FUNCTIONAL CONSEQUENCES OF TGFB1 POLYMORPHISMS IN REGULATORY T CELLS AND HAEMATOPOIETIC STEM CELL TRANSPLANTATION

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Introduction

Haematopoietic stem cell transplantation (HSCT) is used to treat malignant and non-malignant diseases. Apart from clinical and Human Leukocyte Antigen (HLA)-related factors, non-HLA Immunogenetics is being increasingly recognized to play a role in the outcome of HSCT. A gene that may be relevant for the outcome of HSCT is TGFB1, which encodes Transforming Growth Factor β 1 (TGF-β1), a cytokine that is central in the regulation of numerous immune processes. Several polymorphisms in TGFB1 have been identified and some of them are known to cause alterations in cytokine secretion. We and others have previously shown that polymorphisms within TGFB1 affect the outcome of HSCT. Regulatory T cells (Treg) are major producers of TGF-β1 and play a fundamental role in immune events in HSCT. Consequently, the aim of this project is to study the effect of TGFB1 polymorphisms on the function of Treg and their role in HSCT complications such as graft-versus-host disease.

Materials and Methods

Peripheral blood from healthy volunteer donors was obtained by venipuncture. DNA was extracted by an in-house salting-out method. Molecular typing techniques were used to type TGFB1 +29 T>C polymorphism. Volunteer donors were grouped in three genotype categories for further experiments. Peripheral blood mononuclear cells (PBMC) were isolated by ficoll gradient, and Treg ($CD4^+CD25^{++}CD127^{lo}FOXP3^+$) and effector ($CD4^+CD25^-CD127^{hi}FOXP3^-$) cells were isolated with a microbead-based method (Figure 1).

Isolated Treg and effector cells were stimulated with antiCD3/CD28 antibodies and cultured *in vitro* in 96-round bottom well plates. Latency-associated-peptide (LAP, membrane-bound TGF-β1) expression was measured on resting and stimulated cells by flow-cytometric analysis at specific time-points.

Figure 1 Example of a regulatory T cell isolation procedure. PBMC (top row) obtained from healthy volunteer donors were used to isolate CD4+CD25- (third row) and CD4+CD25+ (bottom row) cells. The second row shows the CD4-depleted fraction. The first column shows the forward and side scatter plots (*y* axis) and lymphocyte gate. The second and third columns show scatter plots for CD4 (*y* axis) vs. CD25 expression and forward scatter on the lymphocytes, respectively. The fourth column shows CD127 (*y* axis) vs. CD25 expression scatter plots gated on the CD4+ lymphocytes. Plots generated with Flojo software (v. 6.4.7, Tree Star Inc., Ashland, USA).

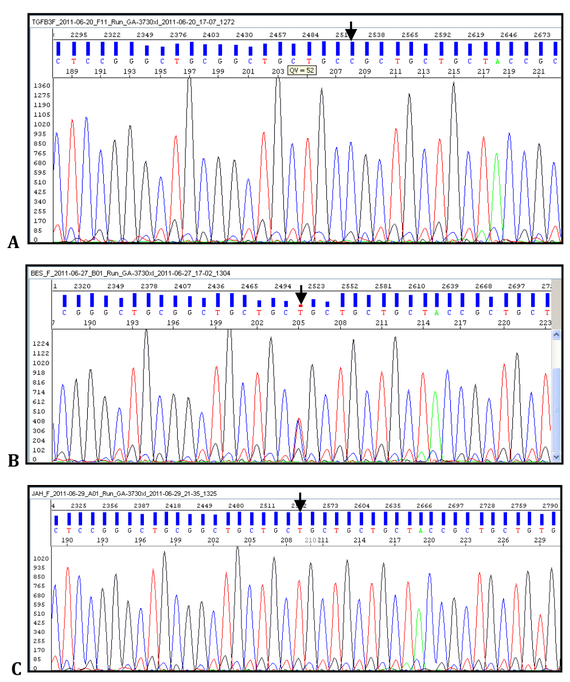

Figure 2 Three TGFB1 signal peptide region sequencing results showing +29T>C. (A) CC individual. (B) TC individual. (C) TT individual. Sequences analysed with Sequencing Analysis 5.2 (PE Applied Biosystems, Foster City, CA, USA).

Results

Molecular typing techniques for the detection of TGFB1 +29T>C SNP have been employed to type 31 healthy blood donors recruited for functional experiments. Figure 2 shows representative results for the 3 TGFB1 +29 T>C genotypes as identified by sequencing.

Membrane-bound TGF-β1 (LAP positivity) upon TCR stimulation of isolated Treg has been confirmed to be specific to this cell subset (Figure 3). LAP levels have been assessed in 5 donors per TGFB1 +29T>C genotype. The *in vitro* kinetics of TGF-β1 induction on these cells after TCR stimulation with soluble antibodies against CD3 and CD28 was shown to peak at 24h of incubation and to reach an average of 25.7% of the $CD4^+CD25^{++}CD127^{lo}$ cells (range 11.0-51.2%), followed by a reduction to low levels by 48h, which remain constant up to 96h (Figure 4). TCR stimulation with plate-bound antibodies produces maximal and sustained LAP expression on Treg (Figure 5). A trend towards a higher percentage of LAP+ Treg generated after soluble activation when the cells bear a +29C allele (Pro10 on its signal peptide) was identified (p=0.066) (Figure 6).

