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Final report

Single chain Fv constructs of anti-ganglioside GD2 antibodies for radioimaging and radioimmunotherapy

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Abstract:

T-lymphocytes are ideal targeting vehicles because (1) they are naturally equipped with trafficking capabilities, (2) they can undergo clonal expansion when they come in contact with antigen if given the appropriate mitogenic signals, (3) they release cytokines which recruit other inflammatory/immune cells, (4) they can initiate other arms of immunity at the tumor site and (5) they are capable of being engineered with powerful cytotoxins or enzymes. Both clonal expansion and recruitment of T-cells can greatly magnify targeted delivery, improving the therapeutic index of the intended diagnostic or treatment modality. Although lymphokine activated killer cells (LAK) and tumor infiltrating lymphocytes (TIL) have been tested in tumor targeting, the clonal frequency of tumor-specific lymphocytes is generally very low and <0.016% of administered lymphocytes arrive at the tumor sites, accounting for the limited efficacy and generalized toxicity of LAK/TIL adoptive immunotherapy. Until the specific delivery of these cells is optimized, the potential of cell targeting cannot be explored. Quantitative analysis and trace-labeling of human lymphocytes for homing studies to human tumors in vivo have been limited by the unavailability of cell-labeling techniques and efficiency of gene transduction into T-cells. Issues unique to cellular targeting include homing properties of lymphocyte subpopulations (CD4+ vs CD8+ T-cells vs NK cells), clonal expansion of antigen-specific cells, and the cytotoxic potential of gene-modified T-cells.

T-lymphocytes die of apoptosis when their TCR is engaged, unless costimulatory molecules on their cell surface are activated. To enhance the survival and proliferation of T lymphocytes reacting against tumor cells that lack costimulatory molecules, we previously reported the successful transduction of G_{D2}-specific-scFv-CD28 chimeric immune receptor (CIR) into primary human peripheral blood CD8+ T-cells, which became selectively expanded when cultured with cells expressing both the MHC class I and G_{D2}. Thus, the transduced CIR carries out a functional costimulatory response that enhances their survival and selective expansion in the absence of natural costimulatory molecules. During the last funding period we have developed a novel affinity chromatography technique to rapidly clone efficient retroviral packaging cell lines. Using the pMSCVneo vector to carry the CIR and the packaging line GP+envAM12, we can now transduce scFv-CD28-zeta-chain efficiently into primary human T-cells. The bulk culture achieves CIR monoclonality by 20 days of in vitro culture, achieving a 40-fold expansion in cell number within 2 months. The transduced T-cells kill tumors in vitro in an antigen specific manner and suppressed tumor growth when injected iv into SCID mice bearing human tumor xenografts. We have achieved CIR gene transduction in two separate antigen systems, one for GD2 (5F11 scFv) and one for the antigen p58 (8H9scFv). The novel antigen p58 was chosen because of its broad and usually homogeneous distribution in human solid tumors, and most importantly, their absence on cell membranes of normal human tissues. In separate experiments, we have shown that T-cells transduced with the herpes simplex viral thymidine kinase (HSV-tk) gene can be radiolabeled with ^{131}I-FIAU to a safe nuclear radiation dose. Using a dicistronic construct we are inserting chimeric immune receptor plus HSV-tk into T-cells to allow such their trafficking to be radioactively monitored. We plan to study the role of cytokines, chemoreceptors and CD4 helper T-cells in recruiting CD8+ transduced T-cells to the tumor site. These studies should provide us with an adoptive cell therapy approach to target cytotoxicity to human tumors, and a lymphocyte tracking tool to study delivery to the tumor sites, to determine if they proliferate locally and/or recirculate. Such pharmacologic information is crucial for optimizing gene-modified T-cells in future clinical trials.

