Expression of two markers of germinal center T cells (SAP and PD-1) in angioimmunoblastic T-cell lymphoma

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ABSTRACT

Background and Objectives

In the present paper we report that SAP, an intracytoplasmic molecule that is involved in cell signaling, is an immunohistologic marker for germinal center T cells in paraffin-embedded tissue. We document its expression, and also that of PD-1 (another recently described marker of germinal center T cells to which a new antibody has been raised), in normal and neoplastic lymphoid tissue to evaluate the suggestion that helper T cells within the germinal centers of human lymphoid tissue are the cell of origin of angioimmunoblastic T-cell lymphoma (AITL), and to assess the diagnostic value of these two markers.

Design and Methods

Expression of SAP and PD-1 was investigated by immunohistochemistry in paraffin-embedded tissue sections and in cell lines. Western blotting was performed on cell lines, and antibody specificity was confirmed by immunostaining of transfected cells.

Results

Screening on more than 500 lymphoma biopsies showed that 95% (40/42) of cases of AITL expressed at least one of these markers. SAP was also expressed on many cases (15/21) of acute T lymphoblastic leukemia, in keeping with its presence in cortical thymocytes. However, PD-1 and SAP were also found in a minority of cases of peripheral T-cell lymphoma other than AITL, in contrast to a report that the former marker is specific for AITL. This observation raises the possibility that such *non-angioimmunoblastic* cases may be related to germinal center helper T cells.

Interpretation and Conclusions

These two markers provide additional evidence that AITL arises from germinal center T cells. They may also prove of value in the diagnosis of this disease since a negative reaction was rarely observed in this disorder.

Key words: lymphoma, AITL, germinal center T cells, immunohistochemistry, western blotting.

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The germinal centers of human lymphoid B-cell follicles contain a specialized population of T helper cells,[1-3] and recent gene expression studies[4,5] have yielded extensive information on their molecular profile and emphasized their important role in the context of normal and pathologic antibody production.[6] The importance of these cells in the human immune response is demonstrated by the immunodeficiency states caused by genetic defects of germinal center T-cell-associated molecules (e.g. the absence of the co-stimulator molecule ICOS results in common variable immunodeficiency).[7]

It has been proposed that germinal center T cells represent the cell of origin of angioimmunoblastic T-cell lymphoma (AITL).[8-10] One argument is based on the observation that cases express the inhibitory receptor PD-1 (programmed death-1) (CD279) (a marker of germinal center T cells),[11] whereas other non-Hodgkin's lymphomas are PD-1-negative. On these grounds, it was suggested that PD-1 represents a new diagnostic marker for angioimmunoblastic lymphoma.

In the present study, we evaluated the expression of PD-1 using a new monoclonal antibody specific for this molecule. We also report an additional immunohistologic marker (SAP) of germinal center T cells.

Design and Methods

Full details of the antibodies used in this study, the tissue samples studied and the immunostaining and western blotting techniques used are given in the *Supplementary section*.

Results

Immunostaining of normal lymphoid tissue

The expression of the markers PD-1 and SAP in human lymphoid tissue (studied by single and double labeling techniques) is illustrated in Figures 1 and 2 and summarized in Table 1. Staining was performed on paraffin-embedded tissue sections but immunostaining of cryostat sections of human tonsil showed the same reactivity patterns. We confirmed that PD-1 was preferentially expressed by scattered cells in germinal centers, and these cells were shown by double labeling for CD3 to be T cells. Outside lymphoid follicles, the great majority of T cells were PD-1-negative (or only weakly positive). Additional double immunostaining showed that many PD-1-positive follicular T cells lacked CD57, CD8, the B-cell transcription factor PAX-5 and the proliferation marker Ki67 (Figure 1). However, approximately 20% of the PD1-positive follicular T cells expressed BCL6 (Figure 1) (although the proportion of BCL6-positive cells varied between germinal centers). The pattern of staining observed for the adaptor molecule SAP (Figure 2) was very similar to the pattern observed for PD-1, and double staining for the two markers showed essentially identical labeling within germinal centers. In extrafollicular regions SAP was expressed by occasional T cells that were PD-1-negative (Figure 2).

