

Expression of soluble human signaling lymphocytic activation molecule in vivo

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Background: Signaling lymphocytic activation molecule (SLAM) is a novel glycoprotein expressed on activated T and B cells. Ligation of cell surface SLAM, either by anti-SLAM mAbs or the recombinant soluble form of SLAM (sSLAM), enhanced the proliferation of T and B cells in vitro. In addition, the engagement of SLAM on T cells preferentially induced IFN- γ production even by allergen-specific T_{H2} clones. **Objective:** In this study we investigated the expression of sSLAM in vivo in healthy individuals and in disease conditions that are associated with increased T_{H1}- or T_{H2}-cell responses.

Methods: The expression of mRNA encoding sSLAM in peripheral blood and synovial fluid (SF) lymphocytes was studied by using reverse transcriptase-PCR, and the presence of sSLAM protein in serum and SF samples was investigated by using a specific ELISA.

Results: Lymphocytes from patients with rheumatoid arthritis (RA) and healthy individuals consistently expressed mRNA encoding sSLAM. In addition, sSLAM protein was present in 38% of serum and 54% of SF samples from patients with RA and in 47% of serum samples from healthy individuals. The levels of sSLAM in positive serum and SF samples from patients with RA and in positive serum samples from healthy individuals were not significantly different. In contrast, the levels of sSLAM were significantly lower in patients with reactive arthritis or in patients with elevated IgE levels than in patients with RA. Similarly, the frequency of positive SF samples was significantly lower in reactive arthritis (28%) than in RA (54%).

Conclusion: These results indicate that sSLAM is present in serum and SF, further suggesting that sSLAM regulates T- and B-cell function in vivo. Moreover, these data suggest an association between low sSLAM production and the occurrence of T_{H2} responses in vivo. (*J Allergy Clin Immunol* 1999;103:114-8.)

Key words: Arthritis, allergy, lymphocyte

Signaling lymphocytic activation molecule (SLAM) is a novel 70-kd glycoprotein belonging to the CD2 family of

Abbreviations used

mSLAM:	Membrane-bound form of SLAM
RA:	Rheumatoid arthritis
ReA:	Reactive arthritis
SF:	Synovial fluid
SFMC:	Synovial fluid mononuclear cells
SLAM:	Signaling lymphocytic activation molecule
sSLAM:	Soluble form of SLAM

the Ig gene superfamily. It is constitutively expressed on peripheral blood CD45RO⁺ T cells, T-cell clones, immature thymocytes, and a proportion of peripheral blood B cells, and it is rapidly upregulated in T and B cells after activation.^{1,2} In addition to the membrane-bound form of SLAM (mSLAM), activated lymphocytes express mRNA encoding a soluble, secreted form of SLAM (sSLAM), which lacks 30 amino acids encompassing the entire 22 amino acid transmembrane region.^{1,2} An alternatively spliced form of mSLAM with a truncated cytoplasmic tail and an intracytoplasmic form of SLAM lacking the leader sequence have also been identified.

Recently, SLAM was shown to be a high-affinity self-ligand with an affinity constant of 0.1 nmol/L (Cocks et al, submitted). Engagement of SLAM by a specific mAb or recombinant sSLAM enhances antigen-specific T-cell proliferation and directly induces the proliferation of pre-activated T cells in a CD28-independent manner (Cocks et al, submitted).^{1,3} Interestingly, ligation of SLAM preferentially increases the production of IFN- γ by CD4⁺ T-cell clones, even by T_{H2} clones.^{1,3} In addition, treatment of T_{H2} cells derived from the skin of patients with atopic dermatitis with agonistic anti-SLAM mAbs redirects the differentiation of these cells to the T_{H0} or T_{H1} phenotype.⁴ Furthermore, recombinant sSLAM or L cells transfected with mSLAM induce proliferation and Ig production by activated B cells.²

We have previously shown that the expression of mSLAM is upregulated on synovial T cells from patients with rheumatoid arthritis (RA).⁵ In this study we investigated the expression of sSLAM in patients with RA, reactive arthritis (ReA), or atopy, as well as in healthy individuals. Our data indicate that sSLAM can be detected in vivo in biologic fluids. A proportion of both patients with RA and healthy individuals expressed sSLAM protein, and the median level of sSLAM in positive serum samples in these 2 groups was not significantly different. In contrast, the levels of sSLAM in samples from patients with ReA or atopy were significantly lower than those found in samples from patients with RA.

