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ORIGINAL ARTICLE

Lymphocyte Apoptosis and FAS Expression in Patients with 22q11.2 Deletion Syndrome

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Abstract

Purpose Immunodeficiency is one of the key features of 22q11.2 deletion syndrome (del), and it is seen in approximately 75% of the patients. The degree of immunodeficiency varies widely, from no circulating T cells to normal T cell counts. It has been hypothesized that the low number of T cells may at least in part be due to increased apoptosis of T cells. Increased spontaneous T cell apoptosis has been reported in one patient with 22q11.2del, but this has not been further investigated.

Methods A national cohort of patients with a proven heterozygous deletion of chromosome 22q11.2 diagnosed by FISH or MLPA and a group of age and sex matched controls were studied. Spontaneous and activation-induced apoptosis, in addition to FAS expression on lymphocytes, were measured using flow cytometry. Serum levels of FASL were analyzed using ELISA.

Results There was no increased spontaneous apoptosis in patients with 22q11.2del. Upon activation, anti-FAS-induced apoptosis was significantly increased in patients compared to those in controls, while there was no difference in activation induced cell death or activated cell autonomous death. We also found a significant increase in expression of FAS on freshly isolated lymphocytes from patients, while there was no difference in serum levels of FASL. Patients with congenital heart defects (CHD) had significantly higher serum levels of FASL compared to non-CHD patients.

Conclusion We have shown increased FAS expression on lymphocytes from patients with 22q11.2del as well as increased levels of FASL in patients with CHD. Those changes may contribute to the pathophysiology of the 22q11.2del.

Keywords 22q11.2del · 22q11.2 deletion syndrome · FAS · FAS-L · primary immunodeficiencie · apoptosis

Introduction

The 22q11.2 deletion syndrome (DS) is the most common microdeletion syndrome in humans with an estimated prevalence from 1:3000 to 1:6000 live births [1]. Due to clinical variability and heterogeneity, the occurrence of the disorder may be underestimated [2]. The prevalence is also expected to rise due to improved patient survival [1].

In 22q11.2del, most of the patients have a typical 3 mega base (Mb) deletion on the long arm of chromosome 22 containing over 30 different genes. Approximately 8% of the patients have a smaller, 1.5 Mb, deletion and 2% have atypical deletions. The inheritance of the syndrome is autosomal dominant, but 90% of cases appear to be de novo. There is no apparent correlation between the size of the deletion and the clinical phenotype [3, 4]. However, distal deletions that do not include T-box 1 gene were found to be associated with better T cell counts [5].

22q11.2del displays a very wide phenotypic spectrum including immunodeficiency, hypoparathyroidism, cardiac

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defects, autoimmunity, facial anomalies, velopharyngeal insufficiency, developmental delay, learning disabilities, psychiatric disorders, and renal, ocular, and skeletal malformations [4]. The various clinical problems are age- dependent and typically appear in a chronologic manner. First, the organ malformations dominate, and later, neuropsychological problems appear [3].

Immunodeficiency is one of the key features of 22q11.2del and is seen in approximately 75% of the patients. The degree of immunodeficiency varies widely, from no circulating T cells to normal T cell counts. However, most patients have as infants mildly to moderately decreased number of circulating T cells [4, 6, 7]. The frequency and severity of the immunodeficiency do not appear to be related to other phenotypic features [8]. The immunodeficiency is thought to arise secondary to aplasia, hypoplasia, or aberrant migration of the thymus gland [4, 6]. It has also been hypothesized that the low number of T cells may at least be in part due to increased apoptosis of T cells. Thus, Gupta et al. observed increased spontaneous apoptosis of both CD4+ and CD8+ T cells from one patient with 22q11.2del. Peripheral blood mononuclear cells (PBMCs) of that patient expressed increased levels of FAS and FASL and decreased levels of BCL-2 [9]. At the same time, Pierdominici et al. found a reduction of both spontaneous, as well as CD3- and FAS-mediated apoptosis of lymphoid cells from thymic tissue obtained from two patients with 22q11.2del compared to controls [10]. Further, Zhou et al. found no difference in FAS, BCL-2, and P53 expression in the hypoplastic thymuses in 22q11.2del compared to non-22q11.2del thymic tissue [11].

T cell apoptosis plays an essential role in the immune system. During an immune response, naïve T cells undergo expansion and differentiate into effector cells. Naïve T cells and T cells in the early expansion phase are resistant towards apoptosis. After reaching the peak of the immune response, T cells become sensitive towards cell death and T cell numbers decline during the contraction phase. Induction of activation induced cell death (AICD) depends on restimulation via the T cell receptor (TCR). Activated T cells which are not restimulated, die by activated cell autonomeous death (ACAD). Some T cells surviving AICD enter the memory T cell pool [12, 13]. Dysregulation of apoptosis in the immune system may lead to immunodeficiency, autoimmunity, or cancer [13, 14]. For example, increased AICD of T cells was described in patients with cartilage-hair hypoplasia [15], and several studies have reported increased spontaneous apoptosis and AICD in lymphocytes from patients with common variable immunodeficiency and selective immunoglobulin A deficiency [16]. On the other hand, mutations affecting FAS apoptotic pathway are associated with a loss of apoptotic signaling and lead to an autoimmune disorder called autoimmune lymphoproliferative syndrome [17]. Further, several studies have reported that T cells undergo increased spontaneous apoptosis and AICD in aged individuals as compared with young controls. This can explain a decline in T cell functions during aging, along with decreased thymic output and reduced proliferative potential, which leads to increased frequency of infections, autoimmunity, and cancer [18]. Interestingly, the age-dependent decline in T cell numbers is slower in patients with 22q11.2del compared to those in controls due to a homeostatic proliferation [4]. One could hypothesize that it could be, at least partly, explained by reduced, rather than increased, T cell apoptosis in 22q11.2del.

