IPH4102, a Humanized KIR3DL2 Antibody with Potent Activity against Cutaneous T-cell Lymphoma

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Abstract

Advanced cutaneous T-cell lymphoma (CTCL) remains an unmet medical need, which lacks effective targeted therapies. In this study, we report the development of IPH4102, a humanized monoclonal antibody that targets the immune receptor KIR3DL2, which is widely expressed on CTCL cells but few normal immune cells. Potent antitumor properties of IPH4102 were documented in allogeneic human CTCL cells and a mouse model of KIR3DL2+ disease. IPH4102 antitumor activity was mediated by antibody-dependent cell cytotoxicity and phagocytosis. IPH4102 improved survival and reduced tumor growth in mice inoculated with KIR3DL2+ tumors. Ex vivo efficacy was further evaluated in primary Sézary patient cells, sorted natural killer–based autologous assays, and direct spiking into Sézary patient peripheral blood mononuclear cells. In these settings, IPH4102 selectively and efficiently killed primary Sézary cells, including at unfavorable effector-to-target ratios characteristic of unsorted PBMC. Together, our results offer preclinical proof of concept for the clinical development of IPH4102 to treat patients with advanced CTCL.

Introduction

Cutaneous T-cell lymphomas (CTCL) comprise a group of heterogeneous diseases that vary greatly in their biologic behavior, their histologic and immunophenotypic aspects and, consequently, in prognosis and survival (1, 2). Mycosis fungoides is the most frequent CTCL subtype: mycosis fungoides presents with an indolent clinical course in many cases; however, tumor-stage disease and cell transformation are usually associated with dramatically poorer prognosis. Sézary syndrome is an aggressive leukemic variant of CTCL with unfavorable prognosis, defined by erythroderma, lymphadenopathy, and the presence of atypical T cells in skin and peripheral blood. The detection of the same malignant clonal T cells, identified by their rearranged T-cell receptor (TCR) gene, in blood and skin lesions, is a key component of Sézary syndrome diagnosis (1–3). There is currently no standard-of-care for late-stage CTCL such as transformed mycosis fungoides (tMF) or Sézary syndrome and both approved and unapproved agents are used, in monotherapy or in combination (reviewed in refs. 4 and 5).

Among these agents, several monoclonal antibodies (mAbs) directed to tumor antigens are currently prescribed or developed in advanced CTCL, including alemtuzumab, mogamulizumab, and brentuximab vedotin (BV). Alemtuzumab (MabThera) exerts consistent long-term efficacy as salvage therapy in a few relapsed/refractory (r/r) Sézary syndrome patients, whereas it is less effective in mycosis fungoides and transformed diseases (6, 7). The benefit of alemtuzumab is limited by its poor safety profile as it induces profound long-lasting immunosuppression that results in hematologic toxicity and severe infectious complications (8). Mogamulizumab (Poteligeo) targets CCR4, a receptor involved in the homing of T lymphocytes to the skin. It is currently undergoing several clinical trials in CTCL (9). Early clinical results of mogamulizumab in CTCL are promising, although not significantly improved as compared with currently available therapies (10, 11). Finally, BV (Adcetris) is the second antibody–drug conjugate (ADC) ever approved by the FDA and the European Medicines Agency [for the treatment of r/r Hodgkin’s lymphoma (HL) and systemic anaplastic large-cell lymphoma (ALCL)]. BV preliminary efficacy data in CTCL are encouraging, but somewhat less impressive than in HL and ALC (12, 13). Its safety profile in CTCL is similar in nature and frequency of adverse events to what was previously observed in other brentuximab-treated patient populations, with 60% of patients peripheral neuropathy, experiencing sometimes long lasting (13), attributed to the cytotoxic drug component of the ADC. Interestingly, these mAb-based therapies are all directed to receptors expressed on immune cells, and therefore display imperfect specificity in tumor targeting. Indeed, the
unrestricted patterns of expression of their respective targets actually affect their safety profiles. Furthermore, none of these mAbs was developed to target primarily CTCL tumors. Overall, safer and more effective agents are still greatly needed in advanced CTCL, and this could be achieved with the development of more targeted therapies.

The lack of CTCL-specific mAb therapeutic strategies can be explained by the heterogeneity of the diseases and the scarcity of patients—for instance, as compared with B-cell–derived malignancies—but also mirrors the historical absence of accessible common marker to be targeted. The classical immunophenotype of Sézary and mycosis fungoides tumor cells in flow cytometry is CD3⁺CD4⁺CD8⁻ but these cells also frequently lose T-cell markers (3, 14). More recent studies have reported several promising novel surface markers, that could facilitate diagnosis and be targeted by mAb, such as CD164 and FCRL3 (15), Nkp46 (16, 17), CD60 (18), and PD-1 (19). Among the potential markers expressed on Sézary and tMF cell surface, KIR3DL2 is one of the most extensively studied and the relevance of its expression in these diseases has been established: KIR3DL2 is detected both on skin lesions and on leukemic cells (20–24); the number of KIR3DL2⁺ cells enumerated thanks to TCR-Vβ expression (27, 28); finally, KIR3DL2⁺ cells in the blood correlate with disease relapse in the skin (29). KIR3DL2 (alias CD158k) is a member of the family of killer immunoglobulin-like receptors (KIR) expressed on cytotoxic lymphocytes, which modulate their effector functions through binding to their cognate MHC class I ligands (30). KIR3DL2 contains three extracellular immunoglobulin (Ig) domains and a long cytoplasmic tail shared by the inhibitory receptors of the family. Its expression on normal immune cells is restricted to a fraction of blood natural killer (NK) cells and to less than a few percent CD8 and CD4 T cells (31).

