Expression of Costimulatory Molecules in Low-Grade Mucosa-Associated Lymphoid Tissue-Type Lymphomas *in Vivo*

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B-cell lymphomas of mucosa-associated lymphoid tissue (MALT) type develop against a background of chronic inflammation and have functional autoantigen receptors. Because they respond to environmental factors *in vivo,* the expression of costimulatory molecules, which play a key role in the differentiation of normal B-lymphocytes and in T-/B-cell interaction, may be critical in early MALT-type lymphoma pathogenesis until further chromosomal aberration leads to progression. We found a high number of tumor-infiltrating T-lymphocytes (TITLs) in all low-grade MALT-type lymphomas. The TITLs in low-grade lymphomas were activated and expressed a memory and immunocompetent phenotype. Reverse transcriptase-polymerase chain reaction analyses and immunohistochemistry confirmed the presence of CD40-ligand and Fas-ligand in 80% of low-grade lymphomas. In contrast to the TITLs, the tumor B cells did not express CD40-ligand or Fas-ligand *in vivo* or *in vitro*. Moreover, the cytokine profile *in vivo* suggested a Th2/Th3-weighted profile (interleukin-10, interleukin-13, transforming growth factor β_1) rather than Th1-weighted (interferon-γ, interleukin-2). By interphase fluorescence *in situ* hybridization analysis the translocation t(11;18)(q21;q21) was found in four of nine (44%) cases studied. Interestingly, there was a four times higher proliferation and survival rate of purified t(11;18)-positive tumor B cells *in vitro*, although there were no significant profile differences from the TITLs *in vivo*. The finding of essential costimulating molecules in low-grade MALT-type lymphomas *in vivo* indicates a locally directed cognate T-/B-cell interaction. Consequently, a potentially equipped inflammatory background may not only determine the fate of autoreactive B-cells, but is also crucial to lymphoma maintenance and progression. *(Am J Pathol 1999, 155:2019–2027)*

B-cell lymphomas of mucosa-associated lymphoid tissue (MALT-type) arise in sites primarily devoid of lymphoid tissue and against a background of long-lasting chronic inflammation.[1] Their propensity to remain localized may be dependent on continuous antigen stimulation and/or help provided by the residual lymphoid tissue.[2] Both histopathological and clinical features of MALT-type lymphomas suggest that lymphomagenesis is, at least in part, T-cell dependent. This idea has gained considerable support from clinical observations that most human low-grade gastric MALT-type lymphomas can regress at early stages on *Helicobacter pylori* eradication[3,4] and that some low-grade MALT-type lymphomas respond to autologous T-cell help, resulting in tumor cell proliferation *in vitro*.[5]

Recently, *in vitro* and molecular studies suggested that MALT-type lymphoma B cells are antigen sensitive but autoreactive[6–8] and thus have escaped a tolerance mechanism that is normally checked by various T-cell subsets through interactions of cell surface molecules and cytokines. Experimental observations suggest that antigen and Fas-ligand/CD40-ligand expressed on activated T-cells act during cognate T-/B-cell interaction and are crucial checkpoints for germinal center B-cell maturation, generating marginal zone B cells, but act to delete harmful B cells[9,10]. The maturation of B cells to effective producers of high-affinity and high-specificity antibodies is controlled by defined T-cell subsets through cell surface molecules and soluble cytokines.[11–13] In particular, whereas Th1-type cytokines (ie, interleukin-2 (IL2), interferon-γ (INF-γ)) are important in cell-mediated immune responses and the eradication of microbial pathogens,[14] Th2- and Th3-type cytokines (IL4, IL6, IL10, and transforming growth factor β_1 (TGF-β_1)) have been demonstrated to be crucial for B-cell maturation[15,16] but may also support the growth of otherwise uncontrolled B cells in autoimmune disorders[17] and suppress cell-mediated autoimmunity (ie, TGF-β_1). Therefore, the discovery of functionally heterogeneous CD4+ T-cell subsets that secrete different cytokines may determine not only the type

Table 1.   Specimen Data

Patient	Site of origin	Diagnosis	t(11;18) (q21;q21)	Stage*
1	Stomach	Low-grade MALT	No	EI2
2	Stomach	Low-grade MALT	n.d.	EII1
3	Stomach	Low-grade MALT	Yes	EI2
4	Stomach	Low-grade MALT	No	EI2
5	Stomach	Low-grade MALT	No	EI1
6	Stomach	Low-grade MALT	No	EI2
7	Stomach	Low-grade MALT	Yes	EII1
8	Thyroid	Low-grade MALT	Yes	n.d.
9	Stomach	Low-grade MALT	Yes	EII1
10	Stomach	Low-grade MALT	No	EI2
11	Stomach	Gastritis	No	—

* Stage according to modified classification of Musshoff.[53]
n.d., not determined.

of B-cell immune response but may also provide key molecules in B-cell lymphoma initiation and progression.

