Plasma Cell Survival Is Mediated by Synergistic Effects of Cytokines and Adhesion-Dependent Signals[1]

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Recent results suggest that plasma cell longevity is not an intrinsic capacity, but depends on yet unknown factors produced in their environment. In this study, we show that the cytokines IL-5, IL-6, TNF-α, and stromal cell-derived factor-1α as well as signaling via CD44 support the survival of isolated bone marrow plasma cells. The cytokines IL-7 and stem cell factor, crucially important for early B cell development, do not mediate plasma cell survival, indicating that plasma cells and early B cells have different survival requirements. As shown in IL-6-deficient mice, IL-6 is required for a normal induction, but not for the maintenance of plasma cell responses in vivo, indicating that the effects of individual survival factors are redundant. Optimal survival of isolated plasma cells requires stimulation by a combination of factors acting synergistically. These results strongly support the concept that plasma cell survival depends on niches in which a combination of specific signals, including IL-5, IL-6, stromal cell-derived factor-1α, TNF-α, and ligands for CD44, provides an environment required to mediate plasma cell longevity. ***The Journal of Immunology,*** **2003, 171: 1684–1690.**

Upon antigenic stimulation, B cells can proliferate and differentiate into Ab-secreting plasma blasts and eventually into nondividing plasma cells, constitutively secreting thousands of Ab molecules per second (1). Primary Ab responses usually cease within days or weeks after immunization. However, following repeated immunization with T-dependent Ag, specific Ab titers can persist for years (2). Maintained Ab responses mainly consist of Ab of the IgG subclasses and constitute an essential part of the protective immune memory (3). The average lifetime of Ab-secreting plasma cells is shorter than 3 wk (4). However, some plasma cells can live much longer to produce persistent memory Ab titers (5, 6). The estimated $t_{1/2}$ of these long-lived plasma cells in mice is ~6 mo (7). Like early B cells, most of these plasma cells reside in the bone marrow, although a smaller population can be found in the splenic red pulp (7–9). Recent evidence suggests that plasma cell longevity is not an intrinsic capacity of the cells, but is regulated by their environment (9). In consequence, a current concept of plasma cell homeostasis suggests that the survival of individual plasma cells depends on specific survival signals produced in a limited number of ecological niches, most of them present in the bone marrow, but also in inflamed tissues (10, 11).

The development of B cells from their precursors depends on the environment of the bone marrow (12, 13). It has been suggested that this process occurs in specific niches (14) in which a stepwise progression through a set of well-defined differentiation stages takes place that is controlled by survival and differentiation signals provided by the environment (15). Mature B cells leave the bone marrow to circulate through the periphery and secondary lymphoid tissues. At that stage of their development, cell fate is mainly determined by whether or not they encounter their cognate Ag, and costimulatory signals from T cells and dendritic cells (16).

Stimulation by survival factors results in the induction of anti-apoptotic molecules, e.g., Bcl-2 or Bcl-x_L, which maintain mitochondrial integrity and are required for the homeostasis of lymphocyte subpopulations. (17, 18). This work is aimed to identify the mechanisms by which the survival of mature plasma cells is regulated.

Materials and Methods

Mice and immunizations

IL-$5^{-/-}$ and IL-$6^{-/-}$ mice were purchased from The Jackson Laboratory (Bar Harbor, ME). All mice were housed in specific pathogen-free conditions in the institute's animal facility. Each mouse received 100 μg alum-precipitated OVA in 100 μl PBS i.p. Subsequently, 3–5 wk later, mice were boosted by i.v. injection of 100 μg OVA in 100 μl PBS.

ELISA and ELISPOT

For measurement of OVA-specific Ab in sera, 96-well plates were coated with OVA (Sigma-Aldrich, St. Louis, MO; 5 μg/ml in PBS). Sera were incubated for 1 h at 37°C at various dilutions. For detection, biotin-conjugated goat anti-mouse IgG (Southern Biotechnology Associates, Birmingham, AL) was added. The ELISA was developed with streptavidin-conjugated alkaline phosphatase (Boehringer Mannheim, Indianapolis, IN) and *p*-nitrophenylphosphate disodium salt (Merck, Darmstadt, Germany). IgG- and anti-OVA-secreting cells were detected by ELISPOT, as described (19).