The aim of our study was to study if patients with 22q11.2del in general have increased spontaneous apoptosis as well as activated cell death by AICD or ACAD.

Materials and Methods

Patients

Sixty-seven patients, 31 males, median age 9 years (IQR 4–15 years) from all over Norway with a proven heterozygous deletion of chromosome 22q11.2 by fluorescent in situ hybridization or multiplex ligation-dependent probe amplification were included in the study. They all attended the Pediatric Outpatient Clinic at Rikshospitalet, Oslo University Hospital during the time period 2011–15. Patients with atypical and known additional deletions were not included. Fifty-seven healthy individuals, 26 males, median age 10 years (IQR 5.5–19.5 years) were included in a control group. The patients and controls did not have any clinical apparent infection when sampled. In some patients, a restricted subset of tests were performed due to insufficient blood volume. This is indicated in the figure legend. The age and sex matching was preserved in those cases.

The study was conducted according to the guidelines at our hospital and was approved by the Regional Committee for Research Ethics, reference number 2011/1741. Before inclusion, written informed consent was obtained from the participants and/or their parents.

Blood Sampling and Cell Culture

Peripheral venous blood was drawn into tubes containing Serum Sep Clot Activator or Sodium Heparin.

For serum preparation, blood was allowed to clot before centrifugation at 2000 × *g* for 15 min. All serum samples were aliquoted, stored at − 80 °C, and thawed < 3 times.

PBMCs were isolated from heparinized whole blood with Lymphoprep (Axis-Shield PoC, Norway), counted, and resuspended in RPMI 1640 medium (Lonza, Belgium), containing penicillin/streptomycin (Pen-Strep, Lonza, Belgium) and 10% heat inactivated fetal bovine serum (Lonza, Belgium).

For activation, freshly isolated PBMCs (1×10^6 cells/ml) were cultured in 24-well cell culture plates (Corning Inc., NY) in the presence or absence of monoclonal anti-CD3 antibody (OKT3) (Sigma-Aldrich, MO) at concentration 1 μg/ml and recombinant human interleukin 2 (IL-2) (BD Biosciences, CA) at concentration 30 U/ml for 72 h at 37 °C in a humidified 5% CO_2 atmosphere. Cells were then washed and restimulated with a secondary monoclonal antibody (2.MoAb) anti-CD3 Ab (OKT3, 1 μg/ml) or with human activating anti-FAS antibody clone CH11 at concentration 500 ng/ml (Millipore, CA) or left untreated for 24 h at 37 °C in a humidified 5% CO_2 atmosphere (Fig. S1).

For analysis of FASL in supernatants, cell supernatants obtained after incubation were aliquoted and stored at − 80 °C.

Determination of Cell Death

During apoptosis, phosphatidylserine (PS) translocates from the inner part of the plasma membrane to the out layer and becomes exposed on the external surface of the cell. Annexin-V has a high affinity for PS and is therefore suited to detect apoptotic cells. In conjunction with a permeability probe 7-AAD, a distinction can be made between viable AnnV− 7-AAD− cells, apoptotic cells with intact plasma-membrane integrity (early apoptotic AnnV+ 7-AAD− cells), and cells with leaky plasma membrane (late apoptotic/necrotic AnnV+ 7AAD+ cells) [19]. For analysis of cell death, cells were stained with PE annexin-V Apoptosis Detection Kit I (BD Biosciences, CA) according to the manufacturer's instructions. Briefly, cells were harvested, washed twice in ice cold phosphate-buffered saline (PBS), and then resuspended in 1× binding buffer. Then, the solution was transferred to BD Truecount Tubes (BD Biosciences, CA) and phycoerythrin (PE) annexin-V and 7-amino-actinomycin (7-AAD) were added. Cells were gently vortexed and incubated for 15 min at room temperature in the dark. After incubation, more binding buffer was added. Samples were then kept on ice and analyzed within 1 h using FACSCalibur (BD Biosciences, CA). Data were collected on 50,000 Truecount bead events and analyzed using CXP Analysis software (Beckman Coulter, CA) gated on lymphocytes.

Measurement of FAS

For analysis of FAS expression on cell surface, PBMC were stained with FITC Mouse Anti-Human FAS antibody or FITC Mouse IgG1, κ Isotype Control (BD Biosciences, CA) according to the manufacturer's instructions. Briefly, cells were washed with ice cold PBS, resuspended in FBS Stain Buffer (BD Biosciences, CA), and preincubated for 15 min with purified human IgG (Sigma-Aldrich, MO) before staining in order to avoid unspecific MoAb binding. FAS antibody or isotype control was then added, and cells were incubated for 30 min on ice in the dark. After incubation, cells were washed in staining buffer and analyzed immediately using FACSCalibur (BD Biosciences, CA). Data were collected on 45,000 events and analyzed using CXP Analysis software (Beckman Coulter, CA) gated on lymphocytes.

Measurement of FASL

Human FAS ligand was measured both in serum and cell culture supernatants by human FAS ligand enzyme-linked immunosorbent assay (ELISA) kit obtained from R&D Systems (Minneapolis, MN). Manufacturer's instructions were followed during the procedure. The optical density was determined using a microplate reader (Multiscan Ascent) set to 450 nm and the wavelength correction set to 540 nm. Results were analyzed using Ascent Software (Thermo Electron, Finland).

