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Determinants of response to daratumumab in Epstein-Barr virus-positive natural killer and T-cell lymphoma

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ABSTRACT

Background The potential therapeutic efficacy of daratumumab in natural killer T-cell lymphoma (NKTL) was highlighted when its off-label usage produced sustained remission in a patient with highly refractory disease. This is corroborated recently by a phase II clinical trial which established that daratumumab monotherapy is well tolerated and displayed encouraging response in relapsed/refractory NKTL patients. However, little is known regarding the molecular factors central to the induction and regulation of the daratumumab-mediated antitumor response in NKTL.

Methods CD38 expression was studied via immunohistochemistry, multiplex immunofluorescence and correlated with clinical characteristics of the patient. The therapeutic efficacy of daratumumab was studied in vitro via CellTiter-Glo (CTG) assay, complement-dependent cytotoxicity (CDC), antibody-dependent cell cytotoxicity (ADCC), and in vivo, via a patient-derived xenograft mouse model of NKTL, both as a single agent and in combination with L-asparaginase. Signaling mechanisms were characterized via pharmacologic treatment, RNA silencing, flow cytometry and corroborated with public transcriptomic data of NKTL.

Results Epstein-Barr virus-positive NKTL patients significantly express CD38 with half exhibiting high expression. Daratumumab effectively triggers Fc-mediated ADCC and CDC in a CD38-dependent manner. Importantly, daratumumab monotherapy and combination therapy with L-asparaginase significantly suppresses tumor progression in vivo. Ablation of complement inhibitory proteins (CIP) demonstrate that CD55 and CD59, not CD46, are critical for the induction of CDC. Notably, CD55 and CD59 expression were significantly elevated in the late stages of NKTL. Increasing the CD38:CIP ratio through sequential CIP knockdown, followed by CD38 upregulation via All-Trans Retinoic Acid treatment, potently augments complement-mediated lysis in cells previously resistant to daratumumab. The CD38:CIP ratio consistently demonstrates a statistically superior correlation to antitumor efficacy of daratumumab than CD38 or CIP expression alone.

Conclusion This study characterizes CD38 as an effective target for a subset of NKTL patients and the utilization of the CD38:CIP ratio as a more robust identifier for patient stratification and personalisation of treatment. Furthermore, elucidation of factors which sensitize the complement-mediated response provides an alternative approach toward optimizing therapeutic efficacy of daratumumab where CDC remains a known limiting factor. Altogether, these results propose a strategic rationale for further evaluation of single or combined daratumumab treatment in the clinic for NKTL.

INTRODUCTION

Natural killer T-cell lymphoma (NKTL) is a highly malignant subtype of mature T and NK neoplasm characterized by Epstein-Barr virus (EBV) infection and often associated with destructive lesions in the sinonasal cavity and the upper aerodigestive tract.[1] This neoplasm makes up 5%–15% of all non-Hodgkin's lymphoma (NHL). Prognosis remains relatively dismal with a 5-year overall survival rate of 54% in patients with nasal disease and 34% in patients with extranasal disease.[2 3] Epidemiologically, this disease is predominant in East Asia and South America although its increasing incidence in USA and Europe emphasizes its relevance globally.[4–6]

Although NKTL has a dismal prognosis, treatment options have improved over the years. In a recent prospective study of an international cohort of patients, a combination of radiotherapy and chemotherapy was the preferred first line treatment, with the SMILE protocol (dexamethasone, methotrexate, ifosfamide, L-asparaginase) most commonly administered in the patients.[3] Nonetheless, chemotherapy still induces serious side effects from adverse reactions, and radiation therapy in the head and neck region predisposes to oral mucositis which can progress to an acute life-threatening

stage.[7] Relapsed and refractory NKTL patients who do not respond to salvage chemotherapy also fail to benefit from autologous hematopoietic stem cell transplantation (HSCT).[8] Comparatively, while allogeneic HSCT demonstrates slightly improved response rates particularly in combination with L-asparaginase based regimens, it is also associated with high treatment mortality rates and should only be reserved for high-risk patients.[9 10] Thus, current clinical outcomes of NKTL remain suboptimal.

Targeted immunotherapy is currently a novel and active area of research in NKTL. NKTL tumors express programmed death protein ligand 1 (PD-L1), which ligates programmed cell death protein 1 (PD-1) and transmits a negative regulatory signal on T-cells thereby providing a potential mechanism to evade immunosurveillance.[11] This is supported by a clinical study which demonstrated that administration of a small group of patients with the anti-PD-1 antibody pembrolizumab appears to induce an effective treatment response.[12] This study highlights that the various immunotherapeutic-based treatment strategies warrant thorough investigation in NKTL so as to better overcome this aggressive malignancy.

Daratumumab, a human anti-CD38 antibody, is currently approved by US Food and Drug Administration (FDA) in combination with velcade or lenalidomide or as monotherapy for MM patients who have not responded to at least three prior lines of therapy.[13–16] These patients had shown profound responses in clinical trials with an unprecedented improvement in progression-free survival of 8.5–22.6 and 4.0 months, respectively.[17] Other preclinical studies have also supported the antitumor efficacy of daratumumab treatment. In both chronic lymphocytic leukemia (CLL) and NHL, daratumumab triggered Fc-mediated cytotoxicity via antibody-dependent cell cytotoxicity (ADCC) and ADCP in vitro as well as inhibited tumor cell dissemination in patient-derived xenograft (PDX) mouse models.[18 19] Daratumumab treatment is similarly effective against T-cell acute lymphoblastic leukemia (T-ALL) although via immune-independent mediated mechanisms of cytotoxicity such as CD38 crosslinking-induced apoptosis or the dysregulation of the ectoenzymatic activity of CD38, as Daratumumab could suppress tumor progression in completely immunodeficient mice models of T-ALL.[19 20]

The efficacy of Daratumumab in NKTL is lesser known. A previous study had demonstrated that CD38 positive expression was detected in a majority of NKTL tumors and that elevated expression of CD38 correlated significantly with inferior outcomes.[21] Separately, a clinical study was published describing a highly successful outcome with the off-label administration of Daratumumab in a patient with relapsed/refractory NKTL.[22] Despite receiving prior lines of radiotherapy, asparaginase-based combination chemotherapy as well as allogeneic hematopoietic-cell transplantation, the disease persisted until the patient was started on daratumumab. EBV and residual extranodal NKTL was undetected for the longest period of sustained remission since diagnosis. Importantly, flow cytometric analysis showed that this patient exhibited high CD38 expression.[22] More recently, the phase 2 clinical study of daratumumab monotherapy in relapsed/refractory NKTL patients was completed. Daratumumab monotherapy was well tolerated with no new safety concerns and achieved an overall response rate of 25.0%.[23]

While these reports separately highlight CD38 receptor as a promising novel therapeutic target in NKTL, there are no studies performed to date which seek to elucidate the determinants of response to Daratumumab in this aggressive blood malignancy. This study uncovers factors which are critical to the induction and optimization of the daratumumab-mediated response in NKTL thereby proposing rational strategies for patient stratification and the incorporation of daratumumab in effective combinations for future clinical trials.

MATERIALS AND METHODS

Immunohistochemistry

CD38 antibody (Cell Marque #118R-18) was used to detect CD38 expression on formalin fixed paraffin embedded slides derived from NKTL cell lines and commercially acquired NKTL patient samples (n=68), (Pantomics, USA). The immunohistochemistry (IHC) staining was performed on Leica Bond RX autostainer. Membrane staining was scored as 0, 1+, 2+ or 3+, according to its intensity. H-score was then calculated by the formula (% [1+] x 1) + (% [2+] x 2) + (% [3+] x 3) as described previously.[24 25] Cells are categorized as CD38hi with a score ≥250, CD38mid with a score ≥50 and CD38lo with any score <50.

Multiplex immunofluorescence

For the multiplex immunofluorescence (MIF), a cohort of patient samples were retrieved from the archives of the Department of Pathology, National University Hospital of patients diagnosed with NKTL between 1992 and 2017. The patient study group and preparation of samples for MIF are further described in online supplemental methods.

Image acquisition and analysis were done with the Vectra V.2 multispectral automated imaging system (PerkinElmer) and in Form V.2.0 image analysis software. Nuance software (PerkinElmer) was employed to build the spectral libraries for the chromogens (Opal 520, Opal 690 and DAPI). These chromogen signature profiles were later used to spectrally unmix and quantitate CD3 and CD38 staining intensity, with appropriate regions for analysis chosen by two pathologists. For each case, four images containing at least 10 000 cells were analyzed. The absolute optical density of each chromogen indicating the intensity of each antibody was obtained for every image and normalized against respective positive cut-offs.

