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Lymphomatoid granulomatosis, a single institute experience: pathologic findings and clinical correlations

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Abstract

Lymphomatoid granulomatosis (LYG) is a rare angiocentric and angiodestructive EBV-associated B-cell lymphoproliferative disorder. It is hypothesized that these patients have dysregulated immune surveillance of EBV. We reviewed the biopsies of 55 patients with LYG who were referred for a prospective trial at the NCI (1995–2010) and evaluated the histologic, immunohistochemical, in situ hybridization, and molecular findings of these biopsies in conjunction with clinical information. Grading of the lesions was based on morphologic features and the number of EBV-positive B cells.

The median age was 46 years (M:F 2.2:1). Clinically, all patients had lung involvement (100%) with the next most common site being the central nervous system (38%). No patient had nodal or bone marrow disease. All patients had past EBV exposure by serology but with a low median EBV viral load. We reviewed 122 biopsies; the most common site was lung (73%) followed by skin/subcutaneous tissue (17%); other sites included kidney, nasal cavity, gastrointestinal tract, conjunctiva, liver, and adrenal gland. Histologically the lesions showed angiocentricity, were rich in T cells, had large atypical B cells, and were positive for EBV. Grading was performed predominantly on the lung biopsy at diagnosis; they were distributed as follows: LYG grade 1 (30%), grade 2 (22%), and grade 3 (48%). Necrosis was seen in all grades with a greater degree in high-grade lesions. Immunoglobulin gene rearrangement studies were performed and a higher percentage of clonal rearrangements were seen in LYG grade 2 (50%) and grade 3 (69%) as compared to grade 1 (8%). LYG is a distinct entity that can usually be differentiated from other EBV-associated B-cell lymphoproliferative disorders based on the combination of clinical

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presentation, histology, and EBV studies. Grading of these lesions is important because it dictates the treatment choice.

Keywords

Lymphomatoid granulomatosis; Epstein Barr virus; immunodeficiency; immunosurveillance; lung lymphoproliferative disorder

INTRODUCTION

Lymphomatoid granulomatosis (LYG) is a rare angiocentric and angioinvasive EBV-associated B-cell lymphoproliferative disorder (LPD) primarily involving the lungs that was first described by Liebow *et al.* in 1972 (1). LYG initially was believed to be a T-cell lymphoproliferative disorder (2, 3) based on the predominance of T-cells. Katzenstein *et al.* later reported that these lesions were EBV-associated (4). In 1994, Guinee *et al.* demonstrated that EBV was present in the atypical B cells, which were clonal at least in some cases, by immunoglobulin rearrangement (5). LYG was included as a distinct disease entity in the 2001 WHO classification (6) and considered a lymphoproliferative disorder of indeterminate malignant potential. The initial grading of LYG was based on cellular composition, cytologic atypia, and necrosis (7) and later based on the density and number of EBV positive large atypical B cells. However, in practice, the grading can be inconsistent and poorly reproducible among observers. The greatest difficulty for the diagnosis of LYG has been with grade 1 lesions because of the possible absence or paucity of EBV-positive atypical B cells.

Currently, it is still uncertain whether LYG represents a unique and rare disease or is part of the spectrum of EBV B-cell LPDs. Earlier studies were likely confounded by the inclusion of T and NK cell lymphomas (formerly included in the diagnosis of angiocentric immunoproliferative lesion) with cases of LYG (3, 8–10). More recently, it has been hypothesized that patients with LYG have defective immune surveillance of EBV-infected B cells particularly by the CD8-positive T cells (7, 11). In the current study we have examined the distribution of regulatory T cells (Tregs), since these cells have been implicated in suppressing normal T-cell immunity and the immune response to viral infections (12). In regards to treatment, historical outcomes of patients treated with steroids and/or chemotherapy have been poor (median survivals of 14 months) (4). At the National Cancer Institute (NCI) in a prospective clinical trial, patients with LYG grade 1 and 2 have been treated with interferon (IF) α and grade 3 with immunochemotherapy (dose-adjusted etoposide, prednisone, vincristine, cyclophosphamide, doxorubicin, rituximab, DA-EPOCH-R). This therapy stratification has improved progression-free survival (PFS) to 56% (median follow-up 5.1 years) in patients with LYG grade 1–2 and PFS to 44% (median follow-up 32 months) in patients with LYG grade 3 (13).

Since therapy for LYG is guided by grading, it is imperative to have a consistent, accurate, and adequate evaluation of these lesions. We report our institution's unique experience, the histologic, molecular, and immunophenotypic findings with this rare disease.

MATERIALS AND METHODS

Tissue specimens and immunohistochemical studies

Biopsies from patients referred to the National Cancer Institute (NCI) over a 15-year period (1995–2010) with a potential diagnosis of LYG were retrieved from the archives of the Hematopathology Section of the Laboratory of Pathology. We reviewed biopsy material from 69 patients; 3 patients were previously reported in an earlier study (14), which focused on the cutaneous manifestations. All formalin-fixed paraffin embedded (FFPE) tissue biopsies were reviewed by the authors (E.S.J, S.P., and J.Y.S.), utilizing the criteria of the 2008 WHO classification (15). 14/69 patients that were initially referred as possible LYG were excluded. The diagnoses on review were: NK/T cell lymphoma, nasal type (1 case), peripheral T-cell lymphoma, not otherwise specified (1 case), classical Hodgkin's lymphoma (4 cases), and EBV positive polymorphic or monomorphic B-cell lymphoma, lacking features of LYG (8 cases).

Of the 55 patients that were included in the study, 34 of the patients (62%) had prior therapy before enrolling in the LYG clinical trial, while 19 patients (35%) did not, and for 2 patients it was unknown whether there was prior treatment (4%). For the patients that had prior treatment, both the pre and post therapy biopsies were reviewed.

