

Latent Cytomegalovirus Infection in Rheumatoid Arthritis and Increased Frequencies of Cytolytic LIR-1+CD8+ T Cells

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Objective. Leukocyte immunoglobulin-like receptor 1 (LIR-1) is up-regulated by cytomegalovirus (CMV), which in turn, has been associated with premature aging and more severe joint disease in patients with rheumatoid arthritis (RA). The aim of this study was to investigate the expression and functional significance of LIR-1 in CMV-positive RA patients.

Methods. We determined the phenotype, cytolytic potential, CMV-specific proliferation, and HLA-G-triggered, LIR-1-mediated inhibition of interferon- γ secretion of LIR-1+ T cells in RA patients and healthy controls.

Results. We found increased frequencies of CD8+ T cells with CMV pp65-specific T cell receptors in CMV-positive RA patients as compared to CMV-positive healthy controls. CMV-specific CD8+ T cells in these patients were preferentially LIR-1+ and exhibited a terminally differentiated polyfunctional phenotype. The numbers of LIR-1+CD8+ T cells increased with age and disease activity, and showed high levels of reactivity to CMV antigens. Ligation of LIR-1 with soluble HLA-G molecules in vitro confirmed an inhibitory role of the molecule when expressed on CD8+ T cells in RA patients.

Conclusion. We propose that latent CMV infection in the context of a chronic autoimmune response induces the recently described “chronic infection phenotype” in CD8+ T cells, which retains anti-infectious effector

features while exhibiting autoreactive cytolytic potential. This response is likely dampened by LIR-1 to avoid overwhelming immunopathologic changes in the setting of the autoimmune disease RA. The known deficiency of soluble HLA-G in RA and the observed association of LIR-1 expression with disease activity suggest, however, that LIR-1+ T cells are insufficiently controlled in RA and are still likely to be involved in the pathogenesis of the disease.

The human memory T cell compartment is shaped not only by antimicrobial immune responses, but also by autoimmunity and by latent infections with viruses such as cytomegalovirus (CMV) (1). The latter drive the generation of terminally differentiated T cells, which are characterized by the loss of costimulatory molecules such as CD27 and CD28, shortened telomeres, and by the expression of inhibitory natural killer (NK) cell receptors (2). CMV infection in immunocompetent hosts usually runs an asymptomatic course but has been reported to cause massive clonal expansions involving up to 40% of the global T cell pool (3). This increase over time in CMV-reactive T cells specific for antigens derived from latent CMV has been called memory inflation and involves both the CD4+ and the CD8+ T cell compartment (4,5). As a consequence, a stable CMV-reactive T cell compartment with an extremely dynamic cell turnover is established.

Clinically, CMV infection can cause organ-specific or systemic infections in immunocompromised patients. We and other investigators (6–8) have shown that the presence of a latent CMV infection influences the clinical course and outcome of rheumatoid arthritis (RA), the prototypical T cell-mediated autoimmune disease with severe perturbations of immune homeostasis, particularly in various T lymphocyte compartments. Similar observations have been reported in other autoimmune diseases, such as psoriasis (9), granulomatosis with polyangiitis (10,11), Alzheimer's disease (12), and systemic lupus erythematosus (13).

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Latent CMV infection has been associated with increased expression of the inhibitory NK cell receptor leukocyte immunoglobulin-like receptor 1 (LIR-1; also known as immunoglobulin-like transcript 2 and CD85j, with the gene name LILRB1) on CMV-reactive CD8+ T cells (14). LIR-1 belongs to a group of immunoregulatory receptors containing 2–4 immunoreceptor tyrosine-based inhibitory motifs within the cytoplasmic region. Upon tyrosine phosphorylation, LIR-1 recruits the SH2 domain-containing phosphatase 1 (SHP-1) tyrosine phosphatase or SH2 domain-containing inositol-5'-phosphatase (SHIP), both of which are involved in negative signaling and inhibition of cell activation (15). Furthermore, LIR-1 is expressed on almost all immune cells, including antigen-presenting cells and subsets of CD4+ and CD8+ T cells (16).

During the process of establishing latency following an acute CMV infection, the expression of LIR-1 on T cells is up-regulated (17,18), which results in reduced T cell proliferation in the autologous mixed lymphocyte reaction (19). The increase in LIR-1 expression after CMV infection is sustained throughout life and is regarded as a marker of premature immune senescence. It has been proposed that in otherwise healthy individuals, up-regulation of LIR-1 limits collateral tissue damage due to the sustained, long-term anti-CMV immune response (20), or it regulates T cell homeostasis (21). In conjunction with autoimmune conditions, however, LIR-1 expression appears to have additional and varying implications.

Diminished LIR-1 expression on B cells and altered functionality on T cells has been reported in systemic lupus erythematosus patients (22). Increased LIR-1 expression was found on the lymphocytes of patients with autoimmune thyroid disease (23) and multiple sclerosis (24). Genetic polymorphisms of LIR-1 were found to be associated with RA in patients not expressing RA-associated HLA-DRB1 alleles (25).

Since the effects of latent CMV infection and chronic immune response converge in patients with RA, we hypothesized that LIR-1 might be involved in the pathogenesis of the disease. The aim of this study, therefore, was to investigate the expression and functional significance of LIR-1 in CMV-positive RA patients. Based on the reported relevance of latent CMV infection to RA disease severity, we focused our study on the phenotype and function of polyfunctional and terminally differentiated CMV-specific lymphocytes positive for LIR-1, which is expressed not only on CD4+ T cells, but also, and more prominently, on CD8+ T cells.