NKTL xenograft model

Animal studies were performed in accordance with IACUC policies. Five weeks old C.B-Igh-1b/

GbmsTac-Prkdc^{scid}-Lyst^{bg} N7 (Beige-Scid) mice were injected subcutaneously with NKS1 resuspended in matrigel (Corning, USA). When the tumor was palpable, the mice were randomized into two treatment groups: IgG 10 mg/kg (control), daratumumab (5 mg/kg and 10 mg/kg). Treatments were administered once a week, intraperitoneal (i.p). Tumor size was measured three times a week using a caliper and derived using the formula: ½×length of tumor × (width of tumor)^2.

Please refer to online supplemental materials and methods for all further information.

RESULTS

CD38 is expressed in NKTL patient samples

It was first important to understand and characterize the pattern of CD38 expression in NKTL. This analysis was performed in two different cohorts of patients. In the first cohort, commercially available whole tumor tissue (n=68) from Chinese NKTL patients were processed by IHC and stained for CD38 expression. The IHC scores demonstrated that almost all NKTL samples show CD38 positivity. Seventy-eight per cent of the patients presented with a CD38 H-score higher than 100 and the median H-score of CD38 expression in the patient samples was 190 (figure 1A).

A second cohort was evaluated to further characterize CD38 expression specifically in the CD3 +NKTL subpopulation of the tumor sample. This cohort consists of samples (n=50) from patients diagnosed with extranodal NKTL and primary nodal NKTL, the latter of which has been notably classified as EBV +peripheral T-cell lymphoma (PTCL) based on the 2017 WHO recommendations.[26] As they are both associated with EBV and characterized by a cytotoxic T or NK-cell infiltrate, many cases of primary nodal NKTL were previously diagnosed as extranodal NKTL in the past. However, we and other groups have shown that nodal NKTL, shows features distinct from extranodal NKTL and often present with advanced stage disease in addition to poorer survival outcomes.[27 28] It was therefore of interest to include these cases in our study.

The percentage of double positive CD38+CD3+ cells was calculated from the multispectral imaging analysis (figure 1B) and the summary of these cases represented in table 1. Forty-nine out of 50 patient samples exhibited more than 1% CD38+CD3+ positive staining. Twenty-four per cent (12 cases) demonstrated ≥50% CD38+CD3+ staining, and the highest percentage of CD38+CD3+ staining detected in a patient was 84%. Seventy per cent of the patients (35 out of 50) exhibited greater than 10% CD38+CD3+ expression in the tumor sample (table 1).

Subsequently, we studied if there was any association between CD38+CD3+ staining in the tumor sample to the clinical characteristics of the patient (online supplemental table 1). Our analysis showed that the percentage of CD38+CD3+ expression in the tumor showed no significant association with (1) the subset of EBV-positive extranodal NKTL versus EBV-positive PTCL (previously primary nodal NKTL) (figure 1C), (2) patient diagnosis at stages 1–4 (figure 1D) and (3) patients of different genders (figure 1E). Kaplan-Meier analysis comparing the survival curve of patients with tumors exhibiting the top and bottom 25th percentile of CD38+CD3+ expression demonstrated a p value of 0.081, where high CD38+CD3+ expression confers a trend for shorter overall survival of the patients (figure 1F). Previously, we had published the results of a microarray analyses on these same patient tumor samples (GSE90784).[27] On study, we established that the percentage of CD38+CD3+ positive cells indeed correlated significantly with the mRNA expression of CD38 in corresponding tumor samples (figure 1G). This implies that relatively meaningful correlations may be made between the CD38+CD3+ population in NKTL and CD38 mRNA expression in prospective patient samples.

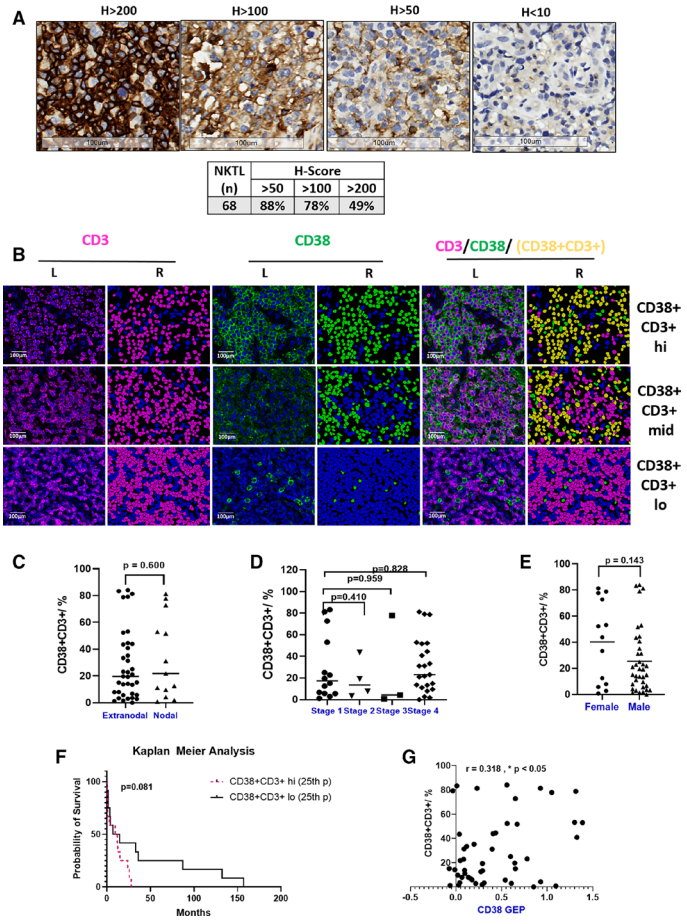
NKTL cell lines express varying levels of CD38 similar to patient samples

As patient NKTL samples are relatively rare, the mechanism of action of Daratumumab in NKTL can be more rigorously evaluated by studying a representative panel of NKTL cell lines. A panel of 8 NKTL cell lines and 2 T-cell Lymphoma cell lines were selected and measurement of mRNA expression by qPCR showed that these cell lines express varying levels of CD38 which can be stratified into CD38hi, CD38mid and CD38lo groups (figure 2A). This was corroborated by fluorescence-activated cell sorting (FACS) analyses whereby the number of molecules of CD38 expressed per cell was determined (figure 2B). Daudi is a Burkitt's lymphoma cell line which was originally used[29] to demonstrate the in vitro efficacy of daratumumab and thus is included here as a positive control. The results show that Daudi, NKS1, and NK92 cells express more than 50 000 molecules per cell and can be categorized as CD38hi. HANK1, NKYS and SNT-8 cells express more than 7000 molecules of CD38 per cell and are designated CD38mid cell lines. The rest of the panel, KHYG, SNK6, SNK1, SNK10 and HUT78 cells express between 1000 and 3000 molecules of CD38 per cell and are considered CD38lo. We also found that healthy primary NK cells also express a low number of CD38 molecules similar to CD38lo cell lines.

Further confirmation of the classification of the panel of NKTL cell lines was determined by IHC (figure 2C) where CD38hi expressing cell lines, NKS1 and NK-92 were indeed highly positive with a H-Score of >250, NKYS and HANK1 are CD38mid with H-score of 100–200, while the CD38lo cell lines showed little CD38 positive staining (online supplemental table 2).

Anti-CD38 antibody Daratumumab triggers Fc-mediated immune responses in NKTL

Daratumumab prominently triggers Fc-receptor mediated immune responses including ADCC and complement dependent cytotoxicity (CDC). Therefore, we cultured target NKTL cell lines with immune effector cells in the presence of daratumumab or control IgG.



