Activation of Resting Human Primary T Cells with Chimeric Receptors: Costimulation from CD28, Inducible Costimulator, CD134, and CD137 in Series with Signals from the TCRζ Chain

Helene M. Finney,[1]* Arne N. Akbar,[†] and Alastair D. G. Lawson*

Chimeric receptors that include CD28 signaling in series with TCRζ in the same receptor have been demonstrated to activate prestimulated human primary T cells more efficiently than a receptor providing TCRζ signaling alone. We examined whether this type of receptor can also activate resting human primary T cells, and whether molecules other than CD28 could be included in a single chimeric receptor in series with TCRζ to mediate the activation of resting human primary T cells. Human CD33-specific chimeric receptors were generated with CD28, inducible costimulator, CD134, or CD137 signaling regions in series with TCRζ signaling region and transfected by electroporation into resting human primary T cells. Their ability to mediate Ag-specific activation was analyzed in comparison with a receptor providing TCRζ signaling alone. Inclusion of any of the costimulatory signaling regions in series with TCRζ enhanced the level of specific Ag-induced IL-2, IFN-γ, TNF-α, and GM-CSF cytokine production and enabled resting primary T cells to survive and proliferate in response to Ag in the absence of any exogenous factors. Inclusion of CD28, inducible costimulator, or CD134 enhanced TCRζ-mediated, Ag-specific target cell lysis. Chimeric receptors providing B7 and TNFR family costimulatory signals in series with TCRζ in the same receptor can confer self-sufficient clonal expansion and enhanced effector function to resting human T cells. This type of chimeric receptor may now be used to discover the most potent combination of costimulatory signals that will improve current immunotherapeutic strategies. ***The Journal of Immunology,*** **2004, 172: 104–113.**

The chimeric receptor or T-body (1, 2) immunotherapy strategy involves engineering a T cell to express a chimeric receptor consisting of an Ab-binding region linked via a transmembrane region to an intracellular signaling domain involved in cellular activation. This approach provides MHC-unrestricted recognition of target cells by T cells. Removal of the MHC restriction of T cells facilitates the use of these molecules in any patient, and also, in both $CD8^+$ and $CD4^+$ T cells, usually restricted to MHC class I or II epitopes, respectively. The use of Ab-binding regions allows T cells to respond to epitopes formed not only by protein, but also carbohydrate and lipid. This chimeric receptor approach is especially suited to immunotherapy of cancer, being able to bypass many of the mechanisms by which tumors avoid immunorecognition, such as MHC down-regulation, lack of expression of costimulatory molecules, CTL resistance, and induction of T cell suppression (3), and where the use of both $CD8^+$ CTL and $CD4^+$ T cells are best combined for optimum antitumor efficacy (4). This approach has been demonstrated to be applicable to a wide range of tumor Ags (reviewed in Ref. 3), in addition to viruses such as HIV (5, 6).

Most chimeric receptors have been designed to deliver only a primary activation signal by the employment of the signaling region from either the ζ-chain of the TCR/CD3 complex (TCRζ), or the γ-chain from the FcεRI receptor (FcRγ). Provision of such signaling through this type of receptor, although capable of anti-tumor activity in various murine models, has been shown to be insufficient to induce proliferation in primary T lymphocytes (7, 8) and has led to only limited clinical success (9, 10). In accordance with the two-step hypothesis of T cell activation (11), the observation that stimulation through the TCR alone may lead to anergy or death rather than activation (12, 13), and the importance of costimulation in tumor immunotherapy (14), recent effort has focused on the introduction of costimulatory signaling to this type of receptor.

The CD28 signaling domain was first introduced as an additional construct in parallel (15, 16). We demonstrated that the CD28 signaling region could be provided in the same construct in series with the TCRζ chain to enhance the activation of a human T cell line (17). Provision of CD28 costimulatory signaling in such constructs has subsequently been shown to enhance the activation of primary T cells in vitro (18–22) and enhance tumor cell killing in vivo (23, 24).

To date, this approach has been limited to the inclusion of CD28, the best characterized costimulatory molecule (reviewed in Refs. 25–27). Signaling via CD28 is required for optimum IL-2 production, cell cycle progression, and survival (13, 28–31). CD28 costimulation is essential in naive T cells, but thought to be of less importance in memory and effector T cell responses (32–34). Additional costimulatory molecules may play a more significant role in different T cell subsets and at different stages of T cell activation (35–37).

Molecules such as inducible costimulator (ICOS)[2] (38), CD134 (39), and CD137 (40), expressed following the induction of CD28 signaling, are thought to be important for prolonging the response and the generation of T cell memory. ICOS is a B7 receptor family member, structurally similar to CD28, expressed on activated T

*Celltech R&D, Slough, United Kingdom; and [†]Windeyer Institute of Medical Sciences, London, United Kingdom

Received for publication May 22, 2003. Accepted for publication October 22, 2003.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] Address correspondence and reprint requests to Dr. Helene M. Finney, Celltech R&D, 208 Bath Road, Slough, Berkshire, SL1 3WE, United Kingdom. E-mail address: hfinney@celltech.co.uk

[2] Abbreviations used in this paper: ICOS, inducible costimulator; PI3, phosphatidylinositol 3; TRAF, TNFR-associated factor.

0022-1767/04/$02.00

cells (reviewed by Ref. 41). Costimulation by ICOS augments cytokine production and proliferation (38, 42–45). Costimulation via ICOS has been shown to play an important role in humoral immunity (46–49). CD134 (OX-40) and CD137 (4-1BB) are members of the TNFR family, are structurally distinct from CD28 and ICOS, and expressed on activated T cells. Costimulation via CD134 has been shown to enhance proliferation and cytokine production (50–53), enhance tumor immunity (54), and enhance memory T cell development (55, 56). CD134 promotes Bcl-x_L and Bcl-2 expression and is thought to be essential for the long-term survival of $CD4^+$ T cells (53). Activation of CD137 has been demonstrated to increase TCR-induced proliferation, survival, and cytokine production by both $CD4^+$ and $CD8^+$ T cells and to enhance CTL generation and rejection of poorly immunogenic tumors (57–62).

The majority of chimeric receptor studies have used retroviral vectors as the method of primary T cell transduction. As retrovirus only infects dividing cells (63), these studies have required the preactivation of the T cells. To avoid the necessity of such nonspecific activation, and to facilitate the analysis of the full potential of the coprovision of costimulatory signaling, we used electroporation, using the recently developed Nucleofector device, to transduce freshly isolated resting human primary T cells.

In this investigation, we studied whether a TCRζ/CD28 fusion receptor, known to be capable of efficiently activating prestimulated human primary T cells, was capable of activating resting human primary T cells. Additionally, we examined whether costimulatory molecules other than CD28 could be provided in series with TCRζ in a single receptor, and whether such receptors were able to activate resting human primary T cells. Chimeric receptors were generated with CD28, ICOS, CD134, or CD137 signaling regions membrane proximal to and in series with TCRζ signaling region. These chimeric receptors were transfected into resting human primary T cells, and their ability to mediate Ag-specific cytokine production, Ag-specific target cell lysis, and induction of survival and proliferation was analyzed in comparison with a receptor providing TCRζ signaling alone.

Materials and Methods

Abs and reagents

Soluble CD33 extracellular region was purified by affinity chromatography from supernatant from an NS0 cell line, as described previously (17). HRP-conjugated anti-human IgG rabbit polyclonal Ab was purchased from DAKO (Hambourg, Germany). HRP-conjugated anti-phosphotyrosine mAb and anti-human p85 phosphatidylinositol 3 (PI3)-kinase rabbit polyclonal Ab were purchased from Upstate Biotechnology (Lake Placid, NY). HRP-conjugated anti-rabbit IgG goat polyclonal Ab was purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). PE-conjugated hamster anti-Bcl-2 Ab was purchased from BD PharMingen (San Diego, CA).

Construction of chimeric receptor genes

Each component of the chimeric receptor constructs was either PCR cloned and then cut with restriction endonucleases or generated by annealing two oligonucleotides such that single-stranded restriction endonuclease compatible ends were generated. Each component was subcloned in a cassette format into pBluescript SK^+ (Stratagene, La Jolla, CA) to generate full-length constructs. The cassette was designed to facilitate the exchange of each component of chimeric receptor genes, and is illustrated in Fig. 1. The binding region was subcloned as a *Not*I to *Spe*I fragment, the extracellular spacer as a *Spe*I to *Nar*I fragment, and the transmembrane region as a *Nar*I to *Mlu*I fragment. Signaling region components were subcloned as a *Bcl*I to *Bam*HI fragment into a *Bam*HI site such that insertion in the correct orientation destroys the 5′, but retains the 3′ *Bam*HI site. This allows subsequent insertion of a second signaling region.

Single chain Fv cassette. Leader sequence and V_L from the engineered human P67 Ab were PCR cloned with 5′ oligonucleotide A5267 (introducing a *Not*I and *Hind*III site) and 3′ oligonucleotide F22785 (introducing a *Bgl*II site). V_H was PCR cloned with 5′ oligonucleotide F22786 (introducing a *Bgl*II site) and 3′ oligonucleotide F22785 (introducing a *Spe*I site). These two fragments were coligated into the cloning cassette, and then a linker fragment comprising $(Gly^4Ser)^4$ amino acid sequences was cloned in the *Bgl*II site between them. This spacer was generated by annealing oligonucleotides F22966 and F22967.