Taking advantage of the expression of KIR3DL2 at the surface of tMF and Sézary syndrome tumor cells, and of its uniquely narrow pattern of expression on normal cells, we thought to develop an anti-KIR3DL2 therapeutic mAb to treat patients with advanced CTCL. Our objective was to select and design a humanized anti-KIR3DL2 IgG1 with potent-depleting activity against KIR3DL2-expressing tumors in different in vitro and in vivo models, including against primary Sézary patient cells. The present study provides detailed overview of the preclinical efficacy features of IPH4102, our lead anti-KIR3DL2 mAb candidate.

Materials and Methods

Generation of anti-KIR3DL2 antibodies

Anti-KIR3DL2 mAbs were generated after immunization of Balb-C mice (Elevage Janvier) with recombinant human KIR3DL2 fused to human fragment crystallizable (Fc) region (ref. #2878-KR-050, R&D Systems). After initial selection based on binding to KIR3DL2-expressing cells and absence of cross-reactivity to other human KIR receptors, including KIR3DL1 and KIR2D molecules, a dozen murine mAb candidates were chimerized to obtain human IgG1 with effector functions. On the basis of its overall in vitro preliminary efficacy profile, anti-KIR3DL2 clone 2B12 was selected and humanized by CDR grafting: 16 sequence variants were constructed that showed unchanged binding capacity to KIR3DL2 as compared with their parental counterpart (by flow-cytometry titration on KIR3DL2⁺ cells and Surface Plasmon Resonance analysis on recombinant KIR3DL2 protein). In our selection process, we also identified anti-KIR3DL2 mAb clone 13E4 for its optimal staining of KIR3DL2⁺ cells in flow cytometry. Originally, 13E4 was a mouse IgG2b: It was chimerized to obtain a mouse IgG1 that allows more convenient production, purification, and conjugation to fluorochromes, and minimizes the risk of binding to human Fc receptors (FcR) in staining experiments.

Other antibodies

Alemtuzumab (MabCampath), humanized IgG1 directed to CD52, and rituximab (Rituxan), chimeric human IgG1 directed to CD20, were purchased from Theradis Pharma. The isotype-matched control (IC) mAb used in all assays is a humanized IgG1 directed to an irrelevant antigen, designed and produced internally.

Flow cytometry

Unless otherwise stated, KIR3DL2 detection in flow cytometry was performed using anti-KIR3DL2 mAb clone 13E4 (produced internally) conjugated to R-Phycoerythrin (13E4-PE; PhycoPro #PB31; ProZyme). IPH4102 was also coupled to RPE (IPH4102-PE) to allow convenient monitoring in flow cytometry of the cells targeted by the mAb lead candidate. Multicolor flow-cytometry analyses were performed using the following commercial reagents: anti-CD3 (or TCR-Vβ)-FITC, anti-CD4-PC7, anti-CD56-PE, anti-CD14-PE (all from Beckman-Coulter), and anti-CD206-APC (BioLegend). TCR-Vβ rearrangement of the malignant T-cell clone was determined using the TCR-Vβ Repertoire Kit from Beckman-Coulter. Flow-cytometry analyses of Sézary patient samples and autologous assays were performed on a FC500 apparatus (Beckman-Coulter). For in vitro allogeneic antibody-dependent cell cytotoxicity (ADCC) and phagocytosis (ADCP) assays, flow-cytometry analyses were performed on a FACS Canto apparatus and acquired with BD FACSDiva software (Becton-Dickinson). All data were analyzed with FloJo software (Tree Star Inc.).