NKTL (n)	H-Score		
	>50	>100	>200
68	88%	78%	49%

Figure 1 CD38 is expressed significantly in EBV positive extranodal natural killer T-cell lymphoma (NKTL) and primary nodal NKTL (EBV +PTCL) patient samples. (A) IHC was performed in a cohort of Chinese NKTL patients (n=68, Pantonomics, California, USA) with an anti-CD38 antibody (cell Marque). To evaluate CD38 expression, membrane staining was scored as 0, 1+, 2+ or 3+, according to its intensity. H-score was then calculated by the formula (% [1+] x 1) + (% [2+] x 2) + (% [3+] x 3) as described in the Methods section. (B) This figure shows CD38/CD3/DAPI multiplexed immunofluorescence (MIF) staining on a representative panel of NKTL patient samples (n=50, NUH). Top row case shows high level of CD38 expression, with CD38+/CD3 +cells (yellow) accounting for 90% of CD3+ tumor cells (magenta). Middle row case shows medium level of CD38 staining, with CD38+/CD3 +cells (yellow) account for 30% in tumor cell population (magenta). Bottom row case represents low CD38 staining, with 0% CD38+/CD3 +cells (yellow). (L) Refers to left panel: immunofluorescence images: CD3—magenta (membrane); CD38—green (membrane); DAPI—blue (nuclear) an (R) He right panel: with corresponding marks: magenta—CD3 +cells; green—CD38+ cells; yellow—CD3+/CD38 +cells; blue—negative cells. (C) CD38 +CD3+ percentage levels from the MIF study in samples from patients diagnosed with EBV positive extranodal NKTL and primary nodal NKTL (EBV positive PTCL) were analyzed and compared. Welch T test was performed to study statistical differences. (D) The CD38 +CD3+ percentage scores were analyzed according to the staging of the cancer that patients received at diagnosis. No significant difference was observed of the CD38 +CD3+ expression between stage 2, 3 and 4 and stage 1. (E) Patient samples harvested from female and male patients were studied. Welch T test detected no significant difference between the percentage expression of CD38 +CD3+ expression in male vs female patients. (F) Kaplan-Meier analyses was performed on the overall survival between patients expressing the top 25th percentile score of CD38 +CD3+ and the lowest 25th percentile score. (G) CD38 +CD3+ percentage expression on the cell surface elucidated from multiplex immunofluorescence was correlated with CD38 gene expression levels from a microarray analyses published previously and performed on the same patient samples. The percentage surface expression of CD38+CD3+ demonstrated significant correlation with mRNA expression (Graphpad prism), where *p<0.05. EBV, Epstein-Barr virus; IHC, immunohistochemistry; NUH, National University Hospital; PTCL, peripheral T-cell lymphoma.

CD38hi-expressing cells displayed the highest induction of ADCC, with 40%–50% of NKS1 targeted to ADCC. 10%–20% lysis was observed in CD38mid-expressing cell lines while CD38lo-expressing cell lines did not undergo ADCC at all (figure 2D). Effector and target only controls were unaffected by Daratumumab treatment (online supplemental figure 1).

Daratumumab had been selected for its ability to trigger potent CDC. The highest induction of CDC was detected in the NK92 cell line which expresses less CD38 molecules than KMS12BM or NKS1 (figure 2E). Additionally, HANK1 but not NKYS was targeted to CDC although both expressed similar levels of CD38 molecules. This implies an additional level of regulation mediating daratumumab-induced CDC.

Table 1 Extranodal NKTL and primary nodal NKTL (EBV-positive PTCL) tumors from patient samples show significant expression of CD38

CD38 +CD3+staining	No of cases
>50%	12
>10%	23
>1%	14
<1%	1
Total no of cases	50

Tumor samples from NKTL patients (n=50) were analyzed for CD38 and CD3 expression by multiplexed immunofluorescence. The percentage of CD38+CD3+ staining was calculated by taking the percentage of CD38+CD3+ positive cells over total CD3+ positive cells in the tumor sample.
EBV, Epstein-Barr virus; NKTL, natural killer T-cell lymphoma; PTCL, peripheral T-cell lymphoma.

It was previously reported that out of 14 multiple myeloma (MM) and 19 NHL cell lines, 27 were completely resistant to daratumumab-mediated CDC assays. Susceptibility to CDC seemed to correlate with elevated levels of CD38 and lower levels of complement inhibitory proteins (CIPs) CD59 and CD55.[30]

Additionally, daratumumab does not show any direct cytotoxic effect on NKTL and requires the recruitment of partner immune factors to mediate its antitumor activity (figure 2F).

All-trans retinoic acid increases CD38 surface expression in NKTL via mRNA upregulation and potently augments daratumumab-mediated cytotoxic effects

The level of CD38 expression is critical in mediating the overall cytotoxic effects of daratumumab. All-trans retinoic acid (ATRA) has been shown to upregulate CD38 expression and enhance daratumumab-mediated induction of ADCC and CDC in MM through the binding of the retinoic-acid-responsive element within the first intron of the CD38 gene.[31 32]

To study the singular effect of ATRA in Daratumumab-mediated lysis in NKTL, we selected a non-cytotoxic concentration (100 nM) of ATRA (figure 3A), which can be clinically achieved in patients.[33] At this concentration, we observed a higher fold upregulation of CD38 mRNA levels and surface protein expression in CD38mid-lo vs CD38hi cell lines particularly in HuT78 (figure 3B–C). ATRA pretreatment indeed resulted in increased daratumumab-mediated ADCC (figure 3D) and CDC (figure 3E). The importance of CD38 is evident in the HuT78 cell line which was originally CD38lo but transformed to CD38hi with ATRA pretreatment and displayed correspondingly drastic increases in the functional responses of ADCC and CDC to daratumumab.

NKYS, a CD38mid cell line, did not undergo CDC even with ATRA pretreatment unlike HANK1. Again, this suggests that increasing CD38 levels alone is insufficient to overcome inhibitory factors that supress CDC in some NKTL cell lines.

Panobinostat, a pan-histone deacetylase inhibitor, is another small molecule that has demonstrated synergism with Daratumumab through the upregulation of CD38 expression in MM.[34] However, Panobinostat treatment only enhanced CD38 expression in one of the three cell lines evaluated, NKYS. Additionally, this was not accompanied by an augmentation in CDC-mediated lysis of NKTL (figure 3F–G).

Daratumumab-induced CDC in NKTL is regulated at an additional level by the expression of CD55 and CD59, which are also significantly associated with the later stage of this cancer

Cancers cells safeguard from accidental lysis primarily through the expression of membrane bound complementary regulatory proteins CD46, CD55 and CD59.[35–37] The CIPs CD46 and CD55 block the complement cascade at the upstream C3 activation stage,[38 39] while CD59 interferes with the formation of the membrane attack complex and usually acts additively or synergistically with CD46 and CD55.[40 41]

We have earlier observed that although the cell lines KMS12BM and NKS1 express higher levels of CD38 in comparison to NK92, NK92 however demonstrates the highest sensitivity to Daratumumab-induced CDC. This suggests the involvement of complement inhibitory factors which may mitigate the effectiveness of daratumumab-induced CDC in CD38hi expressing cells.

To distinguish the role of CIPs CD46, CD55 and CD59 in regulating the efficacy of Daratumumab in NKTL, we first evaluated the level of expression of CIPs in all NKTL cell lines by FACS. Indeed, KMS12BM and NKS1 exhibited significantly higher levels of CD55 and CD59 molecules as compared with NK92 (figure 4A), while CD46 expression showed less difference. This seemingly inverse correlation between expression of CIPs (CD55 and/or CD59) and CDC, is shown in figure 4B, where cell lines which expressed higher levels of CIPs displayed a lower induction of CDC across the CD38hi, mid or low categories. The relative expression levels of CD46, CD55 and CD59 in these cell lines were also confirmed by IHC (online supplemental table 3).

In order to examine the specific role each CIP may contribute to CDC, a single knockdown was performed in CD38 positive (CD38hi-mid) NKTL cell lines. As observed in figure 4A, these cell lines express different levels of each of the CIP and thus were selected for the knockdown of CD46/CD55/CD59 based on levels of CIP being expressed. NK92 and HANK1 express significantly high levels of CD46 and almost negligible levels of CD55 or CD59, thus making these cell lines excellent candidates to study the role of CD46 in daratumumab-mediated CDC. We observed that suppressing the levels of CD46 in these cell lines does not have any significant effect on the percentage of CDC induced (figure 4C). NKYS and NKS1 cell lines express highest levels of CD55

