| Common Name       | Latin Name                     | Accession Number | Sequence                     |
|-------------------|--------------------------------|------------------|------------------------------|
| MOUSE             | Mus musculus                  | AAA3796.1        | WLVVYGVLFGGLLYVALCIWV       |
| HUMAN             | Homo sapiens                  | NM_006139        | WLVVYGVLGACGLLYVAIFIW       |
| BABOON            | Papio anubis                  | XP_009181099.1   | WLVVYGVLGCGLLYVAIFSIFW      |
| MACAQUE           | Macaca fascicular             | KM_0015432133   | WLVVYGVLGCGLLYVAIFCIFW      |
| GREEN MONKEY      | Chlorocebus sabaeus           | KM_0017965942   | WLVVYGVLGCGLLYVAIFCIFW      |
| GIBBON            | Nomascus leucogenys           | KM_003253970    | WLVVYGVLGCGLLYVAIFCIFW      |
| MARMOSET          | Callithrix jacchus            | KM_008999126    | WLVVYGVLGCGLLYVALSWF        |
| TARSIER           | Tarsius syrichta              | KM_008874308    | WLVVYGVLGVLSEMVALCTFW       |
| TREE SHREW        | Tupaiota chinensis            | KM_00615077     | WLVVYGVLGVLSEMVALAFNY       |
| RAT               | Rattus norvegicus             | NP_037253.1      | WLPVLVYGVLGCGLLYVTLICIW     |
| WOODCHUCK         | Marmota monax                 | AAF36590.1       | WTLVYFSQVLGSLYLMLCILW       |
| RABBIT            | Oryctolagus cuniculus         | XP_017198104.1   | WLVVYGAVLFGSMLVVALSCSW      |
| DOG               | Canis familiaris              | NP_001003087.2   | WLVVYGAVLFGSMLVVALCAYN      |
| CAT               | Felis catus                   | NP_001009844.1   | WLVVYGGLGFLSMLVAIGC         |
| SIBERIAN TIGER    | Panthera tigris altaica       | XP_007900030.2   | WLVVYGGLGFLSMLVAICAWC       |
| FERRET            | Mustela putorius              | KM_03062342     | WLVVYGVLGVLSEMVALAFNY       |
| GIANT PANDA       | Alluropoda melanoleuca        | KM_002919949    | WLVVYGVLGVLSEMVALAFNY       |
| ALPACA            | Vicugna pacos                 | XP_006202628.1   | WLVVYGVLGVLSEMVALAFNY       |
| SHEEP             | Ovis aries                    | KM_012111193    | WLVVYGVLGVLSEMVALCNCW       |
| BROWN FLYING FOX  | Pteropus alecto               | XP_006921437.1   | WLVVYGVLGVLSEMVALCNCW       |
| BROWN BAT         | Myotis lucifugus              | KM_01441937     | WLVVYGVLGVLSEMVALCNYW       |
| HORSE             | Equus caballus                | KM_001100179    | WLVVYGVLGVLSEMVALCNYW       |
| WHITE RHINOCEROS  | Ceratotherium simum           | XP_008426878.1   | WLVVYGVLGVLSEMVALCNYW       |
| AFRICAN ELEPHANT  | Loxodonta africana            | KM_00340929     | WLVVYGVLGVLSEMVALCNYW       |
| ARMADILLO         | Dasypus novemcinctus          | KM_012523351    | WLL-ALLGLGSLYVCLAFLFWY      |
| KILLER WHALE      | Orcinus Orca                  | XP_004262874    | WLVVYGVLGVLSEMVALCNCW       |
| BOTTLENOSE DOLPHIN| Tursiops truncatus            | XP_004131514    | WLVVYGVLGVLSEMVALCNCW       |
| GREY-TAILED OPOSSUM| Monodelphis domestica        | XP_007501953.1   | WPVVAALCFAFSMLVYAFCNW       |
| TASMANIAN DEVIL   | Sarcophilus harrisi           | KM_003766002    | WAVAALCFLASMLEAFCNW         |
| KOALA             | Phascolarctos cinereus        | XP_020828504.1   | WAVAALCFLASMLEAFCNW         |
| STARLING          | Sternum vulgaris              | KM_014880339    | WILGTVGLSLYVLCAINVY          |
| GREAT TIT         | Parus major                   | XP_013549801.1   | WLIATIALILGLSMVALAFNY       |
| CHICKEN           | Gallus gallus                | NM_053511        | WVAWATGALGSMVLAFNY          |