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TABLE I. Characteristics of the study groups and the expression of sSLAM*

Study groups	n	Female/male	Age, y (mean ± SD)	Disease duration (mean ± SD)	Samples positive for sSLAM		Median sSLAM level in positive samples (ng/mL)	
					Serum	SF	Serum	SF
RA								
All	82	53/29	59 ± 14	12 ± 10 y	31 (38%)	44 (54%)	35 (18-67)†	37 (22-83)
Duration >1 y	69	47/22	60 ± 13	14 ± 10 y	25 (36%)	36 (52%)	35 (19-56)	39 (28-100)
Duration <1 y	13	6/7	50 ± 19	8 ± 4 mo	6 (46%)	8 (62%)	36 (18-67)	28 (16-47)
ReA	29	6/23	34 ± 13	3 ± 3 wk	8 (28%)	8 (28%)‡	17 (15-30)§	14 (13-16)§
Atopy	69	43/26	37 ± 17	ND	31 (45%)	ND	20 (14-27)§	ND
Healthy individuals	70	40/30	24 ± 5	NA	33 (47%)	ND	28 (15-53)	ND

ND, Not done; NA, not applicable.

*Expression of sSLAM was studied with a specific ELISA.

†Values in parentheses indicate interquartile ranges.

‡P < .05 compared with all patients with RA by using chi-square test.

§P < .05 compared with all patients with RA by using Mann-Whitney U test.

METHODS

Patient samples

Serum and synovial fluid (SF) samples were concurrently collected from patients with RA or ReA. SF samples were obtained by needle aspiration from inflamed knee joints and placed into heparinized tubes. Serum samples from atopic patients and healthy volunteers were also studied. After centrifugation, serum samples and SF supernatants were stored at -20°C until examined by ELISA. PBMCs and SF mononuclear cells (SFMCs) were isolated by Ficoll-Hypaque density-gradient centrifugation. Synovial tissue was obtained during orthopedic surgery.

The characteristics of the patient groups are presented in Table I. RA was determined according to the criteria of the American College of Rheumatology.⁶ The mean (± SD) erythrocyte sedimentation rate was 53 ± 26 mm/h (n = 67), and the mean C-reactive protein level was 45 ± 32 mg/L (n = 74) at the time the samples were taken. The diagnosis of ReA was made on the basis of the clinical picture, positive serology, and/or stool culture. ReA was triggered by *Salmonella enteritidis* in 16 patients, by *Chlamydia trachomatis* in 4 patients, by *Yersinia enterocolitica* O:3 in 1 patient, and by an unknown agent in 8 patients. Seventeen of the patients with ReA were HLA-B27 positive, 5 were HLA-B27 negative, and 7 had an unknown HLA-B27 status. The samples were taken during a postinfectious arthritis phase, and all patients had active disease. Atopy was defined on the basis of the total IgE levels only, and the term "patients with elevated IgE levels" is used in addition to atopy. Adult patients demonstrating total IgE levels greater than 100 U/mL (330 ± 355 U/mL, mean ± SD) were included. This study was approved by the ethical committee of Turku University.

sSLAM ELISA

The levels of sSLAM in the serum and SF samples were determined by using a SLAM-specific sandwich ELISA. The wells of microtiter plates were coated with a mixture of 2 anti-SLAM mAbs (A12, 1B12; 2 µg/mL each) in carbonate buffer (pH 9.6) overnight at 4°C and washed 3 times with PBS and 0.05% Tween. The wells were then blocked with RPMI supplemented with 10% FCS for 1 hour at room temperature, after which 100 µL of samples and standards diluted in RPMI supplemented with 10% FCS were added and incubated for 3 hours at 37°C. After 3 washes with PBS-Tween, a secondary biotinylated anti-SLAM mAb (5G4, 2 µg/mL) diluted in PBS supplemented with 0.5% BSA and 0.05% Tween was added. The plates were then incubated overnight at 4°C and washed 3 times with PBS-Tween. Finally, the wells were incubated with peroxi-

dase-conjugated streptavidin in PBS-BSA-Tween for 1 hour at room temperature and developed with 2,2'-azino-bis (2-ethylbenzotriazoline-6-sulfonic acid) substrate at 37°C. The detection limit of the ELISA was 10 ng/mL. Duplicate determinations were performed for each sample.

