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*J Immunol* published online 30 July 2012
http://www.jimmunol.org/content/early/2012/07/29/jimmunol.1103495

Supplementary Material
http://www.jimmunol.org/content/suppl/2012/07/30/jimmunol.1103495.DC1

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An NKp30-Based Chimeric Antigen Receptor Promotes T Cell Effector Functions and Antitumor Efficacy In Vivo

Tong Zhang, Ming-Ru Wu, and Charles L. Sentman

NKp30 is a natural cytotoxicity receptor that is expressed on NK cells and recognizes B7-H6, which is expressed on several types of tumors but few normal cells. To target effector T cells against B7-H6+ tumors, we developed several chimeric AgRs (CARs) based on NKp30, which contain the CD28- and/or CD3ξ-signaling domains with the transmembrane domains from CD3ξ, CD28, or CD8α. The data show that chimeric NKp30-expressing T cells responded to B7-H6+ tumor cells. The NKp30 CAR-expressing T cells produced IFN-γ and killed B7-H6 ligand-expressing tumor cells; this response was dependent upon ligand expression on target cells but not on MHC expression. PBMC-derived dendritic cells also express NKp30 ligands, including immature dendritic cells, and they can stimulate NKp30 CAR-bearing T cells to produce IFN-γ, but to a lesser extent. The addition of a CD28-signaling domain significantly enhanced the activity of the NKp30 CAR in a PI3K-dependent manner. Adoptive transfer of T cells expressing a chimeric NKp30 receptor containing a CD28-signaling domain inhibited the growth of a B7-H6+ expressing murine lymphoma (RMA/B7-H6) in vivo. Moreover, mice that remained tumor-free were resistant to a subsequent challenge with the wild-type RMA tumor cells, suggesting the generation of immunity against other tumor Ags. Overall, this study demonstrates the specificity and therapeutic potential of adoptive immunotherapy with NKp30 CAR-expressing T cells against B7-H6+ tumor cells in vivo. The Journal of Immunology, 2012, 189: 000–000.
(which can induce immune tolerance and suppression) express NKp30 ligands (15, 16). Therefore, NKp30 ligand-targeting provides a fairly specific means to engage T cells against multiple types of tumors and to promote antitumor immunity. In this study, we determined the function and efficacy of these NKp30-based CARs.

Materials and Methods

Mice

C57BL/6 (B6; wild-type [wt]) mice were purchased from the National Cancer Institute (Frederick, MD). Perforin-deficient mice C57BL/6-PtprC<sup>−/−</sup> (Ptpr<sup>−/−</sup>) were obtained from The Jackson Laboratory (Bar Harbor, ME). All experiments were conducted according to protocols approved by Dartmouth College’s Institutional Animal Care and Use Committee.

Cells and cell culture

Bosc23, GP+E86, IT67, K652, L937, HeLa, U266, and Jurkat cell lines were obtained from the American Type Culture Collection (Rockville, MD). Breast cancer cell lines MCF-7 and T47D were provided by Dr. James Direnzo (The Geisel School of Medicine at Dartmouth). Pancreatic cancer cell line Panc-1 was provided by Dr. Murray Korc (School of Medicine, Indiana University, Indianapolis, IN). Prostate cancer cell line DU145 and melanoma cell line A375 were provided by Dr. Marc Ernstoff (The Geisel School of Medicine at Dartmouth). An RNA subtype RMA/B7-H6 that expresses an NKp30 ligand, B7-H6, was generated by retroviral transduction using dualtropic retrovector constructs containing the B7-H6 gene, according to our previous protocol (22). Packaging cells Bosc23, GP+E86, and PT67 were grown in DMEM with a high glucose concentration (4.5 g/l), supplemented with 10% heat-inactivated FBS (Atlanta Biologicals, Lawrenceville, GA), 100 U/ml penicillin, 100 μg/ml streptomycin, 1 mM pyruvate, 10 mM HEPES, 0.1 mM nonessential amino acids, and 50 μM 2-ME. All other cell lines were cultured in RPMI 1640 plus the same supplements as in DMEM. Human dendritic cells (DCs) were generated from blood mononuclear cells that were obtained from cell cones from the Dartmouth-Hitchcock Medical Center Blood Donor Center for leukapheresis cell donations. CD14<sup>+</sup> cells were selected using magnetic beads (Miltenyi Biotec) and were cultured in six-well plates (5 × 10<sup>6</sup> cells/ml) in 2 ml complete RPMI 1640 media with recombinant human IL-4 (100 ng/ml; PeproTech, Rocky Hill, NJ) and recombinant human GM-CSF (100 ng/ml; PeproTech). On days 4 and 6, 2 ml fresh media with IL-4 and GM-CSF was added to the cultures. On day 8, the nonadherent cells were collected and used as iDCs. To generate mDCs, the media were replaced with fresh media containing LPS (1 μg/ml; Sigma, St. Louis, MO) and CD40L (200 ng/ml; PeproTech) on day 6 for 2 d.

