

ILDR2 is a Novel B7-like Protein that Negatively Regulates T Cell Responses

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Abbreviations: CIA, collagen-induced arthritis; CTLA-4, cytotoxic T lymphocyte-associated protein 4; DC, dendritic cell; ECD, extracellular domain; IBD, inflammatory bowel disease; IgSF, immunoglobulin superfamily; IgV, V-type immunoglobulin domain; IHC, immunohistochemistry; ILDR1, Immunoglobulin-like domain-containing receptor 1; ILDR2, Immunoglobulin-like domain-containing receptor 2; LSR, lipolysis-stimulated receptor; Mφ, macrophage; PD-1, Programmed death 1; PD-L1, programmed death ligand 1; PVR, poliovirus receptor; RA, rheumatoid arthritis; R-EAE, relapsing-remitting experimental autoimmune encephalomyelitis; Tck, cytokine activated T cells; TIGIT, T cell immunoreceptor with Ig and ITIM domains, tTJs, tricellular tight junctions; VISTA, V-domain Ig suppressor of T cell activation

Abstract

The B7-like protein family members play critical immunomodulatory roles and constitute attractive targets for the development of novel therapies for human diseases. We identified ILDR2 as a novel B7-like protein with robust T cell inhibitory activity, expressed in immune cells, and in immune privileged and inflamed tissues. The ILDR2-Fc fusion protein, which binds to a putative counterpart on activated T cells, showed beneficial effect in the collagen-induced arthritis model, and abrogated the production of pro-inflammatory cytokines and chemokines in autologous synovial-like co-cultures of macrophages and cytokine stimulated T cells. Collectively, these findings point to ILDR2 as a novel negative regulator for T cells, with potential roles in the development of immune-related diseases, including autoimmunity and cancer.

51 **Introduction**

52 The immune system is tightly regulated by a network of positive and negative immune
53 regulators, including the B7/CD28 family of proteins (1, 2) which belong to the immunoglobulin
54 superfamily (IgSF), and play pivotal roles in fine tuning of immune responses and in maintenance
55 of peripheral self-tolerance. The importance of these proteins is further emphasized by their
56 involvement in pathological processes and by their clinical application in a variety of human
57 diseases, including autoimmunity and cancer (3, 4). Due to their fundamental biological
58 importance and therapeutic potential, there has been considerable interest in the identification
59 of additional members of the extended B7/CD28 family of ligands and receptors.

60 The human proteome encompasses hundreds of IgSF proteins, yet only a few are key
61 modulatory components of the immune system, and of T cell regulation in particular (4, 5).
62 Proteins of the IgSF tend to evolve quickly (6), and therefore sequence similarity to an
63 annotated member of the IgSF is a poor predictor of its function. In order to discover novel
64 immune modulatory proteins, we set to identify remote paralogs of known members of the B7-
65 like family that modulate T cell activity (e.g. PD-L1 ('programmed death ligand 1'), CD86, B7-H3,
66 B7-H4 and VISTA ('V-domain Ig suppressor of T cell activation')), based on similar genomic and
67 proteomic characteristics among known B7 family members, such as gene structure, protein
68 domains, predicted cellular localization and expression pattern. Using this search strategy, we
69 identified an IgSF protein family member known as ILDR2 (immunoglobulin-like domain-
70 containing receptor 2) as a new predicted B7-like protein with potential immunomodulatory
71 function. Experimental work presented herein confirmed this prediction.

72 ILDR2 and its two paralogs, ILDR1 and LSR (lipolysis-stimulated receptor; also named ILDR3)
73 have been designated as angulin family proteins (angulin-1/LSR, angulin-2/ILDR1, and angulin-
74 3/ILDR2) following their identification as protein components of tricellular tight junctions (tTJs),
75 which are required for recruitment of tricellulin to tTJs (7). Tricellular tight junctions are
76 specialized structures of tricellular contacts, i.e. where the corners of three epithelial cells meet,
77 that are required for full barrier function of the cellular sheet (8).

78 *Ildr2*, the gene encoding the murine orthologue (formerly designated "*Lisch-like*") was originally
79 identified as a modifier of susceptibility to type 2 diabetes in obese mice, and was associated
80 with reduced β -cell mass and replication rates, and persistent mild hypoinsulinemic

hyperglycemia (9) . The human gene encoding ILDR2, originally named C1orf32, is located in a region on Chr1q23-25 that has been associated with type 2 diabetes (9).

Roles in hepatic clearance of lipoproteins and in lipid homeostasis have been described for LSR (10) and for ILDR2 (11). ILDR1 was shown to regulate gastrointestinal hormone secretion through a mechanism dependent on fatty acids and lipoproteins (12). In addition, involvement in aggressive cancer behavior has been reported for LSR (13).

A recent publication reported upregulation of ILDR2 in human DC2 cells, a subpopulation of polarized dendritic cells (DCs) that promote Th2 differentiation (14). However, no immune-related function has been reported previously for any of the angulin family members.

Here we demonstrate that ILDR2 displays negative regulatory functions on human and mouse T cells in various experimental systems. Furthermore, ILDR2-Fc, a fusion protein of ILDR2 extracellular domain (ECD) with an Fc fragment, displays therapeutic effects in collagen-induced arthritis (CIA), a mouse model of rheumatoid arthritis (RA). We conclude that ILDR2 represents a novel B7-like ligand, which exerts negative immune modulation via interaction with a putative counterpart receptor expressed on activated T cells, thereby defining it as a novel immune checkpoint.

Materials and Methods

Mice

SJL/J (Harlan Labs; Indianapolis, IN); DO11.10 and BALB/c (Jackson Laboratories; Bar Harbor, ME) were housed under SPF conditions in the Northwestern University Center for Comparative Medicine. All of the procedures performed on animals within this study were conducted in accordance with the guidelines of Northwestern University Animal Care and Use Committee. Male DBA/1 mice (Harlan Labs; Indianapolis, IN) were used at 8-12 weeks of age. All procedures were approved by the Clinical Medicine Ethical Review Board and the UK Home Office.

Bioinformatic analysis of ILDR2

Bioinformatic characteristics, such as genomic structure and splice variants identification, were determined using Compugen's LEADS platform (15). Prediction for transmembrane domain was performed with TMHMM (16). Signal peptide was predicted with SignalP 4.0 (17) and validated experimentally by N-terminal sequencing. Immunoglobulin (Ig) domains identification was performed using InterProScan (18). Secondary structures were predicted using PSIPRED (19) and SCRATCH (20). Multiple sequence alignments were performed with MAFFT (21). EMBOSS-Needle was employed on globally aligned sequences to calculate the level of sequence identity and similarity (22, 23). The IgV domains of known B7 related proteins were extracted from SwissProt sequence annotation features (24).

Immunohistochemical analysis

IHC was carried out at Rockland Immunochemicals Inc. (Gilbertsville, PA) on formalin-fixed paraffin-embedded (FFPE) sections of normal or disease human tissue samples. IBD biopsies (2 samples of Crohn's disease and 2 samples of ulcerative colitis, obtained from USBiomax) were analyzed and compared to samples of normal small intestine and colon. A rabbit polyclonal anti-ILDR2 antibody, Ab-13, was used (at 2.5ug/ml) as the primary antibody, and the principal detection system consisted of a secondary Goat anti-rabbit IgG antibody (Vector Laboratories, BA-1000), Vectastain ABC-AP Staining KIT (Vector Laboratories, AK-5000), and Red Alkaline Phosphatase Substrate Kit (Vector Laboratories, SK-5100), which was used to produce a fuchsia-colored deposit. Stained slides were imaged with a DVC 1310C digital camera coupled to a Nikon microscope.

FACS and qRT-PCR analyses for ILDR2 expression on immune cells

Human PBMCs were isolated by Ficoll gradient from buffy coats of healthy donors. Monocytes were purified using RosetteSep Monocyte Enrichment Kit (Stem Cell Technologies). To generate macrophages, monocytes were cultured in 100ng/ml M-CSF (R&D Systems) for 5 days.

For FACS analysis, we used CGEN-Ab-10, an anti-ILDR2 hlgG1 mAb identified by phage display, and Synagis (Palivizumab, a hlgG1 anti-RSV mAb, Caligor Rx.) as isotype control. Each antibody was conjugated to AF647 using Alexa Fluor 647 Conjugation Kit (Life Technologies) according the manufacturer's instructions. Fluorophor conjugated mAbs against the following human proteins were purchased from Biolegend: Pacific Blue-conjugated anti-CD14, PE-conjugated anti-CD56, FITC-conjugated anti-HLA-DR. PerCp-Cy5.5-conjugated anti-CD16 was obtained from BD Bioscience. Prior to staining of cells with antibody, Fcy receptors were blocked by incubating 4×10^5 cells with 400ug/ml human IgG (Jackson ImmunoResearch) in FACS Buffer, at 4°C for 30min. Conjugated antibody was used at 5ug/ml in FACS buffer to stain cells at 4°C for 30 minutes, followed by two washes in FACS Buffer, and analysis by FACS CANTO. Captured data was analyzed using FlowJo software.

For quantitative RT-PCR analysis RNA was purified from cells using RNeasy RNA purification kit (Qiagen) according to manufacturer's protocol. cDNA was generated using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). qRT-PCR was performed using Taqman Universal PCR Master Mix (Life Technologies) and probes for human ILDR2 (Hs01025494_m1) and beta actin (HS1060665_g1). Analysis was performed using the ddCt method.

Ectopic expression of ILDR2 on 'Stimulator Cells' and T cell activation

Human ILDR2 was ectopically expressed in T cell stimulator cells (short designation within this work 'stimulator cells'). Briefly, these cells are Bw5147 cells stably expressing a membrane-bound anti-human CD3 single chain antibody fragment (described in detail by Leitner et al.(25)). Expression constructs encoding for the long or the short ILDR2 isoforms were cloned in the retroviral expression vector pCJK2, and expressed in these 'stimulator cells'. The expression of these isoforms, which have an identical extracellular domain, was examined by FACS analysis using a specific anti-ILDR2 polyclonal Ab (Ab-13). Cells expressing empty vector and no human costimulatory molecule were used as control. As positive controls for positive and negative costimulation, 'stimulator cells' expressing a known positive costimulatory protein (ICOSL) or a

known coinhibitory protein (PD-L1) were used. The use of ICOSL, CD70 or B7-H3 expressing 'stimulator cells' to activate human T cells have been described previously (25-27).

'Stimulator cells' were irradiated (2x3000 rad) and seeded in flat-bottom 96-well plates at 2×10^4 cells/well, in the presence of 1×10^5 untouched CD3+ human T cells, previously purified from buffy coats derived from healthy donors using Ficoll-Paque (GE-Healthcare) and MACS-depletion of CD11b, CD14, CD16, CD19, CD33 and MHC-class II-bearing cells, as described previously (28). After 48 hours of co-culture with the 'stimulator cells', T cell proliferation was measured by ^3H -thymidine incorporation (as described in (29)).

