Down-regulation of leucocyte immunoglobulin-like receptor expression in the synovium of rheumatoid arthritis patients after treatment with disease-modifying anti-rheumatic drugs

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Objectives. To compare the expression of leucocyte immunoglobulin-like receptors (LILRs) also known as ILTs and LIRs in rheumatoid arthritis (RA) synovial membrane before and after treatment with disease-modifying anti-rheumatic drugs (DMARDs) and investigate regulation of LILR-expression and function in vitro.

Methods. A study was performed on serial synovial biopsies obtained from 10 RA patients before and after treatment with DMARDs. Expression of the activating LILRA2 (ILT1 or LIR-7) and inhibitory LILRB2 (ILT4 or LIR-2) and LILRB3 (ILT5 or LIR-3) was evaluated by immunohistochemical staining, and quantified by a validated scoring system. Peripheral blood mononuclear cells and in vitro derived macrophages were used to determine effects of DMARDs on expression and function of LILRs.

Results. Abundant expression of LILRB2, B3 and A2 was found in synovial tissue of all patients before treatment. Number of inflammatory cells expressing both inhibitory and activating LILRs dramatically decreased in patients who responded to treatment, but remained high in those who did not. However, treatment of macrophages with DMARDs in vitro did not down-regulate LILR expression. On the other hand, reduction in LILR expression in RA synovia was associated with decreased inflammatory infiltrates in those who responded to treatment. Cross-linking of LILRA2 on macrophages caused substantial production of tumour necrosis factor (TNF-α) in a dose- and time-dependent manner that was strongly inhibited by dexamethasone.

Conclusions. We show that expression of LILRs in RA synovium was significantly reduced only in patients who responded to treatment. However, clinical responses may not be due to direct effects of DMARDs on LILR expression but due to partial inhibition of LIRA2-mediated TNF-α production by steroids leading to suppression of inflammation.

KEY WORDS: Leucocyte immunoglobulin-like receptors, Rheumatoid arthritis, Disease modifying anti-rheumatic drugs.

Rheumatoid arthritis (RA) is a chronic inflammatory disease of the synovial joints with varying clinical activity [1]. The key events in RA involve activation of macrophages and fibroblast-like cells in the intimal lining of the synovium leading to synovial hyperplasia [2] and migration of activated leucocytes into the affected joint where they release inflammatory mediators, amplifying ongoing inflammation and joint destruction [3]. A great deal of evidence supports the benefits of early intervention with disease-modifying anti-rheumatic drugs (DMARDs) on the disease course and outcome [4]. However, many second-line DMARDs currently used were discovered serendipitously with disease-modifying anti-rheumatic drugs (DMARDs) on the disease course and outcome [4]. However, many second-line DMARDs currently used were discovered serendipitously with little knowledge of their mechanism of action [5].

Leucocyte immunoglobulin-like receptors (LILRs), also known as LIRs, ILTs and CD85 antigens [6], are a family of immunoglobulin-like receptors that regulate cellular activation in vitro [7–11]. We recently observed selective co-expression of an activating LILRA2, and inhibitory LILRB2 and B3, by macrophages, fibroblast-like synoviocytes and endothelial cells in synovial tissue from patients with active RA [12] but not LILRB1, B4, A1 or B5 (unpublished data). Since there is substantial evidence indicating that LILRs and related molecules may determine the threshold and extent of leucocyte activation [9, 12], it is possible that some DMARDs used to successfully treat RA may alter the expression and/or function of activating and/or inhibitory LILRs in the synovial membrane. Changes in the relative balance of activating and inhibitory LILRs expressed by a particular cell in the synovium could determine its activation state.

Here, we demonstrate that patients who responded to treatment with DMARDs had substantially reduced LILR expression in synovial tissue, and this was associated with decreased number of inflammatory and endothelial cells that are the major source of LILRs. We show that in vitro treatment of macrophages with dexamethasone significantly inhibited LILRA2-induced production of tumour necrosis factor (TNF-α) without significantly altering levels of LILR expression. Our results suggest that clinical improvement in response to DMARDs may not be due to direct effects on LILR expression or function. The effects of glucocorticoids may be mediated in part by reducing LILR2-dependent TNF-α production leading to decreased recruitment and activation of inflammatory cells. LILRs may be potential therapeutic targets in RA and other inflammatory diseases.