PATIENTS AND METHODS

Patients and tissue samples. Patients with RA (n = 63) according to the American College of Rheumatology/

European League Against Rheumatism 2010 criteria (26) were recruited from the rheumatology unit of the University of Leipzig. The control group consisted of age-matched healthy subjects (n = 70). The CMV status of the RA patients and healthy controls was determined by serologic analysis using enzyme-linked immunosorbent assay (ELISA; Medac). This study was approved by the local ethics committee. Samples of synovium were obtained from patients undergoing synovectomy at the Department of Orthopedics at the University of Leipzig. Synovial tissue T cells were isolated as described previously (27).

Immunofluorescence staining and flow cytometry. T cell phenotyping was performed on freshly isolated peripheral blood mononuclear cells (PBMCs). The following antibodies were used in different fluorescent conjugates: anti-CD3 (BW264/56), anti-CD8 (BW135/80), anti-CD4 (M-T466), anti-CD28 (15E8), anti-CD27 (M-T271), anti-CD31 (AC128), anti-CD45RA (T6D11), anti-CD45RO (UCHL-1), anti-CCR7 (FR11-11E8), anti-CD57 (TB03), anti-programmed death 1 (anti-PD-1; PD1.3.1.3) (all from Miltenyi Biotec), anti-LIR-1 (292305 [R&D Systems] and GHI/75 [Miltenyi Biotec]), anti-IgG1 (11711 [R&D Systems] and IS6-11E5.11 [Miltenyi Biotec]), anti-CD3 (SP34-2; BD Biosciences), and anti-HLA-A2 (BB7.2; AbD Serotec).

Analysis of cells for the expression of surface markers was performed using FACSCalibur and LSR II flow cytometers (BD Biosciences). Data were analyzed with FlowJo software (Tree Star) and CellQuest (BD Biosciences) software. Doublets and dead cells were removed by exclusion of propidium iodide-positive cells.

Dextramer staining. PBMCs from RA patients were isolated by Ficoll-Paque density-gradient centrifugation. For determination of CMV-specific CD8+ T cells, PBMCs from CMV-positive RA patients and healthy controls were stained for HLA-A2 molecules. PBMCs (5×10^5) from HLA-A2-positive, CMV-positive RA patients (n = 8) and healthy donors (n = 12) were incubated with fluorescence-labeled monoclonal antibodies and an appropriate concentration of dextramer complexes for CMV proteins pp65 (CMV pp65/HLA-A*0201_{NLVPMVATV}) and IE-1 (CMV IE1/HLA-A*0201_{VLEETSVMML}) (both from Immudex) in a small volume for 30 minutes at 4°C with protection from the light.

Cytotoxicity assay. Cytolytic CD8+ T cells were analyzed by cell surface mobilization of CD107a (lysosome-associated membrane protein 1). CD8+ T cells were isolated by positive selection using magnetic-activated cell sorting (Miltenyi Biotec). CD8-depleted PBMCs (5×10^6) were loaded with 5 µg of CMV pp65 peptide mixture or control peptide (15-mers, 11-amino acid overlap; Jerini Peptide Technologies) for 2 hours at 37°C. After washing, target cells and effector CD8+ T cells were seeded at an effector cell-to-target cell ratio of 4:1. Cytotoxicity assays were performed at 37°C for 4 hours in the presence of 0.0125 µg of CD107a antibody (Alexa Fluor 488-conjugated; BioLegend). Staphylococcal enterotoxin B (SEB; 1 µg/ml) (Sigma) was used as a positive control. Medium alone was used as an unstimulated control. After 1 hour of coculture, monensin (2 µM; eBioscience) or GolgiStop (2 µM; BD Biosciences) was added for the last 3 hours of cell culture. Subsequently, cells were stained and measured by fluorescence-activated cell sorting (FACS). Dead cells were removed by exclusion of propidium iodide-positive cells. Only experiments with >0.2% CD107a+CD8+ T cells were included in the statistical analysis.

Proliferation assay. The fluorescence-based proliferation analysis was performed by labeling PBMCs with 3 µg/ml of

5,6-carboxyfluorescein succinimidyl ester (Molecular Probes) or 10 μ M Cell Proliferation dye eFluor 670 (eBioscience). Cells (2×10^6) were cultured for 7 days in the presence of 1 μ g/ml of CMV lysate or control lysate (Microbix Biosystems) or 1 μ g/ml of SEB (Sigma) as a positive control, in X-Vivo 15 medium (Lonza) containing 2 mM L-glutamine, 100 units/ml of penicillin, and 100 μ g/ml of streptomycin.

Soluble HLA-G inhibition assay. The HLA-G positive choriocarcinoma cell line JEG-3 (HTB-36; ATCC) was stably transfected with the expression vectors of the HLA-G targeting microRNA-152 and with the respective mock vector (as a control), as previously described (28). Soluble HLA-G-containing and HLA-G-free cell culture supernatants were collected and stored at -80°C until time for use. A protein concentration step was applied, and the soluble HLA-G content was determined by ELISA (Exbio). For inhibition assays, CD8+ T cells were stimulated with 2 μ g/ml of anti-CD3 for 6 hours in the presence of 5 μ l of supernatant containing soluble HLA-G. Supernatant without soluble HLA-G was used as a control. Cells were subsequently stained and measured by

FACS analysis. Dead cells were removed by exclusion of propidium iodide-positive cells. Intracellular staining of interferon- γ (IFN γ) was performed using an Inside Staining kit (Miltenyi Biotec).

Statistical analysis. GraphPad Prism 5.0 software was used for statistical analysis. Prior to all comparisons, a normality test was performed. Between-group differences in medians or means were analyzed by Student's *t*-test or the Mann-Whitney rank sum test as appropriate. Correlations were evaluated using Pearson's product-moment correlation or Spearman's rank correlation coefficient.

