Interleukin-16 expression in The Immunopathogenesis of Rheumatoid Arthritis

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Abstract: Rheumatoid Arthritis is a chronic inflammatory disease characterized by hyperplasia of the synovium and excessive cellular infiltration, which leads to progressive joint destruction. We analyzed, interleukin 16 (IL16), in relation to disease activity to characterize its biologic function in RA. Secreted IL-16 was measured by enzyme immunoassay in sera from 30 RA patients and 30 healthy controls. And also, in synovial fluid (SF) from 16 RA patients and 15 patients with non- RA synovitis as controls. IL-16 expression in peripheral mononuclear cells (PBMC) was characterized by flow cytometric analysis after intracellular cytokine staining for IL-16. In synovial tissue specimens, both were done:- immunohistochemistry for localization of IL-16, and histopathology, in which the tissue scored semiquantitatively for synovial hyperplasia and cellular infiltration. IL-16 was detected at significantly higher levels in sera and sf of RA patients in comparison to HC and non-RA synovitis ($P < 0.001$ and $P < 0.0001$ respectively). Also, IL-16 was detected significantly higher in SF in comparison to sera in RA patients ($P < 0.001$). Flow cytometry of PBMC showed that a great proportion of both CD4+ and CD8+ cells expressed IL-16 protein. Also, immunohistochemistry revealed more CD4+ and less frequency of CD8+ cells in synovial infiltration. A significant correlation between IL-16 expression and local inflammatory activity could not be established ($P > 0.21$) by microscopic analysis of the synovial cells infiltrate. In addition, no significant association was observed between serum, sf, and synovial tissue expression of IL -16 and clinical disease activity in RA ($P > 0.61$, $P > 0.51$, and $P > 0.42$ respectively). This indicated that, IL-16 played a regulatory rather than a proinflammatory role in the immunopathogenesis of RA.

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1. Introduction:

Rheumatoid Arthritis (RA) is an autoimmune disease characterized by chronic inflammation of synovium, which leads to polyarticular destruction.

In RA synovium, various types of immune competent cells including CD4 + T cells, Macrophage derived synovial cells, and fibroblast – like synovial cells accumulate and produce various inflammatory mediators such as cytokines, chemokines and metalloproteinases that contribute to the development of synovitis and resorption of bone and cartilage, thus destroying joints ability to function (Panayi, *et al.,* 2002).

Interleukin 16 (IL-16) is a T cell derived cytokine originally identified and purified as lymphocyte chemoattractant factor from concanavalin A stimulated human peripheral blood mononuclear cells (PBMC) (Cruikshank, 2001). CD8 + T cells synthesize and store the bioactive protein (Center *et al.,* 2002). While CD4 + T cells store the precursor molecule (pro-IL-16) (Hermann *et al.,* 2003). IL-16 acts via the CD4 molecule as its receptor (Franz *et al.,* 2001).

IL-16 exerts a broad spectrum of both pro- and anti-inflammatory biologic activities, these functions include the selective chemotactic and competence growth factor activity for CD4 + T cells, stimulation of IL-2 receptor (CD 25) and MCH Class II expression on resting T- cells (Parada *et al.,* 2002) and activation of tyrosine kinase P56 (Ryan *et al.,* 2002).

However, IL-16 has been shown to suppress CD3/TCR mediated T-Cell activation in mixed lymphocyte reactions (transient induction of T cell anergy) (Theodor, et al., *et al.,* 2001), and to inhibit mitogen induced IL- 2 production by CD4 + T cells (Ogasawara *et al.,* 1999).

The aim of this work was to analyze the expression of IL-16 in relation to disease activity, to characterize its biologic function in RA.

2. Material and Methods

The study was carried out on the patients attending to Rheumatology, Orthopedic and Internal Medicine Departments, in El-Hussein Hospital, Al-

Azhar University in the period from October 2001 to December 2003.