Construction of chimeric NKp30 receptors

The full-length human NKp30, CD8, and CD8α cDNAs were purchased from Open Biosystems (Huntsville, AL). Human CD3<sub>ε</sub>-chain–signaling domain and full-length B7-H6 cDNAs were cloned by RT-PCR using RNAs from Jurkat cells as templates. The chNKp30 constructs used in this study are illustrated in Fig. 1A. wt NKp30 contains the full-length human NKp30. Chimeric receptor NKp30-3<sub>ε</sub> comprises the extracellular domain (aa 1–139) of human NKp30 fused to the TM domain (aa 31–51) and the extracellular ligands. The NKp30-CD8<sub>ε</sub>-3<sub>ε</sub> receptor was constructed by joining the extracellular domain of NKp30 to the TM domain of human CD8α (aa 183–203) and the signaling domain of CD3<sub>ζ</sub>-chain. The CD8α-CD3<sub>ζ</sub>-chain. As a control receptor, NKp30-28(TM)-3<sub>ε</sub> is similar to NKp30-3<sub>ε</sub>-3<sub>ε</sub>, except that the CD8α-signaling domain was removed. All PCR reactions were performed using a high-fidelity DNA polymerase Phusion (New England BioLabs, Ipswich, MA). All oligonucleotides were synthesized by Sigma-Genosys (The Woodlands, TX). All genes were cloned into a retrovector pFB-neo (Stratagene, Palo Alto, CA).

Retroviral transduction

Production of retroviral vectors and retroviral transduction were performed according to modified protocols, as described previously (22, 23). In brief, transiently transduced primary T cells were infected with recombinant retrovirus collected from vector-transfected GP+E86 cells, whereas dualtropic retrovirus vectors were generated by vector-transfected PT67 cells were used to infect human primary T cells. Primary T cells from spleens of B6 mice were infected 18–24 h after Con A (1 μg/ml; Sigma) stimulation.

Two days postinfection, transduced primary T cells (0.5–1 × 10<sup>5</sup>/ml) were selected in RPMI media containing G418 (1 mg/ml) plus 25 U/ml recombinant human IL-2 for three additional days. Viable cells were isolated using Histopaque-1083 (Sigma), washed extensively, and expanded for 2 d without G418 before functional analyses or i.v. injection. Primary human cells were stimulated with anti-CD3 mAb OKT3 (40 ng/ml; eBioscience, San Diego, CA) for 3 d before retroviral transduction. G418 selection of retrovirally transduced human T cells followed the same procedures for selecting CAR-transduced murine T cells.

Production of soluble human NKp30-mIgG2a fusion protein

To make a soluble human NKp30-mIgG2a fusion protein, the extracellular portion of human NKp30 (aa 1–262) was fused to the mouse IgG2a hinge/CH2-CH3 portion. NKp30-mIgG2a gene was cloned into pFBB-neo. NKp30-mIgG2a fusion protein was expressed in retroviral vector stably transduced B16F10 cells. The production and purification of NKp30-mIgG2a protein were performed according to previous protocols (22, 23).

Generation of anti-B7-H6 mAbs

Eight- to twelve-week-old B6 mice were immunized (i.p.) with mitomycin C-treated RMA/B7-H6 cells (× 10<sup>5</sup>). Two weeks after the initial immunization, mice were boosted with 50 μg recombinant B7-H6 (extracellular domain) prepared from Escherichia coli and IFA (Sigma), three times at weekly intervals. Three days after the last boosting immunization, mice splenocytes were fused to NS1 cells (provided by Dr. William Wade, The Geisel School of Medicine, Dartmouth College) using standard techniques, and hybridomas that expressed anti-CAR mAbs were screened for reactivity to both B7-H6–negative and –positive cell lines by flow cytometry. Two clones (47.39 and 127.4; both of the IgG2a subclass) were isolated.

Flow cytometry

For flow cytometry analysis of NKp30 ligand expression, cells were stained with either NKp30-mIgG2a or anti-B7-H6 mAb, followed by DyLight 649-conjugated goat anti-mouse IgG (BioLegend, San Diego, CA). Cell surface phenotyping of transduced primary T cells was determined by staining with FITC-conjugated anti-CD4 (clone OKT4; BioLegend), PE-conjugated anti-NKp30 (clone P30-15; BioLegend) mAbs. The following mAbs were used to analyze the cell surface phenotype of DCs: allophycocyanin-conjugated anti-CD86 (BioLegend), PE-conjugated anti-CD11c (BioLegend), FITC-conjugated anti-CD83 (BioLegend), and FITC-conjugated anti-HLA-DR (eBioscience). Intracellular staining of Bcl-x<sub>L</sub> in T cells was performed using anti–Bcl-xL–FITC (Southern Biotech, Birmingham, AL), based on the protocol described previously (24). All samples were preincubated with either FcR block Ab (anti-mouse CD16/CD32, 2.4G2; Bio X Cell, Lebanon, NH) for mouse cells staining or human γ globulins (Cohn’s fraction, G4386; Sigma) for human cell staining. Cell fluorescence was monitored using an Accuri C6 cytometer. Flow cytometry analysis was performed using either Accuri or FlowJo software.

