3. Ergebnisse

3.1 Die Lebensspanne von Plasmazellen ist nicht Antigen-abhängig

Manz, et al, *Int Immunol* 1998. 10: 1703-1711.

Der Ausgangspunkt dieser Arbeit [33] waren kurz zuvor beschriebene Experimente, die zeigten, dass persistierende Gedächtnis-Antikörpertiter von langlebigen Plasmazellen produziert werden können. Diese Zellen waren damit als Träger des humoralen Antikörper-Gedächtnisses identifiziert. Es stellte sich die Frage, wie das Überleben dieser Zellen reguliert ist. Naheliegend war eine Abhängigkeit dieser Zellen von dem Vorhandensein von Antigen, denn zu diesem Zeitpunkt wurde noch davon ausgegangen, dass sowohl die Aufrechterhaltung von Gedächtnis-Antikörpertitern, als auch das Überleben von Gedächtnis B-Zellen Antigen-abhängig ist. Um diese Hypothese zu überprüfen wurden BALB/c Mäuse wiederholt mit dem Protein-Antigen Ovalbumin immunisiert. Dies führte zur Bildung Ovalbumin-spezifischer Gedächtnis B-Zellen und Plasmazellen. Die Phänotypisierung dieser Zellen mittels Durchflusszytometrie ergab, dass MHCII exprimierende frühe Plasmazellstadien nur bis etwa zwei Wochen nach der Immunisierung zu finden sind. Danach zeigen alle Ovalbumin-spezifischen Plasmazellen den Phänotyp von terminal differenzierten, schwach MHCII ausprägenden, B220 negativen Plasmazellen. Etwa 90% dieser Zellen befindet sich im Knochenmark, wo wir sie noch Monate später in etwa unveränderter Zahl nachweisen konnten. Unabhängig vom Ko-transfer von Ovalbumin (Antigen) konnte die B220 negative Zellfraktion aus dem Knochenmark immunisierter Tiere über Monate stabile Ovalbumin-spezifische Antikörpertiter übertragen. Dieses Ergebnis zeigte, dass weder das Überleben langlebiger Plasmazellen, noch die Aufrechterhaltung spezifischer Antikörpertiter vom Vorhandensein von Antigen abhängt. Kurze Zeit später konnte eine andere Arbeitsgruppe zeigen [60], dass auch das Überleben von Gedächtnis B-Zellen nicht Antigen-abhängig ist. Obwohl Antigen entscheidend ist für die Initiierung spezifischer Immunantworten, zeigen diese Experimente jedoch, dass das Vorhandensein von Antigen für die Aufrechterhaltung weder des humoralen, noch des zellulären B-Zellgedächtnisses, erforderlich ist.

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Survival of long-lived plasma cells is independent of antigen

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Abstract

Recent studies have shown that persistent specific antibody titer is provided by long-lived plasma cells (PC) which constitute a new kind of 'memory-providing cells'. In the present study, we examine the role of antigen for the long-term survival of PC and the maintenance of specific serum antibody titers. Using a novel cytometric technology, to identify and isolate antigen-specific PC, we analyzed long-lived PC of BALB/c mice, during their development (between day 1 and 10) after secondary immunization with ovalbumin (OVA) and in the phase of the established immune reaction. Most if not all OVA-specific PC were generated within a few days after immunization. Within ~3 weeks, they matured, as indicated by down-regulation of expression of MHC class II. These PC are long lived and located in spleen and bone marrow. Upon adoptive transfer, OVA-specific PC from bone marrow, but not memory B cells, conferred specific and long-lasting antibody titers to antigen-free IgH syngeneic recipients. In response to antigenic challenge, new OVA-specific antibody-secreting cells were generated from transferred memory B cells. Antibody secretion by long-lived PC was not affected. Our results confirm that persistent antibody titers are provided by long-lived PC, independent of memory B cells and demonstrate that this humoral memory is inert to antigen.

Introduction

Antigen-specific memory is one of the essential characteristics of the immune system. It is mediated by antigen-experienced T and B lymphocytes, and characterized by fast and efficient immune reactions upon re-exposure to antigen (1–3), but also by the persistence of antigen-specific antibodies in the body fluids, the humoral memory. Humoral antibody can eliminate a pathogen from circulation before it can elicit an immune reaction, thus providing passive protection (4,5). In humans, protective antibody titers can last for >20 years (6). In mice, after immunization with T-dependent antigens, high titers of specific antibody are maintained for >1 year, i.e. the murine lifetime (6,7). Since *in vivo* antibodies in the serum decay with a half-life of a few days (8,9), their continuous production by antibody-secreting plasma cells (PC) is required to maintain a specific titer. Recently, it has been demonstrated that persistent antibody titers are provided by long-lived PC (10,11). Here, we address the question whether long-lived PC react to antigen, i.e. whether antigen is required for their survival or antibody production. It has been shown that protein antigens can be retained in spleen and draining lymph nodes of immunized individuals in the form of antigen–antibody complexes for a long time (12,13). Persistence of antigen has been suggested to be essential for long-term survival of memory B cells (14). A role for antigen–antibody complexes has also been discussed for the regulation and maintenance of humoral memory (15,16). In addition, engagement of the antigen receptor on naive B cells seems to be required for their survival (17). For long-lived PC providing humoral immunity, the need for persistence of antigen is not clear.

Methods

Animals and immunization

BALB/c mice were obtained from Bomholtgard (Ry, Denmark). CB20 mice were bred at the animal facility of the Institute for Genetics, University of Cologne. For primary immunization, each mouse received 100 μg alum-precipitated ovalbumin (OVA) in 100 μl PBS i.p. After 3–5 weeks, primed mice were boosted by i.v. injection of 100 μg soluble OVA in 100 μl PBS.

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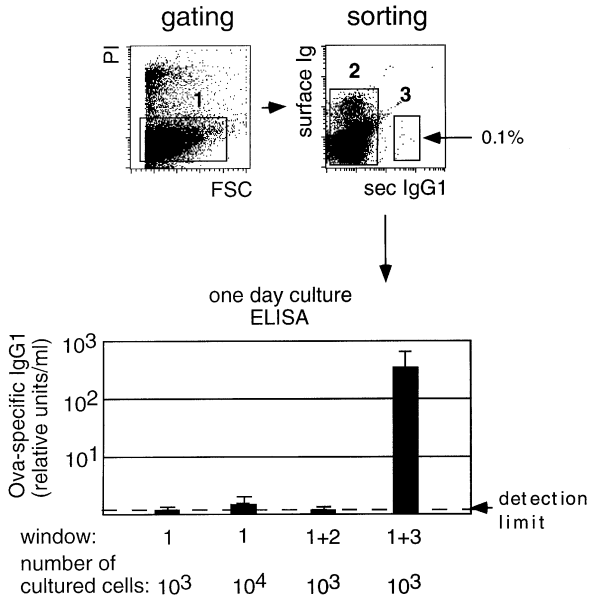

Fig. 1. Cells stained for secreted IgG1 contain all the capacity to secrete OVA-specific IgG1 in culture. Bone marrow cells were stained for total surface Ig and for secreted IgG1 (sec IgG1) 12 days after secondary immunization with OVA. Cells were sorted by FACS according to the windows shown above. Staining with propidium iodide was used to exclude dead cells (window 1, live cells; window 2, cells not stained for secreted IgG1; window 3, cells stained for secreted IgG1 expressing no or only low level of surface Ig). All cells sorted through windows 2 and 3 also passed the live gate window 1. Cells were cultured in 96-well plates for 24 h. Then OVA-specific IgG1 in the supernatants was measured by ELISA.

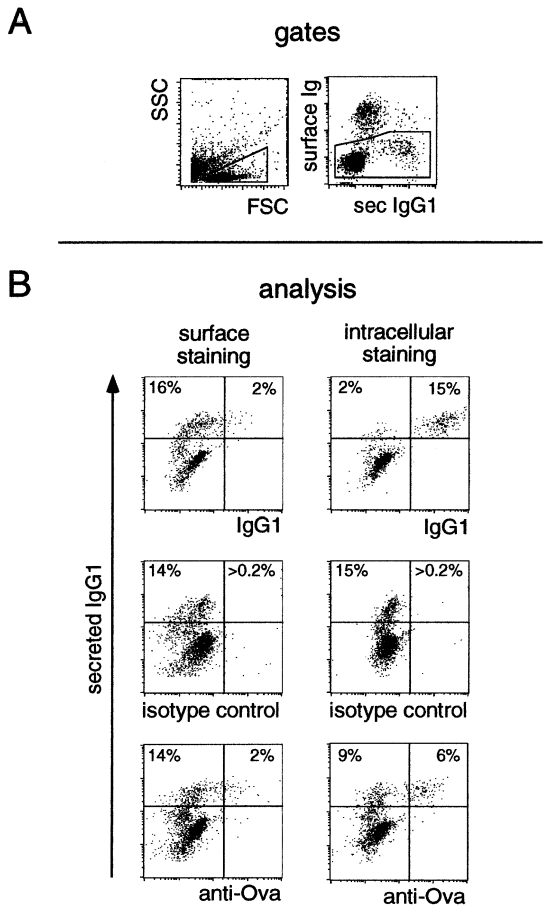

Fig. 2. Flow cytometric identification of OVA-specific PC. Bone marrow or spleen cells from mice immunized and boosted with OVA (data shown here: spleen cells from mice boosted 6 days earlier) were stained for surface Ig expression and secreted IgG1. Cells stained for IgG1 were enriched by MACS and fixed with formaldehyde, which preserves the cell membrane widely undisrupted. Intracellular staining is obtained after incubation with the detergent saponin. (A) Exclusion of surface Ig^+ and resting (small) cells from the analysis. (B) Secreted IgG1 versus intracellular (with saponin) or surface (without saponin) counterstaining for IgG1, isotype control (anti-IgE) or OVA-specific antibody production as indicated.

Reagents and antibodies

Biotin-labeled goat anti-mouse Ig and IgG1 were from Southern Biotechnology Associates (Birmingham, AL). MACS beads, monoclonal rat anti-mouse IgG1 antibodies and the magnetic cell sorter MACS were provided by Miltenyi Biotec (Bergisch Gladbach, Germany). mAb R33.18.10 (anti-murine □ light chains, 18) was a gift from Claudia Uthoff (Institute for Genetics, University of Cologne). Anti-B220 mAb RA3-6B2 (19), mAb HP6054 and anti-MHC class II (M5/114) were purified from hybridoma supernatants. R33.18.10 was coupled to avidin by Miltenyi Biotec. FITC-labeled rat (IgG1) anti-mouse IgE (isotype control), anti-syndecan-1, anti-IgG1a, anti-IgG1b and CyChrome-conjugated streptavidin were from PharMingen (San Diego, CA). Murine anti-OVA (IgG1, □) antibody was purified from hybridoma supernatant in our laboratory. Gelatin (75 bloom) was purchased from Sigma (St Louis, MO). Cells were cultured in RPMI from Gibco (Eggenstein, Germany) with 5% FCS supplement (Gibco). OVA (Sigma) was dialysed against PBS and coupled to phycoerythrin (PE; a gift from Miltenyi Biotec) and FITC (FLUOS; Boehringer Mannheim, Mannheim, Germany).

Labeling and enrichment of antibody-secreting cells

Total spleen or bone marrow cells were washed, filtered through a 70 □m nylon filter and resuspended in PBS/5% BSA. Cells were labeled with goat anti-mouse Ig–biotin at a concentration of 5 □g/ml (10 min on ice), washed once in PBS/5% BSA and incubated with streptavidin–CyChrome (1:1000). Then cells were spun down for 10 min at 350 *g* and resuspended in 1 ml of freshly prepared NHS-biotin (Sigma; 0.5 mg/ml) in PBS (37°C). After 15 min incubation, cells were washed 3 times in cold PBS/BSA. The tube was changed at every washing step. Subsequently cells were resuspended in 30 □g/ml unconjugated R33.18.10 mAb in PBS/BSA and incubated for 5 min on ice. Then, R33.18.10–avidin conjugate (30 □g/ml) was added. After 5 min on ice, the cell suspension was mixed with prewarmed gelatinous medium as described (20) and kept for 30 min at 37°C. Gelatinous medium was removed by washing with a 10-fold excess of warm (37°C) PBS. Secreted antibody was labeled with anti-IgG1-coupled

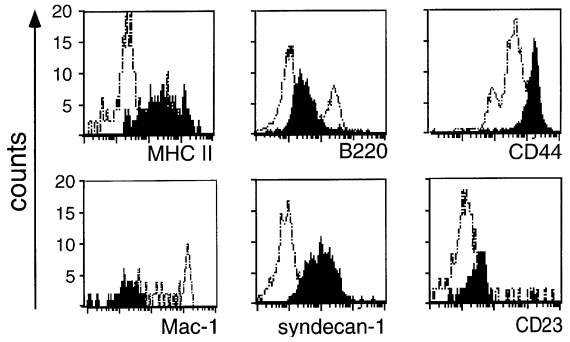

Fig. 3. Expression of surface markers on antibody-secreting PC. Spleen or bone marrow were prepared and stained for secreted IgG1 versus surface Ig as described in Fig. 2 (spleen cells shown). Aliquots were stained for the surface markers indicated at each histogram. Surface Ig^+ and resting cells were excluded from the analysis as shown in Fig. 2. Histograms show cells which stained positive (filled) or negative (open) for secreted IgG1.

magnetic beads and subsequently with anti-IgG1–PE. Cells were washed and $IgG1^+$ cells were enriched with a Mini-MACS column as described in the manufacturer's instruction. Aliquots were stained with FITC-labeled antibodies against additional markers (alive or after fixation with 2% formaldehyde). To prevent unspecific binding of the staining antibody to the cellular-affinity matrix, the affinity matrix was saturated with murine mAb HP6054 previous to the labeling with anti-MHC class II (M5/114). Analysis of stained cells was performed with a FACScalibur (Becton Dickinson, Mountain View, CA) using CellQuest software (Becton Dickinson).

Fixation and intracellular staining

Cell samples were stained for surface Ig and secreted IgG1 or surface Ig alone, washed and resuspended in PBS at 10^6 cells/ml and mixed with an equal volume of 4% formaldehyde in PBS. After 10 min incubation at room temperature, cells were washed twice in PBS and resuspended in PBS/5% BSA. For intracellular staining 0.5% saponin (Sigma) was added; antibodies and FITC-conjugated OVA were used at a concentration of 5 μg/ml, PE-conjugated OVA was used at 0.2 μg/ml.

Cell transfer

Prior to adoptive transfer, bone marrow cells from BALB/c mice boosted at least 3 months before were stained with anti-Thy-1.2-coated magnetic beads and anti-Thy-1.2–PE. Thy-1.2^+ cells were separated using the Mini-MACS magnetic cell sorter. The fraction depleted of Thy-1^+ cells was labeled with biotin-conjugated anti-B220, then incubated with streptavidin-conjugated magnetic beads and subsequently stained with streptavidin–FITC. Separation according to B220 expression was done using the Mini-MACS system. The fraction positive for Thy-1.2 was distributed equally upon the $B220^+$ and $B220^-$ fractions. The same number of cells (6×10^6/mouse) was transferred by injection into the tail vein of irradiated (500 rad) CB20 IgH syngeneic recipients.

Enumeration of OVA-specific PC

Cells were stained for surface Ig by goat anti-mouse Ig and for intracellular anti-OVA antibody using OVA–PE as staining reagent. Between 2 and 5×10^5 spleen or bone marrow cells were measured by FACS. Absolute numbers of surface Ig low/OVA-binding cells were calculated from the frequency of these cells among total cells in spleen and bone marrow, and from the number of total cells in both tissues. This calculation was based on the assumption that the femur contains 12.7% of total bone marrow cells (21).

ELISA

For measurement of OVA-specific antibody in sera or supernatants, 96-well plates were coated with OVA (5 μg/ml in PBS). As a standard, murine anti-OVA IgG1 antibody purified in our laboratory from hybridoma supernatant was used. Sera or supernatants were prediluted 1:3 to 1:10 and incubated for 1 h at room temperature. For detection, biotin-conjugated goat anti-mouse IgG1 or allotype-specific monoclonal anti-IgG1 (PharMingen) were used. The ELISA was developed with streptavidin-conjugated alkaline phosphatase (Boehringer Mannheim) and 4-nitrophenylphosphate disodium salt (Merck, Darmstadt, Germany).

Results

Identification of antibody-secreting PC by cellular-affinity matrix technology

Non-dividing PC and their direct precursors, proliferating plasma blasts, are characterized by their unique feature of antibody secretion. This was used for their analysis by classical plaque-forming assays (22). However, this approach does neither allow physical isolation of PC for functional studies nor direct multiparameter analysis of cellular marker expression. To improve the analysis of secreting cells, e.g. PC, we have developed a novel technique, cellular-affinity matrix technology (20), allowing immunofluorescent labeling of viable cells for products secreted by them. To this end, total cells from spleen or bone marrow were coated artificially with an antibody specific for Ig light chain (by biotinylation of the cell surfaces and subsequent incubation with avidin-coupled anti-κ antibody). This affinity matrix binds the secreted antibody of each individual antibody-secreting cell and relocates it to its cell surface. To identify PC after secondary immunization with OVA, we used an Ig κ chain-specific affinity matrix and detected secreted Ig with anti-IgG1-specific antibody (in BALB/c mice, >90% of the PC formed during this immune response are of the IgG1, κ subtype, data not shown). Staining of membrane-bound Ig with saturating amounts of antibody before usage of the affinity matrix technology allowed to distinguish between membrane-bound and secreted Ig.

