METHODS FOR THE TREATMENT AND THE PROGNOSIS OF CANCER

The invention relates to an inhibitor of IL4I1 for use in a method for treatment of cancer displaying IL4I1-expressing in the human or animal body, provided that said cancer is not follicular lymphoma. In another aspect, the invention relates to a method for determining the prognosis of a subject suffering from a cancer, provided that said cancer is not follicular lymphoma, comprising the step of detecting the presence of IL4I1 in a sample obtained from said subject, wherein the presence of IL4I1 indicates that the subject has a poor prognosis. The invention also relates to a method for determining the prognosis of a subject suffering from a follicular lymphoma, comprising the step of detecting the presence of IL4I1 in a sample obtained from said subject, wherein the presence of IL4I1 indicates that the subject has a good prognosis.
METHODS FOR THE TREATMENT AND THE PROGNOSIS OF CANCER

FIELD OF THE INVENTION
The present invention relates to methods for the treatment and the prognosis of cancer.

BACKGROUND OF THE INVENTION
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. 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).

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. The principle TAM and MDSC cancer promoting mechanisms consists of the expression of amino-acid catabolizing enzymes allowing tumor escape from the immune response. Examples of such amino-acid catabolizing enzymes are Indoleamine-2,3-dioxygenase (IDO), Arginasel and Inducible Nitric Oxid Synthase, all associated with the inhibition of T cell responses. In line with this, observations of the negative impact of IDO expression on survival in pre-clinical models have lead to the development of a specific inhibitor, which is currently under clinical investigation as an adjuvant therapy.

An object of the invention is thus to find alternative inhibitors of amino-acid catabolizing enzymes for use in methods of treatment of cancer.

SUMMARY OF THE INVENTION
The invention relates to the inhibition of an amino-acid catabolizing enzyme, called IL4I1 (Interleukin 4 Induced gene 1), which has been found by the inventors to be expressed in a large set of human cancers including lymphoma. Furthermore, the inventors have also shown that IL4I1 does not play the same role in all types of
cancers. Indeed, they have shown that the expression of IL4I1 is indicative of poor prognosis in all cancers excepting follicular lymphoma, for which the expression of IL4I1 is indicative of good prognosis.

The present invention thus provides a method for treating cancer displaying IL4I1-expressing cells provided that said cancer is not follicular lymphoma, comprising the step of administering a therapeutically effective amount of an inhibitor of IL4I1 to a subject in need thereof.

In another aspect, the invention relates to a method for treating Hodgkin lymphoma or follicular lymphoma, wherein said Hodgkin lymphoma or follicular lymphoma displays at most 10% of IL4I1-expressing cells, said method comprising the step of administering a therapeutically effective amount of a mTOR inhibitor to a subject in need thereof.

In another aspect, the invention relates to a method for determining the prognosis of a subject suffering from a cancer provided that said cancer is not follicular lymphoma, comprising the step of detecting the presence of IL4I1 in a sample obtained from said subject, wherein the presence of IL4I1 indicates that the subject has a poor prognosis.

In still another aspect, the invention relates to a method for determining the prognosis of a subject suffering from follicular lymphoma, comprising the step of detecting the presence of IL4I1 in a sample obtained from said subject, wherein the presence of IL4I1 indicates that the subject has a good prognosis.

**DETAILED DESCRIPTION OF THE INVENTION**

The present invention provides a method for treating cancer displaying IL4I1-expressing cells provided that said cancer is not follicular lymphoma, comprising the step of administering a therapeutically effective amount of an inhibitor of IL4I1 to a subject in need thereof.

Also provided is an inhibitor of IL4I1 for use in a method for treatment of cancer displaying IL4I1-expressing cells in the human or animal body, provided that said cancer is not follicular lymphoma.

In particular, the present invention provides a method for treating cancer displaying IL4I1-expressing cells provided that said cancer is not follicular lymphoma, comprising the step of administering a therapeutically effective amount of an
inhibitor of IL4I1 to a subject in need thereof, wherein said inhibitor of IL4I1 is selected from the group consisting of
- an inhibitor of the enzymatic activity of IL4I1 of formula (I),
- an antibody against IL4I1 or a fragment thereof which binds IL4I1 and wherein said antibody against IL4I1 or fragment thereof inhibits IL4I1 enzymatic activity, and
- an agent down-regulating the expression of IL4I1 in IL4I1-expressing cells.

In an embodiment, the invention provides a method for treating cancer displaying IL4I1-expressing cells provided that said cancer is not follicular lymphoma, comprising the step of administering a therapeutically effective amount of an inhibitor of the enzymatic activity of IL4I1 of formula (I) to a subject in need thereof.

Also provided is an inhibitor of the enzymatic activity of IL4I1 of formula (I) for use in a method for treatment of cancer displaying IL4I1-expressing cells in the human or animal body, provided that said cancer is not follicular lymphoma.

The inhibitors of the enzymatic activity of IL4I1 of formula (I) can bind IL4I1, as its natural substrate, phenylalanine, and inhibit its enzymatic activity. Preferably, the inhibitors of the enzymatic activity of IL4I1 of formula (I) have a $K_1$ (dissociation constant for binding of inhibitor to enzyme) lower or equal to 3mM, more preferably lower or equal to 30µM, and more preferably lower or equal to 10µM.

The enzyme-inhibitor constant $K_1$ can be measured directly by various methods well known by the man skilled in the art; one accurate method is measurement of enzymatic activity, in the presence of the inhibitor, whereby the inhibitor is titrated into a solution of enzyme and the $H^\Delta C^\Delta$ released by the enzymatic degradation of phenylalanine is measured by oxidation of colorimetric or fluorimetric substrate of peroxidase.

The invention thus relates to an inhibitor of the enzymatic activity of IL4I1 of formula (I)
wherein

- $X$ is an aromatic moiety selected from the group consisting of phenyl and pyridyl; said aromatic moiety being optionally substituted with NH$_2$ or COOH,
- $Y$ is a linear or branched C$_1$-C$_6$ alkyl,
- $R_1$ is selected from the group consisting of H, methyl, vinyl, propargyl, F and CN,
- $R_2$ is selected from the group consisting of NH$_2$, NH-Z, NH-Boc, NH-methyl, NH-Cl, NH-ter-butyl, NH-OH and NH-COCH$_3$,
- $R_3$ is selected from the group consisting of COOH; COOR$_4$, wherein $R_4$ is a linear or branched C$_1$-C$_6$ alkyl; CONH$_2$; CO$_2$H-P(OH) and CO$_2$H-SO$_3$H.

The invention also relates to an inhibitor of the enzymatic activity of IL4I1 of formula (I)

wherein

- $X$ is an aromatic moiety selected from the group consisting of phenyl and pyridyl; said aromatic moiety being optionally substituted with NH$_2$ or COOH,
- $Y$ is a linear or branched C$_1$-C$_2$ alkyl,
- $R_1$ is selected from the group consisting of H, methyl, vinyl, propargyl, F and CN,
- $R_2$ is selected from the group consisting of NH$_2$, NH-Z, NH-Boc, NH-methyl, NH-Cl, NH-ter-butyl, NH-OH and NH-COCH$_3$,
- $R_3$ is selected from the group consisting of COOH; COOR$_4$, wherein $R_4$ is a linear or branched C$_1$-C$_6$ alkyl; CONH$_2$; CO$_2$H-P(OH) and CO$_2$H-SO$_3$H.

The invention further relates to an inhibitor of the enzymatic activity of IL4I1 of formula (I)
wherein

- X is an aromatic moiety selected from the group consisting of phenyl and pyridyl; said aromatic moiety being optionally substituted with NH₂ or COOH,

- Y is a linear or branched C₁⁻C₂ alkyl,

- R₁ is selected from the group consisting of H, methyl, vinyl, propargyl, F and CN,

- R₂ is selected from the group consisting of NH₂, NH-Z, NH-Boc, NH-methyl, NH-Cl, NH-ter-butyl, NH-OH and NH-COCH₃,

- R₃ is selected from the group consisting of COOH; COOR₄, wherein R₄ is a linear or branched C₁⁻C₃ alkyl; CONH₂; CO₂H-P(OH) and CO₂H-SO₃H.

The invention also relates to an inhibitor of the enzymatic activity of IL4I1 of formula (I)

wherein

- X is an aromatic moiety selected from the group consisting of phenyl and pyridyl; said aromatic moiety being optionally substituted with NH₂ or COOH,

- Y is CH₂,

- R₁ is selected from the group consisting of H, methyl, vinyl, propargyl, F and CN,

- R₂ is selected from the group consisting of NH₂, NH-Z, NH-Boc, NH-methyl, NH-Cl, NH-ter-butyl, NH-OH and NH-COCH₃,

- R₃ is selected from the group consisting of COOH, COOCH₂CH₃, CONH₂, CO₂H-P(OH) and CO₂H-SO₃H.

The invention also relates to an inhibitor of the enzymatic activity of IL4I1 of formula (I)

wherein

- X is a phenyl optionally substituted with NH₂ or COOH,

- Y is CH₂,
- $R_1$ is selected from the group consisting of H, methyl, vinyl, propargyl, F and CN,

- $R_2$ is selected from the group consisting of NH$_2$, NH-Z, NH-Boc, NH-methyl, NH-Cl, NH-ter-butyl, NH-OH and NH-COCH$_3$,

- $R_3$ is selected from the group consisting of COOH, COOCH$_2$CH$_3$, CONH$_2$, CO$_2$H-P(OH) and CO$_2$H-SO$_3$H.

