c-Myb is required for plasma cell migration to bone marrow after immunization or infection

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Plasma cell migration is crucial to immunity, but little is known about the molecular regulators of their migratory programs. Here, we detail the critical role of the transcription factor c-Myb in determining plasma cell location. In the absence of c-Myb, no IgG+ antigen-specific plasma cells were detected in the bone marrow after immunization or virus infection. This was correlated with a dramatic reduction of plasma cells in peripheral blood, mislocalization in spleen, and an inability of c-Myb–deficient plasma cells to migrate along a CXCL12 gradient. Therefore, c-Myb plays an essential, novel role in establishing the long-lived plasma cell population in the BM via responsiveness to chemokine migration cues.

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throughout the immune response (Fig. 1 C), there were few or no BM IgG1+ ASCs detected in the absence of c-Myb. This was not due to a lack of production, as assessment of NP+IgG1+ ASCs in the spleen demonstrated comparable frequencies over time (Fig. 1 D). Similarly, affinity maturation of splenic NP+IgG1+ ASCs appeared normal (Fig. 1 E).

c-Mybfl/fl mice carrying an AicdaCre/+ allele, in which c-Myb was deleted after Ag activation of mature B cells (Kwon et al., 2008), also revealed a lack of NP+IgG1+ ASCs in the BM during an immune response (Fig. 1 F). This indicates a role for c-Myb during the processes of ASC differentiation and migration rather than in establishing a preexisting condition in naive B cells.

NP+ GC B cells formed normally in the absence of c-Myb at day 7 after immunization, but by day 14 there was a twofold decrease, suggesting persistence of these cells was not optimal in the absence of c-Myb. This was not due to a lack of production, as assessment of NP+IgG1+ ASCs in the spleen demonstrated comparable frequencies over time (Fig. 1 D). Similarly, affinity maturation of splenic NP+IgG1+ ASCs appeared normal (Fig. 1 E). c-Mybβ/β mice carrying an AicdaCre/+ allele, in which c-Myb was deleted after Ag activation of mature B cells (Kwon et al., 2008), also revealed a lack of NP+IgG1+ ASCs in the BM during an immune response (Fig. 1 F). This indicates a role for c-Myb during the processes of ASC differentiation and migration rather than in establishing a preexisting condition in naive B cells. NP+ GC B cells formed normally in the absence of c-Myb at day 7 after immunization, but by day 14 there was a twofold decrease, suggesting persistence of these cells was not optimal in the absence of c-Myb (Fig. 1 G). Within the c-Myb−deficient NP+ GC compartment, however, the frequency of IgG1+ cells was increased at day 14 and 28 after immunization compared with controls (Fig. 1 H). Thus, the number of IgG1+NP+ ASCs was decreased in the absence of c-Myb.

RESULTS AND DISCUSSION
c-Myb is required for establishing Ag-specific ASCs in the BM during a TD response

To assess the contribution of c-Myb to a humoral response, we generated c-Mybβ/β mice carrying a Cd23Cre/+ transgene that deletes c-Myb at the T2 stage of B cell development (Emambokus et al., 2003; Kwon et al., 2008). Strikingly, immunized c-Mybβ/βCd23Cre/+ had no IgG1+ Ag-specific ASCs in the BM (Fig. 1 A–C). Although the frequency of Ag-specific IgG1+ ASCs in the BM of Cd23Cre/+ control mice increased...
GC B cells, which are arguably the precursors to IgG1+ ASCs in the BM, was similar between knockout and controls at days 7 and 14, and significantly decreased at day 28 (Fig. 1 I). Thus, the lack of ASC in the BM of c-Myb–deficient mice was not caused by an absence of IgG1+NP+ GC B cells.