Production of ILDR2-Ig fusion proteins

ILDR2-Ig fusion proteins were produced by cloning the ECD of human ILDR2 (aa 1-184) fused to the Fc region (constant and hinge regions) of mouse IgG2a (ILDR2-mFc) or human IgG1 (ILDR2-hFc) in mammalian expression vectors. ILDR2-mFc contains a TEV linker at the ECD-Fc junction. ILDR2-hFc carries a C220S mutation at the hinge domain of human IgG1 Fc. Various batches of these proteins were produced from stable pools of CHO-S cells at Catalent Pharma Solutions, Middleton, WI USA or at Cobra Biologics, Sweden. All proteins were produced using fed-batch technology and purified from culture supernatants using standard protein A column affinity chromatography.

Activation of Human T cells

Total human PBMCs were obtained from healthy donors' buffy coats by standard density centrifugation using Ficoll-Paque Plus (GE HealthCare). Human naïve CD4+ T cells were further isolated using CD4+ Human T cell Isolation Kit II (Miltenyi) according to manufacturer's instructions. Purified naïve human CD4+ T cells were co-cultured with previously irradiated autologous PBMCs (at 3000rad) at a ratio of 1:1 (5×10^5 T cells with 5×10^5 irradiated PBMCs per well), and activated with anti-CD3 (0.5ug/ml, clone OKT3, eBioscience) and anti-CD28 (2ug/ml, clone CD28.2, eBioscience) antibodies, in the absence or presence of stated concentrations of soluble ILDR2-hFc or control Ig (hIgG1, Synagis). The amount of protein in each well was balanced to a total of 10ug/ml with Control Ig. After 24 hr in culture, cells were pulsed with ^3H -thymidine and were harvested after 72 hours in culture. Each sample was tested in duplicate.

For T cell reactivation, human T cell blasts were generated from PBMCs following stimulation with PHA (2ug/m) and hIL-2 (4ng/ml) for 10 days. T cell blasts were harvested and frozen for later use. Anti-CD3 (UCHT-1) was used to coat 96-well plates at 0.5ug/ml, 4°C, overnight. Plates were washed with PBS, and tested proteins were immobilized on the plates at different concentrations. T cell blasts were added at 1×10^5 per well, and supernatants were collected 24h later for analysis of IL-2 secretion using a hIL-2 Quantikine kit. Recombinant Human PD-L1-Fc (B7- H1-Fc, R&D Systems) was used as positive control, and rhIgG1-Fc (R&D Systems) as negative control. These T cell reactivation assays were carried by R&D Systems.

Activation of Murine T cells

Naïve CD4+ T cells were isolated from total lymph node and splenocyte populations of wild type mice (BALB/c or SJL) via automax sort (CD4-negative sort plus anti-CD25 to remove CD25+ cells from the isolated CD4+ T cells, followed by CD62L-positive sort; Miltenyi). Polyclonal T cell activation was carried out using naive CD4+ T cells and synthetic beads (Dynabeads M-450 Epoxy, Invitrogen) coated with anti-CD3 (0.5ug/ml; clone 2C11) and anti-CD28 (2ug/ml; clone 37.51 eBioscience), following the manufacturer's protocol. The beads were coated with the above mentioned mAbs in the absence or presence of the stated concentrations of ILDR2-mFc, or mouse IgG2a (BioXCell) as negative control. The total amount of protein used for bead coating was completed with mIgG2a to 10ug/ml. CD4+ T cells were activated with the coated beads at a ratio of 1:1 (beads to cells) in flat bottomed 96-well plates, and cellular proliferation was evaluated by ^3H -thymidine incorporation after 72 hours in culture.

For Ag-specific T cell activation, naive CD4+ T cells were isolated from spleens of DO11.10 mice (transgenic for an OVA323-339-specific TCR; Jackson Laboratory) via automax sort (CD4-negative sort plus anti-CD25 to remove CD25+ cells from the isolated CD4+ T cells, followed by CD62L-positive sort; Miltenyi). Splenocytes from a syngeneic BALB/c mouse were collected in parallel, irradiated with 3000rads and used as APCs. Naive DO11.10 CD4+ T cells (0.5×10^6 T cells/well) were cultured with irradiated APCs at a 1:1 ratio in flat-bottomed 96-well plates, and were activated in the presence of OVA323-339 peptide at 20ug/ml. Soluble ILDR2-mFc and Ig control (mouse IgG2a, BioXCell) were added at the stated concentrations. Cultures were pulsed with 1uCi of ^3H -thymidine at 24 hours and harvested at 72 hours.

Binding of ILDR2-hFc to resting and activated human T cells

Human primary CD4⁺ or CD8⁺ T cells were enriched from healthy donors derived buffy coats using RosetteSep™ Human T Cell Enrichment Cocktail (Stem Cell Technologies), according to the manufacturer's instructions. For T cell activation, 96-well plates were pre-coated with PBS in the absence or presence of 1 µg/ml anti-CD3 (clone UCHT-1; R&D Systems) for 4h at 37°C. Cells were plated at 0.1x10⁶ per well and cultured in a humidified, 5% CO₂, 37°C incubator for 72 hours, harvested and stained with viability dye (Fixable Viability Stain 450, BD Biosciences). PD-1 expression on anti-CD3 treated T cells, but not on resting cells, confirmed the activated status of these cells (not shown). Resting and activated T cells were incubated for 1hr at room temperature with ILDR2-hFc or control Ig (hIgG1, Synagis) at 100 µg/ml in FACS buffer (0.5% BSA, 2mM EDTA, 0.05% NaN₃ in PBS), and ILDR2-hFc binding was detected using PE-conjugated anti-hIgG (Jackson Laboratory). Cells were washed three times, gated for singlets (FSC-H vs. FSC-A), and for live cells, and ILDR2-hFc binding was determined within this gated population. Data acquisition was performed with MACSQuant Analyzer 10 (Miltenyi) and analyzed using FlowJo software (version 10).

Binding of ILDR2-mFc to mouse CD4 T cells

Untouched CD4⁺CD25⁻ T cells were isolated from pools of spleens of BALB/c mice, using a T cell Isolation Kit (Miltenyi) according to the manufacturer's instructions. Following isolation, cells were left untreated (resting) or activated using polyclonal activation of T cells, with plate bound anti-CD3 antibody (clone-145-2C11, BD-Pharmingen, coated on 96-well plates at 2 µg/ml, for 4 hours at 37°C) and 2 µg/ml of soluble anti-CD28 antibody (clone 37.51, eBioscience). Cells were harvested at different time points after activation (12h, 24h and 48 h) and stained with 2 µg/ml anti-CD25 antibody (clone PC61, Biolegend) to evaluate T cell activation. For binding of tested proteins, biotinylated (using DSB-X™ Biotin Protein Labeling Kit, Molecular Probes, D-20655) ILDR2-mFc or Ig control (mIgG2a) were used. In order to prevent binding to Fcγ receptors, the ILDR2-mFc protein used for this analysis contained the N297A mutation, resulting in an aglycosylated Fc domain. Cells were incubated for 30 min at room temperature with biotinylated ILDR2-mFc or control Ig at 60 µg/ml in FACS buffer (0.5% BSA and 2mM EDTA in PBS), washed and stained with 0.5mg/ml streptavidin-PE (Jackson ImmunoResearch) for 30min at room temperature. Resting or activated CD4⁺ cells were first gated for lymphocytes (FSC-A vs. SSC-A), followed by live cells gate, and further gated for singlets (FSC-H vs. FSC-A). Further gating

on CD25 was carried out to focus on the population of activated T cells. Data acquisition was performed with MACSQuant Analyzer 10 (Miltenyi) and data analyzed using FlowJo software.

Collagen induced arthritis mouse model

DBA/1 mice were immunized with type II bovine collagen in CFA. No collagen boost was further used in this model. The following proteins were used: ILDR2-mFc, mIgG2a as isotype control (BioXCell, BE0085), Enbrel (etanercept, ECD of human TNFR fused to human IgG1Fc, Wyeth Pharmaceuticals). Tested proteins were administered i.p. at 100ug/mouse from the day of disease onset (day 1) for each mouse individually and followed at 3 times per week until day 10 after disease onset (total of four administrations). Arthritis severity was assessed using the following clinical scoring system: 0 = normal, 1 = slight swelling and/or erythema, 2 = pronounced edematous swelling and 3 = joint rigidity. Each limb was graded, thus allowing a maximum score of 12 per mouse. The animals were sacrificed at day 10 post onset of disease. Statistical analysis was carried out using two-way ANOVA with repeated measures for all groups followed by two-way ANOVA with repeated measures for each test group vs. control mIgG2a.

Autologous co-cultures of cytokine activated T cells and macrophages from healthy donors or RA patients

Monocytes and CD4+ T cells were isolated from healthy donors or RA patients' blood or buffy coats. T cells were cultured with the following cytokines (obtained from Peprotech): TNF α (25 ng/ml), IL-6 (100ng/ml) and IL-15 (100ng/ml) for 6 days to induce cytokine activated T cells (Tck). Macrophages (M ϕ) were differentiated from monocytes with M-CSF (50ng/ml, Peprotech) for 6 days. Autologous Tck and M ϕ were co-cultured for 24hrs in the presence of various concentrations of ILDR2-hFc or control Ig (hIgG1, Synagis). TNF α secretion was evaluated by ELISA using Human TNF- α Cytoset (Invitrogen). Additional Cytokines were evaluated by Luminex, using human 30-Plex Panel (ThermoFisher Scientific).

Quantitative RT-PCR analysis on normal human tissues

RNA samples from human normal tissues were obtained from different sources (ABS, BioChain, Ambion, Asterand, Genomics Collaborative Inc, Tel Aviv Sourasky Medical Center and Chaim Sheba Medical Center). cDNA was synthesized by reverse transcription (RT) on each total RNA sample, previously treated with DNaseI (Ambion), using Random Hexamer primers (Invitrogen

Corporation), RNasin (Promega Corporation) and MultiScribe™ polymerase. Quantitative RT-PCR analysis (qRT-PCR) was carried out using these cDNA samples as template in the SYBR Green I assay (Applied Biosystems) with UNG Enzyme.

Primers were specific for the short transcript: forward primer spanning the junction of exons 5 and 6: 5'-TGTGGAGATTATGCCAGAGTGG-3', and reverse primer derived from the intracellular region: 5'-GACATTTCTCTCGATCGCTCTGT-3'. Fluorescence detection was performed using the PE Applied Biosystem SDS 7000. The cycle in which the reactions achieved a threshold level of fluorescence (Ct= Threshold Cycle) was registered and was used to calculate the relative transcript quantity (Q) in the RT reactions, using the equation $Q = \text{efficiency}^{\text{Ct}}$. The efficiency of the PCR reaction was calculated from a standard curve, created by using serial dilutions of several RT reactions. To minimize inherent differences in the RT reactions, the resulting relative quantities were normalized using a normalization factor calculated by assessing the relative quantity (Q, as described above) for several housekeeping genes in each cDNA sample and dividing by the median quantity of this gene in all panel samples to obtain the "Relative Q rel to MED". Then, for each sample the median of the "relative Q rel to MED" of the selected housekeeping genes was calculated and served as normalization factor of this sample for further calculations. For each RT sample, the expression of the specific amplicon was normalized to the normalization factor calculated from the expression of different housekeeping genes (HSKPs). The HSKPs that were used for the calibration of the 'Normal panel' were HPRT1, SDHA and G6PD.

