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Reduced Ability of Neonatal and Early-Life Bone Marrow Stromal Cells to Support Plasmablast Survival[1]

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In human infants (<1 year), circulating IgG Abs elicited in response to most T-dependent Ags rapidly decline and return to baseline within a few months after immunization for yet-unknown reasons. In mice immunized between 1 and 4 wk of age, a limited establishment of the bone marrow (BM) pool of long-lived plasma cells is observed. In this study, we show that tetanus toxoid (TT)-specific plasmablasts generated in the spleen are efficiently attracted in vitro and in vivo toward early-life BM stromal cells, which express adult levels of CXCL12. Similarly, adoptively transferred TT plasmablasts efficiently reach the BM compartment of 2-wk-old and adult mice. In contrast, TT plasmablasts fail to persist in the early-life BM compartment, as indicated by the persistence of a significantly lower number of TT plasmablasts in the early-life compartment than in the adult BM compartment 48 h after transfer. This limited persistence is associated with an increased rate of in vivo apoptosis of TT-specific plasmablasts that have reached the early-life BM and with a significantly lower survival rate of TT-specific plasmablasts cocultured on early-life BM stromal cells compared with adult BM stromal cells. Thus, early-life BM stromal cells fail to provide the molecular signals that support plasmablast survival and differentiation into surviving plasma cells. *The Journal of Immunology,* **2006, 176: 165–172.**

To protect against infectious diseases that occur in the first year of life, immunization should ideally be done in the neonatal period or in early infancy. However, such strategies are somewhat impeded by the immaturity of the immune system (1, 2). With regard to Ab responses, it has long been recognized that IgG responses elicited in infants following natural infection or immunization are weaker than those elicited at a later stage of immune maturation (3–12). Importantly, it was recently recognized that the persistence of these Ab responses is much shorter than those elicited in older children or adults. Even when high Ab titers have eventually been generated through repeat infant immunization, serum Abs rapidly decline and disappear within a few months (13–17). This may be associated with a resurgence of vulnerability to infection (17, 18), requiring the administration of vaccine boosters already in the second year of life.

Serum IgG Abs essentially result from plasma cells that have undergone activation, proliferation, selection, and differentiation into Ab-secreting plasmablasts within germinal centers of secondary lymphoid organs (19). Whereas some Ag-specific B cells differentiate in germinal centers toward memory B cells (20), short-lived plasmablasts acquire the capacity to migrate to specific niches (21, 22) where they receive the signals required for their final differentiation into long-term-surviving plasma cells (23). A growing body of evidence indicates that the survival of plasma cells is not an intrinsic property but depends on their ability to respond to a combination of environmental signals (24, 25) provided within specific niches throughout the bone marrow (BM)[3] (26). Numerous signals supporting plasma cell survival have been identified by ex vivo studies. Protection of plasma cells from apoptosis (27, 28) requires direct contact with BM cells (24, 25, 27) recently identified as $CXCL12^+$, $VCAM\text{-}1^+$ stromal cells (26). CXCL12 is required for the chemoattraction and/or retention of $CXCR4^+$ plasma cells in the BM, as demonstrated by a 3-fold reduction of BM plasma cells in CXCR4-deficient mice (29). Adhesion molecules such as VCAM-1 may contribute to the retention of plasma cells expressing $\alpha_4\beta_1$ integrins into BM niches (30–32) providing the required signals. Numerous survival signals have been identified by studying the ex vivo survival of BM plasma cells, including IL-6, CXCL12, and TNF-α, as well as B lymphocyte stimulator/B cell activating factor and April (24, 25, 33). However, the relative contribution of adhesion molecules and survival factors to the final differentiation of apoptosis-prone plasmablasts into long-term-surviving factor plasma cells is still poorly understood (19, 34).

Because ethical issues prevent the direct study of Ab-secreting cells (ASCs) in the spleen, nodes, and BM of human infants, we have developed neonatal murine immunization models that reflect to a large extent the human immune maturation process observed in neonates and infants (1, 35–39). We previously observed that early-life B cell responses are limited by a delayed induction of germinal centers that results from a delayed postnatal maturation of follicular dendritic cells until the third week of age (40). Despite these limitations, strong immunogens such as tetanus toxoid (TT) elicit neonatal B and T cell memory responses, such that boosting at 4 wk of age generates adult-like numbers of TT-specific ASCs in the spleen (40). Unexpectedly, in contrast to what occurs in adults, these strong splenic responses are not associated with the appearance of TT-specific ASCs in the early-life BM compartment

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[3] Abbreviations used in this paper: BM, bone marrow; ASC, Ab-secreting cell; TT, tetanus toxoid; BMSC, BM stromal cell; BMSCC, BM stromal cell culture.

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(41). This homing defect could not be circumvented by using other Ags (our unpublished observations) or by immunization strategies, including the use of adjuvants as strong as CpG oligonucleotides that markedly enhance TT-specific ASCs in the adult but not in the early-life BM compartment (42).

In this study, we investigated the mechanisms of this deficient establishment of the BM ASC pool by assessing the relative contribution of plasmablast migration and of ASC survival in the BM. We conclude that plasmablasts generated in the spleen efficiently migrate into the early-life BM compartment but fail to establish themselves as surviving ASCs. This appears to directly reflect the failure of early-life BM stromal cells to support plasmablast survival. Thus, the postnatal development of the immune system includes the acquisition by BM stromal cells of functions supporting plasmablast survival.

Materials and Methods

Mice

Specific pathogen-free adult BALB/c mice (BALB/cByJ, H-2^d) were purchased from Charles River, bred, and kept under specific pathogen-free conditions in the zoo technology unit of the University of Geneva. Breeding cages were checked daily for births. Pups were kept with mothers until weaning at the age of 4 wk. Adult mice were used at 8–16 wk of age. Manipulations of mice were conducted according to Swiss and European guidelines, and all experiments were approved by the Geneva Veterinary Office.