Recently, in a larger series a t(11;18)(q21;q21) chromosome translocation was found to be the characteristic aberration in low-grade MALT-type lymphoma[18] involving a novel gene *MLT* and the *HIAP1* gene, encoding an inhibitor of apoptosis.[19] Although its biological and clinical significance is so far unknown, it is suggested that the rearranged genes may support increased B-cell survival and decrease T-cell-dependent help.[20]

We present herein a detailed characterization of the microenvironment of low-grade MALT-type lymphomas, which is different from chronic gastritis, and show that essential costimulatory molecules are present in all cases irrespective of the t(11;18) alteration, which may provide an important background for tumor progression.

Materials and Methods

Patients and Specimens

Fresh tissues were received within 2–6 hours after surgery for the preparation of cell suspensions, snap-freezing in liquid nitrogen, and routine fixation for histological examination. Nonneoplastic tonsillar tissues with reactive tonsillitis and resection samples from patients with primary low-grade MALT-type lymphoma of the stomach were selected from the lymph node registry in Würzburg. The tumors were always confined to the stomach and regional lymph nodes without clinical evidence of generalized disease (Table 1). Additionally, one low-grade MALT-type lymphoma of the thyroid gland and one case of gastritis (no. 11; as a control) were investigated (Table 1). For diagnostic purposes, the morphological and immunophenotypical analyses of paraffin-embedded and freshly frozen sections were performed by standard methods as recently described.[1] In all cases the diagnosis was confirmed according to the criteria of the Revised European American Lymphoma (REAL) Classification.[21]

Immunostaining

Immunohistochemical staining was performed on 4-μm cryostat sections of fresh frozen surgical specimens. Bi-

opsy tissues were kept at $-70°C$ as snap-frozen blocks until sections were prepared at the time of the experiments. The immunoperoxidase method was applied by a three-step incubation procedure with diluted affinity-purified anti-rabbit, anti-rat, anti-mouse antibodies (all from Dako, Hamburg, Germany) and serum from each species as a control, as described in detail elsewhere.[22] APAAP staining was used to visualize mouse anti-human CD40-ligand (kind gift from RA Krozcek, Berlin, Germany) as described.[23] The following primary antibodies were used in this study: rabbit anti-human Fas-ligand (SC-834-G; Santa Cruz Biotechnology, Santa Cruz, CA), rat anti-human IL-2 (MQ1–17H12; Pharmingen, Heidelberg, Germany), mouse anti-human IL-10 (mAb 2,17; R&D Systems, Wiesbaden, Germany), rat anti-human IL-13 (JES10-5A2; Pharmingen), and TGF-β_1 (BMS4435; Boehringer Mannheim, Mannheim, Germany). CD40-ligand and Fas-ligand served in immunostained cytospins as positive controls for cytokines from different cell lines (Jukat, Raji, THP-1) stimulated with phorbol myristic acetate/ionomycin or lipopolysaccharide over 24 hours.[24,25]

Flow Cytometry

Flow cytometric analysis was performed on a FACScan (Becton Dickinson) with an argon ion laser tuned at 488 nm, using LYSIS II for data acquisition and analysis and triple immunostaining with directly conjugated antibodies (anti-CD19 (HD 37; Sigma); anti-κ (KP-53; Sigma), anti-λ (HP6054; Sigma), anti-CD3 (UCHT-1; Sigma), anti-CD14 (Leu-M3; Becton Dickinson), anti-CD40 (mAb89),[26] anti-CD4 (R-8886; Sigma), anti-CD8 (R-806; Dako), anti-CD38 (MHCD3804; MEDAC), anti-IgD (I-2768; Sigma), anti-IgM (P-9295; Sigma), anti-CD25 (R-811; Dako), anti-CD69 (BD 347823; Becton Dickinson)). Each measurement contained 20,000 cells. As a specificity control and for instrument setup isotype controls were used in parallel for each directly conjugated antibody regarding species, isotype, and type of fluorochrome (all from Sigma, Deisenhofen, Germany).

B-Cell Purification, Cell Culture, Stimulation, and Cytogenetic Analysis

Viable lymphocyte single-cell suspensions were isolated by density-gradient centrifugation and negative depletion using magnetic beads coupled with anti-CD2 and anti-CD14 (Dynal, Hamburg, Germany) to remove macrophages and T cells. Thereafter, as a control, nonneoplastic memory B cells from tonsils were depleted of CD38$^+$ (clone ACT 13.5; Serotec, Wiesbaden, Germany) and IgD$^+$ (clone HJ9; Sigma) cells to remove germinal center cells, plasma cells, and mantle zone cells as described elsewhere.[27,28] Lymphoma cells were further purified by negative depletion of nonneoplastic bystander B cells, using beads coated with antibodies to immunoglobulin heavy and light chains not expressed by the lymphoma, including IgD. Tumor-infiltrating T cells were isolated from primary cell suspensions, using anti-CD4$^+$- and anti-CD8$^+$-coupled magnetic beads and Detach-a-beads (all

