Potential involvement of IL-9 and Th9 cells in the pathogenesis of rheumatoid arthritis

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Abstract

Objective. IL-9 has been shown to be upregulated before the clinical onset of articular disease in RA. The exact role of IL-9 and Th9 cells in RA, however, has not yet been adequately studied. The aim of this study was to evaluate the expression of IL-9 and IL-9-expressing cells in RA patients.

Methods. IL-9, IL-9R, PU.1, IL-9, thymic stromal lymphopoietin (TSLP), IL-4 and TGF-β expression was assessed by real-time-PCR in the synovial tissues of RA and OA patients. IL-9, IL-9R, IL-4, TSLP and TGF-β were also investigated by immunohistochemistry. Peripheral CD4+ T cell subsets were studied by flow cytometry analysis before and after incubation with citrullinated peptides.

Results. IL-9 was overexpressed in RA synovial tissues and correlated with the degree of histological organization of B and T cells in ectopic lymphoid structures. The majority of IL-9-producing cells were identified as CD3+ cells. Increased mRNA and protein expression of IL-9R, IL-4, TSLP and TGF-β was also observed in RA synovial tissue. Blood peripheral Th9 cells were expanded by citrullinated peptides.

Conclusion. These results indicate that Th9 cells and IL-9 were frequently detected in peripheral blood mononuclear cells and synovia of RA patients. A possible pathogenic role for Th9 in RA is discussed.

Key words: IL-9, Th9 cells, rheumatoid arthritis, citrullinated peptide.
cells, with a potential role in T-cell-dependent B-cell differentiation, expansion and antibody production, has also been described [2, 3]. The development of Th9 cells requires a balance of signals from cytokines such as IL-4 and TGF-β [2], and from epithelial cytokines such as thymic stromal lymphopoietin (TSLP) [4].

All these cytokines have been demonstrated to be increased in RA [5–7] and to induce the transcription factor PU.1 that acts in recruiting chromatin-modifying enzymes and activating gene expression [8]. A role for IL-9 in RA is also suggested by the demonstration of its increased levels before the clinical onset of articular disease [9]. Although these observations overall suggest a potential role for IL-9 in RA, the exact role of Th9/IL-9 has not yet been adequately studied.

The aim of the present study was to assess whether the Th9 subset occurs in the synovial tissue of patients with RA and correlates with the degree of histological organization and to analyse the frequency of Th9 cells in the peripheral blood of RA patients. The role of a citrullinated peptide in in vitro Th9 activation was also evaluated.

Here we show for the first time that IL-9 and Th9 cells are overexpressed in RA synovial tissue and correlate with the degree of histological organization of B and T cells in ectopic lymphoid structures. We also show that peripheral Th9 cells are increased in RA and specifically activated by citrullinated peptides. Altogether, these results suggest that Th9 cells and IL-9 may play an important role in the pathogenesis of RA.
Materials and methods

Immunohistochemistry in synovial tissues

Immunohistochemical analysis for CD3, CD19, CD21, IL-9, IL-9R, IL-17, PU.1 and TSLP was performed on 5-μm-thick paraffin-embedded sections from synovial biopsies and from tonsils (used as positive controls), as previously described [10]. Each sample was obtained by arthroscopy or arthroplasty from 36 RA biologic-naive patients [28 female, mean duration of disease 51 (s.d. 12) months, DAS28 6.6 (s.d. 2.8)] who fulfilled the 2010 ACR/EULAR classification criteria [11]. At the time of synovial biopsy, all patients were receiving a low dose of steroids (<7.5 mg daily prednisone), 7 were not receiving any DMARDs, 21 were receiving MTX (>10 mg weekly) and 8 were receiving LEF therapy. OA synovial biopsies were

