Immune Recovery after Allogeneic Hematopoietic Stem Cell Transplantation following Flu-TBI versus TLI-ATG Conditioning

**Category of the manuscript:** original manuscript.

**Authors:** Muriel Hannon¹, Yves Beguin¹, Grégory Ehx¹, Sophie Servais¹,², Laurence Seidel³, Carlos Graux⁴, Johan Maertens⁵, Tessa Kerre⁶, Coline Daulne¹, Muriel de Bock¹, Marianne Fillet¹, Aurélie Ory², Evelyne Willems², André Gothot⁷, Stéphanie Humblet-Baron¹,⁸, and Frédéric Baron¹,²

¹Groupe Interdisciplinaire de Génoprotéomique Appliquée (GIGA)-I3, University of Liège, Liège, Belgium; ²Department of clinical Hematology, CHU of Liège, Liège, Belgium; ³Department of statistics, SIME, CHU of Liège, Liège, Belgium; ⁴Mont-Godine University Hospital (UCL), Yvoir, Belgium; ⁵AZ Gasthuisberg Leuven, Leuven, Belgium; ⁶Ghent University Hospital, Ghent, Belgium; ⁷Department of Laboratory Medicine, University of Liège, Liège, Belgium; ⁸Currently in autoimmune genetics laboratory, University of Leuven, Leuven, Belgium.

**Running title:** Immune recovery after Flu-TBI vs ATG-TLI

**Keywords:** immune recovery, nonmyeloablative conditioning, TBI, TLI, GVHD

**Fundings:** GE is Télévie PhD student and FB is senior research associate of the National Fund for Scientific Research (FNRS) Belgium. SHB is research associate and TK clinical researcher of the national Fund for Scientific Research Flanders (FWO). This study was supported by funds from: the FNRS, the Leon Fredericq fund and Anti-Cancer Center at the University of Liège, and from Genzyme-Sanofi.

**Address for correspondence:** Muriel Hannon; University of Liège, department of Hematology, CHU Sart-Tilman 4000 Liège Belgium. Tel +32 - 4 - 366 72 01; Fax +32 - 4 - 366 88 55; E-mail: m.hannon@chu.ulg.ac.be

**Conflicts of interests:** YB and FB have received lecture honoraria and research support from Genzyme / Sanofi.

**Word count:** abstract: 246 words; manuscript: 4148 words; Figures: 3
**Translational relevance:**

Over the last 2 decades, new conditioning regimens for allogeneic hematopoietic cell transplantation (HCT) have been developed. These approaches, termed nonmyeloablative HCT, can be performed in patients up to 75 years of age and rely nearly exclusively on immune-mediated graft-versus-tumor effects for tumor eradication. Unfortunately, graft-versus-tumor effects have been associated with the development of graft-versus-host disease (GVHD), a redoubtable complication of allogeneic HCT consisting of recipient healthy organ destruction by donor immune cells contained in the graft. A new conditioning regimen for allogeneic HCT combining total lymphoid irradiation (TLI) and anti-thymocyte globulin (ATG) has been developed with the aim of inducing strong graft-versus-tumor effects without GVHD. In the current study, we demonstrate that, in comparison to patients receiving a nonmyeloablative conditioning combining fludarabine and low-dose TBI, patients conditioned with the TLI-ATG regimen had a lower incidence of GVHD, a higher frequency of CMV-reactivation, and a higher incidence of relapse. These phenomena might be explained by slower recoveries of CD4+ T cells and naïve CD8+ T cells but higher regulatory T-cells/naïve CD4+ T cell ratios in TLI-ATG recipients.
Abstract

Purpose: A conditioning regimen for allogeneic hematopoietic cell transplantation (HCT) combining total lymphoid irradiation (TLI) plus anti-thymocyte globulin (ATG) has been develop to induce graft-versus-tumor effects without graft-versus-host disease (GVHD). Experimental Design: We compared immune recovery in 53 patients included in a phase II randomized study comparing nonmyeloablative HCT following either fludarabine plus 2 Gy total body irradiation (TBI arm, n=28) or 8 Gy TLI plus anti-thymocyte globulin (TLI arm, n=25). Results: In comparison to TBI patients, TLI patients had a similarly low 6-month incidence of grade II-IV acute GVHD, a lower incidence of moderate/severe chronic GVHD (P=0.02), a higher incidence of CMV reactivation (P<0.001), and a higher incidence of relapse (P=0.01). While recovery of total CD8+ T cells was similar in the two groups, with median CD8+ T cell counts reaching the normal values 40-60 days after allo-HCT, TLI patients had lower percentages of naïve CD8 T cells. Median CD4+ T cell counts did not reach the lower limit of normal values the first year after allo-HCT in the two groups. Further, CD4+ T cell counts were significantly lower in TLI than in TBI patients the first 6 months after transplantation. Interestingly, while median absolute regulatory T cell (Treg) counts were comparable in TBI and TLI patients, Treg/naïve CD4+ T cell ratios were significantly higher in TLI than in TBI patients the 2 first years after transplantation. Conclusions: Immune recovery differs substantially between these two conditioning regimens possibly explaining the different clinical outcomes observed (NCT00603954).
INTRODUCTION

