µm; also applies to magnified panel in G. BC, bridging channel; F, Follicle; RP, red pulp; T, T zone.
Figure 7. **GTIRC produce cytokines and chemokines supporting GC-derived plasmablasts.** (A) Expression levels of Ccl19, Ccl21, and Cxcl12 mRNA in total lymph node cells (LN). CD45<sup>-</sup> Ter119<sup>-</sup> EpCAM<sup>-</sup> stromal cells subdivided into PDPN<sup>+</sup> CD31<sup>-</sup> CD157<sup>+</sup> TRCs, PDPN<sup>+</sup> CD31<sup>-</sup> CD157<sup>-</sup> MeFRC, PDPN<sup>+</sup> CD31<sup>+</sup> LECs, PDPN<sup>-</sup> CD31<sup>+</sup> BECs, or other stroma not staining for these marker combinations. CD19<sup>+</sup> CD5<sup>+</sup> lymphocytes (Lymphoc.), CD11c<sup>+</sup> MHCII<sup>+</sup> CD86<sup>+</sup> DCs, and CD11b<sup>+</sup> CD11C<sup>-</sup> macrophages (Mφ) were also sorted. (B) Gene expression of Baff, April, and Il6. Each diamond represents pooled poplitelal lymph nodes from for mice. (C) Chemokine mRNA and (D) cytokine mRNA expression after subdivision of T zone PDPN<sup>+</sup> CD31<sup>-</sup> CD157<sup>+</sup> TRC into CD157<sup>high</sup> GTI RC and CD157<sup>int</sup> TRC. *, P = 0.01; ***, P = 0.0004. Gating, see Fig. S3. Each diamond represents pooled cells from 12 lymph nodes. All values are relative to the b2m mRNA. Data are representative of two independent experiments. Two-tailed unpaired Student’s t test. (E) Expression of VCAM1, ICAM1, and CD44 on the same stromal cell groups as in C and D. (F) Live cells numbers after 48 h culture of isolated stroma cell populations in vitro. ***, P = 0.0046; ****, P < 0.0001. Each diamond represents one field; data merged from three independent culture wells. Kruskall-Wallis test comparing nonparametric multiple groups.
early extrafollicular PC response (Sze et al., 2000). Indeed, earlier experiments show that PC output from GCs is reduced once GC B cells incur higher-stringency antibody feedback (Zhang et al., 2013).

GC B cell selection depends on antigen access and antigen presentation–dependent Tfh cell signals. Although PC output from the GC is skewed toward higher affinity (Chan and Brink, 2012), B cell receptor signaling seems to have a minor role on GC B cell selection (Khalil et al., 2012). T cell interactions are a major regulator of GC B cell differentiation (Victora and Nussenzweig, 2012), and these interactions are limited by the efficiency with which B cells are able to take up antigen and present this to T cells. Tfh cell signals induce GC B cell recirculation into the dark zone for further proliferation and immunoglobulin gene hyper-mutation (Victora et al., 2010; Gitlin et al., 2015; Liu et al., 2015). Tfh cells produce IL-21 and IL-4 when interacting with B cells (Shulman et al., 2014).

Several functions are attributed to IL-21. IL-21 shapes Tfh cell differentiation, influencing their capacity to produce IL-4 and IL-21 (McGuire et al., 2015). IL-21 may regulate extrafollicular PC generation during initial B cell activation, but also GC development (Linterman et al., 2010; Zotos et al., 2010). In naive B cells IL-21 can induce GC differentiation and inhibit TACI expression (Goenka et al., 2014a), but it can also cooperate with IL-4 to induce Blimp1, leading to PC differentiation (Ozaki et al., 2004). Therefore, the main function of IL-21 seems to be more a general trigger of B cell differentiation, rather than being instructive for differentiation toward a specific direction (Ozaki et al., 2004). Other factors, e.g., IL-4, may be able to provide additional instructive co-stimuli. The rapid effect of IL-21 on plasmablast differentiation shown here makes it likely that at least part of the action of IL-21 is on B cells directly. Therefore, the reduced affinity maturation seen in IL-21–deficient animals (Zotos et al., 2010) may at least partly be a result of a direct reduction of PC output from the GC. Other models are possible: increased antigen presentation by GC B cells to Tfh cells not only increases recirculation to the GC dark zone, but also PC generation (Victora et al., 2012). Once arrived in the dark zone, asymmetric cell division may drive some cells into PC output (Meyer-Hermann et al., 2012; Thaunat et al., 2012), or contact with stroma at the edge of the GC may signal PC differentiation.