Clinical Chemistry and Immunology

Leukocyte differential count and quantitative immunoglobulin testing were routinely performed at the Department of Medical Biochemistry, Oslo University Hospital. Lymphocyte subpopulation phenotyping was routinely assayed at the Department of Immunology, Oslo University Hospital.

Statistics

SPSS for Windows release 25 (Chicago, IL) was employed for the statistical analysis. For comparison of two groups, the non-parametric Mann-Whitney *U* test was used. When more than two groups of individuals were compared, the non-parametric Kruskal-Wallis test was used. If a significant difference was found, Mann-Whitney *U* test was used to calculate the difference between each pair of groups. Coefficients of correlation (r) were calculated by the non-parametric Spearman's rank test. The strength of correlation was interpreted as previously described [20]. Categorical data were compared with a Chi-squared test. Data are given as median and interquartile range (IQR) unless otherwise stated. Results were considered significant when $p < 0.05$. Figures were generated using GraphPad Prism version 7.04 for Windows (GraphPad Software, La Jolla, CA).

Results

Cohort of Patients

A total of 67 patients with a proven deletion of chromosome 22q11.2 were included in the analysis. Thirty-seven patients had congenital heart defects (CHD), and 31 of those were of a conotruncal type. Eighteen patients had hypoparathyroidism

at the moment of blood sampling. Fifteen patients had submucous cleft palate, and six patients had hypothyreosis. All patients received standard treatment according to their additional diagnosis. Immunological profile of patients is summarized in Table 1.

Spontaneous Apoptosis in Patients and Controls

Increased T cell apoptosis has previously only been reported in one patient with 22q11.2del [9]. To further investigate if this is generally related to the disorder, we performed FACS analysis using annexin/7-AAD staining in freshly isolated PBMC gaiting on lymphocytes. Analysis of spontaneous apoptosis was performed on 26 patients, 15 males, median age 12 years (IQR 6–17 years) and 19 controls, 12 males, median age 10 years (IQR 7–23 years). As shown in Fig. 1, there was no difference ($p = 0{,}301$) in the percentage of viable cells in patients (median value 96%, IQR 94%–97%) compared to those in controls (median 95%, IQR 94–96%). The amount of early apoptotic cells was 2.7% (IQR 1.6–3.3%) in patients and 3.0% (IQR 2.1–4.2%) in controls ($p = 0{,}132$). We neither found any difference ($p = 0{,}809$) in the amount of late apoptotic cells in patients (median value 1.6%, IQR 1.1–1.9%) compared to those in controls (median 1.5%, IQR 1.0–1.9%).

Activated Cell Death in Patients and Controls

In order to investigate activated cell death in lymphocytes from patients ($n = 13$, 6 males, median age 12 years [IQR 4–20 years]) and controls ($n = 9$, 4 males, median age 8 years [IQR 10–22 years]), we performed flow cytometry analysis after in vitro culture with anti-CD3 mAb and IL-2 and secondary restimulation with either anti-CD3 mAb or anti-FAS or left untreated.

When investigating ACAD, we found no difference in the percentage of viable (median 64% [IQR 58–71%] vs 68% [IQR 59–72%], $p = 0{,}815$), early (median 11% [IQR 8–16%] vs 14% [IQR 7–16%], $p = 0.920$), or late (median 19% [IQR

Table 1 Immunological profile of patients with 22q11.2del

Parameters	Values[a]	Unit
Leukocyte differential count ($n = 65$)		
Neutrophils	3.4 (2.3–4.7)	$\times 10^9$/L
Lymphocytes	2.0 (1.5–3.0)	$\times 10^9$/L
Monocytes	0.4 (0.3–0.5)	$\times 10^9$/L
Eosinophils	0.3 (0.1–0.4)	$\times 10^9$/L
Basophils	0.0(0.0–0.1)	$\times 10^9$/L
Lymphocyte subpopulations ($n = 57$)		
CD3+ T lymphocytes	1280 (937–1720)	Cells/μL
CD4+ T lymphocytes	713 (533–1042)	Cells/μL
CD8+ T lymphocytes	424 (330–622)	Cells/μL
CD19+ B lymphocytes	451 (279–968)	Cells/μL
CD16+/CD56+/CD3− NK cells	375 (249–598)	Cells/μL
T lymphocytes subpopulations ($n = 43$)		
CD3+/CD4−/CD8−/TCR alpha/beta+ double negative	1,3 (0,9-1,6)	% of CD3+ T lymphocytes
CD4+/CD45RO+ memory	45 (35–57)	% of CD4+ T lymphocytes
CD4+/CD45RO+/CXCR5+ follicular-like	10 (8–12)	% of CD4+ T lymphocytes
CD4+/CD45RA+ naïve	63 (48–75)	% of CD4+ T lymphocytes
CD4+/CD45RA+/CD31+ recent thymic emigrants	70 (62–76)	% of CD4+/CD45RA+ T lymphocytes
CD4+/CD45RO+/CD127low/CD25+ regulatory	6,0 (4,5-6,5)	% of CD4+ T lymphocytes
CD8+/CD27+/CD28+ naïve	66 (52–80)	% of CD8+ T lymphocytes
CD8+/CD27+/CD28+ early effector/memory	10 (7–17)	% of CD8+ T lymphocytes
CD8+/CD27−/CD28− late effector/memory	17 (4–33)	% of CD8+ T lymphocytes
Immunoglobulins ($n = 66$)		
IgA	1.3 (0.7–1.8)	g/L
IgG	9.5 (7.5–11.3)	g/L
IgM	0.6 (0.4–0.8)	g/L

[a] Values are expressed as median (interquartile range)

