Neogenesis of Lymphoid Structures and Antibody Responses Occur in Human Melanoma Metastases

Arcadi Cipponi, Marjorie Mercier, Teofila Seremet, Jean-François Baurain, Ivan Théate, Joost van den Oord, Marguerite Stas, Thierry Boon, Pierre G. Coulie, and Nicolas van Baren

Abstract

Lymphoid neogenesis, or the development of lymphoid structures in nonlymphoid organs, is frequently observed in chronically inflamed tissues, during the course of autoimmune, infectious, and chronic graft rejection diseases, in which a sustained lymphocyte activation occurs in the presence of persistent antigenic stimuli. The presence of such ectopic lymphoid structures has also been reported in primary lung, breast, and germline cancers, but not yet in melanoma. In this study, we observed ectopic lymphoid structures, defined as lymphoid follicles comprising clusters of B lymphocytes and follicular dendritic cells (DC), associated with high endothelial venules (HEV) and clusters of T cells and mature DCs, in 7 of 29 cutaneous metastases from melanoma patients. Some follicles contained germinal centers. In contrast to metastatic lesions, primary melanomas did not host follicles, but many contained HEVs, suggesting an incomplete lymphoid neogenesis. Analysis of the repertoire of rearranged immunoglobulin genes in the B cells of microdissected follicles revealed clonal amplification, somatic mutation and isotype switching, indicating a local antigen-driven B-cell response. Surprisingly, IgA responses were observed despite the nonmucosal location of the follicles. Taken together, our findings show the existence of lymphoid neogenesis in melanoma and suggest that the presence of functional ectopic lymphoid structures in direct contact with the tumor makes the local development of antimalanoma B- and T-cell responses possible.

Introduction

Many if not all human tumors express antigens that can be recognized by autologous T lymphocytes (1). Cancer patients who have circulating antitumoral antibodies and T lymphocytes (2, 3). Tumors are often infiltrated by inflammatory cells, principally lymphocytes and macrophages. Many studies support the notion that this inflammation accompanies an immune reaction mounted by the host against its tumor. It has been shown that tumor-infiltrating B and T lymphocytes can be directed at tumor antigens (4–7). In several types of tumors such as melanoma and ovarian, colorectal and breast carcinomas, the presence of tumor-infiltrating T cells has been associated with a more favorable clinical evolution (8–11). Altogether these observations indicate that cancer patients can mount spontaneous adaptive immune responses against their tumor. These responses have been extensively studied mainly through the identification of their target antigens. However, little is known about the mechanisms that trigger off these responses, at which stage of the disease and where they originate, and how they are maintained. It is commonly thought that these events take place in the lymph nodes that drain tumor sites.

The secondary lymphoid organs, which include the lymph nodes, the spleen, and mucosal associated lymphoid tissues, are the major sites of lymphocyte activation during adaptive immune responses (12). In the absence of antigenic stimulation, resting B lymphocytes aggregate around a network of follicular dendritic cells (FDC), forming clearly recognizable structures called follicles. Outside of these follicles, the T-cell zones contain mainly resting T cells, interdigitating dendritic cells (DC), and fibroblastic reticular cells. To reach the lymph nodes, resting lymphocytes circulating in the blood adhere to specialized blood vessels located near the B- and T-cell zones, the high endothelial venules (HEV). The specificity of this binding is mainly mediated by L-selectin (CD62L) expressed on naive and central memory lymphocytes, and their receptors, the peripheral node addressins (PNAd), which are sulfated glycoproteins expressed selectively on the endoluminal side of HEV. Migrating B cells are attracted toward the follicles by the CXCL13 chemokine, secreted mainly by FDC, whereas T lymphocyte migration toward the T-cell zones is mainly controlled.
by CCL19 and CCL21, produced principally by fibroblastic reticular cells.

Following antigenic stimulation, B cells proliferate vigorously in germinal centers within the follicles. Activated helper T cells migrate within the follicle, and interaction with these follicular helper T cells induces expression of activation-induced cytidine deaminase (AID) in the germinal center B cells (13). This enzyme controls somatic hypermutation in the variable region of immunoglobulin (Ig) genes, which introduces additional diversity in the antibody repertoire (14, 15). Competitive interaction with FDC, which present the antigen in limited amount, results in the clonal selection of the B cells with the highest Ig affinity. AID also directs isotype switching, resulting in the preferential production of either IgG, IgA, or IgE (14, 15).