Progress Report

1. Single chain v-Fragment (scFv) technology (Table 2)[80] 3G6, 3F8 and 5F11 are all anti-GD2 murine monoclonal antibodies developed in our laboratory. 3G6 is a class switch variant of 3F8. 8H9 is a murine IgG1 antibody specific for the antigen gp58. The heavy and light chain cDNA of 3G6, 5F11, and 8H9 were joined by the linker $(GGGGS)_3$ into scFv in a VH-VL orientation for 5F11 and 8H9, and VL-VH orientation for 3G6. Rat anti-idiotypic monoclonal antibodies were: A1G4, idio2, C4E4, A2A6, C2H7 (for 3F8 and 3G6), 2E9 (for 8H9), and 1G9 (for 5F11).[81]

Table 2: Parallel developments for anti-GD2 and anti-gp58 MoAb

MoAb	Antigen	Rat anti-idiotype	ScFv	ScFv-CD28	ScFv-CD28-zeta chain
3G6, 3F8	GD2	A1G4, C4E4, idio2, C2H7, A2A6	yes*	yes	-
5F11	GD2	1G8, 1C1	yes*	yes	yes
8H9	p58	2E9, 1E12, 1F11	yes	-	yes

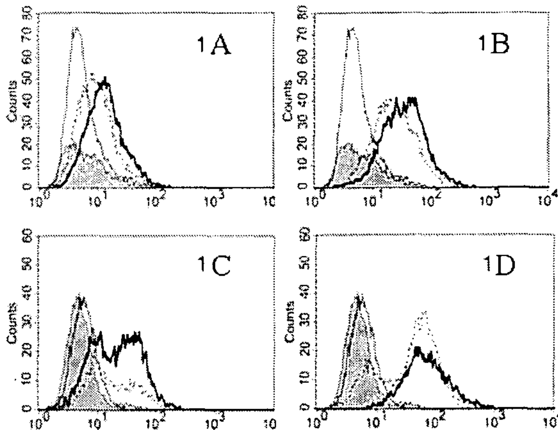
2. Retroviral mediated gene transfer of chimeric immune receptor into primary human T-cells