RT-PCR and quantitative PCR

Extraction of total RNA and preparation of cDNAs from human tumor cell lines and PBMCs were performed as described (23). The resulting cDNA, corresponding to 50 ng total RNA, was subjected to PCR amplification in a total volume of 20 μl, including 0.5 μmol/l each primer, 0.2 mmol/l each deoxynucleotide triphosphate, and 1 U Taq DNA polymerase (New England Biolabs, Ipswich, MA). Amplification conditions were as follows: 55°C for 5 min, followed by 30 cycles of 95°C for 30 s (denaturation), 60°C for 30 s (annealing), and 72°C for 30 s (extension), with a 3-min incubation at 72°C at the end. The PCR products were run on agarose gels and visualized by staining with SYBR Safe (Invitrogen). For quantitative real-time PCR of human IL-2 mRNA, triplicates of cDNA samples from T cells were mixed with SYBR Green I (Applied Biosystems) and 1 U Taq DNA polymerase (New England Biolabs) and IFA (Sigma), three

Cytotoxicity assay

Cytotoxicity of T cells against target cells was determined by an LDH-Cytotoxicity assay using the Cytotox96 Non-Radioactive Cytotoxicity Assay kit (Promega, Madison, WI). Specific lysis was determined using the following equation: percentage of specific lysis = [(experimental – effector spontaneous – target spontaneous)/(target maximum – target spontaneous)] × 100. In the cytotoxicity-blocking experiments, K562
target cells were preincubated with a soluble NKp30 receptor, NKp30-mlgG2a (10 μg/ml), for 30 min before coculture with T cells in an LDH-release assay.

Cytokine production by T cells

To determine whether CAR T cells responded to tumor cells with production of IFN-γ, T cells (10⁵) were cocultured with suspension tumor cells at an E:T ratio of 1:1 or with adherent tumor cells at an E:T ratio of 1:0.25 (10², 2.5 × 10⁵) in 96-well V-bottom or flat-bottom plates, respectively, for 24 h. Cell-free supernatants were assayed for IFN-γ by ELISA using DuoSet ELISA kits (R&D Systems).

Treatment of lymphoma-bearing mice with chimeric NKp30-modified T cells

As a systemic mouse lymphoma model, B6 mice were injected with 10⁵ RMA/B7-H6 cells in 400 μl HBSS via tail veins. For treatment with T cells, mice were administered 5 × 10⁶ wt NKp30 (wtNKp30) or chNKp30-modified T cells i.v. starting on day 5 posttumor inoculations. T cell transfer was repeated on days 7 and 9 using T cells from the same T cell preparation that were expanded for additional days in vitro. Mice were monitored closely and sacrificed when they became moribund.

Tumor rechallenge

Mice that survived for 60 d after initial tumor inoculation without signs of disease were regarded as tumor free and were inoculated s.c. with 10⁶ wt RMA tumor cells on the shaved right flank. Naive B6 mice were used as controls. Tumor size was monitored every 2 d, and mice were sacrificed when tumor burden became excessive.

Statistical analysis

Differences between groups were analyzed using a Student t test or ANOVA; p values < 0.05 were considered significant. Kaplan–Meier survival curves were plotted and analyzed using Prism software (GraphPad Software, San Diego, CA).

Results

Construction and expression of CARs based on the human NKp30 receptor

A series of chNKp30 receptor-expressing retroviral vectors was generated in which all receptors share the common extracellular domain of NKp30. The structures of the chNKp30 and wtNKp30 receptors used are shown in Fig. 1A. In NKp30-3ζ, the TM domain and cytoplasmic (CYP) tail of NKp30 was replaced by the corresponding region of the CD3ζ-chain. To facilitate surface expression and dimerization of chimeric receptors, the human CD8a TM domain was used instead to make NKp30-CD8(TM)-3ζ. To determine whether the CD28 signaling domain can enhance the overall activation signal upon engagement of chNKp30 to its ligand, the human CD28 TM and CYP domains were inserted between the extracellular domain of NKp30 and the CD3ζ CYP tail. The resulting receptor is referred to as NKp30-CD8(TM)-3ζ. A control receptor, NKp30-CD28(TM)-3ζ, has the CD28 TM portion but not the CYP region of the CD28 molecule included.

Surface expression of chNKp30 receptors on human T cells was analyzed by flow cytometry using anti-NKp30 and anti-CD4 mAbs. As shown in Fig. 1B, retroviral transduction of human T cells with wtNKp30 gene did not lead to significant surface expression. Insufficient expression of wtNKp30 on the cell surface may be due to an absence of FcγR expression on T cells, which is associated with CD3ζ and NKp30 on human NK cells. Replacement of the NKp30 TM domain with the CD3ζ TM domain improved CAR expression. The hinge and TM domains of CD8a have been widely used for expressing CARs on T cells (7, 26). Our results showed that the CD8a TM domain efficiently allows surface expression of chNKp30 receptor on T cells, which is consistent with these previous studies. Similar to the CD8a TM domain, the human CD28 TM domain resulted in higher surface expression of chNKp30, with or without the CD28 CYP domain.

