ORIGINAL ARTICLE

NKp46 is a diagnostic biomarker and may be a therapeutic target in gastrointestinal T-cell lymphoproliferative diseases: a CELAC study

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ABSTRACT

Objectives Primary GI T-cell lymphoproliferative diseases (T-LPD) are heterogeneous entities, which raise difficult diagnosis and therapeutic challenges. We have recently provided evidences that lymphomas complicating coeliac disease (CD) arise from innate-like lymphocytes, which may carry NK receptors (NKRs).

Design NKRs expression was compared by flow cytometry in intraepithelial lymphocytes (IEL) from CD, type I or type II refractory CD (RCD). NKp46 was next assessed by immunohistochemistry in paraffin-embedded biopsies from 204 patients with CD, RCDI, RCDII or GI T-cell lymphomas and from a validation cohort of 61 patients. The cytotoxic properties of an anti-NKp46 monoclonal antibody conjugated to pyrrolobenzodiazepine (PBD) was tested *ex vivo* in human primary tumour cells isolated from fresh duodenal biopsies.

Results NKp46 (but not CD94, NKG2A, NKG2C, NKG2D) was significantly more expressed by malignant RCDII IEL than by normal IEL in CD and RCDI. In paraffin biopsies, detection of >25 NKp46+ IEL per 100 epithelial cells discriminated RCDII from CD and RCDI. NKp46 was also detected in enteropathy-associated T-cell lymphomas (EATL, 24/29) and in monomorphic epitheliotropic intestinal T-cell lymphomas (MEITL, 4/4) but not in indolent T-LPD (0/15). Treatment with anti-NKp46-PBD could efficiently and selectively kill human NKp46+ primary IEL *ex vivo*.

Conclusion NKp46 is a novel biomarker useful for diagnosis and therapeutic stratification of GI T-LPD. Strong preclinical rationale identifies anti-NKp46-PBD as a promising therapy for RCDII, EATL and MEITL.

Significance of this study

What is already known on this subject?

- Type II refractory coeliac disease (RCDII) is a severe complication of coeliac disease (CD), frequently progressing to a fatal enteropathy-associated T-cell lymphoma (EATL).
- RCDII is difficult to discriminate from benign conditions such as CD and type I RCD (RCDI).
- RCDII arise from innate-like lymphocytes carrying NK receptors (NKRs).

What are the new findings?

- Expression of a single NKR, NKp46, is a hallmark of the malignant intestinal lymphocytes, which characterise monoclonal RCDII and aggressive forms of intestinal T-cell lymphomas (EATL and MEITL).
- In paraffin biopsies, detection of >25 NKp46+ intraepithelial lymphocytes (IEL) per 100 epithelial cells discriminated RCDII from CD and RCDI.
- Coherent with the severe outcome of NKp46+ lymphoproliferations, NKp46 expression was associated with shortened survival in both CD complications and in overt intestinal T-cell lymphomas.

How might it impact on clinical practice in the foreseeable future?

- As NKp46 can be easily detected in formol-fixed tissue sections, it provides a useful diagnostic marker adapted to immediate patient management to identify RCDII and aggressive versus indolent intestinal T-cell lymphomas.
- As therapeutic options available in primary gastrointestinal T-cell lymphomas are currently disappointing, the benefit of targeting NKp46 using antibody drug conjugated should be further investigated.

INTRODUCTION

Primary GI T-cell lymphoproliferative diseases (T-LPD) are heterogeneous and diagnostically challenging entities.[1] Best defined entities are two highly aggressive lymphomas characterised by a 5-year survival rate shorter than 20%,[2] the

enteropathy-associated T-cell lymphoma (EATL), commonly associated with coeliac disease (CD), and the monomorphic epitheliotropic intestinal T-cell lymphomas (MEITL), not associated with CD and more prevalent in Asia.[1] Because of their epitheliotropism and CD103 expression, both EATL and MEITL are thought to derive from intraepithelial lymphocytes (IEL).[3] However, MEITL display a predominant positive T-cell receptor (TCR) phenotype associated with CD8, CD56 and SYK expression[3] that are generally lacking in EATL. In 2016, the revised WHO classification of lymphoid neoplasms included a novel entity named indolent T-cell lymphoproliferative disease of the GI tract (indolent T-LPD) that is characterised by a good outcome.[1 4–8] In contrast to EATL and MEITL, indolent T-LPD tumour cells are made of small and mature TCRαβ+ T cells that express CD4 or CD8 and infiltrate predominantly the *lamina propria* where they likely arise.[1 4–8] Clinical and histological presentation may however mimic EATL or MEITL, leading to misdiagnosis and unnecessary aggressive treatment. Finally, besides these three malignant entities, an additional condition is type II refractory CD (RCDII). As EATL, which it often precedes, this condition develops in a small subset of patients with CD, who become refractory to the gluten-free diet (GFD). In contrast with EATL, malignant cells have a normal cytological appearance and they do not divide actively. As they predominantly infiltrate the gut epithelium, RCDII can be viewed as an intraepithelial lymphoma. More specifically, RCDII is characterised by a clonal expansion of IEL displaying clonal TCRγ rearrangements and intracellular CD3 (iCD3) but no surface CD3 (sCD3)-TCR complexes. Prognosis is poor due to the frequent severe malnutrition refractory to GFD and to the risk of transformation into overt clonally related EATL.[9–15] Diagnosis of RCDII is very challenging, as regular morphological analysis does not allow differentiating RCDII malignant IEL from the normal IEL, that infiltrate the gut in CD. RCDII can notably be difficult to differentiate from slow response to GFD and from type I refractory CD (RCDI), a non-malignant complication of CD of as yet poorly understood mechanism.[13 16]

In order to allow the accurate diagnosis of GI T-LPD that is indispensable to adjust therapy, we have developed a multidisciplinary diagnostic approach combining immunohistochemistry and molecular *TCR* gene rearrangement analyses in biopsies with multiparameter flow cytometry (MFC) on lymphocytes isolated from fresh duodenal biopsies.[11 12 17 18] However, this approach is time consuming and IEL MFC is only available in a small number of specialised centres, emphasising the need to develop diagnostic criteria adapted to individual patient management. We recently demonstrated that RCDII IEL arise from a subset of innate-like lymphocytes with dual T and natural killer (NK) cell traits that are imprinted during their differentiation in response to combined signals from NOTCH and interleukin-15 (IL-15).[19]

We therefore investigated whether NK receptors (NKRs) might be used as a diagnosis marker to simplify the diagnosis of lymphomas complicating CD but also to stratify primary GI T-LPD according to their origin and/or prognosis. In addition, we hypothesised that NKRs could serve as novel therapeutic targets in these debilitating diseases with a dismal prognosis.

