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A transforming growth factor-β1-mediated bystander immune suppression could be associated with remission of chronic idiopathic thrombocytopenic purpura

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Abstract Bystander immune suppression has been demonstrated in experimental models of oral immune tolerance induction. This phenomenon is associated with expression of transforming growth factor (TGF)-β1 and T-helper cell (Th) 2 cytokines. We have studied serum levels of Th cytokines and B- and T-lymphocyte subsets in chronic idiopathic thrombocytopenic purpura (ITP), a disorder in which the production of platelet autoantibodies might be caused by a cytokine network dysregulation. Forty-six patients with ITP were separated into three groups depending on the platelet count (pltc): (1) $<50\times10^9$/l, (2) 50–150×10^9/l and (3) $>150\times10^9$/l. We found significantly elevated plasma levels of the Th3 cytokine TGF-β1 in patients with pltc $>150\times10^9$/l (23.5 ± 2.8 ng/ml), compared with patients with pltc $<50\times10^9$/l (2.3 ± 0.6 ng/ml; $P<0.0001$), patients with pltc 50–150×10^9/l (7.2 ± 1.7 ng/ml; $P<0.0001$) and healthy volunteers (9.8 ± 1.3 ng/ml; $P<0.01$). The serum levels of the Th1 cytokines interleukin (IL)-2 and interferon (IFN)-γ were below the detection limits of the assays. Likewise, the Th2 cytokine IL-4 was not detectable or was very low both in patients and controls. The serum levels of IL-10, a Th2 cytokine, were within the assay range and patients with pltc $<50\times10^9$/l had significantly lower levels (0.6 ± 0.1 pg/ml) than both patients with pltc 50–150×10^9/l (1.8 ± 0.1 pg/ml; $P<0.005$) and healthy volunteers (1.4 ± 0.1 pg/ml; $P<0.005$). Furthermore, patients with pltc $<50\times10^9$/l and splenectomised patients had significantly higher levels of CD4+CD25+ activated T cells [26.2 ± 14.8% ($P<0.05$) and 26.7 ± 11.9% ($P<0.005$), respectively] than healthy controls (16.5 ± 4.0%). Also, the number of natural killer (NK) cells among patients with pltc $>150\times10^9$/l were significantly elevated (26.6 ± 16.0%; $P<0.05$) compared with controls (17.4 ± 7.6%). In conclusion, our data corroborate previous findings of elevated numbers of activated T cells in chronic ITP patients with active disease, but neither a clear-cut Th1 nor a Th2 serum cytokine profile could be established. However, ITP in remission was associated with elevated TGF-β1, which might be a part of a bystander immune suppression. We propose that the effect of possible expression of TGF-β1 by oral immune tolerance induction deserves to be explored in ITP patients with an active disease.

Key words TGF-β1 · ITP · Bystander immune suppression · T-cell activation

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Introduction

Chronic idiopathic thrombocytopenic purpura (ITP) is an immune disorder characterised by enhanced platelet destruction in the reticuloendothelial system due to platelet autoantibodies [28]. The targets for the platelet autoantibodies are most frequently the membrane glycoprotein (GP) IIb/IIIa and GP Ib/IX [25, 49, 50]. There is increasing evidence that regulatory T lymphocytes play an important role in the pathogenesis in this disorder, and a variety of abnormalities within the T-cell population have been described in ITP [13, 24, 30, 31, 34, 40, 41, 44, 46]. However, the results reported are inconsistent. Several investigators have found increased numbers of human leukocyte antigen (HLA)-DR+ and CD25+ T lymphocytes, suggesting that the T cells are abnormally activated in ITP [13, 31, 41]. Furthermore, several reports on serum cytokines in ITP have pointed towards a specific CD4+ T helper (Th) cell and macrophage activation [8, 9, 18, 35, 43, 51]. Some

investigators have described a Th1 response [9, 41, 51], whereas others have described either a less prominent Th1 response [18, 35] or a mixed Th0/Th1 response [43]. Conversely, Crossley et al. [8] found a Th2 response in patients with chronic ITP. The possible relationship of therapy and acute and secondary forms of the disorder on these seemingly inconsistent results is unclear. The objective of the present study was to form a base for future specific immune therapies by describing the Th1–Th3 cytokine profile and the lymphocyte immunophenotype using well-characterised patients with chronic ITP and highly sensitive and standardised techniques.