Evaluation of murine T cell apoptosis

CD4+CD25- murine T cells were stimulated with plate bound anti-CD3 (2µg/ml) alone or in the presence of 10µg/ml ILDR2-mFc or control Ig (mIgG2a). Live gated and ungated CD4 T cells were analyzed by FACS at 24 and 48 hrs post-stimulation for the cell death marker 7-AAD (aminoactinomycin D) and for the expression of the early apoptosis marker annexin V, using Annexin V-PE Apoptosis Detection Kit I (BD Biosciences).

310 Results

311 Structural analysis of ILDR2

312 The human ILDR2 mRNA (GenBank accession: NM_199351) encodes a 639 a.a. type I membrane
313 protein containing a signal peptide at its N terminus, followed by a large V-type Ig (IgV) domain
314 of 148 a.a, a stalk region of 19 a.a., a transmembrane domain of 20 a.a., and an intracellular tail
315 of 433 a.a. (**Fig. 1a**). This protein shows 94% overall homology to its murine ortholog, *Ildr2*
316 (GenBank accession: NM_001164528), with 96% identity and 98% similarity in the ECD.
317 Alignment of the protein sequences of ILDR2 and its two paralogs reveals overall homology of
318 39% and 44% to ILDR1 and LSR, respectively. There is 36% identity and 52% similarity between
319 the ECDs of ILDR2 and ILDR1; and 47% identity and 66% similarity between the ECDs of ILDR2
320 and LSR (**Fig. 1b**). A stretch of amino acids enriched in cysteine and proline residues (CCP-rich
321 domain) is located at the juxtamembrane region of the intracellular domain of human and
322 mouse ILDR2 (**Fig. 1a**). This CCP-rich domain is also found in the other two paralogs, ILDR1 and
323 LSR (**Fig. 1b**).

324 The gene structure of the human *ILDR2* gene is depicted in **Fig. 1c**. Shown are the splice variants
325 of ILDR2, with the longest transcript (comprising 10 coding exons) encoding the membrane
326 protein described above and presented in Fig. 1A. A novel shorter isoform, revealed by
327 Compugen's LEADS platform (15), encodes a type I membrane protein with the same ECD but a
328 significantly shorter and distinct intracellular tail of 48 a.a. Both the long and short isoforms
329 contain the CCP-rich domain. A third splice variant predicted by LEADS encodes a protein lacking
330 the transmembrane domain and is thus expected to be a secreted isoform. In addition, RT-PCR
331 analysis revealed another transcription variant, in which the 4th exon (an exon of 57 bp which
332 codes for the 19 a.a. stalk region) is spliced out. This variant contains the transmembrane
333 domain but has a shorter ECD without the stalk region. A similar alternative exon of 57 bp
334 coding for the stalk region appears in LSR (7). Interestingly, an exon of 57 bp which codes for the
335 stalk region is also present in the gene coding for VISTA, another B7-like protein (30).

336 Splice isoforms of the mouse *Ildr2* gene have been previously described. Four mouse isoforms
337 were mentioned in Dokmanovic-Chouinard et al (9), three of them have the same structure as
338 the human long isoform, soluble isoform and Skip 4 variant, depicted in Fig. 1B. Seven mouse

splice isoforms were later described by Higashi et al (7), two of which have the same structure as the human long and soluble isoforms shown in **Fig. 1c**. In this publication, the Skip 4 variant murine version was also detected but in the context of the long isoform (exons 1,2,3,5-10), while the human Skip 4 variant that we identified was in the context of the short isoform (exons 1,2,3,5-5').

Aligning the sequence of ILDR2 to NCBI NR using Blast revealed low sequence homology to other B7-like proteins. Pair alignments between ILDR2 and these proteins show various similar signatures along the IgV domain. This domain was found to mediate the interaction of B7-like ligands with their counterpart receptors on T cells, as shown for example in the case of CD80 and CD86 (see pdb|1i85 and pdb|1i8l) (31, 32). The sequence identity and similarity between the ILDR2 IgV and that of other B7-like proteins, ranges between 15%-21% and 24-36%, respectively (**Table 1**). This degree of homology is similar to that exhibited among other members of the B7 family (33).

Multiple sequence alignment between the IgV domains of ILDR2 and that of other B7-like proteins (**Fig. 1d**) depicts shared canonical cysteine residues and the DxGxYxC motif. In addition, a unique long helical loop containing two non-canonical cysteines is evident between the C and C' strands of the ILDR2 IgV structure (**Fig. 1d** and **Supplementary Fig. 1**). The two ILDR2 paralogs, LSR and ILDR1, also have an extended loop with two non-canonical cysteines between the C and C' strands.

Expression of ILDR2 in human and mouse tissues

Information on expression of Human ILDR2 mRNA was extracted from the Genotype-Tissue Expression (GTEx) database (34). Results presented in **Fig. 2a** indicate high expression in testis, and high to moderate expression in brain. Lower expression is observed in other tissues, such as kidney, heart and colon. Quantitative RT-PCR analysis using primers specific to the short ILDR2 transcript on RNA samples from various human normal tissues (see **Supplementary Fig. 2**) also showed highest expression in brain and testis, moderate expression in kidney, peripheral blood mononuclear cells (PBMCs) and stomach, and low expression in various other tissues. Support for the expression of ILDR2 protein was obtained by querying the Proteomics Database (<https://www.proteomicsdb.org/proteomicsdb/#overview>) and the Human Proteome Map

(<http://www.humanproteomemap.org/query.php>) of protein mass spectrometry data, showing that peptides derived from ILDR2 were identified in testis, brain frontal cortex and retina.

Relative expression pattern of murine *Ildr2* mRNA was extracted from the data available at the GNF gene expression atlas (35). Results, shown in **Fig. 2b**, point to roughly similar expression pattern as that described above for human ILDR2 mRNA, indicating high to moderate expression in various regions within the brain. High to moderate expression was also observed in different structures in the eye (retina, iris, eye cup, lens, ciliary bodies), which are not part of the data available at GTEx for human RNA expression. Moderate expression also appears in kidney, and significantly lower or no expression in other tissues. Unlike the high ILDR2 mRNA expression observed in human testis, no expression of murine *Ildr2* mRNA was detected in mouse testis.

Expression of ILDR2 on immune cells

Expression of the ILDR2 protein on inflamed samples obtained from inflammatory bowel disease (IBD) patients (ulcerative colitis and Crohn's disease) was evaluated by immunohistochemistry (IHC) analysis using an anti-ILDR2 polyclonal antibody. Results point to increased ILDR2 expression in reactive epithelium and immune cells in IBD samples compared to normal colon and small intestine (**Fig. 3**).

A strong upregulation of ILDR2 mRNA was recently reported in human DC2 cells (polarized DCs capable of promoting the differentiation of Th2 cells) compared to other DC subtypes(14). We further evaluated the expression of ILDR2 on immune cells by FACS analysis using an anti-ILDR2 monoclonal antibody. Human PBMCs were gated to lymphocytes and monocytes, and stained for immune markers (**Fig. 4a**) to identify immune cell subtypes. Further staining with the anti-ILDR2 Ab (shown in **Fig. 4b**) indicated ILDR2 expression on the three monocytes subtypes, identified based on their relative expression of CD14 and CD16 (as reviewed in Wong et al(36)). Highest ILDR2 expression was observed in the CD16+ monocytes subsets i.e. CD14+ CD16+ 'Intermediate Monocytes' and CD14low CD16+ 'Non-classical Monocytes'. Weak staining for ILDR2 was also observed on CD56+ lymphocytes (mostly, NK & NKT cells). The other immune cell subsets tested did not show significant or consistent staining.

ILDR2 protein expression was also detected on human monocyte-derived macrophages (**Fig. 4c**). RT-PCR analysis indicated that the expression of ILDR2 mRNA was higher in monocytes treated

with M-CSF (i.e. differentiated macrophages) compared to monocytes incubated in media alone (**Fig. 4d**), suggesting that ILDR2 is upregulated following differentiation of human monocytes into macrophages by M-CSF.

ILDR2 inhibits T cell activation

The functional effect of the ILDR2 protein on T cell activation was tested following ectopic expression of the full ILDR2 protein in 'T cell stimulator cells'. These are Bw5147 cells (a murine thymoma cell line) engineered to express membrane-bound anti-human CD3 single chain antibody fragments (25). Both the long and short isoforms of ILDR2, which have the same ECD, were tested. T cell stimulators expressing the known costimulator ICOSL served as positive control for positive costimulation, while cells expressing the coinhibitory ligand PD-L1 served as positive control for coinhibition. Expression of the long or short isoforms of ILDR2 on T cell stimulators exerted an inhibitory effect on human T cell activation, which was somewhat similar to that exerted by the known coinhibitory ligand PD-L1 (**Fig. 5a**). The stronger T cell inhibitory effect observed with cells expressing the short isoform most likely stems from a ~10 fold higher surface expression of this isoform compared to the long isoform on the T cell stimulator cells (see **Supplementary Fig. 3**).

In order to further examine the immunomodulatory role of ILDR2 on human and mouse T cell responses we used ILDR2-Fc, a fusion protein consisting of the ECD of human ILDR2 fused to the Fc region of human IgG1 (ILDR2-hFc) or of mouse IgG2a (ILDR2-mFc).

A clear dose dependent reduction of T cell activation by ILDR2-hFc was observed on anti-CD3/CD28 activated human CD4+ T cells (**Fig. 5b**). A pronounced inhibitory effect of ILDR2-hFc was also evident using human T cell blasts, following their reactivation with anti-CD3 (**Fig. 5c**). The inhibition displayed by ILDR2-hFc was substantially stronger than that observed with PD-L1-Fc, used as positive control (ED50 = 0.54 µg/ml for ILDR2-hFc, 1.3 µg/ml for PD-L1-Fc).

Similar results were obtained with mouse T cells activated with anti-CD3/CD28 coated beads. Upon co-immobilization on beads, ILDR2-mFc suppressed murine CD4+ T cell activation manifested as reduction in T cell proliferation, in the two mouse strains tested (SJL and BALB/C, **Fig. 5d**). ILDR2-mFc also displayed pronounced and dose dependent inhibition of antigen-

specific T cell activation, following OVA₃₂₃₋₃₃₉-induced activation of CD4⁺ T cells from DO11.1 (OVA-TCR transgenic) mice in the presence of syngeneic irradiated APCs (**Fig. 5e**).

The inhibitory effect of ILDR2-Fc on human or murine T cell activation, was also evident in a different experimental setup involving co-immobilization of ILDR2-Fc with plate bound anti-CD3, using additional readouts including cytokine secretion and expression of T cell activation markers (data not shown).

