nature immunology

Eosinophils are required for the maintenance of plasma cells in the bone marrow

Van Trung Chu[1], Anja Fröhlich[1,2], Gudrun Steinhauser[1], Tobias Scheel[1], Toralf Roch[1,4], Simon Fillatreau[1], James J Lee[3], Max Löhning[1,2] & Claudia Berek[1]

Plasma cells are of crucial importance for long-term immune protection. It is thought that long-lived plasma cells survive in specialized niches in the bone marrow. Here we demonstrate that bone marrow eosinophils localized together with plasma cells and were the key providers of plasma cell survival factors. *In vitro*, eosinophils supported the survival of plasma cells by secreting the proliferation-inducing ligand APRIL and interleukin-6 (IL-6). In eosinophil-deficient mice, plasma cell numbers were much lower in the bone marrow both at steady state and after immunization. Reconstitution experiments showed that eosinophils were crucial for the retention of plasma cells in the bone marrow. Moreover, depletion of eosinophils induced apoptosis in long-lived bone marrow plasma cells. Our findings demonstrate that the long-term maintenance of plasma cells in the bone marrow requires eosinophils.

© 2011 Nature America, Inc. All rights reserved.

Memory B cells and plasma cells are the two arms of the humoral immune response that ensure long-term protection against pathogens[1–3]. Plasma cells are essential contributors to this protection through their secretion of antigen-specific antibodies. Plasma cells can develop through different pathways[4]. Naive B cells challenged with antigen may directly differentiate into short-lived plasma cells, whereas long-lived plasma cells are generated predominantly in germinal centers during T cell–dependent immune responses[5,6]. Plasma blasts migrate to the bone marrow, where they find an appropriate environment that allows them to differentiate into mature long-lived plasma cells[7–9]. During primary immune responses only a few antigen-specific plasma cells accumulate in the bone marrow, although many more are found after secondary challenge with antigen[10].

Bone marrow stromal cells provide a specific microenvironment that supports the survival of plasma cells through cell contact–dependent signals and cytokines[4,7,11,12]. Bone marrow reticular stromal cells express CXCL12, a chemokine that both attracts plasma cells into the bone marrow and supports their maintenance in this location[13–15]. In addition, cytokines such as APRIL (a proliferation-inducing ligand), IL-6, tumor necrosis factor, IL-10, IL-4 and IL-5 are known to prolong the survival of plasma cells *in vitro*[16]. Although *in vitro* experiments suggest that IL-6 is a crucial cytokine for the long-term survival of plasma cells, the bone marrow of IL-6-deficient mice does not have a lower number of plasma cells[11,16]. In contrast, in mice deficient in APRIL, although the generation of plasma cells is normal, the ability of the bone marrow compartment to support the survival of plasma cells is considerably impaired[17]. Blocking APRIL by treatment of immunized animals with TACI-Ig (a fusion of the APRIL receptor TACI and human immunoglobulin G (IgG)) has been shown to diminish the number of plasma cells in the bone marrow by more than 60% (ref. 18). Furthermore, mice deficient in BCMA, the high-affinity receptor of APRIL expressed by plasma cells, have considerably fewer plasma cells[18]. This suggests a critical role for APRIL in the long-term survival of plasma cells in the bone marrow.

Little is known about the cellular sources of cytokines required for the survival of plasma cells *in vivo*[11,16–19]. In the mucosal-associated lymphoid tissue, APRIL is produced locally by neutrophils and binds to heparin proteoglycans, thus providing a survival niche for plasma cells[20]. Neutrophils that infiltrate tumor lesions in the human bone marrow constitutively produce APRIL[21]. In the murine lymph node, dendritic cells and in particular F4/80$^+$Gr-1^{lo} monocytes-macrophages are the main producers of IL-6 and APRIL[22].

Here we investigated which cells in the bone marrow provide plasma cell survival factors and contribute to the establishment of a niche for the long-term survival of plasma cells. We found that F4/80$^+$Gr-1^{lo} eosinophils were the main source of plasma cell survival factors in the bone marrow. Eosinophils were present in close association with plasma cells, which suggested they are part of the plasma cell survival niche. Eosinophil-deficient mice had considerably fewer plasma cells both before and after antigen injection. Eosinophil reconstitution transiently increased the frequency of plasma cells. In addition, depletion of eosinophils drove long-lived plasma cells into apoptosis. Our findings demonstrate that eosinophils are required for the retention and long-term maintenance of plasma cells in the bone marrow.

[1]Deutsches Rheuma-Forschungszentrum, Institut der Leibniz-Gemeinschaft, Berlin, Germany. [2]Experimental Immunology, Department of Rheumatology and Clinical Immunology, Charite-University Medicine, Berlin, Germany. [3]Department of Biochemistry and Molecular Biology, Division of Pulmonary Medicine, Mayo Clinic Arizona, Scottsdale, Arizona, USA. [4]Present address: Centre for Biomaterial Development, Helmholtz-Zentrum Geesthacht, Berlin, Germany. Correspondence should be addressed to C.B. (berek@drfz.de).

Received 21 July 2010; accepted 6 December 2010; published online 9 January 2011; doi:10.1038/ni.1981

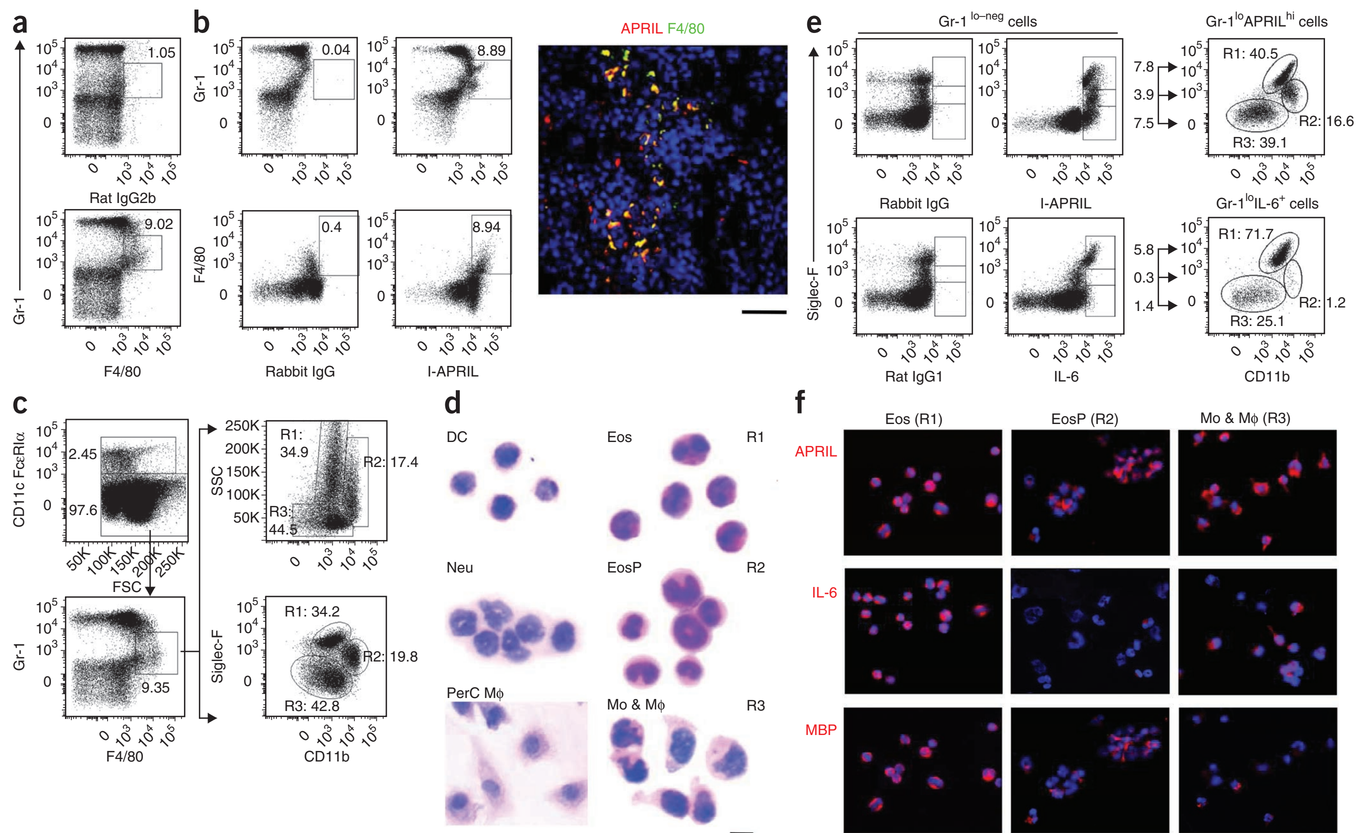

Figure 1 Bone marrow eosinophils provide plasma cell survival factors. (**a**) Flow cytometry of bone marrow cells; numbers adjacent to outlined areas indicate percent Gr-1^{lo}F4/80^{+} cells. (**b**) Staining of intracellular APRIL (I-APRIL) in Gr-1^{lo} (top) and F4/80^{+} (bottom) bone marrow cells (left) and immunohistology showing colocalization of staining for APRIL and F4/80 in the bone marrow (right). Numbers adjacent to outlined areas (left) indicate percent cells in each. Scale bar, 75 μm. (**c**) Flow cytometry identifying three different CD11b^{+} subsets (R1, R2 and R3) among CD11c^{-}FcεRIα^{-}Gr-1^{lo-neg} cells. Numbers in plots indicate percent cells in each outlined area (left) or subset (right). (**d**) Hematoxylin and eosin staining of bone marrow cell subsets sorted and prepared by cytospins. Eos, eosinophil; Neu, neutrophil; EosP, immature eosinophil; PerC Mϕ, peritoneal cavity macrophage (control); Mo & Mϕ, monocyte-macrophage. Scale bar, 15 μm. (**e**) Intracellular expression of APRIL (top) or IL-6 (bottom) in bone marrow cells gated on Gr-1^{lo-neg} cells (left) and frequency of R1, R2 and R3 cells in the fraction of Gr-1^{lo}APRIL^{hi} or Gr-1^{lo}IL-6^{+} cells (right). Numbers adjacent to outlined areas indicate percent cells in each. (**f**) Immunofluorescence of sorted eosinophils (Eos (R1)), immature eosinophils (EosP (R2)) and monocytes-macrophages (Mo & Mϕ (R3)) stained with antibodies specific for APRIL, IL-6 or MBP; nuclei were counterstained with the DNA-intercalating dye DAPI. Scale bar, 45 μm. Data are representative of three to five experiments.