RESULTS

Analysis of CMV-specific CD8+ T cells in RA patients and controls. We reported previously that CMV-reactive CD4+ T cells, which secrete IFN γ in response to CMV antigen, are more frequent in RA patients than in healthy controls (7). To investigate the

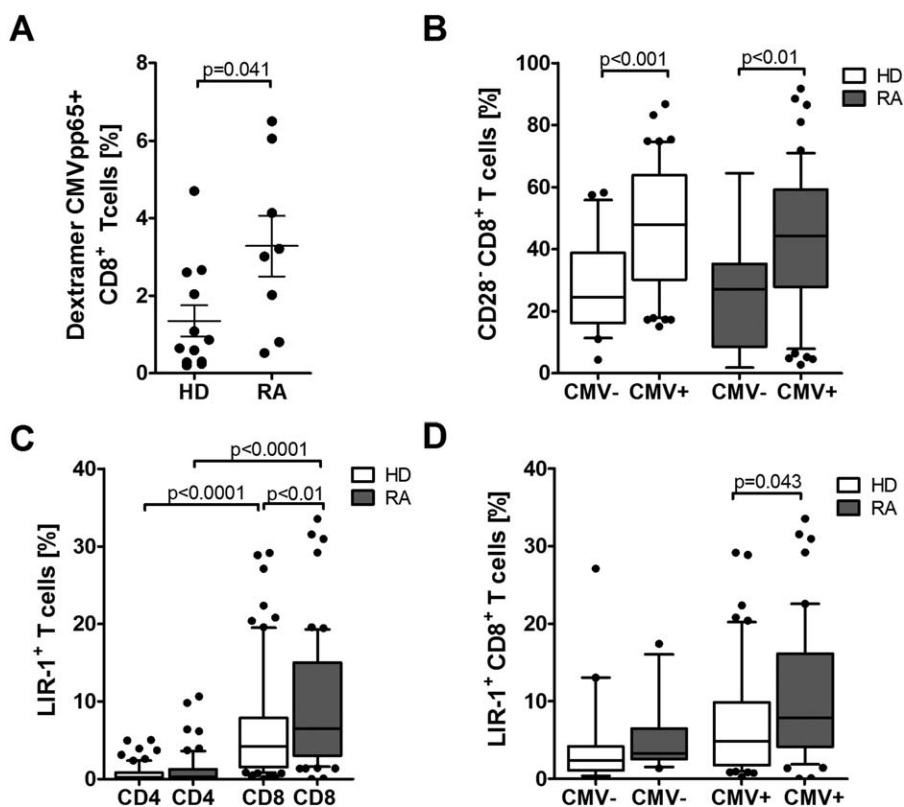


Figure 1. Increased frequencies of cytomegalovirus (CMV)-specific dextramer-positive CD8+ T cells and LIR-1+CD8+ T cells in rheumatoid arthritis (RA). **A**, Frequency of CMV pp65/HLA-A*0201_{NLVPMVATV} dextramer-positive CD8+ T cells in healthy donors (HD; n = 12) and RA patients (n = 8). Each symbol represents an individual sample; horizontal lines and error bars show the mean \pm SEM. **B**, Frequency of CD28-CD8+ T cells in CMV-negative and CMV-positive healthy donors (n = 8 and n = 57) and CMV-negative and CMV-positive RA patients (n = 20 and n = 50). **C** and **D**, Frequency of LIR-1+ T cells in CD4+ and CD8+ T cells from healthy donors (n = 63) and RA patients (n = 70) (**C**) and in CD8+ T cells from CMV-positive and CMV-negative healthy donors (n = 49 and n = 11) and RA patients (n = 51 and n = 19) (**D**). Data in **B-D** are shown as box plots. Each box represents the 25th to 75th percentiles. Lines inside the boxes represent the median. Whiskers represent the 10th and 90th percentiles. Solid circles indicate outliers. Except where indicated otherwise, frequencies are given as the percentage of total CD8+ T cells.