Allogeneic hematopoietic cell transplantation (allo-HCT) following nonmyeloablative conditioning is frequently used in patients with hematological malignancies who are not eligible for a myeloablative conditioning because of age, comorbidities, or prior high dose allo-HCT. This approach has relied on optimization of pre- and post-transplant immunosuppression to overcome host-versus-graft reactions, thereby allowing engraftment and eradication of tumors nearly exclusively through immune-mediated graft-versus-tumor effects (1-5). Main causes of failure of nonmyeloablative HCT include disease relapse, graft-versus-host disease (GVHD) and infections, stressing the need for research focusing at immune reconstitution after nonmyeloablative HCT.

T cell recovery after allo-HCT following high-dose conditioning depends on both homeostatic peripheral expansion (HPE) of donor T cells contained in the graft (6), and T cell neo-production from donor hematopoietic stem cells (thymo-dependent pathway)(7-10). In young patients undergoing myeloablative allo-HCT, most circulating T cells during the first months following allo-HCT are the progeny of T cells infused with the graft through HPE (6). Beyond day 100, neo-generation of T cells by the thymus is progressively set up and plays an increasing role in reconstituting the T cell (8, 11-13). However, previous studies have demonstrated that the thymo-dependent pathway plays a significant role only in patients younger than 60 years of age at transplantation and is affected by the occurrence of acute and extensive chronic GVHD(14-16). Since HPE allows the expansion of both NK cells and non-tolerant T cells, it has been postulated that HPE is the driving force of graft-versus-tumor effects, but also of acute GVHD, and to a lesser extent, chronic GVHD(6).

One of the most widely used nonmyeloablative conditioning regimens associates fludarabine (90 mg/m² total dose) with 2 Gy total body irradiation (TBI)(1, 2). This
regimen can be safely performed in an outpatient setting but is associated with a relatively high incidence of GVHD(1-3, 5, 17). In an effort at preventing GVHD, the Stanford group has developed another nonmyeloablative conditioning that combines total lymphoid irradiation (TLI, 8 Gy total dose) with anti-thymocyte globulin (ATG; 7.5 mg/kg Thymoglobulin® total dose)(18-23). This approach allowed sustained engraftment with a low incidence of GVHD. In murine models of transplantation, this was achieved through Th2 polarization of donor T cells by recipient invariant NK/T cells (iNKT, still present at transplantation thanks to their relative resistance to ionizing radiation)(18-21, 24), and through expansion of donor regulatory T cells (Treg) by recipient iNKT(24).

Here we compared immune recovery in a cohort of 53 patients included in a phase II randomized study carried out through the Belgian Hematological Society (BHS)-transplantation committee comparing nonmyeloablative allo-HCT with either fludarabine plus 2 Gy TBI (TBI arm) or 8 Gy TLI + ATG (TLI arm) conditioning. The aim was to explore whether differences in clinical outcomes between TBI and TLI patients could be explained by different patterns of immune recovery following these two conditioning regimens.

PATIENTS AND METHODS

Study population

This study includes data from 53 patients (from 4 centers; out of a total cohort of 94 patients included in 8 centers) included in a phase II randomized study comparing nonmyeloablative allogeneic peripheral blood stem cell (PBSC) transplantation with either fludarabine plus 2 Gy TBI (TBI arm, n=28) or 8 Gy TLI + ATG (TLI arm, n=25). The study was approved by the Ethics Committee of the University of Liège, and all patients signed a written informed consent form. The study was registered in clinicaltrial.gov
(NCT00603954). The clinical results of the study (including the data from the 94 patients) have been reported elsewhere (25).