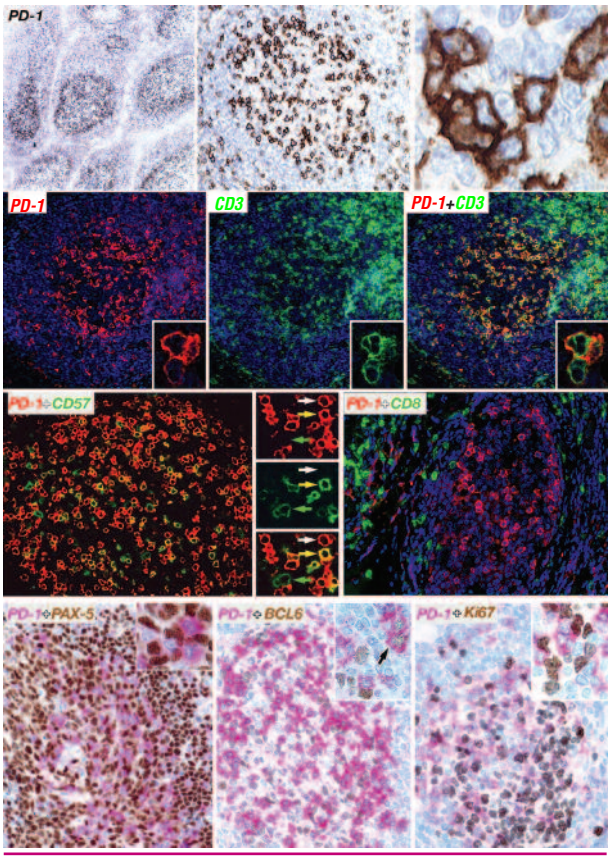

Figure 1. Expression of PD-1 by lymphoid cells. *First row*: Immunoperoxidase staining of human tonsil (seen at low, intermediate and high power) shows strong labeling of many cells within germinal centers. *Second row*: Double immunofluorescent staining shows that many of the PD-1-positive cells (red) in the germinal center co-express CD3 (green), and that very few PD-1-positive cells are found in the T-cell-rich area outside the follicle. The inset shows germinal center T cells co-expressing PD-1 and CD3. *Third row* (left): Double labeling for PD-1 (red) and CD57 (green) in a germinal center (left) shows that many cells express PD-1 alone (white arrow) and that a minority express both markers (yellow arrow) or CD57 (green arrow) alone. Double labeling for PD-1 (red) and CD8 (green) shows reciprocal expression of the two molecules in intrafollicular T cells (right). *Fourth row*: Double immunoenzymatic staining shows (left) the reciprocal expression of PD-1 (red) and PAX-5 (nuclear, brown) in B cells, and also that a proportion of PD-1-positive germinal center T cells (red) express nuclear BCL6 (brown - example arrowed), and (right) that they lack the proliferation marker Ki-67.

Immunostaining of neoplastic lymphoid tissue

Neoplastic cells. Among T-cell neoplasms, PD-1 and SAP were expressed over three quarters of the cases of AITL tested (42/49 and 59/69 cases, respectively) (Table 2 and Figure 3). The two markers were expressed independently of each other, with the result that almost 95% of cases (40/42) expressed at least one of these markers (Tables 2 and 4). In addition to angioimmunoblastic lymphoma, both PD-1 and SAP were found in a minority of

