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Research paper

Advanced flowcytometric analysis of regulatory T cells: CD127 downregulation early post stem cell transplantation and altered Treg/CD3$^+$CD4$^+$-ratio in severe GvHD or relapse ☆

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ABSTRACT

Regulatory T cells (Tregs) are of crucial importance to suppress graft versus host disease (GvHD) post allogeneic stem cell transplantation (SCT), but are also known to impair antitumor immunity. However, Treg longitudinal studies are rare and in this respect advanced flowcytometric approaches for Treg characterization are necessary.

To investigate the relation of both the percentage and the absolute numbers of Tregs on GvHD or relapse we measured CD4$^+$CD25$^{+/hi}$CD127$^{lo/-}$ Tregs in 239 peripheral blood (PB) samples of 16 patients during the first two years post-SCT. A 10-color flowcytometric panel was established to evaluate Treg subpopulations and has been tested in ten healthy individuals.

In patients we demonstrated a decrease in CD127 expression on T cells early post-SCT which increases during the first year. Moreover, Tregs reached higher absolute numbers in patients with GvHD ≤ grade I compared to those with GvHD grades II–IV. In contrast, the percentage of Tregs was significantly higher in patients with GvHD grades II–IV or disease relapse compared to those without GvHD. These patients fit into the range of healthy individuals where a median value of 7.5% and 6.4% of T helper cells were characterized as CD4$^+$CD25$^{+/hi}$CD127$^{lo/-}$ and CD4$^+$CD25$^{+/hi}$ Tregs, respectively. Furthermore, Tregs could be further subdivided into 40% naïve, 51% central memory and 9% effector memory Tregs.

Our results showed for the first time a downregulation of CD127 expression on T cells including Tregs in patients early post-SCT. Additionally, new insights into the recovery of Tregs regarding GvHD and relapse were provided.

Abbreviations: ALL, acute lymphoblastic leukemia;AML, acute myeloid leukemia;APC, allophycocyanin;BM, bone marrow;BMT, bone marrow transplantation; BW, body weight;CsA, cyclosporin A;DLI, donor lymphocyte infusion;ECD, phycoerythrin—Texas Red;FITC, fluorescein isothiocyanate;GvHD, graft versus host disease;GMP, good manufacturing practice;hi, high;int, intermediate;lo, low;mAb, monoclonal antibody;MFD, matched family donor;MFI, mean fluorescence intensity;MMF, mycophenolate mofetil;MMFD, mismatched family donor;MMUD, mismatched unrelated donor;MUD, matched unrelated donor;MTX, methotrexate;NK, natural killer;PB, peripheral blood;PBSC, peripheral blood stem cells;PC5, phycoerythrin–cyanine 5.1;PC7, phycoerythrin–cyanine;PE, phycoerythrin;SCT, stem cell transplantation;Treg, regulatory T cell.

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1. Introduction

Tregs play a pivotal role in maintaining self-tolerance and controlling adaptive immune responses (Hoffmann et al., 2004; Liu et al., 2006; Crellin et al., 2007; Terme et al., 2008). They suppress conventional T cell function, cytokine production and proliferative capacity in a contact dependent manner (Clark et al., 2004; Banham, 2006; Miyara and Sakaguchi, 2007; Terme et al., 2008). After allogeneic hematopoietic stem cell transplantation (SCT) for high risk malignancies, Tregs are key players in the prevention of graft rejection (Meloni et al., 2004; Somerset et al., 2004; Codarri et al., 2007; Miyara and Sakaguchi, 2007) and the alleviation of graft versus host disease (GvHD) (Hoffmann et al., 2004; Ermann et al., 2005; Miyara and Sakaguchi, 2007; Wolf et al., 2007). However, Tregs are also known to impair antitumor immunity forming a potential tumor escape mechanism (Beyer and Schultze, 2006; Strauss et al., 2007; Zwirner et al., 2007; Brinkrolf et al., 2009). Natural Tregs originate from the thymus as $CD3^+CD4^+$ cells expressing high levels of CD25 together with the transcription factor FoxP3. In addition, CD127 is proposed to be inversely correlated to CD25 expression. Therefore CD127 is suggested as a biomarker for human Tregs when combined with CD25 (Ardon et al., 2010; Liu et al., 2006; Seddiki et al., 2006; Codarri et al., 2007; Hartigan-O'Connor et al., 2007; Hoffmann et al., 2007; Miyara and Sakaguchi, 2007).

To date, different flowcytometric strategies are proposed to determine the amounts of Tregs in peripheral blood (PB) such as $CD4^+CD25^+FoxP3^+$ and $CD4^+CD25^{+/hi}$ Treg definition or the recently used $CD4^+CD25^+CD127^{lo/-}$ approach (Liu et al., 2006; Seddiki et al., 2006; Codarri et al., 2007; Hartigan-O'Connor et al., 2007). Hence, there is an ongoing discussion whether both the $CD4^+CD25^{+/hi}$ and the $CD4^+CD25^+FoxP3^+$ subset include only one major part of Treg cells (Hartigan-O'Connor et al., 2007; Miyara and Sakaguchi, 2007).

