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Human anti-NKp46 antibody for studies of NKp46-dependent NK cell function and its applications for type 1 diabetes and cancer research

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Natural killer (NK) cells are innate lymphocytes that efficiently eliminate cancerous and infected cells. NKp46 is an important NK activating receptor shown to participate in recognition and activation of NK cells against pathogens, tumor cells, virally infected cells, and self-cells in autoimmune conditions, including type I and II diabetes. However, some of the NKp46 ligands are unknown and therefore investigating human NKp46 activity and its critical role in NK cell biology is problematic. We developed a unique anti-human NKp46 monocloncal antibody, denoted hNKp46.02 (02). The 02 mAb can induce receptor internalization and degradation. By binding to a unique epitope on a particular domain of NKp46, 02 lead NKp46 to lysosomal degradation. This downregulation therefore enables the investigation of all NKp46 activities. Indeed, using the 02 mAb we determined NK cell targets which are critically dependent on NKp46 activity, including certain tumor cells lines and human pancreatic beta cells. Most importantly, we showed that a toxin-conjugated 02 inhibits the growth of NKp46-positive cells; thus, exemplifying the potential of 02 in becoming an immunotherapeutic drug to treat NKp46-dependent diseases, such as, type I diabetes and NK and T cell related malignancies.

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Keywords: NK cells · NKp46 · antibody · cancer · type 1 diabetes

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Introduction

Natural killer (NK) cells are innate effector lymphocytes, first identified for their ability to kill tumor cells without prior stimulation [1]. To date, NK cells have emerged as one of the most crucial first responders to tumors, viral, bacterial, or fungal infections [2–7]. They are also involved in autoimmune diseases such as type I and type II diabetes [8, 9]. NK cells have cytotoxic activity and can secrete cytokines; hence they are part of the newly characterized family of innate lymphoid cells (ILCs). They are classified as Group 1 ILCs, mainly due to their production of type 1 cytokines (IFNγ and TNFα) [10, 11]. NK cells have the distinct ability to recognize diverse targets due to their numerous germline encoded activating and inhibitory receptors [12, 13]. A balance of signals received by these receptors ultimately determines whether the NK cells act against a given target cell, or remain neutral [14].

Three activating receptors found on NK cells, NKp30, NKp44, and NKp46, are collectively known as natural cytotoxicity receptors (NCRs) [12]. These receptors are crucial in NK cells' antitumor and antiviral activities [12, 15]. NKp46 has been established as a critical activating receptor since it is expressed almost exclusively by NK cells and is the only NCR with a mouse orthologue, denoted Ncr1 [12, 16, 17]. Its ligand repertoire ranges from viral ligands, such as, hemagglutinin (HA) and hemagglutinin-neuraminidase (HN) of influenza virus [18], Sendai virus [18], Newcastle disease virus [19], and poxvirus [20], to fungal ligands [7], to unknown ligands found on tumors [2], adipose cells [9], human pancreatic beta cells [8], hepatic stellate cells [21], and bacteria such as *Fusobacterium nucleatum* [4]. Recently, a soluble NKp46 ligand was identified [22]. The identification of the unknown membrane-bound ligands, in particularly the tumor ligands of NKp46, has been intensely investigated for over two decades.

NKp46 is part of the IgG superfamily, and consists of two C2-type Ig-like domains: D1 is the membrane distal domain and D2 is the membrane proximal domain [23, 24]. Interestingly, the D1 domain was shown to not be involved in NKp46 ligand recognition, and binding was mostly mediated by its D2 domain (which also contains stalk and hinge regions) [25]. Different features within the D2 domain determine binding to various ligands. For example, there are three glycosylation sites, Asn216, Thr125 and Thr225 which are commonly involved in the binding to different ligands. HA recognition by NKp46 was shown to be sialic acid dependent, primarily mediated by the residue Thr225 [25, 26], which is also involved in the recognition of some but not all tumors [25]. Asn216 and Thr125 both participate in the recognition of human beta cells [27], but do not require sialyation. Similarly, sialyation is not required for the recognition of the unknown ligand on *F. nucleatum* [4].

The unique capacity of NKp46 to bind different ligands using diverse features and binding sites, and the elusive identity of NKp46 cellular ligands has hindered research involving the function of NKp46. Furthermore, the biological relevance of the D1 domain of NKp46 remains largely unknown.

Consequently, in order to gain insight into how human NKp46 influences NK cell biology we developed a unique anti-NKp46 mAb, denoted hNKp46.02 (referred to as 02 throughout), which impairs all NKp46-dependent activities. Our study also presents the great potential of 02 in becoming a leading immunotherapeutic strategy against NKp46-dependant diseases, such as, type I diabetes and NKp46-expressing malignancies, such as T and NK cell lymphomas.

Results

Generation of anti-NKp46 mAbs

To develop antibodies against NKp46 we injected NKp46-deficient mice ($Ncr1^{gfp/gfp}$, [16]) with a fusion protein consisting of the extracellular portion of NKp46 fused to human IgG (NKp46-Ig). Many newly generated anti-NKp46 mAbs were evaluated for their ability to bind NKp46 (data not shown), however we will present data regarding one unique antibody, 02. Gating strategies for flow cytometry assays are consistent throughout our work and presented in Supporting Information Fig. 1. Binding was initially examined on mouse thymoma BW transfectant cells expressing NKp46 (BW NKp46). As controls we used a commercially available anti-NKp46 mAb (denoted 9E2) and an anti-NKp46 mAb previously developed in our lab, 461-G1 [18, 25]. Like 9E2 and 461-G1, 02 specifically interacted with BW NKp46 but not with the parental BW cells (Fig. 1A). To demonstrate that 02 recognizes NKp46 naturally expressed by human NK cells, we stained IL-2 activated primary bulk human NK cells (activated NK cells) (Fig. 1B). The activated NK cells used throughout our experiments were isolated from PBMCs. Our purification protocol reached approximately 97% purity and we verified their identity as $CD56^{+}CD3^{-}$ cells (Supporting Information Fig. 2). We also stained freshly isolated PBMCs to confirm that 02 specifically identifies NKp46 found on NK cells (Fig. 1C). Indeed, like 461-G1, 02 positively stained almost all the NK cells, which made up approximately 8% of the total PBMCs of this particular donor, with minimal to no staining of either NKT or T cells (which made up 6% and 70% of the total PBMCs, respectively). Similar results were obtained with PBMCs derived from additional donors.