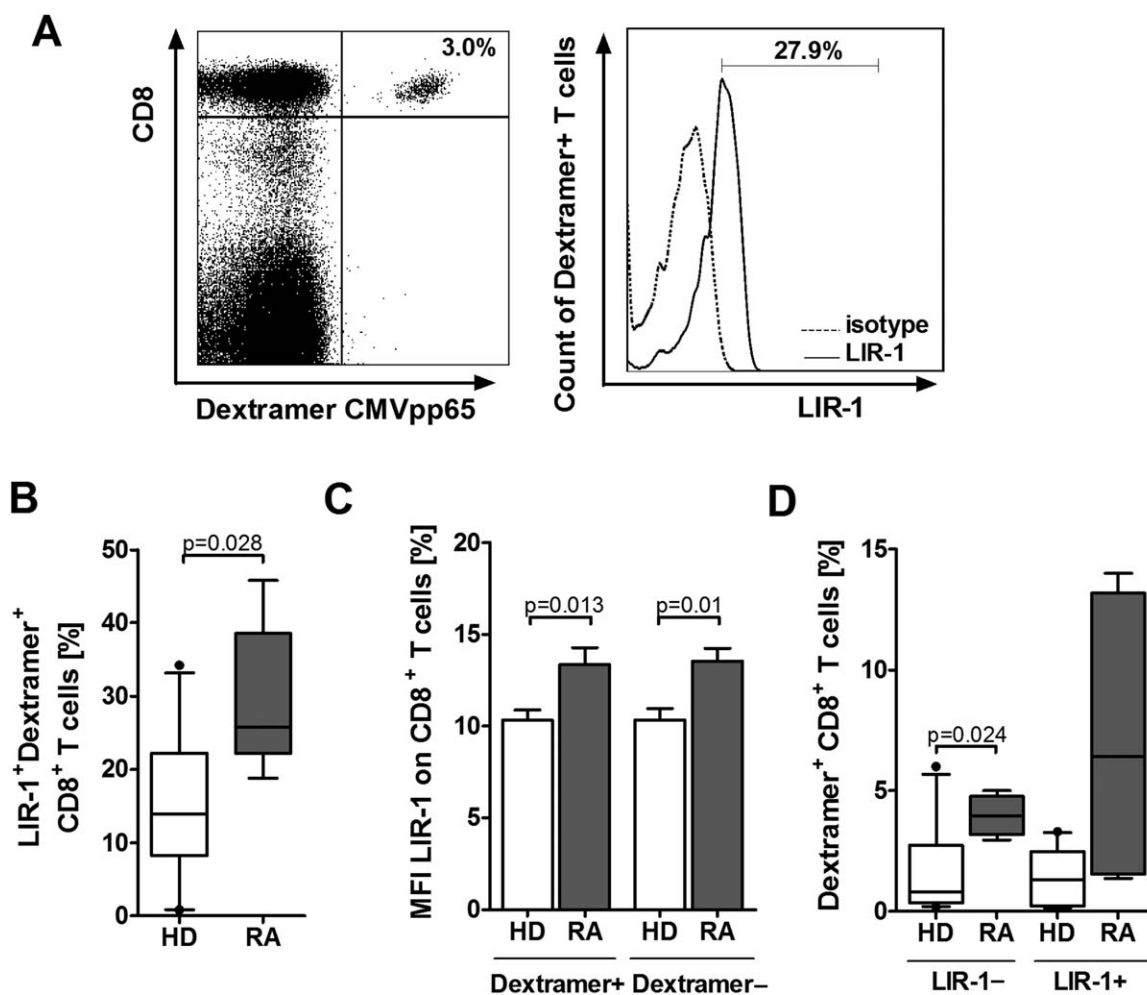


Figure 2. Increased frequencies of leukocyte immunoglobulin-like receptor 1 (LIR-1)-positive cells among cytomegalovirus (CMV)-specific dextramer-positive CD8+ T cells in rheumatoid arthritis (RA). **A**, Representative dot plot of dextramer CMV pp65 binding to CD8+ T cells (left) and representative histogram of LIR-1 expression on dextramer-positive CD8+ T cells (right). **B**, Frequencies of LIR-1+ cells among dextramer-positive CD8+ T cells from healthy donors (HD; n = 10) and from anti-cyclic citrullinated peptide-positive RA patients (n = 5). **C**, Fluorescence intensity of LIR-1 expression on dextramer-positive and dextramer-negative CD8+ T cells. **D**, Frequency of CMV pp65-specific dextramer-positive cells among LIR-1+ and LIR-1- CD8+ T cells from healthy donors (n = 10) and RA patients (n = 4). Values are the mean \pm SEM. Data in **B** and **D** are shown as box plots. Each box represents the 25th to 75th percentiles. Lines inside the boxes represent the median. Whiskers represent the 10th and 90th percentiles. Solid circles indicate outliers.

consequences of latent CMV infection for the CD8+ T cell compartment in RA, the frequency of CMV-specific CD8+ T cells was determined using CMV pp65-specific dextramers and was found to be increased in RA patients positive for CMV and anti-cyclic citrullinated peptide as compared to CMV-positive healthy controls (Figure 1A). Latent CMV infection is known to cause memory inflation, with a concomitant increase in poly-functional, terminally differentiated CD4+ and CD8+ T cells with pathologic phenotypes (5,29). As an example of such a phenotype, we analyzed the frequencies of CD28- T cells and found an increase in both the CD4

and the CD8 T cell compartments in CMV-positive as compared to CMV-negative RA patients (Figure 1B).

Analysis of LIR-1+CD8+ T cells in RA patients and controls. CMV infection is known to induce overexpression of LIR-1. In RA patients and healthy control subjects, CD8+ T cells express LIR-1 more frequently than do CD4+ T cells, as previously reported (20) and as confirmed by the findings of our present study (Figure 1C). Surprisingly, LIR-1 expression on CD8+ T cells in RA patients was even higher than that in healthy controls. This increase was most pronounced in CMV-positive RA patients as compared to CMV-positive controls (Figure 1D).

