The establishment of resident memory B cells in the lung requires local antigen encounter

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Memory B cells are found in lymphoid and non-lymphoid tissues, suggesting that some may be tissue-resident cells. Here we show that pulmonary influenza infection elicited lung-resident memory B cells (BRM cells) that were phenotypically and functionally distinct from their systemic counterparts. BRM cells were established in the lung early after infection, in part because their placement required local antigen encounter. Lung BRM cells, but not systemic memory B cells, contributed to early plasmablast responses following challenge infection. Following secondary infection, antigen-specific BRM cells differentiated in situ, whereas antigen-non-specific BRM cells were maintained as memory cells. These data demonstrate that BRM cells are an important component of immunity to respiratory viruses such as influenza virus and suggest that vaccines designed to elicit BRM cells must deliver antigen to the lungs.

Durable humoral immunity is maintained by long-lived, antibody-secreting plasma cells that home to the bone marrow¹⁴, as well as resting memory B cells that are poised to respond rapidly following a secondary encounter with antigen¹⁵. The generation of both cell types requires help from T cells, typically in the germinal center (GC), where B cells rapidly proliferate and are selected for high-affinity antigen receptors²⁶⁻²⁷. As GC B cells differentiate into memory cells, they stop proliferating²⁸ and, in some cases, home to mucosal surfaces²⁹⁻³⁰. For example, pulmonary infection with influenza virus elicits memory B cells in the lung that express CXCR3⁹, whereas memory B cells responding to intestinal infection with rotavirus express mucosal homing receptors, such as α4β7 integrin and CCR9⁹.

Early studies of memory B cells in the lung used limiting dilution and in vitro differentiation to indirectly enumerate memory B cells by enzyme-linked immunospot (ELISPOT)³⁰ but did not directly characterize the memory B cells themselves. More recent studies use fluorescently labeled hemagglutinin (HA) to identify influenza-specific B cells in the lung and show that these cells have hallmarks of resident memory cells, such as the expression of CD69. Importantly, following adoptive transfer, memory B cells from the lung respond more quickly to challenge infection than do those from lymphoid organs⁹, suggesting that memory B cells in different locations may be functionally distinct.

Although these studies imply that resident memory B (BRM) cells are generated following influenza infection, they do not test whether potential BRM cells in the lung recirculate or whether they alter the properties of a secondary B cell response following infection. In fact, it is not clear whether BRM cells in the lung would benefit pulmonary immunity. For example, lung-resident memory T (TRM) cells are a critical element of pulmonary immunity because they must physically contact MHC-expressing target cells in a secondary response in order to exert their effector functions³⁰. In contrast, memory B cells either differentiate into antibody-secreting cells (ASCs) or re-enter the GC and undergo additional rounds of proliferation and selection³¹⁻³³. Both of these functions can be efficiently accomplished in draining lymph nodes. Given that antibodies generated in the lymph node circulate systemically and efficiently protect the lung³⁴, it is not clear whether BRM cells might be useful or if they even exist at all.

Here we used parabiosis to definitively demonstrate the presence of BRM cells in the lung following influenza infection. We found that influenza-specific BRM cells, particularly IgM⁺ BRM cells, were established early after influenza infection and were phenotypically distinct from their lymphoid counterparts. The formation of BRM cells that colonized the lung was dependent on early CD40-dependent interactions with T cells in lymphoid organs. However, the placement of BRM cells in the lung also required encounter with antigen in the lung itself. Importantly, the presence of BRM cells in the lung led to a rapid secondary ASC response in the lung following a challenge infection. This rapid secondary response was maintained when mice were treated with the sphingosine-1-phosphate receptor 1 (S1PR1) agonist FTY-720 to prevent lymphocyte recirculation, suggesting that BRM cells differentiated in situ rather than in draining lymph nodes. Taken together, these data suggest that, along with resident memory T cells, BRM cells in the lung contribute to protection against secondary pulmonary infections.

Results

Identification of influenza-specific B cells. In order to identify influenza-specific B cells, we expressed recombinant nucleoprotein (NP) monomers with a biotinylation domain and a 6x his tag in Escherichia coli and expressed recombinant HA from the A/PR8/34 (PR8–H1N1) and A/X31 (X31–H3N2) viruses with a GNC4 trimerization domain and either a 6x his tag or a biotinylation (Avi) domain in 293 cells (Fig. 1a). Following purification over a nickel column (Fig. 1b), we enzymatically biotinylated the purified recombinant HA protein, and ‘tetramerized’ the recombinant HA and NP proteins with fluorochrome-conjugated streptavidin to generate reagents that could be used in flow cytometry assays.

To determine whether our B cell tetramers could identify antigen-specific cells, we infected C57BL/6 mice with either PR8 or X31 and determined the frequency of tetramer-binding cells in the GC population (complete gating strategy in Supplementary Fig. 1) from

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the mediastinal lymph nodes (mLN). In mice infected with PR8 for 15 days, we found that about 10% of the GC B cells bound the HA(PR8) tetramer and 40% bound the NP tetramer, but very few B cells bound the HA(X31) tetramer (Fig. 1c). Conversely, in mice infected with X31, about 4% of the GC B cells bound the HA(X31) tetramer and about 43% bound the NP tetramer, but very few bound the HA(PR8) tetramer (Fig. 1d). We also observed both NP-specific and HA(PR8)-specific cells in the CD138+ plasmablast population and in the CD38+IgD−PNA− memory B cell population in the mLN of PR8-infected mice (Fig. 1c, f).

To further confirm that we were identifying antigen-specific memory B cells rather than non-specific binding to sulfonic acid (for HA tetratmers), we next gated on isotype-switched memory B cells in the mLN of naive, PR8-infected and Schistosoma mansoni-infected mice (Fig. 1g-i). We found essentially no binding of either NP or HA(PR8) tetramers to isotype-switched memory B cells in either the naïve or S. mansoni-infected mice but observed about 11% NP-specific and 5% HA(PR8)-specific isotype-switched memory B cells in PR8-infected mice (Fig. 1h). Both IgM+ memory B cells and isotype-switched memory B cells could be found in lungs, spleen and mLN of PR8-infected mice, but not in those of naïve mice, on day 30 and day 70 after infection (Supplementary Fig. 2). These results demonstrate the specificity of the B cell tetramer reagents.