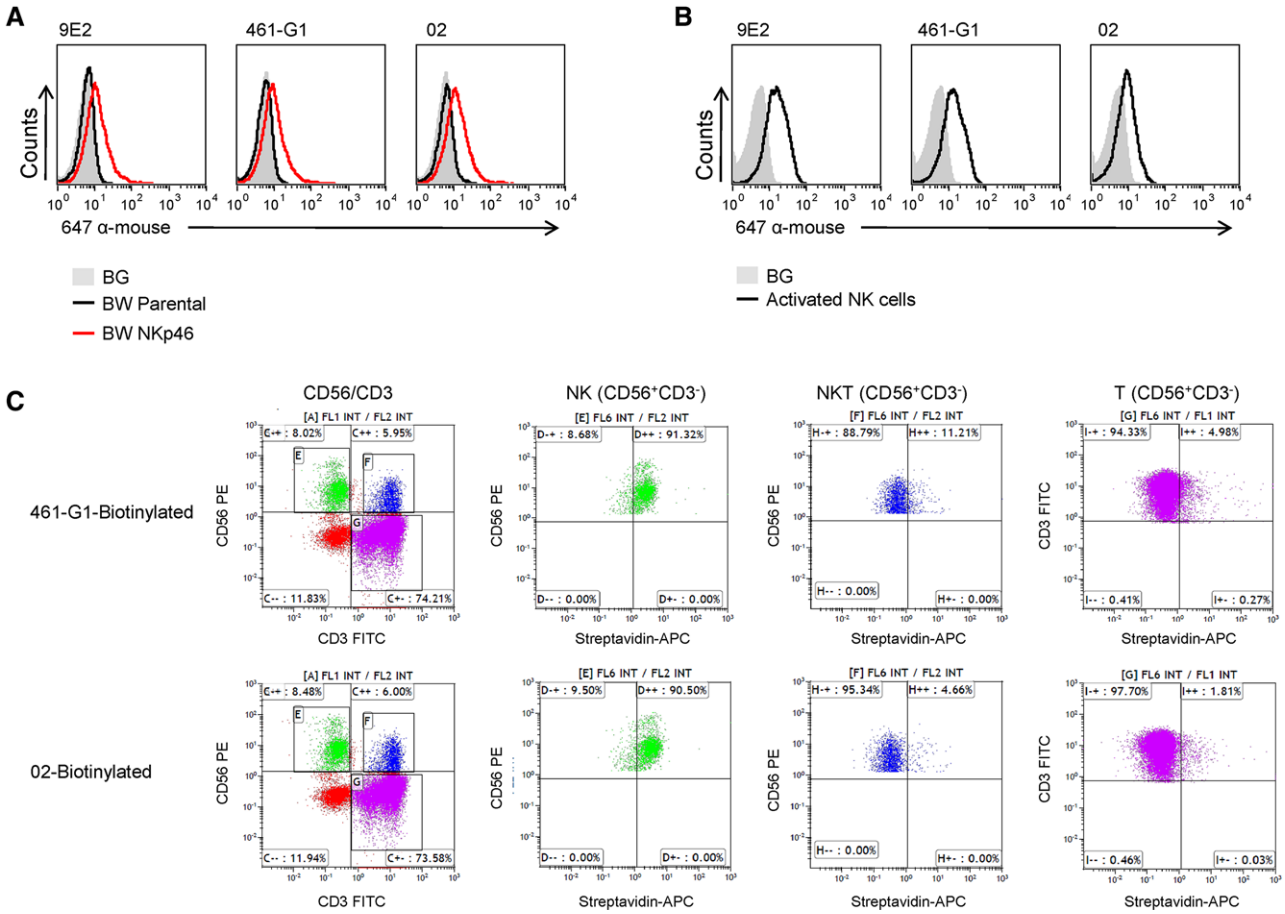

Figure 1. Several novel anti-NKp46 mAbs bind NKp46. (A) Flow cytometry analysis using anti-NKp46 mAbs (9E2, 461-G1, 02) of BW parental versus BW transfected cells expressing NKp46 (black and red histograms, respectively). Staining with 9E2 and 461-G1, was done with 0.5μg/50 000cells, and 50 μL/50 000cells of supernatant from the hybridoma of 02 was used for staining. After an hour at 4°C, cells were washed and stained with a conjugated secondary antibody (647 α-mouse) followed by FACS analysis. The filled gray histogram represents staining with secondary antibody only of the BW parental cells (BG). The background of BW NKp46 transfectants was similar and is not shown in the figure. Figure shows one representative experiment out of 6 performed. (B) Flow cytometry analysis of sorted activated NK cells. Staining with 9E2 and 461-G1 was done with 0.5 μg/50 000 cells, and 50 μL/50 000 cells of supernatant from the hybridoma of 02 was used for staining. After an hour at 4°C, cells were washed and stained with 647 α-mouse followed by FACS analysis. The filled gray histogram represents staining of NK cells with secondary antibody only. Figure shows one representative experiment out of 6 performed. (C) Freshly isolated PBMCs (200 000 cells/well) were incubated at 4°C with biotinylated 461-G1 and 02 mAbs (40 μg/mL), followed by secondary staining with Streptavidin-APC, α-CD56 PE and α-CD3 FITC. Cell populations were divided as follows: NK cells ($CD56^+/CD3^-$), NKT ($CD56^+/CD3^+$), T cells ($CD56^-/CD3^+$). Figure shows one representative experiment out of two performed, with a total of four different donors.

Binding of NKp46 by the 02 mAb led to NKp46 downregulation from the surface of NK cells

We initially checked whether any of the anti-NKp46 mAbs could block the interaction of NKp46 with its ligands. For this we used BJAB tumor cells which express an unknown ligand for NKp46 (Fig. 2A). NKp46-Ig was incubated either alone or with the various anti-NKp46 mAbs on ice. Subsequently, the treated NKp46-Ig fusion proteins were used to FACS stain BJAB cells. None of the anti-NKp46 mAbs were able to block the binding of NKp46-Ig to the cells (Fig. 2A). Additional tumor cell lines were assessed, and similar results were received (Supporting Information Fig. 3A).

We next tested whether any of the anti-NKp46 mAbs lead to reduced NKp46 expression on the surface of NK cells. In these assays we initially treated NK cells with anti-NKp46 antibodies (9E2, 461-G1, and 02) followed by staining with a conjugated form of 9E2 (the commercial anti-NKp46 antibody, APC α-NKp46). To confirm that neither 461-G1, nor 02, blocks the binding of 9E2 to NKp46 we treated NK cells with 9E2, 461-G1, and 02 for an hour at 4°C, followed by staining with APC α-NKp46 (Supporting Information Fig. 3B). From these assays we saw that APC α-NKp46 was able to bind NKp46 in the presence of 461-G1 and 02, hence the antibodies do not have overlapping epitopes, whereas pre-incubation with purified 9E2 blocked APC α-NKp46 binding. Next, we treated NK cells with the anti-NKp46 mAbs for 8 hours either at 4°C or 37°C. After incubation, small samples of cells were evaluated for viability by Trepan blue staining, to confirm that the different antibody treatments did not induce cell death (data not shown). The cells were then washed and stained with

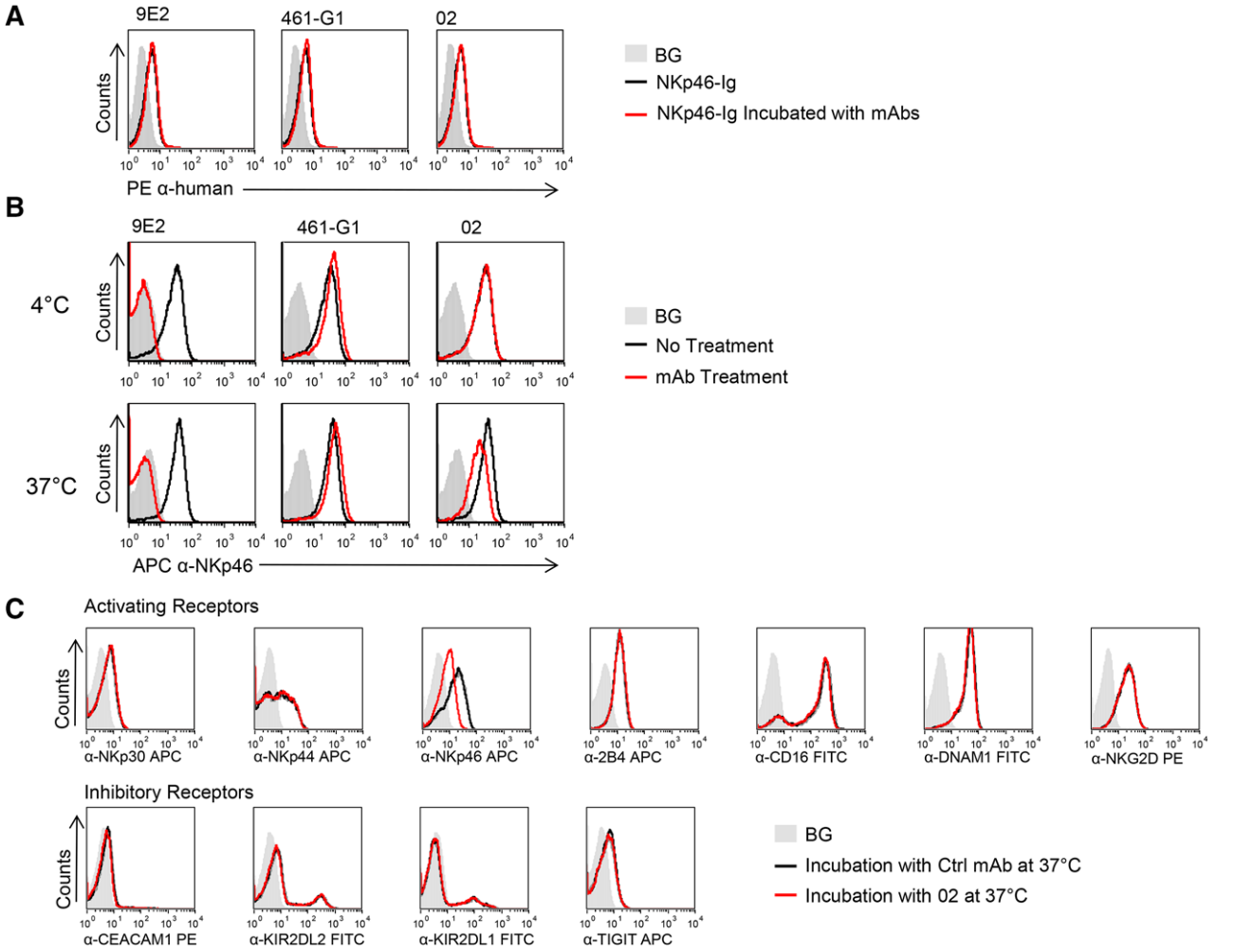

Figure 2. Characterization of several anti-NKp46 mAbs. (A) NKp46-Ig was pre-incubated either alone (black histogram) or with anti-NKp46 mAbs (9E2, 461-G1, or 02 – red histograms; for concentrations used please see Materials and methods) at 4°C, followed by staining of BJAB cells with the pre-treated NKp46-Ig. The filled gray histogram represents staining of BJAB with secondary antibody only. Figure shows one representative experiment out of 2 performed. (B) Sorted activated NK cells (50 000 cells/well) were incubated either alone (No treatment, black histograms) or with 5 μg/mL of the indicated anti-NKp46 mAbs (9E2, 461-G1, or 02, red histograms) at 4°C or 37°C for 8 hours, followed by staining with APC α-NKp46. The filled gray histogram represents staining with isotype control (IgG APC) of cells treated at 4°C. The background of cells treated at 37°C was similar and is not shown in the figure. Figure shows one representative experiment out of 5 performed, with a total of 6 different NK donors. (C) Sorted activated NK cells (50 000 cells/well) were incubated with 5μg/mL of control (black histogram) and 02 (red histogram) mAbs at 37°C for 16 hours, followed by staining of NK cells with the indicated conjugated antibodies. The filled gray histogram represents staining of NK cells with isotype control antibody. Figure shows one representative experiment out of 2 performed.

