Multiplex B Cell Characterization in Blood, Lymph Nodes, and Tumors from Patients with Malignancies

A. Ali Zirakzadeh, Per Marits, Amir Sherif and Ola Winqvist

*J Immunol* 2013; 190:5847-5855; Prepublished online 29 April 2013;
doi: 10.4049/jimmunol.1203279

http://www.jimmunol.org/content/190/11/5847
Multiplex B Cell Characterization in Blood, Lymph Nodes, and Tumors from Patients with Malignancies

A. Ali Zirakzadeh,* Per Marits,* Amir Sherif, † and Ola Winqvist*

B lymphocytes contribute to immune surveillance, by tumor-specific Abs and Ag presentation to T lymphocytes, but are insufficiently studied in humans. In this article, we report a flow cytometric investigation of B lymphocyte subpopulations in blood, lymph nodes (LNs), and malignant tissues from 20 patients operated on because of advanced solid tumors. The CD19* compartment in peripheral blood was essentially unaltered in patients, as compared with healthy control subjects. In metastatic LNs, signs of B lymphocyte activation were observed, as evidenced by increased proportions of plasmablasts and CD86-expressing cells. In tumor-infiltrating B lymphocytes (TIL-B), both switched memory cells and plasmablasts were expanded, as compared with nonmalignant epithelium. Moreover, pronounced skewing of IgA/Igκ ratio was evident among TIL-Bs. By spectratype analysis on IgH, we confirmed a monoclonal expansion of the Vh7 family in TIL-B, also present in a tumor-associated LN. Sequencing the clonally expanded Vh7 revealed signs of somatic hypermutation. In conclusion, B lymphocytes in cancer patients exhibit signs of activation in tumor-associated tissues, likely induced by recognition of tumor Ags. Increased numbers of switched memory cells and plasmablasts in combination with clonal expansion and signs of somatic hypermutation suggest a CD4+ T lymphocyte–dependent antitumoral response, which may be exploited for immunotherapy. The Journal of Immunology, 2013, 190: 5847–5855.

Lymphocytic infiltrates within solid tumors are well-recognized positive predictors of survival (11). Notably, B cells are a significant component of these infiltrates. For example, they have been reported to be present in ~25% of breast cancers and constituted up to 40% of the tumor-infiltrating lymphocyte (TIL) population (12–14). Furthermore, tumor-infiltrating B lymphocytes (TIL-Bs) have been correlated with survival in ovarian cancer. The survival was higher when tumors contained both CD20+ and CD8+ cells than either of the TILs alone, which suggests an immunological cooperation between the two cell populations (15). B cells in lymph node (LNs) may also have a major impact on tumor immune responses. In a murine model, 30–35% of freshly harvested tumor-draining LN (TDLN) cells consisted of CD19* B cells. CD40 targeting of the TDLN B cells and dendritic cells, in combination with CD3 targeting of T cells, induced a strong antitumor response. However, depleting either B cells or DCs significantly diminished the antitumor response (16). This study illustrates the potential of TDLN B cells to be effective as APCs with generation of more potent effector cells for adoptive immunotherapy. Moreover, in vitro activated TDLN B cells produce a strong humoral tumor response (17).

However, most studies of B cells in cancer have been performed in mouse models. Comparatively little is known about human tumor-associated B cells and their role in cancer biology. It is therefore of great interest to characterize different subpopulations of B cells in cancer patients. For this purpose, we emulated the Freiburg classification of B cells, categorizing them into six distinct subpopulations, including transitional, naive, marginal zone–like, switched memory, CD21low B cells, and class-switched plasmablasts (18–20). By comparing B cell subpopulations from blood, metastatic lymph node, and tumor from patients with malignancies using the Freiburg panel, L chain discrimination, and also BCR cloning, we present evidence for clonal expansion of tumor recognizing B cell clones.

Materials and Methods

Subjects

Twenty patients were included in the study: 5 with colon cancer; 9 with urinary bladder cancer (UBC); 4 with malignant melanoma, 1 with pancreatic...
cancer, and 1 with prostate cancer. Specimens from LNs, the primary tumor, and in some cases, macroscopically normal urinary bladder mucosa were obtained (Table I), immediately put in RPMI 1640 medium (Sigma), and put on ice, awaiting subsequent preparation (see Cell Preparation section). In addition, peripheral blood was collected from the patients and from 14 healthy donors. The remaining part of LNs and primary tumor were subjected to routine histopathological examination. The study was approved by the local ethical committee, and informed consent was given by the patients.

