European Journal of Immunology

Contribution of stromal cells to the migration, function and retention of plasma cells in human spleen: potential roles of CXCL12, IL-6 and CD54

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Plasma cells (PC) localize to discrete areas of secondary lymphoid tissue and bone marrow (BM). The positioning of PC in different sites is believed to be regulated by chemokines and adhesion molecules expressed by accessory cells in the lymphoid tissue microenvironment. However, the mechanisms responsible for the positioning of PC within the red pulp (RP) of human spleen have not been elucidated. Therefore, we examined the contribution of human splenic stromal cells to the migration and function of human PC. Splenic PC expressed the chemokine receptor CXCR4 and responded to its ligand CXCL12. In contrast, PC lacked CXCR5 and CCR7, and consequently exhibited minimal migration towards CXCL13 and CCL21. Splenic stromal cells proved to be a rich source of CXCL12, and could induce the migration of human B cells. Furthermore, they supported Ig production by splenic PC mainly by secreting IL-6. Lastly, a striking difference between splenic and BM PC was the constitutive expression of CD11a by only splenic PC. Notably, splenic stromal cells expressed high levels of CD54, the counter-structure of CD11a, and splenic PC were positioned adjacent to stromal cells in the RP. Thus, we propose that stromal cells attract PC to the RP and contribute to their retention and function through the combined expression of CXCL12, CD54 and IL-6.

Received 1/7/04
Revised 22/12/04
Accepted 10/1/05

[DOI 10.1002/eji.200425442]

Key words: Human spleen · Plasma cells · Chemotaxis · Adhesion · Humoral immunity

Introduction

Long-lived plasma cells (PC) are generated within secondary lymphoid tissues in response to T cell-dependent Ag and are responsible for the production of protective Ig [1–4]. Once generated, PC migrate to discrete areas of lymphoid tissue, such as the splenic red pulp (RP) [5, 6], tonsillar mucosal epithelium [7], other mucosa-associated lymphoid tissues (small and large intestine [8]), and BM [9]. PC have also been detected within inflamed tissue, such as synovium of rheumatoid arthritis patients [10] and kidneys of autoimmune mice [11].

The positioning of PC within different anatomical sites appears to be regulated by the concerted actions of chemokines and adhesion molecules. For example, murine B cells alter their responsiveness to different chemokines as they differentiate into PC. Thus, while mature B cells express the chemokine receptors CCR7, CXCR4 and CXCR5, and migrate towards the corresponding ligands CCL19/21, CXCL12 and CXCL13, PC down-regulate CCR7 and CXCR5, but not CXCR4, and consequently exhibit selective migration towards CXCL12 [3, 4, 12]. Similarly, IgA-secreting PC, but not those secreting IgM or IgG, migrate towards CCL28 and CCL25 by virtue of their selective expression of CCR9 and CCR10 [8, 13]. CXCL12 is produced by stromal cells present in the RP of murine spleen [12] and BM [14, 15],

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Abbreviations: PC: Plasma cells · **GC:** Germinal center · **RP:** Red pulp · **SA:** Streptavidin · **BAFF:** B cell-activating factor belonging to the TNF family · **BAFF-R:** BAFF receptor · **BCMA:** B cell maturation Ag · **TACI:** Transmembrane activator of CAML interactor

while CCL25 and CCL28 are produced by epithelial cells in the mucosal tissue of the small intestine and salivary glands [4, 16–18]. Accordingly, it has been proposed that the unique responsiveness of PC to these chemokines guides their movement from the lymphoid areas of spleen and lymph nodes to their final microenvironments. These studies complemented earlier investigations of the role of adhesion molecules expressed by PC generated following administration of Ag via different routes [19, 20]. Mucosal immunization resulted in an increased proportion of IgA-PC expressing the integrin α4β7 that eventually localized to the intestinal mucosa [20, 21], whereas systemic immunization yielded IgG-producing PC that persisted in the blood [19, 22]. Because the ligand for α4β7, MAdCAM-1, is expressed on intestinal endothelium, it was proposed that interactions between α4β7 and MAdCAM-1 retain IgA-PC within mucosal lymphoid tissue [4, 8].

Despite these studies, the mechanisms responsible for the positioning of human splenic PC in the RP alongside stromal cells [6, 12] have not been fully elucidated. For this reason, we examined the contribution of stromal cells to the positioning and function of human splenic PC. Based on our results, we propose that splenic stromal cells contribute to the positioning and function of splenic PC through the combined production of CXCL12 and IL-6, as well as the expression of CD54.

Results

Human splenic PC express CXCR4 and exhibit selective migration to CXCL12

We recently identified a population of germinal center (GC)-derived PC in the RP of human spleen [6], analogous to long-lived murine splenic PC [5, 12, 23]. These cells can be identified by high expression of CD38 and CD27 and low CD20 ($CD38^{++}CD20^{+/-}$), and distinguished from splenic naive ($CD20^{+}CD27^{-}$) and memory ($CD20^{+}CD27^{+}$) B cells (Fig. 1a, [6, 24]).

The positioning of PC in murine spleen is regulated by their responsiveness to CXCL12, which is produced by stroma within the RP [12]. The chemokine receptors CXCR4, CXCR5, CCR7 have important roles in coordinating migration of mature murine B cells [3, 4]. CXCR3 also induces migration of murine PC *in vitro* in response to CXCL9, CXCL10 and CXCL11 [13, 25]. To investigate the distinctive positioning of mature human B cells in the follicles and PC in the RP, the expression of these receptors was determined. CXCR3 was expressed by a subset of memory B cells, but was absent from naive B cells, and splenic and BM PC (Fig. 1b). All B cell subsets expressed CXCR4 (Fig. 1b). In contrast, although naive and memory B cells expressed comparable levels