The invention further relates to an inhibitor of the enzymatic activity of IL4I1 of formula (I) wherein

- $X$ is a pyridyl optionally substituted with NH$_2$ or COOH,
- $Y$ is CH$_2$,
- $R_1$ is selected from the group consisting of H, methyl, vinyl, propargyl, F and CN,

- $R_2$ is selected from the group consisting of NH$_2$, NH-Z, NH-Boc, NH-methyl, NH-Cl, NH-ter-butyl, NH-OH and NH-COCH$_3$,
- $R_3$ is selected from the group consisting of COOH, COOCH$_2$CH$_3$, CONH$_2$, CO$_2$H-P(OH) and CO$_2$H-SO$_3$H.

In an embodiment, the inhibitor of the enzymatic activity of IL4I1 of formula (I) is typically selected from the group consisting of L-phenylalanine ethyl ester (or 2-amino-3-phenyl-propionic acid ethyl ester), N-acetyl-phenylalanine (or 2-acteylamino-3-phenyl-propionic acid) and aza-phenylalanine (i.e. a phenylalanine wherein one of the -CH= of the phenyl group is replaced by -N=). In particular, the compound of formula (I) is 3-(2-pyridyl)-alanine (or 2'-aza-phenylalanine).

According to the invention, NH-Z and NH-Boc refer to the following protective groups:

-NH-Z:
In another aspect, the invention provides a method for treating cancer displaying IL4I1-expressing cells provided that said cancer is not follicular lymphoma, comprising the step of administering a therapeutically effective amount of an antibody against IL4I1 or a fragment thereof which binds IL4I1 and wherein said antibody against IL4I1 or fragment thereof inhibits IL4I1 enzymatic activity.

Also provided is an antibody against IL4I1 or a fragment thereof which binds IL4I1 and wherein said antibody against IL4I1 or fragment thereof inhibits IL4I1 enzymatic activity, for use in a method for treatment of cancer displaying IL4I1-expressing cells in the human or animal body, provided that said cancer is not follicular lymphoma.

The person skilled in the art will be aware of standard methods for production of such specific antibody or fragment thereof. For example, specific antibodies or fragments thereof may be generated by immunizing an animal with IL4I1 and by selecting the antibodies, which inhibit the enzymatic activity of IL4I1.

The person skilled in the art will be aware of standard methods for production of both polyclonal and monoclonal antibodies and fragments thereof which bind to IL4I1. Antibody fragments, particularly Fab fragments and other fragments which retain epitope-binding capacity and specificity are also well known, as are chimeric antibodies, and "humanized" antibodies, in which structural (not determining specificity for antigen) regions of the antibody are replaced with analogous or similar regions from another species. Thus antibodies generated in mice can be "humanized" to reduce negative effects, which may occur upon administration to
human subjects. Chimeric antibodies are now accepted therapeutic modalities with several now on the market. The present invention therefore comprehends use of antibody specific for IL4I1 which includes F(ab')2, F(ab)2, Fab, Fv and Fd antibody fragments, chimeric antibodies in which one or more regions have been replaced by homologous human or non-human portions. The person skilled in the art will also be aware that fragments such as for example ScFv fragments and divalent or multivalent ScFv-type molecules can be prepared using recombinant methods.

The inhibition of IL4I1 activity may be measured by any method known by the skilled person. For example, the inhibition of the enzymatic activity may be measured, as described in the experimental section, by quantification of H2O2 produced by IL4I1 phenylalanine oxidative deamination in the presence of an inhibitor as described previously and compared to a control (i.e. without inhibitor).

In another aspect, the invention provides a method for treating cancer displaying IL4I1-expressing cells provided that said cancer is not follicular lymphoma, comprising the step of administering a therapeutically effective amount of an agent down-regulating the expression of IL4I1 in said IL4I1-expressing cells.

Also provided is an agent down-regulating the expression of IL4I1 in IL4I1-expressing cells for use in a method for treatment of cancer displaying IL4I1-expressing cells in the human or animal body, provided that said cancer is not follicular lymphoma.

Typically, an agent down-regulating the expression of IL4I1 can be a nucleic acid, which interferes with the expression of IL4I1.

Examples of such agents are antisense molecules or vectors comprising said antisense molecules. Antisense molecules are complementary strands of small segments of mRNA. Methods for designing effective antisense molecules being well known (see for example US6165990), it falls within the ability of the skilled artisan to design antisense molecules able to downregulate the expression of IL4I1 in IL4I1-expressing cells. Further examples are RNA interference (RNAi) molecules such as, for example, short interfering RNAs (siRNAs) and short hairpin RNAs (shRNAs). RNAi refers to the introduction of homologous double stranded RNA to specifically target a gene's product, in the present case IL4I1, resulting in a
null or hypomorphic phenotype. Methods for designing effective RNAi molecules being well known (see for review π ), it falls within the ability of the skilled artisan to design RNAi molecules able to downregulate the expression of IL4I1 in IL4I1-expressing cells.

A particular agent down-regulating the expression of IL4I1 in IL4I1-expressing cells is a siRNA comprising the nucleotide sequence as shown in SEQ ID NO: 1.

Another particular agent down-regulating the expression of IL4I1 is a siRNA having a nucleotide sequence as shown in SEQ ID NO: 2 and a complementary nucleotide sequence as shown in SEQ ID NO: 3.

SEQ ID NO:2: [5'r(GGGUGGAGACGCCGCACG)d(TT)3'],
SEQ ID NO:3: [5'r(UUGACCGGCGUCCACCC)d(AG)3']

This particular siRNA comprises a double stranded region of 19 nucleotides and a single stranded region of 2 nucleotides at the 3' end of each one of the strands of siRNA.

As used in SEQ ID NO:2 and SEQ ID NO:3, "r" means ribonucleotide, and "d" means deoxyribonucleotide.

Typically, the down-regulation of the IL4I1 expression may be measured by various methods known by the skilled person, such as for example immunohistochemistry, binding assay, mRNA level for IL4I1 and radio-immunoassay or Enzyme link immunosorbert assay for IL4I1.

The down-regulation of the IL4I1 expression may further be detected indirectly by the measurement of its enzymatic activity since it was found by the inventors that enzymatic activity of IL4I1 paralleled IL4I1 expression. The enzymatic activity of IL4I1 may be measured by quantification of H2O2 produced by IL4I1 phenylalanine oxidative deamination, as described in the experimental section.

The invention also relates to a pharmaceutical composition comprising:

- an inhibitor of the enzymatic activity of IL4I1 of formula (I), and/or
- an antibody against IL4I1 or a fragment thereof which binds IL4I1 and wherein said antibody against IL4I1 or fragment thereof inhibits IL4I1 enzymatic activity, and/or
- an agent down-regulating the expression of IL4I1 in IL4I1-expressing cells, together with a pharmaceutically-acceptable carrier.
In one embodiment, the invention relates to the pharmaceutical composition as defined previously for use in a method for treating cancer displaying IL4I1-expressing cells in the human or animal body, provided that said cancer is not follicular lymphoma.

A person skilled in the art will be aware of suitable carriers. Suitable formulations for administration by any desired route may be prepared by standard methods, for example by reference to well-known text such as Remington; The Science and Practice of Pharmacy.

In an embodiment of the invention, the cancer to be treated according to the invention is selected from the group consisting of non-lymphoid cancers displaying IL4I1-expressing cells and lymphomas displaying IL4I1-expressing cells, provided that said lymphoma is not follicular lymphoma.

As used herein, "non lymphoid cancer" means cancers, which are not derived from lymphoid cells. As used herein, "lymphoid cells" means lymphocytes and plasma cells.

In an embodiment, the non-lymphoid cancers are solid tumors.

In a particular embodiment, non-lymphoid cancers are typically selected from carcinomas, sarcomas, mesotheliomas, blastomas and germ cell tumors.

In another particular embodiment, non-lymphoid cancers are typically selected from the group consisting of mesotheliomas, non small-cell lung carcinomas, colon carcinoma, breast carcinoma, thyroid carcinoma, testicular germ cell tumors and ovarian carcinoma, displaying IL4I1-expressing cells.

In a specific embodiment, the cancer to be treated is selected from the group consisting of non-lymphoid cancers displaying IL4I1-expressing cells, provided that the cancer is not breast carcinoma.

In another specific embodiment, the cancer to be treated is selected from the group consisting of lymphomas displaying IL4I1-expressing cells provided that said lymphoma is not follicular lymphoma. Lymphomas are typically selected from B-cell lymphomas displaying IL4I1-expressing cells provided that said lymphoma is not follicular lymphoma.

In a specific embodiment, the cancer to be treated is selected from the group consisting of PMBL (Primary Mediastinal large B-cell Lymphoma), classical
Hodgkin lymphomas (cHL), NLPHL (Nodular lymphocyte predominant Hodgkin's lymphoma), non-mediastinal Diffuse Large B-Cell Lymphoma (DLBCL) and SLL/CLL (Small Lymphocytic Lymphoma / Chronic Lymphocytic Leukemia), displaying IL4I1-expressing cells.

