Expression patterns of Th1 and Th2 cytokine genes in childhood idiopathic thrombocytopenic purpura (ITP) at presentation and their modulation by intravenous immunoglobulin G (IVIg) treatment: their role in prognosis

Athanasia Mouzaki, Maria Theodoropoulou, Ioannis Gianakopoulos, Vassiliki Vlaha, Maria-Christina Kyrtsonis, and Alice Maniatis

Childhood idiopathic thrombocytopenic purpura (ITP) resolves usually after the first episode, although it may recur, and in 10% to 20% of patients develops into a chronic disorder. Evidence of the immunoregulatory role of Th1/Th2 responses in autoimmune diseases prompted us to perform a prospective study of Th1/Th2 gene expression profiles and transforming growth factor β (TGF-β) plasma levels in 18 children (median age, 6.4 years) with acute ITP, before and after intravenous immunoglobulin G (IVIg) infusion, and during a follow-up period (0.5-5 years). Initially, 12 of 18 patients had either low Th0/Th1 plus interleukin 10 (IL-10) or no in vivo cytokine gene expression (0). At 24 hours after IVIg infusion this pattern became 0 or Th2 (9 of 12) or remained low Th0/Th1 (3 of 12). During follow-up these patients did not relapse and maintained 0 or Th2 pattern without IL-10. Of the remaining 6 patients, 4 presented with a Th1 or Th0/Th1 pattern plus IL-10 that persisted after IVIg treatment (although interferon-γ [IFN-γ] expression diminished) and stabilized to Th1 plus IL-10 at follow-up, which was marked by infrequent episodes of ITP. Two patients presenting with a strict Th1 pattern characterized by high expression of IFN-γ, which remained unchanged after IVIg and at follow-up, can be characterized as chronic ITP. TGF-β plasma levels were low in patients with active disease and increased in remission. Overall, acute ITP presents with Th1, Th0/Th1, or 0 in vivo cytokine gene expression. Stable remission is associated with a 0 or Th2 pattern. A 0 or Th2 pattern after IVIg gave the best prognosis, whereas sustained high expression of IFN-γ and refractoriness to IVIg were the main indicators of poor prognosis. (Blood. 2002;100:1774-1779)

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In recent years, numerous studies have shown that patients suffering from autoimmune diseases have polarized Th1 or Th2 responses. Childhood ITP does not seem to fit the definition of an established autoimmune disease and although spontaneous remission occurs in the majority of children, it still remains impossible to predict at the time of diagnosis which child will develop an acute self-resolving disorder and which a chronic disorder. To this end, we studied the expression of a panel of Th1 and Th2 cytokine genes in a group of children who presented with ITP and assessed the effect of IVIg administration on the cytokine gene expression of the above patients. In addition, we performed follow-up studies in the same children 0.5 to 5 years later to investigate whether the primary findings can be of prognostic value. Although the data obtained are based on a small number of cases studied, it appears that the Th cytokine profile at presentation and after IVIg infusion can predict the clinical course of childhood ITP.

### Patients, materials, and methods

#### Patients

Eighteen patients with acute ITP and 14 healthy children as controls were studied. All subjects presented to the Pediatric Department of the Patras University Hospital (PUH). Samples of heparinized blood (0.5-5 mL) were drawn from patients prior to any treatment and 24 hours after completion of IVIg administration, and from controls only once during a routine visit to PUH for minor problems. Samples were also collected at follow-up visits at least once for each patient. Informed consent was obtained from each participating patient (when old enough) and the patient’s parent or guardian.

Human experimentation guidelines were submitted to and approved by the internal review board and scientific advisory committee of PUH. PUH abides by the Helsinki declaration on ethical principles for medical research involving human subjects.

#### IVIg

The IVIg preparation used was Sandoglobulin (Novartis, Basel, Switzerland). When reconstituted for therapeutic use it contained 50 mg/mL IgG, 25 to 35 mg/mL sucrose, 6 to 10 mg/mL glucose, and 40 to 100 mM NaCl. For in vitro studies, 1 mL of 15 different reconstituted IVIg preparations was kept stored at −20°C.