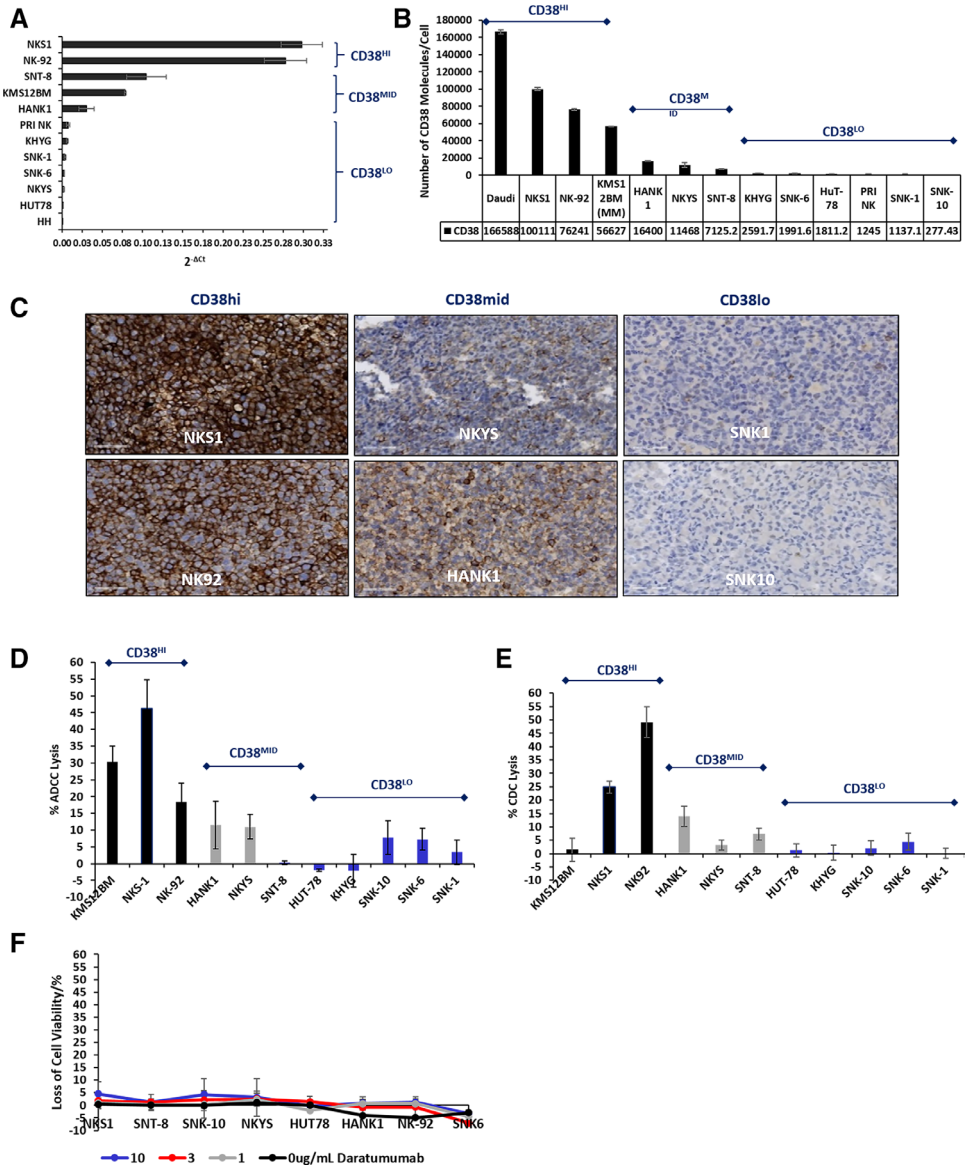


Figure 2 Daratumumab promotes anticancer activity in EBV-positive NK and T cell lymphomas through the induction of antibody-dependent cell cytotoxicity (ADCC) and complement dependent cytotoxicity (CDC). (A) Basal CD38 mRNA expression levels were studied in a panel of NKTL cell lines and one MM cell line (KMS12BM) by qRT-PCR. (B) CD38 (Pharmingen) expression on the surface of the cell lines were detected by FACS where at least 10 000 events were collected. The fluorescence of Quantibrite-PE beads (Pharmingen) was measured in parallel to create a standard curve for the elucidation of number of molecules expressed on surface of the cell. Cells were categorized into CD38hi, CD38mid and CD38lo cell lines as described in results. (C) CD38 expression on NKTL cell lines were evaluated via IHC and scored as described in 1A. (D) NKTL cell lines were preincubated with 10 µg/mL of Daratumumab or IgG. Subsequently, NKTL was cocultured with healthy donor effector primary NK cells at an effector:target ratio of 4:1. Cell lysis was measured 4 hour later (CytoTox-One, Promega). (E) NKTL cell lines were preincubated with 10 µg/mL of Daratumumab or IgG and then cultured with 20% human complement serum. Two hours later, cell viability was assessed (CTG, Promega). (F) NKTL cell lines were treated at the indicated concentrations of daratumumab for 48 hours and cell viability measured by Cell-Titer Glo (Promega). All experiments were performed at least n=3. EBV, Epstein-Barr virus; IHC, immunohistochemistry; MM, multiple myeloma; NK, natural killer; NKTL, natural killer T-cell lymphoma.

among the CD38hi-mid NKTL panel. Here, silencing CD55 in CD38mid NKYS was able to further sensitize the cells to daratumumab-induced CDC. Additionally, down-regulation of CD55 was also able to significantly enhance complement-mediated lysis of NKS1 cells similar to levels of that stimulated in NK92 in figure 2E (figure 4D). Except NKS1, all the CD38hi-mid NKTL cell lines express relatively low levels of CD59. Thus, in addition to NKS1, KMS12BM was selected for CD59 knockdown due high expression levels of CD59 and CD38. It is noteworthy that the suppression of CD59 resulted in only NKS1 but not KMS12BM cells exhibiting an enhancement in CDC (figure 4E). And interestingly, while each single knock-down of the CIP CD55 or CD59 in NKS1 results in an