In another specific embodiment, the cancer to be treated is selected from the group consisting of lymphomas displaying IL4I1-expressing cells, provided that said cancer is not PMBL (Primary Mediastinal large B-cell Lymphoma) or follicular lymphoma.

In a further specific embodiment, the cancer to be treated is selected from the group consisting of B-cell lymphomas displaying IL4I1-expressing cells, provided that said cancer is not PMBL (Primary Mediastinal large B-cell Lymphoma) or follicular lymphoma.

The invention also concerns mTOR inhibitors for use in methods for treating particular types of Hodgkin and follicular lymphomas. Indeed, it has been shown that the nature of the T lymphocyte populations present within the inflammatory infiltrate of Hodgkin lymphoma and follicular lymphoma is associated with the prognosis of these tumors\textsuperscript{21,22}. In contrast to what has been observed in non-lymphoid tumors, the magnitude of the regulatory T cell infiltrate is associated to a better prognosis, whereas the presence of cytotoxic T cells constitutes a pejorative factor. The inventors have shown that both Hodgkin lymphoma and follicular lymphoma can express high levels of IL4I1 in the intratumoral macrophages or in the tumor cells. The inventors have also shown that at least some of the toxicity mediated by IL4I1 on T cells is achieved via the antiproliferative effect of H\textsubscript{2}O\textsubscript{2} liberated by the enzymatic activity. Since regulatory T cells are more resistant than effector T cells to H\textsubscript{2}O\textsubscript{2} toxic effect\textsuperscript{23}, the results of the inventors indicate that IL4I1 may participate to the selection of regulatory T cells in the tumor microenvironment.

In addition, since IL4I1-mediated degradation of the essential amino acid phenylalanine may contribute to its local depletion, the results of the inventors indicate that said T lymphocyte amino acid deficiency triggers a signaling pathway that inactivates the mTOR complex leading to inhibition of T lymphocyte proliferation (response to amino acid depletion by inactivation of the mTOR
signaling pathway is for instance described by Cobbold SP et al.\textsuperscript{24).} Since the mTOR inactivation has been shown to alter functional polarization of T lymphocytes leading to the loss of their effector functions in favor of regulatory ones\textsuperscript{25}, the results of the inventors indicate that IL4I1 expression in Hodgkin lymphoma and follicular lymphoma participate to the enrichment in regulatory T cell populations at the expense of CD4\(^+\) and CD8\(^+\) effector T cells. When intratumor IL4I1 expression is low or negative in these pathologies (i.e. when said Hodgkin lymphoma or follicular lymphoma displays at most 10\% of IL4I1-expressing cells), treatments using mTOR inhibitors such as rapamycin and its analogs could be considered to mimic the effect of amino acid depletion.

The invention thus concerns a method for treating Hodgkin lymphoma or follicular lymphoma, wherein said Hodgkin lymphoma or follicular lymphoma displays at most 10\% of IL4I1-expressing cells, said method comprising the step of administering a therapeutically effective amount of a mTOR inhibitor to a subject in need thereof.

Also provided is a mTOR inhibitor for use in a method for treatment of Hodgkin lymphoma or follicular lymphoma in the human or animal body, wherein said Hodgkin lymphoma or follicular lymphoma displays at most 10\% of IL4I1-expressing cells.

According to the invention, the percentage of IL4I1-expressing cells in Hodgkin lymphoma or follicular lymphoma is typically measured by immunohistochemistry (immunostaining) performed on a sample obtained by biopsy. Examples of samples obtained from the subjects are any type of tumor biopsy, including lymph nodes. The percentage of IL4I1-expressing cells may also be measured by immunological techniques such as ELISA on tumor cell suspensions or by the measurement of its enzymatic activity (by quantification of H\(_2\)O\(_2\) produced by IL4I1 phenylalanine oxidative deamination).

A mTOR inhibitor is a compound which targets intracellular mTOR ("mammalian Target Of Rapamycin"). mTOR is a family member of phosphatidylinositol 3-kinase(P13-kinase) related kinase. The compound rapamycin and other mTOR inhibitors inhibit the mTOR pathway via a complex with its intracellular receptor FKBP12 (FK506-binding protein 12). mTOR modulates translation of specific mRNAs via the regulation of the phosphorylation state of several different translation proteins, mainly 4E-PB1, P70S6K (p70S6 kinase 1) and eEF2.
A mTOR inhibitor of (according to) the present invention e.g. includes rapamycin, which is a known macrolide antibiotic produced by *Streptomyces hygroscopicus*, and rapamycin derivatives, e.g. rapamycin substituted in position 40 and/or 16 and/or 32, for example compounds of formula I disclosed in WO 2007/131689 (incorporated by reference), such as for example 40-O-(2-hydroxyethyl)-rapamycin (also known as everolimus), 32-deoxorapamycin, 16-0-substituted rapamycins such as 16-pent-2-ynoxy-32-deoxorapamycin, 16-pent-2-ynoxy-32 (S or R)-dihydro-rapamycin, 16-pent-2-ynoxy-32 (S or R)-dihydro-40-0- (2-hydroxyethyl)-rapamycin, 40-[3- hydroxy-2- (hydroxy- methyl)-2-methylpropanoate]-rapamycin (also known as CCI779), 40- epi-(tetrazolyl)-rapamycin (also known as ABT578), or 40-O-ethoxyethyl-rapamycin (also known as biolimus 9). mTOR inhibitors also include the so-called rapalogs, e.g. as disclosed in WO9802441 (incorporated by reference), WO01 14387 (incorporated by reference) and WO0364383 (incorporated by reference), such as AP23573, e.g. 40-O-(dimethylphosphinoyl)-rapamycin, compounds as disclosed disclosed in WO2005047295 in Example 1 (incorporated by reference), also designated as biolimus A9 and compounds disclosed under the name TAFA-93. Other mTOR inhibitors are e.g. disclosed in WO2004101583, WO9205179, WO9402136, WO9402385, WO9613273 (all incorporated by reference).

Typically, before applying a method of treatment according to the present invention to a subject suffering from cancer, a diagnostic test may be performed in order to determine whether the cancer displays IL4I1-expressing cells. By performing such a pre-treatment diagnostic test, it is possible to determine whether a subject would be responsive to a method of treatment according to the invention.

Accordingly, the invention also concerns a method for determining the responsiveness of a subject suffering from cancer to an inhibitor of IL4I1 as defined previously, comprising the step of detecting the expression of IL4I1 in a cancer sample obtained from said subject, provided that said cancer is not follicular lymphoma.

Typically, the cancer sample is a tumor biopsy.
It falls within the ability of the skilled artisan to carry out such a diagnostic test, since detection of the expression of IL4I1 in the above mentioned cancers can be easily carried out by any usual method known by the skilled person. Typically, IL4I1 expression may be measured through the detection of IL4I1-mRNA in the tumor cell lysate, for example by RT-PCR.

IL4I1 expression may also be measured by immunohistochemistry performed on a sample obtained by biopsy, as described in the experimental section. Examples of samples obtained from the subjects are any type of tumor biopsy, including lymph nodes, and optionally on whole blood sample.

The expression of IL4I1 may also be detected by immunological techniques such as ELISA and Western Blot on whole blood sample, plasma sample or serum sample. The expression of IL4I1 may further be detected by the measurement of its enzymatic activity since it was found by the inventors that enzymatic activity of IL4I1 paralleled IL4I1 expression. The enzymatic activity of IL4I1 may be measured by quantification of H₂O₂ produced by IL4I1 phenylalanine oxidative deamination, as described in the experimental section. This test can be carried out on a sample obtained from the patient, such as for example any type of tumor biopsy, whole blood sample, plasma sample or serum sample or other biological fluids such as bronchoalveolar liquid. The *in vivo* enzymatic activity of IL4I1 may also indirectly be measured by quantitation of phenylalanine and phenylpyruvate in biological fluids.

In the context of the invention, the term "treating" or "treatment", as used herein, means reversing, alleviating, inhibiting the progress of, or preventing the disorder or condition to which such term applies, or reversing, alleviating, inhibiting the progress of, or preventing one or more symptoms of cancer. As used herein, "subject" refers to a human or animal that may benefit from the administration of an inhibitor of IL4I1, a composition or a method as recited herein. Most often, the subject will be a human but can be any mammals.

By a "therapeutically effective amount" of a compound according to the invention, an antibody against IL4I1, a fragment thereof which binds IL4I1 and wherein said antibody against IL4I1 or fragment thereof inhibits IL4I1 enzymatic activity, of an agent down-regulating the expression of IL4I1, or of a mTOR inhibitor, is meant a
sufficient amount to treat cancer, at a reasonable benefit/risk ratio applicable to any medical treatment. It will be understood, however, that the total daily usage of the compound of the invention, the antibody against IL4I1, the fragment thereof which binds IL4I1 and wherein said antibody against IL4I1 or fragment thereof inhibits IL4I1 enzymatic activity, of the agent down-regulating the expression of IL4I1, or of the mTOR inhibitor, will be decided by the attending physician within the scope of sound medical judgment. The specific therapeutically effective dose level for any particular subject in need thereof will depend upon a variety of factors including the stage of cancer being treated and the activity of the specific inhibitor/cytotoxic agent employed, the age, body weight, general health, sex and diet of the subject, the time of administration, route of administration, the duration of the treatment; drugs used in combination or coincidental with the and like factors well known in the medical arts. For example, it is well known within the skill of the art to start doses of the compound at levels lower than those required to achieve the desired therapeutic effect and to gradually increase the dosage until the desired effect is achieved.

