Pediatric Cancers Are Infiltrated Predominantly by Macrophages and Contain a Paucity of Dendritic Cells: a Major Nosologic Difference with Adult Tumors

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Abstract

Purpose: Adult cancer is frequently preceded by a period of prolonged chronic inflammation caused by infectious microbial agents or physical or chemical irritants. By contrast, an association between the classic pediatric neoplasias and inflammatory triggers is only rarely recognized. We hypothesized that the difference could be reflected in the inflammatory cell infiltrates of pediatric and adult cancer.

Experimental Design: Three investigators retrospectively studied 27 pediatric and 13 adult cancers at first diagnosis by immunohistochemistry. Inflammatory cells were identified and counted, and their location in relation to tumor tissue was analyzed.

Results: A majority of tumor-associated leukocytes (TAL) in adult tumors were located at the edges of tumor islands forming inflammatory foci between the supporting stroma and the malignant infiltrate. In contrast, TALs in pediatric tumors were scattered within the malignant tumor islands. In adult tumors, TALs were composed of diverse leukocyte types; but in pediatric tumors, the infiltrating cells were predominantly macrophages that accumulated in areas of necrosis within the tumors. The most striking feature in the pediatric tumors was the virtual absence of dendritic cells. The proportion of intratumoral dendritic cells in pediatric samples was 4.1%; whereas in adult tumors, they formed 36.9% of TALs within the tumor islands and 25.1% around the tumors.

Conclusions: We conclude that TALs in pediatric cancers are composed mainly of macrophages and largely devoid of dendritic cell. The findings may provide a major nosologic difference reclassifying pediatric and adult tumors based on nominal inflammatory and noninflammatory etiologies.

Immunohistochemical analysis of adult cancers often reveals a significant infiltration of leukocytes, either intratumoral or peritumoral, at the edges of the tumor tissue between the tumor islands and their stromal component (1). Heavier infiltration of leukocytes is commonly associated with a better prognosis, such that in patients with ovarian cancer, the 5-year overall survival rate is 38% in patients whose tumors contained T cells and 4.5% in those patients whose tumors contain no T cells within the islands, making this one of the most important of all prognostic factors in this disease (2). Similarly, in patients with gastric cancer, the 5-year survival rates for patients with high natural killer (NK) or dendritic cell infiltration were 75% and 78%, respectively; whereas in patients with low numbers of infiltrating cells, the respective survival figures were only 50% and 43% (3).

Adult cancer is frequently preceded by a period of prolonged chronic inflammation caused by infectious microbial agents (Hepatitis B or C, human papillomavirus, EBV, *Helicobacter pylori*) or other physical agents or irritants (repeated solar exposure, prolonged exposure to tobacco smoke, or asbestos fibers; ref. 4). Such preneoplastic inflammatory periods may persist for several decades before a state of genomic instability, and the full neoplastic phenotype is acquired. On the contrary, with the notable exception of EBV-induced Burkitt's lymphoma presenting in children in parts of Africa, an association between typical pediatric neoplasias (i.e., small round blue cell tumors) and inflammatory triggers has not been recognized. Furthermore, the preneoplastic period in pediatric cancer cannot be decades but is much shorter, often only weeks or months.

We hypothesized that the abovementioned differences in the etiologies of adult and pediatric cancers could be reflected in the quality and quantity of the inflammatory cell component of pediatric tumors. Because chronic inflammation is characterized by the presence of leukocytes and histiocytes readily demonstrable within tissue samples, we critically and quantitatively examined the lymphoid and myeloid (dendritic cells and tissue macrophages) content of several typical pediatric tumors at diagnosis and contrasted these with representative adult tumors.

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Materials and Methods

Tumor samples. Five representative specimens of individual pediatric tumor types assessed at original biopsy that were followed later by tumor resection were attained from the archives of the Children's Hospital of Pittsburgh. The careful identification and analysis of the inflammatory cell content of the original biopsies is the subject of this report. The pediatric tumor types examined were neuroblastoma, Wilms' tumor, Ewing's sarcoma, hepatoblastoma, osteosarcoma, and rhabdomyosarcoma (Table 1). Three of the tumor samples were discarded because the tissue was insufficient to completely evaluate the immune cell infiltration. Thus, 27 pediatric tumor samples were eligible. Tissues from 13 adult primary tumors (breast, colon, and esophagus) served as controls for the methodology and to confirm that our observations were similar to those reported for adults in the literature (1). The study was exempted from local institutional review board review based on the preexisting tissue used anonymously with patient deidentification.