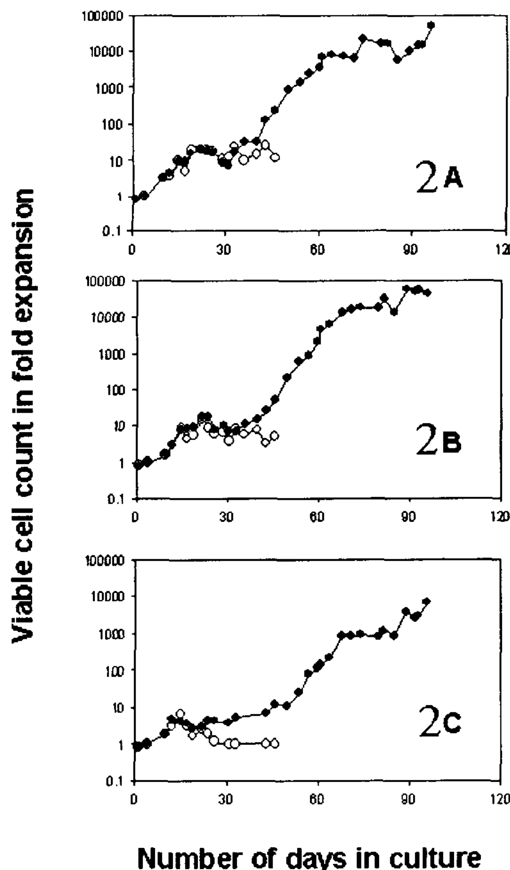
3G6-scFv-CD28 A CIR consisting of 3G6-scFv and the signal transduction domain of CD28[58] was constructed and high-titer producer cells were generated from the PG13 packaging cell line, with a titer of 7×10^5 infectious particles per ml. PBMC were activated with phytohemagglutinin A and exposed to virus for 18 hours in the presence of polybrene (4 ug/ml). Three days after infection 28% to 40% of the CD3+ PBMC cells stained positively for the vector-encoded receptor in both CD4+ and CD8+ subsets. By FACS the transduced cells reacted with all 5 of 5 anti-idiotypic antibodies (i.e. Idio-2, C2H7, A2A6, C4E4, A1G4).[81] By western blots, the scFv-CD28 chimeric protein (36 kDa in NIH 3T3 fibroblasts and ~40 kDa in Jurkat cells) was expressed as a dimer. The engagement of the 3G6-CD28 on Jurkat cells by the anti-idiotypic antibody A1G4 triggered the association 85 kDa regulatory subunit of PI3-kinase with the phosphorylated CD28. IL-2 secretion by transfectants stimulated with immobilized anti-CD3 MoAb required costimulation by either anti-CD28 MoAb 9.3, or A1G4, or short term culture with tumor cells expressing GD2. In contrast, in transfectants with the truncated 3G6-CD28 where CD28 signaling is disabled, or irrelevant transgene such as NTP ($NGFR^{la}$, low affinity nerve growth factor receptor) A1G4 had no effect. The costimulatory effect of GD2-positive EL4 was completely abolished by the addition of GD2-specific F(ab')2. Thus, 3G6-CD28 specifically recognizes cell-surface GD2, leading to CD28-dependent signaling, including IL-2 secretion. 3G6-CD28-transduced T lymphocytes selectively survived CD3-dependent cell death in the presence of anti-idiotype A1G4. In addition, A1G4 MoAb induced preferential expansion of 3G6-CD28-transduced primary T lymphocytes. Corecognition of MHC-peptide complexes and GD2 selectively activated PBMC expressing 3G6-CD28. CD8+ T cells transduced with 3G6-CD28 remained a constant fraction of all T cells (10±2%) when cocultured either with 3T3 cells alone, 3T3-A2.1, 3T3-A2.1/B7, or 3T3-GD2, but steadily increased to 32±4% by day 12 if exposed to 3T3-A2.1/GD2. In control cultures, cells expressing 3G6-CD28TR or NTP remained unchanged. Thus, the increase in the fraction of 3G6-CD28-positive CD8+ T cells required both HLA A2.1 and GD2, which, on the other hand, had no effect on the transduced CD4+ subset. The same result was achieved in cultures of transduced CD8+ T cells in the absence of CD4+ cells. These data established that the engagement of 3G6CD28 with cell-surface GD2 antigen provides a powerful costimulatory signal to T cells specific for GD2-positive target cells.

sc8H9-CD28-hTCR-ζ and sc5F11-CD28-hTCR-ζ The *hCD8a leader-scFv-CD28* were ligated to *hTCR-ζ-chain* to produce the gene hCD8-leader-8H9scFv-$hCD28_{TM}$-$hCD28_{cyto}$-TCRζ and hCD8-leader-5F11scFv-$hCD28_{TM}$-$hCD28_{cyto}$-TCRζ. These sequences were verified and ligated into the

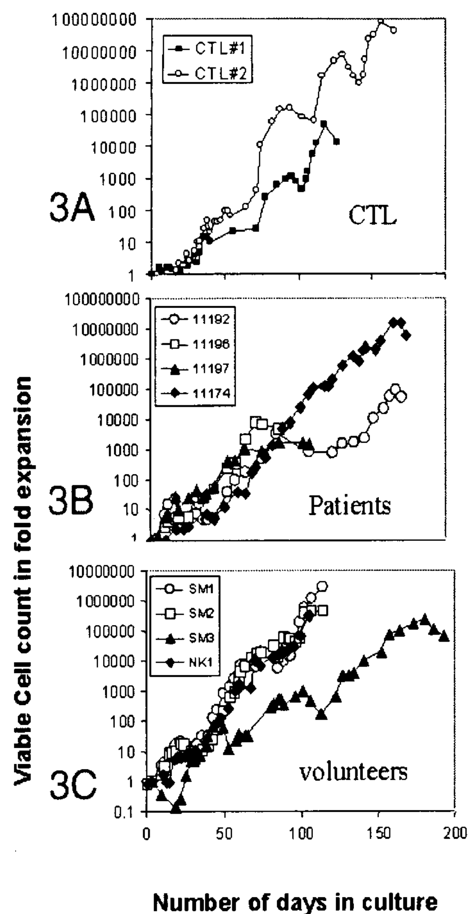
pMSCVneo vector, and used to transfect the packaging line GP+envAM12 bearing an amphotropic envelope and selected in G418.