Ags, adjuvants, and immunization procedures

TT (gift of Berna Biotech) was used at a dose of one limit of flocculation per mouse. TT was absorbed to $Al(OH)_3$ (gift of Chiron) before immunization. Based on previous observations (35), the dose of $Al(OH)_3$ was weight-adjusted to 0.5 or 0.25 mg for immunization of 2- and 1-wk-old mice, respectively. Mice were immunized i.p. In some experiments, mice received a secondary immunization 3 wk after priming, with the same dose of TT in 1 mg of $Al(OH)_3$ as indicated.

Enumeration of ASCs by ELISPOT

The TT-specific ASC number was assessed by ELISPOT as described previously (41). For calculation of the number of ASCs in the total BM, the number of ASCs per million cells was multiplied by the number of cells recovered from both femurs and tibias and by a coefficient of 5.3 (43).

CFSE labeling and purification of plasmablasts

Splenocytes were labeled with 1 μM CFSE (Molecular Probes) for 10 min at 37°C as described previously (44). Plasmablasts were positively selected using anti-syndecan-PE (BD Pharmingen) followed by anti-PE beads (Miltenyi Biotec) and sorted using an autoMACS separator according to the manufacturer's instructions (Miltenyi Biotec). TT-specific plasmablasts ($2–3 \times 10^6$) were transferred i.v. to 2-wk-old or adult mice.

Cell staining and flow cytometry

Single-cell suspensions from spleen and BM were incubated for 20 min with 20% of 2.4G2 (anti-Fc) supernatant in PBS/1% FCS/0.02% azide and stained with anti-syndecan-PE (BD Pharmingen) followed by staining for annexin V-Cy5 according to the manufacturer's instructions (BD Pharmingen). Cells were analyzed using the FACSCalibur (BD Biosciences) and CellQuest software (BD Immunocytometry Systems). An electronic gate was set on $CFSE^+$syndecan-PE^+ cells on acquisition.

Immunohistochemistry

BM from 1-, 2-, and 4-wk-old or adult mice were obtained by flushing femurs and tibias with PBS. Whole BM plugs were frozen directly in Tissue-Tek OCT compound (Sakura) and cut into 10-μm cryosections. Sections were fixed in acetone for 10 min and stored at −20°C until staining. In some experiments, BM plugs were fixed in 4% paraformaldehyde in PBS, incubated in 5, 15, and 30% sucrose in PBS, and frozen in Tissue-Tek OCT as described previously (45). Staining for CXCL12 was performed on acetone-fixed BM sections with goat anti-mouse stromal cell-derived factor 1 (Santa Cruz Biotechnology), followed by biotinylated donkey anti-goat IgG (Jackson ImmunoResearch Laboratories) and streptavidin-Quantum Red (Sigma-Aldrich). Staining for VCAM-1 was performed on paraformaldehyde-fixed BM sections. Endogenous peroxidase activity was inhibited by incubating slides for 5 min with 0.3% H_2O_2/methanol. Nonspecific binding was blocked with 5% BSA in PBS, and sections were then stained with biotinylated anti-CD106 (BD Pharmingen) followed by ExtrAvidin-HRP (Sigma-Aldrich) before the addition of substrate (3-amino-9-ethylcarbazole from BioGenex). Sections were visualized with a Zeiss Axiophot 1 microscope and photographed with an AxioCam digital camera (Zeiss).

Transfer of memory B cells to adult and 1-wk-old mice

Adult and 1-wk-old mice were primed with TT/$Al(OH)_3$. Three weeks later, splenocytes were applied to a Ficoll Hypaque gradient centrifugation (Lympholyte-M; Cederlane Laboratories) and washed, and $4–5 \times 10^7$ cells were transferred i.p. to 1-wk-old or i.v. to adult mice. Recipients were boosted 24 h later with TT/$Al(OH)_3$. Spleen and BM were recovered 6 days after the boost for evaluation of TT-specific IgG ASC by ELISPOT.

Transfer of plasmablasts to adult and 2-wk-old mice

Adult and 1-wk-old mice were primed with TT/$Al(OH)_3$ and boosted 3 wk later. Four days after boosting, splenocytes were treated with ammonium chloride, potassium carbonate (ACK) lysis buffer to eliminate erythrocytes and washed, and 1×10^8 cells were transferred i.v. to adult or 2-wk-old mice. Eighteen or 48 h later, as indicated in the figures, spleen and BM were recovered for evaluation of TT-specific IgG ASC by ELISPOT.

BM stromal cell (BMSC) cultures

BMSC cultures (BMSCCs) were prepared according to a protocol adapted from the Whitlock and Witte method (46). Single-cell suspensions were obtained from the BM of 2-wk-old and adult naive mice and seeded at a concentration of 1×10^7 cells/ml in RPMI 1640/5% FCS. Cultures were prepared in 24-well (1 ml/well) or 6-well plates (5 ml/well) (Corning). After 45 min, cells were resuspended and transferred to another well, and on day 1, culture wells were gently washed to remove nonadherent cells as described (47). At confluence (day 7–8), supernatants (600 μl) were collected and cells were lysed with TRIzol reagent (Invitrogen).

Migration assay

Chemotaxis assays were performed in 24-well plates with Transwell inserts (6.5-mm diameter, 5-μm pores; Corning) as described previously (48), with minor modifications. The inserts were saturated with RPMI 1640/2% FCS for at least 1 h at 37°C. The lower Transwell chamber was filled with 600 μl of solution containing recombinant mouse CXCL12 (R&D Systems) in RPMI 1640/2% FCS or BM culture supernatant. Spleen cell suspensions were diluted at 1.2×10^7 cells/ml, and 100 μl was placed into the inserts. Cells were allowed to migrate for 90 min at 37°C. Finally, the cells were collected from the lower Transwell compartment, and TT-specific ASCs were quantified by ELISPOT. Percentages of migration were calculated as follows: percentage of migrated ASC = migrated ASC/total ASC × 100. Desensitization of the CXCR4 chemokine receptor was performed with 500 nM CXCL12 as described (49).

