Expression of a Variant of CD28 on a Subpopulation of Human NK Cells: Implications for B7-Mediated Stimulation of NK Cells

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Expression of a Variant of CD28 on a Subpopulation of Human NK Cells: Implications for B7-Mediated Stimulation of NK Cells

Joanna Galea-Lauro,† David Darling,‡ Shu-Uin Gan,§ Leonid Krivochtchapov,* Marcel Kuiper,* Joop Gäken,* Bernard Souberbielle,*§ and Farzin Farzaneh*²

The ability of NK cells to kill tumor cells is controlled by a balance between activating and inhibitory signals transduced by distinct receptors. In murine tumor models, the costimulatory molecule B7.1 not only acts as a positive trigger for NK-mediated cytolysis but can also overcome negative signaling transduced by MHC class I molecules. In this study, we have evaluated the potential of human B7.1-CD28 interaction as an activating trigger for human blood NK cells. Using multiparameter flow cytometric analysis and a panel of different CD28 mAbs, we show that human peripheral blood NK cells (defined by CD56+/CD16−, and CD3− surface expression) express the CD28 costimulatory receptor, with its detection totally dependent on the mAb used. In addition, the level of CD28 varies among individuals and on different NK cell lines, irrespective of CD28 steady-state mRNA levels. By performing Ab binding studies on T cells, our data strongly suggest that binding of two of the anti-CD28 Abs (clones 9.3 and CD28.2) is to a different epitope to that recognized by clones L293 and YTH913.12, which is perhaps modified in the CD28 molecule expressed by the NK cells. We also show that B7.1 enhances the NK-mediated lysis of NK-sensitive but not of NK-resistant tumor cells and that this increased lysis is dependent on CD28-B7 interactions as shown by the ability of Abs to block this lysis. Coculture of the B7.1-positive NK-sensitive cells also led to the activation of the NK cells, as determined by the expression of CD69, CD25, and HLA class II. The Journal of Immunology, 1999, 163: 62–70.

Natural killer (NK) cells are a subpopulation of lymphoid cells that do not require prior sensitization or MHC restriction for target recognition. The ability of these cells to kill their targets is controlled by a fine balance between distinct receptors mediating inhibitory and activating signals (1–3). NK cell triggering receptors, represented by the NKR-P1 family of C-type animal lectins, recognize oligosaccharide ligands on NK-sensitive target cells and signal cytotoxic activity (4, 5). Killer cell inhibitory receptors, represented by p58 and p70 receptors of the Ig superfamily, or C-type lectins (e.g., CD94/NKG2) analogous to murine Ly-49, recognize autologous MHC class I molecules and by contrast mediate suppressive signals for cytotoxicity (3). Tilting this balance, for instance in favor of activatory signals, leads to cytotoxicity, as is the case with aggregation of CD16 molecules, which leads to Ab-dependent cellular cytotoxicity (ADCC)² (6, 7).

A number of studies using murine tumor models have now shown that de novo expression of B7.1 can render NK-resistant tumor cells susceptible to NK lysis by binding to either CD28 (8, 9) or to an as yet unidentified molecule (10). For example, transfection of the B7.1 molecule into the NK-resistant BW-Li T lymphoma variant cell rendered them NK-sensitive (despite high cell surface expression of class I H-2Dk) and concomitantly reduced their metastasis, which was restored in SCID-beige mice, which are devoid of NK function (9). However, B7.1 costimulation does not always lead to NK activation and NK-mediated lysis. For example, P815 plasmacytoma and EL4 thymoma cells remained resistant to NK-mediated killing (11, 12), as did the murine NC mammary adenocarcinoma cells after B7.1 expression (13).

In contrast to these studies, parallel data for the involvement of CD28-mediated stimulation of human blood NK cells lag behind. While expression of CD28 on murine NK cells is clearly established, expression of CD28 on human NK cells is somewhat unclear. For example, fetal NK cells, unlike adult peripheral blood NK cells, are positive for CD28 expression (14). In addition, the widely used YT2C2 NK cell line (15) is CD28 positive whereas the NKL cell line recently derived from an aggressive human NK cell leukemia is CD28 negative (16). Expression of B7.1 by several human tumor lines, and by the mouse P815 tumor line, renders these tumor cells sensitive to lysis by the YT2C2 NK human cell line, in a CD28-dependent manner. Suggesting that CD28-B7.1 interactions may also be important in human NK cytotoxic activity (15). A number of different human tumor cells expressing B7.1 have already been evaluated for their ability to enhance NK-mediated cytotoxicity using freshly isolated human blood NK cells (17, 18). For example, the H125 human lung adenocarcinoma carcinoma enhances NK-mediated cytotoxicity when modified to express B7.1, whereas other cell lines remain NK resistant (17, 18). However, detailed analysis of CD28 on the blood NK cells was not performed in these studies, making it difficult to evaluate the role of CD28-B7 interactions in these models.
Human CD28 is a complex gene and consists of a single copy gene organized into four exons, each corresponding to a functional domain (19, 20). At the mRNA level, the CD28 gene transcribes four RNA species; a smaller pair consisting of a 1.5-kb transcript and a 1.3-kb transcript, and a larger pair consisting of a 3.5- and a 3.7-kb transcript (19, 20). All four mRNA transcripts are inducible to varying degrees during T cell activation (20). The larger 3.5 and 3.7 kb mRNA species arise from the use of an alternate candidate nonconsensus polyadenylation signal (GATAAA) located 2167 bp downstream from the first signal. This accounts for the size difference between the larger (3.7/3.5 kb) and the smaller (1.5/1.3 kb) mRNA species. In addition, the gene has an internal splicing event that uses an unusual splice donor/acceptor site, resulting in the CT/AC splice junction leading to an in frame deletion of 252 bp of the coding region. This accounts for the size difference between the 3.7- and 3.5-kb mRNA species and the 1.5- and 1.3-kb mRNA species and suggests that alternate products of the CD28 gene may be expressed as cell surface molecules with potentially different physiologic roles (19, 20). cDNA cloning reveals that the 1.5-kb or the 3.7-kb transcript encodes the surface expressed 44-kDa form of CD28 recognized by the CD28-specific mAb 9.3. There is yet no direct evidence that the protein products of the 1.3- or 3.5-kb transcripts exist or have a physiological role. In addition, a variant form of CD28 (45 kDa) is expressed by PHA-activated T cells that is recognized by a polyclonal antiserum but not by the anti-CD28 mAb 9.3 (21, 22).

