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Elodie Belnoue, Chantal Tougne, Anne-Françoise Rochat,  
Paul-Henri Lambert, Daniel D. Pinschewer and Claire-Anne  
Siegrist

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# Homing and Adhesion Patterns Determine the Cellular Composition of the Bone Marrow Plasma Cell Niche

Elodie Belnoue, Chantal Tougne, Anne-Françoise Rochat, Paul-Henri Lambert, Daniel D. Pinschewer, and Claire-Anne Siegrist

According to commonly held concepts, plasma cell (PC) longevity in bone marrow (BM) depends upon their access to survival niches. These are thought to exist in nursery cell types, which support PCs by secreting PC survival factors. To better define PC survival niches and their functioning, we adoptively transferred traceable Blimp-1<sup>GFP</sup> PCs into recipient mice lacking a proliferation-inducing ligand (APRIL), IL-6, or macrophage migration inhibitory factor. Transferred BMPCs were preferentially associated with Ly-6C<sup>high</sup> monocytes (normalized colocalization index: 9.84), eosinophils (4.29), and megakaryocytes (2.12). Although APRIL was essential for BMPC survival, PC recruitment into the proximity of nursery cells was unimpaired in APRIL-deficient mice, questioning the concept that the same factors account for attraction/retention of PCs as for their local survival. Rather, the order of colocalization with BMPCs (monocytes > eosinophils > megakaryocytes) reflected these cells' relative expression of CXCR4, VLA-4, and LFA-1, the homing and adhesion molecules that direct/retain PCs in the BM. This suggests a scenario wherein the cellular composition of the BMPC niche is defined by a common pattern of attraction/retention on CXCL12-abundant reticular docking cells. Thereby, PCs are directed to associate in a functional BM niche with hematopoietic CXCR4<sup>+</sup>VLA-4<sup>+</sup>LFA-1<sup>+</sup> nursery cells, which provide PC survival factors. *The Journal of Immunology*, 2012, 188: 1283–1291.

Long-lived Ab-secreting plasma cells (PCs) are critical for long-term protection postinfection or immunization. PCs may persist for decades throughout the body, namely at sites of inflammation, but predominantly in the bone marrow (BM) compartment. A complex orchestration guides Ag-specific B cells to exit the germinal centers of lymphoid organs and migrate toward the BM, a privileged location where they receive additional signals required for their final differentiation and prolonged survival (1). There is strong evidence that the longevity of BMPCs is dependent on environmental factors (2) (i.e., their access to specific survival niches).

Given the importance of long-term PC persistence to both protection by immunological memory as well as autoimmunity, considerable effort has been devoted to the definition of the BMPC survival niche. Previous approaches were oriented toward identifying the cell types, which: 1) display preferential contacts with PC; and 2) express putative PC survival factors. The widely held view is thus that BMPCs' proximity to other cell types in the niche is

a direct consequence of the survival factors these cells produce. Numerous signals may support PC survival in vitro (3), although their contribution to the establishment and survival of BMPC in vivo is less clear. Defining which cell types and factors play critical or minor roles for in vivo PC survival is complicated by the complexity and heterogeneity of BM cell populations and the diversity of methodological approaches (4–6).

Several different BM cell types have recently been claimed as providing critical contributions to BMPC survival, including reticular stromal cells (7, 8), osteoblasts (9), osteoclasts (10), Gr1<sup>int</sup>CD11b<sup>+</sup> monocytes (11), eosinophils (12), megakaryocytes (MGKs) (13), and basophils (14). CXCL12-abundant reticular (CAR) cells, CXCL12<sup>+</sup> reticular cells, and osteoblasts (15) were unambiguously demonstrated as key compartments of the BMPC niche when it was demonstrated that PCs lacking CXCR4 (the receptor for CXCL12) fail to home to the BM compartment (7). But whether CXCL12 provides critical PC survival signals in vivo is unknown. CXCL12<sup>+</sup> cells all express VCAM-1, but BM VCAM-1<sup>+</sup> cells are abundant (35%), and only 17% of VCAM-1<sup>+</sup> express CXCL12 (7). It remains unclear whether all VCAM-1-expressing cells participate in the BM niche or whether only those expressing CXCL12 do. MGKs were recently described as providing critical BMPC support based on a preferential colocalization with PC compared with granulocytes (13). Yet, only 30% (and as few as 10% in some mice) of BMPCs are close to MGKs, which produce only low levels of the PC survival factor a proliferation-inducing ligand (APRIL) but are an abundant source of BM IL-6, which efficiently supports PC survival in vitro (3, 16, 17). Doubts casted, however, over their critical in vivo role when BMPCs were found to be normal in IL-6<sup>-/-</sup> mice (3). BM-resident dendritic cells produce the macrophage migration inhibitory factor (MIF), which supports mature B cells in perivascular BM niches (18). This led to the hypothesis that the antiapoptotic activity of MIF may contribute to the longevity of BMPCs (19). This postulate has, however, not been experimentally tested. Lastly, both BAFF and APRIL support PC survival (11, 20) in vitro. A critical in vivo role of APRIL, but not BAFF, was demonstrated using APRIL<sup>-/-</sup>

Department of Pathology-Immunology, World Health Organization Collaborating Center for Vaccinology and Neonatal Immunology, University of Geneva, CH-1211 Geneva 4, Switzerland; and Department of Pediatrics, World Health Organization Collaborating Center for Vaccinology and Neonatal Immunology, University of Geneva, CH-1211 Geneva 4, Switzerland

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Address correspondence and reprint requests to Dr. Elodie Belnoue, Departments of Pathology-Immunology and Pediatrics, World Health Organization Collaborating Center for Vaccinology and Neonatal Immunology, University of Geneva, CH-1211 Geneva 4, Switzerland. E-mail address: elodie.belnoue@unige.ch

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Abbreviations used in this article: APRIL, a proliferation-inducing ligand; ASC, Ab-secreting cell; BM, bone marrow; BMPC, bone marrow plasma cell; CAR, CXCL12-abundant reticular; MBP, major basic protein; MFI, mean fluorescence intensity; MGK, megakaryocyte; MIF, macrophage migration inhibitory factor; PC, plasma cell; TT, tetanus toxoid; WT, wild-type.

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and BAFF<sup>-/-</sup> mice (11). Conversely, the use of blocking reagents concluded that either APRIL or BAFF could support PC survival (21, 22). We reported the role of APRIL-producing BM Gr1<sup>int</sup> CD11b<sup>+</sup> cells in the support of BMPCs, referring to these cells as resident BM monocytes (11). However, these cells are a heterogeneous population including monocytes, neutrophil precursors, and eosinophils, which were recently claimed as being the major source of APRIL and IL-6 (12).

Given the importance of these questions for the induction of long-lived vaccine- or infection-induced PCs and for treatment of Ab-mediated autoimmune manifestations, we undertook a comprehensive analysis of the constituents of the BMPC niche. To this end, we established an adoptive transfer model of immunization-induced Blimp-1<sup>GFP/+</sup> fluorescent reporter PCs into wild-type (WT) or APRIL-, IL-6-, or MIF-deficient mice to define the relative contribution of each factor on BMPC survival. Having confirmed in this model that APRIL is required for BMPC survival (11), APRIL-deficient recipients were used to assess the influence of APRIL on the preferential association of BMPCs with putative nursery cells (monocytes, neutrophils, eosinophils, and MGKs). As the preferential colocalization of BMPCs was unaffected by the absence of APRIL, we assessed the expression of the CXCR4, VLA-4, and LFA-1 homing and adhesion molecules by BMPCs and monocytes, eosinophils, and MGKs. Our observations led us to propose a new concept of a functional BMPC niche, in which a central role is attributed to CAR docking cells attracting CXCR4<sup>+</sup>/VLA-4<sup>+</sup>/LFA-1<sup>+</sup> PCs alongside with other hematopoietic cell lineages (including monocytes, eosinophils, and MGKs) that express the same set of homing/adhesion molecules and are thus recruited in the proximity of BMPCs, regardless of the factors they express.