Survival assays

BMSCCs were initiated as described above. On day 6, stromal cells were replated in 96-well plates at 5×10^4 cells/well in RPMI 1640/1.4% FCS. The next day, plasmablasts from primed and boosted adult mice (day 4 after boost) were positively selected using anti-syndecan-PE followed by anti-PE beads as described above. Purified plasmablasts were resuspended in RPMI 1640/1.4% FCS, and 5×10^3 cells were added to the BMSCCs. In some experiments, anti-VCAM (M/K-2; Southern Biotechnology) was added to the cultures at 5 or 20 μg/ml. The total number of TT-specific IgG ASCs added per well was determined by ELISPOT as described above. Forty-eight hours later, cells were recovered from the cocultures, and TT-specific IgG ASC were quantified by ELISPOT. Percentages of survival were calculated as follows: percentage of surviving ASC = surviving ASC/total ASC × 100.

Real-time quantitative PCR assay

Total cellular RNA was isolated by TRIzol reagent according to the manufacturer's instructions (Invitrogen). Quality of RNA was verified using an Agilent 2100 bioanalyzer. cDNA was synthesized from 1 μg of total RNA using random hexamers and Superscript II reverse transcriptase following the supplier's instructions (Invitrogen). SYBR Green assays were designed using the program Primer Express, version 2.0 (Applied Biosystems), with default parameters. Amplicons were designed over exon boundaries, and sequences were aligned against the mouse genome by BLAST to ensure that they were specific for the gene being tested. Oligonucleotides were obtained from Invitrogen. The efficiency of each design was tested with

serial dilutions of cDNA and was >90% for all primer pairs. PCR (10-μl volume) contained diluted cDNA, 2× SYBR Green Master Mix (Applied Biosystems), and 300 nM forward and reverse primers. PCR were performed on a SDS 7900 HT instrument (Applied Biosystems) with the following parameters: 95°C for 10 min and 45 sequential cycles of 95°C for 15 s and 60°C for 1 min. Each reaction was performed in three replicates in 384-well plates.

EEF and *TBP* were chosen as internal control genes for the normalization of data (50). The following primers were used: CXCL12, 5′-CCG CGC TCT GCA TCA GT-3′ (forward) and 5′-TCT CGA AGA ACC GGC AGG-3′ (reverse); VCAM-1, 5′-CTC TGG GAA GCT GGA ACG AA-3′ (forward) and 5′-GCC ACT GAA TTG AAT CTC TGG AT-3′ (reverse); EEF1A1, 5′-TCC ACT TGG TCG CTT TGC T-3′ (forward) and 5′-CTT CTT GTC CAC AGC TTT GAT GA-3′ (reverse); and TBP, 5′-TTG ACC TAA AGA CCA TTG CAC TTC-3′ (forward) and 5′-TTC TCA TGA TGA CTG CAG CAA A-3′ (reverse). Normalization factors and relative RNA expression were calculated according to geNorm as described previously (50). The relative RNA expression was multiplied by 100.

Statistical analysis

Statistical differences between two groups were analyzed by Mann-Whitney *U* test, and differences between multiple groups were analyzed by one-way ANOVA followed by the Tukey multiple comparison test. Differences with probability values of >0.05 were considered insignificant.

Results

Age-dependent limitations of the establishment of plasma cells in BM

To further define the age at which BALB/c mice reach a stage of immune maturation sufficient to establish an adult-like pool of BM ASCs, mice were immunized at 1, 2, or 4 wk of age with TT adsorbed to alum (TT/AL(OH)$_3$). Confirming our previous results (41), a very low level of TT-specific ASCs were detected 10 days after immunization in the BM of mice primed at 1 wk of age compared with adults (Fig. 1). Remarkably, TT-specific ASCs remained rare in the BM compartment even when immunization was performed at 4 wk of age (Fig. 1), when the total number of white blood cells in the BM has reached adult levels (not shown). This finding is in contrast to the demonstrated capacity of 2-wk-old mice to raise adult-like, TT-specific ASC in their spleens (Ref. 40 and data not shown) and suggests that plasmablasts generated in the spleen fail to efficiently home into the early-life BM compartment.

Adult-like expression of CXCL12 by early-life BMSCs

The migration of adult plasmablasts across the BM endothelium toward specific BM niches has been found to rely on chemoattraction by CXCL12 produced by stromal cells (29). Therefore, the level of expression of CXCL12 in the early-life BM compartment was assessed ex vivo on BM cryosections. Staining for CXCL12 was similarly detected throughout the BM of infant and adult BALB/c mice (Fig. 2*A*). The presence of similar levels of CXCL12 mRNA in the BM of 2-wk-old and adult mice was confirmed by quantitative real-time PCR (Fig. 2*B*). Primary short-term BMSCCs were derived from 1-, 2-, and 4-wk-old or adult mice and were shown to maintain their capacity to produce CXCL12 after in vitro expansion (Fig. 2*C*). CXCL12 was expressed at a lower level in BMSCs from 1- and 2-wk-old mice, but adult levels of expression were observed in BMSCs harvested from 4-wk-old mice.

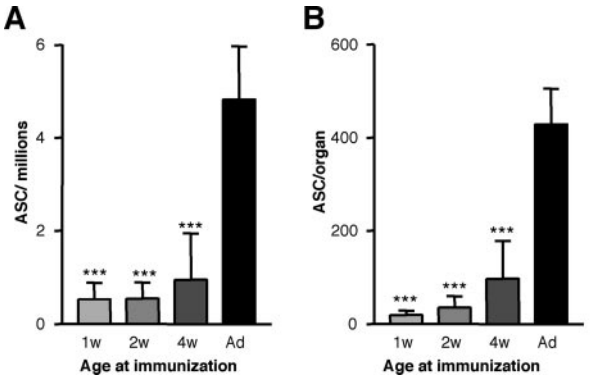

FIGURE 1. Age-dependent deficiency of plasma cells in early-life BM. BALB/c mice received injections i.p. at 1 wk (1w), 2 wk (2w), 4 wk (4w), or 14 wk (Ad) of age with alum-adsorbed TT. At 10 days after immunization, the mice were sacrificed, and TT-specific IgG ASCs in the BM were measured by ELISPOT. Results are expressed as mean ± SD obtained in groups of eight immunized mice, pooled two by two. ASCs per millions of cells (*A*) and ASCs per organ (*B*) from one experiment of two are shown. ***, $p < 0.001$ when comparing mice immunized at 1, 2, or 4 wk of age vs adults.