During this study, we used a panel of CD28 mAbs, including the widely used 9.3 mAb, used in the initial study of CD28 on NK cells (14) and show by multiparameter flow cytometry that human blood NK cells (defined by CD56+CD3−CD16−) stain for CD28. The detection of CD28 is absolutely dependent on the mAb used, suggesting that a variant of CD28 is being expressed by the NK cells. In addition, the ability to express this variant appears to vary among individual adults. Using RT-PCR, we also show evidence of CD28 transcripts from two established human NK cell lines and from purified blood NK cells isolated from individuals who express different levels of the CD28 protein. Studies reported here also show that engagement of CD28 on NK cells leads to their activation. For example, coculture of NK cells with B7.1-positive NK-sensitive tumor cells, but not NK-resistant tumor cells, results in increased expression of NK activation markers and enhanced tumor cell lysis, which could be inhibited by anti-B7.1 and CD28 mAbs. In conclusion, the present data suggest that CD28 functions as a positive putative signal for human NK-mediated cytotoxicity.

Materials and Methods

NK cells and Methods

PBMCS (i.e., lymphocytes and monocytes) were used as the source of NK cells and were isolated by standard methods involving Ficoll centrifugation. Briefly, blood from 18 healthy volunteers aged 21–45 was collected into heparinized tubes, diluted 1:1 in PBS (without Ca2+ or Mg2+ salts) layered onto Ficoll Paque, and centrifuged for 20 min at 400 × g in an MBE bench top centrifuge. Cells that collected at the interface were aspirated, washed twice in HBSS, and resuspended either in ice-cold PBS/2% FCS for flow cytometry experiments or in RPMI 1640 medium/10%FCS for in vitro NK assays. Highly purified NK cells were obtained through three rounds of depletion of T cells, B cells, and monocytes by the following Dynabeads: M-450 Pan-T (CD3), M-450 Pan-B (CD19), and M-450 CD14 (monocytes) used according to the manufacturer’s instructions (Dynal, Wirluk, U.K.). The use of these beads in combination allowed the routine isolation of 99.9% pure NK cells, as assessed by phenotypic characterization of the cells using flow cytometry. Activated NK cells were generated by culturing these cells in the presence of 6000 IU/ml of human rIL-2 (Chiron, Harefield, U.K.) for 24–48 h. The YT2C2 cell line was obtained from Dr. Kendall Smith at Cornell University (Ithaca, NY), whereas the NKL cell line was obtained from Dr. Michael Robertson at the Indiana Cancer Research Institute (Indianapolis, Indiana). Both cell lines were maintained in RPMI 1640/10% FCS with the NKL line supplemented with 100 IU/ml of human rIL-2.

Cell lines

The cell lines used for this study were Jurkat (human T cell line), U937 (human monoblastoid cell line), K562 (human erythroleukemia cell line), RPMI-1788 (human B cell line), A431 (human vulva epithelial cells), and RPMI-8226 (human monoblastoid cell line). The cell lines were confirmed to be mycoplasma free using the Gen-Probe mycoplasma detection kit (Gen-Probe, San Diego, CA). The RPMI-1788 cells were maintained in IMDM medium, whereas all other cell lines were maintained in RPMI 1640 medium, supplemented with 10% FCS, 1 mM sodium pyruvate, 100 U/ml penicillin, and 100 μg/ml streptomycin.

RT-PCR analysis of CD28

Total RNAs were isolated from T and NK blood cells of three individuals, from Jurkat T cells, from the NKL and YT2C2 NK cell lines, and from U937 cells using TriZol (Life Technologies, Grand Island, NY). Next, 1 μg of RNA was denatured at 70°C in 12 μl of diethyl pyrocarbonate-treated water containing 0.5 μg of oligo(dT) primers for 10 min and quick chilled on ice. Reverse transcription was performed in a 20-μl total volume with 500 μM dNTP (Pharmacia, Piscataway, NJ), 200 U Superscript II reverse transcriptase (Life Technologies) in 1× first strand buffer (10 mM DTT, 50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM MgCl2). The reaction mixtures were incubated at 42°C for 1 h, heat inactivated at 70°C for 15 min, and stored at −20°C until further use. PCRs in 50 μl contained 2 μl of cDNA, 10 pmol of each primer, 100 μl of each dNTP, 5 μl of 10× PCR buffer (HT Biotech, Cambridge, U.K.) and 0.2 U Supertaq (HT Biotech).

For amplification of CD28, a sense primer 5′-CACGACAAAGAT-GGCCTAGGCTGCTCT-3′ spanning the translation start codon at position 182 in exon 1 and an antisense primer 5′-TCAAGGCGATAAGGCGTG-3′ directed to the translation stop codon at position 126 in exon 4 were used. Using this primer pair, two products are expected, a 674-bp transcript representing the full-length coding region of human CD28 and a 422-bp transcript representing amplification of mRNA transcripts with the 252-bp exon internal deletion (20). PCR conditions were as follows: 35 cycles with denaturation at 94°C for 1 min, annealing at 70°C for 1 min, and extension at 72°C for 1 min followed by a 10-min final extension at 72°C. PCR products were separated on a 1% agarose gel. For testing the integrity of the RNA and normalizing the sample variation of cDNA synthesis, PCR was performed with the housekeeping gene GAPDH.

