Soluble LILRA3, a Potential Natural Antiinflammatory Protein, Is Increased in Patients with Rheumatoid Arthritis and Is Tightly Regulated by Interleukin 10, Tumor Necrosis Factor-α, and Interferon-γ

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ABSTRACT. ***Objective.*** Leukocyte immunoglobulin-like receptor A3 (LILRA3) belongs to a family of cell-surface receptors with inhibitory or activating functions. LILRA3 lacks transmembrane and cytoplasmic domains, suggesting that it may be secreted. LILRA3 has high homology to activating LILRA1 and A2, hence may act as a soluble agonist/antagonist to these receptors. Individuals lacking the LILRA3 gene have higher incidence of multiple sclerosis and Sjögren's syndrome, suggesting LILRA3 may be antiinflammatory. LILRA3 mRNA was detected in monocytes and mast cells but no protein expression has ever been described. Our aim was to examine LILRA3 protein expression in serum and synovial fluid of patients with rheumatoid arthritis (RA) and determine its *in vitro* regulation.

Methods. We developed a new ELISA to examine levels of LILRA3 in serum, synovial fluid, and/or culture supernatants from controls and patients with RA, degenerative arthritis, or gout. We used qRT-PCR and flow cytometry to determine the expression and cytokine-mediated regulation of LILRA3.

Results. LILRA3 protein is constitutively present in normal serum, with significantly higher concentrations in patients with RA. Serum LILRA3 concentrations from RA patients correlated with disease activity and levels in synovial fluid. Treatment of monocytes with interleukin 10 or interferon-γ significantly upregulated while tumor necrosis factor-α significantly downregulated LILRA3 mRNA and protein expression.

Conclusion. We show for the first time that LILRA3 is significantly increased in serum of patients with RA and is tightly regulated by key cytokines involved in pathogenesis of RA. These results suggest that LILRA3 may play a role in chronic inflammatory conditions such as RA. (First Release July 1 2010; J Rheumatol 2010;37:1596–606; doi:10.3899/jrheum.091119)

Key Indexing Terms:
LEUKOCYTE IMMUNOGLOBULIN-LIKE RECEPTOR A3 — CYTOKINES
ENZYME-LINKED IMMUNOSORBENT ASSAY — RHEUMATOID ARTHRITIS

Leukocyte immunoglobulin-like receptors (LILR), also termed Ig-like transcripts (ILT), are a family of 13 highly homologous molecules localized within the leukocyte receptor complex in chromosome 19q13.4[1,2]. They are primarily expressed by leukocytes and are increasingly recognized as critical regulators of leukocyte activation by providing negative or positive signals[1,2,3]. LILR have two or four C-2-type Ig-like extracellular domains and are classified as inhibitory, activating, or soluble receptors based on their transmembrane and cytoplasmic domain structures[4,5]. Inhibitory LILR (B1, B2, B3, B4, and B5) have long cytoplasmic tails containing immunoreceptor tyrosine-based inhibitory motifs (ITIM) that transmit inhibitory signals[4,6,7,8]. Activating LILR (A1, A2, A4, A5, ILT8, ILT1-like) have short cytoplasmic domains lacking signaling motifs but contain a charged arginine residue in their transmembrane domain that links with immunoreceptor tyrosine-based activation motifs (ITAM) of the FcR common γ chain, facilitating activation signals[1,2,3,6,9,10].

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LILRA3 (also known as LIR4, ILT6, or CD85e) has no transmembrane domain and is predicted to be secreted[1,2,3].

LILRA3 is the only LILR showing diversity in mRNA expression, with functional LILRA3 absent in some individuals[2,6,11,12]. It is presently unknown whether the LILRA3-null allele has arisen due to environmental-specific pathogen disruption[13,14] or due to some form of balancing selection[15]. Although the latter seems consistent with the observation that the 6.7-kb LILRA3 deletion is detected worldwide, the extremely high frequency of this deletion in Japanese compared to other populations[15] may indeed point to disruptive selection. Functionally, LILRA3 that bears close sequence similarity to the extracellular domains of activating LILRA1 and LILRA2, and inhibitory LILRB1 may bind the same or similar ligands, thus acting as an antagonist or agonist to these LILR[1,6]. However, ligand(s) for LILRA3 and most of the activating and inhibitory LILR remain unknown.

Inhibitory LILRB1 and LILRB2 have been demonstrated to bind classical and nonclassical MHC class I molecules[16] and a viral class I homologue (UL-18)[16,17]. LILRA1 was shown to bind HLA-B27[18]. Nevertheless, a number of recent studies show that several LILR can also functionally bind to non-MHC class I molecules including binding of *Staphylococcus aureus* to LILRB1[19], Nogo 66 to LILRB2[20], and bone marrow stromal cell antigen-2 to LILRA4[21]. These indicate that regulatory functions of LILR may not be restricted to diseases associated with MHC class I molecules. Interestingly, the extensive expression of most activating and inhibitory LILR by cells primarily involved in innate immunity, namely monocytes and neutrophils[22], suggest that LILR may play a key role in regulation of Toll-like receptor (TLR)-mediated cellular activation. Indeed, recent studies show that both activating and inhibitory LILR strongly inhibit TLR-mediated monocytes (Tedla, *et al*, unpublished data) and dendritic cell[23] activation, and modulate dendritic cell functions[24,25]. Moreover, mice lacking gp49B1 (an ortholog of LILRB4) expression on neutrophils and monocytes have exaggerated systemic responses to lipopolysaccharide (LPS) *in vivo*[26].

Rheumatoid arthritis (RA) is a chronic inflammatory synovitis mediated by a range of activated inflammatory leukocytes and stromal cells[27,28]. The mechanisms regulating leukocyte activation in RA are not fully understood[27,28]. Previously, we reported extensive expression of activating LILRA2 and LILRA5 and inhibitory LILRB2 in synovium of patients with active RA but not in osteoarthritis (OA), suggesting that these receptors may play a role in the pathogenesis of chronic joint inflammation[29]. Further, levels of LILR expression correlated significantly with disease severity[30] and were reduced in synovial tissue from patients who responded to treatment with disease-modifying antirheumatic drugs, consistent with regulated expression of LILR in inflammation[31]. We propose that LILRA3 may be a soluble antagonist of activating LILRA1 and/or A2. Although no protein expression has ever been described for LILRA3, its mRNA is constitutively expressed in immune cells[6]. The regulation of LILRA3 mRNA expression *in vivo* and *in vitro* is poorly defined. We describe for the first time that LILRA3 protein is constitutively expressed in serum of healthy subjects and is significantly increased in patients with RA. Consistent with its possible antiinflammatory function, LILRA3 was abundantly expressed in peripheral blood mononuclear cells (PBMC) of healthy subjects, and was significantly upregulated by immune-suppressive [interleukin 10 (IL-10)] and immune-regulatory cytokines [interferon-γ (IFN-γ)] and significantly downregulated by proinflammatory cytokine [tumor necrosis factor-α (TNF-α)].