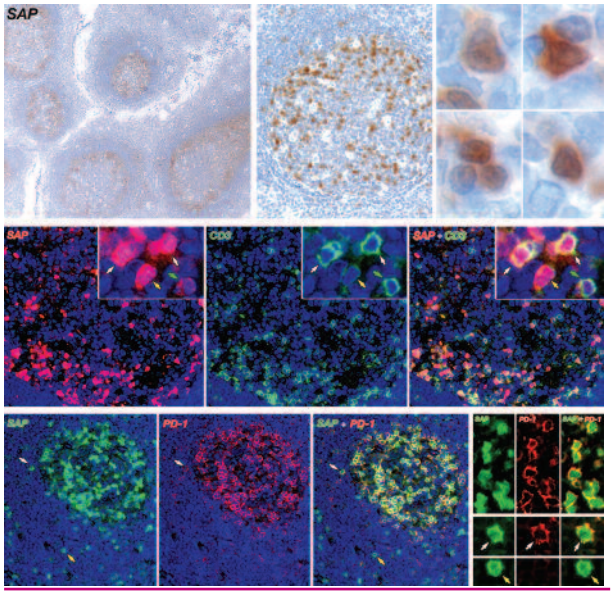

Figure 2. SAP expression in normal human tonsil. *Upper row*: Immunperoxidase staining (viewed at low, intermediate and high magnification) shows a population of strongly stained cells within germinal centers with cytoplasmic and nuclear labeling. *Second row*: Double immunofluorescent staining for SAP (red) and CD3 (green) shows that many cells co-express these markers (white arrows). Cells expressing SAP with little or no CD3 are also present (yellow arrows) together with SAP-weak/negative CD3-positive cells (green arrows). *Third row*: Double immunofluorescence labeling for SAP (green) and PD-1 (red) shows that many cells in the germinal center co-express both molecules. Examples of double positive germinal center cells are shown at higher magnification on the far right (upper panels). However, scattered cells lying outside the germinal center express SAP alone (yellow arrow) (see also higher magnification view of two of these cells on the far right (lower panels). The white arrow indicates a double stained cell outside the germinal center that has probably migrated from this site.

Table 1. Immunostaining patterns of PD-1 and SAP in normal lymphoid tissue.

Marker	*Tissue staining distribution*			*Subcellular localization pattern*
	Tonsil	*Thymus*	*Spleen*	
PD-1	Germinal center T cells. Scattered extrafollicular cells, usually more weakly stained.	Scattered cells in the medulla.	T cells in lymphoid areas. Rare cells in the red pulp.	Membrane-associated.
SAP	Germinal center T cells. Scattered extrafollicular cells, usually more weakly stained.	Majority of cortical thymocytes. Scattered cells in the medulla.	T cells in lymphoid areas. Scattered cells in the red pulp.	Cytoplasmic, sometimes nuclear.

Table 2. Immunostaining of PD-1 and SAP in lymphoid neoplasms (positive cases/ total cases).

	PD-1	*SAP*
Lymphoma/leukemia type		
T/NK cell non-Hodgkin's		
Lymphoblastic (T)	0/20	15/21
Peripheral	5/30[a]	13[d]/37
Intestinal	1/10	3[e]/10
Angioimmunoblastic T cell (AITL)	42/49	59/69
Natural killer (NK)	1[b]/8	3/5
Mycosis fungoides	5/9	0/6[f]
ALK-positive	0/4	1[g]/13
ALK-negative, ALCL	0/1	1[h]/7
B cell non-Hodgkin's		
Lymphoblastic (B)	0/10	0/11
Chronic lymphocytic (CLL)	0/13[c]	0/20
Mantle cell	0/14	0/20
Follicular (Grade 1, 2, 3)	0/70	0/114
Burkitt's	0/21	0/14
Diffuse large	3/98	3[i]/115
Marginal zone (nodal and splenic)	0/14	0/23
MALT	0/8	0/12
Hairy cell	0/1	0/1
Myeloma/plasmacytoma	0/2	0/10
Hodgkin's		
Classical	0/18	1/21
Lymphocyte predominant	0/11	4[j]/14

aTwo out of 30 cases, in which only small clusters of atypical cells were positive, were scored as negative; b In this case approximately 50% of the tumor cells were positive; c In six out of 13 cases, PD-1-positive large cells were found in proliferation centers (but the cases were scored as negative); d One of the 13 cases showed only weak staining; e In one of these three cases only 50% of tumor cells were weakly positive; f In three out of six cases scored as negative, up to 10% of tumor cells were positive; g The tumor cells were weakly positive in this case; hThe tumor cells were very weakly positive in this case; i In two out of the three cases, approximately 60-70% of the tumor cells were very weakly positive; j In these four cases weak immunostaining was seen in approximately 50% of the tumor cells.