Immunostaining and cell blocks. The antibodies for immunohistochemistry and their sources, working dilutions, and antigen retrieval have been described in detail previously (5). Slides were deparaffinized, hydrated, and treated with a 3% H_2O_2/methanol solution for 20 minutes. If required, the slides were then treated with a prewarmed heat antigen retrieval solution for 30 minutes in a steamer, cooled for 10 minutes, and rinsed in distilled water for 10 minutes. The antigen retrieval solution was 10 mmol/L citrate buffer (pH 6). Antibody was applied for 30 minutes at 37°C. The appropriate anti-mouse or anti-rabbit biotinylated antibody was applied for 15 minutes, and avidin-biotin complex was applied (ABC Vector, Burlingame CA) for 15 minutes. Diaminobenzidine was the chromogen, and slides were counterstained with hematoxylin, dehydrated, and coverslipped.

Controls. Staining controls (i.e., tissues known to contain the antigen of interest) were identified and staining was optimized by testing antigen retrieval methods and times as well as antibody dilutions. An optimized control-positive tissue slide was included with every batch of immunostaining, as was the control for endogenous staining by omission of the antibody and replacement with an irrelevant mouse or rabbit serum.

Quantitation of tumor-infiltrating leukocytes. Automated quantitation was abandoned when it became obvious that cell counts were quite low. Three of the investigators reviewed the immune stains simultaneously and made independent counts of the numbers of cells in peritumoral or intratumoral locations in five fields that represented peritumoral site and five that represented intratumoral areas, where possible.

Table 1. Patient ages and outcomes

Cancer	Patient no.	Age (y), mean (range)	Survival (*n*)
Colon cancer	4	58 (48-69)	NA
Breast cancer	6	61 (38-70)	NA
Esophagus cancer	3	62 (41-76)	NA
Ewing's sarcoma	5	12.5 (10-16)	4
Osteosarcoma	5	15.5 (10-17)	4
Rhabdomyosarcoma	4	7.5 (4-12)	3
Hepatoblastoma	4	1.6 (0.5-4)	4
Neuroblastoma	4	2.0 (0.25-5)	3
Wilms' tumor	5	3.3 (1-9)	5

NOTE: Selected tumor samples representing typical adult and pediatric cancers were analyzed by immunohistochemistry for the presence of tumor-infiltrating leukocytes. Samples were taken at diagnosis.
Abbreviation: NA, not applicable.

Quadrants of the tissue and central areas were selected. Two blocks of the same control adult tumor were included to assess the variation of counts within an individual tumor, and three independent cell counts were compared for consistency of CD3, S100, and CD57 and then merged. The rest were counted by a single observer (R.J.). Counting was possible but difficult where the target antigen also occurred in the tumor, as for CD57 and S100. The total number of tumor-associated leukocytes (TAL) in each section was calculated by summing up the counts of $CD3^+$, $CD57^+$, $CD68^+$, and $S100^+$ cells.

Statistical analysis. A leukocyte count for each tumor sample was an average of counts derived from 15 high-power fields (HPF) per each tumor sample (5 HPF per researcher). Data were further analyzed by two-tailed Mann-Whitney *U* test using SPSS for Windows (version 11.01, SPSS Inc., Chicago, IL). *P*s < 0.05 were considered statistically significant.

Results

The total number of TALs was variable both in adult (range, 21-385 cells per HPF) and pediatric (range, 13-221 cells per HPF) tumor sections and was not significantly different between the groups ($P > 0.05$; Fig. 1). Wilms' tumor sections contained less TALs than the breast cancer sections (medians, 32 versus 66.5 cells per HPF; $P = 0.045$) or the Ewing sarcoma samples (32 versus 62 cells per HPF; $P = 0.009$). No other differences were found in the total number of TALs.