RESULTS

Monocytes, macrophages and eosinophils produce APRIL

To determine which cells in the bone marrow provide plasma cell survival factors, we prepared suspensions of bone marrow cells from naive BALB/c mice and analyzed the cells by multiparameter flow cytometry. Staining with antibodies specific for the granulocyte marker Gr-1 and the macrophage marker F4/80 showed that in unimmunized BALB/c mice, about 10% of bone marrow cells were F4/80^{+}Gr-1^{lo}, and these cells expressed APRIL, as assessed by intracellular staining (**Fig. 1a**,**b**). Costaining of bone marrow tissue sections with antibodies specific for F4/80 and APRIL confirmed that all F4/80^{+}Gr-1^{lo} cells expressed APRIL (**Fig. 1b**, right).

Analysis of side scatter (SSC) and CD11b expression showed that F4/80^{+}Gr-1^{lo} cells in the bone marrow were a heterogeneous cell population (**Fig. 1c**). Only about half of the F4/80^{+}Gr-1^{lo}CD11b^{+} cells had low SSC, as expected for monocytes and macrophages (**Fig. 1c**). Cells with more SSC (fractions R1 and R2) were in addition positive for the eosinophil marker Siglec-F (**Fig. 1c**), which indicated that these cells were eosinophils. It is known that bone marrow eosinophils express F4/80 (ref. 23).

For further characterization we sorted the F4/80^{+}Gr-1^{lo} R1, R2 and R3 cell fractions, as well as conventional dendritic cells (Gr-1^{lo-neg}CD11c^{hi}) and neutrophils (Gr-1^{hi}), from bone marrow and macrophages from the peritoneal cavity (**Supplementary Fig. 1a**,**b**,**c**). Staining with hematoxylin and eosin confirmed that cells in fraction R3 were monocytes and macrophages (F4/80^{+}Gr-1^{lo}CD11b^{int}Siglec-F^{-}SSC^{lo}; **Fig. 1d**), whereas R1 cells (F4/80^{+}Gr-1^{lo}CD11b^{int}Siglec-F^{hi}SSC^{hi}) and R2 cells (F4/80^{+}Gr-1^{lo}CD11b^{hi}Siglec-F^{+}SSC^{int}) were eosinophils, as indicated by the staining of eosin in the cytoplasm. The smaller size and the bilobed nucleus with compact chromatin identified the R1 cells as mature eosinophils, whereas the larger size and the doughnut or ring-shaped nucleus with loose chromatin indicated that the R2 cells were immature eosinophils (**Fig. 1d**). These results show that both monocytes-macrophages and eosinophils produce APRIL in the bone marrow.

Bone marrow eosinophils produce plasma cell survival factors

To directly assess whether eosinophils are a major source of plasma cell survival factors, we stained bone marrow cells for Siglec-F and for intracellular APRIL and IL-6 (**Fig. 1e**). Siglec-F^{hi}Gr-1^{lo} eosinophils had high coexpression of APRIL and IL-6, whereas Siglec-F^{int} Gr-1^{lo}

© 2011 Nature America, Inc. All rights reserved.

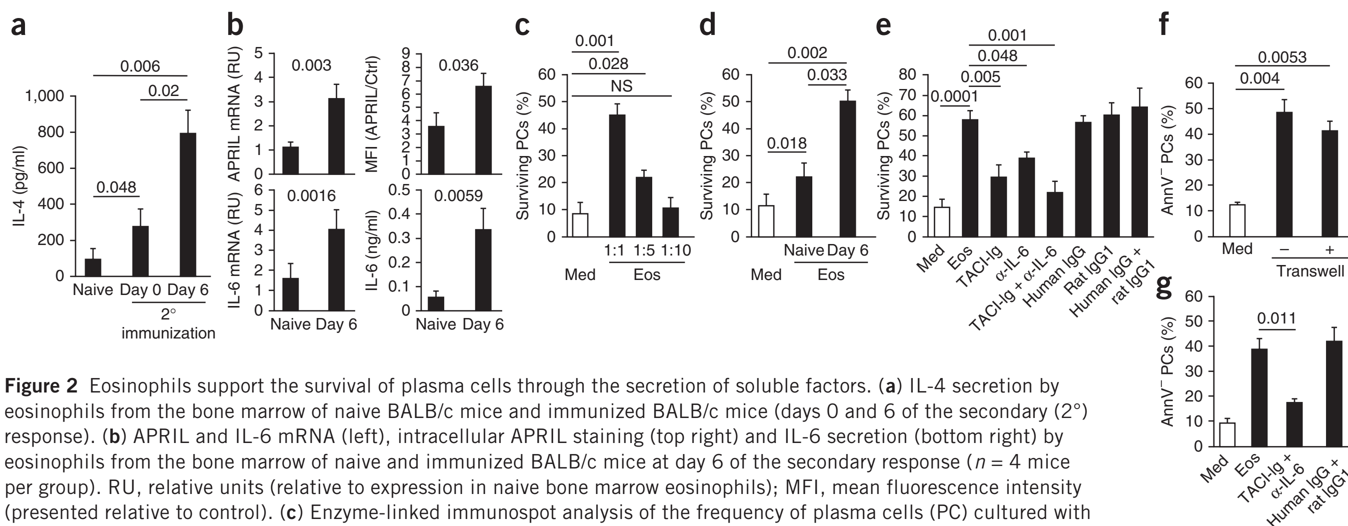

Figure 2 Eosinophils support the survival of plasma cells through the secretion of soluble factors. (**a**) IL-4 secretion by eosinophils from the bone marrow of naive BALB/c mice and immunized BALB/c mice (days 0 and 6 of the secondary (2°) response). (**b**) APRIL and IL-6 mRNA (left), intracellular APRIL staining (top right) and IL-6 secretion (bottom right) by eosinophils from the bone marrow of naive and immunized BALB/c mice at day 6 of the secondary response ($n = 4$ mice per group). RU, relative units (relative to expression in naive bone marrow eosinophils); MFI, mean fluorescence intensity (presented relative to control). (**c**) Enzyme-linked immunospot analysis of the frequency of plasma cells (PC) cultured with medium alone (Med) or various numbers of eosinophils from day 6 of the secondary response. (ratio, PC/Eos horizontal axis) (**d**) Frequency of plasma cells cultured at a ratio of 1:1 with eosinophils from naive and immunized mice (day 6 of secondary response). (**e**) Frequency of plasma cells cultured at a ratio of 1:1 with eosinophils alone from immunized mice (Eos) or along with various inhibitors (horizontal axis). α-IL-6, antibody to IL-6. (**f**) Plasma cells negative for annexin V (AnnV⁻) after culture at a ratio of 1:1 with eosinophils from immunized animals in Transwells with (+) or without (–) inserts. (**g**) Plasma cells negative for annexin V after culture as in **e**. NS, not significant. *P* values, Student's *t*-test. Data are from one of two experiments (error bars, s.d. of triplicate cultures).

© 2011 Nature America, Inc. All rights reserved.

immature eosinophils expressed only APRIL. Siglec-F$^-$Gr-1^{lo} monocytes-macrophages expressed APRIL; however, only a small fraction of them, if any, had low expression of IL-6 as well (**Fig. 1e**). Gating on Gr-1^{lo}APRIL^{hi} or Gr-1^{lo}IL-6$^+$ cells showed that most of the APRIL^{hi} cells (fractions R1 and R2) and IL-6$^+$ cells (fraction R1) were eosinophils (**Fig. 1e**). We confirmed those findings by staining cytospins of eosinophils (R1), immature eosinophils (R2) and monocytes-macrophages (R3) with antibodies specific for APRIL and IL-6 (**Fig. 1f**). We controlled the purity of the sorted populations by using antibodies specific for the major basic protein (MBP), a marker specific for eosinophils[24].

We compared the expression of plasma cell survival factors in bone marrow eosinophils with that in monocytes-macrophages, Gr-1^{hi} neutrophils and CD11c^{hi} conventional dendritic cells (**Supplementary Fig. 2**). Real-time PCR analysis showed that in the bone marrow, APRIL and IL-6 mRNA were expressed mainly in F4/80$^+$Gr-1^{lo} eosinophils and monocytes-macrophages, and this analysis confirmed that eosinophil precursors did not express IL-6 (**Supplementary Fig. 2a**). IL-6 mRNA expression corresponded with IL-6 secretion *in vitro,* for which the highest expression was in mature eosinophils (**Supplementary Fig. 2b**). These data show that bone marrow eosinophils are a major source of cytokines required for the survival of plasma cells.