#### Cell cultures

Heparinized venous blood was collected from the ITP patients at presentation, 24 hours after IVIg treatment, and at follow-up visits as well as from healthy pediatric controls. Peripheral blood mononuclear cells (PBMCs) were prepared by centrifugation over a ficoll-paque gradient (Pharmacia, Uppsala, Sweden). Plasma was collected and stored at −80°C. The cells (10⁶ PBMCs/group) were processed immediately or cultured for 8 hours in RPMI 1640 culture medium (Gibco BRL, Gaithersburg, MD) containing 10% fetal calf serum (FCS; Gibco BRL) and other supplements as previously described, in the presence of 5 ng/mL phorbol myristate acetate (PMA) and 1 μM ionomycin (Sigma, St Louis, MO). The cells were counted using a Sysmex NE-8000 counter (Kyoto, Japan) and their viability was estimated by the trypan blue exclusion method as described previously.

#### TGF-β1 ELISA

Determination of TGF-β1 levels in the plasma and also in different IVIg preparations was performed by an enzyme-linked immunosassay (ELISA) as instructed by the manufacturer (Quantikine; R & D Systems, Minneapolis, MN).

#### Reverse transcriptase–polymerase chain reaction (RT-PCR)

Total cellular RNA isolated by the guanidinium thiocyanate-phenol-chloroform extraction procedure, as described by Chomczynski and Sacchi, was reverse transcribed (RT) after heat denaturation and annealing, with Random Hexamer (Promega, Madison, WI), in the presence of 200 U Superscript RT (Gibco BRL) and 0.5 mM of each deoxynucleotide (Promega), in 50 μL, for 1 hour at 37°C. Then 1 μL of the RT mixture was submitted to polymerase chain reaction (PCR), in a volume of 50 μL, in the presence of 150 μM of each deoxynucleotide, 2.5 U Taq polymerase (Gibco BRL), and 0.25 μM of the upstream and downstream primers (Institute of Molecular Biology, Crete, Greece). Each reaction was carried out with RNA extracted from 6.5 × 10⁶ viable PBMCs. The PCR products were run on 2% agarose gels and stained with ethidium bromide for UV light visualization and photography. For quantitative evaluation, the bands were scanned and the data analyzed using ImageTool V1.28 software (University of Texas Health Science Center, San Antonio, TX). The RT-PCR signal generated by β2-microglobulin (β2m) mRNA was chosen to estimate the amounts of cDNA obtained from different cell samples. Each RT-PCR included controls for RNA extraction (tissue buffer alone treated as a normal sample), RT (RT reagents without RNA), and PCR (PCR reagents without cDNA). The PCR primer pairs used in this study are shown in Table 1.

#### EMSAs

Electrophoretic mobility shift assays (EMSAs) were performed to assay for the presence and function of the lymphotrophic transcription factors (TFs) nuclear factor-κB (NF-κB), activator protein-1 (AP-1), and nuclear factor of activated T cells (NFAT) as described previously with modifications. Briefly, the cellular membranes were broken by sonication on ice and a 3 to 4 × ρellet volume of a buffer containing 5 mM HEPES (N-2-hydroxyethylpiperazine-n’-’-ethanesulfonic acid), pH 7.9, 26% glycerol, 1.5 mM MgCl₂, 0.2 mM EDTA (ethylenediaminetetraacetic acid), 0.5 mM DTT (dithiorhodophenilirichlorothione), 0.5 μM PMSF (phenylmethyl-sulfonyl fluoride), and 1 μg/mL leupeptin (all from Sigma) was added and then salt (KCl) adjusted to 300 mM to elute the proteins from chromatin. After an incubation of 30 minutes on ice, the cell lysates were centrifuged at 27 000g, for 60 minutes, at 4°C to sediment chromatin. Protein concentration was determined using the Bradford assay (Bio-Rad, Hercules, CA). As an oligonucleotide probe we used the sequences AGTGTAGGGATTGTAGGCACTTTG for NF-κB, GTGACTCAGGGCG for AP-1, and AAGAGAACACACTGTTT for NFAT.

### Results

#### Patients

Table 2 shows the age, sex, and platelet counts of patients and controls. Table 3 shows clinical and laboratory parameters of patients that the Th cytokine profile at presentation and after IVIg infusion can predict the clinical course of childhood ITP.

