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

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Abstract

Purpose: A conditioning regimen for allogeneic hematopoietic cell transplantation (HCT) combining total lymphoid irradiation (TLI) plus anti-thymocyte globulin (ATG) has been developed 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 ATG (TLI arm, $n = 25$).

Results: In comparison with 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 to 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. Furthermore, $CD4^+$ 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). *Clin Cancer Res; 1–9. ©2015 AACR.*

Introduction

Allogeneic hematopoietic cell transplantation (allo-HCT) following nonmyeloablative conditioning is frequently used in patients with hematologic 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 posttransplant 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; refs. 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). Because HPE allows the expansion of both NK cells and nontolerant 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/m^2 total dose) with 2 Gy total body irradiation (TBI; refs. 1, 2). This regimen can be safely

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Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

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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 with patients receiving a nonmyeloablative conditioning combining fludarabine and low-dose total body irradiation (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 phenomenons 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.

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; refs. 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; refs. 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-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 (Liège, Belgium), 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/m^2 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 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 through levels between 15 ng/mL and 20 ng/mL the first 28 days and between 10 ng/mL and 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 days 180 (sibling donors) or 365 (unrelated donors).

Clinical management

G-CSF (5 μg/kg/d) was generally administered when the granulocyte counts dropped below 1.0×10^9/L. Acute GVHD and late acute GVHD were graded using international criteria, whereas chronic GVHD was graded according to the NIH criteria (26). Treatment for acute GVHD usually consisted of prednisolone 2 mg/kg/d. 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. PCR for cytomegalovirus (CMV) was performed weekly until day 100 and every 2 to 4 weeks thereafter. Patients with a positive PCR received preemptive ganciclovir. Chimerism among T cells, isolated with the RosetteSep (Stem Cell Technologies) 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 1,095 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

IL7 and IL15 levels were measured by ELISAs following the manufacturer's protocol (high sensitivity IL7 and IL15 quantikine, R&D Systems). The concentrations of IL2, IL4, IL10, TNFα, and IFNγ on day 28 were determined in patient sera using Bio-Plex Pro Human Cytokine 27-plex Assay (Bio-Rad Laboratories) according to the manufacturer's recommendations.

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Measurements of ATG concentrations

Concentrations of ATG capable of binding to human lymphocytes were assessed using the method developed by Kakhniashvili and colleagues (28) with minor modifications as reported by Podgorny and colleagues (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 ($CD4^+CD45RA^{high}$ lymphocytes), memory $CD4^+$ T cells ($CD4^+CD45RO^+$ 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 nonspecific staining. Absolute counts were obtained by multiplying the percentages of positive cells by the white blood cell counts (Advia 120 hematology analyzer, Bayer Technicon). 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/effector-memory T cells ($CD4^+CD45RA^-CCR7^-$ and $CD8^+CD45RA^-CCR7^-$), regulatory T cells (Treg, $CD4^+CD25^{high}CD127^{low}FoxP3^+$), proliferating Treg ($CD4^+CD25^{high}CD127^{low}FoxP3^+KI67^+$), *$CD56^{dim}$ NK cells* ($CD3^-CD56^{low}$), CD56bright NK cells ($CD3^-CD56^{high}$), *iNKT cells* ($CD3^+CD56^+$TCR $V\alpha24J\alpha18^+$ TCR $V\beta11^+$), naïve B cells ($CD19^+CD27^-IgD^+$), unswitched memory B cells ($CD19^+CD27^+IgD^+$), and 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 Vα24Jα18 6B11, TCR Vβ11 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) according to the manufacturer's instructions. Cells were acquired on a FACSCanto II (Becton Dickinson) and data were analyzed with FlowJo software (7.0, TreeStar Inc.). A minimum of 100 events in the parent population were 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 assay

T-cell receptor excision circles (TREC) assays were performed on blood samples collected on days 100, 365, and then yearly after HCT, as previously described. Briefly, PBMCs were isolated using Ficoll–Paque Plus gradient centrifugation and then cryopreserved. SjTRECs 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 two groups was performed using the Fisher 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) and SAS version 9.3 for Windows (SAS Institute).