Materials and methods

Patients

Forty-six patients with chronic ITP (30 females and 16 males), aged 17–90 years (mean 48 years), were enrolled in the study. The diagnosis of ITP was based on thrombocytopenia lasting more than 6 months, normal or increased number of megakaryocytes in a bone marrow biopsy, absence of splenomegaly and exclusion of other known causes of thrombocytopenia [such as connective tissue disease, malignancy, human immunodeficiency virus (HIV) or drug-induced disorders]. All patients were regularly followed up by an experienced haematologist. The patients were separated into three groups based on their platelet count (pltc): (1) active disease $<50\times10^9$/l (range 18–49×10^9/l; mean $29\pm13\times10^9$/l) ($n=8$), (2) stable disease 50–150×10^9/l (range 60–142×10^9/l; mean $101\pm26\times10^9$/l) ($n=22$) and (3) in remission $>150\times10^9$/l (range 163–351×10^9/l; mean $233\pm59\times10^9$/l) ($n=16$). Patients in remission were stable, with pltcs persisting above 150×10^9/l. The control material consisted of 18 healthy volunteers, aged 20–55 years (11 females and 7 males) with normal pltcs (range 177–385×10^9/l; mean $243\pm52\times10^9$/l). Further patient characteristics are given in Table 1. A standard venipuncture technique for multiple blood sampling (Vacutainer, Becton Dickinson, San Jose, Calif.), avoiding venous occlusion, was employed. Approximately 5 ml blood was collected into a tube containing ethylenediaminetetraacetate (EDTA) and used for determination of pltc by an automatic blood cell counter (Technicon H2, Technicon, N.Y.).

Cytokine analysis

Sera from the patients and the controls were separated from 10 ml whole blood by centrifugation at 2000 *g* for 20 min and stored at –20 °C until assay. The serum levels of interleukin (IL)-2, interferon (IFN)-γ, IL-4 and IL-10 were determined using commercially available enzyme-linked immunosorbent assay (ELISA) kits (R & D Systems, Minn.). For the plasma transforming growth factor (TGF)-β1 assay (R & D Systems), platelet-poor plasma was prepared by centrifuging EDTA-anticoagulated whole blood for 20 min at 2000 *g* in order to minimise the contribution of platelet degranulation. Briefly, recombinant human IFN-γ, IL-2, IL-4, IL-10 and TGF-β1 standards and sera or plasma were added in duplicates to wells of a microtitre plate precoated with a monoclonal antibody specific for IFN-γ, IL-2, IL-4 or IL-10. For the TGF-β1 assay, the soluble TGF-β receptor type II, which binds TGF-β1, was precoated onto the microtitre wells. The plate was then incubated at room temperature for up to 3 h. After washings, an enzyme-conjugated anti-cytokine antibody was added to the wells, and the plate was incubated for another 45–60 min. After removal of unbound antibody–enzyme reagent, the colour was developed and then stopped by adding an acid solution to each well. The absorbance was recorded at 450 nm or 490 nm. The sample cytokine concentration was calculated from the corresponding standard curve.

Flow cytometry analysis

Heparinised Vacutainer tubes were used to collect 10 ml of venous blood (Becton Dickinson). After dilution with an equal volume of 0.01 M phosphate buffer saline (PBS), pH 7.4, 4 ml blood suspension was layered on 3 ml ficoll-isopaque (Pharmacia AB, Uppsala, Sweden) and centrifuged at 400 *g* for 30 min. The mononuclear cell fraction was harvested, washed twice with 0.01 M PBS and resuspended in 1 ml 0.01 M PBS. The cell suspension (100 µl), approximately 1×10^6 leukocytes, was mixed with 10 µl rabbit serum (Dakopatts, Glostrup, Denmark) and incubated in the dark for 30 min at 37 °C. After one washing, the cells were resuspended in 100 µl 0.01 M PBS, pH 7.4, containing 0.5% bovine serum albumin (BSA) and 0.1% NaN_3. Thereafter, aliquots of the cell suspension were incubated with the following combinations of fluorescein isothiocyanate (F)- or phycoerythrin (PE)-conjugated monoclonal antibodies: CD45 (F), CD14 (PE), anti-glycophorin-A (PE), CD3 (F), CD3 (PE), CD4 (F), CD4 (PE), CD8 (PE), CD16+56 (PE), CD19 (PE), CD25 (F) and CD122 (PE). Appropriate isotype-matched control antibodies were used. All monoclonal antibodies were from Becton Dickinson, except for the anti-glycophorin-A that was purchased from Dakopatts (Glostrup, Denmark).

