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Abstract

We previously reported a strong IL4I1 gene expression in primary mediastinal B-cell lymphoma (PMBL) and recently identified the protein as a secreted L-phenylalanine oxidase, physiologically expressed by myeloid cells, which inhibits T cell proliferation in vitro. Here, we analyzed the pattern of IL4I1 protein expression in 315 human lymphoid and non lymphoid malignancies. Besides PMBL, IL4I1 expression in tumors was very frequent. IL4I1 was detected in tumor-associated macrophages from most of the tumors and in neoplastic cells from follicular lymphoma, classic and nodular lymphocyte predominant Hodgkin lymphomas and small lymphocytic lymphoma, three of which are germinal center-derived. IL4I1-positive tumor cells were also detected in rare cases of solid cancers, mainly mesothelioma. The enzymatic activity paralleled protein expression, suggesting that IL4I1 is functional in vivo. Depending on the tumor type, IL4I1 may impact on different infiltrating lymphocyte populations with consequences on tumor evolution. In the particular case of follicular lymphoma cells, which are susceptible to antitumor cytotoxic T cells killing but depend on interactions with local T helper cells for survival, a high level of IL4I1 expression seems associated with the absence of bone marrow involvement and a better outcome. These findings plead for an evaluation of IL4I1 as a prognosis factor.

MESH Keywords B-Lymphocytes ; enzymology ; Cohort Studies ; Enzyme-Linked Immunosorbent Assay ; Female ; Germinal Center ; enzymology ; pathology ; Humans ; Immunoenzyme Techniques ; L-Amino Acid Oxidase ; metabolism ; Lymphoma, B-Cell ; enzymology ; pathology ; Macrophages ; enzymology ; pathology ; Male ; Middle Aged ; Neoplasms ; enzymology ; pathology ; Neoplastic Cells, Circulating ; pathology ; Tumor Cells, Cultured

Author Keywords lymphoma ; cancer ; immunosuppression ; myeloid-derived suppressor cells ; tumour-associated macrophages.

INTRODUCTION

An immediate-early IL4-inducible gene called Interleukin Four Induced Gene 1 (IL4I1) was first described in the mouse and has subsequently been characterized in human B cells. IL4I1 mRNA expression is restricted to lymphoid tissues and induced by IL4. We have demonstrated that high expression levels of this gene is characteristic of Primary Mediastinal Large B cell Lymphoma (PMBL), a specific subtype of diffuse large B cell lymphoma (1). More recently, we have shown that the IL4I1 protein is a secreted L-amino acid oxidase which inhibits T cell proliferation through phenylalanine degradation in vitro. Moreover, we have observed high IL4I1 expression in antigen-presenting cells of myeloid origin (macrophages and dendritic cells) from normal and inflammatory lymphoid tissue (2).

These observations highlight similarities between IL4I1 and other amino acid-catabolizing enzymes, such as Indoleamine-2,3-dioxygenase (IDO), Arginase1 and Inducible Nitric Oxid Synthase, all associated with the inhibition of T cell responses. The expression of these enzymes has been demonstrated in several tumor types, both in the malignant cell and/or the microenvironment. Tumors produce several soluble factors responsible for aberrant myelopoiesis, which result in the differentiation of myeloid cells with immunosuppressive properties called myeloid-derived suppressive cells (MDSC) which are comprised of monocytes, dendritic cells, polymorphonucleocytes and immature progenitors (3–5). In cancer-bearing individuals, MDSC are expanded in the blood and secondary lymphoid tissue and can be recruited to the tumor bed via chemokines such as CCL2 where some differentiate into tumor-associated macrophages (TAM) (6). Different types of leukocytes infiltrate tumors and can impact on local immune response, but TAMs predominate and their number is associated with poor prognosis in several types of cancer including lymphoma (7–9). The principle TAM and MDSC cancer promoting mechanisms consists of the expression of amino-acid catabolizing enzymes allowing tumor escape from the immune response (10). In line with this, observations of the negative impact of IDO expression on survival in pre-clinical models (11) have lead to the development of a specific inhibitor which is currently under clinical investigation as an adjuvant therapy (12).

Aside from IL4I1 mRNA overexpression in PMBL, little is known about IL4I1 expression in human tumors and their microenvironment. In this work, we describe for the first time the pattern of IL4I1 protein expression in a large set of human cancers including lymphoma and verify IL4I1 activity in some tumoral samples. We confirmed the expression at the protein level in PMBL. We also found expression in other B cell lymphomas, in particular those known to derive from germinal-center B cells. Moreover, the presence of an inflammatory infiltrate was generally associated with the detection of IL4I1-positive macrophages, both in solid tumors and lymphomas. Finally, we selected follicular lymphoma for an evaluation of the correlations between IL4I1 expression and clinical parameters, since IL4I1 expression varies quantitatively both in the TAM and in the tumor cell populations in this pathology. In our small cohort, a high level of IL4I1 expression was associated with characteristics predictive of better prognosis, pleading for a careful evaluation of IL4I1 impact on both non lymphoid and lymphoid cancer prognosis.

MATERIAL AND METHODS

Histological samples

Three hundred fifteen cases consisting of malignant solid tumors (n=121) and lymphoid malignancies (n=194) classified according to the World Health Organization criteria were retrieved from the files of the Departments of Pathology of Henri Mondor hospital and Intercommunal hospital, Créteil, France. Non-lymphoid malignancies included colorectal carcinomas (n =13), gastric carcinomas (n =5), non small-cell lung carcinomas (n=13), small-cell lung carcinomas (n=2), bladder carcinoma (n=7), ovarian carcinomas (n=3), mesotheliomas (n=10), renal cell carcinomas (n=11), melanomas (n=4), breast carcinomas (n=11), thyroid carcinomas (n=6), hepatocellular and/or cholangiocellular carcinomas (n=6), testicular germ cell tumors (n=10), prostatic carcinomas (n=4), soft tissues sarcomas (n=16). The 194 cases of B- and T/NK-cell neoplasms comprised 28 PMBL, 36 diffuse large B cell lymphoma (DLBCL) of germinal center and non-germinal center cell type, 10 small lymphocytic lymphoma/chronic lymphoid leukemia (SLL/CLL), 12 marginal zone lymphoma (MZL), 36 follicular lymphoma, 24 classical Hodgkin lymphoma (cHL) and 11 nodular lymphocyte predominant Hodgkin lymphoma (NLPHL). In addition, for comparison, twenty-five samples of reactive lymphoid tissues were selected (1 tonsil, 4 spleens, 18 reactive lymph nodes and 2 neonatal thymuses).