In a particular embodiment of the invention, the methods for treatment of cancer according to the invention are applied to the human or animal body simultaneously, separately or sequentially with another method for treatment of cancer, said another method for treatment of cancer being preferably selected from the group comprising surgery, external radiotherapy, chemotherapy, hormone therapy and cytokine therapy.

In an embodiment, the method of treatment according to the invention is combined with a chemotherapy, wherein said chemotherapy comprises the administration of at least one anti-cancer agent.

As used herein, the expression "anti-cancer agent" refers to compounds, which are used in the treatment of cancer.

Anti-cancer agents include but are not limited to fludarabine, gemcitabine, capecitabine, methotrexate, taxol, taxotere, mercaptopurine, thioguanine, hydroxyurea, cytarabine, cyclophosphamide, ifosfamide, nitrosoureas, platinum complexes such as cisplatin, carboplatin and oxaliplatin, mitomycin, dacarbazine, procarbazine, etoposide, teniposide, campathecins, bleomycin, doxorubicin,
idarubicin, daunorubicin, dactinomycin, plicamycin, mitoxantrone, L-asparaginase, doxorubicin, epimicm, 5-fluorouracil, taxanes such as docetaxel and paclitaxel, leucovorin, levamisole, irinotecan, estramustine, etoposide, nitrogen mustards, BCNU, nitrosoureas such as carmustme and lomustine, vinca alkaloids such as vinblastine, vincristine and vinorelbine, imatimbmesylate, hexamethylhnelamine, topotecan, kinase inhibitors, phosphatase inhibitors, ATPase inhibitors, tyrhostins, protease inhibitors, inhibitors herbimycin A, genistein, erbstatin, and lavundustin.

In one embodiment, the anti-cancer agent is selected for the group consisting of taxol; taxotere; platinum complexes such as cisplatin, carboplatin and oxaliplatin; doxorubicin; taxanes such as docetaxel and paclitaxel; vinca alkaloids such as vinblastine, vincristine and vinorelbine; genistein; erbstatin; and lavundustin.

In another aspect, the invention relates to a method for determining the prognosis of a subject suffering from cancer, provided that said cancer is not follicular lymphoma, comprising the step of detecting the presence of IL4I1 in a sample obtained from said subject, wherein the presence of IL4I1 indicates that the subject has a poor prognosis.

Without wanting to be bound by a theory, and excepting in follicular lymphoma, 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. Consequently, a patient having a cancer expressing IL4I1 will have a poor prognosis. In line with this, the inventors have shown a correlation between IL4I1 expression and tumor escape in a murine model of cancer.

In still another aspect, the invention relates to a method for determining the prognosis of a subject suffering from a follicular lymphoma, comprising the step of detecting the presence of IL4I1 in a sample obtained from said subject, wherein the presence of IL4I1 indicates that the subject has a good prognosis. Indeed, and without wanting to be bound by a theory, in follicular lymphomas, the malignant B cell depends upon signalling 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. IL4I1-mediated T cell inhibition in this setting may suppress the positive stimuli
exerted by follicular T helper cells. In line with this, the inventors have shown a correlation between higher IL4I1 expression and clinical parameters indicative of good prognosis.
Examples of samples obtained from the subjects are any type of tumor biopsies, including lymph nodes, whole blood sample, plasma sample or serum sample.
The detection of the expression of IL4I1 may be carried out by detecting the presence of IL4I1 mRNAs in the tumor cells, notably by RT-PCR, or any other method known by the skilled person.
The detection of the presence of IL4I1 in the sample may be also notably carried out by immunochemistry techniques, using anti-IL4I1 antibodies, optionally tagged, as disclosed previously.
As previously mentioned, the expression of IL4I1 may also be indirectly detected by the measurement of its enzymatic activity since it was found by the inventors that enzymatic activity of IL4I1 paralleled IL4I1 expression. Measurement of the enzymatic activity of IL4I1 is fully described in the experimental section. The in vivo enzymatic activity of IL4I1 may be also indirectly measured by quantitation of phenylalanine and phenylpyruvate in biological fluids.
The term "detecting" as used above includes qualitative and/or quantitative detection (measuring levels) with or without reference to a control.
The term "prognosis" is used herein to refer to the prediction of the likelihood of cancer-attributable death or progression, including recurrence, metastatic spread, and drug resistance.
As used herein, "poor prognosis" indicates an increased likelihood of cancer-attributable death or progression, including recurrence, metastatic spread, and drug resistance.
As used herein, "good prognosis" indicates a decreased likelihood of cancer-attributable death or progression, including recurrence, metastatic spread, and drug resistance.
The prognosis results obtained according to the method of the invention can also be correlated to, or serve as a basis for, a "risk classification" of the patients. As used herein, "risk classification" means the level of risk or the prediction that a subject will experience a particular clinical outcome. A subject may be classified into a risk group or classified at a level of risk based on the predictive methods of the present
invention. A "risk group" is a group of subjects or individuals with a similar level of risk for a particular clinical outcome. Examples of risk classifications are the International Prognostic Index (IPI) and the Follicular Lymphoma International Prognostic Index (FLIPI, 13).

FIGURE LEGENDS

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 in human tumors and tumor cell lines
(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 H2O2 produced in 2 hours. One representative experiment is shown. (e) Total activity of cell lysates from three melanomas compared with autologous normal tissue (for two of them).

Figure 4: Relation between IL4I1 expression, regulatory T cell infiltrate 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 (Band 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). E and F, distribution of patients according to percentage of FoxP3+ T cells (Tregs) in the tumour biopsy at diagnosis.

Figure 5: IL4I1 expression by monocytes inhibits T cell proliferation and inflammatory cytokine and chemokine secretion. Human PBMC from two different donors co-cultured with irradiated THP1 or THP1-IL4I1 cells were stimulated with an anti-CD3 antibody (A) or with PPD (B). Proliferation was measured by 3H-thymidine incorporation during the last 16 hours of a 4-day culture. Results are expressed as the average cpm of quadruplicates ± standard deviation (SD). A representative experiment (of 5) is shown. (C) PBMC were co-
cultured as in A and day 1 and day 3 culture media analyzed respectively for IL2 and IFNγ secretion by ELISA. (D) PBMC were co-cultured as in A and culture media harvested at day 3 analyzed on a Raybiotech cytokine array. Dot blots were quantified using the Labwork software (UVP, United Kingdom). In B, C and D, white bars and gray bars represent results obtained with THP1 and THP1-IL4I1 cells, respectively.

**Figure 6:** Dichotomy of IL4I1 activity induction by IFNγ and IL4. (A) $10^5$ monocytes, monocyte-derived Mφ, monocyte-derived DC and B cells were stimulated with IFNγ or IL4 and IL4I1 enzymatic activity against phenylalanine measured after 48h. The mean of 3 experiments ± SD is shown. (B) Kinetics of IL4I1 enzymatic activity of IFNγ-treated Mφ and DC (left and center) and U4-treated B lymphocytes (right). Representative experiments are shown. Enzymatic activity is expressed as pmoles H$_2$O$_2$ produced by 105 cells per hour using phenylalanine as substrate.

**Figure 7:** Hodgkin cells instruct monocytes to produce IL4I1 through IFNγ and IL13 secretion. (A) Monocytes from the monocytic cell line THP1 were grown in 48-h conditioned media either from two different Hodgkin lymphoma cell lines (KMH2 and L428) or from a large B cell lymphoma cell line (SU-DHL-4) or in unconditioned medium. IL4I1 activity was measured in the THP1 cells 48 h later. Results are expressed as pmoles H$_2$O$_2$ produced per hour by $10^5$ cells. (B) THP1 cells were grown as in A with addition of neutralizing antibodies against IL10, IFNγ and IL13 or control IgG. Results are expressed as percent inhibition of IL4I1 activity compared to activity measured in cells cultivated with IgG control.

**Figure 8:** Measurement of the in vivo growth of tumors displaying IL4I1-expressing-cells in mice
(A) Mice were vaccinated with a melanoma tumor cell epitope. Seven (left graph) or 28 days (right graph) after vaccination, mice were challenged with tumor cells expressing murine IL4I1 (square) or transfected with control vector (circle), and then the percentage of tumor-free mice was determined. (B) Mice were vaccinated
as in (5). Seven days after vaccination, mice were challenged with tumor cells expressing murine IL4I1 (tumor 1, square, and tumor 2, triangle) or transfected with control vector (control tumor, circle), and then the percentage of tumor-free mice was determined. (C) Mice were challenged with tumor cells expressing murine IL4I1 (tumor 1, square, and tumor 2, triangle) or transfected with control vector (control tumor, circle), and then the percentage of tumor-free mice was determined.

**Figure 9: IL4I1 activity in the sera of mice challenged with IL4I1 expressing tumors**

Mice vaccinated against the gp33 tumor epitope as in figure 8 were challenged 7 days after immunization with different proportions of an IL4I1 expressing tumor (tumor 2) admixed with the control tumor. IL4I1 activity was measured on a pool of three sera, 30 days after tumor challenge, i.e. before tumor appearance.