cases of peripheral T-cell lymphoma (5/30 and 13/37, respectively) (Table 2, Figure 3). Twenty-two cases that had been investigated for both markers were reviewed, and two cases, each of which was positive for both PD-1 and SAP - Table 3, showed some features consistent with atypical AITL, but a further six cases (two double positive and four positive for SAP alone) appeared to be classic examples of peripheral T-cell lymphoma. Among the samples tested for only marker, a further six cases expressing PD-1 or SAP were identified, and two of these (one PD-1-positive, one SAP-positive) also had some features on review suggestive of possible AITL. All cases of AITL were immunostained for the CXCL13 chemokine, and many cases contained CXCL13-positive neoplastic cells. However, background extracellular staining was often present and labeling in many positive cells was restricted to a single intracytoplasmic dot. In consequence, evaluation of immunostaining in the majority of cases was more difficult than for PD-1 and SAP.

SAP was expressed in 15/21 cases of T lymphoblastic lymphoma (Table 2 and Figure 3) (whereas PD-1was not found in this tumor type). In contrast, PD-1 was found in 5/9 cases of mycosis fungoides, whereas SAP expression was limited to a small minority of the neoplastic cells in 3/6 cases (Table 2 and Figure 3). SAP and PD-1 were absent from almost all of the non-Hodgkin's B cell neoplasms investigated (Table 2). PD-1 was found on the tumor cells in three diffuse large B-cell lymphomas (out of 98) and was also present in a minority of larger cells in cases of chronic lymphocytic leukemia/lymphoma (CLL). In six out of the 13 CLL cases, proliferation centers were recognizable and it was clear that the

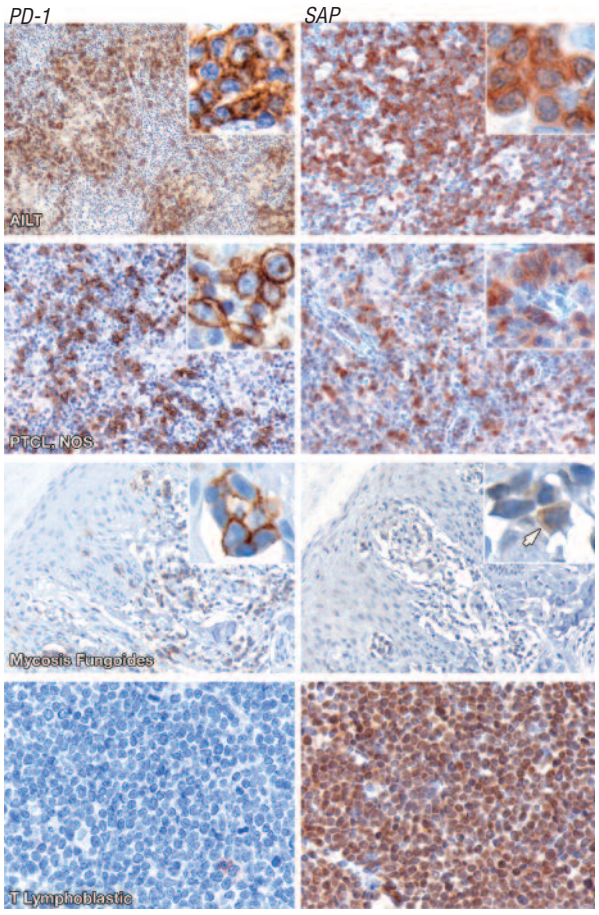

Figure 3. Immunostaining of T cell lymphomas for PD-1 and SAP. *Top row*: Two angioimmunoblastic T-cell lymphomas (AITL) were positive for both markers. *Second row*: a case of peripheral T cell lymphoma expresses PD-1 and SAP. *Third row*: neoplastic cells in a case of mycosis fungoides express PD-1 (inset, high magnification) but are negative for SAP, with the exception of a few weakly positive cells (arrowed) (inset, high magnification). *Fourth row*: a case of T lymphoblastic lymphoma shows extensive nuclear staining for SAP.