Table 1. Primers and conditions for the RT-PCR experiments performed in this study

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence of primers (5’ → 3’)</th>
<th>T (°C)</th>
<th>Product</th>
</tr>
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<tbody>
<tr>
<td>IL-2</td>
<td>GCCAAGTCCCCCTTGGCATTTG</td>
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<tr>
<td>IFN-γ</td>
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<td>492</td>
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<tr>
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<tr>
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</tr>
<tr>
<td>IL-10</td>
<td>ACCGGTCCAGAGACCTG</td>
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<td>260</td>
</tr>
<tr>
<td>IL-13</td>
<td>CTCTGTTCTGCCCTCTGTCTCTCT</td>
<td>61</td>
<td>234</td>
</tr>
</tbody>
</table>

| β2m    | GCCCACTGAAAGAATGAG         | 56     | 150     |

T (°C) indicates annealing temperature; product, PCR product size (bp).
The results are from RT-PCR performed on PBMCs isolated from children (P1-P12) and processed immediately (ex vivo) and the patients. The results are from RT-PCR performed on PBMCs isolated from all the pediatric controls expressed none of the cytokine genes tested ex vivo, whereas all expressed these cytokines when cultured with mitogens. (2) IL-3, IL-6, and IL-13 expression was within control range in all patients (data not shown). (3) All cytokine genes tested in this study were able to be induced by mitogens in all the patients (data not shown). (4) For the Th pattern analysis of the patients we used the data obtained for the cytokines IL-2, interferon-γ (IFN-γ), and IL-4 (Figure 1); IL-10 data are shown separately (Figure 2) and were not included in this analysis because non-T cells such as monocytes can produce high amounts of this cytokine also.23

Patients P1 to P12 (Figure 1, group 1) are those who had one acute episode only. They presented with a low Th0/Th1 pattern (7 of 12) or no ex vivo cytokine expression (5 of 12). After IVIg infusion the majority of these patients expressed no cytokine in vivo or IL-4 only (Th2) exactly like patients P8 and P9 of this group who required no therapy. Patients P1, P2, and P5, who required steroids after a moderate response to IVIg infusion, are the ones who presented with a Th1/Th2 pattern with the highest values of IL-2/IFN-γ of this group; this pattern was maintained after IVIg infusion though the intensities of IL-2 and IFN-γ gene expression were lower. At follow-up, 1 to 4 years later, all 12 patients expressed none of the cytokines tested in vivo or they expressed IL-4 only (Th2).

Patients P13 to P16 (Figure 1, group 2, gray histograms) are the ones with an occasional episode of thrombocytopenia triggered by viral infections, who require no maintenance treatment. They presented with Th1 or Th0/Th1 pattern that was maintained after IVIg infusion but with lower expression of IFN-γ. At follow-up, 0.5 to 5 years later, they all have a Th1 (IFN-γ) in vivo pattern.

Patients P17 and P18 (Figure 1, group 2, black histograms) are those with chronic disease requiring maintenance steroid therapy.

Ex vivo Th1/Th2 cytokine gene expression in the ITP patients versus healthy pediatric controls

Figures 1 and 2 depict the data obtained from the pediatric controls and the patients. The results are from RT-PCR performed on PBMCs isolated from blood and processed immediately (ex vivo) and are given in pixels (mean values ± SEM).

For the sake of brevity, we state at this point that (1) PBMCs isolated from all the pediatric controls expressed none of the cytokine genes tested ex vivo, whereas all expressed these cytokines when cultured with mitogens. (2) IL-3, IL-6, and IL-13 expression was within control range in all patients (data not shown). (3) All cytokine genes tested in this study were able to be induced by mitogens in all the patients (data not shown). (4) For the Th pattern analysis of the patients we used the data obtained for the cytokines IL-2, interferon-γ (IFN-γ), and IL-4 (Figure 1); IL-10 data are shown separately (Figure 2) and were not included in this analysis because non-T cells such as monocytes can produce high amounts of this cytokine also.23

Patients P1 to P12 (Figure 1, group 1) are those who had one acute episode only. They presented with a low Th0/Th1 pattern (7 of 12) or no ex vivo cytokine expression (5 of 12). After IVIg infusion the majority of these patients expressed no cytokine in vivo or IL-4 only (Th2) exactly like patients P8 and P9 of this group who required no therapy. Patients P1, P2, and P5, who required steroids after a moderate response to IVIg infusion, are the ones who presented with a Th1/Th2 pattern with the highest values of IL-2/IFN-γ of this group; this pattern was maintained after IVIg infusion though the intensities of IL-2 and IFN-γ gene expression were lower. At follow-up, 1 to 4 years later, all 12 patients expressed none of the cytokines tested in vivo or they expressed IL-4 only (Th2).