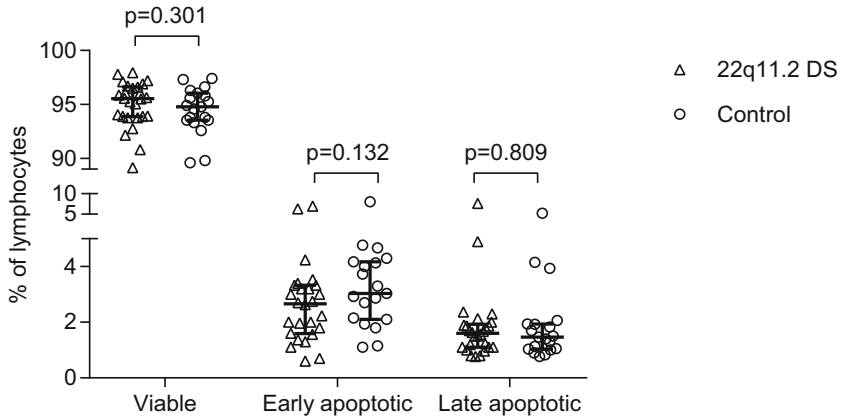

Fig. 1 Analysis of spontaneous apoptosis in 22q11.2del patients ($n = 26$) and controls ($n = 19$). Proportion of viable (AnnV− 7-AAD−), early apoptotic (AnnV$^+$ 7-AAD−), and late apoptotic/necrotic (AnnV+ 7AAD+) freshly isolated lymphocytes. Individual symbols identify single subjects. Horizontal line represents median value; error bars represent interquartile range

16–26%] vs 17% [IQR 15–27%], $p = 0{,}616$) apoptotic cells in patients compared to those in controls on stimulated, but not reactivated cells (Fig. 2a).

When studying AICD, we found no difference in the percentage of viable (median 64% [IQR 57–71%] vs 65% [IQR 58–68%], $p = 0{,}920$), early (median 12% [IQR 9–16%] vs 16% [IQR 9–18%], $p = 0{,}664$), or late (median 20% [IQR 17–27%] vs 20% [IQR 16–26%], $p = 0{,}663$) apoptotic cells in patients compared to those in controls on culture reactivated with anti-CD3 mAb (Fig. 2b).

After reactivation with anti-FAS, the percentage of viable cells was significantly decreased (median 43% [IQR 37–47%] vs 54% [IQR 43–58%], $p = 0.035$), and the percentage of late apoptotic cells was significantly increased (median 37% [IQR 33–42%] vs 26% [IQR 24–38%], $p = 0.030$) in patients vs healthy controls (Fig. 2c).

Expression of FAS on Lymphocytes

Since FAS-mediated apoptosis was increased in patients, we wanted to investigate the expression of FAS on lymphocytes. As shown in Fig. 3, the patients ($n = 23$, 12 males, median age 12 years [IQR 5–17 years]) had significantly ($p = 0.004$) increased expression of FAS on freshly isolated lymphocytes with a median value of 73% (IQR 64–75%) compared with controls ($n = 18$, 12 males, median age 10 years [IQR 7–27 years]) where the corresponding values were 55% (IQR 50–70%).

In order to investigate if upregulation of FAS is impaired in patients with 22q11.2del, we investigated the expression of FAS on lymphocytes after in vitro culture with anti-CD3 mAb and IL-2 and secondary reactivation with either anti-CD3 mAb or anti-FAS. We found no difference in the expression of FAS in patients ($n = 4$, one male, median age 14 years [IQR 3–35 years]) compared to those in controls ($n = 9$, four males, median age 10 years [IQR 8–22 years]) on activated and reactivated cultures. In cultures with PBMC left untreated, the patients had a tendency ($p = 0.064$) to increased expression of FAS with a median value of 79% (IQR 67–94%) compared with controls where the corresponding values were 60% (IQR 53–69%) (Fig. S2).

Levels of FASL

In order to clarify if expression of FASL is affected, we analyzed levels of FASL in serum. There was no difference ($p = 0.269$) in FASL levels in patients ($n = 63$, 28 males, median age 9 years [IQR 5–15 years]) with a median value 97 pg/mL (IQR 75–136 pg/ml) compared to those in controls ($n = 56$, 26 males, median age 10 years [IQR 5–18 years]) where the corresponding values were 91 pg/ml (IQR 70–109 pg/ml) (Fig. 4a). Further, we found no difference in levels of FASL in patients ($n = 10$, five males, median age 11 years [IQR 5–21 years]) compared to those in controls ($n = 11$, five males, median age 10 years [IQR 8–24 years]) in supernatants obtained after stimulation and reactivation with anti-CD3 mAb or left untreated (Fig. 4b).

Characteristics of FAS

Since expression of FAS on freshly isolated lymphocytes from patients was significantly higher than those in controls, we wanted to investigate if there was any association between expression of FAS and clinical features of 22q11.2del. We did not find any difference in FAS expression in patients with CHD ($p = 0.482$), submucous cleft palate ($p = 0.841$), hypoparathyroidism ($p = 0.759$), or hypothyroidism ($p = 0.308$) when compared to patients without those features (data not shown).