Reagents and Ab

mAb IM7 (anti-CD44), IRAWB (anti-CD44), and KM81 (anti-CD44) were kindly provided by A. Hamann (Charité University Hospital, Berlin, Germany). mAb 90 (anti-CD38), 281-2 (anti-CD138), 1D3 (anti-CD19), R35-38 (isotype standard rat IgG2b), and R-3595 (isotype standard rat IgG2a) were obtained from BD PharMingen (San Diego, CA). Goat anti-mouse Ig-biotin and Fluoromount G were purchased from Southern Biotechnology Associates. MACS beads, mAb X-56 (anti-mouse IgG1), and

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the magnetic sorter MACS were provided by Miltenyi Biotec (Bergisch Gladbach, Germany). mAb R33.18.10 (anti-κ light chain), 2.4G2 (anti-CD16/32), and 1C10 (anti-CD40) were purified from hybridoma supernatants. CyChrome-conjugated streptavidin, recombinant murine IL-1β, IL-2, IL-4, IL-5, IL-7, IL-10, IL-18, and TNF-α were obtained from BD PharMingen; IL-6 and LIF from Cell Concepts (Umkirch, Germany); stromal cell-derived factor (SDF[3])-1α was purchased from R&D Systems (Minneapolis, MN); hyaluronic acid (HA) from Sigma-Aldrich. IL-12 was a gift from the Genetics Institute (Boston, MA). Sulfate polystyrene latex microspheres (5 $\pm$ 0.1 μm mean diameter) were obtained from Interfacial Dynamics (Portland, OR).

Plasma cell isolation

Bone marrow cells were labeled with goat anti-mouse Ig-biotin (Southern Biotechnology Associates; 5 μg/ml), and subsequently incubated with streptavidin-CyChrome. Subsequently, staining of IgG1-secreting plasma cells was performed with minor modifications of the original protocol (20). Briefly, cells stained for surface Ig were washed and resuspended in 1 ml of freshly prepared EZ-link Sulfo-NHS-LC-biotin (Pierce, Rockford, IL; 0.5 mg/ml) in PBS (37°C, for 10 min). One milliliter of complete medium was added, and cells were incubated for another 10 min. After three washing steps with cold PBS/0.5% BSA (PBS/BSA), cells were incubated with anti-CD16/CD32 (10 μg/ml) and unconjugated R33.18.10 (30 μg/ml) for 5 min on ice. Then R33.18.10-avidin conjugated (30 μg/ml) was added. After 7 min on ice, the cell suspension was resuspended in complete medium and kept at room temperature for 10 min. Within 15 min, cells were slowly cooled down to 4°C. Subsequently, cells were incubated with digoxigenin-conjugated anti-IgG1 for 10 min, followed by incubation with anti-digoxigenin magnetic beads (10 min), and finally with anti-digoxigenin FITC (10 min). Then cells were washed, and IgG1-positive cells were enriched with a MiniMACS column (described by the manufacturer) and isolated by FACS.

Cell culture

Cell preparation and culture were done in RPMI 1640 medium (Life Technologies, Karlsruhe, Germany) supplemented with 10% FCS (Sigma-Aldrich) and referred to as complete medium. Plasma cells were sorted into 96-well plates (round bottom; Costar, Cambridge, MA) by FACS. One hundred plasma cells were cultured per well in 200 μl at 37°C in a humidified incubator.

Coating of latex microspheres with Abs

Beads were resuspended in PBS containing the indicated Ab (80 μg/ml) and incubated for 1.5 h at 37°C. Then beads were washed with PBS and incubated with complete medium for 30 min at room temperature.

RT-PCR

Cells were directly FACS sorted into tubes (10 or 100 cells/tube) and RT-PCR was performed. Plasma cells were identified, as described above. Mature B cells (B220 positive/CD24 intermediate) were sorted from spleen cell suspensions. For B cell stimulation, spleen cells were cultured at the density of 10^6 cells/ml in complete medium with LPS (25 μg/ml; Sigma-Aldrich). After 3 days of culture, activated B cells (CD138 positive/surface IgM positive) were sorted. RT-PCR was performed by using One-Step RT-PCR kit (Qiagen, Valencia, CA), according to the producer's instructions. The PCR protocol consisted of 35 cycles (30 cycles for β-actin) of 94°C for 45 s, 58°C for 45 s, and 72°C for 1 min using Master Cycler Personal (Eppendorf, Hamburg, Germany). The PCR amplification products were separated in 1.5% agarose gel and visualized by ethidium bromide staining. Bcl-2, Bcl-x_L, Mcl-1, Bax, CD95, and β-actin primers were as reported (21, 22).