Of note, ILDR2-Fc present in a soluble form did not exert inhibitory effects on purified T cells (data not shown). Such effects were displayed only upon immobilization of the protein on beads or plates, or when APCs were also present in the T cell assays. This observation implies a requirement for crosslinking of ILDR2-Fc to a surface such as beads or onto APCs via their Fcγ receptors. Further support for this notion was obtained from experiments using a N297A mutant of ILDR2-mFc resulting in an aglycosylated Fc domain with greatly reduced ability to bind Fcγ receptors. This mutant showed inhibitory activity only upon its crosslinking to beads or plates, but no such activity was displayed when it was tested in the presence of APCs (data not shown)

In order to assess whether induction of T cell apoptosis contributes to the inhibitory effect of ILDR2-Fc on T cell proliferation, we evaluated the levels of Annexin V marker of apoptosis, and the cell death marker 7AAD. Results indicate that the levels of early and late apoptosis were not enhanced, but were rather somewhat reduced in the presence of ILDR2-Fc (**see Supplementary Fig. 4**), implying that apoptosis does not underlie the inhibitory effect of ILDR2-Fc on T cell responses.

ILDR2-Fc binds activated T cells

Based on the robust inhibitory activity observed for ILDR2 on T cells, we asked whether T cells express a putative counterpart for ILDR2, and tested the binding of ILDR2-Fc to resting or activated T cells. Results, presented in **Fig. 6**, show binding of ILDR2-Fc to human or mouse activated T cells, but no detectable binding to resting non-activated T cells. Binding of ILDR2-hFc was detectable on either CD4⁺ or CD8⁺ activated human T cells (**Fig. 6a**). The kinetics of such binding was tested using ILDR2-mFc and mouse CD4⁺ T cells, and indicates weak binding already evident by 12h post cell activation, which increased and leveled off at 24h (**Fig. 6b**). These

findings suggest inducible expression of a counterpart receptor of ILDR2 following T cell activation.

ILDR2-Fc displays beneficial effects in the CIA model of rheumatoid arthritis

The robust inhibitory effect on T cell activation displayed by the ILDR2-Fc protein in various in vitro experimental systems, prompted us to test its effect in mouse models of autoimmune diseases. Therapeutic administration of ILDR2-mFc showed beneficial effects in the CIA mouse model of RA, manifested in reduction of clinical score (**Fig. 7**), to a similar extent as that obtained with Enbrel (TNFR-Fc). Furthermore, short-term administration of ILDR2-mFc in the relapsing-remitting experimental autoimmune encephalomyelitis (R-EAE) model of multiple sclerosis and the NOD model of type 1 diabetes resulted in long term beneficial effects, as reported elsewhere (Podojil et al, submitted).

Inhibitory effect of ILDR2-Fc in a translational assay mimicking interactions of T-cells and macrophages in the RA synovium

The therapeutic potential of ILDR2-Fc for treatment of RA was further studied using co-cultures of cytokine activated T cells (Tck) and macrophages from healthy donors and from RA patients. Such co-cultures have been suggested to mimic the deleterious interaction of these cells in the RA synovium, which drive the secretion of pro-inflammatory cytokines (in particular TNF α) and play a major role in the pathology of RA. This experimental system provides a translational tool to evaluate potential therapies for RA (37).

As shown in **Fig. 8a**, addition of ILDR2-hFc to these synovial-like co-cultures induced a dose dependent inhibition in TNF α secretion. ILDR2-hFc downregulated additional pro-inflammatory and regulatory cytokines and chemokines in these cultures with pronounced inhibitory effect on MIP-1 α , RANTES, GM-CSF, IL-12, IFN γ , IL-1RA, IL-5 and IL-6 (**Fig. 8b**). Mild inhibition was observed for secretion of IL17, IL-13 and soluble IL-2R, whereas the production of IL-10, IL-15, IFN α and IL-7 was not affected (data not shown). ILDR2-hFc inhibited the secretion of TNF α also in such co-cultures from RA patients (**Fig. 8c**), demonstrating that the ILDR2 pathway is functional and responsive in these patients.

482 Discussion

483 This work presents ILDR2 as a novel member of the B7-like family of immunomodulators. Taken
484 together, our data show that ILDR2 exerts robust inhibitory effects on human and mouse T cells,
485 when tested as an Fc fusion protein (ILDR2-Fc) or upon overexpression of the full membrane
486 protein. Similar to other B7-like negative regulators, e.g. B7-H4 (38) and VISTA (30), ILDR2
487 negatively regulates T cell responses by suppressing early TCR activation and cell division but
488 without enhancing apoptosis. Furthermore, we have also found that ILDR2 affects T cell
489 differentiation (Podojil et al, submitted), leading to reduction of pro-inflammatory responses
490 (Th1/Th17), enhancement of iTreg differentiation, and promotion of anti-inflammatory
491 responses (Th2, and secretion of IL-10). In this regard, it is of interest to point the recently
492 reported enhanced expression of ILDR2 on DC2 cells (14), which are polarized DCs capable of
493 promoting the differentiation of Th2 cells.

494 The robust inhibition of T cell activation exerted by ILDR2 is most likely due to its agonistic
495 activity on an inhibitory receptor, rather than inhibition of a T cell stimulatory pathway. The
496 need to crosslink ILDR2-Fc is in line with the mode of action of an agonistic ligand and further
497 supports this notion.

498 While the counterpart receptor of ILDR2 has not been identified, the binding studies presented
499 herein indicate expression of an inducible counterpart for ILDR2 on activated T cells, which can
500 be detected during early TCR activation. This observation is reminiscent of other negative
501 receptors of the B7-like family which are upregulated on T cells following activation, such as
502 CTLA4, PD-1 and TIGIT ('T-cell immunoreceptor with Ig and ITIM domains').

503 We describe the protein structure of ILDR2 and its two paralogs, ILDR1 and LSR (ILDR3) and
504 demonstrate that while these proteins show similarities to those of known B7-like family
505 members, ILDR2 and its paralogs share unique structural features, such as the presence of a CCP
506 (cysteine and proline residues) rich domain located at the juxtamembrane region of the
507 intracellular domain, and a unique extended loop with non-canonical cysteine residues in the
508 IgV within their ECD.

509 ILDR2 and its two paralogs, ILDR1 and LSR have been identified as protein components of
510 tricellular tight junctions, specialized structures where the corners of three epithelial cells meet

that are required for full barrier function of the cellular sheet (8), and thus function as a type of cell adhesion molecules. Members of the IgSF account for a large proportion of cell adhesion molecules (39). Some IgSF proteins that have properties of adhesion molecules have been shown to also perform important immunological functions, including immune checkpoint regulators. For example, PVR/CD155 belongs to the family of nectin-like adhesion proteins and is a ligand of TIGIT (also designated as WUCAM - Washington University Adhesion Molecule), a known immune checkpoint in NKs and T cells. The PVR-TIGIT interaction plays dual roles in cell adhesion and cell signaling (40), including adhesion of T follicular helper cells to follicular DCs (41). An additional example in this family is PVRL2/CD112/Nectin-2, a member of the nectin family of adhesion molecules, which is a ligand of CD226 (a prominent costimulatory receptor in the TIGIT pathway) and of a novel checkpoint CD112R/PVR-Ig (42). Recently, another known adhesion molecule, PSGL-1, was characterized as a novel negative regulator of T cell function that is upregulated in activated T cells (43). Similar to these IgSF proteins, ILDR2 seems to play dual roles, as tricellular tight junction component and as a novel immunomodulator, presented here.

The higher expression observed for ILDR2 in immune privileged tissues such as brain, eye and testis is reminiscent of the expression pattern observed for other negative immunomodulators of the B7/CD28 family, including PD-L1, which are part of the mechanisms of immune-tolerance induction operating in immune privileged sites (44, 45).

Herein we also present the therapeutic effect of ILDR2-mFc in CIA in mice, a model of rheumatoid arthritis. This protein was extensively studied further in other models of autoimmune diseases (Podojil et al, submitted), and the results indicated that short-term administration of ILDR2-mFc resulted in long term beneficial effect in the R-EAE model of multiple sclerosis (MS) and the NOD model of type 1 diabetes. ILDR2-Fc was also shown to promote transplant engraftment in a model of minor mismatch bone marrow transplantation. Furthermore, ILDR2-Fc displays a unique mode of action, by combining immunomodulation and regulation of immune homeostasis, as well as Tregs induction and reestablishment of antigen-specific immune tolerance, leading to durable amelioration of autoimmunity.

Using a translational assay that mimics the deleterious interaction of immune cells in the RA synovium, ILDR2-hFc was shown to decrease secretion of TNF α and other pro-inflammatory cytokines that play a major role in the pathology of RA. Moreover, in another translational assay

using PBMCs from MS patients (Podojil et al, submitted) ILDR2-Fc reduced antigen specific T cell activation and secretion of inflammatory cytokines and increased anti-inflammatory and Treg-related cytokines. These translational assays further substantiate the immunomodulatory activity of ILDR2-Fc and its potential to affect autoimmune processes and inflammation in patients. Together with the in vivo data from mouse models of autoimmunity, our findings point to ILDR-Fc as a promising novel approach for treatment of autoimmune diseases.

To summarize, ILDR2 is a novel negative regulator of T cell responses, with potential roles in the modulation of immune tolerance, and critical implications for the development of autoimmunity and of immune responses to cancer.