Patients and methods

Reagents

Ciclosporin A, methotrexate, dexamethasone, 1α,25-dihydroxyvitamin D3 (vitamin D3) and lipopolysaccharide (LPS) were purchased from Sigma (Sigma Aldrich, St Louis, MO, USA). Normal goat serum, biotinylated goat anti-mouse or goat anti-rabbit secondary antibodies, avidin–biotin–alkaline phosphatase complex (Vectastain kit) and alkaline phosphatase substrate (vector red) were from Vector laboratories, Burlingame, CA, USA. Fluorescein isothiocyanate (FITC)-conjugated goat F(ab')2 anti-mouse IgG [F(ab')2 specific] with minimum cross-reactivity to human, rat and bovine serum was purchased from Jackson ImmunoResearch (West Grove, PA, USA). Tissue culture media (RPMI-1640), l-glutamine and fetal bovine serum (FBS) were from Invitrogen (Carlsbad, CA, USA). Tissue culture...
Expression of LILRs in RA synovium

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the number of fields varied from patient to patient (range 4–26) and there were obvious regional variations in staining, the median count for the whole section is reported as a conservative measure of the staining for each antibody.

A standard haematoxylin and eosin (H&E) stain was used to determine the degree of inflammation by counting inflammatory cell infiltrate and the number of small blood vessels in tissue sections as described earlier. All sections were stained and counted by an independent observer who was blinded to all treatment details, source of the biopsies and outcome measurements.

The expression of LILRs in peripheral blood monocytes

Peripheral blood mononuclear cells (PBMCs) were prepared from 40 ml of anti-coagulated whole blood obtained from a new group of RA patients (n=14) and healthy volunteers (n=11) using standard Ficoll-Paque gradient (Amersham Pharmacia, Piscataway, NJ, USA) [8]. Surface expression of LILRB2, B3 and A2 was determined by flow cytometry as described [8, 12]. In brief, PBMCs were washed with cold phosphate-buffered saline (PBS), re-suspended in PBS containing 10% human serum at 2 x 10^6/ml and 50 μl were incubated for 30 min at room temperature with IgG1 mAbs to LILRB2, B3, A1 or control mouse IgG1 (5 μg/ml). After two washes with cold PBS containing 1% BSA and 0.05% NaN₃ (PAB buffer), 10 μl (10 μg/ml) of FITC-conjugated F(ab')₂ goat anti-mouse IgG [F(ab')₂-specific] antibody was added to cell suspensions and samples placed on ice for 45 min. Cells were washed twice with PAB buffer, fixed with 1% parafomaldehyde in PBS, and analysed using a FACScan flow cytometer (Becton Dickinson). Staining with directly conjugated anti-CD45-FITC/CD14-PE/CD3-Percp and corresponding isotype controls were used to delineate the monocyte gate (≥95% CD14+ve cells). A unimodal shift in mean fluorescence intensity (MFI) of cells stained with specific antibodies compared with cells stained with the isotype-matched negative control antibody was considered positive.

Modulation of LILR expression by DMARDs in vitro

An in vitro system using vitamin D₃-differentiated monocyctic cell line (THP-1) was established to assess the direct effect of DMARDs on LILRB2, B3 and A2 expression. Differentiated THP-1 cells (macrophages) were used in this system because macrophages were the major cell type expressing inhibitory and activating LILRs in the rheumatoid synovium and these cells are key players in the pathogenesis of RA [18]. In brief, THP-1 cells were cultured in RPMI 1640 (GIBCO-Invitrogen, Carlsbad, CA, USA) complete medium containing 10% heat-inactivated FBS (GIBCO-Invitrogen) and 1% penicillin/streptomycin (GIBCO-Invitrogen) in LPS minimized conditions. Cells were then differentiated into macrophages with 10⁻^7 M vitamin D₃ for 48 h, and then transferred into complete medium for a further 24 h as described elsewhere [19]. Differentiated cells were treated with ciclosporin A (10, 100 and 1000 ng/ml), methotrexate (5, 50 and 500 ng/ml) or dexamethasone (10⁻², 10⁻³ and 10⁻⁴ M) for 24, 48 and 72 h. The range of concentrations of DMARDs used was selected empirically from the literature and was further optimized on the basis of a cytotoxicity study performed in our laboratory. Optimal concentrations of DMARDs or dexamethasone used in this study were similar to most published reports [20–23] and were within the pharmacological doses (ciclosporin A: 5–10 mg/kg/day, methotrexate: 12.5 mg/week and dexamethasone: 0.75 mg/kg/day) [24, 25]. After treatment with DMARDs, cells were stained with mAbs directed against LILRB2, B3 and A1 using a standard two-step staining protocol as described earlier [8, 12].