MATERIALS AND METHODS

Patients and subjects. Archival serum and synovial fluid from 26 patients with RA, 10 with OA, 10 with gout, and 68 healthy donors were used for LILRA3 ELISA. All patients and controls were Caucasian. RA, OA, and gout patients were defined in accord with the American College of Rheumatology criteria[32] or clinical diagnosis, respectively. Clinical assessment of RA activity utilized the Disease Activity Score 28-joint count (DAS28)[33], with DAS28 < 2.6 designating low disease activity[34].

This study was approved by the institutional ethics committee, and informed consent was obtained from all patients and controls.

Buffers and reagents. Phosphate buffered saline (PBS), PBS + 0.05% sodium azide (PBSN), PBS + 0.05% Tween-20 (PBST), pH 7.4, tris-buffered saline (TBS), pH 8.0, TBS + 0.1% Tween-20 (TBST); bovine serum albumin (BSA), *Escherichia coli* LPS and Brefeldin A (Sigma, Sydney, Australia); streptavidin-HRP, recombinant TNF-α, IL-10 and IFN-γ (R&D Systems, Minneapolis, MN, USA); TMB chromogen substrate (Nalgene, Panbio, Brisbane, Australia); Western chemiluminescence reagent (Perkin Elmer, Waltham, MA, USA); biotinylated goat anti-mouse secondary antibody; FITC-conjugated goat F(ab')2 anti-mouse IgG [F(ab')2-specific] (Jackson ImmunoResearch, West Grove, PA, USA); irrelevant mouse IgG1-and IgG2B negative control monoclonal antibodies (mAb; Sigma, Australia); flurochrome-conjugated primary mAb against CD14-PE, CD4–PE, CD8-Percp and CD3-APC and corresponding controls (Pharmingen); 2 specific anti-LILRA3 antibodies, a mouse IgG1 mAb clone 2E9, and mouse IgG2B mAb raised against the full-length protein, were purchased from Abnova and R&D Systems, respectively. Zenon mouse IgG1 labeling kit was used to conjugate anti-LILRA3 with Alexa 488 (Molecular Probes Inc., Eugene, OR, USA). In-house rhLILRA3 proteins were produced in *Escherichia coli* and mammalian cells. Specificity of anti-LILRA3 antibody (Abnova) was confirmed by ELISA and Western blotting against a panel of purified LILR-APtag fusion proteins expressed by 293T cells that had been transfected with the full extracellular domains of LILR cDNA (Figure 1D). FACS analysis of nonpermeabilized peripheral blood monocytes that express all surface inhibitory and activating LILR[22] was used to further confirm specificity of both anti-LILRA3 antibodies (Figure 1E).

Detection of LILRA3 in serum and synovial fluid by ELISA. A new ELISA for LILRA3 was developed after extensive optimization. In brief, 96-well Nunc Maxisorb plates were precoated with 100 μl of 0.05 M bicarbonate buffer, pH 9.6, for 30 min at room temperature (RT). Recombinant LILRA3 standards were serially diluted, serum or synovial fluid samples were diluted 1 in 10 in TBS + 0.1% BSA, and culture supernatants were used neat. 100 μl of diluted samples and standards were added to wells in duplicate, and incubated 2 h at 37°C, then left overnight at 4°C. The following day, plates were blocked with 300 μl/well of 5% skim milk powder in PBST for 30 min at RT. Plates were washed 3 times with strong agitation using 200 μl/well PBST, then 0.5 μg/ml mouse anti-LILRA3 mAb (Abnova) diluted

