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

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CTD SUPPLEMENTARY APPENDIX 1

All subjects were treated with combined IS according to the protocols of the SAA WP of the EBMT which included: ATG (1.5 vial/10 Kg from day 1 to day 5), oral Cyclosporine A (CsA) (5 mg Kg/day from day +1 to day 180 or 240), Steroids (Methylprednisolone or Prednisone 1 or 2mg/kg/day) from day 5 to 15 and then tapering the dose during the next two weeks, ± G-CSF (5 or 10 mcg/Kg/day). Low-dose steroids and/or CsA were also given before ATG in a small minority of patients, during the diagnostic work-up as "ad interim" treatment.

"In this study some samples belonged to the same patients who were tested at diagnosis and when they responded to IS. This explains why the sum of the DO and RT samples is superior[66] to the number of patients.[53]

*The RT (Responsive to Treatment) samples belonged to subjects who, after a first course of combined IS, have achieved partial (platelets >20×10^9/L, Hb >8g/dL without transfusions plus PMN > 0.5×10^9/L on or off G-CSF) (N°9 patients) or complete (platelets >150×10^9/L, Hb >12 g/dL without transfusions plus PMN >1.5×10^9/L on or off G-CSF) (N°21 patients) hematological response. All RT patients had received ATG (anti-Thymocyte globuline) at least three months before the study.

°Also in this study some samples belonged to the same patients who were tested at diagnosis and when they responded to IS. This explains why the sum of the DO and RT samples is superior (31) to the number of patients.[28]

^The DO (Disease at Onset) samples belonged to subjects at diagnosis who did not received ATG.

&In G-CSF, Transfusions, CsA, Steroids boxes numbers indicate the number of subjects who were on (yes) or off (no) G-CSF, CSA or steroids at the moment of marrow sampling, and who had received (yes) or not (no) any transfusion within 30 days prior to marrow sampling.

§Non Responders were those with Transfusion dependence from Platelets and red cells and/or platelets < 20×10^9/L and Reticulocytes <2% and PMN <0.5×10^9/L on or off G-CSF.

SUPPLEMENTARY APPENDIX 2

Flow cytometry analysis of intracellular expression of TNF-α, IFN-γ, IL- 4 in marrow CD3$^+$ cells

Bone marrow samples from patients and controls were harvested in sterile heparin and shipped on ice within 24 hour to the Laboratory of Oncology of the G.Gaslini Institute. BM MNCs were isolated by separation on Ficoll-Hypaque, cryopreserved and thawed for use at study. After checking viability by trypan blue dye exclusion test, cells were cultured for 5 hours at 37°C in a 5% CO2 atmosphere in RPMI 1640 medium supplemented with 10% FBS in the presence of 50 ng/mL phorbol 12-myristate 13-acetate (PMA; Sigma, St Louis, MO), 250 ng/mL calcium ionophore (A23187; Sigma), and 5 ng/mL brefeldin A (Sigma), which inhibits intracellular transport of cytokines into the Golgi complex thus impeding their release in culture supernatants.

After culture, cells were washed twice in PBS with 1% FBS and subsequently incubated for 30 minutes at 4°C with anti-CD3–tricolor (Caltag, Burlingame, CA). In all the experiments, to minimize background staining, Fc receptor blockade was achieved by incubation of cells with excess purified human immunoglobulin before staining. Stained cells were re-suspended for 20 minutes at 4°C in Cytofix/Cytoperm fixation/permeabilization solution (Caltag), according to the manufacturer's instructions. Once permeabilized, cells were washed twice and stained for intracellular cytokines with the following monoclonal antibodies (mAbs): mouse anti-IFN-phycoerythrin (PE; Caltag), rat anti-inteleukin (IL)-4-PE (Caltag), and rat anti-TNF-PE (Caltag).

Negative controls were PE-conjugated isotype-matched mAbs of irrelevant specificity and a ligand-blocking control in which fluorochrome-conjugated antibodies were preincubated with appropriate concentrations of matched recombinant cytokines, according to the manufacturer's instructions. Flow cytometric analysis was performed using a FACScalibur cytometer (Becton Dickinson, San Jose, CA, USA), the gate was set on CD3$^+$ cells and results expressed as absolute number of CD3$^+$/TNF-α$^+$, CD3$^+$/IFN-γ, CD3$^+$/IL4$^+$ cells per ml of marrow.