Identification of the metastatic LNs

Identification of metastatic LNs (MLNs) was done by pathological examination or, alternatively, by intracellular flow cytometry against epithelial cell markers, as previously described (21).

Cell preparation

All the obtained specimens were taken care of within 2 h and treated as follows: PBMCs were separated from peripheral blood using density centrifugation (Ficoll-Paque plus; GE Healthcare). Single-cell suspensions from LNs around the tumors, primary tumors, and nonmalignant tissues were isolated by gentle pressure using a glass homogenizer. Primary tumors and urinary bladder specimens were homogenized using GentleMACS Dissociator (Miltenyi Biotec) in 10 ml RPMI 1640 medium (Sigma), containing 1% collagenase/Hyaluronidase solution (StemCell Technologies).

Flow cytometry analysis

After cell preparation, the cells were freshly used for flow cytometry. They were washed with FACS buffer containing PBS, 2.5% bovine growth serum, and 0.05% NaCl, followed by staining with fluorophore-conjugated Abs against B cell surface markers. The following Abs were used: CD19 allophycocyanin-Cy7, IgD FITC, IgM PE-Cy5, CD38 PE-Cy7. Igκ allophycocyanin, Igκ PE, IgG PE-Cy5, CD27 PE, CD21 allophycocyanin, CD40 FITC, CD86 PE-Cy5, CD69 FITC (Becton Dickinson), and IgG FITC (Miltenyi Biotec). The stained cells were investigated using a FACSaria (Becton Dickinson); 2 × 10^3 to 1 × 10^6 events/sample were collected. The data were analyzed using FACSDivia software. The following Abs were used for identification of MLNs: EpCam AF488 (Fujirebio Diagnostic), mouse anti-human Cytokeratin 20, Cytokeratin 19, Cα19-9 (Dako), and mouse anti-human melanoma (HMAB45 + DT101 + BC199; Abcam). The secondary Abs and isotype controls were as follows: mouse IgG2a AF488 (Becton Dickinson), goat anti-mouse IgG allophycocyanin (Jackson), negative control mouse IgG2a, and negative control mouse IgG1 (Dako).

Spectratype analysis

DNA extraction. DNA from PBMC, LNs, tumor, and nonmalignant tissue of a UBC patient (61-y-old woman) was extracted using DNA lysis buffer, pH 8.0 (10 mM Tris, 1 mM EDTA, 0.1% SDS) and protease K before incubation in 56°C for 10 min. The DNA was then purified, using phenol:chloroform:isoamyl alcohol 25:24:1 saturated with 10 mM Tris, pH 8.0, 1 mM EDTA (Sigma-Aldrich). After centrifugation, sodium acetate (NaOAc) 3 M and 100% ethanol were added to the upper phase of the samples, stepwise rehydration, and resuspension in ultrapure water.

PCR. The 46–52 functional Vh gene segments were grouped into 7 Vh subgroups based on their homology, as previously described (22). Seven forward primers against the frame 3 (FR3) of each of the seven subgroups were used in conjunction with a universal, 6-FAM–conjugated reverse primer against Jh region (22). The PCR was performed with GoTaq master mix (Promega), according to the manufacturer’s recommendations. Spectratyping. For spectratype analysis, Gene Scan 400 HD size standard, Hi-Di Formamide, and ABI 3730 DNA Analyzer (Applied Biosystems) was used as described previously (23). The samples were prepared according to the manufacturer’s protocol (Applied Biosystems). The software, Peak Scanner (Applied Biosystems), was used to analyze the data.

Sequencing

The PCR product from the earlier spectratype analysis was purified with QIAquick PCR purification kit (Qiagen). The purified PCR product was then cloned using TOPO TA Cloning Kit for Sequencing with TOP10 chemically competent cells (Invitrogen) and purified using Plasmid Mini Kit (Qiagen). The purified plasmids were sequenced using ABI 3730 DNA analyzer (Applied Biosystems).

Statistical analysis

The flow cytometry data and comparisons between the specimens were analyzed using Student two-tailed unpaired t-test and Mann–Whitney U-test in GraphPad Prism software. The comparisons of Igκ/Igκ L chain distributions were analyzed using Fisher’s F-test.