Time and dose-dependent activation of macrophages by LILRA2 cross-linking in vitro

Macrophages differentiated from THP-1 cells were activated by cross-linking LILRA2 using plate bound anti-LILRA2 antibody
as described elsewhere [8, 26]. In brief, wells of 96-well flat-bottom Costar® 3596 tissue culture plates (Corning Incorporated, Corning, NY, USA) were coated overnight at 4°C with 100 μl (5 μg) F(ab')2 goat anti-mouse IgG, Fc-specific (Jackson ImmunoResearch), in PBS. After aspiration, 50 μl mAb to LILRA2, diluted to the desired concentrations in PBS containing 2.5% BSA-fraction V (Boehringer, Mannheim, Germany), was added. Irrelevant mouse IgG1 mAb was used as negative control. After incubation for 2 h at 37°C with 5% CO2 in air, wells were washed twice with 0.9% NaCl before use. In the meantime, cells were harvested, washed twice with PBS, re-suspended in RPMI 1640 supplemented with 10 mM HEPES (Sigma) and 0.1% BSA (Boehringer), and 1 × 10⁶ cells in 200 μl added to each well. After incubation at 37°C and 5% CO2 in air, cell-free supernatants were collected at various time points and stored at −80°C for measurement of TNF-α by a DuoSet ELISA Kit (R&D Systems, Minneapolis, MN, USA).

**The effect of DMARDs on LILRA2-mediated activation of macrophages in vitro**

LILRA2-mediated TNF-α production at the maximal time point (24 h) was assessed by cross-linking of LILRA2 on the surface of in vitro-derived macrophages with an optimal dose of anti-LILRA2 mAb (0.1 μg/ml) in the presence or absence of three doses of ciclosporin A (100, 10 and 10 ng/ml), dexamethasone (10⁻⁷, 10⁻⁸, 10⁻⁹ M) or methotrexate (500, 50 and 5 ng/ml).

**The effect of in vivo steroid treatment on the expression of LILRA2 and LILRA2-mediated TNF-α production**

PBMCs were obtained from three RA patients undergoing treatment with 7.5–10 mg/day oral prednisolone for the last 6 months and from three patients who had not received steroid treatment in the last 2 months. Surface expression of LILRA2 on monocytes collected from each group was assessed by flow cytometry and compared with healthy controls. Levels of LILRA2-mediated TNF-α production by PBMCs (adjusted to contain 5 × 10⁴ monocytes per well) from each group were assessed after cross-linking of LILRA2 as described earlier.

**The effect of DMARDs on cell viability**

THP-1 cells and THP-1-derived macrophages were treated with optimal doses of ciclosporin A (100 ng/ml), methotrexate (50 ng/ml) or dexamethasone (10⁻⁸ M) for 72 h and viability of cells in suspension assessed by flow cytometry using Annexin V-FITC Apoptosis Detection Kit (BD BioSciences). The viability of PBMCs, THP-1 cells and THP-1-derived macrophages in 96-well plates during LILRA2 cross-linking experiments with or without DMARDs was assessed using a fluorescence microplate protocol using the LIVE/DEAD® Viability assay kit (Molecular Probes, Invitrogen).