APC α-NKp46 (Fig. 2B). We observed that incubation with the 02 mAb at 37°C led to reduced levels of NKp46 surface expression. The MFI values of the experiment presented in Fig. 2B, and those of additional experiments performed are provided in Supporting Information Table 1. Taken together, an average 60% downregulation of NKp46 expression was observed following treatment with 02 for 8 hours at 37°C (Supporting Information Table 1).

To confirm that the downregulation was specific to NKp46, we repeated the assay on activated NK cells with 02 and a control mAb, followed by FACS staining for a large repertoire of NK cell receptors, both activating and inhibitory. In these assays we doubled our assay time to assess whether longer incubations times would alter the expression levels of the various receptors. The results demonstrated that the 02-mediated downregulation was specific to NKp46 (Fig. 2C). Similar results were obtained using activated NK cells from various donors (data not shown).

Our next step was to optimize the NKp46 downregulation mediated by 02. We began with a dose-dependent assay, where we incubated activated NK cells with increasing concentrations of 461-G1 (used as control), and 02 mAbs for 8 hours at either 4°C or 37°C (Fig. 3A and B, respectively). As the concentration increased, neither 461-G1 nor 02 affected NKp46 expression at 4°C; however, at 37°C, 02 induced NKp46 downregulation in a dose-dependent manner. The minimum amount of 02 that induced the maximum NKp46 downregulation (an approximate 40% decrease in surface expression) was 5 μg/mL (Fig. 3B). Significantly larger amounts

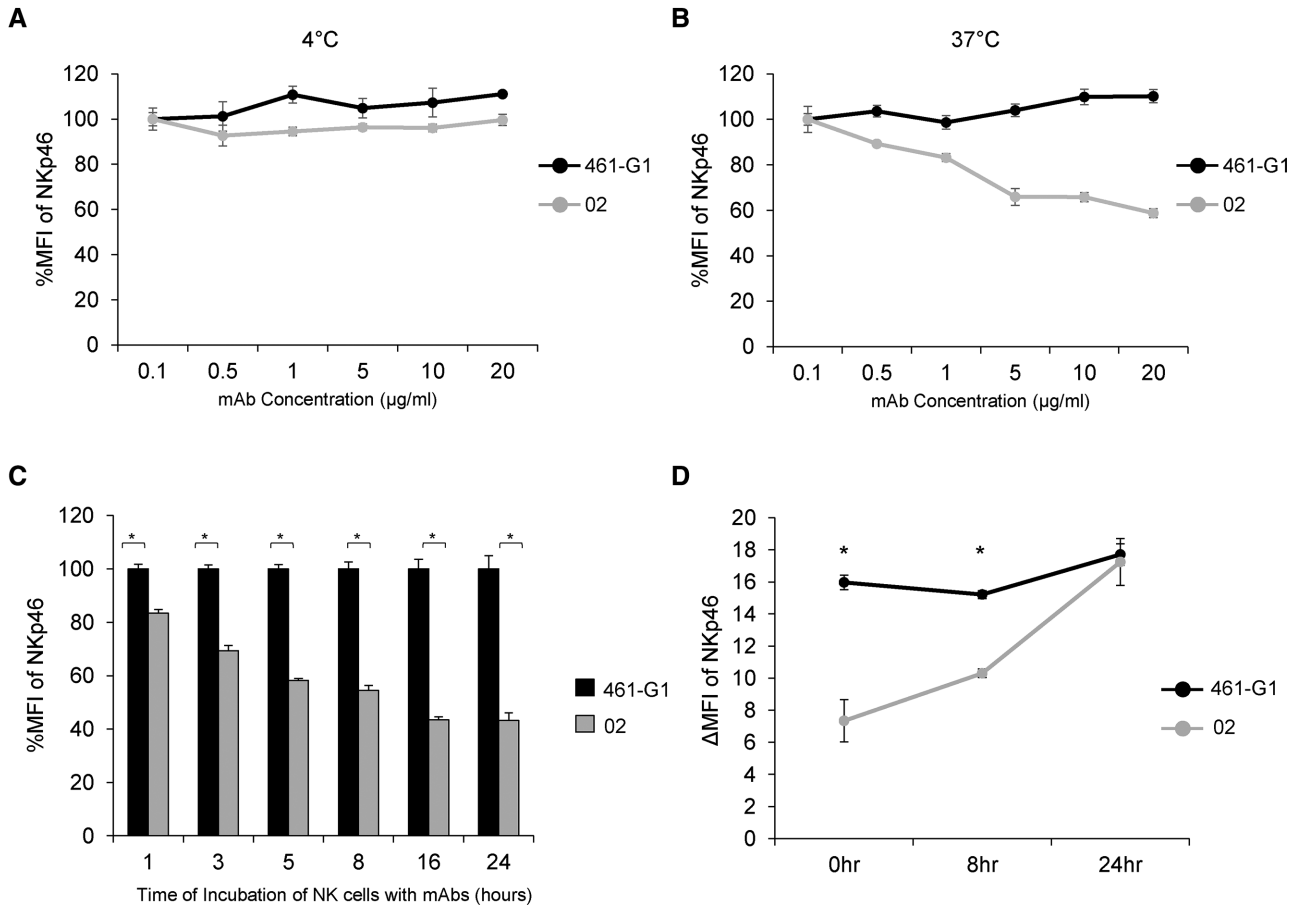

Figure 3. Time and concentration-dependent 02-mediated NKp46 downregulation (A and B). Sorted activated NK cells (50 000 cells/well) were incubated with 461-G1 or 02 mAbs for 8 hours at the indicated concentrations either at 4°C (A) or 37°C (B), followed by staining with APC α-NKp46. The MFI values of the isotype control IgG APC were averaged and subtracted from all samples. Adjusted MFI values were then averaged and normalized to the lowest concentration checked (0.1 μg/mL) for each antibody and set as 100%. Shown are the mean values and SD derived from triplicates. Figure shows one representative experiment out of 3 performed. (C) Incubation of activated NK cells (50 000 cells/well) with 5μg/mL of either 461-G1 or 02 mAbs at 37°C was performed for 1, 3, 5, 8, 16, and 24 hours, followed by staining with FITC α-NKp46. The MFI values of the isotype control IgG FITC staining's were averaged and subtracted from all samples. Adjusted MFI values were then averaged and normalized to 461-G1 (set as 100%). Shown are the mean values and SD derived from triplicates. $^*p < 0.0005$ by paired Student's *t*-test. Figure shows one representative experiment out of 4 performed. (D) Recovery of NKp46 expression. Activated NK cells (50 000 cells/well) were incubated with 5 μg/mL of either 461-G1 or 02 mAbs at 37°C for 16 hours followed by a wash and resuspension in RPMI and return to 37°C. NKp46 expression was assessed 0, 8, and 24 hours after removal of the antibodies by FACS staining with FITC α-NKp46. The MFI values of the isotype control IgG FITC staining's were averaged and subtracted from all samples (ΔMFI). Adjusted MFI values were averaged. Shown are the mean values and SD derived from triplicates. $^*p < 0.005$ by paired Student's *t*-test. Figure is one representative experiment out of three performed.

of 02 did not yield further downregulation of the receptor from the surface. Taking these results into consideration, we performed a time-course assay with activated NK cells incubated with the 461-G1 and 02 mAbs at 5μg/mL. We observed that after 16 hours, 02 led to a decrease in NKp46 surface expression by approximately 60%, which was maintained even after 24 h (Fig. 3C).