Results

Isolation of bone marrow plasma cells

Following immunization with OVA, the majority of bone marrow plasma cells in BALB/c mice secrete Ab of the IgG1 isotype (own unpublished data). This characteristic was used to identify them in bone marrow cell suspensions by using the cellular affinity matrix technology (20) allowing immunofluorescent labeling of Ab-secreting cells (Fig. 1*A*). Staining of membrane-bound Ig with saturating amounts of anti-Ig before use of the affinity matrix technology allowed to discriminate between membrane-bound and secreted Ig. To increase the numbers of plasma cells, mice were immunized and boosted with OVA. About 2 wk after the secondary immunization and stable thereafter, the frequency of IgG1-secreting plasma cells had increased to ~0.1%. These cells were enriched by MACS and isolated by FACS (Fig. 1*A*). When analyzed by intracellular immunofluorescence microscopy, the sorted cells were found to be Ab-containing cells having the typical morphology of plasma cells, with enlarged cytoplasm and an eccentric nucleus (Fig. 1*B*). As confirmed by ELISPOT, >90% of the cells stained for secreted IgG1 were Ab-secreting cell (ASC).

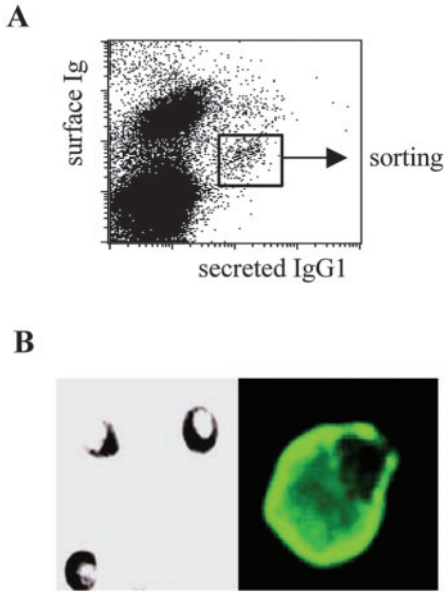

FIGURE 1. Purification of bone marrow plasma cells. *A*, FACS analysis of IgG-1-secreting bone marrow cells after MACS enrichment. Bone marrow cell suspensions were prepared from femurs of at least five mice. The cellular affinity matrix technology was used to label IgG1-secreting cells, as described in *Materials and Methods*. Subsequently, these cells were enriched by MACS and isolated by FACS. Dead cells were excluded according to propidium iodide staining. *B*, Cytospin analysis of isolated cells. FACS-sorted IgG1-secreting cells were cytocentrifuged onto slides, fixed, and stained with FITC-conjugated anti-IgG1 mAb. The cell preparate was then analyzed either by phase microscopy (*left*) or by fluorescence microscopy (*right*).

Isolated bone marrow plasma cells disappear rapidly in culture

The number of plasma cells in culture was determined by ELISPOT. When cultured without feeder cells, ~3% of the original plasma cell numbers were recovered after 3 days and none after 1 wk (Fig. 2*A*). In contrast, in cultures of nonprocessed total bone marrow cells, a less pronounced decline in the numbers of IgG-secreting cells was found. The frequency of surviving plasma cells in suspensions containing total bone marrow cells after 7 days was ~70%, with a high degree of variation. To test whether plasma cells are better supported in the intact organ, we determined the numbers of plasma cells in both femurs of the same mice either directly, or after 7 days of culture of intact bone. Remarkably, no decline of plasma cell numbers could be observed under this condition (Fig. 2*A*). Culture of isolated plasma cells together with a feeder layer derived from bone marrow of SCID mice resulted in the survival of 30% plasma cells until day 3 (Fig. 2*B*). These findings show that the survival of mature bone marow plasma cells depends on factors produced by their environment.

[3] Abbreviations used in this paper: SDF, stromal cell-derived factor; ASC, Ab-secreting cell; HA, hyaluronic acid; OH-U, hydroxyurea.