After 30-min incubation at 4 °C, the cells were washed twice with 0.01 M PBS and resuspended in 300 µl 0.01 M PBS containing 1% paraformaldehyde. Analysis was performed on a flow cytometer [fluorescence-activated cell sorter (FACS), Becton Dickinson, Mountain View, Calif.], equipped with a 15-mW argon-ion laser. The immunofluorescent data were collected in list mode. Logarithmic amplifiers were used for fluorescence signals, and 10,000 events were collected. The data were analysed using the CellQuest software (Becton Dickinson). The lymphocyte population was identified by the back-gating method according to Loken et al. [27], and a lymphocyte gate was set in the forward (FSC) and side-scatter (SSC) dot plot. The fraction of T cells or CD4-positive T cells expressing CD25 or CD122 was determined by setting a gate in the fluorescence dot plot excluding CD3– or CD4– cells.

Table 1 Clinical characteristics, treatment at the time of analysis and previous therapy of idiopathic thrombocytopenic purpura patients with active disease [platelet count (pltc) $<50\times10^9$/l], stable disease (pltc 50–150×10^9/l) and remission (pltc $>150\times10^9$/l)

	Age (mean years ± SD)	Gender (male/female)	Splenectomised (no. of patients)	Corticosteroid therapy at time of study (no. of patients)	Other immunosuppressive therapy at time of study (no. of patients)	Previous therapy, i.e. corticosteroids or other immunosuppressive drugs (no. of patients)
pltc <50 ($n=8$)	50 ± 23	3/5	3	2	None	8
pltc 50–150 ($n=22$)	47 ± 23	5/17	3	3	None	17
pltc >150 ($n=16$)	45 ± 22	8/8	9	5	1 (azathioprine)	16

Statistics

Standard statistic methods were used for calculation of mean values and standard deviation. Unless otherwise stated, the mean value ± SD is reported. The difference between mean values was evaluated using the two-tailed Students' *t*-test for unpaired data, and a P value <0.05 was considered statistically significant.

Results

Th1 cytokines

The serum concentration of IFN-γ and IL-2 were below the detection limit for the assay (>3 pg/ml and >7 pg/ml, respectively). This was true both for the ITP patients and the healthy controls.

Th2 cytokines

The serum level of IL-4 was either present at a very low concentration or below the detection limit (0.13 pg/ml) in both patients and controls. The serum levels of IL-10 were within the assay range, and statistically significant differences were seen between the patient groups and healthy volunteers (Fig. 1). Patients with pltc $<50\times10^9$/l had a significantly reduced mean serum concentration of IL-10 (0.6 ± 0.1 pg/ml) both compared with patients with pltc 50–150×10^9/l (1.8 ± 0.1 pg/ml; $P<0.005$) and the healthy controls (1.4 ± 0.1 pg/ml; $P<0.005$). A difference was also found between patients with pltc $<50\times10^9$/l and those with pltc $>150\times10^9$/l (0.6 ± 0.1 and 1.4 ± 0.4 pg/ml, respectively); however, this difference did not reach statistical significance ($P=0.16$).

Th3 cytokine

The mean plasma level of TGF-β1 was found to be significantly higher in patients in clinical remission, i.e. pltc $>150\times10^9$/l (23.5 ± 2.8 ng/ml) than in patients with pltc $<50\times10^9$/l (2.3 ± 0.6 ng/ml; $P<0.0001$), patients with pltc 50–150×10^9/l (7.2 ± 1.7 ng/ml; $P<0.0001$) or healthy controls (9.8 ± 1.3 ng/ml; $P<0.01$) (Fig. 2). Also, the patients with pltc $<50\times10^9$/l had significantly lower mean plasma TGF-β1 than the controls ($P<0.001$). However, there was no statistically significant difference in mean plasma TGF-β1 between patients with pltc $<50\times10^9$/l and pltc 50–150×10^9/l ($P=0.09$). In splenectomised patients ($n=15$) regardless of pltc, the mean plasma TGF-β1 level was 16.2 ± 3.6 ng/ml. In patients in remission, there was no statistical difference ($P=0.62$) in plasma levels of TGF-β1 between splenectomised ($n=9$; 24.9 ± 4.6 ng/ml) and non-splenectomised ($n=7$; 22.0 ± 3.6 ng/ml) patients.

