Supplementary Material 1: Materials and methods

S1.1 Cell culture

CHO-S cells in suspension were cultured in shaker incubators in PowerCHO-2CD medium (Lonza, Switzerland) containing 8mM Ultraglutamine (Lonza, Switzerland) and HT supplement (Lonza, Switzerland). F9 cells were cultured on 0.1% gelatin-coated tissue flasks in DMEM (Gibco) (Dulbecco's modified Eagle Medium) supplemented with 10% FCS (Fetal Calf Serum), CT26 cell were cultured with DMEM (Gibco) supplemented with 10% FCS, A20 cell were cultured in RPMI supplemented with 10% FCS, 5mM Ultraglutamine and 50µM β-Mercaptoethanol. If not otherwise specified cells were incubated at 37 °C and 5% CO_2.

S1.2 Cloning of fusion proteins

For the cloning of hIL12-F8-F8 the gene codifying for the F8 moiety was PCR amplified from F8-mIL7 (1), whereas the genes encoding hp40 and hp35 were PCR amplified from the heterodimeric form of F8hIL12 (F8-hup35/hup40-F8) (2). In a first step, the hp40 gene was PCR amplified using the primers SW3 (5'-TCCTGTTCCTCGTCGCTGTGGCTACAGGTGTGCACTCGATATGGGAACTGAAGAAAG ATGTTTATGTC-3') and SW4 (5'-CGATCCGCCACCGCCAGAGCCACCTCCGCCTGAACCGCCTCCACCACTGCAGGGCAC AGATGCCCATTC-3'); the PCR product obtained was amplified again using the primer SW1 and SW4 in order to append the leader sequence and a NheI restriction site at the 5' end and a 45 nucleotides linker at the 3' for the annealing on the hp35 gene. The hp35 gene was PCR amplified using the primers SW5 (5'-GGTGGAGGCGGTTCAGGCGGAGGTGGCTCTGGCGGTGGCGGATCGAGAAACCTCCC CGTGGCCACT-3') and SW6 (5'-CACCTCACCTCCATCAGCGCTACCGGAAGCATTCAGATAGCTCAT-3'), which append a 45 nucleotides linker at the 5' for the annealing on hp40 and an 18 nucleotides linker at the 3' for annealing on F8. The first F8 diabody gene (named F8A) was PCR amplified with the primers SW7 (5'-TGCTTCCGGTAGCGCTGATGGAGGTGAGGTGCAGCTGTTGGAGTCT-3') and SW8 (5'-

GGACGATGAGCCGGAAGAGCTACTTCCGGATGAGGAAGATTTGATTTCCACCTTGGT CCCTTG-3') in order to add a 18 nucleotides linker at the 5' end for the annealing on hp35 and a 45 nucleotides linker at the 3' containing a BspEI restriction site. The F8 diabody gene was also amplified with the primers SW9 (5'-TCATCCGGAAGTAGCTCTTCCGGCTCATCGTCCAGCGGCGAGGTGCAGCTGTTGGAG TCT-3') and SW1 (5'-TTTTCCTTTTGCGGCCGCTCACTATTTGATTTCCACCTTGGTCCCTTG-3'), which append at the 5' end a 45 nucleotides linker containing a BspEI restriction site and at the 3' end a double stop codon and a NotI restriction site: this second PCR product was named F8B.

In a second step hp40 and hp35 were assembled by PCR using the primers SW2 (5'-CTCCGCTAGCGTCGACCATGGGCTGGAGCCTGATCCTCCTGTTCCTCGTCGCTGTGGC -3') and SW6, whereas hp35 and F8A were PCR assembled using the primers SW5 and SW8. The PCR products hp35-F8A and F8B were digested with BspEI and ligated together in order to obtain hp35-F8A-F8B. Since hp35 contains a BamHI restriction site the PCR product hp40-hp35 and the assembled hp35-F8A-F8B were digested with BamHI and ligated together. The final assembly was then double-digested NheI/NotI and ligated into the mammalian cell expression vector pcDNA3.1(+) (Invitrogen).

For cloning hIL12-F8diabody, hp35 gene was PCR amplified with the primers SW5 and SW6, whereas F8 gene was amplified using the primers SW7 and SW1 (the generated fragment was named F8C). The PCR products hp35 and F8C were assembled by PCR using the primers SW5 and SW1. The previously described F8hIL12 was double-digested BamHI/NotI: the vector containing hp40 and hp35/BamHI was gel extracted and named pcDNA3.1(hp40-hp35/BamHI). Subsequently, the PCR product hp35-F8C was double-digested BamHI/NotI and ligated into pcDNA3.1(hp40-hp35/BamHI).