Eosinophils support the survival of plasma cells

To address whether eosinophils support the survival of plasma cells, we cocultured sorted bone marrow eosinophils and plasma cells (**Supplementary Fig. 1b**,**d**). We prepared eosinophils from unimmunized and immunized BALB/c mice. Injection of the T cell–dependent antigen phOx (2-phenyloxazolone) resulted in a greater frequency of eosinophils in the bone marrow (**Supplementary Fig. 3a**) and induced an activated phenotype, as shown by their enhanced secretion of IL-4 (**Fig. 2a**). There was even greater activation after secondary immunization with soluble phOx (**Fig. 2a**). Together with the enhanced expression of IL-4, the expression of APRIL and IL-6 mRNA and protein was also significantly higher (**Fig. 2b**). We obtained similar results whether we delivered the antigen intraperitoneally in aluminum hydroxide or emulsified in complete Freund's adjuvant (**Fig. 2a**,**b** and **Supplementary Fig. 3a**,**b**).

Cocultures of plasma cells and eosinophils isolated 6 d after secondary immunization showed that eosinophils supported the survival of plasma cells in a dose-dependent manner (**Fig. 2c**). Plasma cell survival was greater when they were cultured with eosinophils isolated from the bone marrow of primary immunized BALB/c mice, and even more so for those isolated after secondary immunization (**Fig. 2d**). The addition of the soluble fusion protein TACI-Ig[25], which blocks APRIL, or of neutralizing antibody to IL-6 to the culture medium resulted in a significantly lower number of surviving antibody-secreting cells (**Fig. 2e**). We noted the greatest effect when we added both inhibitors, which suggested that both APRIL and IL-6 are required for the survival of plasma cells and that both factors are provided by eosinophils.

In addition, approximately 90% of plasma cells were positive for annexin V after 48 h of culture in the absence of eosinophils (**Supplementary Fig. 4a**). Apoptosis of plasma cells was prevented not only by direct contact with eosinophils but also by culture with eosinophils in a two-chamber Transwell system (about 50% of plasma cells were negative for annexin V; **Fig. 2f** and **Supplementary Fig. 4a**). Plasma cell survival was much lower after the addition of TACI-Ig and antibodies specific for IL-6 to the culture medium (**Fig. 2g** and **Supplementary Fig. 4b**), which provided further evidence that eosinophils support the survival of plasma cells by supplying soluble APRIL and IL-6. These data suggest that eosinophils support the survival of plasma cells by secreting APRIL and IL-6.

Eosinophils and plasma cells colocalize in the bone marrow

To determine whether eosinophils and plasma cells interact in the bone marrow, we immunized BALB/c mice with phOx and prepared tissue sections 6 d after the secondary immunization, when newly induced plasma blasts home to the bone marrow. Staining with antibody specific for laminin, the basement membrane protein of endothelial cells, showed that plasma cells localized along the thin-walled venous sinusoids (**Fig. 3a**). Costaining with IgG- and APRIL-specific antibodies showed closely associated pairs of plasma cells and APRIL$^+$ cells (**Fig. 3a**). The association between eosinophils and plasma cells was even greater at day 60 after the secondary immunization. Staining

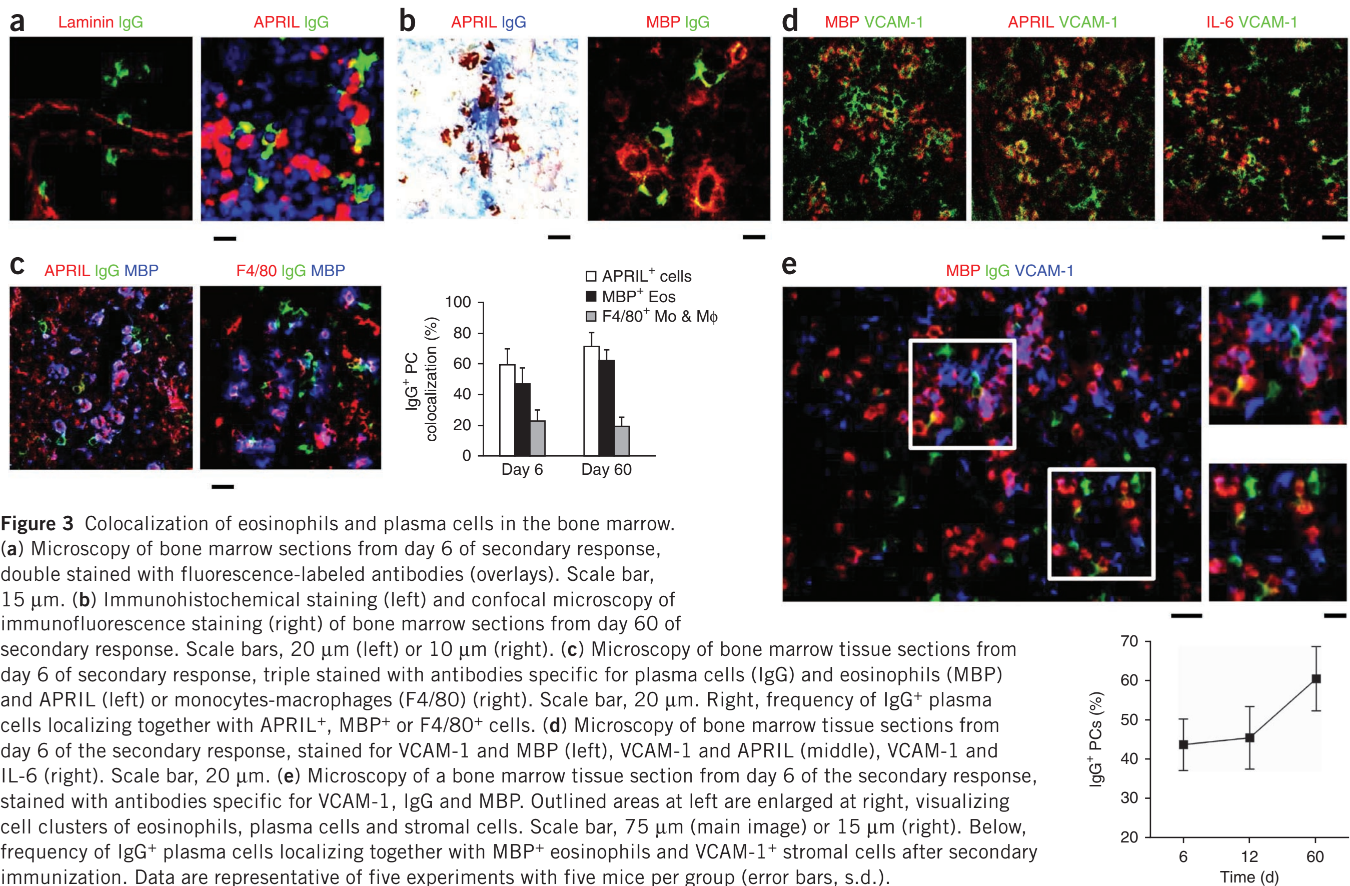

Figure 3 Colocalization of eosinophils and plasma cells in the bone marrow. (**a**) Microscopy of bone marrow sections from day 6 of secondary response, double stained with fluorescence-labeled antibodies (overlays). Scale bar, 15 μm. (**b**) Immunohistochemical staining (left) and confocal microscopy of immunofluorescence staining (right) of bone marrow sections from day 60 of secondary response. Scale bars, 20 μm (left) or 10 μm (right). (**c**) Microscopy of bone marrow tissue sections from day 6 of secondary response, triple stained with antibodies specific for plasma cells (IgG) and eosinophils (MBP) and APRIL (left) or monocytes-macrophages (F4/80) (right). Scale bar, 20 μm. Right, frequency of IgG$^+$ plasma cells localizing together with APRIL$^+$, MBP$^+$ or F4/80$^+$ cells. (**d**) Microscopy of bone marrow tissue sections from day 6 of the secondary response, stained for VCAM-1 and MBP (left), VCAM-1 and APRIL (middle), VCAM-1 and IL-6 (right). Scale bar, 20 μm. (**e**) Microscopy of a bone marrow tissue section from day 6 of the secondary response, stained with antibodies specific for VCAM-1, IgG and MBP. Outlined areas at left are enlarged at right, visualizing cell clusters of eosinophils, plasma cells and stromal cells. Scale bar, 75 μm (main image) or 15 μm (right). Below, frequency of IgG$^+$ plasma cells localizing together with MBP$^+$ eosinophils and VCAM-1$^+$ stromal cells after secondary immunization. Data are representative of five experiments with five mice per group (error bars, s.d.).

of bone marrow tissue sections showed large clusters containing many eosinophils and plasma cells (**Fig. 3b**). Triple staining for MBP and IgG together with either APRIL or F4/80 showed that at day 6 after the secondary immunization, nearly 60% of plasma cells localized together with APRIL$^+$ cells (**Fig. 3c**). Approximately half of the plasma cells were involved in association with eosinophils and only 20% were involved in association with macrophages (**Fig. 3c**). At day 60 after the secondary immunization, the frequency of plasma cells in association with eosinophils was even greater (**Fig. 3c**).