BRM cells in the lung and lymphoid tissues are distinct. To characterize non-circulating memory B cells in the mLN, lung and spleen, we infected mice with PR8 and, 56 days later, infused them with fluoroochrome-conjugated anti-B220 5 min prior to euthanasia. We subsequently gated on CD19+B220− (non-circulating) CD38+ isotype-switched memory B cells and finally gated on NP-binding cells (Fig. 2a–d). We found that the majority of NP-specific isotype-switched memory B cells in the mLN and spleen expressed CD73 (Fig. 2e), a marker noted in previous memory B cell studies, whereas most isotype-switched memory B cells in the lung did not express CD73. NP-specific memory B cells in the lung also

Fig. 1 | Identification of influenza-specific B cells. a, Schematic of recombinant NP and HA proteins. b, Coomassie-stained sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE) gel showing recombinant HA(PR8) (lane 2), HA(X31) (lane 3) and NP (lane 4). Image is representative of 12 independent preparations of recombinant protein. c–f, Cells from the mLN of day 15 influenza-infected mice were first gated on live, singlet CD19+ lymphocytes (Supplementary Fig. 1a) and then on PNA+CD95+ GC B cells (c–d), or first gated on live, singlet lymphocytes (Supplementary Fig. 1a) and then on CD138+ plasmablasts (e), or first gated on live, singlet CD19+IgD+ lymphocytes (Supplementary Fig. 1b) and then on PNA+CD38+ memory B cells (f). Data are representative of four independent experiments with five mice. g–i, Cells from the mLN of naive mice (g), day 23 PR8-infected mice (h), and week 9 S. mansoni-infected mice (i) were gated on live, singlet CD19+CD38+ lymphocytes (Supplementary Fig. 1c) and analyzed for NP-specific and HA-specific isotype-switched (ISW) memory B cells. Data are representative of four experiments with five mice. eFluor 450; PE, phycocerythrin.
differed from those in the mLNs and spleen based on the expression of CD80 and PD-L2 (Fig. 2f).

Resident memory T cells in the lung are often characterized using CD69 and CD103\(^+\). Although most NP-specific memory B cells in all three tissues expressed CD69, none of them expressed CD103 (Fig. 2g). Moreover, although approximately 12% of the BRM in the mLNs and spleen expressed the LN homing receptor CD62L, almost none of the BRM in the lung expressed this marker (Fig. 2h). Finally, NP-specific memory B cells in the lung uniformly expressed high amounts of CXCR3, whereas those in the mLNs and spleen had a substantial population of CXCR3- cells (Fig. 2i). These characteristics were generally shared by NP-specific isotype-switched memory B cells in the non-circulating (B220\(^-\)) and total (B220\(^+\)) populations (Supplementary Fig. 3a–b), although we should note that more than 95% of NP-specific isotype-switched memory B cells in the lung and mLNs are in the B220\(^-\) fraction. Despite the phenotypic differences between memory B cells in the lungs and lymphoid organs, the distribution of isotypes was remarkably similar, except for an increase in the frequency of IgM\(^+\) BRM cells in the spleen (Fig. 2j) and Supplementary Fig. 3c–e). These results demonstrate that BRM cells in the lung are phenotypically different from their lymphoid counterparts.

BRM cells in the lung do not recirculate. To determine whether the non-circulating, influenza-specific memory B cells permanently resided in the lung, we infected C57BL/6 (CD45.2\(^+\)) mice with influenza and, on day 44, surgically paired them with CD45.1\(^+\) partner mice for an additional 15 days to allow recirculating cells to equilibrate between the partners. To determine whether the pairs had reached equilibrium, we enumerated both donor (CD45.2\(^+\)) and partner (CD45.2\(^-\)) naïve B cells in the mLNs and spleen. We found that naïve B cells had equilibrated between donor mice and partner mice in the mLNs and spleens of both mice in each pair (Fig. 3a,b).

We next gated on non-circulating IgM\(^+\) or isotype-switched memory B cells in the lung and examined whether the NP-specific memory B cells had equilibrated (Fig. 3c). When PR8-memory mice were paired with naïve mice, NP-specific IgM\(^+\) memory B cells as well as NP-specific isotype-switched memory B cells remained in the previously infected lung and did not migrate to the naïve lung (Fig. 3d,e). We also paired previously infected CD45.2\(^+\) mice with CD45.1\(^+\) mice that had previously received intranasal lipopolysaccharide (LPS) to promote pulmonary inflammation. Again, we found that NP-specific IgM\(^+\) memory B cells as well as NP-specific isotype-switched memory B cells remained in the previously infected lung and did not migrate to the LPS-treated lung (Fig. 3f). Finally, we paired previously infected CD45.2\(^+\) mice with previously infected CD45.1\(^+\) mice so that both lungs had been exposed to virus. Again, we found that NP-specific IgM\(^+\) memory B cells as well as NP-specific isotype-switched memory B cells remained in the lung of origin and did not migrate to the opposite lung (Fig. 3h,i). These data...
suggest that antigen-specific memory cells in the lung are maintained as tissue resident cells (BRM cells) and do not migrate, even to previously infected lungs.

We also tested whether tissue residence was restricted to the lung or also occurred in the mLNs and spleen. Using PR8-infected CD45.1+ mice paired with PR8-infected CD45.2+ mice, we found that both HA-specific and NP-specific memory B cells in both the IgM+ and isotype-switched fractions were strongly biased to remain in the lung of origin (Supplementary Fig. 4a–d). This bias was less pronounced in the mLNs and was only significant in the isotype-switched memory B cells (Supplementary Fig. 4e–h). The bias was even less evident in the spleen but did achieve significance for NP-specific IgM+ and isotype-switched memory B cells (Supplementary Fig. 4i–l).

Fig. 3 | Identification of influenza-specific, non-circulating BRM cells in the lung. Mice were infected on day 0, surgically paired with partner mice on day 44 and analyzed on day 59. a, b, Cells from the mLNs (a) or spleen (b) of each partner mouse were gated on live, singlet lymphocyte CD19+CD38+IgD+CD45.1 naive B cells (Supplementary Fig. 1a), and the frequency of CD45.2+ cells was determined in each partner. Data are combined from four independent experiments with three mice each. Graph shows individual points as well as mean ± s.d. c, Cells from the lung were gated on live, singlet lymphocytes (Supplementary Fig. 1a) and subsequently gated on CD19+B220− (non-circulating), CD38+IgD+CD45.2− (ISW) memory B cells. d, e, The frequencies and numbers of NP-specific IgM+ (d) and ISW (e) memory B cells from host and partner mice were determined in the lungs of naïve mice paired with PR8-infected mice. f, g, The frequencies and numbers of NP-specific IgM+ (f) and ISW (g) memory B cells from host and partner mice were determined in the lungs of LPS-treated mice paired with PR8-infected mice. h, i, The frequencies and numbers of NP specific IgM+ (h) and ISW (i) memory B cells from host and partner mice were determined in the lungs of PR8-infected mice paired with PR8-infected mice. Data are representative of three independent experiments, each with three pairs of mice (d,e), two experiments with three pairs of mice (f,g), or three experiments combined, with a total of 11 pairs of mice (h,i). Graphs show individual data as well as mean ± s.d. Significance was determined using one-way ANOVA (paired) followed by the Bonferroni–Sidak method for multiple comparison: *P = 0.0428 (d), ***P = 0.0001 (e), ****P = 0.0001 and **P = 0.0040 (f–h), and ****P = 0.0001 and **P = 0.0022 (i). P < 0.05 is considered significant. BV-421, Brilliant Violet 421; BV-510, Brilliant Violet 510; FITC, fluorescein isothiocyanate; SSC-A, side scatter area.
Memory B cells are established early after immunization. To determine whether BRM cells in the lung are also established early after infection, we infected both CD45.1+ mice and CD45.2+ mice with PR8, surgically paired them 15 days later and evaluated the presence of BRM cells 15 days after joining. We found that naïve B cells in mLNs and spleen had equilibrated in the parabiotic pairs 20 days after joining. We found that naïve B cells in mLNs and spleen had equilibrated in the parabiotic pairs 15 days after joining. We found that naïve B cells in mLNs and spleen had equilibrated in the parabiotic pairs 15 days after joining. We found that naïve B cells in mLNs and spleen had equilibrated in the parabiotic pairs 15 days after joining. We found that naïve B cells in mLNs and spleen had equilibrated in the parabiotic pairs.

