Selective Depletion of Alloreactive Donor T Cells Leads to Elimination of Graft-Versus-Host Reactivity and Stimulates Graft-Versus-Leukaemia/Myeloma Effect

Original Article

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Abstract. Graft-versus-host disease is a severe complication of allogeneic stem cell transplantation. The major role is played by alloreactive donor T-cell clones leading to host tissue damage. Selective depletion is a strategy to eliminate host-reactive donor T cells from haematopoietic stem cell allografts to prevent graft-versus-host disease while conserving useful donor immune functions. We have used irradiated peripheral blood mononuclear cells from cancer patients and healthy donor cells as responder cells in primary mixed leukocyte reaction. To prepare graft-versus-leukaemia/myeloma-specific T cells, alloreactive T cells in primary mixed leukocyte reaction were depleted with anti-CD25 immunotoxin. The remaining T cells had insignificant alloreactivity in secondary mixed leukocyte reaction. Then, allodepleted donor T cells were repeatedly stimulated using purified leukaemia/tumour cells from the same cancer patient. Leukaemia/tumour-reactive donor T cells were purified using cell sorter on the basis of CD4 and CD8 activation. Their specificity was tested in nonradioactive cytotoxicity test. We performed 22 reactions (15 samples with leukemic and 7 samples with multiple myeloma cells). Selective depletion of alloreactive donor T cells with anti-CD25 immunotoxin led to significant depletion (99.2–100 %, median 99.7%). The effect of donor T cells was well preserved, while the graft-versus-host reactivation of donor cells was negligible, even after repeated stimulation with patient’s non-tumour cells. Thus, it is possible to selectively deplete donor alloreactive T cells with anti-CD25 immunotoxin. In the cases of leukaemia patients, a strong graft-versus-leukaemia reactivity was noticed in allodepleted donor T cells; in myeloma patients, graft-versus-myeloma reactivity was less significant.

Introduction

Allogeneic haematopoietic stem cell transplantation (HSCT) represents effective treatment of many types of haematological malignancies and other non-malignant diseases that are otherwise untreatable (Weisdorf et al., 2012). Currently, there is the prevalent opinion that the potential of treatment by HSCT consists essentially in use of antitumor activity of allogeneic donor T cells that are able to activate the graft-versus-leukaemia (GVL) and graft-versus-myeloma (GVM) reaction.

The most compelling evidence for a GVL response in humans is the observation of complete remissions in recipients with relapsed malignancy after HSCT who receive donor leukocyte infusions. Such remissions have been observed in 70–80 % of recipients with chronic myeloid leukaemia in chronic-phase relapse (Mavroudis et al., 1998), and to a lesser extent in recipients with acute myeloid leukaemia (AML), chronic lymphoid leukaemia (CLL), multiple myeloma (MM) and low-grade
lymphomas (van Dijk et al., 1999; Kolb et al., 2004; Mielke et al., 2008; Pasquini et al., 2012). Although graft-versus-host disease (GVHD) is usually associated with a GVL response (Barrett and Battiwalla, 2010), GVL can occur in the absence of clinical GVHD (van Dijk et al., 1999; Pasquini et al., 2012). Regression of tumour growth was also noticed in patients with other malignancies (Amrolia et al., 2006).

Despite the progress of supportive therapy in patients after haematopoietic stem cell transplantation during the last 25 years, an undesirable GVH reaction remains one of the major problems of allogeneic HSCT; GVHD is one of the main causes of morbidity and mortality in post-transplantation period (Weisdorf et al., 2012). Acute GVHD is mediated by allogeneic T cells (Vaclavkova et al., 2006). Activated T cells can also be characterized by the presence of CD25 (IL-2 receptor α) on the cell surface. These populations of effector cells cause damage to recipient tissue, especially skin, liver and gastrointestinal tract, via deregulated cytokine production. As the GVH reaction is primarily caused by donor T cells, their depletion should prevent GVHD. In clinical trials, while using T-cell depletion to prevent GVHD in HLA-matched related donors, a dose of T cells lower than 10^5 per kilogram of the recipient’s body weight has rarely been associated with severe GVHD (Chen et al., 2002). Depletion of all T cells from the graft prevented development of GVHD but also led to a delay in immune reconstitution and an increase of potentially lethal opportunistic infections and leukemic relapses (Ferrara et al., 1999; Barrett and Battiwalla, 2010). In myeloma patients, severe GVHD as a consequence of HSCT is a major limitation of allogeneic approach (Amrolia et al., 2006; Ritchie et al., 2010).