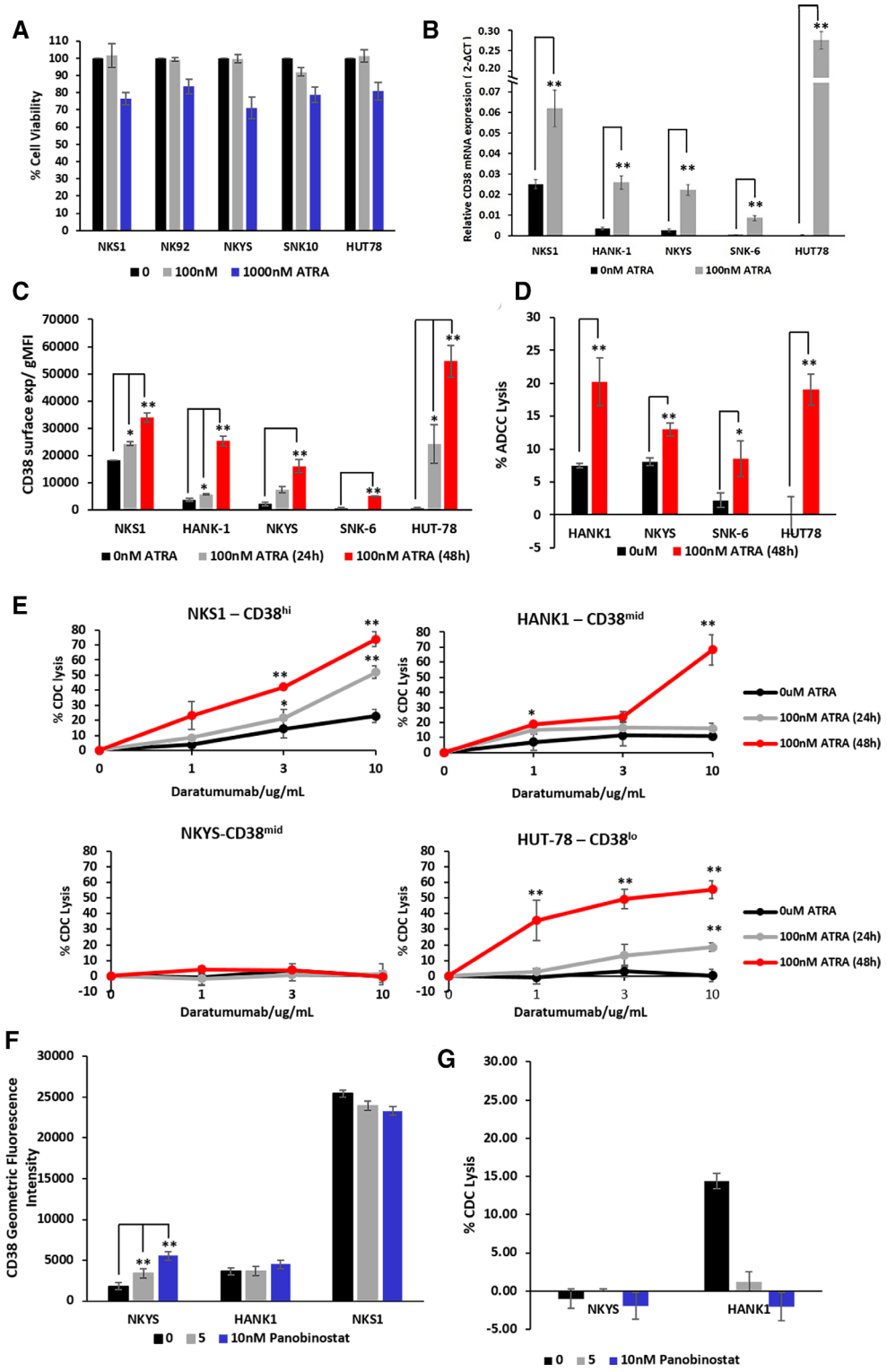


Figure 3 All-trans retinoic acid (ATRA) enhances anti-NKTL activity of Daratumumab by enhancing ADCC and CDC through upregulation of CD38. (A) NKTL cell lines were treated with varying doses of atra for 48 hours and cell viability (CTG, Promega) assay was performed. (B) qPCR analysis was performed in NKTL cell lines 18 hours after treatment with atra. **P<0.01 (C) CD38 cell surface expression was assessed 24 hours and 48 hours after treatment with 100 nM atra and analysis performed by FACS. At least 10 000 events were studied. *P<0.05, **p<0.01. (D, E) NKTL cell lines were treated with 100 nM of atra at the stated timepoints and (D) ADCC and (E) CDC analyzed as described previously *p<0.05, **p<0.01. (F) NKTL was treated with panobinostat for 48 hours and analyzed for CD38 surface expression by FACS. At least 10 000 events were collected. (G) NKTL was pretreated with panobinostat for 48 hours and subsequently incubated with 10 µg/mL of Daratumumab or IgG. Twenty per cent complement human serum was then added and viability of cells measured after 2 hours. All experiments were performed at least n=3. ADCC, antibody-dependent cell cytotoxicity; ATRA, all-trans retinoic acid; CDC, complement-dependent cytotoxicity; NKTL, natural killer T-cell lymphoma.

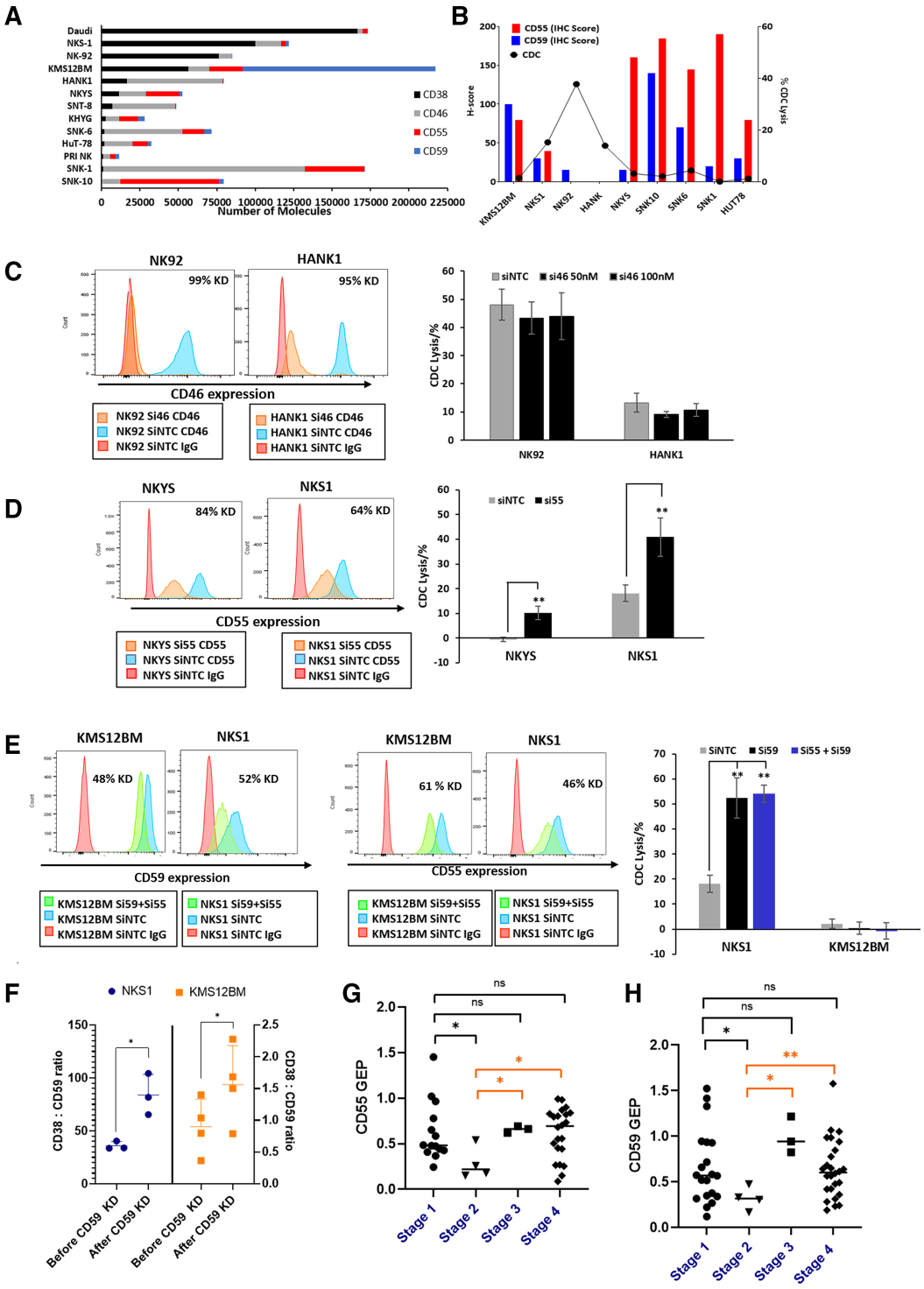


Figure 4 Complement inhibitory proteins (CIP) CD55 and CD59, can suppress the potency of Daratumumab-induced CDC in NKTL and are significantly associated with the later stage of this malignancy. (A) NKTL cell lines were analyzed for surface expression of membrane CIPs CD55, CD59 and CD46 by FACS and Quantibrite beads (BD) were concurrently used to identify number of molecules per cell. (B) A comparison of the IHC score of NKTL cell lines and CDC lysis induced suggests that CIPs CD55 and CD59 may be involved in regulating daratumumab-mediated CDC. (C–E) CIPs in indicated cell lines were silenced with Si Non Targeting Control (NTC) and (C) siCD46 (D) siCD55 (E) siCD55+siCD59 via electroporation (NEON). After 72 hours, CIP surface expression was detected by FACS and the effect on Daratumumab mediated CDC measured as described in Methods. **P<0.01. All experiments were performed at least n=3. (F) The ratio of CD38:CD59 expression was analyzed in NKS1 and KMS12BM before and after CD59 knockdown. The y axis on the left denotes the CD38:CD59 ratio in NKS1 and the y-axis on the right denotes the CD38:CD59 ratio of KMS12BM. The increase in CD38:CD59 ratio after CD59 knockdown was significant. *P<0.05. (G) CD55 and (H) CD59 GEP levels extracted from GSE90784 database was plotted according to the stage of cancer the patient was diagnosed, *p<0.05, **p<0.01. CDC, complement-dependent cytotoxicity; IHC, immunohistochemistry; NKTL, natural killer T-cell lymphoma; ns, not significant.

augmentation of the CDC activity of daratumumab compared with non-targeting control, the simultaneous double knockdown of CD55 and CD59 does no result in any further additive or synergistic lysis of the cells. Additionally, despite the double knockdown of the CIPs, KMS12BM is remains resistant to daratumumab-mediated CDC (figure 4E). This led us to hypothesize that the ratio of CD38 to CIP surface expression is an overriding factor regulating the efficacy of Daratumumab activity over the absolute number of molecules of CD38 or CIP CD55 or CD59 expressed by the NKTL. A closer analysis demonstrates that although the individual ratios of CD38:CD59 expression increases by approximately 2-fold after CD59 knockdown in both cell lines, the values of the ratio are vastly different, 83.8 in NKS1 and 1.82 in KMS12BM (figure 4F). Previously, only total levels of expression of either CD38, CD55 or CD59 were studied in patients, little has been done to evaluate the ratio of these levels. Our observations led us to hypothesize that the CD38: CIP ratio may instead constitute a more accurate and critical factor determining the susceptibility of a tumor to daratumumab as opposed to the absolute expression of each regulatory protein.