**Figure 10: Antitumor immune response modulation by tumor IL4I1 expression.** Two days after adoptive transfer of splenocytes from transgenic mice expressing a gp33 specific T cell receptor (2x10^6 cells/mouse), mice were immunized against the gp33 tumor epitope as in figure 5. Seven days after immunization, mice were challenged with a high number of cells from IL4I1-expressing tumor 1 and tumor 2 or control tumor (10^7 cells/mouse, n=3 per group, representative experiment out of 3). All mice developed tumors synchronously at day 10 post-challenge. (A) gp33-41-specific CD8+ T cells were measured in the blood, using H2d-gp33 tetramers. Results are presented as % of H2d-gp33 positive cells amongst the CD8 positive T cells. (B) Mice were sacrificed at day 13 and gp33-specific splenocytes were characterized for CD44 (memory marker) and surface CD107a (marker of cytotoxic activity) expression and analyzed for IFNγ production by ELISPOT.

**Figure 11: Inhibition of IL4I1 enzymatic activity by L-phenylalanine ethyl ester**

The enzymatic activity of IL4I1 was measured in the presence of 0 mM (circle, control), 0.7 mM (triangle), 1.5 mM (square) or 5mM (lozenge) of L-phenylalanine ethyl ester.
**Figure 12: Inhibition of IL4I1 enzymatic activity by N-acetyl-phenylalanine**
The enzymatic activity of IL4I1 was measured in the presence of 0 mM (circle, control), 0.7 mM (triangle), 1.5 mM (square) or 5mM (lozenge) of N-acetyl-phenylalanine.

**Figure 13: Inhibition of IL4I1 enzymatic activity by 2'-aza-phenylalanine**
The enzymatic activity of IL4I1 was measured in the presence of 0 mM (circle, control), 0.6 mM (triangle), 1.2 mM (square) or 5mM (lozenge) of 2'-aza-phenylalanine.

**Figure 14: Inhibition of IL4I1 activity in Hodgkin lymphoma cells by a specific siRNA.** The Hodgkin lymphoma cell line L428 (2x10⁶ cells) was transfected with 6 µg of IL4I1 siRNA or control siRNA by nucleofection (Nucleofector technology®, Lonza). IL4I1 activity was measured 3, 5 and 7 days later.

**EXAMPLES**
In the following description, all molecular biology experiments for which no detailed protocol is given are performed according to standard protocols.

**Material and methods**

**Material**
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, Creteil, 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 (Primary Mediastinal large B-cell Lymphoma), 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 microarray blocks were stained with hematoxylin-eosin for histological studies.

**Antibodies, cytokines, peptide and siRNA**

Human recombinant IL4 (50ng/ml) was purchased from R&D systems (Lille, France) IFNγ (50ng/ml), GM-CSF (50 mg/ml), M-CSF (100ng/ml) were purchased from Preprotech France (Levallois Perret, France). Monoclonal functional grade anti-CD3 (OKT3) was purchased from eBioscience (San Diego, USA). Neutralising antibodies against IL13 (clone B-B 13) was purchased from Abeam and those anti IFNγ (clone MMHG-I) was purchased from Calbiochem. The 33-41 amino acid peptide from the glycoprotein of lymphochoriomenengitis virus (KAVYNPATM, H2-D^b restricted) was purchased from Polypeptide group. IL4I1 siRNA [5’(GGUGGAGAAGCGCGGUCAAG)3’] was purchased from Qiagen.

**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 (FCS), L-glutamine and antibiotics. The monocytic THP1 cell line was cultivated in DMEM and RPMI 1640 (Invitrogen, Cergy Pontoise France) containing 10% FCS. All media were supplemented with 2mM L-glutamine, 100 UI/ml penicillin and 100 µg/ml streptomycin (all from Invitrogen). Human
peripheral blood mononuclear cells (PBMC) from healthy subjects were obtained after informed consent from the French Blood Bank (EFS) and purified on a lymphocyte separation gradient (Eurobio, France). Human monocytes were enriched by adherence from PBMC. DC were differentiated in AIM-V serum free medium (Invitrogen) supplemented with glutamine and 50ng/ml IL4 and 50 ng/ml GM-CSF. Cells obtained after 5-6 days in culture were designated as immature DC (iDC). Mature DC (mDC) were obtained by culturing 1x10^6 iDC with different maturation stimuli in AIM-V serum-free medium in 6-well plates for up to 48-72 h. Adherent mDC were removed by gentle scraping. Monocyte-derived macrophages were seeded in sealed gas-permeable bags (Baxter Healthcare corporation, Deerfield, USA) under non-adherent conditions at a density of 1x10^6 cells/ml containing 100ng/ml of M-CSF. After 6 days of culture, macrophages were recovered, counted and seeded in 6 well plates at 1x10^6 cells/ml and activated with different stimuli. B lymphocytes were obtained from human tonsils obtained from tonsillectomies performed at the Intercommunal Hospital of Creteil with ethical approval. Tissue was cut into small pieces in RPMI 1640 containing 10% FCS and forced through a 100µm mesh nylon screen to disrupt the pieces into singles cells. After two washes in RPMI/FCS, the cells were counted. The viability of all cells or cell lines was established by Trypan blue exclusion.

For enzymatic activity studies, viable cryopreserved cells from 5 follicular lymphomas, 1 MZL, 2 traumatic normal spleens and three frozen biopsies of melanoma tumors with non invaded adjacent skin 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 antibody (1/400 dilution, rabbit polyclonal abl8524, Abeam, 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.

Regulatory T cells (FoxP3+ cells) were detected on paraffin embedded total tissue sections using an anti-FoxP3 Ab (1/50e dilution, mouse monoclonal 236A/E7, kindly provided by Teresa Marafioti, Oxford, UK) and an indirect immunoperoxidase method (Vectastain kit, Vector Laboratories, Burlingame USA) after antigen retrieval performed by water bath heating in EDTA buffer pH=9. The percentage of FoxP3+ cells among the total number of lymphoid infiltrating cells was evaluated by a semi-quantitative method.

For double immunostaining, a 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 KPI, Dako, Glustrup, Denmark) or CD30 (diluted 1/30, clone Ber-H2, Dako, Glustrup, Denmark) antibodies and labelled using the alkaline phosphatase-conjugated ABC procedure (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 peroxidase-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 (Adobe 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 14. 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 (2.5x10^5 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.

**Proliferation assays**

All functional assays were performed in complete RPMI 1640 supplemented with 10% heat-inactivated human AB-serum. PBMC (2x10^5 cells/well) stimulated with anti-CD3 (2µg/ml) were co-cultured 4 days in 96 well round bottom plates with 2.5x10^4 to 10^5 irradiated (150 Gy) THP1 and THP1-IL4I1 cells in various proportions (as indicated in figure 5A). Alternatively, the PBMC were incubated with 100U/ml of purified protein derivative (PPD, Aventis Pasteur) and irradiated THP1 or THP1-IL4I1 cells for 5 days. ³H-thymidine (1 µCi/well, Amersham, Saclay, France) was added for the final 18 h of coculture. T cell proliferation is expressed as cpm ³H-thymidine incorporated.

**Cytokine measurements**

Cytokine and chemokine secretion in proliferation experiments was detected using the human cytokine antibody array 1 from RayBiotech according to manufacturer's instructions. Human IL2 and IFNγ were measured with OptEIA ELISA kits from...
BD Biosciences (Le Pont de Claix, France) according to manufacturer's instructions.

*Measurement of the in vivo growth of tumors displaying IL4I1-expressing-cells in mice*

5 Figure 8A: C57BL/6 mice were vaccinated with the gp33-41 T cell epitope expressed by melanoma tumor cells (the model used is the B16 tumor cell line transfected with the 33-41 amino acid peptide from the glycoprotein of lymphochoriomeningitis virus) emulsified in incomplete Freund adjuvant. Seven (left graph) or 28 days (right graph) after vaccination, mice were challenged with $10^5$ tumor cells expressing murine IL4I1 or transfected with control vector. The number of tested animals is indicated in parenthesis. The percent of tumor-free mice is represented as Kaplan Meier's survival curves and statistical analysis was performed using the log rank test.

Figure 8B: C57BL/6 mice were vaccinated as in figure 8A. Seven days after vaccination, mice were challenged with $10^5$ tumor cells expressing murine IL4I1 (tumor 1 and tumor 2) or transfected with control vector (control tumor). The number of tested animals is indicated in parenthesis. The percent of tumor-free mice is represented as Kaplan Meier's survival curves and statistical analysis was performed using the log rank test comparing tumor 1 and tumor 2 with control.

Figure 8C: C57BL/6 mice were challenged with $5 \times 10^4$ tumor cells expressing murine IL4I1 (tumor 1 and tumor 2) or transfected with control vector (control tumor). The number of tested animals is indicated in parenthesis. Data are expressed as percent of tumor-free mice represented as Kaplan Meier's survival curves and statistical analysis was performed using the log rank test.

