Changes in cytokine profile pre- and post-immunosuppression in acquired aplastic anemia

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ABSTRACT

Cytokine expression assessed by flow cytometry in 53 acquired aplastic anemia patients before and after combined immunosuppression (EBMT WPSAA protocols) showed that $CD3^+$ marrow cells containing TNF-α, IFN-γ and IL4 were similar in subjects with disease at onset (DO) and responsive to treatment who had more $CD3^+$/TNF-α^+ and CD 3^+/IFN-γ^+ cells than normal controls. *In vitro* block of TNF-α and/or IFN-γ significantly increased BFU-e over baseline in 28 patients. In responsive to treatment patients only TNF-α block significantly incremented colonies over normal controls. Absolute marrow $CD3^+$/TNF-α^+ and $CD3^+$/IFN-γ^+ cells prospectively tested in a group of 21 subjects declined significantly more in Responders than in Non Responders to immunosuppression at Response Evaluation Time respect to Diagnosis. Both in Responders and in Non Responders these cells remained higher than in normal controls. This study suggests that immunosuppression does not fully clear excess TNF-α and IFN-γ from marrow of patients with good outcome and raises the hypothesis that additional cytokine blockade might be useful in immunosuppression for acquired aplastic anemia.

Key words: TNF-α, IFN-γ, IL4, aplastic anemia, immunosuppression.

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Introduction

Idiopathic acquired aplastic anemia (AAA) is due to an immune destruction of hematopoietic stem cells in the bone marrow. Whereas events initiating immune aggression have not been fully clarified, pathogenic steps more closely associated with damage of marrow cells are better understood.[1,2] Current evidence indicates that auto reactive T cells, activated by different stimuli, release myelosuppressive cytokines including TNF-α and IFN-γ which cause hematopoietic stem cell death by blocking mitosis and increasing apoptosis[3-7] pointing to these cytokines as late effectors of the hematopoietic cell depletion of idiopathic AAA. In the absence of an HLA matched sibling donor, combined immunosuppression (IS) with ATG and cyclosporine A is the first-line treatment producing a response rate of about 80%.[1,8] However, responsive patients retain a more defective hematopoiesis compared to transplanted and normal individuals[9] and have an up to 30% risk of non-response/disease recurrence.[10-12] Mechanisms underlying the risk of relapse have not been fully clarified at the level of marrow biology. It can be postulated that treatment does not sufficiently clear TNF-α and IFN-γ, the late mediators of the hematopoietic damage, from the marrow of responding patients. To explore this hypothesis we assessed, in the marrow $CD3^+$ cells of idiopathic AAA patients treated with combined IS, the intracellular expression of TNF-α, IFN-γ and IL4, as effectors involved in the immune response, and the effect of *in vitro* block of TNF-α and IFN-γ on the growth of hematopoietic progenitors.

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The online version of this article contains a supplementary appendix.

Design and Methods

Cytokine expression in CD3+ cells assessed at diagnosis and in responders

From December 2000 to June 2006 in the AIEOP (Associazione Italiana di Ematologia ed Oncologia Pediatrica) Centers and in the Hematology Unit of Ospedale S. Martino, Genova, 36 patients with AAA diagnosed according to international criteria[13] were sampled at disease onset (DO) for evaluation of absolute numbers of marrow $CD3^+$ and of $CD3^+$/TNF-α, $CD3^+$/IFN-γ, $CD3^+$/$IL4^+$ cells.

Over the same period in the same Centers, 30 patients were sampled for the same parameters after having achieved hematologic response following a first course of combined IS as from WP SAA EBMT Protocols (group of Responsive to Treatment - RT). At study entry no patients had either marrow cytogenetic abnormalities or dysplastic features and all had been infection-free for at least two previous weeks. Patients' characteristics and treatment details are shown in *Online Supplementary Appendix 1*. Controls were 10 (6 males and 4 females) healthy marrow voluntary donors enrolled over the study period in Centers participating in the study.

