Tumor-Infiltrated Immune Response Correlates with Alterations in the Apoptotic and Cell Cycle Pathways in Hodgkin and Reed-Sternberg Cells

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Abstract

Purpose: To analyze tumor-microenvironment relationships in Hodgkin lymphoma (HL) as potential determinants in the decision-making process related to the alterations in cell cycle and apoptotic pathways of Hodgkin/Reed-Sternberg (H/RS) cells.

Experimental Design: Based on a cohort of 257 classic HL patients, we carried out a global descriptive correlational analysis and logistic regression study to identify tumor-infiltrated immune cell rate in HL that could be interconnected with genes involved in the regulation of apoptotic/proliferative pathways in H/RS cells.

Results: Our results reveal the existence of a connection between the reactive microenvironment and molecular changes in apoptotic/proliferative pathways in H/RS cells. A lesser incidence of infiltrated cytotoxic cells in the tumor ($CD8^+$ T lymphocytes, $CD57^+$ natural killer, and granzyme B^+ cells) was associated with overexpression of antiapoptotic proteins (Bcl-X_L, survivin, caspase-3, and nuclear factor-κB) in tumoral cells. Increased incidence of general infiltrated immune cells, such as $CD4^+$ T lymphocytes, $CD57^+$ natural killer cells, activated CTL, and dendritic cells, in the microenvironment of the tumor was associated with increased growth fraction of tumoral cells, including G_1-S checkpoint (cyclin D and cyclin E) and tumor suppressor pathways (p16 and SKP2), and with the presence of EBV (signal transducers and activators of transcription 1 and 3 expression; STAT1/STAT3).

Conclusions: A lower level of cytotoxic cells correlated with an increase of antiapoptotic mechanisms in H/RS cells, whereas the global infiltrated immune population correlated with the growth fraction of the tumor. Our collective data suggest a causal relationship between infiltrated immune response and concurrent changes of the different proliferative checkpoints, tumor suppressor, and apoptotic pathways of H/RS cells in HL.

Hodgkin/Reed-Sternberg (H/RS) cell clones of 25% of classic Hodgkin lymphoma cases have nonfunctional immunoglobulin gene rearrangements, whereas the remaining cases seem to have acquired somatic mutations preventing immunoglobulin expression (1, 2). Previous studies have described alterations in genes controlling apoptosis and proliferation of H/RS cells, such as p53 expression (3, 4), deregulation of the Bcl2 family (3–5), proliferative and/or apoptotic indices (3, 6), activation of the nuclear factor-κB (NF-κB) pathway (7), and biological factors such as EBV detection (8, 9), which influence the clinical aggressiveness of HL (5, 10–12).

The tumor microenvironment has been considered to be a manifestation of host immune reactions to malignant cells (13). The immune response in HL is likely to be inadequate because of the poor immunogenicity, the immunosuppressive effect of tumoral cells (14), or the poor response of the host immune system. The abnormal cytokine pattern in HL may contribute not only to the proliferation of H/RS cells but also to the maintenance of an inappropriate environment in which an effective host immune response to H/RS cells cannot be achieved. The role of the reactive microenvironment was found to be associated with the number, subset type, and activation state of the reactive immune cells (15, 16), specifically the cytotoxic and regulatory T cells (17–19). However, the full significance of infiltrating immune cells in the pathology of HL continues to be controversial (20).