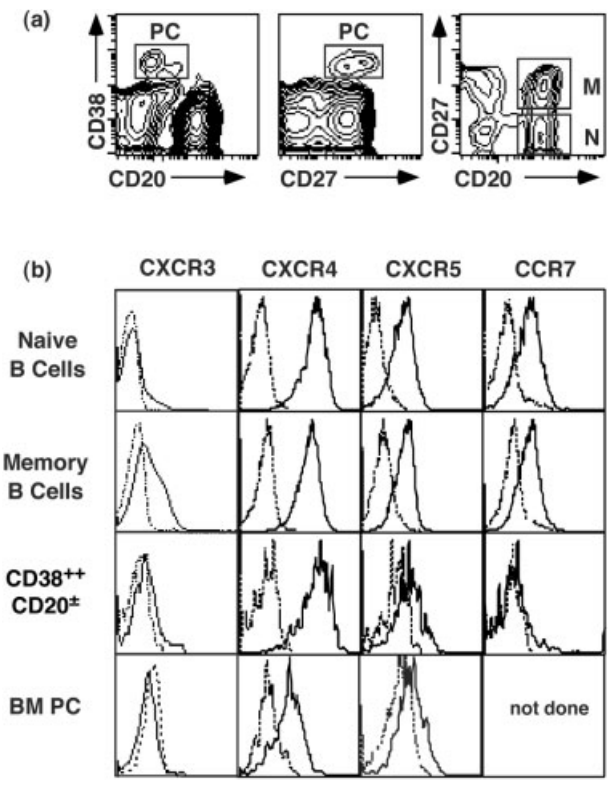

Fig. 1. Alterations in the expression of chemokine receptors during B cell differentiation. (a) Splenic MNC were stained for CD27, CD38 and CD20. Plasma cells (PC; $CD38^{++}CD27^{++}CD20^{+/-}$), naive (N; $CD20^{+}CD27^{-}$) and memory (M; $CD20^{+}CD27^{+}$) B cells could then be resolved. (b) The fourth fluorescence channel was used to assess expression of CXCR3, CXCR4, CXCR5 or CCR7. Gates were set on splenic naive, memory and PC. BM MNC from different donors were stained for CD20, CD38 and the indicated molecule, and a gate set to identify BM PC ($CD38^{++}CD20^{+/-}$). Dotted line represents negative control, solid line represents marker of interest. Histograms represent cells from one donor, and represent multiple donors ($n>3$).

of CXCR5 and CCR7, expression of both of these was markedly decreased on splenic and BM PC (Fig. 1b, [15]).

To correlate expression with function, chemotaxis of splenic B cells in response to ligands for CXCR4 (CXCL12), CXCR5 (CXCL13) and CCR7 (CCL21) was assessed. Total B ($CD20^{+}$) cells underwent basal migration in the absence of any exogenous chemokine (10.9±1.8% migrating cells; Fig. 2a–c). However, an increasing proportion of $CD20^{+}$ cells migrated towards CXCL12, CXCL13 and CCL21 in a dose-dependent manner (Fig. 2a–c). Both naive and memory B cells migrated towards CXCL12, CXCL13 and CCL21; however, significantly more memory cells migrated at most chemokine concentrations (CXCL12, CCL19 $p<0.01$, CXCL13 $p<0.05$; Fig. 2a–c). Indeed, the chemotactic index of memory cells generally exceeded that of naive cells (Fig. 2d–f). Splenic PC underwent significantly less basal migration than naive or memory B cells (3.9% *vs.*

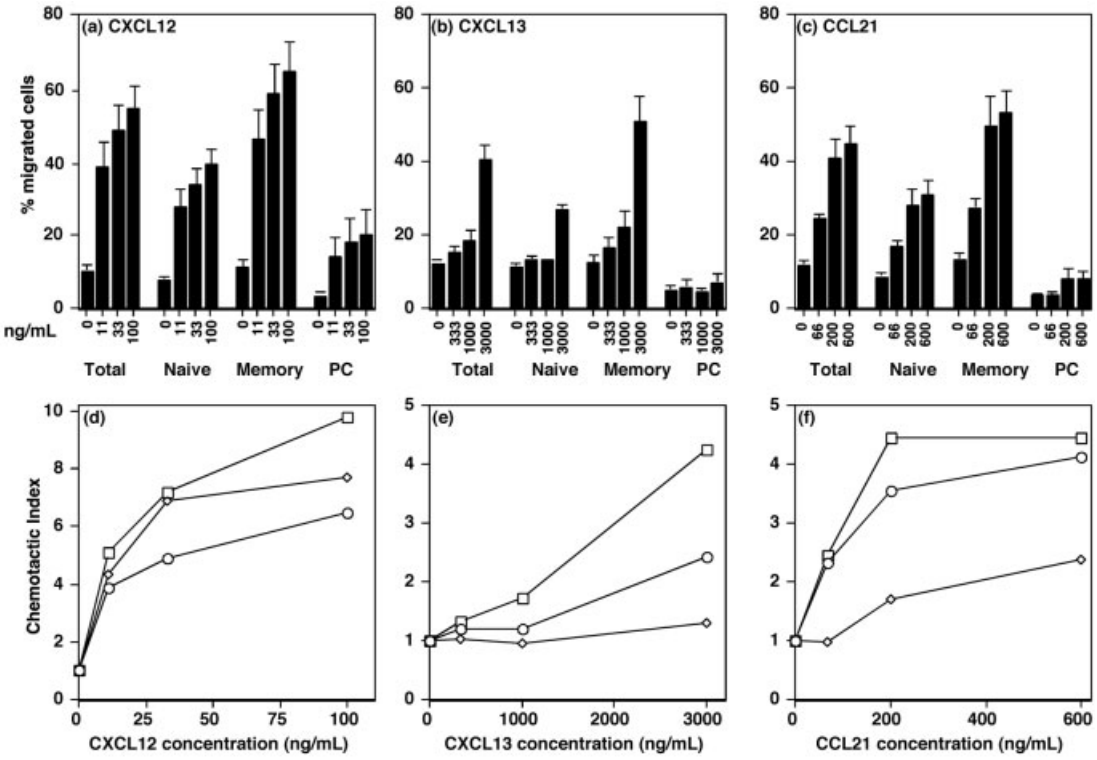

Fig. 2. Splenic PC exhibit selective migration towards CXCL12. Splenic MNC (10^6/well) were loaded into the upper chamber of a transwell, and media or different concentrations of (a, d) CXCL12, (b, e) CXCL13 or (c, f) CCL21 were added to the lower wells. Cells were allowed to migrate for 4 h. Migrated cells were harvested from the lower wells and stained with mAb specific for CD27, CD38 and CD20, enabling resolution of total, naive, memory and PC. (a–c) The values represent the mean percentage of cells (± SEM) that migrated to each chemokine concentration. (d–f) The chemotactic index of naive (○), memory (□) and PC (◇) B cells in response to the different concentrations of each chemokine was calculated as chemokine-induced migration/basal migration. These results are the mean (± SEM for a–c) of 6 (CXCL12), 3 (CXCL13) or 5 (CCL21) experiments performed using cells from different donor spleens.