Very few spleen and bone marrow cells from mice immunized and boosted with OVA were stained for secreted IgG1. These cells showed only a low level of surface Ig expression (Fig. 1). When isolated by FACS, without further stimulation exclusively these cells secreted OVA-specific IgG1 antibodies *in vitro* (Fig. 1) and were found to be antibody-containing cells as analyzed by intracellular immunofluores-

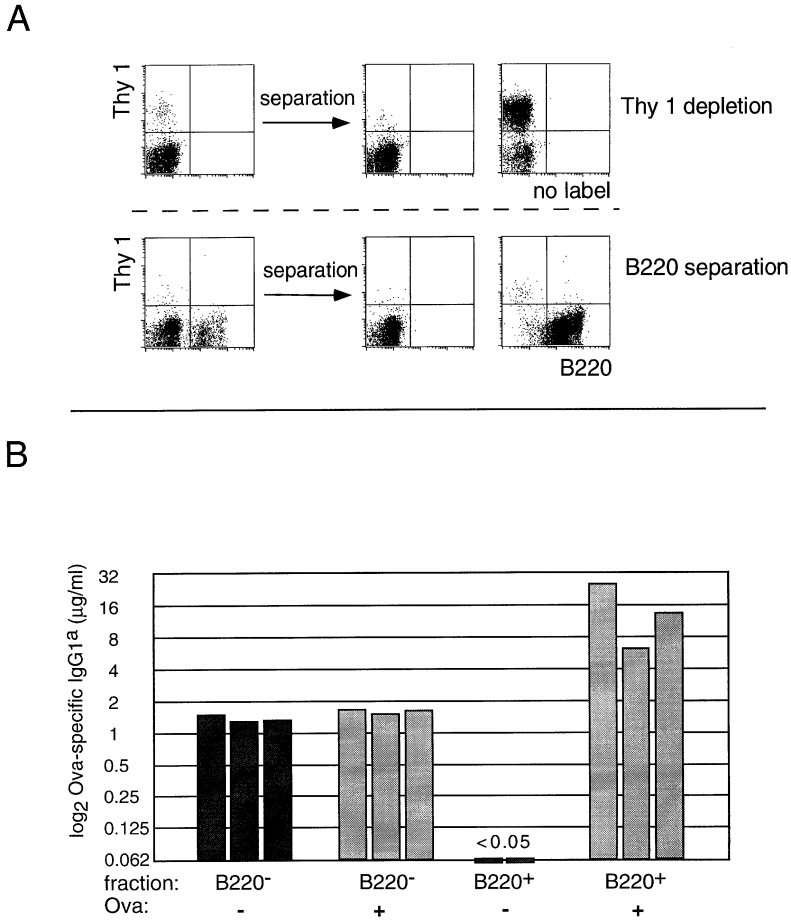

Fig. 4. Separation and adoptive transfer of memory B cell- and PC-containing cell fractions. (A) Bone marrow cells prepared from BALB/c mice immunized and boosted with OVA 3 months before were stained with anti-Thy-1.2 magnetic beads and subsequently with anti-Thy-1.2–PE. Dot-plots were gated for live cells according to light scatter and by exclusion of propidium iodide-binding particles as shown in Fig. 1. About 1.5% of the cells expressed Thy-1.2. These cells were depleted using the Mini-MACS magnetic cell sorter. The fraction depleted of Thy-1.2 was labeled with biotin-conjugated anti-B220, and then stained with streptavidin-conjugated magnetic beads and streptavidin–FITC. By Mini-MACS separation, $B220^+$ cells were depleted to >99% purity of the $B220^-$ cell fraction. (B) In contrast to $B220^+$ cells, $B220^-$ bone marrow cells, containing PC, transfer antibody titer without co-transfer of antigen. Thy-1.2^+ cells were added in equal numbers to all cell fractions before transfer. Cells were transferred i.v. (6×10^6 cells/mouse) with or without addition of OVA (2 □g per mouse) into IgH^b syngeneic CB20 recipients. One week after transfer, OVA-specific IgG1 of the donor (IgH^a) was measured in the sera of the recipients (IgH^b) by ELISA (detection limit was 50 ng/ml for $IgG1^a$).

cence microscopy (not shown). Among these IgG1-secreting cells, those specific for OVA were identified by their capacity to bind OVA intracellularly, due to their antibody specificity. For this experiment, the antibody-secreting cells had been enriched, using high gradient MACS (23), fixed and stained intracellularly with fluorescein-labeled OVA. The frequency of OVA-specific PC among IgG1-secreting cells varied depending on the kinetics of the immune reaction. During the first 2 weeks after antigenic challenge, a high percentage of the cells that stained for IgG1 secretion were OVA-specific (Fig. 2). While the absolute number of OVA-specific PC in the bone marrow remains constant (Fig. 6A), the frequency of OVA-specific cells among all PC decreased rapidly during time (not shown). This effect likely reflects immune responses against other antigens, inducing formation of PC with other specificities than OVA.

The phenotype of 'young' PC, 6 days after boost with OVA, is shown in Figs 2(A) and 3. These cells express no or little B220, some surface Ig, high levels of CD44, MHC class II and syndecan (CD138). No differences were found between PC resident in the spleen or bone marrow.

Separation of PC and memory B cells

To compare the relative contribution of memory B cells versus PC for the maintenance of long-lasting antibody titer, we transferred both types of cells separately into antigen-innocent recipients. We used B220 (murine CD45R) to separate memory B cells and PC. B220 is expressed on memory B cells

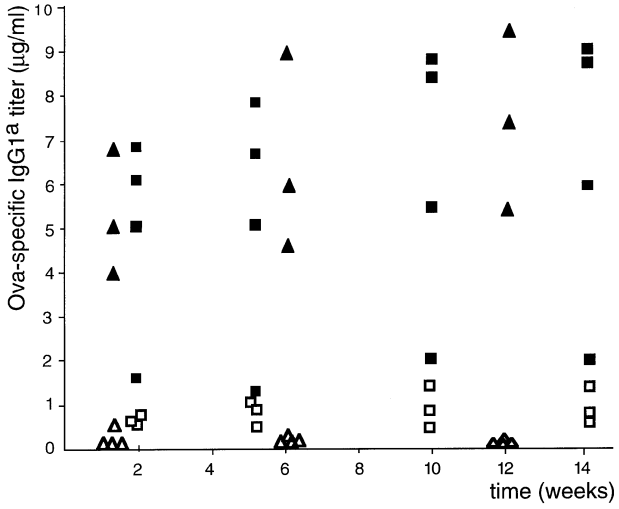

Fig. 5. B220$^-$ bone marrow cells transfer long-term antibody titer without need for antigen. Bone marrow cells were prepared from IgH^a-expressing BALB/c mice, <3 months after secondary immunization with OVA. Separation of cell fractions containing memory (B220$^+$) or PC (B220$^-$) and transfer without addition of OVA but with equal numbers of Thy-1.2$^+$ cells added was performed as described in Fig. 4. At the time points indicated, sera were prepared from the recipients (IgH^b) of B220$^-$ (black squares/black triangles for two independent experiments) and B220$^+$ (open squares/open triangles) cell fractions, and were analyzed for OVA-specific IgG1^a by ELISA.

(24,25), but not on PC (26). Donor cells were obtained from bone marrow of BALB/c mice expressing IgH^a, immunized and boosted with OVA 3–7 months earlier. At the time point of transfer, donor mice showed a stable OVA-specific antibody titer (data not shown) and the population of OVA-specific PC in the bone marrow of these mice did not proliferate any longer (10).

Thy-1.2$^+$ cells were depleted from the bone marrow by MACS. Then, Thy-1.2$^-$ cells were separated into B220$^+$ and B220$^-$ fractions magnetically (Fig. 4A). Equal numbers of B220$^+$ or B220$^-$ cells were transferred together with Thy-1.2$^+$ cells into unimmunized syngeneic (IgH^b) CB20 mice, with or without antigen (OVA). Donor (IgH^a)- and recipient (IgH^b)-derived OVA-specific IgG1 antibody titers were determined in the sera of the recipients by ELISA. As early as 1 week after transfer, B220$^-$ bone marrow cells containing PC, but not memory B cells provided OVA-specific serum antibody titers of 1–2 µg/ml IgG1^a (donor), irrespective of whether antigen had been co-transferred or not. At this time point, however, we could not detect any OVA-specific donor antibody titer (detection limit ~50 ng/ml) in mice which had received B220$^+$ cells without co-transfer of OVA (Fig. 4B). Thus, confirming our previous phenotyping, PC were almost exclusively contained within the B220$^-$ cell fraction. The presence of memory B cells in the B220$^+$ bone marrow cell fraction was demonstrated by antigenic challenge. Obviously and as expected (14), the memory cells were activated by co-transferred antigen and formed antibody-secreting cells, providing a clearly detectable antibody titer in the recipients that had received the B220$^+$ cell fraction together with antigen. Transfer of OVA together with the transferred B220$^+$ cells led to a >100-fold increase in the antibody titer compared to transfer of B220$^+$ cells without antigen (<50 ng/ml compared to an average of ~10 µg/ml). Co-transfer of antigen did not alter the donor-derived anti-OVA IgG1 serum levels in mice which had received B220$^-$ cells, showing that this cell fraction contained no significant numbers of memory B cells.

Long-lived PC provide humoral memory independent of antigen

To demonstrate that long-lived PC indeed can provide persistent and stable antigen-specific serum antibody titers in the absence of antigen, we measured OVA-specific IgG1^a for >3 months following transfer of B220$^-$ cell fractions into antigen-free recipients (Fig. 5). During the entire period of observation, six out of seven mice showed high, constant OVA-specific antibody titers of ~4–9 µg/ml IgG1^a. Over time, recipients of B220$^+$ bone marrow cells developed lower or undetectable anti-OVA antibody titers (<0.05–1.5 µg/ml IgG1^a), in all likelihood reflecting contamination with B220$^-$ PC. OVA-specific IgG1^b (recipient) was not detectable at any time point of observation (data not shown).

PC dynamics in spleen and bone marrow

For cells expressing little or no surface Ig, intracellular binding of OVA was restricted to antibody-secreting cells (Fig. 2). Thus, OVA-specific PC were identified according to intracellular binding of OVA and low expression of surface Ig. We used this marker combination to determine the frequency of OVA-specific PC in time, after secondary immunization in spleen and bone marrow. In the spleen, OVA-specific PC were detectable in numbers of up to 1.5×10^5 within the first week after antigenic challenge. Afterwards, their number declined rapidly, to low but constant numbers of ~1×10^4 per spleen. In the bone marrow, the number of OVA-specific PC increased continuously within 10 days after secondary immunization, then their number remained stable at a plateau of ~7×10^4 cells per mouse for the whole period of observation (3 months, Fig. 6A).

MHC class II expression marks equal maturation status of PC from spleen and bone marrow

Most cytometric markers analyzed in this study were not differentially expressed during the maturation of early PC to long-lived PC, as reflected by equal quantitative expression at different time points after immunization or in different organs, except for surface Ig, B220 and MHC class II antigen. Expression of surface Ig was down-regulated within the first 2 days after secondary antigenic challenge (data not shown). B220, a marker expressed on all B cells, naive and memory, was also lost as early as 2 days after secondary immunization on nearly all antibody-secreting cells. In contrast, MHC class II expression was rapidly up-regulated upon activation of B cells and during their differentiation to PC. Later, expression of MHC class II decreased to a low level again, within several weeks after secondary immunization. Then, neither bone marrow-derived nor splenic PC showed considerable MHC class II expression (Fig. 6B). Thus, the loss of MHC class II expression is characteristic of late PC. Throughout the immune response, at the time points studied, OVA-specific PC in the

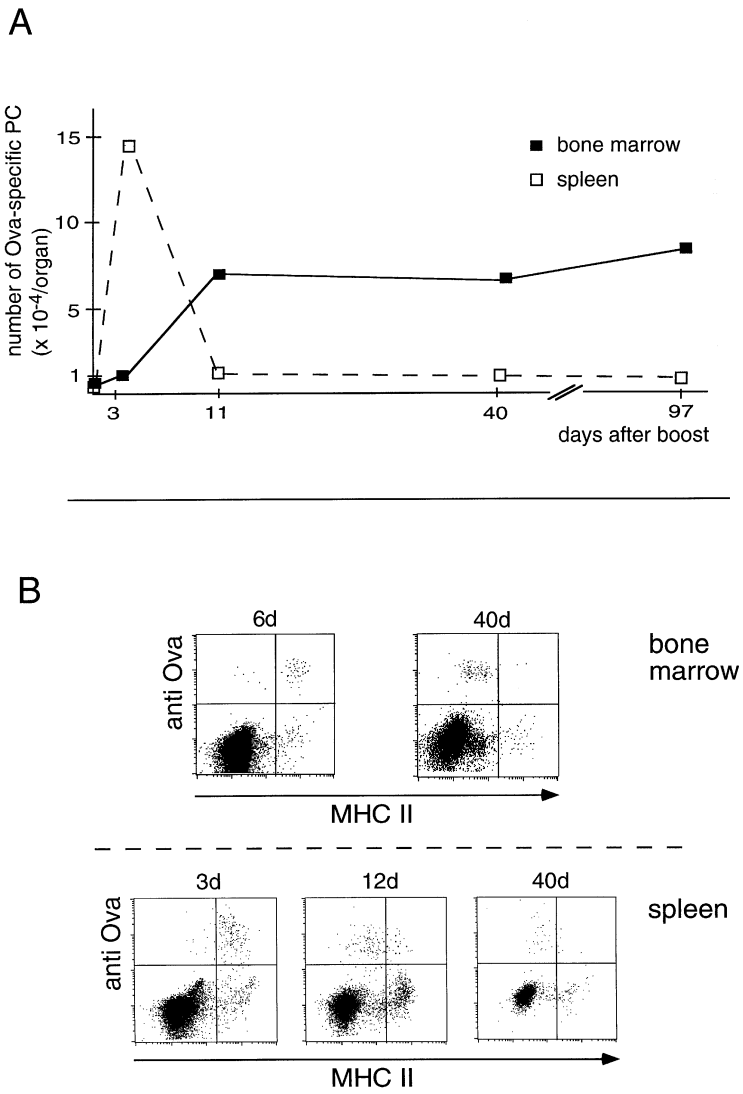

Fig. 6. Persistent splenic OVA-specific PC show the same level of maturation as PC residual in the bone marrow. (A) Absolute numbers of OVA-specific PC in spleen and bone marrow. Frequencies of OVA-specific PC defined as surface Ig low/intracellular OVA-binding blasts were analyzed in a time course after secondary immunization. Pooled cells from three mice for each time point and organ were analyzed. (Unimmunized mice showed less than one event in 1×10^5 cells which corresponds to ~1000 cells on this scale as lower detection limit, not shown.) Absolute numbers were calculated from frequencies and the numbers of total cells per organ as described in Methods. **(**B) Low MHC class II expression on mature PC. Total spleen and bone marrow cells were stained for surface Ig and enriched for IgG1-secreting cells without fluorescent staining (only magnetic label was used). Cells were fixed and stained for intracellular expression of anti-OVA antibody and for MHC class II. Surface Ig^+ cells were excluded from the analysis by gating as shown in Fig. 2. Thus anti-OVA antibody-expressing cells represent OVA-specific PC. The respective timepoint after boost immunization is indicated for each dot-plot.

spleen showed the same phenotype as those in the bone marrow, thus suggesting that late splenic PC are not formed continuously *de novo*.

Discussion

The present data demonstrate that neither antigen nor memory B cells are necessary to maintain humoral memory, i.e. an established, specific titer of serum antibodies. Such antibodies are secreted by recently described, long-lived PC, residing predominantly in the bone marrow (10,11). Induction, differentiation and conditions of survival of the newly described, long-lived 'memory PC' are poorly understood. Using a new technology for the cytometric identification of viable PC, the cellular-affinity matrix technology (20), we here provide a phenotypic analysis of long-lived PC during their differentiation and demonstrate that their survival is independent of antigen. The affinity matrix technology served to demonstrate that the cytometric parameters 'intracellular binding of fluorescent OVA' and 'low surface Ig' unambigu-

ously identify OVA-specific PC. This was demonstrated by cytometric correlation and cell sorting for secretion analysis in short-term culture.

In the spleen, a few days after secondary immunization with OVA, a major fraction of the IgG1-secreting cells was OVA-specific, with up to 1.5×10^5 cells per spleen at day 3. This number rapidly declines to $<10^4$ per spleen from day 10 onwards. In the bone marrow, the number of OVA-specific PC increased from 10^4 to 7×10^4 between days 3 and 10, and remained constant thereafter. These kinetics resemble those obtained by other groups (7,26), and reflect the fact that PC are generated in reactive organs of the immune system, like spleen, and migrate to the bone marrow thereafter (17,27,28). Here, we have analyzed the phenotype of PC in both organs at different time points after immunization. Within 3 days, PC lose expression of B220 (murine CD45R) and surface Ig, start to express syndecan-1 (CD138), and up-regulate expression of MHC class II. Such cells are found in the spleen and in the bone marrow within the first 12 days after immunization. Interestingly, from about day 6 onwards, OVA-specific PC with down-regulated MHC class II expression appear, again both in spleen and bone marrow. After day 40, all PC in spleen and bone marrow are MHC class II^{low}, including those that we had shown before to be long-lived (10). This result is in accordance with the recent analysis of Slifka *et al*. (11) and identifies MHC class II^{low} as a combinatorial marker for mature PC. Moreover, it shows that, long after an immune reaction, PC in reactive lymphoid tissue and bone marrow have the same, mature phenotype, suggesting that PC from reactive lymphoid organs are not necessarily constantly generated *de novo*, e.g. by residual antigen (29,30). In functional terms, the low expression of surface Ig and MHC class II molecules is expected to render mature PC inefficient in antigen presentation. Together with the elimination of circulating antigen by secreted antibody, this suggests that an efficient humoral memory does provide a high threshold for reactivation of memory B cells.