Lymphocyte subsets and activation

The results are summarised in Table 2. There were no statistically significant differences between the five groups with regard to the percentage of B cells (CD19+) or cytotoxic T cells (CD3+CD16+CD56+). All groups had similar numbers of CD3+CD122+ cells, i.e. activated pan-T cells. However, patients with pltc $<50\times10^9$/l and splenectomised patients had significantly higher levels of CD4+CD25+ cells ($26.2\pm14.8\%$; $P<0.05$ and $26.7\pm11.9\%$; $P<0.005$, respectively), i.e. activated Th-cells, than healthy controls ($16.5\pm4.0\%$). The number of T cells (CD3+) were lower ($61.8\pm16.4\%$; $P<0.05$), while NK cells (CD3–CD16+CD56+; $28.7\pm17.9\%$; $P<0.05$), acti-

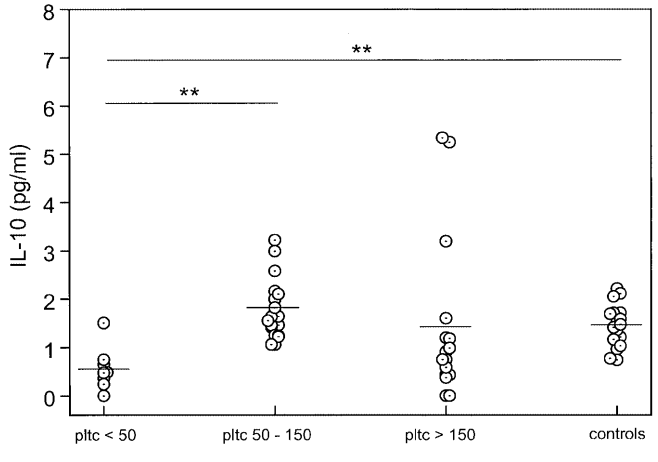

Fig. 1 The serum levels of interleukin-10 in idiopathic thrombocytopenic purpura patients with active disease [platelet count (pltc) $<50\times10^9$/l], stable disease (pltc 50–150×10^9/l), in remission (pltc $>150\times10^9$/l) and healthy controls. The *horizontal lines* denote the mean values. $^{**}P<0.01$

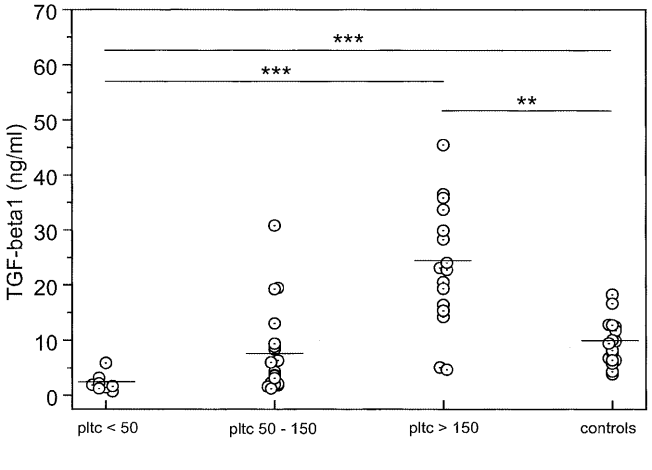

Fig. 2 The plasma levels of transforming growth factor-β1 in idiopathic thrombocytopenic purpura patients with active disease [platelet count (pltc) $<50\times10^9$/l], stable disease (pltc 50–150×10^9/l), in remission (pltc $>150\times10^9$/l) and healthy controls. The *horizontal lines* denote the mean values. $^{**}P<0.01$; $^{***}P<0.001$

Table 2 Lymphocyte subsets in idiopathic thrombocytopenic purpura patients with active disease [platelet count (pltc) $<50\times10^9$/l], stable disease (pltc 50–150 $\times10^9$/l), in remission (pltc $>150\times10^9$/l), after splenectomy and healthy controls

