Immunization-Induced Perturbation of Human Blood Plasma Cell Pool: Progressive Maturation, IL-6 Responsiveness, and High PRDI-BF1/BLIMP1 Expression Are Critical Distinctions between Antigen-Specific and Nonspecific Plasma Cells[1]

Inés González-García, Esther Ocaña, Gema Jiménez-Gómez, Antonio Campos-Caro, and José A. Brieva

The present study shows that reimmunization with tetanus toxoid (tet) caused a transient increase of the human blood plasma cell (PC) pool, detectable from 6th to 15th day postboost, as well as the temporal alteration of several PC features. Labeling of specific PC with FITC-tet C fragment (tetC) allowed kinetics analysis of the tetC$^+$ and tetC$^-$ PC, and revealed remarkable differences between them: 1) the kinetics of tetC$^+$ PC occurrence was exponential, and most of them appeared in a narrow time frame (5th to 8th day postboost), whereas the tetC$^-$ PC increase was lower (three to five times) and more prolonged (4th to 15th day postboost). 2) The tetC$^+$ PC subset contained a fraction of cycling cells, expressed high levels of DR, CD138, and CD126, and responded to IL-6 by improving their survival and Ig secretion; in contrast, the tetC$^-$ PC showed higher CXCR4 and lower DR and CD138, did not respond to IL-6, and contained a fraction of apoptotic cells. 3) Sequential phenotypic analysis revealed maturational changes within the tetC$^+$, but not tetC$^-$, PC subset; sequencing of tetC$^+$ PC *IgVH* genes showed clear features of Ag selection. 4) The tetC$^+$ PC expressed several times more positive regulatory domain I- binding factor 1/B lymphocyte-induced maturation protein 1 transcription factor than the tetC$^-$ PC. 5) The tetC$^-$ PC and bone marrow resident PC similarly expressed low DR and high CXCR4, but differed in that the latter exhibited higher levels of CD31, CD138, and positive regulatory domain I- binding factor 1/B lymphocyte-induced maturation protein 1. These findings support the view that tetC$^+$ PC contain bone marrow PC precursors, and tetC$^-$ PC probably belong to a removable compartment of aged PC. ***The Journal of Immunology,*** **2006, 176: 4042–4050.**

Plasma cells (PC)[3] are the final step of the B lymphocyte differentiation process and, as such, they are responsible for the effector phase of humoral immune responses, i.e., the synthesis and secretion of Ig or Abs. There is increased evidence that the biology of mammals PC is rather complex. Thus, when Ags drain into secondary lymphoid organs, specific PC are first generated in inductive areas of these tissues, i.e., in T cell area Ag-activated foci and germinal centers (1–5). Most of these early PC undergo rapid apoptosis (5, 6). Later on and on the arrival of circulating precursors (7, 8), PC accumulate in special niches mainly located in deposit organs, such as the bone marrow (BM) and mucose lamina propria (for systemic and mucosal responses, respectively) (5, 7–10). BM PC secrete high affinity Ab for prolonged periods of time, thereby becoming the main cells responsible for the systemic defensive humoral response and serum Ig levels (11–14). The nature of the circulating precursor of BM PC remains uncertain, although accumulating findings support the view that circulating Ag-specific PC might accomplish this role. This is first based on the observation of transient occurrence in the human blood of specific Ab-secreting cells, after immunization to a variety of Ag (15–18), an event that is immediately followed by the phase of fast elevation of serum-specific Ab titles (15, 19). These Ab-secreting cells have been identified as PC (20). In addition, human and mouse circulating PC exhibit intermediate phenotypic and functional properties, when compared with those of early PC and BM PC, thus indicating a maturational gradient for PC in the direction: inductive organs→blood→BM (20, 21). More recently, Blink et al. (22) have reported kinetics, mutational Ig maturation, and phenotypic and functional data that strongly indicate that the Ag-specific PC transiently present in the mice blood are the most probable candidate for the establishment of the BM long-lived and high affinity Ab-secreting PC compartment.

Because high affinity Ab-secreting PC are enriched in the BM (13, 14, 22), Ag-recognition strength at earlier developmental phases of the BM PC precursors might determine their capacity for survival and migration into specific survival niches of this deposit area. In this context, two independent molecular interactions have been demonstrated in the formation of BM survival niches for PC, including VLA4 integrin (CD49d-CD29) and CXCR4 expression by PC and the recognition of their corresponding ligands provided by BM stromal cells (20, 23–27). Despite the relevance of these findings, knowledge of the multistep process that drives the

Servicio de Inmunología and Unidad de Investigación, Hospital Universitario Puerta del Mar, Cádiz, Spain

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[2] Address correspondence and reprint requests to Dr. José A. Brieva, Servicio de Inmunología, Hospital Universitario Puerta de Mar, Avenida Ana de Viya 21, 11009 Cádiz, Spain. E-mail address: josea.brieva.sspa@juntadeandlucia.es

[3] Abbreviations used in this paper: PC, plasma cell; Blimp-1, B lymphocyte-induced maturation protein 1; BM, bone marrow; BMMC, BM mononuclear cell; MFI, mean fluorescence intensity; PB, peripheral blood; PRDI-BF1, positive regulatory domain I-binding factor 1; tet, tetanus toxoid; tetC, tet C fragment.

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selection, retention, and establishment of the long-lived PC compartment remains incomplete.

Several pieces of evidence support the notion that IL-6 plays an important role at the terminal stages of B cell differentiation. Thus, IL-6 induces the proliferation and survival of normal human PC either elicited by a mitogenic stimulus in vitro (28) or obtained from the blood of patients with reactive plasmocytosis (29). More importantly, IL-6 produced by BM stromal cells exerts a pronounced effect on the survival and/or Ig secretion capacity of human BM PC (23), a finding that has been reported also in mouse systems (26, 30). Therefore, the acquisition of IL-6 responsiveness appears to be an important event in the maturational PC program.

It is now well established that the transcription factor positive regulatory domain I- binding factor 1 (PRDI-BF1), the human homologue of the murine B lymphocyte-induced maturation protein 1 (Blimp-1), plays a pre-eminent role in controlling terminal B cell differentiation into the PC stage, for several reasons: in humans and mice, its presence has been demonstrated in PC, but not in memory B cells (31). Its forced expression is sufficient to induce PC differentiation in B cell models (32). In addition, mice lacking Blimp-1 in their B cells fail to mount humoral responses, lack PC, and exhibit very low levels of serum Ig (33). PRDI-BF1/Blimp-1 essentially functions as a gene repressor, causing the exit from cell cycle and the extinction of the expression of several genes critical for B cell development at earlier stages, notably the *Pax-5* gene (34). This latter has been shown to repress the expression of *XBP1*, a factor that also accomplishes important functions in the PC differentiation program (35). In addition, by introducing a *gfp* reporter gene into the *Blimp-1* locus, it has been shown recently that PC expressing the largest amount of this factor were recruited predominantly into the BM compartment, whereas splenic early PC and blood PC exhibited lower levels of *Blimp-1* expression (21). In consequence, the quantity of this factor seems to correlate with the level of PC maturation.