In most cases of infectious aggression, the coordinated action of innate and adaptive immunity results in the elimination of the pathogen and restoration of tissue integrity. Failure to do so leads to chronic inflammation, characterized by lymphomonocytic infiltration and attempts to confine the infection by encapsulation or granuloma formation. In addition, ectopic lymphoid structures, also called tertiary lymphoid structures or organs, develop within the inflamed area, a phenomenon called lymphoid neogenesis. These structures resemble the B- and T-cell areas of lymph nodes, in that they contain follicles comprising B cells and FDC, and distinct T-cell areas comprising mature DC, T-cell clusters, and neighboring HEV-like blood vessels. This phenomenon is thought to improve adaptive responses against persisting antigens, by bringing the whole immune response machinery to the site of the aggression (16, 17). Ectopic lymphoid structures are found in many chronic infectious diseases such as Helicobacter-associated gastritis, chronic HCV hepatitis, and Lyme arthritis. Lymphoid neogenesis is also frequently observed in noninfectious chronic inflammatory diseases, including many autoimmune diseases such as thyroiditis, rheumatoid arthritis, and multiple sclerosis, as well as in chronic allograft rejection (16). It has been shown that these structures can host B- and T-cell responses directed at antigens expressed in the diseased tissue (18–20).

The molecular mechanisms that lead to lymphoid neogenesis are only partially understood. They seem to be similar to those that lead to the formation of new secondary lymphoid organs during fetal development. This development is orchestrated by cells of hematopoietic origin, the lymphoid tissue inducer cells (LTi), which drive endothelial cells to express PNAd and become HEV, and stromal cells to differentiate into FDC and fibroblastic reticular cells. It is not known whether LTi-like cells contribute to the formation of ectopic lymphoid structures. An alternative mechanism has been proposed, by which activated lymphocytes substitute for LTi (16, 21).

Malignant tumors resemble chronic infections, autoimmune diseases and chronic allograft rejection in that they host a persistent immune infiltration that fails to clear the antigenic insult. Consistent with this, the presence of ectopic lymphoid structures has been reported in several neoplastic diseases. Non–small cell lung cancers were found to host such structures, combining B-cell follicles, HEV associated with enriched naive T cells, and clusters of mature DC and T cells whose density was associated with a better prognosis (22, 23). B-cell follicles containing germinal centers were observed in primary medullary and ductal breast cancer. In these tumors, molecular characterization of Ig gene rearrangements showed that clonal proliferation, somatic Ig mutation, and clonal selection all occurred (24–26). The analysis of primary germ cell cancers has revealed the presence of lymphoid follicles with germinal centers, in which ongoing antigen-driven antibody responses occurred (27). Finally, ectopic lymphoid structures have been detected in colorectal cancer, with their presence being correlated to a better prognosis (28). In all these tumors, lymphoid neogenesis seems to be associated with an inflammatory infiltrate.

In this work, we report the presence of ectopic lymphoid structures in metastatic lesions of melanoma, a tumor type that is frequently considered as particularly immunogenic, and characterize the B-cell responses that take place in these structures.

Materials and Methods

Patients and tissue samples

Tumors and healthy tissues were obtained as surgical discard samples, or as research-aimed surgery or biopsy after informed consent, under approval of the Commission d’Ethique Biomédicale Hospitalo-Facultaire, Cliniques universitaires Saint-Luc, Brussels, Belgium. Fresh tissue samples were snap frozen on dry ice or in isopentane shortly after surgical resection, embedded in Tissue-Tek OCT Compound (Sakura) and stored at −80°C.

Immunohistochemistry

Seven μm-thick cryosections were obtained from frozen OCT-embedded tissue samples, air dried, stored at −80°C until use, then thawed and fixed in 4% paraformaldehyde for 5 minutes. Endogenous peroxidase activity was blocked with Peroxidase Blocking Reagent (Dako Cytomation). For PNAd immunostaining, sections were treated with Biotin Blocking System (Dako) to neutralize endogenous biotins, incubated for 30 minutes with the biotinylated MECA-79 antibody, washed, and incubated for 30 minutes with streptavidin coupled to horseradish peroxidase (Vector Laboratories). For all other antigens, sections were incubated for 30 minutes with unlabelled primary antibody (see list in Supplementary Table S1), washed, and incubated for 30 minutes with a secondary polyclonal goat anti-mouse antibody coupled to horseradish peroxidase. After washing, bound antibodies were detected with 3-aminio-9-ethylcarbazole and sections were counterstained with hematoxylin. All reactions were carried out at room temperature. A reactive lymph node and a reactive tonsil from noncancerous persons were used as positive controls for all antigens except Melan-A, for which a lymph node melanoma metastasis with confirmed expression of the Melan-A gene by reverse transcriptase PCR (RT-PCR) was used. Negative controls included omission of primary antibody on the control tissue sections. Stained slides were digitized by automated whole-slide image capture, using a Mirax Midi scanner (Carl
One-fortieth of the reaction volume was used for PCR. Quantitative reverse transcriptase (Invitrogen) and a random hexamer (Eurogentec), 300 nmol/L of each primer, 100 nmol/L of probe, and qPCR RNA extraction from tissue samples, cDNA synthesis, and qPCR