Results

To characterize the tumor-associated B cell compartment in humans, we studied 20 patients with various solid tumors. Details regarding the diagnoses, clinical data, and surgical samples obtained for investigation are shown in Table I.

Signs of clonal expansion of circulating B cells from patients with malignant disease

To investigate B cell subpopulations in peripheral blood from patients with malignant disease, we used the validated Freiburg panel, initially introduced for common variable immunodeficiency diagnosis, using cell-surface markers to categorize CD19B cells into six subpopulations (18–20). By flow cytometry, the following CD19B cell subpopulations are distinguished in peripheral blood: naïve B cells (IgD^-CD27^-), marginal zone–like/natural effector B cells (IgD^-CD27^), class-switched memory B cells (IgD^+CD27^), the rare CD21^lowCD38^low B cell population, and transitional B cells (IgM^-CD38^+), and plasmablasts (IgM^-CD38^+). An example from a healthy blood donor is demonstrated in Fig. 1.

The B cell surface phenotypes of PBMCs from the cancer patients were first compared with that of PBMCs from 10 healthy blood donors (Fig. 2). None of the patients exhibited lymphopenia or abnormal total numbers of B cells in peripheral blood (Supplementary Table I) (15), and there was no significant difference in the proportion of CD19^+ lymphocytes, as compared with healthy donors (Fig. 2A, top left panel); nor was there any discernible difference in the distribution of B cell subpopulations in patient blood according to the Freiburg panel, with the exception of plasmablasts. The proportion of plasmablasts was significantly lower (p < 0.05) in the blood of patients with malignant disease (Fig. 2A, bottom panel). Skewing of Igκ/Igκ L chain usage may indicate clonal expansion of B lymphocytes. The Igκ/Igκ L chain ratio on peripheral blood B lymphocytes from healthy donors revealed the expected Igκ dominance, with an Igκ/Igκ L

Table I. Information about the patients, severity of their disease, and number of samples

<table>
<thead>
<tr>
<th>Cancer Types</th>
<th>Patients</th>
<th>Age, y</th>
<th>Sex, M:F</th>
<th>MLN:Patients</th>
<th>NMLN:Patients</th>
<th>Blood</th>
<th>Tumor</th>
<th>Nonmalignant Tissue</th>
<th>Chemotherapy</th>
</tr>
</thead>
<tbody>
<tr>
<td>CC</td>
<td>5</td>
<td>47–69</td>
<td>4:1</td>
<td>9:5</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>N</td>
</tr>
<tr>
<td>UBC</td>
<td>9</td>
<td>53–86</td>
<td>7:2</td>
<td>4:2</td>
<td>17:4</td>
<td>8</td>
<td>7</td>
<td>4</td>
<td>2 Y, 7 N</td>
</tr>
<tr>
<td>MM</td>
<td>4</td>
<td>50–76</td>
<td>3:1</td>
<td>5:3</td>
<td>3:3</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>N</td>
</tr>
<tr>
<td>PC</td>
<td>1</td>
<td>72</td>
<td>1:0</td>
<td>1:1</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>N</td>
</tr>
<tr>
<td>PrC</td>
<td>1</td>
<td>81</td>
<td>1:0</td>
<td>2:1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>N</td>
</tr>
<tr>
<td>Total</td>
<td>20</td>
<td>47–86</td>
<td>16:4</td>
<td>19:11</td>
<td>22:8</td>
<td>9</td>
<td>8</td>
<td>5</td>
<td>2 Y, 11 N</td>
</tr>
</tbody>
</table>

*aNumber of metastasized and nonmetastasized LNs versus number of patients from whom they were acquired.

*bEach sample is taken from one patient.

CC, Colon cancer; MM, malignant melanoma; N, no previous neoadjuvant chemotherapy; PC, pancreatic cancer; PrC, prostate cancer; Y, with previous neoadjuvant chemotherapy.
chain ratio of 0.7 (Fig. 2B, top panel). However, the Ig\(_\lambda\)/Ig\(_κ\) L chain ratio among peripheral blood B lymphocytes from patients with malignant disease displayed a significantly increased spreading (p < 0.05) compared with the ratios on B lymphocytes from healthy donors, indicating clonal expansions of B lymphocytes in some of the patients (Fig. 2B, top panel). However, the proportion of circulating IgG\(^+\) B lymphocytes was unaffected (Fig. 2B, middle panel). In addition, circulating B lymphocytes from patients with malignant disease did not demonstrate any signs of recent activation because no significant differences with regard to expression of CD69 (Fig. 2B, bottom panel), CD86, and CD40 were observed (data not shown).