The IL-9 source in RA and OA synovial tissue was assessed by confocal microscopy analysis in 10 RA patients and 10 OA patients. Experiments were performed in triplicate. (A–C) CD3+ cells were the major source of IL-9; (A and B) single staining for CD3 (A) and IL-9 (B), respectively. (C) Merged double staining for CD3 (green) and IL-9 (red). (D–F) Th17 in RA synovial tissues. (D and E) Single staining for IL-17 and IL-9, respectively. (F) Merged double staining for IL-17 (green) and IL-9 (red). (G–I) Th9 in RA synovial tissues. (G and H) Single staining for PU.1 and IL-9, respectively. (I) Merged double staining for PU.1 (green) and IL-9 (red). (J–L) Th9 cells in OA synovial tissue. (J–K) Single staining for PU.1 and IL-9, respectively. (L) Merged double staining for PU.1 (green) and IL-9 (red).
IL-9R expression in RA synovial tissue was assessed by immunohistochemistry in 36 RA patients and 15 OA patients. Experiments were performed in triplicate. Representative microphotographs showing CD3 (A, D and G), CD20 (B, E and H) and IL-9R+ cells (C, F and I) in paraffin-embedded synovial samples obtained from RA patients without (A-C) and with (D-F and G-I) T-B cell aggregates. Diffuse positive cells were detected outside T-B cell aggregates (C, F and I), with few cells detectable at the outside border of lymphoid aggregates (F and I). (A-I) original magnification ×250.

obtained from 15 patients with end-stage disease undergoing knee joint replacement surgery and were included as controls. All participants gave their informed consent, and the study was approved by the university hospital ethics committee (Azienda Ospedaliera Universitaria Paolo Giaccone, University of Palermo, Palermo, Italy). Synovial biopsy samples were fixed in formalin, and haematoxylin and eosin-stained sections were studied by the same pathologist (A.R.) using a microscope equipped with an ocular micrometer. Evaluation of the presence of lymphocytic infiltrates and/or synovial hyperplasia was performed by two investigators independently (F.C. and A.R.). Biopsies were grouped according to the following criteria: T/B cell aggregates with germinal centres (GCs), T-B cell aggregates without GCs or diffuse infiltration of T and B cells without lymphoid organization. The presence of GCs within lymphoid aggregates was determined according to standard histological criteria [12].

Sections were incubated with unlabelled rabbit-anti-human-IL-9 (Novus Biologicals, Littleton, CO, USA), rabbit-anti-human-IL-17 (Santa Cruz, CA, USA) and rabbit anti-human TSLP (Novus Biologicals, Littleton, CO, USA). To characterize Th9 cells, double staining was performed on paraffin-embedded sections of human synovial tissues. Sections were incubated with unlabelled anti-human IL-9 and PU.1 antibodies (Th9 cells), and IL-9 and IL-17 (Th17 cells), then treated with FITC- or rhodamine red-conjugated anti-mouse or anti-rabbit antibodies plus RNasi (200 ng/ml) and counterstained using Toto-3 iodide (642/660; Invitrogen). Confocal analysis was used to acquire fluorescence staining.

RNA isolation and quantitative real-time PCR
Synovial biopsies obtained from 18 of the 36 patients with RA and from all OA patients, soon after removal, were stored in RNAlater solution (Applied Biosystems, Foster City, CA, USA). Real-time PCR was performed as previously described [13]. Master mix and Taqman gene expression assays for GAPDH control and target genes IL-9 (Hs00914237_m1), IL-9R (Hs01108522_m1), IL-4 (Hs00174122-m1), TGF-β (Hs00998133_m1), TSLP

Fig. 3 IL-9R expression in RA synovial tissues
(Hs00263639_m1), PU.1 (Hs00162150_m1), IFN-γ (Hs00989291_m1), IL-21 (Hs00222327_m1) and IL-27 (Hs00377366_m1) were obtained from Applied Biosystems (Foster City, CA, USA). Final values were expressed as fold induction.

Patients and culture conditions

Peripheral blood samples were obtained from 15 of the above-described RA patients with established disease [12 female, mean age 48 (s.d. 6)], from 20 new patients with early untreated RA [14 female; mean age 32 (s.d. 14), mean disease duration 11 (s.d. 4) months] who fulfilled the 2010 ACR/EULAR classification criteria [14] and from 16 sex- and age-matched healthy controls. These patients had active disease as defined by the presence of six or more swollen joints and six or more tender joints at baseline, and either a CRP level of ≥2 mg/dl and/or an ESR of ≥30 mm/h (DAS > 5.1) at screening. All participants gave their informed consent, and the study was approved by the university hospital ethics committee. Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized blood samples by Ficoll-Hypaque (Sigma) density-gradient centrifugation. RPMI 1640 medium supplemented with 2 mM L-glutamine, 5 mM HEPES, 100 U/ml penicillin or 10 μg/ml streptomycin, 0.5 mM sodium pyruvate, 0.05 mM of non-essential amino acids (both obtained from Life Technologies) and 10% fetal calf serum (obtained from BioWhittaker) were used to assess all experiments. Cell viability (trypan blue dye exclusion) was always >95%. PBMCs were cultured with phorbol myristate acetate (50 ng/ml; Sigma, St Louis, MO, USA) and ionomycin (1 μg/ml; Sigma, St Louis, MO, USA) and incubated at 37°C in 5% CO₂. After 2 h of incubation, Brefeldin A (10 μg/ml; Sigma, St Louis, MO, USA) was added, and