Material consisted of routinely-fixed pathological specimens. Most tumor samples analyzed were issued from tissue microarrays constructed with 2 to 3 representative 0.6-mm tissue cores taken from the initial tumor sample using a Beecher instrument. Paraffin-embedded tissue sections obtained from conventional paraffin blocks and from tissue microarrays blocks were stained with hematoxylin-eosin for histological studies.

The present study was approved by the French ethical committee “Comité de Protection des Personnes” (CPP) Ile de France IX.

Cell lines and cell suspensions

The Lymphoma cell lines L428, KM-H2 and SU-DHL-4 were purchased from DSMZ (Braunschweig, Germany). L428 were cultivated in ISCOVE supplemented with 20% fetal calf serum, L-glutamine, non essential amino acids and antibiotics. KM-H2 and SU-DHL-4 were cultivated in RPMI 1640 supplemented with 20% fetal calf serum, L-glutamine and antibiotics.

For enzymatic activity studies, viable cryopreserved cells from 5 follicular lymphomas, 1 MZL and 2 traumatic normal spleens were thawed and used to generate whole cell lysate or submitted to magnetic cell sorting using anti-human CD19 MACS beads according to the manufacturer's instructions (Miltenyi Biotec, Paris, France).

Immunohistochemistry

All lymphoma cases had been evaluated for B- and T-cell differentiation antigens and other appropriate antigens, by immunohistochemistry performed on 3 μm paraffin-embedded tissue sections using the corresponding diaminobenzidine detection kit on the Ventana automated immunostainer (Nexes, Ventana Medical Systems, Tucson, AZ, USA), according to the manufacturer's recommendations.

Deparaffinized tissue sections were evaluated for IL4I1 protein expression using anti-IL4I1 mAb antibody (1/400 dilution, rabbit polyclonal ab18524, Abcam, Cambridge, United Kingdom) and an indirect immunoperoxidase method (ImmPRESS, Vector Laboratories, Burlingame USA) after antigen retrieval performed by water bath heating in EDTA buffer pH8. In lymphoma tissue samples, internal positive controls consisted of positive myeloid cells (tingible-bodies macrophages and/or histiocytes). IL4I1 protein expression was analyzed in tumor and stromal cells. A semi-quantitative evaluation was performed to assess the density of IL4I1-positive tumor cells (<10 %, 10–50%, >50% of total tumor cells) and the percentage of IL4I1-positive stromal cells (<10%, 10–50%, >50% of total stromal cells, which were considered as TAM after CD68 or CD14 staining). Cases with few IL4I1-positive cells were scored as negative.

For double immunostaining, first antigen retrieval was performed by water bath heating in citrate buffer pH6 (Dako). Slides were then incubated 10 min with horse serum (Vectastain kit, Vector Laboratories, Burlingame USA), then incubated 1 hour with anti- CD14 (diluted 1/50, mouse monoclonal Ab-2, clone7, Labvision, Fremont, CA, USA), CD68 (diluted 1/50, clone KP1, Dako, Glustrup, Denmark) or CD30 (diluted 1/30, clone Ber-H2, Dako, Glustrup, Denmark) antibodies and labelled using the ABC procedure (biotinylated anti-mouse IgG followed by streptavidin-conjugated alkaline phosphatase, Vectastain kit, Vector Laboratories, Burlingame USA). In a second step, after microwave heating in EDTA buffer pH8 (5min at 750W), slides were incubated 1 hour with anti-IL4I1 antibody (diluted 1/400) followed by anti-rabbit peroxydase-conjugated antibody (ImmPRESS, Vector Laboratories, Burlingame USA).

Images were captured with a Zeiss Axioskop2 microscope (Zeiss, Oberkochen, Germany) and Neofluar 100x/0.1 NA optical lenses (Zeiss). Photographs were taken with a DP70 Olympus camera (Olympus, Tokyo, Japan). Image acquisition was performed with Olympus DP Controller 2002, and images were processed with Adobe Photoshop v7.0 (Adope Systems, San Jose, CA).

Enzymatic activity measurement

IL4I1 activity was measured on 10^6 total lymph node cells, splenocytes or sorted cell populations. For cell lines, the activity was measured on 10^5 cells. Lysates in Phosphate Buffer Saline containing protease inhibitors (Complete mini, Roche, Meylan, France) were obtained by 3 freeze-thaw cycles. IL4I1 phenylalanine oxidative deamination was performed against phenylalanine as in (2). Fluorimetric quantification of H_2O_2 produced by the enzymatic reaction was performed by Ultra Amplex Red (Invitrogen, Cergy-Pontoise, France) oxidation analysis using an Optima Fluostar plate reader (BMG Labtech, Champigny, France). For secreted activity, cells were plated in PBS containing 5% fetal calf serum (2.5×10^5 cells in 100 µl) and 10X reaction mix was directly added to the cells.