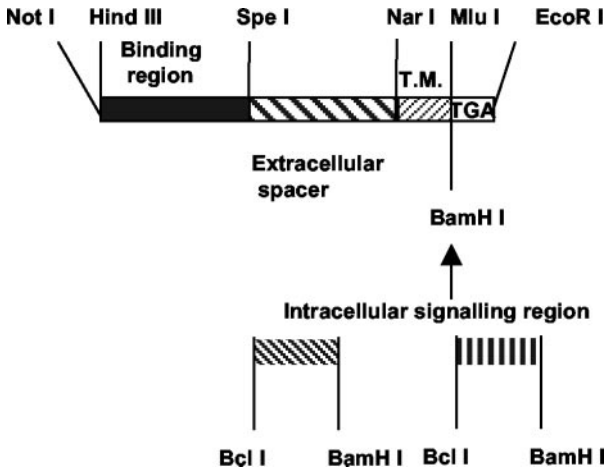

FIGURE 1. Diagram of the chimeric receptor cloning cassette.

G1 spacer and TCRζ transmembrane cassette. Human IgG1 hinge, CH2 and CH3, and TCRζ, transmembrane region, were PCR cloned with 5′ oligonucleotide F24675 (introducing a *Spe*I site) and 3′ oligonucleotide F24676 (introducing a *Nar*I site 3′ to the spacer, the TCRζ transmembrane sequence, and a 3′ *Mlu* I site).

CD28 transmembrane cassette. Human CD28 transmembrane region was generated by annealing oligonucleotides F2801 and F2802, which when annealed generate a 3′ *Nar*I overhang and a 5′ *Mlu*I overhang.

TCRζ cassette. Human ζ intracellular region was PCR cloned with 5′ oligonucleotide F34729 (introducing a *Bcl*I site) and 3′ oligonucleotide F34730 (introducing a *Bam*HI site).

CD28 cassette. Human CD28 intracellular region was generated by annealing oligonucleotides B0735 and B0736, which when annealed generate a 5′ *Bcl*I overhang and a 3′ *Bam*HI overhang, both compatible with a *Bam*HI site.

ICOS cassette. Human ICOS intracellular region was generated by annealing oligonucleotides F34731 and F34732, which when annealed generate a 5′ *Bcl*I overhang and a 3′ *Bam*HI overhang, both compatible with a *Bam*HI site.

CD134 cassette. Human CD134 intracellular region was generated by annealing oligonucleotides F18605 and F18606, which when annealed generate a 5′ *Bcl*I overhang and a 3′ *Bam*HI overhang, both compatible with a *Bam*HI site.

CD137 cassette. Human CD137 intracellular region was generated by annealing oligonucleotides F25568 and F25569, which when annealed generate a 5′ *Bcl*I overhang and a 3′ *Bam*HI overhang, both compatible with a *Bam*HI site.

All of the above cassettes were completely sequenced (Applied Biosystems *Taq* DyeDeoxy Terminator Cycle sequencing, 373A XL, Foster City, CA) in pBluescript SK^+ before subcloning on a *Hind*III to *Eco*RI fragment into an expression vector derived from pQBI-AdCMV5 (QBIOgene, Carlsbad, CA).

All oligonucleotides were supplied by Oswell (Southampton, U.K.) and are listed in the 5′ to 3′ orientation in Fig. 2.

Using standard molecular biology techniques, the cassettes described above were assembled to generate chimeric receptors with the specificity of the engineered human Ab P67, directed against human CD33. These chimeric receptors are illustrated in Fig. 3.

1) P67/G1/ζ chimeric receptor consists of a single chain Fv linked to an extracellular spacer comprising human IgG1 hinge, CH2 and CH3, linked to the transmembrane and intracellular regions of human TCRζ. The single chain Fv consists of the leader sequence and V region of the L chain of the Ab linked a $(Gly^4Ser)^4$ linker to The V region of the H chain of the Ab. The extracellular spacer consists of residues 234–478 of human IgG1 (64). This is linked to residues 10–30 of human TCRζ transmembrane region and residues 31–142 of human TCRζ intracellular region (65, 66).

2) P67/G1/CD28.ζ fusion chimeric receptor consists of a single chain Fv

A5267:ATATAGCGGCCGCAAGCTTCCACCATGTCTGT
CCCCACCCAAGTCCTC
F22785:CCCAGATCTTTTTACTTCTACTTTAGTACCCTG
F22786:GGGAGATCTGAGGTGCAGCTGGTGCAGTCTG
GAGCAGAG
F22787:TTTGTCACTAGTAGAAGACACTGTCACCAGTG
TTCCCTGTCCCCAGTAAGCCAGCCAAGGATTTCCATT
CACACAGTAGTAGAATGCTGTGTCCTCAGACCTCAG
AGAAG
F22966:GATCTGGTGGCGGAGGGTCAGGAGGCGGAGG
CAGCGGAGGCGGTGGCTCGGGAGGCGGAGGCTCGA
F22967:GATCTCGAGCCTCCGCCTCCCGAGCCACCGC
CTCCGCTGCCTCCGCCTCCTGACCCTCCGCCACCA
GGCAGTGAGAATG
F2801:CGCCTTTTGGGTGCTGGTGGTGGTTGGTGGAGT
CCTGGCTTGCTATAGCTTGCTAGTAACAGTGGCCTTT
ATTATTTTCTGGGTGA
F2802:CGCGTCACCCAGAAAATAATAAAGGCCACTGT
TACTAGCAAGCTATAGCAAGCCAGGACTCCACCAAC
CACCACCAGCACCCAAAAGG
F34729:CCCTGATCAAGAGTGAAGTTCAGCAGGAGCG
CAG
F34730:CCCGGATCCGCGAGGGGGCAGGGCCTGCATG
TG
B0735:GATCAAGGAGTAAGAGGAGCAGGCTCCTGCA
CAGTGACTACATGAACATGACTCCCCGCCGCCCCGG
GCCCACCCGCAAGCATTACCAGCCCTATGCCCCACC
ACGCGACTTCGCAGCCTATCGCTCCG
B0736:GATCCGGAGCGATAGGCTGCGAAGTCGCGTGG
TGGGGCATAGGGCTGGTAATGCTTGCGGGTGGGCCC
GGGGCGGCGGGGAGTCATGTTCATGTAGTCACTGTG
CAGGAGCCTGCTCCTCTTACTCCTT
F34731:GATCAAAAAAGAAGTATTCATCCAGTGTGCA
CGACCCTAACGGTGAATACATGTTCATGAGAGCAGT
GAACACAGCCAAAAAATCTAGACTCACAGATGTGAC
CCTAG
F34732:GATCCTAGGGTCACATCTGTGAGTCTAGATTT
TTTGGCTGTGTTCACTGCTCTCATGAACATGTATTCA
CCGTTAGGGTCGTGCACACTGGATGAATACTTCTTTT
TT
F18605:GATCACGGAGGGACCAGAGGCTGCCCCCCGA
TGCCCACAAGCCCCCTGGGGGAGGCAGTTTCCGGAC
CCCCATCCAAGAGGAGCAGGCCGACGCCCACTCCAC
CCTGGCCAAGATCG
F18606:GATCCGATCTTGGCCAGGGTGGAGTGGGCGT
CGGCCTGCTCCTCTTGGATGGGGGTCCGGAAACTGCC
TCCCCCAGGGGGCTTGTGGGCATCGGGGGGCAGCCT
CTGGTCCCTCCGT
F25568:GATCAAAACGGGGCAGAAAGAAACTCCTGTA
TATATTCAAACAACCATTTATGAGACCAGTACAAACT
ACTCAAGAGGAAGATGGCTGTAGCTGCCGATTTCCA
GAAGAAGAAGAAGGAGGATGTGAACTGG
F25569:GATCCCAGTTCACATCCTCCTTCTTCTTCTTCT
GGAAATCGGCAGCTACAGCCATCTTCCTCTTGAGTAG
TTTGTACTGGTCTCATAAATGGTTGTTTGAATATATA
CAGGAGTTTCTTTCTGCCCCGTTTT

FIGURE 2. Oligonucleotides used in the generation of chimeric receptor constructs.

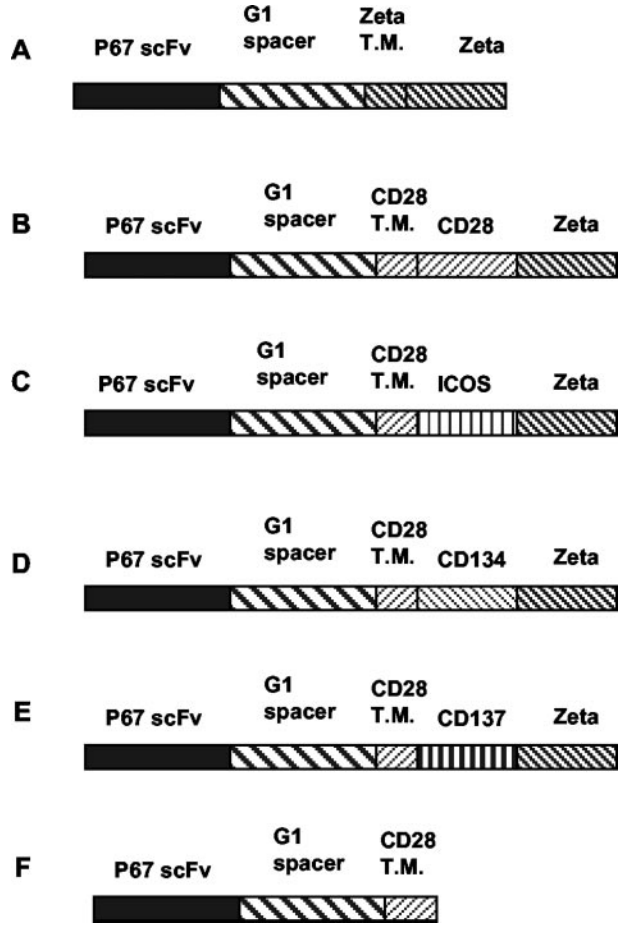

FIGURE 3. Diagram of the chimeric receptors generated. *A*, P67/G1/ζ; *B*, P67/G1/CD28.ζ; *C*, P67/G1/ICOS.ζ; *D*, P67/G1/CD134.ζ; *E*, P67/G1/CD137.ζ; *F*, P67/G1/−.

linked to an extracellular spacer comprising human IgG1 hinge, CH2 and CH3, linked to the transmembrane and intracellular regions of human CD28 linked to the intracellular region of human TCRζ. The single chain Fv and extracellular spacer are the same as in 1 above. The extracellular spacer is linked to residues 135–161 of human CD28 transmembrane region and residues 162–202 of human CD28 intracellular region (67). This is linked to residues 31–142 of human TCRζ intracellular region as in 1 above.