Flow cytometry analysis of intracellular expression of TNF-α, IFN-γ, IL- 4 in marrow $CD3^+$ cells was performed as described elsewhere[14] and is detailed in the *Online Supplementary Appendix 2*.

Cytokine blockade in marrow culture

We assessed the growth of BFU-e in the absence and in the presence of agents blocking TNF-α and/or IFN-γ on 31 marrow samples obtained from patients studied for cytokine expression. Controls were those of the above study. Committed progenitor assays was performed as described elsewhere[14] and as detailed in the *Online Supplementary Appendix 3*.

Cytokine expression in CD3+ cells in patients with sampling at diagnosis and during follow-up

From May 2002 to July 2006, 21 consecutive patients were prospectively tested at diagnosis and at time of response evaluation after combined IS as from WP SAA EBMT protocols, for intracellular expression of TNF-α, IFN-γ, and IL-4 in $CD3^+$ marrow cells. Patients' details are shown in *Online Supplementary Appendix 1*. Response was evaluated at a median of 120 days (min 90 - max 180) from treatment start. At response evaluation time (RET) 11 patients were responders (partial and complete) and 10 non responders (see the *Online Supplementary Appendix 1*). Controls were 13 (6 males and 7 females) healthy marrow voluntary donors with a median age of 23 years (min 5-max 45) enrolled from January to December 2004 in the participating Centers. Flow cytometry analysis of intracellular expression of TNF-α, IFN-γ, IL4 in marrow $CD3^+$ cells was performed as described. Studies were approved by the institutional Ethics Committees.

Normal controls were healthy voluntary donors. Informed consent was obtained from parents of patients and controls or, whenever eligible, from patients and controls themselves in accordance with the Helsinki declaration of principles. Bone marrow was taken from the patients during aspirations required for diagnosis or disease monitoring.

Statistics

Due to small sample size and non-normal distribution, quantitative data were described as medians and comparisons were performed by means of non-parametric tests (Friedman, Wilcoxon, Kruskal Wallis and the Mann-Whitney U tests) using Bonferroni correction (see the *Online Supplementary Appendix 4*).

Results and Discussion

Cytokine expression in CD3+ cells assessed at diagnosis and in responders

Absolute $CD3^+$ cells in DO were higher than in controls (p=0.003) but similar (p=0.776) in RT and normal subjects. Consistently, DO had more $CD3^+$ cells than RT patients (p= 0.011). $CD3^+$/TNF-α^+ cells in both RT and DO subjects were more numerous than in normal controls (p=0.001 and <0.001, respectively) whereas the difference between DO and RT was not significant (p= 0.083) (Table 1). $CD3^+$/IFN-γ^+ cells were more numerous in RT (p=0.001) and DO (p<0.001) than in normal subjects but similar among each other (p=0.592) (Table 1).

After Bonferroni correction, $CD3^+$/$IL4^+$ cells were similar in paired comparisons between normal controls and RT (p=0.240), RT and DO (p=0.370), and DO and normal subjects (p=0.067) (Table 1).

Cytokine expression analyzed by the presence/absence of treatment with G-CSF, transfusions, CsA and steroids showed no differences in either RT or DO patients except for the following: (i) $CD3^+$/IFN-γ^+ cells were higher (p=0.026) in RT subjects who received (3 patients) versus those not receiving (24 patients) steroids; (ii) $CD3^+$/TNF-α^+ cells were more numerous in DO subjects not receiving (25 patients) versus those receiving (8 patients) steroids (p=0.0428).

Cytokine blockade in marrow culture

BFU-e growth without the addition of etanercept and/or IFN-γ blocking antibody, was different in controls, compared to RT, and DO (p=0.0001). DO patients had remarkably less colonies versus both RT (p<0.001) and normal controls (p=0.001), whereas progenitor growth was similar in RT and normal subjects (p=0.551) (Table 1).