### Conditioning regimen and GVHD prophylaxis

In the TBI arm, conditioning consisted of fludarabine 30 mg/m² on days -4, -3 and -2, followed by a single dose of 2 Gy TBI administered on day 0 (TBI administration on day -1 was also permitted). In the TLI arm, conditioning consisted of 8 Gy TLI (80 cGy daily, starting 11 days before transplantation, until a total of 10 doses (8 Gy) has been delivered) and ATG (Thymoglobulin®, Genzyme) given i.v. at a dose of 1.5 mg/kg/d from days -11 through -7. Postgrafting immunosuppression was similar in both arms and included mycophenolate mofetil (MMF) administered orally from the evening of day 0 through day 28 (HLA-identical sibling donors) or day 42 (10/10 HLA allele-matched unrelated donors) at a dose of 15 mg/kg t.i.d., and tacrolimus administered orally from day -3. Tacrolimus doses were adapted to achieve whole blood trough levels between 15 and 20 ng/ml the first 28 days and between 10-15 ng/ml thereafter. Full doses were given until day 100 (sibling recipients) or 180 (unrelated recipients). Doses were then progressively tapered to be discontinued (in the absence of GVHD) by day 180 (sibling donors) or 365 (unrelated donors).

### Clinical management

G-CSF (5 µg/kg/d) was generally administered when the granulocyte counts dropped below 1.0 x 10⁹/L. Acute GVHD and late acute GVHD were graded using international criteria, while chronic GVHD was graded according to the NIH criteria(26). Treatment for acute GVHD usually consisted of prednisolone 2 mg/kg/day. Extensive chronic GVHD
was treated according to the investigator's choice, usually with prednisolone and a calcineurin inhibitor.

Infection prophylaxis generally consisted of acyclovir (400 mg t.i.d. orally), oral fluconazole (400 mg/day) and co-trimoxazole or aerosolized pentamidine. Polymerase chain reaction (PCR) for cytomegalovirus (CMV) was performed weekly until day 100 and every 2-4 weeks thereafter. Patients with a positive PCR received preemptive ganciclovir. Chimerism among T cells, isolated with the RosetteSep (StemCell Technologies, Vancouver, Canada) technology or by flow-cytometry, was assessed at days 28, 40, 100, 180 and 365 after HCT using PCR-based analysis of polymorphic microsatellite regions (multiplex PCR). Graft rejection was defined as occurrence of < 5% T cells of donor origin after HCT, as previously described(27). Disease evaluation was routinely carried out on days 40, 100, 180, 365, 730 and 1095 after HCT. Relapse and progression were defined according to the criteria proposed by the European Group for Blood and Marrow Transplantation (EBMT, https://portal.ebmt.org/sites/clint2/clint/Documents/Statistical%20Endpoints_CLINT %20Project_final%20version.pdf).

**Cytokine levels**

IL-7 and IL-15 levels were measured by ELISAs following the manufacturer's protocol (High sensitivity IL-7 and IL-15 quantikine, R&D Systems, Minneapolis, MN, USA). The concentrations of IL-2, IL-4, IL-10, TNF-α, and IFN-γ on day 28 were determined in patient sera using Bio-Plex Pro™ Human Cytokine 27-plex Assay (Bio-Rad Laboratories, Nazareth Eke, Belgium) according to the manufacturer's recommendations.

**Measurements of ATG concentrations**
Concentrations of ATG capable of binding to human lymphocytes were assessed using the method developed by Kakhniashvili et al. (28) with minor modifications as reported by Podgorny et al. (29) around days -7, -4, 0, 3, 7, 10, 14, 17 and 20 after transplantation.

**Immune recovery**

Immune recovery was prospectively assessed as previously described(16). Briefly, fresh patients’ PBMC were phenotyped on days 28, 42, 60, 80, 100, 120, 180, 365, 540, 730 and yearly thereafter using 4-color flow cytometry after treatment with a red blood cell lyzing solution. The analyzed cell subsets were T cells (CD3+), CD4+ T cells (CD3+CD4+ lymphocytes), CD8+ T cells (CD3+CD8+ lymphocytes), naïve CD4+ T cells (CD4+CD45RAhigh lymphocytes), memory CD4+ T cells (CD4+CD45R0+ lymphocytes), NK cells (CD3-CD56+ lymphocytes) as well as B cells (CD19+ lymphocytes). The percentage of positive cells was measured relative to total nucleated cells, after subtraction of non-specific staining. Absolute counts were obtained by multiplying the percentages of positive cells by the white blood cell counts (Advia 120 hematology analyzer, Bayer Technicon, Tarrytown, USA). Lower and higher limits of normal values for each cell subset were defined respectively as 5 and 95 percentiles of values obtained in 47 age-matched healthy volunteer donors.