*Monitoring of the mouse immune response with IFNγ-ELISPOT and flow cytometry immunophenotyping*

An aliquot of 100 µl of heparinised blood was collected from each mouse. The gp33-41 specific CD8+ lymphocytes were labelled with H2-Db-gp33-41 tetramers coupled to phycoerythrin, an anti-CD8 antibody (clone KT15, fluorescein coupled) and an anti-CD44 antibody (clone KM201, APC coupled) for 30 min at room temperature (all reagents from Beckman-Coulter). After red blood cell lysis using iTag MHC tetramer lysis Reagent, cells were washed in PBS and fixed with 1% formaldehyde prior analysis by a Cyan flow cytometer (DAKO-Cytomation). For
surface CD107a labelling, blood cells were incubated with gp33-41 tetramers and an anti-CD107a antibody (clone 1D4B, APC coupled) for 30 minutes as above, before a 5-hour culture at 37°C to allow cell stimulation by the tetramers. One hour after the beginning of the stimulation, 2 µM monensin were added. Then, CD8 labelling was performed for 15 minutes at room temperature, red blood cells were lysed and cells were fixed as above. For splenocytes, 5x10^5 cells were labelled using the same protocol as above, except that red blood cell lysis was performed before labelling using ACK solution (NH₄Cl 0.15 M, KHCO₃, 1 mM, Na₂EDTA 0.1 mM, pH = 7.4). At least 30,000 events were acquired and data was analysed with Summit 4 software (DAKO-Cytomation).

For IFNγ-ELISPOT, ethanol treated PVDF microplates (Millipore) were coated in PBS with an anti-mouse IFN-γ monoclonal antibody (clone AN-18 15 µg/ml). Duplicate aliquots of spleen cells (2 x 10^5/well) were added together with the gp33-41 peptide or a control H2-D^b restricted peptide from the influenza virus nucleoprotein (1 µg/ml) in culture medium supplemented with 30 U/ml of human rIL-2. After overnight culture, plates were washed in PBS with 0.05% Tween 20 and a biotinylated anti-mouse IFN-γ antibody (clone R4-6A2) was added for 18 h at 4°C. Spots were visualized by adding, successively, alkaline-phosphatase-labeled ExtrAvidin (diluted 1/6000, Sigma) for 1 h and substrate (BCPI/NBT, Bio-Rad) for 30 min. The number of spots, each of them representing an IFN-γ-secreting cell, was counted with a transmitted-light stereomicroscope using image-analyzing software connected to a camera (KS ELISPOT system; Carl Zeiss Vision). Results are expressed as the number of spots per 100 CD8+ cells after subtraction of the background obtained with an irrelevant peptide.

**Inhibition of IL4I1 enzymatic activity**

Inhibition of IL4I1 activity was measured on conditioned media from IL4I1 expressing and secreting HEK 293 cells preincubated 5 min on ice with the different inhibitors. IL4I1 phenylalanine oxidative deamination was performed against phenylalanine (control) as in 14, or against phenylalanine in presence of different concentrations of inhibitors of IL4I1 of formula (I). Inhibitors tested are the followings:
Fluorimetric quantification of H$_2$O$_2$ 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). Global non-linear regression analysis and computation of best fit Ki values was performed using the Prism 5 software package (Graphpad).

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

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Concentration (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-phenylalanine ethyl ester (Fig.11)</td>
<td>0.7  1.5  5</td>
</tr>
<tr>
<td>N-acetyl-phenylalanine (Fig.12)</td>
<td>0.7  1.5  5</td>
</tr>
<tr>
<td>2’-aza-phenylalanine (Fig.13)</td>
<td>0.6  1.25  5</td>
</tr>
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</table>
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 Ia 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 Id), mesotheliomas, colorectal carcinomas and testicular germ cell tumors.

**TABLE 1: Immunohistochemical analysis of IL4I1 protein expression in non lymphoid malignancies.**

<table>
<thead>
<tr>
<th>Histology</th>
<th>No. Cases studied</th>
<th>No. Cases with IL4I1 positive tumor cells (% cases)</th>
<th>Proportion of IL4I1 positive TAM (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>&lt;10</td>
<td>10-50</td>
</tr>
<tr>
<td>Colorectal carcinomas</td>
<td>13</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>Gastric carcinomas</td>
<td>5</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>Non small-cell lung carcinomas</td>
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<td>1</td>
</tr>
<tr>
<td>Small-cell lung carcinomas</td>
<td>2</td>
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</tr>
<tr>
<td>Bladder carcinomas</td>
<td>7</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>Ovarian carcinomas</td>
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<td>Mesotheliomas</td>
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<tr>
<td>Renal cell carcinomas</td>
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<tr>
<td>Melanomas</td>
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<tr>
<td>Breast carcinomas</td>
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<tr>
<td>Thyroid carcinomas</td>
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<td>Hepatocellular and/or cholangiocellular carcinomas</td>
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</tr>
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<td>Testicular germ cell tumors</td>
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<td>Prostatic carcinomas</td>
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</tr>
<tr>
<td>Sarcomas</td>
<td>16</td>
<td>8</td>
<td>0</td>
</tr>
</tbody>
</table>
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 14). 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 15, 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.

**TABLE 2:**

Immunohistochemical analysis of IL4I1 protein expression in lymphoid malignancies

<table>
<thead>
<tr>
<th>Histology</th>
<th>No. Cases studied</th>
<th>No. Cases with IL4I1 positive tumor cells (% cases)</th>
<th>Proportion of IL4I1 positive TAM (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>&lt;10</td>
</tr>
<tr>
<td>B-cell lymphoma</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SLL/CLL</td>
<td>10</td>
<td>4 (40%)</td>
<td>2</td>
</tr>
<tr>
<td>Mantlecell</td>
<td>8</td>
<td>0</td>
<td>7</td>
</tr>
<tr>
<td>Follicular</td>
<td>36</td>
<td>23 (64%)</td>
<td>20</td>
</tr>
<tr>
<td>MZL of MALT-type*</td>
<td>4</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>Splenic or nodal MZL</td>
<td>8</td>
<td>0</td>
<td>7</td>
</tr>
<tr>
<td>Plasmacytoma/myeloma</td>
<td>6</td>
<td>0</td>
<td>6</td>
</tr>
<tr>
<td>Burkitt's lymphoma</td>
<td>5</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>PMBL</td>
<td>28</td>
<td>25 (89%)</td>
<td>11</td>
</tr>
<tr>
<td>Non-mediastinal DLBCL</td>
<td>36</td>
<td>6 (17%)</td>
<td>12</td>
</tr>
<tr>
<td>T and NK cell lymphoma</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Peripheral T-cell unspecified</td>
<td>3</td>
<td>0</td>
<td>3</td>
</tr>
</tbody>
</table>
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
NLPHEL 11 10 (91%) 1 4 6

**IL4I1 activity in reactive lymphoid tissues, lymphoid malignancies, melanoma biopsies 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 (fig. 3a), 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 (fig. 3b). 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 or negative lymphoma.

Data from Ma *et al* demonstrated that IL4I1 can be detected in the secretome of HL cell lines *in vitro* 16. 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 tumors presenting a strong expression by TAM, whereas L428 might derive from cHL with strongly IL4I1-positive HRS. We also evaluated IL4I1 activity in frozen biopsies from non-lymphoid tumors. Three different melanoma cases were tested and for
two of them the activity was compared to the activity of normal tissue adjacent to the tumor, since for this type of tumor we could not isolate the IL4I1+ TAM from the IL4irtumor cells (fig. 3e). The results indicated significant differences in IL4I1 activity in melanoma compared to normal skin, confirming that TAM overexpress IL4I1 in comparison to normal tissue macrophages.

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

10 **IL4I1 expression, Treg infiltrate and clinical parameters in follicular lymphoma**

Baseline characteristics and follow-up were available for 23 follicular lymphoma patients (table 3).

**TABLE 3:**

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

<table>
<thead>
<tr>
<th>Parameter</th>
<th>No Patients with &lt;10% IL4I1+ tumor cells</th>
<th>No Patients with &gt;10% IL4I1+ tumor cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>6</td>
<td>4</td>
</tr>
<tr>
<td>Male</td>
<td>7</td>
<td>6</td>
</tr>
<tr>
<td>Age</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;60 years</td>
<td>10</td>
<td>7</td>
</tr>
<tr>
<td>&gt; 60 years</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Bone marrow</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Involved</td>
<td>12</td>
<td>4</td>
</tr>
<tr>
<td>Not involved</td>
<td>1</td>
<td>6</td>
</tr>
<tr>
<td>FLIPI</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0-1</td>
<td>7</td>
<td>5</td>
</tr>
<tr>
<td>2</td>
<td>6</td>
<td>2</td>
</tr>
<tr>
<td>3-5</td>
<td>4</td>
<td>7</td>
</tr>
<tr>
<td>Median follow-up</td>
<td>28 (18-80)</td>
<td>36 (19-106)</td>
</tr>
</tbody>
</table>
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.

We observed a relation between IL4I1 expression and the level of the FoxP3+ infiltrate in the diagnostic biopsy (fig. 4E and 4F). This relation was statistically significant when considering IL4I1 expression in macrophages (\(p=0.04, \chi^2\) method, fig. 4F), suggesting that T regs are less susceptible to IL4I1 toxic activity on T cells. This result is in accordance with recent data from Mougiakakos et al. showing that Tregs present reduced sensitivity to oxidative stress. A high number of Tregs in the tumor infiltrate has previously been associated with a better prognosis. 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).