Immunohistochemical studies were performed on FFPE tissue sections using the following panel of antibodies (CD20, CD3, CD30, CD15, CD4, CD8, CD56, LMP1, FOXP3, and CD25) on cases with available material according to previously published techniques (16–19). Selected cases were studied to assess the latency phenotype of EBV with additional stains for EBNA2, clone PE2, mouse monoclonal, 1:500 dilution, Abcam (Cambridge, MA) and ZEBRA, clone BZ1, mouse monoclonal, 1:1600 dilution, Santa Cruz Biotechnology (Dallas, TX). Detection was performed with Ultraview-DAB from Ventana (Tucson, AZ). CD4, FOXP3 and CD25 were used to assess the frequency of Tregs in tissue sections, and enumerated as the number of positive cells/10 hpfs. The same panel was applied to 3 cases of interstitial pneumonitis as controls. The study was approved by the NCI Institutional Review Board (protocol number NCI-94-C-0074, clinical trial number NCT00001379).

In situ hybridization for Epstein Barr virus (EBV) encoded RNA (EBER)

In situ hybridization for EBER was performed on FFPE tissue sections using a FITC labeled oligonucleotide probe supplied by Ventana on an automated stainer (Ventana-Benchmark, Tuscon, AZ, USA). Visualization was achieved using the ISH iView system with Alkaline-Phosphatase and nitro-blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate substrate, with nuclear fast red as contrast. A positive control was run with all cases and stained appropriately.

Molecular studies

Immunoglobulin Polymerase Chain Reaction (PCR)—DNA extraction was performed on FFPE with QiaCUBE extraction platform using their proprietary buffer and proteinase K digestion. PCR was performed using consensus primers directed to V_H framework (FR) III and joining region (J_H) of the immunoglobulin heavy chain gene (FRIII-

IGH PCR) and to V_H FR region II and the joining region (FR_{II}-IGH PCR), according to the method of Ramasamy *et al.* (20). Testing for the IG κ locus was additionally performed using the Biomed II primer set described by van Dongen *et al.* and supplied by *InVivoScribe* Technologies (*IGK@* Gene Clonality Assay-ABI Fluorescence Detection, Cat#: 1-102-0031) (21). These reactions interrogate rearrangements involving the V κ loci and J κ (tube A), the V κ locus and the κ DE locus (tube B), and the κ -intron RSS locus and the κ DE locus (tube B). The products from all reactions were separated by capillary electrophoresis on an ABI 3130xl Genetic Analyzer and the electropherograms analyzed using GeneMapper software version 3.7 (ABI). For cases prior to 2008, PCR amplification for detecting monoclonal immunoglobulin heavy chain gene rearrangements were performed as previously described (22).

T-cell receptor gene rearrangement—DNA was extracted using the QIAamp DNA FFPE Tissue Kit according to the manufacturer's instructions with minor modifications. PCR for detection of T-cell receptor gene rearrangements (*TRG*) were performed as described by Lawnicki *et al.* (23) using primers that interrogate all of the known V γ family members, and the J γ 1/2, JP1/2 and JP joining segments. To allow for fluorescence detection, each joining region primer was covalently linked to a unique fluorescent dye. The products were analyzed by capillary electrophoresis on an ABI 3130xl Genetic Analyzer, and electropherograms were analyzed using GeneMapper software version 3.7 (ABI). For cases prior to 2008, PCR amplification for detecting monoclonal *TRG* were performed as previously described (24).

EBV virus nuclear antigen (*EBNA2*) PCR—PCR for detection of EBV was performed on an ABI 7900 using TaqMan technology and primers targeting the *EBNA2* gene. Prior to 2007, the PCR detection of EBV was performed using the quantitative real-time PCR on 7900TH Sequence Detection System (Applied Biosystems). The products were analyzed using a fluorogenic internal probe specifically targeted to the *EBNA2* gene.

RESULTS

Clinical Features

The median age of 55 patients included in this analysis was 46 years (range 21–74 years) comprising 38 males and 17 females (M:F 2.2:1). Clinically at presentation, all patients had lung involvement (100%), with the next most common site being the central nervous system (CNS) (38%), followed by liver (19%), skin (17%), and kidney (15%) (Table 1). CNS and liver involvement by LYG were determined by radiography, while skin involvement was determined by physical exam and biopsies. Serum samples were assayed on admission for antibodies to EBV in 34/51 patients and 32/34 patients (94%) had evidence of prior infection confirmed by viral capsid antigens for IgG (VCA-IgG). The median EBV viral load was 18 copies per 10⁶ genome equivalent (range 0–22,727). In addition, 51 patients also had evaluation of their cerebrospinal fluid (CSF) by *EBNA2* PCR as part of their initial workup since the CNS is a common site of involvement by LYG. There were 8 positive CSFs by *EBNA2* PCR (1 LYG grade 1, 1 LYG grade 2, 5 LYG grade 3, 1 consistent with LYG), while the remaining 43 samples were negative.

We reviewed a total of 122 biopsies with features of LYG. The most common biopsy site was the lung (89/122, 73%) followed by the skin/subcutaneous tissue (21/122, 17%). Other sites included kidney (3), nasal cavity (4), gastrointestinal (GI) tract (2), conjunctiva (1), liver (1), and adrenal gland (1) (Table 2). Staging bone marrow biopsies were performed in 35 patients, all of which were negative for involvement.

The distribution of the lung biopsies was as follow: 6/60 right middle lobe (10%), 16/60 right lower lobe (27%), 5/60 right upper lobe (8%), 21/60 left lower lobe (35%), and 12/60 left upper lobe (20%). The majority of biopsies were performed in the lower lobes (37/60, 62%). There were 29 lung biopsies that did not further specify the site. Pre and post therapy (e.g. steroids, chemotherapy) biopsies received from referring hospitals to a large degree showed similar morphologic findings.