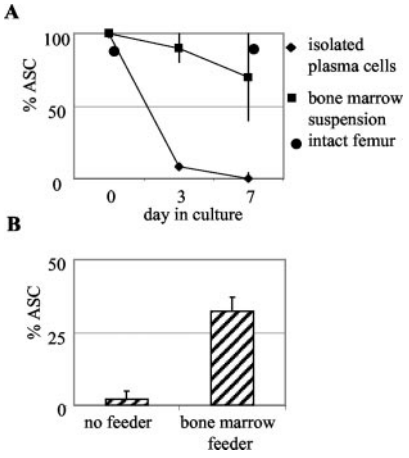

FIGURE 2. Bone marrow environment maintains plasma cell function. *A*, Isolated bone marrow plasma cells disappear rapidly in culture. Kinetics of plasma cells in cultures consisting of isolated plasma cells (100 cells/well), total bone marrow single cell suspension (10^6 cells/ml), or intact femurs were analyzed in culture by measuring the numbers of ASC by ELISPOT. Plasma cell numbers in intact femur cultures at days 0 (referred to as 100%) and 7 were compared in both femurs of individual mice ($n = 4$). Values are expressed as the mean ± SD of ASC numbers in duplicate cultures. One representative experiment is shown ($n = 3$). *B*, Isolated plasma cells are rescued by bone marrow cells. FACS-sorted IgG1-secreting cells (100 cells/well) were cultured alone or on a bone marrow feeder layer (10^5 cells/ml) prepared from SCID mice. The numbers of plasma cells in cultures were analyzed at day 3 by ELISPOT. The mean + SD of one representative experiment is shown ($n = 2$).

Pro- and antiapoptotic gene expression in isolated plasma cells

Several reports suggested that the survival of normal and malignant plasma cells is regulated by the expression of pro- and antiapoptotic molecules (23–25). In this study, the presence of transcripts for Bcl-2, Bax, Bcl-x_L, Mcl-1, and CD95 was investigated in isolated bone marrow plasma cells, resting mature B cells, and 3-day LPS-activated B cells by RT-PCR. All three cell types expressed Bax, Bcl-x_L, and Mcl-1 (Fig. 3). We detected neither Bcl-2 nor CD95 transcripts in plasma cells. The observed absence of CD95 expression is in accordance with the described lack of this molecule on human bone marrow plasma cells (26).

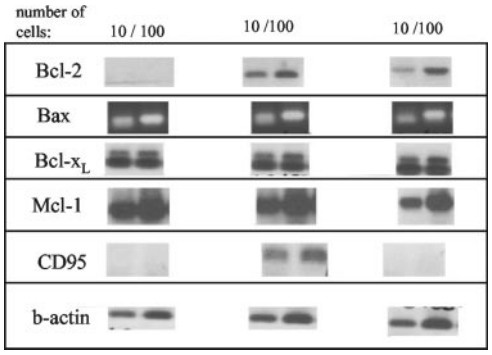

FIGURE 3. Pro- and antiapoptotic gene expression in isolated plasma cells. Mature B cells ($B220^+$, CD24 intermediate), LPS-activated B cells (3 days cultured, $CD138^+$, IgM^+), or plasma cells (IgG1-secreting cells, as identified in Fig. 1) were FACS sorted into tubes, and RT-PCR was performed, as described in *Materials and Methods*. PCR bands obtained for the indicated molecules for plasma cells (*left*), LPS-activated B cells (*middle*), and mature B cells (*right*) are shown for 10 and 100 sorted cells, respectively. In Bcl-x_L lanes, the *lower and upper bands* consist of the primer pairs and Bcl-x_L transcripts, respectively.

Identification of factors mediating plasma cell survival

The effect of cross-linking of various cell surface receptors or stimulation with cytokines produced in the bone marrow was investigated on isolated plasma cells (Table I). Addition of IL-5, IL-6, SDF-1α, TNF-α, or bone marrow supernatant resulted in 30–70% plasma cell recovery after 3 days. Additionally, we tested whether cross-linking of CD19, CD38, CD40, CD44, or CD138 affected plasma cell recovery. Although stimulation of CD44 resulted in ~60% surviving plasma cells, cross-linking of the other receptors with stimulating Ab did not result in significantly increased frequencies of surviving plasma cells compared with the isotype controls. Among the cytokines tested, IL-6 showed the most pronounced effect on plasma cell survival. Its effectiveness was concentration dependent (Fig. 4*A*). The addition of this cytokine immediately following isolation rescued 70% plasma cells until day 3 in culture, while the addition of IL-6 later did not rescue plasma cells (Fig. 4*B*). This observation is in accordance with the idea that plasma cells require constitutive signals to survive. To ensure that in our culture system IL-6 induced plasma cell survival rather than proliferation, bone marrow plasma cells were cultured in presence of hydroxyurea (OH-U), an inhibitor of proliferation (Fig. 4*C*). At the concentrations used in our experiments, OH-U completely blocks the proliferation of plasma blasts, i.e., the direct precursors of the nonproliferating plasma cells (27) (data not shown). However, OH-U did not reduce the numbers of plasma cells cultured together with IL-6 (Fig. 4*C*). Similar results were obtained for IL-5, SDF-1α, and stimulation by the CD44 ligand HA (data not shown). This observation indicates that these factors stimulate the survival, but not the proliferation of these cells.