Table 2. Primer Sequences Used for RT-PCR

mRNA	Sense primer (5′ → 3′)	Antisense primer (5′ → 3′)	Annealing temperature (°C)	MgCl$_2$ (mmol/L)
GAPDH	TGAAGGTCGGAGTCAACGGATTTGGT	CATGTGGGCCATGAGGTCCACCAC	65	2.0
CD40-L	CCTCAAATTGCGGCACATGT	GACAAACACCGAAGCACCTGG	60	1.5
Fas-L	AACTCAAGGTCCATGCCTCTG	GGTGAGTTGAGGAGCTACAGACA	60	1.5
IFN-γ-RE	GCCGTCCTCAGTGCCTACACCA	TTATACTGGATCTCACTTCCGTTCA	65	1.5
IFN-γ	TTGGAAAGAGGAGAGTGACAG	TACTGGGATGCTCTTCGACCTC	60	1.5
IL2	ATGTACAGGATGCAACTCCTGT	GTTTCAGATCCCTTTAGTTCCAG	60	1.5
IL4	ATGGGTCTCACCTCCCAACTGCT	CGAAACACTTTGAATATTTCTCTCTCAT	65	1.5
IL10	AGACTTTCTTTCAAATGAAGGATC	TCAGTTTCGTATCTTCATTGTCA	60	1.5
IL13	TGGCGCTTTTGTTGACCACGGT	AAGCTGGAAAACCCAGCTGAGA	60	1.5
TGF-β$_1$	AACCCACAACGAAATCTATGAC	AACCCACAACGAAATCTATGAC	60	1.5

from Dynal). If the purity of cell isolates was >95%, CD19$^+$/IgD$^-$/CD38$^-$/sIg$^+$ cell suspensions were subjected to further analyses. Purified B cells were stimulated for reverse transcriptase-polymerase chain reaction (RT-PCR) and Western blot analyses with 500 ng/ml ionomycin and 10 ng/ml 12-*O*-tetradecanoylphorbol-13-acetate for 6 or 24 hours. To measure the proliferation the B cells were stimulated in the CD40-system as described previously.[6]

Cytogenetic analyses were performed as described.[18] The presence of the t(11;18)(q21;q21) was determined either by conventional karyotyping or by applying a fluorescence *in situ* hybridization (FISH) assay that was recently developed to detect the chromosomal breakage in 11q21 in interphase cells (A. Rosenwald, manuscript submitted for publication). Karyotypes were described according to the International System for human cytogenetic nomenclature.

Western Blot

For each sample total protein extraction was prepared from 1×10^6 cells from cell culture. The cells were resuspended in 100 μl of Laemmli buffer with 1 μmol/L leupeptin (Sigma), 1 μmol/L pepstatin (Sigma), and 1 mmol/L pefabloc (Boehringer Mannheim)[29] and boiled for 5 minutes, and the DNA was shared with a cannula. The solution was spun down, and the protein supernatant was stored at −20°C. The protein extractions were monitored by sodium dodecyl sulfate-polyacrylamide gel electrophoresis.[29] In each lane 30 μl of protein extract mixed with bromphenol blue was applied. Proteins were transferred to a nitrocellulose membrane, which was probed with anti-CD40-ligand (TRAP cell culture supernatant; dilution 1:5) or anti-Fas-ligand (clone G247-4; Pharmingen) as the primary antibody, respectively. For detection the ECL system (Amersham, Freiburg, Germany) was used according to the manufacturer's instructions.

RNA Extraction

All tumor tissue blocks were snap-frozen in liquid nitrogen and stored at −70°C until extraction of RNA. Total RNA was prepared (TRIzol reagent; Life Technology, Paisley, UK) from 20 sections of about 10 μm from the frozen tumor tissue blocks. The integrity of the RNA was checked by electrophoresis on a 2% formaldehyde-agarose gel, and the yield of RNA was quantitated by measuring the optical density. To check for carry-over material during the isolation step, extraction buffer without tissue was used as a negative control.

cDNA Synthesis

First-strand cDNA was synthesized with 1 μg of total cellular RNA. RNA, 2 μg of dT-15 primer, and diethyl-pyro-carbonate (DEPC)-treated water at a final volume of 8 μl were incubated for 10 minutes at 65°C. After chilling on ice, a master mix consisting of deoxynucleoside triphosphates and dithioreitol (final concentrations of 1 mmol/L each and 10 mmol/L, respectively), 25 U of recombinant RNase inhibitor (Promega, Heidelberg, Germany), RT-buffer, and 200 U of Moloney-murine reverse transcriptase (GIBCO BRL, Eggenstein, Germany) were added to a final reaction volume of 25 μl. After 70 minutes of incubation at 37°C, the samples were heated to 98°C for 4 minutes. The efficiency of cDNA synthesis was assessed by PCR with glyceraldehyde-3-phosphate dehydrogenase (GAPDH)-specific primers (Table 2). The GAPDH-PCR was performed in a final volume of 25 μl, using 2.5 μl 10× PCR-buffer (USB, Cleveland, OH), 2.0 μl 25 mmol/L MgCl$_2$, 0.5 μl 10 mmol/L dNTPs, 0.5 μl 20 pmol of each primer, 0.5 U *Taq*-polymerase (USB), 18.4 μl PCR-water, and 1 μl cDNA.