**Results**

**LILR expression in RA synovial tissue before and after DMARDs treatment**

After completing the immunohistochemical staining and semi-quantitative analysis of immunoreactive cells in all synovial tissue samples by an independent observer, the clinical data from each patient was unmasked, and their results before and after treatment paired. Seven patients showed clinical improvement (responders), as indicated by a substantial reduction in the DAS (Table 1). Three patients (1, 3 and 4) did not show a clinical response to treatment, characterized by no change, or an increase in DAS (non-responders) (Table 1). There was abundant expression of inhibitory (LILRB2 and B3) and activating LILR (LILRA2) in synovial tissue from all patients before treatment with mean positive cell counts of 26 ± 8.9, 28 ± 9.0 and 18 ± 6.0 per high power field (HPF) for LILRB2, B3 and A2, respectively (Fig. 1A and B). Analysis of LILR expression in synovial tissue before and after treatment revealed a statistically significant decrease in number of LILRB3 (31 ± 10.2 vs 12 ± 4.4 cells/HPF) and LILRA2 (19 ± 6.5 vs 6 ± 3.2 cells/HPF) positive cells in patients who responded to treatment (Figs. 1A and 2A). In contrast, an apparent increase of LILRB3 (25 ± 12.4 vs 30 ± 8.6 cells/HPF) and LILRA2 (16 ± 5.1 vs 22 ± 5.5 cells/HPF) positive cells was observed in samples of non-responders (Figs. 1B and 2B). Similar trends in LILRB2 expression was observed from both responders and non-responders (Fig. 1).

Staining of serial sections confirmed our previous observation [12] that LILRB2, B3 and A2 were primarily expressed by CD68-positive macrophages and to a lesser extent, by other inflammatory cells such as neutrophils, mast cells and fibroblast-like synoviocytes (data not shown). In addition, substantial immuno-reactivity of LILRA2 was seen on the endothelial lining of small blood vessels in 8 of 10 patients (Fig. 1e and f).

**LILR expression on peripheral blood monocytes in vivo**

To determine expression of LILRB2, B3 and A2 on PBMCs, 14 patients with RA receiving various combinations of DMARDs treatment for varying durations and 11 sex- and age-matched controls were recruited. Monocytes obtained from all subjects

**Statistical analyses**

After determining the median number of inflammatory cells, blood vessels and cells expressing LILRs from each individual, mean values were calculated for each group (responders and non-responders). The effect of DMARD treatment on the mean expression of LILRs in synovial tissue of responders and non-responders was analysed by Student’s t-test. A one-way analysis of variance (ANOVA) test with a Dunnett’s multiple comparison post-test was applied to evaluate the effects of DMARDs *in vitro*. A P-value of <0.05 was considered significant.

**Table 1. DAS before and after treatment with DMARDs**

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age</th>
<th>Sex</th>
<th>Treatment</th>
<th>DAS before treatment</th>
<th>DAS after treatment</th>
<th>Time between first and second biopsy (months)</th>
</tr>
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<tbody>
<tr>
<td>RA1*</td>
<td>64</td>
<td>M</td>
<td>IM gold/oral MTX</td>
<td>5.0</td>
<td>5.3</td>
<td>28</td>
</tr>
<tr>
<td>RA3*</td>
<td>75</td>
<td>F</td>
<td>IM gold/oral MTX</td>
<td>6.9</td>
<td>5.9</td>
<td>36</td>
</tr>
<tr>
<td>RA4*</td>
<td>66</td>
<td>F</td>
<td>Sulphasalazine</td>
<td>4.4</td>
<td>4.5</td>
<td>22</td>
</tr>
<tr>
<td>RA2</td>
<td>83</td>
<td>M</td>
<td>IM gold/oral MTX</td>
<td>5.2</td>
<td>1.2</td>
<td>13</td>
</tr>
<tr>
<td>RA5</td>
<td>75</td>
<td>F</td>
<td>Ciclosporin A/prednisolone</td>
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<td>24</td>
</tr>
<tr>
<td>RA6</td>
<td>77</td>
<td>M</td>
<td>Ciclosporin A/prednisolone</td>
<td>5.8</td>
<td>2.1</td>
<td>20</td>
</tr>
<tr>
<td>RA7</td>
<td>76</td>
<td>M</td>
<td>IM MTX/prednisolone</td>
<td>5.5</td>
<td>1.4</td>
<td>23</td>
</tr>
<tr>
<td>RA8</td>
<td>75</td>
<td>M</td>
<td>IM gold/oral MTX</td>
<td>5.6</td>
<td>0.8</td>
<td>18</td>
</tr>
<tr>
<td>RA9</td>
<td>60</td>
<td>F</td>
<td>Ciclosporin A/prednisolone</td>
<td>6.4</td>
<td>2.6</td>
<td>12</td>
</tr>
<tr>
<td>RA10</td>
<td>78</td>
<td>M</td>
<td>IM gold/oral MTX</td>
<td>0.9</td>
<td>0.6</td>
<td>14</td>
</tr>
</tbody>
</table>