Cell lines, human macrophages, and cell culture

All cells were cultured at 37°C in 7% CO₂ in RPMI-1640 (RPIM) supplemented with 10% FCS and 1% glutamine (all purchased from Fisher Scientific). The KIR3DL2⁺ Sézary cell line HuT 78 and the KIR3DL2-negative Burkitt lymphoma B cell line Raji were both purchased from the American Type Culture Collection (ATCC TIB-161 and CCL-86, respectively). Raji cells were transfected to stably express human KIR3DL2 and named Raji-KIR3DL2 thereafter. KHYG-1 is an NK cell line purchased from the Leibniz Institute-German Collection of Microorganisms and Cell Cultures (DSMZ no. ACC 725). The KHYG-1 cell line was transfected and sub-cloned to stably express high levels of human CD16a/FcRIIIa (KHYG-1-FcγRIIIa).
Human macrophages were derived from healthy donors' monocytes. Briefly, human peripheral blood mononuclear cells (PBMC) were prepared from human whole blood by centrifugation on Ficoll (Ficoll Paque PLUS; Fisher Scientific). After several washing steps to eliminate platelets and red blood cell lysis with NH$_4$Cl (STEMCELL; 1 mL ready-to-use solution in 10 mL final RPMI, 5 minutes at room temperature), monocytes were sorted by negative selection with the MACS Monoocyte Isolation Kit II (Miltenyi Biotech). Macrophages were generated from isolated monocytes by incubation in RPMI/10% FCS plus 20 ng/μL M-CSF (Peprotech) for 7 to 10 days. At the end of the culture, the phenotype of macrophages was verified by multicolor flow cytometry (defined as CD206$^+$ CD14$^+$ cells). The day before use in ADCP assay (see below), macrophages were seeded at 10$^5$ cells per well in 96-well flat-bottom plates.

**In vitro allogeneic ADCC and ADCP assays**

ADCC was determined by a 4-hour chromium ($^{51}$Cr) release assay using KHYG-1-FcRIIIa cells as effectors. KHYG-1-FcRIIIa cells were mixed with $^{51}$Cr-loaded HuT 78 cells at the effector-to-target (E/T) ratio of 10/1. Increasing concentrations of IPH4102, alemtuzumab, or IC mAb were added and the mixtures were incubated for 4 hours at 37°C. Supernatants were transferred to a LumaPlate and released radioactivity measured by a TopCount NXT apparatus (both from PerkinElmer). Specific lysis of HuT 78 was calculated according to the formula: specific lysis (%) = 100 x ($E - S$)/($M - S$), where $E$ is the experimental release, $S$ the spontaneous release of $^{51}$Cr by the cell mixture, and $M$ the maximum release of $^{51}$Cr by the target cells lysed in PBS/4% Triton X-100.

To address *in vitro* ADCP mediated by IPH4102, HuT 78 target cells were labeled with PKH67 according to the supplier’s protocol (PKH67GL-1KT kit; Sigma-Aldrich). Briefly, 10 $\times$ 10$^6$ HuT 78 cells were pelletized by centrifugation were suspended in 6 mL PKH67 solution (2 μmol/L in diluent C) and incubated for 2 to 5 minutes at room temperature, protected from light, with gentle shaking. To stop the loading of PKH67, 6 mL FCS was added and the cells were washed three times with RPMI. PKH67-labeled HuT 78 cells were then incubated with human macrophages at an E/T ratio of 4/1. IPH4102, IC mAb, or alemtuzumab was incubated with cell mixture at 10 μg/mL for 1 hour at 37°C. ADCP was assessed by the transfer of PKH67 staining to macrophages by flow-cytometry analysis.

**Animals and xenograft models**

Eight-week-old female CB-17 severe combined immunodeficient (SCID) mice purchased from Elevage Janvier were housed under pathogen-free conditions at our animal facility. For xenograft experiments, 5 $\times$ 10$^6$ Raji-KIR3DL2 were injected either i.v. or s.c. on day 1. In the intravenous disseminated model, mAb treatment was initiated 1 day after tumor cell inoculation. IPH4102 and isotype-matched negative control were administered i.p. at the indicated doses, twice weekly for 3 weeks. Mice were observed daily to monitor clinical signs (hind limb paralysis) and weighed two to three times a week. When required, mice were humanely euthanized according to predefined criteria of disease severity. For the subcutaneous model, mice were weighed weekly, tumor size was measured two to three times a week and tumor volume (in mm$^3$) was calculated as length $\times$ width$^2$/2. When the tumors reached approximately 100 mm$^3$ (day 17), mice were randomized in groups of 10 to 13 and mAb treatment started the day after (day 18).

**Szémary syndrome patients and *ex vivo* autologous ADCC assays**

Diagnosis of Szémary syndrome was done according to the revised international consensus criteria proposed by the ISCL and EORTC-CLTF (2). PBMCs were isolated by gradient centrifugation (LSM; PAA Laboratories). Autologous killing experiments were performed either on sorted cells or by directly incubating unmanipulated whole PBMC with the indicated mAbs. For the sorted cell setting, PBMCs were split into two samples to purify CD4$^+$ T cells and autologous NK cells by negative selection with the respective MACS isolation kits (Miltenyi Biotech). Sorted CD4$^+$ T cells were preincubated for 30 minutes at room temperature with parental anti-KIR3DL2 2B12 clone or IPH4102, negative control rituximab or positive control alemtuzumab at 10 μg/mL and then mixed with the autologous NK lymphocytes at the indicated E/T ratios (varying from one patient to another depending on the obtained number of purified NK cells). Incubation was conducted for 4 to 6 hours at 37°C in RPMI/10% FCS. For ADCC assays on PBMC, NK and KIR3DL2$^+$ CD4$^+$ T-cell contents were determined by flow cytometry to evaluate the initial E/T ratio. PBMCs were then incubated with IPH4102 or control mAbs for 4 days at 37°C in RPMI/10% FCS. In both settings, Szémary cell death was monitored by multicolor flow cytometry through the incorporation of 7-aminoactinomycin D (7-AAD; Becton-Dickinson), a fluorescent DNA dye that incorporates only in cells that have lost their membrane integrity. 7-AAD was added to the cell mixture and incubated for 15 minutes immediately before analysis.

