CD38/CD31, the CCL3 and CCL4 Chemokines, and CD49d/Vascular Cell Adhesion Molecule-1 Are Interchained by Sequential Events Sustaining Chronic Lymphocytic Leukemia Cell Survival

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Abstract

CD38 and CD49d are associated negative prognosticators in chronic lymphocytic leukemia (CLL). Despite evidence that both molecules are involved in interactions occurring between CLL and normal cells in the context of CLL-involved tissues, a functional link is still missing. Using gene expression profiles comparing $CD38^{+}CD49d^{+}$ versus $CD38^{-}CD49d^{-}$ CLL cells, we showed overexpression of the CCL3 and CCL4 chemokines in cells from the former group. These chemokines were also up-regulated by CD38 signals in CLL; moreover, CCL3 was expressed by CLL cells from bone marrow biopsies (BMB) of $CD38^{+}CD49d^{+}$ but not $CD38^{-}CD49d^{-}$ cases. High levels of CCR1 and, to a lesser extent, CCR5, the receptors for CCL3 and CCL4, were found in CLL-derived monocyte-macrophages. Consistently, CCL3 increased monocyte migration, and $CD68^{+}$ macrophage infiltration was particularly high in BMB from $CD38^{+}CD49d^{+}$ CLL. Conditioned media from CCL3-stimulated macrophages induced endothelial cells to express vascular cell adhesion molecule-1 (VCAM-1), the CD49d ligand, likely through tumor necrosis factor α overproduction. These effects were apparent in BMB from $CD38^{+}CD49d^{+}$ CLL, where lymphoid infiltrates were characterized by a prominent meshwork of $VCAM\text{-}1^{+}$ stromal/endothelial cells. Lastly, CD49d engagement by VCAM-1 transfectants increased viability of $CD38^{+}CD49d^{+}$ CLL cells. Altogether, CD38 and CD49d can be thought of as parts of a consecutive chain of events ultimately leading to improved survival of CLL cells. [Cancer Res 2009;69(9):4001–9]

Introduction

The life and death economy of cells is mediated by a balanced net of events ruled by surface receptors, adhesion molecules, and soluble factors, among other parameters. This holds true for chronic lymphocytic leukemia (CLL) cells, which derive a relevant part of their proliferative drive from positive interactions with the neighboring nonneoplastic environment (1). Out of the panoply of surface molecules examined in CLL, attention has been recently given to CD49d, a member of the integrin superfamily (2). CD49d regulates adhesion to extracellular matrix by binding fibronectin and to other cells by interacting with vascular cell adhesion molecule-1 (VCAM-1)/CD106 (2). CD49d-dependent interactions have a role in preventing both spontaneous and drug-induced apoptosis of normal or neoplastic B cells (3–5). Moreover, chemokine-induced transmigration of CLL cells across endothelia depends on CD49d expression by CLL cells and is favored by the production of the matrix metalloproteinase-9 as the result of CD49d engagement (6).

CD49d marks a subset of CLL patients characterized by aggressive and accelerated clinical course (7–10), and its expression is strongly associated with CD38 (11–13), a type II transmembrane glycoprotein acting both as ectoenzyme (EC 3.2.2.5) and receptor for CD31 (reviewed in ref. 14). CD38 also represents a dependable negative prognosticator in CLL (15–18); due to its receptorial features, CD38 is part of a network sustaining growth and survival of CLL cells (19). Interactions of $CD38^{+}$ CLL cells with surrounding nonneoplastic $CD31^{+}$ cells is more likely to occur, although not exclusively (20), in peripheral lymphoid organs and/or bone marrow (BM) for several reasons. First, CD38 expression is usually higher in residential as opposed to circulating CLL cells (21–23). Second, both BM and peripheral lymphoid organs can provide accessibility to CD31, as endothelial, stromal, and the so-called nurse-like cells (NLC) all express high-CD31 levels (1, 24, 25).

Although both CD38 and CD49d may be regarded as molecules deeply involved in the continuous interactions taking place between CLL cells and their environment, a functional link between CD49d and CD38 is still missing. By defining the global gene profile of CLL cells coexpressing CD38 and CD49d, we selected the genes for the chemokines CCL3 and CCL4 as overexpressed by $CD38^{+}CD49d^{+}$ CLL cells. Given this starting point, we provided evidence showing a novel prosurvival circuitry operating in $CD38^{+}CD49d^{+}$ CLL, sequentially involving the CD38/CD31 pair, CCL3 and CCL4 with their receptors, and eventually the CD49d/VCAM-1 axes.

Materials and Methods

CLL patients, healthy donors, and cell lines. Peripheral blood (PB) samples from 101 typical CLL (26) entered this study. The main clinical and

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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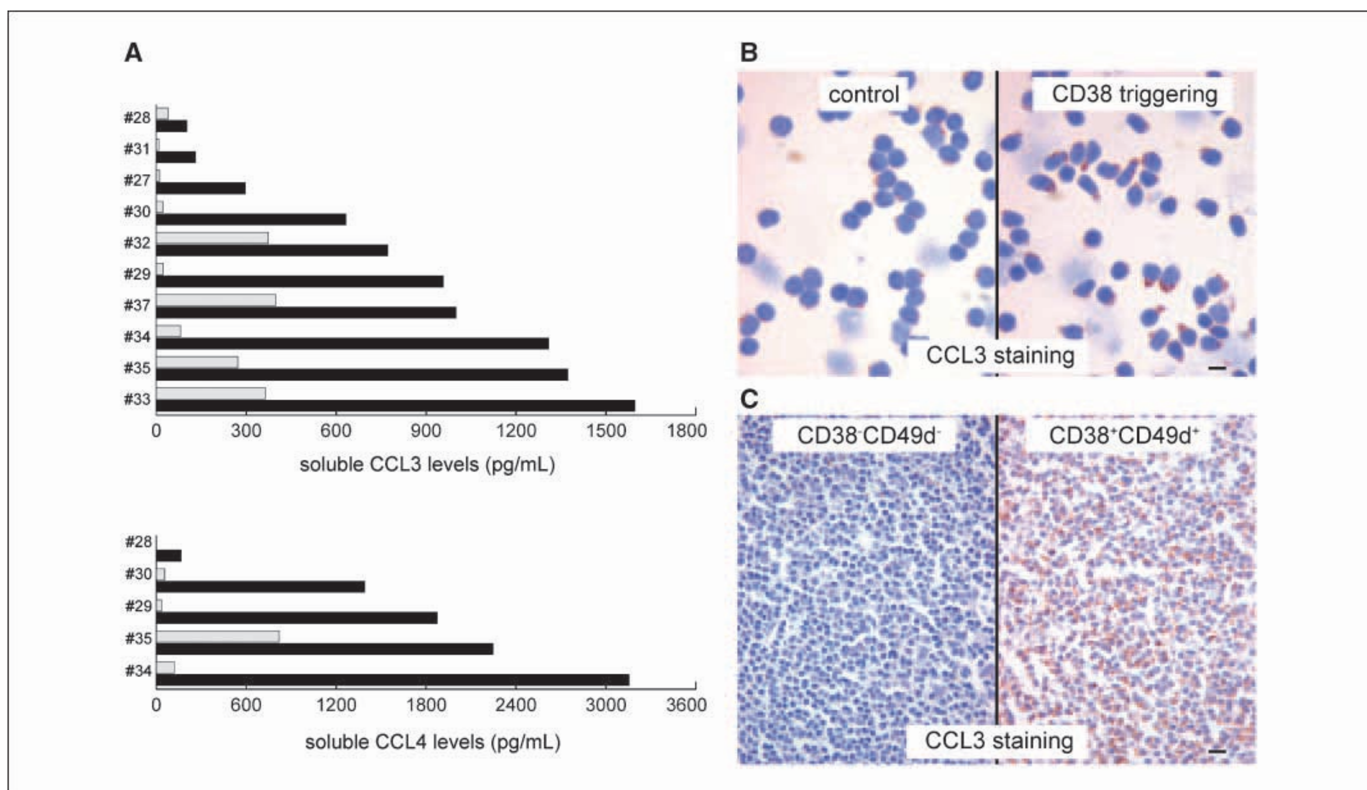