Table 3. Correlation between PD-1 and SAP expression in T-cell lymphomas (positive cases/total cases).

Lymphoma/ leukemia type	*PD-1-pos/ SAP-pos*	*PD-1-pos/ SAP-neg*	*PD-1-neg/ SAP-pos*	*PD-1-neg/ SAP-neg*
T lymphoblastic	–	–	9/13	4/13
Peripheral T-cell lymphoma	4/22	–	4/22	14/22
Intestinal	-	1/10	3/10	6/10
Angioimmunoblastic T cell lymphoma	31/42	4/42	5/42	2/42
Natural killer (NK)	–	1^/3	2/3	–
Mycosis fungoides	–	4/5	–	1/5
ALK-positive	–	–	1*/3	2/3
ALK-negative	–	–	1$/1	–

*^Approximately 50% of the tumor cells were positive; * The tumor cells were weakly positive; $ The tumor cells were very weakly positive.*

large PD-1-positive cells belonged to these structures. SAP was absent from all cases of non-Hodgkin's B cell

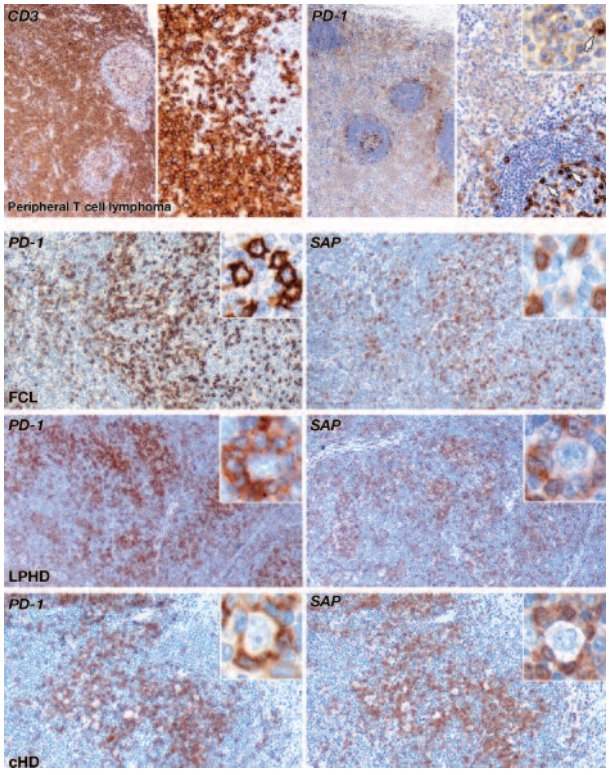

Figure 4. PD-1 and/or SAP in a case of peripheral T-cell lymphoma and on infiltrating cells in B-cell lymphomas. *Top row, left*: The positive CD3 staining of tumor cells in a case diagnosed as typical peripheral T-cell lymphoma highlights its interfollicular growth pattern and the preservation of lymphoid follicles. *Top row, right*: the tumor cells in the same case show weak to moderate expression of PD-1. Note the difference in terms of intensity between the strongly positive normal T cells (arrowed) in a germinal center and the weaker staining of tumor cells (as also illustrated at high magnification in the inset) adjacent to the unstained mantle zone cells. *Second row*: in a case of follicular lymphoma, many PD-1- and SAP-positive cells are seen in a neoplastic follicle. *Third row*: in a case of lymphocyte predominant Hodgkin's disease (LPHD) many reactive lymphocytes are PD-1- and SAP-positive. The insets show cells expressing these markers forming rosettes surrounding the tumor cells. *Third row*: in a case of classical Hodgkin's disease (cHD) PD-1 and SAP are seen in many reactive lymphocytes, and the insets show neoplastic cells surrounded by rosettes of PD-1- and SAP-positive T cells. However, this case is an exception, since the majority of classical Hodgkin's disease biopsies contained only low numbers of PD-1/SAP-positive cells.