**Statistical analyses**

In the allogeneic ADCC assay, differences between the treatment groups and the condition without any mAb were analyzed with a one-way ANOVA. In the autologous PBMC assay, differences between the treatment groups and the condition without any mAb were analyzed with a nonparametric Friedman test. Correlation between KIR3DL2 expression on CD4$^+$ cells and TCR-Vβ clone, in flow cytometry, was analyzed with a nonparametric Spearman correlation test. Survival curves in the mouse intravenous model were compared with the IC group with a log-rank (Mantel–Cox) test. In the mouse subcutaneous model, tumor volumes at selected days (27 and 45) were compared between control and treated groups with a Mann–Whitney $t$ test.

**Human and animal studies**

Blood samples used in the different *in vitro* and *ex vivo* staining and functional studies were obtained from Szémary syndrome patients followed at Saint Louis Hospital (Paris,
France) or from healthy volunteers after written informed consent.

Mice were handled in accordance with the European Union guidelines and French laws for laboratory animal care and use. Study design was approved by the local animal ethical committee.

Results

IPH4102 binds KIR3DL2, a selective marker of Sézary leukemic cells

KIR3DL2 is recognized as relevant and specific marker of advanced CTCL cells, whether they are localized in skin lesions or circulating in patients who present blood involvement (20–22, 24, 26, 29). Taking advantage of the many new anti-KIR3DL2 mAb we had generated, we selected among them the best mAb clone for monitoring KIR3DL2 in flow cytometry. Anti-KIR3DL2 mAb clone 13E4 allows excellent detection of KIR3DL2 in flow cytometry, does not cross-react with any other human KIR protein and binds to a different epitope on KIR3DL2 than IPH4102 (not shown). Thus, 13E4 directly coupled to R-phycocerythrin (13E4-PE) was used to analyze blood samples from a cohort of 42 different Sézary patients. Immuno-stainings for detection of the KIR3DL2+CD4+ T-cell blood content (using 13E4-PE mAb) and identification of the TCR-Vβ rearrangement of the circulating tumoral clone were performed in parallel. TCR-Vβ rearrangement of the malignant T-cell clone was unambiguously identified for 32 Sézary patients. In this group, a very good correlation between the percentages of KIR3DL2+ and TCR-Vβ+ cells within the CD4+ T-cell population was observed (Spearman r = 0.6609; P < 0.0001; Fig. 1A). Indeed, the majority of patients (27 of 32, 84.4%) had very similar percentages of circulating KIR3DL2+ CD4+ T cells and CD4+ TCR-Vβ+ cells. For one patient, even higher percentage of KIR3DL2+CD4+ T lymphocytes was detected as compared with TCR-Vβ+ cells, whereas more clonal T cells than KIR3DL2+CD4+ T cells were seen in 4 of

![Figure 1](image.jpg)

**Figure 1.** IPH4102 binds KIR3DL2, a key marker of malignant Sézary cells. A, correlation of KIR3DL2 expression and TCR-Vβ clonality on circulating CD4+ T cells from 32 Sézary patients. Each plot represents the individual percentages of CD3+CD4+ KIR3DL2+ and CD4+ TCR-Vβ+ cells within the total CD4+ T-cell population for one Sézary patient. The correlation between the two factors was analyzed with a Spearman nonparametric correlation test. Dotted line, the median percentage of CD4+ KIR3DL2+ T cells (1.4%) observed in a cohort of 35 healthy donors. B, KIR3DL2 expression, analyzed in flow cytometry, on circulating CD4+ T cells from Sézary patients with undetermined malignant T-cell clonality (n = 10). The individual percentage of KIR3DL2+ CD4+ T cells is plotted. All patients show more than 30% of KIR3DL2+ cells among their CD4+ T lymphocytes population. For comparison, the median percentage of KIR3DL2+CD4+ T lymphocytes from healthy donors (n = 40) is indicated (dotted line). C, IPH4102 and TCR-Vβ costaining of circulating CD4+ T lymphocytes of Sézary patients. Dot-plot representation of multicolor flow-cytometry analysis of six Sézary patient blood samples stained with IPH4102 and the anti-TCR-Vβ mAb corresponding to each patient’s malignant clone. Shown results correspond to gated lymphocytes.
32 patients. However, for these four patients, the proportion of KIR3DL2⁺ CD4⁺ T cells was still significantly higher than the one observed in healthy donors. Indeed, immunostainings performed on blood samples from 35 healthy volunteers showed a median percentage of CD4⁺ KIR3DL2⁺ T cells of 1.4 (range, 0.2%–8.7%). For the remaining 10 Sézary patients, the TCR-Vβ mAbs led to the detection of 0.9% to 64.2% of the CD4⁺ T-cell population (median% = 23.9), whereas more than 30% of circulating CD4⁺ T cells expressed KIR3DL2, in accordance with their Sézary syndrome diagnosis (Fig. 1B), therefore suggesting that their malignant T-cell clone was not recognized by the commercially available anti-TCR-Vβ mAb panel. These data pointed toward the possibility to detect a significant blood tumor burden in Sézary patients, despite the absence of TCR-Vβ information for the malignant clone, through the use of KIR3DL2 as a single flow-cytometry marker (Fig. 1B).