Results

Patients, donors, and clinical outcomes

Patients' characteristics are summarized in Supplementary Table S1, while comparison of characteristics and outcomes of patients from the clinical publication (25) included or not in the biologic study described here is provided in Supplementary Table S2. Briefly, median patient age was 60 years (range, 38–71 years). Twenty-eight patients received PBSC from HLA-identical siblings, and 25 from a 10/10 HLA allelic-matched unrelated donor. In comparison with TBI patients, TLI patients had a similar (low) incidence of graft rejection (1 vs. 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 NRM (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 Fig. 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 and colleagues (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 IL7 for $CD4^+$ and $CD8^+$ T cells (while IL7 also stimulates B cells ontogenesis), and by IL15 for $CD8^+$ T cells, NK,

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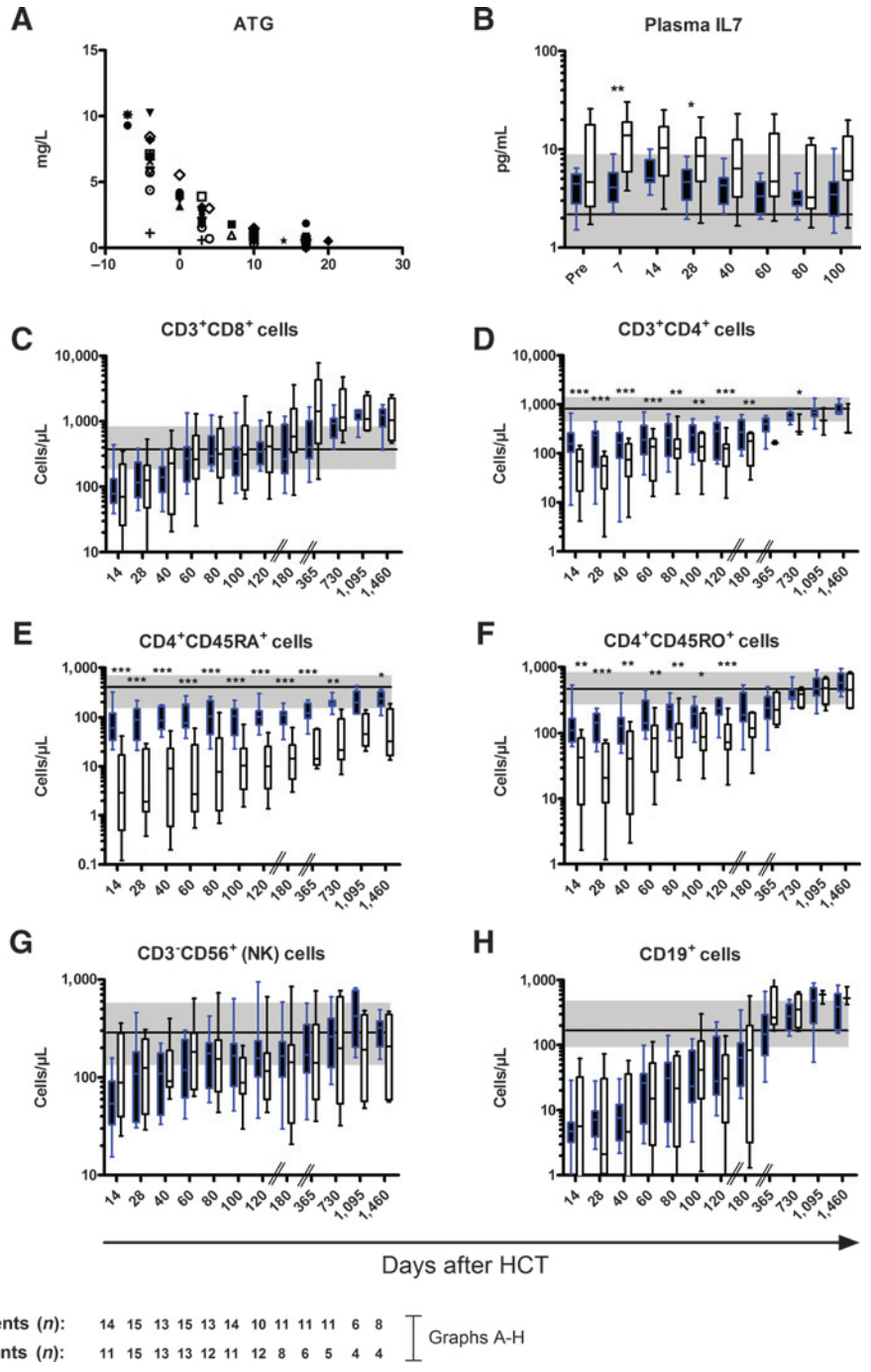