Sources of Samples:

Heparinized peripheral blood samples (15 ml) and sera (1 ml) were obtained from 30 patients with RA and 30 healthy controls (HC). Healthy controls were chosen for lack of any clinical symptoms or signs of inflammation, normal values of C-reactive protein (CRP), a normal blood count, normal erythrocytic sedimentation rate (ESR), and both ages and sexes matched with the study group, All patients with RA fulfilled the American Rheumatism Association 1987 revised criteria (Arnett *et al.,* 1988).

Most of them were receiving disease modifying drugs. Disease activity was evaluated by clinical and laboratory findings described by Van, *et al.*(1990). The clinical judgment of disease activity in RA was based upon the Ritchie score index, number of swollen joints and ESR. The disease activity score (DAS) was determined according to the following equation:-

DAS= 0.53938 x √Ritchie score + 0. 06465 x (no. of swollen joints) + 0. 330 x ESR +0. 224 (Van *et al.,* 1990).

Patients Characteristics are:- Table (1)

Table (1): Base line Characteristics of Patients with RA

Patients	Range	Mean
Sex (7 males : 23 Females)		
Age in years	37 – 79	56. 3
Rheumatoid Factor (RF) IgM = 64.2% Positive		
DAS	1. 92 – 5. 79	3.82
No of swollen Joints	3 – 11	4
ESR (mm/h)	9 – 84	27. 3
CRP (mg/ l)	6. 2 –186. 2	21. 9
Disease duration / year	1 – 12	4

The patients on the following drugs therapy according to their disease condition:-

Eighteen receiving nonsteroidal anti-inflammatory drugs, 22 receiving corticosteroids and 10 receiving methotrexate therapy.

In addition, heparinized synovial fluids (SF) specimen were collected from 16 patients with RA and 15 patients with non- RA synovitis (5 osteo arthritis (OA), 6 reactive artyhritis, 3 psoriatic arthritis, and 1 ankylosing spondylitis) were investigated as controls with age, sex and disease duration matched with RA patients. Also, synovial tissue samples were obtained at the time of surgery from 20 patients with RA and 10 patients with OA under going synovectomy or orthoplasty.

Synovial tissue samples were embedded in paraffain for immunohistochemical analysis.

Immunoassay for IL-16 concentrations in serum and SF specimens were measured by a solid phase sandwich enzyme linked immunosorbant assay (ELISA) (Biosource. Ratingen, Germany) according to manufacturer's instructions as described by Sabine ***et al.*** (2001).

Flow Cytometric Analysis of PBMC:- PBMC were isolated from heparinized blood samples by Ficoll – hypaque density centrifugation (Biochrom, Berlin, Germany). Cells were then characterized by Flow Cytometric analysis after intracellular cytokine staining as described by Chupp *et al.,* 1998, as following: Isolated PBMC were resuspended at a density of 2 x 106 cells / ml in PBS/ 0.1% bovine serum albumin (BSA) and stained for cell surface antigens with fluorochrome labled antibodies. Anti-CD4, anti-CD8, anti-CD3 antibodies and isotype control (IgG1/ IgG2a) were obtained from Becton - Dickinson (Heidelberg, Germany).

Cells are fixed in 4% paraformaldhyde for 15 min. at 4 ºC, washed twice in PBS/ 0.1% BSA, and permeabilized for 1 hour at 4 ºC with 0.1 saponin (w/v) solution (Sigma, Deisenhofen, Germany) containing 5% non fat dry milk to block non specific binding. For intracellular cytokine staining PBMC were incubated with 0. 25 ug of an R-phycoerthrin (PE) conjugated mouse anti-human IL-16 antibody (PharmMingen, Hamburg, Germany) for 30 min at 4 ºC, washed again and resuspended in PBS for Flow Cytometric analysis. To reveal specificity of staining target cells were incubated with an isotype matched immunoglobulin of irrelevant specificity at the same concentration (PE conjugated IgG1 iso type control, PharMingen). List mode data were acquired on a FACSCalibur (Becton – Dickinson) flow cytometer. Dead cells and monocytes were excluded by forward and side scatter gating. Typically, 10,000 events were acquired and analyzed using the CellQuest software. Bivariate dot plots were generated upon data reanalysis to display the frequencies of individual cells coexpressing certain levels of cell surface antigens and intracellular IL-16 (Sabine ***et al.,*** 2001).