Expression of surface Ig on PC is down-regulated to low levels within 3 days after immunization, as is evident from the present analysis and work of other groups (26,31). At present, it is not clear whether this surface Ig is functional, especially whether mature and long-lived PC are reactive to antigen. Slifka *et al*. (11) showed by adoptive transfer of PC-containing bone marrow and simultaneous blocking of memory B cell function that persistent antibody titers are provided by long-lived PC. In that study, replicating lymphocytic choriomeningitis virus was used as antigen, to generate long-lived PC, and unintentional co-transfer of viral antigen in the cell transfer system had not been excluded, e.g. by comparison to transferred memory B cells. Here, we used immunization with an inert antigen under controlled antigen-free conditions, i.e. non-reactivity of donor memory B cells and host. We adoptively transferred long-lived, mature bone marrow PC immunized with an inert antigen into IgH syngeneic recipients, comparing their reaction to antigen with that of memory B cells. For the transfer experiments, PC were separated from naive and memory B cells according to lack of expression of B220. From the frequency of anti-OVA-secreting cells among IgG1-secreting cells (Figs 1 and 2B, ~0.03%) and the number of cells transferred (6×10^6/mouse), we estimate that the $B220^-$ cell fractions transferred into each mouse contained ~2000 OVA-specific antibody-secreting cells.

Within 7 days, the transferred PC had provided their hosts with a specific serum titer of 1–2 μg/ml, while in animals transferred with $B220^+$ cells, no OVA-specific Ig was detectable. The serum titer provided by the transferred, long-lived PC raised to up to 10 μg/ml and remained stable for >14 weeks, the period of observation. This experiment clearly shows that it is not the $B220^+$ memory B cells, but a population of $B220^-$ cells that provides persistent antibody titers, by secretion of specific antibodies from PC. If each PC would secrete ~0.3 ng Ig/day (Fig. 1), as determined here for PC sorted according to secretion, the transferred population, containing an estimated 2000 OVA-specific PC per mouse, contained within 6×10^6 $B220^-$ cells, would be able to secrete ~0.6 μg/day of specific Ig, Within 7 days and a half-life of IgG1 of ~1 week, this would accumulate to ~3 μg/ml, which is about what is found (~2 μg/ml, Fig. 4B), in all likelihood reflecting constant and continued secretion of OVA-specific IgG1 by the transferred PC. Although the recruitment of non-secretory precursor cells, activated to secrete specific Ig independent of antigen, cannot formally be ruled out, these would clearly be distinct from memory B cells.

The persistence of long-lived PC from bone marrow in antigen-inexperienced hosts already strongly suggests that antigen is not required for their survival. It has been shown by radioactive tracing of inert protein antigen that antigen can persist over long time periods in reactive lymphoid organs, but not in bone marrow (32). In the present experiment, the transfer of $B220^+$ bone marrow cells demonstrated that in any case we transferred less antigen than required to activate naive or memory B cells. That such cells were contained in the $B220^+$ cell population became evident when we challenged them with 2 μg of soluble, co-transferred OVA. Within 7 days after transfer, the activated transferred cells provided OVA-specific donor ($IgG1^a$) titers of ~10 μg/ml. Such titers remained stable for >6 weeks (data not shown). Kinetics, isotype of Ig and differentiation into long-lived PC, i.e. persistent antibody titers, indicate that this antigen response is mounted by transferred memory B cells, rather than naive B cells. The presence of memory B cells in bone marrow, as demonstrated here, has been a matter of debate. While in humans, immigration of memory B cells into bone marrow has been demonstrated (33), other authors have found few if any memory B cells at this site in mice (34).

Long-lived OVA-specific PC are not only independent of antigen for their survival and continued secretion of antibodies, but also do not react detectably to re-stimulation with antigen. Co-transfer of 2 μg of soluble OVA, together with PC, into naive IgH syngeneic recipients did not detectably affect the serum titers generated within 7 days after transfer. Thus, long-lived PC do neither expand nor die upon challenge by antigen, and, as can be inferred from the coexistence of antigen and specific antibodies, also are not up- or down-regulated by antigen–antibody complexes.

The inertness of long-lived PC to antigen and antigen-antibody complexes raises the principal problem of how homeostasis of PC in the bone marrow is regulated, especially in the case of chronic or multiple antigenic stimulations. According to the present view, provocation with antigen at

doses overrunning the protective humoral antibody memory will trigger activation of memory cells, their affinity maturation and differentiation into PC, which will in turn migrate to the bone marrow. There, they co-exist with the pre-existing PC (data not shown), both providing their share of affinity-matured antibodies to the protective serum titer and enhancing the threshold for the activation of memory B cells even further. The pool of specific, long-lived PC will be filled up with new PC until humoral antibody concentrations are protective and prevent further activation of memory B cells. In humoral antibodies, a spectrum of affinities will provide a differential molecular memory. At present the overall limitation of bone marrow for PC numbers is not clear, although it would be surprising if there were none. The work of Haaijman *et al*. (35) suggests that such a limitation may exist at ~0.2% of bone marrow cells. In human bone marrow of aged individuals, we found the frequency of PC to be $<0.5\%$ (syndecan$^+$/intracellular Ig$^+$ cells, data not shown). The differential induction of long-lived versus short-lived PC, the molecular basis of homeostasis of long-lived PC in the bone marrow and the rules determining which long-lived PC are eliminated are a challenge for future research. Already now, our data provide a clear-cut explanation on why antigen-based therapeutic concepts have been and will be problematic in the management of diseases, based on pathological humoral memory, like autoantibodies in autoimmune diseases and allergen-specific IgE in allergy. On the other hand, our results, in conjunction with those of Slifka *et al.*, will have vast implications for the development of vaccination strategies and therapeutic approaches to allergy and autoimmunity.

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Abbreviations

OVA	ovalbumin
PC	plasma cells
PE	phycoerythrin

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3.2 Das Überleben von Plasmazellen wird durch synergistische Effekte von Zytokinen und Kontakt-abhängigen Signalen vermittelt

Cassese et al., J Immunol 2003. 171: 1684-1690.

Nachdem wir gezeigt hatten, dass das Überleben von langlebigen Plasmazellen nicht von dem Vorhandensein von Antigen abhängig ist, stellte sich die Frage, ob diese Zellen per se langlebig sind, d.h. mit einer Halbwertszeit im Bereich von einigen Monaten sterben, oder ob die Lebensspanne dieser Zellen von Faktoren in ihrer Umgebung reguliert wird.

Um die Faktoren zu identifizieren, die für das Überleben von reifen, sich nicht mehr teilenden Plasmazellen im Knochenmark verantwortlich sind, wurden diese Zellen aus dem Knochenmark von BALB/c Mäusen isoliert und ihr Überleben in Kultur in Abhängigkeit von verschiedenen Faktoren und Zellen bestimmt. Es zeigte sich, dass alle isolierten Plasmazellen innerhalb von 3 Tagen gestorben waren, falls keine autologen Knochenmarkszellen oder Kulturüberstand dieser Zellen vorhanden war [61]. Die Gegenwart von autologen Milzzellen zeigte keinen vergleichbaren Effekt auf das Überleben der Plasmazellen. Zugabe von IL-5, IL-6, TNF-a, SDF-1 und Liganden für CD44 (Hyaluronsäure, aktivierende Antikörper) verlängerten das Überleben von Plasmazellen, am effektivsten in bestimmten Kombinationen. So gewährleistet Hyaluronsäure zusammen mit IL-6 das Überleben von etwa 90% der eingesetzten Plasmazellen für mindestens 5 Tage. Diese Ergebnisse zeigen, dass das Überleben von Plasmazellen abhängt von der Verfügbarkeit spezifischer und synergistisch wirkender Faktoren, die im Knochenmark ausgeprägt werden. Dieses Resultat ist vereinbar mit der Beobachtung, dass die Ausbildung von spezifischen Langzeit-Antikörpertitern von einer Akkumulation von Plasmazellen im Knochenmark begleitet wird [62].

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).

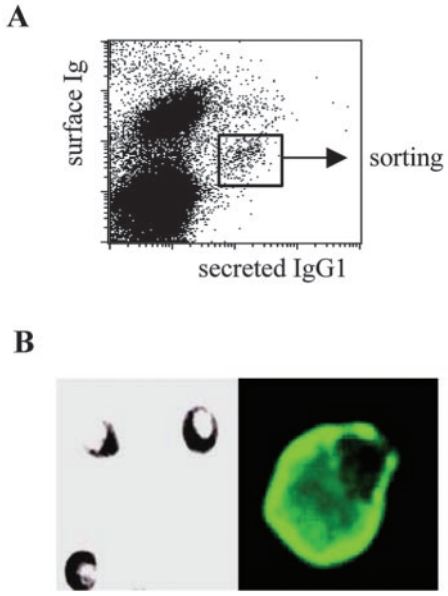

FIGURE 1. Purification of bone marrow plasma cells. *A*, FACS analysis of IgG-1-secreting bone marrow cells after MACS enrichment. Bone marrow cell suspensions were prepared from femurs of at least five mice. The cellular affinity matrix technology was used to label IgG1-secreting cells, as described 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.

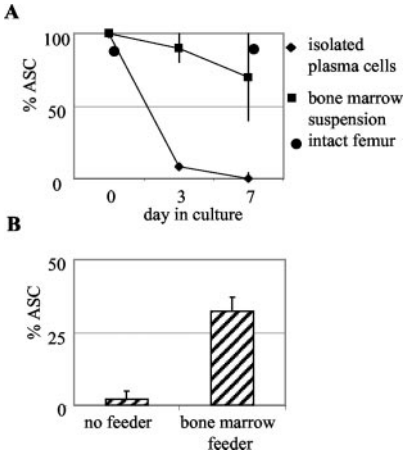

FIGURE 2. Bone marrow environment maintains plasma cell function. *A*, Isolated bone marrow plasma cells disappear rapidly in culture. Kinetics of plasma cells in cultures consisting of isolated plasma cells (100 cells/well), total bone marrow single cell suspension (10^6 cells/ml), or intact femurs 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).

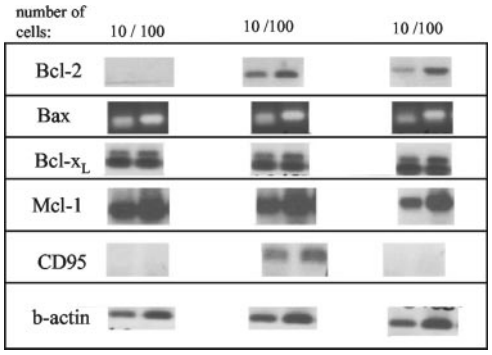

FIGURE 3. Pro- and antiapoptotic gene expression in isolated plasma cells. Mature B cells ($B220^+$, CD24 intermediate), LPS-activated B cells (3 days cultured, $CD138^+$, IgM^+), or plasma cells (IgG1-secreting cells, as identified in Fig. 1) were FACS sorted into tubes, and RT-PCR was performed, as 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.

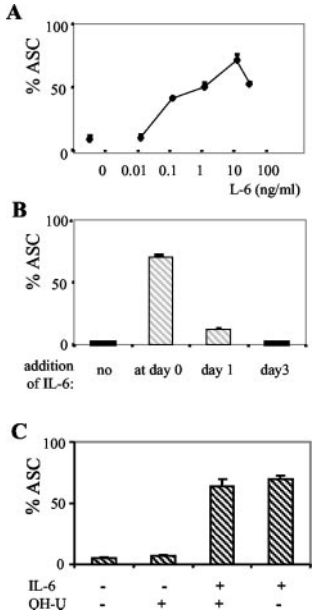

FIGURE 4. Effects of IL-6 on plasma cell function. *A*, Dose-response curve of IL-6. rIL-6 was added to isolated plasma cells (100 cells/well) at the concentrations indicated (0.01–50 ng/ml), and the numbers of ASC were determined after 3 days in culture by ELISPOT. Values are expressed as the mean + 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.

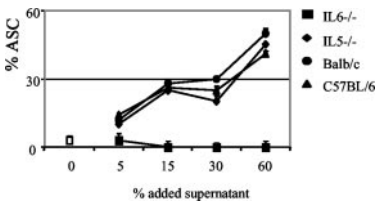

FIGURE 5. IL-6 is required to support plasma cell function in bone marrow cultures. Purified plasma cells (100 cells/well) were cultured together with various concentrations of bone marrow supernatants prepared from C57BL/6, IL-6$^{-/-}$, IL-5 $^{-/-}$, and BALB/c mice, as indicated. The numbers of ASC 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

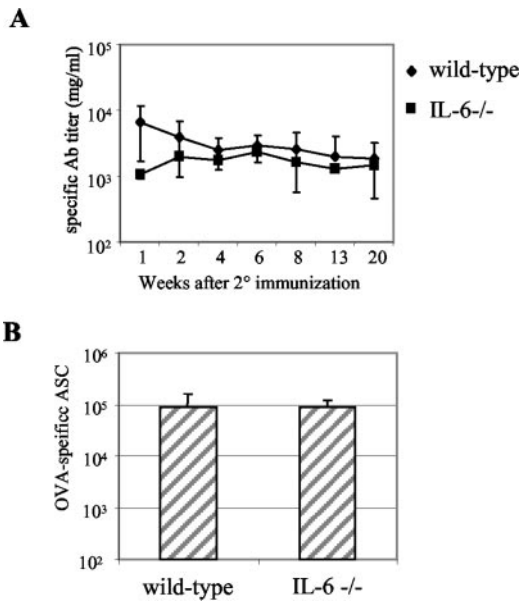

FIGURE 6. IL-6 is required for a normal induction, but not for the maintenance of Ab responses in vivo. *A*, Kinetics of serum Ag-specific IgG titers, determined by ELISA, were compared in IL-6$^{-/-}$ and wild-type mice following boost immunization with OVA (day 0 of analysis). The data shown represent 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.

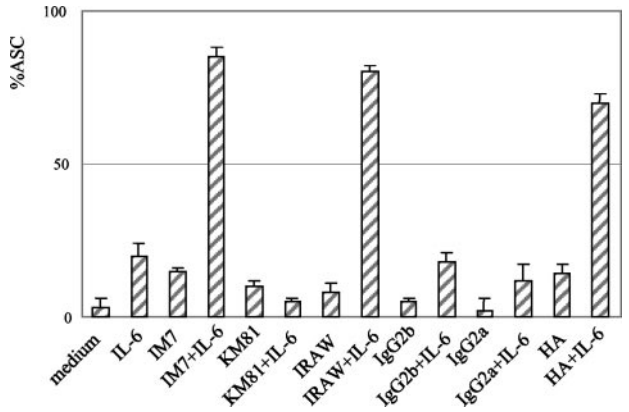

FIGURE 7. Stimulation by IL-6, together with CD44 triggering, acts synergistically in supporting function of plasma cells. Isolated bone marrow plasma cells were cultured together with various stimuli, as indicated. Three different immobilized anti-CD44 Ab (IM7, IRAW, and KM81; 4 μg/ml) and soluble HA (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.

Acknowledgments

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3.3 Die Chemokinrezeptoren CXCR3 und CXCR4 sind an der Regulation des Wanderungsverhaltens IgG-sezernierender Zellen beteiligt

Hauser , et al., J Immunol 2002. 169: 1277-1282.

Die Ergebnisse unserer und anderer Arbeitsgruppen zeigten, dass das Überleben von langlebigen Plasmazellen von spezifischen Faktoren in ihrer Umgebung abhängt. Nur im Knochenmark und in entzündeten Geweben kann eine grössere Anzahl dieser Zellen langfristig überleben. In diesem Teil der Arbeit wurde die Regulation des Wanderungsverhalten Ovalbumin-spezifischer, IgG-sezernierender Zellen (diese Definition umfasst Plasmazellen und ihre direkten Vorläufer) im zeitlichen Verlauf einer sekundären Immunisierung untersucht [63]. In dieser Immunantwort produzieren etwa 90% aller Antikörper-sezernierenden Zellen IgG. Die maximale Anzahl Ovalbumin-spezifischer, IgG-sezernierender Zellen war in der Milz am Tag 6 ereicht. Am Tag 4 nach der Sekundärimmunisierung begannen diese Zellen bereits die Milz zu verlassen, um in das Knochenmark zu wandern. Die Wanderung des grössten Teils der Ovalbumin-spezifischen, IgG-sezernierenden Zellen von der Milz in das Knochenmark fand zwischen Tag 4 bis Tag 6 nach der Immunisierung statt.

Nun sollte untersucht werden, ob Chemokine an der Regulation der Plasmazell-Lokalisation beteiligt sind. Es konnte gezeigt werden, dass Ovalbumin-spezifische, IgG-sezernierende Zellen auf Liganden für die Chemokinrezeptoren CXCR3 und CXCR4, nicht jedoch auf andere Chemokine (Migrationswerte der Basalmigration: < 1,5%) chemotaktisch reagieren. Gegen Konzentrationsgradienten der CXCR3 und CXCR4 Liganden MIG, IP10, ITAC und SDF-1 wanderten 30-70% der Ovalbumin-spezifischen, IgG-sezernierenden Zellen. Die Empfänglichkeit Ovalbumin-spezifischer IgG-sezernierender Zellen für diese chemotaktischen Signale war nur transient, zwischen Tag 4 bis Tag 6, jedoch nicht mehr an Tag 12 nach der Immunisierung, zu beobachten.

Diese Untersuchungen waren in Übereinstimmung mit zeitgleichen Untersuchungen aus Jason Cysters Arbeitsgruppe, die zeigen konnten, dass CXCR4 defiziente Plasmazellen nur noch ineffizient im Knochenmark akkumulieren [64]. Es ist bekannt, dass Liganden des Chemokinrezeptors CXCR3 oftmals

beteiligt sind an der Einwanderung verschiedener Zellarten in entzündetes Gewebe. Über eine Rolle dieses Rezeptors in der Migration von B-Zellen und Plasmazell-Vorläufern war jedoch noch nichts bekannt.