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- 681

683 **Figure 1a**

685

Signal peptide

ILDR2 M-----
LSR MQQDGLGVGTRNGSGKGRSVHPSWPWCAPRPLRYFGRDARARRAQTAAALLAGGLSRGL
ILDR1 M-----AWPK-LPAP-----
*

ILDR2 -----DRVLLRWISL-FWLTAMVEGLQVTVDPDKKKVAMLFQPTVLRCHFTSSSH-Q
LSR GSHPAAGRDVVFVWLLSTWCTAPARATQVTVSNPYHVILFQPVTLPCFYQMTSTPT
ILDR1 -----WLLCTWLPAGCLSLVTVQHTERYVTLFASIIKCDYTTSAQLQ
* : * * * . * : * * * . : . * * . * : : :

ILDR2 PAVVQWKFKSYODRMGESLGMSSSTRAQ---SLSKRNLEWDPYLDCLDSRRTVRVVASQ
LSR QPIVIWKYKSFORDRIADAFSPASVDNQLNAQLAAGNPGYNPYVECDQSVRTVRVVASQ
ILDR1 DVVVTWRFKSFCKDPIFDYYSAS-----YQAALSLGQDPSNDNDNQREVRIVAQR
* : * * : * : * : : : : : : : : * : * : * : * : * : * : * : *

ILDR2 G-STVTLGDFYRGREITIVHDADLQIGKLMWGDGSLYMCITITPDDLEGKNEDESVLLVL
LSR G-NAVTLGDIYQGRITITGNADLTFDQTAWGDSGVYICSVVSAQDLQGNNEAYAEIIVL
ILDR1 GQNEPVLGVDRQRKITIQNRADLVINEVMWWDHGVYMCIEAPGDTSGDPDKEVKLIVL
* . * * * : * : * * * * * : : * * * : : : * . * : : * : * : * : *

Stalk region Transmembrane domain CCP

ILDR2 GRGGLLADLLPSFAVEIMPEWVFGVLVLLGVFLFFVLVIGICWCQCCPHSCCCYVRCPCCP
LSR GRTSGVAELLPGFQAGPIEDWLFVVVCLAAFLIFLLLGICWCQCCPHTCCCYVRCPCCP
ILDR1 -----HWLTVIFIIILGALLLLLIIGVCWCQCCPQYCCCYIRCPCCP
* : * * . : * . : * : * : * : * : * : * : * : * : * : * : * : *

ILDR2 DSCCCPE---ALYEAGKAAKAGYP---PSVSGVPGPYIPSVPL-----GG
LSR DKCCCE---ALYAAGKAATSGVPSIY-APSTYAHLSPAKTPPPAMIPMGPAYNGYPGG
ILDR1 AHCCCEEEALARHRYMKQAQALGFQMMGKPLYWGADRSSQVSSYPM-----
* * * : * : * * : * * * *

ILDR2 AP-----SSGMLMDKPHPPPLAPSDSTGGSHSVRKGYRIQADKERDSMKVLYYVEKELAQ
LSR YPGDVDRSSSAGGQGSYVPLLRDTDSVAS-EVRSGYRIQASQDDSMRVLYYMEKELAN
ILDR1 HP-----LLQRDLSPSSLPQMPMTQTNTQPIANG-----VLEYLEKELRN
* . . . : . : * * * : * : *

ILDR2 FDPAR---RMRGRYNTISELSSLHEEDSNFRQSFHQMRKQFPVSGDLESNPDIWGSV
LSR FDPSPRGPP---SGRVERAMSEVTSIHEDD-----W---
ILDR1 LNLAQPLPDLKGRFGHPCSMLSLSEVVE-RRIIHLP-----PLIRDLSSS-----
: : : * * . * : * * . :

ILDR2 MGGSSGASRGPSAMEYNKEDRESFRHSQPRSKSEMLSRKNFATGVPVAVSMDELAAFADSY
LSR ---RSRPSRGPALTPIRDEEWGGHSPRSPRGWDQEPAREQAGGWRA-----
ILDR1 -----RRTSDSL-HQQWLTPIPSRPWD-----
* . : : : :

ILDR2 GQRPRRADGNSHEARGGSRFERSESRAHSGFYQDDSLSEYYGQRSRS-----R
LSR -RRPRARSVDALDDLTPPSTAESGSRPTS--NGGRSRAYMPPRSRS-----R
ILDR1 -----LREGRSHHHYPDFHQELQDRGPKSWALERRELDPSWSGRHRSRLNGSPIHWSR
* : . : . : : : * * * : *

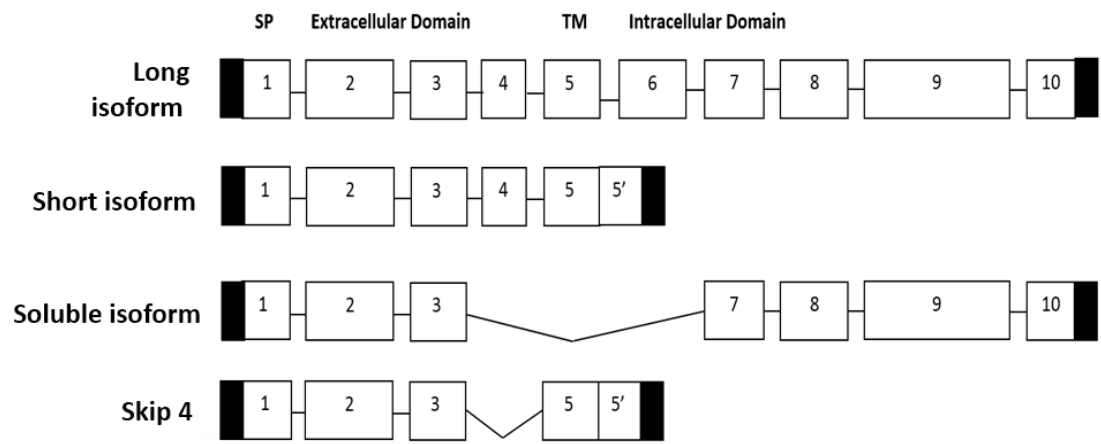
ILDR2 EPLTDADRGWAFSPARRRPAEDAHLPLRVSR---TPGTAPKYDHSYLSARERQARPEGA
LSR DDLYDQDDSRDFPRSR---DPHYDDFRSR--ERP-PADPRSHHH---RTRDPRDNG-
ILDR1 DLSLSDVPSS---SEARWR---PSHPPFRSRCQERPRRPSPRESTQ---RHGRRRRHR-
: * * . . : * . : * * * . * * . *

ILDR2 SRGGSLETSPKRSQAQLGPRASYYAWSPPGTYKAGSSQDDQEDASDDALPPYSELELTRG
LSR SRSGDLPYDGRLLLEAVRKKGSEERRP-----HKEEEEEAYPPAPPPYSE-----
ILDR1 SYSFPLPSGLSSWSSEEDKERQPQSWRA-----HRRGSHSPHWPEEKPP-----
* . * . . : * *

ILDR2 PSYGRDLPLYHSNSEKKRKEPAKKTNDFPTRMSLVV
LSR -----TDSQASRERRLKKNLALSRESLVV
ILDR1 -SYRSLDITPGKNSRKKGSVERRSEKDSHSGRSVVI
: : . * : : : * : *

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688 **Figure 1c**



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690 **Figure 1d**

		C-C' Unique Helical loop
ILDR2	LQVTVPDKKKVAMLFQPTVLR	CHF--STSSHQPAVVQWKFKSYQDRMGESLGMSSSTRAQ
CD80	-----VIHVTKEVKEVATLSCG	---HNVSV-----E
CD86	-----NETADLPCQF	--ANSQN-----Q
ICOSL	---DTQEKEVRAMVGSDVELSCAC	--PEGSR-----F
PDL1	FTVTVPKDLVVEYGSNMFTIECKF	--PVEKQ-----L
PDL2	FTVTVPKELYIIIEHGSNVTLECNF	--DTGSH-----V
B7H3	LEVQVPEDPVVALVGTDATLCCSF	--SPEPG-----F
B7H4	HSITVTTVASAGNIGEDGILSCTF	---EPD-----I
VISTA	FKVATPYSLYVPEGQNVTLTCRLLGPVDKG	-----H
ILDR2	SLSKRNLEWDPYLDCLDSRRTVRVVAS	-----KQGSTVTLGDFYRGR--
CD80	ELAQTRIYWQ-----KEKKMVLTM	-----SGDMNIWPEYKNRTI
CD86	SLSELVVFVWQ-----DQENLVLNEVYL	-----GKEKFDSVHSKYMGRTS
ICOSL	DLNDVYVYWQ-----TSESKTVVTYHIP	-----QNSSLENVDSRYRNRAL
PDL1	DLAALIVYWE-----MEDKNIIQFVH	-----GEEDLKVQHSSYRQRAR
PDL2	NLGAITASLQ-----KVENDT	-----SPHRERAT
B7H3	SLAQLNLIWQ-----LTDTK--QLVH	-----SFAEGDQGSAYANRTA
B7H4	KLSDIVIQWL-----KEGVLGLVHEFKE	-----GKDELSEQDEMFRGRTA
VISTA	DVT-FYKTWY-----RSSRGEVQTCSERRPIRNLTFQDLHLHHGGHQAANTSHDLAQRHG	
ILDR2	---EITIVHDADLQIGKLMWGD	SGLYICIIITPD-DLEGKNEDSVE-----
CD80	FD-----ITNNLSIVILALRPSDEGTYE	CVVLKYE-KDAFKREHLAEVT-----
CD86	FDSD-----SWTLRLHNLQIKDKGLYQCI	IIHHKK-PTGMIRIHQMNSELS---
ICOSL	MSPAGMLRGDFSLRLFNVTPODEQKHF	CLVLSQS--LGFQEVLSVE-----
PDL1	LLKDQLSLGNAALQITDVKLQDAGVYRC	MISYGG-A-DYKRIT-----
PDL2	LLEEQLPLGKASFHIPQVQVRDEGQYQCI	IIYGV-AWDYKYLTLK-----
B7H3	LFPDLLAQGNASLRLQVRVADEGSFT	CFVSIRDFGSAAVSLQVA-----
B7H4	VFADQVIVGNASLRLKNVQLTDAGTYKCYI	ITSK-GKGNANLEYK-----
VISTA	LESASDHGNGFSITMRNLTLDSGLYIC	CLVV-----EIRHHHSEHRVHG

691

692 **Figure 1.** Identification and characterization of ILDR2 as a novel B7-like protein. **(a)** Amino acid
693 sequence alignment and topology of human and mouse ILDR2. The signal peptide and
694 transmembrane domains are marked by blue rectangles, IgV boundaries are marked by
695 brackets, cysteines in black rectangles, and the CCP-rich domain by a red rectangle. **(b)** Amino
696 acid sequence alignment and topology of human ILDR2, LSR (ILDR3) and ILDR1. Protein domains
697 are marked as described in A. **(c)** Gene structure and transcription variants of human ILDR2.
698 Shown are exons of alternative spliced transcripts from human ILDR2. The coding regions are
699 depicted as open boxes, and the untranslated regions (UTRs) are represented in black boxes. **(d)**
700 Multiple sequence alignment of the IgV domains of ILDR2 and of other proteins of the B7 family.
701 The DxGxYxC motif is highlighted in pink. The canonical cysteine residues in the IgV domain,
702 known to form a disulfide bridge, are marked in yellow. Non-canonical cysteine residues are
703 marked in grey.