Figure 1. *A,* expression of CCL3 and CCL4 protein by CLL cells. CCL3 (*top*) and CCL4 (*bottom*) levels were evaluated by ELISA in supernatants of CLL cells from CD38$^+$CD49d$^+$ cases triggered for 24 h by either IB4 agonist (*black columns*) or IB6 nonagonist (*gray columns*) anti-CD38 mAbs. *B,* CCL3 staining of CLL cells (case 30) after 24-h cultures with either IB6 nonagonist (*control*) or IB4 agonist (*CD38 triggering*) anti-CD38 mAbs. Original magnification, 1,000×; *scale bar,* 7.5 μm. *C,* CCL3 staining in BM infiltrating CLL cells from cases 42 (CD38$^-$CD49d$^-$; *left*) or 48 (CD38$^+$CD49d$^+$; *right*). Original magnification, 400×; *scale bar,* 20 μm.

biological features of CLL, the experiments for which each sample has been used, are listed in Supplementary Table S1. In 11 cases, matched PB and BM samples were available (Supplementary Table S1). IGHV mutational status and cytogenetic abnormalities were detected as described (7, 27). Patients provided informed consent in accordance with the local institutional review board requirements and the declaration of Helsinki.

PB samples and buffy coats of healthy donors were kindly provided by the blood bank of Centro di Riferimento Oncologico. Purified CLL cells, monocytes from CLL samples, and normal B cells were obtained by immunomagnetic selection using fresh PB samples as starting material (27, 28). Macrophages were obtained by allowing monocytes to adhere in the presence of 0.5 μg/mL lipopolysaccharide (LPS; Sigma).

Human umbilical vascular endothelial cell (HUVEC), microvascular endothelial cells from subcutaneous adipose tissue (ADMEC), and murine L-fibroblast cells transfected with full-length human CD31 (L-CD31), VCAM-1 (L-VCAM-1; courtesy of Pablo Engel, University of Barcelona, Barcelona, Spain), or vector alone (L-mock) were obtained as described (29–31).

Flow cytometry. Sources, specificities, and fluorochrome combinations of monoclonal antibodies (mAb) used for CLL diagnosis and CD38 or CD49d determination were reported elsewhere (7, 10). A CLL sample was defined CD38$^+$ or CD49d$^+$ when the molecule was found in at least 20% of CD5$^+$/CD19$^+$ cells. CD38 and CD49d expression was also investigated in matched PB and BM samples from six CD38$^+$CD49d$^+$ and five CD38$^-$CD49d$^-$ cases (Supplementary Table S1). Although CD38 expression was slightly higher in BM samples of five of six CD38$^+$CD49d$^+$ cases, average expression levels of CD38 and CD49d were comparable between BM and PB samples (not shown). CCR1/CD191 and CCR5/CD195 expression by CLL and healthy donor PB cell subpopulations was evaluated on fresh samples combining CCR5-FITC, CCR1-PE (R&D Systems) with CD3-PerCP, CD19-APC, and CD14-Pe-Cy7 (Becton Dickinson). Data were acquired on a FACSCanto flow cytometer and analyzed by Diva or CellQuest softwares (Becton Dickinson).

Apoptosis was evaluated by sequentially staining CLL cells with Syto16 (Molecular Probes, Invitrogen), anti–CD19-APC mAbs, and 7-amino-actinomycin-D (7-AAD, Becton Dickinson; ref. 32). Viable cells (Syto16^{bright}/7-AAD$^-$) are distinguished from apoptotic (Syto16^{dim}/7-AAD$^-$) or necrotic (Syto16$^-$/7-AAD$^+$) cells in flow cytometry plots (32).

Immunohistochemical and immunocytochemical analyses. BM biopsies (BMB) sections from 20 CLL cases were tested for the expression of CD10, CD20, CD34, CD49d, CD68, VCAM-1, CCR1, CCL3. CCL3 expression was also tested on cytospin preparations of purified CLL cells treated *in vitro* with anti-CD38 mAbs. Staining procedures are reported in Supplementary Materials and Methods.

Two different semiquantitative four-tier grading systems were used for the immunohistochemical evaluation of CD68 and VCAM-1 staining within CLL-infiltrated areas. Both systems relied on the analysis of positive cells on five randomly selected high-power microscopic fields (magnification, 400×) showing the highest degree of lymphoid infiltration. CD68 was graded as follows: grade 0, absence of CD68$^+$ cells; grade 1, up to 20 CD68$^+$ cells; grade 2, 21 to 40 CD68$^+$ cells; grade 3, >40 CD68$^+$ cells. VCAM-1 grading system was as follows: grade 0, absence of VCAM-1$^+$ cells; grade 1, one or more scattered VCAM-1$^+$ cells forming an incomplete meshwork; grade 2, detection of a diffuse but meager meshwork of VCAM-1$^+$ cells; grade 3, detection of a diffuse and dense meshwork of VCAM-1$^+$ cells.

CCL3 assessment on BMB was similarly performed in CLL-infiltrated areas. CCL3$^+$ cases showed a clear cytoplasmic reactivity to anti-CCL3 antibodies in most CLL cells. Scattered CCL3$^+$ cells (e.g., infiltrating T cells) could be present also in CCL3$^-$ cases.