The present study analyzes the effect of a conventional tetanus toxoid (tet) booster immunization on the human circulating PC pool. The results reveal that the immunization induces the release to the blood of tet-specific as well as tet-nonspecific PC. These two PC subsets share some features, but also exhibit different temporary kinetics and phenotypic and functional characteristics. In addition, the tet C fragment$^+$ (tetC$^+$) PC subset distinctively shows the capacity to undergo maturational changes in vivo, to respond to IL-6, and to express larger quantities of PRDI-BF1/Blimp-1 mRNA. These observations are discussed in light of the PC developmental sequence.

Materials and Methods

Materials

RPMI 1640 culture medium, L-glutamine, FCS, penicillin, and streptomycin were purchased from Invitrogen Life Technologies. Human rIL-6 and rabbit anti-IL-6 Ab were provided by PeproTech. Cycloheximide, mitomycin C, and tetC purified and bound to FITC (FITC-tetC) were purchased from Sigma-Aldrich. Fibronectin and annexin V FITC were provided by Roche. FITC-labeled mAb against CD19, CD20, and BrdU; PE-labeled mAb against CD20, CD31, CD38, CD95, HLA-DR, CXCR4, and ki-67; PerCP-Cy5.5-labeled mAb against CD38; and Alexa Fluor 647-labeled mAb against CD19; and the corresponding isotypic negative controls and BrdU flow kit were provided by BD Biosciences. PE-labeled mAb against CD126, CD138, and APO2.7, and purified mAb against CD95 (CH11 clone) were provided by Beckman Coulter. Cy5-conjugated mouse anti-human IgG were obtained from Jackson ImmunoResearch Laboratories. FITC-conjugated mAb against p63 (VS38c clone) and Intrastain kit were from DakoCytomation. Unconjugated and peroxidase-conjugated goat anti-human IgG, IgA, used for determining human Ig by ELISA and for ELISPOT, were provided by BioSource International. ELISPOT 96-well microtiter plates were provided by Millipore. Purified tet was a gift from Llorente Laboratorios.

Preparation of tonsil, blood, and BM mononuclear cells (BMMC)

Tonsils were obtained from subjects undergoing tonsillectomy for chronic tonsillitis. The tonsillar tissue was chopped into small pieces, and the resulting cell suspension was washed in culture medium. Heparinized peripheral blood (PB) was obtained from volunteers at different times (usually 0, 6, 15, and 30 days) after a conventional tet booster immunization (50 IU), and PBMC were prepared by Ficoll/Hypaque density-gradient centrifugation. An absolute mononuclear cell count was routinely obtained from every blood sample in an automated hemocytometer. BM samples were obtained from rib pieces resected during thoracotomy for lung tumors, and BMMC were obtained and prepared by Ficoll-Hypaque density-gradient centrifugation, as previously reported (23). Approval was obtained from the institutional review board (Comisión Ética). Informed consent was provided according to the Declaration of Helsinki.

Cell staining and flow cytometry analysis

For external marker staining, 200 μl of tonsil, PBMC, and BMMC cell suspension (1–5 $\times$ 10^6 cells/ml) was incubated with optimal concentrations of mAb for 20 min in the dark at 4°C. PC identified by specific external labeling (see below) were subsequently explored for the additional detection of internal markers with FITC-tetC (100 ng/ml) and with mAb for VS38c, APO2.7, IgG, and Ki67, by using an Intrastain kit, according to manufacturers' instructions. Analysis of proliferating cells was performed by detecting BrdU incorporation during a 24-h culture period, using the BrdU Flow kit. FACS analysis was performed on a FACSCalibur cytometer (BD Biosciences) equipped with an air-cooled argon ion laser emitting at 488 nm and with a red diode laser emitting at 635 nm. The instrument was equipped with four fluorescence detector photomultiplier tubes, with green fluorescence (FITC, FL1) being collected through a 530/30-nm bandpass, red/orange (PE, FL2) through a 585/42-nm bandpass, orange (PerCP, FL3) through a 670-nm longpass filter, and red (allophycocyanin, FL4) through 661/16-nm bandpass filter. Cell analysis was performed with CellQuest software (BD Biosciences). CD38^{high} cells were gated on a CD20/CD38 dot plot of tonsillar B cell fraction and on a CD19/CD38 dot plot of PBMC and BMMC using the FL1-FL3, FL2-FL3, or FL3-FL4 plots depending on the mAb combination used. The free channels were used to explore the expression of additional markers on these PC. Isotype- and fluorochrome-matched negative controls were used to establish positive window as that including cells brighter than the brightest 5% of the negative control cells. Data from 2000–5000 CD38^{high} cells/sample were collected, and the percentage as well as the mean fluorescence intensity (MFI) of the CD38^{high} cells positive for each analyzed molecule were monitored.

Isolation of human PC from different territories

For PC isolation, tonsil and PB non-T cell populations were obtained by a previously reported rosette technique (36). Blood non-T cells were further depleted of monocytes by adherence on plastic (36). The tonsil and blood monocyte-depleted non-T cell fraction consisted of B cells (>97% CD19$^+$ cells). The PC present in tonsil and blood B cell fractions and in BMMC were stained with the appropriated combinations of fluorochrome-conjugated mAb, and were identified by flow cytometry analysis as CD20$^{+/-}$ CD38^{high}, CD19$^+$CD38^{high}, and CD19$^{+/-}$CD38^{high}CD138$^+$ for tonsil, blood, and BM PC, respectively, as previously reported (20). Blood PC containing intracytoplasmic anti-tet Ig were labeled by using FITC-tetC staining as above, and blood tetC$^+$ and tetC$^-$ CD19$^+$CD38^{high} PC were also identified. All of these PC populations were purified by sterile FAC sorting on a FACSAria cell sorter (BD Biosciences) equipped with a solid state coherent sapphire laser emitting 13 mW at 488 nm and with an air-cooled JDS Uniphase HeNe laser emitting 11 nW at 633 nm. These purified cell fractions showed morphological features and intracytoplasmic Ig staining (>97%) typical of PC (20).

Cell culture

Cell cultures (10^6 cells/ml) were set up in a culture medium consisting of RPMI 1640 supplemented with 10% FCS, 10 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin, in 96-well flat-bottom plates in a final volume of 250 μl per well. Two different techniques were used to evaluate PC apoptosis. FITC annexin V binding technique was used, as previously reported (37), on PBMC cultured for 48 h in the absence and in the presence of CH11 anti-CD95 mAb (200 ng/ml). In addition, intrastaining with APO2.7 mAb was also used for detecting spontaneous apoptosis in freshly obtained tetC$^-$ and tetC$^+$ PC. PBMC (10^6 cells/ml) were also cultured in the absence and in the presence of IL-6 (5 ng/ml). The effect of IL-6 on tetC$^+$ and tetC$^-$ PC recovery was assessed after 48 h of culture. Then, the cells were collected and labeled for external CD19 and CD38,

and internally with FITC-tetC, and the absolute number of tetC$^+$ and tetC$^-$ PC present in a similar volume of untreated and IL-6-treated cultures was estimated by flow cytometry. The percentage of IL-6-induced change in cell recovery was obtained by the following formula: (number of PC in IL-6-treated cultures/number of PC in control cultures $-$ 1) $\times$ 100. To determine the IL-6 effect on Ig secretion, PBMC (10^6 cells/ml) were cultured in the absence and in the presence of 5 ng/ml IL-6, unless indicated otherwise. After 7 days, cell-free culture supernatants were obtained, and the quantity of IgG-tet, and of total IgG and IgA secretion was determined by ELISA, as previously reported (36). PBMC secreting total IgG and IgG-tet were detected by an ELISPOT technique (38). PBMC were plated in duplicate wells at 5×10^4/ml, 5×10^5/ml, and 5×10^6/ml, and cultured for 24 h. The spots were counted under a stereo-microscope, and the results represent the mean of duplicate wells.