8.5% naive, 11.9% memory B cells; $p<0.05$, $n=8$). Similarly, the proportion of splenic PC migrating towards all concentrations of CXCL12 was less than migrating naive and memory B cells (naive *vs*. PC: $p<0.05$, Memory *vs*. PC: $p<0.001$, $n=6$; Fig. 2a). However, the chemotactic indices of the PC resembled those of mature B cells (Fig. 2d). Consistent with their reduced expression of CXCR5 and CCR7 (Fig. 1b), minimal migration of PC towards CXCL13 and CCL21 was observed (Fig. 2b, c, e, f). Thus, splenic PC exhibit selective migration towards CXCL12.

Splenic stromal cells produce CXCL12

Stromal cells derived from BM and lymph node produce CXCL12 [15, 26], yet expression by human splenic stromal cells is unknown. Stromal cells generated from splenic MNC were found to contain abundant levels of CXCL12 message (Fig. 3a, upper panel, lane 8). The weak signal detected in total MNC may be attributable to small numbers of stromal cells in that population (Fig. 3a, upper panel, lane 5). In contrast, naive B, memory B, PC, activated $CD4^+$ and $CD8^+$ T cells did not express CXCL12 (Fig. 3a, upper panel). The selective presence of CXCL12 message in human splenic stromal cells was consistently observed for all stromal lines generated (Fig. 3b, upper panel, lanes 4–7), indicating they are a unique source of this chemokine.

To determine whether the CXCL12 message was associated with protein production, the amount of CXCL12 secreted by cultured stromal cell lines was determined. All stromal cells ($n=6$) produced detectable amounts of CXCL12 (mean 3 ng/ml; Fig. 3c). Although this amount was less than that tested in chemotaxis assays using purified CXCL12, concentrations as low as 300 pg/ml induced B cell migration (not shown). Next, the biological activity of secreted CXCL12 present in supernatants from cultured stromal cells was investigated. Supernatants from different stromal cell lines contained B cell chemotactic activity that was abolished by neutralizing anti-CXCL12 mAb (Fig. 3d, e). Thus, the ability of PC to respond to CXCL12, but not CXCL13 or CCL21 (Fig. 2), coupled with production of CXCL12 by stromal cells (Fig. 3) and the positioning of PC within the RP [6], strongly suggests that stromal cell-derived CXCL12 guides PC to this region of the spleen.

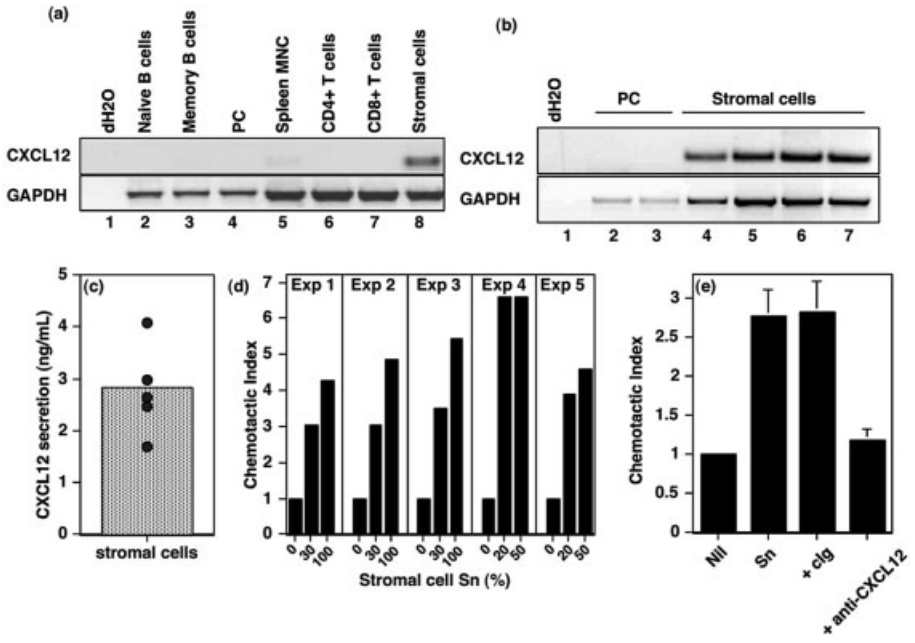

Fig. 3. Human splenic stromal cells are a source of biologically active CXCL12. (a) RNA was extracted from naive or memory B cells, PC, MNC, activated $CD4^+$ or $CD8^+$ T cells or stromal cells and transcribed into cDNA which was used as template to determine the expression of CXCL12 (upper panel) and GAPDH (bottom panel). (b) RNA was extracted from splenic PC (lanes 2, 3) and stromal cells (lanes 4–7) isolated from different donors and transcribed into cDNA to determine the expression of CXCL12 (upper panel) and GAPDH (bottom panel). dH_2O (lane 1) was used as a negative control. (c) The amount of CXCL12 secreted by splenic stromal cells was determined by ELISA. Each dot represents secretion by stromal cells from a different spleen; the column is the mean. (d) Supernatants (Sn) were collected from cultured human stromal cell lines and assayed for the ability to induce chemotaxis of human splenic B cells. The data represent migration of splenic B cells (chemotactic index) in response to increasing concentrations (depicted as % culture medium) of stromal cell Sn. Each experiment (Expt) utilized a Sn generated from a different stromal cell line; different donor B cells were used for Expts 1–3, and 4–5. (e) The chemotactic activity of stromal cell supernatants (Sn) on human splenic B cells was assayed in the presence of a control mAb (+ cIg) or a neutralizing anti-CXCL12 mAb (+ anti-CXCL12). The Nil culture represent basal migration. The data represent the mean chemotactic index ± SEM of experiments using supernatants from three different stromal cell lines.

