bone marrow milieu. Thus, in patients requiring rescue therapy, allo-RIC should be considered as a platform for additional therapeutic strategies after transplantation in order to take advantage of the GVM effect.

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Increase of CCR7$^-$ CD45RA$^+$ CD8 T cells (T_{EMRA}) in chronic graft-versus-host disease

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Among the late effects of hematopoietic stem cell transplantation (HSCT), chronic graft-vs-host disease (cGVHD) still remains as the major determinant of long-term outcome and quality of life. The disease typically appears between 3 months to 1.5 years following an allogeneic transplantation and is characterized by symptoms similar to those of autoimmune disease.

GVHD is caused by donor CD4 and CD8 T cell-mediated anti-host reaction that evolves over several weeks to months, suggesting a requirement for persistent alloreactive T cells.

It has recently been demonstrated that donor-derived, alloreactive CD4 T cells play an important role in the pathogenesis of cGVHD that might be associated with a preponderance of CD4 effector memory cells (CCR7$^-$ CD62L^{low}, $CD4_{EM}$).[1]

Comparatively little is known about the role of CD8 T cells in cGVHD and whether, similarly to CD4 T cells, it is associated with the preponderance of a particular CD8 T-cell subset.

Human CD8 T cells can be divided into at least four different subsets, based on their phenotype and functions: naive (T_{naive}, CCR7$^+$ CD45RA$^+$) and central memory (T_{CM}, CCR7$^+$ CD45RA$^-$) T cells display high proliferative potential and lack of an immediate effector functions whereas effector memory (T_{EM}, CCR7$^-$ CD45RA$^-$) and CD45RA$^+$ effector memory (T_{EMRA}, CCR7$^-$ CD45RA$^+$) T cells have low proliferative capacities but produce cytokines and exert cytotoxic activity, respectively.[2]

CD45RA$^+$ effector T_{EMRA} cells represent the most differentiated type of memory cells; they have high susceptibility to apoptosis and express high levels of cytotoxic molecules such as perforin and Fas ligand.

In order to assess the balance between memory and effector cells and analyze the distribution of CD8 T cells within the four previously described subsets, we have studied peripheral blood samples from cGVHD patients.

In total, 20 adult patients, affected by various hematologic malignancies and treated by allogeneic peripheral blood stem cell (aPBSC) transplantation from HLA-identical related donors, were recruited for this study.

Ten of them had cGVHD and 10 patients had no current cGVHD (>100 days after transplantation). In the group of patients with cGVHD, three had extensive and seven limited disease. A great proportion of patients with cGVHD required immunosoppressive treatment at the time of sampling. Ten patients who underwent kidney transplantation (KT) requiring treatment with immunosuppressive drugs were used as controls for the effects of immunosuppressive drugs. Fifteen age- and sex-matched normal healthy subjects (HS) were used as controls. Patients showing infection at the time of the study were not included. The study was approved by the Institutional Review Board.

Fresh peripheral blood mononuclear cells (PBMCs) were obtained by Ficoll separation (Cedarlane, Ontario, CA, USA).

Table 1 Characterization of CD8 T-cell populations in cGVHD

	HS	*NoGVHD*	*GVHD*	*KT*
T_{naive}	35±16	31±17	**12±6**	34±19
T_{CM}	15±9	9±4	6±3	13±6
T_{EM}	23±8	27±12	28±11	22±13
T_{EMRA}	24±11	26±20	**55±14**	30±18

Values indicate the percentage±standard deviation (s.d.) of T_{naive}, T_{CM}, T_{EM} and T_{EMRA} subsets of CD8T cells in 10 patients with cGVHD (cGVHD), 10 patients who received aPBSC transplantation without cGVHD (No cGVHD), 10 patients who underwent kidney transplantation and received immunosuppressive drugs (KT) and 15 healthy subjects (HS). Statistically significant differences are indicated in bold and underlined (See the text for *P*-values).

Cells were stained with quantum-red (QR)-conjugated anti-human CD8 monoclonal antibody (mAb) (Sigma), phycoerythryn (PE)-conjugated anti-human CCR7 mAb, fluorescein isothiocyanate (FITC)-conjugated antihuman CD45RA mAb and FITC-conjugated anti-human perforin mAb, all from BD Biosciences (San Diego, CA, USA). PE-conjugated Pro™ Pentamer H-Y^{A2} was from ProImmune (Oxford, UK). mAbs were used following the manufacturer's recommendations. Perforin expression was assessed after fixation with 2% paraformaldehyde and permeabilization with permeabilizing solution (BD Biosciences).

Flow cytometry was performed using a FACSCan flow cytometer (BD Biosciences) and analysis was performed with WinMDI 2.8 software.