Patients P13 to P16 (Figure 1, group 2, gray histograms) are the ones with an occasional episode of thrombocytopenia triggered by viral infections, who require no maintenance treatment. They presented with Th1 or Th0/Th1 pattern that was maintained after IVIg infusion but with lower expression of IFN-γ. At follow-up, 0.5 to 5 years later, they all have a Th1 (IFN-γ) in vivo pattern.

Patients P17 and P18 (Figure 1, group 2, black histograms) are those with chronic disease requiring maintenance steroid therapy.

Table 2. Data of study subjects

<table>
<thead>
<tr>
<th>Patient</th>
<th>Sex</th>
<th>Age (years)</th>
<th>PLT (10^9/L)</th>
<th>IVIg (d)</th>
<th>Time after IVIg (y)</th>
<th>Current condition</th>
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Group 1 (P1-P12), patients with acute ITP followed by long-term remission; P8 and P9 received no treatment; group 2 (P13-P18), patients P13 to P16 with acute ITP followed by infrequent relapses triggered by viral infections; P17 and P18 had acute ITP followed by frequent episodes occurring within periods of less than 1 month, regardless of infection, requiring continuous monitoring. Only the times when blood samples were given for the present study are shown in the follow-up phase.

PLT indicates number of platelets (10^9/L); IVIg, 2 g/kg body weight (daily dose); NA, not applicable; time after, time in years after the first episode when blood sample was taken for the follow-up study.
They have a constant Th1-high-IFN-γ pattern in all phases of the disease studied. At follow-up, 0.8 to 1.1 years later, they maintain the highest IFN-γ levels of all patients with ITP.

**Ex vivo IL-10 gene expression**

On the whole, IL-10 gene expression (Figure 2) correlated negatively with disease activity (Figure 2, total). In group 1, it was highly expressed in the acute phase; its expression decreased after IVIg infusion and reached zero levels at follow-up.

In group 2, IL-10 expression was highly expressed at all times in the relapsing category with a transient decrease after IVIg infusion. Its expression decreased after IVIg infusion and reached zero levels at follow-up.

**Absence of a transcriptional silencer in patients with ITP**

The TFs AP-1, NF-κB, and NFAT regulate cytokine transcription.34,35,37 Of the 3 TFs, AP-1 and NF-κB are exclusively positive regulators, whereas NFAT TFs play a dual role. An NFAT-silencer (NFAT-S) down-regulates transcription and an NFAT-transcription activator (NFAT-A) up-regulates transcription. The silencer is only detectable in resting naive CD4 T cells and is lost in recently activated cells (effectors) and resting memory cells. The activator is detectable in activated and memory cells.24,38 It was thus suggested that the presence of a silencer contributes to the more stringent activation requirements of naive CD4 T cells, thus safeguarding against autoimmunity.24 To test this hypothesis, we performed EMSA experiments with AP-1, NF-κB, and NFAT probes and nuclear extracts from ex vivo or mitogenically stimulated peripheral blood lymphocytes (PBLs) from a healthy child and 2 children with ITP from group 1, in the acute phase and in remission.

Phenotypic analysis performed in remission showed that these patients, like the healthy child, had over 50% resting naive CD4 T cells (data not shown). The results are shown in Figure 4. At presentation, the patients, in contrast to the healthy control, had AP-1, NF-κB, and NFAT-A activity in their ex vivo T cells and, similarly to the healthy control, in their mitogenically stimulated cells. In remission, none of these TFs were present in the nucleus of the patients’ ex vivo cells but were present in their stimulated cells, that is, the same pattern with that in the healthy control, with the exception that the NFAT-S was missing.