**Fig. 4 IL-4, TGF-β and TSLP expression in RA synovial tissues**

IL-4, TGF-β and TSLP expression in RA synovial tissue was assessed by real-time PCR and immunohistochemistry in 36 RA patients and 15 OA patients. (A, E and I) real-time PCR expression of IL-4 (A), TGF-β (E) and TSLP (I) in RA patients and controls. Strong IL-4 immunoreactivity was observed in the infiltrating mononuclear cells of RA synovial tissue (B), but not in OA synovial tissues (C). Strong TGF-β expression was found in both the infiltrating mononuclear cells and the synovial layer of RA synovial samples (F), but not in OA synovial tissues (G). TSLP expression was observed mainly in RA synovia in the context of the synovial layer (J) and in endothelial cells of neovessels (J insert); some infiltrating inflammatory mononuclear cells were also TSLP positive. In control synovial samples, TSLP expression was observed only in the context of endothelial cells (K). Isotype control staining for IL-4 (D), TGF-β (H) and TSLP (L). (B-D, F-H, J-L) original magnification ×250. TSLP: thymic stromal lymphopoietin.
after 16 h of incubation, cells were collected and stained with the following mAbs: anti-human-CD4-PerCP (BD Biosciences, San Jose, CA, USA), anti-human-PU.1-FITC (R&D Systems, La Jolla, CA, USA), anti-human IL-9-PE (BD Biosciences, San Jose, CA, USA), anti-human IL-17-APC, anti-human IFN-γ-FITC (BD Biosciences, San Jose, CA, USA) and anti-human FoxP3-FITC (R&D Systems, La Jolla, CA, USA). Isotype-matched irrelevant antibodies were used as negative controls. The cells were incubated with mAbs for 30 min on ice and washed twice in PBS containing 0.1% (w/v) NaN₃ (Sigma Aldrich). After surface staining, the cells were fixed with 2% (w/v) paraformaldehyde (Sigma, St Louis, MO, USA) for 30 min at 4°C and then treated with a permeabilization solution (BD Biosciences, San Jose, CA, USA) for 10 min at room temperature and stained with antibodies for intracellular antigens for 30 min at 4°C. PBMCs (10⁶/ml) were stimulated with aggrecan peptide and with citrullinated arthritogenic aggrecan peptide at a final concentration of 25 μg/ml for 16 h at 37°C in 5% CO₂. Citrullinated and non-citrullinated aggrecan peptide were synthesized using a commercially available synthesis service (Chem Progress, San Vittore Olona, Milan, Italy) with the arginine/citrulline substitution underlined in the following sequence VVLLVATEGR/Cit VRVNSAYQDK; the citrullination was verified by mass spectrometry (Chem Progress, San Vittore Olona, Milan, Italy). Flow cytometry analysis was performed using a FACSCalibur (BD Biosciences, San José, CA, USA). At least 50,000 events, gated on the lymphocyte region, were acquired for each sample.

Statistical analysis
Analyses were performed using GraphPad Prism software (version 5.0; GraphPad Software). Multiple groups were analyzed using one-way analysis of variance and pairwise comparisons using the Mann–Whitney U test, with Bonferroni’s correction to adjust significance levels for multiple comparisons where appropriate. Correlation analyses were performed by using the Spearman’s rank correlation coefficient. P < 0.05 was considered significant.