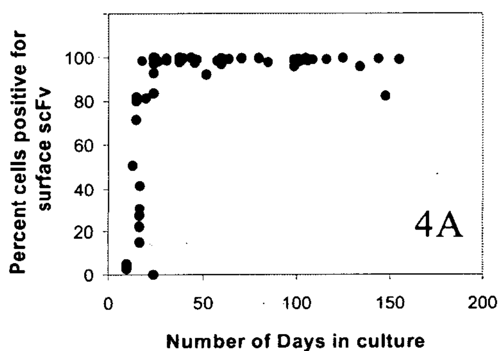
3. Use of anti-idiotype technology for rapid enrichment and cloning of producer lines by affinity column or cell sorting Anti-linker antibody may be useful in monitoring scFv gene expression. However, its efficiency depends on the accessibility of the scFv-linker portion. Although purified antigens can also be used to monitor scFv expression, certain classes (complex carbohydrates or unstable antigens) can be difficult to prepare and their chemistry highly variable. Without a standardized reagent for affinity purification or enrichment of virus producer cells, as well as monitoring and sorting of transduced lymphocytes, CIR technology remains inefficient. A dicistronic construct consisting of scFv-CD28-γ and green fluorescent protein (GFP) exploited the latter to monitor gene transduction and to enrich producer lines.[47] Although GFP can validate the gene transfer process, its added immunogenicity and its safety in clinical applications remain uncertain. Anti-idiotypic antibodies are frequently used as antigen-mimics for infectious diseases and cancer.[82,83] Internal image rat anti-idiotypic antibodies can be conveniently produced against mouse MoAb. Since large scale production of clinical grade MoAb is now routine, anti-idiotypic antibodies may be ideal surrogates especially if the antigen is not readily available. In addition, the biochemistry of immunoglobulins in positive selection (panning, affinity chromatography, sorting) and binding assays is well-known and is easy to standardize. Here we demonstrate that an anti-idiotypic MoAb against 8H9 can be used as a surrogate antigen for cloning CIR into primary human lymphocytes, i.e. a CIR of 8H9scFv, human CD28 and human TCR-ζ chain. While previous studies showed that anti-idiotypic antibody can enhance cytotoxicity of scFv-γR-gene modified murine cytotoxic T-cell line,[84] we now demonstrate that anti-idiotypic MoAb, besides allowing rapid affinity enrichment of producer cell line and monitoring of surface scFv expression, induces clonal expansion of CIR-modified primary human lymphocytes. Highly cytotoxic lymphocytes can be propagated in vitro undergoing 10^6 fold expansion over a period of 6 months.

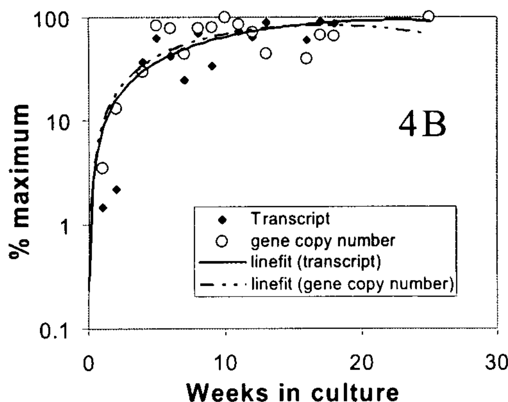




The retroviral producer lines were affinity-enriched using MACS goat anti-rat IgG MicroBeads on the MiniMACS system (Miltenyi Biotec, Auburn, CA). Following each enrichment, viral supernatant from the producer line was used to infect the indicator cell line K562. Surface 8H9scFv expression on both the producer lines and the transfected K562 (4 days after infection) were measured by immunofluorescence using anti-idiotypic antibody 2E9. With each successive affinity enrichment **(Figure 1A and 1C)** of producer line and subsequent successive subcloning (**Figure 1B and 1D**), the surface expression (mean fluorescence) of 8H9-scFv increased and became more homogeneous for the producer clones (Figure 1A and 1B) as well as indicator line K562 (Figure