Table I. *Various cytokine-mediated and contact-dependent signals can support plasma cell survival*

Tested Components	Percentage of Surviving Plasma Cells at Day 3
Medium	4
Bone marrow supernatant	50
IL-1β	3
IL-2	5
IL-4	3
IL-5	40
IL-6	70
IL-7	7
IL-10	8
IL-12	15
IL-18	3
TNF-α	30
TGF-β	4
GM-CSF	7
Stem cell factor	5
LIF	5
SDF-1α	58
Anti-CD19	12
Anti-CD38	10
Anti-CD40	7
Anti-CD44 (IM7)	60
Anti-CD44 (IRAW)	60
Anti-CD138	9
Isotype control Ab IgG2a	12
Isotype control Ab IgG2b	9

[a] FACS-sorted plasma cells (100 cells/well) were cultured in the absence or presence of cytokines (10 ng/ml) or cross-linking Ab (4 μg/ml). The numbers of plasma cells in cultures were analyzed at day 3 by ELISPOT. Variations in doublet cultures and in a second experiment were below 5% for all values.

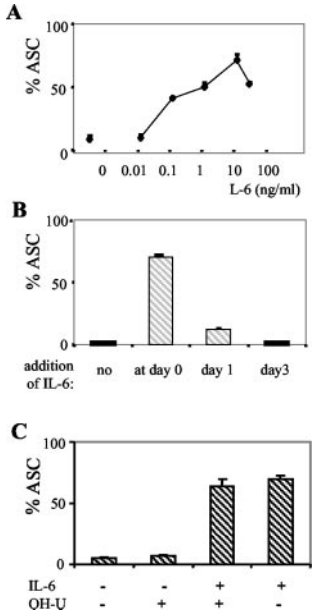

FIGURE 4. Effects of IL-6 on plasma cell function. *A*, Dose-response curve of IL-6. rIL-6 was added to isolated plasma cells (100 cells/well) at the concentrations indicated (0.01–50 ng/ml), and the numbers of ASC were determined after 3 days in culture by ELISPOT. Values are expressed as the mean + SD in duplicate cultures ($n = 3$). *B*, Plasma cells require IL-6 stimulation immediately after isolation. IL-6 (10 ng/ml) was added to isolated plasma cells at the time points indicated (day 0 = onset of culture). The numbers of ASC after 3 days of culture were evaluated by ELISPOT. The mean + SD of one representative experiment is shown ($n = 2$). *C*, IL-6 does not induce cell proliferation. Plasma cells were cultured (100 cells/well) in the absence or presence of OH-U (0.5 mM), with or without IL-6 (10 ng/ml). The numbers of ASC after 3 days of culture were determined by ELISPOT. Values show one representative experiment ($n = 2$) and are expressed as the average + SD in duplicate cultures.

IL-6 is not essential for long-term Ab production in vivo

The active component of the bone marrow supernatant is IL-6, as shown by the uneffectiveness of supernatants prepared from IL-6-deficient (IL-6$^{-/-}$) mice in supporting plasma cell survival (Fig. 5). In accordance, the addition of the neutralizing anti-IL-6 Ab 20F3 severely reduced the positive effect of bone marrow supernatant (data not shown). Supernatants prepared from IL-5$^{-/-}$ mice were as effective as those obtained from wild-type animals. Whereas IL-5$^{-/-}$ and IL-6$^{-/-}$ mice were on C57BL/6 background, plasma cells had been isolated from BALB/c mice. Supernatants derived from BALB/c and C57BL/6 bone marrow cultures equally supported the survival of BALB/c plasma cells, excluding a role of the genetic background. These results demonstrate that IL-6 is an essential component of the supernatants used in our experiments, and led to the question as to whether this molecule might also play a crucial role for the homeostasis of plasma cells in vivo.

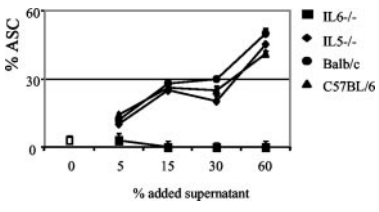

FIGURE 5. IL-6 is required to support plasma cell function in bone marrow cultures. Purified plasma cells (100 cells/well) were cultured together with various concentrations of bone marrow supernatants prepared from C57BL/6, IL-6$^{-/-}$, IL-5 $^{-/-}$, and BALB/c mice, as indicated. The numbers of ASC after 3 days of culture were determined by ELISPOT. Values are expressed as the average + SD in duplicate cultures.