CD38 and CD49d engagement. Purified CD38$^+$CD49d$^+$ CLL cells were incubated with either the agonist IB4 or the nonagonist IB6 (5 μg/10^6 cells) anti-CD38 mAbs or plated onto mytomicin-C–treated L-CD31, L-VCAM-1, and L-mock cells (2.5 × 10^5 per well). Both CLL cells and supernatants were collected after 14 and 24 h. For spontaneous

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apoptosis evaluation, cells were collected after 1 to 3 wk of coculture with L-VCAM-1 or L-mock cells.

ELISA. The amounts of CCL3 and CCL4 proteins in CLL cell culture supernatants and tumor necrosis factor α (TNFα) in macrophage-derived conditioned media (CM) were determined by ELISA (Bender Medsystems GmbH). Expression of CCR1 and CCR5 by adherent macrophages and VCAM-1 by endothelial cells was evaluated by an ad hoc ELISA (30) using unconjugated anti-CCR1, anti-CCR5 (R&D Systems), or anti–VCAM-1 (Chemicon) mAbs.

Macrophage-derived CM and monocyte chemotaxis assay. To obtain macrophage-derived CM, macrophages were cultured for 24 to 48 h with either 50 ng/mL of recombinant human CCL3 (R&D Systems; CCL3-CM), 0.5 μg/mL LPS (LPS-CM), or medium alone (CNT-CM). Subconfluent HUVEC and ADMEC were incubated for 12 h with 100 μL/well of CM and evaluated for VCAM-1 expression. In selected experiments, CM were preincubated with an antihuman TNFα neutralizing mAb (R&D Systems; 20 ng antibody/1 mL CM for 30 min at 37°C).

Responsiveness of purified monocytes to CCL3 and CCL4 (3 ng/mL) was investigated by a 2-h chemotaxis assay, carried out in 24-well 5-μm pore size Transwell plates (Costar), as reported (28). Results were expressed as migration index (MI), i.e., transmigrating cells in the presence of chemokine divided by transmigrating cells in the absence of chemokine.

Results

CCL3 and CCL4 are overexpressed in $CD38^+CD49d^+$ CLL cells. A gene expression profile of purified CLL cells from 26 cases (11 $CD38^+CD49d^+$ and 15 $CD38^-CD49d^-$; Supplementary Table S1) was performed (see Supplementary Materials and Methods). Results indicated 294 differentially expressed genes: 89 down-regulated and 205 up-regulated in the $CD38^+CD49d^+$ group. A hierarchical clustering, generated using these 294 best-correlated genes, clearly split the 11 $CD38^+CD49d^+$ from the 15 $CD38^-CD49d^-$ CLL (Supplementary Fig. S1*A* and Supplementary Table S2). Among the genes up-regulated in $CD38^+CD49d^+$ CLL, we found the *CD38* and *CD49d* genes and several genes previously identified as related either to the $CD38^+$ phenotype and/or poor prognosis, such as lipoprotein lipase, Septin-10, activation-induced cytidine deaminase, and spastic paraplegia 20 (Supplementary Table S2; refs. 33, 34).

To further elucidate the biological functions of genes representing the expression signature of $CD38^+CD49d^+$ CLL, the identifiers for the 294 best-correlated genes were linked to the "Onto-Express" bioinformatics tool (35). Biological processes showing the highest significant enrichment for genes found to be differentially expressed between $CD38^+CD49d^+$ and $CD38^-CD49d^-$ CLL are listed in Supplementary Table S3. Of note, the genes for the chemokines *CCL3* and *CCL4* were constantly selected as overexpressed by the $CD38^+CD49d^+$ CLL group (CCL3 mean fold change, 3.47; CCL4 mean fold change, 4.36; Supplementary Table S3). Quantitative real time–PCR (QRT-PCR) experiments, carried out in 37 CLL samples (22 $CD38^+CD49d^+$ and 15 $CD38^-CD49d^-$; Supplementary Table S1), confirmed the constitutive higher expression of both CCL3 and CCL4 transcripts in $CD38^+CD49d^+$ CLL (Supplementary Fig. S1*B*). Such a higher expression was also confirmed by comparing CCL3 and CCL4 transcript levels in BM samples from six $CD38^+CD49d^+$ and five $CD38^-CD49d^-$ cases, without significant differences with the corresponding PB samples (Supplementary Fig. S1*C*). These data, along with the notion of a role played by chemokines in favoring microenvironmental interactions of CLL cells (28, 36, 37), suggested to investigate in detail the function of CCL3 and CCL4 in the pathophysiology of $CD38^+CD49d^+$ CLL.

CCL3 and CCL4 are up-regulated by CD38 engagement in $CD38^+CD49d^+$ CLL cells. The next step was the analysis of CCL3 and CCL4 mRNA and protein modulation upon CD38 ligation in $CD38^+CD49d^+$ CLL cells. Purified CLL cells from 11 $CD38^+CD49d^+$ CLL cases (Supplementary Table S1) were cultured either with the agonist IB4 mAb or the nonagonist IB6 anti-CD38 mAb as comparative control. After 14 (t14) and 24 (t24) hours, CLL cells and culture supernatants were collected for evaluation of CCL3 and CCL4 at the mRNA and protein levels. As compared with controls, higher levels of transcripts for CCL3 (mean fold increase, 18.0 ± 8.1; $P = 0.041$) and CCL4 (mean fold increase, 13.8 ± 5.2; $P = 0.005$), both peaking at t14, were detected by QRT-PCR upon CD38 ligation (not shown). A parallel increase was observed at the protein level (Fig. 1*A*), although with a later (t24) peak (mean CCL3 protein, 0.9 ± 0.1 ng/mL; mean fold increase, 14.0 ± 4.4; $P = 0.003$/mean CCL4 protein, 1.7 ± 0.5 ng/mL; mean fold increase, 49.0 ± 29.0; $P = 0.01$). Consistently, a clear increase of CCL3-specific staining of CLL cell cytoplasm was observed upon a 24-hour CD38 engagement by agonist mAbs (Fig. 1*B*). Notably, a direct correlation was found between CD38 expression levels by flow cytometry and the levels of CCL3 protein released in culture supernatants both at control conditions ($P = 0.03$; Spearman's ρ rank correlation) and upon CD38 engagement ($P = 0.02$).