**B cell–intrinsic migratory defect**

To rule out potential secondary changes in the microenvironment affecting migration of ASCs in c-Myb/fl/fl Cd23cre/+ mice, mixed BM chimeras were created. Ly5.1 wild-type BM was mixed 1:1 with Ly5.2 c-Myb/fl/fl Cd23cre/+ (or Cd23cre/+ only for control chimeras) and used to reconstitute irradiated recipients (Fig. 2 A). Immunization of these chimeras revealed Ly5.1+NP+IgG1+ ASCs were present in the BM (Fig. 2, B and C), as were Cd23cre/+ Ly5.2+NP+IgG1+ ASCs (Fig. 2 C). In contrast, NP+IgG1+ c-Myb–deficient, BM-resident ASCs were absent (Fig. 2 B). Therefore, although wild-type ASCs migrated to the BM, c-Myb–deficient ASCs did not within the same animal, demonstrating the B cell intrinsic basis of the defect.

**Infection also fails to induce long-lived BM ASC**

To investigate whether the inability of c-Myb–deficient ASCs to seed the BM depended on the immunizing agent, c-Myb/fl/fl Cd23cre/+ and Cd23cre/+ control mice were infected with influenza virus. Whereas IgG1 dominates after an NP-KLH in alum immunization, the response to influenza infection is dominated by IgG2c production due to IFN-γ production (Severinson et al., 1990; Collins and Dunnick, 1993; Peng et al., 2002). Mice were analyzed 6 wk after infection to assess the establishment of the antiinfluenza BM ASC population. As with NP-KLH immunization, the frequency of influenza-specific IgG+ ASCs in spleens of c-Myb–deficient mice was comparable to controls (Fig. 2 D). However, whereas Cd23cre/+ mice generated a population of IgG+ influenza-specific ASCs in BM, no such IgG+ influenza-specific ASCs were detected in the absence of c-Myb (Fig. 2 E). Therefore, c-Myb is essential for Ag-specific BM ASCs in response to immunization and infection.

**Localization defect in the absence of c-Myb**

All data presented so far support the contention that c-Myb–deficient ASCs are defective in migration to the BM. A clear intermediary in migration to the BM would be the presence of ASCs in the blood during an immune response. Detecting ASCs in blood during a primary response is technically limiting, so instead we assessed blood for migrating NP+IgG1+ ASCs after secondary immunization. Mice, immunized with NP-KLH in alum and allowed to rest for at least 4 wk, were boosted with soluble NP-KLH to assess whether secondary
Consistent with this, when we assessed ASC in the spleen by flow cytometry 4 wk after primary immunization, we measured a twofold increase in B220^loCD138^hi cells in the absence of c-Myb at (Fig. 3, D–F), a phenomenon that was B cell intrinsic (Fig. 3 G).

BM ASCs present in c-Myb–deficient naive mice are mutated at a low frequency

To further investigate the ASC defect, c-Myb^fl/flCd23^Cre/+ mice were crossed with Blimp-1^gfp/+ reporter mice (Kallies et al., 2004), within which plasma cell populations can be divided into Blimp-1^int and Blimp-1^hi populations that have distinct characteristics (Kallies et al., 2004). Blimp-1^int plasma cells are short-lived, less mature plasma cells, which is the population that retains migratory potential (Hauser et al., 2002). c-Myb^fl/flCd23^Cre/+ or c-Myb^fl/fl littermates that were Blimp-1^gfp/+ were used to track Blimp-1^intCD138^hi plasmablasts and Blimp-1^hi CD138^hi plasma cells. Whereas both populations still existed in the BM, mesenteric lymph nodes (MLNs), and spleen, there was a significant increase in Blimp-1^int plasmablasts in the absence of c-Myb (Fig. 4, A and B). Furthermore, the majority of BM B220^loCD138^hi cells were IgM (mean ± SEM: 81 ± 3% versus 34 ± 1.7% in controls), indicating a different route of formation.