More detailed T and B cell phenotyping was retrospectively performed using cryopreserved PBMC prospectively collected on days 40, 100, 180 and 365 and then yearly thereafter using 8-color flow cytometry. The following cell subsets were quantified using multicolor staining: naïve T cells (CD4+CD45RA+CCR7+ and CD8+CD45RA+CCR7+ lymphocytes), central memory T cells (CD4+CD45RA-CCR7+ and CD8+CD45RA-CCR7+), effector/effectormemory T cells (CD4+CD45RA-CCR7- and
CD8+CD45RA-CCR7-), \textit{regulatory T cells} (Treg, CD4+CD25^{high}CD127^{low}FoxP3+), \textit{proliferating Treg} (CD4+CD25^{high}CD127^{low}FoxP3*Ki67+), \textit{CD56^{dim} NK cells} (CD3-CD56^{low}), \textit{CD56^{bright} NK cells} (CD3-CD56^{high}), \textit{iNKT cells} (CD3+CD56+TCR \text{V}^{\alpha24}J^{\alpha18}+ TCR \text{V}^{\beta11}+), \textit{naive B cells} (CD19+CD27-IgD+), \textit{unswitched memory B cells} (CD19+CD27+IgD+) and \textit{switched memory B cells} (CD19+CD27+IgD+). The following clones were used: CD4 SK3, CD45RA HI 100, CCR7 3D12, CD8 HiT8a, CD31 L133.1, CD25 BC96, CD127 eBioRDR5, FoxP3 206D, Ki67 B56, CD3 SP34-2, CD56 B159, TCR Va24Ja18 6B11, TCR Vb11 C21, CD19 SJ25C1, CD27 M-T271, IgD IA6-2. Surface staining was performed as previously described (16). Intracellular staining was performed using human intracellular FoxP3 staining kit (Biolegend, San Diego, CA) according to the manufacturer’s instructions. Cells were acquired on a FACSCanto II (Becton Dickinson) and data were analyzed with FlowJo software (7.0, Tree Star Inc., San Carlos, CA). A minimum of 100 events in the parent population was considered mandatory to ensure reliable subsets analyses and data were not considered if the number of cells analyzed was not sufficient. Absolute counts were calculated by multiplying the percentage of positive cells in the lymphoid gate by the absolute lymphocyte count measured in the patients’ peripheral blood the day of PBMC collection. Lower and higher limits of normal values for each cell subset were defined respectively as the 5 and 95 percentiles of values obtained in 45 age-matched healthy volunteer donors.

**T-cell receptor excision circles (TREC) assay**

TREC assays were performed on blood samples collected on days 100, 365 and then yearly after HCT, as previously described. Briefly, PBMC were isolated using Ficoll-Paque™ Plus gradient centrifugation and then cryopreserved. SJTREC were quantified for each sample by nested real-time PCR, as previously described (16).
Statistical analyses

Immunologic data from patients were censored at time of graft rejection, at second transplantation, or at progression of the underlying disease. The Mann-Whitney test was used to compare immune recovery data between TBI and TLI recipients. The Wilcoxon matched pair test was used to compare sjTRECs concentrations evolution. The Spearman correlation coefficient was used to analyze potential associations between lymphocyte subset counts and sjTRECs levels after HCT. Comparison of the number of patients who developed at least one infectious episode in the 2 groups was performed using the Fisher’s exact test. Survival (OS) and progression-free survival (PFS) were estimated by the Kaplan-Meier method. Cumulative incidence curves were used for GVHD, CMV infection, and relapse incidence (RI) with death as a competing risk, and for nonrelapse mortality (NRM) with relapse as a competing risk. Results were significant at the 5% critical level (p<0.05). Statistical analyses were carried out with Graphpad Prism (Graphpad Software, San Diego, CA) and SAS version 9.3 for Windows (SAS Institute, Cary, NC, USA).
RESULTS

Patients, donors and clinical outcomes

Patients’ characteristics are summarized in supplemental table 1, while comparison of characteristics and outcomes of patients from the clinical publication (25) included or not in the biological study described here is provided in supplemental table 2. Briefly, median patient age was 60 years (range, 38 to 71 years). Twenty-eight patients received PBSC from HLA-identical siblings, and 25 from a 10/10 HLA allelic-matched unrelated donor. In comparison to TBI patients, TLI patients had a similar (low) incidence of graft rejection (1 versus 2 patients, P=0.6) and of grade II-IV acute GVHD (at 180 days 12% vs 18%, P=0.8), a lower incidence of chronic GVHD (at 3 years 8% vs 46%, P=0.02), a higher incidence of CMV reactivation (at 100 days: 89% vs 50%, P<0.001), and a comparable incidence of nonrelapse mortality (at 3 years, 16% vs 14%, P=0.9) a higher incidence of relapse/progression (at 3 years, 52% vs 18%, P=0.01). Importantly, overall survival was similar in the two groups.