BRM cells are formed by early CD40-dependent mechanisms. To establish the kinetics of GC formation relative to BRM cell generation, we enumerated antigen-specific GC B cells in the mLNs, as well as isotype-switched memory B cells in the blood and lung. We found that GC B cells appeared in the mLNs as early as 7 days after infection, peaked around 15 days after infection and declined thereafter (Fig. 5a). In contrast, memory B cells in both the blood (Fig. 5b) and lung (Fig. 5c) were barely detectable at day 7, but peaked at day 15 and declined thereafter.

To test whether the appearance of BRM cells in the lung was dependent on CD40 signalling, we infected mice with PR8 and treated them at various intervals with a blocking antibody to CD40L (MR1). We found that CD40L blockade for 2 weeks (regardless of when the treatment started) completely eliminated the GC B cell response in both the mLNs and the lung (Fig. 5d–g). Early CD40L blockade prevented the placement of NP-specific IgM+ and isotype-switched BRM cells in the lung (Fig. 5h). Reductions in BRM cells were less pronounced when the blockade occurred between day 10 and day 20 (Fig. 5i) or between day 20 and day 30 (Fig. 5j). However, BRM cells in the lung were not reduced when CD40L was blocked between day 30 and day 40 (Fig. 5k), despite a complete loss of GC B cells in the mLNs and lung. We obtained a similar result when we examined HA-specific BRM cells (Supplementary Fig. 5).
Fig. 5 | BRM cells in the lung are generated from early CD40-dependent precursors. a. Cells from the mLN of PR8-infected mice were gated on live, singlet lymphocyte, CD19+PNA+CD95+ GC B cells (Supplementary Fig. 1f), and NP-specific as well as HA-specific cells were enumerated. b, c. Cells from the blood (b) and lungs (c) of PR8-infected mice were gated on live, singlet lymphocyte, CD19+CD38+IgM+IgD+ ISW memory B cells (Supplementary Fig. 1c), and the frequency (b) and number (c) of NP-specific as well as HA-specific cells were determined. Data are representative of three independent experiments with five mice at each time point. The data points represent mean ± s.d. d–k. Mice were infected with PR8 and administered anti-CD40L (MR1) or isotype-matched control antibody (CT) every other day for 10 days starting on day 5 (d, h), day 10 (e, i), day 20 (f, j) or day 30 (g, k). Cells from the mLN and lung were gated on live, singlet lymphocyte, CD19+CD138+ cells (Supplementary Fig. 1g) and subsequently gated on GL7+CD38+ GC B cells (d–g). Cells from the lung were gated on live, singlet lymphocyte, CD19+CD138+CD38+IgM+IgD− IgM BRM cells or CD19+CD138+CD38+IgM+IgD− ISW BRM cells (h–k) (gating in Supplementary Fig. 1g). Data in d–k are representative of three independent experiments with five mice per group per timepoint. Graphs show mean ± s.d. as well as individual data points. Significance was determined using a Mann–Whitney U-test: **P = 0.0079 and ***P = 0.0014 (d), ***P = 0.0079 and *P = 0.0462 (e), **P = 0.0079 and **P = 0.0024 (f), *P = 0.0159 and *P = 0.0180 (g); or unpaired, two-tailed t-test: **P = 0.0018 and ***P = 0.0003 (h) and **P = 0.0011 (i). P < 0.05 is considered significant. PerCP, peridinin chlorophyll protein complex.
although the IgM⁺ BRM cells were placed in the lung as early as day 10, whereas the isotype-switched memory B cells required up to 30 days to fill the lung compartment. These data indicated that the seeding of influenza-specific BRM cells in the lung is dependent on early T cell interactions, possibly in the GC, and that IgM⁺ BRM cells are seeded earlier than isotype-switched BRM cells.

**BRM establishment in the lung is dependent on local antigen.** To test whether local antigen was needed for the placement of BRM cells in the lung, we surgically paired naïve mice, waited 15 days for them to attain equilibrium, infected one mouse with PR8 and the other with X31, and analyzed the influenza-specific BRM cells in the lungs 10 days later. After gating on non-circulating isotype-switched memory B cells (Fig. 6a), we found that PR8-infected partners had many more HA(PR8)-specific BRM cells than the X31-infected partners had (Fig. 6b). Conversely, we found that the X31-infected partners had many more HA(X31)-specific BRM cells than the PR8-infected partners had (Fig. 6b). Importantly, both partners had similar numbers of NP-specific BRM cells (Fig. 6b). These data demonstrate that even in concurrently infected partner mice, BRM cells tend to stay in the lungs that express the antigen to which they respond.

We next tested whether antigen-non-specific pulmonary inflammation could recruit pre-existing systemic memory B cells to the lung. To do this, we infected mice with PR8 in the peritoneal cavity (or not), challenged them (or not) 30 days later with X31, and examined the placement of NP-specific, HA(X31)-specific and HA(PR8)-specific memory B cells (Fig. 6c). We found that peritoneal infection with PR8 did not elicit either NP-specific (Fig. 6d) or HA(PR8)-specific (Fig. 6e) isotype-switched memory B cells in the lung. As expected, a pulmonary challenge with X31 elicited both NP-specific (Fig. 6d) and HA(X31)-specific (Fig. 6f) isotype-switched memory B cells in the lung. Importantly, pulmonary challenge with X31 also recruited HA(PR8)-specific isotype-switched memory B cells to the lung (Fig. 6e). We observed similar results on day 10 (Fig. 6d–f) and day 45 (Fig. 6g–i) after the challenge infection. We also examined IgM⁺ memory B cells in the same experiment but found that so few HA(PR8)-specific IgM⁺ memory B cells were recruited to the lungs that the result was not significant (Supplementary Fig. 6c,f). Taken together, these data indicate that BRM cells need to encounter antigen again for their placement in the lung.

