Semaphorin 3A, a potential immune regulator in familial Mediterranean fever

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ABSTRACT

Objective. *Semaphorin 3A (sema3A) plays a regulatory role in immune responses with effects on both T and B regulatory cells. Familial Mediterranean fever (FMF) is an autoinflammatory disease, yet a possible role for regulatory T and B cells has been described.*

Methods. *17 FMF patients during attack and then in remission, 8 FMF patients with smoldering disease and 12 healthy controls were enrolled. Sema3A in serum and its expression on regulatory T and B cells was evaluated. Clinical parameters of FMF patients were assessed.*

Results. *Semaphorin 3A serum level was lower in FMF patients during attack, smoldering disease or remission than healthy controls, (242.3±9.8 ng/ml vs. 258.9±11.5 ng/ml vs. 232.5±22.7 ng/ml vs. 323.3±160.2 ng/ml, respectively p<0.05). This decrease was specifically noted on regulatory B and T cells in FMF patients during attack and in smoldering disease and normalised in remission.*

Conclusion. *Sema3A expression on T and B regulatory lymphocytes is low in FMF patients during attack and in smoldering disease compared to the expression in remission and healthy controls. These results are in line with previous descriptions suggesting a possible role of regulatory T cells in termination of FMF attacks. Further studies are needed to verify these preliminary findings.*

Introduction

Familial Mediterranean fever (FMF) is an autoinflammatory disorder characterised by recurrent attacks of fever, peritonitis, pleuritis, arthritis and erysipelas-like skin lesions (1, 2).

Mutations in the Mediterranean fever gene (MEFV), mapped to the short arm of chromosome 16, encoding a protein called pyrin/marenostrin, are strongly associated with clinical FMF and felt to be pathogenic (4-6). Pyrin is thought to regulate caspase-1 function indirectly and thereby influence IL-1β processing and apoptosis (3). The current understanding is that a defective pyrin protein may be responsible for an exaggerated inflammatory response mediated by IL-1 and a cascade of pro-inflammatory cytokines. Indeed, pro-inflammatory cytokines such as interleukin (IL)-6, IL-8, IL-12, and tumour necrosis factor (TNF) alpha are found elevated in FMF patients during attacks (7). Inflammatory activity is suggested to continue, however, between attacks, as is evident by increased expression of mRNA of IL-6, IL-8 and TNF α (8) and increased expression of T cell activation and proliferation markers in attack free periods (8).

Recently, a role for regulatory T cells and the adaptive immune systems has been suggested in FMF. Regulatory T cells were found to be decreased during FMF attacks and upregulated after FMF attacks, possibly assisting the termination of FMF attacks (9).

Semaphorin 3A (sema3A), a secreted member of the semaphorin family, is now recognised as a potent immunoregulator with a role during all stages of the immune response (10). Sema3A expression on T and B regulatory cells has been recognised to act as a suppressive marker, contributing to the regulatory properties of these cells (8). It was shown in systemic lupus erythematosus (SLE) to increase the regulatory abilities of B cells (*e.g.* decrease TLR9, increase IL-10 and FoxP3) (9, 10). It was also demonstrated to have a regulatory function on T regulatory cells in rheumatoid arthritis (RA) (11). The expression of sema3A in FMF as a possible effector on regulatory T and B cells has not yet been evaluated.

The aim of this study is to evaluate the expression of sema3A in peripheral blood, on B regulatory cells and on regulatory T cells of FMF patients during attack, in remission and with smoldering disease, in comparison with healthy controls.

Material and methods

Patient populations

Four groups of patients were evaluated: a) Seventeen consecutive refractory FMF patients with at least one attack a month who fulfilled the Tel Hashomer criteria for a FMF attack (24-48 hours from symptom onset); b) The same patients 14 days after termination of an attack at clinical remission; c) Eight FMF patients with smoldering disease, as defined by a high C-reactive protein (CRP) level above 20 mg/dL, but without any symptoms of serositis or arthritis and without concurrent known infectious disease; and d) Twelve healthy controls. Demographic characteristics and clinical manifestations of FMF patients were assessed and recorded before blood sampling, including: age, gender, age of onset of FMF, family history of FMF, presence of arthritis, serositis, typical erysipelas-like eruption, amyloidosis, genetic mutations, years of colchicine use and cumulative dose. FMF diagnosis was made according to the Tel Hashomer criteria (12). Severity of FMF was determined utilising the Mor severity score (13). The study was approved by the local Research Ethics Board and all patients gave their informed consent.

Semaphorin 3A serum level

The measurement of sema3A serum level was conducted using a commercial ELISA kit (MBS-MyBiosource, San Diego, California, USA) according to the manufacturer instructions. The serum samples were stored at -20º until ELISA evaluation.