A similar up-regulation of CCL3 and CCL4 transcripts was obtained in four representative $CD38^+CD49d^+$ cases, in which CD38 engagement was performed using CD31-transfected murine L-fibroblasts instead of agonist anti-CD38 mAbs (not shown). Finally, no additional up-regulation of CCL3 and CCL4 transcript levels was observed upon simultaneous engagement of CD38 and

Table 1. Immunohistochemical analysis in BMB from $CD38^+CD49d^+$ and $CD38^-CD49d^-$ CLL

	#	BM*	CCL3†	CD68‡	VCAM-1‡
$CD38^-CD49d^-$ CLL	38	40(N)	−	1	1
	39	45(N + I)	−	2	2
	40	40(N + I)	−	2	2
	41	90(D)	−	2	1
	42	50(N + I)	−	1	1
	43	45(N)	−	0	0
	44	90(D)	+	2	2
	45	50(N + I)	−	1	1
	46	50(N + I)	−	1	1
	47	40(N + I)	−	2	1
$CD38^+CD49d^+$ CLL	48	90(D)	+	3	3
	49	40(N + I)	+	3	2
	50	70(D)	−	2	2
	51	50(N)	+	3	3
	52	50(N + I)	+	2	2
	53	45(N + I)	−	2	2
	54	90(D)	+	3	3
	55	50(N + I)	+	2	2
	56	60(N + I)	−	2	2
	57	40(N + I)	+	3	2

Abbreviations: N, nodular; I, interstitial; D, diffuse.
* Figures refer to percent BM infiltration; patterns of infiltration are reported in parenthesis.
† Negative (−) and positive (+) expression was determined as reported in Materials and Methods.
‡ CD68 and VCAM expression was evaluated by two different semiquantitative four-tier grading systems (from grade 0 to grade 3).

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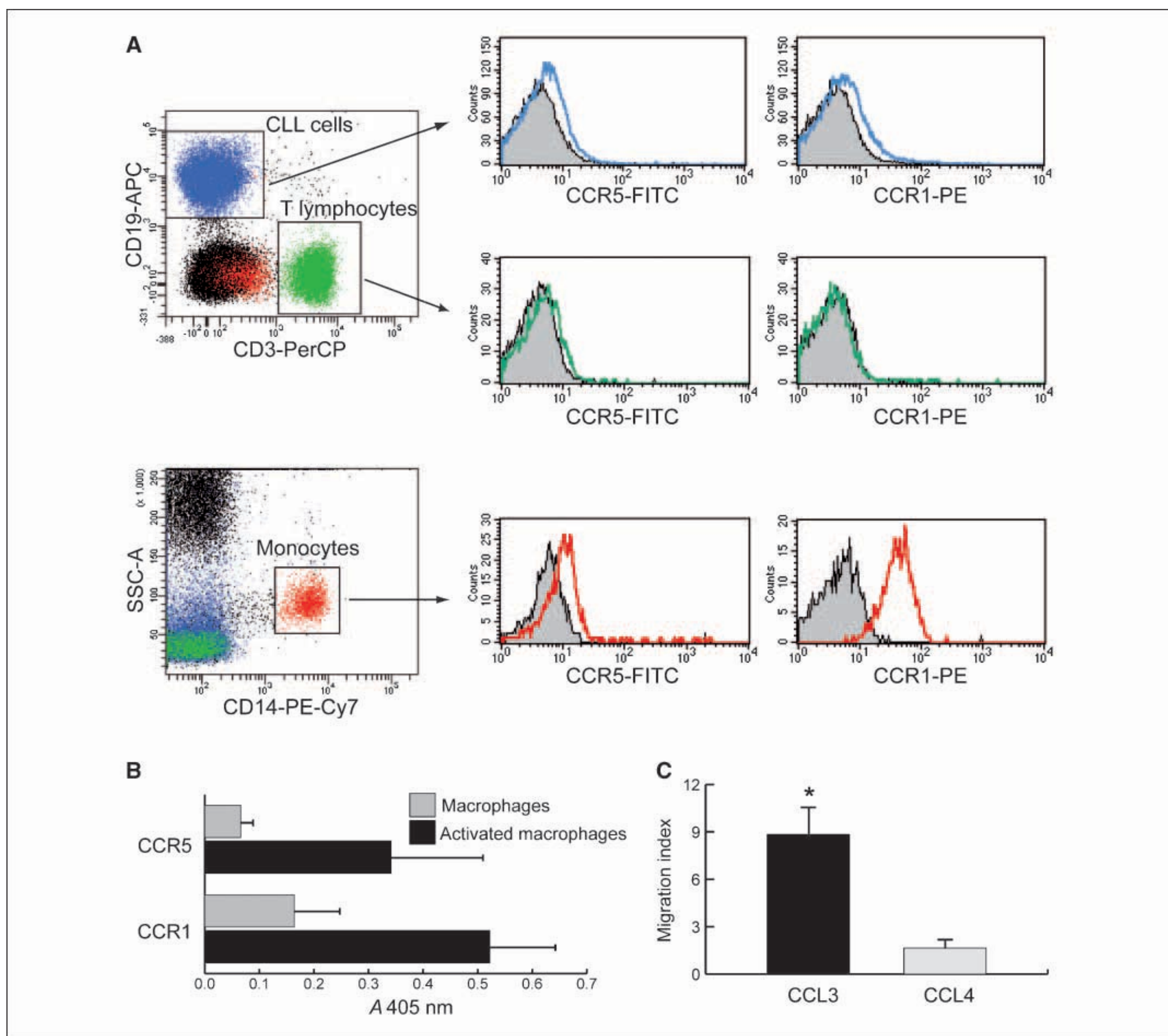

Figure 2. Expression of CCR1 and CCR5 by PB subpopulations of CLL samples and CCL3-mediated or CCL4-mediated monocyte migration. *A,* flow cytometry analysis of CCR5 and CCR1 expression by PB cells of a CLL representative case (case 97). *Left,* CLL cells, T lymphocytes, and monocytes identified on the basis of immunologic gating (*dot plots*). Colored histograms represent CCR5-FITC and CCR1-PE expression by each PB population. Gray histograms refer to isotype controls. *B,* CCR5 and CCR1 expression by macrophages evaluated by ELISA. *Columns,* results from four different resting (*light gray*) and activated (*dark gray*) macrophage populations; *bars,* SE. *C,* purified monocytes from nine CLLs tested for migration toward CCL3 and CCL4. *Columns,* migration indices; *bars,* SE. *, $P < 0.05$.

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CD49d by agonist anti-CD38 mAbs and L-VCAM-1 transfectants, respectively (not shown).

CCL3 is expressed by BM CLL cells from CD38$^+$CD49d$^+$ CLL. BM can provide the optimal microenvironment for CLL cells to interact with endothelial and stromal cells, e.g. via the CD38/CD31 pair (14). We investigated CCL3 expression by BM infiltrating CLL cells from BMB of 10 CD38$^+$CD49d$^+$ and 10 CD38$^-$CD49d$^-$ cases (Supplementary Table S1). By CD20 staining, different infiltration patterns and a variable degree of BM involvement were observed, without significant differences between CD38$^+$CD49d$^+$ and CD38$^-$CD49d$^-$ CLL (Table 1). By limiting analyses to CLL-involved areas, CCL3 protein was detected in eight CLL cases, all but one belonging to the CD38$^+$CD49d$^+$ CLL group ($P = 0.02$; Table 1), with a distinct CCL3-specific cytoplasmic staining observed in the vast majority of infiltrating neoplastic cells (Fig. 1*C*).