	CD19 %	CD3 %	CD3– CD16+ CD56+ %	CD4:CD8 ratio	CD3+ CD16+ CD56+ %	CD3+ CD122+ %	CD3+ CD25+ %	CD4+ CD25+ %
pltc <50 ($n=8$)	8.8±4.3	65.1±17.7	26.1±15.6	2.3±1.8	12.5±8.6	11.6±8.2	16.5±13.5	26.2±14.8[a]
pltc 50 –150 ($n=22$)	11.1±7.3	67.1±14.0	21.8±13.5	1.8±0.9[b]	11.7±11.3	14.4±9.6	14.6±5.5	18.8±7.2
pltc >150 ($n=16$)	9.0±6.5	64.4±16.4	26.6±16.0[a]	1.0±0.9	16.9±12.4	8.7±5.1	15.3±9.6	21.8±12.2
All patients ($n=46$)	9.9±6.6	65.8±15.9	24.3±15.6	1.6±1.3	13.6±11.5	12.0±7.5	15.2±8.3	21.1±10.6
Splenectomy ($n=15$)	9.5±7.6	61.8±16.4[a]	28.7±17.9[a]	1.1±0.8	14.1±11.6	12.2±9.1	18.1±8.8[a]	26.7±11.9[c]
Controls ($n=18$)	11.1±3.3	71.5±9.1	17.4±7.6	1.8±1.5	10.3±5.5	6.1±3.9	13.2±4.6	16.5±4.0

[a] $P<0.05$ compared with healthy controls
[b] $P<0.05$ compared with pltc >150
[c] $P<0.005$ compared with healthy controls

vated pan-T cells (CD3+CD25+; 18.1±8.8%; $P<0.05$) and activated Th cells (CD4+CD25+; 26.7±11.9%; $P<0.005$) were significantly higher in splenectomised patients than in healthy controls (71.5±9.1%, 17.4+7.6% and 13.2±4.6%, respectively). Also, the number of NK cells among patients with pltc $>150\times10^9$/l were significantly elevated (26.6±16.0%) compared with the controls (17.4±7.6%; $P<0.05$). The CD4:CD8 ratio was found to be higher in patients with pltc 50–150 $\times10^9$/l (1.8±0.9) than those with pltc $>150\times10^9$/l (1.0±0.9; $P<0.05$).

Discussion

The present study could not establish an unequivocal Th1 or Th2 serum cytokine profile in ITP patients with active disease. Cytokines are, however, produced and regulated in local compartments, and assessment of serum/plasma concentrations may not be the optimal measure of the ongoing Th response in ITP. Nevertheless, our study shows that patients with ITP in remission, i.e. with a pltc exceeding 150×10^9/l, had significantly higher plasma levels of TGF-β1, a Th3 cytokine, than patients with active disease and controls. In contrast, patients with stable disease (pltc between 50×10^9/l and 150×10^9/l) and controls both had significantly lower levels of plasma TGF-β1 than patients in clinical remission.

Antigen stimulation activates the CD4+ Th cells into different cytokine responses. The Th1 response is characterised by elevated production of IL-2, IFN-γ and tumour necrosis factor (TNF)-β, and promotes the production of opsonising and complement-fixing antibodies, macrophage activation, antibody-dependent cell-mediated cytotoxicity (ADCC) and delayed type hypersensitivity. The Th2 cells, producing IL-4, IL-5, IL-6, IL-9, IL-10 and IL-13, are considered responsible for humoral immune responses such as non-complement fixing immunoglobulin (Ig) G and IgE synthesis and inactivation of several macrophage functions [32, 38]. Th0 cells are instead considered to be less differentiated than those cells mediating Th1 or Th2 responses and are characterised by a mixed Th1/Th2 cytokine pattern [39]. T cells that produce TGF-β appear to be a unique T-cell subset, named Th3 cells, which possess downregulatory properties on Th1 and Th2 cells [3, 5, 12, 20, 33].

The term bystander immune suppression originates from experiments where oral administration of antigen was found to promote activation of T cells producing TGF-β [29]. TGF-β has also been found to be an important inhibitor of B-cell proliferation and antibody production [7, 23]. Although bystander immune suppression was originally described for induction of T cells by orally administered antigen, it has been suggested that this process could, in principle, take place in response to any immune manipulation that induces Th2- or Th3 cells [47]. Hence, our data could indicate that ITP in active stage is associated with a downregulated Th3 response, and remission might be induced by upregulation of the Th3 response and a TGF-β mediated bystander immune suppression. In ITP patients, this response appears to follow any medical therapy that induces a clinical remission, i.e. corticosteroids, immunosuppressive drugs or splenectomy. The underlying mechanism of this upregulation is unknown; however, it has been reported that corticosteroid therapy might enhance NK cell activity in patients with active ITP [42]. Also, the results from other investigators [15] and our own findings indicate that splenectomy might change the NK-cell numbers. Both corticosteroid therapy and splenectomy could thereby induce a possible bystander immune suppression. Th3 cells are also associated with secretion of the Th2 cytokines IL-4 and IL-10 [26, 37]. Indeed, the Th1 cytokines IL-2 and IFN-γ were undetectable in our patients, whereas patients with active ITP (pltc $<50\times10^9$/l) had a significantly diminished mean serum level of IL-10 compared with controls and patients with a stable disease (pltc 50–150 $\times10^9$/l). This finding might be another indicator of a suppressed Th3-type cytokine response in patients with active ITP.