The expression of semaphorin 3A on $CD25^{high}$ $CD4^{+}$T cells

The expression of sema3A on $CD4^{+}$ CD25high T cells from healthy controls and FMF patients was assessed by staining mononuclear cells with monoclonal antibodies, human anti-CD4 PE and CD25 PC5 (Immunotech, Beckman-Coulter, Marsellie, France), and human anti-sema3A AlexaFluor 488 (R&D, Minneapolis, MN, USA), and evaluated in Flow cytometry software (FC500 and CXP software, Beckman Coulter, Brea, CA, USA).

Table I. Demographics and clinical characteristics of the study population.

Variable	FMF (attack and remission) n=17	FMF (smoldering disease) n=8	healthy controls n=12	*p*-value
Age (years), mean±SD	38.1 ± 10.2	48 ± 15	43.1 ± 3.4	NS
Sex: female	8 (47%)	4 (50%)	5 (41%)	NS
Origin Maroccan jew	6 (35%)	4 (50%)		NS
Arab	4 (23.5%)	2 (25%)		NS
Druze	4 (23.5%)	2 (25%)		
Other	3 (18%)			
BMI (kg/m2), mean ± SD	25 ± 4.7	26.1 ± 5.6	23.7 ± 6.3	NS
Smoker current	4 (23.5%)	2 (25%)	3 (25%)	NS
M694V homozygot	5 (29%)	4 (50%)		NS
V726A homozygote	5 (29%)	1 (12.5%)		NS
V726A/E148q	4 (23.5%)	1 (12.5%)		NS
Age of onset (years)	10 ± 7.9	15 ± 6.3		NS
Colchcine dose (mg)	1.9 ± 0.48	2.25 ± 0.5		NS
Mor severity score	3.2 ± 1.9	2.8± 1.6		NS

FMF: Familial Mediterrenean fever; BMI: body mass index.

The expression of semaphorin 3A on $CD25^{high}$ $CD19^{+}$ B cells

The expression of sema3A on $CD19^{+}$ $CD25^{+}$high B cells from healthy controls and FMF patients was assessed by staining mononuclear cells with monoclonal antibodies, human anti-CD19 PE and CD25 PC5 (Immunotech, Beckman-Coulter, Marsellie, France), and human anti-sema3A AlexaFluor 488 (R&D, Minneapolis, MN, USA), and evaluated in Flow cytometry software (FC500 and CXP software, Beckman Coulter, Brea, CA, USA).

Statistical analysis

Continuous data were described by means and SD and categorical variables as frequencies and percentages (Table I). Comparisons between the four patient groups were made using one-way ANOVA followed by Tukey's *post-hoc* test.

We further evaluated clinical correlation between sema3A serum level and disease-related covariates. For numerical covariates, the relationship was studied by a correlation test and is reported via Pearson's "r". For binary covariates, the relationship was studied by a two-sample, two-tailed *t*-test and is reported via "Δ", the difference in means. Statistical analysis was performed using the R Foundation for Statistical Computing 3.0.2 2013.

Results

Patient population

Table I summarises the demographic, clinical and genetic data as well as treatments of the patient populations. The 3 groups of patients namely, FMF in attack and remission (a), smoldering disease (b) and healthy controls (c) were similar with regard to age and gender. Sixty-three precent of all FMF patients (25 patients in groups a and b) were of Jewish descent, 23% were of Arab descent and 14% were Druze (there was no ethnic difference between groups, Table I). The age of onset was 10 years ±7.9 in group a) and 15 years ±6.3 in group b). The mean colchicine dose was similar between groups a and b (1.9±0.48 *vs*. 2.25±0.5) as was the MOR severity score (3.2±1.9 and 2.8±1.6), respectively. None of the patients was treated with IL-1 anatagonists.

Thirty-six percent of FMF patients were homozygotes for M694V mutation, 24% were homozygotes for V726A, 20% were compound heterozygotes for V726A/E148q, 4% were homozygote for E148q and 26% were