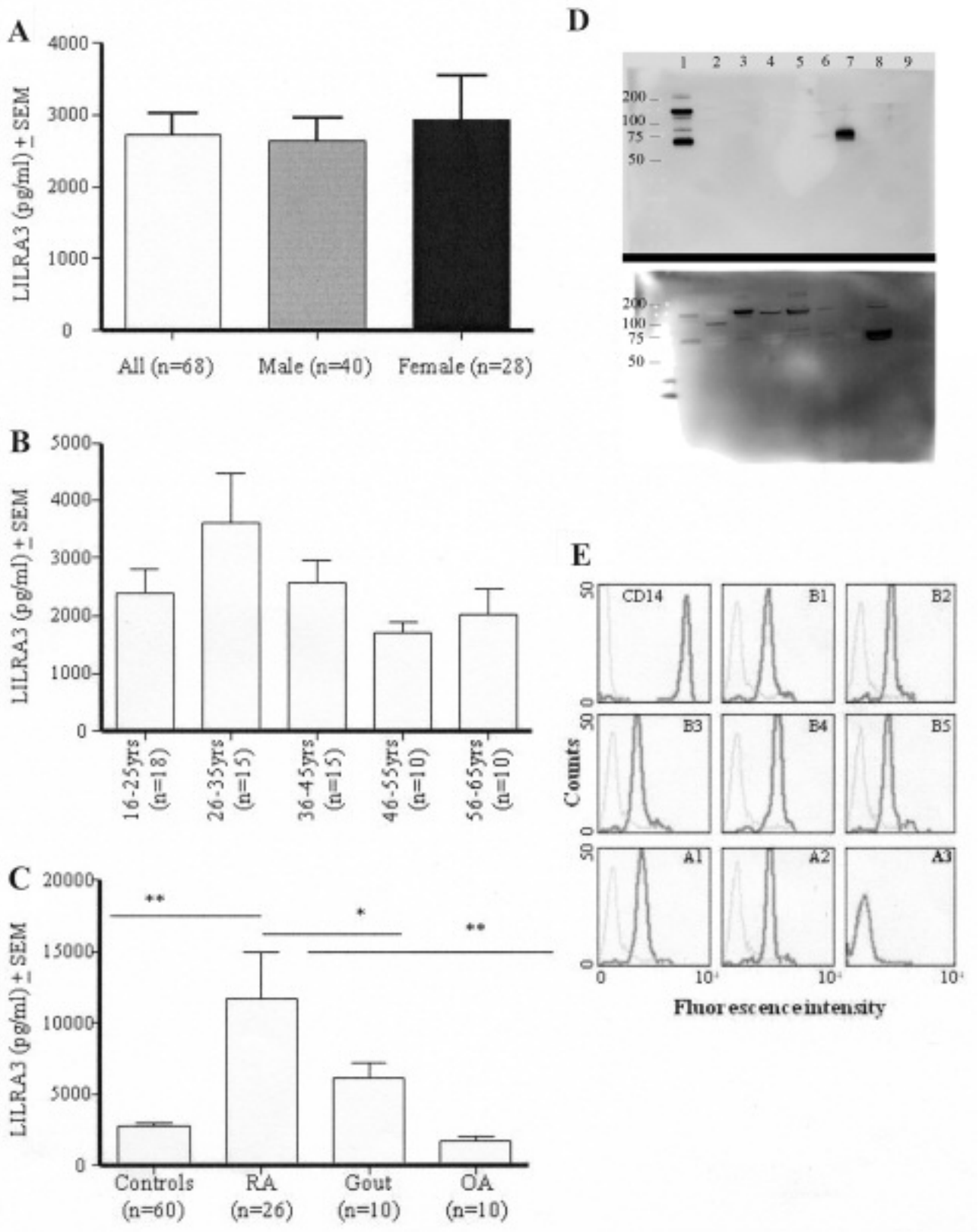

Figure 1.

Figure 1. A. LILRA3 ELISA in serum showing constitutive presence of LILRA3 in serum from healthy subjects. B. Donors in the age group 26–35 years had the highest and those age 46–55 years had the lowest levels of LILRA3, although differences were not statistically significant. C. Serum from patients with RA showed an average of 4.3-fold higher levels of LILRA3 than controls, 2-fold higher than patients with gout, and 6.9-fold higher than patients with degenerative arthritis. The increased expression of LILRA3 in serum of patients with RA was statistically significant compared to controls ($p < 0.01$), patients with gout (*$p < 0.05$), and patients with osteoarthritis (OA; **$p < 0.01$). D. Specificity of anti-LILRA3 mAb was confirmed by Western blot analysis of purified alkaline phosphatase-tagged recombinant mammalian LILRA3 (lane 1), B4 (lane 2), A1 (lane 3), A2 (lane 4), B1 (lane 5), B2 (lane 6) fusion proteins showing specific immunoreactivity to only LILRA3 protein (upper panel, D). Alkaline phosphatase non-tagged mammalian LILRA3 was used as additional positive control (lane 7), and culture supernatant from mock transfected 293T cells was used as negative control (lane 9). Lower panel, D, is the same membrane reblotted with rabbit anti-placental alkaline phosphatase antibody showing all lanes had detectable amounts (200–500 ng) of protein loaded into the gel. As expected, the lanes with non-tagged LILRA3 (lane 7) and negative control (lane 9) did not show immunoreactivity and AP-tag protein showed strong positive band (lane 8). E. Flow cytometric analysis of nonpermeabilized peripheral blood monocytes using specific IgG1 mouse mAb against each surface LILR (Amgen Inc.) and soluble LILRA3 (Abnova) shows surface expression of all LILR, except LILRA3. This further confirmed that LILRA3 mAb not non-specifically detect any of the other LILR despite their abundant expression on the surface of monocytes.

in blocking buffer was added to each well and incubated at 4˚C overnight. Wells were washed 3 times. After 2 h incubation at RT with 1 μg/ml biotinylated goat anti-mouse secondary antibody in PBSN, wells were thoroughly washed and further incubated with 2.5 μg/ml of streptavidin-HRP in TBS for 20 min at RT. After washing, 100 μl of TMB substrate was added and the plates were incubated in the dark for 60 min at RT. The reaction was stopped by adding 50 μl 1 M H_2SO_4 to each well, and optical density measured at 450/540 nm (SpectraMax Plus, Molecular Devices, Sunnyvale, CA, USA).

Flow cytometry for intracellular LILRA3 expression. Peripheral blood mononuclear cells were isolated from patients with RA and healthy donors using standard density gradient centrifugation and stained for intracellular LILRA3 and cell-surface markers as described[35]. In brief, cells were washed with cold PBS containing 0.05% NaN_3 and 1% BSA (PAB buffer) and prefixed with 4% paraformaldehyde in PBS at 5 x 10^6 cells/ml for 10 min at RT. Cells were washed with 10 volumes of cold PAB buffer and resuspended at 2 x 10^6 cells/ml in 0.5% saponin in PBS containing 1% BSA (permeabilization medium) for 20 min at room temperature with constant shaking. 50 μl aliquots of the cell suspension in permeabilization medium were then incubated with unconjugated anti-LILRA3 mAb (5 μg/ml) or control mouse IgG1 (10 μg/ml) for 30 min at RT. After a single wash with the permeabilization medium, cells were incubated with FITC-conjugated F(ab')2 goat anti-mouse IgG [F(ab')2-specific] secondary antibody at 10 μg/ml for 30 min on ice. After 3 washes with PAB buffer, cells were post-fixed in 1% paraformaldehyde in PBS, and analyzed by flow cytometry. The coexpression of LILRA3 with T cell subsets was determined by 4-color flow cytometry using Alexa-488 labeled anti-LILRA3 IgG1 mAb and directly conjugated T cell markers.

Western blot analysis of LILRA3 in PBMC. Cell lysates from fresh normal PBMC were prepared in Western lysis buffer containing 1% NP-40, 150 mM NaCl, 50 mM Tris HCl, pH 7.5, 20 mM iodoacetamide, 5 mM EDTA, and protease inhibitors (Roche Applied Science, Castle Hill, NSW, Australia). Cell lysates (25 μg) were separated in 10% SDS-PAGE reducing gels and transferred to PVDF membranes (Perkin Elmer, Boston, MA, USA). Membranes were then blotted with 5 μg/ml anti-LILRA3 mAb (Abnova) and HRP-conjugated goat anti-mouse secondary antibody (BioRad, Regents Park, NSW, Australia) and immunoreactivity detected by chemiluminescence (Perkin Elmer)[36].