DNA bands were excised from the agarose gel and extracted with the Qnex II extraction kit (Qiagen, Chatsworth, CA). The bands were cloned into PCR2.1 vector using the TA cloning kit according to the manufacturer’s protocol. Sequencing of the PCR insert was performed with an ABI 373 automated sequencer.

Coculture of NK cells with tumor cells

Purified NK cells and tumor cells were added together in a ratio of 10:1 in a 12-well plate in a total volume of 5 ml/well. Twenty-four hours later, the NK cells were analyzed for activation markers by flow cytometry as described below.

Monoclonal Abs

For flow cytometry, the following Abs (directly conjugated to either FITC, R-PE, or peridinin chlorophyll protein (PerCP)) were used: anti-CD56-RPE (clone B-A19; Serotec, Oxford, U.K.), anti-CD3-PerCP (clone SK7; Becton Dickinson, Mountain View, CA), anti-CD16-FITC (clone DJ130c; Dako, Cappertina, CA), anti-CD3-FITC (rat IgG2b, clone YTH913.12; Serotec), anti-CD28-FITC (mouse IgG1, clone CD28.2; Pharmingen, San Diego, CA), anti-CD69-FITC (mouse IgG1, clone L78; Becton Dickinson), anti-HLA-DR-FITC (clone CR3/43; Dako), anti-CD25-FITC (clone ACT-1; Dako), rat IgG2b-negative control FITC (clone LO-DNP-11; Serotec), and mouse IgG1-negative control FITC (Sigma). The cell surface Ag CTLA-4 was detected using a biotin-conjugated Ab (clone BNI3; Becton Dickinson), detected by avidin-FITC (Sigma). Other mAbs used were unconjugated (mouse origin) and included: anti-CD28 (clone L293; Becton Dickinson), anti-CD28 (clone CD28.3 9.3 IA1; kindly donated by Bristol-Myers Squibb, Seattle, WA), anti-HLA-ABC (clone W6/32; Serotec), human HLA class II- DR, DP, DQ (clone WR18; Serotec), anti-B7.1 (clone L307.4; Becton Dickinson), and anti-B7.2 (clone BU 63; Serotec). These Abs were detected indirectly using a rabbit anti-mouse Ig-FITC conjugate (Dako). Cell surface staining for B7 was also detected using the fusion protein CTLA4-Hg (CTLA-4 fused with human IgG1 constant regions but lacking the CH1 domain) obtained from culture supernatant of the J558L cell line.
FIGURE 1. Representative FACS analysis of human blood NK cells isolated from three different adults, identified as CH, AB, and MK. 

A, Percentage of total lymphocytes that express CD56 on their surface. PBMCs were stained with anti-CD56 directly conjugated to PE and analyzed as described in Results. A total of 5000 cells was analyzed for each individual. 

B, A live gate was set such that only those lymphocytes that are CD56$^+$ were collected for analysis. 

C, Three-color analysis of the CD56$^+$ cells for CD3 using an anti-CD3 PerCP Ab (fluorescence on the y-axis) and a negative control FITC-conjugated Ab to control for background fluorescence in the FITC channel. As shown in these three individuals the percentage number of CD56$^+$ cells that also stain for CD3 varied between 17.7% and 38.7%. The background fluorescence by the FITC-Ab was negligible. 

D, Three-color analysis of the CD56$^+$ cells for CD3 using an anti-CD3 PerCP Ab (fluorescence on the y-axis) and an anti-CD28-FITC isotype-matched with the negative control FITC.
cell line (23). CTLA4Ig was detected using goat anti-human Ig-FITC conjugate (Sigma).

For blocking studies the following Abs were used: anti-CD28 (clone L293; Becton Dickinson), anti-B7.1 (clone 1B5; kindly donated by Innogenetics, Gent, Belgium), and anti-CD16 (clone 3G8; Pharmingen). Negative control isotype-matched Abs were used accordingly. For studies using CTLA4Ig, supernatant from J558L cells was collected and purified for CTLA4Ig on a Protein G Plus-Agarose column according to the manufacturer’s instructions (Oncogene Science, Cambridge, U.K.).

**Retrovirus preparation and infection of tumor cells**

Human B7.1 (hB7.1) cDNA was generated and cloned into a disabled retroviral vector containing a selectable hygromycin B marker gene as described previously (24, 25). K562 cells were infected with supernatant from the hB7.1 retrovirally infected PA317 packaging line in the presence of 3 μg/ml polybrene for 48 h. These cells were subjected to 5 rounds of infection and selected in 300 μg/ml of hygromycin B for 2 wk, without cloning. A431 and P815 cells were infected with supernatant from PA317/hB7.1 cells and were selected in 300 μg/ml of hygromycin B for 2 wk, without cloning. CD56 expression was confirmed by staining the cells with a specific mAb for B7.1 (clone L307.4; Becton Dickinson) and with the fusion protein CTLA4Ig, followed by detection with a FITC-conjugated secondary Ab.

**Flow Cytometry**

For the immunophenotyping of NK and T cells, PBMCs were washed in PBS buffer containing 2% FCS and immunolabeled with either anti-CD56-PE, CD3-PerCP, CD16-FITC, or CD28-FITC individually or in combination. Isotype-matched Abs were also used as controls for background staining. Flow cytometry was performed using a FACScan (Becton Dickinson) equipped for five-parameter analysis studies. Leakage of fluorescence from one channel to another was compensated for by using cells stained separately with different fluorochrome-labeled Abs and then subtracting the fluorescence leakage into the different channels for that fluorochrome. After electronic gating, 5000 events were collected only on CD56+ cells. Furthermore, within this region, a fraction of the cells also stained positive for CD3. For this study, NK cells were phenotypically defined as being CD56+ CD3− and further confirmed by CD16 staining. For specific analysis of T cells within the same study, a live gate was set such that 5000 events were collected only on cells that stained positive for CD3. Any cells that were CD56+ within this region were then excluded. FACS analysis of CD28 was also performed on isolated NK cells purified after the immunomagnetic depletion of T cells, B cells, and monocytes.