Semiquantitative Polymerase Chain Reaction

For gross quantitation, we used expression of GAPDH as an external standard, which was amplified in separate reactions. Twenty-five cycles of amplification for each sample were carried out in a DNA thermal cycler 2600 (Perkin-Elmer Centus, Emeryville, CA), and aliquots were taken after 21, 23, and 25 cycles. Each cycle of amplification consisted of 30 seconds of denaturation at 94°C, 30 seconds of annealing at 60°C, and 1 minute of elongation at 72°C. The aliquots were subjected to electrophoresis, and the amount of amplification was assessed by comparing signal intensites. After the cDNA amount in each sample was adjusted to generate an equal result in GAPDH amplification, specific RT-PCRs for CD40-ligand, Fas-ligand, and cytokines were carried out. Specific oli-
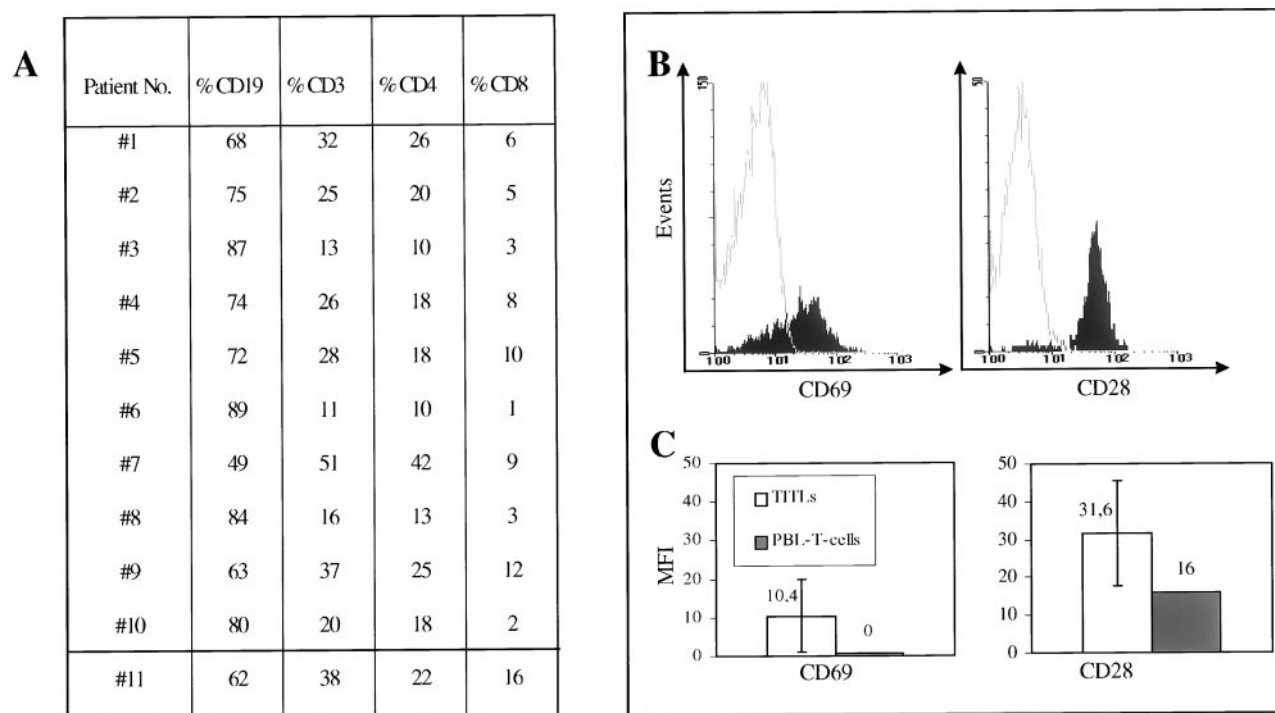
Figure 1. Characterization of tumor infiltrating T cells in low-grade MALT-type lymphomas, using multicolor FACS analysis of freshly isolated tumor cell suspensions. **A:** Summary of the percentage of B and T cells in tumor tissues (1–10, low-grade MALT-type lymphomas; 11, follicular gastritis, *H. pylori*-associated). **B:** Characteristic FACS histograms of TITLs of low-grade MALT-type lymphoma (no. 5) with high expression of CD69 and CD28 (**solid line,** isotype control; **dashed lines**, CD69 and CD28) of TITLs. **C:** Summary of FACS data of TITLs mean fluorescence intensity (MFI) of CD69 and CD28 expression as compared with T cells from PBLs of the same patients.

gonucleotides were selected in such a manner that they were only able to hybridize with cDNA and not with genomic DNA (Table 2). The master-mix conditions for the specific PCRs were the same as already described for GAPDH, except for the $MgCl_2$ concentration (Table 2). PCR fragments were sequenced to ascertain specificity. Ionomycin-stimulated peripheral blood lymphocyte (PBL) cDNA was used as a positive control. The specificity and sensitivity for each primer pair of the cytokine PCRs were seperately tested as described.[30] Two microliters of cDNA from stimulated PBLs was taken as the control cDNA in each test reaction. The primer pairs were tested with different $MgCl_2$ concentrations (1.0, 1.5, 2.0, 2.5, 3.0, 3.5 mmol/L) and different annealing temperatures. Under consideration of the intensity of the PCR product and the purity of the backround analyzed on an agarose gel, we selected the most sensitive combination of $MgCl_2$ concentration and annealing temperature. The control PCRs in Figure 5 showed different intensities due to the different concentrations of cytokine mRNAs in the control PBLs.