Zusammen mit unseren anderen Untersuchungen, die zeigten, dass Plasmazellen in entzündeten Nieren von NZB/W Mäusen akkumulieren [65], legt diese Arbeit nahe, dass CXCR3 für die Wanderung von Antikörper-sezernierenden Zellen in entzündetes Gewebe verantwortlich ist. Wir konnten tatsächlich eine mindestens 100-fach erhöhte Produktion von für IP10 codierende mRNA in der entzündeten Niere von NZB/W Mäusen nachweisen.

Chemotactic Responsiveness Toward Ligands for CXCR3 and CXCR4 Is Regulated on Plasma Blasts During the Time Course of a Memory Immune Response[1]

Anja E. Hauser,* Gudrun F. Debes,† Sergio Arce,* Giuliana Cassese,* Alf Hamann,† Andreas Radbruch,* and Rudolf A. Manz[2]*

Plasma blasts formed during memory immune responses emigrate from the spleen to migrate into the bone marrow and into chronically inflamed tissues where they differentiate into long-lived plasma cells. In this study, we analyze the chemokine responsiveness of plasma blasts formed after secondary immunization with OVA. Starting from day 4 and within ~48 h, OVA-specific plasma blasts emigrate from spleen and appear in the bone marrow. Although these migratory cells have lost their responsiveness to many B cell attracting chemokines, e.g., CXC chemokine ligand (CXCL)13 (B lymphocyte chemoattractant), they migrate toward CXCL12 (stromal cell-derived factor 1α), and toward the inflammatory chemokines CXCL9 (monokine induced by IFN-γ), CXCL10 (IFN-γ-inducible protein 10), and CXCL11 (IFN-inducible T cell α chemoattractant). However, the responsiveness of plasma blasts to these chemokines is restricted to a few days after their emigration from the spleen, indicating a role for these molecules and their cognate receptors, i.e., CXCR3 and CXCR4, in the regulation of plasma blast migration into the bone marrow and/or inflamed tissues. ***The Journal of Immunology,*** **2002, 169: 1277–1282.**

Secretion of Ab into the body humors by plasma cells is a basic defense mechanism of the vertebrate immune system, but also can become an integral part of the pathology in autoimmune diseases and allergies (1). The production of Ab of high affinity for protein Ag requires T-B cell interaction in secondary lymphoid organs (2). Primary immunization results in the formation of Ab-secreting cells (ASC)[3] in extrafollicular foci. Most of these ASC die within ~1 wk in these foci (3). In contrast, most ASC formed during a memory response leave the splenic follicles to migrate to and maintain the humoral immune response either in the bone marrow (4–6) or in chronically inflamed tissues (7). Some plasma blasts migrate into the red pulp of the spleen where these cells also can persist for long periods of time (8). Chemokines and their receptors are crucially involved in the control of lymphocyte trafficking. Although, for example, CXC chemokine ligand (CXCL)12 typically mediates lymphocyte homeostasis, CXCL9, CXCL10, and CXCL11 are associated with lymphocyte migration into inflamed tissue (9–11).

It has recently been suggested that CXCR4 is required for normal accumulation of plasma cells in the bone marrow (12). Whether other chemokine receptors besides CXCR4 are involved in the regulation of ASC migration into the bone marrow and which molecules direct ASC into inflamed tissues, has not been clarified yet. In this study, we analyze the localization and chemokine responsiveness of OVA-specific ASC during the time course of a memory response. At the time point of emigration of these cells from the spleen and only for a few days, ASC migrate toward a limited number of chemokines. Besides CXCL12, the only known ligand for CXCR4, these chemokines include all ligands for CXCR3, i.e., the inflammatory chemokines CXCL9, CXCL10, and CXCL11. These results suggest that CXCR3 mediates the attraction of ASC into inflamed tissues. CXCR3 may also contribute supplementary to CXCR4 for the migration of ASC into the bone marrow.

Materials and Methods

Mice and immunizations

BALB/c female mice were bred at the animal facility of the Deutsches Rheuma Forschungszentrum Berlin (Berlin, Germany) under specific pathogen-free conditions. At the time point of primary immunization, the animals were 6–8 wk of age. Each mouse received 100 μg OVA (Sigma-Aldrich, Steinheim, Germany) precipitated in aluminum-magnesium-hydroxide (Imject-Alum; Pierce, Rockford, IL; 200 μl in PBS) injected i.p. After 3–6 wk, primed mice were immunized again by injection of 50 μg OVA in 100 μl PBS into the lateral tail vein.

Quantification of OVA-specific IgG ASC by ELISPOT

Single-cell suspensions from bone marrow (femurs) and spleen were filtered through a 70-μm cell strainer (BD Falcon, Bedford, MA), washed, and resuspended in RPMI 1640 medium (Life Technologies, Paisley, U.K.), supplemented with 10% FCS (Invitrogen, Carlsbad, CA), penicillin, streptomycin, and glutamine (complete medium). The standard ELISPOT technique (13) was modified as described, briefly: RIA plates (flat-bottom, high-binding; Corning, Corning, NY) were coated overnight at 4°C with OVA (Sigma-Aldrich) in PBS (5 μg/ml). Then, plates were incubated with PBS containing 3% BSA (Biomol, Hamburg, Germany) 1 h before adding the cells in various dilutions in complete medium. The cells were incubated for 2 h in a humid atmosphere with 5% CO_2. Subsequently, cells were removed by vigorous washing with 3% BSA in PBS/0.01% Tween 20 (BSA/PBS/Tween).

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[3] Abbreviations used in this paper: ASC, Ab-secreting cell; PC, plasma cell; CXCL, CXC chemokine ligand; CCL, CC chemokine ligand.

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Between all following steps, plates were washed three times with BSA/PBS/Tween. Biotin-labeled goat anti-mouse IgG (Southern Biotechnology Associates, Birmingham, AL) in PBS (1 μg/ml) was added and plates were incubated for 30 min at room temperature. Following that, 50 μl streptavidin-alkaline phosphatase (Sigma-Aldrich) was added after a 30-min incubation at room temperature. 5-bromo-4-chloro-3-indolyl-phosphate (Sigma-Aldrich) was added to visualize spot formation: 5-bromo-4-chloro-3-indolyl-phosphate per was dissolved in 2-AMP-buffer (95 ml 2-amino-2-methyl-1-propanol, 0.1 ml Triton X-405, 150 mg/ml $MgCl_2$, 900 ml Aqua dest, pH 10.25 adjusted with HCl) at a concentration of 1 mg/ml. The solution was mixed with melted agarose (type I, low electroendosmosis; Sigma-Aldrich) to obtain a final agarose concentration of 0.6%. The mixture was kept at 65°C for 30 min. After addition of the substrate, plates were cooled down to room temperature for solidification of the agarose. The plates were allowed to develop for 2 h at 37°C. Spots were counted under an inverted microscope. Total numbers of OVA-specific ASC in the bone marrow were calculated assuming that both femurs together contain 12.6% of the total bone marrow cells (14).

Chemokines and chemotaxis assay

All chemokines except for CC chemokine ligand (CCL)17 (thymus- and activation-regulated chemokine) were purchased from R&D Systems (Minneapolis, MN). Recombinant TARC was produced as recently described (15). TARC-producing cell line was kindly provided by I. Förster (Technical University of Munich, Munich, Germany). Chemotaxis assays were performed as described (16). Briefly, 24-well plates with Transwell inserts (6.5-mm diameter, 5 μm pores; Corning) and RPMI 1640 medium (Life Technologies) supplemented with 0.5% BSA (low endotoxin; Sigma-Aldrich) was used (assay medium). The inserts were coated with 50 μl of murine fibronectin (Invitrogen) at a concentration of 10 μg/ml in water and incubated for 1 h at 37°C in a humid atmosphere with 5% CO_2. The solution was removed and the inserts were dried for 2 h at 37°C. Lymphocytes were isolated by gradient centrifugation (Histopaque-1083; Sigma Diagnostics, St. Louis, MO), washed, counted, and diluted in assay medium at a final concentration of 5×10^6 cells/ml. Before the chemotaxis assays, freshly harvested cells were kept in prewarmed (37°C) medium during the cell isolation procedure, taking ~90 min (density gradient centrifugation and washing). The lower Transwell chamber was filled with 600 μl of chemokine solution, and 100 μl of the cell suspension was added to the upper chamber. Cells were allowed to migrate for 90 min at 37°C in a humid atmosphere with 5% CO_2. Finally, the cells were collected from the lower Transwell compartment and from control samples containing total cells before migration. In both fractions, OVA-specific ASC were quantified by ELISPOT assay as described above and percentages of migrated cells were calculated.

Functionality of the chemokines was tested by analyzing the migration of CD4-positive cells and eosinophils. Eosinophilic granulocytes were quantified in the input and after migration as follows: the cells were brought on glass slides by cytospin and stained with Giemsa's solution, then they were assessed and counted under a microscope. The numbers of CD4-positive cells were quantified in the input and migrated population: triplicates of 500 μl aliquots for each chemokine and the medium control were added to a fixed amount of beads (TruCount; BD Biosciences, Mountain View, CA), Abs used for gating (anti-mouse CD4, H129.19) were added, and the number of cells and beads was counted without washing by FACS. Samples were analyzed on a FACSCaliber using CellQuest software (BD Biosciences).

Analysis of OVA-specific plasma cells (PC) by FACS

Single-cell suspensions prepared from bone marrow were washed twice in PBS/0.5% BSA. Cells were incubated with the primary staining reagents at titrated concentrations for 10 min on ice. Primary staining reagents were: FITC-conjugated OVA (OVA was purchased from Sigma-Aldrich and labeled with FITC according to the manufacturer's protocols), anti-CD138-PE (clone 281-2; BD PharMingen, San Diego, CA), anti-CXCR4-biotin (anti-murine-CXCR4, clone 1D9, a generous gift from R. Förster, Institute of Immunology, Hannover Medical School, Hannover, Germany) was labeled with sulfosuccinimidyl-6-biotinamido-hexanoate (Pierce) according to the manufacturer's protocols. Biotinylated, isotype-matched rat anti-mouse IL-12 (clone C17.8) has been used as control. For blocking of the CXCR4 staining, cells were preincubated with 100-fold excess of unlabeled anti-CXCR4 Ab. After washing, the cells were stained with streptavidin coupled to allophycocyanin as secondary reagent. Cytometric analysis was performed using a LSR cytometer (BD Biosciences). Samples were stained with propidium iodide. Dead cells, debris, and RBCs were electronically excluded by gating. For analysis, CellQuest software (BD Biosciences) was used.

Results and Discussion

At the time OVA-specific IgG-ASC leave the spleen, these cells migrate toward CXCR3 and CXCR4 ligands

The numbers of OVA-specific IgG-secreting cells, i.e., plasma blasts, in spleen and bone marrow were determined by ELISPOT at daily intervals between days 3 and 6 after secondary immunization (Fig. 1). In the spleen, where the immune response was initiated, these cells reached peak numbers at days 4 and 5, before decreasing 10- to 50-fold during the following 2 days. At the same time, i.e., between days 4 and 6 after secondary immunization, OVA-specific ASC appeared in the bone marrow where their numbers peaked at day 6. These data show that the migration of OVA-specific ASC from spleen into the bone marrow occurred basically within the narrow time window between days 4 and 6 after secondary immunization. The numbers of ASC in the blood peaked at that time but were altogether <1000 cells in 1 ml. The obvious lack of accumulation in the blood suggests that individual plasma blasts circulate only for a short period of time and are already determined to migrate to their final destinations. Similar results had earlier been observed after immunization with lymphocytic choriomeningitis virus, hapten-coupled protein, and sheep erythrocytes (3, 17–19), indicating that the rapid PC formation and migration into the bone marrow observed in our study is a general phenomenon.

To identify the chemokines and their cognate receptors involved in this migratory process, we analyzed OVA-specific ASC isolated at the time point when the cells start to leave the spleen, i.e., at day 4 after immunization, for their chemotactic response in vitro. A recent report by Bowman et al. (20) indicates that chemokines selectively recruit PC secreting Ab of different isotypes. It should be noted that the studies presented focus on the migration of IgG-secreting cells, the predominant isotype of the persistent humoral memory response against OVA in our immunization protocol (our unpublished results). Chemokines were tested at concentrations between 0.1 and 300 nM. About 60% of the IgG-ASC migrated toward CXCL12 (stromal cell-derived factor 1α) at the optimal concentration ranging between 10 and 100 nM (Fig. 2). About 30% of these cells migrated toward CXCL9 (monokine induced by IFN-γ) at a concentration of 100 nM. Migration toward medium alone was <1.5% (basal migration). Migration toward CXCL9 and CXCL12 was blocked to basal level (<1.5%) by addition of these chemokines into the upper and lower Transwell chamber

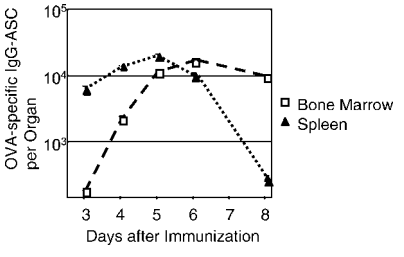

FIGURE 1. Within 2 days, IgG-ASC emigrate from the spleen and appear in the bone marrow. BALB/c mice were immunized and boosted with OVA. At the time points indicated, mice were sacrificed and OVA-specific IgG-ASC were identified by ELISPOT. Total numbers of these cells in spleen and bone marrow were calculated as described in *Materials and Methods*. Pooled cells from three to five mice for each time point and organ were analyzed, the data show one representative experiment of three.

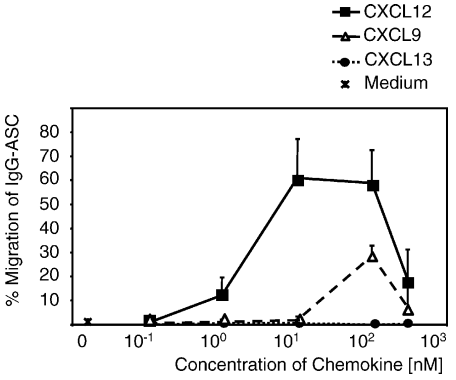

FIGURE 2. Chemotaxis of IgG-ASC toward chemokine gradients. Cells were isolated from murine spleen at day 4 after secondary immunization with OVA. Chemotaxis was determined by the Transwell system, OVA-specific IgG-ASC were enumerated by ELISPOT. The percentage of input OVA-specific IgG-ASC that migrated to the lower chamber of the Transwells is shown. Data shown are the average of three independent experiments tested in triplicates with cells pooled from two mice per experiment. Error bars represent SD. The numbers of IgG-secreting cells were 548 ± 153 cells/5 × 10^5 total spleen cells plated.

in equivalent concentrations (checkerboard assay), indicating that the migration could be due to chemotaxis and not to chemokinesis. Notably, and in accord with two recent reports (12, 21), no response has been detected to CXCL13 (B lymphocyte chemoattractant), CCL19 (EBI 1 ligand chemokine), and CCL21 (secondary lymphoid tissue chemokine), as shown in Fig. 2 and Table I, respectively. These chemokines are important for B cell localization in the various zones of secondary lymphoid organs (22).

About 30 and 10% of OVA-specific IgG-ASC migrated in response to 100 nM CXCL10 (IFN-γ-inducible protein 10) and 100 nM CXCL11 (IFN-inducible T cell α chemoattractant), respectively (data not shown). For all other chemokines tested, no migration above the basal level could be detected (Table I). As far as possible, the functionality of the chemokines used was tested on other cell types, as shown in Table II. Although our results cannot formally exclude the possibility for other chemokines to be involved in the regulation of plasma blast migration, our study has identified a number of chemokines which can attract plasma blasts formed in secondary immune responses, i.e., CXCL9, CXCL10, CXCL11, and CXCL12. The cognate receptors for these chemokines are CXCR3 and CXCR4, respectively (19, 23–25). Our results indicate that CXCR3 and CXCR4 are functionally expressed on IgG-secreting plasma blasts formed in secondary immune responses, at a time point when they leave the spleen.

Table I. *Summary of IgG-ASC chemokine responsiveness*

Receptor	Chemokine	Chemotaxis[a]
CCR1	CCL3	−
CCR2	CCL2	−
CCR3	CCL11	−
CCR4	CCL17	−
CCR5	CCL4	−
CCR6	CCL20	−
CCR7	CCL19	−
CCR8	CCL1	−
CCR9	CCL25	−
CCR10	CCL27	−
CXCR1	CXCL6	−
CXCR2	CXCL6	−
CXCR3	CXCL9, 10, 11	+
CXCR4	CXCL12	+
CXCR5	CXCL13	−
XCR1	XCL1	−
CX3CR1	CX3CL1	−

[a] A negative value (−) indicates migration below the basal level of 1.5%, positive results (+) are 30, 27, 11, and 60% for CXCL9, CXCL10, CXCL11, and CXCL12, respectively. XCR1, XC chemokine receptor 1; XCL1, XC chemokine ligand 1.

Table II. *Chemotactic response of various leukocyte populations*[a]

Chemokine	Cell Type	Chemotaxis (%)[b]
CCL4	$CD4^+$ T cells[c]	40
CCL11	Eosinophils	>50
CCL17	$CD4^+$ T cells[d]	12–15
CCL21	$CD4^+$ T cells[d]	75
CCL19	$CD4^+$ T cells[d]	75
CXCL9	$CD4^+$ T cells[d]	25
CXCL12	$CD4^+$ T cells[d]	40
CXCL13	$CD4^+$ T cells[d]	12–15

[a] At optimal chemokine concentrations.
[b] Percentage of input.
[c] In vitro-activated Th1 cells.
[d] Freshly isolated from spleen.