Results
IL-9, IL-4, TGF-β, TSLP and Th9 expression in RA synovial samples
Synovial tissues from RA patients were first analyzed for the topographical organization of the inflammatory infiltrates. Fifteen RA patients (41.6%) displayed diffuse infiltrates of inflammatory cells without aggregates of specific microstructures. T-B cell aggregates without GC organization were observed in 10 patients (27.7%), whereas aggregation of inflammatory cells in secondary follicles with GC formation was observed in 11 patients (30.5%). We next evaluated the synovial expression of IL-9, IL-9R, PU.1, IFNγ, IL-21 and IL-27 in patients with RA and OA. A significant upregulation of IL-9, IL-9R and...
PU.1 m-RNA was observed in the synovium of RA patients compared with OA patients (Fig. 1A–C), IL-9 expression being correlated with the level of inflammatory infiltrate organization (Fig. 1D). IFN-γ and IL-27, but not IL-21, were also significantly upregulated in RA synovia (~4- and ~6-fold increase, respectively), but their levels were not correlated with IL-9 (data not shown). IL-9 was detected by immunohistochemistry in all the RA biopsies, mainly among synovial fibroblasts and infiltrating mononuclear cells (Fig. 1E–G). Conversely, weak IL-9 expression was observed in OA patients (Fig. 1H). In RA, IL-9 expression was directly correlated with the degree of inflammatory infiltrate and lymphoid organization (Fig. 1I–O). IL-9-expressing cells were confirmed to be mainly Th9.
cells by confocal microscopy analysis, which demonstrates the predominant CD3 (Fig. 2A–C), but not CD19/CD68 (data not shown), expression of IL-9+ cells and the predominant co-expression of IL-9 with PU.1 (Fig. 2G–I). A weak degree of co-localization was, however, observed between IL-9 and IL-17 (Fig. 2D–F), suggesting that Th17 cells produce IL-9 in RA. No Th9 cells were detected in OA synovial samples (Fig. 2J–L). IL-9R protein expression was confirmed in all RA synovial tissues (Fig. 3). Positive cells showed a diffuse distribution in the sublining, with few positive cells detectable in the proximity of lymphoid aggregates (Fig. 3D–I). Since Th9 polarization has been demonstrated to be dependent on IL-4, TGF-β and TSLP [2, 4], we next evaluated their expression in synovial tissues from patients and controls. A significant upregulation of IL-4 (Fig. 4A–D), TGF-β (Fig. 4E–H) and TSLP (Fig. 4I–L) was demonstrated in RA synovial tissues compared with OA synovial tissues, at both m-RNA and protein level, and the number of IL-4/TGF-β/TSLP-positive cells was directly correlated with the number of IL-9-positive cells (Fig. 5). Strong IL-4 staining was observed in the infiltrating mononuclear cells of RA patients (Fig. 4B), but not in OA synovial tissues (Fig. 4C). Strong TGF-β expression was found in both the infiltrating mononuclear cells and the synovial layer of RA patients (Fig. 4F) but not in OA synovial tissues (Fig. 4G). TSLP expression was observed mainly in RA in the context of the synovial layer (Fig. 4J) and endothelial cells (Fig. 4J insert); some infiltrating inflammatory mononuclear cells were also TSLP positive. In control synovial samples, TSLP expression was observed only among endothelial cells (Fig. 4K).

Peripheral blood Th9 detection and in vitro expansion with aggrecan citrullinated peptide

We next evaluated the frequency of IL-9-producing cells in RA patients and controls. In order to characterize the phenotype of circulating IL-9-positive cells, the co-expression of IL-9/IL-17 (Fig. 6A and B), IL-9/PU-1 (Fig. 6C and D) and IL-9/FoxP3 (Fig. 6E and F) was first evaluated. In RA, the majority of IL-9-producing cells did not co-express IL-17 or FoxP3 (Fig. 6A, B, E, F and H); a high percentage of these cells PU.1 positive (Fig. 6C, D and H). Th9 cells were significantly increased among PBMCs isolated from established RA [0.5 (s.d. 0.08)] and early RA [0.3 (s.d. 0.05)] patients compared with controls [0.017 (s.d. 0.006)] (Fig. 6G). PBMCs from RA patients and controls were also stimulated with the citrullinated aggrecan peptide, with the non-citrullinated aggrecan peptide as control. In RA patients (Fig. 6I and J), but not in controls (data not shown), after stimulation with the citrullinated aggrecan peptide we observed a strong and significant expansion of Th9 and Th17 cells. The response to citrullinated aggrecan (cit-aggrecan) was significantly correlated with the presence and titre of ACPA (r² = 0.45, P < 0.05) and was more intense in patients displaying synovial GCs (data not shown).