ATG levels

We first assessed the kinetics of functional ATG serum levels in 15 TLI patients. As shown in figure 1A, median functional ATG levels were 4.0 (range, 3.2-5.6) mg/L on day 0, 2.2 (range, 0.6-3.9) on day 3, and 0.95 (range, 0.34-1.49) on day 10 after transplantation. These day 0 levels are below the threshold of ATG levels associated with a lower incidence of chronic GVHD in a recent paper by Chawla et al. (8.12 mg/L) in patients given PBSC after conditioning with fludarabine, busulfan and ATG (Thymoglobulin®, 4.5 mg/kg given from day -2 to day 0 before PBSC infusion) with or without TBI (30).
Cytokine levels

Previous studies in humans have demonstrated that HPE is mainly driven by IL-7 for CD4+ and CD8+ T cells (while IL-7 also stimulates B cells ontogenesis), and by IL-15 for CD8+ T cells, NK and NK/T cells(6, 31, 32). This prompted us to assess their levels among TBI and TLI recipients. As shown in figure 1B, IL-7 plasma levels were higher in TLI than in TBI recipients the first 100 days after transplantation (p values ranged from <0.001 to 0.5), suggesting more pronounced T cell lymphopenia in TLI recipients, given that IL-7 levels after allo-HCT depend mainly on consumption by T cells(32-34). In contrast, IL-15 levels were comparable between TBI and TLI recipients from preconditioning to day 28 after transplantation, suggesting similar CD8+ T cell and NK cell recovery in the two groups of patients (supplemental figure 1A), although post-transplant IL-15 (and to a lesser extend Il-7) levels have also been correlated with other factors such as CRP levels (32, 33).

On day 28 after transplantation, IL-2 serum levels were below the threshold for detection (2.1 pg/ml) in the 2 groups of patients. IL-4 levels were comparable in the 2 groups of patients, but IL-4/CD4 cell ratios were significantly higher in TLI than in TBI patients (P=0.0004) (supplemental figure 1B-C). Further, IL-10 levels were significantly higher in TLI patients (P=0.0481), possibly suggesting a TH-2 polarization of donor T cells (as observed by Lowsky et al.(18)) or a higher production of IL-10 by Treg in TLI patients (as observed in a preclinical murine model(24)) (supplemental figure 1B). Finally, IFN-gamma serum levels were similar between the 2 groups of patients, while TNF-alpha levels were significantly higher in TLI than in TBI patients (P=0.0493, supplemental figure 1B).

T cell chimerism levels and immune recovery (4-color flow cytometry)

Hannon et al., IR after TBI vs TLI,
Previous studies have demonstrated that faster establishment of donor T cell chimerism correlated with lower risk of graft rejection, higher incidences of acute and chronic GVHD, but also with a lower risk of relapse in patients transplanted with a nonmyeloablative conditioning(19, 27). Interestingly, in comparison to TBI patients, TLI patients had lower donor T cell chimerism levels on day 180 (median 82% versus 95%, P=0.09) and 365 (median 88% versus 97%, P=0.04) after transplantation (supplemental figure 2A).

Immune recovery in TBI and TLI patients is described in Figure 1 C-H. Recovery of CD8+ T cells was similar in the two groups, with median CD8+ T cell counts reaching the normal values 40-60 days after allo-HCT. The same was observed for NK cells, with median NK cell counts reaching normal values 60-80 days after transplantation. In contrast, recovery of CD4+ T cells was slower than for CD8+ T cells with median CD4+ T cell counts not reaching the lower limit of normal values the first year after allo-HCT in the two groups. Further, CD4+ T cell counts were significantly lower in TLI than in TBI patients the first 6 months after transplantation. This was due to both slower recovery of CD4+CD45RA+ T cells and memory CD4+CD45RO+ T cells. Finally, B cell recovery was similar in the 2 arms, with median B cell counts reaching the normal range 1 year after allo-HCT.