PC differentiation and survival happens in niches of secondary lymphoid tissues (Sze et al., 2000) and bone marrow (Manz et al., 2002) that provide homing signals and survival factors (Chu et al., 2011; Chu and Berek, 2013). Higher-affinity PCs continuously replace the initially formed low-affinity PCs in these compartments (Smith et al., 1997). The composition of these compartments is probably homeostatic in nature, i.e., simply dependent on influx, because of the emergence of new cells, and...
death, because of limited niche capacity, rather than PC affinity or developmental origin (Sze et al., 2000; Manz et al., 2002; Mohr et al., 2009). Early output of plasmablasts from GC described here may contribute to the replacement of PCs in local niches from a very early stage (Sze et al., 2000).

Stroma in the GTI represents a new niche supporting differentiation of GC-derived PCs. CD152<sup>high</sup> PDPh<sup>+</sup> GTI stroma is rich in CXCL12 and CCL19/21. GTIRC may be related to CXCL12-expressing reticular cells (CRCs) located in the dark zone that have been shown to also express CCL19 (Bannard et al., 2013; Rodda et al., 2015). However, CRCS characteristically form long protrusions into the GC dark zone (Rodda et al., 2015), whereas GTIRCs are typically located outside the GC and in the spleen form strands parallel to the border between the GC and T zone. Cells committed to plasmablast differentiation may be attracted by chemokines produced by GTIRC, and plasmablasts in the GTI express high levels of CXCR4 and also CCRe. Plasmablasts in the GTI express TACI, which is not expressed inside the GC (Goenka et al., 2014a). Although BAFF has been shown to regulate affinity-dependent selection in the GC (Goenka et al., 2014a), stroma in the GTI is the initial microenvironment that provides APRIL. TACI ligation by APRIL inhibits B cell proliferation and can induce PC differentiation (Mackay and Schneider, 2008), whereas BAFF present in the GC seems to have the opposite effect (Goenka et al., 2014a). Additionally, GTIRC produce Il6 mRNA, which collaborates with IL-21 inducing B cells to differentiate into PC (Dienz et al., 2009). APRIL and IL-6 are also produced by downstream stromal niches where PCs differentiate after emerging through the GTI, e.g., macrophages in the lymph node medulla (Mohr et al., 2009).

A simple model for the regulation of PC output summarizing the data presented here would be that B cells successfully selected by Thh cells (dependent on how well they were able to access and present antigens) and being exposed to Thh-derived IL-21 plus possible cofactors such as IL-4 (Shulman et al., 2014) will get stimulated to recirculate to the dark zone. There they will proliferate, and some will differentiate into PCs. Whether this is directional differentiation caused by specific instructive signals, asymmetric cell division (Meyer-Hermann et al., 2012; Thaunat et al., 2012), or simply a result of cells getting into contact with chemokine-expressing GTIRCs producing APRIL and IL-6 remains to be seen. In any case, the local environment in the GTI will support these cells to undergo further replication and differentiation, before moving toward longer term survival niches and differentiating into mature nonproliferating antibody-secreting PCs.