To further characterize the cellular composition of the putative bone marrow plasma cell survival niche, we stained tissue sections with antibodies specific for the adhesion marker VCAM-1 and MBP. VCAM-1 was expressed on stromal cells and also on a fraction of eosinophils (**Fig. 3d**, left). This analysis showed clustering of eosinophils in the underlying reticulum of stromal cells (**Fig. 3d**, left). Higher magnification showed that eosinophils were the main source of APRIL and IL-6 (**Fig. 3d**, middle and right). At 60 d after the secondary immunization, more than 60% of bone marrow plasma cells were present in close association with eosinophils and stromal cells (**Fig. 3e**). Both the colocalization and the provision of APRIL and IL-6 suggested that eosinophils are an essential part of the plasma cell survival niche.

Plasma cells express the chemokine receptor CXCR4 and respond to its ligand, CXCL12 (ref. 13). RT-PCR analysis showed that neither monocytes-macrophages nor eosinophils express CXCL12 (**Supplementary Fig. 5a**). On the basis of that observation, it is unlikely that these cells directly attract plasma cells. However, both monocytes-macrophages and eosinophils expressed CXCR4 at the level of mRNA and protein (**Supplementary Fig. 5b**). The mean fluorescence intensity at the cell surface was greatest for mature eosinophils, whereas there was less intense staining on eosinophil precursors and on monocytes-macrophages (**Supplementary Fig. 5b**). Thus, plasma cells and mature eosinophils localize together in the bone marrow, perhaps by being attracted to the same CXCL12-secreting sites.

Expression of APRIL and IL-6 in eosinophil-deficient mice

To directly address whether eosinophils are required for the maintenance of plasma cells in the bone marrow, we analyzed mice lacking mature eosinophils[26]. For this, we used ΔdblGATA-1 mice, which have a deletion in the high-affinity GATA-binding site of the promoter of the gene encoding the transcription factor GATA-1, which allows normal hematopoiesis but specifically blocks the development of mature eosinophils. The frequency of F4/80$^+$Gr-1^{lo} cells was about 50% lower in these mice than in wild-type BALB/c mice (**Fig. 4a**). Staining for CD11b and Siglec-F demonstrated the specific absence of mature eosinophils (**Fig. 4a**). Furthermore, we confirmed the absence of eosinophils in ΔdblGATA-1 mice by staining bone marrow tissue sections with MBP-specific antibodies (**Fig. 4b**). We observed only a few weakly MBP$^+$ cells, presumably eosinophil precursors.

In contrast to wild-type BALB/c mice, ΔdblGATA-1 mice had low APRIL expression in the bone marrow (**Fig. 4b**) and, in line with those observations, APRIL mRNA was much lower in abundance (**Fig. 4c**). The weak APRIL staining was restricted to monocytes-macrophages and endothelial cells. In addition, eosinophil-deficient mice had fewer IL-6-expressing cells (**Fig. 4b**). Accordingly, we extracted less IL-6 mRNA from total bone marrow cells of ΔdblGATA-1 mice than from that of BALB/c mice, and when we cultured bone marrow cells from eosinophil-deficient mice *in vitro*, IL-6 secretion was almost undetectable (**Fig. 4c**).

© 2011 Nature America, Inc. All rights reserved.

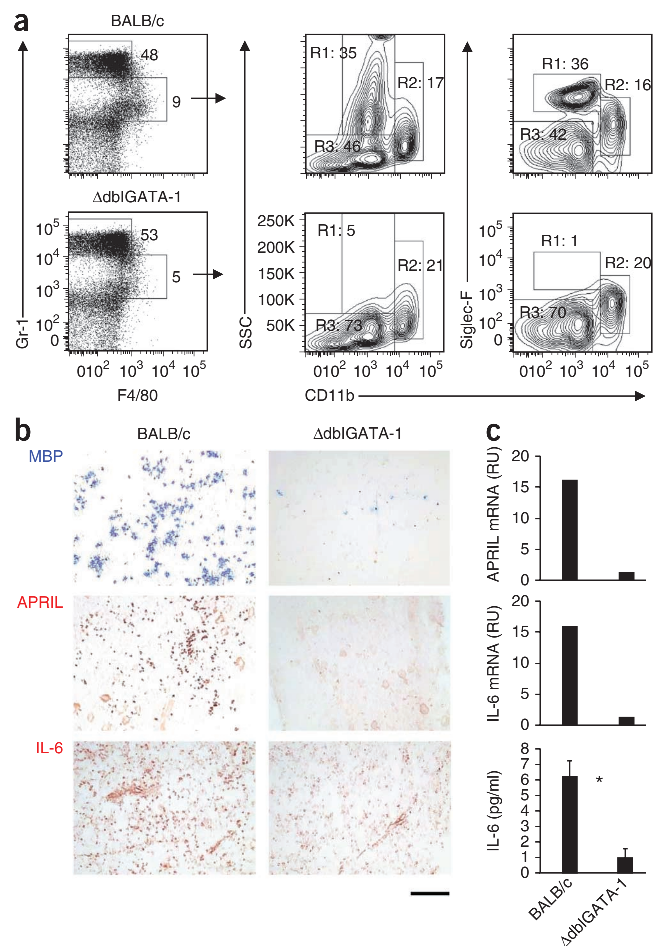

Figure 4 Lower expression of APRIL and IL-6 in the bone marrow of eosinophil-deficient mice. (**a**) Flow cytometry of eosinophils (R1), immature eosinophils (R2) and monocytes-macrophages (R3) from BALB/c and ΔdblGATA-1 mice, with gating on bone marrow F4/80$^+$Gr-1^{lo} cells. Numbers adjacent to outlined areas indicate percent cells in each. (**b**) Immunohistological staining of bone marrow tissue sections from BALB/c and ΔdblGATA-1 mice for MBP, APRIL and IL-6. Scale bar, 150 μm. (**c**) Expression of APRIL mRNA (top) and IL-6 mRNA (middle) and IL-6 secretion (bottom) by total bone marrow cells from BALB/c and ΔdblGATA-1 mice. RU, relative units (relative to expression in bone marrow cells from ΔdblGATA-1 mice). $*P < 0.002$ (Student's *t*-test). Data are representative of four experiments with four mice per genotype (error bars, s.d.).

We obtained similar results with PHIL mice, another mouse strain which lacks mature eosinophils[27]. In these mice, expression of the cytocidal diphtheria toxin A chain under the control of the eosinophil peroxidase promoter prevents the development of mature eosinophils[27]. We found much lower expression of APRIL and IL-6 in the bone marrow of these mice, with the remaining APRIL expression being restricted to monocytes and macrophages (**Supplementary Fig. 6**). These data indicate that mature eosinophils are the main source of the plasma cell–survival factors APRIL and IL-6 in the bone marrow.

Plasma cells in bone marrow of eosinophil-deficient mice

We investigated whether the absence of eosinophils affected the number of plasma cells in bone marrow. At steady state, ΔdblGATA-1 mice had a much lower number of plasma cells in the bone marrow (**Fig. 5a**). The frequency of CD138$^+$ plasma cells (**Fig. 5a**, left) and the absolute number of IgG$^+$ plasma cells (**Fig. 5a**, middle) were one third lower, as shown by flow cytometry and enzyme-linked immunospot assay. Those observations correlated with significantly less secretion of total IgG by cultured bone marrow cells from ΔdblGATA-1 mice (**Fig. 5a**, right). Staining of bone marrow tissue sections provided further evidence that the number of plasma cells was significantly lower in the bone marrow of ΔdblGATA-1 than in that of wild-type BALB/c mice (**Supplementary Fig. 7a**: $P = 0.001$). We found significantly lower bone marrow plasma cell numbers at steady state in PHIL mice as well (**Supplementary Fig. 7a**; $P = 0.005$).

We found normal development of B cells in the bone marrow of ΔdblGATA-1 mice (**Supplementary Fig. 8a**) and normal differentiation into newly formed transitional B cells from type 1 (T1) and type 2 (T2), marginal zone B cells and follicular B cells (**Supplementary Fig. 8b**). Furthermore, staining of bone marrow sections from ΔdblGATA-1 mice for laminin and VCAM-1 demonstrated typical organization of the bone marrow structure and normal development of the venous and arterial sinus architecture (**Supplementary Fig. 9a**). The expression of CXCL12 mRNA in total bone marrow cells suggested that stromal cells had normal expression of chemokines (**Supplementary Fig. 9b**). These observations suggest that although deficiency in eosinophils does not affect the development and organization of the bone marrow, it does impair the maintenance of plasma cells at steady state.

Plasma cell accumulation in immune responses

Next we investigated the maintenance of plasma cells in bone marrow and spleen after immunization with phOx. To exclude the possibility of a general defect in the generation of antigen-specific plasma cells, we analyzed the splenic immune response in ΔdblGATA-1 mice. We found that ΔdblGATA-1 mice responded with normal germinal center development (**Supplementary Fig. 10a**). As described for wild-type BALB/c mice[28,29], antibodies with a variable region kappa-Ox1 (Vk-Ox1) light chain dominated the phOx-specific immune response. Sequencing showed normal accumulation of somatic mutations and selection for high-affinity variants (**Supplementary Fig. 10b**). Furthermore, the frequency of splenic plasma cells was similar to that in BALB/c mice (**Supplementary Fig. 10c**). Nevertheless, the frequency and number of plasma cells in bone marrow of ΔdblGATA-1 mice were significantly lower (**Fig. 5b** and **Supplementary Fig. 7b**).