**Lymphocyte subset reconstitutions (8-colors flow-cytometry)**

*CD8+ and CD4+ lymphocyte subsets (Figure 2)*

Among CD8+ T cells, TLI patients had lower percentages of naïve CD8+ T cells but higher percentages of effector/effector-memory CD8+ T cells (TEM CD8+) than TBI patients. Similar observations were made for CD4+ T cells where TLI patients had dramatically
lower percentages of naïve CD4+ T cells but higher percentages of effector/effector-memory CD4+ T cells (TEM CD4+).

**Treg and NK/T- cell recovery (Figure 3)**

Previous studies have demonstrated that Treg play a pivotal role in preventing GVHD after allo-HCT both in mouse-to-mouse and in humanized mouse models of GVHD (35, 36). Given the lower incidence of chronic GVHD observed in TLI patients, study of Treg recovery in our patient population was of particular interest. Median absolute Treg numbers reached the lower limit of normal values 6 months and 2 years after transplantation in TBI and TLI patients, respectively (NS), confirming the previously reported slow Treg recovery after allo-HCT(37, 38). A prior study by the Stanford group in a preclinical mouse model has demonstrated that the balance between Treg and naïve CD4+ T cells was associated with tolerance induction after TLI/antithymocyte serum (ATS) conditioning (39). This prompted us to compare Treg/naïve CD4 T cell ratios in TBI and TLI patients. Interestingly, Treg/naïve CD4+ T cell ratios were significantly higher in TLI than in TBI patients the 2 first years after transplantation, possibly explaining a higher tolerance in TLI patients leading to lower incidence of chronic GVHD.

Data from mouse models (40) as well as recent clinical observations in humans have demonstrated that Treg depend mainly on IL-2 for their homeostasis (41, 42). Although IL-2 levels were below the limit of detection on day 28 after transplantation, Treg proliferation was relatively high with a mean of 23.5±18.0% of Treg expressing KI67 on day 40 after transplantation (compared to 21.2±18.0% for conventional T cells (Tconv), P=0.1). Interestingly, there was a trend for higher Treg proliferation in TLI than in TBI patients on day 40 after allo-HCT, with 29.9±15.4% Treg from TLI patients expressing KI67, versus 19.7±18.7% for those in TBI patients (P=0.07).
As mentioned above, iNKT cells play a pivotal role in preventing GVHD following TLI/ATS conditioning in mice (43). Unfortunately, we did not assess bone marrow iNKT levels on day 0 because we did not want to expose the patients to an additional bone marrow aspiration. With this limitation, median iNKT levels in the peripheral blood were similar in TBI and TLI patients and remained below the lower limit of normal from day 40 to 2 years after transplantation. Further, the iNKT/T cell ratio (a parameter recently demonstrated to be predictive of acute GVHD in allogeneic HCT recipients(44)) was also comparable in the two groups.

*B & NK cell subset recovery (Supplemental figure 3)*

B cell recovery was superimposable in TBI and TLI patients. In contrast to what was observed for T cell subsets, naïve B cell counts recovered before memory B cells as previously reported by other groups of investigators (7, 45-47), including after HLA-haploidentical stem cell transplantation (48). Specifically, median naïve B cell counts were already within the normal ranges 180 days after transplantation while unswitched memory B cells remained below normal ranges the first year after transplantation in both arms, and switched B cells reached the lower limit of normal values 2 years after transplantation.

Finally, NK cell reconstitution was similar in both arms, with faster recovery of CD56bright NK cells in comparison to CD56dim NK cells, as reported previously by other groups of investigators (49) and particularly after HLA-haploidentical stem cell transplantation (50).

*Thymic function (sjTREC levels)*
SjTREC levels were significantly higher in TBI than in TLI patients on day 100 as well as 2 and 3 years after transplantation. Indeed, while median sjTREC levels reached the lower limit of normal 2 years after transplantation in TBI patients, they remained below that limit throughout the study period in those given TLI conditioning (Figure 3G). As observed previously in another cohort of patients(16), the sjTREC levels increased significantly from day 100 to 1 year (P=0.027), 2 years (P=0.039) and 3 years (P=0.06) after transplantation in patients < 60 years of age at transplantation, while they did not (P values ranged from 0.11 to 0.6) among patients 60 years of age or older (Figure 3H), reflecting an impaired thymic function in the latter group.

Infections

First 100 days after transplantation

During the first 100 days after transplantation, 11 TBI patients experienced 21 episodes of bacterial infection while 12 TLI patients had a total of 20 episodes of bacterial infection (P=0.7).