**BRM cells in the lung respond rapidly to infection.** To directly compare the ability of systemic or pulmonary infection to elicit BRM cells in the lung, we infected (or mock infected) mice in either the peritoneal cavity or the lung and, after 30 days, enumerated NP-specific B cells. As expected, naïve mice lacked NP-specific GC B cells (Fig. 7a) and isotype-switched memory B cells (Fig. 7b), whereas the spleens of mice infected in the peritoneal cavity or the lung had numerous NP-specific GC and isotype-switched memory B cells. In contrast, only the lungs of mice intranasally infected with PR8 had NP-specific isotype-switched memory B cells (Fig. 7c). To test the effect of lung BRM cells on the outcome of a challenge infection, we intraperitoneally, intranasally or mock infected mice with PR8, challenged all groups with X31 on day 30, measured weight loss over 2 weeks and assayed viral titers on day 35. We found that intranasally primed mice had the lowest drop in weight (Fig. 7d) and the lowest viral titers (Fig. 7e).
Fig. 6 | Establishment of BRM cells in the lung requires local antigen encounter. a,b. Naïve mice were surgically joined, and on day 15, one was infected with PR8 and the other was infected with X31. BRM cells in the lung were analyzed on day 25. Cells from the lung were gated on live, singlet lymphocytes (Supplementary Fig. 1a) and subsequently gated on B220–CD19–CD38hiIgM–IgD– ISW BRM cells (a). The frequency and number of HA(PR8)-specific (top row), HA(X31)-specific (middle row) and NP-specific (bottom row) BRM cells were determined in each partner (b). Data are representative of three independent experiments with five mice per timepoint. Significance was determined using one-tailed, paired t-test: ****P = 0.0001 and **P = 0.0477. d-i. Mice were peritoneally infected with PR8 on day 0, intranasally challenged with X31 on day 30 and analyzed on day 40 (d-f) and day 75 (g-i). Cells from the lung were gated on live, singlet lymphocytes (Supplementary Fig. 1a) and subsequently gated on CD19+ B220+ CD38hi IgM– IgD– ISW memory B cells (c). NP-specific (d,g), HA(PR8)-specific (e,h) and HA(X31)-specific (f,i) ISW BRM cells were enumerated on day 40 (d-f) and day 75 (g-i). These data are representative of two independent experiments with five mice per timepoint. Data were analyzed with an unpaired t-test: ***P = 0.0001 (d), ****P = 0.0009 (e), **P = 0.0013 (f), **P = 0.0047 (g), **P = 0.0011 (h) and ***P = 0.0004 (i). P < 0.05 is considered significant. i.n., intranasal.

Although intranasally infected mice have BRM cells, they also likely have resident memory T cells, which undoubtedly contribute to secondary immunity. To test whether BRM cells participate in pulmonary recall responses, we enumerated ASCs by ELISPOT in mice that were intraperitoneally, intranasally or mock infected for 30 days and subsequently challenged with X31 (or not) for 4 days. We found that naïve and peritoneally infected mice completely lacked NP-specific ASCs in their lungs before and after challenge, whereas intranasally infected mice had some NP-specific ASCs in the lung prior to challenge and had significantly more ASCs 4 days later (Fig. 8a).

We performed a similar experiment in which BLIMP-1–yellow fluorescent protein (YFP) reporter mice were intraperitoneally, intranasally or mock infected for 30 days and subsequently challenged with X31 (or not) for 3 days. Given that ASCs strongly
express BLIMP-1, we gated on the reporter, YFP, to enumerate ASCs in the lung. We found that naive and peritoneally infected mice completely lacked NP-specific ASCs in their lungs before and after challenge, whereas intranasally infected mice had some NP-specific ASCs in the lung prior to challenge and had significantly more ASCs 3 days later (Fig. 8b). To test whether the ASCs remaining in the lung following a primary infection declined over time, we performed the same experiment, but challenged the mice with X31 at day 60. We found a few remaining ASCs in the lungs of previously infected mice but a large increase in the pulmonary ASCs response in mice with lung BRM cells, but not in mice with systemic memory B cells (Fig. 8c).

To ensure that the ASCs that we observed in the lungs of challenged mice were due to responding BRM cells and not recirculating memory B cells, we treated PR8 memory mice with fingolimod (FTY720) to block lymphocyte recirculation just before and after challenge with X31. We found that FTY720 did not reduce the NP-specific ASC response in the lung (Fig. 8d), demonstrating that the observed spots were derived from BRM cells that differentiated in situ. We also examined the fate of the BRM cells that reacted with the challenge virus (NP specific) and the BRM cells that did not react with the challenge virus (HA(PR8) specific). We found that the NP-specific BRM cells declined after challenge (Fig. 8e), whereas the HA(PR8)-specific BRM cells did not (Fig. 8f). Collectively, these findings demonstrate that BRM cells in the lung are important for rapid secondary ASC responses and that inflammatory signals alone are insufficient to trigger the differentiation of BRM cells in the lung16,17.

Discussion
Our data show that pulmonary infection with influenza virus elicits non-recirculating, lung-resident BRM cells. Unlike memory B cells in lymphoid organs, BRM cells in the lung uniformly express...
**Fig. 8** BRM cells are required for rapid secondary ASCs in the lung. **a.** NP-specific ELISPOTs in the lung 4 days after challenge infection. Graph shows data points as well as mean ± s.d. Data are representative of five independent experiments with four mice per group. Data were analyzed with one-way ANOVA and Tukey’s test for multiple comparisons: **P = 0.0037, **P = 0.0002 and P = 0.003. **b,** c. BLIMP-1 reporter mice were infected with PR8 on day 0 and challenged with X31 on day 30 (b) or 60 (c), and ASCs were enumerated 3 days later. Cells in the lung were gated on live, singlet lymphocytes (Supplementary Fig. 1a) and subsequently gated on NP-specific YFP+ cells. Data are representative of three independent experiments (b) or two experiments (c) with three to five mice per group. Graphs show individual data points as well as mean ± s.d. Data are analyzed by one-way ANOVA with Bonferroni–Sidak test for multiple comparisons: ****P = 0.0001, ****P = 0.0001 and P = 0.0001 (b); or Tukey’s test for multiple comparisons: ****P = 0.0001, ****P = 0.0001 and P = 0.0001 (c). **d.** Mice were infected with PR8 on day 0, treated with FTY720 and challenged with X31 on day 30, and ELISPOTs in the lung were analyzed 4 days later. Data are representative of two independent experiments with six mice per group (control) or four mice per group (FTY720). Graph shows data points as well as mean ± s.d. Data were analyzed with a Mann–Whitney U-test. **e,** f. Mice were infected with PR8 on day 0, challenged with X31 on day 30 and analyzed on day 33. Cells from the lung were gated on live, singlet lymphocyte, CD19+B220 CD38–IgD–IgM– ISW memory B cells (Supplementary Fig. 1e), and NP-specific (e) and HA(PR8)-specific (f) memory B cells were enumerated. Graphs show individual data points as well as mean ± s.d. Data are representative of three independent experiments with three mice per group. Data were analyzed with Student’s t-test: *P = 0.0488, P < 0.05 is considered significant.