METHODS AND PATIENTS

Patients and clinical data

The records of 84 CD or RCD and 48 GI T-cell lymphoma patients enrolled prospectively in the French national centres experts des lymphomes associés à la maladie coeliaque (CELAC) registry until May 2017 were reviewed (see online supplementary figure 1). Intestinal biopsies were obtained according to INSERM protocol C08-34. The observation time was the interval between diagnosis and last contact (last follow-up or death). Mean follow-up was 4.7 years. Diagnosis of CD was based on HLA-DQ2/8 typing, detection of coeliac-specific antibodies, such as antitissue transglutaminase, anti-endomysial antibody and presence of villous atrophy with an increase of IEL in intestinal biopsies. RCD was defined by persistent malnutrition syndrome and villous atrophy after 1 year of strict adherence to GFD and was further divided into RCDI and RCDII. RCDI was diagnosed in the absence of detectable monoclonal TCRγ rearrangement with normal CD8+ TIEL phenotype. Conversely, RCDII was characterised by monoclonal TCRγ rearrangement associated with abnormal IEL phenotype, defined by >50% CD3+ but CD8− IEL by IHC of formalin-fixed paraffin-embedded (FFPE) sections, and/or >25% CD45+ IEL negative for sCD3 by MFC after isolation from fresh biopsies.[11–13 16] Flow cytometry can notably differentiate normal T cells with sCD3-TCR complexes from RCDII malignant IEL, which display iCD3 but generally neither sCD3 nor CD8. EATL and MEITL were diagnosed according to the revised WHO classification of lymphoid neoplasia.[1] While EATL generally manifests with CD30+ cells and wide range cytology features, MEITL was monomorphic, with marked epitheliotropism and usually CD8+CD56+ small-sized to medium-sized cells. Indolent T-LPD was diagnosed as previously described[7 8] and is characterised by a dense but non-destructive lymphoid infiltrate of small CD4+ or CD8+ lymphoid cells, with monoclonal *TCRγ* rearrangement. Intestinal biopsies from healthy controls (n=13), as well as from patients with non-coeliac enteropathies (n=12) were obtained in accordance with National guidelines. The validation cohort consisted of 61 CD, RCDI and RCDII patients from VU University Medical Center (Amsterdam, the Netherlands) diagnosed between June 2002 and January 2017 (45 cases) and from Necker Hospital (Paris, France) from May to October 2017 (16 cases). An expert panel of pathologists (JB, NB, VV) blinded to NKp46 status reviewed all cases.

Multiparameter flow cytometry

IEL were isolated from fresh duodenal biopsies and multiparameter staining of lymphocytes was performed as previously described[11 12 17] with 8-colour mixes of antibodies against CD3ε, CD4, CD8α, CD45, CD103, TCRαβ, TCRγδ, CD122, CD127, CD56, CD16, NKp46, NKG2A, CD94, NKG2C, NKG2D. Data were analysed on a FACSCanto II using FlowJo V.10.2 software (BD Biosciences).

Immunohistochemistry

A minimum of four separate FFPE (10% formalin) GI biopsies were stained with H&E and reviewed by three expert pathologists (JB, NB and VV). Villous atrophy was graded and counts of IEL enumerated according to Oberhuber *et al*.[20 21] Antigen detection included CD3, CD8, CD30, CD4, Granzyme B, CD103 and NKp46 (8E5B, Innate Pharma). For production of anti-NKp46 antibody, BALB/c female mice (aged 12 weeks) were immunised with the human-NKp46-Fc protein. Serum and hybridoma supernatants were screened by IHC on FFPE RAJI and RAJI-NKp46 cell pellets. The RAJI-NKp46 cells are RAJI cells transfected with lentiviral particles encoding human NKp46. Following screening, one candidate was selected and the monoclonal antibody (clone 8E5B) was produced (see online supplementary figure 2). The number of NKp46+ IEL per 100 epithelial cells (NKp46+ IEL/100 EC) was assessed on well-orientated serial sections by counting about 500 EC. In EATL, MEITL and

indolent T-LPD, NKp46 expression, count of NKp46+ large cells and distribution (membrane and intracellular) were assessed on neoplastic cells.

Conjugate preparation

8B6A-PBD is a mouse monoclonal anti-NKp46 antibody drug conjugated (ADC) to the selective DNA alkylating compound pyrrolobenzodiazepine dimers (PBD, SG-1882 ref Kung Sutherland Blood 2013) with a drug antibody ratio of 1.9, according to a protocol adapted from Lhospice *et al*.[22]

Apoptosis and internalisation assays

For *ex vivo* cytotoxicity assays, primary cells isolated from fresh duodenal biopsies were incubated for 96 hours with IL-15 added or not with 8B6A-PBD, control isotype (IC)-PBD, deglycosylated anti-NKp46 antibody (two negative controls) or PBD (positive control). Cell viability was measured using Annexin V-FITC and red-fluorescent propidium iodide. To study internalisation, anti-NKp46 and IC were labelled with the pH-sensitive fluorescent dye CypHer5E. The CypHer5E signal was analysed in sCD3 CD103+NKp46+ cells, enabling robust discrimination of antibody internalisation from surface binding.

Statistical analysis

Continuous data were summarised using median and compared using the non-parametric Mann-Whitney U test because of skewed distributions. CIs were estimated using a continuity correction. Graphics and statistical analysis were performed using GraphPad/Prism7 and R software. P<0.05 was considered to be statistically significant.

Analysis on CD and RCD discovery cohort

Receiver operating characteristic (ROC) curve analysis was performed to calculate the performance of the NKp46 marker to predict RCDII and to define the NKp46+ IEL/100 EC threshold optimising sensitivity and specificity. Thereafter, two groups were defined in the patients with CD and RCD, depending on this threshold. Survivals were estimated using the Kaplan-Meier method on these two groups and comparisons were made using the log-rank test.