Materials and methods

Mice and immunizations

6- to 12-wk-old sex-matched C57BL/6 mice were obtained from Harlan Laboratories and kept in specified pathogen-free conditions. For intravital microscopy, lightm2Cgn (B1-8i<sup>+/+</sup>) mice (gift from C.-A. Reynaud, Institute Necker, Paris, France; Dogan et al., 2009), were crossed with ROSA<sup>mT/mG</sup> mice (007576; Jackson Laboratory), which contain a Cre-inducible membrane-tagged version of eGFP (Muzumdar et al., 2007). R26R–Confetti/CreERT2 (Snippert et al., 2010) spleen sections were from Thomas Winkler (Friedrich-Alexander-University Erlangen-Nuremberg, Erlangen, Germany).

Animal studies were performed with approval of the Birmingham Ethical Review Subcommittee and under a UK Home Office project license. Intravital imaging experiments were conducted according to German animal protection laws and approved by the appropriate governmental authority (Landesamt für Gesundheit und Soziales) in Berlin.

For primary immunizations, mice were injected i.p. with 50 µg alum-precipitated NP-coupled to CGG at a molar ratio of 18:1 plus 10<sup>7</sup> chemically killed B. pertussis (Lee Labs; Becton Dickinson) (Sze et al., 2000) or 100 µg alum-precipitated NP-KLH at a ratio of 17:1 i.p. (Zotos et al., 2010), i.v. with 2 × 10<sup>8</sup> SRBCs (TCS Biosciences), or freshly prepared NP-haptenated SRBCs (NP-SRBCs) in PBS. Carrier-primed responses were induced by injecting 50 µg soluble NP<sub>18</sub>-CGG i.p. into mice that were primed with CGG 4 wk earlier (Sze et al., 2000). Recombination of the mT/mG allele in Aicda<sup>CreERT2</sup> mice was induced by a single gavage of 6 mg tamoxifen (Sigma) dissolved in corn oil at 20 mg/ml 4 d after SRBC immunization, and tissues were analyzed 24 or 48 h later. The R26R–Confetti/CreERT2 mice were induced three times by gavage of 4 mg tamoxifen and on the fourth day immunized with SRBCs i.v. GCs were analyzed 5 d after immunization.

Immunohistology

Spleen sections were prepared and double-stained as described previously (Marshall et al., 2011). The following additional antibodies were used: goat anti–mouse IRF4 (M-17; Santa Cruz Bio-tech) and rabbit anti–mouse active caspase 3 (C92-605; BD Biosciences), or freshly prepared NP-haptenated SRBCs (NP-SRBCs) in PBS. Carrier-primed responses were induced by injecting 50 µg soluble NP<sub>18</sub>-CGG i.p. into mice that were primed with CGG 4 wk earlier (Sze et al., 2000). Recombination of the mT/mG allele in Aicda<sup>CreERT2</sup> mice was induced by a single gavage of 6 mg tamoxifen (Sigma) dissolved in corn oil at 20 mg/ml 4 d after SRBC immunization, and tissues were analyzed 24 or 48 h later. The R26R–Confetti/CreERT2 mice were induced three times by gavage of 4 mg tamoxifen and on the fourth day immunized with SRBCs i.v. GCs were analyzed 5 d after immunization.

For fluorescence staining, IgD-FITC, B220–FITC (RA3-6B2), CD21–FITC (7G6), BCL6–Alexa488 (K12-91), CD138 (281-2), and CXCR4 (2B11) were from BD Biosciences, followed by biotinylated goat anti–sheep or swine anti–rabbit antiserum (Dako) and StreptABComplex/AP as described (Sze et al., 2000). Aicda CreERT2 mice (gift from C.-A. Reynaud, Institute Necker, Paris, France; Dogan et al., 2009), were crossed with ROSA<sup>mT/mG</sup> mice (007576; Jackson Laboratory), which contain a Cre-inducible membrane-tagged version of eGFP (Muzumdar et al., 2007). R26R–Confetti/CreERT2 (Snippert et al., 2010) spleen sections were from Thomas Winkler (Friedrich-Alexander-University Erlangen-Nuremberg, Erlangen, Germany).