Follicular lymphoma clinical data and statistical analysis

Specific clinical information and follow-up were available in 23 follicular lymphoma cases (table 3). The correlation between IL4I1 expression in tumor cells and/or in the microenvironment and the bone marrow involvement was evaluated using the Fisher's exact test for binary parameters after tabulation in contingency tables (Staview, version 5, SAS Institute Inc., Cary, NC, USA). The correlation between IL4I1 expression and Follicular Lymphoma International Prognosis Index (FLIPI) was similarly performed after stratification of patients in low risk (FLIPI 0-1) and intermediate-high risk (FLIPI 2-5).

RESULTS

IL4I1 expression in non lymphoid malignancies

IL4I1 protein expression was analyzed in 121 cases of solid tumors (Table 1). Expression in tumor cells was observed in only 9 cases (7.4%), including 5/10 mesotheliomas, 2/13 non small-cell carcinomas, 1/6 thyroid carcinoma and 1/3 ovarian carcinoma. In tumor cells, IL4I1 immunoreactivity consisted of intracytoplasmic granular staining. Among these 9 cases, the percentage of positive tumor cells was heterogeneous. One mesothelioma and one ovarian carcinoma (figure 1a) showed high IL4I1 expression in more than 50% of the malignant cells whereas the other cases demonstrated usually less than 10% positive tumor cell expression. In all of them, IL4I1 staining was also detectable in a variable percentage of the infiltrating histiocytes. Interestingly, the IL4I1-positive ovarian carcinoma was characterized by an intense intratumoral granulomatous reaction and numerous IL4I1-positive epithelioid and giant cells were observed (figure 1b). Moreover, in one case of thyroid carcinoma, IL4I1 expression in tumor cells was focal and restricted to an inflammatory area with calcifications (figure 1c). Both of these observations suggest a possible role for inflammatory processes in the induction of IL4I1 expression.

In the remaining 112 cases, IL4I1 protein expression was either scored as negative on both malignant cells and stromal cells (n= 55) or in most cases IL4I1 expression was restricted to large cells of the tumor microenvironment, indicative of histiocytes (n=57). Negative cases were more frequent on tissue microarrays which were sampled in the tumor-cell rich zone and may not contain an inflammatory infiltrate. Double immunostaining with anti-CD68 and anti-IL4I1 antibodies was difficult to interpret because of superposition of the granular structures containing the two antigens. However, the percentage of IL4I1 positive reactive cells usually correlated with the density of CD68 positive cells and there was no evidence of IL4I1 expression in reactive small lymphocytes. Moreover, the use of an antibody directed against the membrane antigen CD14 confirmed that all of these cells were of monocyte/macrophage origin (figure 1a inset). The percentage of IL4I1-positive TAM was variable and in 72% of the cases, less than 10% were positive. The highest proportion of IL4I1-positive TAM was seen in non small-cell lung carcinomas (figure 1d), mesotheliomas, colorectal carcinomas and testicular germ cell tumors.

IL4I1 protein expression in reactive lymphoid tissue and lymphoid malignancies

In reactive lymph nodes and hyperplastic tonsils, IL4I1 expression was observed in tingible bodies macrophages located in the germinal centers of lymphoid follicles (figure 2a). In addition, scattered positive histiocytes were found in interfollicular areas. In reactive lymph nodes with peripheral sinusal histiocytosis, numerous IL4I1-positive histiocytes were observed in the sinuses (previously reported

in ref (2)). No expression was observed in centrocytes and centroblasts of the germinal centers. In the spleen, tingible bodies macrophages of germinal centers of lymphoid follicles located in the white pulp were positive as well as occasional histiocytes of the red pulp. In the thymus, scattered IL4I1-positive histiocytes were observed in the medulla.

Among lymphomas, no significant expression of IL4I1 was observed in tumor cells of T/NK neoplasms (Table 2). In contrast to non lymphoid malignancies and T/NK lymphomas, IL4I1 expression was observed in tumor cells of several B-cell lymphoma subtypes. In accordance with our previous study (1), most PMBL (89%) were positive for IL4I1 with strong intracytoplasmic staining in up to 100% of the tumor cells (range 10%–100%) (Figure 2b). In contrast to PMBL, most non-mediastinal DLBCL were IL4I1-negative, with only a minority of cases (17%) showing occasional IL4I1-positive tumor cells (less than 10% tumor cells, figure 2c). There was no correlation between the IL4I1 expression by malignant cells and the germinal center or non-germinal center phenotype of DLBCL established according to Hans algorithm.

Among small B-cell lymphomas, neoplastic cells of MZL, mantle cell lymphoma and plasmacytoma/myeloma did not express IL4I1, while 64% of follicular lymphomas were IL4I1- positive with a percentage of positive tumor cells ranging from 10% to 80%. Positive tumor cells were located predominantly in the intrafollicular areas (figure 2d) except for one case of follicular lymphoma with marginal zone differentiation where cells were mainly observed in the perifollicular marginal zone of neoplastic follicles (figure 2e). No correlation between IL4I1 staining and the histological grade of the follicular lymphomas was found. In small lymphocytic lymphoma/CLL, 40% (4 out of 10) of the cases were positive and interestingly this positivity tended to be restricted to paraimmunoblasts of proliferation centers (figure 2f).

Among Hodgkin lymphomas, 42% (10 out of 24) of cHL displayed IL4I1-positive Hodgkin and Reed Sternberg cells (HRS). Double immunostaining for IL4I1 and CD30 showed CD30-positive HRS with a paranuclear granular IL4I1 staining (figure 2g). The percentage of IL4I1 expressing HRS ranged from 10% to 70%. IL4I1 expression in cHL was not correlated with the EBV status of HRS. In addition, most NLPHL (91%) contained IL4I1- positive tumor cells (figure 2h).