Chimeric NKp30-expressing T cells produce IFN-γ upon coculture with NKp30 ligand-positive tumor cells

A panel of human tumor cell lines was screened for NKp30 ligand expression using a soluble human NKp30-mIgG2a fusion protein and an anti-B7-H6 mAb (called 47.39). As shown in Fig. 2A, K562, A375, HeLa, and T47D cells expressed high amounts of NKp30 ligands, whereas U937, RPMP8226, DU145, and Panc-1 cells expressed low amounts of NKp30 ligands. Some tumor cells (IM9, U266, and MCF-7) did not express NKp30 ligands on the cell surface. All tested human tumor cells have detectable levels of BAT3 mRNA, as detected by RT-PCR (Fig. 2B). In contrast, B7-H6 mRNA levels were correlated to surface expression of NKp30 ligands, suggesting that B7-H6 is the major surface ligand of NKp30 in tumors. The result was also consistent with the fact that BAT3 is a nuclear protein, which is usually not expressed on the cell surface. In addition, RT-PCR results showed that PBMCs lacked the mRNAs of B7-H6 and Bat3 (Fig. 2B).
To determine whether chimeric NKp30-transduced human T cells were able to recognize NKp30 ligand-positive tumor cells, the chNKp30 CAR-bearing T cells were cultured with different tumor cells, and IFN-γ responses measured by ELISA. As shown in Fig. 3, NKp30-3ζ, NKp30-CD8(TM)-3ζ, NKp30-CD28(TM)-3ζ, or NKp30-CD28-3ζ T cells produced significant amounts of IFN-γ after coculture with NKp30 ligand-positive cells but not when cultured with ligand-negative cells, indicating that these NKp30 CAR-modified T cells could functionally recognize NKp30 ligand-bearing tumor cells. In contrast, wtNKp30-modified T cells did not show any significant response to the stimulation by NKp30 ligand-negative cells. NKp30-CD28-3ζ receptor bestowed T cells with significantly higher lytic activity than did NKp30-3ζ, NKp30-CD8(TM)-3ζ, or NKp30-CD28 (TM)-3ζ in the presence of NKp30 ligand-positive tumor cells, especially when the ligand expression was low. The reason was likely due to better surface expression of this CAR and/or the presence of the CD28 costimulatory signaling domain in NKp30-CD28-3ζ.

Human DCs express NKp30 ligands and can stimulate chimeric NKp30-expressing T cells to produce IFN-γ

It is known that NKp30 plays an important role in human NK–DC interactions (27, 28). At low NK/DC ratios, DCs promote IFN-γ production and cytotoxicity by NK cells in an NKp30-dependent manner (28, 29), which suggests that DCs express NKp30 ligands. With the use of NKp30-mIgG2a, we confirmed that both iDCs and mDCs can bind to soluble NKp30, which is consistent with DCs expressing ligands for NKp30 (Fig. 4A). The level of cell surface staining on iDCs was higher than on mDCs. However, there was no significant expression of B7-H6 on DCs as determined with mAb 47.39, a specific anti–B7-H6 mAb. To determine whether the NKp30 CAR-modified T cells can respond to DCs, T cells were cocultured with either iDCs or mDCs at a 5:1 ratio for 24 h. IFN-γ production (200–800 pg/ml) was observed by NKp30-CD28-3ζ–modified T cells after coculture with DCs (Fig. 4B). Compared with mDCs, iDCs induced higher amounts of IFN-γ, which reflected their greater binding to soluble NKp30.

Chimeric NKp30-bearing human T cells kill NKp30 ligand-positive tumor cells

The cytotoxic activity of chNKp30-modified human T cells against various tumor cell lines was determined. As shown in Fig. 5A, NKp30 CAR-bearing T cells were able to lyse NKp30 ligand-positive target cells (RMA/B7-H6, T47D, Panc-1, A375, K562, and RPMI8226) but not the ligand-negative cell lines RMA and MCF-7 in vitro. Similar to cytokine production, no significant killing was observed when wtNKp30-modified T cells were used. NKp30-CD28-3ζ receptor bestowed T cells with significantly higher lytic activity than did NKp30-3ζ, NKp30-CD8(TM)-3ζ, or NKp30-CD28(TM)-3ζ. Because RMA/B7-H6 tumor cells lack expression of human MHC class I and II molecules, these data indicate that the chNKp30 receptor-modified T cell-mediated killing of these tumor cells was ligand dependent and MHC independent. To confirm that chNKp30-mediated killing is dependent on the interactions between NKp30 and its ligands, soluble NKp30 (NKp30-mIgG2a) was incubated with K562 target cells prior to the
coculture with T cells. As shown in Fig. 5B, NKp30-mIgG2a significantly reduced NKp30-28-3ζ-bearing T cell-mediated cytotoxicity. These results demonstrated that chNKp30-bearing T cells killed ligand-positive tumor cells, and the interaction between chNKp30 receptors and NKp30 ligands was essential for chNKp30-mediated T cell function.