As the efficacy of monoclonal antibodies such as daratumumab in NKTL rely considerably on the expression levels of CIPs, we sought to investigate the relationship between the gene expression levels of CD59 and CD55 (from GSE90784) and clinical characteristics of our patients. To our knowledge, this has not been described in recent reports in NKTL. We did not observe any significant correlation between the gene expression levels of CD55 or CD59 with the gender, versus EBV-positive extranodal NKTL versus EBV-positive PTCL (primary nodal NKTL), CD38+CD3+ expression of the patient or overall survival (online supplemental figure 2A–D). However, one interesting observation was the significant elevation of the expression of CIP CD55 and CD59 from stage 2 to late stage 4 NKTL (figure 4G,H). This may have considerable impact in decreasing the NKTL tumor CD38:CD55/CD59 ratio and corresponding effectiveness of Daratumumab therapy as the disease progresses.

NKTL can be further sensitised to daratumumab-mediated complement-dependent lysis by regulating the ratio of CIPs to CD38

ATRA did not significantly affect the expression of CD55 and CD59 while it could drastically increase the expression of CD38 (figure 5A). We had earlier hypothesized that increasing the ratio of CD38: CIPs may particularly sensitize CD38mid-lo cells to further CDC. To test this hypothesis, we increased the expression ratio of CD38: CIP by downregulating the expression of CIPs with siRNA followed by upregulating CD38 expression with ATRA.

The CD38mid-expressing cell line NKYS displays increase in CDC with single CD55 knockdown. Subsequent treatment with ATRA after CD55 silencing results in the doubling of amount of CDC stimulated (figure 5B). We observed a similar effect in NKS1 where silencing of CD55 and CD59, followed by the addition of ATRA induced almost a total lysis of all NKS1 cells (figure 5C). Again, an identical trend was observed in a CD38lo and CD55hiCD59hi cell line Hut78. The dual silencing of CD55 and CD59 in HuT78 cells was insufficient to trigger CDC. However, preincubation with ATRA overcame the inhibitory effect of the CIPs through a dramatic increase in the CD38:CIP ratio which then subsequently triggered a massive amount of Daratumumab-mediated CDC even higher than ATRA treatment alone too (figure 5D).

These experiments showed that by increasing the CD38:CIP ratio, we could strongly sensitize cells to further lysis via CDC. To statistically study this, a Spearman's rank correlation study was performed in two ways (1) Between the absolute number of molecules of CD38, CD46, CD55 and CD59 to CDC and (2) Between the ratios of the number of molecules of CD38: CIP (CD46/CD55/CD59) to CDC. The data points are gathered based on the results obtained from the previous CDC experiments performed (figures 2–5).

Our analysis shows that, the number of molecules of CD38 positively correlates with CDC and that of CD55 inversely correlates with CDC while CD46 and CD59 do not show any significant correlation (figure 5E).

However, when we studied the correlation between the ratios of CD38:CD46, CD38:CD55 or CD38:CD59 and the extent of Daratumumab-mediated CDC, we observe that the CD38: CIP ratio consistently exhibits a significant and positive correlation with the extent of CDC lysis. In addition, the correlation coefficient values of CD38:CD55 or CD38:CD59 show robust associations closer to 1, thereby implying that the CD38: CIP ratio may more accurately predict the efficacy of daratumumab than absolute expression level of the molecules alone (figure 5F).

Daratumumab can strongly inhibit NKTL growth in vivo

NKS1 is a cell line that was developed from a patient via serial transplantation in a mouse xenograft.[42] As myeloma patients show evidence of a depletion in primary NK cells after daratumumab treatment, SCID Beige mice were selected to capture this clinical scenario leaving only a functional complement system to exert the antitumor effect of the antibody. Daratumumab treatment significantly inhibited the growth of NKTL in the NKS1 xenograft model and in some mice almost completely reversed tumor formation (figure 6A). This suggests that the complement system and potentially other non-immune modulatory effects of Daratumumab are sufficient to induce the potent antitumor in NKTL during the period of study. Survival studies also show that mice treated with 10 mg/kg of daratumumab has longer survival rates as compared with control mice (figure 6B).

To gain insight as to the mechanisms of resistance against daratumumab treatment, we selected three mice which developed exponential tumor growth after 32 days of last dose of 10 mg/kg daratumumab treatment (daratumumab 10 mg/kg R—average tumor size 1646 mm^3 ±172) and three mice which maintained a good

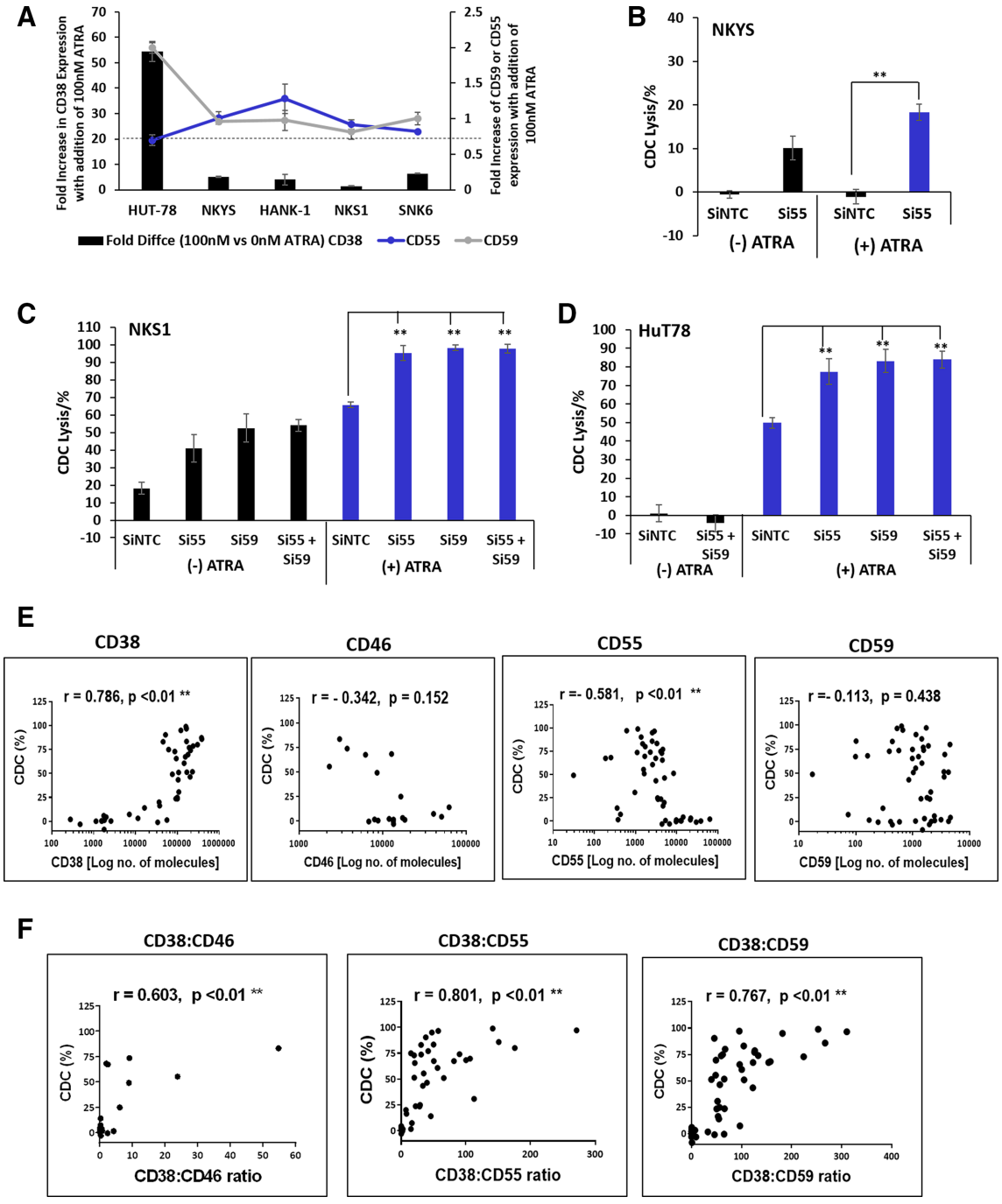


Figure 5 Sequential knockdown of CD55 or CD59 followed by the addition of ATRA potently enhances daratumumab-mediated cell lysis through the increase of the CD38:CIP ratio. (A) NKTL cell lines were pretreated with 100 nM atra and expression levels of CD38, CD55 and CD59 measured by FACS. At least 10 000 events were collected. Fold difference of the expression before and after atra treatment was plotted accordingly. (B) CD55 in NKYS cell lines were silenced by electroporation with siRNA for 72 hours. 24 hours after CD55 knockdown, NKYS was treated with 0 nM or 100 nM atra for the next 48 hours. subsequently, cells were harvested and analyzed for Daratumumab mediated CDC lysis. (C, D) CD55 and CD59 in (C) NKS1 cells and (D) HuT78 cells were silenced by electroporation of siRNA for 72 hours. Twenty-four hours after CD55/CD59 knockdown, cells were treated with 0 nM or 100 nM atra for the next 48 hours. After this, cells were harvested and analyzed for Daratumumab mediated CDC lysis. **P<0.01. All experiments were performed at least n=3. (E) The Spearman's rank correlation coefficient was evaluated between CDC lysis induced and number of molecules of CD55/CD59/CD46/CD38 in the cell surface. A significant and positive correlation was observed with CD38 and a significant negative correlation associated with CD55 expression levels. Conversely, no significant correlation was observed with CD46 and CD59. (F) All the ratios of CD38:CIP that is CD38:CD46, CD38:CD55 and CD38:CD59 expression display not only a significant but also a high correlation coefficient with CDC lysis. (Graphpad Prism). ATRA, all-trans retinoic acid; CDC, complement-dependent cytotoxicity; CIP, complement inhibitory proteins; NKTL, natural killer T-cell lymphoma.