The leukocyte pool in adults was diverse and contained equal numbers of $CD3^+$ T cells, $CD68^+$ macrophages, and $S100^+$ dendritic cell (Fig. 2; $P > 0.05$). Tumor sections contained more $CD3^+$ cells than $CD57^+$ NK cells (43 versus 9 cells; $P = 0.009$); otherwise, no significant differences were found in cell numbers in adult tumors. In contrast, a predominant cell type among pediatric tumors was $CD68^+$ macrophages ($P \leq 0.002$), forming 68% (median) of TALs. T cells were present in low numbers, but only few $CD57^+$ NK cells could be detected. Strikingly, dendritic cells (identified by S100, CD1a, CD83, CD123, or DC-LAMP staining) were almost completely absent, and a detectable infiltration of $S100^+$ interdigitating, dendrite-forming cells were observed only in one neuroblastoma tumor (45 cells per HPF) and in the peritumoral area of one nasopharyngeal rhabdomyosarcoma. In sum, adult tumors contained more $CD3^+$ (medians, 43 versus 6 cells per HPF; $P = 0.028$), $CD57^+$ (9 versus 2 cells per HPF; $P = 0.049$), and S100+ (13 versus 0 cell per HPF; $P < 0.001$) cells and less $CD68^+$ macrophages (14 versus 31.5 cells per HPF; $P = 0.003$) than pediatric tumors (Fig. 2). Representative photomicrographs from a pediatric cancer (Wilms' tumor) and an adult carcinoma (breast carcinoma) are shown (Fig. 3).

The distribution of leukocytes between tumor islands and the adjacent stroma was studied in those pediatric cancers where such areas were clearly distinguishable ($n = 15$). This excluded all osteosarcoma and hepatoblastoma and two neuroblastoma tumors because diagnostic needle biopsies did not reflect margins, and the growth pattern was such that tumor cells (especially osteosarcomas) were interspersed with nonneoplastic stromal cells. Diametrically different distribution patterns were found in the pediatric and adult cancers. Whereas majority of TALs in adult carcinomas were found peritumoral, adjacent to the tumor islands, forming focal inflammatory cell aggregates (see Fig. 3), 74% (median) of leukocytes in pediatric tumors resided within the tumor islands. This was significantly more than the proportion of intratumoral cells in adult cancers

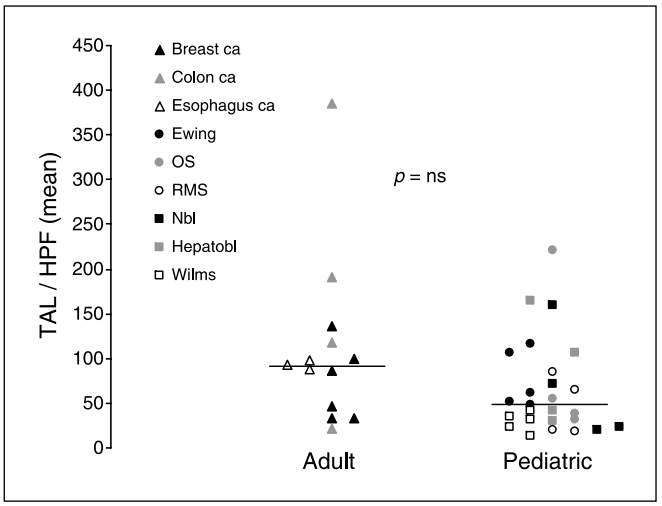

Fig. 1. Number of TALs in analyzed tumors; 5 HPF from 13 adult and 27 pediatric tumor samples biopsied at the time of diagnosis were analyzed by three investigators. Total number of leukocytes was counted as described and an average of leukocytes per HPF calculated for each tumor. Points, individual mean counts for each tumor sample. Black lines represent medians for study groups. TAL counts in the studied groups were not significantly different ($P > 0.05$).

(20%; $P < 0.001$; Fig. 4). Various pediatric cancers did not differ from each other in this respect (Fig. 4). The most numerous cell type, both inside and outside tumor islands in pediatric cancer, was a CD163/CD68$^+$ macrophage that constituted 69% (mean) intratumoral and 62% of cells peritumoral. The respective figures for S100$^+$ cells were 4% and 8%. In adult carcinomas, intratumoral and peritumoral TALs were composed of S100$^+$ dendritic cell, macrophages, and T cells in similar proportions. A significantly smaller population of cells expressed the NK cell surface marker, CD57. The ratio of CD68$^+$ macrophages/S100$^+$ dendritic cell in pediatric cancers was 15.5, and in adult cancers, it was 1.2.

The infiltrate of macrophages in pediatric tumors associated strongly with the presence of necrosis within these tumors. The only exception was osteosarcoma, where macrophages and osteoclasts were well represented also in the nonnecrotic areas of the tumors, around the osteoid/chondroid matrix.