**Discussion**

Established autoimmune diseases have polarized Th1 and Th2 responses.19-23 Acute ITP is a childhood autoimmune disorder that either resolves after the first episode or develops into a relapsing form with rare episodes triggered by viral infections or, rarely, into a more serious chronic form that requires maintenance treatment.2,3,5 Studies performed with a selected population of chronic adult ITP patients with active disease observed neither a clear-cut Th1 nor a Th2 serum cytokine profile,39 whereas studies performed with a mixed population of patients with acute or chronic or...
The patients with high expression of IFN-γ at presentation (group 2) had either a transient response to IVIg (low IFN-γ; Th1, or Th0/Th1) or were refractory to IVIg (high IFN-γ; Th1). An important differentiating factor between the milder relapsing form and the aggressive chronic form of ITP in group 2 is the presence or absence of the anti-inflammatory cytokine IL-10. IL-10 provides a negative feedback mechanism that counteracts the activation of Th1 cells and monocytes/macrophages, so the complete absence of IL-10–expressing cells in the peripheral blood of patients P17 and P18 probably contributes to the enhanced Th1 cytokine synthesis by the autoreactive T-cell clones, which, in turn, inhibits IL-4 synthesis in these patients.

Based on the above, we propose the model depicted in Figure 5. Multicenter studies of children presenting with ITP (RT-PCR/cytokine gene expression) and receiving none or IVIg-only treatment, and followed for at least 6 months, will test the validity of our pilot study.

The TGF-β levels in the IVIg preparations tested were negligible, thus appearing inadequate to influence cytokine expression in the patients. The very low plasma TGF-β concentrations at the time of disease onset increased after IVIg treatment. We may assume that the low TGF-β levels during active disease leave the autoantibody production against autologous platelets uncontrolled. In addition, because TGF-β promotes thrombopoietin production, which, in turn, leads to increased platelet production, low TGF-β levels may also account directly for low platelet numbers during active disease. The relative increase of TGF-β levels in the patients after treatment and in remission may mediate a bystander immune suppression, which may be adequate for the majority of the patients who recover or have rare episodes of thrombocytopenia but inadequate for the patients with chronic active disease.

A noteworthy aspect of this study is the EMSA finding (Figure 4) that the NFAT transcriptional silencer was absent from the nucleus of the ex vivo T cells of the 2 patients tested who were in remission. The positive TFs AP-1 and NF-κB were not active in these cells, indicating that the cells were not in vivo activated, whereas all positive factors including NFAT-A were activated in the in vitro–stimulated cells indicating that the cells were not anergic. Acquisition of the NFAT-S, NFAT-binding silencer.

chronic-complex ITP showed a Th0/Th1 pattern of T-cell activation.

We analyzed the Th cytokine expression patterns in 18 children with acute ITP, before and after IVIg administration, and at follow-up. In parallel, we determined the expression pattern of IL-10 and plasma levels of TGF-β.

Compared to the healthy pediatric controls who expressed no Th cytokines in vivo, the majority of the children who presented with acute ITP were expressing IL-2, IFN-γ with or without IL-4 cytokine genes in vivo (72% of the sample), suggesting an early CD4 Th0 and Th1 cell activation, in accordance with an earlier study. A minority of the patients (28% of the sample) presented with no cytokine expression and these were 5 patients of group 1 who went into complete remission after IVIg infusion or with no treatment at all (2 patients).

Of the patients expressing Th1 or Th0/Th1, the first differentiating factor was their response to IVIg infusion. Those who expressed no cytokine or Th2 after IVIg treatment required no other therapy and went into long-term remission maintaining the 0 or Th2 pattern at follow-up. Those who maintained the Th1 or Th0/Th1 pattern after IVIg treatment were either refractory to IVIg or had a transient response to it. The differential effect of IVIg treatment in ITP patients probably reflects different degrees of imbalance of the immune system of patients, which could, in turn, reflect the relative frequency of autoreactive clones in the periphery. It has been shown that IVIg induces direct apoptosis of activated T and B lymphocytes and also monocytes/macrophages. We may thus hypothesize that the effectiveness of IVIg treatment correlates negatively with the severity of the disease.

The second differentiating factor was the relative intensity of IFN-γ gene expression: The patients with low IFN-γ expression at presentation (group 1) either responded completely to IVIg (0 or Th2 pattern after termination of treatment) or required steroids in addition (maintenance of low Th0/Th1 expression after termination of treatment), but then went into stable remission.
converting proliferating precursors into resting cells during T-cell maturation. We hypothesize that this at this stage of lymphocyte maturation causes a failure in the acquisition of the NFAT-S by some otherwise mature thymocytes and results in an increased number in the periphery of naïve T cells, which are more prone to develop into autoreactive clones on antigenic stimulation.

**Noted added in proof.** Since this manuscript was submitted for publication a year has passed during which the patients included in this study were monitored, and their follow-up diagnosis remains the same.

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**References**

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