RNA extraction for real-time polymerase chain reaction (RT-PCR) and quantitative PCR (qPCR). Monocytes from patients and controls were negatively selected from freshly isolated PBMC with > 95% purity (Miltenyi Biotec, Auburn, CA, USA). CD3, CD4, CD8, and CD19 were positively and natural killer cells negatively selected from PBMC using dynabeads with > 98% purity (Invitrogen). Total RNA was extracted from the purified cells using RNA Extraction Kit (Promega, Sydney, Australia). Reverse transcription was performed on 1 μg of RNA using SuperScript III Kit (Invitrogen). Aliquots (1 μl) of cDNA were then used for PCR using primers designed to specifically identify LILRA3 or control GAPDH as described[35]. For quantitative analysis, LILRA3 transcript levels were determined by RT-PCR. Aliquots (5 μl) of cDNA were amplified using SYBRGreenER qPCR SuperMix and 200 nM of LILRA3 primer set (forward 5'-AAT CAA AGC GCC AAT CTC AT-3'; reverse 5'-GAG TCA GCA GGT AGG GGT TG-3') or 100 nM of ß-actin control primers (forward 5'-CAT GTA CGT TGC TAT CCA GGC-3'; reverse 5'-CTC CTT AAT GTC ACG CAC GAT-3') in an ABI Prism 7700 sequence detector (Applied Biosystems). A 2 min start at 50˚C and then 2 min at 95˚C was followed by 45 cycles of PCR (95˚C, 15 s; 60˚C, 45 s). The reactions finished with 2 min at 25˚C and results analyzed as described[36]. The integrity of amplification indicated by a single melt peak for each product was verified by a dissociation curve analysis at 95˚C for 15 s, 60˚C for 45 s, and 95˚C for 15 s, and further confirmed by detection of the expected single 120-bp product after agarose gel analysis and DNA sequencing.

Modulation of LILRA3 mRNA and protein expression by cytokines. 1 × 10^6/ml of negatively selected monocytes from healthy subjects were incubated in RPMI media containing 10% autologous serum with rhTNF-α (25 ng/ml), rhIL-10 (25 ng/ml), rhIFN-γ (25 ng/ml), or a combination of rhTNF-α (25 ng/ml) and rhIL-10 (25 ng/ml), for 1–12 h at 37˚C in 5% CO_2. Cells were then harvested for detection of LILRA3 mRNA using qPCR. For protein detection, cell supernatants were collected 12–72 h after incubation with the above reagents plus or minus 5 μg/ml Brefeldin A and used for intracellular LILRA3 staining and LILRA3 ELISA.

Statistical analysis. Kruskal-Wallis test and Dunn's post-test were used to compare serum LILRA3 within healthy subjects and Bonferroni-corrected Mann-Whitney U test was used to compare control serum to patient samples. Spearman r was used correlate disease activity score to the amounts of LILRA3 in the serum of patients with RA and to correlate levels in synovial fluid to serum of patients. The expression of LILRA3 in PBMC of patients was compared to controls using one-way ANOVA. One-way ANOVA with Dunnett's post-test was used to compare LILRA3 mRNA and protein expression *in vitro*. A p value < 0.05 was considered significant.

RESULTS

Patients with RA have significantly higher levels of LILRA3 in serum. LILRA3 protein was present in sera from all healthy subjects (mean age 41.2 ± 10.6 yrs; male:female ratio 1.4:1) with a mean concentration of 2700 ± 300 pg/ml (Figure 1A). Although LILRA3 protein levels in females (2900 ± 420 pg/ml) were higher than in males (2600 ± 200 pg/ml) and in the 26–35-year age group in both sexes, the differences were not statistically significant (Figure 1A). The average serum LILRA3 in patients with RA (mean age 49 ± 17.9 yrs; M:F 1:6) was 11700 ± 2951 pg/ml, which was significantly higher than in controls (2700 ± 300 pg/ml), patients with gout (6000 ± 100 pg/ml; mean age 61.7 ± 11.3 yrs; M:F 1:1), and patients with OA (1700 ± 324 pg/ml;

mean age 77.3 ± 7.5 yrs; M:F 2:1; Figure 1C). Patients with RA in remission showed levels comparable to healthy subjects, whereas those with severe disease had levels 10 times higher than those patients in remission (Figure 2A). Moreover, serum LILRA3 in patients with RA correlated significantly with their Disease Activity Score (Spearman r = 0.806, $p < 0.01$; Figure 2B), and the amount of serum LILRA3 in 9 patients correlated to the levels found in their corresponding synovial fluid samples regardless of disease activity (Spearman r = 0.402); however, this did not reach statistical significance (p = 0.059; Figure 2C).

LILRA3 is constitutively expressed in normal PBMC and is upregulated in patients with RA. Both anti-LILRA3 mAb detected high levels of LILRA3 expression in permeabilized lymphocytes and monocytes (Figure 3A) of healthy subjects, but as expected not on the cell surface (data not shown). Double-staining with anti-CD4 or anti-CD8 T cells showed expression of LILRA3 in both subsets (Figure 3A). Moreover, Western blots showed LILRA3 protein of ~50 kDa in PBMC lysates from 2 subjects (Figure 3A), further confirming constitutive expression of LILRA3 protein in healthy subjects. Lymphocytes and monocytes from 10 patients with active RA (DAS28 > 2.6) showed marginally higher intensity intracellular staining for LILRA3 compared to control subjects (n = 15) with mean fluorescence intensity (MFI) of 82.7 ± 7.6 versus 63.1 ± 4.2 and 80.5 ± 9.4 versus 69.3 ± 4.8, respectively (Figures 3B, 3C). The proportions of LILRA3-positive monocytes (76.1% ± 2.1% vs 79.9% ± 3.4%) and lymphocytes (81.8% ± 3.3% vs 72.8% ± 4.2%) were similar in patients and controls (Figure 3C). LILRA3 mRNA was ubiquitously expressed in peripheral blood leukocytes from RA and control subjects (Figure 4A). LILRA3 levels were significantly higher in B cells and monocytes of patients with active RA by 3-fold and 2.3-fold, respectively, but there was no significant difference in the levels expressed by T cells and natural killer cells (Figure 4B).