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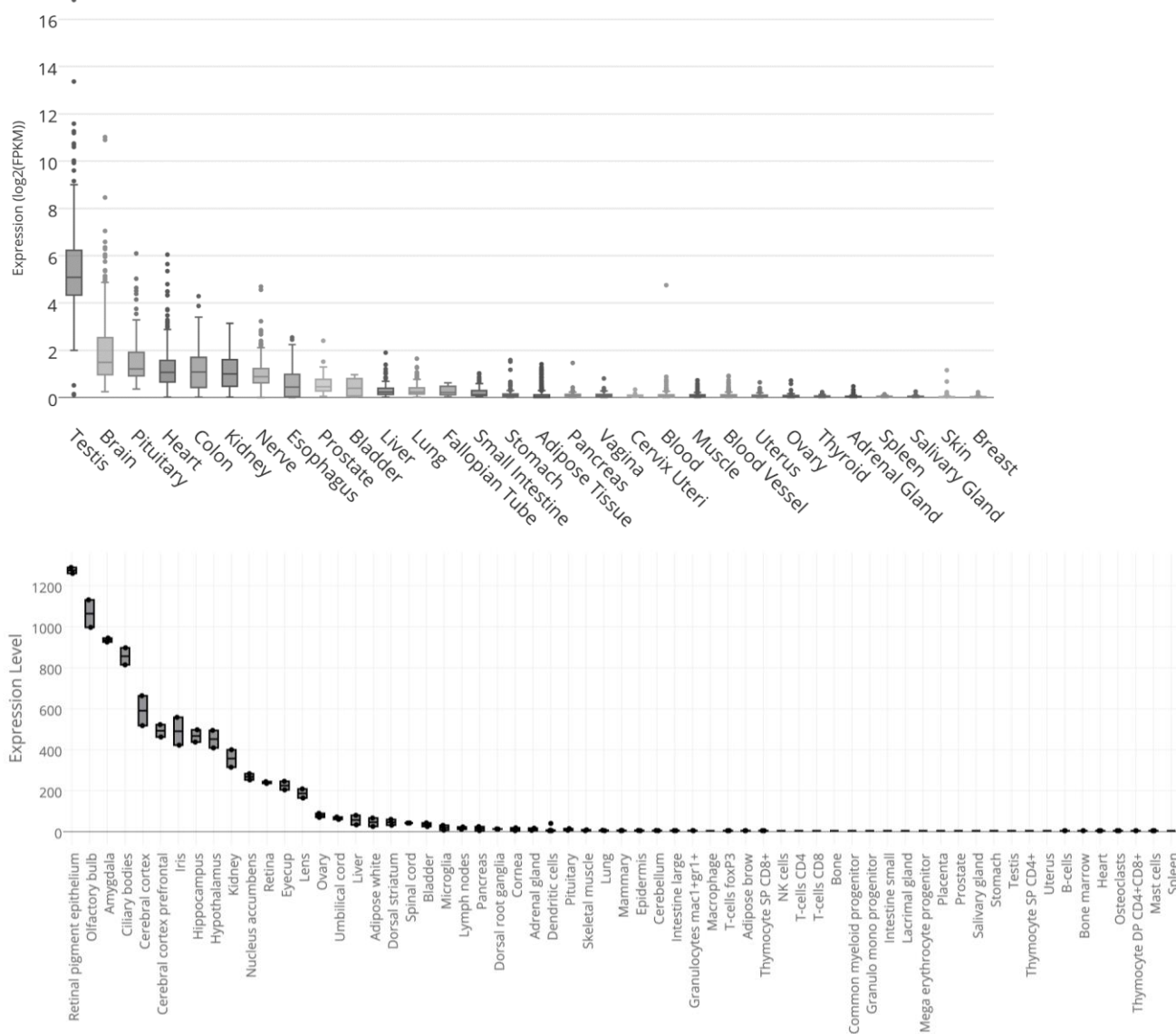
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717 **Figure 2a**

718 **Figure 2b**

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720 **Figure 2.** Expression profile of ILDR2 mRNA (a) ILDR2 mRNA expression data from normal human
721 tissues was extracted from the Genotype-Tissue Expression (GTEx) database. The Y axis depicts

expression levels as FPKM (Fragments per kilobase of exon per million fragments mapped). (b)
Expression data of murine Ildr2 mRNA in normal mouse tissues was extracted from the GNF
Gene Expression Atlas. Values shown in the Y axis are arbitrary units of microarray gene
expression levels.

Figure 3

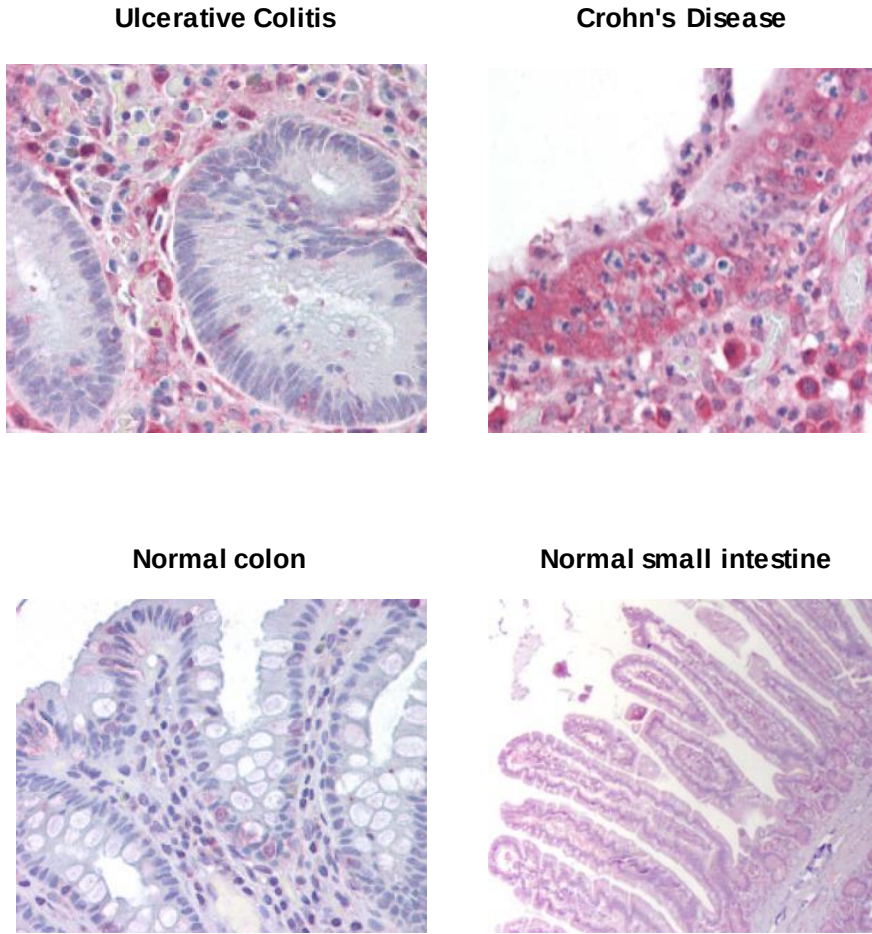


Figure 3. ILDR2 protein expression in inflamed tissues. Immunohistochemistry of ILDR2
expression within inflamed tissue samples of ulcerative colitis and Crohn's disease, as well as
normal controls of colon and small intestine, was carried out using anti-ILDR2 polyclonal

antibody, Ab-13, as described in Methods. Shown are representative staining out of two samples of each tissue and condition. Magnification X40.

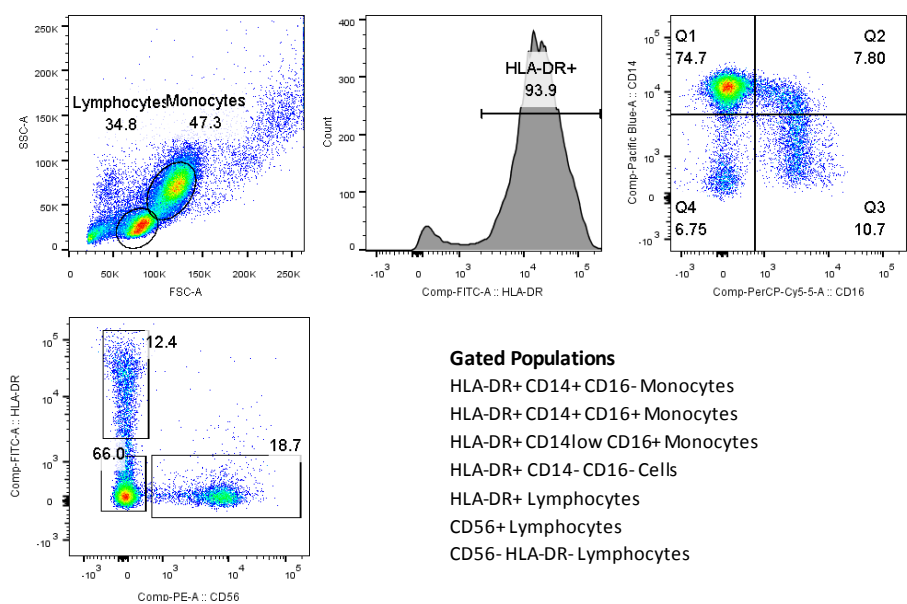
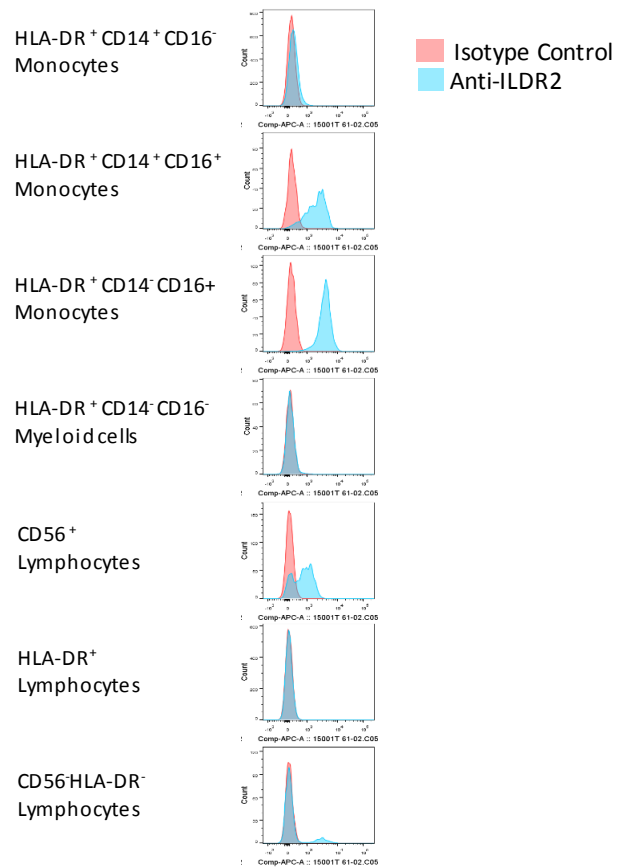
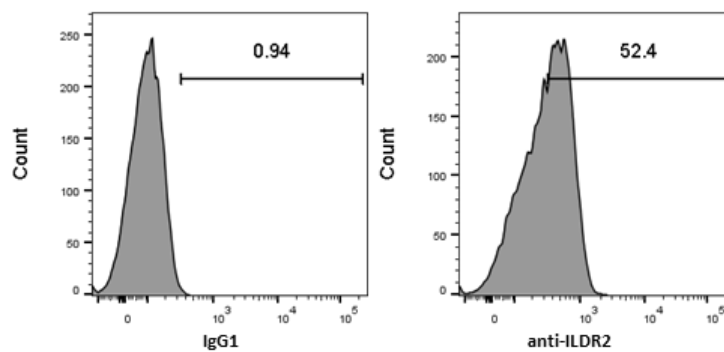