We found no correlation between expression of FAS and CD16+/CD56+/CD3− NK cells ($r = 0.157$, $p = 0.497$) and CD19+ B lymphocytes ($r = -0.396$, $p = 0.076$), as well as CD3+ ($r = 0.017$, $p = 0.943$), CD4+ ($r = -0.039$, $p = 0.867$), CD8+ ($r = 0.065$, $p = 0.779$), CD3+/CD4\/CD8\/TCR alpha/beta+ double negative ($r = 0.207$, $p = 0.442$), CD4+/CD45RO+ memory ($r = 0.279$, $p = 0.295$), CD4+/CD45RO/CXCR5+ follicular-like ($r = 0.308$, $p = 0.245$), CD4+/CD45RA+ naïve ($r = -0.372$, $p = 0.156$), CD4+/CD45RA+/CD31+ recent thymic emigrants ($r = -0.158$, $p = 0.560$), CD4+/CD45RO+/CD127 low/CD25+ regulatory ($r = -0.304$, $p = 0.252$), CD8+/CD27+/CD28+

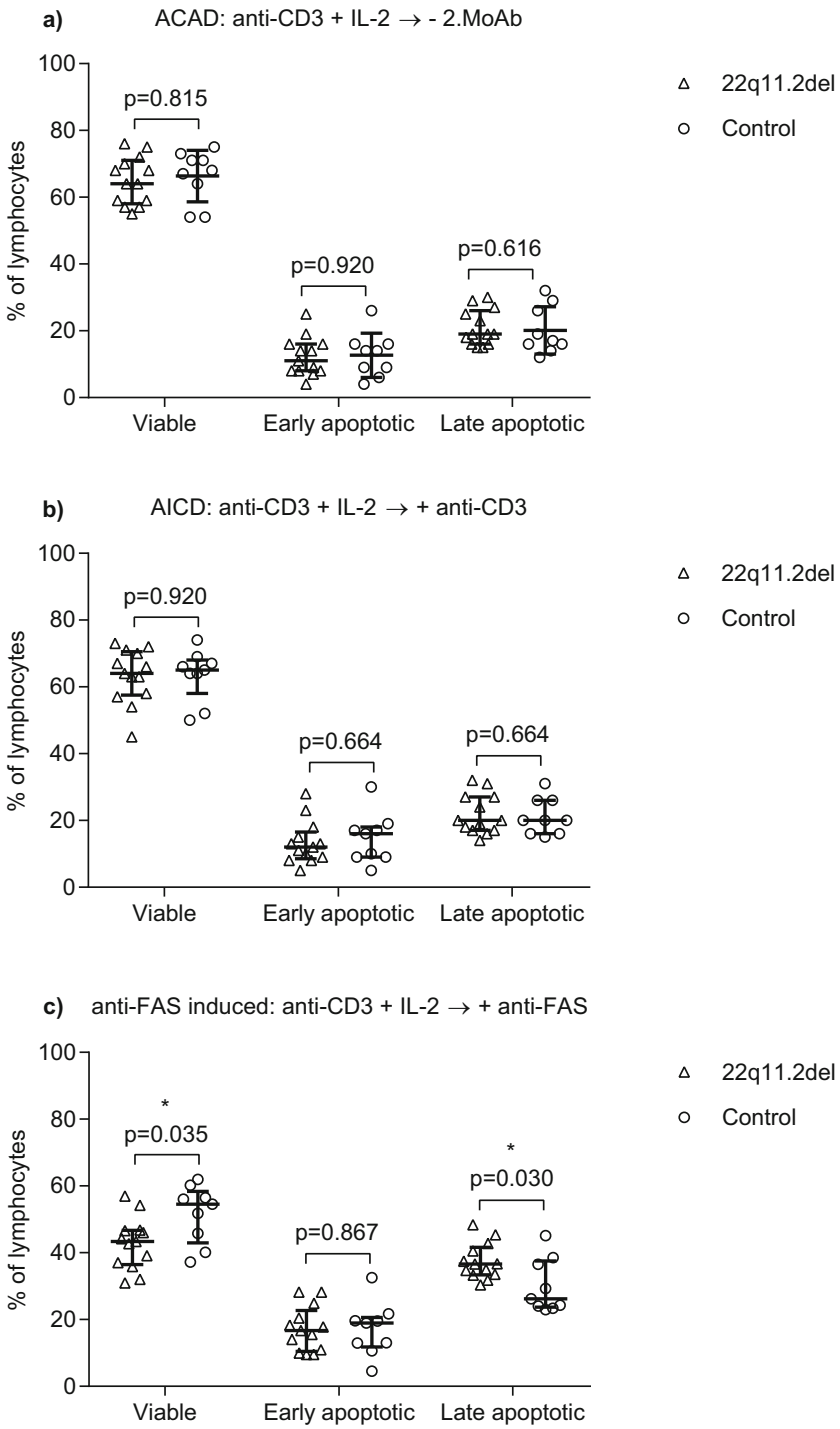

Fig. 2 Analysis of cell death in 22q11.2del patients ($n = 13$) and controls ($n = 9$). Proportion of viable (AnnV− 7-AAD−), early apoptotic (AnnV+ 7-AAD−), and late apoptotic/necrotic (AnnV+ 7AAD+) lymphocytes on in vitro culture with anti-CD3+ IL-2 and **a** left untreated (ACAD), **b** restimulated with anti-CD-3 (AICD), or **c** anti-FAS (anti-FAS-induced apoptosis). Individual symbols identify single subjects. Horizontal line represents median value; error bars represent interquartile range

naïve ($r = -0.294$, $p = 0.268$), CD8+/CD27+/CD28+ early effector memory ($r = -0.166$, $p = 0.540$), and CD8+/CD27−/CD28− late ($r = 0.308$, $p = 0.247$) T lymphocytes (data not shown).

Further, we found a moderate to strong positive correlation between age in months and FAS expression on lymphocytes ($r = 0.427$, $p = 0.042$ in patients versus $r = 0.840$, $p = 0.001$ in controls) (data not shown).