The apparent discordance between the lack of Ag-specific ASCs in the BM after immunization, but a resident, steady-state BM plasma cell population was investigated by sort-purification of Blimp-1^hiCD138^hi cells and analysis of the genes encoding the Ag receptor to determine whether these cells originated within a GC. Comparison of the DNA sequence of variable heavy-chain Igh gene segments from c-Myb^fl/flCd23^Cre/+ mice...
after isotype-switching and thus does not affect the migration of IgM+ plasma cells, that it acts primarily on GC-derived ASC, or that the IgM+ plasma cells in the bone marrow are generated in situ. Taken together, BM plasma cells that develop in the absence of c-Myb appear to be less reliant on the GC and may experience different development or selection conditions than control mice.

Differential responsiveness to chemokine signals is regulated by c-Myb
We investigated the hypothesis that dysregulated chemokine receptor expression or function may contribute to the lack of Ag-specific ASCs in the blood and BM in the absence of c-Myb. Given that Cxcr4 is regulated by c-Myb in hematopoietic progenitors (Lieu and Reddy, 2009; Quintana et al.,...
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no difference in their migration toward CXCL10 (Fig. 5 D).

Migration to S1P has been shown to be essential for plasma cells to migrate to the BM (Kabashima et al., 2006). However, the efficiency of the in vitro migration assay to S1P for splenic plasma cells is relatively low (0.5% input; Kabashima et al., 2006), and as such we could not attain a reliable assessment of c-Myb–deficient plasma cells to migrate to S1P. Therefore, we cannot rule out that migration to this chemokine is also defective. We have, however, assessed transcript levels of S1pr1 in c-Myb–deficient and control plasmablasts and found no significant difference (unpublished data).

Histological assessment revealed that c-Myb–deficient ASCs were mislocalized in the spleen (Fig. 5, E–G). At day 7 after immunization, control plasmablasts were detected mainly in extrafollicular foci and the splenic red pulp. In contrast, c-Myb–deficient plasmablasts had infiltrated T cell areas within the spleen (Fig. 5, E–G), suggesting an inappropriate response

Figure 5. c-Myb regulates migration to CXCL12. (A and B) Cd23cre/+ (dotted gray line or closed squares) and c-Mybfl/flCd23cre/+ (black solid line or open circles) mice were immunized with NP-KLH precipitated in alum and spleens harvested at day 7 after immunization. (A) Splenic plasmablasts (B220loCD138hi) were assessed for CXCR4 expression, representative of plasmablasts assessed at multiple time points after immunization. (B–D) CD138-enriched splenic cells were assessed for the ability of plasmablasts to migrate to CXCL12 (B) representative plot, (C) combined data from three independent experiments, and (D) CXCL10, combined data from two independent experiments. * P < 0.05 (Mann-Whitney nonparametric, two-tailed test; mean ± SEM). (E–G) Cd23cre/+ and c-Mybfl/flCd23cre/+ mice were immunized with NP-KLH precipitated in alum and spleens harvested at day 7 after immunization. Sections were stained with B220 (blue) and IgG1 (red; E), and CD3 (blue) and IgG1 (red; F), representative of three spleens per genotype; ASCs in T cell zones were enumerated (G; n ≥ 7 per genotype of T cell zones assessed). Bars, 200 µm. * P < 0.05 (unpaired two-tailed t test; mean ± SEM).
to localization cues had occurred, similar to CXCR4-deficient mice. It is important to note, however, that it is unclear whether CXCR4-deficient mice have an accumulation of plasma cells in T cell areas as well as the accumulation seen in the marginal zone (Kabashima et al., 2006). As c-Myb–deficient mice are not a phenocopy of CXCR4 deficiency, it is likely that other migration processes may be regulated by c-Myb. This may include migration to S1P, but as discussed above, we were limited in assessing the role of S1P. Collectively, the data presented here led us to conclude that c-Myb has an essential role in the emigration of ASCs from secondary lymphoid organs through regulation of responsiveness to CXCL12 downstream of CXCR4 expression.