An ideal solution would be to eliminate only donor T cells responsible for GVH reactivity while conserving or stimulating useful donor immune functions, especially GVL/GVM and anti-microbial reactivity. This rationale is based on the presumption that GVH and GVL/GVM are mediated by different clones of individual donor T cells which can be separated (Mavroudis et al., 2003a; Chaidos et al., 2012). To eliminate GVH reactivity, the alloreactive donor T cells can be selectively depleted after their activation with recipient non-tumour peripheral blood mononuclear cells (PBMC).

Previously, we have demonstrated that selective depletion of such activated alloreactive donor T cells that express activation markers, including CD25, on their surface can be effectively eliminated using an anti-CD25 immunotoxin (Michálek et al., 2003a). This ricin A chain anti-CD25 immunotoxin not only eliminates alloreactivity, but also CD4/CD25^+ regulatory T cells; thus, preservation of the third-party (anti-leukemic and anti-microbial) reactivity can be further enhanced (Michálek et al., 2000). To simulate the in vivo situation, where donor T cells are repeatedly exposed to recipient cells and antigens, we performed repeated stimulations of allorepleted donor T cells with recipient PBMC in vitro in mixed lymphocyte reaction (MLR). The population of donor T cells recognize a broad spectrum of recipient non-tumour antigens and express several activation markers, including CD25 molecules on their surface. As previously demonstrated, we used an anti-CD25 immunotoxin (IT) (Vaclavkova et al., 2006) to eliminate donor alloreactive T cells effectively and specifically, without significant damage to the remaining populations of donor T cells with regard to GVL and GVM reactivity (Michálek et al., 2003b). Here, we present the results of pre-clinical testing of anti-CD25 immunotoxin in prevention of GVH disease and potentiation of the GVL/GVM reaction for future clinical application.

Material and Methods

Patient characteristics

After receiving signed informed consent approved by the local Ethical Committee, we used bone marrow from seven multiple myeloma patients (MM) and blood from 15 leukaemia patients – nine patients had acute myeloblastic leukaemia (AML), three had acute lymphoid leukaemia (ALL), two had chronic lymphoid leukaemia (CLL) and one had chronic myeloid leukaemia (CML). Twenty donors were HLA-mismatched; healthy volunteers and two sibling donors were HLA-matched with two of the CLL patients. These patients had CLL and underwent allogeneic HSCT from their HLA-matched sibling, but unfortunately they both died after transplantation.

Cell collection

As a source of leukemic cells, we used 40 ml of anticoagulated peripheral blood from patients at the time of diagnosis (9 patients) or at the time of relapse (3 patients) with at least 70 % of leukemic blasts in peripheral blood. In myeloma patients, we collected CD138^+ myeloma cells from the bone marrow aspiration enriched via magnetic columns (Miltenyi Biotec, Bergisch Gladbach, Germany). The purity of CD138^+ myeloma cells was greater than 70 % in all samples tested. Anticoagulated peripheral blood (40 ml) was obtained from these patients at the time of haematological remission (< 1 % of blasts in peripheral blood in leukaemia patients or < 5 % of myeloma cells in bone marrow) to get non-tumour cells. Tumour cells and non-tumour PBMC from the same patient were used in our experiments as antigens during the stimulation of donor cells after irradiation with 30 Gy.

Fresh PBMC from healthy donors were obtained by Histopaque (Sigma-Aldrich, St. Louis, MO) density gradient separation and used immediately in primary MLR. MLRs were performed in complete media which consisted of X-VIVO 15 (Cambrex, Walkersville, MD) supplemented with heat-inactivated 10% AB human se-
rum. PBMC from patients served as stimulator cells after irradiation with 30 Gy.

**Primary mixed lymphocyte reaction**

On Day 1, 100 × 10^6 PBMC of a healthy donor (responder cells) were stimulated with irradiated (30 Gy) non-tumour PBMC of the patient at a 5 : 1 ratio (responder : stimulator). Cells were incubated at 37 °C in 5% CO₂ in complete media in 75 cm² flasks (SARSTEDT, Nümbrecht, Germany) at a concentration of 5 × 10^6/ml responder cells. During primary MLR, activation of donor T cells occurs and activation markers including CD25 molecules are expressed. After 24 h, cells were subjected to selective depletion of alloreactive responder cells using anti-CD25 immunotoxin, RFT5-SMPT-dgA, as previously described (Michálek et al., 2000). Briefly, cells were incubated with 4 µg/ml IT for 24 h, then 2 µg/ml IT was added and cells were incubated for another 24 h; 10 mM NH₄Cl was used as an IT-enhancing agent during both IT incubations.