## Materials and Methods

### Mice

Adult BALB/c, C57BL/6J, and IL-6<sup>-/-</sup> (C57BL/6J background) mice were purchased from Charles River Laboratories (L'Arbresle, France and Sulzfeld, Germany). Blimp-1<sup>GFP/+</sup> (C57BL/6J background) mice were gifts from Stephen L. Nutt (Victoria, Australia), APRIL<sup>-/-</sup> mice from Genentech (San Francisco, CA), and MIF<sup>-/-</sup> mice (BALB/c background) from Thierry Calandra (Lausanne, Switzerland). IgH<sup>a</sup> congenic C57BL/6J mice were obtained through the Swiss Immunological Mouse Repository. Mice were kept under specific pathogen-free conditions, and all experiments were carried out according to Swiss and European guidelines and approved by the Geneva Veterinary Office.

### Ags, adjuvants, and immunizations

BALB/c mice were immunized twice i.p. at a 3-wk interval with tetanus toxoid (TT) (1 Lf per mouse; gift from Sanofi Pasteur, Lyon, France) adsorbed to Al(OH)<sub>3</sub> (gift from Novartis, Siena, Italy). C57BL/6J and Blimp-1<sup>GFP/+</sup> mice were primed with TT/Al(OH)<sub>3</sub> with added CpG<sub>826</sub> oligonucleotides (50 µg; Operon Biotechnologies, Cologne, Germany) (23) and boosted with TT-Al(OH)<sub>3</sub> 5 wk after priming. At different time points after immunization, BM was recovered for evaluation of TT-specific IgG Ab-secreting cells (ASCs) by ELISPOT (23), and serum was taken for the determination of TT-specific IgG titer by ELISA as previously described (23).

### Abs and reagents

Abs to CD8 (clone 53-6.7), CD138/Syndecan-1 (clone 281-2), CD11b/Mac-1 (clone M1/70), Ly-6C (clone AL-21), Ly-6G (clone 1A8), F4/80 (clone F4/80), Siglec-F (clone E50-2440), VCAM (clone 429), CD184/CXCR4 (clone 2B11), CD49d/VLA-4 (clone R1-2), CD11a/LFA-1 (clone M17/4), PerCP-Cy5.5-conjugated streptavidin, and isotype controls were from BD Biosciences (San Jose, CA). Rabbit polyclonal anti-GFP and rat anti-CD41/integrin α 2b (clone MWR30) were from Abcam (Cambridge, U.K.). Anti-CD115/M-CSFR (clone AFS98) and anti-CD93 (clone AA4.1) were from eBioscience (San Diego, CA). Monoclonal anti-MBP (major basic protein; clone MT-14.7) was provided by N. A. Lee and J. J. Lee (Mayo Clinic, Scottsdale, AZ). FITC-conjugated goat anti-rat IgG was from Southern Biotechnology Associates (Birmingham, AL). Alexa 488, cyanin 3, or Qdot 605-conjugated streptavidins, Qdot 605-conjugated goat

anti-rat IgG, and Alexa 488-conjugated anti-rabbit IgG were from Molecular Probes (Eugene, OR).

### Immunohistochemistry

Mice were anesthetized by injection of a lethal dose of pentobarbital and perfused intracardially with PBS followed by 4% paraformaldehyde. Femurs and tibias were incubated overnight in 4% paraformaldehyde then in sucrose before snap freezing in isobutane and mounting in Tissue-Teck OCT compound (Sakura, Zoeterwoude, The Netherlands). The femurs were cut at a thickness of 10 µm. Sections were visualized with a Zeiss Axioskop 2 (Carl Zeiss, Oberkochen, Germany) or Zeiss LSM 510 Meta confocal microscopes (Carl Zeiss). GFP<sup>+</sup> cells were counted 18, 48, or 72 h after transfer on every tenth cut of four bones (two tibias and two femurs) per mouse, and the surface area of each cut was calculated by specifically designed macro functions of the MetaMorph Imaging System software (Universal Imaging, Downingtown, PA). Analysis of cell-cell proximity was performed by quantifying the colocalization of 100–200 GFP<sup>+</sup> cells from two to six cuts per mouse. If each cell type colocalized randomly with PCs, the colocalization frequencies per total surface area would be similar for each cell type. Normalized contacts were thus calculated as previously described (13). Briefly, the total cell surface area was calculated by multiplying the surface area of a single cell (calculated by using the diameter of each cell subset and assuming a circular shape) with the total cell number of the cell subset. Normalized contacts are calculated by division of the absolute number of cell contacts of each population to PCs by the total cellular surface area of the population.

### Flow cytometry analysis and cell sorting

BM cells were stained with the indicated Abs and acquired on an FACS-Canto cytometer (BD Biosciences, Mountain View, CA). Data were analyzed using FlowJo software (Tree Star, Ashland, OR). Surface marker mean fluorescence intensity (MFI) was calculated by subtracting the MFI of isotype control staining from the MFI of the Ab staining.

CD11b<sup>+</sup>Ly-6C<sup>high</sup>Siglec-F<sup>int</sup>, CD11b/Mac-1<sup>+</sup>Ly-6C<sup>int</sup>Siglec-F<sup>neg</sup>, CD11b/Mac-1<sup>+</sup>Ly-6C<sup>int</sup>Siglec-F<sup>int</sup>, and CD11b<sup>+</sup>Ly-6C<sup>int</sup>Siglec-F<sup>high</sup> cells were purified from the BM by cell sorting. BM cells were washed, incubated with 2.4G2 (anti-Fc), and stained with the indicated Abs. The four different subsets were sorted on an FACS Aria sorter (BD Biosciences, Mountain View, CA) to >90% purity.

### Real-time quantitative RT-PCR

Total cellular RNA of FACS-sorted cells was isolated by RNeasy mini kit (Qiagen, Hilden, Germany) and cDNA synthesized as previously described (24). SYBR Green assays (Invitrogen) and amplicons were designed as previously described (24). The following primers (Invitrogen) were used: *IL-6* forward 5'-CTATGAAGTCTCTCTGCAAGAGACT-3' and *IL-6* reverse 5'-GGGAAGGCCGTGGTTGTC-3'; *BAFF* forward 5'-CCGCGG-CCACAGGAA-3' and *BAFF* reverse 5'-TGAGAGGTCTACATCTTGT-CTGTTTC-3'; *APRIL* forward 5'-TTTCACAATGGGTCCAGGTGTA-3' and *APRIL* reverse 5'-AGGCATACTTCTGATACATCGGAAT-3'; *MIF* forward 5'-GCACGCATCGGCAAGATC-3' and *MIF* reverse 5'-CAG-CTTACTGTAGTTGCGGTTCTG-3'; *EEF1A1* forward 5'-TCCACTTGG-TCGCTTTGCT-3' and *EEF1A1* reverse 5'-CTTCTTGTCCACAGCTT-GATGA-3'; and *TBP* forward 5'-TTGACCTAAAGACCATTGCACTTC-3' and *TBP* reverse 5'-TTCTCATGATGACTGCAGCAA-3'. Normalization factors and relative RNA expression were calculated according to geNorm as previously described (11).