Blockade of TNF-α or/and IFN-γ in culture of normal controls' MNCs, did not increase absolute number of BFU-e over baseline. In RT patients, BFU-e became significantly higher than baseline after block of *TNF-α*, (p=0.010), of *IFN-γ* (p=0.003) and double block (p=0.003) (Table 1).

In DO patients, colonies were also significantly more numerous over baseline after block of *TNF-α* (p=0.034), of *IFN-γ* (p=0.009) and of *TNF-α* and *IFN-γ* (p=0.007) (Table 1).

These data show that neutralization of TNF-α and IFN-γ increases the number of BFU-e over baseline, indicating that these cytokines inhibit erythropoiesis *in vitro* in both DO and RT patients. To better understand which

cytokine removal was more effective in increasing *in vitro* erythropoiesis, we compared the increment of growth of BFU-e after cytokine block in the three groups. BFU-e increment in RT patients after TNF-α blockade was significantly greater than in normal controls (p=0.04), whereas in DO patients it was not (p=0.352). No significant difference occurred between RT and DO (p=0.162) (Figure 1A). Blockade of IFN-γ gave a greater, but not significant, increment in RT subjects (p=0.097) versus normal controls. No increment difference between DO patients and normal controls (p=0.966), and between RT and DO groups (p=0.144) was found (Figure 1B). After double block, the increment in the RT group was significantly greater (p=0.019) than in normal controls, whereas in DO patients was not (p=0.067). No significant increment was found in RT *vs.* DO subjects (p=0.414) (Figure 1C).

Intracytoplasmic expression of TNF-α, IFN-γ and IL-4 in marrow CD3⁺ cells in this subset mirrored that of the enlarged group. (*Online Supplementary Appendix 5*).

Intra CD3⁺ cells cytokine expression in patients with sampling at diagnosis and during follow-up

Responders (p=0.041) and non responders (p=0.021) significantly reduced their absolute number of CD3⁺ cells from diagnosis to RET (Figure 2 A and B). Only responders significantly (p=0.013) reduced CD3⁺/TNF-α⁺ cells at

Table 1. Comparison of cytokine expression and BFU-e growth between normal controls and aplastic patients by disease status.

	Marrow cytokine expression			
	CD3⁺ cells*	CD3⁺/ TNF-α+cells*	CD3⁺/ IFN-γ+cells*	CD3⁺/ IL4⁺ cells*
Normal controls	3532 (569-4370)	50 (18-112)	173 (51-245)	881 (102-2054)
RT°	3564 (585-8330)	561 (51-2089)	785 (116-2273)	1450 (386-6080)
DO°	5901 (941-8624)	1154 (47-2502)	945 (47-2832)	3312 (78-7849)
P^	Overall: 0.0008 Normal *vs.* RT: 0.776 Normal *vs.* DO: 0.003 RT *vs.* DO: 0.011	Overall: 0.0001 Normal *vs.* RT: 0.001 Normal *vs.* DO: <0.001 RT *vs.* DO: 0.083	Overall: 0.0002 Normal *vs.* RT: 0.001 Normal *vs.* DO: <0.001 RT *vs.* DO: 0.592	Overall: 0.038 Normal *vs.* RT: 0.240 Normal *vs.* DO: 0.067 RT *vs.* DO: 0.370

	BFU-e growth after cytokine block				
	Baseline‘	after block TNF-α‘	after block IFN-γ‘	after double block‘	P§
Normal=	338 (257-400)	335 (262-404)	341 (262-407)	337 (260-411)	Overall: 0.083 Baseline *vs.* TNF-α block: na Baseline *vs.* IFN-γ block: na Baseline *vs.* Double block: na
RT°=	271 (20-410)	345 (20-515)	331 (20-456)	342 (20-495)	Overall: <0.001 Baseline *vs.* TNF-α block: 0.010 Baseline *vs.* IFN-γ block: 0.003 Baseline *vs.* Double block:0.003
DO°=	10 (0-374)	20 (0-402)	20 (0-460)	28 (1-476)	Overall: 0.0004 Baseline *vs.* TNF-α block: 0.034 Baseline *vs.* IFN-γ block: 0.009 Baseline *vs.* Double block: 0.007