Migratory programs of cell populations are reliant on chemokine receptor expression, their accompanying signal transduction pathways, and corresponding chemokine gradients within organs (Cyster, 2003). Long-lived ASCs produced in secondary lymphoid organs during an immune response rely on chemokines to migrate to the BM. In particular, ASCs expressing CXCR4 on their surface will respond to CXCL12 gradients within the spleen and LN, thus migrating into the blood and to the BM in a directed manner. Here, we demonstrate that the transcription factor c-Myb is essential for ASCs to respond to CXCL12 within the spleen, and without it, long-lived ASCs generated during a TD response do not emigrate and thus cannot establish the stable BM-resident compartment normally required for humoral immunity. This modulation of CXCL12 responsiveness occurred in the context of mislocalization of ASCs in the spleen and a failure of Ag-specific ASCs to enter the blood and migrate to the bone marrow. Collectively, these data reveal c-Myb as an essential regulator of humoral immunity.

Little is known about the transcriptional networks underlying responsiveness of ASCs to migration signals that ultimately lead to the BM. Two studies have correlated the deletion of transcription factors with a deficit or absence of Ag-specific ASCs in the BM. Germline deletion of Aiolos (Cortés and Georgopoulos, 2004) results in defects in the high-affinity BM population; however, it was unclear at what stage (production, migration, or retention) this defect was occurring. Similarly, germline deletion of KLF2 (Hart et al., 2011) demonstrated multiple defects in the formation and responses of the B cell lineage, one of which was a deficiency in Ag-specific ASCs in the BM after boost. Expression of KLF2 or Aiolos was not altered by c-Myb deficiency (unpublished data). In contrast to these studies, we pinpoint a specific role for c-Myb in the directed migration of ASCs out of organs through regulation of responsiveness to CXCL12. This phenomenon was not restricted to the spleen or immunization route, as ASCs accumulated in the organ of formation in response to influenza infection, haptenated proteins, and environmental Ags in unimmunized mice.

This study has identified a transcription factor underpinning the migration of plasma cells that is required to form the long-lived population in the BM. In sum, these experiments have revealed mechanisms underlying the formation of immunity, which will be crucial for understanding the pathogenesis of many antibody-mediated diseases.

**Materials and Methods**

Mice, immunizations, and purification of cells. Cd23-Cre (Kwon et al., 2008) were provided by M. Buslinger (The Research Institute of Molecular Pathology, Vienna, Austria) and c-Mybfl/fl mice were provided by J. Frampton (University of Birmingham, Birmingham, England, UK; Emambokus et al., 2003). Blimp0/0 reporter mice as previously described (Kallies et al., 2004). Ly5.1 mice were maintained at the Walter and Eliza Hall Institute. All mice are on a C57BL/6 background and are backcrossed. Animal procedures were approved by the Walter and Eliza Hall Institute Animal Ethics Committee. For primary responses, mice were injected intraperitoneally with 100 µg of NP conjugated to KLH (molar ratio between 13 and 20), precipitated on 10% alum. For secondary responses, 50 µg of NP-KLH in PBS injected intraperitoneally per mouse. For influenza infections, mice were inoculated with 10⁵ PFU of HKx31 (H3N2) influenza virus as previously described (Flynn et al., 1998; Belz et al., 2006). For sort purification, cells were stained with antibodies and purified by FACSARia or Influx (BD), with purity >98%.

**Flow cytometry and antibodies.** Single-cell suspensions were resuspended in PBS 2% FCS and stained for flow cytometric analysis. Cells were analyzed live (with the addition of propidium iodide) on the FACSCanto (BD) and data was analyzed with FlowJo software (Tree Star). The following antibodies were used: CD95 (IO2), IgG1 (x56), Ly5.2 (104), and CD138 (281) from BD; CD19 (ID3), CXCR4 (2B11) from eBioscience; NIP, G41, (SC5) and B220 (RA3-6B2) were conjugated in-house. FcRHI/III (24G; supernatant) was used to block nonspecific binding.