RNA samples were prepared from frozen tissue sections or tissue fragments using the guanidinium isothiocyanate/cesium chloride procedure (29). RNA quality was verified by manual counting of CD20 cells in the adjacent section. Selected groups of cells were microdissected using a PALM MicroBeam laser capture workstation (Carl Zeiss). Isolated fragments were catapulted on the cap of AdhesiveCap 500 Opaque tubes (Carl Zeiss) and were snap frozen in dry ice and stored at −80°C until further processing.

Laser capture microdissection

Additional cryosections (9–16 μm thick) were cut from the remaining frozen tissues and were mounted onto ultralovietreated membrane-coated slides (PALM Microlaser Technologies). Samples were fixed in 75% ethanol for 1 minute at −20°C, stained for 30 seconds with 1% cresyl violet in ethanol, and dehydrated in consecutive washes of 75% and 95% ethanol for 30 seconds each, and for 2 minutes in 100% ethanol. Slides were allowed to air dry for 5 minutes. Follicular and extrafollicular areas were identified and selected based on their morphology and on CD20 immunostaining of an adjacent tissue section. The number of B lymphocytes in each isolated follicle was estimated by manual counting of CD20+ cells in the adjacent section. Selected groups of cells were microdissected using a PALM MicroBeam laser capture workstation (Carl Zeiss). Isolated fragments were catapulted on the cap of AdhesiveCap 500 Opaque tubes (Carl Zeiss) and were snap frozen in dry ice and stored at −80°C until further processing.

RNA extraction from tissue samples, cDNA synthesis, and qPCR

RNA samples were prepared from frozen tissue sections or tissue fragments using the guanidinium isothiocyanate/cesium chloride procedure (29). RNA quality was verified with an Agilent Bioanalyzer. Total RNA was treated with TURBO DNase (Turbo DNA-freeTM Kit; Ambion). The first-strand cDNA was produced from 2 μg of total RNA with the M-MLV reverse transcriptase (Invitrogen) and a random hexamer primer (Fermentas), according to the manufacturer’s protocol. One-fourth of the reaction volume was used for PCR. Quantitative RT-PCR amplifications were carried out as described (25), using the PCR Core Kit (Eurogentec), in a final volume of 25 μL with 0.625 U of HotGoldStar DNA polymerase (Eurogentec), 300 nmol/L of each primer, 100 nmol/L of probe, 200 μmol/L of each dNTP, and 5 nmol/L of MgCl2 in an ABI 7300 Real-Time PCR system (Applied Biosystems). The following PCR conditions were used: 94°C for 10 minutes, 45 cycles of 94°C for 15 seconds, and 60°C for 1 minute. The qPCR primers and probes (Eurogentec) are reported in Supplementary Table S1. Samples for quantitative PCR were assayed in duplicate. Levels of expression were normalized to β-actin.