Quantification of PRDI-BF1/BLIMP-1 transcripts

Total RNA was extracted from highly purified tonsil, blood, and BM PC using the High Pure RNA Isolation kit (Roche), following the manufacturer's protocol, including an on-column DNase I digestion step. cDNA synthesis was conducted using random hexamer primers and the components from the Transcriptor First Strand cDNA Synthesis kit (Roche). PRDI-BF1/BLIMP-1 and internal reference β-actin transcripts were quantified according to a fluorescence-based real-time detection method performed with an ABI PRISM 7000 sequence detection system (Applied Biosystems). The real-time quantitative PCR was performed from cDNA using TaqMan Universal PCR Master Mix, No AmpErase UNG (Applied Biosystems). Primer sequences and target-specific fluorescence-labeled TaqMan probes were obtained from TaqMan Gene Expression Assays (Applied Biosystems); assay references were as follows: Hs00153357_m1 for PRDI-BF1 and Hs99999903_m1 for β-actin. PCRs were performed in 96-well microtiter plates, according to the manufacturer's instructions (Applied Biosystems). Relative gene expression levels were calculated using the $2^{-\Delta\Delta CT}$ method (39). The data are presented as the fold change in PRDI-BF1 gene expression normalized to the β-actin gene and relative to sample indicated in figure legends. Two independent analyses were performed for each sample and for each gene.

IgVH$_3$ *gene sequences analysis*

Amplification of *IgVH$_3$* gene family was performed by PCR using a specific sense oligonucleotide (5′-TARRAGGTGTCCAGTGT-3′) localized on *IgVH$_3$* signal peptide regions and a specific antisense oligonucleotide (5′-GAAGACSGATGGGCCCTTGGT-3′) complementary for IgG isotype C region. PCR was performed with the Expand High Fidelity Plus PCR System (Roche) using the cDNA obtained as above. Products were agarose gel purified, ligated into pBluescript vector (Stratagene), and bacterial transformed. Individual clones were sequenced in a Genetic Analyzer ABI-310 (Applied Biosystems), and nucleotide sequences were compared with *IgVH$_3$* germline using the IMGT *IgVH* database (⟨http://imgt.cines.fr/⟩).

Statistical analysis

The Friedman test for ANOVA was used to compare sequential changes in multiple related samples. Differences between paired experimental points were established by using the Wilcoxon test. The Mann-Whitney *U* test was used for the analysis of differences between unrelated samples.

Results

Immunization with tet induced sequential changes in the number, proliferative and apoptotic capacity, and the phenotype of blood PC

Previous studies have demonstrated that human circulating PC can be defined as CD38^{high} cells by flow cytometry (20, 40). In a first series of experiments, blood was obtained from volunteers at 0, 6, 15, and 30 days after a conventional tet booster immunization, and PC were detected. Fig. 1*A* shows an example of the reduced, but detectable number of blood PC determined as CD38^{high} cells in a CD19 CD38 dot plot, during this time frame. Fig. 1*B* summarizes the temporary kinetics of the percentage of CD38^{high} cells in several experiments, showing that basal percentages of circulating PC increased on average by 5 times at day 6, still remained high ($\cong$3 times) at day 15, and returned to preboost values 1 mo after immunization. Apoptotic PC, determined as the percentage of annexin V$^+$CD38^{high} cells after 48 h in either nonstimulated (□) or anti-CD95 mAb-treated cultures (▨), also showed an increase of $\sim$3 times at day 6 postboost, stayed high at day 15, and returned to preboost levels after 30 days (Fig. 1*C*). However, when testing the proliferative capacity of blood PC determined by BrdU incorporation into CD38^{high} cells, the temporary pattern observed was slightly different, with a clear increase of this activity assessed as the percentage of BrdU-containing PC on day 6, that quickly returned to preboost values in the samples obtained at 15 and 30 days (Fig. 1*D*). In addition, the pretreatment of blood samples with mitomycin C, an inhibitor of DNA synthesis, blocked most (>90%) BrdU incorporation into PC (data not shown). Similarly to BrdU incorporation, the MFI of DR expression by blood PC was markedly enhanced at day 6 postboost, and declined to preboost values thereafter (Fig. 1*E*). Finally, a different temporary sequential pattern was found when the PC surface expression of CXCR4 was examined. Thus, Fig. 1*F* shows that the percentage of PC exhibiting CXCR4 expression increased at day 6 and remained high even 1 mo after booster. Prebooster percentages of CXCR4$^+$ PC were observed in samples obtained after 6 wk (data not shown). Significant changes were not detected for the PC expression (either the percentage or the MFI) of surface CD19, CD20, CD27, CD29, CD31, CD38, CD40, CD45, CD49d, and CD95, nor for intracellular κ-chain (data not shown).

Effect of tet booster immunization on the kinetics of appearance in the blood of tetC-specific (tetC$^+$) and nonspecific (tetC$^-$) PC

It has been reported recently that, upon tet immunization, specific PC released to the circulation can be detected by the staining of cytoplasmic anti-tet Ab-containing PC with FITC-tetC (24). Accordingly, the kinetics of these specific PC was next assessed. Fig. 2*A* shows an example of the labeling with FITC-tetC of human circulating PC after tet booster. As can be seen, tetC$^+$ CD38^{high} cells were first apparent at day 5, reached a maximum at days 6 and 7, and were very low at day 8, declining even more thereafter, as has been reported previously (24). Staining of CD38^{high} cells with FITC-tetC was specific as the pretreatment of permeabilized cells with unlabeled tetC (1 μg/ml), but not with BSA, prevented tetC$^+$ PC detection. In addition, FITC-tetC treatment of nonpermeabilized blood cell fractions obtained between days 0 and 8 after tet booster showed negative cell staining, ruling out the presence of surface IgG-tetC$^+$ cells during this period. Fig. 2*B* summarizes the results obtained from different donors, regarding the temporary kinetics analysis (0, 6, 15, and 30 days after tet booster) of the absolute numbers of CD38^{high} cells (an estimation of all circulating PC), IgG-secreting cells determined by ELISPOT, tetC$^+$ CD38^{high} cells, and IgG-tet-secreting cells determined by ELISPOT. As can be seen, the number of IgG-secreting cells, which has been generally estimated as $\cong$60–70% of all human blood Ig-secreting cells, was parallel and very close to that of CD38^{high} cells, and both values were clearly increased at day 6 (5 times) and at day 15 (2.5 times), returning to preboost values after 1 mo. The number of both IgG-tet-secreting cells and tetC$^+$ CD38^{high} cells was either very low or undetectable in the preboost sample, showed a dramatic increase at day 6, returned at very low levels at day 15, and was again undetectable 30 days after tet booster (Fig. 2*B*). Four tet-immunized donors were analyzed on a daily basis, and showed that an increase of tetC$^+$ CD38^{high} cells over the preboost level was never detectable before day 5, reached maximum numbers at day 6 and 7 (with peak response at day 6 in all cases), and returned to values from very low to undetectable at day 8 after tet stimulation. According to the numerical data (see legend to Fig. 2*B*), at the peak of the response (day 6), 20–30% of total blood PC were tet specific. Fig. 2*B* also shows that the kinetics of tetC$^+$ CD38^{high} cell number, although parallel, was an average of 44%