### In vivo plasmablast transfer

C57BL/6J, C57BL/6J IgH<sup>a</sup> congenic, or BALB/c adult mice were primed and boosted as described above. Four days after boosting, splenocytes, following ammonium chloride lysis buffer treatment, were transferred i.v. (1 × 10<sup>8</sup> cells) into recipient mice. Eighteen and 48 h later, the BM was recovered for evaluation of TT-specific IgG ASCs by ELISPOT (23). In depletion experiments, 1 mg 1A8 or RB6-8C5 Abs (BioXCell, West Lebanon, NH) was injected i.v. at days -1, 0, and 1. TT-specific IgG titers were determined by ELISA in sera from transferred APRIL<sup>-/-</sup> and C57BL/6 mice at different time points as previously described (23). TT-specific IgG<sub>1</sub><sup>a</sup> and IgG<sub>1</sub><sup>b</sup> titers were determined by ELISA with a similar protocol using biotin-conjugated goat anti-mouse IgG<sub>1</sub><sup>a</sup> or IgG<sub>1</sub><sup>b</sup> Abs (BD Biosciences) and peroxidase-conjugated extravidin.

### Statistical analysis

Statistical differences between two groups were analyzed by Mann-Whitney *U* test; differences between multiple groups were analyzed by one-way ANOVA with the Tukey multiple comparison test, and differences

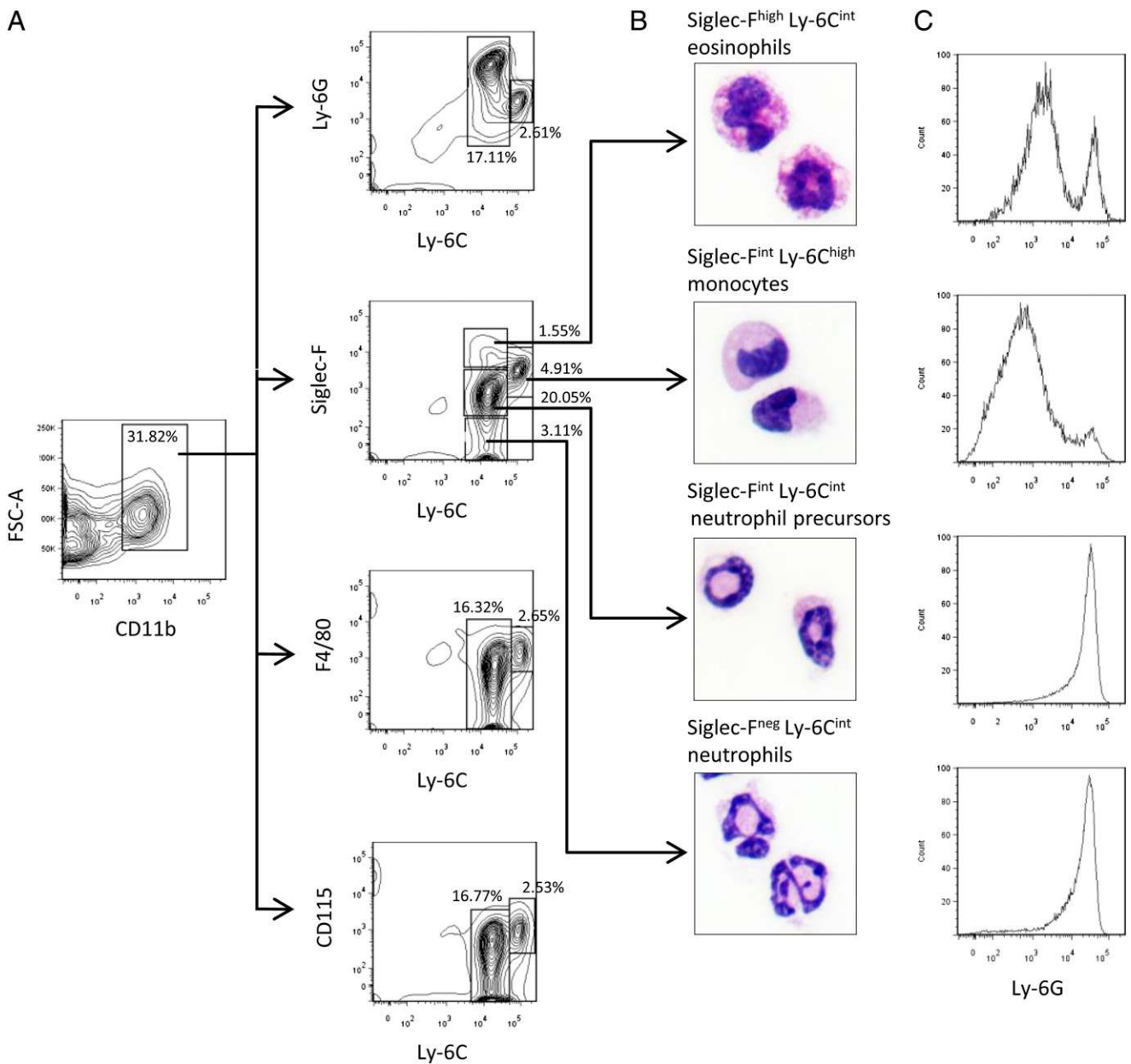
in kinetic studies between two groups were analyzed by two-way ANOVA with the Bonferroni test using Prism (GraphPad). Differences with probability values <0.05 were considered significant.

**Results**

*Distribution and preferential proximity of PCs in the BM compartment*

Earlier attempts at defining the BMPC niche were largely based upon the assessment of cell types in preferential contact with PCs. We therefore established an adoptive transfer model allowing the synchronized homing of Ag-specific PCs into the BM compartment and their analysis by confocal immunohistochemistry. Blimp-1<sup>GFP/+</sup> C57B7/6 mice (1) were immunized with two doses of aluminum hydroxide-adsorbed tetanus toxoid (TT). Their splenocytes were

harvested 4 d after the boost and adoptively transferred i.v. into naive C57BL/6 mice. At this early time point, PCs (defined as CD138<sup>high</sup>GFP<sup>high</sup> cells) constitute <5% of GFP<sup>+</sup> cells such that most CD138<sup>+</sup>GFP<sup>+</sup> splenocytes are plasmablasts (not shown). GFP<sup>+</sup> cells were detected in the BM compartment as early as 18 h after transfer and accumulated further over the subsequent 48-h period (Supplemental Fig. 1A), matching our previous observations with anti-TT ASCs (11). These Blimp-1<sup>GFP/+</sup> cells expressed high levels of CD138, the PC marker, for the largest part were not Blimp-1<sup>GFP/+</sup> CD8<sup>+</sup> T cells (25, 26) and displayed the characteristic morphology of PCs (Supplemental Fig. 1B). Their GFP fluorescence intensity increased with time (not shown), as expected given the increase of Blimp-1 expression in terminally differentiated PCs (1). Altogether, these observations validated the



**FIGURE 1.** Characterization of BM CD11b<sup>+</sup> Gr1<sup>low</sup> cells. **A**, Representative dot plots showing the percentages of different subsets after staining with CD11b, Ly-6C, Ly-6G, F4/80, CD115, and Siglec-F. The CD11b<sup>+</sup> population was first divided into two subsets: Ly6C<sup>high</sup> and Ly-6C<sup>int</sup>. Ly-6C<sup>high</sup> monocytes were highly positive for F4/80 and CD115. The Ly-6C<sup>int</sup> subset was further divided into three subpopulations by the expression of Siglec-F: Ly-6C<sup>int</sup>Siglec-F<sup>low</sup>, Ly-6C<sup>int</sup>Siglec-F<sup>int</sup>, and Ly-6C<sup>int</sup>Siglec-F<sup>high</sup>. Dot plots are representative of six mice. **B**, Pictures of the different subsets after cell sorting and cytopsin (Zeiss Axiophot, original magnification ×40, AxioCam color CCD camera, AxioVision software). **C**, Representative histograms for the Ly-6G expression of the different subsets after cell sorting.