To eliminate the possible influence of rheumatoid factors in sSLAM ELISA, 6 rheumatoid factor-positive serum samples from patients with RA were incubated with fixed *Staphylococcus aureus* cells with high surface levels of protein A (Pansorbin cells; Calbiochem, La Jolla, Calif) for 1 hour at 4°C. Thereafter the samples were centrifuged at 10,000 rpm for 5 minutes, and supernatants were collected. This treatment was performed twice and removed greater than 80% of total Ig from the samples when measured by ELISA, although it did not significantly affect the levels of sSLAM (data not shown).

mRNA isolation and reverse transcriptase-PCR analysis

Total RNA was extracted as described by Chirgwin et al.⁷ Briefly, cells were homogenized in 4 mol/L guanidium isothiocyanate, and RNA was purified by using ultracentrifugation through a cesium chloride cushion. Two micrograms of total RNA was reverse transcribed with Superscript reverse transcriptase (Gibco BRL, Gaithersburg, Md) and oligo(dT)₁₂₋₁₈ as primer (Gibco BRL). An aliquot of the resulting cDNA was amplified with 1 U of AmpliTaq DNA polymerase (Perkin Elmer, Norwalk, Conn) in a Perkin-Elmer-Cetus DNA Thermal Cycler 9600 for 40 cycles (denaturation for 30 seconds at 94°C, annealing for 30 seconds at 60°C, and extension for 60 seconds at 72°C). Twenty microliters of each PCR product was separated by 1.2% agarose gel electrophoresis and visualized as UV fluorescence after staining with ethidium bromide. The following primers were used: 5'-ATCACTGGAGAACAGTGT-3' and 5'-TTCGTTTTACCTGAGGGGTCTG-3' specific for sSLAM¹; 5'-ATCACTGGAGAACAGTGT-3' and 5'-CCCAGCAT-ACACTGCCC-3' specific for mSLAM and the intracytoplasmic form of SLAM¹; and 5'-CTGGCGTCGTGATTAGTGATGATGA-3' and 5'-CTTAGGCTTTGTATTTTGGCTTTTCC-3' specific for hypoxanthine-guanine phosphoribosyltransferase.

Statistical analysis

The data are expressed as medians with interquartile ranges because the levels of sSLAM in positive samples were not normally distributed. Kruskal-Wallis ANOVA was performed to indicate that sSLAM levels in positive samples were significantly different

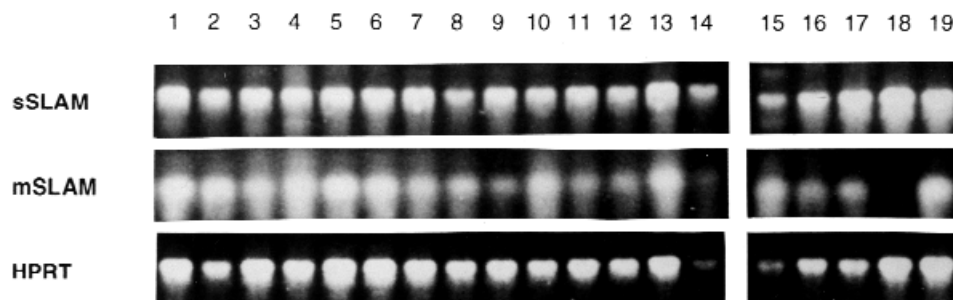


FIG 1. The expression of mRNA encoding sSLAM and mSLAM in SFMCs (samples 1, 3, 5, 7, and 9) and PBMCs (samples 2, 4, 6, 8, and 10) from 5 patients with RA, in PBMCs from 4 healthy individuals (samples 11 to 14), and in 5 synovial tissue samples (samples 15 to 19). Total RNA was isolated and subjected to reverse transcription. cDNA was amplified by using primers specific for sSLAM, mSLAM, and hypoxanthine-guanine phosphoribosyl-transferase (*HPRT*) as described in the Methods section. The amplified cDNAs were detected by using staining with ethidium bromide.