For the study of activation markers on NK cells after coculture with tumor cells, the cells were labeled with CD56-PE, CD3-PerCP, and either CD69-FITC, HLA-DR-FITC, or CD25-FITC. CD56 was used as the marker for NK cells to separate the tumor cells from the NK cells on the FACS machine.

**Preclearance of anti-CD28 Ab by T cells and B cells**

The FITC-conjugated anti-CD28 Ab produced by clone YTH913.12 was precleared by T cells and B cells separately as follows. Next, 5 × 10^6 T cells or 5 × 10^7 B cells were purified by immunomagnetic beads (as described above), incubated with 100 μl of FITC-anti-CD28 (diluted 1:10) for 30 min on ice, and the “precleared” Ab solution was collected by removing cells using a Magnetic Particle Concentrator as described by Dynal. The precleared Ab was then used for staining purified NK cells isolated from the same individual who provided the T and B cells used for the preclearance of the Ab.

**“Epitope mapping” of anti-CD28 Abs**

To “map” the binding site of the different anti-CD28 Abs, T cells were incubated with increasing amounts of the L293 Ab (unconjugated), washed, and incubated with saturating amounts of the FITC-conjugated anti-CD28 (clones CD28.2 and YTH913.12) Abs as described previously (26). Similarly, NK cells were stained with increasing amounts of L293 Ab, washed, and stained with saturating amounts of FITC-conjugated anti-CD28 (clone YTH913.12).

**NK cytotoxic assays**

NK cytotoxicity was measured by standard chromium release assays. Briefly, this involved labeling 2 × 10^5 target cells with 100 μCi of ^51^Cr (in sterile saline; Amersham, Little Chalfont, U.K.) in a slow-shaking (30 turns/min) water bath for 2 h at 37°C. The cells were washed, resuspended at 5 × 10^4 cells/ml, and 100 μl was added to PBMCs to give E:T ratios of 100:1, 50:1, 25:1, 12.5:1, 6.25:1, and 3.125:1 or added to purified NK cells to give E:T ratios of 10:1, 5:1, 2.5:1, 1:2.5, 1:5, and 1:10. The assay was performed in a 96-well U-bottom plate in a final volume of 200 μl per well, in triplicate sets. At the end of a 4-h coculture, 100 μl of supernatant was collected from each well, added to 1 ml of scintillant (High Flash-Point, universal LSC-mixture, Ultima Gold; Packard, Meriden, CT) and counted for radioactivity using a Liquid Scintillation Analyzer (TRI-CARB 2200CA; Packard) as previously described (27). The percentage cytotoxicity was determined using the following formula: % cytotoxicity = 100 (E - S)/(T - S), where S is cpm of the spontaneous release by target cells alone, T is cpm of total release of ^51^Cr from target cells lysed in 5% Triton X-100 (made up in PBS), and E is cpm released from target cells in the presence of effector cells. In all experiments, the spontaneous release of chromium from the tumor cells alone never exceeded 10% of the total chromium released from the same number of tumor cells lysed in the presence of Triton X-100. For functional studies, mAb L293 (anti-CD28) was used at 5 μg/ml, 5B5 (anti-B7.1) was used at 1 μg/ml, and CTLA4Ig was used at 4 μg/ml.

Statistical analysis was performed by Statworks software (Apple Computers), using unpaired student t test (2 tailed p value), at 95% level of confidence.

**Results**

**Peripheral blood human NK cells express a variant of CD28**

CD28 expression was studied on NK cells isolated from 18 healthy individuals. A differential count for CD3+, CD56+, and CD16+ cells was also routinely performed on all the PBMC samples. Briefly as expected, several subtypes of T and NK cells are present within the lymphocyte population. Using a combination of two different markers (CD3 and CD56), the average percentage of T cells (marked by CD3) and of NK cells (marked by CD56) is 67.8 ± 5.6% and 19.1 ± 5.7% of all lymphocytes, respectively (data not shown). These markers are not exclusive; 6.1 ± 2.9% of all lymphocytes express both CD56 and CD3. Therefore, CD16 was included for a more rigorous discrimination between T and NK cells. Using a combination of CD3 and CD16 or CD56 and CD16, a very small subset of cells (3.3 ± 2.8% of all lymphocytes) are shown to express both CD3 and CD16 simultaneously, with CD16 being expressed at low levels (data not shown). The cells expressing high CD16 levels are all CD56+ and lack CD3 expression. A very small number of CD56+ cells lack CD16 but are CD3+ (3.3 ± 2.8% of all lymphocytes). Thus for the purpose of this study T cells are defined as CD3+CD56−CD16− and NK cells defined as CD56+CD3−CD16+.

The FACS data shown for CD28 expression are representative results from three different individuals, marked as CH, AB, and MK. The percentage of CD56+ cells in these individuals varied from 9.8% to 21.5% of total lymphocytes (Fig. 1A). Because this is quite a small fraction of the total PBMCs, an electronic gate was set and only those cells positive for CD56 expression were saved (Fig. 1B). These CD56+ cells were further analyzed for CD3 and CD16 expression using different fluorescence-labeled Abs (CD3-PerCP and CD28-FITC). The labeling by CD28-FITC Ab (clone YTH913.12) (Fig. 1D) was compared with an isotype-matched Ab in C. The CD56+ CD3+ cells in all three individuals are staining positive for CD28 albeit at different levels. E, Three-color analysis of the CD56+ cells (PE channel) for CD3 expression using an anti-CD3 PerCP Ab (fluorescence on the y-axis) and for CD16 expression using an anti-CD16-FITC Ab (x-axis). As shown for two representative individuals, the CD56−CD3+ cells are highly positive for CD16, which classifies them as NK cells. A total of 5000 cells was analyzed per sample.
negative-control-FITC Ab (Fig. 1C), with both Abs having identical florescence:protein ratios. As shown in Fig. 1C, the fraction of CD56^+ cells positive for CD3 expression ranged from as low as 17.7% to as high as 38.7% (upper left quadrant). However, these cells are negative for background staining with the negative-control-FITC-conjugated Ab.