Monocyte macrophages express the CCR1 and CCR5 chemokine receptors and migrate in response to CCL3 and CCL4. Expression of the CCL3-specific and CCL4-specific receptors CCR1 and CCR5 was examined by flow cytometry in PB cell populations from 39 CLL cases (12 CD38$^+$CD49d$^+$, 14 CD38$^-$CD49d$^-$, 10 CD38$^-$CD49d$^+$, and 3 CD38$^+$CD49d$^-$; Supplementary Table S1) and 9 healthy donors. Irrespective of CD38 or CD49d expression by CLL cells, monocytes showed in all cases the highest mean fluorescence intensity levels for CCR1 and CCR5, the latter at a lower intensity (Supplementary Table S4; Fig. 2*A*). Similar CCR1 and CCR5 expression levels were detected in adherent

macrophages from CLL cases, with an increase upon *in vitro* activation (Fig. 2*B*). Conversely, CLL cells and residual T lymphocytes showed low mean fluorescence intensity levels for both chemokine receptors (Supplementary Table S4; Fig. 2*A*). Similar expression patterns were observed in PB cell subsets from healthy donors (Supplementary Table S4).

After showing that monocytes express CCR1 and, to a lesser extent, CCR5, we analyzed the effects exerted by CCL3 and CCL4 on migration of purified monocytes from nine CLL cases (five CD38$^+$CD49d$^+$, one CD38$^-$CD49d$^-$, two CD38$^-$CD49d$^+$, and one CD38$^+$CD49d$^-$; Supplementary Table S1). Both chemokines increased monocyte migration; however, the effects were more apparent with CCL3. Indeed, a statistically significant higher number of monocytes migrated in response to CCL3 rather than CCL4 (MI for CCL3, 8.7 $\pm$ 1.7; MI for CCL4, 1.7 $\pm$ 0.5; Fig. 2*C*); no significant differences were observed between monocyte preparations purified from CLL cases expressing different CD38 or CD49d levels (not shown).

CD68$^+$ cells infiltrate CLL areas of BMB from CD38$^+$CD49d$^+$ CLL. Based on the expression of CCL3 selectively found in CLL cells from BMB of CD38$^+$CD49d$^+$ cases, along with the capability of this chemokine to attract monocytes, we attempted to quantify the extent of infiltration of CD68-expressing monocytoid cells in the context of CLL-involved areas of BMB from CD38$^+$CD49d$^+$ and CD38$^-$CD49d$^-$ CLL (Fig. 3). The number of round-to-spindle–shaped CD68$^+$ cells intermingling with CLL cells was significantly higher in CD38$^+$CD49d$^+$ compared with CD38$^-$CD49d$^-$ cases (P = 0.016). In particular, a grade 3 infiltration was solely found in BMB from CD38$^+$CD49d$^+$ CLL (5 of 10 cases); conversely, all five grade 0 (null) or grade 1 (low) infiltrations were observed in the CD38$^-$CD49d$^-$ group (Table 1). Infiltrating CD68$^+$ cells strongly expressed the CCL3-specific receptor CCR1 (Supplementary Fig. S2).

CM from CCL3-treated macrophages up-regulates VCAM-1 by endothelial cells. In our model, we hypothesized a role for the CD68$^+$ macrophage component recruited in the context of BM lymphoid aggregates by CCL3 released by CD38$^+$CD49d$^+$ CLL cells. In particular, we investigated the ability of CCL3-stimulated macrophages to induce endothelial cells to express the CD49d ligand VCAM-1 (38).

VCAM-1 expression was determined in two endothelial cell models (HUVEC and ADMEC) after 12 hours of incubation with CM collected from CLL-derived macrophages challenged *in vitro* with either CCL3 (CCL3-CM) or culture medium alone (CNT-CM) from four different CD38$^+$CD49d$^+$ CLL macrophage preparations (Supplementary Table S1). As shown in Fig. 4*A*, the mean VCAM-1 expression seemed significantly up-regulated in both cell models upon exposure to CCL3-CM compared with CNT-CM.

It is known that VCAM-1 expression can be up-regulated in different cell types by TNFα (38). Accordingly, a significant enrichment of TNFα protein was detected in CCL3-CM compared with CNT-CM (Fig. 4*B*). The highest levels of TNFα protein were

