Defective FOXP3 expression in patients with acute Kawasaki disease and restoration by intravenous immunoglobulin therapy

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Objective. The aims of this study were: 1) to investigate forkhead box P3 (FOXP3) expression in patients with Kawasaki disease (KD), exploring possible differences during the acute phase and after defervescence; 2) to evaluate a possible association of the FOXP3 single nucleotide polymorphism (SNP) 543 (SNP ID: rs2232367) with KD.

Methods. FOXP3 expression was evaluated on 8 children with KD and 15 healthy children by flow cytometry and Real-Time polymerase chain reaction (RT-PCR). FOXP3 SNP 543 was genotyped by denaturing high-performance liquid chromatography (DHPLC) and sequencing on DNA samples from 58 additional children with KD and 114 healthy donors.

Results. The frequencies of CD4+CD25+FOXP3+ regulatory T cells were significantly (p=0.0002) lower during the acute phase of KD than in sex-and age-matched healthy donors (median % ± SD: 4.8±1.3 vs. 7.7±1.7) and a similar tendency was revealed for FOXP3 mRNA transcripts (p<0.0001). FOXP3 expression increased significantly, at both protein and mRNA levels, after intravenous immunoglobulin (IVIG) therapy treatment and achieving complete remission of disease (at least 48 hrs; median 9.5 days, range 2-30). Of the 58 patients screened, only one female subject (1.7%) carried the presence of 543 SNP in heterozygosis (C>T; for a total of 1 allele out of 88), with no difference between KD patients and controls (0.0%, 0/203 alleles).

Conclusion. Our data reinforce the notion of an impaired immunoregulation in KD, suggesting also a role of IVIG treatment in modifying the Treg compartment. FOXP3 SNP 543 does not seem to be involved in susceptibility to KD in Italian children.

Introduction

Kawasaki disease (KD) is an acute, self-limited vasculitis that occurs in children of all ages but mostly in the first years of life (1, 2). Although more than 40 years have passed since the discovery of this disease (3), the etiology of the illness remains obscure, although clinical and epidemiologic features are mostly consistent with an infectious cause (4, 5). Currently, intravenous immunoglobulins (IVIG) in combination with acetylsalicylic acid (ASA) is the first-line treatment of the acute phase of KD, and early diagnosis and treatment is fundamental in order to reduce the risk of cardiac complications (6, 7). However, a subset of patients experience resistance to IVIG therapy (8). A diverse array of mechanisms has been suggested as being responsible for the anti-inflammatory activity of IVIG, but how exactly these effects are mediated are only beginning to be understood (9). Recently a novel mechanism of action of IVIG based on its immunomodulatory and anti-inflammatory potential has provided an intriguing link between IVIG and regulatory T cells (Treg) (10, 11). The existence of Treg-activating epitopes (tregitopes) in the crystallisable fragment (Fc) portion of the IgG molecule has also been recently reported (12). However, due to the complexity of immune cells interplay, the heterogeneity of regulatory T cells population (13) and of IVIG (9) many questions remain to be addressed.

Recently, CD4+CD25+FOXP3+ regulatory T cells have been extensively studied in the modulation of immune responses during infections (14) and autoimmune diseases (15); however to our knowledge only one study dealing with regulatory T cells in KD has been published (16). In this study we therefore

Competing interests: none declared.

ABBREVIATIONS:
KD: Kawasaki disease
PB: Peripheral blood
IVIG: Intravenous immunoglobulin
FOXP3: Forkhead box P3
Treg: Regulatory T cells
SNP: Single nucleotide polymorphism

Key words: Kawasaki disease, forkhead box p3, regulatory T cells, intravenous immunoglobulin.
aimed to investigate the hypothesis that an impaired immunoregulation could be associated with KD, by investigating the percentage of CD4+CD25+FOXP3+ T cells in KD patients during acute phase and after defervescence, and also monitoring FOXP3 mRNA levels by Real Time PCR. A second aim of this study was to evaluate a possible association with a polymorphism in the FOXP3 gene in patients with KD, to exclude that possible abnormalities in FOXP3-positive cells might be due to this SNP.