Figure 1.
A, kinetics of active ATG levels in the serum of TLI patients ($n = 15$). B, evolution of plasma IL7 levels the first 100 days after transplantation in TBI (black boxes, $n = 14$) and TLI (white boxes, $n = 13$) patients. C–H, recovery of T, B, and NK cells the first 4 years after transplantation 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–95th percentiles) and horizontal lines the medians in 45 aged-matched healthy controls. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

and NK/T cells (6, 31, 32). This prompted us to assess their levels among TBI and TLI recipients. As shown in Fig. 1B, IL7 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 IL7 levels after allo-HCT depend mainly on consumption by T cells (32–34). In contrast, IL15 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 (Supplementary Fig. S1A), although posttransplant IL15 (and to a lesser extend Il7) levels have also been correlated with other factors such as CRP levels (32, 33).

On day 28 after transplantation, IL2 serum levels were below the threshold for detection (2.1 pg/mL) in the two groups of patients. IL4 levels were comparable in the two groups of patients, but IL4/CD4 cell ratios were significantly higher in TLI than in TBI patients ($P = 0.0004$; Supplementary Fig. S1B and S1C). Furthermore, IL10 levels were significantly higher in TLI patients ($P = 0.0481$), possibly suggesting a TH-2 polarization of donor

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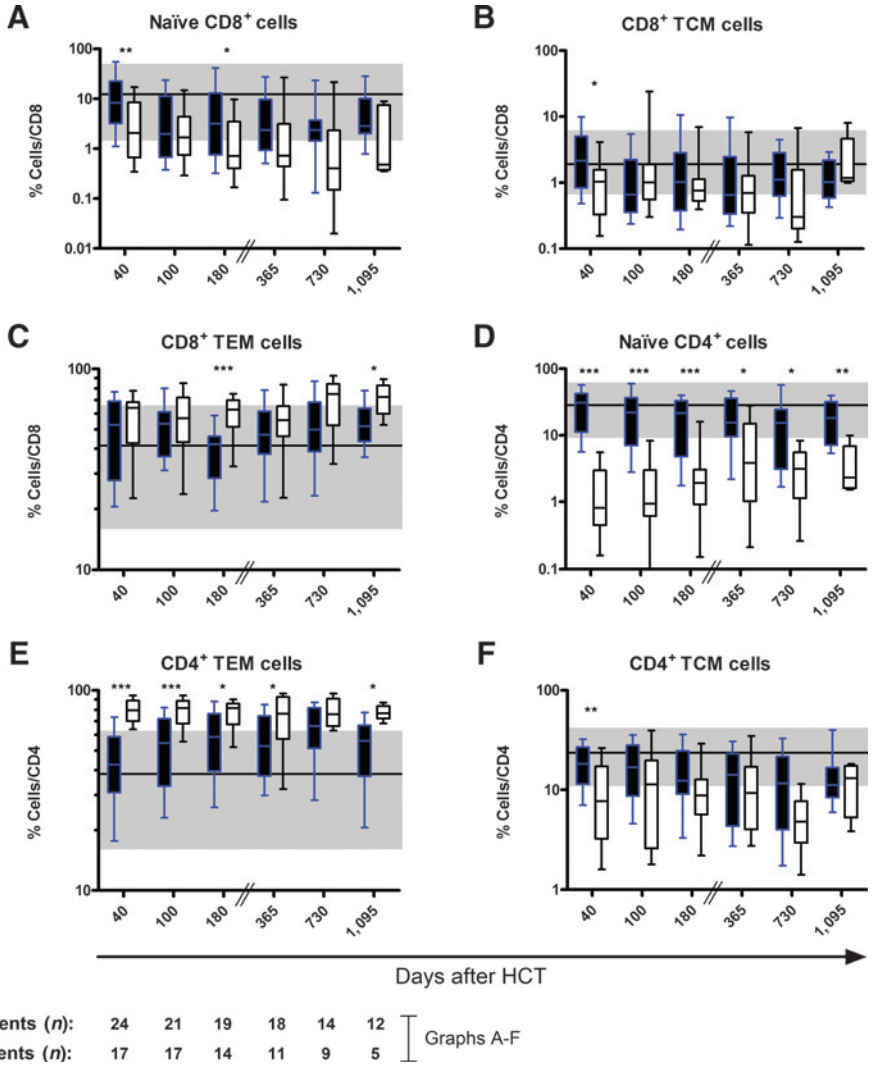