Immunohistochemistry: Serial sections of synovial tissue samples were stained for cell surface markers CD4, CD8, and CD68 by an indirect alkaline phosphtase technique (Tak, et al., 1994). The following primary mouse Mab were used: anti-CD4 (Clone IF6, dilution 1: 20; Novocastra, Dossenheim, Germany), anti-CD8 (Clone C8/144B, dilution 1: 20; Dako, Hamburg, Germany), and anti-CD68 (Clone PG-M1, dilution 1:50; Dako). Secondary antibodies (alkaline phosphatase conjugated rabbit anti-mouse antibodies) and substrate (Naphthol-AS-MX-phosphate/fast red) were obtained from Sigma. Slides were counterstained with Mayer's hematoxylin (Merk,

Darmastadt, Germany) and subjected to microscopic analysis.

Immunohistochemical detection of IL-16 expression in synovial tissues was performed with a mouse anti-human IL- 16 IgG1 antibody (hybridoma supernatant, kidney provided by W. Schlusener, Tubingen. Germany) as described by Blaschke ***et al.*** (1999). As following, paraffin sections were pretreated by microwave and endogenous peroxidase activity was blocked with 3% hydrogen peroxide. After blocking with normal rabbit serum (Dako), sections were incubated at 4C overnight with anti- IL-16 IgG1 antibodies. An iso type control serum was always included as negative control. Slides were washed 3 times in PBS and a biotinylated rabbit anti-mouse IgG polyclonal antibody (Dako) was applied. After incubation for 30 min at room temperature. Slides were washed again and the strepavidin-peroxidase complex (StreptAB complex, HRP;Dako) was added for 10 min at room temperature. Peroxidase activity was then visualized by addition of 3 amino 9 ethylcarbazole substrate solution.

Histopathology: Microscopic analysis was done as described by Tak *et al.* (1994). Hematoxylin and eosin stained sections were done. Tissues were scored semiquantitatively for synovial lining hyperplasia (Score 0-3. 0= 1-2, 1= 3-4, 2= 5-6, 3 > 7 cell layers) and the degree of infiltration with lymphocytes and polymorphonuclear granulocytes (PMN) using a 5 point scale: 0= 0 cells, 1 = 1 – 5 cells, 2 = 5 – 25 cells, 3 = 25 – 50 cells, 4 = > 50 cells per high power field (HPF, magnification 250 x). The inflammatory score was determined by the sum of the scores for the synovial lining hyperplasia and the infiltration with lymphocytes and PMN. Similarly, the presence of CD4, CD8, CD86, and IL-16 positive cells was scored on a 5 point scale. At least 5 separate areas were examined for each biopsy specimen to minimize sampling errors. Results represent the mean of all area scores.

Statistical analysis: Data were expressed as the mean ±SEM. Samples with values below the detection limit of the assay were regarded as negative. Statistical differences between groups were evaluated by Student's test. *P* values were determined by 2 sided calculation. Correlations between IL-16 levels and the disease activity score were quantified applying Spearman's rank correlation test.

3. Results

IL-16 levels were measured by ELISA in sera from 30 patients with RA. The results were compared with IL-16 concentrations in sera from 30 C. As illustrated in table (2), IL-16 was detected at significantly higher levels in RA sera [range (105.3-957.4 pg/ml) and mean (612.1±42.6pg/ml)] than HC sera [range (0.00-229.8 pg/ml) and mean (116.5±9.6pg/ml)] with $P<0.001$. Also, IL-16 levels were analyzed by ELISA in sf of 16 patients with RA and 15 patients with non-RA synovitis as control group. Elevated IL-16 concentrations were found in SF specimens of RA patients [range (2.1-24.6 ng/ml) and mean (13.4±1.3 ng/ml) more than non-RA synovitis [range (0.00-4.9 ng/ml) and mean (1.1±0.4 ng/ml)] with statistically high significant $p < 0.0001$ [Table (1).