LILRA3 is modulated by cytokines in monocytes. *In vitro* treatment of monocytes with IL-10 and IFN-γ caused significant upregulation of LILRA3 in a time-dependent manner (peak 6 h) with 14.6-fold and 7.1-fold increases, respectively, compared to untreated cells (Figure 4C). In contrast, treatment with TNF-α for 1 h significantly downregulated constitutively expressed LILRA3 mRNA by 3.3-fold. Interestingly, *in vitro* culture of monocytes over a 12-h period without treatment caused a progressive decrease in constitutively expressed LILRA3 mRNA (Figure 4C). Treatment of monocytes from healthy subjects with IL-10 or IFN-γ in the presence of Brefeldin A caused a time-dependent upregulation of intracellular LILRA3 protein (Figures 5A, 5B), but there was a substantial decrease in cells treated with TNF-α (data not shown). The MFI from 3 independent experiments after 48-h treatment with IL-10 and IFN-γ were 41.6 ± 1.5 and 88.7 ± 5.1, respectively, which were significantly higher than the untreated control cells (28 ± 2.3;

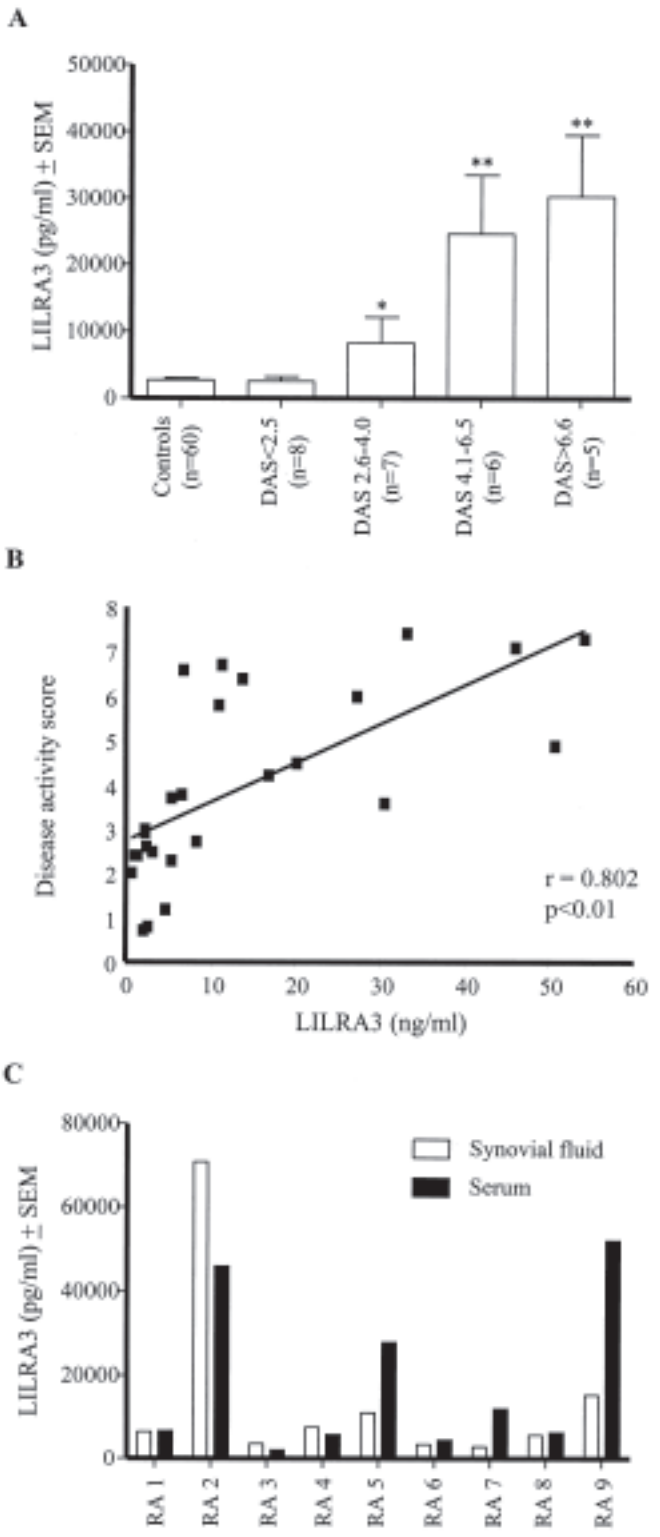

Figure 2. A. Increased levels of LILRA3 in serum of patients with active rheumatoid arthritis (RA; DAS > 2.6) were significantly higher than those with low disease activity scores (*$p < 0.05$, **$p < 0.01$) compared to patients with low disease activity. B. Levels of LILRA3 in serum from patients with RA correlated strongly with disease severity, by DAS (Spearman r = 0.802, $p < 0.01$). C. Amounts of LILRA3 in serum from patients with RA correlated to levels in their corresponding synovial fluid (n = 9).