Categorizing cases based on the highest grade, 16/54 patients (30%) were classified as LYG grade 1, 12/54 patients (22%) as LYG grade 2, and 26/54 patients (48%) as LYG grade 3, with one case not graded due to insufficient material. Grading was determined predominantly on the lung biopsies (50/54, 93%), while extrapulmonary sites (e.g. nasal cavity, GI tract) made up the minority. Altogether, 90 biopsies were graded, and were distributed as: 31/90 (35%) LYG grade 1, 17/90 (19%) LYG grade 2, and 40/90 (45%) LYG grade 3. Skin lesions were not graded, and are separately described below.

Morphologic, In situ hybridization, and Immunophenotypic Findings of LYG

All lesions were angiocentric and had various degrees of angioinvasion and angiodestruction that involved a range of small to large caliber vessels (Figure 1). The infiltrate was polymorphous with an admixture of small lymphocytes, histiocytes; occasionally plasma cells were seen, but were not prominent. Neutrophils, eosinophils, multinucleated giant cells, and well-formed granulomas were not seen. The infiltrates tended to have a patchy distribution, often well circumscribed, and the surrounding lung parenchyma was largely unremarkable. A certain degree of coagulative necrosis was seen in all grades and usually lacked neutrophils and apoptotic bodies. Of note, focal necrosis was seen in 9/31 (29%) cases of LYG grade 1 (Figure 2). However, more extensive necrosis (sometimes >50% of the area) was seen in LYG grade 2 and 3. "Ghost cells" were highlighted with CD20 immunostaining within the necrosis in grade 2 and 3 lesions. Secondary changes such as fibroblastic foci at the periphery of the lesion and intra-alveolar macrophages could be seen as well as edema, but frank organizing pneumonia was not a typical finding. Atypical medium to large lymphoid cells were infrequently seen in H&E stained sections in grade 1 biopsies. LYG grade 2 and 3 more consistently had large atypical cells with irregular nuclear contours, vesicular chromatin, and occasional distinct nucleoli, sometimes reminiscent of Hodgkin/Reed-Sternberg (HRS) cells. Numerous small lymphocytes were always present and admixed with the large atypical lymphoid cells in all grades, and showed prominent angiocentricity and angioinvasion.

We evaluated 11 patients with concurrent (< 3 months after initial diagnosis) lung biopsies involving different lobes/segments and found that 9/11 showed the same grade (3 LYG grade 1, 1 LYG grade 2, 5 LYG grade 3), while 2 showed different grades (2 LYG grade 2 and 3) (Table 3). We also reviewed the material of 10 patients with subsequent biopsies

greater than 3 months after initial diagnosis (range 4–122 months). Four patients continued to have the same grade (1 LYG grade 1, 1 LYG grade 2, 2 LYG grade 3). Two of the patients with an initial diagnosis of LYG grade 1 progressed to grade 2 and grade 3 despite therapy. Four of the patients with an initial diagnosis of LYG grade 3 had lower grade lesions on relapse (3 LYG grade 1, 1 LYG grade 2).

We did not regularly see treatment effect in the biopsies, but there were two patients (Patients 45, 48, Table 3) with a diagnosis of LYG grade 3 who were previously treated at their respective outside institution with chemotherapy (i.e. CHOP; cyclophosphamide and prednisone). The post therapy lung biopsies showed typical features of LYG grade 3, but the necrosis was well-circumscribed and surrounded by a distinct thick band of fibrosis (Figure 7).

Immunohistochemistry with CD20 was essential to highlight the large atypical B cells (LACs) and typically showed variable intensity (mainly with LYG grade 2 and 3), similar to EBV infected B cells in other settings. LYG grade 2 and some LYG grade 3 lesions had a T-cell/histiocyte-rich large B-cell-like pattern or atypical B cells formed small clusters (Figure 3), while grade 3 lesions had larger clusters or small sheets of LAC; however, confluency with obliteration of underlying architecture as with diffuse large B-cell lymphoma (DLBCL), was not seen (Figure 4, 5). In 15 biopsies in which CD30 was performed, 7/15 (47%) contained frequent positive cells, 5/15 (33%) had rare atypical cells, and 3/15 (20%) were negative. CD15 was performed on 6 biopsies with CD30 positivity, and the lymphoid cells were negative in each case.

The majority of small lymphocytes within the vessel walls were T cells seen with CD3 immunostaining, which was a helpful finding leading to a diagnosis of LYG. CD4-positive T cells were predominant in nearly all cases studied (20/21; 95%) compared to CD8. Light chain restriction was seen in 8/21 biopsies studied (38%) and was predominantly kappa (5 cases) compared to lambda (3 cases). Of the 8 light chain restricted biopsies, 4/8 were LYG grade 3, 1/8 LYG grade 2, 1/8 LYG grade 1, and 2/8 could not be further graded.

In situ hybridization for EBV (EBER) stained fewer than 5 cells per high power field (hpf) in LYG grade 1 and was seen in both small and large B cells for all grades. 10/22 (46%) grade 1 biopsies were positive for in situ hybridization for EBER. Overall, LYG grade 1 lesions were the most difficult to diagnose due to the paucity of LACs and lack of EBER positive cells. In LYG grade 2, in situ hybridization for EBER stained fewer than 50 cells per hpf, while grade 3 lesions had more than 50 cells per hpf and focally >100/hpf in some areas (Figure 4D, 5D). Although EBER positivity was mainly seen in the large atypical B cells, positive small also were frequently seen. The vast majority of higher grade LYG biopsies showed expression of EBER. 15/15 biopsies (100%) of LYG grade 2 and 33/35 (94%) of the LYG grade 3 biopsies were positive for EBER. Of the two LYG grade 3 biopsies that were negative for EBER, both were positive for LMP1, with the negative result for EBER due to extensive necrosis and poor preservation of RNA. Overall, considering all of the biopsies for a given patient, 50% of the patients with LYG grade 1 and 100% of the LYG grade 2 and 3 patients had EBV detected in one or more biopsies (Table 3). Of note, EBER was performed on 6 bone marrow biopsies for staging and all were negative.