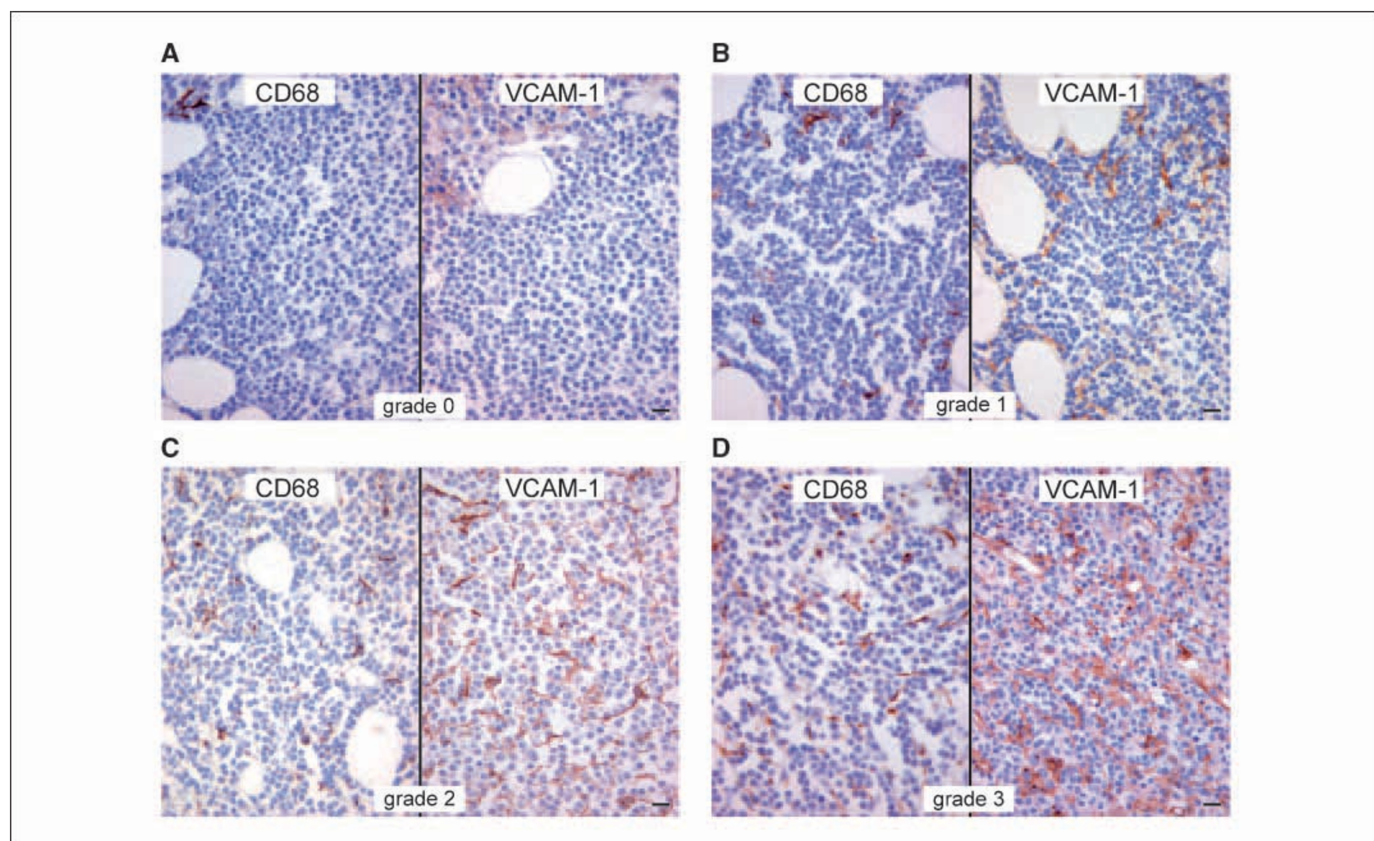

Figure 3. Patterns of CD68$^+$ cell infiltration and distribution of VCAM-1 expression in BM lymphoid aggregates from CD38$^-$CD49d$^-$ and CD38$^+$CD49d$^+$ CLL. The number of CD68$^+$ cells and the pattern of VCAM-1 expression were evaluated in the context of the BM lymphoid aggregates following two different four-tier grading systems (grade 0 through grade 3). Panels represent examples for each grade of CD68 (*left*) and VCAM-1 (*right*) expression. *A,* grade 0 (CD38$^-$CD49d$^-$ CLL; no. 43). *B,* grade 1 (CD38$^-$CD49d$^-$ CLL; no. 38). *C,* grade 2 (CD38$^+$CD49d$^+$ CLL; no. 52). *D,* grade 3 (CD38$^+$CD49d$^+$ CLL; no. 54). Original magnification, 400$\times$; *scale bars,* 20 μm.

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found in CM from LPS-exposed macrophages (LPS-CM), which was also the most effective CM in inducing VCAM-1 up-regulation by endothelial cells (Fig. 4*A* and *B*).

The specific contribution of TNFα to VCAM-1 expression by HUVEC and ADMEC was confirmed by neutralizing its biological activities in CCL3-CM derived from selected macrophage cultures (CLL cases 90 and 97). As shown in Fig. 4*C*, TNFα neutralization was followed by a significant reduction of VCAM-1 up-regulation in both cell models.

VCAM-1 is up-regulated in endothelial/stromal cells from BMB of CD38$^+$CD49d$^+$ CLL. These results were validated by analyzing VCAM-1 expression in BM lymphoid aggregates of CLL cases expressing or not CD38 and CD49d. A significantly more prominent meshwork of VCAM-1$^+$ cells was detected in the context of the lymphoid infiltrates of CD38$^+$CD49d$^+$CLL compared with CD38$^-$CD49d$^-$ cases ($P = 0.002$). No cases graded 0 or 1 for VCAM-1 expression were observed in the CD38$^+$CD49d$^+$ group, whereas high (grade 3) VCAM-1 expression levels were only scored in this group (Table 1; Fig. 3*A–D*).

Inside the lymphoid aggregates, VCAM-1 marked both the sinusoidal endothelia and the meshwork of interdigitating BM stromal cells, the former identified by CD34 and the latter by CD10 staining in serial sections (Supplementary Fig. S3). Moreover, double immunostaining indicated that the VCAM-1$^+$ endothelial/stromal component generally lacked CD68 expression. In turn, CD68$^+$ macrophages were almost constantly VCAM-1$^-$ (Supplementary Fig. S4).

CD49d engagement by VCAM-1 protects CLL cells from apoptosis. Given the high expression of VCAM-1 characterizing BMB of CD38$^+$CD49d$^+$ CLL, and the close proximity in CLL-involved BM microenvironment of CD49d$^+$ CLL cell membranes and VCAM-1$^+$ cellular processes from stromal/endothelial cells (Supplementary Fig. S5), we tested whether engagement of CD49d by VCAM-1 was able to delay the spontaneous apoptosis observed in cultured CLL cells (1).

Purified CLL cells from five CD38$^+$CD49d$^+$ cases (Supplementary Table S1) were cultured on murine L-fibroblast cells transfected with full-length human VCAM-1 or vector alone, and cell viability was determined over a time period spanning from 7 to 21 days. Figure 4*D* shows that CLL cells exposed to VCAM-1 were characterized by a relevant improvement in cell viability. In particular, the effects on protection from apoptosis exerted