During the same period, 2 TBI patients experienced 2 fungal infections (candidiasis) while 5 TLI patients had a total of 6 fungal infections (2 candidiasis, 3 aspergillosis and 1 cryptococcosis) (P=0.16).

Among CMV-seropositive patients and/or donors, 12/21 TBI patients versus 16/18 TLI patients experienced a CMV infection the first 100 days after transplantation (P=0.038). Further the first episode of CMV reactivation occurred sooner in TLI than in TBI patients leading to a very significantly (P<0.001) lower cumulative incidence of CMV reactivation in TBI than in TLI patients when compared using the log-rank test.

Days 101-365 after transplantation
Incidences of infections from day 101 to day 365 were calculated in patients who survived free of progression/graft rejection for at least 350 days after transplantation. Among these patients, 9 out of 23 TBI patients experienced a total of 24 bacterial infections, while 8 out of 14 TLI patients had a total of 10 bacterial infections (P=0.7). Further, 3 out of 23 TBI patients experienced a total of 4 fungal infections, while 1 of 14 TLI patients had a total of 1 fungal infection (P=0.7).

**DISCUSSION**

Main causes of failure after nonmyeloablative HCT are relapse of the underlying disease, infections, and GVHD (5, 51). Given that immune cells are involved in these three transplant complications, studies assessing immune recovery after nonmyeloablative transplantation are important. Indeed, following both HLA-haploidentical and HLA-matched HCT, faster immune reconstitution has been associated with lower relapse rates and better survival (7, 48), while favoring Treg homeostasis improved steroid-refractory chronic GVHD (41). In the current study, we compared immune recovery in a cohort of 53 patients included in a prospective multicenter randomized study comparing two widely used nonmyeloablative conditioning regimens (25)). The main findings of the current analyses are discussed below.

A first observation was that functional ATG persisted up to day 7 after transplantation, as observed by Lowsky et al. (18). This might explain some differences in the pattern of immune recovery that we observed between the 2 arms of the study, such as slower recovery of CD4+ and of naive CD8+ T cells (52). Further, since all TLI but no TBI recipients received ATG, it is impossible to separate the respective role of TLI versus ATG in the observations discussed below. Importantly, ATG is unlikely to be the sole factor responsible for the lower incidence of chronic GVHD observed in TLI patients given that functional ATG levels in day
0 sera of our TLI patients were well below the threshold associated with a lower incidence of chronic GVHD in a recent paper by Chawla et al. (30).

In murine models of allo-HCT, protection from GVHD following TLI/ATS conditioning depends on residual host iNKT cells (located mainly in the bone marrow) that secrete IL-4, which in turn polarizes donor T cells towards a Th2 pattern. Importantly, iNKT cells also promote expansion of donor Treg and drive them to produce IL-10 (43). Several observations support similarities in immune recovery patterns in the mouse model mentioned above (43) and in TLI patients from the current study. First, as observed in the mouse model, Treg/naïve CD4 T cell ratios were significantly higher in TLI than in TBI patients from day 40 to 2 years after transplantation. This might have contributed to the lower incidence of chronic GVHD observed in current TLI patients given that a high Treg/naïve CD4 T-cell ratio has been associated with tolerance induction in mice conditioned with TLI/ATS (39). Secondly, IL-10 serum levels on day 28 were significantly higher in TLI than in TBI patients, possibly translating Th-2 polarization of donor CD4+ T cells in TLI patients or higher secretion of IL-10 by Treg from TLI patients as observed in the mouse model where Treg from TLI/ATS mice produced higher levels of IL-10 than control Treg (24). Third, there was a trend for higher Treg proliferation in TLI than in TBI patients on day 40 (as assessed by Ki67 expression) (p=0.066). Taken together, these observations might support the hypothesis that the TLI/ATG regimen could prevent chronic GVHD in part by increasing the Treg/naïve CD4+ T cell ratio.

Besides ATG and Treg, a third mechanism that might explain protection from GVHD in TLI/ATG patients is their slower establishment of full donor T-cell chimerism (27).

Previous studies have observed a strong correlation between occurrence of chronic GVHD and a lower relapse risk after nonmyeloablative allo-HCT (3-5, 53). In agreement with these observations, the lower incidence of chronic GVHD in current TLI patients was offset...
by a higher risk of disease relapse/progression (although this result should be taken with caution given the relatively low number of patients with various diagnoses).

Finally, the slower CD4+ T cell recovery was associated with a higher incidence of CMV infection the first 100 days after transplantation in TLI patients. This is in line with a prior publication showing early CMV viremia due to impaired viral control in patients given grafts after TLI-ATG conditioning (23). Whether this is due to TLI/ATG conditioning or to the use of ATG itself remains to be determined.