In the tumor microenvironment, a variable proportion of IL4I1-positive cells resembling histiocytes was observed in all lymphoma subtypes. cHL and NLPHL showed particularly high expression of IL4I1 in histiocytes, with a common strong granular staining. Double immunofluorescent staining with IL4I1 and CD5, CD20 or CD68 confirmed the exclusive labelling of macrophages (data not shown). Notably, an inverse correlation was observed in cHL between IL4I1 expression in HRS and infiltrating TAM, respectively. Indeed, cHL with a high number of IL4I1-positive HRS displayed few positive TAM whereas cHL with low/negative HRS presented many IL4I1-positive TAM.

IL4I1 activity in reactive lymphoid tissues, lymphoid malignancies and Hodgkin lymphoma-derived cell lines

To confirm the functionality of IL4I1 detected by immunohistochemistry, we developed a highly sensitive method to detect IL4I1 activity in vitro (figure 3). A minor but detectable activity was found in normal spleen, which could be attributed to tingible bodies macrophages. In accordance with strong IL4I1 expression revealed by immunohistochemistry, a much higher level (7 fold) of IL4I1 activity was measured in tumor cell lysates from follicular lymphoma. Both $CD19^{+}$ tumor B cells and the $CD19^{-}$ compartment displayed such activity. In contrast, negligible IL4I1 activity was detected in total tumor lysate and $CD19^{+}$ tumor MZL cells, whereas some activity could be measured in $CD19^{-}$ stromal cells. Therefore, IL4I1 activity in stromal cells can be detected in the $CD19^{-}$ cell population of both IL4I1-positive and negative lymphoma.

Data from Ma et al demonstrated that IL4I1 can be detected in the secretome of HL cell lines in vitro (13). Our immunohistochemistry results showed that HRS expresses variable levels of IL4I1. We thus compared IL4I1 activity of two HRS-derived cell lines L428 and KM-H2 to that of the DLBCL-derived cell line SU-DHL-4. We detected higher enzymatic activity in L428 and KM-H2 than in SU-DHL-4 (figure 3c & d). While L428 enzymatic activity was very strong and associated with detectable enzyme secretion, KM-H2 activity was much lower. This suggests that KM-H2 might derive from the poorly IL4I1-expressing HRS of cHL tumours presenting a strong expression by TAM, whereas L428 might derive from cHL with strongly IL4I1-positive HRS.

Overall, these results parallel the immunohistochemistry findings and demonstrate that the protein has an L-phenylalanine oxidase activity and therefore is functional in tumors.

IL4I1 expression and clinical parameters in follicular lymphoma

Baseline characteristics and follow-up were available for 23 follicular lymphoma patients (table 3). As we observed a variable level of IL4I1 expression in these patients, we classified IL4I1 staining in tumor cells and TAM as negative/low and medium/high (≤ 10% and >10 % IL4I1 positive cells among the studied population, respectively). We observed a strong relation between IL4I1 expression by tumor B cells and TAM (p=0.0003), indicating that a common stimulus might induce the production of the enzyme by both type of cells.

All patients studied had received treatment combining Rituximab and CHOP-like regimen at induction. Of note, some of them also received a Rituximab maintenance therapy as scheduled as part of a clinical trial. Patients were stratified as higher risk (FLIPI 2–5, n=15) and lower risk (FLIPI 0–1, n=8). Statistical comparison of the relation between IL4I1 expression in the tumor lymph node and progression risk evidenced a slight trend between high levels of IL4I1 and low risk FLIPI (p= 0.22 for tumor cell IL4I1, p=0.38 for TAM IL4I1, data not shown). Interestingly, patients with high expression of IL4I1 generally presented at diagnosis with a lower frequency of bone marrow involvement and relapsed less frequently (figure 4). This correlation was significant when considering bone marrow involvement and IL4I1 expression in tumor cells (figure 4A, p=0.02), as well as relapse and IL4I1 expression in TAM (figure 4D, p=0.03), although a similar tendency was observed for both cell populations (figure 4B & C). These data suggest that IL4I1 expression in tumor B cell and/or TAM might represent an additional prognosis marker in follicular lymphoma.

CONCLUSION

In this study we examined the pattern of expression of the immunosuppressive enzyme IL4I1 in a series of 315 human malignancies. In the majority of the tumors which presented a noticeable inflammatory infiltrate, we detected IL4I1 expression in the macrophage population. Moreover, in several lymphoma subtypes of B-cell origin and in rare solid tumor cases, IL4I1 was found to be expressed in the tumor cell compartment. When measured, enzymatic activity paralleled IL4I1 expression.

We confirm and strengthen here our previous observation that IL4I1 is physiologically expressed in tingible bodies macrophages from secondary lymphoid tissue (Figure 2) and that it is induced in macrophages from peripheral tissues or lymphoid organs during inflammation ((2) and unpublished data). According to these observations, tumors associated with the strongest inflammatory reaction displayed the highest percentage of IL4I1-positive cells. These cells morphologically resembled macrophages and were also positive for CD14 or CD68. Our data thus show that, in addition to previously described immunosuppressive enzymes, TAM can express IL4I1. An abundant IL4I1-positive TAM infiltrate was typically observed in mesothelioma, pulmonary adenocarcinoma, testicular germ cell tumors, colon carcinoma and most B cell lymphomas. Particularly strong expression of IL4I1 was detected in one case of ovarian carcinoma where the significant inflammatory infiltrate was accompanied by giant cells containing granulomas. Moreover, in this case, IL4I1 expression was detected in the ovarian tumor cells, suggesting a putative role for some common inducing soluble factor(s), including potentially estrogens. Indeed, recent transcriptome data indicated that IL4I1 mRNA expression could be induced in the uterus of mice by administration of ethynylestradiol (14).

In accordance with its normal pattern of expression, IL4I1 was more frequently detected in B-cell lymphoid malignancies, not only in infiltrating macrophages, but also in lymphoma cells of cHL and NLPHL, SLL/CLL, follicular lymphoma and PMBL. In follicular lymphoma, these observations were confirmed by measurement of the enzymatic activity on sorted $CD19^{+}$ and $CD19^{-}$ cell populations.