**B lymphocytes in MLNs have an activated phenotype**

We decided to investigate B cell subpopulations, isolated from MLNs and nonmetastatic LNs (NMLNs). An example of an LN investigated using the Freiburg panel is shown in Fig. 3A. The distribution of the B lymphocyte populations in the LN appears similar to that observed in peripheral blood, with the exception of an increased proportion of switched memory cells (Ig\(^D\)/CD27\(^+\)) and a lower proportion of IgM\(^+\)/CD38\(^-\) transitional B cells (Fig. 3A).

When investigating the B lymphocyte subpopulations in lymph nodes using the Freiburg panel, we first noticed that the fraction of CD19\(^+\) B lymphocytes from MLNs was significantly lower than in NMLNs (p < 0.01; Fig. 3B), but the balance between naive (Ig\(^D\)/CD27\(^-\)) and switched memory cells (Ig\(^D\)/CD27\(^+\)) was unaltered. However, the distribution of the other B cell subpopulations was altered in the presence of LN metastasis: in the MLNs, the proportion of marginal zone–like/innate effector B cells (Ig\(^D\)/CD27\(^+\)) was significantly decreased (p < 0.01; Fig. 3B). In addition, there was a significantly increased proportion of transitional B cells (IgM\(^+\)/CD38\(^+\); p < 0.05) and plasmablasts (IgM\(^+\)/CD38\(^-\); p < 0.001) in MLNs (Fig. 3B).

Because we found a significant difference in the distribution of Ig\(_\lambda\)/Ig\(_κ\) L chain usage in peripheral blood (Fig. 2B), we compared

**FIGURE 1.** Multicolor flow cytometry analysis of subpopulations of B cells in blood of cancer patients. A representative example of PBMCs is shown. (A) A lymphocyte gate is established on forward (FSC) and side scatter (SSC) characteristics (left panel, gate 1), followed by gating on CD19\(^+\) cells (right panel, gate 2). (B) In the Freiburg classification, the following CD19\(^+\) B cell subpopulations are defined: Ig\(^D\)/CD27\(^-\) naive (gate 3), Ig\(^D\)/CD27\(^+\) marginal zone–like (gate 4), and Ig\(^D\)/CD27\(^-\) class-switched memory (gate 5). Furthermore, CD21/CD38 and IgM/CD38 dot plots permit distinction of CD21\(^{low}\)/CD38\(^{low}\) (gate 6), IgM\(^-\)/CD38\(^{+}\) transitional B cells (gate 7), and IgM\(^-\)/CD38\(^{+}\) plasmablasts (gate 8).

**FIGURE 2.** The CD19\(^+\) B cell compartment in peripheral blood from patients and healthy donors. (A) Mean percentages of CD19\(^+\) B cells in total lymphocytes and of the B cell subpopulations in CD19\(^+\) lymphocytes are shown. (B, top panel) The Ig\(_\lambda\)/Ig\(_κ\) isotype distribution was determined by flow cytometry. The proportion is calculated by dividing Ig\(_\lambda\) with Ig\(_κ\) percentages of CD19\(^+\) cells. (B, middle and bottom panels) Percentages of CD69\(^+\) and IgG\(^+\) cells in CD19\(^+\) lymphocytes are shown. Numbers of patients and healthy donors in this experiment are indicated in Table I. The SD of the Ig\(_\lambda\)/Ig\(_κ\) ratio was calculated using Fisher’s F-test. All error bars indicate SEM. Percentages of lymphocyte subpopulations were compared with the Student two-tailed t test or Mann–Whitney U test. *p < 0.05.
the L chain usage in LNs with or without metastases. There was no significant difference in the Igλ/Igκ L chain usage when comparing B cell L chain expression from MLNs and NMLNs (Fig. 3C, top panel). The proportion of Igλ/Igκ light chains was investigated by flow cytometry. The proportion is calculated by dividing Igλ with Igκ percentages of CD19+ cells. (C, middle and bottom panels) CD69+ and CD86+ B cells are shown. Numbers of MLNs and NMLNs are indicated in Table I. The SD of the Igλ/Igκ ratio was calculated using Fisher’s F-test. All error bars indicate SEM. Percentages of lymphocyte subpopulations were compared with the Student two-tailed t test or Mann–Whitney U test. *p < 0.05, **p < 0.01, ***p < 0.001.