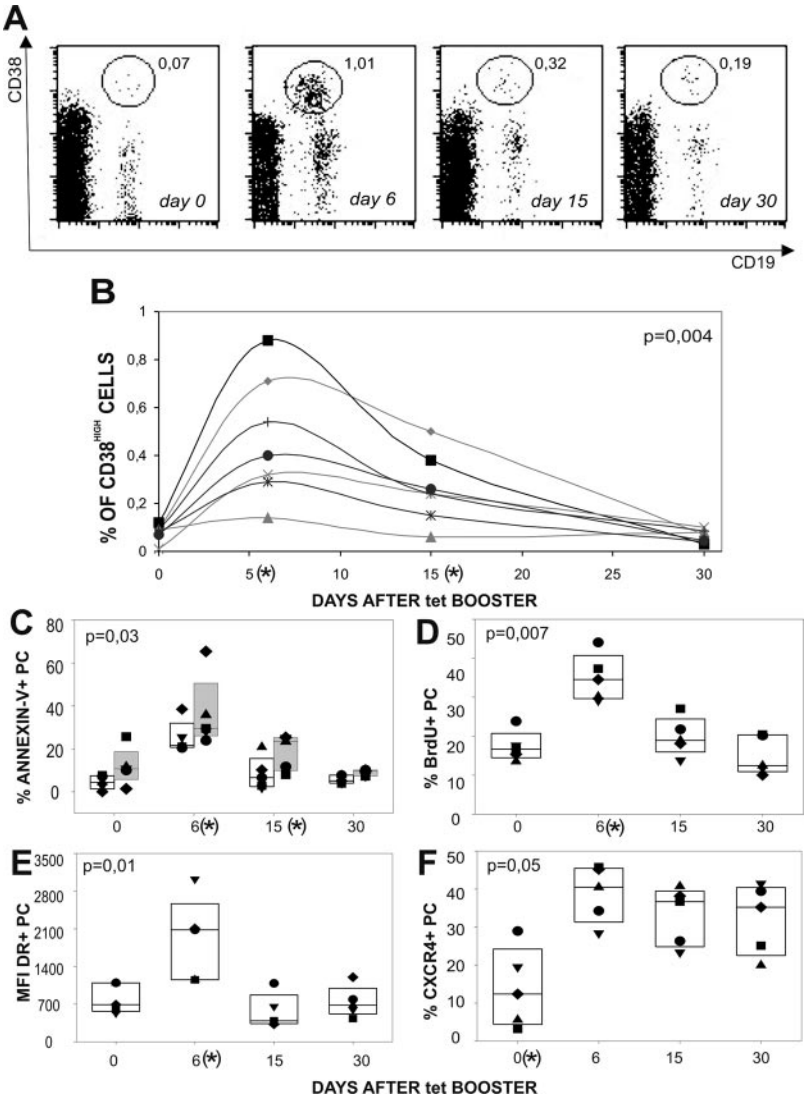

FIGURE 1. Effect of tet booster on human circulating PC. PC were identified as $CD19^+CD38^{high}$ cells present in blood mononuclear cells obtained at 0, 6, 15, and 30 days after a conventional tet booster. *A*, CD19 CD38 dot plots obtained by flow cytometry analysis of labeled blood samples of a representative experiment. $CD38^{high}$ cells are contained in a circular window, and the percentage of positive cells observed at the different time points is depicted. *B*, Temporal kinetics of the percentage of $CD19^+CD38^{high}$ cells after tet booster. Every set of symbols and lines represents one of the seven included donors. *C*, Temporal kinetics of the percentage of blood annexin V^+CD38^{high} cells obtained after 48 h of culture in the absence (□) and in the presence (▨) of the anti-CD95 mAb CH11 (200 ng/ml). *D*, Kinetics of the percentage of BrdU-incorporating blood $CD38^{high}$ cells. *E*, Kinetics of the MFI of DR expression by PB $CD38^{high}$ cells. *F*, Kinetics of the percentage of PB $CD38^{high}$ cells expressing CXCR4. *C–F*, Every set of symbols corresponds to one of the five donors analyzed. Results are represented as a box indicating the median and quartiles. Friedman test *p* values are also included. Asterisks identify time point results that were significantly different from those of the other time points by Wilcoxon test.

lower than that of ELISPOT-detected IgG-tet-secreting cells (2504 ± 703 and 1436 ± 554, for IgG-tet-secreting cells and $tetC^+$ $CD38^{high}$ cells, respectively; mean ± SEM), probably because the tet Ag used in the former technique was only a major part (fragment C) of the whole tet molecule (41), which was used in the ELISPOT. This indicated that, although the $tetC^+$ PC were all tet specific, the $tetC^-$ PC population detected by this method probably contained contamination (10–15%) of tet^+ PC that was not revealed by FITC-tetC. As can be seen in Fig. 2*C*, the kinetics shown by IgG-tet-secreting cells and $tetC^+$ $CD38^{high}$ PC correlated well with that exhibited by the circulating cells capable of spontaneous IgG-tet secretion in vitro. The temporary kinetic of the absolute number of circulating $tetC^-$ $CD38^{high}$ cells was also investigated. As shown in Fig. 2*D*, tet immunization also induced a marked enhancement of $tetC^-$ PC numbers to ~4 times at day 6, remained elevated at an average of 2.5 times at day 15, and returned to preboost levels after 1 mo. Similar results were obtained by subtracting IgG-tet-secreting PC from total IgG-secreting PC (from ELISPOT data contained in Fig. 2*B*). Thus, at day 6, the numbers of IgG-tet- and IgG non-tet-secreting cells estimated by ELISPOT were 2504 ± 703 and 5763 ± 2125, respectively (mean ± SEM).

Comparison of phenotypic, proliferative, and apoptotic features of $tetC^+$ and $tetC^-$ human blood PC

To clarify the contribution of the $tetC^-$ and $tetC^+$ PC subset to the sequential changes described above (Fig. 1), a broad phenotypical study was performed on blood $CD38^{high}$ cells obtained at 6 days after tet immunization. At this time point, $tetC^+$ cells averaged 20% of total $CD38^{high}$ cells. As can be seen in Fig. 3, the two PC populations exhibited marked differences. Although most blood PC were DR^+, $tetC^-$ PC exhibited a broad spectrum of MFI, while $tetC^+$ PC showed a rather homogeneous and markedly higher expression of this molecule. In addition, the percentages of PC expressing CD138 and CD126 were also higher in $tetC^+$ PC. In contrast, the proportion of CD95- and CXCR4-expressing cells was higher in $tetC^-$ PC. We were unable to identify apoptotic PC using the annexin V-binding technique in permeabilized cells, a step required for FITC-tetC costaining. Accordingly, apoptotic cells were examined by using APO2.7 mAb, a method that has been previously shown useful for detecting human apoptotic PC (42). As can be seen, $tetC^+$ PC were negative for this marker, while a fraction of $tetC^-$ PC expressed the molecule. In addition, the comparative analysis of the intracellular content of IgG (Fig. 3) revealed that this parameter was similar in $tetC^+$ and $tetC^-$ PC, as