**FIGURE 4.** Human DCs bind to NKp30 and can stimulate autologous NKp30-CD28-3ζ-modified T cells to produce IFN-γ. (A) The cell surface phenotype and binding to NKp30 of PBMC-derived human DCs (both iDCs and mDCs) was determined by flow cytometry. Specific mAb or NKp30-Ig as indicated (solid line) or an isotype control Ab staining (dashed line) is shown. (B) Five to seven days after retroviral transduction, NKp30 CAR-modified T cells (10⁵ cells) were cocultured with either iDCs or mDCs at a ratio of 5:1 (T/DC) for 24 h. IFN-γ amounts in the supernatants were determined by ELISA. Results shown (mean + SD) are representative of two experiments. *p < 0.05.

**FIGURE 5.** NKp30 CAR-modified T cells lyse NKp30 ligand-positive tumor cells in vitro. (A) Effector T cells derived from human PBMCs were modified with wtNKp30, NKp30-3ζ, or NKp30-CD28-3ζ and cocultured with tumor cells at a ratio of 5:1 in 5-h LDH-release assays. The data are mean + SD of triplicates from two independent experiments. (B) Effector T cells modified with wtNKp30 or NKp30-CD28-3ζ were cocultured with target cells K562 in the presence of 10 μg/ml NKp30-mIgG2a or mouse IgG at a ratio of 5:1; the percentage of specific lysis was determined after a 5-h LDH-release assay. The data are presented as mean + SD and are representative of two independent experiments. (C) PI3K is involved in NKp30-CD28-3ζ receptor-mediated cytotoxicity. NKp30-modified effector T cells were incubated with a PI3K inhibitor LY294002 (10 μM) at 37°C for 1 h before coculture with K562 target cells at an E:T ratio of 5:1 in 5-h LDH-release assays. Vehicle controls are 0.1% DMSO. The data shown are the mean + SD of triplicates and are representative of two independent experiments. *p < 0.05.
Activation of PI3K pathway is involved in enhanced IFN-γ production, as well as cytotoxicity of NKp30-CD28-3ζ+ T cells

Cross-linking of CD28 leads to activation of the PI3K pathway. Therefore, we hypothesized that significantly enhanced IFN-γ production and cytotoxicity by T cells after engagement of the NKp30-CD28-3ζ receptor might be due to activation of PI3K. To test this hypothesis, a PI3K inhibitor, LY294002, was added to the coculture of CAR T cells with irradiated HeLa cells. Compared with vehicle control, the presence of LY294002 (10 μM) inhibited NKp30-CD28-3ζ T cell-mediated IFN-γ production and cytotoxicity, indicating that incorporation of a CD28-signaling domain into chimeric NKp30 receptors can activate T cells via the PI3K pathway (Fig. 5C).

Integration of a CD28 signal into the NKp30 CAR promotes in vitro T cell proliferation

It is known that CD28 signals enhance T cell survival and proliferation. To investigate whether integration of CD28 signaling in the chNKp30 receptor resulted in similar outcomes upon engagement of the NKp30 receptor, CFSE-labeled T cells were cocultured with HeLa cells (NKp30 ligand positive) in the presence of a small amount of IL-2 (25 U/ml) for 3 d. As shown in Fig. 6A, engagement of NKp30-CD28-3ζ-bearing T cells led to more T cell proliferation than did engagement of NKp30-3ζ-bearing T cells. Two important mechanisms through which CD28 signaling promotes T cell survival are through upregulation of IL-2 and an antiapoptotic protein, Bcl-xL. To determine whether NKp30-CD28-3ζ T cells upregulated IL-2 and Bcl-xL in response to cross-linking of the chimeric receptor, T cells were cultured in anti-NKp30 mAb-coated wells for 24 h. IL-2 mRNA and Bcl-xL protein were determined using real-time PCR and intracellular staining, respectively. The results show that NKp30-CD28-3ζ+ T cells increased IL-2 expression by 25-fold after receptor cross-linking compared with a 10-fold induction in NKp30-3ζ+ T cells (Fig. 6B). No significant upregulation of IL-2 was observed in wtNKp30-modified T cells cultured under these conditions. Similarly, we observed greater expression of Bcl-xL in NKp30-CD28-3ζ+ T cells compared with NKp30-3ζ+ T cells (Fig. 6C). These data suggest that NKp30-CD28-3ζ+ T cells can receive a costimulatory signal through CD28 that leads to increased IL-2 and Bcl-xL expression.

Adoptive transfer of NKp30-CD28ζ+ T cells significantly improves the survival of RMA/B7-H6 tumor-bearing mice and induces the generation of immunological memory

Because NKp30 is a pseudogene in inbred mice, we determined whether human NKp30 CARs could be expressed on mouse T cells, which allows the testing of in vivo efficacy of chimeric NKp30-modified T cells against NKp30 ligand-positive tumor cells in immunocompetent mouse tumor models. Similar to the expression profile observed on human T cells, NKp30 CARs were expressed on mouse T cells (Fig. 7A). All chNKp30-modified murine T cells responded to coculture with RMA/B7-H6 cells, but not with RMA cells, by producing IFN-γ. NKp30-CD28-3ζ-bearing T cells produced more IFN-γ than did NKp30-3ζ-bearing T cells (Fig. 7B). However, NKp30-3ζ-bearing T cells were targeted by NKp30-CD28ζ+ T cells (Fig. 7B). In addition, chNKp30-modified mouse T cells were highly targeted by NKp30-CD28ζ+ T cells (Fig. 7B).