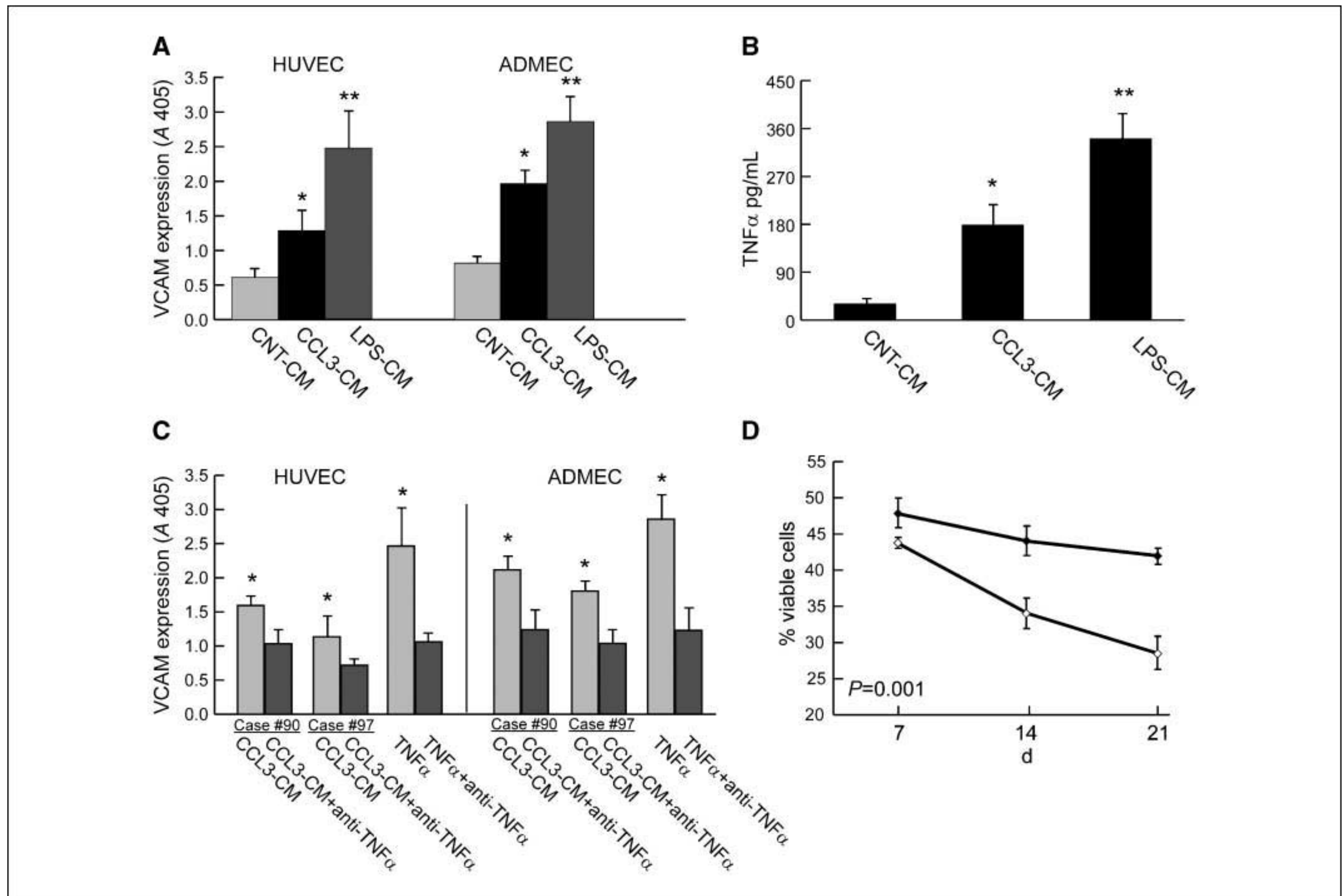

Figure 4. VCAM-1 up-regulation by endothelial cells after exposure to macrophage CM and effects of CD49d engagement via VCAM-1 on CLL cell apoptosis. *A,* expression of VCAM-1 on HUVEC and ADMEC after 12-h incubation with either unstimulated (*CNT-CM*), CCL3-stimulated (*CCL3-CM*), or LPS-stimulated macrophage CM (*LPS-CM*). *B,* TNFα protein levels in CNT-CM, CCL3-CM, and LPS-CM. ELISA values in *A* and *B* are mean ± SE of duplicate samples from four separate macrophage preparations. *, $P < 0.01$ versus control; **, $P < 0.01$ versus both conditions. *C,* the specific contribution of TNFα stimulation on VCAM-1 expression by HUVEC and ADMEC was evaluated by neutralizing (*+anti-TNFα*) or not the activity of TNFα in CM from two selected macrophage preparations before their addition to HUVEC and ADMEC. TNFα was added to both cell lines as positive control for VCAM-1 up-regulation. Values obtained by ELISA are mean ± SE of duplicate samples. *, $P < 0.01$ versus control. *D,* purified CD38$^+$CD49d$^+$ CLL cells from five patients cultured on L-VCAM-1 (*closed diamonds*) or L-mock (*open diamonds*) cells. At the indicated times (7–21 d), cell viability was analyzed by flow cytometry using the syto-16/7-AAD method. *Points,* percentages of viable cells of duplicate samples; *bars,* SE.

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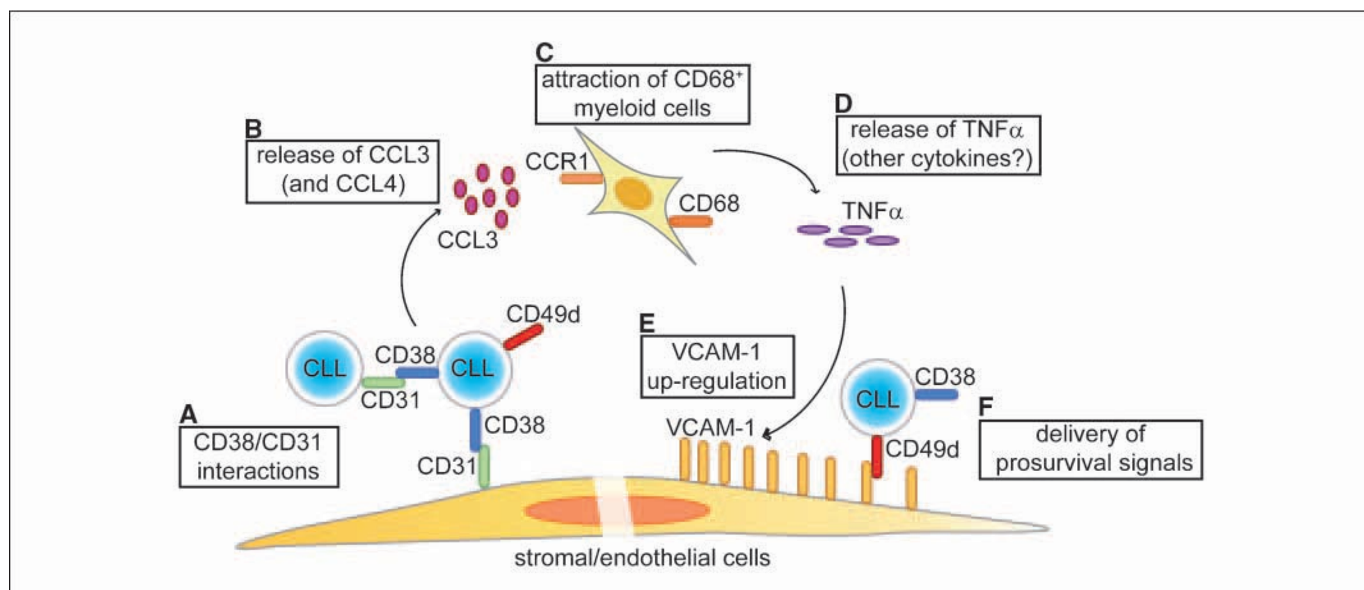

Figure 5. Model for a prosurvival circuitry operating in CD38$^+$CD49d$^+$ CLL. CD38/CD31 interactions (*A*), either homotypic (CLL-CLL) or heterotypic (CLL-stromal/endothelial cells), yield the release of CCL3 and CCL4 by CLL cells (*B*); these chemokines (mainly CCL3) attract CD68$^+$ myeloid cells expressing the CCL3 receptor CCR1 (*C*); CD68$^+$ cells, through the release of TNFα and other cytokines (*D*), determine the overexpression of VCAM-1 by stromal/endothelial cells (*E*); VCAM-1/CD49d interactions can efficiently increase survival of CD49d-expressing CLL cells (*F*).

by CD49d/VCAM-1 interactions became apparent after 7 days of culture and were more evident after 14 and 21 days of culture, reaching 42% mean viable cells compared with 28.5% in nonstimulated conditions ($P = 0.001$; Fig. 4*D*).