Results

Tumor-Infiltrating T-Cells in MALT-Type B-Cell Lymphoma Are CD28 Positive and Express CD69 Irrespective of the Presence of t(11;18)

As determined by FACScan analysis, low-grade lymphomas contained high numbers of tumor-infiltrating T-lymphocytes (TITLs) (for details see Figure 1A). The CD4/CD8 ratio was 3.3 in t(11;18)-positive cases and 3.3 in t(11;18)-negative cases. To investigate whether TITLs were activated (CD69$^+$) and immunocompetent (CD28$^+$), three-color fluorescence-activated cell sorter (FACS) analysis was used. As shown in Figure 1, B and C, low-grade TITLs expressed significantly more CD69 than the T cells from PBLs of the same patient. Moreover, in contrast to PBL T cells, all lymphoma TITLs expressed CD28, with an even higher average mean fluorescence intensity (MFI). Although CD69 and CD28 MFI expression was higher in t(11;18)-negative low-grade lymphoma TITLs (MFI CD69/28: 12/33) as compared with t(11;18)-positive TITLs (MFI CD69/28: 9/31), the differences were statistically insignificant.

Activated TITLs but Not Lymphoma B-Cells Express CD40-Ligand and Fas-Ligand in Vivo and in Vitro

To investigate whether CD40-ligand and Fas-ligand mRNA were present in lymphoma tissues *in vivo*, a semi-quantitative RT-PCR was used. Within the low-grade lymphomas 80% were positive for CD40-ligand and Fas-ligand mRNA, whereas immunohistochemistry found protein expression that paralleled mRNA expression in all cases. Notably, CD40-ligand-positive as well as Fas-ligand-positive cells were found in considerable numbers
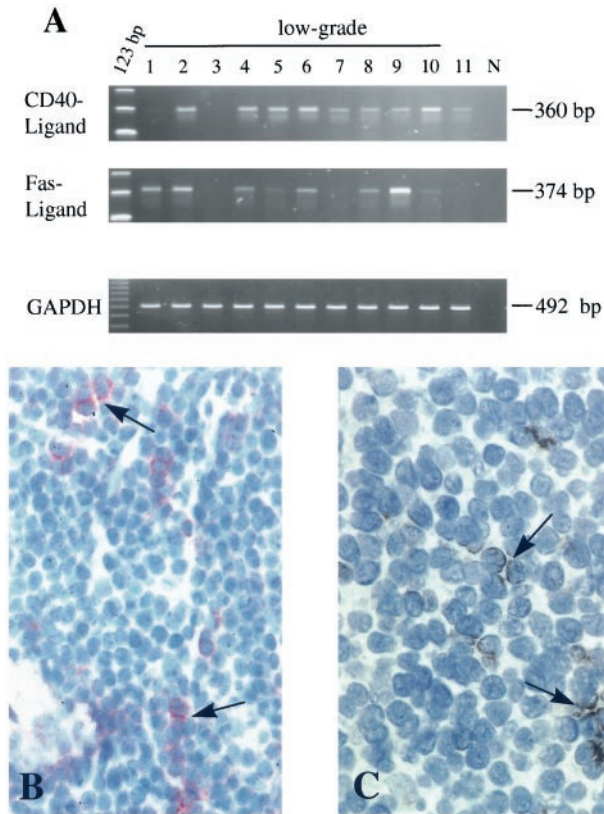
Figure 2. CD40-ligand and Fas-ligand expression in MALT tumor tissues *in vivo.* **A:** A semiquantitative RT-PCR with GAPDH as standard. N, negative control. **B:** Immunohistochemical detection of scattered CD40-Ligand expressing cells within the low-grade tumor infiltrate (no. 6; **arrow**; APAAP, cryostat section, ×250). **C:** Fas-ligand expression in the same tissue, giving brown spotted signals (no. 6; **arrow**; PAP, peroxidase-anti-peroxidase; cryostat section, ×400).
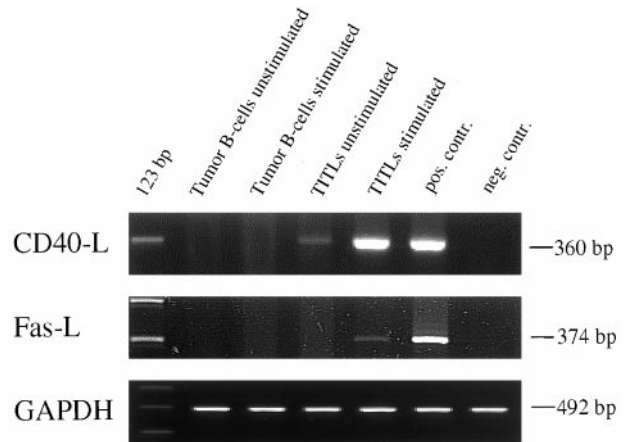

Figure 3. CD40-ligand mRNA is expressed by unstimulated and stimulated TITLs but not by malignant B cells. Fas-ligand mRNA is expressed by stimulated TITLs. Lymphocytes were purified and cultured for 6 hours with or without stimulation, as described in Materials and Methods. GAPDH RT-PCR is given as the control.

by endothelial cells of small vessels (Figure 5C). IL10 mRNA- and protein-expressing cells were present in 60% of low-grade lymphomas. IL13 mRNA/protein was detected in 40% of the cases. Cytokine-expressing cells were found to be loosely distributed within the tumor tissue (Figure 5, D and E).