**Values are expressed as median, minimum and maximum number of absolute cells/mL of marrow. °RT and DO patients are defined as in Legend of Table 1. ^The Kruskal Wallis or the Mann-Whitney U tests were used to compare differences among three groups (Normal, RT, DO). §Intra-patient values were compared using the Friedman or the Wilcoxon tests.= Overall comparison Normal vs RT vs. DO p=0.0001. = RT vs. DO p<0.001, DO vs normal controls p=0.001 = RT vs normal subjects p=0.551. ‘Values are provided as median, minimum and maximum number of absolute BFU-e/10⁵ bone marrow mononuclear cells; na: not applicable because of no significant difference of the overall comparison.*

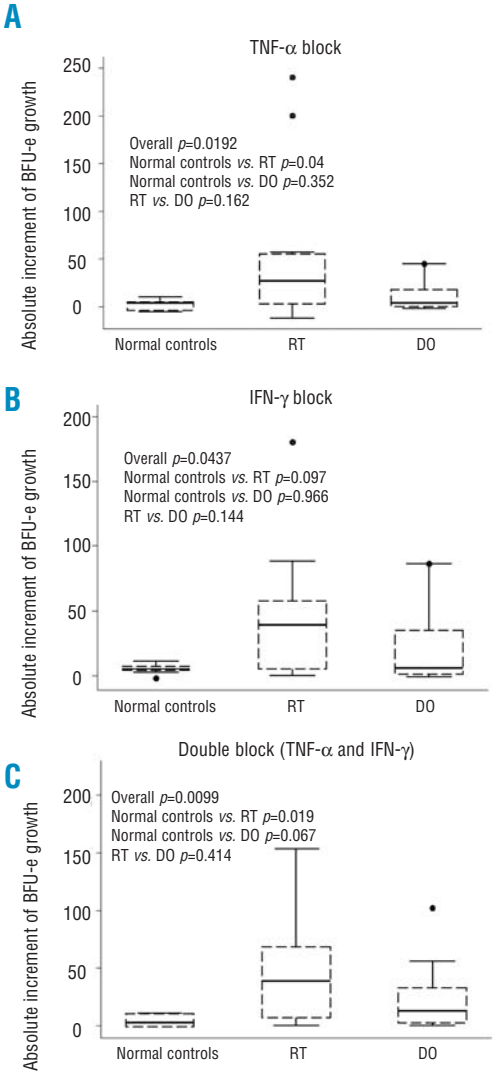

Figure 1. Increment of growth of BFU-e after cytokine block. Absolute increment of BFU-e in normal controls, in Responsive to treatment (RT) and Disease at Onset (DO) patients after block of TNF-α (A), IFN-γ (B) and TNF-α and IFN-γ (double block) (C). In the box plot bars represent median, upper and lower adjacent value of increment of BFU-e/10⁵ BM MNCs.

RET compared to diagnosis whereas non responders did not (p=0.093) (Figure 2 C and D). Again, only responders significantly (p=0.016) diminished $CD3^+$/ IFN-γ^+ cells at RET compared to diagnosis whereas this was not the case for non responders (p=0.074) (Figure 2 E and F). Regarding IL-4 (Figure 2 G and H), responders did not (p=0.96) reduce $CD3^+$/ $IL4^+$ cells at RET compared to diagnosis whereas non responders did (p=0.005), possibly reflecting a prevalent Th1 cell expansion at the expense of Th2 cells in these patients.

Noteworthy, in agreement with data of the first study, $CD3^+$ cells containing TNF-α^+ or IFN-γ^+ at RET were significantly more numerous than in normal subjects not only in non responders but also in responders (Figure 2 C and F). There was no difference between responders and non responders either at diagnosis or at RET for any cellular subset (*Online Supplementary Appendix 6*).