lymphoma, with the exception of three cases (out of 115) of diffuse large B-cell lymphoma (one of these three cases was PD-1-negative, the other two were not evaluated for PD-1) and proliferation centers in CLL (although the staining was weaker than for PD-1 and the number of SAP-positive cells was lower). Moreover, weak labeling of neoplastic cells for SAP was observed in four out of 14 cases of lymphocyte predominant Hodgkin's disease (in approximately just over half of the tumor cells). Reed-Sternberg cells were SAP-negative with the exception of one case (out of 21) of classical Hodgkin's disease in which approximately 50% of the tumor cells were weakly to moderately positive.

Infiltrating cells

In all studied B-cell lymphomas, occasional non-neoplastic cells carrying PD-1 and SAP were observed. The highest percentage of PD-1- and SAP-positive infiltrating cells was found in follicular lymphomas, lymphocyte predominant Hodgkin's disease (Figure 4) and in some cases of diffuse large B-cell lymphoma. In the first disease, the number of these infiltrating cells varied widely from case to case and was independent of histologic grade. In lymphocyte predominant Hodgkin's disease, PD-1 and SAP were positive in infiltrating cells, and in many cases these cells formed rosettes surrounding the neoplastic cells (Figure 4). In contrast, in classical Hodgkin's disease, PD-1-positive or SAP-positive rosettes were found in only a minority of cases (Figure 4).

Western blotting

Western blotting of cell lysates from the YT line with the anti-PD-1 antibody NAT showed a single band with a molecular weight of approximately 47 kDa (Figure 5). Anti-SAP was tested against the YT line and also against a number of T-cell lymphoblastic cell lines (Jurkat, CCRF/CEM and Molt-4). A band of approximately 15 kDa was found in each of these, accompanied in the case of Jurkat and CCRF/CEM with a second band of slightly smaller size (Figure 5). In contrast, the ALK-positive lymphoma cell line Karpas 299 was SAP-negative. β-actin was used as a protein loading control and was present as a band of comparable intensity in each cell line.

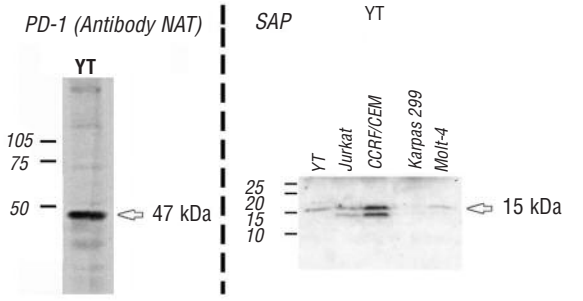

Figure 5. Western blotting analysis of antibody NAT and anti-SAP in lymphoid cell lines. Blotting with the former antibody in the NK cell-derived cell line YT shows a band with a molecular weight of approximately 47 kDa. SAP is detectable as a 15 kDa band in the YT line and also in three T-cell lymphoblastic cell lines (Jurkat, CCRF/CEM and Molt-4). Karpas 299 (an ALK-positive lymphoma cell line) is negative. A second band, slightly smaller than the 15 kDa band, is also visible in the Jurkat and CCRF/CEM cell lines.

Discussion

It was reported in the 1980s that T cells within germinal centers express CD57 (also known as HNK-1 or Leu-7),[12,13] and subsequently that they are helper T cells carrying the chemokine receptor CXCR5.[1-3] In 2004 two laboratories reported that follicular T cells show unique patterns of gene expression compared to other helper T cell subsets.