In a small cohort of patients post-SCT we studied the immune reconstitution of Tregs with regard to GvHD and relapse. Furthermore, we compared the CD127 expression between patients post-SCT and healthy individuals.

To reach further insights in to Tregs, we established a 10-color flowcytometric panel for quantification of Tregs including naïve, central memory and effector memory Treg subpopulations and tested this panel with PB of healthy individuals.

2. Patients and methods

2.1. Patients and healthy individuals

After written informed consent, approved by the local ethic committee (Frankfurt, Germany, Ethical No. 50/07) both ten healthy individuals and 16 patients (aged 3–45 years) were enrolled in our Treg study. The immune reconstitution of Tregs was monitored in the PB post allogeneic SCT in 14 children and two adults suffering from acute leukemia (n = 12) or neuroblastoma (n = 4) over a period of approximately two years post-SCT. Tregs were quantified in 239 PB samples, within the first three months post-SCT weekly, followed by two measurements per months until six months, then monthly up to one year and finally every two months during the second year post-SCT. Table 1 shows patient's characteristics with regard to diagnosis, stem cells source, graft manipulation and therapy. The latter included reduced intensity conditioning consisting of fludarabin, melphalan, thiotepa or full myeloablative regimen. GvHD prophylaxis was given to all patients consisting of OKT3, MMF, MTX or CsA. Thirteen patients received steroids post-SCT, in median for five weeks as a treatment of GvHD or engraftment-syndrome. A donor lymphocyte infusion (DLI) was given to six of 16 patients. In total ten of 16 patients (63%) are alive.

2.2. Grafts

Graft types included BM without manipulation (n = 8), unmanipulated PBSC (n = 2) and CD3/CD19-depleted PBSC (n = 6). In the latter case the grafts were purified immunomagnetically on a CliniMacs device (Miltenyi Biotec, Bergisch Gladbach, Germany) obeying GMP. For quality control after CD3/CD19-depletion a single-platform flowcytometric approach was used as we described previously (Koehl et al., 2007; Koehl et al., 2008; Koenig et al., 2010). Eleven patients received grafts from matched donors (three family donors (MFD) and eight unrelated donors (MUD)). Five patients were transplanted from haploidentical mismatched family donors (MMFD) as indicated in Table 1. Grafts included in average $4.7*10^6$, $5.9*10^6$ and $12.9*10^6$ $CD34^+$ stem cells/kg body weight (BW) and $4.6*10^7$, $3.6*10^8$ and $7.3*10^4$ $CD3^+$ T cells/kg BW for BM, PBSC and CD3/CD19-depleted PBSC, respectively.

2.3. Immunophenotyping of Tregs, Treg subpopulations and the overall $CD3^+CD4^+$ cells

One hundred-200 µl PB were stained for Treg analyses using fluorescence-conjugated monoclonal antibodies (mAbs) in a combination of the following 10- and 5-color panels: CD3 (fluorescein isothiocyanate-FITC/phycoerythrin-Texas Red-ECD/Pacific Orange-PacO; clone UCHT1), CD4 (ECD/phycoerythrin–cyanine 7-PC7; clone T4), CD4 (Pacific Blue-PacB; clone 13B8.2), CD16 (APC-Alexa Fluor® 750-APC-A 750; clone 3G8), CD25 (phycoerythrin–cyanine 5-PC5/PC7; clone 2A3), CD45RA (ECD; clone 2H4), CD56 (allophycocyanin—APC; clone N901), CD62L (FITC/PC5; clone DREG56), CD69 (PC5; clone TP1.55.3), CD117 (phycoerythrin—PE; clone 104D2D1), CD127 (APC-Alexa Fluor® 700-APC-A 700/PE; clone R34.34). CD25-PC7 was produced by BD Biosciences (Heidelberg, Germany); all other surface mAbs were from Beckman Coulter (Krefeld, Germany). In addition appropriate isotype controls were prepared. Treg reconstitution post-SCT was determined with one 5-color panel (CD3-FITC, CD127-PE, CD4-ECD, CD62L-PC5, CD25-PC7 or CD62L-FITC, CD127-PE, CD3-ECD, CD25-PC5, CD4-PC7). An automated lyse/no wash procedure with a fixation step followed using a TQ-Prep™ Workstation (Beckman Coulter, Krefeld, Germany) with the ImmunoPrep reagent as we described for other immune cells previously was applied (Koehl et al., 2007; Huenecke et al., 2008; Koenig et al., 2010). In healthy individuals Tregs were quantified using one 10-color panel (CD62L-FITC, CD117-PE, CD45RA-ECD, CD69-PC5, CD25-PC7, CD56-APC, CD127-APC-A700, CD16-APC-A750, CD4-PacB, CD3-PacO). To avoid background labeling in the 10-color analyses for simultaneous detection of Treg- and NK subpopulations we included a washing step and used VersaLyse Lysing solution including IO-Test Fixative (1:40)(Beckman Coulter, Krefeld, Germany). Finally, absolute cell numbers of Tregs were calculated using a dual-platform approach for both panels. For the evaluation of the 10-color and the 5-color panel, gating strategies