Analysis on RCDII subgroup

Cox's regression was used to assess the association between the NKp46+ IEL/100 EC value and survival and/or EATL transformation. Comparisons between RCDII with NKp46+ IEL/100 EC below and above 70 were made using the log-rank test.

Analysis of the validation cohort

NKp46 expression was assessed blinded on IEL. Using the previously defined cut-off of 25 NKp46+ IEL/100 EC, ROC curve analysis was performed and sensitivity and specificity were calculated.

Analysis on primary GI T-cell lymphoma

The NKp46 was assessed as positive or negative to define two groups. Survivals were estimated using the Kaplan-Meier method on these two groups and comparisons were made using the log-rank test.

Analysis on *ex vivo* experiments

Percentages of remaining alive tumour cells were compared using the non-parametric Mann-Whitney U test.

RESULTS

At diagnosis, NKp46 (CD335) expression discriminates RCDII from patients with CD and RCDI

Expression of NKRs was first compared on IEL sorted from fresh duodenal biopsies in 15 patients with CD, 7 patients with RCDI and 21 patients with RCDII by MFC. IEL were defined by their FSC/SSC properties and CD45 positivity after excluding doublets. RCDII cells were further identified based on their positivity for CD103 and iCD3 and negativity for sCD3. In all tested patients, NKp46 was expressed by the majority of RCDII IEL while it was only rarely expressed by normal T-IEL (see online supplementary figure 3). Accordingly, the frequency of NKp46+IEL in CD45+ cells was significantly higher in RCDII samples (median 63.3% (22.7%–95.2%)) than in CD and in RCDI samples in which the vast majority of IEL are conventional T cells (median 9.2% and 9.7%, respectively, p<0.0001). In contrast, the distribution of other NKRs (CD94, NKG2A, NKG2C and NKG2D) had a less discriminating power between RCDII and RCDI or CD, likely because the latter NKRs are expressed both by RCDII IEL and normal T-IEL in active CD and in RCDI (p=0.0148, p=not significant (ns), p=0.0148 and p=ns for CD94, NKG2A, NKG2C and NKG2D, respectively) (figure 1A).[19 23]

NKp46 expression was next assessed by IHC in FFPE small bowel biopsies from 14 patients with active CD (ACD), 23 patients with RCDI and 34 patients with RCDII at diagnosis, as well as in 13 histologically normal controls and in 12 patients with non-coeliac enteropathies. Morphological and phenotypic characteristics determined by IHC, counts of NKp46+ IEL in CD45+ cells by MFC and *TCR* rearrangement in patients with CD, RCDI and RCDII are shown in supplementary table 1. Median counts of IEL were 53 per 100 EC (20–120) in ACD and RCDI, and 77.5 per 100 EC in RCDII (25–150)). A monoclonal *TCRγ* rearrangement was found in all patients with RCDII and in only one patient suspect of RCDI. In this patient, resistance to GFD was however previously ascribed to the mucosal localisation of leukaemic CD3+TCRαβ+CD8+CD57+ large granular lymphocytes, which explained the detection of the monoclonal *TCRγ* rearrangement.[24] In all patients with ACD and RCDI, the frequency of NKp46+ IEL was low (median: 2.9% among total IEL, (0%–6.4%)). In contrast, frequency of NKp46+ IEL among total IEL was significantly higher in patients with newly diagnosed RCDII (median: 94.7% (80.6%–100%) (p<0.0001)). Abnormal IEL displayed often strong and membranous NKp46 positivity, except in four RCDII cases, which presented with granular NKp46 expression characterised by intracellular dots (figure 1C). Counts of NKp46+ IEL correlated with that of aberrant CD8− IEL (Pearson's correlation coefficient r=0.57, p=0.0004) (see online supplementary table 1 and online supplementary figure 4).

Coherent with these findings, median counts of NKp46+ IEL/100 EC were higher in RCDII than in patients with CD and RCDI (68 (25–150) vs 3 (0–15), p<0.0001) (figure 1B). Median counts of NKp46+ IEL/100 EC were also low in histologically normal controls (5 (0–15)) and in patients with non-coeliac enteropathies (7.5 (0–20)) (figure 2C and online supplementary table 2). Counts of NKp46+ IEL determined by IHC correlated with frequencies of NKp46+ IEL defined by MFC (Pearson's correlation coefficient r=0.76, p<0.0001) (online supplementary figure 5).

ROC analysis showed that a threshold of 25 NKp46+ IEL/100 EC perfectly discriminated RCDII from patients with CD and RCDI, with positive and negative predictive values of 100%