The chemokine receptor CXCR3 and completely lack the lymph node–homing receptor CD62L. Moreover, lung-resident BRM cells are evenly divided into CD73+ and CD73− populations, whereas memory B cells in lymphoid organs are predominantly CD73+. Influenza-specific BRM cells are recruited mainly to the lung in the first few weeks after infection, in part due to local antigen encounter. Finally, the presence of BRM cells in the lung leads to an accelerated ASC response in the lung following a challenge infection. These data demonstrate that like TRM cells, BRM cells are an instrumental component of pulmonary immunity to influenza.
The formation of BRM cells, like that of other memory B cells, is dependent on CD40 signaling, most likely in GCs. Interestingly, GCs are found in both the mLNs and the lungs of influenza-infected mice, and some studies suggest that GCs in the lung select mainly for influenza-specific memory B cells in the lung. However, we find that the GC response in the mLNs develops very rapidly after infection, whereas the GC response in the lung develops more slowly, likely because it takes time to form inducible bronchus-associated lymphoid tissue (iBALT). Given our observation that BRM cells in the lung are placed very early after infection and that CD40L blockade at late times has a minimal effect on lung-resident BRM cells, despite eliminating GCs in both the mLNs and the lung, it seems likely that most lung-resident BRM cells formed in response to a primary infection coming from GCs in the mLNs.

Interestingly, not all memory B cells transit through GCs, as mice lacking follicular helper T cells, and therefore GCs, still generate memory B cells. Moreover, GC-independent memory B cells poorly express CD73. Given our observation that more than half of the BRM cells in the lung lack CD73, it is possible that some of these cells were generated independently of the GC reaction either in the mLNs or in the lung. These data are also consistent with our observation that many BRM cells, particularly IgM+ BRM cells, seed the lung very early after infection, and with previous studies showing that systemic memory B cells are also formed mainly early after immunization.

Although memory B cells are generated mainly early after infection, we still observe them in the blood as late as 70 days after infection, suggesting that recirculating memory B cells could be recruited to the lungs for extended periods of time. In fact, we find that memory B cells established at systemic sites during a primary response can be ‘called’ into the lung by a subsequent inflammatory response in the lung, such as a non-specific infection. However, during a primary response, the placement of newly generated BRM precursors in the lung appears to be restricted to a limited time after infection. Moreover, as shown by our experiments with parabiotic mice concurrently infected with two different viruses, the ability to seed the lung is not dictated exclusively by inflammation, which is present in the lungs of both partners, but by the availability of local antigen, suggesting that BRM precursors need to contact antigen in the lung in order to stay (or survive) as lung-resident BRM cells. These data are similar to those showing that the placement of TRM cells in the skin and the lung requires contact with local antigen.

Where might this encounter with antigen occur? One possibility is that BRM precursors encounter antigen in iBALT. We know that iBALT is formed in the lungs of influenza-infected mice and that mice lacking conventional secondary lymphoid organs generate and maintain long-lived B cell responses. Moreover, GC-like B cells are observed in the lungs of infected mice, even though the existence of pulmonary follicular T cells remains controversial, possibly due to the altered phenotype of helper T cells in peripheral non-lymphoid tissues. Interestingly, GCs in the lung seem to preferentially select for broadly reactive memory B cells, although it is unclear whether the broad reactivity of BRM cells is due to site-specific properties of the GC response or to the early placement of less stringently selected (and perhaps more broadly reactive) BRM cells. This latter idea is consistent with data showing that rapamycin treatment of influenza-infected mice leads to curtailed GC responses but a more broadly reactive repertoire of memory B cells.

Our data highlight another qualitative difference between BRM cells in the lung and memory B cells in lymphoid organs: the rapidity of secondary responses. Mice with NP-specific BRM cells in the lung generate secondary ASC responses in the lungs more rapidly than do mice with memory B cells at systemic sites. Importantly, the accelerated secondary response in the lungs is maintained in the presence of FTY-720, a functional antagonist of the chemotactic molecule S1P1, demonstrating that the response is generated in situ from precursors already in the lung. This change in secondary response time may be simply due to location, with BRM cells in the lung encountering antigen and inflammation earlier than their lymphoid counterparts and accelerating their response accordingly. Alternatively, BRM cells may be qualitatively different from those in lymphoid organs, as their phenotype suggests, and may be poised to rapidly differentiate into ASCs with minimal stimulation. Consistent with this idea, memory B cells from the lung (presumably BRM cells) adoptively transferred to recipient mice provide more efficient protection than that provided by memory B cells isolated from secondary lymphoid organs.

In summary, our data demonstrate that influenza-specific BRM cells are formed in the lungs following pulmonary influenza infection and that these cells are necessary for an accelerated ASC response following challenge infection. BRM cells are formed early after infection and seed the lung in a process that depends on local encounter with antigen. These data suggest that vaccines designed to elicit highly effective, long-lived protection against influenza virus infection will need to deliver antigens to the respiratory tract.

Online content
Any methods, additional references, Nature Research reporting summaries, source data, statements of data availability and associated accession codes are available at https://doi.org/10.1038/s41590-018-0260-6.

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**Author contributions**

S.R.A., F.E.L. and T.D.R. designed the experiments. J.E.B., B.A.G. and T.D.R. designed the recombinant influenza proteins and J.E.B. and B.A.G. expressed, purified and characterized the tetramers. U.M. and S.R.A performed the surgeries. M.D.S. performed the intratracheal infections. S.R.A performed and analyzed the experiments and generated the figures. S.R.A. and T.D.R. wrote the manuscript. All authors edited the manuscript.

**Competing interests**

The authors declare no competing interests.