Figure 3 LAP expression on activated Treg (and not on effector CD4 cells) peaks at 24h of incubation. Isolated Treg and effector CD4 cells were activated with soluble anti-CD3 and anti-CD28 in the presence of IL-2. LAP positivity was determined at t=0, 24 and 48h of incubation by flow cytometry. (A) LAP (*y* axis) vs. CD4 (top row) and CD127 (bottom row) staining on isolated Treg after 0, 24 and 48h of incubation. Plots generated by gating on live cells (top row) and on CD4+ cells (bottom row). (B) Histogram for LAP staining on Treg at different time points after activation. Cells gated on lymphocytes. (C) Histogram for LAP staining on effector CD4 cells at different time points after activation. Cells gated on lymphocytes.

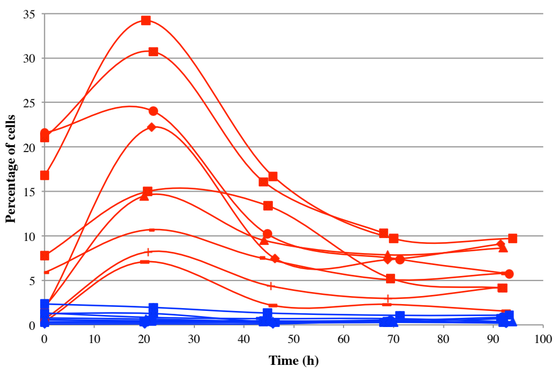

Figure 4 LAP+ cells after TCR stimulation in isolated Treg (CD4+CD25+CD127lo) and Effector (CD4+CD25-CD127hi) lymphocytes. In a series of independent experiments, isolated Treg (red) and effector (blue) cells were activated with soluble anti-CD3 and anti-CD28 in the presence of IL-2 and cultured for different periods. The cells were harvested at specific time points and surface LAP expression was assessed by flow cytometry.

Figure 5 LAP expression on Treg is maximal and sustained after plate-bound TCR stimulation. Isolated Treg (red) and effector CD4 (blue) cells were stimulated with plate-bound anti-CD3 and anti-CD28 in the presence of IL-2. LAP staining (*y* axis) was assessed at different time points after incubation. Gated on live cells.

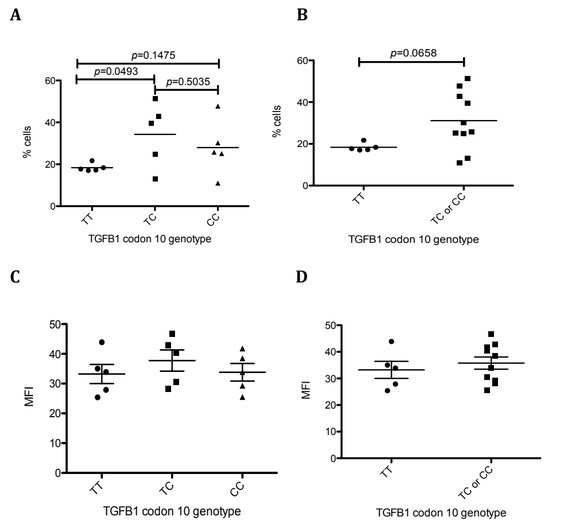

Figure 6 LAP expression on Treg upon TCR stimulation differs according to TGFB1 codon 10 +29T>C genotype. A total of 15 independent Treg activation experiments (5 per genotype) were performed and LAP expression was assessed at 24h of incubation with soluble anti-CD3 and anti-CD28. (A) Percentage of LAP+ cells within the CD4+CD25+CD127lo gate according to their TGFB1 +29 T>C genotype. (B) The same results grouped according to the presence or absence of a C allele on TGFB1 +29 T>C. (C) Median fluorescence intensity (MFI) of the LAP+ cells according to their TGFB1 +29 T>C genotype. (D) The same results grouped according to the presence or absence of a C allele at TGFB1 +29 T>C.

Conclusions

TGFB1 +29 T>C genotype seems to influence the amount of membrane-bound TGF-β1 expressed exclusively by in vitro TCR-stimulated Treg. The presence of a C allele confers a trend towards higher production of LAP by these cells in a dominant model. Differences in the levels of LAP on Treg may affect the function of these cells in an inflammatory context such as that present in HSCT patients. Consequently, this possibility must be explored and further experiments are being developed in order to address these questions.

Future work

We are currently undertaking RT-Q-PCR experiments in order to characterise the effect of TGFB1 +29 T>C polymorphism on the expression of this cytokine by Treg and effector CD4+ cells at the RNA level. Additionally, we are developing optimised autologous suppression assays in order to evaluate the functional effect of TGFB1 polymorphisms on immune modulation by Treg.

In the future, we will extend our analysis to other polymorphisms within the regulatory region of TGFB1, and perform haplotype analysis on the joint effect of these polymorphisms on the outcome of HSCT and on the function of Treg.

These data will hopefully help to identify the risk factors associated with TGFB1 expression in patients and donors and to generate clinically useful algorithms of genetic risk assessment in HSCT.

Acknowledgements

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