**Figure 6.** Engagement of NKp30-CD28ζ receptor led to increased T cell proliferation and upregulation of IL-2 and Bcl-xL. (A) NKp30 CAR (either wtNKp30 or chNKp30)-modified human T cells were labeled with CFSE, as described in Materials and Methods, and cocultured with HeLa cells (NKp30 ligand positive) in the presence of a small amount of IL-2 (25 U/ml) for 3 d. Analysis of T cell proliferation (i.e., CFSE dilution) was performed on both NKp30ζ+ (FLA) and NKp30ζ− cells within the same mixed T cell population by flow cytometry. (B) NKp30ζ-modified T cells (2.5 × 10^5 cells) were cultured in anti-NKp30 mAb (4 μg)-coated 24-well plates for 24 h. Mouse IgG was used as a negative control. IL-2 gene expression was determined by real-time PCR, as described in Materials and Methods. Results are shown as fold increase, in which the IL-2 gene expression in the control mAb-treated T cells was normalized to 1. Data are presented as mean ± SD from two independent experiments. (C) Twenty-four hours after cross-linking with immobilized anti-NKp30 mAbs, as described above, T cells were collected. Bcl-xL expression was determined by flow cytometry after intracellular staining with anti-Bcl-xL–FITC (solid line) or isotype control mAbs (dashed line). *p < 0.05.
Even at an E:T ratio of 1:1, T cells expressing either NKp30-CD28-3ζ or NKp30-3ζ receptor killed RMA/B7-H6 cells at an efficiency of 80% (Fig. 7C). No significant killing of NKp30 ligand-negative RMA cells was observed. Perforin had a role in the killing process, because NKp30 CAR-modified T cells (10⁵ cells) were cocultured with irradiated RMA/B7-H6 cells (10⁵ cells) for 24 h. Mouse lymphoma cell line RMA was used as a negative control. IFN-γ amounts in the supernatants were analyzed by ELISA. Results are shown as mean ± SD. (C) Effector T cells derived from wt B6 mice and perforin-deficient mice (Pfp⁻/⁻) that were modified with wtNKp30, NKp30-3ζ, or NKp30-CD28-3ζ were cocultured with RMA or RMA/B7-H6 cells, respectively, at a ratio of 1:1 in 5-h LDH-release assays. The data are presented as mean ± SD of triplicates from a representative experiment of two independent experiments. *p < 0.05.

Previous results showed that i.v. injection of RMA cells leads to systemic lymphoma in B6 mice (30). Therefore, we tested whether RMA/B7-H6 cells could develop systemic lymphoma after i.v. inoculation into immunocompetent B6 mice. Although B7-H6 is a human molecule, its expression did not significantly alter the growth of RMA cells. Intravenous administration of 10⁵ RMA/B7-H6 cells led to the development of systemic lymphoma, with a median survival of 18 d, which is the typical growth for a similar dose of RMA tumor cells in B6 mice (30). To determine the efficacy of NKp30 CAR-bearing T cells in eliminating established tumors in this model, 5 × 10⁶ T cells (transduced with either

**FIGURE 7.** Human NKp30 CARs can be expressed and function on murine T cells. (A) Human NKp30 expression on mouse T cells 7 d after transduction. NKp30 expression was detected using the PE-conjugated anti-NKp30 mAb in combination with the anti-mouse CD4-FITC mAb. CD4⁻ T cells are CD8⁺ T cells. The data are representative of three experiments. (B) Seven days after retroviral transduction, NKp30-modified T cells (10⁵ cells) were cocultured with irradiated RMA/B7-H6 cells (10⁵ cells) for 24 h. Mouse lymphoma cell line RMA was used as a negative control. IFN-γ amounts in the supernatants were analyzed by ELISA. Results are shown as mean ± SD. (C) Effector T cells derived from wt B6 mice and perforin-deficient mice (Pfp⁻/⁻) that were modified with wtNKp30, NKp30-3ζ, or NKp30-CD28-3ζ were cocultured with RMA or RMA/B7-H6 cells, respectively, at a ratio of 1:1 in 5-h LDH-release assays. The data are presented as mean ± SD of triplicates from a representative experiment of two independent experiments. *p < 0.05.
wtNKp30 or chNKp30 genes) were administered i.v. on days 5, 7, and 9 posttumor inoculations. As shown in Fig. 8A, treatment with NKp30-CD28-3ζ T cells significantly improved median survival from 18 to 30 d, and 4 of 23 mice (17%) became long-term survivors (Fig. 8A). Surprisingly, although wtNKp30, NKp30-3ζ, and NKp30-CD8(TM)-3ζ allowed T cells to respond to RMA/B7−H6 cells in vitro, the murine T cells modified with these receptors showed little effect on the survival of tumor-bearing mice in this aggressive lymphoma model.