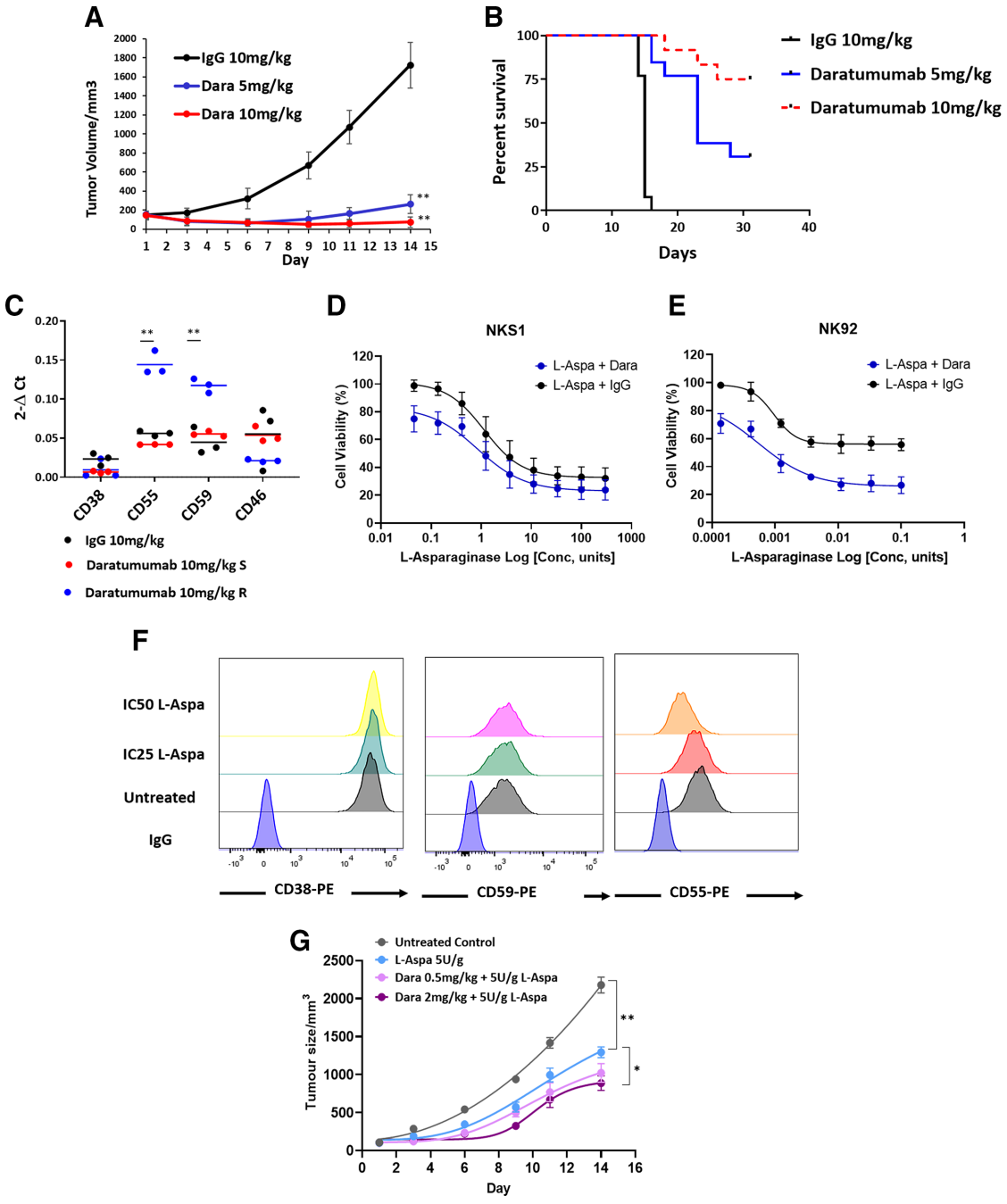


Figure 6 Daratumumab significantly inhibits tumor progression in a patient-derived NKTL tumor xenograft model both as a single agent and in combination with L-Asparaginase. (A) Scid beige mice were injected with patient-derived NKS1 cells and when tumors were palpable, mice were divided into three treatment groups where each group is n=10. Mice were treated with 10 mg/kg of IgG, 5 mg/kg and 10 mg/kg of daratumumab, i.p. Tumor volume was measured over time. **P<0.01. (B) Viability of the mice with Daratumumab treatment were monitored up to 30 days and plotted as a survival curve (C) mice which developed exponential tumor growth with Daratumumab treatment at the end of 30 days, **'R'** (n=3) and mice with almost complete regression of tumor growth with Daratumumab (**'S'**) were analyzed more closely. these tumors were harvested and mRNA extracted to perform a qPCR analysis for CD38, CD55, CD59 and CD46 expression. (D) NKS1 and NK92 were treated simultaneously with indicated concentrations of L-asparaginase and 3 µg/mL Daratumumab over a 24-hour period and then complement serum added and cell lysis measured by CTG (Promega). (E) NKS1 cell line was treated at IC25 and IC50 concentrations for 24 hours and the surface expression levels of CD38, CD59 and CD55 measured by FACS. At least 10 000 events were collected. (F) CB17 mice (C.B-Igh-1b/IcrTac-Prkdcscid) were injected with patient-derived NKS1 cells and when tumors were palpable, mice were divided into respective treatment groups where each group consist of n=10 mice and the mice were treated with L-Aspa 5 U/g, DarA 0.5 mg/kg+5 U/g L-Aspa, DarA 2 mg/kg+5 U/g L-Aspa, i.p. tumor volume was measured over time. **P<0.01, *p<0.05. NKTL, natural killer T-cell lymphoma.

response (daratumumab 10 mg/kg S - average tumor size 348 mm^3 ±302), and compared the mRNA expression of these tumors to three untreated (IgG 10 mg/kg – 2442 mm^3 ±471) mice which were harvested on day 15. The qPCR analysis showed that there was a slight decrease in CD38 mRNA expression regardless of tumor sensitivity or resistance to daratumumab. However, there was a significant upregulation of CD55 and CD59 in 'resistant' tumor tissues compared with 'sensitive' tumor tissues and a downregulation in CD46 mRNA levels (figure 6C). This interesting response implies that CD55 and CD59 may contribute to mechanisms of resistance which arise from the tumor in response to daratumumab treatment.

Daratumumab can enhance the anticancer activity of L-asparaginase in vivo

L- asparaginase-based regimens feature increasingly as first-line treatment for newly diagnosed or advanced stage NKTL.[43] L-asparaginase, unlike anthracyclines, cannot be effluxed by the multidrug resistant P-glycoprotein overexpressed in NKTL.[44] It breaks down and removes Asparagine, a non-essential amino acid that cannot be synthesized by the cancer cell. The combination of daratumumab and L-asparaginase in vitro is able to further suppress the survival of NKTL over L-asparaginase treatment alone (figure 6D). As drugs such as panobinostat and ATRA are able to increase CD38 expression, we studied the effect of L-Asparaginase on CD38, CD55 and CD59 surface expression levels. There was no significant change in CD38 or CD59 expression, however, a 18%±1.6% and 48%±6.3 decrease in CD55 expression was detected at IC25 and IC50 concentration of L-asparaginase, respectively (figure 6E). Subsequently, the combination treatment of daratumumab and L-asparaginase was investigated in vivo. We found that the addition of daratumumab at 2 mg/kg to L-asparaginase was able to significantly further inhibit tumor growth over L-asparaginase alone, congruent with what was observed in vitro (figure 6F). Interestingly, daratumumab treatment alone seemed more effective than the combination of L-asparaginase and daratumumab (data not shown).