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IgD-Alexa647 (11-26; eBioscience) were used. Secondary antibodies were FITC-conjugated donkey anti–rat or donkey anti–rabbit, Cy3 or Cy5 conjugated donkey anti–sheep, and Cy3-conjugated goat anti–hamster (Jackson Immunoresearch). The slides were mounted in antifade mounting medium (Prolong Gold; Invitrogen). Images were taken on fluorescence microscope (DM6000; Leica). Image data were analyzed using Fiji (Schindelin et al., 2012) or point counting using a microscope with an eyepiece containing a counting graticule (Weibel, 1963). Plasmablasts at the GTI were quantified on IRF4/IgD double-stained tissue sections by counting all IRF4high cells in a 40-µm wide strip along the GTI (Fig. S4). Cell numbers were divided by the area of IgD+ GCs on the same tissue section.

Confetti spleen sections were stained with goat anti–mouse IRF4, followed donkey anti–goat Alexa Fluor 594 (Invitrogen). Some sections were counterstained with IgD APC (BD Bioscience). A confocal microscope (LSM880; Zeiss) was used to separate six colors with excitation/detection wavelengths: mCerulean (CFP) 405/420 ± 5 nm, hrGFPII (GFP) 488/503 ± 13 nm, mYFP (YFP) 514/530 ± 13 nm, tdimer2(12) (dsRFP) 561/583 ± 13 nm, Alexa Fluor 594 594/630 ± 10, and APC 633/692 ± 43 nm.

Laser capture microdissection for semiquantitative RT-PCR (qRT-PCR)

qRT-PCR gene expression analysis from laser capture microdissected tissue was done from snap-frozen acetone-fixed spleen sections, taken 5 d after i.p. NP-CGG immunization of carrier-primed C57BL/6 mice. Between 10 and 20 8-µm thick serial sections were collected on photoactivated localization microscopy membrane slides (NF; Zeiss) hydrated in 100, 70, and 50% ethanol and stained for 3 min with 1% wt/vol cresyl violet (Sigma-Aldrich). Slides were then dehydrated by quick washes in 50, 70, and 100% ethanol and air-dried. Laser capture microdissection was performed using a Microbeam HT microscope (Zeiss). To identify GCs and GTI unequivocally, four sections taken from the series were stained immunoenzymatically for IgD and IRF4 (Zhang et al., 2016). A photograph of each section was printed and used as a reference to identify GC (IgD+ area within IgD+ follicles), follicles (IgD+), T zone (IgD+ areas surrounded by follicles with central arteriole), the GTI (between GC and T zone containing IRF4+ cells), and plasmablast/PC-rich extrafollicular foci (IRF4+ between T zone and red pulp; Fig. S2). Membrane-only areas were selected as a negative control. Serial microdissected areas were catapulted into RNeasy buffer (Qiagen) in the nuclease-free microtiter plate lids. RNA was isolated immediately using RNeasy Micro kit (Qiagen). cDNA was stored at −20°C, and qRT-PCR gene expression analysis was done as described (Zhang et al., 2013). Sequence of primers and probes are listed in Table S1.

Two-photon laser-scanning microscopy

B cells from B10.A/2 × /Pdrm1 (GTP)/Cdtm x2 mice were isolated using EasySep B cell untouched isolation kit (StemCell Technologies). 3 × 106 B cells were injected i.v. into C57BL/6J recipients 1 d before immunization with 10 µg NP-CGG emulsified in complete Freund’s adjuvant into the right foot. To label follicular dendritic cells, 10 µg Alexa Fluor 633–labeled CD21/35 Fab fragments were injected into the same foot 12–24 h before imaging. To identify B cell follicles, naive B cells from C57BL6 spleens labeled with 2.5 µM Hoechst 33342 (Invitrogen) were injected i.v.