3) P67/G1/ICOS.ζ fusion chimeric receptor consists of a single chain Fv linked to an extracellular spacer comprising human IgG1 hinge, CH2 and CH3, linked to the transmembrane of human CD28 linked to the intracellular regions of human ICOS and TCRζ. The single chain Fv, extracellular spacer, and transmembrane region are the same as in 2 above. The transmembrane region is linked to residues 166–199 of human ICOS intracellular region (38). This is linked to residues 31–142 of human TCRζ intracellular region, as above.

4) P67/G1/CD134.ζ fusion chimeric receptor consists of a single chain Fv linked to an extracellular spacer comprising human IgG1 hinge, CH2 and CH3, linked to the transmembrane of human CD28 linked to the intracellular regions of human CD134 and TCRζ. The single chain Fv, extracellular spacer, and transmembrane region are the same as above. The transmembrane region is linked to residues 213–249 of human CD134 intracellular region (39). This is linked to residues 31–142 of human TCRζ intracellular region, as above.

5) P67/G1/CD137.ζ fusion chimeric receptor consists of a single chain Fv linked to an extracellular spacer comprising human IgG1 hinge, CH2 and CH3, linked to the transmembrane of human CD28 linked to the intracellular regions of human CD137 and TCRζ. The single chain Fv, extracellular spacer, and transmembrane region are the same as in 2 above. The transmembrane region is linked to residues 197–238 of human CD137 intracellular region (40). This is linked to residues 31–142 of human TCRζ intracellular region, as above.

6) P67/G1/− control chimeric receptor consists of a single chain Fv linked to an extracellular spacer comprising human IgG1 hinge, CH2 and CH3, linked to the transmembrane of human CD28. The single chain Fv, extracellular spacer, and transmembrane region are the same as in 2 above. The transmembrane region is followed by the stop codon. This is the control receptor necessary for the intracellular ELISA analysis.

Isolation of total, CD4$^+$, and CD8$^+$ human primary T cells

PBMC were isolated from the blood of healthy volunteers by Ficoll-Paque PLUS (Amersham Biosciences, Uppsala, Sweden) density-gradient centrifugation. The interface cells were washed three times with PBS, and T cells were isolated by MACS depletion (Miltenyi Biotec, Bergisch Gladbach, Germany). PBLs were labeled with microbeads using a pan T cell, CD4$^+$ T cell, or CD8$^+$ T cell isolation kit and separated on magnetic columns in a VarioMACS separator, according to the manufacturer's recommendations (Miltenyi Biotec). The purity of CD4$^+$ and CD8$^+$ T cells was tested by flow cytometry and was found to be >95%.

Transfection of human primary T cells

Purified human primary T cells were transfected by electroporation. Plasmid DNA was prepared by p500 columns, according to the manufacturer's instructions (Qiagen, Chatsworth, CA). A total of 3–6 μg of DNA was added to 4×10^6 T cells resuspended in 100 μl of Nucleofector solution for T cells (Amaxa Biosystems, Cologne, Germany), electroporated using the U-13 program of the Nucleofector device (Amaxa Biosystems), and immediately transferred into prewarmed RPMI 1640 medium supplemented with 10% (v/v) FCS and 4 mM glutamine.

Immunofluorescence analysis

T cells expressing chimeric receptors were identified by immunofluorescence, labeling cells with FITC-conjugated CD33 Ag (1 μg/ml) 24 h after transfection. Immunofluorescence was analyzed using a FACScan cytofluorometer equipped with the CellQuest research software (BD Biosciences, Mountain View, CA).

Stimulation and cytokine production assays

Transfected T cells were left to rest for 3 h after transfection and then stimulated by either solid-phase Ag or target cells. For solid-phase stimulation, cells were plated at 2×10^5 cells/well in 96-well plates (Nunc Immunol, Naperville, IL) left uncoated, or precoated with soluble CD33 at 5 μg/ml in 0.1 M $NaHCO_3$ (pH 9.6). Target cells comprised the mouse myeloma cell line NS0, engineered to express human CD33 (Ag-positive target cells), or engineered to express only the empty expression vector (Ag-negative target cells). T cells were plated at 2×10^5 cells/well in 96-well plates with 1×10^6 target cells. Ninety-six-well plates were then incubated for 48 h in a humidified 37°C incubator, and cell culture supernatants were harvested and assayed for human IL-2, IFN-γ, TNF-α, and GM-CSF using Duoset ELISA sets, according to the manufacturer's directions (R&D Systems, Abbingdon, U.K.).

Intracellular ELISA

The association of cytosolic molecules with the intracellular region of the recombinant receptors was analyzed by intracellular ELISA. Transfected T cells were left to rest for 3 h after transfection and then stimulated by incubation in 96-well plates (Nunc Immunol) precoated with soluble CD33 extracellular region at 5 μg/ml in 0.1 M $NaHCO_3$ (pH 9.6) and then blocked with PBS/1% (w/v) PEG600. Cells were plated at 5×10^5 cells/well and incubated for 3 h in a humidified 37°C incubator. Cell supernatant was then removed, and 200 μl/well cold lysis buffer (50 mM HEPES, pH 7, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Zwittergent 3-12 (Calbiochem, La Jolla, CA) and one protease inhibitor Complete tablet/20 ml buffer (Roche Diagnostics, Lewes, U.K.)) were added. The cells were incubated in this lysis buffer on ice for 5 min before washing plates six times with PBS/0.1% Tween. Receptors bound to the plate were detected by incubation with an HRP-conjugated anti-human IgG Fc rabbit polyclonal Ab to the G1 spacer (1/1000). Tyrosine phosphorylation was detected by incubation with an HRP-conjugated anti-phosphotyrosine mAb. PI3-kinase p85 subunit association was detected by incubation with a rabbit polyclonal anti-p85 Ab (1/2000), followed by a further four washes and a second incubation with an HRP-conjugated anti-rabbit IgG goat polyclonal Ab. Ab incubations were conducted in lysis buffer/1% (w/v) PEG600 for 20 min at room temperature. Plates were washed again, and color was detected with tetramethylbenzidine substrate, followed by absorbance reading at 630 nm using a Labsystems Multiskan Ex plate reader and data analyzed using Genesis II software (Thermolife Sciences, Basingstoke, U.K.).

Cell proliferation

Ag-induced cell proliferation was analyzed by immunofluorescence. Purified human primary T cells at 2×10^7 cells/ml were labeled by incubation with 5 μM CFSE (Molecular Probes, Eugene, OR) at 37°C for 15 min in RPMI 1640 medium. Cells were then washed twice with PBS and transfected, as described above. Transfected T cells were left to rest for 3 h after transfection and then stimulated by incubation in 96-well plates (Nunc Immunol) precoated with soluble CD33 at 5 μg/ml in 0.1 M $NaHCO_3$ (pH 9.6). Cells were plated at 2×10^5 cells/well and incubated in a humidified 37°C incubator. CFSE immunofluorescence was analyzed using a FACScan cytofluorometer equipped with the CellQuest research software (BD Biosciences) after 7 days of stimulation.

Induction of Bcl-2 expression

T cells were isolated and transfected, as described above, left to rest for 3 h after transfection, and then stimulated by incubation in 96-well plates (Nunc Immunol) precoated with soluble CD33 at 5 μg/ml in 0.1 M $NaHCO_3$ (pH 9.6) or left unstimulated in 96-well tissue culture plates. Cells were plated at 2×10^5 cells/well and incubated in a humidified 37°C incubator for 8 days. Cells were washed, fixed, and permeabilized using Cytofix/Cytoperm solution, and then washed and labeled in Cytoperm/Cytowash buffer with anti-Bcl-2 Ab, according to the manufacturer's recommendations (BD PharMingen). Cells were washed again and analyzed by flow cytometry, as above.

Target cell lysis

T cells were isolated and transfected, as described above, and left to rest for 3 h after transfection, and then 2×10^5 cells were stimulated by incubation in round-bottom 96-well plates with target cells at E:T ratios of 10:1, 20:1, 40:1, and 80:1. Target cells, mouse myeloma NS0 transfected with a human CD33 construct (Ag positive), or control vector (Ag negative) were labeled with CFSE, as described above, for T cells. Cells were incubated for 40 h in a humidified 37°C incubator, and target cell viability was analyzed by propidium iodide exclusion/flow cytometry. The percentage of target cell lysis was calculated to be: (percentage of viable target cells in the absence of effector cells) − (percentage of viable target cells in the presence of effector cells).