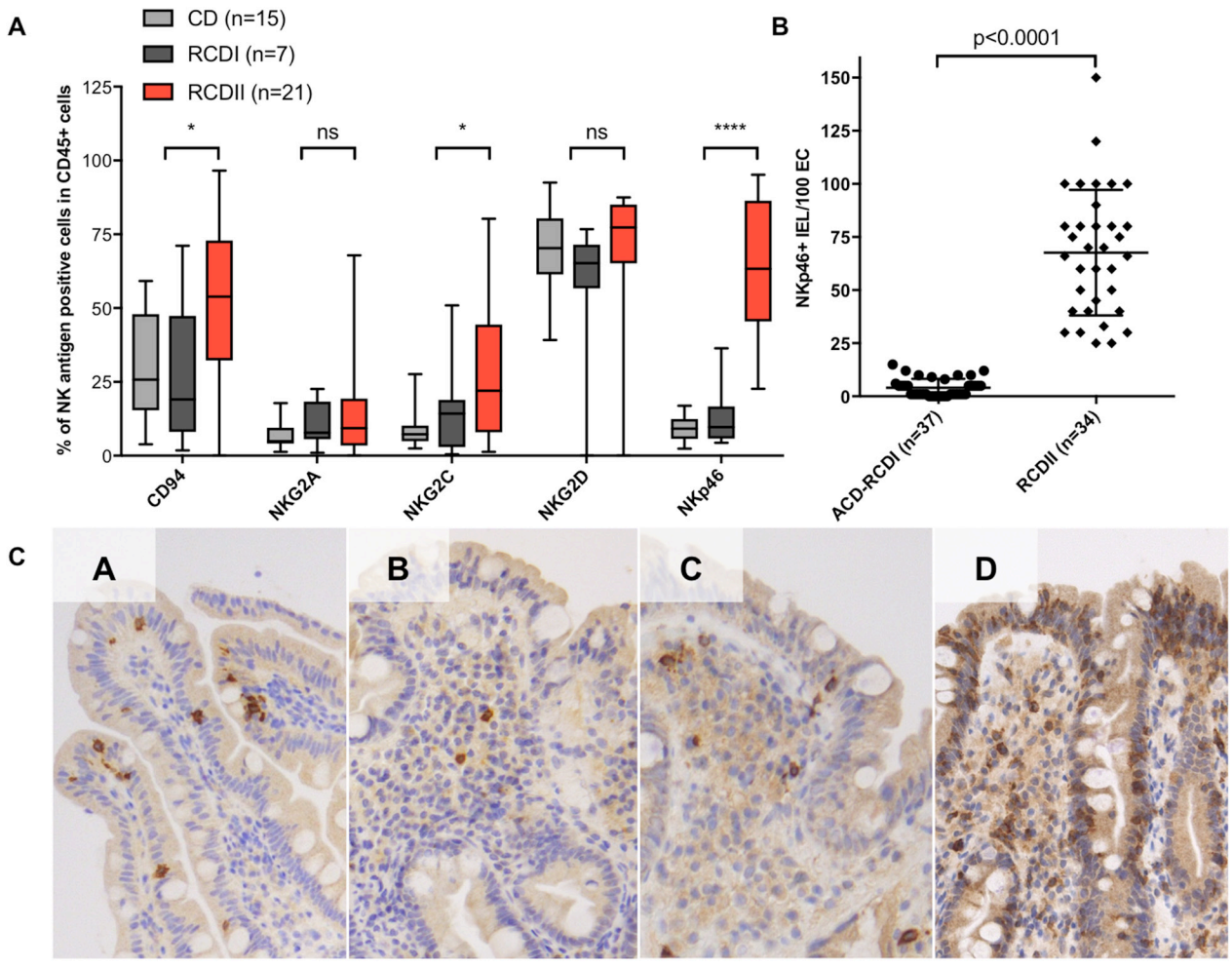

Figure 1 Expression of natural killer receptors (NKRs) by type II refractory coeliac disease (RCDII) intraepithelial lymphocytes (IEL). (A) Frequency of cells expressing indicated NKRs among CD45+ IEL isolated from fresh duodenal biopsies in CD (n=15), type I refractory CD (RCDI, n=7) and RCDII (red, n=21) by multiparameter flow cytometry (MFC). Medians and ranges are shown. (B) Number of NKp46+ IEL/100 epithelial cells (EC) in patients with CD, RCDI or RCDII at diagnosis, by immunohistochemistry (IHC). (C) NKp46 expression by IHC in representative intestinal paraffin-embedded biopsies (×200) from control (A), patients with CD (B), patients with RCDI (C) and patients with RCDII (D). ns, not significant.

(95% CI 88 to 100) and area under curve of 1 (figure 2A). Accordingly and in contrast with patients with CD or RCDI, all patients with RCDII had >25 NKp46+ IEL/100 EC (figure 1B and online supplementary table 1). In the validation cohort, which consisted of 26 patients with RCDII and 35 patients with CD or RCDI, ROC area under curve was estimated to be 0.916, p<0.0001 (figure 2B). Using the cut-off of 25 NKp46+ IEL/100 EC, the predictive performance in the validation cohort assessed blinded was high, with positive and negative predictive values of 95% (95% CI 75 to 100) and 87% (95% CI 72 to 95), respectively (figure 2C and online supplementary table 3). According to NKp46 expression, there were five false negative cases, as five RCDII cases were misclassified as patients with CD or RCDI (online supplementary figure 6). However, extensive analysis on these cases showed that in fact two cases could be reclassified as CD/RCDI, as case A12 was polyclonal and case A13 had only 20% abnormal IEL by MFC with negative coeliac serology. One case (A29) was classified as RCDII given the increased frequency of malignant IEL by MFC (37% CD7+sCD3−iCD3+IEL), but had partial villous atrophy and low counts of IEL by IHC (30 IEL/100 EC), that was concordant with the count of NKp46+ IEL assessed blinded by IHC (20 NKp46+ IEL/100 EC). Despite the apparent subnormal biopsy, this patient was considered as a false-negative case because the count of NKp46+ IEL/100 EC was under the threshold of 25. Of note, >50% of IEL were NKp46+ by IHC that is concordant with RCDII diagnosis definition[12] (see online supplementary table 4). According to NKp46 expression, there was also one false-positive case, as one RCDI case was misclassified as patient with RCDII. In conclusion, after centralised review by both teams (Necker and VU hospital), NKp46 expression assessment was able to reclassify most equivocal cases.

In line and as expected with the reduced survival of patients with RCDII,[13] counts of NKp46+ IEL over 25 NKp46+ IEL/100 EC correlated with significantly reduced overall survival (OS) (5-year rate of OS 74.8% vs 96.4%, p=0.0007) (see online supplementary figure 7A). Analysis using Cox model did not however reveal any association between NKp46+ IEL counts and either EATL occurrence or death in patients with RCDII (see online supplementary figure 8). Twenty sequential biopsies of six patients with RCDII were analysed after specific treatments. Median NKp46+ IEL/100 EC counts decreased from 68 (25–150) in newly diagnosed patients with RCDII to 30 (0–100) (p=0.0008) after treatment (figure 2C).

