Characterization of the genomic, cDNA and Amino Acid sequences of PILAR.

The predicted Amino Acid sequence of the following genes encoding a C-type Lectin domain (Smart #00034; http://smart.embl-heidelberg.de) were aligned with ClustalW software (http://www.ebi.ac.uk/clustalw/) to create a pattern with the residues conserved in at least 6 of 9 sequences and coded by a single exon: AF097358, AF461811, AY486483,Z22576, AF175206, AY358499, DQ049594, HSA133532 and AF133299.

Genomic sequences at chromosome 12p12-13 were translated into the 6 possible ORFs by using ORF Finder software (http://www.ncbi.nlm.nih.gov/gorf/gorf.html) and were scanned for the presence of the pattern with PattinProt software (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa_pattinprot.html).

RACE-PCR was performed with the human spleen marathon-ready cDNA kit (Clontech, Carlsbad, CA), following manufacturer's instructions. The following internal primers were used: Forward.Outer: 5′-CAGGGGACTGGCTTGGAGTGAGAGAT-3′; Forward.Inner: 5′-TCTGATGATACCAGAAATTGGACAGCCAGT-3′; Reverse.Outer: 5′-ATGGGCCCTCACCAGAGGTTCCGTAT-3′; Reverse.Inner: 5′-TGCTGCAAATCCACTTGATATCAATAAA-3′. The sequence was deposited at the Genbank (Accession#EF127467).

Real-Time Quantitative PCR.

PILAR expression was analyzed by TaqMan PCR analysis as described previously (8, 22, 23). The *PILAR* system consisted of the following primers: PILAR.F, 5′-GTTAGCGCCTTGCCATGATTA -3′, PILAR.R, 5′- AGGAAGCACATAAGGCCAATCTT -3′; and the probe PILAR.P, 5′-(FAM) CTTCATACATCGGATAGTTCCCAAGTTGATACA (TAMRA)-3′. We normalized the cDNA load to human *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) with primers GAPDH F: 5′-CCTGCACCACCAACTGCTTA -3′ and GAPDH R: 5′-CATGAGTCCTTCCACGATACCA-3′ and the probe GAPDH.P: 5′(FAM)CCTGGCCAAGGTCATCCATGACAAC(TAMRA)-3′.

Generation of soluble fusion proteins

The cDNA that encodes the extracellular domain of CD161 (160 AA) was PCR amplified from mRNA isolated from human spleen using the primers CD161.F: aagcttATACAGAAATCATCAATAGAAAAATGC; and CD161.R: ggatccGAGTCAGGATACACTTTATTTCTCACA, which introduced restriction sequences for HindIII and BamHI. The fragment was subcloned into pSecTag (Invitrogen) in frame with a human Ig leader sequence (upstream) and a Myc epitope (downstream), followed by a 6 x His-tag, to generate the constructs Ig-PILAR-Myc and Ig-CD161-Myc. The sequence amplified with primers BTN.F: 5′-aagctTCAGTTTTCTGTGCTTGGAC-3′ and BTN.R: 5′-gaattcTCTGGGCGCTCCTGAAGAAG-3′, corresponding to the extracellular domain of BTN3A1 (Genbank#NM_007048) was cloned, expressed and purified in the same manner and used as a negative control.

293 cells were independently transfected with the plasmids pIg-CD161-Myc or pIg-BTN-Myc using Lipofectamine (Invitrogen), following manufacturer's instructions. Supernatants from the 2 transfections were harvested at day 3 and 7 and the secreted fusion proteins were purified from culture medium by using a standard Ni cloumn (GE Healthare), following manufacturer's instructions. After dialysis against PBS protein concentration determined by the Bradford assay.

ELISPOT analysis

For ELISPOT analysis, $2x10^6$ PBMCs/ml from an $A2^+$ donor were incubated for 7 days (10:1) ratio with CEF peptide pool-pulsed (2 μg/ml) $A2^+$ aAPCs or autologous monocyte-derived dendritic cells (generated by incubating magnetically purified $CD14^+$ cells for 7 days with Granulocyte–macrophage colony stimulating factor (20 ng/ml, Peprotech) and IL-4 (50 ng/ml, R&D)). Interferon-γ ELISPOT was performed on day 8 against irradiated $A2^+$ aAPCs or autologous monocyte-derived dendritic cells (10:1 ratio), pulsed with 2 μg/mL antigens.

Flat-bottomed, 96-well nitrocellulose-lined plates (Millipore MultiScreen, Millipore, Bedford, MA, USA) were coated with IFN-γ mAb (MD-1; eBioscience, San Diego, CA) and incubated overnight at 4°C. After washing with Coating Buffer (eBioscience, San Diego, CA), plates were blocked with 10% FBS serum for 2 h at 37°C. Effector and target cells were incubated together for 20 h in RPMI medium 1640 supplemented with 10% FBS. After incubation, the plates were thoroughly washed with 0.05% Tween 20 in PBS to remove cells, and biotinylated secondary IFN-γ mAb (4S.B3; eBioscience) was added to each well. After incubation for 2 h at 37°C, the plates were washed and developed with Avidin-horseradish peroxidase (eBioscience) for 1 h at room temperature. After washing, fresh substrate (3-amino-9-ethyl carbazole, Sigma, St. Louis, MO) was added and the plates incubated for approx. 20 min.

Nucleofection of T cells

Unstimulated T cells were nucleofected using the Human T Cell Nucleofector Kit (Amaxa Inc., Gaithersburg, MD) and a pcDNA3.1 plasmid encoding the PILAR open-reading frame, following the manufacturer's protocol. Control lymphocytes were treated in an identical manner in the absence of the expression plasmid. Forty-eight hours post nucleofection the cells were stained for PILAR and analyzed by flow cytometry.