Results

Expression of chimeric receptors in resting human primary T cells

A panel of chimeric receptors comprising the same Ag-binding specificity and extracellular spacer region, but different signaling regions, was generated (Fig. 3). These chimeric receptors each comprised the signaling component from human TCRζ; additionally, a costimulatory signaling component from human CD28, ICOS, CD134, or CD137 is fused in series. We previously reported that for a TCRζ/CD28 fusion receptor, placing CD28 in the membrane-proximal position was most efficient (17); we also found the same preferred orientation in similar studies with TCRζ/CD137 fusion receptors in Jurkat cells (data not shown), so for analysis in human primary T cells, chimeric receptors were constructed with the costimulatory signaling region in the membrane-proximal position. These chimeric receptors were transfected into freshly isolated human primary total T cells by electroporation. Cell surface expression of functional chimeric receptors was determined 24 h later by incubation with fluorescent soluble CD33 Ag. Fig. 4 shows the histograms of FL1 signal plotted against frequency for transfectants expressing the five constructs described, compared with an empty expression vector control. Dead cells were excluded by gating forward/side scatter profiles. All constructs were expressed in greater than 60% of human resting T cells with similar

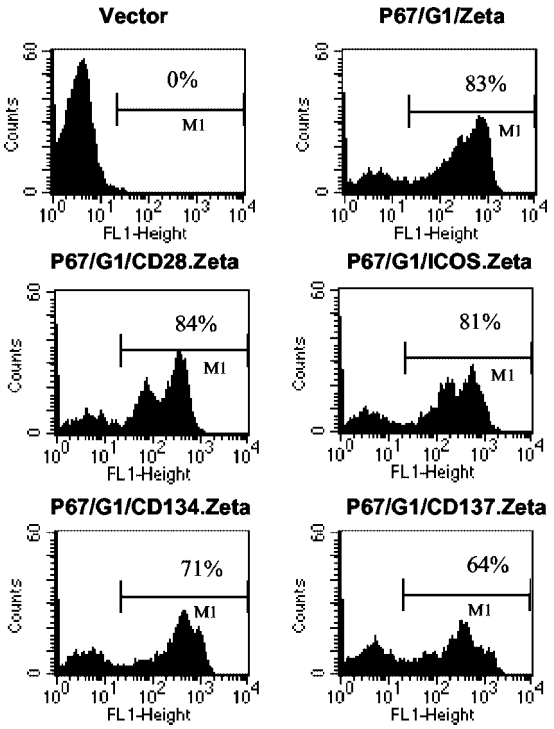

FIGURE 4. Surface expression of chimeric receptors. Human primary T cells were transfected with chimeric receptor constructs, and 24 h later cells were labeled with FITC-conjugated CD33 before analysis in a FACScan (BD Biosciences). Results are representative of four experiments using different donors.

efficiency. Construct expression was maintained in these cells, although at a lower level, for at least 8 days. Chimeric receptor constructs were expressed with equal efficiency in both $CD4^+$ and $CD8^+$ cells, demonstrated by similar expression profiles obtained in either $CD4^+$ or $CD8^+$ isolated subsets (data not shown).

Cytokine production mediated by specific activation of chimeric receptors

To assess the ability of the inclusion of costimulatory signaling regions to enhance TCRζ-mediated T cell activation, cytokine production in response to Ag stimulation was compared for the different receptors. Fig. 5 shows the concentrations of IL-2, IFN-γ, TNF-α, and GM-CSF measured in the cell culture supernatants of human primary T cell transfectants after 48 h of solid-phase CD33 Ag or CD33 Ag-positive target cell stimulation. Inclusion of each of the costimulatory signaling regions in series with TCRζ led to enhanced Ag-induced IL-2, IFN-γ, TNF-α, and GM-CSF production compared with the chimeric receptor with TCRζ alone. This cytokine production was Ag specific, demonstrated by low level constitutive cytokine production in the absence of solid-phase Ag or presence of Ag-negative target cells. Cell-expressed CD33 Ag exhibited similar cytokine profiles induced by solid-phase Ag, albeit at a reduced level. Inclusion of CD28 was most potent for the induction of IL-2. Inclusion of either CD28 or ICOS led to greater induction of IFN-γ, TNF-α, and GM-CSF than inclusion of either CD134 or CD137.

Therapeutically, the combination of both $CD4^+$ and $CD8^+$ T cells is preferred; hence, the majority of these investigations were conducted in isolated total T cells. However, to demonstrate receptor function in both subsets, and to see whether cytokine profiles might be beneficially manipulated by adjusting the natural ratio of $CD4^+$ to $CD8^+$ cells, Ag-induced cytokine production was compared for equal numbers of isolated $CD4^+$ and $CD8^+$ T cells from the same donors. Fig. 6 shows the concentrations of IL-2, IFN-γ, and TNF-α measured in the cell culture supernatants of human primary $CD4^+$ and $CD8^+$ T cell transfectants after 48 h of

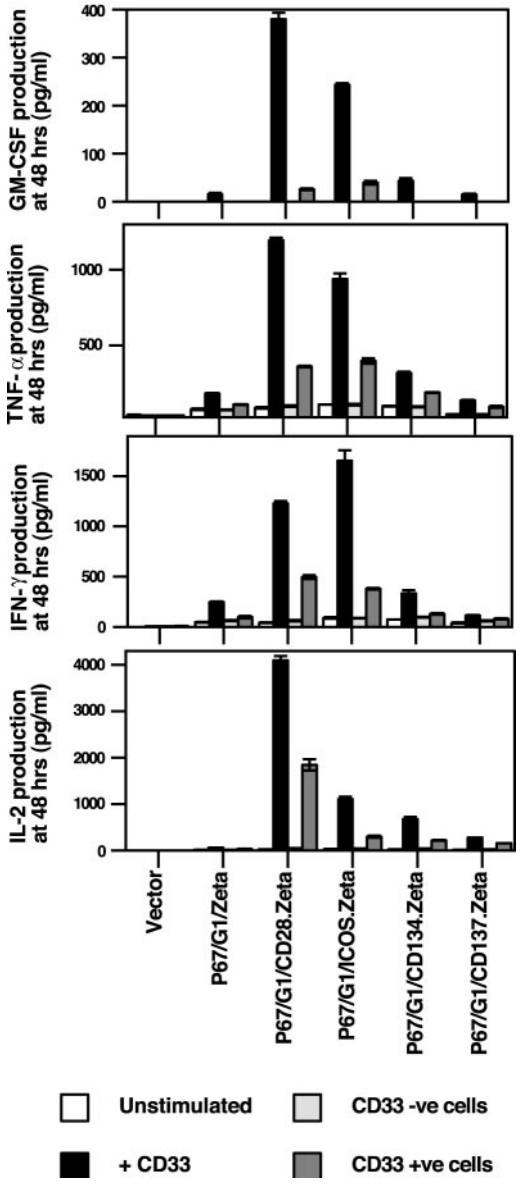

FIGURE 5. Stimulation of chimeric receptors with solid-phase CD33 Ag and cell surface-expressed CD33 Ag. Human primary T cells transfected with chimeric receptors were incubated for 48 h on Ag-coated plates or target cells; supernatants were then harvested and assayed for IL-2, IFN-γ, TNF-α, and GM-CSF by ELISA. Stimulations were done in triplicate. Results shown are representative of three experiments using different donors.

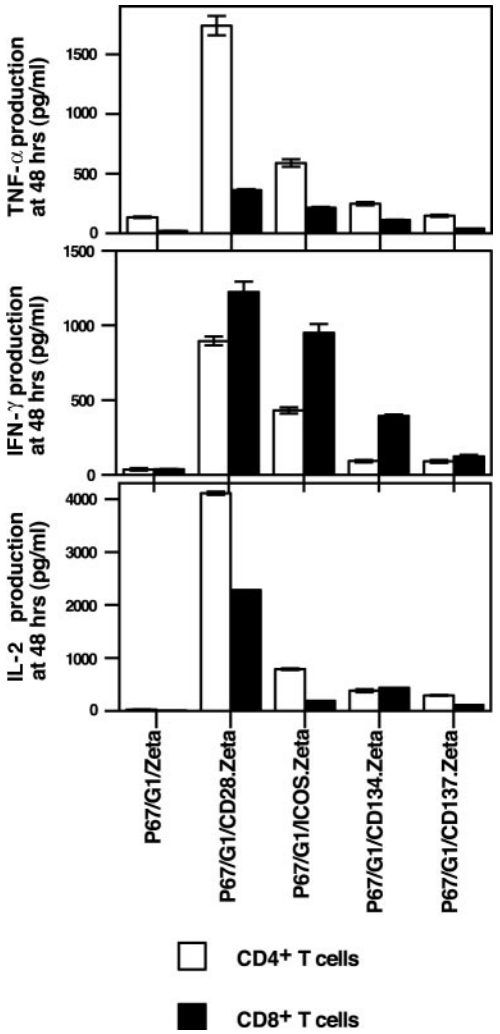

FIGURE 6. Comparison of Ag-stimulated cytokine production from $CD4^+$ and $CD8^+$ primary T cells. Human primary $CD4^+$ and $CD8^+$ T cells transfected with chimeric receptors were incubated for 48 h on Ag-coated plates; supernatants were then harvested and assayed for IL-2, IFN-γ, and TNF-α by ELISA. Stimulations were done in triplicate. Results shown are representative of three experiments using different donors.

solid-phase CD33 Ag stimulation. In both $CD4^+$ and $CD8^+$ T cells, inclusion of each of the costimulatory signaling regions in series with TCRζ led to enhanced Ag-induced IL-2, IFN-γ, and TNF-α production compared with the chimeric receptor with TCRζ alone. Chimeric receptor-mediated production of IL-2 and TNF-α was favored by $CD4^+$ T cells, whereas the production of IFN-γ was favored by $CD8^+$ T cells. We found that GM-CSF production could be induced in either subset and was donor dependent (data not shown).