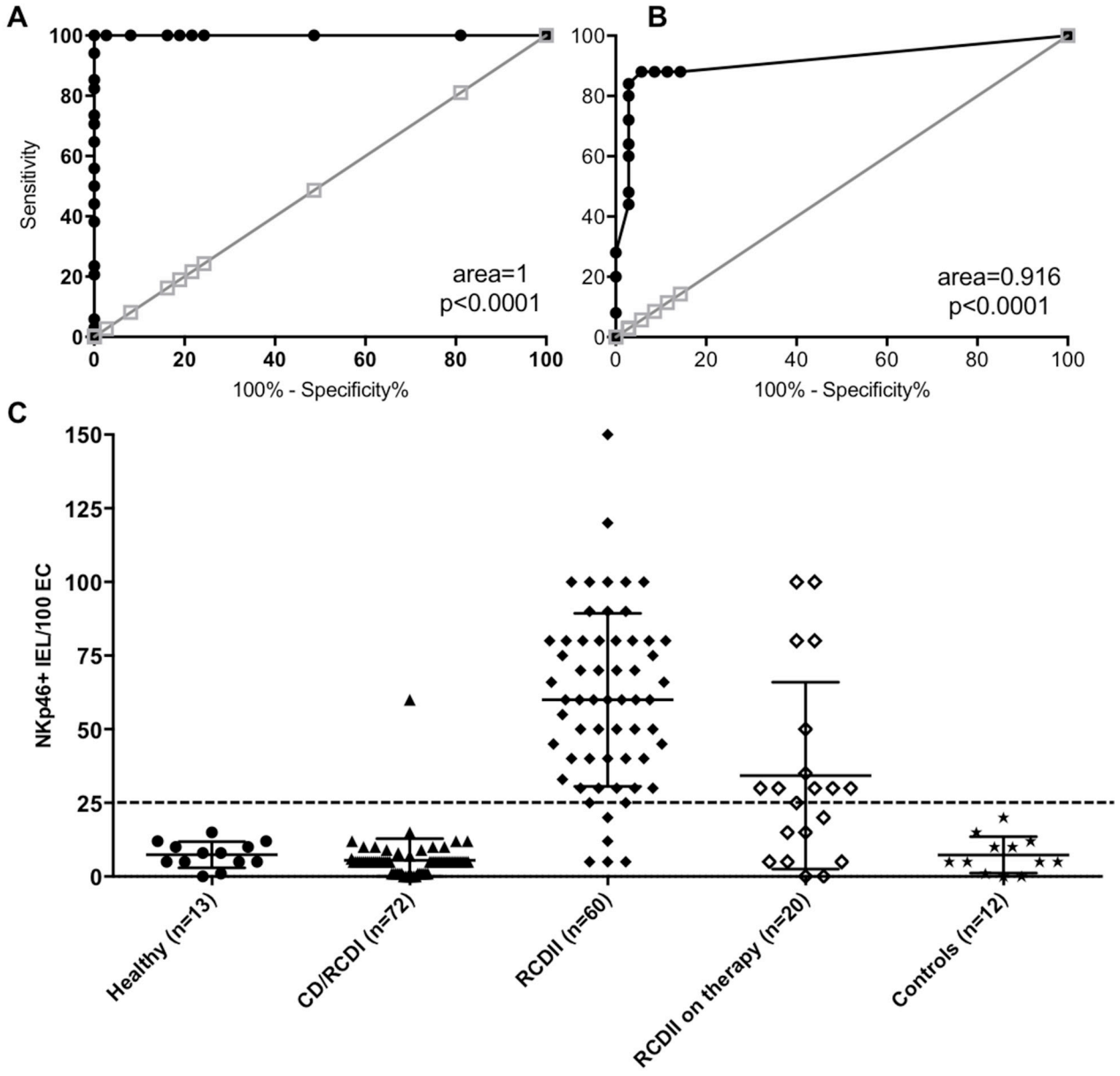

Figure 2 Selective expression of NKp46 by type II refractory coeliac disease (RCDII) intraepithelial lymphocytes (IEL). (A) Receiver operating characteristic (ROC) curve analysing the predictive value of counts of NKp46+ IEL/100 epithelial cells (EC) for differentiating RCDII from CD and RCDI at diagnosis in the discovery cohort. The estimated area under the ROC curve is 1 for a threshold of 25 NKp46+ IEL/100 EC count (p<0.0001). (B) ROC curve analysing the predictive value of counts of NKp46+ IEL/100 EC for differentiating RCDII from CD and RCDI at diagnosis in the validation cohort. The estimated area under the ROC curve is 0.916 for a threshold of 25 NKp46+ IEL/100 EC count (p<0.0001). (C) Discovery and validation cohorts: numbers of NKp46+ IEL/100 EC in histologically normal controls (n=13), CD and RCDI (n=72), RCDII at diagnosis (n=60) and after treatment (n=20), and in control patients with non-coeliac enteropathies (n=12). Each dot represents a different patient. The dotted line represents the diagnostic cut-off identified by ROC analysis.

NKp46 is selectively expressed in aggressive subtypes of primary GI T-cell lymphoma

A total of 48 patients with newly diagnosed EATL (n=29), MEITL (n=4) or indolent T-LPD (n=15) were included. Histologically normal lymph node (LN) and tonsils were used as control tissues. The 29 EATL (19 intestine, 5 LN and 5 other tissues) were all characterised by an infiltrate of large tumour cells that were usually CD8− and CD56− but CD30+. The four MEITL cases presented with CD3+CD8+CD56+ small-sized to medium-sized tumour cells. The 15 patients with indolent T-LPD were characterised by a non-destructive intestinal T-cell infiltrate that expressed CD4 in 13 and CD8 in 2 cases.

While NKp46 was only expressed by rare lymphocytes in normal lymphoid tissues, NKp46 was expressed in 83% of EATL (n=24/29) and 100% of MEITL cases (n=4/4) (figure 3). In EATL cases, frequencies of tumour NKp46+ large cells varied between 50% and 100% of tumour cells with a majority of patients having >80% of NKp46+ tumour cells (n=21/24) (see online supplementary table 5). NKp46 staining was heterogeneous with weak to strong expression. NKp46 staining was