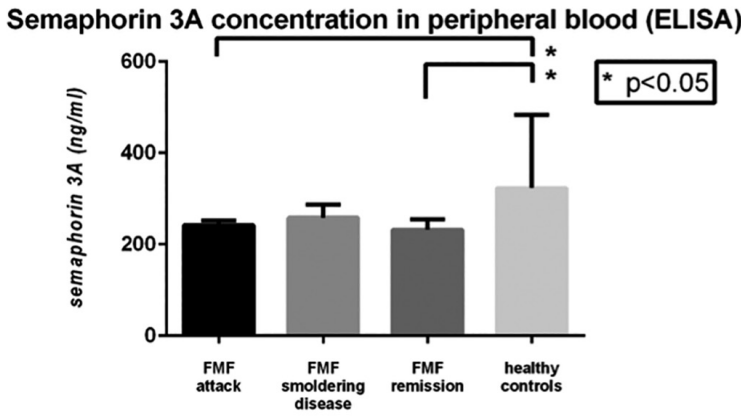

Fig. 1. Semaphorin 3A concentration in peripheral blood.
Semaphorin 3A concentration in peripheral blood as evaluated by ELISA is lower in FMF patients in an attack, smoldering disease or remission than healthy controls (242.3±9.8 ng/ml *vs*. 258.9±11.5 ng/ml *vs*. 232.5±22.7 ng/ml *vs*. 323.3±160.2 ng/ml, respectively, $p<0.05$).

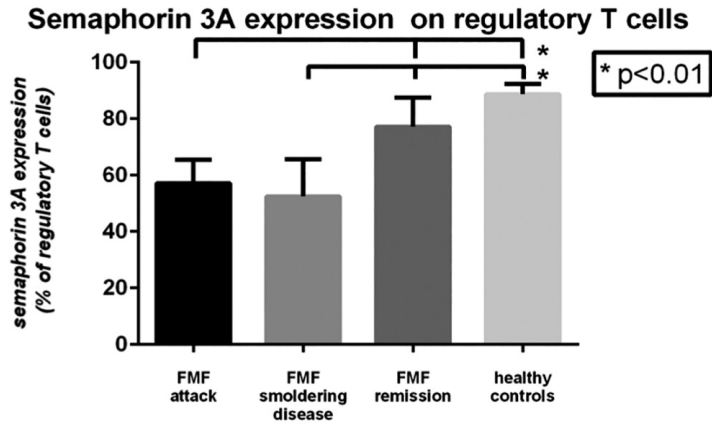

Fig. 2. Semaphorin 3A expression on regulatory T cells.
Semaphorin 3A expression on regulatory T cells in FMF patients in an attack is lower than in remission and than in healthy controls (57.2%±8.3 *vs*. 77.2%±10.3 *vs*. 88.7%±3.6%, respectively, $p<0.01$). Semaphorin 3A expression on regulatory T cells is also lower in FMF patients with smoldering disease compared with FMF patients in remission and healthy controls (52.5%±13.1 *vs*. 77.2%±10.3 *vs*. 88.7%± 3.6%, respectively, $p<0.01$).

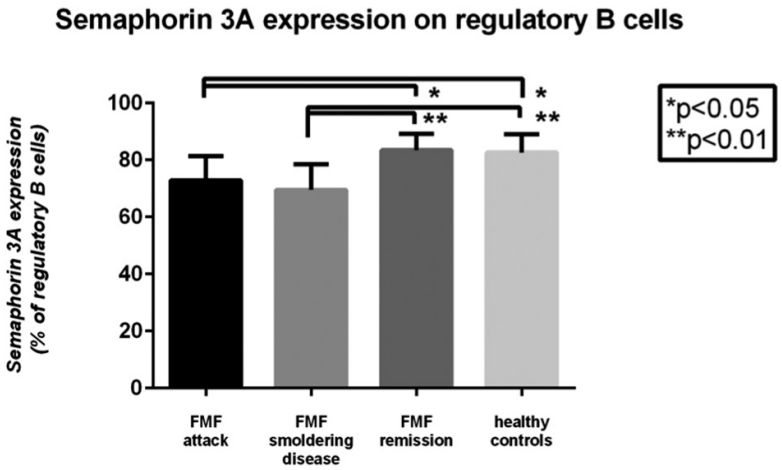

Fig. 3. Semaphorin 3A expression on B cells.
Semaphorin 3A expression on B cells in FMF patients in an attack is lower than FMF in remission and healthy controls (72.9%±8.5 *vs*. 83.4%±5.8 *vs*. 82.6%±6.4, respectively, $p<0.05$). Semaphorin 3A expression on B cells in FMF patients with smoldering disease is also lower than FMF patients in remission and healthy controls (69.5%±9 *vs*. 83.4%±5.8 *vs*. 82.6%±6.4, respectively, $p<0.01$).

not tested for MEFV gene mutation (there was no significant differences in distribution of gene mutations between the groups, Table I).

Semaphorin 3A serum levels

As seen in Figure 1, the serum levels of sema3A were lower in FMF patients, whether in attack, remission or smoldering disease, compared to healthy controls 242.3±9.8 ng/ml *vs*. 258.9±11.5 ng/ml *vs*. 232.5±22.7 ng/ml *vs*. 323.3±160.2 ng/ml, $p<0.05$).