Tyrosine phosphorylation of and PI3-kinase association with the intracellular regions of chimeric receptors

To see whether the chimeric receptors transduce intracellular signals in a similar manner to their natural counterparts, tyrosine phosphorylation and association with PI3-kinase were analyzed using an intracellular ELISA. This method takes advantage of the relatively high affinity of the interaction between the P67scFv binding site of the chimeric receptor and the CD33 Ag. We analyzed the binding of FITC-conjugated CD33 to cell-expressed chimeric receptors, and calculated the affinity of Ag binding, by Scatchard analysis (68), to be 2.7 nM. This affinity allowed cell-expressed chimeric receptors to be bound to and stimulated by Ag-coated plates, for cells to be lysed, and cell debris washed away so that the association of molecules with the intracellular region of the chimeric receptor could be analyzed in a standard ELISA format. ELISA OD values for each chimeric receptor were compared with a control receptor with an identical extracellular region, but no intracellular signaling region (Fig. 3*F*), which represents the negative control background OD value. Fig. 7 demonstrates that all the receptors, including the control receptor, bound the Ag-coated plate with similar efficiency. All of the receptors containing the TCRζ signaling region had phosphorylated tyrosine residues compared with the control receptor with no signaling region. Receptors containing a B7 receptor family signaling region, either CD28 or ICOS, showed significant association with PI3-kinase compared with the control receptor. Receptors containing a TNFR family signaling region, either CD134 or CD137, showed no association with PI3-kinase.

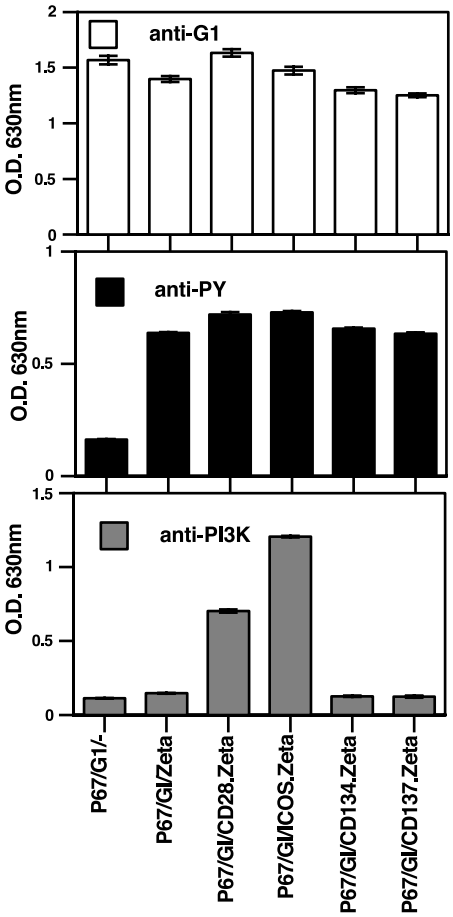

FIGURE 7. Analysis of chimeric receptor signaling in response to CD33 stimulation. Human primary T cells expressing chimeric receptors were incubated for 2 h on Ag-coated plates, cells were lysed, and plates were washed. Receptor bound to the plate (anti-G_1), tyrosine phosphorylation (anti-PY), and PI3-kinase association (anti-PI3K) were detected by ELISA. Stimulations were done in triplicate. Results shown are representative of three experiments using different donors.

Proliferation mediated by specific activation of chimeric receptors

Chimeric receptors providing TCRζ signaling alone are insufficient to induce proliferation of resting murine primary T cells (7, 8). To see whether the same is true in resting human T cells, and to determine which costimulatory regions might induce proliferation, we analyzed by flow cytometry the ability of the chimeric receptors to respond to Ag. T cells were labeled before transfection with CFSE, and Ag-induced proliferation was analyzed by FACS after 7 days of Ag stimulation. Fig. 8 shows that the chimeric receptor comprised of TCRζ alone was incapable of mediating Ag-induced proliferation of resting human T cells, as depicted by no reduction in CFSE FL1 signal. Chimeric receptors that included a signaling region derived from CD28, ICOS, CD134, or CD137 were all able to mediate proliferation when stimulated in the same way. No proliferation was observed with any chimeric receptor in the absence of Ag stimulation (data not shown).

Induction of Bcl-2 expression

To analyze whether the inclusion of costimulatory signaling regions in chimeric receptors is able to deliver antiapoptotic signals, the induction of Bcl-2 expression in response to Ag stimulation was examined. Transfected resting T cells were stimulated with solid-phase CD33 or left unstimulated for 8 days. Bcl-2 expression was then measured by flow cytometry. As Fig. 9 demonstrates, the chimeric receptor comprised of TCRζ alone did not up-regulate Bcl-2 expression; however, inclusion of CD28, ICOS, CD134, or CD137 led to an Ag-specific induction of Bcl-2 expression.

Target cell lysis

To examine whether chimeric receptor-mediated activation of resting human primary T cells could facilitate specific target cell killing, we examined the viability of both Ag-positive and Ag-negative target cells after incubation for 40 h with transfected T cells. The target cells used were a mouse NS0 cell line generated by transfection with a vector expressing human CD33. A matched negative control target cell line was transfected with an empty expression vector. The target cells were prelabeled with CFSE, both to ensure that each target cell line went through a similar degree of division during the experiment (data not shown), and to facilitate isolation of target cells from effector T cells on viability analysis by flow cytometry. Fig. 10 demonstrates the chimeric receptor-mediated Ag-specific lysis of target cells. Inclusion of CD28, ICOS, or CD134 enhanced target cell killing mediated by TCRζ alone. Inclusion of ICOS enhanced target cell killing to a

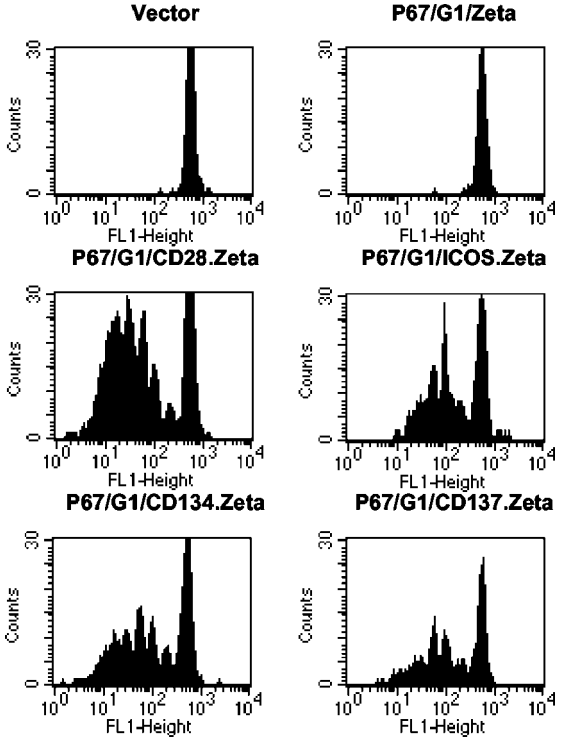

FIGURE 8. Ag-induced proliferation via chimeric receptors. Human primary T cells were labeled with CFSE, transfected with chimeric receptor constructs, and stimulated for 7 days with solid-phase CD33 before analysis in a FACScan (BD Biosciences). Results are representative of three experiments using different donors.

greater degree than inclusion of either CD28 or CD134. CD137 inclusion led to the same level of target cell lysis as TCRζ alone (data not shown).

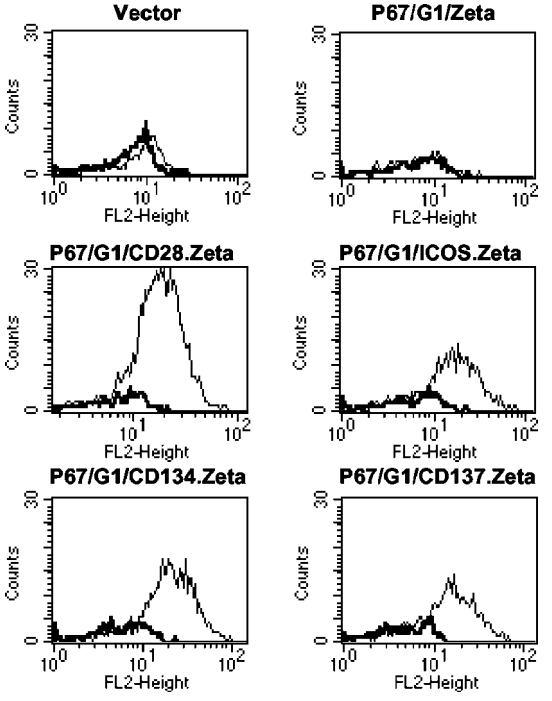

FIGURE 9. Ag-induced Bcl-2 expression. Human primary T cells were transfected with chimeric receptor constructs, then stimulated for 8 days with solid-phase CD33 (thin lines) or left unstimulated (bold lines). Cells were labeled with a PE-conjugated anti-Bcl-2 Ab before analysis by flow cytometry.

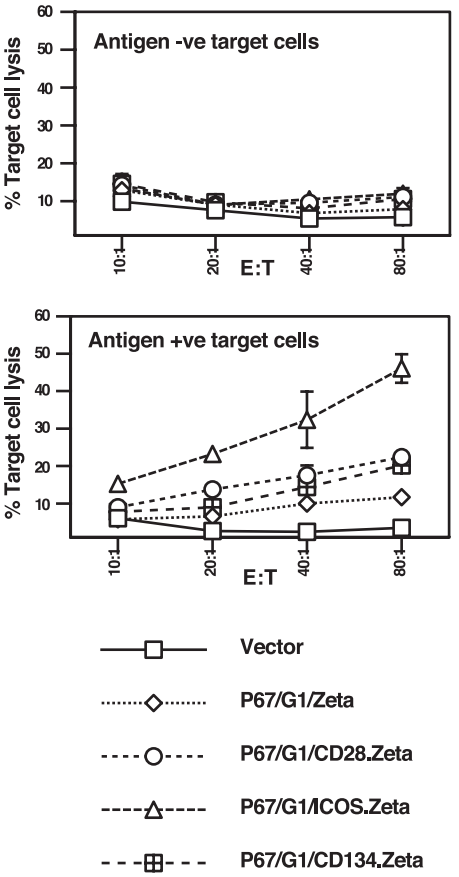

FIGURE 10. Ag-specific target cell lysis. Human primary T cells were transfected with chimeric receptor constructs, then stimulated for 40 h with CD33 Ag-positive or matched Ag-negative target cells. Target cell viability was analyzed by flow cytometry. Percentage of target cell lysis was calculated to be: (percentage of viable target cells in the absence of effector cells) − (percentage of viable target cells in the presence of effector cells). Transfections and stimulations were conducted in triplicate. Results are representative of three experiments using different donors.