Similar in vitro migration of ASCs toward early-life or adult BMSC supernatants

To address the possibility that a chemokine other than CXCL12 may be missing in early life or that other BMSC-derived products may inhibit ASC migration, BMSCC supernatants were assessed for their capacity to attract TT-specific ASCs in Transwell assays. The ELISPOT quantification of TT-specific ASCs demonstrated that the BMSCC supernatants of 2-wk-old mice exert a chemoattractant influence on adult TT-specific ASCs that was as strong as the one observed when CXCL12 was added at an experimentally defined optimal concentration (Fig. 2*D*). This effect was even stronger than that seen with adult BMSC supernatants, but this can be explained by a reproducible 2-fold higher cell number in BMSCCs derived from infant rather than from adult mice. Preexposure of splenic ASC to a high concentration (500 nM) of CXCL12 to "desensitize" the CXCR4 receptor completely abrogated plasmablast migration toward both adult and early-life BMSC supernatants. (Fig. 2*D*). The migration toward CXCL12 was as efficient when using TT-specific ASC primed in 1-wk-old instead of adult mice (Fig. 2*E*). Altogether, these experiments demonstrate that TT-specific plasmablasts of 1-wk-old or adult mice are efficiently attracted by CXCL12, which is expressed at adult levels by murine BMSCs from the age of 2–4 wk onwards.

Lack of in vivo persistence of ASCs in the early-life BM compartment

The adult-like migration of plasmablasts toward early-life BMSCs in vitro was contrasting with the low level of specific BM ASCs that can be achieved after immunization in early life. This discrepancy was addressed by adoptive transfer experiments in which mice were adoptively transferred i.v. (or i.p. for 1-wk-old mice) with splenocytes from TT/AL(OH)$_3$ immune mice and immunized 24 h later with TT/AL(OH)$_3$ to generate high numbers of plasmablasts capable of migration toward the BM compartment (Fig. 3). Six days after in vivo boosting, the number of TT-specific ASC was 8-fold higher in the BM of adult compared with the BM of infant recipients ($p = 0.01$), whereas ASCs were present at significantly higher numbers in the spleens of infant recipients ($p = 0.02$). The relative contribution of the BM to the total pool of TT-specific ASCs was therefore 17-fold higher ($p = 0.01$) following the adoptive transfer of ASCs to adult compared with the transfer to 1-wk-old mice (Fig. 3*C*). Reverse experiments demonstrated that TT-specific cells primed in 1-wk-old mice established themselves into the BM compartment of adult recipients as efficiently as cells generated in adult donors (Fig. 3, *D–F*). This result suggested that the homing defect was attributable to the BM compartment but did not allow distinguishing between impaired migration and persistence of ASC in the early-life BM.

To exclude a differential influence of the route of injection on subsequent homing patterns, the next series of experiments included a direct i.v. transfer to adult or 2-wk-old BALB/c mice of

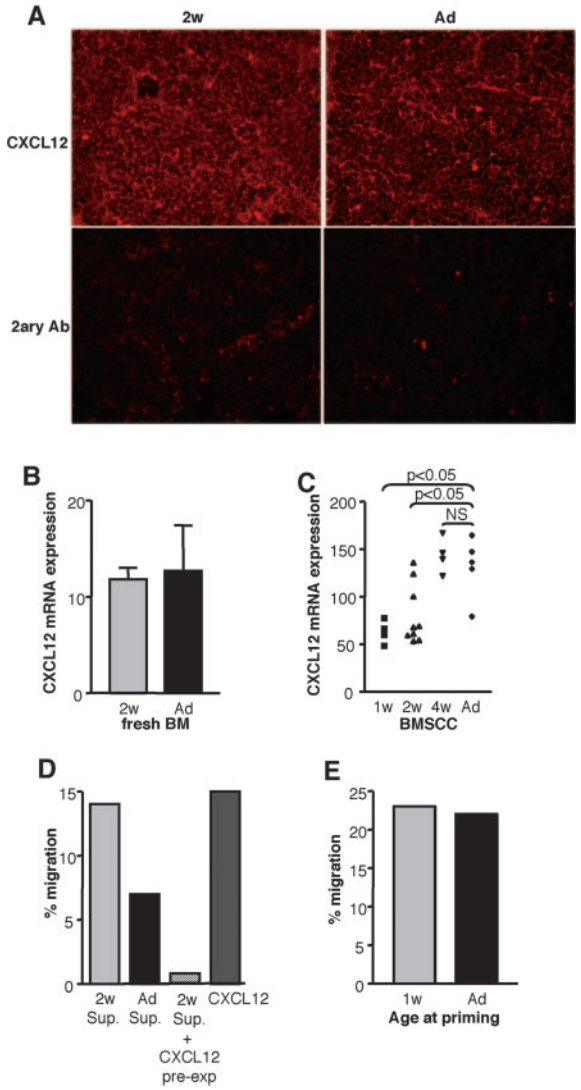

FIGURE 2. CXCL12 is not a limiting factor of early-life BM. *A*, Cryostat sections were prepared from the BM of naive 2-wk-old or adult mice and stained for CXCL12 as described in *Materials and Methods*. Controls omitting the primary Ab are shown for comparison. Photos were taken with a ×40 objective. *B*, The expression of mRNA for CXCL12 was evaluated on fresh BM cells from 2-wk-old or adult mice by quantitative real-time PCR. Results are expressed as mean ± SD obtained from groups of six or seven mice. *C*, The expression of mRNA for CXCL12 was evaluated by quantitative real-time PCR on BMSCCs prepared from 1-, 2-, and 4-wk-old and adult mice. Results are shown for four to nine independent cultures per age group, each representing a pool of two to four mice. *D*, The migration of TT-specific plasmablasts toward BM stromal cultures from 2-wk-old or adult mice was evaluated in a Transwell assay. Adult mice were immunized with TT/Al(OH)$_3$ and boosted on day 21. Splenocytes were isolated at day 4 postboost, and 1.2×10^6 splenocytes were put in the upper compartment. BM cultures were prepared from 2-wk-old and adult mice. Supernatants were collected after 1 wk and transferred to the lower compartment for the migration assay. CXCL12 was used as a positive control. Desensitization of the CXCR4 chemokine receptor was performed by incubating cells with 500 nM CXCL12 before the Transwell assay. TT-specific ASCs were enumerated by ELISPOT. The percentage of input TT-specific ASC that migrated to the lower chamber is shown. The numbers of total TT-specific ASC were $5900/10^6$ spleen cells. One experiment of five is shown. *E*, The migration of adult and early-life TT-specific plasmablasts toward CXCL12 was evaluated in a Transwell assay. One-week-old or adult mice were immunized with TT/Al(OH)$_3$ and boosted on day 21. Splenocytes were isolated at day 4 postboost, and migration toward 30 nM CXCL12 was evaluated as in *D*. One experiment of four is shown.