SUPPLEMENTARY APPENDIX 3

Committed progenitor assay

After cells were thawed and viability checked, the in vitro assay for the growth of burst-forming unit-erythroid (BFU-Es) from patients and controls was performed using methyl cellulose media (MethoCult; StemCell Technologies, Vancouver, BC, Canada). In brief, 1×10^5 BM MNCs/dish were tested in triplicate (i.e. a total of 3×10^5 seeded BM MNCs) in semisolid

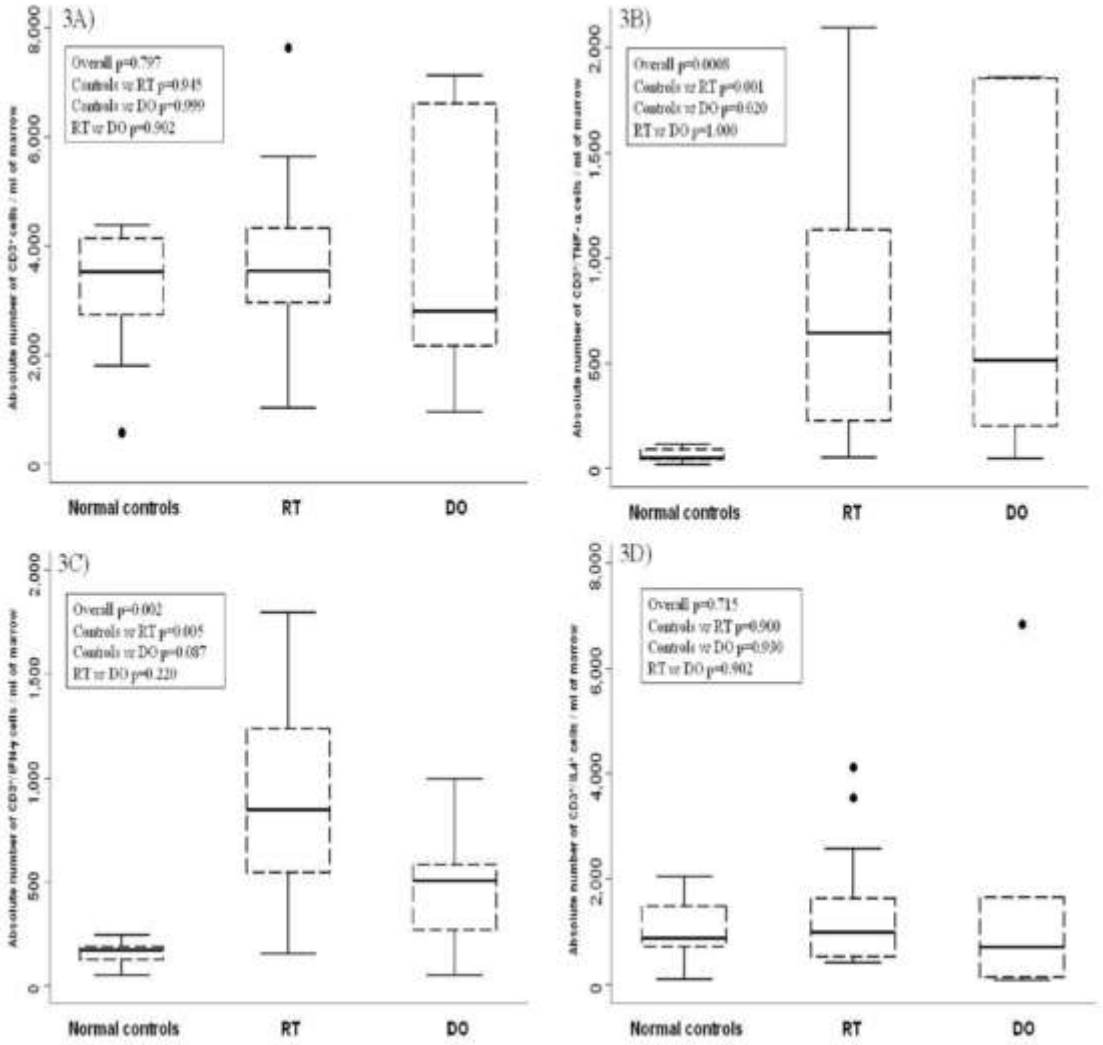
Online Supplementary Appendix 1. Intra CD3 cytokine expression assessed at diagnosis and in responders.