Responsiveness toward CXCR3 and CXCR4 ligands on ASC in the time course after secondary immunization

Fig. 1 indicates that OVA-specific plasma blasts, formed within the first 4–5 days after secondary immunization in the spleen, enter the bone marrow between days 5 and 6. We tested CXCL12 and CXCL9 for their potential to attract ASC harvested from bone marrow at various time points (Fig. 3). At day 6 after immunization, OVA-specific ASC from bone marrow migrated as well toward optimal concentrations of CXCL9 and CXCL12 as ASC from spleen. The percentage of responsive ASC at that time point was not different from that observed in splenic ASC at day 4. However, at day 12 after immunization, bone marrow ASC had completely lost their capacity to migrate toward CXCL9 and CXCL12 at those concentrations optimal for day 4 splenic ASC (Fig. 3), and in concentrations between 0.1 and 300 nM (data not

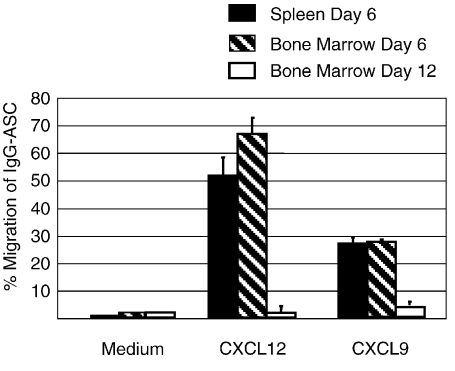

FIGURE 3. Within 6 days after arrival in the bone marrow, IgG-ASC lose the responsiveness toward CXCR3 and CXCR4 ligands. Cells were isolated from murine spleen and bone marrow at days 6 or 12 after secondary immunization with OVA. Chemotaxis was determined by the Transwell system, OVA-specific IgG-ASC were enumerated by ELISPOT. CXCL9 and CXCL12 were used at a concentration of 100 and 10 nM, respectively. The percentage of input OVA-specific IgG-ASC that migrated to the lower chamber of the Transwells in one representative experiment of three is shown. The numbers of IgG-secreting cells per 5 × 10^5 total cells plated were 398 (bone marrow cells at day 12), 374 (bone marrow cells at day 6), and 166 (spleen cells at day 6).

shown). A 3-h period of preincubation at 37°C before the chemotaxis assay did not restore the ability of day 12 bone marrow ASC, nor did this prevent day 6 bone marrow ASC from migrating toward these chemokines.

The change in the chemotactic responsiveness of PC and their limited time period of migration into the bone marrow argue against a continuous formation of short-lived PC renewing the bone marrow PC population as hypothesized earlier (1), and are in accord with experiments showing that the population of PC persisting in the bone marrow consists of long-lived cells (4, 5, 26).

CXCR4 expression on bone marrow PC

To determine whether PC down-regulate surface CXCR4 expression after entry into the bone marrow, CXCR4 expression was analyzed by FACS on OVA-specific PC at days 6 and 12 after secondary immunization. OVA-specific PC were identified by staining for the PC surrogate marker CD138 together with Ag (3, 27). A population of ~0.1% of these cells was detected in the bone marrow after secondary immunization (Fig. 4*A*). The staining "background" in nonimmunized controls was below a frequency of 0.01%. Despite the absence of chemotactic responsiveness toward CXCL12, CXCR4 is expressed on 77% of the OVA-specific PC derived from the bone marrow at day 12 after immunization (Fig. 4*B*). Specificity of the CXCR4 staining was controlled by staining with an isotype-matched irrelevant Ab and by incubation with unlabeled Ab before CXCR4 staining. CXCR4 seemed to be rather up-regulated at day 12 compared with day 6 bone marrow PC. Uncoupling of CXCR4 surface expression to migration and signaling by an unknown mechanism has been described for hematopoietic stem cells and B cells, respectively (28, 29). We assume that CXCR4 on these bone marrow PC may serve a function different from mediating chemotaxis, possibly mediating anti-apoptotic signals and supporting cellular survival, as shown for T cells and peritoneal B cells, respectively (30, 31). The presence of mRNA for CXCR4 has been detected on PC derived from peripheral lymph nodes (21). However, these cells did not migrate toward CXCL12. In another report, splenic PC migrated well toward CXCL12 (12). Our results show that the chemotactic responsiveness toward CXCL12, but not expression of CXCR4, depends on the time point after immunization and PC localization, possibly explaining the apparently contradicting results on CXCR4 expression and responsiveness to CXCL12.

Plasma blast migration into the bone marrow and inflamed tissue

Memory Ab titers are produced by a population of PC formed in secondary lymphoid organs migrating and persisting in the bone marrow (6, 19, 32). The capacity of PC to migrate into the bone marrow seems to be critical for the development of persisting Ab responses (33). Chemokines and their cognate receptors usually are important regulators of cell migration. Changes of chemokine responsiveness had been observed during differentiation of PC derived from lymph nodes after primary immunization (21), indicating that these changes may be crucial for emigration from this tissue. To gain information about the regulation of PC migration into the bone marrow, we analyzed the migration and chemokine responsiveness of IgG-secreting PC specific for the protein Ag OVA during the time course of a secondary immune response.

In this study, we show that the responsiveness of plasma blasts toward CXCR3 and CXCR4 ligands is restricted to the

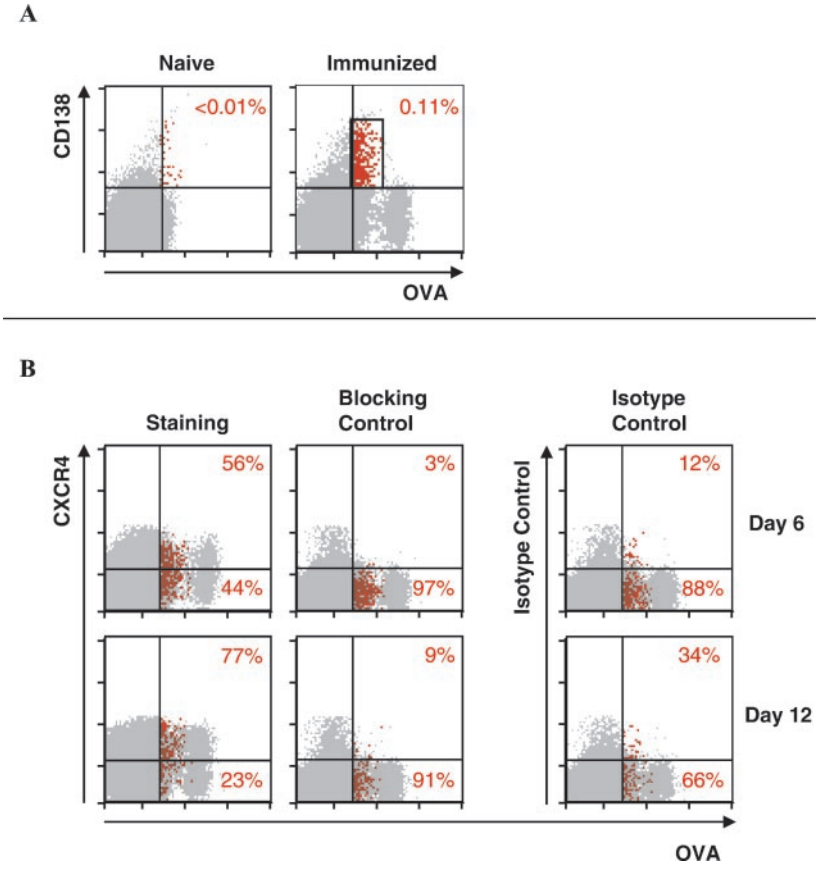

FIGURE 4. *A*, Expression of CXCR4 on total bone marrow cells. OVA-specific PC were identified by the expression of CD138 together with their ability to bind Ag in immunized and control animals. *B*, Cells were stained for CXCR4, specificity of the staining was confirmed by preincubation of the cells with an excess of unlabeled Ab (blocking control) and by an isotype-matched control. For each sample, 500,000 total cells were acquired, cells were gated electronically to exclude dead cells and debris. Fluorescence intensity is shown in a logarithmic scale. Red dots represent OVA-specific PC. Frequencies of OVA-specific PC are indicated by red numbers in the various quadrants.

period of their emigration from spleen and migration into the bone marrow, suggesting a role for these receptors in the regulation of this process. It has been shown recently that plasma cells in CXCR4-deficient fetal liver chimeras accumulate in the bone marrow in ~3-fold reduced numbers, showing that this receptor is responsible for either migration into or retention of PC in the bone marrow (12). Whether CXCR3 is also involved in directing plasma blasts into the bone marrow and responsible for the remaining accumulation of these cells in the bone marrow of CXCR4-deficient chimeras remains to be elucidated.

In several autoimmune diseases, plasma cells have been detected in chronically inflamed tissue (7, 34, 35). Among these cells are those secreting autoantibodies (7, 34). Through the generation of high local autoantibody concentrations, plasma cells that lodge in the affected inflamed tissue may contribute specifically to the pathogenesis of the disease. We have recently described the accumulation of plasma cells formed in the spleen in the inflamed kidneys of NZB/W F_1 mice (36), a model for autoimmune systemic lupus erythematosus (37). Due to the effective migration of plasma blasts into these inflamed organs, plasma cells are found in the kidneys of these mice in numbers comparable to those in the bone marrow. The specificity of the immigrating plasma blasts was not restricted to self-Ag. OVA-specific plasma cells formed a few days after secondary immunization in the spleen lodged in the inflamed kidneys at later time points, indicating that, independent of their specificity, plasma blasts have the capacity to migrate into inflamed tissues (36). In this study, we show that during their migratory phase, such plasma blasts migrate toward the CXCR3 ligands CXCL9, CXCL10, and CXCL11. Using quantitative PCR, we detected an at least 1000-fold increase in the mRNA expression of CXCL10 in the inflamed kidneys of NZB/W mice as compared with the kidneys of healthy controls (data not shown). It has been shown that the ligands for CXCR3 are expressed in various inflamed tissues, including the kidneys of patients suffering from glomerulonephritis (38). It is also well documented that CXCR3 is a key player in the recruitment of T cells to sites of inflammation (9, 39–41).

CXCR3 has been detected recently on a subset of human tonsillar PC of unknown descent and designation (42). We suppose that during their short period of circulation through the blood, CXCR3 and its ligands crucially contribute to directing plasma blasts into inflamed tissues. At least 30% of these cells migrate toward ligands for CXCR3; and therefore, are likely attracted to sites of inflammation while ~60% of circulating plasma blasts migrate toward CXCL12 (Fig. 2) and possibly can migrate into the bone marrow. Whether both receptors are expressed together on an individual cell, or whether CXCR3 and CXCR4 are expressed on distinct plasma blast subpopulations remains to be investigated.

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3.4 Plasmazell-Homöostase in den entzündeten Nieren von NZB/W Mäusen.

Cassese et al., Eur J Immunol 2001. 31: 2726-2732.

Neben unseren Untersuchungen der Regulation der Wanderung und des Überlebens von Plasmazellen im Verlauf von Immunreaktionen gegen Fremdantigen wurde hier auch die Homöostase von Plasmazellen in den an murinem Lupus erkrankten NZB/W Mäusen untersucht [65]. Ähnlich wie beim Menschen geht die Entstehung des systemischen Lupus in diesen Tieren einher mit stark erhöhten gesamt Serum-Antikörpertitern, der Produktion von Anti-Doppelstrang-DNA-Antikörpern und einer daraus resultierenden Nephritis. Wir fanden, dass sich die Anzahl von Plasmazellen im Knochenmark von erkrankten NZB/W Mäusen nicht signifikant von der von nicht erkrankten Tieren unterscheidet. In der Milz dagegen wurde eine ca 50-fach erhöhte Anzahl dieser Zellen nachgewiesen, hierdurch war die Gesamtzahl von Plasmazellen in Milz und Knochenmark von NZB/W Mäusen jeweils etwa gleich. Eine vergleichbar grosse Anzahl von Plasmazellen wie in diesen Organen befand sich auch in der entzündeten Niere, wo normalerweise keine Plasmazellen zu finden sind. Dagegen fanden wir keine Anzeichen für die Akkumulierung von aktivierten B-Zellen in diesem Organ. Nach Immunisierung mit Ovalbumin wurde beobachtet, dass die Ovalbumin-spezifischen Plasmazellen zuerst in der Milz auftraten, später aber sowohl im Knochenmark als auch in der entzündeten Niere akkumulierten und über den gesamten Beobachtungszeitraum von drei Monate dort verblieben. Diese Befunde sprechen dafür, dass Plasmazellen nicht in den Nieren aus aktivierten B-Zellen entstehen, sondern dass in der Milz gebildete Plasmazellen sowohl in das Knochenmark als auch in das entzündete Nierengewebe einwandern. Diese Befunde waren in guter Übereinstimmung mit unseren Ergebnissen die zeigten, dass in der Milz entstandene Ovalbumin-spezifische Plasmablasten zu ansteigenden Konzentrationen des CXCR3 Liganden IP10 wandern und dass dieses Chemokin in der entzündeten Niere von NZB/W Mäusen stark erhöht ausgeprägt wird (siehe 3.3).

Inflamed kidneys of NZB/W mice are a major site for the homeostasis of plasma cells

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(NZB × NZW)F1 (NZB/W) mice develop a disease similar to human systemic lupus erythematosus (SLE), including autoantibody production, hypergammaglobulinaemia and inflammation of the kidneys. It is known that large numbers of lymphocytes infiltrate the kidneys of these mice. Here, we compare the roles of bone marrow, spleen and inflamed kidneys of NZB/W mice in the activation of B cells and the persistence of antibody-secreting cells (ASC). ASC are present in the kidneys of NZB/W mice with full-blown disease, as many as in the spleen and bone marrow. The specificity of the ASC in the inflamed kidneys is not restricted to self-antigens. After immunization of NZB/W mice with ovalbumin (OVA) the OVA-specific ASC are found initially in the spleen. Weeks later, OVA-specific ASC are found in high numbers in the bone marrow and the kidneys of these mice, but no longer in the spleen. As determined by FACS, B cells with a germinal center phenotype ($B220^+/PNA^+$) are found only in very low numbers in the kidneys, but in high numbers in the spleen of NZB/W mice. Germinal centers could not be detected in the kidneys, but in the spleen, and plasma cells appear to be scattered over the tissue. These data suggest that in autoimmune NZB/W mice, plasma cells generated in immune reactions of secondary lymphoid organs, later accumulate and persist in the inflamed kidneys, were they enhance the local concentrations of Ab and immunocomplexes. These experiments identify the inflamed kidneys of NZB/W mice as a site of prime relevance for the homeostasis of plasma cells, irrespective of their specificity.

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1 Introduction

In normal individuals, antibody-secreting cells (ASC) are found exclusively in secondary lymphoid organs, where these cells are formed, and in the bone marrow, where these cells migrate to and persist to produce long-term antibody titers [1–3]. The survival of these cells seems to be tightly regulated by yet unknown survival factors produced by cells present in their microenvironment [4].

In several autoimmune diseases, ASC have been detected in the affected, chronically inflamed organs [5, 6]. Among these ASC are cells secreting autoantibodies [6–8]. Although there is evidence that ASC are generated in inflamed tissue [9], it is not clear whether these tissues in addition provide a site of persistence for immigrated ASC. In the latter case, one would expect that the specificity of the ASC in chronically infected organs is not restricted to self antigens present here. This issue has not been clarified yet.

Here, we analyze activated B cell populations, ASC and follicles in autoimmune NZB/W mice. These mice suffer from a disease which closely resembles human SLE, including autoantibody production, inflammation and nephritis [10]. In the NZB/W model, immunoglobulin G (IgG) with high affinity to double-stranded DNA plays an important role in the pathogenesis of the disease, especially in the destruction of the kidneys [11]. The pathogenic autoantibodies in murine and human SLE are mostly somatically mutated and isotype-switched, sug-

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The first two authors contributed equally to this work.

Abbreviations: ASC: Antibody-secreting cell **PNA:** Peanut agglutinin **NZB/W:** New Zealand Black/White

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gesting that the respective ASC had been selected according to the affinity of their antibodies in germinal centers [12, 13]. At about 3 to 4 months of age NZW/B mice start to express anti-DNA antibodies in the serum and to develop proteinuria. Serum titers increase and proteinuria becomes more severe with time, until a plateau is reached at about 6 months. Although pathogenesis of SLE is not restricted to particular tissues, inflammation of the kidneys is a hallmark of this disease in mice and humans. Here, we show that in autoimmune NZB/W mice the inflamed kidneys themselves are a major site for the survival of immigrated ASC of any specificity.

2 Results and discussion

2.1 ASC are present in kidneys of autoimmune NZB/W mice in equally high numbers as in spleen and bone marrow

The ELISPOT technique was used to detect and quantify IgG-secreting ASC from spleen, bone marrow and kidneys. We compared 5–9-month-old NZB/W females to age-matched, healthy CB20 and BALB/c female control mice. At this age, the NZB/W mice had developed full-blown disease. In the control mice, the bone marrow contained 5–10 times more IgG-secreting ASC than the spleen (Fig. 1). This is in accordance with earlier studies [14–16]. Numbers of ASC in the bone marrow of the NZB/W mice were increased about twofold compared to the controls. The difference between the numbers of ASC in the NZB/W and control mice was much more pronounced in the spleen. In this organ, the autoimmune animals showed about 40 times more ASC than control animals, *i. e.* the same numbers as in bone marrow. It is likely that these high numbers of splenic ASC are due to the autoimmune disease in NZB/W mice. Surprisingly, ASC were detected in the kidneys of NZB/W mice, in numbers comparable to spleen and bone marrow. In control mice, approximately 50 times less ASC are found in the kidneys. This experiment identifies the kidneys of NZB/W mice as a location which is quantitatively relevant for the production of immunoglobulins.