Discussion

The demonstration of the enhanced ability of a single chimeric receptor, including CD28 signaling in series with TCRζ to induce Ag-specific proliferation and effector function in prestimulated human primary T cells (18–24), led us to investigate whether similar effects could be demonstrated in resting human primary T cells. With the expanding number of known costimulatory receptors and an increased understanding of their importance in mounting and maintaining an immune response, we examined whether molecules other than CD28 could be included in a single chimeric receptor in series with TCRζ to mediate T cell activation in resting human primary T cells.

A chimeric receptor with the P67 Ab specificity for human CD33 (69), an extracellular spacer comprised of human IgG1 hinge, CH2 and CH3 sequences, and an intracellular TCRζ signaling region was generated; the ability of this receptor to mediate T cell activation was compared with chimeric receptors with identical extracellular domains, but with costimulatory signaling regions fused in series with TCRζ. These costimulatory sequences were derived from members of both the B7 receptor family (CD28 and ICOS) and the TNFR family (CD134 and CD137). The chimeric receptors were constructed with the costimulatory signaling regions membrane proximal and the primary activation signaling region of TCRζ membrane distal. All sequences were human derived or humanized to limit as much as possible the potential risk

of immunogenicity in a clinical setting. We chose to examine the overall performance of the chimeric receptors in isolated total T cells, rather than purified $CD8^+$ CTLs only, as $CD4^+$ T cells have also been shown to play an important role in antitumor immunity (4). However, additionally, we demonstrate that the receptors function in both $CD4^+$ and $CD8^+$ T cell subsets.

The chimeric receptors were transfected into resting human primary T cells by electroporation, and equivalent, efficient functional expression was detected for all of them. The percentage of viable cells transfected with the chimeric receptor constructs was between 64 and 84%. This efficiency of transfection compares favorably both with the 22–42% obtained by retroviral transduction of prestimulated human primary T cells with similar constructs (19) and our attempts to transduce resting primary T cells with adenovirus (15%; data not shown).

Inclusion of both B7 receptor and TNFR family member costimulatory signaling regions in series with TCRζ enhanced the level of specific Ag-induced IL-2, IFN-γ, TNF-α, and GM-CSF cytokine production compared with TCRζ alone. Inclusion of CD28 elicited much higher levels of IL-2 production than ICOS, CD134, or CD137, consistent with the known importance of CD28 for optimal IL-2 production (29). Inclusion of CD28 or ICOS led to higher levels of IFN-γ, TNF-α, and GM-CSF than inclusion of either CD134 or CD137. Although ICOS, CD134, and CD137 signals are known to be capable of eliciting these cytokines (42–44, 50–52, 57, 58, 60), these costimulatory receptors would not naturally be engaged in the absence of CD28, and so no previous comparison of their cytokine profiles in resting human primary T cells has been made. The ability to transfect these molecules into resting human primary T cells, in which their natural counterparts are not expressed, facilitates the study of aspects of costimulation not possible by other methods. The results presented in this work demonstrate the potential of other costimulatory molecules to compensate for CD28 in the activation of resting human primary T cells. This type of chimeric receptor will therefore be particularly useful in clarifying the role of costimulatory receptor down-regulation in the function of memory T cell subsets (70–72).

Appropriate signal transduction via the chimeric receptors was confirmed by intracellular ELISA analysis. This technique was developed in preference to Western blotting, which we used for similar studies in stable cell lines (17), because the intracellular ELISA method was found to be more sensitive and required fewer cells, which allowed the direct comparison of different receptors in cells isolated from the same donor. The activated receptors demonstrated tyrosine phosphorylation associated with the TCRζ signaling region, consistent with the successful recruitment of Zap-70 and phosphorylation of the immunoreceptor tyrosine-based activation motifs (73). PI3-kinase was associated with activated receptors containing B7 receptor family members, CD28 and ICOS, in accordance with its reported association with the natural CD28 (74, 75) and ICOS molecules (76). PI3-kinase was not associated with receptors containing the TNFR molecules, CD134 and CD137, which initiate signaling via the recruitment of various TNFR-associated factor (TRAF) signaling molecules (77). We tried to examine the association of TRAF 2 and TRAF 3 with the chimeric receptors in this format, as these molecules have both been demonstrated to associate with CD134 and CD137 (78–81); however, we have been unable to source suitable Abs for this study to date.

The chimeric receptor that featured primary activation signaling through TCRζ only was not able to induce Bcl-2 expression or proliferate in response to Ag. However, inclusion of any one of CD28, ICOS, CD134, and CD137 signaling regions enabled resting primary T cells to express Bcl-2 and proliferate in response to Ag. This occurred in the absence of addition of any exogenous factors, such as phorbol esters, stimulatory Abs, or cytokines, demonstrating that these chimeric receptors can confer self-sufficient clonal expansion to resting human T cells. The ability of CD28, CD134, and CD137 to induce Bcl-2 and provide survival signals has been reported for the natural receptors (30, 31, 53, 57, 61).

Resting human primary T cells transfected with the TCRζ chimeric receptor were able to elicit Ag-specific target cell lysis within 40 h of stimulation by these cells. Target cell lysis was enhanced by the inclusion of CD28 signaling consistent with the enhanced in vivo tumor cell killing demonstrated using preactivated primary T cells (23, 24). Target cell lysis was also enhanced by the inclusion of CD134 and ICOS signaling. Target cell lysis was enhanced to the greatest degree by the use of the previously unreported fusion receptor providing both TCRζ and ICOS signaling.

Signaling via ICOS, CD134, or CD137 naturally occurs in addition to CD28 signaling, and optimal T cell activation might be achieved by combining these receptors either in parallel by the cotransfection of two different fusion receptors or in series by the generation of triple fusion receptors. The cloning cassette designed for this investigation facilitates the exchange of each component and the generation of multicomponent signaling regions, and hence will permit both approaches. Cotransfection of two receptors in parallel will require the exchange of spacer and transmembrane regions to allow generation of two different homodimers and the prevention of heterodimer formation. The in-series fusion approach will require the in-frame insertion of a third signaling region.

We have demonstrated that the inclusion of costimulatory signals in series in one receptor with TCRζ is applicable to both B7 receptor and TNFR family members, and that these receptors can elicit enhanced Ag-specific cytokine production and target cell killing compared with signaling via TCRζ alone. Inclusion of costimulatory signaling regions prevents apoptosis and facilitates the Ag-specific expansion of human primary resting T cells. Inclusion of different types of costimulatory signals and hence the use of multiple signaling pathways will also differentially affect properties not measurable in vitro. Characteristics such as the ability of engineered T cells to traffic to tumor sites, expand, survive, and generate memory in vivo need now to be studied with chimeric receptors, which provide TCRζ signaling and different costimulatory signals either in addition to, or instead of, CD28.

References

1. Gross, G., and Z. Eshhar. 1992. Endowing T cells with antibody specificity using chimeric T cell receptors. *FASEB J. 6:3370.*
2. Eshhar, Z., N. Bach, C. J. Fitzer-Attas, G. Gross, J. Lustgarten, T. Waks, and D. G. Schindler. 1996. The T-body approach: potential for cancer immunotherapy. *Springer Semin. Immunopathol. 18:199.*
3. Sadelain, M., I. Riviere, and R. Brentjens. 2003. Targeting tumors with genetically enhanced T lymphocytes. *Nat. Rev. Cancer 3:35.*
4. Pardoll, D. M., and S. L. Topalian. 1998. The role of $CD4^+$ T cell responses in antitumor immunity. *Curr. Opin. Immunol. 10:588.*
5. Bitton, N., F. Verrier, P. Debre, and G. Gorochov. 1998. Characterization of T cell-expressed chimeric receptors with antibody-type specificity for the CD4 binding site of HIV-1 gp120. *Eur. J. Immunol. 28:4177.*
6. Roberts, M. R., L. Qin, D. Zhang, D. H. Smith, A. C. Tran, T. J. Dull, J. E. Groopman, D. J. Capon, R. A. Byrn, and M. H. Finer. 1994. Targeting of human immunodeficiency virus-infected cells by $CD8^+$ T lymphocytes armed with universal T-cell receptors. *Blood 84:2878.*
7. Brocker, T., and K. Karjalainen. 1995. Signals through T cell receptor-ζ chain alone are insufficient to prime resting T lymphocytes. *J. Exp. Med. 181:1653.*
8. Brocker, T. 2000. Chimeric Fv-ζ or Fv-ϵ receptors are not sufficient to induce activation or cytokine production in peripheral T cells. *Blood 96:1999.*
9. Mitsuyasu, R. T., P. A. Anton, S. G. Deeks, D. T. Scadden, E. Connick, M. T. Downs, A. Bakker, M. R. Roberts, C. H. June, S. Jalali, et al. 2000. Prolonged survival and tissue trafficking following adoptive transfer of CD4ζ gene-modified autologous $CD4^+$ and $CD8^+$ T cells in human immunodeficiency virus-infected subjects. *Blood 96:785.*