Approximately 20% to 30% of HL patients fail therapy and eventually die as a result of progressive disease or complications of therapy (21). Our knowledge of the molecular biology of HL is incomplete and has not been practically translated into an improvement in the treatment of this disease. Recently, a gene expression profile study in advanced classic HL identified signatures of tumoral growth/apoptosis that are associated with

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Table 1. Protein, threshold, pattern of reactivity, and percentage of positive immune and H/RS cells: results from immunohistochemistry and *in situ* hybridization

Immune response	Protein	Threshold of positivity*	Pattern of reactivity †	Positive cases (%)
T lymphocytes	CD4	>70 positive cells/field	Any positive	147/187 (78.6)
	CD8	>150 positive cells/field	Any positive	45/191 (24.1)
NK cells	CD56	>10 positive 7cells/field	Any positive	27/198 (13.6)
	CD57	>70 positive cells/field	Any positive	20/216 (9.3)
CTL	Granzyme B	>8 positive cells/field	Any positive	114/195 (58.5)
	TIA-1	>60 positive cells/field	Any positive	74/200 (37.0)
Dendritic cells	CD21	>0.3 positive area/field	Any positive	10/201 (5.0)
	S-100	>40 positive cells/field	Any positive	38/157 (24.2)
Regulatory T cells	FOXP3	>25 positive cells/field	Any positive	42/219 (19.2)
H/RS cells	**Protein**	**Threshold of staining***	**Pattern of reactivity †**	**Positive cases (%)**
Apoptosis	Bcl2	>50% positive H/RS cells with strong expression	High/low	52/257 (20.2)
	Bcl-X_L	>50% positive H/RS cells with strong expression	High/low	63/257 (24.5)
	Mcl1	>50% positive H/RS cells with strong expression	High/low	14/239 (5.9)
	Survivin	>50% positive H/RS cells with strong expression	High/low	188/233 (80.7)
	Bax	>10% positive H/RS cells	High/low	239/248 (96.4)
	NF-κB	Nuclear expression in H/RS cells	Positive/negative	194/241 (80.5)
	Caspase-3	Cytoplasmic and nuclear expression in H/RS cells	Positive/negative	132/249 (53.0)
Proliferation	Bcl6	>10% positive H/RS cells	Positive/negative	24/242 (10.0)
	Cyclin A	>10% positive H/RS cells	Positive/negative	246/252 (97.6)
	Cyclin B1	>10% positive H/RS cells	Positive/negative	225/247 (91.1)
	Cyclin E	>10% positive H/RS cells	Positive/negative	212/242 (87.6)
	Cyclin D1	Any positive H/RS cells	Positive/negative	15/250 (6.0)
	Cyclin D3	>10% positive H/RS cells	Positive/negative	104/250 (41.6)
	CDK1	>10% positive H/RS cells	Positive/negative	212/238 (89.1)
	CDK2	>10% positive H/RS cells	Positive/negative	222/250 (88.8)
	CDK6	>10% positive H/RS cells	Positive/negative	55/247 (22.3)
	p16	Expression in H/RS cells similar to benign cells	Positive/negative	177/242 (73.1)
	p18	Any positive H/RS cells	Positive/negative	117/210 (55.7)
	p21	>50% positive H/RS cells	High/low	25/252 (10.0)
	p27	>50% positive H/RS cells	High/low	52/242 (9.4)
	p53	>80% positive H/RS cells	High/low	44/248 (17.8)
	Rb	>50% positive H/RS cells	High/low	110/245 (44.9)
	SKP2	>50% positive H/RS cells	High/low	209/249 (83.9)
	Hdm2	>10% positive H/RS cells	High/low	174/235 (74.0)
	STAT1	Nuclear expression in H/RS cells	Positive/negative	213/245 (86.9)
	STAT3	Nuclear expression in H/RS cells	Positive/negative	155/244 (63.4)
	EBV	Cytoplasmic/membranous expression in H/RS cells	Positive/negative	90/257 (35.0)

*The thresholds of positivity for immune markers and the thresholds of staining apoptotic/proliferative markers were established as mentioned in Materials and Methods (3, 18, 19).
† Pattern of reactivity of immune cells represents the number of positive cells in each field analyzed and the pattern of reactivity of apoptotic/proliferative marker was considered as positive/negative or high/low in function of the thresholds established in internal controls.