Biopsies from 7 patients were additionally studied for EBNA2 and ZEBRA by immunohistochemistry to assess the latency phenotype of EBV. Five of the 7 cases tested showed positivity for EBNA2, consistent with latency type III. Focal staining for ZEBRA was seen in 2 of the EBNA2-positive cases, while all others were negative.

25 biopsies (5 LYG grade 1; 4 LYG grade 2; 13 LYG grade 3; 3 not further graded) were evaluated with FOXP3 and CD25 immunohistochemistry. There was no difference in the mean number of FOXP3-positive T cells/10 hpfs in grade 1 (18.0) versus grade 2 LYG (18.5). However, there appeared to be more FOXP3-positive T cells in grade 3 lesions (mean 39.2). CD25 stained relatively few lymphocytes/10 hpfs, with no difference among grades (LYG grade 1 (2.8); LYG grade 2 (3.3); and grade 3 (6.4). Control samples of interstitial pneumonitis were stained for FOXP3 and CD25. These contained a mean of 10 FOXP3-positive cells/10 hpfs, fewer than seen in LYG, but the number of cells positive for CD25 did not differ (2.6/10 hpfs).

Biopsies from other extrapulmonary sites such as the kidney (3), GI tract (2), liver (1), nasal cavity (4), conjunctiva (1), and adrenal gland (1) showed similar morphology as LYG in the lung with angioinvasion, polymorphous infiltrate, and EBV positive LACs (Figure 6). Many of the extrapulmonary sites had extensive necrosis with morphologic and immunophenotypic features consistent with LYG grade 3 (7/12, 58%).

21 skin biopsies were evaluated from 15 patients. Of these, three patients (Patients 9, 10, 25, Table 3) were described in our previous study (14). Clinically, 2/21 skin lesions were described as plaques, 2/21 as papules, 6/21 as nodules, and 11/21 lesions were not further characterized. All 21 biopsies showed a dense dermal or subcutaneous lymphohistiocytic infiltrate composed of small T-lymphocytes and histiocytes, with associated non-necrotizing granulomatous inflammation in 15/21 (71%) of the biopsies. The infiltrate was frequently confined to the subcutaneous tissue (10/21, 48%), manifesting features of lobular panniculitis (Figure 8). Rimming of fat spaces by T cells was absent, and cytophagocytosis was not observed. Focal necrosis involving subcutis or dermis was seen in 5/21 (24%) of the biopsies. However, the epidermis was spared. Only 4 cases had admixed atypical large B cells with CD20 immunostains; these were generally sparse and distributed as single cells. EBER was positive in 3/13 (23%) of the biopsies, but a fourth EBER-negative case was positive for *EBNA2* by PCR.

Lung biopsies in patients with underlying known immunodeficiencies or autoimmune disease with immunosuppressive therapies

Three patients had a history of immunosuppressive therapy for underlying disease (rheumatoid arthritis, Crohn's disease), and one patient had a history of common variable immunodeficiency (CVID) (Table 3, Patients 52–55). The lung biopsies from these patients were histologically indistinguishable from the LYG patients without known history of immunodeficiency. All cases were classified as LYG grade 3.

Molecular Findings

Immunoglobulin gene rearrangement studies were performed by PCR on a total of 44 biopsies. 19 (43%) had a clonal rearrangement, 11 (25%) were polyclonal, 6 (14%) had a suspicious or abnormal rearrangement, and 8 (18%) failed to amplify. Of the clonally rearranged cases, 1/12 (8%) were LYG grade 1, 4/8 (50%) grade 2, and 11/16 (69%) grade 3 (Table 4).

T-cell receptor gene rearrangement studies (*TRG*) were performed on a total of 32 biopsies (Table 4). 2/32 cases were clonal (6%), 6/32 showed a restricted or abnormal pattern (19%), 22/32 had a polyclonal rearrangement (69%), and 2/32 had no amplification (6%). The two biopsies with clonal *TRG* rearrangement were LYG grade 3 involving the lung.

EBNA2 PCR was performed on 13 biopsies (6 positive; 7 negative). In two of the positive cases, *EBNA2* PCR detected EBV, while EBER by in situ hybridization was negative. Those biopsies showed marked necrosis, compromising RNA integrity for the EBER stain.

DISCUSSION

In practice, the diagnosis of LYG is challenging because the disease is exceedingly rare and the histological features can be very subtle. We are in a unique position to describe the pathological spectrum of disease, as a prospective clinical trial for LYG that has spanned over 20 years is ongoing at the NCI. We have had the opportunity to review 122 biopsies from 55 patients and describe the major clinical and histological findings of this entity.

LYG is unique clinically and histologically from other B-cell LPDs. Unlike other EBV-associated B-cell LPDs, patients with LYG typically have no clearly defined underlying immunodeficiency state. The lung biopsies show an angiocentric polymorphous infiltrate composed of histiocytes, small lymphocytes, and variable numbers of LACs. The lesions have a patchy distribution and are remarkably well-circumscribed within a largely normal surrounding lung parenchyma. The histological features exhibit a spectrum with overlapping features between each subsequent grade.

Histologic examination of the LACs in LYG grades 1 and 2 on H&E sections was difficult because the cells were inconspicuous within the background inflammatory cells. Staining with CD20 or CD79a to highlight these LACs was usually necessary to evaluate their density and distribution, and to evaluate for angiocentricity. LYG grade 3 had larger aggregates of LACs and occasional small sheets but still could be distinguished from EBV-positive DLBCL, as seen in the elderly. While the latter condition often involves extranodal sites, including the lung, the neoplastic cells exhibit a diffuse growth pattern, with obliteration of the underlying architecture. When necrosis is present it tends to be focal with a patchy distribution and not centered around vessels (25). Angioinvasion of vessels by T cells is another distinguishing feature of LYG, and helpful in the differential diagnosis with other EBV-related B-cell lymphoproliferative disorders. LYG is generally T-cell rich, in comparison with EBV-associated DLBCL.