A subset of CD56^-CD3^+ cells were either CD28^- (ranging between 3.0% and 24.3% of all CD56^- cells; upper left quadrant, Fig. 1D) or CD28^+ (ranging between 8.6% and 13.8% of all CD56^- cells, upper right quadrant). The CD56^-CD3^- cells, shown in the lower quadrants of Fig. 1D varied in CD28 expression according to the individual studied. In the first example presented here (marked as CH), only 36.8% of the total CD56^-CD3^- cells are weakly CD28 positive (lower right quadrant), a result typical of 12 of the 18 individuals examined; whereas in the second individual (marked as AB) 62.4% are CD28^+, typical of 4 of 18 individuals, and in the third individual (marked as MK) 92.5% of all the CD56^-CD3^- cells are CD28^+, a result typical of only 2 of 18 individuals. The level of CD28 detected by the YTH913.12 Ab on these CD56^-CD3^- cells was always lower than the level of CD28 detected by the same Ab on T cells isolated from the same individuals (mean fluorescence intensity 21.3 ± 16.4 and 93.3 ± 15.3, respectively). The pattern of CD28 staining on the NK cells was reproducible for each individual over a period of 12 mo. The CD56^-CD3^- cells expressed high levels of CD16, thus confirming their NK identity, whereas the CD56^-CD3^- cells were mostly CD16^- or CD16^dim (Fig. 1E).

CD28 expression was examined further with three more anti-CD28 Abs on NK cells from three separate donors. Ab clone L293, gave a similar pattern of staining to clone YTH913.12, but labeled the CD56^-CD3^- cells with less intensity (MFI of 12 ± 4.3 as compared with 21.3 ± 16.4). In contrast, Abs 9.3 and CD28.2 did not stain the NK cells, despite clear detection of CD28 expression by these Abs on the CD56^-CD3^- cells and on the T cells (CD56^-CD3^+) from the same individuals (data not shown). NK cells were also studied for surface expression of CTLA4 and found to be negative (data not shown). Expression of CD28 was also examined on the NKL and the YT2C2 and cell lines. YT2C2 cells stained positive with all four mAbs whereas the NKL was negative for CD28. These results are in accordance with their published data.

Binding activity of clone YTH913.12 to NK cells is removed by preclearance with T cells but not B cells

NK cells were further stained with the YTH913.12 clone after it had been precleared with either T cells or B cells. The mean fluorescence intensity of the stained NK cells decreased from 28.6 (uncleared Ab) to 6.3 after it had been precleared with T cells, a reduction of 77.9%. By contrast, staining with the YTH913.12 precleared with B cells decreased from 28.6 to 23.2, a reduction of only 18.6%.

Abs CD28.2, YTH913.12, and L293 bind to different epitopes of CD28

To investigate the epitopes recognized by the different anti-CD28 Abs, binding inhibition experiments were conducted. Fig. 2 shows that the binding of FITC-conjugated YTH913.12 (MFI 86.2) and FITC-conjugated CD28.2 (MFI 94.6) to T cells was reduced up to 80.4% (MFI 16.8) and 17.7% (MFI 77.8), respectively, after preincubation of the cells with increasing amounts of unconjugated L293. Binding of the FITC-conjugated Abs was unaffected by irrelevant isotype-matched Abs (data not shown). These data suggest that YTH913.12 and L293 either bind to the same or closely spaced epitopes and that the binding of L293 competes for the binding with YTH913.12. In contrast, CD28.2 and L293 bind unrelated epitopes, as shown by the inability of L293 to significantly inhibit the binding of FITC-conjugated CD28.2. The binding of unconjugated L293 Ab to NK cells inhibited the binding of FITC-conjugated YTH913.12 by 76%, suggesting once again that these two Abs bind related epitopes.

Purified blood NK cells express CD28 mRNA transcripts

Molecular identity of CD28 on NK cells was further confirmed by RT-PCR using RNA isolated from purified NK cells from individuals with the three different levels of CD28 expression. In addition, we also used T cells from the same individuals as a positive control for CD28 in each subject. As shown in Fig. 3, all the different NK sources express RNA coding for full-length human CD28 (band 674 marked with upper arrow in lanes b, d, and f), irrespective of the protein levels seen by FACS analysis. This band is also present in the YT2C2 (lane j) and the NKL (lane g) cell lines, despite the fact that the NKL cell line shows no CD28 protein staining by the CD28 Abs in this study. T cells isolated from the same individuals (lanes a, c, and e, respectively) and the Jurkat T cell line (lane i) express both predicted bands, i.e., full-length and deleted version (band size 422 marked with lower arrow). The 422-bp band is also expressed in the YT2C2 cell line (lane j) and in one of the NK samples (lane d). The U937 cells (lane h) used as a negative control for CD28 lacks all the CD28 bands, as does the water control in lane k. GAPDH used for normalization was constant throughout all samples (data not shown). Both the 674- and the 422-bp bands have been cloned and sequenced and found to be identical with the published full-length and deleted CD28 sequences.

The additional bands resolved on the agarose gel in Fig. 3 have been cloned and sequenced by us. These represent novel alternative splice variants, all of which use previously described splice acceptor/donor sequences. These are now the subject of further study.