**Secondary mixed lymphocyte reaction**

On Day 4, selectively depleted donor cells were harvested, washed twice, and the cell culture was split into two equal parts. The first part was mixed with irradiated (30 Gy) patient’s tumour cells (stimulators in secondary MLR) in a 5 : 1 ratio (responder : stimulator). In this MLR, GVL/GVM-specific donor T cells should be activated. Weekly restimulations with the same patient’s irradiated tumour cells were performed on Day 10 and Day 17 of MLR. Due to a lack of myeloma cells, Day 17 restimulation was not performed in myeloma samples. The second part of MLR was mixed with irradiated (30 Gy) patient’s non-tumour PBMC at the same 5 : 1 cell ratio (responder : stimulator) to detect residual alloreactivity of selectively depleted donor T cells. Restimulation with patient’s irradiated non-leukemic cells was also performed on Day 10 and Day 17 of MLR.

**Control mixed lymphocyte reactions**

MLR without selective depletion of alloreactivity served as a positive control demonstrating activation of donor alloreactive T cells. The amount of 5 × 10^6/ml of donor PBMC were cultivated in complete media and stimulated on Day 4, Day 10 and Day 17 of the cultivation using irradiated non-tumour PBMC of the patient. Donor T cells cultivated in complete media without any antigenic stimulation served as a negative control.

**Flow cytometry**

Cells were analysed by flow cytometry using the following combination of directly conjugated antibodies (BD Biosciences, San Diego, CA) for surface staining: CD3-FITC, CD25-PE, CD8-APC-Cy7 and CD4-PerCP. A minimum of 50,000 cells was acquired in FACS Canto II flow cytometer using BD FacsDivA software (BD Biosciences). Flow-Jo software (Ashland, OR) version 7.1.2 was employed for graphical presentation.

**Cell sorting**

Activated GVL/GVM-reactive T cells were purified by immunomagnetic separation or by FACS cell sorting 24 h after restimulation, on Day 11 or Day 18, according to manufacturer’s instructions. We used immunomagnetically labelled cells with anti-CD25 DynaBeads and VarioMACS (Miltenyi Biotec, Bergisch Gladbach, Germany). This method was performed in seven experiments in leukemic patients; in the rest of experiments, we used FACS Aria cell sorter (BD Biosciences). Cells for FACS sorting were stained with monoclonal antibodies, and both CD3⁺CD4⁺CD25⁺ and CD3⁺CD8⁺CD25⁺ T-cell fractions were sorted out for further expansions and analyses.

**Cytotoxicity assay**

We used a flow cytometry-based assay (Molecular Probes, Eugene, OR) to monitor cytotoxic T-lymphocyte (CTL) activity of GVL/GVM T-cell clones (effector cells) against leukaemia/myeloma cells (target cells) according to manufacturer’s instructions. Briefly, target cells were labelled for 20 min with (3)3,3'-dioctadecyloracarbocyanine (DiOC 6), a green fluorescent membrane stain, then washed twice with PBS and combined at various ratios with effector cells. After 120 min incubation of target cells with effector cells, propidium iodide was added to label dying target cells.

Cell-mediated cytotoxicity was analysed by flow cytometry in FACSCanto flow cytometer (BD Biosciences) using BD FacsDivA software (BD Biosciences). The percentage of specific lysis was calculated as follows: ([cpm released experimental – cpm spontaneous]/[cpm total lysis – cpm spontaneous]) × 100 % (Mielke et al., 2008).

**Statistical analysis**

A descriptive statistics of means and standard deviation was used. All analyses were done using Statistica for Windows 7.1 and Microsoft Office Excel 2003.

**Results**

**Primary mixed lymphocyte reaction and selective depletion of alloreactivity**

A total of 22 MLRs were performed using samples from 22 different patients – seven of them suffered from multiple myeloma and 15 from various types of leukaemia (9× AML, 3× ALL, 2× CLL, 1× CML).

PBMC from healthy HLA-matched donors were used as responders in primary MLR in two cases (two patients with CLL). The remaining responders were PBMC from healthy HLA-mismatched donors.