DISCUSSION

The approach toward the eradication of cancer must always be multipronged, and immunotherapeutic strategies are now recognized as an effective and safe addition to the treatment armamentarium. Daratumumab has been applied successfully in relapsed/refractory MM leading to profoundly improved patient responses.[16 45] This success has led to further preclinical testing in other hematological malignancies such as T-ALL,[20] B-cell ALL (B-ALL),[46] acute myeloid leukemia,[47] NHL[19] and even lung cancer due to Daratumumab' s immune-promoting effects.[48 49] In light of studies that demonstrating CD38 predicts poorer prognosis in NKTL and the clinical efficacy of daratumumab in a single refractory patient, we sought to preclinically evaluate the determinants of daratumumab response in NKTL.

We first characterized in two independent patient cohorts that there is indeed a subset of CD38 mid to high expressing patients that may benefit from CD38-targeting via Daratumumab treatment. There was, however, no clear clinical association between CD38+CD3+ expression and gender, age, stage of disease or overall survival. Due to a rarity of patient samples, our local cohort size may not be sufficient to fully evaluate this relationship and it may benefit more from an international cohort study in future. Similar to the previously published study though, CD38hi expressing patients exhibited a trend towards poorer overall survival.

NKTL cell lines were subsequently used to demonstrate that direct binding of daratumumab antibody only to CD38 does not suppress NKTL survival. However, the addition of immune cofactors potently triggered cytotoxic responses via ADCC and CDC. Enhancement of CD38 expression with ATRA strengthened the positive association between CD38 expression and daratumumab-mediated cytotoxicity in NKTL. This is congruent with reports from the GEN501 and SIRIUS clinical trials in MM which showed that CD38 expression in patients with partial responses are significantly higher than those without partial response.[30] In particular, the potent increase in ATRA-mediated CDC lysis in HuT78 which was originally Daratumumab-resistant suggests that elucidating factors regulating CDC in NKTL may allow development of a strategy to sensitize even CD38 low expressing NKTL to daratumumab. Furthermore, previous reports in MM have focused more on augmenting ADCC and ADCP-mediated cytotoxicity triggered by daratumumab while factors which further sensitize tumor cells to CDC-mediated cytotoxicity induced by daratumumab are lesser studied.

Cells are protected from complement-induced lysis by inhibitory proteins, such as CD46, CD55 and CD59 which also play a crucial role in the immunological escape of tumor cells and in the development of drug resistance.[50 51] Analysis of MM patients enrolled in the GEN501 study showed an increased expression of CD55 and CD59 on MM cells during disease progression, suggesting that their overexpression is associated to the MM resistant phenotype.[52] Interestingly, we also observed this in tumors harvested from the NKTL xenograft mice resistant to daratumumab, where CD55 and CD59 levels were elevated but not CD46. Furthermore, the analysis of NKTL patient data indicates that CD55 and CD59 seem to be more highly expressed in patients at stage 4 as compared with stage 2.

In MM, CLL, and NHL, Daratumumab exerts an antitumor response primarily through ADCC and ADCP, while CDC remains a known limiting factor. However, our preclinical investigations suggest that elucidating the factors which can sensitize the CDC-mediated response provides an alternative approach towards understanding and optimizing the therapeutic efficacy of daratumumab. From the series

of knockdown experiments in NKTL cell lines, we confirm that we CD55 and CD59 but not CD46 play significant role in inhibiting daratumumab-mediated CDC in NKTL. In conjunction with the observation that CD55 and CD59 are upregulated in the later stages of NKTL, this particularly highlights the potential importance in overcoming the inhibitory effects of CD55 and CD59.

When double knockdown of CIPs did not sensitize CD38lo cell line HuT78 to CDC as effectively as ATRA treatment, we asked if targeting/manipulating the CD38:CIP ratio instead of CD38 or CIP molecules individually is a more effective and rational therapeutic strategy which can be incorporated to sensitize recalcitrant cancers to daratumumab. Increasing the CD38:CIP ratio by silencing CIPs and sequentially adding ATRA successfully triggered CDC in these CDC-resistant cell lines and completely lysed all cells in CDC-sensitive cell line NKS1. In line with this, the significantly lower ratio of CD38:C59 molecules exhibited after CD59 knockdown in KMS12BM as compared NKS1 provides further evidence of the importance of the level of CD38:CIP ratio over CD38 expression alone especially where NKS1 and KMS12BM express similar levels of CD38.

The Spearman's rank correlation studies subsequently confirmed the significant and strong association of the CD38:CIP ratio with CDC, stronger than with CD38 or CD55, CD59 alone. This ratio could be a useful predictive biomarker in future clinical trials. These are also important parameters that should be evaluated pretreatment and post-treatment in clinical trials. Recently, Huiqiang Huang *et al* have completed a Phase 2 study of Daratumumab monotherapy treatment in relapsed/refractory extranodal nasal, type NKTL patients.[23] In this patient cohort, the median H-score of CD38 expression was 67.5 with 10/22 expressing a H-Score of 0–49 and 11/22 a H-score of ≥50. Based on the categories of CD38 expression levels defined by the range of H-Score (0–300) observed in our patient cohort (where the median H-score was 190), the CD38 H-score expression of patients in this clinical trial falls in the range of lo-mid levels of CD38 expression. Even so, at a mid-lo CD38 expression range, a 25% ORR was observed in NKTL patients. In line with our preclinical findings, the level of CD38 expression has a gatekeeping effect on the magnitude of response. Additionally, while the levels of CD38 expression on the tumor are important, our study suggests that the CD38:CD55/CD59 ratio on patient samples is a more accurate predictor of Daratumumab efficacy in NKTL. Given the large range of CD38 expression observed in NKTL patient tumor samples as compared with the relatively high levels on MM patients, this will also help to stratify patients who will respond optimally to daratumumab treatment in future trials.

It may also be promising to explore CIP silencing therapeutic strategies such as the utilization of CD59 inhibitors as a therapeutic adjuvant[53] or bispecific antibodies that can target both CD38 and CIP[54] that can potentially be used in conjunction with ATRA in the clinic with daratumumab treatment.

The in vivo NKTL xenograft study also demonstrated a highly successful tumor regression with as low as 5 mg/kg weekly regimen and a prolongation in survival. Furthermore, in vitro and in vivo evaluation of the combination of daratumumab with L-asparaginase suggests that adding daratumumab to the treatment regimen mediates at least an additive effect in suppressing the tumor.

It has been proposed that the clinical efficacy of daratumumab may also be on account of its inhibitory effect on immune-suppressor populations such as T regulatory cells as well as on the immune-suppressor molecule adenosine, resulting in elevated T-cell expansion and functional immune responses monitored in responding patients.[55 56] This suggests that NKTL patients may also well benefit from the therapeutic combination of Daratumumab with inhibitors that block the PD-1/PD-L-1 axis which is especially promising given the recent efficacy of PD-1 blockade in NKTL failing L-Asparaginase regimens.[12]

We conclude that this study characterizes CD38 as a serious candidate therapeutic target for a subset of NKTL patients via the utilization of daratumumab as an anti-CD38 targeting antibody. It addresses the gap in the identification and elucidation of factors that influence the preclinical efficacy of CD38-mediated anti-NKTL targeting. In particular, given that Daratumumab was selected on the basis of its propensity to trigger CDC, it highlights the utilization of the CD38:CIP ratio as a far more robust identifier for patient stratification and proposes that this ratio provides a more cogent understanding as to the mechanisms of patient response to daratumumab as compared with CD38 or CIP expression alone. Importantly, we demonstrate equivalent potency of daratumumab in a PDX model as well as further additional tumor suppressive effects in combination with L-asparaginase which is a main component of first line chemotherapy in NKTL patients. Our findings propose a novel rational approach for the clinical evaluation and optimization of daratumumab response in future clinical trials particularly with ATRA in NKTL patients.

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Contributors NM designed the study, performed the experiments, analyzed the data and wrote the manuscript. WJC, and SBN designed the study, analyzed the data and wrote the manuscript. AHFN and SF performed the experiments, analyzed

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Competing interests LZ and JY are staff of Janssen Pharmaceuticals.

Patient consent for publication Not required.

Ethics approval Animal studies were performed in accordance with IACUC policies of the National University of Singapore (R16-1468). The patient study was approved by the NHG Domain Specific Review Board B (2009/212). This study was approved by NUS institutional ethics review board.

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