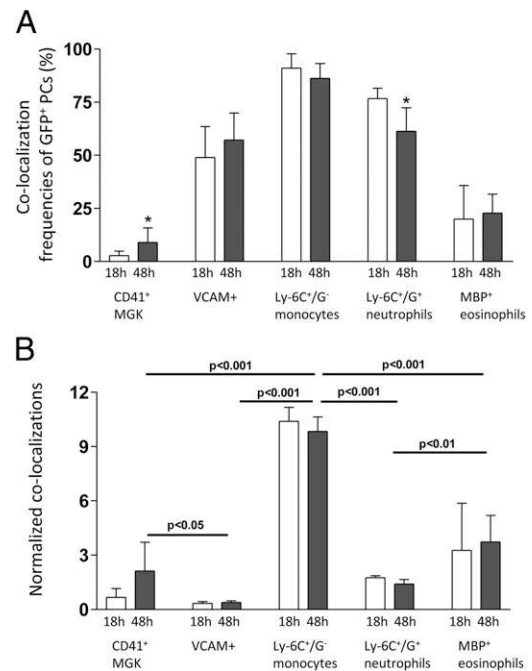
use of this adoptive transfer model for the study of PC establishment and survival in the BM. We first assessed whether Blimp-1<sup>GFP/+</sup> PCs were preferentially localized in certain anatomic localizations by counting all Blimp-1<sup>GFP/+</sup> PCs present in 100 consecutive sections of one femoral bone collected 48 h after adoptive transfer. Normalizing the number of GFP<sup>+</sup> cells per surface area confirmed that Blimp-1<sup>GFP/+</sup> PC were scattered throughout the longitudinal axis of the bone without preferential gross anatomical distribution at bone extremities or center (Supplemental Fig. 2).

Hence, we have investigated the microanatomical environment of transferred PCs. Having previously demonstrated the importance of Gr-1<sup>int</sup> CD11b<sup>+</sup> cells for PC survival, we studied the lineage specificities of this heterogeneous population by FACS using anti-Ly-6G and Ly-6C Abs and H&E staining of BM cytopins. Four subsets were identified within CD11b<sup>+</sup> cells (Fig. 1): Siglec-F<sup>high</sup> Ly-6C<sup>int</sup> cells (1.4% of total cells) were eosinophils (Fig. 1B); immature (Siglec-F<sup>int</sup>Ly-6C<sup>int</sup>, 14.3%) and mature (Siglec-F<sup>neg</sup>Ly-6C<sup>int</sup>, 2.1%) cells expressed the Ly-6G<sup>high</sup> neutrophil marker (Fig. 1C); and Siglec-F<sup>int</sup>Ly-6C<sup>high</sup> cells (2.9%) expressed the M-CSF (M-CSFR/CD115<sup>+</sup>) monocytic marker (Fig. 1A). These Siglec-F<sup>int</sup>Ly-6C<sup>high</sup> monocytes expressed F4/80 (Fig. 1A) but not CD19 or CD11c, nor the CD40, CD86, or MHC class II activation molecules (data not shown). We thus included neutrophils, eosinophils, and monocytes, in addition to MGKs (13) and VCAM-1<sup>+</sup> cells, in our studies of the preferential colocalization of BMPCs.

First, we counted the proportion of Blimp-1<sup>GFP/+</sup> PCs observed in the microenvironment of Ly-6C<sup>+</sup>Ly-6G<sup>+</sup> neutrophils, Ly-6C<sup>+</sup>Ly-6G<sup>neg</sup> monocytes, MBP<sup>+</sup> eosinophils, or CD41<sup>+</sup> MGKs. The environments of 100–200 PCs were included in each quantification. At 18 and 48 h after transfer, most Blimp-1<sup>GFP/+</sup> PCs were observed in the proximity of Ly-6C<sup>+</sup>Ly-6G<sup>+</sup> neutrophils (mean of two experiments: 76.7 and 61.3%, respectively), Ly-6C<sup>+</sup>Ly-6G<sup>neg</sup> monocytes (91.1 and 86.2%), and MBP<sup>+</sup> eosinophils (20 and 22.8%), and few were close to MGKs (2.8 and 9.0%) (Fig. 2A). Proximity with VCAM-1<sup>+</sup> cells was also frequent (49.0 and 57.2%). To assess the significance of these interactions, the colocalizations observed were normalized by the total surface area of each population (number × cell size), as previously described (13). After normalization, the relative PC colocalization frequencies with MGKs 48 h after transfer were calculated as 2.1 (Fig. 2B), confirming the observations of Winter and colleagues (13). Ly-6C<sup>+</sup>Ly-6G<sup>+</sup> neutrophils and VCAM-1<sup>+</sup> cells are abundant in the BM, such that their relative normalized PC colocalization frequencies were lower. In striking contrast, the normalized relative colocalization frequency with Ly-6C<sup>+</sup>Ly-6G<sup>neg</sup> monocytes was 4.6-fold higher than with MGKs ( $p < 0.001$ ) and 2.3-fold higher than with MBP<sup>+</sup> eosinophils ( $p < 0.001$ ). This suggested that Ly-6C<sup>+</sup>Ly-6G<sup>neg</sup> monocytes were preferentially associated with PCs in their BM niche.

#### Resident monocytes are the main source of APRIL in the BM

To identify the relative contribution of specific cell lineages and the PC survival factors they express, cell populations were sorted by FACS from the BM compartment of naive mice, and their expression of IL-6, MIF, BAFF, and APRIL was analyzed. IL-6 mRNA was only expressed at low levels by BM neutrophils, eosinophils, and monocytes (Fig. 3A) and not detected by ELISA in the supernatant of BM CD11b<sup>+</sup> cells (11, 17). It was not possible for us to successfully sort a specific population of MGKs, but these cells were reported by others as the main source of BM IL-6 (13). MIF mRNA was expressed at a significantly higher level in BM eosinophils than in other cell types (Fig. 3B). BAFF mRNA was expressed at high levels by BM neutrophils (Fig. 3C), and