Two-group comparison was carried out on the analysis of variance and Student's *t*-test. A *P*-value less than 0.05 was considered statistically significant. Data were analyzed using the SYSTAT 11 software.

CCR7 and CD45RA expression was analyzed on CD8 T cells from patients who received aPBSC transplantation both with and without cGVHD and healthy subjects. Additionally, as all cGVHD patients were receiving immunosuppressive treatment at the time the blood samples were collected, we also analyzed CD8 T-cell subsets in patients receiving cyclosporine A (CsA) and steroids treatment following KT in order to evaluate the impact of immunosuppressive drugs on CD8 subsets.

Table 1 shows a significant reduction of CD8 T_{naive} cells in patients with cGVHD, compared with stem cell transplantation patients without cGVHD ($P=0.008$), with patients who underwent KT ($P=0.006$) and with healthy subjects ($P=0.008$). The T_{CM} subset was also decreased in cGVHD patients, but the values' difference in patients without cGVHD, kidney transplated and healthy subjects was not statistically significant. Effector CD8 T cells' distribution showed no statistically significant difference in the T_{EM} subset in the tested groups, but patients with cGVHD had a significantly higher percentage of T_{EMRA} cells, compared with stem cell transplantation patients without cGVHD (respectively; $P=0.003$), with patients who underwent KT ($P=0.004$) and with healthy subjects ($P=0.005$).

These results suggest that the decrease of the T_{naive} and the strong increase of the T_{EMRA} CD8 T-cell subsets are a peculiarity of cGVHD and are not an effect of immunosuppressive drugs administration.

Previous studies showed that $CD45RA^+$ T_{EMRA} cells express high levels of perforin notably associated with CCR7 down-regulation, as the acquisition of cytotoxicity properties is paralleled with loss of lymph node homing ability.[2] Accordingly, we detected increased perforin levels in CD8 $CCR7^-$ cells from cGVHD patients (38.8%±15.4) compared with patients without GHVD (12.5%±5.9) and healthy subject (10.4%±5.4).

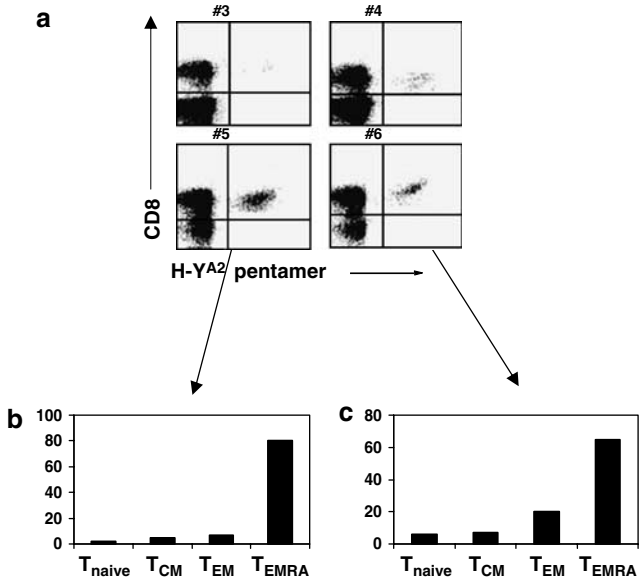

Figure 1 Distribution of different subsets of H-Y-specific CD8 T cells. **(a)** PBMC were obtained from two non-HLA-A0201 male patients receiving transplant from an identical female donor (patients #3 and #4) and two HLA-A0201 male patients in whom cGVHD occurred and who received transplant from HLA-identical female sibling (patients #5 and #6). PBMCs were stained with anti-CD8 and H-Y^{A2} pentamer. On analysis within CD45RA and CCR7, most CD8 H-Y^{A2+} T cells from both patient #5 **(b)** and patient #6 **(c)** are predominantly contained within the $CD45RA^+$ $CCR7^-$ (TEMRA) cell subset. In **(b)** and **(c)**, numbers on the *y*-axis indicate the percentage of total $CD8^+$ H-Y^{A2+} T cells.

These data are consistent with previous studies showing increasing perforin gene expression by quantitative RT-PCR in GVHD patients.[3]

As in HLA-identical transplantation GVHD may be induced by minor histocompatibility antigen (mHags) differences between the donor and the recipient, with the antigen being present in the recipient and not in the donor, we analyzed the distribution of CD8 T lymphocyte subsets specific for the male-related mHag H-Y in PBMCs from two HLA-A0201 male patients in whom cGVHD occurred and who received transplant from an HLA-identical female sibling. Previous studies have reported that H-Y is a target of cytotoxic T lymphocytes (CTLs) in GVHD, as demonstrated using a skin-explant assay and tetrameric HLA-mHag peptide complexes,[4] and, in particular, there is evidence for the priming of CD8 T-cytotoxic lymphocytes against H-Y antigen.[5]

As shown in Figure 1a, the frequency of H-Y-specific CD8 T lymphocytes, as detected by staining with an H-Y^{A2} pentamer, in these two patients is notably higher (4.7 and 2.16%, respectively) than the frequency in a non-HLA-A0201 male patient receiving transplant from an identical female donor (0.06%). In both patients, the vast majority of $pentamer^+$ CD8 T cells were $CD45RA^+CCR7^-$ (Figure 1b), thus confirming in an alloantigen-specific system the skewed distribution of CD8 T-cell subset observed in the whole CD8 population.