*Non-responders.
expressed high levels of LILRB2, B3 and A2 on their surface regardless of the type and duration of treatment with DMARDs (Fig. 3). The levels of LILR expression on monocytes from patients with RA were not significantly different to those expressed by control subjects (Table 2).

**LILR expression by macrophages after treatment with DMARDs in vitro**

To further investigate whether the reduction of LILRA2 expression in synovial tissue after treatment could be due to direct regulation of LILR expression by DMARDs, LILRA2 levels were measured on macrophages treated with various doses of ciclosporin A, methotrexate and dexamethasone for 24, 48 and 72 h. These cells expressed basal levels of LILRA2 on their surface (Fig. 4A). There was no significant change \( (P > 0.05) \) in expression of LILRA2 on DMARD-treated cells at various time points when compared with untreated controls (Fig. 4B and C), suggesting that these drugs may not directly decrease the expression of LILRA2 observed in synovial tissue. Consistent with this, evaluation of inflammatory cell infiltrates in synovial tissue before and after treatment showed significantly fewer inflammatory cells in synovial tissue of patients who responded to treatment \((36.2 \pm 11.8 \text{ cells/HPF} \text{ before and 13.2} \pm 4.2 \text{ cells/HPF after treatment; } P < 0.5)\), but not in non-responders (Fig. 5).

However, there were few or no differences in numbers of apoptotic/necrotic cells in synovial tissue obtained from responders or non-responders (data not shown). Furthermore, flow cytometric analysis of macrophage apoptosis after treatment with high doses of DMARDs in vitro consistently showed \(<6.0 \pm 1.2\% \) of cell death as compared with \(5.6 \pm 1.7\% \) for untreated controls (mean ± s.d., \( n = 6 \)) (Fig. 6). These results suggested that reduced LILR-expression in the synovial tissue of responders might be due to decreased infiltration of inflammatory cells as a result of attenuated macrophage activation.

**Activation of macrophages via cross-linking of LILRA2**

To investigate whether LILRA2 ligation could activate macrophages, LILRA2 on THP-1-derived macrophages and PBMCs were cross-linked with anti-LILRA2 mAb, and TNF-\( \alpha \) production was evaluated. Cross-linking triggered significant TNF-\( \alpha \) production \((800–1000 \text{ pg/ml})\) in a dose- and time-dependent manner with a peak antibody dose of \(0.1 \mu \text{g/ml}\) (Fig. 7A) and an optimal response after \(12–16\) h (Fig. 7B), which did not return to baseline over \(48\) h. Levels of TNF-\( \alpha \) produced in response to LILRA2 cross-linking were comparable with those induced by \(100\) ng/ml of LPS \((851.1 \pm 47.5 \text{ pg/ml within 24 h})\).

The dose-dependent production of TNF-\( \alpha \) in response to LILRA2 cross-linking was confirmed using PBMCs from four healthy

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**Fig. 1.** Immunohistochemical analysis of LILR expression in rheumatoid synovial tissue. Changes in the expression of LILRB2 (a, b), LILRB3 (c, d) and LILR A2 (e, f, g) as a result of DMARD treatment \((250 \times \text{ magnification})\) in patient RA8 who responded to treatment (A) and in patient RA4 who did not respond to treatment (B). Panels a (LILRB2), c (LILRB3), e and g (LILRA2) show immunohistochemical staining before treatment (positive cells stained red). Panels b, d and f are staining of tissue for LILRB2, B3 and A2 18 or 22 months after treatment. Panel h is a section from same patients stained with isotype-matched negative control antibody.
volunteers (Fig. 7C). Cross-linking of LILRA2 on PBMCs (~5 x 10^4 monocytes per well) with 0.001, 0.01 or 0.1 µg/ml of anti-LILRA2 antibody for 18 h caused the release of 4470 ± 39.4, 5469 ± 11.0 and 6405 ± 42.2 pg/ml of TNF-α, respectively, whereas, equivalent doses of isotype-matched IgG control induced minimal TNF-α production from PBMCs and in vitro-derived macrophages. 