Figure 4a



739 **Figure 4b**

740 **Figure 4c**



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Figure 4d

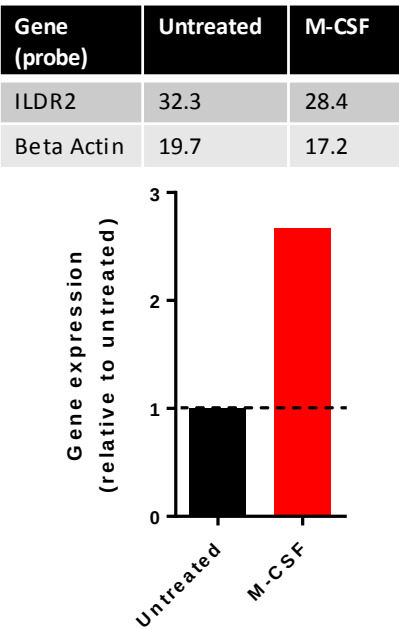


Figure 4. ILDR2 protein expression on immune cell subpopulations. (a) Gating strategy for monocyte and lymphocyte subpopulations derived from human PBMCs. Lymphocytes were identified by low FSC/SSC, and monocytes by high FSC/SSC and by HLA-DR staining. Monocyte subtypes were defined based on relative expression of CD14 and CD16 (see text for details). HLA-DR+ CD14-CD16- most likely consist of various other myeloid cells. Lymphocytes populations were defined based on CD56 or HLA-DR expression (CD56+, mostly NK and NKT cells; HLA-DR+, mostly B cells; and CD56- HLA-DR-, mostly T cells). (b) PBMCs from human healthy donors were subjected to FACS analysis and gated as in A, following staining with AF647 conjugated anti-ILDR2 Ab (Ab-10) or with isotype control (hIgG1). Shown is one representative healthy donor representative out of 7. (c-d) ILDR2 expression on human monocyte-derived macrophages. Human macrophages were obtained by *in vitro* differentiation of blood monocytes using M-CSF, as described in Methods. (c) ILDR2 protein expression was evaluated

on monocyte-derived macrophages by FACS analysis using anti-ILDR2 mAb (Ab-10) or isotype control (hIgG1). (d) ILDR2 mRNA expression was evaluated by qRT-PCR. Ct values indicated in the table were obtained for ILDR2 and for beta actin (housekeeping gene) in monocyte-derived macrophages or in untreated monocytes (incubated in media only). Bars show ILDR2 expression (normalized to beta actin) in monocyte-derived macrophages relative to untreated monocytes.

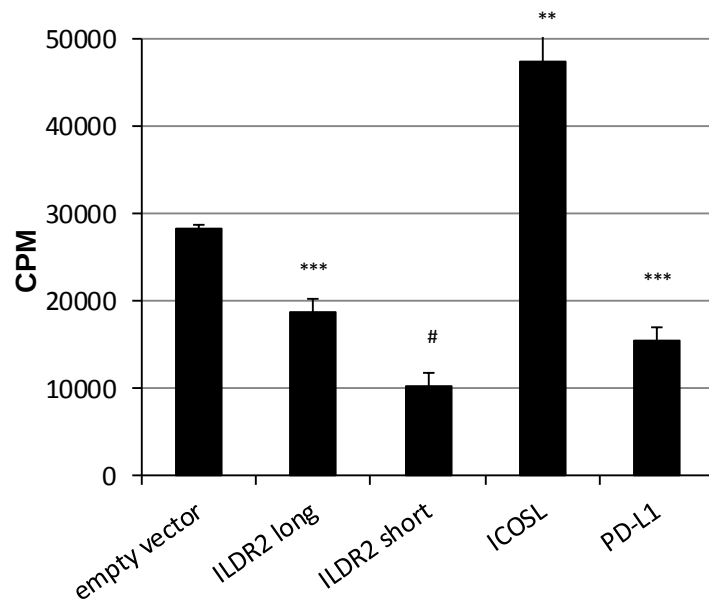
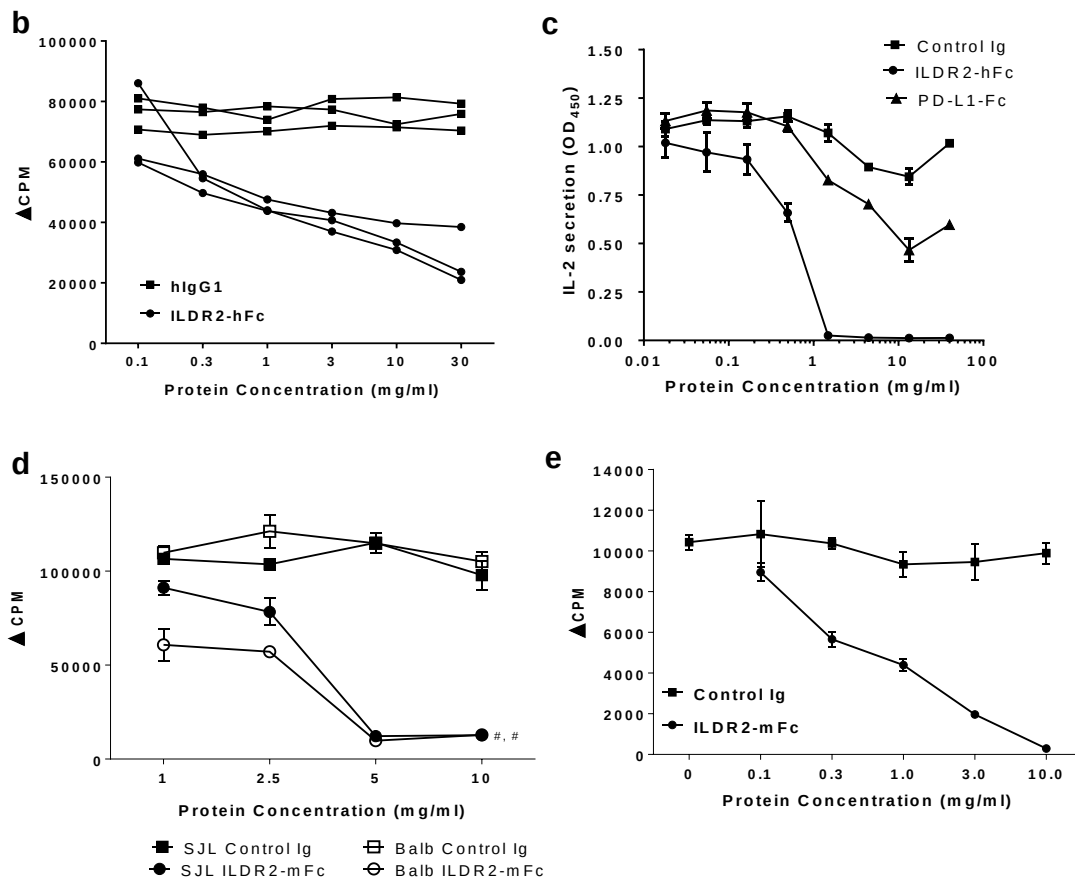


Figure 5a

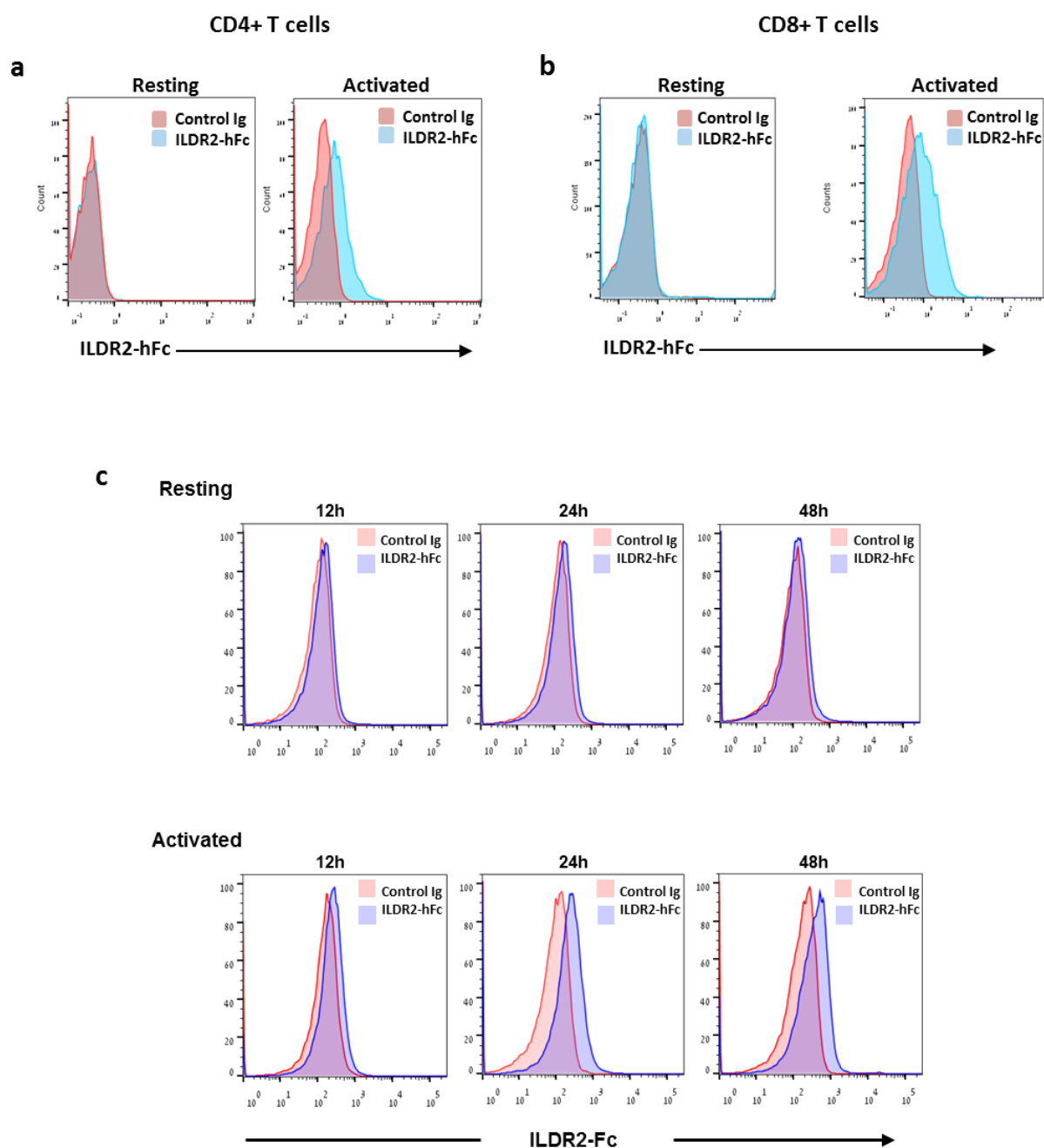
Figure 5b-e

Figure 5. ILDR2 inhibits activation of murine and human T cells. (a) Membrane expressed ILDR2