Finally, as our lead candidate IPH4102 aims to treat patients with CTCL, we determined its binding profile on fresh-blood samples from Sézary patients. Representative examples of costaining with IPH4102-PE and anti-TCR-Vβ mAb on blood samples from 6 patients with known malignant T-cell clonality are presented on Fig. 1C. As expected, IPH4102 showed consistent and homogeneous staining of the identified Sézary TCR-Vβ⁺ clone within the lymphocyte population. A minor subpopulation of TCR-negative cells was also detected by IPH4102 in patients’ blood (Fig. 1C) that was identified as a NK cell subset (data not shown).

**IPH4102 exerts potent ADCC and ADCP against a KIR3DL2⁺ CTCL cell line**

IPH4102 is a humanized IgG1 selected to exert potent antitumor effector functions. To dissect the various modes of action used by IPH4102 to kill KIR3DL2⁺ tumors, we performed *in vitro* ADCC assays using the KHYG-1 NK cell line as effectors, *in vitro* ADCP assays using monocyte-derived macrophages and *in vitro* killing assays using human complement (complement-dependent cytotoxicity, CDC). The HuT 78 Sézary cell line was selected as common target in all three *in vitro* assays. Anti-CD52 alemtuzumab mAb was used as positive control, and an isotype-matched humanized IgG1 directed against an irrelevant antigen as isotype (negative) control (IC).

In the ADCC experiment, IPH4102 mediates potent and concentration-dependent killing of HuT 78 Sézary cells, leading to more than 60% of specific lysis at the highest concentration. In contrast, the IC mAb did not induce any ADCC, as expected (Fig. 2A). Strikingly, the profile of IPH4102-mediated ADCC against HuT 78 was very similar to the one mediated by alemtuzumab, the referent anti-CD52 mAb known for its potent cytotoxic activity in many models (32) and in cancer patients (6, 8, 33). The ADCP experiment was repeated with macrophages derived independently from monocytes of 8 different healthy donors (Fig. 2B). IPH4102 induced robust and reproducible ADCP of HuT 78, with all healthy donor–derived macrophages tested (Fig. 2B). In these experimental conditions, the alemtuzumab-dependent ADCP against HuT 78 using the same macrophage batches was slightly more effective.

Finally, we questioned whether IPH4102 was able to recruit complement to kill KIR3DL2⁺ tumor target cells, which is another mode-of-action reported for therapeutic mAbs conveniently studied *in vitro* (34–36). In our experimental conditions, using human complement, IPH4102 was devoid of such CDC activity (not shown).

Altogether, these results emphasize the potent antitumor activity of IPH4102 against the Sézary cell line HuT 78, as IPH4102 effector functions and antitumor modes of action include ADCC and ADCP, using allogeneic NK cells and monocyte-derived macrophages, respectively, but not CDC.

![Figure 2](https://cancerres.aacrjournals.org)
IPH4102 improves survival and reduces tumor growth in mice engrafted with KIR3DL2⁺ tumors

To study the antitumor efficacy of IPH4102 in vivo, we set up two xenograft models of KIR3DL2⁺ tumors in the SCID mouse. This immunocompromised mouse strain retains fully functional NK cells and macrophages (37). In addition, mouse FcRs effectively bind human Fc (38), allowing murine effector cells to be recruited by humanized mAb and perform ADCC or ADCP in vivo (39). Raji cells transfected to stably express KIR3DL2 (Raji-KIR3DL2) were inoculated to SCID mice either i.v. (disseminated model) or s.c. (to obtain a solid tumor). In the disseminated intravenous model (n = 8 mice/group), IPH4102 was administered i.p., twice weekly for 3 weeks, at 30, 100, or 300 μg per injection, starting the day after tumor engraftment. The IC mAb was given as often, only at the highest dose (300 μg). A supplementary group of mice was treated with 300 μg IPH4102 with a 5-day delay after tumor inoculation, in a curative setting. Mice were monitored for clinical signs (hind limb paralysis and weight loss) for 92 days. IPH4102 significantly improved survival at all doses and schemes of administration tested (Fig. 3A) as compared with IC mAb. Median survival of the IC-treated control group was 17 days (range 16–19 days). In the 30, 100, and 300 μg IPH4102 groups, median survivals reached 39, 31, and 36.5 days, respectively. At the end of our study (day 92), 2 mice were still alive in both the 30 and 300 μg IPH4102 dose groups. There was no apparent dose-related effect, suggesting that maximal efficacy was achieved with as little as 30 μg of IPH4102 per injection. IPH4102 also significantly improved survival in the curative setting (median survival was 23 days, range 20–42 days) although tumors were left to grow for one third of the time they needed to kill the mice, before treatment initiation (Fig. 3A).