In this project we investigated, in different phases of disease, the changes in myelosuppressive cytokines TNF-α and IFN-γ, the final mediators of the damage on marrow hematopoietic cells in idiopathic AAA and of IL4, a cytokine characterizing Th2 polarization of T cells.

Intracytoplasmic expression indicates that in RT patients, myelosuppressive cytokine load was significantly greater than in normal controls. On the contrary, IL4 was not differentially expressed in patients' subgroups and in normal controls, suggesting a non-primary involvement of this cytokine in the pathogenesis of the disease. The expression of inflammatory cytokines might be influenced by transfusions, steroids, CsA and G-CSF.[15,16] In this study, we did not find any effect of these factors except for some influence of steroids on restricted cell subsets and in small subgroups. The limited size of some groups does not allow firm conclusions to be drawn but our finding, consistent with others from the literature,[5,17] seems to confirm that intralymphocytic TNF-α and IFN-γ overexpression is related to the disease.

Cytokine blockade study showed that in the same patients in whose marrow cells TNF-α or IFN-γ were over-expressed, the neutralization of these effectors enhanced the growth of erythroid progenitors. This is a new finding confirming that TNF-α and IFN-γ contribute to erythroid failure of AAA.[3-7]

In DO patients, BFU-e increment was not significant with any cytokine block, probably due to the severely reduced stem cell potential as witnessed by the low number of colonies of these patients compared to normal and RT groups. In RT patients, TNF-α block was the most effective in increasing erythropoiesis *in vitro* (Figure 1 A and C) suggesting that in the marrow of these subjects an important excess of this cytokine persists. This does not manifestly harm erythropoiesis probably because of compensatory proliferation of stem cell compartment as witnessed by BFU-e numbers comparable to normal controls. It is possible that in case of re-activation of the autoimmune attack this smoldering activity becomes effective and damages hematopoietic cells which then results in an overt relapse.

Prospective study of cytokine expression suggests that responders and non responders can not be differentiated by the number of marrow $CD3^+$/TNF-α^+ and $CD3^+$/IFN-γ^+ cells present at diagnosis (*Online Supplemetary Appendix 5*). This is in contrast with other data,[3] indicating that at diagnosis responders to IS displayed $CD8^+$/IFN-γ^+ cells whereas non responders had $CD8^+$ staining negative for IFN-γ. There are differences between the two studies,