Stromal cells increase Ig secretion by splenic PC through production of IL-6

Ig production by human PC can be increased by culturing with BM or synovial stromal cells [7, 27]. Therefore, the effect of spleen-derived stromal cells on Ig production by PC was determined. When cultured *in vitro*, splenic PC spontaneously secreted significant amounts of Ig (Fig. 4a–c). Addition of stromal cells enhanced the levels of all Ig isotypes by 3.0±1.1 fold (n=6, range 1.8–5.4; Fig. 4a–c).

It was previously demonstrated that human BM stromal cells supported PC survival through cell-cell interactions [28] and production of cytokines [27, 29]. To determine how stromal cells increased Ig production by splenic PC, co-cultures of these cells were performed in the presence of neutralizing mAb to IL-6 and IL-10, or a blocking VLA-4 mAb. Anti-IL-6 mAb reduced the stromal cell-induced increase in IgM, IgG and IgA secretion by 65–90% (Fig. 4b). On the other hand, neutralizing IL-10 or blocking VLA4 had minimal effect (data not shown). To determine whether IL-6 is produced by stromal cells or PC, supernatants were collected from cultured stromal cells and assayed for their ability to support Ig secretion. Ig secretion by splenic PC was increased >3-fold in the presence of stromal cell supernatants compared to cultures performed in media alone. This activity was comparable to that of intact stromal cells (Fig. 4c) and abrogated by neutralizing anti-IL-6 mAb (Fig. 4d). In contrast, mAb to other soluble factors (IL-10, CXCL12) had no effect (not shown). The amount of IL-6 secreted by cultured stromal cell lines was in the range previously found to enhance Ig production by human splenic PC (~10 ng/ml [6]; Fig. 4e). Thus, stromal cells increase PC Ig secretion primarily through IL-6 production.

BAFF does not enhance PC function

BAFF (B cell-activating factor belonging to the TNF family) is a potent B cell survival factor [30], and its production by stromal cells is sufficient for the development of mature B cells [31]. BAFF achieves its affect by binding one of three receptors: BAFF-R, TACI (transmembrane activator of CAML interactor), and BCMA (B cell maturation Ag) [30]. To determine

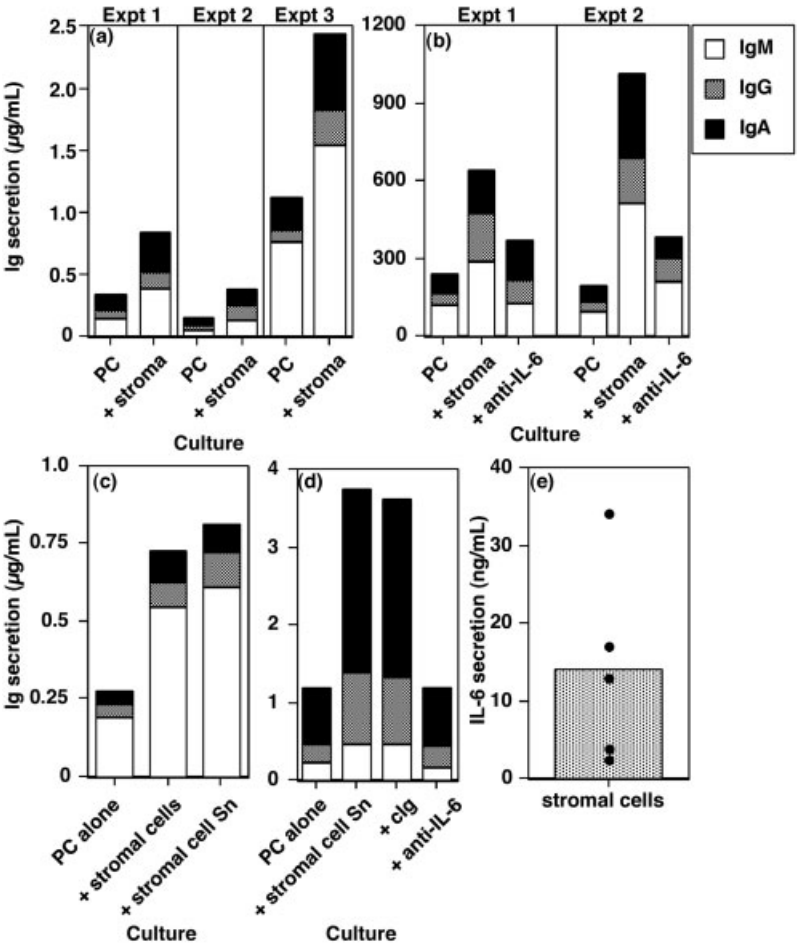

Fig. 4. Stromal cells augment Ig production by PC by the production of IL-6. PC were isolated and cultured (3×10^3–5×10^3 cells/well/200 μl) in medium only (PC), or with (a) irradiated stromal cells alone (+ stroma) or (b) in the presence of an anti-IL-6 mAb (+ anti-IL-6), (c) irradiated stromal cells, or cell-free supernatant (Sn) collected from cultured stromal cells, or (d) stromal-cell supernatant in the absence of presence of a control Ig (cIgG) or neutralizing anti-IL-6 mAb. After 5 days, the secretion of IgM (□), IgG (■) and IgA (■) was determined by ELISA. The values represent the mean of duplicate cultures from experiments performed using $CD38^{++}CD20^{+/-}$ cells and stromal cell lines obtained from different donor spleens. (e) Stromal cells were expanded from spleen MNC and the amount of IL-6 produced was determined by ELISA. Each dot represents secretion by stromal cells from a different spleen; the column is the mean.

whether BAFF contributes to the survival and function of human PC, we examined expression of BAFF receptors on B cell subsets. Soluble BAFF bound to naive and memory B cells (Fig. 5a, b). This was likely mediated by BAFF-R as these cells expressed high levels of BAFF-R but neither TACI nor BCMA (Fig. 5a, b). In contrast, PC in spleen (Fig. 5c) and BM (not shown) did not bind BAFF or express BAFF-R, TACI or BCMA. Consistent with this, the basal levels of Ig secreted by PC were not enhanced by BAFF (Table 1). Together, these data demonstrate that PC lack expression of BAFF receptors and, consequently, BAFF has no effect on their function.