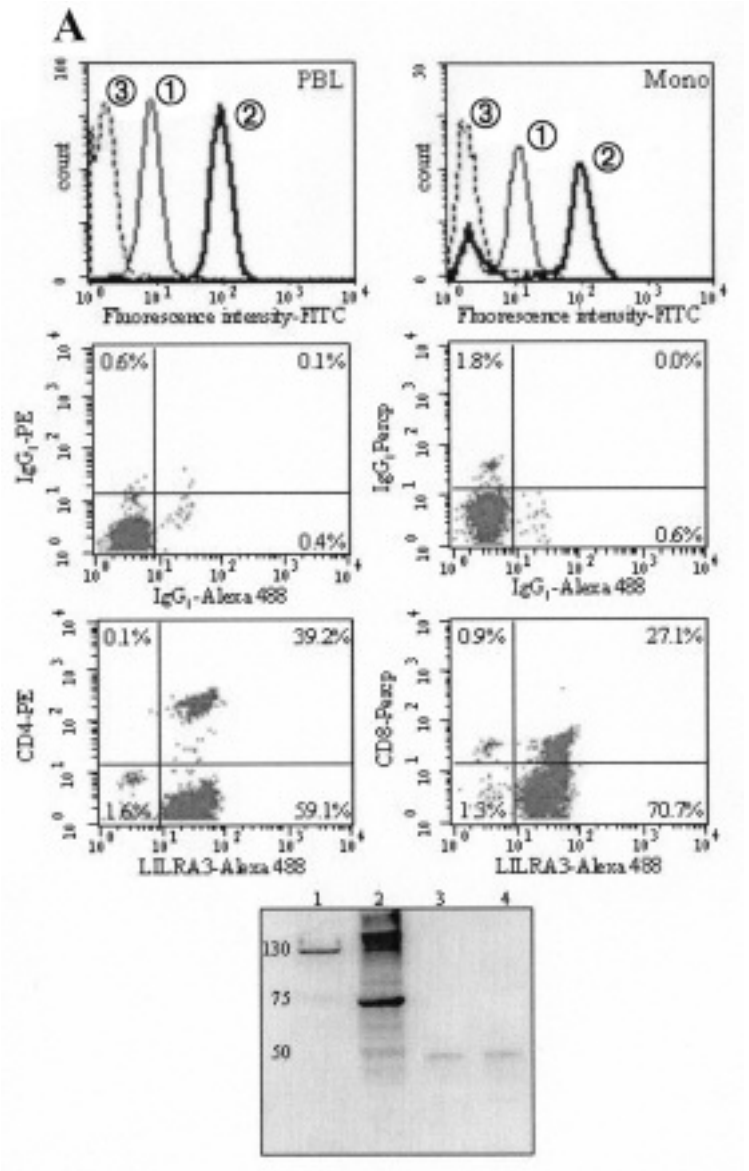

Figure 3. A. Flow cytometry of permeabilized normal peripheral blood lymphocytes (PBL) and monocytes using 2 different mAb [R&D Systems, histogram (1); Abnova, histogram (2)] showing constitutive intracellular expression of substantial amounts of LILRA3. Histograms on the left of each panel (3) are cells incubated with isotype-matched control mAb. Dot plot analysis of CD4 and CD8 T cells show both subsets of T cells are positive for LILRA3. Bottom panel: Western blot analysis of 2 independent PBMC lysates using anti-LILRA3 mAb (Abnova) shows the expected 50-kDa LILRA3 protein (lanes 3, 4). Lanes 1 and 2 are LILRA3 fusion proteins produced in mammalian cells and *E. coli*, respectively, used as positive controls.

Figure 5B). Consistent with the intracellular staining, cells treated with IL-10 or IFN-γ without Brefeldin secreted significantly more LILRA3 protein into the culture supernatant in a time- and dose-dependent manner, with 490 ± 110, 1160.9 ± 150.9, and 2200.1 ± 237.3 pg/ml, respectively, after 24-h incubation; and 480.2 ± 80.9, 1780.0 ± 330.6, and 1370.1 ± 413.5 pg/ml, respectively, after 48-h (Figures 5C, 5D). In contrast, TNF-α significantly downregulated constitutively produced LILRA3 protein by up to 50% (47%–53%) and inhibited IL-10-induced LILRA3 protein production by up to 70% (55%–74%) at any time point (Figure 5C).

DISCUSSION

Membrane-bound inhibitory and activating LILR are constitutively coexpressed in peripheral blood leukocytes[1,2,3,7,22],

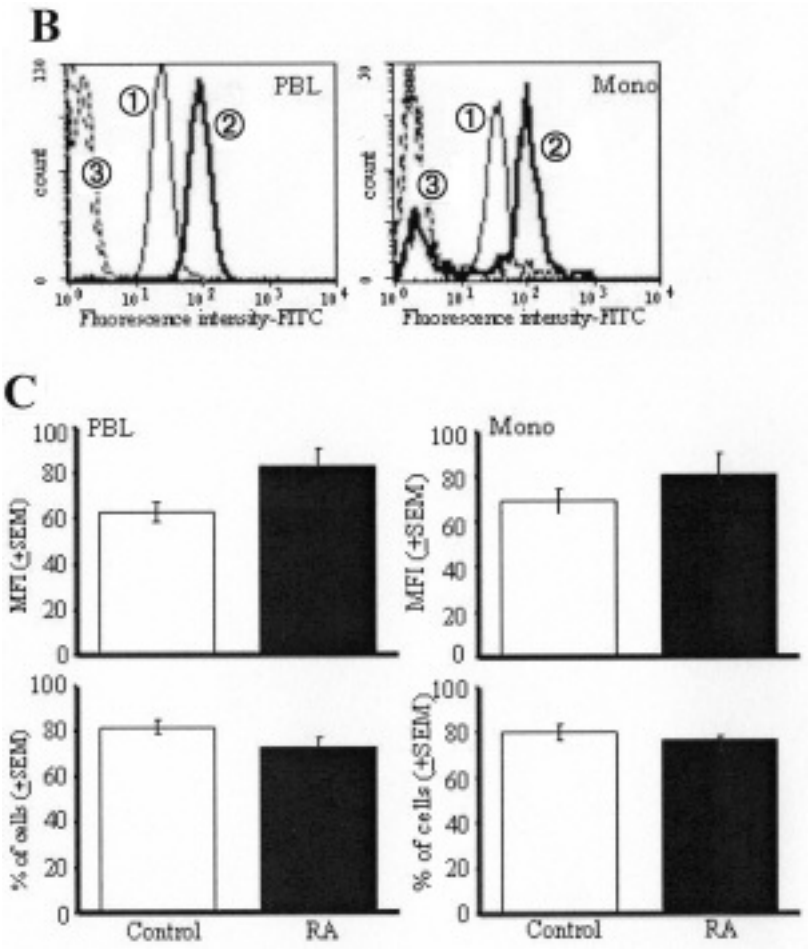

Figure 3. B. Representative flow cytometry from 2 patients with active RA (2) and 2 control subjects (1) shows increased expression of intracellular LILRA3 in PBL and monocytes from patients determined using mAb from Abnova. Histograms on the left of each panel (3) show cells incubated with isotype-matched control mAb. C. Quantification of mean fluorescence intensity (MFI; upper panels) and proportions of LILRA3-positive PBL and monocytes (lower panels) from 10 patients with active RA and 15 controls: intensity is marginally higher in patients.

suggesting that a balance between inhibitory and activating LILR determines the threshold and amplitude of cellular activation. LILRA3 is a soluble receptor that bears close sequence similarity to activating LILRA1 and LILRA2, and inhibitory LILRB1[1,6], thus it may bind the same or similar ligand(s) acting as antagonist or agonist to these membrane-bound receptors. To date there are limited data on LILRA3 mRNA[1,6], but no data on protein expression.

We developed a new robust ELISA and demonstrated the existence of LILRA3 protein. We show that it is constitutively expressed in serum of normal healthy individuals, at a range of 100–4800 pg/ml. This is comparable to the amounts of other soluble receptors involved in immune regulation found in serum of healthy donors including soluble IL-1ßR[37], sTNFRI, sTNFRII[38,39], and sCD4[40], but substantially lower than soluble CD14[41]. A measurable higher level of serum LILRA3 was observed in healthy female subjects compared to males (Figure 1A).