between the study groups ($P < .001$). The comparison of sSLAM levels between 2 individual groups was performed with the Mann-Whitney U test. The chi-square test was used to compare the frequencies of positive samples. Correlations were calculated with Spearman's rank correlation method. Probability (P) values less than .05 were considered statistically significant. In all statistical analysis, patients with RA were analyzed as one group and were not divided into 2 groups on the basis of disease duration.

RESULTS

Lymphocytes from patients with RA and healthy individuals consistently express sSLAM

To study the production of sSLAM *in vivo*, we first investigated the expression of mRNA encoding sSLAM and mSLAM in 8 PBMC and SFMC samples (5 paired samples) from patients with RA and in 4 PBMC samples from healthy individuals by using reverse transcriptase-PCR. Synovial tissue samples from 5 patients with RA were also studied. mRNA encoding sSLAM was present in all PBMC, SFMC, and synovial tissue samples studied (Fig 1 and data not shown). Expression of mSLAM could be detected in all peripheral blood and SF samples and in 4 of 5 synovial tissue samples. No major differences in the expression levels of sSLAM or mSLAM between PBMCs, SFMCs, or synovial tissue samples were observed (Fig 1). These data suggest that sSLAM is consistently expressed by PBMCs and SFMCs from patients with RA and healthy individuals and indicates that sSLAM is not produced by cleavage of mSLAM.

sSLAM protein is present in serum and SF samples from patients with RA and healthy individuals

Because mRNA encoding sSLAM was readily detectable in PBMCs and SFMCs, we studied the presence of sSLAM protein in serum and SF samples from patients with RA and in serum samples from healthy individuals by using a SLAM-specific ELISA. As shown in Table I, sSLAM could be detected in 31 (38%) serum

and in 44 (54%) SF samples from patients with RA. The median level of sSLAM in positive serum and SF samples was comparable (Table I, Fig 2). The levels of sSLAM in SF samples from patients with short disease duration (<1 year) tended to be lower, but this difference did not reach statistical significance. The levels of sSLAM in serum or SF samples did not correlate with age, disease duration, or the erythrocyte sedimentation rate or C-reactive protein level (data not shown).

A significant proportion (47%) of serum samples from healthy individuals was also positive for sSLAM, and the levels of sSLAM in positive serum samples were not significantly different in healthy individuals and in patients with RA.

Low levels of sSLAM are detected in patients with ReA or atopy

Engagement of SLAM has been shown to regulate the cytokine production profile of T_H cells.¹ Therefore we also studied the levels of sSLAM in patients with ReA and in patients with elevated IgE levels because, in contrast to RA, these conditions are associated with increased frequency of T_{H2} cells.⁸⁻¹¹ As shown in Table I and Fig 2, sSLAM protein could be detected in some serum and SF samples from patients with ReA, but the proportion of positive SF samples was lower than that found in RA ($P = .016$). In addition, the levels of sSLAM in positive serum ($P = .049$) and SF ($P = .001$) samples from patients with ReA were significantly lower than those found in patients with RA. In atopic patients the frequency of serum samples positive for sSLAM was comparable with that found in patients with RA, but the levels of sSLAM in positive samples were significantly lower in atopic patients than in patients with RA ($P = .003$; Table I, Fig 2). The difference in the mean levels of sSLAM in positive serum samples from patients with ReA (21 ± 3 ng/mL, mean $\pm$ SEM) or atopy (21 ± 1 ng/mL) when compared with healthy individuals (42 ± 8 ng/mL) did not reach statistical significance.

DISCUSSION

We demonstrate for the first time that sSLAM protein can be detected in vivo. A proportion of both serum and SF samples from patients with RA and healthy individuals was positive for sSLAM, suggesting that sSLAM produced by lymphocytes themselves may be involved in the regulation of T- and B-cell function. sSLAM could also be detected in samples from patients with ReA or from patients with elevated IgE levels. Interestingly, the median level of sSLAM in patients with ReA and atopy was significantly lower than that found in patients with RA.