Contrary to prior case series (3) describing the absence of necrosis in grade 1 lesions, we found that necrosis was more common (29%) than previously thought, but usually remained focal (Figure 2). Therefore, necrosis can be seen throughout the spectrum of LYG, from grades 1 to 3 and its mere presence does not necessarily indicate a higher grade. However, the presence of “ghost” B cells, as seen by CD20 immunostaining, is usually associated with higher grades, particularly grade 3. The type of necrosis seen was coagulative, lacking inflammatory cells (e.g., neutrophils and eosinophils) and apoptotic bodies or “nuclear dust.” Extrapulmonary sites were commonly involved and included CNS, liver, skin, and kidney (descending order). Although there were no brain biopsies in this series, CNS involvement was confirmed based on the radiologic characteristics and multifocality of the brain lesions by magnetic resonance imaging (MRI) in 38% of cases (26). Evaluation of the cerebrospinal fluid (CSF) by cytomorphology, flow cytometry, and *EBNA2* PCR was also performed in many of the cases, but was less sensitive (16% positive cases) compared to MRI (38%) (Table 3). The lower sensitivity of CSF PCR may be due to the deeply parenchymal nature of the lesions, sparing the meninges. Besides the skin, the majority of extrapulmonary biopsies were diagnosed as LYG grade 3 based on the number of LACs and EBER positive B cells (Figure 6). This finding was likely skewed because the majority of these biopsies were performed to confirm the clinical suspicion of disease progression or therapy resistance.

The histological manifestations of LYG in the skin differ from findings observed in the lung and other extranodal sites. The most common finding is subcutaneous panniculitis, with frequent non-necrotizing granulomas. Skin lesions are less often positive for EBER than the lung (23% in the current series), and EBV-positivity is usually associated with necrosis in nodular lesions. The findings in our current study are similar to those previously reported by our group in a series of patients studied prior to 1990 (14). These observations suggest that the cutaneous manifestations are at least in part an epiphenomenon, and have a different pathobiology. EBV is known to upregulate the release of cytokines and chemokines, and prior studies have linked the vasculitis of LYG to this phenomenon (27). Similarly, the cutaneous lesions may be a result of the systemic, rather than local, effects of EBV. Interestingly, the presence of cutaneous lesions did not coincide directly with the presence of active disease in the lung. The pathological findings in the skin are not specific, although they might suggest the diagnosis of LYG. We have not encountered any case in which LYG was confined to the skin. Other EBV-associated lymphoproliferative disorders may present with cutaneous disease, and should be considered in the differential diagnosis (28, 29).

Similar to prior studies (1, 4, 5), the spleen, bone marrow, and lymph nodes were not involved by LYG in our patients. Katzenstein *et al.* also described that enlargement of the spleen and lymph nodes were not common findings. In 42 cases, the lymph nodes biopsied were either normal or had non-specific findings such as reactive lymphoid hyperplasia (4). In our study of 35 patients who had bone marrow biopsies, all were negative for involvement by LYG and only 8 cases had non-specific findings such as intertrabecular lymphoid or lymphohistiocytic aggregates. Caution should be used in diagnosing LYG in a patient with enlarged lymph nodes, and splenomegaly.

In a recent review by Katzenstein *et al.* (30) it was hypothesized that LYG grade 1 may represent improper sampling and that higher grade lesions are eventually seen with multiple concurrent biopsies. Whether LYG grade 1 exists or is due to sampling is debatable. In our patients that had multiple lung biopsies for monitoring of disease, we found LYG grade 1 could be seen in other lung fields that were biopsied (3 cases). We also appreciated in the periphery of many LYG grade 2 cases (best seen on wedge biopsies) small vessels typically had a rich T-cell infiltrate with rare scattered LACs, consistent with a LYG grade 1 lesion (Figure 3E, 3F). In 3/6 patients with LYG grade 3, we found that their post therapy lung biopsies showed persistent disease with LYG grade 1, suggesting that the low grade LYG lesions may not be eradicated with chemotherapy. It is important to perform multiple lung biopsies if the patient has multiple lesions in order to properly grade and choose the most appropriate therapeutic approach. Biopsy of the larger dominant nodules is also advised.

Many of our patients (34/55, 62%) had various therapies at their original institution, prior to being treated at the NCI. The remaining patients had no prior therapy. Patients were then treated with the therapy choice as mentioned above (i.e. IF α or DA-EPOCH-R) based on the grade. High grade lesions with prominent LACs typically responded to chemotherapy, but some LYG grade 3 cases recurred with a lower grade disease.

Currently, the 2008 WHO classification uses the number of EBV positive B cells/hpf as part of the criteria for grading. However, one must be aware of the impact of necrosis in assessing the number of EBER positive cells. Since the areas of necrosis are always negative by in situ hybridization for EBER, this can greatly underestimate the number of EBER positive cells especially when the necrosis is extensive, as in high grade lesions. It should be noted that EBV was not only seen in the large transformed B cells. The EBER positive cells usually showed a spectrum in cell size with numerous small B cells were positive for EBER as well. The total number and density of EBER-positive cells were both considered in determining grade in the WHO system. These exceptions, although few, emphasize the importance of factoring in multiple findings to properly grade these lesions.

The greatest difficulty was with the diagnosis of grade 1 lesions. This is not surprising as these lesions usually had a paucity of LACs and may have lacked EBV by in situ hybridization/immunohistochemistry or by PCR. 46% of the LYG grade 1 biopsies had EBER positive cells and three of the negative cases were also negative by *EBNA2* PCR.