Discussion

Our pilot study material consisted of archival tissue samples collected at diagnosis from 40 patients (27 pediatric and 13 adult cancers). We examined TAL number, type, and distribution in tissue sections and confirmed earlier published results regarding the number, composition, and distribution of leukocytes in adult carcinomas (1). To our surprise and to our knowledge previously not recognized by others, pediatric cancers were substantially different from adult cancers in respect to composition and distribution of TALs. Whereas a majority of TALs in adult tumors located at the edges of tumor islands, forming inflammatory foci between the supporting stroma and the malignant cell infiltrate, TALs in pediatric tumors were scattered evenly within the malignant tumor islets. Furthermore, in adult tumors, the TALs were composed of diverse cell types, including T cells, NK cells, macrophages, and dendritic cells; but in pediatric tumors, the infiltrating cells were predominantly macrophages with rare other cell types. The intratumoral number of macrophages within the pediatric tumors was 2-fold higher than the T cells that were next abundant in frequency (69.3% versus 26.9%; $P < 0.001$). A striking feature was the absence of dendritic cells in these pediatric tumors. The proportion of intratumoral dendritic cell in the pediatric samples was only 4.1%; whereas in adult tumors, they formed on average 36.9% of TALs within the tumor islands and 25.1% within the peritumoral component. Thus, in adults the ratio of CD68$^+$/S100$^+$ was around 1:1; but in children, the number of macrophages (CD68$^+$) was 15 times higher than the number of dendritic cell in the tumor sections.

In our previous study, we validated several antibodies (e.g., CD14, CD68, CD163, CD1a, CD83, CD123, DC-LAMP, and S100) to enable us to distinguish dendritic cells and macrophages in tissue sections (5). These closely related cell types share several differentiation antigens; in addition, the expression of certain markers is strongly dependent on the state of maturation or activation of the cells. Thus, CD1a and CD83 are expressed only by immature and mature dendritic cells, respectively; whereas expression pattern of S100 in dendritic cell is slightly broader, and certain members of the S100 family are expressed also by activated macrophages (6–9). Using a panel of dendritic cell–antibodies validated for tissue sections, only a very few dendritic cell–like cells could be shown in the pediatric cancers. It is, therefore, likely that the presence of significant dendritic cell infiltration in pediatric cancers is distinctly unusual. The S100 antibody, on the other hand, detected several dendritic type cells in adult cancers, in line with earlier reports. It is not certain, however, that all S100$^+$ cells in adult tissue sections were true dendritic cells because molecules, such as lipopolysaccharide, interleukin-10, and IFN-γ, induce S100A8, S1009, and S10012 in activated macrophages (6–9).

Cell counting was done manually by three investigators. Computerized morphometrics were attempted but abandoned because of the extreme variability of cell distribution and the

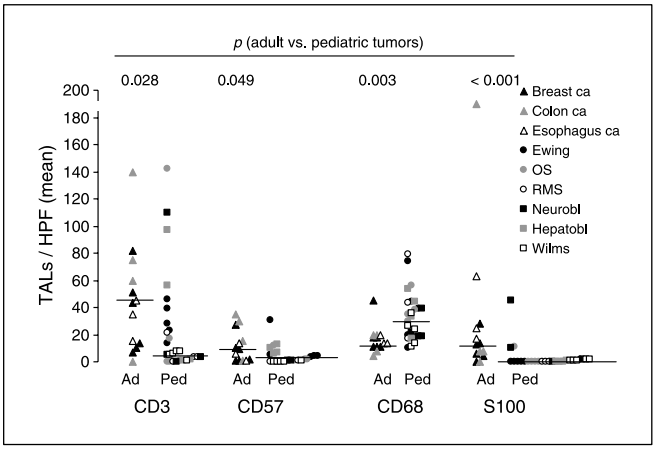

Fig. 2. Leukocyte types in pediatric and adult cancers; 5 HPF from 13 adult and 27 pediatric tumor samples biopsied at the time of diagnosis were analyzed by three investigators. Tumor sections were stained with the indicated antibodies, and the average number of labeled cells per HPF counted. Points, individual mean counts for each tumor sample. Black lines represent medians for study groups. Values for pediatric and adult tumors are significantly different for all cell types (*P*s are presented). Within pediatric group, the numbers of CD68$^+$ and S100$^+$ cells are significantly different from others. Within adult group, no significant differences existed in the number of infiltrating CD3$^+$, CD68$^+$, and S100$^+$ cells. The number of CD3$^+$ cells was higher than that of CD57$^+$ cells ($P = 0.009$).