**Additional information**

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Methods

Mice and parabiosis. Male and female C57BL/6j (CD45.2) and B6.SJL-PpcrePepc/Bom (CD45.1) mice were purchased from the Jackson Laboratory, and BLIMP-1-YFP reporter mice were obtained from S. Crotty (La Jolla Institute for Immunology) and bred in the University of Alabama at Birmingham vivarium, as noted in the Life Sciences Reporting Summary associated with this paper. Swiss Webster mice infected with Schistosoma mansoni (strain NMRI) were obtained from the Schistosomiasis Resource Center and distributed through BEI resources, National Institute of Allergy and Infectious Diseases. Parabiosis surgery was performed as described using pairs of female mice that had been cohoused for at least 2 weeks prior to surgery. In brief, anesthetized mice were shaved and disinfected with betadine, and longitudinal incisions in the skin were made from approximately 1 cm behind the ear to just past the hind limb without opening the peritoneal cavity. The skin was loosened from the connective tissue, and the two mice were sutured together at the scapulae, flank and thigh. The dorsal edges of the skin were joined using 9 mm stainless steel wound clips. Body temperature was maintained using a heating pad during surgery and recovery. Mice were provided with buprenorphine (0.05 mg/kg) prior to surgery and carprofen (5 mg/kg) prior to surgery and 24 h postsurgery. All animal procedures were approved by the University of Alabama Institutional Animal Care and Use Committee and were performed according to guidelines outlined by the National Research Council.

Infections and viral foci assay. Influenza A/PR8/34 (PR8) was used at 15,000 viral foci units (VFU) for intranasal infection and at 1 × 106 VFU for intraperitoneal (i.p.) infection. In Fig. 7a–d, the i.p. infections were repeated for 6 days at 1 × 106 VFU, 1 × 105 VFU, 1 × 104 VFU, 1 × 103 VFU, 1 × 102 VFU and 1 × 101 VFU. Influenza X31 was used at 1.25 × 105 VFU for primary infection and 1.25 × 104 VFU for secondary infections. Mice were anesthetized with isofluorane. The i.p. injections were performed according to guidelines outlined by the National Research Council. Under the aseptic conditions specified by the National Institute of Allergy and Infectious Diseases and approved by the Institutional Animal Care and Use Committee and were maintained using a heating pad during surgery and recovery. Mice were provided with buprenorphine (0.05 mg/kg) prior to surgery and carprofen (5 mg/kg) prior to surgery and 24 h postsurgery. All animal procedures were approved by the University of Alabama Institutional Animal Care and Use Committee and were performed according to guidelines outlined by the National Research Council.

Antibody infusion, blocking antibodies and FTY-720 treatment. To identify circulating B cells, mice were intravenously administered 2.4 mg of fluorescein-conjugated anti-CD19 antibody (BD Biosciences), followed by staining with fluorescein-conjugated streptavidin (Life Technologies) and 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (BCIP/NBT) substrate (Moss Substrates). Samples were plated in duplicates and the mean foci count was used to calculate the VFU per ml of sample.

Tissue preparation and flow cytometry. Lungs were isolated, cut into small fragments and digested for 30 min at 37 °C with 0.06 mg/ml collagenase A (Sigma) and 30 µg/ml DNase I (Sigma) in RPMI-1640 medium (GIBCO). Digested lungs, mLNs or spleens were mechanically disrupted by passage through a wire mesh. Red blood cells were lysed with 150 mM NH4Cl, 10 mM KHCO3 and 0.1 mM ethylene diamine tetraacetic acid (EDTA). Fe receptors were blocked with 5 µg/ml anti-CD16/32 (BD Biosciences), followed by staining with fluorescein-conjugated antibodies. Analytic flow cytometry was performed on an LSR II (BD Biosciences) instrument available through the Comprehensive Flow Cytometry Core at University of Alabama, Birmingham (UAB).

Production of influenza proteins, immunoblot and tetramers. The coding sequences of PR8 HA (accession number: P03452) and X31 HA (accession number: P03438) were synthesized in frame with the human CD5 signal sequence upstream and the GCN4 isoleucine zipper trimerization domain downstream (GeneArt). These cDNA were fused in frame with either a 6XHis tag or an AviTag at the C-terminus and cloned into the pCXPoly(+) mammalian expression vector. Constructs encoding HA-6XHis and HA-AviTag were cotransfected using 293Fectin Transfection Reagent into Freestyle 293-2F Cells (Thermo Fisher Scientific) at a 2:1 ratio. Transfected cells were cultured in Freestyle 293 Expression Medium for 3 days, and the supernatant was recovered by centrifugation. Recombinant HA molecules were purified by fast protein liquid chromatography (FPLC) using a HiTrap HP Column (GE Healthcare) and eluted with 250 mM of imidazole. The coding sequence of NP from PR8 was synthesized in frame with the coding sequence for a 15–amino acid biotinylination consensus site on the 3’ end (GeneArt). The modified NP sequence was cloned in frame to the 6XHis tag in the pTRE–Has2e expression vector (Invitrogen). NP protein was expressed in E. coli strain CVB1901 (Avidity), purified by FPLC using a HiTrap HP Column (GE Healthcare) and eluted with a 50–250 mM gradient of imidazole. Purified HA was biotinylated by addition of biotin–protein ligase (Avidity). Biotinylated proteins were then tetramerized with fluorescent-labelled streptavidin (Prozyme). Labelled tetramers were purified by size exclusion on a HiPrep 16/60 Sephacryl S-300 column (GE Healthcare).

ELISPOT. For ELISPOT assays, multiscreen HA plates (MAHAS4510, Millipore) were coated with purified NP at 1 µg/ml in PBS overnight at 4 °C. Plates were washed with PBS and blocked with complete medium (RPMI supplemented with 10% fetal bovine serum (FBS), 0.5% Penicillin (100 U/ml), 0.5% streptomycin (100 µg/ml), 1% L-glutamine (200 mM), 1% sodium pyruvate (100 mM), 1% HEPES pH 7.4 (1 M), 0.15% sodium bicarbonate, 1.2% amino acids (50X), 1.2% non-essential amino acids (100X), 1.2% vitamins (100X), 0.7% glucose, 0.1% 2-mercaptoethanol (1000X, 55 mM). Single-cell suspensions were washed, diluted in complete medium and cultured on coated plates for 5 h. Cells were aspirated, and plates were washed with 0.2% Tween 20 in PBS. Bound IgG was detected using alkaline phosphatase–conjugated goat anti–mouse kappa (Southern Biotech) diluted (1:1000) in 0.5% BSA, 0.05% Tween 20 in PBS for 1 h at 37 °C. Plates were washed with 0.2% Tween 20 in PBS and developed with BCIP/NBT (Moss Substrates) substrate for 1 h. Spots were recorded using a CTL Immunospot S6 Macroplate Imager Reader (New Life Scientific) and counted manually.

Statistical analysis. GraphPad Prism software (Version 7.0) was used for data analysis. Datasets that did not follow a Gaussian distribution were analyzed using non-parametric tests. Comparisons between two samples were performed with Student’s t-test or the Mann–Whitney U-test. Parabiosis experiments were analyzed using one-way analysis of variance (ANOVA) (paired) followed by the Bonferroni–Sidak method for multiple comparison. One-way ANOVA, followed by analysis-specific post-tests, was carried out when more than two variables were compared; P < 0.05 was considered statistically significant.

Reporting Summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

The data supporting the findings of this study are available from the corresponding author upon reasonable request.