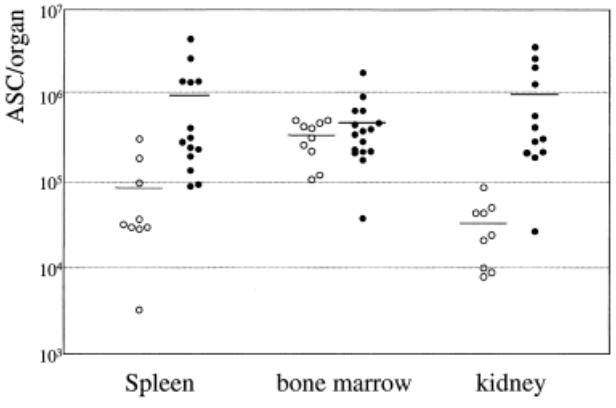

Fig. 1. Numbers of ASC in various organs. Organs from 5–9-month-old and control mice (BALB/c and CB20) were analyzed. ASC were detected by ELISPOT. Each dot (filled circles: NZB/W mice; open circles: control mice) represents one individual animal and horizontal bars represent the mean of each group.

2.2 Germinal center reactions take place in the spleen but not in the kidneys of NZB/W mice

Our data show that besides spleen and bone marrow, inflamed kidneys are a major site of ASC residency in autoimmune NZB/W mice. However, these data do not show whether the ASC in the kidneys have differentiated from B cells within the organ, or whether they had immigrated into the kidneys after differentiation in secondary lymphoid organs. A recent study suggests that B cell activation, ectopic germinal center formation, and plasma cell differentiation can take place in chronically inflamed synovial tissue of rheumatoid arthritis patients [17]. Experimental evidence also has been provided for the recruitment of activated B cells and plasma cell precursors into inflamed tissues [18]. We determined the absolute numbers of $B220^+/PNA^+$ germinal center B cells as a measure for the germinal center activity in the spleen and in the kidneys (Fig. 2). Less than 3×10^5 lymphocytes were counted in the kidneys from individual healthy control mice, likely due to contamination of cells from peripheral blood. Kidneys from NZW/B female mice contained between 10–50 fold more lymphocytes with high frequencies of $B220^+/Thy\text{-}1^-$ B cells. However, the numbers of $B220^+/PNA^+$ germinal center B cells in the kidneys were about 100 times lower than in the spleen, and did not exceed 10^4. Thus, while the numbers of ASC are about equal in spleen and kidneys of NZB/W mice, germinal center B cells are found predominantly if not exclusively in the spleen.

Tissue sections of kidneys were analyzed for the presence of plasma cells and germinal center cells. We used PNA and antibodies specific for B220 (murine CD45R) on consecutive sections to identify germinal center B cells, and antibodies against syndecan-1 (CD138) for the detection of plasma cells [19, 20]. In inflamed kidneys, syndecan-1-positive cells could be detected frequently in the cortex close to the glomeruli, but also in the outer medulla, here mostly in the vicinity of blood vessels (Fig. 3). The syndecan-1-positive cells show the characteristic morphology of mature plasma cells, with a small excentric nucleus and a large extended basophilic cyto-

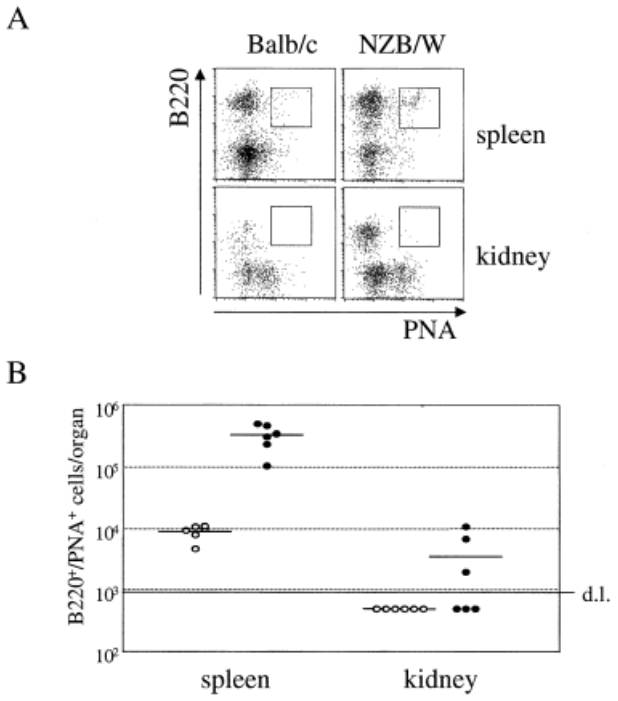

Fig. 2. Numbers of B220$^+$/PNA$^+$ B cells in spleen and kidneys. (A) Single-cell suspensions from kidney and spleen were analyzed for the presence of B220$^+$/PNA$^+$ B cells by FACS. (B) The number of B220$^+$/PNA$^+$ B cells/organ was calculated from the total lymphocyte count and the frequency of these cells in one particular organ. Each dot represents one individual mouse and horizontal bars represent the mean of each group (d. l. detection limit).

plasm. Such cells could not be detected in kidney sections from BALB/c mice (data not shown).

By histology, we could not find any evidence for the formation of germinal centers in the kidneys of NZB/W mice, neither at the age of 3 months when disease is initiated, nor at the age of 5–7 months, when ASC cells are present in high numbers in these organs (Fig. 4). The numbers of germinal center B cells, as determined by cytometric analysis of single-cell suspensions of the entire organ (Fig. 2), are, in all likelihood, an overestimate. The frequencies and absolute numbers of these signals are at the limit of analytical sensitivity of the technology and probably represent background signals.

We can draw the following conclusions from the histological analysis of kidneys. First, this analysis confirmed the ELISPOT analysis (Fig. 1), showing that plasma cells are present in high numbers in the kidneys of NZB/W mice. Second, these plasma cells are scattered throughout the organ. Third, in accordance with the calculated low numbers of germinal center B cells in the kidneys (Fig. 2), B200$^+$/PNA$^+$ cells are virtually absent, and do not form germinal center-like structures.

2.3 ASC specific for the antigen OVA home to and persist in the kidneys of NZB/W mice

The presence of high numbers of ASC and the absence of B cell clusters and germinal centers in the kidneys of NZB/W mice suggest that the ASC found here were formed elsewhere but have migrated into these organs, and persist here like in bone marrow. To test this hypothesis directly we determined the numbers of OVA-specific ASC in spleen, kidney and bone marrow, after secondary immunization with OVA (Fig. 5). NZB/W and BALB/c mice, 4–5 months of age, were immunized and boosted with OVA. In the spleens of BALB/c and NZB/W mice, about 10^5 OVA-specific ASC appeared within 4 days of the secondary immunization. At this time point, only about 10^4 OVA-specific ASC could be detected by ELISPOT assay in the kidneys of both, BALB/c and NZB/W mice. The presence of OVA-specific ASC in the kidneys of control mice was unexpected. At this time, it is not clear, whether these few ASC were derived from kidney blood or tissue, *i. e.* whether they reflect a general capacity of kidneys to harbor plasma cells. The high numbers of OVA-specific ASC in the spleen compared to bone marrow and kidney of BALB/c and NZB/W mice, early after immunization, strongly suggests that the generation of OVA-specific plasma cells initially occurs in the spleen, as expected, and to a lesser extent or not at all in the kidneys, irrespective of whether the kidneys are inflamed or not. In accordance with earlier results [2, 15], 30–50 days after secondary immunization, in the BALB/c mice most OVA-specific ASC are found in the bone marrow and no longer in the spleen. At this time, 30 (data not shown) and 50 days (Fig. 5) after secondary immunization, several hundred thousand OVA-specific ASC are detectable in the bone marrow of NZB/W as well as BALB/c mice, suggesting that long-lived plasma cells home to the bone marrow of NZB/W mice, and survive there for long time periods, as has been shown for normal mice. In addition, however, at this time point, several hundred thousands OVA-specific ASC are also found in the kidneys of the NZB/W mice, i.e. about the same number as in the bone marrow. These OVA-specific ASC were not detectable in the kidneys of control mice. Apparently, ASC can accumulate and persist in the inflamed kidneys of NZB/W mice, like in the bone marrow, but unlike in normal kidneys. This accumulation is not restricted to self antigens and is independent of the specificity of the plasma cells.

It is interesting to note the high numbers of total ASC (Fig. 1) and germinal center B cells (Fig. 2) in the spleens

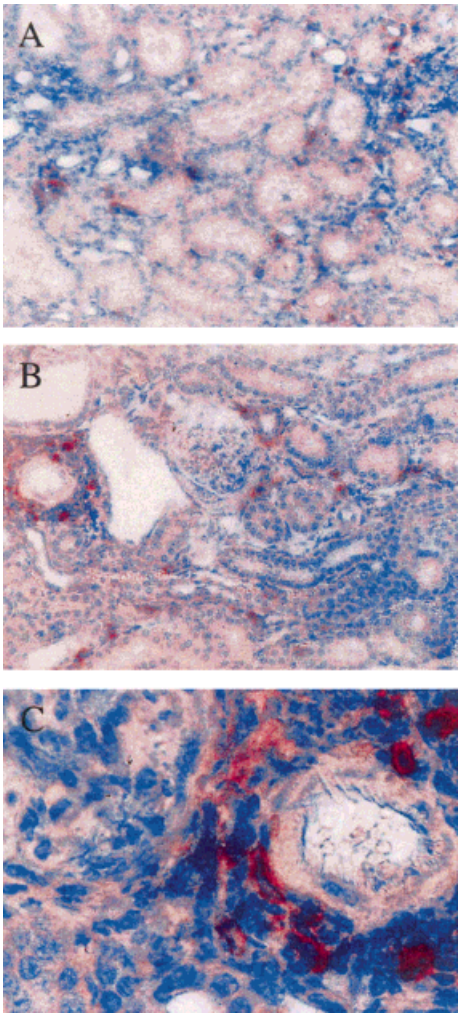

Fig. 3. Histological analysis of renal plasma cell infiltrates. Sections were stained with antibodies against syndecan-1 (CD138) (red) for plasma cells and hematoxilin (blue). Renal plasma cell infiltrates were found in the medulla (A) as well as in the cortex (B, C) of autoimmune NZB/W but could not be detected in control mice (not shown). The morphology of syndecan-positive cells resembles that of mature plasma cells with an excentric nucleus and enlarged cytoplasm (C). Original magnifications: A, B: ×80; C: ×250.

of NZB/W mice. OVA-specific ASC are also present in the spleen early after secondary immunization, while they are virtually absent 50 days later (Fig. 5). This suggests that ASC specific for a particular antigen are formed in NZB/W mice similarly as in healthy individuals in one wave within a few days. Thus, the continuous presence of ASC in the spleen of the autoimmune mice is probably due to the continuous formation of ASC specific for various and persisting antigens.

It has been shown in animal models and patients with various autoimmune diseases, that plasma cells

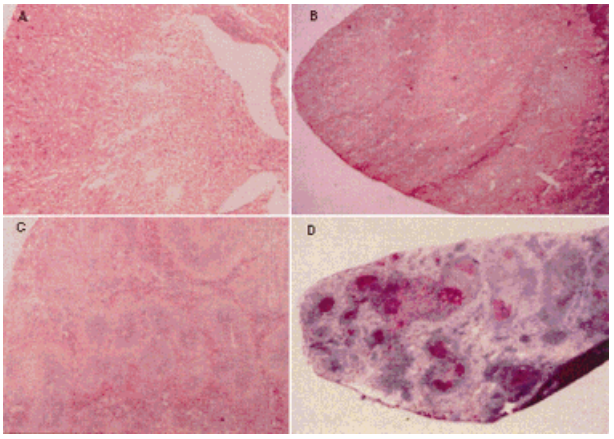

Fig. 4. Histological analysis of lymphoid follicles. Sections from 3–6 month-old NZB/W mice were stained with hematoxilin (A, C, original magnification ×80), or with PNA (B, D, original magnification ×25) as described in Sect. 3. Kidney (A, B) and spleen (C, D) sections shown are representative for organs from 20 mice.

in chronically inflamed tissues can produce antibodies specific for self-antigens [21–23]. The results presented here clearly demonstrate that the ASC in the kidneys of NZB/W mice are not restricted to self-antigens.

Our results strongly suggest that during the chronic phase of the autoimmune disease in NZB/W mice, plasma cells home to and persist equally well in the bone marrow and the inflamed kidneys, while they do not persist in the kidneys of healthy control mice. In established disease of NZB/W mice, these plasma cells are not generated in the kidneys but rather seem to be immigrants from secondary lymphoid organs and react to arbitrary antigens. Similar, the migration of ASC cells to and the persistence of these cells in inflamed tissue has been reported recently by Kreen and coworkers in inflamed synovial tissue of patients suffering from osteoarthritis [24], suggesting that the persistence of ASC in inflamed tissues might be a general phenomenon. The number of ASC in an individual is restricted by the availability of survival niches for these cells [4]. The ASC-specific survival factors produced in these niches are not yet identified. Our data suggest that such factors are produced in chronically inflamed tissues. Indeed, experiments in our laboratory show that the survival of mature, non-malignant plasma cells isolated from bone marrow can be prolonged by pro-inflammatory cytokines, like by IL-6 and TNF-α (Cassese et al. Manuscript in preparation).

The involvement of kidney plasma cells in the pathogenesis of the disease remains to be elucidated. Clearly, due to their high numbers, the renal plasma cell infiltrates in NZB/W mice contribute to the development of hyper-

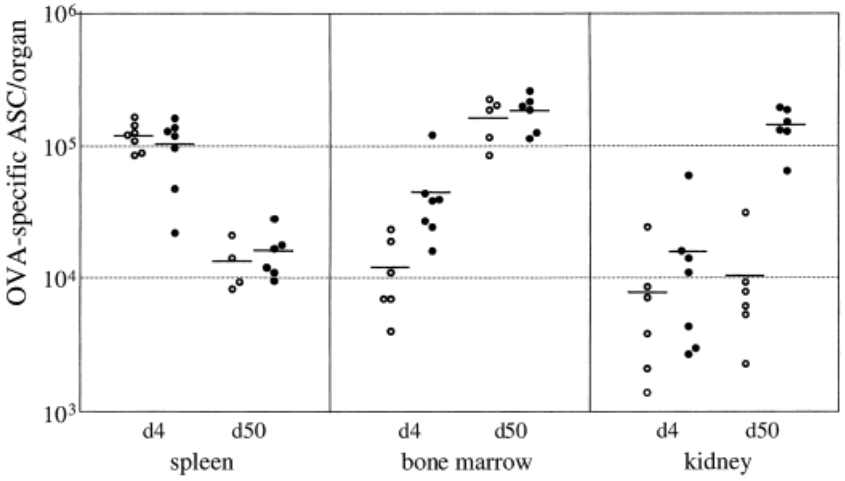

Fig. 5. OVA-specific ASC appear in the kidneys from NZB/W mice with the same kinetics as in bone marrow. Mice were primed with OVA and boosted 3 weeks later (day 0 of analysis). Numbers of OVA-specific ASC per organ were determined by ELISPOT 4 days and 50 days after the boost immunization. Each dot (closed circles: NZB/W mice; open circles: control mice) represents one individual animal and horizontal bars represent the mean of each group.

gammaglobulinemia. Probably, they also contribute to the production of autoantibodies in these animals, as it has been shown for plasma cells located in chronically inflamed tissues of other autoimmune diseases, *e. g.* in BALB/c mice immunized with heparan sulfate, another animal model for SLE [23]. Indeed they may enhance the local concentrations of antibodies and immuncomplexes which finally lead to the observed severe destruction of the kidneys.

3 Materials and methods

3.1 Mice and immunization

NZW and NZB breeder pairs were obtained from Harlan Winkelmann (D-33176 Borchen, Germany). NZB/W, CB20 and BALB/c mice were bred at the animal facility of the Deutsches Rheuma Forschungszentrum Berlin. Mice were analyzed between 5 and 9 months of age when disease had already started in NZB/W mice. The mice were immunized by injection of 100 mg alum-precipitated OVA per mouse and boosted 3–4 weeks later by intravenous injection of 100 mg OVA in PBS.

3.2 Histology

Organs were fixed in formalin and embedded in paraffin to be stained with hematoxilin. To stain for B cells, plasma cells and germinal centers, frozen tissue sections were stained with anti-B220 (RA3-6B8), anti-syndecan-1 (Pharmingen) or biotinylated PNA (Vector). The B220 and syndecan-1 stainings were then incubated with biotinylated mouse anti-rat IgG (Jackson). PNA stainings were performed on frozen tissue sections (liquid nitrogen for 10 min). All combinations were finally labeled with ExtrAvidin"-alkaline phosphatase (Sigma). The red color was developed using sigma-fast red^{tm} (Sigma). All sections were counterstained with hematoxilin.

3.3 Quantification of ASC by ELISPOT

Spleen, bone marrow and kidney single-cell suspensions were filtered through a Falcon cell strainer (70 mm), washed and resuspended in RPMI 1640 (Gibco, Germany) supplemented with 5% fetal calf serum (Gibco), penicillin, streptomicin and β-mercaptoethanol (complete culture medium). Remaining particles were removed by sedimentation (1 min). Single IgG-secreting cells were detected by a modification of the ELISPOT technique [25]. Ninety-six-well flat-bottom plates (high binding, Costar) were coated overnight at 50 ml per well with 5 μg/ml goat anti-mouse IgG (Biozol) in PBS. The plates were then blocked with PBS containing 3% BSA (Biomol, Hamburg, Germany) for 1 h at room temperature. Subsequently the blocking buffer was replaced by different cell dilutions in complete culture medium, incubated for 2–4 h at 37°C in a humid atmosphere with 5% CO_2. Between all steps, plates were flicked and washed 3–6 times with PBS/3% BSA and PBS/3% BSA/0.01% TWEEN before and after the incubation with cells respectively. Plates were incubated with 1 μg/ml biotin-labeled goat anti-mouse IgG (Biozol) in PBS/3% BSA/0.01% Tween for 30 min at room temperature. Subsequently, 50 μl avidin-alkaline phosphatase (Sigma) was added. After 30 min at room temperature, detection was carried out by addition of 5-bromo-

4-chloro-3-indolyl phosphatate (BCIP, Sigma) substrate. The substrate was prepared by dissolving of 1 mg BCIP per 1 ml 2-AMP buffer (95 ml M 2-amino-2methyl-1-propanol, 0.1 ml Triton X-405, 150 mg/ml $MgCl_2$, 900 ml water, pH 10.25 adjusted with HCl) and mixing of this solution with 0.2 ml 3% agarose (type I, low EEO, Sigma). The mixture was kept at 65 °C for 30 min. After addition of the substrate, plates were cooled down to room temperature to allow solidification of the agarose. After 2 h at 37°C spots were enumerated under the microscope. The specificity of the ELISPOT assay was tested by inhibition of spot formation during the secretion period with 1% azide, resulting in a reduction in spot formation of 70–90%. Absolute numbers of ASC were calculated by the number of spots/well multiplied by the dilution factor of the cell suspension. Since both femurs together contain about 12.6% of total bone marrow cells [26], the numbers of ASC detected in the two femurs were multiplied by a coefficient of 7.9 to extrapolate the absolute numbers of ASC in total bone marrow.