A deficiency in mounting humoral immune responses has been reported in IL-6$^{-/-}$ mice (28). In these animals, 10 days after boost immunization, specific Ab titers were reduced ~10-fold compared with wild-type controls. To test whether this effect was due to a reduced plasma cell lifetime, we followed the kinetics of OVA-specific Ab titers for 5 mo after secondary immunization (Fig. 6*A*). The specific Ab titers measured following this immunization protocol are maintained by long-lived plasma cells (8, 29). IL-6$^{-/-}$ mice required a longer period of time to mount a specific Ab response. However, ~4 wk after immunization, Ab titers were equally high and stable during the following 20 wk in both groups of animals. Also, as long as 21 wk after secondary immunization, the absolute numbers of OVA-specific bone marrow plasma cells were similar in IL-6$^{-/-}$ and wild-type mice (Fig. 6*B*). These results show that IL-6 is not essential to support plasma cell survival in vivo.

Synergistic signals are required to support plasma cell longevity

Stimulation of isolated plasma cells by a single survival factor did not support the function of these cells for longer than 3 days. After 5 days in the presence of IL-6 or cross-linking of CD44, the percentages of viable plasma cells did not exceed 25% (Fig. 7). However, together with IL-6, the activating anti-CD44 Ab IM7 and IRAWB and the natural ligand HA, but not the blocking anti-CD44 Ab KM81, led to the recovery of 70–85% plasma cells after 5 days of culture. Isotype control Ab together with IL-6 did not show any effect other than IL-6 alone. Additive effects of CD44 stimulation together with IL-6 would have been much lower than the observed

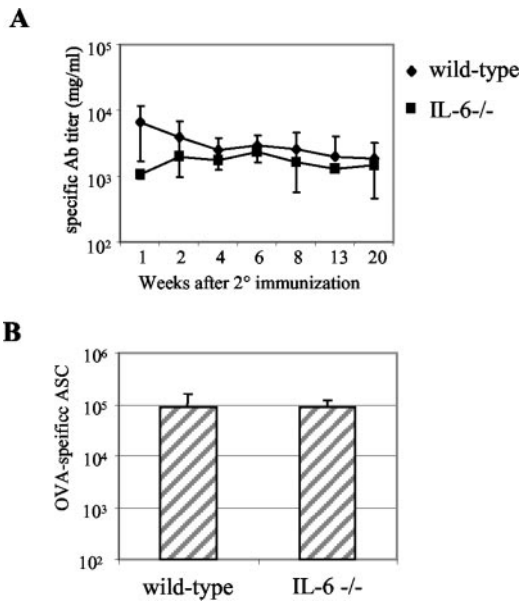

FIGURE 6. IL-6 is required for a normal induction, but not for the maintenance of Ab responses in vivo. *A*, Kinetics of serum Ag-specific IgG titers, determined by ELISA, were compared in IL-6$^{-/-}$ and wild-type mice following boost immunization with OVA (day 0 of analysis). The data shown represent the average ± SD of 8–10 mice in each group. *B*, IL-6$^{-/-}$ and wild-type mice have equal numbers of OVA-specific plasma cells in their bone marrow. At 21 wk after boost immunization, numbers of OVA-specific plasma cells were measured by ELISPOT. Values expressed are the average + SD of six to eight mice in each group.

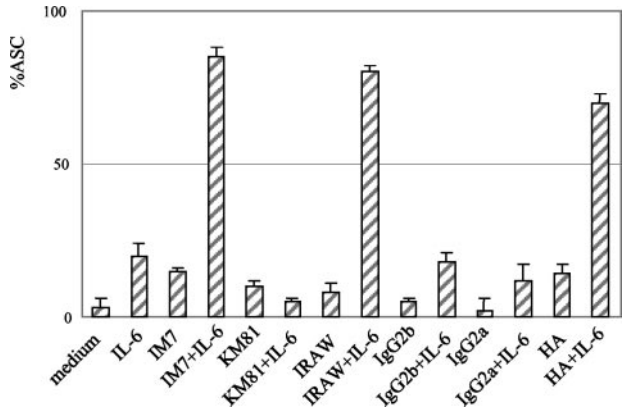

FIGURE 7. Stimulation by IL-6, together with CD44 triggering, acts synergistically in supporting function of plasma cells. Isolated bone marrow plasma cells were cultured together with various stimuli, as indicated. Three different immobilized anti-CD44 Ab (IM7, IRAW, and KM81; 4 μg/ml) and soluble HA (10 μg/ml) were used. IM7 and IRAW are stimulating anti-CD44 mAb; KM81 is a blocking anti-CD44 mAb. HA is a component of extracellular matrix and is the major ligand of CD44 in vivo. IL-6 was added when indicated at 10 ng/ml. After 5 days of culture, the numbers of surviving plasma cells were determined by ELISPOT. The mean + SD in duplicate cultures of one representative experiment ($n = 3$) is shown.

percentages of surviving plasma cells, showing that both stimuli act synergistically.