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☐ The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement
☐ An indication of whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
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☐ Only common tests should be described solely by name; describe more complex techniques in the Methods section.
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☐ A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons
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☐ For null hypothesis testing, the test statistic (e.g. F, t, r) with confidence intervals, effect sizes, degrees of freedom and P value noted
☐ Give P values as exact values whenever suitable.
☐ For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
☐ For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
☐ Estimates of effect sizes (e.g. Cohen's d, Pearson's r), indicating how they were calculated
☐ Clearly defined error bars
☐ State explicitly what error bars represent (e.g. SD, SE, CI)

Our web collection on statistics for biologists may be useful.

Software and code

Policy information about availability of computer code

| Data collection | Flow cytometry data was collected using BD FACSDivia Software Version 8.0.1 from BD Biosciences |
|-----------------|--------------------------------------------------------------------------------------------------|
| Data analysis   | Treestar Flowjo 9.9.6. was used for viewing and analysis of flow cytometry files (FCS format).  |
|                 | Microsoft Excel version 14.5.2 was used to organize tabulated data                               |
|                 | GraphPad Prism 7 was used for all the graphical displays and statistical analysis                 |

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Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

| Sample size | Sample size was determined to obtain a significant difference between groups, with a power of 80%, when the statistical significance was set at less than a p value of 0.05. The sample size chosen withstands the standard deviation within groups to give us information about the true mean of the group and takes into account expected attrition (due to death of animals) based on our previous studies. Parabiosis experiments were started with 8-10 pairs per group to account for the expected attrition, while all other experiments were with 5 animals per group. |
| --- | --- |
| Data exclusions | no data were excluded |
| Replication | To test whether our experiments were reproducible, we performed each experiment a minimum of 2 times and a maximum of 5-10 times. We found that the results of each experiment we performed the experiments. |
| Randomization | Mice were bred at UAB and pups of same age and sex (within a week) were pooled and randomly weaned into cages of 5. When mice were old enough for experiments (8+ weeks), they were assigned to a group per cage. |
| Blinding | The studies were not blinded because they did not involve subjective measurements. For example, the gates for flow cytometry analyses were automatically applied to both experimental and control samples. Cell counts were standardized using defined concentrations of fluorescent beads on a flow cytometer. ELISPOTs were counted and the data entered before assigning the samples to each group from the plate design. |

Reporting for specific materials, systems and methods

| Materials & experimental systems | Methods |
| --- | --- |
| n/a | n/a |
| Involved in the study | Involved in the study |
| Unique biological materials | ChIP-seq |
| Antibodies | Flow cytometry |
| Eukaryotic cell lines | MRI-based neuroimaging |
| Palaeontology | |
| Animals and other organisms | |
| Human research participants | |

Unique biological materials

Policy information about availability of materials

Obtaining unique materials Recombinant influenza protein "tetramers" are available upon request following the completion of an MTA

Antibodies

Antibodies used

| Antibodies | Vendor | Catalog number |
| --- | --- | --- |
| B220 RA3-6B2 Invitrogen AF647, AF488 14-0452-81 A10235 | | |
| CD95 Jo2 BD FITC 554257 4080693 | | |
| CD19 6D5 Biolegend APC-Cy7, APC Fire 115529, 115558 B253924, B243046 | | |
| CD138 281-2 Biolegend BV605 142516 B240942 | | |
| CD38 90 eBiosciences APC, AF700 17-0381-82, 56-0381-82 4316984 | | |
| IgD 11-26 eBiosciences eFluor450 48-5993-82 4316984 | | |
| IgM II/41 eBiosciences PerCP-eFlour710 46-5790-82 4321765 | | |
| CD73 TY11.8 Biolegend BV605 127215 B240318 | | |
| PD-L2 (CD273) 122 eBiosciences FITC 11-9972-82 E00936-1632 | | |
| CD80 16-10A1 eBiosciences Biotin 13-0801-82 E023084 | | |
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| Cell line source(s) | State the source of each cell line used. |
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**Specimen provenance**

Provide provenance information for specimens and describe permits that were obtained for the work (including the name of the issuing authority, the date of issue, and any identifying information).

**Specimen deposition**

Indicate where the specimens have been deposited to permit free access by other researchers.

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If new dates are provided, describe how they were obtained (e.g. collection, storage, sample pretreatment and measurement), where they were obtained (i.e. lab name), the calibration program and the protocol for quality assurance OR state that no new dates are provided.

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### Animals and other organisms

**Policy information about studies involving animals; ARRIVE guidelines** recommended for reporting animal research

#### Laboratory animals

Laboratory mice (mus musculus) strains C57BL/6, B6.SJL–PtprcPepcb/BoyJ and BLIMP–1–YFP reporter mice were used between 8 weeks and 12 weeks of age. Both males and females were used, except in parabiosis experiments, in which we used only females because the males would fight. All mice were bred at the UAB vivarium and all procedures were approved by IACUC.

#### Wild animals

No wild animals were used.

#### Field-collected samples

No field collected animals were used.

### Human research participants

**Policy information about studies involving human research participants**

#### Population characteristics

Describe the covariate-relevant population characteristics of the human research participants (e.g. age, gender, genotypic information, past and current diagnosis and treatment categories). If you filled out the behavioural & social sciences study design questions and have nothing to add here, write "See above."

#### Recruitment

Describe how participants were recruited. Outline any potential self-selection bias or other biases that may be present and how these are likely to impact results.
## ChIP-seq

### Data deposition

- Confirm that both raw and final processed data have been deposited in a public database such as [GEO](https://www.ncbi.nlm.nih.gov/geo/).
- Confirm that you have deposited or provided access to graph files (e.g. BED files) for the called peaks.

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Provide a list of all files available in the database submission.

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(e.g. UCSC)

Provide a link to an anonymized genome browser session for "initial submission" and "Revised version" documents only, to enable peer review. Write "no longer applicable" for "Final submission" documents.

### Methodology

#### Replicates

Describe the experimental replicates, specifying number, type and replicate agreement.

#### Sequencing depth

Describe the sequencing depth for each experiment, providing the total number of reads, uniquely mapped reads, length of reads and whether they were paired- or single-end.

#### Antibodies

Describe the antibodies used for the ChIP-seq experiments; as applicable, provide supplier name, catalog number, clone name, and lot number.

#### Peak calling parameters

Specify the command line program and parameters used for read mapping and peak calling, including the ChIP, control and index files used.

#### Data quality

Describe the methods used to ensure data quality in full detail, including how many peaks are at FDR 5% and above 5-fold enrichment.

#### Software

Describe the software used to collect and analyze the ChIP-seq data. For custom code that has been deposited into a community repository, provide accession details.