Lastly, we wanted to investigate the amount of time it would take NKp46 expression to recover after the removal of the 02 mAb. We incubated activated NK cells with 5μg/mL of the antibodies for 16 hours at 37°C. The cells were washed and resuspended in culture medium, and returned to 37°C. After 24h we observed the reconstitution of NKp46 expression back to regular levels, represented by the control antibody, 461-G1, which does not affect basal NKp46 surface expression (Fig. 3D).

NKp46 downregulation mediated by 02 impedes NK cell activation against target cells

To determine whether NKp46 downregulation by 02 would influence the functionality of NK cells, we first needed to find target cells that would lead to NKp46-dependent NK cell activation. For this, we used a cell-based reporter system which involves BW NKp46 cells. These cells were transfected with a chimeric protein composed of the extracellular part of NKp46 fused to the mouse ζ-chain (BW NKp46). Engagement of the receptor with its ligand leads to secretion of mouse IL-2 (mIL-2), which is then quantified by ELISA. Various cancer cell lines were assayed in this system, and a select few, 721.221, BCBL1, BJAB, C1R, Jurkat, and K562 were chosen based on their consistent and significant activation of

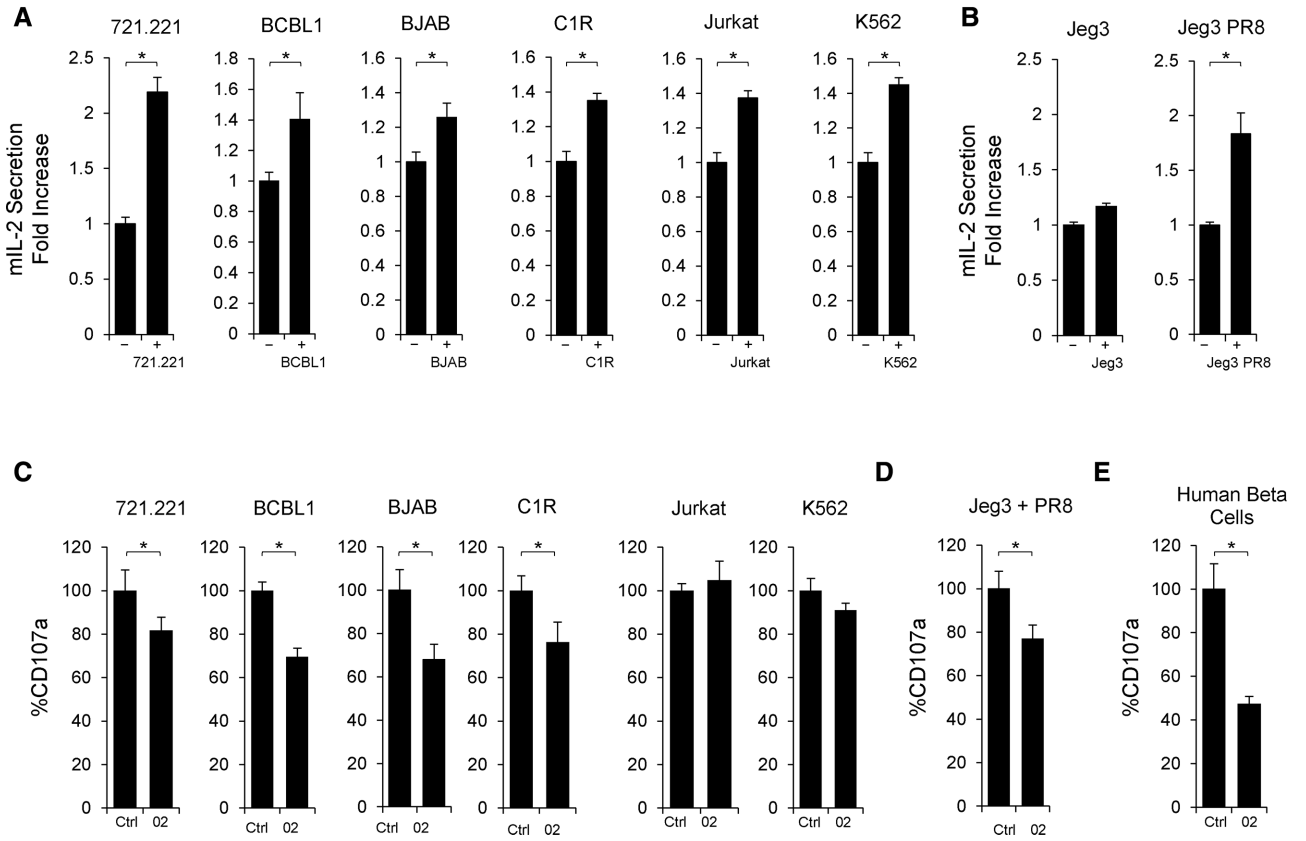

Figure 4. Effect of 02-mediated NKp46 downregulation on NK cell function. (A and B). BW NKp46 transfectants (50 000 cells/well) were incubated either alone (-) or with the indicated cancer cell lines (A) or with Jeg3 and Jeg3 incubated with influenza strain PR8 (Jeg3 PR8) (B) (indicated by (+)) for 48 hours at 37°C, followed by ELISA for mIL-2 secreted by BW NKp46 transfectants. mIL-2 secretion was measured at OD650nm, and results were normalized to basal mIL-2 secretion from BW NKp46 transfectants (set as 1). A and B show one representative experiment out of 3 performed. Shown are the mean values and SD derived from triplicates. $^*p < 0.05$. (C--E) Activated bulk NK cells (50 000cells/well) were initially incubated with 5 μg/mL of either control or 02 mAbs for 16 hours at 37°C. Cancer cell lines (C), Jeg3 in the presence of influenza (D), and human pancreatic beta cells (E), were added to the pre-incubated NK cells, along with anti-CD107a APC and anti-CD56 PE antibodies for 2 h at 37°C. Quadruplicate samples were plated and analyzed by flow cytometry. %CD107a positive cells of NK cells alone were averaged and subtracted from all samples. Adjusted %CD107a values were then averaged and normalized to control mAb (set at 100%). For C, D, and E, all experiments were performed at least twice with one representative replicate presented. Shown are the mean values and SD derived from quadruplicates. $^*p < 0.05$ by paired Student's *t*-test.

BW NKp46 (Fig. 4A). Jeg3 cells, which are known to not express NKp46 ligands [26, 28], were incubated with the influenza strain PR8, and used as a positive control for the assay (since influenza virus hemagglutinin is a ligand of NKp46 [18]). As can be seen in Fig. 4B, Jeg3 cells incubated with BW NKp46 induced no significant mIL-2 secretion, as opposed to Jeg3 PR8 cells which lead to a significant increase in mIL-2 secretion.

The above described target cells were then used to examine the functionality of 02. CD107a degranulation assays were performed on activated NK cells immediately after they were incubated with either a control or 02 mAbs for 16 hours. In the absence of target cells, NK cells pre-incubated alone or with the antibodies did not degranulate (data not shown). When incubated with target cells the NK cells degranulated; and similar levels of %CD107a positive cells were observed between NK cells pre-incubated alone or with the control antibody (data not shown). As such, in our results we compared the %CD107a positive NK cells, pre-incubated with either a control antibody or the 02 antibody. As can be seen in Fig. 4C, activated NK cells pre-incubated with 02 displayed a significant decrease in %CD107a levels when in the presence of certain target cells (721.221, BCBL1, BJAB, and C1R). Similarly, activated NK cells previously incubated with 02, had lower levels of %CD107a when incubated with Jeg3 PR8 cells (Fig. 4D). These results indicate that the decrease in NKp46 expression led to lower NK cell activation against specific targets. Interestingly, NK cells pre-incubated with the control or 02 antibody responded similarly to Jurkat and K562 cells (Fig. 4C), suggesting that the NKp46 ligand they express is not critically important for their elimination by NK cells. %CD107a levels of the above experiments are presented in Supporting Information Table 2. Taken together, these results display the ability of 02 to distinguish between target cells which express an NKp46 ligand to those whose elimination is NKp46-dependent.