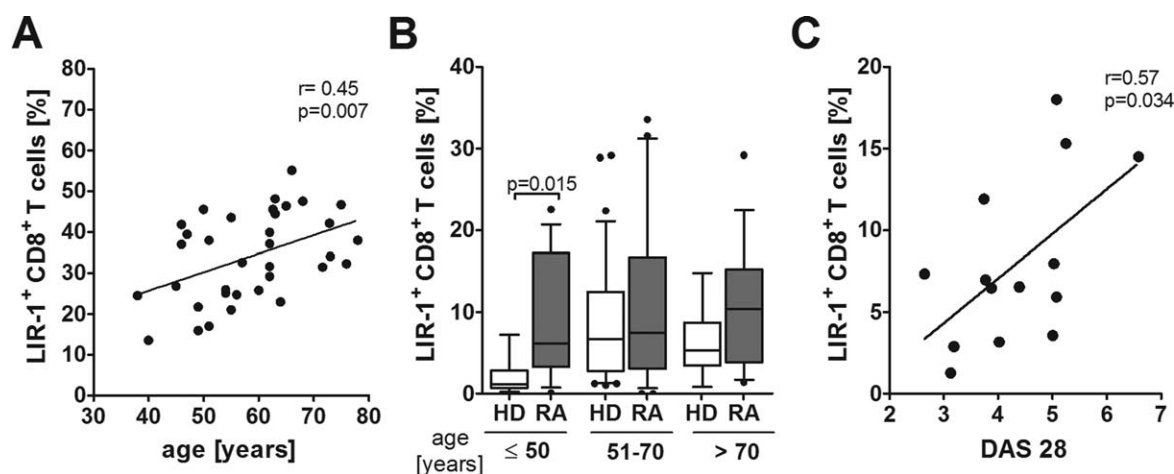


Figure 3. Influence of age and disease activity on frequencies of LIR-1+CD8+ T cells in rheumatoid arthritis (RA). **A**, Correlation of the frequencies of LIR-1+CD8+ T cells in the peripheral blood of 35 RA patients by age group. **B**, Frequency of LIR-1+CD8+ T cells in the peripheral blood of RA patients and healthy donors (HD) in 3 age groups: ≤ 50 years ($n = 13$ and $n = 8$, respectively), 51–70 years ($n = 24$ and $n = 37$, respectively), and >70 years ($n = 16$ and $n = 7$, respectively). Data are shown as box plots. Each box represents the 25th to 75th percentiles. Lines inside the boxes represent the median. Whiskers represent the 10th and 90th percentiles. Solid circles indicate outliers. **C**, Correlation of the frequencies of LIR-1+CD8+ T cells in the peripheral blood of 14 RA patients by Disease Activity Score in 28 joints (DAS28). All frequencies are given as the percentage of total CD8+ T cells.

In CMV-negative RA patients, a trend toward increased frequencies of LIR-1+CD8+ T cells was discernible, but the difference did not reach statistical significance ($P = 0.085$).

LIR-1+CD8+ T cells were also quantified in the affected joints of RA patients and were found in significant numbers, both in rheumatoid synovium and in synovial fluid, although the frequencies in the synovial membrane were lower than those in the peripheral blood (P not significant) (data not shown).

Analysis of LIR-1 expression on CMV pp65-specific CD8+ T cells revealed increased LIR-1+ cells among dextramer-positive CD8+ T cells from RA patients as compared to controls (Figures 2A and B). Importantly, LIR-1 expression levels were also significantly higher on dextramer-negative CD8+ T cells from RA patients as compared to controls (Figure 2C). In RA patients, more CD8+ T cells were CMV pp65 specific, both among LIR-1+ and LIR-1- cells (Figure 2D). Determination of CD8+ T cells specific for CMV IE-1 revealed very low frequencies, both in RA patients and in healthy individuals (data not shown).

The frequencies of LIR-1+CD8+ T cells were found to increase with age (Figure 3A), as reported previously (18). Of note, however, the frequencies of LIR-1+CD8+ T cells were also higher when only subjects younger than age 50 years were compared (Figure 3B). Clinically, increased frequencies of LIR-1+CD8+ T cells were found to be associated with higher levels of disease activity, as indicated by a significant positive correla-

tion with the Disease Activity Score in 28 joints (Figure 3C).

Phenotyping of LIR-1+CD8+ T cells. Repeated chronic immune responses often result in polyfunctional, terminally differentiated, and possibly exhausted T cell phenotypes, such as the CD4+CD7-CD28- T cell subset originally described in RA (30) or the CD8+CD27-CD28- T cell subset (1). Phenotype characterization of LIR-1+CD8+ T cells from RA patients by flow cytometry revealed them to be preferentially CD27-CD28- and CD28-CD57+ and to contain increased frequencies of CD45RA+CCR7- effector T cells. Furthermore, LIR-1+CD8+ T cells more frequently express the NK cell marker CD56 than do LIR-1- T cells. Analysis of the “exhaustion” marker PD-1 revealed no difference in its expression on LIR-1+CD8+ T cells as compared to LIR-1- T cells (Figure 4A). Expression of the fractalkine receptor CX₃CR1, which enables T cells to migrate into the rheumatoid synovium (31), was also increased on LIR-1+CD8+ T cells from RA patients as compared to LIR-1-CD8+ T cells from RA patients and from healthy controls (Figure 4B). The chemokine receptor CCR5, which is also associated with latent virus infection and an effector phenotype of T cells (32), was not differentially expressed on LIR-1+ T cells from RA patients as compared to healthy controls (Figure 4C).

Functional characterization of LIR-1+CD8+ T cells in RA. Inhibitory immune receptors, such as LIR-1, are expressed on T cells, where they control the magnitude of the immune response after activation. We therefore analyzed the regulation of LIR-1 expression in vitro, the