After secondary immunization, we observed a distinctly detectable influx of antigen-specific plasma cells into the bone marrow (**Fig. 5b**), in line with the normal expression of CXCR4 on splenic plasma cells (**Supplementary Fig. 9c**). The overall kinetics for the increase and decrease in plasma cell numbers in the bone marrow were similar for ΔdblGATA-1 and wild-type BALB/c mice. We detected the greatest number of total and antigen-specific plasma cells 6 d after antigen boost, followed by a steep decrease (**Fig. 5b** and **Supplementary Fig. 7b**). In eosinophil-deficient ΔdblGATA-1 mice, the number of antigen-specific plasma cells in the bone marrow 12 d after secondary immunization was no longer significantly different from the number before boost (**Fig. 5c**). In contrast, even 60 d after booster injection of antigen, the frequency and absolute number of antigen-specific plasma cells remained significantly greater in the bone marrow of immunized wild-type mice (**Fig. 5b**,**c**).

As eosinophils are thought to be mainly associated with T helper type 2 (T_H2) immune responses[30], we sought to determine whether the survival of plasma cells in the bone marrow was affected by the absence of eosinophils in a T_H1 response. We infected BALB/c and ΔdblGATA-1

© 2011 Nature America, Inc. All rights reserved.
npg

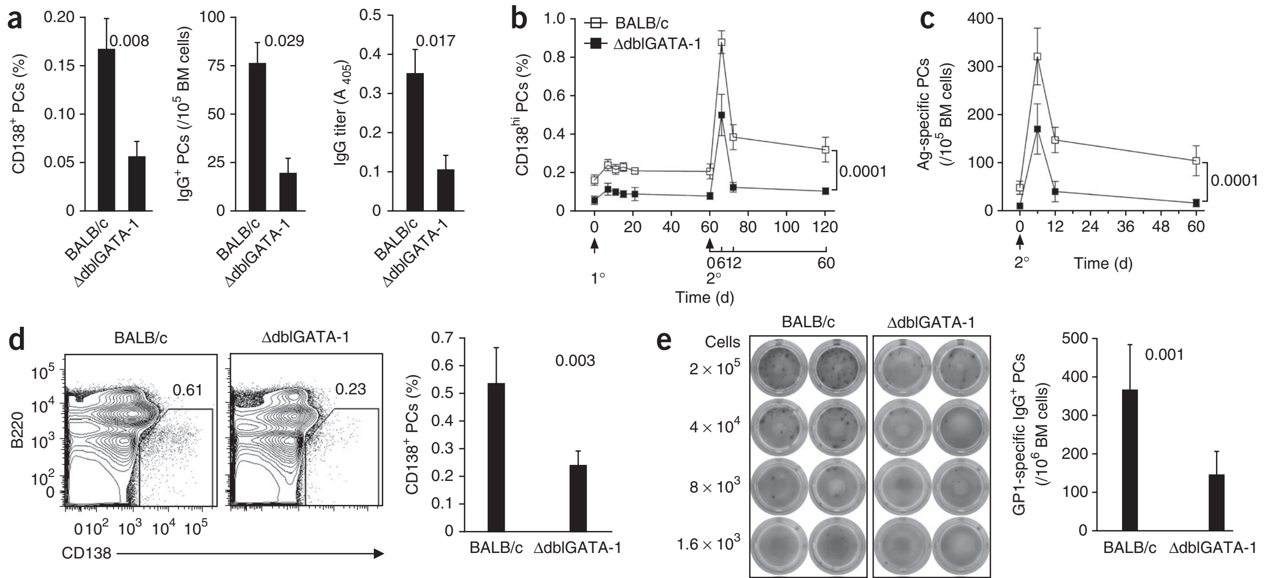

Figure 5 Impaired accumulation of plasma cells in the bone marrow of eosinophil-deficient mice. (**a**) Frequency of $CD138^+$ plasma cells (left), number of IgG^+ plasma cells (middle) and IgG secretion (right), assessed for total bone marrow (BM) cells of unimmunized BALB/c mice ($n = 3$) and ΔdblGATA-1 mice ($n = 4$). Concentration of IgG in supernatants of total bone marrow cell cultures. A_{405}, absorbance at 405 nm. (**b**,**c**) Frequency of $CD138^{hi}$ plasma cells (**b**) and antigen-specific plasma cells (**c**) in the bone marrow of phOx-immunized BALB/c and ΔdblGATA-1 mice during the primary (1°) and secondary (2°) response ($n = 4$ mice per group per time point). (**d**,**e**) Immune response in the bone marrow of BALB/c and ΔdblGATA-1 mice 7 months after LCMV infection. (**d**) Frequency of $CD138^+$ plasma cells. Numbers in plots indicate percent cells in outlined area. (**e**) Enzyme-linked immunospot analysis of IgG^+ plasma cells specific for glycoprotein 1 GP1. Right (**d**,**e**), data summaries. *P* values, Student's *t*-test and two-way ANOVA. Data are representative of three experiments with six mice per group (error bars, s.d.).

mice with lymphocytic choriomeningitis virus (LCMV), which induces a strong T_H1 response[31]. Eosinophil-deficient ΔdblGATA-1 mice responded as well as BALB/c mice to LCMV (**Supplementary Fig. 11**). Nevertheless, the frequency of plasma cells was significantly lower in the bone marrow of ΔdblGATA-1 mice (**Fig. 5d**). This was reflected in the total number of IgG^+ plasma cells (data not shown) and in the number of plasma cells specific for the glycoprotein 1 antigen of LCMV (**Fig. 5e**). These results show that eosinophils are required for the maintenance of plasma cells in the bone marrow in both T_H1- and T_H2-dependent immune responses.

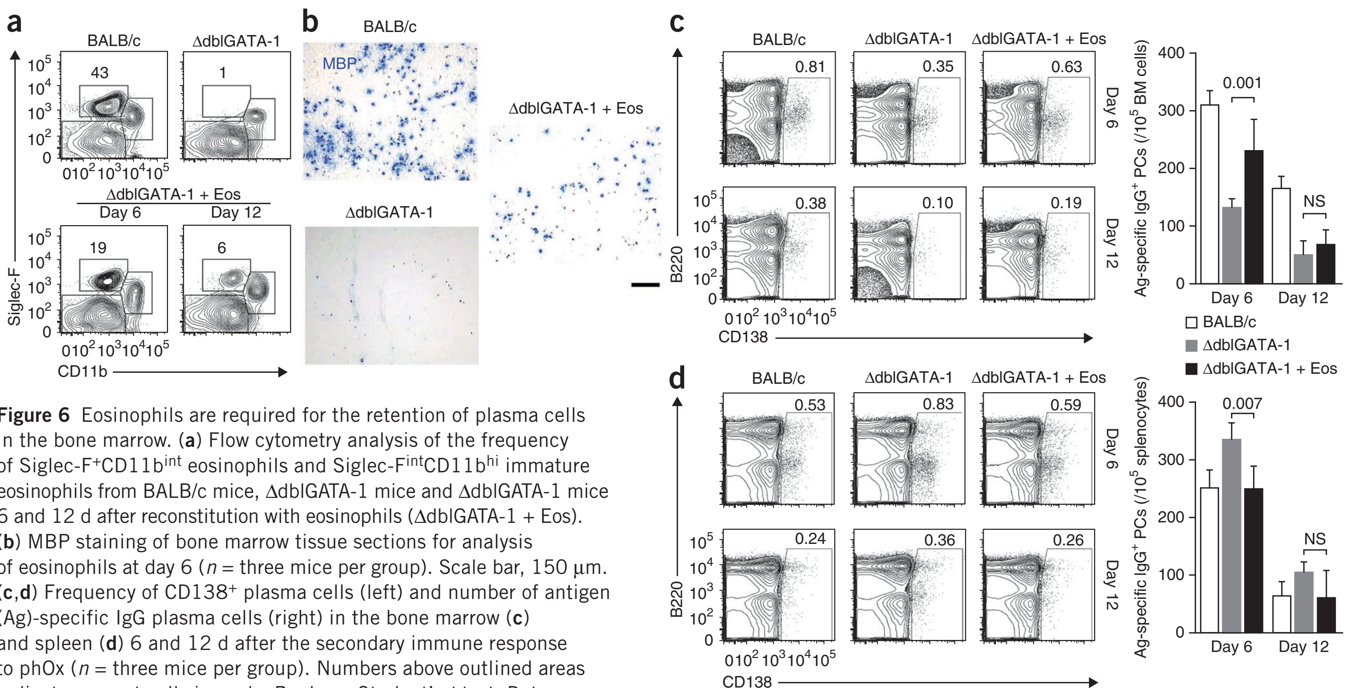

Figure 6 Eosinophils are required for the retention of plasma cells in the bone marrow. (**a**) Flow cytometry analysis of the frequency of Siglec-$F^+$$CD11b^{int}$ eosinophils and Siglec-$F^{int}$$CD11b^{hi}$ immature eosinophils from BALB/c mice, ΔdblGATA-1 mice and ΔdblGATA-1 mice 6 and 12 d after reconstitution with eosinophils (ΔdblGATA-1 + Eos). (**b**) MBP staining of bone marrow tissue sections for analysis of eosinophils at day 6 (n = three mice per group). Scale bar, 150 μm. (**c**,**d**) Frequency of $CD138^+$ plasma cells (left) and number of antigen (Ag)-specific IgG plasma cells (right) in the bone marrow (**c**) and spleen (**d**) 6 and 12 d after the secondary immune response to phOx (n = three mice per group). Numbers above outlined areas indicate percent cells in each. *P* values, Student's *t*-test. Data are representative of two experiments (error bars, s.d.).

© 2011 Nature America, Inc. All rights reserved.