Discussion

There is increasing evidence that the lifetime of plasma cells is determined by their microenvironment rather than intrinsic factors (9–11). As an initial study in the field, this work was aimed to identify the signals and mechanisms that regulate plasma cell survival in vitro.

Bone marrow plasma cells were identified by their ability to secrete IgG1, the most abundant isotype expressed in this cell population. The isolated Ab-secreting cells expressed only little MHCII (data not shown), indicating that these cells were terminally differentiated plasma cells activated at least 12 days earlier (8). In culture, ASC could be detected for a prolonged period of time in the presence of other bone marrow cells. Because plasma cells are considered to produce and secrete large amounts of Abs constitutively, the observed loss of ASC in the absence of feeder cells or their products is most likely due to cell death by neglect. In accordance, it has been shown that stromal cells can prevent apoptosis of plasma cells isolated from tonsils (23). However, it could not be formally excluded that the factors identified stimulate plasma cell function rather than plasma cell survival. Studies on mice overexpressing the antiapoptotic molecule Bcl-2 as a transgene demonstrated sustained survival of Ab-secreting cells (30). In human bone marrow plasma cells, Bcl-2 protein has been detected in high levels (26). The absence of mRNA for Bcl-2 in isolated plasma cells observed in this study may explain the rapid disappearance of these cells when cultured without survival factors or feeder cells. It can be speculated, that upon isolation, the lack of survival factors may immediately lead to the loss of Bcl-2 expression, while the protein, with its longer $t_{1/2}$, could still be present. This idea is supported by the finding that IL-6 rescued plasma cells when added immediately after isolation, but not when added later. The potential of IL-6 to induce Bcl-2 expression has been demonstrated earlier (31). Independent of whether the survival factors identified in this study induce the expression of Bcl-2 or not, the requirement for IL-6 immediately following detachment of plasma cells from their normal environment shows that to survive, these cells require the continuous stimulation by specific factors, e.g., IL-6.

In the present work, we showed that among the many molecules tested, only IL-5, IL-6, TNF-α, SDF-1α, and HA had the capacity to support plasma cell survival. These factors are produced by bone marrow stromal cells, suggesting a role for these molecules in the regulation of plasma cell survival in the bone marrow microenvironment. These molecules have also been described as modulators of inflammation (32, 33), indicating that inflamed tissues may offer optimal conditions for plasma cells. Indeed, plasma cells can persist quite well in inflamed tissue (19).

It has been suggested that IL-6 enhances the terminal differentiation of B lymphocytes (34, 35) and promotes the growth of malignant plasma cells (36). Whether IL-6 acts as a factor promoting survival, proliferation, or differentiation has not been clarified in these studies. In our experiments, IL-6 appeared to act as survival factor for mature plasma cells, because its effect on maintaining cell numbers in culture did not depend on DNA synthesis. As shown in IL-6$^{-/-}$ mice, IL-6 is not essential for the maintenance of serum Ab titers and numbers of OVA-specific plasma cells. Because the OVA-specific Ab titers induced with application of our immunization protocol were maintained by long-lived plasma cells (8, 29), this result indicates that IL-6 is not required for supporting the survival of plasma cells in vivo. Interestingly, the onset of the Ab response in the IL-6$^{-/-}$ mice was delayed compared with that of wild-type animals. However, the IL-6$^{-/-}$ mice had normal long-lasting memory Ab titers and numbers of OVA-specific plasma cells in the bone marrow. These data are in accordance with the observation that induction and strength of an Ab response are regulated independently (37). Although the time period required for the initiation of the response depends on the numbers of Ag-specific memory B cells available, the final strength of the Ab response is regulated by the amount of Ag that possibly determines the number of plasma cells initially formed. Thus, one possible explanation for the delayed humoral immune response in the IL-6$^{-/-}$ mice is that the impaired germinal center formation in these mice (28) results in reduced numbers of Ag-specific memory B cells and consequently in a delayed response. Alternatively, IL-6 might be crucial for supporting the survival or proliferation of plasma blasts, which is required to mount the final plasma cell numbers.