Among lymphoid malignancies, both cHL and NLPHL are characterized by an extensive inflammatory infiltrate. In cHL cases, this infiltrate classically represents more than 98% of the tumor cell content and comprises T cells, B cells, eosinophils, neutrophils, mast cells and numerous macrophages (15). Interestingly, two opposite patterns of IL4I1 expression were observed in HL. When IL4I1 was highly expressed (more than 50% of the malignant cells), the inflammatory infiltrate was mostly IL4I1-negative. On the contrary, when the HRS was IL4I1-negative or –low, nearly all the HL associated macrophages strongly expressed IL4I1. Accordingly, we detected IL4I1 enzymatic activity in two HRS-derived cell lines, with L428 displaying a high level associated with the secretion of the enzyme. The latter result confirms data obtained by Ma et al (13) and suggests that L428 may represent a suitable model of IL4I1-positive HRS. Robust IL4I1 expression by HRS and/or TAM may contribute to the anergic phenotype of the HL infiltrating T cells, in addition to previously described mechanisms such as infiltration by regulatory T cells (16).

NLPHL constitutes a rare entity characterized by atypical tumor B cells embedded in dense population of small T and B lymphocytes mixed with histiocytes. This stroma differs markedly from the cHL infiltrate by an absence of eosinophils, granulocytes and plasmocytes. However, histiocytes from NPLHL strongly express IL4I1 in the large majority of the cases. Moreover, this expression is always accompanied by a high level of IL4I1 expression in tumour cells.

We have previously demonstrated high expression of IL4I1 mRNA in PMBL, a distinct entity among DLBCL (1, 17). We now confirm its detection at the protein level in 89 % of the cases. Most tumor cells strongly express IL4I1, indicating that IL4I1 can represent a reliable marker for this type of tumor amongst DLBCL. Indeed, only scattered IL4I1-positive cells can be detected in a small percentage of non mediastinal DLBCL.

The only IL4I1-inducing cytokine in B cells known to date is IL4, which acts through stimulation of the IL4/IL13 receptor and subsequent phosphorylation of the transcription factor STAT6 (18). IL4I1 expression may thus be induced by constitutive STAT6 activation in cHL (19) and PMBL (20). Yet, in contrast to PMBL, IL4I1 expression by HRS is not consistently observed. Involvement of

IL4 in follicular lymphoma pathogenesis has been recently reported, along with Erk and STAT6 activation (21). However, we could not correlate expression of the IgE receptor CD23, a STAT6 target, by tumor cells, with IL4I1 expression (data not shown). In cHL and follicular lymphoma, IL4I1 might thus be induced by other signals in addition to the IL4/IL13/STAT6 pathway.

Classical HL, NLPHL, and follicular lymphoma display a common characteristic: the tumor B cell in all cases is supposedly derived from a germinal center B cell. This origin, although controversial, has also been suggested for PMBL, which shares several common features with cHL (22). It is tempting to speculate that the microenvironment associated with the germinal center may favor IL4I1 expression, partially via STAT6 activation. Although our immunohistochemical method did not allow detecting IL4I1 expression in normal germinal center B cells, we cannot exclude low expression at some stage of their development which might participate in the regulation of B-T helper cell interactions. However, no IL4I1 expression was detected in tumor cells from germinal-center type DLBCL and Burkitt's lymphoma. This discrepancy might be explained in the case of Burkitt's lymphoma by the presence of myc expression, as the IL4I1 gene was recently shown to be strongly repressed by myc expression in murine bone marrow cells in vitro (23). Among other B-cell lymphoma, IL4I1 was not detected in non germinal centre-derived small B cell lymphomas, i. e. splenic and MALT-type MZL, mantle cell lymphoma and plasmacytoma/myeloma, with the exception of SLL/CLL. Indeed, IL4I1 was expressed in 40% of SLL/CLL cases with a peculiar pattern of expression restricted to cells of proliferation centers. The reason for this observation needs to be clarified. However, some SLL/CLL might derive from germinal center selected B cells (24).

Various levels of IL4I1 production by tumor and inflammatory cells in association with other immunoregulatory mechanisms may dictate the magnitude of T lymphocyte inhibition in the tumor microenvironment. Moreover, the T cell target affected by this immune modulation might differ between solid tumors and lymphoid malignancies. Indeed in solid tumors, IL4I1, as for other immunosuppressive enzymes, should preferentially affect the number and/or the functionality of anti-tumor T lymphocytes, facilitating tumor escape from immune surveillance. In support of this hypothesis, recent data from Finak et al. identified IL4I1 as a poor outcome-associated gene among 163 prognosis-predictive genes differentially expressed between normal breast tissue and tumor stroma (25). In contrast, in a number of lymphomas, the malignant B cell depends upon signaling by its environment for survival. For example, maintenance of interactions between follicular lymphoma B cells and follicular T helper cells has been proposed to support proliferation of the malignant clone (26). IL4I1-mediated T cell inhibition in this setting may suppress the positive stimuli exerted by follicular T helper cells. In line with this, we found a correlation between higher IL4I1 expression and clinical parameters indicative of better outcome. Since IL4I1 expression in TAM and follicular lymphoma cells is strongly correlated, a large cohort will be necessary to determine if one of these cell populations actually exerts the prominent effect and if IL4I1 expression favorably impacts on survival in this pathology.

Overall, IL4I1 expression is found in TAM of most human malignancies studied and in neoplastic cells of several tumors of B-cell origin, in particular PMBL and germinal-centre derived lymphomas. Presence of IL4I1-positive cells and/or detection of enzymatic activity in the majority of the tumors studied suggest that IL4l1 expression could be a general mechanism affecting the relation of tumor cells with the immune system.