Construction of Ig heavy chain cDNA libraries

RNA was purified from microdissected tissue sections using the TriPure isolation reagent (Roche) according to the manufacturer’s protocol, except for the addition of 20 ng tRNA at the start, and 20 μg of glycogen before the precipitation step. Recovered RNA was resuspended in 5 or 10 μL of RNase-free water and stored at −80°C. The mRNA encoding Ig heavy chain genes was selectively retrotranscribed using Superscript II reverse transcriptase (Invitrogen) and a set of degenerate primers matching the DNA region encoding the transmembrane region of the IGHV, IGHM, IGHG1, IGHG2, IGHG3, IGHG4, IGHAI, and IGH2A genes (see Supplementary Table S1 for primer sequences). After RNaseH treatment (Fermentas), the resulting cDNA was divided in 4 equal parts, one for each isotype family (except IgE). Each part was amplified by 2 rounds of nested PCR, according to a previously reported method (30). The first PCR used a set of degenerate forward primers matching the leader (L) sequence of the IGHV family genes, and a reverse primer matching the respective Ig heavy chain constant gene. Amplification was carried out with Taq DNA polymerase (Takara) in 1x PCR Buffer n’1 from the Expand Long Template PCR System Kit (Roche), with 5 μL of cDNA in a 50 μL volume, and started with 4 minutes at 94°C, followed by 30 cycles of 45 seconds at 95°C, 45 seconds at 54°C, and 45 sec + 2 sec/cycle at 72°C, followed by 10 minutes at 72°C. The second PCR used a set of degenerate forward primers matching the Framework 1 region of the IGHV family genes and a more proximal reverse primer matching the respective Ig heavy chain constant gene. Amplification was carried out with 5 μL of a 1/100 dilution of the product of the first PCR in a 50-μL volume, using the same enzyme and buffer, and started with 4 minutes at 94°C, followed by 30 cycles of 45 seconds at 94°C, 30 seconds at 55°C, and 45 seconds + 2 sec/cycle at 72°C, followed by 10 minutes at 72°C. The PCR products were separated by electrophoresis on an agarose gel, and a gel slice containing DNA in the desired range was excised. The DNA was purified with the Genejet Gel Extraction Kit (Fermentas) and cloned into TOP10 bacteria using the TOPO TA Cloning Kit (Invitrogen). Sequencing of individual DNA clones was carried out using the Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems). The sequences were compared with published germline Ig genes using the international ImMunoGeneTics information system (IMGT) database (website http://www.imgt.org; ref. 31). The VH, Dβ, and Jβ segments in each sequenced clone were assumed to be derived from the most homologous corresponding germline genes. Comparison between cloned and germline sequences allowed to identify junctional diversities and somatic mutations. In addition, as the PCR product included the 5’ end of the Ig constant region, the Ig isotype of each clone could be determined, including within the IgG and IgA subfamilies.

Results

Sample collection and processing

A series of 29 tumor samples was obtained by surgical excision of skin metastases from melanoma patients. Samples were selected prospectively or retrospectively on the basis of tissue integrity and RNA quality. The cutaneous or subcutaneous origin of the tumors was distinguished from possible superficial lymph node metastases by anatomical location, presence of skin structures, and absence of residual lymph node tissue and capsule on histologic examination. Lymph nodes and a tonsil from noncancerous patients were used as positive controls. All the tissue samples were frozen shortly after resection. Sequential cryosections were used either for immunohistochemistry or for gene expression testing by RT-
PCR or quantitative RT-PCR (qRT-PCR). Additional cryosections were obtained for laser capture microdissection. The main characteristics of the patients and tumor samples are reported in Supplementary Table S2.

**Cutaneous melanoma metastases contain fully developed ectopic lymphoid structures**

We observed on conventionally stained sections of some cutaneous melanoma metastases structures resembling lymphoid follicles. To confirm this, we used immunohistochemistry to study the type and distribution of immune cells in skin metastases from melanoma patients. Using an anti-CD20 antibody specific for B lymphocytes, we detected B-cell aggregates in 14 of the 29 tumor samples. These aggregates often formed dense structures resembling lymph node follicles. Plasma cells stained for CD138 were distributed more diffusely and irregularly outside the B-cell aggregates. We then searched for the presence of FDCs, which in lymph nodes form an interdigitating network in the center of B-cell follicles. In 10 of the 29 tumor samples, we observed that dense B-cell clusters were associated with more centrally located cells expressing CD21, a complement receptor expressed selectively by FDC. These cells were also stained by anti-VCAM1 and anti-FDCag antibodies, 2 other FDC markers (data not shown). We concluded that these structures correspond to lymphoid follicles. An illustrative example of follicles in a melanoma metastasis is shown in Fig. 1. Another example can be viewed in Supplementary Fig. S1 and control stainings in Supplementary Fig. S2.

To determine whether some follicles contained germinal centers, we stained adjacent cryosections against AID, a protein that is specifically expressed in the nuclei of germinal center B cells. Several follicles were found to host a small group of cells with nuclear expression of AID (see Fig. 1). The largest of these cell clusters was also stained for nuclear Ki-67, indicating intense B-cell proliferation, as is frequently observed in germinal centers from secondary lymphoid organs (data not shown). Germinal centers were only observed in follicles that were in close contact with melanoma cells.

We next looked whether HEV-like blood vessels were present in our tumor samples. Immunostaining with the MECA-79 antibody, which is specific for PNAd, revealed the presence of small PNAd+ blood vessels in the neighborhood of most of the melanoma associated follicles, but not in other parts of the tumors. We observed that most follicles were also intimately associated with neighboring concentrations of CD3+ T cells, including CD8+ T cells often clustered around DC-LAMP+ mature DCs and surrounded by PNAd+ HEVs, as occurs in T-cell zones in secondary lymphoid organs (see Fig. 1 and Supplementary Fig. S1 and S2). DC/T-cell clusters were also repeatedly observed in or outside most of the tumor samples, including those in which B-cell follicles and HEV-like blood vessels were absent (data not shown).