Many factors contributed to the diagnosis. Emphasis was placed on the presence of prominent angioinvasion by small T cells in the absence of other causes for the pulmonary infiltrate. Correlation with clinical features is equally important. Additionally, one must rule out technical factors for the absence of EBV. Therefore, is it possible to diagnose LYG in an EBV negative biopsy? Yes, only in grade 1 lesions with prominent angiocentricity and in conjunction with a high clinical suspicion. Grade 2 and 3 lesions usually did not pose a problem in grading because the LACs were more prominent and EBV was detectable in all cases (see Table 3).

The EBV latency pattern of LYG was previously reported in a single series of 4 cases (31). Those authors identified expression of LMP1 and EBNA2 in 2 of 3 EBV-positive cases,

suggesting a type III latency pattern. In our study we confirmed type III latency in 5 of 7 cases tested; ZEBRA was focally positive in 2, suggesting some ongoing lytic activity. The latency III phenotype is associated with defective immune surveillance, such as occurs in PTL. The EBV-infected B cells in LYG may avoid immune surveillance, not by a physical barrier as in pyothorax-associated lymphomas, but rather because the T cells are dysfunctional in regulating the virally infected cells.

Morice *et al.* reported that the background small T cells were predominantly CD8 compared to CD4 (32). In our study we found just the reverse. Out of the 21 biopsies that had CD4 and CD8 immunostains, we found that nearly all (20/21, 95%) of the cases tested had predominantly CD4 positive T cells (Figure 1D, 1E). Regulatory T cells (Tregs) are characterized by a specific phenotype (CD4+CD25+ FOXP3+) and have been shown to suppress tumor-specific T-cell immunity in the tumor cell microenvironment, which may contribute to disease progression (33, 34). Tregs have also been associated with decreased survival in certain solid tumors (35). We performed FOXP3 immunostaining and found that there were more FOXP3-positive T cells in LYG grade 3 (median 39/10 hpfs) versus grades 1 or 2 (median 18/10 hpfs) suggesting that an increased number of Tregs in grade 3 may contribute to disease progression. Interestingly, Karube *et al.* also showed an increased frequency of EBV-positive cells in FOXP3-positive adult T-cell leukemia/lymphoma cases versus negative suggesting suppression by Tregs on the T-cell immunosurveillance for EBV (36). Although our sample size is small, the data are suggestive of a possible role in progression, and a larger group of cases should be investigated.

The differential diagnosis for LYG includes inflammatory disorders such as Wegener granulomatosis, EBV-associated B-cell LPDs, such as post-transplant lymphoproliferative disorder (PTLD), senile or age related B-cell LPD, Hodgkin lymphoma, and T cell or NK cell lymphomas (e.g. extranodal NK/T-cell lymphoma, nasal type; PTCL, NOS). Extranodal NK/T-cell lymphomas, nasal type, can be difficult to differentiate from LYG because both lesions are EBV-associated, angiocentric, and can involve sites such as the lung, but other sites such as GI tract and nasal cavity are unusual for LYG. Additionally, the pattern of necrosis differs with more numerous apoptotic debris not generally seen in LYG. Additional studies for immunoglobulin gene rearrangement or double-labeling to determine the immunophenotype of the EBER positive cells can be helpful in such cases.

There were two cases that had a clonal T-cell gene rearrangement (*TRG*). Both of these cases were LYG grade 3 involving the lung. Although T cells were abundant, the large neoplastic cells were clearly of B-cell lineage. Restricted or even clonal *TRG* may be associated with EBV infection (25, 37). This phenomenon is believed to be the result of the expansion of a few dominant T-cell clones reacting to EBV viral epitopes (37). Paradoxically, restricted or clonal T-cell responses are more likely to be seen in patients with less effective control of EBV, and chronic or persistent EBV infection (38).

Occasionally the differential diagnosis for LYG may include classical Hodgkin lymphoma (CHL). The EBER-positive LAC in LYG are often CD30-positive, but CD15 is negative. However, variable expression of CD20 may be present, as often seen in CHL. In CHL positive for EBV, the EBER positive cells do not show variation in cell size, as is typical in

LYG. In addition, Hodgkin lymphoma should be favored if there is evidence of lymph node involvement, which is virtually never seen in LYG.

Patients with an underlying acquired or inherited immunodeficiency syndrome may be diagnosed with LYG (e.g. Wiskott Aldrich syndrome, X-linked lymphoproliferative syndrome, HIV/AIDS, solid organ transplant) under the current WHO classification. In this study we focused on the biopsies from patients without an underlying acquired or inherited immunodeficiency. However, a few cases from patients who had an underlying immunodeficiency (i.e. CVID) or receiving prolonged immunosuppressive regimens related to underlying autoimmune disease (e.g. rheumatoid arthritis) showed features indistinguishable from LYG.

Other EBV-associated B-cell LPDs such as PTLTD may present more of a challenge and at times may show overlap with LYG. There were some findings that were helpful in this distinction, such as lack of T-cell angioinvasion, relatively T-cell “poor” lesions in relation to the B cells (Table 5), and prominent plasmacytic differentiation, all features characteristic of polymorphic PTLTD. Also, in contrast EBV B-cell LPDs, the nodules in LYG were typically well-circumscribed, with smaller nodules rich in small T cells in an angiocentric distribution often seen at the periphery (*****Figure 3E, F).

Nearly all patients studied by EBV serology showed evidence of past exposure but had a low median viral load (18 copies/10⁶ genome equivalents). This is in contrast to patients with PTLTD who typically have elevated median viral loads (mean of 544,750 EBV copies/mL, range 74,000–3,200,000) (39). If the clinical picture is consistent with LYG (multiple lung lesions, sparing of the lymph nodes, spleen, bone marrow, and low peripheral blood viral load) and the lesions have morphologic and immunophenotypic features consistent with LYG, we feel that a diagnosis of LYG can be made in the post transplant setting.