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Abbreviations

cHL: classical Hodgkin lymphoma
NLPHL: nodular-predominant Hodgkin lymphoma
MZL: marginal zone lymphoma
MALT: Mucosa-Associated Lymphoid Tissue
PMBL: primary mediastinal B-cell lymphoma
SLL/CLL: small lymphocytic lymphoma/chronic lymphocytic leukaemia
MDSC: myeloid-derived suppressive cells
TAM: tumor-associated macrophage

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Figure 1

IL4I1 protein expression in non lymphoid malignancies

(a&b) Ovarian carcinoma with granulomatous reaction. **(a)** Double staining using IL4I1 (brown) and CD14 (red) antibodies shows IL4I1 expression in tumor cells (arrow) and in CD14 positive reactive cells (arrow-head and small inset) (HPF x200, inset HFPx1000). **(b)** IL4I1 is strongly expressed in epithelioid cells of the granulomatous reaction (HPF X 200). **(c)** Thyroid carcinoma. IL4I1 expression in tumor cells is restricted to an inflammatory area with calcifications (HPF X 400). **(d)** Lung adenocarcinoma. IL4I1 is expressed in reactive cells: intra-alveolar macrophages (arrow) and histiocytic cells within the reactive infiltrate (arrow head) (HPF X 400).

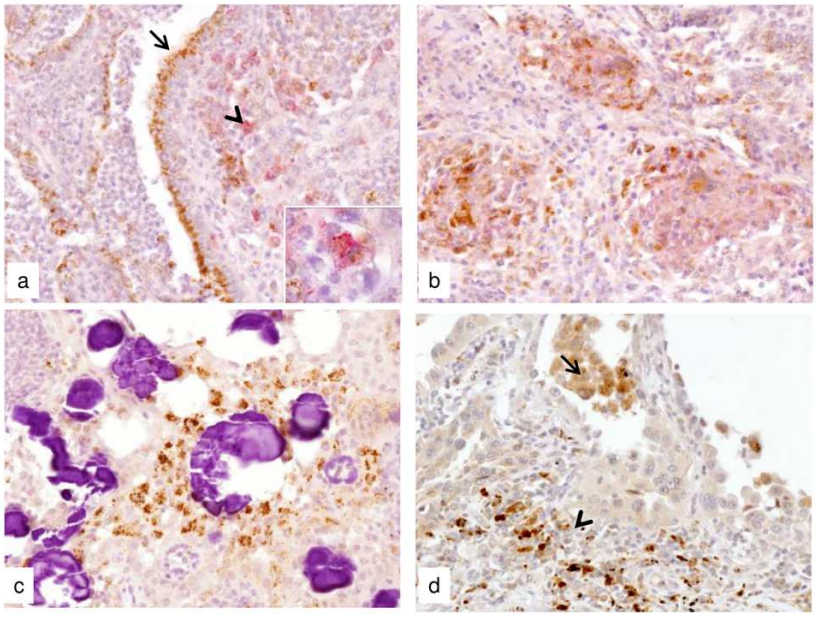

Figure 2

IL4I1 protein expression in reactive lymphoid tissue and lymphoid malignancies

(a) Reactive lymph node. IL4I1 is expressed in tingible bodies macrophages located in the germinal centers of lymphoid follicles (HPFx100, inset, HPFx1000). **(b)** PMBL. All tumor cells display strong IL4I1 intracytoplasmic positivity (HPFx400). **(c)** DLBCL. One of the 17% cases demonstrating occasional IL4I1 positive cells is shown. (HPFx400). **(d&e)** Follicular lymphomas. In case **d**, IL4I1 positive tumor cells are located predominantly in the intrafollicular areas (HPFx100, inset HPFx1000). In case **e** with marginal zone differentiation, numerous IL4I1 positive tumor cells are observed in the perifollicular marginal zone of neoplastic follicles (HPFx100, inset HPFx1000). **(f)** Small lymphocytic lymphoma/CLL. IL4I1 positive paraimmunoblasts are detected in proliferation centers (HPFx100, inset HPFx1000). **(g)** Classical Hodgkin lymphoma. Double immunostaining with IL4I1 (brown) and CD30 (red) shows paranuclear granular IL4I1 positivity in CD30 positive Reed-Sternberg cells (arrow) (HPFx400). **(h)** Nodular lymphocyte predominant Hodgkin lymphoma. Pop-corn–like tumour cells are strongly IL4I1 positive (HPFx200).

