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CDNA CLONING OF THE NKTα AND NKTβ CHAIN GENES

A bulk human NKT cell line was generated by autologous stimulation of PBMCs with α-GalCer. RNA was extracted with TRIzol (Invitrogen) and reverse transcribed using oligo-dT and Superscript II (Invitrogen). cDNA encoding the variable and a portion of the constant extracellular domains of the NKTα (TRAV10) and the NKTβ (TRBV25-1) chains was amplified by PCR using the oligonucleotides: 5'-cgccatatgaaaaaccaagtggagcag-3' and 5'-gtacacggcaggatccgggttctggat-3'; and 5'-cgccatatg-gaagctgacatctaccag-3' and 5'-ggtgtgggagatctctbcttctga-3', respectively, and subsequently cloned into pGEM Easy (Promega). Genes were verified by sequencing, before transfer as NdeI–BamHI or NdeI–BglII fragments into either of two pET30 expression vectors (Novagen), containing the sequences encoding the Cα or Cβ domains (1). Codons for residue Thr48 (Cα constant domain) and residue Ser57 (Cβ constant domain) were mutated to encode cysteine by QuikChange Site-Directed Mutagenesis (Invitrogen). The genes were thus predicted to encode the extracellular domains of NKTα and NKTβ, terminating immediately before the cysteines that normally form an interchain disulphide bond. Instead, interchain disulphide-bond linkage was mediated through the two introduced constant-domain cysteines. Genes encoding the mutated NKT15'98AAA100' and NKT15/LC13 (CDR3β swap) β chains were engineered by QuikChange Site-Directed Mutagenesis (Invitrogen).

CLONING AND EXPRESSION OF CD1D

Human CD1d cDNA was derived from peripheral blood mononuclear cell RNA extracted with TRIzol (Invitrogen), and reverse transcribed using oligo-dT and Superscript II (Invitrogen). cDNA encoding the three extracellular domains of human CD1d (encoding residues 1-275) was amplified by PCR using the oligonucleotides: 5'-tcccccggggtcccgcaaaggcttttccccctcc-3' and 5'-cggggtaccttagcagtgatggtgatgatgatgccgcggccagtagaggacgatgtcc-3'. The codon for residue Cys10 was mutated to encode serine by QuikChange Site-Directed Mutagenesis (Invitrogen). Mouse CD1d cDNA was a gift from M. Kronenberg. Soluble mouse and human CD1d were co-expressed with β microglobulin (β2M) from the same source using the pFastBac Dual expression vector and the BAC-TO-BAC baculovirus expression system (Invitrogen). The mCD1d protein was designed for downstream biotinylation through the incorporation of a BirA tag at the COOH terminus of the protein (2), whereas the hCD1d was biotinylated via an engineered free cysteine at the COOH terminus. Hexa-histidine–tagged protein was expressed in HIGH FIVE insect cells and harvested 3 d after infection. Secreted, recombinant protein was dialyzed extensively in either 10 mM Tris, pH 8, or PBS before purification using Ni-agarose beads (QIAGEN). The Ni-agarose beads were washed extensively in a buffer containing 10 mM tris pH 8, 20 mM imidazole and 0.3 M NaCl and prior to purification the dialysate containing the secreted CD1d was spiked with 20 mM imidazole. CD1d protein was bound to the Ni-agarose beads, washed, and eluted in a buffer containing 10 mM Tris, pH 8, 250 mM imidazole, 0.3 M NaCl.

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