inhibits human T cell activation. T cell 'stimulator cells' (a murine thymoma cell line engineered to express membrane-bound anti-human CD3 single chain antibody fragments) were transfected with expression constructs encoding the long or the short splice variant of human ILDR2. Cells transfected with the empty vector served as negative control. Proliferation of human CD3+ T cells was evaluated upon co-culture with 'stimulator cells' expressing the ILDR2 short and long variants, and ICOSL or PD-L1, as reference. Shown is a representative experiment out of 4 experiments carried out with a total of 3 donors. Presented are Means +/- SEM of triplicate samples. **p<0.01, ***p<0.001, and #p<0.0001 compared to empty vector. **(b)** Soluble ILDR2-hFc inhibits activation of human T cells. Naïve CD4+ T cells obtained from human PBMCs were activated by soluble anti-CD3 and anti-CD28, in the presence of irradiated autologous PBMCs. ILDR2-hFc or isotype control (hIgG1, Synagis) were added at the specified final concentrations. Cell proliferation was evaluated by ³H-thymidine incorporation at 72 hours. Shown are representative results obtained with three human donors. **(c)** Immobilized ILDR2-hFc has inhibitory effect on reactivation of stimulated human T cells. Human T cell blasts, obtained by stimulation of human PBMCs with PHA and hIL2 for 10 days, were reactivated for 3 days in 96-well plates coated with anti-CD3, in the presence of ILDR2-hFc or isotype control (rhIgG1-Fc) at the indicated concentrations, as described in Methods. IL-2 secretion was measured by ELISA after 24hrs. PD-L1-Fc (rhB7-H1/Fc) was used as positive control. Shown are representative results for one out of three experiments. **(d)** Bead-immobilized ILDR2-mFc reduces polyclonal murine T cell proliferation. Naïve CD4+ T cells were isolated from two mouse strains, SJL or BALB/c, and activated in the presence of beads coated with anti-CD3 and anti-CD28, as described in Methods. The beads were co-coated with the indicated concentrations of ILDR2-mFc or isotype control (mIgG2a). Cells were activated with the coated beads at a ratio of 1:1 (T cells to beads), and cell proliferation was evaluated by ³H-thymidine incorporation after 72 hours. **(e)** Soluble ILDR2-mFc reduces antigen-specific murine T cell activation. Naïve CD4+ T cells from DO11.1 mice were activated by 20ug/ml OVA₃₂₃₋₃₃₉ peptide and co-incubated with irradiated APCs at ratio of 1:1 (APCs to T cells) in the presence of different concentrations of ILDR2-mFc or isotype control (mIgG2a). Proliferation was evaluated by ³H-thymidine incorporation after 72 hours.

Figure 6a-c



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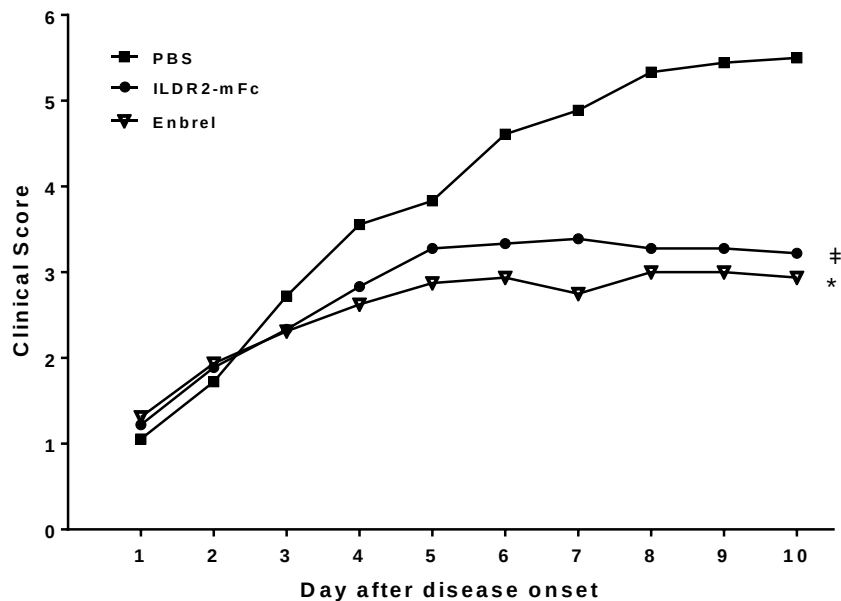
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Figure 6. ILDR2-Fc binds to activated T cells. Binding of ILDR2-hFc was analyzed on resting and activated human CD4+ (a) and CD8+ (b) T cells. Isolated human CD4+ or CD8+ T cells were left untreated or stimulated with plate bound anti-CD3 for 72hrs, as described in Methods. Cells were incubated with ILDR2-hFc or isotype control (hIgG1, Synagis) at 100µg/ml, followed by staining with PE-conjugated anti-hIgG Fc and evaluated by flow cytometry. Results, shown as histograms of IDLR2-hFc binding or of isotype control hIgG1, are representative of two independent experiments. (c) Binding kinetics of ILDR2-mFc to resting and activated mouse CD4+ T cells. CD4+CD25- T cells were isolated from Balb/C splenocytes, and activated using plate bound anti-CD3 and soluble anti-CD28, as described in Methods. Binding of biotinylated ILDR2-mFc and isotype control (mIgG2a) was tested at different time points of T cell activation and evaluated by flow cytometry. Results are shown as histograms of IDLR2-mFc binding and of isotype control mIgG2a.

839 **Figure 7**



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841 **Figure 7.** Therapeutic effect of ILDR2-mFc in CIA model of RA. DBA/1 mice were immunized with
842 bovine type II collagen in CFA and treated from the day of disease onset (day 1) with ILDR2-mFc,
843 Enbrel (100ug/mouse each) or PBS (vehicle control), 3 times per week for 10 days. Average
844 clinical scores are shown for n = 8-9 mice/ group. Shown is a representative study out of two.
845 ‡p=0.079, *p=0.047 vs. PBS.

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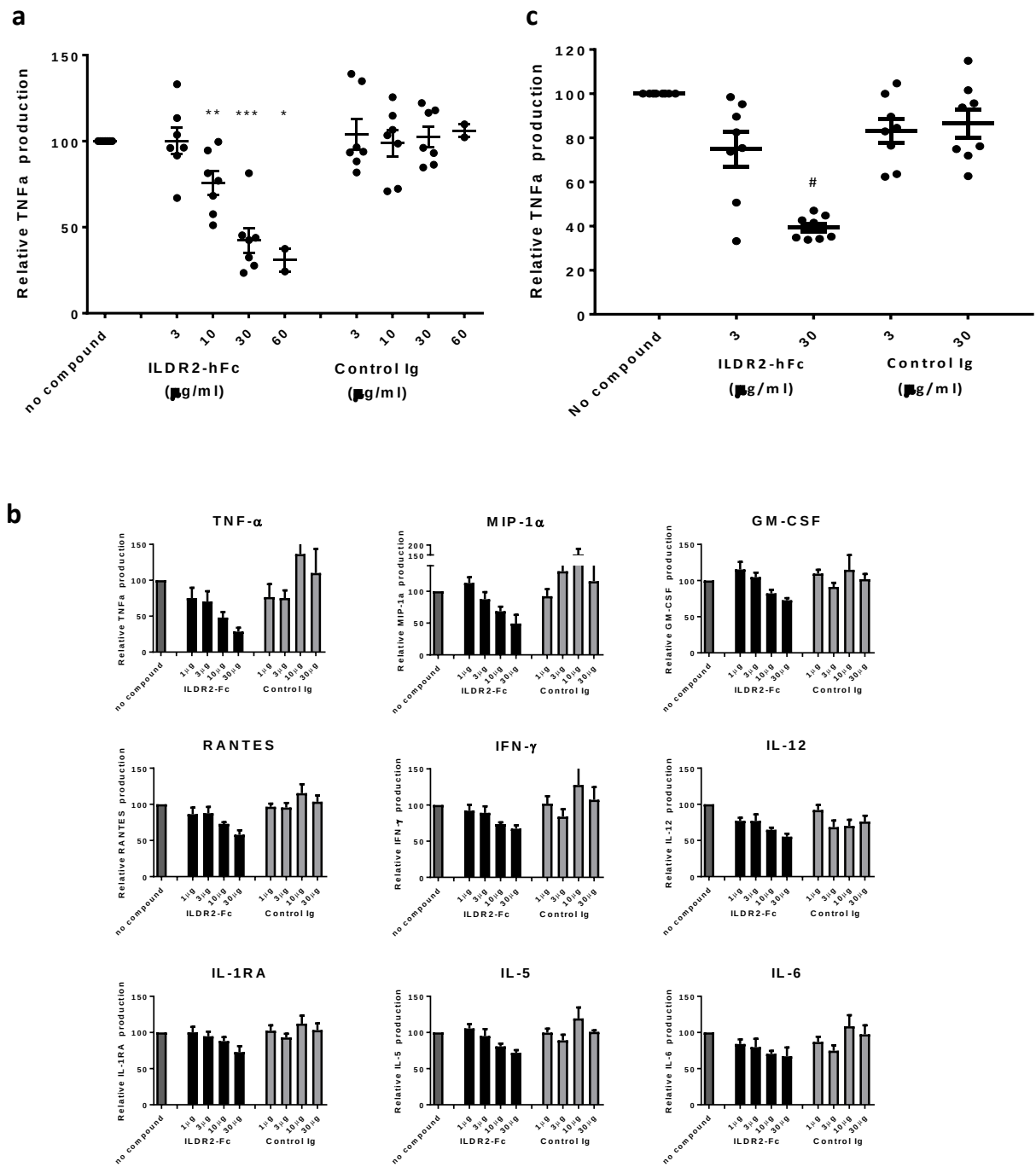


Figure 8

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857 **Figure 8.** ILDR2-hFc inhibits RA-related cytokines in translational autologous co-cultures of
858 'synovial like' cytokine stimulated T cells (Tck) and monocyte-derived macrophages (Mφ)
859 established from PBMCs of healthy controls or RA patients. ILDR2-hFc or isotype control (hIgG1)
860 were added at the indicated concentrations to Tck and Mφ co-cultures derived from healthy
861 donors PBMCs, as described in Methods. After 24 hrs, supernatants were analysed for TNFα
862 secretion by ELISA (**a**). Supernatants from 4 donors were also evaluated for secretion of
863 additional RA-related cytokines and chemokines by Luminex (**b**). TNFα secretion in Mφ and Tck
864 co-cultures derived from PBMCs of RA patients was evaluated by ELISA (**c**). Cytokine values were
865 normalized to those obtained in cultures without any added compound ('No compound'). Dots
866 represent individual donors; Bars represent average ± SEM. *p<0.05, **p<0.01, ***p<0.001,
867 #p<0.0001 vs. corresponding concentration of Control Ig.

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884 **Table 1. Identity and Similarity of ILDR2 IgV to other B7 family proteins**

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Protein IgV	Identity	Similarity
VISTA	19.5%	34.0%
PDL1	17.9%	30.8%
PDL2	17.5%	24.4%
B7H3	20.8%	35.6%
B7H4	16.9%	27.1%
ICOSL	17.2%	35.2%
CD80	16.4%	31.5%
CD86	15.3%	27.0%