Together with previous reports, the work presented in this study suggests that SDF-1α and its cognate receptor, i.e., CXCR4, play multiple roles in the regulation of plasma cell homeostasis. In fetal liver chimeras, the lack of CXCR4 on plasma cells leads to a >3-fold reduction of Ag-specific plasma cells found in the bone marrow at day 7 after immunization (38). In wild-type mice, Ab-secreting cells migrate toward SDF-1α (39) at the time point of their translocation from spleen to the bone marrow. These data suggest that this chemokine is important for the migration of early plasma cells into the bone marrow. Once there, the cells completely lose the capacity to migrate toward this chemokine. However, they maintain the expression of CXCR4 (39). These results indicate that on mature plasma cells, this receptor mediates another function than the regulation of migration. As suggested by the work presented in this study, this function is to support the survival of plasma cell. The reduced numbers of bone marrow plasma cells in CXCR4-deficient chimeras could be mainly due to reduced migration of plasma cells/plasma blasts into this organ. However, this result may in part also be due to reduced survival of these cells.

Bone marrow plasma cells express high levels of CD44 (8, 26), a cell surface glycoprotein whose principal ligand is HA, a component of the extracellular matrix (40). CD44 is widely expressed

and participates in lymphopoiesis, adhesion to the extracellular matrix, homing to lymph nodes, and lymphocyte activation (41, 42). It has been proposed that the interaction of CD44 present on myeloma cells with extracellular matrix produced by stromal cells localizes myeloma cells to the bone marrow. In addition, it stimulates IL-6 production by the stromal cells (43). In this study, we show that CD44 engagement could represent per se a crucial survival stimulus for nonmalignant plasma cells.

As far as tested in this study, one signal alone was not able to extend the lifetime of isolated plasma cells for more then 3 days. However, stimulation with a combination of survival factors, e.g., stimulation with anti-CD44 and IL-6, synergistically supported the survival of the vast majority of plasma cells for 5 days. Also, plasma cell survival requires the continuous presence of survival factors, as shown by the requirement for IL-6 immediately following isolation. This observation is in accordance with the notion that Blimp-1, one of the key molecules required for plasma cell differentiation (44), induces apoptosis, if not counterbalanced by antiapoptotic members of the Bcl-2 family (45). Together, these results support the hypothesis that the survival of plasma cells depends on the presence of niches, in which a specific combination of factors is present.

We were not able to sustain plasma cell survival in vitro for much longer than 5 days. This is by far shorter than the estimated $t_{1/2}$ of these cells in vivo, i.e., ~6 mo. We may have missed another factor that is essential for long-term plasma cell survival. Alternatively, the conditions in culture possibly vary from those in vivo in several aspects relevant for cell survival. These differences could include suboptimal stimulation by the survival factors tested, e.g., due to their degradation, but could also be due to other parameters, such as glucose concentration, pH, or nutrients, which may not resemble physiological conditions. As indicated by the finding that plasma cell survived best in the intact femur, the three-dimensional microarchitecture of the bone marrow tissue might be required for normal plasma cell homeostasis. Plasma cell survival studies in an organ culture system are necessary to clarify this issue.

Plasma cell survival niches are not necessarily exclusive for this cell type. An attractive speculation is that they might be shared with early B cells (11). This idea is supported, e.g., by the observation that a stromal cell-mediated feedback loop between pre-B cells and plasma cells seems to exist in the bone marrow (46). In the present work, the cytokines IL-7 and stem cell factor, both essential for the survival and differentiation of early B cell stages in the bone marrow (47), did not act on plasma cells, arguing against identical survival requirements supporting B cell precursors and plasma cells. However, our data do not exclude the existence of a general mechanism regulating B cell and plasma cell homeostasis.

Our results suggest that mature bone marrow plasma cells are prone to apoptosis, but can be rescued by survival factors produced in their environment. They strongly support the concept that the homeostasis of plasma cells requires specific survival niches and identify the cytokines IL-5, IL-6, TNF-α, and SDF-1α as well as HA as likely components of these niches. Importantly, our data show that IL-6, to date the most important growth factor described for malignant plasma cells, is a powerful, however not essential survival factor also for normal plasma cells. The observed redundancy in the function of IL-6 to support plasma cell survival might be expected because of the importance of persisting Ab responses to maintain immune protection. However, this finding is important for our understanding of the mechanism regulating the homeostasis of plasma cells and may explain why anti-IL-6 Ab therapy alone is not sufficient to deplete multiple myeloma cells efficiently. Additionally, it stresses the complexity of the regulation of plasma cell formation and survival. In this initial study, specific factors were identified supporting plasma cell survival in vitro. The detailed role that these factors play for the homeostasis of plasma cells in vivo remains to be elucidated.

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