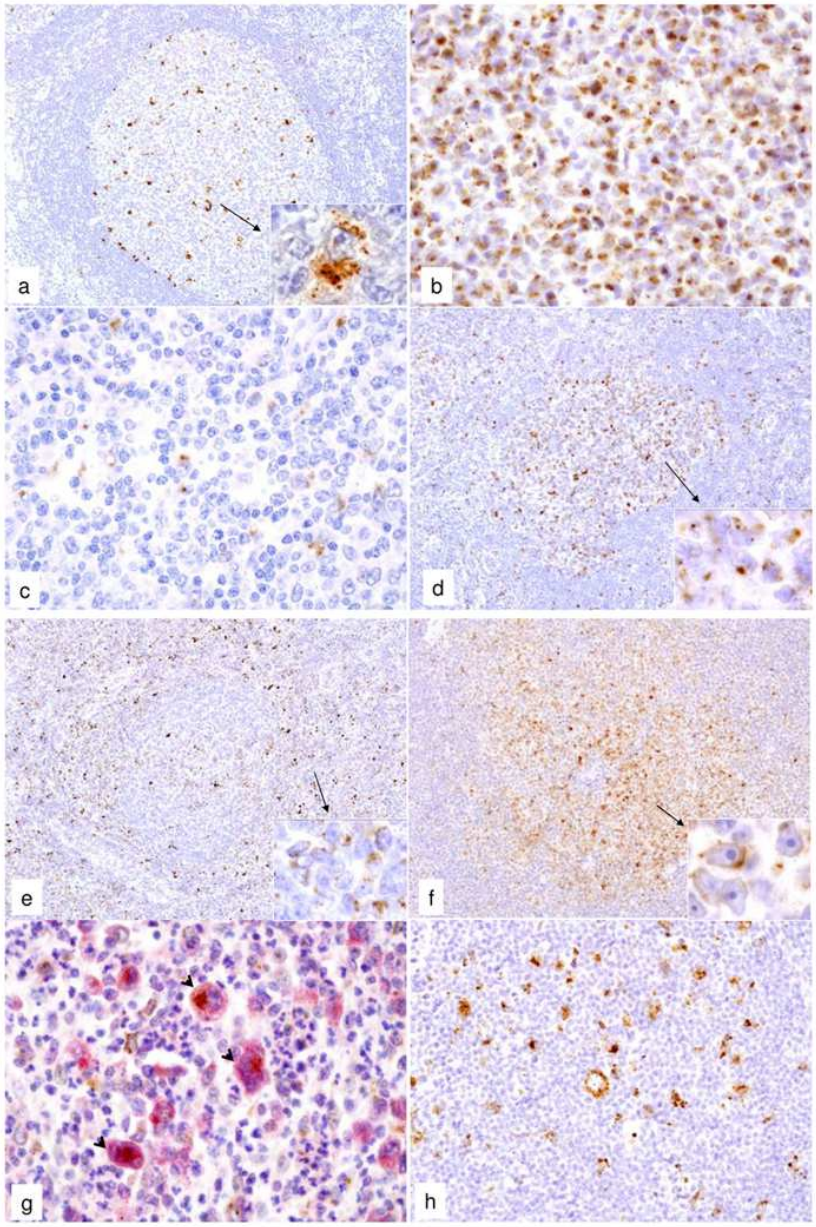

Figure 3
IL4I1 enzymatic activity

(a) Activity of total cell lysates from a normal spleen, splenocytes from a MZL and lymph node cells from a follicular lymphoma (FL-1). **(b)** Activity of $CD19^{+}$ and $CD19^{-}$ MACS-sorted cell lysates from splenocytes from a MZL and lymph node cells from a follicular lymphoma (FL-2). **(c)** Activity of total cell lysates of SU-DHL-4 (DLBCL), KM-H2 (cHL) and L428 (cHL) cell lines. **(d)** Secreted activity of SU-DHL-4 and L428 cell lines. Activity is expressed as pMoles of H_2O_2 produced in 2 hours. One representative experiment is shown.

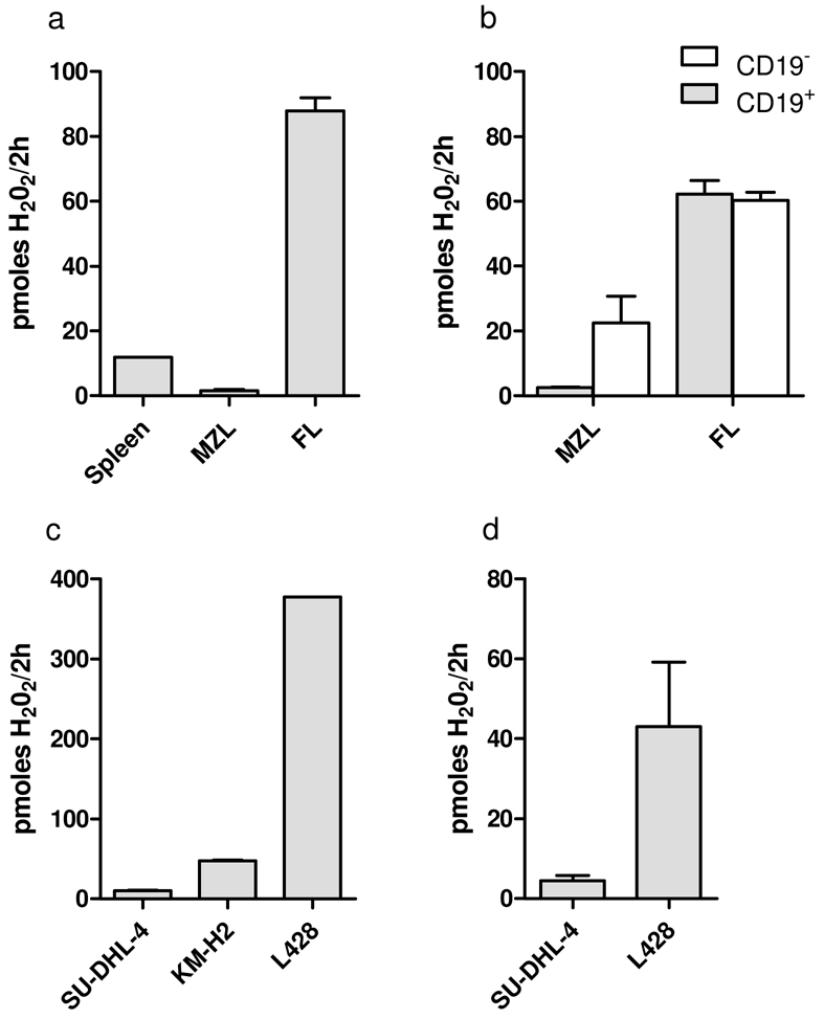

Figure 4

Relation between IL4I1 expression and bone marrow involvement in follicular lymphoma patients

The 23 patients described in table 3 were classified according to the level of IL4I1 expression in tumor cells and TAM (≤10 % or >10% positive cells among the corresponding cell population). The association between IL4I1 expression in tumor cells (A and C) or TAM (B and D) and clinical parameters was analyzed using Fisher's exact test (p values are indicated). A and B, distribution of patients according to bone marrow involvement at diagnosis. C and D, distribution of patients according to detection of a relapse during the follow-up (see table 3 for median follow-up).