3.4 Antibodies

All antibodies used for the ELISPOT method and to stain tissue sections are described above. To characterize lymphocytes in spleen and kidney cell suspensions the following reagents were used: monoclonal antibodies RA3.6B2 (anti B220) and HO13.4.9 (anti Thy-1) were purified from hybridoma supernatants and coupled to Cy5 and R-phycoerythrin respectively. PNA-fluorescein-iso-thiocyanat was purchased from Vector.

3.5 Cytometric analysis and quantification of $B220^+/PNA^+$ cells

Single-cell suspensions were prepared from spleen and kidneys. Large particles were removed by sedimentation. Cells were washed twice in PBS/BSA and total cell numbers determined in a Neubauer chamber. Cells were stained by immunofluorescence for B220 and PNA binding. In some experiments, cells were also stained for Thy-1. Immunofluorescence and cytometric analysis were essentially performed as described [27]. Briefly, staining was performed in PBS/BSA for 10 min on ice, using titrated concentrations of the reagents. Cells were analyzed on a FACSCalibur (Becton Dickinson Immunocytometry Systems; San Jose). Dead cells were excluded from the analysis by propidium iodide. Forward and side scatter was used to exclude red blood cells and debris. Frequencies of $B220^+/PNA^+$ cells were calculated with the cell quest program (Becton Dickinson Immunocytometry Systems). Absolute numbers of $B220^+/PNA^+$ organ were calculated on the basis of their frequency determined by FACS and the number of total cells/organ.

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3.5 Schwach CD38 ausprägende IgG-sezernierende Zellen sind die Vorläufer verschiedener Plasmazell-Populationen

Arce et al., J Leucocyte biology, In Press (erscheint voraussichtlich im Juni 2004).

Unsere Untersuchungen protektiver und autoreaktiver Immunreaktionen in verschiedenen Mausmodellen führten zur Identifizierung von Molekülen, die wesentlich an der Differenzierung, Migration und Regulation der Lebensspanne von Plasmazellen beteiligt sind. Mit dieser Arbeit sollte nun begonnen werden, die Homöostase von Plasmazellen im Menschen zu untersuchen. Die Methode der "Zellulären Affinitätsmatrix" [66]wurde angewandt um humane IgG-sezernierende Zellen, d.h. Plasmazellen und Plasmablasten *ex vivo* und *in vitro* durchflusszytometrisch zu identifizieren und ihren Phänotyp zu bestimmen. Untersucht wurden Zellen (I) aus der Tonsille, einem sekundären lymphatischen Organ, in dem die Plasmazelldifferenzierung eingeleitet wird; (II) dem Blut, durch das direkte Plasmazell-Vorläufer von sekundären lymphatischen Organen in das Knochenmark oder entzündete Gewebe wandern; und (III) im Knochenmark, in dem langlebige Plasmazellen zu finden sind. Verschiedene deutlich abgrenzbare Subpopulationen IgG-sezernierender Zellen wurden identifiziert. Sie unterscheiden sich in der Ausprägung von CD9, CD19, und CD38. Nur wenig CD38 ausprägende IgG-sezernierende Zellen fanden sich ausschliesslich in der Tonsille. Ihr weiterer Immunphänotyp, ihr frühes Erscheinen Kulturen aktivierter B-Zellen sowie die histologisch ermittelte Lokalisation dieser Zellen in Follikeln, zeigt, dass es sich hierbei um sehr frühe Stadien der Plasmazelldifferenzierung handelt. Im Blut wurden keine deutlich abgrenzbaren Subpopulationen IgG-sezernierender Zellen identifiziert, was darauf hindeutet, dass die verschiedenen im Knochenmark gefundenen Plasmazell-Differenzierungsstadien von einem gemeinsamen migratorischen Vorläufer abstammen. Diese Arbeit schuf die Grundlage für eine eingehendere Untersuchung der Plasmazell-Homöostase im Menschen. Die Befunde aus diesem Teil der Arbeit sind in Übereinstimmung mit einer stufenweisen Differenzierung humaner Plasmazellen beginnend mit einer schwach CD38 ausprägenden Zelle.

CD38-low IgG-secreting cells are precursors of various CD38-high expressing plasma cell populations

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Despite the important role immunoglobulin (Ig)G-secreting plasma cells play in memory immune responses, the differentiation and homeostasis of these cells is not completely understood.

Here, we studied the differentiation of human IgG-secreting cells *ex vivo* and *in vitro*, identifying these cells by the cellular affinity matrix technology. Several subpopulations of IgG-secreting cells were identified among the cells isolated from tonsils and bone marrow, particularly differing in the expression levels of CD9, CD19, and CD38. CD38-low IgG-secreting cells were present exclusively in the tonsils. A major fraction of these cells appeared to be early plasma cell precursors since (I), upon activation of B cells *in vitro*, IgG-secretion preceded up-regulation of CD38 and (II), on tonsillar sections IgG-containing, CD38-low cells with a plasmacytoid phenotype were found in follicles, were plasma cell differentiation starts. A unitary phenotype of migratory peripheral blood IgG-secreting cells suggests that all bone marrow plasma cell populations share a common precursor cell. These data are compatible with a multi-step model for plasma cell differentiation and implies that a common CD38-low IgG-secreting precursor gives rise to a diverse plasma cell compartment.

Introduction

Eventually, activated B cells can differentiate into non-proliferating mature plasma cells. Directed by chemokines [1, 2] early plasma cells can leave the secondary lymphoid organs to migrate into the bone marrow [3], or into inflamed tissues [4]. While most plasma cells found in secondary lympoid tissue are short-lived, bone marrow plasma cells can live for extended periods of time [5] with an estimated half-live of about 6 months in mice [6].

Human plasma cell differentiation has been studied extensively in vitro [7-9] and on plasma cells isolated from blood [10, 11]. The studies on normal plasma cells isolated from tonsils, blood and bone marrow indicate that these tissues contain plasma cells expressing high levels of CD38 but heterogeneous levels of other surface molecules [12, 13]. While early plasma cells and their precursors are found in tonsils, more mature plasma cells are present in the bone marrow possibly reflecting the different functions of these tissues in inducing and maintaining plasma cell responses, respectively. IgG is the dominant Ig isotype secreted by these cells in T-dependent immune responses [14]. It has been indicated that most IgG-secreting cells present in the tonsils are proliferating cells (B cell/plasma blasts), while IgG-secreting cells in the bone marrow are non-proliferating cells (plasma cells) [12]. However, the exact relationship between phenotypically distinct plasma cell populations is not clear yet.

Here, we used the cellular affinity matrix technology, to identify human IgG-secreting cells, i.e. plasma cells and their immediate precursors. This technology had been initially applied to identify murine antibody-secreting plasma cells and cytokine secreting T-cells [15]. We identified subpopulations of IgG-secreting cells, including a CD38-low plasma cell precursor population present in tonsillar germinal centers.

Materials and Methods

Tissues and lymphocyte isolation. Peripheral blood mononuclear cells (PBMC) were isolated from buffy coats by Ficoll-Hypaque (Pharmacia, Piscataway, NJ) density-gradient centrifugation [16]. Tonsils and bone marrow were obtained from patients undergoing tonsillectomy and surgical removal of the head of the femur, respectively. After cutting the tonsils and bone into pieces, tissue cells were washed out by vigorous pipeting with PBS/0.5%BSA and filtered through a cell strainer (Becton Dickinson, USA). Erythrocytes were lysed with ammonium chloride. All samples were taken with the patient's consent. To minimize background staining, PBMC were depleted of CD3, CD14 and CD16-positive cells prior to the staining for IgG. The depletion was achieved

by using direct-microbeads to these antigens and a type-CS column as indicated by the manufacturer (Miltenyi Biotech, Germany). To analyze IgG-secreting cells from tonsils and bone marrow no depletion step was necessary.

Cellular affinity matrix technology. Single cell suspensions were biotinylated with 0.5 mg of sulfo-NHS-LC-biotin (Pierce, Rockford, IL, USA) in 1 ml of PBS (37°C, for 10 min), then the same volume of RPMI 10% FCS was added and cells were incubated for another 10 min. After 3 washing steps with PBS/0.5%BSA, cells were incubated with a Cy5 coupled anti-human IgG (Pharmingen, BD 10 µg/ml, 15 min on ice). Subsequently, the cells were washed and unconjugated anti-human lambda (clone HP6054, ATCC, 50 µg/ml, 0.2 ml, 4°C) was added, 5 min later, 50 µg/ml of HP6054 conjugated to avidin (catching antibody), was added. After 10 min incubation on ice, cells were resuspended in 2 ml RPMI/10% FCS at a final concentration of 3 million cells/ml and incubated for 30 min at 37°C. Under these conditions, Ig were secreted and those bearing lambda light chains were captured on the surface of the Ig-secreting cells. Thereafter, the cells were cooled on ice for 10 min and 5 µg/ml of digoxigenized (DIG)-anti human IgG conjugate was added (Pharmingen, BD). After washing, cells were stained with a titrated concentration of anti-DIG-phycoerythrine (PE). To allow discrimination between membrane bound and secreted and captured IgG, cells were stained for surface IgG prior to capturing the secreted IgG.

Monoclonal antibodies and FACS analysis. The following fluoresceine-labeled antibodies were used for phenotyping: CD3, CD5, CD14, CD16, CD19, CD20, CD22, CD25, CD27, CD28, CD35, CD40, CD44, CD45, CD49d, CD49e, CD70, CD71, CD72, CD86, CD95, CD100, CD154, HLA-DR (Pharmingen, BD), CD9, CD38 (Immunotech, Coulter, FL, USA) and CD10, CD138 (AMS Biotechnology GmbH, Germany). A FACScalibur (BD) flow cytometer was used for analysis. Propidium iodide (1 µg/ml) was used to exclude dead cells according to uptake of the dye. For intracellular staining, cells were fixed in 2% formaldehyde and incubated with anti-IgG-fluoresceine (FITC) in PBS/BSA containing 0.5% saponin.

In vitro stimulation of PBMC. CD38/CD138-depleted PBMC were labeled with CFSE (Molecular probes, Eugene, OR) as described [17] and subsequently stimulated with 1µg/ml of tetanus toxoid [18]. To deplete CD38/CD138-positive cells, PBMC were labeled with CD38-PE (Pharmingen, BD) and thereafter with anti-PE and CD138 microbeads. Cells were depleted by MACS (Miltenyi Biotech, Germany). The depleted fraction did not contain any detectable IgG-secreting plasma cell as tested by the IgG-secretion assay. CD38-high cells were also not detectable by FACS analysis.

Histology. Freshly ectomized human tonsils were cut in convenient pieces of approximately 300 mm^3 and embedded in Tissue-Tek® Cryomold® Intermediate. Seven µm cryosections were performed, and acetone fixed. Before specific antibody staining, unspecific binding sites were blocked with PBS/2% BSA for 20 min at RT. Sections were stained with FITC labeled mouse anti-human CD38 monoclonal antibody (DAKO, clone AT13/5) and biotinylated mouse anti-human IgG monoclonal antibody (BD Pharmingen, clone G18-145), respectively. After washing, sections were incubated with rhodamin-labeled streptavidin. Nuclei were counterstained with DAPI.

PCR. Cells were sorted directly into PCR tubes by FACS. PCR was performed for beta actin and Blimp-1 (primers: Blimp-1: 5'-TCGGGTCGTTTACCCCATC-3'
and 5'-CACAGCGCTCAGGCCATTA-3'). Reactions were annealed at 55°C and amplified for 35 cycles.

Results

In order to detect all IgG-producing plasma cell and plasma cell precursor populations, including those possibly not expressing commonly used markers, we used the cellular affinity matrix technology [15]. This technique allowed us to identify cells secreting Ig of the IgG isotype. Single cell suspensions from tonsils, blood and bone marrow were analyzed for the presence of IgG-secreting cells. In all samples including the controls without catch-ab, a population of "background"-stained cells appeared which was excluded from the further analysis by using the gate as shown in Fig. 1 A. The population that was brightly stained for secreted IgG, but was absent in control samples lacking catch-ab was considered to be plasma cells because all these cells exhibited intense staining for cytoplasmic IgG (Fig. 1 B). Following isolation by fluorescence-activated cell sorting (FACS), the capacity of these cells to secrete IgG was confirmed by ELISPOT technique [19].

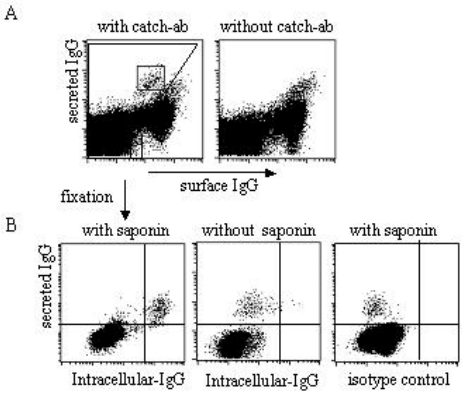

Figure 1. Detection of IgG-secreting cells. Bone marrow, blood or tonsil derived single cell suspensions (here, cells from bone marrow are shown) were stained for surface and secreted IgG as described in the text. (A) Left dot plot: cells were stained for surface and subsequently for secreted IgG as described in the materials and methods section. Right dot plot: negative control treated with all staining reagents but lacking catch-ab. The IgG-secreting cell population (left plot) is indicated by the square. (B) Cells stained for surface and secreted IgG as shown in A, were fixed and stained for intracellular IgG. Saponin was used to allow the staining ab to penetrate the cell membrane. Non-secreting/surface IgG^+ cells were excluded from the analysis by gating (gate shown in A, left plot). The cells stained for secreted IgG were specifically labeled for intracellular IgG (left), but neither for an isotype control (right), nor for IgG without permeabilization of the cell membrane (middle). Dead cells were excluded from the analysis by PI staining. About 2 x 10^5 cells are shown in each dot plot. Representative data of of 12 independend experiments are shown.

Spots were formed by more than 90 % of cells stained for secreted-IgG (data not shown). Thus, the cells identified by the cellular affinity matrix technology were IgG-secreting plasma cells or immediate plasma cell precursors. In healthy individuals, the average frequencies of these cells were about 0.47± 0,2 %, 0.01± 0,1% and 0.2± 0,1% (n ≥ 8 for all tissues) among the nucleated cells in tonsils, peripheral blood and in bone marrow, respectively. While frequencies in tonsils and bone marrow showed little variation between different donors, frequencies of blood IgG-secreting cells could reach up to 0.1% in a few samples. The expression of various surface markers on IgG-secreting cells was analyzed (Fig. 2).

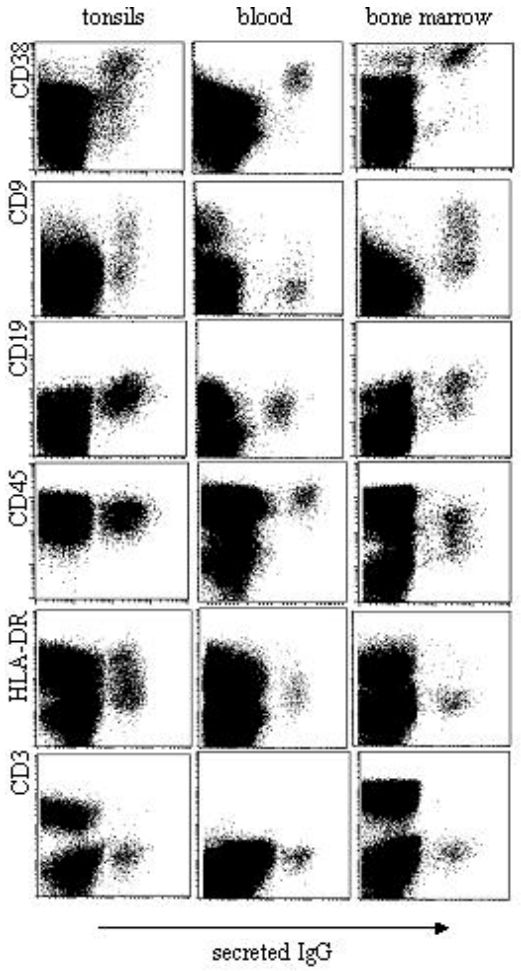

Figure 2. Identification of IgG-secreting plasma cell subpopulations. Single cell suspensions from tonsils (left columns), blood (middle column) and bone marrow (right columns) were stained for surface and secreted IgG and counterstained for the markers indicated. Dead cells were excluded from the analysis by PI staining. Non- secreting/surface IgG^+ cells were excluded from the analysis by gating (shown in Fig. 1 A, left plot). About 2 x 10^5 cells are shown in each dot plot. Representative data of 6 independent experiments are shown.