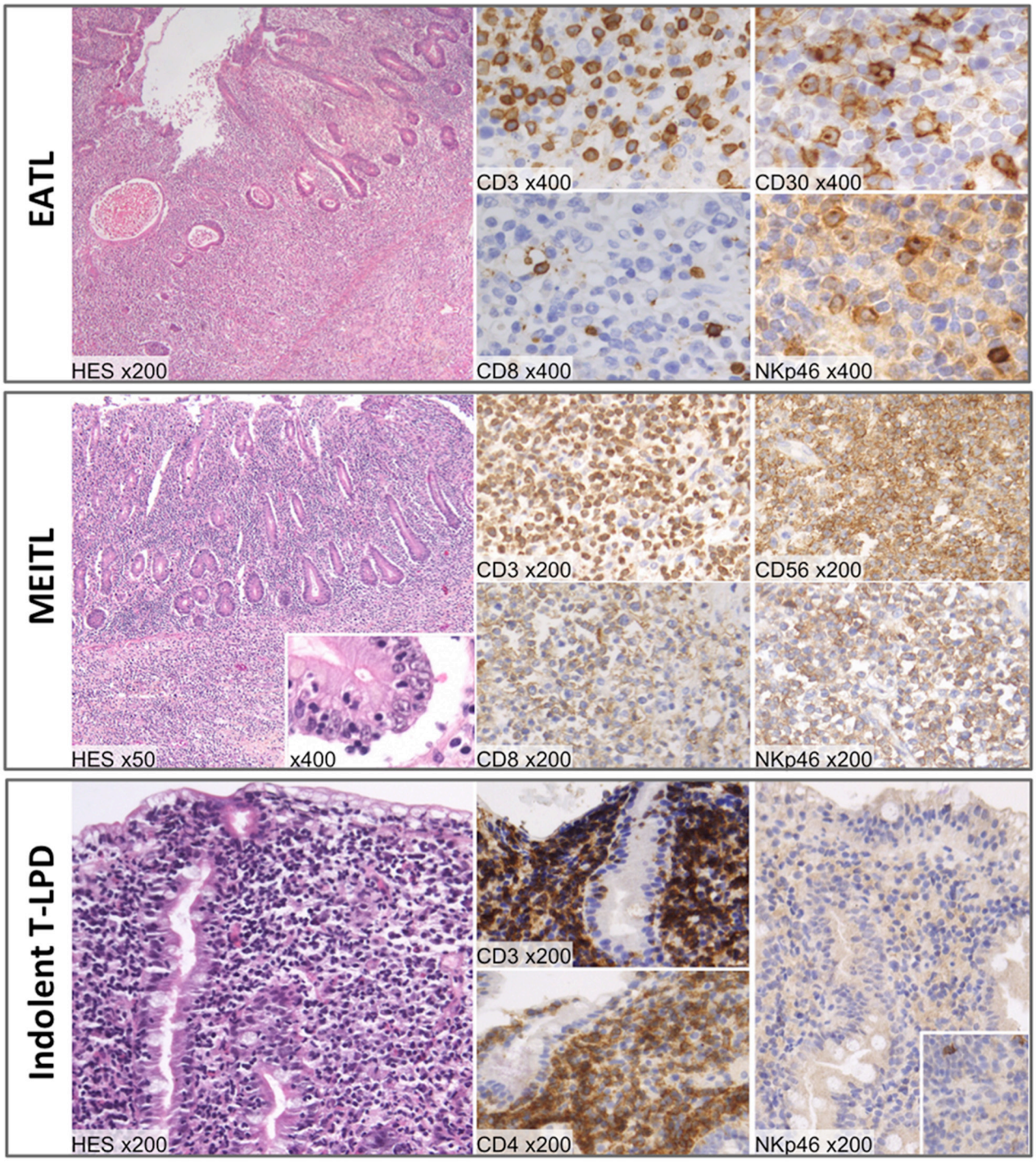

Figure 3 NKp46 immunohistochemical staining in primary GI T-cell lymphomas. Representative histology (H&E) and immunohistochemical staining of paraffin-embedded biopsies in primary GI T-cell lymphomas. Enteropathy-associated T-cell lymphoma (EATL): architecture is disrupted by diffuse infiltrate (H&E ×200) of large lymphoid tumour cells positive for CD3, CD30 and negative for CD8. Tumour cells strongly express NKp46, with a dotted pattern. Monomorphic epitheliotropic intestinal T-cell lymphoma (MEITL) in one duodenal biopsy: infiltrate of lymphoid tumour cells displaying epitheliotropism (H&E ×400) and positive for CD3, CD8, CD56 and NKp46. Indolent T-LPD in one duodenal biopsy: massive *lamina propria* infiltrate of lymphoid tumour cells positive for CD3 and CD4 but negative for NKp46.

either membranous (n=12/24), intracellular with positive dots (n=6/24) or both membranous and intracellular (n=6/24). Among the 24 NKp46+ EATL, 11 cases complicated CD or RCDI and 13 RCDII. Among the 5 NKp46− EATL, two complicated RCDII and two CD or RCDI (in one patient, history was not available) (see online supplementary figure 9). We were able to assess *TCRβ*, *TCRδ* and *TCRγ* gene rearrangements in 13 EATL. In 9 out of 13 cases, *TCR* rearrangements were incomplete. Complete VDJ rearrangement of *TCRβ* was observed only in three patients, including the two NKp46− EATL cases which were analysed for *TCR* gene rearrangement (see online supplementary table 5). All four cases of MEITL expressed NKp46. The frequencies of MEITL NKp46+ tumour cells varied between 50% and 100% of tumour cells (see online supplementary table

5. NKp46 expression was membranous with an intermediate to strong intensity in the four patients. In contrast, NKp46 was never expressed by indolent T-LPD cases (n=0/15) (figure 3).

In line with the selective expression of NKp46 on EATL and MEITL, which have a much poorer prognosis than indolent T-LPD cases, the 5-year rate of OS was significantly shorter in NKp46+ cases compared with NKp46− cases (5-year OS 5.4% vs 46.8%, p=0.0023) (see online supplementary figure 7B). Of note, in patients with EATL, prognosis was similar regardless of NKp46 expression (see online supplementary figure 10).

Finally, we analysed the NKp46 expression in other peripheral T-cell lymphoma (PTCL) as they may occasionally affect the GI tract. NKp46 was strongly expressed by NK/T-cell lymphomas (n=32/38) and weakly by anaplastic large-cell lymphomas (n=5/7). NKp46 was rarely expressed in other PTCL (PTCL, NOS n=1/10; angioimmunoblastic T-cell lymphoma n=0/9; adult T-cell leukaemia/lymphoma n=2/8) (data not shown).

8B6A-PBD efficiently eliminates NKp46+ primary tumour cells

As a proof of concept, a monoclonal antibody conjugated to PBD, directed against NKp46, was assessed on NKp46+ primary malignant IEL. *Ex vivo* analysis of RCDII IEL freshly isolated from duodenal biopsies demonstrated efficient internalisation of anti-NKp46-CypHer5E as shown by the sixfold increase of median fluorescent intensity after a 24-hour incubation compared with cells incubated with IC-CypHer5E (figure 4A). Accordingly, freshly isolated primary NKp46+ RCDII IEL were efficiently killed by 8B6A-PDB but not by IC-PDB and, after 96 hours, apoptosis induced by 8B6A-PBD in NKp46+ cells was similar to that induced by PBD alone at a 50-fold higher molar concentration (p=0.03) (figure 4B). The effect was target specific, since normal residual sCD3+CD7+ IEL which did not express detectable levels of the NKp46 antigen, were unaffected by the conjugate (see online supplementary figure 11).