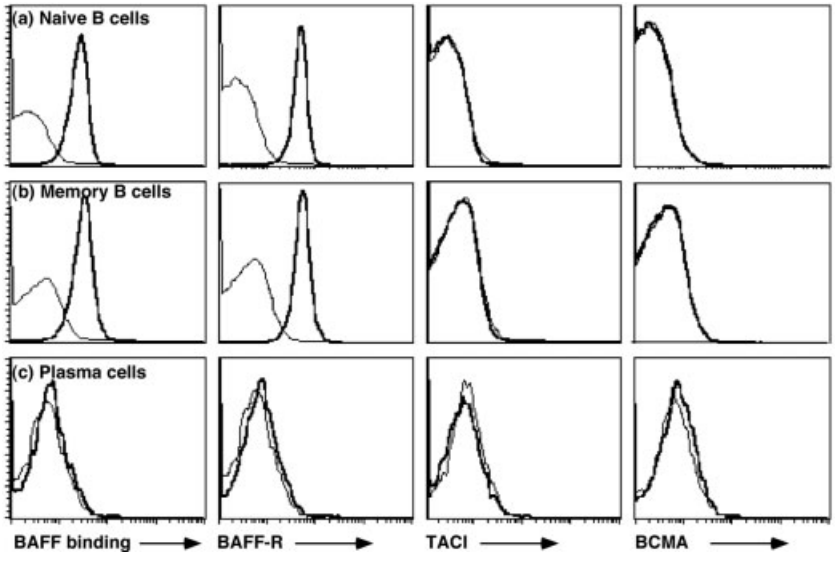

Fig. 5. Human PC down-regulate expression of BAFF receptors. Splenic MNC were incubated with anti-CD38, anti-CD20 and anti-CD27 mAb plus soluble BAFF protein or mAb specific for BAFF-R, TACI or BCMA. (a–c) Binding of soluble BAFF to, and expression of BAFF-R, TACI and BCMA on, (a) naive B cells, (b) memory B cells and (c) PC was determined by gating on $CD20^{+}CD27^{-}$, $CD20^{+}CD27^{+}$ and $CD38^{++}CD20^{+/-}$ B cells, respectively. For each plot, the thick and thin histograms represent the fluorescence of cells incubated with the specific and control reagent, respectively.

Table 1. BAFF does not enhance Ig production by primary human PC[a)]

	Ig secretion (ng/ml)					
	IgM		IgG		IgA	
	– BAFF	+ BAFF	– BAFF	+ BAFF	– BAFF	+ BAFF
Expt 1	170	138	3.8	3.0	10.0	5.0
Expt 2	287	250	9.7	7.0	136	117
Expt 3	123	80	40	28	93	99
Expt 4	301	409	21.3	24.3	87	73
Mean ± SEM	220±43	219±72	18.7±8	15.6±6.2	82±26	74±25
Expt 5	7.3	10	133	152	28.5	24.0

a) $CD38^{++}CD20^{+/-}$ cells ($\sim 5\times10^3$) from human spleens (Expt 1–4; mean ± SEM) or BM (Expt 5) were isolated by cell sorting and cultured for 5 days in media in the absence (– BAFF) or presence of soluble BAFF (+ BAFF). The levels of secreted IgM, IgG and IgA were determined by ELISA. Each value represents the mean of duplicate cultures.

Splenic, but not BM, PC express CD11a

The data presented so far indicate that stromal cells could attract splenic PC to the RP and support their function by producing CXCL12 and IL-6. However, PC in human spleen (Fig. 2), tonsil, small intestine [8], and BM [15] all migrate towards CXCL12. Thus, the exact mechanism by which PC are retained within the splenic