Coculture of B7^+ NK-sensitive tumor cells with NK cells induces NK activation as marked by induction of CD69, CD25, and HLA class II expression

The human K562 cell line (NK-sensitive cells) and the A431 cell line (NK-resistant cells) were infected with a retroviral vector encoding hB7.1. The infected cells (named K562hB7.1 and...
A431hB7.1) were selected as described above, but were not cloned. Both cell lines become highly positive for B7.1 as demonstrated by the binding of both a mAb specific for human B7.1 and the CTLA4Ig fusion protein, showing that the expressed molecule retained binding activity for its cognate receptor (Fig. 4). Parental K562 cells express very low levels of B7.1; however, the level of this molecule on the K562hB7.1 cells is much higher (mean fluorescence intensity (MFI) of 10.22 compared with 138.42 using anti-B7.1 and MFI of 7.9 compared with 63.4 using CTLA4Ig).

The K562 and the A431 cell lines were assessed for their ability to activate NK cells before and after B7.1 expression. After a 24-h coculture, the cells were labeled with a combination of CD56-PE, CD3-PerCP, and either CD69-FITC, CD25-FITC, or HLA class II-FITC Abs; FACS data were collected on CD56+ cells only. Coculture of NK cells with K562 cells leads to the activation of a subpopulation of the CD56+CD3- NK cells as marked by the induced expression of CD69, which is absent in the NK cells cultured alone (Fig. 5, lower right quadrant, 12.06% positive compared with 0.98% in control). Expression of CD69 on the NK cells was further enhanced if the K562 cells expressed B7.1 on their surface (27.82% positive). This enhanced expression was inhibited if the K562hB7.1 cells were precoated with anti-B7.1 Ab (9.6% positive cells only; data not shown). Furthermore, not only was the percentage of cells staining for CD69 higher, but the intensity of staining was higher in the NK cells cocultured with K562hB7.1 as compared with those cocultured with K562 cells (MFI of 76.54 as compared with MFI of 33.4, respectively). The activated NK cells were also positive for CD25 and MHC class II, in comparison to control NK cells, which were negative (data not shown). The CD56+CD3- cells in the same study (Fig. 5, upper quadrants in the FACS profiles) or the T cells (data not shown) were not activated by the coculture of PBMCs with the tumor cells, as shown by the absence of activation markers.

Coculture of NK cells with the “NK-resistant” A431 tumor cells failed to activate the NK cells, as shown in Fig. 4 by the lack of CD69 on the CD56+CD3- cells, whether or not the A431 cells expressed surface B7.1. As a positive control for activation, NK

![FIGURE 3. RT-PCR analysis of human CD28 in blood T cells (lanes a, c, and e) and blood NK cells (lanes b, d, and f) isolated from individuals typical of CH (lanes a and b), AB (lanes c and d), and MK (lanes e and f). Other samples shown are the NKL cell line (lane g), U937 monoblastoid cell line (lane h), Jurkat T cell line (lane i), YT2C2 cell line (lane j), and water control (lane k). A 1-kb ladder is used for markers (lane l). The upper arrow represents the 674-bp band (full-length CD28), and the lower arrow represents the 422-bp band (splice variant of CD28). The other transcripts seen in the figure are novel variants of CD28 that we have cloned and are sequenced and are the subject of further studies not discussed here.](http://www.jimmunol.org/)

![FIGURE 4. FACS analysis of tumor cells for B7 expression. Cells were stained with either an Ab to B7.1 or B7.2 and detected with a FITC-conjugated rabbit anti-mouse Ig. The fluorescence intensity obtained by these Abs (histogram marked by solid line) is compared with the background staining (histogram marked by gray area) obtained by isotype-control-matched Abs. Parental K562 cells are weakly positive for B7.1, but become highly positive after infection for B7.1. Parental A431 and P815 cells are negative for B7.1 but become positive for B7.1 after infection. Expression of B7.1 is also detected using CTLA4Ig, showing that the transduced protein retains binding activity to one of its natural ligands. The histograms also show that the human B cell line RPMI 1788 expresses high levels of both B7.1 and B7.2. The MHC class I status of the tumor cells was also determined by flow cytometry as shown in the first panel.](http://www.jimmunol.org/)
induced expression of CD69 (Fig. 5, lower right quadrant) cells were cultured in the presence of rhIL-2, which resulted in the NK-mediated killing of K562 and K562hB7.1 cells. RESULTS show that CTLA4 Ig induces the NK-mediated lysis of A431hB7.1 cells but not of parental cells. In addition, this lysis is induced by ADCC as shown by the ability of anti-CD16 Ab to block it. The data are representative of three experiments using NK cells isolated from three different individuals.

B7.1 expression enhances the NK-mediated killing of the human K562 erythroleukemia cell line

The K562 cell line, confirmed here to be class I negative (Fig. 4), is used universally as an NK-sensitive target. Expression of B7.1 on K562 cells augments their susceptibility to NK-mediated lysis at different E:T ratios (Fig. 6A). The results are plotted as mean ± SD of five experiments each done in triplicate. A, K562hB7.1 cells are more sensitive to NK-mediated lysis than the parental K562 cells, as shown. Results are similar whether PBMCs or purified NK cells are used as effectors, showing that the killing is mediated by NK cells. The relative number of NK cells in the two assays are identical. B, The enhanced killing of K562hB7.1 cells is directly dependent on B7.1, while Abs to either CD28 or B7.1 block this enhanced killing, as shown. The data shown are the mean ± SD of three different experiments and was performed with purified NK cells at an E:T ratio of 10:1.

cells were cultured in the presence of rhIL-2, which resulted in the induced expression of CD69 (Fig. 5, lower right quadrant, 58% positive cells, MFI of 60.06).