Switched memory B cells and plasmablasts accumulate in tumors

From eight patients, mainly with UBC, samples from tumor and corresponding nonmalignant tissue were available (Table I), which enabled us to compare TIL-B with the presence of B cell subpopulations in nonmalignant epithelial tissue. Representative plots from these investigations are shown in Fig. 4A and 4B where samples from a UBC patient are shown. There was no difference in the proportion CD19+ B cells retrieved from tumors when compared with B cells from normal tissues. However, the distribution of B cell subpopulations within tumor tissue appeared right-shifted, that is, shifted toward more developed stages of maturation, with significantly increased representation of IgD CD27+ switched memory cells (p < 0.05) and IgM CD38+ plasmablasts (p < 0.01). Albeit not reaching significance, there was a corresponding tendency toward decreased representations of naive, marginal zone–like and CD21low B cells in the tumor samples (Fig. 4C). Furthermore, the Igλ/Igκ L chain ratio displayed pronounced skewing among the TIL-Bs (p < 0.05) compared with the L chain usage among B cells from normal epithelia (Fig. 4D, upper panel). There were no significant differences between CD69 (Fig. 4D, lower panel), IgG+, CD40+, and CD86 (data not shown) TIL-Bs when compared with B cells from corresponding nonmalignant epithelial tissue.

UBC specimens display the same distribution of B cell subtypes

To investigate whether there was a difference between distributions of B cell subtypes in specimens from a certain cancer type compared with the original data from all patients, we analyzed data from UBC separately. There were no differences between circulating B cell subtypes in UBC compared with healthy control and B cell subtypes in the original data (Fig. 5A). No differences of other markers and distribution of Igλ/Igκ L chain ratio in the two groups of specimens could be seen (Fig. 5B). When comparing B cell subtypes, CD86+, CD69+ B cells, and Igλ/Igκ L chain ratio in MLN with
NMLN, no differences could be seen between the two groups of patients (Fig. 6A, 6B). The distributions of B cell subtypes in tumor from UBC patients compared with their counterparts in normal urinary bladder epithelium did not display any differences with data from all patients (Fig. 7A). The same observation could be noticed when CD69+ B cells and Igλ/Igκ ratio in tumors versus nonmalignant tissue were compared in the two groups of patients (Fig. 7B).

**Corresponding clonal B cell expansion in tumor and adjacent LNs**

The observed skewing of Igλ/Igκ ratios in B cells from blood (Fig. 2B), MLNs (Fig. 3C), and TILs (Fig. 4D) suggested clonal expansion of B cells in response to recognition of tumor Ags. To confirm the presence of clonal expansion, we performed a spectratype analysis investigating the recombination of seven IgH families in B cells from a UBC patient, where B cells from tumor and LN were available for analysis. The spectratype analyses investigating the usage of the heavy chains Vh1–6 did not display any major expansion in any particular CDR3 length, when comparing peaks from tumor, LN, nonmalignant tissue, or PBMCs (Supplemental Figs. 1–3). However, we found one outstanding CDR3 peak when the Vh7 H chain was investigated among B cells from the tumor (Fig. 8). The dominant peak in the LN displayed the same CDR3 length as demonstrated in the overlay plot of tumor and LN (Fig. 8B), thus suggesting the same clonal expansion in the TDLN and among tumor-infiltrating B cells. In a second, more distant LN from the same patient, there was no dominant CDR3 peak. In comparison, B cells from nonmalignant tissue displayed a Gaussian-like distribution in their CDR3 lengths (Fig. 8, bottom panel).
To further characterize the clonally expanded H chain in the tumor tissue, we sequenced three clones of the Vh7 family (P1, P3, and P6). Comparing the sequence data from the three clones with published sequences revealed the V-D-J region subfamilies to be IGHV7-04-1*02, IGHD1-07*01, and IGHJ5*02, respectively (Fig. 9A–C). Three bases in FR3 of all the three clones differed compared with the corresponding bases reported from germline sequences (Fig. 9A). One of these mutations was found in a WRCY hotspot motif (Fig. 9A, rectangle) and caused an amino acid change from serine to glycine, whereas the other two were silent mutations. Clone P1 displayed a G-to-A mutation in position 108 of the CDR3, resulting in an amino acid switch from glycine to glutamic acid (Fig. 9D, 9E). The sequencing was carried out twice with forward and reverse primers, and both experiments confirmed the mutations.