In vitro treatment of macrophages and PBMCs with dexamethasone (Fig. 8A and B, respectively) but not ciclosporin A or methotrexate significantly inhibited LILRA2-mediated TNF-α production (Fig. 8C and D). Inhibition in response to dexamethasone was dose-dependent (Fig. 8A and B), and was not due to drug toxicity. The proportions of dead cells in wells treated with dexamethasone and activated with anti-LILRA2 were 5.6–6.8% compared with 5.1–7.5% for cells activated with anti-LILRA2 without dexamethasone (n = 5). PBMCs from RA patients who were on regular doses of oral prednisolone released marginally lower (~20%) amounts of TNF-α in response to LILRA2 cross-linking compared with those not treated with steroids (Fig. 9A). Addition of dexamethasone in vitro caused similar dose-dependent inhibition of LILRA2-mediated TNF-α production in PBMCs obtained from patients on or off steroid (Fig. 9B). Interestingly, there was no difference in LILRA2 expression on blood monocytes from both groups, and levels

TABLE 2. Expression of LILRB2, B3 and A2 on the surface of PBMCs from patients with RA and healthy controls

<table>
<thead>
<tr>
<th></th>
<th>Patients (n = 14)</th>
<th>Controls (n = 11)</th>
</tr>
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<tbody>
<tr>
<td>LILRB2</td>
<td>49.3 ± 4.47</td>
<td>51.2 ± 5.75</td>
</tr>
<tr>
<td>LILRB3</td>
<td>42.8 ± 3.27</td>
<td>36.9 ± 3.77</td>
</tr>
<tr>
<td>LILRA2</td>
<td>51.7 ± 5.86</td>
<td>53.1 ± 4.39</td>
</tr>
</tbody>
</table>

*At the time of blood collection, seven patients were on oral MTX and IM gold; five were on CsA and prednisolone; two were on prednisolone and IM MTX.
Results presented as mean fluorescent intensity (MFI) ± s.e.m.
were comparable with those observed on monocytes from age- and sex-matched controls (Fig. 9C).

Discussion

The mechanisms of action of many drugs currently used in the treatment of RA, including DMARDs, are not completely understood. Several studies have examined the effects of treatment on synovial membrane pathology and demonstrated changes in numbers of T-lymphocytes, macrophages, microvessels and/or synovial fibroblasts [27–33]. However, the mechanisms responsible for these changes are poorly defined. We previously reported abundant expression of inhibitory LILRB2 and B3 and an activating LILRA2 in synovial tissue from patients with active RA [12]. There was limited expression of these molecules in the late fibrotic stage of RA or in synovium obtained from degenerative arthritis [12]. We also demonstrated that upon cross-linking or co-ligation, LILRs can activate or inhibit leukocytes [8, 9]. These observations confirm that these molecules are present in active inflammatory conditions and may determine the threshold and/or extent of activation of leukocytes and other inflammatory cells.

Interestingly, our earlier studies showed no expression of other inhibitory LILRs (B1, B4 and B5) and the activating LILRA1 in rheumatoid synovia (unpublished data) suggesting a unique association of LILRB2, B3 and A2 to this disease. Hence, the focus of this study was the regulation of these LILRs by DMARDs. We proposed that modulation of LILRB2, B3 and/or A2 expression and/or function by DMARDs may regulate the activation state of inflammatory cells in synovial tissues from patients with active RA. We compared their expression in the synovial tissue from a single index knee joint in a group of patients who had never received DMARDs with tissue obtained from the same subjects 18–36 months after treatment and changes in LILR expression were correlated with clinical outcomes.