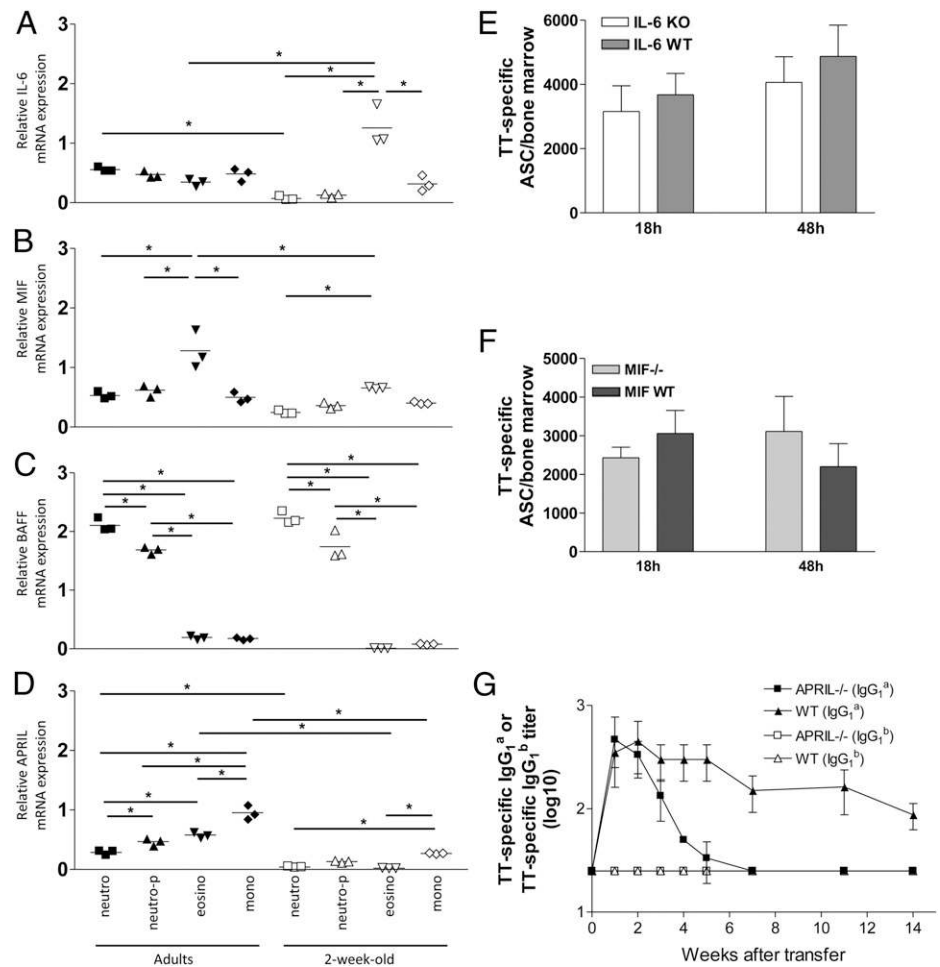
**FIGURE 2.** Preferential contacts of Blimp-1<sup>GFP/+</sup> PCs with MGKs, monocytes, and eosinophils. Splenocytes from immunized adult Blimp-1<sup>GFP/+</sup> mice were transferred into C57BL/6 WT recipients as previously described. Eighteen and 48 h after transfer, the percentages of GFP<sup>+</sup> cells in contact with different cell types were calculated as described in the *Materials and Methods* section with two to three mice per time point. **A**, Colocalization frequencies (mean ± SD) of GFP<sup>+</sup> PCs with the different cell subsets. **B**, Normalized contacts (mean ± SD) of GFP<sup>+</sup> PCs with the different cell subsets, calculated as described in the *Materials and Methods* section. \* $p < 0.05$ .

APRIL mRNA was mainly expressed by BM monocytes, followed by eosinophils and neutrophil precursors (Fig. 3D). Regrettably, we were unable to use the only commercially available anti-APRIL mAb (clone CSA-836; Stressgen) for these analyses, owing to indiscriminate staining of WT and APRIL<sup>-/-</sup> cells (data not shown). In addition to their higher level of expression of APRIL, BM monocytes (total  $2.1 \times 10^6$  cells) were 3-fold more numerous than BM eosinophils (total  $7.2 \times 10^5$  cells) in C57BL/6 mice, suggesting a predominant role for BM monocytes as the source of APRIL.

#### APRIL-producing monocytes are critical for PC survival in the BM

The individual role of each survival factors on the survival of IgG-producing BMPCs was assessed by the adoptive transfer of TT-specific PCs into gene-deficient mice. Eighteen and 48 h after transfer, the number of TT-specific BMPCs assessed by ELISPOT was similar in IL-6<sup>-/-</sup> and WT recipients (Fig. 3E). Similar results were obtained after transfer into MIF<sup>-/-</sup> and WT recipients (Fig. 3F). We have previously published a marked reduction of TT-specific BMPCs 48 h after transfer in APRIL<sup>-/-</sup> and not in BAFF<sup>-/-</sup> mice (11). Two additional experiments were performed in this study, confirming the critical role of APRIL. First, TT-specific PCs were induced in IgH<sup>a</sup> mice and transferred into IgH<sup>b</sup> WT and APRIL<sup>-/-</sup> mice. TT-IgG<sub>1</sub><sup>a</sup> Abs declined rapidly in APRIL<sup>-/-</sup> mice, reaching undetectable levels 7 wk after transfer (Fig. 3G) and thus confirming insufficient PC survival for sustained Ab production. Host-derived TT-IgG<sub>1</sub><sup>b</sup> were not detected at any time point. Second, Blimp-1<sup>GFP/+</sup> PCs were adoptively transferred into WT or APRIL<sup>-/-</sup> mice. Seventy-two hours later,

**FIGURE 3.** Resident monocytes are the main BM source of APRIL, which is critical for PC survival. Neutrophils, neutrophil precursors, monocytes, and eosinophils were sorted by FACS and analyzed for their expression of mRNA transcripts of IL-6 (A), MIF (B), BAFF (C), and APRIL (D). E, Splenocytes from immunized adult C57BL/6 mice were transferred into C57BL/6 IL-6<sup>-/-</sup> and WT recipients. Eighteen and 48 h after transfer, TT-specific IgG BMPC were quantified by ELISPOT. Results (mean ± SD) expressed as TT-ASCs per BM were obtained in one of two independent experiments including six mice per group. F, Splenocytes from immunized adult BALB/c mice were transferred into C57BL/6 MIF<sup>-/-</sup> and BALB/c WT recipients. Eighteen and 48 h after transfer, TT-specific IgG BMPC were quantified by ELISPOT. Results (mean ± SD) expressed as TT-ASCs per BM were obtained in one experiment including five mice per group. G, Splenocytes from immunized adult congenic Igh<sup>b</sup> C57BL/6 mice were transferred into C57BL/6 APRIL<sup>-/-</sup> and WT recipients. Serum titers of TT-specific IgG<sub>1</sub><sup>a</sup> and TT-specific IgG<sub>1</sub><sup>b</sup> were determined by ELISA at different time points. Results are depicted from one of two representative independent experiments including four to six mice per group. \**p* < 0.05.



significantly fewer Blimp-1<sup>GFP/+</sup> PCs were detected (*p* < 0.0001) in APRIL<sup>-/-</sup> mice (Supplemental Fig. 3). Thus, APRIL is essential for recruitment and/or survival of PCs and thereby for sustained Ab production.