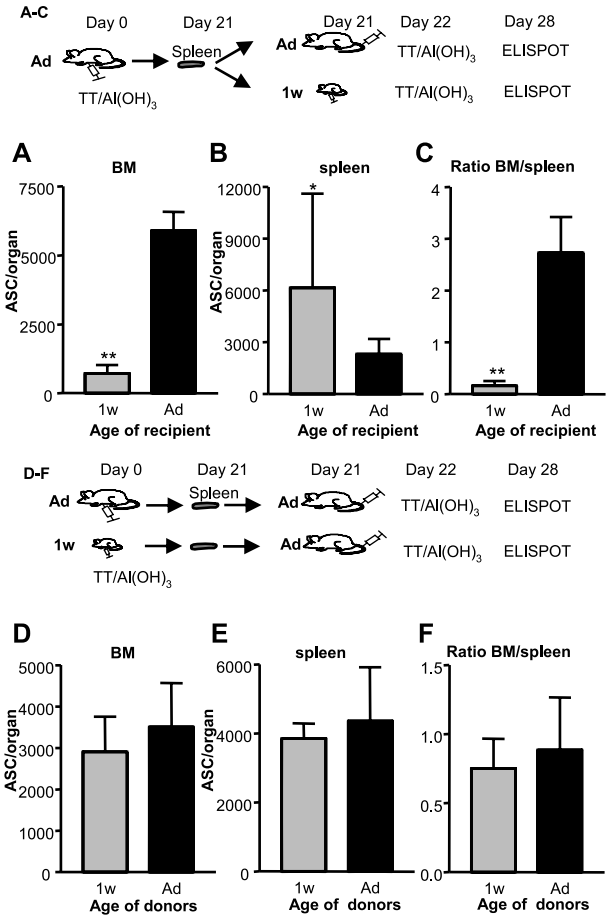

FIGURE 3. Deficiency of early-life BM and not early-life plasma cells. *A–C*, Adult BALB/c mice received injections i.p. with alum-adsorbed TT. Three weeks after immunization, splenocytes were transferred to 1-wk-old or adult recipients that were "boosted" with alum-adsorbed TT 24 h later. Six days after the boost, the mice were sacrificed, and TT-specific IgG ASC in the spleen and BM were measured by ELISPOT. Results are expressed as mean ± SD obtained in groups of three to six mice. ASCs per organ from one experiment of three are shown. **, $p < 0.01$; *, $p < 0.05$ when comparing 1-wk-old recipients vs adults. *D–F*, Adult or 1-wk-old BALB/c mice received injections i.p. with alum-adsorbed TT. Three weeks after immunization, splenocytes were transferred to adult recipients that were boosted with alum-adsorbed TT 24 h later. Six days after the boost the mice were sacrificed, and TT-specific IgG ASC in the spleen and BM was measured by ELISPOT. Results are expressed as mean ± SD obtained in groups of three to six mice. ASCs per organ from one experiment of three are shown.

plasmablasts previously generated by the priming and boosting of adult donors. Donor TT-specific plasmablasts were harvested 4 days after boosting, experimentally defined as the peak of the plasmablast response (not shown). Cells were adoptively transferred i.v. to adult or 2-wk-old recipient mice, and analyses were performed either early (18 h) or late (48 h) after transfer. At 18 h, similar numbers of TT-specific ASC were recovered from the BM compartment of 2-wk-old and adult recipients (Fig. 4, *A–C*). At 48 h, the number of TT-specific ASCs in the BM of adult recipients was similar to that observed 18 h after transfer (Fig. 4, *D–F*). In contrast, the BM of 2-wk-old recipients contained 3-fold-lower TT-specific ASCs ($p = 0.05$) compared with adult recipients despite similar numbers of TT-ASC in the spleens of both age groups. In fact, 70% of the transferred adult TT-specific ASCs that were detected at 18 h in the BM compartment of 2-wk-old mice had disappeared 30 h later. Thus, the establishment of the BM plasma