At least three of the molecules shown in these gene expression studies to be upregulated, in follicular T cells, i.e. BCL6, CXCL13 and PD-1 (CD279), can be detected by immunohistologic labeling of paraffin sections.[8,10,11,14,15] In the present paper we report that an additional molecule upregulated in follicular T cells,[4] namely SAP (SLAM-associated protein, also known as SH2D1A) can also be detected at the protein level in paraffin-embedded tissue. SAP is essential in T cells for providing late B-cell help and the establishment of long-term humoral immunity,[16] and its clinical importance is demonstrated by the fatal lymphoproliferative disorder that frequently develops in patients with mutations in this X-linked gene.[17,18]

Double labeling for SAP in combination with PD-1, a known marker of germinal center T cells, confirmed that these two molecules are usually co-expressed in the same cells, although the presence of scattered SAP-positive, PD-1-negative T cells in interfollicular areas suggested that SAP is induced slightly in advance of PD-1, before helper T cells enter the germinal center. Approximately one fifth of SAP- and PD-1-positive follicular T cells co-expressed BCL6, in keeping with previous reports that a subset of intrafollicular T cells express this transcription factor.[10,19,20] However, it was of interest that there was only limited overlap between SAP/PD-1 expression and that of CD57. Microarray analysis has shown that CD57-positive and -negative follicular helper T cells are very similar in terms of gene expression,[5] but it appears from the present work that SAP and PD-1 tend to be preferentially expressed by CD57-negative germinal center T cells.

Among T-cell lymphomas, SAP and/or PD-1 were expressed by neoplastic cells in the majority of cases of AITL. This provides independent support for earlier proposals that this neoplasm arises from germinal center T cell.[10,14,20-23] The unique clinical and pathologic features of this disorder are fully in keeping with an origin from germinal center T cells, given the potent physiologic influence of these cells on the maturation of germinal center B cells. It should be added that not all AITL cases in this study expressed SAP and/or PD-1. This is in disagreement with a recent report[11] that PD-1 is expressed in *all* cases (23/23) of AITL. However, our findings are comparable to the observations relating to another marker of germinal center T cells (CXCL13) which was reported by Grogg *et al.*[14,15] to be absent in at least 10% of AITL. Furthermore, CD10 is also absent in a minority of cases.[22,24]

We also observed that SAP and/or PD-1 are expressed in a minority of T-cell lymphomas other than AITL. This can be interpreted as indicating that these cases do

indeed derive from germinal center T cells but that they lack for some reason typical clinicopathologic features of AITL. In keeping with this view, Dupuis *et al*. reported that CXCL13 was expressed in a minority (6/20) of peripheral T-cell lymphomas, and then noted that these six cases showed some morphologic similarities to AITL on review.[22] They therefore argued that CXCL13 may identify an *AITL-like* subgroup of peripheral T-cell lymphoma. Similarly, Grogg *et al*. also observed CXCL13 expression in 8/26 cases classified as peripheral T-cell lymphoma, and found that on review six could be reclassified as AITL.[14] However, the other two cases showed no angioimmunoblastic features, and this is in keeping with our own observations that two out of eight cases of peripheral T-cell lymphomas expressing SAP (with or without PD-1) showed features suggestive of AITL on review, but that the remainder appeared to be classical peripheral T-cell neoplasms. The same tendency was seen in cases in which only one of the two markers were evaluated (*see Results*). The remaining SAP/PD-1-positive peripheral T-cell lymphoma cases showed features associated with AITL (although evaluation of some cases was limited, e.g. because of lack of immunostaining for review, or a small sized sample in a tissue array). Furthermore, we observed expression of PD-1 in a minority of cases of mycosis fungoides, a cutaneous neoplasm that is unlikely to arise from germinal center helper T cells.

There is thus an alternative to the view that the expression of SAP and/or PD-1 (or of markers such as CXCL13) indicates a derivation from germinal center T cells (even when histologic features of AITL are absent), namely that these markers might occasionally be aberrantly upregulated in non-germinal center T cells as a result of neoplastic transformation. Follicular T cells probably do not represent a distinct lineage but rather a subpopulation of helper T cells that upregulate a range of inducible markers (e.g. SAP, PD-1, BCL6, ICOS) when they enter the germinal center. Furthermore, a minority of cells that lie outside this site express these inducible markers (usually at lower levels). Consequently, these molecules are within the potential repertoire of molecules that helper T cells can express and their occasional expression in peripheral T-cell neoplasms other than AITL may, therefore, not indicate an origin from classical germinal center T cells.