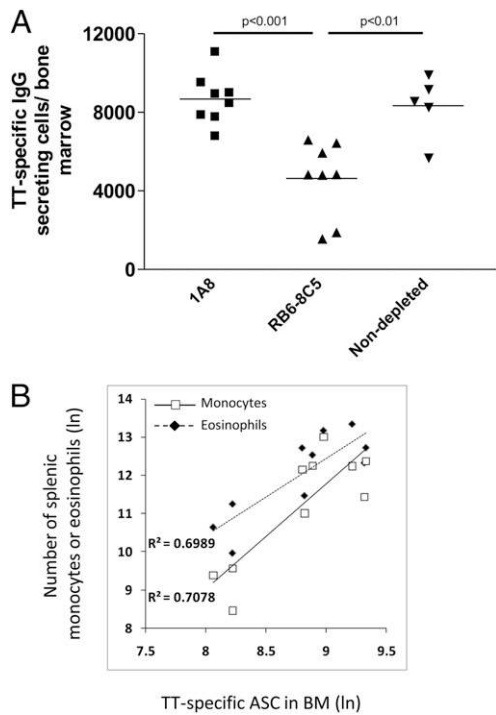
To further assess the critical role of APRIL-producing Ly-6C<sup>+</sup> Ly-6G<sup>neg</sup> BM monocytes and/or eosinophils for PC survival, we combined adoptive transfer and cell-depletion experiments. Injecting 1 mg of the anti Ly-6G 1A8 Ab at days -1, 0, and 1 after transfer reduced Ly-6G<sup>high</sup> cells (i.e., neutrophils and a fraction of eosinophils) (Fig. 1C) from spleen and BM compartments (Supplemental Fig. 4; compare marker profiles in Fig. 1C). This treatment, which does not affect Ly-6G<sup>low</sup> monocytes, did not affect the survival of Blimp-1<sup>GFP/+</sup> PCs (Fig. 4A). Treatment with the anti-Gr1 RB6-8C5 Ab only partly reduced Ly-6C<sup>high</sup> monocytes in the spleen and BM compartment (Supplemental Fig. 4). Yet, a significant 2-fold reduction of Blimp-1<sup>GFP/+</sup> PCs was observed in RB6-8C5 Ab-treated mice (Fig. 4A). This provided additional evidence that within BM CD11b<sup>+</sup> cells, the major PC supporting population is represented by Ly-6C<sup>high</sup> cells. In the absence of a depleting Ab allowing the specific and selective elimination of only BM monocytes or eosinophils, we assessed in each individual mouse the correlation between the number of residual eosinophils and monocytes to that of Blimp-1<sup>GFP/+</sup> BMPCs. The splenic compartment served as a surrogate for variable depletion efficiency in the BM (due to hematopoiesis) of individual mice. We observed a significant positive correlation between the number of PCs surviving in the BM and the residual number of both Ly-6C<sup>high</sup> monocytes and eosinophils 48 h after transfer (Fig. 4B), confirming their role of nursery cells.

Taken together, these results define APRIL as an essential survival factor for BMPC establishment and monocytes as the main, but not exclusive, source of APRIL in the BM.

#### Similar composition of the PC BM niche in adult and early life

We have previously shown that TT-producing PCs inefficiently established themselves in the BM of infant mice and that this impairment correlated with limited APRIL production by early-life BM CD11b<sup>+</sup> cells. To define whether this resulted from differences in the composition of the BMPC niche during ontogeny, adult Blimp-1<sup>GFP/+</sup> PCs were transferred into 2-wk-old and adult control mice. As expected, fewer adult Blimp-1<sup>GFP/+</sup> PCs were recovered from 2-wk-old recipient mice than from adult controls (data not shown).

Remarkably though, the frequency of proximity and normalized colocalizations between Blimp-1<sup>GFP/+</sup> PCs and MGKs was similar in both age groups (Fig. 5A, 5B). Fewer monocytes, eosinophils, and neutrophils were present in the BM of infant mice, resulting in less frequent proximity with these cell types (Fig. 5A). Upon normalization for cell type-specific abundance, however, the proximity between Blimp-1<sup>GFP/+</sup> PCs and Ly-6C<sup>+</sup> Ly-6C<sup>+</sup> monocytes was markedly increased in infant mice (Fig. 5B). This result was unexpected given the reduced APRIL levels in neonates and the seemingly predominant contribution of Ly6C<sup>+</sup> monocytes as an APRIL source. Specifically, this finding questioned the widely held view that BMPCs' proximity to other cell types in the niche was a direct consequence of the survival factors these cells produce. To define how ontogeny affected the production of PC survival factors by BM CD11b<sup>+</sup> cells, the four subsets of CD11b<sup>+</sup>

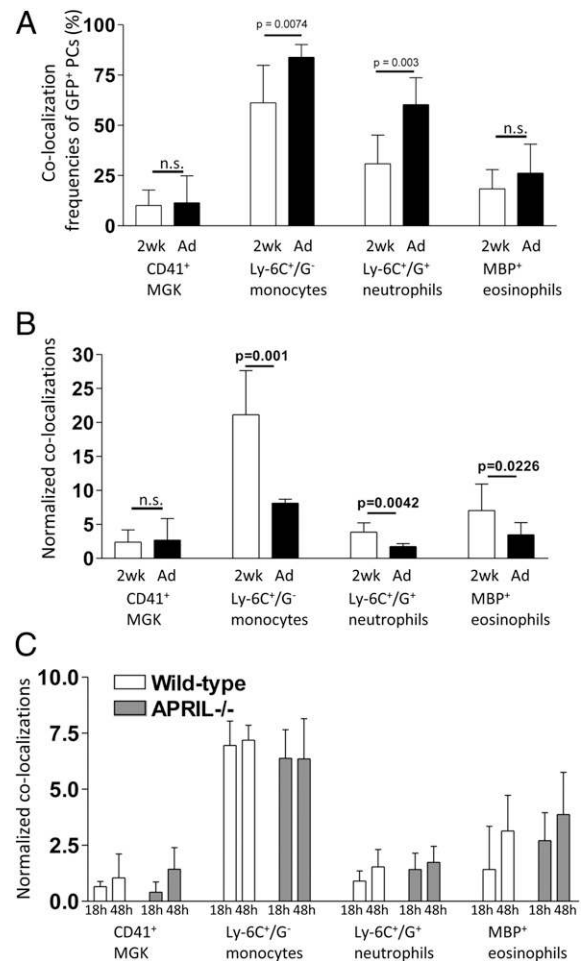


**FIGURE 4.** Critical role of APRIL-producing Ly-6C<sup>high</sup> BM monocytes in the BMPC pool. *A*, Splenocytes from immunized adult C57BL/6 were transferred into WT recipients treated with 1A8 or RB6-8C5 Abs (days -1, 0, and 1) as described in the *Materials and Methods* section. TT-specific IgG BMPC were quantified by ELISPOT 48 h after transfer. Results (mean ± SD) expressed as TT-ASCs per BM were obtained in one of two representative experiments including at least five mice per group. *B*, Correlations between the number of TT-specific BMPCs and the number of residual spleen monocytes or eosinophils in depleted and control mice.

BM cells were FACS sorted from naive adult and infant mice, and their expression of PC survival factors was compared. BAFF expression by BM neutrophils was similar in both age groups (Fig. 3C). The IL-6 mRNA expression was significantly higher (3.6-fold;  $p < 0.001$ ) in infant eosinophils (Fig. 3A). Yet, this was apparently insufficient for optimal support of PC survival in the early-life BM compartment (11). In contrast, adult BM eosinophils expressed more MIF than infant cells (Fig. 3B). Remarkably, APRIL mRNA expression was profoundly downregulated in all four CD11b<sup>+</sup> BM cell subsets of 2-wk-old mice (Fig. 3D). Thus, the limited survival of BMPCs in the early-life BM compartment could not be accredited to a failure to establish in the proximity of BMPC-supporting cells (nursery cells) but was related to a downregulation of APRIL production by several cell populations in the infant BM.

#### *Preferential PC proximity to nursery cells establishes independently of APRIL expression*

The BMPC niche is commonly thought of as an entity, which is primarily defined by availability of nursery cell contacts and thus of survival factors they produce. Doubts were cast over this concept when we found similar distribution of BMPC in adult and early life, irrespective of the marked downregulation of APRIL RNA levels in infant cells. We thus used APRIL<sup>-/-</sup> mice to assess the influence of APRIL production on the proximity of Blimp-1<sup>gfp/+</sup> PCs to putative nursery cells. Surprisingly, the normalized colocalizations between Blimp-1<sup>gfp/+</sup> PCs and MGKs, neutrophils, monocytes, and eosinophils were remarkably similar irrespective of the APRIL competence of the recipient BM (Fig. 5C). Taken together, these results demonstrate that the preferential proximity of BMPCs



**FIGURE 5.** Preferential contacts of Blimp-1<sup>gfp/+</sup> PCs in infant WT and adult APRIL<sup>-/-</sup> mice. *A* and *B*, Splenocytes from immunized adult Blimp-1<sup>gfp/+</sup> mice were transferred into adult and 2-wk-old WT recipients. Forty-eight hours later, the percentages of GFP<sup>+</sup> cells in contact with different cell types were calculated as previously described, with two to three mice per time point. *A*, Colocalization frequencies (mean ± SD) of GFP<sup>+</sup> PCs with the various cell subsets. *B*, Normalized contacts (mean ± SD) of GFP<sup>+</sup> PCs. *C*, Splenocytes were similarly transferred into adult C57BL/6 APRIL<sup>-/-</sup> and WT recipients and the preferential contacts of GFP<sup>+</sup> cells quantified as previously described.

with monocytes, MGKs, or eosinophils occurred independently of the essential PC survival factor APRIL.