**Discussion**

During the past few years, increasingly important roles of B cells in the immune system of cancer patients have been reported (12–17, 24). In this study, we describe an increased frequency of plasmablasts in MLNs compared with NMLNs, as well as in tumor versus nonmalignant tissue. Together with the demonstration of a common clonal expansion of tumor-infiltrating B cells in a tumor-associated LN, these observations argue for the presence of tumor-specific B cell responses in cancer patients.

Categorizing subpopulations of B cells has contributed to the understanding of diverse clinical conditions in which B cells play a role, such as common variable immunodeficiency (19) and chronic graft-versus-host disease (25). In this study, we use for the first
time, to our knowledge, the well-described Freiburg panel to characterize B cell subpopulations from cancer patients. Comparison between CD19+ B cells in healthy blood and in patient blood illustrated no significant difference (Fig. 2A), nor was any difference observed when comparing TIL-Bs and nonmalignant tissue–infiltrating B lymphocytes (NTIL-Bs; Fig. 3A). However, the CD19+ cells occupied a smaller proportion of the lymphocyte compartment in MLNs compared with NMLNs (Fig. 4A). Unfortunately, whether this is due to reduced absolute numbers or is secondary to T cell expansion cannot be deduced from available data. Likewise, the reduced proportion of marginal zone-like B cells in MLNs (Fig. 3A) remains unexplained. As expected, the mean percentage of naive B cells was higher in peripheral blood than in LNs and lower still within the tumor and nonmalignant tissue (Figs. 2–4), whereas the reverse is true for switched memory cells. Interestingly, a higher mean percentage of switched memory B cells in tumors than in normal epithelium was evident, which may indicate tumor Ag encounter and/or recruitment of Ag-specific B cells (Fig. 4A). This interpretation is reinforced by the higher mean percentage of plasmablasts within tumors as compared with their counterparts in nonmalignant tissue (Fig. 4A).

It is tempting to speculate that the lower representation of plasmablasts in peripheral blood of cancer patients as compared with healthy blood donors reflects homing of these cells to tumor sites. Comparison of plasmablasts in MLNs and NMLNs also shows a higher mean percentage in the former (Fig. 3A). Likewise, the proportion of CD86-expressing B cells increased in the presence of metastasis, indicating tumor-specific activation of B cells. Notably, the proportion of CD69+ B cells was lower in MLNs compared with NMLNs and there was no significant difference between any of the activation markers in tumor tissues as compared with normal epithelium. This may be explained by the different kinetics displayed by the markers upon Ag-specific activation, where CD69 is rapidly downregulated, whereas CD86 expression is more long-lasting (26, 27). Interestingly, the mean percentage of transitional B cells was higher in MLNs than in NMLNs. Although the absolute cell numbers in this compartment is low, the observation is intriguing. One possible explanation is increased de novo recruitment of precursor B cells, and alternatively that an immunosuppressive milieu is created by the tumor, which hampers their maturation to B cells.

The origin and function of the CD21low B cells in the Freiburg panel has been debated. Their numbers are increased during chronic viral infection, as well as in certain immunodeficiencies and autoimmune diseases (19, 28). Some authors have suggested that they resemble innate-like tissue homing B cells (29), but their exact role is likely dependent on the underlying condition. In the setting of HIV infection, two distinct subpopulations have been described, namely, CD27+–activated mature B cells and CD27−exhausted tissue-like memory B cells (30). In this study, CD21low B cells were mostly present in tumors and nonmalignant tissues, although the absolute cell numbers in this compartment is low, the observation is intriguing. One possible explanation is increased de novo recruitment of precursor B cells, and alternatively that an immunosuppressive milieu is created by the tumor, which hampers their maturation to B cells.