Materials and methods

Patients
Eight patients (3 females and 5 males) were admitted during 2008 for acute KD. Venous blood samples of acute febrile disease were collected during routine venipuncture before any medication was given. All patients were treated with an IVIG infusion at the dosage of 2 g/kg. Six out of eight patients did promptly respond to the first IVIG infusion; in one case a second IVIG dose was needed and in another patient two steroid pulses were added to IVIG treatment to achieve clinical remission. During the acute phase of disease, three patients presented coronary arteries abnormalities (two coronary dilations, one coronary ectasia that ultimately resolved within 2 weeks). An additional blood sample was collected for repeated analyses in clinical remission, after at least 48 hours (median 9.5 days, range: 2-30). A control group consisting of 28 females and 30 males (89 females and 25 males) were studied (203 total alleles). All patients were of Caucasian origin. FOXP3 exon 5 specific amplifications were performed with Platinum Taq DNA Polymerase High Fidelity (Invitrogen) according to the manufacturer’s recommendations using the following primers forward 5'-TTATTGGGATGAAGCCTGAC-3' and reverse 5'-TTATGGGATGAAGCCTGAC-3'.

DNA extraction, denaturing high performance liquid chromatography (DHPLC) and sequencing

Genomic DNA was extracted from whole blood (200 μL) from 58 patients with KD and 114 healthy controls using QIAamp DNA blood Mini Kit (Qiagen). Patients and controls were all of Caucasian origin. FOXP3 exon 5 specific amplifications were performed with Platinum Taq DNA Polymerase High Fidelity (Invitrogen) according to the manufacturer’s recommendations using the following primers forward 5'-TGGGAGT-CAGGGGTTCGAGG-3' and reverse 5'-TTATTGGGATGAAGCCTGAC-3'. Polymorphism at position 543 of FOXP3 mRNA levels were quantified by quantitative real-time PCR using the ABI PRISM 7500 Sequence Detection System (Applied Biosystems). Briefly, total RNA was extracted from PBMCs using TRIzol reagent (Invitrogen, CA, USA) according to the manufacturer’s instructions and stored at -80°C until use. The first strand of cDNA was synthesised using oligo(dT)12-18 primers and Superscript II Reverse Transcriptase (both from Invitrogen, Life Technologies, Carlsbad, CA, USA). Relative transcripts of FOXP3 mRNA were determined using TaqMan Universal Master Mix reagent (Applied Biosystem, Branchburg, New Jersey, USA), in accordance with the manufac


turer’s protocol. FOXP3 primers and TaqMan probe were purchased as an Assay-On-Demand from Applied Biosystems (assay identification numbers: Hs 00203958_m1). Each sample was assayed in duplicates or triplicates with the housekeeping gene RNaseP (Taqman Rnase P control reagents, Applied Biosystem, Foster City, CA, USA) as internal reference in 25 μl final reaction volume. The PCR conditions were as follows: 95°C for 10 min followed by 45 cycles of two-stage temperature profile of 95°C for 15 s and 60°C for 1 min. The threshold cycle number (Ct) was determined for all PCR reactions and in most cases the threshold was manually adjusted to lie within the exponential phase using ABI 7500 Fast System SDS software. Levels of mRNA expression were calculated using the 2-ΔΔCt method (17).

Quantitative real-time PCR

FOXP3 mRNA levels were quantified by quantitative real-time PCR using the ABI PRISM 7500 Sequence Detection System (Applied Biosystems). Briefly, total RNA was extracted from PBMCs using TRIzol reagent (Invitrogen, CA, USA) according to the manufacturer’s instructions and stored at -80°C until use. The first strand of cDNA was synthesised using oligo(dT)12-18 primers and Superscript II Reverse Transcriptase (both from Invitrogen, Life Technologies, Carlsbad, CA, USA). Relative transcripts of FOXP3 mRNA were determined using TaqMan Universal Master Mix reagent (Applied Biosystem, Branchburg, New Jersey, USA), in accordance with the manufac

ter’s protocol. FOXP3 primers and TaqMan probe were purchased as an Assay-On-Demand from Applied Biosystems (assay identification numbers: Hs 00203958_m1). Each sample was assayed in duplicates or triplicates with the housekeeping gene RNaseP (Taqman Rnase P control reagents, Applied Biosystem, Foster City, CA, USA) as internal reference in 25 μl final reaction volume. The PCR conditions were as follows: 95°C for 10 min followed by 45 cycles of two-stage temperature profile of 95°C for 15 s and 60°C for 1 min. The threshold cycle number (Ct) was determined for all PCR reactions and in most cases the threshold was manually adjusted to lie within the exponential phase using ABI 7500 Fast System SDS software. Levels of mRNA expression were calculated using the 2-ΔΔCt method (17).