Altogether, among the 29 cutaneous melanoma metastases analyzed, 7 contained complete ectopic lymphoid structures, combining follicles, HEV-like blood vessels, and T-cell mature DC clusters. Four of these tumors hosted at least one follicle containing a germinal center. Of the remaining 22 tumors, 3 contained HEV associated with B-cell clusters without evidence of FDC, 3 others had complete follicles without HEV, and the 16 remaining tumors had no evidence of lymphoid neogenesis.

**Confirmation of FDCs and germinal centers in follicles**

We microdissected 7 individual follicles from cryosections cut from 3 selected metastases with AID+ cells and carried out qRT-PCR amplification of the CD21L transcript, a long CD21 mRNA isoform that is selectively expressed by FDCs (32). CD21L was detected in the follicles, but not in tumor areas taken outside the follicles from the same cryosection (Fig. 2). Similarly, we detected the expression of the AID gene in most microdissected follicles, but not in the extrafollicular areas (Fig. 2). Altogether, these data confirmed that cutaneous melanoma metastases contain B-cell follicles and suggested that some of them are reactive and host B-cell responses.

**Lymphoid neogenesis is irregularly distributed among different types of melanoma lesions**

We assessed whether ectopic lymphoid structures were also present in primary melanomas and in other types of metastases. We used qRT-PCR to quantify the CD21L transcript as a molecular marker for the presence of FDCs, and thus also of lymphoid follicles, in a large series of melanoma samples. Lymph node metastases were not included, because they often contain normal lymphoid tissue and thus would give false positive results. Consistent with our previous observations, CD21L transcripts were detected in 9 of 63 cutaneous metastases. In contrast, none of 25 primary cutaneous melanomas were positive, neither was normal skin. In visceral metastases, FDC were detected in some lung, muscle, and gut metastases, but intriguingly in neither of 12 liver and 12 brain metastases (Fig. 3).

**Molecular characterization of the immunoglobulin gene repertoire expressed in melanoma-associated ectopic lymphoid structures**

To assess whether active B-cell responses are taking place in B-cell follicles present in melanoma metastases, we analyzed the diversity of rearranged heavy chain immunoglobulin genes expressed in 4 different follicles isolated from 3 selected tumors, T3 (2 follicles), T4, and T9 (1 follicle each). Four consecutive cryosections were obtained from each tumor, and whole follicular areas were isolated from each section by laser capture microdissection. We chose to isolate whole follicles instead of germinal centers, because the latter were often undetectable or limited to very few AID+ cells, difficult to identify in tissue sections for microdissection. The selected follicles all contained AID+ cells, as confirmed by immunostaining or qRT-PCR of close cryosections (data not shown). Ig heavy chain cDNA libraries were constructed independently from each follicle section as illustrated in Supplementary Fig. S3. Brieﬂy, RNA extracted from each follicle section was reverse transcribed with a mix of primers specific for the transmembrane exons of all the constant region genes except IGHE. IgE-encoding transcripts were excluded from the libraries because they had not been detected by RT-PCR in our tumor samples, as opposed to the other Ig isotypes (data not shown). By
excluding secreted Ig, we restricted our analysis to naive, memory, and germinal center B cells and largely excluded plasma cells, as the latter can migrate from distant secondary lymphoid organs via the blood to inflammatory sites, and thus might have infiltrated the tumors independently of local B-cell responses (33). The resulting cDNA was amplified separately for each IgD, IgM, IgG, and IgA isotype by nested PCR, based on the approach described by Wang and colleagues (30). The amplified products, which included the 5’ extremity of the first exon of the constant region genes (CH1) to allow isotype identification, were cloned and sequenced. Thus, 64 independent VDJCH1 cDNA libraries were obtained, from each of 4
different follicles, 4 adjacent sections, and for each of 4 different isotypes.

In total, we obtained 672 interpretable VDJCH1 sequences corresponding to 304 individual B-cell clones defined by their unique VDJ rearrangement, junctional diversity, and common somatic mutations. None of these 304 clones was present in more than one follicle. We first looked whether repeated clones, that is, represented by more than one B cell in an individual follicle, were present. These clones are likely to have resulted from local clonal proliferation. A clone was considered repeated if its VDJ sequence was amplified independently from 2 nonadjacent sections from the same follicle. This conservative definition excludes occurrences of single B cells divided among 2 adjacent sections. It clearly underestimates the real number of repeated clones in our samples. In total, 17 distinct repeated clones were identified among the 4 follicles (Table 1).