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treatment response and outcome in these patients and two other signatures representing the tumor microenvironment and host immune response (22). This suggests that not only the biology of the tumor cell but also the characteristics of the immune response of the host could be factors determining the treatment response and clinical behavior of the tumor. Among these complex interactions, immune cells present in the infiltrate are able to modulate apoptosis and proliferation via death receptors, cytotoxic granule liberation, withdrawal of growth factors, and production of immunosuppressive cytokines (23–25). On the other hand, apoptosis and proliferation observed in H/RS cells have also been partially associated with the presence of alterations of these genes and microenvironmental signals due to the intervention of the reactive immune infiltrate (26). The evaluation of the possible interaction of H/RS cells with components of their microenvironment may be useful for determining new markers of therapeutic response, which should provide more accurate information pertinent to patient care. Bearing these considerations in mind, this study set out to explore the association between the tumor-infiltrated cells and the different genes involved in the defective regulation of apoptosis and cell cycle observed in H/RS cells of a series of 257 HL patients, with a view to thinking about new strategies for the design of future therapies.

Materials and Methods

HL patients. The studied population consisted of 257 patients with the histologic and subtype confirmation (WHO classification) of HL who had been diagnosed between 1994 and 1998 by members of the Spanish Hodgkin Lymphoma Study group (3). All of the samples included represent at-diagnosis biopsies and all of the patients were treated following standard protocols (3, 12). The alterations in the

major tumor suppressor pathways and cell cycle checkpoints, the biological markers, and the tumor-infiltrating cells of these tumors and patients have been described elsewhere (3, 12, 17, 18).

Immunohistochemistry and* in situ *hybridization. Immunohistochemistry and *in situ* hybridization of the different markers in H/RS and immune cells have been previously assessed using tissue microarray technology (18). Immunohistochemical staining of the cellular infiltrate surrounding tumoral H/RS cells was done in the Immunohistochemistry Unit of the Department of Pathology of the Hospital de Tortosa Verge de la Cinta. Infiltrated immune cells were quantified manually on two digital images of representative areas selected based on the presence of H/RS cells with an appropriate inflammatory background as previously described (18, 19). The mean number of positive cells/field for each antibody was calculated. For CD21, the positive zones were measured as areas, the mean was calculated, and the final result was expressed as total positive surface area (μm^2)/field. The thresholds established for each marker, calculated after a careful preliminary evaluation and estimation of different cutoffs, and the related numbers of positive cases observed in the series for each of them were shown in Table 1. The cutoffs were established according to statistical principles and based on the frequency of distribution of each of the immune-positive cells (histogram) in the series (18, 19).

Immunohistochemical expression of the different markers of apoptosis and proliferation in H/RS cells was done using the LSAB Visualization System (DAKO) in the Immunohistochemistry and Histology Unit of the Centro Nacional de Investigaciones Oncológicas (Madrid, Spain). The different patterns of staining of each apoptotic and proliferative markers were recorded as positive/negative or high/low expression, taking into account the expression in H/RS cells (Table 1). The different cutoffs for each marker were previously established by the reactivity of accompanying lymphocytes and macrophages and by internal controls included in the tissue microarray (3). The numbers of positive cases observed in the series in function of the threshold of each marker were shown in Table 1.

Statistical methods. Statistical analysis was done using Statistical Package for the Social Sciences 11.0 (SPSS). All possible relationships between apoptosis and proliferative markers of tumoral cells and each one of the components of the immune response were evaluated using the unpaired *t* test or Mann-Whitney *U* test, as appropriate. For continuous variables, the results are expressed as means and SDs. Statistical relationships for dichotomized immune variables were also analyzed by the Pearson χ^2 test or Fisher's exact test, as appropriate. To find the best-fitting model that describes these relationships, we also did a logistic regression analysis using the forward Wald variable selection method. The dependent variable consisted of the dichotomous levels of the immune infiltrate components. The independent variables included the complete set of proliferative and apoptotic markers in tumoral cells (independent variables). Odds ratios are presented with 95% confidence intervals. For all tests, values of $P < 0.05$ were considered to be statistically significant.