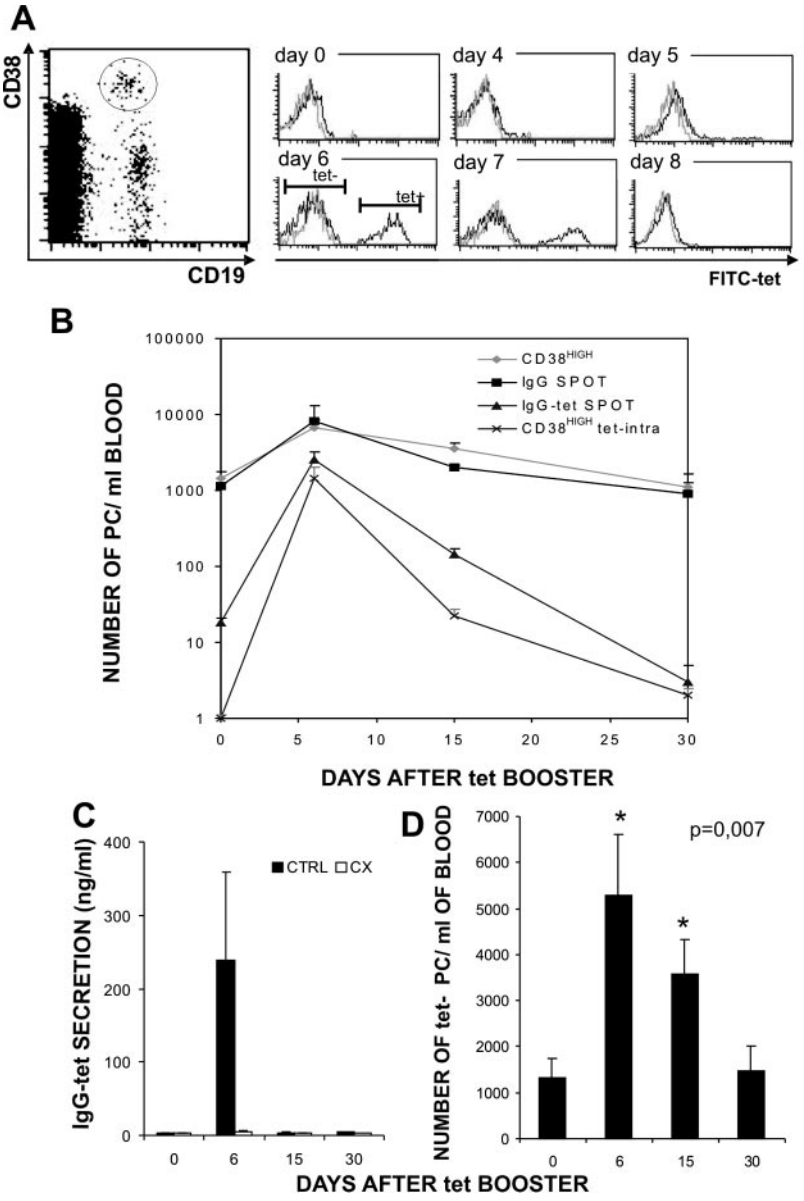

FIGURE 2. Study of temporal kinetics of the effect of tet booster on the occurrence of circulating tetC-specific ($tetC^+$) and nonspecific ($tetC^-$) PC. *A*, PC were identified as $CD38^{high}$ cells in the CD19 CD38 dot plot obtained by flow cytometry analysis, as in Fig. 1 (*left panel*). The presence of PC specific for tet was established by the labeling of permeabilized $CD38^{high}$ cells containing cytoplasmic Ig that recognize FITC-tetC. Paler histograms represent the corresponding negative controls. A representative experiment of the FITC-tetC expression histograms obtained at different days after tet booster is depicted (*right panel*). Usual windows containing $tetC^-$ and $tetC^+$ PC are shown in the histogram corresponding to day 6. *B*, The numbers of circulating total $CD19^+CD38^{high}$ cells and $tetC^+$ $CD19^+CD38^{high}$ cells determined by flow cytometry, as well as total IgG-secreting cells and IgG-tet-secreting cells determined by ELISPOT, were obtained at days 0, 6, 15, and 30 after tet booster. Values were expressed as the number of the PC population under analysis present in 1 ml of blood, and was derived from multiplying the percentage of the particular PC by the absolute number of mononuclear cells/ml blood. Results represent the mean ± SEM of seven experiments. Results (PC number per ml of blood) at day 6 were 6719 ± 1676, 1436 ± 554, 8267 ± 3010, and 2504 ± 703, for $CD38^{high}$ cells, $tetC^+$ $CD38^{high}$ cells, IgG-secreting cells, and IgG-tet-secreting cells, respectively. *C*, Kinetics study of blood PC capable of IgG-tet secretion in vitro. Blood mononuclear cell fractions obtained at indicated times were cultured for 7 days, and the IgG-tet secreted to the supernatant was assessed by ELISA (■). Cycloheximide (10 μg/ml) was added to similar cultures (CX; □). Results are expressed as the mean ± SEM of IgG-tet secretion (ng/ml) of seven experiments. *D*, Analysis of temporal kinetics of the absolute number of circulating $tetC^-$ $CD38^{high}$ cells detected at different times after tet booster. Values were expressed as in *B*. Results are expressed as the mean ± SEM of seven experiments. Friedman test *p* values are also included. Asterisks identify time point results that were significantly different from those of the other time points by Wilcoxon test.

evidenced by an equivalent MFI of expression. The percentage of IgG-containing PC was higher for $tetC^+$ PC, as almost all (>90%) of them were IgG-secreting cells, indicating that specific PC producing other isotypes were scarcely induced after tet reimmunization. VS38c was identically expressed by $tetC^+$ and $tetC^-$ PC (Fig. 3), suggesting that both PC populations exhibited a similar and highly developed endoplasmic reticulum (43). However, BrdU incorporation and the expression of the intracellular protein Ki67, two parameters related to the proliferative cell status, were both significantly increased in $tetC^+$ PC (Fig. 3). Interestingly, the proportion of proliferating cells declined considerably if the BrdU pulse was delayed 12 h, probably indicating that this activity only occurred early in the culture (data not shown). Other surface molecules present on human blood PC not showing distinctive expression in $tetC^+$ or $tetC^-$ PC included CD19, CD27, CD29, CD31, CD38, CD45, and CD49d (data not shown). The re-evaluation of the analysis included in Fig. 1, but distinguishing between $tetC^-$ and $tetC^+$ PC subsets, allowed the observed alterations to be easily attributed. Thus, elevation of DR expression and BrdU incorporation detected at day 6 (Fig. 1, *D* and *E*) was due to changes occurring in the $tetC^+$ PC, whereas increased apoptosis and CXCR4 expression (Fig. 1, *C* and *F*) were only present on $tetC^-$ PC (data not shown).