Analysis of IL-16 serum and sf levels in relation to disease activity scores for RA patients revealed no significant correlation ($P > 0.61$ and $P > 0.51$ respectively).

IL-16 levels were found at higher concentrations within the SF specimens in comparison to mean serum levels in RA patients with Statistically Significant $P <0.001$ (Table 2).

IL-16 expression in PBMC from 30 RA patients and 30 HC was analyzed by flow cytometric analysis after intracellular staining with a fluorochrome conjugated anti-IL-16 antibody. To characterize the immunophenotype of IL-16 positive cells, PBMC were also stained for CD4 and CD8 surface antigens. Frequencies of IL-16 positive PBMC were obtained by gating on CD4 and CD8 cells, respectively. Two color flow cytometry for CD4 or CD8 versus IL-16 of unstained PBMC from RA patients revealed that a great proportion of both CD4 (69.3±3.1%) and CD8 cells (81.3 ± 2.6%) expressed IL-16 protein, compared with HC samples of PBMC which showed, CD4 (61.9±1.4%) and CD8 cells (75.1 ± 3.1%) expressed IL-16 protein and the differences between RA patients and HC statistically insignificant ($P >0.54$ and $P > 0.51$ respectively).

These data indicated that the majority of resting CD4 and CD8 T cells express IL-16 in both patients and control groups. On the other hand, no significant correlation between % IL-16 positive CD4 and CD8 T cells and DAS in RA patients ($\boldsymbol{P} > 0.69$).

Immunohistochemistry of synovial tissue sections with a polyclonal anti- IL-16 antibody. IL-16 positive cells were detected at high frequency within the inflamed rheumatoid synovium, where as rare immunoreactive cells stained positive for IL-16 in the control group. To determine which cell type are predominantly responsible for synovial IL-16 production, serial synovial tissue section were analyzed by immunohistochemical staining for CD4 and CD8 surface antigens. Results indicated that a great proportion of infiltrating CD4, but also CD8 T-lymphocytes present in less frequencies within the synovial lining layer.

IL-16 expression within the synovial cell infiltrate in relation to disease activity. Synovial tissue samples from RA patients were semiquantitatively

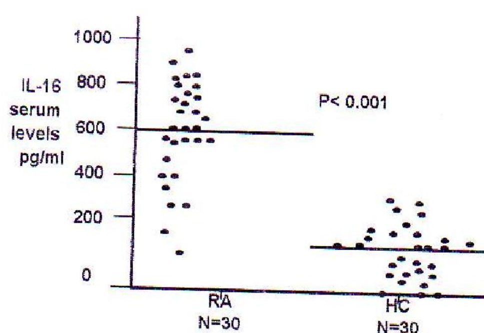
scored for histopathology and immunohistologic features by microscopic analysis. Results were compared between 2 patients subgroups with low (DAS ≤ 2.5) and high disease activity (DAS ≥ 3.5), to analyze the potential dependence of IL-16 expression on scores for local inflammatory activity and clinical disease activity (Table 3). Histopathology analysis revealed a differences between inflammatory scores: local inflammatory activity tended to be higher in patients with high DAS, but this difference did not reach statistical significance (***P*** > 0.39) in comparison with low DAS RA patients.

And, also, immunohistologic scores for surface antigen CD4 and CD8 showed strong variations between patients: semiquantative assessment of CD4 and CD8 positive cell revealed more ore less similar scores for synovial tissues of RA patients with low and high disease activity and the differences statistically insignificant (***P*** > 0.51, ***P*** > 0. 38 respectively).