A substantial fraction of the tonsillar IgG-secreting cells expressed HLA-DR and thus can be considered to be early plasma cells [20, 21]. CD38 was expressed at high levels on all IgG-secreting cells derived from blood and bone marrow. However, an additional IgG-secreting cell population was detected in the tonsils, expressing only low levels of CD38. A third population of IgG-secreting cells, present in only very low numbers, expressed even less CD38.

Heterogeneity within the CD38-high plasma cell compartment had been described [13]. However, IgG-secreting cell populations expressing no or low levels of CD38 have been missed in earlier analyzes of tissue samples because these studies had been restricted to CD38-high cells. Recently and in accordance with our findings, it had been demonstrated that stimulation of memory B cells in culture could result in the formation of CD38-low IgG-secreting cells [22].

To further characterize IgG-secreting subpopulations, as a measurement for cell size, we compared the mean forward scatter profile of these cells to that of B cells (Table 1). Tonsilar CD38-neg/very low IgG-secreting cells had about the same forward scatter profile than B cells, i.e. about 245 linear units. Both, the CD38-low and CD38-high population showed an increased scatter profile with 302 and 317 linear units, respectively. The differences in scatter between all three populations were statistically significant ($p < 0.05$). These values were still below those measured for IgG-secreting cell populations isolated from blood or bone marrow. Since an increase in cell size is probably associated with an increase in plasma cell maturation [23], these results already suggested that the relatively small, CD38-low antibody-secreting cell were early plasma cell precursors.

Table 1. Mean Forward Light Scatter analysis [a]

Tissue	cell type	mean FSC
Tonsils	B cells	242 ±13
	IgG-sec cells:	
	CD38 negative	246 ±10
	CD38 intermediate	302 ± 3
	CD38 high	317 ± 5
Blood	B cells	266 ± 29
	IgG-sec cells	376 ± 36
Bone Marrow	B cells	279 ± 4
	IgG-sec cells:	
	CD19 low	373 ± 5
	CD19 high	439 ± 24

a) Mean forward light scatter (FSC) of various cell populations from the indicated tissues are shown (n ≥ 6). Mixtures of cells of at least 3 donors were used in each experiment. Each value represents the average of 3 independent experiments. Differences observed in the forward scatter profile between CD38 intermediate and CD38 high IgG-secreting cells are statistically significant (t test, p < o.o5).

To further test whether CD38-low IgG-secreting cells resemble a temporary stage of the early plasma cell development rather than a distinct plasma cell subpopulation, we analyzed the expression of this marker upon stimulation of B cells in vitro. PBMCs were depleted of plasma cells and activated B cells by MACS using CD38 and CD138 magnetic beads. The remaining cells, consisting mainly of resting B cells (memory and naive),

were activated by tetanus toxoid as described [18]. When IgG-secreting cells first appeared at day 3 in culture, these cells expressed no or only low levels of CD38 (Fig. 3 A). Later, at day 6, all IgG-secreting cell had up-regulated CD38 to high levels. During the same period of time, the frequencies of these cells increased from 0.2 to 1.8 %. The staining for secreted-IgG on individual cells also increased between day 3 and day 6 in culture suggesting an increase in IgG-secretion and level of maturation. Labeling of the cells with carboxyfluorescein diacetate succinimidyl ester (CFSE) allowed the analysis of their proliferation profile [17].

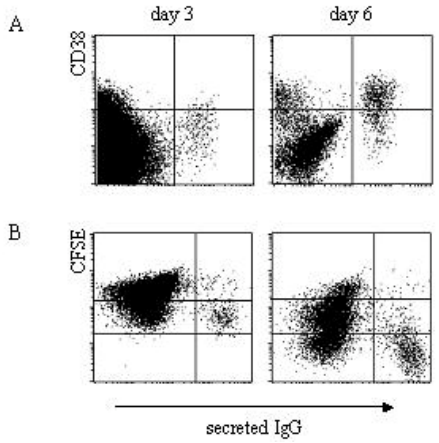

Figure 3. Plasma cell differentiation in vitro. (A) PBMC were depleted of plasma cells and stimulated with tetanus toxoid. IgG-secreting cells were identified by the cellular affinity matrix technology as described in the text and figure 1. At the time points indicated, the IgG-secreting cells were stained for CD38. (B) PBMC were labeled with CFSE and cultures were initiated as described in A. Cells were analyzed at the indicated time points. Dead cells were excluded from the analysis by PI staining. Representative data of 3 independent experiments are shown.

Already at day 3, the early appearing IgG-secreting cells had lost some CFSE, indicating that proliferation precedes differentiation into IgG-secreting cells (Fig. 3 B). Until day 6, the IgG-secreting cells underwent further rounds of proliferation indicated by a further decrease in CFSE labeling. These data support the idea that plasma cell differentiation starts already at a CD38-negative stage.

CD9 is expressed at high levels on murine plasma cells in spleen [24]. Expression of this marker was also characteristic for both, the tonsilar IgG-secreting CD38-high and CD38-intermediate populations described here (data not shown). Based on this result, we aimed in identifying plasma cells and their precursors by staining tonsilar cells for CD9 together with CD19. This marker combination stains for less than 3% tonsilar CD19-positive B cells, all of them expressing CD38 either at high or intermediate levels (Fig 4 A). All CD38-intermediate CD9 positive tonsilar B cells express CD27 at intermediate levels while CD38-high CD9-positive plasma cells express CD27 at high levels. CD138, a marker for plasma cells, was expressed on a subfraction of 8 % of CD38-intermediate CD9-positive tonsilar B cells and on 59 % CD38-high CD9-positive plasma cells. As determined by PCR, the transcription factor blimp-1, which is specifically up-regulated during plasma cell differentiation and not expressed on mature B cells, was also expressed in both populations (Fig. 4 B).

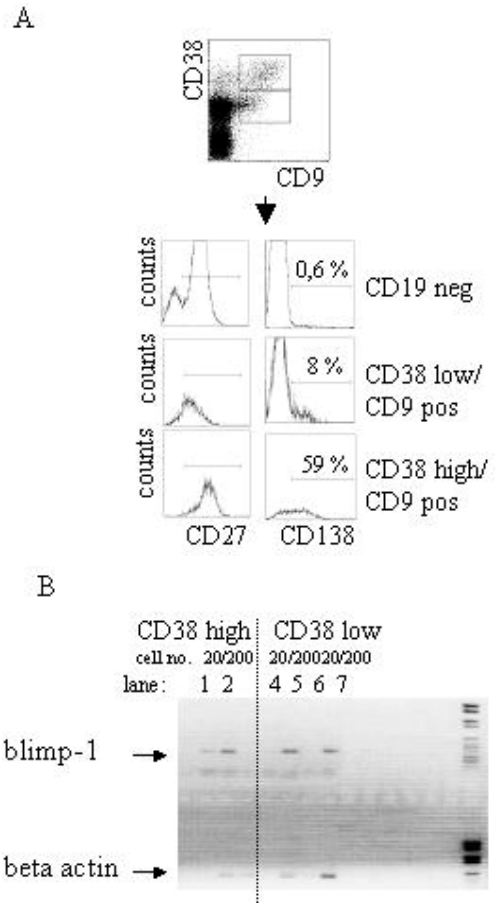

Figure 4. Phenotype of CD38-intermediate CD9/CD19-positive tonsilar B cells. (A) Tonsillar cells were stained for CD9, CD19 and CD38 together with either CD27 or CD138. CD19-negative cells were excluded from the analysis. (B) Tonsilar cell populations were sorted by FACS and PCR was performed on the indicated number of cells. Lane 4/5 and 6/7 represent probes obtained from two different donors analysed in one experiment. Data shown are representative data of 4 independent experiments.

Thus, with respect to the expression of CD27, CD138 and blimp-1, the population of CD38-intermediate, CD9-positive B cells resemble intermediates between B cells and plasma cells. In accordance with the identification of a CD38-intermediate

IgG-secreting cells this results shows that tonsilar plasma cell differentiation starts before CD38 is expressed at high levels.

The enlarged cytoplasm of IgG-secreting cells containing high amounts of Ig allows the identification of these cells on tissue sections. Localization of CD38-high cells and IgG-expression were compared on tonsilar sections by immunofluorescence staining (Fig. 5). In accord with previous studies [25], the vast majority of CD38-high plasma cells in the tonsils were found in extrafollicular regions. Only a few were detected within the follicles. The majority of IgG-containing cells were also found in the extrafollicular regions. Additionally, IgG-containing cells were found in the light zone of some, but not all germinal centers. These cells were co-located with interstitial IgG.

As shown by double staining for IgG (rhodamin, red) and CD38 (FITC, green), the majority of cells containing large quantities of IgG located inside the follicles were not brightly stained for CD38 (Fig. 5). In contrast, the IgG-containing cells found in a ring adjacent to the follicular areas express high levels of CD38, as indicated by the yellow color resulting of an overlay of the green staining for CD38 and the IgG staining in red. Although differing in their levels of CD38 expression, IgG-containing cells inside and outside the follicular areas exhibited a plasmacytoid phenotype, suggesting that both cell types resemble plasma blasts or plasma cells.

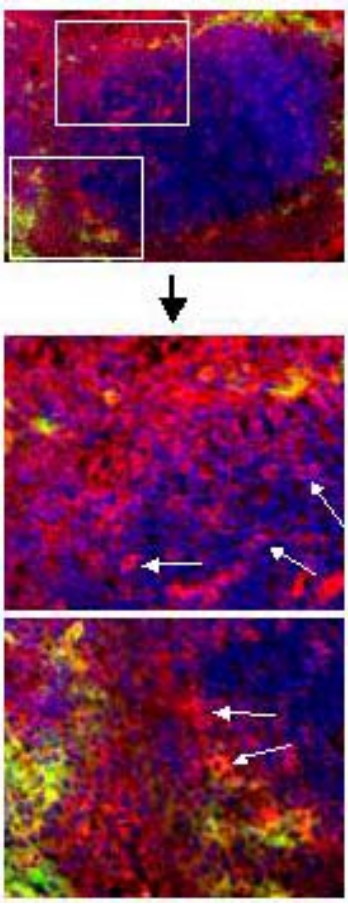

Figure 5. CD38 and IgG staining on tonsillar sections. Cryosections were stained with mouse anti-human monoclonal antibodies for CD38 (FITC, green) and IgG (rhodamin, red), nuclei were stained with DAPI (blue). Cells expressing high levels of CD38 together with IgG appear in yellow.

Co-localization of interstitial IgG and CD38-low IgG-containing cells indicates that these cells already had started to secrete IgG and thus were committed to plasma cell differentiation. This result is in accord with the observation that Blimp-1, a master factor for plasma cell differentiation, is expressed in a fraction of about 4-15% of germinal center B cells [26].

We further characterized the phenotype of blood IgG-secreting cells in donors not recently vaccinated. In contrast to the observed heterogeneity within the tonsilar and bone marrow IgG-secreting cell compartment, IgG-secreting cells in the blood were homogenous with respect to the expression of most markers analyzed (Table 2). The phenotype of steady state peripheral blood IgG-secreting cells observed here does not differ from that described for blood plasma cells induced by immunization or that described for peripheral blood plasma cells in SLE patients [10, 13, 27].

Table 2. Phenotype of peripheral blood Ig secreting cells [a)]

Antibody	Intensity of staining
CD3	-
CD5	-
CD9	-
CD10	-
CD14	-
CD16	-
CD19	+
CD20	-
CD21	-
CD22	-
CD25	-
CD27	++
CD28	-
CD35	+/-
CD38	++++
CD40	+
CD44	++++
CD45	+++
CD49d	+
CD49e	-
CD70	+/-
CD71	++
CD72	-
CD86	+
CD95	++
CD100	-
CD138	+/-
CD154	-
HLA-DR	+/-
surf-IgG	+

a) Single cell suspensions were stained for surface and secreted IgG and counterstained for the markers indicated ($n \geq 6$). Dead cells were excluded from the analysis by PI staining.

Discussion

Here, for the first time, we used the cellular affinity matrix technology, which allows the direct identification of antibody

secreting cells, to study human plasma cells and their immediate precursors, i.e. plasma blasts, in tonsil, blood and bone marrow samples. In secondary lymphoid tissues, e.g. tonsils, B cells are activated to form plasma cells. We show that the differentiation of IgG-secreting cells in the tonsils starts before CD38 is expressed. IgG-secreting cells expressing only little CD38 are present in human tonsils and early in B cells cultures, where these cells precede IgG-secreting cells with a CD38-high phenotype. As shown on tissue sections, cells containing high quantities of IgG in their enlarged cytoplasm and expressing only little CD38 are located in tonsillar follicles. In contrast, CD38-high IgG-containing plasma cells are mainly present outside the follicular areas. The early IgG-secreting plasma cell precursors seem to be present particularly in the light zone of germinal centers. This idea is in accord with the finding that in mice, antigen-specific cells with a plasmacytoid phenotype are present in splenic follicles early following immunization, i.e. within 36-60 hours [28, 29]. During their course of differentiation, CD38-low plasma cell precursors possibly leave the follicles and increase the expression of CD38. A few CD38-high plasma cells were detected in the follicles, possibly indicating that up-regulation of CD38 precedes their translocation into the extrafollicular areas. The presence of IgG in the interstitium adjacent to the IgG-containing CD38-low plasma cell precursors in the tonsillar light zone led us speculate that these cells are the source of the soluble IgG molecules involved in the selection of germinal center B cells. Tangye and colleagues recently identified antibody-secreting cells with a CD38-negative phenotype in cultures of B memory cells [22]. In these cultures of CD40Ligand activated cells, IgA, IgM and IgG-secreting cells had been detected in comparable numbers among the CD38-positive and CD38-negative populations. Interestingly, it had been shown in these experiments that the two populations differing in the expression of CD38 have different survival requirements allowing the assumption that CD38-negative antibody-secreting cells may resemble short-lived plasma cells. Tangye could not resolve the question whether the CD38-negative antibody-secreting cells resemble precursors of the CD38-high cells or whether both cell types belong to different lineages. Our data suggest that CD38-high plasma cells are descendants of CD38-low antibody-secreting cells. However, we also detected some CD38-low, plasmacytoid IgG containing cells outside the follicles. It remains to be elucidated whether these cells belong to a distinct plasma cell linage, e.g. of short-lived plasma cells.

A fraction of the plasma cells formed in secondary lymphoid tissues can enter the blood stream to migrate to the bone marrow, an organ, which is of particular importance for the survival of long-lived plasma cells. That bone marrow plasma cells are the progeny of B cells activated in secondary lymphoid tissues is known for long [3]. Also, it has been shown that ab secreting cells present in secondary lymphoid tissues following immunization are equipped with chemokine receptors allowing their emigration into the bone marrow, intestinal lamina propria or into inflamed tissues [1, 2, 30], suggesting that the formation of antibody-secreting cells and their early differentiation occurs in secondary lymphoid tissue. It is interesting to note that peripheral IgG-secreting cells in the blood showed a homogeneous phenotype while their progeny and their precursors, i.e. bone marrow plasma cells and tonsillar plasma cells are heterogeneous with respect to CD9, CD19, CD38 and CD45. This suggests that plasma cell interaction with stromal cells, extracellular matrix components and/or cytokines present in lymphoid tissues may regulate the expression of various surface molecules on plasma cells. That plasma cells are responsive to extrinsic signals had been shown earlier. Tonsillar plasma cells e.g. can be rescued from apoptosis by stromal cells [31]. Also, the interaction with their environment allows the survival and proliferation of plasma blasts [32] and the entrance of a certain number of these cells into the long-lived plasma cell compartment in the splenic red pulp [33].

Due to the absence of CD19 on myeloma cells, plasma cell differentiation has been considered to be accompanied by the loss of this molecule [34, 35]. In accord with other reports [13, 27] we show that CD19 is expressed on all non-malignant IgG-secreting cells including the later plasma cell stages in the bone marrow. However, the level of CD19 expression on IgG-secreting cells differs in the analyzed tissues. All tonsillar IgG-secreting cells at least partially resembling early plasma cell stages, express CD19 at high levels, comparable or higher than other CD19-positive cells in this tissue. IgG-secreting cells in the blood, possibly on their way from secondary lymphoid tissues to the bone marrow and other peripheral tissues, show reduced levels of CD19. In the bone marrow, however, two populations of plasma cells are found expressing either high or low levels of CD19. The later seem to express CD19 in levels comparable to those expressed on plasma cells isolated from blood. Since plasma cells migrating through the blood are at least partly precursors of bone marrow plasma cells, this may indicate that CD19 is lost transiently during plasma cell translocation through the blood but is up-regulated on mature bone marrow plasma cells. This hypothesis is in accord with the observation that the level of CD19 expression on bone marrow plasma cells correlates with their cell size (table 1) and thus with an increase in the level of maturation [23]. However, this issue remains to be further elucidated.

Our data show that plasma cell differentiation can be dissected in distinct developmental stages of CD38-low and CD38-high cells. The presence of distinct sequential steps during early plasma cell differentiation in the tonsils may possibly mark controlled checkpoints regulating this process in secondary lymphoid tissues. The presence of distinct subpopulations of bone marrow IgG-secreting cells representing later stages of the plasma cell development may indicate the presence of distinct

types of plasma cells, e.g. short-lived and long-lived ones. However, we did not find subpopulations among the bone marrow plasma cell precursors, i.e. the IgG-secreting cells in the peripheral blood, arguing against different plasma cell lineages. An alternative explanation for the presence of multiple subpopulations of bone marrow plasma cells is the presence of a "checkpoint" controlling the entrance into the long-lived plasma cell compartment. Such a mechanism would explain the distinct bone marrow plasma cells by the presence of a population, which have entered the bone marrow just recently but are not allowed to enter the long-lived compartment yet, and a plasma cell population resembling the long-lived compartment. This idea is in accord with the hypothesis that plasma cell longevity is not guaranteed by entering the bone marrow but depends on specific niches providing survival signals [36-39].

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