Previous work done in our lab demonstrated in both human and mouse models of type I diabetes, that NK cells interact with and destroy pancreatic beta cells, in an NKp46-dependent manner [8, 27]. Consequently, we tested whether NKp46 downregulation by 02 on activated NK cells would prevent killing of fresh

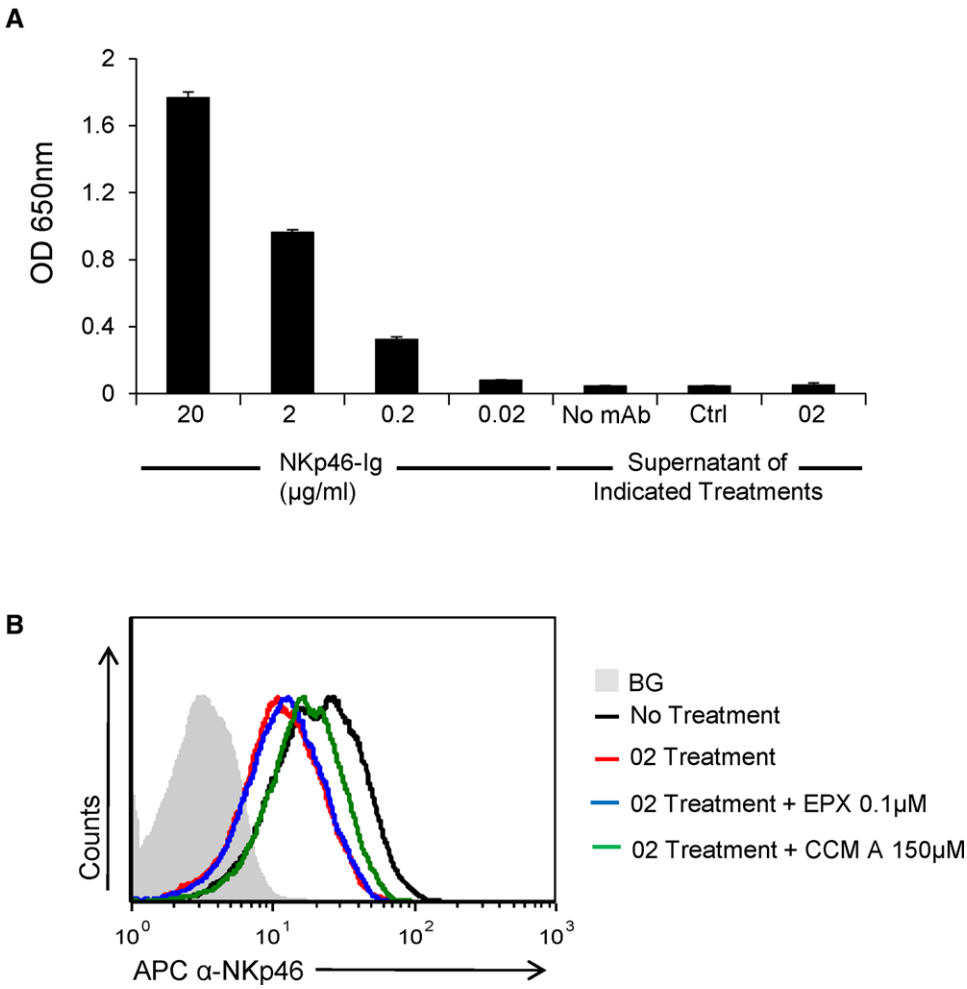

Figure 5. Determining the fate of NKp46 on primary NK cells following binding of anti-NKp46 mAbs. (A) Sorted activated NK cells (50 000 cells/well) were initially incubated either alone (No mAb) or with 5 µg/mL of control or 02 mAbs for 16 h at 37°C. To determine if NKp46 is shed by the anti-NKp46 mAbs, supernatants of each treatment were collected and plated onto an ELISA plate. As a positive control for the direct ELISA, additional wells on the ELISA plate were coated with NKp46-Ig at the indicated concentrations. A biotinylated anti-NKp46 mAb was then used for detection of NKp46, and ELISA assays were performed. Figure shows one representative experiment out of 2 performed. Shown are the mean values and SD derived from triplicates. (B) Sorted activated NK cells (50 000 cells/well) were initially incubated either alone or with proteasomal (EPX 0.1µM) or lysosomal (CCM A 150 µM) inhibitors for 20 minutes before the addition of 5 µg/mL of the 02 mAb for 6 h at 37°C. This was followed by staining of the cells with APC α-NKp46. The filled gray histogram represents staining of untreated NK cells with isotype control APC IgG. The background of treated cells was similar and is not shown in the figure. Figure shows one representative experiment out five of performed.

human beta cells. FACS analysis revealed a significant decrease in %CD107a positive NK cells, of approximately 50%, indicating that 02 strongly inhibited NK cell activation against human pancreatic beta cells (Fig. 4E, %CD107a values provided in Supporting Information Table 2).

Mechanism of 02-mediated NKp46 downregulation

The 02-mediated downregulation of NKp46 can either be due to receptor shedding or internalization. To test whether NKp46 is shed following incubation with 02 we monitored the amount of NKp46 in the supernatants. Activated NK cells were incubated either alone, with control or with 02 mAb for 16 hours at 37°C. Supernatants were collected and plated onto ELISA plates. As a positive control for the assay, NKp46-Ig was also plated on the ELISA plate in 10 fold decreasing concentrations, reaching concentrations as low as 0.02 µg per mL. Figure 5A shows that we were able to detect NKp46-Ig; however, no NKp46 was detected in the supernatants of the treated NK cells. The ELISA assay may not have been sensitive enough, yet due to our detection of low concentrations of NKp46-Ig we concluded that it is highly unlikely that 02 causes shedding of NKp46 from the surface of NK cells.

To test whether NKp46 is internalized and degraded, we treated activated NK cells with either proteasome or lysosomal inhibitors (epoxomicin (EPX) and concanamycin A (CCM A), respectively) prior to incubation with the 02 mAb. As shown in Fig. 5B, 02 reduced NKp46 expression on all the NK cells; however, while the untreated and EPX treated cells showed similar levels of NKp46 downregulation (red and blue histograms, respectively),

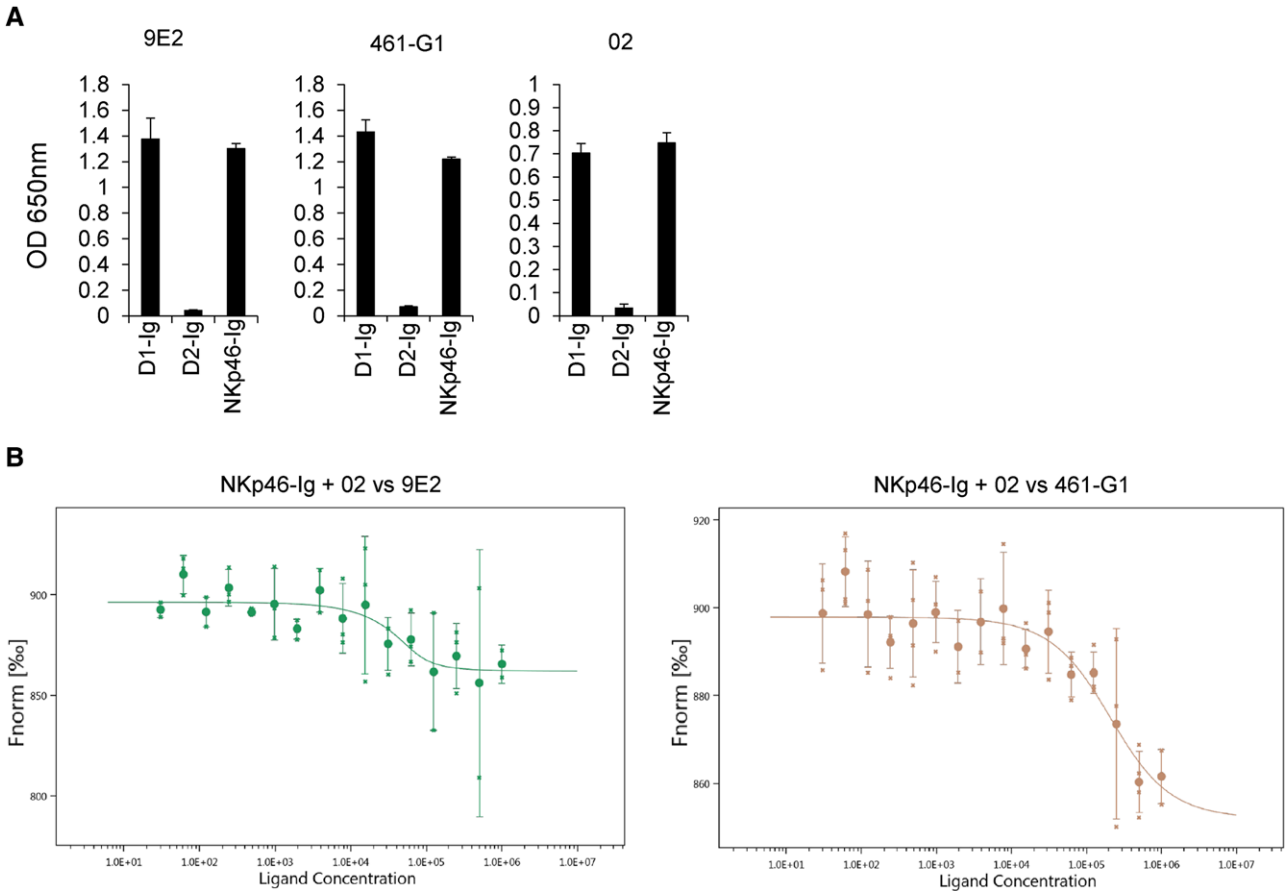

Figure 6. Identifying and characterizing the binding domain of the anti-NKp46 antibodies. (A) ELISA plates were coated overnight at 4°C with the fusion proteins D1-Ig, D2-Ig, and NKp46-Ig. The anti-NKp46 antibodies (9E2, 462-G1, 02) were subsequently added, followed by detection with a biotin anti-mouse antibody at OD650nm. Figure shows one representative experiment out of 2 performed. Shown are the mean values and SD derived from quadruplicates. (B) Competition binding assay of antibodies to NKp46. Labeled NKp46-Ig (70nM) was pre-saturated with 02 (0.25μM) followed by incubation with decreasing concentrations of either 9E2 or 461-G1 (1 μM–30 pM). Interactions were quantified by MST and the recorded fluorescence was normalized (Fnorm). Data represents three to five independent experiments.