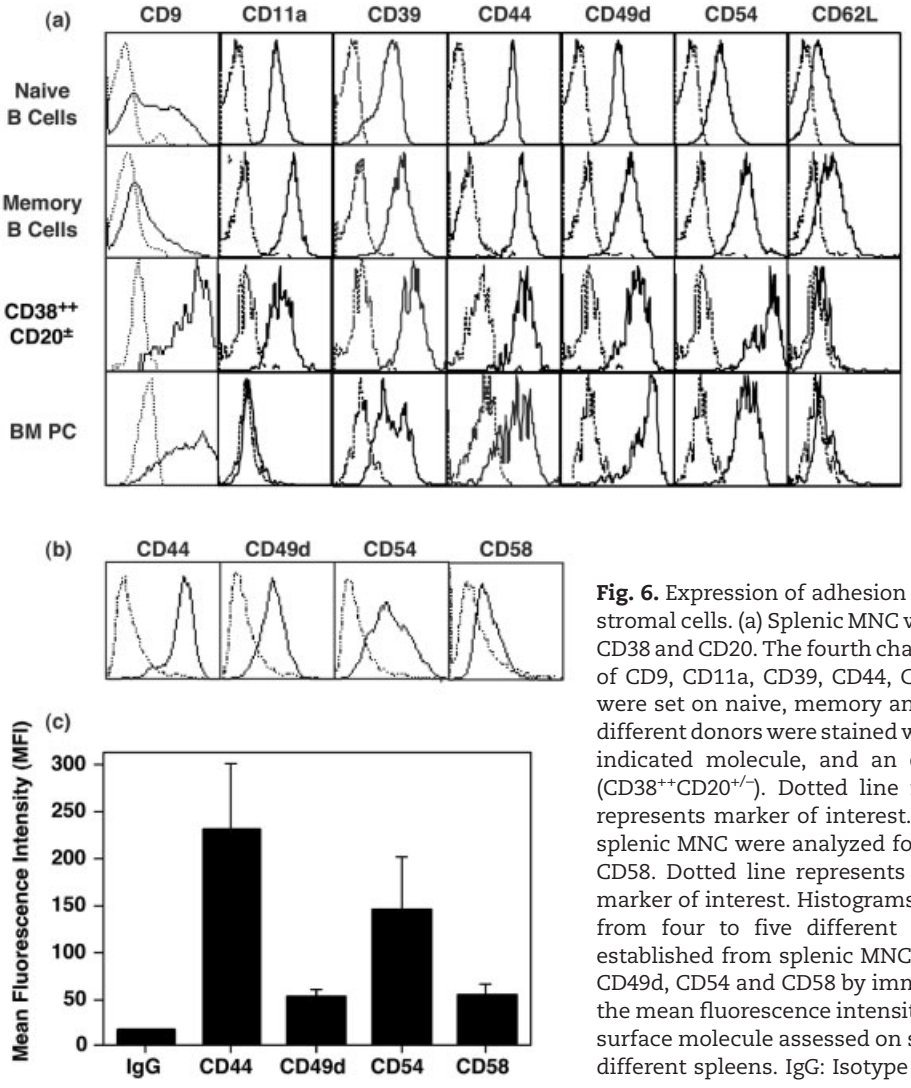

Fig. 6. Expression of adhesion molecules by B cell subsets and splenic stromal cells. (a) Splenic MNC were stained with mAb specific for CD27, CD38 and CD20. The fourth channel was then used to assess expression of CD9, CD11a, CD39, CD44, CD49d, CD54 or CD62L. Electronic gates were set on naive, memory and $CD38^{++}CD20^{+/-}$ B cells. BM MNC from different donors were stained with mAb specific for CD20, CD38 and the indicated molecule, and an electronic gate set to identify BM PC ($CD38^{++}CD20^{+/-}$). Dotted line represents negative control, solid line represents marker of interest. (b) Stromal cell lines established from splenic MNC were analyzed for expression of CD44, CD49d, CD54 and CD58. Dotted line represents negative control, solid line represents marker of interest. Histograms are representative of stromal cell lines from four to five different donor spleens. (c) Stromal cell lines established from splenic MNC were analyzed for expression of CD44, CD49d, CD54 and CD58 by immunofluorescence. The values represent the mean fluorescence intensity (± SEM) of expression of the indicated surface molecule assessed on stromal cell lines established from up to different spleens. IgG: Isotype control.

RP, rather than migrating to other tissues, is unclear. Because PC in mucosal tissues, but not in BM, express α4β7 [20, 21], it has been proposed that interactions between α4β7 and MAdCAM-1 on intestinal endothelium mediate the retention of PC within these sites [4, 8]. We therefore hypothesized that differential expression of adhesion molecules on splenic and BM PC might be responsible for the alternative positioning of these cells. Our analysis revealed that CD11a was present on naive B cells, memory B cells and PC in spleen, but was undetectable on PC in the BM (Fig. 6a). Other molecules involved in cell interactions and homing (CD39, CD44, CD49d, CD54) were expressed on naive and memory B cells as well as splenic and BM PC, the only difference being the elevated levels of CD39 and CD54 on splenic compared to BM PC (Fig. 6a). CD62L was expressed by B cells, but not PC, while CD9 was expressed by some naive and memory B cells, but all splenic and BM PC (Fig. 6a). Thus, CD11a is uniquely expressed by PC in human spleen.

Expression of adhesion molecules on splenic stromal cells was also examined to determine whether specific receptor-ligand interactions could potentially occur between PC and stromal cells. Stromal cells from different donor spleens were found to express high levels of CD44, which recognizes hyaluronan [32], and CD54, the counter-structure of CD11a/CD18 (Fig. 6b, c). These cells also expressed CD49d and CD58, albeit at lower levels than CD44 and CD54 (Fig. 6b, c). Thus, the selective expression of CD11a by splenic, but not BM, PC may contribute to their retention in the spleen by interacting with CD54-expressing stromal cells.

Splenic PC localize with CD54$^+$ stromal cells in the red pulp

It was next examined whether stromal cells and PC co-localize in the spleen. Sections of human spleen were labeled with Ab specific for CD54, to identify stromal cells in the red pulp [33, 34], and IgM, which identifies sIgM$^+$ B cells in the follicles and intracellular IgM^{hi} PC in the RP (Fig. 7a) [6]. This analysis demonstrated that PC were adjacent to, and surrounded by, CD54$^+$ stromal cells in the RP (Fig. 7a, b). In fact, CD54$^+$ stromal cells and PC appear to form cell contacts (Fig. 7b). Thus, CD54 expression by stromal cells in the splenic RP may facilitate the retention of CD11a-expressing PC in this region of the spleen.

Discussion

The unique positioning of cells in defined anatomical compartments contributes to the development of specific and appropriate immune responses. Here, we examined the mechanisms underlying the localization of PC within the RP of human spleen. Splenic PC expressed the chemokine receptors CXCR4, but not CXCR5 or CCR7 (Fig. 1b) and exhibited chemotactic responses similar to PC from murine spleen or human BM [12, 13, 15, 25] as they migrated towards CXCL12, while responses to CXCL13 and CCL21 were minimal (Fig. 2). In mice, CXCR5 and CCR7 ligands are produced by stromal cells and DC, respectively, present in lymphoid follicles [3, 4]. On the other hand, CXCL12 is expressed by splenic stromal cells positioned alongside PC in the RP (Figs. 3, 7; [12]). Thus, reduced expression of CXCR5 and CCR7 and responsiveness to their ligands, coupled with continued expression of CXCR4 and responsiveness to CXCL12, is consistent with the positioning of human splenic PC in the RP rather than the white pulp [6]. The positioning of intestinal PC is believed to result from the concentrated expression of CCL25 and CCL28 in mucosal tissues and the acquired expression of CCR10 and CCR9 by IgA-PC [3, 4, 8, 13]. Interestingly, microarray analysis of human B cell