### Flow Cytometry

#### Plots

- Confirm that:
  - The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).
  - The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a 'group' is an analysis of identical markers).
  - All plots are contour plots with outliers or pseudocolor plots.
  - A numerical value for number of cells or percentage (with statistics) is provided.

#### Sample preparation

- **Lymph nodes:** mechanically disrupted through wire mesh, filtered and resuspended at 10^6/mL of PBS(10%FBS)
- **Spleen:** mechanically disrupted through wire mesh, lysed RBCs, filtered and resuspended at 10^6/mL of PBS(10%FBS)
- **Lung:** mechanically disrupted through wire mesh, lysed RBCs, filtered and resuspended at 10^6/mL of PBS(10%FBS)
- **Blood:** ~500μL of blood collected in heparin coated tubes. Lysed RBCs (2x), filtered and resuspended in 200μL of PBS(10%FBS)

Single cells suspensions were plated at 1-2x10^6 in 96-well v-bottom plates and stained with 50μL of the mix of tetramer, antibodies and Fc block antibody in PBS (10% FBS) for 30min at 4 degrees centigrade. The cells were washed twice with PBS (10% FBS) and fixed with 10% Formalin for 5min at room temperature and washed in PBS (10% FBS). The cells were resuspended in PBS (10% FBS).

#### Instrument

- **BD LSR II Flow cytometer**

#### Software

- Flow cytometry data was collected using BD FACSDiva Software Version 8.0.1 from BD Biosciences
- FlowJo 9.6: Analysis of flow cytometry data
- Microsoft Excel: Organization of numerical data and calculation of numbers from proportions.
- GraphPad Prism 7: Graphing and statistical analysis.

#### Cell population abundance

Sorted populations are not included in this study

#### Gating strategy

All samples were initially gated on the lymphocytes in the FSC-A/SSC-A plot. The gate is placed above a threshold of 50,000 on the FSC-A axis. Doublets are excluded, and live cells are selected using a dead cell dye. The following are sequential gates for germinal center cells (GCs), antibody secreting cells (ASCs), Resident memory by parabiosis, resident memory by infusion and phenotyping of resident memory cells (identified by infusion):
| GGBs                                                                 | CD138-CD19+CD38-GL7+NP+  |
|---------------------------------------------------------------------|--------------------------|
| CD138-CD19+CD38-GL7+PR8+                                           | CD138-CD19+CD38-PNA+GL7++NP+  |
| CD138-CD19+CD38-PNA+GL7++NP+                                       | CD138-CD19+CD38-PNA+GL7++NP+  |

**ASCs**

C57BL/6 mice:

CD138+CD19+/lo

Blimp-YFP reporter mice:

YFP+CD19+/lo

YFP+CD19+/loCD138+

Resident memory (parabiosis)

Ex. Donor CD45.2+

aB220-CD19+CD38+IgM+IgD-CD45.2+NP+

aB220-CD19+CD38+IgM+IgD-CD45.2+HA+

aB220-CD19+CD38+IgM-IgD-CD45.2+NP+

aB220-CD19+CD38+IgM-IgD-CD45.2+HA+

Resident memory (infusion)

aB220-CD19+CD38+IgD+isotype+

aB220-CD19+CD38+IgD-PR8+ isotype+

Infused BRM phenotyping, isotyping

Gating strategy (except lymphocyte gates, doublet exclusion and live/dead exclusion) is presented in most figures.

Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.

### Magnetic resonance imaging

#### Experimental design

| Design type | Indicate task or resting state; event-related or block design. |
|-------------|---------------------------------------------------------------|
| Design specifications | Specify the number of blocks, trials or experimental units per session and/or subject, and specify the length of each trial or block (if trials are blocked) and interval between trials. |
| Behavioral performance measures | State number and/or type of variables recorded (e.g. correct button press, response time) and what statistics were used to establish that the subjects were performing the task as expected (e.g. mean, range, and/or standard deviation across subjects). |

#### Acquisition

| Imaging type(s) | Specify: functional, structural, diffusion, perfusion. |
|-----------------|-------------------------------------------------------|
| Field strength  | Specify in Tesla                                      |
| Sequence & imaging parameters | Specify the pulse sequence type (gradient echo, spin echo, etc.), imaging type (EPI, spiral, etc.), field of view, matrix size, slice thickness, orientation and TE/TR/flip angle. |

| Area of acquisition | State whether a whole brain scan was used OR define the area of acquisition, describing how the region was determined. |
|---------------------|------------------------------------------------------------------------------------------------------------------|

| Diffusion MRI |   | Used |   |
|--------------|---|------|---|
|              |   | No   |   |

#### Preprocessing

| Preprocessing software | Provide detail on software version and revision number and on specific parameters (model/functions, brain extraction, segmentation, smoothing kernel size, etc.). |
|------------------------|------------------------------------------------------------------------------------------------------------------|
| Normalization          | If data were normalized/standardized, describe the approach(es): specify linear or non-linear and define image types used for transformation OR indicate that data were not normalized and explain rationale for lack of normalization. |
| Normalization template  | Describe the template used for normalization/ transformation, specifying subject space or group standardized space (e.g. original Talairach, MNI305, ICBM152) OR indicate that the data were not normalized. |
| Noise and artifact removal | Describe your procedure(s) for artifact and structured noise removal, specifying motion parameters, tissue signals and physiological signals (heart rate, respiration). |
| Volume censoring       | Define your software and/or method and criteria for volume censoring, and state the extent of such censoring. |

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Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.
**Statistical modeling & inference**

**Model type and settings**
Specify type (mass univariate, multivariate, RSA, predictive, etc.) and describe essential details of the model at the first and second levels (e.g. fixed, random or mixed effects, drift or auto-correlation).

**Effect(s) tested**
Define precise effect in terms of the task or stimulus conditions instead of psychological concepts and indicate whether ANOVA or factorial designs were used.

**Specify type of analysis:**
- Whole brain
- ROI-based
- Both

**Statistic type for inference**
(See Eklund et al. 2016)
Specify voxel-wise or cluster-wise and report all relevant parameters for cluster-wise methods.

**Correction**
Describe the type of correction and how it is obtained for multiple comparisons (e.g. FWE, FDR, permutation or Monte Carlo).

**Models & analysis**

| n/a | Involved in the study |
|-----|-----------------------|
|     | Functional and/or effective connectivity |
|     | Graph analysis |
|     | Multivariate modeling or predictive analysis |

**Functional and/or effective connectivity**
Report the measures of dependence used and the model details (e.g. Pearson correlation, partial correlation, mutual information).

**Graph analysis**
Report the dependent variable and connectivity measure, specifying weighted graph or binarized graph, subject- or group-level, and the global and/or node summaries used (e.g. clustering coefficient, efficiency, etc.).

**Multivariate modeling and predictive analysis**
Specify independent variables, features extraction and dimension reduction, model, training and evaluation metrics.