| TURKEY            | Meleagris gallopavo           | CAP04926.1       | WVAWATGALGSMVLAFNY          |
| DALMATIAN PELICAN | Pelecanus crispus             | XP_009488081.1   | WTVTAVGLFLSMVALAFNY         |
| BALD EAGLE        | Haliaeetus leucocephalus      | XP_010563861.1   | WIMAVATGLFSLVAFNY           |
| RUFF              | Calidris pugnax              | KM_014954243    | WIMAVATGLFSLVAFNY           |
| ALLIGATOR         | Alligator mississippiensis    | KM_006261310    | WLMMAAIGFAKSSILVACIHCS      |
| SALTWATER CROCODILE | Crocodylus porosus           | XP_023410247    | WLMMAAIGFAKSSILVACIHCS      |
| GREEN SEA TURTLE  | Chelonia mydas                | XP_007071905.1   | WLVVYGVLGVLSEMVALAFVWC      |
| SOFTSHELL TURTLE  | Pelodiscus sinensis           | KM_014577908    | WPIJVLQFLGLVSLYVAIFCIFW     |
| CLAWED FROG       | Xenopus tropicalis            | KM_012969996    | WLPVILVQVWGLSMVALAFNY       |
| BURMESE PYTHON    | Python bivittatus             | XP_025031234.1   | VATIGPLLGFLVSLVAAFCVW       |
| PIT VIPER         | Protobothrops mucrosquamatus  | KM_015819766.1   | MPNAIPLGFVFSMLIAAYYY       |
| ANOLE             | Anolis carolinensis           | KM_008114951    | MLMAIAIFIFIYVSVTAAFCVY     |
| BEARDED DRAGON    | Pogona vitticeps              | XP_020670148.1   | WTVGTLVGLGIAFECYVW         |
| RAINBOW TROUT     | Oncorhynchus mykiss           | NP_002118604.1   | WMMLGFYWTTGLVVLVFAYV       |
| ATLANTIC SALMON   | Salmo salar                  | AC133726.1       | WMMLGFYWTTGLVVLVFAYV       |
| PUFFER FISH       | Takifugu rubripes             | NP_002167008.1   | GWMMLFLGVLGSMVLVAAACIW      |
| KILLIFISH         | Fundulus heteroclitus         | XP_02712711.2    | WIIIMMVLAGLGLSVAIFAIFW      |
| COELACANTH        | Latimeria chalumnae           | KM_014490127    | WLPICLATTFLGAMTAMCYNLQ      |

Supplemental Table 1. The YxxxxT motif in the CD28 TM domain is evolutionarily conserved. The TM domain sequences of CD28 homologs from 51 different species are shown for mammals, marsupials, birds, reptiles, and fish. The conserved YxxxxT motif is highlighted in green and the single T variant in the alpaca is highlighted in yellow.
Supplemental Figure 1. Analysis of intermolecular FRET by flow cytometry. (A) Diagram of the CD28 dimer fused at the C-terminus to CER and YFP. When the tails of a CER-YFP containing dimer are within 100Å of each other, CER acts as a donor and YFP as an acceptor for FRET when excited by a 405nm laser and sensitized FRET emission is detected at 550nm. (B) CD28 deficient DO11.10 CD4 T cell hybridoma cells were retrovirally transduced with either CD28-CER, CD28-YFP, or both and were gated on expressing cells. Left, cell expressing only CD28-CER (blue box); middle, cell expressing only CD28-YFP (yellow box); right, cells expressing both CD28-CER and CD28-YFP (green box). (C) Fluorescent profiles of CD28-CER, CD28-YFP, or dual expressing cells as gated in (B) for the CER, YFP, or FRET channels. (D-F) Relative FRET efficiency (FRETe) was calculated and plotted by YFP or CER for dual expressing cells (D) or by YFP for YFP only cells (E) or CER for CER only cells (F).
Supplemental Figure 2. FRET analysis of hCD25 chimeras expressed in T cells. Relative FRET efficiency (FRET_e) was calculated for T cells expressing YFP and CER hCD25 chimeras containing the WT mouse CD28 TM domain (A) hCD25 chimeras containing the mouse CD28 Y7/LL mutated TM domain (B) or unmodified hCD25 (C). Relative FRET_e was plotted by YFP or CER for dual expressing cells (YFP + CER) or by YFP for YFP only cells or CER for CER only cells.