In summary, from our experience, the following findings were helpful in confirming a diagnosis of LYG (Table 6). Clinically, the patient must have pulmonary involvement, usually multifocal lesions with a predilection for the lower lobes. Extrapulmonary sites are common and may be seen with disease progression. Histologically, the infiltrate is polymorphous infiltrate composed of predominantly small T cells and LACs (number dependent on grade) with an angiocentric distribution. Immunohistochemistry for CD20 is imperative to highlight the LACs in LYG grades 1 and 2 and CD3 is very helpful in highlighting the angioinvasion by the T cells. CD4 positive T cells usually predominate over CD8 and the surrounding lung parenchyma should be largely normal. For grading, the number of LACs and EBER positive cells should be used, and while necrosis (coagulative-type) is more extensive in higher grades, it can also be seen in grade 1 lesions. If EBER is negative by in situ hybridization and clinical suspicion for LYG is high, one might consider PCR for EBV/EBNA2 sequences for confirmation; otherwise we recommend additional lung biopsies if multiple lesions are seen clinically.

LYG is an extremely rare EBV-associated B-cell LPD in which grading has important treatment implications. Since the discovery of this disease as a B-cell LPD, rare small case

series have been published to elucidate and define this disease. Based on our study and histologic review of LYG at the NCI, the morphologic and immunophenotypic findings of the different grades have some overlap and underscore the idea that the different grades in LYG are part of a histologic spectrum and may be indistinguishable from other EBV-associated B-cell LPD without a comprehensive clinical workup.

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Figure 1.
LYG grade 1. (A) Angiocentric and angioinvasive lesion with inconspicuous large atypical cells. (B) Prominent small T-cell infiltrate seen with CD3. (C) Rare large atypical B-cells highlighted with CD20. The T-cells are predominantly (D) CD4 positive compared to (E) CD8.

Figure 2.

LYG grade 1 with focal necrosis. **(A)** The lesion is angiocentric and angiodestructive. **(B)** Focally there is coagulative-type necrosis. **(C)** CD20 highlights rare scattered large atypical B-cells that are **(D)** EBER positive (< 20 cells/high powered field) consistent with a diagnosis of LYG grade 1.

Figure 3.

LYG grade 2. (A) Large nodular infiltrate with largely normal adjacent lung parenchyma (left). (B) Polymorphous infiltrate with large atypical Hodgkin-like B-cells that are prominent on H&E and are positive for (C) CD20. (D) In situ hybridization for EBER stains many B-cells (>20/hpf) consistent with a diagnosis of LYG grade 2. (E) At the periphery the lung parenchyma is largely normal and many of the grade 2 lesions (F) had patchy angiocentric infiltrates consistent with LYG grade 1.

Figure 4. LYG grade 3. **(A)** Large nodule with extensive necrosis (top). **(B)** Atypical lymphoid cells with **(C)** some cells reminiscent of Hodgkin/Reed-Sternberg cells. **(D)** Numerous EBER positive B-cells (>50/hpf) but negative in the areas of necrosis (left).

Figure 5.

LYG grade 3. (A) Numerous atypical cells are highlighted by (B) CD20 with an (C) angiocentric distribution and (D) positivity for EBER by in situ hybridization. Note the areas of necrosis (C, D, top) are negative for EBER but CD20 highlights the ghost cells. (E) Shows ghost cells at higher power, necrotic lymphoid cells still expressing the CD20 antigen, that palisade around a vessel.

Figure 6. Adrenal gland biopsy with LYG grade 3. (**A, B**) Similar to the lung, there is an angiocentric lesion with associated coagulative necrosis. The LACs are (**C**) EBER positive (**D**) and CD20 positive.

Figure 7.

Post therapy lung biopsies. (A) Lung biopsy from a 23 year-old male that was previously treated with CHOP showing (B) dense fibrosis around coagulative necrosis. (C) Lung biopsy from a 33 year-old male that was treated with prednisone and cyclophosphamide showing walled off necrosis. (D) There were still numerous large atypical B-cells seen with CD20 and (inset) numerous EBER positive cells (>50/hpf) consistent with a grade 3 lesion.

Figure 8.

LYG of the skin. (A) A dense dermal infiltrate extending deep into the subcutaneous tissue with associated (B) lobular panniculitis. (C) Granulomatous inflammation can be seen with (D) vessels showing angioinvasion.

Table 1

Sites Involved by LYG at Clinical Presentation

Site of Involvement	Percentage
Lung	100%
CNS	38%
Liver	19%
Skin	17%
Kidney	15%

Table 2

Pathologic findings of 122 biopsies in 55 patients diagnosed with LYG

M:F 2.2:1		
Median age 46 years (range 21–74 years)		
Biopsy site	N (%)	Grade [‡]
Lung	89 (73%)	I-30, II-17, III-33
Skin	21 (17%)	N/A *
Other extrapulmonary sites **	12 (10%)	I-1, III-7

* Grading of skin lesions was not performed, as discussed.

** Other sites included liver, kidney, nasal cavity, adrenal gland, GI tract, and eyelid/conjunctiva.

[‡] Not all cases were graded (9 lung biopsies, 4 extrapulmonary sites) due to insufficient biopsy material

Table 3

Concurrent and Subsequent Biopsy Distribution, Extrapulmonary Sites, Prior Therapy, and EBNA PCR in the CSF of 55 patients with LYG