The origin and function of the CD21low B cells in the Freiburg panel has been debated. Their numbers are increased during chronic viral infection, as well as in certain immunodeficiencies and autoimmune diseases (19, 28). Some authors have suggested that they resemble innate-like tissue homing B cells (29), but their exact role is likely dependent on the underlying condition. In the setting of HIV infection, two distinct subpopulations have been described, namely, CD27+–activated mature B cells and CD27−exhausted tissue-like memory B cells (30). In this study, CD21low B cells were mostly present in tumors and nonmalignant tissues, although the absolute cell numbers in this compartment is low, the observation is intriguing. One possible explanation is increased de novo recruitment of precursor B cells, and alternatively that an immunosuppressive milieu is created by the tumor, which hampers their maturation to B cells.

The origin and function of the CD21low B cells in the Freiburg panel has been debated. Their numbers are increased during chronic viral infection, as well as in certain immunodeficiencies and autoimmune diseases (19, 28). Some authors have suggested that they resemble innate-like tissue homing B cells (29), but their exact role is likely dependent on the underlying condition. In the setting of HIV infection, two distinct subpopulations have been described, namely, CD27+–activated mature B cells and CD27−exhausted tissue-like memory B cells (30). In this study, CD21low B cells were mostly present in tumors and nonmalignant tissues, although the absolute cell numbers in this compartment is low, the observation is intriguing. One possible explanation is increased de novo recruitment of precursor B cells, and alternatively that an immunosuppressive milieu is created by the tumor, which hampers their maturation to B cells.
influenced by the presence of tumor Ag. On the contrary, the increased numbers of plasmablasts in tumors and MLNs, and the higher percentage of switched memory B cells in MLNs, obviously indicate tumor-specific T cell–dependent activation and differentiation of B cells. This process, signified by somatic hypermutation and germinal center formation, also explains the increased skewing and spreading of Igλ/Igκ ratio in patient samples (Figs. 2B, 3B, 4B). Somatic hypermutation is characterized by single base substitutions resulting in a characteristic mutation pattern (31–33). Our sequencing of the Vh7 clone showed a WRCY hotspot motif, which often appears after a mutation in WRC motif caused by activation-induced cytidine deaminase (34, 35). This potential activation-induced cytidine deaminase–dependent mutation strengthens the hypothesis of B cell somatic hypermutation in TIL and in MLNs, and again suggests a T cell–driven response. Certainly, this response may be because of a microbial peptide or stress ligands expressed by the malignant urothelium, but given its context, we consider it more likely to be elicited by tumor Ags. One of our three sequenced clones, P1, contained a single base difference in CDR3 compared with the other clones, which also may represent a somatic hypermutation.

The presence of clonal B cell expansions is formally demonstrated by the spectratype analysis, where we were able to demonstrate a monoclonal expansion of TIL-Bs also present in a tumor-associated LN (Fig. 8). Restricted Ig gene expression in TIL-Bs has been described by others (36, 37). Some investigators have suggested that this expansion occurs within the tumor tissue and in tumor-associated germinal centers (36). By contrast, our result shed light to another option where a tumor-draining LN could be the origin of the B cell response in our study. Merely spectratype analysis is only suggestive. Sequencing of the H chain in the tumor-associated LN is necessary to demonstrate this issue. Notably, we also observed skewing of Igλ/Igκ ratio in some NMLNs (Fig. 3B). This is not surprising; because LNs were in close proximity to the tumor, some, or most of these, are likely tumor draining, and thus may have received soluble tumor debris in vivo, resulting in B cell activation.

In many reports, Ag-experienced TIL-Bs correlate with survival time of patients with different cancer types (38–41). In studies with TIL-Bs in ovarian cancer and breast carcinomas, somatic hypermutation and clonal B cell expansion were shown, using Vh gene analysis. In addition, TIL-Bs displayed class-switching and Ag-presenting phenotypes (40, 41). These observations correlate with our results from TIL-Bs, indicating Ag-dependent tumor response in malignancies.

Taken together, our results suggest that B cell activation patterns reflect a T cell–dependent humoral response to tumors. This underscores the role of B cells as APCs, which is also highlighted.
by the higher percentage of CD68+ B cells in MLNs. It has been demonstrated that TDNL B cells in mice can be efficient APCs (16, 17). Yet, it remains to be demonstrated whether they play this role in humans with solid tumors. We have previously described that a tumor-specific Th1 response is present in sentinel LN in colon and bladder cancer (42, 43), and that sentinel node–acquired CD4+ T cells are promising for use in adoptive immunotherapies (1). In this study, we emulated sentinel node–resident total APCs for ex vivo T cell activation and expansion. Enrichment of tumor-specific B cells as an alternative APC population is a theoretically appealing alternative that deserves to be investigated.