| Common name       | Latin name                  | Accession Number | Sequence                      |
|-------------------|-----------------------------|------------------|-------------------------------|
| MOUSE             | Mus musculus                | NM_009843        | WILAVA2SLGFSSFLYAVLS          |
| HUMAN             | Homo sapiens                | NP_05205.2       | WILAVARSSGLFSFLYAVLS          |
| BABOON            | Papio anubis                | NP_001106104.1   | WILAVA2SSGLFSFLYAVLS          |
| MACAQUE           | Macaca fascicularis         | XP_005574071.1   | WILAVASGLFSFLYAVLS            |
| GREEN MONKEY      | Chlorocebus sabaeus         | XP_007964134.1   | WILAVASGLFSFLYAVLS            |
| GIBBON            | Nomascus leucogenys         | XP_003254019.1   | WILAVASGLFSFLYAVLS            |
| MARMOSET          | Callithrix jacobus          | XP_002749715.1   | WILAVASGLFSFLYAVLS            |
| TARSIER           | Tarsius syrichta            | XP_008072501.1   | WILAVASGLFSFLYAVLSS           |
| TREE SHREW        | Tupaja chinensis            | XP_006153140.1   | WILAVASGLFSFLYAVLS            |
| RAT               | Rattus norvegicus           | NM_031674.1      | WILAVASGLFSFLYAVLS            |
| MARMOT            | Marmota marmota             | XP_015336776.1   | WILAVASGLFSFLYAVLS            |
| RABBIT            | Oryctolagus cuniculus       | NP_001076154.1   | WILAVASGLFSFLYAVLS            |
| DOG               | Canis familiaris            | NP_001003016.1   | WILAVASGLFSFLYAVLS            |
| CAT               | Felis catus                 | NM_001009236     | WILAVASGLFSFLYAVLS            |
| SIBERIAN TIGER    | Panthera tigris altaica     | XP_006921439.1   | WILAVASGLFSFLYAVLS            |
| FERRET            | Mustela putorius            | XP_004764261.1   | WILAVASGLFSFLYAVLS            |
| GIANT PANDA       | Ailuropana melanoleuca      | XP_002919994.1   | WILAVASGLFSFLYAVLS            |
| ALPACA            | Vicugna pacosapaca          | XP_006205269.1   | WILAVASGLFSFLYAVLS            |
| SHEEP             | Ovis aries                  | NP_001009214.1   | WILAVASGLFSFLYAVLS            |
| BROWN FLYING FOX  | Pteropus alecto             | XP_006921439.1   | WILAVASGLFSFLYAVLS            |
| BROWN BAT         | Myotis lucifugus            | XP_006082485.1   | WILAVASGLFSFLYAVLS            |
| HORSE             | Equus caballus              | XP_023478240.1   | WILAVASGLFSFLYAVLS            |
| AFRICAN ELEPHANT  | Loxodonta africana          | XP_003406166.1   | WILAVASGLFSFLYAVLS            |
| ARMADILLO         | Dasypus novemcinctus        | XP_004453677.1   | WILAVASGLFSFLYAVLS            |
| KILLER WHALE      | Orcinus Orca                | XP_004262872.1   | WILAVASGLFSFLYAVLS            |
| BOTTLENOSE DOLPHIN| Tursiops truncates          | XP_019794455.1   | WILAVASGLFSFLYAVLS            |
| OPOSSUM           | Monodelphis domestica       | XM_007501888.2   | WILAVASGLFSFLYAVLS            |
| TASMANIAN DEVIL   | Sarcophilus harrisii        | XM_003766000.2   | WILAVASGLFSFLYAVLS            |
| PLATYPUS          | Ornithorhynchus anatinus     | XM_001514865.2   | WILAVASGLFSFLYAVLS            |
| STARLING          | Sturnus vulgaris            | XP_014735824.1   | WVLGATASGFLLSIISAILVG         |
| GREAT TIT         | Parus major                 | XP_015490579.1   | WVLGATASGFLLSIISAILVG         |
| CHICKEN           | Gallus gallus              | NM_001040091.1   | WVLGATASGFLLSIISAVG           |
| TURKEY            | Meleagris gallopavo         | XM_019617059.1   | WVLGATASGFLLSIISAIW            |
| DALMATIAN PELICAN | Pelecanus crispus          | XP_009478785.1   | WVLGATASGFLLSIISAVG           |
| BALD EAGLE        | Haliaeetus leucocephalus    | XP_010563675.1   | WVLGATASGFLLSIISAIW           |
| RUFF              | Calidris pugnax             | XP_014809732.1   | WVLGATASGFLLSIVISAILVG        |
| PIT VIPER         | Protobothrops mucrosquamatus| XM_015818576.1   | WILVPVSGLGSILIFTIMV           |
| ANOLE             | Anolis carolinensis         | XM_008125603.2   | WISVMIASGLGSILITYMY           |
| ALLIGATOR         | Alligator mississippiensis  | XM_006267243.3   | WILGAASGLSSILLISAFLLS         |
| SALTWATER CROCODILE| Crocodylus porosus          | XP_019409174.1   | WILGAASGLSSILLISAFLLS         |
| GREEN SEA TURTLE  | Chelonia mydas              | XM_007071927.1   | WILGAASGLSSILLICAVLSS         |
| BURMESE PYTHON    | Python bivittatus           | XP_007421444.2   | WILILVASGLGSILITIFISS         |
| BEARDED DRAGON    | Pogona vitticeps            | XP_020634619.1   | IIVTVVAVVGFLLSVIFSITVYIT      |
| CLAWED FROG       | Xenopus tropicalis          | XM_018097345.1   | ILLVVCVMFLSFSIFAVLLC          |

Supplemental Table 2. CTLA4 also contains an evolutionarily conserved YxxxT motif. The TM domain sequences of CD28 homologs from 44 different species are shown for mammals, marsupials, birds, and reptiles. The conserved YxxxT motif is highlighted in green, conservative aa variants (T/S and Y/F) are highlighted in yellow, and the single nonconservative aa variant in the bearded dragon (T/L) is highlighted in red.
Supplemental Figure 3. C123S disrupts CD28 disulfide bond formation. Retroviruses expressing WT and C123S CD28 containing a C-terminal HA tag were transfected into CD28-deficient, DO11.10 T cells (KO) and lysates were analyzed by nonreducing and reducing SDS-PAGE and western blotting with anti-HA Ab.