1C and 1D).Producer lines were stained with anti-idiotypic MoAb 2E9 before (shaded peak, A and B), and after first (dotted line peak, A) and second (thick solid line, A) affinity purification, and after first (dotted line, B) and second (solid line B) subcloning, showing improved scFv expression. **Table 3** summarized the length of time (in weeks) required to clone high efficiency producer cell line.

4. Long term propagation of CIR-transduced primary human lymphocytes. Following in vitro activation with anti-CD3 and anti-CD28, primary human PBMC were infected with the virus from producer line supernatant by centrifugation at 1000 xg for 60 minutes at room temperature. Using PBMC from normal volunteers, the in vitro requirement of IL2 and anti-idiotypic antibody for lymphocyte expansion was studied. On day 10 after gene transduction, 17-40% of cells became scFv-positive by FACS analysis. By day 15, 75-80% became positive and by day 24, 99% of the cells became positive. This clonal evolution to homogeneity was found in CD4+, CD8+ and the small CD56+ populations. IL-2 concentration of 50 to 100 U/mL appeared optimum, and anti-idiotypic MoAb 2E9 was absolutely necessary to maintain prolonged T-cell growth (**Figure 2**). Clonal expansion was expressed as fold expansion of initial viable lymphocyte number. IL-2 (100 U/ml fig 2A, 50 U/ml fig 2B and 20 U/ml, fig 2C) was added after retroviral infection and was present throughout the entire in vitro culture period, in the presence (solid circles) or absence (open circles) of solid-phase anti-idiotypic antibody. Viable cell count was performed using trypan blue assay. These experiments were repeated twice with similar results.

In the presence of 100 U/ml of IL2 and solid-phase anti-idiotypic antibodies, PBMC from 4 patients with stage 4 neuroblastoma off chemotherapy (**Figure 3B**), two CTL samples[85] (Figure 3A) and 4 fresh blood specimens from two normal volunteers (Figure 3C), were expanded in vitro following CIR-gene transduction. Continual expansion (10^3 to 10^8 fold) was achieved after 150-200 days of culture, with a doubling time ranging from 5 to 10 days. 8H9scFv average gene copy number, transcript level, and surface expression were studied in these samples (**Figure 4**). The scFv-positive population enriched quickly during the first 20 days of culture in the absence of 2E9 (Figure 4A). As expected, the gene copy number and transcript level also plateaued with similar kinetics (Figure 4B). When the scFv-positive population became >95%, an average of 4.5 gene copies per cell

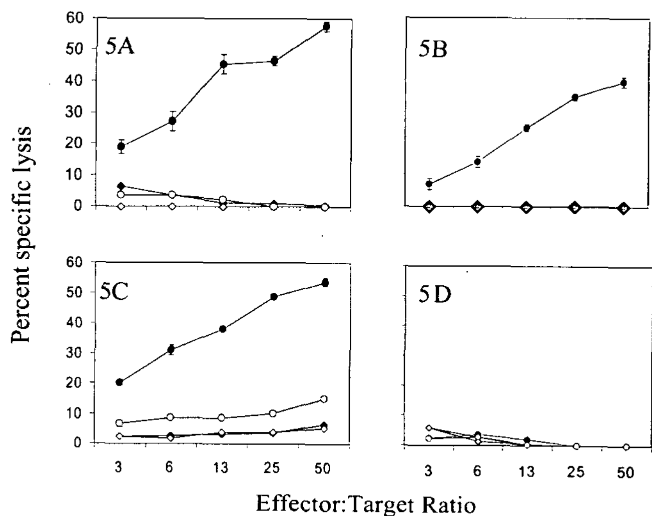
(range 2-9) was detected, which remained relatively stable throughout the extensive length of in vitro culture. ScFv expression was typically >95% throughout 6 months of culture (Figure 4A). The proportion of CD8+ cells versus CD4+ cells increased steadily to >50% by day 40 of culture, and decreased slowly over 3-4 months. At concentrations of IL-2 <50 U/mL, CD4+ cells outgrew the CD8+ population even faster (data not shown).