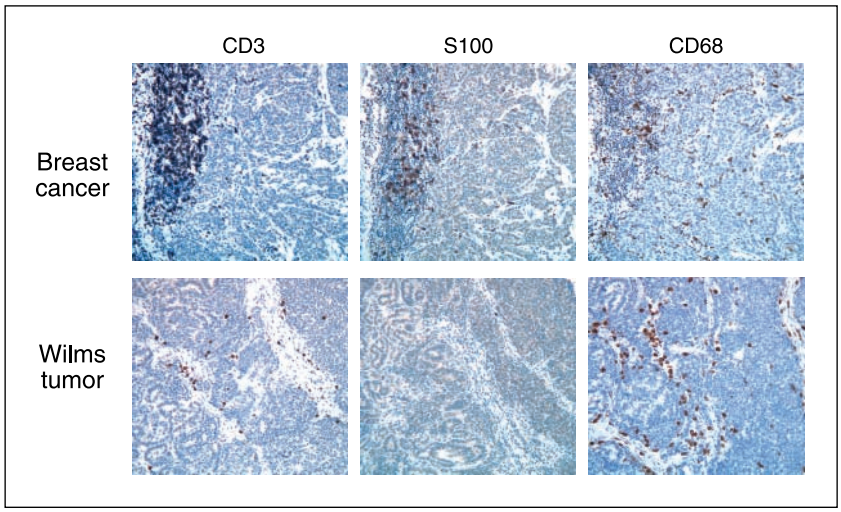

Fig. 3. CD68, CD3, and S100 stained cells in Wilms' tumor and adult breast carcinoma. Indicated tumors were stained with anti-CD68, anti-CD3, and anti-S100 antibodies, and representative areas were photographed. (Original magnification, × 200).

tiny number of events (data not shown). No consistent accounting of peritumoral and intratumoral clusters could be achieved; the numbers of staining cells were generally so small that visual counting was far more efficient. There is also an element of subjective interpretation, and in some instances, informed judgments about background or cross-reactions were required.

The presence of inflammatory or immune cells in pediatric tumors has not been thoroughly studied. Lymphocytic infiltration on occasion has been described in neuroblastomas (10, 11), and some early studies have shown a positive correlation between the number of infiltrating lymphocytes and patient prognosis (12, 13). Clonal analysis showed that the majority of lymphocytes in neuroblastomas were CD3-positive T cells; in addition, of 90 neuroblastoma-derived clones, only three represented NK cells (11). Infiltrating T cells in pediatric cancers are infrequent, difficult to clone, and functionally ineffective (14). We are not aware of a previous study documenting the presence or absence of dendritic cells among the TALs in pediatric cancers, or whether that finding has any association with prognosis. In our pilot study, the pediatric material was too heterogenous to attempt evaluating tumors with good versus bad outcome. However, because the prognosis for survival among pediatric solid tumor patients is generally around 70% and taking into account the absence of dendritic cells in all these pediatric tumors examined, it is unlikely that the positive correlation between dendritic cell and prognosis found in adults would exist in the pediatric patients. Wilms' tumor patients, for example, have in general a >90% chance of survival; yet, in this study, only rare cells with characteristics of dendritic cells could be found in the tumor samples. The selected patient material in this study represented typical pediatric patients in this regard; 23 of 27 patients were alive at the time of data analysis (Table 1).

Although immature dendritic cells and macrophages both are active in phagocytosis and present antigen at the cell surface, their functions are quite different. Dendritic cells reside or are recruited into tissues in the immature state (immature dendritic cells) and are actively phagocytic and macropinocytotic, taking up many times their cell volumes in the microenvironment, stimulated by damage- and pathogen-associated molecular pattern ligands interacting with various cell surface receptors, including the Toll-like receptors. When such danger signals are encountered in the form of microbial antigens or cell death, immature dendritic cells are driven to differentiate to mature dendritic cell, leaving affected tissues and migrating to local or central lymphoid tissue to present their antigens to a variety of cell types, including naive T cells (15). Macrophages, on the other hand, are metabolically active and are responsible for the local control of damage and active phagocytosis and removal of apoptotic and necrotic corpses. Although the functional dichotomy seems to be clear, local regulatory signals, including IFN-γ and interleukin-10, modulate the transformation between immature dendritic cells and macrophages (16–21). The absence of dendritic cells and the presence of macrophages in pediatric cancers suggest a relative lack of dendritic cell precursors or differing anlage of tumors in these patients.