10. Walker, R. E., C. M. Bechtel, V. Natarajan, M. Baseler, K. M. Hege, J. A. Metcalf, R. Stevens, A. Hazen, R. M. Blaese, C. C. Chen, et al. 2000. Long-term in vivo survival of receptor-modified syngeneic T cells in patients with human immunodeficiency virus infection. *Blood 96:467*.
11. Bretcher, P. A., and M. A. Cohn. 1970. A theory of self and non-self discrimination. *Science 169:1042*.
12. Tan, P., C. Anasetti, J. A. Hansen, J. Melrose, M. Brunvand, J. Bradshaw, J. A. Ledbetter, and P. S. Lindsley. 1993. Induction of alloantigen-specific hyporesponsiveness in human T lymphocytes by blocking interaction of CD28 with its natural ligand, B7/BB1. *J. Exp. Med. 177:165*.
13. Harding, F. A., J. G. McArthur, J. A. Gross, D. H. Raulet, and J. P. Allison. 1992. CD28-mediated signalling co-stimulates murine T cells and prevents induction of anergy in T-cell clones. *Nature 6370:607*.
14. Liebowitz, D. N., K. P. Lee, and C. H. June. 1998. Costimulatory approaches to adoptive immunotherapy. *Curr. Opin. Oncol. 10:533*.
15. Alvarez-Vallina, L., and R. E. Hawkins. 1996. Antigen-specific targetting of CD28-mediated T cell co-stimulation using chimeric single-chain antibody variable fragment-CD28 receptors. *Eur. J. Immunol. 26:2304*.
16. Krause, A., H.-F. Gou, J.-B. Latouch, C. Tan, N.-K. V. Cheung, and M. A. Sadelain. 1998. Antigen-dependent CD28 signaling selectively enhances survival and proliferation in genetically modified activated human primary T lymphocytes. *J. Exp. Med. 188:619*.
17. Finney, H. M., A. D. G. Lawson, C. R. Bebbington, and A. N. C. Weir. 1998. Chimeric receptors providing both primary and costimulatory signaling in T cells from a single gene product. *J. Immunol. 161:2791*.
18. Eshhar, Z., T. Waks, A. Bendavid, and D. G. Schindler. 2001. Functional expression of chimeric receptor genes in human T cells. *J. Immunol. Methods 248:67*.
19. Hombach, A., A. Wieczarkowiecz, T. Marquardt, C. Heuser, L. Usai, C. Pohl, B. Seliger, and H. Abken. 2001. Tumor-specific T cell activation by recombinant immunoreceptors: CD3ζ signalling and CD28 costimulation are simultaneously required for efficient IL-2 secretion and can be integrated into one combined CD28/CD3ζ signaling receptor molecule. *J. Immunol. 167:6123*.
20. Geiger, T. L., P. Nguyen, D. Leitenberg, and R. A. Flavell. 2001. Integrated *src* kinase and costimulatory activity enhances signal transduction through single-chain chimeric receptors in T lymphocytes. *Blood 98:2364*.
21. Maher, J., R. J. Brentjens, G. Gunset, I. Riviere, and M. Sadelain. 2002. Human T-lymphocyte cytotoxicity and proliferation directed by a single chimeric TCRζ/CD28 receptor. *Nat. Biotechnol. 20:70*.
22. Gilham, D. E., A. O'Neil, C. Hughs, R. D. Guest, N. Kirillova, M. Lehane, and R. E. Hawkins. 2002. Primary polyclonal human T lymphocytes targeted to carcino-embryonic antigens and neural cell adhesion molecule tumor antigens by CD3 ζ-based chimeric immune receptors. *J. Immunother. 25:139*.
23. Haynes, N. M., J. A. Trapani, M. W. Teng, J. T. Jackson, L. Cerruti, S. M. Jane, M. H. Kershaw, M. J. Smyth, and P. K. Darcy. 2002. Rejection of syngeneic colon carcinoma by CTLs expressing single-chain antibody receptors codelivering CD28 costimulation. *J. Immunol. 169:5780*.
24. Haynes, N. M., J. A. Trapani, M. W. Teng, J. T. Jackson, L. Cerruti, S. M. Jane, M. H. Kershaw, M. J. Smyth, and P. K. Darcy. 2002. Single-chain antigen recognition receptors that costimulate potent rejection of established experimental tumors. *Blood 100:3155*.
25. Lenschow, D. J., T. S. Walunas, and J. A. Bluestone. 1996. CD28/B7 system of T cell costimulation. *Annu. Rev. Immunol. 14:233*.
26. Sansom, D. M. 2000. CD28, CTLA-4 and their ligands: who does what and to whom? *Immunology 101:169*.
27. Alegre, M.-L., K. A. Frauwirth, and C. B. Thompson. 2001. T-cell regulation by CD28 and CTLA-4. *Nat. Rev. Immunol. 1:220*.
28. June, C. H., J. A. Ledbetter, M. M. Gillespi, T. Lindsten, and C. B. Thompson. 1987. T-cell proliferation involving the CD28 pathway is associated with cyclosporin-resistant interleukin 2 gene expresssion. *Mol. Cell. Biol. 12:4472*.
29. Jenkins, M. K., P. S. Taylor, S. D. Norton, and K. B. Urdahl. 1991. CD28 delivers a costimulatory signal involved in antigen-specific IL-2 production by human T cells. *J. Immunol. 8:2461*.
30. Boise, L., A. J. Minn, P. J. Noel, C. H. June, M. A. Accavitti, T. Lindsten, and C. B. Thompson. 1995. CD28 costimulation can promote T cell survival by enhancing the expression of Bcl-x_L. *Immunity 3:87*.
31. Radvanyi, L. G., Y. Shi, and H. Viziri. 1996. CD28 costimulation inhibits TCR-induced apoptosis during a primary T cell response. *J. Immunol. 156:1788*.
32. Lucas, P. J., I. Negishi, K. Nakayama, L. E. Fields, and D. Y. Loh. 1995. Naive CD28-deficient T cells can initiate but not sustain an in vitro antigen-specific immune response. *J. Immunol. 154:5757*.
33. Schweitzer, A. N., and A. H. Sharpe. 1998. The complexity of the B7-CD28/CTLA-4 costimulatory pathway. *Agents Actions Suppl. 49:33*.
34. London, C. A., M. P. Lodge, and A. K. Abbas. 2000. Functional responses and costimulator dependence of memory $CD4^+$ T cells. *J. Immunol. 164:265*.
35. Watts, T. H., and M. A. DeBenedette. 1999. T cell co-stimulatory molecules other than CD28. *Curr. Opin. Immunol. 11:286*.
36. Salazar-Fontana, L. I., and B. E. Bierer. 2001. T-lymphocyte coactivator molecules. *Curr. Opin. Hematol. 8:5*.
37. Sharpe, A. H., and G. J. Freeman. 2002. The B7-CD28 superfamily. *Nat. Rev. Immunol. 2:116*.
38. Hutloff, A., A. M. Dittrich, K. C. Beier, B. Eljaschewitsch, R. Kraft, I. Anagnostopouls, and R. A. Kroczek. 1999. ICOS is an inducible T-cell costimulator structurally and functionally related to CD28. *Nature 397:263*.
39. Latza, U., H. Durkop, S. Schnittger, J. Ringeling, F. Eitelbach, M. Hummel, C. Fonatsch, and S. Stein. 1994. The human OX40 homolog: cDNA structure, expression and chromosomal assignment of the ACT35 antigen. *Eur. J. Imuunol. 24:677*.
40. Alderson, M. R., C. A. Smith., T. W. Tough, T. Davis-Smith, R. J. Armitage, B. Falf, E. Roux, E. Baker, G. R. Sutherland, W. S. Din, and R. G. Goodwin. 1994. Molecular and biological characterization of human 4-1BB and its ligand. *Eur. J. Immunol. 24:2219*.
41. Carreno, B. M., and M. Collins. 2002. The B7 family of ligands and its receptors: new pathways for costimulation and inhibition of immune responses. *Annu. Rev. Immunol. 20:29*.
42. Beier, K. C., A. Hutloff, A. M. Dittrich, C. Heuck, A. Rauch, K. Büchner, B. Ludewig, H. D. Ochs, H. W. Mages, and R. A. Kroczek. 2000. Induction, binding specificity and function of human ICOS. *Eur. J. Immunol. 30:3707*.
43. McAdam, A. J., T. T. Chang, A. E. Lumelsky, E. A. Greenfield, V. A. Boussiotis, J. S. Duke-Cohan, T. Chernova, N. Malenkovich, C. Jabs, V. K. Kuchroo, et al. 2000. Mouse inducible costimulatory molecule (ICOS) expression is enhanced by CD28 costimulation and regulates differentiation of $CD4^+$ T cells. *J. Immunol. 165:5035*.
44. Gonzalo, J. A., J. Tian, T. Delaney, J. Corcoran, J. B. Rottman, J. Lora, A. Al-Garawi, R. Kroczek, J. C. Gutierrez-Ramos, and A. J. Coyle. 2001. ICOS is critical for T helper cell-mediated lung mucosal inflammatory responses. *Nat. Immun. 2:597*.
45. Riley, J. L., P. J. Blair, J. T. Musser, R. Abe, K. Tezuka, T. Tsuji, and C. H. June. 2001. ICOS costimulation requires IL-2 and can be prevented by CTLA-4 engagement. *J. Immunol. 166:4943*.
46. McAdam, A. J., R. J. Greenwald, M. A. Levin, T. Chernova, N. Malenkovich, V. Ling, G. J. Freeman, and A. H. Sharpe. 2001. ICOS is critical for CD40-mediated antibody class switching. *Nature 409:102*.
47. Dong, C., A. E. Juedes, U. A. Teman, S. Shresta, J. P. Allison, N. H. Ruddle, and R. A. Flavell. 2001. ICOS co-stimulatory receptor is essential for T cell activation and function. *Nature 409:97*.
48. Tafuri, A., A. Shahinian, F. Bladt, S. K. Yoshinaga, M. Jordana, A. Wakeham, L. M. Boucher, D. Bouchard, V. S. F. Cha, G. Duncan, et al. 2001. ICOS is essential for effective T-helper-cell responses. *Nature 409:105*.
49. Grimbacher, B., A. Hutloff, M. Schlesier, E. Glocker, K. Warnatz, R. Drager, H. Eibel, B. Fischer, A. A. Schaffer, H. Mages, et al. 2003. Homozygous loss of ICOS is associated with adult-onset common variable immunodeficiency. *Nat. Immun. 4:261*.
50. Flynn, S., K. M. Toeller, C. Raykundalia, M. Goodall, and P. Lane. 1998. CD4 T cell cytokine differentiation: the B cell activation molecule, OX40 ligand, instructs CD4 T cells to express interleukin 4 and up-regulates expression of the chemokine receptor, blr-1. *J. Exp. Med. 188:297*.
51. Gramaglia, I., A. D. Weinberg, M. Lemon, and M. Croft. 1998. OX40 ligand: a potent costimulatory molecule for sustaining primary CD4 T cell responses. *J. Immunol. 161:6510*.
52. Oshima, Y., L. P. Yang, T. Uchiyama, Y. Tanaka, P. Baum, M. Sergerie, P. Herman, and G. Delespesse. 1998. OX40 costimulation enhances interleukin-4 (IL-4) expression at priming and promotes the differentiation of naive human $CD4^+$ T cells into high IL-4-producing effectors. *Blood 92:3338*.
53. Rogers, P. R., J. Song, I. Gramaglia, N. Killeen, and M. Croft. 2001. OX40 promotes Bcl-x_L and Bcl-2 expression and is essential for long-term survival of CD4 T cells. *Immunity 15:445*.
54. Weinberg, A. D., M. M. Rivera, R. Prevell, A. Morris, T. Ramstead, J. T. Vetto, W. J. Urba, G. Alvord, C. Bunce, and J. Shields. 2000. Engagement of the OX-40 receptor in vivo enhances antitumor immunity. *J. Immunol. 164:2160*.
55. Gramaglia, I., A. Jember, S. D. Pippig, A. D. Weinberg, N. Killeen, and M. Croft. 2000. The OX40 costimulatory receptor determines the development of CD4 memory by regulating primary clonal expansion. *Eur. J. Immunol. 165:3043*.
56. Maxwell, J., A. D. Weinberg, R. A. Prell, and A. T. Vella. 2000. Danger and OX40 receptor signaling synergize to enhance memory T cell survival by inhibiting peripheral deletion. *J. Immunol. 164:107*.
57. Hurtado, J. C., Y. J. Kim, and B. S. Kwon. 1997. Signals through 4-1BB are costimulatory to previously activated splenic T cells and inhibit activation-induced cell death. *J. Immunol. 158:2600*.
58. Shuford, W. W., K. Klussman, D. D. Tritchler, D. T. Loo, J. Chalupny, A. W. Liadak, T. J. Brown, J. Emswiler, H. Raecho, C. P. Larsen, et al. 1997. 4-1BB costimulatory signals preferentially induce $CD8^+$ T cell proliferation and lead to the amplification in vivo of cytotoxic immune responses. *J. Exp. Med. 186:47*.
59. Melero, I., W. W. Shuford, S. A. Newby, A. Aruffo, J. A. Ledbetter, K. E. Hellstrom, R. S. Mittler, and L. Chen. 1997. Monoclonal antibodies against the 4-1BB T-cell activation molecule eradicate established tumors. *Nat. Med. 3:682*.
60. Tan, J. T., J. K. Whittmire, R. Ahmed, T. C. Pearson, and C. P. Larsen. 1999. 4-1BB ligand, a member of the TNF family, is important for the generation of anti-viral CD8 T cell responses. *J. Immunol. 163:4859*.
61. Takahashi, C., R. S. Mittler, and A. T. Vella. 1999. Cutting edge: 4-1BB is a bona fide CD8 T cell survival signal. *J. Immunol. 162:5037*.
62. Wen, T., J. Bukczynski, and T. H. Watts. 2002. 4-1BB ligand-mediated costimulation of human T cells induces CD4 and CD8 T cell expansion, cytokine production, and the development of cytolytic effector function. *J. Immunol. 168:4897*.
63. Miller, D. G., M. A. Adam, and A. D. Miller. 1990. Gene transfer by retrovirus vectors occurs only in cells that are actively replicating at the time of infection. *Mol. Cell. Biol. 10:4239*.
64. Kabat, E. A., T. T. Wu, H. M. Perry, K. S. Gottesman, and C. Foeller. 1991. *Sequences of Proteins of Immunological Interest*. National Institutes of Health, Bethesda.