Because ligand-negative tumor cells could selectively grow out after CAR T cell therapy, it would be beneficial if treatment with NKp30-CD28-3ζ T cells induced host immunity against other tumor Ags. The four mice that remained tumor-free after 60 d (from Fig. 8A) were rechallenged s.c. with wt RMA tumor cells, which do not express any NKp30 ligands. These tumor-free mice were resistant to a subsequent challenge of RMA cells, whereas all control naive mice had aggressive tumors after 18 d (Fig. 8B). These data are consistent with the idea that adoptive transfer of NKp30-CD28-3ζ T cells allowed hosts to generate immunological memory against RMA tumor Ags. In addition, we observed that NKp30-CD28-3ζ+ T cells persisted longer than did either NKp30-3ζ+ or NKp30-CD8(TM)-3ζ+ T cells (data not shown), which correlated with their enhanced antitumor efficacy.

Discussion

The specificity of a therapeutic agent is a key factor in cancer immunotherapy. T cells use their highly specific TCR to recognize a specific antigenic peptide in the context of MHC. However, T cell-mediated antitumor immunity is hindered by many factors, such as low frequency of tumor-specific T cells, low affinity of TCR to its ligand as the result of central deletion of T cells with high affinity to self-Ags in the thymus, and downregulation of MHC or Ag presentation on tumor cells (31). As an important component of innate immunity, NK cells respond to cancers with broader specificities using receptors, such as the NCRs (9, 32). NKp30 is one promising NCR that can be harnessed for T cell-based cancer immunotherapy because its primary ligand B7−H6 is expressed on NK-insensitive tumors, even when high amounts of NK cells are present in the blood after transfer (37, 38). In comparison with NK cells, T cells are much more abundant, proliferate faster in response to stimulation, and have a longer half-life. Therefore, the combination of recognizing many types of tumor cells by NK cell receptors, such as NKp30, with these advantageous biological properties of T cells is expected to translate into enhanced antitumor efficacy.

When expressed on NK cells, NKp30 associates with CD3ζ and FeRγ-chains as a monomer (13). Retroviral transduction of wtNKp30 gene into human T cells does not lead to significant surface expression, which may be due to the absence of FeRγ-chain expression in human γδ T cells. In NKp30-3ζ, the ζ, CDP TM and CYP domains were replaced with the counterparts of the CD3ζ chain to minimize the need for association with the FeRγ-chain; improved surface expression of chimeric NKp30 was observed. It is unclear whether the NKp30-3ζ receptor expresses as a homodimer or hybridimer with the CD3ζ chain; this will be determined in future studies. Because both CD8α and CD28 can form homodimers without the need to associate with other proteins, inclusion of either CD8α or CD28 TM domains into chimeric NKp30 receptors gave rise to high surface expression (>50% of cells) with these chimeric receptors. Greater expression of CD28-containing chimeric receptors was shown to correlate with better functional activity (e.g., cytokine production and cytotoxicity) (39). CD28 TM-containing CARs often show greater surface expression than do CARs with CD3ζ TM (40). The explanation for this may be that CD28 TM-containing CARs tend to predominantly form homodimers independent of the TCR–CD3 complex, whereas CD3ζ TM-containing CARs can form heterodimers of CAR with the endogenous CD3ζ chain that may be limited by TCR–CD3 complex expression (40, 41).

Chimeric NKp30-modified T cells responded to NKp30 ligand-positive tumor cells by producing IFN-γ. In antitumor immunity, IFN-γ production is often essential to mediate antitumor effects by adoptively transferred T cells in vivo, because IFN-γ coordinates a diverse array of cellular programs (42, 43). In some models, IFN-γ is critical in inhibiting tumor growth by inducing apoptosis or inhibiting angiogenesis (44, 45). Corthay et al. (46) showed that...
IFN-γ was critical for CD4+ T cell-mediated macrophage activation and tumor rejection. NKp30-3Cζ+ T cells showed a similar in vitro cytotoxicity against RMA/B7-H6 cells to NKp30-CD28-3Cζ+ T cells. However, IFN-γ production by NKp30-CD28-3Cζ+ T cells in response to the stimulation of RMA/B7-H6 cells was much greater, suggesting that the threshold for chimeric NKp30 receptors to exert cytotoxicity is lower than IFN-γ production. There was a general correlation between B7-H6 expression and IFN-γ production, which was not observed for cytotoxicity. Cytotoxicity was observed by NKp30 CAR T cells if B7-H6 was expressed on the cell surface, and it did not differ within the variation of ligand expression of the tumor cells tested. Faroudi et al. (47) reported a similar observation: the threshold for CTL-mediated cytotoxicity was 100-fold less than that for IFN-γ production, and the differences were likely due to the formation of distinct immunological synapses (lytic synapse versus stimulatory synapse). Much more IFN-γ was produced when CAR T cells were cultured with suspension tumor cells than with adherent tumor cells, which may reflect differences in the assay conditions. Differences in responses against individual tumor cell lines may also reflect variations in the tumor cells’ production of inhibitory or stimulatory factors that may also affect CAR T cell function. In addition, only NKp30-CD28-3Cζ+–bearing T cells conferred significant in vivo antitumor efficacy, suggesting that IFN-γ production, in addition to direct killing, by chimeric NKp30-modified T cells may be critical for in vivo efficacy.