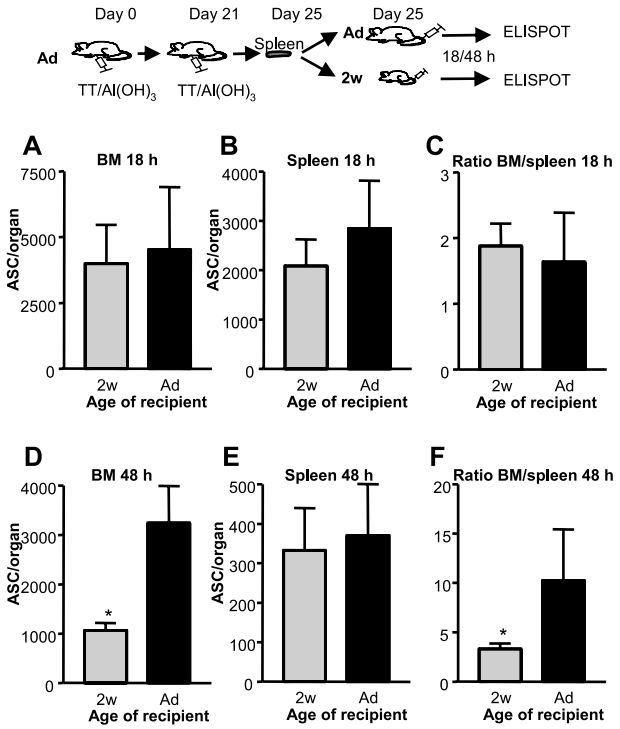

FIGURE 4. Efficient migration but impaired persistence of plasmablasts in early-life BM. *A–C*, Adult BALB/c mice received injections i.p. with alum-adsorbed TT and boosted 3 wk later. Four days after the boost, splenocytes were transferred to 2-wk-old or adult recipients. Eighteen hours later, the mice were sacrificed, and TT-specific IgG ASCs in the spleen and BM were measured by ELISPOT. Results are expressed as ASCs per organ (mean ± SD) obtained in groups of three mice and represent one of three experiments. *D–F*, Adult BALB/c mice received injections i.p. with alum-adsorbed TT and boosted 3 wk later. Four days after the boost, splenocytes were transferred to 2-wk-old or adult recipients. Forty-eight hours later, the mice were sacrificed, and TT-specific IgG ASCs in the spleen and BM were measured by ELISPOT. Results are expressed as ASCs per organ (mean ± SD) obtained in groups of three mice and represent one of two experiments. *, $p < 0.05$ when comparing 2-wk-old recipients vs adults.

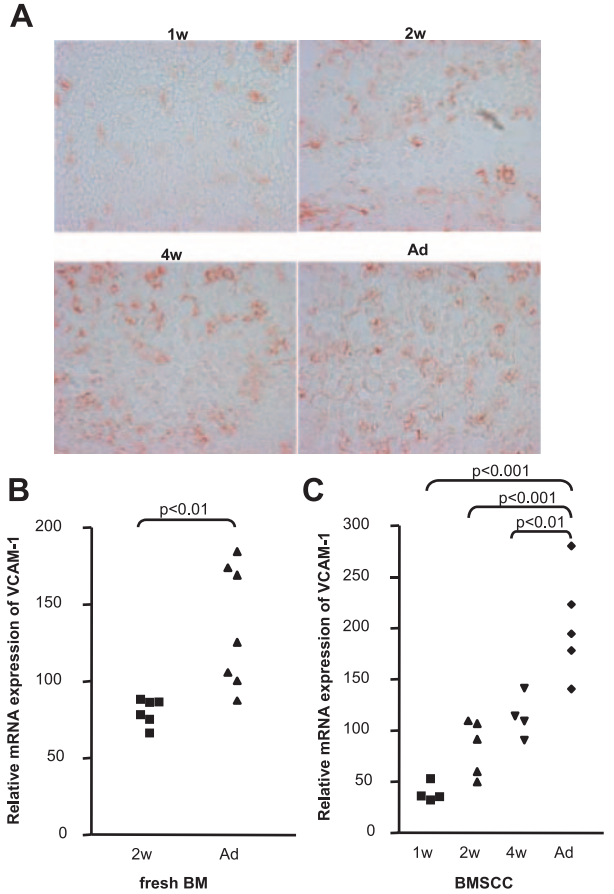

FIGURE 5. Limited VCAM-1 expression on early-life BM. *A*, Cryostat sections were prepared from the BM of naive 1-, 2-, and 4-wk-old and adult mice and stained for VCAM-1 as described in *Materials and Methods*. Photos were taken with a ×40 objective. *B*, The expression of mRNA for VCAM-1 was evaluated on fresh BM cells from 2-wk-old or adult mice by quantitative real-time PCR. *C*, The expression of mRNA for VCAM was evaluated by quantitative real-time PCR on BMSCCs prepared from 1-, 2-, and 4-wk-old and adult mice. Results are shown for four or five independent cultures per age group representing each a pool of two to four mice.

cell pool is limited in early life by factors that affect ASC persistence in the BM rather than plasmablast migration and initial homing.

Limited VCAM-1 expression on early-life BMSCs

The differentiation of apoptosis-prone plasmablasts into surviving plasma cells requires their localization into specific niches providing anchoring and survival factors acting synergistically. As the BM niches for plasma cells have been characterized by the presence of as $CXCL12^+$, $VCAM\text{-}1^+$ stromal cells (26), we studied the ontogeny of VCAM-1 expression in the BM compartment. The level of VCAM-1 expression was markedly weaker on BM cryosections of 1- and 2-wk-old mice compared with that of adult mice (Fig. 5*A*). This finding was confirmed by the observation of significantly lower ($p = 0.002$) mRNA levels in total BM cell suspension of 2-wk-old mice compared with adult mice (Fig. 5*B*). Because VCAM-1 is expressed by BM endothelial and stromal cells (51), BMSCCs generated from mice of different ages were used to assess VCAM-1 expression. VCAM-1 was expressed at significantly lower levels in BMSCs from 2-wk-old compared to adult mice (Fig. 5*C*; $p < 0.008$), and its expression only progressively increased with age. The strong correlation between the age-dependent limitation of BM ASCs and the limited expression of VCAM-1 by BMSCs suggested that VCAM-1 may be one of the factors involved in the persistence of ASCs in adult BM.

Short survival of plasmablasts in the early-life BM compartment

The insufficient adhesion of hemopoietic stem cells to BMSCs is associated with their mobilization and appearance in the circulation (52). To define whether the rapid disappearance of plasmablasts from the early-life BM compartment (Fig. 4) might reflect their return to circulating blood, TT-specific ASCs were enumerated in blood at various times after the adoptive transfer. However, very few ASCs (<10 ASCs per milliliter of blood) were found in the blood of adult and 2-wk-old mice 18 h after transfer and none after 48 h. Thus, the rapid disappearance of Ag-specific ASCs from the early-life BM compartment is not associated with their release into the blood.

Altogether, the previous experiments suggested that the early-life BM compartment may fail to support the differentiation of plasmablasts into surviving plasma cells. Plasmablasts elicited by $TT/AL(OH)_3$ immunization of adult mice were thus harvested 4 days after boosting, labeled with CFSE, and selected by $CD138^+$ expression. This cell suspension, which contained 30–50% TT-specific ASCs (measured by ELISPOT; data not shown), was adoptively transferred by i.v. injection to 2-wk-old and adult mice.

