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Cloning of VH147 H and L chains and generation of VH147 and mk147 tg mice

RNA was isolated from the KRN5-147 hybridoma using the RNeasy kit. cDNA was generated using primers in the constant region, Cγcross or Cκ, and amplified using a degenerate 5′ primer, VH5′1 and Cγcross or L3′ and Cκ, for the H and L chains, respectively (Roark, J.H., C.L. Kuntz, K.A. Nguyen, A.J. Caton, and J. Erikson. 1995. *J. Exp. Med.* 181:1157–1167.). The resulting PCR products were directly sequenced using a terminator mix (Big Dye; Applied Biosystems) and the Cγcross or Cκ reverse primers. To assist in the cloning of the H and L chain genes, the H and L chain protein molecules were sequenced. Supernatants containing KRN5-147 Ig were purified on a protein G column, the H and L chains were separated using SDS-PAGE, and amino terminal sequencing was performed using Edman degradation. The amino acid sequences were then blasted against the mouse genomic sequence database to design primers for use in PCR to clone the KRN5-147 H and L chain genomic fragments.

A 5.1-kb fragment containing the rearranged H chain gene and a 6-kb fragment containing the rearranged L chain gene were amplified. These fragments include ?1.5 kb upstream of the leader exon to ensure the expression fidelity of the transgenes. The H and L chain fragments were then cloned into the EcoRI site in a H chain expression vector and the NotI and HpaI sites in an L chain expression vector containing the IgM or Igκ constant regions, as well as downstream regulatory elements, provided by D. Nemazee (The Scripps Research Institute, La Jolla, CA) (Nemazee, D.A., and K. Burki. 1989. *Nature*. 337:562–566.). The H chain transgene construct uses the BALB/c IgM constant region, which is of the IgM^a allotype. This will allow for the distinction of the tg Ig from the endogenous C57BL/6 IgM^b Ig. The VH147 H chain transgene has an identical V region to a germline gene found in the C57BL/6 chromosome 12 contig (nt 110627.1, region 231977–232270 minus strand) and uses D_HQ52 J_H3 segments, and the L chain transgene uses Vκ14-111/Jκ2 as determined by comparison to the National Center for Biotechnology Information Ig BLAST and IMGT/V-Quest databases. The transgene constructs were confirmed by sequencing and transfection together into the Ig^- NSO cell line. The constructs were then linearized by cutting with SalI and PvuI or NotI and SalI for the H and L chains, respectively, and injected either as H chain alone or H+L chains into pronuclei from C57BL/6 mice in the Washington University Department of Immunology transgenic/knockout core facility (run by M. WHite, Washington University, St. Louis, MO). The H and L chains cointegrated; therefore, both H alone and H+L mice were screened for the presence of the transgene by PCR using the primers VH147 3′ (CATTCCTACCTCCACTCTGTCCCTC) and psv2neo5′ (CAGGGTTATTGTCTCATGAGCGG). H chain tg protein expression and allelic exclusion of endogenous Ig was determined by flow cytometry on peripheral blood using anti-IgM^a–FITC (DS-1) and anti-IgM^b–PE (AF6-78; BD Biosciences). Five founder lines of H chain alone (VH147) and four of H+L chain (mk147) tg mice were obtained. Two lines each of VH147 (3902 and 3907) and mk147 (7451 and 7452) were crossed onto a B6.K background and expanded for further analysis. Results from the two founder lines were similar; therefore, experiments are presented only from the 3907 line for VH147 and the 7452 line for mk147.

Recombinant GPI-his protein

The coding sequence of GPI was amplified from B6.K spleen cDNA, tagged at the 3′ end with a 6-histidine tag, cloned into the pET-23 (+) vector (termed GPI-his), and confirmed by sequencing. Cultures of BL21 *E. coli* pET-23 GPI-his were grown at 37°C with shaking to OD 0.6 and then induced for 3 h with 1 mM IPTG (Sigma-Aldrich). GPI-his protein was recovered using Talon resin (CLONTECH Laboratories, Inc.) and eluted using 250 mM imidazole (Sigma-Aldrich). The eluted protein was then dialyzed into 20 mM piperazine, filtered, and further purified using an FPLC monoS column. Purified GPI-his was dialyzed into 5 mM citric acid buffer (pH 7), lyophilized, and stored at −70°C until further use.

Antibodies used for flow cytometry

FITC-conjugated CD1d (clone 1B1; eBioscience), PNA (Vector Laboratories), CD11b (M1/70), CD21/35 (7G6), CD22 (Cy34.1), CD24 (M1/69), CD44 (IM7), CD80 (16-10A1; BD Biosciences), CD69 (H1.2F3), CD86 (GL1; Biolegend), PE-conjugated CD23 (B3B4), MHC class II (M5/114.15.2), B220 (RA3-6B2), CD5 (53–7.3; BD Biosciences), APC- or PE-conjugated B220 (RA3-6B2; Biolegend), or streptavidin-PE (Invitrogen) or -APC (Vector Laboratories) were used for flow cytometry.

Immunofluorescence

Spleens were suspended in OCT, frozen in 2-methyl-butane cooled with liquid nitrogen, sectioned, and fixed with acetone, and sections were stored at −20°C before staining. The sections were blocked with 5% normal goat serum (Vector Laboratories)/0.1% Tween 20 and then stained with anti-CD22–FITC and anti–MadCAM-1 (BD Biosciences), or anti-B220–PE and PNA-biotin (Vector Laboratories) or GPI-his-biotin. Anti–Rat_{Ig}-$Alexa_{546}$ (Invitrogen) or streptavidin-FITC was used as a secondary antibody. Immunofluorescence was visualized under a fluorescent microscope (Eclipse E800; Nikon), and pictures were taken with a digital camera (DXM1200; Nikon) and ACT-1 software (Nikon).

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