IL2 was found in three low-grade cases (nos. 1, 6, and 8 (weak)), with some scattered positive cells surrounding small blood vessels (Figure 5B). IFN-γ was found at a very low level in one low-grade case (no. 1), whereas no protein expression was found by immunohistochemistry (data not shown). IFN-γ receptor mRNA (as a control) was always detecable.

t(11;18) May Affect Tumor B-Cell Proliferation and Survival in Vivo

In one t(11;18)-negative and t(11;18)-positive case, cell culture material was compared with normal memory B cells *in vitro.* Strikingly, the basal proliferation (without any stimulation) and the survival rate were significantly higher
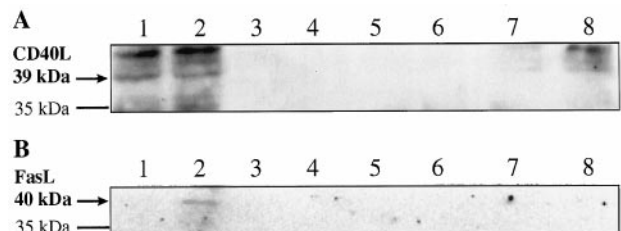
outside the reactive residual germinal centers within the tumor mass (Figure 2,B and C).

To prove that the TITLs and not the tumor B cells were the source of CD40-ligand or Fas-ligand, TITLs and lymphoma B cells were purified and investigated seperatly *in vitro*. After ionomycin/12-*O*-tetradecanoylphorbol-13-acetate stimulation, purified TITLs were induced to express CD40-ligand mRNA as well as Fas-ligand mRNA, whereas tumor B cells could not be triggered, even after stimulation to express Fas-ligand and CD40-ligand (Figure 3). Because B cells were recently found to be able to express low basic levels of CD40-ligand mRNA,[31] we also measured the protein expression by Western blot assay and could not detect any CD40-ligand protein in normal memory B cells and tumor B cells as compared with the positive control (Figure 4A). In addition, neither Fas-ligand mRNA nor protein was found in MALT tumor B cells (Figure 4B).

Cytokines Expressed in Vivo *Suggest a TH2/3-Weighted but Not a TH1-Weighted Profile*

The most prominent cytokine found in all lymphoma tissues by RT-PCR and immunohistochemistry was TGF-β_1, which was expressed in the cytoplasm of tumor cells and