To explore the possibility that $tetC^+$ PC subset changed their maturational status in the time frame during which they were clearly detectable in the blood, the expression of suggestive markers was studied at day 5 and 7 postboosting. As can be seen in Fig. 4, $tetC^+$ PC from day 7 showed lower expression of DR and CD95. In contrast, the CD138 expression clearly increased from day 5 to 7. No changes were observed in the $tetC^-$ PC. To establish the nature of the $tetC^+$ PC population, these cells were isolated from blood samples obtained from three donors 6 days after tet booster immunization, and their *IgVH* gene was sequenced. Thirty-five sequences of the *IgVH3* gene family were analyzed, showing on average 18.03 ± 1.68 mutations per sequence (mean ± SEM). Most of these mutations accumulated in the CDR 1 + 2 regions, as evidenced by a mean mutation frequency of 10.63 and 3.85, for CDR and framework regions, respectively. In addition, many of the CDR mutations give rise to amino acid replacement (206 of 246 total mutations).

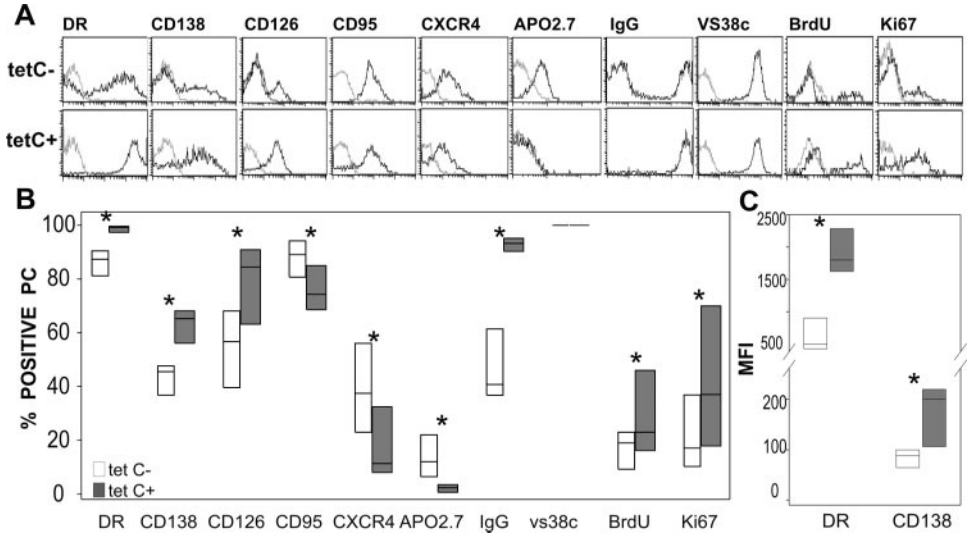

FIGURE 3. Comparative analysis of blood tetC$^-$ and tetC$^+$ PC. *A*, A representative experiment showing the expression histograms of a variety of surface proteins (DR, CD138, CD126, CD95, and CXCR4) and intracellular markers (APO2.7, IgG, Vs38c, BrdU, and Ki67) by tetC$^-$ and tetC$^+$CD38^{high} cells, present in the blood 6 days after tet booster. Paler histograms represent the corresponding negative controls. *B* and *C*, The results observed in several donors are summarized. Values are expressed as the percentage (*B*) and the MFI (*C*) of the expression of the indicated markers. Results are expressed as boxes representing the median and quartiles of seven experiments. Asterisks indicate results that were significantly different by Wilcoxon test.

Response to IL-6 by human blood tetC$^+$ and tetC$^-$ PC

The distinctive expression of CD126, the receptor of IL-6, by tetC$^+$ and tetC$^-$ PC suggested that this cytokine may be relevant for understanding the biology of human blood PC. Accordingly, PBMC obtained 6 days after tet immunization were cultured in the presence and in the absence of IL-6 for 7 days, and the IgG, IgA, and IgG-tet secreted into the supernatant were evaluated. These cells actively produced IgG, IgA, and IgG-tet, as cycloheximide addition (10 μg/ml) to these cultures inhibited >90% of the secretion (data not shown). Fig. 5*A* shows that the addition of IL-6 (5 ng/ml) enhanced basal IgG-tet secretion by these cells by an average of two times ($p < 0.01$). This quantity of IL-6 was shown to be optimal in previous concentration curve experiments (data not shown). In fact, basal IgG-tet production was dependent on endogenous IL-6 secretion in these cultures, because the inclusion of anti-IL-6 Ab (1 μg/ml), but not of anti-IFN-α Ab, reduced a 78.2 ± 20% of the basal IgG-tet secretion (mean ± SEM; $n = 4$). In contrast, IgG-non-tet secretion, a datum obtained by subtracting the quantity of IgG-tet from the total IgG production in each experiment, remained unaffected by IL-6 (Fig. 5*A*). Likewise, IgA secretion was not modified by IL-6 addition either (Fig. 5*A*). The effect of IL-6 on the recovery of tetC$^-$ and tetC$^+$ PC was examined after 48 h of culture. Fig. 5*B* shows that the number of recovered tetC$^-$ PC was not modified by IL-6, while the number of recovered tetC$^+$ PC increased by ~60% on average ($p < 0.03$), after the cytokine treatment.

Comparison between blood tetC$^-$ and tetC$^+$ PC and human PC from other territories

The possible relationship between circulating tetC$^+$ and tetC$^-$ PC and resident BM PC was next explored by analyzing the expression of several surface markers. Fig. 6, *A–C*, shows that BM PC exhibited an expression of CD31 and CD138 clearly higher than both circulating PC subsets. In addition, BM PC and tetC$^-$ PC shared similar expression of CXCR4 and DR, two markers that were distinctively expressed by tetC$^+$ PC, which exhibited lower expression of CXCR4 and markedly higher expression of DR.

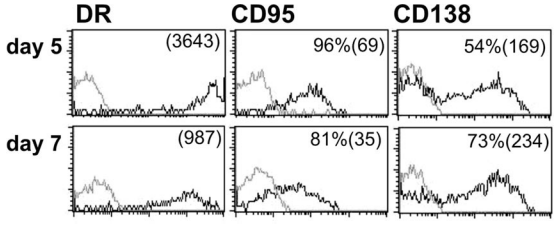

FIGURE 4. Temporary sequence of maturational changes within the tetC$^+$ PC subset. Comparative expression of DR, CD95, and CD138 by tetC$^+$ CD38^{high} obtained 5 and 7 days after tet booster immunization. Paler histograms represent the corresponding negative controls. Representative results of one of three similar experiments are shown. The percentage and the MFI (in parentheses) of positive PC are included for each histogram. The results of three experiments were (day 5 vs day 7, mean ± SEM): 92 ± 2 vs 65 ± 7% and 54 ± 5 vs 69 ± 8%, for the percentage of tetC$^+$ CD38^{high} cells expressing CD95 and CD138, respectively; 68 ± 5 vs 20 ± 2 and 3167 ± 382 vs 1037 ± 523 for MFI of CD95 and DR expression by tetC$^+$CD38^{high} cells, respectively ($p < 0.05$).