In the subcutaneous model, administration of IPH4102 or IC mAb (each, 300 μg per injection, twice a week for 3 weeks) started when tumors reached approximately 100 mm³, with 10 and 13 mice per group, respectively. Figure 3B shows the individual tumor growth curves in both treatment groups. IPH4102 significantly reduced tumor growth in the subcutaneous model as compared with IC mAb. At the last day analyzed (day 55), all tumors in the control group had grown larger than 500 mm³, all but one even larger than 1,000 mm³, whereas in the IPH4102-treated group, 4 mice of 10 still had tumors smaller than 500 mm³ (Fig. 3B and C). Altogether, these in vivo experiments demonstrate that IPH4102 has reproducible and potent antitumor efficacy, in both disseminated and solid tumor models and in curative settings.

Figure 3. In vivo antitumor efficacy of IPH4102 in disseminated intravenous and localized subcutaneous models of KIR3DL2⁺ xenograft in SCID mice. A, Kaplan–Meier survival curves of mice inoculated i.v. with Raji-KIR3DL2. Eight mice per group received IPH4102 at the indicated doses twice weekly for 3 weeks, starting the day after tumor injection (black lines) or isotype control mAb at the highest dose (gray dotted line). One group (black dotted line) was treated with 300 μg of IPH4102, with a 5-day delay after tumor engraftment. Log-rank Mantel-Cox test: ***P < 0.0002 for each of the four treatment groups as compared with the IC group. Arrows, mAb injections (dotted line arrows, 5-day delayed; black arrows, dose ranging). B, individual tumor growths in mice engrafted s.c. with Raji-KIR3DL2. Treatment started when tumors reached approximately 100 mm³ and mice were randomized 1 day before treatment initiation. Isotype control mAb (left; n = 13) or IPH4102 (right; n = 10) was given twice weekly for 3 weeks, with 300 μg mAb per injection. Arrows, mAb injections. C, same data as in B but with tumor volumes represented as means ± SD. Gray curve, isotype control; black curve, IPH4102. Statistical comparison of tumor volumes was performed with the Mann–Whitney t test: day 27, ***P < 0.0002 and day 45, ****P < 0.0001.
IPH4102 mediates targeted killing of malignant Sézary cells by autologous NK cells

We then evaluated the antitumor efficacy of IPH4102 using tumor cells purified from Sézary patient as targets (T) and autologous NK cells as effectors (E). This autologous model is more relevant than the allogeneic settings described above, as it takes into account both the actual functional state of NK cells in Sézary patients, and the intrinsic sensitivity of tumor cells to IPH4102-mediated ADCC. This type of experiment was performed with the anti-KIR3DL2 clone 2B12, i.e., IPH4102 parental chimeric human IgG1 (see Materials and Methods), then with IPH4102. 2B12 and IPH4102 showed no significant difference in efficacy in all the allogeneic assays performed, as expected from their equivalent binding affinity to KIR3DL2 (not shown). For each patient tested, incubations were conducted for 4 to 6 hours with a single concentration of 2B12 or IPH4102 (10 μg/mL), and with varying E/T ratios. Depending on the number of cells recovered after sorting, E/T ratios typically ranged from 1/10 to 5/1. Anti-CD20 mAb rituximab and anti-CD52 mAb alemtuzumab were used as negative and positive controls, respectively. Incorporation of 7-AAD was monitored by flow cytometry as a marker of cell death. Antitumor activity of IPH4102 against primary Sézary cells was observed in all patient samples tested (n = 8) and increased with the E/T ratio (Fig. 4). In most cases, IPH4102 antitumor effect is evidenced at an E/T ratio equal or even lower than 1/1. Regardless of the E/T ratio used, the unspecific baseline of NK-mediated cytotoxicity remained negligible, as shown by the absence of increased killing with rituximab. Alemtuzumab positive control confirmed the functional capacity of Sézary patient NK cells to kill.