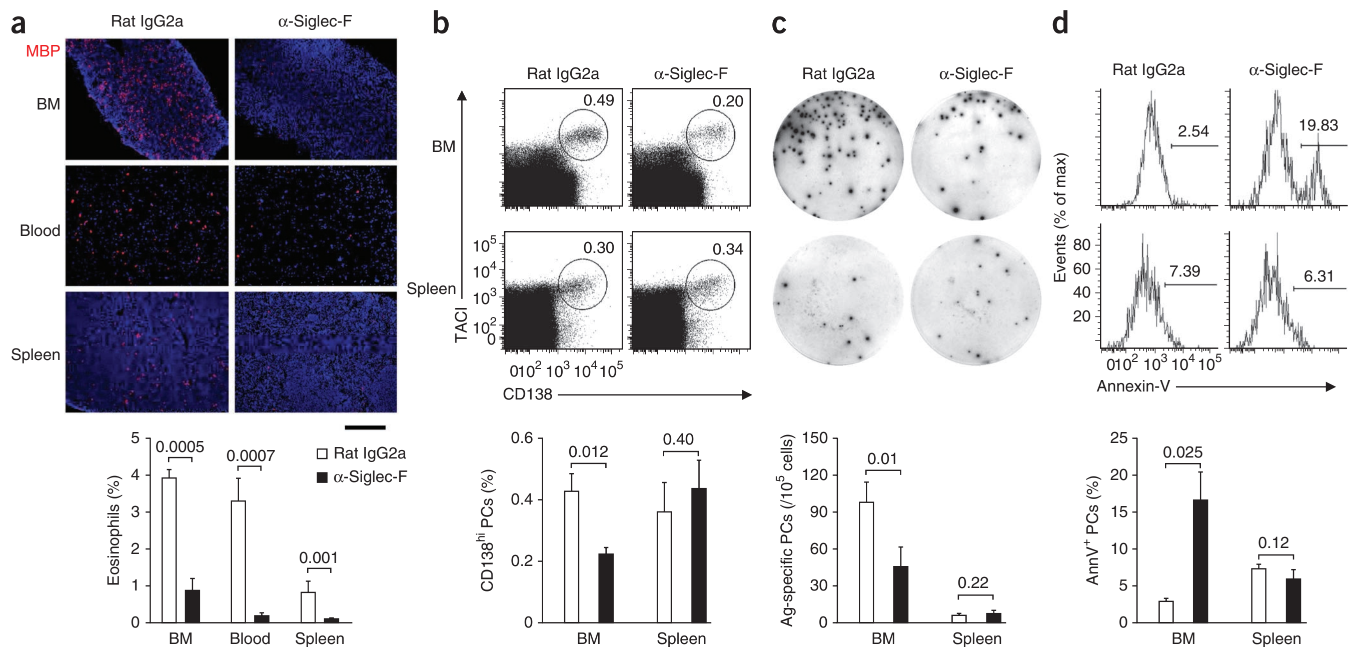

Figure 7 The long-term survival of plasma cells is dependent on eosinophils. (**a**) MBP+ eosinophils in tissue sections from bone marrow and spleen and in cytospins from blood samples of control BALB/c mice (Rat IgG2a) or BALB/c mice depleted of eosinophils (α-Siglec-F; $n = 3$ mice per group). Scale bar, 300 μm. Below, data summary. (**b**–**d**) Frequency of CD138+TACI+ plasma cells (**b**), absolute number of antigen-specific plasma cells (**c**) and frequency of annexin V–positive (AnnV+) apoptotic plasma cells (**d**) in the bone marrow and spleen of mice as in **a** ($n = 6$ mice per group). Below, data summaries. *P* values, Student's *t*-test. Data are representative of two experiments (error bars, s.d.).

© 2011 Nature America, Inc. All rights reserved.

Eosinophil-dependent bone marrow retention of plasma cells

We set up reconstitution experiments to directly investigate whether the retention of plasma cells in the bone marrow required the presence of eosinophils. We sorted bone marrow eosinophils 6 d after secondary immunization of BALB/c mice, and transferred the cells into recipient ΔdblGATA-1 mice. Analysis of reconstituted ΔdblGATA-1 bone marrow showed that transferred eosinophils migrated back into the bone marrow; however, only about one third of them survived 12 d after transfer (**Fig. 6a**,**b**).

Eosinophil reconstitution of ΔdblGATA-1 mice affected the number of plasma cells in bone marrow and spleen after immunization (**Fig. 6c**,**d**). At day 6 after secondary immunization, the presence of eosinophils was sufficient to result in a significantly greater number of antigen-specific plasma cells in the bone marrow of ΔdblGATA-1 mice (**Fig. 6c**) and concomitantly resulted in a lower number in the spleen, to nearly normal numbers (**Fig. 6d**). However, the effect was transient, as the number of plasma cells in the bone marrow of reconstituted mice was similar to that of unreconstituted ΔdblGATA-1 mice 12 d after secondary immunization, when transferred eosinophils numbers had decreased. These data demonstrate a direct role for eosinophils in the retention of plasma cells in the bone marrow.

Eosinophil-dependent long-term survival of plasma cells

To determine whether eosinophils are required for the long-term survival of plasma cells, we immunized BALB/c mice with phOx. At day 60 after secondary immunization, long-lived antigen-specific plasma cells are found mainly in the bone marrow[7]. Injection with a Siglec-F-specific antibody, which is known to induce apoptosis in eosinophils[32], resulted in nearly complete depletion of eosinophils in the blood, spleen and bone marrow of BALB/c mice 4 d after the final injection (**Fig. 7a**). Depletion of eosinophils affected the total number of plasma cells and the number of antigen-specific plasma cells in the bone marrow to an equal extent (**Fig. 7b**,**c**). Just 1 week after the first injection of antibody to Siglec-F, the frequency of bone marrow plasma cells was 50% lower and on average, 16% of the remaining plasma cells were positive for annexin V (**Fig. 7d**), which suggested that in the absence of eosinophils, plasma cells undergo apoptosis. We observed a lower number of plasma cells after depletion of eosinophils only in the bone marrow. We observed no effect on the number of plasma cells (**Fig. 7b**,**c**) and no greater frequency of annexin V–positive plasma cells (**Fig. 7d**) in the spleen. These data demonstrate that eosinophils have a pivotal role for the long-term survival of plasma cells in the bone marrow. As the main source of plasma cell survival factors, eosinophils are an essential part of the plasma cell survival niche.

DISCUSSION

The bone marrow provides a microenvironment that supports the long-term maintenance of plasma cells by providing survival factors such as IL-6 and APRIL[16–18]. Our work here has shown that F4/80+Gr-1lo eosinophils were the main source for these survival factors in the bone marrow. *In vitro* coculture showed that eosinophils supported plasma cell survival by secreting APRIL and IL-6. The analysis of eosinophil-deficient mice showed that in the absence of eosinophils, the maintenance of plasma cells in the bone marrow was impaired. We have demonstrated that eosinophils were required both for plasma cell retention and also for the long-term survival of these cells in the bone marrow. Thus, our data indicate eosinophils are central players in the long-term maintenance of immune protection.

Expression of CXCR4 on mature eosinophils and plasma cells may confer similar homing properties to both cell types, resulting in their colocalization to sites of CXCL12 secretion. During hematopoiesis, CXCR4 is required for the retention of eosinophil precursors in the

bone marrow[33,34], and during immune responses, CXCL12 secreted by bone marrow stromal cells may guide resident eosinophils into the plasma cell survival niche[12,13]. Although both eosinophils and monocytes and macrophages express CXCR4, we found that eosinophils colocalized mainly with plasma cells. Lower surface expression of CXCR4 on monocytes and macrophages may explain this preferential association of eosinophils with plasma cells.

We assessed the importance of eosinophils for the long-term maintenance of plasma cells in the bone marrow using ΔdblGATA-1 mice, a mouse strain that has normal immune structures but lacks mature eosinophils. We used phOx antigen precipitated in aluminum hydroxide for primary immunization. This adjuvant induces antigen-independent activation of eosinophils measurable as an enhanced IL-4 secretion[35,36]. Activated eosinophils promote the early population expansion and differentiation of antigen-specific B cells into IgM-secreting plasma cells[35]. However, after secondary immunization with soluble phOx, we noted even greater activation, and enhanced IL-4 secretion by eosinophils was accompanied by higher expression of the plasma cell survival factors APRIL and IL-6. This probably accounted for the finding that eosinophils isolated from mice after secondary immunization had improved ability to prolong the survival of plasma cells.

The finding that immunization of ΔdblGATA-1 mice with the T cell–dependent antigen phOx or infection with LCMV induced normal antigen-specific immune responses supports the idea that eosinophils are not required for the induction of either T_H2 or T_H1 responses. However, eosinophils are needed for the retention and long-term maintenance of plasma blasts in the bone marrow. Our data suggest that eosinophils are required as a continuous source of survival factors. As soon as eosinophils are depleted, plasma cells rapidly die by apoptosis. Once plasmablasts have settled in the bone marrow and developed into mature plasma cells, they are apparently unable to leave the bone marrow. In line with that interpretation, we found no substantial increase in the number of plasma cells in the spleen of BALB/c mice depleted from eosinophils for 1 week. We observed relocation of plasma cells to the spleen only at the early phase of the immune response, when the newly generated plasma blasts were unable to accumulate in the bone marrow of eosinophil-deficient ΔdblGATA-1 mice. Whereas eosinophils seem to provide a constitutive source of plasma cell survival factors in the bone marrow, dendritic cells and monocytes-macrophages, together with neutrophils and epithelial cells, may provide sufficient APRIL and IL-6 to support the maintenance of plasma cells at peripheral sites, such as lymph nodes and lamina propria[20,37,38].