In conclusion, immune recovery differs substantially between these two conditioning regimens, possibly explaining the different clinical outcomes observed.

ACKNOWLEDGEMENTS
We are very grateful to Sandra Ormenese from the Imaging and Flow Cytometry Platform of the GIGA for help with flow cytometry analyses. The authors are also grateful to Olivier Dengis and Sophie Dubois for excellent technical assistance.

CONTRIBUTION
Study design: FB, SHB, MH; Participation in study design: YB, GE; Flow-cytometry analyses: MH, SHB, AG, GE; TREC analyses, MH, CD; Cytokine assessment, MdB, MF, GE; Inclusion and follow-up of patients: YB, FB, EW, CG, JM, TK, KT and SS; manuscript writing MH, FB; manuscript editing: SHB, TK, SS, GE, YB; manuscript review and approval: all authors.
REFERENCES


FIGURE LEGENDS

Figure 1. A) Kinetics of active ATG levels in the serum of TLI patients (n=15). B) Evolution of plasma IL-7 levels the first 100 days after transplantation in TBI (black boxes, n=14) and TLI (white boxes, n=13) patients. C-H) Recovery of T, B and NK cells the first 4 years after transplantation in TBI (black boxes) and TLI (white boxes) patients. Plots display the median, 25th and 75th percentiles of the distribution (boxes), and whiskers extend to the 10th and 90th percentiles. Gray zones show normal ranges (from 5th to 95th percentiles) and horizontal lines the medians in 45 aged-matched healthy controls. In the different subfigures, * means p<0.05, ** means p<0.01 and *** means p<0.001.

Figure 2. A-F) Recovery of CD8+ and CD4+ T cell subsets in TBI (black boxes) and TLI (white boxes) patients. TCM, central memory T cell; TEM, effector /effector memory T cells. Plots display the median, 25th and 75th percentiles of the distribution (boxes), and whiskers extend to the 10th and 90th percentiles. Gray zones show normal ranges (from 5th to 95th percentiles) and horizontal lines the medians in 45 aged-matched healthy controls. In the different subfigures, * means p<0.05, ** means p<0.01 and *** means p<0.001.

Figure 3. A-F) Recovery of regulatory T cells (Treg) and invariant NK/T cells (iNKT) in TBI (black boxes) and TLI (white boxes) patients. G) Thymic function (assessed by
sjTREC blood concentrations) in TBI and TLI patients. **H** Evolution of sjTREC levels according to recipient age in the whole cohort (<50 years (blue boxes), 50-60 years (yellow boxes), >60 years (red boxes)) after transplantation. Plots display the median, 25th and 75th percentiles of the distribution (boxes), and whiskers extend to the 10th and 90th percentiles. Gray zones show normal ranges (from 5th to 95th percentiles) and horizontal lines the medians in 45 aged-matched healthy controls. In the different subfigures, * means p<0.05, ** means p<0.01 and *** means p<0.001.
Figure 1

A. ATG

B. Plasma IL-7

C. CD3+CD8+ cells

D. CD3+CD4+ cells

E. CD4+CD45RA+ cells

F. CD4+CD45RO+ cells

G. CD3+CD56+ (NK) cells

H. CD19+ cells

Days after HCT

# of TBI patients: 14 15 13 13 10 11 11 6 8

# of TLI patients: 14 15 15 14 13 11 11 8 8

Graphs A-H
Figure 2

(A) Naive CD8+ cells

(B) CD8+ TCM cells

(C) CD8+ TEM cells

(D) Naive CD4+ cells

(E) CD4+ TEM cells

(F) CD4+ TCM cells

Days after HCT

# of TBI patients: 24 21 19 18 14 12

# of TLI patients: 17 17 14 11 9 5

Graphs A-F
Immune Recovery after Allogeneic Hematopoietic Stem Cell Transplantation following Flu-TBI versus TLI-ATG Conditioning

Muriel Hannon, Yves Beguin, Gregory Ehx, et al.

*Clin Cancer Res* Published OnlineFirst March 16, 2015.

Updated version
Access the most recent version of this article at:
doi:10.1158/1078-0432.CCR-14-3374

Supplementary Material
Access the most recent supplemental material at:
http://clincancerres.aacrjournals.org/content/suppl/2015/03/18/1078-0432.CCR-14-3374.DC1

Author Manuscript
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.