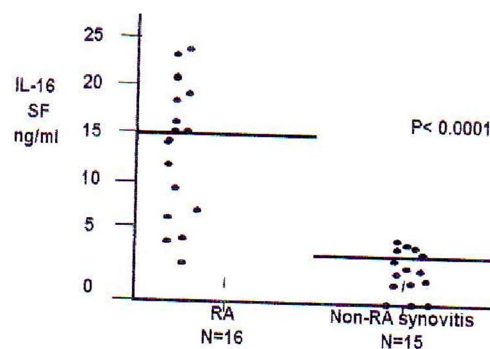
Also, there was no statistical significance between IL-16 expression and clinical disease activity (***P*** > 0. 42). On the other hand, there was statistically significant difference between the 2 patients subgroubs only for the immunohistologic scores of CD68 expression (***P*** < 0. 001). No significant correlation was found between local inflammatory activity and IL-16 expression (***P*** > 0. 21).

Table (2) serum and SF levels of IL-16 in patients with R.A. and their control groups

IL-16	Range	Mean	*P*-value
Serum (N=30)	105.3 – 957.4	612.1±42.6	< 0.001
			< 0.001
S.F. (N= 16)	2.1 – 24. 6	13.4 ± 1. 3	< 0.0001
Controls:			
·HC (N=30)	0.00-229.8	116.5±9.6	
·Non-RA synovitis (N=15)	0.00-4.9	1.1±0.4	



Fig(1): Comparison between Il-16 in sera from RA patients and HC



Fig(2): Il-16 levels in SF from RA patients and non-RA synovitis

Table 3: Histopathologic Immunohistologic Features of synovial tissues from RA patients with low (DAS ≤ 2.5) and high (DAS ≥ 3.5) disease activity.

	Disease activity		
	DAS < 2.5 N = 13 Mean	DAS < 3. 5 N = 17 Mean	***P***
Inflammatory Score	6. 12 ± 0. 23	7. 42 ± 0. 51	> 0. 39
CD4	1. 98 ± 0. 17	2. 51 ± 0. 32	> 0. 51
CD8	1. 56 ± 0. 08	1. 49 ± 0. 21	> 0. 38
CD68	1. 41 ± 0. 17	2. 51 ± 0. 31	< 0. 001
IL-16	1. 84 ± 0. 16	1. 99 ± 0. 34	> 0. 42

4. Discussion

Genetic, clinical and biological heterogeneity of RA are reported by Piotr *et al.* (2001). Also, the inflammatory reactions in the joints are regulated and controlled by T-cells. The cytokines produced by these cells may enhance or suppress the inflammatory process (Yocum, 2003). In the present study, we investigated the relationship between expression of IL-16 a novel T-cell derived cytokine, and disease activity in RA to further characterize its biologic function during the immunopathogenesis of RA.

In our study, analysis of serum IL-16 concentrations revealed significantly higher IL-16 levels in sera from RA patients in comparison to HC ($P < 0.001$). These results in agreement with the results reported by Sabine ***et al.*** (2002). However, in contrast to results obtained by Franchim ***et al.*** (2001). They found that the concentration of T-cells derived cytokines included IL-16 in peripheral blood of RA patients is low, They explained their results that the patients in the initial stages of RA exhibited a reduced percentage of stimulated T-cells in peripheral blood (Mangge, ***et al.,*** 2001).

In this study, SF IL-16 levels were statistically significant in a higher concentration in RA patients compared with non-RA synovitis ($p < 0.0001$).

These results in agreement with the results obtained by Cruikshank ***et al.*** (2002). According to these results, IL-16 is certainly not specific for RA, since preliminary analysis of non-RA synovitis yielded presence of IL-16 in their SF.

In the present study, IL-16 levels were significantly elevated in SF in comparison to peripheral blood in RA patients ($p < 0.001$). Our results in agreement with the results reported by Franz ***et al.*** (2001). An explanation for our finding may be that the majority of RA patients were under immunosuppressive treatment with corticosteroids and methotrexate and these drugs may exert an inhibitory effect on T-cell function and thus alter IL-16 secretion (Barrera ***et al.,*** 2002). Another explanation is the selective migration of theCD4+cells into the joints from peripheral blood, where they play the crucial role in Rheumatoid synovitis (Van Room ***et al.,*** 2003).