In the same assay, we also evaluated the detrimental cell death of patients’ effector NK cells induced by the incubated antibodies. To do so, 7-AAD incorporation in the CD4⁺ cell population, corresponding to NK cells, was analyzed (Fig. 4). For all patients and at all E/T ratios tested, neither rituximab nor 2B12/IPH4102 was found to promote NK cell death. In contrast, NK survival appeared compromised in the presence of alemtuzumab, reflecting its potent yet noneffective cytotoxic activity known to lead to profound immunosuppression in alemtuzumab-treated patients (40). Altogether, these

Figure 4. Targeted ex vivo antitumor efficacy of IPH4102 and its parental anti-KIR3DL2 2B12 clone against primary Sézary cells in an autologous ADCC assay. A, CD4⁺ T cells from Sézary patients (n = 4) were preincubated with alemtuzumab (squares, positive control), rituximab (triangles, negative control), or anti-KIR3DL2 mAb 2B12 (circles) at a unique concentration of 10 μg/mL. After the addition of autologous NK cells at the indicated E/T ratio, incubation was conducted for 4 to 6 hours and Sézary (top) or NK (defined as CD4⁺ cells, bottom) cell death was monitored through the incorporation of 7-AAD. Results are expressed as the percentage of 7-AAD⁺ cells among each population at a given E/T ratio. B, similar experiments as in A were performed with CD4⁺ T cells and autologous NK cells from Sézary patients (n = 4) but using humanized anti-KIR3DL2 mAb IPH4102 instead of 2B12.
findings demonstrate unambiguously that (i) malignant Sézary cells are sensitive to ADCC mediated by IPH4102 through KIR3DL2 targeting at their surface; (ii) Sézary patients circulating NK cells are functional and able to mediate potent IPH4102-driven ADCC; and (iii) IPH4102 antitumor activity is selectively targeted against KIR3DL2-expressing tumor cells and spares NK effectors.

**IPH4102 promotes ex vivo killing of tumor cells in Sézary patient primary PBMC**

Finally, we questioned whether IPH4102 was able to mediate killing of leukemic Sézary cells when directly incubated with PBMC from Sézary patients, i.e., in conditions in which the NK to tumor cell ratio was representative of the clinical situation.

First, we determined by flow cytometry the actual NK to tumor cell ratios in PBMC from 8 different Sézary patients. NK cells were defined as CD3⁺CD56⁻ cells and Sézary cells as CD3⁺ (or TCR-Vβ⁺, when available) CD4⁺KIR3DL2⁺ lymphocytes. In this group of patients, median E/T was 1/7.33 (range, 1/43–1/2). Sézary patient PBMC samples were left untreated or incubated with 10 µg/mL of IPH4102, rituximab, or alemtuzumab. After 4 days of incubation, Sézary cell death was analyzed in flow cytometry through the incorporation of 7-AAD whereas NK cell content was monitored in parallel samples by CD3/CD56 labeling. For all patients tested, IPH4102 induced significant Sézary cell killing, as compared with negative control conditions (absence of mAb or addition of rituximab; Fig. 5A and B, left). Alemtuzumab also showed an antitumor effect against the targeted cells confirming that Sézary patients’ NK cells were functional, although with higher variability than when activated by IPH4102. Furthermore, no or minor depletion of the NK cell population was detected in PBMC incubated with IPH4102 or rituximab when compared with untreated conditions, whereas a significant decrease in this lymphocyte subset was detected following incubation with alemtuzumab (Fig. 5A and B, right). Altogether, these results obtained in a challenging experimental setting confirm the excellent antitumoral potential of IPH4102 for the treatment of Sézary patients.

**Figure 5.** Ex vivo antitumor efficacy of IPH4102 toward Sézary tumor cells within patients’ PBMC. After determination of NK and tumor Sézary cells contents, fresh PBMC samples from eight Sézary patients were left untreated (no mAb, white bar) or incubated in the presence of 10 µg/mL rituximab (negative control, gray striped bar), IPH4102 (black bar), or alemtuzumab (positive control, black striped bar) for 4 days. A, left, Sézary tumor cell death was monitored by flow cytometry through incorporation of 7-AAD. Killing efficacy is expressed as the percentage of 7-AAD⁺ tumor cells among the tumor cell population defined as CD3⁺CD4⁺TCR-Vβ⁺ (for patients 11 and 12) or CD3⁺CD4⁺KIR3DL2⁺ cells. The percentages of NK and Sézary cells within the lymphocyte population and before incubation (day 0) are also indicated for each patient to allow estimation of the initial E/T ratio. Right, the percentage of NK cells (defined as CD3⁺CD56⁻ cells) within total PBMC was monitored in parallel at the end of the incubation time. B, schematic representation as median ± SD and statistical analysis of the data obtained in A for Sézary tumor cell death (left) and NK cell content (right). Data under each treatment condition were compared with the condition without mAb with a nonparametric Friedman test (Sézary cells, n = 8; NK cells, n = 6). **, P < 0.05; ***, P < 0.001. n.d., not determined; n.s., not significant.
Discussion

The present study describes the results of the preclinical efficacy studies of IPH4102, the first-in-class humanized anti-KIR3DL2 mAb selected for the development of advanced CTCL patients treatment. This report also provides the largest dataset of KIR3DL2 expression on Sézary syndrome patient samples, generated with novel staining reagents.