There was a 5–10-fold increase in the amount of LILRA3 detected in patients with RA, in which patients with the highest disease activity showed the most dramatic increase (Figure 2). In contrast, there were significantly lower levels of LILRA3 in serum of patients with degenerative and crystal-induced arthritis, suggesting that this receptor might be selectively important in the pathogenesis of RA. In support of this suggestion, preliminary immunohistochemical studies of synovial tissue from patients with RA (n = 12) and controls with traumatic meniscus rupture (n = 8) showed substantially increased expression of LILRA3 protein in macrophages and synovial lining cells in patients, compared to limited expression by endothelial cells in controls (data not shown). Further investigation of synovial fluid from

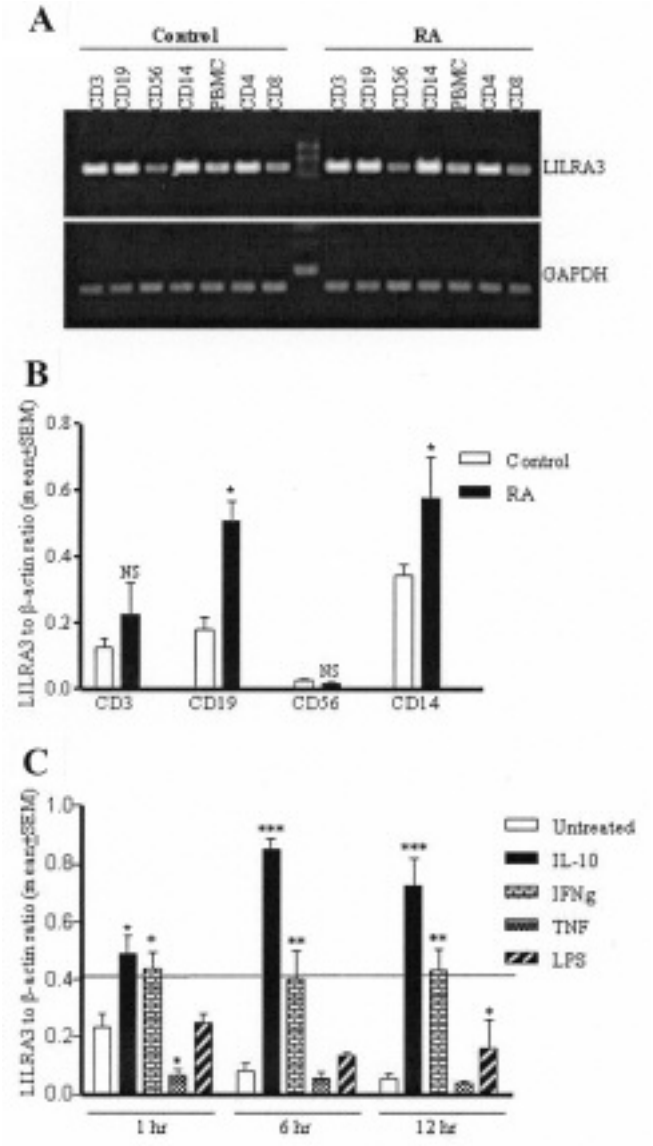

Figure 4. A. Representative RT-PCR results on freshly-isolated PBMC subsets showed widespread constitutive expression of LILRA3 mRNA. B. Quantitative RT-PCR showed significantly more LILRA3 mRNA in CD14-positive monocytes and CD19-positive B cells from patients with RA compared to age and sex-matched controls. Data in B are average of mean ß-actin/LILRA3 ratios of quantitative-PCR performed in triplicates from monocytes of 3 controls and 3 patients with active RA (*$p < 0.05$ compared to controls). C. Time-dependent regulation of LILRA3 mRNA by cytokines showing sustained upregulation by IL-10 and IFN-γ, early downregulation by TNF-α and late upregulation by LPS in monocytes from 3 independent healthy subjects performed in triplicate quantitative-PCR (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ compared to corresponding untreated monocytes). Horizontal line shows the level of LILRA3 mRNA expression at 0 timepoint.

other diseases with an inflammatory arthritis component such as psoriatic arthritis, septic arthritis, inflammatory bowel disease, and/or systemic lupus erythematosus (SLE) would confirm the specificity of this response to RA. Future analysis of homozygote or heterozygote LILRA3 gene deletion and/or the expression of various LILRA3 isoforms would provide further insights into whether the amounts of LILRA3 protein and/or the expressed isoform correlate directly with disease pattern or severity.

The increased production of LILRA3 in serum of patients with RA is similar to previous studies that showed 4–10-fold increases in serum levels of soluble receptors involved in inflammation. These include increased levels of soluble TNFRI, TNFRII, CD4, and/or CD14 receptors in patients with RA[40,42,43,44], SLE[45,46], multiple sclerosis[47], and sepsis[38,41]. Further, increased serum levels of these soluble receptors have been shown to be associated with response to treatment and disease severity and/or mortality in patients with SLE[45], multiple sclerosis[47], sepsis[38,41], and RA[42,43,44]. This is comparable to our findings that show a strong positive correlation between serum LILRA3 and disease severity (Figure 2).

In previous studies, we reported extensive expression of LILRA2 in synovial tissue from patients with active RA[29,30], and cross-linking of this receptor caused increased production of TNF-α[31]. Our data strongly suggest that in patients with RA, a disease characterized by recurrent stages of remission and relapse, LILRA3 may increase during active disease to counteract excessive LILRA1 and/or A2-mediated activation of leukocytes, acting as part of a regulatory loop. This proposal is consistent with the observation that lack of LILRA3 gene is associated with increased incidence of multiple sclerosis[48,49] and Sjögren's disease[50]. Further, LILRA3 was upregulated in patients with psoriasis who responded to IL-10 treatment[51], despite lack of genetic association between this disease and LILRA3 disruption[52]. Based on the high degree of structural homology between activating LILRA1 and A2 receptors with LILRA3, it is tempting to speculate that LILRA3 might be a soluble antagonist to these molecules. However, lack of known ligand(s) to these receptors precludes functional studies that would address this proposal.

We also showed significant upregulation of LILRA3 in monocytes by IL-10, a known immunosuppressive cytokine, while there was strong downregulation by a proinflammatory cytokine, TNF (Figures 4 and 5), indicating that it is tightly regulated. This further supports our proposal that LILRA3 may be an important antiinflammatory molecule. Further, activation of monocytes with LPS caused time-dependent upregulation of LILRA3 mRNA (data not shown), reminiscent of the increased production of soluble CD14, TNFRI, and TNFRII by monocytes in response to LPS that is believed to prevent excessive activation of these cells[38,41].