Mice were anesthetized by i.p. injection of 0.1 mg ketamine and 0.01 mg xylazine (Rompun; Bayer Healthcare) per gram body weight. If necessary, anesthesia was topped up by further i.m. injection of anesthetic. Surgical preparation of the mouse popliteal lymph node was performed as already published (Mempel et al., 2004). In vivo imaging was performed with a two-photon laser-scanning system (LaVision BioTec) equipped with an optical parametric oscillator (APE). The system was pumped with a femtosecond-pulsed titanium-sapphire laser, and excitation wavelength was 930 nm. An objective lens for deep tissue imaging (20× dipping lens, NA 0.95, WD 2 mm; Olympus) was used. XYZ-stacks were collected within a scan field of 500 × 500 µm at 512 × 512-pixel resolution and a Z-plane distance of 3 µm. The fluorescence signal was detected with photomultiplier tubes with the following interference filters: 460 ± 30 nm, 525 ± 25 nm, 593 ± 20 nm, and 655 ± 20 nm.

Cell sorting for qRT-PCR

For sorting of activated B and T cells, 106 QM eYFP cells were adaptively transferred into C57BL6 hosts 1 d before immunization with NP-SRBCs i.v. Splenocytes were stained using Hoechst 33258 (Sigma-Aldrich), B220 APCCy7 (RA3-6B2, Biologend), anti–Fas–PECy7 (Jo2), CD4-APC (RM4-5), CD62L-PE (MEL-14; BD Biosciences), PD-1-PE (J43, eBioscience), CXCR5-biotin, and Streptavidin-PerCP-Cy5.5 (BD Biosciences). Cell populations were sorted in a high-speed cell sorter (MoFlo; Beckman-Coulter). Until 4 d after immunization, activated B cells were sorted as B220hi eYFP+, activated T cells as CD4+ CD62Llow, and nonactivated T cells as CD4+ CD62L+.

Lymph node stromal cell populations were done with variations as described (Link et al., 2007). In brief, popliteal lymph nodes dissected into small pieces and digested by shaking for 45 min at 35°C in 1 ml RPMI 1640 medium containing 10% FCS, 1% penicillin-streptomycin, 0.1 mg DNase (Sigma-Aldrich), and 2.5 mg Collagenase D (Roche). Not fully digested tissue was incubated for another 20 min with 1 ml of fresh digestion buffer. Enzymatic digestion was completed by adding 15 µl 0.5 M EDTA left on ice for 5–10 min. Cells were filtered and washed with PBS (0.5% FCS and 2 mM EDTA). To enrich the stromal cell fraction, hematopoietic cells were depleted by incubating the cell suspension with MACS anti–CD45 microbeads and passing over a MACS LS column (Miltenyl). The enriched cells were incubated for 20 min at 4°C in PBS containing 0.5% BSA and 2 mM EDTA with the following fluorescently labeled antibodies: CD45-PerCP-Cy5.5 (C363-16A), Ter199-PerCP-Cy5.5 (Ter199), EpCAM-PerCP-Cy5.5 (G8.8), CD157-APC (BP-3), VCAM-1-PECy7 (429), CD44 Alexa Fluor 700 (IM7), Str BV605 (BioLegend), PDPN-PE (eBio8.11), CD31-FITC (390), MadCAM-1-biotin (MECA-367), ICAM-1 pacific blue (YNI/1.74; eBioscience), and Str-PECy7 (BD Biosciences).

CD4+ Ter199 EpCAM+ were gated as stroma. From this population, further subpopulations were sorted as follows: TRCs as CD31+ PDPN+ CD157+ and MadCAM+ (Mempel et al., 2004), GTIRCs as MadCAM+ CD31+ PDPN+ CD157hi, MeFRC as PDPN+
CD31+ CD157+, LECs as PDPN+ CD31+, BECs as PDPN+ CD31+, and other stroma as cells not staining for these marker combinations. Further populations sorted were: other lymphocytes as CD19+ CD5+, DCs as CD11c+ MHCII+ CD86+ and macrophages as CD11b+ CD11c+.

Stromal cells were sorted by using low-pressure in a MoFlo Astrios (Beckman Coulter), cDNA preparation was as described before (Link et al., 2007). Real-time PCR from cDNA (qRT-PCR) was done in multiplex with β2-microglobulin (β2m) and gene expression related to β2m expression levels. Primers and probes are listed in Table S1.