Figure 2.
A–F, recovery of $CD8^+$ and $CD4^+$ T cell subsets in TBI (black boxes) and TLI (white boxes) patients. TCM, central memory T cell; TEM, effector/effector memory T cells. Plots display the median, 25th and 75th percentiles of the distribution (boxes), and whiskers extend to the 10th and 90th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

T cells (as observed by Lowsky and colleagues; ref. 18) or a higher production of IL10 by Treg in TLI patients (as observed in a preclinical murine model; ref. 24; Supplementary Fig. S1B). Finally, IFNγ serum levels were similar between the two groups of patients, whereas TNFα levels were significantly higher in TLI than in TBI patients ($P = 0.0493$; Supplementary Fig. S1B).

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

Previous studies have demonstrated that faster establishment of donor T-cell chimerism correlated not only 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 with TBI patients, TLI patients had lower donor T-cell chimerism levels on day 180 (median 82% vs. 95%, $P = 0.09$) and 365 (median 88% vs. 97%, $P = 0.04$) after transplantation (Supplementary Fig. S2A).

Immune recovery in TBI and TLI patients is described in Fig. 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 to 60 days after allo-HCT. The same was observed for NK cells, with median NK cell counts reaching normal values 60 to 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. Furthermore, $CD4^+$ 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 two 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 (Fig. 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 $CD4^+$).

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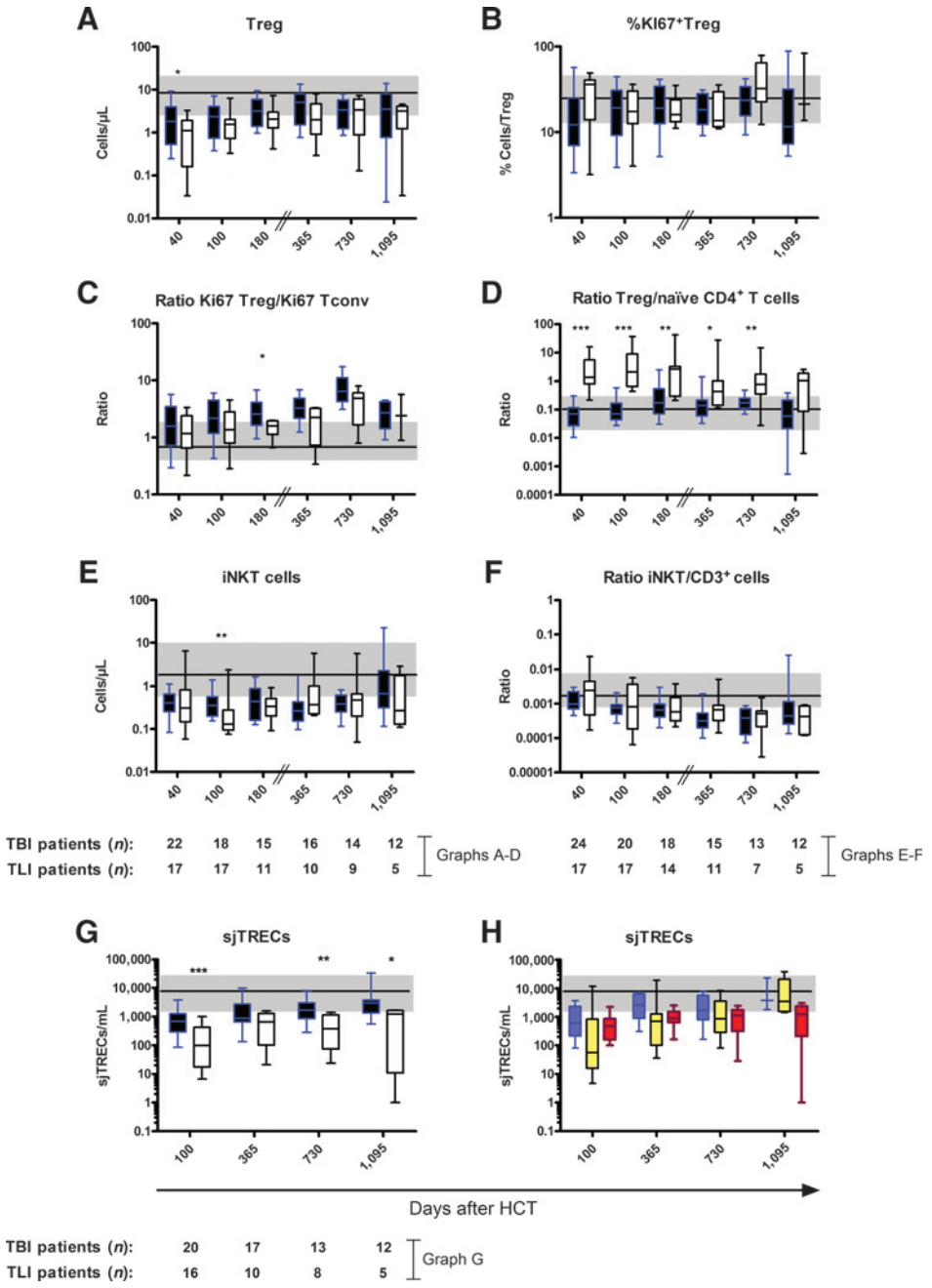