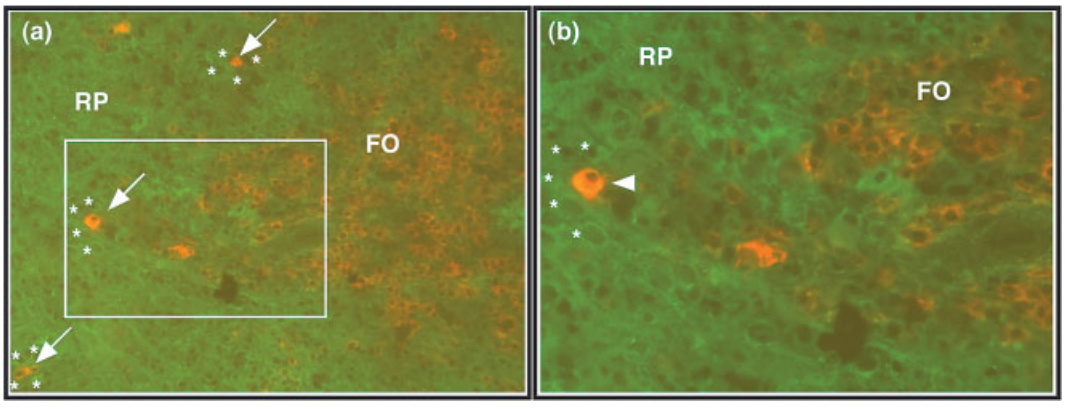

Fig. 7. Splenic PC and stromal cells co-localize in the red pulp. Immunofluorescence staining was performed on spleen sections obtained from a normal healthy donor using anti-IgM (red) and anti-CD54 mAb (green). The follicle (FO), red pulp (RP), PC (arrows) and stromal cells (*), are indicated. Original magnification: (a) ×20; the boxed region of (a) is presented at ×40 magnification in (b).

subsets revealed that splenic PC, but not naive or memory B cells, expressed CCR10 (manuscript submitted), in addition to CXCR4 (Fig. 2). However, the CCR10 ligand, CCL28, exhibits minimal expression in spleen ([15–18], not shown), thus making it unlikely that interactions between this receptor/ligand pair would contribute to the localization of PC to the splenic RP.

Analysis of mature B cells revealed that some memory B cells, but not naive B cells or PC, expressed the inflammatory chemokine receptor CXCR3 (Fig. 1b). Preferential expression of CXCR3 by memory B cells may explain their increased frequency in inflammatory sites in human autoimmune diseases [35]. In contrast, there was no difference in expression of CXCR4, CXCR5 and CCR7 by naive and memory B cells (Fig. 1b), and both populations underwent migration towards the corresponding ligands (Fig. 2); however, the response of memory cells exceeded that of naive cells. The lack of expression of CCR10 by naive and memory B cells (manuscript submitted) is consistent with the lack of chemotactic activity of CCL28 on these B cells [18].

The importance of adhesion molecules and integrins, in addition to chemokine receptors, in the positioning of lymphocytes within lymphoid tissues is well recognized [3, 4, 33]. Our results also point to an important role for adhesion molecules in the positioning of splenic PC in the RP. Splenic PC expressed high levels of CD9, CD11a, CD44, CD49d and CD54 (Fig. 6a). By contrast, CD11a was lost from the surface of PC that migrated to the BM (Fig. 6a). CD11a heterodimerizes with CD18 to form LFA-1 which interacts with CD54, while CD49d and CD29 associate to form VLA-4, which recognizes VCAM-1 and fibronectin [33]. CD54 is expressed on stromal cells in splenic RP (Figs. 6, 7) [33, 34]. Consequently, PC may be retained in the RP of human spleen, rather than migrating to the BM, by adhesive interactions between CD11a/CD18 and CD54. This parallels the acquired expression of $\alpha4\beta7$ by intestinal-homing PC and of its ligand MadCAM-1 on intestinal endothelium as a mechanism for the positioning of PC within mucosal tissue [4, 8, 20, 21]. This is also reminiscent of the increased level of expression of specific integrins, including CD11a, on marginal zone (MZ) B cells, compared to follicular B cells, and their contribution to retaining B cells in the splenic MZ [33]. Notably, increased expression of CD11a by MZ B cells resulted in their increased adhesion to ICAM-1 compared to follicular B cells [33]. Extrapolating to PC, it would be anticipated that the selective expression of CD11a on splenic, but not BM, PC would contribute to their positioning and retention alongside $CD54^+$ stromal cells within the splenic RP (Fig. 7). A striking difference between PC from different anatomical sites is the expression of CD62L by those in peripheral blood [19, 22], but not spleen (Fig. 6a). CD62L is required for entry into lymphoid tissues through HEV [22]; the differential expression of CD62L suggests that circulating PC are tissue-homing cells, most likely to the BM, while those in the spleen were generated in that tissue and remain there as resident PC.

Stromal cells may also provide a microenvironment conducive to PC survival and Ig secretion, an effect achieved mainly through production of IL-6 (Fig. 4) [27, 29, 36–38]. Stroma-derived factors other than IL-6 are also likely to play important roles in maintaining the survival and function of PC, evidenced by the finding that (i) IL-6-deficient stromal cells could modestly support the survival of BM PC [38], and (ii) neutralizing IL-6 mAb did not completely abolish the ability of splenic stromal cells to improve Ig secretion by PC (Fig. 4). Although stroma-derived factors such as BAFF and CXCL12 are important survival factors for murine BM PC [37, 39], we did not find an effect of these molecules on Ig production by human splenic PC (Table 1). The lack of an effect of BAFF was consistent with reduced binding of soluble BAFF and expression of BAFF receptors on human PC (Fig. 5c). The ability of BAFF to enhance survival of murine, but not human, PC may reflect species-specific differences in the expression pattern of BAFF receptors (Fig. 5) [39]. BAFF can indeed support survival of human Ig-secreting cells (ISC); however, the specific target of BAFF in this situation appears to be plasmablasts [40], rather than PC (Table 1). Thus, ISC alter their requirements for survival as plasmablasts differentiate into PC. This change in sensitivity to different survival factors is underscored by the discrete anatomical location of plasmablasts and PC within secondary lymphoid tissues. Thus, plasmablasts associate with DC capable of producing BAFF in extrafollicular areas of the spleen [41], while PC migrate to the RP of the spleen, or BM, where their longevity likely becomes dependent on resident stromal cells producing IL-6 (Fig. 4) [38].