Maintenance of interactions between follicular lymphoma B cells and follicular T helper cells has been proposed to support proliferation of the malignant clone. IL4I1-mediated T cell inhibition in this setting may suppress the positive stimuli exerted by follicular T helper cells. In line with this, our data suggest that IL4I1 expression in tumor B cell and/or TAM might represent an additional prognosis marker in follicular lymphoma associated with better outcome.
**IL4I1 as a putative anti-inflammatory gene expressed by myeloid cells**

We have previously shown that the purified IL4I1 enzyme was able to inhibit T lymphocyte proliferation *in vitro*. In the current study, we have chosen the THP1 monocytic cell line to overexpress human IL4I1 (THP1-IL4I1), as IL4I1 expression is mainly detected in cells of myeloid origin *in vitro* and *in vivo*. IL4I1 activity in the transfected cells was 895±363 pmoles H₂O₂/10⁵ cells (mean from 5 independent tests). As expected, when THP1-IL4I1 cells were added to a culture of anti-CD3 stimulated PBMC, the level of PBMC proliferation was significantly decreased (37.6 ± 13.9 %, mean of 5 different experiments) in accordance with a decrease in IL2 secretion at day 1 (Fig. 5A & C). Moreover, this inhibition was proportional to the number of THP1-IL4I1 cells added to the culture and detectable for THP1-IL4I1/IZPBMC ratios of as little as 1 per 32 cells (Fig. 5A and data not shown). A similar effect was observed using PBMC stimulated with purified protein derivative (PPD, Fig. 5B). Interestingly, we also measured a decrease in T cell secretion of proinflammatory cytokines (IL6, GM-CSF), IFNγ and inflammatory chemokines (IL8, CXCL8, GROα, CXCL1, MCP1, CCL2 and MCP2, CCL8) as shown in Fig. 5C & D. These results suggest that IL4I1 may exert an immunoregulatory function in inflammatory conditions.

**Differential role of IL4 and IFNγ in IL4I1 induction in B lymphocytes and myeloid cells**

Our results were unexpected since IL4I1 has been originally described in B cells as an IL4-inducible gene. This led us to explore *in vitro* the level of IL4I1 activity in myeloid cells, i.e. monocytes, monocyte-derived DC (Dendritic Cells) and Mφ (Macrophages), versus B cells after stimulation with IL4 and IFNγ (Fig. 6A). IL4 was able to induce a two-fold increase in IL4I1 activity in B lymphocytes, whereas IFNγ did not have any effect. Conversely, IFNγ was a strong inducer of IL4I1 activity in all the myeloid populations, particularly in monocytes, which have a low basal activity (5 folds). No increase of IL4I1 activity in myeloid cells was observed in the presence of IL4, although we were not able to evaluate the IL4 effect on DC since this cytokine is necessary for their differentiation. Thus, IL4I1 can be induced
both in B lymphocytes and myeloid cells, with IL4 and IFNγ exerting dichotomous roles in these populations. However, Mø and DC display much higher levels of IL4I1 activity than B lymphocytes, with activities in a similar range of those measured in the THP1-IL4I1 cell line. As IL4I1 mRNA is known to be induced from the first hours post-IL4 stimulation in B cells, we determined the kinetics of IL4I1 production in myeloid cells. IL4I1 activity continued to increase after 48h of stimulation of DC, Mø or B cells with their respective stimulating cytokine (Fig. 6B), indicating that IL4I1 protein accumulated over time.

**Induction of IL4I1 production in monocytes by Hodgkin cells**

The monocytic cell line THP1 was cultivated for 48h in conditioned media from the Hodgkin lymphoma cell lines KM-H2 and L428 or from the Large B cell lymphoma cell line SU-HDL-4 as control or normal medium (Fig. 7). The media from both Hodgkin cell lines were able to induce a level of IL4I1 expression that was comparable to stimulation of THP1 cells by IFNγ. This expression was partially blocked when neutralising antibodies against IL13 or IFNγ were added to the culture. These results show that soluble factors produced by Hodgkin cells instruct monocytes to express IL4I1. Amongst these factors IL13 and IFNγ, which are known to be produced by Hodgkin cells, could play a role.

**Measurement of the in vivo growth of non-lymphoid tumors displaying IL4I1-expressing-cells in mice**

IL4I1-expressing tumors escape the anti-tumoral immune response (Figure 8A): mice challenged with the IL4I1+ cell line develop tumors with a significantly higher frequency than mice challenged with control tumors in both experimental settings. These results indicate that IL4I1 expression by the tumor facilitate escape from the CD8 T cell response induced by the gp33-41 vaccination.

Confirmation of the role of IL4I1 expression in tumors using a second IL4I1+ B16 clone (Figure 8B): mice challenged with the IL4I1+ cell line develop tumors with a significantly higher frequency than mice challenged with control tumors in both experimental settings. These results confirm that IL4I1 expression by the tumor facilitate escape from the CD8 T cell response induced by the gp33-41 vaccination.
Tumors developed more rapidly in the group challenged with tumor 2 cells than in the group challenged with tumor 1 cells, although the difference was not statistically significant. This might be in accordance with tumor 2 displaying a higher IL4I1 activity in vitro.

There is no intrinsic growth difference between the 3 tumor clones tested (Figure 8C): all mice developed tumors between day 20 and 30 independently from IL4I1 expression. These data indicate that IL4I1 expression does not affect tumor growth in the absence of a specific pre-existing anti-tumor response. Interestingly, the IL4I1 activity was detected in the sera of mice challenged with variable proportions of tumor 2 admixed with control tumor cells, before macroscopic tumor appearance. Thus IL4I1 is secreted and available for systemic T cell inhibition (Figure 9).

**Modulation of the immune response against gp33 by the expression of IL4I1 in tumors**:

The systemic immune response against the tumor epitope gp33-41 was evaluated in the blood and spleen of mice vaccinated with gp33-41 and challenged with high numbers of control tumor cells or IL4I1 expressing tumors (Figure 10). The number of gp33-41 specific T cells increased rapidly (6 days) after control tumor injection, due to restimulation of memory cells (Figure 10A). In contrast, this increase was absent (tumor 2) or considerably less prominent (tumor 1) in mice challenged with the two IL4I1 expressing tumors. The importance of this effect correlated with the level of IL4I1 activity of these tumors. There was no difference between the three groups of mice in the relative number of gp33-41 specific CD44+ memory T cells or surface CD107a+ cytotoxic T cells. However, the decrease in T cells producing IFNγ in response to gp33-41 was significantly more profound than the decrease of total gp33-41 specific T cells (Figure 10B). These data suggest that IL4I1 expression by the tumor inhibit both the proliferation of tumor-specific T cells and their IFNγ-producing capacity.

**Inhibition of IL4I1 enzymatic activity**

Several inhibitors have been tested on the enzymatic activity of IL4I1. As disclosed in figures 11-13, L-phenylalanine ethyl ester (Fig. 11), N-acetyl-phenylalanine
(Fig. 12) and 2'-aza-phenylalanine (Fig. 13) are inhibitors of IL4I1 enzymatic activity.

For each inhibitor, the $K_i$ value has been measured:

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>$K_i$ (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-phenylalanine ethyl ester</td>
<td>3.1</td>
</tr>
<tr>
<td>N-acetyl-phenylalanine</td>
<td>2.1</td>
</tr>
<tr>
<td>2'-aza-phenylalanine</td>
<td>2.8</td>
</tr>
</tbody>
</table>

When tested at inhibitory doses L-phenylalanine ethyl ester and 2'-aza-phenylalanine were toxic on the Jurkat T cell line, whereas N-acetyl-phenylalanine did not affect Jurkat proliferation *in vitro*. The toxic effect on T cells was alleviated by phenylalanine addition indicating that this effect is due to competition of the inhibitor with phenylalanine. Thus T cells seem to be particularly sensitive to variations of phenylalanine concentration.

Another mechanism of IL4I1 T cell suppression might depend on phenylalanine depletion in T cell microenvironment. Moreover, IL4I1 activity naturally produced by the Hodgkin lymphoma cell line L428 was efficiently inhibited by a specific siRNA (Figure 14) with a maximum effect at day 3 after transfection.

In addition to showing that the inhibitors of IL4I1 of the invention are effective, these results confirm that the inhibition of IL4I1 is a promising pathway for the treatment of cancers (excepting follicular lymphoma) displaying IL4I1 expressing cells.
REFERENCES.


Throughout this application, various references describe the state of the art to which this invention pertains. The disclosures of these references are hereby incorporated by reference into the present disclosure.
CLAIMS

1. An inhibitor of IL4I1 for use in a method for treatment of cancer displaying IL4I1-expressing cells in the human or animal body, provided that said cancer is not follicular lymphoma.