Immunophenotypic analysis

Peripheral blood mononuclear cells (PBMCs) were isolated with lymphocyte separation medium (Eurobio, France) by standard density gradient centrifugation. For flow cytometry, 0.5-1 x 10^6 PBMCs were incubated for 15 min with CD4-peridinin chlorophyll protein-cyanin 5.5 (PerCP-Cy5.5) and CD25 phycoerythrin (PE)-conjugated Abs (both from BD Biosciences, San Jose, CA). FOXP3 intracellular staining was performed with anti-human FOXP3 flow kit (Clone 259D, Biolegend, San Diego, CA, USA) according to the manufacturer’s protocol. Briefly, surface stained cells were first fixed and permeabilised with FOXP3 1X Fix/Perm Buffer. After washing, permeabilised cells were stained with Fluorescein isothiocyanate (FITC-) conjugated anti-human FOXP3 mAb or mouse IgG1 isotype control for 30 minutes in the dark. Cells were acquired on a FACSAnstrument flow cytometer and analyzed using FACSdiva software v 5.0.2 (BD Biosciences) and FlowJo software v 7.1.2 (Tree Star, Inc.). Gates for CD4+FOXP3+ T cells were set according to isotype negative control. The gate for whole CD4+CD25+ T cells was set using as a control their respective unstimulated cells. A total of 100,000 events were acquired for each sample, and an initial lymphocyte gate was defined according to forward scatter (FSC) and side scatter (SSC).

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Statistical analysis

Comparison of CD4+CD25+FOXP3+ percentages and of the amount of FOXP3 mRNA was performed using Student t-test. Fisher’s exact test was used to compare the frequency of 543 SNP between KD patients and controls
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A

CD4 PerCP-Cy5.5

CD25 PE

FOXP3 FITC

B

CD25 PE

FOXP3 FITC

Fig. 1. Phenotypic characterisation of CD4+CD25+, CD4+FOXP3+ (panel A) and CD4+CD25+ FOXP3+ T cells (panel B) in a Kawasaki Disease patient during the acute phase (top) and a healthy control (bottom). In each plots CD25 and FOXP3 expression was determined on CD4+ gated cells. Gate for FOXP3+ T cells were set according to isotype control IgG1 antibody. An initial lymphocyte gate was defined according to forward scatter and side scatter.

Fig. 2. (A) Percentage of CD4+CD25+FOXP3+ T cells in Peripheral Blood (PB) of Kawasaki Disease (KD) patients compared to healthy controls. Data are shown as box plots. The horizontal lines of the boxes denote the 25th, 50th (median) and 75th percentiles. The lines outside the boxes represent the minimum and the maximum value (excluding outliers); (B) Relative expression of FOXP3 in PBMCs as determined by Real-Time PCR. FOXP3 expressions levels were determined with the 2^ΔΔCT methods (20) and presented as mean ± SEM.

or allele frequency of Italian population. A p-value of less than 0.05 was considered to be statistically significant.

Results

**FOXP3 expression in KD patients during acute disease phase**

At the time of enrollment, during the acute phase of KD disease, we observed a lower percentage of CD4+CD25+ T cells in peripheral blood of patients compared to healthy controls (median % ± SD: 10.1±2.9 vs. 16.0±3.9; p=0.0012; Figure 1, panel A). Moreover, the percentage of CD4+CD25+ T cells that co-express FOXP3 (see Fig. 1. Panel B for a representative dot plot between KD patients and controls) was significantly (p=0.0002) lower in KD patients when compared with healthy controls (median % ± SD: 4.8±1.3 vs. 7.7±1.7; Fig. 2A). Similarly, at the baseline, during acute phase of disease, the amount of FOXP3 transcripts in KD patients was 2.9±0.6 median fold lower than in control subjects (p<0.0001; Fig. 2B).

**FOXP3 expression in KD patients after clinical remission**

After at least 48 hours from last treatment (median 9.5 days; range: 2-30) and after achieving complete remission of disease (defervescence of fever; decrease of C-reactive protein and white blood cell counts) the percentage of CD4+CD25+FOXP3+ cells and the relative mRNA levels of FOXP3 showed a significant increase, returning to levels comparable to those observed in healthy controls (Fig. 3A and 3B). A representative flow cytometric analysis of FOXP3 expression on CD4+CD25+ T cells during acute phase disease and after IVIG treatment and complete clinical remission is shown in Figure 3C. No difference in FOXP3 expression levels between patients with or without coronary arteries abnormalities was seen at both protein and molecular level (data not shown).

**Evaluation of 543 (C>T) polymorphism**

Of the 58 patients screened, only one female subject carried the presence of 543 SNP in heterozygosis (C>T) with no statistical difference between KD patients and controls (0.0%, 0/203 alleles) or a Caucasian population (C>T control allele frequency 0.03, n=371) (18).