NK cells treated with CCM A downregulated NKp46 to a lesser extent (green histogram). We consistently observed that the presence of a lysosomal inhibitor on 02-treated cells led to a significant increase in NKp46 expression as opposed to no inhibitor or the proteasomal inhibitor (Supporting Information Fig. 4). These results clearly suggest that after 02 binds NKp46, it undergoes internalization and degradation via the lysosomal pathway.

02 binds an epitope on the D1 domain of NKp46

Next, we looked to characterize the binding domain of 02 on NKp46. NKp46 is composed of two domains, D1 and D2. Fusion proteins comprised of the D1 or D2 domains fused to human IgG were prepared (D1-Ig and D2-Ig, respectively) and used in a direct ELISA assays (Fig. 6A). Interestingly, 02 bound the D1-Ig, the same domain bound by 9E2 and 461-G1, which do not induce receptor downregulation (Fig. 2B). Consequently, we wanted to determine whether 02's binding site on the D1 domain is distinct to that of 9E2 and 461-G1. We applied the use of Microscale Thermophoresis (MST), a method used for quantitative analysis of protein interactions within a solution [29, 30]. In these assays we pre-saturated NKp46-Ig with the 02 antibody, followed by addition of decreasing concentrations of either 9E2 or 461-G1 (Fig. 6B, green and brown graphs, respectively). Indeed, binding curves were generated by both antibodies; hence, even in the presence of saturating concentrations of 02, both 9E2 and 461-G1 bind NKp46. Accordingly, 02's binding site is distinct to that of 9E2 and 461-G1. All possible combinations of binding were evaluated and the results consistently demonstrated that 02, 9E2, and 461-G1 all have distinct binding sites on NKp46 which do not block the binding of one another (data not shown).

02 as a therapeutic mAb

NKp46 has been implicated as either a critical player in the progression and severity of different diseases, including type I diabetes, or as a prognostic marker in NK cell malignancies [31–34], and approximately 20% of T cell lymphomas [35, 36].

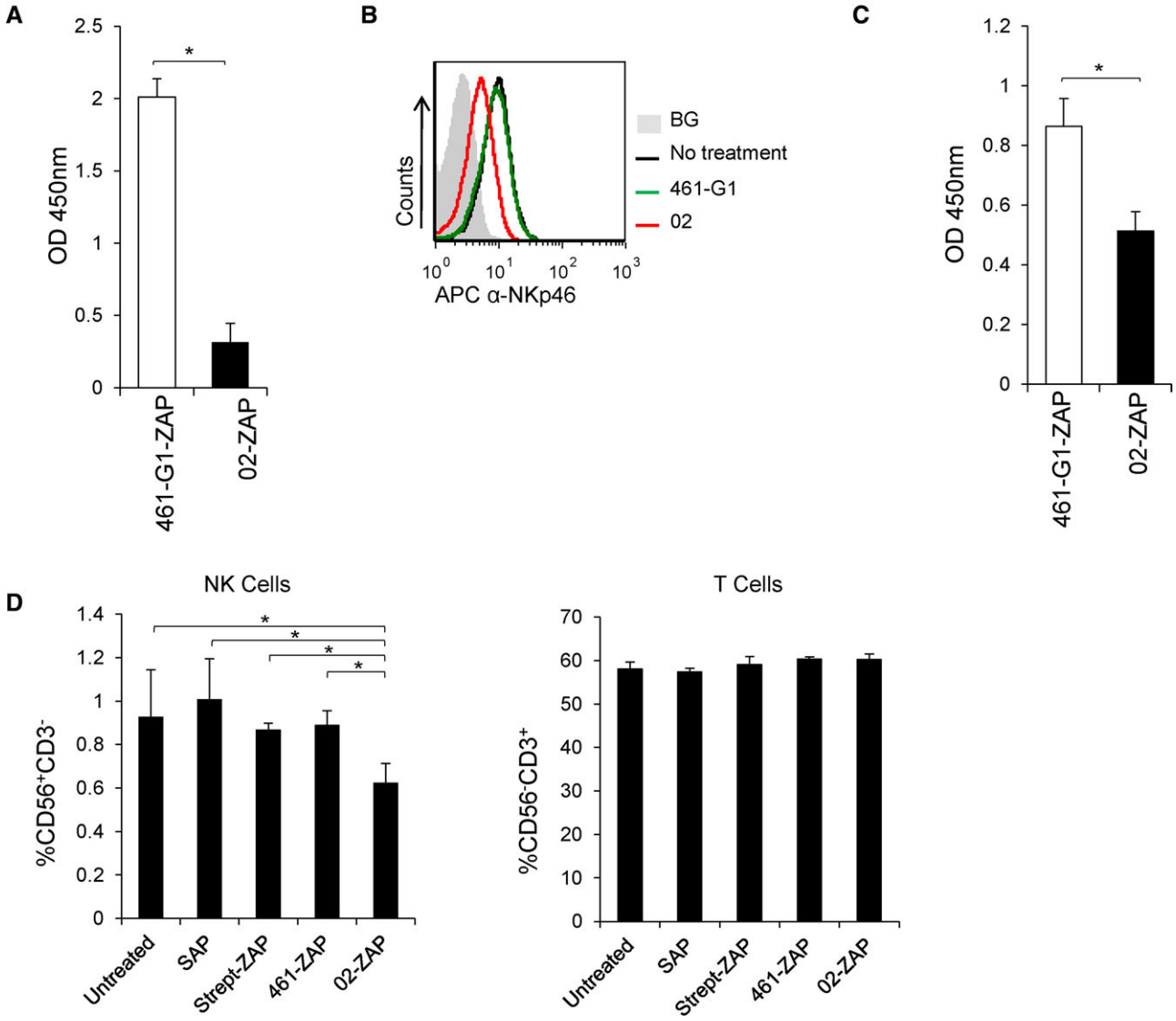

Figure 7. 02 conjugated to a toxin inhibits cell proliferation. (A) Sorted activated NK cells were plated at 2500 cells/well in RPMI overnight at 37°C. The next day, biotinylated 461-G1 and 02 were conjugated to streptavidin-ZAP (461-G1-ZAP and 02-ZAP) in an equal molar concentration of 10 nM. The conjugates were added to the NK cells and placed at 37°C for 72 hours, after which an XTT cell viability assay was performed. Shown are the mean values and SD derived from sextuplicates. One representative assay is shown out of two performed. *$p < 0.05$ by paired Student's *t*-test (B) YTS cells were incubated for 16 hours at 37°C either alone (black histogram) or with 5 ug/mL of either 461-G1 or 02 (green and red histograms respectively) followed by FACS staining with APC α-NKp46. The filled gray histogram represents staining of non-treated YTS cells with isotype control antibody (BG). The background of treated cells was similar and is not shown in the figure. One representative assay is shown out of two performed. (C) YTS cells were plated at 2500 cells/well in RPMI overnight at 37°C. The next day, biotinylated 461-G1 and 02 were conjugated to streptavidin-ZAP in an equal molar concentration of 0.01pM. The conjugates were added to the YTS cells and placed at 37°C for 72 hours, after which an XTT cell viability assay was performed. Shown are the mean values and SD derived from sextuplicates. *$p < 0.005$ by paired Student's *t*-test. (D) Freshly isolated PBMCs were plated at 100 000 cells/well in NK culture medium (please see Materials and Methods for specific details), followed by addition of 10 nM of the indicated treatments. Cells were placed at 37°C for 72 hours, after which flow cytometry analysis was performed with α-CD56 PE and α-CD3 APC. Shown are the mean values and SD derived from sextuplicates. *$p < 0.05$ by paired Student's *t*-test.