KIR3DL2 has been reported as a relevant marker for skin-resident and leukemic cells in tMF and Sézary syndrome (20–22, 24, 26, 27). Using the newly generated anti-KIR3DL2 mAb clone 13E4, that exhibits more specificity and sensitivity than previously available reagents, we analyzed the largest cohort of Sézary patients ever gathered in a single study (42 subjects). We confirmed that KIR3DL2 is homogenously expressed on most Sézary patients’ tumor cells in peripheral blood. Furthermore, we corroborated the excellent correlation between TCR-Vβ clonality and KIR3DL2 expression. This substantial dataset supports the relevance of KIR3DL2 as a marker to improve advanced CTCL diagnosis and strengthen the rationale to target KIR3DL2 in these patients.

The antitumor efficacy of our lead candidate IPH4102 was evaluated in several complementary preclinical in vivo, in vitro, and ex vivo models. Mouse models of CTCL xenografts require strongly immunosuppressed mouse strains for efficient tumor growth (41, 42), and thus are not suitable to study IPH4102 activity that relies on functional effector cells. As a consequence, to address IPH4102 efficacy in vivo, we developed xenograft models in the SCID mouse, using the B-NHL cell line Raji transplanted to express KIR3DL2. Those KIR3DL2 transfectants were inoculated s.c. or i.v., to display solid and leukemic tumors as for patients with CTCL, and provide information on IPH4102 ability not only to inhibit tumor growth but also to improve overall survival. To make these models more challenging, we tested IPH4102 in curative settings by starting IPH4102 treatment with significant delay after tumor cells subcutaneous implantation or intravenous inoculation. In vivo, IPH4102 was active in both the solid and the disseminated KIR3DL2+ tumor models, in which it significantly reduced tumor growth and improved survival in curative settings.

In addition, we tested IPH4102-mediated cytotoxic activity against KIR3DL2+ Sézary cells. In all assays, we used the anti-CD20 mAb alemtuzumab as a positive control to confirm effector NK cell function and provide efficacy comparison. Indeed, although alemtuzumab is not approved for the treatment of CTCL, it is given off-label to patients with CTCL with refractory disease and after multiple treatment failures (7, 8, 43) and shows high response rate and nonnegligible proportion of complete and long-lasting responses (4–6).

Our results show that IPH4102 is able to recruit human NK cells or human macrophages as effectors to mediate ADCC and ADCP, against the Sézary cell line HuT 78 with a level of efficacy comparable with that of alemtuzumab. However, IPH4102 did not promote CDC in our experimental conditions.

Beyond functional assays using effectors prepared from healthy donors against tumor cell lines, and to strengthen our confidence that IPH4102 could be a promising therapeutic candidate, we performed ex vivo autologous assays using samples from patients with CTCL. Indeed, primary patient cells are more representative of the future clinical situation, with the intrinsic potential resistance of tumors to any therapy and the possible defects borne by the patient’s immune system (34, 44, 45). Sézary syndrome was chosen as a model as patients’ blood samples represented a convenient source of leukemic cells and autologous effector NK cells. IPH4102 was effective in autologous killing assays in which it induces NK cell–based lysis of autologous Sézary cells, demonstrating both the functional integrity of patients’ NK cells, and the sensitivity of primary tumors to ADCC through KIR3DL2 targeting. ADCC is known to contribute to the clinical efficacy of many approved therapeutic mAbs, including anti-HER2 trastuzumab (46), anti-CD20 rituximab and obinutuzumab (44, 47–50), anti-CCR4 mogamulizumab (45), or anti-EGFR cetuximab (51). Moreover, IPH4102 was found to reproducibly promote Sézary tumor cells lysis when directly incubated with patients’ unsorted PBMC, hence at much less favorable effector to target ratios, unequivocally confirming the potency of this therapeutic candidate. In addition, the killing of KIR3DL2+ Sézary tumor cells mediated by IPH4102 is selective, as efficient killing of Sézary cells was achieved whereas NK effectors were totally spared despite KIR3DL2 expression by some of them. In sharp contrast, in the same experimental conditions, alemtuzumab induces not only the elimination of Sézary tumor cells but also of NK effectors that are key elements for the generation of antitumor cytotoxicity. The detrimental nontargeted effects of alemtuzumab exemplified in our ex vivo autologous killing assays are in agreement with clinical findings, as alemtuzumab therapy is known to lead to complications and severe side effects due to profound immunosuppression. Our results strongly suggest that IPH4102 will be devoid of such immunosuppressive adverse effects. It is widely admitted that preserving a fully active immune system while eliminating tumor cells is a key element of a successful therapeutic strategy in patients with CTCL (14, 52, 53). Taken together, our preclinical staining and efficacy data emphasize the relevance of therapeutically targeting KIR3DL2 and fully supporting the development of IPH4102, the first-in-class anti-KIR3DL2 mAb, for the treatment of patients with advanced CTCL. IPH4102 is currently undergoing regulatory studies, including nonclinical safety evaluation, to allow the conduct of a phase I clinical trial.

Disclosure of Potential Conflicts of Interest

C. Patarel has ownership interest (including patents) in Innate Pharma. H. Sicard is a senior director and has ownership interest (including patents) in Innate Pharma. No potential conflicts of interest were disclosed by the other authors.

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