	Patients" N=53"	Samples-RT"* N=30"	Samples DO"^ N=36"
Gender, n (%)			
Male	31 (58)		
Female	22 (42)		
Median age at sampling, years (min-max)	12.8 (2.1– 56.2)		
VSAA n (%)	11 (21)		
SAA	37 (70)		
NSAA	5 (9)		
GCSF, n (%) &			
Yes		5 (17)	1 (3)
No		24 (80)	32 (89)
Not available		1 (3)	3 (8)
Transfusions, n (%)&			
Yes		7 (23)	24 (67)
No		22 (74)	9 (25)
Not available		1 (3)	3 (8)
CSA, n (%)&			
Yes		25 (83)	4 (11)
No		4 (14)	29 (81)
Not available		1 (3)	3 (8)
Steroids, n (%)&			
Yes		3 (10)	8 (22)
No		24 (80)	25 (70)
Not available		3 (10)	3 (8)
	Cytokine blockade in marrow culture		
	N=28°	**N=16°**	**N=15°**
Gender, n (%)			
Male	20 (71)		
Female	8 (29)		
Median age at sampling, years (min-max)	14.3 (3.6 – 47.1)		
GCSF, n (%)&			
Yes		3 (19)	0
No		12 (75)	10 (67)
Not available		1 (6)	5 (33)
Transfusions, n (%)&			
Yes		1 (6)	8 (53)
No		14 (88)	2 (14)
Not available		1 (6)	5 (33)
CSA, n (%)&			
Yes		12 (75)	1 (7)
No		3 (19)	9 (60)
Not available		1 (6)	5 (33)
Steroids, n (%)&			
Yes		3 (19)	1 (7)
No			12 (75) 9 (60)
Not available		1 (6)	5 (33)
	Intracytoplasmic cytokine expression in patients with sampling at diagnosis and during follow-up		
	N=21	**-**	**-**
Gender, n (%)			
Male	10 (48)		
Female	11 (52)		
Median age at sampling, years (min-max)	10.1 (2.1 – 32.8)		
Response n (%)			
Responders	11 (52)		
Non Responders§	10 (48)		

cultures containing 0.9% methyl cellulose, 30% FBS, 1% bovine serum albumin, 10-4 M 2-mercaptoethanol, and 2 mM L-glutamine. To support erythroid colony growth, 50 ng/mL recombinant human stem cell factor (rhSCF), 20 ng/mL recombinant human granulocyte-macrophage colony-stimulating factor (rhGM-CSF), 20 ng/mL recombinant human interleukin 3 (rhIL-3), 20 ng/mL rhIL-6, 20 ng/mL recombinant human granulocyte colony-stimulating factor (rhG-CSF), and 4 U/mL erythropoietin were added to the culture system (Stem Cell Technologies, Vancouver, BC, Canada). Cultures were incubated for 14 to 16 days under humidified conditions in a 5% CO2 atmosphere at 37°C, in the presence and in the absence of 10 ng/mL of the recombinant soluble human tumour necrosis factor (TNF) receptor-Fc fusion protein Etanercept (Immunex, Seattle, WA) in which the human p75 TNF receptor is fused with the Fc portion of human IgG1, and/or 10 ng/mL of monoclonal anti-human IFN-γ (R&D System, Minneapolis, USA). Control cultures were carried out in the presence of the purified Fc fragment of human IgG1 (Alexis, Del Mar, CA, USA). BFU-E from both patients and controls were counted at the indicated times. The term BFU-E was used to identify immature progenitors that give rise to larger, burst-configured erythroid colonies made up of 3 to 8 closely arranged clusters of hemoglobinized erythroblasts per colony, whose number peaks after 14 to 16 days of incubation.

For each patient BFU-e growth was calculated as the mean count of three dishes, i.e. BFU-e number was expressed out of 105 BM MNCs. For purposes of comparison, values of different groups (Normal controls, Disease at Onset [DO], Responsive to treatment [RT]), median, maximum and minimum values were adopted.

Some subjects had received G-CSF that directly acts on myeloid progenitors and G-CSF treatment details were not available for a number of patients (Table 1). In addition, baseline CFU-GM (Colony Forming Units-Granulocyte-Macrophages) growth was definitely reduced as compared with that of erythroid progenitors. This precluded the use of CFU-GMs for appropriate comparisons and led us to focus the



Legend to Supplementary Appendix 5 (Figure 3 A-D) Marrow cytokine expression in controls and 28 patients tested for BFU-e growth after cytokine block. Absolute number of $CD3^+$ (A), $CD3+/TNF\text{-}a^+$ (B), $CD3^+/$ $IFN\text{-}\gamma^+$ (C) and $CD3^+/IL4^+$ (D) cells in the marrow of normal controls, patients Responsive to Treatment (RT) and with Disease at Onset (DO) who were tested for BFU-e growth after cytokine block. In the box plot bars represent median, upper and lower adjacent value of positive cells/mL of marrow.

analysis on erythroid progenitors which were reckoned less directly influenced by treatments and more representative of the hematopoietic activity.