Characteristics of FASL

We also investigated if there was any association between FASL levels in serum and clinical features of 22q11.2del. We found that patients with CHD ($n = 35$) had significantly ($p = 0.022$) raised serum levels of FASL with median value of 100 pg/ml (IQR 79–158 pg/ml) compared to patients without CHD ($n = 28$) where the corresponding values were 88 pg/ml

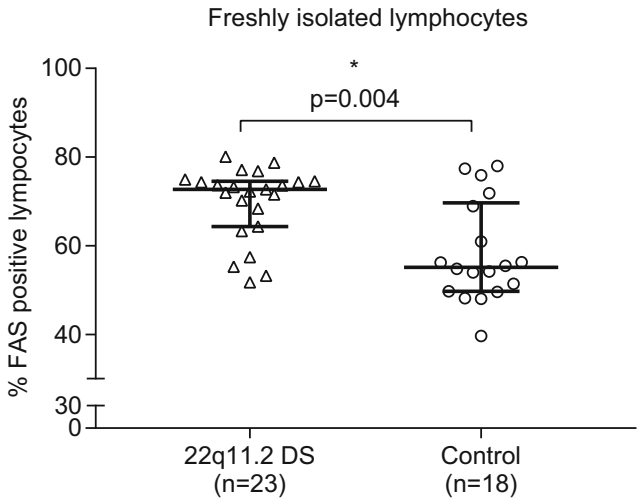

Fig. 3 Analysis of FAS expression on freshly isolated lymphocytes from patients ($n = 23$) and controls ($n = 18$). Individual symbols identify single subjects. Horizontal line represents median value; error bars represent interquartile range

(IQR 63–105 pg/ml) (Fig. 5). FASL levels were significantly higher in CHD subgroup ($p = 0.037$) compared to those in controls. There was no difference in serum FASL levels between non-CHD ($p = 0.479$) subgroup when compared those in controls (median 91 pg/ml, IQR 71–109 pg/ml).

We did not find any difference in serum FASL levels in patients with submucous cleft palate ($p = 0.569$), hypoparathyroidism ($p = 0.665$), or hypothyroidism ($p = 0.069$) when compared to patients without those features.

We found no correlation between serum levels of FASL and CD3+ ($r = 0.175$, $p = 0.220$), CD4+ ($r = 0.230$, $p = 0.104$), CD8+ ($r = 0.003$, $p = 0.984$), CD3+/CD4\/CD8\/TCR alpha/beta+ double negative ($r = 0.206$, $p = 0.222$), CD4+/CD45RO/CXCR5+ follicular-like ($r = -0.247$ $p = 0.141$), CD4+/CD45RA+ naïve ($r = 0.354$, $p = 0.031$), CD4+/CD45RA+/CD31+ recent thymic emigrants ($r = 0.081$, $p = 0.632$), CD4+/CD45RO+/CD127 low/CD25+ regulatory ($r = 0.085$, $p = 0.615$), and CD8+/CD27+/CD28+

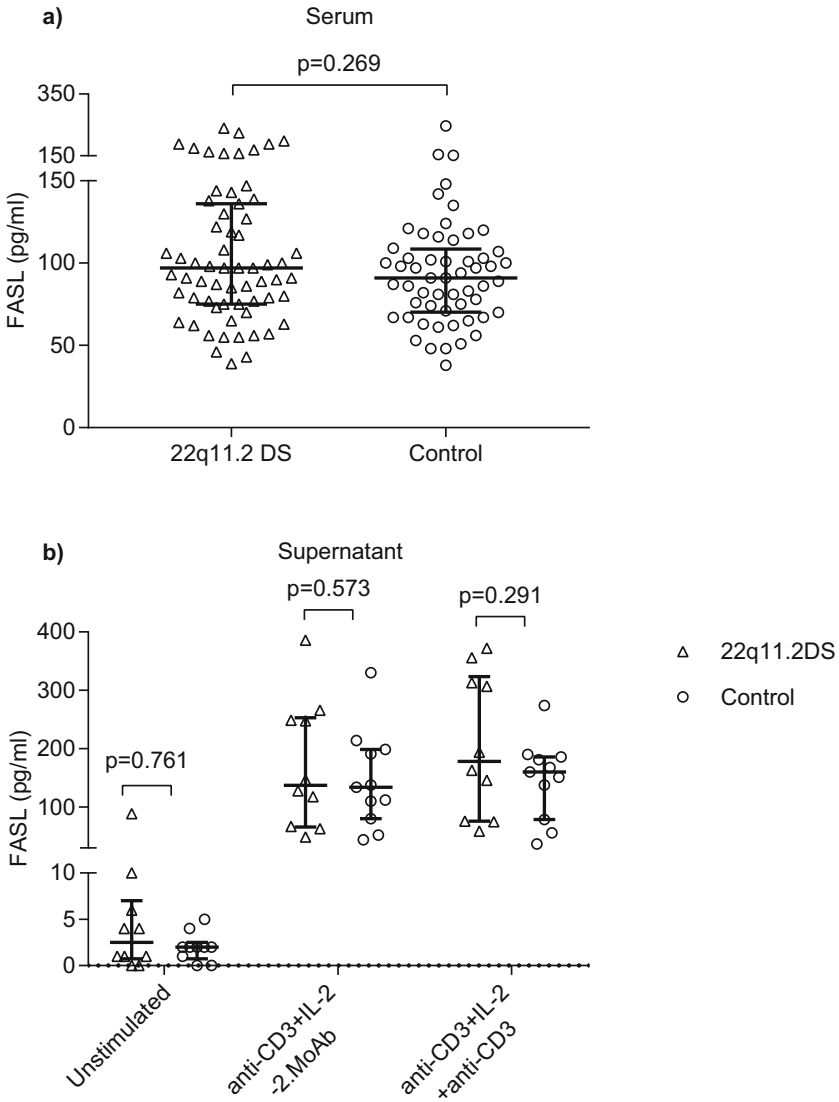

Fig. 4 Analysis of FASL levels in **a** serum from patients ($n = 63$) and controls ($n = 56$) and **b** in supernatants obtained after stimulation and reactivation with anti-CD3 mAb or left untreated from patients ($n = 10$) and controls ($n = 11$). Individual symbols identify single subjects. Horizontal line represents median value; error bars represent interquartile range