We next looked at local occurrence of class switch recombination. We identified 8 clones in which a unique VDJ sequence was coupled to either of 2 different constant regions. We observed IgD to IgG1, IgG1 to IgG2, IgG1 to IgA1, and IgA1 to IgA2 transitions (Table 1). Clones with more than 2 different isotypes were not detected. Finally, we also searched for clonal variants within individual B-cell clones. Two or more VDJCH1 sequences were considered to be clonal variants derived from a single B-cell precursor if they shared the same VDJ rearrangement and junctional diversity, and if they differed by more than 5 nucleotides. The latter definition was deduced from the error rate of retrotranscription and PCR amplification in our experimental setting, according to the law of Poisson. This rate was 0.003, measured as the number of mismatches observed in a large number of CH1 sequences. In total, 17 different B-cell clones had evidence of clonal variation (Table 1). Altogether, 27 B-cell clones (8.9%) showed evidence of affinity maturation, including 13 that had more than one of the 3 cardinal features

Figure 2. Expression of CD21L (top) and AID (bottom) by qRT-PCR in 7 microdissected follicles (F1, F3 to F8) isolated from cutaneous melanoma metastases T3, T4, and T9. Extrafollicular areas microdissected in the same tumor sections were used as negative controls (EF) and follicles containing germinal centers microdissected from a normal reactive lymph node as positive controls (gray boxes). Results are numbers of cDNA copies normalized to β-actin and with the mean level of expression in normal follicles set to 100. Error bars indicate one SD. n, the number of biologic replicates tested. Replicates are the same follicle in adjacent tumor sections or different follicles isolated from the same lymph node section. No PCR product was amplified from extrafollicular areas microdissected from the same tumor sections, indicating less than 5 cDNA copies for CD21L and less than 2 for AID.
Melanoma Tumors Host Functional Ectopic Lymphoid Structures

![Graph](https://example.com/graph.png)

**Figure 3.** Expression of CD21L measured by qRT-PCR in primary tumors and different types of metastases from melanoma patients. The y-values represent numbers of CD21L cDNA copies, normalized to β-actin and with the level of expression in normal lymph node set to 100.

of Ag-driven B-cell responses. The aligned VDJ\(_{C1}\)1 sequences of B-cell clone B2 are shown in Fig. 4 as an illustrative example. Among all the Ig sequences derived from the 27 B-cell clones undergoing affinity maturation, about 10% seemed to be nonfunctional because of frame shifts resulting either from out-of-frame VDJ rearrangements or from base insertion or deletion (data not shown). These features are characteristic of ongoing germinal center B-cell responses, during which a number of irrelevant Ig gene rearrangements occur before being selected negatively (26).

We concluded that part of the B cells present in melanoma-associated ectopic lymphoid structures have undergone clonal expansion and selection, somatic hypermutation, class switch recombination, and abortive Ig rearrangements, displaying the characteristics of an Ag-driven B-cell response that takes place in secondary follicles. These Ig responses are oriented toward the IgM, IgG1, IgG2, IgA1, and IgA2 isotypes.

**Primary melanomas contain incomplete ectopic lymphoid structures**

Lymphoid follicles are usually not observed in conventionally stained histologic sections of primary melanomas, with the exception of desmoplastic/neurotropic melanomas (34), suggesting that these tumors do not contain ectopic lymphoid structures. Consistent with this, we failed to detect CD21L expression in 25 primary melanomas. However, the presence of tumor-associated HEV-like blood vessels expressing PNAd has recently been reported in 11 of 18 primary melanoma samples (35). We addressed this paradox by testing 10 primary melanomas with known lymphocyte infiltration for the presence of lymphoid follicles, HEV, and clusters of mature DC and T cells by immunohistochemistry. Nine tumors contained CD20\(^+\) B cells forming a loose infiltrate rather than a dense follicle-like structure. No CD21\(^+\) FDC were observed (Table 2 and Supplementary Fig. S4). All tumors contained a CD8\(^+\) T-cell infiltrate associated with mature DC. Six of 10 tumors contained PNAd\(^+\) blood vessels, closely associated with the T- and B-cell tumor infiltrate. This suggested that primary melanomas host HEV, but no FDC and no follicles, and thus that the process that leads to full scale lymphoid neogenesis is incomplete in these tumors.