Results

Immunohistochemical analysis. The results about the immunohistochemical detection of immune cells and apoptotic/proliferative protein of H/RS cells are summarized in Table 1. Classic HL tumors were principally infiltrated by $CD4^+$ T lymphocytes (78% of cases), granzyme B^+ cells (58%), and $TIA\text{-}1^+$ cells (37%). The H/RS cells seem to express cyclins and cyclin-dependent kinases (CDK) involved in the G_1-S and G_2-M transitions, and molecules involved in apoptosis control, and exhibit multiple alterations in the expression of the CDK inhibitors that are involved in the various tumor suppressor pathways, such as p53-$p21^{WAF1}$, $p16^{INK4a}$-Rb, and $p27^{KIP1}$. These data have been partially presented in previous articles (3, 18). In comparison with the patterns of staining of the different internal controls previously established according to the different thresholds, H/RS cells of this series present (*a*) an important increase in the expression (overexpression) of cyclin E, CDK2, CDK6, Bcl2, Bcl-X_L, and survivin; (*b*) an increased nuclear expression of NF-κB, and signal transducers and activators of transcription 1 and 3 (STAT1 and STAT3); and (*c*) the loss of expression of $p16^{INK4a}$ $p27^{KIP1}$, and the antiapoptotic Bax.

Relationship between tumor-infiltrated immune cells and H/RS cell apoptotic markers. The number of infiltrated immune cells, considered as continuous or dichotomized variables, varied

Table 2. Number of infiltrated immune cells present in the reactive microenvironment of tumors of HL patients according to the staining patterns of the apoptotic markers expressed by H/RS cells

Reactive cells	Immune markers	Apoptotic markers*	Mean number ± SD of immune cells †	*P*
T lymphocytes	CD4	Bcl-X_L low	198.35 ± 133.14	0.014 ‡
		Bcl-X_L high	257.75 ± 142.67	
		Mcl1 low	168.53 ± 138.71	0.022§
		Mcl1 high	223.97 ± 135.21	
	CD8	Bcl-X_L low	115.94 ± 74.41	0.014§
		Bcl-X_L high	84.85 ± 59.87	
NK cells	CD56	Bcl2 low	6.18 ± 6.09	0.013§
		Bcl2 high	3.65 ± 3.45	
	CD57	Bcl2 low	43.75 ± 57.42	0.006§
		Bcl2 high	21.95 ± 15.51	
		NF-κB negative	62.45 ± 76.72	0.002§
		NF-κB positive	34.49 ± 44.20	
Dendritic cells	S-100	Bax low	51.00 ± 10.39	0.034 ‡
		Bax high	29.52 ± 17.31	

*Patterns of staining for apoptotic markers recorded as positive/negative or low/high expression, taking into account the expression in H/RS cells and the different cutoffs previously established for each marker (see Table 1).
† Mean number and SD of infiltrated immune cells quantified in HL sections and distributed in function of the patterns of staining of the apoptotic markers of H/RS cells.
‡ Evaluated by unpaired *t* test.
§ Evaluated by Mann-Whitney *U* test.

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Table 3. Number of infiltrated immune cells present in the reactive microenvironment of tumors of HL patients according to the staining patterns of the proliferative markers expressed by H/RS cells