Consequently, we wanted to investigate whether our antibody has the potential to become a novel immunotherapeutic drug for the treatment of these diseases. As a proof of concept, we proceeded to conjugate a toxin to our antibody, hypothesizing that once it undergoes internalization it will introduce the toxin into the cell and thereby lead to cell death. The toxin used, saporin, is a ribosome inactivating protein which cannot enter cells on its own [37, 38]. We also conjugated the control antibody, 461-G1, which we showed binds NKp46 but does not induce internalization (Fig. 2B). The conjugates (461-G1-ZAP and 02-ZAP) were incubated with either activated NK cells (Fig. 7A) or the NK tumor cell line, YTS (Fig. 7B-C) for 72 hours, followed by a cell viability assay. We confirmed the ability of 02 to induce NKp46 internalization on YTS cells (Fig. 7B) before proceeding with the assay. As can be seen in Fig. 7A, NK cells treated with 02-ZAP were severely inhibited, indicating the potential of this conjugate in treating

NKp46-dependent autoimmune diseases, such as type I diabetes. In Fig. 7C we demonstrated the significant capability of 02-ZAP in inhibiting cell growth of tumors expressing NKp46.

To show specific activity of the conjugates against NK cells we proceeded to treat PBMCs for 72 hours followed by FACS staining against CD56 and CD3 (Fig. 7D). Indeed, only the 02-ZAP led to a significant decrease in the percent of NK cells, while T cells remained unharmed. Hence, 02-ZAP can specifically target NK cells within a population of PBMCs and markedly hinder their proliferation.

Discussion

NKp46 is one of the most important activating receptors found on all NK cells. Its role in the identification and elimination of various NK cell targets, which include viruses, bacteria, fungi, human beta cells, stellate cells, adipose cells, and tumor cells has been well established [2, 4, 7–9, 18, 21]. The recognition of all of these highly diverse targets is mediated by the membrane proximal domain D2 [23–25]. NKp46 utilizes different features and binding sites within its D2 domain to identify a large and dynamic repertoire of ligands [25]. In contrast, the biological significance of NKp46's membrane distal domain, D1, remains largely unknown.

Accordingly, research centered on the human NKp46, including identification of its ligands and its involvement in NK cell function during physiological and pathological processes has been hindered. Since NKp46 is the only NCR which has a mouse orthologue, Ncr1, a mouse model where Ncr1 has been knocked out ($Ncr1^{gfp/gfp}$) was previously generated by our lab [16]. This model has been used extensively to investigate the role Ncr1 plays in tumor transformation [39–41], metastases [42], pathogenic infection [4, 5, 16, 28, 43], graft versus host disease (GVHD) [44], fibrosis [21], and type I and type II diabetes [8, 9, 27]. However, a similar model for investigating the function and biology of human NKp46 is, of course, not available.

Since NKp46 utilizes different features and binding sites to recognize its ligands, a single blocking monoclonal antibody will most likely be unsuccessful in inhibiting binding of NKp46 to all its ligands. The 02 mAb that we developed downregulates NKp46 from the surface of NK cells and leads to its degradation. Thus, using this mAb we are now able to uncover the extent to which it influences NK cell behavior in any given circumstance. In addition, the identities of NKp46 self-ligands, tumor ligands, and bacterial ligands have remained elusive for over two decades. This antibody could identify targets whose elimination by NK cells is NKp46 dependent, thereby bringing us closer to identifying additional and important ligands of NKp46.

We were able to determine that the 02-mediated NKp46 downregulation was not due to receptor shedding, but rather receptor internalization and degradation via the lysosomal pathway. This antibody-mediated receptor internalization was achieved by 02 binding to the D1 domain of NKp46. Interestingly, the commercially available anti-NKp46 antibody, 9E2, and another anti-NKp46 mAb developed in our lab, 461-G1 [18, 25], also bound the D1 domain; however, this interaction did not affect the surface levels of NKp46. We confirmed that 9E2, 461-G1, and 02 have distinct binding sites on the D1 domain. The D1 domain of Ncr1 was previously shown to contain a residue which when mutated lead to receptor accumulation in the ER and to unstable expression of the mutated NKp46 receptors on the cell surface [45]. Therefore, based on our and previous data we propose that the D1 domain may be important for receptor localization and stabilization.

We demonstrated that treatment with the 02 antibody of NK cells affected their activity against different targets. Target cell lines were selected based on their ability to consistently and significantly induce mIL-2 secretion by BW NKp46 transfectants, thus indicating that they express a ligand for NKp46. However, this assay does not test whether NKp46 is dominantly involved in target recognition and elimination when other NK cell receptors are present. CD107a degranulation assays revealed that 02 was able to inhibit NK cell activity against certain targets (721.221, BCBL1, BJAB, and C1R), whereas other targets such as Jurkat and K562 were not affected. These results exemplify the use of 02 as a scientific tool to identify target cells whose elimination by NK cells is mediated by NKp46, and the extent to which it influences NK cell killing of a given target. Previous work in our lab showed that NK cells contributed to the progression of type I diabetes by eliminating beta cells in an NKp46-dependent manner [8, 27]. As such, when the 02-treated NK cells were incubated with human beta cells, their activation by these targets was severely diminished.

Additional diseases directly involving NK cells include rare forms of cancer: extranodal NK/T-cell lymphoma, aggressive NK cell leukemia, and large granular lymphocyte (LGL) leukemia [31–34]. It has also been shown that approximately 20% of T cell lymphomas express NKp46 [35, 36]. Since 02 induces receptor internalization, it may be considered for use as an antibody-drug conjugate (ADC). ADCs link cytotoxic drugs to antibodies targeted against a given receptor, thus antibody-mediated receptor internalization would be followed by release of the cytotoxic agent within the cancerous cells [46, 47]. Currently two ADCs have been approved by the FDA and over 40 additional clinical trials are underway [47]. Consequently, we set out to prove the ability of 02 in becoming an ADC, by conjugating it to the toxin, saporin. The 02-ZAP conjugate was significantly successful in impeding the proliferation of NK cells either alone or within a population of PBMCs, and the NK tumor cell line, YTS. Together, these results establish the use of a conjugated form of 02 in treating NKp46-dependent autoimmune diseases, such as type I diabetes, fibrosis and NKp46-expressing tumors. Interestingly, a study by Vely et al. from 2016 demonstrated that the absence of ILCs (including NK cells) did not confer increased susceptibility to disease in humans [48]. Therefore, the partial downregulation of NKp46 mediated by the 02 antibody would most probably not have a detrimental effect on the anti-viral or anti-tumor activity of the NK cells. Similarly, the use of a toxin conjugated 02 antibody to eliminate NKp46-expressing malignancies would not have destructive consequences on one's immune system. Taken together, these results exemplify the inordinate potential of 02 in becoming a leading immunotherapeutic drug, which can be used

to treat NKp46-dependent diseases and various NKp46-expressing malignancies, such as NK cell lymphomas and T cell lymphomas.

In conclusion, we have developed a unique anti-NKp46 mAb whose applications range from a scientific tool which will further our understanding of human NK cell biology, to a therapeutic drug with the potential of treating life-threatening conditions.

Materials and methods

Cells and viruses

The human cell lines used in this study were 721.221, BCBL1, BJAB, C1R, Jurkat, K562, Jeg3, YTS, and the murine thymoma BW cell line. NK cells were isolated from peripheral blood lymphocytes and IL-2 activated bulk NK cells were cultured as previously described [49]. Briefly, PBMCs were collected from heparinized blood by centrifugation on Lymphoprep (StemCell Technologies). NK cells were isolated using the EasySep human NK cell enrichment kit (StemCells Technologies). Activated primary NK lines were generated by culturing the isolated NK cells with irradiated feeder cells (allogeneic PBMCs from two donors and 8866 cells) and 20 μg/mL PHA (Roche). The cultures were maintained in DMEM:F-12 Nutrient Mix (70:30), 10% human serum (Sigma), 2mM glutamine (Biological Industries [BI]), 1mM sodium pyruvate (BI), 1x nonessential amino acids (BI), 100 U/mL penicillin (BI), 0.1 mg/mL streptomycin (BI), and 500 U/mL rhIL-2 (PeproTech). The NK cells were cultured for approximately 4 weeks. Primary human beta cells were a kind gift from Prof. Yuval Dor. The human influenza virus used in this study was the A/Puerto Rico/8/34 H1N1 (PR8) strain, and was generated as previously described [50].