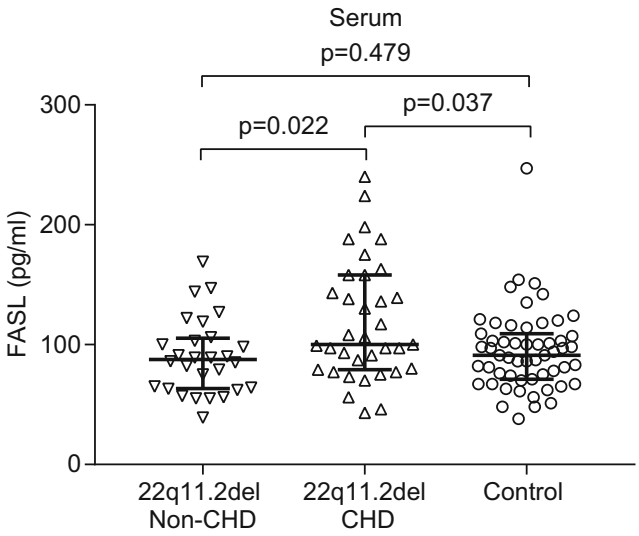

Fig. 5 Serum levels of FASL measured by ELISA in 22q11.2del with congenital heart defects ($n = 35$), without congenital heart defects ($n = 28$), and in controls ($n = 55$). Horizontal line represents median value; error bars represent interquartile range

early effector memory ($r = -0.001$, $p = 0.996$) T lymphocytes. A weak, but significant, correlation were found between FASL and CD16+/CD56+/CD3− NK cells ($r = 0.415$, $p = 0.002$), CD19+ B lymphocytes ($r = 0.415$, $p = 0.002$), CD4+/CD45RO+ memory ($r = -0.444$, $p = 0.006$), CD8+/CD27+/CD28+ naïve ($r = 0.442$, $p = 0.007$), and CD8+/CD27−/CD28− late ($r = -0.439$, $p = 0.007$). We found no correlation between serum levels of FASL and expression of FAS ($r = 0.092$, $p = 0.800$) (data not shown). Further, we found a weak to moderate negative correlation between age in months and FASL in serum ($r = -0.424$, $p = 0.001$ in patients versus $r = -0.531$, $p < 0.001$ in controls) (data not shown).

In patients, there was no correlation between levels serum FASL and FASL measured in supernatants in the ACAD group ($r = 0.097$, $p = 0.790$) and in AICD group ($r = 0.092$, $p = 0.800$). However, serum levels of FASL were moderate correlated with FASL measured in supernatants in the ACAD group ($r = 0.689$, $p = 0.027$) as well as in AICD ($r = 0.705$, $p = 0.023$) in the control group. Levels of FASL in AICD group were strongly correlated with FASL levels in ACAD group ($r = 0.959$, $p < 0.001$ in patients and $r = 0.924$, $p < 0.001$ in controls) when measured in supernatants (data not shown).

Discussion

In the present study, we investigated spontaneous apoptosis, AICD, and activated cell autonomous death (ACAD) in lymphocytes from patients with 22q11.2del compared to healthy individuals.

We did not find increased spontaneous apoptosis in patients with 22q11.2del. Upon activation, anti-FAS-induced apoptosis was significantly increased in patients with 22q11.2del compared to those in controls, while there was no difference in AICD or ACAD. We also found a significant increase in expression of FAS on freshly isolated lymphocytes from patients. There was no difference in serum levels of FASL in patients versus controls, but patients with CHD had significantly higher levels of FASL compared to those in patients without CHD.

Increased spontaneous T cell apoptosis has previously been shown in one patient with DiGeorge syndrome [9]. We analyzed spontaneous apoptosis in freshly isolated lymphocytes from 26 patients with 22q11.2del and 19 controls. In our hands, we did not find any increase in spontaneous apoptosis in the patients. Thus, we cannot confirm the finding of Gupta et al. This deviation can be explained by a number of factors. Firstly, different techniques were employed. Secondly, while Gupta et al. performed analysis in one patient, we investigated spontaneous apoptosis in 26 patients with 22q11.2del and 19 healthy controls. Thus, we could perform statistical analysis. Thirdly, the patient described in the article by Gupta et al. was a 15-month-old girl, which underwent hospitalizations every 4–6 weeks due to various infections. Thus, the spontaneous apoptosis result could represent the contraction phase after an infection.