For the cloning of mIL12-F8-F8 the gene for F8 in diabody format was PCR-amplified from the previously described clone of F8-mIL7 immunocytokine (1) using primers NP68 (5'-CTCATCCGGAAGTAGCTCTTCGGGATCCTCGTCCAGCGGCGAGGTGCAGCTGTTGGA GTCTGG-3') which appends a 15 amino acid linker containing a BamHI restriction site at the N-terminus of the antibody moiety and SW1 that contains two stop codons and a NotI restriction site at the 3'. The double-digested BamHI/NotI PCR product was cloned into the mammalian cell expression vector pcDNA3.1(+) (Invitrogen). The gene for mp40 was PCR amplified from the

previously described L19-IL12 clone (3) using primers NP59 (5'-TCCTGTTCCTCGTCGCTGTGGCTACAGGTGTGCACTCGATGTGGGAGCTGGAGAAAG ACGTT-3') containing the C-terminus of the leader sequence and NP60 (5'-tgtgttccctgcagggtccgatccGGTGGAGGCGGTTCAGGCGGAGGTGGCTCT-3'), which appends a 15 amino acid linker at the C-terminus of the mp40 subunit. The gene for mp35 was PCR amplified from the previously described L19-IL12 clone (3) using primers NP61 (5'-GGTGGAGGCGGTTCAGGCGGAGGTGGCTCTGGCGGTGGCGGATCGAGGGTCATTCC AGTCTCTGGACCT-3'), which inserts a 15 amino acid linker at the N-terminus of the mp35 subunit and NP62 (5'-ccagagaaaaGctTaaacattatt-3'), which inserts a HindIII restriction site in the mp35 gene. The mp40 and mp35 genes were PCR-assembled using primers SW2, containing a NheI restriction site upstream of the leader sequence and NP62. The double-digested NheI/HindIII PCR product was cloned into the mammalian cell expression vector pcDNA3.1(+) (Invitrogen). The gene for mp35 was PCR amplified from the previously described L19-IL12 clone (3) using primers NP63 (5'- CCAGAGAAAAGCTTAAACATTATT-3') and NP64 (5'-GTGATGGGCTATCTGAGCTCCGCCGGTAGCGCTGATGGAGGT-3'), which appends a 6 amino acid linker at the C-terminus of the mp35 subunit. The gene for F8 diabody was PCR amplified from the previously described clone of F8-mIL7 immunocytokine (4) using primers NP65 (5'- GGTAGCGCTGATGGAGGTGAGGTGCAGCTGTTGGAGTCTGGG-3'), which inserts a 6 amino acid linker at the N terminus of the diabody and NP66 (5'-CAAGGGACCAAGGTGGAAATCAAATCTTCCTCATCCGGAAGTAGCTCTTCGGGATCC TCGT-3'), which inserts a 15 amino acid linker containing a BamHI restriction site at the C terminus of the antibody moiety. The mp35 and F8 diabody genes were PCR-assembled using primers NP63 and NP66. The double-digested HindIII/BamHI PCR product was cloned into the previously constructed pcDNA3.1(+) vector containing the F8 diabody gene.

Finally, the mp40-mp35 gene was obtained by NheI/HindIII double-digestion of the pcDNA3.1(+) vector containing the right sequence and inserted into the previously constructed pcDNA3.1(+) vector containing the mp35-F8 diabody – F8 diabody gene.

For the cloning of mIL12-KSF-KSF, the KSF diabody gene (5, 6) was PCR-amplified with primers NP65 and NP71 (5'-AATCGGTCTGGAATGCCTGAGGGC-3') which removes the BamHI restriction site present in the KSF sequence and with primers NP70 (5'-GCCCTCAGGCATTCCAGACCGATT-3') and NP67 (5'-GACCAAGCTGACCGTCCTAGGCTCTTCCTCATCCGGAAGTAGCTCTTCGGGATCCTC

GT-3'), which inserts a 15 amino acid linker containing a BamHI restriction site. The genes encoding for KSF were then PCR assembled. The gene for mp35 was PCR-amplified using primers NP63 and NP64, it was then PCR-assembled with the KSF diabody gene using primers NP63 and NP67. The double-digested HindIII/BamHI PCR product was cloned into the previously constructed pcDNA3.1(+) vector containing the mp40-mp35 gene.

The KSF gene was PCR-amplified from the new vector using primers NP68 and NP53 (3'-ATGCGGCCGCTCATTAGCCTAGGACGGTCAGCT-5'), which appends two stop codons and a NotI restriction site at the 3' end. The double-digested BamHI/NotI PCR product was cloned into a pcDNA3.1(+) vector. Finally the mp40-mp35-KSF gene was obtained by NheI/BamHI double-digestion of the pcDNA3.1(+) vector containing the right sequence and inserted into the previously constructed pcDNA3.1.(+) vector containing the KSF diabody gene.

All the restriction enzymes and the T4 Ligase used were from New England Biolabs (USA).

Expand High Fidelity PCR System from Roche (Germany) was used for all the PCR reactions.

References:

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