Reactive cells	Immune markers	Proliferative markers*	Mean number ± SD of immune cells †	P
T lymphocytes	CD4	p16 negative	249.43 ± 142.45	0.006 ‡
		p16 positive	190.15 ± 131.61	
		SKP2 low	154.85 ± 109.55	0.013 ‡
		SKP2 high	221.37 ± 139.91	
		STAT3 negative	244.85 ± 134.12	0.009§
		STAT3 positive	190.80 ± 134.88	
	CD8	Cyclin D3 negative	96.72 ± 61.20	0.009§
		Cyclin D3 positive	128.70 ± 81.91	
		p16 negative	127.69 ± 76.62	0.020 ‡
		p16 positive	100.49 ± 69.33	
		p27 low	114.89 ± 74.67	0.023 ‡
		p27 high	86.47 ± 60.28	
		p53 low	115.62 ± 76.39	0.017§
		p53 high	81.34 ± 45.95	
NK cells	CD56	CDK2 negative	8.11 ± 6.47	0.013 ‡
		CDK2 positive	5.21 ± 5.59	
		p27 low	6.48 ± 6.23	0.015§
		p27 high	3.87 ± 3.33	
	CD57	Cyclin D1 negative	35.13 ± 41.65	0.011§
		Cyclin D1 positive	102.60 ± 17.04	
		p16 negative	53.08 ± 74.44	0.013§
		p16 positive	34.05 ± 38.12	
CTL	Granzyme B	Cyclin D3 negative	12.91 ± 14.76	0.021 ‡
		Cyclin D3 positive	18.60 ± 19.36	
		p16 negative	19.84 ± 17.10	0.010 ‡
		p16 positive	13.08 ± 16.79	
		p18 negative	19.82 ± 21.49	0.006§
		p18 positive	12.70 ± 12.55	
		STAT3 negative	9.80 ± 12.49	<0.0001 †
		STAT3 positive	18.52 ± 18.55	
		Bcl6 negative	13.50 ± 13.92	0.001§
		Bcl6 positive	31.00 ± 31.00	
	TIA-1	SKP2 low	104.13 ± 97.37	0.035 ‡
		SKP2 high	67.63 ± 85.89	
Dendritic cells	CD21	p16 negative	0.18 ± 0.47	0.020§
		p16 positive	0.08 ± 0.24	
		Cyclin D1 negative	0.09 ± 0.29	0.010§
		Cyclin D1 positive	0.42 ± 0.65	
Regulatory T cells	FOXP3	Rb low	14.46 ± 12.78	0.030 ‡
		Rb high	18.20 ± 12.97	
		p21 low	10.50 ± 8.45	0.004§
		p21 high	16.95 ± 13.34	
		CDK2 negative	10.72 ± 8.82	0.017§
		CDK2 positive	16.90 ± 13.30	
		STAT1 negative	10.92 ± 9.70	0.031 ‡
		STAT1 positive	16.69 ± 13.01	

*Patterns of staining for proliferative markers in H/RS cells (see Table 1). Markers implicated in G_1-S checkpoints and $p16^{INK4a}$-Rb pathway (in red), $p53\text{-}p21^{WAF1}$ and $p27^{KIP1}$ pathways (in green), and STAT pathway (in blue).
† Mean number and SD of infiltrated immune cells quantified in HL sections and distributed in function of the patterns of staining of the proliferative markers of H/RS cells.
‡ Evaluated by unpaired *t* test.
§ Evaluated by Mann-Whitney *U* test.

between the apoptotic markers in H/RS cells. As illustrated in Table 2, a significantly greater number of $CD4^+$ T lymphocytes and fewer $CD8^+$ T lymphocytes were clearly associated with the overexpression of the antiapoptotic genes *Bcl-X_L* and *Mcl1*. A reduced number of $CD56^+$ and $CD57^+$ natural killer (NK) cells in the tumor were also significantly related to the overexpression of the antiapoptotic genes *Bcl2* and *NF-κB*. Finally, a greater number of infiltrated $S\text{-}100^+$ dendritic cells were significantly associated with the loss of expression of Bax. Using dichotomized immune variables, the significant relationship between $CD8^+$ T lymphocytes and the antiapoptotic gene *Bcl-X_L* was confirmed ($P = 0.016$, Pearson χ^2 test), whereas the $CD57^+$ NK cells seem related to the others antiapoptotic genes *Bcl2*, *survivin*, and *NF-κB* ($P = 0.016$, 0.031, and 0.008, respectively, Fisher's exact test). No significant relationship was found between lymphocytes and NK cells and the expression of the proapoptotic markers or between cytotoxic cells (identified by granzyme B and TIA-1) and the global markers of apoptosis.