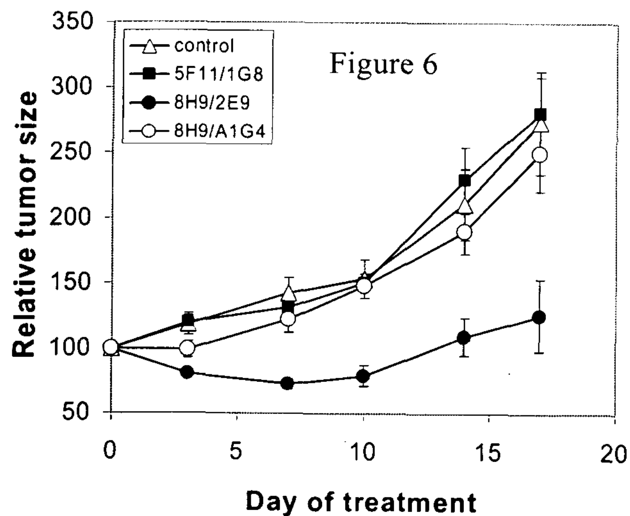
Transduced lymphocytes mediated non MHC-restricted antigen-specific cyto-toxicity in vitro against neuroblastoma and rhabdomyosarcoma cell lines In vitro cytotoxicity against NMB-7 (**Figure** 5A) and LAN-1 (Figure 5B) neuro-blastoma, or rhabdomyosarcoma HTB-82 (Figure 5C) were efficient. Antigen-dependence was demonstrated by the inhibition of cytotoxicities by MoAb 8H9. Daudi cell line (Figure 5D) was not killed because it was antigen-negative. This cytotoxicity was independent of target HLA expression or HLA types (data not shown). Unmodified lymphocytes from the same donor, cultured under the same conditions (100 U/ml of IL2), did not show antigen-specific killing (Figures 5). Control (5F11scFv) CIR modified lymphocytes also did not show antigen-specific killing of HTB82 (data not shown). In Elispot assays, IFN-γ secretion was detected when transduced lymphocytes were stimulated with antigen-positive tumors (NMB7 and HTB82) but not antigen-negative controls (Daudi, data not shown). Tumor cytotoxicity was demonstrated in CIR-gene modified T-lymphocytes whether they were derived from volunteers or from patients. This observation is important since T cells in patients and animals with growing tumors can have be defective in signal transduction.[86] Whether these cells will consistently demonstrate anti-tumor effect in vivo, especially in the tumor environment, will require further studies.

Table 3: Enrichment of producer line	Time (wks)	% scFv+ day 4
No affinity purification		
PG13/8H9scFv-CD28-MSCV		
No subcloning	0	7
1 subcloning	4	7
2 subclonings	8	15
3 subclonings	12	21
4 subclonings	16	25
With affinity purification		
PG13/8H9scFv-CD28-MSCV		
1 affinity purification	1	10
2 affinity purifications	2	18
3 affinity purifications	3	29
4 affinity purifications	4	33
+ 1 subcloning	8	42
+ 2 subclonings	12	54
GP+envAm12/8H9scFv-CD28-ζ		
1 affinity purification	1	15
2 affinity purifications	2	30
+ 1 subcloning	6	36
+ 2 subclonings	10	57
Cell sorting	2	69
+ 1 subcloning	6	74
GP+envAm12/5F11scFv-CD28-ζ		
1 affinity purification	1	18
2 affinity purifications	2	25
+ 1 subcloning	6	37
+ 2 subclonings	10	50
Cell sorting	2	55
+ 1 subcloning	6	63