Discussion

CCL3 and CCL4 are small (8–10 kDa), structurally related, and secreted proteins, which are inducible and produced by mature hematopoietic cells (reviewed in ref. 39). Biologically, CCL3 and CCL4 have overlapping effects and act as potent chemoattractants for monocyte macrophages, dendritic, T, and natural killer cells (39). Despite indirect suggestions of a putative role for CCL3 and CCL4 in CLL (13, 40, 41), their involvement in this disease has not been investigated in detail.

Here, we show a distinctive overexpression of transcripts for the CCL3 and CCL4 genes in CD38$^+$CD49d$^+$ CLL cells and a clear-cut transcript up-regulation and increased release of CCL3 and CCL4 by CD38$^+$CD49d$^+$ CLL cells upon CD38 triggering. Of note, a direct correlation between the levels of CD38 and CCL3 protein was shown by considering both the basal CCL3 levels in CD38$^+$CD49d$^+$ CLL, and the increased amount of CCL3 released upon CD38 engagement.

These results are in keeping with the known capacity of CD38 engagement to mediate, along with proliferative and/or antiapoptotic signals (19, 31), cytokine release in other cellular systems (14, 42). Because CD31 is expressed by various cell types, including monocytes, endothelial cells, and CLL cells themselves irrespective of CD38 expression (20, 43), it is conceivable that interaction of CD31$^+$ cells with CD38$^+$ CLL cells might frequently occur *in vivo*, contributing to increased levels of CCL3 and CCL4 in biological fluids. In this regard, a preliminary survey of CCL3 protein concentration in plasma samples from 53 CLL revealed almost 3-fold higher CCL3 levels in CD38$^+$ (25 cases) compared with CD38$^-$ (28 cases) CLL (mean values of 43.8 versus 15.1 pg/mL, respectively).[8]

These findings raise the issue of the role played by CCL3 and CCL4 in CLL. According to our results, CLL-derived CCL3, more than CCL4, significantly contributes to the recruitment of cells from the monocyte macrophage lineage to BM microenvironmental sites. These conclusions are supported by distinct lines of evidence. First, CCR1 and CCR5 (the receptors for CCL3 and CCL4) are strongly expressed, the former more than the latter, by PB monocytes and macrophages (44) from healthy and CLL samples, but not by the majority of T or neoplastic B cells. Second, PB monocytes from CLL samples are uniquely sensitive to CCL3 signals *in vitro*, in keeping with the strong expression of CCR1 (39, 45). A third line of evidence is represented by the *in vivo* observation of a higher number of infiltrating CD68$^+$ cells in the context of CLL-involved areas of BMB from CD38$^+$CD49d$^+$ CCL3-producing cases compared with CD38$^-$CD49d$^-$ CLL. CD68 was shown to mark a heterogeneous population of myeloid cells, including macrophages, dendritic cells, and NLC, a distinctive cell population arising from monocytes cultured *in vitro* in close contact with CLL cells (1, 24, 25). According to costaining studies, this CD68$^+$ component infiltrating the BM stroma of CD38$^+$CD49d$^+$ CLL, in addition to being CCR1$^+$ in keeping with their putative capacity to respond to CCL3, expressed CD14[9] and lacked VCAM-1, a phenotype also reported for NLC (1, 24, 25).

The observation of a preferential monocyte macrophage infiltration in CLL-involved BM stroma from CD38$^+$CD49d$^+$ cases raises the additional question regarding the functional role of these cells in the pathophysiology of CD38$^+$CD49d$^+$ CLL. We addressed this issue by considering (*a*) the parallel increase of CD68$^+$ cells and

[8] D. Benedetti, unpublished observation, March 2008.
[9] C. Tripodo, personal observation, June 2008.

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neovasculature, as detected in lymph nodes of lymphoproliferative disorders (46); (*b*) the association in CLL lymph node sections between high CD38 expression by tumor cells and increased tumor vascularity (22); and (*c*) the known feature of VCAM-1, the CD49d ligand, to be induced on the surface of stromal/endothelial cells upon several stimuli (38). This background prompted us to evaluate the expression of VCAM-1 in CLL-involved BM tissues from cases expressing CD38 and CD49d or not. Results indicate a close correlation among the $CD38^+CD49d^+$ phenotype by CLL cells, infiltration of $CD68^+$ macrophages, and presence of a stromal/endothelial component strongly expressing VCAM-1. A functional link between CD68 and VCAM-1, as expressed in CLL-involved BMB, was indirectly found by showing the capacity of CM produced by CCL3-stimulated macrophages from $CD38^+CD49d^+$ CLL cases to up-regulate VCAM-1 expression in endothelial cell models (29, 30). As indicated by our experiments and in keeping with literature data, TNFα turned out to be among the major cytokines released by CCL3-stimulated macrophages likely responsible for VCAM-1 up-regulation by endothelial cells (47). However, as suggested by an incomplete reversion of VCAM-1 overexpression upon TNFα blocking, other cytokines allegedly released by CCL3-stimulated macrophages (e.g., interleukin-1) may also contribute to VCAM-1 up-regulation (47).

Microenvironmental interactions in marrow or secondary lymphoid organs have been shown to confer growth advantage and extended survival to CLL cells and play a role in disease progression and resistance to therapy (1, 48). Operationally, growth and prosurvival signals are delivered through a number of different receptor-ligand pairs. As an example, interactions occur between CLL cells, expressing CD40, and $CD40L^+$ T cells, the latter specifically attracted by CLL-derived CCL22 (1, 49). Moreover, NLC interact with CLL cells using the CD38/CD31 axis usually associated with CD100/plexinB1–mediated contacts (31) and by producing soluble factors, such as CXCL12 and BAFF/APRIL, which bind CLL cells, in turn expressing their respective receptors (50). These interactions are favored by the release by NLC of chemokines (e.g., CCL19, CCL21, and CXCL12) capable of attracting CLL cells, especially if expressing CD38, ZAP-70, and CD49d (28, 36, 37).

Here we showed that $CD68^+$ NLC or monocyte macrophage cells can also be attracted by specific chemokines (e.g., CCL3) produced by CLL cells when expressing high levels of surface CD38 to allow efficient CD38/CD31 interactions. These attracted $CD68^+$ cells, in addition to directly interact with CLL cells, can be active in determining, through the release of TNFα and other cytokines, the overexpression of VCAM-1 by the stromal/endothelial component of BM microenvironment. Specific VCAM-1/CD49d interactions can efficiently increase survival of CD49d-expressing CLL cells (Fig. 5). The proposed circuitry may represent the cellular basis explaining the aggressive clinical course of CLL coexpressing CD38 and CD49d.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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