#### *Supposed PC nursery cells display characteristic homing and adhesion molecules resembling those of PCs*

Our results confirm an essential role of APRIL as BMPC survival factor. The adoptive transfer experiments in APRIL<sup>-/-</sup> mice indicated, therefore, that the composition of the BMPC niche was not driven by the need for contacts with APRIL-producing cells. We therefore sought other potential explanations for specific cell types' preferential proximity to BMPCs. We hypothesized that supposed nursery cells may be recruited into proximity of BMPCs owing to common homing and adhesion patterns. Given the importance of CXCL12 for PC homing and the expression of VCAM-1 by all CXCL12<sup>+</sup> cells (7), we assessed CXCR4 and VLA-4 expression on BMPCs and eosinophils, monocytes, and neutrophils that are not observed in preferential association with BMPCs (controls). The highest CXCR4 expression was observed on PCs and monocytes, followed by eosinophils (Fig. 6A, 6B). Similarly, VLA-4 expression



was highest on monocytes followed by PCs and then eosinophils, with lowest expression on neutrophils. This generated a double gradient of CXCR4 and VLA-4 expression, with CXCR4<sup>hi</sup>VLA-4<sup>hi</sup> BM monocytes, CXCR4<sup>int</sup>VLA-4<sup>int</sup> BM eosinophils, and CXCR4<sup>low</sup>VLA-4<sup>low</sup> BM neutrophils (Fig. 6A, 6B). Both LFA-1 and CD93 expression, which reportedly contribute to the retention of PCs in BM (27, 28), was also significantly higher on PCs and monocytes than on eosinophils and neutrophils (Fig. 6B). Overall, the expression of CXCR4, VLA-4, LFA-1, and CD93 by BMPCs, monocytes, and eosinophils confirmed that the surface expression of these migration and adhesion molecules was most similar in BMPCs and Ly-6C<sup>high</sup> monocytes (Fig. 6B, 6C), followed by eosinophils and neutrophils. This declining gradient of homing/adhesion pattern resemblance found a direct correlate in their normalized relative proximity frequencies (compare Fig. 2B). Altogether, these data show that PCs are directed to associate in a docking station with hematopoietic CXCR4<sup>+</sup>VLA-4<sup>+</sup>LFA-1<sup>+</sup> nursery cells, which provide PC survival factors (Fig.7).

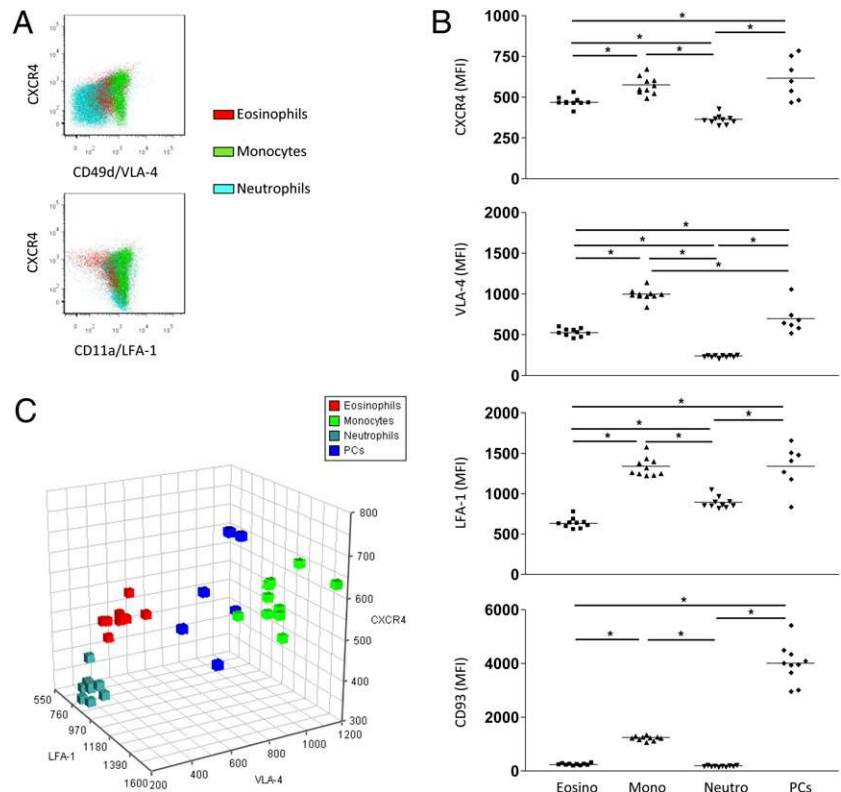
**Discussion**

The presented study suggests that the cellular composition of the BMPC niche is primarily determined by a pattern of specific homing (CXCR4) and adhesion (VLA-4 and LFA-1) molecules. These are shared between BMPCs and specific hematopoietic lineages, which therefore are recruited to the same microenvironment. Accordingly, high levels of CXCR4/VLA-4/LFA-1 characterize the cell types, which preferentially colocalize with PCs, namely CD11b<sup>+</sup> Ly-6C<sup>high</sup> F4/80<sup>high</sup> monocytes. These monocytes also share with PCs the expression of CD93, which is required for PC retention in the BM (28) and for which the function could thus be to retain PCs and monocytes into the BMPC niche. Additional adhesion molecules are likely involved in jointly recruiting PCs and their nursery cells to their BM niche.

The possibility must be considered that the long quest for the most important cell lineage in BMPC survival has its conceptual limitations. Despite the critical role of APRIL in BMPC survival, PCs are not directed toward specific nursery cells solely because these latter ones produce APRIL. The microenvironment of BMPCs (predominantly monocytes and eosinophils) is similar in adults and in early life, despite APRIL concentrations as low as to limit PC survival. Even the complete absence of APRIL in the respective gene-targeted mice did not alter the niche composition of the transiently surviving cells (Fig. 5). Hence, the recruitment of PCs to BM niches and their local survival represent independently governed processes. They seem intertwined, however, in that the recruitment process assures appropriate contacts of the cellular players, which need to dock together for long-term Ab production by BMPCs (2, 8). In this concept, additional players such as dendritic cells shown to support PC function (29) could also be recruited in this functional BMPC niche.