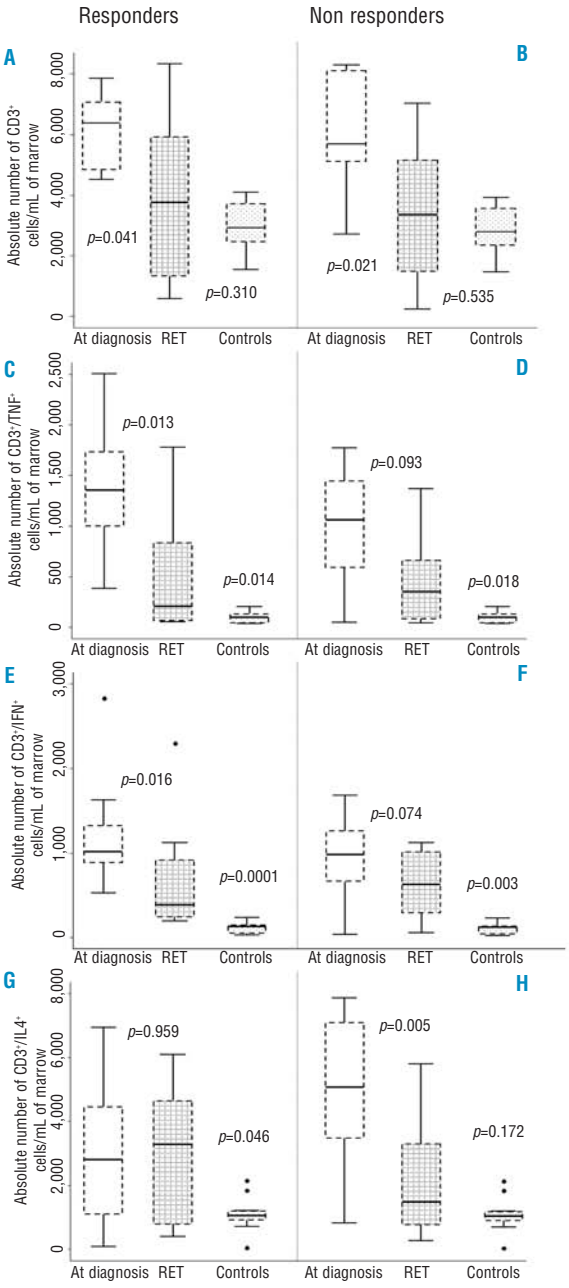

Figure 2. Intracytoplasmic cytokine expression in patients with sampling at diagnosis and during follow-up. Absolute number of: $CD3^+$ cells in responders (A) and non responders (B) $CD3^+$/TNF-α^+ cells in responders (C) and non responders (D), $CD3^+$/ IFN-γ^+ cells in responders (E) and non responders (F) $CD3^+$/$IL4^+$ cells in responders (G) and non responders (H), normal controls are included in the comparisons. In all panels the left sided p refers to comparisons between diagnosis and RET both in responders and in non responders, and the right sided p to comparisons between RET and normal controls both in responders and non responders. In the box plot bars represent median, upper and lower adjacent value of positive cells/mL of marrow.

represented by diverse tested cell populations (CD 3$^+$ *vs.* CD8$^+$), different age of patients (median age in our cohort was ten years) and earlier time (four *vs.* six months) of response assessment after treatment, that may explain the diverse findings. Even if our responders to IS, consistent with the clinical outcome, cleared marrow CD3$^+$/TNF-α$^+$, CD3$^+$/IFN-γ$^+$ cells at RET versus diagnosis more efficiently than non responders, it is of note that myelosuppressive cytokine load of responders at RET was still greater than in normal controls. Although aspiration technique might have influenced the number of cells/mL, the consistency of cytokine expression in first and in paired sample studies seems to attenuate the role of this potentially disturbing effect. Overall, these new findings, consistent with results of BFU-e study, confirm that IS does not clear excess TNF-α and IFN-γ from the marrow of patients with good outcome. It can then be speculated that a more intense combined IS including targeted agent against key molecules like TNF-α, might reduce the harmful potential on the marrow of AAA patients, increase response to IS and, hopefully, diminish relapse risk. This study confirms that TNF-α and IFN-γ have an important role in the pathogenesis of AAA, and indicates that these cytokines decline to a lower extent in non responders than in responders to IS in whose marrow they are not cleared to normal but still persist and reduce erythropoiesis. This might contribute to relapse in case of immune attack re-activation and raises the hypothesis of the clinical usefulness of adopting additional cytokine blockade aiming to reduce the risk of marrow failure recurrence.

Authorship and Disclosures

CD designed the study, coordinated the sample and data collection, interpreted the results, wrote the paper; AC, EF, ML performed the flow cytometry analysis and the colony studies, participated in the result interpretation and revised the paper; FB did the statistical analysis, most of the imaging and revised the manuscript; OB contributed to the sample and data collection, participated in the result interpretation and revised the manuscript; JS co-coordinated the sample collection, contributed to the data collection, participated in the result interpretation, contributed to the writing of some parts of the manuscript and to the imaging, and revised the manuscript; UR, PS, MTvL, DL, GFT, MP, AL, AM and MLS contributed to the sample and data collection, shared the result interpretation and revised the manuscript; AB and VP shared the design of the study, participated in the result interpretation and revised the paper.

The authors reported no potential conflicts of interest.

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