Semaphorin 3A expression on T regulatory cells

The percent of regulatory T cells expressing sema3A was lower in FMF patients in an attack than in remission and than healthy controls (57.2%±8.3 *vs*. 77.2%±10.3 *vs*. 88.7%±3.6%, $p<0.01$) respectively, but similar to patients with smoldering disease (Fig. 2). FMF patients in remission had expression of sema3A on regulatory T cells comparable to healthy controls (77.2%±10.3 *vs*. 88.7%± 3.6%), Fig. 2. The Mean Fluorescence Intensity (MFI) of sema3A expression on regulatory T cells was also significantly lower during attack than remission (3.5±0.4 *vs*. 4.96±1.1, $p<0.01$).

Semaphorin 3A expression on B regulatory cells

Finally, the expression of sema3A on regulatory B cells was lower in FMF patients in an attack than FMF in remission and healthy controls (72.9%±8.5 *vs*. 83.4%±5.8 *vs*. 82.6%±6.4%, $p<0.05$). Sema3A expression on regulatory B cells in FMF patients with smoldering disease was lower than FMF patients in remission and healthy controls as well (69.5%±9 *vs*. 83.4%±5.8 *vs*. 82.6%±6.4%, $p<0.01$) respectively, Fig. 3. The Mean Fluorescence Intensity (MFI) of sema3A expression on regulatory B cells was also significantly lower during attack than remission (2.87±0.47 *vs*. 4.39±1.5, $p<0.05$).

Clinical correlation

Semaphorin 3A expression on regulatory T cells was inversely correlated with CRP concentration r=-0.5640; $p=0.002$ (Fig. 4) but did not correlate with age,

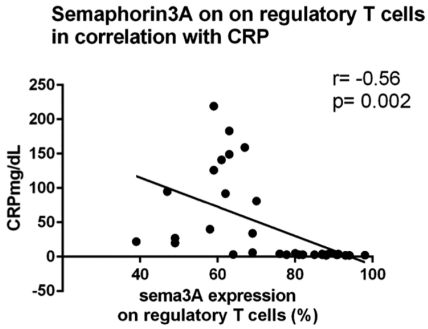

Fig. 4. Semaphorin 3A expression in regulatory T cells was inversely correlated with CRP concentration r= -0.5640 ; *p*=0.002.

gender, age of onset of FMF, genetic mutations, years of colchicine use nor its cumulative dose, or FMF severity score.

Discussion

In this study we have demonstrated for the first time that the serum level of sema3A is reduced in FMF patients compared to healthy controls and, furthermore, that the expression of sema 3A specifically on regulatory B and T cells is reduced during attacks of FMF, normalising thereafter in remission.

FMF is considered to be an autoinflammatory disease in which the inflammation is mediated by a defect in the pyrin protein that eventually leads to unopposed or over-activation of the inflammasome and subsequently a cascade of proinflammatory cytokines, inteleukin 1β (IL-1 β), IL-6, TNF and IL-18. Pyrin is expressed in neutrophils, eosinophils, monocytes, dendritic cells, and synovial fibroblasts but not at significant levels in lymphocytes and indeed the lymphocytes are not considered to be key player in FMF (14). Nevertheless, the immune system is complex and although lymphocytes probably do not initiate inflammation in FMF they may play a secondary part in the inflammatory cascade. Several studies have demonstrated such involvement of the adaptive immune system including activation of Th17+ lymphocytes (15), a low neutrophil-to-lymphocyte ratio that correlated with severity of FMF disease (16) and reduced T helper cells (17). We have previously demonstrated that regulatory T cells are reduced in the first days of FMF attack and recover subsequently (6). Regulatory T cells have a potent anti-inflammatory effect and hence were speculated to have a possible role in termination of the inflammation in FMF attacks. In our current study a low expression of sema3A on regulatory B and T cells that normalises in remission is in line with that theory and may help to explain the activation of T regs and B regs in FMF attack. A clear negative correlation between the expression of sema3A on regulatory T cells and CRP levels further supports the concept that sema3A has a direct role in inhibiting inflammation in FMF.

Moreover, sema3A upregulates the expression of IL-10 from Tregs that inhibits neutrophils function, the main effector cell in FMF (18). Further studies are needed in order to understand the possible role of regulatory T and B cells and semphorin 3A as regulators of inflammation in FMF. Semaphorin 3A, which has been advanced as a putative target for therapy in autoimmune disease, should be considered and studied in autoinflammatory diseases as well.

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