We found abundant LILRB2, B3 and A1 expression in all patients prior to commencement of treatment with DMARDs. Consistent with our previous study [12], the major cellular sources of LILRB2 and B3 were macrophages and infiltrating neutrophils. LILRA2 was variably expressed on macrophages, neutrophils, mast cells fibroblasts and endothelial cells. After treatment with DMARDs for 18–36 months, the activating and inhibitory LILRs were significantly reduced in those patients who attained clinical improvement as indicated by DAS score but not in those who failed to respond.

To investigate whether the reduced expression of LILRs in responders was due to a direct effect of the DMARDs, an in vitro model was established whereby THP-1-derived macrophages expressing LILRs were treated with varying concentrations of these agents and changes in LILRs expression determined.
DMARDs did not alter the constitutive surface expression of LILRB2, B3 or A2 at either protein (Fig. 4) or mRNA levels making a direct effect unlikely. Furthermore, we were unable to significantly modulate expression of LILRB2, B3 and A2 mRNA or protein in THP-1-derived macrophages using various doses of cytokines (IL-1, TNF-α, IFN-γ and IL-10) that are known to be involved in the pathogenesis of RA or with synovial fluid obtained from patients with active RA (data not shown). Therefore, the mechanisms of LILR regulation in vivo remain unclear. The identity of ligands recognized by activating LILRs, and the effect of DMARDs and inflammatory mediators on these ligands has yet to be determined. It is also noteworthy that most of the patients (9/10) in this study received combination therapy with more than one drug and 5 out of 10 patients had intramuscular gold injections (Table 1). Thus, the effect of gold and/or a combination of the various drugs on in vitro expression of LILRs requires further investigation.

Our preliminary results using peripheral blood showed high expression of LILRB2, B3 and A2 on monocytes from RA patients treated with various DMARDs (Fig. 3). Furthermore, the levels of LILR expression on monocytes from patients were comparable with those on monocytes from sex- and aged-matched controls (Fig. 3; Table 2). Similar to previous reports, inflammatory cell numbers in synovial tissue from patients who responded to treatment were significantly decreased [27], and associated with reduced LILR expression (Figs. 2 and 5). In contrast, number of inflammatory cells in tissues from non-responders increased and this was associated with significantly increased LILR expression (Figs. 2 and 5). These observations suggested that the effect of DMARDs on LILR expression may be indirect and subsequent to improvement in active inflammation that is associated with decreased recruitment and/or proliferation of the inflammatory cells that are the principal sources of these molecules. Further studies on the expression of LILRs on the surface of circulating leucocytes collected from a large number of patients before and after treatment might provide a better representation if these molecules are directly regulated by DMARDs in vivo. It is possible that functions of LILRs may be modulated by the effect of DMARDs on LILR-ligands that are yet to be identified.

Fig. 6. Annexin V and PI staining of in vitro-derived macrophages treated with optimal doses of DMARDs for 48 h. Right-upper quadrant shows PI and Annexin V double-positive cells (late apoptosis) and right lower quadrant shows Annexin V-positive cells (early apoptosis) of untreated cells (A) or cells treated with 10⁻⁸ M Dex (B) 50 ng/ml MTX (C) 100 ng/ml CsA (D) showing little difference in percentages of apoptotic cells between untreated and drug-treated cells. These are representative data from six independent experiments.

Glucocorticoids are thought to exert anti-inflammatory effects via glucocorticoid receptors that inhibit genes for a number of inflammatory mediators by blocking nuclear factor (NF)-κB and AP-1 transcription factors [35, 36]. The anti-inflammatory effects of steroids are also linked to their ability to induce apoptosis of inflammatory cells [37]. Our results clearly indicate that inhibition of LILRA2-induced NF-κB production by macrophages and PBMCs in response to dexamethasone is not due to increased cell death or down-regulation of LILRA2 expression on the cell surface. Whether dexamethasone inhibits LILRA2-mediated cell activation by blocking NF-κB and/or AP-1 remains to be
monocytopaenia seen after treatment with oral prednisolone was only marginally lower (20%) than those not treated with this drug. Nevertheless even a modest reduction of LILRA2-mediated TNF-α production but can also systemically deplete monocytes that are the major source of this cytokine. Therefore, the net suppression of monocyte-derived TNF-α by glucocorticoids in vivo might be greater than the 20% observed in these ex-vivo experiments.