We observed SAP expression in more than two-thirds of the cases of acute lymphoblastic leukemia of T-cell origin (and in three cell lines arising from this disorder). This is in keeping with our observation that cortical thymocytes contain this protein, a finding that raises the question of its physiologic role in immature T cells. SAP and PD-1 were not expressed in the great majority of B-cell neoplasms. It has been reported in the past that SAP is expressed, as assessed by flow cytometry, in about 15% of tonsillar B cells[25] and it is clear that the molecule can be detected in B-cell lines and in lines derived from Hodgkin's disease and from Burkitt's lymphoma.[25-59] In the latter category, SAP expression was associated with the presence of Epstein-Barr virus. In contrast, the only data on SAP expression in biopsied lymphoma samples are found in a report by Mikhalap *et al*.[30] who stated that SAP was detectable in 12/12 cases of diffuse large B-cell lymphoma and in 14 cases of Hodgkin's disease (but not other categories of lymphoma) according to immunohistologic staining of paraffin-embedded biopsies. This is in discordance with our own observations that only three out of 115 cases of diffuse large cell lymphoma were SAP-positive, and that SAP was found (in only a proportion of the neoplastic cells) in 4/14 cases of lymphocyte predominant Hodgkin's disease (weakly), and in 1/21 cases of classical Hodgkin's disease. These observations are, however, in keeping with a paper reporting that SAP transcripts are not expressed in diffuse large B-cell lymphoma but are detectable in peripheral T-cell lymphoma,[31] and with a report from Sanchez Aguilera *et al*.[32] that SAP expression by neoplastic cells was not observed in classical Hodgkin's disease.

The other marker investigated in this study (PD-1) was not detected in cases of B-cell neoplasia in a study by Dorfman *et al*.,[11] and this is in keeping with our own observation that PD-1 was detectable in only 3/98 diffuse large B-cell lymphomas (out of a total of more than 250 samples covering the spectrum of B-cell lymphomas). However, it was of interest that PD-1 (and, to a lesser degree, SAP) was detectable in cells in the proliferation centers seen in cases of chronic lymphocytic leukemia. It has been reported that mRNA encoding SAP (but not protein) is found in mouse B cells[26,33,34] and human memory B cells,[35] but the degree to which PD-1 and SAP play a role in B cells requires further study.

In conclusion, our observations provide additional support for the idea that AITL derives from intrafollicular T cells since the great majority of cases expressed SAP and/or PD-1 (two molecules selectively expressed by this type of T cell). The histologic diagnosis of AITL is notoriously difficult,[9] as evidenced in one study in which 50% of cases referred to a specialist center had been misdiagnosed,[8] and any selective immunohistologic markers are of potential value. Further studies are needed to establish the role of PD-1 and SAP in the diagnosis of this disorder, but it seems that they are clear candidates for inclusion in the panel of diagnostic markers. Further studies using these markers may help to re-define the borderline between classical AITL and cases of peripheral T-cell lymphoma that lack the classical features of AITL. In this context, it will be of interest to investigate whether PD-1, SAP and other germinal center T-cell markers are expressed by the rare cases of BCL6-positive, CD10-positive T-cell lymphomas that show a tendency to home to lymphoid follicles.[21,36,37]

Authors' Contributions

GR, J-FG and ST were responsible for most of the experimenal work; WK, SP and M.-L.H. provided tissue material. JCP and LM helped in the performance of immunohistochemistry. MAP provided cases of lymphomas and also made a major contribution to the study design; DYM and TM reviewed the immunostaining, checked and analyzed the data, and wrote the manuscript.

Conflict of Interest

The authors reported no potential conflicts of interest.

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