Figure 4. There is neither CD40-ligand nor Fas-ligand protein expression in tumor B cells, as demonstrated by Western blot analyses. CD40-ligand (**A**) and Fas-ligand protein (**B**) was detectable in a stimulated C8166 T-cell line (**lane 2**) as the positive control, and CD40-ligand protein was detectable in a Jurkat T-cell line (**lanes 7** and **8**). Neither protein expression was found in unstimulated nor in stimulated normal and tumor B cells (**lanes 3–6**). The cells were stimulated for 24 hours as described in Materials and Methods. **Lanes 1** and **2:** Cell line C8166 unstimulated and stimulated. **Lanes 3** and **4:** Purified memory B cells unstimulated and stimulated. **Lanes 5** and **6:** Tumor B cells (no. 8) unstimulated and stimulated. **Lanes 7** and **8:** Jurkat cell line, unstimulated and stimulated, respectively.
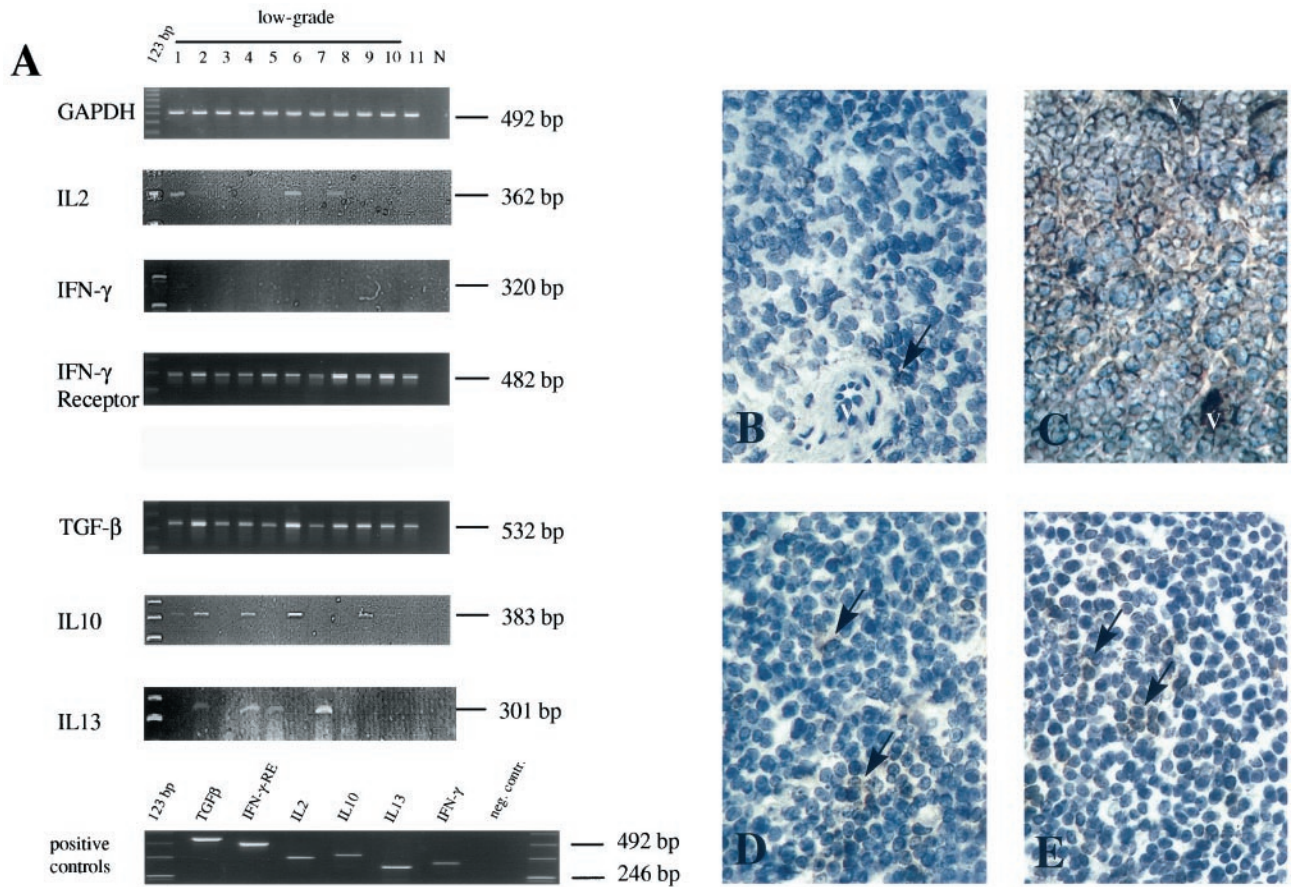
Figure 5. A: Semiquantitative RT-PCR analysis of cytokine expression in MALT-type lymphoma tissues. N, negative control. Th1-weighted cytokines were normally absent, despite weak expression of IL2 in cases 1, 6, and 8. In contrast, Th2/Th3-weighted cytokines (TGF-β_1, IL10, and IL13) were present in low-grade lymphomas. Ionomycin-stimulated normal PBLs from healthy donors were taken as positive controls. Protein expression in immunohistochemistry paralleled mRNA expression. **B:** Only a few IL2-positive cells (**arrow**) (V, vessels) with a faint cytoplasmatic staining were found perivascularly and within the tumor tissue (case 1; PAP, cryostat section, ×400). **C:** Strong cytoplasmic staining for TGF-β_1 in tumor cells as well as in small blood vessels (case 6; PAP, cryostat section, ×200). **D** and **E:** IL10 (case 6; PAP, cryostat section, ×250; **D**) and IL13 (case 7; PAP, cryostat section, ×200; **E**). Numerous positive brown staining cells (**arrows**) were found loosely distributed within the tumor tissues.
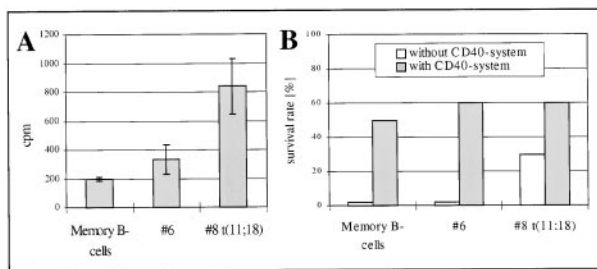

Figure 6. There is a higher proliferation of purified tumor B cells in case 8 with t(11;18) as compared with case 6 (no translocation) or normal memory B cells without any stimuli after 3 days of cell culture (**A**). Data are expressed as mean of triplicate [³H]thymidine cpm uptake values, with SD as an error bar. The different proliferation in each case was paralleled by the survival rate as determined by trypan blue staining and lowest in normal memory B cells and case 6 and highest in case 8 after 7 days of cell culture without any stimuli (**B, open bars**). However, these differences disappeared by triggering with anti-CD40 (**B, closed bars**) and resulted in increased survival of tumor and normal B cells.

in the t(11;18)-positive case as compared with the t(11;18)-negative case or the control memory B cells (Figure 6A). Notably, the differences in survival could be reversed by triggering the lymphocytes in the CD40 system (Figure 6B).

Discussion

Tumor regression after eradication of a chronic stimulus is unique to MALT-type lymphomas[3,4,32] and indicates an intricate relationship between the tumors and their hosts. However, the presence of TITLs alone, even with a high CD4/CD8 ratio, is not sufficient to explain the symbiotic interaction between MALT-type lymphoma B cells and the TITLs. Because *in vitro* activated T cells can promote MALT-type lymphoma growth,[33] our *in vivo* findings that TITLs of low-grade lymphomas consisted of activated T cells (CD69+), which coexpress immunocompetence markers (CD28+), became significant.