Relationship between tumor-infiltrated immune cells and H/RS cell proliferative markers. As shown in Table 3, the principal

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relationships encountered in this study were restricted to the proteins of the G_1-S transition. T lymphocytes, CTL, NK, dendritic cells, and regulatory T cells exhibited significant relationships with proteins involved in the G_1-S checkpoint and tumor suppressor pathways defined by the $p16^{INK4a}$-Rb, p53-$p21^{WAF1}$, and $p27^{KIP1}$ pathways. Most of these relationships were verified with the dichotomized immune variables (data not shown). This suggests that multiple components of the innate immune system are actively involved, presumably through a coordinated response with the apoptotic and cell cycle mechanisms of H/RS cells.

In particular, a generally higher number of infiltrated T lymphocytes, regulatory T cells, cytotoxic cells, and dendritic cells and a reduced number of NK cells in the tumors seemed significantly associated with the positive expression of cyclin D and the overexpression of CDK2 in H/RS.

A higher number of global reactive cells (T lymphocytes, NK, cytotoxic cells, dendritic cells, and regulatory T cells) were significantly associated with the alterations in the $p16^{INK4a}$-Rb pathway, whereas a lower number of $CD8^+$ T lymphocytes and $FOXP3^+$ regulatory T cells were significantly associated with the alterations observed in the p53-$p21^{WAF1}$ pathway. A greater infiltration of $CD8^+$ T lymphocytes, NK, and activated cytotoxic cells was associated with the $p27^{KIP1}$ pathway and the infiltration of granzyme B cytotoxic cells was associated with the expression of the *Bcl6* gene.

The presence of $CD4^+$ T lymphocytes and granzyme B^+ cytotoxic cells was associated with the expression of the transcription factor STAT3, whereas a higher number of $FOXP3^+$ regulatory T cells were significantly associated with the expression of the transcription factor STAT1.

Logistic regression analyses. Relationships encountered between each one of the components of the immune response with the different apoptotic and proliferative pathways of tumoral cells were verified with the logistic regression model (Table 4). In this model, the analysis indicates that a low level of infiltrated cytotoxic cells in the reactive microenvironment of HL patients ($CD8^+$ T lymphocytes, $CD57^+$ NK cells, and granzyme B^+ cytotoxic cells) seems associated with the overexpression of antiapoptotic proteins observed in H/RS cells ($Bcl\text{-}X_L$, survivin, caspase-3, and NF-κB). On the other hand, the analysis indicates also that a general high level of immune infiltrate $CD4^+$ T lymphocytes, $CD57^+$ NK cells, activated CTL, and dendritic cells in the same time that a low level of nonactivated CTL ($TIA\text{-}1^+$ CTL) and dendritic cells in the reactive microenvironment of these patients was associated with the abnormal expression of the proliferative proteins regulating the transition from G_1 to S in H/RS cells (cyclin D and cyclin E) and involved in the tumor suppressor pathways (p16 and SKP2). A low level of infiltrated CD4 T lymphocytes and a high level of activated CTL were associated with the STAT3 expression in tumoral cells, whereas a low level of infiltrated dendritic cells was associated with the STAT1 expression.

Discussion

Tumor-microenvironment interactions seem to be possible determinants in the evolution of the tumor (27, 28). In this study, for the first time, we have undertaken a descriptive associational analysis of human HL to determine the possible relationships between the number and type of immune cells that have infiltrated the tumor and the expression of proteins involved in the tumoral apoptotic/proliferative pathways. Our results indicate that (*a*) there is a relationship between cells of the global reactive microenvironment and molecular changes in the cytoplasm and nucleus of H/RS cells, (*b*) these molecular changes affect the apoptotic/proliferative pathways of tumoral cells, (*c*) the cytotoxic immune response seems to be associated with the apoptotic pathway, (*d*) the global immune response seems to be associated only with the G_1-S checkpoint and tumor suppressor pathways of the cell cycle, and (*e*) $CD4^+$ T cells but also CTL and dendritic cells seem associated with the STAT pathway, which is involved in EBV infection.