To our knowledge, cell death of lymphocytes upon activation has never been reported in patients with 22q11.2del. Removal of activated and expanded T cells by AICD in vivo can be mimicked by an in vitro model system using TCR-activated T cells cultured in vitro with IL-2 and restimulated through the TCR [21]. It is commonly believed that AICD involves the engagement of death receptors like FAS, tumor necrosis factor (TNF), or TNF-related apoptosis-inducing ligand (TRAIL). T cell activation itself influences the susceptibility to FAS-induced apoptosis because initial T cell activation leads to upregulation of FAS [13]. Moreover, TCR restimulation results in the expression of FASL. FASL induces FAS-mediated apoptosis either of the same cell that expresses FAS or of neighboring cells [21]. Thus, the apoptosis pathway is triggered by FASL binding and clustering of surface FAS, which will trigger the extrinsic apoptosis pathway. Death receptor-independent, intrinsic pathways are also described for AICD [13]. In contrast to AICD, ACAD does not require restimulation of the TCR upon activation and is not mediated by death receptors [13]. We found no difference in the percentage of viable, early, or late apoptotic cells in patients compared to those in controls after restimulation of TCR-activated lymphocytes through the TCR with anti-CD3 mAb or left untreated. These findings suggest that AICD as well as ACAD are not impaired in 22q11.2del.

We found a statistical significant increase in apoptosis induced upon restimulation of TCR-activated lymphocytes with commercial anti-FAS reagent in patients compared to those in

controls. Further, we found a highly statistical significant increase in expression of FAS on freshly isolated lymphocytes in patients compared to those in controls confirming the previous finding of Gupta et al. [9] who reported increased levels of FAS as well as higher level of FAS mRNA in PBMC from one patient with 22q11.2del. Interestingly, we did not find any significant difference in expression of FAS on lymphocytes upon TCR activation and restimulation, but the number of patient studied is low. This can indicate that TCR induced upregulation of FAS is not impaired in 22q11.2 patients. Since we neither found a difference in AICD nor in the expression of FAS in patients compared to those in controls on activated and restimulated cultures, as well as no difference in levels of FASL in patients compared to those in controls in supernatants obtained after stimulation and reactivation with anti-CD3 mAb or left untreated, it is not clear if this increase in FAS induced apoptosis represents a defect in the apoptotic machinery, contributing to the immunodeficiency seen in the syndrome. Although FAS has been viewed primarily as a death-inducing receptor, accumulating evidence suggests that ligation of FAS also mediates a variety of non-apoptotic activities, such as cellular activation, differentiation, and proliferation [22].

It has been reported that FAS can activate multiple survival pathways including NF-κb, extracellular-signal-regulated kinase 1 and 2, p38, JUN N-terminal kinase, and AKT [21]. Moreover, FAS triggering has been shown to enhance the proliferation of human T lymphocytes [21]. It is well known that low T cell count seen in early infancy in 22q11.2del is normally corrected by homeostatic proliferation, and most adult patients have normal or nearly normal blood T cell count [4]. Further, McLean-Tooke et al. suggested that increase in T cell counts in 22q11.2del with age is due to increase in memory or effector cells [23]. Klebanoff et al. demonstrated that FAS accelerates differentiation of naïve T cells into effector memory cells [24]. Thus, increased FAS expression on lymphocytes may contribute to the proliferation of T cells seen in 22q11.2del. We did not find any correlation between FAS and T cell subpopulations. We found weak, but significant positive correlation between FASL and NK cells, CD19+ B lymphocytes, and CD8+ naïve T lymphocytes in patients. We also found a weak, but significant negative correlation between CD4+ memory and CD8+ late T cells in 22q11.2 patients. We observed a moderate to strong positive correlation between age in months and FAS expression on lymphocytes, while we found a weak to moderate negative correlation between age in months and FASL in serum. We did not find any correlation between FAS and FASL.

Another role of FAS is reported by Arai et al. who demonstrated that a FAS-mediated signal is required for optimal production of CXCL10 and CXCL9 under limiting IFN-γ concentrations [25]. Moreover, Choi et al. demonstrated that FASL do not kill cultured astrocytes, but instead induce a variety of chemokines, such as CCL3, CC CCl-2, CXCL2, and CXCL10 [26]. We have previously shown increased serum levels of CXCL10 in 22q11.2 patients, but no increase in IFN-γ compared to those in controls [27]. Thus, a link between increased levels of FAS and CXCL10 in patients with 22q11.2del may exist.

Interestingly, although we did not observed any difference in serum levels of FASL between patients and controls, we found that patients with CHD had significantly higher serum levels of FASL compared to non-CHD patients, as well as healthy individuals. This increase could possibly be explained by structural changes of the heart. Thus, FASL has been detected in the serum of patients with both myocarditis [28] and congestive heart failure [29]. It has been shown that plasma FASL levels are significantly increased in the early phases of acute myocardial infarction and that the levels of FASL are correlated with its severity [30]. Further, Huby et al. identified that FASL is a key molecule in the development of dilated cardiomyopathy and heart failure [31]. Moreover, FASL have been shown to be associated with higher coronary atherosclerotic burden, independent of traditional cardiovascular risk factors [32]. On the other hand, it has been shown that maternal serum FASL was significantly higher when fetuses had heart failure than when they did not. [33]. Further, Sallee et al. demonstrated that *FASL* gene transfer to the embryonic heart induced programmed cell death and outflow tract defects similar to those described in congenital human conotruncal heart defects [34]. Thus, it is a possibility that CHD seen in 22q11.2del can be due to increased FASL. We found no difference in FAS expression between CHD and non-CHD group. However, it can be due to the fact that we investigated the FAS expression on lymphocytes. The distribution can be different on the myocytes.

To conclude, we have shown increased FAS expression on lymphocytes in patients with 22q11.2del. We did not observe an increase in either spontaneous or activation induced apoptosis, except after restimulation with anti-FAS. We also observed that patients with CHD had significantly higher serum levels of FASL compared to patients without CHD. Further studies are needed in order to clarify the role of FAS and FASL in the pathogenesis of 22q11.2del.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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