Beyond the ability of CD4+ T cells to provide help in the priming of tumor-specific CD8+ CTLs, evidence from mouse models and cell lines indicates that tumor-specific CD4+ T cells can orchestrate additional effector functions in tumor immunity.[34–37] Therefore, the observation that CD40-ligand was expressed within the MALT tumor tissues of at least low-grade lymphomas is important in three ways. First, as shown recently,[2,6] CD40-ligand is the most potent stimulus for proliferation of MALT-type lymphoma B cells *in vitro* and may also promote tumor growth *in vivo*. Second, CD40-ligand is able to inhibit

drug-induced apoptosis in lymphoma cell lines *in vitro*,[38,39] suggesting that it may induce the resistance of tumor cells to proapoptotic and antiproliferative effects of cytotoxic drugs *in vivo*. Third, CD40-ligand is known to be expressed on T cells after triggering by specific antigens.[40] Its detection in tumor tissues thus favors the idea of antigen-mediated tumor promotion by activated T cells. This notion is further strengthened by the observation that the TITLs but not the tumor B cells expressed CD40-ligand, in contrast to the recently described aberrant CD40-ligand expression of tumor-B cells in some low-grade non-Hodgkin's lymphoma (eg, chronic lymphocytic leukemia[41,42]). Therefore, the presence of CD40-ligand in tumor tissues points to the possibility of nonautonomous tumor growth supported by activated CD40-ligand-positive T cells and is consistent with tumor regression after antibiotic treatment.

In addition to CD40-ligand, we also found Fas-ligand to be expressed *in vivo.* During normal T-/B-cell interaction Fas-ligand expression is commonly detectable on highly activated but not on resting T cells and is important to the deletion of harmful autoreactive B cells.

Given that most MALT-type B-cell lymphomas were found to express antigen receptors with specificity for autoantigens,[7,8,29,43,44] the presence of Fas-ligand in the tumor tissues was surprising. A possible explanation for this result could be either an aberrant expression of Fas-ligand by the tumor cells as described recently for some carcinomas[45] or a so far unknown escape mechanism in which the tumor B cells become resistant to Fas triggering. Our results favor the latter idea, because we could not detect any Fas-ligand mRNA or protein expression in normal or tumor B cells, even under rigorous stimulation *in vitro*. Furthermore, it is possible that impaired Fas-mediated apoptosis or Fas mutations as described recently in some B-cell lymphomas[46] are operative in MALT-type lymphomas as well and may lead to a functional Fas resistance.[20,47]

The finding of Th2- and Th3-type cytokines but only low levels of Th1-type cytokines in MALT tumor tissues *in vivo* is in contrast to a recent report on four cases in which TITLs secreted a Th1 cytokine (INF-γ) after *H. pylori* stimulation *in vitro*.[48] This discrepancy may be explained either by TGF-β_1 expression in the tumor cells *in vivo*, which may cause a deviation of the T-cell response to a Th2 phenotype, as shown recently in mice,[49] or by *in vitro* selection (due to culture conditions) of an otherwise (eg, major histocompatibility complex-restricted) controlled Th1 population, although it cannot be ruled out that a few cells in the tumor microenvironment may be functionally significant. However, the existence of Th1 cytokines would argue against tumor promotion and favor antitumor immunity,[37] whereas Th2-type (IL10, IL13) and Th3-type (TGF-β_1) cytokines were shown to be potent promoters for B-cell proliferation and differentiation.[40,50,51]

The observation that activated T cells secrete Th2-type cytokines in MALT-type lymphomas *in vivo* may attribute to them a function that goes beyond solely translating *H. pylori*-specific signals into growth signals for MALT lymphoma B cells. Thus Blanckenstein et al[52] recently proposed that presentation of tumor antigens by B cells to CD4$^+$ T cells diverts antitumor immunity toward a "nonproductive" Th2-type humoral immune response and away from a "productive" Th1-type cellular response. Therefore, it is possible that a Th1-weighted immunity established during *H. pylori*-associated chronic inflammation as described recently[53,54] may support antitumor immunity, whereas a Th2-weighted profile *in vivo* favors progression of lymphoproliferative disease and aberrant B-cell clones. If this indeed turns out to be a general phenomenon it will be important to design strategies that will break Th2-mediated T-cell tolerance and/or polarization to promote the development of an effective tumor immunotherapy, even in low-grade MALT-type lymphomas progressively growing after *H. pylori* eradication. In this context, it may be important that the negative and the positive t(11;18) cases did not differ regarding their expression profiles and appear to be dependent on the same environment. A reason for this could be that the t(11;18) translocation, which represents the most frequent structural abnormality in MALT-type lymphomas,[18] involves genes that do not impair the T-/B-cell interaction. This may explain why purified low-grade tumor B cells respond uniformly in the context of CD40 triggering and cytokine stimulation[2] but behave differently without exogenous stimuli *in vitro*. Therefore, it will be interesting to compare tumor regression *in vivo* with respect to the presence of t(11;18) because tumor B cells may still benefit from the immunological setting but have developed sophisticated survival strategies, even without a need for continuous T-cell collaboration.

Acknowledgments

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