Since IL-16 represent a T- cell derived cytokine, expression of IL-16 in RA was next characterized in PBMC by flow cytometric analysis after intracellular cytokine staining. Results revealed that IL-16 is produced by a great proportion of both CD4+ and CD8+ T lymphocytes. These results in agreement with the results reported by Sabine ***et al.*** (2001).The production of IL-16 by T cells can be explained by: Resting CD8 T cell lymphocytes express active caspase-3 for cleavage of pro-IL-16 into its bioactive 13 KDa form(Zhang ***et al.,*** 1998). Whereas CD4 T cells require antigenic stimulation for processing of pro-caspase-3 into its 20 KDa active form and subsequent release of bioactive IL-16, this occurred in RA and leaded to elevation of IL-16 serum level in RA patient than HC group (Wu Dm ***et al.,*** 1999).

Immunohistochemical for cell surface antigens on serial synovial tissue sections clarified that, IL-16 producing cells were identified within the lining layer of synovium and within the inflammatory infiltrates of the sublining layer in a great proportion of CD4+ T cells, but also in less frequencies of CD8+ T cells. These findings correspond well with the results obtained by Franz, ***et al.***(2001). The functions of CD4+ cell are chemoattractant, competence growth factor, and local production of IL-16 by infiltrating T lymphocytes within the rheumatoid synovium which considered to play a critical role in the immunopathogenesis of RA (Cruikshank ***et al.,*** 1998).

Histopathological study showed, local inflammatory activity tended to be higher in RA patients with high DAS, but this statistically non-significant ($P > 0.39$) in comparison with low DAS patients. Different Results have been reported for the relationship between histopathological changes and clinical disease activity:- A close association between histopathologic feature of rheumatoid synovial tissue and disease activity with statistical significant ($P < 0.001$) has been reported by Waxman and Sledge (2003), but a significant reduction of synovial inflammation without clinical improvement has also been described by Tak ***et al.*** (1999). On another studies have shown a close association between the degree of synovial tissue macrophage infiltration, the expression of monokines (IL-16, TNF), and local disease activity (Tak ***et al.,*** 1997). These finding support that, synovial tissue macrophages and monocyte derived cytokines play a central role in the immunopathogenesis of chronic inflammation and joint erosion in RA (Sabine ***et al.,*** 2001).

The failure to prove a significant relationship between IL-16 expression and both clinical and local inflammatory activity in RA patients. This explained by IL-16 production might be controlled by local regulatory mechanisms or that IL-16 itself might exert additional regulatory function in chronic synovial inflammation (Hermann ***et al.,*** 2003). Also, IL-16 mediates pleiotropic functions: chemoattration of CD4+ cells (T cells, eosinophils, monocytes), activation of resting CD4+ T cells as indicated by regulation of HLA-DR antigens and IL- 2R expression, anti-inflammatory function of IL-16 as reflected by transient induction of CD4+ T cell anergy, inhibition of TCR/CD3 mediated T cell

stimulation in mixed lymphocyte reactions, and suppression of mitogen induced IL-2 production [(Ogasawara ***et al.*** (1999) and Ryan ***et al.*** (2001)]. In addition, IL-16 exerted a regulatory function in chronic synovial inflammation by down modulating further T cell activation. Negative feedback regulation of T cell activation in RA could explain impaired synovial T cell proliferation and low level expression of other T cell derived cytokines [Feldman ***et al.,*** 2003].

In conclusion, IL-16 is detected in abundance concentration in SF of RA patients. The lack of correlation between IL-16 expression, local inflammatory activity, and clinical disease activity indicates that IL-16 has a regulatory role rather than a proinflammatory role in the immunopathogenesis of RA.

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