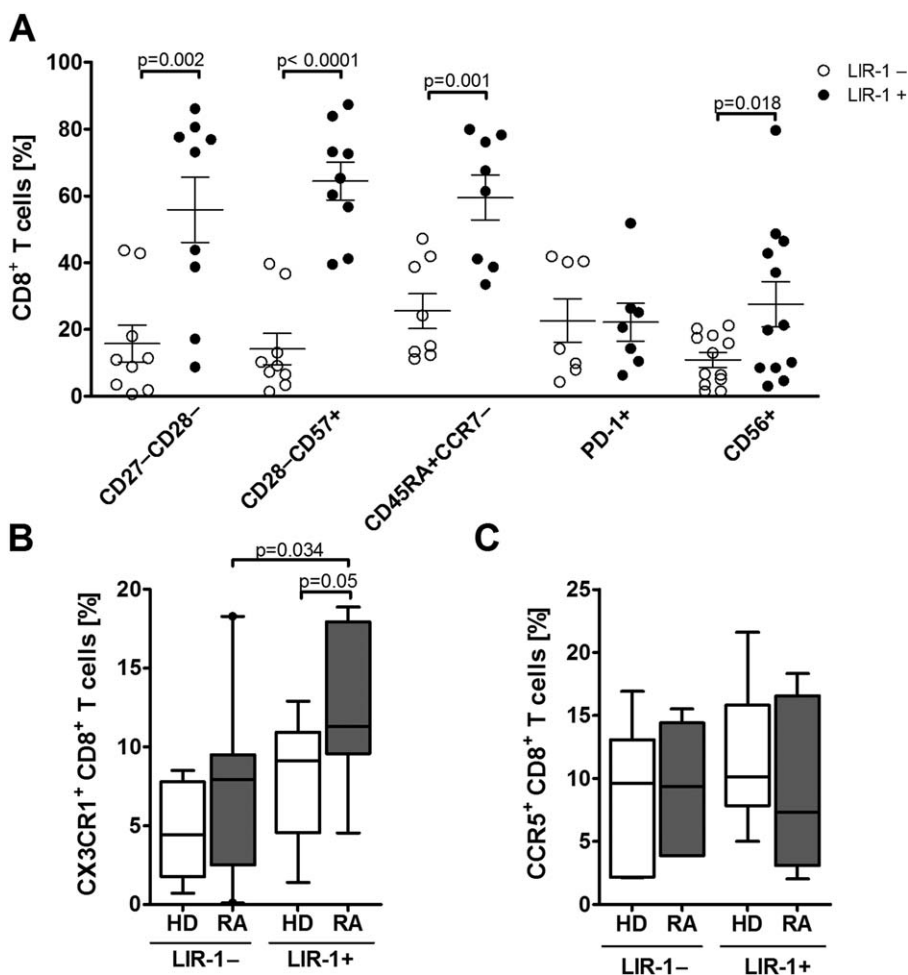


Figure 4. Phenotypic characterization of LIR-1⁺CD8⁺ T cells in rheumatoid arthritis (RA). **A**, Frequencies of CD27⁻CD28⁻ T cells, CD28⁻CD57⁺ T cells, CD45RA⁺CCR7⁻ T cells, programmed death 1 (PD-1)-positive T cells, and CD56⁺ T cells in LIR-1⁺CD8⁺ and LIR-1⁻CD8⁺ T cell subsets in RA patients. Each symbol represents an individual sample; horizontal lines and error bars show the mean \pm SEM. **B** and **C**, Frequencies of chemokine receptors CX₃CR1⁺ (**B**) and CCR5⁺ (**C**) among LIR-1⁺CD8⁺ T cells and LIR-1⁻CD8⁺ T cells from RA patients (n = 9) and healthy donors (HD; n = 8). Data are shown as box plots. Each box represents the 25th to 75th percentiles. Lines inside the boxes represent the median. Whiskers represent the 10th and 90th percentiles. Solid circle indicates an outlier. LIR-1 = leukocyte immunoglobulin-like receptor 1.

effector status of LIR-1⁺CD8⁺ T cells, and the inhibitory effect of the LIR-1 molecule on CD8⁺ T cells from RA patients.

To gain insight into the regulation of LIR-1, PBMCs from RA patients were stimulated *in vitro* for 6 days using various stimuli. The expression of LIR-1 on CD8⁺ T cells was down-regulated after 2 days without exogenous stimuli. Expression could be maintained for 4 days with anti-CD3, and it remained detectable even after 6 days of culture in the presence of CMV lysate or phytohemagglutinin (data not shown).

Functionally, the cytolytic potential of LIR-1⁺CD8⁺ T cells was determined by CD107a mobiliza-

tion assay using flow cytometry. Significantly higher expression of CD107a in response to CMV pp65-loaded PBMCs was detected on CD8⁺ T cells from RA patients as compared to healthy controls, indicating increased CMV-specific cytolytic potential (Figures 5A and B). Control peptide-loaded antigen-presenting cells could not induce CD107a expression in CD8⁺ T cells (data not shown). In healthy controls and RA patients, increased cytolytic potential was mainly found in LIR-1⁺CD8⁺ T cells.

The proliferative potential of LIR-1⁺CD8⁺ T cells after stimulation with CMV lysate was also quantified *in vitro* (Figures 5C and D). LIR-1⁺CD8⁺ T cells were not found to be proliferatively exhausted, since