2. The inhibitor of IL4I1 according to claim 1, wherein said inhibitor of IL4I1 is selected from the group consisting of
   - an inhibitor of the enzymatic activity of IL4I1 of formula (I)
     \[
     \text{Formula (I)}
     \]
     wherein
     - \(X\) is an aromatic moiety selected from the group consisting of phenyl and pyridyl; said aromatic moiety being optionally substituted with \(\text{NH}_2\) or \(\text{COOH}\),
     - \(Y\) is a linear or branched \(\text{C}_1-\text{C}_6\) alkyl,
     - \(R_1\) is selected from the group consisting of \(\text{H}, \text{methyl}, \text{vinyl}, \text{propargyl}, \text{F}\) and \(\text{CN}\),
     - \(R_2\) is selected from the group consisting of \(\text{NH}_2, \text{NH-Z}, \text{NH-Boc}, \text{NH-methyl}, \text{NH-Cl}, \text{NH-ter-butyl}, \text{NH-OH}\) and \(\text{NH-COCH}_3\),
     - \(R_3\) is selected from the group consisting of \(\text{COOH}; \text{COOR}_4\), wherein \(R_4\) is a linear or branched \(\text{C}_1-\text{C}_6\) alkyl; \(\text{CONH}_2\); \(\text{CO}_2\text{H-P(OH)}\) and \(\text{CO}_2\text{H-SO}_3\text{H}\),
   - an antibody against IL4I1 or a fragment thereof which binds IL4I1 and wherein said antibody against IL4I1 or fragment thereof inhibits IL4I1 enzymatic activity, and
   - an agent down-regulating the expression of IL4I1 in IL4I1-expressing cells.

3. The inhibitor of IL4I1 according to claim 2, wherein the agent down-regulating the expression of IL4I1 in IL4I1-expressing cells is a nucleic acid which interferes...
with the expression of IL4I1, preferably selected from the group comprising antisense molecules and RNA interference molecules.

4. The inhibitor of IL4I1 according to claim 2, wherein the agent down-regulating the expression of IL4I1 in IL4I1-expressing cells is a siRNA comprising the nucleotide sequence as shown in SEQ ID NO: 1.

5. An inhibitor of the enzymatic activity of IL4I1 of formula (I)

   ![Formula Image]

   \( R_1, R_2, R_3 \)

   \( Y \)

   \( X \)

   \( (I) \)

   wherein

   - \( X \) is an aromatic moiety selected from the group consisting of phenyl and pyridyl; said aromatic moiety being optionally substituted with \( \text{NH}_2 \) or \( \text{COOH} \),

   - \( Y \) is a linear or branched \( C_1-C_6 \) alkyl,

   - \( R_1 \) is selected from the group consisting of \( \text{H} \), methyl, vinyl, propargyl, \( \text{F} \) and \( \text{CN} \),

   - \( R_2 \) is selected from the group consisting of \( \text{NH}_2 \), \( \text{NH}-\text{Z} \), \( \text{NH}-\text{Boc} \), \( \text{NH}-\text{methyl} \), \( \text{NH}-\text{Cl} \), \( \text{NH}-\text{ter-butyl} \), \( \text{NH}-\text{OH} \) and \( \text{NH}-\text{COCH}_3 \),

   - \( R_3 \) is selected from the group consisting of \( \text{COOH} \); \( \text{COOR}_4 \), wherein \( R_4 \) is a linear or branched \( C_1-C_6 \) alkyl; \( \text{CONH}_2 \); \( \text{CO}_2\text{H-P(OH)} \) and \( \text{CO}_2\text{H-SO}_3\text{H} \),

   for use in a method for treatment of cancer displaying IL4I1-expressing cells in the human or animal body, provided that said cancer is not follicular lymphoma.

6. The inhibitor of the enzymatic activity of IL4I1 according to claim 5, wherein said inhibitor has a \( K_i \) lower or equal to 3mM, preferably lower or equal to 30µM, and more preferably lower or equal to 10µM.

7. The inhibitor of the enzymatic activity of IL4I1 according to anyone of claims 5-6, wherein said inhibitor is selected from the group consisting of 2-amino-3-phenyl-
propionic acid ethyl ester, 2-acteylamino-3 -phenyl-propionic acid and 2'-aza-
phenylalanine.

8. The inhibitor of the enzymatic activity of IL4I1 according to anyone of claims 5-
6, wherein said inhibitor is 3-(2-pyridyl)-alanine.

9. A pharmaceutical composition comprising an inhibitor of IL4I1 as defined in
anyone of claims 1-8, together with a pharmaceutically-acceptable carrier, for use in
a method for treatment of cancer displaying IL4I1-expressing cells in the human or
animal body, provided that said cancer is not follicular lymphoma.

10. The inhibitor according to anyone of claims 1-8 or the pharmaceutical
composition according to claim 9, wherein said cancer displaying IL4I1-expressing
cells is selected from the group consisting of mesotheliomas, non small-cell lung
carcinomas, colon carcinoma, breast carcinoma, thyroid carcinoma, testicular germ
cell tumors and ovarian carcinoma, displaying IL4I1-expressing cells.

11. The inhibitor according to anyone of claims 1-8 or the pharmaceutical
composition according to claim 9, wherein said cancer displaying IL4I1-expressing
cells is selected from the group consisting of Primary Mediastinal large B-cell
Lymphoma, classical Hodgkin lymphomas, Nodular lymphocyte predominant
Hodgkin's lymphoma, non-mediastinal Diffuse Large B-Cell Lymphoma and Small
Lymphocytic Lymphoma / Chronic Lymphocytic Leukemia, displaying IL4I1-
expressing cells.

follicular lymphoma in the human or animal body, wherein said Hodgkin
lymphoma or follicular lymphoma displays at most 10% of IL4I1-expressing cells.

13. The inhibitor according to anyone of claims 1-8 and 10-12, or the
pharmaceutical composition according to anyone of claims 9-11, wherein said
method for treatment is applied to the human or animal body simultaneously,
separately or sequentially with another method for treatment of cancer selected
from the group comprising surgery, external radiotherapy, chemotherapy, hormone therapy and cytokine therapy.

14. A method for determining the responsiveness of a subject suffering from cancer to an inhibitor of IL4I1 as defined in anyone of claims 1-8, comprising the step of detecting the expression of IL4I1 in a cancer sample obtained from said subject, provided that said cancer is not follicular lymphoma.

15. A method for determining the prognosis of a subject suffering from a cancer, provided that said cancer is not follicular lymphoma, comprising the step of detecting the presence of IL4I1 in a sample obtained from said subject, wherein the presence of IL4I1 indicates that the subject has a poor prognosis.

16. A method for determining the prognosis of a subject suffering from a follicular lymphoma, comprising the step of detecting the presence of IL4I1 in a sample obtained from said subject, wherein the presence of IL4I1 indicates that the subject has a good prognosis.
FIGURE 4

Tumor cells

A

\[ p = 0.02 \]

\[ \star \]

\[ \text{Number of cases} \]

\[ > 10\% \quad \leq 10\% \]

\[ \text{BM+} \quad \text{BM-} \]

B

\[ p = 0.09 \]

\[ \text{n.s.} \]

\[ \text{Number of cases} \]

\[ > 10\% \quad \leq 10\% \]

\[ \text{BM+} \quad \text{BM-} \]

IL4I1 expression

C

\[ p = 0.07 \]

\[ \text{n.s.} \]

\[ \text{Number of cases} \]

\[ > 10\% \quad \leq 10\% \]

\[ \text{Relapse} \quad \text{No relapse} \]

D

\[ p = 0.03 \]

\[ \star \]

\[ \text{Number of cases} \]

\[ > 10\% \quad \leq 10\% \]

\[ \text{Relapse} \quad \text{No relapse} \]

IL4I1+ cells

E

\[ p = 0.22 \]

\[ \text{Number of cases} \]

\[ > 10\% \quad 5-10\% \quad < 5\% \]

\[ \text{FoxP3+ infiltrate} \]

F

\[ p = 0.04 \]

\[ \text{Number of cases} \]

\[ > 10\% \quad 5-10\% \quad < 5\% \]
FIGURE 9

FIGURE 10

A

B

control

IFNg

CD44*
H2-D^b gp33^*
CD107^*
H2-D^b gp33^*
FIGURE 13

[Graph showing pmoles H2O2/2h vs. phe with different concentrations of KI (0, 1.2 mM, 0.6 mM, 5 mM).]

FIGURE 14

[Bar graph showing pmoles H2O2/h/10 μg proteins vs. days post-transfection for control siRNA and IL4I1 siRNA.]
# INTERNATIONAL SEARCH REPORT

**International application No**

PCT/EP2009/066873

## A. CLASSIFICATION OF SUBJECT MATTER

<table>
<thead>
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<th>INV.</th>
<th>Classification</th>
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According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, CHEMABS Data, WPI Data

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

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* Special categories of cited documents

A* document defining the general state of the art which is not considered to be of particular relevance

E earlier document but published on or after the international filing date

L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

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P* document published prior to the international filing date but later than the priority date claimed

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X* document of particular relevance the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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X* document member of the same patent family

Date of the actual completion of the international search

9 February 2010

Date of mailing of the international search report

16/02/2010

Name and mailing address of the ISA/

European Patent Office, P B 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel (+31-70) 340-2040
Fax (+31-70) 340-3016

Authorized officer

Dami ani, Federica
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<td>METZ RICHARD ET AL: &quot;Novel tryptophan catabolic enzyme ID02 is the preferred biochemical target of the antitumor indoleamine 2,3-di oxygenase inhibitory compound D-1-methyl-tryptophan.&quot; CANCER RESEARCH 1 AUG 2007, vol. 67, no. 15, 1 August 2007 (2007-08-01), pages 7082-7087, XP002519668 ISSN: 0008-5472 cited in the application abstract page 7082</td>
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