In vivo treatment with antibodies, fusion proteins, or cytokines

250 μg of hamster anti-mouse CD40L blocking antibody (MR1; gift from N. Jones, University of Birmingham, Birmingham, England, UK), control antibody hamster IgG (Jackson ImmunoResearch), soluble IL-21R fusion protein, or mock protein (mouse IgG2a, gift from L. Walker, University College London, London, England, UK) were injected in 150 ml PBS (i.v.); 1 µg of IL-21 cytokine (Peprotech) was injected i.v.

TACI-Fc is a fusion protein comprising the extracellular portion of TACI and the FC portion of mouse IgG2c, which had been mutated to eliminate complement fixating capacity and binding to FC receptors. Mouse Fc from IgG2c (mFc) was used as control protein. 100 μg of TACI fusion protein (TACI-Fc) or mFc as control or 90 µg of anti-APRIL (Apyr-1-1; AdipoGen), or isotype control mouse IgG2b (Jackson ImmunoResearch) were injected i.v.

Antibody, cytokines or soluble receptors were injected 4 d after SRBC immunization. Tissues were harvested 24 h later. TACI-Fc and control Fc were injected 48 h or 24 h before endpoint at 5 d after NP-CGG immunization of carrier-primeed mice.

Statistical analysis

All statistical analysis was performed on Prism 6 using nonpaired two-tailed Student’s t test from log transformed data or two-sided Wilcoxon Mann-Whitney U Test where indicated. Statistics were done by including data from all independent replicates. P values are indicated throughout with * for P < 0.05, ** for P < 0.01, *** for P < 0.001, and **** for P < 0.0001.

Online supplemental material

Fig. S1 shows the frequency and principal component analysis of florescent protein cells in GCs and adjacent GTIs. Fig.S2 shows the validation of microdissection and qRT-PCR method for different areas of immunized spleen. Fig. S3 shows the isolation of fibroblastic reticular cell subpopulations. Fig. S4 shows the manual stereological analysis of tissue sections and its validation by digital image analysis.

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The authors declare no competing financial interests.

Author contributions: Y. Zhang designed the study, performed experiments, analyzed data, and wrote the manuscript. L. Tech performed experiments on in vivo migration of plasmablasts and analyzed data. L.A. George performed experiments and analyzed data. A. Acs performed the Confetti immunization. R.E. Durrett advised on bioinformatics analysis. H. Hess provided reagents and suggested experiments. L.S.K. Walker provided reagents and transgenic mice. D.M. Tarlinton provided experimental tissues from transgenic mice. A.L. Fletcher advised on stroma isolation experiment and did in vitro stromal cell cultures. A.E. Hauser designed intravitral migration experiments. K.-M. Toellner designed the study, analyzed data, and wrote the manuscript.