The balance between T_{H1} and T_{H2} cells is thought to be important in the initiation and the outcome of autoimmune and allergic disorders. Results with animal models of autoimmune diseases indicate a proinflammatory role for T_{H1} cells in autoimmunity.¹² There is evidence for preferential activation of T_{H1} cells in the rheumatoid synovium,^{13,14} suggesting that T_{H1} cytokines are involved in the pathogenesis of RA. Studies on the expression of T_{H1} versus T_{H2} cytokines in ReA have yielded somewhat contrasting results. Freshly isolated synovial tissue cells or mitogen-activated SF CD4⁺ cells from patients with ReA expressed significantly more IL-4 than those from patients with RA, suggesting that ReA may be a T_{H2} -mediated disease.^{8,9} In contrast, synovial T cells cloned from patients with *Yersinia*-triggered ReA were predominantly T_{H1} .^{15,16} However, procedures used to clone T cells may bias toward a T_{H1} phenotype because of IL-12 produced by the feeder cells. Atopy is thought to be a prototypic T_{H2} -driven disease. IL-4 is present in skin biopsy specimens from allergen-induced cutaneous reactions in atopic patients, whereas IFN- γ is undetectable.¹¹ In addition, IL-4, but not IFN- γ , is produced by bronchoalveolar T cells from patients with asthma and by allergen-specific T-cell clones from atopic patients.^{10,17} Our atopic patients were defined on the basis of elevated serum IgE levels, demonstrating a predominant T_{H2} phenotype in these patients, and the levels of sSLAM in all atopic patients were less than 40 ng/mL. Therefore on the basis of our results it appears that low levels of sSLAM may be associated with T_{H2} responses, which is in agreement with the findings that the engagement of SLAM preferentially increases the production of IFN- γ by T-cell clones belonging to the different T-helper cell subsets^{1,3} and by SFMCs from patients with RA.⁵

We have previously demonstrated a significantly increased expression of mSLAM on synovial T cells from patients with RA when compared with peripheral blood T cells from the same patients.⁵ The present data indicates that sSLAM, in contrast to mSLAM, is not preferentially expressed in the joints. In addition, no significant correlation between the levels of mSLAM and sSLAM in 15 patients with RA could be observed (data not shown). Therefore our results suggest differential expression of sSLAM and mSLAM in vivo.

The relative importance of sSLAM and mSLAM in the regulation of lymphocyte function remains to be determined. The effects of recombinant sSLAM and mSLAM transfectants on the proliferation and Ig pro-