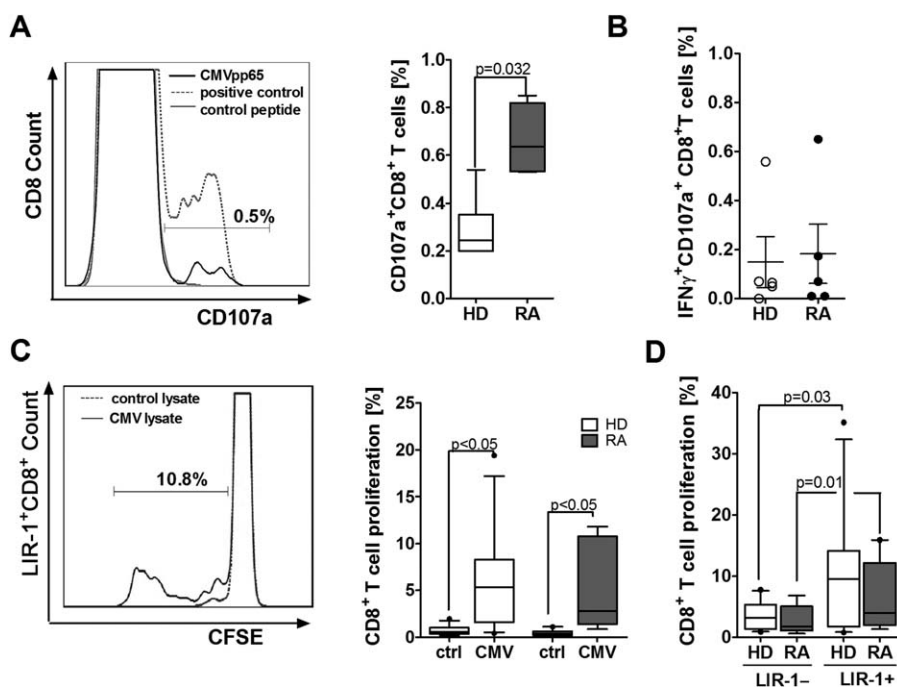


Figure 5. Functional analysis of LIR-1⁺CD8⁺ T cells in rheumatoid arthritis (RA). **A** and **B**, CD107a-degranulation assay. The representative histogram in **A** shows CD107a expression on CD8⁺ T cells after restimulation with cytomegalovirus (CMV) pp65 peptide-loaded, staphylococcal enterotoxin B (SEB; positive control)-loaded, or control peptide-loaded antigen-presenting cells (left). The percentage of CD107a⁺CD8⁺ T cells is also shown (right). In **B**, the percentage of IFN γ +CD107a⁺CD8⁺ T cells from RA patients (n = 5) and healthy donors (HD; n = 5) is shown. **C** and **D**, Proliferation of LIR-1⁺CD8⁺ T cells from CMV-positive healthy donors and RA patients. The representative histogram in **C** shows proliferation of LIR-1⁺CD8⁺ T cells after restimulation with CMV lysate (1 μ g/ml) or control lysate (left). The proliferation rate of CD8⁺ T cells incubated with CMV lysate or control lysate from healthy donors (n = 12) and patients with RA (n = 8) is also shown (right). In **D**, the percentage of proliferated LIR-1⁻CD8⁺ and LIR-1⁺CD8⁺ cells from healthy donors (n = 12) and RA patients (n = 8) in response to CMV lysate is shown. Data in **A** (right), **C** (right), and **D** are shown as box plots. Each box represents the 25th to 75th percentiles. Lines inside the boxes represent the median. Whiskers represent the 10th and 90th percentiles. Solid circles indicate outliers. In **B**, each symbol represents an individual sample; horizontal lines and error bars show the mean \pm SEM.

they mounted a robust proliferative response to CMV lysate, which exceeded the proliferation rates of LIR-1⁻CD8⁺ T cells both in RA patients and in healthy controls (Figure 5D). Despite their enhanced cytolytic potential, however, CD8⁺ T cells from RA patients did not show an increased proliferation rate as compared to healthy controls (Figure 5C).

Soluble HLA-G is a ligand of LIR-1 and is known to evoke an inhibitory signal in most cell types. In patients with RA, decreased serum levels of soluble HLA-G have been reported (33), which may contribute to autoimmunity. Hence, we investigated the responsiveness of RA CD8⁺ T cells by triggering LIR-1. Addition of exogenous soluble HLA-G in vitro led to decreased frequencies of CD107a⁺CD8⁺ T cells (Figures 6A and B), indicating that the inhibitory signal triggered by ligation of LIR-1 would be effective if sufficient soluble HLA-G molecules were available in RA. Furthermore, intracellular IFN γ expression in CD8⁺ T cells was also down-regulated in cultures with soluble HLA-G (Figure 6C).

DISCUSSION

Latent CMV infection and RA share several phenotypical features in the T cell compartment. In addition, the clinical impact of CMV infection on the RA disease course has previously been reported (6–8). We describe herein a population of CD8⁺ T cells in CMV-positive RA patients that exhibits proinflammatory, cytolytic, and antiviral features and is functionally inhibited due to up-regulated expression of LIR-1.

In healthy individuals, expression of LIR-1 on CD8⁺ T cells is up-regulated by CMV infection, possibly with the goal of limiting collateral tissue damage due to the longstanding immune response against the latent virus or, alternatively, as a homeostatic mechanism (6–8). We found that the frequency of LIR-1⁺CD8⁺ T cells was significantly higher in CMV-positive RA patients than in CMV-positive healthy controls and that it increased not only with age, but also with higher levels of disease activity. In addition, LIR-1 was up-regulated on CD8⁺ T cells that were not specific for CMV antigens.

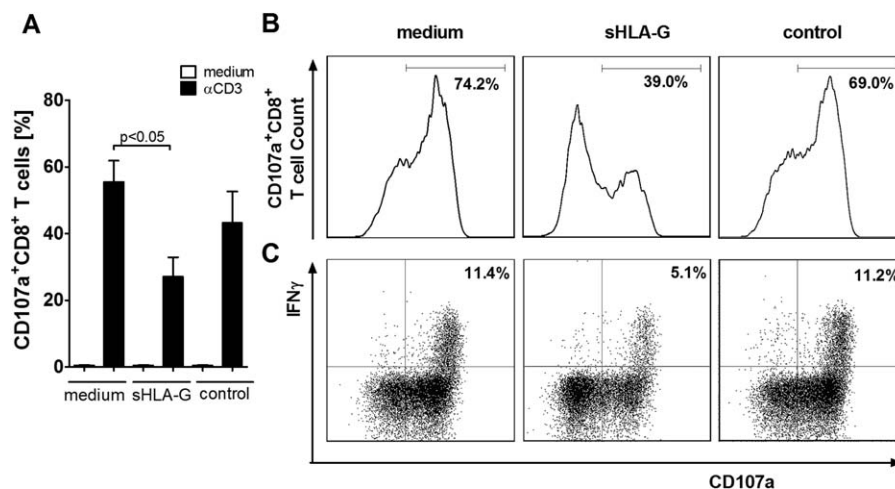


Figure 6. Inhibition of cytotoxic CD8⁺ T cells in patients with rheumatoid arthritis (RA) by soluble HLA-G (sHLA-G). **A**, Inhibition of CD107a expression after the addition of medium alone, soluble HLA-G (5 μ l), or anti-CD3 control to in vitro cultures of CD8⁺ T cells. Values are the mean \pm SEM of 3 samples per group. **B** and **C**, Representative histograms showing the frequencies of CD107a⁺CD8⁺ T cells (**B**) and the expression of intracellular interferon- γ (IFN γ) on CD8⁺ T cells (**C**) from RA patients after in vitro activation in medium or soluble HLA-G-containing or control supernatants.