Are eosinophils of importance in human immune responses? One indication that this may indeed be the case is provided by a report describing a patient with common variant immune deficiency who lacked eosinophils and had very few plasma cells in the bone marrow[39]. That finding supports the idea that like their mouse counterparts, human eosinophils have a major role in the survival of plasma cells. Eosinophils have an important role in chronic asthmatic inflammation, being critically involved in T cell activation in allergic inflammatory responses both as antigen-presenting cells and also as a major source of T_H2 cytokines such as IL-4 (refs.30,40). In allergic or asthmatic situations, eosinophils attracted into the inflamed mucosal tissues may not only support the T_H2-mediated inflammatory process but also prolong the ectopic maintenance of plasma cells and thus further enhance the allergen-specific immune responses. As our data have shown that eosinophils are required for the long-term survival of plasma cells, eosinophil-directed therapy might provide a new therapeutic approach for the treatment of autoimmune diseases and other diseases in which plasma cells contribute to the pathogenesis.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/natureimmunology/.

Note: Supplementary information is available on the Nature Immunology website.

ACKNOWLEDGMENTS

We thank N.A. Lee (Mayo Clinic, Scottsdale, Arizona, USA) for MBP-specific antibody; the members of the Deutsches Rheuma-Forschungszentrum, in particular T. Kaiser, for technical support; and R.S. Jack, A. Hegazy and A. Radbruch for critical reading of the manuscript. Supported by Deutsche Forschungsgemeinschaft (BE 1171/2-1 to C.B.; SFB 618 and SFB 650 to M.L.), Bundesministerium für Bildung und Forschung (0315267D to M.L.), the Volkswagen Foundation (Lichtenberg Professorship to M.L.) and the Berlin Senate of Research and Education (to Deutsches Rheuma-Forschungszentrum).

AUTHOR CONTRIBUTIONS

V.T.C., C.B. and M.L. designed experiments and analyzed the data; V.T.C. did most of the experiments; A.F. and M.L. analyzed the immune response to LCMV; T.S. analyzed the GC response; J.J.L. contributed the PHIL mouse and eosinophil-specific antibodies; T.R. and G.S. helped with data acquisition; and V.T.C., C.B., M.L., A.F. and S.F. prepared the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

Published online at http://www.nature.com/natureimmunology/.
Reprints and permissions information is available online at http://npg.nature.com/reprintsandpermissions/.

1. Ahmed, R. & Gray, D. Immunological memory and protective immunity: understanding their relation. *Science* **272**, 54–60 (1996).
2. Mamani-Matsuda, M. *et al.* The human spleen is a major reservoir for long-lived vaccinia virus-specific memory B cells. *Blood* **111**, 4653–4659 (2008).
3. Manz, R.A., Hauser, A.E., Hiepe, F. & Radbruch, A. Maintenance of serum antibody levels. *Annu. Rev. Immunol.* **23**, 367–386 (2005).
4. Fairfax, K.A., Kallies, A., Nutt, S.L. & Tarlinton, D.M. Plasma cell development: from B-cell subsets to long-term survival niches. *Semin. Immunol.* **20**, 49–58 (2008).
5. Smith, K.G., Light, A., Nossal, G.J. & Tarlinton, D.M. The extent of affinity maturation differs between the memory and antibody-forming cell compartments in the primary immune response. *EMBO J.* **16**, 2996–3006 (1997).
6. Shapiro-Shelef, M. & Calame, K. Regulation of plasma-cell development. *Nat. Rev. Immunol.* **5**, 230–242 (2005).
7. Radbruch, A. *et al.* Competence and competition: the challenge of becoming a long-lived plasma cell. *Nat. Rev. Immunol.* **6**, 741–750 (2006).
8. Blink, E.J. *et al.* Early appearance of germinal center-derived memory B cells and plasma cells in blood after primary immunization. *J. Exp. Med.* **201**, 545–554 (2005).
9. Dilosa, R.M., Maeda, K., Masuda, A., Szakal, A.K. & Tew, J.G. Germinal center B cells and antibody production in the bone marrow. *J. Immunol.* **146**, 4071–4077 (1991).
10. Benner, R., Hijmans, W. & Haaijman, J.J. The bone marrow: the major source of serum immunoglobulins, but still a neglected site of antibody formation. *Clin. Exp. Immunol.* **46**, 1–8 (1981).
11. Minges Wols, H.A., Underhill, G.H., Kansas, G.S. & Witte, P.L. The role of bone marrow-derived stromal cells in the maintenance of plasma cell longevity. *J. Immunol.* **169**, 4213–4221 (2002).
12. Tokoyoda, K., Egawa, T., Sugiyama, T., Choi, B.I. & Nagasawa, T. Cellular niches controlling B lymphocyte behavior within bone marrow during development. *Immunity* **20**, 707–718 (2004).
13. Hargreaves, D.C. *et al.* A coordinated change in chemokine responsiveness guides plasma cell movements. *J. Exp. Med.* **194**, 45–56 (2001).
14. Hauser, A.E. *et al.* Chemotactic responsiveness toward ligands for CXCR3 and CXCR4 is regulated on plasma blasts during the time course of a memory immune response. *J. Immunol.* **169**, 1277–1282 (2002).
15. Nie, Y. *et al.* The role of CXCR4 in maintaining peripheral B cell compartments and humoral immunity. *J. Exp. Med.* **200**, 1145–1156 (2004).
16. Cassese, G. *et al.* Plasma cell survival is mediated by synergistic effects of cytokines and adhesion-dependent signals. *J. Immunol.* **171**, 1684–1690 (2003).
17. Belnoue, E. *et al.* APRIL is critical for plasmablast survival in the bone marrow and poorly expressed by early-life bone marrow stromal cells. *Blood* **111**, 2755–2764 (2008).

© 2011 Nature America, Inc. All rights reserved.

18. O'Connor, B.P. *et al.* BCMA is essential for the survival of long-lived bone marrow plasma cells. *J. Exp. Med.* **199**, 91–98 (2004).
19. Lamb, R.J., Capocasale, R.J., Duffy, K.E., Sarisky, R.T. & Mbow, M.L. Identification and characterization of novel bone marrow myeloid DEC205$^+$Gr-1$^+$ cell subsets that differentially express chemokine and TLRs. *J. Immunol.* **178**, 7833–7839 (2007).
20. Huard, B. *et al.* APRIL secreted by neutrophils binds to heparan sulfate proteoglycans to create plasma cell niches in human mucosa. *J. Clin. Invest.* **118**, 2887–2895 (2008).
21. Mhawech-Fauceglia, P. *et al.* The source of APRIL up-regulation in human solid tumor lesions. *J. Leukoc. Biol.* **80**, 697–704 (2006).
22. Mohr, E. *et al.* Dendritic cells and monocyte/macrophages that create the IL-6/APRIL-rich lymph node microenvironments where plasmablasts mature. *J. Immunol.* **182**, 2113–2123 (2009).
23. McGarry, M.P. & Stewart, C.C. Murine eosinophil granulocytes bind the murine macrophage-monocyte specific monoclonal antibody F4/80. *J. Leukoc. Biol.* **50**, 471–478 (1991).
24. Lee, J.J. *et al.* Interleukin-5 expression in the lung epithelium of transgenic mice leads to pulmonary changes pathognomonic of asthma. *J. Exp. Med.* **185**, 2143–2156 (1997).
25. Chu, V.T., Enghard, P., Riemekasten, G. & Berek, C. In vitro and in vivo activation induces BAFF and APRIL expression in B cells. *J. Immunol.* **179**, 5947–5957 (2007).
26. Yu, C. *et al.* Targeted deletion of a high-affinity GATA-binding site in the GATA-1 promoter leads to selective loss of the eosinophil lineage in vivo. *J. Exp. Med.* **195**, 1387–1395 (2002).
27. Lee, J.J. *et al.* Defining a link with asthma in mice congenitally deficient in eosinophils. *Science* **305**, 1773–1776 (2004).
28. Berek, C., Berger, A. & Apel, M. Maturation of the immune response in germinal centers. *Cell* **67**, 1121–1129 (1991).
29. Berek, C. & Milstein, C. Mutation drift and repertoire shift in the maturation of the immune response. *Immunol. Rev.* **96**, 23–41 (1987).
30. Spencer, L.A. & Weller, P.F. Eosinophils and Th2 immunity: contemporary insights. *Immunol. Cell Biol.* **88**, 250–256 (2010).
31. Löhning, M. *et al.* Long-lived virus-reactive memory T cells generated from purified cytokine-secreting T helper type 1 and type 2 effectors. *J. Exp. Med.* **205**, 53–61 (2008).
32. Zimmermann, N. *et al.* Siglec-F antibody administration to mice selectively reduces blood and tissue eosinophils. *Allergy* **63**, 1156–1163 (2008).
33. Nagase, H. *et al.* Expression of CXCR4 in eosinophils: functional analyses and cytokine-mediated regulation. *J. Immunol.* **164**, 5935–5943 (2000).
34. Ma, Q., Jones, D. & Springer, T.A. The chemokine receptor CXCR4 is required for the retention of B lineage and granulocytic precursors within the bone marrow microenvironment. *Immunity* **10**, 463–471 (1999).
35. Jordan, M.B., Mills, D.M., Kappler, J., Marrack, P. & Cambier, J.C. Promotion of B cell immune responses via an alum-induced myeloid cell population. *Science* **304**, 1808–1810 (2004).
36. McKee, A.S. *et al.* Alum induces innate immune responses through macrophage and mast cell sensors, but these sensors are not required for alum to act as an adjuvant for specific immunity. *J. Immunol.* **183**, 4403–4414 (2009).
37. He, B. *et al.* Intestinal bacteria induce T cell-independent IgA2 class switching by linking epithelial cells with lamina propria B cells through APRIL. *J. Immunol.* **28**, 812–826 (2007).
38. Cerutti, A. Location, location, location: B-cell differentiation in the gut lamina propria. *Mucosal Immunol.* **1**, 8–10 (2008).
39. Juhlin, L. & Michaelsson, G. A new syndrome characterised by absence of eosinophils and basophils. *Lancet* **1**, 1233–1235 (1977).
40. Hogan, S.P. Recent advances in eosinophil biology. *Int. Arch. Allergy Immunol.* **143** (Suppl. 1), 3–14 (2007).