DISCUSSION

The classification, pathophysiology and prognosis of primary intestinal T-LPDs merit further investigation since therapeutic options are not yet standardised. Based on the retrospective analysis of the CELAC cohort, a large French National Registry, and on a validation cohort from the Netherlands, we show that NKp46 expression is a useful biomarker for identifying malignant complications of CD and for refining diagnosis in the heterogeneous group of GI T-LPD. In addition, we provide

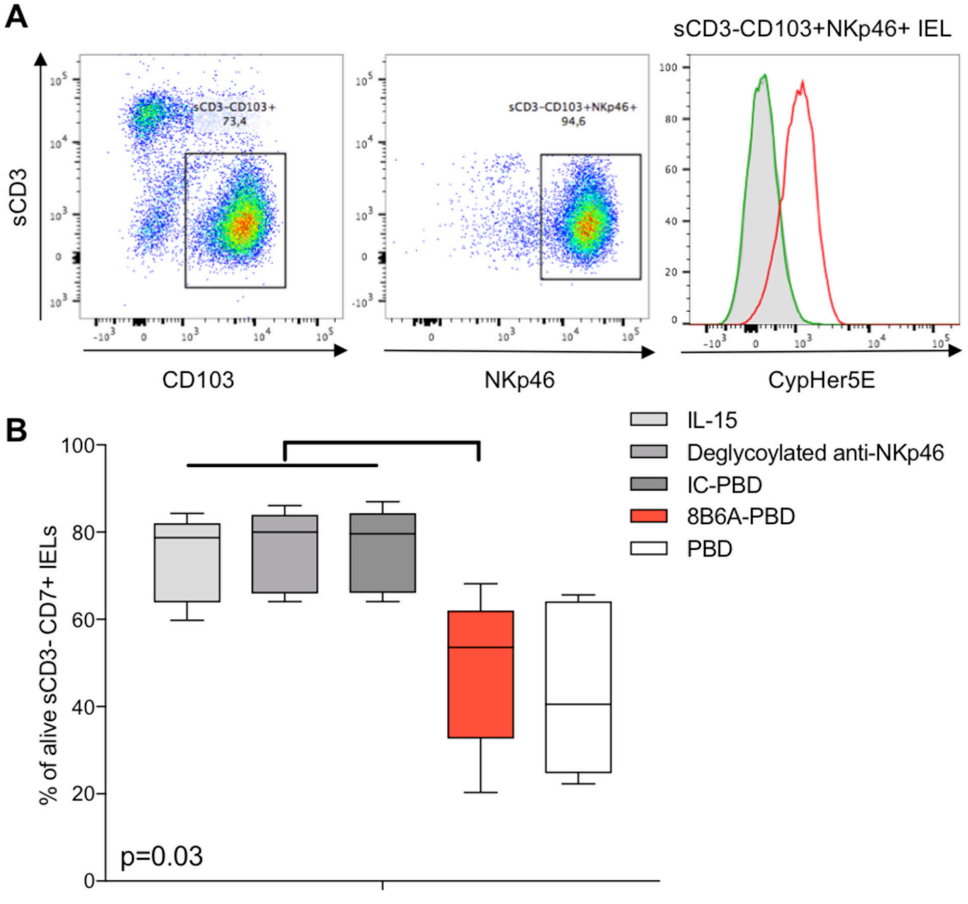

Figure 4 Anti-tumour efficacy of 8B6A-PBD antibody. (A) Representative experiment of internalisation assay of the anti-NKp46 antibody by NKp46+ primary tumour cells *ex vivo*. The CypHer5E signal was assessed on sCD3−CD103+NKp46+ intraepithelial lymphocytes (IEL) untreated (in grey), treated with the anti-NKp46 (in red) or the corresponding isotype control (in green) labelled with the pH-sensitive fluorescent dye CypHer5E after 24 hours of incubation. (B) *Ex vivo* cytotoxicity of 8B6A-PBD against primary tumour NKp46+ IEL isolated from fresh duodenal biopsies of five patients with type II refractory coeliac disease (RCDII). IEL were incubated in the presence of interleukin-15 (IL-15) (grey) alone or in combination with anti-8B6A-PBD (red), IC-PBD (grey), deglycosylated anti-NKp46 (grey) or PBD alone (white). Box plots compare the frequencies of malignant sCD3−CD7+ IEL negative for annexin V and propidium iodide and therefore still alive after 96 hours. Horizontal bars indicate median values obtained in five experiments performed with IEL from patients with distinct RCDII. PBD, pyrrolobenzodiazepine.

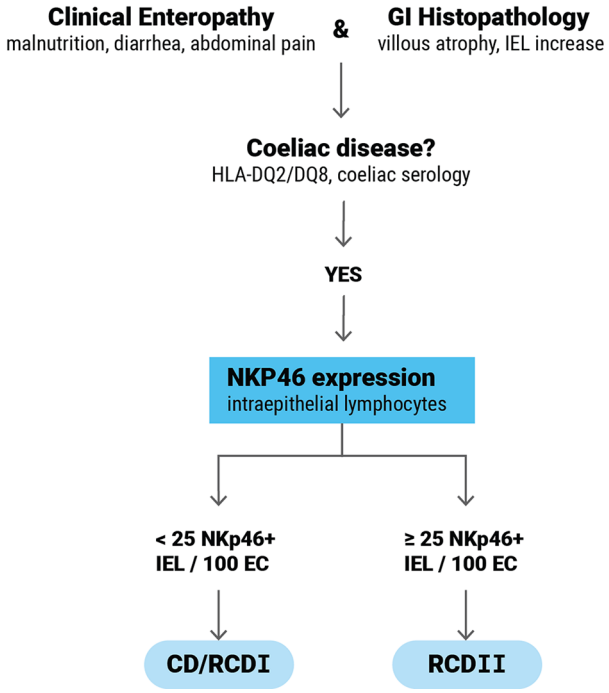

Figure 5 Scheme proposing an algorithm based on NKp46 expression for primary GI T-cell lymphoproliferative diseases diagnosis orientation. CD, coeliac disease; EC, epithelial cells, IEL, intraepithelial lymphocytes; RCDI and II, type I or type II refractory CD.

evidence that NKp46 might represent a novel therapeutic target in NKp46-positive intestinal LPD, a group of severe diseases lacking efficient therapy.