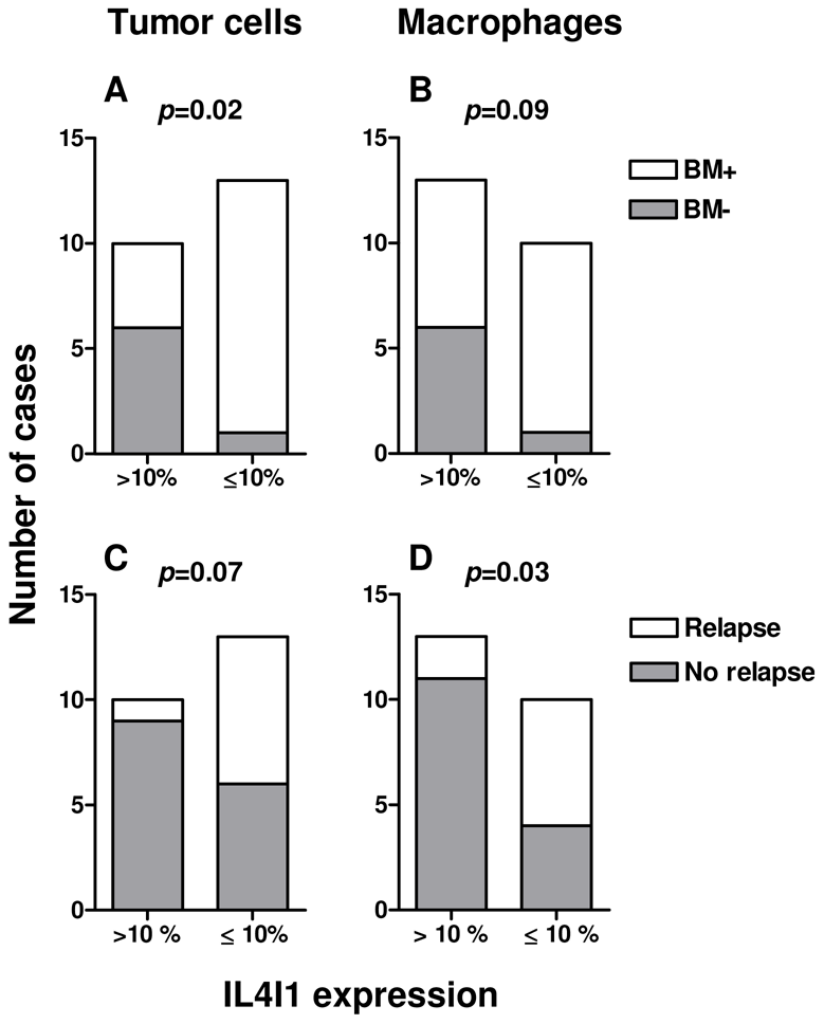

TABLE 1

Immunohistochemical analysis of IL4I1 protein expression in non lymphoid malignancies.

Histology	No. Cases studied	No. Cases with IL4I1 positive tumor cells (% cases)	Proportion of IL4I1 positive TAM (%)		
			<10	10–50	>50
Colorectal carcinomas	13	0	5	4	0
Gastric carcinomas	5	0	3	0	0
Non small-cell lung carcinomas	13	2(15%)	8	1	1
Small-cell lung carcinomas	2	0	0	0	0
Bladder carcinomas	7	0	3	0	0
Ovarian carcinomas	3	1 (33%)	0	0	1
Mesotheliomas	10	5 (50%)	2	5	2
Renal cell carcinomas	11	0	0	1	0
Melanomas	4	0	2	0	0
Breast carcinomas	11	0	5	0	0
Thyroid carcinomas	6	1 (16%)	2	0	0
Hepatocellular and/or cholangiocellular carcinomas	6	0	0	0	0
Testicular germ cell tumors	10	0	6	1	2
Prostatic carcinomas	4	0	2	0	0
Sarcomas	16	0	8	0	0

TABLE 2

Immunohistochemical analysis of IL4I1 protein expression in lymphoid malignancies

Histology	No. Cases studied	No. Cases with IL4I1 positive tumor cells (% cases)	Proportion of IL4I1 positive TAM (%)		
			<10	**10–50**	**>50**
B-cell lymphoma					
SLL/CLL	10	**4** (40%)	2	8	0
Mantle cell	8	0	7	1	0
Follicular	36	**23** (64%)	20	14	2
MZL of MALT-type*	4	0	4	0	0
Splenic or nodal MZL	8	0	7	1	0
Plasmacytoma/myeloma	6	0	6	0	0
Burkitt's lymphoma	5	0	4	1	0
PMBL	28	**25** (89%)	11	14	3
Non-mediastinal DLBCL	36	**6**(17%)	12	18	6
T and NK cell lymphoma					
Peripheral T-cell unspecified	3	0	3	0	0
Angioimmunoblastic	6	0	3	1	2
Anaplastic large cell	2	0	1	1	0
Nasal-type NK/T-cell	1	0	0	1	0
Hepatosplenic	4	0	4	0	0
Enteropathy-type	2	0	1	1	0
Hodgkin lymphoma					
cHL	24	**10**(42%)	4	12	8
NLPHL	11	**10**(91%)	1	4	6

* Marginal zone lymphoma of mucosa-associated lymphoid tissue, which included 2 gastric and 2 small bowel cases

TABLE 3

Distribution of clinical and pathologic variables between negative/low and medium/high IL4I1-expressing follicular lymphoma patients

Parameter	No Patients with ≤10% IL4I1$^+$ tumor cells	No Patients with >10% IL4I1$^+$ tumor cells
Sex		
Female	6	4
Male	7	6
Age		
≤60	10	7
>60 yr	3	3
Bone marrow		
Involved	12	4
Not involved	1	6
FLIPI		
0–1	3	5
2	6	2
3–5	4	3
Median follow-up	28(18–80)	36(19–106)