© 2011 Nature America, Inc. All rights reserved.

© 2011 Nature America, Inc. All rights reserved.

ONLINE METHODS

Mice. BALB/c mice were from Charles River Laboratories; ΔdblGATA-1 mice (BALB/c background) were from The Jackson Laboratory; and PHIL (C57BL/6 background) and C57BL/6 mice were from Mayo Clinic Arizona, US. For primary immunization, mice were immunized intraperitoneally with 100 μg phOx coupled to chicken serum albumin (CSA), precipitated with aluminium hydroxide or emulsified in complete Freund's adjuvant. After 6–8 weeks, mice were boosted intravenously with 100 μg soluble antigen. BALB/c and ΔdblGATA-1 mice were infected with 2×10^6 plaque-forming units of LCMV strain WE. Animal experiments were approved by the animal care and use committee (Landesamt für Gesundheit und Soziales, Berlin).

Antibodies and reagents. Antibody to CD11b (anti-CD11b; M1/70), anti-CD11c (N418), anti-Ly6G (Gr-1, RB6-8C5), anti-F4/80, anti-VCAM-1 (6C71), anti-IL-6 (MP5-20F3), anti-B220 (RA3.6B2), anti-IgD (11.26c), anti-GL-7, anti-IgM (M41), anti-CD4 (GK1.5) and anti-CD21 (7G.6) were from Deutsches Rheuma-Forschungszentrum. Anti-Siglec-F (E50-2440), anti-CD138 (281-2), anti-CD23 (B3B4), anti-CXCR4 (2B11/CXCR4) and annexin V were from BD Pharmingen. Anti-FcεRIα (MAR-1) was from eBioscience; anti-APRIL (CSA-836) was from Stressgen; anti-MOMA-1 (T-2012) was from BMA Biomedicals; and peanut agglutinin (PNA, B-1075) was from Vector. Rat IgG2a (KLH/G2a-1-1), IgG2b (KLH/G2b-1-2), IgG1 (KLH/G1-2-2) and rabbit IgG (0111-01) (Southern Biotech) were used as isotype-matched control antibodies. Rabbit anti–mouse laminin (L 9393) was from Sigma; monoclonal rat anti–mouse MBP (MT-14.7) was from N.A. Lee and J.J. Lee. Fluorescent dye–conjugated secondary goat–anti rabbit IgG (A11010), anti-mouse IgG (A11001) and anti-rat IgG (A21247) (Molecular Probes), streptavidin (S32354, 554064; Molecular Probes or BD) and sheep anti-Dig (Deutsches Rheuma-Forschungszentrum) were also used. Cell suspensions from spleen or bone marrow were stained for surface and intracellular expression as described[25]. Stained cells were analyzed with an LSR II and data were analyzed with FlowJo software.

Cell preparation and cell culture. Single-cell suspensions from bone marrow and spleen were prepared. For isolation of bone marrow dendritic cells, cells were stained with phycoerythrin–conjugated anti-CD11c, incubated with anti-phycoerythrin beads, enriched by magnetic-activated cell sorting and sorted by flow cytometry. For sorting of neutrophils, the $CD11c^-$ fraction was stained with anti-Gr-1 and anti-CD11b (**Supplementary Fig. 1a**). For isolation of eosinophils (R1), immature eosinophils (R2) and monocytes-macrophages (R3), bone marrow cell suspensions were depleted of B and T cells by magnetic-activated cell sorting and cells were stained with anti-CD11c anti-FcεRIα, anti-Gr-1, anti-F4/80 and anti-CD11b. Bone marrow dendritic cells, basophils–mast cells and neutrophils were excluded by gating. The R1, R2 and R3 cell subsets were sorted based on SSC and CD11b (**Supplementary Fig. 1b**). Peritoneal cavity cells were plated for 2 h in RPMI 1640 medium supplemented with 10% (vol/vol) FCS (Gibco), 50 μM 2-mercaptoethanol (Sigma), 100 U/ml of penicillin and 100 μg/ml of streptomycin in 6-cm plastic dishes (Costar). Nonadherent cells were removed by washing four times with PBS and adherent macrophages were isolated (**Supplementary Fig. 1c**). Total bone marrow cell populations (1×10^6 cells/ml) or sorted bone marrow cell populations (2×10^5 cells per 200 μl) were cultured for 24 h in RPMI 1640 medium supplemented as described above. Secretion of cytokines was measured by Bio-Plex (Bio-Rad), the concentration of total IgG and antigen-specific IgG was measured by standard enzyme-linked immunosorbent assay.

Cytospins and immunostaining. For cytospins, 1×10^5 sorted bone marrow cells in 150 μl complete medium were deposited into 48-well plates (Costar) containing poly-L-lysine-coated 12-mm round cover slips (BD). After 3 h, plates were centrifuged, washed with PBS and fixed for 10 min with 100% cold ethanol.

Femurs and tibias were carefully flushed with DMEM (Gibco), then intact bone marrow and spleen were frozen immediately in Tissue-Tec optimum cutting temperature compound and stored at −70 °C. Tissue sections 5–7 μm in thickness were prepared and used for fluorescence or immunohistochemical staining.

Analysis of the immune response. BALB/c and ΔdblGATA-1 mice were immunized with phOx. Germinal center B cells ($GL7^+PNA^{hi}$) were sorted at day 15 and the variable region of the V_kOx1 light chain genes sequenced[28]. Enzyme-linked immunospot analysis (Immunspot Analyzer; CTL) was used to determine the number plasma cells. Spleen or bone marrow cells (2×10^5) were diluted fivefold and incubated for 3 h on plates coated with anti–mouse immunoglobulin, phOx-CSA or GP1 (Millipore). After incubation with secondary antibody (Southern Biotech), spots were counted. Immunoglobulin titers were measured by standard enzyme-linked immunosorbent assay.

Gene expression analysis. Total RNA was extracted from 5×10^5 to 1×10^6 cells with NucleoSpin RNA II (Macherey-Nagel) and was reverse-transcribed into cDNA with a Sensiscript RT kit (Qiagen). The expression of APRIL, IL-6, CXCR4 and CXCL12 was measured by RT-PCR and real-time PCR[25] (primer sequences (TibMolBiol), **Supplementary Table 1**).

Co-culture of plasma cells and eosinophils. BALB/c mice were immunized with phOx, and 6 d after boosting, bone marrow $CD138^+$ plasma cells were purified with an isolation kit (Miltenyi Biotec; **Supplementary Fig. 1d**). Plasma cells (1×10^2) were cultured with eosinophils in 96-well U-bottomed plates. After 24 h of coculture, cells were transferred to plates-coated with anti–mouse immunoglobulin and were incubated for additional 24 h at 37 °C. Spots were counted by enzyme-linked immunospot. Other plasma cells (1×10^4) were cultured for 48 h together with eosinophils separately in Transwells (pore size, 5 μm; Costar) and the frequency of annexin V–positive cells was determined. Blocking experiments included the soluble fusion protein TACI-Ig or anti-IL-6 (R&D Systems) or both. Human IgG or rat IgG1 was used as an isotype-matched control antibody.

Adoptive transfer of eosinophils. At day 6 after antigen boost, bone marrow cells from BALB/c mice underwent depletion by magnetic-activated cell sorting with a 'cocktail' of antibodies (biotinylated anti-B220, anti-IgM, anti-CD4 and anti-CD8) and eosinophils were sorted as described (**Supplementary Fig. 1**). Sorted eosinophils were transferred into ΔdblGATA-1 mice by intravenous injection on 2 consecutive days (1×10^7 cells per day), and 6 or 12 d later, the number and frequency of eosinophils in bone marrow was measured. For analysis of whether reconstitution with eosinophils restored the accumulation of plasma cells, BALB/c and ΔdblGATA-1 mice were immunized with phOx-CSA in aluminium hydroxide and, 6 weeks later, ΔdblGATA-1 mice were reconstituted with eosinophils (daily intravenous injection of 1×10^7 eosinophils between days −2 and +1 relative to boosting). On day 0, mice were boosted with soluble antigen and the number and frequency of plasma cells determined 6 and 12 d later.

Eosinophil depletion. BALB/c mice were injected intraperitoneally three times (every other day) with 20 μg soluble anti-mouse Siglec-F. Rat IgG2a was used as an isotype-matched control antibody (R&D Systems). At day 4 after the final injection, eosinophils were analyzed in bone marrow, blood and spleen and the number of plasma cells in bone marrow and spleen determined.

Statistical analysis. A paired two-tailed Student's *t*-test and two-way analysis of variance with the assumption of equal variance of samples were used for statistical analysis.

doi:10.1038/ni.1981