Two sets of MLR were started for each type of responder cells, i.e. selective depletion arm and positive control arm (untreated with anti-CD25 immunotoxin) as described in Methods.
As shown in Fig. 1, the selective depletion procedure effectively reduced alloreactive CD25+ donor T cells. In leukaemia patients (Fig. 1A, B), this proportion dropped to 0–0.14 % (median 0.12 %) of CD4+CD25+ donor T cells and 0–0.10 % (median 0.05 %) of CD8+CD25+ T cells. In myeloma patients (Fig. 1C, D), the drop was to 0–0.12 % (median 0.04 %) of CD4+CD25+ donor T cells and 0–0.81 % (median 0.21%) of CD8+CD25+ donor T cells. In contrast, untreated cells reacted strongly against their allostimulators. In leukaemia patients, the alloreactivity reached 10.75–16.03 % (median 11.91 %) of donor CD4+CD25+ T cells, and 4.6–11.9 % (median 6.73 %) of CD8+CD25+ donor T cells (Fig. 1A, B). In myeloma patients, MLR without selective depletion of alloreactivity contained 5.0–8.93 % (median 7.91 %) of donor alloreactive CD4+CD25+ T cells, and 2.3–6.2 % (median 4.26 %) of CD8+CD25+ T cells (see Fig. 1C, D). Figure 2 shows a representative flow cytometric analysis of primary MLR in a leukaemia patient.

**Secondary mixed lymphocyte reaction**

On Day 3, selectively allosedepleted donor T cells were harvested, washed and split into two secondary MLRs, each containing the equal number of cells. The first part of cells was then stimulated using irradiated non-tumour cells (SD+GVH culture) to monitor residual alloreactivity. The second part of cells was stimulated using irradiated tumour cells of the same patient (SD+GVL/GVM culture) to initiate activation of allosedepleted tumourspecific T-cell clones. The activation and reactivation of CD25+ T cells was measured repeatedly in both cultures after 24 h of antigen stimulation (see Fig. 3).

As shown in Fig. 3, selective depletion with anti-CD25 immunotoxin effectively minimized alloreactivity to negligible values even after repeated stimulation with the same alloantigen, while the graft-versus-leukaemia was well preserved and even enhanced with repeated leukaemia antigen stimulation (Fig. 3A, B). The graft-versus-myeloma effect was also noticed, but to a lesser extent than the GVL effect (Fig. 3C, D). Non-IT-treated cells reacted strongly against alloantigen, confirming the critical role of anti-CD25 immunotoxin in powerful
selective depletion of alloreactivity. Figure 4 shows a representative flow-cytometric analysis of secondary MLR in a leukaemia patient. For myeloma patients, the third restimulation was not performed due to the lack of myeloma cells.

We can summarize that the anti-leukemic effect of selectively depleted T cells was enhanced by repeated stimulations with leukaemia antigens. Both CD4+ and CD8+ leukaemia-reactive donor T cells can be clearly identified with negligible GVH reactivity. Mainly CD4+ selectively depleted donor T cells were activated by leukemic cells. The situation in multiple myeloma samples was similar in effectiveness of selective depletion with the anti-CD25 IT, and the presence of desirable GVM reactivity of donor T cells was recognizable, but less dominant. Short treatment with the anti-CD25 immunotoxin shortly after initial allostimulation led to profound and long-lasting allodepletion even despite repeated stimulation with the same alloantigens.

**Cell separation, expansion and cytotoxicity assay**

Twenty-four hours after the last restimulation with leukemic/myeloma antigens, the leukaemia/myeloma reactive T cells were sorted with at least 90% purity of desired CD4+CD25+ T and CD8+CD25+ T cells; the obtained cells were expanded for two weeks. Harvested

**Fig. 4.** Stimulation of alloreactive and leukaemia-reactive CD4+CD25+ T cells
Flow-cytometric analysis of activated CD4+CD25+ T cells in a secondary MLR without selective depletion of alloreactivity using non-leukemic cells as stimulators (left panel), after anti-CD25 IT treatment and repeated GVH stimulation (central panel), and after anti-CD25 treatment and repeated GVL stimulation (right panel). The percentages of activated CD4+CD25+ T cells are shown.
cells were used as effector cells (E) in non-radioactive cytotoxicity assay with DiOC and propidium iodide. Leukaemia or myeloma cells from the appropriate patient were used as target cells (T). As negative control, PBMC from another healthy donor were used in a non-radioactive cytotoxicity assay.