TNF-α plays an important role in RA disease progression and is currently a therapeutic target in this and other inflammatory diseases [41]. TNF-α up-regulates the activity and production of tissue degrading proteases such as MMP-9 in cells abundantly present in rheumatoid synovium and facilitates migration of some of these cells through the extracellular matrix [42]. Furthermore, the demonstration of LILRA2 on endothelial cells suggests functions beyond cell activation such as its potential involvement in leucocyte adhesion and recruitment. We previously demonstrated expression of LILRA2 by cells other than haematopoietic origin (endothelial cells) [12], and confirmed our finding in this study. Although this is the first demonstration of expression of an activating LILR by endothelial cells, recent studies showed expression of inhibitory LILRB2 and LILRB4 by activated endothelial cells in transplants undergoing rejection [43, 44]. Conversely, it is possible that over-expression of an activating LILRA2 on endothelial cells in active RA is induced by inflammatory cells and could lead to propagation of inflammation.

It is tempting to speculate that LILRs may regulate protease and cytokine production in the inflammatory infiltrate in RA, and thereby, regulate the process of pannus formation and joint destruction. It is possible that treatment of patients with steroids, but not ciclosporin A or methotrexate, may alter cytokine production in response to signalling through LILRA2, hence reducing the activation and recruitment of leucocytes the inflamed synovium.

Given the recognition of a diverse array of major histocompatibility complex (MHC)-I molecules by some members of the LILR family [39] and the well-established link between certain MHC-I molecules and RA, it is reasonable to speculate that interaction of certain LILRs and their ligands, including MHC-I molecules, may determine the state of macrophage activation in rheumatoid synovium. This is supported by studies on the killer cell immunoglobulin-like receptors (KIRs), a family of immunoregulatory receptors that are closely related to LILRs. Expression of KIRs in conjunction with HLA inheritance have been linked to development of RA-related vasculitis [45], scleroderma [46] and psoriatic arthritis [47]. The killer inhibitory receptors, expressed by natural killer cells and T-cell subpopulations, transduce activating or inhibitory signals through HLA class I molecules [48]. Selective and varied expression of certain KIRs and their corresponding ligands could contribute to the heterogeneous nature of this disease. For example, KIR2DS2 is an activating receptor, implicated in RA susceptibility in an HLA class I-dependent manner [45], and similarly, the inhibitory KIR3DL2 and HLA-B27 are implicated in spondyloarthritides [49]. In this study, we have established a strong association of LILRB2, B3 and A2 with active RA and their expression is down-regulated in synovial tissue obtained from patients with clinical remission. However, the relative role of LILRs, KIRs and other related molecules and their potential ligands, in the diverse nature of the disease requires further studies using a large sample size.

The down-regulation of LILRA2-mediated TNF-α production by dexamethasone is consistent with the known potent and pleotropic effects of glucocorticoids in regulating inflammation. They suggest that the efficacy of DMARD treatment does not relate to a direct effect on LILR expression and function. Nevertheless LILRs may be potential novel therapeutic targets in pathological inflammatory processes such as RA. Definitive demonstration of a role for LILRs in regulating inflammation in vivo requires identification and/or development of specific agonists and antagonists.
FIG. 8. In vitro effects of DMARDs on macrophages activated by LILRA2 cross-linking. Changes in LILRA2-induced TNF-α production in macrophages after treatment with various doses of Dex (A), MTX (B) or CsA (C) for 24 h (n = 3). (D) Dose-dependent inhibition of LILRA2-mediated TNF-α production in response to treatment of PBMCs (n = 4) with Dex in vitro. *P < 0.05, **P < 0.01 when compared with untreated controls.

FIG. 9. LILRA2-induced TNF-α production by PBMC from healthy subjects and RA patients on or off prednisolone treatment (n = 3 for each group). Release of TNF-α by PBMCs without (A) or with (B) additional in vitro Dex treatment. (C) Representative expression of LILRA2 on the surface of PBMCs obtained from a healthy subject (a), RA patients off (b) or on (c) steroid therapy. *P < 0.05, **P < 0.01 when compared with untreated controls.
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