Figure 3.
A–F, recovery of regulatory T cells (Treg) and invariant NK/T cells (iNKT) in TBI (black boxes) and TLI (white boxes) patients. G, thymic function (assessed by sjTREC blood concentrations) in TBI and TLI patients. H, evolution of sjTREC levels according to recipient age in the whole cohort (<50 years [blue boxes], 50 to 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. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Treg and NK/T-cell recovery (Fig. 3). Previous studies have demonstrated that Treg plays 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 with 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 depends mainly on IL2 for their homeostasis (41, 42). Although IL2 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 Ki-67 on day 40 after transplantation [compared

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with 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 Ki-67, 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. Furthermore, the iNKT/T cell ratio (a parameter recently demonstrated to be predictive of acute GVHD in allogeneic HCT recipients; ref. 44) was also comparable in the two groups.

B- and NK cell subset recovery (Supplementary Fig. S3). B-cell recovery was superimposable in TBI and TLI patients. In contrast with 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 $CD56^{bright}$ NK cells in comparison with $CD56^{dim}$ 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, although 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 (Fig. 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 (Fig. 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, whereas 12 TLI patients had a total of 20 episodes of bacterial infection ($P = 0.7$).

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

Among CMV-seropositive patients and/or donors, 12 of 21 TBI patients versus 16 of 18 TLI patients experienced a CMV infection the first 100 days after transplantation ($P = 0.038$). Furthermore, 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 to 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, whereas 8 out of 14 TLI patients had a total of 10 bacterial infections ($P = 0.7$). Furthermore, 3 out of 23 TBI patients experienced a total of four fungal infections, whereas 1 of 14 TLI patients had a total of one 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 and colleagues (18). This might explain some differences in the pattern of immune recovery that we observed between the two arms of the study, such as slower recovery of $CD4^+$ and of naive $CD8^+$ T cells (52). Furthermore, because 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 and colleagues (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 IL4, which in turn polarizes donor T cells toward a Th2 pattern. Importantly, iNKT cells also promote expansion of donor Treg and drive them to produce IL10 (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). Second, IL10 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 IL10 by Treg from TLI patients as observed in the mouse model where Treg

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from TLI/ATS mice produced higher levels of IL10 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 Ki-67 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.

Disclosure of Potential Conflicts of Interest

Y. Beguin reports receiving a commercial research grant and speakers bureau honoraria from Sanofi. No potential conflicts of interest were disclosed by the other authors.

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