B7.1 does not induce the NK-mediated lysis of NK-resistant tumor cells

Our next set of experiments was designed to investigate whether B7.1 could be used to induce killing of NK-resistant tumor cells. The adherent A431 cell line, which expresses high levels of MHC class I, but no B7.1 or B7.2 (Fig. 4), were used. After infection with the hB7.1 retroviral vectors, A431 cells express high levels of (clone 5B5) Abs. The assays were done in triplicate for three different individuals, at an E:T ratio of 10:1, using purified NK cells as the only effectors. Results are plotted as mean ± SD of the three experiments. The NK-mediated killing of parental K562 cells is not dependent on B7.1-CD28 interaction, despite the low levels of B7.1 by the K562 cells. This was shown by the lack of inhibition of killing by either anti-CD28 Ab (45.6% compared with 51.3%; p = 0.146), anti-B7.1 Ab (47.9% compared with 51.3%, p = 0.356), or combined Abs (48.1% compared with 51.3%, p = 0.373) (Fig. 6B). In contrast, the increase in the NK-mediated lysis of K562hB7.1 cells was completely blocked by the L293 anti-CD28 (47.9% killing compared with 80.62%, p = 0.002) or anti-B7.1 Ab (49.3% compared with 80.62%, p = 0.003). Anti-CD28 and anti-B7 when used together were not synergistic. The level of cytotoxicity observed in the presence of either Ab was similar to that obtained with the parental K562 cells, i.e., reducing the level of killing to the B7-independent baseline. These results also demonstrate that the anti-CD28 Ab does not provide a “negative signal” or interfere per se with cytolytic function of NK cells, but that the interaction of CD28 with B7 is essential for the augmented killing seen in the B7.1 transfectants.

In these studies, the soluble CTLA4 Ig fusion protein was also used to block any effects of B7.1. However, we consistently found that CTLA4 Ig induced even more killing rather than inhibiting the increased killing of these cells, a similar finding to that reported previously (28). Presumably, the CTLA4 Ig protein binds to B7.1 on the tumor cell by the CTLA4 portion of the protein and to Fc receptors on the NK cells by the Ig portion of the molecule, thus bridging the two cells together and allowing the killing of the tumor cell to occur, in a manner similar to that which occurs in ADCC. This was confirmed by the ability of an Ab to CD16 (FcRIII) to completely block the ADCC-induced killing (data not shown).
B7.1 as shown by the anti-B7.1 Ab and by the increased binding of CTLA4Ig (Fig. 4). A431 cells were “resistant” to NK lysis, and the expression of B7.1 did not induce or enhance their NK-mediated lysis (data representative of five different experiments) (Fig. 7). Similar results were obtained when the assay was performed overnight instead of 4 h (data not shown).

However, in the presence of CTLA4Ig, NK cells could efficiently lyse the A431hB7.1 cells (Fig. 7). This ADCC indicates that A431 cells are not inherently resistant to NK-mediated lysis. It is important to note that the binding of CTLA4Ig to the NK cells (via the Ig portion) is not enough for the induction of NK-mediated lysis of A431 or U937 cells. This is shown by the inability of the NK cells to lyse the parental A431 cells in the presence of CTLA4Ig (Fig. 7). Therefore, the formation of a CTLA4Ig-mediated bridge between these tumor cells and NK cells overcomes the resistance (perhaps due to MHC class I expression) of these cells to NK-mediated lysis.

The RPMI 1788 human B cell line expresses high levels of both B7.1 and B7.2 and is highly positive for MHC class I (Fig. 4). Therefore, we were interested to determine whether these high levels of B7 expression were enough to overcome negative signaling (perhaps due to MHC class I) and induce the killing of these cells (as has been shown for the Bw-Li cells in the murine system (9)). However despite the high levels of B7 on their surface, these cells were resistant to NK killing in either a 4-h or an overnight coculture experiment. Similar results were obtained using NK cells obtained from five different individuals (data not shown).

**Human B7.1 does not confer sensitivity to mouse tumor cells for human blood NK cells**

Previous studies have shown that murine P815 cells can be lysed by the YT2C2 human NK cell line following expression of human B7.1 on their surface (15). Therefore, we examined whether P815 cells expressing human B7.1 (see Fig. 4 for the expression of hB7.1 on these cells) also become targets for human blood NK cells. Neither the parental P815 cells nor P815hB7.1 cells could be lysed by human blood NK cells (data not shown). This suggests that even though B7.1 might allow the transfected P815 cells to bind more efficiently to the human NK cells, other recognition mechanisms are needed for cytotoxicity to occur. Interestingly, the CTLA4Ig fusion protein induced the killing of the P815hB7.1 cells (49.5% killing in the presence of CTLA4Hg as compared with 4.66% in its absence, i.e., 10.6-fold increase at an E:T ratio of 50:1) but not of the P815 cells, presumably in a manner similar to the ADCC-mediated lysis. These data suggest that under the appropriate conditions, NK cells can kill tumor cells from a different species, thus emphasizing the innate and “nonspecies specific” nature of NK-mediated lysis.

**Discussion**

The ability of NK cells to kill their targets is controlled by a balance between inhibitory and activating signals (1–5). In contrast to the inhibitory pathway of NK cell regulation, little is known about stimulatory or activation signals in NK cells. Data emerging from several murine studies provide evidence that CD28 can act as a positive signal and induce NK-mediated cytotoxicity of B7-positive, but not -negative, tumor cells (8, 9). These studies show that B7.1 can overcome the inhibitory effect of MHC class I on NK cells (9). In addition, in murine NK cells, CD28-mediated costimulation is necessary for optimal proliferation (11). In contrast, the only convincing study to date showing CD28 involvement in the lysis of human B7-positive cells has been with the YT2C2 NK cell line (15, 29). In the present study, we have directly addressed the ability of CD28 to trigger human NK cell-mediated cytotoxicity of B7-positive tumor cells.