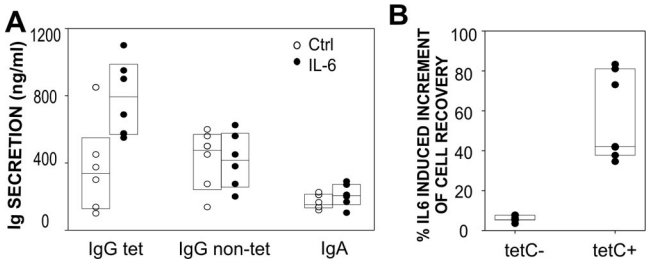

FIGURE 5. Effect of IL-6 on blood tetC$^+$ and tetC$^-$ PC obtained 6 days after tet booster. *A*, Effect of IL-6 on Ig secretion by cultured circulating PC. Blood cells were cultured for 7 days in the absence and in the presence of IL-6 (5 ng/ml) and the IgG-tet; total IgG and IgA secreted to the supernatant were determined by ELISA. *B*, Effect of IL-6 on blood tetC$^-$ and tetC$^+$ CD38^{high} cells recovered after 24 h of culture. Values were calculated as the percentage increase of the cells recovered in IL-6-treated compared with that of control cultures. Results are expressed as individual data as well as boxes representing the median and quartiles of six and seven experiments, for *A* and *B*, respectively.

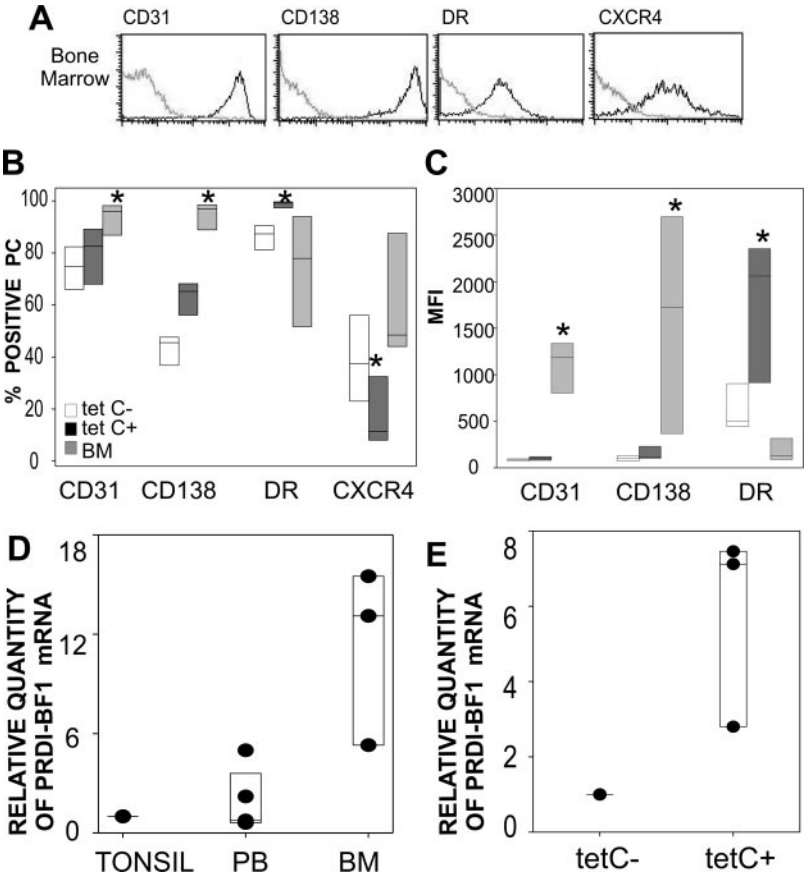

FIGURE 6. Comparative analysis of blood tetC$^+$ and tetC$^-$ PC and PC from other territories. *A*, The figure shows a representative experiment of the expression histograms of CD31, CD138, DR, and CXCR4 by BM CD38^{high} cells, obtained by flow cytometry analysis. Paler histograms represent the corresponding negative controls. Representative histograms of expression of these markers by PB tetC$^-$ and tetC$^+$ PC are depicted in Fig. 3, and have not been repeated here for simplicity. *B* and *C*, Summarized comparison of the expression of the indicated molecules by blood tetC$^-$ and tetC$^+$ (data obtained from Fig. 3) and BM CD38^{high} cells. Results are expressed as boxes representing the median and quartiles of the percentage and the MFI of marker expression in seven and five experiments, for blood and BM, respectively. An asterisk located on a given population denotes a p value lower than 0.05 obtained in the comparison with the other two populations. *D* and *E*, Comparison of the quantity of mRNA for PRDI-BF1/Blimp-1 expressed by PC isolated from tonsil, blood, and BM obtained from unrelated donors (*D*), and blood tetC$^-$ and tetC$^+$ PC (*E*), as determined by real-time PCR using the $2^{-\Delta\Delta CT}$ method. Values were normalized using as endogenous calibrators those of tonsil PC and blood tetC$^-$ PC (that were considered 1) for *D* and *E*, respectively. Individual results are depicted as well as boxes representing the median and quartiles of five, five, and three experiments, for tonsil, blood, and BM PC, respectively (*D*), and of three experiments (*E*).

Previous studies have established that, in humans, there is a gradient of increasing maturity from early PC (tonsil), to transitional/circulating PC (blood) to PC obtained from deposit organs (BM and mucose lamina propria) (20, 43). Although all of these PC populations expressed detectable amounts of mRNA for PDRI-BF1/Blimp-1 (20, 44), the relative quantities of this important factor present in each of them have not been analyzed yet. Accordingly, mRNA for this transcription factor was determined in PC isolated from the different territories by quantitative real-time RT-PCR. Fig. 6*D* shows that when the amount of PRDI-BF1/Blimp-1 present in tonsil PC was considered as 1, blood PC showed twice as much factor, and BM PC exhibited an even higher quantity, thus following the maturation gradient direction mentioned above. In addition, the expression of this transcription factor in purified human blood tetC$^-$ and tetC$^+$ PC was also explored. As can be seen in Fig. 6*E*, blood tetC$^+$ PC expressed on average 4 times more PRDI-BF1/Blimp-1 than blood tetC$^-$ PC ($p < 0.01$).

Discussion

The reduced number of PC occurring in the human blood of most healthy adults has usually been seen as negligible. Nevertheless, recent reports indicate that this cellular pool undergoes alterations that reveal its participation in the PC biology. Thus, 6–7 days after a conventional booster immunization, Ag-specific as well as nonspecific PC subsets are increased in the blood (19, 24). The aim of the present study was to gain a deeper insight of these two human circulating PC subsets. Comparative flow cytometry analysis performed at day 6 after tet booster, when both subsets were easily demonstrable in the blood, showed that tetC$^-$ and tetC$^+$ PC shared some important characteristics, such as exhibiting a similar amount of intracytoplasmic IgG and an equal development of the endoplasmic reticulum. In this regard, it has been shown recently that human blood and colon mucose lamina propria PC expressed a similar pattern and level of soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor exocytic components (45), suggesting that, once the PC stage has been reached, they possess similarly developed Ig synthesizing and secretory machinery. Therefore, both human blood PC subsets under study have acquired an equivalent functionality. Despite this fact, present data revealed the existence of substantial differences between them. These distinctive features help to understand some physiological aspects of the complex PC compartment.