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Supplemental material

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Figure S1. Frequency of CFP-, GFP-, YFP-, and RFP-positive cells in 23 GCs and adjacent GTIs. PC1 is the principle component 1 of a principle component analysis of the four color frequencies using Clustvis (http://bit.cs.ut.ee/clustvis/, accessed December 11, 2017). #GC and #GTI PC are the number of IRF4+ XFP+ cells detected in each zone that went into the analysis.
Figure S2. **Validation of microdissection and qRT-PCR method for different areas of immunized spleen.** (A) Photomicrographs of two proximate spleen sections 5 d after NP-CGG immunization of carrier-primed mice. Left, immunohistochemical staining for IgD and IRF4 identifies B cell follicle (F), GC, GTI, PC areas in red pulp (PC), and T zone (T). Right, cresyl violet staining revealing intense staining of the B cell follicle and GTI and weaker staining of the GC, T zone, and red pulp. Red lines indicate examples of areas microdissected for qRT-PCR analysis. (B) Relative expression levels of mRNA coding for Cxcl13, Cxcr5, Pax5, Cd3 and Cd19, Igj, Xbp1 mRNA and Iμ-Cγ1 hybrid transcript, Bcl6, Aicda, and IgG1 germline transcript (IgG1 GT) from follicle, GC, GTI, PCs in red pulp, and T zone. Each spot represents signal from one specific area taken from several consecutive sections. All values are relative to expression of β2m mRNA. Data and statistics are representative of three different spleens. Nonpaired two-tailed Student’s t test; *, P = 0.03; **, P = 0.0052; ***, P < 0.0002; ****, P < 0.00001. Two sided Mann-Whitney test for Bcl6, IgG1 GT; *, P = 0.04; **, P = 0.002.
Figure S3. **Isolation of fibroblastic reticular cell subpopulations.** (A) Sorting protocol. Lymph nodes were harvested 8 d after s.c. NP-CGG in alum foot immunization. CD45<sup>-</sup> Ter119<sup>-</sup> EpCAM<sup>-</sup> MadCAM<sup>-</sup> stromal cells were subdivided into PDNP<sup>+</sup> CD31<sup>-</sup> TRCs, PDNP<sup>-</sup> CD31<sup>-</sup> LECs and PDNP<sup>-</sup> CD31<sup>-</sup> as BECs. Right, subdivision of TRCs into CD157<sup>high</sup> GTI RC, CD157<sup>int</sup> TRC, and CD157<sup>–ve</sup> MeFRC. (B) Representative microphotographs from CD157<sup>–ve</sup> MeFRCs, CD157<sup>int</sup> TRCs, and CD157<sup>high</sup> GTI RCs after 96 h culture. Reticular cells were sorted into three populations according to the protocol, 10,000 freshly sorted cells plated into αMEM with 10% FBS and 1% pen/strep, and incubated at 37°C/10% CO₂. (C) Cells were counted using a light microscope (20×) after 48 h, 1 field was counted. Cells were scored as attached and elongated (alive) or round, often with membrane blebbing evident (Fletcher et al., 2011). Each diamond represents one field. Data merged from three independent culture wells. Kruskall-Wallis test comparing nonparametric multiple groups. **, P = 0.0046; ****, P < 0.0001.
Figure S4. **Manual stereological analysis of tissue sections and its validation by digital image analysis.** (A) Manual quantification of IRF4\(^{hi}\) at the GC-T zone interface. A 100-field 1 cm\(^2\) ocular counting grid was moved over the center of the GTI projected using a 25× microscope lens. Using a 25× lens the width of one field is 40 µm. Cells were counted if they fell into this 40-µm wide band. GC area was quantified using point counting and the same counting grid (one intercept corresponds to 1,600 µm\(^2\)). Tissues were blinded and randomized before quantification. Some tissues were analyzed by an independent examiner with good reproducibility. (B) To test the reliability of the manual counting method, a semiautomated analysis method was designed to measure blue staining area within the GTI from whole spleen sections. In brief, spleen sections immunoenzymatically stained for IRF4 and IgD were scanned using a 10× lens of an AxioScan microscope scanner (Zeiss). Using Fiji (Link et al., 2007), brown and blue colors were separated by color deconvolution. Follicular areas were segmented from the brown channel smoothed using a median filter. The GTI was segmented by manually drawing a line along the GC–T zone border. The area of the adjacent GC was measured as the IgD negative compartment enclosed by IgD staining and the line defining the GTI. The area covered by IRF4 staining was measured by widening the line defining the GTI to 40 µm, and measuring segmented blue staining area within this band in the blue channel. This was done for all GCs on a section, and data were exported into Excel and Prism for statistical analysis.
Table S1. Sequence of primers and probes used for qRT-PCR

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All primers and probes are from Eurofins Genomics (Ebersberg Germany). CD3e (Mm00599683_m1), CD19 (Mm00515420_m1), and IL21R (Mm0060319_m1) are TaqMan gene expression assay (Thermo Fisher).

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