65. Weissman, A. M., M. Baniyash, D. Hou, E. Samelson, W. H. Burgess, and R. D. Klausner. 1988. Molecular cloning of the ζ chain of the T cell antigen receptor. *Science 239:1018*.
66. Moingeon, P., C. C. Stebbins, L. D'Adamio, J. Lucich, and E. L. Reinherz. 1990. Human natural killer cells and mature T lymphocytes express identical CD3 ζ subunits as defined by cDNA cloning and sequence analysis. *Eur. J. Immunol. 20:1741*.
67. Aruffo, A., and B. Seed. 1987. Molecular cloning of a CD28 cDNA by a high-efficiency COS cell expression system. *Proc. Natl. Acad. Sci. USA 84:8573*.
68. Krause, D., C. Shearman, W. Lang, E. J. Kanzy, and R. Kurrle. 1990. Determination of affinities of murine and chimeric anti α/β-T-cell receptor antibodies by flow cytometry. *Behring Inst. Mitt. 87:56*.
69. Andrews, R. G., B. Torok-Storb, and I. D. Bernstein. 1983. Myeloid-associated differentiation antigens on stem cells and their progeny identified by monoclonal antibodies. *Blood 62:124*.
70. Dunne, P. J., J. M. Faint, N. H. Gudgeon, J. M. Fletcher, F. J. Plunkett, M. V. Soares, A. D. Hislop, N. E. Annels, A. B. Rickinson, M. Salmon, and A. N. Akbar. 2002. Epstein-Barr virus-specific $CD8^+$ T cells that re-express CD45RA are apoptosis-resistant memory cells that retain replicative potential. *Blood 100:933*.
71. Appay, V., P. R. Dunbar, M. Callan, P. Klenerman, G. M. Gillespie, L. Papagno, G. S. Ogg, A. King, F. Lechner, C. A. Spina, et al. 2002. Memory $CD8^+$ T cells vary in differentiation phenotype in different persistent virus infections. *Nat. Med. 8:379*.
72. Wills, M. R., G. Okecha, M. P. Weekes, M. K. Gandhi, P. J. Sissons, and A. J. Carmichael. 2002. Identification of naive or antigen-experienced human $CD8^+$ T cells by expression of costimulation and chemokine receptors: analysis of the human cytomegalovirus-specific $CD8^+$ T cell response. *J. Immunol. 168:5455*.
73. Isakov, N. 1998. ITAMs: immunoregulatory scaffolds that link immunoreceptors to their intracellular signaling pathways. *Receptors Channels 5:243*.
74. Pages, F., M. Ragueneau, R. Rottapel, A. Truneh, J. Nunes, J. Imbert, and D. Olive. 1994. Binding of phosphatidylinositol-3-OH kinase to CD28 is required for T cell signalling. *Nature 369:327*.
75. Prasad, K. V. S., Y.-C. Cai, M. Raab, B. Duckworth, L. Cantley, S. E. Shoelson, and C. E. Rudd. 1994. T-cell antigen CD28 interacts with the lipid kinase phosphatidylinositol 3-kinase by a cytoplasmic Tyr(P)-Met-Xaa-Met motif. *Proc. Natl. Acad. Sci. USA 91:2834*.
76. Coyle, A. J., S. Lehar, C. Lloyd, J. Tian, T. Delaney, S. Manning, T. Nguyen, T. Burwell, H. Schneider, J. A. Onzalo, et al. 2000. The CD28-related molecule ICOS is required for effective T cell-dependent immune responses. *Immunity 13:95*.
77. Inoue, J., T. Ishida, N. Tsukamoto, N. Kobayashi, S. Azuma, and T. Yamamto. 2000. Tumor necrosis factor receptor-associated factor (TRAF) family: adapter proteins that mediate cytokine signalling. *Exp. Cell Res. 254:14*.
78. Arch, R. H., and C. B. Thompson. 1998. 4-1BB and OX-40 are members of a tumor necrosis factor (TNF)-nerve growth factor receptor subfamily that bind TNF receptor-associated factors and activate nuclear factor κB. *Mol. Cell. Biol. 18:558*.
79. Kawamata, S., T. Hori, A. Imura, A. Takori-Kondo, and T. Uchiyama. 1998. Activation of OX-40 signal transduction pathways leads to tumor necrosis factor receptor-associated factor (TRAF) 2- and TRAF5-mediated NK-κB activation. *J. Biol. Chem. 273:5808*.
80. Jang, I. K., Z. H. Lee, Y. J. Kim, and B. S. Kwon. 1998. Human 4-1BB (CD137) signals sre mediated by TRAF2 and activate nuclear factor-κB. *Biochem. Biophys. Res. Commun. 242:613*.
81. Saoulli, K., S. Y. Lee, J. L. Cannons, W. C. Yeh, A. Snatana, M. D. Goldstein, N. Bangia, M. A. DeBedette, T. W. Mak, Y. Coi, and T. H. Watts. 1998. CD28-independent TRAF2-dependent costimulation of resting T cells by 4-1BB ligand. *J. Exp. Med. 187:1849*.