**Discussion**

We report here for the first time the presence of ectopic lymphoid structures in human melanoma metastases. These structures associate lymphoid follicles, comprising mainly B lymphocytes clustered around FDC, with HEV and with clusters of DCs and T cells. The presence of germinal centers in some follicles and the mature phenotype of neighboring DCs and T cells suggest that adaptive immune responses take place in these structures. Here we confirm this for B-cell responses, by showing that the Ig transcript repertoire in melanoma follicles is enriched for clones that display the main characteristics of affinity maturation. Our observations do not allow to determine whether these local immune responses are directed at melanoma antigens, but we feel that this is a realistic possibility. Tumor-associated ectopic lymphoid structures are presumably not connected to the specialized networks of afferent lymph and blood vessels that carry antigens to B- and T-cell zones in lymph nodes and spleen and are therefore more likely to sense antigens present locally, as in the case of mucosal associated lymphoid tissue. It must be noted that all the follicles containing germinal centers that we observed were invaded by or were in close contact with tumor cells.

Primary melanomas sometimes host HEV and neighboring clusters of DC and T cells, but in contrast to metastases they never contain lymphoid follicles, which seem to be replaced by less organized accumulations of B lymphocytes. The absence of FDC is the likely explanation for this absence of follicles. The
accumulated B cells could be naïve or memory B cells that migrated from the blood to the tumor via the neighboring HEV, but even though some of them might have encountered their cognate antigen, an appropriate B-cell response ought to be impaired because of the absence of FDC. It will be important to understand why FDC are missing in primary melanomas.

The antibody responses that take place in melanoma follicles are switched toward the IgG1, IgG2, IgA1, and IgA2 isotypes. To the best of our knowledge, this is the first time that IgA responses are documented in ectopic lymphoid structures, presumably because they were not specifically searched for in previous studies, many of which point to IgG as the major isotype produced. Could antimalanoma IgA influence tumor development? As opposed to IgG, IgA do not activate the complement and do not mediate antibody-dependent cellular cytotoxicity. It seems therefore unlikely that such IgA responses could have an antitumoral effect. Why do antibody responses in melanoma metastases switch toward IgA? Isotype switching is mainly driven by specific cytokines, usually produced by follicular helper T cells (Th) in germinal centers. One such cytokine, TGF-β, has been identified as the key inducer of T-cell–dependent IgA class switching (36–38).

The detection of IgA responses in all of the 4 follicles that we have analyzed suggests that TGF-β modulates the microenvironment of melanoma metastases. This cytokine plays a dual role in cancer progression. In early stages, it acts negatively by inhibiting cellular proliferation, whereas in more advanced disease it stimulates cellular dedifferentiation and invasiveness and acts as a potent inhibitor of inflammation. Its capacity to inhibit T-cell activation makes it a frequently cited candidate to explain the resistance that tumors display against immune-mediated destruction (39).

Because lymphoid neogenesis develops as a consequence of ongoing adaptive responses in contact with persistent antigens, its presence in melanomas indicates that these tumors host an active immune reaction, and that melanoma-infiltrating lymphocytes are not completely inhibited by the tumor

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<td>T9/F4/S3,4</td>
<td>IgG1, IgA1</td>
<td>No</td>
</tr>
<tr>
<td>B16</td>
<td>V3-15 D4-17 J5</td>
<td>T9/F4/S1,2,3</td>
<td>IgA1</td>
<td>Yes</td>
</tr>
<tr>
<td>B17</td>
<td>V3-23 D5-24 J4</td>
<td>T9/F4/S2,4</td>
<td>IgA1, IgA2</td>
<td>Yes</td>
</tr>
<tr>
<td>B18</td>
<td>V3-23 D3-9 J6</td>
<td>T9/F4/S1,2,3,4</td>
<td>IgA2</td>
<td>Yes</td>
</tr>
<tr>
<td>B19</td>
<td>V3-33 D3-3 J4</td>
<td>T9/F4/S2,4</td>
<td>IgA2</td>
<td>Yes</td>
</tr>
<tr>
<td>B20</td>
<td>V3-66 D4-17 J6</td>
<td>T9/F4/S1,4</td>
<td>IgG1</td>
<td>Yes</td>
</tr>
<tr>
<td>B21</td>
<td>V3-66 D7-27 J6</td>
<td>T9/F4/S2,3,4</td>
<td>IgA1, IgA2</td>
<td>Yes</td>
</tr>
<tr>
<td>B22</td>
<td>V4-59 D5-5 J3</td>
<td>T9/F4/S2,3,4</td>
<td>IgG1</td>
<td>Yes</td>
</tr>
<tr>
<td>B23</td>
<td>V5-51 D1-7 J4</td>
<td>T9/F4/S1,3</td>
<td>IgA1</td>
<td>No</td>
</tr>
<tr>
<td>B24</td>
<td>V5-51 D5-5 J6</td>
<td>T9/F4/S1,2,4</td>
<td>IgG1</td>
<td>Yes</td>
</tr>
<tr>
<td>B25</td>
<td>V5.a D3-22 J2</td>
<td>T9/F4/S1,3,4</td>
<td>IgA1</td>
<td>No</td>
</tr>
<tr>
<td>B26</td>
<td>V3-30 D7 J3</td>
<td>T9/F4/S1,2</td>
<td>IgM</td>
<td>Yes</td>
</tr>
<tr>
<td>B27</td>
<td>V3-66 D6-19 J6</td>
<td>T9/F4/S2,3,4</td>
<td>IgG1</td>
<td>Yes</td>
</tr>
</tbody>
</table>