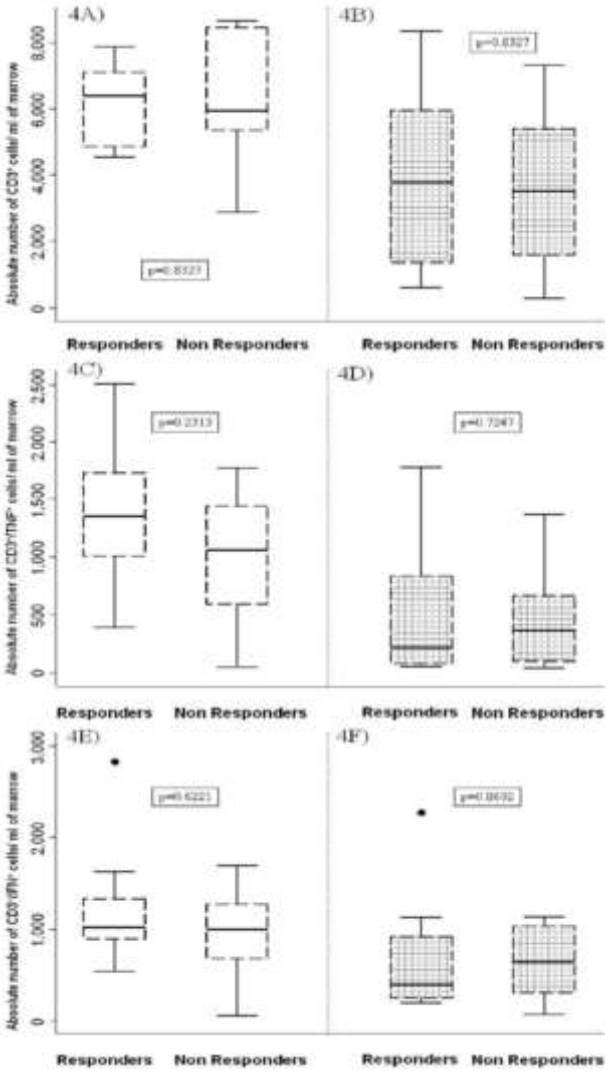
SUPPLEMENTARY APPENDIX 4

Statistics

The intra-patient values of different outcomes were compared using the Friedman and the Wilcoxon tests, while the Kruskal Wallis and the Mann-Whitney U tests were used to measure differences among groups (RT, DO, Responders, Non Responders and controls). The same analyses were used after stratification of AAA patients by their treatment with transfusions G-CSF, steroids or CSA.

The effect of block of TNF-α and of INF-γ alone or in combination, on the growth of BFU-e was measured as absolute values and as absolute difference in comparison to the baseline values and amongst RT, DO and control group. In more detail, for each subject the absolute increment of BFU-ε growth with respect to baseline conditions was calculated by subtracting the baseline number of colonies from the number obtained after the addition of TNF-α, and/or IFN-γ blocking agent.

All tests were two-tailed and a p value < 0.05 was considered as statistically significant. The statistical software "Statistica" (release 6.0, StatSoft Corporation, Tulsa, OK) and the software "*Stata*" (release 7.0, StataCorp 2001, College Station, TX, USA) were used.



Legend to Supplementary Appendix 6. Figure 4 A-H Comparisons of marrow cytokine expression of Responders and Non Responders at Diagnosis and RET. Absolute number of: $CD3^+$ in Responders vs Non Responders at Diagnosis (A) and at RET (B). $CD3^+/TNF\text{-}\alpha^+$ in Responders vs Non Responders at Diagnosis (C) and at RET (D). $CD3^+/IFN\text{-}\gamma^+$ in Responders vs Non Responders at Diagnosis (E) and at RET (F). $CD3^+/IL4^+$ cells in Responders vs Non Responders at Diagnosis (G) and at RET (H). In the box plot, bars represent median, upper and lower adjacent value of positive cells/ml of marrow.