Table 4. Logistic regression analysis that best fits the relationship between the immune infiltrate and the set of apoptotic and proliferative markers of tumoral cells in HL patients

Reactive cells	Immune markers	H/RS markers associated*	OR (95% CI)	*P*
T lymphocytes	CD4 (≤70 cells/field)	p16 positive	3.468 (1.155-10.413)	0.027
		STAT3 positive	3.508 (1.229-10.014)	0.019
	CD8 (≤150 cells/field)	$Bcl\text{-}X_L$ high	6.474 (1.439-29.129)	0.015
NK cells	CD57 (≤70 cells/field)	Survivin high	5.428 (1.231-23.931)	0.025
		NF-κB positive	6.552 (1.300-33.014)	0.023
		Cyclin D1 negative	25.778 (4.809-138.171)	<0.0001
CTL	Granzyme B (≤8 cells/field)	Survivin high	4.680 (1.253-17.477)	0.022
		Caspase-3 positive	4.645 (1.772-12.179)	0.002
		Cyclin D3 negative	4.887 (1.934-12.347)	0.001
		STAT3 negative	3.787 (1.633-8.784)	0.002
	TIA-1 (≤60 cells/field)	SKP2 high	8.100 (2.162-30.341)	0.002
Dendritic cells	CD21 (≤0.5 area/field)	Cyclin E positive	60.500 (5.450-671.627)	0.001
		Cyclin D1 negative	26.889 (2.220-325.662)	0.010
	S-100 (≤40 cells/field)	Cyclin D3 negative	4.229 (1.535-11.652)	0.005
		STAT1 positive	8.138 (1.111-59.633)	0.039

Abbreviations: OR, odds ratio; 95% CI, 95% confidence interval.
*Markers implicated in the apoptosis (in black), G_1-S checkpoints and $p16^{INK4a}$-Rb pathway (in red), p53-$p21^{WAF1}$ and $p27^{KIP1}$ pathways (in green), and STAT pathway (in blue).

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In the complex scenario of immune response against the tumor, cumulative data on the descriptive changes of genes and proteins involved in the host immune response but also in the apoptotic/proliferative pathways of H/RS cells are currently available. Some of these data, included in the present study, have been correlated separately with the clinical outcome of HL patients (3, 17, 18, 22). Theoretical works are necessary to identify how immune response interacts with the apoptotic/proliferative mechanisms of H/RS cells that could participate and promote changes of the morphology, treatment response, and outcome in HL. Our results provide some evidences that the clinicobiological behavior of HL patients is subjected to the direct cross-talk between the immune response and H/RS cells. An up-to-date literature review (*in vitro* studies) indicates that the immune cells, by direct contact or by signals delivered within the tumor microenvironment, may be able to induce some of the changes involved in the apoptotic/proliferative pathways of H/RS cells that could explain the clinicobiological variability in this disease.