In addition to B7-H6, BAT3 has been identified as a ligand for NKp30 receptor (17, 18). However, BAT3 is a nuclear protein, which is not normally expressed on the cell surface. BAT3 mRNA tumor cells (e.g., MCF-7, DU145, and IM9) lack surface staining of NKp30 ligands, which is consistent with BAT3 being a nuclear protein. In addition, the responsiveness of NKp30-CD28-3Cζ+ T cells to tumor cells correlated with the expression of B7-H6 mRNA in the target cells, suggesting that B7-H6 is the major NKp30 ligand being recognized on the tested tumor cells. In this study, freshly isolated human PBMCs were deficient in B7-H6 based on flow cytometry and RT-PCR data. However, PBMC-derived DCs (both iDCs and mDCs) that were generated from in vitro culture (7–10 d) with GM-CSF and IL-4 expressed NKp30 ligands, although B7-H6 was not expressed by DCs. BAT3 was expressed on the surface of iDC-released exosomes, which can stimulate NK cells to produce IFN-γ (19). It is not clear whether BAT3 is directly expressed on the surface of iDCs. NKp30 plays a role in NK cell-mediated killing of iDCs, but NK cell killing of monocytes and mDCs is minimal (28). In this study, NKp30-CD28-3Cζ+ T cells produced significantly less IFN-γ upon coculture with mDCs compared with iDCs, which is in agreement with previous findings. Lower levels of NKp30 ligands on mDCs may contribute to reduced stimulatory effects on NKp30-CD28-3Cζ+ T cells. B7-H6–specific CARs can be developed to further minimize the reactivity of CAR-modified T cells against normal cells, such as DCs.

Heparan sulfate epitope(s) on tumor cells may also bind to NKp30 (48, 49). Removal of heparan sulfate from the surface proteins on some tumor cells with heparin lyases resulted in ~50% reduction in the binding to an NKp30-3g fusion protein (48). The glycosylation status of the NKp30 receptor affects its binding to some of the ligands (50). NKp30 receptor has several potential N-linked glycosylation sites. Treatment of NKp30 receptor with protein N-glycanase F, which removes the N-glycans from glycoproteins, significantly reduces the binding of NKp30 to its ligands (49). However, N-linked glycosylation may not affect the NKp30–B7-H6 interaction, because the glycosylation sites of NKp30 are located outside of the interface of NKp30 with B7-H6 (21).

Incorporation of domains from costimulatory molecules (e.g., 4-1BB, OX40, and CD28) into chimeric receptors was also shown to improve the antitumor efficacy of T cells both in vitro and in vivo (3, 26, 50, 51). The signaling domain of CD28 in chimeric receptors is particularly important in IL-2 production, proliferation, and cell survival of lymphocytes (51–53). In this study, we confirmed that, upon cross-linking of NKp30 receptor by anti-NKp30 mAbs or engagement with NKp30 ligand-positive tumor cells, the CD28 signaling domain in the NKp30-CD28-3Cζ receptor enhanced T cell functions, such as upregulation of IL-2 and Bcl-xL and cell proliferation.

This study showed that adoptive therapy of NKp30-CD28-3Cζ+ T cells into RMA/B7-H6 tumor-bearing mice elicited the hosts to generate memory responses against NKp30 ligand-negative RMA cells, suggesting the development of “epitope spreading.” Initial targeting of B7-H6 on RMA cells by NKp30-CD28-3Cζ+ T cells is expected to lead to tumor cell death, followed by efficient presentation of tumor Ags by professional APCs (such as DCs), probably as a result of the presence of proinflammatory cytokines (e.g., IFN-γ, TNF-α, and GM-CSF) and chemokines. Cross-priming of host T cells by these APCs may further lead to expansion of polyclonal tumor-specific T cells (i.e., epitope spreading). For tumors to evade immune surveillance, tumor cells are selected with mutations or deletions of targeted Ags (54, 55). Therefore, induction of polyclonal tumor-specific T cells will minimize the chances for tumor cells to “escape.” Generation and maintenance of memory responses are important for reducing recurrent tumor disease.

In summary, a chNKp30 receptor can be used to redirect T cells against NKp30 ligand-expressing tumors. Incorporation of a CD28-signaling domain into chimeric NKp30 receptors can stimulate both primary and costimulatory signals for enhanced anti-tumor activities. NKp30 can recognize its ligands on several different types of tumor cells, and this study demonstrates a potential broad therapeutic usefulness of this chNKp30 CAR approach for the treatment of cancer.

Acknowledgments
We thank the National Cancer Institute Biological Resource Branch for providing recombinant human IL-2 and the staff of the Animal Resources Center for assistance with animal care.

Disclosures
Dartmouth College has filed for patent protection for the chimeric NKp30 receptors and anti-B7H6-specific Abs described in this study.

References