NOTE: These clones display one or more of the following characteristics: Clonal amplification illustrated by repeated occurrences of clonal B cells in the same follicle, isotype switch, and clonal variation. T3, T4, and T9 identify the tumor samples, F1 to F4 the follicles, and S1 to 4 the tissue sections from which the sequences were isolated. Ig sequences from clone B2 are shown in Fig. 4 as a representative example.
Figure 4. Ig sequences derived from B cell clone B2 isolated from lymphoid follicle F1 present in melanoma metastasis T3. DNA sequences of the 12 bacterial colonies (S01 to S12) with a V3-11/01, D3-22/01, J4’02 gene rearrangement are aligned to the corresponding germline immunoglobulin VDJ and IGHGl genes (bottom, in black and bold characters). Matched and mismatched nucleotides are displayed in gray and black, respectively. All these sequences originate from a single B cell precursor, as they share the same junctional diversities as well as many somatic mutations. Sporadic mismatches are assumed to result from reverse transcription or PCR errors. Underlined mismatches represent somatic mutation variants that identify B cell subclones. All sequences have the IgG1 isotype, except sequence S02, which has the IgG2 isotype (IgG2 nucleotides appear in small characters).

Table 2. Immunohistochemical analysis of primary melanomas with known lymphocyte infiltration

<table>
<thead>
<tr>
<th>Tumor no.</th>
<th>HEV (PNAd⁺)</th>
<th>B cells (CD20⁺)</th>
<th>FDC (CD21⁺)</th>
<th>CTL (CD8⁺)</th>
<th>Mature DC (DC- LAMP⁺)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T30</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>NT</td>
<td>+</td>
</tr>
<tr>
<td>T31</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>T32</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>+</td>
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<tr>
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<td>+</td>
<td>+</td>
<td>–</td>
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<td>T34</td>
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<tr>
<td>T36</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>NT</td>
<td>+</td>
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<tr>
<td>T37</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>+</td>
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<td>T38</td>
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<tr>
<td>T39</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

NOTE: The presence (+) or absence (−) of the indicated cells is shown. An illustrative example (T33) is shown in Supplementary Fig. S4.

Abbreviation: NT, not tested.
Coronella-Wood J, Hersh E. Naturally occurring B-cell re-

Punt CJ, Barbuto JA, Zhang H, Grimes WJ, Hatch KD, Hersh EM. Anti-


mediated cytotoxicity. The detrimental effect of such an IgA

with IgG for binding to tumor cells, thereby inhibiting IgG-

can also hypothesize that locally secreted IgA might compete

mechanisms have been proposed to explain this effect, such as

the impairment of antitumoral T-cell responses by blocking

antibodies, and the modulation of the inflammatory response

by immune complexes (42, 43). On the basis of our findings, we

can also hypothesize that locally secreted IgA might compete

with IgG for binding to tumor cells, thereby inhibiting IgG-

mediated cytotoxicity. The detrimental effect of such an IgA–

IgG competition has been shown recently with HIV vaccines

(44). To assess whether lymphoid neogenesis helps or impairs

tumor progression, it will be important to evaluate its prog-

nostic impact in a large series of melanoma patients. This study

does not allow to answer this question, as it was designed to

provide a proof-of-principle that lymphoid neogenesis is present

in melanoma and that it hosts ongoing adaptive responses.

References


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tumor antibody produced by human tumor-infiltrating and peri-


11. Taylor RC, Patel A, Panageas KS, Busam KJ, Brady MS. Tumor-


Melanoma Tumors Host Functional Ectopic Lymphoid Structures


Neogenesis of Lymphoid Structures and Antibody Responses Occur in Human Melanoma Metastases

Arcadi Cipponi, Marjorie Mercier, Teofila Seremet, et al.

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