Discussion

In the present study we demonstrated, for the first time, that IL-9 and Th9 cells are overexpressed in RA synovial tissue and correlate with the degree of histological organization of B and T cells in ectopic lymphoid structures. Peripheral blood Th9 cells were found to be increased in RA, and expanded after in vitro exposure to a citrullinated peptide.

Although initially characterized as Th2 cell derived, a new IL-9-producing Th cell subset, namely Th9 cells, has been demonstrated [2]. Th9 effector cells are distinct from Th2 cells and can be differentiated in vitro via TCR stimulation in the presence of TGF-β and IL-4, or directly in the presence of TSLP [2, 4]. Given the pleiotropic functions of IL-9, Th9 cells might be involved in several types of inflammatory diseases. IL-9 has recently been found to be increased before the clinical onset of articular disease, and is associated with the presence of RA-related autoantibodies and circulating biomarkers of inflammation [9]. These results and our findings suggest that IL-9 and Th9 cells may make an important contribution to adaptive immune responses occurring in the inflamed joints of RA patients.

In our study, a significant expansion of Th9 cells was observed in the circulation of patients with established and early RA, independently of disease duration and treatment. IL-9 overexpression was also demonstrated in the synovial samples of patients with established RA, mainly expressed by cells of the synovial lining layer and by infiltrating CD4+ T cells expressing the Th9-specific factor PU.1 (identified as Th9 cells). The development of Th9 cells requires a balance of signals from cytokines that would otherwise generate distinct Th subsets, such as IL-4 and TGF-β [2], and epithelial cytokines such as TSLP [4], which results in the induction of PU.1. IL-9 overexpression in RA was accompanied by a significant increased expression of IL-4, mainly among infiltrating mononuclear cells—indicating an involvement of adaptive immunity in regulating Th9 development, and highlighting the complexity of immune system dysregulation in RA. In this study, we also showed that TSLP is strongly upregulated in the synovial layer of RA tissues and is directly correlated with the IL-9 expression. TSLP has been demonstrated to belong to the innate branch of immunity and to drive, also independently of canonical cytokines, the development of Th9 cells. In relation to this, TSLP levels have been found to be significantly increased in SF of RA patients and capable of stimulating TSLP receptor-expressing CD11c+ myeloid dendritic cells to secrete chemokines, causing influx and subsequent activation of CD4+ T cells [7].

The strong connection between IL-9 and autoimmunity has also recently been highlighted by the demonstration of its potential role in T-cell-dependent B-cell differentiation, expansion and antibody production [3]. The correlation between IL-9 and lymphoid organization that we observed in the present study further confirms, in our opinion, the importance of IL-9 and IL-9-producing cells in the induction of autoimmunity in RA.
regard, our demonstration of a strong expression of IL-9R (m-RNA and protein) in the synovium of RA patients, especially in those synovial samples displaying the presence of GCs, seems to be relevant. IL-9R has been demonstrated by Fawaz et al. [3] to be expressed in GC B cells, and its expression seems to be functional, because stimulation with IL-9 results in upregulation of phosphorylation of the transcription factors signal transducer and activator of transcription (STAT)3 and STAT5 and the ability of IL-9 to potentiate IgE production.

As for other human autoimmune diseases, RA susceptibility and severity are linked to multiple genes [15], but the strongest genetic association exists with the HLA-DRB1 genes, and in particular the HLA-DR4 alleles. There is a clear association between these alleles and peptide citrullination, as recently demonstrated by Scally et al. [16]. Citrullinated arthritogenic aggrecan peptide has been considered to be a biomarker of RA, having been identified in the peripheral blood of RA patients and shown to stimulate a specific CD4 response. It is considered a candidate autoantigen for RA, being one of the principle proteoglycans for cartilage extracellular matrix functioning in cushioning synovial joints, self-antigens that share a common sequence at position 70–74 of the HLA-DR1 chain in 90% of RA patients. Our demonstration that stimulation with the arthritogenic aggrecan peptide results, in RA patients, in a strong and significant expansion of Th9 cells might suggest the existence of an association between citrullination and autoreactive Th9 cells. In conclusion, in this study we provide data on a potential role for IL-9 and IL-9-producing cells in the pathogenesis of RA. More studies are, however, required to better elucidate the functional and pathogenic role of IL-9 and Th9 cells in RA.

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