Based on our data, we propose a scenario whereby splenic stroma sequentially provide a chemotactic gradient for migration of PC from their site of generation within lymphoid follicles to the RP and then cytokines, such as IL-6, play a role in their survival and function. Although human PC present in diverse anatomical locations can all migrate towards CXCL12, the selective positioning of these cells is likely regulated by the co-ordinated expression of adhesion molecules and chemokine receptors.

Materials and methods

Reagents

The following were used in this study: purified anti-CD54 mAb, FITC-anti-CD20 and CD27, PE-anti-CD54 and CD58 mAb (Becton Dickinson, San Jose, CA); purified anti-CCR7 mAb, FITC- and PE-isotype controls, PE-anti-mouse IgM, PE-anti-human CD11a, CD27, CXCR3 and CXCR4 mAb, biotinylated anti-CD44 mAb, streptavidin (SA)-conjugated PerCp (BD PharMingen, San Diego, CA); PE-anti-CD38, anti-CD62L mAb, biotinylated isotype control, biotinylated anti-CD38 mAb, APC-conjugated isotype control, anti-CD20 and CD38 mAb (Caltag, Burlingame, CA); PE-anti-CD9 mAb (e-Bioscience, San Diego, CA); biotinylated anti-CXCR5 mAb, (R&D Systems, Minneapolis, MN). Recombinant human BAFF, anti-BAFF-R, anti-TACI and anti-BCMA mAb [40] were provided by Susan Kalled (Biogen, Cambridge, MA). IL-6, CXCL12 and CCL21 (Peprotech, Rocky Hill, NJ); CXCL13, anti-IL-6 and anti-CXCL12 mAb (clone 79014.111; R&D Systems).

Cells

MNC were prepared from normal human spleens and BM [6, 40]. PC were isolated by sorting $CD38^{++}CD20^{+/-}$ cells [9] on a FACStar^{Plus} or FACSVantage after labeling MNC with anti-CD20 and anti-CD38 mAb [6]. Stromal cells were generated by culturing splenic MNC (4×10^6/ml) in RPMI 1640 containing 20% FCS [34]. Nonadherent cells were removed after 4–7 days, and medium replaced. Cells were collected 3–4 weeks following initial culture by treating the culture flasks with PBS/0.5 mM EDTA. The expanded stromal cells were $CD40^{dim}$, $CD44^+$, $CD49d^+$, $CD54^+$, $CD58^+$ and lacked expression of CD3, CD14, CD19, CD21, CD45, CD62L and HLA-DR, (not shown) [34], demonstrating they are neither macrophages or DC. To generate conditioned supernatant, stromal cells were seeded ($\sim10^6$) in T25 culture flasks in RPMI 1640/20% FCS [34]. After 2 weeks the adherent cells were washed, and then cultured for an additional 4 days in RPMI 1640 containing 0.5% BSA (chemotaxis media; see below). FCS was avoided because it inhibited chemotaxis.

Immunofluorescence staining

Phenotypic analysis of spleen and BM MNC, and histological analysis of spleen sections, were performed as described [6, 40].

Chemotaxis assays

Chemotaxis assays were performed using 5 μm Transwell plates (Corning, NY). CXCL12, CXCL13, CCL21 or stromal cell supernatant, in the presence and absence of neutralizing anti-CXCL12 mAb (100 μg/ml) or an isotype control mAb, were diluted in chemotaxis media and added to wells of a 24-well plate in 600 μl. Chemotaxis media was used as a control for basal cell migration. Splenocytes were cultured overnight, resuspended in chemotaxis media, and 10^6 cells were added to the upper chamber of the transwell in 100 μl. Plates were incubated at 37°C in a humidified atmosphere containing 5% CO_2 for 4 h. A known number of Calibrite beads (Becton Dickinson) was then added to each bottom well before harvesting and the number of migrated B cells was calculated as a function of the ratio of beads to live cells. The migrated population was stained with anti-CD27, CD38 and CD20 mAb to determine the proportions of naive, memory and $CD38^{++}CD20^{+/-}$ B cells, which were then used to calculate the absolute number of cells of each subset that had migrated. Cell migration was calculated as the percentage of input cells. Chemotactic indices were calculated as the number of cells migrating in the presence of chemokine divided by the number of cells migrating in its absence.

Expression of CXCL12 and IL-6 by stromal cells

Total RNA was extracted from different cell populations and then transcribed into cDNA [6]. CXCL12 cDNA was detected by PCR (primers: 5′ GAA CGC CAA GGT CGT GGT, 3′ CAG CCG GGC TAC AAT CTG A; Sigma-Aldrich). Amplification of GAPDH [6] served as a positive control. All reactions were performed using REDTaq (Sigma) in a Thermal Cycler (MJ Research, Waltham, MA). Secretion of CXCL12 and IL-6 by stromal cells was determined by ELISA.

Ig ELISA

Sorted PC were cultured ($\sim5\times10^3$ cells/well) in round-bottom 96-well plates (Becton Dickinson Labware, Franklin Lakes, NJ) alone or with irradiated (5,000 rad; [34]) stromal cells. In some cultures, neutralizing anti-IL-6 mAb or an isotype control mAb was included (20 μg/ml), or the cultures were supplemented with soluble human BAFF (2.5 μg/ml), or stromal cell supernatants. After 5 days, the level of secreted IgM, IgG and IgA determined by ELISA [6, 40].

Statistics

Data were analyzed by ANOVA using Prism software (GraphPad Software, San Diego, CA).

Acknowledgements: We thank the Australian Red Cross Blood Service for providing human spleens, Dr. Joy Ho for providing normal human BM, Susan Kalled for reagents, Joseph Webster and Tara Macdonald for cell sorting, Tri Phan for assistance with immunofluorescence, and Tony Basten, Robert Brink, Phil Hodgkin and Tri Phan for critical review of this manuscript. This work was supported by the National Health and Medical Research Council (NHMRC) of Australia. S.G.T. is an NHMRC RD Wright Biomedical Research Fellow.

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