Specificity of expanded leukaemia/myeloma-reactive T-cell clones was tested against the original autologous leukaemia/myeloma cells in five patients (1 MM, 2 CLL, 2 AML) with sufficient numbers of remaining tumour cells. Allodepleted T-cell clones showed specific cytotoxicity to autologous tumour cells in all five individuals. In leukemic patients, specific cytotoxicity of expanded donor T cells reached 21–42 % (median 37 %) at 20 : 1 E : T ratio and 12–25 % (median 20 %) at 2 : 1 E : T ratio. In a representative myeloma patient, a specific cytotoxicity effect was also noticed: 34 % at 20 : 1 E : T ratio and 19 % at 2 : 1 E : T ratio. Data from all five independent experiments are shown in Fig. 5.

Discussion

Allogeneic HSCT, including donor lymphocyte infusion (DLI), is currently considered standard treatment for patients suffering from haematological malignancies, with short long-term survival or ineffective chemotherapy treatment (Mielke et al., 2011). Cells from the donor immune system significantly affect the therapeutic result of HSCT, which is usually a compromise between eligible GVL/GVM reaction and undesirable and life-threatening GVHD. GVL/GVM and GVH effects manifest themselves simultaneously very often; current transplantation protocols are not able to differentiate between these two reactions.

Selective depletion of alloreactive donor T cells is an effective approach to minimizing GVHD while preserving GVL/GVM reactivity. For selective depletion, immunomagnetic separation, photodepletion (PD) (Mielke et al., 2008) or anti-CD25 immunotoxin can be used. FACS sorting of activated alloreactive cells has also been proposed. However, similarly to immunomagnetic depletion of activated alloreactive CD25+ T cells, its effect is only temporary and not as profound as with PD (Mielke et al., 2008) or anti-CD25 immunotoxin (Michálek et al., 2003b).

PD can effectively eliminate alloreactive T cells with the same potency as anti-CD25 immunotoxin; in addition, anti-CD25 immunotoxin also eliminates CD4+CD25+ regulatory T cells, thus enabling and accelerating maximal activation of allodepleted GVL/GVM T-cell clones (Michálek et al., 2003a). Two clinical trials using RFT5-SMPT-dgA anti-CD25 IT were performed, verifying the clinical benefit of selective depletion in the case of HLA-identical related donors (Amrolia et al., 2006), as well as in case of haploidentical HSCT (Mavroudis et al., 1998; Aversa, 2011). These studies have demonstrated that alloreactive CD25+ cells could be selectively depleted in vitro using anti-CD25 IT while preserving
third-party reactivity (Michálek et al., 2003b; Solomon et al., 2005).

In this study, we were able to separate alloreactivity from tumour-specific reactivity of alloreduced T cells. This study also analyses immune responses to different cancer types, demonstrating that leukaemia-derived antigens elicit stronger immune responses than myeloma-derived antigens. Previously, we were able to demonstrate that individual tumour-reactive T-cell clones are different from alloreactive T-cell clones from the same individual (Michálek et al., 2003a). Alloreduced T cells can be depleted selectively, and donor-derived GVL/GVM T-cell clones can be used for in vivo treatment without regulatory T-cell immunosuppressive effect.

We achieved at least 2-log depletion of alloreactive T cells and regulatory T cells using anti-CD25 IT. This depletion was permanent and lasted even after repeated restimulations with patient’s PBMC. This proves complex elimination of undesirable alloreactivity. Alloreduced T cells repeatedly stimulated with autologous leukemic cells were able to specifically kill their target leukemic cells.

Based on these encouraging results, we are planning to perform a clinical trial using alloreduced GVL-reactive T cells as donor lymphocyte infusions in patients relapsing after HSCT. We are able to eliminate or at least significantly suppress GVHD, and therefore, significantly improve the quality of life of patients undergoing transplantation. Furthermore, safe HSCT without significant risk of GVHD could be offered to a wide spectrum of non-cancer patients, especially those suffering from severe autoimmune diseases (inflammation of connective tissue, multiple sclerosis) and other diseases where a positive effect of HSCT could be expected.

Another advantage of this method lies in individualization of adoptive immunotherapy for each patient.

In conclusion, we have shown that anti-CD25 immunotoxin (IT) RFT5-SMPT-dgA causes permanent and effective selective depletion of donor alloreactive T-cell clones. The GVL effect of alloreduced T cells seems to be more pronounced than the GVM effect, both leading to specific cytotoxic reaction against tumour cells.

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References