In healthy individuals, lymphocytes involved in memory inflation tend to acquire a specific phenotype, which was previously described as “exhaustive.” More recently, it has been suggested that this “chronic infection phenotype” represents a population of T cells that can still efficiently control latent infection, while certain levels of effector function are diminished to prevent overwhelming immunopathologic changes due to collateral auto-reactivity (34). Increased LIR-1 expression on CD8⁺ T cells is likely to represent such a CMV-induced chronic infection phenotype, since it is linked to latent CMV infection in healthy individuals (18). Our results show that in comparison to healthy controls, LIR-1 was further up-regulated on CD8⁺ T cells in RA patients. In this autoimmune disease, increased LIR-1 expression could result from an insufficiently controlled latent CMV infection, leading to higher numbers of T cells, which are required and recruited, or it could represent a regulatory mechanism aimed at controlling autoimmunity in RA in the context of latent CMV infection.

The functional analysis confirmed that LIR-1⁺ T cells in RA are polyfunctional and have cytolytic potential. Their higher expression of CD56 and CD57, which is associated with increased cytotoxicity in CMV-seropositive healthy individuals (1,35), might further increase their cytolytic potential in RA. Phenotype analysis using the T cell differentiation markers CCR7 and CD45RA confirmed that LIR-1⁺CD8⁺ T cells are effector T cells. In addition, their expression of CX₃CR1, which is known to be up-regulated on CMV-specific CD4⁺ and CD8⁺ T cells from healthy individuals (36), might enable them to

migrate toward fractalkine gradients, which has been reported to occur in the rheumatoid synovium (31). Differences in PD-1 expression between LIR-1⁺ and LIR-1⁻CD8⁺ T cells were not significant.

In phases of CMV reactivation or of relevant suppression of the controlling immune response, the CMV-specific LIR-1⁺CD8⁺ T cells appear to be able to mount a cytolytic immune response. Numerically, only 25% of the CMV pp65-specific CD8⁺ T cells are LIR-1⁺, and <10% of LIR-1⁺ cells are specific for the immunodominant CMV antigen pp65. Even assuming that another 10% of LIR-1⁺ cells recognize other CMV antigens, it still leaves the majority of LIR-1⁺CD8⁺ T cells unreactive to CMV. The observation that LIR-1 overexpression is also found on T cells that are not reactive to CMV antigens and possibly even on T cells from CMV-negative RA patients indicates that CMV-independent factors related to the chronic autoimmune disease are also involved.

Our finding of increased proliferative activity is evidence against the interpretation that expression of LIR-1 on CD8⁺ T cells is the equivalent of T cell exhaustion, since T cell exhaustion is generally regarded to be accompanied by low proliferative capacity (37,38). Our observation is consistent with a similar study showing increased proliferative capacity of LIR-1⁺CD8⁺ T cells from healthy controls (14). In RA, exaggerated proliferative replication of LIR-1⁺CD8⁺ T cells could even contribute to the observed increase in the frequency of CMV-specific dextramer-positive CD8⁺ T cells, possibly due to a failure to “switch off” ongoing immune responses. Alternatively, the increased frequency of CMV-specific T cells could be a

consequence of globally increased T cell proliferation leading to replicative senescence in this disease (39), but this latter explanation is partially contradicted by the increased proliferation among LIR-1+CD8+ T cells.

LIR-1 remains functional as an inhibitory receptor in RA, since we found LIR-1 ligation by soluble HLA-G to block cytotoxicity in our experiments. Antigen recognition by CD8+ T cells requires antigen-presenting major histocompatibility complex (MHC) class I molecules to interact with T cell receptors and CD8 coreceptors within the tightly organized and spatially focused immunologic synapse. Those MHC class I molecules are ligands for LIR-1, and when LIR-1 is recruited to the immunologic synapse, it exerts a strong inhibitory effect, even more so if it encounters the viral high-affinity ligand UL18, which is expressed on the juxtaposed cells in latent CMV infection (40). Accordingly, up-regulation of the inhibitory receptor LIR-1 in RA could be an attempt to limit auto-reactivity in order to alleviate the autoimmune disease.

Soluble HLA-G has been reported to be reduced in RA (33) and has been implicated in the pathogenesis of RA by the associations of its genetic polymorphisms with disease susceptibility (41). Latent CMV infection down-regulates HLA-G expression (42), and decreased levels of soluble HLA-G, in turn, could cause LIR-1+CD8+ T cells in RA to become hyperactive.

Taken together, our data suggest that the intricate network and finely tuned crosstalk of UL18, HLA-G, and classic MHC class I molecules with LIR-1 is disturbed during the course of a CMV infection in RA. As a consequence, deficiency of soluble HLA-G might diminish the inhibitory effects of LIR-1 on CD8+ T cells in CMV-positive RA patients. LIR-1+CD8+ T cells, in turn, could also be involved in the pathogenesis of RA by contributing directly to chronic inflammation. The observed significant correlation of the frequency of LIR-1+CD8+ T cells with disease activity strongly supports this hypothesis. At the same time, those cells might still be involved in the immunologic control of the latent CMV infection, which illustrates possible unwanted side effects of immunosuppression in this disease.

AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Wagner had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design. Rothe, Quandt, Pierer, Wagner.

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