TGF-β1 is, however, produced by a variety of cells, and significant amounts are found in the platelet α-

granules [10]. Thus, the elevated plasma levels of TGF-β1 observed could partially be explained by in vitro platelet activation/degranulation. However, our analyses of TGF-β1 were performed on EDTA-anticoagulated blood to prevent platelet activation during processing of the samples. Also, the finding that ITP patients in remission had significantly higher levels of plasma TGF-β1 than controls, despite similar pltcs, indicates an explanation other than in vitro platelet degranulation. In vivo platelet activation in ITP patients, with release of TGF-β1, could also account for the differences observed for this cytokine. However, conflicting results are reported on the degree of platelet activation in ITP. Some investigators have described a low degree of platelet activation [6, 43], whereas others have reported increased platelet activation in patients with active disease, followed by a decrease when therapy induces an elevation of the pltc [4, 19]. Another possible explanation for high plasma levels of TGF-β1 in patients in remission could be a release of TGF-β1 derived from NK cells. Finally, increased platelet turnover in vivo in patients in remission seems to be a less probable explanation for elevated plasma levels of TGF-β1. To our knowledge, there are no data supporting a state of compensated thrombocytolysis in ITP patients in remission. Branehög [2] showed that ITP patients had a lower degree of platelet turnover after splenectomy than before splenectomy (mean pltc before splenectomy $37 \pm 39 \times 10^9$/l and after splenectomy $322 \pm 136 \times 10^9$/l). Conversely, the low TGF-β1 levels in active ITP probably cannot be explained by the low platelet number, since active ITP is frequently associated with an increased platelet turnover.

Regarding the lymphocyte immunophenotype, we found an elevated percentage of CD4+CD25+ cells, i.e. activated Th cells, in patients with active disease. These data corroborate previous findings of other investigators [13, 31, 41]. However, activated T cells were not found in our patients with stable disease or patients in clinical remission. The activation of Th cells in patients with active disease could reflect an ongoing antigen stimulation by platelet antigens and thus a self-maintaining process.

Moreover, we found a statistically significant increase in the percentage of NK cells (CD3–CD16+CD56+) in ITP patients in remission. Such an increase was also observed for patients with active disease, but without statistical significance. This result seems to be in contradiction to the results of Garcia-Suarez et al. [14], who found an elevated percentage of NK cells only in patients with refractory/active disease. NK cells have been shown to possess a suppressive effect on B cells and antibody production [22]. Furthermore, recent findings show that NK cells are a major source of TGF-β [17]. TGF-β derived from NK cells could then, in co-stimulation with CD8+ T cells, develop suppressor activity [16, 17]. These investigators also suggest that NK cells may play an important role in the downregulation of anti-self and non-self immune responses in vivo, via TGF-β production [21]. Hence, an expansion of NK cells in ITP patients might be a mechanism by which the production of platelet autoantibodies can be downregulated.

The splenectomised patients were found to have a reduced percentage of T cells, elevated percentages of NK cells and activated pan-T cells and Th (CD3+CD25+ and CD4+CD25+, respectively) compared with healthy controls. The mechanism by which these T-cell subsets are changed is unknown, but this finding could indicate that the splenectomy procedure not only removes the major site of platelet destruction but also changes the cellular immune system. The eventual contribution of the disease process per se on this finding, i.e. that splenectomised patients have a more active disease, cannot be answered by the available data.

Inducing a Th3 cell cytokine response and bystander immune suppression with orally administered antigen (oral tolerance induction) has been the topic of several studies of autoimmune disorders. Promising results have been obtained in both animal and human models [1, 5, 12, 36, 45, 48]. Also, it has recently been pointed out that studies of oral immune tolerance induction in ITP are highly warranted and still await to be conducted [11]. Such therapy appears to be associated with fewer side effects than immunosuppressive treatment frequently recommended for refractory ITP patients. In view of our data revealing low levels of TGF-β1 in active ITP, most probably due to a downregulation of the Th3 response, it seems consequent to evaluate such an approach.

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