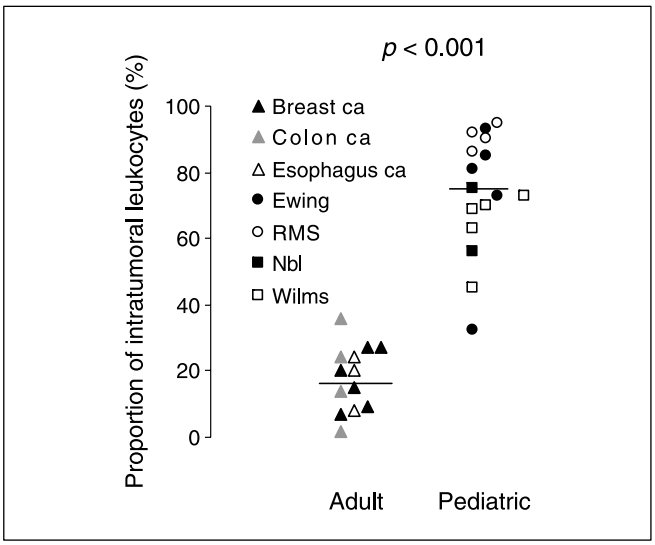

Fig. 4. Proportion of intratumoral cells in pediatric and adult tumors; 5 HPF of 15 eligible pediatric and 13 adult tumor samples were analyzed for the distribution of leukocytes between peritumoral and intratumoral areas. Percentage of intratumoral cells in each analyzed tumors. Different tumors are marked with separate symbols. The proportion of intratumoral leukocytes in pediatric tumors is significantly higher ($P < 0.001$).

Our earlier studies support this suggestion because we have found the number of monocytoid dendritic cell precursors in the blood of pediatric cancer patients to be significantly reduced (22, 23). It is also possible that the microenvironment in pediatric tumors favors macrophage rather than dendritic cell differentiation from monocytes. Local signals in the form of cell hypoxia, tumor necrosis, apoptosis, and resulting inflammation are evidently important in providing chemotactic and differentiating signals to dendritic cell/macrophage precursor cells (4, 24–26). Until now, the characterization and quantitation of inflammation in tumor samples has largely been based on a descriptive analysis of TALs. However, recent advances in microarray and microRNA technology will almost certainly provide additional and complementary information about their role of inflammation in pediatric and adult cancers.

Unlike in adults, in children, carcinogenesis occurs simultaneously with maturation of immune system. In theory, this may lead into total ignorance of cancer by immune cells. On the other hand, the capacity to promote immune responses to various antigens is weaker during early childhood. Production of several cytokines, including interleukin-6, interleukin-12, tumor necrosis factor-α, and IFN-γ, are reduced by neonatal leukocytes compared with adult cells (27–29). Furthermore, unlike adult naive T cells, neonatal T cells do not down-regulate CCR7 expression after activation and show impaired chemotactic migratory response to several inflammatory chemokines, suggesting that T cells in children are limited in their capacity to traffic to nonlymphoid tissue sites of inflammation (30). Thus, a physiologic immunodeficiency of immaturity in pediatric patients may partially explain the low T-cell and dendritic cell content in pediatric tumors.

This study was designed to clarify the inflammatory cell content in typical pediatric tumors (i.e., sarcomas and blastomas). The results were related to those found in the most common adult type cancers (i.e., epithelial carcinomas). Our important and previously unrecognized notion is that composition and location of TALs are significantly different in pediatric and adult cancers. Whether the differences are related to the tissues from which the cancers arise, or to the inflammatory/noninflammatory etiologies of the cancer, or to other contributing factors, including the physiologic immunodeficiency of immaturity, is presently unknown. It is possible that the numerical predominance of macrophages and the absence of dendritic cell could be shown also in adult sarcomas and blastomas. In fact, a prolonged chronic inflammation preceding the cancerous growth in affected tissue is typical finding in adult carcinomas, not in adult sarcomas. Thus, the immunobiology of sarcomas and blastomas might be different from that of epithelial carcinomas.

In conclusion, our results show that the leukocyte infiltrate in pediatric cancer is relatively monotonous, composed largely of macrophages in necrotic areas of the tumor islands. The presence of other leukocyte types, especially dendritic cells, is quite sparse. In adult cancers, the leukocytes form inflammatory foci adjacent to tumor islands and are more diverse, having T cell, NK cell, and macrophage representation in addition to $S100^+$ dendritic cell–like cells. These observations support our hypothesis of the importance of prolonged chronic inflammation in the etiology of adult but not pediatric cancer, and that this difference may be reflected in the quality and quantity of immune cell infiltration within the tumor site. The findings serve as an important and novel means to distinguish these two quite different diseases that are each called cancer, differing in origin, response to therapy, and prognosis.

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