The cell cycle and apoptotic functions are coordinately regulated (29, 30) and the balance between the two signaling cascades can be influenced by the reactive microenvironment of the tumor. The deregulation of H/RS cell apoptotic pathway seems to be significantly associated with infiltrated CTLs ($CD8^+$ T cells, NK cells, and cytotoxic cells) that are able to activate apoptotic caspase proteolytic cascade through tumor necrosis factor receptor superfamily interactions (FasL/Fas and CD40/CD40L). The different members of this superfamily share common cell signaling pathways that mediate the activation of nuclear factor NF-κB and mitogen-activated protein kinases. In this case, CD40/CD40L interactions are known to induce the up-regulation of Bcl-X_L and Mcl1 expression (26, 31) and to mediate the activation of NF-κB (32, 33), which induces changes in the expression of a set of proteins regulating apoptosis, such as survivin and Bcl-X_L (7, 34). CTLs are also able to trigger a second proapoptotic pathway through the protease granzyme B, which, once released from CTLs, is translocated into the target cell by perforin, where it activates the effector caspase cascade (35). On the other hand, the wide variety of cytokines and chemokines present in HL tumoral tissue (interleukin-2, interleukin-4, interleukin-6, and interleukin-13), responsible for the massive influx of activated immune cells (36), has been shown to regulate the expression of the various members of Bcl2 family, such as the antiapoptotic Bcl2 homologues Bcl-X_L and Mcl1 and the proapoptotic Bax (37–41).

Alterations observed in the G_1-S checkpoint of H/RS cell cycle and in the principal tumor suppressor pathways Rb-$p16^{INK4a}$ and $p27^{KIP1}$ are significantly associated with the global immune infiltrate present in the tumor. Likewise for the apoptotic markers, the physiologic signals present in the reactive microenvironment also interfere with components of the G_1-CDK checkpoint (cyclin D3, CDK6, and p27; refs. 42, 43). The constitutively activated NF-κB has also been shown to induce changes in the expression of a set of proteins regulating cell cycle progression and gene transcription, including cyclin D1, p53, $p16^{INK4a}$, and $p27^{KIP1}$ (7, 44). Cytotoxic cells are able to induce directly the permanent down-regulation of $p27^{KIP1}$, probably as a consequence of increased degradation mediated by SKP2, a ubiquitin ligase for $p27^{KIP1}$ (43, 45, 46). Related with the heightened proliferative state in these tumors is the high level of expression of Bcl6, a multifunctional regulator that is able not only to down-regulate cyclin D2 and $p27^{KIP1}$ expression (47) but also to repress Bcl-X_L (40).

The presence of EBV was significantly associated with the overexpression of STAT1 and STAT3. STAT3 was found to be associated with a low infiltration of CD4 T lymphocytes and a high infiltration of activated cytotoxic cells. Although STAT1 is considered to be a potential tumor suppressor (promoting apoptosis), STAT3 is thought to be an oncogene because it leads to the activation of cyclin D1 and Bcl-X_L expression and is involved in promoting cell cycle progression and cellular transformation and in preventing apoptosis (48).

The physiologic relevance of these relationships could be related to the different probabilities of survival of HL patients. Previous results from this cohort showed that lower infiltration of $CD8^+$ T cells, NK cells, and regulatory T cells and greater infiltration of cytotoxic cells (granzyme B^+ and TIA-1^+) are unfavorable prognostic factors (18) and that shorter survival is associated with Bcl2, p53, Bcl-X_L, and Bax expression in H/RS cells (3, 12). Recently, four different sets of these deregulated genes have shown to be associated with a therapeutically unfavorable response in HL patients (22). Under these conditions, the concomitant analysis of the immune infiltrate and the apoptotic/proliferative pathways of tumoral cells should provide more accurate information about the specific molecular pathways critical for cancer cell growth. Possible molecules that interfere with these molecular links, particularly some enzymes representative of the immune metabolic state or tumoral cell cycle (22), might be pharmaceutically manipulated and could be candidates for new therapeutic targets pertinent to patient care.

In conclusion, our results highlight the possible causal relationship between the characteristics of the tumor microenvironment and the expression of proteins that regulate apoptosis and cell cycle in H/RS cells. This interaction could probably explain the chain of events with clinicobiological implications for HL patients established in several investigations relating immune response markers and apoptotic/proliferative molecules separately with clinicobiological data. These results should be further evaluated by experimental observations of functional models to put in evidence the possible cross-talk between the immune response and the apoptotic/proliferative mechanism in tumoral cells and should be considered for new strategic therapeutic approaches.

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