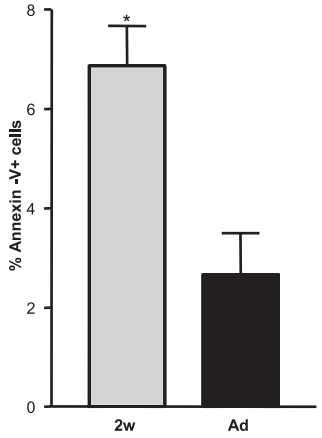

FIGURE 6. Apoptosis of plasmablasts in early-life BM. Adult mice were primed with TT/$Al(OH)_3$ and boosted 3 wk later. Four days after the boost, plasmablasts were labeled with CFSE and purified by positive selection of syndecan$^+$ cells using autoMACS. Two to three million TT-specific IgG plasmablasts were transferred into an adult mouse and into a 2-wk-old mouse. Forty to 44 h later, BM cells were recovered and stained with syndecan-PE and annexin V-Cy5. Percentages of annexin V-positive cells of CFSE$^+$ syndecan$^+$ cells are shown. Three independent experiments are shown. Results are expressed as mean ± SD obtained from three independent experiments. *, $p < 0.05$ when comparing 2-wk-old recipients vs adults.

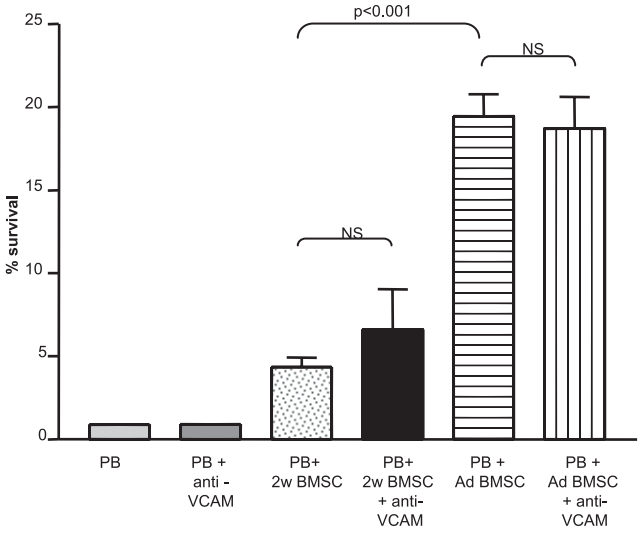

FIGURE 7. Impaired ability of early-life BMSCs to support plasmablast survival. The survival of TT-specific IgG plasmablasts was evaluated in cocultures with BMSC from 2-wk-old or adult mice. BMSCCs were prepared from 2-wk-old and adult mice, and the stromal cells were reseeded into 96-well plates at 5×10^4 cells/well 6 days after the start of culture. Adult mice were primed and boosted with TT/$Al(OH)_3$. Four days after the boost, plasmablasts were purified by positive selection of syndecan$^+$ cells using autoMACS, and 5×10^3 cells were added to wells containing only medium or to wells containing BMSCs that had been reseeded the previous day. Anti-VCAM-1-blocking mAb was added to some BMSCCs as indicated. After 48 h of coculture, the cells were recovered, and surviving TT-specific IgG ASCs were enumerated by ELISPOT. The percentage of surviving plasmablasts was calculated as follows: ASC recovered/ASC put into culture × 100. Results are expressed as the percentage of surviving plasmablasts (mean ± SD) in four independent cultures per age group representing each a pool of four mice. One experiment of four is shown.

Within the BM cells collected 40–44 h after transfer, the fraction of CSFE$^+$, CD138$^+$, and annexin V$^+$ apoptotic cells was found in three separate experiments to be 2.1- to 3.1-fold higher in 2-wk-old mice than in adult controls (Fig. 6). Thus, plasmablasts that reach the early-life BM compartment undergo an increased rate of apoptosis in vivo.

To directly assess the capacity of early-life BMSC to facilitate or maintain plasmablasts survival, TT-specific plasmablasts elicited in adult mice were cultured in vitro on BMSCCs generated from either 2-wk-old or adult mice. In accordance with their high susceptibility to apoptosis, few plasmablasts survived after 48 h of culture in the absence of supporting stromal cells (Fig. 7). The survival rate was much higher when plasmablasts were seeded on adult BMSCs. In contrast, the addition of BMSC from 2-wk-old mice only slightly increased the percentage of surviving plasmablasts at a level that was significantly lower ($p < 0.001$) than that seen using adult stromal cells as seed layer. Plasmablast survival on early-life or adult BMSCs was not influenced by the addition of a blocking Ab directed against VCAM-1 (Fig. 7). This result indicates that VCAM-1 may not play an essential role in the limited plasmablast-supporting capacity of early-life BMSCs as observed previously for plasma cell survival (24). These results confirm that early-life BMSCs have a limited capacity to generate the signals required to support plasmablast survival.

Discussion

The present study demonstrates that plasmablasts generated in the spleen efficiently migrate to the early-life BM compartment but that early-life BMSCs lack some functional properties that are required to support their survival.

We had previously reported the delayed establishment of the long-term BM plasma cell pool following neonatal murine immunization (41). Most of our studies have used TT, a strong immunogen being required to elicit detectable primary ASC responses in early life. However, similar results were also obtained with OVA as a model Ag (our unpublished observation). In addition, total IgG-secreting plasma cells, which are likely to reflect responses to environmental Ags in nonimmunized mice, were also shown to increase very slowly in the BM compartment during the first 6 wk of life (41). The present studies demonstrate that this delay reflects the existence of limiting factors affecting the early-life BM compartment itself rather than early-life plasmablasts. Postponing immunization until 4 wk after birth, an age at which the administration of an immunogen as strong as TT elicits adult-like numbers of splenic plasmablasts (40), failed to induce adult-like ASC responses in the BM. The adoptive transfer of adult TT-specific memory B cells, followed by in vivo Ag exposure to drive B cell differentiation toward high number of ASC in the spleen, resulted into the appearance of ASCs in the adult but not the early-life BM. Last, the direct transfer of adult splenic plasmablasts to 2-wk-old or adult recipients resulted into similar number of ASCs into the spleen of both age groups, but 2 days after transfer, these cells could only be recovered from the adult BM compartment. In contrast, neonatally primed splenocytes efficiently established themselves into the BM of adult recipients. Thus, there is a postnatal immune maturation of the BM compartment that extends over a period of several weeks (>4 wk), i.e., much later than the age at which efficient ASC responses may be elicited in secondary lymphoid organs.