In this study, analysis of CD28 protein expression on blood NK cells was examined with a panel of different anti-CD28 mAbs. NK cells were found to be positive for CD28, this being crucially dependent on the Ab used. Two of four Abs (clones YTH913.12 and L293) give a positive staining, strongly suggesting that the CD28 molecule expressed on the NK cells is different to the CD28 on T cells. As discussed above, the CD28 gene is transcribed into four RNA species; a smaller pair consisting of a 1.5-kb transcript and a 1.3-kb transcript, and a larger pair consisting of a 3.5- and a 3.7-kb transcript arising from the use of an alternate, nonconsensus polyadenylation signal located 2167 bp downstream from the first signal (19, 20). In addition, the gene has an internal splicing event using an unusual splice donor site, resulting in the deletion of 252 bp in the coding region. The cDNA library used for the initial characterization of the CD28 mRNA transcript (1.5 kb) from T cells was screened by the 9.3 mAb used in the initial characterization of the CD28 Ag (30) and used in later studies for the characterization of CD28 on blood NK cells, which were found to be negative (14). Later studies using mutants of the CD28 Ig fusion protein show that the MYPPPY motif, which is part of the deleted region, is a requirement for the epitope of the 9.3 Ab (31). Therefore, we predicted that the expressed CD28 molecule might be the splice variant, whereby the splicing event leads to the loss of epitopes recognized by the other two anti-CD28 Abs (clone 9.3 and CD28.2). However, the molecular studies performed here show that this not the case as all the different NK cells express the full-length CD28 transcript.

Alternatively, it is possible that the discrepancy in Ab staining is due to posttranslational modifications (such as glycosylation events) of the CD28 protein within the NK cells, with masking of some epitopes and not others. As already discussed in the introduction, a variant form of CD28 has already been described that is not recognized by the 9.3 Ab (21, 22). Results from the NKL line show that the presence of CD28 transcripts does not necessarily lead to CD28 protein expression as shown by lack of Ab staining by FACS. On the contrary, the YT2C2 cell line expresses both the CD28 transcripts and the CD28 protein, suggesting that perhaps the stability of the mRNA varies within different NK populations, and the mechanism for this control is not yet understood. Our data also show the presence of novel CD28 transcripts (Fig. 3), which we have cloned and sequenced, and shown to previously described splice acceptor/donor sequences. These transcripts are being analyzed in further detail.

Our data also show that the affinity of the L293 Ab is lower than that of the YTH913.12, based on the level of staining of the NK cells. During our study, we also found that different individuals varied in the level of expression of the CD28 molecule on their NK cells, with only 2 of 18 individuals giving high expression. In addition, we monitored the expression of CD28 on all the individuals over 1 year and the results remained consistent, showing that the staining pattern was not a consequence of “subclinical” infections or other causes that might regulate CD28 expression.

The data reported here also show that the binding activity of clone YTH913.12 to NK cells is removed by T cells but not by B cells. This means that the Ab is binding to a common Ag on T cells and NK cells but not B cells, and is thus unlikely to be due to a contaminant in the YTH913.12 Ab. The binding inhibition studies of the different anti-CD28 Abs to T cells strongly suggest that clones L293 and YTH913.12 bind to closely related epitopes, which is different to that recognized by CD28.2. The results were similar for NK cells, where L293 competes for the binding of YTH913.12. Molecular identity of the CD28 expressed by NK cells.
cells using immunoprecipitation and immunoblotting techniques is now in progress.

Our data also demonstrate that B7.1 expressed on MHC class I-negative NK-sensitive target cells (K562 cells) induces the enhanced activation of the NK cells and increases the sensitivity of the tumor cells to NK-mediated killing. This is seen in Fig. 5 by the expression of the activation markers CD69, CD25, and class II on NK cells, when cocultured with K562b7.1, and in Fig. 6 by the increased killing of the K562b7.1 cells compared with parental K562 cells. It is important to point out that these assays were performed with a mixed population of K562b7.1 cells after selection and not with cloned cells. Therefore, the increased NK lysis of K562b7.1 cells is not due to selection of a subtype with increased sensitivity to NK cells. Thus, these data suggest that hB7.1 can act as a putative triggering molecule in human NK cells, an observation that is similar to that reported in some murine models.

In contrast, MHC class I-positive NK-resistant target cells (A431) remain resistant to NK cell lysis despite high levels of B7.1 expression on their surface. These data are in accordance with that published recently demonstrating that squamous cell carcinomas of the head and neck (SCCHN) cell lines, which are highly class I positive, remain resistant to NK killing after B7.1 expression (18). In these cells, B7.1 is unable to overcome the negative signaling by the tumor cells, due possibly to MHC class I-mediated protection against NK-mediated lysis. Our data also suggest that if the triggering of cytotoxicity does not occur, then the NK cells fail to become activated as shown by the lack of CD69, CD25, and class II expression on NK cells after coculture with A431b7.1 cells (Fig. 5). However, the inhibition of NK cell cytotoxicity can be overcome in these NK-resistant target cells by an ADCC-like mechanism involving CD16 molecule on the NK cells, suggesting that the signaling pathways are via different mechanisms.

Despite the high levels of both B7.1 and B7.2, the RPMI 1788 B cells were resistant to blood NK cells. This is in contrast to the B7.1-positive human B cell lines JY, Arent, CCRF-SB, and LL2 (EBV-transformed B lymphoblastic cell lines), Raji and Daudi (Burkitt's lymphoma cell lines), as well as ARH77 (plasma cell leukemia cell line), which were shown to be lysed efficiently by the YT2C2 human NK line in a CD28-B7-dependent manner (15).

In conclusion, the present study shows that human NK cells express a variant of CD28 that allows the activation of NK cells by B7.1-positive NK-sensitive tumor cells. It is also possible that human and mouse NK cells may have fewer differences than previously thought.

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