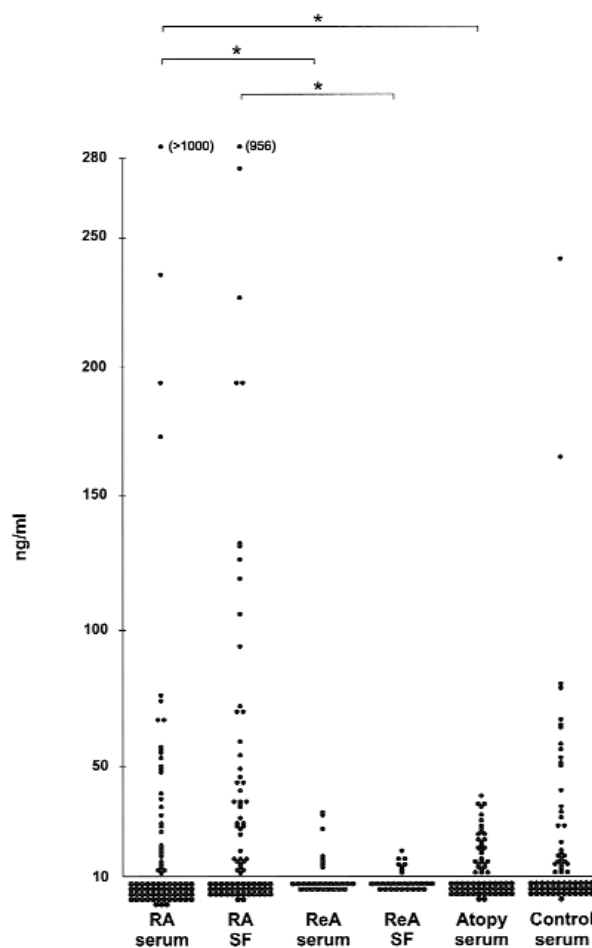


FIG 2. The levels of sSLAM in serum and SF samples from patients with RA, ReA, or atopy and healthy individuals (control). sSLAM was detected by using a specific sandwich ELISA. The detection limit of the ELISA was 10 ng/mL. Asterisk denotes a significant difference ($P < .05$) in mean level of sSLAM in positive samples determined by using Mann-Whitney U test.

duction by B cells were shown to be comparable.² High concentrations of sSLAM (10 to 20 μ g/mL) are needed to stimulate T or B cells in vitro (Cocks et al, submitted).² This is likely to be due to the fact that in solution approximately 99% of sSLAM is present as a dimer, and the monomer appears to be the primary functional form of the molecule (Cocks et al, submitted). We demonstrate that some serum and SF samples from patients with RA and healthy individuals contain relatively high levels of sSLAM, up to 1 μ g/mL. At present, there is no data on the amount of the monomeric form of sSLAM in vivo. Nevertheless, the high amount of sSLAM that is needed for in vitro function suggests that mSLAM may be the dominant functional form of the molecule. However, activated lymphocytes secrete increased levels of sSLAM, resulting in high local sSLAM concentrations. This is particularly true during the activation of lymphocytes in the lymphoid organs where lymphocytes may amplify their own proliferation by means of sSLAM-

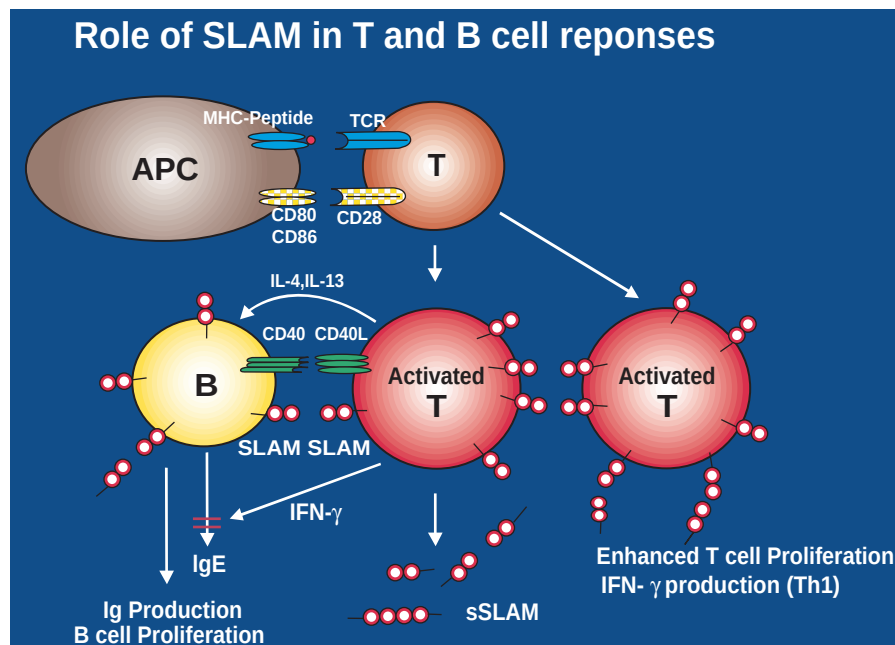


FIG 3. Schematic diagram illustrating the roles of sSLAM and mSLAM in the regulation of T- and B-cell responses.

mSLAM interactions without the requirement of direct cell-cell contact.

Taken together, our demonstration of sSLAM expression *in vivo* indicates that, in addition to homophilic mSLAM-mSLAM interactions, lymphocytes may regulate the function of other lymphocytes by means of secretion of sSLAM. The roles of sSLAM and mSLAM in the regulation of lymphocyte functions are further illustrated in Fig 3. In addition, the present results suggest that the relatively low expression of sSLAM may be associated with the occurrence of predominant T_H2 responses.

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