**Discussion**

Although a number of immunoregulatory abnormalities have been demonstrated during the acute phase of KD disease, including activation of monocytes, macrophages, helper T cells and B cells and deficiency of suppressor/cytotoxic T cells, (19-21), none of these alterations appear to be specific for the disease or has been identified as a potential target for therapy. An increased production of proinflammatory cytokines such as IL-1, IL-6, tumour necrosis factor α, TNF-α, have also been reported during the acute phase of KD disease and correlated with vascular endothelial cell activation and antibody-mediated injury (22, 23).
Taken together, these studies suggest that the acute phase of KD may be accompanied by alterations of immune regulation which may result in an impaired inflammatory response. Intravenous immune globulin have a beneficial effect in KD and in many other autoimmune and systemic inflammatory conditions, and it is generally accepted that the immunomodulatory effects of IVIG are directed against a wide range of targets (24-25). One of the major mechanisms of the antiinflammatory effects of immune globulins is likely to be the modulation of cytokine production (26), and decreased levels of the inflammatory cytokine interleukin-1 have been reported in Kawasaki disease after the administration of intravenous immune globulin (23). In addition, infusion of IVIG has also been reported to interfere with activation of T and B lymphocytes in patients with KD (27). As mentioned, IVIG has been shown to expand regulatory T cells both in vitro and in vivo (9, 10), but only one study has thus far addressed this issue in KD (16).

In this paper, the authors have analysed the percentage of CD25+CD4+ T cells and the amounts of FOXP3 mRNA in patients with KD during acute-phase disease and after IVIG treatment. It should be emphasised that, although CD25 (the interleukin 2 receptor alpha chain) is considered the best surface marker of regulatory T cells, it can be expressed on any T cell following activation (17, 28). So, the percentage of T lymphocytes co-expressing CD4 and CD25 should not be interpreted as frequency of Treg, especially in those disease, such as KD, where abnormalities in T cell activation may be involved.

In contrast, the transcription factor forkhead box p3 (FOXP3) appears to be the most specific marker for CD4+CD25+ Treg cells and allows a more precise identification of the CD4 T-cell subset with a regulatory/suppressive phenotype (29). Nevertheless a precise definition of “regulatory CD4+ T cells” still remains controversial. Indeed it has become clear that effector T cells can transiently express FOXP3 following TCR-mediated activation (30). In the present study we show for the first time that Treg cells are significantly decreased in patients with clinically active KD. Similarly, FOXP3 mRNA gene expression levels were found decreased in the pre-IVIG samples compared with control samples. We also found, not surprisingly, that FOXP3 mRNA levels strongly correlated with the percentages of CD4+FOXP3+ and CD4+CD25+FOXP3+ T cells (data not shown). Interestingly, the proportion of regulatory T cells and the amounts of FOXP3 mRNA increased in the deferescence phase, after IVIG treatment. These changes appeared not to be related to clinical variables such as coronary involvement, but our numbers are too small to draw any definite conclusions. This is of importance considering that other investigators have already shown that the presence of IVIG with Treg in culture is significantly able to increase FOXP3 mRNA expression (10), and that expansion of CD4+CD25+ regulatory cells by IVIG results in a significant protection from the development of MOG-induced EAE (11). In one patient who did not respond to conventional treatment with IVIG, but to steroid pulses, we also found restored expression of FOXP3, at both protein and molecular levels. Although the effect of steroids on regulatory T cells was already known in other autoimmune conditions in both mice and human (31, 32), it has never been reported in Kawasaki disease. The restoration of FOXP3 expression following steroid treatment is consistent with the conclusion that the absence of the inflammatory state may be the most reasonable mechanism to explain normalisation of Tregs. In fact, and as discussed above, general inflammatory and composition of the inflammatory milieu, as well as autoimmune genetic background, may all contribute to the observed Tregs defects seen during the acute phase. Although our study did not provide a direct effect of IVIG on Treg cells (and the modulation of the inflammatory state by IVIG should be also taken into account for a more complete description and interpretation of our result) this phenomenon cannot be excluded (10, 11).

The current study also documented that an individual SNP (rs2232367) in the FOXP3 gene, responsible for a C>T transition in the first base of exon 5, that has been reported to likely affect splicing process and, consequently, FOXP3
mRNA expression, and that might be of importance in autoimmune disorders (33) does not differ between KD patient and healthy controls. The dysregulation in FOXP3 expression in the acute phase is therefore not secondary to this particular SNP.

We are aware of the limits of our study. First of all, the small sample size, due to the relative rarity of this disease in western countries and to the need to draw additional amounts of blood in young children. Second, as we did not include febrile controls we cannot exclude that inflammation per se could have played a role in the abnormalities seen. However, the results were consistent in all eight patients tested and therefore unlikely due by chance. However, to our knowledge the FOXP3 expressing cells have never been studied in KD and our preliminary findings provide novel theories for the disease pathogenesis and IVIG mechanisms of action.

References