5. Targeting CIR-modified T cells to xenografts in SCID mice: developing an adoptive cell therapy model. 8H9scFv-CIR gene-modified lymphocytes suppressed HTB82 tumor growth, when mixed at 1:1 ratio at the time of tumor implantation (data not shown). The in vivo anti-tumor effect of 8H9scFv-CIR gene-modified lymphocytes was then tested in an established tumor model. Human rhabdomyosarcoma was strongly reactive with 8H9, but not with 5F11 (anti-G_{D2}) antibodies. 5F11scFv-CIR gene-modified and IL-2 cultured lymphocytes were used as controls cells in adoptive therapy. 5F11scFv-CIR contained the same CD28-TCRζ construct used for 8H9scFv-CIR. Experiments were initiated when tumors grew to around 0.8 cm diameter. Control groups (**Figure 6**) were injected with either (1) no T cells but anti-idiotype 2E9 ip [Δ], (2) 5F11scFv-CIR modified lymphocytes

intravenously plus anti-idiotype 1G8 (specific for 5F11 idiotype) ip [■] or (3) 8H9scFv-CIR modified lymphocytes i.v. plus A1G4 (irrelevant anti-idiotypic antibody) ip [●]. Suppression of tumor growth was most significant with lymphocytes transduced with the 8H9scFv-CIR gene [O], $p<0.05$), and only if the specific anti-idiotype 2E9 was administered. 5F11scFv-CIR modified lymphocytes or 8H9scFv-CIR plus A1G4 did not show significant anti-tumor effect when compared to control. This in vivo effect of gene-modified lymphocytes was demonstrated in 3 separate experiments.





Publications and manuscripts

1. Krause A, Guo HF, Tan C, et al: Antigen-dependent CD-28 signaling enhances survival and proliferation in genetically modified activated human primary T lymphocytes. J. Exp. Med. 188:619-626, 1998

2. Modak S, Kramer K, Humayun G, et al: Monoclonal antibody 8H9 targets a novel cell surface antigen expressed by a wide spectrum of human solid tumors. Cancer Research 61:4048-4054, 2001

3. Cheung NKV, Guo HF, Modak S, Cheung IY. Anti-idiotypic antibody as the surrogate antigen for cloning scFv and its fusion proteins. Hybridoma and hybridomics 21:433-443, 2002.

4. Cheung NK, Guo HF, Modak S, Cheung IY. Anti-idiotypic antibody facilitates scFv chimeric immune receptor gene transduction and clonal expansion of human lymphocytes for tumor therapy. 2002 (submitted)

5. Cheung NKV, Guo HF, Modak S, Larson SM. Single chain Fv-streptavidin substantially improved therapeutic index in multi-step targeting directed at disialoganglioside GD2. 2002 (in preparation)

Patent applications and PCTs

1. Single chain Fv constructs of anti-ganglioside GD2 antibodies. Nai-Kong V. Cheung, Steven M. Larson, Hong-fen Guo, Kenneth Rivlin, Michel Sadelain (Memorial Sloan-Kettering, SK714, SN 09/142,974, patent filed in 9/18/1998, approved 9/17/2002, patent #6,451,995, Inventor # 2301144)

2. Uses for monoclonal antibody 8H9. Nai-Kong V. Cheung (Memorial Sloan-Kettering, MSK 948, Dkt#638A: SN: 09/982,645 (10/18/2001), PCT Int'l App'l No. PCT/US01/32565 (10/18/2001), Dkt#638B: SN 10/097558 (3/8/2002)

3. Method for preparation of single chain antibodies. Nai-Kong V. Cheung (Memorial Sloan-Kettering Cancer Center, MSK 948) Dkt#676, Int'l . App'l. No. PCT/US02/ 33331 (10/17/2002), US SN 10/273762 (10/17/2002)

4. Fusion proteins of a single-chain antibody and CD28 and uses thereof. Michel Sadelain, Nai-Kong V. Cheung, Anja Krause, Hong Feng Guo. SN 08/940,544 (filed 9/30/1997)