Although generally considered an inhibitor of proinflammatory cytokines, IL-10 is increasingly recognized as having multiple immunomodulatory functions[53,54,55,56,57,58]. The molecular mechanisms of its functions are not fully elucidated. We observed that it significantly regulated expres-

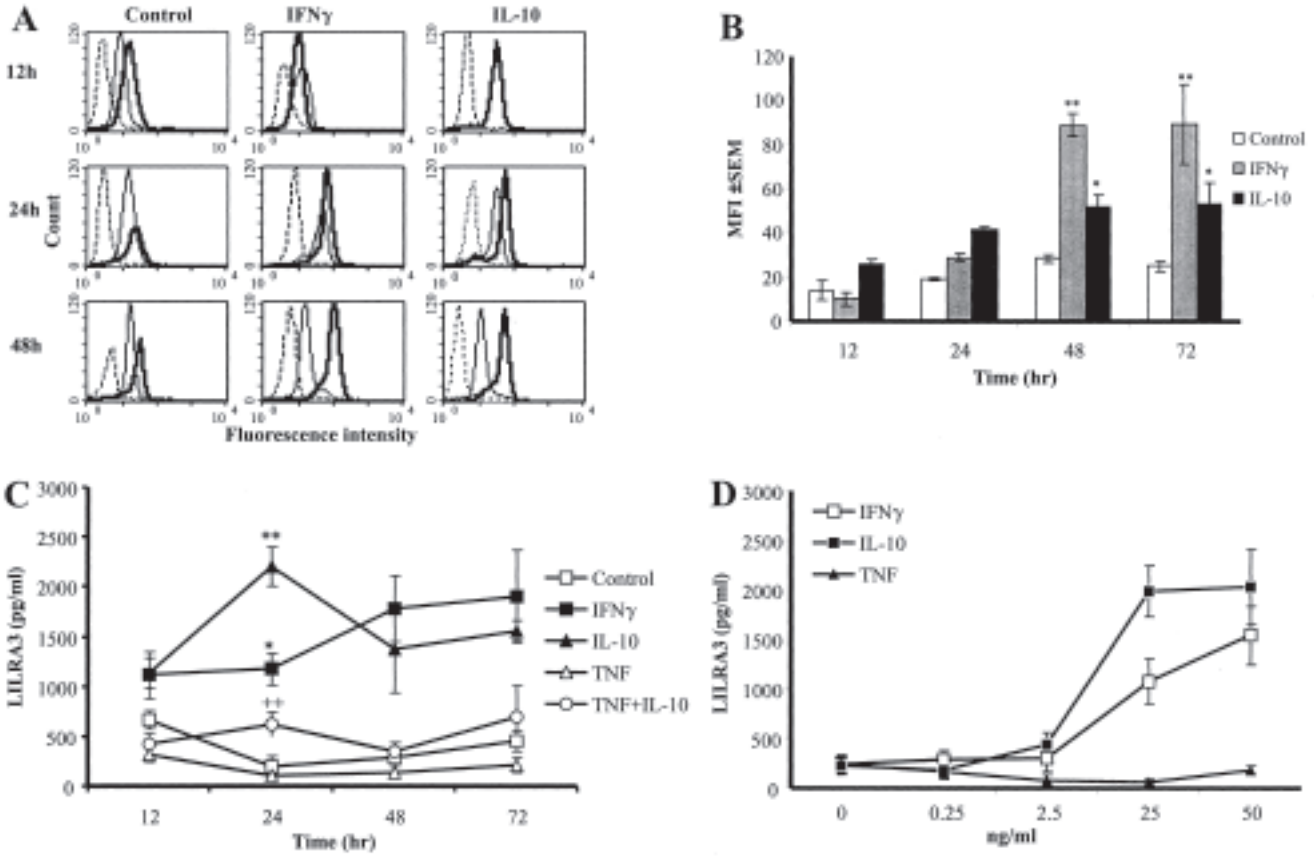

Figure 5. A. Representative flow cytometry results in permeabilized peripheral blood monocytes from a healthy subject treated with IL-10 or IFN-γ with (bold histogram line) or without (medium histogram line) Brefeldin A showing time-dependent intracellular accumulation of LILRA3 protein in cells treated with 25 ng/ml of these cytokines. B. Mean fluorescence intensity (MFI) of LILRA3 expression in monocytes from 3 independent donors treated with cytokines in the presence Brefeldin A showing time-dependent accumulation of LILRA3 protein in cells treated with IL-10 or IFN-γ (*$p < 0.05$; **$p < 0.01$ compared to corresponding untreated control cells). C. LILRA3 ELISA results of culture supernatant from monocytes treated with 25 ng/ml IL-10, IFN-γ, TNF-α or a combination of IL-10 and TNF-α without Brefeldin A, showing time-dependent increased secretion of LILRA3 in cells treated with IL-10 or IFN-γ and time-dependent inhibition of LILRA3 secretion in cells treated with TNF-α or combination of TNF-α and IL-10 (*$p < 0.05$; **$p < 0.01$ compared to corresponding untreated control cells; $^{\dagger\dagger}p < 0.01$, IL-10 + TNF-α treated cells compared to cells treated with IL-10 alone, n = 3). D. Dose-dependent increase in LILRA3 production in IL-10 or IFN-γ but not TNF-α treated PBMC culture supernatants at 24-h timepoint (n = 1).

sion of a potential novel immune-regulatory receptor. Consistent with our finding, IL-10 has been shown to strongly upregulate a number of activating and inhibitory LILR and other soluble inflammatory receptors in monocytes[36,51] and dendritic cells[36,51,59,60]. It is interesting that *in vitro* treatment of monocytes with IFN-γ, an important cytokine in activation, differentiation, and survival of macrophages, seems to prevent the progressive downregulation of LILRA3 mRNA observed in untreated cells, and it caused significant increase in protein production in a time- and dose-dependent manner. The mechanisms and functional significance for this IFN-γ-mediated regulation of LILRA3 expression require further investigation. T and B cells play important roles in the perpetuation of joint inflammation in RA[27,28]; however, their role in regulation of chronic inflammation is not well established. We show for the first time that in addition to significantly increased levels of LILRA3 in serum of patients with RA, monocytes and B cells from patients with RA show increased expression of LILRA3 mRNA. These suggest that lymphocyte and/or monocyte-derived LILRA3 might play a role in modulating the magnitude of inflammation in RA. Together, our results provide insight into the expression and regulation of a novel immune-regulatory receptor that may potentially play a role in the pathogenesis of chronic inflammatory conditions such as RA.

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