CXCL12, the main chemoattractant of plasmablasts toward the BM (29, 48), is expressed at adult levels by BMSC from the age of 2–4 wk onwards, as assessed ex vivo and after in vitro expansion in short-term BMSCCs. In addition, early-life BMSCs efficiently attract TT-specific plasmablasts in vitro and exert no inhibitory influence on the migration of adult plasmablasts. Last, plasmablasts were present at similar number in the BM compartments of adult and 2-wk-old mice when assessed early (18 h) after adoptive

transfer. Altogether, these results demonstrate that plasmablasts generated in the spleen efficiently migrate toward CXCL12-expressing BMSCs already at an early maturation stage but fail to establish themselves as surviving ASCs in early-life BM niches.

The differentiation of apoptosis-prone plasmablasts into surviving plasma cells requires their localization into specific niches, providing anchoring and survival factors acting synergistically (25). BM niches for plasma cells have been characterized as containing CXCL12$^+$, VCAM-1$^+$ stromal cells (26), and this led us to study the postnatal dynamics of CXCL12 and VCAM-1 in BMSC. In contrast to the early acquisition of CXCL12 expression, we identified a delayed postnatal maturation of VCAM-1, which was not yet expressed at adult levels by BMSC at the age of 4 wk. Adhesion molecules have been shown to work synergistically with chemokines and cytokines to promote plasma cell survival, and it is tempting to correlate this delayed acquisition with the delayed capacity of supporting the establishment of ASCs into the BM ASCs. The importance of VLA-4/VCAM-1 interactions for the survival of plasmacytoma cells has been demonstrated (53). However, although blocking VLA-4 decreased Ab secretion in a plasma cell/stromal cell coculture system, thus suggesting an effect on plasma cell survival, inhibiting contact with VCAM-1 did not have the same inhibiting effect (24). Similarly, we did not observe a reduction of plasmablasts survival when the VCAM-1 Ab was added to adult or early-life BMSCCs (Fig. 7). This observation suggests that the significantly reduced expression of VCAM-1 on early-life BMSCs may not play an essential role in the limitation of the capacity of BMSCs to support the survival of plasmablasts and/or their differentiation into surviving plasma cells.

Whereas most TT-specific ASCs that migrated to an adult BM were still producing TT-specific Abs 2 days after adoptive transfer, $>$70% of TT-specific ASCs had disappeared from early-life BM within 30 h. These ASCs did not return to the blood or the spleen compartments. In contrast, we observed within the same time frame a 2- to 3-fold increase in the proportion of plasmablasts undergoing in vivo apoptosis in the early-life BM. Plasmablasts survival was significantly increased by coculture on a layer of adult BM stromal cells, but this effect was much more limited on early-life BMSCs. Numerous factors have been shown to provide signals supporting plasma cell survival, including IL-6, CXCL12, IL-5, TNF-α, CD44 ligands, and APRIL and/or BAFF (24, 25, 33, 34). In contrast, little is yet known about the signals required for the early survival of apoptosis-prone plasmablasts in the BM and their differentiation into long-lived plasma cells (34). As an example, blocking B lymphocyte stimulator both increases the apoptosis of splenic plasmablasts and reduces plasma cell numbers in the BM (54, 55). Whether B cell maturation Ag (BCMA) signaling is required for the survival of plasmablasts, its establishment in the BM or its requirement for the survival of plasma cells thus remains to be established. Similarly, the absence of specific plasmablast/plasma cell markers does not allow us to formally assess whether missing BM-derived signals limit the survival of plasmablasts or their end-stage differentiation into plasma cells. More work is thus needed to identify the molecular and cellular factors that better support plasmablast survival in the adult rather than the early-life BM compartment. The availability of adult and early-life BMSC populations with distinct functional properties provides new opportunities toward the identification of such factors and the study of their ontogeny. The use of short-term primary BMSCs was necessary to limit the risk of a spontaneous differentiation of neonatal cells during a prolonged in vitro culture period. These short-term cultures contain several cell populations, such that differences between neonatal and adult BMSCs could result from differences in their cellular composition and/or in their function. Despite the technical limitations resulting from the low number of BMSCs that can be retrieved from infant mice and expanded in short-term cultures, studies comparing the cellular composition and the genetic profiles of early-life and adult BMSCs have thus been initiated.

The observation of a developmental acquisition of BM niches capable of supporting the survival of plasmablasts and their differentiation into plasma cells is of significant interest. A limited number of plasma cell BM niches is indeed postulated as regulating the frequencies of BM plasma cells in different species, including mice and humans, and hence as controlling serum Ab levels (56, 57). We currently do not know whether the limitations of the BM compartment reported in our early-life murine models affect human infants. However, the relative stability during several years of most Ag-specific serum IgG levels in adults is in striking contrast to their rapid decline when immunization is performed during the first year of life (13–16). In addition, it is striking that the stage of immune maturation that is sufficient for the induction of strong short-lived Ab responses is reached in both species much earlier in life than the one associated with the induction of sustained Ab levels. We therefore suggest that the slow increase in serum IgG titers may not only reflect the progressive exposure to a growing number of environmental Ags but also the progressive access of plasmablasts to BM niches that support their survival. This limitation could reflect the requirement for early-life stromal cells to essentially support hemopoietic stem cells. It is tempting to postulate that evolution could have resulted into a delayed development of plasma cell BM niches so as to preferentially support rapid changes in serum Ab profiles rather than the long-term persistence of Abs against neonatally encountered Ags. A delayed induction of functional BM niches would result in enhanced competition between plasmablasts elicited in response to early encountered Ags and thus play an important role in the shorter duration of early-life Ab response to infections and immunization (13–16). This will need to be taken into careful consideration for the design of immunization program requiring the maintenance of sustained Ab responses following early-life immunization.

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