Disclosures

The authors have no financial conflicts of interest.

References

Supplemental legends

**Table SI. Range of total acquired flow cytometry events and percentage of lymphocytes.**
The table displays the range of total number of all events and number of acquired cells in the lymphocyte gate in the samples. It also shows the range of lymphocyte percentage of all events and the range of CD19+ events in lymphocyte gates of all samples.

**Figure S1. Spectratype analysis of B cell Vh1 and Vh2 in PBMC, tumor, lymph nodes and non-malignant tissue.** Spectratypes of Vh1 and Vh2 are demonstrated in PBMC, tumor (T), lymph node 1 (LN1), lymph node 2 (LN 2), non-malignant tissue (NT) of a patient with urinary bladder cancer. The size range of the gene products are given in base pairs (bp).

**Figure S2. Spectratype analysis of B cell Vh3 and Vh4 in PBMC, tumor, lymph nodes and non-malignant tissue.** Spectratypes of Vh3 and Vh4 are demonstrated in PBMC, tumor (T), lymph node 1 (LN1), lymph node 2 (LN 2), non-malignant tissue (NT) of a patient with urinary bladder cancer. The size range of the gene products are given in base pairs (bp).

**Figure S3. Spectratype analysis of B cell Vh5 and Vh6 in PBMC, tumor, lymph nodes and non-malignant tissue.** Spectratypes of Vh5 and Vh6 are demonstrated in PBMC, tumor (T), lymph node 1 (LN1), lymph node 2 (LN 2), non-malignant tissue (NT) of a patient with urinary bladder cancer. The size range of the gene products are given in base pairs (bp).
### Table S1. Range of total acquired flow cytometry events and percentage of lymphocytes.

<table>
<thead>
<tr>
<th></th>
<th>ALL EVENTS</th>
<th>LYMPHOCYTES TOTAL EVENTS</th>
<th>LYMPHOCYTES %</th>
<th>CD19 TOTAL EVENTS</th>
</tr>
</thead>
<tbody>
<tr>
<td>HEALTHY BLOOD</td>
<td>$1 \times 10^4 - 1.3 \times 10^5$</td>
<td>$4015 - 7.6 \times 10^4$</td>
<td>40.20 – 88.20</td>
<td>251 – 11568</td>
</tr>
<tr>
<td>PATIENT BLOOD</td>
<td>$1 \times 10^4 - 1 \times 10^5$</td>
<td>$5172 - 49512$</td>
<td>34.60 – 64.10</td>
<td>246 – 5123</td>
</tr>
<tr>
<td>METASTASIZED LN</td>
<td>$1 \times 10^4 - 844799$</td>
<td>$1766 - 70.4 \times 10^4$</td>
<td>2.30 – 81.10</td>
<td>164 – 9413</td>
</tr>
<tr>
<td>NON-METASTASIZED LN</td>
<td>$2 \times 10^4 - 1 \times 10^5$</td>
<td>$9228 - 60523$</td>
<td>46.10 – 84.00</td>
<td>3590 – 2.3 \times 10^4</td>
</tr>
<tr>
<td>TUMOR</td>
<td>$1 \times 10^4 - 9.0 \times 10^4$</td>
<td>$1096 - 3.3 \times 10^4$</td>
<td>5.10 – 42.90</td>
<td>203 – 1782</td>
</tr>
<tr>
<td>NON_MALIGNANT TISSUE</td>
<td>$2 \times 10^4 - 5 \times 10^5$</td>
<td>$1931 - 1.9 \times 10^5$</td>
<td>9.70 – 37.10</td>
<td>176 – 902</td>
</tr>
</tbody>
</table>

The table displays the range of total number of all events and number of acquired cells in the lymphocyte gate in the samples. It also shows the range of lymphocyte percentage of all events and the range of CD19+ events in lymphocyte gates of all samples.
Figure S3

Vh5

PBMC

T

LN1

LN2

NT

Vh6

120 160 bp

120 160 bp