The subset of blood tetC$^+$ PC was necessarily connected to the in vivo Ag stimulation process and the corresponding humoral response. Kinetically, these cells were undetectable or present at very low levels, in the preboost sample, underwent an exponential and fast appearance at days 6–7, and returned to very low numbers at day 15 after tet triggering. In fact, most of these cells (>90%) were present in the blood for only 48–72 h (days 5–8 after booster). Parallel results were obtained by ELISPOT detection of IgG-tet-secreting cells (Fig. 2*B*), as well as by examining the presence of blood cells capable of spontaneous IgG-tet secretion in culture (Fig. 2*C*), which is in agreement with previous reports (15, 19, 24). Furthermore, an elevated proportion of tetC$^+$ PC were cycling cells, an activity that appeared to be transient and, probably, residual. This datum is consistent with the idea that these cells were exiting from a recent proliferative phase. Moreover, tetC$^+$ PC expressed as much DR as small B lymphocytes, a feature revealing their recent entry into the PC differentiation program, because marked down-regulation of this molecule is known to occur in mature PC, as a result of *CIITA* gene repression by PRDI-BF1/Blimp-1 (46). In addition, IgVH3 transcripts obtained from sorted blood tetC$^+$ PC harbored an elevated number of somatic mutations, with a clear CDR predominant distribution and high

level of amino acid replacement. These mutational features are consistent with the notion that blood tetC$^+$ PC had undergone Ag selection and might secrete high affinity IgG-tet Ab (47). Collectively, the present findings indicate that the tetC$^+$ PC subset represents the release to the circulation of a relatively synchronous wave of PC, recently generated as a result of tet stimulation of memory B cells in inductive areas of local lymphoid tissues.

Although the fate of blood tetC$^+$ PC remains uncertain, present results lead to assume that, totally or partly, they are probably recruited into specific survival niches present in the BM. This assumption is reinforced by three distinctive properties of these cells observed in the present study. The first of these results was the observation of the high expression of CD126 (the specific IL-6-recognizing protein) by tetC$^+$ PC in comparison with that exhibited by tetC$^-$ PC. Present data reveal that this cytokine seemed to act by inducing the survival and the Ig-secreting capacity of tetC$^+$, but not tetC$^-$, PC. Interestingly, IL-6 is produced by stromal cells present in PC-survival niches in the BM (23, 26, 30) and spleen (48, 49), and IL-6-inducing effect has been clearly demonstrated on PC present in these locations. In fact, early PC from human tonsils were found to be unable to respond to IL-6 (36, 50). In consequence, blood tetC$^+$ PC have acquired the capacity to respond to a cytokine that is present in survival niches and that is required for PC survival there. The second distinctive feature relates to the quantitative analysis of PRDI-BF1/Blimp-1. As mentioned above, the expression of this transcription factor is a hallmark in the PC development, and, in mice models, PC containing the largest quantity of this factor are recruited into the BM (21, 22). Present results confirm in humans that mRNA for PRDI-BF1/Blimp-1 increased in the direction early PC→blood PC→BM PC. Notably, when this study was performed on blood PC distributed into tetC$^+$ and tetC$^-$ PC subsets, the former showed a PRDI-BF1/Blimp-1 expression several times higher than that of tetC$^-$ PC, a finding that assimilated blood tetC$^+$ PC with BM PC. Finally, the finding that DR and CD95 expression decreased while that of CD138 increased in tetC$^+$ PC between days 5 and 7 postboost was of particular relevance, because these changes have been clearly associated with the progression in the PC differentiation program, as well as with the PC present in the BM (20–22, 33, 48). These observations reveal the sequential recruitment into the blood tetC$^+$ PC subset of increasingly mature cells, as an expression of the ongoing in vivo tet-induced humoral immune response. Progressive differentiation observed in blood tetC$^+$ PC subset suggests that PC showing higher maturity and secreting Ab with improved Ag affinity are probably released beyond day 8 postbooster, as has been demonstrated in mice (14, 22). Taken together, all these data strongly support the notion that the blood tetC$^+$ PC population contains immediate precursors of BM PC.

The origin and nature of the blood tetC$^-$ PC subset are less clear. They seem to be at an advanced stage of the PC developmental program, as indicated by high CXCR4 and low DR expression similar to that of resident BM PC (20, 27, 48), observations recently reported by Odendahl et al. (24) in this human in vivo tet-induced system. These authors also found proximate numbers of blood tetC$^+$ and tetC$^-$ PC 6–7 days after tet immunization, and based on these data they propose the attractive hypothesis that tetC$^-$ PC may represent aged BM PC dislodged from survival niches by new immigrant tetC$^+$ PC. Present results add new precision to the understanding of the tetC$^-$ PC subset. In contrast to the rapid temporal kinetics of the tetC$^+$ PC subset, tetC$^-$ PC showed a basal preboost level detectable in most donors, which increased by several times at days 6 and 15, returning to basal figures by day 30 postboost. In fact, in four of five donors analyzed, increased levels of tetC$^-$ PC were already detectable at day 3 postboost (number/ml of blood: 1532 ± 275 and 3296 ± 351, for days 0 and 3, respectively; mean ± SEM; $p < 0.05$), a time at which tetC$^+$ PC increases had not been yet observed. These results indicated that tet immunization-mediated induction of the blood tetC$^-$ PC subset showed a lower increase, which was spread out over 9–10 days, and that the quantities of tetC$^-$ PC outnumbered those of tetC$^+$ PC (Fig. 2, *B* and *D*). These findings suggested that a concomitant phenomenon, additional to that of mere displacement by direct competition for survival niches, might also act in the release to the blood of tetC$^-$ PC. In this regard, it has been reported recently in mice that vaccine adjuvant-mediated inflammation provokes temporary disruption of CXCR4/stromal cell-derived factor 1-dependent BM pre-B cell niches, and the corresponding transient release to the circulation of BM resident pre-B cells (51). Therefore, it is tempting to speculate that adjuvant stimulation occurring along with the tet-specific activation would contribute to loosen resident PC from survival niches, because their retention also depends on CXCR4-stromal cell-derived factor 1 interaction (25, 49). This idea is reinforced by the recent observation that BM resident pre-B cells and PC share, at least in part, the same anatomical niches (52). Furthermore, despite the mentioned similar expression of CXCR4 and DR, present results indicate that blood tetC$^-$ PC differed from BM PC in several important aspects. First, blood tetC$^-$ PC exhibited low levels of CD31 and CD138, two adhesion molecules highly expressed on human BM PC (20). Moreover, in contrast to BM PC, blood tetC$^-$ PC showed lack of response to IL-6 and expressed low levels of PRDI-BF1/Blimp-1. Taken together, all the data presented suggest that the circulating tetC$^-$ PC subset consists of mature PC, recently displaced from survival niches by the possible disruptive activity of adjuvant stimulation. As a result, blood tetC$^-$ PC may undergo changes that probably reflect their disconnection from signals provided in the survival niches, a situation that might be temporally reversible upon homing to a suitable new location. It is conceivable that emptied niches will be more readily occupied by tetC$^+$ PC, because they exhibited several features that could confer to them clear advantages in this process, including higher IL-6 responsiveness and expression of CD138 and, more importantly, higher levels of PRDI-BF1/Blimp-1. Experimental data show that BM PC survival is not an inherent event, but requires continuous signals provided by niche stromal cells (23, 26, 30), and that the number of survival niches appears to be limited (53). In this regard, present results show that freshly obtained blood tetC$^-$ PC expressed increased CD95 and already contained a relevant fraction of PC that had initiated apoptosis (a phenomenon not observed in tetC$^+$ PC), thus indicating that a part of these cells will probably be definitively removed by this mechanism. Further work will be required to ascertain whether all PC survival niches are equally susceptible to these removal events. This will give us a clearer view about the physiological relevance of the described phenomena for the humoral immune system.

Disclosures

The authors have no financial conflict of interest.

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