Thus, our data represent the first demonstration of a correlation between the increase of T_{EMRA} subset in CD8 T cells and the occurrence of cGVHD in patients who underwent a PBSC transplantation.

Although it is possible that reduction in size of the T_{naive} and T_{CM} CD8 subsets in cGVHD might be the consequence of the reduced production of naive recent thymic T-cell emigrants and/

or impaired survival in the periphery,[6] the mechanisms leading to the increase of CD8 T_{EMRA} cells are unknown.

As the percentage of $CD8^+$ T cells in PBMCs is not modified in each patients' group (cGVHD = 32.4 ± 16.5%; no cGVHD = 32.2 ± 20%; KT = 33.9 ± 12.7%; HS = 30.8 ± 14.5) and the increase in the percentage of T_{EMRA} subset is paralleled by a glaring decrease of the T_{naive} and T_{CM} subsets, we speculate that in cGVHD there is a progressive differentiation from T_{naive}/T_{CM} to T_{EMRA} cells as a consequence of prolonged alloantigen exposure or IL-15 stimulation; T_{EMRA} cells, in fact, can be generated by IL-15-stimulated T_{CM} cells even in the absence of an antigen. In support of such a possibility, patients with cGVHD have elevated serum IL-15 levels,[7,8] and *in vivo* activation and expansion of human T lymphocytes in response to IL-15 causes exacerbation of human T-cell-mediated xenogeneic GVHD.[9]

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Activating mutations of *JAK2*V617F are uncommon in *t*-MDS and *t*-AML and are only observed in atypic cases

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Activating mutations of genes in the receptor tyrosine kinase *RAS-BRAF* (RTK/*RAS-BRAF*) signal transduction pathway are common in myelodysplasia (MDS) and acute myeloid leukemia (AML)[1–4] and in the therapy-related subsets of these diseases (*t*-MDS/*t*-AML).[5] Recently, a specific activating mutation of the *JAK2* tyrosine kinase was detected in 154/201 cases of polycythemia vera (77%), in 21/44 cases of idiopatic myelofibrosis (48%) and in 50/144 cases of essential thrombocythemia (35%).[6,7] The *JAK2*V617F mutation was subsequently observed only sporadically in 3/119 cases of chronic myelomonocytic leukemia, including a case with a history of B cell lymphoma, and in 5/101 cases of MDS including four patients with a normal karyotype.[8]

In our systematic search for genetic abnormalities in *t*-MDS and *t*-AML and their association to clinical parameters and cytogenetic characteristics, we examined 140 unselected patients with *t*-MDS or *t*-AML previously examined for mutations in the RTK/*RAS-BRAF* pathway[5] for the *JAK2*V617F mutation. PCR amplification and direct sequencing of *JAK2* exon 12 was performed as previously reported[5] using primers described elsewhere.[8]

Two out of 89 patients presenting as *t*-MDS (cases 43 and 179) harboured a 1849G > T substitution resulting in a

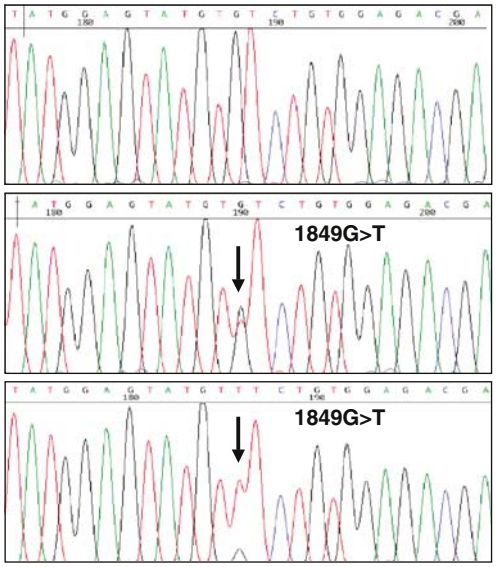

Figure 1 Direct DNA sequence analysis of *JAK2* exon 12. At the top, a wild-type sequence from a normal control is shown for comparison. The middle sequence shows equal fluorescence intensities of the mutated and the wild-type alleles in patient 43, whereas at the bottom the sequence of the wild-type allele of patient 179 is only faintly visible.