Antibodies and fusion proteins

The anti-NKp46 mAbs (461-G1 [25] and 02) generated by us required immunization of $Ncr1^{gfp/gfp}$ mice with NKp46-Ig fusion proteins. All antibodies generated by us are of the IgG1 isotype. For experiments 1A-B, supernatants of 02 hybridoma were used for FACS staining. Positive staining thus led to the purification of the 02 antibody, which was used throughout the rest of the study. Control mAb used was anti-HLA.B7 (purified from hybridomas). FACS stainings were performed with the following antibodies (Abs): purified α-NKp46 (9E2), α-NKp46 APC (9E2), α-NKp46 FITC (9E2), α-NKp44 APC (P44-8), α-NKp30 APC (P30-15), α-2B4 APC (C1.7), α-CD16 FITC (3G8), α-DNAM1 FITC (TX25), α-NKG2D PE (1D11), α-CEACAM1 PE (ASL-32), α-KIR2DL2 FITC (DX27), α-KIR2DL1 FITC (HP-MA4), α-CD107a APC (H4A3), α-CD56 PE (HLD56), α-CD3 APC (HIT3a), and Streptavidin-APC which were purchased from Biolegend. Anti-TIGIT APC (MBSA43) was purchased from eBioscience. Isotype control Abs used in FACS stainings were: IgG FITC (IgG_1), IgG PE (IgG_2), and IgG APC (IgG_1) were all clone MOPC-21 and purchased from Biolegend. Additional secondary Abs used in FACS stainings were AlexaFluor 647-conjugated AffiniPure goat anti-mouse IgG and PE-conjugated AffiniPure donkey anti-human IgG, both purchased from Jackson ImmunoResearch. Purified α-mouse IL-2 (JES6-1A12) and biotinylated α-mouse IL-2 (JES6-5H4) mAbs were purchased from Biolegend. The fusion proteins NKp46-Ig, D1-Ig, and D2-Ig were generated in HEK293T cells and purified, as previously described [18, 25, 51].

FACS staining

Flow cytometry was performed with the abovementioned antibodies and detailed protocols are found in the Figure Legends. Flow cytometry guidelines were followed according to Cossarizza et al. [52]. Analysis was performed using the FACS-Calibur flow cytometer (BD Biosciences) and CellQuest software.

FACS Staining of PBMCs

PBMCs were isolated from heparinized blood by centrifugation on Lymphoprep (StemCell Technologies). 200 000 cells were incubated at 4°C with 20 μg/mL of either biotinylated 461-G1 or 02 mAbs. Biotinylation of the purified antibodies was done using the EZ-Link NHS-PEG4 Biotinylation Kit (ThermoFisher Scientific, 21455), and following the manufacturer's instructions. Cells were washed and then incubated simultaneously with Streptavidin-APC, α-CD56 PE, and α-CD3 FITC (triple staining) at 4°C. Samples were analyzed using the Gallios Flow Cytometer (Beckman Coulter) and Kaluza Analysis software and FCS Express 4.

Blocking experiments

NKp46-Ig fusion proteins (3 μg/sample) were incubated either alone, with anti-NKp46 mAbs (9E2 and 461-G1 at 1 μg/sample; 02 at 100 μL/sample of supernatant from hybridomas) diluted in PBSx1, for 1 hour at 4°C. FACS staining with treated fusion proteins was then performed on BJAB, MCF7, and C1R cells.

NKp46 receptor downregulation assays

Activated NK cells (50 000 cells/100 μL) were incubated for different time periods with either control or anti-NKp46 mAbs at 4°C or 37°C (see figure legends for incubation times and concentrations used). After the allotted time period, cells were washed and stained with a conjugated anti-NKp46 mAb clone 9E2 (APC or FITC α-NKp46) as indicated in the figure legends, followed by FACS analysis.

BW reporter assay

Murine thymoma BW cells were used to generate BW NKp46 transfectants which were subsequently used in BW assays, as previously

described [18]. Briefly, BW NKp46 cells were incubated with irradiated (6000 rad) target cells at differing E:T ratios at 37°C. After 48 hours, supernatants were collected and mIL-2 secretion levels were quantified by sandwich ELISA using anti-mIL-2 mAbs.

CD107a degranulation assays

Analysis of CD107a on the surface on NK cells has been previously described [53]. Activated NK cells were initially incubated with 5 μg/mL of either 461-G1 or 02 mAbs for 16 hours at 37°C. Target cells at different E:T ratios (all E:T ratios were 1:2, except BJAB cells which had an E:T of 1:1), anti-CD107a and anti-CD56 mAbs were then added to the samples for 2 hours at 37°C. %CD107a positive NK cells was determined by FACS analysis.

Proteasome and lysosome inhibition

Activated bulk NK cells were incubated either alone or with the proteasome inhibitor, epoxomicin (EPX, 0.1 μM), or the lysosomal acidification inhibitor, concanamycin A (CCM A, 150 μM), (both from Merck Milipore) for 20 min at 37°C. The control and 02 mAb were then added for an additional 8 hours at 37°C, followed by FACS staining with APC α-NKp46.

ELISA

NKp46 surface shedding

50 000 cells/well of activated NK cells were plated either alone (No mAb), or with a control antibody (anti-HLA.B7 0.5 μg/well) or the 02 antibody (0.5 μg/well) at 37°C. After 16 hours incubation the plates were centrifuged and supernatants (150 μL) were collected and placed on ELISA plates overnight at 4°C. As a positive control to identify NKp46, we used NKp46-Ig which was plated on the same ELISA plates at the indicated concentrations. Blocking was performed with (PBSx1/Tween 0.05%/BSA 1%), followed by addition of a biotinylated anti-NKp46 mAb at 1μg/mL diluted in blocking buffer. Streptavidin-HRP was then added and detection with TMB substrate (SouthernBiotech) was measured at OD 650 nm.

Determining the binding domain of anti-NKp46 mAbs

The fusion proteins (NKp46-Ig, D1-Ig, D2-Ig, and W32R-Ig) were first plated at 1 μg/mL diluted in PBSx1. Blocking was performed with (PBSx1/Tween 0.05%/BSA 1%), followed by addition of the anti-NKp46 mAbs at 1 μg/mL diluted in blocking buffer. Biotin anti-mouse was subsequently added followed by streptavidin-HRP, and detection with TMB substrate was measured at OD 650 nm.

Determining distinct binding sites using microscale thermophoresis (MST)

The MST experiments were performed with labeled NKp46-Ig. Labeling was performed using the Monolith NT Protein Labeling Kit RED-NHS from NanoTemper Technologies. The experiments were carried out on a blue/red Monolith NT.115 (NanoTemper Technologies) using the red filter set. The minimum amount of fluorescence (above 200 fluorescent units) was determined by capillary scan (at 95% LED-light-emitting diode-power). The labeled fusion proteins were mixed with the antibodies and measured at 20% MST power for 30 seconds with 95% LED power in standard capillaries at 25°C. In competition assays, pre-saturated NKp46-Ig (70 nM) with antibody was kept at a constant concentration, followed by addition of decreasing concentrations of another antibody (1:1 dilutions, starting from 1 μM to 30 pM). Titration of the non-fluorescent antibodies resulted in a gradual change in thermophoresis, which was plotted as a change in the normalized fluorescence (Fnorm), to yield a binding curve [54].

02 and 461-G1 conjugation to toxin followed by XTT viability assay and FACS staining of PBMCs

The antibodies, 461-G1 and 02, were initially biotinylated using EZ-Link NHS-PEG4 Biotinylation Kit (ThermoFisher Scientific, 21455) according to the manufacturer's instructions. Once biotinylated, the antibodies were conjugated to the toxin saporin, which itself was attached to streptavidin (Strept-ZAP), as purchased from ATSbio. Conjugation of the antibodies to the toxin (denoted as antibody-ZAP), treatment of the cells, and the XTT cell viability assay were performed according to the manufacturer's instructions (Biotin-Z Internalization Kit KIT-27-Z100, ATSbio).

For the assays with PBMCs, 100 000 cells were plated per well followed by the addition of 10 nM: medium (untreated), saporin (SAP), Stretpt-ZAP, 461-ZAP, and 02-ZAP. Cells were incubated at 37°C for 72 hours, followed by staining of the cells with α-CD56 PE and α-CD3 APC. During FACS analysis, live cells were gated and counted for 60 s. Within the gate of live cells were two additional gates: $CD56^+CD3^-$ (NK cells) and $CD56^-CD3^+$(T cells). The percent of NK or T cells was evaluated by dividing the number of NK or T cells by the total number of live cells and then multiplied by 100.

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Abbreviations: **ADC**: antibody-drug conjugate · **BW NKp46**: BW transfectant cells expressing NKp46 · **CCMA**: concanamycin A · **EPX**: epoxomicin · **HN**: hemagglutinin-neuraminidase · **ILC**: innate lymphoid cell · **LGL**: large granular lymphocyte · **mAb**: monoclonal antibody · **mIL-2**: mouse IL-2 · **MST**: microscale thermophoresis · **NCR**: natural cytotoxicity receptor · **NK**: Natural killer · **SAP**: saporin

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