The functional BMPC niche is thus essentially defined by CXCL12<sup>high</sup>VCAM-1<sup>+</sup>ICAM-1<sup>+</sup> BM-resident cells, which attract PCs together with the appropriate BM myeloid cells. Whether CXCR4 expression remains critical for BMPC survival/maintenance once PCs have reached their BMPC niche is yet unknown. It would require assessing contacts between CXCL12<sup>+</sup> cells and PCs at a later time after transfer. Osteoblasts were initially suggested as the main source of CXCL12 in the BM (30), but their CXCL12 expression was found to be low (31). Nestin<sup>+</sup> cells constitute the vascular BM niche (32), and they also express modest levels of CXCL12 (31, 32). In contrast, CAR cells are the main BM source of CXCL12, and they display a homogeneous expression of adhesion molecules, namely including VCAM-1, ICAM-1, and CD44 (a molecule also implicated in PC survival) (3). CAR cells, which are the main component of the reticular BM niche (33) and the best candidate for the niche of hematopoietic stem cells (34), have been shown to retain BMPCs (7). We thus

**FIGURE 6.** Expression of CXCR4, VLA-4, LFA-1, and CD93 on BM cell subsets. A, Representative dot plots showing the expression of CXCR4, VLA-4, and LFA-1 gated on eosinophils (red), monocytes (green), and neutrophils (blue). B, Dot plots (representative of 10 individual mice) representing the MFI of CXCR4, VLA-4, LFA-1, and CD93 for eosinophils, monocytes, neutrophils, and PCs. Each symbol represents one individual mouse. C, Three-dimensional scatter plot showing the expression of CXCR4, VLA-4, and LFA-1 on BMPCs, eosinophils, monocytes, and neutrophils. \**p* < 0.05.





consider it likely that CAR cells also act as the docking station for those hematopoietic lineages, which produce PC survival factors.

Our adoptive PC transfer model established an essential role of APRIL but not of IL-6, MIF, or BAFF in PC survival *in vivo* (Fig. 3) (11). This is not meant to exclude a contribution of these or other factors to PC longevity, although our test setting could not detect it. It deserves mentioning that Ag-specific PCs slowly but steadily accumulated in vaccinated APRIL<sup>-/-</sup> mice and that the BM of APRIL<sup>-/-</sup> mice contains normal total PC numbers (E. Belnoue, C. Tougne, and C.-A. Siegrist, unpublished observations) (22), whereas adoptively transferred PC rapidly disappeared (Fig. 3G). This suggests that alternative factors and pathways can facilitate BMPC survival in the absence of APRIL. It thus seems likely that these factors may also play an auxiliary role in APRIL-competent individuals.

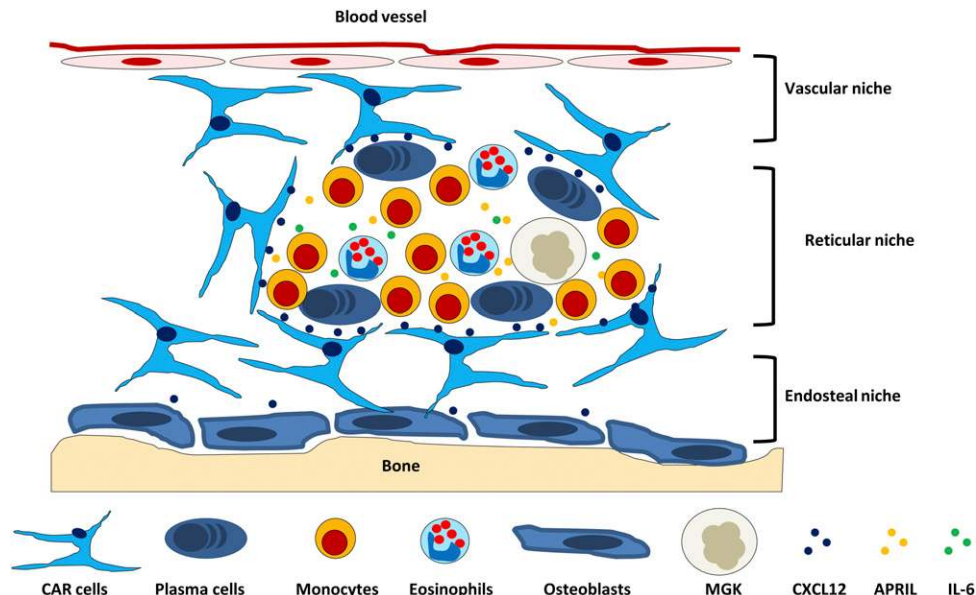
The main cellular sources of APRIL in BM remain controversial. We confirm that random association cannot account for the frequency of contacts between MGKs and BMPCs (13). However, MGK levels of APRIL mRNA are low (35). Given the relative paucity of BMPCs, they are unlikely to represent a major local source of APRIL. MGKs are, however, known to express CXCR4 (36, 37), VLA-4 (37, 38), and LFA-1 (37), offering a likely explanation for their presence in the BMPC niche. Eosinophils are more frequently found in direct vicinity of BMPCs than MGKs, and they express higher levels of APRIL than the latter ones (12). Still, both parameters are even higher for Ly-6C<sup>high</sup> monocytes. Earlier depletion experiments had attempted to demonstrate a critical role of eosinophils in BMPC survival (12). These experimental results however, may not contradict our present interpretation when revisiting the technical details: Chu and colleagues (12) used the Siglec-F marker to identify eosinophils, and this marker is also expressed on a subset of monocytes (Fig. 1). In further support of our interpretation, the Ly-6G-specific mAb 1A8, which depletes a fraction of eosinophils, did not affect the survival of Blimp-1<sup>GFP/+</sup> PCs. Regrettably, specific Abs for *in vivo* depletion of Ly-6C<sup>high</sup> BM monocytes are unavailable. This prevented us from formally demonstrating the individual role and contribution of APRIL<sup>+</sup>Ly-6C<sup>high</sup> monocytes to BMPC survival. Partial depletion by the Ly-6G/C-specific mAb RB6-8C5 reduced

Blimp-1<sup>GFP/+</sup> PCs by half, but we are aware of the fact that the same Ab also partly depletes eosinophils. Similar difficulties are encountered when the monocytic marker CD115 is used as a target for depletion, owing to its expression on granulocytes (39). The conditional depletion of mononuclear phagocytes in macrophage Fas-induced apoptosis transgenic mice or by the administration of clodronate liposomes was considered, but it is known to markedly reduce CXCL12 and thus drastically alter the composition of the BMPC niche (31, 40). These side effects would have hampered the interpretation of such approaches, which thus were not pursued in this study. Regardless of these technical limitations, we can conclude that Ly-6C<sup>high</sup> monocytes are the main source of APRIL in the BM, both on a per-cell basis and a numerical abundance. A contribution of eosinophils and MGKs to BM APRIL levels remains likely, too.

We thus propose a refined scenario for the constitution of the BMPC niche, which may impact on our concepts and definition of the entity both in terms of function and as a structural entity. The reticular niche made up by CAR cells represents a docking station (Fig. 7) for both PCs and hematopoietic cells (monocytes > eosinophils > MGKs) producing PC survival factors. In this scenario, the critical determinants of BMPC niche are neither lineage-specific nor directly related to the expression of PC survival factors. It is merely a shared set of homing and adhesion molecules, which accounts for homing and docking to CXCL12<sup>high</sup>VCAM-1<sup>+</sup>ICAM-1<sup>+</sup> cells, thereby recruiting PCs and their nursery cells to the reticular niche. The formal demonstration that CXCR4<sup>+</sup>VLA-4<sup>+</sup>LFA-1<sup>+</sup>CD93<sup>+</sup> homing/adhesion molecules are the sole determinants of the BMPC niche remains, to date, an unmet challenge. However, these findings have obvious implications and offer novel molecular strategies to prolong vaccine responses but also for the treatment of Ab-mediated autoimmune disorders.

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**FIGURE 7.** A model for the BM niche, identified as the reticular niche in which CAR cells act as a docking station ensuring that hematopoietic cells (primarily monocytes, followed by eosinophils and MGKs but not neutrophils) migrate within the PC microenvironment and produce PC survival factors.

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## Disclosures

The authors have no financial conflicts of interest.

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