We recently demonstrated that RCDII malignant lymphocytes, which develop in the gut epithelium of patients with CD, arise from innate-like lymphocytes with dual T and NK traits. In line with these findings, we show that NKp46 expression is a hallmark of RCDII IEL, shared by EATL regardless the underlying enteropathy RCDII or more rarely CD or RCDI. Since NKp46 can be easily detected in FFPE tissue sections by the novel anti-NKp46 8E5B antibody, NKp46 represents a robust marker to facilitate the diagnosis of RCDII. This rare but severe complication of CD can be considered as an intraepithelial lymphoma. Due to their normal cytology and intracellular expression of CD3, RCDII IEL can be easily mistaken for normal T-IEL. As a consequence, current diagnosis of RCDII is very challenging in the absence of flow cytometry and *TCR* clonality analyses.[25] Herein, we show that a threshold of 25 NKp46+ IEL/100 EC can differentiate RCDII from uncomplicated CD and from non-malignant cases of CD refractory to GFD (RCDI) with high sensitivity and specificity. NKp46 (CD335, NCR1) is a highly conserved member of the natural cytotoxicity receptor (NCR) family that is specific to NK and innate lymphoid cells.[26] NKp46 may also identify a functionally distinct cellular subset in mice and humans that is susceptible to leukaemic transformation in the presence of excessive IL-15.[27] Our study indicates that NKp46 is a marker for malignant RCDII cells and, as such, could be considered a tumour-associated antigen. We therefore propose a simplified algorithm demonstrating how the addition of an NKp46 expression assessment to classical clinical, biological and histological criteria may improve diagnosis and therapeutic management (figure 5). In patients with CD, assessment of NKp46 in addition to other immunohistochemical markers may clarify differential diagnoses between benign and malignant intraepithelial lymphoproliferation, notably when flow cytometry and molecular studies including *TCR* gene rearrangement and NGS are not available.

Given the clonal relationship and common cellular origin between RCDII and EATL,[11 13] it is not surprising that NKp46 was also often expressed in EATL tumour cells. Only rare EATL cases did not express NKp46. This may reflect a loss in surface antigens during the malignant evolution process, as suggested by one case with NKp46 expression in RCDII cells with no NKp46 expression in EATL tumour cells. Alternatively but not exclusively, NKp46 expression reflects the cellular origin of the malignant IEL. Thus, the capacity of expressing NKp46 is lost when T-cell precursors progress towards the TCRαβ lineage.[19] In line with our previous findings in RCDII[19] and with the frequent silent TCR phenotype[28–32] of EATL, most NKp46+ EATL displayed incomplete *TCR* rearrangements. In contrast, complete *TCRβ* rearrangements were observed in the two tested NKp46 negative EATL cases. Future studies will be necessary to define whether heterogeneity in NKp46 expression may reflect heterogeneity in the cellular origin of EATL. Interestingly, NKp46 was also expressed by the four MEITL cases which, similarly to EATL, are very aggressive lymphomas that may be derived from IEL.[3] In contrast, none of the indolent CD4+ or CD8+ T-LPD expressed NKp46. Since indolent T-LPD are very rare, they remain largely unrecognised and are often misdiagnosed either as RCDII or as aggressive PTCL, leading, in the latter case, to unnecessary intensive therapy.[7] In contrast with MEITL and EATL, which have an extremely poor prognosis despite intensive chemotherapy and autologous stem cell transplantation,[33–37] indolent T-LPD generally have a mild course and can be controlled by corticosteroids.[7] Since NKp46 negative primary GI T-cell lymphomas were mainly indolent T-LPD, our results confirm the relatively good prognosis usually associated with this entity (5-year rate of OS 57% in our cohort, online supplementary figure 12).[7] NKp46 thus emerges as a powerful biomarker to discriminate primary GI T-LPD with very distinct outcomes, particularly indolent T-LPD from RCDII, EATL and MEITL. However, it has been suggested that NKRs expression is restricted to true NK cell lymphomas and a subset of intestinal enteropathy-type T-cell lymphomas with a cytotoxic phenotype.[38–41] We observed that NKp46 can also be expressed by a subset of extraintestinal lymphomas which may localise to the gut (data not shown), stressing the need to integrate all clinical, pathological and phenotypical criteria to establish a definitive diagnosis in GI primary lymphomas.

As in most PTCLs, there is no satisfactory standard of care for patients with newly diagnosed EATL and MEITL.[33–37] In addition, although patients with RCDII can generally be improved by corticosteroids for variable periods of time, a curative treatment is still lacking.[13] This highlights the need for novel, and, if possible, targeted therapies. Since NKp46 is frequently expressed by severe GI T-LPD but only by minor subsets of normal cells, NKp46 emerges as a promising therapeutic target. Consequentially, an endocytosed version of the anti-NKp46 monoclonal antibody was linked to the PBD cytotoxic drug to generate an ADC that can selectively deliver the drug into tumour cells. As a proof of concept, we showed that 8B6A-PBD can selectively and efficiently kill freshly isolated human primary NKp46+ RCDII IEL *ex vivo*. Overall, these data provide an experimental rationale for considering clinical studies using anti-NKp46 ADC to treat NKp46+ GI T-LPD.

In conclusion, our study shows that NKp46 is a novel and handy biomarker to clarify diagnosis of GI lymphoproliferations and to guide individual patient management. In addition, our

findings open the path to a novel therapy for RCDII, EATL and MEITL.

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Contributors MC, JB, GM, OH and NC-B conceptualised, supervised the study and wrote the manuscript. MC, JB, NG, TvG, SC provided data and data analysis. A-SJ, MC contributed to statistical interpretation. MC, JB, GM, CC, NG, DS, TvG, CJJM, RD, FS, AM, LF, SK, BM, TJM, VA, EM, VA, LP, AT, NB provided patient material and clinical data. MC, JB and OH obtained fundings. All authors edited and approved the final version of the paper. MC, NC-B and OH were responsible for the final version of the manuscript.

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