Patient	Age/Sex	Initial lung biopsy (LYG grade)	Concurrent lung biopsy grade (<3 months)	Subsequent lung biopsy grade (>3 months)	Extrapulmonary Site/Grade	Prior Therapy	EBNA PCR CSF	EBV detected***
1	50/M	1	1			None	Negative	+
2	59/M	1				None		-
3	55/M	1				None	Negative	-
4	41/F	1				None	Negative	-
5	34/M	1				None	Negative	+
6	62/M	1	1			Yes	Negative	+
7	56/M	1			Adrenal/3, Skin *	Yes	Positive	+
8	46/F	1			Kidney/3	Yes	Negative	+
9	43/F	1		1	Skin *	Yes	Negative	-
10	41/M	1		2	Nasal cavity/1	Yes		+
11	49/M	1				Yes	Negative	-
12	52/F	1				Yes	Negative	+
13	33/F	1			Skin *	Yes	Negative	-
14	21/M	1	1			Yes		-
15	36/M	1		3		Yes	Negative	+
16	47/M	1				Yes	Negative	-
17	46/F	2			Kidney/3	None	Negative	+
18	58/M	2	3			None	Negative	+
19	51/F	2			Skin *	None	Negative	+
20	37/F	2	2			Yes	Negative	+
21	41/F	2		2		Yes	Negative	+
22	45/M	2			Skin *	Yes	Negative	+
23	66/F	2				Yes		+
24	52/F	2				Yes	Positive	+
25	20/M	2				Yes	Positive	+

Patient	Age/Sex	Initial lung biopsy (LYG grade)	Concurrent lung biopsy grade (<3 months)	Subsequent lung biopsy grade (>3 months)	Extrapulmonary Site/Grade	Prior Therapy	EBNA PCR CSF	EBV detected***
26	28/M	2				Yes	Negative	+
27	42/M	2				Yes	Negative	+
28	39/M	2			Skin*	Yes	Negative	+
29	31/M	3				Unknown	Negative	+
30	74/M	3				Unknown		+
31	43/M	3	2		Liver/3, Colon*†	None	Negative	+
32	47/M	3			Stomach/3	None	Positive	+
33	32/F	3	3		Skin*	None	Negative	+
34	65/M	3	3			None	Negative	+
35	47/M	3		1		None	Negative	+
36	52/M	3		1	Eye/lid/3†	None	Negative	+
37	42/M	3		2	Skin*	None		+
38	60/M	3				None	Negative	+
39	25/M	3			Skin*	None	Negative	+
40	38/M	3				Yes		+
41	41/M	3				Yes	Negative	+
42	53/M	3	3		Kidney*†	Yes	Negative	+
43	43/M	3		1	Skin*	Yes	Negative	+
44	47/M	3		3	Conjunctiva/3	Yes	Negative	+
45	23/M	3				Yes	Positive	+
46	28/F	3			Skin*	Yes		+
47	40/F	3				Yes	Negative	+
48	33/M	3	3			Yes	Positive	+
49	54/M	3				Yes	Negative	+
50	30/F	3		3	Skin*	Yes	Negative	+
51	50/M	c/w LYG			Skin*	None	Negative	-

Patient	Age/Sex	Initial lung biopsy (LYG grade)	Concurrent lung biopsy grade (<3 months)	Subsequent lung biopsy grade (>3 months)	Extrapulmonary Site/Grade	Prior Therapy	EBNA PCR CSF	EBV detected ^{***}
52	42/F ^{**}	3				None	Negative	+
53	46/F ^{**}	3				Yes	Positive	+
54	57/M ^{**}	3	3			Yes	Negative	+
55	34/M ^{**}	3				Yes	Positive	+

* Not further graded due to lack of EBV positive cells and large atypical B-cells.

[†] Initial biopsy site was extrapulmonary

** Patients with an underlying known immunodeficiency or autoimmune disease on immunosuppressive therapy

*** Presence of EBV in one or more of the patient's biopsies by EBV in situ, LMP1, or EBNA by PCR.

Table 4

Grade Distribution and PCR Results of All LYG biopsies

Grade	N (%)	IG PCR +	TRG PCR +
1	31 (35%)	1/12 (8%)	0/7
2	17 (19%)	4/8 (50%)	0/4
3	40 (45%)	11/16 (69%)	2/16 (13%)*
TOTAL	88	16/36 (44%)	2/27 (7%)

Abbreviations: IG PCR, immunoglobulin gene rearrangement; TRG PCR, T-cell receptor gene rearrangement.

* 2 TRG positive cases were classified as LYG, Grade 3.

Table 5

Features of Lymphomatoid granulomatosis (LYG) versus EBV-associated B-cell lymphoproliferative disorder (LPD)

Features	LYG	EBV B-LPD*
Clinical	May have history of inherited/acquired immunosuppression Normal EBV viral load	History of inherited/acquired immunosuppression Elevated EBV viral load
Morphology and Phenotype	<ul style="list-style-type: none"> • Angiocentricity/angioinvasion • Well circumscribed nodules within the lung parenchyma (best seen in wedge biopsy) • Atypical B-cells in a T-cell rich background • In higher grade lesion (grade 2 or 3) the periphery may show LYG grade 1 • Plasmacytosis with light chain restriction typically not prominent 	<ul style="list-style-type: none"> • No angiocentricity/angioinvasion • More diffuse with ill-defined nodules • B-cells are predominant compared to T-cells with full range of B-cell maturation to plasma cells • Prominent plasmacytosis with light chain restriction

EBV = Epstein Barr virus

* These cases resemble polymorphic B-cell lymphoproliferative disorder as seen in the post-transplant setting, but are not associated with transplantation.

Table 6**Diagnosis of Lymphomatoid Granulomatosis**

Clinical	<p>High clinical suspicion</p> <p>Multiple lung lesions (predilection for the lower lobes)</p> <p>Extrapulmonary sites common (CNS, kidney, liver, skin)</p> <p>Absence of lymph node, spleen, or bone marrow involvement</p> <p>Normal or minimally-elevated EBV viral load</p>
Morphologic	<p>Angiocentric/angioinvasive</p> <p>Polymorphous infiltrate (histiocytes, small T-cells, plasma cells)</p> <p>Small T-cells are prominent with CD4>CD8</p> <p>Large atypical B-cells present (density dependent on grade)</p> <p>Presence of EBV (by EBER or EBNA PCR), number of EBER positive dependent on grade</p> <p>Necrosis seen in all grades but more prominent in LYG grade 2 and 3</p> <p>Patchy infiltrate with largely normal surrounding lung parenchyma</p>