**BM chimeras.** For 50:50 chimera, lethally irradiated Ly5.1 mice (2 × 5.5 Gy) were reconstituted with 50% Ly5.1 BM and 50% c-Mybfl/fl/Cd23lox−/lox or Cd23lox−/lox BM. Mice were reconstituted for 7–8 wk before NP-KLH/alum immunization as described above. Mice were bled before immunization to test chimerism by flow cytometry.

For µMT chimeras, lethally irradiated Ly5.1 mice (2 × 5.5 Gy) were reconstituted with 80% µMT BM and 20% c-Mybfl/flERlox−/− or ERlox−/− BM. Mice were reconstituted for at least six weeks before NP-KLH/alum immunization as described above. 4 wk after immunization, estrogen receptor–activated deletion of lacP-flanked c-Myb alleles was triggered by oral gavage of tamoxifen (5 mg [Sigma-Aldrich] in 83 µl of a solution of 90% peanut oil [Sigma-Aldrich] and 10% ethanol) on two successive days. Deletion was assessed by PCR on sort-purified BM ASCs.

**Chemotaxis.** ASCs were enriched from spleens of pooled mice per genotype using CD138 magnetic beads (Miltenyi Biotec). 10⁵ cells were resuspended in 100 µl RPMI, supplemented with 0.5% BSA and containing PE-labeled beads to facilitate the enumeration of migrating cells. Cells were applied to the top of trans-wells containing CXCL12 (0, 0.1, 0.4, or 1 µg/ml), CXCL10 (0.1 µg/ml), or medium alone in the bottom chamber. Migrated cells were stained with antibodies to B220 and CD138, and total cell count was assessed by flow cytometry with the addition of a known number of beads to each sample. Migration to nil was also assessed and subtracted from each sample when calculating frequency of input.

**Histology, ELISPOT, and ELISA.** Portions of spleens were frozen in OCT (Tissue-Tek), 7-µm sections were cut using a microtome (Leica) and stained for immunohistochemistry as detailed previously (Zotos et al., 2010). ASCs or antibody was analyzed by ELISPOT and ELISA, respectively, as previously described (Zotos et al., 2010). For influenza-specific ELISPOTs,
purified HKx31 influenza virus was disrupted in a 1/10 dilution of lysis buffer (0.05 M Tris, pH 7.5, 0.5% Triton X-100, and 0.6 M KCl) in PBS, pH 7.2, and viral lysate was used to coat wells.

**VH sequencing.** Blimp-1^hi^ CD138^hi^ cells were pre-enriched with CD138 beads and sort-purified on a FACSAria (BD). Three mice per genotype were pooled pre-enrichment. RNA was isolated using the MicroAid Plus (QIAGEN) according to the manufacturer’s guidelines, cDNA was prepared using SuperScript II Reverse transcription (RT Life Technologies) and VH7183 family transcripts amplified with PFU DNA polymerase (Promega) using a degenerate VH7183-forward primer (5'-CCTTAGMAGCCTGGAV-RKTC-3') in combination with the IgG CH1 (5'-GGACAGGGMTC-CAKAGTTCCA-3'). Gel-purified PCR products were 3'-adenylated using Taq polymerase (Applied Biosystems) and cloned into pCR2.1-TOPO (Invitrogen) according to the manufacturer’s instructions. Standard M13 primers were used to amplify cloned inserts from individual colonies and PCR products were purified over columns (QIAGEN) in preparation for sequencing with the relevant CH1 reverse primer (Australian Genome Research Facility). Germline VH7183 sequences were identified using IMGT/V-QUEST (Brochet et al., 2008).

**Statistical analysis.** The Mann-Whitney nonparametric, two-tailed test or an unaired, two-tailed Student’s t test was used for statistical analyses, using GraphPad Prism software.

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